PRODUCTION OF A NEW COMPOUND BY METABOLISM OF THEOPHYLLINE IN *COFFEA ARABICA* L.

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PRODUCTION OF A NEW COMPOUND BY METABOLISM

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LIST OF ABBREVIATIONS

TLC - thin-layer chromatography

^{14}C - carbon 14

CO_2 - carbon dioxide

cm - centimeter

D_2O - deuterium oxide

HPLC - high performance liquid chromatography

IR - infrared

MeOH - methanol

µCi - microcurie

µl - microliter

mg - milligram

ml - milliliter

M - molar

M.W. - molecular weight

nm - nanometer

N - normal

NMR - nuclear magnetic resonance

NaAc - sodium acetate

NaN_3 - sodium azide

BRRMS - Biomedical Research Resource in Mass Spectrometry

CEC - Consolidated Electro Dynamics Corp. (No longer in existence)

IUPAC - International Union of Pure and Applied Chemistry

CA - Chemical Abstracts

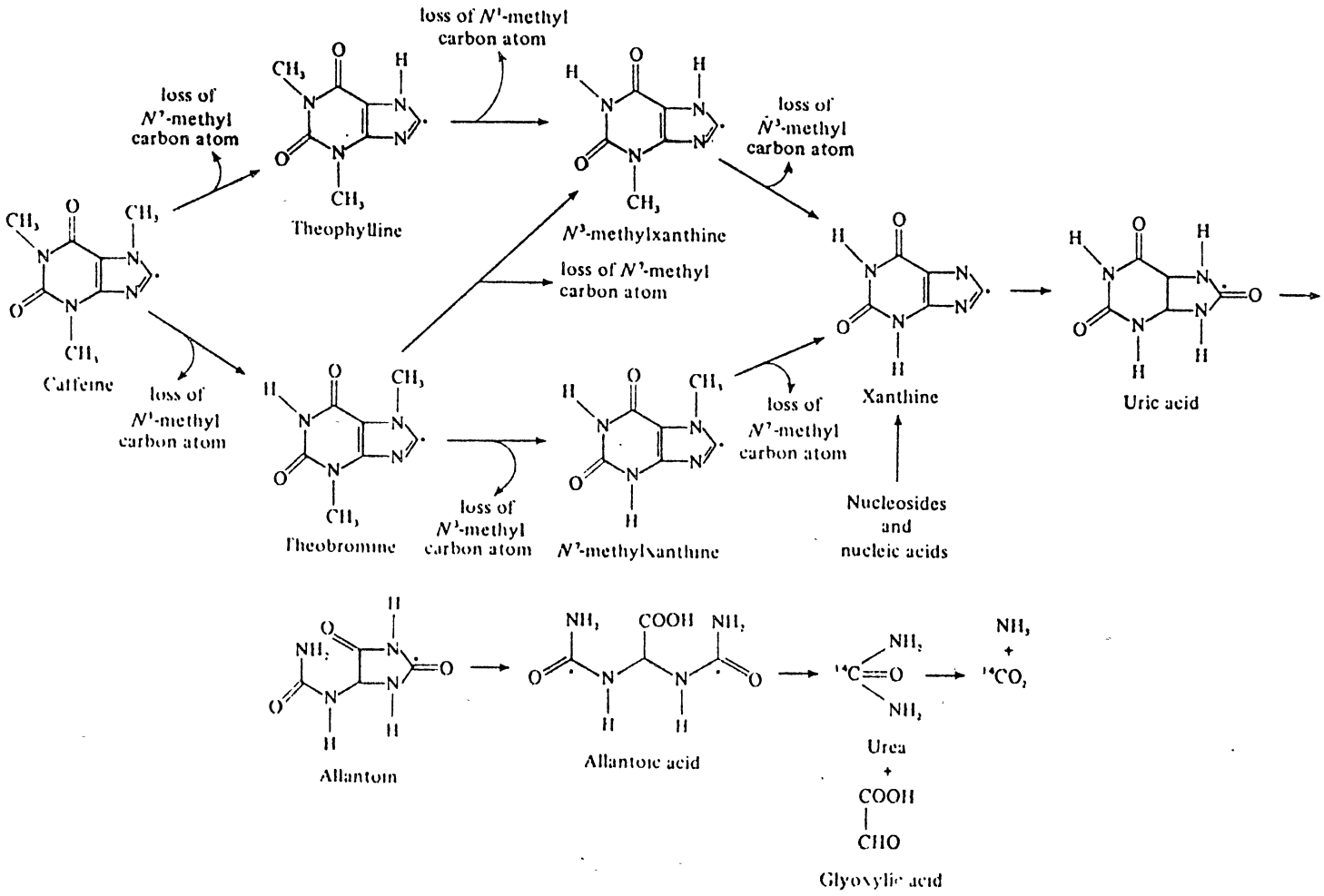
CHAPTER I

INTRODUCTION

Biodegradation of caffeine (1,3,7-trimethylxanthine) occurs in Coffea arabica L. plants through theophylline (1,3-dimethylxanthine) and theobromine (3,7-dimethylxanthine) as the first biodegradation products (Figure 1) (1). Theophylline is associated primarily with caffeine biodegradation whereas theobromine is involved in both biosynthesis and biodegradation of caffeine. Theophylline and theobromine are found as minor components whereas the caffeine is the major purine alkaloid produced. Xanthine is produced by removal of the $\underline{N}^1$-, $\underline{N}^3$- and $\underline{N}^7$-methyl groups of the dimethylated xanthine and converted to uric acid which undergoes hydrolytic cleavage to urea and glyoxylic acid in the coffee plant.

The objective of this work was to find out if theophylline was metabolized in the predicted pathway of Coffea arabica. In the present study an unknown was found to be produced by metabolism of radioactive theophylline. Its purification was by thin-layer chromatography, ion-exchange chromatography, and high performance liquid chromatography. Its molecular formula was determined by high resolution mass spectrometry. This study also shows how the unknown compound can fit into the biodegradation of theophylline.

Figure 1. Biodegradation of Caffeine by Coffea arabica L.


loss of N¹-methyl carbon atom
loss of N⁷-methyl carbon atom
Theophylline
N³-methylxanthine
loss of N³-methyl carbon atom
loss of N⁷-methyl carbon atom
Caffeine
loss of N¹-methyl carbon atom
Theobromine
loss of N³-methyl carbon atom
N⁷-methylxanthine
loss of N⁷-methyl carbon atom
Xanthine
Nucleosides and nucleic acids
Uric acid
Allantoin
Allantoic acid
Urea
+
Glyoxylic acid
+

CHAPTER II

LITERATURE REVIEW

A. Biodegradation of Caffeine

The biodegradation products of [8-^{14}C]caffeine were reported to be theophylline, theobromine, $\underline{N}^3$- and $\underline{N}^7$-methylxanthines, allantoin, allantoic acid, and urea. For [8-^{14}C]theophylline, the degradation products were $\underline{N}^3$-methylxanthine, allantoin, allantoic acid, urea, and an unknown compound, but no $\underline{N}^1$-methylxanthine (1). The pattern of biodegradation is also shown in Figure 1. Suzuki also reported that in tea and coffee, hypoxanthine and xanthine are metabolized to urea and CO_2 (2). Kalberer synthesized four different labelled caffeine molecules, two with ^{14}C in the purine ring (C-2 and C-8) and two with ^{14}C in the methyl groups ($\underline{N}^3$-methyl-^{14}C and $\underline{N}^7$-methyl-^{14}C). Radioactive caffeine solutions were fed through the mid-rib of coffee leaves, which degraded the caffeine to 3- and/or 7-methylxanthine, but no radioactivity was detectable in the mono- and dimethyluric acids. Large amounts of radioactive allantoin and smaller amounts of active allantoic acid and urea were produced. No radioactivity was found in amino acids connected with C-1 metabolism (serine, citrulline, arginine, methionine) (3). Kalberer's results are also shown in Figure 1. It was proved that methyl transferases catalyze the transfer of methyl groups from $\underline{S}$-adenosylmethionine to 7-methyl-, 3-methyl-, and 1-methylxanthine with

the production of theobromine, theophylline, and paraxanthine for the biosynthesis of caffeine (4-6).

Xanthine oxidase (EC 1.2.3.2) is known to oxidize hypoxanthine to xanthine and then to uric acid (Figure 1). This enzyme utilizes oxygen, which acts as an electron acceptor. When molecular oxygen was the oxidizing agent and the reaction was carried out at pH 7.5, ^{18}O was incorporated into uric acid from ^{18}O-labeled water (7). This is in contrast to the enzymic reaction which forms xanthine from $\underline{N}^3$-methylxanthine and $\underline{N}^7$-methylxanthine (1-3).

Hohnloser, Osswald, and Lingens studied the enzymatic demethylation of caffeine (1,3,7-trimethylxanthine) by Pseudomonas putida C-1. Cells showed an activity in the range of 30-70 µmol caffeine demethylated per hour and per milliliter of crude extract. The presence of NADH or NADPH was absolutely necessary for activity. The only demethylation product detectable was theobromine. Formaldehyde was formed during the process (8).

The preferential incorporation of ^{14}C from $^{14}CO_2$ into C-6 of xanthine, which indicated the involvement of phosphoribosylaminoimidazole carboxylase (EC 4.1.1.21) in the synthesis of purines in nodules of soybeans, was reported by Boland and Schubert (9). This enzyme catalyzes the incorporation of CO_2 into 5'-phosphoribosyl-5-amino-4-imidazole carboxylate in the conventional purine biosynthetic pathway. In these studies Boland and Schubert demonstrated that the xanthine accumulation in allopurinol-treated plants due to de novo purine biosynthesis and not breakdown of pools of nucleotides, nucleic acids, or other purine-containing substances (9).

Frischknecht et al. demonstrated that the temperature had the

strongest effect on purine alkaloid biosynthesis compared to another environmental factor in Coffea arabica (10). A decrease in temperature lowers caffeine production and an increase enhances it. However, high caffeine production was observed with high dark respiration in very young leaves or in leaves exposed to raised temperatures. Frischknecht et al. concluded that adenine-containing nucleotides were the most likely precursors of the purine alkaloids.

B. Ureides: Allantoin and Allantoic Acid

Uricase (EC 1.7.3.3) has been reported to catalyze the oxidation of uric acid to allantoin, producing also hydrogen peroxide, in the root of soybean plants (11) and in nodules of Sesbania exalta, soybean, and alfalfa (12). Allantoinase (allantoin amidohydrolase, EC 3.5.2.5) catalyzes the conversion of allantoin to allantoic acid. Vogels et al. found that allantoinases from different sources: animal livers, plant seeds, Pseudomonas species, and bacteria do not follow a pattern in their behavior. For example, they show differences in pH optimum curve, stability upon storage, heating and acid pretreatment, behavior during purification, and Km (13). The presence of a metal-ion-independent allantoinase, which proved to be a sulfhydryl enzyme, was reported to be present in Lathyrus sativus by Nirmala and Sastry (14). Vogels et al. also reported that none of the enzymes tested catalyzed the degradation of 3-methylallantoin nor 1-acetylallantoin but did degrade methylolallantoin and 5-aminohydantoin (13). Allantoicase (allantoate amidinohydrolase, EC 3.5.3.4) catalyzes the degradation of allantoate to urea and glyoxylate. Rattan detected allantoicase activity in germinating peanut cotyledons, but it could not be found in resting seeds

(15). Drewes and Van Steden mentioned that allantoin might be a) a storage product for nitrogen, b) the form in which nitrogen is translocated in plants, or c) a product in the detoxification of ammonia in plant tissue (16). A number of leguminous plants synthesize ureides in nodules and use these compounds for transport and storage of nitrogen whereas legumes in which ureides are not synthesized transport the nitrogen from the root as amides (17, 18). For instance, by inoculating seeds of cluster bean (Cyanopsis tetragonoloba L. cv FS-277) and pigeonpea (Cajanus cajan cv UPAS-120) with a Rhizobium culture, Sheoran demonstrated that nitrogen is transported from roots of cluster beans in the form of amides in the form of asparagine and glutamine and from roots of pigeonpea in the form of ureides (19). Such results are in agreement with those of Shearer et al., who reported that legumes can be classified as ureide transporters and amide transporters (20). Boland and Schubert found that ureides were synthesized via de novo purine synthesis followed by oxidation and hydrolysis (9). The specific activities of glutamine synthetase (EC 6.3.1.2), glutamate synthase (EC 1.4.1.14), and aspartate aminotransferase (EC 2.6.1.1), the enzyme involved in the initial assimilation of ammonia in developing soybean nodules, were determined by Reynolds and others who demonstrated that such assimilation proceeded via a pathway involving biosynthesis of purines and their breakdown with ureide formation (21).

Fujihara and Yamaguchi investigated the assimilation of $^{15}NH_3$ by crude breis prepared from crushed soybean nodules, bacteroid fractions, and supernatant plant fractions of such breis (22). In the crude breis the incorporation of ^{15}N was highest in alanine and next highest in the amide-N of asparagine and glutamate; the incorporation of ^{15}N in

allantoic acid was low, although higher than that of other amino compounds. In the bacterial fraction from nodule breis the labelling of glutamate, alanine, and glycine was high but the labelling of allantoic acid was low. The addition of azaserine (a glutamine antagonist) or allopurinol (an inhibitor of xanthine oxidase) inhibited the incorporation of ^{15}N into allantoic acid; on the other hand, the addition of some dicarboxylic acids, fumarate, succinate, and malate, increased this incorporation. Those results suggested that part of the glutamine produced from fixed nitrogen flowed into the pathway of purine biosynthesis in host plant cells and contributed to the formation of allantoin and allantoic acid via xanthine-uric acid as an intermediate (22).

