THE CHEMISTRY AND BIOCHEMISTRY OF RICININE AND SOME RELATED PYRIDINE COMPOUNDS

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RICHARD ALLEN HILES
1970

This is to certify that the

thesis entitled

THE CHEMISTRY AND BIOCHEMISTRY OF RICININE

AND SOME RELATED PYRIDINE COMPOUNDS

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Richard Allen Hiles

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of the requirements for

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R. U. Byerrum
Major professor

Date Feb. 19, 1970

ABSTRACT

THE CHEMISTRY AND BIOCHEMISTRY OF RICININE AND SOME RELATED PYRIDINE COMPOUNDS

By Richard Allen Hiles

The role of NAD as a necessary intermediate in the biosynthesis of ricinine from quinolinic acid in Ricinus communis var. Cimarron was evaluated. This evaluation was accomplished through a study of the ability of NAD to cause a dilution of the incorporation of quinolinic acid-6-^{14}C into the alkaloid. When ten times as much NAD as quinolinic acid-^{14}C were co-fed, there was no suppression of ^{14}C incorporation. Instead, there was an elevation in radioactivity incorporated into ricinine from labeled quinolinic acid. Data previously presented in the literature were reinterpreted in light of this new finding. It was concluded that NAD and most probably the pyridine nucleotide cycle are not necessary intermediates in the synthesis of ricinine. This conclusion implies that ricinine and NAD are made from quinolinic acid by separate and more or less independent pathways.

In attempting to elucidate an early intermediate between quinolinic acid and ricinine the following possible precursors were prepared: 3-cyanopicolinic acid (I), 3-amidopicolinic acid (II), 4-hydroxyquinolinic acid (III),

and 3-carboxy-2-pyridone (IV). Important changes were made in the reported synthesis of I. The synthesis of II and III have not been previously reported.

Compounds I through IV were tested for an intermediary role in ricinine biosynthesis by the competitive feeding method and reverse isotope dilution method using quinolinic acid-6-^{14}C as the radioactive source compound. Only IV caused a decrease in the radioactivity incorporated into ricinine from labeled quinolinic acid and became itself radioactive. Synthetic IV-^{14}C was prepared and fed to the plants. The ricinine which was later isolated contained an insignificant amount of radioactivity. It was concluded that I to IV are not precursors of ricinine.

The microbial metabolism of ricinine was also investigated. Three types of soil bacteria which can use ricinine as a sole source of carbon were isolated and tentatively identified as belonging to the mycobacterium-nocardia group. A cell free extract of each of the bacteria was found to cause the conversion of ricinine to ricinine acid. The mechanism of this ricinine nitrilase reaction was shown to be analogous to acid or alkaline catalyzed nitrile hydrolysis. Using whole cells and specifically labelled ricinine-^{14}C, it was determined that carbons 2,6 and 8 were oxidized directly to CO_2, that carbons 7 and 9 also directly formed volatile compounds, and that only carbons 3,4 and 5 were actually used for the synthesis of cellular materials.

Richard Allen Hiles

During attempts to varify the structures of I, II, III and V (a by-product in the synthesis of I shown to be quinolinimide) mass spectrometry was used. It was noted that the parent ion was almost undetectable with I, II and III. The utilization of mass spectrometry for the structural determination of pyridine acids, amides and nitriles was further explored using the following compounds: Nicotinic acid (VI), isonicotinic acid (VII), picolinic acid (VIII), cinchomeronic acid (IX), quinolinic acid (X), dipicolinic acid (XI), nicotinamide (XII), nicotinonitrile (XIII) and methyl 3-amidopyridine-2-carboxylate (XIV). The mass spectrometer was shown to be a powerful tool for the detection of a carboxyl or carboxyl ester group adjacent to the ring nitrogen of pyridine (I, II, III, VIII, X, XI, and XIV). The method could not readily differentiate between carboxyl groups which were located on the β or γ position on the ring (VI, VII, IX, XII, and XIII).

THE CHEMISTRY AND BIOCHEMISTRY OF RICININE AND SOME RELATED PYRIDINE COMPOUNDS

By

Richard Allen Hiles

A THESIS

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

DOCTOR OF PHILOSOPHY

Department of Biochemistry

1970

"The true worth of an experimenter consists in his pursuing not only what he seeks in his experiment, but also what he did not seek."

Claude Bernard

ACKNOWLEDGMENTS

I wish to express my gratitude for the encouragements and consultations of Dr. Richard U. Byerrum during the investigations presented in this thesis. I gratefully acknowledge the assistance of Meade A. Pearlman, David A. VanDyke, Dr. James M. Tiedje and Dr. Peter Hirsch in portions of the microbial studies, and Jack E. Harten and Dr. C. C. Sweeley in procurement of the mass spectra. I would also like to publicly thank my wife, Susie, for her support and sometimes technical assistance over the past four years. The financial assistance from the National Institutes of Health is acknowledged.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

ABBREVIATIONS

NAD	nicotinamide adenine dinucleotide
nicotinic acid	3-pyridinecarboxylic acid
nicotinamide	3-pyridinecarboxamide
quinolinic acid	2,3-pyridinedicarboxylic acid
quinolinic acid anhydride	2,3-pyridinedicarboxylic anhydride
3-cyanopicolinic acid	3-cyanopyridine-2-carboxylic acid
3-amidopicolinic acid	3-amidopyridine-2-carboxylic acid
4-hydroxyquinolinic acid	2,3-dicarboxy-4-pyridone
4-chloroquinolinic acid	4-chloropyridine-2,3-dicarboxylic acid
quinolinimide	pyridine-2,3-imide
2-hydroxynicotinic acid	3-carboxy-2-pyridone
TLC	thin layer chromatography

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THE PLANT ANABOLISM OF RICININE

INTRODUCTION

Numerous studies have shown that quinolinic acid is a common precursor to many of the pyridine ring compounds in plants (1), animals (2) and microorganisms (3). Among these pyridine compounds are NAD and the other pyridine nucleotide cycle intermediates (4), and the alkaloids nicotine (5) and ricinine (6).

The metabolic pathway which causes the extensive modification of quinolinic acid into ricinine (N-methyl-4-methoxy-3-cyano-2-pyridone) has remained obscure. Known are the facts that the pyridine ring of the acid becomes the ring of ricinine (7), that the methyl groups can come from methionine (8), that the cyano carbon can come from the number 3 carboxyl group of nicotinic or quinolinic acid (9, 10), and that it is possible for both the cyano carbon and nitrogen to come from the amide of nicotinamide (7).

In a search for possible ricinine intermediates, T. Robinson (11) reported that an extract from castor beans would cause the conversion of N-methylnicotinonitrile to the corresponding 4- and 6-pyridones. The lack of activity against N-methylnicotinamide, N-methylnicotinic acid, nicotinamide, nicotinonitrile or NAD demonstrated a rather high degree of specificity. The level of enzymatic activity

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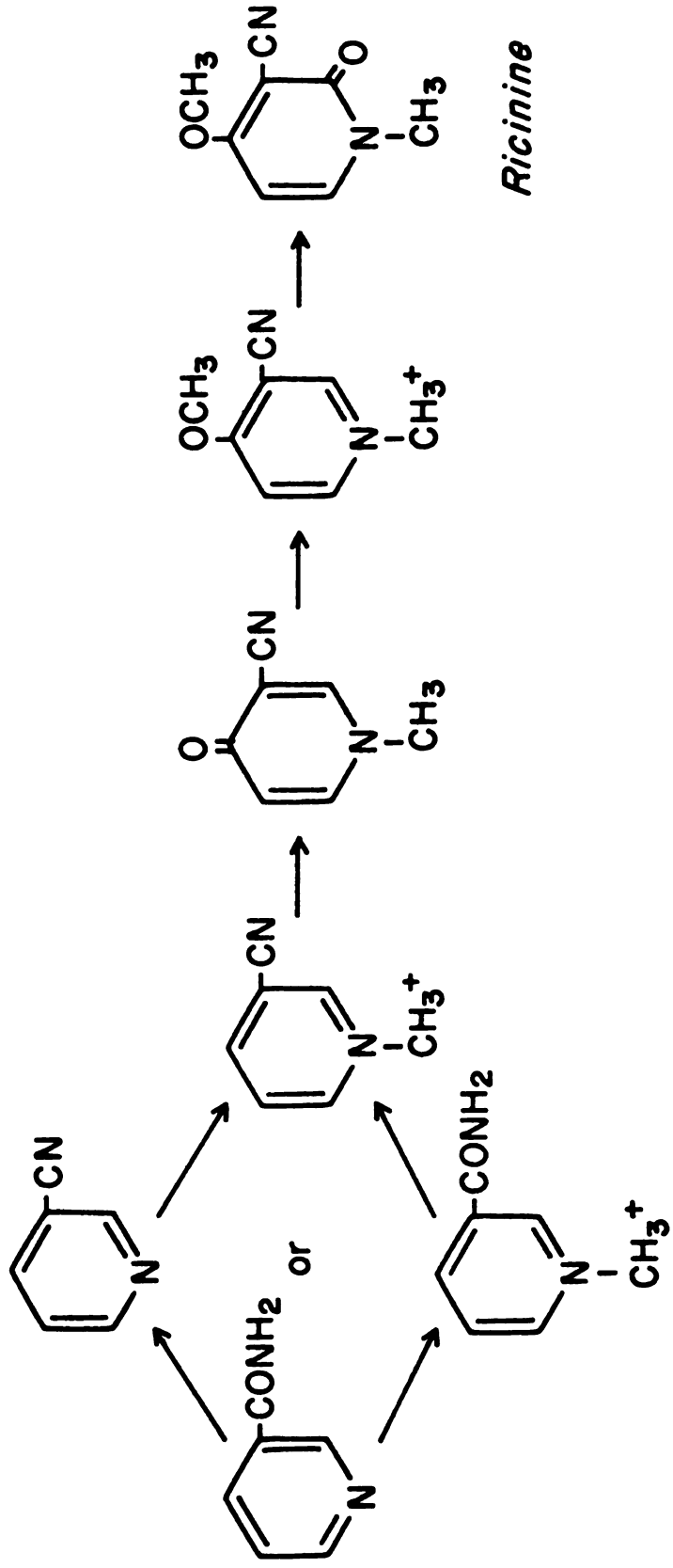
in the extracts was roughly proportional to the level of ricinine accumulation in the plant. The pathway for the biosynthesis of ricinine as presented by the author is in Figure 1.

In strong contradiction to this proposed pathway are the observations that quinolinic acid is a better precursor for ricinine than nicotinamide, and that neither nicotinonitrile or N-methylnicotinonitrile are incorporated into ricinine to a significant extent (12). In additional searches for possible intermediates, the following compounds have also been shown to be less efficient precursors of ricinine than quinolinic acid under optimum condition of quinolinic acid incorporation: Nicotinic acid (13), nicotinamide, N-methylnicotinamide, N-methylnicotinic acid (12), 4-hydroxy-3-cyano-2-pyridone, N-methyl-4-hydroxy-3-cyano-2-pyridone (ricinic acid), N-methyl-4-methoxy-3-amido-2-pyridone, and N-methyl-4-methoxy-3-carboxy-2-pyridone (ricinine acid) (14).

More recent experiments have shown the compound 4-methoxy-3-cyano-2-pyridone (N-demethylricinine) to be an excellent precursor (60% conversion) of ricinine in the green leaves of castor beans (15). Thus, it appears that the addition of the N-methyl could well be the final step in the synthesis of ricinine.

Leete and Leitz (9) first suggested the intermediates of what is now called the pyridine nucleotide cycle

Figure 1. The pathway for the biosynthesis of ricinine as proposed by T. Robinson (11)


CN
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CONH2
or
CONH2
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CH3+
CN
O
N
CH3
OCH3
CN
N
CH3+
OCH3
CN
O
N
CH3
Ricinine

might be involved in the biosynthesis of ricinine. Three studies (6, 12, 16) have now been reported which were interpreted as being in support of the hypothesis that this cycle was a necessary intermediate in the biosynthetic pathway between quinolinic acid and ricinine in the castor bean as well as between quinolinic acid and nicotine in the tobacco plant. This conclusion was based on the relative amounts of radioactivity incorporated into ricinine or into nicotine after feeding labeled quinolinic acid, nicotinic acid mononucleotide, and/or NAD to the plants.

One of the purposes of the experiments described in this chapter was to critically evaluate the role of NAD and the pyridine nucleotide cycle in the biosynthesis of ricinine. This was accomplished by competitive feeding experiments and subsequent comparison of the effects of NAD on the extent of the incorporation of quinolinic acid-6-^{14}C into ricinine in Ricinus communis var. Cimarron. In addition, using this same competitive feeding approach, several previously untested compounds were screened for their possible precursor role in ricinine biosynthesis. Those compounds which gave a positive indication of a precursor role were further evaluated by a direct incorporation experiment.

MATERIALS AND METHODS

Procedures for Organic Analysis

Infrared spectrophotometric analysis was performed using the KBr pellet method. Each pellet consisted of 2 mg of vacuum-$CaSO_4$ dried sample and 410 mg of dry potassium bromide. The samples were run on a Perkin-Elmer 337 Grating Infrared Spectrophotometer using an air blank.

Ultraviolet spectra were determined against a water blank using a Beckman "DK-2" Ratio Recording Spectrophotometer with a 1 cm path length.

Mass spectral analyses were performed by Dr. C.C. Sweeley or Mr. Jack Harten on a LKB 9000 with a direct probe. The ionizing current was 60 uA, and the accelerating voltage was 70 eV. The ion source temperature was normally 290° except for the more volatile samples for which it was lowered to 210°.

Elemental analysis was done on vacuum-$CaSO_4$ dried samples by Spang Microanalytical Laboratory, Ann Arbor, Michigan.

Preparation and Characterization of Organic Compounds

Methyl 3-Carboxypyridine-2-Carboxylate:

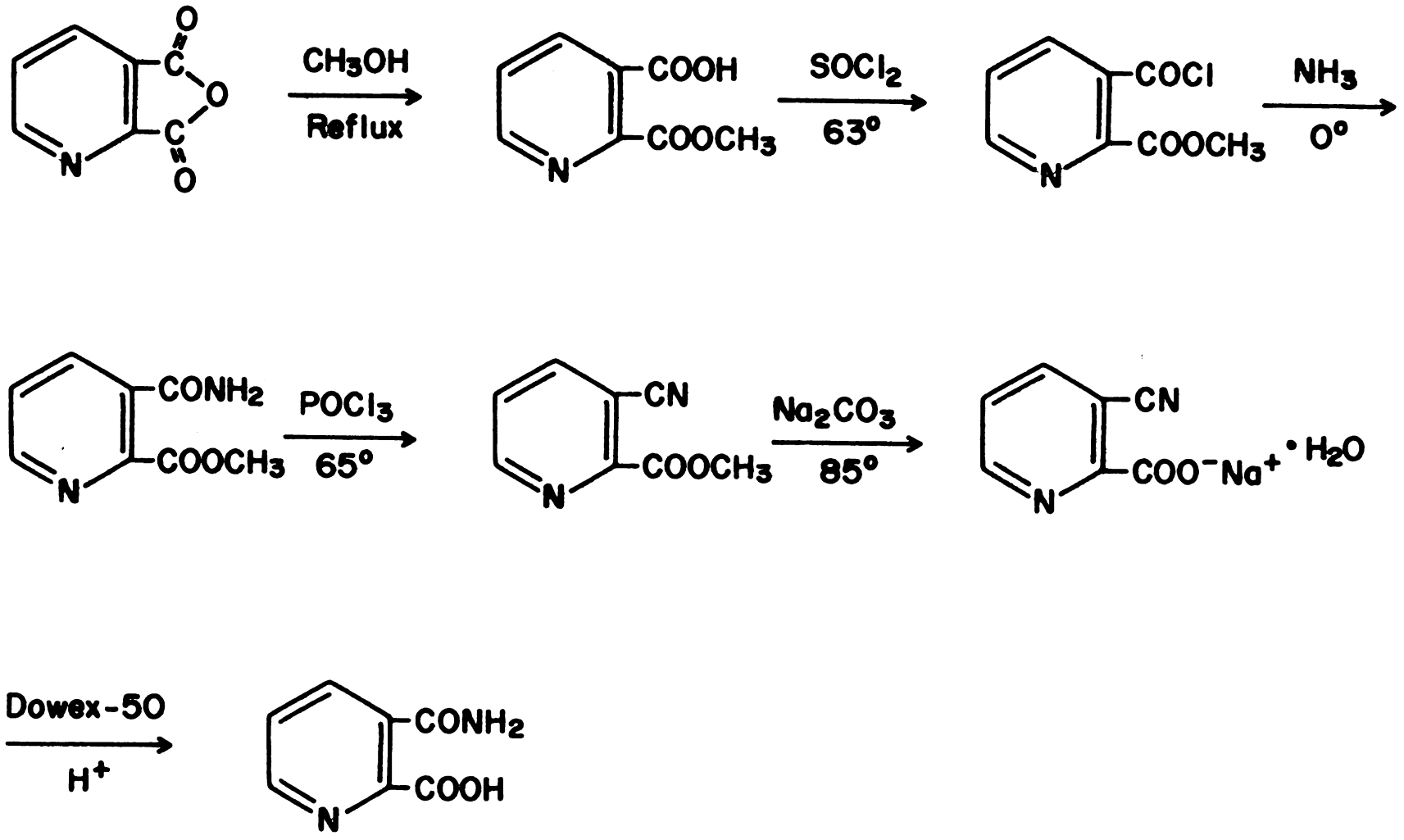
This compound was prepared by an adaptation of the procedure of Kenyon and Thaker (17). Quinolinic acid

anhydride was refluxed on a steam bath with a 4:1 molar ratio of absolute methanol to anhydride for 4 hours. The excess alcohol was removed under reduced pressure and the residue dissolved in ethyl acetate and refluxed on the steam bath for one hour. Slow cooling to room temperature yielded white crystals which after collecting and complete removal of the ethyl acetate melted at 125-126°. Additional crops of the 2-methyl ester were taken until the melting point of the crystals fell below 120° at which point it was considered that there was too high a contamination of the 3-methyl ester for easy isolation. All crystals were purified so as to melt with gas evolution at the reported temperature of 125 to 126°.

Methyl 3-Amidopyridine-2-Carboxylate and Quinolinimide:

The acid chloride of methyl 3-carboxypyridine-2-carboxylate was obtained by warming 4 g of the above finely powdered methyl ester compound with freshly purified thionyl chloride (18, 19, 20) in a flask fitted with a condenser. During the 4 to 6 hours required for complete solution to occur the oil bath temperature was carefully maintained at 62 to 64° with rapid stirring. A $CaSO_4$ drying tube was used at all times while working with the $SOCl_2$ or the acid chloride derivative. The excess thionyl chloride was removed on a rotary evaporator system fitted with a vacuum pump. The syrupy residue was quickly transferred to a one liter Erlenmeyer flask with 200 ml of anhydrous ethyl ether,

Figure 2. The scheme for the synthesis of 3-cyanopicolinic acid and 3-amidopicolinic acid


CH3OH
Reflux
COOH
COOCH3
SOCl2
63°
COCl
COOCH3
NH3
0°
CONH2
COOCH3
POCl3
65°
CN
COOCH3
Na2CO3
85°
CN
COO-Na+ • H2O
Dowex-50
H+
CONH2
COOH
N

dissolved by warming and then cooled to ice bath temperature. In a separate flask, saturated NH_3-ether was prepared by passing ammonia gas through 100 ml of ethyl ether at zero degrees for one hour. The mixing of the two cold ether solutions resulted in the immediate formation of a precipitate which was rapidly removed by filtration in a 19 cm Buchner funnel. The precipitate was rinsed with ether to remove any NH_3 and dried. The NH_4Cl was eliminated by packing the finely ground powder into a small Buchner funnel and sucking 40 ml of ice cold water through it. The methyl 3-amidopyridine-2-carboxylate could be separated from a major impurity (later shown to be the cyclic imide of quinolinic acid) by a carefully watched procedure of fractional crystallization from water. The long, clear plates of the amide melted at 126-128° and after sublimation at 133°. The appearance of small feather like crystals of impurity during the crystallization process indicated the necessity of the rapid collection of the amide plates. After this first crop of crystals, all other attempts to obtain an additional amide compound free from imide impurity failed.

The melting point of the amide was in agreement with reported values (18). However, the elemental analysis more closely resembled that of the monohydrate.

