AN ABSTRACT OF THE THESIS OF

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Title: THE CONVERSION OF SOME MONOALKYLHYDRAZINES TO THEIR CORRESPONDING HYDROCARBONS BY A HEPATIC MICROSOMAL ENZYME SYSTEM IN RAT

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Abstract approved:
Donald J. Reed

An aspect of the metabolism of monoalkylhydrazines has been investigated using both intact rats and rat liver microsomal enzyme preparations. Intact rats respired propane and n-butane on administration of 0.3-0.5 mmoles of isopropyl- and n-butylhydrazine, respectively. Based upon dose administered, a five to ten percent yield of hydrocarbon was obtained.

In vitro experiments were conducted to further characterize the reaction. The conversion of alkylhydrazines to alkanes occurred predominantly in the microsomal fraction of liver cells. All of the simple monoalkylhydrazines, methyl-, ethyl-, n-propyl-, isopropyl- and n-butylhydrazine, were converted to the corresponding alkanes at a rate of ca. 20 mμmoles/120 min/10 mg of microsomal protein. Other substrates yielding methane were the azo derivative of

Procarbazine (N-isopropyl-α-[2-methylhydrazino]-p-toluamide), the azoxy derivative of Procarbazine and methylazoxymethanol acetate. The reaction specifically required oxygen, NADPH and liver microsomes. Rat and guinea pig microsomal preparations converted the alkylhydrazines to alkanes at a rate higher than bovine, porcine and ovine microsomal preparations.

Various parameters typical of enzyme reactions indicated that the reaction was enzymic. The apparent Michaelis constant for ethylhydrazine was found to be 4.5×10^{-5} M with a maximal enzyme velocity of 32.0 mμmoles of ethane/120 min/10 mg of microsomal protein. The enzymic reaction was linear with time to 120 min, with microsomal protein to 10 mg and with NADPH to 30 μmoles. Some enzyme inhibitors, p-chloromercuribenzoate and N-ethylmaleimide, caused 50% inhibition at 10^{-6} M and 10^{-5} M, respectively. Cyanide, carbon monoxide, chelating agents and a mixed-function oxidase inhibitor (SKF 525-A) had no great effect on the enzyme reaction.

Cytochromes b_5 and P_{450} did not appear to play a role in the reaction. The lack of correlation between loss of enzyme activity and cytochrome content during tryptic digestion, lack of enzyme induction with P_{450} induction by phenobarbital and 3-methylcholanthrene administration and lack of correlation between b_5 content and enzyme activity on lipase solubilization indicate strongly that cytochrome P_{450} and b_5 are not required for alkylhydrazine oxidase

activity. Only a marginal purification could be performed.

A methylhydrazine derivative, Procarbazine, was converted to the azo derivative with the same microsomal preparations and conditions. The possibility of an alkyldiazene (alkyldiimide) intermediate is discussed.

The Conversion of Some Monoalkylhydrazines to Their Corresponding Hydrocarbons by a Hepatic Microsomal Enzyme System in Rat

by

Russell Allen Prough

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TABLE OF CONTENTS

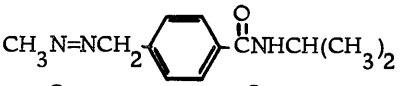
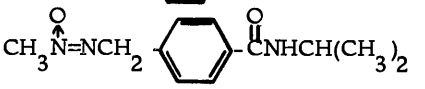
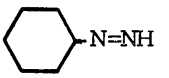
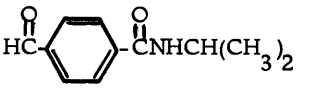
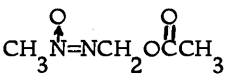
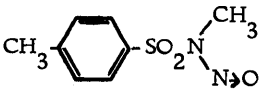
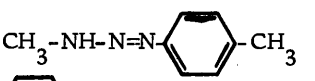
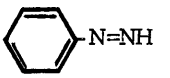
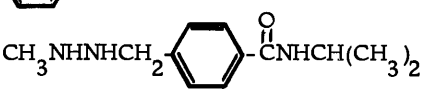
LIST OF TABLES

LIST OF FIGURES

ABBREVIATIONS

2-Diethylaminoethyl diphenylpropylacetate	SKF 525-A
Deoxynucleic acid	DNA
Ethylenediaminetetracetic acid, disodium salt	EDTA
N-Ethylmaleimide	NEM
Flavin adenine dinucleotide	FAD
Flavin mononucleotide	FMN
Microns (10^{-6} moles)	μ
Nanometers (10^{-9} meters)	nm
Nicotinamide adenine dinucleotide, reduced	NADH
Nicotinamide adenine dinucleotide phosphate, oxidized form	$NADP^{\oplus}$
Nicotinamide adenine dinucleotide phosphate, reduced form	NADPH
Ribonucleic acid	RNA
Sodium 4-chloromercuribenzoate	pCMB

ABBREVIATIONS AND STRUCTURE OF PERTINENT
HYDRAZINES AND HYDRAZINE DERIVATIVES

Azoprocarbazine (azo-PCZ)	
Azoxyprocarbazine (azoxy-PCZ)	
t-Butyldiazene	$(CH_3)_3CN{=}NH$
n-Butylhydrazine (BH)	$CH_3CH_2CH_2CH_2NHNH_2$
Cyclohexyldiazene	
sym-Dimethylhydrazine or 1,2 dimethylhydrazine (SDMH)	$CH_3NHNHCH_3$
unsym-Dimethylhydrazine or 1,1 dimethylhydrazine (UDMH)	$(CH_3)_2NNH_2$
Dimethylnitrosamine (DMNA)	$(CH_3)_2NN{\rightarrow}O$
Ethylhydrazine (EH)	$CH_3CH_2NHNH_2$
N-isopropyl-p-formylbenzamide	
Isopropylhydrazine (IpH)	$(CH_3)_2CHNHNH_2$
Methylazoxymethanol acetate (MAM)	
Methylhydrazine (MMH)	CH_3NHNH_2
N-Methyl-N-nitroso-p-toluenesulfonamide (Diazald)	
3-Methyl-p-tolyltriazene (MTT)	
Phenyldiazene	
Procarbazine (PCZ)[1]	
n-Propylhydrazine (PH)	$CH_3CH_2CH_2NHNH_2$

[1] A registered trade name by Hoffmann-LaRoche, Nutley, New Jersey.

THE CONVERSION OF SOME MONOALKYLHYDRAZINES TO THEIR CORRESPONDING HYDROCARBONS BY A HEPATIC MICROSOMAL ENZYME SYSTEM IN RAT

I. INTRODUCTION

With the development of the chemistry of the common elements, a multitude of new inorganic and organic compounds have been discovered and characterized. Medical and industrial science, in the quest of new drugs and products, have sponsored a great part of these investigations. Although the effects of many of these substances on man and his environment have been studied, many common compounds have not been extensively investigated with regard to their toxicity and metabolism. The need for study in these areas is becoming increasingly important as air, land and water pollution become a serious threat to man's existence.

Purpose of the Study

The following discussion and tables will show how widely distributed nitrogen-nitrogen (N-N) compounds are in occurrence and use. In light of these facts and the small amount known about their metabolism, this thesis will attempt to elucidate a small but possibly significant area of the biochemistry of these compounds. The metabolism of monoalkylhydrazines and their derivatives will be investigated and related to the current knowledge about hydrazine

metabolism. This thesis will show that the conversion of monoalkylhydrazines to alkanes is enzymic as judged by various criteria common for most enzymes. The characteristics and partial purification of the enzyme are described. The involvement of some biological constituents such as cytochromes are also reported and discussed. Throughout this thesis, the term alkylhydrazines will refer to the monoalkylhydrazines.

The toxic properties of compounds containing N-N bonds were recognized by several German workers as early as 1887; particularly irritation of the nose, throat and eyes (Curtius, 1887; McAulay, 1946). The effect of hydrazines and azo compounds on plants was also noted quite early (Loew, 1890). However, as late as 1953, the exposure level of hydrazine required to cause the various physiological effects had not been determined (Clark, 1953). Three quarters of a century passed before adequate toxicological data were compiled for even the simplest hydrazine compounds. Less yet is known about the metabolism of hydrazines or the effects they have on the metabolic functions of the body.

Hydrazine and azo compounds are used extensively as industrial materials, drugs and pesticides. Industrially, hydrazines are used in the manufacture of analytical reagents, anti-oxidants, chemiluminescent materials, diesel fuel additives, dyes, explosives, fuel cells, hair wavers, metal films, photographic chemicals, plastics, rubber,

soldering fluxes, textiles and many others (Audrieth and Ackerson-Ogg, 1951; Clark, 1953; Orth et al., 1965; Zahn and Wiedersich, 1958). They have also been used as fungicides, gas absorbents, plant growth regulators and rocket fuel (Clark, 1953; Konig, 1968). Many hydrazine and azo compounds are used in organic syntheses. Table 1 briefly summarizes their use for synthesis purposes.

Pharmaceutical compounds containing nitrogen-nitrogen bonds are numerous. Table 2 lists some of the most common drugs containing a N-N bond. Much use has been made of hydrazines as chemotherapeutic agents and immunosuppressants (Amiel et al., 1967; Bollag, 1963).

Within the last decade several naturally-occurring antibiotics have been found to contain N-N bonds. Other naturally-occurring compounds containing the N-N bond have recently been isolated and characterized. These natural products are briefly summarized in Table 3. The extreme small number of these compounds and their toxicity make them unique among all of the natural products. To date very little is known of their bio-synthesis and less is known about their metabolism.

Effect of Hydrazines on Gross Metabolism

To date no concise compilation has been made of the effects of hydrazine and its derivatives on gross metabolism. Since J. A. Wittkop

Table 1. Uses of hydrazines in organic syntheses.

Reaction Name	Use	Reference
Curtius Reaction	Preparation of amines from esters or acid chlorides	Clark, 1953
Fischer Indole Synthesis	Preparation of indoles from the cyclization of the arylhydrazone of aliphatic ketones	Smith, 1966
McFayden-Stevens Reaction	Preparation of aldehydes from aromatic acids or esters	Clark, 1953
Schmidt Reaction	Preparation of primary amines from organic acids, amides from ketones and nitriles or formyl derivatives of amines from aldehydes	Clark, 1953
Wolff-Kishner Reaction	Reduction of carboxyl groups to methylene groups	Clark, 1953
	Alkylation using diazomethane	Cram & Hammond, 1959
	Dehalogenation (limited)	Clark, 1953
	Formation of cyclopropane ring	Todd, 1949
	Hydrolysis of substituted imides	Clark, 1953
	Preparation of azo derivatives by oxidation of hydrazines	Clark, 1953
	Preparation of hydrazones	Clark, 1953
	Reduction of unsaturated C-C bonds, N-oxides, nitrites, esters, oximes, nitroso groups, sulfonyl compounds and azo compounds	Furst, Berlo & Hooton, 1965

Table 2. Typical pharmaceutical compounds containing N-N bonds (Goodman and Gillman, 1965).

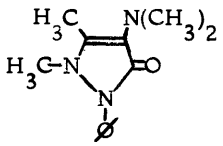
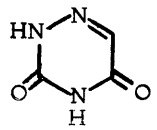
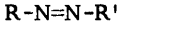
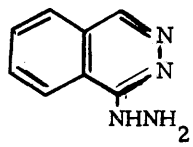
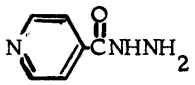
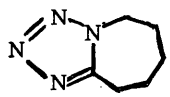
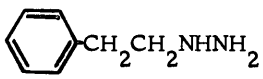
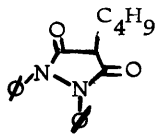
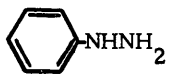
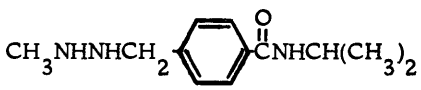
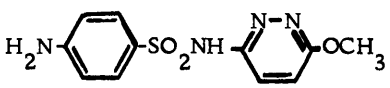
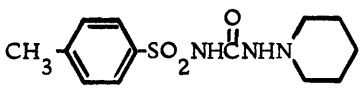
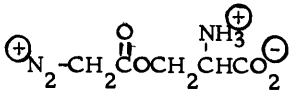
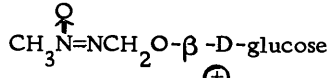
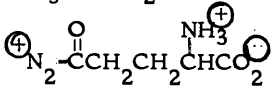
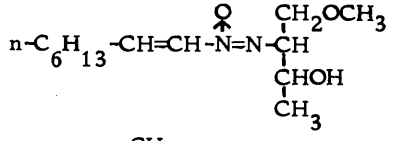
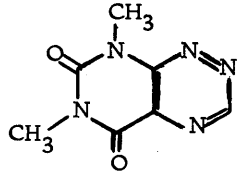
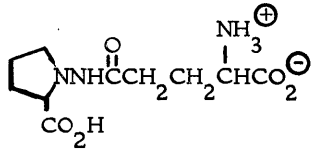
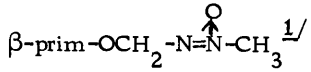
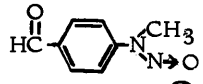
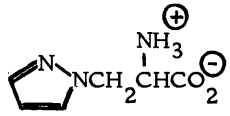
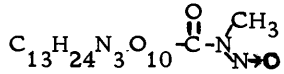
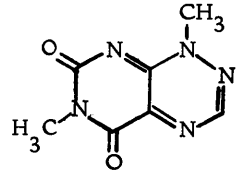
Name	Structure	Use
Aminopyrine (Pyramidon)		Analgesic and anti-inflammatory agent
6-Azauracil (and nucleotide)		Pyrimidine analogue
Azo dyes		Bactericide and tissue-proliferating agent
Hydralazine		Hypotensive agent
Iproniazid (Marsilid)		Antidepressive, rheumatic and antitubercular agent
Isoniazid		Antitubercular agent
Metrazol		Central nervous system stimulant
Phenelzine sulfate (Nardil)		Stimulant, antidepressive agent
Phenylbutazone (Butazolidin)		Analgesic (gout and rheumatoid arthritis)
Phenylhydrazine (and acetyl derivative)		Polycythemia ruba, p. vera agent
Procarbazine (Natulan, Ibenzmethyzin)		Hodgkin's disease agent
Sulfamethoxypyridazine (Kynex)		Bactericide
Tolazamide (Tolinase)		Hypoglycemic agent (diabetes)

Table 3. Naturally occurring N-N compounds.

Name	Structure	Source	Reference
Agaritine (β -N-[γ-L(+)-glutamyl] -4-hydroxymethylphenylhydrazine)		Agaricus bisporus	Levenberg, 1961 Daniels, Kelly and Hinman, 1961
Alazopeptin	L-alanyl-(6-diazo-5-oxo)-L-norleucyl-(6-diazo-5-oxo)-L-norleucine	Streptomyces griseoplanus	Devoe et al., 1957 Patterson et al., 1965
Azaserine (O-diazoacetyl-L-serine)		Streptomyces fragilis	Coffey et al., 1954 Bartz et al., 1954
Cycasin		Cycas cincinalis L & C.revoluta	Riggs, 1956
6-Diazo-5-oxo-L-norleucine (DON)		Streptomyces (unidentified)	Dion et al., 1956
Duazomycin A	N-acetyl-DON	Streptomyces ambofaciens	Rao, 1961
Duazomycin B	Peptide containing one residue of glutamic acid and two residues of DON	Streptomyces ambofaciens	Rao, 1962
Elaiomycin		Streptomyces hepaticus	Ehrlich et al., 1954 Stevens et al., 1956
Fervenulin (6, 8-dimethyl-5, 7-dioxo-5, 6, 7, 8- tetrahydropyrimido-[5, 4e] -as-triazene)		Pseudomenas cocovenenans	Davis, Robins and Cheng, 1962

Continued

Table 3. Continued.

