METABOLISM OF 2,3-DIMETHYLPYRAZINE

By

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1971

Submitted to the Faculty of the Graduate College
of the Oklahoma State University
in partial fulfillment of the requirements
for the Degree of
DOCTOR OF PHILOSOPHY
December, 1976

METABOLISM OF 2,3-DIMETHYLPYRAZINE

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ACKNOWLEDGEMENTS

The author wishes to express his appreciation to his major advisor Dr. George V. Odell for his assistance in this study and in the preparation of this manuscript. Thanks are extended to Drs. Kermit L. Carraway, Ta-Hsiu Liao, Earl D. Mitchell and Ernest M. Hodnett for their suggestions and assistance as members of the advisory committee and to Oklahoma State University, Department of Biochemistry for financial assistance and facilities.

The author is also grateful to Dr. Tak K. Chan for assistance and advice during the course of these investigations. Thanks are extended to Mr. Hanford Miller and Dr. Daryl Long who encouraged the author to attend graduate school. The assistance of Kevin Castleberry in the preparation of figures is also acknowledged. A very special gratitude and appreciation is extended to my wife, Barbara Reed, for her suggestions and encouragement during this study.

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CHAPTER I

INTRODUCTION

Pyrazine compounds are present in a variety of foods, beverages and pharmaceuticals. Various pyrazines are known to have antibacterial, antituberculosis, antidepressant, diuretic, anticonvulsant, pesticidal and herbicidal activity (1). Alkylpyrazine compounds are present in a variety of roasted foods and are responsible for many of the characteristic flavors and odors of these foods. Beef, cocoa, potato and soybean products, coffee, tomatoes and peppers are but a few examples of foods that have been shown to contain pyrazine compounds (2). It has been suggested that these compounds are probably formed via complex reactions between alpha amino acids and carbohydrates (3).

In recent years patents have been granted for the use of various pyrazines as flavor additives for food and tobacco products (4, 5, 6, 7, 8, 9). In the near future meat substitutes, such as vegetable and "single cell" protein, coupled with flavor additives, are likely to play a key role in feeding the world's increasing population. Thus it is possible that pyrazine compounds may be consumed in larger amounts.

Aside from their strong organoleptic properties mono- and dimethylpyrazines are also central nervous system depressants having hypnotic and anticonvulsant activities. Of a series of pyrazines tested in this regard, Nishie *et al*. (10) have shown that 2,3-dimethylpyrazine has the strongest activity. Its hypnotic activity is one-thirteenth that

of sodium phenobarbital. Much work has been published on the presence of alkylpyrazines in various foods, but little on the metabolism of these compounds. In view of this, the objectives of this work are as follows:

1) To synthesize and determine the toxicity of 2,3-dimethylpyrazine.
2) To define to metabolic fate of the compound in a mammal; that is, to determine how fast and in what form(s) it is excreted.
3) To localize the probable site of metabolism in the animal.
4) To attempt to induce the metabolizing system and monitor any changes in the metabolic fate of the compound.

CHAPTER II

LITERATURE REVIEW

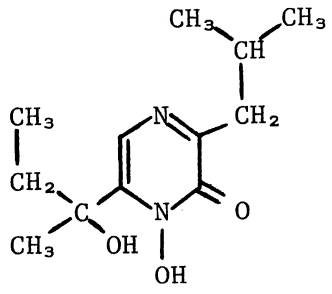
Pyrazines, or 1,4-diazines, are based on the six-membered pyrazine nucleus having two nitrogen atoms para to each other. There are two other classes of diazines. They are pyridazines and pyrimidines which have their two nitrogens situated ortho and meta, respectively.

Pyrazine compounds are somewhat similar to their pyridine analogs in reactivity at their alpha carbon atoms. When compared to the pyridines, they have a higher pi-electron density at the nitrogen atoms and lower electron density at the carbon atoms. The pKa of pyrazine is 0.65. Compared to pyridine (5.2), pyridazine (2.33) and pyrimidine (1.30) it is noted that a second ring nitrogen has a strong base-weakening effect and that the basicity varies inversely to the separation between the two nitrogens in the diazines (1). Each methyl group increases the basic strength by approximately 0.7 pK units (11).

The reactivity of the pyrazines is somewhat similar to that of the other diazines and pyridine. However, they are stable to strong acid and strong alkali which could destroy the pyrimidine ring. The pyrazine ring is more resistant to alkaline permanganate than benzene. Pyrazines are resistant to electrophilic substitution at ring carbon atoms, so electrophilic attack usually takes place at the ring nitrogens. Pyrazines undergo a wide variety of reactions, including reduction, quaternization, N-oxidation, alkylation and halogenation (1).

Pyrazines are synthesized by two general methods. These are the reaction of other heterocycles and the condensation of aliphatic components. Examples of the first type are dehydrogenation of piperazines and permanganate oxidation of phenazines. Examples of the second type are reactions of alpha, beta-dicarbonyl compounds, alpha-aminocarbonyl compounds, alpha-halogenoketones and alpha, beta-diamines (1).

Pyrazines occur in both foods and natural products. They are found in fusel oil and as products of microbial metabolism (1, 12). The aspergillic acids such as hydroxyaspergillic acid (I) are a group of antibiotic pyrazine compounds produced by cultures of Aspergillus (13). 2-Methoxy-3-isobutylpyrazine gives the characteristic odor to green bell peppers and is a major component in the steam distillate of the oil of those peppers (14). The presence of this compound in peppers indicates that biosynthetic pathways for pyrazines occur in higher plants as well as in molds and bacteria.



I

Standinger and Reichstein (15) were the first to report of

pyrazines in foods. They found alkylpyrazines in the aroma fraction of coffee. Gianturco et al. (16) indicated the probable importance of pyrazines in the flavor of cooked foods. In 1973 Maga and Sizer (2) published a review article listing pyrazines found in various food products. They gave an extensive list of references for the pyrazines found in foods. 2,3-Dimethylpyrazine was found to occur in 17 different food products. More recently pyrazines have been found in baked potato aroma and Gouda cheese (17, 18).

Many papers have been published on the origins of pyrazines in roasted foods. In 1893 Stoehr produced 2,6-dimethylpyrazine by heating solutions of ammonia and glucose (19). In 1966 both Dawes (20) and Mason (21) concluded that pyrazines were formed by reactions of amino acids and sugars. In 1969 Wang et al. (22) proposed a mechanism for pyrazine formation. This mechanism involved thermal decomposition of sugars into dicarbonyl fragments which then reacted with amino acids. This adduct was then degraded to form alpha-amino carbonyl compounds which could condense to form pyrazines through dihydropyrazine intermediates. The presence of dihydropyrazines in foods, however, has never been established, possibly because of the harsh treatments used for isolating food volatiles. Koehler (23) later showed that in the formation of pyrazines, carbohydrates provide the carbon atoms and amino acids provide the nitrogens. Further work on pyrazine formation in model systems was done by Koehler (24), Rizzi (3) and Wang and Odell (25).

Pyrazine compounds exhibit a wide range of biological activity. Various pyrazines have been shown to have diuretic, anti-leprosy, anthelmintic, hypoglycemic, antimalarial and antibiotic activities (26,

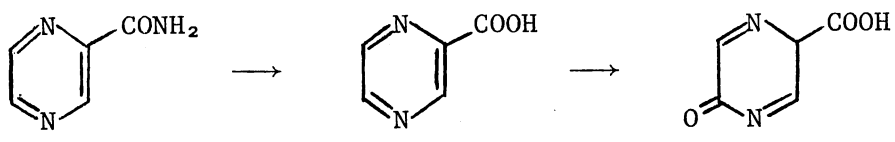
27, 28, 29, 30, 13). Pyrazinamide has been used as an antituberculosis drug at dosages of 35 mg/kg/day (31). Some pyrazine nucleosides have been prepared in attempts to develop new anti-tumor agents. It was hoped that such unnatural nucleosides could be incorporated into nucleic acids and block cell division and growth (32). The pyrazine analog of uracil has been shown to inhibit the growth of bacterial cells at 10^{-5} molar (33). Of course pyrazines have strong organoleptic properties. 2-Methoxy-3-isobutylpyrazine which has the odor of green peppers has an odor threshold of 0.002 parts per billion in water (34).

For many years there has been interest in the use of ammoniated molasses as a protein substitute in cattle feed. A problem is that toxic by-products are formed. The convulsant 4-methylimidazole was suspected to be a possible toxin. In 1970 Nishie et al. (10) found that 4-methylimidazole was produced by ammoniation of glucose. This compound mimicked the toxic effects in cattle produced by ammoniated molasses. Mono- and dimethylpyrazines were also produced in the reaction. They had anticonvulsant activity. 2,3-Dimethylpyrazine had the strongest activity, about five per cent as strong as sodium phenobarbital. Since the convulsions induced in mice by 4-methylimidazole were prevented by alkylpyrazines, it was suggested that the ammoniation of feeds might be done under conditions favorable to the formation of such pyrazines.

The alkylpyrazines in general have low toxicity. 2,3-Dimethylpyrazine has an LD_{50} in mice of approximately 1.4 mg/g as tested by Nishie (10). The dihydropyrazines are probably more toxic. The LD_{50} for 2,3-dimethyl-5,6-dihydropyrazine was shown to be 0.36 mg/g by Smith (35). He noted extreme muscular contractions immediately after

injection.

The antituberculosis drug pyrazinamide has been shown to have many other biological activities. It was observed to increase pyridine coenzyme levels, increase prothrombin time and increase alkaline phosphatase (36, 37, 38). Both pyrazinamide and pyrazine carboxylic acid have been found to inhibit lipolysis (39). The main side effect of pyrazinamide encountered clinically is urate retention. In 1957 Cullen et al. (40) found that the drug could cause hyperuricemia through actions on the tubular transport of uric acid. This has been implicated in the precipitation of some clinical gout (41). In fact pyrazinamide is the most powerful clinically encountered drug for causing urate retention, causing an 80-90 per cent reduction in the renal clearance of urate (42). In 1972 Weiner and Tinker (42) showed that pyrazinamide (II) exerted its uricosuric effect through its metabolite, pyrazinoic acid (III), which acts by inhibition of tubular secretion of urate. They found that the metabolism of pyrazinamide involves a slow hydrolysis to pyrazinoic acid followed by oxidation of pyrazinoate to 5-hydroxypyrazinoic acid (IV), catalyzed by xanthine oxidase. The primary excretion product is pyrazinoic acid.



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Since not much has been published on the metabolism of alkylpyrazines, studying the metabolic fate of similar compounds may be useful. For example in 1887 His (43) observed that pyridine was N-methylated in dogs to produce an N-methylpyridinium metabolite. N-Methylation as well as N-oxidation are involved in the metabolism of nicotine (44, 45). Nicotinamide is excreted after N-oxidation or formation of a 2- or 4-pyridone (46). The sulfonamide drug sulfapyrazine is excreted in rabbits as an acetyl derivative, with the pyrazine ring remaining intact (47). Both histamine and imidazole-4-acetic acid are metabolized in mice and rats by the formation of an N-riboside. This type of conjugation has been implicated in the metabolism of other heterocyclic nitrogen compounds also (48).

From work on 2,3-dimethylpyrazine Elgin (49) has found that 80 per cent of an injected dose is excreted in the urine within 24 hours. Less than one per cent of the dose was expired as $^{14}CO_2$ during the same 24 hours. In earlier work Koehler (50) reported on the urinary excretion of unchanged dimethylpyrazine. He found that 2.5 per cent and 1.1 per cent of the injected dose was eliminated unchanged in the urine in 24 hours after 75 and 50 mg doses, respectively. Forty-three hundredths per cent of a 70 mg dose was eliminated unchanged through the lungs. Smith (35) studied the urinary metabolites of 2,3-dimethyl-5,6-dihydropyrazine. He found 43 per cent of an injected dose to be excreted in the urine in 24 hours. Three major and five minor urinary metabolites were separated using column chromatographic techniques. Thus, part of the higher toxicity of the dihydro compound may be due to its slower metabolism.

Recently Hawksworth and Scheline (51) reported on the metabolism of

several pyrazine derivatives that are important to food flavor. They studied the urinary metabolites of alkyl and alkoxypyrazines in rats. They found that alkyl-substituted compounds were oxidized to their corresponding acids and excreted as acids or their glycine conjugates. In the case of 2,3-dimethylpyrazine which has two adjacent alkyl groups, alkyl oxidation was less extensive and the major metabolite found was 2,3-dimethyl-5-hydroxypyrazine. Using an intragastric dose of 100 mg/kg they found that aliphatic oxidation and ring hydroxylation accounted for 13 and 37 per cent of the dose, respectively. No glycine conjugates were observed. Analogous studies on pyridine compounds showed the same pattern of alkyl oxidation predominating except when two adjacent alkyl groups were present. They found that 2-methoxypyrazine and 2-methoxypyridine were both metabolized by O-demethylation and ring hydroxylation. No evidence was found for either N-oxidation or biliary excretion of any of the pyrazine derivatives.

Whenever compounds normally considered as foreign (xenobiotics) gain entry to a mammalian system they are usually metabolized to other substances. These reactions are commonly referred to as detoxification reactions (48). In reality the foreign compound is either metabolized to a less toxic and usually more polar compound, metabolized to a more toxic compound, or excreted unchanged. Increasing the polarity of a compound increases its solubility and usually increases its rate of excretion. Detoxification mechanisms can involve two phases. The first phase involves oxidation, reduction or hydrolysis, while the second phase is a synthetic one, usually involving a conjugation. An example of this is benzene which is oxidized to phenol and excreted as the glucuronide conjugate (48).

Most foreign compounds are metabolized in the liver but metabolism may also occur in other organs such as the kidneys, lungs, intestinal mucosa, blood and skin. Most of the oxidative reactions take place in the endoplasmic reticulum (microsomes) of hepatocytes but the exact intracellular location of metabolism varies with respect to the reaction type and animal species involved. Glycine conjugation occurs in the liver mitochondria of rats but only in the kidneys of dogs. Glucuronic acid conjugation occurs in the liver microsomes (48). Some other reactions, such as alcohol oxidation occur in the soluble fraction (52).

In all tissues capable of metabolizing xenobiotics the endoplasmic reticulum is the site where most of the oxidation reactions are centered. The cytochrome P-450 system is responsible for a large number of these reactions. This system transfers an oxygen atom from molecular oxygen to the compound metabolized. The cytochrome itself is present in the cell in a protein-bound form and it is the nature of this protein that is thought to determine the specificity of these reactions.

Xenobiotics are mainly excreted in the urine, bile and feces. Recent studies have shown that the mode of excretion depends on molecular weight. Compounds under a certain molecular weight are excreted mainly in the urine, while compounds above that weight are excreted in the bile. This number, defined as the point at which there is at least ten per cent biliary excretion, varies with different species. For anionic compounds, it is about 325 in rats and probably about 500 in man (53, 54). For monoquaternary ammonium cations, the figure is about 200±50 (55).

A number of factors can affect the metabolism of foreign compounds. Many of these are discussed in articles by Williams and Milburn (50)

and Vessel et al. (56). Some of the basic factors are species, age, strain, route of administration, time of day, sex and nutritional state. One of the most important affectors is the presence of another foreign compound. Many such compounds induce or inhibit the detoxifying enzymes. This phenomena was first observed by Brown, Miller and Miller (57). They found that the oxidation of carcinogenic dyes was enhanced in animals pre-treated with the dyes. It has since been shown that this induced system is located in the liver microsomes. This effect, known as microsomal enzyme induction, appears to involve an increased rate of enzyme synthesis (58). The components of the P-450 system have also been shown to increase in amounts during induction (52).

Since the speed in which a drug is metabolized determines the duration and intensity of its action, enzyme induction is very important pharmacologically. For example barbiturates decrease the effect of coumarin anticoagulants by increasing their rate of metabolism. The effect is also important since the natural steroid hormones are hydroxylated by the same inducible system (59). The inducers vary widely in structure. Phenobarbital is the classic inducer, but hundreds have been identified. As an example a diet of charcoal-broiled meat has been shown to significantly alter the rate of phenacetin metabolism in man (60).

CHAPTER III

MATERIALS AND METHODS

A. Materials

Ethylenediamine and diethyl ether were obtained from Fisher Scientific Company, Chemical Manufacturing Division, Fair Lawn, New Jersey. 2,3-Butanedione was purchased from Eastman Kodak, Rochester, New York. Pfaltz and Bauer, Inc. of Flushing, New York supplied catalytic copper chromite. ^{14}C-ethylenediamine was obtained from New England Nuclear of Boston, Mass. Alumina was from ALCOA Chemicals, Bauxite, Arkansas. Dowex 50WX8 and Dowex 1X8 were from J. T. Baker Chemical Co., Phillipsburg, New Jersey and Bio-Gel P-2 was supplied by Bio-Rad Laboratories, Richmond, California. Cellulosepulver MN300, Silica Gel G and Suprapur potassium bromide were purchased from Brinkman Instruments, Westbury, New York. ChromAR 500 and magnesium chloride were from Mallinkrodt Chemical Works, St. Louis, Missouri. Aldrich Chemical Co. of Milwaukee, Wisconsin supplied pyrazinamide, pyrazine-2-carboxylic acid and 2,3-pyrazine dicarboxylic acid. 2,3-dimethylpyrazine was from Chemicals Procurement Laboratories, Inc. of State College, Pennsylvania (used only as a reference compound). Propylene glycol was supplied by the Oxirane Chemical Company, Houston, Texas. Injection sodium phenobarbital U.S.P. was from Wyeth Laboratories, Inc., Philadelphia, Pennsylvania. Sigma Chemical Co. of St. Louis, Missouri

supplied NADH, NADP, glucose-6-phosphate, glucose-6-phosphate dehydrogenase, and nicotinamide. CD-1 strain albino male mice were from the Charles River Co., Wilmington, Mass. and Holtzman albino rats were from the Holtzman Co., Madison, Wisconsin. All animals were housed in plastic cages with metal lids and a bedding of crushed corn cobs. The animals were given Purina Rat and Mouse Pellets (Eckroat Seed Co., Oklahoma City, Oklahoma) and water ad libitum.

B. Synthesis of 5,6-^{14}C-2,3-Dimethylpyrazine

The synthesis of both 2,3-dimethylpyrazine and 5,6-^{14}C-2,3-dimethylpyrazine involved the same two step reaction. The first step, based on the method described by Flament and Stoll (61), was the condensation of ethylenediamine with 2,3-butanedione. The resulting compound, 2,3-dimethyl-5,6-dihydropyrazine, was then catalytically dehydrogenated in the second step, as described by Kitchen and Hanson (62). The product, 2,3-dimethylpyrazine, was then distilled in vacuo and stored dessicated under nitrogen at -6° C.

The first reaction step was conducted in a half-liter three-neck round-bottom flask. A twelve-inch water-cooled condenser was placed in one neck of the flask and a 125 milliliter separatory funnel was placed in the other neck. The center neck was fitted via ground glass connectors to a motor driven Teflon stirrer. The motor was powered by compressed air. The flask was two-thirds submerged in an ice-water bath. 89.7 millimoles of ethylenediamine was reacted with a twenty-five per cent excess of 2,3-butanedione (112.1 millimoles), each reactant diluted with diethyl ether to a 1:10 weight-volume ratio. Both the ethylenediamine and the 2,3-butanedione were purified by fractional

distillation before use. The same reactant amounts were used for the synthesis of unlabelled and ^{14}C-labelled dimethylpyrazine. For the labelled compound the ethylenediamine was spiked with 250 microcuries of ethylene-1,2-^{14}C-diamine dihydrochloride, having a specific activity of 1.82 millicuries per millimole. In the synthesis the ethylenediamine was placed in the cooled reaction flask and allowed to cool for a few minutes. The 2,3-butanedione was then added dropwise with stirring, taking one hour for the addition. The mixture was then stirred an additional fifteen minutes at 0° C, allowed to come to room temperature and stirred for fifteen minutes at room temperature. Using a separatory funnel, the ether layer was removed and the aqueous layer was extracted with six fifteen-milliliter washes of diethyl ether. The ether fractions were then pooled and concentrated on a rotary evaporator.

For the second reaction step the concentrated ether fraction containing the dihydropyrazine was slowly added from a dropping funnel through a three-fourths by five-inch catalytic column of ten per cent copper chromite on eight to fourteen mesh F-1 alumina at 300° C. The bottom of the column was connected to a two-neck flask with a twelve-inch water-cooled condenser fitted in the other neck. This flask was mounted in an ice-water bath. The catalytic column was heated with an asbestos heating tape wrap controlled by a rheostat.

The resulting 2,3-dimethylpyrazine was purified by fractional distillation under reduced pressure. The round-bottom flask from the second reaction step was fitted with a three-inch reflux condenser attached to a four-inch water-cooled condenser. The condenser was coupled through a vacuum nipple to a water aspirator. The end of the condenser was connected through a ground glass joint to a collection

flask with three 50 milliliter round-bottom flasks attached. The flask containing the impure dimethylpyrazine was heated with a heating mantle coupled to a rheostat. The solution was distilled at 12-14 millimeters of mercury absolute pressure. The second fraction, boiling at 47.5 to 49° C was collected as the purified 2,3-dimethylpyrazine.

C. Gas-Liquid Chromatography

A Perkin-Elmer 990 gas-liquid chromatograph with a flame ionization detector was used. A one-fourth inch by six foot column packed with five per cent carbowax 20M (wt./wt.) on 100/120 mesh Gas Chrome Q was used. The column was operated isothermally at 130° C with nitrogen as the carrier gas at seven ml/min. Both the injector and detector temperatures were 250° C. Two microliter samples at a concentration of 500 micrograms per ml. in methylene chloride were injected.

D. Liquid Scintillation Counting

Packard Model 3320 liquid scintillation spectrometers were used for the majority of this study. A Beckman LS-3150T instrument was also used. Standard glass counting vials with ten ml. of counting cocktail mixed according to Bray (63) were used. Quench corrections were made using the channels ratio method.

E. Toxicity Studies

The LD_{50} (lethal dose 50%) of the synthesized 2,3-dimethylpyrazine was determined using the classic method of Reed and Muench (64). Albino male mice (25-30 gram) were injected intraperitoneally with 0.25 ml. of different dilutions of 2,3-dimethylpyrazine in sterile 0.9% saline.

Four mice were used at each dilution. A one c.c. tuberculin syringe equipped with a 26-gauge one-half inch needle was used. The mice were observed for three days after injection, but all the mice that died did so within an hour after injection.

F. Injection and Urine Collection

For the purpose of metabolite isolation, adult (at least 250 gram) male rats were each injected intraperitoneally with one ml. of a solution containing 3.53×10^6 dpm (about 50 mg) of 5,6-^{14}C-2,3-dimethylpyrazine in 0.9 per cent sterile saline. The animals were then placed in a round metabolic cage fitted over a large glass funnel with a sheet of window screen on the top of the funnel to trap feces. The funnel was fitted on the bottom with an Erlenmeyer collection flask mounted in an ice-water bath. The animals had free access to water only. At the end of the 24 hour collection period, the urine was suction-filtered using a Buchner funnel with Whatman No. 4 paper and stored at -10° C.

G. Column Chromatography

Several types of columns were used for the isolation and characterization of the various metabolites of dimethylpyrazine. All samples were concentrated by lyophilization prior to use. For preparative anion exchange chromatography Dowex 1X8 formate form 100-200 mesh resin was used. The 1.9 X 65 cm. columns were operated with a head pressure of 110 cm. and a flow rate of one ml/min. The columns were eluted with a gradient of increasing formate ion concentration by using a two-chamber reservoir. A 500 ml. quantity of water was added initially to both the mixing and upper reservoirs. This was followed by successive

additions of 500 ml. each of the following eluents to the upper reservoir: 2N formic acid, 4N formic acid, 4N formic acid plus 0.2N ammonium formate and 4N formic acid plus 1.0N ammonium formate. For analytical purposes, 0.6 X 17 cm. columns of 200-400 mesh Dowex 1X8 and 200-400 mesh Dowex 50WX8 were used with 44 cm. head pressure, 0.2 ml. per minute flow rate and 15 ml. of each eluent. The eluents for Dowex 50 were deionized water, 1N, 2N and 3N hydrochloric acid. The eluting system for the Dowex 1 columns was modified for different sample types.

Sephadex G-50 medium and Bio-Gel P-2 100-200 mesh columns were used for molecular sieve chromatography. The G-50 column was 1.9 X 73 cm. with a head pressure of 19 cm. The P-2 column was 1.5 X 83 cm. with a head pressure of 90 cm. A smaller 1.2 X 45 cm. P-2 column with a 30 cm. head pressure was used in the in vitro studies. All molecular sieve columns were eluted with 0.02 molar ammonium bicarbonate.