Calculated - no H_2O:	53.34% C	4.47% H	15.55% N
Calculated - 1 H_2O:	48.49% C	5.09% H	14.13% N
Found:	48.67% C	5.03% H	14.04% N

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The large feather like crystals of the impurity found during the amidation of the acid chloride of methyl 3-carboxypyridine-2-carboxylate melted at 241°. There was a broad peak of absorption as seen in Figure 3 at 268 nm ($E^{1\ M}_{1\ cm}$ 2.08 x 10^3). The elemental analysis of the compound was in agreement with the formula $C_7H_4N_2O_2$.

Calculated: 56.76% C 2.72% H 18.91% N

Found: 56.39% C 2.78% H 18.99% N

The infrared spectrum shown in Figure 4A supported a 5 member cyclic imide because of the strong absorption at 1770 and 1700 cm^{-1} and lack of ester characteristics at 1440 to 1435 cm^{-1} and 1250 to 1200 cm^{-1} as compared to the spectrum of methyl 3-amidopicolinate (Figure 4B). The mass spectrum (Figure 20K) with a parent peak at m/e 148 supported $C_7H_4N_2O_2$. The peaks characteristic of esters (M-31 and M-59) and the M-44 typical of amides were absent. From the strong peaks at M-43 perhaps due to a loss of a $\overset{O}{\overset{\|}{C}}$-NH and M-71 from a -$\overset{O}{\overset{\|}{C}}$-NH-$\overset{O}{\overset{\|}{C}}$- loss one must conclude the impurity to be quinolinimide.

Methyl 3-Cyanopyridine-2-Carboxylate:

The dehydration of methyl 3-amidopyridine-2-carboxylate to the corresponding 3-cyano compound was accomplished essentially by the procedure of Fallab and Erlenmeyer (18). Two grams of methyl 3-amidopyridine-2-carboxylate were warmed at 65° with 25 ml of analytical grade $POCl_3$ for 12 hrs in a flask fitted with a condenser and a drying tube.

Figure 3. The ultraviolet spectra of quinolinimide (---) and 3-amidopicolinic acid (——) in water

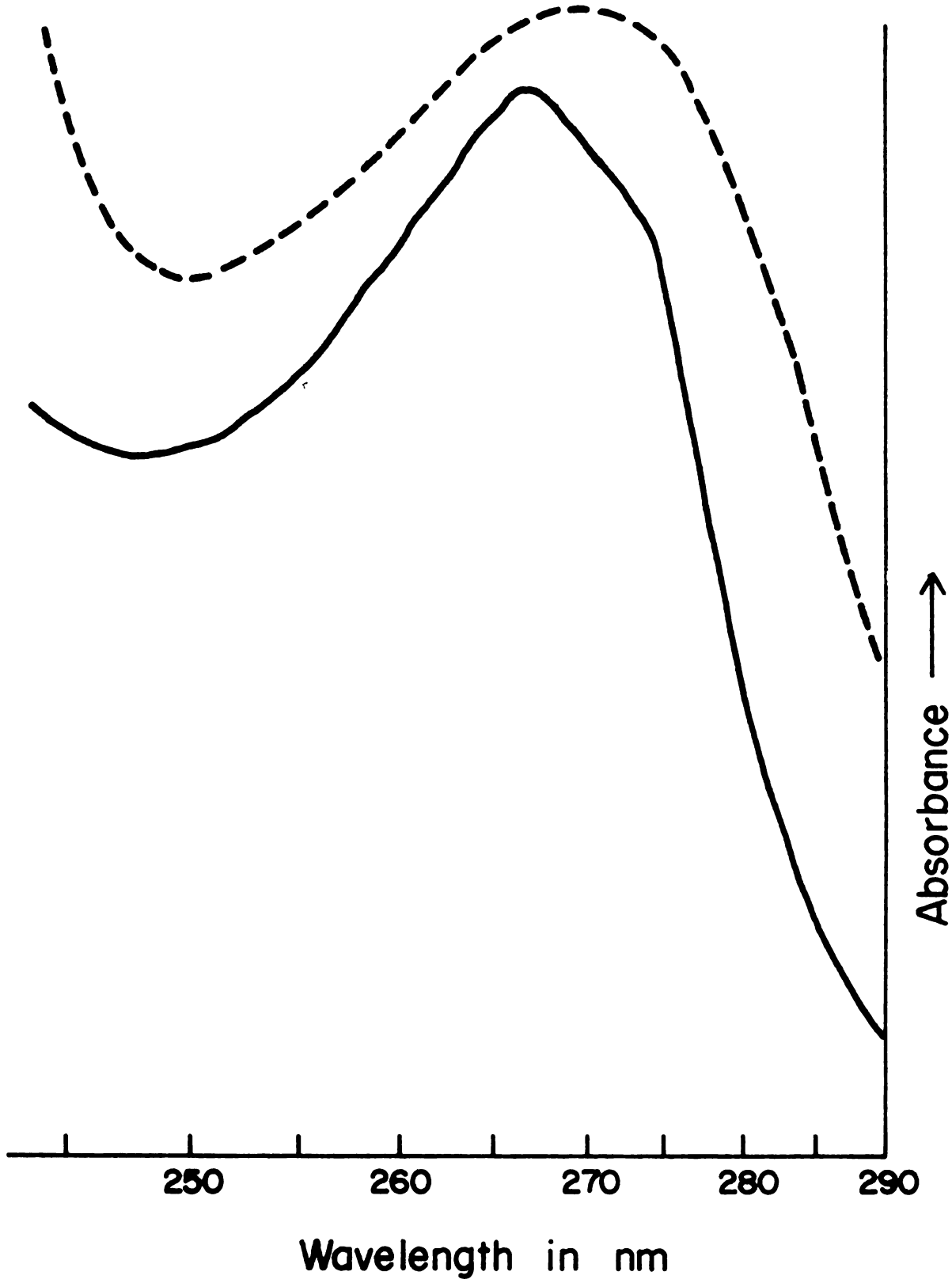
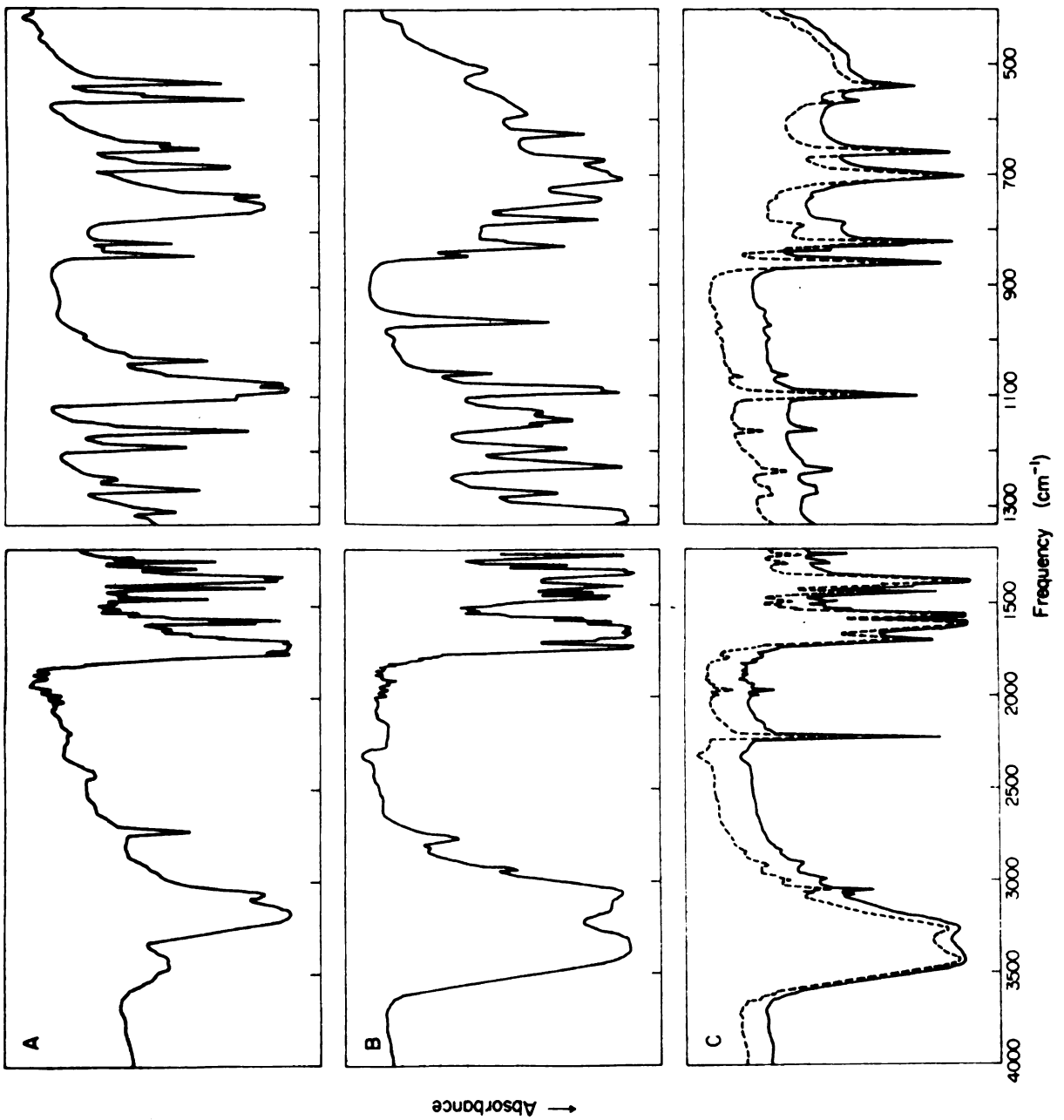

Absorbance
250
260
270
280
290
Wavelength in nm

Figure 4. The KBr pellet infrared spectra of the following compounds:

A. quinolinimide

B. methyl 3-amidopicolinate

C. sodium salt of 3-cyanopicolinic acid monohydrate
m.p. 260-262° (——); m.p. 246-248° (---)


A
B
C
Absorbance
4000
3500
3000
2500
2000
1500
1300
1100
900
700
500
Frequency (cm⁻¹)

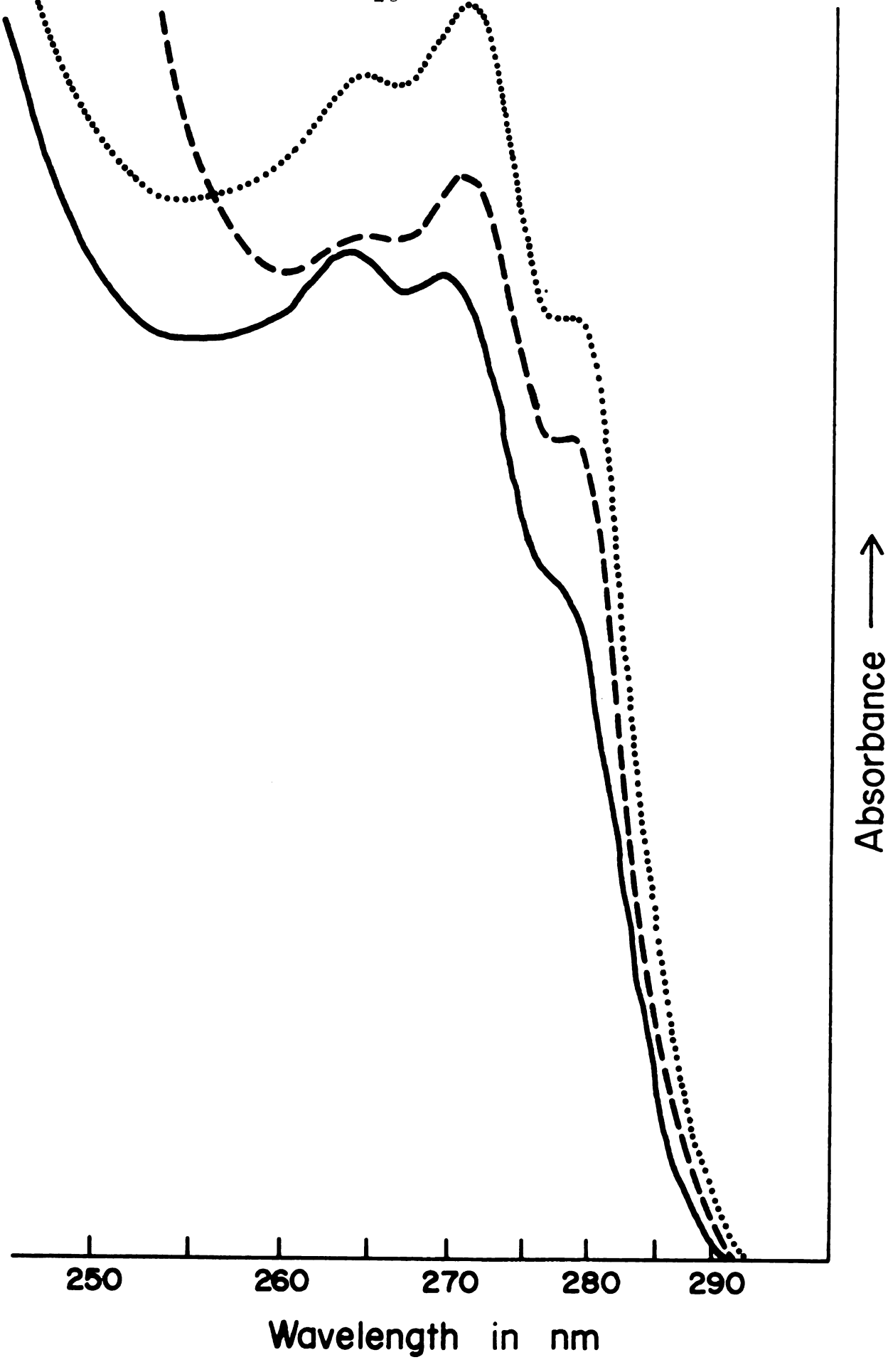
The reaction remained biphasic. After evaporating under reduced pressure the excess $POCl_3$, small portions of water were added so that a complete solution remained when cooled to room temperature and then brought to pH 7 with 2 N Na_2CO_3. The fine needles of precipitate were collected. Recrystallization from water yielded long clear needles which had the reported melting point (150 to 151°) for methyl 3-cyanopyridine-2-carboxylate.

The dehydration reaction could also be successfully performed using a mixture of methyl 3-amidopyridine-2-carboxylate and the previously mentioned quinolinimide impurity. The amount of $POCl_3$ used was calculated assuming the total amount of the finely powdered mixture was all amide compound. On neutralization of the reaction, the majority of the imide remained in the solution while the methyl 3-cyanopyridine-2-carboxylate precipitated. Carefully watched recrystallization from water completed the purification and isolation of the methyl 3-cyanopyridine-2-carboxylate.

3-Cyanopicolinic Acid:

The removal of the ester group from the methyl 3-cyanopyridine-2-carboxylate was accomplished by hydrolysis for 20 min. in 2 N Na_2CO_3 (0.023 ml/mg) at 85° (18). Rapid bubbling of SO_2 through the hot solution brought the reaction to neutrality. At about pH 7 a small amount of solid began to form even while the solution was still quite hot

Figure 5. The ultraviolet spectra of the sodium salt monohydrate of 3-cyanopicolinic acid, m.p. 260-262° (•••), the free acid of 3-cyanopicolinic acid, m.p. 164-164.5° (---) and the methyl ester of 3-cyanopicolinic acid, m.p. 150-151° (——) in water


Absorbance
250
260
270
280
290
Wavelength in nm

and here the neutralization was stopped. The solid platelets which formed at room temperature had a melting point of 260 to 262° in agreement with that reported for the sodium salt of 3-cyanopicolinic acid monohydrate. (The literature reported this neutralization precipitate to be fine needles with m.p. 184° (18).) However, upon recrystallization from water a new melting point of 246 to 248° was observed. The infrared spectra of the two compounds (Figure 4C) were superimposable. Elemental analysis of the compounds showed them both to be in agreement with the monohydrated sodium salt of 3-cyanopicolinic acid.

Calculated:	44.69% C	2.68% H	14.89% N
Found - m.p. 246:	44.83% C	2.83% H	14.41% N
Found - m.p. 260:	44.39% C	2.62% H	14.03% N

The extinction coefficients were $E_1^{1\%}{}_{cm}^{M}$ 1.61 x 10^3, 2.06 x 10^3, and 1.95 x 10^3 at 278 nm, 270 nm and 264 nm respectively (Figure 5). The sodium salt of 3-cyanopicolinic acid monohydrate thus exists in two allotropic forms as perhaps does the free acid discussed in the next paragraph.

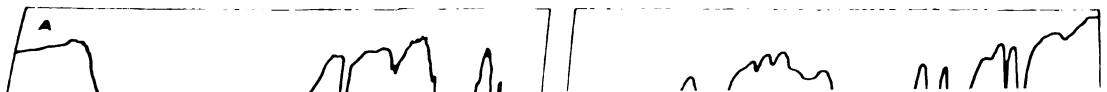
The free acid form of 3-cyanopicolinic acid was obtained by the HCl acidification of a solution of the sodium salt. The fine needle precipitate was collected and recrystallized from hot water--a process which destroyed most of the 3-cyanopicolinic acid. The melting point of 164° was in contrast to the reported 184° (18). As seen in Figure 5 the ultraviolet spectrum of this compound is

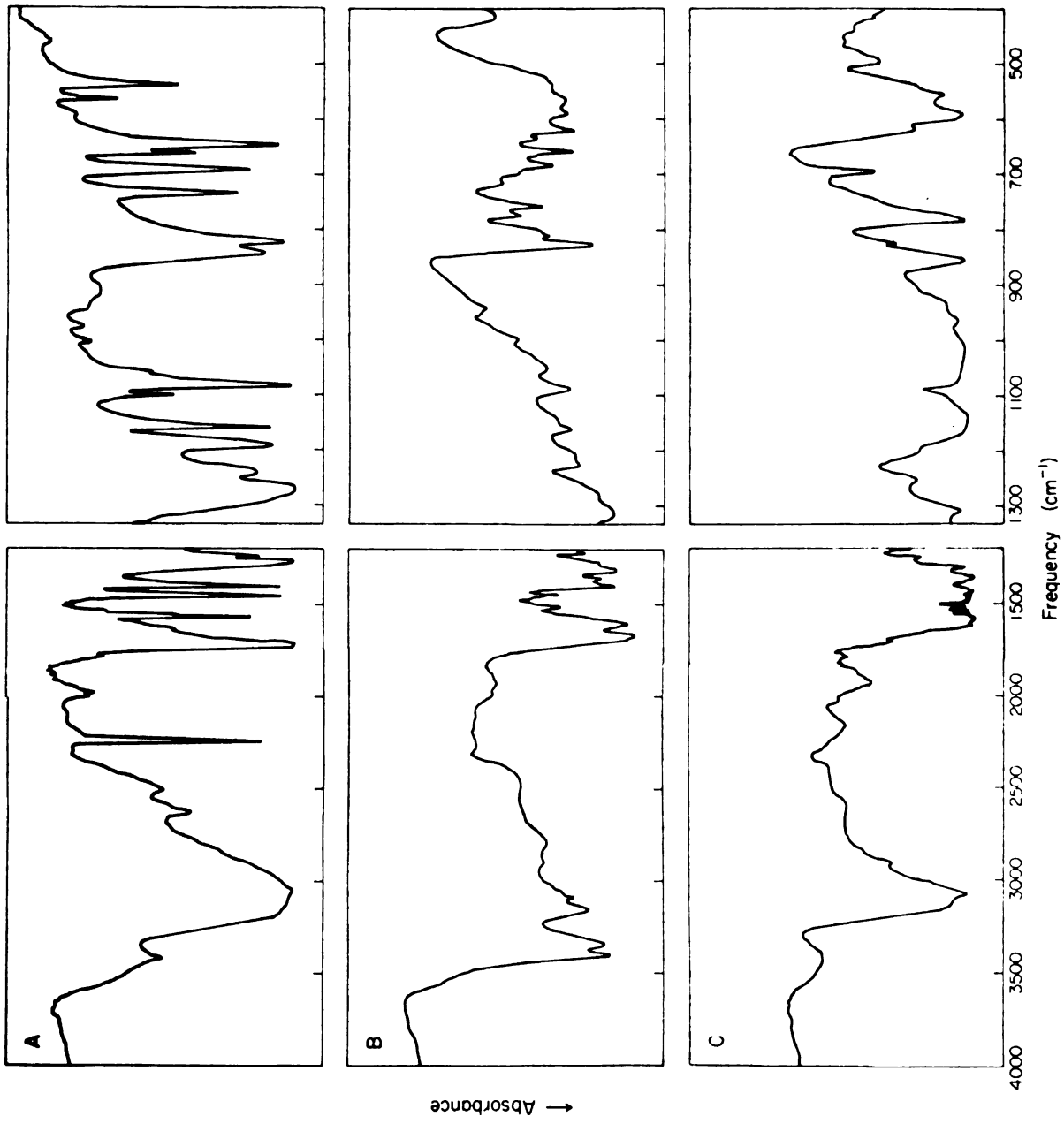
Figure 6. The KBr pellet infrared spectra of the following compounds:

A. 3-cyanopicolinic acid

B. 3-amidopicolinic acid

C. 4-hydroxyquinolinic acid




A
B
C
Absorbance
4000
3500
3000
2500
2000
1500
1300
1100
900
700
500
Frequency (cm⁻¹)

almost identical to that of the sodium salt and similar to the methyl ester. In addition, the elemental analysis is in agreement with the compound 3-cyanopicolinic acid ($C_7H_4N_2O_2$).

