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Metabolism, pharmacological disposition, and modes of action of the antineoplastic agent procarbazine, N-isopropyl-[alpha]-(2- methylhydrazino)-p- toluamide

Author

Shiba, David Alan

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METABOLISM, PHARMACOLOGICAL DISPOSITION, AND MODES OF ACTION OF THE ANTINEOPLASTIC AGENT PROCARBAZINE, N- ISOPROPYL-α-(2- METHYLHYDRAZINO) -p- TOLUAMIDE

by

David Alan Shiba

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DISSERTATION

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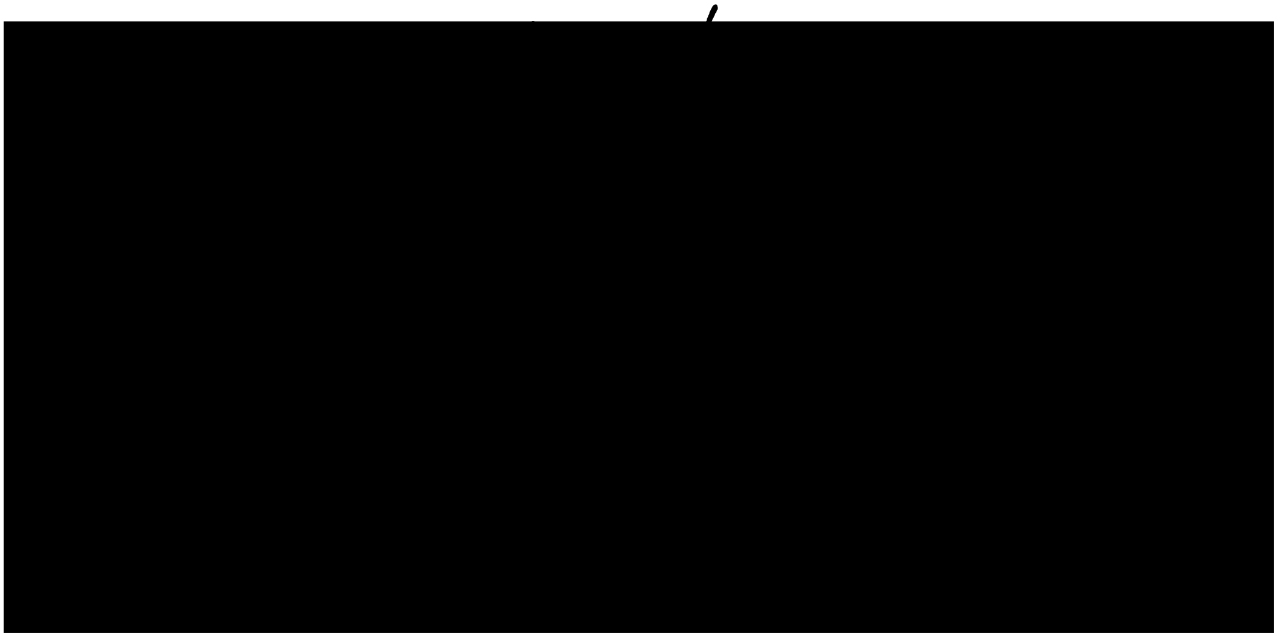

In memory of my grandfather, Aunt Ruby, Uncle Fred, wife's grandmother, and my Boy Scout leader, Mr. Greene. Each has played a major role in my life and development as a person and each lost their battle against cancer. Their memory will always be the driving force behind my intensive pursuit of improved medical treatment and prevention of this debilitating disease.

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Abstract

Procarbazine chemical degradation, *in vitro* and *in vivo* metabolism, antineoplastic activity including the activity of several metabolites, and the effects of pretreatment with Procarbazine and several other drugs upon Procarbazine antineoplastic activity were investigated.

The chemical degradation and metabolism were quantitated using an assay procedure of a combination of direct-probe insertion selected-ion monitoring chemical ionization mass spectrometry and reverse-phase high-performance liquid chromatography. The mass spectrometric internal standards N-isopropyl ($^2H_6$)-α-(2-methylhydrazino)-*p*-toluamide (Procarbazine-d_6) and N-isopropyl ($^2H_6$) terephthalamic acid (acid-d_6) were synthesized. The internal standard for the liquid chromatographic assay was 4-methylacetophenone. Procarbazine chemical degradation and metabolic products including N-isopropyl-α-(2-methylazo)-*p*-toluamide (azo), N-isopropyl-α-(2-methyl-NNO and ONN-azoxy)-*p*-toluamide (benzyl and methyl-azoxy isomers), N-isopropyl-α-(2-methylhydrazone)-*p*-toluamide (hydrazone), N-isopropyl-*p*-toluamide (aldehyde), N-isopropylterephthalamic acid (acid), N-isopropyl-*p*-hydroxymethylbenzamide (alcohol), and N-isopropyltoluamide (methyl) were synthesized and used as standard references.

Procarbazine was rapidly chemically decomposed in buffer solution in the presence of oxygen. No decomposition occurred under nitrogen atmosphere. Procarbazine was rapidly oxidized to the azo derivative, half-life of 12 minutes. The isomerization to hydrazone and its subsequent hydrolysis to the aldehyde was slow. The oxidation of the aldehyde to the acid derivative was very slow. The chemical reaction products of Procarbazine probably play only a minor role in its *in vivo* antineoplastic activity.

In vitro Procarbazine metabolism was investigated using rat liver homogenate fractions. The metabolism required microsomal enzymes contained in either the 9,000 x g supernatant or 100,000 x g microsomal fractions. The kinetics of the *in vitro* Procarbazine disappearance was similar to the chemical decomposition kinetics, half-life of 7 minutes. The azo was slowly stereoselectively metabolized to methyl and benzyl-azoxy isomers. After 10 minutes, the methylazoxy isomer was present at a 9-fold higher concentration. The further metabolism of the azoxy isomers was also stereoselective. The benzylazoxy isomer was metabolized 1.4 times faster. The further metabolism of azoxy proceeds at approximately the same rate as the metabolism of azo to azoxy. The azoxy compounds were hydroxylated in a manner which may be analogous to 1,2-dimethylhydrazine. This metabolic pathway is further supported by data obtained from other *in vitro* investigations including the metabolism of each intermediate, the metabolism of a mixture of Procarbazine and Procarbazine-d_6, and a kinetic isotope study of a mixture of Procarbazine and Procarbazine-d_1 (N-isopropyl-α-(2-methylhydrazino)-*p*-toluamide ($^2H_1$)).

In vivo Procarbazine metabolism was studied in rat, mouse, and man. The *in vivo* rat metabolism was similar to the *in vitro* metabolism. An additional metabolic pathway was observed *in vivo*. This suggests that Procarbazine may be converted to free radical intermediates that decompose to give methane and N-isopropyltoluamide. Phenobarbital pretreatment induced Procarbazine metabolism. Disulfiram partially inhibited the metabolism of azo to azoxy and, possibly, further metabolism of azoxy. Metabolism in mice was similar to that observed in rat.

In vivo Procarbazine antineoplastic activity was evaluated in a L-1210 ascites-bearing CDF_1 mouse assay. Procarbazine and azo were

equally active. A 1.75:1 mixture of methyl:benzylazoxy was 38% more active than either Procarbazine or azo.

Phenobarbital and diphenylhydantoin pretreatment induced microsomal cytochrome P-450 content, and, correspondingly, increased Procarbazine antineoplastic activity. Methylprednisolone had no effect upon activity. Procarbazine pretreatment produced no clear correlation between pretreatment, cytochrome P-450 content, and activity. Procarbazine pretreatment produced no irreversible inhibition of cytochrome P-450 content. Disulfiram pretreatment, while inhibiting Procarbazine metabolism in vitro, did not significantly alter Procarbazine activity. Disulfiram did increase Procarbazine toxicity.

The metabolism of Procarbazine is similar to that proposed for 1,2-dimethylhydrazine. The hydroxylated methylazoxy compound is structurally related to methylazoxy methanol, which is known to transfer its intact methyl group. The in vitro metabolism of the analog benzyl-NNO-azoxymethane under conditions to prevent reduction of the aldehyde to alcohol produced benzyl alcohol. This suggests that a benzylating agent might also be produced from Procarbazine.

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MOLECULAR FORMULA	CHEMICAL NAME	ABBREVIATED NAME
$CH_3NHNHCH_2C_6H_4CONHCH(CH_3)_2$	N-Isopropyl-α-(2-methyl-hydrazino)-P-toluamide	Procarbazine
$CH_3NHNHCHDC_6H_4CONHCH(CH_3)_2$		Procarbazine-d_1
$CH_3NHNHCH_2C_6H_4CONHCH(CD_3)_2$		Procarbazine-d_6
$CH_3N{=}NCH_2C_6H_4CONHCH(CH_3)_2$	N-Isopropyl-α-(2-methyl-azo)-P-toluamide	Azo
$CH_3N{=}NOCH_2C_6H_4CONHCH(CH_3)_2$	N-Isopropyl-α-(2-methyl-NNO-azoxy)-p-toluamide	Benzylazoxy
$CH_3NO{=}NCH_2C_6H_4CONHCH(CH_3)_2$	N-Isopropyl-α-(2-methyl-ONN-azoxy)-P-toluamide	Methylazoxy
$OHCC_6H_4CONHCH(CH_3)_2$	N-Isopropyl-p-toluamide	Aldehyde
$CH_3NHN{=}CHC_6H_4CONHCH(CH_3)_2$	N-Isopropyl-α-(2-methyl-hydrazone)-p-toluamide	Hydrazone
$CH_3C_6H_4CONHCH(CH_3)_2$	N-Isopropyl-p-toluamide	Methyl
$HOCH_2C_6H_4CONHCH(CH_3)_2$	N-Isopropyl-p-hydroxy-methylbenzamide	Alcohol
$HO_2CC_6H_4CONHCH(CH_3)_2$	N-Isopropyltereph-thalamic acid	Acid
$HO_2CC_6H_4CONHCH(CD_3)_2$		Acid-d_6

Introduction

Procarbazine·HCl, N-isopropyl-α-(2-methylhydrazino)-p-toluamide hydrochloride, Figure 1, is one of over 200 substituted hydrazine and hydrazide compounds synthesized by Zeller, et al.[1] at the Hoffmann-La Roche laboratories in 1963.

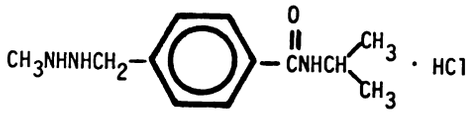


Figure 1. Procarbazine·HCl, N-isopropyl-α-(2-methylhydrazino)-p-toluamide hydrochloride.

These compounds were synthesized and tested as potential monamine oxidase inhibitors. During this testing, Bollag and Grunberg[2] discovered that 1-methyl-2-benzylhydrazine had pronounced antitumor activity against several transplantable tumors in mice and rats. Since 1-methyl-2-benzylhydrazine damaged the liver and had a low therapeutic index, other hydrazine derivatives were tested. Approximately 40 hydrazine derivatives were found to have antineoplastic activity against the rodent tumors. The active compounds had the general structure of R-NH-NH-CH_3. The most effective antineoplastic agents had a substituted benzyl group as the R group. The methyl group was an absolute requirement for activity since desmethyl and N-ethyl hydrazines were inactive.[3] Of the 40 compounds, Procarbazine·HCl was one of the compounds chosen for further screening. Procarbazine·HCl was the least toxic and was studied further.

Procarbazine was first used clinically by Martz and his co-workers[4] in Switzerland in 1963. Significant response was seen in 16 of 17 patients with malignant lymphoma. Therapeutic failures occurred in 14 patients with solid tumors. During the period of 1963 to 1969, Procarbazine was used in clinical trials in the United States. Procarbazine was very effective in the treatment of Hodgkin's disease. In 480 evaluatable patients, 57% obtained objective and subjective positive response to Procarbazine chemotherapy.[5] In all other malignancies, Procarbazine resulted in only a 15% response rate. More importantly, since 95% of the Hodgkin's disease patients were refractory to other forms of therapy, and therefore it was important that Procarbazine was not cross-resistant to other therapeutic agents. In 1969, Procarbazine was approved for noninvestigational clinical use in the treatment of Hodgkin's disease and in other cancer patients who have become resistant to other forms of therapy. Procarbazine is primarily useful in combination regimens such as nitrogen mustard, vincristine (Oncovin), and prednisone (MOPP) and in secondary and tertiary single agent therapy of Hodgkin's disease.[4,6]

Recently, Procarbazine has been shown to be useful in the treatment of brain tumors. As a single agent in the treatment of patients with recurrent brain tumors, Procarbazine produces a 50% response rate for a median duration of 6 months.[7] Procarbazine in combination with vincristine and CCNU (1-(2-chloroethyl)-2-cyclohexyl-1-nitrosourea) achieved a 60% response rate for a median duration of 9 months in patients with recurrent malignant gliomas.[8]

Procarbazine has many toxic side effects which are similar to other antineoplastic agents. Reversible leukopenia and thrombocytopenia occur in 50-70% of all treated patients.[6f] Nausea

and vomiting frequently occur after initial treatment, but subside with continued use. Procarbazine can also cause central nervous system toxic effects that can be eliminated if the dose is kept below 300 mg/day for adult patients.[5] Disorders such as somnolence, depression, agitation, and psychosis occur in up to 31% of the patients.[9] These disorders can be partially due to the weak monamine oxidase inhibitory properties of Procarbazine[10] or decreased levels of pyridoxal phosphate.[11] Other side effects occur in less than 10% of the patients. None of the side effects are serious and all are reversible when drug treatment ends.[5]

Procarbazine is also carcinogenic. Kelly and co-workers[12] reported that mice receiving Procarbazine developed pulmonary tumors and/or leukemia. Rats receiving Procarbazine had a high incidence of mammary adenosarcomas. Recently, long term Procarbazine administration lasting up to 12 years produced acute myolongenous leukemia, osteogenic sarcomas, lymphocytic leukemia, and hemangiosarcomas in several species of monkeys.[13] Hansch[14] found that Procarbazine is mutagenic in the Salmonella typhimurium assay for mutagenic activity in the presence of the S-9 fraction. Procarbazine at a concentration of 2.5 uM produces 30 revertants above background; the equipotent concentration of Aflatoxin-B is 0.0003 uM.

Although Procarbazine has been extensively studied and is used in the clinical treatment of several tumors in man, little is known about its bioactivation and mode of action. Procarbazine metabolism has been intensely investigated, but most of the emphasis has been on the metabolic fate of the N-methyl group. Several ultimate metabolic products including carbon dioxide, methane, and the major human urinary metabolite, N-isopropylterephthalamic acid, have been isolated and identified. However, little is known about the metabolic intermediates

and pathways leading to their production. More importantly, the pathways leading to potential cytotoxic species remain unclear.

The interests in our laboratory and the work described in this dissertation focus upon the relative roles of the chemical degradation and metabolism of Procarbazine in the activation of Procarbazine.

The analytical methods of selected-ion monitoring chemical ionization mass spectrometry and reverse-phase high-performance liquid chromatography are developed to provide sensitive assays to quantitate the chemical degradation and metabolic products. Procarbazine-d_6 and acid-d_6 (Figure i) are synthesized for use as internal standards in the mass spectrometric assay. The azo, aldehyde, hydrazone, methyl, alcohol, and mixture of methyl and benzylazoxy compounds (Figure i) are synthesized for use as reference compounds for the identification and quantitation of the major chemical degradation and metabolic products of Procarbazine by the liquid chromatographic assay.

The chemical degradation of Procarbazine is initially investigated since the proposed pathway for Procarbazine metabolism is identical to the chemical degradation pathway. No previous attempts to quantitate this pathway had been made and knowledge of the kinetics of each chemical degradation reaction will enable the evaluation of the role of chemical reactions in the activation and metabolism of Procarbazine.

The *in vitro* and *in vivo* Procarbazine metabolism is studied to identify the metabolic intermediates between Procarbazine and the major *in vivo* urinary metabolite, N-isopropylterephthalamic acid (Figure i). Since previous studies of Procarbazine metabolism have primarily focused upon the metabolic fate of the methyl group, the metabolism of the remainder of the Procarbazine molecule is studied. These studies might

help elucidate the metabolic pathway(s) leading to the production of cytotoxic agents. Initially, the in vitro metabolism is studied in rat liver homogenate fractions. This eliminates the complexities associated with in vivo metabolic studies. The 9,000 x g and 100,000 x g rat liver supernatant fractions and 100,000 x g microsomal fraction are used to determine which metabolic reactions are catalyzed by soluble and which by membrane-bound enzyme systems. The metabolism of a Procarbazine-d_6 and Procarbazine mixture (Figure i) is analyzed to show that each metabolite observed arises from Procarbazine. Each metabolic and chemical degradation intermediate is further metabolized to determine the sequence of the metabolic pathway. The kinetic isotope effects during the metabolism of a mixture of Procarbazine and Procarbazine-d_1 (Figure i) is analyzed to determine which metabolic steps involve hydroxylation at the benzylic carbon. The in vitro kinetics is studied to evaluate the relative contributions of chemical reactions to the in vitro metabolism. The in vitro metabolism of benzyl-NNO-azoxymethane is analyzed for the production of benzyl alcohol resulting from the production of a benzylating agent.

The in vivo metabolism is studied in rat, CDF_1 mouse, and man. A comparison of the in vivo and in vitro metabolism kinetics in rat should help evaluate the extent of the metabolism occurring in the liver with its microsomal enzyme system and the metabolism catalyzed by other enzymes in the intact animal. The metabolism in the mouse is studied since the mouse is used in the animal tumor model to evaluate the anti-neoplastic activity of Procarbazine and its metabolic and chemical degradation products. The role of the cytochrome P-450 liver microsomal enzymes in the in vivo metabolism is further evaluated through the study

of the effects of phenobarbital induction and disulfiram inhibition upon Procarbazine metabolism.

The animal tumor model is developed to provide a definitive assay for the antineoplastic activity of Procarbazine and its metabolic and chemical degradation products.

The effects of prolonged Procarbazine administration and the administration of methylprednisolone, diphenylhydantoin and phenobarbital upon the antineoplastic activity of Procarbazine is evaluated since current clinical protocols commonly require administration of Procarbazine for 20-30 consecutive days and the administration of these other drugs during chemotherapy. Several of these drugs are known to affect microsomal drug metabolism and the liver microsomal mixed-function oxidase enzymes. Since Procarbazine requires bioactivation, knowledge of possible alterations in Procarbazine antineoplastic activity is essential.

Finally, disulfiram has been shown to inhibit the metabolism of a known carcinogenic methylating agent 1,2-dimethylhydrazine through inhibition of the metabolic pathway leading to alkylating intermediates. The effects of disulfiram upon Procarbazine antineoplastic activity is undertaken in an effort to investigate the cytotoxic effects of alkylating and free radical products.

I. Literature Reviews

1. The Chemistry of 1,2-Dialkylhydrazo, Azo, and Azoxy Compounds

The chemistry of 1,2-dialkylhydrazo, azo, and azoxy compounds has been extensively studied, documented, and reviewed. A comprehensive review of synthesis and preparative procedures is contained in several chapters in a 2-volume treatise by Sandler and Karo.[15] The chemistry is reviewed in several chapters by Smith[16] and in an extensive 2-volume series edited by Patai.[17] Most of the literature deals with aryl or arylaliphatic hydrazo, azo, and azoxy compounds. Since Procarbazine is a 1-methyl-2-alkylhydrazo compound, this summary will be confined to the chemistry and those reactions which are pertinent to 1,2-dialkyl compounds.

Simple dialkylhydrazo compounds are named as 1,2-dialkylhydrazines according to the International Union of Pure and Applied Chemistry (I.U.P.A.C.) rules for nomenclature. These hydrazines have an ammonical odor and are stable if protected from air. They normally exist in a gauche conformation[18] unless they are fully substituted with very electronegative groups.[19] Most alkylhydrazines are bases which have a strength between ammonia and aniline and which form stable salts with one equivalent of mineral acid.[20]

1,2-Dialkylhydrazines may be prepared according to several methods. Direct alkylation of either hydrazine itself or an alkylhydrazine with alkylhalide, sulfate, epoxide, aziridine, or active olefin is a facile reaction due to the strong nucleophilic character of hydrazine.[21] This procedure often yields mixtures of mono- and polysubstituted hydrazines.

Due to the increased nucleophilicity of the substituted nitrogen,[22] alkylation of methylhydrazine normally occurs at the substituted nitrogen[23] and does not give the desired 1,2-dialkyl compound. Specific alkylhydrazines can be synthesized by alkylation of 1,2-diacyl- and monoacylhydrazines followed by hydrolysis to the corresponding hydrazines.[24] Zeller, et al.[1] prepared Procarbazine and other 1,2-dialkylhydrazines by alkylation of the sodium salt of 1-methyl-1,2-bis (benzyloxycarbonyl)-hydrazine with methyl p-(bromomethyl) benzoate.

A more direct method involves the reduction of C=N bonds of azines and hydrazones and N=N bonds of azo compounds using catalytic hydrogenation,[25] lithium aluminum hydride,[26] lithium in liquid ammonia,[27] sodium borohydride,[28] and sodium, sodium amalgam, Raney nickel-hydrazine.[29]

Hydrazo compounds undergo numerous reactions including several which are characteristic of aryl- or arylalkylhydrazines. These include the benzidine rearrangement,[30] coupling with diazonium salts,[31] disproportionation upon heating to an azo compound and an amine,[32] and reductive cleavage of the N-N bond to form 2 molecules of amine.[33] Tertiary alkylhydrazines, upon warming in the presence of acid, will eliminate the tertiary alkyl group.[34] Some β-aminoalkyl- and β-hydroxyalkyl-hydrazines undergo N-N cleavage[35] under vigorous conditions, a few cases of cleavage of other alkylhydrazines have been reported.[36]

Aliphatic hydrazo compounds are unstable in air and are easily oxidized to the corresponding azo compound by halogens, hypohalites, peroxides, mercuric oxide, molecular oxygen, nitric acid, and N-bromosuccinimide.[37] Air oxidation or "autooxidation" to give hydrogen peroxide[38] and an azo compound is rather slow unless traces of heavy

metal compounds such as Cu(II) are present.[39] Peroxyacetic acid is the only reagent reported to perform direct oxidation of hydrazines to azoxy compounds.[40] Mild oxidizing reagents stop at the azo stage ($2e^{\ominus}$ oxidation) or the unstable azo fragments with the elimination of nitrogen gas.[41]

Hydrazines are stronger nucleophiles than the corresponding amines because of an "α-effect" which is usually observed with two adjacent atoms with unshared electron pairs. Edwards and Pearson[42] have suggested that electronic repulsion raises the ground state energy and that the transition state energy is lowered during bond formation as electronic repulsion is decreased. Hydrazine reactions which involve the nitrogen non-bonded electrons include alkylation,[43] acylation,[44] reactions with alkenes and alkynes,[45] nitrosation,[46] and hydrazone formation with aldehydes and ketones.[47]

No recent work has been done on the photochemistry of aliphatic hydrazo compounds. Kay and Taylor[48] reported that the vapor of 1,2-dimethylhydrazine is photolyzed to hydrogen, nitrogen, methane, ammonia, methylamine, and a dimer of methyl methylene imine.

1,2-Dialkylazo compounds, which are named by placing the term "azo" between the name for each of the substituents according to the I.U.P.A.C. system, are light yellow liquids or solids. They can exist as either cis or trans geometric isomers with the trans configuration preferred.[49] Azo compounds are very weak bases. The pK of aliphatic azo compounds have not been measured since they rapidly isomerize to the hydrazone under acidic conditions.[50]

1,2-Dialkylazo compounds have been prepared in several ways. Many of the methods used in the preparation of aromatic azo compounds, such as the coupling with diazonium salts,[51] may not be applied to the

aliphatic series. Only a few of the numerous methods reported for the preparation of aliphatic azo compounds are widely used. The preferred procedure is oxidation of 1,2-dialkylhydrazo compounds to their corresponding azo derivatives. Aliphatic azo compounds may be thermally unstable and are subject to further oxidation to azoxy compounds. As a consequence, the oxidation requires careful control of the reaction conditions and careful choice of the oxidizing agent. The reagent of choice is mercuric oxide,[52] either the red or yellow form.

An alternate method utilizes lead tetracetate to isomerize readily prepared alkyl hydrazones to the desired azo compound. However, with ketohydrazones one usually gets azoacetates[53] and with aldehyde hydrazones, the reaction is complex and not well characterized.[54]

Azo compounds exist predominantly as trans geometric isomers. The cis isomer can be prepared through the irradiation of the trans azo compound with light of a wavelength slightly longer than that absorbed by the trans chromophore. This method has been reported only for aromatic azo compounds such as trans-azobenzene and trans-p,p'-azotoluene.[55] The application of this method to 1,2-dialkylazo compounds has not been reported.

Alkylazo compounds undergo numerous reactions and possess a more varied chemistry than the more stable arylazo compounds. In the presence of acid, alkylazo compounds with acidic α-protons rapidly tautomerize to hydrazones. In polar solvents, Ioffe and Stopskij[56] found that the 1,2-dialkylazohydrazone equilibrium favored the hydrazone by 1.4-3.2 kcal/mole. If acidic β-protons are also present in the molecules, a second tautomerization can occur to form ene-hydrazines. Ene-hydrazines are normally present in very low equilibrium concentrations and are

unisolatable unless special structural features are present. Ene-hydrazines are postulated intermediates in the Fischer indole synthesis.[57]

Azo compounds are both heat and light sensitive. In general, azo alkanes undergo smooth thermal and photochemical decomposition to produce radical pairs with the loss of nitrogen gas.[58] The ease of decomposition varies with the stability of the newly formed radical. These radicals may recombine to form dimers as a major reaction product since they are generated in the same "solvent cage". Other products result from disproportionation, hydrogen atom abstraction, or addition reactions.

Oxidation of the azo function to azoxy may be performed with peroxy-acids such as peroxybenzoic or peroxyacetic acid as the preferred route.[59] These weak peroxyacids, when used in conjunction with suitable solvents, diminish the possibility of isomerization to the corresponding hydrazone, and stereochemistry of the azo function is preserved.[60] Unsymmetrical azo alkanes produce unequal amounts of both positional azoxy isomers upon oxidation. The site of oxygen addition remains unpredictable as reports on the position of oxygen addition to azo alkanes conflict. Steric hinderance should favor the addition to the nitrogen nearest the methyl group in 1-methyl-2-alkylazo compounds,[61] however, reports of addition to the nitrogen farthest from the methyl group also exist.[62]

Less attention has been given to the reduction of azo compounds to hydrazines and amines. Carefully controlled conditions and very mild reducing agents are required to stop the reduction at the hydrazo stage. Reagents such as platinum oxide,[63] palladium on charcoal in methanol,[64] sodium amalgam, sodium, and alcohol,[64b,65] aluminum amal-gam,[65b] zinc and alkali,[65b] hydroquinone,[66] and other hydrazines[67]

have been used. More vigorous reactions and reagents such as catalytic hydrogenation,[68] hydrogen iodide,[69] and sodium dithionite[70] result in the cleavage of the N-N bond to form amines.

Aliphatic azoxy compounds can exist as either cis or trans (syn or anti) geometric isomers as well as asymmetric isomers depending upon the position of the oxygen. They may be named according to the tentative I.U.P.A.C. rules using "azoxy" between the parent hydrocarbons and using -NNO- and -ONN- before "azoxy" to specify the position of the oxygen.[71] Azoxy compounds are only weakly basic and protonation is believed to occur on the oxygen atom.[72] The azoxy group is analogous to a nitro group such that the C-H bond on the carbon bound to the azoxy N-O nitrogen exhibits weakly acidic properties, pKa >14.[59a]

The most general and widely used method of synthesis of dialkylazoxy compounds is the previously discussed oxidation of the corresponding azo compound with peroxyacids. Unsymmetrical azo compounds may yield a mixture of structural isomers. Many early literature reports fail to identify the isomeric composition of the mixture since the asymmetric nature of the azoxy function was not defined until the early 1960's.

An alternative synthetic method involves coupling of C-nitroso compounds with hydroxylamines.[73] This method does not work well for the preparation of unsymmetrical arylazoxy compounds. A pair of symmetrically substituted arylazoxy compounds are obtained. Purely aliphatic azoxy or alkylarylazoxy unsymmetrical compounds can be prepared using this method. Major problems with this method are that low yields are obtained and the position of the oxygen atom in the azoxy product cannot be predicted in the unsymmetrical product.

Freeman [60a] reported that the source of the oxygen appears to be the nitroso compound. However, the position of the oxygen appears to be determined by the nature of the substituent groups of the azoxy compound. Oae, et al.[74] proposed a mechanism for the condensation which involves an intermediate dihydroxyhydrazine in which both nitrogens are similar.

Moss, et al.[75] recently developed a procedure for the unambiguous synthesis of unsymmetrically substituted azoxyalkanes containing primary or secondary alkyl groups. The reaction involves alkylation of an alkyl diazotate with an alkyl halide. This synthetic procedure yields the trans-azoxy isomer with the oxygen on the nitrogen bearing the alkyl group from the alkyl halide. The position of the oxygen can be assigned through the use of proton nuclear magnetic resonance spectroscopy.[60a,76]

Several examples of the oxidation of hydrazones to the corresponding azoxy compounds have been reported. Peracetic acid oxidation of phenylhydrazones of aromatic aldehyde gives the corresponding azoxy compound with the oxygen on the NH-position.[77] Aliphatic hydrazones of aromatic aldehydes such as methyl- and benzylhydrazones yield azoxy products at 0°C but give the corresponding hydrazide at higher temperature. Peroxyacid oxidation of p-tolualdehyde benzylhydrazone is reported to give a mixture of the azoxy compound and 1-benzoyl-2-methylhydrazine. Furthermore, in acidic solutions, azoxy compounds rearrange to the corresponding hydrazides.[78]

The reported chemistry of azoxy compounds is restricted to reduction and rearrangement reactions. Treatment of azoxybenzenes with strong acid, such as 85-95% sulfuric acid, leads to the Wallach

rearrangement.[79] Alkylazoxy compounds undergo other rearrangements in the presence of acid. The presence of an adjacent primary carbon atom leads to the formation of the corresponding hydrazide.[59a] If the conditions are vigorous, acid and hydrazine are isolated instead of the hydrazide.[77] When the two adjacent carbon atoms are primary, the azoxy oxygen migrates to the nearest carbon.[77b] With two secondary adjacent carbons, ketones, nitrogen, and some hydrazine are obtained.[59a] This presumably occurs via the dehydration to an azine followed by hydrolysis. In the absence of water, the corresponding hydrazenoyl chloride (if HCl is the acid) can be isolated.[77b]

Aliphatic azoxy compounds are stable towards bases under mild conditions. Benzylic azoxy compounds, even in the absence of air, rapidly dimerize in the presence of strong base to form a mixture of d,l and meso dimers.[80] Heating with strong base destroys azoxyalkanes with azoxymethane and leads to the formation of cyanide ion and volatile bases.[59a]

Simple aliphatic azoxy compounds can undergo alkylation in the presence of alkyllithium to form mono-C-alkyl products. Alkyllithium forms an anion alpha to the azoxy group which can subsequently react with the alkylating agent followed by reduction to give alkylated azo compounds.

Aliphatic azoxy compounds can be reduced by ethyl phosphite,[82] lithium aluminum hydride,[83] and magnesium turnings in dry ethanol or methanol[59a] to form the corresponding azo and/or hydrazo product. With lithium aluminum hydride, the trans-azo product is obtained regardless of the geometric configuration of the initial azoxy compound. Zinc dust,[84] stannous chloride,[59a] catalytic hydrogenation over plantinum oxide catalyst[64a] or electrochemical reduction[85] produces hydrazo compounds.

The hydrazine is cleaved to the corresponding amine if acidic solutions are used during catalytic hydrogenation.[59a]

Aliphatic azoxy compounds also can undergo photochemical reactions. A comprehensive review of the photochemistry of azoxy compounds was prepared by Spence, Taylor, and Buchardt.[86] Azoxymethane in a gas-phase photochemical reaction forms nitrogen, nitrous oxide, methane, and ethane.[87] Higher aliphatic azoxy compounds can form oxaziridines when irradiated in solutions, although these may be too unstable to isolate.[81,88] With alkylarylazoxy compounds, irradiation can lead to an oxygen migration from the aryl nitrogen to the alkyl nitrogen as well as isomerization from the more stable trans-isomer to the cis-form.[89] Migration to the aryl nitrogen atom has never been observed.

2. The Chemistry of Procarbazine

Procarbazine hydrochloride (N-isopropyl-α-(2-methyl-hydrazino)--p-toluamide·hydrochloride) is a stable white to pale yellow crystalline solid of molecular weight 257.76 grams per mole. It decomposes upon heating at about 223°C.[90] Procarbazine exhibits an ultraviolet absorption maximum at 232 nm ($E=1.3 \times 10^4$) and a minimum at 213 nm as shown in the ultraviolet absorption spectrum of Procarbazine·hydrochloride in Figure 2. The infrared, proton nuclear magnetic resonance, and low- and high-resolution electron-ionization mass spectras are shown in Figures 3-5 with the corresponding assignments for the spectral data.[91]

Procarbazine was first synthesized by a group at Hoffmann-La Roche headed by Zeller.[1] Methylhydrazine was treated with benzyl chloroformate to form the 1-methyl-1,2-bis (benzyloxycarbonyl)hydrazine. The sodium

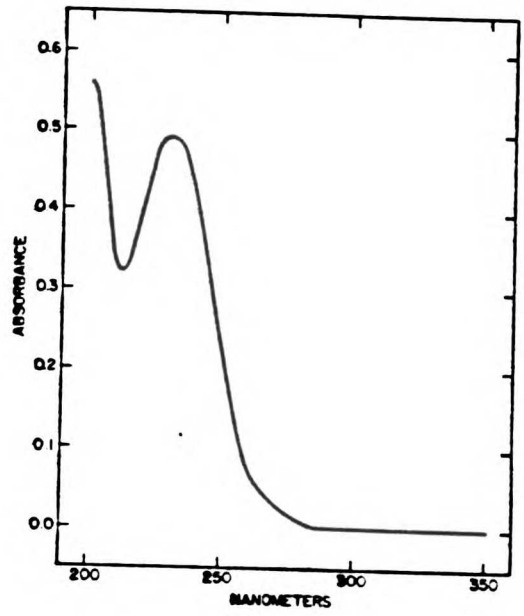


Figure 2. Ultraviolet Spectrum of Procarbazine·HCl

The spectrum has a maximum at 232 nm ($E = 1.3 \times 10^4$) and a minimum at 213 nm. The concentration was 0.01 mg/ml in 0.1N hydrochloric acid.

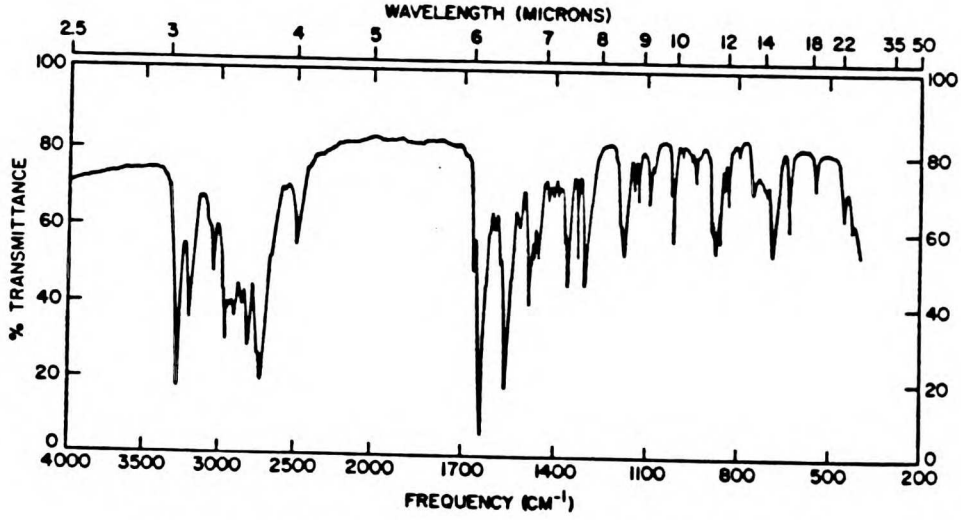


Figure 3. Infrared Spectrum of Procarbazine·HCl

The sample was suspended in Fluorolube^R to obtain spectrum from 4000-1340 cm^{-1} and in mineral oil from 1340-400 cm^{-1}. The spectrum was obtained on a Perkin-Elmer Model 621 Grating Spectrophotometer.

Assignments:	Band(cm^{-1})	Assignment
	3277 and 3200	NH Stretch
	3035	Aromatic CH Stretch
	2961 and 2853	Aliphatic CH Stretch
	2760-2300, main band at 2725	NH_2^+
	1660 and 1636	C=O Stretch(Amide I)
	1556	Amide II
	1299	Amide III
	1361 and 1351	Isopropyl deformation
	857	Aromatic CH out-of-plane Bending

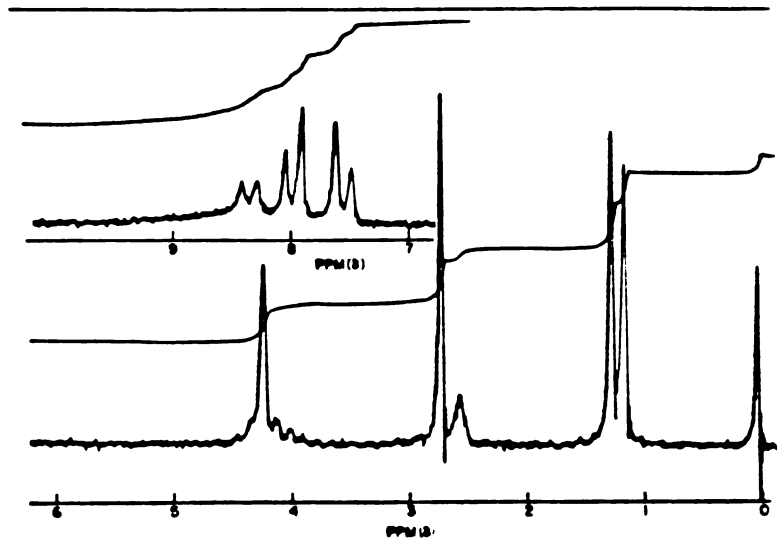
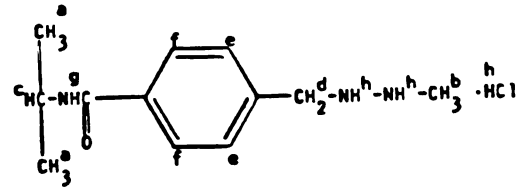


Figure 4. Proton Nuclear Magnetic Resonance Spectrum of Procarbazine·HCl

The sample concentration was 100.8 mg/ml in DMSO-d_6 containing tetramethylsilane as the internal reference.

Assignments:



Protons	Chemical Shift δ (ppm)	Multiplicity	Coupling Constant, J (in Hz)
a	1.20	Doublet	6.0
b	2.72	Singlet	--
c	4.18	Multiplet	6.0
d	4.23	Singlet	--
e	7.55	Doublet	8.5
f	7.99	Doublet	8.5
g	8.36	Doublet	7.0
h	6.33-10.33	Singlet (broad)	--

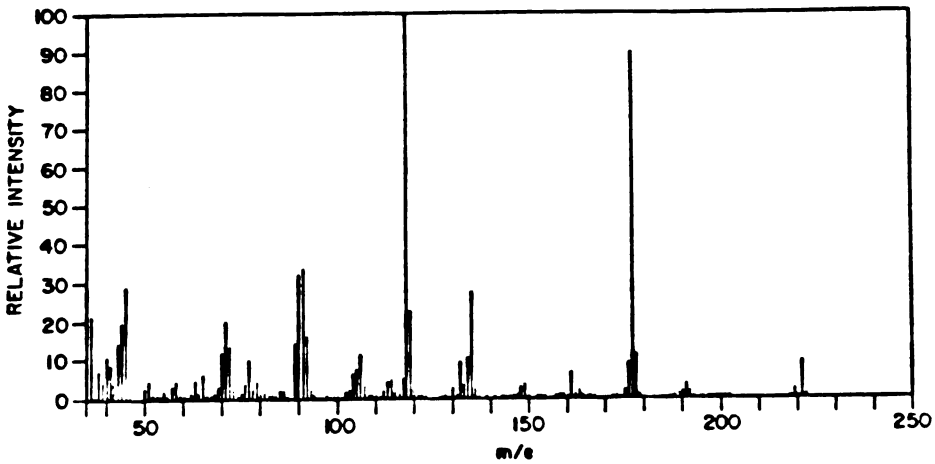


Figure 5. Low Resolution Electron Impact Mass Spectrum of Procarbazine·HCl

The spectrum was obtained using a Varian MAT CH5 mass spectrometer interfaced with a Varian data system. The ionizing energy was 70 eV.

Assignments:	m/e	Assignment
	221	Molecular ion of free base
	191	Loss of $NHCH_3$ from free base
	177	Loss of $NHNHCH_3$ from free base
	163	Loss of $NHCH(CH_3)$ from free base
	149	Loss of C_3H_6 by McLafferty rearrangement from m/e 191
	135	Loss of C_3H_6 by McLafferty rearrangement from m/e 177
	118	Loss of $NHNHCH_3$ from m/e 163
	36	HCl moiety

High Resolution Mass Spectral Assignments

Found Mass	Calcd. Mass	C	H	N	O
118.0428	118.0419	8	6	0	1
135.0698	135.0684	8	9	1	1
149.0753	149.0715	8	9	2	1
163.0870	163.0872	9	11	2	1
177.1170	177.1154	11	15	1	1
191.1288	191.1311	12	17	1	1
221.1533	221.1529	12	19	3	1

salt was formed with sodium hydride and treated with methyl-p-(bromoethyl)benzoate to give 1-methyl-2-p-(methoxycarbonyl) benzyl-1,2-bis (benzyloxycarbonyl)hydrazine. After hydrolysis of the ester and treatment with thionyl chloride, the resulting acid chloride was reacted with isopropylamine. The carbobenzyloxy-protecting groups were removed with hydrogen bromide in acetic acid or catalytic hydrogenation to produce Procarbazine. In an alternate synthetic procedure developed by Gurien and Sinkowski,[91,92] N-isopropyl-p-formylbenzamide was converted to the corresponding methylhydrazone with methylhydrazine and reduced to N-isopropyl-α-(2-methylhydrazino)-p-toluamide by catalytic hydrogenation over palladium/charcoal. Addition of hydrogen chloride to the reaction mixture produced the desired Procarbazine hydrochloride salt. Both synthetic pathways are shown in Figure 6.

Procarbazine hydrochloride is stable under anhydrous conditions and this stability is increased if it is protected from ultraviolet light and kept under a nitrogen atmosphere. Procarbazine is readily water soluble. In the presence of dissolved oxygen under neutral conditions, Procarbazine reacts to give the more lipophilic azo compound and hydrogen peroxide.[1] Aebi, et al.[93] found that Procarbazine in 0.05M phosphate buffer at pH 7.0 was relatively stable and that the presence of metal ions, especially Cu(II), catalyzed the rapid autooxidation of Procarbazine. Erlenmeyer, et al.[94] and Brintzinger, et al.[95] also studied the autooxidation of Procarbazine in aqueous solutions and found that the reaction was catalyzed by Mn(II) ion and its sulfophtalocyanine complex. Metalloproteins such as myoglobin, siderophilin, and, to a much less extent, hemoglobin, also can act as catalysts for the oxidation process.[93,96] Chelating compounds such as deoxyribonucleic acid,

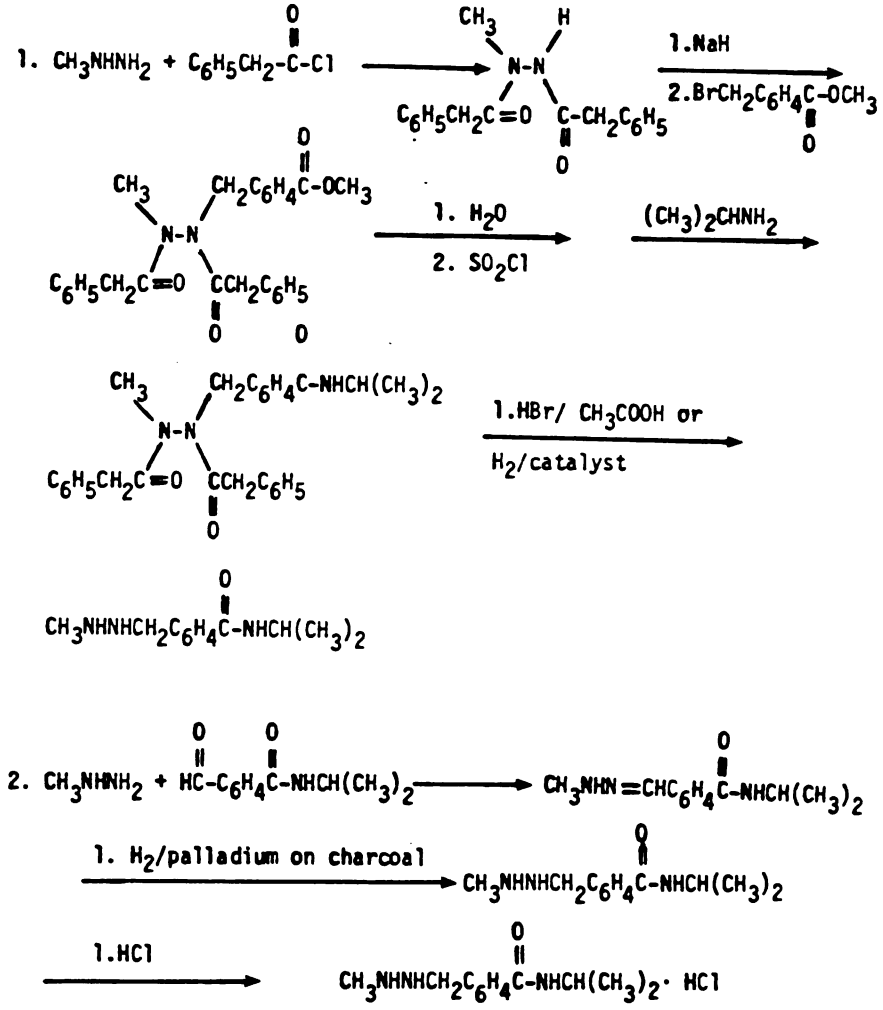


Figure 6. Pathways For the Synthesis of Procarbazine.

ethylenediaminetetraacetic acid (EDTA), albumin, and certain buffer solutions such as pyrophosphate and Tris (2-amino-2-hydroxymethyl-1,3-propane diol) compete for the Cu(II) ion and strongly inhibit the oxidation reaction.[93]

In aqueous solutions the azo derivative slowly isomerizes to the hydrazone of monomethylhydrazine and N-isopropyl-p-formylbenzamide. Rapid isomerization occurs at pH 1-2 with the subsequent hydrolysis of the hydrazone to monomethylhydrazine and N-isopropyl-p-formylbenzamide.[97] Less than 1% of the isomeric formaldehyde hydrazone and subsequent hydrolysis to formaldehyde and the corresponding hydrazine occurs under acidic conditions.[98]

Monomethylhydrazine may be oxidized in the presence of oxygen in alkaline solutions to the unstable monomethyldiazene.[98] Tsuji, et al.[99] prepared several compounds in this class including monomethyldiazene. They decompose rapidly in the presence of oxygen through a free-radical chain reaction. The ultimate product of the decomposition is the corresponding hydrocarbon.

The Procarbazine chemical decomposition pathway is shown in Figure 7.

3. The Metabolism of Procarbazine

The in vivo and in vitro metabolism of Procarbazine has been extensively studied. Although many metabolic intermediates and pathways have been proposed (Figure 8), the metabolic fate of Procarbazine remains unclear.

Raaflaub and Schwartz[100] and Oliverio and co-workers[101] found that in vivo metabolism proceeds in a similar manner in humans, dogs,

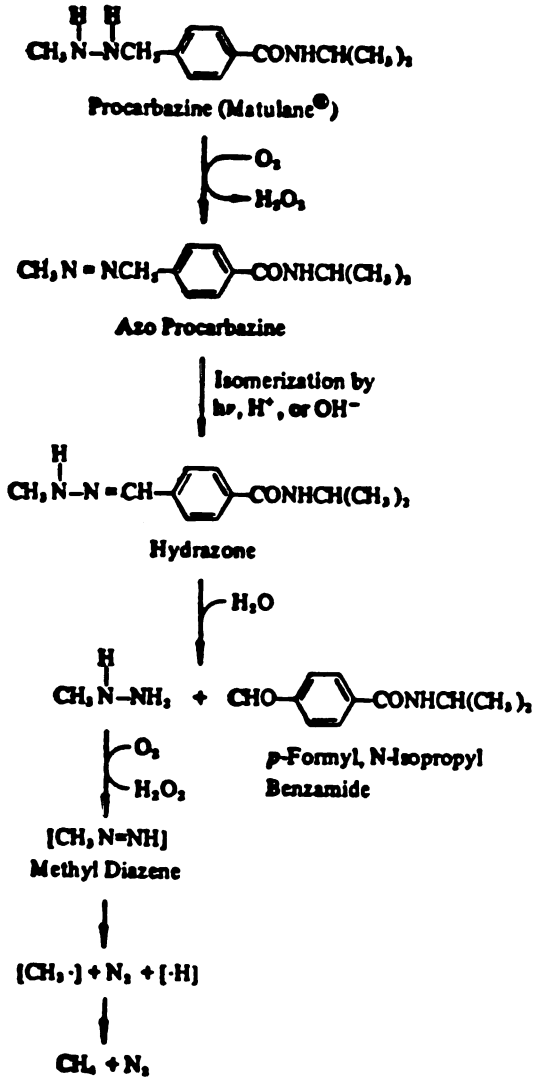


Figure 7. Chemical Decomposition Pathway of Procarbazine in Aqueous Solution.

The brackets denote postulated but not identified intermediates.

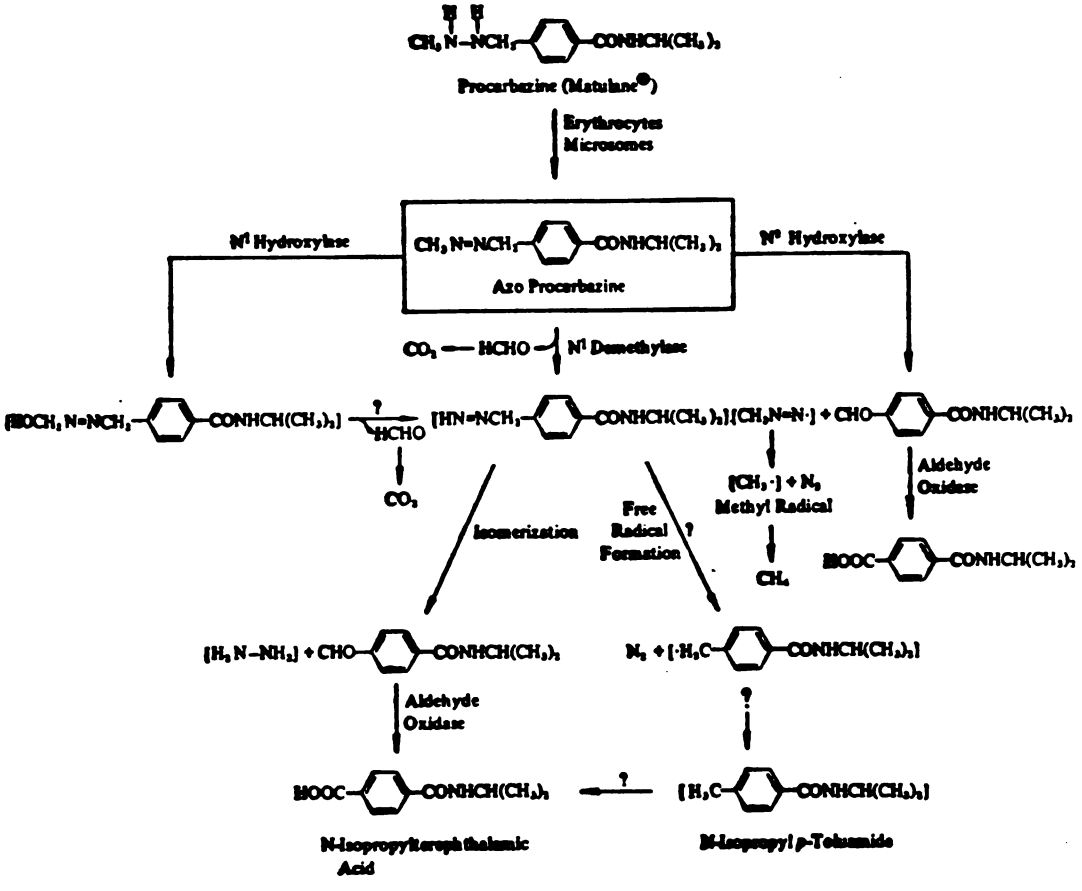


Figure 8. The Postulated Pathways of In Vitro and In Vivo Metabolism of Procarbazine.

The brackets denote postulated but not identified intermediates.

and rodents. Procarbazine is rapidly cleared from blood *in vivo*. The half-life in plasma is about 7 minutes in man and 12-14 minutes in dogs and rodents. Raaflaub and Schwartz[100] found that the decrease in plasma concentration of ^{14}C-carbonyl-labeled Procarbazine corresponded to an increase in azo concentration.

Azo formation from Procarbazine is also the first step found in the *in vitro* metabolism. Baggiolini and co-workers[97] studied this oxidation in isolated perfused rat livers. Procarbazine, azo, and the major urinary metabolite, N-isopropylterephthalamic acid, do not accumulate in the liver nor are they excreted to any significant extent into the bile, therefore, their concentrations in the perfusion medium can be regarded as representative of their metabolism. The *in vitro* oxidation of Procarbazine to azo is about 28 umoles/hour/100 grams of body weight. This reaction proceeds several times more rapidly than the other known reactions to Procarbazine. Removal of the liver from the perfusion system decreased the rate of oxidation of Procarbazine to azo to about 2-3% of the perfusion rate. Baggiolini, *et al.*[97] also found that the amount of azo formed during rat liver perfusion corresponded to the decrease in Procarbazine levels. After 45-60 minutes, the azo levels reached steady state in normal liver. In the presence of SKF 525-A, the azo levels continued to increase during the 2 hour liver perfusion, although the rate of azo formation diminished. Pretreatment with 3-methylcholanthrene resulted in an increase in the azo peak concentration followed by a rapid decrease to an extremely low level. These data suggest that Procarbazine is converted initially to the azo compound which is subsequently metabolized to other metabolites apparently by microsomal hydroxylases. It does not appear that theses enzymes are involved to a major extent in the initial oxidation of Procarbazine.

However, Reed, et al.[102] reported that Procarbazine may be enzymatically and non-enzymatically oxidized by microsomes. Prough, et al.[103] found that boiled rat liver microsomes in the presence of NADPH-regenerating system oxidized 1030 nanomoles of Procarbazine to azo in 30 minutes. Microsomes with the NADPH-regenerating system oxidized 1318 nanomoles of Procarbazine during the same 30 minute incubation. Furthermore, the microsomal oxidation of Procarbazine is cytochrome P-450 dependent.[104] Phenobarbital pretreatment resulted in a 2.7-fold increase in the specific activity of Procarbazine oxidation. Pretreatment with 3-methylcholanthrene did not increase the oxidation rate. Inhibitors of cytochrome P-450 dependent reactions such as carbonmonoxide, metyrapone, or specific antibodies to microsomal NADPH-cytochrome C reductase were effective inhibitors of the oxidation. Methimazole, an inhibitor of the cytochrome P-450 independent amine oxidase was not. Thus, the oxidation could occur by dehydration or by a cytochrome P-450 dependent N-hydroxylation followed by dehydration. N-hydroxylations of this type have been reported previously for 2-acetylaminofluorene[105] and acetaminophen.[106]

The azo compound is further metabolized to the acid which is the major urinary metabolite.[100,101] After intravenous administration of ^{14}C-carbonyl-labeled Procarbazine, 70-80% of the administered radioactivity is excreted in the urine after 24 hours. Raaflaub and Schwartz[100] reported that 42% of the initial dose was the acid, while Oliverio and Kelley[101] found approximately 20% as the acid and less than 5% excreted as the unchanged drug. These results were similar in man, dog, and rodent. Bollag[107] reported that in humans, urinary excretion of radioactive label was approximately 70% of the administered dose. Most of the radioactivity is found during the first six hours. Baggiolini, et al.[97] studied the

formation of the acid *in vitro* using rat liver perfusion. SKF 525-A reduced the rate of the formation of the acid to 55% of the control and 3-methylcholanthrene increased the rate by almost 2.7-fold. SKF 525-A is a potent inhibitor and 3-methylcholanthrene a potent inducer of the cytochrome P-450 liver microsomal enzyme system. These data suggest that the splitting of the $N-CH_2$ bond of Procarbazine or azo is catalyzed by a microsomal hydroxylase. Although both Procarbazine and the azo compound could be a substrate for the $N-CH_2$-splitting hydroxylase, the inter-relationship between the azo and the acid concentrations suggests that the azo compound is the substrate. When the acid production rate is low, the azo levels increase continuously during the 2 hour rat liver perfusion. At an acid production rate 5 times higher, the azo concentration rapidly decreases. As Procarbazine levels decrease, the azo levels increase. Direct cleavage of Procarbazine to acid has not been evaluated. However, the rapid conversion of Procarbazine to azo suggests that the azo compound undergoes a $N-CH_2$ cleavage via a microsomal hydroxylase to produce the acid as the major urinary metabolite.

