CHEMICAL SYNTHESIS AND METABOLISM OF

2,3-^{14}C-2,3-DIMETHYLPYRAZINE

By

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CHEMICAL SYNTHESIS AND METABOLISM OF

2,3-^{14}C-2,3-DIMETHYLPYRAZINE

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CHAPTER I

INTRODUCTION

Alkylpyrazine compounds occur at varying levels in a variety of roasted food products as well as in several uncooked food products. Pyrazine compounds are utilized as drugs. Pyrazinamide is a drug used in large dosages (0.5 - 1.0 gm orally) to treat tuberculosis (1). Further, there exists a recent patent for the use of pyrazines as food flavor additives (2). It is conceivable that pyrazine compounds can be consumed in greater amounts than the levels encountered in natural foods. It would be advantageous to perform long studies on the possible effects of repeated subtoxic doses. Little information on the metabolism or toxicity of these compounds has been reported in the literature.

Among food additives approved by the United States Food and Drug Administration on the basis of "generally-recognized-as-safe," (GRAS) substances (3) are several alkylpyrazines.

It is important to be aware that the definition of toxicity is established at a given time; what is called a "safe" compound of yesterday, by newer standards and methods, may afterwards be shown as harmful.

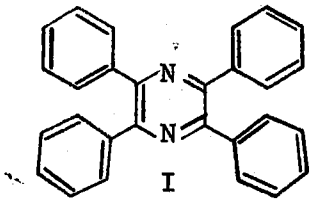
This thesis will report the multistep synthesis of a radioactive dialkylpyrazine. When purified 2,3-^{14}C-2,3-dimethylpyrazine was injected into a rat intraperitoneally, little or none of the

radioactivity was eliminated as respired $^{14}CO_2$, a small amount of the radioactivity was found in the feces, and 84 per cent of the label was located in the urine. In addition, this study verifies Koehler's observation that a portion of the dimethylpyrazine was excreted unchanged (4).

CHAPTER II

LITERATURE REVIEW

The preparation of "amarone" was first described in 1844 by Laurent (5) and was recently reviewed by Krems and Spoerri (6). Pratt noted that "amarone" was identified as tetraphenylpyrazine (I) (7).



I

Krems and Spoerri, in their review on the chemistry of pyrazines, listed several methods of preparation of pyrazines: autocondensation of α-primary aminocarbonyls, autocondensation of α-hydroxyimines, condensation of α, β-diamines with α, β-dicarbonyls, the action of ammonia (or its salts) on polyhydroxy compounds, the action of hydrazine or hydroxylamine on dihydroxymorpholine, the dehydrogenation of piperazines and other lesser known methods (6). Oxidation of piperazines over a suitable catalyst is used commercially to form pyrazines. According to Pratt an indirect route of synthesis of pyrazine is decarboxylation of pyrazinecarboxylic acids (7). Until recently the most

efficient method for pyrazine synthesis was that of Wolff and Marburg from the acetal of iminodiacetaldehyde (8). Treatment with hydrochloric acid forms 2,6-dihydroxymorpholine which reacts with hydroxylamine to yield pyrazine. The fact that the starting material is difficult to obtain is the principle disadvantage. Examples of autocondensation of α-primary aminocarbonyls include reduction of amino acids, oxidation of amino alcohols, replacement of the hydroxyl in a hydroxycarbonyl with an amino acid group, replacement of one carbonyl group in a dicarbonyl compound with an amino group and the action of acetic anyhydride and pyridine on amino acids (6).

This author used direct formation of pyrazine by condensation of an α, β-diamine with an α, β-dicarbonyl. Synthesis was begun with ethylmagnesium bromide formation suggested by Skattebøl, et al., in ether instead of tetrahydrofuran (9, 10). Gilman and Nelson reported that ethylmagnesium bromide will react with anhydrous cadmium chloride to form diethyl cadmium (11). Cason contributed the next step of reacting diethyl cadmium to an acid chloride such as acetyl chloride (12). The ketone produced is 2-butanone. An oxime can be produced from 2-butanone by a method described by Hartung and Crossley (13). Hydrolysis of the oxime with dilute acid resulted in the formation of diacetyl (10). Flament and Stoll showed how diacetyl can condense with ethylenediamine producing 2,3-dimethyl-5,6-dihydropyrazine (14). Two, three-dimethyl-5,6-dihydropyrazine can be catalytically dehydrogenated to 2,3-dimethylpyrazine at high temperatures as reported by Ishiguro, et al. (15).

Kikkoji and Neuberg reported that rabbits fed doses of aminoacetaldehyde hydrochloride converted the compound to pyrazine which

can be isolated from the urine (16). Other pyrazine-like compounds have been isolated from plants, indicating a biosynthetic pathway. Kosuge, et al. proposed the biosynthesis of tetramethylpyrazine from ammonia and acetoin (17).

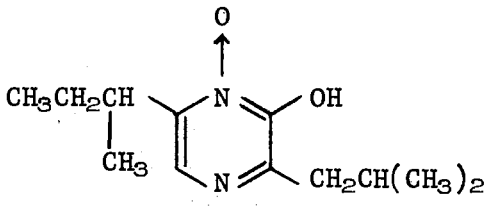
In 1962, Japanese workers reported tetramethylpyrazine to be a flavor component of natto, a fermented soybean product consumed in considerable quantities by the Japanese (18). More recently Kosuge, et al. detected tetramethylpyrazine in many Japanese foodstuffs, Miso and soy sauce in particular, suggesting that alkylpyrazines may play an important role as flavor components of these foodstuffs (17). Tetramethylpyrazine was isolated from Bacillus subtilis and Bacillus natto, which ferments soybean to produce "natto" (17). Another pyrazine, 2-methoxy-3-isobutylpyrazine, is responsible for the aroma of the California green bell pepper and is the major component of that pepper's oil when obtained by steam-distillation (19). Bramwell, et al. found 2-methoxy-3-sec-butylpyrazine and other pyrazines in Galbanum oil (20). Murray, et al. observed 3-isopropyl-2-methoxypyrazine, 3-s-butyl-2-methoxypyrazine and 2-methoxy-3-isobutylpyrazine in green peas (21). Biosynthesis from α-amino acids is suggested. White and Hill found aspergillic acid (II) to be of relatively high toxicity. It is a product of a strain of Aspergillus flavus grown on a certain liquid medium (22). The above pyrazines found in non-roasted foods indicates biosynthesis of pyrazines in plants and microorganisms.

The first report of pyrazine in foods was that of Standinger and Reichstein in which pyrazines were isolated from coffee (23). Gianturco confirmed their results for coffee and, in addition, discovered the presence of several dimethylpyrazines (24, 23). Mason, et al.

recently showed that a major portion of the volatile components of roasted peanuts are alkylated pyrazines, and evidence has accumulated to indicate the presence of compounds substituted with alkyl groups containing up to five carbons (25). Deck and Chang reported the isolation of dimethylpyrazine from potato chips in addition to several other alkylated derivatives (26). Buttery, et al. recently confirmed that many pyrazines contributing to odor are present in the basic fraction of the steam volatile oil from potato chips (27, 26). Dietrich, et al. reported 2,6-dimethyl- and tetramethylpyrazine in cocoa and Rizzi has found a number of alkylated pyrazines in cocoa butter including 2,3-dimethylpyrazine (28, 29). There is a French patent to add methylpyrazines (1-200 ppm) to enhance flavor of chocolate and cocoa preparations (30). Neurath and Duenger have isolated weakly basic heteroaromatic compounds from tobacco smoke (31). Duenger patented a method for improving and enriching tobacco smoke flavor by adding mono-, di-, tri-, or tetra-substituted derivatives of pyrazine (0.005 - 5.0% by wt.) to the tobacco (32). Seifert, et al. reported that since 2-isobutyl-3-methoxypyrazine has an extremely potent aroma and is already in commercial use as a flavoring material, compounds with related structures are being studied as possible flavoring material for nutritious, high protein foods which may have flavor deficiencies (33). Sapers, et al. noted that the toasted off-flavor of puffed dehydrated potato is due, in part, to the presence of several alkylpyrazines (34).

Gas chromatography-mass spectrometer analysis of roasted meat flavor prepared by pyrolysis of water-soluble components of fresh beef has yielded 17 alkylpyrazines to Flament and Ohlaff (35). Watanabe

and Sato obtained at least nine alkyl-substituted pyrazines from the basic fraction of the total volatiles of shallow fried beef and proposed a reaction between sugars and amino acids to produce a specific flavor in beef (36). Koehler, et al. demonstrated the production of alkylated pyrazines in model systems consisting of mixtures of amino acids and sugars heated in diethyleneglycol (37). The fact that pyrazines are produced when reducing sugars and a nitrogen source are heated together is not new, but these workers have succeeded in quantitatively reproducing the pyrazines found in roasted peanuts by heating, in the model systems, a mixture of certain amino acids and sugars found in raw peanuts.



II

Davidson and Wiggans reported that during the course of studies on high temperature ammoniation of molasses, steam distillable heterocyclic base fractions appeared to contain both pyridine and pyrazine bases (38). The major constituent of a non-steam volatile fraction was 4(5)-methylimidazole, but the steam volatile fraction contained compounds similar to 2,5- and 2,6-dimethylpyrazines. Further work by Wiggans on ammoniated molasses revealed the production of five

pyrazine components (39).

In more recent studies of by-products formed in the ammoniation of glucose, Nishie, et al. showed that pyrazine, several methylpyrazines, hydroxypyrazines and related compounds were produced (40). Mono- and dimethylpyrazines exhibit weak central nervous system depressant activity (hypnotic and anticonvulsant). Two, three-dimethylpyrazine was the most potent in this regard, producing approximately 1/13 the hypnotic activity of phenobarbital sodium. Pyrazines with either OH, CH_2OH or $-(CH_2OH)_3-CH_2OH$ substituents were relatively nontoxic (40). Pyrazine derivatives because of their selectivity for bacterial cells hold possible value in antibacteria chemotherapy. Babek and Block prepared various pyrazine analogs of pyrimidines because of the marked antitumor activity which derives from the structural modification of the heterocycle of the natural pyrimidine nucleosides (41). Among these, the uracil analog 1,2-dihydro-2-oxopyrazine 4-oxide, a compound which has been isolated as the antibiotic emimycin, was synthesized by an improved method involving the oxidation of benzoyloxypyrazine (41).

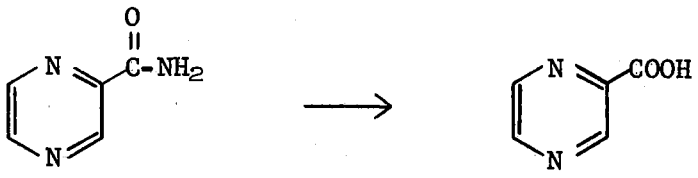
Established metabolic pathways of related compounds may serve as a guide in studying metabolism of pyrazines. Oxidative ring cleavage may yield urea, CO_2 and water. Reductive cleavage would result in primary and secondary amines that would most likely be conjugated when excreted in the urine. For example, the drug sulfanilamide is excreted as the N-acetyl derivative. Formation of N-oxides or N-methylpyrazines may represent major metabolites. N-methylation as a detoxication process is limited in the body, but pyridine derivatives are excreted as N-methyl conjugates. Oxidation and conjugation with glucuronide is another possible route of metabolism. Also pyrazines

may be excreted unchanged. The tranquilizer chlorpromazine is hydroxylated and excreted as a glucuronide (42). Koehler found that a small percentage of an injected dose of 2,5-dimethylpyrazine was not metabolized (4).

In the sulfonamide drug sulfapyrazine, the pyrazine ring is stable and the drug is excreted by rabbits mainly as the acetyl derivative, there being little, if any, hydroxylation of either ring (43).

Stolte reported that fructosazine was oxidized to 2-methylol-5-pyrazinoic acid and excreted in the urine (44).

Pyrazinoic acid (IV) is rapidly absorbed from the alimentary tract and remains unchanged by the kidneys; one research group reports that one-fourth of an oral dose of 1 to 3 grams appears in the urine within two hours (45). Some work with pyrazinamides (III) is reported and hydrolysis of the amide was observed in elimination products, primarily as pyrazinoic acid (46).