Atkins et al. (23) examined transfer of nitrogen and carbon of ureides to amino acids and proteins of leaflets, stems, and petioles, apices, peduncles, pods, and seeds of detached shoots of nodulated cowpea (Vigna unguiculata L. Walp. cv. Caloona) by using ^{14}C,-^{15}N-labeled allantoin. Their studies indicated a preferential utilization of ureide nitrogen in pathways of nitrogen assimilation in the shoot, and a distribution of allantoinase activity in vegetative and reproductive tissues. The finding of ^{14}C-urea in tissue extracts of the shoots suggested an involvement of allantoicase in the cleavage of ^{14}C-allantoic acid. Atkins et al. found that intact tissues of cowpea, especially mature leaflets and stems, metabolized [2-^{14}C]allantoin to form $^{14}CO_2$, and this, coupled with the capacity of extracts from all tissues of cowpea to hydrolyze urea, indicated that allantoin is metabolized via allantoic acid and urea to ammonia and CO_2. They also found a wide range of compounds labeled with [^{15}N]allantoin, indicating

that ureide nitrogen was assimilated (23). Serres reported that in nodulation soybean, allantoin and allantoic acid were very abundant during the reproductive stage and accumulated in stems and pods (24). These results agree with those of Zengbe and Salsac (25), who reported that during the development of soybean plants (Glycine max) ureides constitute the predominant part of the soluble nitrogen. There was a maximum in the ureide content at flowering and during the pod formation period. Throughout the culture period, ureide N in the stem formed nearly half of the total soluble nitrogen, whereas it was very low in the nodules, roots, and leaves. The enzymes of purine catabolism were present in all parts of the plant. The root nodules contained high enzymatic activity except that of allantoicase, which was found only in the stem and leaves. Xanthine dehydrogenase, uricase, and also allantoinase were found in the soluble fraction of nodules, suggesting that allantoin and allantoic acid are produced in nodule cells, but not exclusively in the bacteriods (25). Moreover, Hanks et al. reported that in the nodules of Glycine max L. Merr. cv Amsoy 71, the peroxisomal enzymes, uricase and catalase, were present at much higher specific activity in the uninfected cell fraction and that allantoinase in the endoplasmic reticulum also had a greater specific activity in the uninfected cell fraction (26). In addition, Newcomb and Tandon (18) found that in soybean root nodules, which fixed nitrogen mainly as ureides, cells uninfected by Rhizobia undergo a pronounced ultrastructural differentiation not shown by the infected cells, including enlargement of the microbodies and proliferation of smooth endoplasmic reticulum. Thus the uninfected cells may participate in ureide synthesis because the required enzymes occur in these organnelles (18).

CHAPTER III

EXPERIMENTAL MATERIALS AND METHODS

A. Materials and Chemicals Used

1. Plants

Coffea arabica trees were obtained through the United States Department of Agriculture, Germplasm Resources Laboratory, Beltsville, Maryland; from George A. White, Plant Introduction number 435156, July 2, 1980. The plants were approximately 6 months of age when received in Stillwater. These plants were approximately 33 months of age at the time the experiment was started. On September 21, 1982 at 3:00 PM, 130 μCi [8-^{14}C]theophylline was injected into stems of the plant. The plant height was 57" and had 130 fruit and had no branches with small seeds, 37 branches without seeds, and 13 branches with large seeds. A record of the sampling time, dates and hours from the time of injection, is found in Table I and II. The complete branch was taken for a single sample. The fruit collected during the first two days were washed six times by immersing in distilled water, and no radioactivity was found. Afterwards no preliminary washing of fruit was done.

2. Radioactive Compounds

[8-^{14}C]Theophylline (47.5 Ci/mole) was purchased in a solution of

TABLE I

COFFEE FRUIT SAMPLING

Sample Number	Sample Description	Time (p.m.)	Date	Time Elapsed from Injection
40-1	1 red fruit	3:00	9/22/82	1 day
40-2	1 green fruit	3:00	9/22/82	1 day
40-3	1 green fruit	4:00	9/23/82	2 days
40-4	1 yellow fruit	4:00	9/23/82	2 days
40-5	1 green fruit (lost)	3:00	9/25/82	4 days
40-6	1 yellow fruit	3:00	9/25/82	4 days
40-7	3 green fruit	1:30	9/26/82	4 days and 22.5 hours
40-8	3 yellow fruit	1:30	9/26/82	4 days and 22.5 hours
40-9	1 green fruit	4:30	9/28/82	6 days and 1.5 hours
40-10	1 yellow fruit	4:30	9/28/82	6 days and 1.5 hours
40-11	2 green fruit	4:45	9/29/82	7 days and 1.75 hours
40-12	2 yellow fruit	4:45	9/29/82	7 days and 1.75 hours
40-13	1 yellow fruit	5:00	10/4/82	12 days and 2 hours
40-15	4 yellow fruit	7:00	10/8/82	16 days and 4 hours

TABLE II

COFFEE TWIG AND MAIN TRUNK SAMPLING

Sample Number	Sample Description	Time (p.m.)	Date	Time Elapsed from Injection
40-16*	23 green fruit and small twig	3:00	9/24/82	72 hours
40-17	2 branches, 1 yellow green fruit, and 1 green fruit	4:00	9/26/82	5 days and 1 hour
40-18*	2 branches	4:00	9/27/82	6 days and 1 hour
40-19*	2 branches with 4 green fruit	5:00	9/30/82	9 days and 2 hours
40-20	4 green fruit, leaves	6:00	10/1/82	10 days and 3 hours
40-21*	2 green fruit, leaves	5:00	10/5/82	14 days and 2 hours
40-22	1 branch	4:00	10/7/82	16 days and 1 hour
40-23	17 branches, 1 green fruit, 14 yellow green fruit	4:00	10/12/82	21 days and 1 hour
40-24	8 branches, 35 fruit	4:00	10/15/82	24 days and 1 hour
40-25	22 fruit and the remaining plant material	4:00	10/21/82	30 days and 1 hour

*These samples were taken for analysis in this study.

ethanol from New England Nuclear, Boston, Ma. The manufacturer's radiochromatograms, paper chromatography on Whatman No. 1 paper using n-butanol:acetic acid:water (25:4:10) and thin layer chromatography on silica gel using the following systems: a) chloroform:methanol (9:1), b) chloroform:methanol:acetic acid (50:50:2.5), and c) n-butanol:acetic acid:water (25:4:10) were greater than 99% purity on September 30, 1978 with a projected rate of decomposition of less than 1% per year when stored in ethanol solution at $-10^{o}C$. The sample was used without further purification; however, it was subjected to TLC using $CHCl_3$:CH_3OH (90:10). It was found to be in excess of 98% pure theophylline.

3. Chemical Reagents

The solvents used were of high performance liquid chromatography reagent grade purchased from either J. T. Baker Chemical Co., Phillyssburg, N.J. or Burdick and Jackson Laboratories, Muskegon, MI. Allantoin, allantoic acid, uric acid, xanthines, and theophylline used as standards were purchased from Sigma Chemical Co., St. Louis, MO. Dowex 1 x 8 chloride form, 200-400 mesh was purchased from J. T. Baker Chemical Co., Phillyssburg, N.J. The Dowex 1 x 8 chloride form was converted to the formate form by washing successively with several volumes of H_2O, 1 N NaOH, H_2O, 2 N HCl, H_2O, 8 N HCOOH and then with deionized water until neutral pH was reached. 2,4-Dinitrophenylhydrazine was obtained from Eastman Kodak Company, Rochester, N.Y.

B. Apparatus

1. High Performance Liquid Chromatography

A Model 6000 chromatographic pump from Waters Associate Co., Medford, MA, a Model 2-2919 sample injection valve from Supelco Co., Bellefonte, PA, and an Ultrasphere ODS, reversed phase column (250 x 4.6 mm I.D.) obtained from Rainin Instrument Co., Woburn, MA were used. The effuent was monitored by an UV detector from Waters Associates, Model 440 (254 nm and 280 nm) and another from Isco, Lincoln, NE, Model 82133, and recorded on a 214 nm chart recorder from Isco (214 nm, 0.7 cm/min), one from Houston Instrument, Houston, TX (254 nm, 0.2 cm/min) and another from Sargent-Welch, Baton Rouge, LA (280 nm, 0.1 cm/min). The column was eluted at 25°C with a linear gradient formed in a custom-made gradient maker. The limiting solvent chamber contained 30 ml of 90% methanol and the mixing chamber contained 30 ml of 0.1% H_3PO_4 (28). The flow rate was 1.0 ml/min. After each chromatographic run the column was washed with 20 ml of methanol.

2. Low and High Resolution Mass Spectrometry

Low resolution mass spectra were obtained using an LKB-9000 combination gas chromatograph/mass spectrometer made by LKB Produkter AB, Stockholm, Brómma, Sweden. The conditions of operation were: pressure 10^{-7} torr, electron energy 70 eV, trap current 60 mA, accelerating voltage 3.5 kv. The probe was heated from 25°C to 150°C. For mass calibration the electron impact mass spectrum of perfluorokerosene was taken. This type of instrument has been described by Waller (27).

Fast atom bombardment with a suitable gun coupled with high resolution mass spectrometry analysis was done by Herianna Pang, Department of Chemistry, Massachusetts Institute of Technology, Cambridge, MA. The Varian/MAT 731 equipped with a B-11 N Neutral Atom Gun (i.e., a fast atom bombardment gun) manufactured by Ion Tech Ltd., Headington, England was used as the gas for the gun.

3. Nuclear Magnetic Resonance Spectrometer

The spectrum of the metabolite was taken on a Varian XL-300 NMR, Varian, Palo Alto, CA, spectrometer using frequency 299.944 MHz for protons. D_2O, from Sigma Chemical Co., St. Louis, MO, 100 atom % D, was used as the solvent.

4. Liquid Scintillation Counter

Radioactivity measurements were made on a PRIAS liquid scintillation counter using Instagel from the Packard Instrument Co., Downers Grove, IL.

C. Biosynthesis

1. Administration of Labelled Theophylline

Labelled theophylline was administered using a regular syringe. One hundred thirty millicuries of the compound was injected slowly in the branch point of the stems from the main trunk () of a coffee plant previously described. Care was taken to inject all upper stems of the plant, because the tissue in the lower stems was quite woody and absorption required a very long time. The syringe was rinsed out

three times with distilled water and each time the rinse solution was injected into the plant. The injections were completed in thirty minutes.

2. Isolation of the Metabolite (1)

Fruits, leaves, and stems were detached at 72 hr (Sample No. 40-16), 145 hr (Sample No. 40-18), 218 hr (Sample No. 40-19), and 388 hr (Sample No. 40-21) after injection. The samples had been stored at -18°C. The samples were weighed and dried in an oven for 24 hr, weighed again and ground to a powder with a micro Wiley Mill, A. H. Thomas, Philadelphia, PA. Fifty ml of distilled water was added to the dry, ground plant residue, shaken on a gyratory shaker for four hr at 150 r.p.m. The mixture was filtered with Whatman 41 paper, the residue was washed with distilled water, placed in a round-bottom flask with 25 ml of 0.124 N H_2SO_4, refluxed for 20 min, filtered and washed in the same vacuum flask. The solution was extracted three times with equal volumes of $CHCl_3$. The chloroform was evaporated to 1-2 ml on a steam hot plate and the aqueous extract was evaporated to about 10 ml.

3. Chromatography

a. Development of an Ion-Exchange Column for Coffee Metabolites. Two ml of the aqueous extract was placed on a Dowex 1 x 8 formate column, 1.5 x 16 cm. Elution of the compounds were carried out by use of a concentration gradient as follows(29): 150 ml of deionized water, 250 ml of 0.25 N formic acid, 250 ml of 2 N formic acid, and 250 ml of 4 N formic acid. A 100-µl portion of the contents of each tube, which contained 3 ml, was used for the measurement of radioactivity.

b. Thin Layer Chromatography. The chloroform extract was applied to preparative thin layer chromatography plates, 0.2 mm thick, precoated with silica gel 60F-254 from EM Reagents Co., Cincinnati, OH. The plates were developed using a solvent system containing $CHCl_3$:EtOH (9:1). The compounds were detected on the chromatograms by observing their fluorescence at 254 nm. Sections of 2 cm in width were cut from the plates, they were cut again in areas of 2 cm^2, roughly corresponding to the particular compounds which were used for radioactivity measurements. The remaining theophylline zones on the thin layer chromatography plates were scraped off into a glass Büchner funnel (60 ml), medium porosity and eluted with chloroform and used mass spectrometry to make sure that this was theophylline.

The aqeeous extract (0.4 ml) was spotted on thin layer chromatography plates, 0.25 mm, cellulose MN 300F, Analtech, Newark, DE. The solvent system was EtOH:HOAc:H_2O (81:5:4). The plate was observed under $UV_{254\ nm}$, and each fraction was scraped and eluted with water. The so extracted compounds were evaporated to dryness and 2-ml solutions were prepared. From these solutions 0.5 ml were used for radioactivity measurements, and the solution which contained the radioactive compound was used for HPLC.