Calculated:	56.76% C	2.72% H	18.91% N
Found:	56.65% C	2.90% H	18.93% N

The mass spectrum (Figure 20M) as discussed later also supports a pyridine nitrile with an alpha carboxylic acid. The infrared spectrum is seen in Figure 6A. It should be noted that the free acid was extremely unstable in solution under acidic conditions. Chromatographic properties are found in Table 4.

3-Amidopicolinic Acid:

One gram of the sodium salt of 3-cyanopicolinic acid was dissolved in 40 ml of water and passed onto a neutral Dowex-50W x 8 column (30 cm x 2 cm) in the hydrogen form. The column was water washed until no more ultraviolet absorbing material came off as assayed at 265 nm. The water wash was evaporated to dryness under reduced pressure and the residue crystallized from water.

The feather like white product melted at 159° with gas evolution. An $E^{1\%}_{1\,cm}$ 3.50×10^3 was calculated for the λ_{max} of 266.5 nm (Figure 3). As seen in the infrared spectrum there is support for the complete loss of CN (no absorption at 2250 to 2200 cm^{-1}) while there is absorption at the amide areas of 3340 cm^{-1}, 3150 cm^{-1}, 1660 cm^{-1}, and

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1600 cm^{-1} (Figure 6B). The elemental analysis supported the formula $C_7H_6N_2O_3$ of 3-amidopicolinic acid.

Calculated: 50.61% C 3.64% H 16.86% N

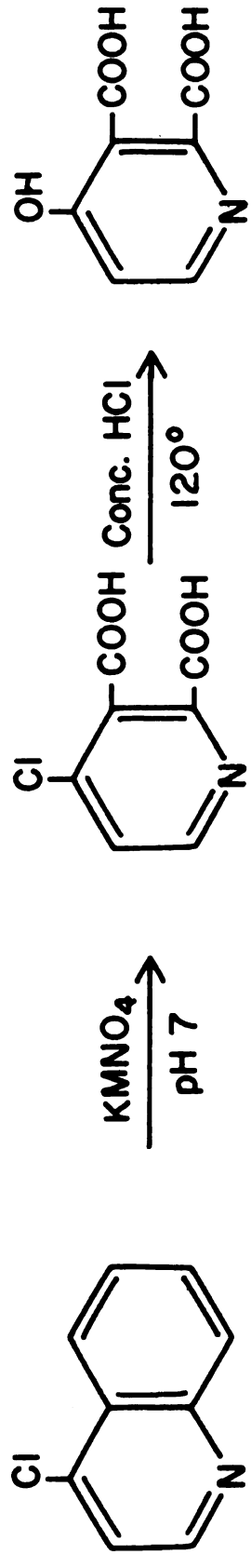
Found: 50.59% C 3.59% H 17.07% N

The mass spectrum of the compound as discussed in more detail later supports a pyridine amide with an alpha carboxylic acid (Figure 20J). The R_f values are found in Table 4.

4-Chloroquinolinic Acid:

The intermediate, 4-chloroquinolinic acid was obtained essentially by the procedure of Späth and Koller (21). Fifteen grams of 4-chloroquinoline were oxidized under refluxing conditions with 3 liters of 4 per cent $KMnO_4$. A stream of CO_2 was passed through the solution to keep the reaction neutral, but slow enough so that no visible vapors of 4-chloroquinoline were carried out the top of the condenser. Two thirds of the permanganate were added in 50 ml portions during the first hour and the remainder during the second hour. After stopping the reaction with ethanol, the solution was hot filtered and made strongly basic with KOH. Steam was passed through the solution until no more ultraviolet absorbing material passed over as assayed at 282 nm. The solution was filtered clear, the pH adjusted to two with HCl and evaporated to dryness. To assure complete dryness the residue was twice suspended in absolute alcohol and evaporated to dryness. This

Figure 7. The scheme for the preparation of 4-hydroxyquinolinic acid


Cl
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material was then extracted five times with 500 ml of absolute ethanol and the combined extracts evaporated to a syrup. After dissolving in hot water and cooling, small little clumps of needles slowly formed. These yellowish to brown crystals were redissolved in hot water and decolorized with activated charcoal. The recrystallized 4-chloroquinolinic acid melted at 173° with gas evolution and a color change to yellowish-brown.

4-Hydroxyquinolinic Acid:

The refluxing of 400 mg of the 4-chloroquinolinic acid in concentrated HCl at 120 to 122° for a period of five hours caused the chloro group to be replaced by a hydroxyl group. After cooling the reaction, 2 ml of water were added and the solution evaporated to dryness. Water addition and evaporation were repeated. Finally 3 ml of water were added and the pH adjusted to 5 or 6 with NaOH in order to achieve complete solution. Lowering to pH 2 with HCl and cooling caused a solid to precipitate. This solid was recrystallized from water.

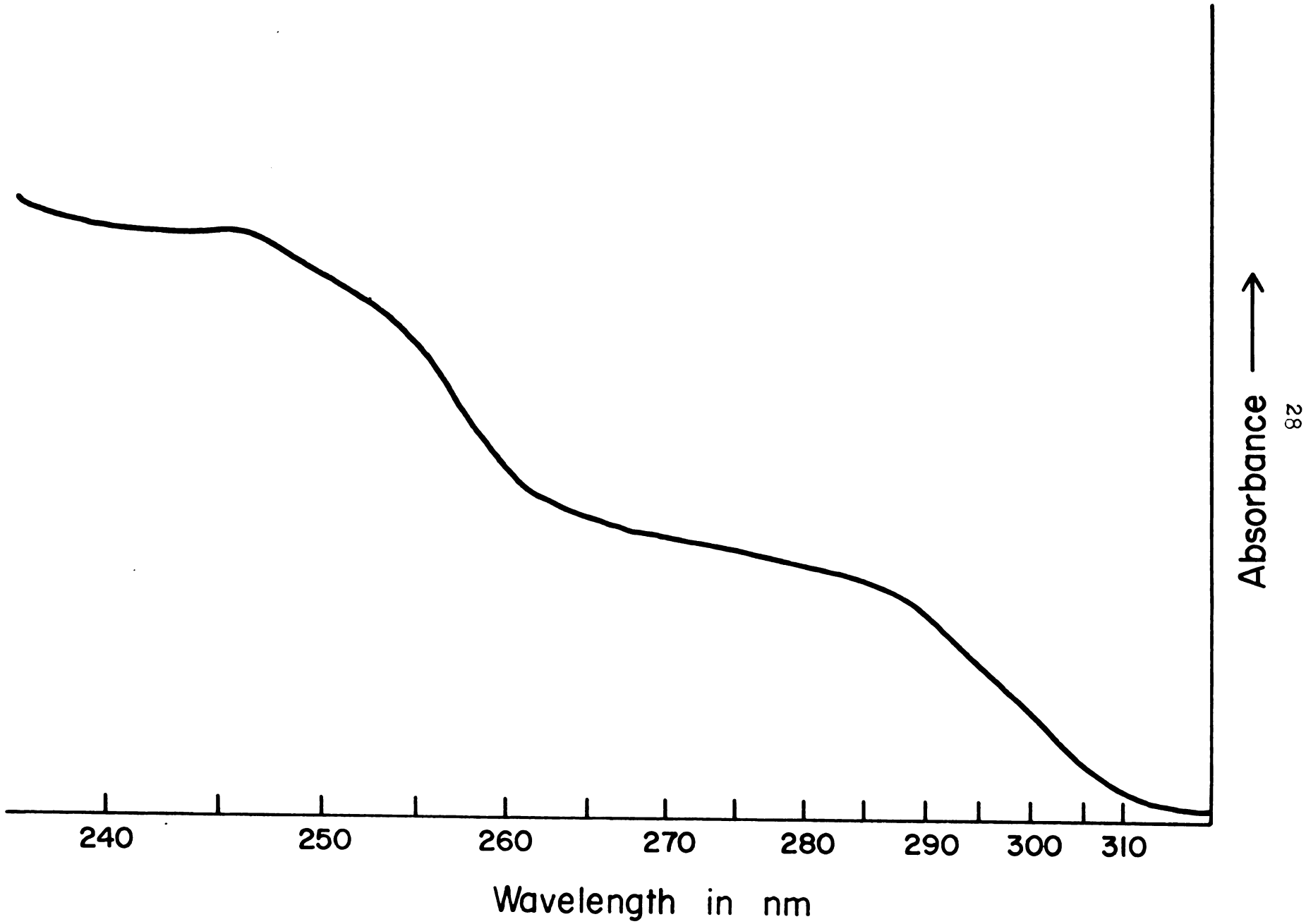
The yellowish prism-like crystals melted with decomposition and gas evolution at 256 to 258°. Elemental analysis was in agreement with the formula $C_7H_5NO_5$.

Calculated: 45.92% C 2.75% H 7.65% N

Found: 45.75% C 2.48% H 7.48% N

The mass spectrum (Figure 20G) was of little assistance in identification of the compound due to an extremely low

Figure 8. The ultraviolet absorption spectrum of 4-hydroxyquinolinic acid in water


Absorbance
240
250
260
270
280
290
300
310
Wavelength in nm

amount of the parent ion expected at m/e 183. The infrared spectrum shown in Figure 6C supports a carboxylic acid with the broad absorption band at 3250 to 2900 cm^{-1} and an aromatic hydroxyl with the weak band at 3600 to 3300 cm^{-1}. The ultraviolet extinction coefficients (Figure 8) calculated for the inflection points at 275 nm and 245 nm were $E_1^{1\%}{}_{cm}$ 3.37 x 10^3 and $E_1^{1\%}{}_{cm}$ 7.53 x 10^3 respectively. The chromatographic properties are given in Table 4.

Preparation of 3-Carboxy-2-Pyridone-7-^{14}C:

3-Carboxy-2-pyridone-7-^{14}C was prepared in low yield from nicotinamide-7-^{14}C by an adaptation of the procedure of Taylor and Crovetti (22). To 20 mg of radioactive nicotinamide was added 10 ml of acetic acid and 2 ml of H_2O_2 (30%). This mixture was heated 4 hours on a steam bath. The reactants were removed and the solid residue of pyridine-N-oxide dried overnight in the round bottom flask. To this oxide was added 2 g of PCl_5 and 2.7 ml of $POCl_3$ and refluxed at 115 to 120° for 1.5 hr. The fluid was removed by evaporation, ice water added and the pH adjusted to 10. The 2-Cl-3-cyanopyridine was then extracted into anhydrous ether. After removal of the ether, 10 ml of concentrated HCl were added and the mixture heated for 5 hrs. at 130 to 140°. The cooled solution was diluted and the pH adjusted to 7 before application to a 25 cm x 2.5 cm Dowex-1 x 8 formate column. The fractions containing the 3-carboxy-2-pyridone (similar to Figure 9D) were pooled

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and taken to dryness. The 3-carboxy-2-pyridone-7-^{14}C was crystallized from water and chromatographically found to be 97% pure through the solvent systems of methanol/H_2O/ acetone/HCl 60:10:20:5 and isobutyric acid/NH_4OH/H_2O 60:1:10 with Silica F_{254} TLC and ethanol (95%)/H_2O 83:17 on cellulose TLC. The impurity corresponded to the positions of nicotinic acid.

Preparation and Feeding of Plants

Castor bean seeds (Ricinus communis var. Cimarron) were washed with a 1.3 per cent solution of NaOCl (Chlorox) and each seed placed approximately one half inch beneath sterilized wet vermiculite contained in 150 ml beakers. The beakers were placed in covered enamel pots and germinated etiolated 6 days at 28°. After this schedule, the vermiculite was removed from around the stem down to the root branch point and the height determined from the stem bottom to the peak of the stem's curvature. Plants not between 4.0 and 5.7 cm or which had split their endosperm so as to expose the cotyledons were not used for feeding experiments (11, 23).

To facilitate injection of the feeding solution, a cavity was made in each plant by running a No. 22 gauge needle down the center of the plant from the top of the stem's curvature toward the roots. Care was taken to insure that the needle did not cause an opening in the side or bottom of the plant. The needle was withdrawn and the

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1.7 x 10
5 ul. A

incision point dry mopped (6).

The feeding solutions as described below were injected into the previously prepared stem cavity with a micro syringe. In groups of three plants (unless otherwise stated) each plant was injected with 7 µl of feeding solution at time zero, 40 minutes later 2 µl and in 20 minutes a final 1 µl. Between injections the plants were kept in the dark at room temperature. Ten minutes after the last injection the plants were extracted. Each control plant received a total of 5 µc of quinolinic acid-6-^{14}C (1.7×10^{-7} moles) at a calculated pH of 6.8 as adjusted with NaOH. In the solution containing the 5 µl of quinolinic acid, each experimental plant was given 1.4×10^{-6} moles of NAD or 1.7×10^{-6} moles of 4-hydroxyquinolinic acid, 3-cyanopicolinic acid, 3-amidopicolinic acid or 2-hydroxynicotinic acid all adjusted to pH 6.8.

One study involved the co-feeding of nicotinic acid-7-^{14}C and 3-carboxy-2-pyridone. Here 9 plants were each given 1.7×10^{-7} moles of nicotinic acid and 1.7×10^{-6} moles of the pyridone. The total amount of radioactivity and method of administration was identical to that used when quinolinic acid was fed.

When 3-carboxy-2-pyridone-7-^{14}C was directly tested for incorporation into ricinine, 14 plants were each given 1.7×10^{-7} moles of pyridone containing 1.14×10^{5} cpm in 5 µl. After 24 hr. at 28°C in the dark ricinine was

harvested from the plants.

Extraction and Isolation Procedures

The procedure for obtaining the ricinine or the ricinine and test compound following competitive feeding and reverse isotope dilution experiments varied depending upon which compound was being tested. For the ricinine controls or when the NAD was the tested compound the plants were severed at the branch point of the roots after the feeding period and these aerial portions weighed and then ground in a Waring Blender with approximately 40 g of cold plants to serve as carrier material. The plants were blended twice with 200 ml of hot water and filtered through a course sintered-glass funnel into a suction flask cooled in a water-ice bath. The funnel was rinsed with hot water. After evaporation under reduced pressure to a volume of 100 ml, the solution was extracted three times with 100 ml of ethyl ether. The flocculent interface was kept with the aqueous layer and removed later by fine filtration. The volume was further reduced to 10 ml and placed on a Sephadex G-10 column (4 cm x 110 cm), eluted with water at the rate of 2.4 ml/cm^2/hr and collected in 3 ml fractions. The fractions were assayed for ultraviolet absorption at 260 nm and for radioactivity by counting 0.2 ml of a fraction in 5 ml of Bray's liquid scintillation fluid. The ricinine, as identified by paper chromatography, TLC, ultraviolet and infrared absorption properties, was contained in fractions

475 to 525. These fractions were pooled, evaporated to dryness, transferred to a volumetric flask and diluted to 25 ml. From this solution two 100 µl samples were removed and streaked on two 10 cm by 20 cm Silica F_{254} TLC plates. The plates were developed with a solvent system of $CHCl_3$/ MeOH/NH_4OH (60:10:1) for one hour. The ricinine (R_f 0.50) was completely separated from pigments and nicotinamide (R_f 0.50) was completely separated from pigments and nicotinamide (R_f 0.38). The area corresponding to ricinine was scraped from the plate, ground to a fine powder in an agate mortar and suspended in 15 ml of Cab-O-Sil/toluene liquid scintillation fluid (24). Each sample was counted for six 10 min. periods on a Packard Model 3310 Tri-carb Liquid Scintillation Spectrometer and the total cpm per 100 µl of ricinine solution taken as a measure of the amount of ricinine made by the plants from quinolinic acid during the feeding time.*

When 3-amidopicolinic acid, 4-hydroxyquinolinic acid, or 2-hydroxynicotinic acid was tested, the three plants were again cut at the roots and weighed and then twice blended with 30 ml to 40 ml of hot water in a Servo Omni mixer to which had been added 20 mg of ricinine and cold carrier compound. This extract was course filtered, evaporated to

---

*There was no quenching of the counting efficiency by ricinine up to 1 mg in 5 ml of Bray solution or by up to 200 mg Silica F_{254} in 15 ml of Cab-O-Sil/toluene solution. Both the amounts of ricinine and Silica F_{254} used in each sample were below these values.

70 ml and extracted three times with an equal volume of ether. After fine filtration, the volume was further reduced to 60 ml, the pH adjusted to 7 and the solution divided into two equal parts. One fraction was evaporated to less than 10 ml and applied to a Sephadex G-10 column as previously described for the ricinine determination with the exception that after dilution of the isolated ricinine to 25 ml, two 200 µl samples were run on the TLC instead of two 100 µl samples. The remaining half of each extract was placed on a separate Dowex-1 x 8 formate column (2.3 cm x 34 cm). The elution of the various columns are shown in Figure 9. Column A contained 3-amidopicolinic acid in peak II, column B contained 4-hydroxyquinolinic acid in peak III and column D contained 3-carboxy-2-pyridone in peak II.

When 3-cyanopicolinic acid was tested, the procedure was identical to the above paragraph except that after equal division of the extract solution the portion which was to be used on the Dowex-1 column was first passed through a 10 cm x 2 cm Dowex-50W x 8 column in the hydrogen form in order to convert the 3-cyanopicolinic acid into the 3-amidopicolinic acid. The acid solution was neutralized and then passed onto a Dowex-1 x 8 formate column and eluted as shown in Figure 9C. Peak II contained the 3-cyanopicolinic acid now in the form of 3-amidopicolinic acid.

The appropriate peak from each of the Dowex-1

Figure 9. The elution patterns from a 2.3 cm x 34 cm Dowex-1 x 8 formate column

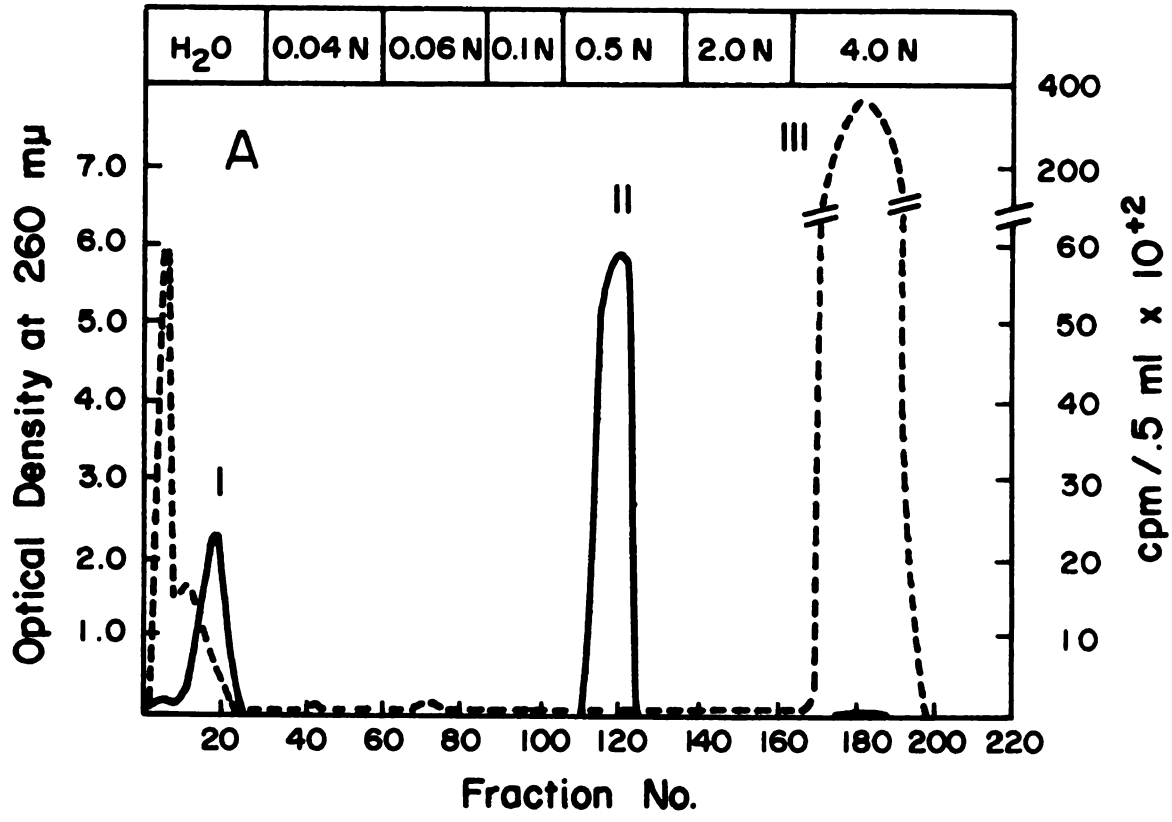
The solid lines (——) represent the ultraviolet absorption and the broken lines (---) the ^{14}C. The fractions were 20 ml each. Each column was eluted with formic acid unless otherwise designated.