Raaflaub, *et al.*[100] and Oliverio, *et al.*[101] have suggested that the azo compound isomerizes to N-isopropyl-*p*-formylbenzamide methylhydrazone which can then be hydrolyzed to the aldehyde and methylhydrazine. The aldehyde is subsequently oxidized to the acid. Baggiolini, *et al.*[97] reported that *in vitro* studies failed to show any evidence of enzyme-catalyzed isomerization of the azo to the hydrazone. The non-enzymatic conversion was so slow that the levels of azo and hydrazone remained essentially unchanged during a several hour saline incubation at pH 7.4 and 37°C. Since the formation of the acid is strongly inhibited by SKF 525-A and enhanced by 3-methylcholanthrene, cytochrome P-450-dependent catalysis appears to be involved at some step between the azo compound and the acid.

Further studies on the metabolism of Procarbazine have focused primarily upon elucidating the metabolic fate of the N-methyl group. This function has been shown to be essential for antineoplastic activity[2,107] since the desmethyl-[108] and N-ethylhydrazine[3] analogs are inactive. The methyl group has been found to be converted to carbon dioxide, formaldehyde, and methane during both *in vitro* and *in vivo* metabolism.

Weitzel, *et al.*[109] postulated the N-demethylation of Procarbazine after they found that formaldehyde was released during the *in vitro* oxidation of Procarbazine with potassium hexacyanoferrate (III). Baggiolini and co-workers[110] used ^{14}C-methyl-labeled Procarbazine to determine the fate of the methyl group *in vivo* in rats and *in vitro* during rat liver perfusion. The N-methyl group is oxidized to CO_2 during liver perfusion. Removal of the liver from the perfusion circuit decreased the CO_2 levels to 2% of the liver perfusion levels. Aebi, *et al.*[111] found that slices of normal human liver metabolized Procarbazine to CO_2 at a rate of about three times slower than rat liver slices. Baggiolini and his co-workers[110] reported that CO_2 expiration in rats reached a steady state level after 10 minutes and continued for 3-5 hours. The amounts of CO_2 and acid formed *in vivo* in 24 hours were of the same order of magnitude. Phenobarbital-pretreatment caused a 2.5-fold increase in the rate of CO_2 production. Baggiolini and Bickel[112] reported that 3-methylcholanthrene-pretreatment increased the rate of CO_2 production by a factor of 3.5 while SKF 525-A reduced the rate by ½ in rats.

Wittkop, *et al.*[113] characterized the N-demethylase enzyme and found that the microsomal fractions of rat liver contained almost all of the enzymatic activity for formaldehyde formation with the nuclear, mitochondrial, or soluble cellular fractions exhibiting much less activity. The rate of formaldehyde formation was linear for up to 40 minutes for

monomethylhydrazine and 120 minutes for Procarbazine, was linear for protein concentrations up to 2.5 mg. of microsomal protein/milliliter, and was optimal at 37°C. Procarbazine and methylhydrazine gave cytochrome P-450 binding spectral changes which are typical of Type II compounds such as aniline with an absorption maximum at 430 nm and a minimum at 390 nm. Demethylation is dependent upon the presence of NADPH or a NADPH-generating system and oxygen since gassing the assay medium for 2 minutes with nitrogen results in a 74-80% decrease in formaldehyde formation. Both Procarbazine and azo undergo demethylation *in vitro* to form formaldehyde. Azoxyprocarbazine is reported to produce significantly amounts of formaldehyde non-enzymatically.[102] A similar compound, methyl azoxy methanol is a good substrate for enzymatic N-demethylation and formaldehyde production.[102]

The effect of SKF 525-A pretreatment is described in conflicting results from several laboratories. Baggiolini and Dewald[114] and Dewald, Baggiolini, and Aebi[115] reported that SKF 525-A added to the liver perfusate reduced the rate of conversion of Procarbazine and methylhydrazine to formaldehyde to 50% and 15% of the control values respectively. Treatment with 3-methylcholanthrene produced a 3-fold increase in the rate of Procarbazine conversion to formaldehyde. Wittkop, *et al.*[113] found that SKF 525-A pretreatment of rat liver microsomes actually stimulated Procarbazine, methylhydrazine, and azo demethylation by 5% as measured by formaldehyde release. Phenobarbital pretreatment increased the cytochrome b_5 content 1.9-fold and cytochrome P-450 content 2.15-fold. Azo and methylhydrazine demethylation rates increased 1.8-2.5-fold[103,113] and Procarbazine demethylation rate increased by 5.7-fold.[115] 3-Methylcholanthrene pretreatment was less effective than phenobarbital.

Pretreatment with Procarbazine, which reduced the cytochrome P-450 content, had no effect upon demethylase activity.[103]

There is evidence which suggests that the N-demethylase activity is both cytochrome P-450 dependent and independent. Steapsin and deoxycholate which convert cytochrome P-450 to the inactive cytochrome P-420 inhibit only the phenobarbital-inducible demethylation.[103,113] Procarbazine and monomethylhydrazine demethylation rates were reduced to the level of non-induced microsomes. Trypsin digestion of non-induced microsomes showed little correlation between the loss of microsomal cytochrome P-450 and demethylase activity. Such a correlation did occur in microsomes from induced rats. The demethylase activity is not dependent upon cytochrome b_5 since trypsin digestion of both induced and non-induced microsomes resulted in a greater rate of enzyme activity loss than cytochrome b_5 content loss. Reed, et al.[102] found that carbon monoxide did not inhibit demethylation of methylhydrazine or azo in non-induced microsomes, but inhibited demethylation in phenobarbital-induced microsomes by about 50%.

Finally, Prough, et al.[116] have studied the N-demethylation reactions utilizing purified pig liver microsomal amine oxidase. Although Procarbazine and methylhydrazine are poor substrates for the amine oxidase, the latter does produce formaldehyde and methane. Using specific inhibitors, they showed that the enzyme responsible for the N-demethylase activity was liver microsomal amine oxidase. The metabolism of Procarbazine resulted in the production of an, as of yet, unidentified nucleophilic metabolite which could be trapped using a carbonyl reagent. This compound is apparently a mono-substituted hydrazine derivative.

The detection of formaldehyde and respiratory CO_2 as metabolites from Procarbazine has led to the proposal of three possible routes of metabolism:[98]

1. The direct demethylation of Procarbazine or azo or the hydrolysis of formaldehyde hydrazone via cleavage of the $N=CH_2$ bond.

2. The cleavage of the N-N bond releasing methylamine which is then further metabolized via N-demethylation.

3. The cleavage of the $N-CH_2$ bond of Procarbazine or azo or the hydrolysis of N-isopropyl-p-formyl-benzamide methylhydrazone to release methylhydrazine and then the subsequent demethylation of methyl-hydrazine.

The metabolic pathway leading to CO_2 and formaldehyde remains uncertain despite numerous work which has focused upon the determination of which, if any, of these pathways plays a major role in the metabolism of Procarbazine.

Direct demethylation of Procarbazine probably does not contribute significantly to the overall demethylation since Procarbazine is rapidly oxidized to the azo compound.[115] The N-isopropyl-p-formylbenzamide methylhydrazone is known while the formaldehyde N-isopropyl-p-formyl-benzamide hydrazone is unknown. Both hydrazones are at this time still hypothetical metabolies of Procarbazine.[117] Little, if any, formaldehyde hydrazone would be formed non-enzymatically. The more stable ring conjugated N-isopropyl-p-formylbenzamide methylhydrazone has been reported to slowly form by isomerization of the azo compound.[97]

Cleavage of the N-N bond *in vivo* was reported in 1937 in the metabolism of Prontosil.[118] Conflicting reports for a similar metabolic N-N bond cleavage of Procarbazine to release methylamine have been published. Baggiolini and Bicket,[112] using rat liver perfusion experiments, found that ^{14}C-methyl-labeled Procarbazine and ^{14}C-labeled

methylhydrazine gave no detectable ^{14}C-methylamine in either the perfusion medium or the bile. N-Demthylation of the amine *in vivo* is almost completely inhibited by relatively small amounts of Procarbazine and methylhydrazine. *In vitro*, the rate of CO_2 production from methylamine is 5-6 times slower than from Procarbazine and 8 times slower than monomethylhydrazine. Schwartz[119] reported that the CO_2 production rates from Procarbazine and methylamine in rats were similar. Furthermore, SKF 525-A administered intraveneously 30 minutes prior to methylamine or Procarbazine did not alter the pattern of excretion, but slowed CO_2 release from Procarbazine. This indicates that the pathways of CO_2 production from these compounds are different. Schwartz also reported detecting methylamine, isolated as the dinitrophenyl derivative, in rat urine 24 hours after parenteral administration of ^{14}C-methyl-labeled Procarbazine.

Evidence for the N-CH_2 bond splitting to give methylhydrazine is provided not from the isolation of methylhydrazine, but rather from the detection of pyridoxalmethylhydrazone in mice injected intraperitoneally with pyridoxal 30 minutes prior to a single subcutaneous injection of Procarbazine. There is a rapid and prolonged decrease in plasma pyridoxal phosphate concentration after Procarbazine administration and methylhydrazine administration. This could be due to either the direct combination of the hydrazine with pyridoxal phosphate or the inhibition of pyridoxal kinase which phosphorylates pyridoxal to pyridoxal phosphate. Raaflaub and Schwartz[100] and Oliverio, *et al.*[101] postulated the release of methylhydrazine during the metabolism of Procarbazine to the acid compound. However, neither group was able to detect its presence. Baggiolini and Bickel[112] reported that methyl-

hydrazine itself is demethylated *in vivo* and *in vitro*. In perfused rat liver experiments, the CO_2 production rate from methylhydrazine is significantly higher than that of Procarbazine. This could explain the inability of several groups to detect methylhydrazine *in vitro*. *In vivo*, however, methylhydrazine is demethlyated at a lower rate than Procarbazine. This lower demethylation rate could be attributed to methylhydrazine's rapid renal excretion or to the combination with pyridoxal phosphate. Reed and Dost[120] and Dost, *et al.*[121] concluded that methylhydrazine must be an intermediate in the production of CO_2 and methane from Procarbazine because the *in vivo* methylhydrazine and Procarbazine metabolic rates were similar. Also, as previously mentioned, Prough, *et al.*[116] were able to trap a nucleophilic metabolite with a carbonyl reagent during the metabolism of Procarbazine. This mono-substituted nucleophilic hydrazine could be methylhydrazine.

Dewald and co-workers,[115] from studies in the intact rat and in rat liver perfusion experiments, concluded that the metabolism of Procarbazine preceeds primarily through the direct demethylation of either azo or Procarbazine. 3-Methylcholanthrene-pretreatment increased and SKF 525-A decreased the rate of CO_2 formation both *in vivo* and *in vitro*. Phenobarbital increased the rate *in vivo* by a factor of about 2.5. Upon pretreatment with 3-methylcholanthrene, the rate of methylhydrazine demethylation is less than half that of Procarbazine and less than the control in isolated perfused livers and the same as the control *in vivo*. Both SKF 525-A and 3-methylcholanthrene slightly lowered the demethylation rate of methylhydrazine in perfused livers. Hence, Dewald and co-workers concluded that direct microsomal demethylation of Procarbazine or the azo compound occurs and that methylhydra-

zine is not demethylated by a microsomal hydroxylase. Methylhydrazine could be a substrate in a minor pathway. It is possible that the decrease in the methylhydrazine demethylation in 3-methylcholanthrene-pretreated rats could be due to the induction of a competitive pathway such as that leading to methane production. However, Dost and Reed found no induction of methane formation from Procarbazine after phenobarbital-pretreatment.[120b]

The azo is demethylated during Procarbazine metabolism. Procarbazine is probably not directly demethylated. Wittkop, Prough, and Reed[113] reported that the azo is demethylated by the O_2-, NADPH-dependent rat liver microsomal demethylase system at a rate of 2-3 times faster than Procarbazine. Furthermore, Procarbazine is rapidly enzymatically and non-enzymatically converted to the azo. Therefore, the azo is undoubtedly the substrate for the demethylase enzyme during formaldehyde and CO_2 production.

The production of methane *in vivo* was studied by Reed and Dost.[120] Intraperitoneal administration of ^{3}H- and N-$^{14}CH_3$-labeled Procarbazine resulted in a 7-10% conversion to methane after 8 hours and 11-22% conversion to CO_2. The ^{3}H-labeled methyl group of Procarbazine is converted to tritiated methane at about the same rate as the ^{14}C-labeled methyl group is to $^{14}CH_4$. This suggests that the methyl group is converted to methane as an intact entity. Phenobarbital-pretreatment results in an increase in the rate and extent of CO_2 formation *in vivo*, but no change in methane production. Schwartz, *et al.*[108] found that about 20% of the initial dose of methylhydrazine was metabolized to methane 1 hour after intraperitoneal injection as compared to less than 5% when Procarbazine was administered. Methane was observed as soon as

3 minutes after injection of Procarbazine and reached a maximum 10 minutes after injection of monomethylhydrazine.

Prough[122] and Reed[102] and their co-workers have studied the in vitro metabolism of alkylhydrazines to methane. Rat liver microsomal preparations along with oxygen and a NADPH-generating system slowly convert monoalkylhydrazines to their corresponding hydrocarbon compounds. Flavins such as FMN (flavin mononucleotide) and FAD (flavin adenine dinucleotide) enhanced the rate up to 50%, but the dependency upon flavins remains inconclusive. Although methylhydrazine can be chemically oxidized to methane,[123] the small amounts of hydrocarbons formed in boiled-liver microsomal preparations suggest that the non-enzymatic oxidation does not contribute much to the total hydrocarbon production. Monoalkylhydrazines can probably be enzymatically dehydrogenated to the unstable monoalkyldiazene, which subsequently collapses to form the alkane and nitrogen. This collapse is very rapid in the presence of oxygen[124] and could occur via an ionic or free-radical mechanism.

The monoalkylhydrazine oxidase system which is responsible for methane formation has been further characterized.[103] It is found in rat, beef, guinea pig, pork, and sheep liver and is not a cytochrome P-450 enzyme. The oxidase requires oxygen, but carbon monoxide, which inhibits cytochrome P-450-dependent reactions by binding to cytochrome P-450, only slightly inhibits the oxidation of methylhydrazine. Neither SKF 525-A nor 3-methylcholanthrene had any effect upon the oxidase activity. Procarbazine-pretreatment, which resulted

in a 30% decrease in microsomal cytochrome P-450 content, did not affect the activity. In non-induced microsomes, trypsin digestion studies showed a parallel loss of oxidase activity with cytochrome b_5 content. In phenobarbital-induced microsomes, the loss of oxidase activity was much greater than the loss of cytochromes P-450 or b_5. Hence, a relationship between b_5 content and alkylhydrazine oxidase remains unclear. Procarbazine does not appear to be a substrate, while the azo compound does produce methane.

Ziegler, et al.[125] characterized and purified a pig liver amine oxidase. This enzyme contained flavoprotein and catalyzed the NADPH- and oxygen-dependent N-oxidation of a variety of sec- and tert-amines to the corresponding hydroxylamines and N-oxides. This enzyme was insensitive to SKF 525-A and CO, did not contain cytochrome P-450, and was not induced by phenobarbital. This enzyme, in the presence of a hepatic microsomal N-oxide dealkylase, released formaldehyde and an amine from a number of N,N-dialkylarylamines. Prough and his co-workers[126] noted the similar characteristics of this enzyme and alkylhydrazine oxidase but found that methylhydrazine and Procarbazine were poor substrates. Methane and formaldehye were formed in small amounts from methylhydrazine.

The azo compound may also serve as an intermediate for the release of methane by a possible free-radical mechanism to liberate methane and nitrogen gas.

In addition to respiratory methane and CO_2, the ultimate fate of the methyl group is apparently the C-1 formate pool and direct and

indirect methylation of DNA and RNA. This ultimate fate of the methyl group will be discussed in the next section, which reviews the modes of action of Procarbazine.

4. The Mode of Action of Procarbazine

The exact mechanism of action of Procarbazine remains uncertain despite numerous studies. Procarbazine reportedly exerts many effects upon cells including degradation of cellular deoxyribonucleic acid (DNA), inhibition of mitosis, protein synthesis, and nucleic acid synthesis, and suppression of several enzyme systems.

In 1963, Berneis and co-workers[127] suggested that the formation of hydrogen peroxide may be responsible for the cytotoxic activity of Procarbazine. Aqueous solutions of DNA, Procarbazine or other 1-methyl-2-benzylhydrazines, and molecular oxygen produced a decrease in the viscosity of DNA over a period of several days. Inert gases such as nitrogen inhibited this viscosity change. Hydrogen peroxide is formed from Procarbazine in buffered solutions at 37^{o}C and pH of 7.0. Formation of hydrogen peroxide occurs in 78% yield after 140 hours. Similar oxidation-reduction reactions occurred with other hydrazines. The addition of peroxidase, catalase, or cysteamine prevented the observed DNA fragmentation into shorter double-helical segments. Cysteine had little effect. These results suggested that hydrogen perioxide was responsible for the DNA damage. Hydrogen peroxide in the presence of ferrous ions can degrade DNA.[128] Hydrogen peroxide is produced from Procarbazine during the oxidation to the azo compound. Strong

reducing agents produce OH-radicals from hydrogen peroxide. The OH-radicals are very reactive and can degrade DNA.

Despite these known deleterious effects of hydrogen peroxide upon DNA, it is very unlikely that hydrogen peroxide alone could account for the antineoplastic activity of Procarbazine. Hydrogen peroxide is a common metabolic product of many compounds which do not show antitumor activity. The abundance of catalases and peroxidases in mammalian tissues would undoubtedly degrade any hydrogen peroxide formed *in vivo* so that very little would reach the tumor cells. Sartorelli and Tsunamura[129] reported that no hydrogen peroxide-induced degradation of pre-existing DNA occured in L-5178y lymphoma cells. Gale and co-workers[130] reported similar results in Ehrlich ascites tumor cells. Gale also found that DNA synthesis was inhibited by Procarbazine to a similar extent in the presence and absence of catalase. The concentrations required for 50% inhibition of DNA synthesis was 2.2×10^{-4} *M* for Procarbazine in the presence of a catalase, 9×10^{-5} *M* for hydrogen peroxide, and 1.5×10^{-3} *M* for methylhydrazine. Furthermore, Gale[130] observed that treatment of tumor cells *in vitro* with solutions of Procarbazine stored for intervals up to 10 days resulted in a 400% increase in peroxide levels after day 4, while resulting in only an 8% increase in the inhibition of DNA synthesis.

Finally, hydrogen peroxide is produced during the oxidation of Procarbazine to the azo compound. Both Procarbazine and the azo exhibit about the same tumor inhibitory effect.[131] Thus, the role of Procarbazine is at most only minor.

Procarbazine can affect cells in several other ways. Gutterman and co-workers[132] reported that a single dose of Procarbazine resulted in a complete block of cellular mitosis of Ehrlich ascites tumor cells implanted intraperitoneally into albino Swiss-Webster male mice. The maximal inhibition occurred after 8 hours. Mitosis remained suppressed beyond the period when DNA synthesis was restored. Rutishauser, et al.[133] studied the in vivo effects of a single Procarbazine dose in a similar animal tumor model. A single dose decreased the percentage of cells undergoing cell division (mitosis) from between 4.9-9.0% to 0.5% depending upon the dose and elapsed time since administration of the drug. The duration of either the period of DNA synthesis (S-phase) or the period between completion of DNA synthesis and the initiation of mitosis (G_2-phase) increased. The antimitotic effects could be observed even at 24 and 48 hours after administration of the drug. However, Llombart and Mínguez[134] reported that there was a direct relation between a decrease in mitotic index (the ratio of the number of cells undergoing division to the total number of cells) and dose and that the cytostatic effect was limited to 12 hours. Between 18 and 24 hours after drug administration, the mitotic index increased above the average pretreatment level just prior to leveling off. The interphase of the cell cycle consisting of the period after cell division (G_1) through S-phase and G_2-phase is the primary target of Procarbazine's action during the cell division process. Llombart and Mínguez[134] reported that the percentage of cells in all phases of cell division was decreased, while Rutishauser[133] found that ana- and telophases remained about the same. Unlike the controls where the number of cells in prophase exceeds those in metaphase, Procarbazine

results in a reversal with metaphase dominating. The duration of metaphase relative to prophase is increased. Llombart and Mínguez [134] occasionally observed mitosis C (blockage of chromosomal division in metaphase and anaphase resulting from interference with the formation of the spindle apparatus in a manner similar to that produced by colchicine) only in those cells already undergoing cell division at the time of Procarbazine administration. These cells disintegrate and never complete mitosis. Rutishauser[133] reported that Procarbazine treatment resulted in breaks in the chromatid (the pair of filaments comprising each chromosome after DNA synthesis) and rejoining of the broken fragments in a random manner. No chromosome breaks occurred. This suggests that the breaks are introduced during or after DNA synthesis. The number of breaks is dependent upon the dose and elapsed time since treatment with Procarbazine.

Huang and Kremer[135] reported that the azo derivative is a potent mitotic inhibitor. If given before active DNA synthesis begins, the azo compound can completely suppress lymphocyte transformation at a concentration of 3×10^{-4} M. No effect was observed if the azo was given after DNA synthesis had begun. This suggests that the azo compound is active in the early phases of cell division.

Llombart and co-workers[136] further studied the antimitotic effects of Procarbazine and presented evidence showing a direct relation between these effects and the alteration of enzyme systems. After a single intraperitoneal injection of 300mg/kg of Procarbazine into Ehrlich ascites-bearing mice, several enzymes of the tricarboxylic acid cycle (Kreb's cycle) were inhibited. This inhibition coincided temporally with the depression of mitosis. NADH (reduced nicotinamide

adenine dinucleotide) and NADPH (reduced nicotinamide adenine dinucleotide phosphate) tetrazolium reductase, lactate dehydrogenase, succinic dehydrogenase, isocitrate dehydrogenase, and cis-aconitase were maximally inhibited at 8 hours after treatment. Both the recovery of the enzymatic and mitotic depressions converged at 24 hours.

Finally, Therman[137] reported that Procarbazine had no *in vitro* effect upon the chromosomes of normal human lymphocytes or mouse spleen cells. Likewise, no *in vitro* effect was observed with Ehrlich ascites or HeLa cells. *In vivo* chromatid translocations (rearrangement of a segment of a chromosome either to a non-homologous chromosome or to a different location on the same or another chromosome) were observed in three different mouse tumors (Ehrlich ascites; 2 BF, and L-1210 leukemia). Normal cells were unaffected. These results suggest that Procarbazine is converted by normal mouse tissue to a compound capable of chromosome breaking. The difference in activity against normal and tumor cells might be the result of (a) selective permeability of the chromosome breaking substance into cancer cells; (b) a smaller level of enzymes capable of destroying the active compound in cancer cells; (c) differences in the rates and extent of DNA repair; or (d) selective activation by cancer cells either of Procarbazine or one of its derivatives.

Procarbazine can cause both single- and double-strand breaks in DNA. Audubert[138] observed that exposure of Procarbazine cells in culture for 2 hours to 11 m*M* of Procarbazine decreased the sedimentation coefficient of DNA on an alkaline gradient from 57 to 45s. At 113 m*M* of Procarbazine, the sedimentation coefficient decreased from 57 to 10s, which corresponds to 1 chain break every 1200 nucleotides. Thus, at the lower concentrations, single-strand breaks occurred and at

the higher concentration, double-strand breaks also were observed. Since the cell culture did not contain a metabolic activating system, the observed activity resulted from either compounds formed during the chemical degradation of Procarbazine or its bioactivation by the tumor cells' enzyme systems. Procarbazine activation resulting from the chemical degradation of Procarbazine was observed by Belova and co-workers.[139] Calf thymus DNA incubated at pH 6.8 and 37°C with 10^{-2} to 10^{-4} M Procarbazine underwent single-strand breakage. These breaks occurred only in the presence of oxygen and not in a nitrogen atmosphere. Using proton nuclear magnetic resonance spectroscopy, they found that Procarbazine is hydrolyzed to release methylhydrazine. Belova and co-workers postulated that Procarbazine can undergo oxidation and hydrolysis in the presence of DNA and that the subsequent oxidation of methylhydrazine may result in DNA strand scission.

Besides degradation of DNA, the N-methyl group of Procarbazine can become incorporated into nucleic acids and proteins. Kreis and Yen[140] observed a rapid uptake of ^{14}C-methyl label into the acid soluble fraction of P815 leukemia implanted intraperitoneally into BDF_1 mice. The labeled Procarbazine was also administered intraperitoneally. The radioactivity of the acid soluble fraction decreased linearly for 24 hours, while the radioactivity in DNA correspondingly increased in almost identical fashion. Radioactive incorporation into ribonucleic acid (RNA), phospholipids, and proteins increased for 5 hours and then plateaued. Analysis of the DNA showed that only adenine, guanine, and thymine contained radioactivity.

Incorporation can occur by either direct transfer of the methyl group (alkylation) or by oxidation of the methyl group to formaldehyde

which can subsequently enter the formate pool.

This incorporation may occur through the formation of a methyldiazonium ion intermediate which alkylates nucleophilic functionalities such as phosphate, amino, sulfhydryl, hydroxyl, carboxyl, or imidazole groups present on biomacromolecules.[141] Purine bases are the primary targets of alkylation, althogh pyrimidines also can be alkylated. The N-7 position of the guanine is strongly nucleophilic and is the most frequently attacked. The N-1 and N-3 positions of adenine, N-3 position of cytosine, and the O-6 position of guanine are less frequently attacked. The classical alkylating agents, such as nitrogen mustards, are bifunctional alkylators. Procarbazine is not cross-resistant with these classical alkylating agents and structurally does not possess the ability to act as a bifunctional alkylating agent. Instead, Procarbazine is apparently one of the few monoalkylating antineoplastic agents. Methylation can result in abnormal base-pairing in DNA, misreading of the DNA template during replication, depurination of the DNA which leads to strand scission of the depurinated DNA chain, or ring cleavage of the methylated purine ring.[141]

The N-methyl group can undergo oxidation and enter the formate pool and into the normal pathway for de novo synthesis of purines and pyrimidines. The N-methyl group can also indirectly methylate through the homocysteine methionine guanine route.[142]

Weitzel and co-workers[109] observed that oxidation of Procarbazine with aqueous potassium hexacyanoferrate (III) gave formaldehyde. They suggested that azomethines and N-hydroxymethyl derivatives from the reaction of formaldehyde with endogenous succinimide and diketopiperazine were responsible for the cytostatic activity of Procarbazine.

Brookes and Lawley[143] reported observing both entrance into the formate pool and methylation by the N-methyl group. HeLa cells growing in culture used Procarbazine-derived formaldehyde in de novo purine biosynthesis. Cellular protein contained little radioactivity, while both DNA and RNA were significantly labeled. Approximately 10 Procarbazine N-methyl groups were associated with each RNA molecule of molecular weight of 2×10^6 daltons and 30 methyl groups with each DNA molecule of molecular weight of 7×10^6 daltons. Almost all of the radioactivity was in normal purines, mainly guanine and adenine. No significant alkylated purines, namely 7-methyl guanine were found. No alkylation by the N-methyl group occurred. Using Landschultz tumor cells implanted in mice, they found significant amounts of labeled 7-methyl guanine after administration of methyl-labeled Procarbazine. Approximately 1 methylated guanine group occurred per 7×10^6 molecular weight of DNA. This corresponds to about 1.2×10^6 alkylated guanine moieties in a mammalian cell containing 10^{13} daltons of total DNA. Brookes and Lawley[143] concluded that this amount is too little to be lethal and probably results in mutation.

Kreis[131,140,142,144] studied the incorporation of methyl-radiolabeled Procarbazine into RNA in an attempt to evaluate the relative roles of alkylation and its effects upon de novo purine synthesis. After an intraperitoneal injection of $^{14}CH_3$-labeled Procarbazine into P-815 ascites-bearing mice, trace amounts of radioactivity were found in methylated purines of cytoplasmic RNA. Though the major methylated base was 7-methylguanine, other methylated bases included thymine, 5-methylcytosine, 1-methylguanine, and 1-methyladenine (tentative identification). Transfer RNA was the major site labeled 2 and 4 hours after the injection

of Procarbazine. Analysis of the ratio of the specific radioactivity of methylated (7-methylguanine, 1-methylguanine, and 1-methylhypoxanthine) versus the non-methylated purines found in the urine after Procarbazine administration as compared to after sodium formate-^{14}C administration suggests that Procarbazine either directly alkylates the purine bases or transfers its methyl group via the homocysteine ⟶ methionine ⟶ purine route. Administration of Procarbazine labeled with both ^{14}C and ^{3}H in the N-methyl groups showed that in the case of 7-methylguanine and, possibly, 5-methylcytosine, the transfer of the intact methyl group occurs. The transfer of the intact methyl group is only a minor pathway. The major pathway for incorporation of the N-methyl group into purines is through the formate pool. Only in the case of 7-methylguanine is there sufficient evidence for the transfer of the intact methyl group of Procarbazine. Injection of a mixture of L-methionine-$C^{3}H_3$ and Procarbazine $^{14}CH_3$ showed that only 7-methylguanine was extensively methylated by Procarbazine through transfer of the intact N-methyl group. Furthermore, experiments using the mixture of methionine and Procarbazine showed that methylation of guanine at the N-7 position is not merely additive. Procarbazine selectively overmethylates guanine in comparison to administration of methionine alone. This results in an abnormal excess of 7-methylguanine in cytoplasmic RNA. This over methylation of guanine may lead to methylation at RNA sites normally not methylated and could disrupt the normal pattern of methylation. Although the effect of this selective overmethylation is not known, Kreis suggested that it might affect the release of the RNA from the DNA template or might also affect the DNA replication or protein synthesis. Furthermore, Revel and Littauer[145]

reported that the lack of methylation of t-RNA leads to alteration in its function so that one might expect overmethylation to also affect its function.

In addition to either direct alkylation or incorporation via the formate pool, several groups presented evidence for the formation of methyl free radicals from Procarbazine *in vivo*. Dost and Reed[120b] studying the *in vivo* metabolism of N-methyl-radiolabeled Procarbazine in rats found significant amounts of methane in the respiratory air. They postulated that the importance of the methyl group to Procarbazine's antineoplastic activity might be due to the transitory existence of methyl free radicals. Further support for the formation of free radicals is provided by the observation by Berneis, *et al.*[146] of a synergism between ionizing radiation and Procarbazine. Schwartz and co-workers,[147] after observing methane formation, suggested that the azo compound could serve as the intermediate to the free radical formation with the liberation of nitrogen gas. However, the azo compound would not be expected to be cleaved to methyl free radical under physiological conditions because of its chemical stability. Instead, enzymatic catalysis of the cleavage would be necessary. Baggiolini and co-workers[97] proposed that the azo compound is hydroxylated on the benzylic carbon by microsomal hydroxylase. This intermediate decomposes to the aldehyde and methyldiazene free radical. Methyldiazene free radical rapidly decomposes to liberate the methyl free radical and nitrogen gas. The methyl radical could react with other cellular components including purines. This could account for some of Procarbazine's carcinostatic activity.

Procarbazine can inhibit *de novo* RNA synthesis. Kreis[131,144c] reported that a single intraperitoneal injection of Procarbazine into

P-815 ascites-bearing mice followed by an intraperitoneal injection of tritiated uridine resulted in a large decrease in tumor t-RNA synthesis after 2 hours of pretreatment. If 24½ hours elapsed between pretreatment and uridine administration, almost no inhibition occurred. Nuclear RNA synthesis was inhibited by only 50% of that occurring to t-RNA synthesis. In most cases, nuclear RNA synthesis increased. The inhibition of tumor t-RNA synthesis is preceded by the inhibition of normal t-RNA methylation. When Kreis administered unlabeled Procarbazine followed 1 hour later by a mixture of ^{14}C-methyl-labeled methionine and tritiated uridine, methylation was already inhibited while de novo synthesis was increased by 38%. If the mixture was given after 2½ hours, inhibition of synthesis was detected. The de novo synthesis of t-RNA returned to 94.5% of normal 24 hours after administration of the mixture. Methylation was still 29% inhibited.

Several other groups reported similar inhibition of RNA synthesis. Gale, et al.[130] reported that a 3-day old catalase-treated Procarbazine solution administered with tritiated uridine depressed RNA synthesis in Ehrlich ascites tumor cells by 28%. If the isotopic precursors were administered 3 hours after the Procarbazine, 73% inhibition occurred. Sartorelli and Tsuanamura[129,148] found that a single 300mg/kg. dose of Procarbazine given intraperitoneally to L-5178y lymphoma-bearing mice depressed the incorporation of orotic-6-^{14}C acid into RNA uracil by 65% if the precursor was given 3 hours later. No inhibition was detected if 12 hours elapsed before administration of the labeled orotic acid. Furthermore, a metabolic imbalance resulted with accumulation of cellular RNA. Weitzel, et al.[149] found that ^{14}C-uridine incorporation by lymphatic leukemia cells was inhibited by Procarbazine,

the azo compound, and nor-Procarbazine. They suggested that RNA synthesis was inhibited by the N-isopropylamide group in the para-position of the Procarbazine aromatic ring. N-Isopropyl-p-formyl-benzamide (aldehyde) and N-isopropyl-p-hydroxymethylbenzamide (alcohol) also inhibited this incorporation. They further hypothesized that the N-isopropylamide substitution at the para-position blocked nucleoside and purine transport into the cell, while the aldehyde inhibited the formation of the di- and tri-phosphates of the nucleoside. However, Gutterman and his fellow workers[132] found that a single 200 mg/kg dose of Procarbazine given to Ehrlich ascites-bearing mice transitorily enhanced the incorporation of tritiated uridine into RNA. This enhancement reached a maximum if the labeled Precursor was given 8-16 hours after Procarbazine. If given 42 hours after Procarbazine, the levels of incorporation had returned to normal. Also, like Gale's finding,[130] a freshly prepared Procarbazine solution had no effect when the cells were treated *in vitro*.

Procarbazine not only inhibits RNA synthesis, but also has been shown to inhibit DNA and protein synthesis. Gutterman, *et al.*[132] found that a single dose of 200 mg/kg of Procarbazine given to Ehrlich ascites-bearing mice inhibited the incorporation of tritiated thymidine into DNA. This inhibition reached a maximum of 35-40% when the *in vivo* treated cells were pulsed *in vitro* 4-8 hours later with labeled precursor. After an 8 hour lapse between Procarbazine treatment and the administration of the labeled precursor, the thymidine utilization returned to normal. After 72 hours, no difference from control remained. The inhibition of tritiated arginine incorporation into protein was delayed and reached a maximum at a 16 hour interval.

No inhibition occurred prior to 8 hours or after 48 hours after Procarbazine treatment. Similar results were reported by Sartorelli and Tsunamura[129,148] using a single 300 mg/kg dose given to L-5178y lymphoma-bearing mice. Inhibition of thymidine incorporation reached a maximum of 70% at the 1-3 hour interval. Inhibition was at 37% at 12 hours and was totally absent after 24 hours. This inhibition was apparently dose related and is not due to inhibition of thymidine kinase, monophosphate kinase, or monophosphate nucleotidase, or DNA nucleotidyl-transferase. Administration of formate-^{14}C and deoxycytidine-^{3}H to trace the formation of DNA through both the purine and pyrimidine nucleotide synthetic routes showed that Procarbazine blocks both routes. Furthermore, a mixture of deoxycytidine, deoxyadenine, and deoxyguanosine did not reverse the decrease in DNA synthesis. This showed that the decrease was not due to the unavailability of a supply of one or more of the deoxyribonucleotides. DNA cellular content was also unchanged. d,l-Leucine-1^{14}C incorporation into protein was inhibited 42% after 12 hours. Cellular protein content significantly accumulated during the Procarbazine imbalance of cellular metabolic processes caused by Procarbazine.

Koblet and Diggelmann[149] found no evidence that Procarbazine acts *in vivo* upon DNA or the biosynthesis of *m*-RNA, r-RNA or s-RNA, interaction which could lead to the decrease in protein synthesis. The mechanisms for protein synthesis were normal including the number of polysomes, the ability of ribosomes to accept polyuridylic acid, the ability of enzymes to load s-RNA at pH 5, and the ability of s-RNA to accept amino acids. Although the incorporation of labeled leucine into peptides was decreased in polysomes in Procarbazine-pretreated animals, the loaded s-RNA of pretreated animals could enter normally into the polysomes of untreated animals.

Similar inhibition of DNA and protein synthesis was shown *in vitro*. Rutishauser and Bollag [150] and Hoelzel and co-workers [151] showed that Procarbazine inhibited the uptake of tritiated thymidine *in vitro* by DNA in Ehrlich ascites carcinoma cells which had been pretreated *in vivo*. Gale and co-workers[130] reported that DNA synthesis was depressed 30% and protein synthesis was unaffected when isotopic precursors for DNA and protein synthesis and a 3-day old Procarbazine solution were added simultaneously to Ehrlich ascites tumor cells *in vitro*. However, if Procarbazine was given 3 hours prior to the precursors, DNA synthesis was reduced 92% and protein synthesis 52%. The inhibition of DNA synthesis was reversed by washing with cell culture medium or drug solution. These data suggest that DNA synthesis is not inhibited by alkylation or covalent binding of Procarbazine or one of its degradation or enzyme-catalyzed products in the tumor cells. This inhibition was also not readily antagonized by exogenous nucleosides, DNA, or RNA. Furthermore, Volm and co-workers[152] found that *in vitro* inhibition of nucleic acid synthesis in tumor cell suspensions or in tumor pieces of many tumor lines, including Walker carcinosarcoma, rat adenocarcinoma, hamster melanoma, mouse sarcoma 180, and a number of human tumors, during a 3 hour incubation period correlates with the antineoplastic effectiveness of Procarbazine.

Weitzel[149,153] studied the effects of Procarbazine upon DNA and protein synthesis and postulated which of the metabolites and decomposition products were responsible for the effects. Although non-methylylated hydrazines inhibited ascites tumor growth and reduced the rate of incorporation of ^{14}C-thymidine into DNA and leucine into proteins, the N-methyl hydrazines showed increased activity. The azo

and azoxy derivatives of Procarbazine can undergo metabolic formation of formaldehyde. Procarbazine is oxidized to the azo compound with the formation of hydrogen peroxide. Both formaldehyde and hydrogen peroxide can inhibit DNA polymerase and DNA-dependent RNA polymerase. Formaldehyde also interferes with the synthesis of nucleoside triphosphates and deoxynucleoside di- and triphosphates. Hydrazine inhibits the formation of purine deoxynucleosides. Formylhydrazine (an azomethine) can react with thiols at 20°C and pH 7.2. It reacts with thioglycolic acid (58%), glutathione (24%), and N-acetylcysteine (22%). Formylhydrazine and the N-hydroxymethyl compounds formed from the reaction of formaldehyde with succinimide and diketopiperazine show high cytostatic activity *in vivo* and *in vitro* by affecting both DNA polymerase and DNA-dependent RNA polymerase. These compounds also can inhibit the synthesis of nucleoside triphosphates and appear to act directly upon DNA. Huang and Kremer[135] found that the azo compound could suppress DNA and protein synthesis.

Besides the effects of Procarbazine upon DNA and the synthesis of DNA, RNA, and protein, several reports of other effects have been published. Gutterman and co-workers[132] found no change in oxygen uptake (respiration) or the production of CO_2 from labeled glucose-1-^{14}C or glucose-6-^{14}C in Ehrlich ascites-bearing mice after a single 200 mg/kg dose of Procarbazine. However, aerobic glycolysis was depressed 25%. Since classical alkylating agents have been observed to decrease the levels of NAD and, consequently, inhibit anaerobic glycolysis in tumor cells, Obrecht and co-workers[154] studied the biochemical effects of Procarbazine upon respiration and metabolism in rapidly proliferating normal tissue and Jensen sarcoma tumor cells. A single 200 mg /kg

intraperitoneal dose of Procarbazine, which produces a 40% remission rate for the Jensen tumor, decreased cellular respiration 15%. No decrease in NAD, aerobic glycolysis, or anaerobic glycolysis was observed. In contrast, in normal rapidly proliferating tissue such as intestinal mucosa and testes, Procarbazine decreased NAD and inhibited anaerobic glycolysis. This inhibition results in an accumulation of the phosphorylated intermediates, triose phosphate and fructose-1,6-diphosphate. Respiration was also decreased while aerobic glycolysis increased in the intestinal mucosa due to a disturbed Pasteur effect. These results on the effects of Procarbazine upon metabolism suggest that Procarbazine is not acting as a classical alkylator. However, Weitzel, et al.[109b] studying the in vitro effects of Procarbazine upon Ehrlich carcinoma cells reported that the concentrations of nicotinamide, fructose diphosphate, and triose phosphate increased, while the NAD concentration decreased. These changes peaked 2-4 hours after treatment. After 8-16 hours, glyceraldehyde-phosphate-dehydrogenase was completely inactivated. Thus, contrary to the findings of Obrecht, these data suggest that Procarbazine acts like other alkylators.

II. Synthetic Work

The study of the metabolism and mode of action of Procarbazine requires the synthesis of several groups of compounds. The use of selected-ion monitoring chemical ionization mass spectrometry as an analytical tool necessitates the synthesis of deuterated-analogs of the compounds undergoing analysis. Reported here are the synthesis of N-isopropyl ($^2H_6$) terephthalamic acid (acid-d_6) and N-isopropyl ($^2H_6$)-α-(2-methylhydrazino-p-toluamide (Procarbazine-d_6). N-Isopropyl-p-formylbenzamide (aldehyde), N-isopropyl-p-toluamide, N-isopropyl- -(2-methylhydrazone)-p-toluamide (hydrazone), N-isopropyl-p-hydroxymethyl-benzamide (alcohol), N-isopropyl-α-(2-methylazo)-p-toluamide (azo), and the mixture of N-isopropyl-α-(2-methyl-NNO-and ONN-azoxy)-p-toluamide (benzyl- and methylazoxy) are synthesized for use as reference compounds for the identification of the major metabolic and chemical decomposition products by chromatographic analysis. Several of these compounds are also tested for antineoplastic activity. Finally, N-isopropyl-α-(2-methylhydrazino)-p-($^2H_1$)-toluamide (Procarbazine-d_1) is synthesized for use in a kinetic isotope study to determine if hydroxylation occurs on the benzylic carbon during the metabolism of Procarbazine.

The syntheses of these compounds are based upon previously reported methods for similar compounds. The purpose of each synthetic procedure is to obtain the desired compound in pure form and in sufficient quantities for the biological studies and therefore optimalization of reaction conditions was not always pursued. Better synthetic procedures might be devised in many cases.

Identification and purity of each synthetic product is essential. Chemical-ionization mass spectra of the compounds were obtained using either a Finnigan 3200 or an Associated Electrical Industries MS-902 mass spectrometer equipped with a chemical ionization source. Isobutane was used as the reagent gas. Proton nuclear magnetic resonance (PNMR) spectra were obtained using a Perkin-Elmer R-12B or Varian A-60 NMR spectrometer or a Varian XL-100 Fourier-transform nuclear magnetic resonance spectrometer. A Perkin-Elmer 457 infrared spectrophotometer and a Cary 14 ultraviolet spectrophotometer are also used. Chromatographic analysis are performed using silica gel thin-layer chromatographic plates visualized with U.V. light and a Chromatronix Model 3500 single pump high-performance liquid chromatograph, equipped with a Chromatronix Model 220 mixed-wavelength ultraviolet detection set at 254 nm, a Chromatronix MA-10 20 ul flow cell, and a Waters Associate's 30 cm/3.9 mm I.D. C_{18}-u bonapak reverse-phase column. Melting point determinations are made using a Thomas melting point apparatus.

N-Isopropyl-p-formylbenzamide

The synthesis of N-isopropyl-p-formylbenzamide used thionyl-chloride to form the acid chloride followed by displacement of the chloride by isopropylamine to give the desired product. 4-Carboxy-benzaldehyde, 6.0 g., 0.04 moles (Aldrich Chemical Company) was refluxed 16 hours with approximately 35 ml. of thionyl chloride, 0.48 moles (Aldrich Chemical Company) in a 100 ml. round-bottom flask equipped with a water-cooled condenser and a $CaCl_2$-filled drying tube. Excess thionyl

chloride is distilled at 79°C. The acid chloride, a viscous dark yellow-brown liquid, was mixed with approximately 20 ml of anhydrous ethyl ether. Over a period of 5-10 minutes, approximately 10.2 ml of isopropylamine, 7.1 g , 0.12 moles (Eastman Kodak) dissolved in 10 ml of dry ether was added to the above solution with stirring and cooling. Excess isopropylamine was used since the reaction liberates hydrogen chloride, which forms the insoluble salt of the amine. The resulting mixture was refluxed for 16 hours. The yellow-gray reaction mixture contained some white floculate material which is presumably the isopropylamine salt. Approximately 50 ml of water was added and extracted with 4 aliquots of 20-30 ml of ether. A thin-layer chromatograph of the combined yellow ether extracts (silica gel developed with ethylacetate; hexane, 35:10) showed 4 spots when visualized with ultraviolet light. The major spot, R_f=0.50, corresponds to the desired aldehyde. The second major spot, R_f=0.58, corresponds to the isopropylamine Schiff's base of aldehyde. The Schiff's base is hydrolyzed to the aldehyde by treatment of the residue remaining after evaporation of the ether with a 10% HCl solution at room temperature for 24 hours. This HCl solution is extracted 4 times with 30 ml of ether. The extract was first washed with 5% $NaHCO_3$ to remove any terephthalamic acid, then with water, and finally it was dried over anhydrous Na_2SO_4. The ether was evaporated and the residue was dried in a vacuum dessicator. Recrystallization from ethylacetate and hexane gave 5.66 g , 74% of a white crystalline solid, m.p. 123-125°C. Thin-layer chromatographic analysis showed only a single spot, R_f=0.50. Chemical ionization mass spectrometry showed the expected MH^+ peak of 192. The 60 MHz NMR spectrum is shown in Figure 9. The NMR data corresponds to that expected for the aldehyde: aldehydic proton

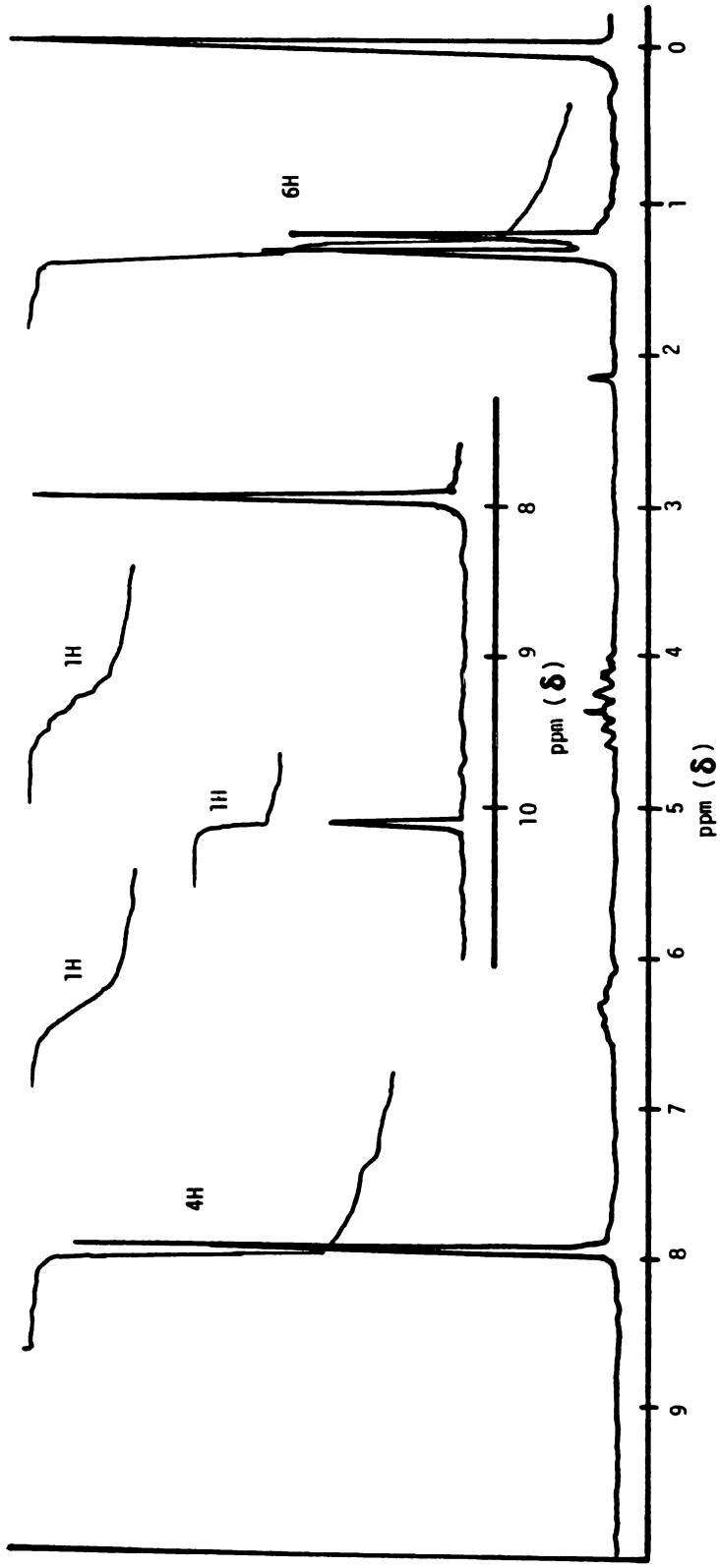


Figure 9. NMR Spectrum of N-Isopropyl-p-formylbenzamide.

Sample dissolved in $CDCl_3$ with tetramethylsilane as the internal reference. Instrument used was a Perkin-Elmer R-12B 60 MHz. NMR.

at 10.1 p.p.m. (1H, singlet); aromatic protons at 7.95 (4H's, singlet); amide proton at 6.2 (1H, broad); isopropyl methine proton at 4.3 (1H, multiplet, J=6.5 Hz); and the isopropyl methyl protons at 1.3 (6H's, doublet, J=6.5 Hz). The infrared spectrum was run as a KBr pellet and is shown in Figure 10. The aldehyde at a concentration of 1 mg /100 ml in ethanol had an ultraviolet absorption maximum of 248 nm.

N-Isopropyl ($^2H_6$)-p-formylbenzamide

The synthesis of the d_6-analog followed the above precedure using isopropyl ($^2H_6$) amine. Isopropyl ($^2H_6$) amine·HCl, 2.5 g , 0.03 moles was dissolved in 5 ml. of water and added to a stirred 10 ml. solution of 2.65 g of Na_2CO_3 (0.03 moles) cooled by an ice bath. The reaction vessel was equipped with a dry ice-acetone cold-finger condenser to prevent the loss of the volatile free-amine. 4-Carboxybenzaldehyde acid chloride, 1.85 g , 0.01 moles dissolved in approximately 20 ml of ether was slowly added. The resulting biphasic mixture was stirred at room temperature for 6 hours. The yield of pure white recystallized solid was 0.6 g (26%). The chemical ionization mass spectrum contained the correct MH^+ peaks at 198.3. The isotopic purity was 100% since no deuterated aldehyde was observed in the mass spectrum. The d_6-aldehyde was subsequently used to synthesize N-isopropyl ($^2H_6$)-α-(2-methyl-hydrazone)-p-toluamide as described later. The NMR analysis of the hydrazone confirmed that no residual isopropyl methyl protons remained. The deuterated and non-deuterated aldehyde had the same R_f when analyzed by thin-layer chromatography.

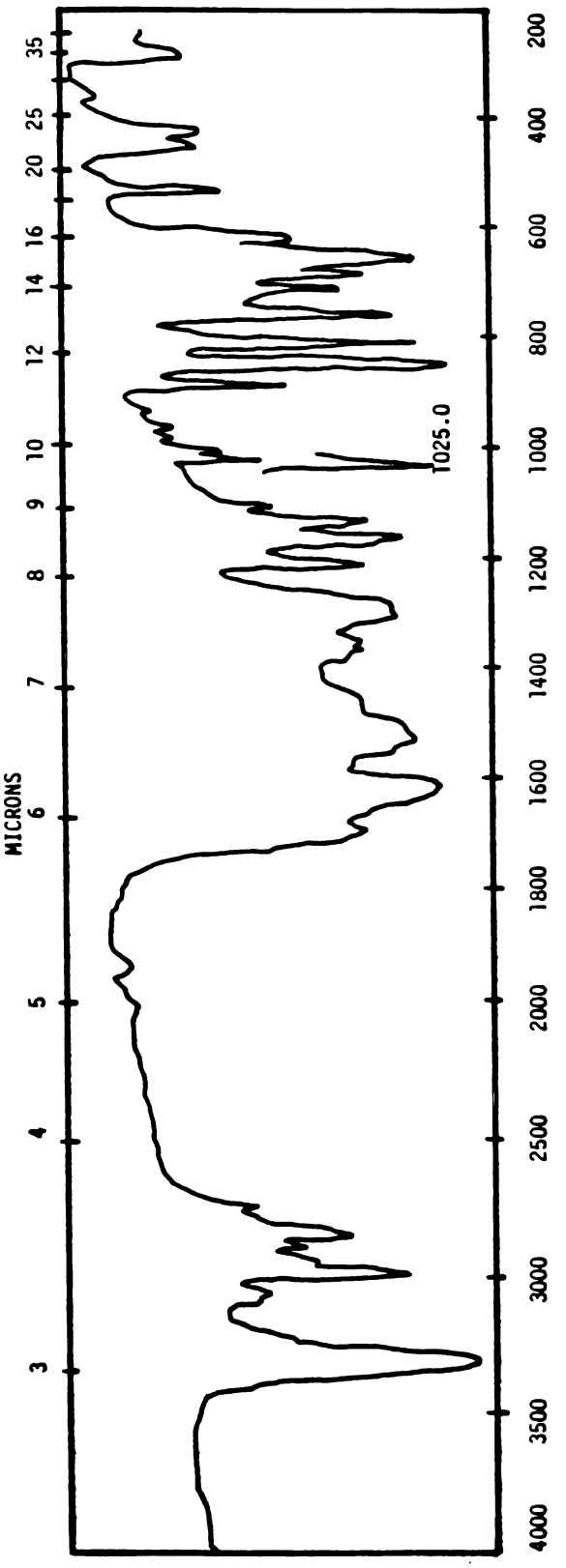


Figure 10. Infrared Spectrum of N-Isopropyl-p-formylbenzamide

The sample was run as a KBr pellet. Polystyrene was the reference.

Isopropyl ($^2H_6$) amine

The synthesis of isopropyl ($^2H_6$) amine followed the procedure used by Colombini and co-workers[155] for the synthesis of ^{14}C-labeled isopropylamine. Hydroxylamine hydrochloride, 5.97 g , 0.09 moles (Eastman-Kodak); 8.1 ml of carbonate-free sodium hydroxide, 3.4 g , 0.09 moles (Dilut-It Analytical Concentrate 5N, J.T. Baker Chemical Company); and 20 ml of distilled water were stirred with cooling with an ice bath. Acetone ($^2H_6$), 5.0 g , 0.09 moles (Gold Label, 99.5 atom % D, Aldrich Chemical Company) was added slowly and stirring was continued for 24 hours. A heavy white precipitate formed after several hours. The aqueous mixture was extracted 5-times with 10 ml portions of ether. The ether solution was dried over anhydrous $MgSO_4$, filtered, and the ether filtrate evaporated using a rotary evaporator to give a white crystalline solid. This solid sublimes at room temperature. The yield of the acetone ($^2H_6$) oxime was 4.16 g (67%). A cold ethereal solution of the oxime was slowly added to a cold solution of 8.17 g , 0.22 moles of $LiAlH_4$ (Alfa-Ventron) suspended in 60 ml of dry ether. The temperature was kept at 0^oC. using an ice bath. The resulting solution was refluxed with stirring under nitrogen for 3 hours using a dry ice-acetone cold-finger condenser to prevent the loss of the volatile isopropyl ($^2H_6$) amine. Excess $LiAlH_4$ was slowly and carefully destroyed by adding dilute hydrochloric acid over a period of about 2 hours, while cooling using an ice bath. The ether layer was removed by rotary evaporation. An excess of 20% NaOH was added to the residue through a dropping funnel and the free isopropyl ($^2H_6$) amine was distilled at $33-34^oC$ into a falsk containing frozen concentrated hydrochloric acid cooled in a dry ice-acetone bath. After lyophilization, 2.4 g (27%) of the white powdered isopropyl ($^2H_6$) amine was recovered, m.p. $148-151^oC$ (literature m.p.= 139.5, $153-5^oC$ [156]).

