THE BIOACTIVATION OF SLAFRAMINE

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ABSTRACT

THE BIOACTIVATION OF SLAFRAMINE

By

Thomas E. Spike

Bioactivation of slaframine by rat liver microsomes has been demonstrated by its ability to stimulate contraction of the guinea pig ileum. The enzymatic bioactivation process has been shown to require reduced nicotinamide dinucleotide phosphate (NADPH) but not oxygen and is induced by pretreating the animals with phenobarbital. The production of the active factor has also been accomplished nonenzymatically with various flavins in the presence of light. The active factor causes a prolonged contraction of the ileum by acting directly on the acetylcholine receptor with an apparent high affinity for the receptor. Its action is prevented by prior application of atropine, but is not reversed by atropine once administered.

The metabolite has not been identified but its properties have been studied. Even though EPR has not shown the presence of any free radicals, it is believed that the active metabolite is either an N-oxide or loss of one electron by the tertiary amine, both of which would give the nitrogen a positive charge. The active compound has been shown to be present at very low levels and is quite unstable as heat and

pH changes. The profound effects that are observed in animals treated with the active compound are described. In addition, some of the structural requirements for the observed response have been determined.

THE BIOACTIVATION OF SLAFRAMINE

By

Thomas E. Spike

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LIST OF ABBREVIATIONS

NADP = nicotinamide dinucleotide phosphate

NADPH = nicotinamide dinucleotide phosphate, reduced

NAD = nicotinamide dinucleotide

NADH = nicotinamide dinucleotide, reduced

FAD = flavin adenine dinucleotide

FMN = flavin mononucleotide

THF = tetrahydrofolic acid

GLC = gas-liquid chromatography

TLC = thin-layer chromatography

DPEA = 2,4-dichloro (6-phenylphenoxy) ethylamine

QD = optical density

i.p. = intraperitoneally

i.v. = intraveineously

INTRODUCTION AND REVIEW OF LITERATURE

During the early 1950's, there were a number of reports throughout the Midwest of excessive salivation by cattle which had been fed certain legumes (1). Cattle eating these forages would refuse further feed after one to three feedings. This presented a significant economic problem due to the decrease in production as well as the cost of replacing the forages. In 1958, when the reports reaching the Agricultural Experiment Station in Illinois became so numerous, Byers and Broquist initiated a study of these "slobber forages" (2, 3). The most severe outbreak of this problem was reported in 1959 in Missouri where animals displayed additional symptoms including diarrhea, bloat, stiff joints and, occasionally, death (4).

Smalley *et al.* (5) and Crump *et al.* (6) observed that the forages were usually red clover and, although they did not visibly display any mold, proved upon microscopic examination to be infested with the heavy mycelium of a dark brown-colored fungus. They, furthermore, showed the fungus to be *Rhizoctonia leguminicola*.

Aust *et al.* (7) were able to grow pure cultures of *Rhizoctonia leguminicola* on cold water extracts of red clover

hay and showed the production of the salivation factor by the fungus. Salivation factor activity was assayed by injecting extracts of the mature mycelium into guinea pigs via intraperitoneal injections and following the degree of salivation which ensued. They were successful in crystallizing the picrate salt of this factor from ethanol and named it "slaframine." The structure and absolute stereochemistry were determined from information obtained by nuclear magnetic resonance, infrared, and mass spectroscopy. The alkaloid was found to be 1-acetoxy-6-aminoocta-hydroindolizine (8, 9).

The biosynthesis of slaframine by Rhizoctonia leguminicola was studied with various ^{14}C-precursors (7). Only lysine and serine were found to be incorporated. Portions of the biosynthetic pathway have since been determined (10).

It has been suggested that cystic fibrosis syndrome may be caused by nonfunctioning of cholinergic nerve fibers, since the glandular sites involved are predominately supplied by fibers coming from the parasympathetic nervous system. Clinical symptoms of cystic fibrosis are malfunction of mucous-secreting membranes resulting in irritation followed by a toughening of these membranes to a fibrous tissue. Sufferers of the disease lack sufficient pancreatic enzymes to utilize dietary protein and, thus, suffer from malnutrition. The exocrine glands are stimulated by cholinergic drugs, and the use of these compounds has, therefore, been studied with regard to therapy. However, the side effects of cholinergic

drugs makes their use almost impossible. The specificity with which slaframine stimulates exocrine gland function without affecting other vital body functions, such as heart rate (11), eliminates the problems associated with the use of other parasympathomimetic agents for the treatment of cystic fibrosis. The administration of slaframine to animals results in sustained secretory activity by exocrine glands (12). For example, studies on the pancreas following administration of slaframine have shown that slaframine increases the activity of the digestive enzymes and maintains a high level of secretion for prolonged periods of time (11).

In all studies with the compound in vivo, there was invariably a substantial delay before the onset of salivation, and no activity was seen with in vitro test systems (12). This suggested that the compound might have to be metabolized to an active form (12). Evidence that the liver was the site of activation of slaframine was obtained by injecting the compound directly into the portal vein which resulted in a faster response than when it was given into the inferior vena cava (13). Furthermore, when slaframine was given into the inferior vena cava, all activity could be prevented by first ligating the portal vein (13). Evidence that the bioactivation process was being accomplished by the drug-metabolizing enzymes of the liver was obtained by pretreating animals with compounds which are known to induce drug-metabolizing activity in liver microsomes and, thus, decrease the lag time.

Likewise, the delay could be increased by giving known inhibitors of drug-metabolizing systems. Goats, which are excellent metabolizers of xenobiotics, have a much shorter period of delay prior to the onset of salivation than does a calf which has much less ability to alter drugs (12, 13).

The biotransformation of most drugs occurs mainly in the liver, but may also take place in plasma, kidney, and other tissues. Drugs are eliminated from the body either unchanged or as metabolites. Generally, the more polar compounds are excreted unchanged. The less polar, lipid-soluble compounds must be transformed before elimination can take place. There are two main types of transformations accomplished in the liver, nonsynthetic and synthetic. Nonsynthetic reactions include oxidations, reductions, or hydrolysis and may result in activation, change in activity, or inactivation of the parent drug. Synthetic reactions involve conjugations between the drug or its metabolite and an endogenous substrate that is usually a carbohydrate, an amino acid, or derivatives of these. Synthetic reactions almost invariably result in inactivation and excretion of the parent drug.

These transformations have been shown to be carried out by certain enzymes which are associated with the endoplasmic reticulum of a number of tissues particularly the liver (14). This enzyme system has been classified as a mixed-function oxidase system according to the terminology of Mason (15) because of its requirement for NADPH and molecular oxygen.

The highest level of enzymic activity is found in the lipoprotein membrane fragments, particularly those of the smooth endoplasmic reticulum (16). Synthesis of the enzymes appears to occur in rough reticulum, but this, when saturated with enzyme, appears to lose its ribosomes to become smooth reticulum (17). The amount of enzymes present can be increased by chronic administration of various compounds. Recent studies have shown that benzpyrene hydroxylase activity is increased by phenothiazines and polycyclic hydrocarbons in organ and tissue cultures, indicating the lack of hormonal control of the induction process (18, 19). Many foreign compounds stimulate their own metabolism of other drugs. Among these are phenylbutazone, chlorcyclizine, probenecid, tolbutamide, hexobarbital, pentobarbital, phenobarbital, aminopyrine, meprobamate, glutethimide, chlorpromazine, chlordiazepoxide, DDT, methoxyflurane, 3,4-benzpyrene and 9,10-dimethyl-1,2-benzanthracene (20). Many chemicals in our environment also stimulate the metabolism of drugs and other foreign substances. These include insecticides, cigarette smoke, and some polycyclic hydrocarbons found in polluted city air. Treatment of animals with such inducing agents increases the apparent concentration of the cytochrome P-450, the oxygen-activating component of the mixed-function oxidases (21, 22).

Just as the mixed-function oxidase system can be induced by various compounds it can also be inhibited. Inhibition can occur either competitively or noncompetitively. The microsomal

enzyme system is rather nonspecific, and frequently one drug will competitively inhibit the metabolism of another which bears no structural resemblance to the inhibitor (23). This is difficult to understand in view of the common concept of substrate specificity found in other enzyme systems. Many drugs which are effective inhibitors in in vitro studies fail to have an inhibitory effect in vivo (24). Conceivably, the failure of these drugs to inhibit in vivo could be due to failure of these drugs to reach effective concentrations at the metabolic site (24). Support to this idea has been obtained by using an isolated liver perfusion system. Ethylmorphine, codeine, morphine, and diphenylphenoxyvalerate (SKF-525A) will inhibit the metabolism of hexobarbital in the perfused liver, whereas only the first two of these are effective in vivo inhibitors of its metabolism (24).

Some compounds have been shown to preferentially inhibit the metabolism of certain drugs while having little or no effect on the metabolism of others (25). An example of this is 2,4-dichloro(6-phenylphenoxy)ethylamine (DPEA). Various steroids have also been shown to be alternative substrates for a common microsomal mixed-function oxidase and, thus, competitively inhibit the alteration of certain drugs such as ethylmorphine and hexobarbital (26). They have also been shown to be less potent inhibitors of chlorpromazine oxidation and inhibition was not competitive (27). Carbon monoxide, which binds to cytochrome P-450 with

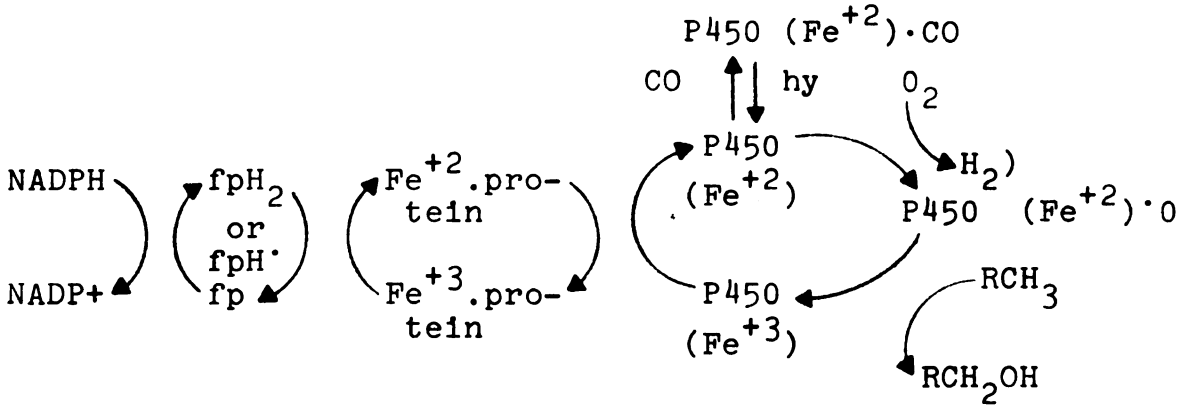
a greater affinity than molecular oxygen will inhibit, non-competitively, the metabolism of those drugs which use P-450 in their terminal oxidation step (28).

The hepatic mixed-function oxidase system has proved too labile for solubilization or separation of its components, but a hemoprotein known as the CO-binding pigment or cytochrome P-450 (32) has been shown to be involved in the terminal oxidation step of the metabolism of drugs. P-450 was discovered by Klingenberg (29) and Garfinkel (30) and was partially characterized by Omura and Sato (31).

In its reduced form, cytochrome P-450 has an affinity for carbon monoxide, although the normal ligand is oxygen (14, 32). P-450 is found in the microsomes of liver and the mitochondria of adrenal cortex, but brain and skeletal muscle are devoid of this cytochrome (17). When foreign compounds are added to hepatic microsomes, certain spectral changes occur, indicating that the compounds have interacted with a microsomal pigment, probably cytochrome P-450. Two types of spectral change occur, one exemplified by that induced by phenobarbital, aminopyrine, or the inhibitor, SKF-525A and the other seen with such substrates as aniline or the inhibitor, DPEA (33). This suggests that there are two different cytochromes with different substrate specificities and is in agreement with an observation that pretreatment of rats with methylcholanthrene produces a hepatic microsomal cytochrome

(P_1-450) which appears to contain only one of two components of the normal cytochrome (P-450) (35).

A similar system, the steroid hydroxylating system of beef adrenal cortex mitochondria, has been solubilized and separated into a flavoprotein NADPH-diaphorase, a non-heme iron protein, and cytochrome P-450 (33). The electron transport system involved in the liver microsomal system has consequently been formulated (on the basis of what is known about the adrenal system) as follows (34):



fp = flavoprotein

The second area of interest in the bioactivation of slaframine is its site of action, the cholinergic nervous system. The action of cholinergic, or parasympathomimetic, drugs is to augment or duplicate the effects of stimulating a parasympathetic nerve. This class of drugs consists of those agents which directly stimulate effector cells, such as pilocarpine, arecoline, muscarine, and certain choline esters, and those which inhibit acetylcholinesterase and

thus permit the acetylcholine released to persist in its action (eserine, neostigmine, and many others).

The relationship between chemical structure and biological activity is particularly fascinating with regard to cholinergic drugs. The chemical grouping which is common to drugs having direct effect on cholinergic receptors consists of a nitrogen atom to which three or four methyl groups are attached. As in the case of ammonium or quaternary ammonium ions, such a chemical grouping carries a net positive charge. Because phosphorus and arsenic have nuclear properties which are similar to nitrogen, the tetramethylphosphonium and tetramethylarsonium ions also have cholinergic properties.

Choline is the simplest methonium compound which occurs naturally and is a member of the B vitamin family. It can act at cholinergic sites but very large, unphysiological quantities are required. The acetate ester of choline is the normal chemical mediator of cholinergic nerves. It has been estimated that this substance is effective at cholinergic receptors at concentrations as low as 10^{-9} molar. This makes it one of the most potent physiological substances known.

Acetylcholine is rapidly hydrolyzed by acetylcholinesterase. The physiological role of acetylcholinesterase is to terminate the transmitter action of acetylcholine at the junctions of various cholinergic nerve endings with their effector organs. Drugs that inhibit or inactivate acetylcholinesterase are called "anticholinesterases." They cause

acetylcholine to accumulate at cholinergic sites and, thus, are capable of producing effects equivalent to continuous stimulation of cholinergic fibers.

Physostigmine, also called "eserine" (an alkaloid obtained from the seed of Physostigma venenosum) is perhaps the most common anticholinesterase. Its pharmacoligical properties were investigated by Christionson (36), Fraser (37), and Argyll-Robertson (38). The elucidation of the chemical basis of the activity of eserine was accomplished by Stedman (39). Binding studies have shown that even though eserine binds to the active site of acetylcholinesterase reversibly, only a negligible amount of the inhibitor is released from the enzyme due to the extremely slow hydrolysis of its ester moiety by the cholinesterase (40).

Atropine is a highly selective antagonist of cholinergic agents on smooth and cardiac muscle and exocrine gland cells. This antagonism is so selective for cholinergic agents that atropine blockade of the action of other types of drugs has been taken as evidence that they act indirectly through cholinergic mechanisms (40).

Atropine, which binds reversibly with specific acetylcholine receptors, has a much higher affinity for the receptor molecule than acetylcholine. Thus, the effects of acetylcholine can readily be reversed by atropine (40).

Studies on slaframine thus far have indicated its potential value as both a medicinal and research tool. The

bioactivation of such an unusual compound is intriguing in itself, but the understanding of the complete mechanism by which it acts so selectively is the ultimate goal. The comprehension of the mode of action necessitates knowing what the active species is and how to handle it. It was therefore the intent of this thesis to isolate and identify the active form of slaframine and to determine some of its chemical properties. Localization and characterization of the enzyme system responsible for the bioactivation process were also intended.