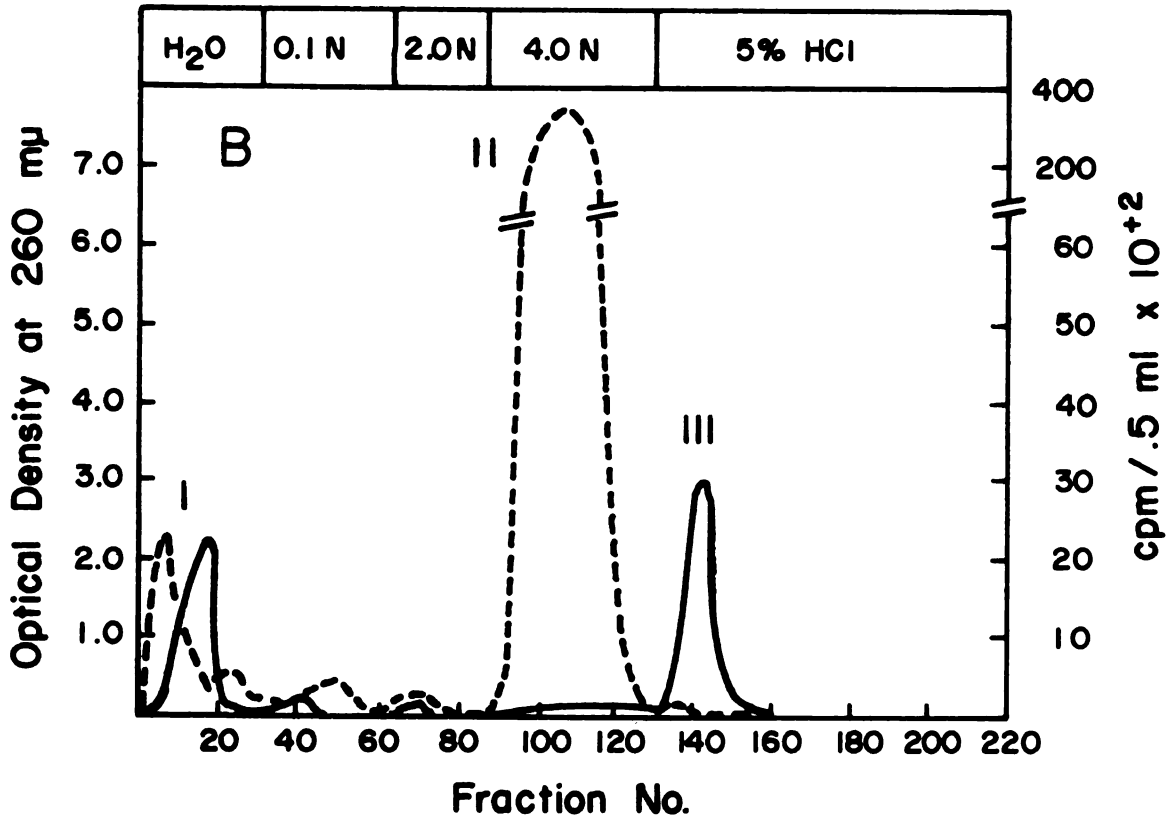
A. the elution pattern when 3-amidopicolinic acid was tested

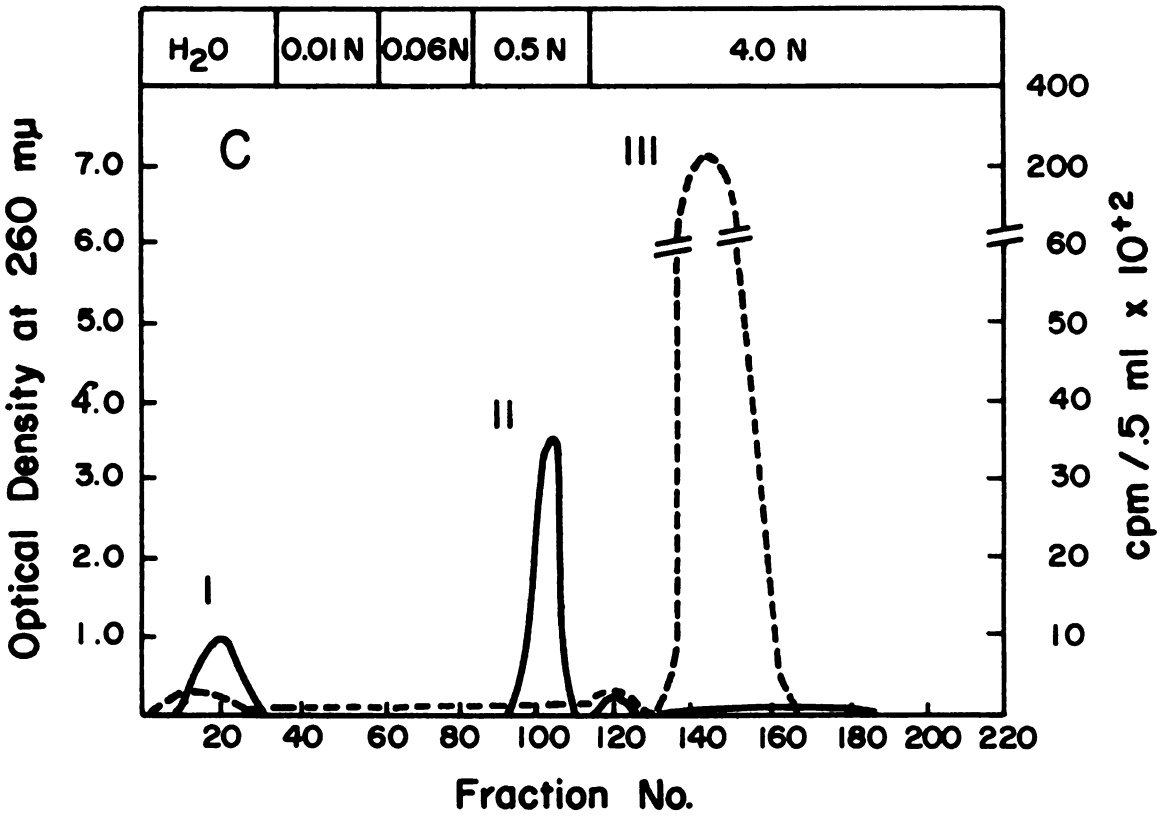
B. when 4-hydroxyquinolinic acid was tested

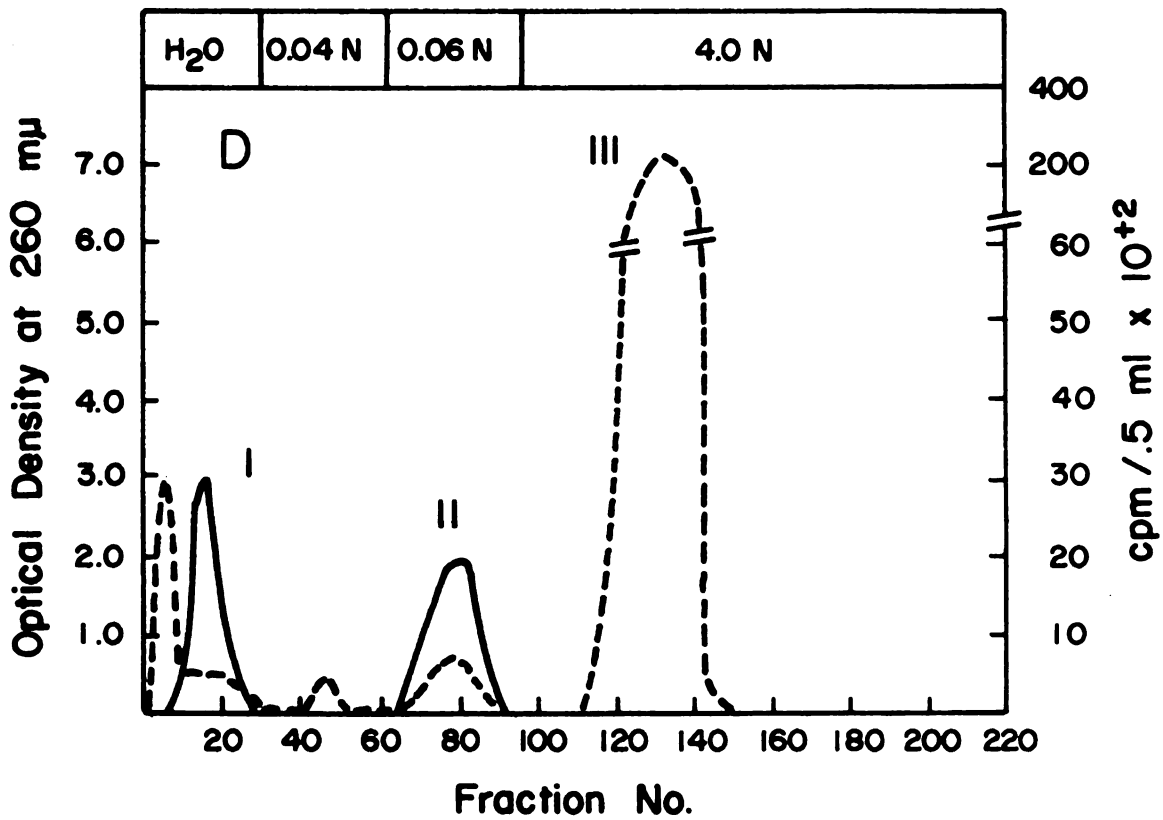
C. when 3-cyanopicolinic acid was tested

D. when 3-carboxy-2-pyridone was tested


H2O
0.04 N
0.06 N
0.1 N
0.5 N
2.0 N
4.0 N
A
I
II
III
Optical Density at 260 mμ
cpm / .5 ml x 10+2
Fraction No.


H2O
0.1 N
2.0 N
4.0 N
5% HCl
B
I
II
III
Optical Density at 260 mμ
cpm / .5 ml x 10+2
Fraction No.


H2O
0.01 N
0.06N
0.5 N
4.0 N
C
I
II
III
Optical Density at 260 mμ
cpm / .5 ml x 10+2
Fraction No.


H2O
0.04 N
0.06 N
4.0 N
D
I
II
III
Optical Density at 260 mμ
cpm / .5 ml x 10+2
Fraction No.

columns was evaporated to dryness under reduced pressure and then diluted in a volumetric flask to 10 ml. Samples were then chromatographed in the solvent systems as given in Table 4 and the radioactive areas detected by autoradiography.

RESULTS

The effects of NAD when co-fed with quinolinic acid-6-^{14}C on the incorporation of radioactivity into ricinine are shown in Table 1. It is obvious that in an approximate 10 fold molar excess of exogenous NAD caused an increase rather than a decrease in the ^{14}C level in the alkaloid.

When co-fed to castor bean plants in a 10 molar excess over quinolinic acid-6-^{14}C only the compounds 3-carboxy-2-pyridone and 3-cyanopicolinic acid caused a decrease in the total radioactivity incorporated into ricinine (Table 2). The compound 4-hydroxyquinolinic acid caused little change in incorporation while the presence of 3-amidopicolinic acid caused an unexplained elevation. However, of the co-fed compounds as isolated from Dowex-1 columns and screened by autoradiography, only the 3-carboxy-2-pyridone showed any incorporation of the radioactivity. Because the amount of autoradiographic exposure seemed weak and because this pyridone chromatographed very close to the heavily exposed area corresponding to nicotinic acid it was decided to more critically evaluate the significance and level of the radioactivity through crystallization. A 7 ml sample and a 3 ml sample from the #1 (see note in Table 2) and #2 quinolinic acid-^{14}C/3-carboxy-2-pyridone co-feedings were added to approximately 50 mg of carrier 3-carboxy-2-pyridone

Table 1.

Feeding Group	
I	
II	
III	
I	
II	
III	
Avg	

*0.17 umol

†0.17 umol
NAD in 10

‡After pur

Table 1. The effect of exogenous NAD on the incorporation of radioactive quinolinic acid into ricinine

Feeding Group	Quinolinic Acid-6-^{14}C Alone*	Quinolinic Acid-6-^{14}C + NAD†	B/A	Average B/A
	A	B		
	Total cpm per 100 µl ricinine solution‡			
I	325	1621	4.9	
II	442	685	1.5	2.9
III	497	1231	2.5	
	Total cpm in the ricinine			
I	71,250	405,250		
II	110,500	171,250		
III	124,250	307,750		
	Avg. 102,000	Avg. 294,750		

*0.17 µmoles in 10 µl solution containing 5 µc per plant.

†0.17 µmoles of quinolinic acid-6-^{14}C (5 µc) + 1.4 µmoles NAD in 10 µl of solution per plant.

‡After purification and dilution to 25 ml.

Table

P
Quinol
3-carb
Quinol
3-carb
Nicoti
3-carb
Quinol
3-cyan
Quinol
3-amid
Quinol
4-OH-q

†All p
other

*A fee
15 uc

‡See p.
ricin

Table 2. The total cpm incorporated into various compounds isolated after competitive feeding experiments

Feeding†	Ricinine‡	Co-Fed Compound	Nicotinic Acid
#1*			
Quinolinic acid-^{14}C + 3-carboxy-2-pyridone	37,500	24,041	97,800
#2			
Quinolinic acid-^{14}C + 3-carboxy-2-pyridone	17,000	37,402	210,350
Nicotinic acid-^{14}C + 3-carboxy-2-pyridone	not determined	16,244	not determined
Quinolinic acid-^{14}C + 3-cyanopicolinic acid	38,500	none	not determined
Quinolinic acid-^{14}C + 3-amidopicolinic acid	238,000	none	not determined
Quinolinic acid-^{14}C + 4-OH-quinolinic acid	79,500	none	not determined

†All plants received the same amount of ^{14}C per group unless otherwise noted (*).

*A feeding in which an unknown amount of less than the normal 15 μc of quinolinic acid-6-^{14}C was fed.

‡See p. 40 for the control levels of incorporation into ricinine (100,000 cpm).

and repeatedly crystallized to a constant specific activity as determined by the ultraviolet absorption in 0.05 N HCl at 322.5 nm and liquid scintillation counting. In addition, the total 3-carboxy-2-pyridone isolated from the competitive feeding experiment with nicotinic acid-7-^{14}C and a control consisting of 10 mg of nicotinic acid-7-^{14}C and 45 mg of 3-carboxy-2-pyridone were also crystallized. The results are shown in Table 3. Because a computation of the total radioactivity in the pyridone indicated a relatively high level of incorporation (Table 3), it was decided to further test the relationship of the 3-carboxy-2-pyridone to ricinine by a direct incorporation experiment. Following the administration of 3-carboxy-2-pyridone-7-^{14}C to castor bean plants and a 24 hr. metabolism period the quantitatively isolated ricinine contained only 3.75×10^3 cpm or 0.2% of the original 1.59×10^6 cpm administered to the plants.

Table 3. The results of the attempts to crystallize the 3-carboxy-2-pyridone obtained from various feeding experiments to a constant specific activity

Sample	Crystallization Specific Activity (cpm/mg 3-carboxy-2-pyridone) and Per Cent of Original Activity				
	Original	First	Second	Third	Final
Fed #1*					
Quinolinic acid-^{14}C + 3-carboxy-2-pyridone	806 cpm/mg 100% (54 mg)	199 cpm/mg 24.6%	177 cpm/mg 22.0%	151 cpm/mg 18.7%	159 cpm/mg 19.6%
Fed #2					
Quinolinic acid-^{14}C + 3-carboxy-2-pyridone	689 cpm/mg 100% (54 mg)	150 cpm/mg 21.8%	117 cpm/mg 17.0%	104 cpm/mg 15.1%	104 cpm/mg 15.1%
Fed					
Nicotinic acid-^{14}C + 3-carboxy-2-pyridone	 100% (62 mg)	583 cpm/mg	278 cpm/mg	258 cpm/mg	266 cpm/mg
Control-Unfed					
Nicotinic acid-^{14}C + 3-carboxy-2-pyridone	45,800 cpm/mg 100% (45 mg)	82 cpm/mg 0.18%	40 cpm/mg 0.09%	37 cpm/mg 0.07%	 0.07%

*A feeding in which an unknown amount of less than the normal 15 uc of quinolinic acid-6-^{14}C was used.

Table 4. The R_f values of some compounds tested for a possible role in the biosynthesis of ricinine

Compound \ Solvent System	A	B	C	D
4-Hydroxyquinolinic acid	0.41	*	0.13	0.18
3-Carboxy-2-pyridone	0.78	*	0.75	*
3-Amidopicolinic acid	0.51	0.50	0.26	0.12
Ricinine	0.88	0.76	0.91	0.71
Quinolinic acid	0.53	*	0.25	0.12
Nicotinic acid	0.81	0.82	0.75	0.74

*Sample streaked excessively.

The solvent systems were:

A. n-Butanol/propionic acid/propanol/H_2O on cellulose TLC 24:18:13:24

B. Methanol/H_2O/acetic acid on Silica F_{254} TLC 70:30:0.5

C. Ethanol (95%)/1 M NH_4 acetate pH 5 on cellulose TLC 75:25

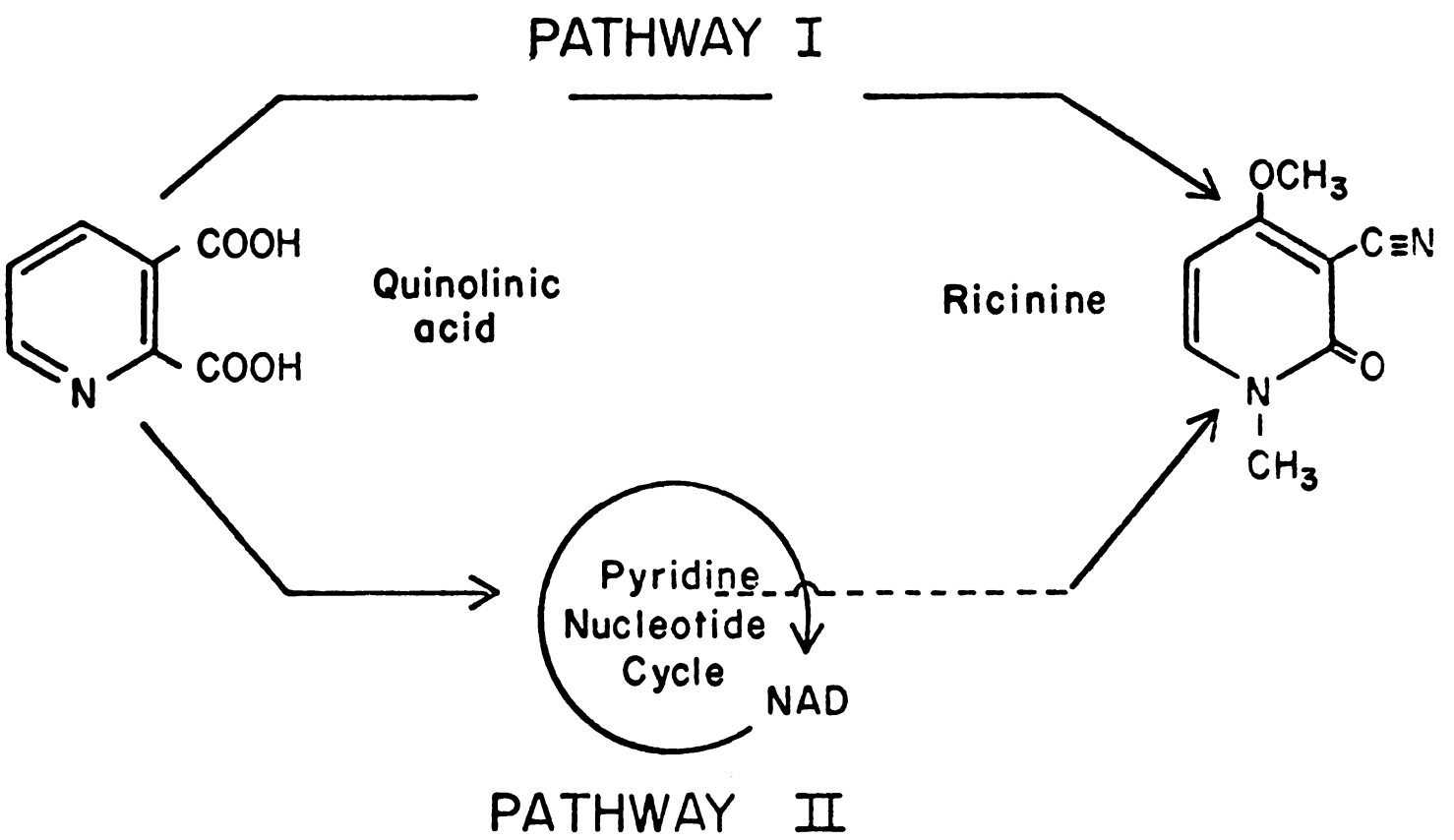
D. Ethanol (95%)/1 M NH_4 acetate pH 5 on Silica F_{254} TLC 75:25

DISCUSSION

The present lack of knowledge concerning the biological role of the alkaloid ricinine in the castor bean plant is in part due to a lack of knowledge of the biosynthetic pathway intermediates and enzymes. Recent experiments which were interpreted to support a compulsory intermediary role for the important compound NAD between quinolinic acid and ricinine required more critical evaluation before any attempts were made to identify unique ricinine intermediates (6, 12, 16).