4. Attempted Preparation of 2,4-Dinitrophenylhydrazone Derivative

To 1.0 ml of the aqueous sample purified by HPLC was added 0.4 ml of 0.5% 2,4-dinitrophenylhydrazine in 6 N HCl (30). The solution was vortexed and incubated for 30 min at room temperature (23°C). Three extractions of 1.0 ml each were done using a solvent system of

$CHCl_3$:EtOH (4:1). The combined solvent layers were extracted with 1.0 ml of 1 N Na_2CO_3 and the solvent was discarded. The Na_2CO_3 solution was washed with 0.6 ml of chloroform-ethanol, then acidified with 0.5 ml of 6 N HCl. The aqueous layer was extracted with three 1-ml portions of $CHCl_3$:EtOH solvent with the same composition. The combined layers were evaporated at room temperature (23^oC). The precipitate was dissolved in 500 µl of absolute ethanol and spotted on a TLC plate, 0.2 mm thick, precoated with silica gel and developed in the solvent system $CHCl_3$:EtOH (9:1). Autoradiography was made to observe the radioactivity.

5. Attempted Preparation of a Methylated Derivative

To the radioactive water-soluble compound eluted from the cellulose plate several drops of diazomethane were added. The diazomethane was prepared from diazald (CH_3-C_6H_4-$SO_2N(CH_3)NO$ reacted with sodium hydroxyde, the solvent used was ether.

CHAPTER IV

RESULTS AND DISCUSSION

A. Isolation and Purification of the Metabolite from Radioactive Theophylline

The isolation procedure used for the preparation of the metabolite fraction is described under "Experimental Methods" and was the one used by Suzyki and Waller (1), the only modification being that the dried plant powder was extracted thoroughly with water before extraction with dilute sulfuric acid. After extraction of the combined supernatant with chloroform the radioactivity of both the aqueous layer and the organic layer was measured, and those values are reported in Table III. As observed in this table, the only sample which showed appreciable radioactivity was the one obtained from the stems after 72 hr of exposure with [8-^{14}C]theophylline. To purify and identify the products of metabolism in coffee fruit, stems, and leaves of the radioactive samples, they were chromatographed on thin-layer plates as described in Chapter III. The radioactivity of the chloroform layer was found due to the labelled theophylline, and it was presumed not to be metabolized by the plant in each sample. Theophylline was recognized on the silica gel plates by ultraviolet quenching at 254 nm. All radioactive theophylline was metabolized by the coffee plants within the period of 218 hr.

TABLE III

TOTAL AMOUNT OF RADIOACTIVITY RECOVERED FROM LEAVES, STEMS AND FRUIT OF *COFFEA ARABICA*

Plant Sample	Time of Exposure (hours)	Plant Part	Grams of Wet Material	Grams of Dried Material	% Dry Material	Radioactivity (nCi) Recovered	
						$CHCl_3$ Layer	H_2O Layer
No. 40-16:		leaves	3.32	1.15	34.6	0.3	0.32
9-24-82	72	stems	2.78	1.09	39.2	55.22	306.71
		fruit	5.67	1.94	34.2	0.3	1.69
No. 40-18:		leaves	4.01	1.41	35.2	0.03	0.0
9-27-82	145	stems	4.56	1.64	36.0	0.06	0.03
		fruit	4.88	1.53	31.4	0.07	0.0
No. 40-19:		leaves	4.27	1.45	34.0	0.0	0.0
9-30-82	218	stems	3.98	1.63	40.9	0.0	0.0
		fruit	1.64	0.53	32.3	0.0	0.0
No. 40-21:		leaves	3.25	1.07	32.9	0.0	0.0
10-5-82	338	stems	2.42	0.90	37.2	0.0	0.0
		fruit	1.55	0.60	38.7	0.0	0.0

The radioactivity found in the aqueous layer was due exclusively to an unknown compound biosynthesized in the stems of the coffee plant from the radioactively labelled theophylline. This compound was detected on the cellulose plate(s) by locating radioactivity of the different fractions distinguished by ultraviolet quenching at 254 nm, as shown in Figure 2. The R_f of the unknown was 0.87. This compound was eluted according to the method described in Chapter III. The purification was complicated because there were many other compounds in the aqueous extract as will be shown later in this chapter. The radioactivity distribution of the different fractions on TLC can be observed in Table IV. An ultraviolet absorbance spectrum was recorded for the fraction of interest which was eluted from the cellulose plate; this can be observed in Figure 3. The spectrum of the unknown compound was taken at two different concentrations and was compared with the theophylline spectrum. Theophylline showed an absorbance maximum at 265 nm, while the unknown compound did not show any distinctive absorbance peak. It was concluded that this broad spectrum in the unknown was likely due to a peptide bond or another type of oxygen-carbon-nitrogen-hydrogen linkage with a minimum of unsaturation such as in amides. The low absorption observed may be due to the lack of aromaticity or to the small amount of the compound present in the eluted fraction. Also, the spectrum of the unknown compound was compared to these of allantoin, allantoic acid, xanthine, and uric acid (Figure 4), which was similar to the spectrum of allantoin or allantoic acid. Consequently, at first it was thought that the radioactive unknown compound was one of those ureides. As a step in identification, the LKB-900 mass spectrum was taken with fruitless results

Figure 2. Location of the Unknown on the Cellulose Plates. The solvent was the same as described in Chapter III.

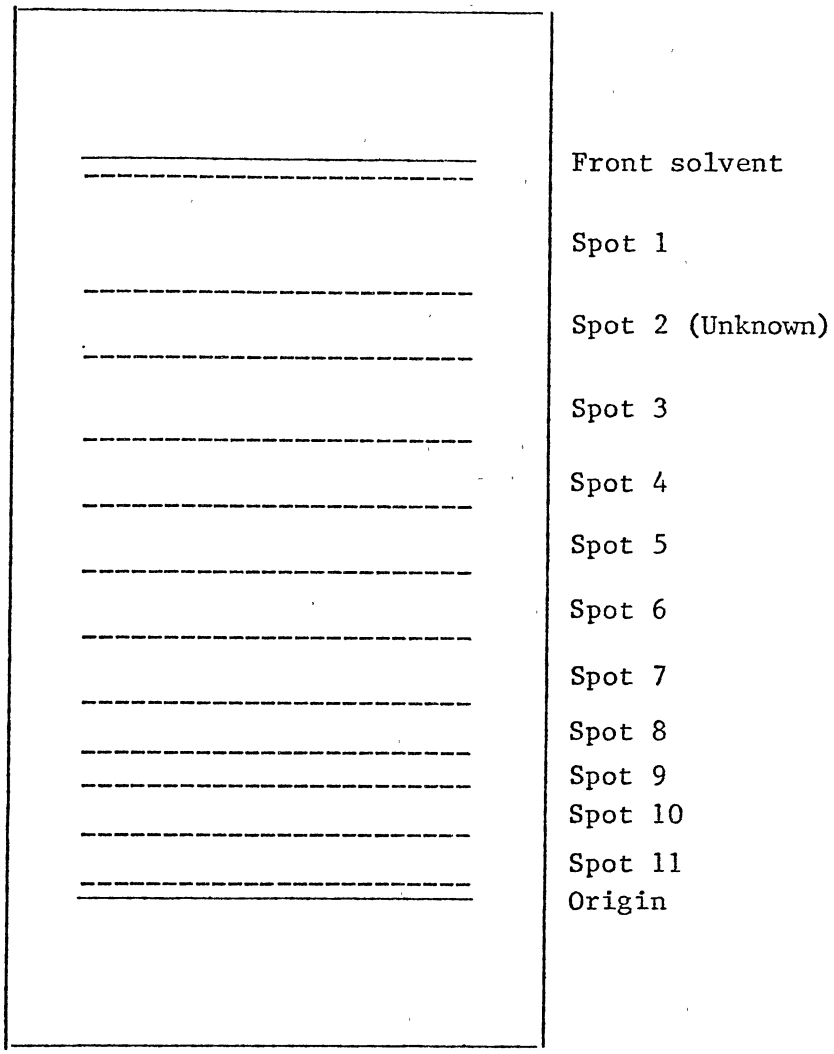

Front solvent
Spot 1
Spot 2 (Unknown)
Spot 3
Spot 4
Spot 5
Spot 6
Spot 7
Spot 8
Spot 9
Spot 10
Spot 11
Origin

TABLE IV

DISTRIBUTION OF RADIOACTIVITY FROM THE DIFFERENT FRACTIONS ELUTED FROM THE CELLULOSE PLATE(S)

Fraction	DPM
Solvent Front	0.0
Spot 1	40
Spot 2	958
Spot 3	84
Spot 4	0.0
Spot 5	0.0
Spot 6	0.0
Spot 7	0.0
Spot 8	0.0
Spot 9	0.0
Spot 10	0.0
Spot 11	0.0
Origin	82

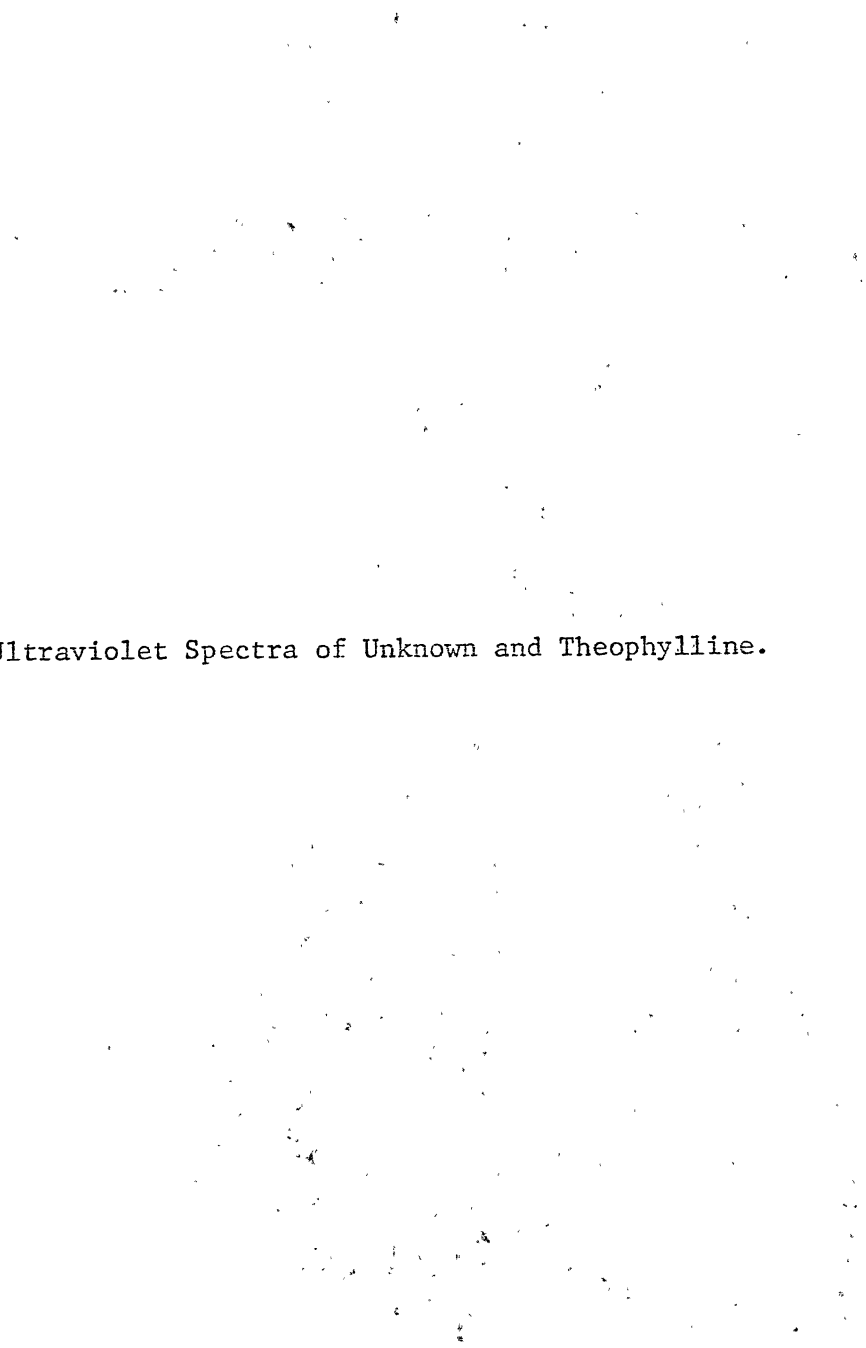


Figure 3. Ultraviolet Spectra of Unknown and Theophylline.