H. Thin Layer Chromatography

Coated glass plates (20 X 20 cm.) were prepared according to Stahl (65). A slurry of 15 grams Cellulosepulver MN 300, 6 grams Silica Gel G and 120 ml. water was mixed in a Waring blendor for 60 seconds and poured to a thickness of 0.75 mm. with a commerical applicator (Brinkman Instruments Co.) (66). The plates were allowed to air dry and were activated for 30 minutes in an 80° C drying oven immediately before use. Two solvent systems were used. Solvent system number one was n-butanol, acetic acid, acetone, five per cent ammonium hydroxide and water in a 7:3:5:3:2 ratio. Solvent number two was t-amyl alcohol, formic acid and water in a 3:2:1 ratio. For preparative chromatography samples were applied using a Burkard type S. A. 20 TLC applicator (Burkard Mfg. Co.,

Ltd., Rickmansworth, England). Using this method the sample was applied in a long "streak" four millimeters wide across each plate. After development the separate bands were located with ultraviolet light, scraped off, eluted with deionized water on a Buchner funnel and lyophilized. The same procedure was then used for the second solvent system.

For final purification, each metabolite fraction was streaked onto 20 X 20 cm. sheets of ChromAR No. 500 sheets, which are composed of 70% SilicAR-TLC 7F and 30% glass fiber. The sheets were developed with chloroform, methanol and concentrated ammonium hydroxide in ratios of 30:25:1 or 100:50:3. After development each sheet was cut to a 2.5 cm. horizontal strip centered at the Rf of the metabolite. These strips were then eluted via descending chromatography into glass vials until no ultraviolet absorbing material could be observed on the strip. The resulting 0.5 to 1.0 ml. of effluent was combined with 5 ml. of deionized water and lyophilized.

I. Ultraviolet Spectroscopy

The ultraviolet spectra of metabolites number one, two, three and four and various pyrazine standards were obtained using a Cary Model 14 double-beam spectrophotometer. Samples of proper concentration to yield maximum absorbances of about 0.8 were scanned from 340 to 220 nanometers. The buffers used for pH 1, 7 and 11, respectively, were 0.1 N hydrochloric acid, 0.1 M sodium phosphate buffer and 0.001 N sodium hydroxide.

J. Mass Spectrometry

Low resolution mass spectra were obtained using a prototype LKB-9000 mass spectrometer (67). Samples were introduced using a direct probe. Spectra were obtained under the following conditions: ionizing voltage, 70 electron volts; trap current, 60 microamps; electron multiplier voltage, 1.9 or 2.1 kilovolts; source temperature, 250° C; scan speed, 5; paper speed, 4 in/sec and filter, 120.

High resolution spectra were obtained using a Consolidated Electrodynamics Type 21-110B instrument (Consolidated Electrodynamics, Monrovia, California). The spectra were recorded on Ilford type Q2, 2 X 15 inch glass photographic plates, under the following conditions: ionizing voltage, 70 volts; source temperature, 270° C; trap current, 70 microamps; accelerating voltage, 7.2 kilovolts; background pressure, 2×10^{-6} torr; electron multiplier, 182 volts. Individual peak masses were calculated by comparison to perfluorokerosene standard peaks which were simultaneously exposed onto the plate.

K. Infrared Spectroscopy

Infrared spectra were obtained using a Perkin-Elmer Model 457 grating infrared spectrophotometer. KBr micropellets (1.5 mm. diameter) were made using a Perkin-Elmer micropellet press. Samples were scanned at medium speed and slit setting six.

L. Magnetic Resonance Spectroscopy

Proton magnetic resonance spectra were obtained using a Varian XL-100 NMR spectrometer. Samples were placed in five mm. tubes either

neat or in 99.87 mole per cent deuterium oxide. To lessen interference from contaminating H_2O and HOD, some samples were lyophilized, taken up in one ml. of D_2O, lyophilized and taken up again in D_2O. Spectra were obtained under the following conditions: spin rate, 30 r.p.s.; sweep width, 1000 Hz; sweep offset, 86025 Hz from D_2O or 83701 Hz from tetramethylsilane; scan time, 250 seconds; filter, 2 Hz; temperature, 25° C.

M. Metabolite Hydrolysis

Approximately one micromole of metabolite number three was mixed with one milliliter of hydrochloric acid of such strength as to yield a final concentration of six normal. This solution was hydrolyzed in a sealed evacuated 18 X 150 mm. test tube for 22 hours at 110° C. It was then lyophilized in a vacuum dessicator over potassium hydroxide and stored at -10° C. A known amount of norleucine was often added as an internal standard.

N. Amino Acid Analysis

Amino acid analyses were obtained using an instrument constructed by Dr. Ta-Hsiu Liao of the Biochemistry Department of Oklahoma State University. The instrument was an adaption of the design of Spackman, Stein and Moore (68) using small ion exchange columns to detect amino acids in the nanomolar range.

O. Separation and Esterification of Metabolite Fragments

The hydrolyzed fragments of metabolite number three were separated into acidic and basic fractions by chromatographing the 6 N HCl

hydrolyzate on a 0.5 X 8.0 cm. column of Dowex 50WX8 proton form 20-50 mesh resin in a disposable pipette. The acidic fraction was eluted with 20 ml. of deionized water and the basic fraction was eluted with 20 ml. 6 N HCl. The fragment fractions were lyophilized and then esterified using a modification of the method of Biemann et al. (69). Each fraction was refluxed with two ml. of 2.2 N ethanolic HCl for two hours, evaporated to dryness over potassium hydroxide in a vacuum dessicator and resuspended in two ml. of methylene chloride. Gaseous ammonia was bubbled through the solution for a few seconds to yield the free esters and precipitate the chloride as ammonium chloride. The solution was then filtered through a disposable pipette with a glass wool plug and taken to dryness in a vacuum dessicator. The ethanolic HCl was made by bubbling gaseous hydrogen chloride through absolute ethanol and calibrating the product by titration with 1.00 N sodium carbonate.

P. Determination of Urinary Excretion Rates

Two albino male 400 gram rats were each injected intraperitoneally with 3.55 X 10^6 dpm (approximately 50 mg) of ^{14}C-dimethylpyrazine in one ml. of sterile saline. Three albino male 30-35 gram mice were given 1.18 X 10^5 dpm (1.7 mg.) in 0.2 ml. Urine was collected as described in section F at fixed intervals, except that after each collection period the apparatus was flushed with about 30 ml. of deionized water. Food and water were given ad lib. Each urine sample was counted in triplicate using liquid scintillation with 0.2 ml. deionized water and 10 ml. counting cocktail.

Q. Determination of Metabolite Percentages

To determine the percentage of excreted radioactivity accounted for by each metabolite, two albino male 400 gram rats were each injected intraperitoneally with 3.92×10^6 dpm (approximately 50 mg.) of ^{14}C-dimethylpyrazine. Urine was collected daily for five days. Two ml. of each urine sample was chromatographed in duplicate on 0.6 X 17 cm. Dowex 1 columns as previously described. The eluting solutions were 15 ml. each of deionized water, 3 N formic acid, 4 N formic acid and 4 N formic acid plus 0.2 N ammonium formate, and 40 ml. of 4 N formic acid plus 1.0 N ammonium formate. The four metabolite peaks of each run were pooled, lyophilized, dissolved in three ml. of water and counted with liquid scintillation.

R. Dimethylpyrazine Metabolism in the Mouse

Three albino male 30-35 gram mice were injected intraperitoneally with 5.09×10^5 dpm (7.5 mg.) of ^{14}C-dimethylpyrazine in 0.15 ml. saline. The urine from the first 24 hours was filtered, lyophilized and dissolved in 50 ml. deionized water. A rough determination of the metabolite percentages was made with a single Dowex 1 run as described in section Q.

S. Mouse Tissue Time Course Studies

Albino male 30-36 gram mice were each injected with 1.20×10^5 dpm (about 2 mg.) of ^{14}C-dimethylpyrazine. At several fixed times after injection a mouse was sacrificed by decapitation. Blood (0.5 to 1.0 ml) was allowed to flow into a 20 ml. beaker containing 20 U.S.P. units

of heparin in 20 microliters of deionized water (6 mg/ml). Within two minutes after sacrifice both kidneys and the liver were removed and placed on ice. After weighing the removed organs they were processed as follows. The kidneys were minced and then homogenized with ten passes in three minutes on a Potter-Elvehjem type Teflon in glass homogenizer after adding one ml. of 0.6 N perchloric acid per gram tissue. After setting five minutes the homogenate was centrifuged for 10 minutes at 700 X g using a Sorvall RC-2B centrifuge with an SS-34 rotor. All centrifugations mentioned in this thesis were done at 4° C. Fifty per cent perchloric acid was then added to the supernatent, 0.6 ml. acid per ml. supernatent. After setting for five minutes the solution was centrifuged again. The collected blood was processed in the same manner except that only one ml. of 0.6 N acid was added. The liver was processed in the same manner as the kidneys except that an equal volume of 95 per cent ethanol was added to remove glycogen before the final centrifugation. The resulting supernatents were then counted using liquid scintillation.

T. Rat Organ Homogenate Studies

Male 250 gram rats were fasted for twelve hours and then sacrificed by decapitation. The liver, kidneys, lungs and the first 15 cm. of the small intestine were removed, weighed and put on ice. Before weighing the small intestine was cleaned by gently dispensing 20 ml. of ice-cold 0.25 M sucrose through it with a syringe. The tissues were then minced and homogenized in four volumes of 0.25 M sucrose (8 volumes for lung and small intestine). The homogenization was done with 12 passes in five minutes on a motor driven Potter-Elvehjem homogenizer at 425 rpm

(640 for lung and small intestine). Aliquots of each homogenate were incubated in duplicate with ^{14}C-dimethylpyrazine. The 5.04 ml. incubation mixtures were prepared by mixing 1 ml. homogenate, 3 ml. 0.1 M sodium phosphate buffer pH 7.4, one unit of glucose-6-phosphate dehydrogenase (Sigma type XV) in 20 microliters of water, 7.88 X 10^4 dpm of ^{14}C-dimethylpyrazine in 20 microliters water, and 1 ml. of a cofactor solution. The cofactor solution contained 5 micromoles NADP, 30 micromoles glucose-6-phosphate, 50 micromoles magnesium chloride and 25 micromoles nicotinamide in one ml. of phosphate buffer. This is an adaption of the incubation medium used by Holcslaw et al. (70). Two controls were run in duplicate for each sample. For one control, the homogenates were denatured for two minutes in boiling water before use. For the other 3500 dpm of purified urinary metabolite number three was substituted for the ^{14}C-dimethylpyrazine. The incubations were done in 18 X 150 mm. test tubes on a Controlled Environment Incubator Shaker (New Brunswick Scientific Co., New Brunswick, New Jersey) for 30 minutes at 37° C and 120 oscillations per minute. The tubes were each sealed with a piece of Parafilm "M" laboratory wrap to retard pyrazine evaporation. The reaction was stopped by the addition of one ml. of 50 per cent perchloric acid, stirred and centrifuged for 10 min. at 1000 X g with an RC-2B centrifuge equipped with an SS-34 rotor. The supernatent was neutralized to pH 6.5 to 7.5 with 8 N potassium hydroxide using a pH meter and then centrifuged as before. The resulting supernatent was lyophilized for at least 24 hours and taken up in 3 ml. deionized water. All samples were counted in duplicate with liquid scintillation using 0.5 ml. sample, 1.5 ml. water and 10 ml. counting cocktail.

U. Rat Liver Subfraction Studies

Rats were fasted and sacrificed as described in section T. Five grams of liver was homogenized in 40 ml. of 0.25 M ice-cold sucrose with six passes in three minutes with a loose-fitting Potter-Elvehjem type homogenizer at 425 rpm. The homogenate was centrifuged for 10 minutes at 750 X g (2500 rpm) with an RC-2B centrifuge equipped with 50 ml. tubes in an SS-34 rotor. The pellet was washed with 20 ml. of sucrose solution and re-centrifuged. The resulting pellet was resuspended to 25 ml. final volume with sucrose solution to yield the nuclear fraction. The supernatents were then combined and centrifuged for 20 minutes at 10,000 X g (9200 rpm on the SS-34 rotor). The pellet was washed with 20 ml. of sucrose solution, re-centrifuged and resuspended to 25 ml. to yield the mitochondrial fraction. The 10,000 X g supernatents were then combined and centrifuged for 30 min. at 113,700 X g (40,000 rpm) in a Beckman L5-65 ultracentrifuge with a 60 Ti rotor and 2.5 X 8.9 cm. Polyallomer tubes. The pellet was resuspended to 25 ml. to yield the microsomal fraction and the supernatent was used as the soluble fraction.

The incubation, processing and counting were done as described in section T with the following exceptions: 2 ml. of each cellular fraction was used in each incubation, 5 micromoles of NADH were added to the cofactors, and 2000 X g centrifugations were used to prepare the sample for liquid scintillation. Only boiled tissue fractions were done as controls.

Another subfraction experiment was done to compare the pyrazine metabolizing activity of the 10,000 X g pellet and supernatent of rat

liver. The procedures were the same as those used for the previously described subfraction except for the tissue preparation. Five grams of liver were homogenized with 35 ml. of either 0.25 M sucrose pH 7.4 or 0.1 M sodium-phosphate buffer pH 7.4. After five passes in two minutes with the loose-fitting Potter-Elvehjem homogenizer, the homogenate was centrifuged for 20 minutes at 10,000 X g (9200 rpm on the RC-2B with SS-34 rotor). After decanting the supernatent, the pellet was resuspended with sucrose or sodium-phosphate buffer to a volume equal to that of the supernatent. Protein was determined using a modification of the Lowry method using bovine serum albumin as the standard protein (71).

V. Induction Studies

Albino female rats (250±25 g) were injected daily with either sodium phenobarbital, 2,3-dimethylpyrazine or a control solution. They received 80 mg/kg in 0.15 ml. on day one and 60 mg/kg in 0.11 ml. on days three, five, six and seven. The control injection solution of 10 per cent ethyl alcohol and 95 per cent propylene glycol was the solvent used for the other two solutions. Four rats were used for each solution type. On day eight, two animals from each group were used for liver homogenate experiments. The tissue preparations, incubations, processing and counting were done as described for liver tissue in section T with the following exceptions: two units of glucose-6-phosphate dehydrogenase were used, six passes in two to three minutes were used for homogenization and 2000 X g centrifugations were used to prepare the samples for liquid scintillation. Also, the samples were lyophilized and reconstituted twice before counting.

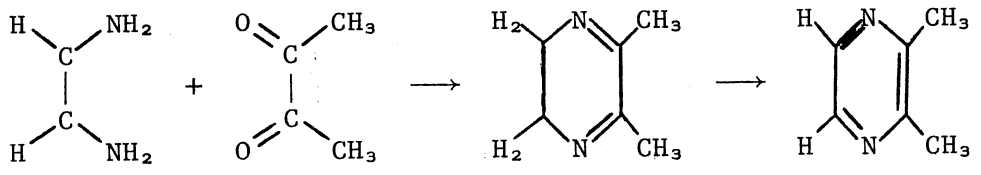
On day nine the remaining animals were used for an *in vivo* induction study. Each animal was injected intraperitoneally with 3.13 X 10^6 dpm (about 50 mg.) of ^{14}C-dimethylpyrazine in sterile saline. The pairs of animals preinjected with phenobarbital, pyrazine and control solutions were housed separately and were given only water during the twenty-one hour urine collection period. The urine was filtered on a Buchner funnel with Whatman No. 4 paper, lyophilized and dissolved in 50 ml. of deionized water. The reconstituted urine was then analyzed using liquid scintillation with duplicate samples of 0.5 ml. urine, 1.5 ml. water and 10 ml. counting cocktail. The reconstituted urine samples were also used to determine the per cent of the excreted radioactivity accounted for by each metabolite. Duplicate 0.6 X 17 cm. Dowex 1X8 column runs were done with each of the three urine samples and the resulting peaks were assayed for ^{14}C, all as described in section Q.

CHAPTER IV

RESULTS AND DISCUSSION

A. Synthesis of ^{14}C-Dimethylpyrazine

The synthesis of 5,6-^{14}C-2,3-dimethylpyrazine (IV) was accomplished by the condensation of ^{14}C-ethylenediamine (I) with 2,3-butanedione (II) followed by the dehydrogenation of the resulting compound, 2,3-dimethyl-5,6-dihydropyrazine (III). The catalytic dehydrogenation method used was found to be quite satisfactory. One pass through the catalytic column yielded a conversion so complete that no trace of the dihydropyrazine could be detected by either gas-liquid chromatography (GLC) or nuclear magnetic resonance (NMR). The overall procedure for synthesis yielded a product of very high purity, as shown by the GLC chromatogram in Figure 1. In other GLC runs, the product was found to give only one peak when co-chromatographed with a sample of commercially produced 2,3-dimethylpyrazine.



(I) (II) (III) (IV) (1)

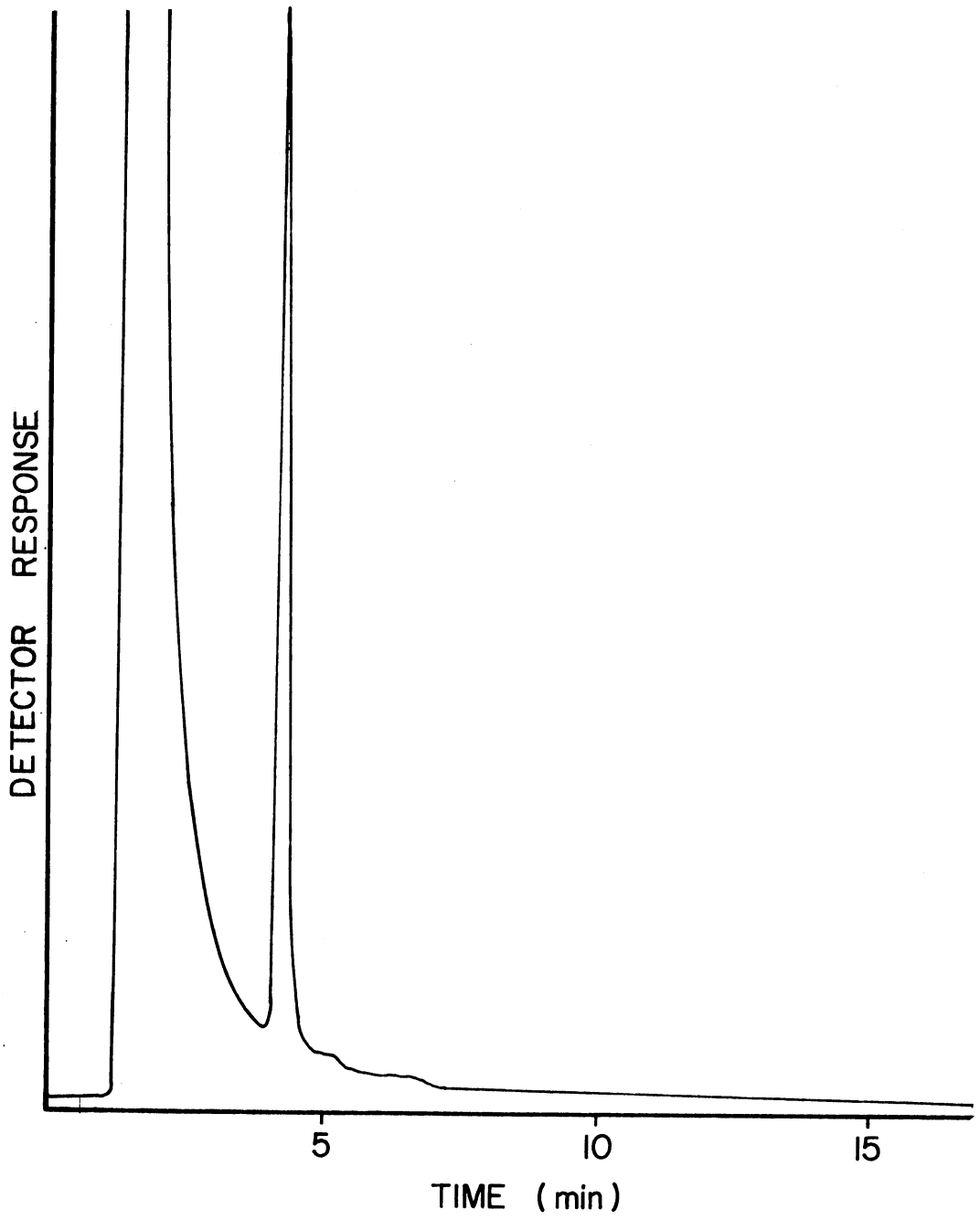


Figure 1. Gas-Liquid Chromatogram of Synthesized 2,3-Dimethylpyrazine in Methylene Chloride (see p. 15)

When the product was co-chromatographed with a sample of synthesized 5,6-dihydro-2,3-dimethylpyrazine two peaks were observed. The retention times of dimethyldihydropyrazine and dimethylpyrazine were 4.8 and 4.2 minutes, respectively. The product of fractional distillation was collected over a narrow temperature range since the objective was high purity instead of high yield. Approximately 3.5 ml. was obtained, constituting about a 36% yield. The product was almost colorless.

As a further proof of identity and purity the product was analyzed with NMR and mass spectrometry. As shown in Figure 2, the product yielded two peaks in the proper ratio of 1:3 with only a small water peak at 4.11 ppm. The peaks at 2.42 and 8.22 ppm are attributable to the methyl and aromatic protons of dimethylpyrazine, respectively. Smith (35) reported peaks at 2.05 and 3.19 ppm in the spectrum of 2,3-dimethyl-5,6-dihydropyrazine.

The mass spectrum of ^{14}C-dimethylpyrazine is shown in Figure 3. The peak at 108 m/e represents the molecular ion. No peak was observed at 110 m/e, which would be the molecular ion of dimethyldihydropyrazine.

B. Toxicity Studies

The toxicity of some alkylpyrazines, including 2,3-dimethylpyrazine have been reported. However it was decided that a study was needed for the exact conditions of injection to be used in this work. Differences in impurities in the injection mixture, animal strain, weight and age could have profound effects.