METHODS

Chemicals

Aminopyrine and hexobarbital were purchased from K and K Laboratories, Inc., Plainview, N. Y. Phenobarbital was purchased from Merck and Co., Inc., Rahway, N. J. NADPH, $NADP^+$, FAD, FMN, riboflavin, NADH, NAD^+, tetrahydrofolic acid, biopterin, and folic acid were all purchased from Sigma Co., St. Louis, Mo. Tritiated acetic anhydride was purchased from Amersham/Searle Corporation, Des Plaines, Illinois. Carbon monoxide was obtained from Matheson Co., Inc., Joliet, Illinois. Acetylcholine iodide, eserine sulfate, pilocarpine HCl, atropine sulfate (monohydrate) were all purchased from Cal Bio Chem, Los Angeles, California. Slaframine was isolated from Rhizoctoria leguminicola in our laboratory by the method of Aust, et al.(7). Slaframine was repeatedly recrystallized as the dipicrate before converting to the dicitrate for use. Conversion to the dicitrate was accomplished by dissolving the dipicrate in .01 N HCl and extracting the picric acid out with diethyl ether. The pH was then raised to 10 and the solution was extracted with two volumes of purified chloroform. The chloroform extract was dried over sodium sulfate and removed under vacuo. The residue was

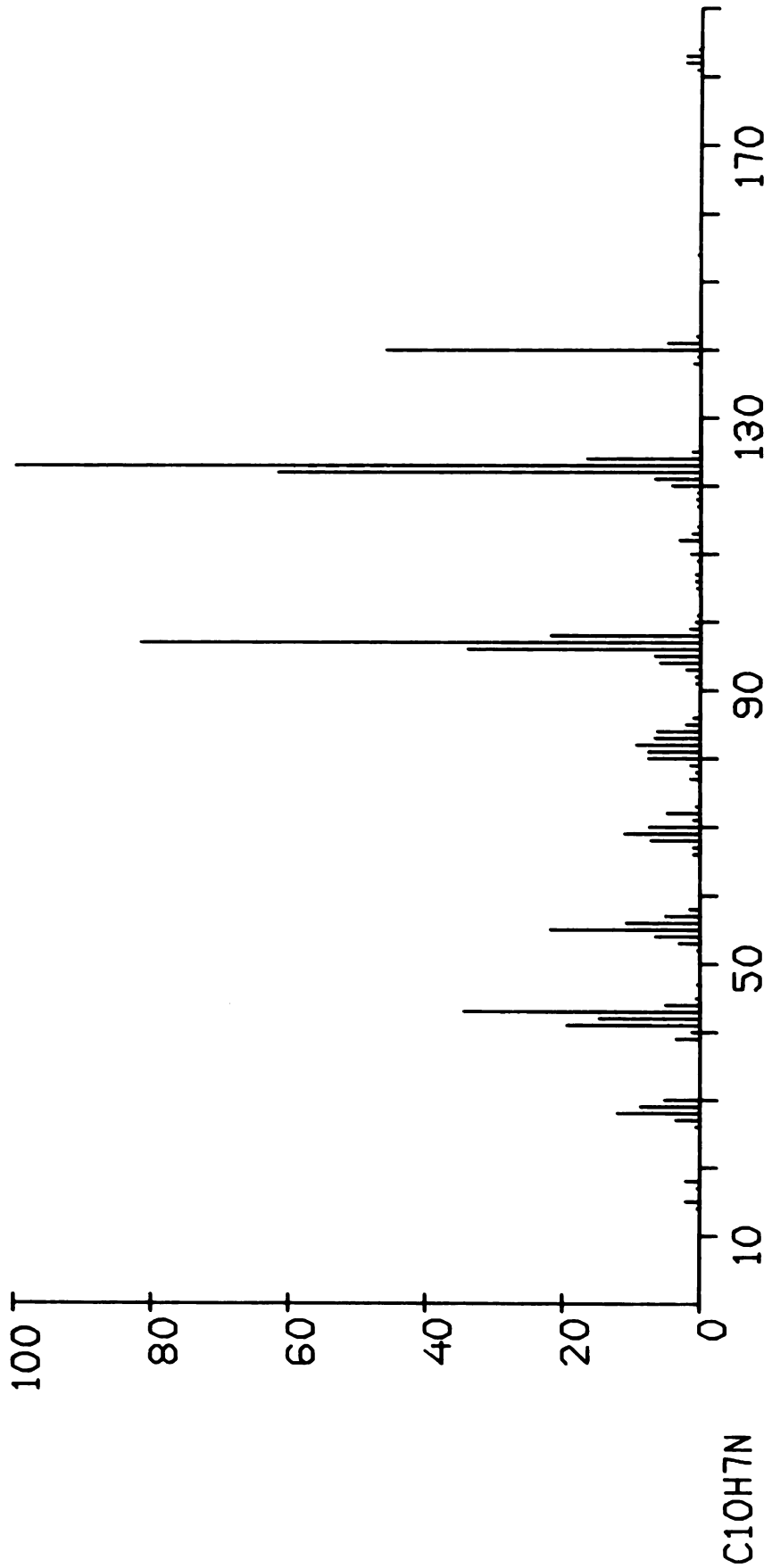
dissolved in a minimum amount of dry diethyl ether and added to a saturated solution of citric acid in diethyl ether. The precipitate was washed with diethyl ether and dried under vacuo. 8-Aminooctahydroindolizine and 1-hydroxyoctatydroindolizine were compliments of Robert Gardener, Department of Chemistry, University of Illinois, Urbana, Illinois.

1-Acetoxyoctahydroindolizine was synthesized by refluxing 50 mg of 1-hydroxyoctahydroindolizine in 10 ml of acetic anhydride and 10 µl of pyridine for one hour. The reaction mixture was then mixed with 50 ml of 0.1 N HCl and extracted with an equal volume of chloroform. The pH was then raised to 10 with sodium carbonate and the solution was extracted twice with equal volumes of chloroform. The chloroform was dried and removed under vacuo. Gas chromatography of the product showed one peak and the structure of 1-acetoxyoctahydroindolizine was confirmed by mass spectrometry (Figure 1).

N-Acetylslaframine was synthesized by the following method. The crude pH 10 chloroform extract from the isolation of slaframine was dried over sodium sulfate and used as the source of slaframine. An excess amount of acetic anhydride was added, and the solution was refluxed for ten minutes. The unreacted acetic anhydride was removed by distillation under vacuum. Pure N-acetylslaframine was obtained by sublimation of the residue.

N-Acetyl-O-deacetyl slaframine was synthesized by heating a solution of N-acetylslaframine in 2 N NaOH in a boiling water

Figure 1.--Mass Spectrum of 1-acetoxy-octahydro-indolizine. Mass Spectrum obtained on an LKB 9000 combination Gas Chromatograph mass Spectrometer of 1-acetoxyoctahydroindolizine dissolved in chloroform. The column was packed with 2% OV-1 and the column temperature was 150°. The ion source temperature was 290°, the filament current was 60 μamps, the electron energy was 70 eV, and the accelerating voltage was 3500 volts.


100
80
60
40
20
0
10
50
90
130
170
C10H7N

bath for ten minutes and extracting the product with chloroform. Deacetylslaframine was prepared by the same method used to prepare N-acetyl-O-deacetylslaframine.

Gas-liquid Chromatography

The majority of gas chromatography was done on a Barber Colman Model 5000 Gas Chromatograph. Columns were six feet long and had an inside diameter of 5mm. Column packings used were OV-1 and OV-17 both 3%, pretested, and on a solid support of chromsorb Q (100/120 mesh). Column temperatures used ranged between 150° and 185°. At 185°, slaframine had a retention time of 1.65 minutes. N-Acetylslaframine, N-acetyl-O-deacetylsalframine, and deacetylslaframine had retention times of 9.8 minutes, 6.3 minutes and 1.1 minutes, respectively. Slaframine had a retention time of one minute on an OV-1 volumn at a column temperature of 175°.

Thin-Layer Chromatography

All thin-layer chromatography was done on precoated Silica Gel F_{254} plates obtained from Brinkmann Instruments, Inc., Westbury, L. I., N. Y. Samples were applied to the plates in the basic form dissolved in chloroform. A list of the various solvent systems and the observed R_F values are shown in Table 1.

TABLE 1.--Thin Layer Chromatography of Slaframine and Its Derivatives.

Solvent System					R_F*			
C	M	P	17%A	58%A	Slaframine	deacetyl-Slaframine	N-acetyl-Slaframine	N-acetyl-O-deacetyl-Slaframine
1	1				.15	.04		
8	8		4		.86	.92		
10	7		4		.85	.52		
10	7		3		.85	.54		
7		6		0.05	.13	.04	.59	.42
10	10		1		.9			
6	10			1	.72			
7	10		3		.68			
6	10		3		.71			
6	12		3		.68			

C - chloroform
M - methanol
P - n-propanol
A - ammonium hydroxide

*Spots were developed with Dragendorff's reagent or Ninhydrin.

Preparation of Slaframine-^{3}H Acetate

Slaframine Free Base

A mixture of 310 mg of slaframine dipicrate, four ml 6 N hydrochloric acid and 16 ml water was stirred at room temperature for two hours. The aqueous mixture was extracted with diethyl ether until the ether extracts were colorless. The pH was adjusted to 10 with 10% sodium hydroxide and the alkaline solution was extracted with three 20 ml portions of chloroform. The chloroform extracts were combined, dried, and evaporated to yield a residue which was used directly in the next step.

N-Carbobenzoxy-Slaframine

A mixture of slaframine free base (from 200 mg of slaframine dipicrate), carbobenzoxy chloride (60 mg), and sodium carbonate (50 mg), in 20 ml water was prepared and stirred at room temperature for three hours. A pH of 9 was maintained by the addition of 2 M sodium carbonate. The aqueous mixture was made acidic with 1 N hydrochloric acid and then extracted with two 20 ml portions of benzene. The aqueous layer was made basic with 10% sodium hydroxide and extracted with five 20 ml portions of chloroform. The combined, dried chloroform extracts were evaporated to yield 81.6 mg of N-Carbobenzoxyslaframine as a viscous oil.

N-Carbobenzoxy-O-Deacetylslaframine

A mixture of 81 mg of N-Carbobenzoxyslaframine and 60 mg potassium carbonate in 10 ml of methanol was stirred at room temperature for seven hours. The methanol was removed under vacuum and the residue treated with 15 ml of chloroform. A small amount of sodium sulfate was added to the chloroform solution and the inorganic salts were removed by filtration. The chloroform was evaporated to yield 46 mg of a white solid, which could be recrystallized from ether to give needles. M.P. 156 - 157°.

Acetylation of Carbobenzoxy-O-deacetylslaframine

The acetylation process was carried out in vacuo (P<1μ) in the laboratory of Dr. R. Neistrom at the University of Illinois. Twenty-nine mg of the alcohol was placed in a break seal vial and connected to the vacuum line. Twenty-five millicuries of tritiated acetic anhydride (specific activity 4000 mc/m mole) in a break seal vial was connected to the vacuum line.

The tritiated acetic anhydride was transferred to the carbobenzoxy-O-deacetylslaframine which had been cooled to 77° K with liquid nitrogen.

The vial which contained the radioactive acetic anhydride was removed and a second container, the contents of which was 20 μl of acetic anhydride and two μl pyridine, was connected to the vacuum line. The contents of this

vessel were transferred to the 77° K vial containing the alcohol and tritiated acetic anhydride. This reaction vessel was sealed, removed from the vacuum line, and allowed to react for 24 hours.

The break vial was broken and the acetic acid was removed by vacuum using a potassium hydroxide trap. Benzene (one ml) was added and the reaction mixture was lyophilized to dryness. The residue was dissolved in 10 ml water made alkaline (pH 10) with sodium carbonate and extracted with five 20 ml portions of chloroform. The combined, dried chloroform extracts were evaporated until all chloroform had been removed.

Removal of N-Carbobenzoxy group from N-Carbobenzoxyslaframine

A mixture of N-Carbobenzoxyslaframine and hydrogen bromide in glacial acetic acid were stirred at room temperature for one hour. The mixture was poured into ice water and the pH was adjusted to 10 with solid sodium carbonate. The alkaline solution was extracted with two 20 ml portions of chloroform. The compound was then converted to the dipicrate and recrystallized to constant specific activity before converting to the dicitrate for use. The resulting labeled compound had a specific activity of 350 mC/m mole. Thin layer chromatography employing two different solvent systems revealed only one peak upon counting 5mm sections which had been scraped from the plate (Figures 2 and 3). The silica

Figure 2.--TLC of ^{3}H-(acetate)-slaframine. The slaframine was spotted on a TLC plate and developed in a propanol:chloroform:ammonium hydroxide (6:7:0.05) solvent system. The plate was scraped in 5mm sections and counted in a scintillation counter.

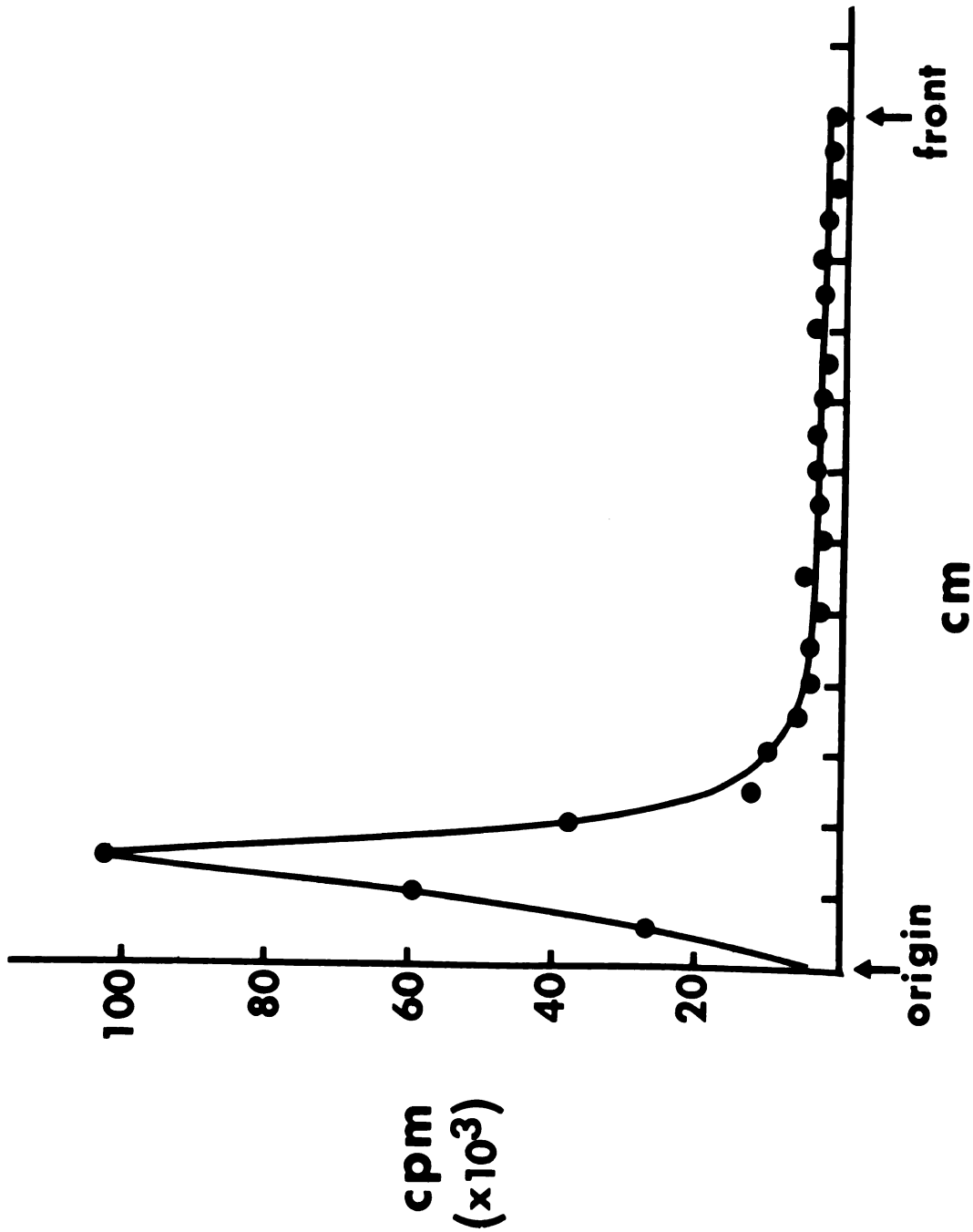
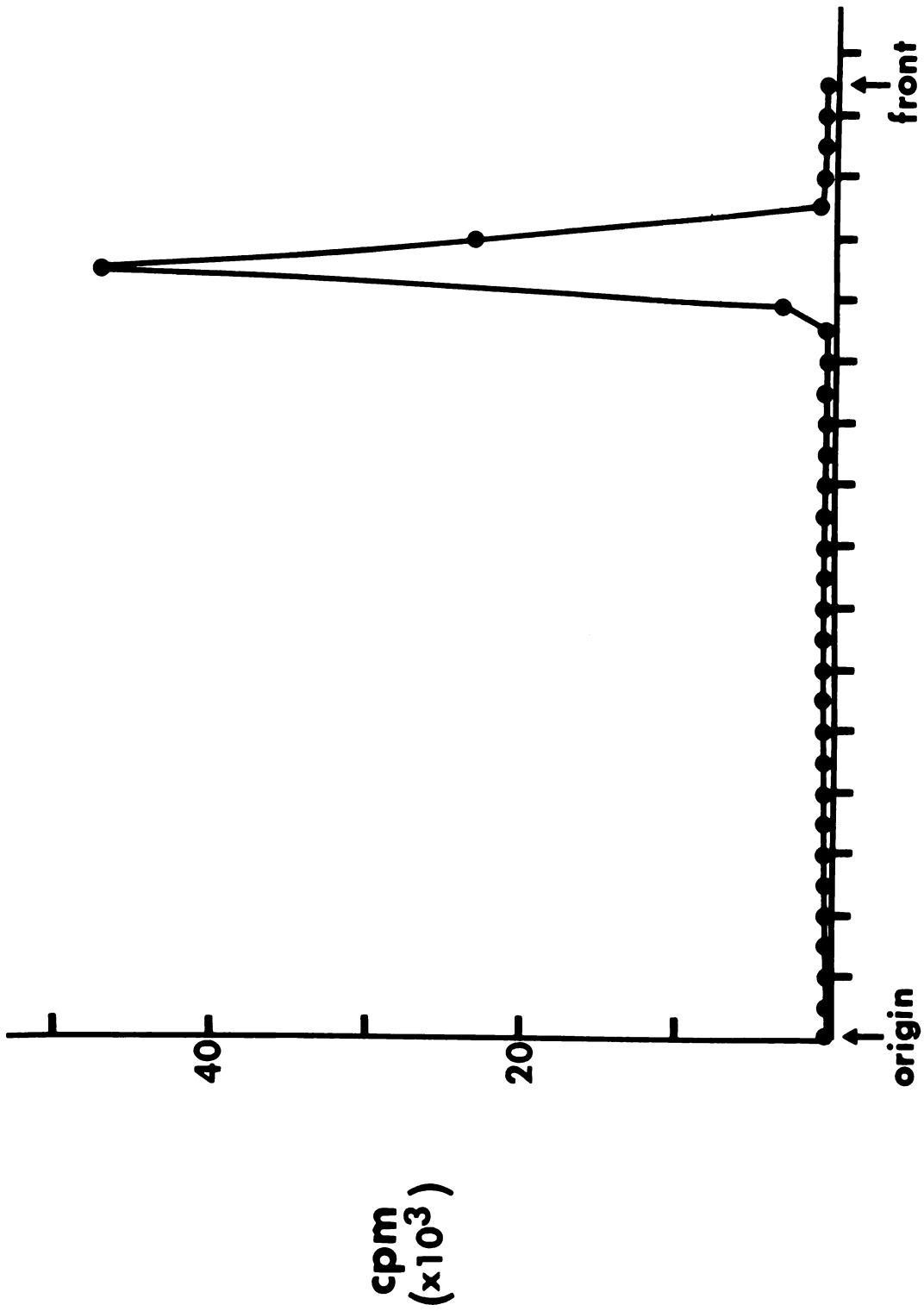

cpm
(x10³)
100
80
60
40
20
origin
front
cm

Figure 3.--TLC of ^{3}H-(acetate)-Slaframine. The slaframine was spotted on a TLC plate and developed in a chloroform: methanol (1:1) solvent system. The plate was scraped in 5mm sections and counted in a scintillation counter.


cpm
(x10³)
40
20
origin
front
cm

gel was suspended in 15 ml of scintillator containing 4% w/w Cab-o-sil in toluene, 9.5% PPO, and 0.03% POPOP[2] and counted in a scintillation counter.