Name	Structure	Source	Reference
Linatine (1-[N-(γ-L-glutamyl)-amino]-D-proline)		Linum usatatissimum (flax seed)	Klosterman, Lamoureux and Parsons, 1967
Macrozamin	 [1/]	Macrozamia spiralis	Riggs, 1956
4-Methylnitrosaminebenzaldehyde		Clitocyte sauveolens	Herrman, 1960
β-Pyrazol-1-ylalanine		Citrullis vulgaris	Noe & Fowden, 1960
Streptozotocin		Streptomyces achromogenes	Herr et al., 1959
Toxoflavine (1,6-dimethyl-5,7-dioxo--1,5,6,7-tetrahydropyrimido-[5,4e]-as-triazene)		Pseudomonas cocovenenans	Davis, Robins and Cheng, 1961

[1/] The abbreviation, prim, is for primeverose or 6-β-D-xyloside-D-glucose.

(1969) has recently reviewed the use of certain hydrazines as drugs; this topic is not discussed here.

Toxicity

Hydrazine exposure has several distinct symptoms (Clark, 1953; Kulagina, 1962). Eye and respiratory irritation followed by a state of excitement describe the first symptoms of hydrazine poisoning. Eye irritation may be followed by swelling and pus discharge. However, permanent eye injury occurs only by contact with the very concentrated vapor of hydrazine. Dizziness and nausea often follow inhalation. The sharp, irritating odor is generally intolerable enough that exposure to damaging quantities is unlikely. Dermatitis and allergetic symptoms are often apparent. The minimum lethal dosage for hydrazine in man has been calculated to be 100 mg per kg of body weight or daily repeated doses of 10 mg per kg of body weight.

Effects on Central Nervous System

Another symptom of administration of hydrazines is convulsions. The time at which the first convulsion occurred in rats has been reported to be roughly dependent on the condition of the animal, i.e., glycogen levels, blood glucose levels, etc. Hydrazine, MMH and UDMH caused convulsions in rats when large doses were given (Medina, 1963). Vitamin B_6 was found to be effective in preventing

the convulsions induced by several hydrazines. However, convulsions from phenelzine (β-phenylethylhydrazine) in rats could not be prevented by pyridoxine or pyridoxal (Dubnick, Leeson and Scott, 1960; Minard, 1967). In the case of phenelzine, the β-phenylethylhydrazone of pyridoxal induced convulsions in shorter time. Other carbonyl reagents such as semicarbazide, thiosemicarbazide, and isoniazid caused convulsions and could be counteracted by pyridoxine injection. Inhibition of B_6-requiring enzymes possibly accounts for the onset of convulsions. The injection of glucose also prevented convulsions by blocking the hydrazine-induced hypoglycemia (Fortney, 1966).

Methemoglobinemia

As early as 1936, workers observed that hydrazines caused methemoglobin to form in guinea pig, mice and rat (Nagazumi, 1936a, b). Up to 30% conversion of oxyhemoglobin to methemoglobin was noted. Phenylhydrazine (4% solution) caused methemoglobin formation from oxyhemoglobin and liberated molecular nitrogen in quantities equal to the amount of original oxyhemoglobin (Nizet, 1946). Beaven and White (1954) detected the formation of benzene and nitrogen during the reactions of phenylhydrazine and acetylphenylhydrazine with oxyhemoglobin. These findings suggest a possible oxidation-reduction reaction between hydrazines and oxygenated hemes. This

aspect of metabolism will be discussed later. Methylhydrazine was found to be a more potent methemoglobin-producing agent than any of the other hydrazines (Fortney and Clark, 1967). In fact, methemoglobinemia has been used as an index for methylhydrazine exposure (Clark and Fortney, 1967).

Alterations of Glucose, Amino Acid and Fat Metabolism

Hydrazines affected glucose metabolism by initially causing hyperglycemia, an increase in blood sugar, in rats, guinea pig and rabbit (Muchnik, 1937). The hyperglycemia was followed by increases in blood lactate and pyruvate levels and decreases in gluconeogenesis (Fortney and Clark, 1967; Fortney, Clark and Stein, 1967). Added amino acids did not prevent hyperglycemia. Also, conversion of ^{14}C-pyruvate, ^{14}C-alanine and ^{14}C-aspartic acid to ^{14}C-glucose was retarded. After all storage carbohydrate (glycogen) was depleted, hypoglycemia, a depression in blood sugar levels, set in (Trout, 1965a). This hypoglycemia seemed related in some manner to convulsions.

The plasma amino acid levels were observed to increase upon hydrazine dosage in rat (Lewis and Izume, 1926; Korty and Coe, 1968). Conversion of ^{14}C-glycine to $^{14}CO_2$ was inhibited by hydrazine, MMH, UDMH and iproniazid (Amenta and Dominquez, 1965a). However, the ^{14}C-acetate to $^{14}CO_2$ conversion was not inhibited.

The increase of amino acid incorporation but decrease of amino acid catabolism indicates that the transaminase reactions might be inhibited (Amenta and Johnston, 1963; Cornish and Wilson, 1968). This inhibition of transaminases by carbonyl reagents was typical of many enzymes requiring pyridoxal phosphate. Added pyridoxal phosphate should reverse the inhibition.

Simonsen and Roberts (1967) reported that hydrazine caused citrulline to accumulate in mouse liver, especially if arginine was administered prior to hydrazine. The inhibition would appear to be due to partial blockage of the ornithine cycle. Hydrazine, UDMH, MMH and SDMH significantly inhibited glutamic acid decarboxylase and γ-aminobutyric acid transaminase in rat brain (Medina, 1963). Pyridoxine prevented convulsions but no correlation was found between the protective effect of pyridoxine and the inhibition of glutamic acid decarboxylase and γ-aminobutyric acid transaminase. UDMH-pyridoxal hydrazone and UDMH-pyridoxal phosphate hydrazone were isolated from brain after injection of UDMH, pyridoxal and pyridoxal phosphate. These findings point to the involvement of pyridoxal-containing enzymes and transaminases in some of the gross effects of hydrazines on metabolism.

Like many compounds foreign to the body, hydrazines caused fat to accumulate in liver (Wells, 1908). Within 24 hours after hydrazine administration, the blood levels of nonesterified fatty acids,

cholesterol and phospholipid in rat were found to be higher than control blood levels (Amenta and Dominquez, 1965b; Bitter, Clark and Lackey, 1967). These increased fatty acid levels caused lipid to accumulate in the liver even faster than when other hepatotoxic agents such as carbon tetrachloride are administered. The lipid levels in these rats returned to normal after 72 hours. Glucose infusion reduced the effect of hydrazine on free fatty acid and liver fatty acid levels (Trout, 1965a, b). The increase in fatty acid transport appears to be due to the increase in the free fatty acid pool. The hepatic uptake of lipid is likewise increased.

Effect on Protein Synthesis

Simple hydrazines were shown to stimulate protein synthesis and liver RNA in rat (Banks, Clark and Stein, 1967; Banks and Stein, 1965). Hydrazine caused a liver enlargement associated with increased protein and RNA content. Similar effects are seen in kidney and gonad. The excess amino acid levels present may stimulate protein synthesis.

However, some other hydrazine derivatives retarded protein synthesis. Neal, Libman and Smulson (1968) have shown that γ-glutamylhydrazides can be inhibitory at high concentration and stimulatory in low concentration to ^{14}C-valine incorporation into protein in Pseudomonas aeruginosa. The methylhydrazine derivative,

Procarbazine (PCZ), has been used as an immunosuppressive agent in dog and man (Amiel et al., 1967; Kayibandi, Amiel and Berardet, 1968). RNA and protein synthesis are decreased in the liver on administration of PCZ. In isolated perfused rat liver, PCZ nearly stopped protein synthesis while no effect on RNA synthesis could be seen (Koblet and Diggelmann, 1968). Polysomes from PCZ treated rats did not bind ^{14}C-labeled amino acids as well as control polysomes. Procarbazine itself was bound to microsomal tissue.

The inhibition of protein synthesis was originally thought to be partly due to formation of hydrogen peroxide from Procarbazine oxidation (Berneis, Kofler and Bollag, 1964). PCZ was found to be slowly converted to azo-PCZ in aqueous solutions. Gale, Simpson and Smith (1967) have shown that in the presence of catalase, an enzyme which destroys hydrogen peroxide, aged Procarbazine solutions were still as inhibitory as in the presence of hydrogen peroxide. The inhibiting metabolite could be washed free from the protein-synthesizing apparatus. Unlike Procarbazine, the metabolite did not bind to microsomes.

Reaction with Heme Compounds

Hydrazines in aqueous solution have been noted to form hydrogen peroxide (Beaven and White, 1954; Rostorfer and Cormier, 1957). Phenylhydrazine and acetylphenylhydrazine reacted with erythrocyte

oxyhemoglobin and methemoglobin to give hydrogen peroxide (H_2O_2) faster than hydrazine. In the presence of ferric ions, 1-methyl-2-benzylhydrazine gave H_2O_2 (Berneis, Kofler and Bollag, 1964). In healthy erythrocytes (containing glucose-6-phosphate dehydrogenase) reduced glutathione (GSH) decreased the peroxide levels (Cohen, 1966; Liebowitz and Cohen, 1968). Glucose -6-phosphate dehydrogenase is thought to be required so that the NADPH:GSSG oxidoreductase can produce more GSH. In turn the excess GSH reduced H_2O_2 possibly via a GSH-dependent peroxidase (Hochstein and Utley, 1968).

Other heme components which react with hydrazines besides oxyhemoglobin and methemoglobin were found (Asami, 1968; Andrejew, Gernez-Rieux and Tarquet, 1959; Lotti and Galoppini, 1961). Catalase and peroxidase, both heme-containing enzymes, were inhibited by hydrazine, phenylhydrazine and isoniazid. Asami showed that phenylhydrazine inactivates cytochromes c and c_1. Some oxidation-reduction reactions may occur between the heme groups or some alkylation may occur.

Effect on Electron Transport System

The electron transport system was found to be inhibited by hydrazines (Asami, 1968; Fortney, 1967; Krulick, 1966). At concentration levels lower than mmolar, the inhibition was found to be due to removal of substrate; i. e., pyruvate and α-ketoglutarate formed

hydrazones which were incapable of being electron transport system substrates. However, at higher concentrations phenylhydrazine inactivated cytochromes c and c_1 with no serious chemical change. The radical formed from phenyldiazene was suspected to interact with the heme group (Asami, 1968).

Effect on DNA and RNA

As previously mentioned, hydrazine caused increases in RNA and possibly DNA levels (Banks, Clark and Stein, 1967; Banks and Stein, 1965). Contrarily, Berneis, Kofler and Bollag (1964) showed that 1-methyl-2-benzylhydrazine was more effective at degrading nucleic acids *in vitro* than H_2O_2. In fact, this compound speeded the H_2O_2 breakdown of nucleic acids. Various transforming DNAs were inactivated by H_2O_2-producing hydrazines (Freese, Sklarow and Freese, 1968). It was postulated that the production of H_2O_2 produced radicals capable of inactivating DNA.

In a previous paper (Gale, Simpson and Smith, 1967), aged solutions of catalase-treated Procarbazine inhibited protein, DNA, and RNA synthesis. This suggests that some metabolite other than H_2O_2 is involved. Other workers have also shown that PCZ inhibits DNA and RNA synthesis (Sartorelli and Tsunamura, 1966).

Hydrazine, MMH and UDMH chemically decompose nucleosides, nucleotides, and RNA (Lingens and Schneider-Bernloehr, 1966). At

37°C and pH 10.5, these hydrazines converted uridine to pyrazolone and ribosylurea. This reaction is specific for uridine and uracil.

Plant Growth Regulators

Certain hydrazine derivatives are effective plant growth regulators (Riddell et al., 1962). Maleic acid 2,2-dimethylhydrazide and succinic acid 2,2-dimethylhydrazide retard the growth of many plants. Plant height is retarded but not rate of development. Reed, Moore and Anderson (1965) correlated the inhibition of shoot elongation in peas to the inhibition of the conversion of tryptamine to indolacetic acid (IAA) when the succinic hydrazide was administered to the plant. The succinic acid 2,2-dimethylhydrazide is slowly converted to succinate and UDMH. The UDMH was found to inhibit tryptamine oxidation. Reed (1967) also showed that β-hydroxyethylhydrazine inhibits tryptamine oxidation in peas in the manner cited above.

Metabolism of Alkylhydrazines

The preceding discussion dealt with the many effects of hydrazines on gross metabolism. Some means of hydrazine release from the body as the free base or a derivative will be reviewed. Hydrazines metabolism will also be discussed. The following will briefly review the known metabolism of hydrazines.

Distribution

Organ distribution of administered ^{14}C-labeled methylhydrazine and its analogue, Procarbazine or N-isopropyl-α-(2-methylhydrazino)-p-toluamide, has been determined in man and rat (Pinkerton, Hagan and Back, 1967; Schwarz, Bollag and Obrecht, 1967). Tissue distribution of the carbon-14 label of ^{14}C-methylhydrazine (15 mg/kg in rat) showed that the highest concentration of label resided in liver, kidney, bladder, pancreas and blood serum. Two hours after injection of a high concentration of ^{14}C-Procarbazine (46 mg/kg in man), the majority of the carbon-14 label was found in liver, kidney, intestine and skin.

Multi-enzyme complexes occur in liver and catalyze the metabolism of xenobiotic compounds (Greek, xenos and bios, for "stranger to life") such as drugs, steroids and carcinogenic compounds (Mason, North and Vanneste, 1965; Omura et al., 1965). These enzymes are associated with the membranous endoplasmic reticulum of the cell. They are described as the pellet resulting from high-speed centrifugation of the supernatant fluid of mitochondrial fractions at 100,000 to 250,000g for several hours (Siekovitz, 1965). The high-speed pellet consists of membranes with and without ribosomes. The ribosomes are ribonucleoprotein granules and are concerned exclusively with protein synthesis.

The enzymic functions of the microsomes are varied as seen by the following microsomal reactions: N-dealkylation, side-chain oxidation, thioester oxidation, hydroxylation, desulfuration, methylation, O-dealkylation and others. These reactions are reviewed in detail by Shuster (1964). The enzymes seem to concentrate in the ribosome-less microsomes called smooth-surfaced endoplasmic reticulum. It seems very likely that xenobiotic compounds such as the hydrazines could be metabolized in the microsomes.

Some of the microsomal oxidative enzymes are limited to those substrates which are lipid soluble (Gaudette and Brodie, 1959). Since 20% of the dry weight of microsomes is phospholipid (Mahler and Cordes, 1966), it is entirely possible that only lipid-soluble compounds can enter the microsomal matrix.