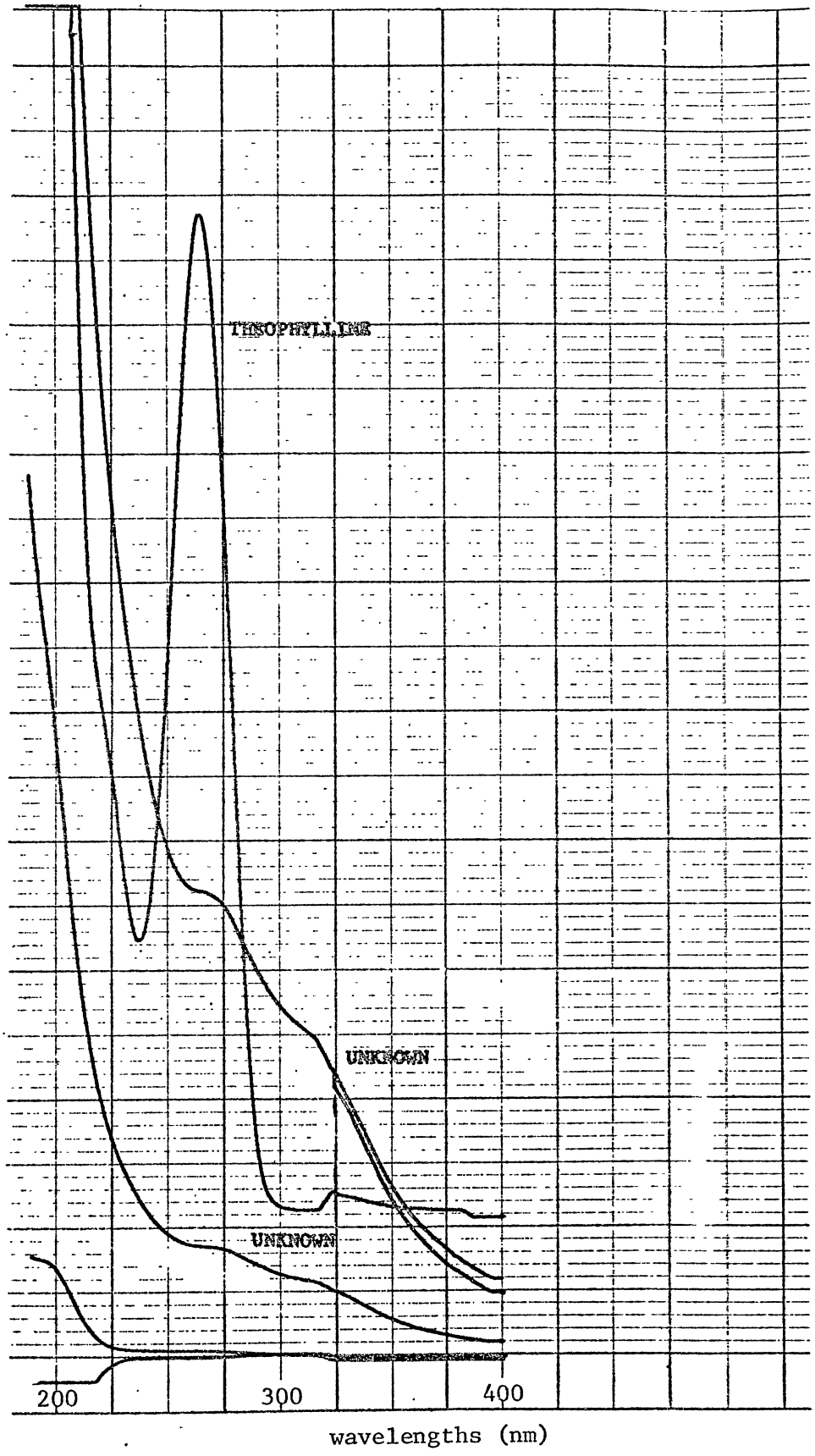
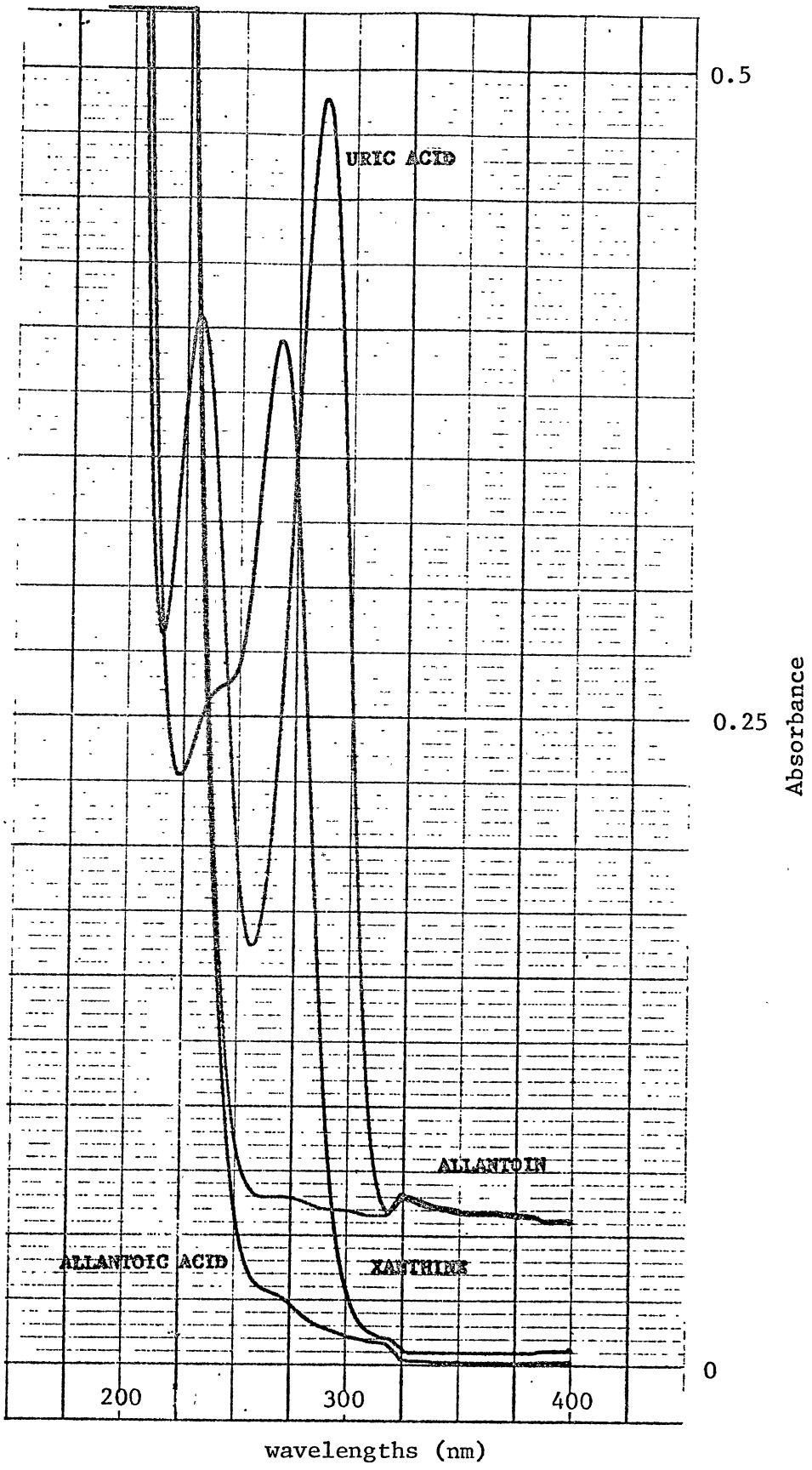

0.5
0.25
0
Absorbance
THEOPHYLLINE
UNKNOWN
UNKNOWN
200
300
400
wavelengths (nm)

Figure 4. Ultraviolet Spectra of Uric Acid, Xanthine, Allantoin, and Allantoic Acid.


0.5
URIC ACID
0.25
Absorbance
ALLANTOIN
ALLANTOIC ACID
XANTHINE
0
200
300
400
wavelengths (nm)

because of the impurities.

The impure preparation was analyzed by high performance liquid chromatography and fractions monitored at 214 nm, 254 nm, and 280 nm which are shown in Figure 5. About seven compounds including the radioactively labelled unknown could be observed from those chromatograms. Collections were made using 1-ml increments. To locate the radioactivity 3175 dpm of the unknown were put into the HPLC, and the eluted compounds were collected and divided into five different fractions, evaporated under nitrogen until dryness, and 1-ml solutions were prepared for counting of the radioactivity. 250-µl aliquots were used for the measurement of radioactivity; the observed results are shown in Table V. To locate the tube which contained the radioactivity 689 dpm were put into the HPLC, and the results are shown in Table VI. The radioactive compound was collected in tubes number 22 and 23. The low molecular absorptivity and/or the small amount of the compound might be the cause of the low absorption observed on the chromatograms.

The crude aqueous layer (2.0 ml) was passed through the Dowex 1 x 8 column, the radioactivity was monitored and the combined radioactive fractions were put in the HPLC as before; the chromatograms were taken at 214 nm, 254 nm, and 280 nm (Figure 6). Following elution from HPLC of the unknown compound, it was reduced to dryness by evaporation over nitrogen. Two nuclear magnetic resonance spectra (proton) were taken (Figures 7 and 8).

The unknown compound was treated with 2,4-dinitrophenylhydrazine. The crude product was spotted on the TLC plate (silica gel), and an autoradiogram was made by which it was theoretically possible to observe

Figure 5. High Performance Liquid Chromatograph Tracings of the Unknown Previously Purified by Cellulose Thin Layer Chromatography. a) At 280 nm, b) At 254 nm, and c) At 214 nm. The number of tubes were recorded at 254 and 280 nm, and they apply to the recording taken at 214 nm.

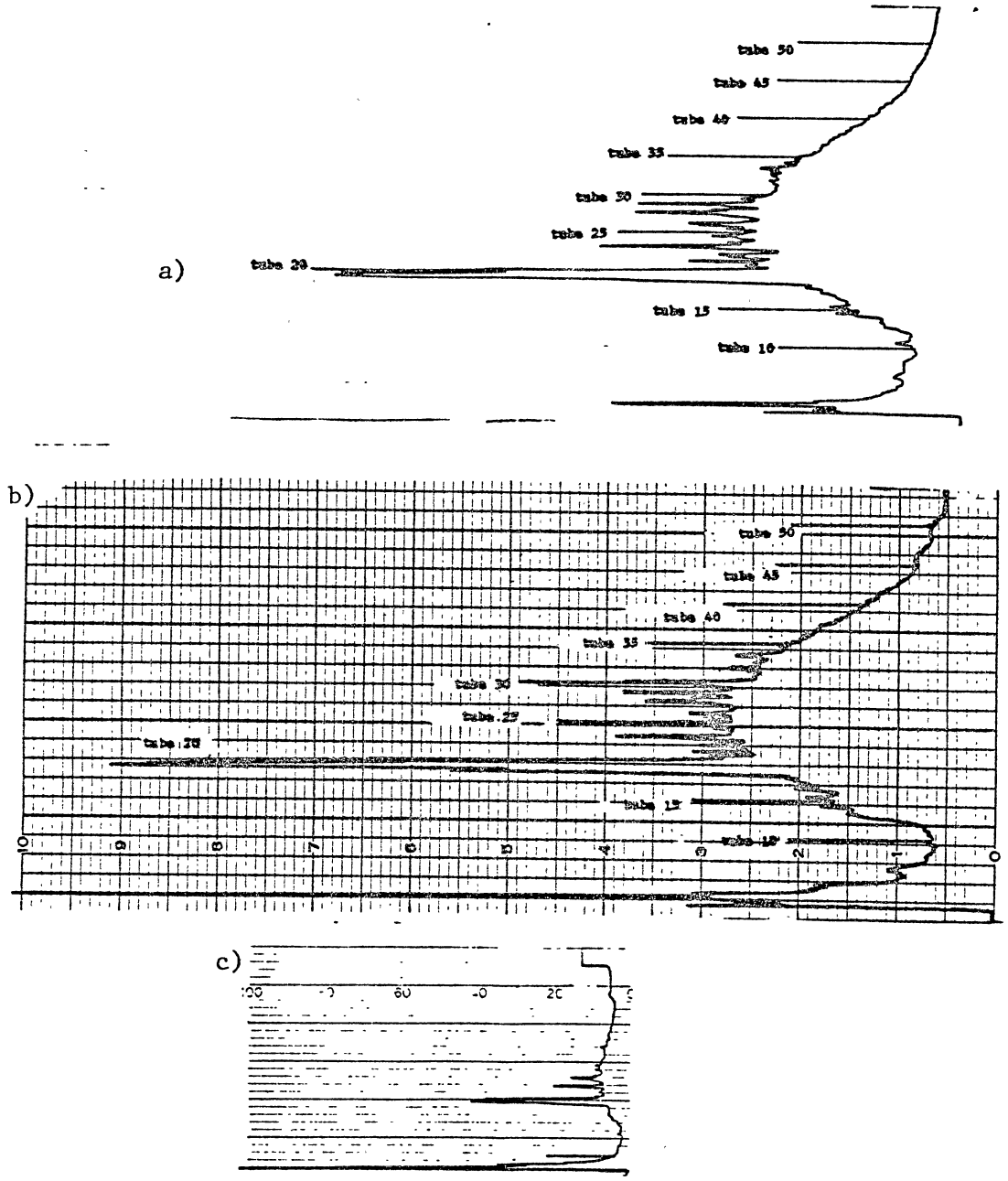

a)
tube 50
tube 45
tube 40
tube 35
tube 30
tube 25
tube 20
tube 15
tube 10
b)
tube 50
tube 45
tube 40
tube 35
tube 30
tube 25
tube 20
tube 12
c)

TABLE V

RADIOACTIVITY DISTRIBUTION OF THE FIVE FRACTIONS COLLECTED FROM THE HPLC

Fraction	Total DPM	Volume (ml)
#1	25	1.0
#2	24	1.0
#3	2540	1.0
#4	0.0	1.0
#5	0.0	1.0

Note: The solvent wash was not radioactive.