Using synthesized dimethylpyrazine the LD_{50} was determined to be 0.7 mg/g body weight when injected intraperitoneally into albino male 30-35 gram mice (Table I, Figure 4). The method of interpreting the

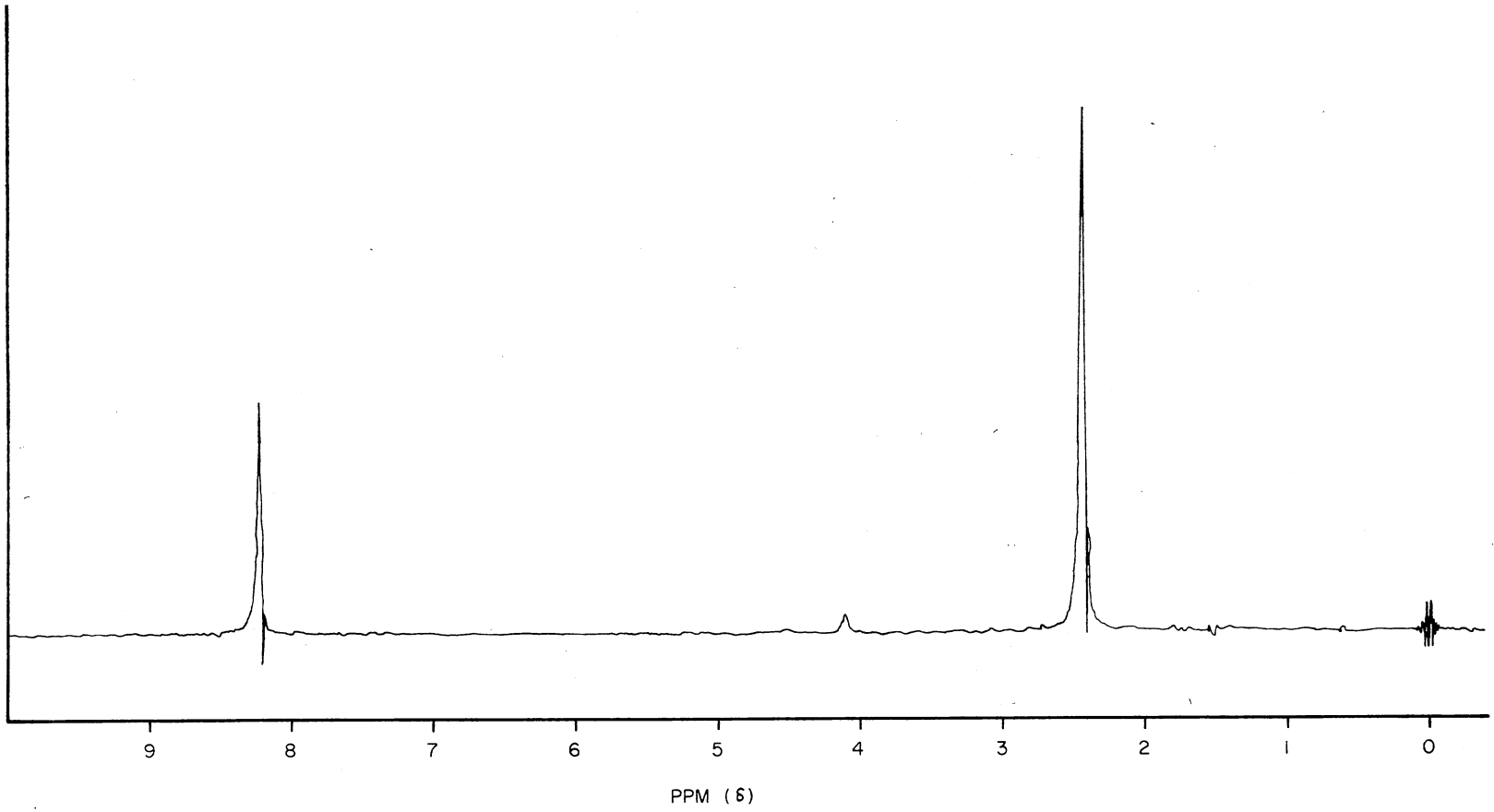


Figure 2. Nuclear Magnetic Resonance Spectrum of Synthesized 2,3-Dimethylpyrazine (see p. 19)

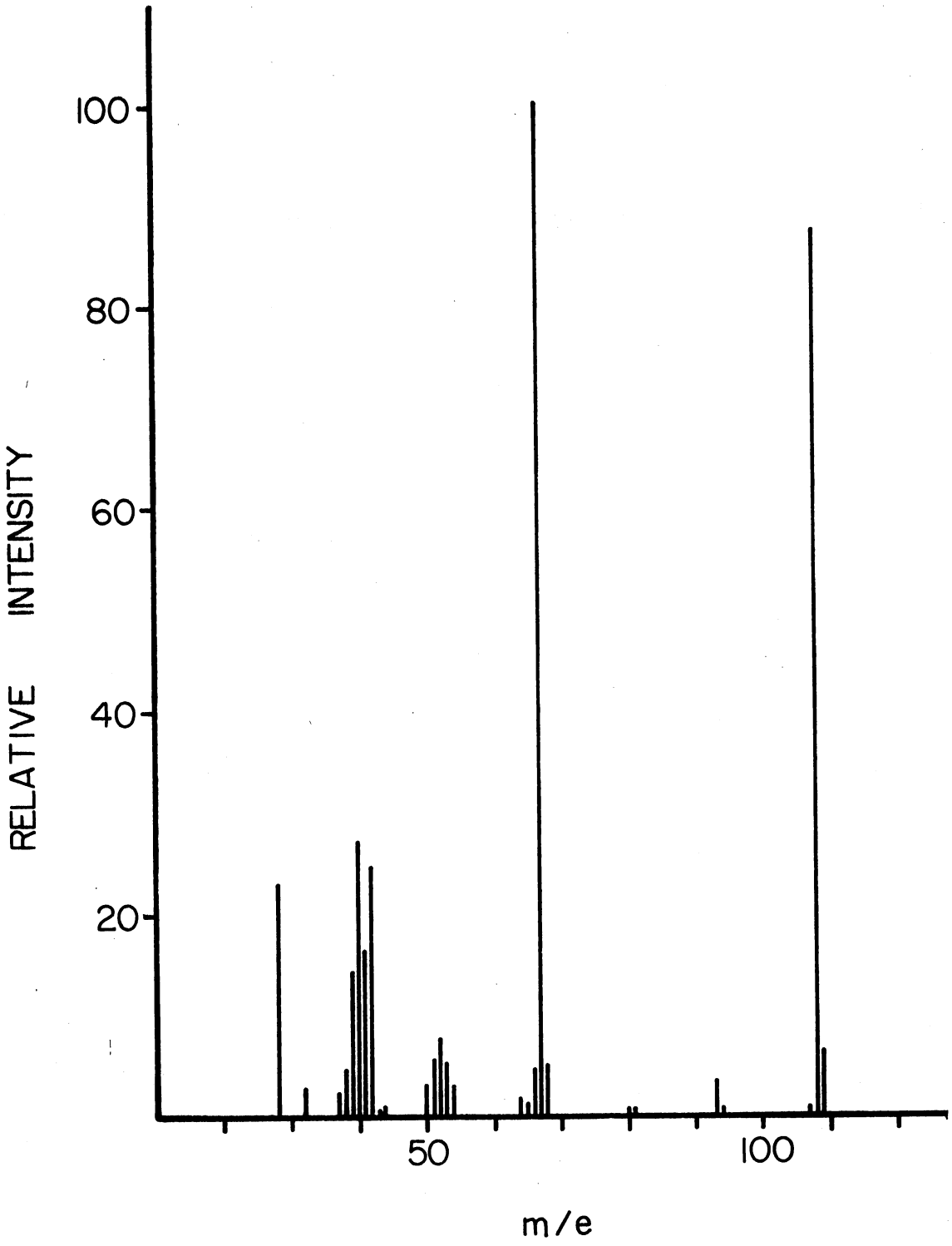


Figure 3. Mass Spectrum of Synthesized 2,3-Dimethylpyrazine (see p. 19)

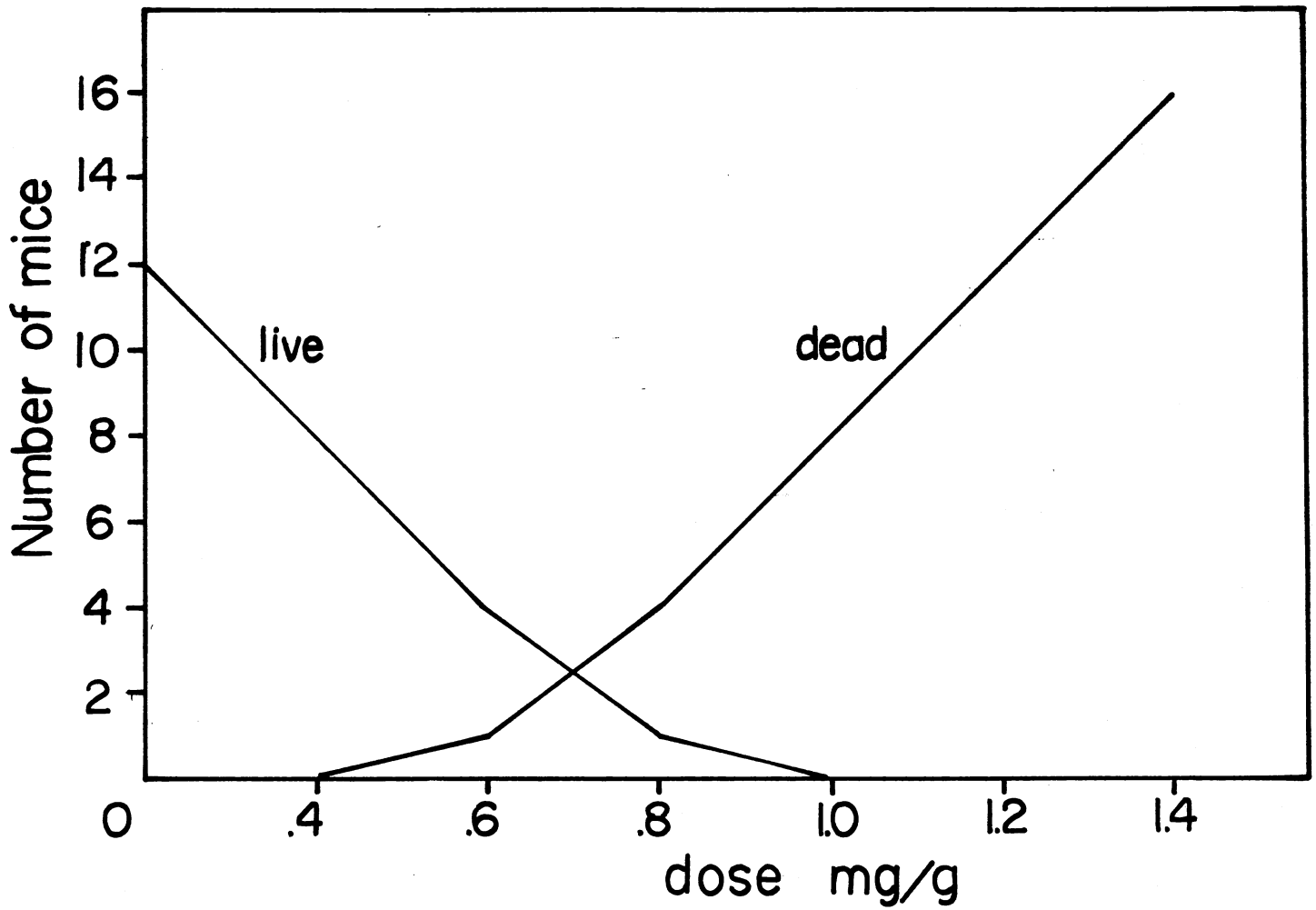


Figure 4. LD_{50} of 2,3-Dimethylpyrazine for Mice (see p. 34)

data was that of Reed and Meunch (64). Deaths usually occurred within 15 minutes. The LD_{50} of 2,3-dimethylpyrazine for 18-25 gram mice was reported to be 1.39 mg/g by Nishie et al. (10). Smith (35) has determined the LD_{50} of 2,3-dimethyl-5,6-dihydropyrazine to be 0.36 mg/g. He reported extreme muscular contractions immediately after injection. No such contractions were observed in this study.

TABLE I

LD_{50} OF 2,3-DIMETHYLPYRAZINE FOR MICE*

Dose (mg/g)	# Alive	# Dead	Total Alive	Total Dead	% Mortality
0.2	4	0	12	0	0
0.4	4	0	8	0	0
0.6	3	1	4	1	20
0.8	1	3	1	4	80
1.0	0	4	0	8	100
1.2	0	4	0	12	100
1.4	0	4	0	16	100
1.6	0	4	0	20	100

*The LD_{50} value was determined to be 0.7 mg/g body weight using the graphical method of Reed and Meunch (64) (see p. 15).

C. Metabolite Isolation

Initial studies of the urinary metabolites of 2,3-dimethylpyrazine revealed four reproducible metabolite fractions. Several chromatographic techniques were used to purify these fractions and to determine how many actual metabolites were present in each fraction. Through such studies an isolation scheme was developed to produce purified metabolites in quantities large enough for characterization. The urine from three separate experiments of three rats each was used for the bulk of the metabolite characterization work.

The animals were each injected with 50 mg of 5,6-^{14}C-2,3-dimethylpyrazine. The urine collected in the first twenty-four hours after injection was filtered, lyophilized and dissolved in about 20 ml. water. The entire reconstituted sample from three rats was then chromatographed on a 1.9 X 65 cm. column of Dowex 1X8 formate form resin. The column was sufficient to separate all four metabolites. Figure 5 shows the metabolite profile. The radioactive peaks obtained were designated one, two, three and four in order of their elution. Peaks two, three and four were anionic while peak one eluted with the water wash. Some of the preliminary studies with urinary metabolites on Dowex 1X8 used 2,3-^{14}C-2,3-dimethylpyrazine. The same four ^{14}C peaks were observed using either 2,3- or 5,6-labelled compound. Thus, it is likely that ring scission has not taken place.

For a control urine run five ml. of urine from an uninjected rat was chromatographed on Dowex 1. To determine where unchanged dimethylpyrazine would elute 50 mg of ^{14}C-dimethylpyrazine was chromatographed on Dowex 1. This resulted in a single peak, eluting at tube number 43.

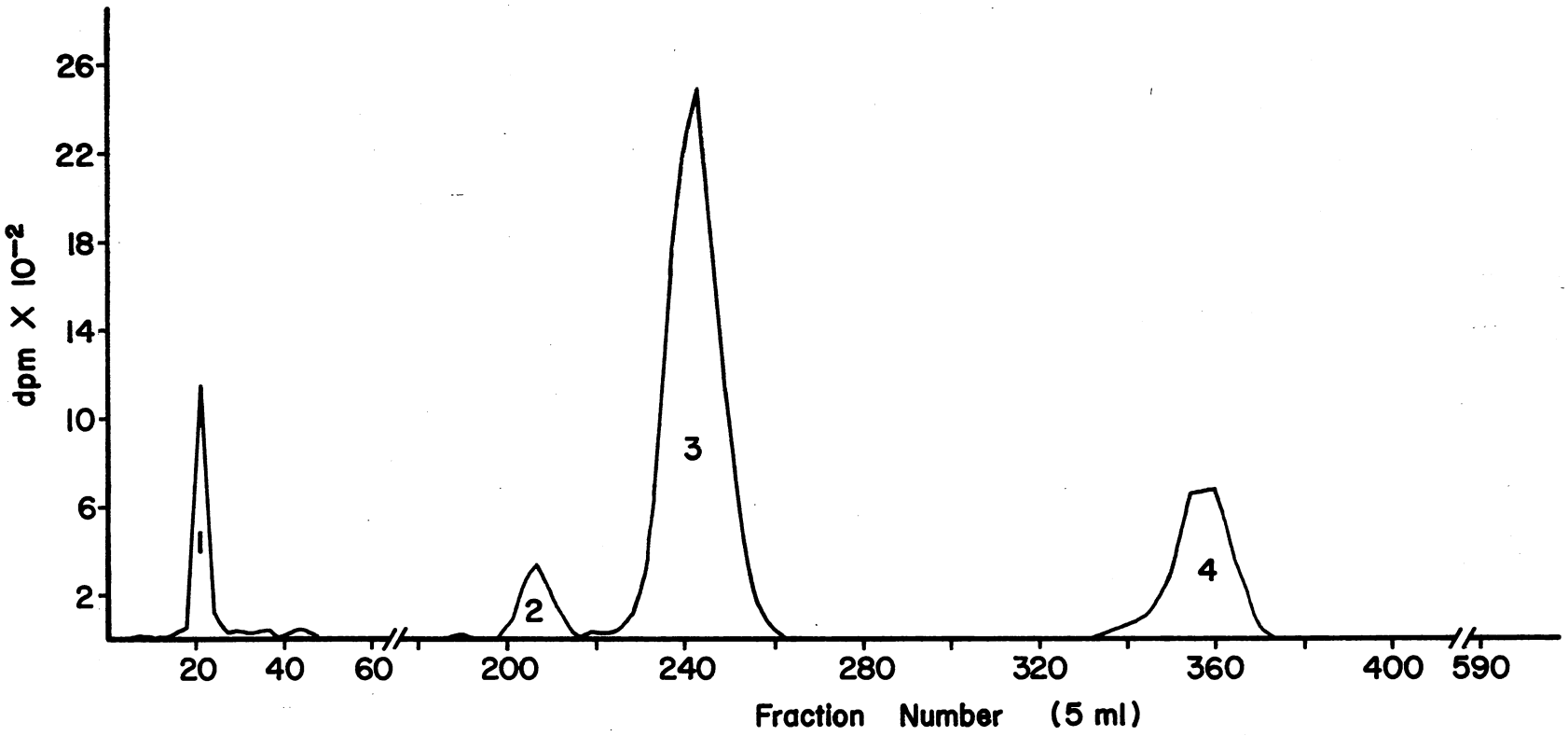


Figure 5. Dowex 1X8 Chromatogram of ^{14}C-Labelled Urinary Metabolites (see p. 16)

The control pyrazine run suggested that none of the four metabolite peaks were due to unchanged pyrazine since each metabolite peak eluted in positions different from that of dimethylpyrazine. The fact that ^{14}C-dimethylpyrazine was lost when lyophilized provided further support. Because of its low boiling point (156° C) only a trace of radioactivity was recovered when a known amount of ^{14}C-dimethylpyrazine was lyophilized.

Each radioactive peak from Dowex 1 was pooled, lyophilized and dissolved in 3-5 ml. 0.02 M ammonium bicarbonate. Each fraction was then passed through a gel filtration column. Previous studies had shown that the radioactive component of peak one was well centered in the elution profile of Bio-Gel P-2, while Sephadex G-50 worked well for peaks two, three and four. A typical chromatographic profile of peak one on P-2 is shown in Figure 6. The radioactive peak is separated from a number of other compounds. Figure 7 shows the elution profile of metabolite three on Sephadex G-50. Metabolites two and four elute at the same position. Although the radioactive peaks aren't separated from many ultraviolet-absorbing impurities, the gel filtration step is useful for removing the salts acquired from anion exchange chromatography. There also may be some non-UV-absorbing compounds separated by this method.

The reconstituted lyophilized peaks from gel filtration were each chromatographed twice using two different solvent systems. The developed chromatograms were observed under short wave ultraviolet light to observe the bands. Small circular samples of 5 mm. diameter were scraped from the plate at one cm. intervals from the origin to the solvent front using disposable pipettes. Each circular spot was

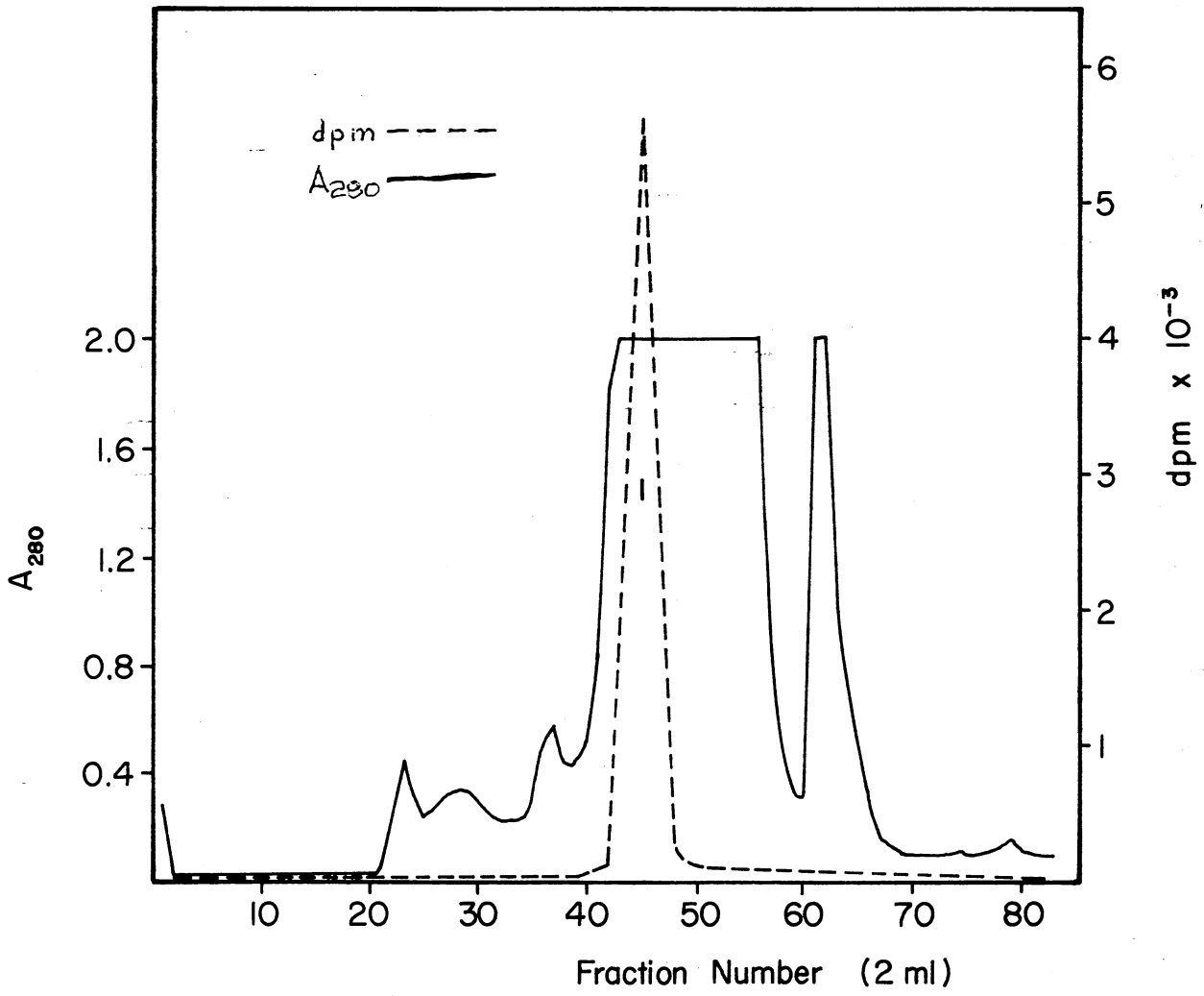


Figure 6. Bio-Gel P-2 Chromatogram of Peak One from p. 36

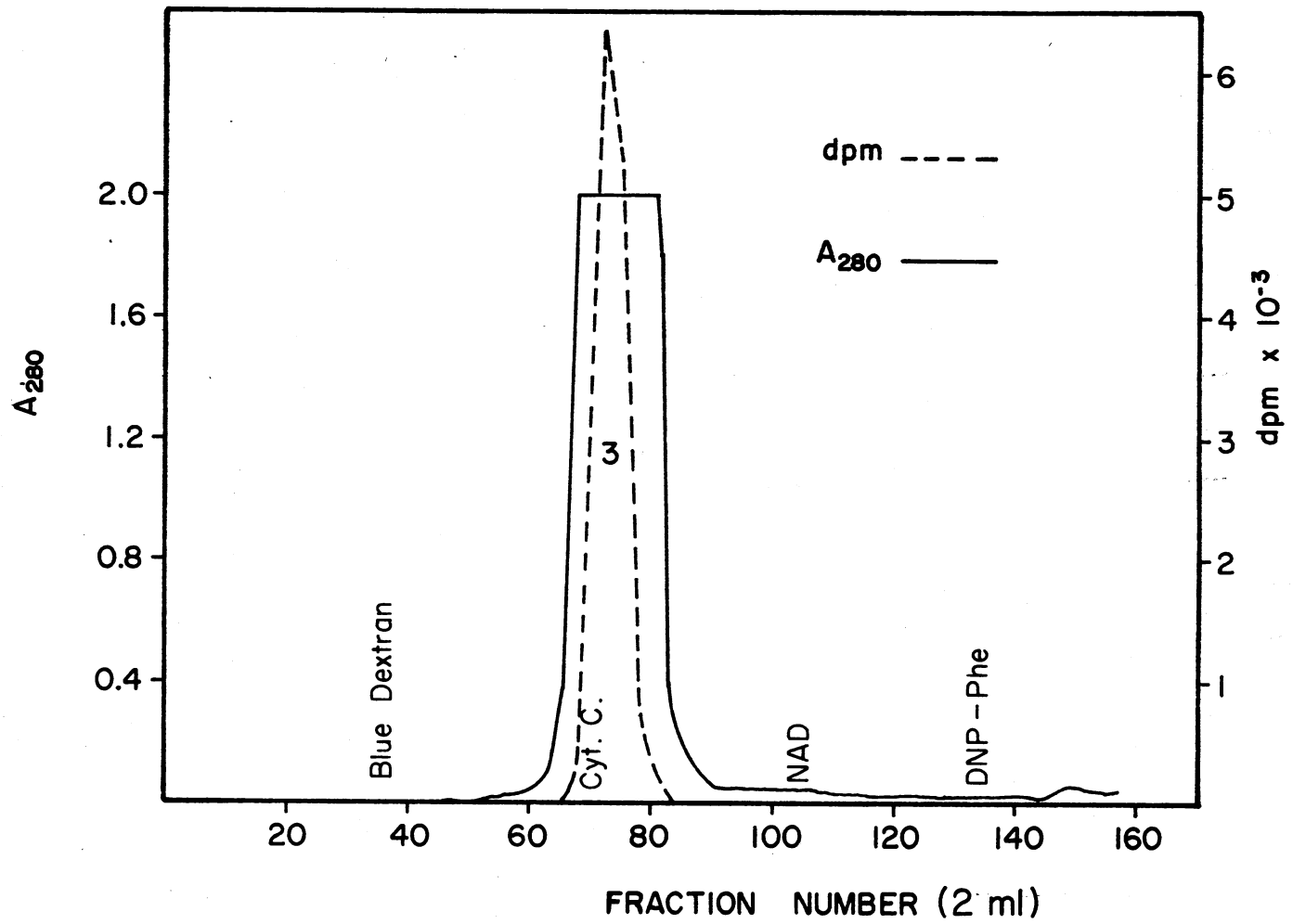


Figure 7. Sephadex G-50 Chromatogram of Peak Three from p. 36

checked for radioactivity with liquid scintillation. A typical profile is shown in Figure 8. Figure 9 shows a conventional chromatogram of all four metabolites using the butanol based system. Many impurities are shown to be separated using this technique. The pentanol based solvent system removed some additional impurities.

After chromatographing each peak with both solvent systems the peaks were each re-chromatographed on gel filtration columns as before. This was found to be necessary since a large amount of silica gel dissolved in the water wash used to elute the metabolites from the thin-layer material.