Animals

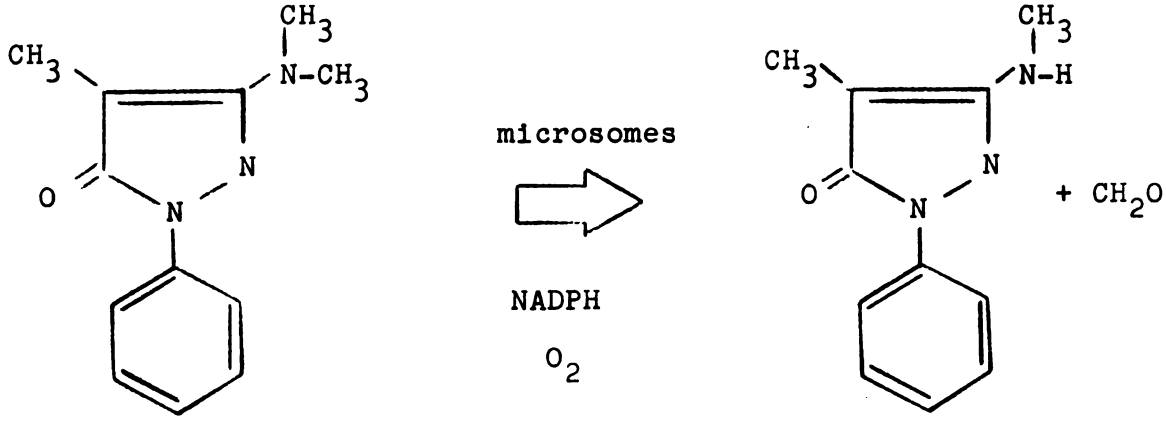
All rats used were of the Holtzman strain and purchased from Spartan Research Animals, Inc., Haslett, Michigan. Animals used as a source of microsomes were male rats weighing 300-400 grams. Phenobarbital induction was accomplished by including 0.1% phenobarbital in the drinking water; the pH adjusted to 7 with sodium hydroxide. Animals used to obtain livers for the liver perfusion experiments were 500-600 g rats of either sex induced with phenobarbital. Blood donors for the liver perfusion experiments were 500-600 grams rats of either sex and blood was taken at two week intervals. Blood was taken by heart puncture after ether anesthesia.

Preparation of Microsomes

The animals were exsanguished and the livers perfused with 10 ml of ice cold 1.15% KCl via the portal vein. The livers were removed and placed in 1.15% KCl on ice. The tissue was blotted dry with filter paper, weighed, and minced by chopping with a scissors. The minced tissue was homogenized in four volumes of 1.15% KCl with five strokes in a Potter-Elvehjen homogenizer equipped with a motor driven Teflon pestle The homogenate was centrifuged at 10,000 g for 20 minutes and the precipitate containing the nuclear and mitochondrial

fractions discarded. The microsomal fraction was isolated as a pellet by centrifuging the 10,000 g supernatant fraction at 105,000 g for 90 minutes in a Spinco Model L ultracentrifuge. The supernatant fraction was discarded and the microsomes were resuspended in 0.05 M Tris-HCl buffer (pH 7.5) containing 50% glycerol. In experiments in which the rats were not starved prior to being sacrificed, the microsomal fraction was carefully separated from the glycogen by loosening the pellet in a small volume of buffer with a swirling action. The protein concentration was assayed by the method of Lowry (42). All operations were performed at 0-5°. The microsomes were either used immediately or stored frozen under N_2 at -15°. These microsomes retained their full animopyrine demethylase activity for several weeks providing they were kept anaerobic.

Aminopyrine Demethylase Assay



Reaction mixtures were incubated at 37° under air in a Dubnoff metabolic shaker and contained microsomes (0.7 mg/ml), $MgCl_2$ (7mM), NADPH (0.5mM), Tris-HCl (0.05 M pH 7.5) and Aminopyrine (20mM).

The N-demethylase activity was assayed by measuring the rate at which formaldehyde was produced using the method of Nash (43). One ml aliquots were removed from the incubation mixtures and diluted into one ml of 10% trichloroacetic acid (TCA). After allowing time for protein coagulation (about five minutes) two ml of Nash reagent (2 M $NH_4C_2H_3O_2$; 0.05 M CH_3COOH; 0.02 M 2,4-pentanedione) were added and the mixtures were heated at 50° for 10 minutes. The assay mixtures were centrifuged at 1000 g to sediment the protein and the O.D. of the supernatant fraction was determined at 412 mμ using a Coleman Jr. Spectrophotometer equipped with a flow cell. The extinction coefficient used was 7.08 O.D. ml^{-1} of assay μM^{-1} of HCHO. Linearity of activity with time (Figure 4) and protein (Figure 5) was shown.

Hexobarbital Hydroxylation Assay

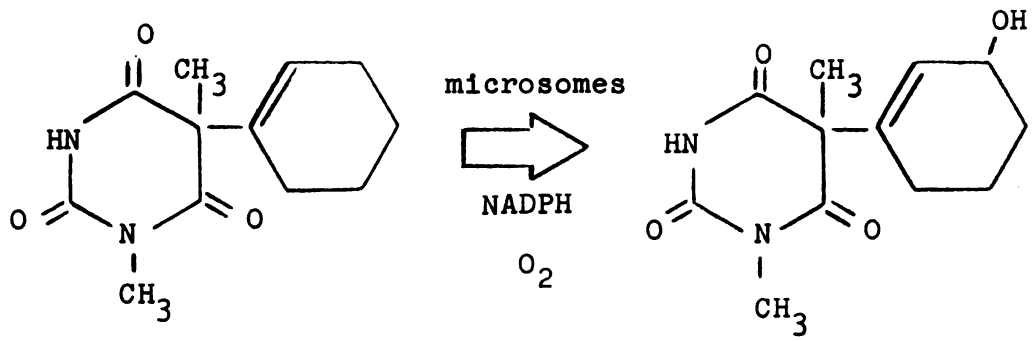


Figure 4.--Activity vs Time for Aminopyrine Demethylase Assay with Rat Liver Microsomes. The metabolizing ability of the microsomes used in all experiments is shown. The reaction is linear for approximately 15 minutes. See text for incubation conditions.

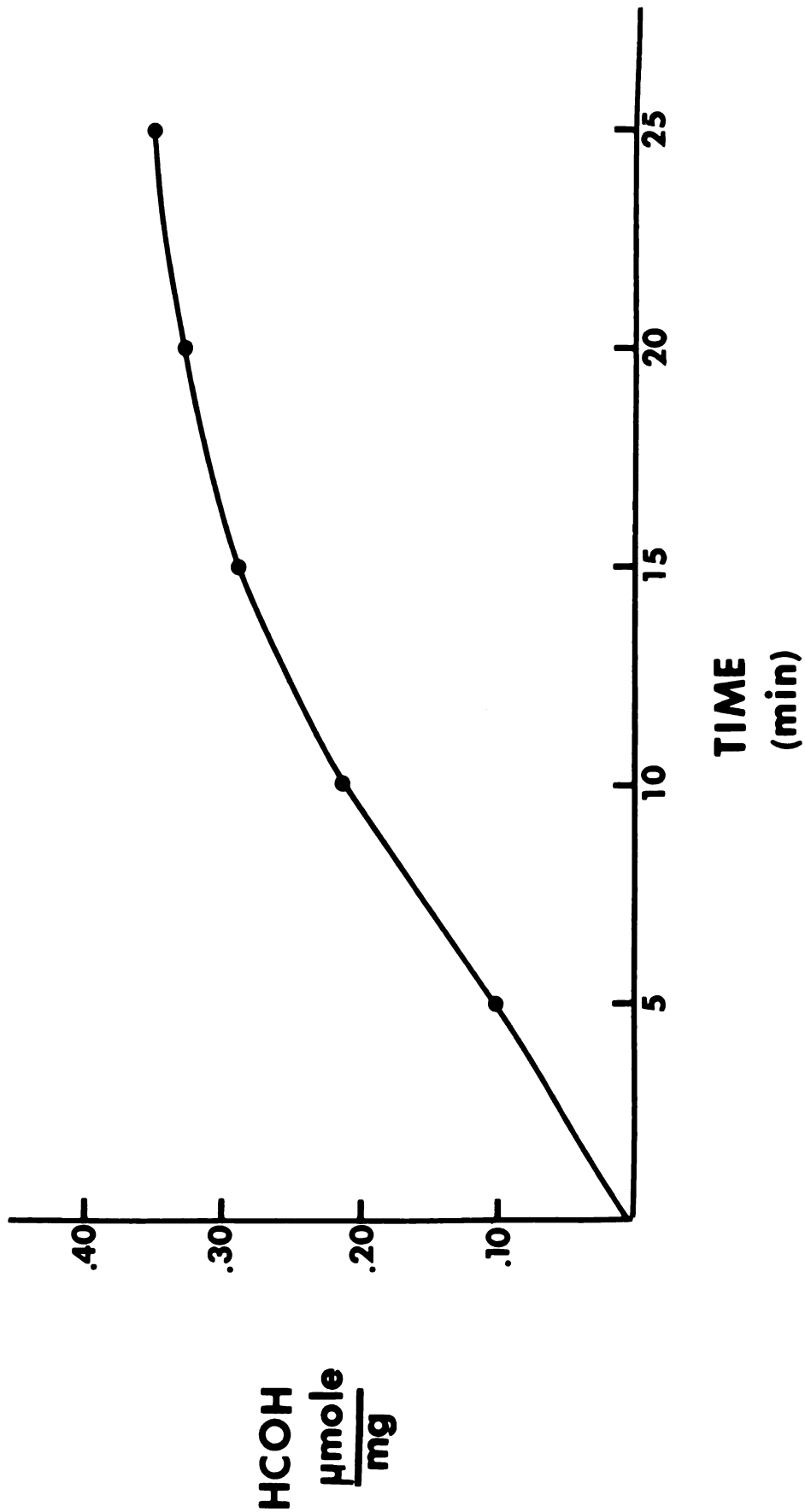
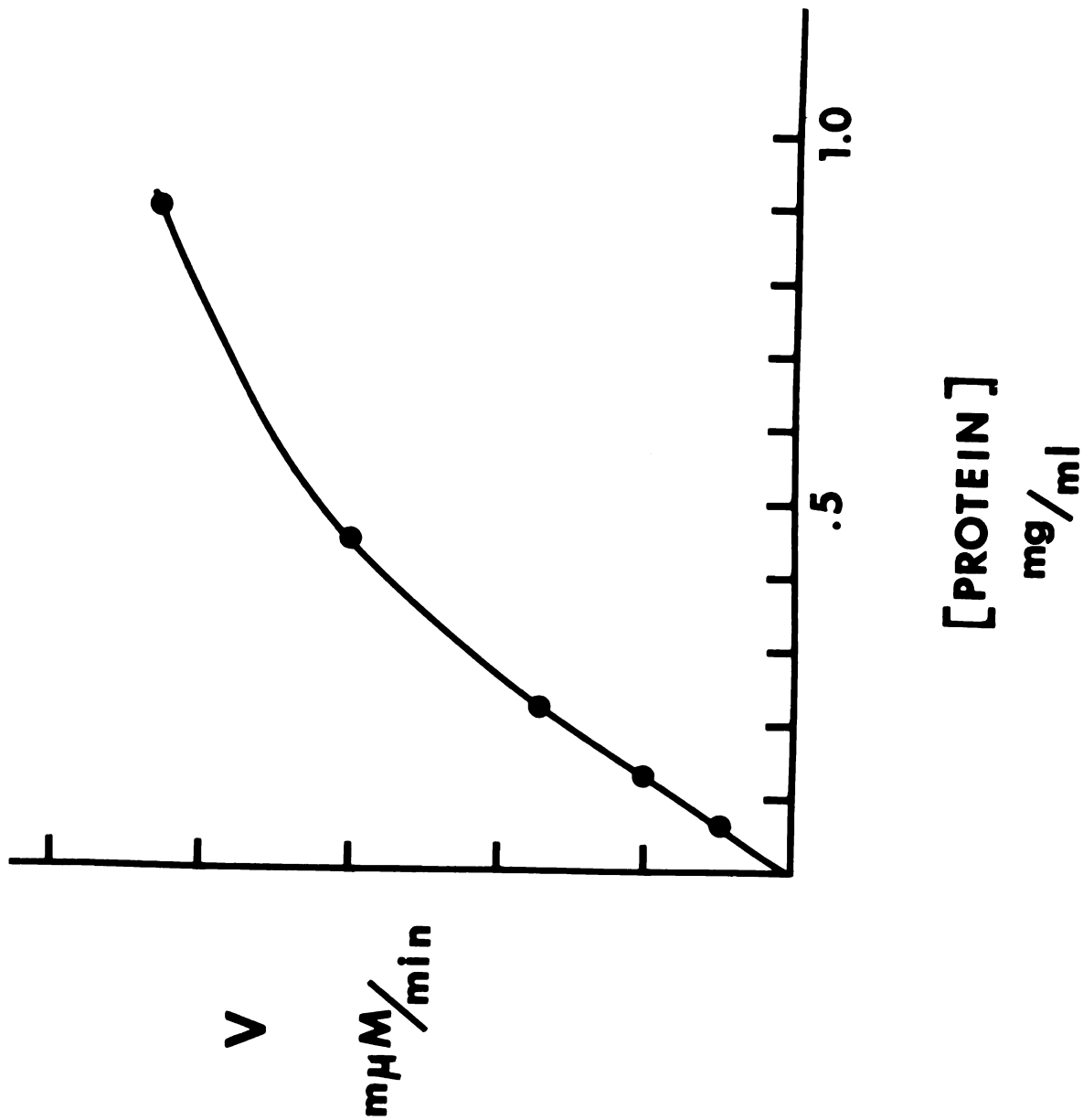

HCOH
μmole
mg
.40
.30
.20
.10
5
10
15
20
25
TIME
(min)

Figure 5.--Velocity vs Protein Concentration for Aminopyrine Demethylase Assay by Rat Liver Microsomes. See text for incubation conditions.


V
mμM/min
.5
1.0
[PROTEIN]
mg/ml

The hydroxylation of hexobarbital by the perfused liver was assayed by the method of Brodie and Cooper (44). This assay involves the disappearance of substrate and is developed on the principle that the more polar metabolite can be extracted out of a nonopolar solvent such as heptane into a basic water solution, while the less polar parent drug remains in the organic solvent.

Two ml samples of the perfusate were added directly to one ml of 6 N HCl and extracted with five ml heptane in a glass stoppered centrifuge tube. The samples were centrifuged in a clinical centrifuge at 3000 RPM for five minutes. A 4 ml aliquot of the organic layer was extracted with two ml of 0.5 N NaOH and the optical density of the organic layer measured on a Beckman DB spectrophotometer at 240 mμ. The extinction coefficient used for Hexobarbital was $10^{-3}M^{-1}cm^{-1}$.

Guinea Pig Ileum Bioassay

About 250 g guinea pigs which had been starved approximately 24 hours, were sacrificed and the ileum was removed immediately, washed, and placed in ice cold Tyrodes solution. Pieces of mid-ileum about 3 cm long were suspended in a vertical 2 x 15 cm organ bath. The organ baths were built into a constant temperature bath maintained at 37°. The organ bath was equipped with two way valve at the bottom such that the sections of ileum could be washed quickly by draining and refilling the bath with fresh Tyrodes solution pre-equilibrated at 37°. The sections of ileum were anchored at one end and

fastened to a force-displacement transducer at the other end. A small capillary tube bubbled air into the organ bath at all times. The organ bath was always filled to the same level which was measured to contain 20 ml.

Treatments were made by injecting the test substances directly into the bathing medium via a syringe. The air bubbling through the bath served to quickly mix the solutions. Contractions were recorded through a force-displacement transducer on a Sargent model SRL recorder.

Partition Chromatography
Assay for Acetate

Silicic acid (100 mesh) was purchased from Mallinckdrodt Chemical Company. The fines were removed by making a slurry in H_2O and decanting off the material unsettled after three minutes. The silicic acid was dried at 100° for 24 hours and stored in a glass stoppered bottle until used. A one cm diameter glass column filled with benzene was packed with 10 gm of silicic acid to which 6.5 ml of 0.5 N H_2SO_4 had been added.

Solvents were equilibrated with 0.5 N H_2SO_4 by shaking in a separatory funnel and then passing the solvent through Whatman #1 filter paper to remove excess sulfuric acid.

The sample (about one ml) was adjusted to pH 2 with 0.5 N H_2SO_4 and absorbed to 1 - 2 g of silicic acid, before applying to the column. The elution pattern used was 50 ml of benzene, 100 ml of chloroform, and 150 ml chloroform

containing 1% tertiary-butyl alcohol. Fractions of five ml were collected from the column and titrated with 0.0163 N alcoholic KOH to the phenolphthalein end point. The samples were then evaporated to dryness and counted in a scintillation counter. The scintillator was dioxane containing 5% naphthalene, 0.7% 2,5-diphenyloxazole (PPO), and 0.05% 1,4-bis [2-(5-phenyloxazolyl)]-Benzene (POPOP).