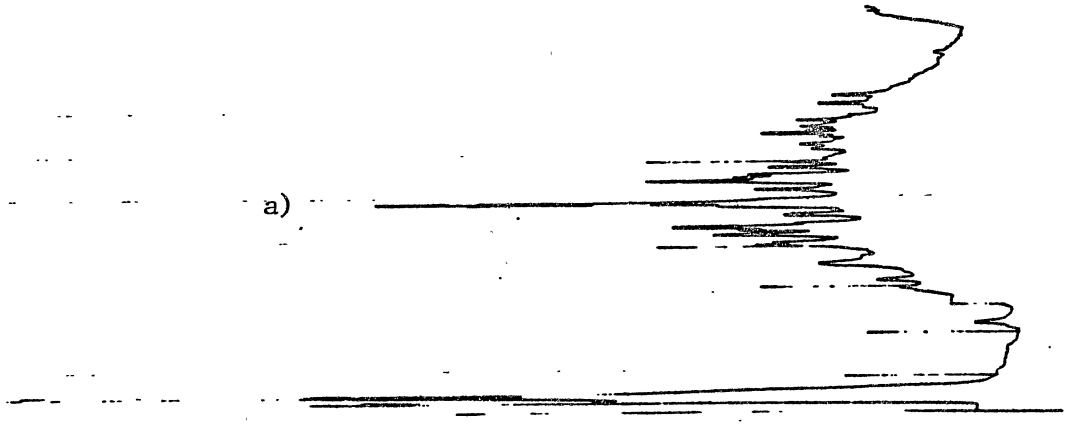
TABLE VI

DISTRIBUTION OF THE RADIOACTIVITY IN THE TOTAL FRACTIONS COLLECTED FROM THE HPLC

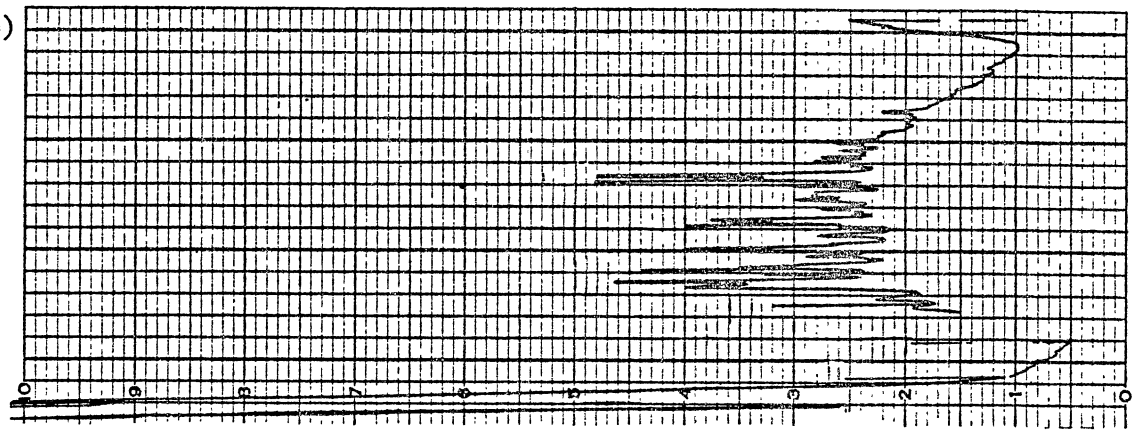
Fraction	DPM	Volume (ml)
#20	0.0	1.0
#21	0.0	1.0
#22	223	1.0
#23	302	1.0
#24	0.0	1.0
#25	0.0	1.0

Figure 6. High Performance Liquid Chromatograph Tracings of Unknown Previously Purified by Ion-Exchange Liquid Chromatography. a) At 280 nm, b) At 254 nm, and c) At 214 nm.

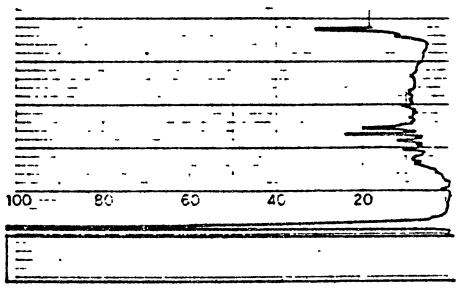
a)



b)



c)



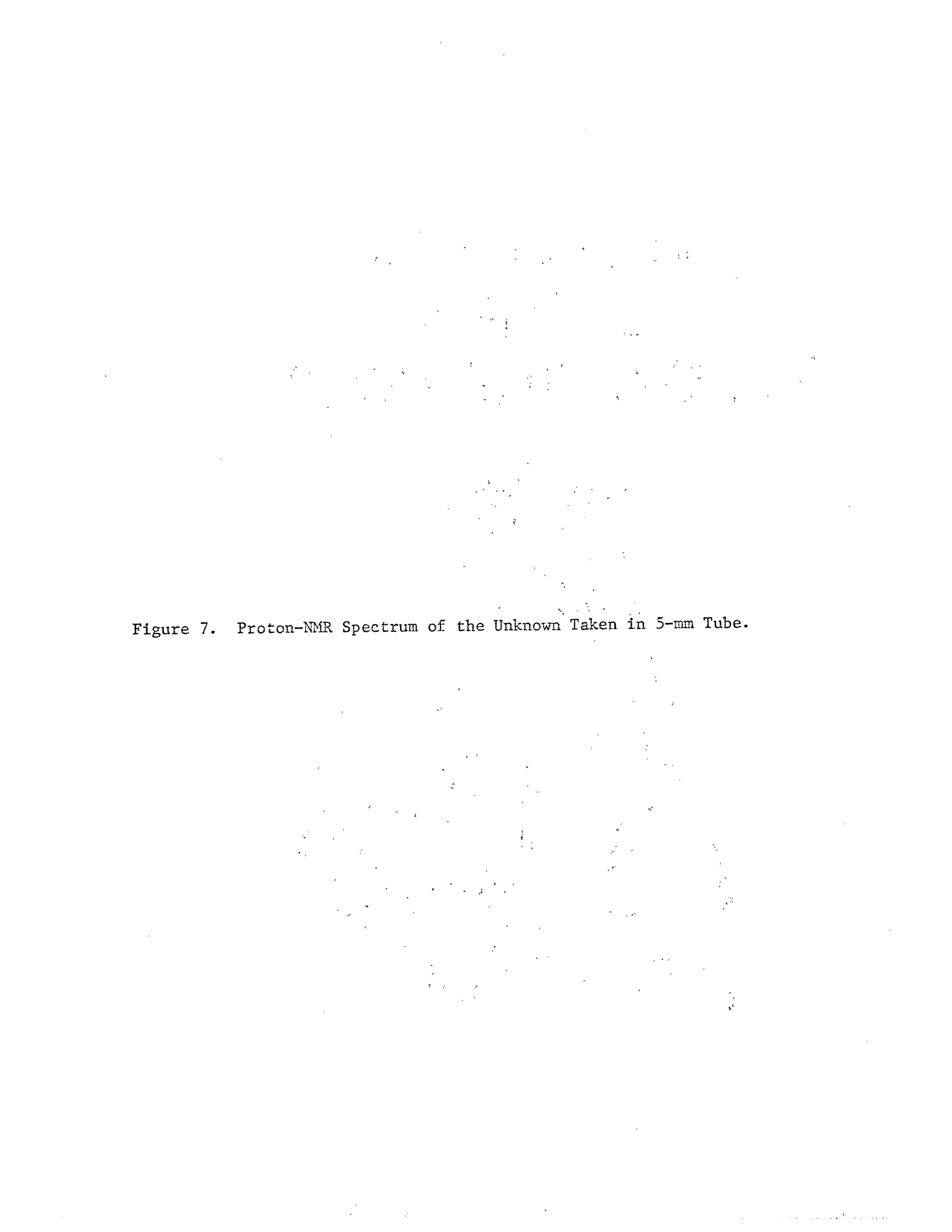


Figure 7. Proton-NMR Spectrum of the Unknown Taken in 5-mm Tube.

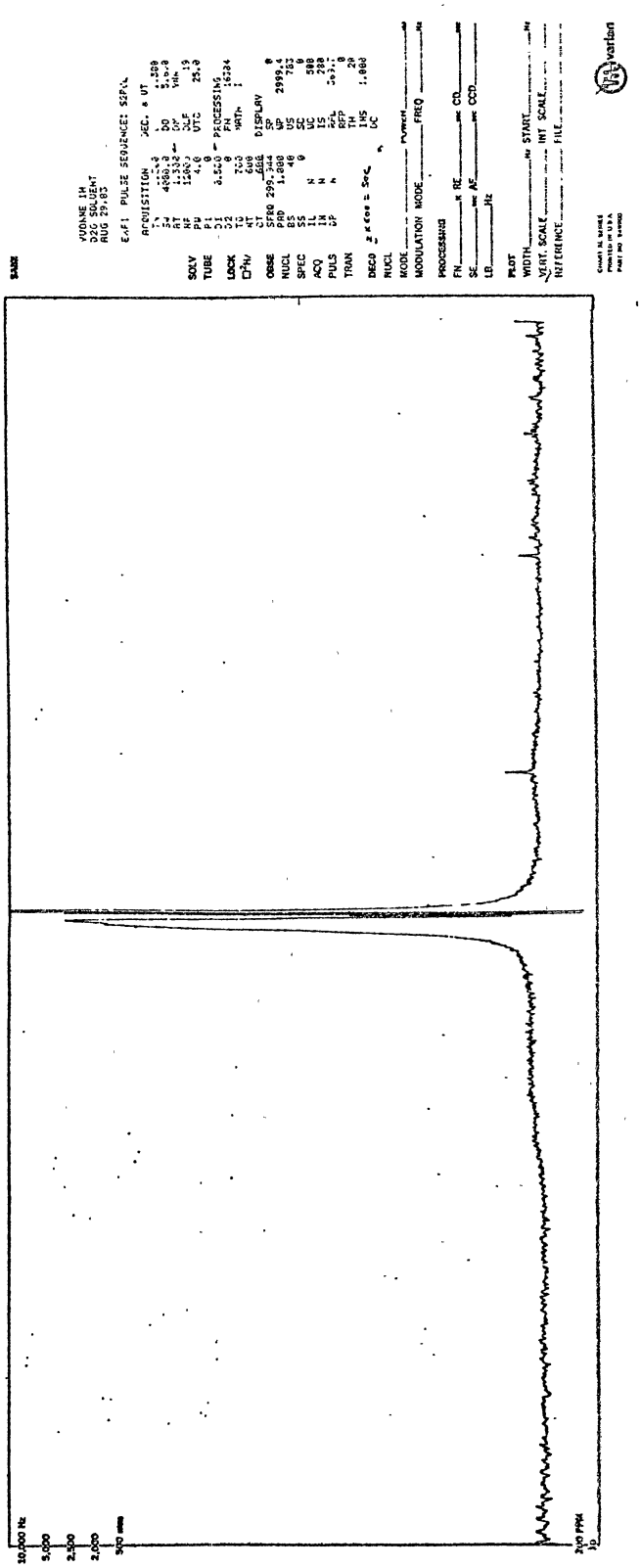
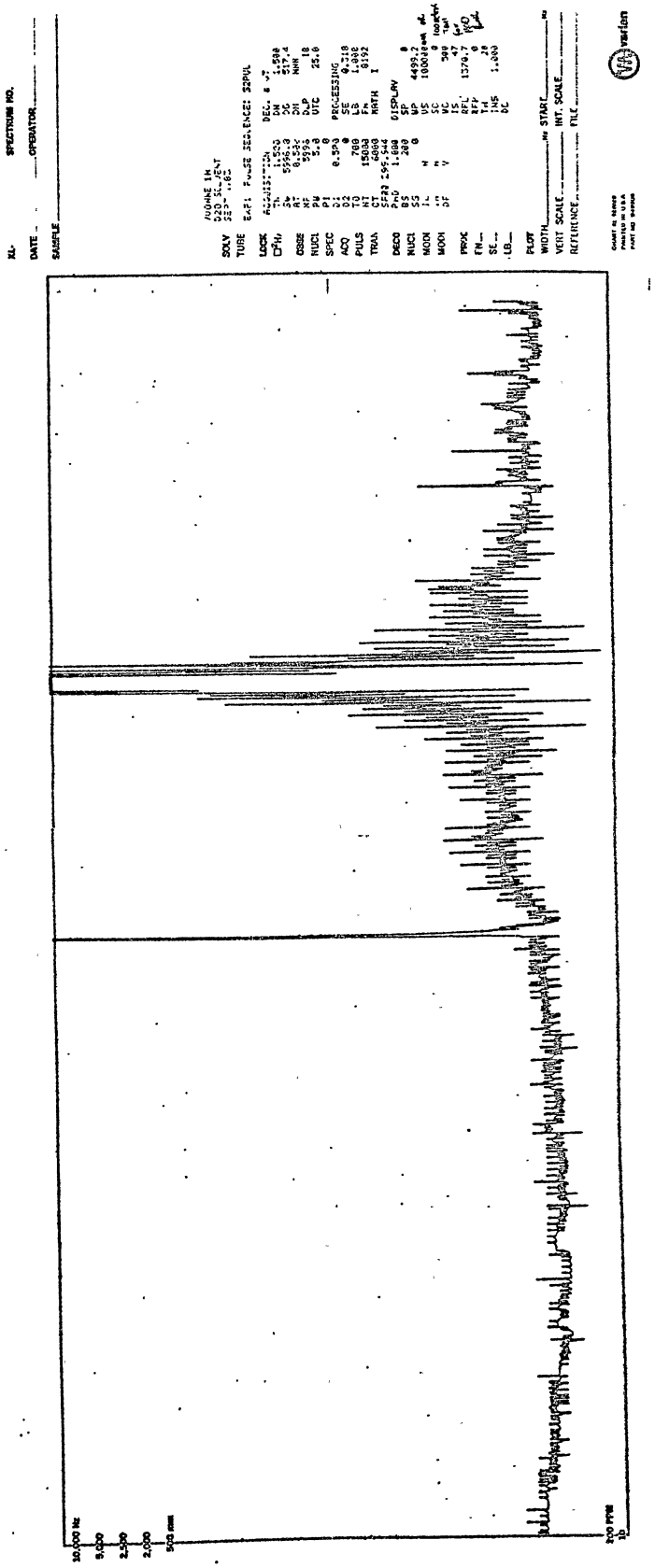