III IV

CHAPTER III

MATERIALS AND METHODS

Synthetic Reagents

Acetyl Chloride - Acetyl-1-^{14}C-Chloride was purchased from Tracerlab Radiochemical, Waltham, Massachusetts. Specific activity was 1.5 mCi/mM and the volume was 0.0475 ml.

Magnesium, Cadmium Chloride, Concentrated Sulfuric Acid and Sodium Nitrite - Purified magnesium turnings, cadmium chloride, concentrated sulfuric acid and sodium nitrite were purchased from J. T. Baker Chemical Company, Phillipsburg, New Jersey.

Ethyl Bromide - Ethyl bromide was a product of Eastman Kodak Company, Rochester, New York

Ether and Ethylene Diamine - Anhydrous ether and anhydrous ethylene diamine were obtained from Fisher Scientific Company, Fair Lawn, New Jersey.

Concentrated Hydrochloric Acid - Concentrated hydrochloric acid was a product of Mallinckrodt Chemical Works, St. Louis, Missouri.

General Reagents

Scintillation Solvent - Scintillation solvent was prepared according to Bray (47). Fifteen milliliters were used when counting samples.

CO_2 Absorber - One normal NaOH was used to absorb CO_2. The air was drawn through this solution via scintered glass bubblers submerged

in a series of four 3.5x20 cm test tubes containing 70 ml, 60 ml, 50 ml, and 40 ml 1 N NaOH. Each day this volume was adjusted to 250 ml.

Synthesis of 2,3-^{14}C-2,3-Dimethylpyrazine

The synthetic scheme outlined in Figure 1 shows the major steps of the synthesis. Two complete series of these reactions were conducted with unlabeled reactants to pretest the synthetic scheme.

Grignard Synthesis

A one liter three-neck round-bottom flask was fitted with a mechanical stirrer, reflux condenser and an equal-pressure dropping funnel. The system was flamed out and dry nitrogen was passed through the system where 0.5 mole of ethylmagnesium bromide was prepared by slow addition of 0.5 mole of ethyl bromide in 375 ml ether to 0.5 mole of magnesium turnings while stirring.

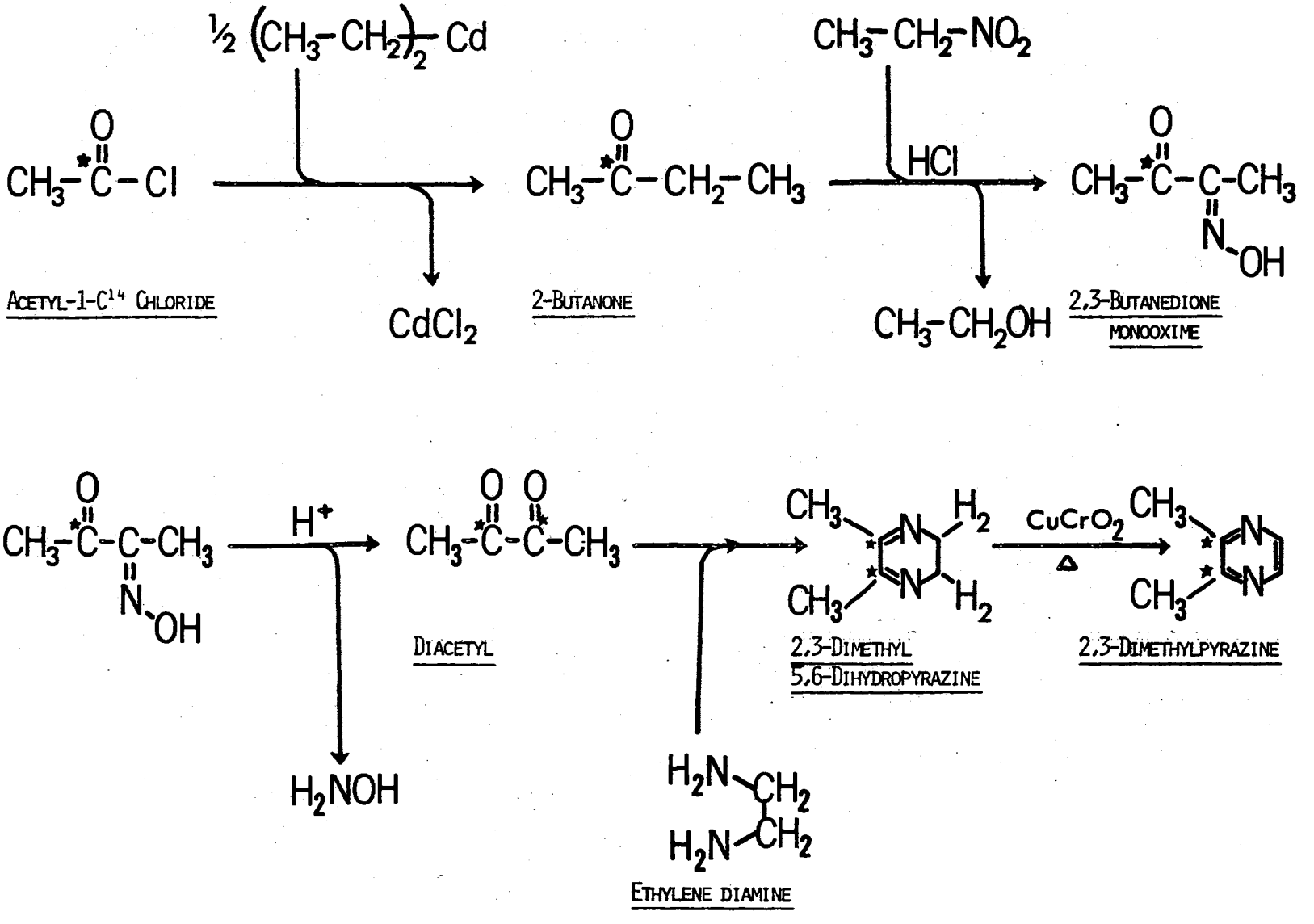
Diethyl Cadmium

Diethylcadmium was formed by slow addition of 0.25 mole anhydrous cadmium chloride to the ethylmagnesium bromide. The flask was cooled with an ice bath during addition of cadmium chloride. After addition of the cadmium chloride heat was applied, with a heating mantel, to reflux the cadmium reagent for six to eight hours.

Formation of 2-^{14}C-2-Butanone

One mCi (0.0475 ml) acetyl-1-^{14}C-chloride was diluted with 5 ml of reagent grade acetyl chloride. This mixture was added slowly to the

Figure 1. Synthesis Scheme of 2,3-^{14}C-2,3-Dimethylpyrazine From Acetyl-1-^{14}C-Chloride


½ (CH3-CH2)2-Cd
CH3-C*(=O)-Cl
CdCl2
ACETYL-1-C14 CHLORIDE
CH3-C*(=O)-CH2-CH3
2-BUTANONE
CH3-CH2-NO2
HCl
CH3-CH2OH
CH3-C*(=O)-C(=N-OH)-CH3
2,3-BUTANEDIONE MONOOXIME
H+
H2NOH
DIACETYL
H2N-CH2-CH2-NH2
ETHYLENE DIAMINE
2,3-DIMETHYL 5,6-DIHYDROPYRAZINE
CuCrO2
Δ
2,3-DIMETHYLPYRAZINE

diethyl cadmium. Additional acetyl chloride was added to equal 0.5 mole then the mixture was refluxed for three hours. Ice water, then 5 per cent H_2SO_4 was used to dissolve the semisolid mass into two phases. To the upper, ether layer, 50 ml of 5 per cent Na_2CO_3 was added and then 50 ml of saturated NaCl. Both the Na_2CO_3 and NaCl were discarded after shaking. Two-butanone was isolated and partially purified from the solution by fractional distillation. The boiling point of 2-butanone is 79.6°C at 1 atm. pressure. The two-step addition of acetyl chloride permitted the maximum utilization of ^{14}C label.

Formation of Oxime

Ethyl nitrite (formed from dropping a solution of 6.2 ml H_2SO_4, 6.6 ml 95 per cent ethanol and H_2O into a solution of 16.1 g $NaNO_2$, 6.6 ml 95 per cent ethanol and H_2O) was bubbled through the 20.0 ml of 2-butanone with a small amount of concentrated HCl to form 2,3-butanedione monooxime. This was salted out as a white flaky crystalline precipitate and collected on a Buchner funnel.

Diacetyl Production

The 16.8 g of monooxime was hydrolysed by slowly adding 23.4 ml of cooled concentrated H_2SO_4 diluted to 67 ml with H_2O. A solution of 27.5 g $NaNO_2$ dissolved in 67 ml H_2O was also added to the solution. This was allowed to stand for three days to insure complete hydrolysis producing diacetyl. The 3.82 ml diacetyl from the synthesis was mixed with 25.45 ml reagent diacetyl to equal 0.33 mole.

Condensation with Ethylene Diamine

To 0.33 mole ethylene diamine in 100 ml ether the 0.33 mole diacetyl also in 100 ml ether was added. The condensation reaction produced 2,3-dimethyl-5,6-dihydropyrazine which was collected under vacuum distillation at 53°-54°C, 12 mm Hg. The undried amount was 12.3 ml. A 1.0 ml sample was removed for other experiments.

Formation of 2,3-Dimethylpyrazine

The remaining 11.3 ml 2,3-dimethyl-5,6-dihydropyrazine was slowly dropped through a catalytic column of 10 per cent copper chromite on 8-14 mesh F-1 alumina at 300°C. Recovery of 9.3 ml of 2,3-dimethylpyrazine was made. Anhydrous Na_2SO_4 was added to absorb any water in the solution leaving 8.0 ml pyrazine.

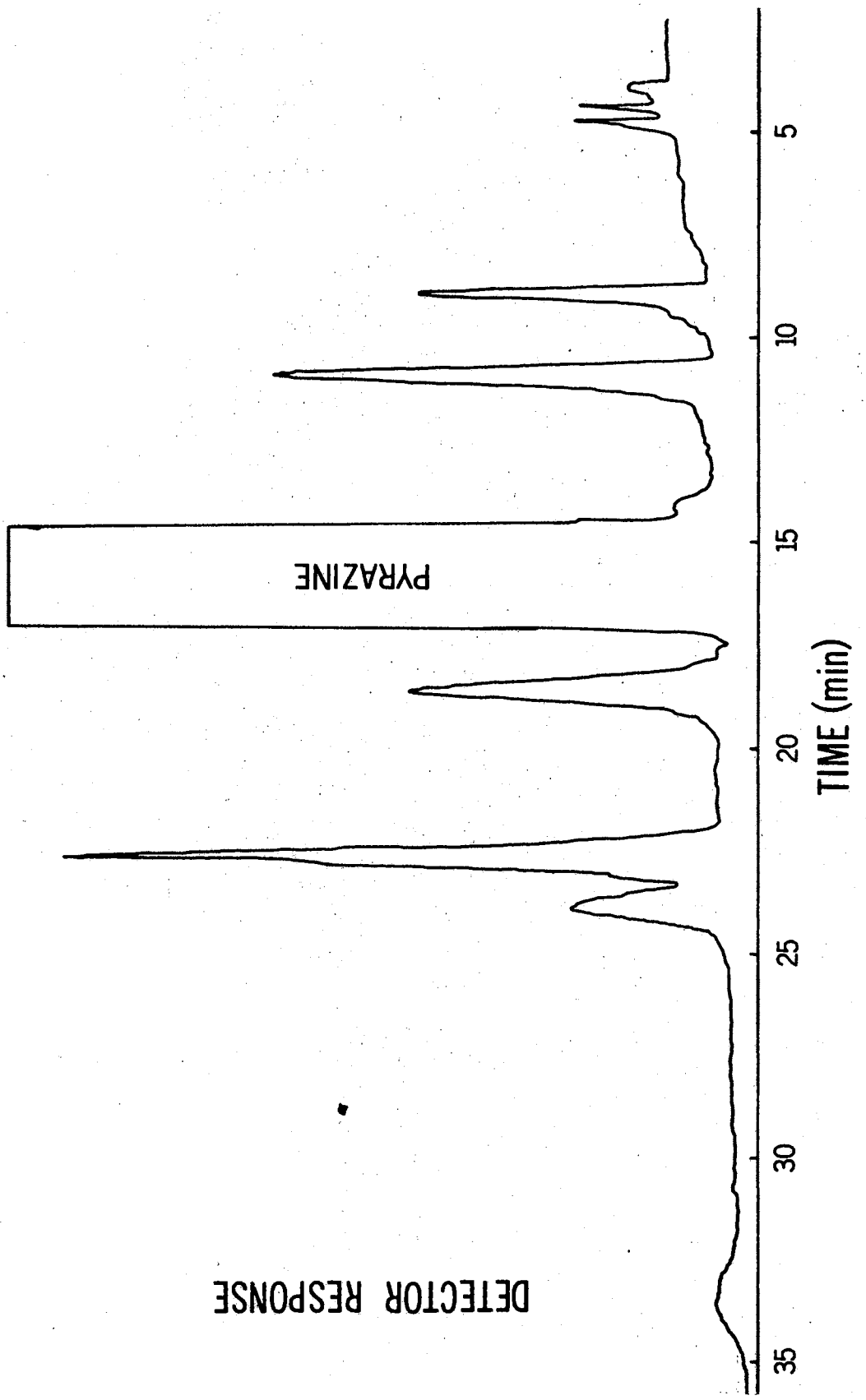
Purification of 2,3-^{14}C-2,3-Dimethylpyrazine

A Varian Aerograph preparatory gas chromatograph, model 711, equipped with a Sargent recorder was used to purify the 2,3-^{14}C-2,3-dimethylpyrazine. The column was 3/8 inch by 16 feet containing 15% Carbowax on Gas Chrom Q. Column conditions are described in the legend of Figure 2. The 2,3-dimethylpyrazine was trapped in dichloromethane cooled with dry ice.