N-isopropyl-p-toluamide

N-Isopropyl-p-toluamide was synthesized by the same methods used for the synthesis of N-isopropyl-p-formylbenzamide. Isopropylamine hydrochloride, 0.37 g , 0.004 moles was mixed with 15% NaOH and extracted several times with approximately 50 ml of ether. The ether layer was dried over anhydrous $MgSO_4$, filtered, and added slowly to a stirred solution of p-toluoylchloride, 0.21 g , 0.001 moles (Aldrich Chemical Company) dissolved in 50 ml. of anhydrous ether. The reaction mixture was refluxed for 16 hours. Thin-layer chromatographic analysis (silica gel, ethylacetate:hexane, 35:10) gave a single spot, R_f=0.63. The ether solution was washed 3-4 times with 10 ml. of water, twice with 10 ml of 10% potassium bicarbonate to remove any toluic acid side-product, and finally, with 3-4 25 ml aliquots of water. The ether layer was dried over anhydrous $MgSO_4$, filtered, and evaporated using a rotary evaporator. The resulting white residue was recrystallized from a minimal amount of benzene. Only the first batch of crystals was collected to give 0.11 g (46%) of a fine white crystalline solid, m.p. 131-131.5°C. The chemical ionization mass spectrum had the MH^+ peak at 178.3.

N-Isopropyl ($^2H_6$)-p-toluamide

N-Isopropyl ($^2H_6$)-p-toluamide was synthesized using the same procedure with isopropyl ($^2H_6$) amine. After recrystallization from ethylacetate-hexane and washing with cold hexane, the first batch contained white crystalline solid in a yield of 22%. The melting point and TLC analysis were the same as the non-deuterated compound. The chemical ionization mass spectrum gave the MH^+ peak at 184.3. A small

amount of the crystalline solid was analyzed by 100 MHz. Fourier-transform NMR spectroscopy. The NMR spectrum shown in Figure 11 shows no residual isopropyl methyl protons. The NMR data corresponds to that expected for N-isopropyl ($^{2}H_{6}$)-p-toluamide: aromatic protons at 7.5 p.p.m. (4H's, pair of doublets, J=4.0 Hz.); amide proton at 5.8 (1H, broad); isopropyl methine proton at 4.2 (1H, singlet); and benzylic protons at 2.4 (3H's, singlet).

N-Isopropyl-α-(2-methylhydrazone)-p-toluamide

The synthesis of N-isopropyl-α-(2-methylhydrazone)-p-toluamide was achieved through the use of a modified procedure of the method described by Todd[157] for the synthesis of benzaldehyde methylhydrazone. N-Isopropyl-p-formylbenzamide synthesized as previously described, 1.0 g , 0.005 moles was dissolved in 50 ml of absolute ethanol. Methylhydrazine, 0.48 g , 0.01 moles (Aldrich Chemical Company) dissolved in 15 ml of absolute ethanol was added dropwise with stirring after flushing the flask with nitrogen gas. The reaction mixture was refluxed with stirring under nitrogen for ½ hour. Analysis of the mixture by TLC (silica gel, ethylacetate:hexane, 35:10) showed that no aldehyde remained. Only a single more polar spot with a R_f=0.43 appeared. The ethanol was removed by rotary evaporation and the remaining white residue was washed with dry ether, filtered, and dried in a vacuum dessicator. The yield was 0.97 g (84%). The white crystalline solid gave a single TLC spot, R_f=0.43. The hydrazone did not cleanly melt. At 137°C , it turned pale yellow. At 157-60°C , it softened but did not completely melt even at temperatures exceeding 220°C. The chemical ionization mass spectrum showed the MH^+ peak at 220. The

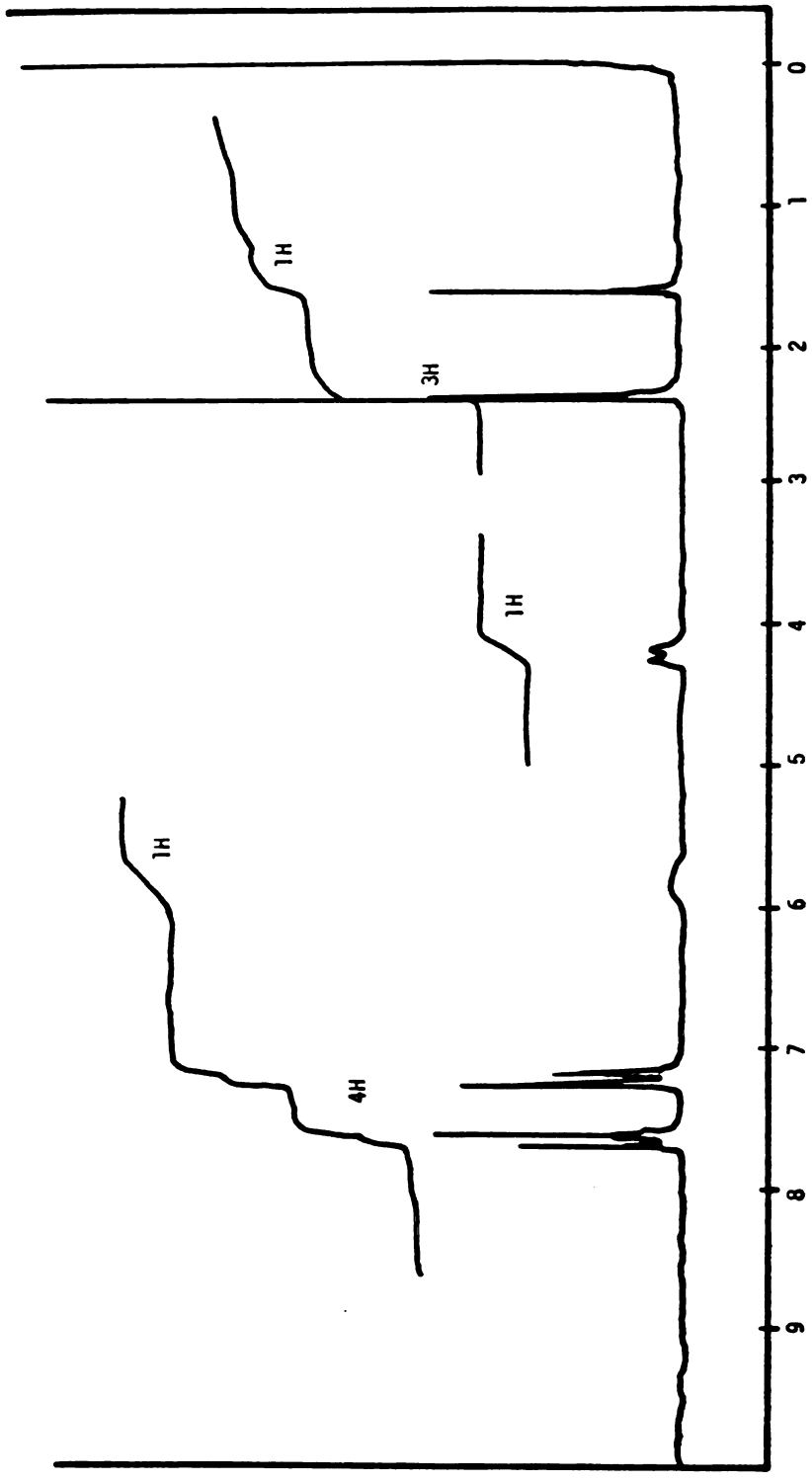


Figure 11. NMR Spectrum of N-Isopropyl($^2H_6$)-p-toluamide

Sample dissolved in $CDCl_3$ with tetramethylsilane as the internal reference. Instrument used was a Varian XL-100 FT 100 MHz. NMR.

60 MHz. NMR spectrum shown in Figure 12 corresponds to that expected for the hydrazone: aromatic protons at 7.65 p.p.m. (4H's, doublet, J=3.5 hz.); imine proton at 7.4 (1H, singlet); amide and hydrazine protons at 5.3 and 6.0 (2H's, broad); isopropyl methine proton at 4.3 (1H, multiplet, J=6.5 Hz.); methyl proton at 2.95 (3H's, singlet); and the isopropyl methyl protons at 1.3 (6H's, doublet, J=6.5 Hz.). The KBr pellet infrared spectrum shown in Figure 13 shows the absence of the aldehyde carbonyl C = O stretching band at 1700 cm^{-1}.

N-Isopropyl ($^2H_6$) -α-(2-methylhydrazone)-p-toluamide

N-Isopropyl ($^2H_6$)-α-(2-methylhydrazone)-p-toluamide was synthesized using the same procedure with N-isopropyl ($^2H_6$)-p-formylbenzamide. The yield of white crystalline solid was 91%. The 100 MHz. Fourier-transform NMR spectrum is shown in Figure 14. The spectrum shows the absence of any residual isopropyl methyl protons at 1.3 p.p.m. The chemical ionization mass spectrum gave the expected MH^+ peak at 226. A KBr pellet infrared spectrum shown in Figure 15 shows the absence of the characteristic isopropyl strong doublet for the methyl C-H bending bands at 1385-1380 cm^{-1} and 1370-1365 cm^{-1}.

N-Isopropyl-p-hydroxymethylbenzamide

The synthesis of N-isopropyl-p-hydroxymethylbenzamide was accomplished through the sodium borohydride reduction of the corresponding aldehyde as outlined by Chaikin and Brown.[158]

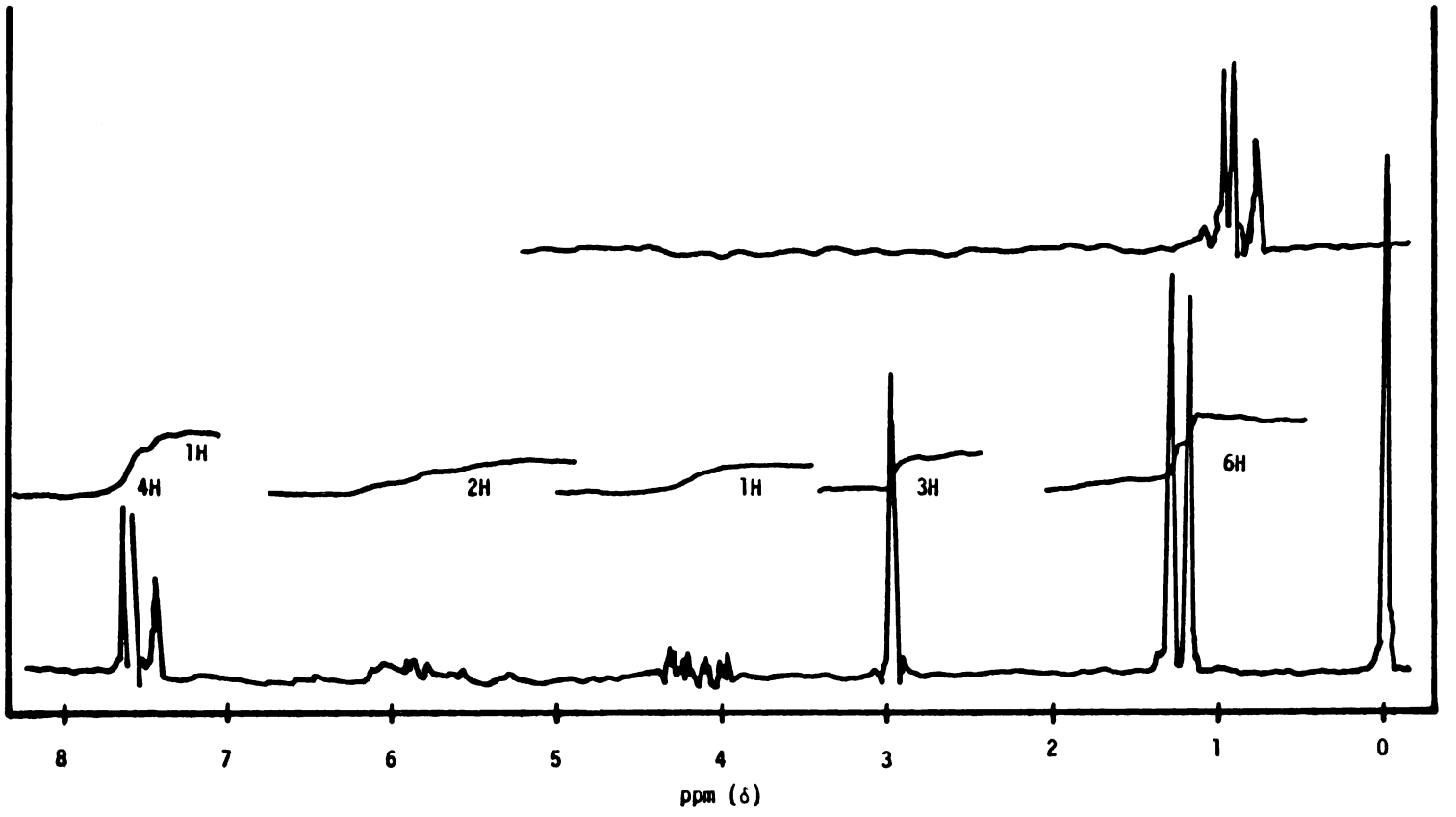


Figure 12. NMR Spectrum of N-Isopropyl-α-(2-methylhydrazone)-p-toluamide.

Sample dissolved in $CDCl_3$ with tetramethylsilane as the internal reference. Instrument used was a Varian A-60 60MHz. NMR.

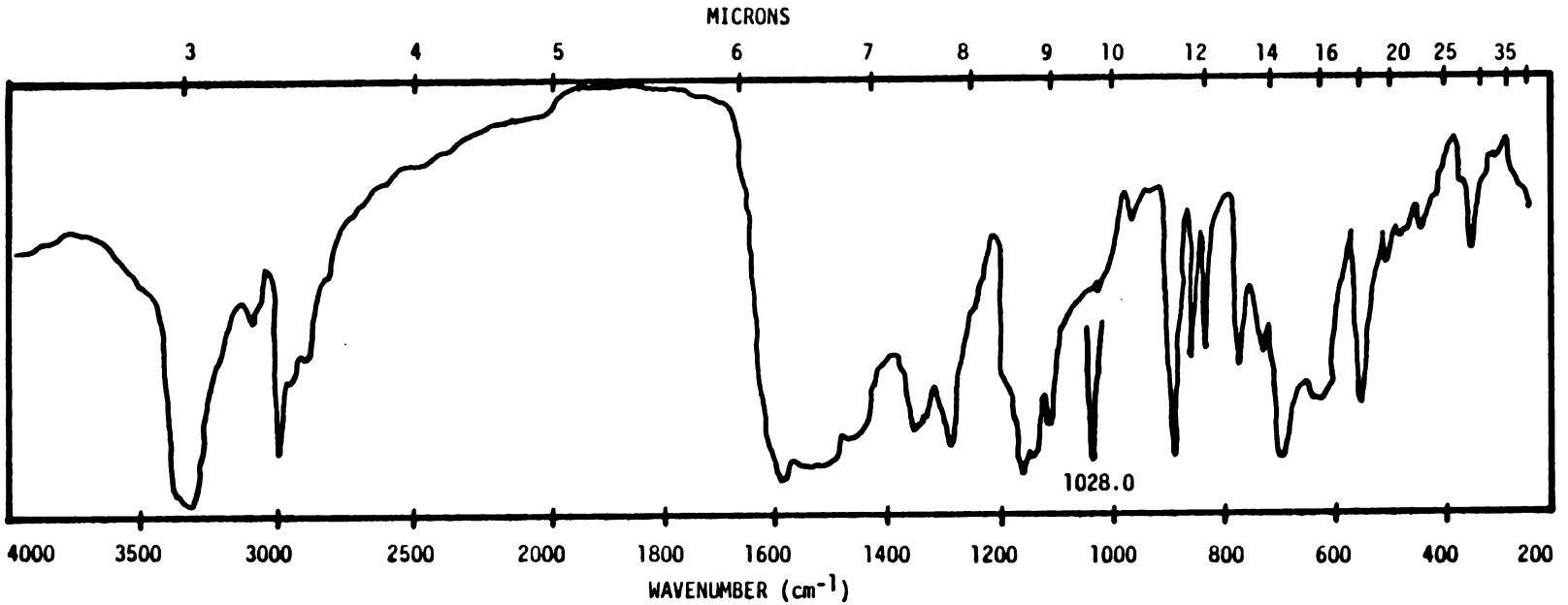


Figure 13. Infrared Spectrum of N-Isopropyl-α-(2-methylhydrazone)-p-toluamide.

The sample was run as a KBr pellet. Polystyrene was the reference.

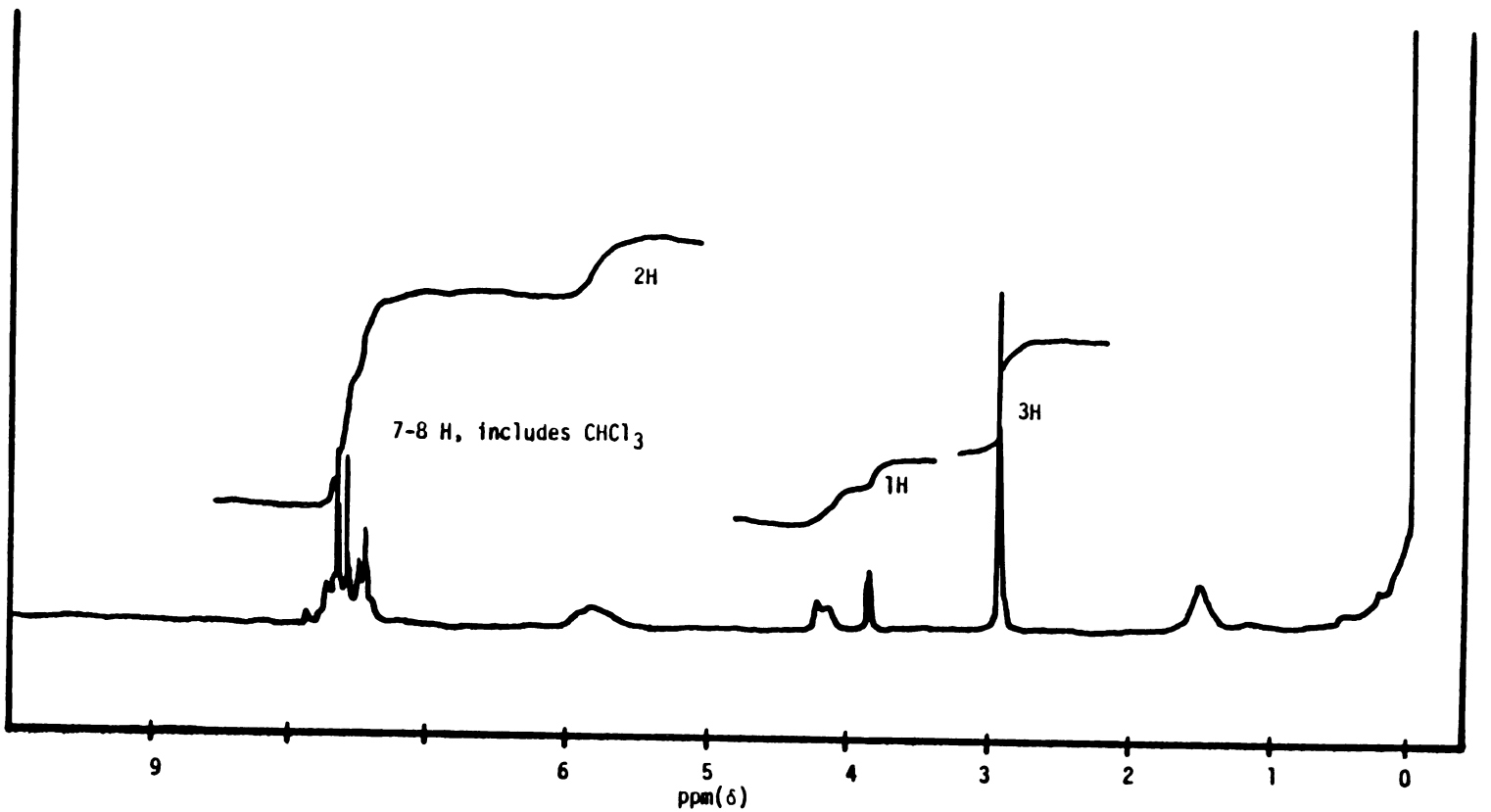


Figure 14. NMR Spectrum of N-Isopropyl($^2H_6$)-α-(2-methylhydrazone)-p-toluamide.

Sample dissolved in $CDCl_3$ with tetramethylsilane as the internal reference. Instrument used was a Varian XL-100 FT 100 MHz.NMR.

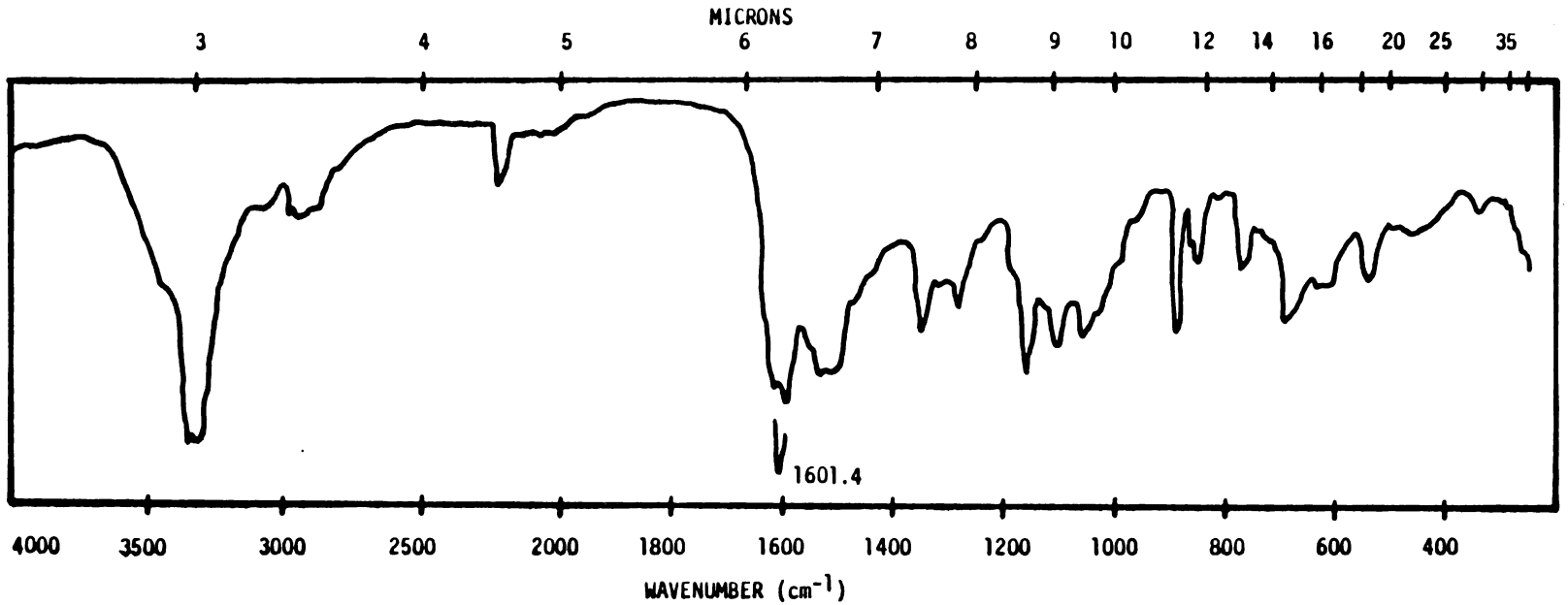


Figure 15. Infrared Spectrum of N-Isopropyl($^2H_6$)-α-(2-methylhydrazone)-p-toluamide.

The sample was run as a KBr pellet. Polystyrene was the reference.

N-Isopropyl-p-formylbenzamide prepared as previously described, 0.1 g , 0.0005 moles was dissolved in methanol and combined with 0.04 g , 0.0001 moles of $NaBH_4$ (Alfa-Ventron) in methanol. After stirring at room temperature for 15 minutes a chemical ionization mass spectrum showed the MH^+ peak at 194 and no residual aldehyde peak. Distilled water was added to the reaction mixture to facilitate ether extraction. The ether extract was dried over anhydrous $MgSO_4$ and the ether was removed by rotary evaporation. The resulting white waxy residue was obtained in essentially quantitative yield. The chemical ionization mass spectrum gave the expected MH^+ peak at 194. The alcohol was dissolved in some methanol and analyzed by reverse-phase high-performance liquid chromatography (20% methanol:water, flow rate of 2.0 ml/minute). The alcohol chromatographed as a single peak with a ratio of the elution time for the alcohol: elution time for the internal standard 4-methylacetophenone of 0.15.

N-Isopropyl-α-(2-methylazo)-p-toluamide

The synthesis of N-isopropyl-α-(2-methylazo)-p-toluamide was performed using a modification of the procedure described by Tsolis, et al.[159] for the synthesis of methylazo-α-phenylethane. The synthesis was done in a glove box under nitrogen atmosphere since chemically the azo compound can isomerize in the presence of oxygen to the more stable hydrazone. All of the solvents and solutions used were depleted of oxygen by vigorously bubbling nitrogen gas through the solution for 15 minutes. Procarbazine hydrochloride 0.1 g , 0.0006 moles (Obtained from Dr. W. E. Scott, Hoffmann-La Roche, Incorporated) was dissolved into 20 ml of 5% potassium bicarbonate. The Procarbazine free-base

extracted with 5 portions of approximately 10 ml. of methylene chloride. After the extraction, the chemical ionization mass spectral analysis showed the presence of the free-base of Procarbazine in the organic layer. The methylene chloride layer was dried over anhydrous $MgSO_4$. This solution was filtered and combined with 0.18 g (0.0009 moles) of red mercuric oxide (Alfa-Ventron) in a 100 ml 3-armed round-bottom flask equipped with a gas inlet tube, water-cooled condenser with a gas outlet tube, and a septum to allow sampling. The reaction flask was flushed with nitrogen gas prior to removing it from the glove box. The reaction was stirred for 1½ hours. The red mercuric oxide turned a gray-black color as the reaction proceeded due to its reduction to elemental mercury. After 1½ hours, chemical ionization mass spectrometric analysis showed the MH^+ peak at 220.2 and no residual Procarbazine. After filtering the solution and rotary evaporation, 0.05 g (41%) of a pale-yellow residue remained. The pale-yellow powder melted sharply at 109-111°C (literature M.P. = 111-115°C[160]). Mass spectral analysis gave the expected MH^+ peak at 220.2 along with a large peak at 177.2. This latter peak corresponds to the tropylium ion fragment. Thin-layer chromatographic analysis (silica gel, ethyl-acetate; hexane, 35:10) gave a faint spot corresponding to the hydrazone and a large spot with a R_f = 0.59. The 60 MHz. NMR spectrum shown in Figure 16, shows that some isomerization to the hydrazone occurred during the reaction as residual peaks for the hydrazone methyl group at 3.0 p.p.m. and imine proton at 7.4 p.p.m. are present. The remaining peaks correspond to that expected for the azo compound: aromatic protons at 7.7. p.p.m. (4H's, quartet, J=8.0 Hz.); amide proton at 6.0 (1H, broad); benzylic protons at 5.0 (2H's, singlet); isopropyl methine proton at 4.3 (1H, multiplet,

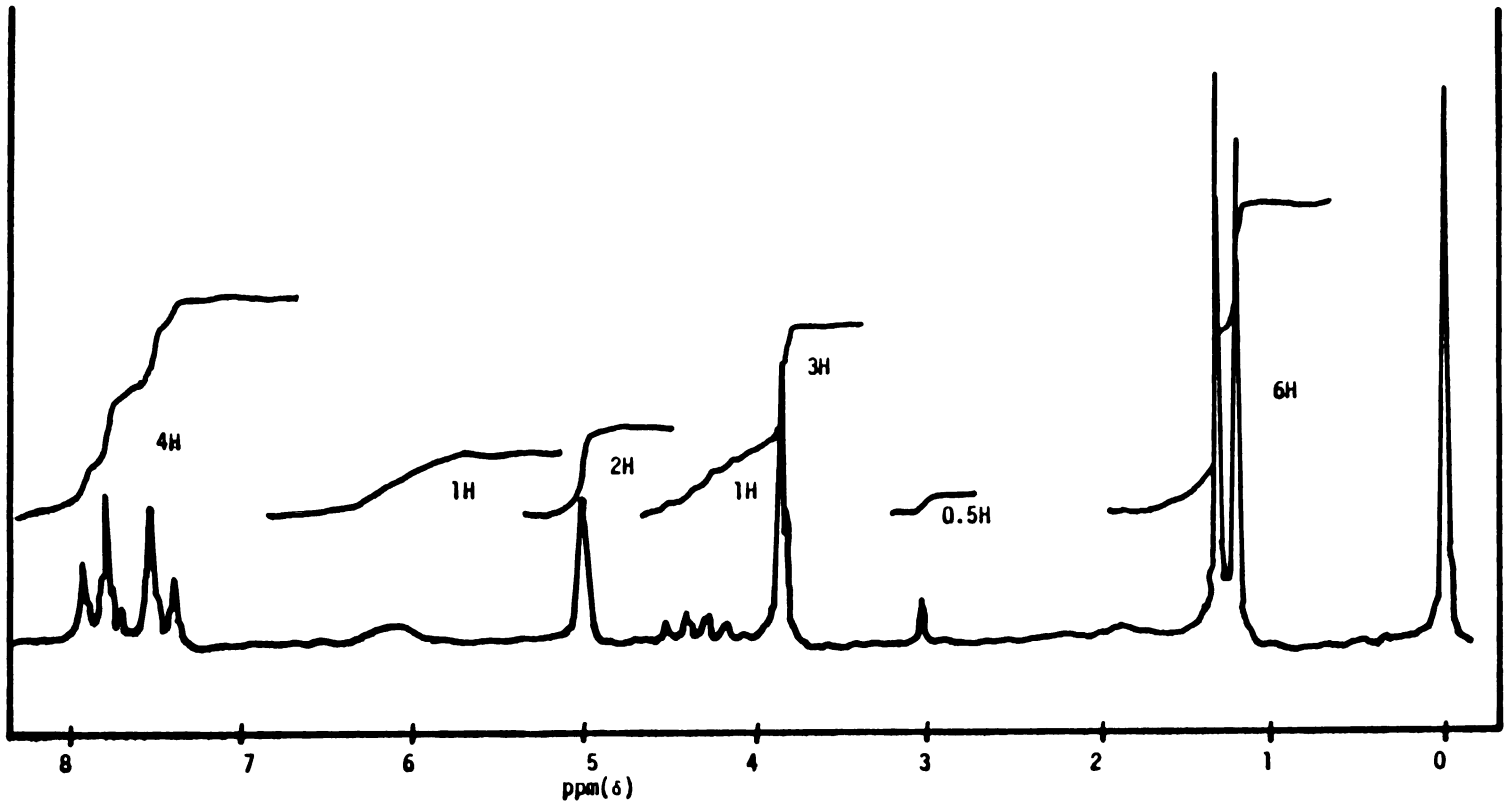


Figure 16. NMR Spectrum of N-Isopropyl-α-(2-methylazo)-p-toluamide.

Sample dissolved in $CDCl_3$ with tetramethylsilane as the internal reference. Instrument used was a Varian A-60 60MHz. NMR.

J = 6.5 Hz.); methyl protons at 3.8 (3H's, singlet); and the isopropyl methyl protons at 1.25 (6H's, doublet, J = 6.5 Hz.). The infrared spectrum, run as a KBr pellet, is shown in Figure 17. A comparison of this I.R. with that for the hydrazone shown in Figure 15 clearly shows this compound is not the hydrazone. The arrows shown on the I.R. show peaks due to the presence of a small amount of the hydrazone.

N-Isopropyl-α-(2-methylazoxy)-p-toluamide

The synthesis of N-isopropyl-α-(2-methylazoxy)-p-toluamide was achieved through modification of the procedure of Brough, et al.[77b] for the synthesis of mixed aliphatic-aromatic azoxy compounds. m-Chloroperbenzoic acid was used in place of perbenzoic acid as the oxidizing agent. All of the synthetic steps were performed in a glove box under nitrogen atmosphere, unless otherwise indicated. All of the solvents were depleted of oxygen by vigorous bubbling of nitrogen gas. The azo compound, 2.0 g , 0.01 moles, synthesized in the previous section was dissolved into 25 ml of dry oxygen-free dichloromethane in a 50 ml. round-bottom flask equipped with a gas inlet tube, dropping funnel, and a water-cooled condenser with a gas outlet tube. After the flask was flushed with nitrogen and cooled with an ice bath to $0^{o}C$, 2.3 g (0.014 moles) of m-chloroperoxybenzoic acid (Aldrich Chemical Company) in 20 ml of dichloromethane was slowly added with swirling over a period of 10 minutes. The flask was removed from the glove box and stirred at room temperature under nitrogen. After 1½ hours, an aliquot was analyzed by mass spectrometry. The expected peak at MH^{+} of 236 was the major peak. No azo remained unreacted. Iodide-starch paper gave a positive indication of excess

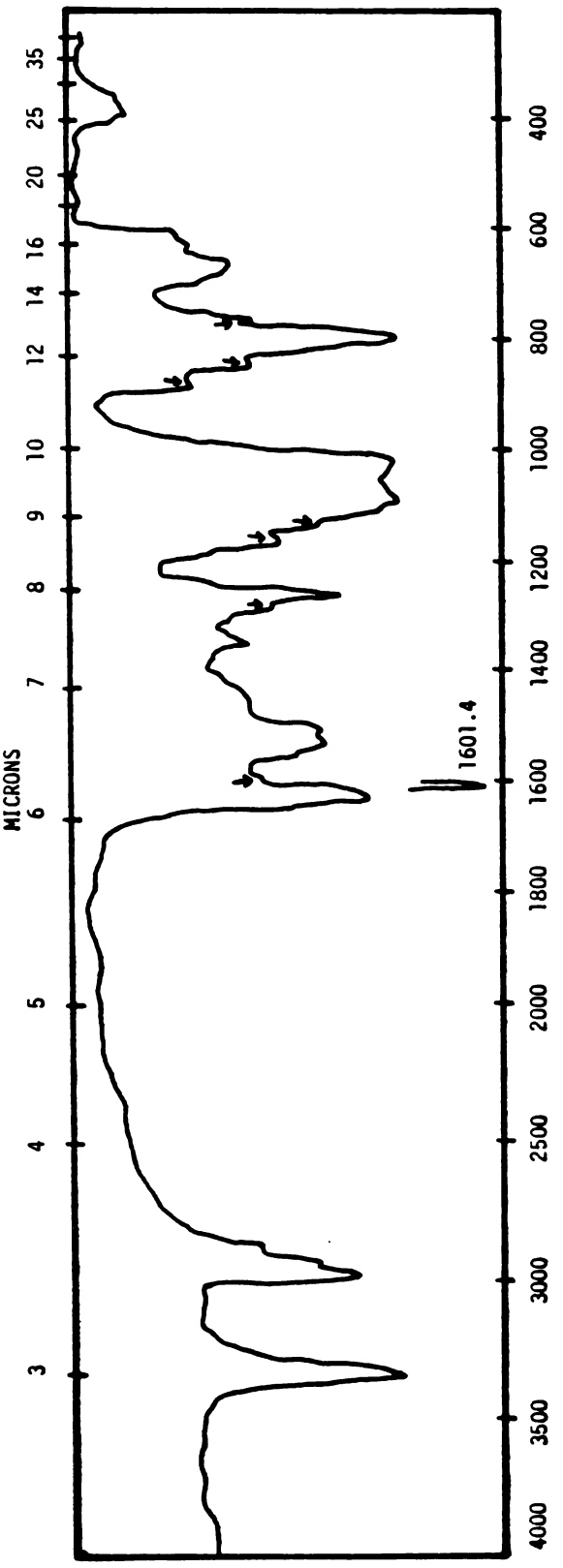


Figure 17. Infrared Spectrum of N-Isopropyl-α-(2-methylazo)-p-toluamide.

The sample was run as a KBr pellet. Polystyrene was the reference.

peracid. The excess peracid was destroyed by shaking with 5% sodium sulfite. Re-testing with starch-iodide paper gave a negative test. The organic layer was removed and the aqueous layer extracted 3-4 times with 25 ml portions of methylene chloride. The organic layers were combined, washed three times with 30 ml of 5% $NaHCO_3$; washed twice with 30 ml of saturated sodium chloride, and finally, washed with water. After drying over $MgSO_4$ the extract was evaporated to dryness on a rotary evaporator. The yield of the pale yellow powder was 0.89 g (41%), m.p. = 95-103°C (literature m.p. = 128-132°C [160] although the composition of the mixture was not specified). Thin-layer chromatographic analysis (silica gel, ethylacetate) of the powder gave a single spot, R_f = 0.42. The mass spectrum of the mixture of methyl- and benzylazoxy isomers gave the expected MH^+ peak at 236.3. The 100 MHz. Fourier-transform NMR spectrum shown in Figure 18, corresponds to that expected of a 2:1 mixture of the methyl :benzylazoxy isomers. The aromatic protons are at 7.8 p.p.m. (4H's, multiplet, J=4.0 Hz.); the amide proton at 6.1 (1H, broad); the benzylic proton of the benzyl- isomer at 5.4 (0.5H, singlet); the benzylic proton of the methyl- isomer at 4.7 (1H, singlet); the isopropyl methine proton at 4.3 (1H, multiplet, J=6.5 Hz.); the N-methyl protons of the methyl- isomer at 4.2 (2H's, singlet); the N-methyl proton of the benzyl- isomer at 3.2 (1H, singlet); and the isopropyl methyl protons at 1.3 (6H's, doublet, J=6.5 Hz.). The assignment of the azoxy benzylic and methyl protons was based upon the values for similar azoxy compounds reported by Freeman[60a] Korsch and Riggs.[76] A comparison of the reported values and those obtained for the synthesized azoxy isomers is shown in Table 1. As shown in the table, the assignments correlate closely with the literature values.

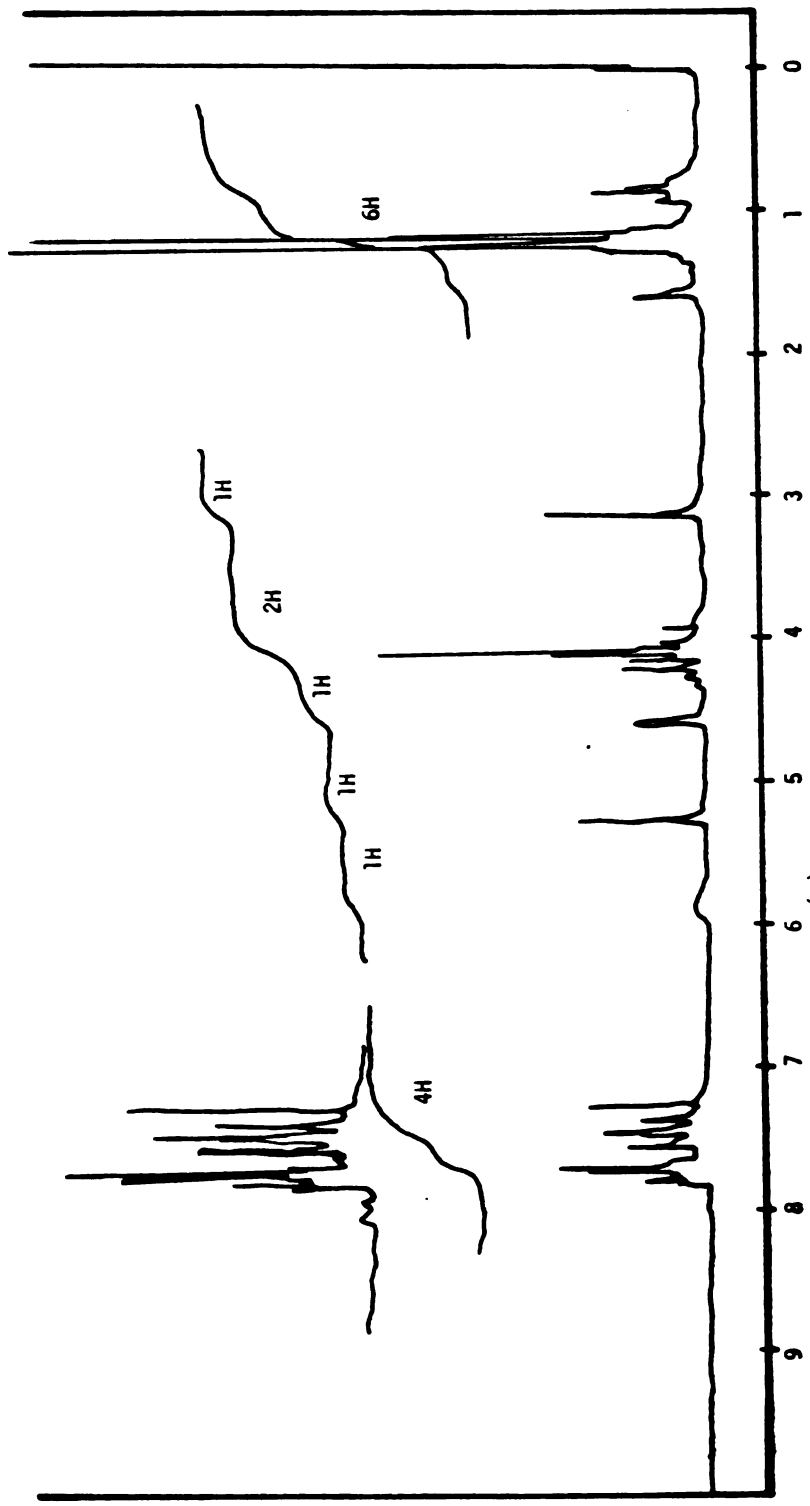


Figure 18. NMR Spectrum of N-Isopropyl-α-(2-methylazoxy)-p-toluamide.

Sample was dissolved in $CDCl_3$ with tetramethylsilane as the internal reference. Instrument used was a Varian XL-100 FT 100MHz. NMR.

TABLE 1.

PROTON MAGNETIC RESONANCE CHEMICAL SHIFTS of the AZO and the AZOXY COMPOUNDS

Compounds	Chemical Shift ppm (δ) $CH_3N=$	$CH_3(O)N=$	$-CH_2N=$	$-CH_2(O)N=$	Ref.
$CH_3N=NCH_3$	3.68				b
$C_6H_5CH_2N=NCH_2C_6H_5$			4.85		b
$CH_3N=NCH_2C_6H_4R$[a]	3.82		5.00		d
$CH_3N=N(O)CH_3$	3.16	4.16			c
$C_6H_5CH_2N=N(O)CH_2C_6H_5$			4.35	5.02	b
$CH_3N=N(O)CH_2C_6H_4R$[a]	3.23			5.38	d
$CH_3(O)N=NCH_2C_6H_4R$[a]		4.20	4.70		d

[a] R= $CONHCH(CH_3)_2$

[b] J. Freeman, J. Org. Chem., 28: 2508-2511 (1963).

[c] B.H. Korsch and N.V. Riggs, Tetrahedron Letters, 10: 523-525 (1964).

[d] Spectra were taken in $CDCl_3$ with tetramethylsilane as the internal standard on a Varian Associates A-60A NMR and XL-100 FT NMR.

Integration of the methyl and benzylic proton resonances give a ratio of 2:1, which inidcates that the mixture contains the methylazoxy and benzylazoxy in a 2:1 ratio. This data served to assign the retention times in the reverse-phase high-performance liquid chromatographic analysis of the mixture. The azoxy mixture was dissolved in methanol and analyzed by reverse-phase high-performance liquid chromatography (20% MeOH/water, flow rate of 2 ml./minute). The compounds were detected using an ultraviolet detector at 254 nm. The chromatograph is shown in Figure 19. The internal standard used was 4-methylacetophenone. The retention ratio, which is the ratio of the elution time for the compound to the retention time for the internal standard, for the methylazoxy isomer is 0.50 and for the benzylazoxy isomer is 0.39.

N-Isopropyl-α-(2-methylhydrazino)-p-toluamide ($^2H_1$) hydrochloride

N-Isopropyl-α-(2-methylhydrazino)-p-toluamide ($^2H_1$) hydrochloride was synthesized by the sodium cyanoborodeuteride reduction of the hydrazone, prepared as previously described. The procedure used is the general procedure of Borch, et al.[161] and Lane[162] for the use of sodium cyanoborohydride in reductions. The previously prepared hydrazone, 0.4 g , 0.002 moles was dissolved in 15 ml of methanol-$^2H_1$ with a few drops of deuterium oxide containing 0.12 g (0.002 moles) of sodium cyanoborodeuteride (98% atom D, Aldrich Chemical Company). The reaction was stirred at room temperature under a nitrogen atmosphere for 7 days. A drop of DCl was added daily to maintain the pH of 4-5. The reaction was analyzed on days 4, 5, and 7 by TLC analysis (silical gel, upper layer of a mixture of butanol:H_2O:Formic acid, 60:35:5). Procarbazine hydrochloride (Hoffmann-La Roche) was used as the standard.

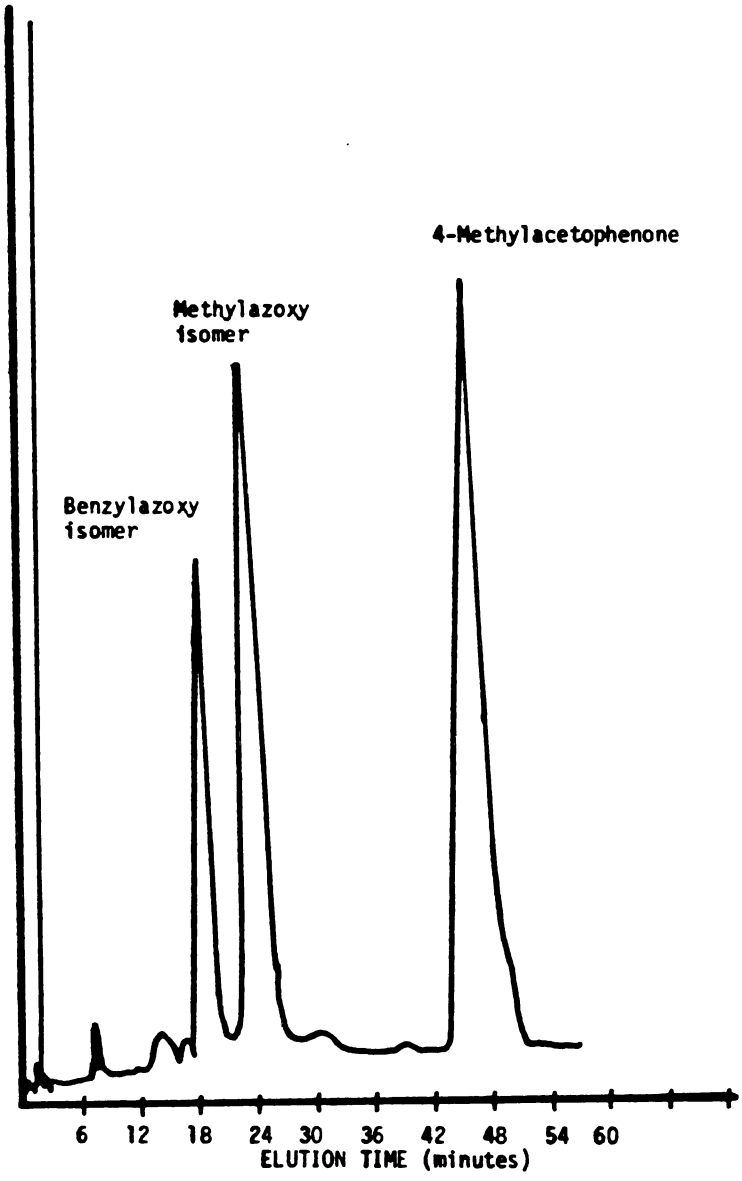


Figure 19. HPLC Chromatograph of N-Isopropyl-α-(2-methylazoxy)-p-toluamide.

Solvent was 20% methanol;water. Flow rate 2ml/min. λ= 254 nm. Spectrum run at room temperature. 4-Methylacetophenone was the internal reference. Water's u-bondapak C_{18} reverse-phase column.

The d_1-compound was present at day 4, R_f = 0.33. Since no further changes occurred after 7 days, the reaction was stopped by addition of DCl until the pH reached 1. Bubbles were evolved during the addition. The remaining steps were done in a glove box under nitrogen atmosphere. All of the solvents were deoxygenated. The acidified reaction mixture was extracted with ethyl acetate to remove any unreduced hydrazone. Thin-layer chromatographic analysis run as above showed no desired compound in the organic layer and the desired compound in the aqueous layer. The aqueous layer was basified with 10% NaOH and extracted 5 times with 5-10 ml. portions of methylene chloride. After drying the extract over anhydrous $MgSO_4$, Procarbazine hydrochloride was precipitated by gaseous hydrochloric acid. The pale yellow precipitate was filtered using a Hirsch funnel with a finely fretted disc. The yield was 0.11 g (28%) after thoroughly drying the pale yellow powder in a vacuum dessicator. The compound partially melted at 182-185°C and turned to a brown liquid upon decomposition at 225°-228°C. The sample was analyzed by thin-layer chromatography as described above. Both authentic Procarbazine and the sample had the same R_f (0.31). Mass spectral analysis gave the expected MH^+ peak at 223.3. A small amount of d_o-Procarbazine was also present.

N-isopropyl ($^2H_6$)-α-(2-methylhydrazino)-p-toluamide hydrochloride

The synthesis of N-isopropyl($^2H_6$)-α-(2-methylhydrazino)-p-toluamide hydrochloride was done according to the procedure described in the previous section for the synthesis of Procarbazine-d_1. In place of hydrazone, the hydrazone-d_6 previously synthesized was used. Sodium cyanoborohydride was used to reduce the hydrazone-d_6.

N-Isopropylterephthalamic Acid

The synthesis of N-isopropylterephthalamic acid was done using the procedure of Shriner and Kleiderer[163] for the synthesis of piperonylic acid N-Isopropyl-p-formylbenzamide, 1.5 g. (0.008 moles) synthesized as previously described was stirred at 70-80°C in 50 ml of distilled water. Potassium permanganate, 1.85 g (0.0117 moles) in 50 ml of water was added over a period of 40-45 mintues. The reaction was stirred at 70-80°C for 1 hour after the addition was complete. A solution of 10% KOH was added until the reaction mixture was alkaline. The solid MnO_2 was filtered hot and the solid was washed 3 times with 5 ml. portions of hot distilled water. Thin-layer chromatographic analysis (silica gel, ethylacetate:hexane, 35:10) of the combined washes and filtrate showed no aldehyde remaining. A single more polar spot remained near the origin. After cooling the filtrate and washes, concentrated HCl was added until no further precipitation occurred. The white precipitate was filtered and washed with cold water. The yield was 1.2 g. (74%) after thoroughly drying in a vacuum dessicator for several days. The white powder melted at 228-231°C. (literature m.p. = 230-231°C The 60 MHz. NMR spectrum is shown in Figure 20. No peak is present for the acid proton because of exchange. The NMR spectrum shows no residual aldehyde is present. The peak for the amide and the isopropyl methine protons did not integrate correctly. Instead of integrating to 2 protons, this region integrated to 5 protons. This is probably the result of residual water in the DMSO solvent. The resonance at 2.5 p.p.m. corresponds to the DMSO residual protons. The aromatic protons are at 8.0 p.p.m. (4H's, singlet), the isopropyl methine proton at 4.2 (1H, multiplet, J = 6.5 Hz.); and the isopropyl methyl protons at 1.3

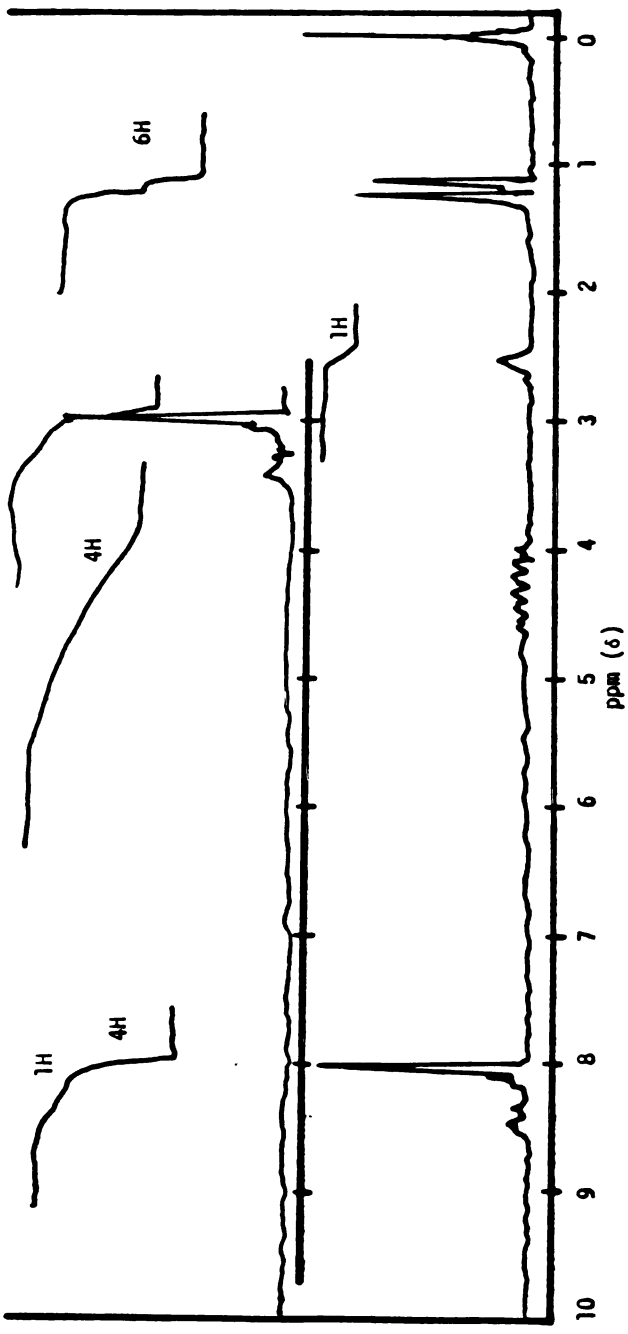


Figure 20. NMR Spectrum of N-Isopropylterephthalamic Acid.

Sample was dissolved in DMSO-d_6 with tetramethylsilane as the internal reference. Instrument used was a Perkin-Elmer R-12B 60 MHz. NMR.

(6H's, doublet, J = 6.5 Hz.). The mass spectral analysis gave the expected MH^+ peak at 208.2. The I.R. spectrum run as the KBr pellet is shown in Figure 21. A comparison of this spectrum with that in Figure for the aldehyde clearly shows the absence of the weak aldehyde C-H stretching bands at 2810 and 2730 cm^{-1}.

N-Isopropyl ($^2H_6$) terephthalamic acid

N-Isopropyl ($^2H_6$) terephthalamic acid was synthesized using the same procedure with N-isopropyl ($^2H_6$)-p-formylbenzamide synthesized as previously described. The yield was 61%. The mass spectrum gave the expected MH^+ peak of 214.2.

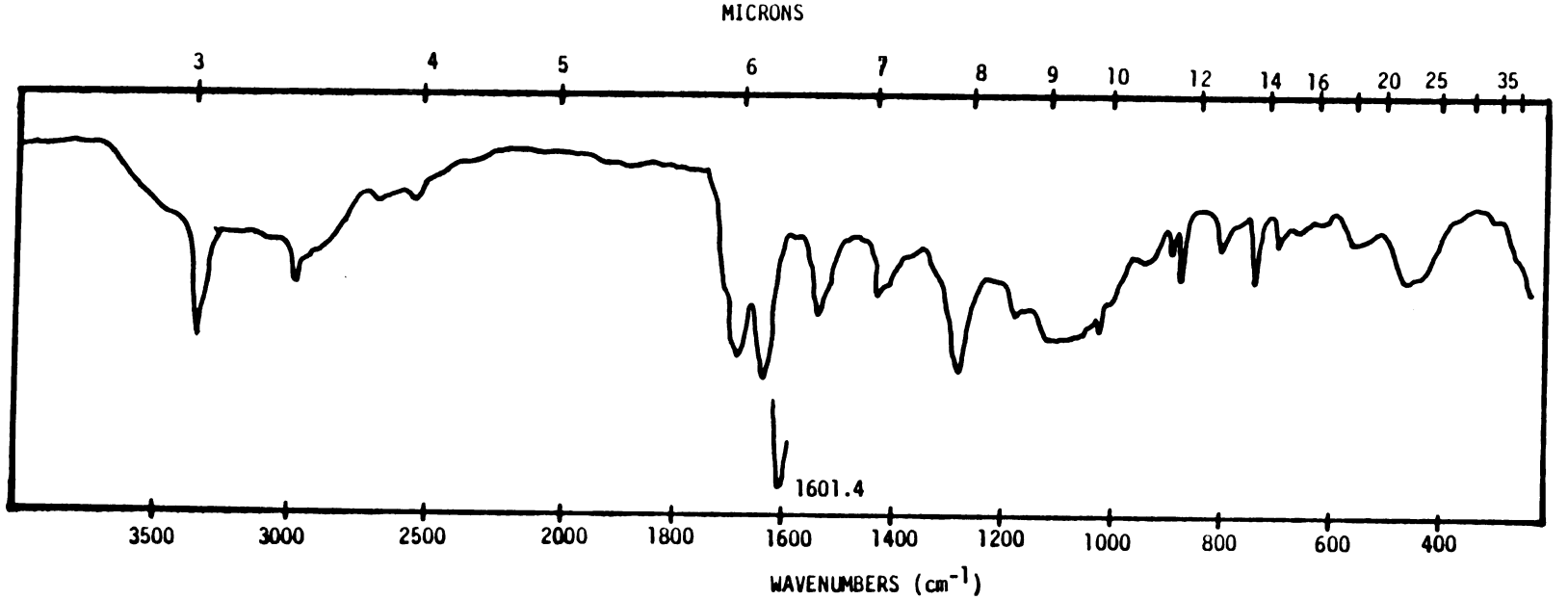


Figure 21. Infrared Spectrum of N-Isopropylterephthalamic Acid.