Precipitation of Protein with Zinc Hydroxide

Since the lability of the metabolite was unknown, a mild procedure for deproteinizing blood and microsomal incubations was required. The procedure was essentially that of Somogyi (45) in which the protein solution was diluted in seven volumes of distilled water. One volume of a 10% solution of $ZnSO_4$:7 H_2O was added and the solution was thoroughly mixed. One volume of 0.5 N NaOH was added slowly with continuous mixing. The flocculant solution was then filtered through Whatman #1 filter paper. The pH did not go below 5.0 and was titrated back to pH 7.0 or 8.0 with the 0.5 N NaOH. A clear solution resulted which did not form emulsions when extracted with chloroform.

Liver Perfusion Experiment

Isolated rat liver perfusions were performed employing a recirculating system similar to that of Van Harken *et al.* (46). Male rats were anesthetized with diethyl ether and the liver exposed by an abdominal incision. The portal vein and

bile duct were cannulated, ligated and the liver carefully removed and attached to the perfusion apparatus as quickly as possible. The apparatus (47) consisted of a stirred blood reservoir connected to a peristaltic pump which circulated the blood to an oxygenator. The blood then flowed through the oxygenator, which had a mixture of oxygen: carbon dioxide (95:1) passing through to the portal vein cannula. The pressure head was regulated by adjusting the height of the oxygenator above the liver. Excess blood flowed, via a bypass, directly to the reservoir.

The perfusate was usually heparinized blood obtained by heart puncture from blood donor rats. The blood was diluted 1:1 with 0.9% sodium chloride. The temperature of the entire system was maintained at 37°. The perfusion system was operated before attaching the liver for 30 minutes to oxygenate the blood. Other perfusion solutions are listed below.

Perfusion Solutions

Tyrode Solution

Solution A	g/10 l	mM
NaCl	80.0	138.00
Glucose	10.0	5.50
KCl	2.0	2.66
$CaCl_2.2\ H_2O$	2.7	1.84
$MgCl_2.6\ H_2O$	1.0	.49

Solution B

$NaHCO_3$	10.0	19.00
NaH_2PO_4	0.5	.36

These two solutions were kept at 0.4° until needed, at which time they were combined in equal volumes.

Krebs-Bicarbonate Ringer Solution

	g/l	mM
NaCl	7.076	122.0
KCl	.222	3.0
$MgSo_4$	.144	1.2
$CaCl_2$	.191	1.3
KH_2PO_4	.054	0.4
Glucose	1.80	10.0
$NaHCO_3$	2.10	25.0

Krebs-Henseleit Solution

Amount	Order of Addition	Solution	M
770.0	(1/2) 4/(1/2)7	0.90% NaCl	0.154
30.8	1	1.15% KCl	0.154
23.1	2	1.62% $CaCl_2.2H_2O$	0.110
7.7	5	2.11% KH_2PO_4	0.154
7.7	3	3.82% $MgSO_4.7H_2O$	0.154
161.7	6	1.30% $NaHCO_3$	0.154

All solutions were made in triple distilled water and kept at 0-4° until needed. The solutions were combined in the order given to prevent precipitation of any salts.

RESULTS

Attempts to Isolate a Metabolite of Slaframine

The following experiments were conducted in attempts to observe a product of slaframine which might be, or be derived from, an active metabolite. The first experiment involved examination of the urine of animals given slaframine. Urine was collected from control and slaframine (2 mg/kg, i.p.) treated animals for 48 hours, adjusted to pH 10 with sodium carbonate and extracted three times with two volumes of chloroform. The extract was dried over sodium sulfate and concentrated to 0.1 ml. It was then analyzed by thin-layer chromatography (TLC) using Dragendorff's reagent to detect tertiary amines. No metabolites of slaframine other than deacetylslaframine could be detected by these methods even when all extractions were done at 4°. The experiment was repeated with ^{14}C-(ring)-slaframine (14 mc/Mole), but, upon analysis by TLC and scintillation counting, all counts appeared to correspond to slaframine.

Attempts to find a metabolite in the blood of animals given slaframine were also unsuccessful. Blood samples were taken at the peak of salivation by heart puncture. Protein

was precipitated by 10% TCA, saturating with ammonium sulfate, or with zinc hydroxide and the solution extracted at pH 10 with chloroform. Analysis of the extract by TLC and scintillation counting failed to show a metabolite of slaframine.

Attempts to produce a metabolite of slaframine by incubations with crude liver homogenates, liver slices, and microsomes were not sucessful. The pH 10 chloroform extracts of these incubations were analyzed by TLC and gas chromatography without any evidence of a metabolite with either radioactive slaframine or with unlabeled slaframine.

It was concluded that the metabolite was present at such low levels that it could not be detected by these methods, or that it was being destroyed by the methods used to precipitate the protein or during extraction into chloroform at pH 10. Therefore some very high specific activity ^{3}H-(acetate)-slaframine (350 mc/m mole) was administered (2 mg/kg, i.p.) to rats and the blood and liver were removed and analyzed by TLC without prior treatment, except for homogenization and centrifugation of the liver protein. Five millimeter sections were subsequently scraped, put in scintillation vials and counted. These experiments also failed to reveal any radioactive metabolites.

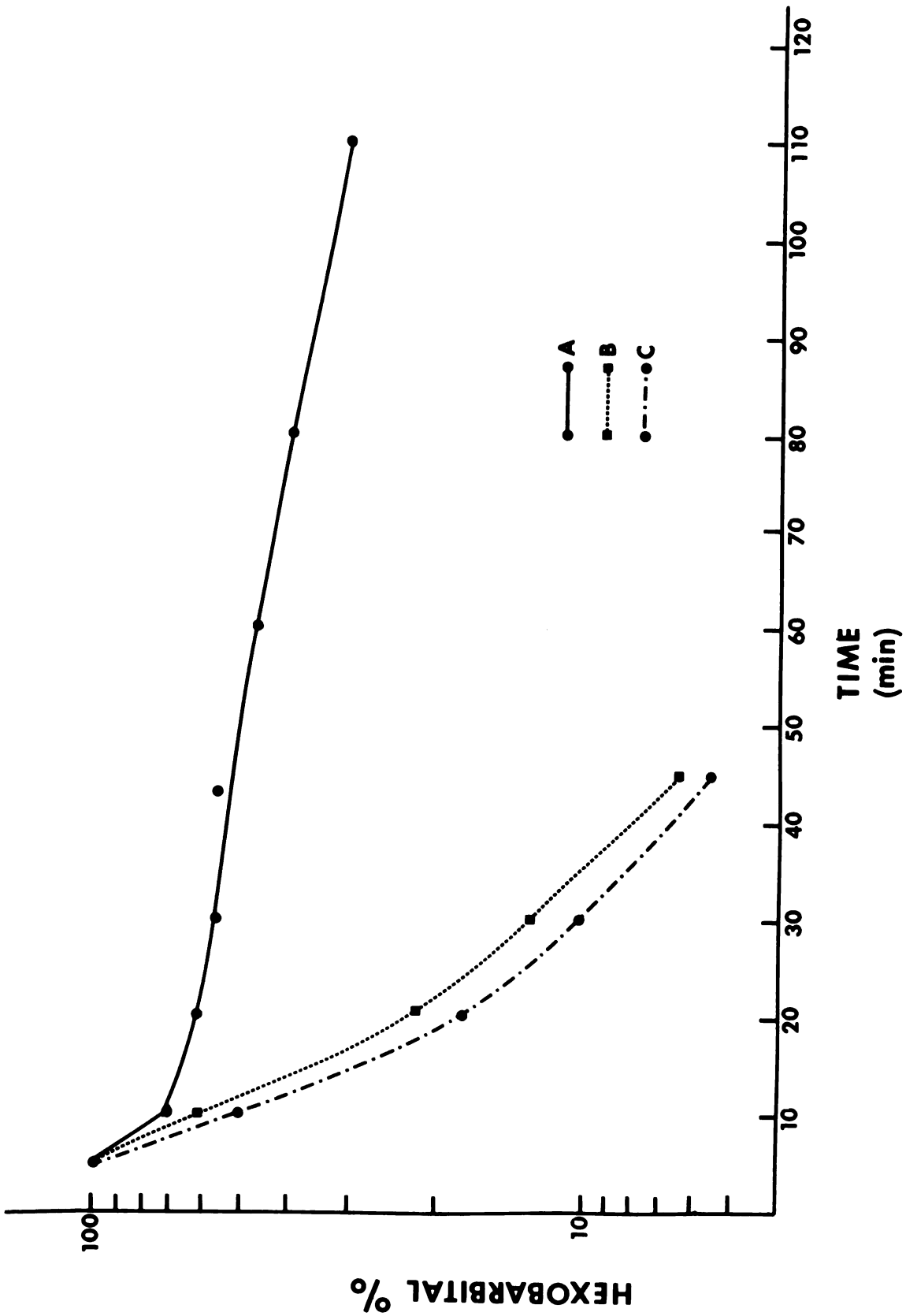
At this time it was decided to use the perfused liver in an attempt to show a metabolite of slaframine. The ability of livers to metabolize drugs was determined under various conditions with hexobarbital as a model substrate.

Forty-seven ml of whole blood was obtained by heart puncture from five male rats (500 g). The whole blood was diluted to 70 ml with 0.85% sodium chloride. A phenobarbital induced, 500 g, male rat served as the liver donor (22.5 g). The portal vein and bile duct were canulated and the liver was connected to the perfusion system as described previously.

After a thirty minute equilibration period, 20 mg of hexobarbital (dissolved in ten ml of 0.5 N sodium hydroxide and neutralized with ten ml of 0.5 N hydrochloric acid) was added to the perfusion system. Aliquots of two ml were taken at 5,10,20,30, and 45 minutes and assayed for the disappearance of hexobarbital by the procedure described in Methods.

In one experiment the bile duct canula was removed and the experiment was repeated with a second addition of 20 mg of hexobarbital. At the end of two hours the bile duct was recanulated and Krebs-Hanseleit solution was substituted for blood in the perfusion system. A fresh liver was also perfused with Krebs-Hanseleit solution to eliminate the possibility that the liver had degenerated in the first experiment and thus account for the observed results. The results of this experiment were, however, the same as the first, showing that the metabolizing ability of the liver decreased when Krebs-Hanseleit solution was used in the place of blood in the perfusion fluid. This is probably because Krebs-Hanseleit solution is less able to carry oxygen to the cells. The results of these experiments are shown in Figure 6. Curve A

Figure 6.--Metabolism of Hexobarbital by the Perfused Rat Liver. The effects of various perfusion fluids on the ability of the perfused liver to metabolize hexobarbital. A- Krebs-Habseleit Solution; B- Blood diluted 1:1 with saline, bile duct ligated; C- Blood diluted 1:1 with saline, bile duct cannulated. The amount of hexobarbital remaining in the perfusate was measured and plotted as the percent of the amount present at five minutes on semilogarithmic paper.

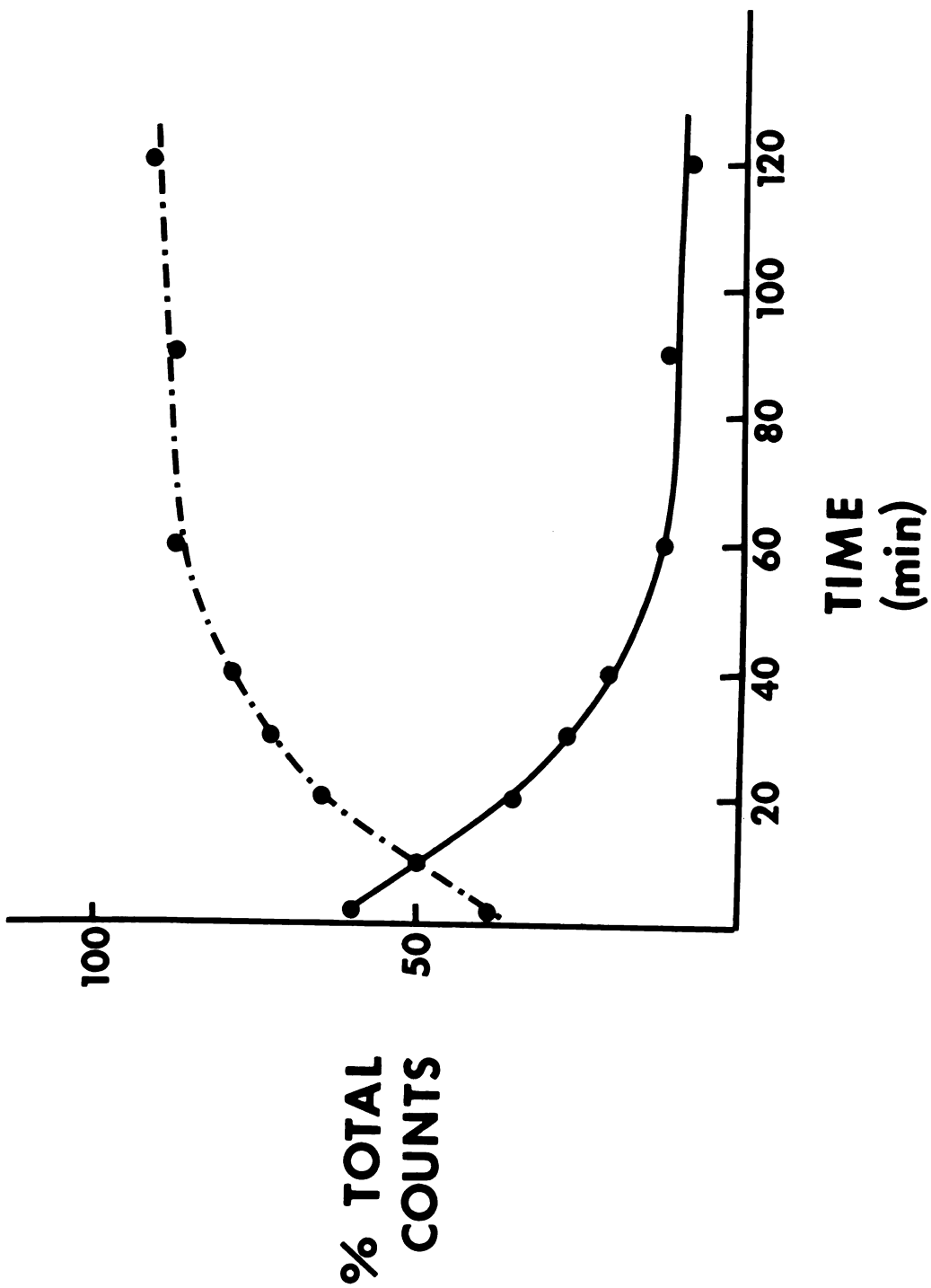

HEXOBARBITAL %
100
10
10
20
30
40
50
60
70
80
90
100
110
120
TIME
(min)
A
B
C

was obtained with Krebs-Hanseleit solution as the perfusate. Curve B was obtained with the bile duct ligated while Curve C was obtained with the bile duct canulated using diluted blood as the perfusate. The liver, perfused with blood, was able to remove roughly half of the hexobarbital from the perfusate in 10 minutes, while roughly one hour was required to remove the same amount of hexobarbital from the Krebs-Hanseleit perfusate.

Metabolism of ^{3}H(Acetate)-Slaframine by the Perfused Rat Liver

From the results of the previous experiment it was decided to use a liver perfused with blood without the bile duct cannulated for the following experiments. After a 30 minute equilibration period 1 mM of the dicitrate salt of ^{3}H-(acetate) slaframine (specific activity 350 mc/m mole) was added to the perfusion system. The total volume of the perfusate was 75 ml. One ml aliquots were removed at 2,10, 20,30,40,60,90, and 120 minutes and placed on ice. The pH of the blood was raised to 10 with solid sodium carbonate and extracted with 10 ml of chloroform. Ten µl aliquots of the chloroform fractions were transferred to scintillation vials and dried under nitrogen. One-hundred µl aliquots of the water fractions were added to scintillation vials. All samples were counted in 15 ml of scintillation fluid which contained 5% naphthalene, 0.7% PPO, and 0.05% POPOP in dioxane. An increase in the fraction of total counts occurred in the water fraction with time (Figure 7).

Figure 7.--Metabolism of ^{3}H-(acetate)-Slaframine by the Perfused Rat Liver. One ml samples were withdrawn from the perfusion fluid reservoir at various time intervals. The pH was raised to 10 with solid sodium carbonate and the solution extracted with 10 ml of chloroform. Aliquots of the water and chloroform fractions were then counted by liquid scintillation (——————, water fraction;—— — ——, chloroform fraction).


100
50
% TOTAL COUNTS
20
40
60
80
100
120
TIME (min)

The radioactivity remaining in the water fraction was shown, upon subsequent analysis, to correspond to acetic acid by chromatography on silicic acid (see Methods).