Administration of 2,3-^{14}C-2,3-Dimethylpyrazine

A normal saline solution, 0.75 to 0.94 ml of 2,3-^{14}C-2,3-dimethylpyrazine, was injected intraperitoneally into a 100 - 200 g white female laboratory rat, approximately 350,000 dpm being administered to each animal.

Figure 2. Preparatory Gas Chromatographic Tracing From Injection of Synthesized 2,3-^{14}C-2,3-Dimethylpyrazine. The operational parameters were: glass column of 3/8" x 16'; 15% Carbowax on Gas Chrom Q; column temperature, 130°C; detector and injector temperature, 250°C; and N_2 flow rate of 55 ml/min. A flame ionization detector was used. Instrument was Varian Aerograph 711.


PYRAZINE
DETECTOR RESPONSE
TIME (min)
5
10
15
20
25
30
35

Metabolism Chamber

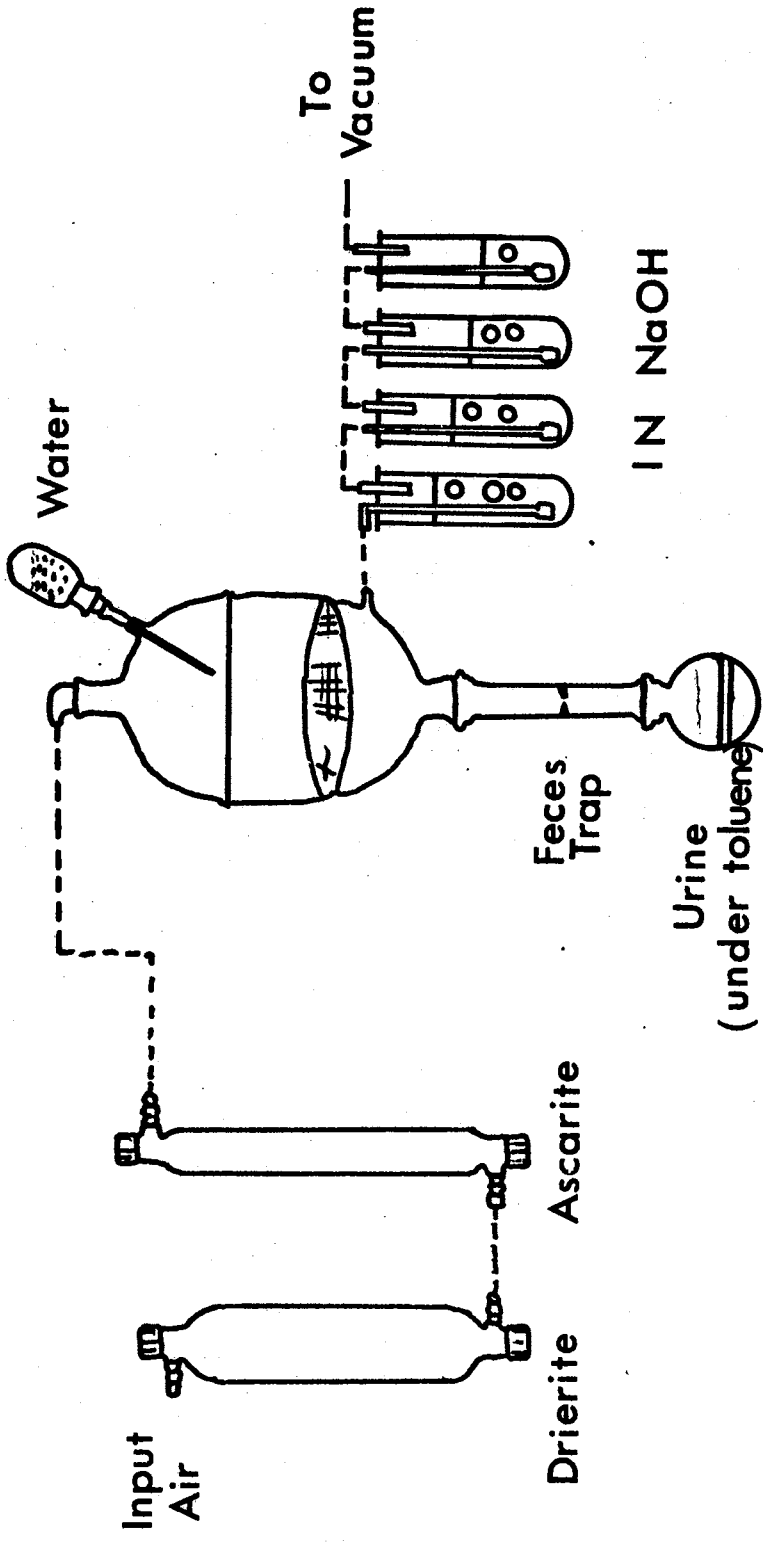
The metabolism chamber was crafted from six-inch glass tubing in the Oklahoma State University glass shop. It was a sealed system in which air was drawn through CO_2-absorbing traps, and urine and feces were collected separately. Food and water were provided ad libitum to the rat. Food was Rockland Complete Mouse/Rat Diet, the same diet that the animals had before the experiment. Figure 3 shows schematic representation of the metabolism chamber. Collection of urine, feces, and respired gases was made for each rat every 24 hours for a duration of six days.

Column Chromatography

Three resins were selected for ion-exchange chromatography: Dowex 1 x 2, chloride form, for the separation of purines and pyrimidines; Dowex 1 x 2, formate form, for the separation of nucleotides; and Amberlite CG-120, ammonium form, for the separation of nucleosides. Since the metabolites of pyrazines possibly include compounds structurally similar to free bases or base-sugars, these columns were utilized (48).

The solvent schedule followed an increasing concentration of formic acid up to 4 M with increasing ammonium formate concentration to 0.2 M. The usual procedure involved placing 300 ml of deionized water in the mixing chamber with the addition of 0.5 liter of 2 M formic acid, 0.5 liter of 4 M formic acid, and, finally, 0.5 liter 4 M formic acid with 0.2 M ammonium formate. Column sizes are described on the legend of each figure.

Figure 3. Metabolism Chamber Diagram. Air circulation by water aspiration. Twenty-four hour collection for urine, feces and respired gases.


To Vacuum
Water
1 N NaOH
Feces Trap
Urine (under toluene)
Ascarite
Drierite
Input Air

Spectrophotometry

Structural similarities of the pyrazines to purines and pyrimidines was noted, and a spectrophotometric assay (A260) was utilized for urinary metabolites separated by ion-exchange chromatography. Absorbance of each fraction was determined with a Beckman DB spectrophotometer.

Liquid Scintillation Spectrometry

The total radioactivity of urine samples was calculated from radioactive levels found in fractions from ion-exchange columns whereby an aliquot was added to 15 ml Bray's solution. Corrections applied to the raw counts per minute data include subtraction of blank; correction for instrument efficiency; quench correction; and finally, dilution factor.

Ionization Chamber Assay of $^{14}CO_2$

The CO_2 respired by the rat was trapped in 1 N NaOH. The CO_2 of a 2 ml aliquot from a total volume of 250 ml was released with acid and introduced into an ion chamber. The radioactivity was measured with a Cary 31 Vibrating Reed Electrometer.

Mass Spectral Analysis

Two radioactive peaks were purified and dried by lyophylization and a portion introduced by direct probe into the prototype LKB-9000 Gas Chromatograph-Mass Spectrometer. Purification of the radioactive pyrazine metabolite followed the procedure of Pontis which involves adsorption onto activated carbon (Norit A) (49). Background spectra

were taken and used for correction. These mass spectral patterns were examined for pyrazine metabolite structural elucidation.

CHAPTER IV

RESULTS AND DISCUSSION

Synthetic Yield

The radioactive yield of the synthetic 2,3-^{14}C-2,3-dimethylpyrazine from acetyl-1-^{14}C-chloride is shown in Table I. This compound was judged to have the same odor as a commercial reference 2,3-dimethylpyrazine. The retention times of the unpurified synthetic 2,3-^{14}C-2,3-dimethylpyrazine, Figure 4, and the commercial reference, Figure 5, are similar. After purification, minor impurities were removed as outlined below. The radioactivity of the minor impurities was not determined. The preparative purification step was necessary in order for the material to be used in metabolic study. The unpurified synthetic compound gave an overall yield of 2.6 per cent or 26 μCi. A portion of this material was purified for the metabolic study. Specific activity of the purified 2,3-^{14}C-2,3-dimethylpyrazine was 230 mμCi/mM.

Purification Yield

The gas chromatographic tracing of synthetic 2,3-^{14}C-2,3-dimethylpyrazine before purification is shown in Figure 4. From Figure 4 the synthesized mixture was estimated to be 71 per cent pure and of approximately the same purity as the product obtained from Chemicals Procurement Laboratories, Figure 5. Figure 6 shows the gas

TABLE I

SYNTHETIC YIELD OF 2,3-^{14}C-2,3-DIMETHYLPYRAZINE

Compound	Per Cent*
Acetyl,1-^{14}C-Chloride 1.0 mCi	
2-Butanone	64.1
2,3-Butanedione, Mono-oxime	25.1
Diacetyl	12.5
2,3-Dimethyl 5,6-Dihydropyrazine	3.9
2,3-Dimethylpyrazine 26 μc	2.6

* Based on product radioactivity

Figure 4. Gas-Liquid Chromatogram of Synthetic 2,3-^{14}C-2,3-Dimethylpyrazine.
Column - 20' x 1/4", I.D.
Column Packing - 15% Carbowax on Gas Chrom Q
Column Temperature - 130°C
Injection Port Temperature - 250°C
Detector Temperature - 250°C
Carrier Gas - Nitrogen
Detector - Flame Ionization Detector
Hydrogen Pressure - 15 p.s.i.
Air Pressure - 40 p.s.i.
Nitrogen Pressure - 40 p.s.i.
Instrument - Perkin - Elmer 990