Many of these microsomal enzymes require NADPH and molecular oxygen (Mason, North and Vanneste, 1965). These enzymes have a wide specificity for substrates and are designated as mixed-function oxidases. Since an active NADPH oxidase occurs in the microsomes, Gram and Fouts (1968) found that addition of NADPH alone gave spurious results for several enzyme assays. However, when NADPH generating enzymes such as glucose 6-phosphate and isocitric dehydrogenase were coupled with the microsomal enzymes, increased enzyme activity was noted (Gram and Fouts, 1967). The NADPH generating enzymes are not effected by the NADPH oxidase and may

become easily incorporated in the microsomal matrix.

Excretion

One of the principal means by which the body rids itself of hydrazine is by renal excretion (Coe and Korty, 1967). Just as many organic acids and bases are excreted, hydrazine excretion (as the free base) involved glomerular filtration, passive diffusion-mediated reabsorption and tubular secretion. Methylhydrazine (MMH) excretion also in anesthetized dogs was found to be very similar to that of hydrazine (Coe, Howe and Goetting, 1968). Both cause increased renal excretion of sodium, potassium and water due partly to a decrease in net tubular reabsorption in the proximal tubule (Coe and Korty, 1968; Van Stee, 1965). Another closely related hydrazine derivative, 1, 1-dimethylhydrazine (UDMH), was found to be different in its effect on renal excretion (Wong, 1966). While hydrazine decreased glomerular filtration rates and proximal renal tubular function, UDMH posed no problem to total renal function.

Hydrazines can also be excreted by means involving metabolites other than the free base (Jenne, 1965; McIsaac, Parke and Williams, 1958). Although nearly 60% of the carbon-14 label of administered ^{14}C-phenylhydrazine is excreted in the urine of rat, only about 16% is excreted as the free base. Some of the most common metabolites of phenylhydrazine have been found to be 1-acetyl-2-phenylhydrazine,

acetoacetic acid phenylhydrazone, acetone phenylhydrazone, p-hydroxyphenylhydrazine, α-ketoglutaric acid phenylhydrazone, pyridoxal phosphate phenylhydrazone and pyruvic acid phenylhydrazone. Benzoic hydrazide was found to be acetylated initially and later converted to 1, 2-diacetylhydrazine and benzoic acid which is excreted largely as hippuric acid in rat (Turnbull, Yard and McKennis, 1962).

Metabolism

The metabolism of the alkyl substituent of alkylhydrazines is similar to that of other alkyl side chains (Shuster, 1964). Several workers have reported the demethylation of methylhydrazine derivatives by rat hepatic tissue (Aebi et al., 1966; Baggiolini and Bickel, 1966). Baggiolini and Bickel noted that the ^{14}C-methyl derivative of Procarbazine administered to rats yielded $^{14}CO_2$. This enzyme system was typical of the hepatic microsomal demethylating enzymes. Kreis, Piepho and Bernhard (1966) discovered that the ^{14}C-label became incorporated in the purine bases excreted in the urine of treated mice. These same ^{14}C-labeled bases are excreted when ^{14}C-sodium formate is administered. This suggests that Procarbazine metabolism involved a reaction which converted the methyl substituent to formic acid; a reaction similar to demethylation reactions on other methylated nitrogen compounds. Weitzel (1967) demonstrated the in vitro formation of formaldehyde from PCZ. This laboratory has

investigated the in vitro demethylation of PCZ, MMH and UDMH in some detail (Wittkop, 1969; Wittkop, Prough and Reed, 1969).

As previously noted phenylhydrazine can be hydroxylated to form p-hydroxyphenylhydrazine (McIsaac, Parke and Williams, 1958). Presumably this is a typical aromatic hydroxylation reaction (Shuster, 1964). Takeshita, Nishizuka and Hayaishi (1963) found a degradation scheme in watermelon seed for β-pyrazol-1-ylalanine to yield pyruvate, ammonia and pyrazole. This is similar to the reaction catalyzed by tryptophan synthetase in which serine and indole are converted to tryptophan (Yanofsky, 1960).

The inhibition of aminotransferases by hydrazines suggests that hydrazines may participate as pseudo-substrates in transamination reactions. Workers have noted that hydrazine and MMH can serve as substrates for the purified glutamine synthetase enzyme system (Speck, 1949; Willis, 1966). The two possible products which might be formed when MMH is employed as a substrate are 1-methyl-2-(5-glutamyl)-hydrazine or 1-methyl-1-(5-glutamyl)-hydrazine. The Michaelis constant for MMH is 100 times larger than for ammonia, but since the maximum velocity is the same for both, MMH strongly inhibits glutamine synthetase. Gigliotti and Levenberg (1964) discovered a γ-glutamyltransferase which hydrolyzes the γ-L-glutamylhydrazide bond of agaritine in Agaricus bisporus. This is an aminotransferase-type reaction.

Many transacylase reactions have been reported. In the section on metabolite excretion we noted that aroylhydrazides and arylhydrazines became acetylated before being excreted (McIsaac, Parke and Williams, 1958; Turnbull, Yard and McKennis, 1962). Jenne (1965) showed the presence of an enzyme system in man which would acetylate isoniazid. The transacylation of γ-glutamylhydrazide was found to be an enzymic transfer of the hydrazine from the acid hydrazide to some carboxyl group in the enzyme. This converts the γ-glutamylhydrazide to glutamic acid and enzyme-bound hydrazine (Neal, Libman and Smulson, 1968). A similar reaction was reported for conversion of isoniazid to hydrazine and γ-picolinic acid (Toida, 1960; Toida, 1962; Toida, 1963). Such acylation reactions result in a product which may be excreted more readily than the free base.

Amine oxidases have been assigned a very important but little understood role in brain amine metabolism (Davison, 1958). Hydrazines have been extensively used as amine oxidase inhibitors. Iproniazid, isonicotinic acid 2-isopropylhydrazide, was noted to form acetone during its in vivo degradation in man (Koechlin, Schwartz and Oberhaensli, 1962). This result is similar to the reports of several other workers who suggest that hydrazines are substrates for monoamine oxidases (MAO) (Clineschmidt and Horita, 1968; Hucko-Haas, 1968). β-Phenylethylhydrazine was slowly converted to phenylacetic acid in the presence of rat liver mitochondrial

preparations containing MAO activity. Hucko-Haas (1968) noted the formation of a stoichiometric EI complex between purified bovine plasma amine oxidase and hydrazines. With time, the inhibition was reversed. This suggests that hydrazines may actually be a substrate for MAO but have a low turnover. At present, the products are unidentified.

Asami (1968) suggested the formation of a phenyldiazene occurred when cytochromes c and c_1 are in the presence of phenylhydrazine. Other workers have shown that PCZ readily forms the azo derivative *in vivo* in rat (Oliverio *et al.*, 1964; Raaflaub and Schwartz, 1965). An equilibrium between azo compounds and the hydrazine derivatives was suggested to exist for the enzyme, $NADPH_2$: dimethylaminoazobenzene oxidoreductase (1. 6. 6. 7), in rat (Hernandez, Gillette and Mazel, 1967). Levenberg (1962) reported that the p-hydroxymethylphenylhydrazine formed from agaritine by γ-glutamyltransferase enzymes in *Agaricus bisporus* was further degraded to a diazonium-type compound. It seems apparent that oxidation of the hydrazine bond to an azo bond is a common process in biological systems.

Several workers have reported biological reactions of hydrazine which might occur via a diazene-like intermediate (Asami, 1968; Beaven and White, 1954; Levenberg, 1962; Nizet, 1946). Although Nizet did not detect benzene from the interaction of oxyhemoglobin

and phenylhydrazine, Beaven and White did detect it. Workers in our laboratory and in Switzerland have noted that MMH and PCZ yield methane in vivo in rat (Dost and Reed, 1967; Dost, Reed and Wang, 1966; Schwartz, Brubacker and Vecchi, 1968). Palmer et al. (1967) have reported that β-hydroxyethylhydrazine-treated citrus plants respired ethylene.

Chemical Reactions Involving Diazenes

In 1907, Chattaway discovered that alkaline solutions of phenylhydrazine yielded benzene and nitrogen. Although he had no direct evidence, he postulated the presence of phenyldiazene as an intermediate in the reaction. Other workers noted that MMH could be chemically oxidized to methane by heat or various oxidizing agents (Cambi and Dubini-Paglia, 1963; Neuman and Nadeau, 1964). No mechanism was proposed. Research on Wolff-Kishner reactions also suggested that diazenes might be involved in the formation of alkanes from hydrazines or hydrazones.

In the Wolff-Kishner reduction, the carbonyl carbon of a hydrazone is reduced to its corresponding hydrocarbon with the loss of molecular nitrogen as seen in Equation 1 (Todd, 1949).

$$R_2C{=}N\text{-}NH_2 \rightarrow R_2CH_2 + N_2 \qquad (1)$$

Cyclic N-alkylhydrazones were shown to lose nitrogen and form a

cyclopropane ring at 230° in a basic solution with a platinum catalyst. It was also noted that benzaldehyde methylhydrazone under the same conditions yielded ethylbenzene. In the absence of solvent, it is possible that all of these reactions have the same mechanism.

Balandin and Vaskevitch (1936) investigated the kinetics of the decomposition of cyclohexanone hydrazone under the preceding conditions and concluded that the most reasonable intermediate was the cyclohexyldiazene. Todd (1949) indicated that the free radical reaction in Equations 2 and 3 was the most likely. The equilibrium step was thought to require alkali as a catalyst for the Wolff-Kishner reaction.

$$C_6H_5\text{-}CH{=}N\text{-}N\begin{matrix}H\\R\end{matrix} \rightleftharpoons C_6H_5\text{-}CH_2\text{-}N{=}N\text{-}R \rightarrow C_6H_5\text{-}CH_2^{\bullet} + R^{\bullet} + N_2 \qquad (2)$$

$$C_6H_5\text{-}CH_2^{\bullet} + R^{\bullet} \rightarrow C_6H_5\text{-}CH_2\text{-}R \qquad (3)$$

where

$$R = H \text{ or } CH_3$$

However, Wolff-Kishner reactions employing milder conditions suggested that diazenes are intermediates but the collapse of diazene is an ionic reaction (Cram and Bradshaw, 1963; Cram, Sahyun and Knox, 1962). The following Equations (4, 5) summarize the ionic reaction.

$$R\text{-}N{=}N\text{-}H + B^{\ominus} \rightarrow R\text{-}N{=}N^{\ominus} + BH \rightarrow R^{\ominus} + N_2 + BH \qquad (4)$$

$$R^{\ominus} + BH \rightarrow RH + B^{\ominus} \qquad (5)$$

A classic study has been made of the decomposition of phenyldiazene (Huang and Kosower, 1965, 1967a, 1968a, b, c, d). Phenyldiazene was found to be somewhat unstable; its half-life being very pH and O_2 sensitive. Phenyldiazene formation from phenylazoformic acid is unobservable in solutions containing dissolved O_2 due to rapid decomposition of the phenyldiazene. Increasing pH from 7.34 to 13.7 destroyed a solution of 2.5×10^{-4} M phenyldiazene in less than two minutes. An alkyldiazene, t-butyldiazene, decomposed some 65 times faster than phenyldiazene at pH 7.34 and in a nitrogen atmosphere (Huang and Kosower, 1967b). The cyclohexyldiazene of Balandin and Vaskevitch (1936) was found to have a half-life of 34.5 sec at 150°. Less branched diazenes such as methyldiazene are probably very unstable. Kosower's data strongly implicated the ionic decomposition of diazenes in the absence of oxygen. However, in the presence of oxygen, the reaction proceeds very rapidly, but at present no mechanism has been postulated.

Disubstituted hydrazines, like UDMH and SDMH, would not be expected to form diazenes (Smith, 1966). Sym-disubstituted hydrazines such as SDMH easily lose two hydrogen atoms and form relatively stable azo compounds. Oxidation of unsym-disubstituted hydrazines like UDMH presents a more complex oxidation scheme.

Oxidation to remove two hydrogen atoms produce charge-separated diimides, $R_2N^{\oplus}=N^{\ominus}$, called azamines. Azamines can rapidly dimerize to form tetrazenes, $R_2N\text{-}N=N\text{-}NR_2$. It might be expected that severe conditions would be required to cause the stable oxidation products of these compounds to form hydrocarbons.

Biological Activity of Procarbazine

The ability of PCZ to inhibit several transplantable tumors spurred investigation of the biochemistry of the compound (Bollag, 1965). PCZ was found to have a favorable therapeutic index but caused considerable liver damage. It was noticed that the methylhydrazine moiety was absolutely necessary for activity and other alkylhydrazine moieties lacked biological activity. Currently, much research is being done to develop other methylhydrazine derivatives with antitumor activity but no detrimental effects. Work done in our laboratory suggests that PCZ and MMH may form methyl free radicals capable of inhibiting tumors (Dost, Reed and Wang, 1966; Dost and Reed, 1967).

Many carcinogenic compounds which inhibit tumors are alkylating agents (Walpole et al., 1954). The carcinogenic nature of PCZ may be due to similar alkylating tendencies. In vitro chemical decomposition of azo compounds to yield free radicals and nitrogen has been studied (Calvert and Pitts, 1966; Schulz, 1939). The

biochemical metabolism of PCZ to yield azo-PCZ is well known as previously mentioned (Oliverio et al., 1964). The collapse of azo-PCZ to free radicals and nitrogen might give a stable benzyl free radical capable of reacting with specific sites. The aim of this thesis is to study the reactions of alkylhydrazines in biological systems.

The work of Dost and Reed (1967) suggested that further investigation of the enzymes involved in the transformation of alkylhydrazines to the parent alkanes was needed. This thesis will report work accomplished by the author to characterize and partially isolate the enzyme or enzymes involved in this transformation. Since a significant amount of the hydrazines concentrates in the liver, hepatic enzyme systems have been studied. The criteria for characterization of alkylhydrazine oxidase activity will consist of those parameters normally investigated to characterize enzyme reactions.

Part of this thesis has been published with the permission of the Graduate School of Oregon State University. This work has appeared in Archives of Biochemistry and Biophysics (Prough, Wittkop and Reed, 1969).

II. EXPERIMENTAL PROCEDURE

Materials

Sprague-Dawley male rats (ca. 200-250 grams) were obtained from Pacord Research, Inc., Portland, Oregon and Bio-Science Animal Labs, Oakland, California. Bovine and porcine liver were generously provided by Steen Brothers Meat Packers, Albany, Oregon and ovine liver was donated by Dr. P. H. Weswig, Department of Agricultural Chemistry, Oregon State University. Guinea pigs were purchased from Pacific Northwest Fisheries, Albany, Oregon.