The final step in the purification scheme was chromatography on ChromAR sheets which have sorbent properties similar to the TLC silicas. This medium was chosen for the final purification step because less polar solvents could be used which would dissolve much less silica. Also, these sheets could be cut into strips after development and eluted directly via descending chromatography. A control proved the effectiveness of this step for eliminating silica residue. Blank thin-layer plates passed through the isolation procedure yielded a small amount of silica residue after the gel filtration step but no detectible residue after the ChromAR step. Only metabolites two, three and four were isolated using ChromAR sheets. Metabolite one was only available in such small amounts at this point in the isolation scheme that a satisfactory solvent system was not found for separating it on ChromAR. Solvent systems were developed for metabolites two, three and four which had Rf values of 0.45-0.55. The solvent system for metabolite two consisted of chloroform, methanol and concentrated ammonium hydroxide in a 30:23:1 ratio. The ratio used for metabolites three and four was

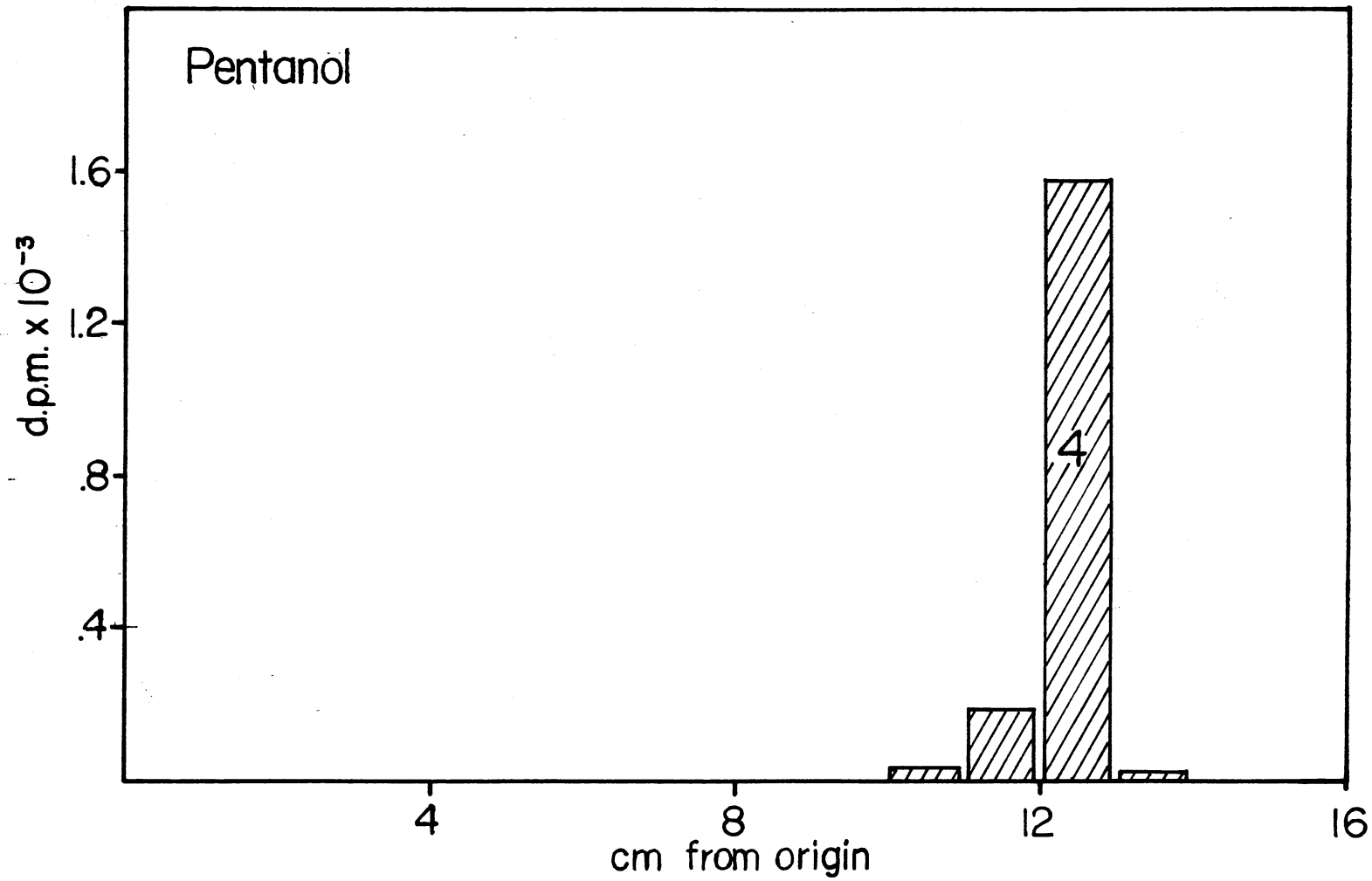


Figure 8. Thin-Layer Chromatogram of Metabolite Fraction Four (see p. 40)

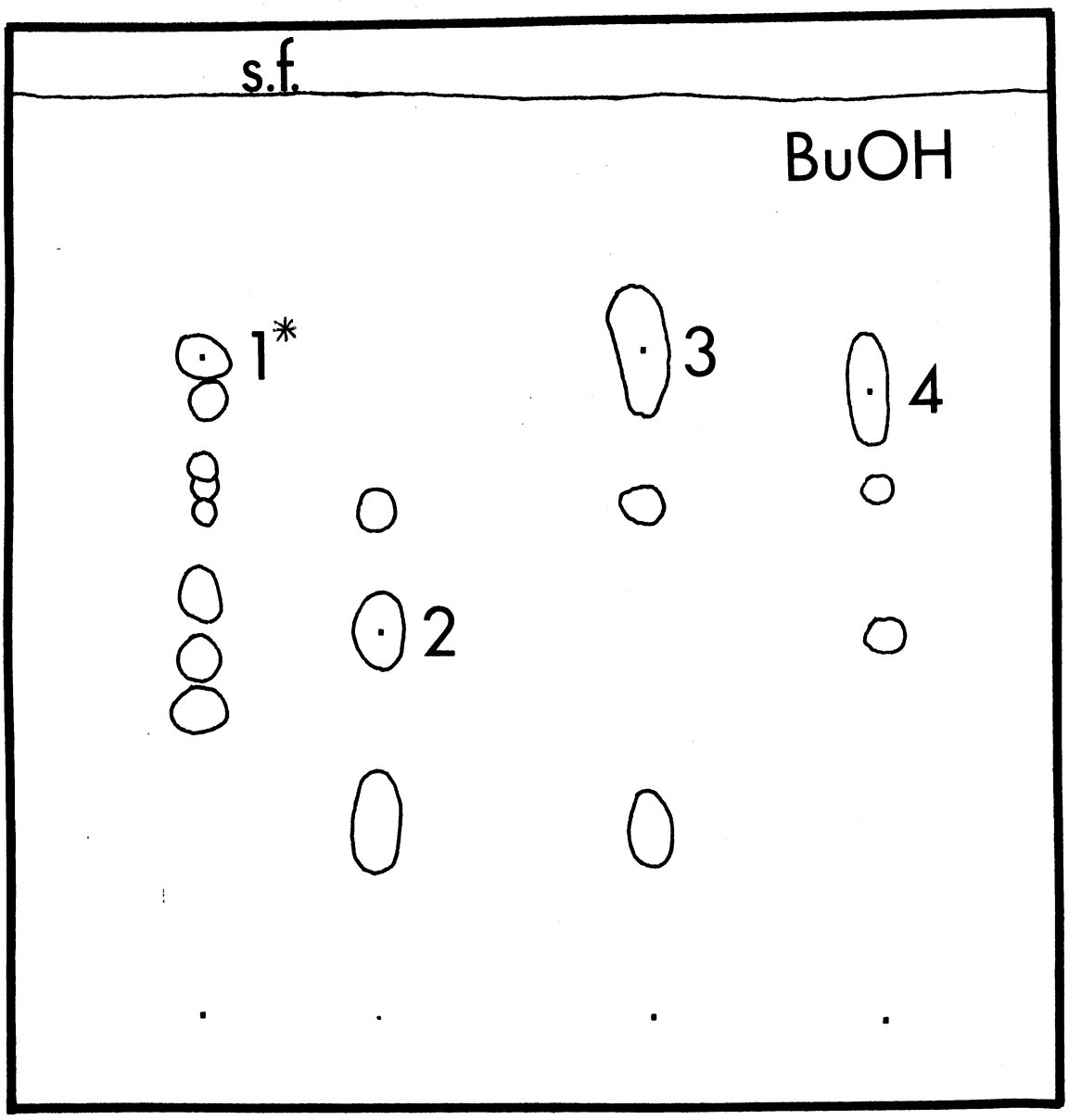


Figure 9. Thin-Layer Chromatogram of Urinary Metabolite Fractions from Gel Filtration Step (see p. 40)

*Numbers 1, 2, 3, 4 designate the radioactive components.

100:50:3. After chromatography on ChromAR the samples were lyophilized and dissolved in four ml. of deionized water.

The complete isolation scheme is shown in Figure 10. Table II shows the efficiencies of the isolation scheme at various steps. The data presented is the average of three experiments of three rats each. The quantitative yield of ^{14}C on lyophilization again suggests that none of the Dowex peaks are due to unchanged pyrazine. The yields of the ChromAR steps in the procedure were not monitored closely but were only about 50%. Since the object of the ChromAR step was high purity and not high yield, only the center portion of each ChromAR band was eluted.

TABLE II

EFFICIENCY AT EACH STEP OF THE METABOLITE ISOLATION SCHEME (%)

Step	Efficiencies of Each Step				Overall Yield
	M 1*	M 2	M 3	M 4	
Urine Lyophilization**	-	-	-	-	100.0
Dowex 1X8***	-	-	-	-	70.3
Gel Filtration	84.3	92.9	89.5	77.4	60.3
Thin-Layer #1	45.6	68.5	74.7	58.0	41.0
Thin-Layer #2	63.2	81.4	71.3	61.8	29.0
Gel Filtration	-	88.8	92.0	83.4	25.7

*M 1, etc. indicates metabolites one, two, three and four.

**This step was quantitative.

***This step yielded 70.3% of the dpm applied to the column.

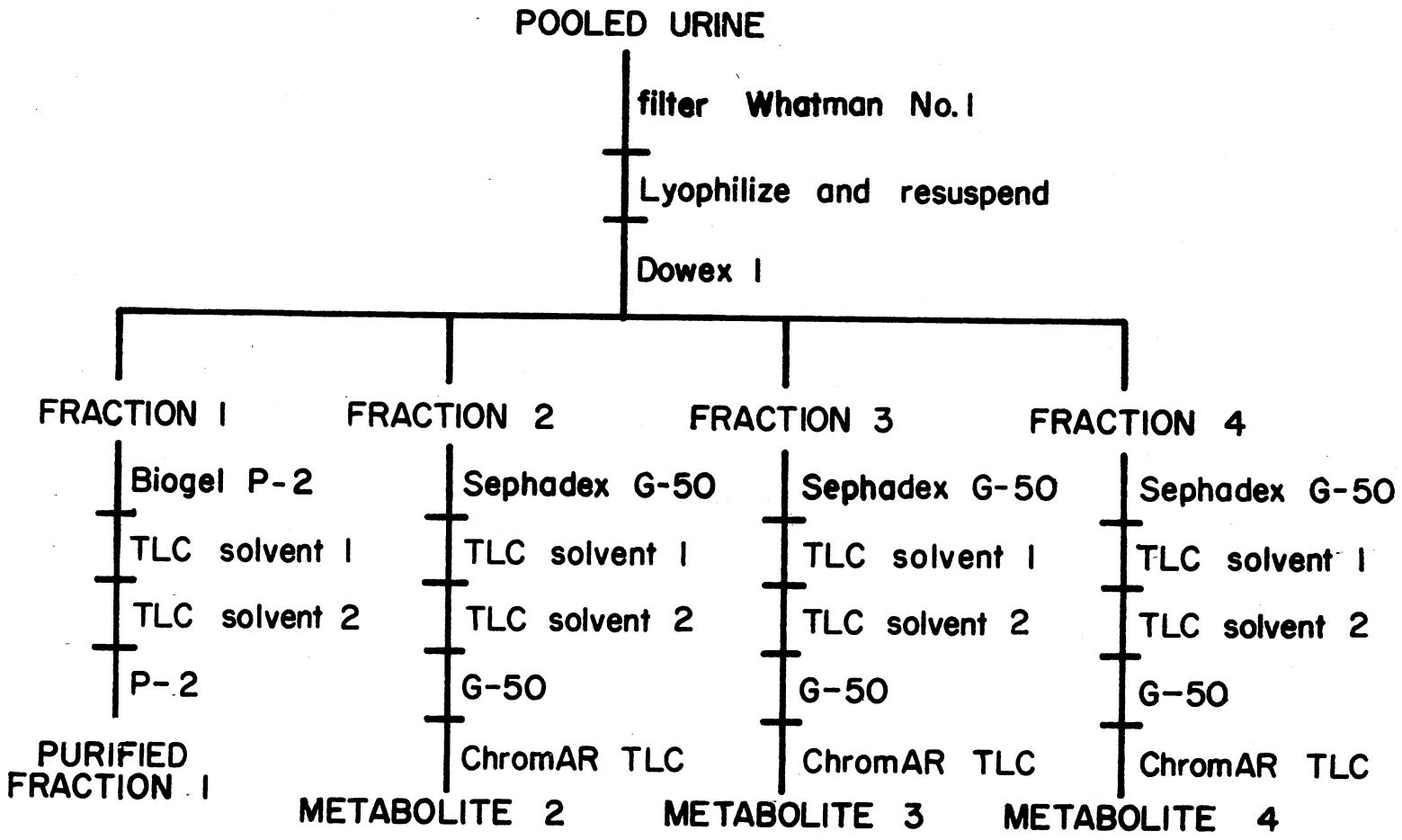


Figure 10. Isolation Scheme for Urinary Metabolites of 2,3-Dimethylpyrazine

From the isolation work it was determined that there were at least four major urinary metabolites of 2,3-dimethylpyrazine. Three of these metabolites were found to be anionic and all four absorbed light at 280 nanometers. None of the metabolite fractions observed on Dowex 1 chromatography were due to the excretion of unchanged dimethylpyrazine in the urine.

To show that the metabolites isolated were true metabolites and not artifacts produced by pyrazine combining with urine components a control experiment was conducted. Twenty-five milligrams (1.77×10^6 dpm) of ^{14}C-labelled dimethylpyrazine was incubated with ten ml. of normal rat urine at 4° C for 24 hours, at room temperature for two hours, and then at -10° C for 24 hours. These conditions simulated the normal handling of urine used in this metabolic study. The urine was then chromatographed on a column of Dowex 1X8 resin, using the same conditions used for metabolite isolation. As shown in Figure 11 only one radioactive peak was obtained. The peak was observed where dimethylpyrazine normally elutes, which was later than where metabolite number one eluted. Since none of the four normal metabolite peaks were obtained it is likely that these four compounds are all actual urinary metabolites. The radioactive peak was then pooled, lyophilized, dissolved in four ml. of water and counted by liquid scintillation. The residual radioactivity accounted for 3.8% of the incubated dose. Much of the residual radioactivity was probably due to the occlusion of some dimethylpyrazine in the sticky urine residue.

D. Metabolite Characterization

After the final purification step of the isolation scheme the

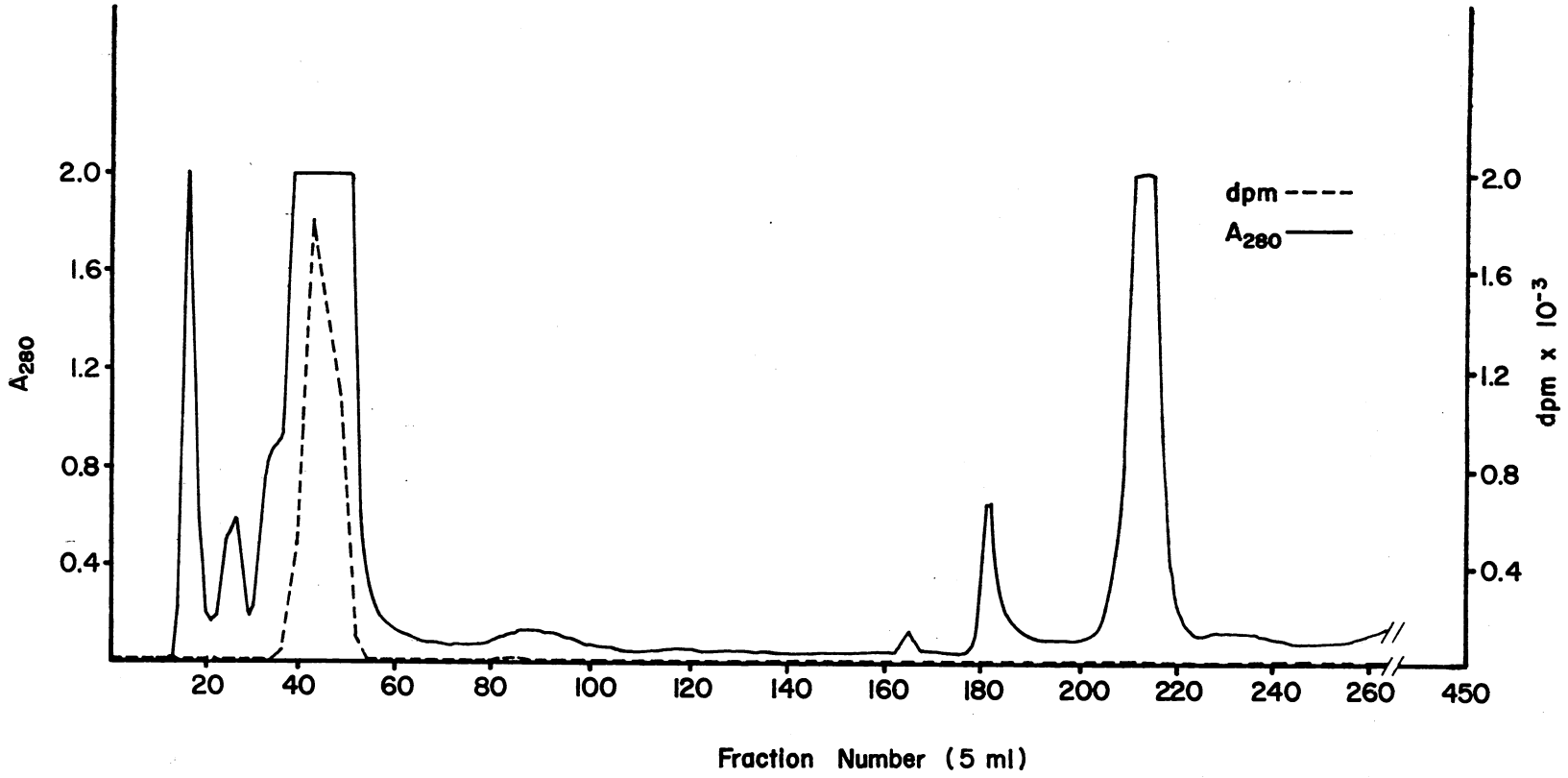


Figure 11. Dowex 1X8 Chromatogram of Urine Incubated With Dimethylpyrazine

purified metabolite fractions two, three and four were each lyophilized and analyzed with mass spectrometry and infrared and nuclear magnetic resonance spectroscopy. Ultraviolet spectra were obtained after dissolving each metabolite in four ml. deionized water.

D.1. Ultraviolet Spectroscopy

The various metabolites of 2,3-dimethylpyrazine are easily monitored during preparatory procedures due to their strong absorbance of ultraviolet light at 265-280 nm. Comparing the ultraviolet spectrum of 2,3-dimethylpyrazine (Figure 12) with those of metabolites two, three and four (Figures 13, 14, and 15), the spectra show the same general pattern. It is probable that during metabolism the pyrazine ring remains intact and aromatic. The absorption maxima differ by no more than four nanometers. Although it is an impure preparation metabolite fraction one also shows this same profile, with an absorption maximum of 276 nm. (Figure 16). Since one possible route of metabolism could be oxidation of the methyl group, the spectra of 2-pyrazinoic acid was also examined (Figure 17). Metabolites two and three both show large spectral shifts at pH 1 while pyrazinoic acid and metabolite four do not.

D.2. Mass Spectrometry

Metabolite number four (M4) was found to be very polar. Since it was the last metabolite to elute from Dowex 1 it seemed likely that it contained a strongly polar group such as a carboxyl. The mass spectrum of M4 is shown in Figure 18. The molecular ion appears at m/e 138. Thirty mass units apparently have been added to the dimethylpyrazine

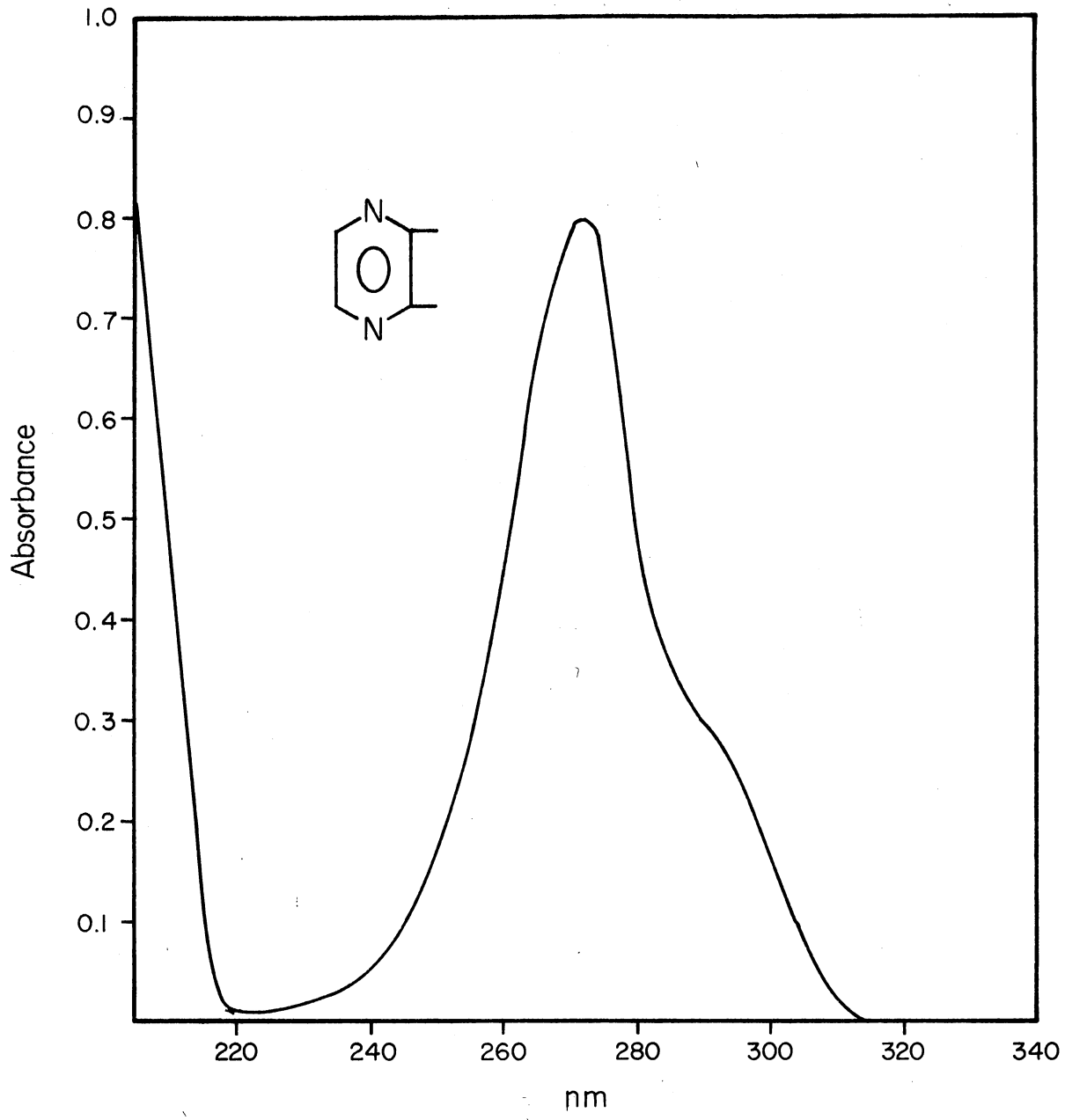


Figure 12. Ultraviolet Spectrum of 2,3-Dimethylpyrazine in 0.02N NH_4HCO_3

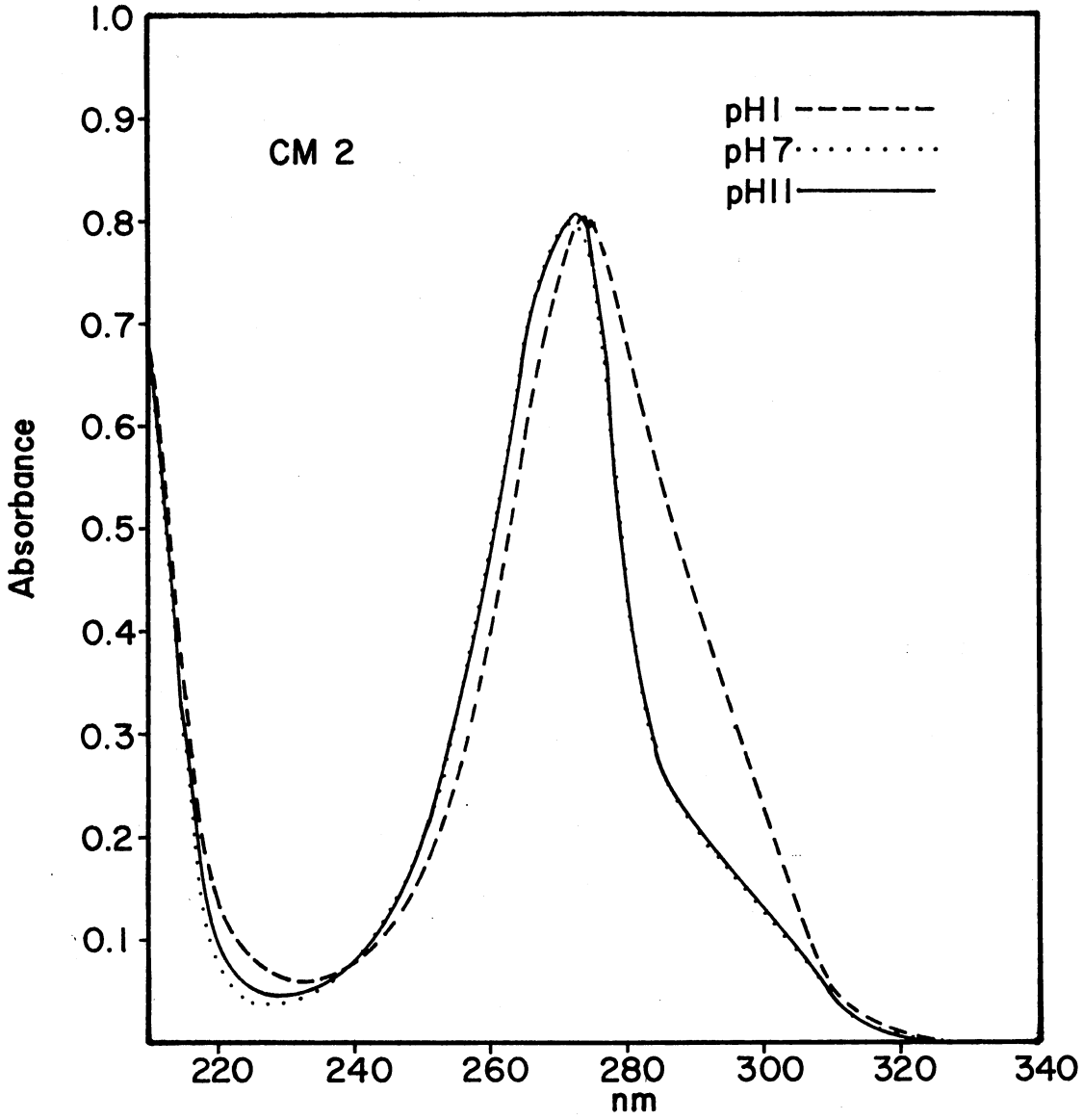


Figure 13. Ultraviolet Spectrum of Metabolite Two (see p. 18)