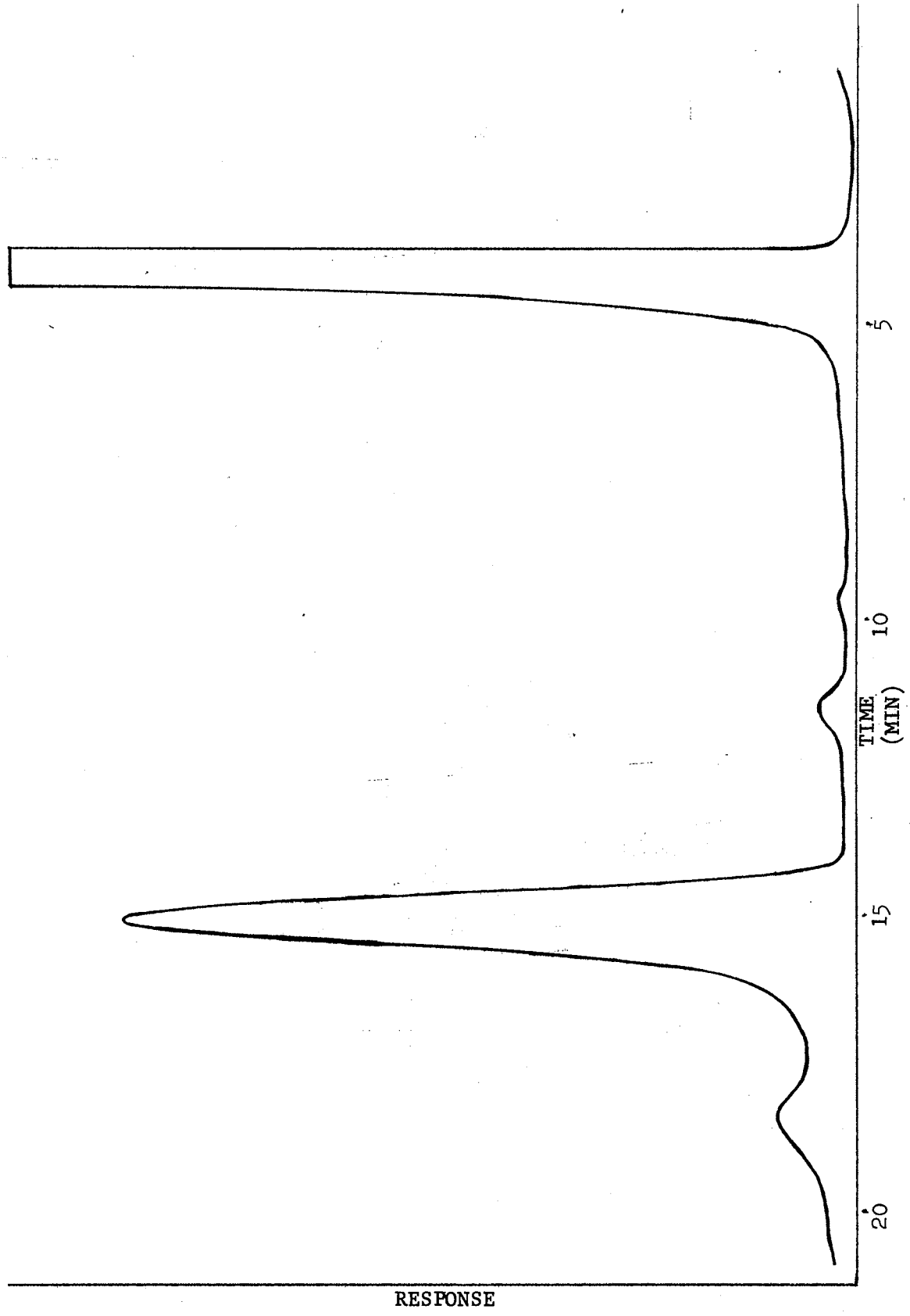

5
10
15
20
TIME (MIN)
RESPONSE

Figure 5. Gas-Liquid Chromatogram of Commercial Reference 2,3-Dimethylpyrazine.
Column - 20' x 1/4", I.D.
Column Packing - 15% Carbowax on Gas Chrom Q
Column Temperature - 130°C
Injection Port Temperature - 250°C
Detector Temperature - 250°C
Carrier Gas - Nitrogen
Detector - Flame Ionization Detector
Hydrogen Pressure - 15 p.s.i.
Air Pressure - 40 p.s.i.
Nitrogen Pressure - 40 p.s.i.
Instrument - Perkin - Elmer 990

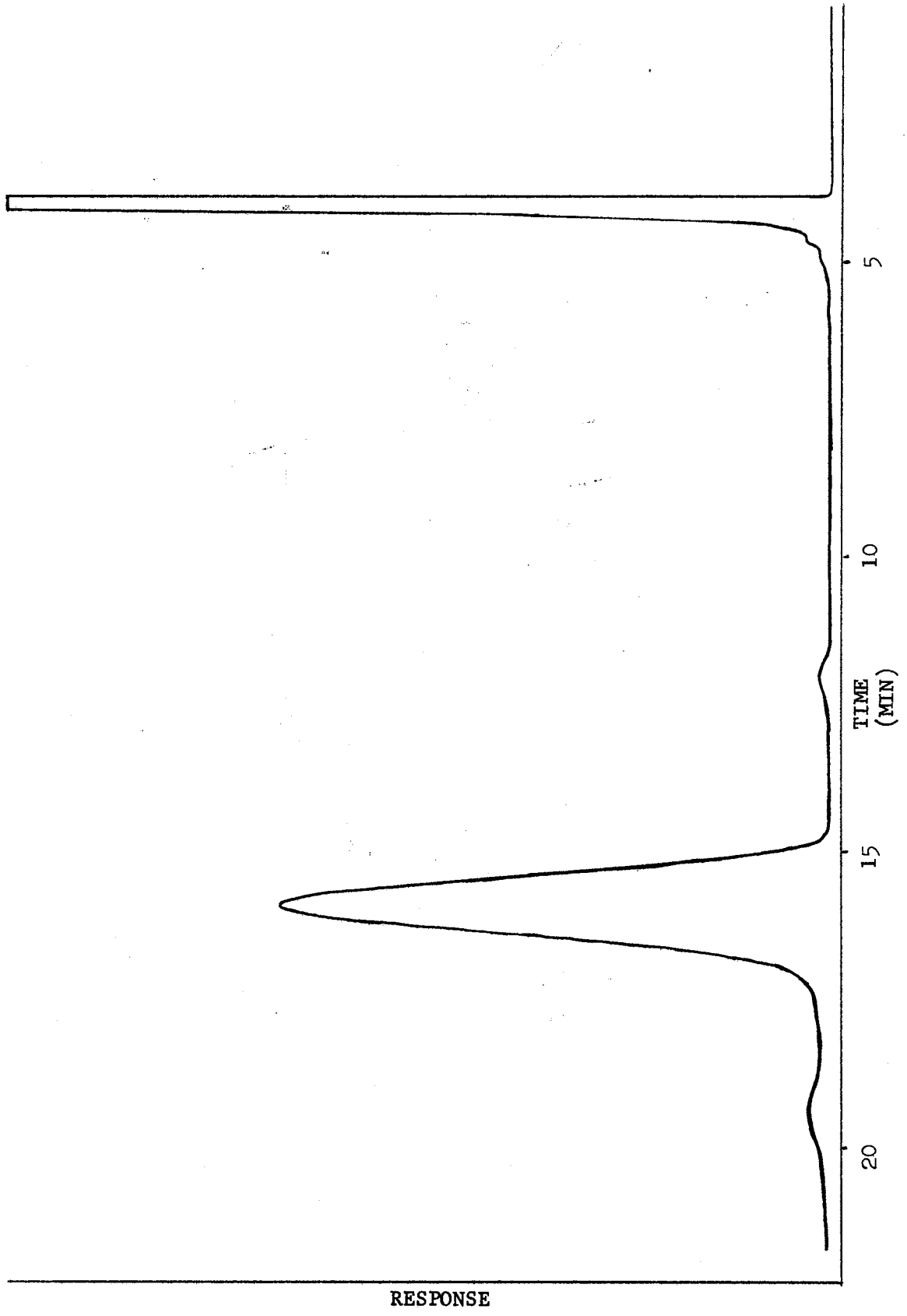
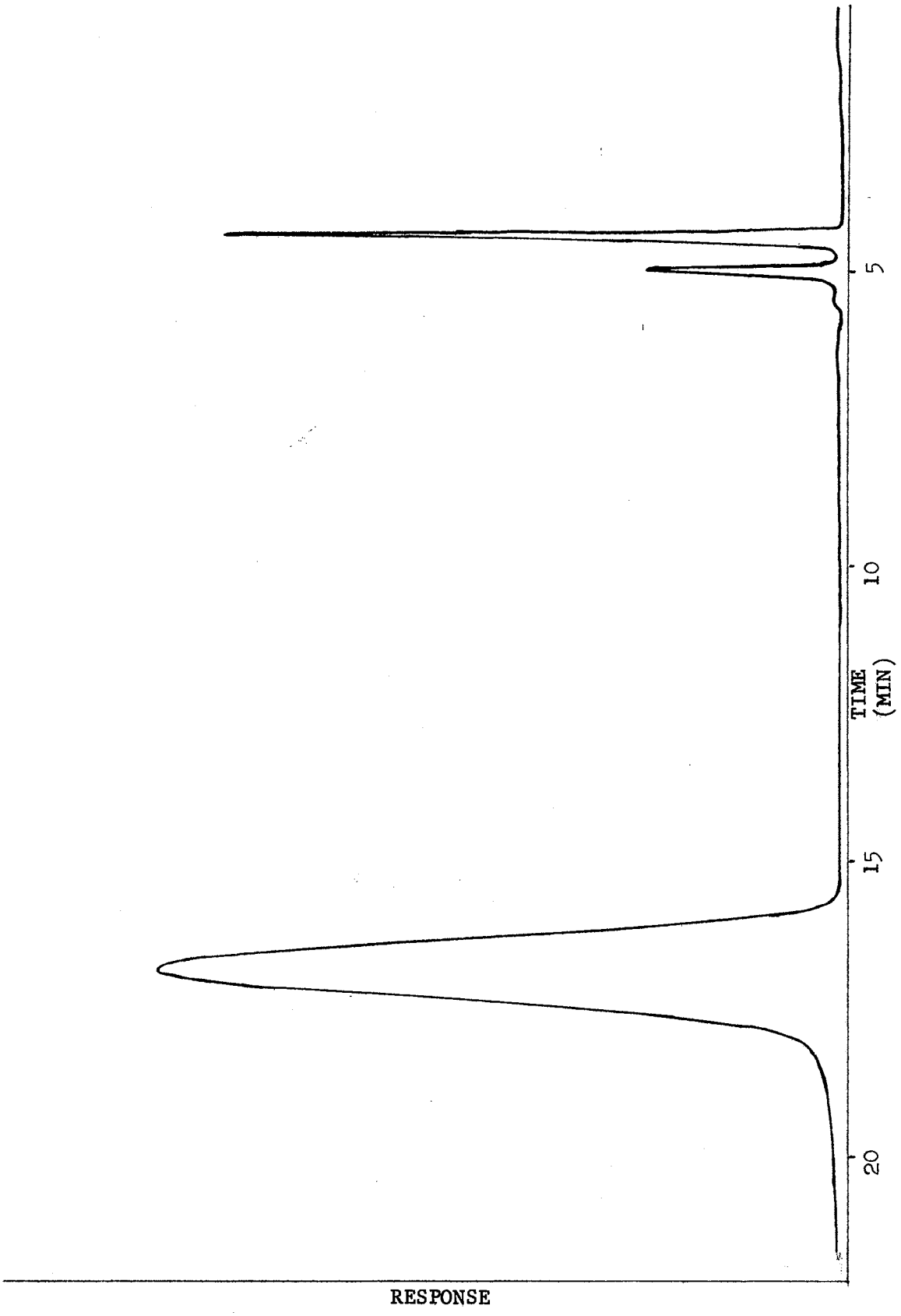

5
10
15
20
TIME (MIN)
RESPONSE

Figure 6. Gas-Liquid Chromatogram of Synthetic, Purified 2,3-^{14}C-2,3-Dimethylpyrazine.
Column - 20' x 1/4", I.D.
Column Packing - 15% Carbowax on Gas Chrom Q
Column Temperature - 130°C
Injection Port Temperature - 250°C
Detector Temperature - 250°C
Carrier Gas - Nitrogen
Detector - Flame Ionization Detector
Hydrogen Pressure - 15 p.s.i.
Air Pressure - 40 p.s.i.
Nitrogen Pressure - 40 p.s.i.
Instrument - Perkin - Elmer 990


5
10
15
20
TIME (MIN)
RESPONSE

chromatographic tracing of 2,3-^{14}C-2,3-dimethylpyrazine collected by preparative gas-liquid chromatography from the mixture. Minor impurities seen on the synthetic 2,3-^{14}C-2,3-dimethylpyrazine gas chromatographic tracing were apparently removed by this purification step. The mass spectra of both unpurified synthesized 2,3-^{14}C-2,3-dimethylpyrazine and commercial reference 2,3-^{14}C-2,3-dimethylpyrazine are shown in Figure 7. The method of introduction into the mass spectrometer was via the molecular separator which reduces the introduction of some impurities. The spectra are observed to be essentially the same.