Sample was run a KBr pellet. Polystyrene was the reference.

III. Development of Quantitative Analytical Methods

The analytical methods used in these studies must possess sufficient sensitivity to detect compounds in biological media at amounts which are only a small fraction of the total administered dose, the ability to detect and resolve structural isomers as well as a wide spectrum of different compounds, and the simplicity that permits use in routine clinical analysis.

One of the major problems in the analysis of Procarbazine is that Procarbazine is rapidly oxidized, especially in the presence of base, to the corresponding azo compound. This, coupled with the basic property of the hydrazine group, presents difficulties in extracting the unoxidized parent compound from the aqueous biological samples. At physiological pH, the basic hydrazine moiety (pK_a = 6.8 for the dissociation of Procarbazine · HCl[91]) is probably protonated. It is therefore necessary to make the biological samples basic in order to extract Procarbazine with organic solvents. One approach to avoid the analytical error resulting from rapid Procarbazine oxidation is to use a d_6-analog as an internal standard since this analog behaves chemically like the d_o- compound. Addition of the internal standard prior to any extraction procedure would lead to an accurate measure of Procarbazine. However, extensive oxidation could lead to concentrations below the detectable limits of the assay.

Procarbazine and its chemical degradation and metabolic products are analyzed in serum, *in vitro* incubation mixtures, and aqueous buffer solutions by reverse-phase high-performance liquid chromatography and

direct-probe insertion selected-ion monitoring chemical ionization mass spectrometry. The neutral extract of the sample is analyzed by high-performance liquid chromatography using 4-methylacetophenone as an internal standard. This system allows the identification and quantitation of each of the known major metabolites and chemical decomposition products, except N-isopropylterephthalamic acid and Procarbazine. The quantitation of these compounds is achieved by using direct-probe insertion selected-ion monitoring chemical ionization mass spectrometric analysis of the acidic and basic extracts of each sample. d_6-Isopropyl-labeled Procarbazine and acid are used as internal standards. Chemical ionization using isobutane is used to avoid widespread fragmentation of the parent molecule. The introduction of the sample via direct-probe insertion is useful for molecules of low volatility or when the molecules are susceptible to decomposition in a gas chromatography-mass spectrometry system.[165] Thermal decomposition of metabolic intermediates is a concern in studying the pharmacology of Procarbazine.

1. Procarbazine and N-Isopropylterephthalamic Acid: Analysis Using Chemical Ionization Mass Spectrometry

   A. Experimental Methods and Procedures

      Instrumentation and Selection of Internal Standards

      The spectrometric system used is a Finnigan 3200 mass spectrometer equipped with a chemical-ionization source using isobutane as a reagent gas. The spectrometer is interfaced with a Data General Corporation Nova 830 commercial computer and includes the capacity to monitor up to 6 ions simultaneously.

The internal standards selected are N-isopropyl ($^2H_6$)-terephthalamic acid (acid-d_6) and N-isopropyl ($^2H_6$)-α-(2-methylhydrazino)-p-toluamide hydrochloride (Procarbazine-d_6). The d_6-analogs were chosen with the deuteriums placed in the methyl group of the isopropyl moiety to prevent facile exchange or loss of the labels during sample preparations. The synthesis of these d_6-internal standards has been discussed in Section II. Stock solutions of these standards were prepared by dissolving 25 mg. of Procarbazine-d_6·HCl in 50 ml. of absolute ethanol containing 5 drops of concentrated HCl and 12.5 mg. of acid-d_6 in 25 ml. of EtOH. These solutions are stored at -45°C. and are used for the generation of the standard curves and for each assay.

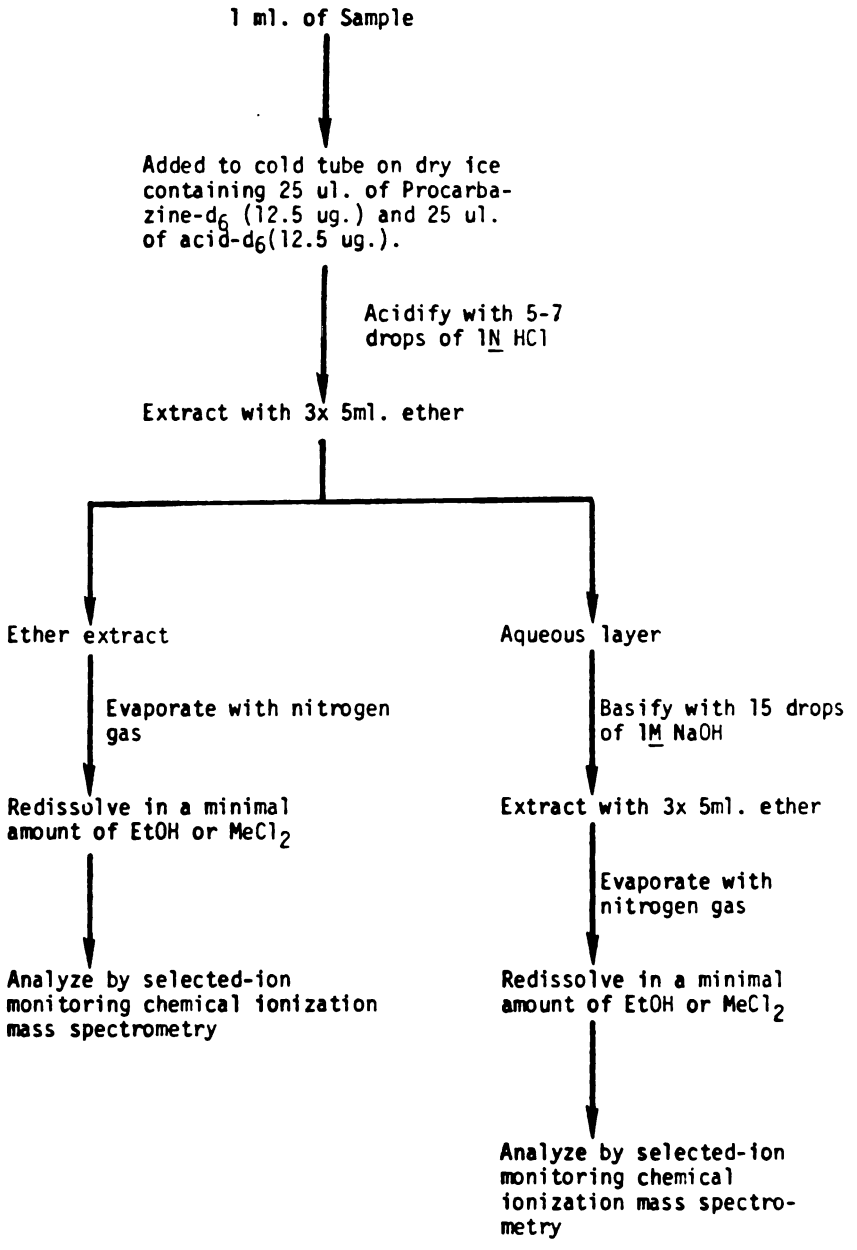
Assay Procedure

A 0.5 to 1.0 ml. sample of aqueous buffer, serum, or incubation mixture is acidified to pH 2 by addition of 5 to 7 drops of 1N HCl. Internal standards, Procarbazine-d_6 and acid-d_6 in ethanol, are added in an amount approximately equal to the anticipated amount of the respective analog in the sample. Typically, 25 ul. (12.5 ug.) of each is added. The acidified sample is extracted with 3 x 5 ml. portions of ether to remove the acid. The pH of the aqueous layer is increased to above pH 11 with the addition of 1 M NaOH. The aqueous solution is immediately extracted with 3 x 5 ml. portions of ether. The acidic and basic extractions of certain biological samples such

as liver supernate require centrifugation for 2 minutes at 2000 r.p.m. and 5°C. in a Damon/IECPR-6000 centrifuge to separate the ether and aqueous layers. The combined ether extracts are evaporated by means of a stream of nitrogen at ambient temperature. The residue is either immediately analyzed or stored frozen at -45°C. in a Forma Bio-Freezer. Samples prepared by this procedure can be kept frozen for over a month. A schematic diagram of the mass spectral assay procedure is shown in Figure 22 .

The residues are redissolved in a small amount (less than 1 ml.) of absolute ethanol or methylene chloride and an aliquot placed on the ceramic tip of the direct-insertion probe. After evaporation of the solvent the sample is inserted into the mass spectrometer at a source temperature of about 175°C. The ion current at masses 228.28 and 228.28, for d_o/d_6-Procarbazine, and 208.22 and 214.22, for d_o/d_6-acid, is monitored for 100 seconds during sample evaporation into the source. A typical selected-ion monitoring scan for Procarbazine and N-isopropylterephthalamic acid is shown in Figure 23 . Also shown are the peak height measurement lines used to determine the d_0/d_6-peak height ratios. These ratios are used in the equations shown in Figure 24 to calculate the concentration of Procarbazine and acid in the sample.

Figure 22. Assay Procedure For Selected-ion Monitoring Chemical Ionization Mass Spectrometric Assay



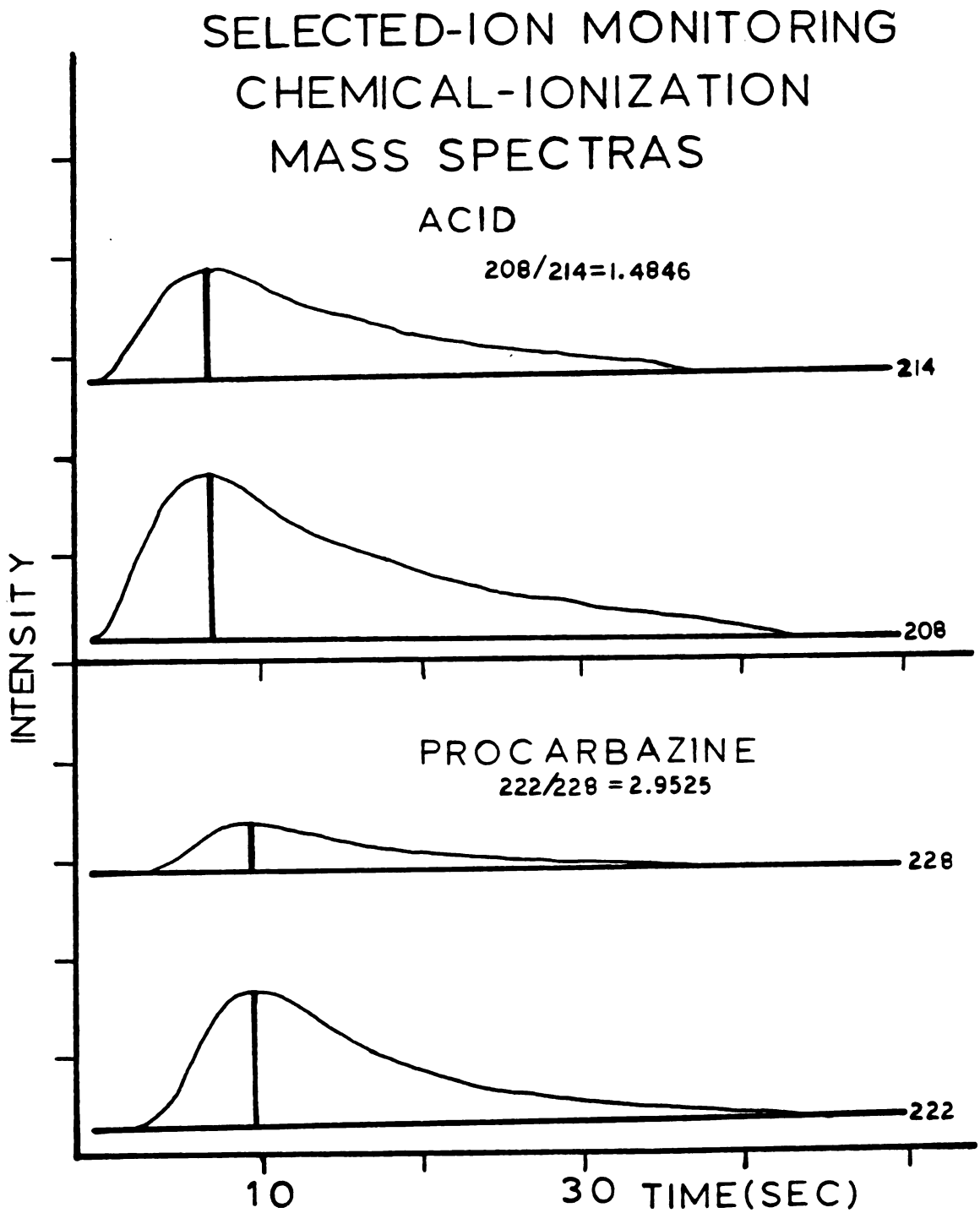


Figure 23. Selected -ion Monitoring Chemical Ionization Mass Spectrometric Profiles of Procarbazine and N-Isopropylterephthalamic Acid.

$$\text{Amount of Procarbazine} = \left[\frac{\text{Peak height of Procarbazine}}{\text{Peak height of Procarbazine-}d_6}\right] \times \left[\text{Amount of Procarbazine-}d_6\text{ added}\right] \times \left[\frac{\text{Volume of incubation}}{\text{Volume analyzed}}\right] \times \left[\text{Slope of std. curve}\right] + \left[\text{Y-intercept of std. curve}\right]$$

$$\text{Amount of Acid} = \left[\frac{\text{Peak height of Acid}}{\text{Peak height of Acid-}d_6}\right] \times \left[\text{Amount of Acid-}d_6\text{ added}\right] \times \left[\frac{\text{Volume of incubation}}{\text{Volume analyzed}}\right] \times \left[\text{Slope of std. curve}\right] + \left[\text{Y-intercept of std. curve}\right]$$

Figure 24. Equations Used For the Calculation of Procarbazine and Acid Concentrations.

Standard Curves

The Procarbazine and acid standard curves were determined from mixtures of Procarbazine and acid with their $^{2}H_{6}$-internal standards as shown in Table 2. After thorough mixing, aliquots of these solutions are analyzed directly according to the above procedures. The mixtures were chosen to give ratios over the range expected when analyzing the *in vitro* and *in vivo* metabolic samples (1.2-19.2).

Human Plasma Standard Curves and Recovery

Standard curves for Procarbazine and N-isopropylterephthalamic acid in human plasma and the recovery of these compounds from plasma were examined. Table 3 shows the amounts of Procarbazine, acid, and their $^{2}H_{6}$-analogs placed

Table 2. Mixtures of Procarbazine, N-Isopropylterephthalamic Acid, and Their d_6- Internal Standards Used to Generate Their Standard Curves.

Procarbazine(ug.)	Procarbazine-d_6(ug.)	d_0/d_6 Calculated Ratio	d_0/d_6 Observed Ratio
24	5	4.8	4.6
48	5	9.6	11.8
72	5	14.6	17.4
96	5	19.2	22.1
24	10	2.4	3.0
24	15	1.6	2.0
24	20	1.2	1.7

Acid(ug.)	Acid-d_6(ug.)	d_0/d_6 Calculated Ratio	d_0/d_6 Observed Ratio
24	5	4.8	4.5
48	5	9.6	8.3
72	5	14.4	11.7
96	5	19.2	14.9
24	10	2.4	2.4
24	15	1.6	1.6
24	20	1.2	1.2

into 500 ul. of human plasma. These mixtures were extracted with ether and analyzed using the above assay procedure.

Table 3. Mixtures of Procarbazine, Acid, and Their d_6-Internal Standards Added to Human Plasma Used to Generate Their Standard Curves and Determine Recovery.

Procarbazine(ug.)	Procarbazine-d_6(ug.)	Observed Ratio	Observed Procarbazine(ug.)	% Recovery
24	10	2.8	24.0	99.8
		2.8	23.8	99.1
72	10	7.7	65.5	91.0
		8.0	68.2	94.8
144	10	15.3	129.7	90.1
		15.7	133.6	92.7
Acid(ug.)	**Acid-d_6(ug.)**	**Observed Ratio**	**Observed Acid(ug)**	**% Recovery**
24	10	2.2	29.0	120.7
		2.0	26.8	111.7
72	10	5.2	68.9	95.7
		5.2	68.3	94.9
144	10	10.6	140.3	97.4
		11.1	147.1	102.2

B. Results

The standard curves generated from the direct-probe insertion selected-ion monitoring chemical ionization mass spectral analysis of a mixture of Procarbazine, N-isopropylterephthalamic acid, and their $^2H_6$-analogs are shown in Figure 25. Figure 26 hows a comparison between

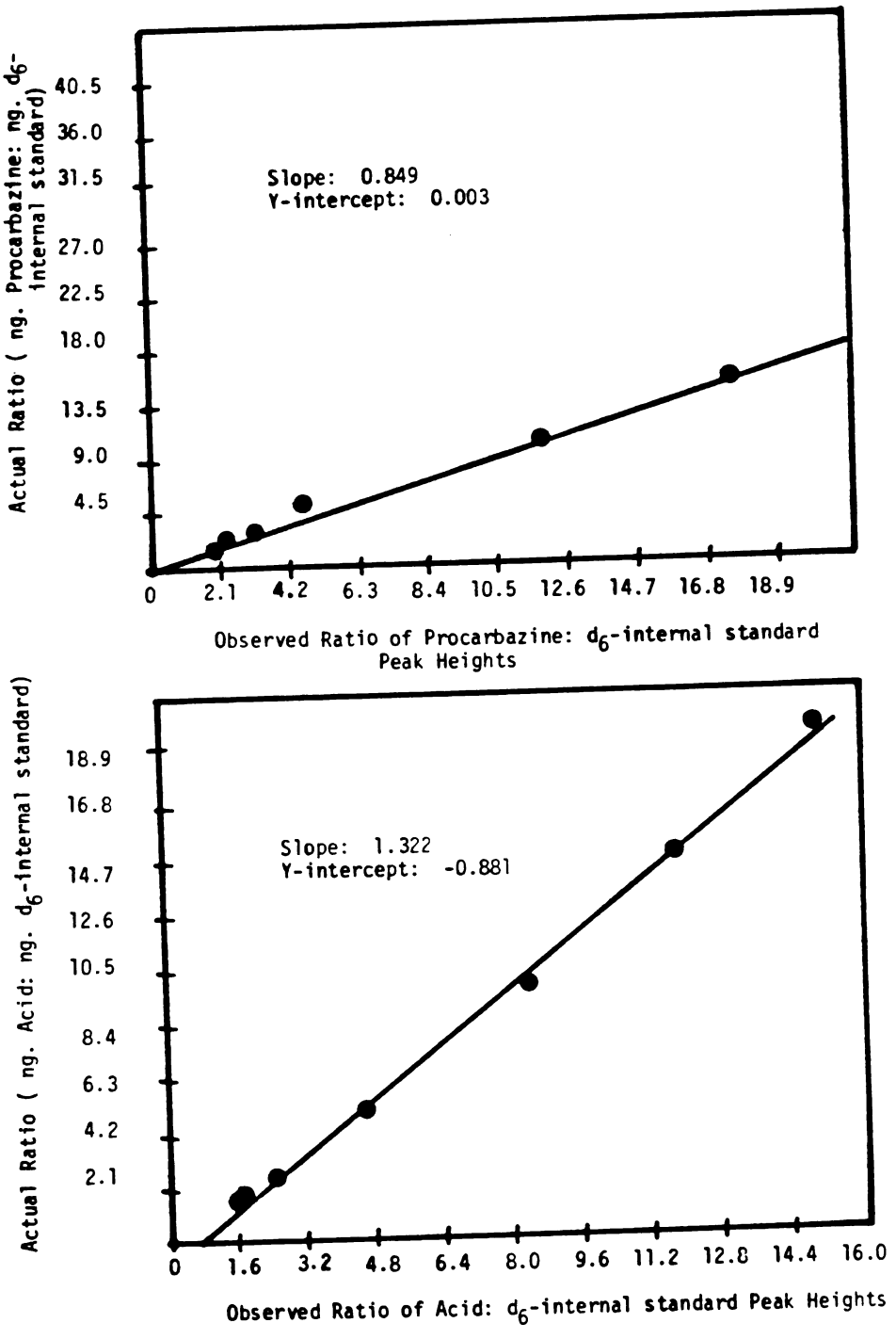


Figure 25. Standard Curves For Procarbazine and N-Isopropylterephthalamic Acid Without Extraction- Selected-ion Monitoring Chemical Ionization Mass Spectrometric Assay.

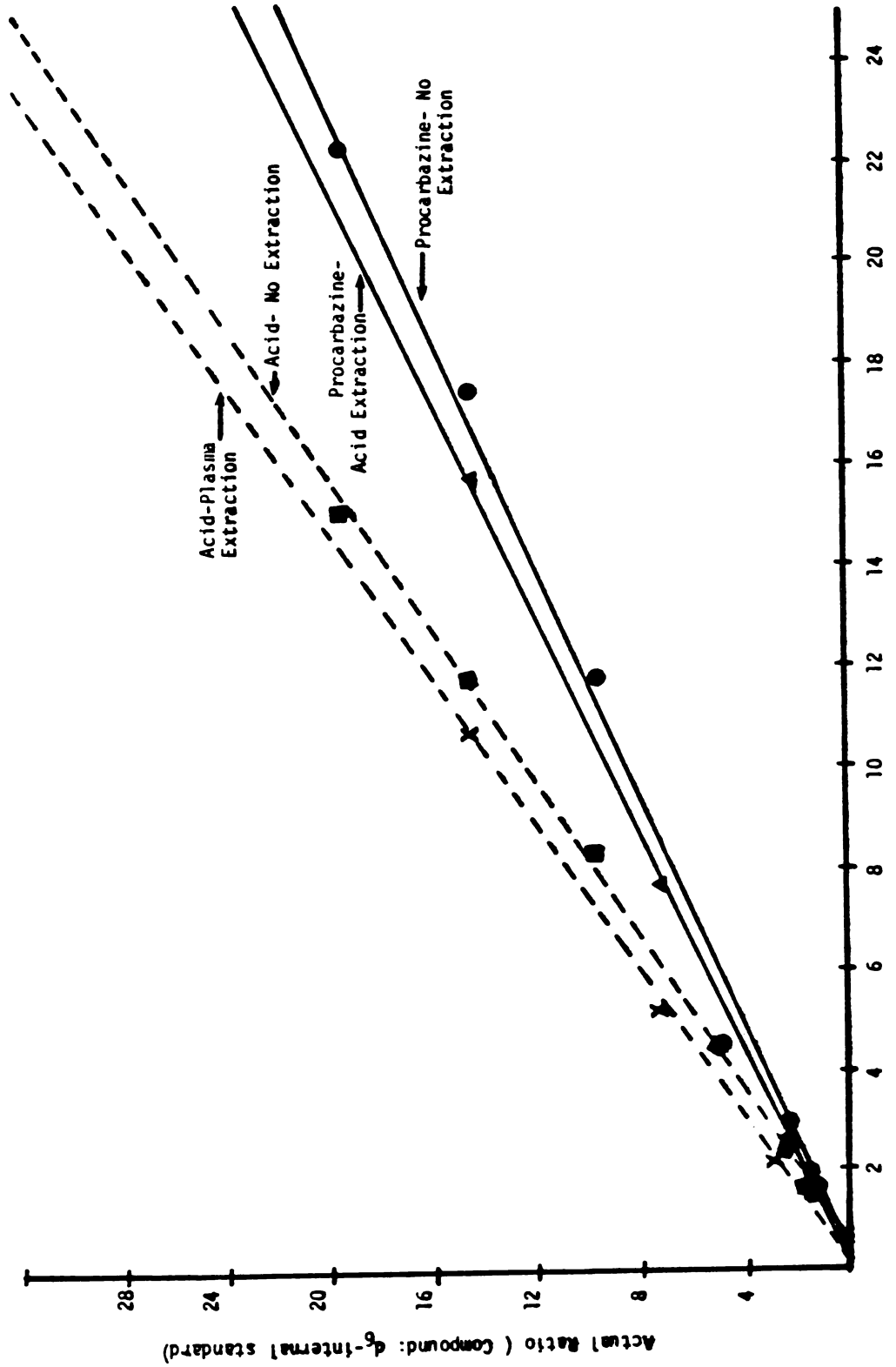


Figure 26. Comparison of Standard Curves Obtained With and Without Extraction From Human Plasma- Procarbazine and N-Isopropylterephthalamic Acid.

these standard curves and those generated using the assay and extraction procedure to quantitate levels of Procarbazine and acid in human plasma. These curves are similar within experimental error and both sets are linear throughout the range of the assay. The percent recovery for the extraction procedure is shown in Table 3. A recovery of 90-100% was obtained using the extraction and assay procedure. The standard curves shown in Figure 25 were used to quantitate the levels of Procarbazine and N-iospropylterephthalamic acid in the human plasma extractions. The sensitivity limit is 1.4 ug. of Procarbazine and acid/1 ml. of sample. However, approximately 500 ng. of compound placed upon the probe is the limit of detection and quantitation.

C. Discussion

The selected-ion monitoring chemical ionization mass spectrometric assay for Procarbazine and N-isopropylterephthalamic acid was successfully developed. The use of $^{2}H_{6}$-labeled internal standards, added prior to the extraction procedure, alleviates the potential analytical errors arising from the instability of Procarbazine in solution in the presence of air. The similarities between the standard curves generated from mixing of Procarbazine, N-isopropylterephthalamic, and their $^{2}H_{6}$-analogs and those using the extraction procedures to extract human plasma samples containing the mixture of deuterated and

non-deuterated compounds show that the extraction procedure and analytical technique can be used to quantitatively estimate the levels of Procarbazine and acid in biological samples. Furthermore, the sensitivity of the assay method will allow its direct application to the study of Procarbazine metabolism in intact animal systems. However, the instability of Procarbazine coupled with several of the metabolic and chemical degradation products having an identical molecular weight and protonated molecular ion mass necessitates the use of an additional analytical system to differentially quantitate the azo and hydrazone and the two azoxy isomers. A high-performance liquid chromatographic assay system was developed to fulfill this need.

2. Non-polar Procarbazine Chemical Degradation and Metabolic Products: Analysis Using Reverse-phase High-performance Liquid Chromatography

A. Experimental Methods and Procedures

Instrumentation and the Selection of Solvents and Internal Standard

The reverse-phase high-performance liquid chromatographic system used is a Chromatronix Model 3500 single pump high-performance liquid chromatograph equipped with a Chromatronix Model 220 mixed-wavelength ultraviolet detector, a Chromatronix MA-10 20 ul. flow cell, and a Linear Model 385 dual-pen recorder. A Waters Associate's 30 cm./3.9 mm I.D. C_{18}-ubondapak reverse-phase column was used at ambient temperature. The solvent system selected was 20% Burdick and Jackson glass distilled methanol in

double-distilled filtered water at a flow rate of 2 ml/min. The water was filtered through a Millipore filter. The solvent was degassed with stirring under a vacuum for 15 minutes. 4-Methylacetophenone was used as an internal standard. This compound is eluted after the methyl compound. No other products from biological samples nor chemical degradation experiments eluted at nor after this internal standard. A stock solution of the internal standard was prepared by dissolving 67 mg. of 4-methylacetophenone in 100 ml. of absolute ethanol. This solution is stored at -45°C. and is used for the generation of the standard curves and for each assay.

Assay Procedure

A 1.0 ml. aliquot of aqueous buffer, serum, or incubation mixture is rapidly extracted with 5 x 3 ml. portions of cold ether. The extract is evaporated by a stream of nitrogen at ambient temperature. The residue is redissolved in 250-500 ul. of methanol and 10 ul. (6.7 ug.) of the internal standard is added. After thorough vortexing of the sample and centrifuging for 1 minute, approximately 15 ul. of the sample is injected into the liquid chromatograph. Rapid analysis or freezing at -45°C. slows any decomposition of the metabolites. The U.V. detector is set at 254 nm and an O.D. of 0.01. The recorder chart speed is 10 cm./hour and the input into the dual pen recorder is set at 5 and 20 millivolts with the detector output of 10 millivolts. A schematic diagram of the liquid chromatographic

assay procedure is shown in Figure 27. A typical high-performance liquid chromatograph of a mixture of the internal standard and the known chemical degradation and metabolic products is shown in Figure 28. Each compound is quantitated using the equations shown in Figure 29. The peak area is the product of the peak height and the peak width at half of the peak height.

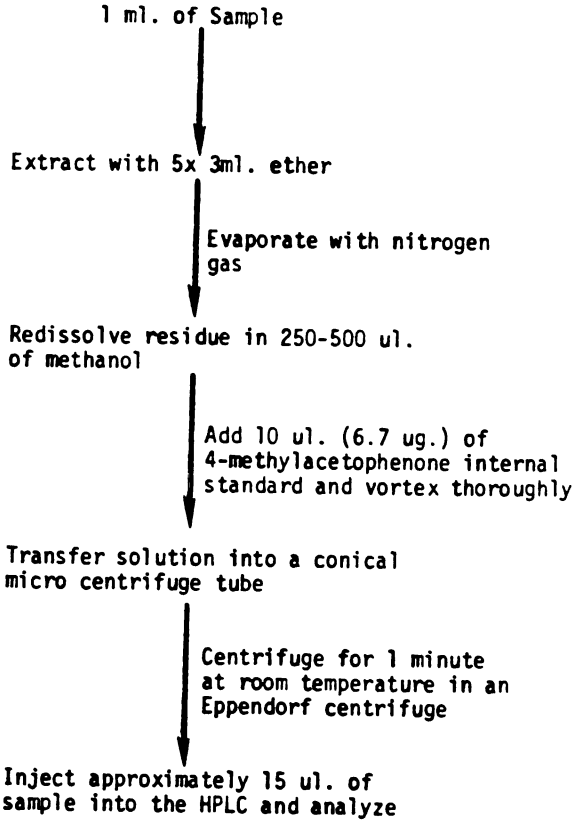
Standard Curves

The aldehyde, azoxy isomers, hydrazones, and N-isopropyl-4-methylbenzamide standard curves were determined from mixtures of these compounds with the internal standard, 4-methylacetophenone, as shown in Table 4 . The mixtures were chosen to give ratios over the range expected when analyzing biological samples (0.08-0.24 and 0.6-1.68).

Standard mixtures of aldehyde, azoxy isomers, and N-isopropyl-4-methylbenzamide compound were evaporated to dryness with a stream of nitrogen gas at ambient temperature. A 10 ul. aliquot of the internal standard (6.7 ug.) was added with 450 ul. of methanol. After thorough mixing, approximately 15 ul. of the mixture was chromatographed.

The standard curves for the azo and hydrazone were generated separately to determine if the azo isomerized to the hydrazone and if the hydrazone was hydrolyzed to the aldehyde during chromatographic analysis. Less than 1% isomerization of azo to hydrazone occurred and no hydrolysis of hydrazone was detected.

Figure 27. Assay Procedure For Reverse-phase High-Performance Liquid Chromatographic Assay



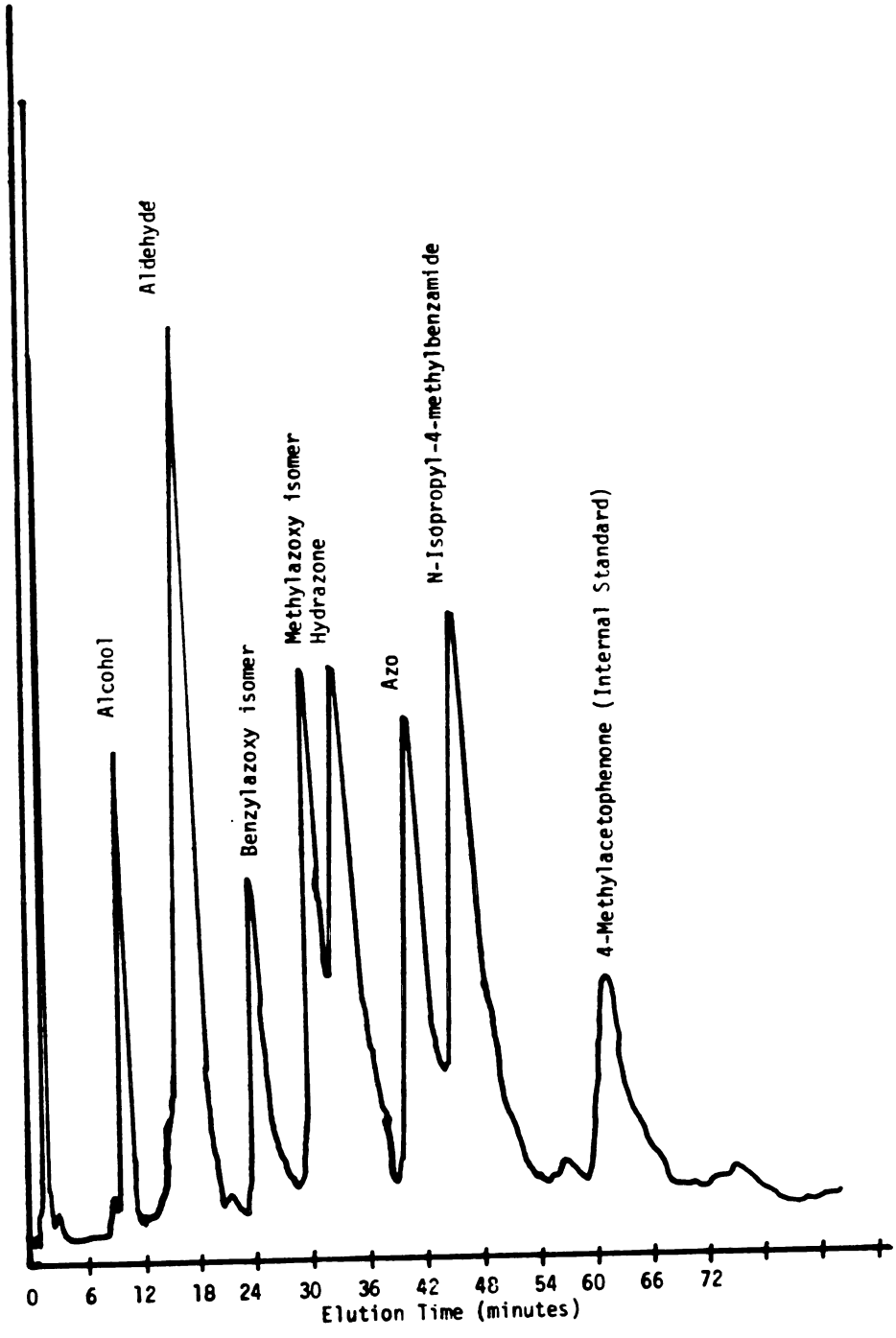


Figure 28. HPLC Chromatograph of a Mixture of the Known Chemical Degradation and Metabolic Products.

Solvent was 20% methanol:water. Flow rate 2ml/min. λ= 254nm. Spectrum run at room temperature. 4-Methylacetophenone was the internal reference. Water's u-bondapak C_{18} reverse-phase column.

$$\text{Amount of Aldehyde} = \left[\frac{\text{Peak area of aldehyde}}{\text{Peak area of internal std.}}\right] \times \left[\text{Amount of internal std. added}\right] \times \left[\frac{\text{Volume of incubation}}{\text{Volume analyzed}}\right] \times \left[\text{Slope of std. curve}\right] \times \left[\text{Y-intercept of std. curve}\right]$$

To calculate the amount of azoxy, hydrazone, or N-isopropyl-4-methyl benzamide, substitute the corresponding peak area for each compound and substitute the slope and y-intercept from each standard curve into the above equation.

To calculate the amount of N-isopropyl-4-hydroxymethylbenzamide, use the equation for N-isopropyl-4-methylbenzamide.

Figure 29. Equations Used For the Calculation of Aldehyde, Azoxy, Hydrazone, N-Isopropyl-4-methylbenzamide, and N-Isopropyl-4-hydoxymethylbenzamide Concentrations.

Table 4. Mixtures of Aldehyde, Azoxy Isomers, N-Isopropyl-4-methylbenzamide, Azo, and Hydrazone and the Internal Standard 4-Methylacetophenone Used to Generate Their Standard Curves.

	ug. Compound	ug. Internal Std.	Ratio	Observed Ratio(Compound:Std.)
Aldehyde	3.1	6.7	0.47	0.17
	6.3	"	0.94	0.39
	12.5	"	1.87	0.80
	25.0	"	3.74	1.33
Methylazoxy	8.0	"	1.18	0.14
	16.1	"	2.36	0.26
	32.3	"	4.73	0.58
	64.5	"	9.46	0.95
Benzylazoxy	4.5	"	0.69	0.08
	8.9	"	1.37	0.15
	17.8	"	2.75	0.34
	35.6	"	5.50	0.57
Methyl	6.3	"	0.93	0.19
	12.5	"	1.87	0.42
	25.0	"	3.74	0.90
	50.0	"	7.48	1.61
Azo	6.3	"	0.93	0.19
	12.5	"	1.57	0.43
	25.0	"	3.44	0.81
	50.0	"	6.64	1.68
Hydrazone	6.3	"	0.93	0.24
	12.5	"	1.57	0.31
	25.0	"	3.44	0.62
	50.0	"	6.64	1.30

Human Plasma Standard Curves and Recovery

Standard curves for the aldehyde, azoxy isomers, hydrazone, and N-isopropyl-4-methylbenzamide compounds in human plasma and the recovery of these compounds from plasma were examined. Table 5 shows the amounts of each compound added to 500 ul. of human plasma. After thorough mixing, the plasma was extracted with ether and analyzed using the above assay procedure.

B. Results

Before the final selection of the solvent and the extraction procedure to be used in the liquid chromatographic assay, many solvents and extraction procedures were tried. Table 6 shows several of the solvents employed and the elution times of each compound analyzed. The different ether extraction volumes and procedures used to determine the optimal number and volume are shown in Table 6 .

The structure and elution time ratio for each of the compounds is shown in Table 7 . The elution time ratio is the ratio of the elution time of each compound to the elution time for the internal standard. The standard curves, Figure 30, for the azoxy isomers, aldehyde, hydrazone, and N-isopropyl-4-methylbenzamide compounds generated from liquid-chromatographic analysis of mixtures of these compounds and the internal standard, 4-methylacetophenone, were analyzed by least squares linear regression analysis. Figures 31-33 show a comparison between these standard curves and those generated using the assay procedure to quantitate

Table 5. Mixtures of Aldehyde, Azoxy Isomers, N-Isopropyl-4-methylbenzamide, Azo, Hydrazone, and the Internal Standard 4-Methylacetophenone Added to Human Plasma Used to Generate Their Standard Curves and Determine Recovery.

	ug. Compound	ug. Internal Std.	Ratio	Observed ug. Compound	% Recovery
Aldehyde	6.3	6.7	0.94	5.5;6.8;5.2	87.2;108.3;83.4
	12.5	"	1.87	12.4;15.0	99.3;120.3
	25.0	"	3.73	22.7;22.6	91.0;90.2
Methylazoxy	16.1	"	2.40	12.9;17.7;19.0	80.4;110.0;118.0
	32.2	"	4.81	34.1;38.8	105.8;120.6
	64.4	"	9.61	68.1;68.8	105.8;106.8
Benzylazoxy	8.9	"	1.32	8.1;10.0;11.8	81.0;112.0;132.6
	17.8	"	2.66	19.3;21.8	108.5;122.5
	35.6	"	5.31	36.7;37.4	103.2;105.0
Methyl	12.5	"	1.87	11.7;15.7;14.8	93.5;125.5;118.2
	25.0	"	3.73	30.8;32.0	123.1;128.0
	50.0	"	7.46	54.2;58.8	108.3;117.5
Azo	12.5	"	1.87	12.6	101.1
	25.0	"	3.73	23.6;25.1	94.3;100.5
	50.0	"	7.46	50.9;49.2	101.7;98.3
Hydrazone	12.5	"	1.87	16.7;11.2;10.8	133.9;89.2;86.6
	25.0	"	3.73	22.8;24.2	91.1;96.8
	50.0	"	7.46	46.3;49.9	92.7;99.8

Table 6. Determination of the Optimal Solvent System and Extraction Procedure For Use in the Reverse-Phase High Performance Liquid Chromatographic Assay

Solvent Selection

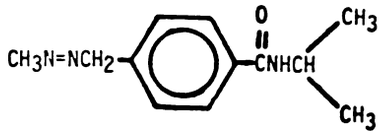
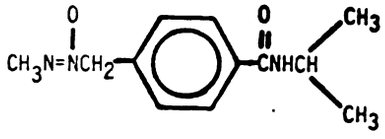
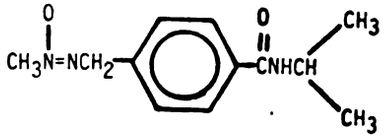
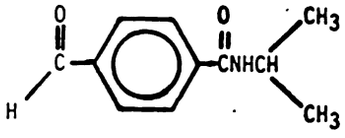
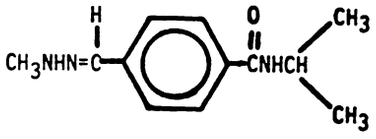
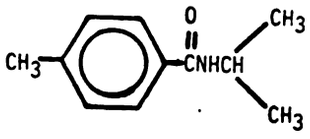
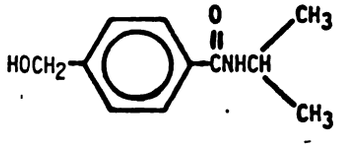
Solvent	Flow Rate (ml/min.)	Elution Times (minutes) Acid	Aldehyde	Benzylazoxy	Methylazoxy	Azo	Hydazone	Methyl
35%EtOH/H_2O	0.8	3.0	7.8					12.8
"	1.0	2.8	7.1	No separation,7.1		8.6	7.4	
90% Acetonitrile/H_2O	1.2		2.3	No separation,2.1				
70% "	1.2		3.6	No separation,3.6				
50% "	1.2		4.3	17.3	19.0			
40% "	1.2	2.1	6.0					
30% "	1.2	1.9	7.0	Not available		11.4	9.4	16.6
15% "	1.2	2.1	25.5	32.3	37.9	47.8	39.0	71.0
10% "	1.2	2.5	49.6	72.5	91.1	112.1	95.8	
" "	2.0	1.6	28.6	41.6	52.4	64.3	55.0	80.8
100% MeOH	1.2							2.3
60%MeOH/H_2O	1.2							7.0
40% "	1.3	2.2	8.1	9.2	10.5	13.9	10.5	18.2
30% "	1.3	2.0	13.5	19.8	23.4	27.5	25.0	35.4
20% "	1.8	2.0	20.0	29.5	37.3	52.5	41.0	57.5

4-Methylacetophenone in 20% MeOH/H_2O eluted at 61.5 minutes.

Optimization of the Extraction Procedure

	Aldehyde	Methylazoxy	Benzylazoxy	Methyl
Amount Added to 500ul. human plasma (ug.)	25.0	64.4	35.6	50.0
% Extracted:				
After 2x3ml.ether	80.5	77.2	91.0	99.7
" additional 2x3ml.	9.0	18.0	15.7	0
Total %	89.5	95.2	105.7	99.7
% Extracted:				
After 5x2ml.ether	91.7	98.4	75.2	102.8
" additonal 5x2ml.	9.8	27.5	22.7	0
Total %	101.5	125.9	97.9	102.8
% Extracted:				
After 4x3ml. ether	97.0	114.3	122.5	107.7
Duplicate	79.0	103.7	98.0	107.7

Table 7. The Structure, Abbreviated Name, and Elution Time Ratio For Each Known Chemical Degradation and Metabolic Product.

Structure	Abbreviated Name	Elution Time Ratio
	Azo	0.67
	Benzylazoxy	0.39
	Methylazoxy	0.50
	Aldehyde	0.27
	Hydrazone	0.55
	Methyl	0.75
	Alcohol	0.15

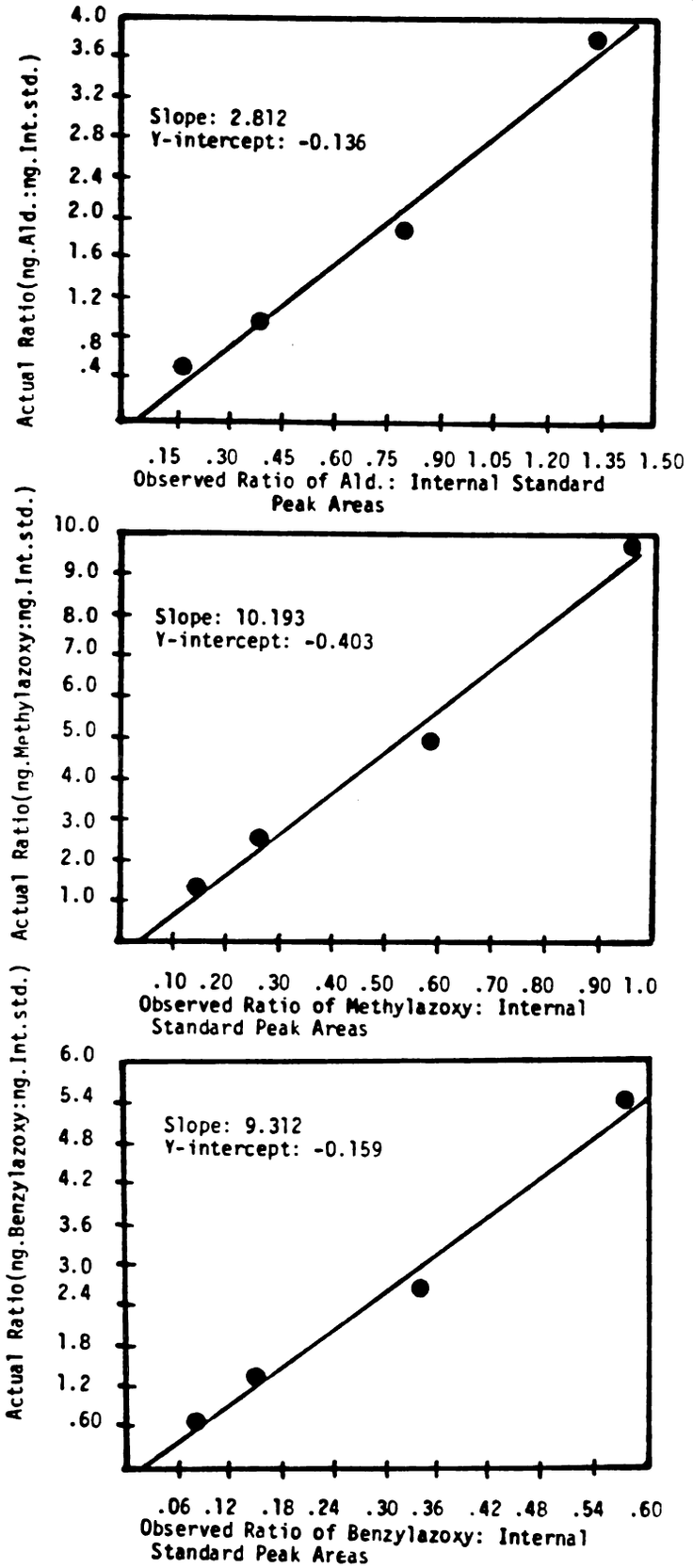
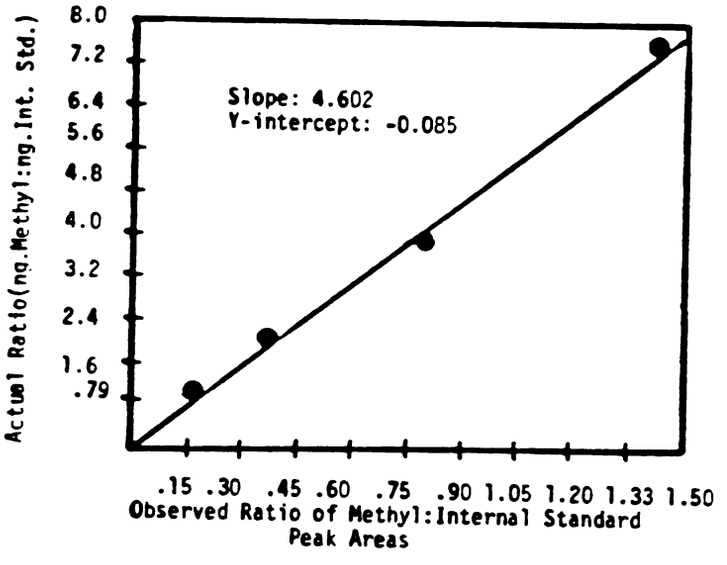
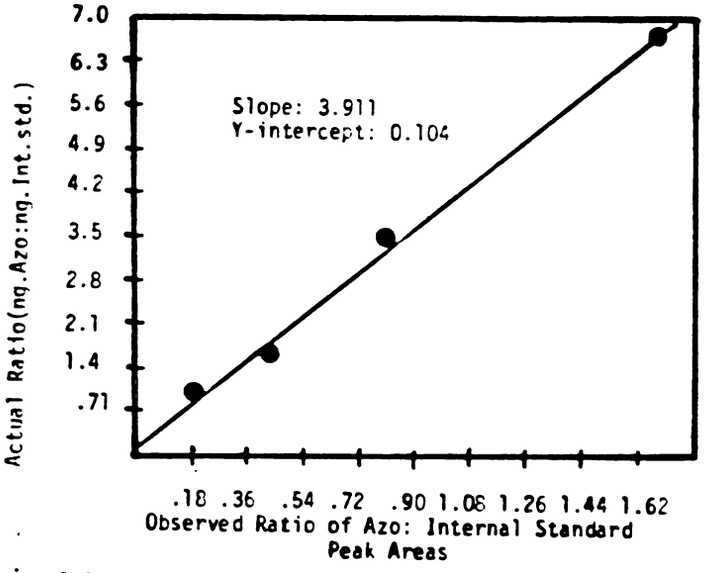
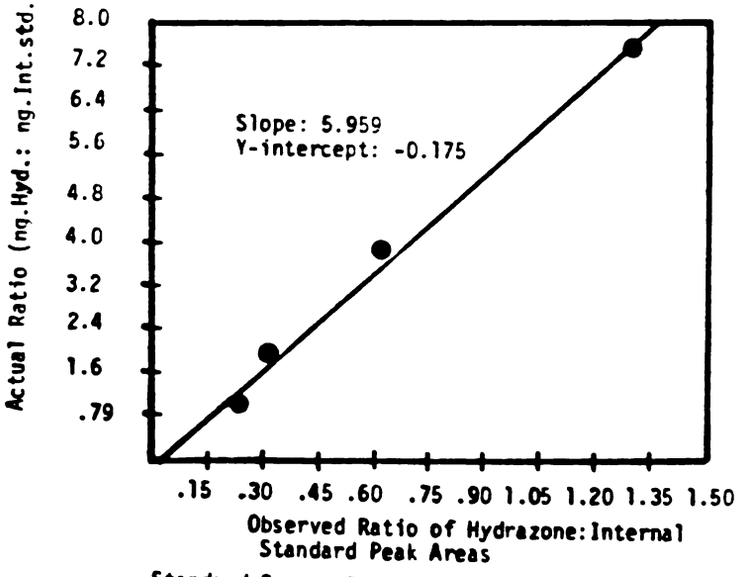


Figure 30. Standard Curves For Aldehyde, Methylazoxy, and Benzylazoxy Without Extraction- Reverse-Phase High-Performance Liquid Chromatographic Assay.







Standard Curves For N-Isopropyl-4-methylbenzamide, Azo, and Hydrazone Without Extraction- Reverse-Phase High-Performance Liquid Chromatographic Assay.

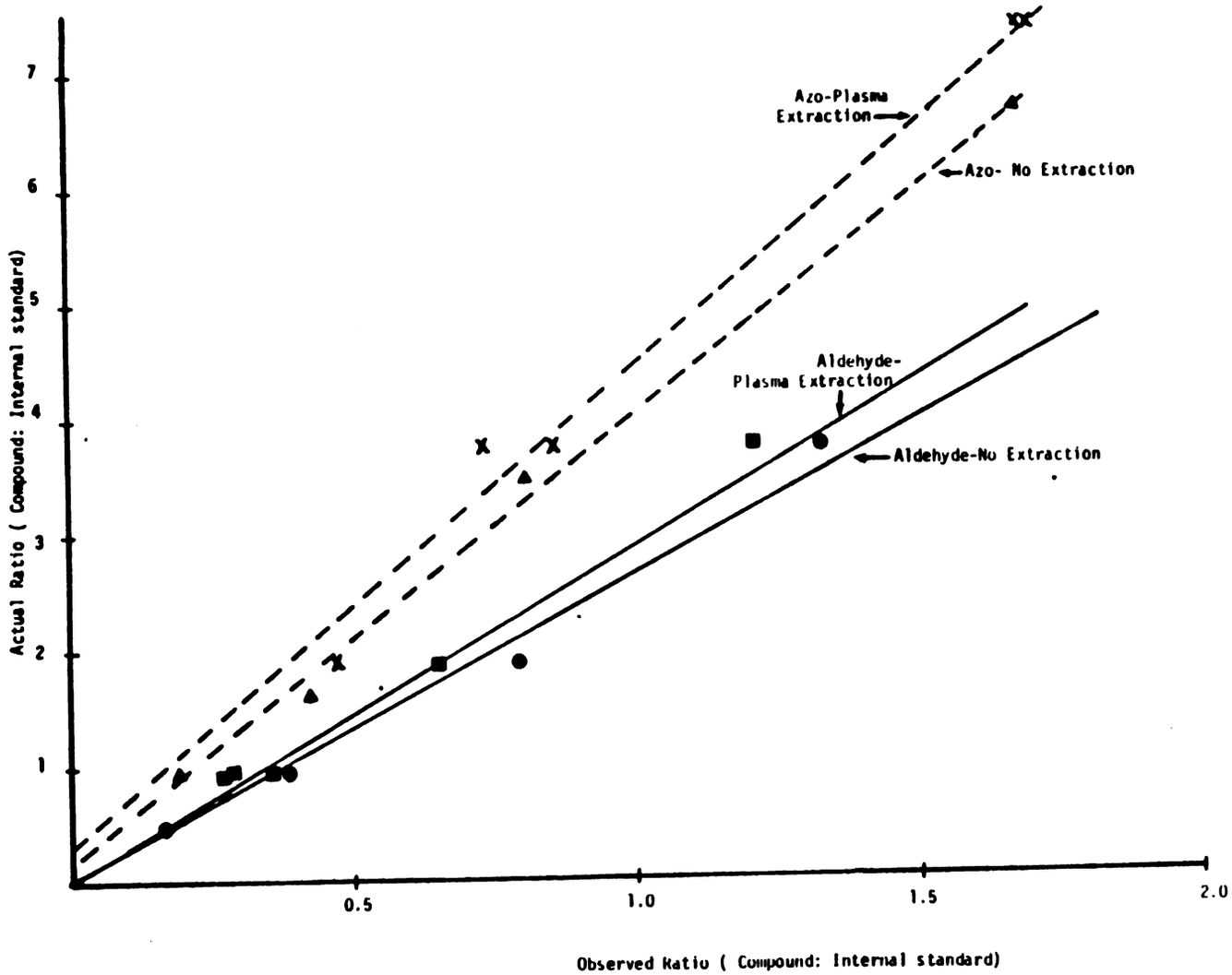


Figure 31. Comparison of Standard Curves Obtained With and Without Extraction From Human Plasma- Azo and Aldehyde.

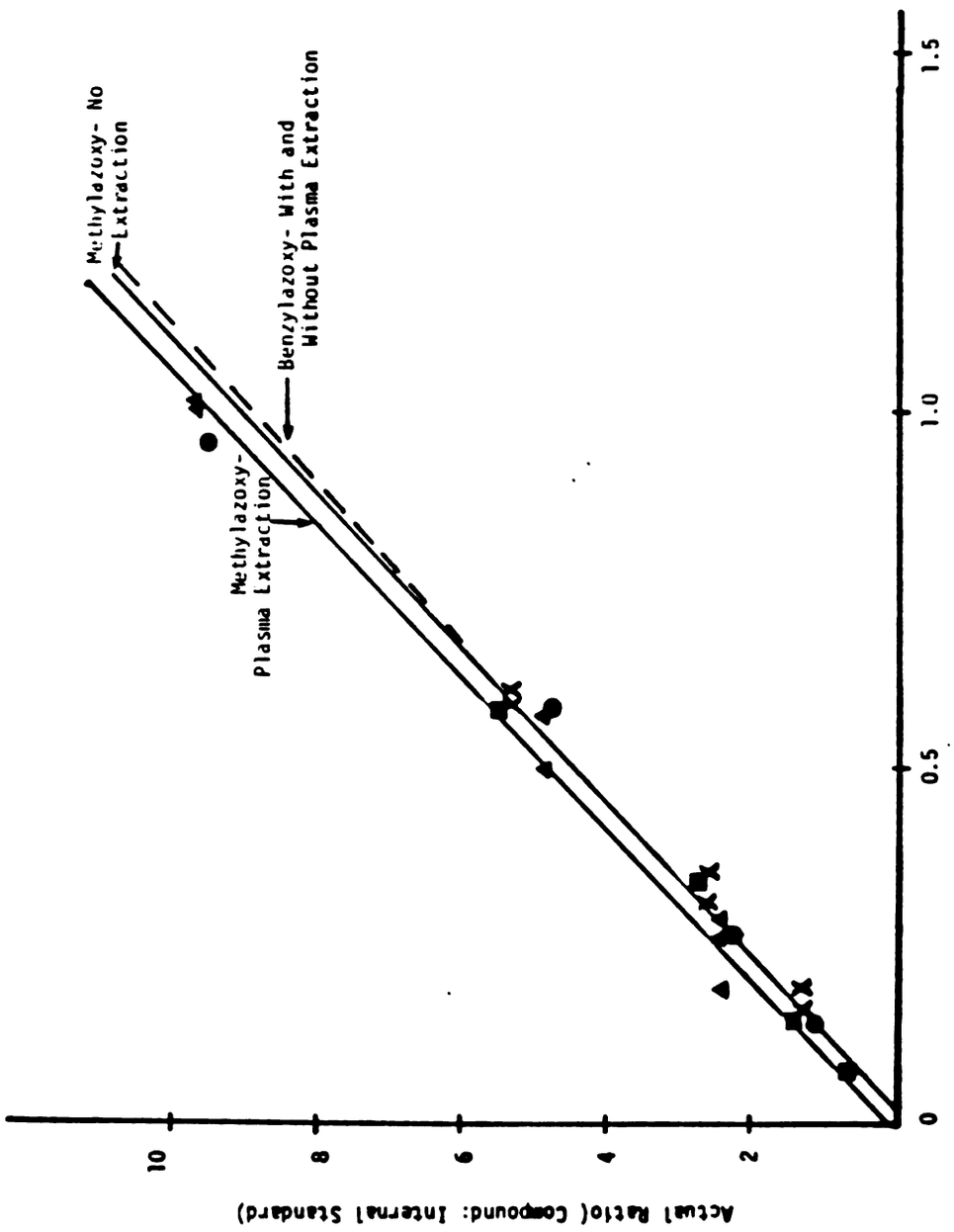


Figure 32. Comparison of Standard Curves Obtained With and Without Extraction From Human Plasma- Methyl- and Benzylazoxy.