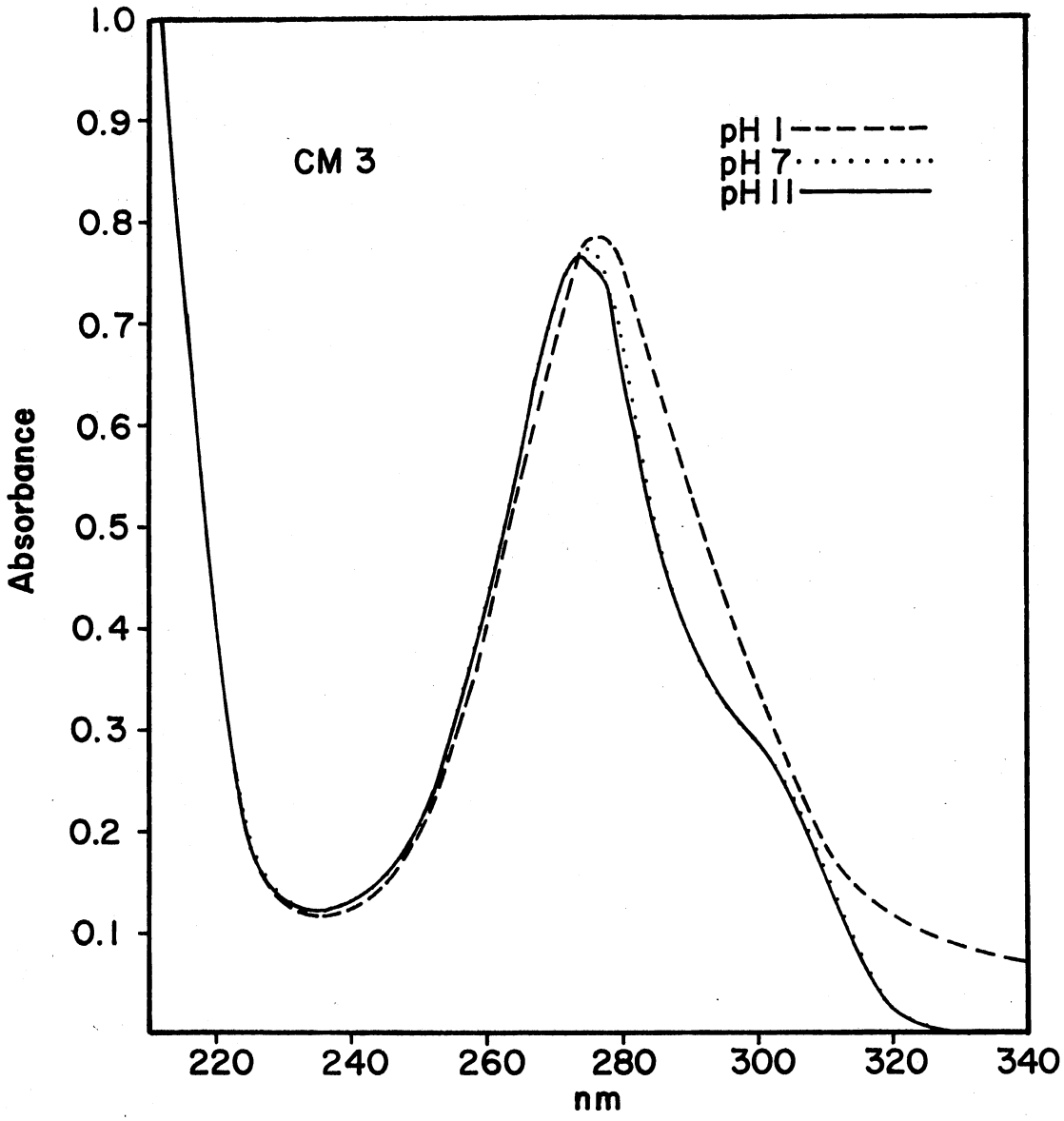


Figure 14. Ultraviolet Spectrum of Metabolite Three (see p. 18)

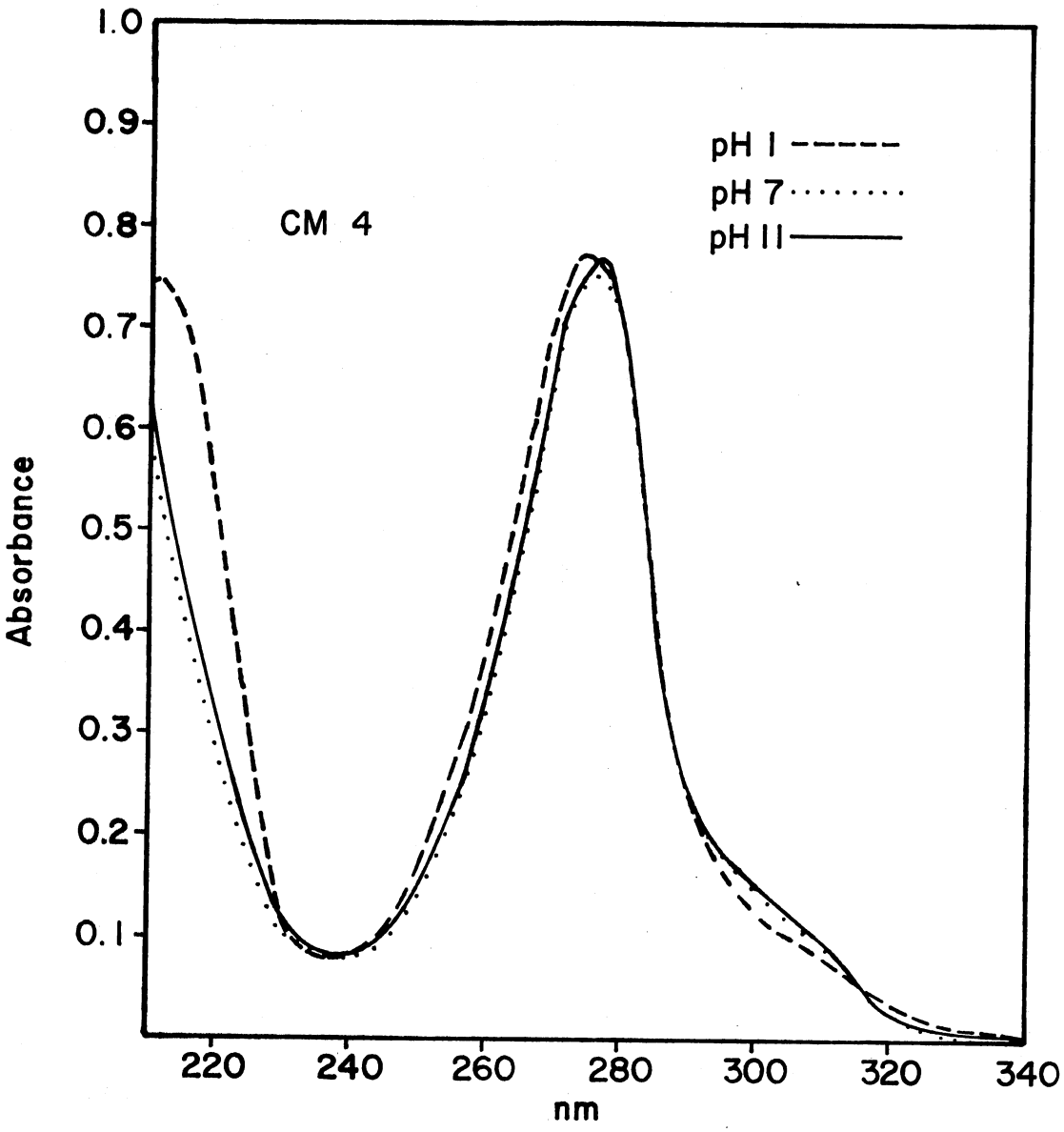


Figure 15. Ultraviolet Spectrum of Metabolite Four (see p. 18)

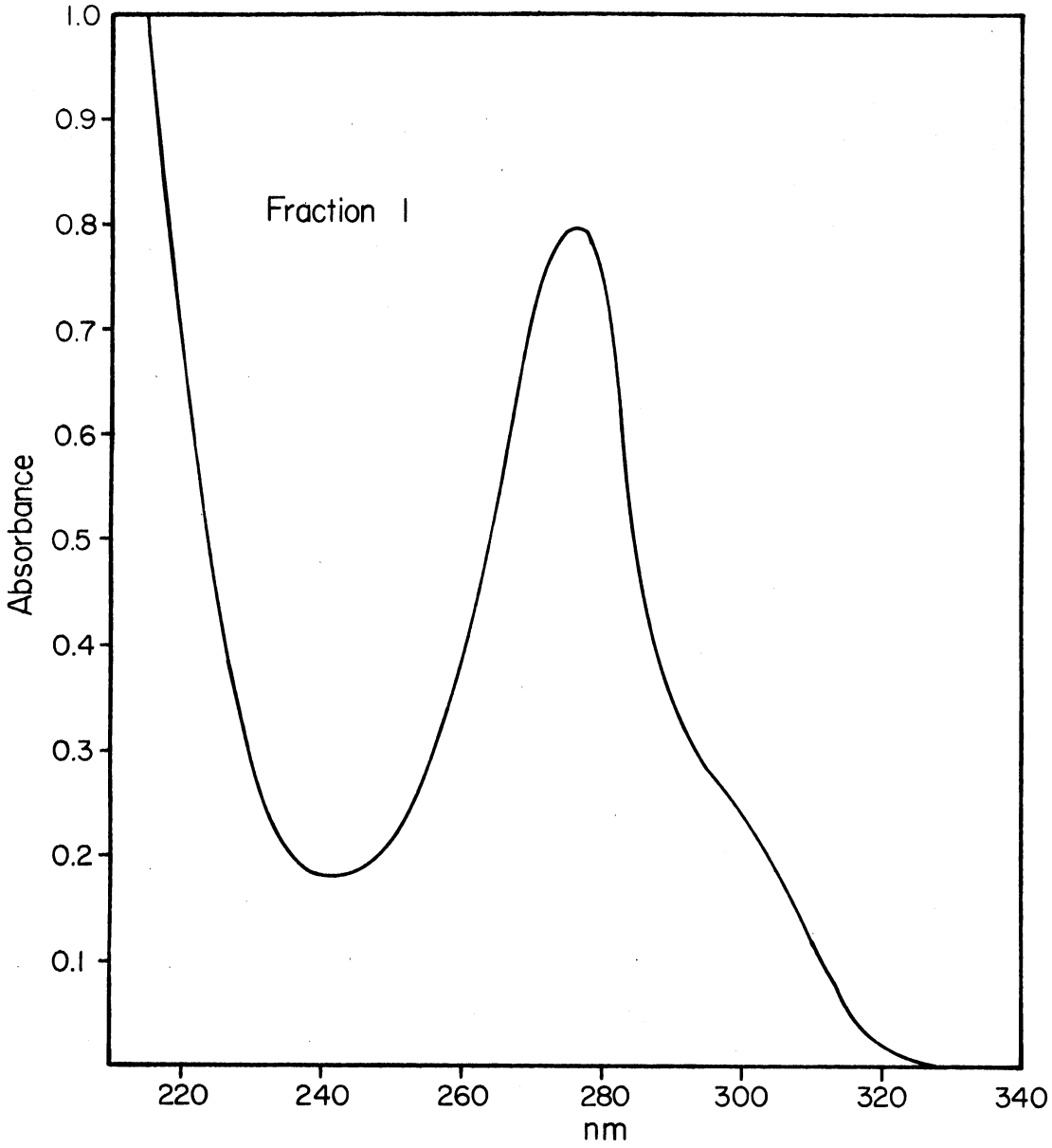


Figure 16. Ultraviolet Spectrum of Purified Metabolite Fraction One in 0.02 M NH_4HCO_3

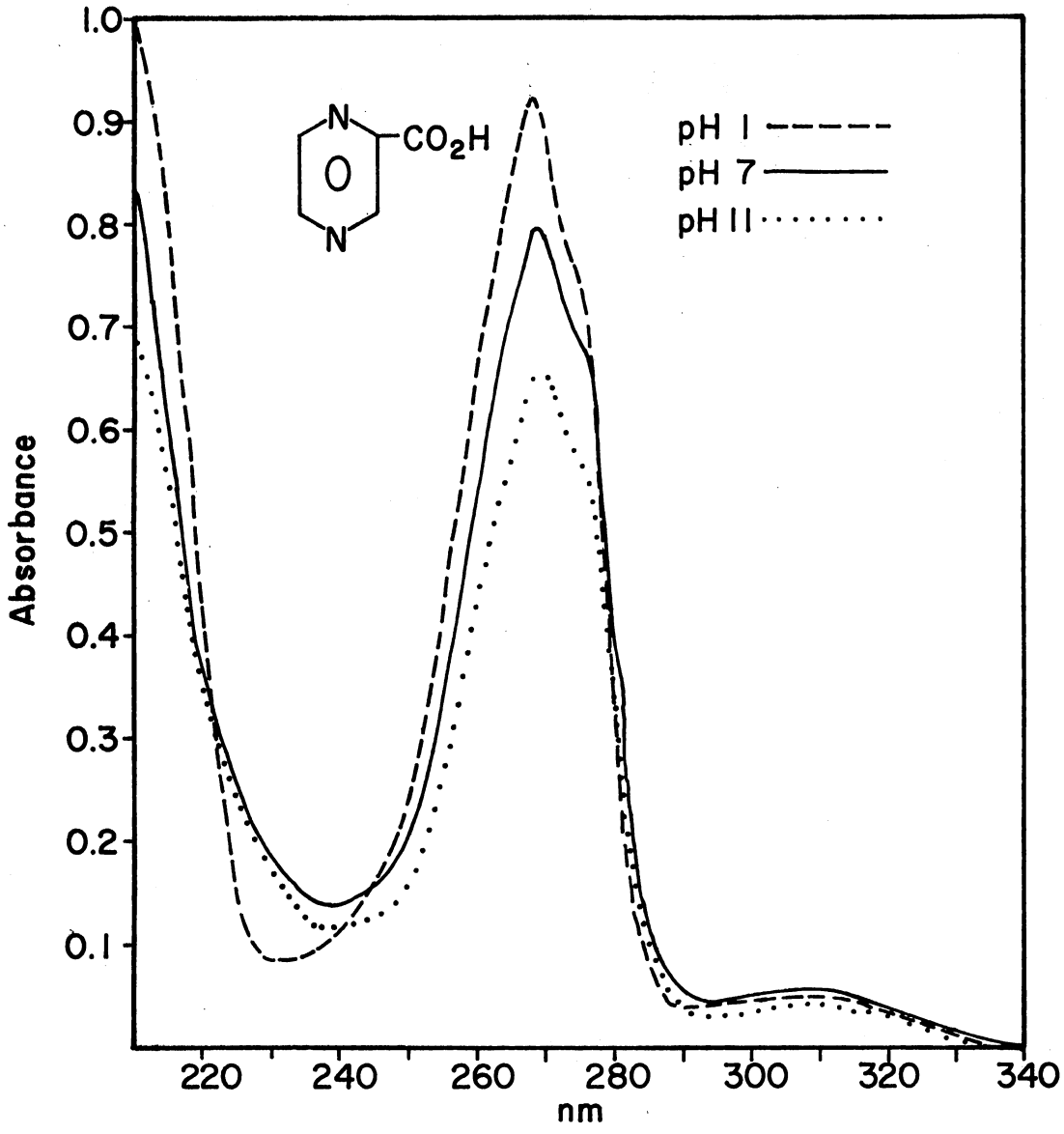


Figure 17. Ultraviolet Spectrum of 2-Pyrazinoic Acid (see p. 18)

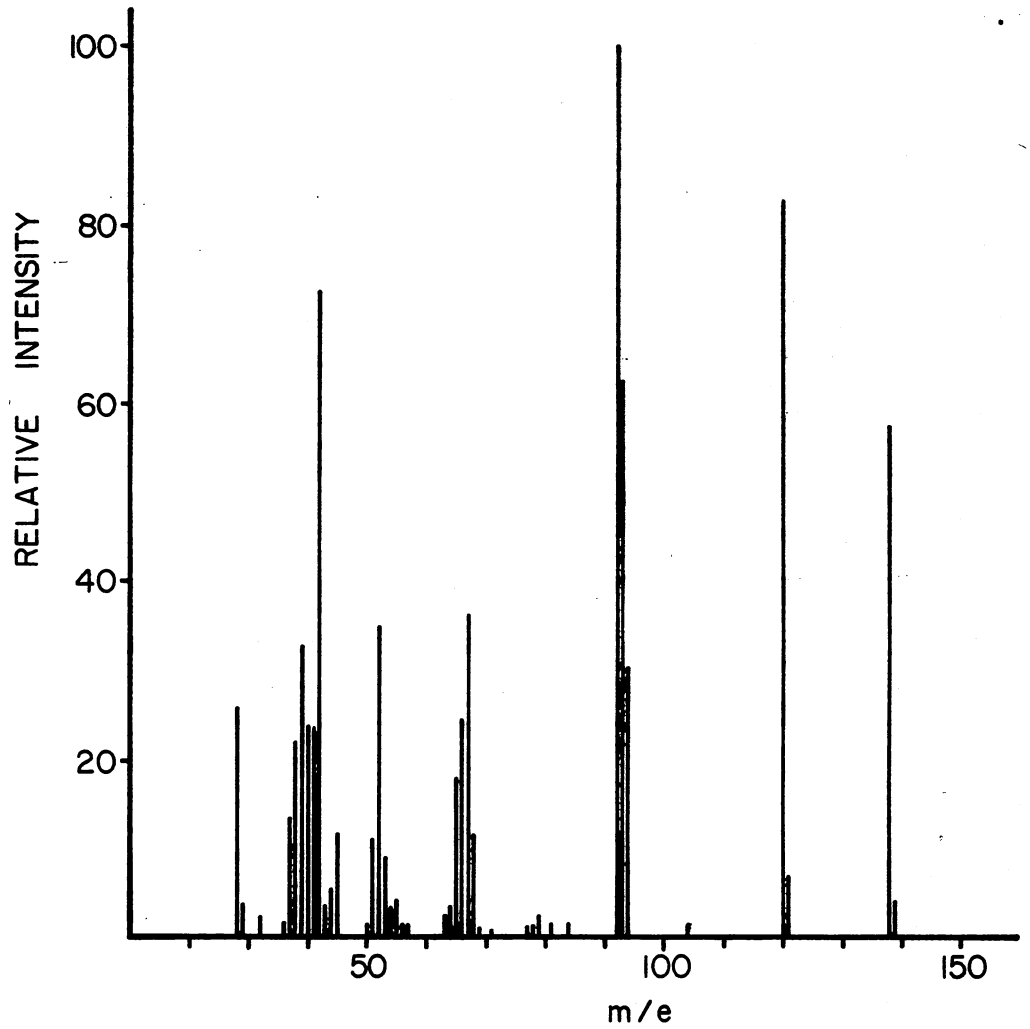


Figure 18. Mass Spectrum of Metabolite Four (see p. 19)

molecule during its metabolism. Two other prominent peaks are found at m/e 120 (M-18) and 92 (M-46). The fragmentation pathway proceeds from 138 to 120 to 92 as evidenced by metastable peaks at m/e 104.4 and 70.5. The calculated values for these metastable transitions are 104.35 and 70.53. The loss of 18 mass units suggests the loss of water. Water could be eliminated from 3-methylpyrazine-2-carboxylic acid via a McLafferty rearrangement. The (M-46) peak is then explained by the further loss of C=O. This type of rearrangement was first observed by McLafferty and Gohlke in studies on ortho-methylbenzoic acid (72). The mass spectrum of M4 was then compared to that of pyrazine-2-carboxylic acid (Figure 19). One would expect similar fragmentation with peaks differing by fourteen mass units since M4 is fourteen mass units heavier. This was found to be the case as shown in Table III.

TABLE III

MASS SPECTRAL COMPARISON OF PYRAZINOIC ACID AND METABOLITE FOUR

Compound	Peaks of Interest					
Pyrazinoic Acid	52*	53*	70	80*	106*	124*
Metabolite Four	66	67	-	94	120*	138*

*Denotes strongest peaks in spectra

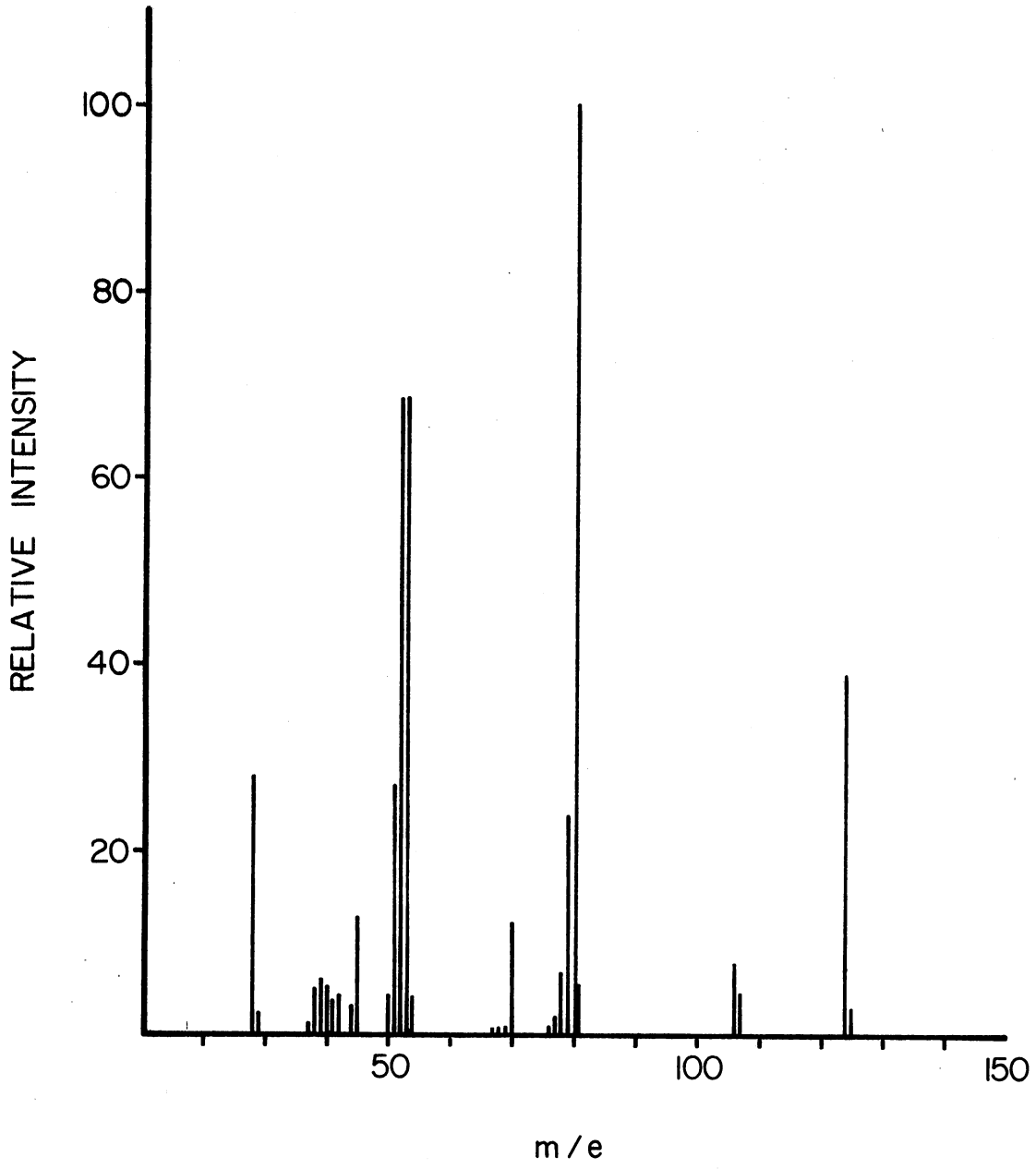
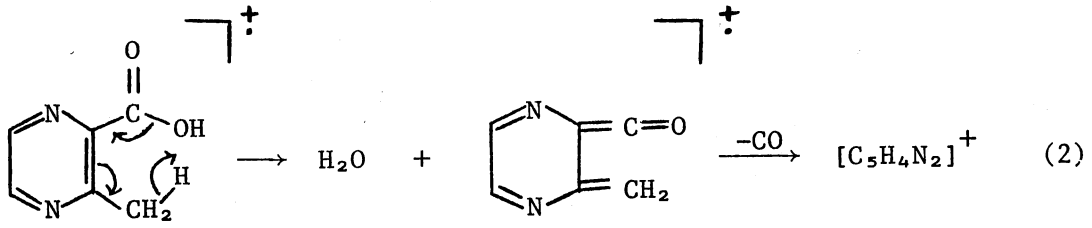


Figure 19. Mass Spectrum of 2-Pyrazinoic Acid (see p. 19)

These compounds show further similarity in that they both volatilize at 25-30° C in the mass spectrometer. The probable fragmentation pathway of M4 is shown in equation two.