Administration of 2,3-^{14}C-2,3-Dimethylpyrazine

Two control and four treatment rats were held individually for a period of six days each in the metabolic chamber. The four treatment rats received approximately 350,000 dpm of the purified labeled 2,3-dimethylpyrazine in normal saline solution. An anesthetic effect was observed for each treatment rat upon injection of 2,3-^{14}C-2,3-dimethylpyrazine. This effect lasted for two to three hours following injection and could have been due to a small amount of dichloromethane having remained in the 2,3-^{14}C-2,3-dimethylpyrazine solution.

Daily Excretion

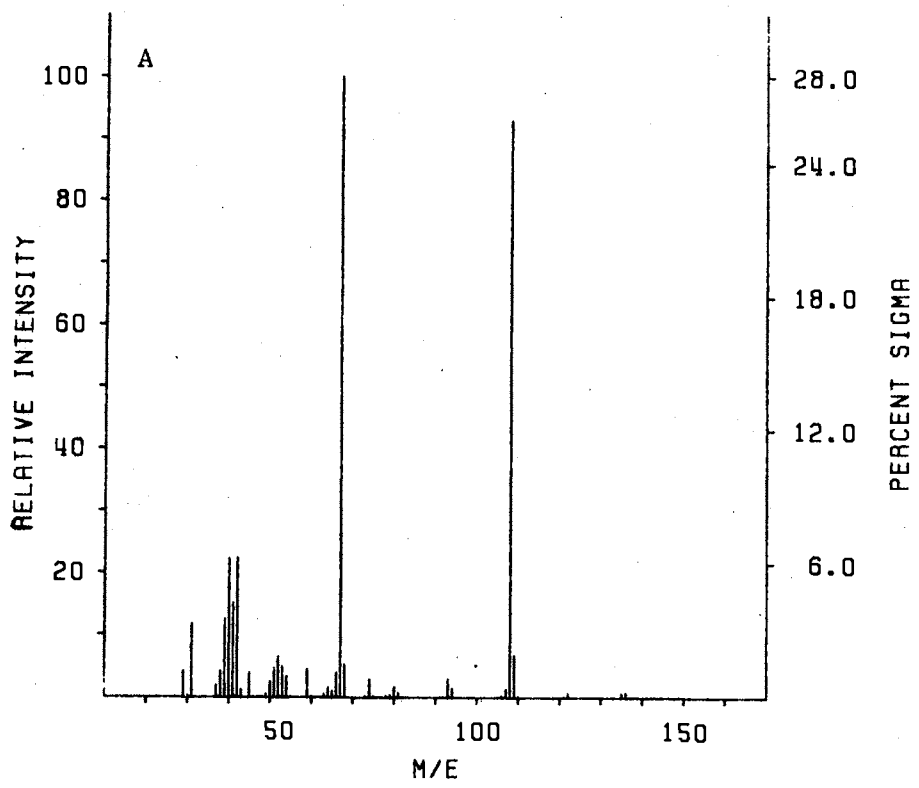
With Rat 1 and Rat 2 no detectible $^{14}CO_2$ was observed in respiration gases; therefore, CO_2 collection procedure was discontinued for Rats 3 and 4. The Cary 31 Vibrating Reed Electrometer was used to assay $^{14}CO_2$, and only a background reading was obtained from 0.8 per cent of the total collection for Rats 1 and 2 during each of the first four days. After the first two experiments the CO_2 collection procedure was discontinued.

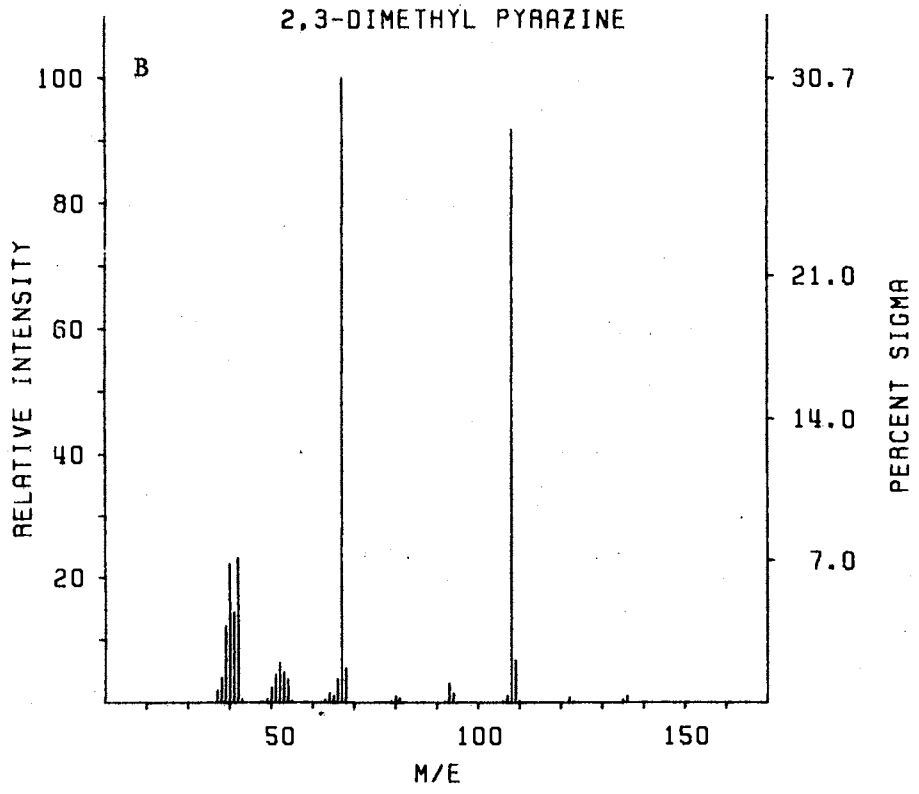
Figure 7. Mass Spectra of Synthesized 2,3-^{14}C-2,3- Dimethylpyrazine and Commercial Reference 2,3-Dimethylpyrazine.

A. Synthesized 2,3-^{14}C-2,3-Dimethylpyrazine Before Purification

B. Commercial Reference 2,3-Dimethylpyrazine

33


A
100
80
60
40
20
RELATIVE INTENSITY
28.0
24.0
18.0
12.0
6.0
PERCENT SIGMA
50
100
150
M/E


2,3-DIMETHYL PYRAZINE
B
100
80
60
40
20
RELATIVE INTENSITY
30.7
21.0
14.0
7.0
PERCENT SIGMA
50
100
150
M/E

Less than ten per cent of the radioactivity was found in the feces by extraction with dichloromethane. Due to the difficulty of completely separating the feces from the urine it would appear that some of the radioactivity found in the feces was from the urine, thus this procedure was discontinued for Rats 3 and 4. Approximately 84 per cent of the administered radioactivity was excreted in the urine. Table II shows the four rats' daily radioactive urinary excretion.

Koehler reported that 0.43 per cent of a dose of 2,5-dimethylpyrazine was respired through the lungs in the first 24 hours and that none was eliminated unchanged in the feces in the first three days. Koehler also found 2.5 per cent and 1.1 per cent of 2,5-dimethylpyrazine eliminated unchanged in the urine in the first 24 hours after intraperitoneal administration (4).

Most of the administered pyrazine was eliminated via the urine within the first 24 hours.

Characterization of Urinary Metabolites

Characterization of urinary metabolites of 2,3-^{14}C-2,3-dimethylpyrazine was attempted.

Some of the radioactivity in the urine of the rats of this experiment was excreted as unchanged 2,3-dimethylpyrazine. This was shown by the mass spectra of Peak I, Figure 11, separated by ion-exchange chromatography (Dowex 1 x 2, formate form column). In the mass spectra, Figure 8, a large m/e 67 and 108 indicates unchanged 2,3-dimethylpyrazine excreted in the urine. Figure 9 shows the mass spectra of the compound in the second major radioactive peak. No conclusions can be made at this time as to structure. Further study is indicated.

TABLE II

URINARY EXCRETION OF RADIOACTIVITY BY RATS ADMINISTERED ^{14}C-DIMETHYLPYRAZINE

	Per Cent Dose			
Day	Rat 1	Rat 2	Rat 3	Rat 4
1	77.7	86.6	82.7	87.6
2	3.8	1.4	0.8	2.1
3	1.3	0.1	0.4	0.0
4	1.8	0.2	0.0	0.1
5	4.0	0.1	0.1	0.0
6	1.6	0.1	0.0	0.0
Total	90.2	88.5	84.0	89.8

Rat 1 received 330,000 dpm.
Rats 2, 3, and 4 received 352,000 dpm.

Figure 8. Mass Spectra of the Purified Compound From Peak I (Figure 11), Rat Urinary Pyrazine Metabolite.