Bovine serum albumin, L-cysteine, DEAE-cellulose anion exchanger, EDTA (disodium salt), FAD, FMN, DL-isocitric acid (trisodium salt), isocitric dehydrogenase (type IV), 3-methylcholanthrene, NADH, $NADP^{\oplus}$, NADPH, nicotinamide, sodium deoxycholate, Triton X-45, Triton X-102, Trizma base (tris-[hydroxymethyl]-aminomethane), trypsin (type III) and trypsin inhibitor (ovomucoid, type II-O) were purchased from Sigma Chemical Company, St. Louis, Mo. Ammonium sulfate and methylazoxymethanol acetate (MAM) were obtained from Mann Research Laboratories, Inc., New York, N. Y. Diazald, sym-dimethylhydrazine (SDMH), dimethylnitrosamine (DMNA), N-ethylmaleimide (NEM) and sodium 4-chloromercuribenzoate (pCMB) were purchased from Aldrich Chemical Company, Inc., Milwaukee, Wis. Sodium phosphate (dibasic), sodium phosphate

(monobasic) and sodium dithionite were obtained from J. T. Baker Chemical Company, Morristown, N. J. Other chemicals purchased were 2 N Folin-Ciocalteau phenol reagent (Fisher Scientific Co., Fair Lawn, N. J.), sucrose and phenobarbital sodium (Mallinckrodt Chemical Works, St. Louis, Mo.), heparin (Eli Lilly & Co., Indianapolis, Ind.), dithiothreitol (P-L Biochemicals, Inc., Milwaukee, Wis.) and 2-mercaptoethanol and 3-methyl-p-tolyltriazene (MTT) (Eastman Organic Chemicals, Distillation Products Industries, Rochester, N. Y.). Peracetic acid was obtained from Dr. E. N. Marvell, Department of Chemistry, Oregon State University. Rubber serum stoppers (14 mm) were purchased from Arthur H. Thomas Co., Philadelphia. All other chemicals, glassware and supplies were obtained from the stores of the Radiation Center, Oregon State University. Porapak Q column support (150-200 mesh) was purchased from Water Associates, Inc., Framingham, Mass.

Methylhydrazine (MMH) and unsym-dimethylhydrazine (UDMH) were purchased from the Matheson-Coleman-Bell Co., Norwood, Ohio. K & K Laboratories, Plainview, N. Y., specially synthesized ethyl- and n-propylhydrazine oxalate, EH and PH respectively, and n-butylhydrazine oxalate (BH) was obtained from California Biochemicals, Co., Los Angeles, Cal. Smith, Kline and French Laboratories, Philadelphia, Penn., furnished 2-diethylaminoethyl diphenylpropylacetate (SKF 525-A) and F. Hoffmann-LaRoche & Co., Nutley,

N. J., generously provided isopropylhydrazine hydrochloride (IpH), N-isopropyl-p-formylbenzamide, Procarbazine (PCZ) and a small amount of the azo derivative of Procarbazine (azo-PCZ). All chemicals were used without further purification.

Argon, compressed air, hydrogen, prepurified nitrogen, oxygen, 1/8 inch stainless steel tubing and fittings were obtained through Salem Steel and Supply Co., Salem, Oregon. Carbon monoxide, n-butane, 2-butylene, ethane, ethylene, methane, propane, propylene, 0.1% oxygen and 1.0% oxygen in nitrogen were purchased from the Matheson Co., East Rutherford, N. J.

Azoprocarbazine (azo-PCZ) and azoxyprocarbazine (azoxy-PCZ) were prepared by a modification of the method reported by Bollag et al. (1964). Mercuric oxide in anhydrous ether:ethanol (1:1 v/v) in equimolar amounts with PCZ yielded azo-PCZ with a ultraviolet absorption maximum at 233 nm in methylene chloride ($\epsilon = 1.46 \times 10^4\ M^{-1} cm^{-1}$). This product had the same absorption coefficient as did the azo-PCZ furnished by Hoffmann-LaRoche & Co. Azoxy-PCZ was synthesized by treating azo-PCZ with peracetic acid in methylene chloride. The infrared spectra of the product showed absorption bands at 6.58 and 7.56 μ as described by Bollag et al. (1964). The reactions were run with an atmosphere of oxygen-free nitrogen.

Methods

Buffers

Four buffers or isotonic solutions were used during the course of this work: Isotonic 0. 25 M sucrose, isotonic 1. 1 M KCl, 0. 05 M Tris buffer, pH 7. 5 and 0. 05 M phosphate buffer, pH 7. 5. Microsome fractions were prepared in all four and the enzyme activity was compared. The sucrose and Tris buffers were best for preparation but phosphate buffer was best for storage. The buffers were prepared by normal procedures.

Respirometry

Respirometry experiments were carried out in a closed recycling system with a total volume of 2 liters. Drierite and soda-lime traps were used to remove water and CO_2. As oxygen was consumed by the rat, a demand valve permitted pure oxygen to enter the respirometer system. Samples of the gaseous atmosphere (1 ml) were injected into the gas chromatograph for analysis (described later in detail).

Preparation of Rat Liver Microsomes

Livers were sliced, carefully washed free of blood, homogenized in a teflon-glass homogenizer in three volumes of 0. 25 M

sucrose solution, and centrifuged at 12,000g for ten min to remove cellular debris, nuclei, and mitochondria. The resulting supernatant was decanted from the precipitate and recentrifuged in an identical manner. The 12,000g supernatant was then centrifuged at 140,000g for four hours at 4° to precipitate the microsomal pellet. The supernatant was decanted and the pellet rehomogenized in 1 ml 0.05 M phosphate buffer, pH 7.5, per gram of original liver. In some earlier work Tris buffer was used, but if the suspensions were not stored for any length of time, no difference was seen in enzyme activity. The preparations were stored at 2-5°.

Protein Determination

During the initial phase of this work, protein concentration of the rehomogenized microsomal suspensions was determined by an E_{280}/E_{260} method. Later the method of Lowry as described by Chaykin (1966) was employed. The E_{280}/E_{260} method gave protein values almost two times higher than the Lowry method.

Enzyme Assay

Since the lower alkanes are not appreciably soluble in water, the conversion of alkylhydrazines to the corresponding alkanes by microsomes was measured by analyzing the gaseous atmosphere which would contain the hydrocarbons produced. The standard assay

mixture used for measuring alkylhydrazine oxidase activity consisted of microsomal protein, 10 mg; monoalkylhydrazine, 2 μmoles; 140,000g supernatant protein (8 mg) or NADPH-regenerating system; and 0.05 M phosphate buffer, pH 7.5, in a total volume of 2 ml. The NADPH-regenerating system was composed of $NADP^{\oplus}$, 0.5 μmoles; DL-isocitric acid, 5 μmoles; nicotinamide, 50 μmoles; $MgCl_2$, 5 μmoles; $MnCl_2$, 0.01 μmoles; and isocitric dehydrogenase (0.5 units per reaction vessel). Unless otherwise stated, these were the concentrations of the components of the assay mixture. Assay mixtures were equilibrated with air and sealed in 10 ml Erlenmeyer flasks with 14 mm rubber serum stoppers. The temperature used for incubation was 25° (see Results section for detail). Aliquots of the gaseous atmosphere of each assay mixture were injected manually into the gas chromatograph using a one ml syringe with one and one-half inch 26-gauge needle and compared with known quantities of standard hydrocarbon gasses.

When a different gaseous atmosphere was used one of two procedures was used. When O_2 concentration itself was varied, the samples were purged with anaerobic nitrogen (N_2) prior to adding microsomes and isocitric dehydrogenase (generally 0.1 ml each). Oxygen-free nitrogen was prepared by passing prepurified nitrogen through heated columns packed with a mixture of diatomaceous earth and reduced copper (Meyers and Ronge, 1939). The microsomes and

isocitric dehydrogenase were made up in buffers through which N_2 had been bubbled and stored under an N_2 atmosphere. The sample flask was flushed with N_2 and quickly sealed with serum stoppers. Using two syringe needles, the N_2 was swept through the flask for five minutes. The volume of O_2 required for a given concentration was calculated and that volume of N_2 removed with a syringe. The desired volume of pure O_2 was quickly added by syringe. The microsomal protein and isocitric dehydrogenase were added to initiate the reaction. A similar procedure of CO flushing was used when CO was used. When smaller amounts of CO were added, the desired volume of CO was added and the excess pressure released by inserting a syringe needle through the serum stopper.

Analysis of Gaseous Atmosphere

The gaseous atmospheres were analyzed with an F & M 810 gas chromatograph equipped with a dual flame detector (F & M Scientific Corporation, Avondale, Pennsylvania, now part of Hewlett-Packard Co., Palo Alto, California). A ten foot 1/8 inch stainless steel column packed with Poropak Q column support (150-200 mesh) was used to separate the samples as seen in Figure 1. The flow rates for the effluent and dual flame detector were as follows: Argon, 90 ml/min; compressed air, 440 ml/min and hydrogen, 63 ml/min. The injection port temperature was ambient and the dual flame detector

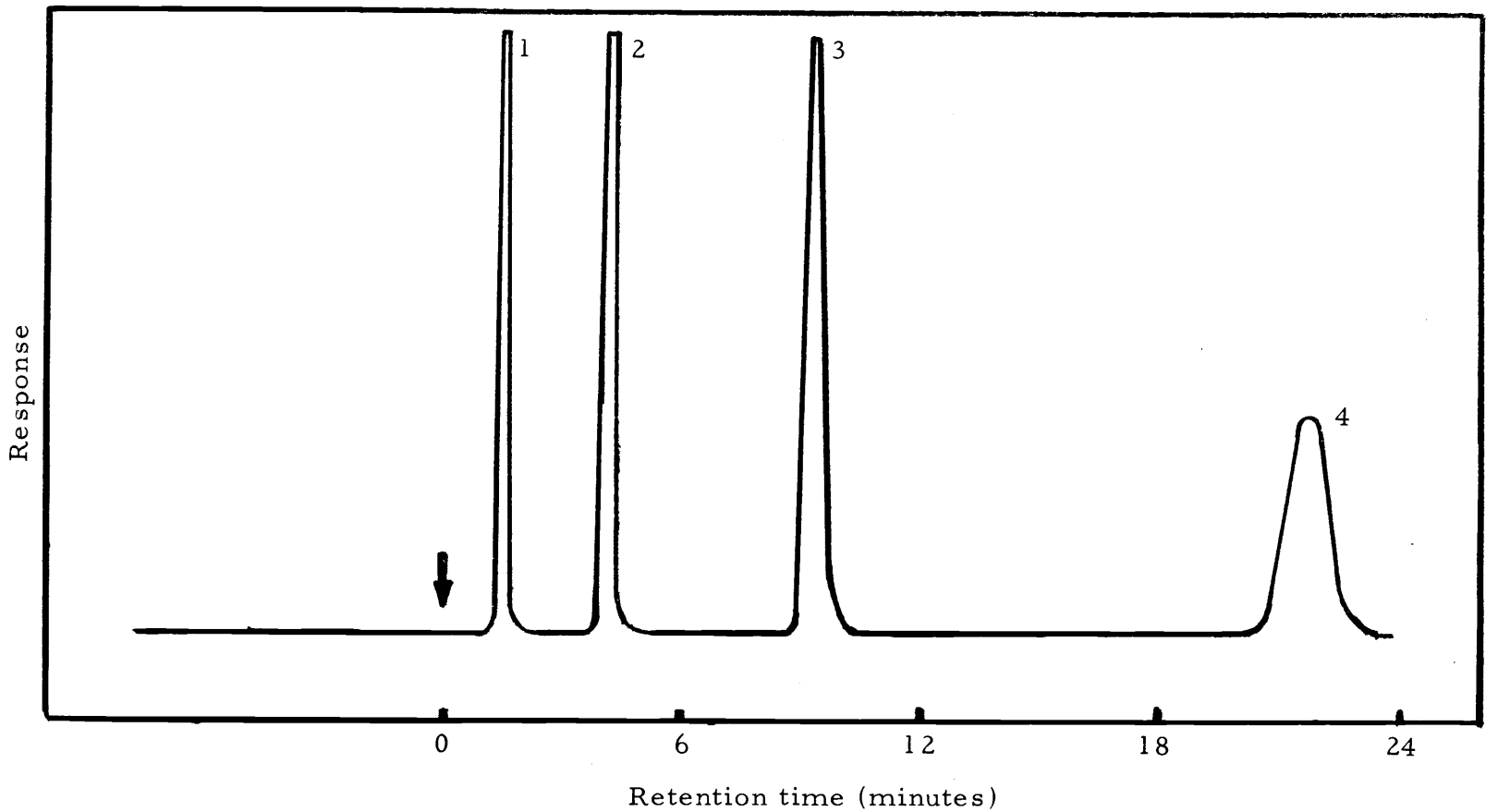


Figure 1. Separation of the four lower alkanes. Gas analysis was of methane (1), ethane (2), propane (3) and butane (4) at 30°, argon flow rate of 40 ml/min, and settings at range 10, attenuation 64.

temperature was 220°. The column temperature depended on what alkane was being analyzed; they are as follows: methane, ambient; ethane, 80°; propane, 100° and butane, 120°. These temperatures were used to keep retention times small and the response peaks as symmetrical as possible. A table of typical retention times can be seen in Table 4. The response settings during assay were range 10, attenuation 4. The gradations in response settings were accurate so that one could use any setting and relate it to the readings at range 10, attenuation 4. The chart speed was always one inch per minute. The preceding conditions were used consistently in all hydrocarbon assays.

Table 4. Typical retention times for pertinent alkanes.

Alkane	Temperature	Retention time Minutes
Methane	ambient	0.18
Ethane	80°	0.26
Ethylene	80°	0.21
Propane	100°	1.30
Propylene	100°	1.20
Butane	120°	3.10
Butylene	120°	3.00

Standard gas mixtures (1 µl/ml) were prepared and quantitated (Figure 2). Calculation of the quantities of hydrocarbon from the peak areas is made as follows:

Calculation of total hydrocarbon: Since the peaks are very

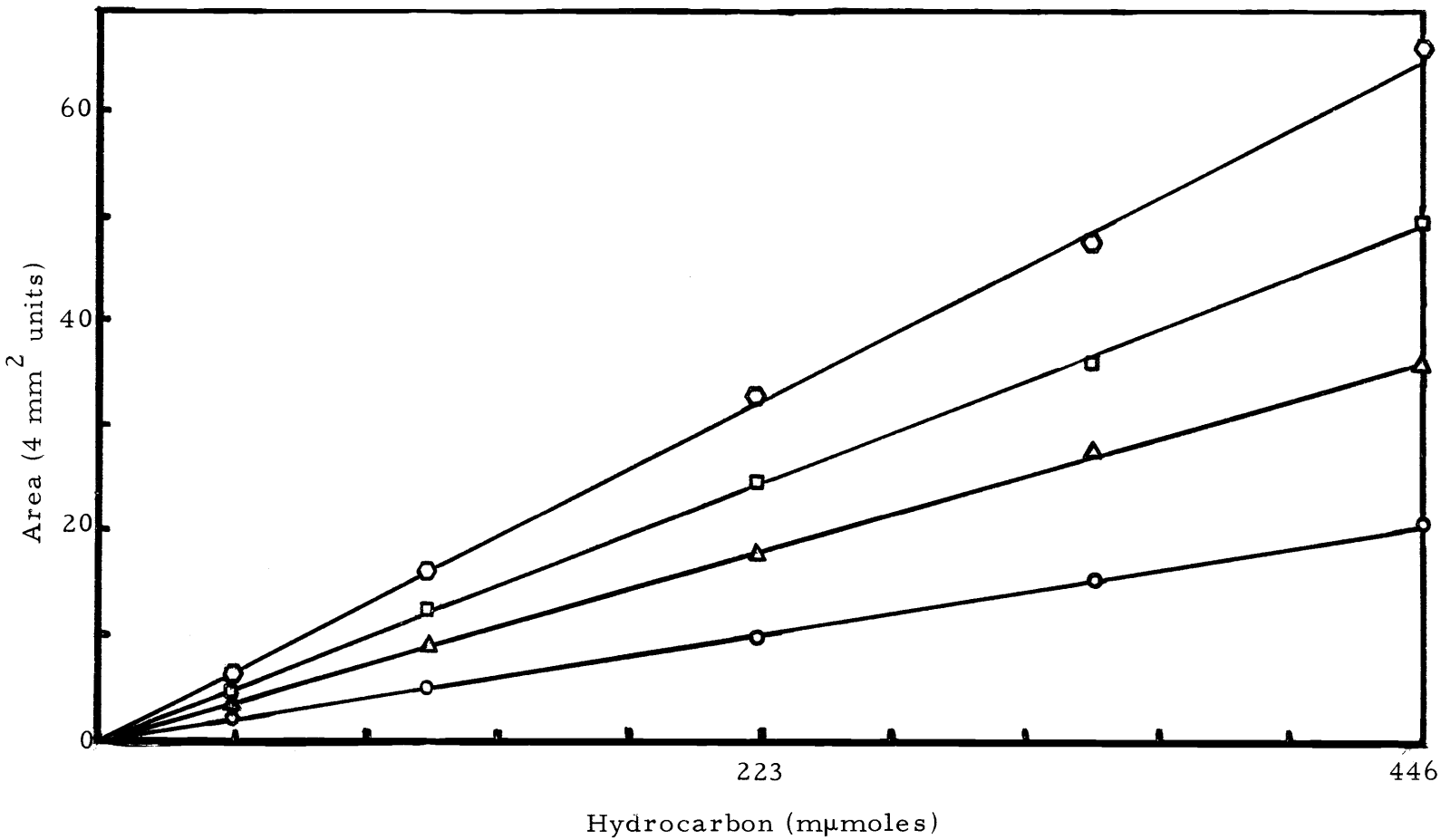


Figure 2. Standard curve of injected quantities of hydrocarbons vs. response area for methane, ○ ; ethane, △ ; propane, □ and butane, ⬡ ; at range 10, attenuation 32.