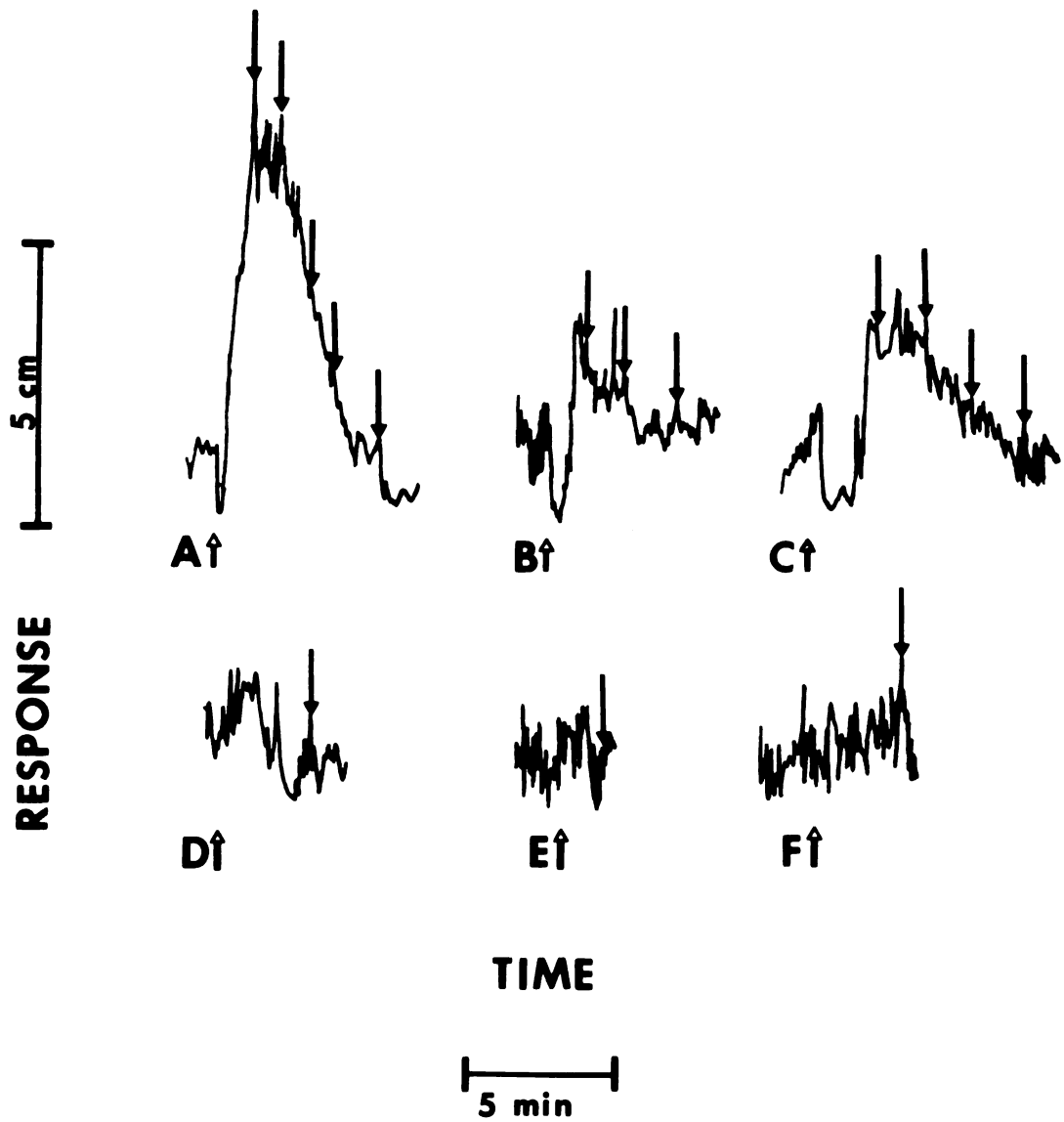
The radioactivity in the chloroform extract was shown to be attributable solely to the slaframine by TLC developed with Dragendorff's reagent. The presence of deacetylslaframine in the chloroform could not be demonstrated since the amount present was too small to detect visually following spraying with Dragendorff's reagent or ninhydrin.

It was realized at this time that perhaps there was no metabolite distinguishable by chemical methods, but that one might be seen physiologically. However, an extremely sensitive bioassay assay would be necessary. The guinea pig ileum, which is a very sensitive and well known bioassay for cholinergic drugs was used to search for physiologically active metabolite of slaframine. As mentioned above, the pharmacological action of slaframine indicated it was acting through the cholinergic nervous system.

Metabolism of Slaframine by Rat Liver Microsomes

Portions of a microsomal incubation mixture containing microsomes, slaframine and NADPH in Tyrodes solution were added to the organ bath containing the ileum. The complete incubation mixture caused a marked contraction of the ileum (Figure 8,A) which persisted much longer (about five minutes) than ones initiated by acetylcholine and could not be reversed by washing.

Figure 8.--Metabolism of Slaframine by Microsomes. Microsomal incubations were tested for their ability to contraction of the guinea pig ileum. A, Complete incubation mixture; B, minus slaframine; C, minus slaframine and microsomes; D, minus NADPH; E, slaframine alone; F, Tyrodes only. Upper arrows indicate washings and lower arrows additions.


5 cm
RESPONSE
A
B
C
D
E
F
TIME
5 min

Various controls were run to gain confirmation that the observed reaction was attributable to the active metabolite of slaframine and required microsomes and NADPH. Figure 8,B displays the results of an injection of the incubation mixture minus slaframine. NADPH and Tyrodes solution gave similar results (Figure 8,C). A requirement for NADPH is shown in Figure 8,D while slaframine alone (dissolved in Tyrodes solution) failed to elicit a response in the ileum (Figure 8,E). Figure 8,F displays the lack of reaction by adding solely Tyrodes solution.

Because of variations in ileum responses all assays were corrected by comparing the response to acetylcholine. By this method a dose response curve for the metabolite produced by liver microsomes could be obtained (Figure 9).

The production of the metabolite was shown to be a function of time removing one ml aliquots from an incubation at minute intervals and placing them on ice until assayed with the ileum. Keeping the samples on ice was necessitated by the fact that the metabolite causes a prolonged contraction of the ileum and subsequent assays must await the return of the ileum to a resting state. In addition acetylcholine had to be given to test the sensitivity of the ileum between each assay. In each experiment there seemed to be some of the metabolite present at zero time. This phenomenon will be explained in a later section. The reaction did seem to be linear for about five minutes (Figure 10) after which the amount of the metabolite present began to decrease. A

Figure 9.--Dose vs Response for the Metabolite Produced by Microsomes. Standard incubation mixtures were incubated for three minutes at 37° and various volumes of the mixture were injected into the organ bath. Results were recorded as percent of the response to 0.1 µg of acetylcholine.

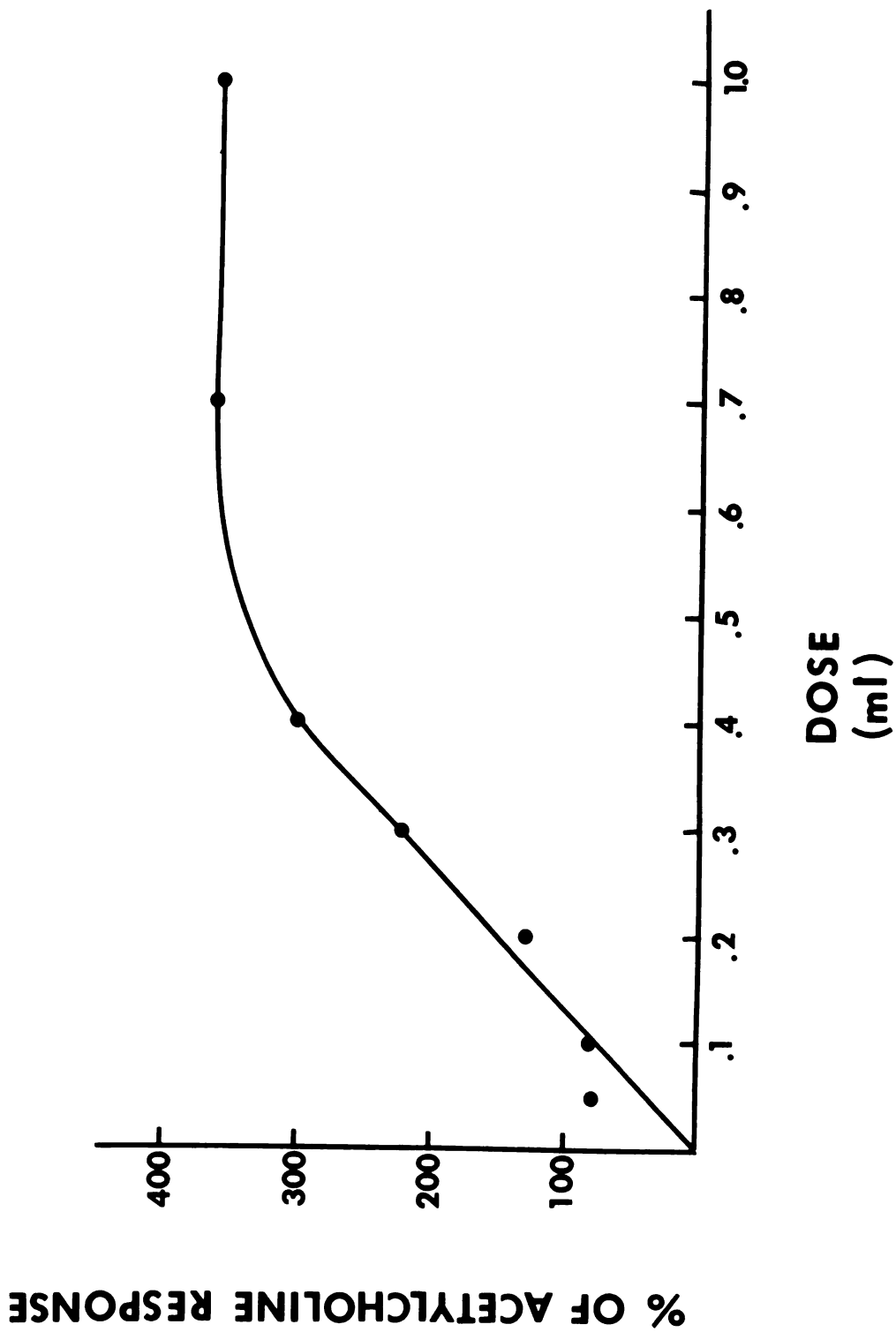
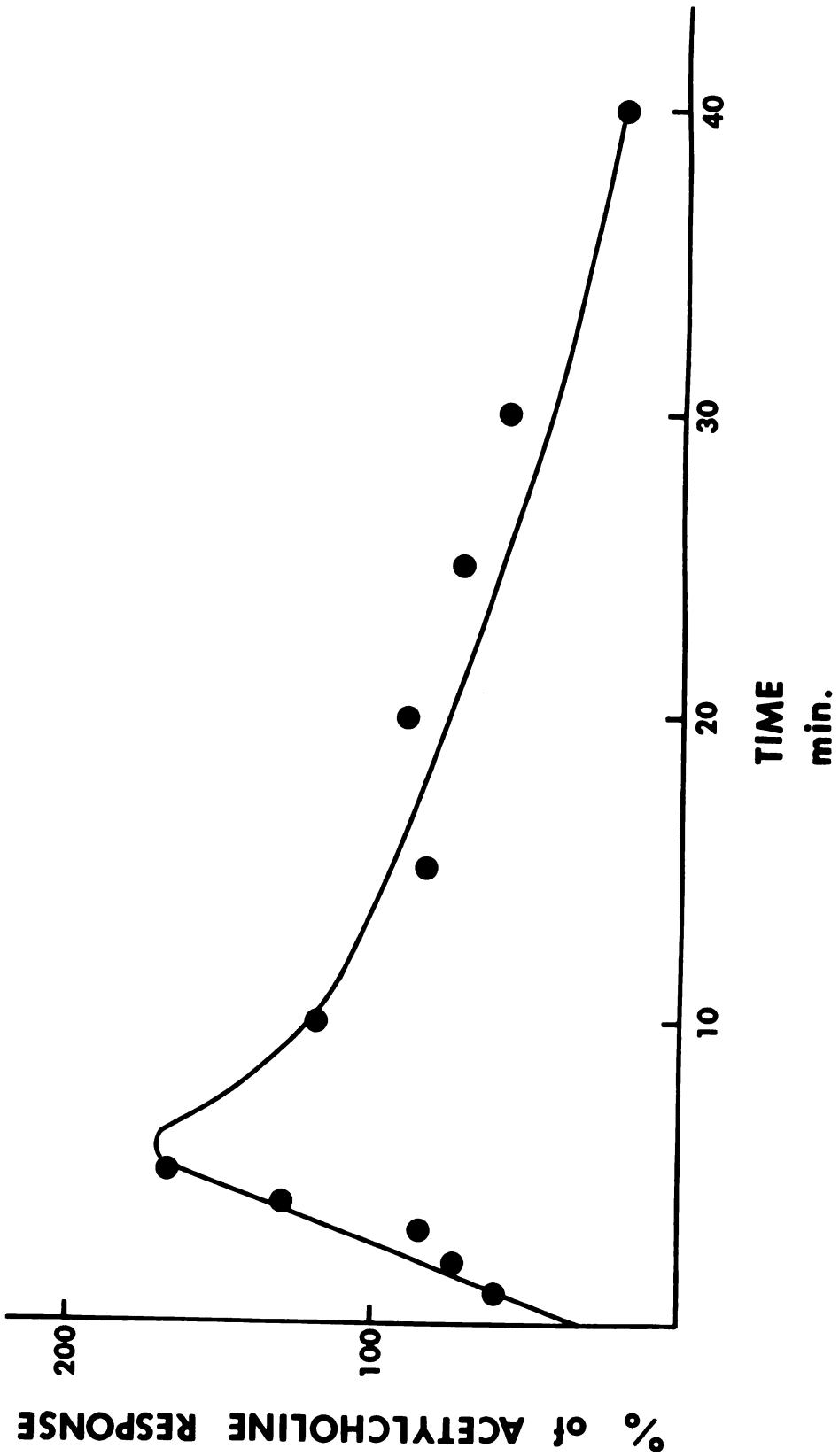

% OF ACETYLCHOLINE RESPONSE
400
300
200
100
.1
.2
.3
.4
.5
.6
.7
.8
.9
1.0
DOSE
(ml)

Figure 10.--Time Course Assay of the Production of Metabolite by Rat Liver Microsomes. Standard incubation mixtures (Methods) were incubated for various time intervals and 0.5 ml of the mixture were introduced into the ileum chamber. Results were recorded as percent of the response realized by 0.1 µg of acetylcholine.


% of ACETYLCHOLINE RESPONSE
200
100
10
20
30
40
TIME
min.

definite increase in activity was realized when incubation mixtures of phenobarbital induced microsomes were compared to control microsomes.

Localization of the Enzyme Responsible for the Metabolism of Slaframine

The livers (28.7 g) from two female rats (400 g each) were homogenized as described in Methods. Slow speed centrifugation (1000g) was used to remove the nuclear fraction and cellular debris. The resulting supernatant fraction was used as a crude homogenate and assayed for its ability to metabolize slaframine to an active form capable of contracting the guinea pig ileum. The mitochondrial fraction was prepared by centrifuging the 1000g supernatant fraction at 10,000g for 20 minutes. The mitochondrial fraction was washed in Tyrodes solution, recentrifuged and then resuspended in Tyrodes for use in the incubations. Further subcellular fractionation was then accomplished as described in Methods for isolation of microsomes. Protein concentration and metabolizing ability were assayed on each resulting fraction. The incubation mixture consisted of one ml of the liver fraction and the incubation mixture used for the aminopyrine demethylation assay except that slaframine (0.2 mg) replaced aminopyrine. Activity (Figure 11) was recorded as response obtained from injecting 0.2 ml of the incubation mixture into the ileum bath, compared to the response obtained from 0.1 µg of acetylcholine just previously injected, per milligram of protein in the incubation mixture (Table 2).

Figure 11.--Ability of Various Subcellular Fractions to Metabolize Slaframine. NADPH (0.4 mM) and slaframine (0.2mM) were added to all incubations. One ml of each fraction was used as the protein source. A, crude homogenate (1000g supernatant fraction); B, 10,000g supernatant fraction; C, mitochondrial fraction (10,000g pellet); D, microsomal fraction (105,000g pellet). Upper arrows indicate washings and lower arrows additions. Time marks are 5 minutes.

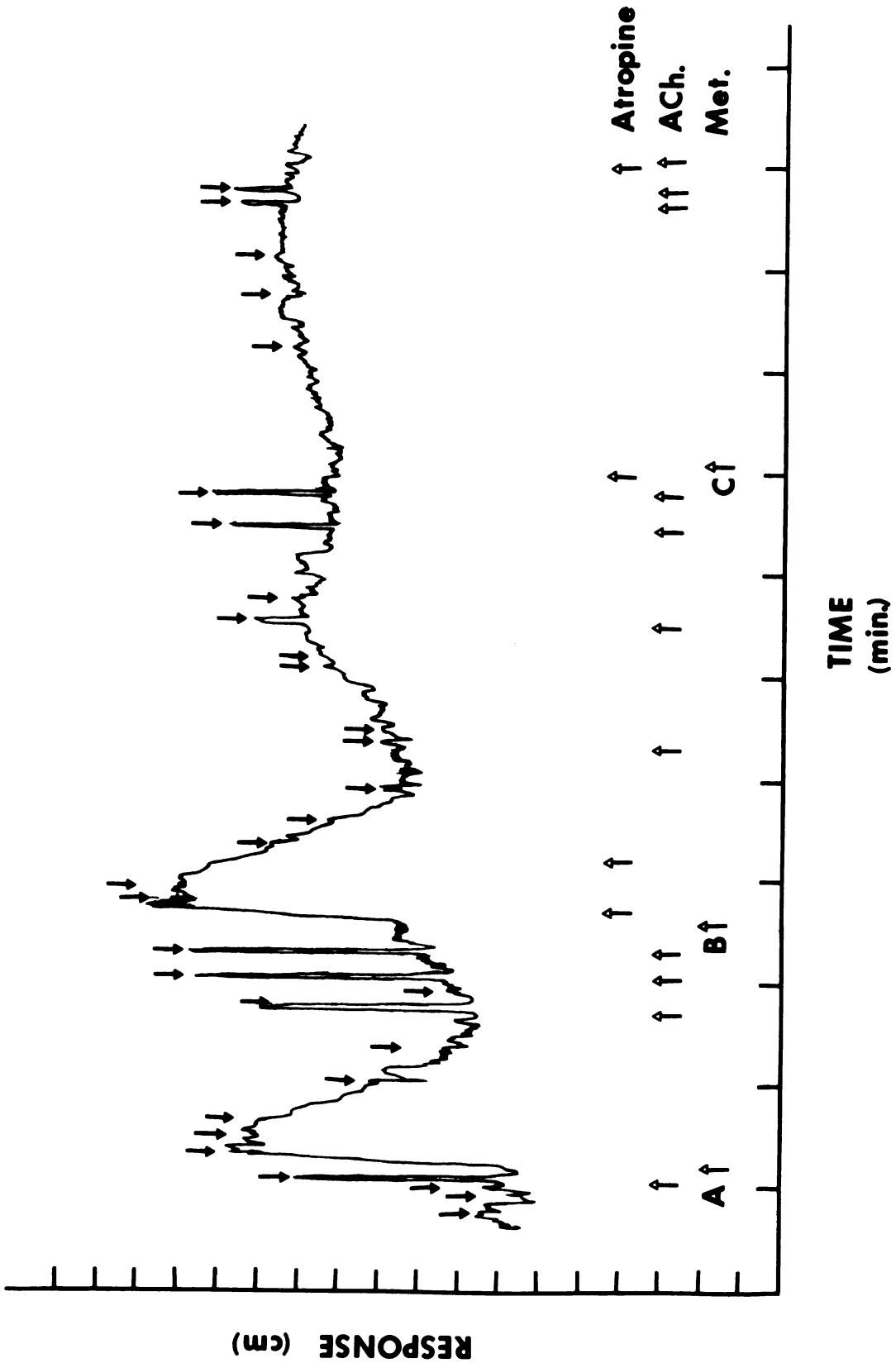

RESPONSE (cm)
TIME (min.)
A
B
C
Atropine
ACh.
Met.