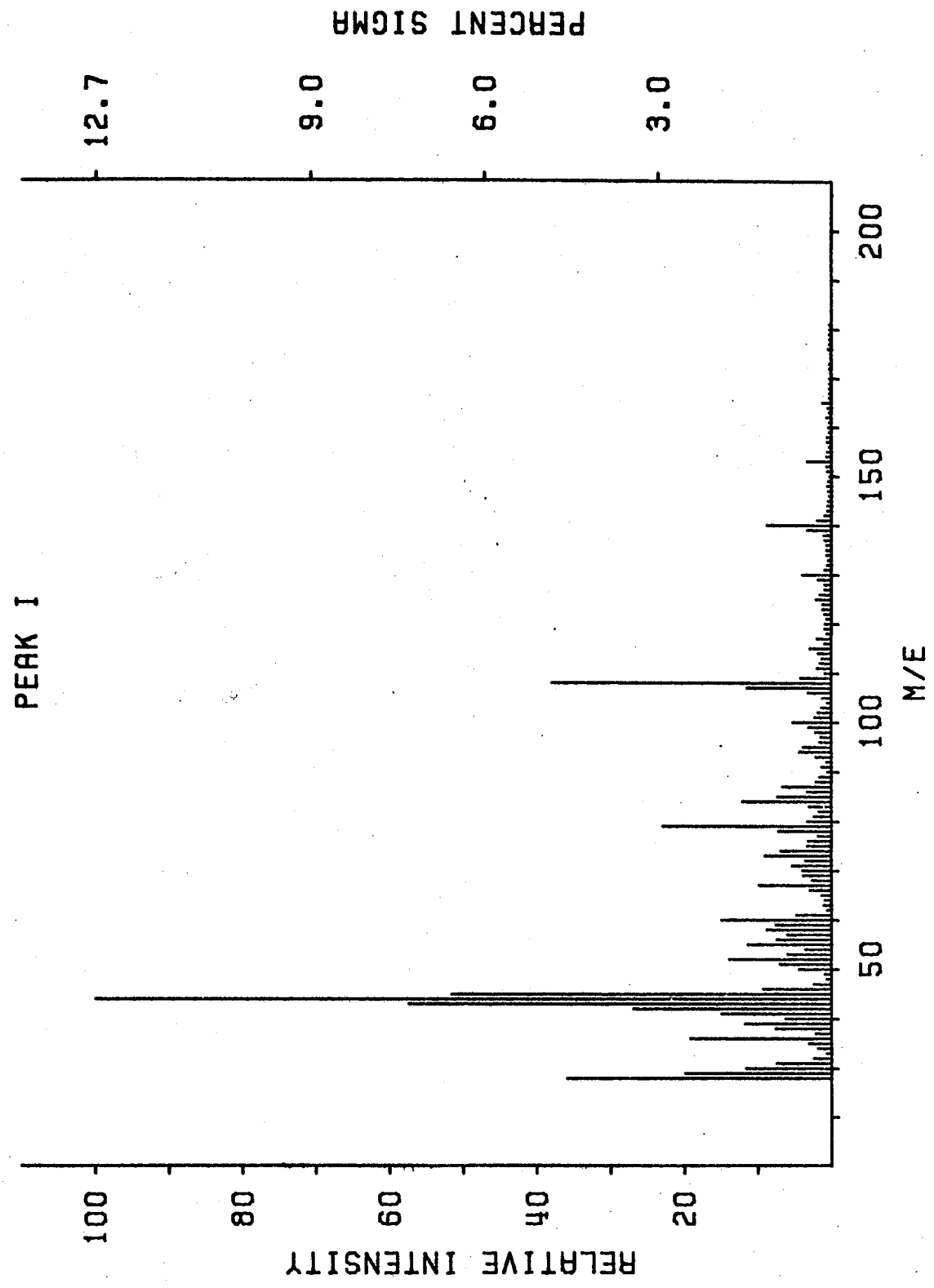
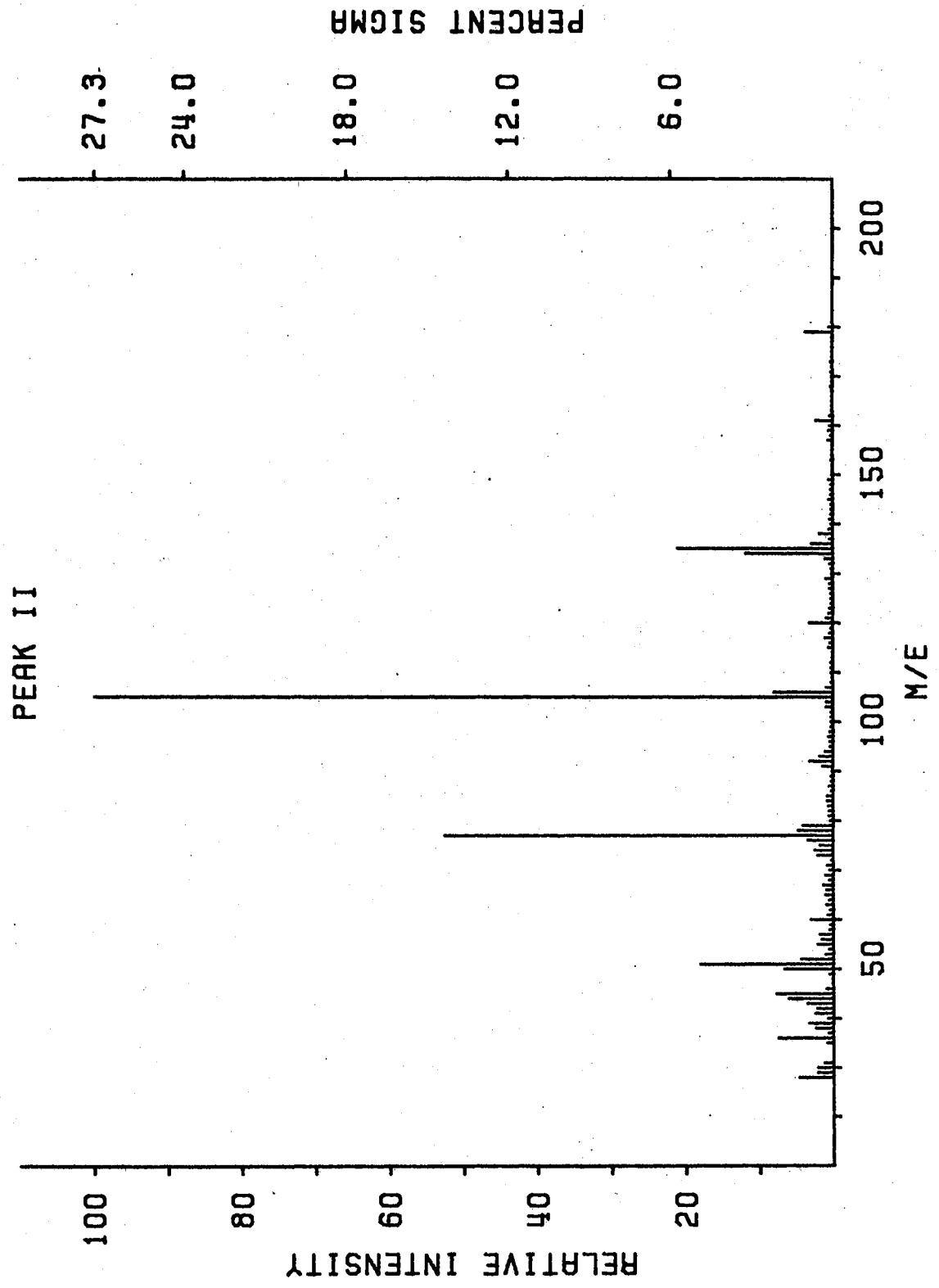

PERCENT SIGMA
12.7
9.0
6.0
3.0
PEAK I
RELATIVE INTENSITY
100
80
60
40
20
50
100
150
200
M/E

Figure 9. Mass Spectra of the Purified Compound From Peak II (Figure 11), Rat Urinary Pyrazine Metabolite.


PERCENT SIGMA
27.3
24.0
18.0
12.0
6.0
PEAK II
RELATIVE INTENSITY
100
80
60
40
20
50
100
150
200
M/E

Three types of ion-exchange columns were used in an attempt to separate urinary metabolites: Dowex 1 x 2, chloride form; Dowex 1 x 2, formate form; and Amberlite CG-120, ammonium form. The absorbance readings and liquid scintillation assays versus fraction number are shown in Figures 10, 11, and 12. The Dowex 1 x 2 formate form columns produced a cleaner separation of metabolites, so a control rat urine sample was also chromatographed using this type of column with results shown in Figures 11, 13, and 14. The first separation (Figure 11) indicates two major radioactive metabolites which absorb at A260. As stated earlier, Peak I contained unchanged pyrazine; it was not possible to structurally characterize Peak II. Figure 14 shows a better separation of urinary metabolites. Time did not permit characterization of the three to four radioactive metabolites shown in Figure 14. A minor change in solvent schedule gave a better separation, as shown in Figure 14. It is of interest to note that there are A260 absorbing compounds present in the control rat's urine occurring at different elution volumes. There was a difference in storage conditions of the rat urine samples chromatographed on Dowex 1 x 2 formate forms as shown in Figures 11 and 14. The sample shown in Figure 11 was stored in a cold room at 5°C for two to three weeks while the sample shown in Figure 14 was stored at -20°C for three to four weeks.

Since 2,3-dimethylpyrazine can be detoxified by hydroxylation of the ring or methyl groups, this would be a possible route of metabolism. The mass spectrometry data of Figures 8 and 9 show no significant m/e 124, thus ruling out any mono-N-oxydimethylpyrazine, monohydroxydimethylpyrazine or monomethylolmonomethylpyrazine. This, however, does not limit the possibility that these three compounds could be metabolic

Figure 10. Separation of Rat Urine Pyrazine Metabolites on a Dowex 1 x 2 Chloride Form Column. Column Size Was 1 x 50 cm. Particle Size Was 100-200 Mesh. Sample Size Was 1/5 of a 6-Day Collection. Solvent Schedule Was: 300 ml H_2O; 1000 ml 0.2M NH_4OH; and 1000 ml 0.2M NH_4OH, 0.025M NH_4Cl.

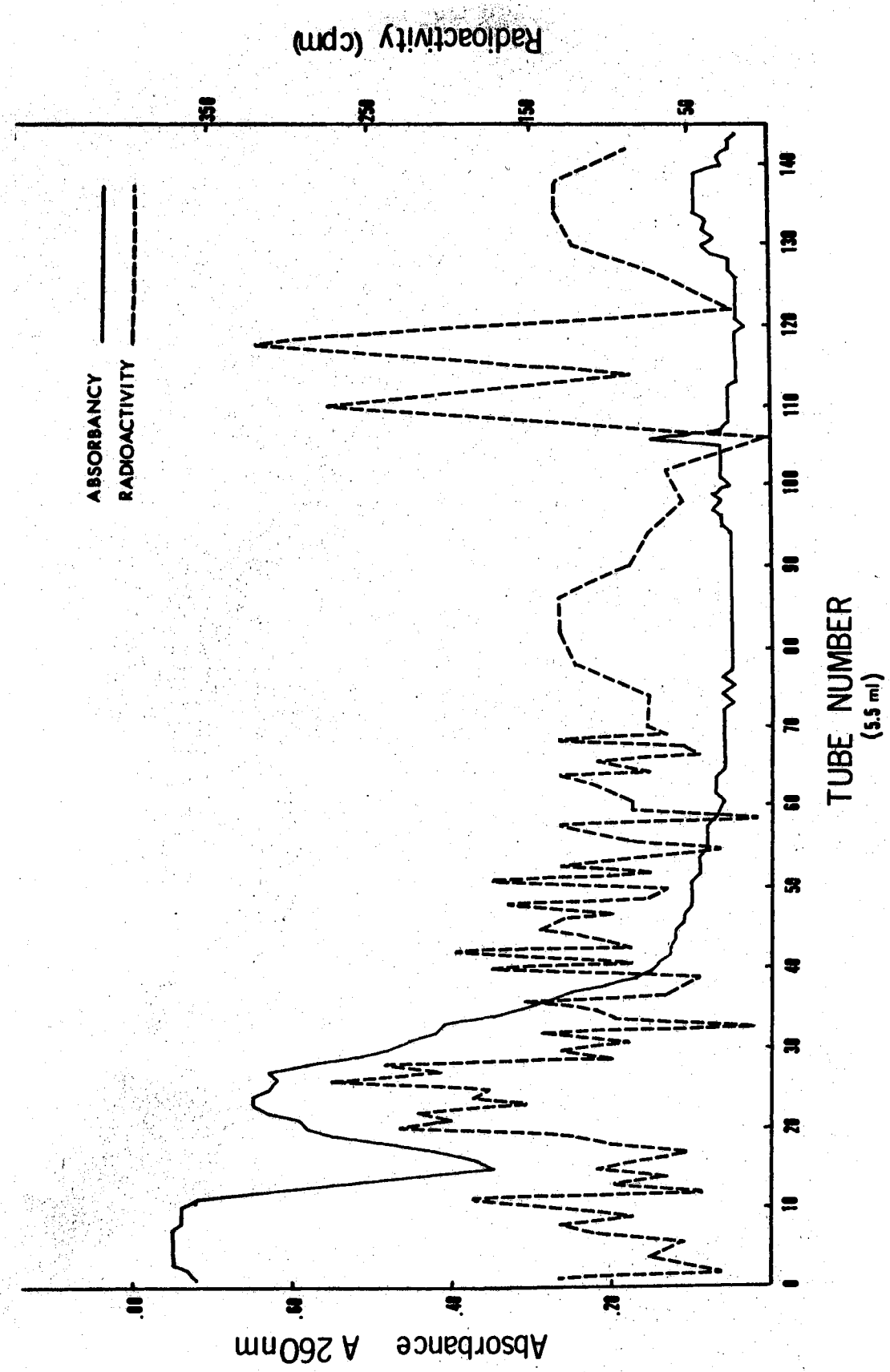

Absorbance A 260nm
Radioactivity (cpm)
TUBE NUMBER
(5.5 ml)
ABSORBANCY
RADIOACTIVITY

Figure 11. Separation of Rat Urine Pyrazine Metabolites on a Dowex 1 x 2, Formate Form Column. Column Size Was 1.8 x 35 cm. Particle Size Was 100-200 Mesh. Sample Size Was 1/5 of 6-Day Collection. Solvent Schedule Was: 300 ml H_2O; 500 ml 2N HCOOH; 500 ml 4N HCOOH; and 500 ML 4N HCOOH, 0.2M NH_4CHO_2.