XL
SPECTRUM NO.
DATE
OPERATOR
SAMP
VNMRS 1H
D2O SOLVENT
AUG 29,05
EXP1 PULSE SEQUENCE: SEPUL
ACQUISITION
PROCESSING
DISPLAY
MODE
MODULATION MODE
PROCESSING
PLOT
WIDTH
VERT. SCALE
REFERENCE
10,000 Hz
5,000
2,500
2,000
500 mm
2x0 PPM
1.0
Varian

Figure 8. Proton-NMR Spectrum Taken in a Microtube.


XL-
SPECTRUM NO.
DATE
OPERATOR
SAMPLE
JOHNNIE LIN
ZOLTAN SZALAY
239-1165
SOLV
TURE
EXPL PULSE SEQUENCE: S2PUL
LOCK
CPW
DEC. 0 0"
DM
DMM
DOSSE
DMF
NUCL
SPEC
PROCESSING
ACQ
PULS
TRAN
DISPLAY
NUCL
MODE
PROC
PLOT
WIDTH
VERT SCALE
INT. SCALE
REFERENCE
FILE
varian
10,000 Hz
5,000
2,500
2,000
500 mm
200 PPM
0

that no derivative was obtained (Figure 9). The spot corresponding to the unknown on the TLC plate was not yellow, but the 2,4-dinitrophenylhydrazone derivatives have yellow colors (31).

Also this compound was reacted with diazomethane, with a negative result being obtained, since there was no bubbling due to evolution of nitrogen. The lack of obtaining a derivative for treatment with 2,4-dinitrophenylhydrazine and diazomethane is taken as evidence that the unknown lacks a carboxylic acid and ketone group.

B. High Resolution Mass Spectrometry and Nuclear Magnetic Resonance Spectra

The high resolution mass spectrum using the CEC-21-110B in the electron impact operational method, obtained through the courtesy of Thomas Dorsey of the NIH-sponsored Biotechnology Research Resource for Mass Spectrometry, Massachusetts Institute of Technology, indicated that the molecular ion peak of the unknown was 157.04874, corresponding to the moelcular formula $C_5H_7N_3O_3$. In Table VII are recorded the absolute abundances of the ions, the ion peaks, the elementary compositions, and the possible fragments lost. (The complete data are reported in the Appendix.) Dr. Henrianna Pang, of Massachusetts Institute of Technology, NIH-sponsored BRRMS facility reported negative results with the unknown compound using the fast atom bombardment source.

From the mass spectrum it could be observed that no nitrogen was lost in the fragmentation peaks obtained which were not taken lower than m/z 69. The molecular ion $M^{+\cdot}$ 157, $C_5H_7N_3O_3$, may lose a hydroxy group, a common occurrence with alcohols, to form the fragment ion at

Figure 9. Drawing of the Autoradiochromatogram of the Supposed Product of Reaction of the Unknown Compound with 2,4-Dinitrophenylhydrazine Viewed Under $UV_{254\ nm}$.

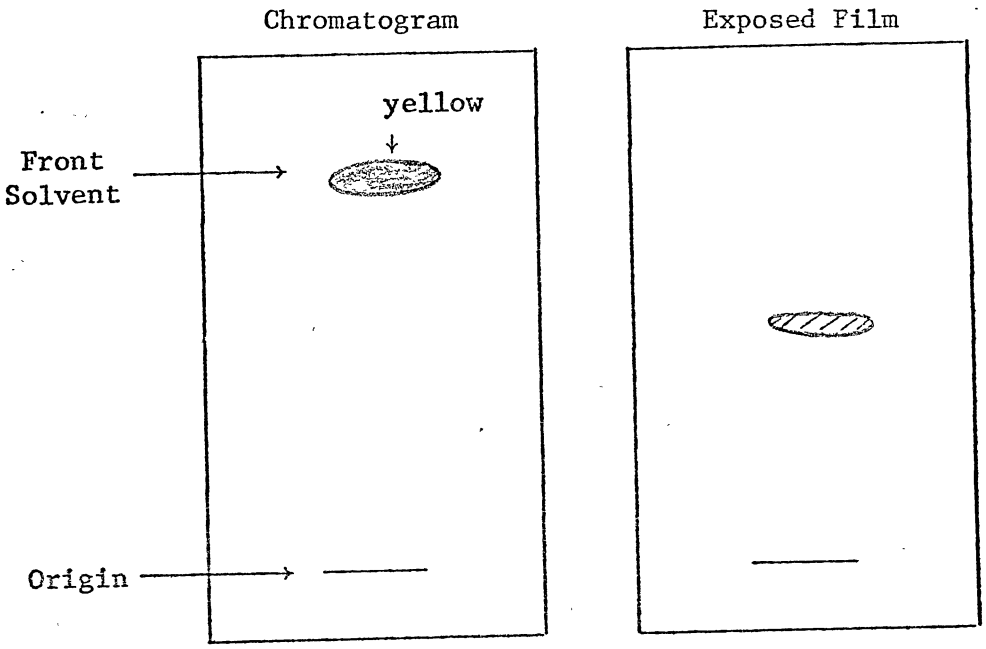

Chromatogram
Exposed Film
yellow
Front
Solvent
Origin

TABLE VII

ELEMENTARY FRAGMENTATION OF UNKNOWN BY ELECTRON IMPACT MASS SPECTROMETRY

Abundance	m/z	Elementary Composition				Ion Peak	Fragment Lost
		C	H	N	O		
405	157	5	7	3	3	$M^{+\cdot}$	
24	156	5	6	3	3	$M^{+\cdot}-1$	-H
179	143	4	5	3	3	$M^{+\cdot}-14$	$-CH_2$
236	140	5	6	3	2	$M^{+\cdot}-17$	-OH
21	129	4	7	3	2	$M^{+\cdot}-28$	-CO
260	127	4	5	3	2	$M^{+\cdot}-30$	$-CH_2O$
551	126	4	4	3	2	$M^{+\cdot}-31$	$-CH_2OH$
609	115	3	5	3	2	$M^{+\cdot}-42$	$-C_2H_2O$
643	99	3	5	3	1	$M^{+\cdot}-30-28$	$-[CH_2O+CO]$
808	98	3	4	3	1	$M^{+\cdot}-30-28-1$	$[CH_2O\_CHO]$
553	85	2	3	3	1	$M^{+\cdot}$ $(C_3H_4O_2)$	—

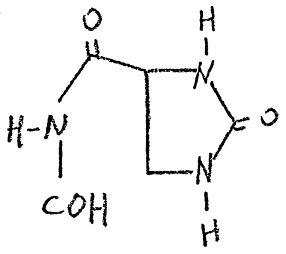
m/z 140. Also it could be observed that CO and CH_2O are lost forming ions at m/z 129 and m/z 127. The ion at m/z is due to the loss of CH_2OH, while ion at m/z 115 shows the loss of a fragment of $-C_2H_2O$. The ions at m/z 99 and 98 show the sequential loss of $-[CH_2O+CO]$ and $-[CH_2O+COH]$. The ion at m/z 85 shows that the initial molecule fragmented to lose $C_2H_3O_2$. This mass spectrum is interpreted to be that the imidazole ring remaining intact through the fragmentation process, but the m/z ion at 85 represents the loss of one carbon from the ring giving a new rearranged ion $C_3H_3N_3O$. These data when combined with the lack of a carbonyl group and NMR give rise to the possible structure shown in Figure 10 with the structure I and II being the most logical based on the mass spectrum.

The proton NMR spectra showed very little about the structure, probably due to the fact that $C_5H_7N_3O_3$ has true hydrogen atoms attached to nitrogen atoms and a hydrogen atom attached to an oxygen (hydroxyl) that will be exchanged with DO to give a deuterium atom on the molecule. The NMR was unable to distinguish between the three other hydrogens.

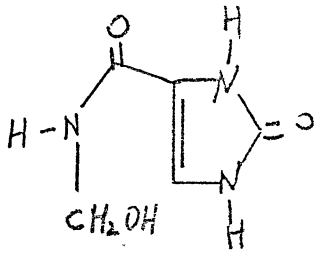
C. Naming of the Metabolite, $C_5H_7N_3O_3$

The nomenclature of these classes of compounds is based on a personal communication with Kurt L. Loening, Director of Nomenclature, Chemical Abstracts Service, American Chemical Society, Columbus, Ohio (32). A survey of the chemical literature was made (33) and no such structure could be found. The compounds are named with the IUPAC rules of organic nomenclature as well as CA practice. Since the radioactivity was retained at position 2 of the imidazolidine ring, it was concluded

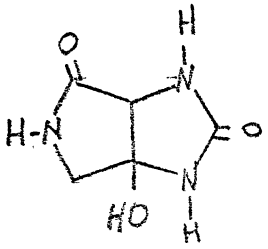
Figure 10. Structures Proposed for the Compound with Molecular Formula $C_5H_7N_3O_3$.



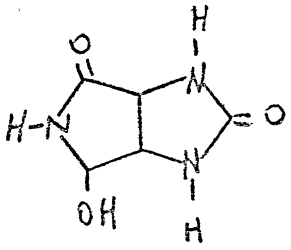
I



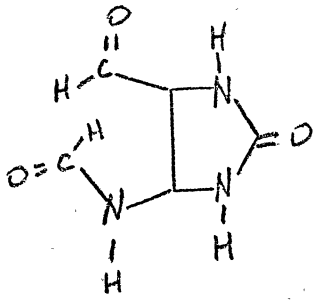
II



III



IV



V

that the coffee plant modified the pyrimidine ring. Uric acid is considered the precursor of the molecule $C_5H_7N_3O_3$. Uric acid, $C_5H_6N_4O_3$ had to lose one nitrogen atom from position 3, Figure 10 to form $C_5H_7N_3O_3$ and the predicted product, on the basis of energy requirements for bond breaking, would produce structure I, N-formyl-2-oxo-4-imidazolidinecarboxamide. Structure II has the name N-hydroxymethyl-2-oxo-4-imidazolidinecarboxamide. Structures III and IV are bicyclic and are probably not formed by the plant. They are a pyrrolo[3,4-d]-imidazole derivative. Compound III is hexahydro-6a-hydroxypyrrolo-[2,3-d]imidazole-2,4-dione and compound IV is named hexahydro-6a-hydroxypyrrolo[3,4-d]imidazole-2,4-dione.

It is possible that the coffee plant could produce $C_5H_7N_3O_3$ that corresponds to loss of nitrogen at position 1, Figure 10, to give the compound with structure V. This compound would be named N-(5 formyl-2-oxo-4-imidazolidinyl)formamide. This structure is unlikely to be correct since it would be predicted to have a different fragmentation pattern.

D. Rationale for the Occurrences of the Unknown

The compound, $C_5H_7N_3O_3$, is a new metabolite of theophylline. Its location in the metabolic pathway of theophylline is based on structures proposed in Figure 11. This places it following uric acid in the biodegradative pathway as is shown in Figure 12.