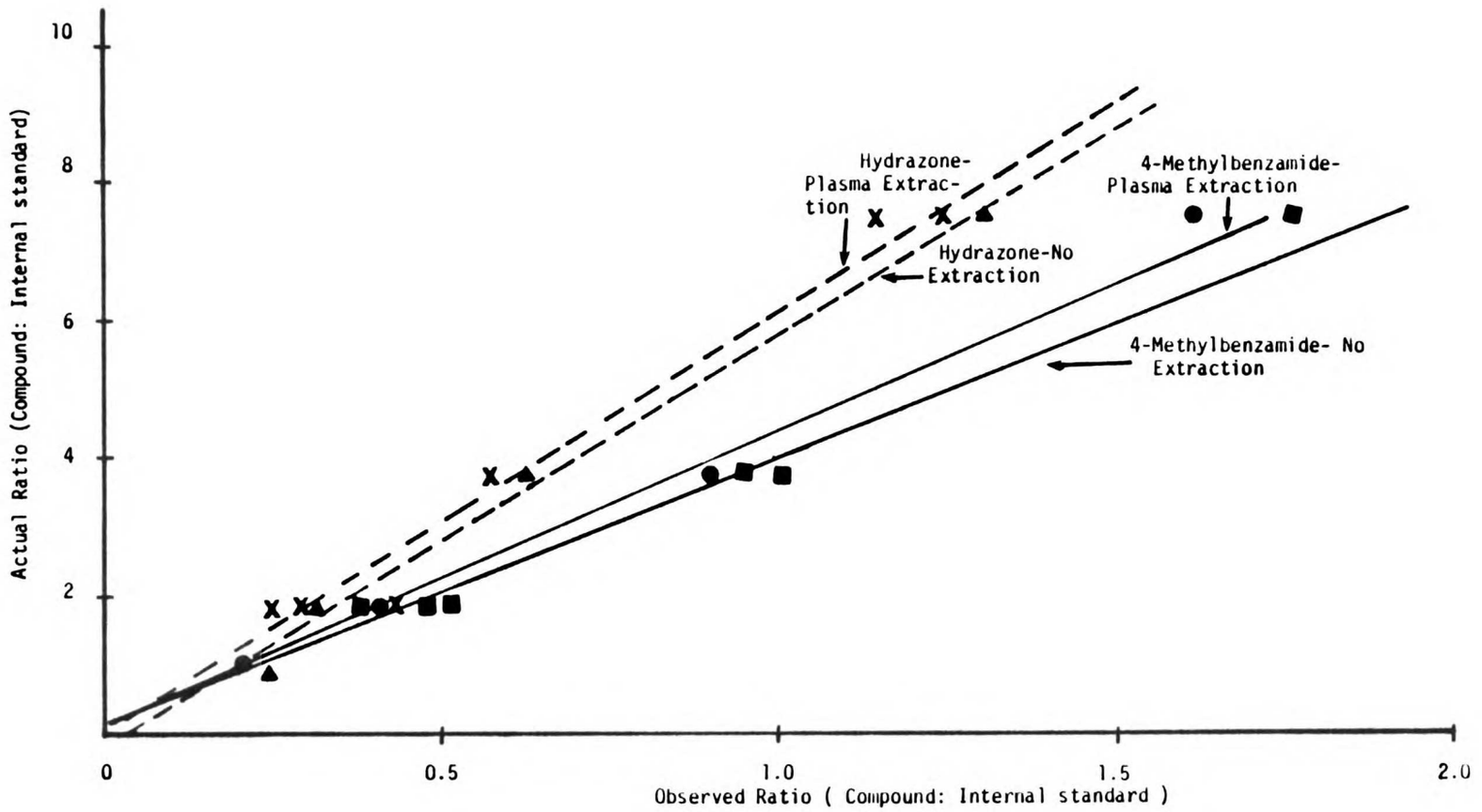


Figure 33. Comparison of Standard Curves Obtained With and Without Extraction From Human Plasma- Hydrazone and N-Isopropyl-4-methylbenzamide.

these compounds in human plasma. These curves are similar within experimental error and both sets are linear throughout the range of the assay. The percent recovery of each compound from human plasma using the assay procedure ranged from 80-134% as shown in Table 5. The standard curves shown in Figure 30 were used to quantitate each compound in the human plasma extractions. The sensitivity limit of the assay system is 0.5 ug. of compound/ml. of sample. However, about 100 ng. of each compound injected onto the column is the limit for detection and quantitation.

Finally, the reverse-phase high-performance liquid chromatographs of a chemical decomposition reaction of Procarbazine in phosphate buffer, pH 7.4, 37°C., 40 minute incubation and a rat liver 9,000 x g. supernatant metabolic incubation of Procarbazine are shown in Figure 34.

C. Discussion

The reverse-phase high-performance liquid chromatographic assay can be used to quantitate the non-polar metabolic and chemical degradation products of Procarbazine. The similarities between the standard curves generated without extraction from human plasma and those generated using the assay procedure show that this assay procedure can be used to quantitate the levels of the aldehyde, azoxy isomers, hydrazone, and N-isopropyl-4-methylbenzamide compounds in biological samples. The addition of the internal standard, 4-methylacetophenone, after the extraction of the

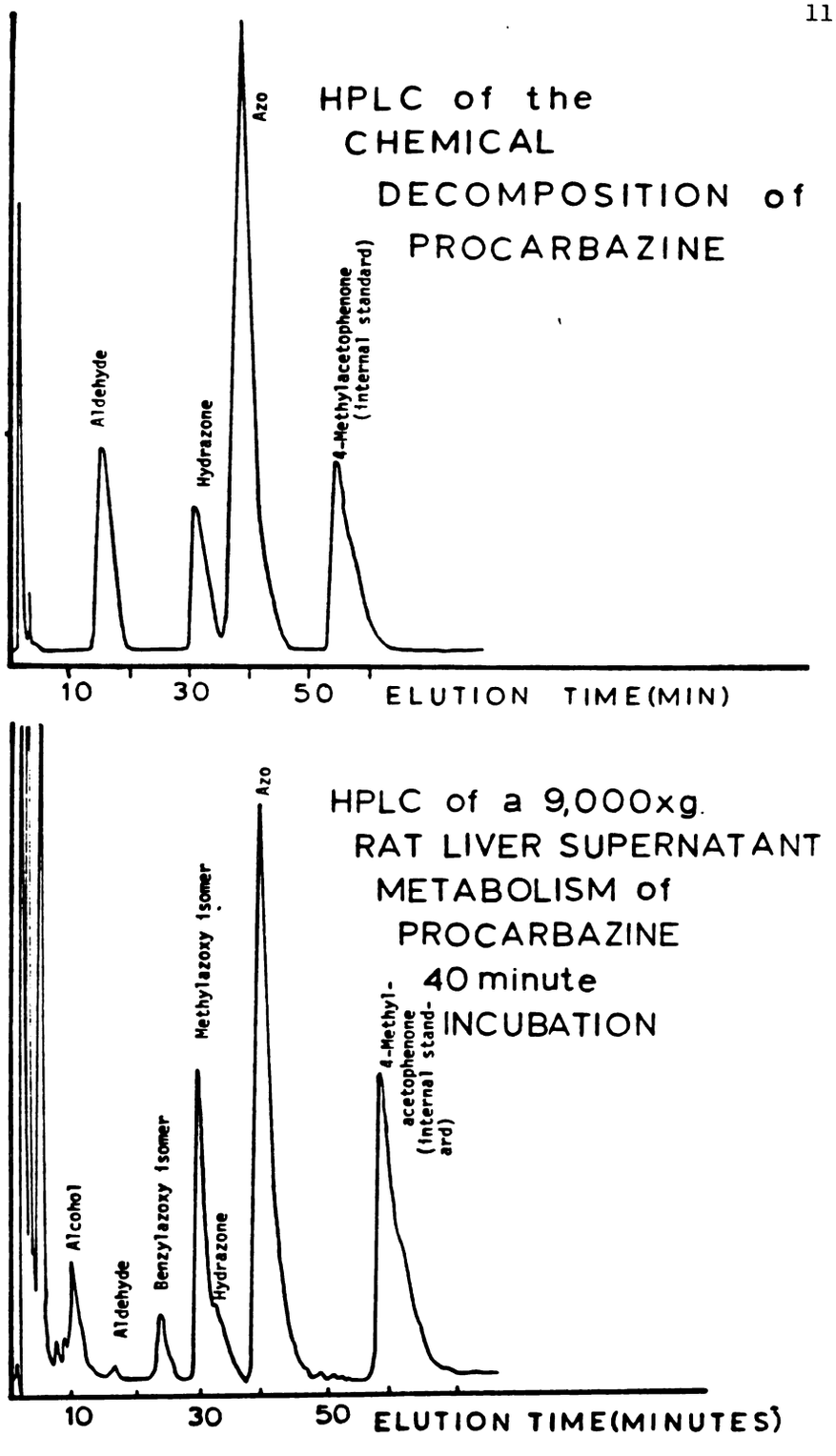


Figure 34. HPLC Chromatographs of a Chemical Decomposition Incubation and a 9,000xg. Rat Liver Supernatant Incubation of Procarbazine.

Chemical decomposition: Phosphate buffer, pH 7.4, 37°C., 40 minute incubation with shaking.

Supernatant: 9,000xg. Rat liver supernate, $MgSO_4$, NADPH, 37° C., 40 minute incubation with shaking.

HPLC conditions: Solvent was 20% methanol: water. Flow rate 2ml/min. λ= 254nm. Spectras run at room temperature. 4-Methyl-acetophenone was the internal reference. Water's u-bondapak C_{18} reverse-phase column.

biological samples did not affect the accuracy of the assay. Furthermore, the sensitivity of the assay allows its direct application to the study of Procarbazine metabolism in intact animals. Greater sensitivity might be obtained by redissolving the dried sample in as little as 50 ul. of methanol instead of 100-500 ul., changing the input into the recorder, or injecting more sample through the use of sample loops larger than 20 ul.

Decomposition of the metabolites is a potential problem in this assay. Rapid extraction of the cold aqueous sample with cold ether followed by either the immediate analysis or the freezing of the sample at -45^{o}C. until analysis might decrease the decomposition rate.

IV. The 9,000 x g. Supernatant, 100,000 x g. Supernatant, and 100,000 x g Microsomal Fractions - Preparation and Related Assays

Procarbazine may be metabolized in several organs and tissues of the body. The principal site of metabolism is the liver, however, with enzymes required for activation localized in the smooth and rough endoplasmic reticulum of hepatocytes.[166] In vitro metabolic studies using homogenized organ and tissue preparations have provided much of the present knowledge of drug metabolism and the role of metabolism upon drug activation and inactivation. The use of in vitro systems eliminates many of the complexities encountered in whole animal metabolic experiments. The in vitro system allows the identification of the tissue involved in the metabolic sequence and pathway, the identification of the co-factors and components of the enzyme systems, involved, the identification of the biochemical properties of the enzyme systems, and the determination of the controlling mechanisms for the activity of each enzymatic step.[167]

The in vitro studies can be carried out using isolated perfused liver preparations, liver slices, or isolated hepatocytes.[168] However, the majority of in vitro studies use the liver homogenate fractions. The 9,000 x g. supernatant fraction contains the membrane-bound enzymes including mixed-function oxidase, soluble enzymes, and the NADPH-generating system. The 100,000 x g. supernatant contains only soluble enzymes and the NADPH-generating system. The 100,000 x g. microsomal fraction contains the membrane-bound enzymes of the endoplasmic reticulum.

The activity of each fraction is greatly influenced by the method of preparation used. Consequently, the determination of the total protein content and the enzymatic activity of each fraction is important in order to compare experiments performed using different preparations. Finally, the determination of the cytochrome P-450 levels as well as P-420 levels is important in assessing the extent of inhibition or induction of the cytochrome P-450-dependent microsomal monooxygenase system.

A. Preparation of the 9,000 x g. Supernatant, 100,000 x g. Supernatant, and 100,000 x g. Microsomal Fractions

The preparation of mouse and rat liver microsomal and supernatant fractions is prepared according to a method published by Fouts.[169] Either Fischer C-344 male rats or CDF_1 male mice are fasted for 24 hours prior to removal of the livers. All of the preparative procedures are done in a cold room and everything which comes into contact with the liver samples are precooled to approximately $0^{\circ}C$. Cold 0.01 M Sorensen's phosphate buffer[170] at pH 7.4 with 1.15% KCl is used as the homogenation medium. The buffer contains monopotassium phosphate (1/15th molar) and disodium phosphate (1/15th molar). The animal was stunned with a blow to the back of the head and sacrificed by decapitation. The blood was drained and the fur wetted with water to prevent hair from entering the abdominal incision. The liver is rapidly removed and the gall bladder if present is separated from the liver. The liver is placed into cold 1.15% KCl - 0.01 M Na/K-phosphate buffer at pH 7.4 and washed to removed hemoglobin,

blotted dry, and placed into a preweighed beaker containing 25 ml. of cold buffer. The wet liver is weighed and 12.5 g. placed in 25 ml. of buffer. The tissue is minced with scissors. The minced tissue is placed into a cold Potter-Elvehjem glass homogenizing tube and homogenized with a cold teflon pestle for about 1 minute (3 up-and-down passages). Warming of the tube during homogenation should be avoided.

The homogenate is centrifuged at 9,000 x g. (8700 r.p.m.) in a Sorvall Superspeed RC2-B centrifuge for 15 minutes at 0°C. The supernatant is removed from below the white lipid upper layer and above the red-tan bottom pellet containing mitochondria and cellular debris. The supernatant is again centrifuged for an additional 15 minutes. If more than 1 centrifuge tube is used, all of the tubes are pooled to minimize differences in the animals which contributed to the supernatant sample. This is the 9,000 x g. supernatant fraction. To prepare the 100,000 x g. supernatant and microsomal fractions, 10 ml. of the 9,000 x g. supernatant is placed into cold Spinco centrifuge tubes using cold buffer to completely fill the tubes. The tubes are then placed into a Beckman 50Ti ultracentrifuge head and centrifuged at 100,000 x g. (43,000 r.p.m.) for 60 minutes at 0°C. in a Beckman L2-65B ultracentrifuge. The clear liquid is decanted and used as the 100,000 x g. supernatant fraction. The microsomal pellet is resuspended into cold buffer and centrifuged for an additional 60 minutes. The clear supernatant is discarded and the pellet rehomogenized in 10.0 ml. of buffer. This is the 100,000 x g.

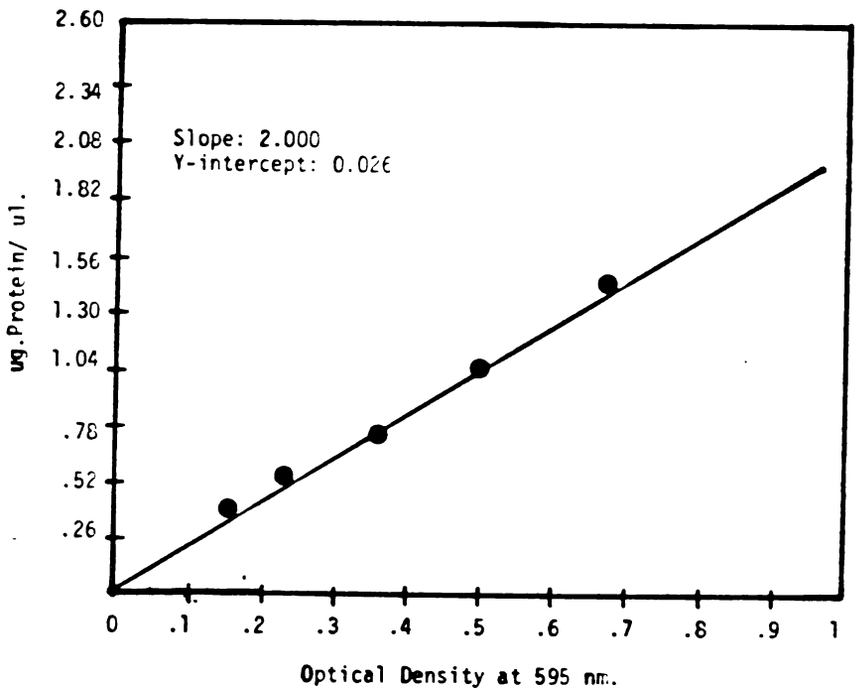
microsomal fraction. Each 10 ml. fraction represents 5 g. of an initial 12.5 g. of wet liver. The fractions may be used immediately or frozen at -45°C. until use. The 100,000 x g. microsomal pellet is stored as a pellet with 2 ml. of buffer layered over it. The 9,000 x g. supernatant fraction and 100,000 x g. microsomal fraction can be stored for 1 month without substantial loss of p-nitroanisole 0-demethylase activity. These stored fractions retain their Procarbazine metabolizing ability.

B. Determination of the Total Protein

The determination of the total protein in each liver homogenate fraction is made by using the Bio-Rad Protein assay. This assay is similar to both the Lowry and Biuret assays and produces comparable results in most cases. The advantages of this assay are that it requires a single reagent and may be completed in 5 minutes. The Bio-rad dye-protein complex is stable for over 1 hour and is not affected by ammonium ion, EDTA, mercaptoethanol, and reducing sugars.[171]

The assay is performed on 0.1 ml. of homogenate prepared as described above. A 5.0 ml. aliquot of the freshly prepared dye reagent is added to each spectrometric tube. The dye reagent is prepared by diluting 20 ml. of the dye concentrate with 80 ml. of distilled water followed by vacuum filtering through a Whatman #1 filter paper. The tube is carefully vortexed to avoid excessive foaming. After 5 minutes to 1 hour, the absorbance of each sample is determined relative to an

appropriate blank of buffer and dye in a Colman Jr. Spectrophotometer at 595 nm. Each sample is prepared in duplicate. A typical least squares linear regression analysis standard curve is shown in Figure 35 . The standard curves are generated using a Bio-Rad lyophilized protein standard at concentrations ranging from 0.35-1.36 ug/ul. The equation used to determine the protein concentration of an unknown sample is also shown.



Protein Concentration(ug/ml.)= [Optical Density at 595 nm.] x [Slope of the standard curve] x [Y-intercept of the standard curve]

Figure 35. Typical Standard Curve and Equation Used For the Quantitation of the Total Protein in the Liver Homogenate Fractions Using the Bio-Rad Protein Assay and Lyophilized Protein Standard.

The 9,000 x g. supernatant, 100,000 x g. supernatant, and 100,000 x g. microsomal samples are prepared by diluting 100 ul. of each homogenate solution described above with 500 ul. of distilled water. After stirring or vortexing, 0.1 ml. of this is used for the assay. This solution is then diluted 1:1 with distilled water and 0.1 ml. of this dilution serves as a second determination of the sample. Further dilutions are made if the readings at 595 nm are beyond the range of the standard curve. The blank contains 5.0 ml. of dye and 0.1 ml. of distilled water.

C. Determination of the Enzyme Activity

Enzyme activity of the liver microsomal enzyme systems is determined by assaying for p-nitroanisole O-demethylase activity. This assay is performed by using a modification of the procedure outlined by Zannoni.[172] According to Zannoni, p-nitroanisole is an excellent "model drug" because the demethylated p-nitrophenol at pH 7.8 absorbs light at 420 nm and can be followed continuously. The O-demethylase reaction is shown in Figure 36.

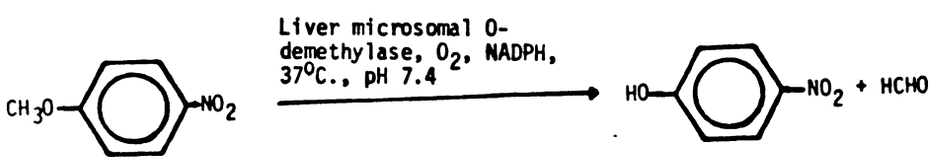
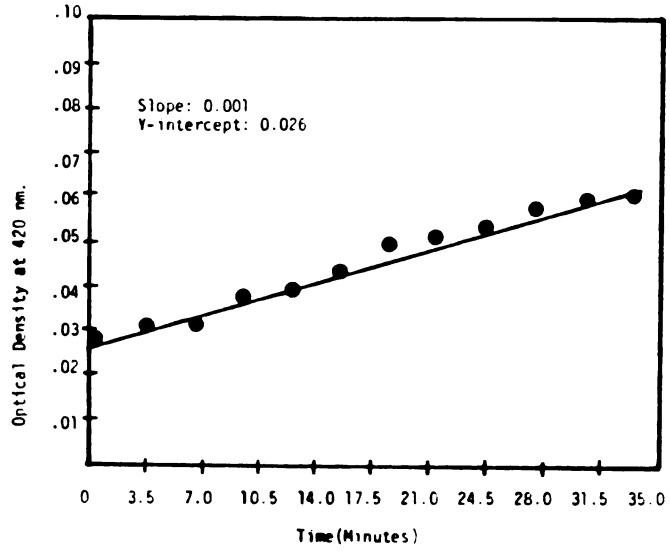


Figure 36. p-Nitroanisole O-Demethylase Reaction

The assay is performed by using 2 Coleman 12 x 75 mm round-bottom cuvettes. The control tube contains 0.8 ml. of phosphate buffer; 0.15 ml. of $MgSO_4$ (12.04 mg./ml.); 1.50 ml. of p-nitroanisole (0.31 mg./ml.); and 0.02 ml. of the supernatant or microsomal sample. The assay tube contains 0.75 ml. of buffer; 0.15 ml. of $MgSO_4$ (12.04 mg./ml.); 1.50 ul. of p-nitroanisole (0.31 mg./ml.); and 0.02 ml. of the supernatant or microsomal sample. Each component is added in the order listed. Before each sample time point, the Coleman Model 60 Jr. Spectrophotometer 420 nm absorbance is zeroed using the control tube. A 0.05 ml. aliquot of 12.42 mg./ml. NADPH is added to the assay tube. The tube is vortexed and the "zero-time" absorbance measurement is made. Both tubes are incubated prior to the start of and during the assay in a Dubnoff metabolic shaker at 37°C. with shaking. The p-nitroanisole absorbance is measured every 3 minutes for 24-30 minutes. The absorbance of 1 ug. of the product p-nitrophenol is determined under the assay conditions by substituting 1 ug. of p-nitrophenol in 1.5 ml. of buffer for p-nitroanisole in the above procedure and measuring absorbance. p-Nitroanisole does not dissolve readily in buffer so the mixture must be heated to 45-51°C. with shaking for over 1 hour. This solution can be used for 8 hours. A 1 ug. (0.003 mM) sample of p-nitrophenol, E = 10,000, gives an absorbance of approximately 0.032. This value is used for each enzyme activity determination.

A typical least squares linear regression analysis of these enzyme kinetic data is shown in Figure 37. Also shown

is the equation used to calculate the enzyme activity expressed as u moles of p-nitrophenol formed/hour/100 mg. of total protein.



Liver microsomal p-nitroanisole O-demethylase activity(umoles of p-nitrophenol formed/hour/100 mg. total protein)=

$$\frac{[\text{Change in optical density/min.}] \times [60 \text{ min./hr.}] \times [100 \text{ mg. total protein}]}{[\text{Optical density of p-nitrophenol}] \times [\text{M.W. p-nitrophenol}] \times [\text{amount of protein}]}.$$

The optical density of p-nitrophenol= 0.032 under the experimental conditions used.
The molecular weight of p-nitrophenol = 139.

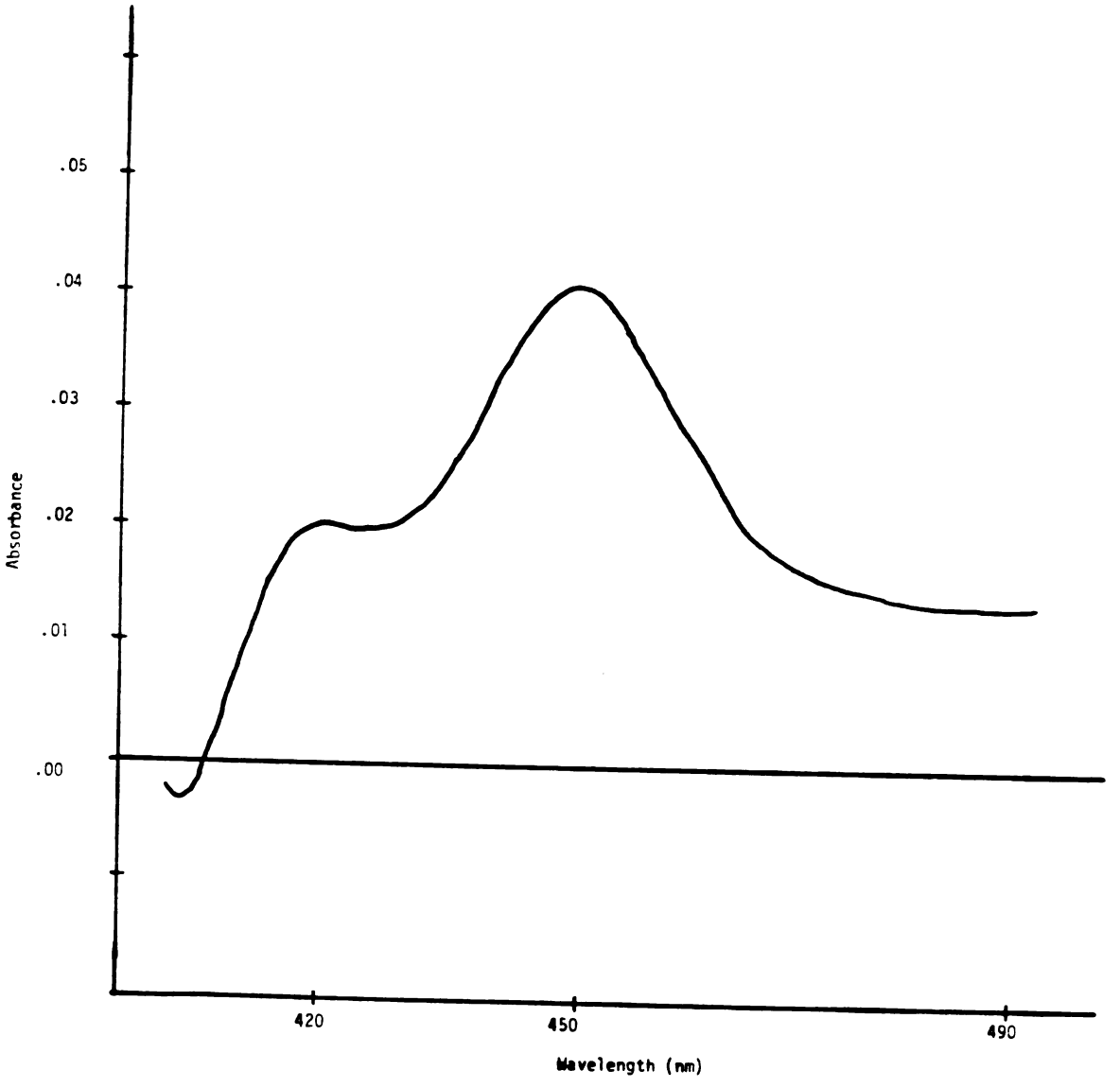
Figure 37. **Typical Standard Curve and Equation for the Spectrometric Quantitation of the Liver Microsomal p- Nitroanisole O-Demethylase Activity.**

D. Determination of the Cytochrome P-450

Cytochrome P-450 is the terminal oxidase in the liver microsomal drug metabolism enzyme system. The levels of cytochrome P-450, under some experimental conditions, have a direct relationship with drug metabolizing activity in hepatic preparations. The levels also provide a convenient

measure of phenobarbital induction and depletion of the microsomal metabolic enzymes by several compounds.

The determination of the cytochrome P-450 levels was performed by Mr. Steve Olson of the University of California at San Francisco Liver Center. The 100,000 x g microsomal fraction is prepared as previously described except that instead of merely washing the liver prior to mincing, the liver lobes are perfused using a syringe with cold phosphate buffer until the lobes are pale pink instead of the hemoglobin-containing red color. The homogeneous microsomal preparation is diluted 1:10 with the cold 1.15% KCl-0.01 M Na/K-phosphate buffer at pH 7.4 used during the microsomal preparation. A 1 ml. aliquot is placed into 2 matched cuvettes and gassed for 20 seconds with carbon monoxide. A few milligrams of sodium dithionite (NaS_2O_4) is added to the assay cuvette and mixed by inversion. The reference and sample absorbance is scanned in an Aminco DW-2 dual-beam recording spectrophotometer from 400-500 nm. A typical spectra is shown in Figure 38. Also shown is the equation used to calculate the amount of cytrochrome P-450 present, expressed as nanomoles of cytochrome P-450/milligram of total protein.



$$\text{Cytochrome P-450 Concentration} = \frac{[\text{Absorbance}] \times [\text{Dilution factor}]}{[E] \times [\text{Cell pathlength}] \times [\text{mg. protein}]}$$

$E = 100 mM^{-1} cm^{-1}$
Cell pathlength= 1 cm.

Figure 38. Typical Visible Difference-Spectrum of the Rat Liver 100,000xg. Microsomal Fraction and the Equation Used For the Quantitation of Cytochrome P-450.

V. The Chemical Degradation of Procarbazine

Procarbazine is chemically unstable in aqueous solutions. In the presence of molecular oxygen, Procarbazine is oxidized to the azo compound and hydrogen peroxide.[1] The azo derivative slowly isomerizes in aqueous solutions to the hydrazone of monomethylhydrazine and N-isopropyl-p-formylbenzamide. The hydrazone is subsequently hydrolyzed to methylhydrazine and N-isopropyl-p-formylbenzamide.[97] The aldehyde may be oxidized to N-isopropylterephthalamic acid.[91] Although these chemical degradation products of Procarbazine have been previously identified, the relative rates of appearance of these compounds is not known. No attempt to quantitate the chemical degradation products has been reported. This information could be important in determining the extent to which chemical decomposition contributes to the *in vitro* and *in vivo* activation or inactivation of Procarbazine. The purpose of the study of the chemical degradation of Procarbazine is to identify and quantitate the chemical degradation products and to determine the kinetics of the decomposition pathway.

A. Experimental Methods and Procedures

Procarbazine, 28.0 u moles of the hydrochloride salt, in 12 ml. of 1.15% KCl/0.01 M Na/K-phosphate buffer at pH 7.4 in an uncovered 25 ml. Erlenmeyer flask was incubated for 80 minutes in a Dubnoff metabolic shaker at 37°C. Another sample, 14.0 u moles of the hydrochloride salt, in 6 ml. of the buffer under nitrogen was also incubated with shaking.

At various times, 1 ml. of each sample was extracted with 5 x 3 ml. of ether, the ether evaporated with nitrogen, and the residue redissolved in approximately 100 ul. of methanol. A 10 ul. portion of the internal standard, 4-methylacetophenone (6.7 ug.), was added and approximately 15 ul. of the vortexed sample was injected into the high-performance liquid chromatograph. Both qualitative and quantitative information is obtained according to the procedure described in Section III.

A 1 ml. aliquot of the reaction mixture was added to a cold tube containing 25 ul. of Procarbazine-d_6 and N-isopropyl-terephthalamic acid-d_6. After acidifying with 6 drops of 1 N HCl, the sample was extracted with 3 x 3 ml. of ether, the ether was evaporated under nitrogen, and the residue redissolved in a minimum volume of methylene chloride. The sample was analyzed and the acid quantitated by the selected-ion monitoring chemical ionization mass spectrometric analytical system according to the procedures in Section III. The remaining aqueous layer was basified with 14 drops of 1 M NaOH, extracted with ether, analyzed, and Procarbazine quantitated according to the procedure described for the acid extraction. Each decomposition product and Procarbazine was identified by comparison of the mass spectral data to that of an authentic compound synthesized according to the procedures in Section III.

In an alternative procedure, a decomposition mixture sample was chromatographed on a silica gel on glass thin-layer plate, developed with ethyl acetate:hexane (35:10) and visualized with ultraviolet light. The R_f values of the decomposition products

were compared to those of the synthetic compounds. Thin-layer chromatographic spots were removed and eluted with methylene chloride. The eluent was analyzed by chemical ionization mass spectrometry and by reverse-phase high-performance liquid chromatography. The spectroscopic data and elution time ratios for each decomposition product were compared to those for the synthetic compounds.

B. Results

Identification of the Chemical Decomposition Products

The identification of the chemical decomposition products was made using both silica gel thin-layer chromatography and reverse-phase high-performance liquid chromatography. Procarbazine decomposes to give the azo, hydrazone, aldehyde, and acid compounds in the presence of air. The R_f values for each of the decomposition products agreed with those of the corresponding synthetic compounds. The mass spectra of both were also identical. The results of the thin-layer chromatographic analysis are shown in Table 8.

Table 8. Thin-layer Chromatographic and Mass Spectrometric Qualitative Analysis of the Chemical Decomposition of Procarbazine in Phosphate Buffer, pH 7.4, 37°C., 40 Minute Incubation in the Presence of Air.

Compound	R_f of Standard	R_f of Incubation Mix.	Standard M.S. MH^+	Incubation M.S. MH^+
Procarbazine	Remains at origin	Remains at origin	222.3	222.3
Azo	0.59	0.59	220.3	220.3
Hydrazone	0.43	0.43	220.3	220.3
Aldehyde	0.50	0.50	192.2	192.2
Acid	Remains at origin	Remains at origin	207.2	207.2

Silica gel glass thin-layer plate, developed in ethylacetate: hexane (35:10), visualized with ultraviolet light.

The reverse-phase high-performance liquid chromatographic spectrum of the decomposition sample is shown in Figure 39. The elution time ratios for the azo, aldehyde, and hydrazone agree with the ratios for the authentic samples. Chemical ionization mass spectral analysis of each chromatographic peak also corresponds to that obtained for the authentic compounds. The results of the liquid chromatographic analysis are shown in Table 9.

Quantitation of Each Chemical Degradation Product and the Kinetics of the Degradation Pathway

The quantitation of azo, aldehyde, and hydrazone was done using the liquid chromatographic analysis. Procarbazine and acid levels were determined using the d_6-internal standards in the selected-ion monitoring chemical ionization mass spectral analysis. The major degradation product is the azo compound which is rapidly formed through the oxidation of Procarbazine. The hydrazone and the hydrolysis product, the aldehyde, are only minor products of the chemical degradation pathway. The acid is present at only trace amounts. The amount of each of the degradation products recovered after incubation at 37°C., pH 7.4 for 90 minutes is shown in Table 10.

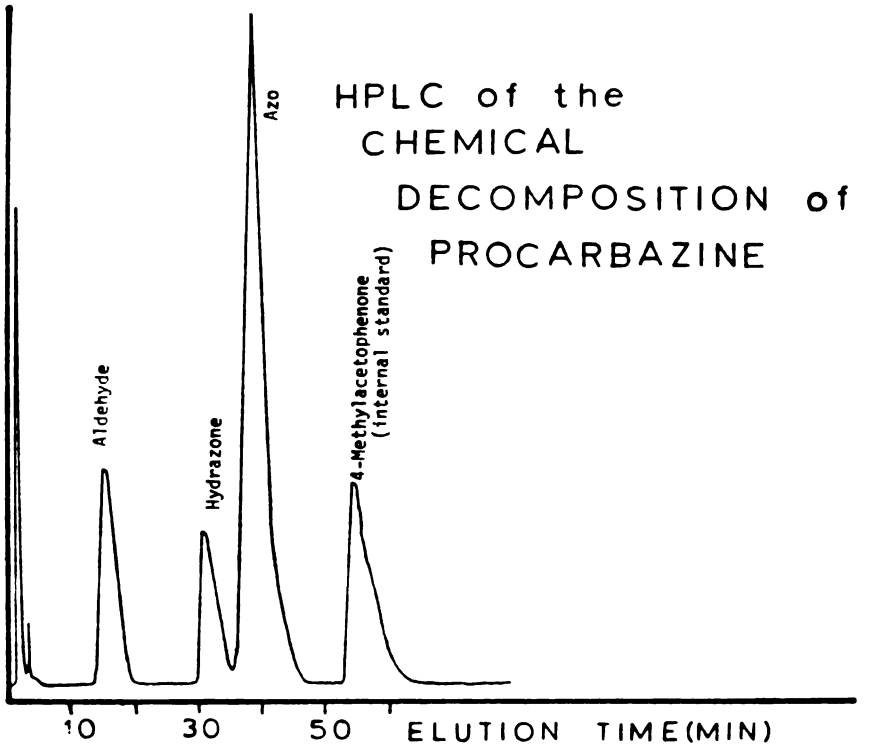


Figure 39. HPLC Chromatograph of a Chemical Decomposition Incubation of Procarbazine in Phosphate Buffer.

Phosphate buffer, pH 7.4, 37°C., 40 minute incubation with shaking in the presence of air.

HPLC conditions: Solvent was 20% methanol:water. Flow rate 2ml/minute. λ= 254nm. Spectrum was run at room temperature. 4-Methylacetophenone was used as the internal reference.

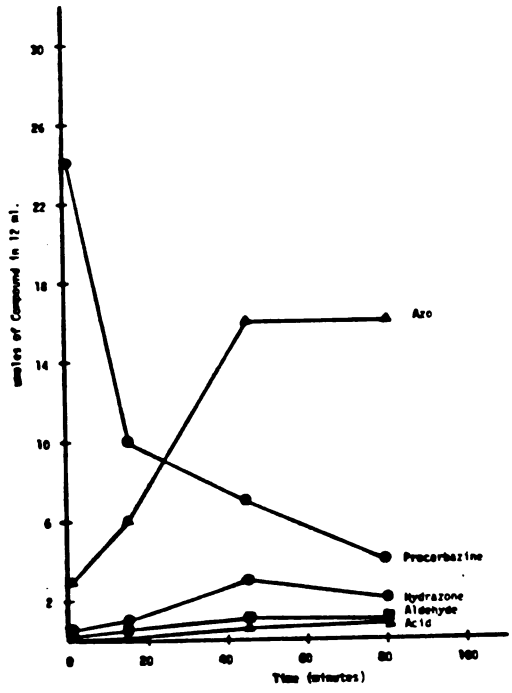
Table 9. HPLC Chromatographic Qualitative Analysis of the Chemical Decomposition of Procarbazine in Phosphate Buffer, pH 7.4, 37°C., 40 Minute incubation in the presence of air.

Compound	Elution Time Ratio of Standard	Incubation Elution Time Ratio	Standard M.S. MH^+	Incubation M.S. MH^+
Azo	0.67	0,67	220.3	220.3
Hydrazone	0.55	0.55	220.3	220.3
Aldehyde	0.27	0.27	192.2	192.2

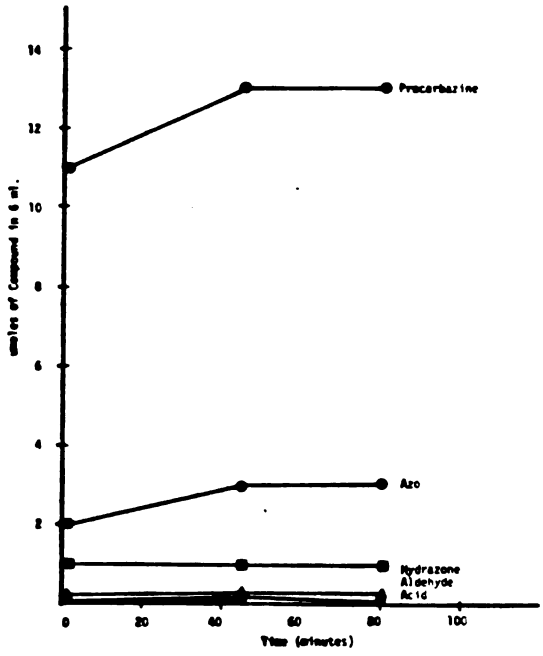
Table 10. Quantitation of the Chemical Decomposition Kinetics of Procarbazine in Phosphate Buffer in the Presence of Air and in the Presence of Nitrogen Gas.

Air:			umoles in 12 ml. Sample				
Time(minutes)	Azo	Hydrazone	Aldehyde	Procarbazine	Acid	Observed Total	% Recovery
0.92	3.0	0.5	0.5	24.0	0.1	28.0	100.8
15.67	6.0	1.0	0.5	10.0	0.2	17.9	63.9
45.83	8.0	2.0	1.0	7.0	0.6	28.6	102.1
80.58	15.0	3.0	1.0	4.0	0.9	24.9	88.9
		Procarbazine initially= 28.0 umoles					
Nitrogen:			umoles in 6 ml. Sample				
1.25	2.0	1.0	0.2	11.0	0.1	14.4	102.9
45.92	3.0	1.0	0.3	13.0	0.1	17.6	125.7
80.58	3.0	1.0	0.3	13.0	0.1	17.6	125.7
		Procarbazine initially= 14.0 umoles					

The appearance curves for the chemical decomposition products of Procarbazine under air and nitrogen atmosphere are shown in Figure40.



The Chemical Decomposition of Procarbazine in the Presence of Air.



The Chemical Decomposition of Procarbazine in the Presence of Nitrogen.

Figure 40. Kinetic Curves For the Chemical Decomposition of Procarbazine in Phosphate Buffer in the Presence of Air and in the Presence of Nitrogen.

Phosphate buffer, pH 7.4, 37°C., incubated with shaking for 80 minutes.

It is clear from these data that the reaction, conversion of Procarbazine to azo requires oxygen. The half-life for the conversion of Procarbazine is approximately 12 minutes. Under nitrogen, 93% of the original Procarbazine dose remains after 40 minutes. After 40 minutes, 27% of Procarbazine remains, the azo represents 50% of the initial dose, and the hydrazone only 9%. This indicates that the isomerization of the azo to the hydrazone is very slow at pH 7.4.

C. Discussion

The oxidative chemical degradation pathway for Procarbazine in phosphate buffer (Figure 41), leads rapidly to the azo compound and hydrogen peroxide. The azo compound, which is the major decomposition product, is slowly isomerized to the hydrazone. Hydrolysis of the hydrazone liberates aldehyde and methylhydrazine. The aldehyde may be further oxidized to the major *in vivo* urinary metabolite, N-isopropylterephthalamic acid. The fate of methylhydrazine has not been determined.

Although the initial oxidation to the azo compound is fast, the remaining chemical reactions are relatively slow. The exact importance of the role of the chemical degradation pathway in the *in vivo* pharmacology and activation of Procarbazine is not defined by these data alone. However, since the isomerization of the azo to the hydrazone and subsequent hydrolysis of the hydrazone to the aldehyde and methylhydrazine are slow, the azo compound may undergo other, more rapid reactions *in vivo* and *in vitro* as will be shown. The contribution of the rearrangement and hydrolysis reaction sequence to *in vivo* antineoplastic activity, therefore, is doubtful.

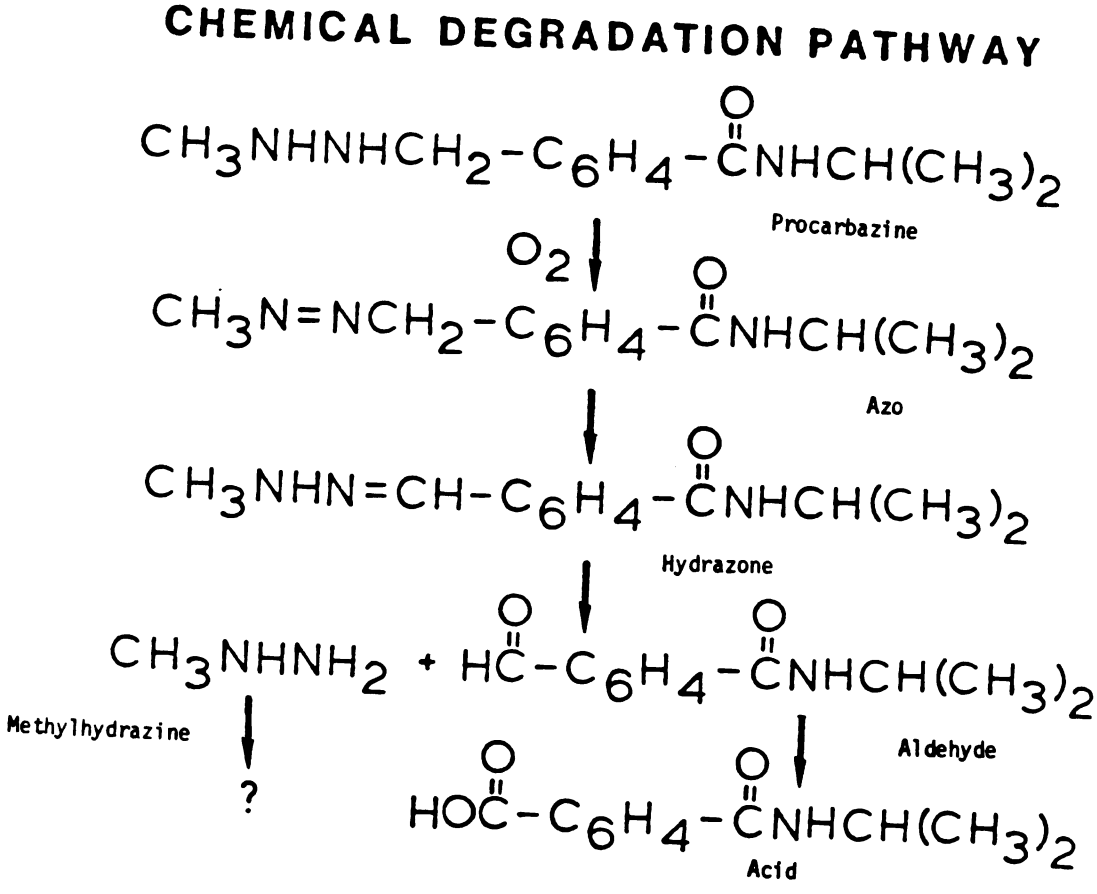


Figure 41. The Chemical Degradation Pathway For Procarbazine.

VI. The In Vitro Metabolism of Procarbazine

The in vitro metabolism of Procarbazine has been studied in perfused rat liver, human liver slices, and rat liver microsomal preparations. The numerous explanations of the in vitro metabolism have been thoroughly discussed in the review of Procarbazine metabolism in Section I. Procarbazine is oxidized to the azo compound. This oxidation is NADPH-dependent, requires liver enzymes, and is several times faster than other reactions of Procarbazine. The azo compound is either further metabolized to the acid or isomerized to the hydrazone. The hydrazone can be hydrolyzed to the aldehyde, which is then further oxidized to the acid. The direct metabolism of azo to acid could result from N^2-C cleavage by microsomal hydroxylase. Both Procarbazine and the azo can also undergo N-demethylation to formaldehyde. Formaldehyde is further metabolized to CO_2.

Despite the numerous studies reported on the in vitro Procarbazine metabolism, little is known about the metabolic pathway after the initial oxidation to the azo compound.

The in vitro metabolism of Procarbazine was studied using the following three fractions of rat liver homogenate: the 9,000 x g. supernatant, the soluble enzyme-containing 100,000 x g. supernatant, and the 100,000 x g. microsomal fraction. Of particular interest are the metabolic pathways leading to the bioactivation of Procarbazine. Procarbazine can act as a potential alkylating species as discussed in the review on the mode of action of Procarbazine. Yet, the pathways leading to

alkylating species have been merely postulated in the past. Finally, since little is known about the kinetics of the in vitro metabolism and the quantitation of each of the metabolic products, the analytical systems described previously in Section III were used to identify and quantitate the metabolic intermediates.

A. Experimental Methods and Procedures

Identification of the In Vitro 9,000 x g. Rat Liver Supernatant Metabolites - 40 Minute Incubation

The rat liver 9,000 x g. supernatant was prepared using male Fischer C-344 rats according to the procedure described in Section IV. A solution of 5.0 ml. of 9,000 x g. supernatant; 0.64 ml. of 9.3 mM Procarbazine solution (final concentration: 0.91 mM); 0.94 ml. of 0.1 M $MgSO_4$ (final concentration: 14.2 mM); and 4.0 mg. of NADPH added every 20 minutes (final concentration: 0.5 mM) was incubated with shaking in a Dubnoff metabolic shaker at 37°C. for 40 minutes. A 1.0 ml. aliquot of the incubation mixture was extracted with 5 x 3 ml. of ether and the ether was evaporated with nitrogen gas. The residue was dissolved in methylene chloride and analyzed by silica gel thin-layer chromatography using ethyl acetate: hexane (35:10) for development. The R_f value of each of the metabolites was compared to the R_f value for the authentic compounds. The aqueous layer was acidified with 6 drops of 1 N HCl and extracted with 5 x 3 ml. of ether. The remaining aqueous layer was made basic with 15 drops of 1 M NaOH and extracted with 5 x 3 ml. of ether. Both ether layers were also evaporated and the residues dissolved in

methylene chloride and analyzed by thin-layer chromatography. All of the spots were collected and eluted with methylene chloride. The eluent was evaporated and analyzed by mass spectrometry and the mass spectra for each spot was compared to that of the corresponding authentic compound.

The incubation sample was also analyzed by reverse-phase high-performance liquid chromatography using the system described in Section III. The elution time ratios were compared to the elution time ratios for the authentic compounds. Each peak was collected and the mass spectra compared to those of the authentic samples. Finally, the neutral, acidic, and basic extracts were analyzed directly by chemical ionization mass spectrometry without separation.

Metabolism of Procarbazine by 9,000 x g. Rat Liver Supernatant, 100,000 x g. Supernatant, and 100,000 x g. Microsomes - 40 minute Incubation

Rat liver 9,000 x g. and 100,000 x g. supernatant and 100,000 x g. microsomes were prepared using Fischer C-344 male rats according to the procedures described in Section IV. The incubation mixture containing 5.0 ml. of the supernatant or microsomes; 0.75 ml. of 9.35 mM drug (final concentration: 1.0 m); 1.0 ml. of 0.1 m $MgSO_4$ (final concentration: 14.3 mM); and 0.25 ml. of 14.0 mM NADPH added every 20 minutes (final concentration: 0.5 mM) was incubated with shaking at 37°C. for 40 minutes. The control incubations contained 5.0 ml. of supernatant or microsomes boiled for 30 minutes at 100°C. and rehomogenized in buffer to give the original volume;

0.38 ml. of 9.35 mM drug (final concentration: 0.6 mM); 0.5 ml. of 0.1 M $MgSO_4$ (final concentration: 8.3 mM); and 0.13 ml. of 14.0 mM NADPH added every 20 minutes (final concentration: 0.3 mM).

A 2.0 ml. aliquot of each incubation mixture was analyzed for the acid and Procarbazine using the mass spectral analysis and for the other metabolites using the reverse-phase high-performance liquid chromatographic analysis described in Section III. The enzyme activity and total protein concentration was determined according to the procedures described in Section IV.

Kinetics of the 9,000 x g. Rat Liver Supernatant Metabolism

The 9,000 x g. rat liver supernatant was prepared as previously described. The incubation mixture containing 10.0 ml. of supernatant; 1.5 ml. of 18.7 mM drug (final concentration: 2.0 mM); 2.0 ml. of 0.1 M $MgSO_4$ (final concentration: 14.3 mM); and 0.5 ml. of 14.0 mM NADPH added every 20 minutes (final concentration: 0.5 mM) was incubated with shaking at 37°C. for 40 minutes. The control incubation contained 5.0 ml. of buffer, 0.75 ml. of drug (final concentration: 2.0 mM); 1.0 ml. of 0.1 M $MgSO_4$ (final concentration: 14.3 mM); and 0.25 ml. of NADPH added every 20 minutes (final concentration: 0.5 mM). Two 1.0 ml. aliquots of the incubation mixture were removed at 1, 10, 25, and 40 minutes. A 1.0 ml. aliquot was analyzed using the liquid chromatographic and another aliquot by the mass spectrometric

assay procedures described in Section III. The control mixture was analyzed only at the end of the incubation. The enzyme activity and total supernatant protein concentration were analyzed as described in Section IV.

The kinetic experiment was repeated using a Procarbazine final concentration of 1.0 mM instead of 2.0 mM. The final concentrations of the other components of the incubation remained the same. This incubation was continued for 60 minutes.

Metabolism of Each Metabolite and Chemical Degradation Product by the 9,000 x g. Rat Liver Supernatant Fraction-40 Minute Incubation

The incubation mixtures contained 5.0 ml. of 9,000 x g. rat liver supernatant, prepared as previously described. Each mixture also contained 0.2 ml. of one of the known metabolic or chemical degradation products at a final concentration of 1.0 mM (Procarbazine was dissolved in buffer, while the other compounds were dissolved in absolute ethanol); 0.55 ml. of buffer; 1.0 ml. of 0.1 M $MgSO_4$ (final concentration: 14.0 mM); and 0.25 ml. of NADPH added every 20 minutes (final concentration: 0.5 mM) and was incubated with shaking at 37°C. for 40 minutes. The control mixtures contained 2.0 ml. of supernatant, 0.04 ml. of drug (final concentration: 0.5 mM); 0.26 ml. of buffer; and 0.4 ml. of 0.1 M $MgSO_4$ (final concentration: 14.8 mM). NADPH was deleted. A better control would be boiled supernatant with all of the components of the experimental incubation. Each reaction was stopped by addition

of an organic solvent. A 1.0 ml. aliquot of each incubation mixture was analyzed by both analytical systems described in Section III. In addition, the stock solutions of each drug were analyzed and quantitated. The enzyme activity and total supernatant protein concentration were also determined as described in Section IV.

Metabolism of Benzyl-NNO-azoxymethane By 100,000 x g. Rat Liver Microsomal Fraction - 40 Minute Incubation

The 100,000 x g. rat liver microsomal fraction was prepared as described in Section IV. The incubation mixture containing 2.0 ml. of 100,000 x g. rat liver microsomes; 0.04 ml. of 68 mM benzyl-NNO-azoxy methane or benzaldehyde in ethanol solution (final concentration: 1.0 mM); 0.4 ml. of 0.1 M $MgSO_4$ (final concentration: 14.8 mM); 0.26 ml. of 0.1 M semicarbazide·HCl (final concentration: 10.0 mM); and 1.2 mg. NADPH added every 20 minutes (final concentration: 0.5 mM) was incubated with shaking at 37°C. for 40 minutes. The control contained the microsomal preparation, boiled for 30 minutes at 100°C., and the other components in the experimental incubation. Each incubation was extracted with 5 x 3 ml. ether at neutral pH. The extract was evaporated with a stream of nitrogen at ambient temperature. The residue was redissolved in methylene chloride and analyzed by gas chromatography - chemical ionization mass spectrometry (GC-MS). The column contained 2% KOH - 2% Carbowax 20M (Applied Science). The gas chromatograph was a Finnigan Model 9500 connected to the Finnigan mass

spectrometer previously described. Benzylalcohol served as a standard compound.

Metabolism of a Mixture of Procarbazine and Procarbazine ($^2H_6$)-Isopropyl By 9,000 x g. Rat Liver Supernatant - 40 Minute Incubation

The 9,000 x g. rat liver supernatant was prepared as previously described. The incubation mixture containing 5.0 ml. of supernatant; 0.75 ml. of buffer; 3.65 g. of a 1.4:1 mixture of Procarbazine $^1H_6/^2H_6$-isopropyl (final concentration: 2.0 mM); 1.0 ml. of 0.1 M $MgSO_4$ (final concentration: 14.3 mM): and 0.25 ml. of 14.0 mM NADPH added every 20 mintues (final concentration: 0.5 mM) was incubated with shaking at 37°C. for 40 minutes. The incubation mixture was extracted with 3 x 6 ml. of ether, evaporated with nitrogen gas, redissolved in absolute EtOH, and analyzed by direct-probe insertion chemical ionization mass spectrometry using an Associate Electrical Industries MS-902 mass spectrometer and a Finnigan 3200 mass spectrometer. The remaining aqueous layer was acidified with 12 drops of 1 N HCl and extracted with 3 x 6 ml. of ether. The aqueous layer was then made basic with 20 drops of 1 M NaOH and extracted. Both acidic and basic extracts were prepared and analyzed according to the procedure used for the neutral extraction. The stock solution of the Procarbazine mixture was also analyzed.