The method presently best suited for this type of evaluation is the competitive feeding experiment in which two compounds, one of which is radioactive, are pitted against each other for incorporation into the end product. The effects of the presence of NAD on the incorporation of quinolinic acid-^{14}C into ricinine as shown in Table 1 demonstrate, that at a concentration level ten times greater than that of quinolinic acid-^{14}C, NAD caused no decrease in the ^{14}C incorporated into ricinine. Instead, there was an increase. If one assumes, as it must have been done previously (6, 12, 16), that exogenous NAD can cross the cellular membrane barriers intact and in addition that NAD is an obligatory intermediate in the biosynthesis of ricinine from quinolinic acid (Figure 10, Pathway II), then there should

Figure 10. Two proposed pathways for the biosynthesis of ricinine from quinolinic acid


PATHWAY I
COOH
COOH
N
Quinolinic acid
Ricinine
OCH3
C≡N
O
N
CH3
Pyridine Nucleotide Cycle
NAD
PATHWAY II

have been a decrease in the incorporation into ricinine. This obviously was not the case (Table 1).

In view of this observation the previous findings of other investigators requires re-examination and interpretation. The pyridine nucleotide cycle operates in much the same manner in a great variety of organisms (25). Castor beans do not seem to be an exception (1). Gholson et al. found that in crude extracts NAD acted as an inhibitor of the first reaction of the pyridine nucleotide cycle (the formation of nicotinic acid mononucleotide from phosphoribosylpyrophosphate and quinolinic acid) (2). If NAD or some product of its metabolism crosses into the cells and acts as such an inhibitor in vivo, then the large excess (endogenous levels have been reported to be from 10^{-8} to 10^{-9} moles/g of tissue) (26, 27, 28) of NAD ($\sim 2 \times 10^{-6}$) given to the experimental plants should have decreased the movement of radioactive quinolinic acid through the pyridine nucleotide cycle. If ricinine and NAD are made by separate pathways (Figure 10, Pathway I), an inhibition of the pyridine nucleotide cycle could increase the incorporation of ^{14}C from quinolinic acid into ricinine. The data in Table 1 are not inconsistent with this latter hypothesis.

It is true that the stimulation by NAD of radioactive incorporation into ricinine might have been due to some other effect(s) not directly connected with the biosynthesis of the alkaloid or the operation of the pyridine nucleotide

cycle. The evaluation, however, of such possible contributing factors as enhancement of transport of quinolinic acid into the cells or general increases in metabolic rates are difficult at this time.

Waller et al. has shown that exogenous NAD can serve as a precursor of ricinine (12). Numerous citations show that NAD is hydrolyzed before it can cross into a cell (29, 30). NAD can also act as a source of nicotinamide and nicotinic acid, both of which can be incorporated into ricinine. When equal molar quantities of radioactive quinolinic acid, nicotinic acid, nicotinamide or NAD were fed in separate experiments to castor beans, 10, 5, 3 and 3 per cent respectively of the label was incorporated into the ricinine (12). Such results suggest that quinolinic acid is a much better precursor for the biosynthesis of ricinine than either of the other three compounds. It seems likely that NAD was incorporated into ricinine as a result of having been first hydrolyzed to nicotinamide or nicotinic acid.

The data of Waller and Henderson (7) concerning the metabolism of nicotinamide in castor beans strongly supports the hypothesis that the pathways of ricinine and NAD biosynthesis from quinolinic acid are separate. They found that the amide nitrogen of exogenous nicotinamide was incorporated intact into the nitrile nitrogen of ricinine. On the other hand, all investigations into the incorporation of nicotinamide into NAD agree that nicotinamide is first

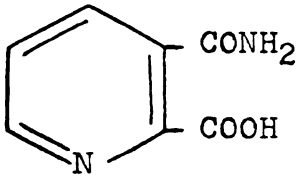
metabolized to some form of nicotinic acid before being incorporated into NAD (31, 32, 33, 34). Furthermore, castor beans clearly have an active complement of enzyme to accomplish the deamidation of nicotinamide (35). It would seem then from these observations the nicotinamide goes into ricinine and NAD by separate pathways.

The true role of the pyridine nucleotide cycle, if any, in the biosynthesis of ricinine will not be clear until the enzymes involved are completely elucidated. At present, however, the data concerning the per cent incorporation of various precursors of ricinine, the amide nitrogen incorporation from nicotinamide and the lack of inhibition by NAD on the incorporation of quinolinic acid into ricinine strongly suggest that NAD and ricinine are made from quinolinic acid by separate and more or less independent pathways. Because of the similarities between the biosynthesis of nicotine and ricinine, it would be surprising to find an involvement of the pyridine nucleotide cycle in the biosynthesis of nicotine (5, 6, 36, 37, 38, 39).

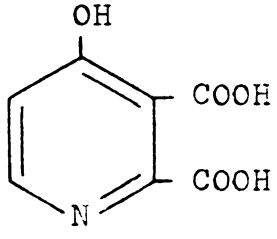
With the supposed role of the pyridine nucleotide cycle returned to its proper perspective, it now seemed correct to focus upon other potential intermediary compounds in the ricinine biosynthetic pathway. As mentioned in the introduction, when compared to quinolinic acid incorporation under optimum conditions, the following compounds do not seem to be intermediates: Nicotinic acid (13), nicotinamide,

N-methylnicotinamide, N-methylnicotinic acid, nicotinonitrile, N-methylnicotinonitrile (and thus the *in vitro* oxidation product N-methyl-3-cyano-4-pyridone) (12). The more highly modified pyridine ring compounds 4-hydroxy-3-cyano-2-pyridone, ricinic acid, N-methyl-4-methoxy-3-amido-2-pyridone and ricinine acid also failed to be significantly incorporated (14). During the latter stages of this thesis research the N-demethylated derivative of ricinine was reported to be an excellent precursor of ricinine. However, the lack of information concerning the specificity of the methylating enzyme still leaves open to question the exact biological role of this new compound (15).

The most significant intermediates to elucidate in a biosynthetic pathway would be the first compounds unique to that system. Assuming that the first unique intermediate is derived directly from quinolinic acid one could suppose one of the following compounds to be a ricinine precursor:

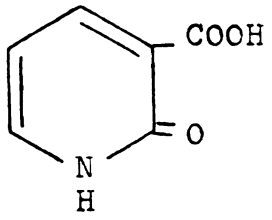


3-amidopicolinic acid



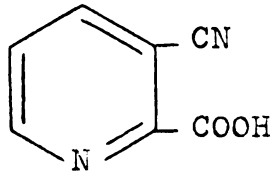
4-hydroxyquinolinic acid

Assuming that the number two carboxyl group could be directly replaced by a hydroxyl group (40, 41) the following compound also becomes a potential first intermediate:



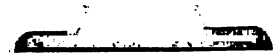
3-carboxy-2-pyridone

If the ricinine nitrile group was formed through an oxime (42) instead of an amide intermediate, then the following compound could be an early intermediate:



3-cyanopicolinic acid

To determine if any of these compounds were intermediates they were prepared according to the procedures previously outlined. The compounds were initially screened for their precursor role by a competitive feeding-reverse isotope dilution experiment with quinolinic acid-^{14}C. In such an experiment a true intermediate will not only cause a decrease in the ^{14}C incorporated into the ricinine but will itself become radioactive. Of the two compounds which caused a depression of the radioactivity in ricinine (3-cyanopicolinic acid and 3-carboxy-2-pyridone), only 3-carboxy-2-pyridone also became radioactive. A more direct evaluation of the relationship between this pyridone and ricinine through a direct incorporation study showed that 3-carboxy-2-pyridone is not an efficient precursor of ricinine.



The question concerning the identity of an early unique precursor of ricinine was not answered in this study. The fact that all of the results proved negative does not rule out the tested compounds as intermediates, for the possibility still exists that there are no free and unbound intermediates in the biosynthetic pathway of ricinine after quinolinic acid.

Perhaps future studies will judge more significant the finding that 3-carboxy-2-pyridone is a significant metabolite of both nicotinic acid and quinolinic acid. An extensive review of the literature elucidated no previous report of the natural occurrence of such an α-pyridone.

In general conclusion, one must say that the areas of ricinine precursor identification and enzymatic level studies aimed towards the elucidation of the biological role of ricinine remains an area open for still additional speculation and research.

THE MICROBIAL CATABOLISM OF RICININE

INTRODUCTION

Numerous naturally occurring and synthetic nitrile compounds have been reported which will serve as substrates for enzymatic reactions. The three observed types of enzymatic reactions towards the cyano group are the cleavage of the alpha carbon-carbon bond to release the intact nitrile group (43), the addition of an oxygen and two hydrogens to form an amide (44-47), and the complete conversion to the corresponding acid and free ammonia (44, 48-55). These latter enzymes, termed "nitrilases" (51), have been found capable of converting indoleacetonitrile to indoleacetic acid in plants (48-51), of converting α-cyano-α-aminoethanol to alanine (54), 4-cyano-4-aminobutyric acid to glutamic acid (55) and β-cyanoalanine to aspartic acid in fungi (44) and of breaking down the alkaloid ricinine (N-methyl-4-methoxy-3-cyano-2-pyridone) into ricinine acid (N-methyl-4-methoxy-3-carboxy-2-pyridone) in bacteria (52, 53).

In examinations of the mechanisms of these "nitrilases," it has been observed that molecular oxygen was not required in the reactions (49, 53). The assumption has thus been made that the conversion of the cyano group to an acid and ammonia was completely analogous to acid or base catalyzed nitrile hydrolysis. Evidence is presented in this chapter which conclusively demonstrates the validity

of this assumption.

The work of Hook and Robinson (52, 53) showed that ricinine nitrilase obtained from a ricinine grown pseudomonad readily hydrolyzed ricinine to ricinine acid at neutral pH without the addition of an outside energy source. It is well established that the nitrile group of ricinine exhibits unusual stability to chemical hydrolysis under both acidic and alkaline conditions (56). Therefore, the mechanism of enzymatic hydrolysis of ricinine becomes of special interest. The extensive investigation of the specificity of the pseudomonad type ricinine nitrilase demonstrated that of the normal ring substitutes of ricinine only the pyridone oxygen is required for at least partial activity (53). The "nitrilase" from plants which will hydrolyze a wide variety of compounds including 3-cyanopyridine was completely inactive against ricinine (51). Thus, in attempting to relate the mechanism of ricinine nitrilase to other "nitrilases," it became imperative to know if the pyridone oxygen has a unique role, i.e. Schiff's base formation to an amino group of the enzyme during the nitrile hydrolysis. Data presented in this study supports the conclusion that the pyridone oxygen functions only in a non-covalent bonding role during the reaction.

The bacterial work of Hook and Robinson was oriented towards the investigation of the enzyme mechanism of ricinine nitrilase (52, 53) and left many questions concerning the

microbial utilization of ricinine as the sole carbon source unanswered. The fact that their crude enzyme extracts were unable to convert ricinine acid to any other compounds and that the conversion of ricinine to ricinine acid actually procured no apparent net carbon gain for the cell, left the interesting thought that ricinine acid might not be an intermediate in the actual reduction of ricinine to some "common" carbon compound. It was not determined if the ricinine metabolizing system was constitutive or inducible. In addition, no insight was given as to the possible "common" carbon end product(s) of ricinine catabolism which could be used for actual cellular growth. Several of the experiments in this chapter were oriented toward answering these and other questions concerning the physiology and biochemistry of microbial utilization of ricinine.

MATERIALS AND METHODS

Growth Media

The mineral medium had the composition as given previously (52). The alkaloid medium contained mineral salts, 0.1% ricinine (sterilized by filtration) and 0.005% yeast extract (Difco). Bacterium F1S was often grown on a medium of 1% glycerol and 0.05% yeast extract while type F5S or F3M was grown on a medium of 1% succinate (pH 6.8) and 0.05% yeast extract. Agar (1.5%) maintenance slants and petri dishes were prepared as 5% glucose and 1% yeast extract or 0.15% ricinine and 0.005% yeast extract with salts medium. Stable storage cultures were prepared by lyophilization of bacteria frozen in powdered milk and kept at -20°.

Isolation of Bacteria

Six samples of sandy soil were obtained from the coastal region of South Carolina, and portions of each incubated at room temperature on salts media containing 0.1% ricinine as the sole carbon source. Half of the samples were obtained from an area where castor bean plants had been previously grown. Samples were removed from the three flasks which showed signs of growth and alternately streaked on ricinine and then glucose agar until three pure

types as judged from their gross morphological characteristics on glucose agar were obtained. These were designated F1S (peach colored and dry with a halo effect around the colony), F5S (off-white and moist in appearance) and F3M (peach colored and mucoid to the point of being runny).

Ricinine Nitrilase Preparation and Assay

Log phase ricinine grown or induced bacteria were isolated by centrifugation for 25 min. at 7,000 x g. These cells were resuspended in salts medium and centrifuged again. The cells were then suspended in a small amount (approximately 4 ml per liter) of pH 7.4 phosphate buffer (0.1 N) being 0.001 M in ricinine, chilled in an ice bath, and sonicated in a shilled 10KC Raytheon Sonic Oscillator at full power for 3.5 min. The sonicate was centrifuged at 20,000 x g for 15 min. and the supernatant decanted. The pellet could then be resuspended for an additional sonication. The sonicate supernatant was maintained in an ice bath or frozen until needed. When it was critical to remove all ricinine, as in the ^{18}O experiments, the cells were washed several times and the ricinine stabilizer omitted from the sonication buffer. Protein was determined by the method of Lowry *et al.* (60).

The standard assay for ricinine nitrilase activity was to follow the loss at room temperature of absorption at 315 nm as compared to a water blank (52). The assay mixture contained 0.3 ml of 0.1 N pH 7.4 phosphate buffer with 0.001 M

ricinine, 2 ml of 0.1 N pH 7.4 phosphate buffer and enough enzyme extract to maintain a linear reaction rate for at least 5 min. (0.1-0.5 ml of an extract of ricinine grown bacteria).

In order to avoid the expense of ricinine when large quantities of ricinine nitrilase were required, the bacteria were first grown on glycerol or succinate medium for 24 hrs at 28°. After harvesting and washing, the cells were induced by suspending them in 150 ml of salts medium containing 150 mg of ricinine and allowed to shake at room temperature for 12 hrs. At the end of this period, 50 mg additional ricinine were added and two hours later the cells were isolated for enzyme extraction.

Ricinine Nitrilase Reaction Product

To determine the nature of the product formed from incubating ricinine with bacterial extracts, 0.1 ml of ricinine-6-^{14}C (0.77 mg/ml at 7.37 x 10^5 dpm/ml) and 3 ml of 0.1 N pH 7.4 phosphate buffer were incubated with a cell extract. At various intervals, samples were removed, the optical density at 315 mm recorded and the reaction stopped with 0.2 ml of 50 per cent trichloroacetic acid. The protein was removed by centrifugation and rinsed with 0.3 ml of 10 per cent trichloroacetic acid. The combined supernatants were evaporated to dryness under reduced pressure and organic compounds taken into chloroform by extracting the residue twice with 2 ml portions of chloroform. These

extracts were then used in the descending paper chromatography systems of Hook and Robinson (52) or the Silica F_{254} TLC system of chloroform/methanol/acetic acid 3:5:2. The radioactivity was located by autoradiography.

For the mass preparation and isolation of the reaction product, the bacterial sonication extract from one liter of succinate grown and ricinine induced F5S cells (40 ml) was shaken in a stoppered flask with 200 mg of ricinine, 8,000 units of penicillin G and 60 ml of 0.1 M, pH 7.4 phosphate buffer. The reaction was stopped after 14 hours by the addition of perchloric acid and the protein removed by centrifugation. After neutralization with KOH and removal of the potassium perchlorate, the supernatant was applied to a 2.5 cm x 25 cm Dowex-1 x 8 formate column and washed with 0.01 N formic acid until no more 260 nm absorbing materials were detected. Subsequent elution with 0.1 N formic acid yielded a large ultraviolet absorbing peak. This fraction was evaporated to dryness under reduced pressure and the residue twice crystallized from hot water. The physical properties were determined according to p. 6.

Analysis of Ricinine Nitrilase Mechanism

A crude enzyme extract of ricinine grown bacteria was incubated with 80 ug of ricinine in phosphate buffer and enough ^{18}O enriched water to make the solution 22 atom % ^{18}O. After 45 min. the completed reaction was stopped

with trichloroacetic acid and the protein removed by centrifugation. The supernatant was removed under a vacuum and the ricinine acid and ricinine extracted into chloroform by twice mixing the residue with 2 ml portions of chloroform. The chloroform was removed under reduced pressure and the residue dissolved in 2 ml of methanol/ether (50 v/50 v) for methylation by the procedure of Schlenk and Gellerman (61). In this procedure the compound Diazald (Aldrich Chem.) was used to generate diazomethane; a methylation method which allows retention of both original carboxyl oxygens. Following the methylation, the solvent was removed in a stream of nitrogen and the residue dissolved in 35 µl of methanol. Two µl of this solution were then gas chromatographed (Supelco SE-30-3% on a 4 ft. column at an oven temperature of 170°) and the mass spectrum determined on a LKB 9000 single focusing gas chromatograph-mass spectrometer. The mass spectrometer was set at 70 eV with an ion source temperature of 290° and the accelerating voltage at 3.5 KV. The mass spectrum of a control sample which had been run in normal water was also determined. Calculations for the number of oxygens incorporated into the ricinine acid were those described in the appendix of Thorp and Sweeley (62).

Radioactive Ricinine

Specifically labelled ricinine was obtained by isolating ricinine from etiolated castor bean seedlings which

had been given a specifically labelled ricinine precursor. The feeding procedure was that given previously (page 30) except with a dark metabolism time of from 24 to 48 hrs at 28°. Different groups of plants were fed L-methionine-(methyl)-^{14}C to obtain ricinine-7,9-^{14}C (8), acetate-(u)-^{14}C to obtain ricinine-2,3,8-^{14}C (57-59), nicotinic acid-7-^{14}C to obtain ricinine-8-^{14}C (7) or quinolinic acid-6-^{14}C to obtain ricinine-6-^{14}C (13). The ricinine was isolated according to Leete and Leitz (9) except that instead of final purification by crystallization, a water solution of the residue was streaked on a preparative Silica F_{254} TLC plate and developed with chloroform/methanol/acetic acid (3:5:2). The area containing ricinine (R_f 0.78) was located with ultraviolet light, scraped from the plate and extracted three times with 100 ml portions of methanol. This extract was taken to dryness and the residue extracted with 50 ml of chloroform. Removal of the chloroform and addition of water yielded a chromatographically pure (52) solution of ricinine as follows: Ricinine-7,9-^{14}C (7.5×10^5 dpm/mg), ricinine-2,3,8-^{14}C (5.3×10^4 dpm/mg), ricinine-8-^{14}C (1.36×10^5 dpm/mg) and ricinine-6-^{14}C (9.55×10^5 dpm/mg). These solutions were stored at -20° until needed.