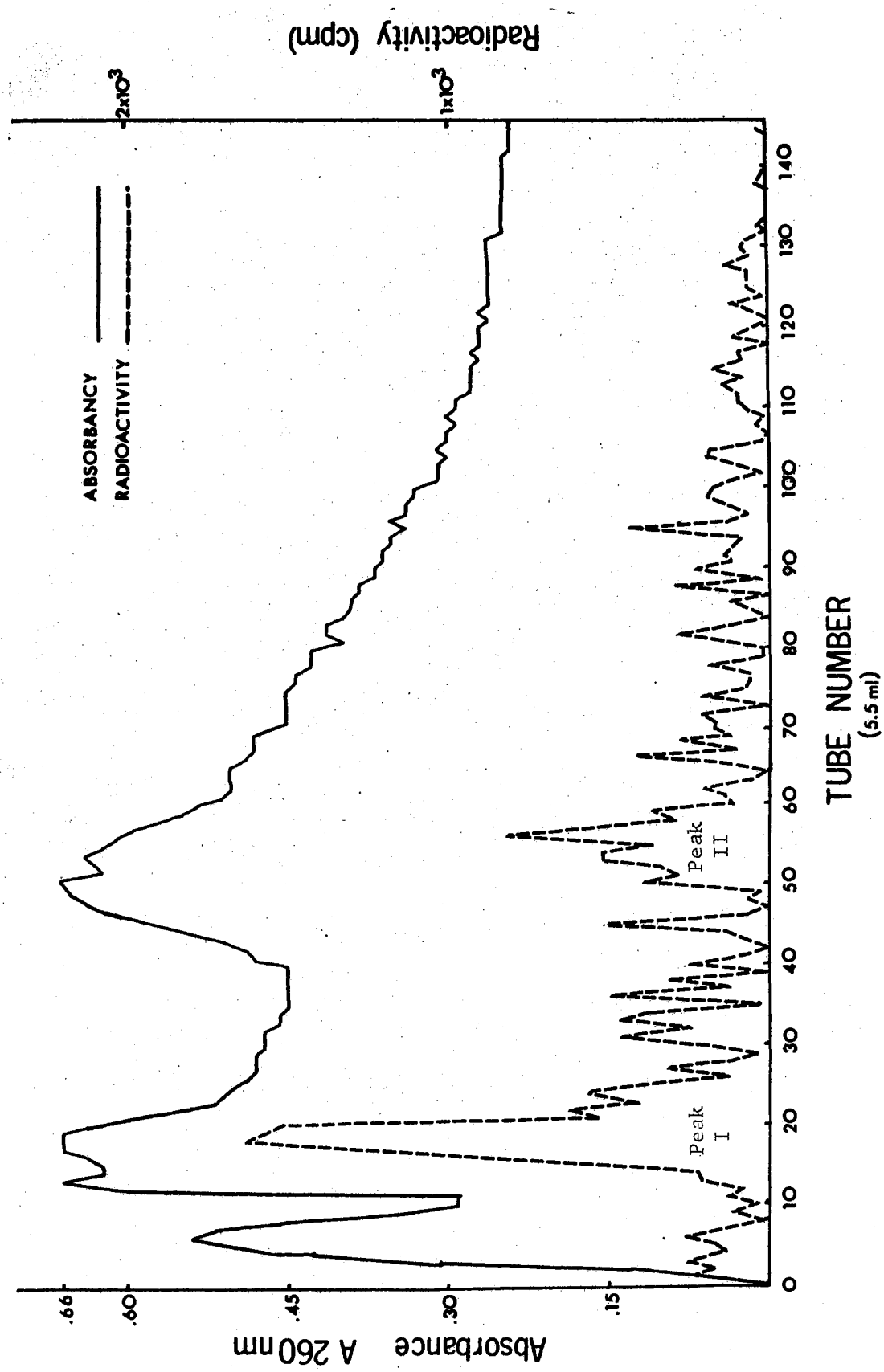

Radioactivity (cpm)
2x10^3
1x10^3
ABSORBANCY
RADIOACTIVITY
Absorbance A 260nm
.66
.60
.45
.30
.15
0
10
20
30
40
50
60
70
80
90
100
110
120
130
140
Peak I
Peak II
TUBE NUMBER
(5.5 ml)

Figure 12. Separation of Rat Urine Pyrazine Metabolites on an Amberlite CG-120, Ammonium Form Column. Column Size Was 2.2 x 35 cm. Particle Size Was 100-200 Mesh. Sample Size Was 1/5 of 6-Day Collection. Solvent Schedule Was: 500 ml H_2O; 500 ml 0.2M NH_4CHO_2; and 500 ml 0.4 NH_4CHO_2, 0.04 HCOOH.

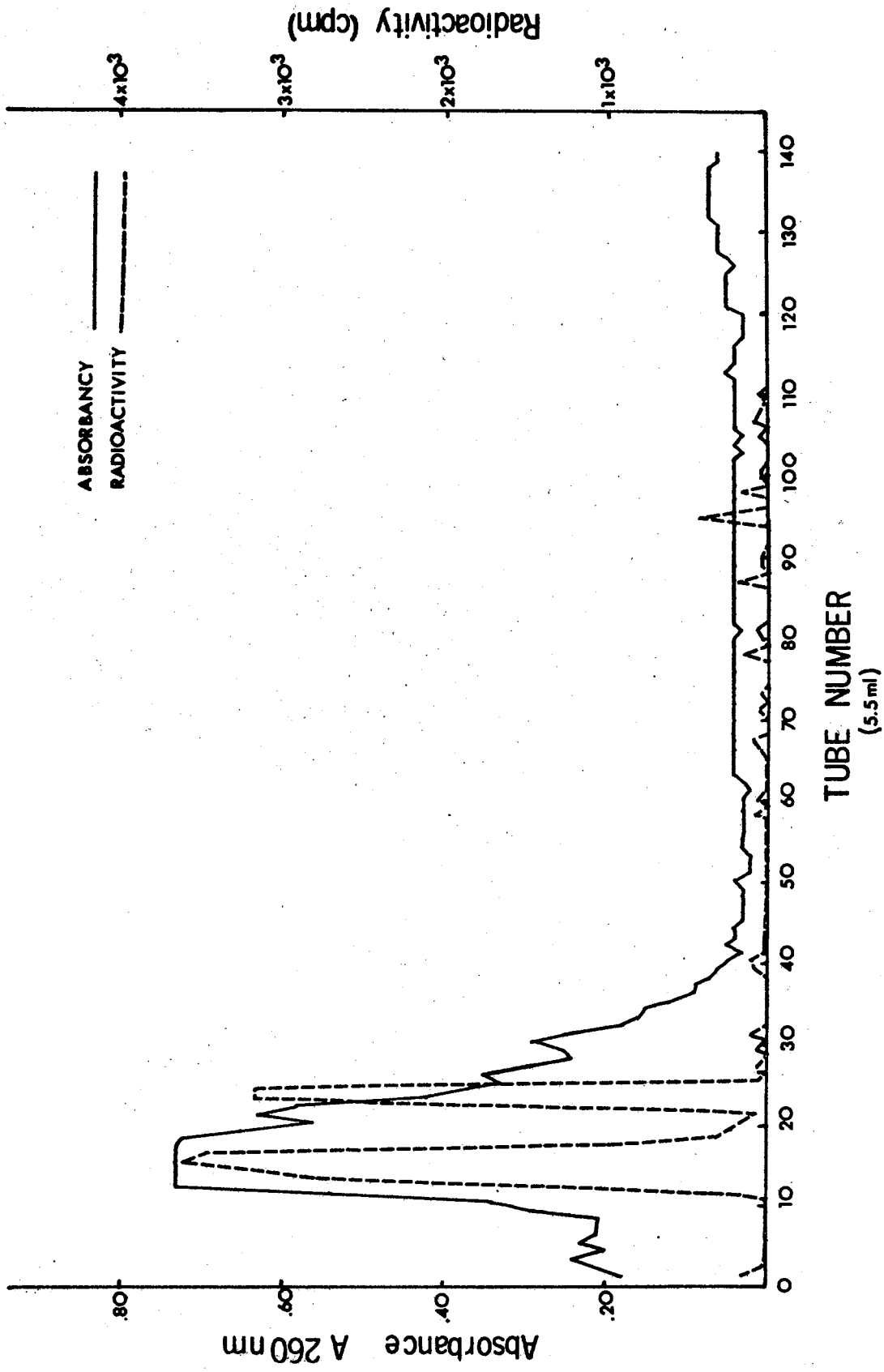

Radioactivity (cpm)
4x10³
3x10³
2x10³
1x10³
ABSORBANCY
RADIOACTIVITY
Absorbance A 260 nm
.80
.60
.40
.20
0
10
20
30
40
50
60
70
80
90
100
110
120
130
140
TUBE NUMBER
(5.5 ml)

Figure 13. Separation of Control Rat Urine Sample on Dowex 1 x 2, Formate Form Column. Column Size Was 1.8 x 60 cm. Particle Size Was 100-200 Mesh. Sample Size Was 2/5 of a 3-Day Collection. Solvent Schedule Was: 500 ml H_2O; 500 ml 2N HCOOH; 500 ml 4N HCOOH; and 500 ml 4N HCOOH, 0.2M NH_4CHO_2.

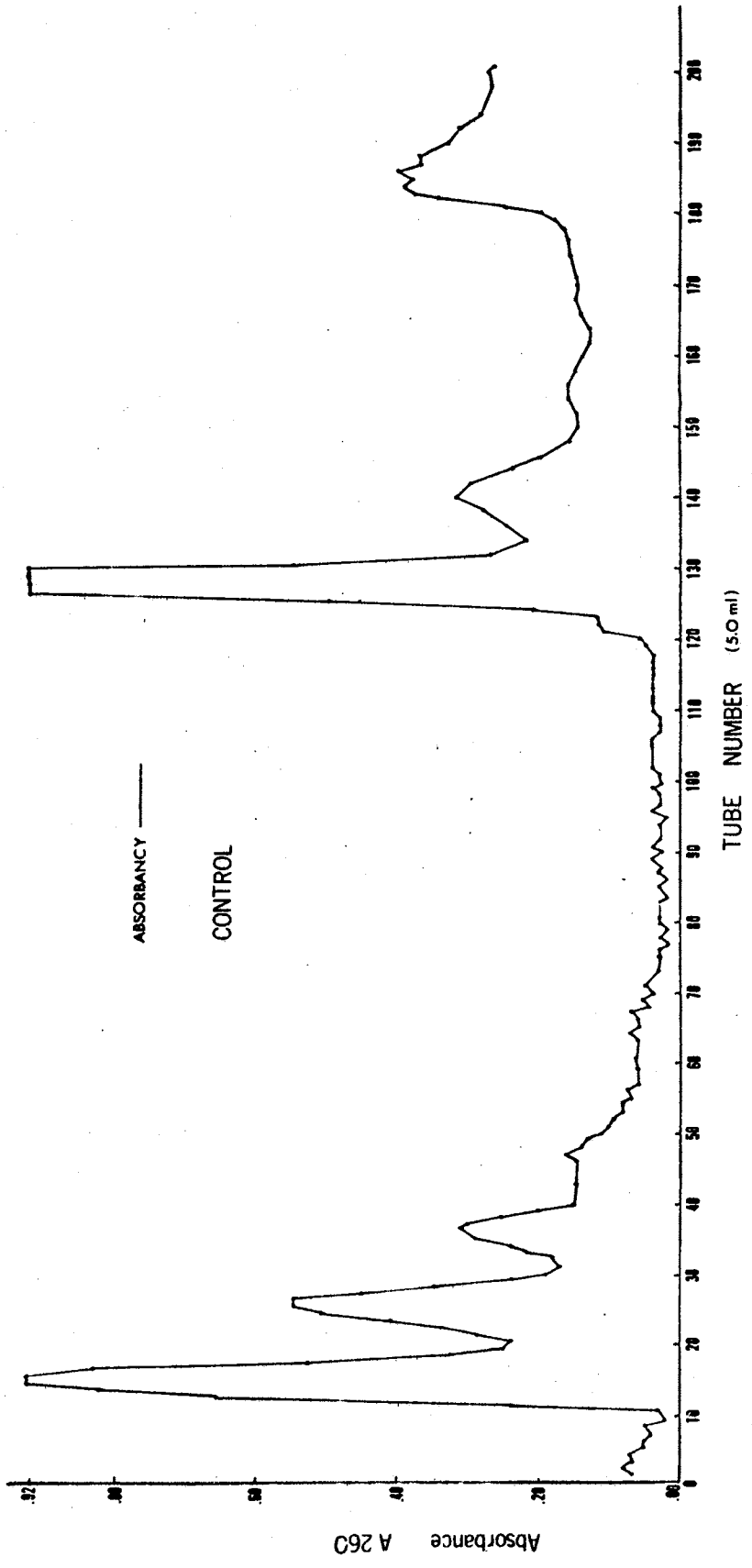
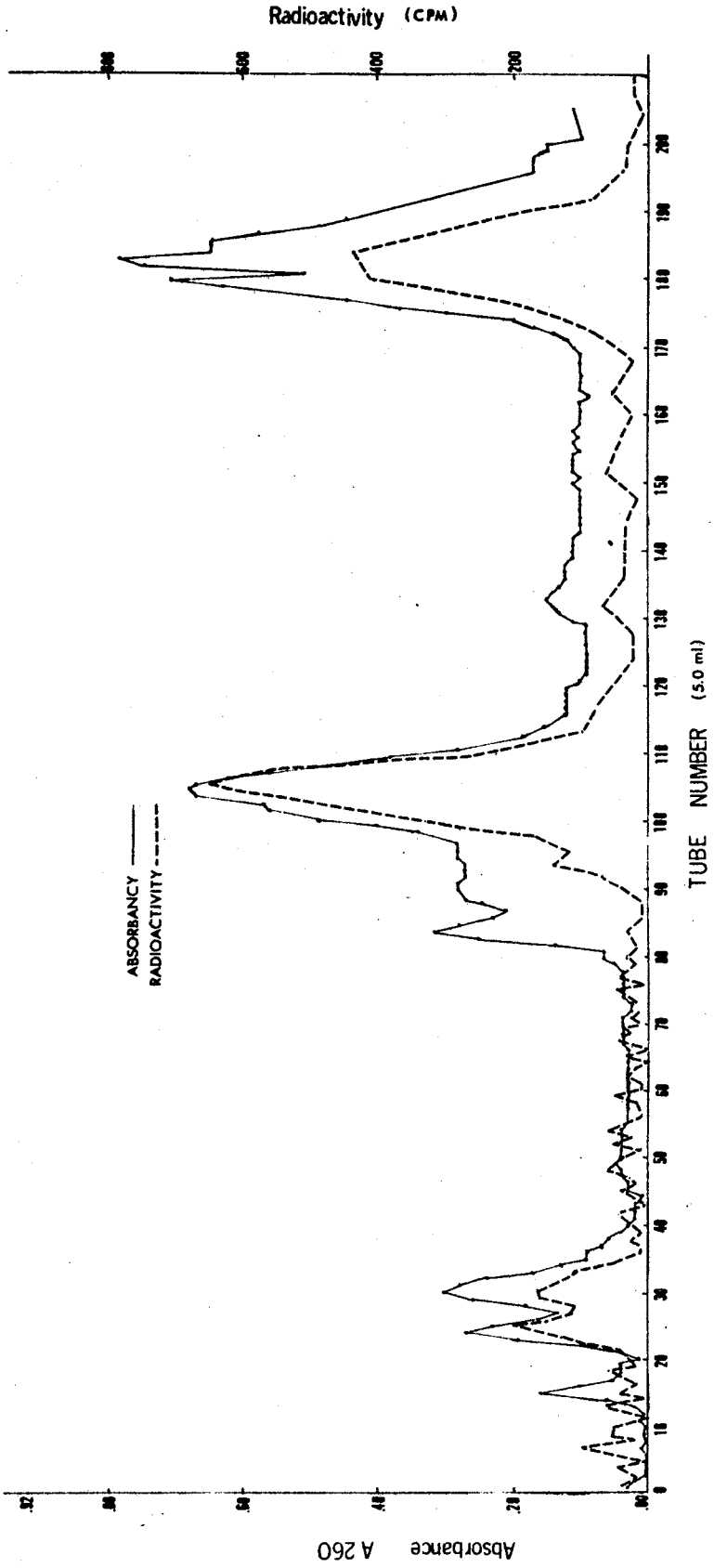