It is likely that this compound is the beginning of a new pathway for the biodegradation of theophylline and caffeine. The possibility exists that this compound is translocated from the stems into the leaves

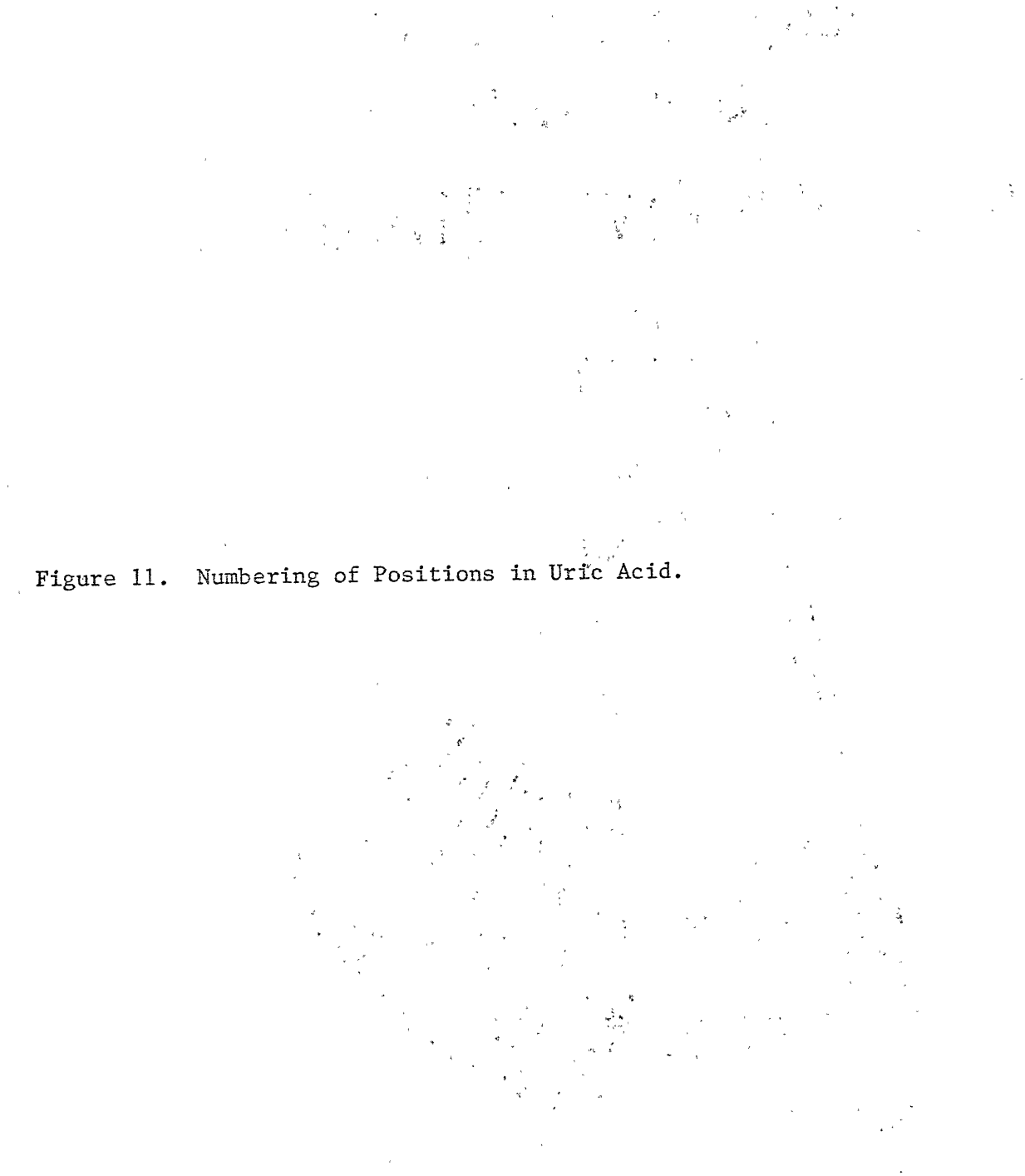


Figure 11. Numbering of Positions in Uric Acid.

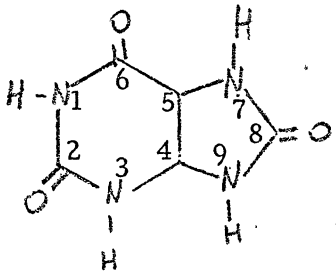
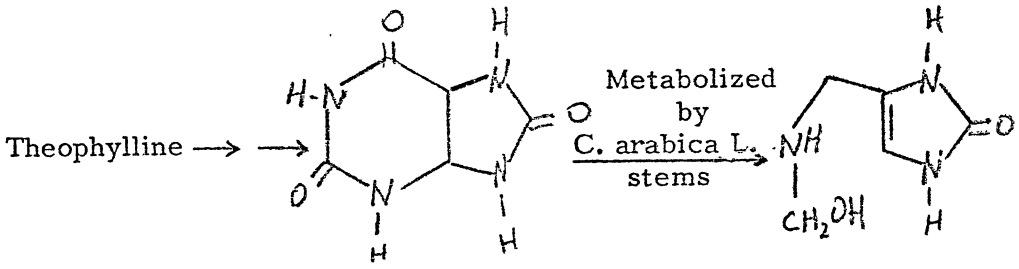

O
H
H - N 1
6
5
N 7
8
= O
2
4
9
O
3
N
N
H
H

Figure 12. Possible Metabolism of Theophylline Producing $C_5H_7N_3O_3$.


Theophylline
Metabolized
by
C. arabica L.
stems
CH2OH

and the fruit; however, none of the other samples taken from 96 to 332 hr showed the presence of such a compound. Serres (24) wrote (34) that no compound other than those reported were found to be produced by uric acid biodegradation in soybeans. This apparent absence is probably due to the fact that the analytical procedure used in the literature report is different from the one used in this work. The fact that it is present only at the 72-hr time after feeding the radioactive theophylline suggests that it may be an intermediate which occurs early (before 72 hr) in the biodegradation of theophylline.

CHAPTER V

SUMMARY AND CONCLUSIONS

In higher plants purines, nucleic acids, nucleosides, nucleotides, and purine alkaloids of cells can be oxidized to xanthine, uric acid and to urea and glyoxylic acid in the process of biodegradation. In most plants xanthine and uric acid are hydrolytically cleaved to glyoxylic acid and urea. Coffee plants contain caffeine, a purine alkaloid, that occurs in leaves, stems, and predominately in fruit. In these plants 7-hydroxyxanthine accepts methyl groups from S-adenosylmethionine to produce caffeine, theobromine, and theophylline. The biodegradation route for caffeine consists of demethylation to give theobromine and theophylline. These dimethylxanthines are again demethylated twice to give xanthine and uric acid. Uric acid is further degraded to glyoxylic acid and urea.

In this study an unknown compound, $C_5H_7O_3N_3$, was isolated as a theophylline metabolite, which was obtained from the aqueous extract and purified by thin-layer chromatography, ion-exchange chromatography and HPLC. The metabolite's absolute structure was determined by high resolution mass spectrometry. In view of the lack of reaction(s) with 2,4-dinitrophenylhydrazic and diazomethane, it was concluded that the new compound had no carbonyl or carboxyl groups present in the unknown. The identification of this compound was not completed due to the small amount of the compound; to finish the identification would require that

the NMR (proton, carbon-13 and nitrogen-15) spectra be taken on a sufficient quantity. It would also be desirable to verify the failures of the dinitrophenylhydrazine and diazomethane reactions.

It is proposed that $C_5H_6N_3O_3$, which most likely has structure I or II shown in Figure 10 (other possible structures are shown in the same figure), is the beginning of a new biodegradative pathway for theophylline.

While it is too early to draw conclusions on $C_5H_7N_3O_3$, it is believed that this compound may be important in Coffea arabica L. plants because:

a) It constitutes a new biodegradation route of theophylline;

b) It is a new ureide which can be biosynthesized from theophylline; and

c) It may be the unknown compound isolated from theophylline reported by Suzuki and Waller (1).

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TABLE VIII

HIGH RESOLUTION MASS SPECTRAL DATA OF THE UNKNOWN*

FILE NAME: R36517 MULTIPLIER OF SIGMA: 2 SLOPE CRITERIA: 15

INT. THIS IS THE STARTING	DEFN. MASS	CALC. MASS TIME: 161241:2	DIF	MAX. HETEROATOMS: TOL	C	C13	H	N	O
65	59.98593	***							
783	70.04147	70.04187	0.4	3.0	4	0	5	0	1
.	.	70.04052	0.9		2	0	4	3	0
453	70.06518	70.06567	0.5		4	0	8	1	0
269	70.07754	70.07825	0.7		5	0	10	0	0
582	71.01474	71.01331	1.4		3	0	3	0	2
.	.	71.01196	2.8		1	0	1	3	1
431	71.05058	71.04969	0.8		4	0	7	0	1
.	.	71.04835	2.2		2	0	5	3	0
57	71.07529	71.07350	1.8		4	0	9	1	0
308	71.08738	71.08508	1.3		5	0	11	0	0
286	72.02047	72.02113	0.7		3	0	4	0	2
.	.	72.01979	0.7		1	0	2	3	1
153	72.05677	72.05752	0.7		4	0	9	0	1
.	.	72.05617	0.6		2	0	5	3	0
278	72.08065	72.08132	0.7		4	0	10	1	0
642	73.03239	73.02896	3.4	6.0	3	0	5	0	2
.	.	73.02761	4.8		1	0	3	3	1
34	73.09181	73.08915	2.7	3.0	4	0	11	1	0
.	.	73.08779	1.0		3	2	11	0	0
41	73.98377	73.98782	4.1	6.0	1	0	0	1	3
143	74.01593	74.01565	0.3	3.0	5	0	2	0	0
21	74.02403	74.02420	0.2		2	0	4	1	2
.	.	74.02286	1.2		0	0	2	4	1
381	74.03653	74.03678	0.1		3	0	5	0	2
.	.	74.03544	1.2		1	0	4	3	1
119	75.02565	75.02348	2.2		5	0	3	0	0
374	75.03313	76.03130	1.8		6	0	4	0	0
365	77.03962	77.03913	0.5		5	0	5	0	0
115	78.04843	78.04695	1.5		6	0	6	0	0
352	79.01771	79.01839	0.7		5	0	3	0	1
.	.	79.01705	0.7		3	0	1	3	0
11	79.04115	79.03952	1.6		2	0	7	0	3
.	.	79.03818	3.0		0	0	5	3	2
.	.	79.03220	1.1		5	0	5	1	0
215	79.05378	79.05478	1.0		4	0	7	0	0
62	79.06843	***							
423	80.02713	80.02622	0.9		5	0	4	0	1
.	.	80.02487	2.2		3	0	2	3	0
48	80.05041	80.05302	0.4		5	0	5	1	0
59	80.06252	80.06260	0.8		6	0	9	0	0
584	80.07473	***							
691	81.03443	81.03408	0.4		5	0	5	0	1
.	.	81.03270	1.7		3	0	3	3	0
317	81.07035	81.07042	0.1		6	0	9	0	0
293	82.03953	82.03187	2.3		5	0	4	0	1
.	.	82.03052	1.0		3	0	4	3	0
.	.	82.02740	2.1		0	1	5	0	1
88	82.06431	82.05567	1.4		5	0	8	1	0
173	82.07684	82.07825	1.4		6	0	10	0	0

INT.	DEFN. MASS	CALC. MASS	DIF	MAX. HETEROATOMS: TOL	C	C13	H	N	O
42	83.03669	83.03711	0.2	3.0	4	0	5	1	1
.	.	83.03577	1.1		2	0	3	4	0
.	.	83.03941	2.5		1	2	3	3	0
95	83.04989	83.04969	0.2		5	0	7	0	1
.	.	83.04835	1.5		3	0	5	3	0
55	83.07344	83.07350	0.1		5	0	9	1	0
321	83.08623	83.08508	0.2		6	0	11	0	0
237	84.04235	84.04494	2.6		4	0	6	1	1
.	.	84.04360	1.2		2	0	4	4	0
.	.	84.04047	1.9		3	1	5	1	1
127	84.05519	84.05752	2.3		5	0	8	0	1
.	.	84.05617	1.0		3	0	5	3	0
746	84.07973	84.08132	1.6		5	0	10	1	0
114	84.09112	84.09390	2.8		6	0	12	0	0
.	.	84.08943	1.7		5	1	11	0	0
553	85.02929	85.02896	0.3		4	0	5	0	2
.	.	85.02761	1.7		2	0	3	3	1
21	85.05199	85.05276	0.8		4	0	7	1	1
.	.	85.05142	0.6		2	0	5	4	0
29	85.06391	85.05534	1.4		5	0	9	0	1
.	.	85.05400	0.1		3	0	7	3	0
509	85.08853	85.08915	0.6		5	0	11	1	0
176	85.10134	85.10173	0.4		6	0	13	0	0
431	85.03677	86.03678	0.0		4	0	6	0	2
.	.	86.03544	1.3		2	0	4	3	1
1020	85.09764	86.09697	0.9		5	0	12	1	0
157	87.04333	87.04461	1.3		4	0	7	0	2
.	.	87.04326	0.1		2	0	5	3	1
435	87.09913	87.10033	1.2		4	1	12	1	0
222	91.05383	91.05478	0.9		7	0	7	0	0
19	92.06072	92.05858	2.1		2	0	8	2	2
.	.	92.05260	1.9		7	0	8	0	0
.	.	92.05913	2.5		5	1	7	0	0
187	93.03303	93.03302	3.0		1	0	5	2	3
.	.	93.03904	1.0		5	0	5	0	1
.	.	93.03270	0.3		4	0	3	3	0
80	93.06941	93.07043	1.0		7	0	7	0	0
7	93.19855	***							
198	94.04095	94.04187	0.9		6	0	6	0	1
.	.	94.04052	0.4		4	0	4	3	0
37	94.07614	94.07825	1.9		7	0	10	0	0
.	.	94.07378	2.6		6	1	8	0	0
13	95.04905	95.04969	0.6		6	0	7	0	1
.	.	95.04835	0.7		4	0	5	3	0
209	95.08565	95.08608	0.4		7	0	11	0	0
88	96.09229	96.09390	1.6		7	0	12	0	0
.	.	96.08943	2.9		6	1	11	0	0
613	97.02881	97.02966	0.1		5	0	5	0	2
.	.	97.02761	1.2		3	0	3	3	1
32	97.05487	97.05534	0.5		6	0	9	0	1
.	.	97.05400	0.9		4	0	7	3	0
136	97.10035	97.10173	1.4		7	0	13	0	0
412	97.27714	***							
809	98.03647	98.03678	0.2		5	0	5	0	2
.	.	98.03548	1.5		3	0	4	3	1
142	98.07295	98.07317	0.2		6	0	10	0	1
.	.	98.07182	1.1		4	0	8	3	0