symmetrical, one can say:

$$\text{area} = \frac{(\text{peak height}) \times (\text{peak width at base})}{2}$$

From the standard curve one can calculate area/μl factors for specific hydrocarbon:

$$\mu\text{l of hydrocarbon} = \frac{(\text{area})}{(\text{area}/\mu\text{l factor})}$$

Since there are 22.4 μl per μmole of gas, each μl would contain 44.6 mμmole of hydrocarbon. One can convert μl to mμmoles by multiplying by 44.6 mμmoles/μl (assuming S. T. P.):

$$\text{mμmoles of hydrocarbon} = (\mu\text{l hydrocarbon}) \times (44.6\ \text{mμmoles}/\mu\text{l})$$

Now one must include a factor for the 1 ml gaseous aliquot. The total volume in a 10 ml Erlenmeyer flask sealed with a serum stopper is 14 ml. Therefore, the gaseous volume would be 12 ml for a 2 ml liquid volume.

$$\text{Total mμmoles of hydrocarbon} = (\text{mμmoles hydrocarbon}) \times (12)$$

or

$$\text{Total mμmoles of hydrocarbon} = \frac{(\text{peak height})(\text{peak width at base})(44.6\ \text{mμ moles}/\mu\text{l})(12)}{(2)(\text{area}/\mu\text{l factor})}$$

Table 5 shows the area/μl factors used for the hydrocarbons measured during the investigations described in this thesis. The conditions are the same as the standard assay for each hydrocarbon. In assuming S. T. P. conditions, the absolute values will vary by a small percentage. However, since all assays were performed under the same conditions, the errors will be consistent.

Table 5. Area/μl factor for pertinent alkanes.

Alkane	Area/μl factor (4 mm^2)
Methane	160
Ethane	280
Ethylene	274
Propane	360
Propylene	354
Butane	520
Butylene	505

Alkylhydrazine Oxidase Activity Assay in Blood

Blood was obtained by heart puncture on live rats. A 5 ml glass syringe with sterile needle was treated with heparin by withdrawing 0. 1 ml of heparin and coating the inside surfaces of the syringe with heparin prior to the heart puncture. The rats were anesthetized with ether. The blood samples were used directly or centrifuged at 10, 000g for 20 min to obtain plasma and erythrocyte fractions. The erythrocytes were washed twice with 10 ml of isotonic KCl.

The assay mixtures were the same as the standard assay except blood or blood fraction was used in place of microsomes and 140, 000g supernatant or NADPH-regenerating system. The buffer used was 0. 05 M Tris buffer, pH 7. 5, which was 10^{-3} M in EDTA.

Purification of Protein Containing Alkylhydrazine Oxidase Activity

A. Triton solubilization: Prior to any column step, the microsomal protein was solubilized with a Triton X-45 and X-102 treatment. The microsomal suspension was made 1. 25% in Triton X-45 and 1. 25% in Trition X-102 and left for 50 min at 4°. The solution was centrifuged at 140, 000g for one hour at 4° and the supernatant decanted off with care.

B. Ammonium sulfate precipitation: The Triton-treated supernatant was treated at 4° with 0. 23 g/ml of ammonium sulfate (40% saturation) to precipitate the active fraction. The $(NH_4)_2SO_4$ was added in ten parts and the pH was maintained at 7. 5 by addition of small amounts of 5 N NaOH. After all the salt was added, the solution was allowed to stand for 30 min and was centrifuged at 20, 000g for 20 min. The pellet was redissolved in 10 to 20 ml of 0. 05 M phosphate buffer. The amount of ammonium sulfate required to give 40% saturation was calculated according to Chaykin (1966).

C. DEAE-cellulose column: DEAE-cellulose was pretreated for

column preparation as described by Peterson and Sober (1962). It was then preequilibrated with 0.001 M phosphate buffer (10^{-3} M EDTA), pH 7.5. A 2.5 x 36 cm column was prepared under ten psi pressure. The redissolved 40% $(NH_4)_2SO_4$ pellet from the preceding step was placed on the column and the column eluted at 4° in a stepwise procedure with the following buffers: 0.001 M phosphate, 200 ml; 0.05 M phosphate, 50 ml; 0.1 M phosphate, 50 ml; 0.15 M phosphate, 50 ml and 0.3 M phosphate, 1,000 ml. The buffers also were 10^{-3} M in EDTA and at pH 7.5.

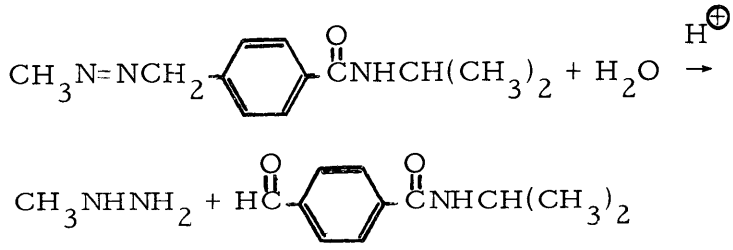
The samples were assayed at each step; however, after 12-24 hours standing, half of the activity was lost at each treatment. Refer to the Results section to see a typical purification table.

Assay for Formation of Azo-PCZ

The assay mixture consisted of $MgCl_2$, 5 μmoles; $MnCl_2$, 0.01 μmoles; nicotinamide, 50 μmoles and 0.05 M phosphate buffer, pH 7.5, in a total volume of 5 ml. Microsomes, NADPH-regenerating system components ($NADP^{\oplus}$, isocitric acid and isocitric dehydrogenase as usual) and Procarbazine, 5 μmoles (extracted with methylene chloride to remove azo-PCZ) were added as designated in the tables. The incubations were conducted in 10 ml centrifuge tubes at

37° for 30 min. The reaction was stopped by adding 5 ml of methylene chloride and centrifuged for three min in a clinical centrifuge at full speed. The aqueous layer was aspirated off and the methylene chloride layer assayed at 232 nm. The quantity of azo-PCZ formed was determined by use of the molar extinction coefficient of $1.47 \times 10^4\ M^{-1} cm^{-1}$. More than 90% of the azo-PCZ was extracted into the methylene chloride in this assay method.

Azo-PCZ formation was also followed by the conversion of azo-PCZ to N-isopropyl-p-formylbenzamide and MMH. Both MMH and N-isopropyl-p-formylbenzamide are rapidly formed (< five min) in nearly quantitative amounts by acid-treatment of azo-PCZ. Equation 6 shows the proposed chemical reaction. The MMH concentration



(6)

was determined by the method of Brown (1968) using the trinitrobenzenesulfonate derivative of MMH (Al Soudi, 1969). Brown's procedure was closely followed using MMH in place of an amino acid. The solutions containing MMH were treated with 2, 4, 6-trinitrobenzenesulfonic acid (one mmole) in buffered solution and the colored derivative determined spectrophotometrically at 484 nm.

The N-isopropyl-p-formylbenzamide was determined using the extinction coefficient at 252 nm. Both the Hoffmann-LaRoche N-isopropyl-p-formylbenzamide and the acid-treated azo-PCZ were found to have identical spectra and an extinction coefficient of ca. 1.75×10^4 $M^{-1} cm^{-1}$.

The methylene chloride assay mixture containing azo-PCZ was treated with 0.1 ml of 6 N HCl followed by addition of 5 ml of water. After 15 min, the water layer was removed by aspiration and the sample assayed at 252 nm. The N-isopropyl-p-formylbenzamide concentration was determined using the 252 nm extinction coefficient. At least 90% of the N-isopropyl-p-formylbenzamide remained in the methylene chloride of the assay mixture. See the Results section for typical assay values.

Trypsin Digestion

The decrease in enzyme activity with decreases in cytochrome P_{450} and b_5 concentration upon tryptic digestion of microsomes was investigated in the following manner. Microsomal suspensions were made ten percent (w/v) in trypsin and incubated at 37°. At 0, 5, 10, 20 and 30 min, 2 ml aliquots were added to trypsin inhibitor solutions (equimolar to trypsin) and centrifuged at 140,000g for one hour at 4°. The pellet was suspended in 2 ml of 0.05 M phosphate buffer, pH 7.5 and tested for enzyme activity, cytochrome b_5 content, and

cytochrome P_{450} content.

Determination of Cytochrome P_{450}

The method of Smuckler, Arrhenius and Hultin (1967) was used to determine P_{450} content. A 0.1 ml sample was added to 5.9 ml of 0.05 M phosphate buffer, pH 7.5, containing a small amount of solid sodium dithionite (amount not critical). The mixture was divided and a stream of CO bubbled through the sample cell prior to recording the spectrum. A difference spectrum was obtained from 380 to 500 nm on the Shamadzu spectrophotometer. The amount of P_{450} was determined from the difference E_{450}-E_{500} after gassing with CO and the increment extinction coefficient of 91 $mM^{-1} cm^{-1}$ (Imai and Sato, 1967). Values were expressed in mμmoles per mg of protein.

Determination of Cytochrome b_5

The method of Smuckler, Arrhenius and Hultin (1967) was also used to determine cytochrome b_5 content. A 1 ml microsomal suspension was treated with 0.3 ml of ten percent (w/v) sodium deoxycholate and 4.7 ml of 0.05 M phosphate buffer, pH 7.5. The suspension was divided into 3 ml portions and a small amount of solid sodium dithionite added to the sample cell to chemically reduce the cytochrome b_5. A difference spectrum from 410 to 427 nm was recorded and the difference E_{427}-E_{410} was used as an arbitrary measure of

cytochrome b_5. Values were expressed as $\Delta(E_{427}-E_{410})$ per mg of protein.

Chemical Oxidation of Hydrazines

The reaction mixtures consisted of alkylhydrazine, 20 μmoles; mercuric oxide, 200 mg and 0.05 M phosphate buffer, pH 7.5, in a total volume of 2 ml. The reaction was run at 25° with an air atmosphere in 10 ml Erlenmeyer flasks sealed with rubber serum stoppers. The gaseous atmosphere was sampled as usual.

Pretreatment of Animals with Inducing Agents

Rats which were pretreated with various compounds were injected intraperitoneally with the inducing agent. Phenobarbital (100 mg/kg body weight) and PCZ (200 mg/kg) in water were administered daily for three days prior to sacrifice. Corn oil containing 3-methylcholanthrene (30 mg/kg) was injected daily for two days prior to preparation of microsomes. The microsomal inhibitor SKF 525-A was injected one hour prior to slaughter in a dose of 50 mg/kg in water.

Equipment

Special equipment used was as follows: Beckman DB spectrophotometer, Beckman DU monochromator equipped with a Gilford

Instrument Laboratories, Inc., model 220 absorbance indicating photometer, Beckman IR-5 spectrometer, Beckman L2-50 ultracentrifuge (generously and graciously provided by Dr. TeMay Ching, Department of Farm Crops, Oregon State University), Beckman #65 fixed angle rotor, Beckman #65 rotor tubes and caps, F & M 810 gas chromatograph, International clinical centrifuge, Shimadzu MPS-50L scanning spectrometer (courtesy of Dr. M. Daniels, Department of Chemistry, Oregon State University) and a Sorvall RC-2 refrigerated centrifuge.

III. RESULTS AND DISCUSSION

In Vivo Metabolism of Alkylhydrazines

The results obtained by workers in our laboratory (Dost, Reed and Wang, 1966; Dost and Reed, 1967) prompted further in vivo studies with other alkylhydrazines. Table 6 presents the yields of respired propane and butane from intact rats administered by intraperitoneal injection of isopropyl- and butylhydrazine, respectively. The yields are between five and ten percent of the dose administered. These values are smaller than the amount of methane respired (20-25%) during MMH metabolism by rats (Dost, Reed and Wang, 1966). However, it would appear that these hydrazines are possibly metabolized in the same manner as MMH. Animals which convulsed released greater quantities of hydrocarbon but expired.

Table 6. In vivo formation of hydrocarbon from alkylhydrazines.

Alkylhydrazine	Dose	Hydrocarbon respired in 120 minutes	
	μmoles	Hydrocarbon	μmoles
Isopropylhydrazine	500	Propane	40
Isopropylhydrazine	200	Propane	17
Isopropylhydrazine	200	Propane	17
n-Butylhydrazine	300	n-Butane	8
n-Butylhydrazine	300	n-Butane	22

Experimental procedures are described in the Methods section.

In Vitro Metabolism of Alkylhydrazines

Species Distribution of Alkylhydrazine Metabolism

A brief species survey showed that guinea pig and rat liver were the most active in converting alkylhydrazines to alkanes (Table 7). However, bovine, pork, and sheep liver microsomes were also capable of the conversion at a smaller rate. The rates of metabolism were between 11-20 mμmoles hydrocarbon/120 min/10 mg microsomal protein.

Table 7. Species survey of alkylhydrazine oxidase activity in liver microsomes.

Species	Ethane formed in 120 min
	mμmoles
Bovine	11.0
Guinea pig	20.5
Porcine	13.5
Rat	17.4
Sheep	15.5

The assay employed a NADPH-regenerating system and ethylhydrazine as the substrate.

Intracellular Localization

In vitro experiments were conducted to investigate the site(s) and their nature which could account for hydrocarbon formation *in vivo* from alkylhydrazines. The organ chosen for investigation was

the liver since appreciable accumulation of hydrazines is known to occur in the liver (Pinkerton, Hagan and Black, 1967; Schwartz, Bollag and Obrecht, 1967). The enzyme activity was found to reside principally in the 140, 000g precipitate as seen in Table 8. This fraction is commonly known as the microsomes (Siekovitz, 1965). Shuster (1964) has reviewed the extensive metabolism of xenobiotic compounds by microsomes and it is not surprising that the conversion of alkylhydrazines to alkanes is specifically catalyzed by liver microsomal preparations.

Table 8. Intracellular distribution of enzymic activity in rat liver tissue.