TABLE 2.--Activation of Slaframine by Rat Liver Fractions.

Fraction	Activity*
Crude Homogenate (1000 g Supernatant fraction)	.132/mg
10,000 g Supernatant fraction	.025/mg
10,000 g pellet	.034/mg
105,000 g Supernatant fraction	0/mg
105,000 g pellet	.098/mg

*Activity = cm response due to metabolite ÷ cm response due to 0.1 µg acetylcholine per mg protein in incubation mixture.

Stability of the Active Metabolite

Experiments preliminary to the isolation of the active metabolite of slaframine (changes in pH, boiling, etc.) consistently resulted in loss of activity. Attempts to deproteinize incubation mixtures, which contained the active metabolite, by placing in a boiling water bath for two minutes, resulted in the destruction of the metabolite. The metabolite was not bound to the protein and could be separated from the microsomes by centrifugation at 105,000g, however, and could be detected in the supernatant fraction at levels equivalent to those observed before centrifuging. Resuspension of the centrifuged protein and subsequent incubation with slaframine and NADPH also gave an equivalent response by the ileum. The conventional methods of precipitating proteins by ionic means could not be used since the ileum is very sensitive to ion concentration. No reaction by the ileum was observed after

raising or lowering the pH of the microsomal incubation to 10 or 2, respectively.

Cofactor Requirements

Since the production of the metabolite appeared to cease by the end of ten minutes, it was thought that some additional cofactor might be required. Therefore a number of cofactors were tried in an attempt to increase the production of the metabolite. To standard incubation mixtures ($2X10^{-4}$ M slaframine, 7 mM $MgCl_2$, 0.4 mM NADPH in 5 ml of Tyrodes solution) 1 mg of various cofactors were added. Controls consisted of identical incubation mixtures without slaframine. Incubations were carried out at 37° for three minutes in a Dubnoff metabolic shaker. None of the controls elicited any response by the ileum. All injections into the ileum chamber were 0.2 ml. A marked increase in activity was observed with all flavins (Table 3). FMN gave the largest increase in activity and FAD the least. NADH gave a small increase, while biopterin, tetrahydrofolic acid, folic acid, NAD^+ and $NADP^+$ had no effect on activity.

The fact that all three flavins tried gave enhanced activity suggested the possibility that the enhancement was not due to a cofactor requirement of an enzyme but rather a reaction of the flavin itself. Further support for this hypothesis was the fact that time assays which contained any of the three flavins did not show a decrease in activity even after one hour incubations. A definite decrease in activity

TABLE 3.--Effect of Various Cofactors on the Microsomal Activation of Slaframine.

Cofactor	Response (cm)	ACH Response (cm)	Fraction of ACH
THF	0.4	5.3	0.07
FAD	3.6	6.0	.60
FMN	8.5	4.5	1.89
NAD	.8	1.5	.53
Folate	1.0	8.2	.12
Biopterin	0.3	6.0	.05
Riboflavin	8.0	4.5	1.95
$NADP^H$	6.0	7.0	.86
THF + NADPH	2.5	6.5	.38
FAD + NADPH	8.2	5.5	1.49
FMN + NADPH	8.0	1.8	4.45
NAD + NADPH	3.7	2.5	1.48
Folate + NADPH	1.1	2.6	.42
Biopterin + NADPH	1.5	2.0	.75
NADH + NADPH	5.5	4.5	1.22

was observed in incubation mixtures containing microsomes, NADPH, and slaframine after 20 minutes (Figure 10).

Carbon monoxide inhibition could not be demonstrated with liver microsomes. Standard incubation mixtures which were degassed and flushed with N_2 and then incubated at 37° for three minutes caused identical contractions by the ileum as when incubations were done aerobically.

The Non-Enzymatic Production of the Slaframine Metabolite

For an additional control in the cofactor requirement experiment (Table 3), only FMN (1 mg) and slaframine (.2 mg) were added to Tyrodes solution with a final volume of five ml

and incubated for three minutes. This solution, which was completely devoid of microsomes or any protein, caused a contraction by the guinea pig ileum which was equivalent to that observed when microsomes were included. Riboflavin and slaframine at similar concentrations have similar results, however, FAD and slaframine alone caused no contraction. Incubations of FAD and slaframine when incubated longer, however, did give activity. The rate of production of the metabolite with FAD could be followed and was linear for about 40 minutes (Figure 12). A similar experiment using FMN reached a maximum at about 15 minutes. The activity with only FMN (1 mg/5 ml) and slaframine (0.2 mg/.5 ml) gave roughly 100 fold greater activity than the microsomal incubation mixture. However, the activity was proportional to the concentration of FMN (Figure 13). A dose response curve for the FMN produced metabolite is shown in Figure 14.

Confirmation that the species responsible for the contraction of the ileum was the same as that which caused the characteristic reactions in the live animal was obtained by injecting some of the compound intravenously in a small rat (100 gm). One ml of a five ml incubation containing 1 mg FMN and 0.2 mg slaframine was administered via the femural vein and caused profuse salivation, lacrimation, and continuous defecation within 30 seconds. These symptoms would normally require nearly 100 times this quantity of slaframine and would have required a much longer time for an observable response (Figure 15).

Figure 12.--Dependence of the Nonenzymatic Production of the Metabolite with FAD upon Time. Injections of 1 µl of a five ml incubation mixture containing 0.2 mg slaframine, and 0.1 mg FAD. Results are recorded as percent of the response realized by the prior injection of 0.1 µg of Acetylcholine.

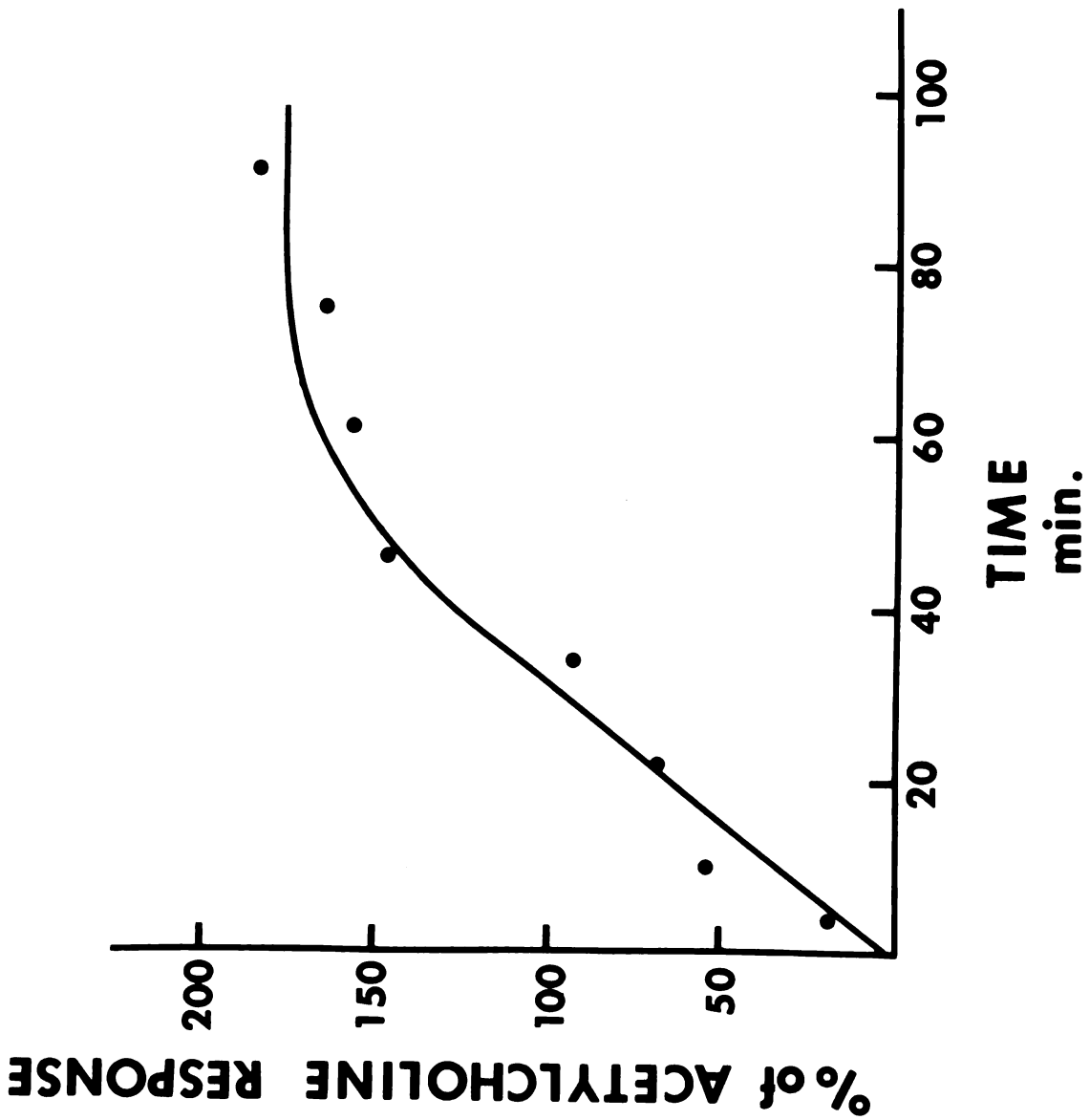

% of ACETYLCHOLINE RESPONSE
200
150
100
50
20
40
60
80
100
TIME
min.

Figure 13.--Dependence of the Production of Metabolite on the Concentration of FMN. Various levels of FMN were incubated with 2×10^{-4} M slaframine. The incubation mixtures had a final volume of five ml. One µl of this incubation mixture was injected into the ileum chamber. Responses were recorded as a percent of the response realized by a prior administration of 0.1 µg of acetylcholine.

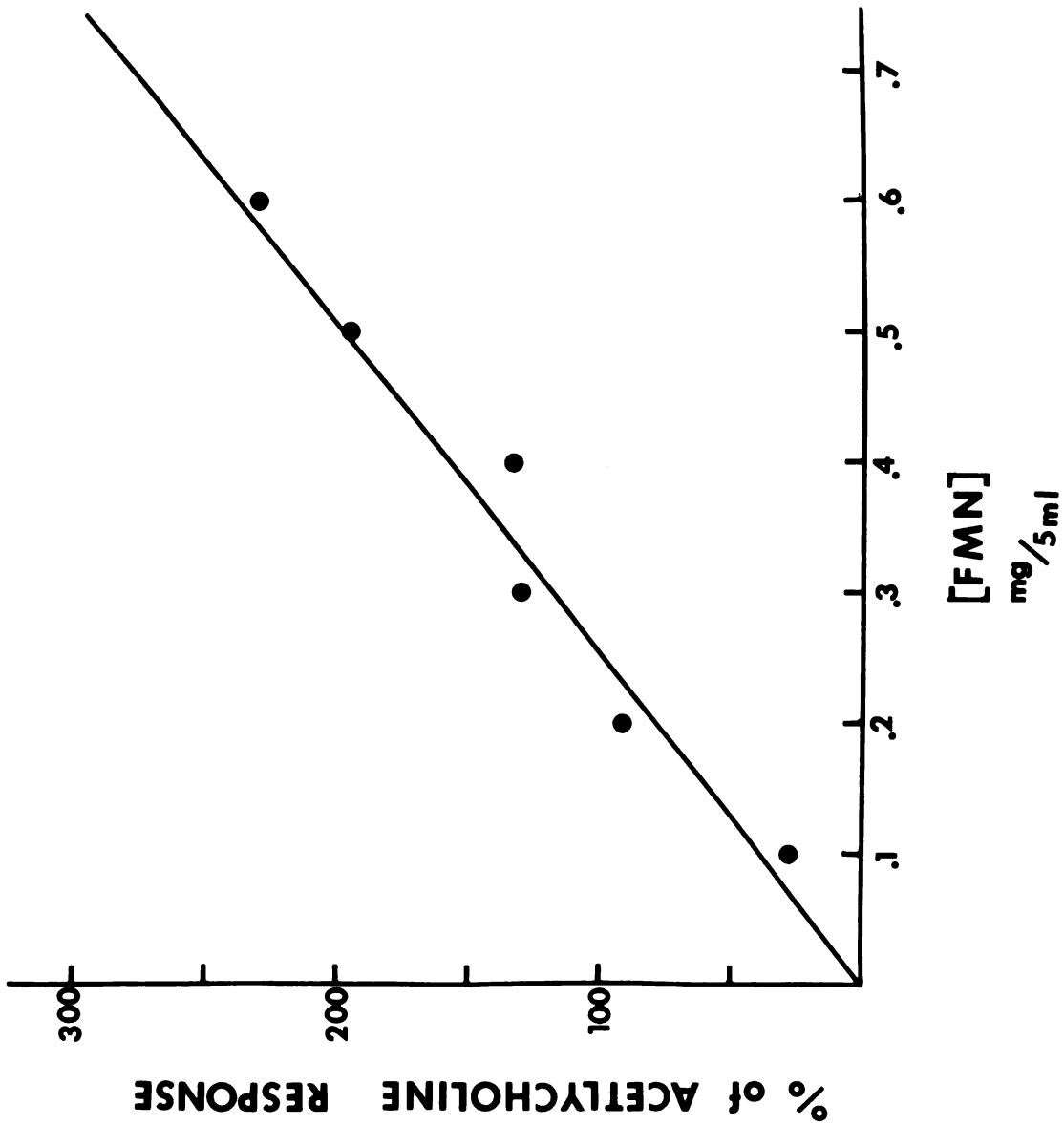

% of ACETLYCHOLINE RESPONSE
300
200
100
.1
.2
.3
.4
.5
.6
.7
[FMN]
mg/5ml

Figure 14.--Dose versus Response for The Non-enzymatically Produced Metabolite. Incubation mixtures, (five ml total volume) containing 0.2 mg of slaframine and 0.1 mg FMN were mixed in the light. One µl of the mixture was injected into the ileum chamber. Results are expressed as percent of response obtained with 0.1 µg of acetylcholine.