Metabolism of a Mixture of Procarbazine and Procarbazine-($^2H_1$)-methylene By 9,000 x g. Rat Liver Supernatant - 40 Minute Incubation

The 9,000 x g. rat liver supernatant was prepared as previously described. The experimental incubation contained 5.0 ml. of supernatant; 0.75 ml. of 9.3 m$\underline{M}$ drug (final concentration: 1.0 m$\underline{M}$); 1.0 ml. of 0.1 $\underline{M}$ $MgSO_4$ (final concentration: 14.0 m$\underline{M}$); and 0.25 ml. of 14.0 m$\underline{M}$ NADPH added every 20 minutes (final concentration: 0.5 m$\underline{M}$). The control incubation contained 2.5 ml. of boiled 9,000 x g. supernatant (boiled for ½ hour at 100°C.); 0.38 ml. of 9.3 m$\underline{M}$ drug (final concentration: 1.0 m$\underline{M}$); 0.5 ml. of 0.1 $\underline{M}$ $MgSO_4$ (final concentration: 14.0 m$\underline{M}$); and 0.13 ml. of 14.0 m$\underline{M}$ NADPH added every 20 minutes (final concentration: 0.5 m$\underline{M}$). The drug solution contained 90.3% Procarbazine-($^2H_1$)-methylene and 9.7% Procarbazine which was determined by chemical ionization mass spectrometry. After shaking at 37°C. for 40 minutes, a 3.0 ml. aliquot was extracted under neutral, acidic, and basic conditions with 5 x 3 ml. of ether. Each extract was evaporated. Another 2.0 ml. aliquot was extracted and analyzed according to the procedures for the liquid chromatographic assay. Each liquid chromatographic peak was collected and evaporated under a stream of nitrogen. All of the evaporated samples were redissolved in a minimal volume of $MeCl_2$ and analyzed by direct-probe insertion chemical ionization mass spectrometry. A 2.0 ml. aliquot of the control incubation was extracted with 5 x 3 ml. of ether, evaporated, and analyzed using the same procedure as above.

B. Results

Identification of the In Vitro Metabolites

Procarbazine is metabolized in an *in vitro* system containing the 9,000 x g. rat liver supernatant fraction, magnesium ions, and the co-factor reduced nicotinamide adenine dinucleotide phosphate. The results of the thin-layer analysis of the metabolic incubation mixture and the synthetic compounds are shown in Table 11. The R_f values and the mass spectral data of each of the thin-layer isolated metabolites corresponds to the corresponding data for the synthetic compounds. The liquid chromatograph of the neutral extract is shown in Figure 42. This chromatograph shows the presence of aldehyde, alcohol, isomeric methyl- and benzylazoxy, azo, and hydrazone in the metabolic mixture. Table 12 presents the comparison of the elution time ratios and mass spectral data for each of these peaks and for authentic compounds. Finally, the chemical ionization mass spectras of the neutral, acidic, and basic extracts of these metabolic incubations are shown in Figure 43. The identified compounds are the predominate peaks.

Metabolism of Procarbazine by 9,000 x g. and 100,000 x g. Rat Liver Supernatant and 100,000 x g. Rat Liver Microsomes - 40 Minute Incubations

The *in vitro* metabolism of Procarbazine was studied using several different preparations of the rat liver homogenates. The initial 9,000 x g. supernatant fraction was further fractionated into its 100,000 x g. supernatant and 100,000 x g. microsomal fractions.

Table 11. Thin-layer Chromatographic and Mass Spectral Qualitative Analysis of a 40 Minute 9,000xg. Rat Liver Supernatant Metabolic Incubation of Procarbazine, pH 7.4, 37°C.

Compound	R_f of the Standard	R_f of the Incubation	Standard M.S. MH^+	Incubation M.S. MH^+
Procarbazine	Remains at origin	Remains at origin	222.3	222.3
Azo	0.59	0.59	220.3	220.3
Methylazoxy	0.41	0.41	236.3	236.3
Benzylazoxy	0.41	0.41	236.3	236.3
Hydrazone	0.43	0.43	220.3	220.3
Aldehyde	0.50	0.50	192.2	192.2
Acid	Remains at origin	Remains at origin	207.2	207.2

Table 12. HPLC Chromatographic and Mass Spectrometric Qualitative Analysis of the 40 Minute 9,000xg. Rat Liver Supernatant Metabolic Incubation of Procarbazine, pH 7.4, 37°C.

Compound	Standard Elution Time Ratio	Incubation Elution Time Ratio	Standard M.S. MH^+	Incubation M.S. MH^+
Azo	0.67	0.67	220.3	220.3
Methylazoxy	0.50	0.50	236.3	236.3
Benzylazoxy	0.39	0.39	236.3	236.3
Hydrazone	0.55	0.55	220.3	220.3
Aldehyde	0.27	0.27	192.2	192.2
Alcohol	0.12	0.15	194.2	194.2

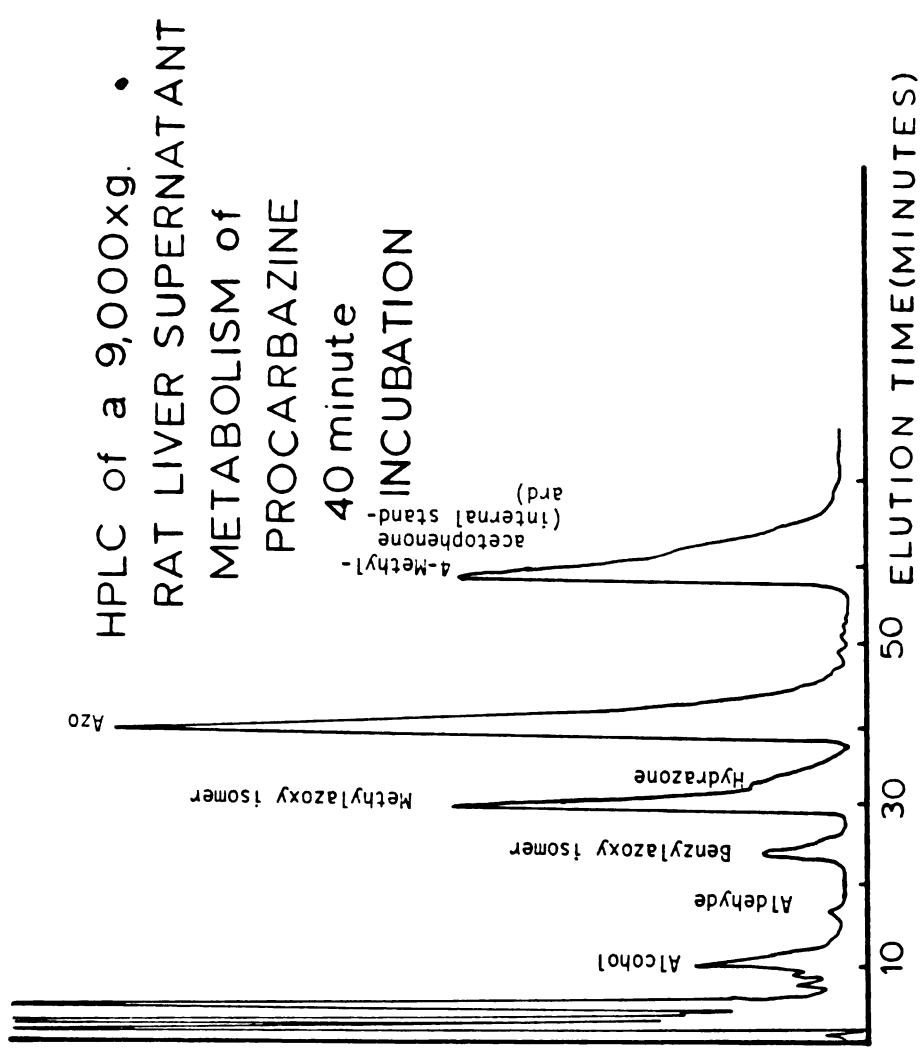


Figure 42. HPLC Chromatograph of the 9,000xg. Rat Liver Supernatant Metabolic Incubation of Procarbazine.

40 Minute incubation with shaking, pH 7.4, 37°C.

HPLC conditions: Solvent was 20% methanol: water. Flow rate was 2 ml/min. λ= 254 nm. Spectrum run at room temperature. 4- Methylacetophenone was the internal reference. Water's u-bondapak C_{18} reverse-phase column.

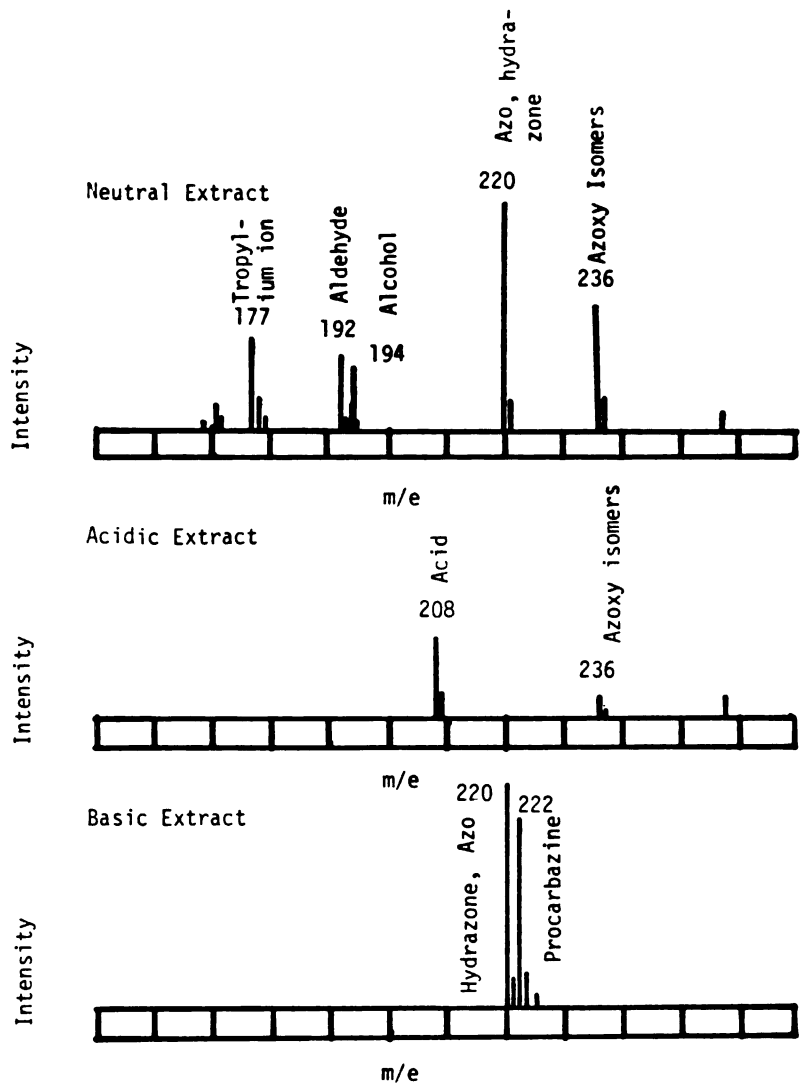


Figure 43. Chemical Ionization Mass Spectral Analysis of the Neutral, Acidic, and Basic Ether Extracts of a 9,000xg. Rat Liver Supernatant Incubation of Procarbazine, pH 7.4, 37°C., 40 Minute Incubation With Shaking.

Table 13 shows the amounts of Procarbazine and each of the metabolites and chemical degradation products for each of the preparations and boiled supernatant blanks after a 40 minute incubation. These levels are expressed in terms of the percentage of the Procarbazine initially added and of the total recovery assuming 100% total recovery.

Also shown are the total percent recovery, total protein concentration of each fraction, and the p-nitroanisole O-demethylase enzyme activity of each fraction. The 100,000 x g. microsomal fraction had 10.8 times more p-nitroanisole O-demethylase activity than the 9,000 x g. supernatant fraction when expressed per unit of total protein. No O-demethylase activity was found in the 100,000 x g. supernatant fraction. This shows that no contamination by the microsomal fraction occurred since the demethylase is a membrane-bound enzyme.

Procarbazine is metabolized primarily by the microsomal enzymes. The major *in vivo* urinary metabolite, N-isopropylterephthalamic acid, was produced only in the 9,000 x g. supernatant and 100,000 x g. microsomal fractions. No measurable amount of the acid was produced by the 100,000 x g. supernatant fraction. In both the 9,000 x g. supernatant and 100,000 x g. microsomal fractions the methyl- and benzylazoxy isomers were produced. The production of the azoxy isomers is apparently stereoselective with the steady-state concentration of the methylazoxy isomer dominating. In the 9,000 x g. supernatant fraction, the ratio of methyl-:benzylazoxy was 3.3:1. The 100,000 x g. microsomal fraction produced only an immeasurable amount of the benzylazoxy

Table 13. Amounts of Procarbazine and Its Chemical Degradation and Metabolic Products Formed After a 40 Minute Incubation of Procarbazine in 9,000xg. and 100,000xg. Rat Liver Supernatant and 100,000xg. Rat Liver Microsomal Fractions and the Corresponding Boiled Fraction Blanks.

Fraction	umoles of Compound (% of Initial Procarbazine/ % of Recovered Products)							%
	Procarbazine	Acid	Azo	Methylazoxy	Benzylazoxy	Aldehyde	Hydrazone	Recovery
9,000xg. supernatant	2.47 (32.2/71.0)	0.17 (2.2/4.9)	0.07 (0.9/2.0)	0.26 (3.4/7.5)	0.08 (1.1/2.3)	0.14 (1.8/4.0)	0.29 (3.8/8.3)	45.5
Blank	3.41 (88.1/81.0)	None detected	0.28 (6.8/6.7)	None detected	None detected	0.14 (3.6/3.3)	0.38 (9.8/9.0)	108.3
100,000xg. supernatant	2.41 (31.5/89.6)	None detected	0.04 (0.5/1.5)	None detected	None detected	0.09 (1.1/3.3)	0.15 (2.0/5.6)	35.1
Blank	3.03 (78.3/91.3)	None detected	0.04 (1.0/1.2)	None detected	None detected	0.14 (3.6/4.2)	0.11 (2.8/3.3)	85.7
100,000xg. Microsomes	0.78 (20.2/45.9)	0.08 (2.1/4.7)	0.15 (3.9/8.8)	0.17 (4.4/10.0)	None detected	0.22 (5.8/12.9)	0.30 (7.7/17.6)	44.1
Blank	3.84 (99.2/85.9)	0.001 (0 / 0)	0.26 (6.8/5.8)	None detected	None detected	0.23 (6.0/5.1)	0.14 (3.6/3.1)	115.6

Initial Procarbazine = 7.64 umoles in experimental incubations.
3.87 umoles in blank incubations.

Enzyme activity: 9,000xg. supernatant = 0.89 umoles/hour/ 100 mg. protein.
100,000xg. microsomes = 9.64 umoles/hour/ 100 mg. protein.

isomer. The absence of measurable amounts of the benzylazoxy isomer could result from the lack or destruction of the necessary enzymes in the microsomal preparation. An alternate explanation would be that the benzylazoxy isomer is more rapidly metabolized than the methylazoxy isomer. This latter hypothesis is supported by the results of the metabolism of the azoxy isomers discussed later in this section. The benzylazoxy isomer was more rapidly metabolized. Since the microsomal preparation was 11-times more active than the 9,000 x g. supernatant fraction, the absence of the benzylazoxy isomer only in the microsomal incubation might be expected.

The further metabolism of the aldehyde to the corresponding alcohol requires the alcohol dehydrogenase present in the soluble fraction. The 100,000 x g. microsomal fraction which lacks the dehydrogenase, produces a much higher amount of the aldehyde than either of the soluble enzyme-containing 9,000 x g. or 100,000 x g. supernatant fractions. Thus, a build-up of the aldehyde occurs in the microsomal fraction.

Finally, the consistent low recovery in the experimental samples as compared to the control samples could be the result of an experimental error in sampling or analysis which occurred only with the experimental samples. The low recovery also could result from a higher percentage of covalently bound product in the experimental samples.

Kinetics of the 9,000 x g. Rat Liver Supernatant Metabolism

The *in vitro* disappearance of Procarbazine and the appearance of its metabolites was studied in 9,000 x g. rat liver supernatant fraction. Table 14 shows the amounts of Procarbazine and each metabolic and degradation product present in the supernatant fraction after incubation of 1, 10, 25, and 40 minutes. Also shown is the amounts in the buffer control after a 45 minute incubation. The kinetic curves are shown in Figure 44. Procarbazine is rapidly metabolized with a half-life of approximately 7 minutes. A concomitant rise in the azo levels occurs throughout the 40 minutes incubations. Further metabolism of the azo compound is slower than the initial oxidation to form the azo. This results in a continued increase in the azo concentration throughout the incubation. Also present, but at decreasing amounts, are the hydrazone, methylazoxy, acid, benzylazoxy, aldehyde, and alcohol. Except for the hydrazone, the amounts of all of the metabolic or decomposition products increased throughout the incubation period. The percent total recovery decreased with increased incubation time. This suggests that some of the Procarbazine could be lost either due to metabolism to a potential alkylating species or some unidentified product(s). Finally, the use of 1 mM drug concentration instead of 2 mM does not signficantly alter the kinetics.

Table 14: Amounts of Procarbazine and Its Chemical Degradation and Metabolic Products Formed During a Kinetic Study of a 9,000xg. Rat Liver Supernatant Metabolic Incubation of Procarbazine.

9,000xg. Supernatant

	umoles of Compound in 14 ml.(% of initial Procarbazine)								
Time (min.)	Aldehyde	Benzylazoxy	Methylazoxy	Hydrazone	Azo	Alcohol	Acid	Procarbazine	% Recovery
1.0	0.18(0.9)	None detected	0.36(1.7)	1.73(8.4)	1.25(6.1)	None detected	0.22(1.1)	19.87(96.4)	114.6
10.5	0.21(1.0)	0.06(0.3)	0.53(2.6)	1.83(8.9)	1.80(8.7	0.04 (0.2)	0.30(1.4)	10.61(51.5)	74.7
25.5	0.21(1.0)	0.15(0.7)	1.05(5.1)	1.96(9.5)	2.82(13.7)	0.07 (0.3)	0.48(2.3)	6.30(30.6)	63.3
40.5	0.23(1.1)	0.37(1.8)	1.46(7.1)	1.50(7.3)	3.77(18.3)	0.18 (0.9)	0.83(4.0)	6.15(29.8)	70.2

Initial Procarbazine= 20.6 umoles.

Buffer Control

45.25	0.15(1.1)	None detected	None detected	0.39(2.8)	2.36(16.9)	None detected	1.12(8.0)	10.19(72.8)	101.5

Initial Procarbazine= 14.0 umoles.

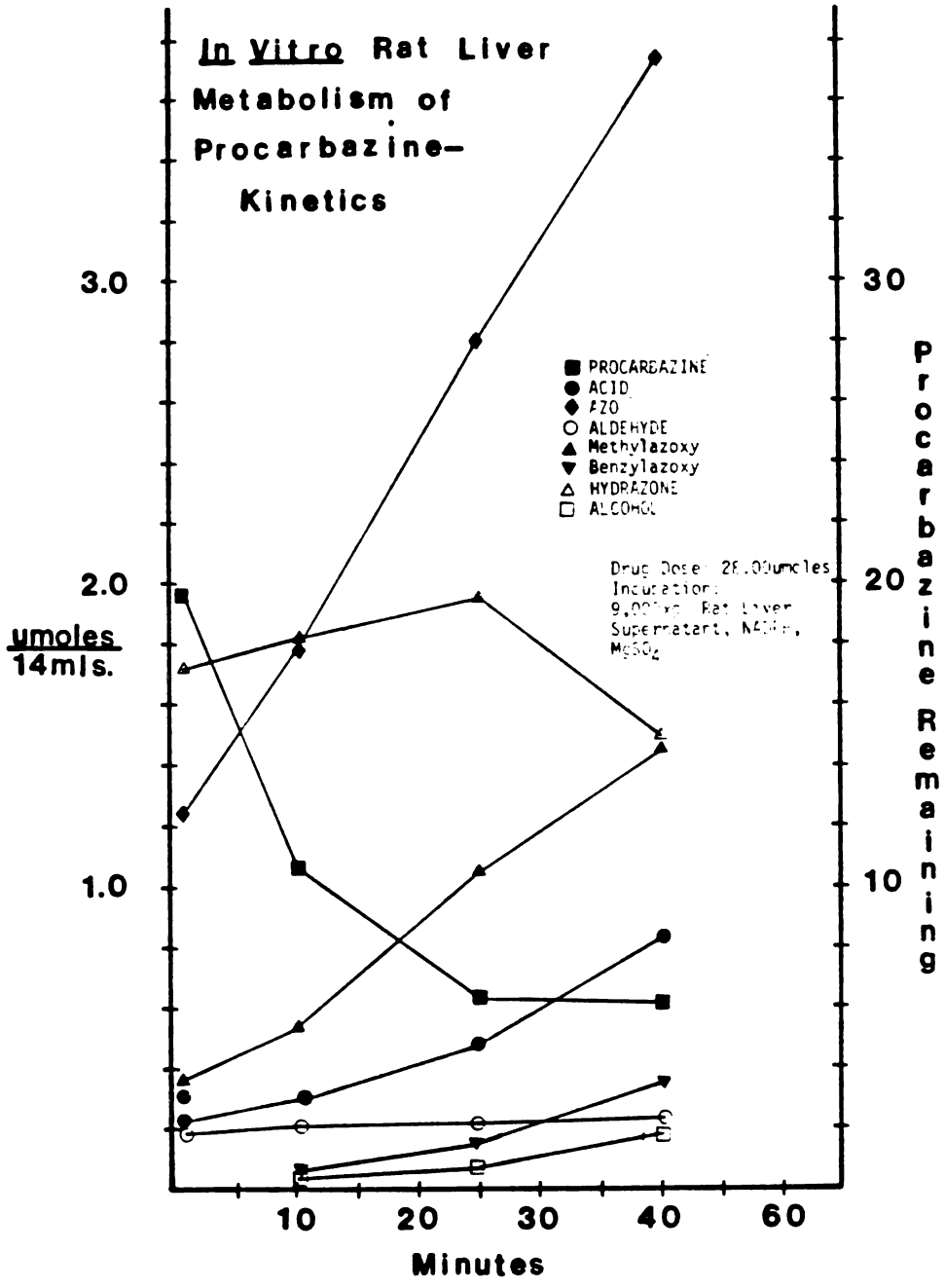


Figure 44. Kinetic Curves For the 9,000xg. Rat Liver Supernatant Metabolic Incubation of Procarbazine.

Metabolism of Each Metabolic and Chemical Degradation Product By 9,000 x g. Rat Liver Supernatant - 40 Minute Incubation

The metabolism of Procarbazine, azo, a 1.5:1 mixture of methyl:benzylazoxy isomers, aldehyde, and hydrazone by 9,000 x g. rat liver supernatant was examined using the same supernatant preparation and incubation procedure. The results of these metabolic incubations are presented in Tables 15 and 16. Procarbazine is rapidly metabolized. The metabolism of Procarbazine produced the azo compound, a pair of azoxy isomers, aldehyde, hydrazone, acid, and alcohol. The production of the isomeric azoxy compounds is stereoselective. The methylazoxy isomer represents 90% of the azoxy mixture after a 40 minute incubation. The dominance of the methylazoxy isomer could also result from a faster rate of further metabolism of the benzylazoxy isomer as compared to the methylazoxy isomer. The metabolism of the azo compound is much slower than the disappearance of Procarbazine. The azo compound is metabolized to the isomeric azoxy compounds, hydrazone, acid, and alcohol. The isomeric azoxy compounds are stereoselectively produced with the methylazoxy isomer representing 76% of the mixture. The 1.5:1 mixture of methyl-:benzylazoxy isomers (60%:40%) is metabolized at about the same rate as the azo compound. The metabolism of the azoxy compound is also stereoselective. The percentage of the methylazoxy compound changes from the initial 60% to approximately 65% after 40 minutes. The benzylazoxy isomer correspondingly decreases. The azoxy compounds are further metabolized to alcohol and acid. A chemical ionization mass spectrum of the 9,000 x g. supernatant incubation of the

Table 15. METABOLISM OF PROCARBAZINE AND ITS INTERMEDIATES BY 9,000xg. RAT LIVER SUPERNATANT DURING A 40' INCUBATION

PROCARBAZINE:

Experimental: Initial Procarb.= 12.5 umoles; 2.0 mM

Procarbazine remaining	65.8
Hydrazine	1.5
Methylazoxy	14.0 Ratio= 8.75
Benzylazoxy	1.6
Azo	15.4
Aldehyde	trace
Alcohol	1.0
Acid	4.0

Total Metabolism= 37.5
Unaccounted= 0

Blank: Initial Procarb.= 2.5 umoles; 0.5 mM

Procarbazine remaining	not determined
Methylazoxy	1.3%
Azo	11.2
Aldehyde	trace
Acid	not determined

AZO:

Experimental: Initial Azo= 6.31 umoles; 1.0 mM

Azo remaining	63.1%
Hydrazone	11.5
Methylazoxy	7.1 Ratio=3.23
Benzylazoxy	2.2
Acid	2.8
Alcohol	0.7

Total Metabolism= 36.9%
Unaccounted= 12.6%

Blank: Initial Azo= 1.26 umoles; 0.5 mM

Azo remaining	77.9%
Hydrazone	14.4
Aldehyde	0.03
Alcohol	0.7

Total Metabolism= 22.1%
Unaccounted= 6.97%

AZOXY MIXTURE: Ratio of Methylazoxy: Benzylazoxy= 1.5:1.

Experimental: Initial azoxy= 11.3 umoles; 1.0 mM

Methylazoxy	57.3%		
Benzylazoxy	44.2%	52.0%	Ratio=1.95
Alcohol		1.5%	
Acid		3.0%	

Total Metabolism= 48.0%
Unaccounted= 43.5%

Blank: Initial Azoxy= 4.5 umoles; 0.5 mM

Methylazoxy	79.6%		
Benzylazoxy	71.7%	76.4%	Ratio=1.69

ALDEHYDE:

Experimental: Initial Aldehyde= 6.32 umoles; 1.0 mM

Aldehyde remaining	0%
Alcohol	39.5
Acid	25.3

Total Metabolism= 100%
Unaccounted= 35.2%

HYDRAZONE:

Experimental:

Qualitatively goes to aldehyde, acid, and alcohol. Much hydrazone remains.

Notes: Blanks contain everything but NADPH.

Enzyme Assay:
Enzyme Activity= 1.67 umoles of p- nitrophenol formed/hr./100 mg total protein

Table 16. PROCARBAZINE AND METABOLITE METABOLISM IN 9,000 XG RAT LIVER SUPERNATE FOR 40 MIN.

	% Initial Substrate Recovered			
	Procarbazine	Azo	40 Benzylazoxy 60 Methyl "	Aldehyde
Procarbazine	66			
Azo	15	63		
Methylazoxy	14	7	34	
Benzylazoxy	2	2	18	
Aldehyde	trace	-	-	0
Alcohol	1	1	2	40
Hydrazone	2	12		
Acid	4	3	3	25
Recovered	104	87	57	65

azoxy mixture is shown in Figure 45. Besides the MH^+ peaks corresponding to the azoxy, alcohol, and acid compounds, a new MH^+ peak of m/e = 252 is present. This peak could correspond to a hydroxylated azoxy compound since it represents the addition of an oxygen atom to the MH^+ of the azoxy compound. The high-performance liquid chromatograph of the incubation mixture is shown in Figure 46. The chromatograph shows several new peaks. These could represent isomeric hydroxylated azoxy compounds. Evidence supporting the hydroxylated azoxy compounds remains to be gathered. The low amounts of these compounds coupled with their instability have made isolation unsuccessful.

The metabolism of the aldehyde by the 9,000 x g. supernatant is very rapid. No aldehyde remains after a 40 minute incubation. The aldehyde is reduced to the alcohol and oxidized to the acid. The reduction apparently proceeds faster. The oxidation reaction is NADPH-dependent. No acid is present when NADPH is deleted. The reduction is apparently NADPH-independent. The levels of alcohol present in the presence or absence of NADPH are almost identical. The reduction might be catalyzed by NAD-dependent alcohol dehydrogenase. Therefore, the presence or absence of NADPH would not affect this reduction.

Finally, metabolism of the hydrazone was found to be slow. Aldehyde, acid, and alcohol were identified as trace products along with large quantities of unreacted hydrazone.

Metabolism of Benzyl-NNO-azoxymethane By 100,000 x g. Rat Liver Microsomal Fraction - 40 Minute Incubation

The metabolism of benzyl-NNO-azoxymethane produced benzyl alcohol. Gas chromatographic analysis of the incubation

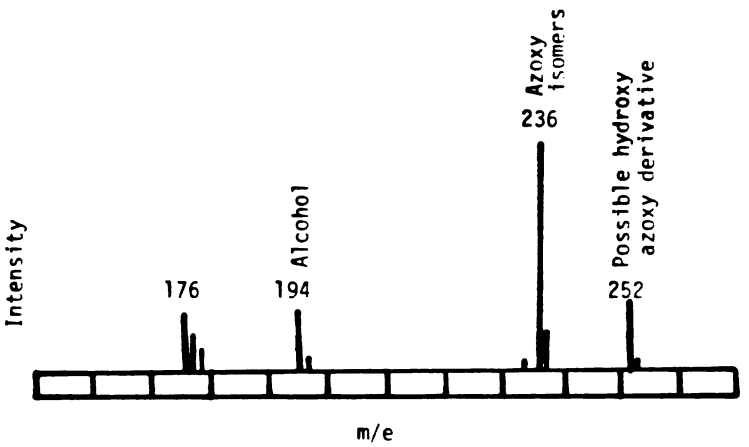


Figure 45. Chemical Ionization Mass Spectrum of a 9,000xg. Rat Liver Supernatant 40 Minute Metabolic Incubation of the Methyl- and Benzylazoxy Mixture.

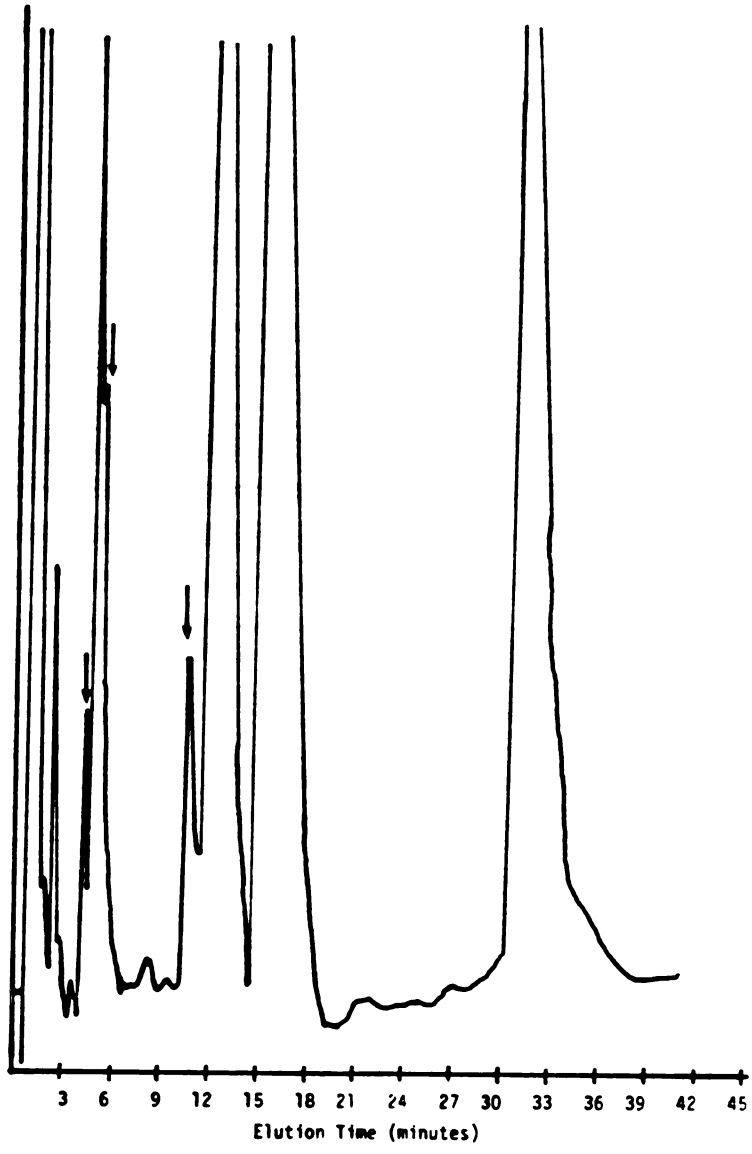


Figure 46. HPLC Chromatograph of a 40 Minute 9,000xg. Rat Liver Supernatant Metabolic Incubation of the Methyl- and Benzylazoxy Isomeric Mixture.

Arrows indicate peaks not present in a chromatograph of a blank using boiled 9,000xg. supernatant.

40 Minute incubation with shaking, pH 7.4, 37°C.

HPLC conditions: Solvent was 20% methanol: water. Flow rate was 2 ml/min. λ= 254 nm. Spectrum run at room temperature. 4- Methylacetophenone was the internal reference. Water's u-bondapak C_{18} reverse-phase column.

mixture contained a peak with the same retention time as benzyl alcohol. Chemical ionization mass spectral analysis confirmed that the peak was benzyl alcohol. No benzyl alcohol was produced in the incubation containing benzaldehyde and semicarbazide or in the control incubation. The semicarbazide inhibits any residual NAD-dependent alcohol dehydrogenase activity in the 100,000 x g. microsomal preparation.

Metabolism of a Mixture of Procarbazine and Procarbazine-($^2H_6$)-isopropyl By 9,000 x g. Rat Liver Supernatant - 40 Minute Incubation

The in vitro metabolism of a mixture of Procarbazine and Procarbazine-($^2H_6$) isopropyl by 9,000 x g. rat liver supernatant was examined to detect additional Procarbazine-derived metabolites. Table 17 lists the chemical ionization mass spectral MH^+ peaks observed along with the structures of the compounds and the $^1H_6/^2H_6$-peak height ratios. The mass spectras of the initial reaction mixture and the neutral, acidic, and basic ether extracts after a 40 minute incubation are shown in Figure 47. The initial mixture contained only Procarbazine and Procarbazine-($^2H_6$) isopropyl with a peak height ratio ($^1H_6/^2H_6$) of 1.4.

The initial $^1H_6/^2H_6$ ratio is used to determine if other compounds of the same mass are contributing to either peak of each of the doublets. Any deviation from this ratio would indicate that the doublet contains other compounds besides the $^1H_6$- and $^2H_6$-metabolites. The neutral extract contained 4-doublets with $^1H_6/^2H_6$ ratios equal to the initial Procarbazine

Table 17. Chemical Ionization Mass Spectral Analysis of a 40 Minute 9,000xg. Rat Liver Supernatant Incubation of a Mixture of Procarbazine and Procarbazine-($^2H_6$)-isopropyl.

MH^+ Peaks	Structure	Ratio $^1H_6$/ $^2H_6$
Neutral Extract		
178	CH_3- R	1.5
184	$^2H_6$- analog	
192	O ‖ HC- R	1.5
198	$^2H_6$- analog	
220	$CH_3N{=}NCH_2$- R CH_3NH-N=C- R H	1.6
226	$^2H_6$- analog	
236	O ‖ $CH_3N{=}NCH_2$- R	1.2 (run on different instrument where initial Procarbazine ratio was 1.4).
242	$^2H_6$- analog $CH_3N{=}NCH_2$- R O	
Acidic Extract		
208	O ‖ HO-C- R	0.43
214	$^2H_6$- analog	
Basic Extract		
222	$CH_3NHNHCH_2$- R	1.4
228	$^2H_6$- analog	

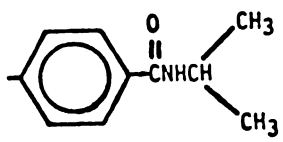
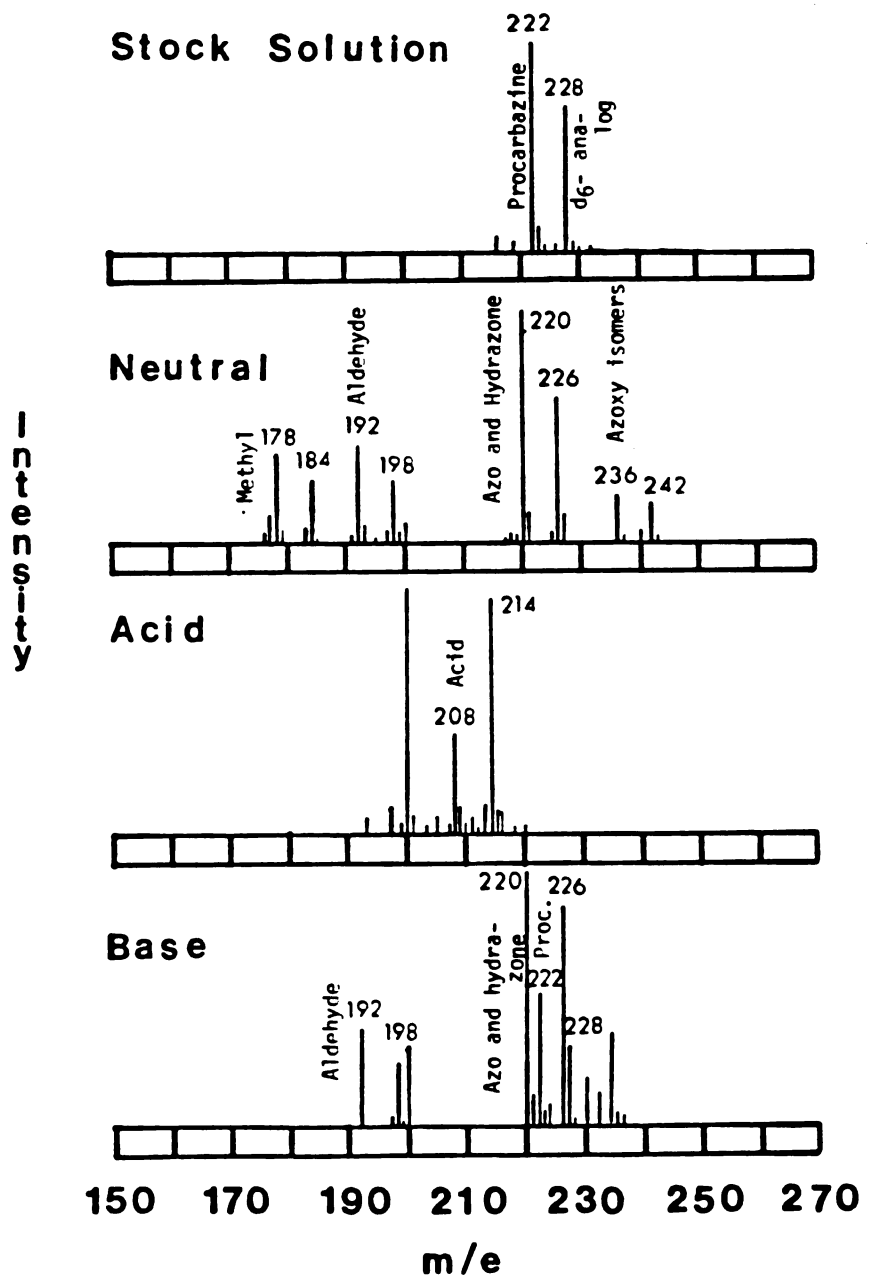
R= 

Figure 47. Chemical Ionization Mass Spectral Analysis of a 40 Minute 9,000xg. Rat Liver Supernatant Incubation of a Mixture of Procarbazine and Procarbazine-($^2H_6$)- isopropyl.



ratio. These doublets correspond to the N-isopropyl-p-methylbenzamide, aldehyde, azo or hydrazone, and the isomeric azoxy compounds. No previously unidentified metabolites were present. The acid extract contained only a single doublet for the acid compound. However, the $^{1}H_{6}/^{2}H_{6}$ ratio is 0.43. This suggests that other compounds with a m/e = 214 are contributing to the $^{2}H_{6}$-acid peak. The basic extract contained doublets for the aldehyde, azo and hydrazone, and the remaining Procarbazine. The $^{1}H_{6}/^{2}H_{6}$ ratios are close to the initial Procarbazine ratio. This again indicates that the doublets represent the $^{1}H_{6}$- and $^{2}H_{6}$-compounds.

Metabolism of Procarbazine and Procarbazine-$^{2}H_{1}$- methylene By 9,000 x g. Rat Liver Supernatant - Kinetic Isotope Effect

The in vitro metabolism of the methylene group of Procarbazine was studied by observing kinetic isotope effects during the 9,000 x g. rat liver supernatant metabolism of a mixture of Procarbazine and Procarbazine-$^{2}H_{1}$ -methylene.

The percent $^{2}H_{1}$-content of each metabolic and chemical degradation product and of Procarbazine was determined. The $^{2}H_{1}$-contents are shown in Table 18. The Procarbazine stock solution contained 90.3% $^{2}H_{1}$. The azo and azoxy isomers also contained the same percentage of $^{2}H_{1}$. This suggests that the oxidation steps leading to the azo and the azoxy isomers do not involve cleavage of a methylene proton. The hydrazone $^{2}H_{1}$-content cannot be calculated because the $^{1}H_{1}$ peak could not be distinguished above the background signal. The same problem occurred for Procarbazine, alcohol, and aldehyde.

Table 18. The Percent $^{2}H_{1}$- Content of Procarbazine and Its Chemical Degradation and Metabolic Products After a 40 Minute 9,000xg. Rat Liver Supernatant Incubation of a Procarbazine and a Procarbazine-($^{2}H_{1}$)- methylene Mixture.

Sample	Compound	%-$^{2}H_{1}$
Stock Procarbazine solution	Procarbazine	90.3
	Azo, hydrazone	82.0
Neutral extract of incubation	Azo	86.6
	Azoxyisomers	89.1

The other metabolic and chemical degradation products could not be analyzed due to the inability to distinguish the peak from the background signal.

C. Discussion

The in vitro metabolism of Procarbazine by rat liver homogenates and microsomal preparations had previously been studied. Procarbazine is chemically and, possibly, enzymatically oxidized to the azo compound. The further metabolism after the azo intermediate was proposed to follow a pathway similar to the chemical degradation pathway shown in Section V. The azo compound could first isomerize to the hydrazone. The hydrolysis of the hydrazone to the aldehyde followed by oxidation of the aldehyde produces the major in vivo urinary metabolite, N-isopropylterephthalamic acid. The present in vitro studies suggest that the generally accepted metabolic pathway plays only a minor role in the metabolism. Instead, the azo compound is stereoselectively oxidized to a pair of isomeric azoxy compounds. The methylazoxy-isomer dominates after a 40 minutes in vitro rat liver 9,000 x g. supernatant incubation. The azoxy isomers might undergo further oxidation to the unstable proposed hydroxyazoxy compounds.

The in vitro metabolism of Procarbazine by 9,000 x g. rat liver supernatant and 100,000 x g. microsomes to the azoxy compounds and, ultimately, to the alkylating species and the major in vivo urinary metabolite, N-isopropylterephthalamic acid, requires NADPH. The enzymes necessary for the production of these metabolites are membrane-bound enzymes present in both the 9,000 x g. supernatant and 100,000 x g. microsomal fractions. None of these metabolites are found in the 100,000 x g. supernatant incubation. The metabolism requires

the microsomal enzymes since either boiled 9,000 x g. supernatant or 100,000 x g. microsomes or the substitution for these fractions by phosphate buffer prevents the production of these metabolites.

The 100,000 x g. supernatant, containing the soluble enzymes, catalyzes the reduction of the aldehyde to the alcohol. This reaction is NADPH-independent and probably is catalyzed by NAD-dependent alcohol dehydrogenase which is present in the soluble fraction. This enzyme is also present in the 9,000 x g. supernatant fraction. This partially accounts for the formation of the alcohol in this fraction. The alcohol could also form during the reaction of water with a potential benzylating agent formed from one of the hydroxy-azoxy isomers. The production of benzyl alcohol during the metabolism of benzyl-NNO-azoxymethane by 100,000 x g. microsomes supports the formation of potential benzylating agents from methylbenzylazoxy compounds. Semicarbazide eliminated any alcohol production due to NAD-dependent alcohol dehydrogenase contamination of the microsomal preparation. The absence of the dehydrogenase in the 100,000 x g. microsomal fraction could account for the relative build-up of the aldehyde as compared to the supernatant preparations.

The <u>in vitro</u> disappearance rate of Procarbazine is comparable to the disappearance rate resulting from chemical decomposition in aqueous buffer at the same pH and temperature. The half-life is approximately 7 minutes as compared to 12 minutes in phosphate buffer. These rates also are comparable to that

reported for the in vivo Procarbazine plasma disappearance in man, 7-10 minutes.[173] The rapid disappearance of Procarbazine corresponds with a rapid increase in the azo levels. The azo is metabolized slowly to the azoxy isomers since the azo level continues to increase throughout the kinetic studies of the in vitro metabolism. The further metabolism of the azoxy isomers proceeds at the same rate as the metabolism of azo to azoxy. After 40 minutes, about 50-60% of the azoxy mixture remained. However, the benzylazoxy isomer is metabolized 1.4 times faster than the methylazoxy isomer. The methylazoxy isomer is also formed faster. After 10 minutes, the methylazoxy isomer is present at a 9-fold higher concentration. Thus, both the production from the azo and further metabolism of the azoxy compounds are stereoselective.

Finally, the previously proposed metabolic pathway through the hydrazone intermediate is not quantitatively important. The chemical isomerization of the azo to the hydrazone is very slow. Only 14% of the hydrazone is formed chemically during a 40 minute incubation in buffer. During the in vitro supernatant incubation, only 11% is formed and only 0.7% of the benzyl alcohol derivative and 3% of the acid that would be formed from the aldehyde during the hydrolysis of the hydrazone were present after 40 minutes.

VII. The In Vivo Metabolism of Procarbazine

The *in vivo* metabolism of Procarbazine has previously been studied in rodents such as rats and mice, in dogs, and in man. The results of these extensive metabolic studies were presented in the review of Procarbazine metabolism in Section I. The previous studies have primarily focused upon the metabolic fate of the N-methyl group. The present experiments were undertaken to elucidate the metabolic fate of the rest of the molecule. Special emphasis was placed upon elucidating the pathway(s) leading to the formation of the alkylating species.

The *in vitro* metabolic experiments discussed in Section VI produced several previously unreported metabolites such as the isomeric methyl- and benzylazoxy and the benzyl alcohol compounds. The *in vivo* studies will further define the overall metabolism of Procarbazine, while determining whether the *in vitro* and *in vivo* Procarbazine metabolism are similar. The *in vivo* metabolism was studied in the rat, mouse, and man. A comparison of the *in vivo* and *in vitro* metabolism in the rat should provide insight into the extent of metabolism occurring in the liver and its microsomal enzyme system and the metabolism which is catalyzed by other enzymes in the intact animal. The study in CDF_1 mice is undertaken because this strain is used in the animal tumor model for the testing of Procarbazine and its chemical degradation and metabolic products for antineoplastic activity. It is important to show that the metabolic pathways in the mouse are similar to

those found in rats. Although the pharmacokinetics in both species are important, this initial study is limited to the qualitation of the major metabolites previously identified during the metabolic studies in the rat. The metabolism of Procarbazine by man is also studied. This preliminary study was designed to evaluate the sensitivity of the assay for each metabolite previously identified in rodents, to determine the appropriate sampling times, and to determine if any of these metabolites also are produced in man.

The evaluation of the role of the cytochrome P-450-liver microsomal enzymes in the in vivo metabolism of Procarbazine is undertaken through studies using the enzyme inducer phenobarbital and the inhibitor disulfiram. The liver microsomal cytochrome P-450 enzyme system is classically induced by either phenobarbital or 3-methylcholanthrene. Phenobarbital induction results in an increase in the metabolism of a broad spectrum of drugs and non-drugs, while 3-methylcholanthrene primarily enhances the metabolism of polycyclic hydrocarbons. Phenobarbital was chosen to study the effects of a cytochrome P-450 inducer upon the kinetics of Procarbazine metabolism.

Finally, disulfiram (tetraethylthiuram disulfide, Antabuse®) whose structure is shown in Figure 48. is used to study the effects of an inhibitor of the cytrochrome P-450 enzyme system upon Procarbazine metabolism.

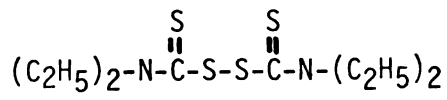


Figure 48. Structure of Disulfiram (Antabuse^{R}), Tetraethylthiuram disulfide.

Stripp, Green, and Gillette [174] reported that a single intraperitoneal or oral dose of disulfiram given to a rat impaired drug metabolism by liver microsomal N-demethylase between 8 hours and 7 days after administration. Cytochrome P-450 content, NADPH-cytochrome C-reductase activity, and cytochrome P-450 reductase activities were correspondingly decreased. The decrease in cytochrome P-450 reductase activity closely paralleled the decrease in ethylmorphine N-demethylase activity. Lang, et al. [175] reported similar findings.

A. Experimental Methods and Procedures

The In Vivo Metabolism in CDF_1 Mice - A Qualitative Study

A single 0.1 ml. intraperitoneal injection of 400 mg. Procarbazine/kg. of body weight was administered to 6 CDF_1 mice. The body weight was assumed to be 20 g. After 30 minutes,

each animal was decapitated and the blood collected into a heparinized centrifuge tube. The blood from all 6 animals was pooled and centrifuged at 2,000 r.p.m. for 10 minutes at 5°C. A 1.5 ml. aliquot of plasma was removed, extracted, and analyzed using the liquid chromatographic assay procedure described in Section III.

In Vivo Metabolism in Man

The patient studied was a 28 year old male undergoing chemotherapy for Hodkgin's disease. He was on day 12 of a 14-day course of chemotherapy with Procarbazine. The only other medication taken was a Nyquil® tablet taken the night prior to the experiment. The patient had eaten 3 hours prior to the beginning of the study. Prior to the oral administration of a 100 mg. capsule of Procarbazine-HCl, a blank plasma sample was taken. Approximately 2 ml. of blood was taken at 0, 14, 26.5, and 43 minutes. Each sample was placed into a heparinized microcentrifuge tube and centrifuged at room temperature for 2 minutes at 15,000 r.p.m. in a Brinkmann Eppendorf Centrifuge, Model 3200. A 1.0 ml. aliquot of the plasma was combined with 50 ul. of N-isopropyl-p-formylbenzamide (200 ng./ul.), which was used as an internal standard. The sample was extracted and analyzed by the liquid chromatographic assay procedure described in Section III. The flow rate was 1.6 ml./minute instead of 2.0 ml./minute.

In Vivo Metabolism Kinetics in Rats

Fischer C-344 male rats received a 1.5 ml. intraperitoneal injection of 150 mg. Procarbazine/kg. body weight. The drug solution was made by dissolving 100 mg. of Procarbazine·HCl per milliliter of normal saline. At various times after injection 2 rats were anesthetized with ether and the maximum amount of blood was removed by cannulation of the femoral artery through an abdominal incision. The blood was placed into a heparinized conical centrifuge tube and centrifuged at 2,000 r.p.m. for 10 minutes at 5°C. Equal aliquots of the plasma from each of the 2 samples were pooled to represent a single post-injection time-point. Either 1.0 ml. or 0.5 ml. of the sample was analyzed using the high-performance liquid-chromatographic and chemical ionization mass spectrometric assay procedures described in Section III.

The Effect of Phenobarbital Upon the Kinetics of In Vivo Procarbazine Metabolism in Rats

The procedure used for the phenobarbital study was identical to that used for the in vivo kinetic study. The use of identical procedures allows the direct comparison between these two studies. Male Fischer C-344 rats received phenobarbital orally over a period of 14 days prior to the metabolic study. The animals received drinking water containing 60 mg. of phenobarbital dissolved in 200 ml. of water. Assuming that each animal drank an average of 20 ml. of water per day, each animal received 6 mg. of phenobarbital per day. Phenobarbital solutions were changed every third day. After 14 days

of pretreatment, each rat was given 150 mg./kg. Procarbazine intraperitoneally. The drug solution contained 100 mg. of Procarbazine·HCl per milliliter of normal saline. Blood was obtained from 2 rats at various times after injection and analyzed as described for the non-treated animals.

Inhibition of the In Vivo Procarbazine Metabolism by Disulfiram in Rats

This experiment was repeated 3 times with a total of 4-pairs of control and experimental groups.

Male Fischer C-344 rats were fasted 12 hours prior to receiving disulfiram. The control animals received 1.5 ml. of a 4% starch solution in distilled water. Oral administration was by gastric intubation after light ether sedation using either a flexible vinyl tube or a stainless steel bent blunt needle manufactured for this procedure. The experimental animals received either a 500 mg./kg. or 1.01 g./kg. dose of disulfiram in 1.5 ml. of 4% starch solution 2 hours prior to Procarbazine. The administration was by the same procedure used for the controls. Since disulfiram was only partially water soluble, a homogenous suspension was administered. The experimental group received a 0.1 ml. intraperitoneal injection of Procarbazine containing 150 mg. Procarbazine/kg. body weight (100 mg. of Procarbazine·HCl dissolved just prior to use in 1 ml. of normal saline). After 30 minutes, the animals were anesthesized with ether and their blood removed by cannulation of the femoral artery through an abdominal incision. Each sample was placed in a

heparinized conical centrifuge tube and centrifuged at 2,000 r.p.m. at 5°C. for 10 minutes. Equal volumes of plasma from 2 animals were pooled. A 1.0 ml. plasma aliquot was prepared for analysis by the liquid chromatographic and mass spectrometric assay systems according to the previously described procedures in Section III.

B. Results

The In Vivo Procarbazine Metabolism in CDF_1 Mice - A Qualitative Study

The in vivo metabolism of Procarbazine in CDF_1 mice is qualitatively similar to that found in Fischer C-344 rats. The liquid chromatograph of the plasma sample taken 30 minutes after administration of Procarbazine is shown in Figure 49. The chromatograph shows peaks from the azo, methyl- and benzyl-azoxy isomers, and a small aldehyde peak. The relative amounts of the two azoxy isomers is similar to that formed in the rat.

In Vivo Procarbazine Metabolism in Man

The liquid chromatograph of the 14 minute human plasma sample is shown in Figure 50. The chromatograph contains an unidentified peak at an elution time of 11 minutes. N-Isopropyl-p-formylbenzamide, the internal standard, elutes at 26 minutes. This unidentified peak was also present in the 43 minute sample. No other peaks were present. This peak is not N-isopropylterephthalamic acid, the major human urinary metabolite, since the acid elutes at 2 minutes. The

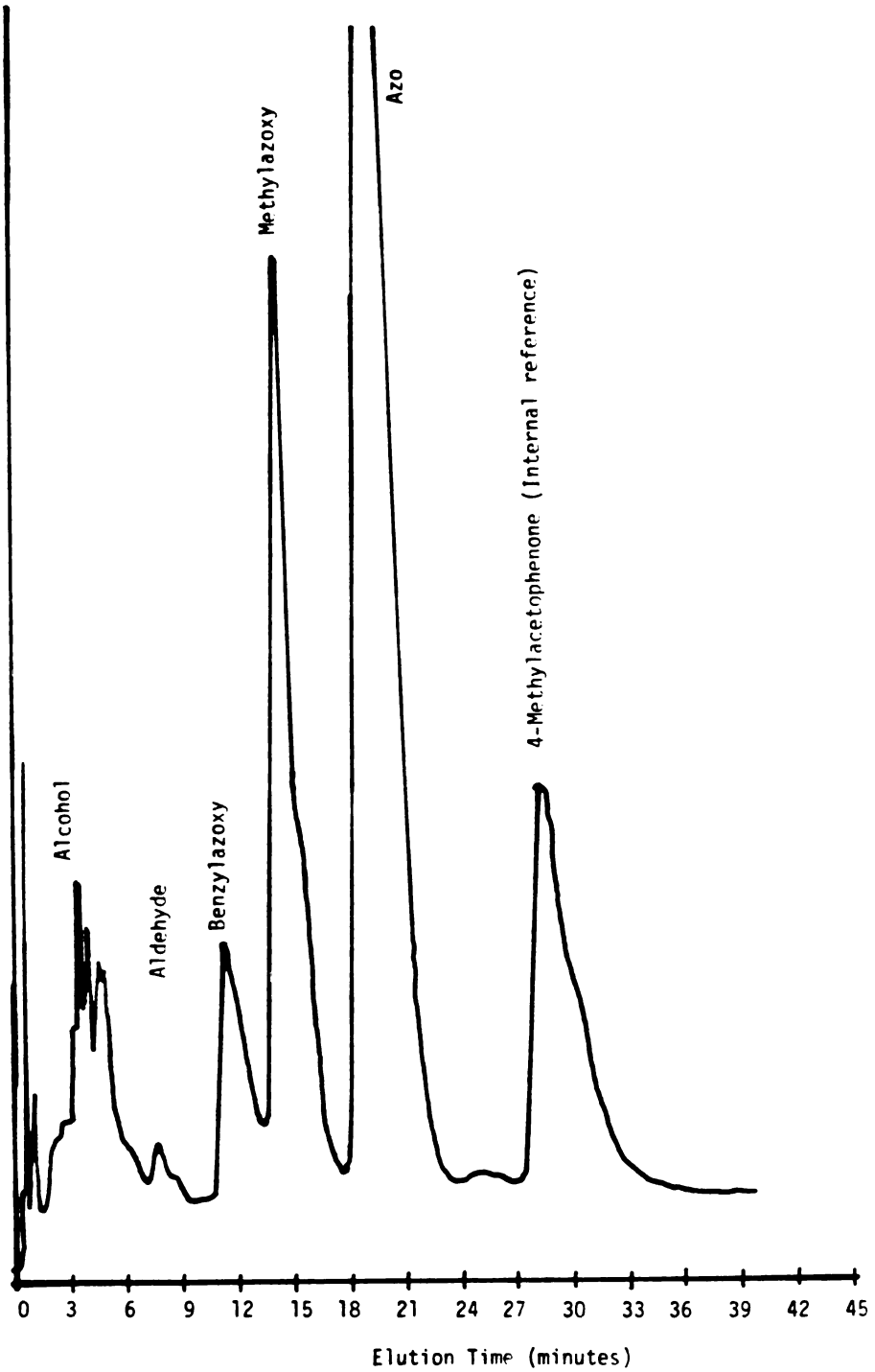


Figure 49. HPLC Chromatograph of a Plasma Sample From a CDF_1 Mouse Administered Procarbazine i.p. 30 Minutes Prior to the Sample.