(2)

m/e 138 m/e 120 m/e 92

The mass spectrum of metabolite number two (M2) is shown in Figure 20. The apparent molecular ion is at m/e 153 with other prominent peaks at m/e 124, 108, 95, 80 and 67. The compound volatilized at 110° C differing markedly from metabolite number four. This indicates the possibility of a very polar compound such as an N-oxide or salt. A metastable peak was observed at 90.6. High resolution mass spectrometry showed the exact mass of the apparent molecular ion to be 153.066. This compares favorably with the theoretical value of 153.067 for the empirical formula $C_7H_9N_2O_2$. Several other fragment peaks match well with logical fragment ions of the $C_7H_9N_2O_2$ parent. These results are shown in Table IV. These major peaks are consistent with the losses of OH, CHO, COOH and $C_2H_4O_2$. Other fragments differ only in the number of hydrogens. One possibe structure for metabolite two would be 3-methyl-4-(N-methyl)-2-pyrazinoic acid (V). This structure could fragment by

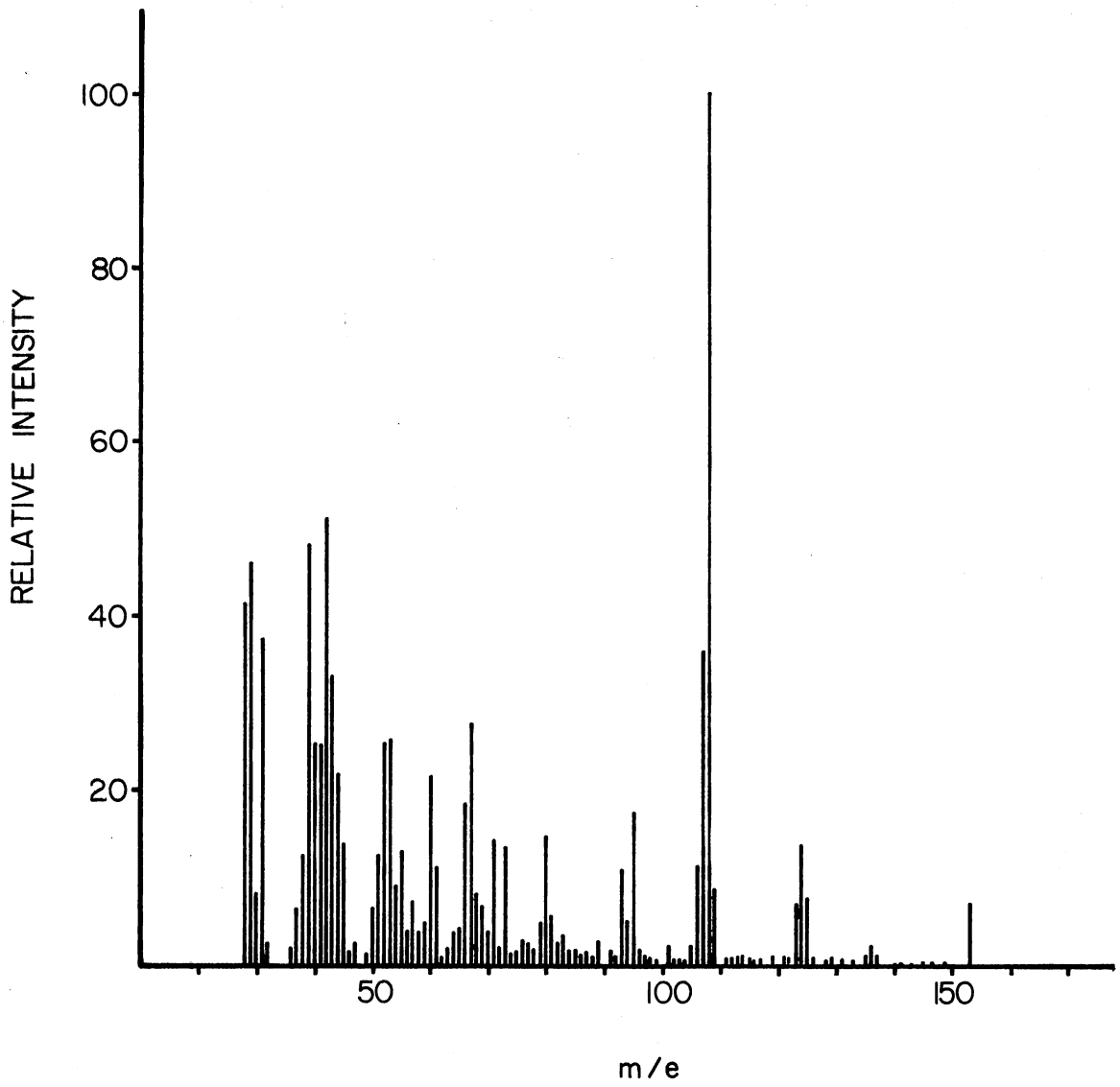
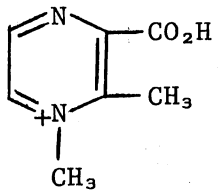
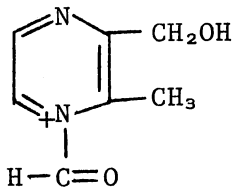


Figure 20. Mass Spectrum of Metabolite Two (see p. 19)

loss of OH to yield m/e 136 or by loss of the whole carboxyl group to yield m/e 108. The loss of the carboxyl group and methyl group would then yield m/e 93. The structure of metabolite two must account for the net addition of ($C_7H_9N_2O_2$ - $C_6H_8N_2$) or CHO_2 to the parent dimethylpyrazine molecule. Another possible route for metabolism would be N-formylation and either ring or side chain hydroxylation. This could also yield the proper molecular weight. A possible structure would be 1-(N-formyl)-2-hydroxymethyl-3-methylpyrazine (VI).



V



VI

Several mass spectral peaks of M2 are also found in the mass spectrum of 2,3-dimethylpyrazine. These are m/e 67, 52, 42 and 40. Bondarovich et al. (16) has determined the empirical formulae of some fragments of alkylpyrazines using high resolution mass spectrometry. For 2,3-dimethylpyrazine they found m/e 42 to be C_2H_4N and m/e 67 to be C_4H_5N. They also found that the peak at m/e 108 was $C_6H_8N_2$ in eight different alkylpyrazines, including 2,3-dimethylpyrazine. The empirical formula for the m/e peak in M2 was also found to be C_6H_8N (Table IV). This is additional evidence that the pyrazine ring

structure has not been destroyed by metabolism to metabolite number two.

TABLE IV

HIGH RESOLUTION MASS SPECTROMETRY RESULTS FOR METABOLITE TWO

m/e	Observed Mass	Calculated Mass	Empirical Formula
153*	153.067	153.066	$C_7H_9N_2O_2$
136*	136.060	136.064	$C_7H_8N_2O$
137	137.073	137.072	$C_7H_9N_2O$
124*	124.062	124.064	$C_6H_8N_2O$
122	122.046	122.048	$C_6H_6N_2O$
123	123.053	123.056	$C_6H_7N_2O$
125	125.067	125.072	$C_6H_9N_2O$
108*	108.070	108.069	$C_6H_8N_2$
105	105.044	105.045	$C_6H_5N_2$
106	106.055	106.053	$C_6H_6N_2$
107	107.059	107.061	$C_6H_7N_2$
109	109.075	109.078	$C_6H_9N_2$
93*	93.046	93.045	$C_5H_5N_2$
94	94.052	94.053	$C_5H_6N_2$
95	95.060	95.061	$C_5H_7N_2$

*Major peaks

The mass spectrum of metabolite number three (M3) is shown in Figure 21. The compound volatilized at 90° C in the mass spectrometer. The base peak is again m/e 108. Metabolites two, three and four all show fragments at m/e 42, 52 and 67. M2 and M3 more closely resemble each other. They share peaks at 153, 108, 107, 80, 67, 53, 52, 43, 42 and 39. The peak of M3 at m/e 269 was the highest peak observed in the spectrum. Although the peak was of low intensity it appeared in every preparation of this metabolite. If the m/e 269 peak is the molecular ion a conjugated metabolite is likely. Simple oxidation could not account for the net addition of 161 mass units. Due to their similar Dowex 1X8 elution positions and mass spectral patterns M3 might be a conjugated form of M2. Some further studies on the possible conjugate structure of M3 are discussed in section D.6.

D.3. Infrared Spectroscopy

The infrared spectra of metabolites two and three are quite similar (Figures 22, 23). Both show strong absorption bands at 1600 and 1400 cm^{-1} and weak bands at 1960, 1300 and 1170 cm^{-1}. The bands at 1600 and 1400 cm^{-1} were also observed for M4. None of the three metabolites yielded any evidence for an N-oxide group which would give a very intense band at 1350-1250 cm^{-1} (73).

D.4. Nuclear Magnetic Resonance Spectroscopy

The proton magnetic spectra of metabolites two and three both show the presence of aromatic ring protons (Figures 24, 25). The values 8.44 and 8.28 ppm both compare favorably with the value of 8.21 observed for 2,3-dimethylpyrazine. The ring protons of a non-aromatic structure

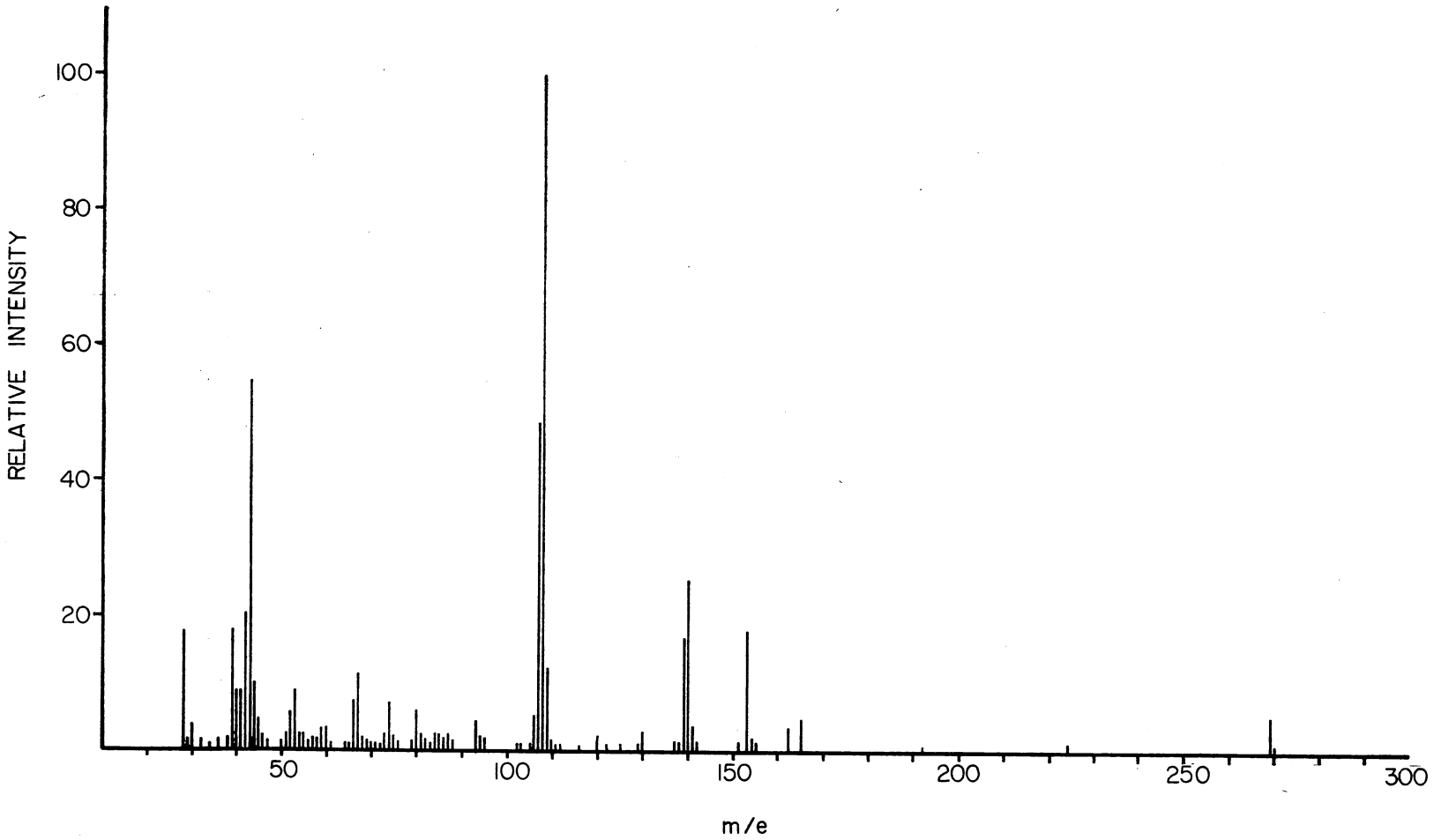


Figure 21. Mass Spectrum of Metabolite Three (see p. 19)

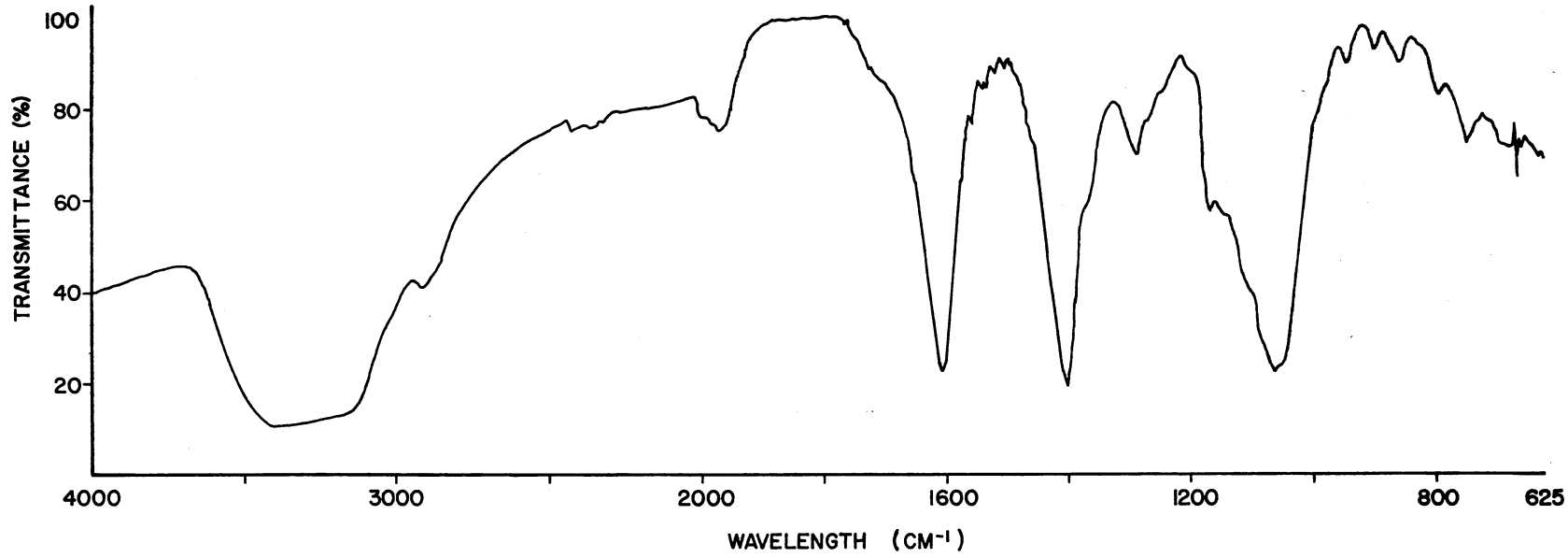


Figure 22. Infrared Spectrum of Metabolite Two (KBr pellet) (see p. 19)

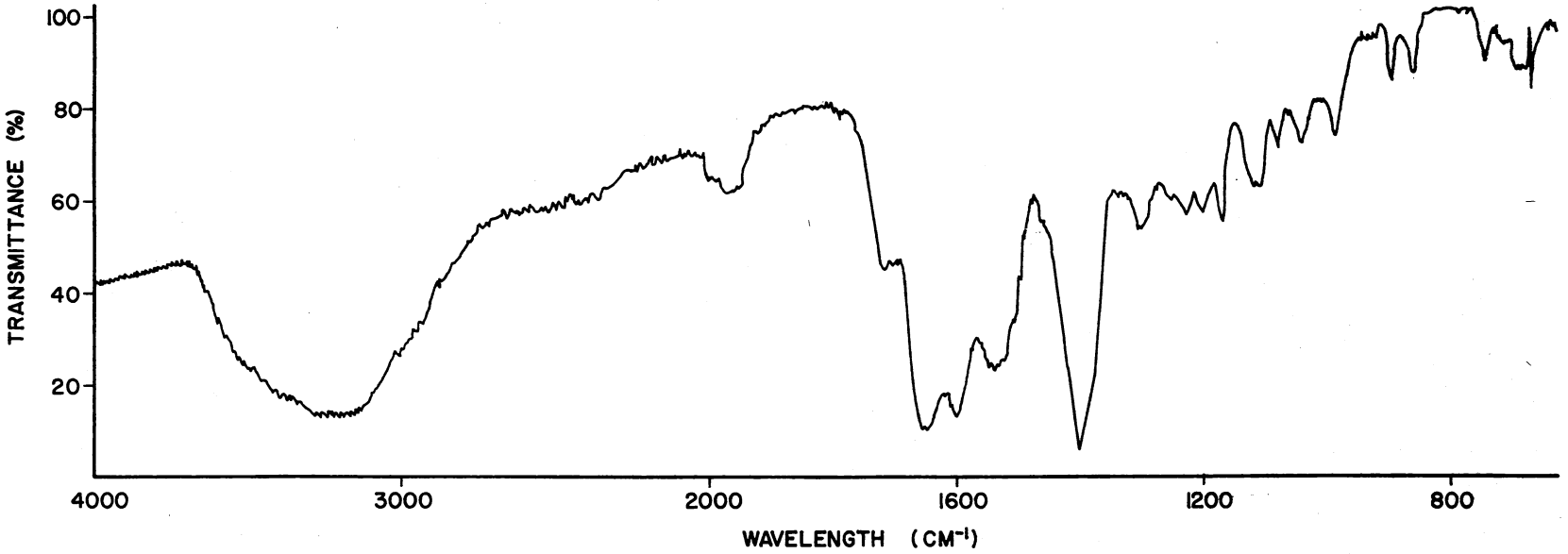


Figure 22. Infrared Spectrum of Metabolite Three (KBr pellet) (see p. 19)

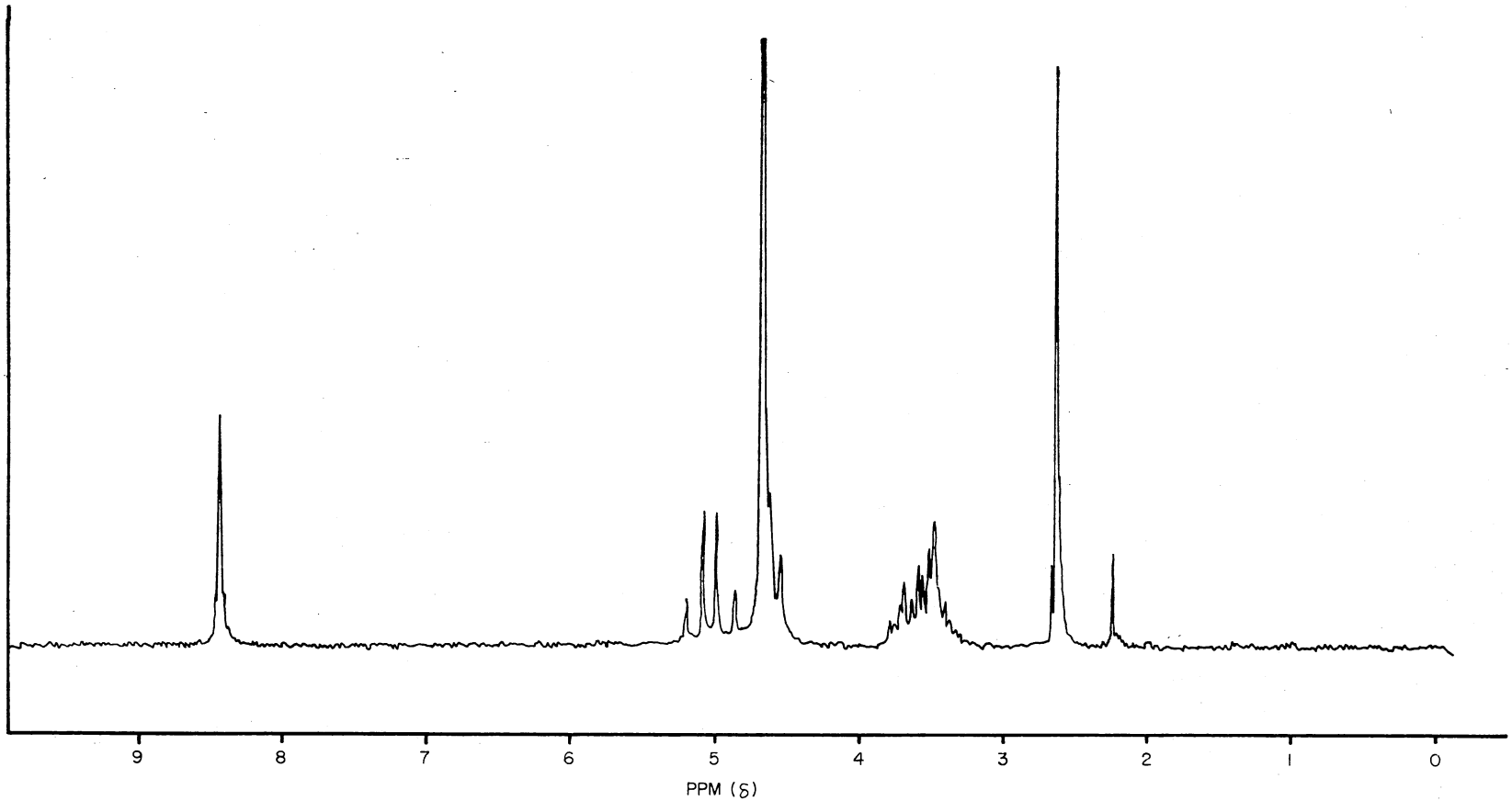


Figure 24. NMR Spectrum of Metabolite Two (see p. 19)

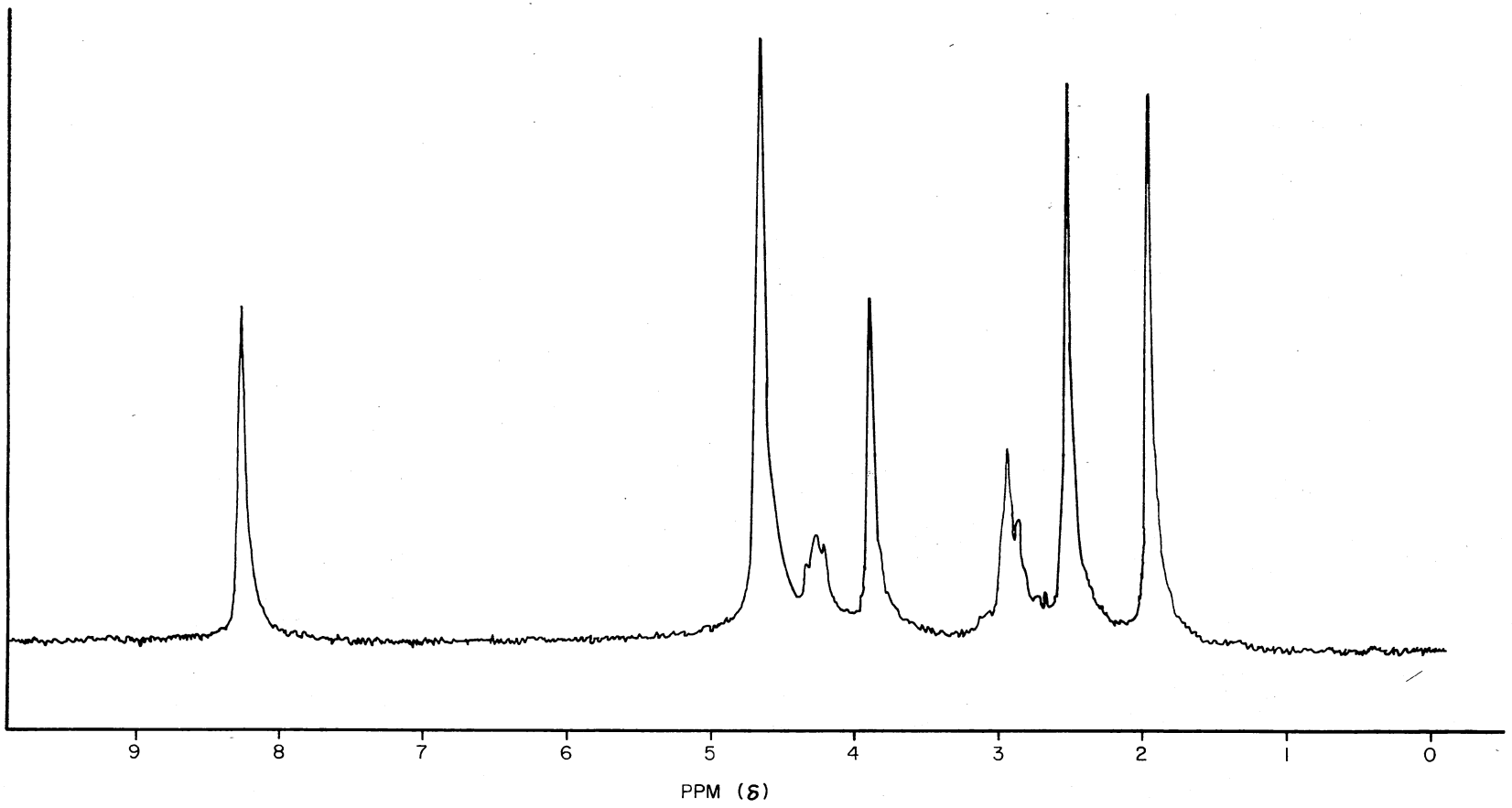


Figure 25. NMR Spectrum of Metabolite Three (see p. 19)

would be much farther upfield. Smith (35) has reported a value of 3.19 ppm for the ring hydrogens of 2,3-dimethyl-5,6-dihydropyrazine. Table V gives a summary of the NMR data obtained for the metabolites and standards used. All the samples were analyzed in D_2O because of solubility problems encountered with less polar solvents. Thus all exchangable protons such as those of hydroxyl and carboxyl groups were not observed.