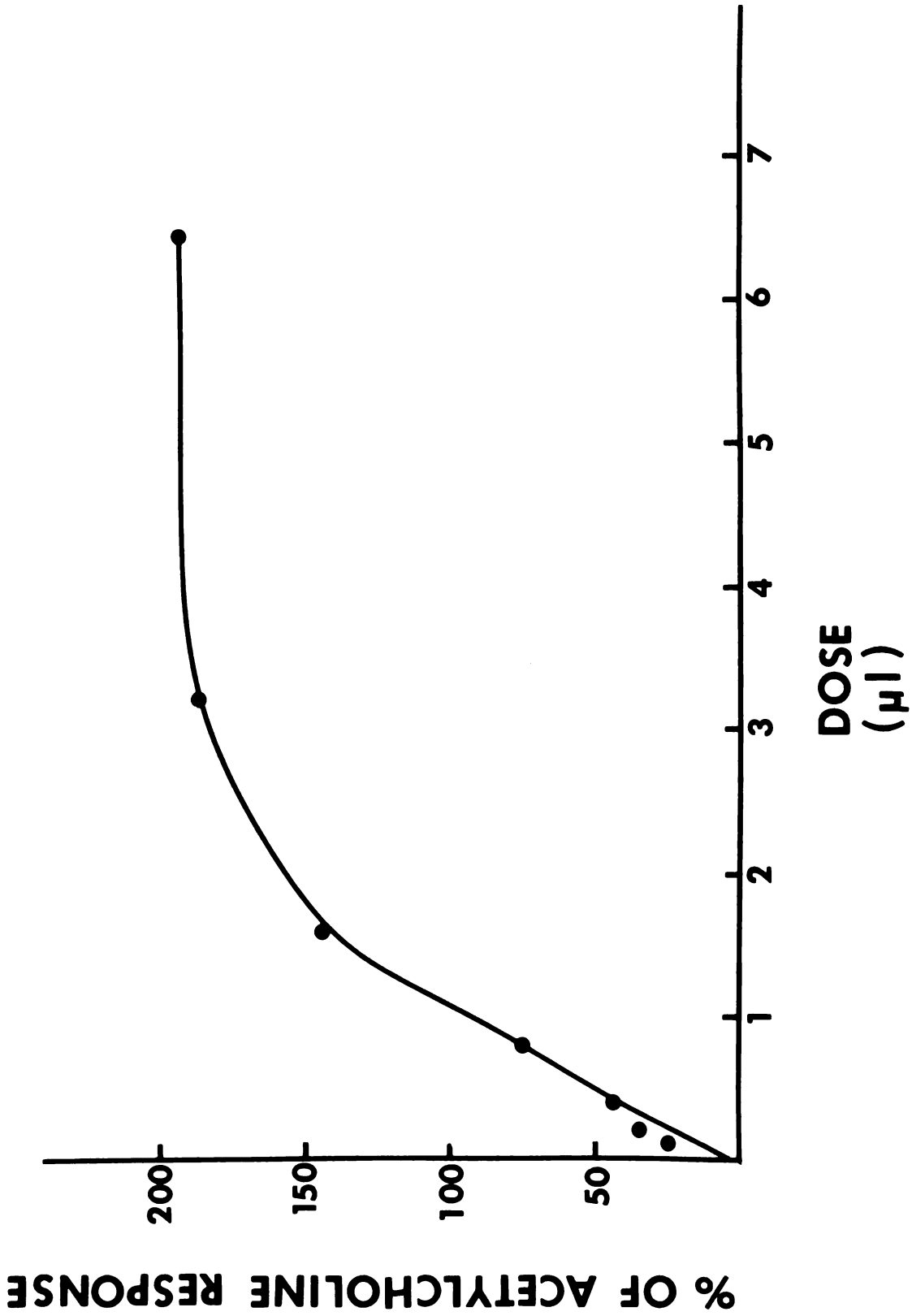
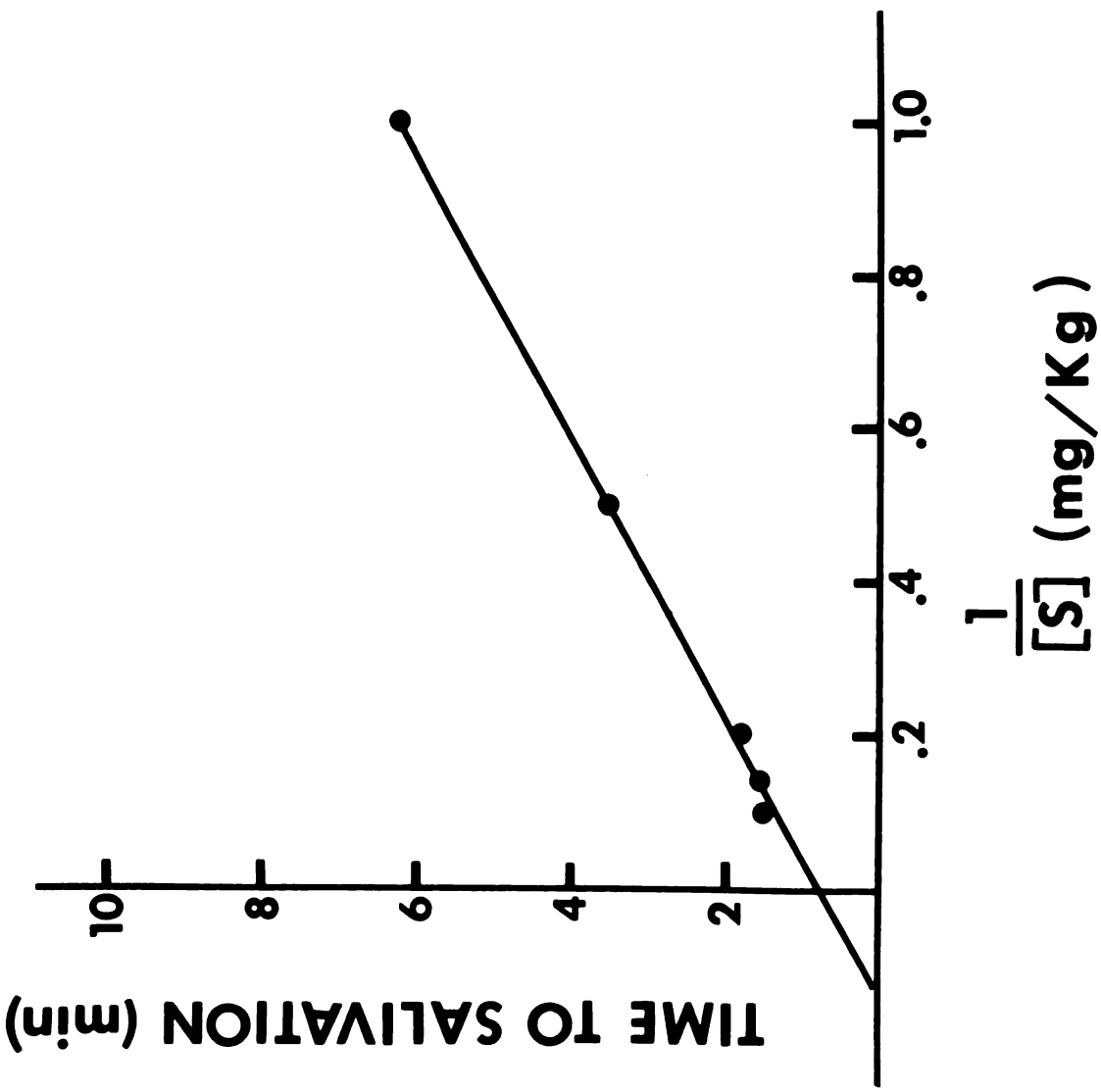

% OF ACETYLCHOLINE RESPONSE
200
150
100
50
1
2
3
4
5
6
7
DOSE
(µl)

Figure 15.--Reciprocal Plot of Dose vs Response for Slaframine in Live Rats. Small rats (100 g) were injected via heart puncture with slaframine and the time between the injection and the onset of salivation was measured.


TIME TO SALIVATION (min)
10
8
6
4
2
.2
.4
.6
.8
1.0
1/[S] (mg/Kg)

Typical reactions of flavins often involve free radicals formed by light. The requirement for light was tested in incubations with FMN and slaframine. No activity was found when the two compounds were combined in the dark. The same mixture, however, when exposed to light did cause the usual response by the guinea pig ileum.

To determine if the flavin carried out the reaction via a H_2O_2 intermediate, 8.6 mg of slaframine were added to 0.5 ml of 30% hydrogen peroxide. The solution was placed in a boiling water bath for ten minutes. Introduction of 10 µl of the mixture resulted in contraction of the ileum while the same amount of 30% hydrogen peroxide caused no response.

Microsomal incubations were carried out in the dark to see if the observed reaction could be attributed entirely to a light catalyzed reaction with a fravin. The activity observed when incubations were accomplished in the dark was equivalent to the amount of activity realized from microsomal incubations in the light. Furthermore when microsomal incubations in the dark were supplemented with FMN, FAD, or riboflavin, no increase in activity was observed. Thus it is shown that the microsomal reaction is indeed a separate reaction from the photochemical reaction.

Stability of the Metabolite Produced by FMN and Light

The metabolite produced by FMN and light was subjected to the same conditions as had the metabolite produced by

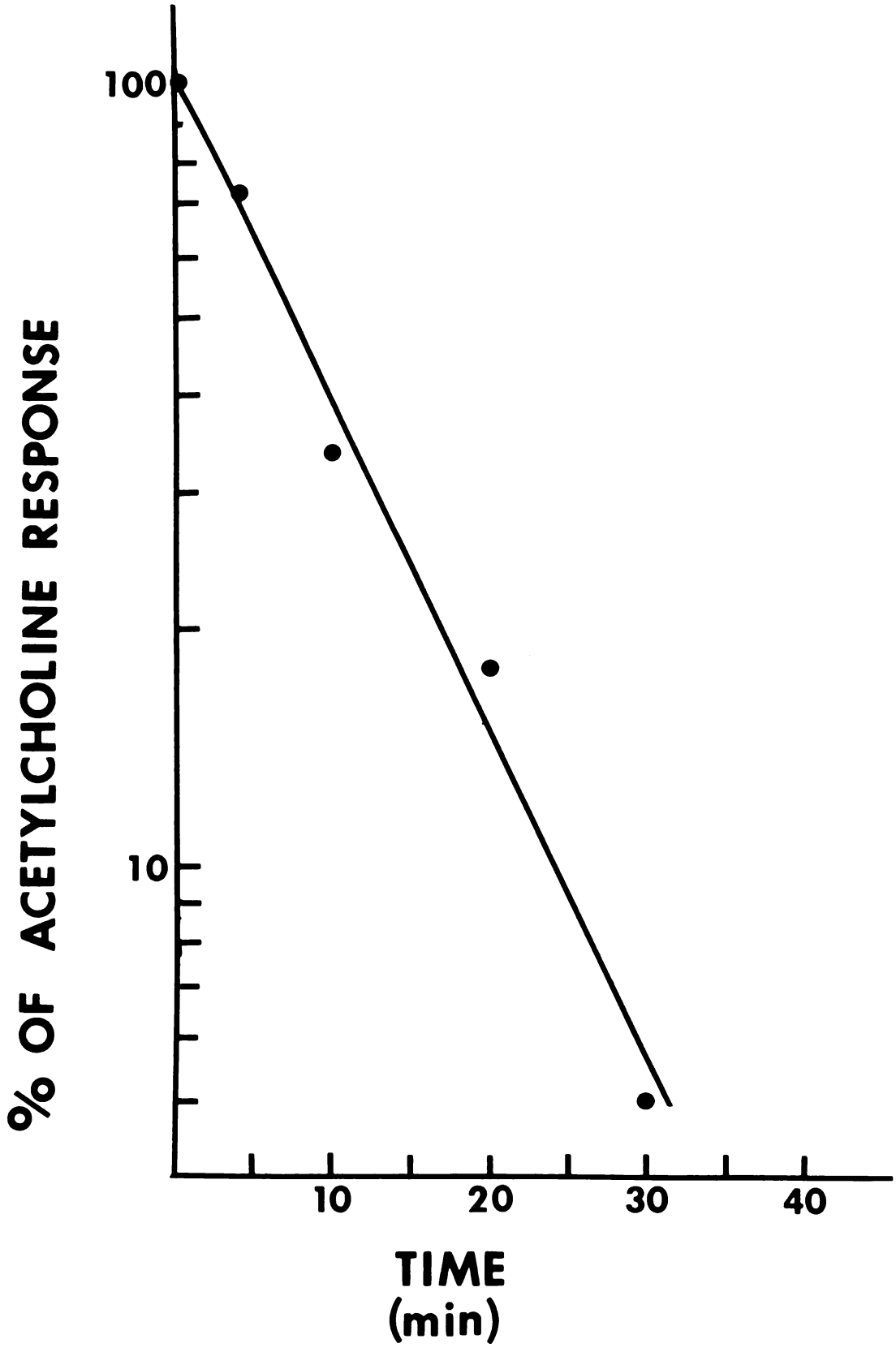
microsomes and NADPH. In all cases (acid pH, basic pH, boiling, etc.) the treatments gave identical results, namely, loss of cholinergic activity.

When active incubations were stored in the dark in the refrigerator for about 24 hours, activity was found to be absent, but could be regained by exposure to light for long periods of time. The reacquisition of activity proved to require longer time periods at night in the absence of sunlight than in the day when both artificial and sunlight were present. Further additions of fresh FMN and/or slaframine had no effect. On the other hand when active incubations were kept in the light and in the cold for 24 hours, activity was immediately observable.

The decay of the active metabolite could be followed by combining FMN and slaframine in the light and then storing in the dark for various periods of time prior to assaying with the guinea pig ileum. A logarithmic plot of the rate of decay yields a straight line indicating a first order rate constant for the decay process (Figure 16). The apparent half-life of the metabolite in the dark is about eight minutes.

Attempts to isolate the metabolite by TLC were unsuccessful. Samples were spotted directly onto TLC plates and developed with two solvent systems. The only Dragendorff positive spot corresponded to slaframine.

Figure 16.--Decay of the Active Metabolite in the Dark. The first order rate constant is demonstrated by the straight line of a plot of time versus the logarithmic of the activity. The apparent half-life of the metabolite is about eight minutes.


100
10
% OF ACETYLCHOLINE RESPONSE
10
20
30
40
TIME
(min)

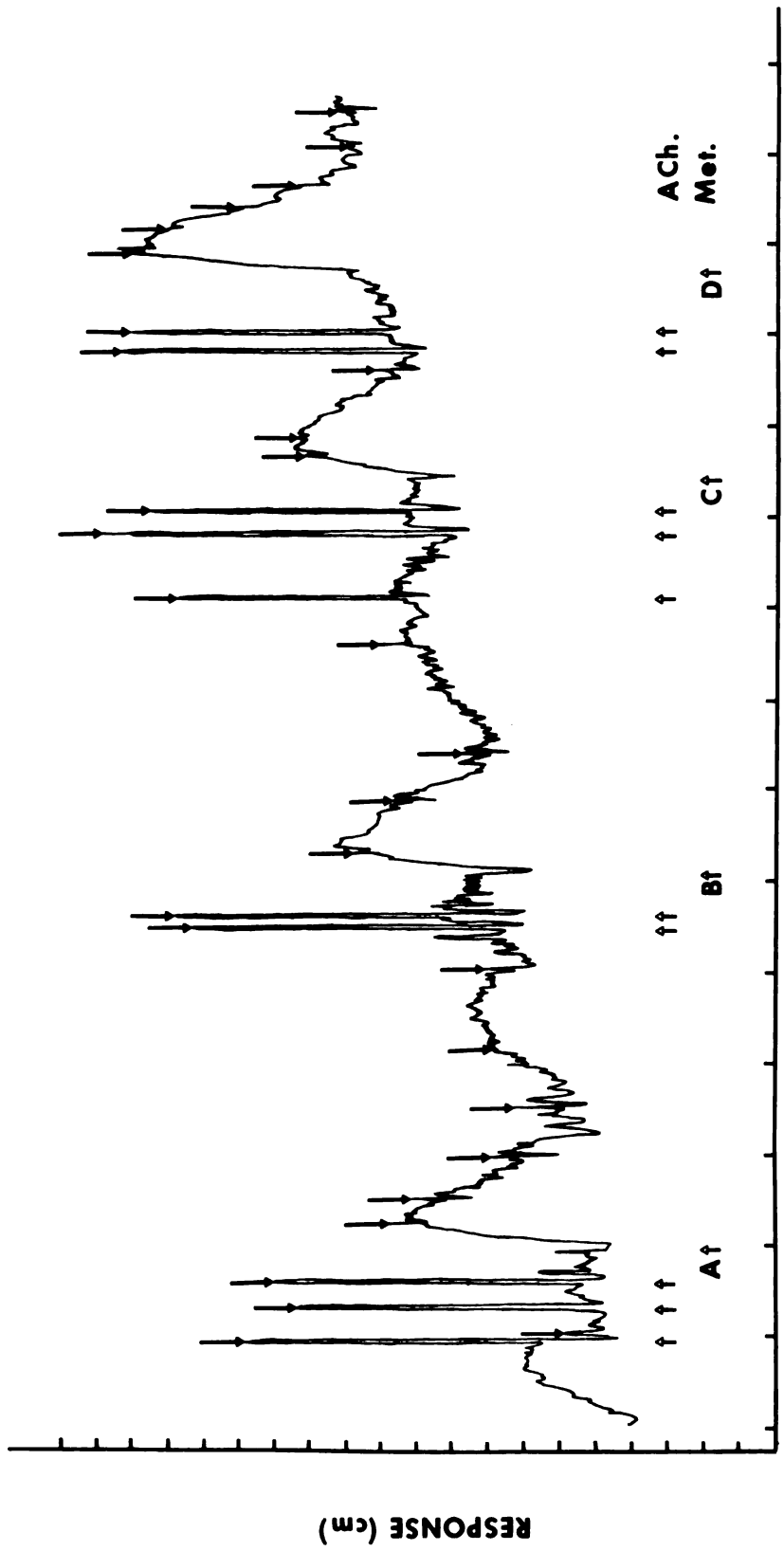
Pharmacological Effects of Activated Slaframine

Some unusual pharmacological actions of this metabolite would suggest that this compound is unlike any other cholinergic compound. Addition of the metabolite, either produced by microsomes and NADPH or FMN and light, caused a rather slow but prolonged contraction (Figure 17,A). The slow contraction was intermediate between the action of acetylcholine and an anticholinesterase such as eserine. Further evidence that the compound was not an anticholinesterase was obtained by the use of atropine. The addition of atropine to the organ bath at levels (8.6 μg), double that required for the reversal of eserine effects, failed to reverse the effect of the metabolite (Figure 17,B). These results suggests a very strong binding of the metabolite with the receptor.

Further experiments with atropine did confirm the fact that the action of the metabolite was cholinergic. Addition of atropine before the addition of the metabolite to the organ bath completely blocked its action, which is consistent with data obtained in animals when observing salivation in response to slaframine.

A gradual but nonetheless significant decrease in sensitivity was observed following administration of the active metabolite. No comparable result was observed when only acetylcholine was used to initiate contractions.

Figure 17.--The Pharmacological Effects of the Active Metabolite of Slaframine. The active metabolite of slaframine produced by microsomes and NADPH was added to the ileum bath alone (A), prior to atropine (B), and after atropine (C). Upper arrows indicate washings and lower arrows additions. Time markings are five minutes.


RESPONSE (cm)
TIME
A↑
B↑
C↑
D↑
ACh.
Met.

Biological Activity of Structurally Related Analogues

The structure-activity relationship of cholinergic compounds makes the study of compounds which are related structurally to slaframine interesting. In addition it should delineate some of the structure requirements for the unusual activity exhibited by the active metabolite of slaframine.

1-hydroxy-octahydroindolizine: The guinea pig ileum did not contract when 1-hydroxy-octahydroindolizine was administered by itself or when in combination with RMN.

1-acetoxy-octahdroindolizine: This compound causes a contraction by the guinea pig ileum whether given in combination with FMN or by itself. The observed contraction did not persist as did the response elicited by the active metabolite of slaframine. After a single wash the ileum relaxes to its resting state as it does following the administration of acetylcholine.

8-amino-octahydroindolizine: Of all the analogues tested, this is the only one which had an observable effect on the live animal. When mice were injected with this amine the animals seemed to be tranquillized. No effect was observed in the ileum, however, when this compound was given alone or with FMN.