Induction Experiments

F1S-glycerol grown and F5S- and F3M-succinate grown cells were harvested while in the log phase of growth and

twice washed with salts medium. A final suspension was made with salts medium of 10 ml per $O.D._{640}$ unit per liter. Duplicate, uncalibrated, single arm Warburg flasks were prepared as follows: 1.5 ml of 0.2% ricinine, 0.8 ml of a two fold concentrate of salts medium in the reaction areas, 0.3 ml of saturated KOH and a wick in the center wells, and 0.2 ml of bacteria in the side arms. Control flask contained no ricinine or 0.5 ml of 7.5×10^{-3} M chloramphenicol. The reaction was initiated by mixing the bacteria with the carbon source after the flask were equilibrated with the 26° water bath. The relative oxygen consumption was recorded after correcting for barometric changes.

Total Oxygen Consumption

The total amount of oxygen consumed per mole of test compound was determined using a constant pressure respirometer. Each reaction flask contained a 0.3 ml saturated KOH wick in the center well and one ml of twice washed ricinine grown bacteria which had been resuspended at 200 ml salts medium per $O.D._{640}$ unit per liter and 0.8 ml of salts medium in the reaction area. The reaction was initiated by mixing the 0.5 ml (0.7 μ moles) of test compound from the side arm. The bath temperature was 25°. The reactions were observed until no more changes in the gas pressure occurred.

Metabolism of Ricinine-^{14}C

In order to elucidate more of the pathway of ricinine metabolism, whole F5S cells were incubated with specifically carbon-14 labelled ricinines. Each 125 ml reaction flask contained 3 ml of ricinine-^{14}C, 10 ml of 0.2% ricinine and 10 ml of ricinine grown F5S cells suspended in enough of a two fold concentration of salts medium such that the final $O.D._{640}$ of the reaction flask was 1.0 to 1.5. The total amount of radioactive ricinine used was 11 x 10^5 dpm of ricinine-6-^{14}C (1.2 mg), 3.8 x 10^5 dpm of ricinine-8-^{14}C (2.8 mg), 9.2 x 10^5 dpm ricinine-7,9-^{14}C (1.2 mg) or 3.6 x 10^5 dpm of ricinine-2,3,8-^{14}C (6.8 mg). In order to collect respired CO_2, four 1 ml samples were removed from each reaction flask and placed in four 25 ml reaction falsks fitted with a rubber stopper and a hanging plastic center well containing a wick and 0.2 ml of Hydroxide of Hyamine 10-X (Packard). At appropriate times, 1 ml samples were removed from the main reaction flasks and pulled through a 0.45 μ filter (Millipore) and the filter immediately rinsed with two 1 ml aliquots of water. The filter was first dried and then the radioactivity determined using 5 ml Bray's solution with 0.5 ml of water. The filtrate was diluted to 5 ml and an aliquot used for ^{14}C determinations. The remainder of the supernatant was evaporated to dryness under reduced pressure, diluted to a standard volume and again the radioactivity on an aliquot determined. At vari-

ous times, 0.2 ml of 10% perchloric acid were injected into the closed system flasks and after standing overnight, the amount of $^{14}CO_2$ collected in the center well determined. The $O.D._{640}$ was determined during the course of the reacting in order to assess the amount of bacterial growth in the reaction cultures. The per cent of the initial radioactivity in each fraction was then determined as a function of time.

RESULTS

General Bacterial Characteristics

Samples of each of the types of the ricinine metabolizing bacterial isolates (F1S, F5S, and F3M) were given to Dr. J. M. Tiedje, Department of Crop and Soil Science and tentatively identified as belonging to the nocardia group. A more extensive investigation by Dr. P. Hirsch, Department of Microbiology, confirmed this finding of the bacteria belonging to the order of Actinomycelates and further classified them as belonging to the mycobacterium-nocardia group. The most distinguishing characteristics of this group are a procaryotic cell with an early rudimentary mycelium vegetative phase.

The cell type F5S was found to give the most consistant growth and yield when grown on ricinine and was, therefore, used most extensively throughout this investigation. None of the cells exhibited an absolute requirement for yeast extract, but under all growth conditions, including non-ricinine carbon media, the growth rate was enhanced by its inclusion. The maximum $O.D._{640}$ was unaffected by the yeast extract addition.

It was often observed that cultures which were in the stationary phase caused the medium to become purple to reddish in color. This pigmentation did not absorb at 640 nm

and thus did not interfere with culture density determinations.

Ricinine Nitrilase Product Formation

When sonication extracts of F1S, F5S or F3M cells which had been grown on ricinine were incubated with ricinine, a decrease in absorption at 315 nm was observed. This loss in optical density was linear with time (Figure 11) for the initial phase of the incubation. There was no change in absorption with a boiled preparation. Using ricinine-6-^{14}C as the substrate, samples of each reaction mixture were chromatographed and the locations of radioactive compounds determined by autoradiography. Table 5 shows that the radioactivity was located at the reported positions of ricinine and ricinine acid (52). A determination of the substrate-product relationship during the course of the reaction is shown in Figure 12. Here a sonication extract of F5S was incubated with ricinine-6-^{14}C and at appropriate time intervals the absorption at 315 nm was recorded and the reaction stopped on an aliquot of the reaction mixture. The material was then chromatographed in the solvent system of Robinson and Hook (52). The radioactive areas, which were located at R_f 0.68 and 0.03, were quantitated by removing the proper sections of the chromatogram and counting them by the liquid scintillation method. It is evident from Figure 12 that for the initial phase of the reaction, the loss of ricinine and the change of absorption at 315 nm are linear and parallel.

Figure 11. The change in optical density at 315 nm when extracts of ricinine grown or induced bacteria were incubated with ricinine and compared to a water blank

The samples were as follows:

△ (——) F5S ricinine grown at a sp. act. of 0.015 μmole/min/mg protein

○ (——) F1S ricinine grown at a sp. act. of 0.110 μmole/min/mg protein

□ (——) F3M ricinine grown at a sp. act. of 0.05 μmoles/min/mg protein

● Boiled enzyme

□ (---) F3M induced with ricinine at a sp. act. of 0.014 μmoles/min/mg protein

△ (---) F5S induced with ricinine at a sp. act. of 0.004 μmoles/min/mg protein

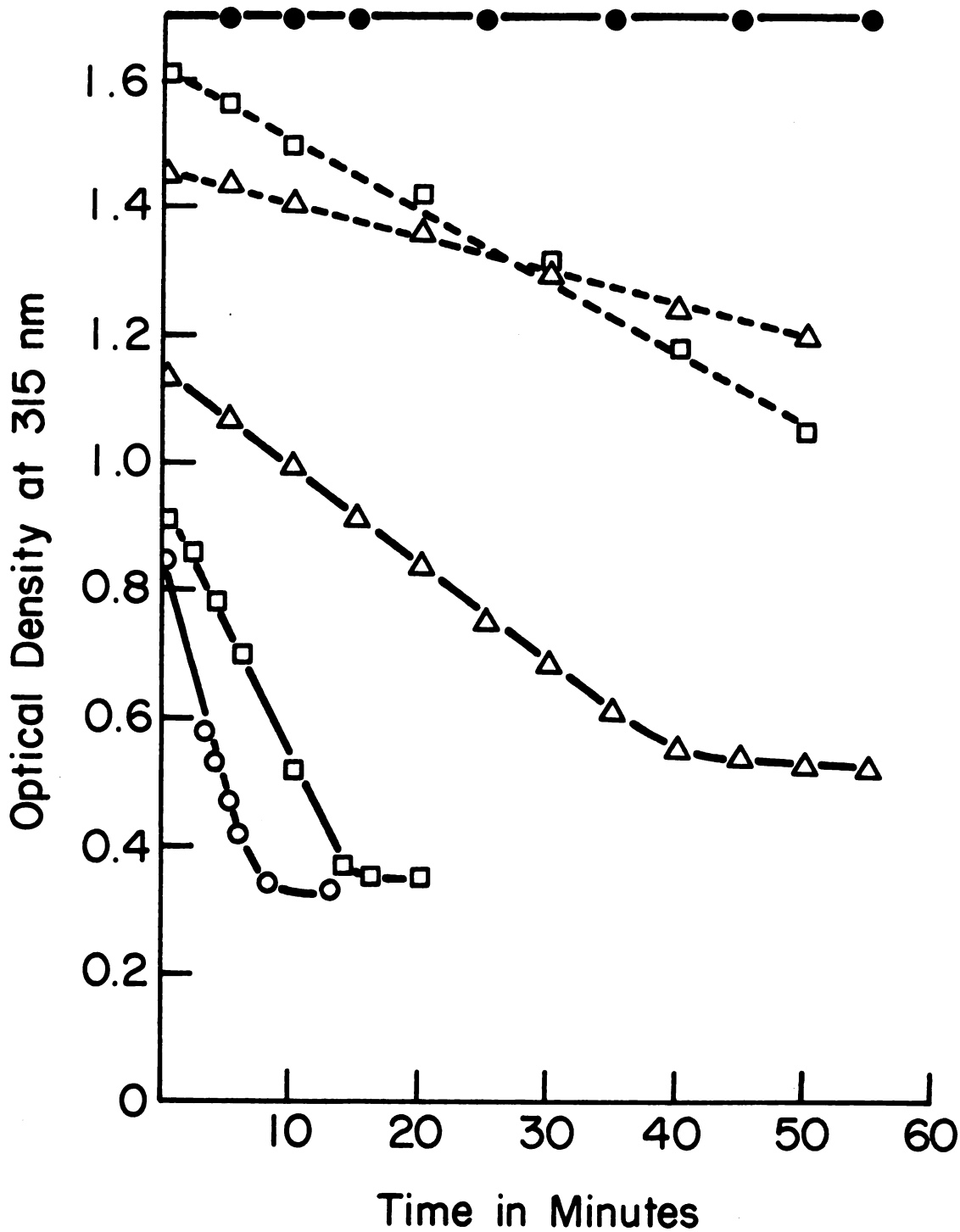

Optical Density at 315 nm
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0
10
20
30
40
50
60
Time in Minutes

Table 5. The R_f values of radioactive compounds following incubation of ricinine-6-^{14}C with samples of bacterial extracts

Chromatography System	Bacterial Source		
	F1S	F5S	F3M
	Observed R_f Values		
Paper*			
BuOH/saturated with 1.5 N NH_4OH	0.03	0.03	0.03
	0.65	0.65	0.65
Paper+			
BuOH/acetic acid/H_2O 4:1:1	0.68	0.68	0.68
	0.72	0.72	0.72
Silica F_{254} TLC‡			
$CHCl_3$/MeOH/acetic acid 3:5:2	0.60	0.58	0.61
	0.76	0.76	0.76

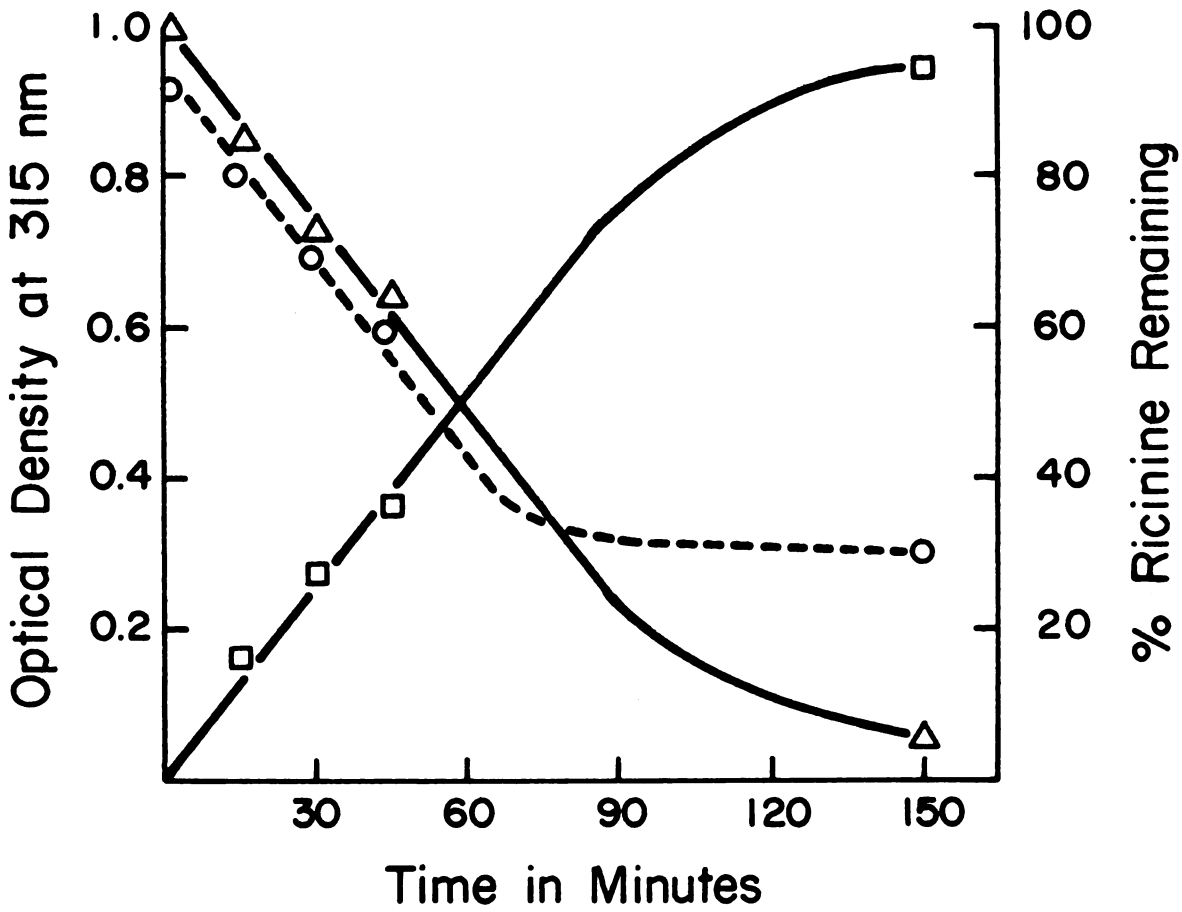
Known R_f values:

*ricinine R_f 0.65, ricinine acid R_f 0.03 (52)

+ricinine R_f 0.72, ricinine acid R_f 0.66 (52)

‡ricinine R_f 0.77

Figure 12. The relationship of optical density change at 315 nm (O), ricinine loss (Δ) and product formation (□) when an extract of ricinine grown F5S cells was incubated with ricinine-6-^{14}C


Optical Density at 315 nm
1.0
0.8
0.6
0.4
0.2
100
80
60
40
20
% Ricinine Remaining
30
60
90
120
150
Time in Minutes

There is a reciprocating relationship for product formation and for the change in optical density.

To complete the tentative identification of the reaction product as ricinine acid, the λ_{max} was determined as well as the elemental analysis and melting point on a twice crystallized isolate of product from a mass preparation reaction. The melting point of 225 to 226° (reported 222 to 223°), the ultraviolet absorption (Figure 13) and the elemental analysis were in good agreement with ricinine acid ($C_8H_9NO_4$) (52).

Calculated:	52.50% C	4.96% H	7.66% N
Found:	52.46% C	4.95% H	7.65% N

Ricinine Nitrilase Mechanism

In using ^{18}O incorporation from enriched water into ricinine acid to study ricinine nitrilase, it was first necessary to isolate small, pure quantities of the acid product. This was accomplished using the differences in the gas chromatographic retention times of the methyl ester of ricinine acid (1.4 min) and ricinine (2.5 min) on a SE-30-3% column run with an oven temperature of 170°. The mass spectrum of methyl ricinate is shown in Figure 14. The peaks of interest for subsequent ^{18}O incorporation analysis are the parent peak at m/e 197 which contains the two oxygens of the carboxyl group plus the pyridone oxygen, the m/e 166 peak corresponding to a loss of the $-OCH_3$ from the ester and the m/e 138 peak due to the loss of the $-COOCH_3$

Figure 13. The ultraviolet absorption spectra of ricinine acid in 0.1 N HCl (——), in 0.1 N pH 7.4 phosphate buffer (---) and in 0.1 N NaOH (-·-)

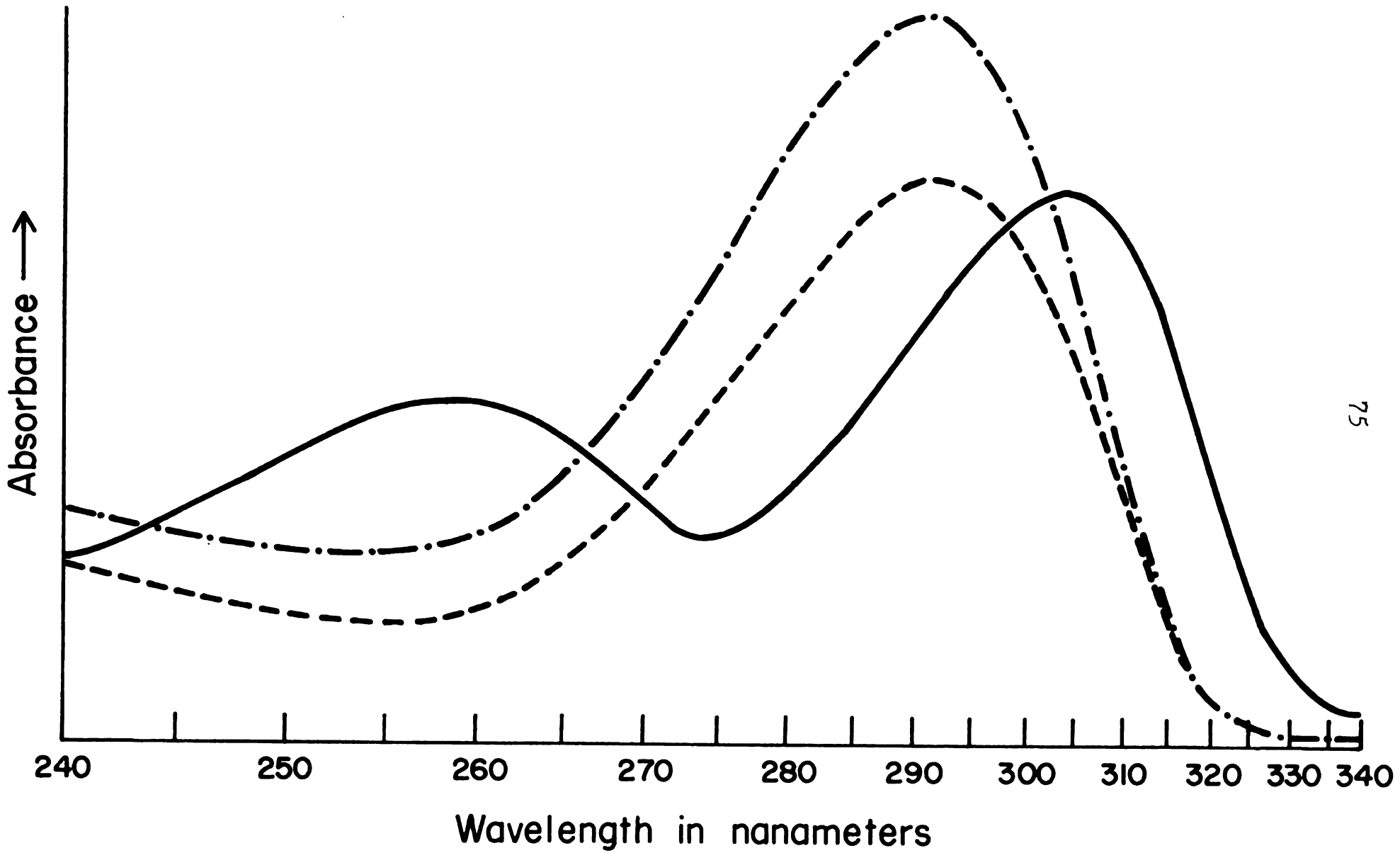
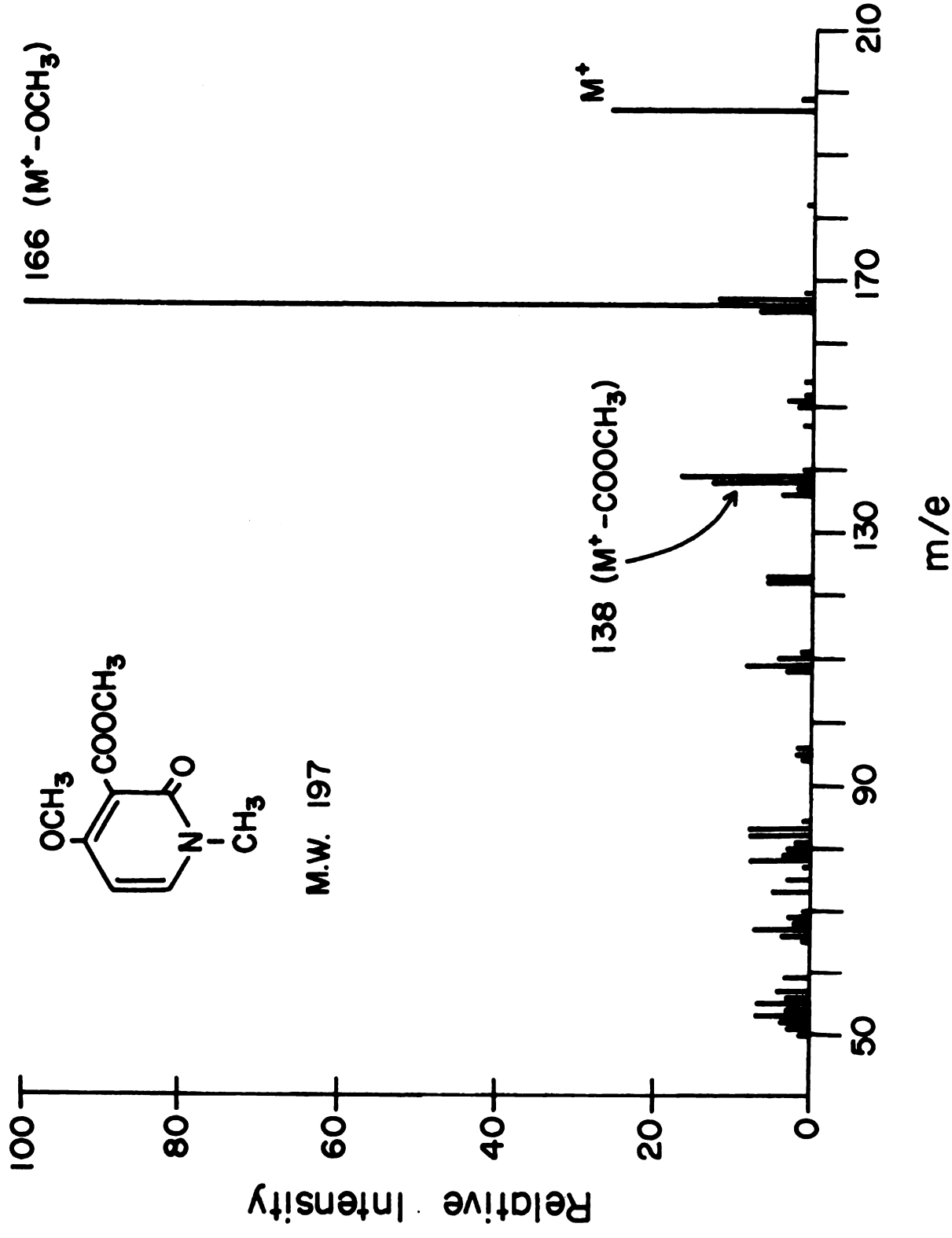