The NMR spectrum of M2 shows peaks at 8.44, 5.04, 3.55 and 2.62 ppm. Integration of the peaks yielded the ratios 2:2-3:6:3. The singlets at 8.44 and 2.62 are consistent with two aromatic ring hydrogens and three ring-methyl hydrogens. This indicates that dimethylpyrazine has been metabolized at one methyl group and possibly also at the ring nitrogens. The groups responsible for the other two peaks have not been identified. Low humps like the one at 3.55 ppm often indicate hydrogen bonded to nitrogen (74).

The data obtained for metabolite three also indicates that two ring-hydrogens and one ring-methyl group have remained intact. This is shown by the peaks at 8.28 and 2.54 in the ratio of two to three. The peak at 1.97 is consistent with three highly shielded protons. This peak and some of the others might be explained by a conjugated moiety. A side chain methyl could be oxidized to an alcohol or acid and then conjugated with a small molecule. Conjugation might also occur at the ring nitrogen.

A good NMR spectrum was not obtained for metabolite four since this metabolite was not produced in large quantities. Figure 26 shows the result of 200 time-averaged scans at a scan speed of 250 seconds per scan. Only one peak was observed other than the D_2O peak. This peak,

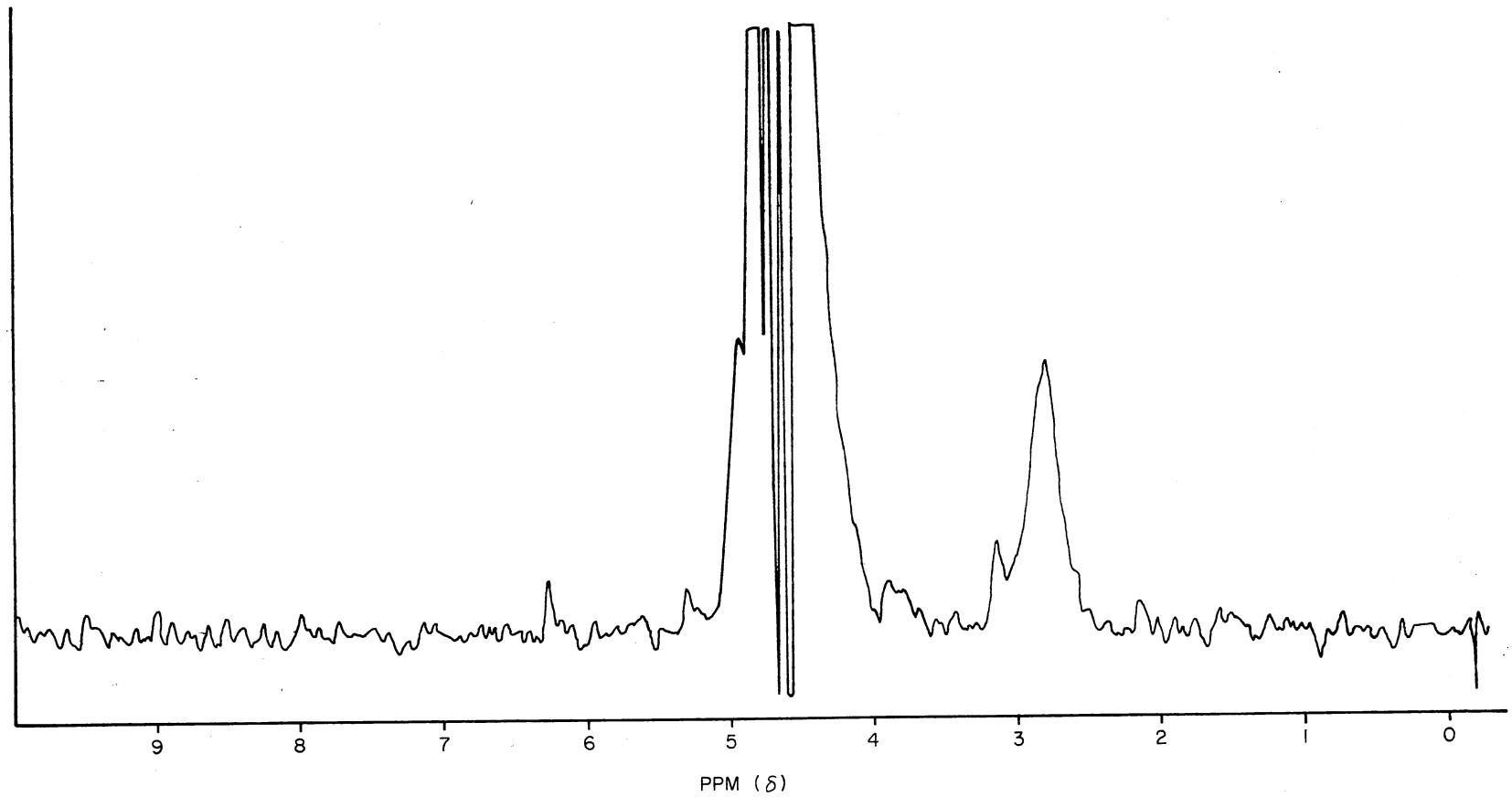
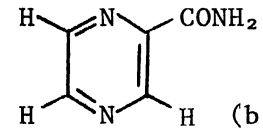
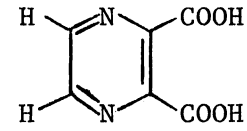
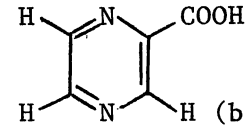
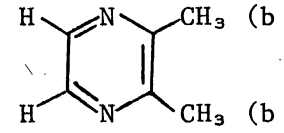


Figure 26. Time-Averaged NMR Spectrum of Metabolite Four (see pp. 19, 67)

located at 2.79 ppm, probably represents the protons of the ring-methyl group.

TABLE V

NMR PEAKS OF VARIOUS PYRAZINE COMPOUNDS

Compound	Peak Values (ppm)	Peak Ratios
a)  a)	a. 8.81 b. 9.17	1:2
a)  a)	a. 8.82	-
a)  a)	a. 8.83 b. 9.27	1:2
a)  a)	a. 8.21 b. 2.49	1:3
Metabolite Two	a. 8.44 b. 5.04 c. 3.55 d. 2.62	2:2-3:6:3
Metabolite Three	a. 8.28 b. 4.28 c. 3.90 d. 2.95 e. 2.54 f. 1.97	2:1-2:2:2:3:3
Metabolite Four	a. 2.79	-

D.5. Further Chromatography of Metabolite Four

The mass spectral pattern of metabolite four (M4) strongly indicated that its structure was 3-methylpyrazine-2-carboxylic acid. To gain further evidence metabolite four and three pyrazine standards were chromatographed using thin layer and anion exchange chromatographic techniques. Approximately 0.8 micromoles each of pyrazinamide, pyrazinoic acid and pyrazine-2,3-dicarboxylic acid were chromatographed using 0.6 X 17 cm. columns of Dowex 1X8 resin. The eluents were 15 ml. each of H_2O, 3N, and 4N formic acid and 30 ml of 4N formic acid plus 1.0N ammonium formate. Figure 27 shows the profile of the three standards. In a separate column run, metabolite four eluted at tube number 47, the same elution position as pyrazinoic acid. The experiment was repeated using Dowex 50WX8 columns of the same size. Both M4 and pyrazinoic acid were eluted in the first six milliliters of the water wash.

Metabolite four and the three standards were chromatographed on ChromAR sheets using the normal solvent system used for M4 isolation. The results shown in Table VI indicate that metabolite four, with an Rf value of 0.43, is slightly less polar than pyrazinoic acid (Rf=0.37). This is to be expected since the methyl group of M4 would be electron donating and decrease the strength of the acid.

D.6. Further Studies on Metabolite Three

The largest ion fragment observed in the mass spectrum of metabolite three was m/e 269. This is 161 mass units higher than the molecular weight of dimethylpyrazine. Because of this M3 is likely to

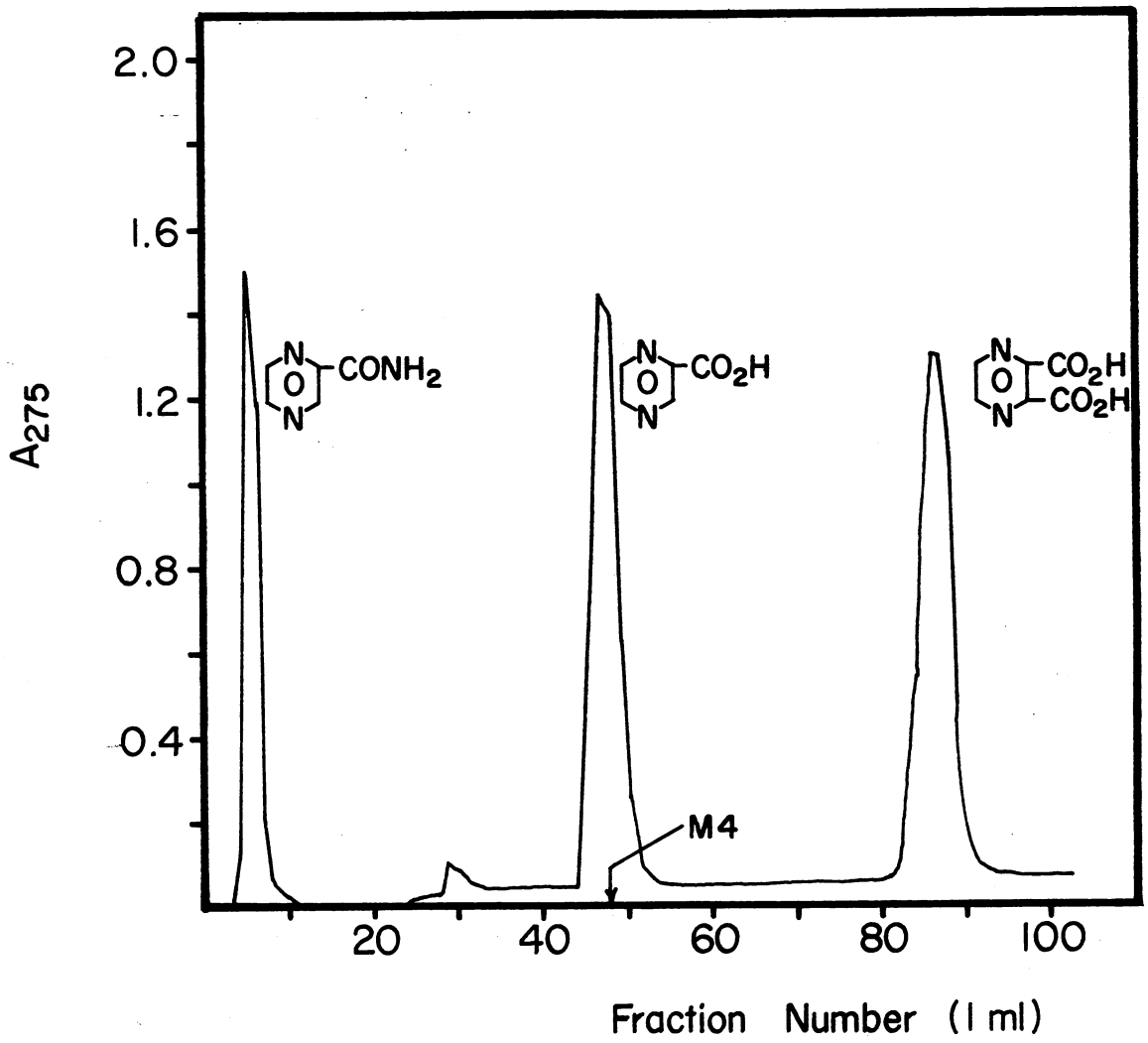


Figure 27. Dowex 1X8 Chromatogram of Pyrazine Standards (see pp. 17, 70)

be a conjugated pyrazine derivative.

TABLE VI

THIN-LAYER CHROMATOGRAPHY OF METABOLITE FOUR*

Compound	Rf Value
Pyrazine-2,3-dicarboxylic acid	0.02
Pyrazinoic acid	0.37
Pyrazinamide	0.92
Metabolite four	0.43

*ChromAR sheets developed in chloroform:methanol:NH_4OH (100:50:3)

Forty-five hundredths micromoles of M3 was hydrolyzed in 6N hydrochloric acid. The resulting hydrolysate was then analyzed for amino acids using the method of Spackman, Stein and Moore (68). Fifteen hundredths micromoles of dl-norleucine was included as an internal standard. The sample yielded only one peak. It eluted exactly at the first buffer change which was after valine and before methionine. The peak thus did not correspond to any of the common amino acids. When an unhydrolyzed sample of M3 was analyzed a very small peak was observed in the same position as in the hydrolyzed sample. Since the metabolite peak eluted exactly at a buffer change further amino acid analyses used only the first buffer for elution. Cystathionine was considered to be

a likely candidate for the unknown since it also eluted at the buffer change. However, when only one buffer was used the elution times were 118 min. for valine, 137 for the M3 fragment and 172 and 181 for the two peaks from cystathionine. Thus it seemed likely that the conjugated moiety was an alpha amino acid with properties similar to valine and methionine. S-methylcysteine and norvaline are similar to methionine and valine, but they eluted differently from the M3 fragment. S-methylcysteine eluted earlier than both valine and the M3 fragment, while norvaline eluted after the fragment.

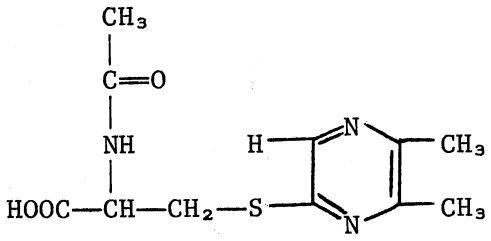
To check the stoichiometry of the conjugation 0.4 micromoles each of hydrolyzed M3 and norleucine were analyzed. Assuming the same ninhydrin color factor for both the M3 fragment and norleucine the ratio found after analysis was 1.18:1.00 (M3 to norleucine). It seems likely that if M3 is a conjugated metabolite the conjugation takes place with a stoichiometry of one to one.

To separate the pyrazine and amino acid-like moieties from the M3 hydrolysate one micromole of hydrolysate was fractionated using a 0.5 X 8 cm. column of Dowex 50WX8 as previously described. The fractions eluting in the water and HCl washes were referred to as the H_2O and HCl fractions. To test the system 0.25 mg. of cystathionine (about one micromole) and 0.25 mg of pyrazinoic acid were chromatographed. All of the pyrazinoic acid and cystathionine was eluted in the H_2O and HCl fractions, respectively, as monitored by ultraviolet absorbance and ninhydrin. Using hydrolyzed M3 the amino acid-like moiety was found only in the HCl fraction as monitored by amino acid analysis. This compound eluted from the analyzer at the same position in both fractionated and unfractionated samples.

The HCl fraction was lyophilized and analyzed using mass spectrometry. The results were very ambiguous. Amino acids often give unacceptable spectra due to decomposition and formation of diketopiperazine dimers (75). Ethyl esters of amino acids can be made with ethanolic-HCl as described by Biemann et al. (69). They found prominent peaks at (M-73) and (M-102) which were caused by cleavage at either side of the alpha carbon. Using the method described in chapter three ethyl esters of alpha-aminobutyric acid and the HCl fraction of M3 were prepared. The spectrum of ethyl alpha-aminobutyrate correlated well with the expected fragments. However the spectrum of the ethylated M3 fragment was poor. The major peaks were at m/e 42, 108, and 139. Better spectra might be obtained by using the amino acid analyzer to purify to HCl fraction and by using minimal amounts of solvents for derivatization.

The amino acid analysis of hydrolyzed M3 also indicated a small amount of cysteine. A cysteine conjugate could be expected to be altered by performic acid oxidation. About 20 nanomoles of hydrolyzed M3 were oxidized with performic acid. One volume of 30% hydrogen peroxide was mixed with nine volumes of 98% formic acid and allowed to stand at room temperature for one hour. The oxidation was conducted at 0° C for one hour using 0.1 ml of the acid solution. Amino acid analysis of the product yielded a peak eluting at 43.6 minutes, compared to 146.9 minutes for the unoxidized sample. A small cysteic acid peak was also observed. This change in elution time closely parallels the shift observed for the oxidation of methionine to methionine sulfone. From these results a possible structure for M3 is 2,3-dimethylpyrazine-5-mercapturic acid (VII).

This structure is consistent with the release of a ninhydrin-positive group upon hydrolysis, the release of small amounts of cysteine and cysteic acid and the change in elution time upon oxidation. The mass spectral peaks of M3 also support this structure (Figure 21). The molecular ion at m/e 269 yields the appropriate molecular weight. Cleavage at either side of the sulfur atom would yield fragments at m/e 107, 130, 140, and 162. Loss of COOH would yield m/e 224 and the m/e 43 peak could be due to the acetyl group.



VII

E. Elimination Rates of Dimethylpyrazine

Work on the isolation of the urinary metabolites of 2,3-dimethylpyrazine utilized the urine excreted in the first 24 hours after injection. It was observed that approximately seventy to eighty per cent of the injected ^{14}C was excreted in the urine during that time period. Further studies were performed to determine more exact excretion rates.

Table VII shows the amounts of radioactivity excreted during each

twelve hour period after injecting two rats with 50 mg. of ^{14}C-2,3-dimethylpyrazine. Most of the radioactivity is excreted during the first twelve hours and very little is excreted after forty-eight hours. These results are in agreement with earlier work done by Elgin (49) with 2,3-^{14}C-labelled dimethylpyrazine. He found that less than one per cent of the dose was excreted after the first two days.

TABLE VII

EXCRETION OF ^{14}C IN THE URINE OF RATS INJECTED WITH 5,6-^{14}C-2,3-DIMETHYLPYRAZINE*

Time (hours)	Percent Dose
12	58.2
24	17.4
48	4.7
72	0.44
96	0.38
96 Hour Total	81.2

*Two rats were administered doses of 3.55 X 10^6 dpm.

The excretion rate of dimethylpyrazine in mice was also checked. Three mice were each injected with 1.7 mg. of ^{14}C-dimethylpyrazine. Nine-tenths per cent of the ^{14}C was excreted in the first ten hours and

49.2 per cent was excreted in the second ten hours. Very little was excreted after twenty hours. A total of 52.4% was excreted in 132 hours. Smith (35) has reported on the urinary excretion of 2,3-dimethyl-5,6-dihydropyrazine in mice. He reported 43% excreted in the first 24 hours and 55% during the first five days. Thus the dihydro compound is excreted at a rate that is similar to that of its aromatic analog. The renal clearance rates of dimethylpyrazine in rats and mice are similar to rates for pyrazinamide in dogs. Weiner and Tinker (42) found that after intravenous administration of one gram of pyrazinamide in dogs 30% was excreted in six hours and 70% was excreted in 24 hours.

The results of Dowex 1X8 chromatography showed that the four metabolites of dimethylpyrazine differed greatly in charge. Since the urinary excretion of compounds depends greatly on their charge, an experiment was done to see if the individual metabolites were excreted at different rates. Two rats were each injected with 50 mg. of ^{14}C-dimethylpyrazine. Their daily urine collections were chromatographed in duplicate on Dowex 1. The metabolite peaks were pooled and analyzed for ^{14}C. Table VIII shows the results of this work. These results show that the percentage appearing as metabolite one sharply increases after the first 24 hours. The more anionic metabolites are excreted much faster. The excretion of metabolite four showed the sharpest decline. Almost all of it was excreted in the first 24 hours. These results are not too surprising since highly polar compounds are more water soluble and often excreted faster than less polar ones.

To determine if mice metabolize dimethylpyrazine to the same metabolites as rats, mouse urine was analyzed using Dowex 1 columns, as done for rat urine. Three mice were each injected with 7.5 mg. of

^{14}C-dimethylpyrazine. Using the urine from the first 24 hours only four radioactive peaks were observed on Dowex 1. These peaks eluted at the same positions in mouse and rat urine. The metabolite percentages were as follows: 4% as M1, 38% as M2, 49% as M3 and 8% as M4.

TABLE VIII

PER CENT OF DAILY EXCRETED ^{14}C IN EACH METABOLITE*

Day	% ^{14}C excreted	% as M1†	% as M2	% as M3	% as M4
1	77.0	5.2	65.6	19.2	10.0
2	2.7	47.6	36.0	15.2	1.3
3	0.5	60.5	24.7	13.4	1.4

†M1, etc. refer to Metabolites 1, etc.

*Two rats were each injected with 50 mg dimethylpyrazine and the urine from each rat was chromatographed in duplicate.

From the urinary excretion studies with rats and mice it was determined that a large portion of an injected dose of ^{14}C-dimethylpyrazine was metabolized and excreted during the first 24 hours after injection. In rats most of the ^{14}C was excreted in the first twelve hours. Collection periods of less than 10-12 hours are not suitable because of the irregularity of urination. To more accurately determine the rate of metabolism a tissue time course study was done. In this

experiment mice were injected intraperitoneally with 2 mg. of ^{14}C-dimethylpyrazine. At fixed times after injection animals were sacrificed and their blood, kidneys and liver were assayed for ^{14}C. The results, shown in Figure 28, indicate that radioactivity is cleared from all three tissues very rapidly. All data points were calculated in terms of dpm per organ. The calculation of total blood volume was based on 78 ml. blood per kilogram body weight (76). The ^{14}C levels reached their peaks in both liver and blood within 20 minutes and possibly before five minutes. A clearing of ^{14}C from the boood by the kidney was readily apparent. The time course for the kidney exhibits a slow rise and fall, peaking at about 40 minutes. It is not possible to conclude whether the compound is metabolized in the liver or kidney since the liver contains a large amount of blood. Drabkina and Ginzburg (77) studied the clearance of pyrazinamide in mice. Using oral doses of five mg. pyrazinamide they found that maximum concentrations occurred in the blood, lungs, kidneys and liver at one to two hours after administration, with the levels decreasing rapidly in five hours.