N-acetyl- and N-acetyl-O-deacetyl-Slaframine: These analogues caused no response in the guinea pig ileum whether administered by themselves or with FMN. It has previously been shown that

deacetylsalframine is inactive in the live animal and thus one would not expect to see activity with analogues without the ester group (47).

EPR Studies of the Metabolite Produced by FMN

EPR spectra were recorded at room temperature with a Varian X-band spectrometer equipped with an optical transmission cavity. The magnetic field strength was calibrated with a Varian F-8 proton resonance flux meter, the frequency of which was monitored by a Hewlett-Packard frequency counter. The microwave frequency was calibrated with a Silverlab wavemeter. Light of a Zenon Lamp was focused on the sample by a quartz lens.

No free radicals were observed in the range where N-oxides absorb even with maximum sensitivity. The sensitivity of the instrument was such that a concentration of 10^{-7}M could be readily detected.

DISCUSSION

The data presented in this thesis confirms the indications obtained by Aust et al. (13) that slaframine, which is inactive itself, is bioactivated to a very potent and long lasting parasympathomimetic substance. Slaframine is activated by liver microsomes, but can also be produced non-enzymatically via a photochemical reaction with flavins.

All attempts to isolate a metabolite of slaframine have been unsuccessful. Both TLC and GLC of basic chloroform extracts of blood or urine of animals given slaframine revealed only the presence of slaframine or deacetyl-slaframine, even when radioactive slaframine was used. Livers of slaframine treated animals were also homogenized and extracted with chloroform with similar results, excluding the possibility that much of the metabolite was remaining in the liver. These results indicated that the metabolite might be either unstable or present in very minute quantities, or both. If the amount of metabolite was substantial but unstable, the decomposition product must be slaframine itself, since nearly all of the slaframine given was recovered as the same. The identical R_f values to authentic slaframine in three TLC solvent systems of the recovered material was taken as evidence that it was actually slaframine.

Results from liver perfusions seemed to confirm the results obtained in live animals, i.e. that no metabolite could be detected by the previously mentioned chemical methods. Radioactivity could be nearly completely recovered when ^{3}H-(acetate)-slaframine was used in the perfusion. All the radioactivity in the pH 10 chloroform extract was shown to be slaframine by TLC. A substantial amount of the tritium was not extractable by chloroform at pH 10, however, and the amount of counts in the water increased with incubation time. The water fractions were analyzed to determine if the tritium in the water was a metabolite of slaframine or just the acetate which had been cleaved. All the counts were shown to correspond to acetate which would indicate that hydrolysis was taking place and was time dependent.

The possibility that deacetylslaframine was the active metabolite of slaframine was rejected because biological activity could not be demonstrated when deacetylslaframine was injected into live animals.

Aust (47) had previously shown that slaframine is not hydrolyzed by a number of cholinesterases. Therefore the results of this experiment may indicate that slaframine may be altered to a species which is susceptible to hydrolysis by some esterases.

Attempts to demonstrate a metabolite of slaframine by TLC after incubation with various liver preparations (slices, crude homogenates and microsomes) were without success.

Since attempts to demonstrate the presence of an active metabolite of slaframine by chemical methods were unsuccessful, attention was shifted to the biological evidence for its presence. Aust (13) had previously shown that slaframine acted as a cholinergic drug in live animals. This along with the probability that the concentration of active metabolite was very low, made the guinea pig ileum an obvious assay.

Microsomal incubations containing slaframine and NADPH caused a sustained contraction of the guinea pig ileum while proper controls (Figure 8) failed to give a similar response. These results were considered conclusive evidence that there is an active metabolite of slaframine and that it is produced by liver microsomes.

The transformation could not be prevented by carbon monoxide inhibition even after repeatedly degassing and flushing with N_2 followed by gassing with carbon monoxide. Therefore cytochrome P-450, commonly involved in the terminal oxidation step in the metabolism of foreign compounds, was shown to be not involved in the activation of slaframine. There was an increase in activity when phenobarbital induced microsomes were used instead of control microsomes. This indicates that some portions of the microsomal drug metabolizing system might be responsible, but not the entire system. Another atypical characteristic was that the production of metabolite reached a maximum after approximately five minutes of incubation. This suggested that another cofactor may be

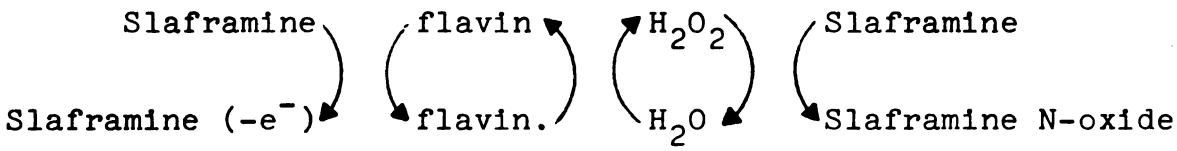
required for further increases in the production of the metabolite. Another explanation would be that the enzyme concentration was limiting, therefore experiments were designed to localize the enzyme system responsible for the bioactivation of slaframine, so that the responsible enzyme could be concentrated. Results showed that the greatest activity was in the microsomal fraction. A considerable amount of activity was also found in the washed mitochondrial fraction suggesting that the reaction may not be carried out solely by the mixed-function oxidases of the microsomal fraction; the usual location for drug transformations.

The search for an additional cofactor resulted in the finding that a marked stimulation in activity was observed with added free flavins (Table 3). Stimulation of metabolism of tertiary amines by added free flavins has previously been reported (48). The lack of a requirement for molecular oxygen and the apparent fast rate constant are also consistent with reports of the transformation of some tertiary amines to N-oxides (48). However, this stimulation, by flavins, was found to be nonenzymatic, which has not previously been reported for the transformation of other tertiary amines.

The nonenzymatic reaction with flavins is thought to proceed via a free radical since an absolute requirement for light was demonstrated. Photoreduction of free flavins goes via rapid dismutation of the semiquinone to the hydroquinone level. Since the photoreduction of flavins by tertiary amines has been reported (49, 50) it is conceivable that

either the half-reduced species of flavins, which is a free radical, or the process of producing it, is responsible for the observed reaction. Evidence for the latter mechanism has been obtained by comparison of the reaction rate with riboflavin, FMN, and FAD. McCormick (50) has reported that photoreduction of FMN and riboflavin to the hydroquinone level is approximately four times faster than the photoreduction of FAD. Similarly, the activation of slaframine by riboflavin or FMN was very rapid, however, then FAD was used as the flavin a much slower rate of production is observed (Figure 12).

If this free radical mechanism is indeed responsible for the activation of slaframine, slaframine may participate in the photoreduction of the flavin to the half-reduced, free radical species. The oxidized (i.e. minus one electron) slaframine perhaps is the active metabolite. Alternatively, the hydroquinone free radical of FMN, could react with slaframine, or with water to form hydrogen peroxide which could react with slaframine by some mechanism and form the active metabolite which may be the N-oxide of the tertiary amine.



Hydrogen peroxide and slaframine, after refluxing for ten minutes gave some activity, indicating that the above mechanism is plausible.

The concentration of the active metabolite is unknown, but is estimated to be very low. In experiments with the guinea pig ileum, one µl of an incubation mixture which is 2×10^{-4} M slaframine is added to the organ chamber which has volume of 20 ml (1×10^{-8} M final concentration). This concentration gave a response approximately equal to 2×10^{-8} M acetylcholine. Thus, if slaframine was completely converted to the active metabolite, the active metabolite would appear to be roughly as active as acetylcholine. However, acetylcholine is readily hydrolyzed by acetylcholinesterase, so its effective concentration is not known but is probably much less than 2×10^{-8} M. Slaframine is insensitive to the action of acetylcholinesterase (47), so its effective concentration is the actual concentration. If such comparison can be made with acetylcholine, then the concentration of the active metabolite is even less. In all probability the active metabolite is more active than acetylcholine, which makes it perhaps the most potent physiological substance known at the present time. The concentration of the free radical, which, in addition to the problem of stability, could easily be present at concentrations below the limits of detection by EPR.

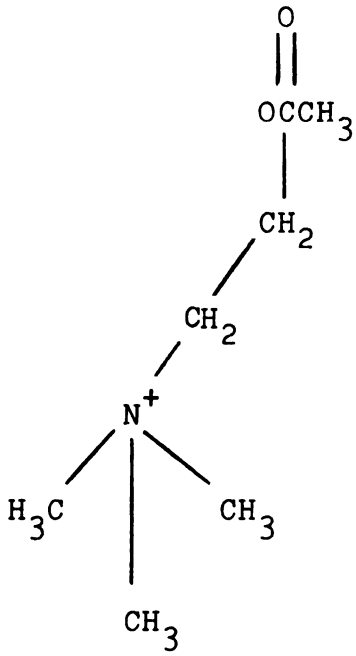
Failure in attempts to increase the amount of metabolite by increasing the irradiation periods suggests that the photodecomposition products may prevent the production of the active metabolite. This also explains why activity cannot be regained in a solution which has lost activity after setting

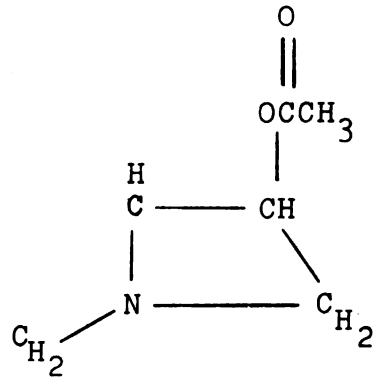
at room temperature in the light, even after additions of fresh slaframine and FMN.

The prolonged contraction observed by the guinea pig ileum even after repeated washings suggests that the active compound has a very high affinity for the receptor. Indeed the binding of the active metabolite to the receptor may be irreversible. After the addition of the active metabolite and repeated washings, the ileum rarely completely relaxes and the response to acetylcholine diminishes. If the active metabolite is a free radical and the anionic site of the receptor had at least a partial negative charge the binding could be covalent.

Proof that the active metabolite binds to the acetylcholine receptor was obtained by experiments with atropine. Since atropine will block the effects of the metabolite if given previously, its action must be elicited by means of the acetylcholine receptor. If it was blocking cholinesterase which would allow acetylcholine to act, atropine would reverse its effects if given at the height of contraction. This does not happen (Figure 17), in fact there is no difference between application of atropine to the contracted ileum and washing. Therefore the metabolite must act directly on the receptor.

One should recognize the structural similarity between acetylcholine and slaframine:





The structure-activity relationship is at present only speculative, but the similarity in structure of slaframine and acetylcholine suggest that the active metabolite is bound in the same manner as acetylcholine. The receptor most likely binds at the ester and tertiary amine positions. It is reasonable that the stronger binding of the metabolite is due to the anionic site rather than the esteratic site. A very plausible explanation of this could be a partial or complete covalent bond is formed between the ring nitrogen of the metabolite and the receptor site.

A possible explanation of the specificity with which the active metabolite of slaframine acts can be built on the structural properties of slaframine. If the receptors at various glands differ in the spatial arrangement between the esteratic and anionic sites, only those which have the exact

structure to compliment the rigid structure of slaframine would be susceptible to binding by activated slaframine. Since acetylcholine is flexible it can accommodate a wide range of spacial differences between the esteratic and anionic sites. Therefore acetylcholine stimulates all cholinergic receptors while the active form of slaframine stimulates only those receptors which have the correct spatial arrangement for these binding sites.

An esthetically pleasing possibility for the bioactivation of slaframine would be to create a positive charge on the tertiary nitrogen. If slaframine gives up an electron from its tertiary amine nitrogen, (to a flavin in the presence of light) the tertiary nitrogen acquires a positive charge. This charge would be stabilized to some extent by the primary amine group in the six position of slaframine. This could give the radical a significant lifetime rather than its probable immediate destruction in the absence of the primary amine. The unpaired electron is subsequently available for sharing with the anionic site of the receptor molecule and could form a partial covalent bond with the receptor. This would explain the extreme persistence of action of the metabolite on the guinea pig ileum despite frequent washings. If this assumption is correct, the active metabolite of slaframine could be an extremely useful tool in the isolation of the receptors for acetylcholine.

Failure in the attempts to isolate the active metabolite in the *in vivo* experiments discussed above could easily be explained if the active species was merely slaframine minus an electron. Changes in pH would certainly affect the stability of such a species so it would be destroyed by the pH 10 chloroform extractions. In addition, the radical would be more polar and thus would be less readily extractable by organic solvents. However, these arguments are probably not necessary, for all attempts to show a product of slaframine and FMN by TLC under very mild conditions (no pH change or extraction) were unsuccessful. TLC of the mixture 24 hours after mixing also failed to show any product of slaframine.

A very likely candidate for the enzyme responsible for the enzymatic activation of slaframine is NADPH-cytochrome c reductase. This FAD containing enzyme has been speculated as being part of the electron transport chain of the mixed-function oxidases of liver microsomes, however, this has not been confirmed. Its candidacy as the enzyme responsible for activation of slaframine is supported by the finding that the reaction requires NADPH which can act as the electron donor to reduce the flavin in the reductase.

Confirmation that the enhancement in activity obtained by added free flavins was nonenzymatic came from experiments employing microsomes in the dark. Microsomes themselves continue to produce the metabolite in the dark but no increase in activity is obtained by supplementing the incubation

mixtures with riboflavin, FMN, or FAD. This clearly points out that two separate reactions are involved. Both reactions could very well be carried out by flavins. The microsomal reaction, as stated above, could involve the flavoprotein, cytochrome c reductase which gets its reducing power from NADPH thus differing from free flavins which abstract an electron from an electron souce, like a tertiary amine, in the presence of light.

The transient nature of the active metabolite is evidenced by the fact that its activity in the dark decays with a half-life of eight minutes. The rate of decay of the metabolite is dependent on pH and temperature. Pathways of decay, however, may be different at low pH than at high pH. Slaframine is known to be readily hydrolyzed by basic conditions. Hydrolysis of the ester moiety is probably not significant in these experiments, however, since slaframine can still be detected by TLC after exposure to pH 10 or pH 2.

It is difficult to understand why the liver carries out any reaction with slaframine. The typical drug metabolizing reactions of the liver make drugs sufficiently polar to be excreted by the kidneys. Slaframine is quite polar and can be excreted readily in the urine as was determined by the live animal experiments. Nearly all of the slaframine could be recovered from the urine. One cannot rule out the possibility that slaframine is in its active form when the kidney

removes it from the blood and then decomposes to slaframine once it is in the urine.

One also cannot eliminate the possibility that the hydrolysis of the active compound may be much more easily accomplished. Thus the actual detoxification of the "toxic" metabolite could occur by hydrolysis while excretion of the "non toxic" slaframine is carried out without alteration. This theory is consistent with results of administration of ^{3}H-(acetate)-slaframine to the perfused liver. The results of this experiment demonstrate the loss of acetate from slaframine with time. Slaframine and deacetylslaframine are also found in the urine of animals given slaframine.

The stability of the metabolite, produced by different systems, was not found to differ. Activity was lost when either the microsomal or flavin incubation mixtures were subjected to acidic conditions (pH 2), basic conditions (pH 10) or boiling. This was taken as additional evidence that the metabolites were the same. No difference could be observed in the pharmacological effects of the enzymatically produced and the nonenzymatically produced metabolite.

Studies with analogues of slaframine help to delineate the necessary structural properties for activity. For example, all attempts (as previously mentioned) to demonstrate activity in analogues which lack the O-acetate group have been unsuccessful. Thus an absolute requirement for the acetate group has been demonstrated. 1-acetoxy-octahydroindolizine showed some biological activity by itself, but

could readily be washed off the receptor sites. The observed action may have been a general response of the acetate ester because the concentration was unknown. If the observed reaction of this analogue is specific, it may point out the necessity of the primary amine for the long lasting effect of the metabolite. This would strengthen the arguments for a free radical since the presence of the primary amine would contribute significantly to the stabilization of a free radical. Further evidence for this is shown by the lack of activity when an electron releasing group is attached to the primary amine as with N-acetylslaframine. This function of the primary amine gains interest in view of the fact that both 1-hydroxy-octahydroindolizine and 1-acetoxy-octahydroindolizine have been implicated as precursors of slaframine (10). It is interesting to speculate about the purpose of the enzyme which places the primary amine in the six position of slaframine during its synthesis by R. leguminicola.

All attempts to find a free radical by EPR have thus far been unsuccessful, but this does not eliminate the possibility of its presence. The sensitivity of EPR for the detection of free radicals is about 10^{-7} M. It is entirely possible that the concentration of the metabolite is less than this. It may also be necessary to lower the temperature of the solution to see the radical. This would require the use of an organic solvent. The production of metabolite has not yet been investigated in organic solvents.

One problem with the use of organic solvent is that they may have an adverse effect on the guinea pig ileum, which is at the present time the only assay method known.

SUMMARY

Slaframine is bioactivated to an active compound which has a stronger affinity for the acetylcholine receptor than either acetylcholine or atropine. This active metabolite can be produced by liver microsomes and NADPH *in vitro*. It can also be produced nonenzymatically by flavins in the presence of light. The production of the active metabolite by the photochemical reaction with flavins results in a much higher (about 200 fold) yield of metabolite than the microsomal production. The active compound appears to be effective at molar concentrations below the minimum level of acetylcholine required to elicit a response.

Isolation attempts have failed thus far because apparently only an extremely small amount of the metabolite is present and it is unstable. Chemical evidence nor the presence of the metabolite of slaframine has not been successful for the same reasons.

The only assay at the present time is the guinea pig ileum. The active form of slaframine has a sustained effect on the ileum and cannot be reversed by atropine. The loss of ability of the guinea pig ileum to contract occurs much faster after exposure to the metabolite than when only exposed to

acetylcholine. This makes the guinea pig assay undesirable and a chemical assay more appealing.

Certain structural properties of slaframine have been shown to be important for the observed effect of the metabolite. The acetate ester and the ring nitrogen (three carbons away) are necessary for activity. The primary amine group may be important, but conclusive evidence awaits the absolute identity of the active compound.

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