Absorbance ⟶
240
250
260
270
280
290
300
310
320
330
340
Wavelength in nanameters

Figure 14. The mass spectrum of the methyl ester of ricinine acid. The ricinine acid was obtained from the reaction of ricinine nitrilase with ricinine in normal water.


166 (M⁺-OCH₃)
M⁺
138 (M⁺-COOCH₃)
OCH₃
COOCH₃
O
N
CH₃
M.W. 197
Relative Intensity
100
80
60
40
20
0
50
90
130
170
210
m/e

group from the parent ion. From the data in Table 6, it can be seen that the m/e 197 and +2 peaks were the result of two originally water contained oxygen atoms being incorporated into the ricinine acid. The m/e 166 and +2 peaks contained only one of these oxygens while the m/e 138 and +2 peaks did not contain any of the water derived oxygen atoms.

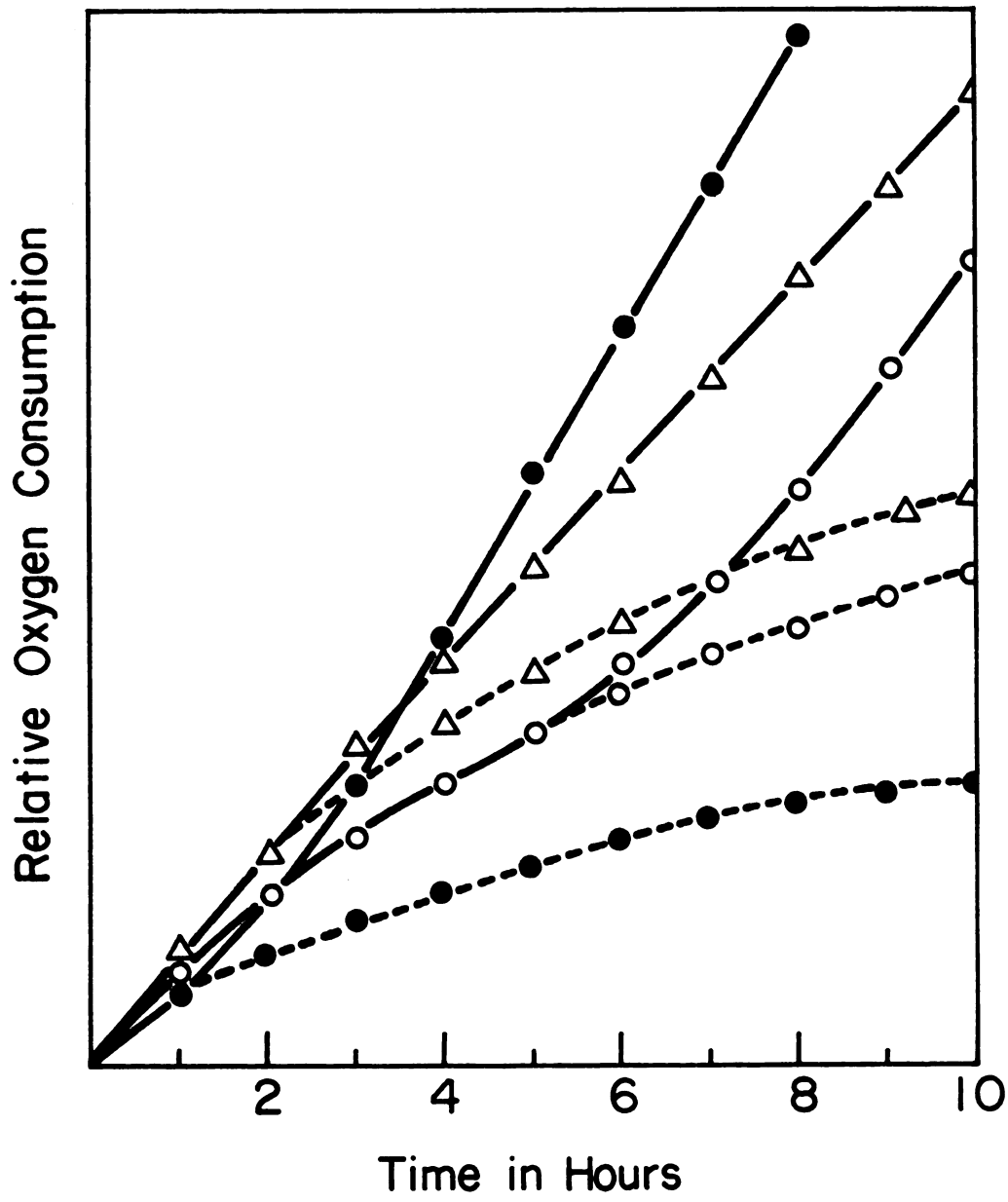
Induction of Ricinine Metabolism

As seen in Figure 15 all three types of bacteria when grown on non-ricinine media were induced by the presence of ricinine to consume oxygen. With no ricinine in the reaction vessel or when chloramphenicol was added there was only a basal level of metabolism. With or without a nitrogen source other than ricinine in the media, the induction times were still 1 to 2 hrs for F1S, 2 to 3 hrs for F5S and 5 to 7 hrs for F3M. The carbon starving of a sample of F3M cells for 24 hrs prior to the initiation of the induction experiment did not cause a decrease in the time required for the oxygen consumption to rise above basal levels. Only bacterial cells which had been grown on ricinine or induced by ricinine had *in vitro* activity against ricinine (Figure 11). However, the specific activity of the induced cell extracts was consistantly lower than that of ricinine grown bacteria (Figure 11).

Table 6. The incorporation of ^{18}O into selected peaks of the mass spectrum of the methyl ester of ricinine acid obtained from the enzymatic hydrolysis of ricinine in ^{18}O enriched water

	Relative Peak Height		Per Cent as ^{18}O Molecules	
	Control (^{16}O)	Experimental (^{18}O)	Found	Calculated
Parent peak (m/e 197)	100.0	100.0	36.1	2 of 4 potential exchangeable oxygens = 36.2
+2 units (m/e 199)	1.4	58.0		
Parent peak $-OCH_3$ (m/e 166)	100.0	100.0	22.2	1 of 3 potential exchangeable oxygens = 22.0
+2 units (m/e 168)	1.5	30.2		
Parent peak $-COOCH_3$ (m/e 138)	100.0	100.0	2.7	0 of 2 potential exchangeable oxygens = 0.0
+2 units (m/e 140)	9.4	12.2		

Figure 15. The inductive oxygen consumption (——) of the bacteria by ricinine. The control samples (---) contained no ricinine or 3.7×10^{-3} moles of chloramphenicol. Time zero was the point where ricinine and bacteria were mixed. The bacteria types consisted of glycerol grown F1S (●——●, ●---●), succinate grown F5S (Δ——Δ, Δ---Δ), and succinate grown F3M (o——o, o---o).


Relative Oxygen Consumption
2
4
6
8
10
Time in Hours

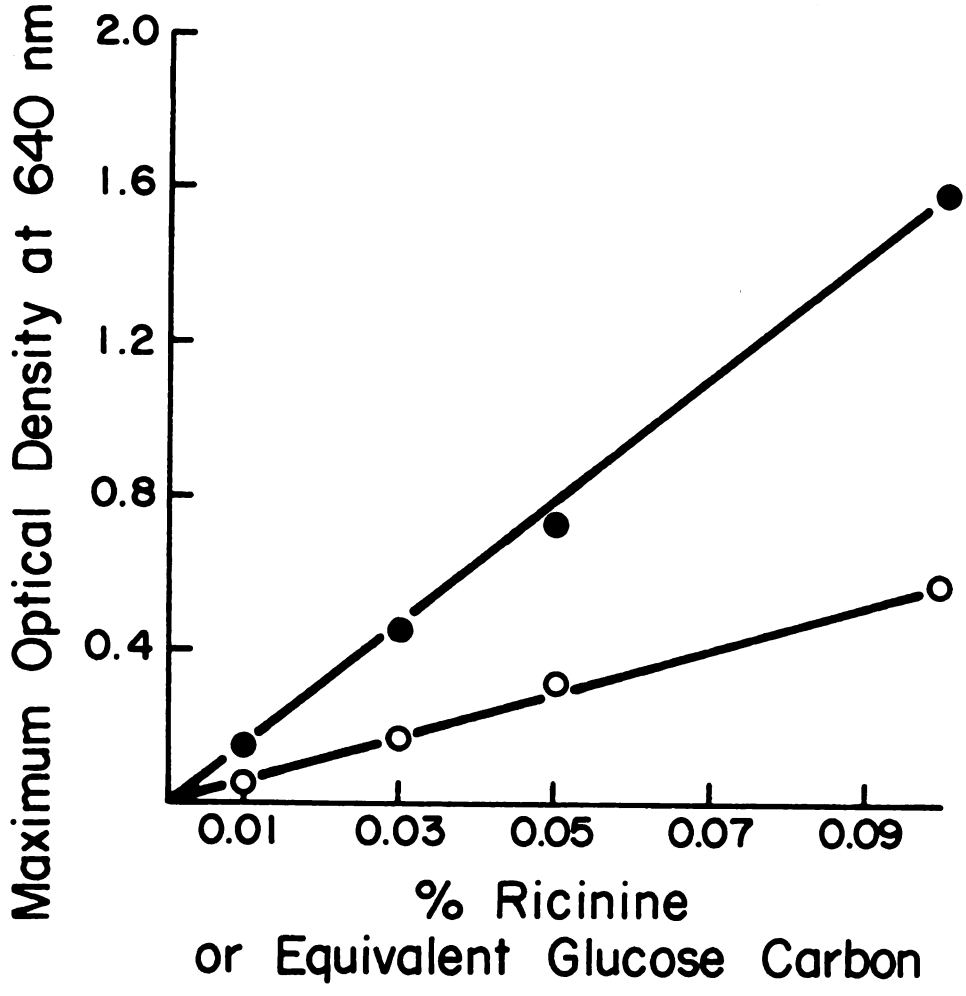
Quantitative Oxygen Consumption

Whole ricinine grown F5S cells consumed 4.8 moles of oxygen per mole of ricinine and 4.6 moles per mole of ricinine acid metabolized. The following compounds were shown not to be metabolized by virtue of the fact that they caused no oxygen consumption when incubated with ricinine grown bacteria: Ricinic acid, N-methyl-4-methoxy-2-pyridone, 3-carboxy-2-pyridone, N-methylnicotinic acid, nicotinic acid, 2-pyridone, and 4-pyridone.

Comparative Growth on Glucose

It was observed that growth of the bacteria on the ricinine medium consistantly gave lower yields of cells than when grown on other carbon sources. To determine if this was due to a toxicity effect of some by-product of ricinine metabolism or to a limited usage of the available ricinine carbon atoms, F5S type cells were grown in separate flasks on ricinine between 0.01 and 0.1 per cent or an equivalent carbon amount of glucose. Figure 16 shows the maximum optical density reading at 640 nm as a function of equivalent carbon amounts of ricinine and glucose. The level of growth was proportional to the amount of carbon given with ricinine being approximately one third as efficient as glucose. The addition of solid ricinine to the stationary phase 0.1% culture resulted in the initiation of additional growth, showing that even at the higher concentration of ricinine, there was no toxic effect.

Figure 16. The variation in maximum optical density at 640 nm obtained by F5S cells when grown on equivalent carbon amounts of ricinine (o) and glucose (●)


Maximum Optical Density at 640 nm
2.0
1.6
1.2
0.8
0.4
0.01
0.03
0.05
0.07
0.09
% Ricinine
or Equivalent Glucose Carbon

In vivo Metabolism of Specifically Labelled Ricinine-^{14}C

The course of the metabolism of ricinine-^{14}C by F5S is seen in Figure 17. In all cases, cellular growth, as indicated by the increase in O.D.$_{640}$, continued up to the same time point as the break point in the solid line (^{14}C loss from solution). In the final distribution of carbon-14 as shown in Table 7, almost all of the 6-labelled ricinine was found as $^{14}CO_2$ as was the ricinine-8-^{14}C. One half of the methyl labelled ricinine was given up as $^{14}CO_2$ while the remaining half of the radioactivity formed a volatile compound which was not incorporated into the cellular material. Ricinine-2,3,8-^{14}C yielded slightly more than two thirds of its radioactivity as $^{14}CO_2$ while the remaining amount was divided between the cellular materials and the supernatant.

Figure 17. The utilization of ricinine-^{14}C by F5S bacteria in which (——) represents the ^{14}C in solution, (---) the ^{14}C in CO_2 and (•••) the ^{14}C in the cells

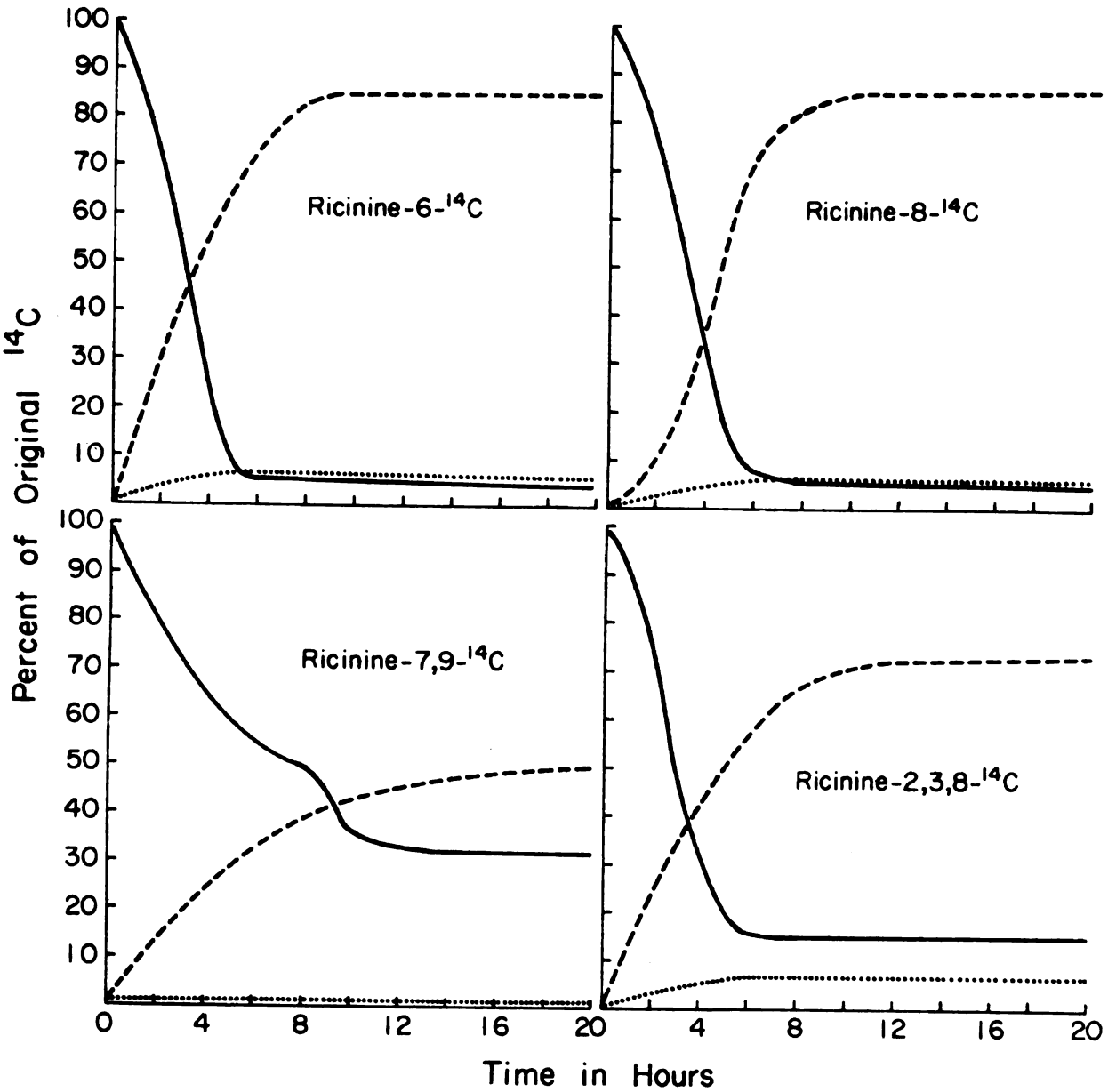

Percent of Original ^{14}C
Ricinine-6-^{14}C
Ricinine-8-^{14}C
Ricinine-7,9-^{14}C
Ricinine-2,3,8-^{14}C
Time in Hours
100
90
80
70
60
50
40
30
20
10
0
4
8
12
16
20

Table 7. The final form of the ^{14}C from specifically labelled ricinine as a per cent of the original amount of ^{14}C given following whole cell metabolism

Form of ^{14}C Recovery	Position of ^{14}C in Ricinine			
	6	8	7,9	2,3,8
% Total recovery	94	95	82	93
% as CO_2	85	84	48	73
% on filter	5	6	2	6
% in supernatant*	4	6	32	13
% in supernatant+	4	6	7	7

*before evaporation

+after evaporation

DISCUSSION

The importance of the enzymatic reactions in which a nitrile group is converted to an acid and ammonia increases as more instances of the "nitrilase" reaction are found. The "nitrilase" type enzyme has now been reported in plants, fungi, a member of the bacterial order of Pseudomnadales (a pseudomonas) and now in three members of the bacterial order of Actinomycetales (mycobacterium-nocardias).