F. In Vitro Studies

Metabolic studies using intact animals provide useful information on the disposition and final metabolites of foreign compounds. However such studies provide little information on the nature and sites of intermediate biotransformation of such compounds since only "terminal" metabolites are collected (78).

Several in vitro experiments were employed in this study of dimethylpyrazine to determine the nature and location of the metabolizing system. The assay procedure made use of the fact that 2,3-dimethyl-

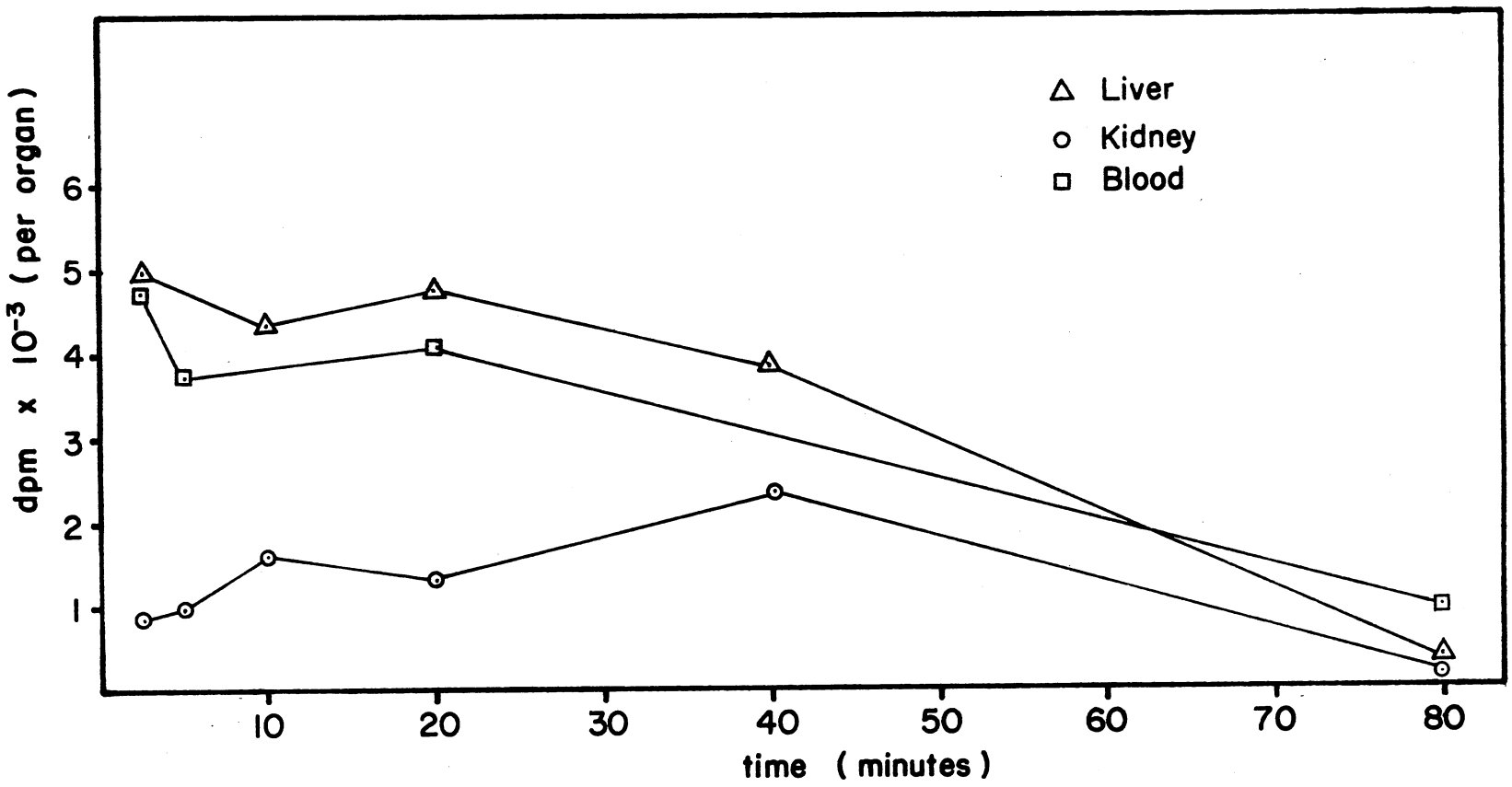


Figure 28. Time Course of ^{14}C-Dimethylpyrazine in Mice (see p. 22)

pyrazine is volatile (boiling point = 156° C) while its metabolites are nonvolatile. The metabolites were shown to be nonvolatile by their quantitative recovery when metabolite-containing urine was lyophilized. In the in vitro experiments ^{14}C-dimethylpyrazine was incubated at 37° C in a buffered mixture containing a tissue homogenate and several cofactors. The mixture included a NADPH-generating system of NADP, glucose-6-phosphate, glucose-6-phosphate dehydrogenase, magnesium chloride and nicotinamide in 0.1 M pH 7.4 sodium phosphate buffer. The nicotinamide was included to protect nucleotides from breakdown by nucleotidase (79). After a thirty minute incubation at 37° C the mixture was processed and lyophilized. The ^{14}C remaining after lyophilization was used to calculate the amount of dimethylpyrazine metabolized. Control incubation mixtures using boiled homogenates were used as blanks. Another control mixture had a known amount of purified metabolite three but no dimethylpyrazine. This control was used to determine the efficiency of metabolite recovery from the incubation mixtures.

The first in vitro experiments were done to determine which tissues metabolize dimethylpyrazine. Isotonic sucrose homogenates were made from the liver, kidneys, lungs and small intestine of 250 gram male rats. Table IX shows the relative metabolizing activities of the four tissues. The values were obtained from duplicate homogenates from each of three rats. Liver has the highest pyrazine metabolizing activity although it is likely that all four tissues have some activity. Although the values are rather imprecise, all four tissues yielded net activity in every rat tested.

TABLE IX

METABOLISM OF DIMETHYLPYRAZINE BY ORGAN HOMOGENATES

Organ	Specific Activity*
Liver	1.81 ± .62
Kidney	0.73 ± .67
Lung	0.37 ± .15
Small Intestine	0.56 ± .27

*Micromoles per gram tissue ± standard deviation (see p. 23)

Since liver yielded the highest homogenate activity it was used to attempt to localize the subcellular fraction responsible for dimethylpyrazine metabolism. Freshly excised rat liver was fractionated into nuclear, mitochondrial, microsomal and soluble supernatent fractions. These fractions were then assayed for activity using an incubation procedure similar to that used for the whole homogenates. No significant activities were found in any of the four fractions. These results do not necessarily preclude the possibility of the activity residing in a particular fraction. Certain necessary conditions may have been lacking in the incubations. Cofactor and protein concentrations are often very critical for such systems. Some loss of activity may have resulted from the long preparation times needed for the fractionation.

A further fractionation effort utilized the precipitates and supernatents from 20 minute centrifugations at 10,000 x g. Significant

activity was found in both fractions. The pellet had an activity of 5.8 nanomoles per mg. protein while the supernatent activity was 8.2. Sucrose buffer was found to stabilize the metabolizing system since the values for the pellet and supernatent derived from a phosphate buffer homogenate were 1.1 and 3.0, respectively. With either buffer the 10,000 X g supernatent had the larger activity. Like many others this metabolizing system could be microsomal. If it is some of the activity of the pellet may be due to the presence of unbroken cells. Further support for a microsomal system could come from induction experiments since some microsomal systems are known to be induced.

Preparative liver homogenate incubations were used to try to determine if the metabolism observed in the tissue homogenate studies was due to formation of the same metabolites observed in urine. These experiments used 50 ml. incubation mixtures containing 10 ml. of liver homogenate and the same procedure used in the previous homogenate experiments. The final product of the second lyophilization was re-suspended in 10 ml. deionized water and two ml. samples were chromatographed on 0.6 X 17 cm. columns of Dowex 1X8 resin. The eluents were 15 ml. each of water, 3 N formic acid, 4 N formic acid and 4 N formic acid plus 1.0 N ammonium formate and 30 ml. of 4 N formic acid plus 2.0 N ammonium formate. A consistent reproducible ^{14}C peak was observed at tube 5-6, although some runs yielded a few scattered peaks between tubes 50-80 (Figure 29). The peak at tube six, which eluted at a position similar to urinary metabolite fraction one was pooled, lyophilized and chromatographed on a 1.2 X 45 cm. Bio-Gel P-2 column. A single radioactive peak was observed (Figure 30). This peak was centered at tube 43 and surrounded by high amounts of UV-absorbing

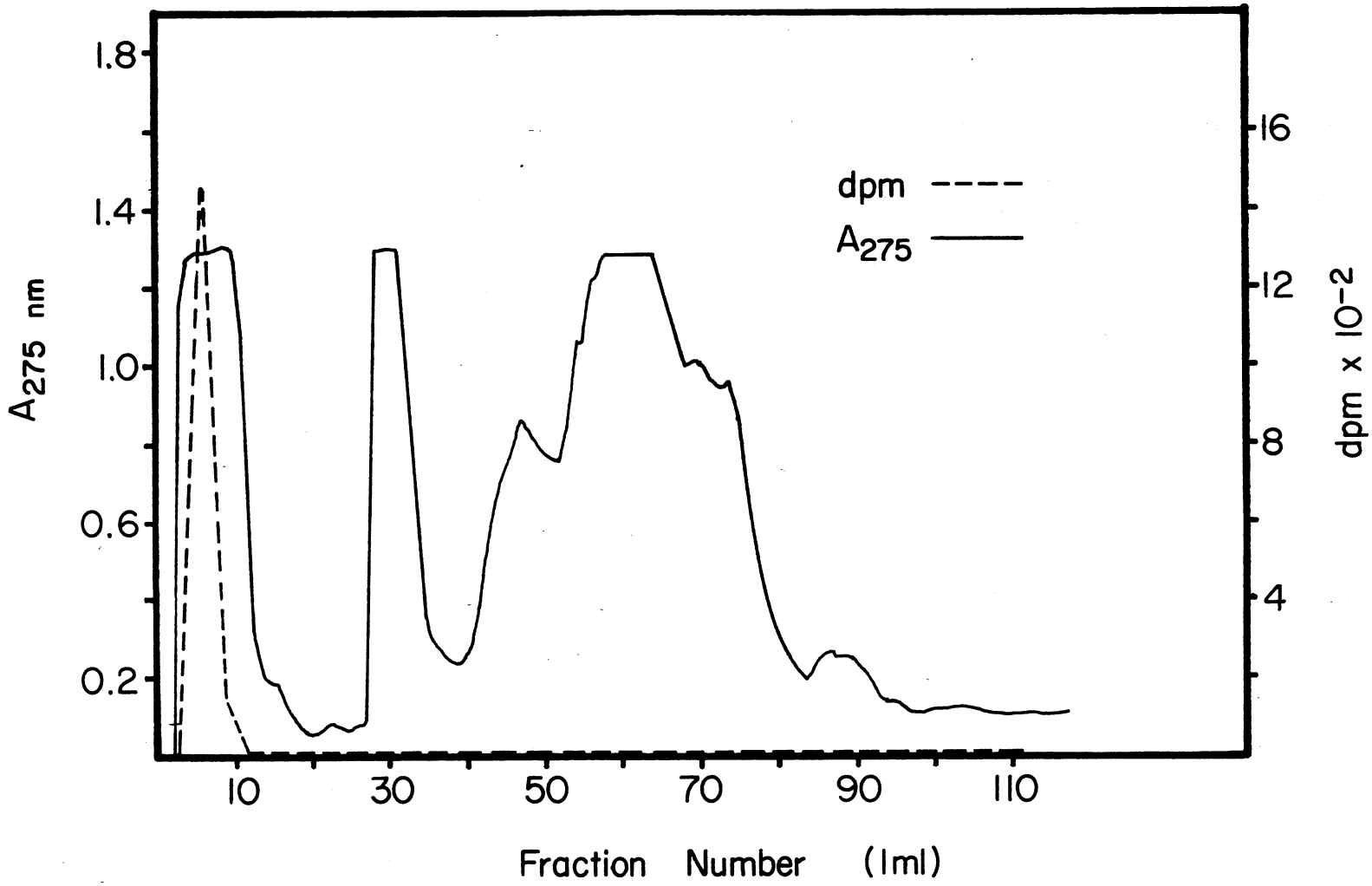


Figure 29. Dowex 1X8 Chromatogram of Homogenate Incubation Mixture (see p. 83)

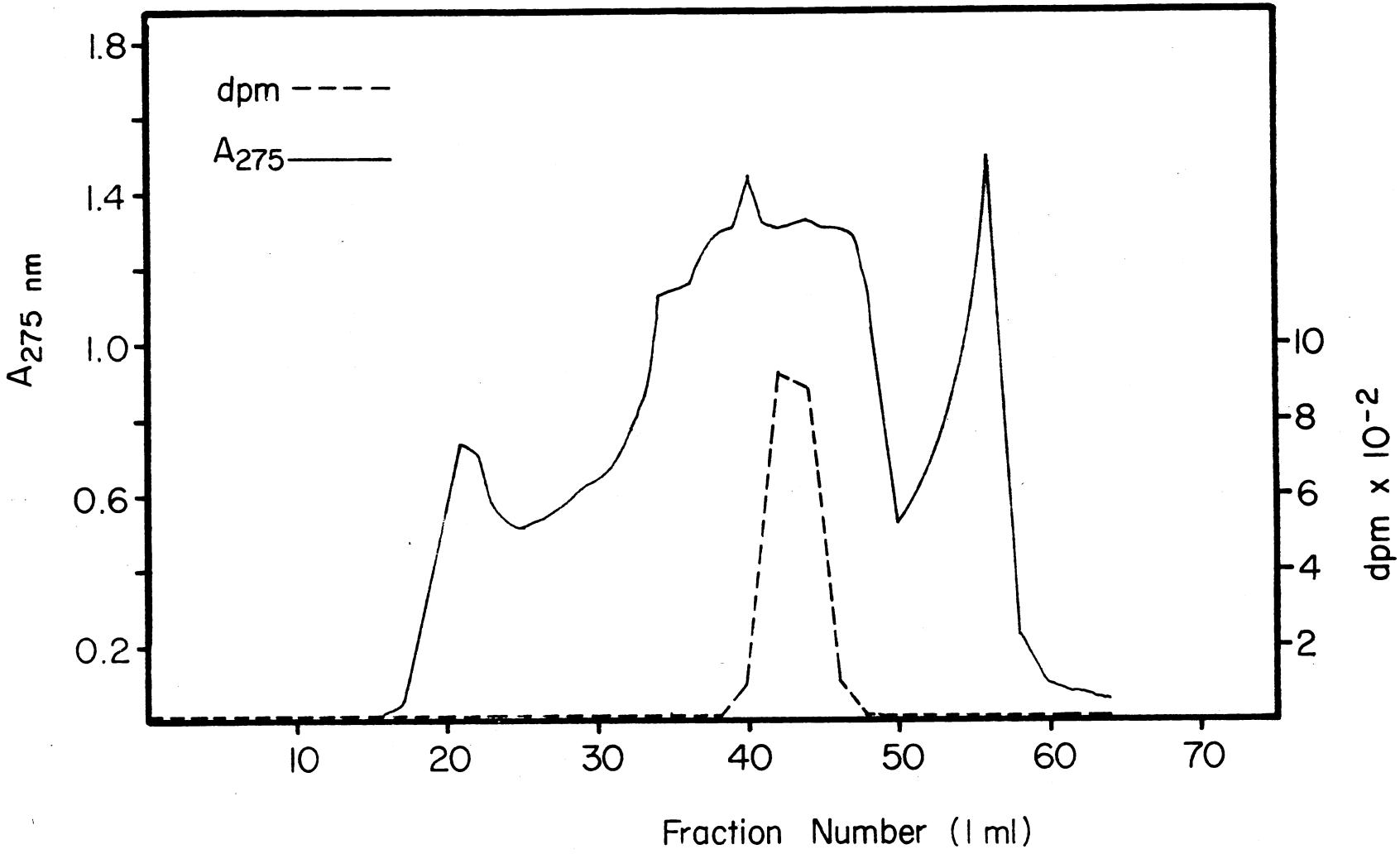


Figure 30. Bio-Gel P-2 Chromatogram of Homogenate Incubation Fraction (see p. 83)

material. When the peak was pooled and rechromatographed on P-2 the ^{14}C peak moved to tube 39 which was close to the eluting position of purified fraction M1. Apparently the large amount of impurities in the homogenate fraction caused the compound to elute abnormally late. Thus the metabolite formed in the liver homogenates seems at least similar to metabolite one of rat urine. The fact that not all of the urinary metabolites were observed is not surprising. With disrupted cells, essential cofactors might not have been present in sufficient quantities. Some compounds utilize more than one organ for their metabolism. Some pathways involve oxidation in one organ and conjugation in another.

G. Induction Studies

The final in vitro studies were conducted to determine if either phenobarbital or dimethylpyrazine can induce the metabolism of dimethylpyrazine. Induction was monitored by checking liver homogenate activities, urinary excretion of metabolites and urinary metabolite percentages. Female albino 250 gram rats were injected daily with either sodium phenobarbital, dimethylpyrazine or a control solution. They were each given 80 mg/kg on day one and 60 mg/kg on days three, five, six and seven. The shift to the lower dose was necessitated by the severe effects observed on days one and two. On day eight a comparison of phenobarbital, dimethylpyrazine and control-injected rats was made by checking the dimethylpyrazine-metabolizing activities of liver homogenates. The procedure was the same used for the liver homogenate experiments. Table X shows the results of an experiment using triplicate homogenates from each of two rats from each group. Boiled homogenate blanks were also run in triplicate. Phenobarbital

produced a significant inductive effect, giving an activity eight times that of normal rats. Dimethylpyrazine caused only a marginal increase in activity.

TABLE X

INDUCTION STUDIES WITH RAT LIVER HOMOGENATES

Inducer	Activity*	Relative Activity
Phenobarbital	2.93 ± .41	8.4
2,3-Dimethylpyrazine	0.56 ± .17	1.6
Control	0.35 ± .15	1.0

*Average micromoles/gram liver ± standard deviation

Two animals from each injection group were used to compare the rates of urinary excretion of ^{14}C. Urine was collected for 21 hours after injection with 40 mg. of ^{14}C-dimethylpyrazine. Both dimethylpyrazine and phenobarbital caused some increase in urinary ^{14}C excretion (Table XI). Again phenobarbital caused the largest effect, yielding a 54% increase in the amount of ^{14}C excreted in the first 21 hours after injection. It is possible that a shorter sampling time than 21 hours would show a greater difference in ^{14}C excretion but irregularity of urination precluded the use of short collection periods.

TABLE XI

URINARY EXCRETION OF ^{14}C IN INDUCER-INJECTED RATS

Inducer	% Dose Excreted (21 hr)	Relative Excretion
Phenobarbital	84.8	1.54
2,3-Dimethylpyrazine	61.4	1.11
Control	55.0	1.00

Each animal was given 40 mg. dimethylpyrazine i.p.

A remaining question was whether the induction caused by phenobarbital and dimethylpyrazine involved selective induction of specific pathways. This would be likely to cause an altered ratio of excreted metabolites. Aliquots of each of the three 21 hour urine samples were chromatographed in duplicate on 0.6 X 17 cm. Dowex 1X8 columns, using the same procedure utilized to obtain the date in Table VIII. The results are shown in Table XII. The metabolite percentages are very similar in all three urine samples. Metabolite four shows the only significant change.

Induction of microsomal enzymes by foreign compounds is very important not only because of the effect on the clearance of these compounds but also because microsomal systems also act on other compounds such as drugs and steroid hormones. Also if the metabolism of two compounds is induced by the same inducer then it is likely that they are metabolized by the same system. There are apparently two

types of inducers. These are the phenobarbital and aromatic hydrocarbon or 3-methylcholanthrene types. These two systems often oxidize the same compound in different positions and probably work by different mechanisms (59).

TABLE XII

METABOLITE PERCENTAGES IN URINE AFTER INDUCTION

Inducer	% as M1*	% as M2	% as M3	% as M4
Phenobarbital	4.1	52.3	40.3	3.2
2,3-Dimethylpyrazine	3.8	56.5	34.1	5.5
Control	4.2	49.9	38.4	7.6

*M1, etc. refers to Metabolite One, etc.

In this study phenobarbital was shown to increase the metabolism of dimethylpyrazine both *in vivo* and *in vitro*. Thus it is likely that dimethylpyrazine is metabolized by a microsomal system. Phenobarbital was found to increase the *in vitro* activity approximately eight-fold. This increase is similar to that found by Conney *et al*. (80). They observed an increase in enzyme activity to a maximum of 3-10 times normal after three twice daily injections of 40 mg. phenobarbital per kilogram. Comparing the results of the control rats in this experiment (using females) to the results of the earlier organ homogenate

experiments (using males), it is evident that the pyrazine metabolizing activity of females is much less than that of males. The male rats had an activity of 1.81 ± .62 micromoles/g liver while the rate for females was 0.35 ± .15. This was to be expected since adult male rats have higher levels of drug metabolizing enzymes. The sex difference in metabolizing activity may be the reason why induction is more easily observed in female rats (59). Although the females had lower basal activity in the present study their level surpassed that of untreated males after exposure to phenobarbital.

CHAPTER V

SUMMARY

5,6-^{14}C-2,3-Dimethylpyrazine was synthesized by the condensation of ethylenediamine with 2,3-butanedione followed by the catalytic dehydrogenation of the resulting dihydro compound. Toxicity studies using 30-35 gram male albino mice yielded an LD_{50} value of 0.7 mg/g body weight. Four metabolites of dimethylpyrazine were isolated from the urine of rats. These metabolites were separated using thin layer and column chromatographic techniques. Using Dowex 1X8 anion exchange resin the same four metabolite peaks were observed in both rat and mouse urine. All four metabolites have ultraviolet absorption spectra closely resembling that of dimethylpyrazine. This indicates that the pyrazine ring has not been cleaved. This is supported by mass spectral and NMR studies and also by the fact that both the 2,3-^{14}C and 5,6-^{14}C-labelled compounds yield the same four metabolite peaks.

Metabolite one is not strongly anionic while the other three metabolites are. Mass spectral studies of metabolite two indicate an empirical formula of $C_7H_9N_2O_2$. Major fragment ions indicate losses of OH, CHO, and $C_2H_4O_2$ from the molecular ion. Possible structures for metabolite two include 3-methyl-4-(N-methyl)-pyrazinoic acid and 1-(N-formyl)-2-hydroxymethyl-3-methylpyrazine. Metabolite three is apparently a conjugate with an alpha-amino acid. Amino acid analysis of hydrolyzed metabolite three yields a ninhydrin positive peak that

elutes between valine and methionine. A possible structure for metabolite three is 2,3-dimethylpyrazine-5-mercapturic acid. The probable structure of metabolite four is 3-methylpyrazine-2-carboxylic acid. Support for this structure comes from a comparison of the mass spectral and chromatographic properties of metabolite four and pyrazinoic acid. Metabolite four fragments by the consecutive losses of 18 and 28 mass units. The probable fragmentation pathway is the loss of water via a McLafferty rearrangement followed by the loss of a carbonyl group.

Seventy to eighty per cent of a 50 mg. dose of ^{14}C-dimethylpyrazine is excreted in the urine of adult rats in the first 24 hours after intraperitoneal injection. Most of the ^{14}C is excreted in the first 12 hours and very little is excreted after 48 hours. Mice excrete about 50% of the dose in the first 24 hours. The more anionic metabolites are excreted much faster than the less anionic ones in rats. Although metabolites two and three are the major metabolites, they are not necessarily the most important pharmacologically. Metabolite one is excreted at the slowest rate and metabolite four resembles a compound known to cause urate retention. After intraperitonial injection dimethylpyrazine is rapidly cleared from the liver, blood and kidneys of mice. The concentration of ^{14}C peaks in the liver and blood before 20 minutes and peaks in the kidney at about 40 minutes.

In vitro tissue homogenate studies showed liver to have the largest pyrazine-metabolizing activity when compared to kidney, lung and small intestine in rats. Attempts to localize the intracellular site of pyrazine metabolism met with little success. The 10,000 X g supernatent showed slightly more activity than the pellet. The metabolite formed in these tissue homogenates is at least similar to

the metabolite number one of urine.

Both phenobarbital and 2,3-dimethylpyrazine can induce the metabolism of dimethylpyrazine. Phenobarbital and dimethylpyrazine show approximately eight and two-fold increases in the activities of liver homogenates, respectively. Phenobarbital causes a significant increase in the amount of ^{14}C excreted after an intraperitoneal dose of ^{14}C-dimethylpyrazine while dimethylpyrazine shows a slight increase. The induction caused by these two compounds probably does not involve a different metabolizing pathway since the metabolite percentages do not significantly change. Since pyrazine metabolism is induced by phenobarbital it is likely that a microsomal system is involved.

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