Procarbazine dose= 400 mg./kg.

HPLC conditions: Solvent was 20% methanol: water. Flow rate was 2 ml/min. λ= 254 nm. Spectrum run at room temperature. 4- Methylacetophenone was the internal reference. Water's u-bondapak C_{18} reverse-phase column.

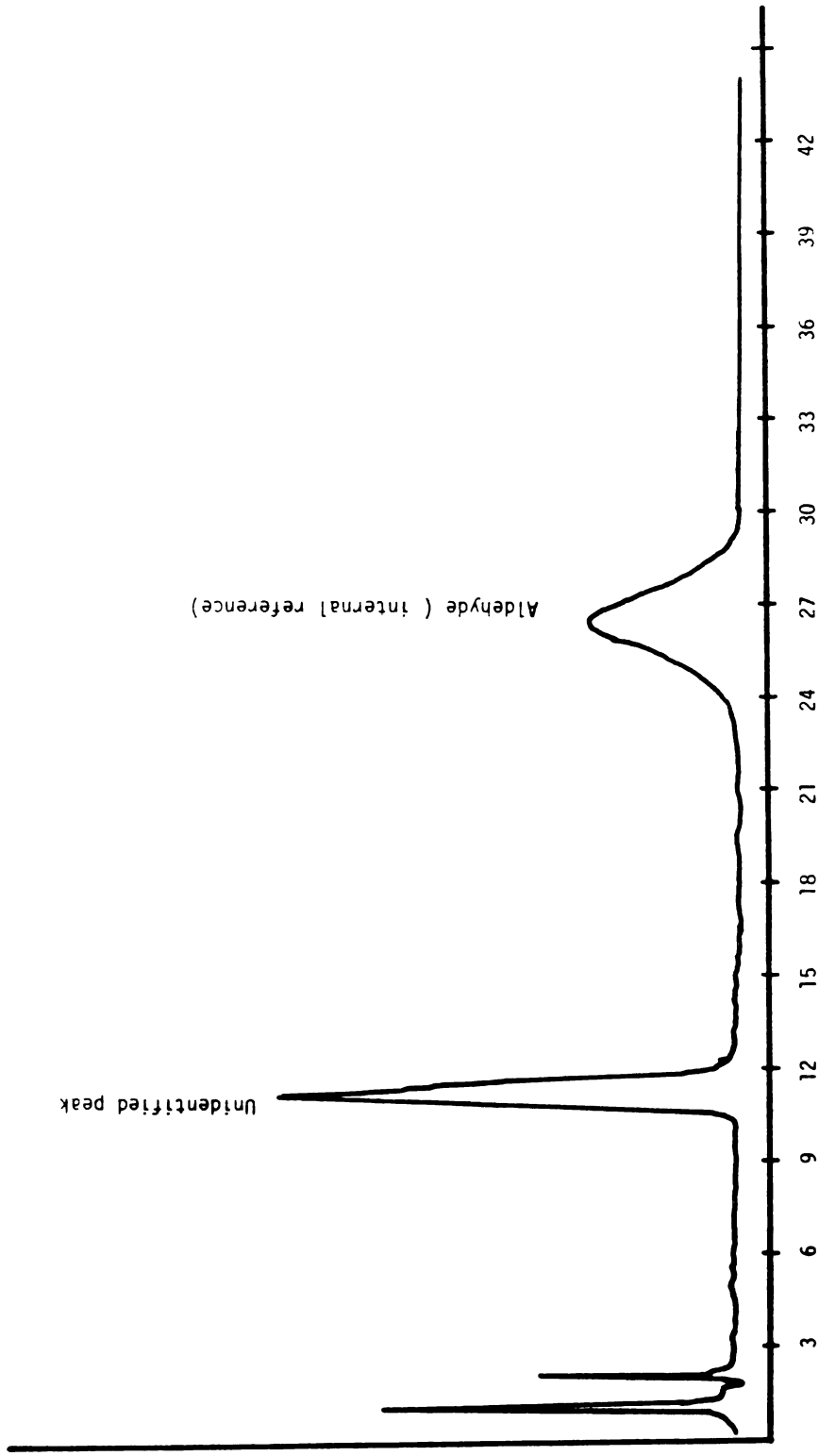


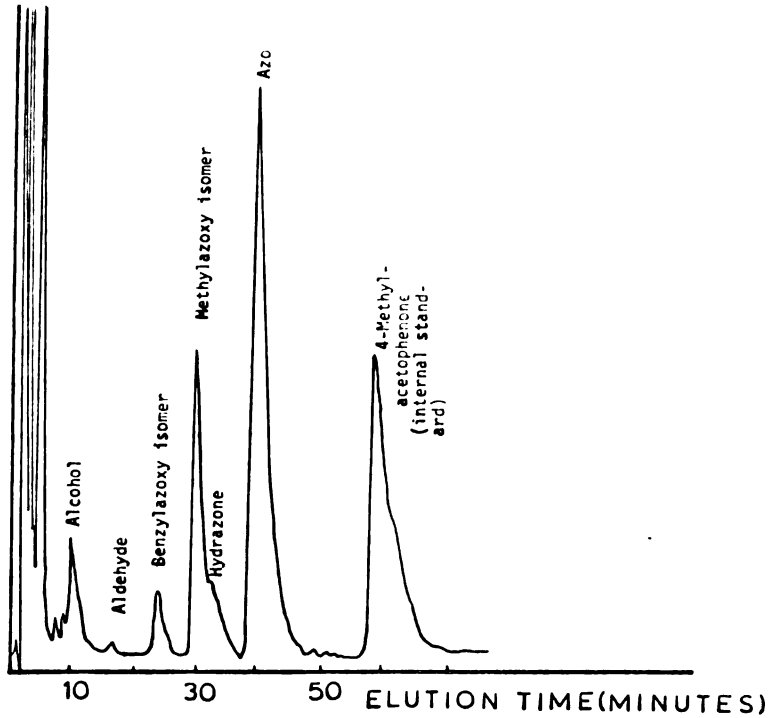
Figure 50. HPLC Chromatographic Analysis of a Human Plasma Sample Taken 14 Minutes After an Adult Patient Took 100mg. of Procarbazine Orally.

HPLC conditions: Solvent was 20% methamol: water. Flow rate was 2 ml/min. λ= 254 nm. Spectrum run at room temperature.N-Isopropyl-4-formyl)benzamide was the internal reference. Water's u-bondapak C_{18} reverse-phase column.

capsule itself nor anything used in its formulation did not account for this peak. Analysis of the capsule and its contents by dissolving the capsule in warm water or by opening the capsule and extraction of its contents under acidic, basic, or neutral conditions with ether did not produce the peak when analyzed by liquid chromatography.

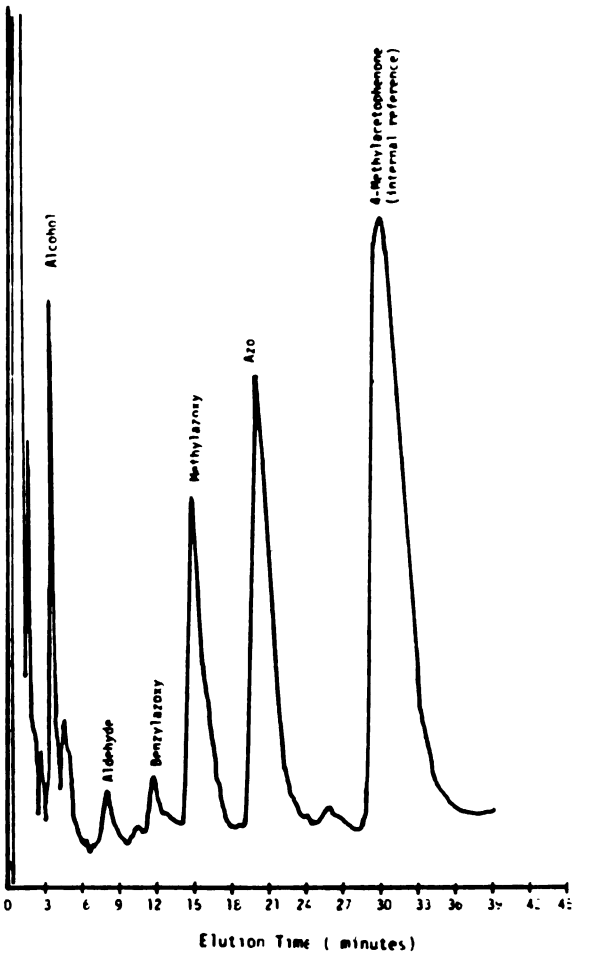
In Vivo Metabolism Kinetics in Rats

The in vivo rat metabolism of Procarbazine is similar to the in vitro metabolism using rat liver homogenates. The same metabolites found in vitro are also present in vivo. Figure 51 shows a comparison of the in vivo and in vitro liquid chromatographs. Figure52 shows the pharmacokinetic curve for the in vivo metabolism. The major difference in the in vivo and in vitro metabolic profiles is that, in vivo, the azo plasma concentration declines after reaching a maximum at 14 minutes. This does not result from slower in vivo oxidation of Procarbazine to azo since the plasma half-life for Procarbazine is approximately 7 minutes. This corresponds to its half-life in the in vitro incubations. A direct comparison of the in vivo and in vitro metabolic kinetics cannot be made since other factors that can affect plasma levels such as excretion, binding to macromolecules including plasma proteins, and absorption by various body tissues and fat depots during distribution occur and complicate the pharmacokinetics in the intact animal.



HPLC Chromatograph of the 9,000xg. Rat Liver Supernatant Metabolic Incubation of Procarbazine.

40 Minute incubation with shaking, pH 7.4, 37°C.



HPLC Chromatograph of the In Vivo Rat Metabolism of Procarbazine.

Figure 51. Comparison of the HPLC Chromatographs of the In Vitro 9,000xg. Rat Liver Supernatant Metabolism and the In Vivo Rat Metabolism of Procarbazine.

HPLC conditions: Solvent was 20% methanol: water. Flow rate was 2 ml/min. λ= 254 nm. Spectrum run at room temperature. 4- Methylacetophenone was the internal reference. Water's u-bondapak C_{18} reverse-phase column.

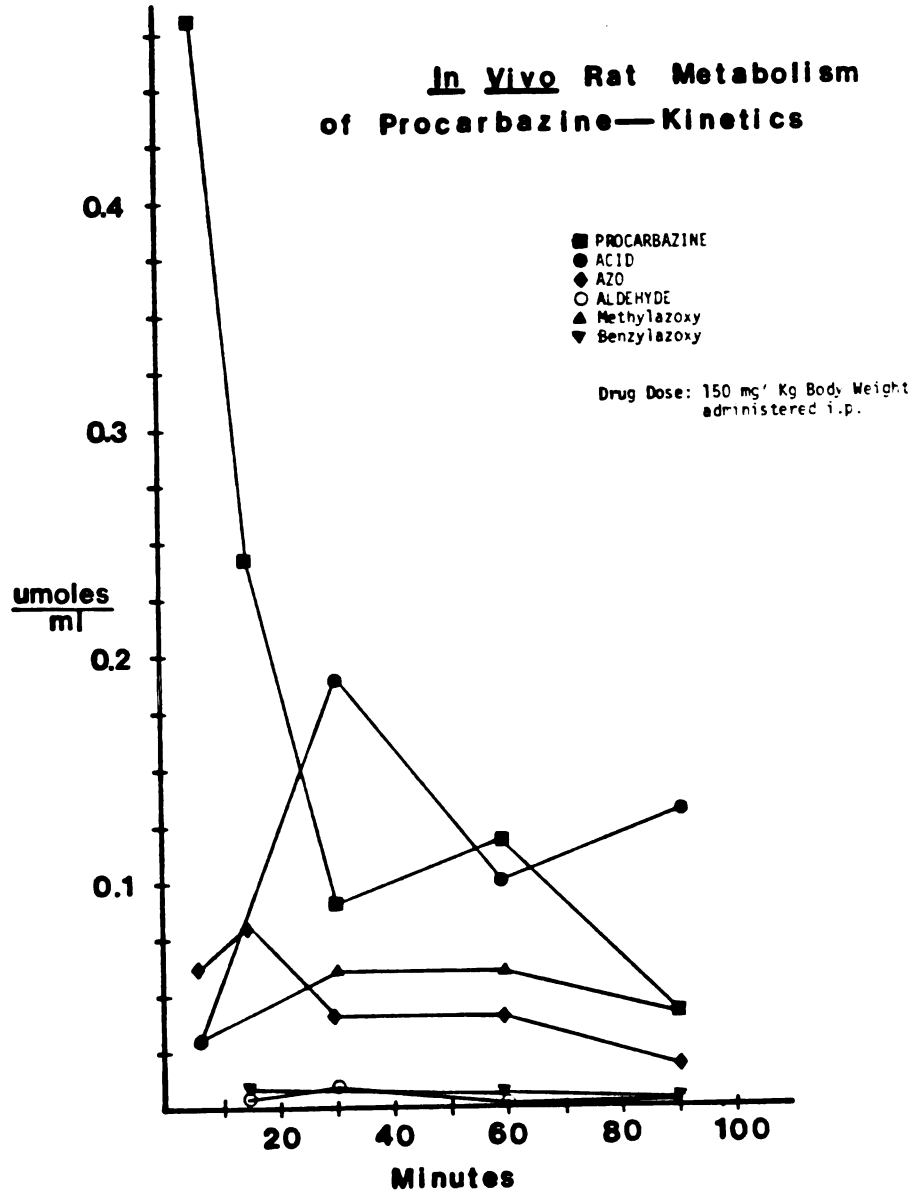


Figure 52. Pharmacokinetic Curves For the In Vivo Metabolism of Procarbazine in the Rat.

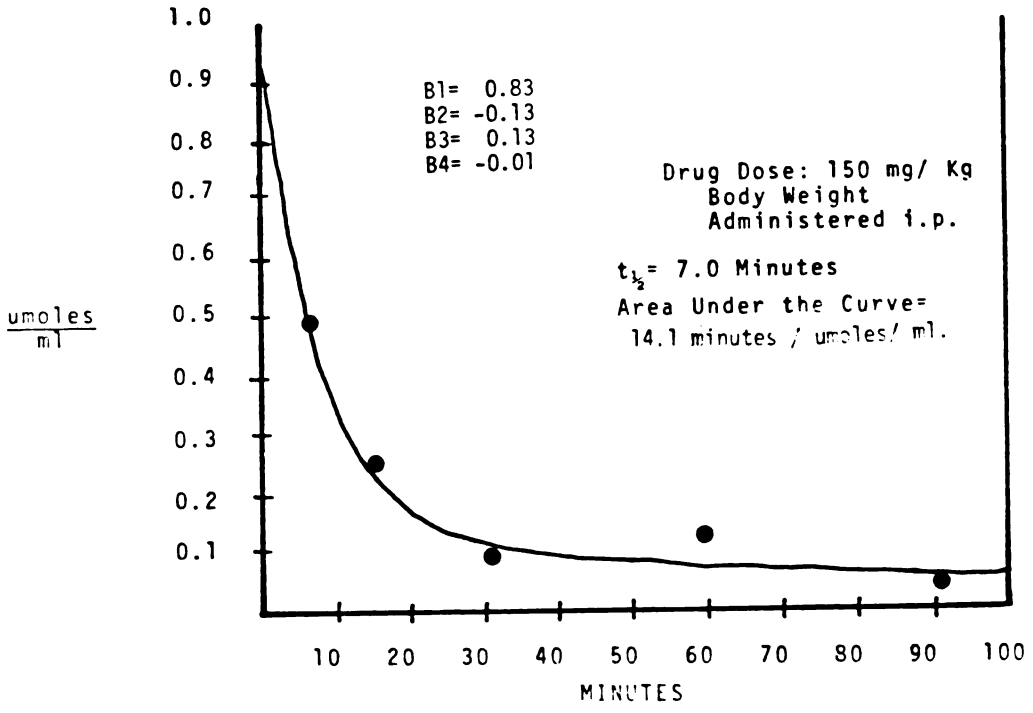


Figure 53. Procarbazine Disappearance From Plasma in Rat. Data is Computer Fit to a 2- Compartment Pharmacokinetic Model.

Computer analysis of the Procarbazine plasma disappearance is shown in Figure 53 . The kinetic data fits a biphasic 2-compartment pharmacokinetic model. After the initial administration of the drug, the plasma Procarbazine concentration rapidly decreased for 10 minutes. This rapid phase was followed by a slower elimination phase. The area-under-the-curve for the plasma disappearance is 14.1 minutes per umoles/ml.

The Effect of Phenobarbital Upon the Kinetics of In Vivo Procarbazine Metabolism in Rats

Phenobarbital-pretreatment results in a very rapid initial decrease in the plasma concentration of Procarbazine. The concentration of each metabolite and degradation product for each time point are shown in Table 19. Figure 54 shows the plasma pharmacokinetic curves for both normal and phenobarbital-pretreated rats. The plasma concentration of Procarbazine remaining at the 6 minute time point suggests that the *in vivo* metabolism is proceeding at about 0.058 umoles/ml/min. in the phenobarbital-pretreated rats as compared to 0.027 umoles/ml/min. for the normal animals. The rate of azo metabolism and the rate of the formation of the azoxy isomers are both increased with phenobarbital-pretreatment. The data for the plasma disappearance of Procarbazine are analyzed by a computer and fits a two-compartment pharmacokinetic model. Figure 55 shows the computer-generated plasma disappearance curves for both the pretreated and non-pretreated experiments. The area-under-the-curve (A.U.C.) values for both groups were determined. The phenobarbital-pretreated group had an A.U.C. of 7.6 minutes umoles/ml. and the normal

Table 19. Amounts of Procarbazine and Its Chemical Degradation and Metabolic Products in the Plasma of Phenobarbital- pre-treated Rats After an i.p. Injection of Procarbazine During a Kinetic Study.

Time(min.)	Procarbazine	Acid	ug/ml.(umoles/ml.) Azo	Methylazoxy	Benzylazoxy	Alcohol
3.6	65.4(0.3)	2.3 (0.01)	6.54 (0.03)	3.40(0.02)	None detected	Trace
6.2	32.3(0.15)	3.92 (0.02)	3.92 (0.02)	8.81(0.03)	" "	"
20.8	33.4(0.15)	23.21 (0.11)	12.29 (0.06)	30.7(0.13)	4.36(0.02)	"
46.3	8.9(0.04)	21.85 (0.11)	2.88 (0.01)	16.4(0.07)	1.2(0.01)	"
76.4	2.6(0.01)	12.32 (0.06)	1.18 (0.01)	6.81(0.03)	0.50(0.002)	"

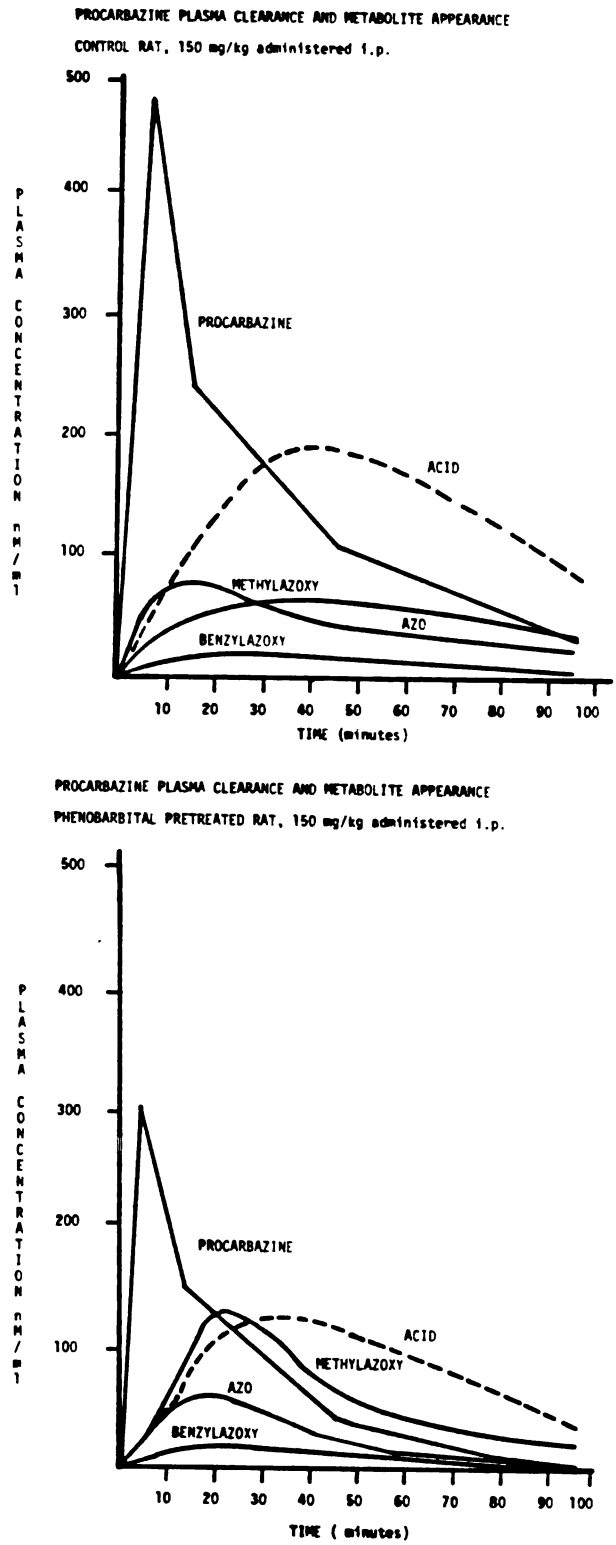


Figure 54. The Pharmacokinetic Curves for the In Vivo Procarbazine Metabolism in Rat With Phenobarbital-pretreatment and With No Pretreatment.

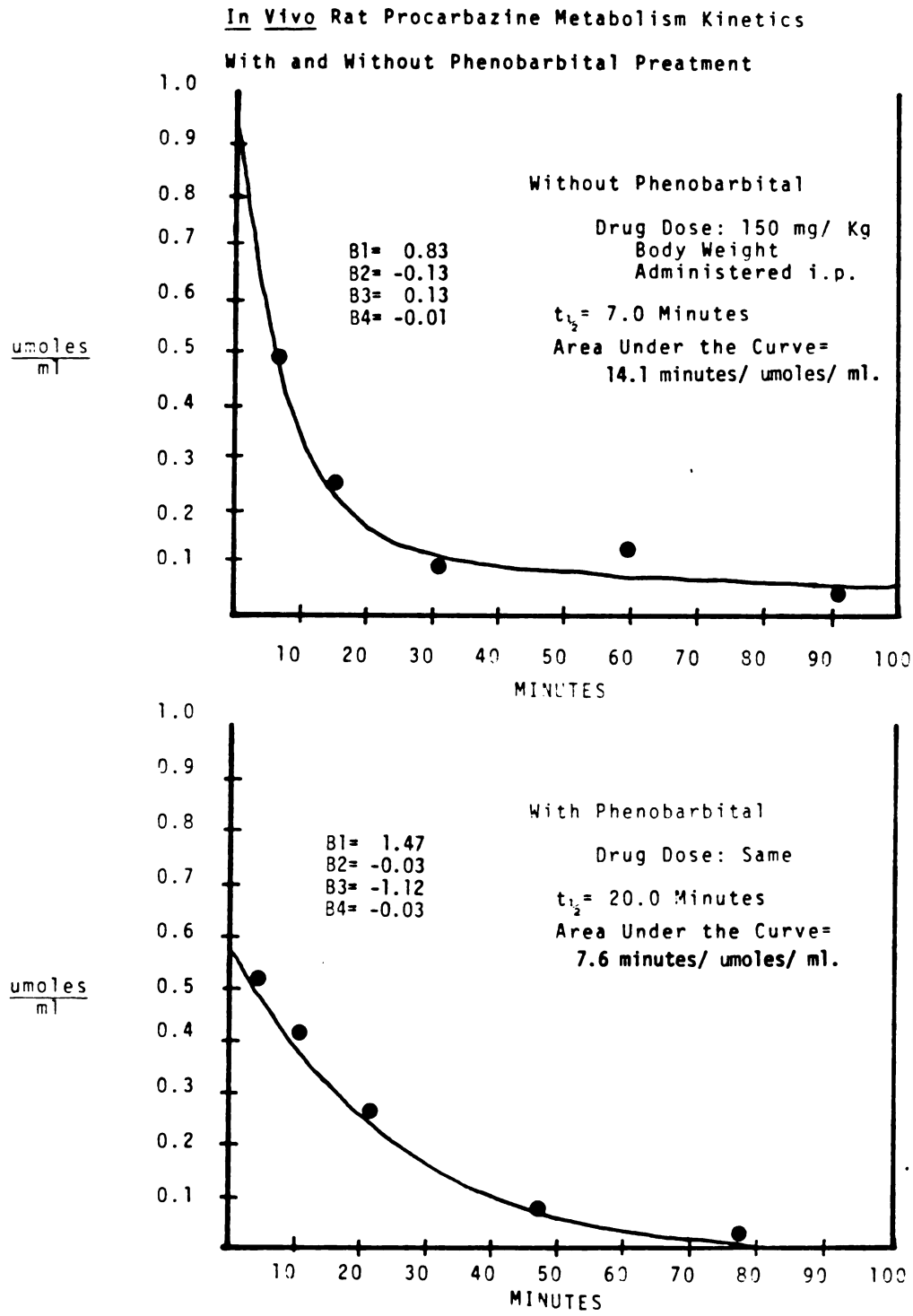


Figure 55. A Comparison of the Procarbazine Disappearance From Plasma in Phenobarbital-pretreated and Unpretreated Rats. Comparison of the Computer-generated Plasma Disappearance Curves.

group an A.U.C. of 14.1 minutes umoles/ml. Hence, phenobarbital-pretreatment doubled the rate of Procarbazine disappearance. This disappearance can be caused by an increase in metabolism, excretion rates, and distribution of Procarbazine into tissue and other areas that sequester the drug from the plasma, possibly related to changes in blood flow.

The Inhibition of the In Vivo Procarbazine Metabolism By Disulfiram in Rats

Disulfiram inhibits the metabolism of Procarbazine after the initial oxidation to the azo compound. The plasma concentration of Procarbazine and its metabolic and chemical degradation products are shown in Table 20. Azo plasma concentration increased up to 2½-fold following disulfiram-pretreatment. Total azoxy plasma concentration decreased slightly. Also shown in Table 20 are the ratios of methyl-: benzylazoxy and the ratio of azo:total azoxy. The ratio of methyl-:benzylazoxy isomers decreased in the disulfiram-pretreated animals. This decrease is mainly attributed to a decrease in the methylazoxy concentration. The azo:total azoxy ratio increased 1.5-2.5-fold with pretreatment. Yet, the total metabolism decreased 7-18% at the higher disulfiram-pretreatment dose and remained unchanged in the groups receiving 500 mg. disulfiram/kg. of body weight. An increase in Procarbazine plasma concentration occurred also at the higher disulfiram dose and remained unchanged at the lower dose.

Table 20. INHIBITION OF THE IN VIVO PROCARBAZINE METABOLISM IN RAT BY DISULFIRAM

EXPERIMENT #	DISULFIRAM	umoles/ ml. AZO	ONN- AZOXY	NNO- AZOXY	TOTAL AZOXY	RATIO AZO/AZOXY	% METABOLISM
1	-	0.07	0.11	0.02	0.13	0.54	68.6
1-D	+	0.14	0.08	0.03	0.11	1.27	68.5
2	-	0.06	0.07	0.05	0.12	0.50	71.8
2-D	+	0.09	0.07	0.02	0.09	1.00	54.5
3	-	0.14	0.07	0.02	0.09	1.56	91.1
3-D	+	0.24	0.06	0.02	0.08	3.00	73.7
4	-	0.13	0.05	0.005	0.055	2.36	87.5
4-D	+	0.18	0.04	0.01	0.05	3.6	80.6

Experiment #	Ratio of ONN/NNO	NET CHANGE
1	5.5	
1-D	2.67	51.5% decrease
2	1.4	
2-D	3.5	150% increase
3	3.5	
3-D	3.0	14.3% decrease
4	10.0	
4-D	4.0	60% decrease

Notes:

Experiment 1&1-D received 500mg/kg disulfiram orally.
Experiments 2-4 and 2-D - 4-D received 1g/kg disulfiram orally.

C. Discussion

The previously identified *in vitro* products such as the azo, methyl- and benzylazoxy isomers, aldehyde, hydrazone, acid, and occasionally, the 4-methylbenzamide compound are also formed in the intact animal. The pharmacokinetic profile of the *in vivo* metabolism in the rat suggests that the rate of metabolism of azo compound is increased compared to the *in vitro* rate. The acid and methylazoxy which are formed from the further metabolism of the azo compound increase with the decrease in azo concentration. This is consistent with the proposed *in vitro* metabolic pathway for Procarbazine in which the rapid initial oxidation of Procarbazine produces the azo compound. The azo is subsequently metabolized stereoselectively to the azoxy isomers, which are further metabolized. The aldehyde produced during this metabolism can itself be further oxidized to the major *in vivo* metabolite, N-isopropylterephthalamic acid. The initial oxidation leading to the azo compound proceeds at a similar rate both *in vivo* and *in vitro*.

The metabolism in CDF_1 mice is qualitatively similar to that observed in the Fischer C-344 rat. The quantitative aspects were not determined, except that the composition of the isomeric azoxy mixture is quantitatively similar.

The *in vivo* Procarbazine metabolism was studied in man. However, none of the previously identified rodent metabolites were detected. The lack of detection of those metabolites could be the result of a complete different metabolic

pathway for Procarbazine in man. However, previous studies, which were discussed in the review in Section I on Procarbazine metabolism, have reported the identification of several of the metabolites which are also formed during the rodent metabolism. The chemical oxidation of Procarbazine to the azo compound occurs rapidly and the azo compound should be present. This single preliminary study of the human metabolism of Procarbazine probably failed to detect any of the compounds formed *in vivo* in rodents because of either incorrect sampling times or a lack of sensitivity of the analytical system employed.

The role of cytochrome P-450 in the *in vivo* Procarbazine metabolism was investigated by studying the effects of the cytochrome P-450-inducer phenobarbital upon Procarbazine pharmacokinetics in rats. Phenobarbital induces the metabolism of Procarbazine. The plasma clearance of Procarbazine determined by the area-under-the-curve computer-fit of the pharmacokinetic data to a two-compartment model is twice as fast in the induced rat than in the normal animals. The production of metabolites also is increased with peak levels occurring approximately 10 minutes earlier in the induced animals. The peak levels also are higher in the induced animals. The further metabolism of metabolites also is accelerated so that the levels of the metabolites 80 minutes after administration of Procarbazine is lower in the induced animals. These results show that Procarbazine metabolism is induced by phenobarbital and are consistent with the general observation that Procarbazine undergoes metabolism by the cytochrome P-450 liver microsomal enzyme systems.

Finally, Procarbazine metabolism was inhibited by disulfiram. Disulfiram partially inhibits the oxidation of the azo compound to the azoxy isomers. This resulted in a 1.5-2.5-fold increase in the azo:total azoxy ratio. The possibility that this ratio change could be the result of increased Procarbazine oxidation to the azo instead of decreased azo metabolism can be ruled out because Procarbazine plasma concentration increased with disulfiram-pretreatment. The metabolism of the azoxy isomers is apparently also partially inhibited.

VIII. The In Vivo Evaluation of the Antineoplastic Acitivity of Procarbazine and Its Metabolic and Chemical Degradation Products Using an Animal Tumor Model

Procarbazine has been shown to be active against a wide variety of tumors implanted in different species of animals. A survey of Procarbazine's activity against several of these animal tumor systems is shown in Table 21 .[98] Most of the systems required multiple Procarbazine doses and evaluated changes in tumor size or weight rather than survival. A more definitive assay of antineoplastic activity based upon increased lifespan of treated versus control animals clearly was desirable.

Two animal tumor systems were designed and evaluated. Ehrlich ascites carcinoma was implanted intraperitoneally into white Swiss mice. Bollag [107] had reported that 400 mg. of Procarbazine/kg. of body weight given intraperitoneally on a schedule of 7 doses in 9 days post-implant resulted in a 300% increase in the mean survival time in this system. The second system used was L-1210 ascites leukemia transplanted intraperitoneally into CDF_1 mice. This tumor line is commonly used by the National Cancer Institute along with P-388 leukemia as a preliminary screen for antineoplastic drugs. Oliverio and Kelley [176] reported that intraperitoneal or oral administration of Procarbazine was active in this system. However, Kreis [117] stated that L-1210 ascites leukemia did not respond to intraperitoneal treatment of Procarbazine and only oral or subcutaneous administration were active.

Table 21. Survey of Procarbazine's Activity Against Animal Tumors.

Host Animal/ Tumor	Dose(mg/kg)	Treatment Protocol	T/C[1]	Reference[2]
Hamster				
Had #1	60(s.c.)	Daily for 14 days	0.37	a
	30(s.c.)	" " 14 "	0.43	a
Mice				
Ehrlich Carcinoma	200 i.p.	5 doses in 7 days	50/529(mg)	b,c
	200 p.o	5 " " 7 "	103/374(mg)	b,c
" Ascites Carcinoma	400 i.p.	7 " " 9 "	40.8/10.2(Ave. Survival,days)	b,c
	400 p.o.	7 " " 9 "	40.6/10 (" " ")	b
S180	200 i.p.	5 " " 7 "	413/950(mg)	b,c
	400 i.p.	5 " " 7 "	281/1554(mg)	b,c
	200 p.o.	5 " " 7 "	356/571(mg)	b
	400 p.o.	5 " " 7 "	191/733(mg)	b
L5178y	300 i.p.	Single dose	-	d
	7.5-120 i.p.	" "	-	d
P815	450 i.p.	3 doses in 7 days	2.3(Ave. Survival)	d
	450 p.o.	3 " " 7 "	2.0(Ave. Survival)	d
L1210(s.c.)	320 i.p.	5 " " 8 "	1.5(" ")	e
	320 s.c.	5 " " 8 "	1.5(" ")	e
L1210 (intracranial)	200 i.p.	3 " " 7 "	1.4(" ")	e
	300 i.p.	2 " " 7 "	1.4(" ")	e
	450 i.p.	1 " " 7 "	1.4(" ")	e
C57 BL/6	50 i.p.	Daily for 12 days	0.12	f
ED 771	25 i.p.	" " 12 "	0.22	e
Rats				
Walker 256	10 i.p.	6 doses in 8 days.	0/4905(mg)	b
	10 p.o.	6 " " 8 "	0/8135(mg)	b

1 Average tumor size, weight(mg), or survival(days) of treated versus control.

2

a Grunberg, E.and H. Prince; Experientia, 22, 324(1966).

b Bollag, W.; Cancer Chemotherapy Reports, 33, 1(1963).

c Bollag, W.; in Proceedings of the International Symposium on the Chemotherapy of Cancer, Lugano, April 28-May 1, 1964, by P. Plattner (editor), Elsevier, New York, 191(1964).

d Sartorelli, A. and S. Tsunamura; Proceedings of the American Association For Cancer Research, 6, 55(1965).

e Oliverio, V. and M. Kelly; in Proceedings of the International Symposium on the Chemotherapy of Cancer, Lugano, April 28-May 1, 1964, by P. Plattner (editor), Elsevier, New York, 221, 1964.

f Grungerg, E. and H. Prince; Chemotherapy, 14, 65(1969).

A. Experimental Methods and Procedures

Ehrlich Ascites Carcinoma in White Swiss Mice

Ehrlich ascites carcinoma was obtained from the Southern Research Center and grown in culture by Dr. Dennis Dean and Mary Williams of the Brain Tumor Research Center, University of California, San Francisco. The white Swiss mice were obtained from the National Cancer Institute. Each mouse weighed approximately 20 g. and received a 0.2 ml. intraperitoneal injection of a homogeneous suspension of 2×10^6 cells/ml. in Hank's balanced salt solution. The 48 animals were non-randomly divided into 8 groups of 5-7 animals. The protocols for treatment of each group is shown in Table 22. Four groups received a single intraperitoneal injection 24 hours after tumor implantation of 200, 300, 400, or 500 mg./kg. Procarbazine. Three groups received intraperitoneal injections of 200, 300, or 400 mg./kg. Procarbazine beginning 24 hours post-implant and repeated for 3 consecutive days. A single group received no treatment after tumor implantation. The median number of days post-implant survival of this group was used as a control and compared to the above groups.

In a separate experiment, 7 mice received 1.9×10^7 cell/0.1 ml. intraperitoneal injection. No treatment was given.

L-1210 Ascites Leukemia in CDF_1 Mice

The L-1210 ascites leukemia and the CDF_1 mice were obtained from the National Cancer Institute. The attempts to grow the L-1210 ascites in culture proved unsuccessful so

Table 22. Protocol For Testing the In Vivo Activity of Procarbazine in Swiss Mice Against Intraperitoneally- Implanted Ehrlich ascites Carcinoma.

Group Number	Number of Animals	Number of Cells	Protocol
1	6	2.0×10^6	Tumor only
2	6	"	Tumor + single dose of 200 mg/kg. Procarbazine
3	7	"	Tumor + single dose of 300 mg/kg. Procarbazine
4	6	"	Tumor + single dose of 400 mg/kg. Procarbazine
5	5	"	Tumor + single dose of 500 mg/kg. Procarbazine
6	6	"	Tumor + 200 mg/kg. Procarbazine on days 1,2,and 3.
7	6	"	Tumor + 300 mg/kg. Procarbazine on days 1,2,and 3.
8	6	"	Tumor + 400 mg/kg. Procarbazine on days 1,2,and 3.
9	7	1.9×10^7	Tumor only

Tumor cells were implanted by intraperitoneal injection. All drug treatments were by intraperitoneal injection beginning 24 hours after tumor implantation.

cells were carried in vivo. Ascites tumor cells were removed from the peritoneal cavity of L-1210 ascites leukemia-bearing CDF_1 mice 4 days after implantation. Removal of the tumor after 4-5 days resulted in hemorrhagic samples which could not be counted accurately; earlier removal resulted in fewer cells in each sample. The peritoneal tumor cell-containing fluid was removed with a syringe and 0.75 ml. of this fluid was diluted with 3.75 ml. of Hank's balanced salt solution (1:6 dilution). A 0.1 ml. aliquot was injected intraperitoneally into CDF_1 mice weighing an average of 20 g. The tumor-bearing mice were completely randomized and treated according to the outline shown in Table 23. Three groups received a single 0.1 ml. intraperitoneal injection of 300, 400, or 500 mg./kg. Procarbazine. The drug was administered 24 hours after tumor implantation. Three groups received 0.1 ml. multiple intraperitoneal treatments on 3 consecutive days beginning 24 hours after tumor implantation. The doses given were 200, 300, or 400 mg./kg./day.

In another experiment, 6 CDF_1 mice received a 0.1 ml. intraperitoneal injection of 1.4×10^6 L-1210 ascites cells which were given in culture and counted. No treatment was given.

Evaluation of the Antineoplastic Activity of Procarbazine, Azo, and An Azoxy Mixture Against Intraperitoneally-Implanted L-1210 Ascites Leukemia in CDF_1 Mice

The L-1210 ascites cells were removed from the peritoneal cavity of tumor-bearing CDF_1 mice. The cells were diluted

Table 23. Protocol For Testing the In Vivo Activity of Procarbazine in CDF_1 Mice Against Intraperitonally-Implanted L-1210 Ascites Leukemia.

Group #	# of Animals	# of Cells	Protocol
1	4	Not counted	Tumor only
2	4	"	Tumor + single dose of 300 mg/kg. Procarbazine
3	5	"	Tumor + single dose of 400 mg/kg. Procarbazine
4	5	"	Tumor + single dose of 500 mg/kg. Procarbazine
5	5	"	Tumor + 200 mg/kg. Procarbazine on days 1,2 and 3.
6	5	"	Tumor + 300 mg/kg. Procarbazine on days 1,2, and 3.
7	5	"	Tumor + 400 mg/kg. Procarbazine on days 1,2, and 3.
8	6	1.4×10^6 (grown in culture.	Tumor only.

All tumor cells were implanted by intraperitoneal injection.
All drug treatments were by intraperitoneal injection beginning 24 hours after tumor implantation.

in Hank's balanced salt solution and counted with a cell counter. The CDF_1 mice that received tumors received a 0.1 ml. intraperitoneal injection of 1.5×10^6 cells. These mice were randomized and divided into 10 groups of animals. The animals not receiving tumors were randomized and divided into a control group of 12 animals and 4 groups of 6 or 7 animals. Fach animal weighed approximately 20 g. The control group received tumor cells and 0.1 ml. intraperitoneal injections of normal saline. The drug solutions were made by dissolving Procarbazine in normal saline and the azo and azoxy compound in Tween 80 with sonication. Each drug solution was prepared immediately before administration and administered intraperitoneally in a volume of 0.1 ml. for 3 consecutive days beginning 24 hours after tumor implantation. Three of the groups of tumor-bearing animals received Procarbazine at a dose of 50, 100 or 200 mg./kg. A non-tumor-bearing group received 200 mg. Procarbazine/kg. Three tumor-bearing groups received the azo compound at doses equivalent to the Procarbazine dose on a molar basis. These groups received 42.5, 85, or 170 mg. azo/kg. A non-tumor-bearing group received 170 mg. azo/kg. Finally, four tumor-bearing groups received a 1.75:1 mixture of the methyl- and benzyl-azoxy isomers at doses equivalent on a molar basis to the Procarbazine dose. These groups received 45.7, 91.3, 182.6, or 273.9 mg. azoxy/kg. The latter dose was double any of the Procarbazine doses. Two non-tumor-bearing groups received either 91.3 or 182.6 mg. azoxy/kg. The non-tumor-bearing

groups which received the drugs served to determine lethal toxicity due to the drugs or the administration medium. All animals which survived the 15 day duration of the experiment were sacrificed.

B. Calculations

The number of animals surviving on each day after tumor implantation was recorded. A computer program was used to generate survival curves and calculate the median and mean lifespan and the percent T/C based upon the median lifespan. The percent T/C is the median lifespan of the treated group divided by the median lifespan of the control group. This ratio is then multiplied by 100 to express the ratio on a percent basis.

C. Results

The Ehrlich Ascites Carcinoma in White Swiss Mice

The results of the treated and non-treated groups are shown in Table 24. Only a few animals which received the 2×10^6 cells died prior to sacrificing on day 37 after tumor implantation. Those animals receiving 1.9×10^7 cells began to die after day 16, but more than 50% survived until they were sacrificed on day 21. The treated animals failed to show any increase in lifespan with either the single or multiple treatments when compared to the control animals. Visually those animals receiving treatment were more active and less bloated due to tumor growth than the controls. Those

Table 24. In Vivo Activity of Procarbazine Against Intraperitoneally-Implanted Ehrlich Ascites Carcinoma in Swiss Mice.

Group#	Protocol	# of Deaths/ Day of Death
1	Tumor only	1/27
2	Tumor + single dose of 200 mg/kg.	1/10; 1/23; 1/29
3	" " " " 300 "	1/25; 1/26
4	" " " " 400 "	1/2(due to injury)
5	" " " " 500 "	1/26
6	" + 200 mg/kg for 3 days	1/18; 1/31
7	" 300 " " " "	2/33
8	" 400 " " " "	1/32; 1/33
9	Tumor only at 10-times the number.	1/9; 1/16; 1/19

Sacrificed remaining animals in groups 1-8 on day 37.
" " " " group 9 on day 22.

receiving multiple doses appeared stronger than those receiving the single dose.

The L-1210 Ascites Leukemia in CDF_1 Mice

The number and day of deaths and the mean and median lifespan for each treated and non-treated group is shown in Table 25. Also shown is the percent T/C based upon the median lifespan. The median lifespan for the controls is 8.5 days for the animals receiving cells transplanted from tumor-bearing mice and 9.5 for the cells grown in culture and injected intraperitoneally at a dose of 1.4×10^6 cells/0.1 ml. injection. Multiple treatment on 3 consecutive days beginning 24 hours after tumor implantation increased lifespan by 40%. The single treatment increased lifespan by 30%. However, except for small increases in the mean lifespan with increased doses, no significant dose response curves can be generated. Figure 56 shows the survival curves for the treatment of L-1210 ascites in CDF_1 mice with intraperitoneally administered Procarbazine.

Evaluation of the Antineoplastic Activity of Procarbazine, Azo, and Azoxy Mixture Against Intraperitoneally-Implanted L-1210 Ascites Leukemia in CDF_1 Mice

The number and day of deaths, the mean and median lifespan, and the percent T/C (median) are shown in Table 26. The median lifespan for the control group was 8.0 days. Treatment with Procarbazine or azo at a dose of 0.8 millimoles of drug/kg. of body weight increased the median lifespan by 25%. The azoxy mixture when given at a dose of 0.8 millimoles/kg.

Table 25. In Vivo Activity of Procarbazine Against Intraperitoneally-Implanted L- 1210 Ascites Leukemia in CDF_1 Mice.

Group #	Protocol	# of Deaths/Day of Death	Lifespan Mean	Lifespan Median	% T/C
1	Tumor only	1/7; 1/8; 1/9; 1/10	8.5	8.5	
2	Tumor + single dose of 300 mg/kg.	2/10; 2/12	11.0	11.0	129.4
3	" " " " 400 mg/kg.	1/10; 2/11; 2/12	11.0	11.2	129.4
4	" " " " 500 mg/kg.	1/10; 2/11; 2/12	11.0	11.2	129.4
5	" + 200 mg/kg. for 3 days	1/11; 4/12	12.0	11.8	141.2
6	" 300 " " " "	1/11; 3/12; 1/13	12.0	12.0	141.2
7	" 400 " " " "	4/12; 1/14	12.0	12.4	141.2
8	Tumor only, 1.4 x 10^6 cells.	1/7; 2/9; 2/10; 1/11	9.5	9.3	

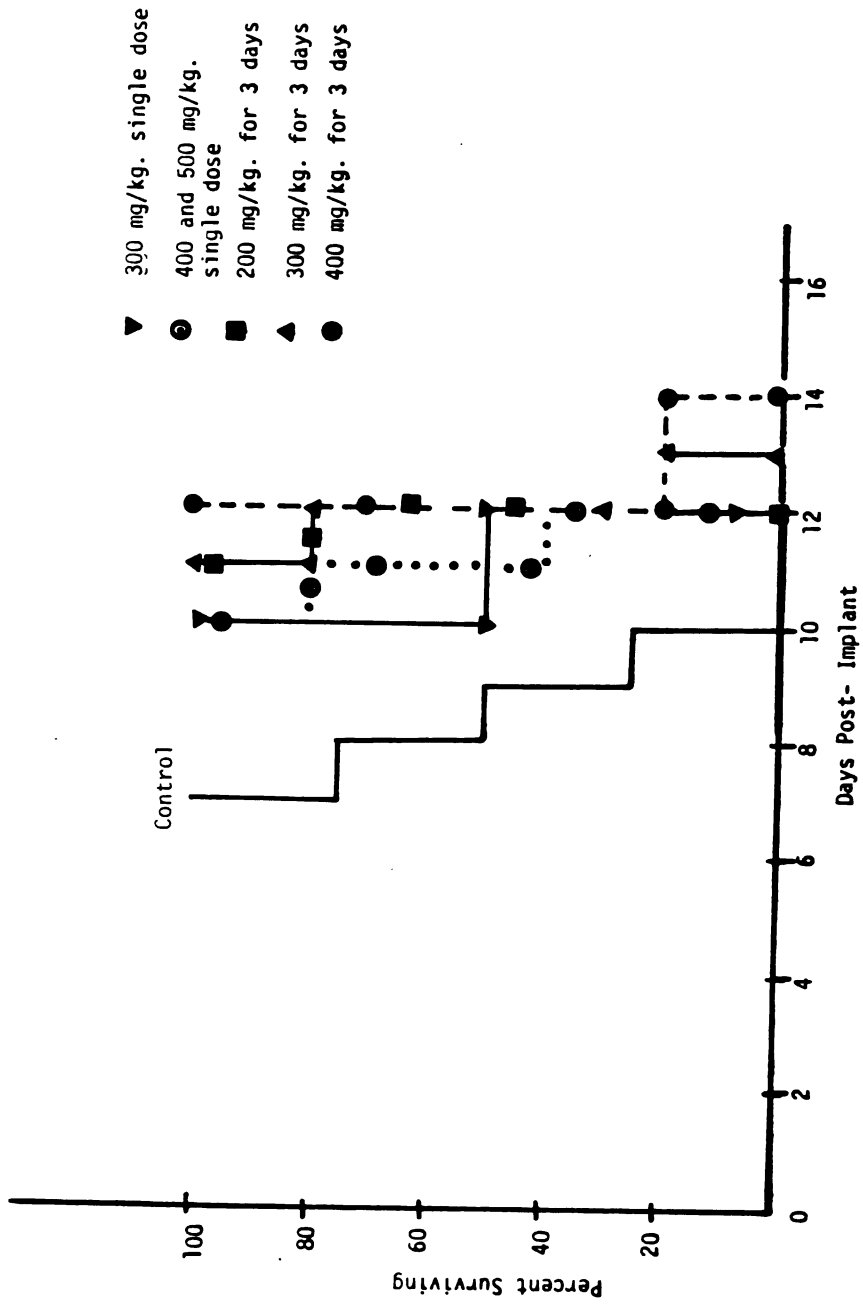


Figure 56. Survival Curves For the Treatment of Intraperitoneally- Implanted L-1210 Ascites Leukemia in CDF1 Mice With Intraperitoneally-Administered Procarbazine.

Table 26. In Vivo Activity of Procarbazine, Azo, and Azoxy Mixture Against Intraperitoneally- Implanted L-1210 Ascites Leukemia in CDF_1 Mice.

Group#	# of Animals	Protocol	# of Deaths/Day	Lifespan Mean	Lifespan Median	% T/C
1	12	Tumor only	1/5; 1/6; 3/7; 6/8; 1/9	8.0	7.4	
2	6	200 mg/kg. Procarbazine	None died in 15 days	-	-	
3	7	Tumor + 200 mg/kg. Procarbazine	1/9; 3/10; 3/11	10.0	10.3	125.0
4	7	" 100 " "	2/8; 1/9; 2/11; 1/12	10.0	9.9	125.0
5	7	" 50 " "	1/6; 1/7; 2/8; 2/10; 1/12	8.0	8.7	100.0
6	6	170 mg/kg. Azo	1/10; 1/11; 4 surviving after 15 days	-	-	
7	7	Tumor + 170 mg/kg. Azo	1/5; 1/6; 1/9; 2/10; 1/11; 1/13	10.0	9.1	125.0
8	7	" 85 " "	1/2; 1/6; 2/9; 2/10; 1/11	9.0	8.1	112.5
9	7	" 42.5 " "	2/2; 2/8; 3/10	8.0	7.1	100.0
10	7	182.6 mg/kg. Azoxy	2/5; 1/13; 1/14; 3 surviving after 15 days	-	-	
11	7	91.3 " "	1/12; 6 surviving after 15 days	-	-	
12	7	Tumor + 182.6 mg/kg. Azoxy	2/5; 4/13; 1/15	13.0	10.4	162.5
13	7	" 91.3 " "	1/9; 3/10; 2/11; 1/13	10.0	10.6	125.0
14	7	" 45.7 " "	1/7; 1/8; 1/9; 3/10; 1/11	10.0	9.3	125.0
15	7	" 273.9 " "	2/4; 3/5; 1/6; 1/7	5.0	5.1	62.5

Animals receiving tumor received 1.5×10^6 cells by an intraperitoneal injection.
All drug treatments were given by intraperitoneal injection beginning 24 hours after tumor implantation.

increased lifespan by 62.5%. Figure 57 shows a dose response curve for each of the drugs. The azoxy data also shows the curves expected if only one of the azoxy isomers is active.

D. Discussion

The antineoplastic activity of Procarbazine was evaluated in both the Ehrlich ascites carcinoma implanted intraperitoneally in white Swiss mice and L-1210 ascites leukemia implanted intraperitoneally in CDF_1 mice. The Ehrlich ascites animal model did not serve as a useful assay since few of the tumor-bearing animals died within 2-3 weeks after tumor implantation. Although visible differences existed between the treated and non-treated animals, this assay did not have a definitive endpoint that eliminates subjective evaluation. The evaluation of differences between the treated and non-treated groups would take an excessively long period of time.

The L-1210 ascites leukemia implanted intraperitoneally into mice appears to be a satisfactory animal tumor model for an assay of the antineoplastic activity of Procarbazine and its metabolic and chemical degradation products. The animals within each group die within a range of 2-3 days. The treated groups die within 2 weeks after tumor implantation. Yet, the median lifespans of the treated and non-treated animals differ by greater than 20%, making the evaluation of activity unambiguous and statistically significant. This assay provides

ACTIVITY OF PROCARBAZINE AND THE AZO AND AZOXY METABOLITES AGAINST L-1210 ASCITES IN CDF_1 MICE, $1.5X\ 10^6$ cells implanted i.p.

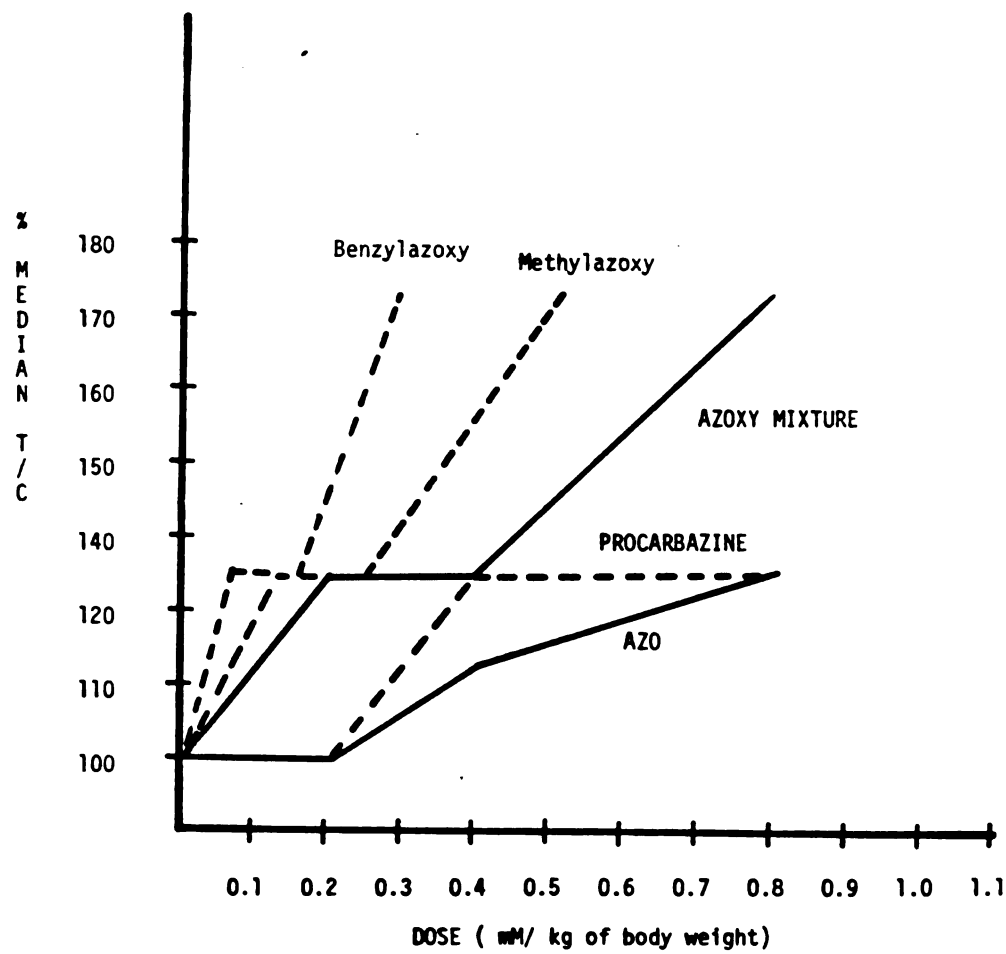


Figure 57. Dose Response Curves For the Treatment of Intraperitoneally- Implanted L- 1210 Ascites Leukemia in CDF_1 With Intraperitoneally- Administered Procarbazine, Azo, and Azoxy Mixture.

a rapid evaluation of antineoplastic activity. Since the results using cultured cells and cells transplanted directly from tumor-bearing animals were similar, the need for the laborious culturing of the cells is eliminated. The removal of the cells for transplantation 4-5 days after implanting into the host animal results in a large number of cells in non-hemorrhagic fluid. These cells can be diluted with Hank's balanced salt solution and accurately counted prior to implantation into the experimental animals. Furthermore, multiple treatment on 3 consecutive days beginning 24 hours after tumor implantation gave a substantially greater percent T/C than single treatment. However, no significant change in percent T/C occurred with increasing doses. Thus, a dose of 200 mg. of Procarbazine per kg. of body weight is administered on 3 consecutive days. For subsequent assays, tumor cells are removed from the peritoneal cavity of the tumor-bearing mice 4 days after implantation, diluted in Hank's solution and counted, diluted to a concentration of 1.5×10^7 cells/ml., and implanted intraperitoneally in a volume of 0.1 ml. Treatment is begun 24 hours after implantation of tumor at a dose of 200 mg. of Procarbazine/kg. or its molar equivalent for other compounds given as a 0.1 ml. intraperitoneal injection on 3 consecutive days.