Cellular fraction	Methane produced in 120 min per mg protein
	mμmoles
700g precipitate	0. 34
9, 000g precipitate	<0. 004
140, 000g precipitate	4. 7

The reaction mixture contained methylhydrazine, 140, 000g supernatant protein (for corresponding precipitate), 8 mg and 0. 05 M Tris buffer, pH 7. 5.

Alkylhydrazine Metabolism in Blood

As pointed out by Oliverio et al. (1964), PCZ can be converted rapidly to azo-PCZ in blood. The possibility of the hydrocarbon formation being due to reaction with blood components in liver prompted

an investigation in two areas. First, can blood convert alkylhydrazines to hydrocarbon and secondly, is this the reaction seen in liver tissue which has been washed free of blood? Table 9 shows that blood was indeed capable of converting alkylhydrazines to the corresponding hydrocarbon. The reaction seemed to occur predominantly with the 10,000g pellet (mostly erythrocytes). It should be noted that boiled 10,000g pellet gave considerable conversion as well. These results are in agreement with reports of early workers in the field who noted that phenylhydrazine reacted with oxyhemoglobin to yield benzene and nitrogen (Beaven and White, 1954; Nizet, 1946).

Table 9. The effect of blood on methane formation from MMH.

Sample	Methane formed in 120 min
	mμmoles
No protein	<0.1
Whole heparinized blood	13.6
Boiled whole heparinized blood	8.5
Blood serum (10,000g supernatant)	<0.1
Red blood cells (10,000g pellet)	19.8
Boiled red blood cells (10,000g pellet)	13.6

The samples consisted of 0.2 ml of the given blood fraction (see the Methods section).

The possibility of hemoglobin contamination accounting for the microsomal activity was ruled out by two criteria. Boiled microsomes were unable to convert MMH to methane (Table 10). In

contrast, boiled blood converted MMH to methane. Microsomes prepared in the standard way had the same activity as microsomes which were repeatedly washed and ultracentrifuged. If hemoglobin accounted for the methane production, the ability of microsomes to interact with alkylhydrazines to give alkanes should have decreased on repeated washing. Hemoglobin can be removed from microsomes by repeated washing (Omura and Sato, 1964).

Table 10. Methane formation from methylhydrazine by rat liver microsomes.

Reaction mixture treatment	Methane formed in 120 min
	mμmoles
Complete	7.5
Nitrogen atmosphere	<0.1
Boiling of microsomes	<0.1
Deletion of methylhydrazine	<0.1
Deletion of 140,000g supernatant	3.8
Boiling of 140,000g supernatant	5.3
Addition of $NADP^{\oplus}$	11.4
Addition of FAD	11.8
Addition of FMN	11.0
Deletion of 140,000g supernatant + addition of NADPH-regenerating system	21.1
Deletion of 140,000g supernatant + addition of NADPH	10.8
Deletion of 140,000g supernatant + addition of NADH	3.9

The complete reaction mixture consisted of 140,000g supernatant, 8 mg, instead of a NADPH-regenerating system, and Tris buffer. All added compounds were 10^{-3} M in concentration.

Cofactor and Oxygen Requirements

From Table 10, one can further characterize the reaction. The 140, 000g supernatant gave about two-fold enhancement and the enhancement was lowered only slightly by boiling. This suggests that some cofactor required for the reaction resides in the 140, 000g supernatant. A similar but larger enhancement was obtained when $NADP^{\oplus}$, FAD and FMN were added. Such a stimulation of activity implies that a NADPH-regenerating system might be required. Similar results led Mueller and Miller (1950) to discover the necessity of a NADPH-regenerating system for azoreductase, an enzyme which reductively cleaves azo dyes to the corresponding amines.

Indeed deletion of the 140, 000g supernatant and addition of the NADPH-regenerating system greatly facilitated the reaction (nearly three fold). The enzyme system also seemed specific for NADPH since added NADH did not stimulate while NADPH did cause some stimulation. The activity with NADPH was not as great as with the NADPH-regenerating system. Nevertheless, the reaction with NADPH was linear with concentrations up to 30 mM (Figure 3). In general, NADPH-regenerating systems are more effective in enhancing NADPH-dependent reactions than added NADPH (Gram and Fouts, 1968). One of the reasons for this difference is that a NADPH oxidase is present in the microsomes; NADPH may be metabolized so

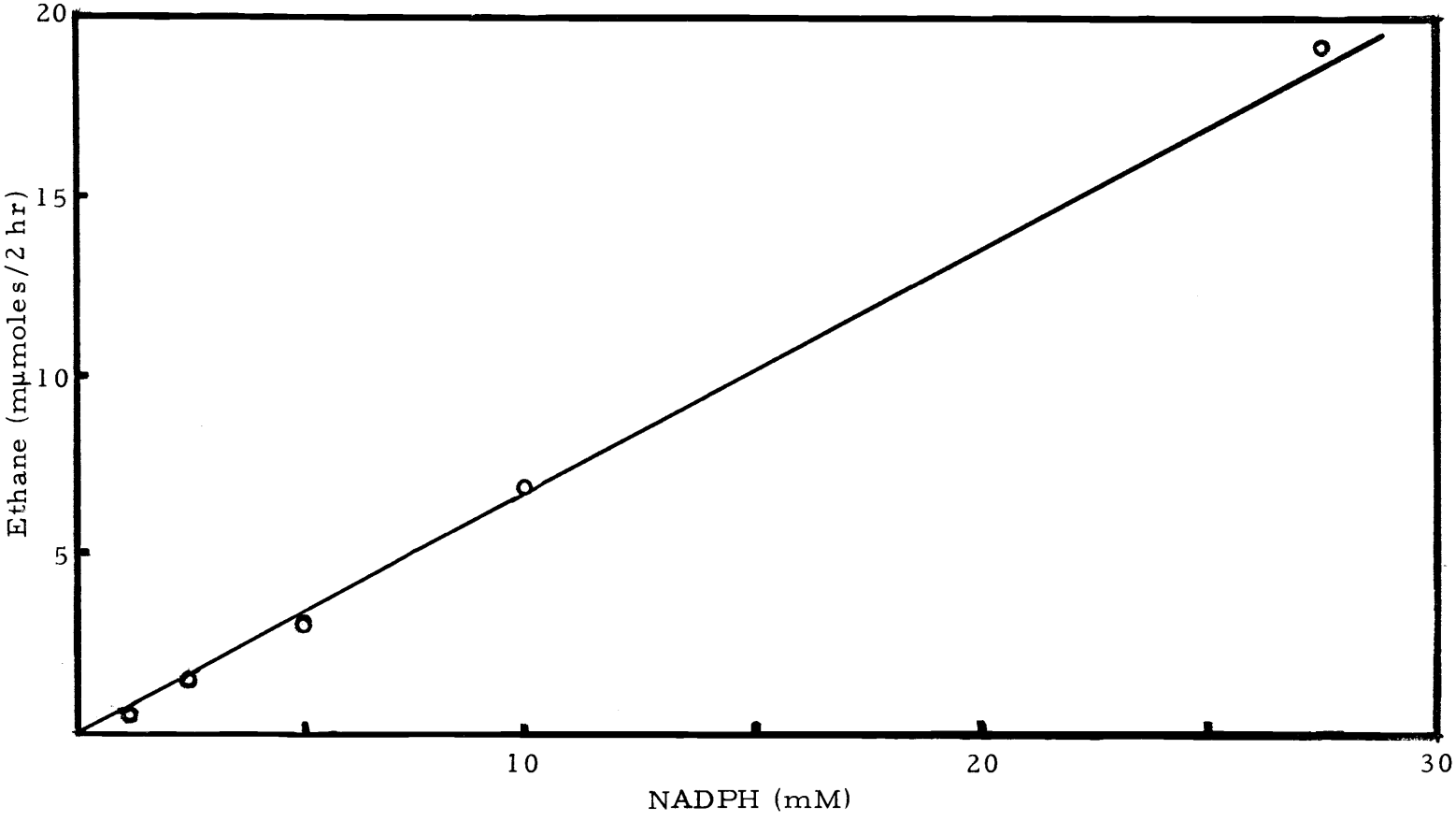


Figure 3. Effect of NADPH on alkylhydrazine oxidase activity in rat liver microsomes.

rapidly that it cannot react as efficiently with the alkylhydrazine substrates.

Although oxygen was required for enzyme activity (Table 10), the effect of oxygen concentration upon alkylhydrazine oxidase activity proved somewhat difficult to study. The enzyme system was not sensitive to decreased oxygen tension until less than one percent oxygen was present in the atmosphere of the reaction vessel (Figure 4). Using CO there was an inhibition (70%) but the activity curve was similar to that with nitrogen. This was not as striking with MMH as a substrate which suggests that a component similar to cytochrome P_{450} might be involved in hydrocarbon formation with substrates other than MMH. Cytochrome P_{450} was found to be one of the heme components which occur in microsomes. Its involvement in oxidative metabolism in the microsomes has been widely studied (Omura et al., 1965). They found that cytochrome P_{450} was found to be 50% inhibited with a CO/O_2 ratio of 0.5-1.0. Experiments employing various CO/O_2 ratios are shown in Table 11. Around 50% inhibition of methane formation from MMH occurred with a CO/O_2 ratio of 5 instead of 0.5 to 1.0. It is concluded that methylhydrazine may have other routes of metabolism which lead to methane formation due to the methyl group metabolism. This would be in agreement with the lower sensitivity of MMH conversion to methane by CO inhibition when compared to the formation of other hydrocarbons. MMH may simply

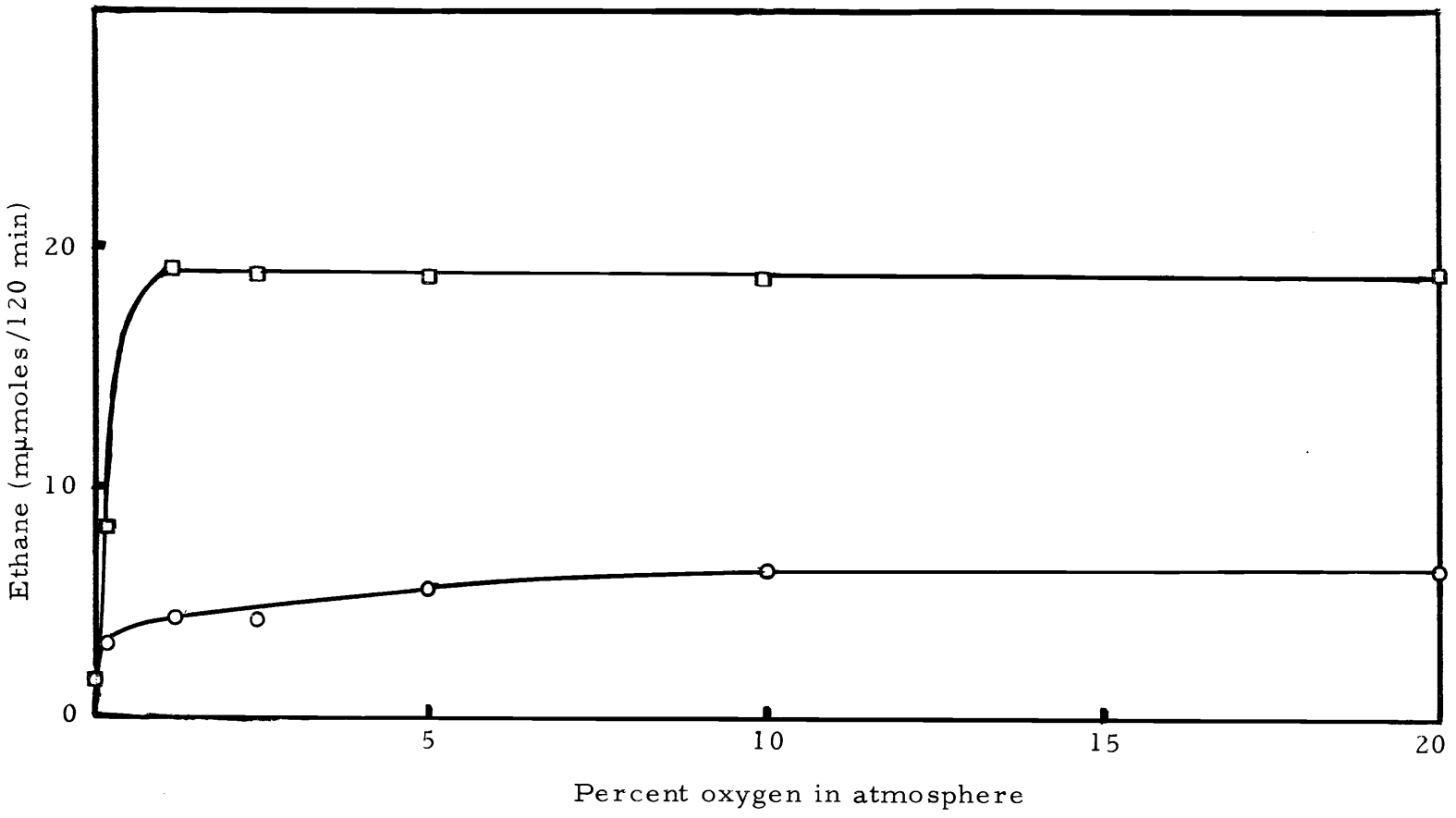


Figure 4. The effect of oxygen concentration on alkylhydrazine oxidase activity in the presence of nitrogen, □ ; or carbon monoxide, ○ . The assay employed a NADPH-regenerating system.

be a poorer substrate for the possible P_{450} dependent oxidation system. However, other paths of metabolism for MMH may be present without involving P_{450}. The possible role of P_{450} will be discussed later.

Table 11. The effect of CO/O_2 ratio on enzyme activity.

Substrate	CO/O_2	Hydrocarbon formed in 120 min	Inhibition
		mμmoles	%
MMH	0	15.0	0
	1	12.3	18
	2	10.1	33
	5	10.0	34
EH	0	6.7	0
	1	6.5	3
	2	6.5	3
	5	2.1	68
BH	0	21.9	0
	1	20.1	8
	2	22.1	0
	5	9.8	52

A NADPH-regenerating system was employed with the standard assay.

Other N-N Compounds as Substrates

In the presence of rat liver microsomes, all of the common alkylhydrazines tested were slowly converted to the corresponding alkanes (Table 12). Small but qualitative formation of ethylene, propylene and butylene was detected using EH, PH, IpH and BH, respectively. Of the other methylhydrazine derivatives only azo-PCZ,

azoxy-PCZ and methylazoxymethanol acetate (MAM) yielded methane (Table 13). However, in the case of azoxy-PCZ, boiled microsomes effectively converted azoxy-PCZ to methane. Procarbazine, UDMH and SDMH yielded no methane. Other methylhydrazine derivatives (diazald, DMNA and MTT) gave no methane. Only two research groups have noted hydrocarbon formation for alkyl- or arylhydrazines (Beaven and White, 1954; Dost, Reed and Wang, 1966; Dost and Reed, 1967). As far as this author knows, the other compounds assayed in Table 13 have not been previously examined for their conversion to hydrocarbon.

The requirement for oxygen and NADPH suggests that the enzyme may be a microsomal mixed-function oxidase (Mason, 1957) or monooxygenase (Hayaishi, 1964). These enzymes have the following stoichiometry as seen in Equation 7. As far as NADPH and O_2 requirements, the conversion of alkylhydrazine to alkane fulfills the stoichiometry if ROH is only a transient intermediate. Besides

$$RH + NADPH + H^{\oplus} + O_2 \rightarrow ROH + NADP^{\oplus} + H_2O \qquad (7)$$

the reaction requirements, the following data support the premise that the rate-controlling step in conversion of alkylhydrazine to the parent hydrocarbon is enzymic.