Absorbance A 260
.92
.80
.60
.40
.20
.00
0
10
20
30
40
50
60
70
80
90
100
110
120
130
140
150
160
170
180
190
200
TUBE NUMBER (5.0 ml)
ABSORBANCY
CONTROL

Figure 14. Separation of Rat Urine Pyrazine Metabolites on a Dowex 1 x 2, Formate Form.Column. Column Size Was 1.8 x 60 cm. Particle Size Was 100-200 Mesh. Sample Size Was 1/10 of Day One. Solvent Schedule Was: 500 ml H_2O; 500 ml 2N HCOOH; 500 ml 4N HCOOH; and 500 ml 4N HCOOH, 0.2M NH_4CHO_2.


Radioactivity (CPM)
ABSORBANCY ——
RADIOACTIVITY - - - -
TUBE NUMBER (5.0 ml)
Absorbance A 260

intermediates. The di-N-oxide, dimethylol, dihydroxydimethylpyrazine or a combination of any two of these three different functional groups holds potential as a final metabolite of 2,3-dimethylpyrazine, since there is an m/e 140 shown in Figure 8. There is no mass spectra evidence from the two metabolite peaks to support a glucuronide conjugate of 2,3-dimethylpyrazine. With a glucuronide m/e 193, 167, and 124 would be expected, but these are not prominent in either Figure 8 or Figure 9.

Williams reported that in the drug sulfapyrazine the pyrazine ring is stable and that the drug is excreted by rabbits in the acetylated form (43). Little, if any, hydroxylation occurs.

An idea of the metabolism of dimethylpyrazine can be derived from the literature and facts presented. Some remains unchanged. Oxidative and reductive ring cleavage is unlikely because of the stability of the pyrazine ring and little or no $^{14}CO_2$ was found in the respired breath of the rats in this experiment. N-methylpyrazines, hydroxypyrazines and the glucuronides are possibilities, but there is no mass spectral evidence for this route of metabolism in this research report. Also, other conjugates, such as acetyl derivatives, are possible. There is some mass spectra evidence to support the possibility that the di-N-oxide, dimethylolpyrazine or a combination of the two functional groups is produced.

The metabolic route of 2,3-dimethylpyrazine from the point of injection to excretion in the urine proceeds from the peritoneal cavity across the mesentary to the abundant capillary supply of the intestinal wall by diffusion, then to the liver by the bloodstream, where it is metabolised.

The 2,3-dimethylpyrazine may be shuttled directly to the kidneys, metabolized there, and then excreted. Future experiments should include the incubation of pyrazines with liver and kidney homogenate or microsomal fractions. Time-location studies of pyrazine in various organs would also indicate specifically where metabolism occurs.

CHAPTER V

SUMMARY

The chemical synthesis and purification of 2,3-^{14}C-2,3-dimethylpyrazine has been completed. The metabolism of this compound in the rat shows that about 80 per cent of the administered dose is excreted in the urine within 24 hours. Biochemical alteration of the compound does occur, and some unchanged pyrazine appears in the urine. Less than 1 per cent of the administered dose is respired as $^{14}CO_2$ in any 24 hour period.

Ion exchange techniques developed for the separation of nucleotide type compounds have been successfully applied to pyrazine urinary metabolites. The metabolites isolated from Dowex 1 x 2, formate form, column have been examined by low resolution mass spectrometry and no reliable structural identifications have been made. Further work is needed to define the major pyrazine metabolites.

Application of the techniques developed in this study might be made in metabolic studies utilizing liver homogenates or subcellular fraction. It is expected that structural identification will require high resolution mass spectrometry.

SELECTED BIBLIOGRAPHY

(1) Stecher, Paul G., (Ed.), The Merck Index, Merck and Co., Inc., Rahway, New Jersey, 1968, p. 888.

(2) Nakel, G. M. and B. M. Dirks, U. S. Patent 3,579,353, 1971.

(3) Hall, R. L. and B. L. Oser, Food Technology, 24, 25 (1970).

(4) Koehler, P. E., "Formation of Alkylpyrazine Compounds and Their Role in the Flavor of Roasted Foods." Ph. D. Thesis, Oklahoma State University, (1969).

(5) Laurent, A., Justus Liebigs Annalen Der Chemie, 52, 356 (1844).

(6) Krems, I. J. and P. E. Spoerri, Chem. Rev., 40, 279 (1947).

(7) Pratt, Y. T., in Elderfield, R. C., (ed.), Heterocyclic Compounds, John Wiley and Sons, Inc., London, 1957, p. 377.

(8) Wolff, L. and R. Marburg, Justus Liebigs Annalen Der Chemie, 363, 169 (1908).

(9) Skattebøl, L., E. R. H. Jones, and M. C. Whiting, Organic Synthesis, John Wiley and Sons, Inc., New York, 1963 p. 792.

(10) Juneja, P. S., Personal Communication, Oklahoma State University.

(11) Gilman, H. and J. F. Nelson, Rec. tran. chim., 55, p. 518, (1936).

(12) Cason, J., Chem. Rev., 40, 15 (1947).

(13) Hartung, W. H. and F. Crossley, Organic Synthesis, John Wiley and Sons, Inc., New York, 1943, p. 363.

(14) Flament, I. and M. Stoll, Helv. Chim. Acta, 50, p. 1754, (1967).

(15) Ishiguro, T., M. Matsumura and H. Murai, Yakagaku Zasshi, 80, 314 (1960); Chem. Abstr., 54, 18531i (1960).

(16) Kikkoji, T. and C. Neuberg, Biochem. Z., 20, p. 464 (1909).

(17) Kosuge, T., H. Zenda, K. Tsuji, T. Yamamoto, and H. Narita, Agr. Biol. Chem., 35, p. 693 (1971).

(18) Kosuge, T., T. Adachi, and H. Kanwija, Nature, 195, p. 1103 (1962).

(19) Buttery, R. G., R. M. Seifert, D. G. Guadagni, and L. C. Ling, J. Agri. Food Chem., 17, p. 1322 (1969).

(20) Bramwell, A. F., J.W.K. Burrell and G. Riezebos, Tetrahedron Lett., 25, p. 3215 (1969).

(21) Murray, K. E., J. Shipton and F. B. Whitfield, Chem. Ind., 897 (1970).

(22) White, E. C. and J. H. Hill, J. Bacteriol., 45, 433 (1943).

(23) Standinger, H. and T. Reichstein, The Structure and Composition of Foods, John Wiley and Sons, Inc., New York, 1939, p. 152.

(24) Gianturco, M. A., Chemistry and Physiology of Flavors, AVI Publishing Co., 1967, p. 431.

(25) Mason, M. E., B. Johnson and [illegible] M. Hamming, J. Agri. Food Chem., 14, 454 (1966).

(26) Deck, R. E. and S. S. Chang, Chem. Ind., 1343 (1965).

(27) Buttery, R. G., R. M. Seifert, D. G. Guadagni, and L. C. Ling, J. Agri. Food Chem., 19, 969 (1971)

(28) Dietrich, P., E. Lederer, M. Winter, and M. Stoll, Helv. Chim. Acta, 47, 1581 (1964).

(29) Rizzi, G. P., J. Agri. Food Chem., 15, 549 (1967).

(30) Van Praag, M. and D. S. Bidmead, Fr. 1,559,547; Chem. Abstr., 72, 41,960t.(1970).

(31) Neurath, G. and M. Duenger, Beitr. Tobakforsch., 5, 1 (1969); Chem. Abstr., 72, 11072n (1970).

(32) Duenger, M. S. African 69 06582; Chem. Abstr., 73, 10647e (1970).

(33) Seifert, R. M., R. G. Buttery, D. G. Guadagni, O. R. Black and J. G. Harris, J. Agri. Food Chem., 20, 135 (1972)

(34) Sapers, G. M., S. F. Osman, C. J. Dooley and O. Panasiuk, J. Food Sci., 36, 93 (1971).

(35) Flament, I. and G. Ohloff, Helv. Chim. Acta, 54, 1911 (1971).

(36) Watanabe, K. and Y. Sato, J. Agri. Food Chem., 19, 1017 (1971).

(37) Koehler, P. E., M. E. Mason, and J. A. Newell, J. Agri. Food Chem., 17, 393 (1969).

(38) Davidson, B. K. and L. F. Wiggans, Chem. Ind., 982 (1956).

(39) Wiggans, L. F., Sugar J., 18, 18 (1956).

(40) Nishie, K., A. C. Waiss, Jr., and A. C. Keyl, Toxicol. Appl. Pharmacol., 17, 244 (1970).

(41) Babek, M. and A. J. Black, Med. Chem., 15, 164 (1972).

(42) Fishman, V. and H. Goldenberg, J. Pharmacol. Expl. Therap., 150, 122 (1965).

(43) Williams, R. T., Detoxication Mechanisms, John Wiley and Sons, Inc., New York, 1959, p. 614.

(44) Stolte, K., Biochem. Z., 12, 449 (1908).

(45) Yu, T. F., L. Berger, D. J. Stone, J. Wolfe, and A. B. Gutman, Exptl. Biol. Med., 96, 264 (1957)

(46) Allen, W. S., S. R. Aronomic, L. U. Brancone and J. H. Williams, Anal. Chem., 25, 895 (1953).

(47) Bray, G. A., Anal. Biochem., 1, 279 (1960).

(48) Heftman, E., Chromatography, 2nd ed. Reinhold Publishing Corp., New York, 1967, p. 632.

(49) Pontis, H. G., J. Biol. Chem., 216, 195 (1955).

VITA

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