This assay was used to calculate the antineoplastic activity of Procarbazine, the azo, and the mixture of azoxy isomers. Bollag, et al.[160] previously tested synthetic azo and azoxy (no mention of which isomer(s) or the composition if a mixture was administered) against Walker carcinosarcoma 256

and Ehrlich carcinoma (solid form) implanted subcutaneously and Ehrlich ascites carcinoma injected intraperitoneally. They reported that the therapeutic indices expressed as a ratio of the maximum tolerated dose (daily dose in a 10 day subactive toxicity test in which no loss in weight or no leukopenia occurred) and the minimum 100% effective dose (lowest daily dose which leads to complete inhibition of the growth of the Walker tumor) were similar for both azo and azoxy treatments and that both were 50% of the therapeutic index of Procarbazine. Unlike these studies, the L-1210 ascites assay showed that Procarbazine and the azo compound have almost identical antineoplastic activity at 3 different dose levels. The mixture of azoxy isomers is 38% more active at a dose of 0.8 millimoles/kg. than either Procarbazine or azo assuming that both azoxy isomers have equal activity. These results are consistent with the in vitro and in vivo metabolic data. Procarbazine is rapidly oxidized to the azo compound in high yield so that both of these compounds should be equally active. The azo is slowly metabolized to the azoxy isomers and these isomers are metabolized to the potential alkylating methydiazonium ion through hydroxylation of the azoxy isomers and, subsequent, decomposition. However, biodistribution factors may be also involved so that a given compound may not have the expected activity.

IX. The Effects of Pretreatment With Procarbazine, Methylprednisolone (Medrol), Diphenylhydantoin (Dilantin), and Phenobarbital Upon the Antineoplastic Activity of Procarbazine

The chronic or concomittant administration of certain drugs can influence the pharmacological and toxicological properties of other therapeutic agents. If a drug undergoes activation or detoxification by the liver microsomal enzyme system, other drugs that can either stimulate or depress these enzymes can markedly influence the duration of action, toxicity, and other important therapeutic parameters. These interactions can become especially critical during clinical chemotherapy in humans.

Several drugs besides those typically used for treatment of the disease itself are chronically administered during the treatment of patients with brain tumors. Since brain tumor patients commonly suffer from convulsions as one of the effects of the progress of the disease, anticonvulsants such as phenobarbital and diphenylhydantoin (Dilantin) are administered. Phenobarbital is commonly administered 3 times daily at a dose of 60 mg./administration. Dilantin is administered at a dose of 300 mg./day. Methylprednisolone (Medrol) is also administered. Methylprednisolone reduces the presence of secondary tumors and improves the neurological status of secondary tumors. These glucocorticoids are administered at a dose of 4-40 mg. given 4 times daily.

Phenobarbital is a classical inducer of the cytochrome P-450 liver microsomal enzyme system. Diphenylhydantoin is also a potent inducer of the activity of the liver microsomal mixed-function

oxidases. Davis, et al. found that after orally feeding squirrel monkeys 10 mg./kg. once daily for 30 days, the levels of cytochrome P-450 increased 5-fold. The activity of the mixed-function oxidases in the metabolism of parathion, the o-demethylation of p-nitroanisole, and azo reductase activity increased 2-3-fold. Furthermore, Bechtel and co-workers [178] reported that after decreasing the levels of liver cytochrome P-450 and b_5 during chronic hypoxia at sea level, the administration of Dilantin increases the levels of these two cytochromes.

Procarbazine is given clinically either singly at a dose of 150 mg./m^2, for 28 consecutive days with a 28 day break before repeating the chemotherapy or in combination with 1-(2-chloroethyl)-3-cyclohexyl-1-nitrosourea (CCNU) at a dose of 60-100 mg./m^2. for 14 days.

The effects of Procarbazine upon metabolism of other compounds and specific enzymes have been reported. Bock, et al.[179] reported that Procarbazine increased the in vitro activity of Δ^4-5 - and Δ^4-5 -reductase in the degradation of cortisol in male rats. Procarbazine decreased the activity of the same enzymes when activity was determined by evaluating the hydration of the C_{4-5}-double bond during cortisone metabolism in both male and female rats. Procarbazine also affects other metabolic enzymes. Eade and co-workers [180] studied the effects of a single Procarbazine dose upon in vitro hepatic microsomal drug metabolism. Procarbazine increased pentobarbital-induced sleep at doses of 25 and 100 mg./kg. by 26 and 71% respectively. At a concentration of 0.5 mM, Procarbazine decreased aminopyrine N-demethylase activity by 44%.

The known effects of phenobarbital, Dilantin, and Procarbazine upon drug metabolism and the cytochrome P-450 microsomal mixed-function oxidase enzyme system coupled with the apparent requirement for bioactivation of Procarbazine make a knowledge of possible interactions between these drugs which are commonly administered chronically or concomittantly during Procarbazine chemotherapy an important objective. The effects of pretreatment with phenobarbital, methylprednisolone, Dilantin, and Procarbazine upon the in vivo antineoplastic activity of Procarbazine against L-1210 ascites was studied. The levels of cytochrome P-450 were determined to correlate antineoplastic activity with the activity of the cytochrome P-450 microsomal enzyme system.

A. Experimental Methods and Procedures

Male CDF_1 mice received either no pretreatment or pretreatment orally in the case of phenobarbital, diphenylhydantoin, and methylprednisolone or by intraperitoneal injection in the case of Procarbazine. The protocol including the number of animals in each group is shown in Table 27. All of the doses were chosen to simulate those clinically received by man. The mice receiving Procarbazine-pretreatment received 0.1 ml. intraperitoneal injections of 15, 30, or 60 mg. Procarbazine in normal saline/kg. of body weight/day. The dose was based upon the assumption that the mice weighed 20 grams. These doses were given daily for either 7 or 14 consecutive days prior to implantation of the tumor. The control group received a 0.1 ml. intraperitoneal injection of normal saline

Table 27. Protocol For the Study of the Effects of Pretreatment With Procarbazine, Methylprednisolone, Diphenylhydantoin, and Phenobarbital Upon the In Vivo Antineoplastic Activity of Procarbazine Against L-1210 Leukemia in CDF_1 Mice.

Group #	# of Animals	Protocol
1	10	1.5×10^6 cells, tumor only
2	6	Tumor + 200 mg/kg. Procarbazine on days 1,2, and 3(3 days).
3	6	" + 100 " " " " 1,2, " 3.
4	10	30 mg/kg. Procarbazine-pretreatment for 7 days + Tumor.
5	6	60 " " " " 7 " + " .
6	6	60 " " " " 14 " + " .
7	6	30 " " " " 7 " + " .
8	10	30 " " " " 14 " .
9	5	30 " " " " 14 ", Sacrificed, plasma removed for HPLC analysis, liver for Cyto. P-450 analysis.
10	10	15 mg/kg. Procarbazine-pretreatment for 7 days + tumor+ 200 mg/kg. Procarbazine treatment for 3 days.
11	10	15 " " " " 7 " + " + 100 mg.kg. Procarbazine " " 3 " .
12	10	30 " " " " 7 " + " + 200 mg/kg. Procarbazine " " 3 " .
13	10	30 " " " " 7 " + " + 100 mg/kg. Procarbazine " " 3 " .
14	6	30 " " " " 14 " + " + 200 mg/kg. Procarbazine " " 3 " .
15	6	30 " " " " 14 " + " + 100 mg/kg. Procarbazine " " 3 " .
16	6	60 " " " " 7 " + " + 200 mg/kg. Procarbazine " " 3 " .
17	6	60 " " " " 7 " + " + 100 mg/kg. Procarbazine " " 3 " .
18	6	60 " " " " 14 " + " + 200 mg/kg. Procarbazine " " 3 " .

Group #	# of Animals	Protocol
19	7	60 mg/kg. Procarbazine-pretreatment for 14 days + Tumor + 100 mg/ kg Procarbazine treatment for 3 days.
20	3	30 " " " " 14 days + 200 mg/kg. Procarbazine treatment for 3 days.
21	4	60 " " " " 7 " + 200 mg/kg. Procarbazine " " 3 ".
22	6	60 " " " " 14 " + 200 mg/kg. Procarbazine " " 3 ".
23	4	60 " " " " 14 " + 100 mg/kg. Procarbazine " " 3 ".
24	6	60 mg/kg. Diphenylhydantoin-pretreatment for 7 days + Tumc
25	6	60 " " " " 7 " + " + 200 mg/kg. Procarbazine treatment for 3 days.
26	10	7.7 mg/kg. Methylprednisolone-pretreatment for 7 days + Tumor.
28	9	7.7 " " " " " 7 " + " + 200 mg/kg. Procarbazine treatment for 3 day
29	9	7.7 " " " " " 7 " + " + 100 mg/kg. Procarbazine treatment for 3 day
30	8	7.7 " " " " " 7 "
31	12	48 mg/kg. Phenobarbital-pretreatment for 7 days + Tumor
32	10	48 " " " " 7 " + " + 200 mg/kg. Procarbazine treatment for 3 days.
33	10	48 " " " " 7 " + " + 100 mg/kg. Procarbazine treatment for 3 days.
34	10	48 " " " " 7 " .
35	5	48 " " " " 7 ", Sacrificed plasma removed for plasma level assay, liver for cytochrome P-450 analisis.
27	5	60 mg/kg. Diphenylhydantoin-pretreatment for 7 days, Sacrificed and plasma removed for plasma level level assay.

Animals receiving tumor received 1.5 x 10^6 cells by an intraperitoneal injection.
All drug treatments were given by intraperitoneal injection beginning 24 hours after tumor implantation.
Procarbazine-pretreatment was given by intraperitoneal injection, all of the other pretreatments were oral.

daily. Diphenylhydantoin was administered orally in the drinking water for 7 days at a concentration at which each mouse received 60 mg./kg./day. All oral concentrations were calculated assuming that each mouse drank an average of 6 ml./day. These solutions were changed every third day. Methylprednisolone was given orally at a dose of 7.5 mg./kg./day. Phenobarbital was also given orally. The phenobarbital dose was 48 mg./kg./day. After completion of the 7 or 14 day pretreatment protocol, Procarbazine administration was discontinued. A period of 48 hours after pretreatment elapsed prior to implantation intraperitoneally of 1.5×10^6 L-1210 ascites cells in a volume of 0.1 ml. The 48 hour period with no pretreatment was chosen to insure that no tumor cells were killed by residual metabolites of Procarbazine. The day after tumor implantation, several animals from the groups receiving Procarbazine-pretreatment for 7 and 14 days at the doses of 30 and 60 mg./kg./day and the group receiving phenobarbital-pretreatment were sacrificed and their livers removed. The 100,000 x g. microsomes were prepared as described in Section IV and the cytochrome P-450 levels and the total microsomal protein levels were determined as described in Section IV.

The plasma from several phenobarbital- and diphenylhydantoin-pretreated animals was pooled and analyzed in the Clinical Laboratories at the University of California, San Francisco, by Dr. Pocar Kabra using a standard assay. Plasma from several animals in the group receiving 30 mg. Procarbazine/kg./day for 7 days was removed and analyzed by the liquid

chromatographic assay system using the conditions described in Section III. This qualitative assay determined that no residual Procarbazine metabolites remained prior to tumor implantation. The experimental animals received either 100 or 200 mg. of Procarbazine/kg. of body weight on 3 consecutive days beginning 24 hours after tumor implantation. Procarbazine was administered in a volume of 0.1 ml. by intraperitoneal injection. Those animal receiving oral pretreatment were gradually withdrawn over the 3 days of Procarbazine treatment by decreasing the dose by 50% on each day. The pretreatment was removed on the day of the final Procarbazine treatment. All animals were returned to normal drinking water. All animals received Purina Lab Chow ad libitum during the experiment and were housed in the animal care facilities at a constant room temperature of 75-80^{o}F. with 12 hours of light and 12 hours of darkness. The animals used to prepare the microsomes received no food for 24 hours prior to sacrificing. The day of death was noted for each animal.

B. Calculations

The mean and median lifespan and the percent median T/C were computed using a computer program.

The equations for the calculations of the cytochrome P-450 levels and the total microsomal protein are shown in Section IV. The determination of the significance of the differences observed in the survival curves is made using a computer program for the Wilcoxon-Gehan statistical analysis of the data.

C. Results

The results of the diphenylhydantoin-, phenobarbital-, and methylprednisolone-pretreatments are shown in Table 28. The 60 mg. diphenylhydantoin/kg./day-pretreatment resulted in a 14% increase in the in vivo antineoplastic activity of Procarbazine against L-1210 ascites leukemia. The plasma levels attained after the 7 day pretreatment was 2 ug./ml.

The 7.5 mg. methylprednisolone/kg./day-pretreatment had no effect upon the activity of Procarbazine in the L-1210 ascites assay system. At both the 100 and 200 mg. of Procarbazine/kg. treatments, the percent median T/C of 111 and 122 respectively were identical to the non-pretreated groups.

Phenobarbital-pretreatment at a dose of 48 mg./kg./day resulted in a significant increase in the activity of Procarbazine at both doses of Procarbazine. The pretreatment resulted in a significant 19% increase in % median T/C at the 200 mg. of Procarbazine/kg. dose and a 13% increase at the 100 mg./kg. dose. The survival curves, Figure 58, of the pretreated and non-pretreated groups at both Procarbazine doses are statistically different when computer analyzed by the Wilcoxan-Gehan statistical analysis. The phenobarbital plasma levels attained after 7 days of pretreatment was 6 ug./ml. This pretreatment resulted in a 52.2% increase in the levels of cytochrome P-450 as shown in Table 28 .

Table 28. Effects of Pretreatment With Methylpredisolone, Diphenylhydantoin, and Phenobarbital Upon the Activity of Procarbazine Against Intraperitoneally- Implanted L-1210 Ascites Leukemia in CDF_1 Mice.

PROTOCOL	MEDIAN	MEAN	T/C	MEDIAN % CHANGE
Tumor	9.0	9.0		
Tumor + 7.5 mg/kg Medrol Pretreatment	9.0	8.8		
Tumor + 7.5 mg/kg Medrol Pretreatment + 200 mg/kg Procarb.	11.0	11.1	122	0%
Tumor + 7.5 mg/kg Medrol Pretreatment + 100 mg/kg Procarb.	10.0	10.2	111	0%
Tumor	7.5	7.8		
Tumor + 60 mg/kg Dilantin pretreatment	7.0	6.7		
Tumor + 200 mg/kg Procarb.	10.0	10.0	133	
Tumor + 60 mg/kg Dilantin pretreatment + 200 mg/kg Procarb.	11.0	10 5	147	14 % increase
Tumor + 48 mg/kg Phenobarb. Pretreatment	8.5	8.5		
Tumor + 48 mg/kg Phenobarb. Pretreatment + 200 mg/kg Procarb.	12.0	11.9	141	19% increase
Tumor + 48 mg/kg Phenobarb. Pre- Treatment + 100 mg/kg Procarb.	10.5	10.5	124	13% increase

Notes: Dilantin studies were run on a different day with a different group of animals.

Cytochrome P-450 Levels:

Control: 0.92 mmoles of P-450/ mg total protein

Procarbazine Pretreated: 0.90 mmoles of P-450/ mg total protein

Phenobarbital Pretreated: 1.40 mmoles of P-450/ mg total protein

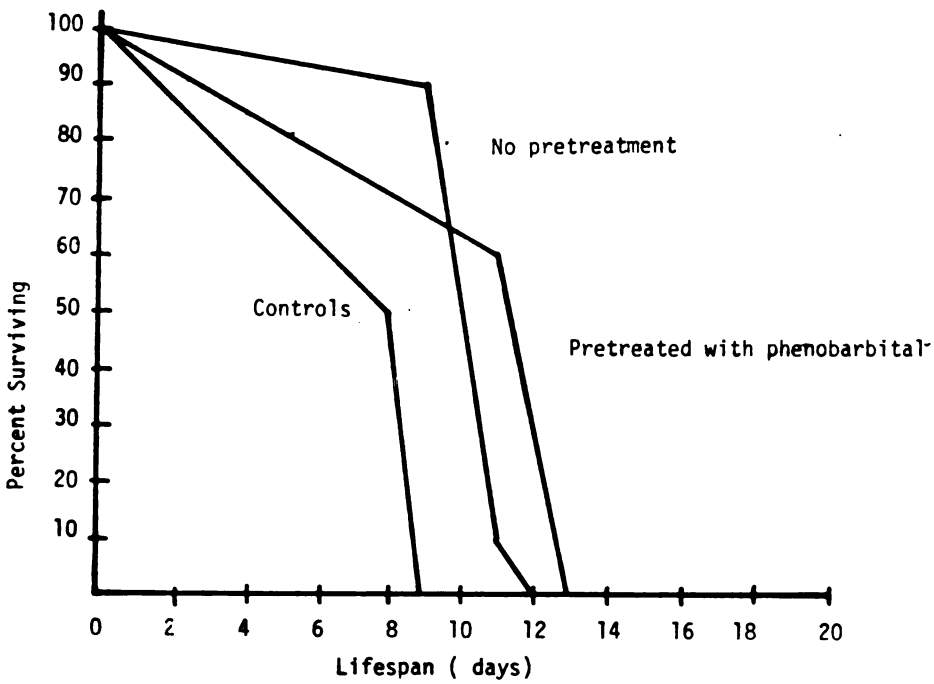


Figure 58. Survival Curves For the Study of the Effects of Phenobarbital-Pretreatment Upon the Activity of Procarbazine Against Intraperitoneally- Implanted L-1210 Ascites Leukemia in CDF_1 Mice.

Procarbazine was given intraperitoneally at a dose of 200 mg/kg. for 3 consecutive days beginning 24 hours after tumor implantation. Phenobarbital was administered orally in drinking water at a dose of 48 mg/kg/day for 7 days.

Table 29. EFFECTS OF PROCARBAZINE PRETREATMENT UPON THE ACTIVITY OF PROCARBAZINE AGAINST I.P. IMPLANTED L-1210 ASCITES IN CDF_1 MICE

PROTOCOL	MEDIAN	MEAN	%T/C	% Change in T/C	Cyto.P-450 (nmoles/mg protein)	TOTAL PRETREATMENT DOSE	TOTAL DOSE(mg)
Tumor	9.0	9.0			0.92		
Tumor + Procarbazine Treatment for 3 days	11.0	11.0	122		0.92		12.0
15 mg/kg Pretreatment for 7 days + Tumor + Procarbazine Treatment	11.0	11.2	122	0	----	2.1	14.1
30 mg/kg Pretreatment for 7 days +	9.0	9.1	100		0.90	4.2	4.2
30 mg/kg Pretreatment for 7 days + " + Procarbazine Treatment	11.5	11.6	128	+6	0.90	4.2	16.1
Tumor	7.5	7.8					
Tumor + Procarbazine Treatment for 3 days	10.0	10.0	133				12.0
30 mg/kg Pretreatment for 14 days + Tumor	7.0	7.3	93		0.96	8.4	8.4
30 mg/kg Pretreatment for 14 days + " + Procarbazine Treatment	10.0	9.8	143	+10	0.96	8.4	20.4
60 mg/kg Pretreatment for 7 days +	7.0	7.0	93		0.80	8.4	8.4
60 mg/kg Pretreatment for 7 days + " + Procarbazine Treatment	9.5	9.7	136	+2	0.80	8.4	20.4
60 mg/kg Pretreatment for 14 days +	7.0	7.3	93		0.98	16.8	16.8
60 mg/kg Pretreatment for 14 days + " + Procarbazine Treatment	8.5	9.0	121	-12	0.98	16.8	28.8

Procarbazine Dose: 200mg/kg given i.p. for 3 days beginning 1 day after tumor implant.

Table 30. EFFECTS OF PROCARBAZINE PRETREATMENT UPON THE ACTIVITY OF PROCARBAZINE AGAINST I.P. IMPLANTED L-1210 ASCITES IN CDF_1 MICE

PROTOCOL	MEDIAN	MEAN	%T/C	% Change in T/C	Cyto.P-450 (nmoles/mg protein)	TOTAL PRETREATMENT DOSE	TOTAL DOSE(mg)
Tumor	9.0	9.0			0.92		
Tumor + Procarbazine Treatment for 3 days	10.0	10.0	111		0.92		6.0
15 mg/kg Pretreatment for 7 days + Tumor + Procarbazine Treatment	10.0	10.4	111	0	----	2.1	8.1
30 mg/kg Pretreatment for 7 days + "	9.0	9.1	100		0.90	4.2	4.2
30 mg/kg Pretreatment for 7 days + " + Procarbazine Treatment	10.0	10.1	111	0	0.90	4.2	10.2
Tumor	7.5	7.8					
Tumor + Procarbazine Treatment for 3 days	8.5	8.5	113				6.0
30 mg/kg Pretreatment for 14 days + Tumor	7.0	7.3	93		0.96	8.4	8.4
30 mg/kg Pretreatment for 14 days + " + Procarbazine Treatment	9.0	9.3	129	+15	0.96	8.4	14.4
60 mg/kg Pretreatment for 7 days + "	7.0	7.0	93		0.80	8.4	8.4
60 mg/kg Pretreatment for 7 days + " + Procarbazine Treatment	8.5	8.3	121	+8	0.80	8.4	14.4
60 mg/kg Pretreatment for 14 days + "	7.0	7.3	93		0.98	16.8	16.8
60 mg/kg Pretreatment for 14 days + " + Procarbazine Treatment	9.0	9.0	129	+15	0.98	16.8	22.8

Procarbazine Dose: 100mg/kg given i.p. for 3 days beginning 1 day after tumor implant.

The results of the different Procarbazine-pretreatment protocols are shown in Tables 29 and 30. The 7 day pretreatment at 15 and 30 mg./kg. did not significantly effect either the cytochrome P-450 level or the Procarbazine activity at either Procarbazine treatment doses. No direct relation exists between the total pretreatment dose and the percent decrease in cytochrome P-450. The greatest total pretreatment dose of 16.8 mg. increased the cytochrome P-450 level by 6.5% as compared to a 4.3% increase and 13% decrease for the 8.4 mg. dose. Pretreatment at 60 mg./kg. for 7 days resulted in a 13% decrease as compared to 43% increase when 30 mg./kg. was given for 14 days although the total pretreatment dose was the same. No clear relationship exists between pretreatment, cytochrome P-450 level, and the % median T/C. Pretreatment increased the lifespan at all doses except at 60 mg./kg. pretreatment for 14 days. This pretreatment resulted in a decrease in survival by the group receiving 200 mg./kg. Procarbazine pretreatment.

D. Discussion

Procarbazine requires bioactivation. The results previously presented suggest that the enzymes in the liver microsomal cytochrome P-450 mixed-function oxidase enzyme system play a crucial role in this activation process.

Phenobarbital and diphenylhydantoin induced the microsomal cytochrome P-450 content and correspondingly increased the antineoplastic activity of Procarbazine. Dilantin-pretreatment is equally as effective in stimulating Procarbazine antiplastic activity as phenobarbital when given at approximately equivalent doses. If similar results occur in man, the use of these anticonvulsants could require an alteration in the Procarbazine chemotherapeutic protocol.

Methylprednisolone had no effect upon the antineoplastic activity of Procarbazine. Its effect in man cannot be ruled out.

The effect of Procarbazine-pretreatment upon its own activity is not completely defined by these preliminary experiments. No clear correlation exists between the total pretreatment dose and the changes in cytrochrome P-450 level. This lack of a correlation could partially be attributed to the method of comparison. The cytochrome P-450 content is expressed in n moles of P-450/mg. of total microsomal protein. If Procarbazine-pretreatment results in a large decrease in total liver weight and total microsomal protein; the cytochrome P-450 content in the intact liver would appear greater when in actuality the pretreatment decreased the cytochrome P-450 content.

Long-term administration of Procarbazine does not significantly alter Procarbazine antineoplastic activity. Further, despite initial decreases in cytochrome P-450 levels during 7 day pretreatment schedules, the cytochrome P-450 levels return to normal with longer pretreatment. This data suggests that Procarbazine reversibly inhibits cytochrome P-450 and does not result in irreversible damage. The increase in the cytochrome P-450 levels after 14 days of pretreatment compared to without pretreatment might result from increased cytochrome P-450 production occurring after cessation of pretreatment. Despite Procarbazine's inhibition of cytochrome P-450 levels, cytochrome P-450 production increases to compensate for the loss. Therefore, the clinical daily administration of Procarbazine during 20-30 day courses apparently does not significantly decrease Procarbazine metabolism to active agents as might be predicted from the literature reports of Procarbazine inhibition of metabolic enzymes and cytochrome P-450.

X. The Effect of Disulfiram-Pretreatment Upon the Antineoplastic Activity of Procarbazine Against L-1210 Ascites Leukemia in CDF_1 Mice

Disulfiram has been shown in Section VII to partially inhibit the *in vivo* metabolism of Procarbazine in rats. It primarily inhibits conversion of the azo metabolite to the azoxy isomers. Subsequent metabolism of the azoxy isomers may also be partially inhibited. This inhibition is analogous to the effect of disulfiram upon the metabolism of the carcinogenic methylating agent 1,2-demethylhydrazine.

Several groups have investigated the effects of disulfiram-pretreatment upon the carcinogenicity of 1,2-dimethylhydrazine, its carcinogenic metabolites azoxymethane and methylazoxymethanol, and the carcinogenic N-nitrosamines. Wattenberg[181] found that a disulfiram-containing diet completely inhibits the formation of large-bowel neoplasia in mice receiving repeated subcutaneous administrations of 1,2-dimethylhydrazine. Nigro and Campbell[182] reported a 40% decrease in the occurance of intestinal tumors in rats fed disulfiram while receiving injections of azoxymethane. Likewise, Schmahl and Krugger[183] observed that a dose of 500 mg./ml. of disulfiram given orally 2 hours before dimethylnitrosamine considerably reduced the toxicity and RNA alkylation by dimethylnitrosamine.

These reports prompted this effort to inhibit the metabolic activation pathway leading to active alkylating intermediates. Elimination of this pathway *in vivo* with the continued formation of free radical products from Procarbazine would provide a means to investigate the cytotoxic effects of both active species. The

effect of disulfiram administered orally prior to each Procarbazine treatment upon the antineoplastic activity of Procarbazine was studied.

A. Experimental Methods and Procedures

The male CDF_1 mice were divided into 7 groups of 5-10 animals each. Each group was treated according to the protocol outlined in Table 31. Six of the groups received 1.5×10^6 L-1210 ascites leukemia cells intraperitoneally. These cells had been removed from the peritoneal cavity of a CDF_1 host mouse 4 days after implantation. The cells were diluted with Hank's balanced salt solution and counted. Each mouse received 0.1 ml. of a 1.5×10^7 cells/ml. suspension. Disulfiram was dissolved in Tween 80 with sonication. Disulfiram was administered by a syringe equipped with a blunt bent needle designed for oral administration of drugs. Each mouse received 0.2 ml. containing 10 mg. of disulfiram 2 hours prior to each Procarbazine treatment. Mice not receiving disulfiram received 0.2 ml. of Tween 80. Procarbazine was dissolved in normal saline and 0.1 ml. containing 100 or 200 mg./kg. Procarbazine was administered intraperitoneally daily for 3 consecutive days beginning 24 hours after tumor implantation to 20 g. average weight mice. All mice received water and Purina Lab Chow ad libitum and were housed in the animal care facilities at a constant room temperature of 75-80°F. with 12 hours of light and 12 hours of darkness. The day of death for each animal was recorded.

Table 31. Protocol For the Study of the Effects of Disulfiram- Pretreatment Upon the Activity of Procarbazine Against Intraperitoneally-Implanted L-1210 Ascites Leukemia in CDF_1 Mice.

Group #	# of Animals	Protocol
1	7	1.5×10^6 Tumor Cells, tumor only.
2	10	Tumor + 200 mg/kg. Procarbazine for 3 days.
3	5	" + 100 " " " 3 " .
4	10	" + 500 mg/kg. Disulfiram.
5	8	500 mg/kg. Disulfiram + 200 mg/kg. Procarbazine for 3 days.
6	10	Tumor + 500 mg/kg. Disulfiram + 200 mg/kg. Procarbazine for 3 days.
7	10	" + 500 mg/kg. Disulfiram + 100 mg/kg. Procarbazine for 3 days.

Animals receiving tumor received 1.5×10^6 cells intraperitoneally.
Disulfiram was administered orally as a Tween 80 solution 2 hours prior to each Procarbazine treatment.
Procarbazine was administered intraperitoneally on 3 consecutive days beginning 24 hours after tumor implantation.

B. Calculations

The mean and median lifespan and the % median T/C were computed. Determination of the significance of the difference observed in the survival curves is made using the Wilcoxan-Gehan statistical analysis of the data.

C. Results

The effect of disulfiram-pretreatment on the antitumor activity is shown in Table 32. The percent median T/C increased by 14.2% at 100 and 200 mg./kg. Procarbazine. Pretreatment with disulfiram without Procarbazine-treatment did not affect the percent median lifespan of tumor-bearing animals. The tumor-bearing groups with or without disulfiram-pretreatment had a median lifespan of 7 days. The survival curves of the disulfiram-pretreated and non-pretreated experimental groups that received tumor and Procarbazine-treatment are shown in Figure 59.

D. Discussion

Disulfiram-pretreatment in rats decreased the metabolism of Procarbazine. The oxidation of the azo to the azoxy isomers and the further oxidation of the azoxy isomers to potential alkylating species are partially inhibited. However, no significant change in the antineoplastic activity of Procarbazine against L-1210 ascites leukemia was observed in CDF_1 mice. Disulfiram-pretreatment did increase the toxicity of Procarbazine. Systemic toxicity increased as shown by increased early

Table 32. The Effects of Disulfiram- Pretreatment Upon the Activity of Procarbazine Against Intraperitoneally- Implanted L-1210 Ascites Leukemia in CDF_1 Mice.

Group #	Protocol	# of Deaths/Day	Lifespan Mean	Lifespan Median	% T/C
1	Tumor only	5/7; 1/9; 1 animal did not receive tumor	7.3	7.0	
2	Tumor + 200 mg/kg. Procarbazine for 3 days	1/9; 7/10; 1/11; 1/12	10.2	10.0	142.9
3	Tumor + 100 mg/kg. Procarbazine for 3 days	1/7; 1/8; 2/9;1/13	9.2	9.0	128.6
4	Tumor + 500 mg/kg. Disulfiram	3/6; 7/7	6.7	7.0	
5	500 mg/kg. Disulfiam + 200 mg/kg. Procarbazine for 3 days.	2/7;1/10;1/17;1/18; 1/20; 1 sacrificed on day 21.	13.2	17.0	
6	Tumor + 500 mg/kg. Disulfiram + 200 mg/kg. Procarbazine for 3 days.	1/3;2/7;1/8;1/10;2/12; 1/13;1/14;1/18	10.4	11.0	157.1
7	Tumor + 500 mg/kg. Disulfiram + 100 mg/kg. Procarbazine for 3 days.	1/2;2/8;1/9;5/10;1/14	9.1	10.0	142.9

THE EFFECTS OF DISULFIRAM PRETREATMENT UPON THE IN VIVO PROCARBAZINE ACTIVITY AGAINST L-1210 ASCITES IN CDF_1 MICE

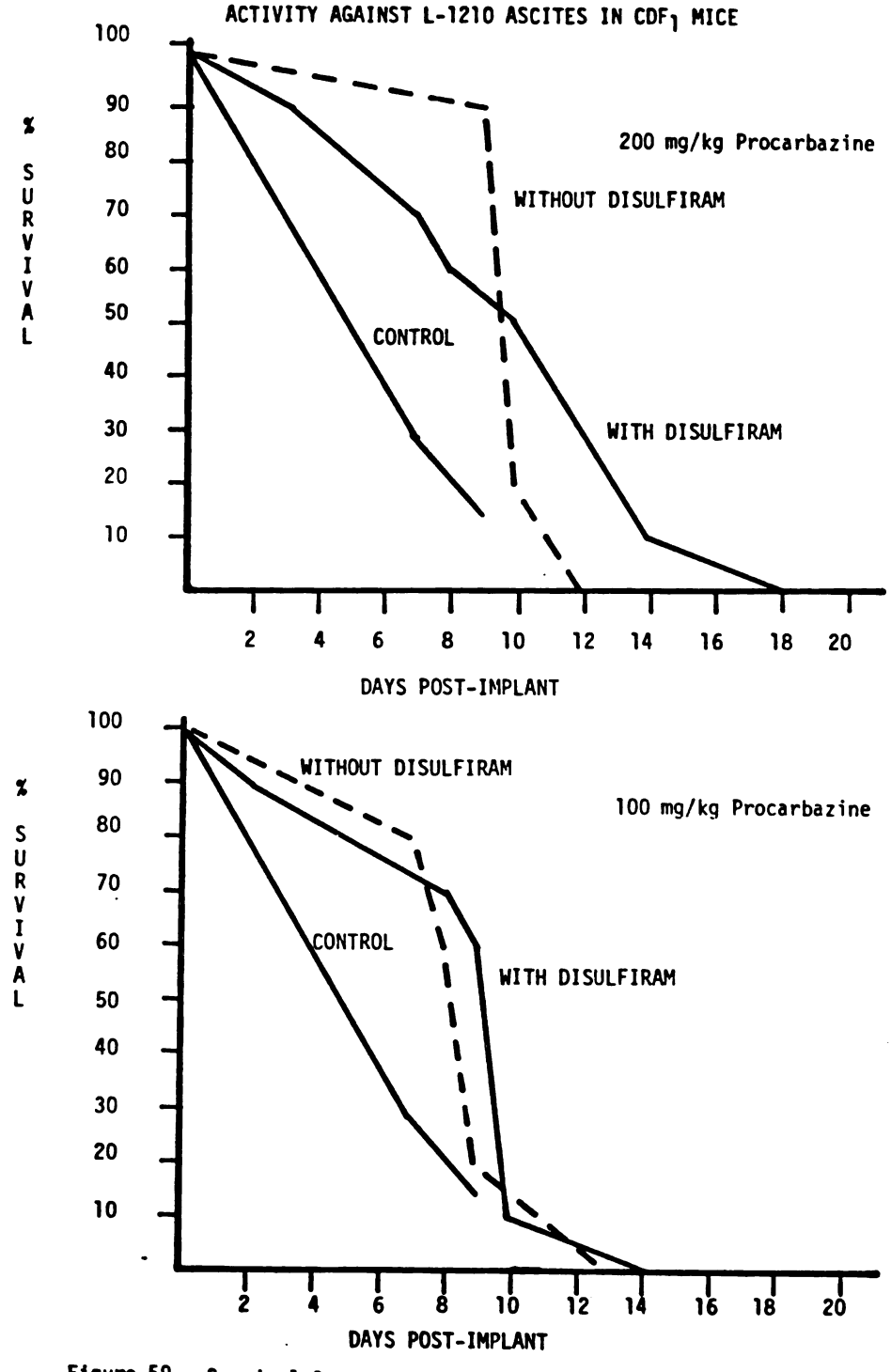


Figure 59. Survival Curves For the Study of the Effects of Disulfiram- Pretreatment Upon the Activity of Procarbazine Against Intraperitoneally- Implanted L-1210 Ascites Leukemia in CDF_1 Mice.

deaths in animals not receiving tumor, but receiving disulfiram and Procarbazine.

The absence of significant differences in Procarbazine's antineoplastic effect despite partial inhibition of its metabolism might result from either continued formation of the same cytotoxic species or production of new species. The presence of both azoxy isomers at the same plasma concentration with or without disulfiram-pretreatment could account for continued cell kill. Disulfiram might also change the metabolism of Procarbazine so that other cytotoxic agents are produced during disulfiram-pretreatment. An alternate explanation is that the partial inhibition of Procarbazine metabolism to cytotoxic species was too small to detect in the animal tumor model. A threshold in cell kill numbers might exist before changes in median lifespan are observable.

Finally, disulfiram could be acting differently in different species. Although similar inhibition of the metabolism of 1,2-dimethylhydrazine by mice and rats have been previously reported,[181,182] disulfiram-pretreatment may not produce the same inhibition of Procarbazine metabolism in CDF_1 mice as was observed in Fisher C-344 rats. Nigro and Campbell[182] suggested that such a species dependency exists in the inhibitory action of disulfiram on azoxymethane carcinogenesis. They reported a several-fold difference in inhibition in rats as compared to that reported by Wattenberg, et al.[184] in CDF_1 mice.

XI. Conclusion

A number of studies indicate that Procarbazine is not active itself. Recent results including those contained in this thesis indicate that Procarbazine requires biological activation for its antineoplastic activity. The current knowledge and concepts of the mode of action and mechanism of activation is the culmination of numerous studies including those described in this thesis.

Four different cytotoxic species have been reported as the active agent derived from Procarbazine. These include hydrogen peroxide, aldehydes (formaldehyde), free radicals (methyl free radical), and alkylating species (methyldiazonium ion).

Early proposals include denaturation of existing DNA by hydrogen peroxide formation during the initial oxidation of Procarbazine to the azo compound. Berneis and co-workers[127] in 1963 suggested that hydrogen peroxide was responsible for DNA fragmentation into short double-helical fragments. The presence of peroxidases, catalase, or cysteamine prevented this damage to DNA in buffered solutions. It is unlikely that hydrogen peroxide production plays a role in the antineoplastic activity of Procarbazine. Hydrogen peroxide is a common metabolic product of many compounds that do not possess antineoplastic activity. Endogenous peroxidases and catalases would efficiently degrade any hydrogen peroxide formed *in vivo*. Gale and co-workers[130] reported that Procarbazine inhibited DNA synthesis in Ehrlich ascites tumor cells *in vitro* to the same extent in the presence or absence of catalase. Sartorelli and Tsunamura[129]

found no hydrogen peroxide-induced degradation of preexisting DNA in L-5178y lymphoma cells. We have shown that the azo compound exhibits the same tumor inhibitory effect as Procarbazine itself.

Another early proposal[109] was that formaldehyde, formed during the enzymatic N-demethylation of Procarbazine, could react with endogeneous succinimide and diketopiperazine to form azomethines and N-hydroxymethyl derivatives. Weitzel[149,153] suggested that these compounds are highly cytotoxic in vivo and in vitro. These compounds can affect both DNA polymerase and DNA-dependent RNA polymerase and can also act as alkylating agents. The hydroxymethyl compounds inhibit the synthesis of nucleoside triphophates and appear to act directly upon DNA. Formaldehyde itself inhibits DNA polymerase and DNA-dependent RNA polymerase and interfers with the synthesis of nucleoside triphosphates and deoxynucleoside di- and triphosphates. It is unlikely that formaldehyde plays a role in the antineoplastic activity of Procarbazine. Formaldehyde is a common metabolic product of many compounds that do not possess antineoplastic activity. Methanol, though toxic, is metabolized to formaldehyde, but is not an antineoplastic agent. Secondly, any formaldehyde formed in vivo from Procarbazine would be immediately oxidized to carbon dioxide. Baggiolini and co-workers [110] reported that the amounts of CO_2 and the major urinary metabolite N-isopropyltereph-thalamic acid formed in vivo in 24 hours were of the same order of magnitude. Thus, too little formaldehyde remains in vivo to play an important role in the antineoplastic activity of Procarbazine.

Another proposed mode of action is the formation of methyl free radical during the in vivo metabolism of Procarbazine. Dost and Reed [120] found that a significant amount of methane was produced from Procarbazine

in vivo in rats. They proposed that methane production resulted from hydrogen atoms abstracted by methyl free radicals. Although Schwartz and co-workers[147] proposed that the azo compound can serve as the intermediate which produces methyl radicals with the liberation of nitrogen gas, the proposed enzymatic hydroxylation of the benzylic carbon of the azo followed by decomposition to the aldehyde and methyldiazene free radical probably occurs under physiological conditions.[97] Methyldiazene free radical rapidly decomposes to methyl free radical and nitrogen gas. Free-radicals can abstract the allylic hydrogen of polyunsaturated fatty acids initiating autooxidation of fatty acids[185] to produce malonoaldehyde.[186] Mukai and Goldstein[187] reported that malonaldehyde can cross-link proteins or DNA. The autooxidation of polyunsaturated fatty acids can lead to production of other radicals and also to cellular necrosis. Although methyl free radical production from Procarbazine might play an important role in Procarbazine's toxicity and/or activity, several arguments cast doubt upon its role.[185] Radicals are rapidly scavenged in vivo by protective agents such as thiols (glutathione) and tocopherol. Enzymes such as superoxide dismutase, catalase, and glutathione-peroxidase keep radical and radical precursor concentrations low. Finally, radicals produced by enzyme systems rarely diffuse out of the enzyme and, if they do, they should be so stable and unreactive that they cannot react with other compounds. To overcome these protective mechanisms, a large amount of radical is required for toxicity. It is known, however, that methane and related metabolites produced through free radical mechanisms are formed in vivo in low yield.

The most widely accepted mechanism for Procarbazine antineoplastic activity is alkylation. Kreis and Yen[140] reported a rapid incorporation

of label from ^{14}C-methyl-labeled Procarbazine into P815 leukemia cells implanted into BDF_1 mice. The label was bound to DNA, RNA, phospholipids and protein. Brookes and Lawley[143] found significant amounts of labeled 7-methylguanine after administration of ^{14}C-methyl-labeled Procarbazine into Landschultz tumor-bearing mice. However, the amount of labeled 7-methylguanine corresponds to approximately 1.2×10^6 alkylated guanine moieties in a mammalian cell containing 10^3 daltons of total DNA. They concluded that this number was too low to be lethal, but could result in mutations. Kreis[140,142,144] showed that the intact ^{14}C- and ^{3}H-methyl group is transfered in this reaction. A comparison of the ratio of the label in methylated versus non-methylated purines after ^{14}C-methyl-labeled Procarbazine and sodium formate-^{14}C administration suggests that the methyl group is transfered directly rather than incorporation via the formate pool and de novo purine biosynthesis. 7-Methylguanine and other bases such as thymine, 5-methylcytosine, 1-methylguanine, and 1-methyladenine (tentative) that are not formed de novo are also labeled, although several of these bases could be methylated indirectly via the homocysteine methionine purine route. Kreis reported that the major site of radiolabel incorporation was into t-RNA. Procarbazine results in an excess of 7-methylguanine into cytoplasmic RNA, possibly at sites normally not methylated. Kreis postulated that overmethylation could affect the release of RNA from the DNA template, DNA replication, or protein synthesis.

Procarbazine is reported to be inactive in fresh preparations.[130] Reports in cell culture also indicate that the parent compound is inactive.

Hansch [14] found that Procarbazine is mutagenic in the Ames' Salmonella typhimurium mutagenicity test when phenobarbital-induced rat liver 9,000 x g. supernatant is added to activate Procarbazine. In the absence of the supernatant, Procarbazine did not exhibit mutagenic activity. It therefore appears that the cytotoxic species are generated in vitro by liver supernatant fractions. Procarbazine and the rapidly formed azo compound exhibit similar antineoplastic activity against L-1210 ascites leukemia implanted into CDF_1 mice. However, a mixture of the metabolically formed methyl- and benzylazoxy isomers is 38% more active. This strongly suggests that metabolism is crucial for the production of cytotoxic species from Procarbazine.

The studies described in this dissertation have identified a metabolic pathway by which Procarbazine may be converted to an active methylating agent. The proposed metabolic pathway leads to a methylating agent by oxidation of Procarbazine to the azo compound, further oxidation to a mixture of the azoxy isomers, and C-hydroxylation to hydroxy-azoxy intermediates. These species are chemically reactive and decompose to an alkylating agent and an aldehyde. This is in contrast to the previously proposed pathway in which Procarbazine is rapidly oxidized to the azo and the azo isomerized to the hydrazone. The hydrazone is hydrolyzed to the aldehyde and methylhydrazine. Methylhydrazine subsequently produces the alkylating species in an undetermined manner. The aldehyde is further oxidized to the major in vivo urinary metabolite, N-isopropylterephthalamic acid. These studies indicate that this metabolic pathway plays only a minor role in the fate of Procarbazine. Chemical isomerization of azo to hydrazone is very slow. Only 14% of the hydrazone is formed chemically during a 40 minute incubation in

buffer. During *in vitro* 9,000 x g. rat liver supernatant incubation, only 11% of the hydrazone was formed and only 0.7% of N-isopropyl-4-hydroxymethylbenzamide and 3% of the acid that are formed from the aldehyde during hydrazone hydrolysis were present after 40 minutes.

The *in vitro* metabolism of Procarbazine rat liver supernatant and microsomal preparations proceeds with the initial rapid oxidation of Procarbazine to the azo compound. This could occur by either direct dehydrogenation to form hydrogen peroxide [1] or by a cytochrome P-450 dependent N-hydroxylation followed by dehydration. Baggiolini and co-workers [97] reported that this initial oxidation proceeds at about 28 umoles/hour/100 grams of body weight in isolated perfused rat liver. This initial oxidation can occur both chemically and enzymatically. The half-life of Procarbazine in the 9,000 x g. rat liver supernatant incubation is approximately 7 minutes as compared to 12 minutes in buffer solution. Baggiolini, *et al.*[97] found that removal of the liver from the perfusion system decreased this oxidation to 2-3% of the perfused rate and that azo production corresponds to the Procarbazine decrease. Prough characterized this initial oxidation reaction. The enzymatic catalysis required NADPH, although its absence decreased the oxidation rate by only 23%. Phenobarbital-pretreatment resulted in a 2.7-fold increase. This suggests that cytochrome P-450 plays a role in the oxidation. 3-Methylcholanthrene did not affect the rate. Inhibitors of cytochrome P-450-dependent reactions such as carbon monoxide, metyrapone, or specific antiboides to microsomal NADPH-cytochrome C reductase inhibited the oxidation, while the cytochrome P-450-independent amine oxidase inhibitor methimazole did not. This work in 9,000 x g. rat liver supernatant has shown that the azo compound in stereoselectively

metabolized to isomeric methyl- and benzylazoxy compounds. The methyl-azoxy represents 73% of the azoxy mixture after 40 minutes and is present at a 9-fold higher concentration after 10 minutes. The further metabolism of the azoxy isomers is also stereoselective. The benzylazoxy isomer is metabolized 12% faster than the methylazoxy isomer. The enzymes necessary for the production of the azoxy isomers are membrane-bound enzymes present in both the rat liver 9,000 x g. supernatant and 100,000 x g. microsomal fractions. The metabolism also requires NADPH.

These proposed Procarbazine in vitro and in vivo metabolic pathways are analogous to the pathway for the metabolism of 1,2-dimethylhydrazine proposed by Fiala and co-workers.[188] A comparison of both pathways is shown in Figure 60 . Both Procarbazine and 1,2-dimethylhydrazine are carcinogenic, as well, as carcinostatic. Dimethylhydrazine is rapidly oxidized to azomethane. Azomethane is further metabolized to azoxy methane. Azoxy methane undergoes subsequent hydroxylation to form methyl azoxymethanol, which can eliminate formaldehyde to form methl-diazohydroxide or methyldiazonium cation. Methyl azoxymethanol is a known methylating agent which has been shown to transfer its intact methyl group. Analogously, Procarbazine is rapidly oxidized to the azo compound. The azo compound is metabolized to methyl- and benzylazoxy isomers. The methylazoxy isomer is a structural analog to azoxy methane. Chemical ionization mass spectrometry and reverse-phase high-performance liquid chromatography provide preliminary evidence that the methyl- and benzylazoxy compounds are hydroxylated on neighboring carbons. The hydroxylated methylazoxy compound is structurally related to methylazoxy methanol, which is know to transfer its intact methyl group. Procarbazine

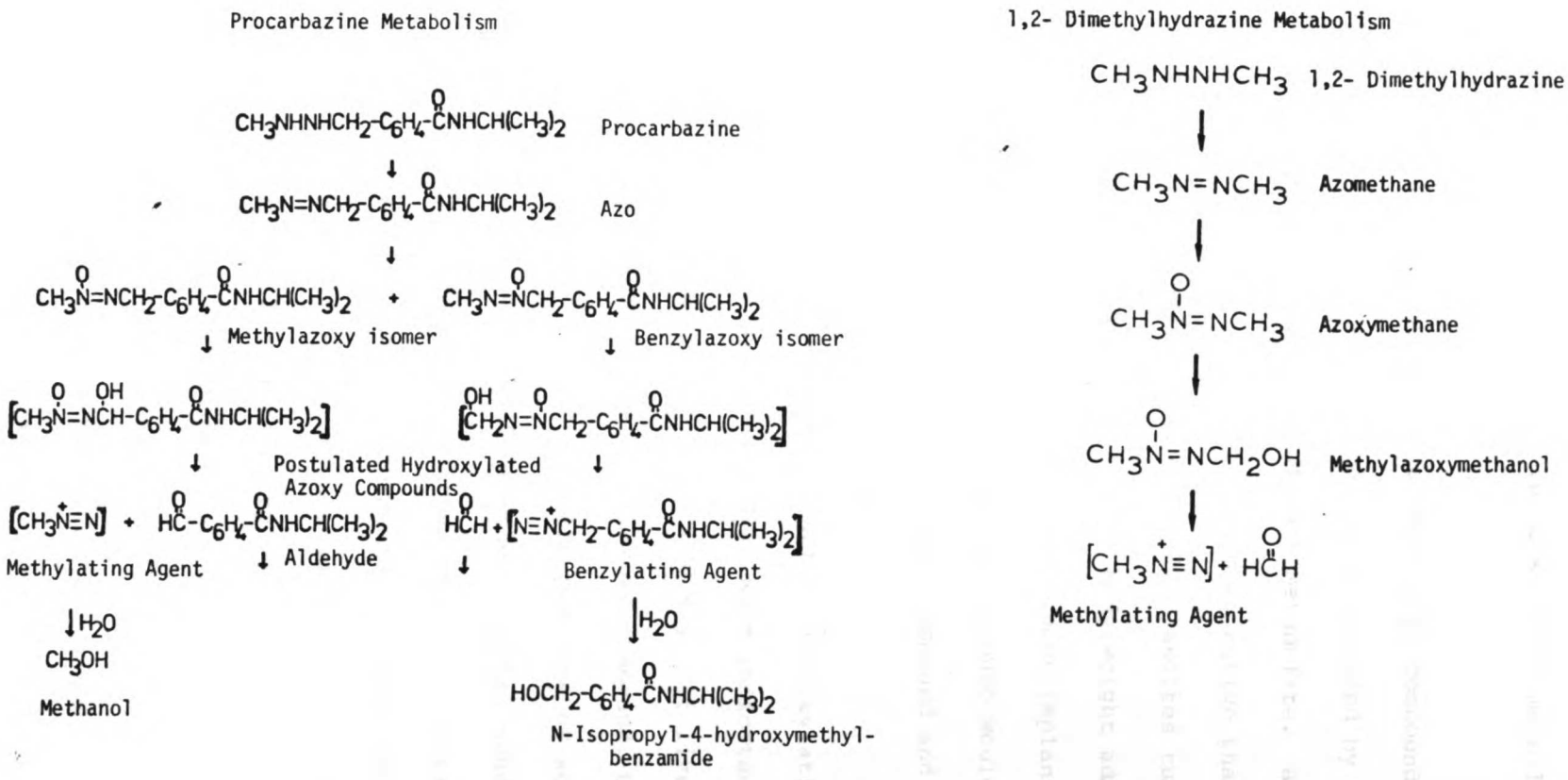


Figure 60. Comparison of the Metabolic Pathways For the Production of Potential Alkylating Agents From Procarbazine and 1,2-Dimethylhydrazine.

has likewise been shown to transfer its intact methyl group and, possibly, benzyl group.

Support for the importance of the azoxy compounds in the production of cytotoxic agents from Procarbazine is provided by the comparison of the antineoplastic activity of each metabolite. A 64%:36% mixture of methyl-:benzylazoxy isomers is 38% more active than Procarbazine against intraperitoneally-implanted L-1210 ascites tumors in CDF_1 mice at a dose of 0.8 millimoles/day/kg. of body weight administered intraperitoneally for 3 consecutive days post-tumor implantation. If only one of the isomers is active, the azoxy compound would give the same T/C but at a 36-64% lower dose. The azo compound and Procarbazine had almost identical neoplastic activity.

Procarbazine has been shown to act as an alkylating agent through the transfer of its methyl group. This work indicates that it may also act as a benzylating agent. The 100,000 x g. rat liver microsomal incubation of an analog of the methylazoxy compound, benzyl-NNO-azoxymethane, produced benzyl alcohol in the presence of semicarbazide, which inhibits any residual NAD-dependent alcohol dehydrogenase activity in microsomal preparations. Procarbazine may act through the formation of multiple active alkylating intermediates. Each intermediate is expected to have a distinct cytotoxic and toxic activity.

Bioactivation of Procarbazine by enzymes of the cytochrome P-450 mixed-function oxidase system described in this work suggested that drug interactions may have an effect upon the effective chemotherapeutic use of Procarbazine. The anticonvulsants, phenobarbital, and diphenylhydantoin, that are commonly administered to brain tumor patients, induced microsomal cytochrome P-450 content and have been shown to increase the

antineoplastic activity of Procarbazine. Phenobarbital has also been shown to increase the disappearance of Procarbazine from plasma in rat, the appearance of metabolites, and their turnover rates. Increased metabolic turnover is consistent with a more rapid formation of active metabolites and a consequent increase in antitumor activity. If similar results occur in man, present Procarbazine chemotherapeutic protocols will require alteration. The chronic or concomitant administration of these microsomal cytochrome P-450 inducers could alter the levels of Procarbazine necessary for clinical therapeutic effectiveness.

Procarbazine is given clinically to brain tumor patients either as a single agent at a dose of 150 mg./m^2 for 28 consecutive days with a 28 day break before repeating the protocol or in combination with 1-(2-chloroethyl)-3-cyclohexyl-1-nitrosourea (CCNU) at a dose of 60-100 mg./m^2/day for 14 days. A preliminary study in L-1210-bearing CDF_1 mice, given 15, 30, or 60 mg./kg. Procarbazine for 7-14 days prior to 3 consecutive doses of Procarbazine 24 hours after tumor implantation, showed a significant decrease in the cytochrome P-450 content in several of the pretreated groups. Procarbazine is known to decrease the activity of several enzyme systems[179,180,189] including those catalyzing the <u>in vitro</u> hepatic microsomal drug metabolism. This preliminary finding suggests that the common clinical protocols for administering Procarbazine daily for 14 or 28 consecutive days may progressively alter the metabolic activation of the agent.

The preliminary animal studies suggest that significant drug interactions may be expected. However, further studies are necessary to define the magnitude and pharmacological significance of these interactions.

Despite the major contributions of the research described in this dissertation, several future experiments might add much to the present knowledge. Each isomer of the azoxy mixture should be synthesized, its antineoplastic activity evaluated, and its further metabolism studied. The human metabolism should be further studied and the analytical methods refined so that the urgently needed routine clinical assay can be developed. The production of a benzylating agent from Procarbazine requires further verification. ^{14}C-ring labeled Procarbazine can be used to study covalent binding by the benzylating agent. The production of free radicals during Procarbazine metabolism remains to be elucidated and its role evaluated. The effects of Procarbazine and other drugs upon Procarbazine antineoplastic activity requires further study before changes in clinical protocols are made. This might require a more sensitive assay for activity than the L-1210 ascites in CDF_1 mice assay. A feasible assay could utilize the in vitro incubation of 9,000 x g. supernatant fraction from pretreated animals, Procarbazine, and tumor cells followed by implantation of these cells into animals or the much more sensitive assay using cell culturing techniques to evaluate changes in the ability of the treated cells to form viable colonies in vitro. Finally, the ability of tumors to activate either Procarbazine or one of its metabolic or chemical degradation products remains unstudied. These studies would provide information as to which of the metabolic steps occur systematically, which within the tumor itself, and which of the compounds serves as the transport form of Procarbazine.

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