Table 12. Hydrocarbon formation from monoalkylhydrazines by rat liver microsomes.

Substrate	Hydrocarbon	Formed in 120 min
		mμmoles
Methylhydrazine	Methane	20.6
Ethylhydrazine	Ethane	17.4
n-Propylhydrazine	Propane	23.1
Isopropylhydrazine	Propane	23.1
n-Butylhydrazine	Butane	22.1

These experiments employed a NADPH-regenerating system in the assay medium. The above values are an average of three or more duplicate determinations.

Table 13. Effectiveness of other N-N compounds as substrates.

Reaction mixture treatment	Methane formed in 120 min
	mμmoles
MMH	18.2
MMH + boiled microsomes	0.9
Procarbazine	<0.1
Azoprocarbazine	15.4
Azoprocarbazine + boiled microsomes	10.2
Azoprocarbazine minus microsomes	0.8
Azoxyprocarbazine	19.2
Azoxyprocarbazine + boiled microsomes	19.4
Azoxyprocarbazine minus microsomes	3.1
MAM	2.0
MAM + boiled microsomes	0.8
Diazald	<0.1
DMNA	<0.1
MTT	<0.1
UDMH	<0.1
SDMH	<0.1

The reaction mixture for the assay employed a NADPH-regenerating system. All compounds were at a concentration of 2×10^{-2}M except MMH which was 10^{-3}M.

Kinetic Analysis of Alkylhydrazine Metabolism

A plot of enzyme velocity vs. substrate yields the typical hyperbolic plot expected from Michaelis-Menten enzyme kinetics. For the reaction,

$$E + S \underset{2}{\overset{1}{\rightleftarrows}} ES \xrightarrow{3} E + P,$$

an expression for the velocity of the reaction, v, or dP/dT, can be obtained where E is enzyme, S is substrate, ES is enzyme-substrate complex and P is product (Mahler and Cordes, 1966). The maximal velocity, V_m, is defined to be $k_3(E)$ and the Michaelis constant, K_m, is defined as $(k_2 + k_3)/k_1$.

$$v = \frac{V_m(S)}{K_m + (S)} \tag{8}$$

One can rearrange this to the double reciprocal form of Lineweaver and Burk (Equation 9). If one plots 1/v vs. 1/S, the slope and intercept can be used to determine V_m and K_m.

$$\frac{1}{v} = \frac{K_m}{V_m}\left(\frac{1}{S}\right) + \frac{1}{V_m} \tag{9}$$

If the velocity vs. substrate curve follows the Michaelis-Menten kinetics, the double reciprocal plot will yield a straight line.

Figure 5 presents the double reciprocal plot for ethyl- and n-butylhydrazine. The maximal velocities were found to be 32.0 and 32.2 mμmoles/120 min/10 mg protein for EH and BH, respectively. The Michaelis constants were found to be 4.5×10^{-5} M and 9.1×10^{-5} M for EH and BH. It should be noted that these values are for microsomal homogenates and not for purified enzyme.

Table 14 shows the linear response of enzyme activity to microsome concentration up to ca. 12.0 mg. Ten mg was chosen as the standard protein concentration per assay. The formation of hydrocarbon with time was also linear using both 140,000g supernatant and NADPH-regenerating system (Table 15).

Table 14. Effect of microsome concentration on hydrocarbon formation.

Microsomes	Ethane formed in 120 min
mg protein	mμmoles
4.0	6.1
8.0	13.5
12.0	19.1
16.0	22.0

The system employed 140,000g supernatant, ethylhydrazine and Tris buffer. Only microsomal protein concentration was varied.

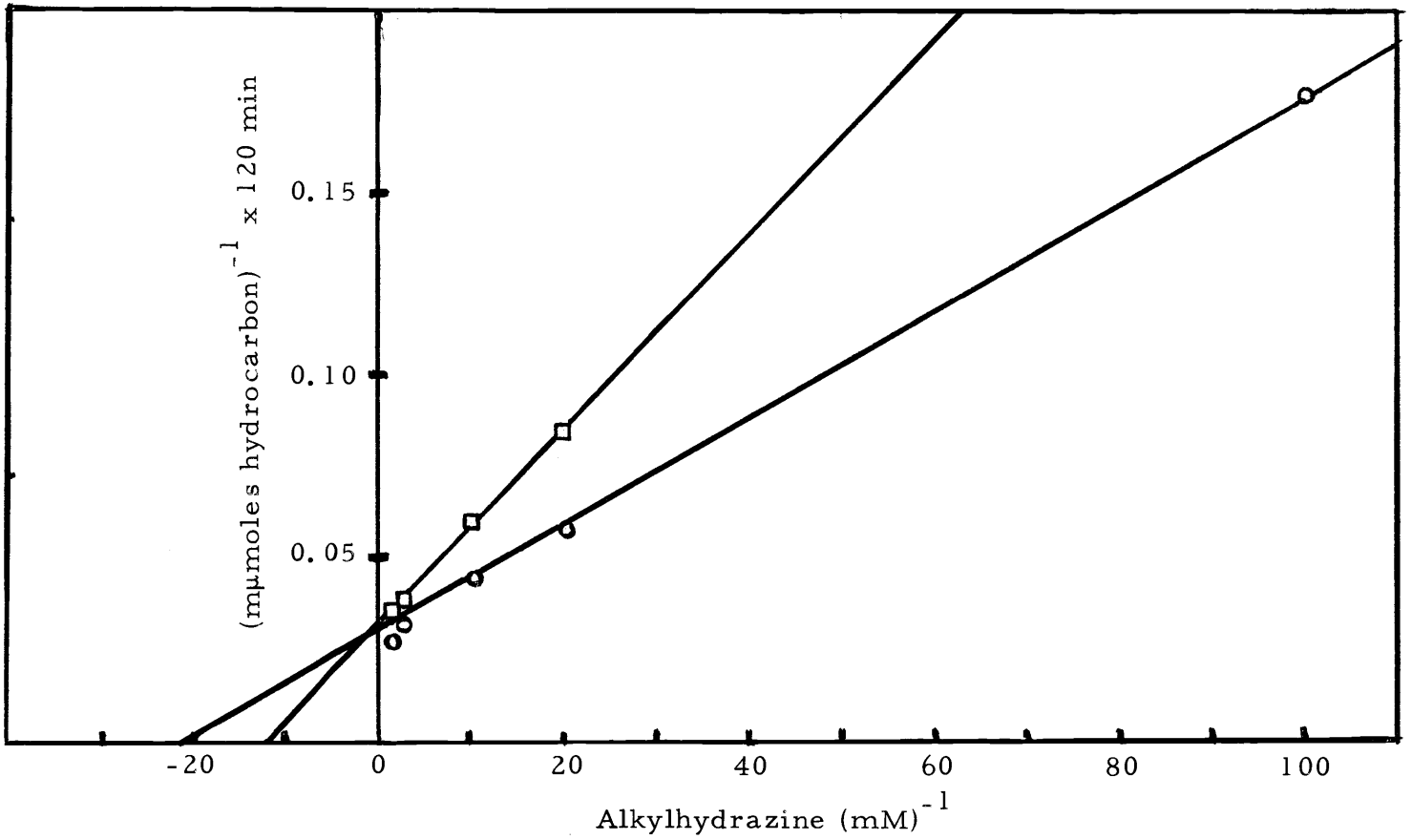


Figure 5. Lineweaver-Burk plot for alkylhydrazine oxidase activity in rat liver microsomes. The assays employed a NADPH-regenerating system, ethylhydrazine, O ; and butylhydrazine, □ .

Table 15. Effect of time on hydrocarbon formation.

Time (min)	Methane formed (mμmoles)		Ethane formed (mμmoles)	
	A	B	A	B
0	0.1	0.1	0.1	0.1
60	6.1	10.1	2.4	7.6
120	13.6	20.6	5.8	15.7
180	15.9			
240	22.5			

Columns A employed 140,000g supernatant, 8 mg and columns B employed a NADPH-regenerating system. The substrates were methyl- and ethylhydrazine.

Temperature Effects

Enzyme activity increased with temperature up to ca. 37° and then decreased above 37° (Figure 6). This is typical for many enzyme reactions (Dixon and Webb, 1964). The standard reaction temperature of 25° was chosen since the non-enzymic activity was found to be very low at 25°. It should be noted that the non-enzymic reaction increased markedly above 35°. This is thought to be due to the increased vapor phase concentration of MMH and the resulting vapor phase autoxidation of MMH.

The data presented thus far support the premise that liver microsomes catalyze the conversion of alkylhydrazines to the corresponding alkane. That the activity requires microsomal protein, O_2, and NADPH-regenerating system, is linear with time and protein, displays typical enzyme kinetics, is specific for NADPH and is

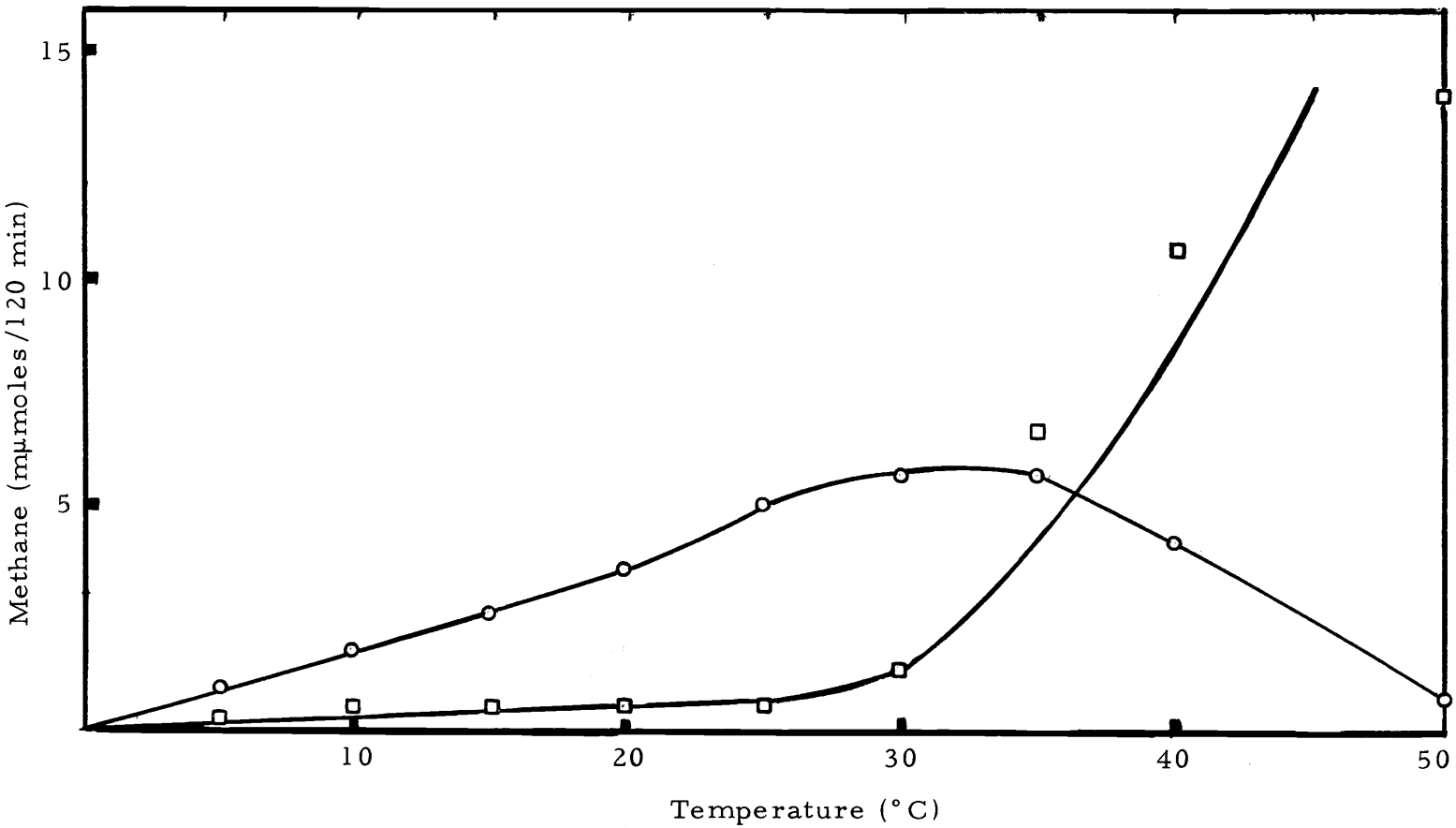


Figure 6. Effect of temperature on the enzymic conversion of methylhydrazine to methane. This experiment employed 140,000g supernatant and 0.05 M Tris buffer. Enzymic reaction, O ; non-enzymic reaction, □ .

temperature sensitive strongly indicate that the reaction is indeed enzymic. Throughout the remainder of this thesis the activity will be designated alkylhydrazine oxidase activity.

Effects of Common Enzyme Inhibitors

Some common enzyme inhibitors were utilized to further characterize the enzyme system (Table 16). Cyanide, a potent electron transport system inhibitor, had no effect on enzyme activity. Carbon monoxide (4:1 mixture with oxygen) did not significantly inhibit the reaction. Any contamination by electron transport components could be ruled out since these two compounds didn't inhibit the reaction and would do so if mitochondrial electron transport components were responsible for an appreciable portion of the observed activity. The involvement of a readily dissociable metal could be ruled out since EDTA did not effect enzyme activity. Compounds which react with sulfhydryl groups caused inhibition at low concentration. Around 50% inhibition occurred when 10^{-6} M pCMB or 10^{-5} M NEM was added to the reaction mixture. This suggests the involvement of a sulfhydryl group in the reaction (Mahler and Cordes, 1966). The common microsomal inhibitor SKF 525-A had no effect on activity. This compound has been extensively used as a non-specific inhibitor of drug metabolism (Brodie, Gillette and LaDu, 1958; Rogers and Fouts, 1964). Some of the drug-metabolizing enzymes of liver are not

inhibited, mainly those involved in intermediary metabolism.

Table 16. The effect of common enzyme inhibitors on alkylhydrazine oxidase activity.

Addition	Methane formed in 120 min
	mμmoles
None	17.1
KCN (10^{-3}M)	17.0
CO/O_2 atmosphere (4:1 v/v)	16.5
EDTA (10^{-3}M)	17.1
pCMB (10^{-6}M)	6.4
NEM (10^{-5}M)	11.0
SKF 525-A (10^{-4}M)	16.5
Dithiothreitol (10^{-3}M)	14.8
2-Mercaptoethanol (10^{-3}M)	7.0
L-Cysteine (10^{-3}M)	11.0

The reaction was conducted with a NADPH-regenerating system and methylhydrazine as substrate.

Free radical scavengers such as dithiothreitol, 2-mercaptoethanol and L-cysteine at a concentration of 10^{-3}M caused only some 50% inhibition. If the solvent cage effect of the proposed free radical intermediate did play a large part in the reaction, the reaction may not be affected by sulfhydryl containing compounds (Pryor, 1966). However, with an enzyme, the solvent cage effect might play a large role and the reaction may be relatively insensitive to radical scavengers. No attempt was made to investigate the possible carbanion

mechanism discussed in the Introduction (Cram and Bradshaw, 1963).