TABLE VIII (Continued)

INT.	OBSD. MASS	CALC. MASS	DIF	TOL	MAX. HETEROATOMS : C	C13	H	N	O
29	98.09681	98.09697	0.2	3.0	6	.	12	1	.
31	98.10931	98.10955	0.2		7	.	14	.	.
19	98.58117	***							
643	99.04446	99.04461	0.1		5	.	7	.	1
.	.	99.04326	1.2		3	.	5	3	1
75	99.10385	99.10480	0.9		5	.	13	1	.
85	100.11029	100.11262	2.3		5	.	14	1	.
.	.	100.10815	2.1		5	1	13	1	.
23	100.00055	***							
14	101.05933	101.05026	0.9		5	.	9	.	2
.	.	101.05891	0.5		3	.	7	3	1
561	102.03200	102.03170	0.3		4	.	5	.	3
.	.	102.03035	1.5		2	.	4	3	2
.	.	102.03437	2.4		7	.	8	1	.
397	103.03809	103.03352	1.5		4	.	7	.	3
.	.	103.03818	0.1		2	.	5	3	2
.	.	103.03505	3.0		1	1	5	.	3
.	.	103.03773	0.3		6	1	8	1	.
266	104.02548	104.02622	0.7		7	.	8	.	1
.	.	104.02987	0.5		5	.	2	3	.
235	105.03302	105.03804	1.0		7	.	5	.	1
.	.	105.03270	0.3		5	.	3	3	.
129	105.06985	105.07043	0.6		8	.	9	.	.
12	105.51184	213.22184***	1.8		14	.	29	.	1
29	107.05015	107.04969	0.5		7	.	7	.	1
.	.	107.04835	1.8		5	.	5	3	.
19	107.08423	107.08205	2.2		3	.	11	2	2
.	.	107.08608	1.8		8	.	11	.	.
93	109.10015	109.10173	1.5		8	.	13	.	.
376	110.03534	110.03276	2.6		1	.	5	2	4
.	.	110.03678	1.4		6	.	5	.	2
.	.	110.03544	0.1		4	.	4	3	1
15	110.10677	110.10955	2.9		8	.	14	.	.
.	.	110.10508	1.7		7	1	13	.	.
56	111.04203	111.04058	1.4		3	.	7	2	4
.	.	111.04461	2.5		5	.	7	2	2
.	.	111.04326	1.2		4	.	5	3	1
.	.	111.04014	1.9		5	1	5	.	2
45	111.11508	111.11738	2.3		8	.	15	.	.
113	111.97902	111.97966	0.6		4	.	.	.	4
378	112.05247	112.05243	0.3		5	.	9	.	2
.	.	112.05109	1.4		4	.	5	3	1
52	112.11337	112.11262	0.7		7	.	14	1	.
21	112.12387	112.12520	1.3		8	.	15	.	.
145	112.98743	112.98749	0.1		4	.	1	.	4
245	113.02315	113.02387	0.7		5	.	5	.	3
.	.	113.02253	0.6		3	.	3	3	2
518	114.03041	114.03170	1.3		5	.	6	.	3
.	.	114.03335	0.1		3	.	4	3	2
.	.	114.02990	0.5		7	1	3	1	.
603	115.03929	115.03952	0.2		5	.	7	.	3
.	.	115.03818	1.1		3	.	5	3	2
.	.	115.04220	2.9		8	.	5	1	.
335	116.04535	116.04735	2.0		5	.	8	.	3
.	.	115.04600	0.5		3	.	6	3	2
.	.	115.04288	2.5		4	1	7	.	3
.	.	116.04555	0.2		7	1	5	1	.
133	118.08269	***							

INT.	OBSD. MASS	CALC. MASS	DIF	TOL	MAX. HETEROATOMS : C	C13	H	N	O
18	119.08909	119.09205	2.0	3.0	9	.	11	2	2
.	.	119.08608	2.0		9	.	11	.	.
84	119.90123	119.98475	3.5	6.0	5	.	.	.	3
121	121.02835	121.02896	0.1	3.0	7	.	5	.	2
.	.	121.02761	1.2		5	.	3	3	1
85	122.03618	122.03678	0.5		7	.	5	.	2
.	.	122.03544	0.7		5	.	4	3	1
361	122.51552	245.03108**	0.0		10	.	5	4	4
207	122.54019	245.08139**	1.0		14	.	13	.	4
.	.	245.03004**	0.3		12	.	11	3	3
.	.	245.09272**	2.3		15	.	3	4	.
94	122.55719	245.11504**	2.1		13	.	15	3	2
77	123.20453	***							
122	123.98035	123.97966	0.7		5	.	.	.	4
153	125.02299	125.02387	0.9		6	.	5	.	3
.	.	125.02253	0.5		4	.	3	3	2
559	126.03083	126.03170	0.9		6	.	5	.	3
.	.	126.01935	0.5		4	.	4	3	2
33	126.97977	***							
269	127.03853	127.03952	1.0		6	.	7	.	3
.	.	127.03818	0.4		4	.	5	3	2
.	.	127.03773	0.9		8	1	4	1	.
34	128.04615	128.04735	1.2		6	.	9	.	3
.	.	129.04600	0.2		4	.	5	3	2
.	.	128.04555	0.6		8	1	6	1	.
133	128.14305	128.14392	0.9		8	.	13	1	.
21	129.05449	129.05517	0.7		5	.	9	.	3
.	.	129.05383	0.6		4	.	7	3	2
.	.	129.05338	1.1		8	1	5	1	.
25	129.09033	129.09156	1.2		7	.	13	.	2
.	.	129.09021	0.1		5	.	11	3	1
471	129.15233	129.15175	0.6		8	.	12	1	.
90	130.15433	130.15510	0.7		7	1	13	1	.
55	130.20397	***							
187	130.21931	***							
25	133.87715	***							
94	133.03409	131.03949	0.3		5	.	7	.	4
.	.	131.03309	1.0		3	.	5	1	1
.	.	131.03577	1.7		6	.	3	4	.
129	131.98345	131.98475	1.3		7	.	.	.	3
9	137.26624	***							
84	133.04035	139.03942	0.5		7	.	7	.	3
.	.	133.03818	1.9		5	.	5	3	2
.	.	139.04220	2.1		10	.	5	1	.
236	140.04811	140.04735	0.9		7	.	8	.	3
.	.	140.04600	2.1		5	.	5	3	2
.	.	140.05002	1.9		10	.	5	1	.
95	141.05550	141.05517	0.3		7	.	9	.	3
.	.	141.05383	1.7		5	.	7	3	2
.	.	141.05785	2.3		10	.	7	1	.
.	.	141.05791	2.9		.	1	9	4	4
.	.	141.05338	2.1		9	1	5	1	.
179	143.03465	143.03444	0.2		6	.	7	.	4
.	.	143.03309	1.5		4	.	5	3	3
.	.	143.03711	2.5		9	.	5	1	1
.	.	143.03577	1.1		7	.	3	4	.
37	143.98643	143.98475	1.7		8	.	.	.	3

TABLE VIII (Continued)

INT.	EXPT. MASS	CALC. MASS	DIF	TOL	MAX. HETEROATOMS = C13	
269	193.04217	194.04226	0.1	3.0	5	8
		194.04092	1.3		6	5
		194.03994	2.8		9	5
		194.04360	1.4		7	1
45	193.99419	194.99257	1.5		8	1
87	195.04827	195.05009	1.9		5	3
		195.04874	0.5		6	7
		195.04562	2.7		5	1
		195.04829	0.0		9	1
		195.04695	1.3		6	1
85	195.04915	***				
127	149.01097	149.01129	0.4		7	3
		149.00995	0.9		5	1
582	149.02435	149.02387	0.5		8	5
		149.02253	1.8		6	3
		149.02555	2.2		11	3
125	149.97652	***				
173	150.02619	150.02723	1.0		7	1
		150.02588	0.3		5	1
39	150.06799	150.05808	0.1		3	0
		150.05574	1.3		7	3
148	150.98833	***				
23	155.04099	156.04226	1.3		7	0
		156.04092	0.1		5	5
		156.04360	2.6		8	4
403	157.05135	157.05009	1.3		7	2
		157.04874	2.5		5	7
		157.05276	1.4		10	2
		157.05142	0.1		8	5
185	159.05659	158.05791	1.3		7	0
		158.05657	0.0		5	8
		158.05925	2.7		8	5
		158.05612	0.5		9	7
		158.05478	1.8		7	1
45	152.92137	***				
55	155.98382	***				
140	153.04549	168.04498	0.5		11	5
		168.04360	1.9		9	4
71	158.06112	168.05607	5.0	6.0	4	10
		168.05473	3.5		5	3
		168.05752	3.5		12	3
		168.05617	4.9		10	6
43	170.07623	170.07725	1.0	3.0	7	1
		170.07591	0.3		5	1
		170.07546	0.8		8	2
64	176.05159	176.05302	1.5		13	5
415	177.05691	177.05517	1.9		10	8
		177.05785	0.9		13	7
95	178.06475	178.05300	1.8		10	10
		178.06567	0.9		13	8
		178.05574	1.0		8	1
303	185.05723	186.05550	1.7		11	3
65	187.06167	187.05931	2.4		5	8
		187.05333	1.7		11	3
		187.05199	0.3		8	7
		187.05886	2.8		10	1
59	199.97742	***				

INT.	EXPT. MASS	CALC. MASS	DIF	TOL	MAX. HETEROATOMS = C13	
254	222.09319	222.09189	1.3	3.0	15	12
		222.09055	2.6		13	13
67	229.07462	229.07389	0.7		13	11
		229.07255	2.1		11	3
		229.07557	2.9		15	7
81	255.24119	256.24023	1.0		15	32
		256.23889	2.3		14	30
9	259.50475	521.01129**	1.8		30	3
		521.00995**	0.4		35	1
72	255.51194	531.02347**	0.4		44	3
15	281.54421	563.03051**	2.2		37	13
		563.02807**	2.1		46	11
6	313.58315	627.17087**	4.6	6.0	49	22
		627.15231**	4.3		43	21
		627.15097**	5.3		46	19
9	441.23239	441.23039	2.0	3.0	29	31
		441.23307	0.7		32	23
13	462.37183	462.37091	0.9		29	50
		462.35957	2.3		27	43
		462.37359	1.8		32	49
		462.37225	0.4		30	45
15	483.95653	***				
81	502.46463	502.46240	2.2		33	53
50	502.48233	502.48353	1.2		30	54
		502.48219	0.1		28	52
25	514.33915	514.34470	5.5	6.0	35	45
		514.34335	4.2		33	44
		514.33980	4.4		37	42
229	515.46551	515.46521	0.7	3.0	29	61
259	515.51438	515.51517	0.8		31	67
59	559.96941	***				

THIS IS THE ENDING TIME: 15:24:44
THE TOTAL NUMBER OF MASSES: 168
THE NUMBER OF C13 EL. COMP.: 35
THE NUMBER OF PTS WITHOUT EL. COMP : 22

*Spectra were obtained by the courtesy of Dr. Klaus Bieman's Mass Spectrometry Laboratory by Thomas Dorsey of Massachusetts Institute of Technology, Cambridge, Mass., using the NIH-sponsored Biotechnology Research Resource for Mass Spectrometry Facility.

VITA 2

Ivonne Chiquinquirá Ludovic De Romero

Candidate for the Degree of

Master of Science

Thesis: PRODUCTION OF A NEW COMPOUND BY METABOLISM OF THEOPHYLLINE IN COFFEA ARABICA L.

Major Field: Biochemistry

Biographical:

Personal Data: Born in Maracaibo, Venezuela, April 11, 1950, the daughter of Mr. and Mrs. Rafael A. Ludovic.

Education: Graduated from Carlos Soublette High School, Caracas, Venezuela in 1967; received a Licenciate degree in Chemistry from Central University, Caracas, Venezuela, in February 1975, completed requirements for the Master of Science degree at Oklahoma State University, in May, 1984.

Professional Experience: Assistant Professor, University of Carabobo, Department of Chemistry, 1975-1981; Fellow, University of Carabobo, 1981-1982 at the English Language Institute, Oklahoma State University; and the Department of Biochemistry, Oklahoma State University, 1982-1983; Graduate Research Assistant, Department of Biochemistry, September, 1983-December, 1983.