Effect of Pretreatment with Inducing Agents

The possibility that the alkylhydrazine oxidase activity might be related in some manner to cytochrome b_5 or P_{450} required further investigation. Induction experiments were performed to change P_{450} levels and measurements of the alkylhydrazine oxidase activity were made (Table 17). Phenobarbital (100 mg/kg for three days) and 3-methylcholanthrene (30 mg/kg for two days) have been shown to increase P_{450} content and some microsomal enzymes activity (Conney, 1967). One can see in Table 17 that increases in P_{450} content due to phenobarbital and 3-methylcholanthrene induction did not correlate with enzyme activity. Since no increase in alkylhydrazine oxidase activity is seen with increases in P_{450} content, it seems unlikely that alkylhydrazine oxidase is a P_{450}-dependent enzyme.

Procarbazine was shown to decrease protein synthesis (Koblet and Diggelmann, 1968). Liver microsomes from PCZ-treated rats (200 mg/kg for three days) had reduced levels of P_{450} but the level of alkylhydrazine oxidase activity remained unchanged. The inhibitor SKF 525-A had no effect on either P_{450} content or alkylhydrazine oxidase activity. Wittkop (1969) noted that pretreatment with SKF 525-A caused no change in P_{450} content.

Table 17. The effect of in vivo induction by pretreatment of rats with various compounds on alkylhydrazine oxidase activity.

Pretreatment compound	P_{450}	Ethane formed in 120 min
	mμmoles/mg	mμmoles
None	0.65	17.5
Phenobarbital	1.45	17.3
3-Methylcholanthrene	0.85	17.4
Procarbazine	0.46	17.7
SKF 525-A	(0.65)	17.5

The reaction was conducted with a NADPH-regenerating system and ethylhydrazine as the substrate (see Methods section). The P_{450} value for SKF 525-A pretreated rats was found not to change in the one hour in vivo incubation; however, other microsomal enzymes have been reported to be inhibited by such treatment (Rogers and Fouts, 1964).

Effects of Detergent Solubilization and Proteolytic Digestion

To further eliminate cytochrome P_{450} involvement, enzyme activity and P_{450} content were followed during a tryptic digest (see Methods section). Figure 7 shows the effect of tryptic digestion on enzyme activity, cytochrome P_{450}, and cytochrome b_5. For graph A (control microsomes), the values for 100% original activity were as follows: Enzyme activity, 32 mμmoles/120 min/10 mg protein; P_{450}, 0.46 mμmoles/mg protein and b_5, 0.006 OD units/mg protein. For graph B (phenobarbital-induced microsomes), the 100% original activity values were as follows: Enzyme activity, 34 mμmoles/120 min/10 mg protein; P_{450}, 0.84 mμmoles/mg protein and b_5,

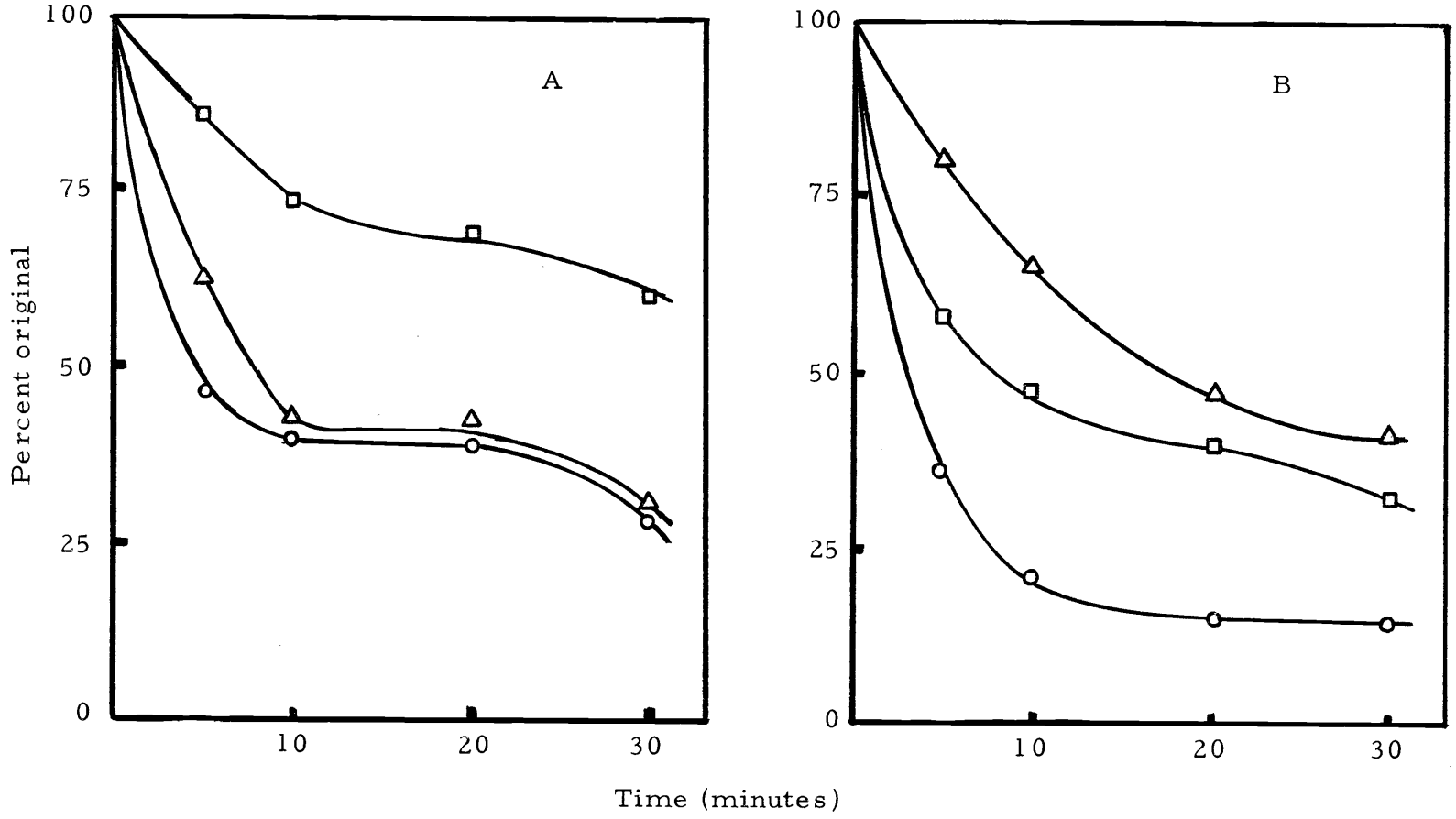


Figure 7. Variation of enzymic activity, O ; cytochrome b_5, Δ ; and cytochrome P_{450}, □ ; with tryptic digestion. Graph A is control microsomes and graph B is microsomes with phenobarbital induction. The reaction mixture included ethylhydrazine as the substrate and a NADPH-regenerating system. (See text for original values.)

0. 009 OD units mg protein. No correlation was seen in graphs A or B between enzyme activity and P_{450} content.

In control microsomes (graph A), cytochrome b_5 content seemed to correspond closely with alkylhydrazine oxidase activity during proteolytic digestion. However, on phenobarbital induction, the same correspondence was not observed. The involvement of cytochrome b_5 was further ruled out by a cytochrome b_5 solubilization experiment (Table 18). The method of solubilization was that of Omura and Sato (1963). While only 32% of the cytochrome b_5 remained in the lipase-treated pellet, 77% of the remaining alkylhydrazine oxidase activity resided in the lipase pellet.

Table 18. Effect of cytochrome b_5 solubilization on hydrocarbon formation.

Sample	Cytochrome b_5	Total protein	Total b_5	Ethane formed in 120 min
	mμmoles/mg	mg	μmoles	mμmoles
Microsomes	15. 3	130. 0	1. 99	11. 0
Lipase supernatant	13. 3	71. 0	0. 94	2. 0
Lipase pellet	9. 7	66. 5	0. 65	8. 5

The microsomes were solubilized with 0. 14% steapsin (pancreatic lipase) and incubated at 4°C for one hour. The sample was centrifuged at 140, 000g for one hour and resuspended in 0. 05 M phosphate buffer. The enzyme assay employed a NADPH-regenerating system and ethylhydrazine as the substrate.

From the preceding information, cytochromes P_{450} and b_5 appeared not to be involved directly in the alkylhydrazine oxidase reaction. The lack of correlation between lose of enzyme activity and cytochrome content during tryptic digestion, lack of induction by phenobarbital and 3-methylcholanthrene and lack of b_5 content with activity on lipase solubilization indicate strongly that cytochrome P_{450} and b_5 are not required for alkylhydrazine oxidase activity.

Partial Purification of Alkylhydrazine Oxidase

Attempts to isolate and purify the alkylhydrazine oxidase gave only marginal results (Table 19). Although a four-step purification resulted in 99.6% loss of the protein, only a 21-fold purification of enzyme activity resulted (Table 19). In addition, alkylhydrazine oxidase activity was totally lost during storage for 48 hours at 5° after the column step. Several storage conditions were tried and none gave retention of enzyme activity beyond 100 hours. This is unlike the purification of azoreductase which gave a preparation which was stable for six months at -60° (Hernandez, Gillette and Mazel, 1967). Part of the difficulties may possibly be due to an absolute necessity of membrane integrity.

Enzymic Formation of Azo-PCZ

PCZ was found to be metabolized in part to methane *in vivo*

Table 19. Isolation and partial purification of alkylhydrazine oxidase.

Treatment	Total protein		Total activity		Specific activity	Purification
	Amount	Recovery	Amount	Recovery		
	mg	%	mμmoles/2hr	%	mμmoles ethane/120 min/mg	Fold
1. Original microsomes	396	100	1571	100	3.97	1.0
2. Triton X-102, X-45 supernatant	144	36	648	41	4.50	1.1
3. 40% $(NH_4)_2SO_4$ precipitate	65	16	455	29	7.00	1.7
4. DEAE-cellulose column	1.5	0.4	131	8	86.0	21.0

The assay employed a NADPH-regenerating system and ethylhydrazine as the substrate. For detail see Methods section.

possibly by the formation of azo-PCZ (Dost and Reed, 1967). Such a conversion may involve an enzyme system which could convert alkylhydrazines to the corresponding alkanes. Since diazenes have been shown to exist, the azo-PCZ might be a similar intermediate in these reactions. An enzyme assay was designed to observe the conversion of PCZ to azo-PCZ (see Methods section). The formation of azo-PCZ was followed directly (spectrophotometric assay for azo-PCZ) and indirectly (spectrophotometric assay for the N-isopropyl-p-formylbenzamide, one of the products formed by acid-treatment of azo-PCZ).

Although considerable azo-PCZ formation occurred in the presence of a suspension of boiled microsomes which was saturated with air, an appreciable enzymic reaction was seen with use of a NADPH-regenerating system (Table 20). Very little enzymic activity was noted without the NADPH-regenerating system. The requirement of reducing equivalents to form the oxidized product is similar to the alkylhydrazine oxidase activity reported here and other oxidative enzyme systems (Mason, 1957).

Chemical Oxidation of Alkylhydrazines

Alkylhydrazines were reacted with mercuric oxide to determine the nature of the reaction and to make a comparison of products with those of the alkylhydrazine oxidase reaction. Small amounts of

methane and propane were formed from the oxidation of MMH and PH, respectively (Table 21). No hydrocarbon was evolved from UDMH and SDMH as is expected (see discussion in Introduction). The other alkylhydrazines yielded the corresponding alkene in relatively large quantities.

The oxidation of substituted hydrazines as discussed in the Introduction section points strongly to one type of intermediate; diazenes. It is conceivable that the alkylhydrazine oxidase reaction might involve a diazene intermediate. The NADPH-dependent formation of azo-PCZ would support this view since azo compounds are actually diazene analogues. It is well known that disubstituted diazenes (azo compounds) are fairly stable while monosubstituted diazenes are unstable (Huang and Kosower, 1968d; Smith, 1966). Phenyldiazene was found to have a short half-life in the presence of oxygen and formed benzene. The possible alkyldiazenes would be even less stable. One alkyldiazene, t-butyldiazene, was studied by Huang and Kosower (1967b) and was noted to be much more unstable than phenyldiazene. Balandin and Vaskevitch (1936) have reported the short half-life of cyclohexyldiazene. Oxygenated intermediates such as azoxyalkanes may exist. Several azoxy compounds occur in nature as noted in Table 3. However, no real attempt was made to isolate such intermediates since only mμmoles of hydrocarbon product are formed in the reactions.

Table 20. In vitro formation of azoprocarbazine from Procarbazine.

Sample	Azo-PCZ	N-isopropyl-p-formylbenzamide
	mμmoles/30 min	mμmoles/30 min
PCZ	237	243
Microsomes	<1	<1
Microsomes and PCZ	1175	830
Boiled microsomes and PCZ	1140	920
Microsomes, PCZ and NADPH-regenerating system	1312	1035
Boiled microsomes, PCZ and NADPH-regenerating system	1030	750

The reaction mixture was as described in the Methods section. Microsomes, a NADPH- regenerating system and Procarbazine were added as designated.

Table 21. The chemical oxidation of various alkylhydrazines.

Substrate (20 μmoles)	Hydrocarbon evolved	mμmoles Hydrocarbon formed		
		2.5 min	5 min	10 min
MMH	Methane	1.70	2.60	4.80
UDMH	None			
SDMH	None			
EH	Ethylene	84.6	272	828
PH	Propane	8.9	42.4	348
	Propylene	271	534	3090
IpH	Propylene	1480	4070	4960
BH	Butylene	88.9	1190	3450

See the Methods section for conditions.

Although the actual mechanism of action of PCZ as an antitumor agent can not be pin-pointed, it seems possible that the diazene intermediate possibly generated in the alkylhydrazine oxidase system might be able to act as an alkylating agent. Either a free radical or ionic species which could be formed from diazenes would act as alkylating agents.

IV. SUMMARY

This thesis has demonstrated that an enzymic process exists which can convert alkylhydrazines to the corresponding alkanes. This enzyme predominantly occurs in the liver microsomes of several animal species. NADPH and O_2 are required for enzyme activity and a NADPH-regenerating system gave maximal activity. The reaction occurred in blood but appeared to be due to interaction with erythrocytes. The reaction in liver is not due to erythrocyte contamination.

Methyl-, ethyl-, n-propyl-, isopropyl- and n-butylhydrazine all gave the corresponding hydrocarbon in this enzyme system. As judged by normal criteria for enzymic reactions, this system was enzymic at least during the rate-determining step. Several azo and azoxy compounds, (azo-PCZ, azoxy-PCZ and MAM), served as substrates to alkylhydrazine oxidase and evolved methane as a product.

The system was not greatly inhibited by electron transport inhibitors, heavy metal chelators, sulfhydryl reagents and common microsomal mixed-function oxidase inhibitors. Cytochrome P_{450} and cytochrome b_5 were not involved as judged by a variety of experiments. Only a limited purification was performed due to the complexity of the microsomal protein.

Microsomes converted PCZ to azo-PCZ which in turn can be

slowly degraded in vivo to yield some methane from the N-methyl group. The corresponding alkenes were obtained by the chemical oxidation of alkylhydrazines. The relation of these reactions to the alkylhydrazine oxidase reaction was discussed.

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