The product obtained by enzymatic hydrolysis of ricinine catalyzed by an extract of ricinine grown cells of F1S, F5S, and F3M types of mycobacterium-nocardias bacteria was shown to chromatograph at the reported R_f values of ricinine acid (52). That this product was ricinine acid was conclusively demonstrated through melting point, mass spectrometry, ultraviolet absorption and elemental analysis. The role of ricinine acid in the metabolism of ricinine was strongly indicated to be that of an intermediate in catabolism by virtue of the fact that both it and ricinine elicited the in vivo utilization of equal amounts of oxygen. Thus, it is evident that not only is ricinine metabolized by more than one order of bacteria, but that at least the first catabolic product is identical in both orders.

The implication would be that the mechanism of ricinine

nitrilase could also be the same in both types of bacterial systems. Hook and Robinson reported that their pseudomonad extract hydrolyzed ricinine to not only ricinine acid and ammonia but also approximately 9 per cent ricinineamide (53). They further demonstrated that the amide was not further metabolized to the acid and was apparently an artifact of the system. Using the autoradiographic method which would have detected a compound containing as little as 1% of the total radioactivity administered, we were unable to detect any ricinineamide during the time-course study with F5S cellular extracts. Occasionally, when in vitro ricinine nitrilase reactions were allowed to stand well past completion of the reaction, a weak radioactive spot was observed following chromatography and autoradiography which roughly corresponded to the reported R_f value of ricinineamide (53). However, the evidence strongly favors the conclusion that the free amide is not an intermediate in ricinine hydrolysis.

As had been previously supposed (52, 53) in crude bacterial extracts, the loss in absorption at 315 nm was shown to be proportional to loss of ricinine and inversely proportional to the formation of ricinine acid. It was further demonstrated that this ability to convert ricinine to the acid was contained completely in the supernatant following centrifugation at 105,000 x g.

For the mass spectrum of ricinine acid methyl ester to be useful in the analysis of incorporation of ^{18}O from

enriched water into ricinine acid during the hydrolysis of ricinine, the origin and content of various mass peaks must be known. The m/e 197 peak was obviously the parent peak and needs no further explanation. That the m/e 166 peak was derived from the loss of an $-OCH_3$ from the ester and not the number four methoxy of ricinine acid follows from the known lability of ester groups (63) during mass spectrometry and the detailed mass spectral study by Waller et al. (64) which showed the methoxy group of ricinine to be stable to loss. Again, based on well documented splitting patterns, it was concluded that the most likely origin of the m/e 138 peak was from loss of the entire carboxyl ester group from the parent ion (63). Thus, it follows that m/e 197 contains all of the original oxygens of the methyl ester of ricinine acid, that m/e 166 has all except one of the carboxyl oxygens and that m/e 138 contains only the number four methoxy and number two pyridone oxygens. The height of the peak at an m/e two units greater than each of these peaks less the background height at that m/e is the contribution due to the incorporation of one ^{18}O from the enriched water.

From this knowledge of the mass spectrum and the isotope analysis data presented in Table 6, the following statements can be made concerning the origin of the ricinine acid oxygens: 1) the pyridone oxygen does not come from water as it would if a Schiff's base had been formed with

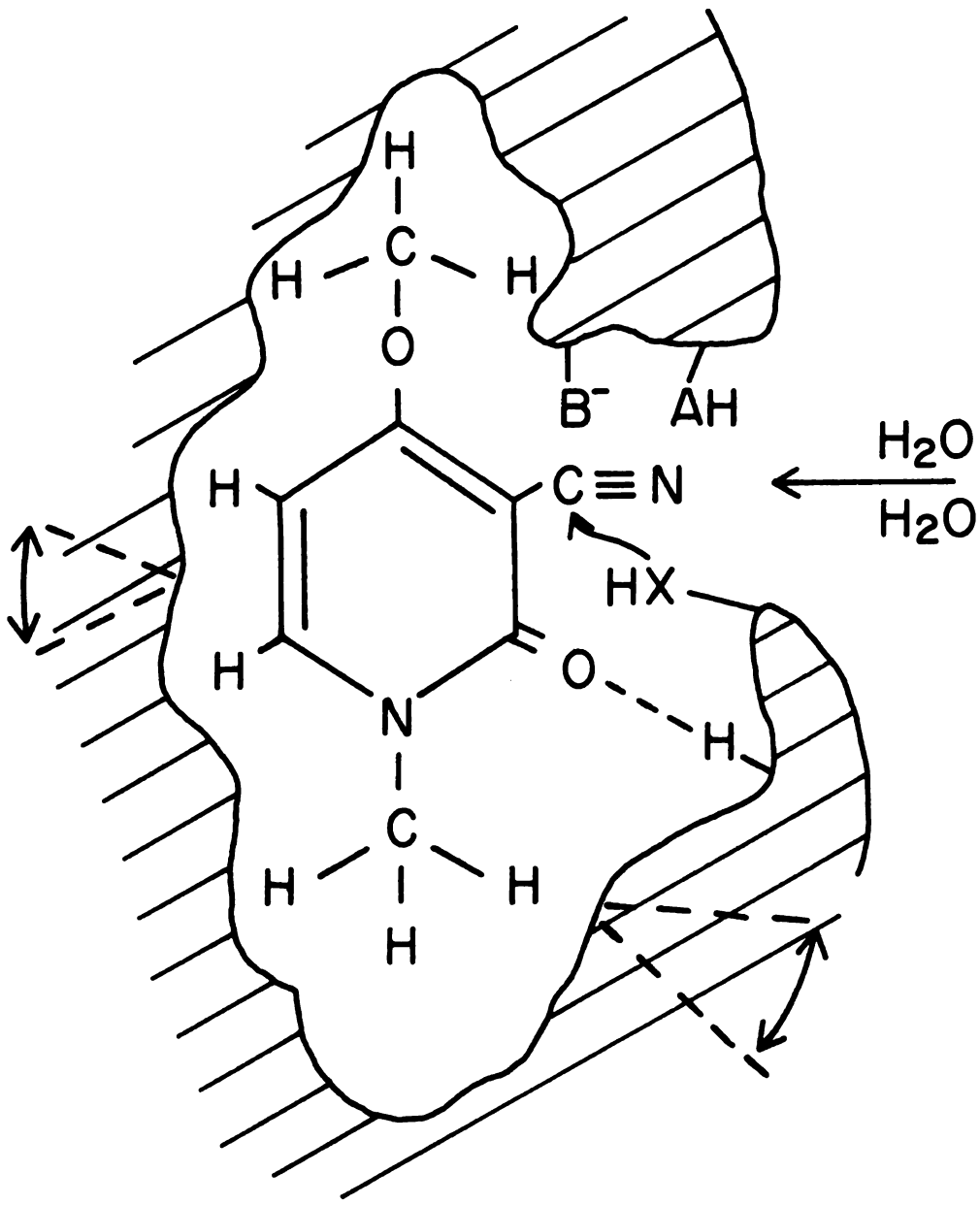
the enzyme during the conversion of ricinine to ricinine acid and 2) both of the oxygens of the carboxyl group are derived from water, a reaction quite analogous to acid or base catalyzed nitrile hydrolysis. It appears that despite the chemical stability of ricinine's nitrile group and the absolute requirement of the pyridone oxygen for enzymatic activity, the enzymatic conversion of ricinine to ricinine acid could proceed by a mechanism similar to other "nitrilases."

From the data presented here and by other investigators, one is able to imagine how the ricinine nitrilase could interact with ricinine to cause the high degree of substrate specificity. Mehadevan and Thimann concluded from their studies of the hydrolysis rate of various nitrile compounds by barley "nitrilase" that the reactions were more analogous to alkaline rather than acid catalyzed nitrile hydrolysis (51). The mechanism for ricinine nitrilase as proposed by Hook and Robinson tends to be closely related to acid catalysis (53). We could find no conclusive evidence to differentiate which type of chemical catalysis is most analogous in ricinine nitrilase. The studies of Hook and Robinson showed that the substitution of a hydrogen or an ethoxy group for the methoxy group of ricinine caused only a decrease in activity. Replacement by a hydrophilic hydroxyl group caused a complete loss of activity. Removal of the N-methyl group still allowed some

activity while replacement by an ethyl group caused an increase rate of nitrile hydrolysis. Coupled with the data concerning the role of the pyridone oxygen, the non-free amide intermediate and the previously proposed nucleophilic covalent Enzyme-XH attack on the substrate (53), one can invision the following lock and key type interaction between substrate and enzyme (Figure 18): The groups on the enzyme required for catalytic activity are not initially located in the enzyme reaction center. In the presence of ricinine, the two para hydrophobic ends of the substrate reorient the enzyme so that the reactive enzyme groups are now in the active center. The pyridone oxygen, perhaps through hydrogen bonding, give a final but critical degree of orientation between the active center and the substrate. The active center of the enzyme can now proceed in the hydrolysis perhaps according to the "nitrilase" mechanism as previously described (53).

Turning to a more physiological approach to the study of ricinine metabolism by the mycobacterium-nocardia type bacteria, it was found that the ability to oxidize ricinine was an inducible property which could be accomplished by degrading existing cellular proteins and subsequently synthesizing the new ricinine enzymes. The time required for this induction was dependent upon the bacterial type. This characteristic time was most likely at the enzyme synthesis level since the prior starving of the cells did not

Figure 18. The proposed lock and key relationship between ricinine and ricinine nitrilase


H
C
H
H
O
B⁻
AH
H₂O
H₂O
H
C≡N
HX
H
O
N
H
C
H
H
H

appreciably alter this time. The specific activities of the ricinine nitrilases obtained from sonication extracts of induced bacteria were slightly less than a third of that obtained with ricinine grown cells.

The specific activities of the ricinine nitrilases in sonication extracts of ricinine grown bacteria were also shown to be dependent upon the bacterial source. F5S was least active, F3M approximately three times more active and F1S approximately seven times more active than F5S. It was observed that the final rate of oxygen consumption of ricinine induced cells was approximately equal. Assuming that the differences in specific activities of the ricinine nitrilases were not due to the sonication extraction procedures, one can conclude that at least for F3M and F1S type bacteria, the conversion of ricinine to ricinine acid is not the rate limiting step in ricinine utilization.

A comparison between the maximum growth attained by F5S on glucose or ricinine indicated that ricinine was only about a third as efficient as was glucose in providing carbon for cellular growth. This effect was not due to a toxic reaction of the bacteria to a ricinine by-product. From this data one can roughly estimate that 2 to 3 carbons of the 8 carbon atoms of ricinine are actually being used by the cells for growth. It is possible that these carbons used for growth could be obtained without opening the pyridone ring, i.e. utilization of the nitrile and/or the

two methyl carbons.

Metabolism of ricinine or ricinine acid by ricinine grown F5S resulted in the consumption of 4.5 to 5 moles of oxygen per mole of substrate. This strongly indicated that ricinine acid is a true intermediate in ricinine catabolism. The theoretical consumption for the complete oxidation to CO_2, H_2O and NH_3 of either compound is 7.5 moles. There was an oxidation efficiency of 60 to 66%. The oxidation efficiency for the oxidation of nicotinic acid by Pseudomonas fluorescens has been reported to be 69 to 72% (41, 65) and for nicotine oxidation by Pseudomonas convexa to be 58% (66). Thus, as with the pyridine ring compounds nicotinic acid and nicotine, the oxidation of ricinine is not complete. The oxidative bacterial metabolism of nicotine (67) and nicotinic acid (68) resulted in an opening of the pyridine ring through a pyridone formation. Because of the similarity in the levels in oxygen consumption between ricinine, nicotinic acid and nicotine, the implication is that the ricinine ring could also be oxidatively opened during metabolism.

The lack of ability of the ricinine grown F5S to oxidize numerous pyridine and pyridone analogues indicates the high degree of specificity of the ricinine metabolizing system. This study also indicates that any ring opening should come early during this ricinine metabolism, i.e. before removal of many, if any, of the ring substitutes.

It should especially be noted that nicotinic acid (41, 65, 69, 70) and N-methylnicotinic acid (66), both of which have been reported to be oxidatively utilized for bacterial growth, were not oxidized by F5S cells which had been prepared for ricinine metabolism.

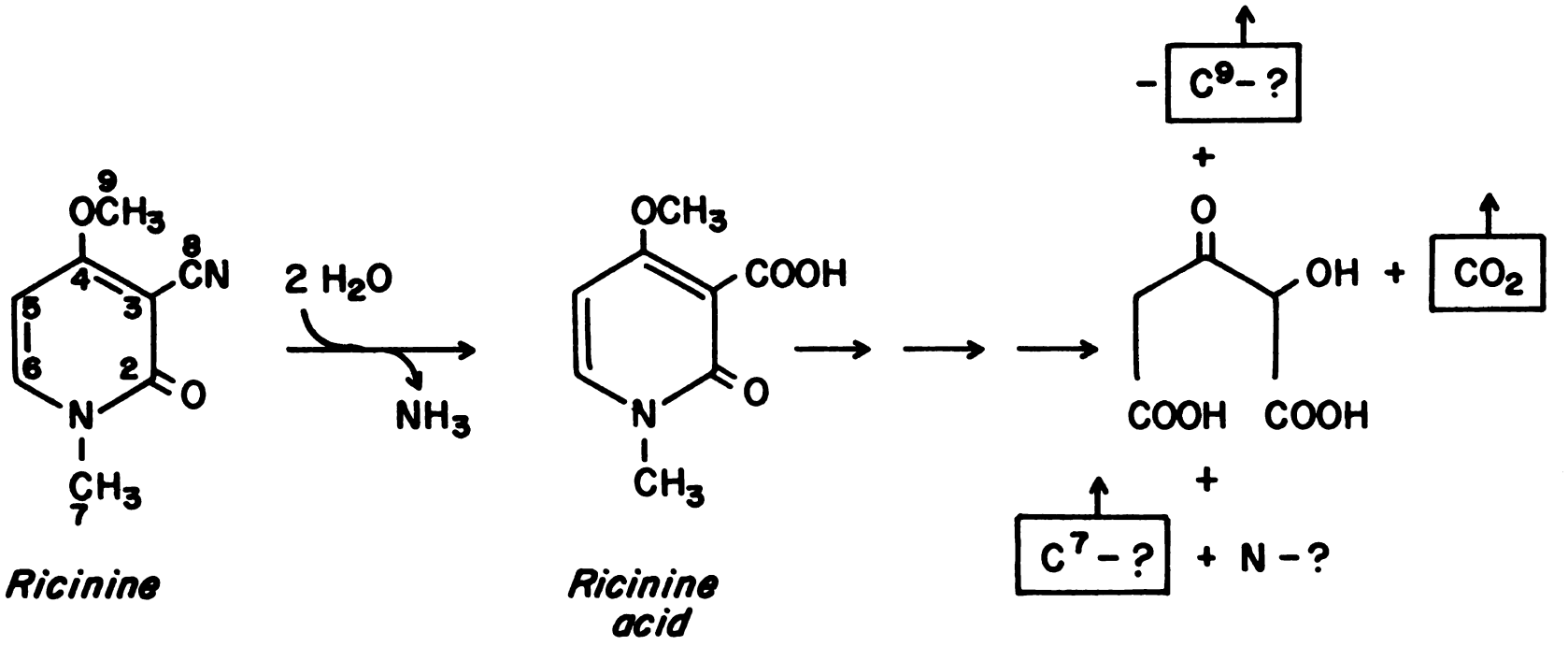
The greatest insight into the overall utilization of ricinine by the bacteria was gained through the utilization of radioactive substrate. The six position in the ring and the carbon of the nitrile group were essentially completely oxidized to CO_2 and at a rate and time only slightly behind the removal of the radioactivity from solution. The indication is that both of these carbons are lost directly from the alkaloid and did not contribute to the actual carbon economy of the cells. Ricinine equally labelled in the two methyl groups also failed to cause the incorporation of radioactivity into the cellular fraction. Half of the label seemed to be oxidized directly to CO_2 and the remaining part formed a volatile compound which was lost into the medium. It is most logical to assume that one of the methyl groups contributed all of the volatile unknown and the other methyl the CO_2. To account for data concerning ricinine-2,3,8-^{14}C one must consider the fact that the labelling of the pyridine ring by the feeding of acetate to plants does result in a low degree of randomization of carbon-14 in positions other than the 2,3 atoms of the ring and the carbon substituent at the 3 position (57-59, 71). Some of

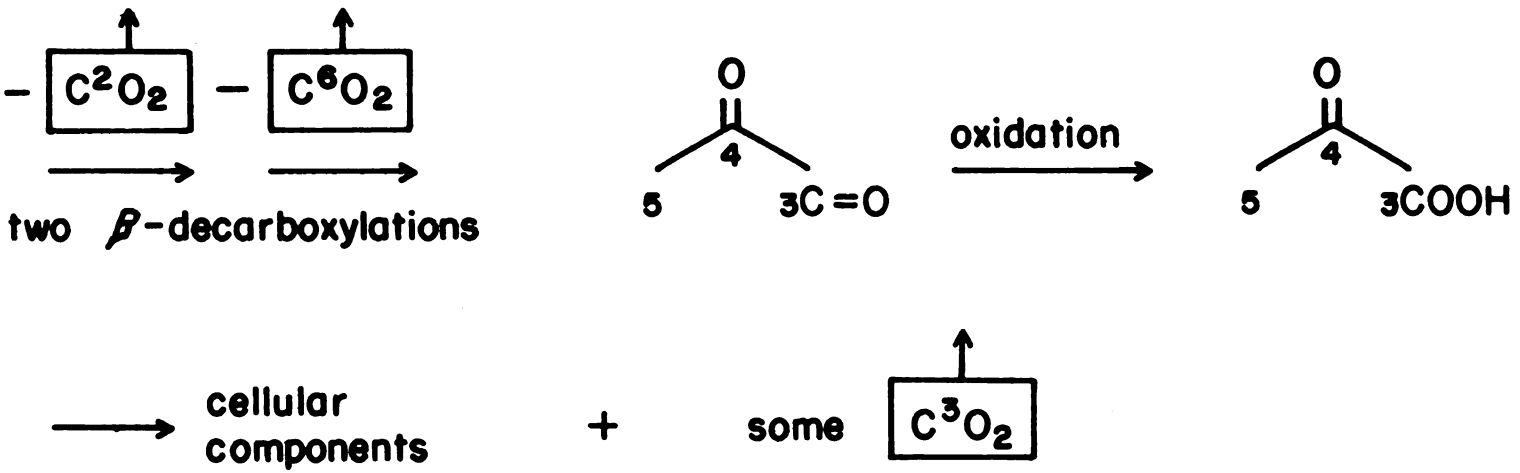
these carbons could contribute to the 73% found at CO_2. With this considered, the value of 73% as CO_2 can be interpreted to mean about two thirds of the 2,3,8 labelled ricinine formed $^{14}CO_2$ directly from ricinine oxidation. The most logical carbons contributing this radioactivity are 2 and 8. The remaining carbon was most likely utilized for cellular growth and the formation of some extracellular products and some CO_2. Considering the comparative study between F5S growth on glucose and ricinine, a reasonable conclusion would be that only carbons 4 and 5 and perhaps much of carbon 3 of ricinine actively contribute to the carbon economy of the bacteria.

Previously, the following carbon products have been shown or proposed as being "common" carbon intermediates derived from the bacterial metabolism of the pyridine portion of pyridine ring compounds: Nicotinic acid to yield $\overset{2}{C}H_3-\overset{3}{C}H_2-\overset{7}{C}OOH + \overset{4}{C}H_3-\overset{5}{C}H_2-\overset{6}{C}OOH$ (72), nicotinic acid to yield $\overset{2}{H}\overset{7}{C}OOH + \overset{7}{C}O_2 + NH_2-\overset{6}{C}(O)-\overset{5}{C}H=\overset{4}{C}H-\overset{3}{C}OOH$ (41, 68), nicotine to give $\overset{2}{H}\overset{7}{C}OOH + NH_2-\overset{6}{C}(O)-\overset{5}{C}H=\overset{4}{C}H-\overset{3}{C}OOH$ (67) and nicotine to give $HOO\overset{6}{C}-\overset{5}{C}H_2-\overset{4}{C}H=\overset{3}{C}H-\overset{2}{C}OOH$ (73). The data concerning the bacterial metabolism of ricinine cannot readily be accounted for through the formation of any of these compounds and thus, necessitates the proposal of a new pathway. On the assumption that the carboxyl carbon is directly replaced by a hydroxyl group as in nicotinic acid metabolism (41) and that the methoxy oxygen is retained on the ring carbon, the

hypothetical scheme seen in Figure 19 can be constructed which accounts for the observed data as well as yields a "common" carbon compound for cellular growth.

Figure 19. The proposed scheme for the microbial utilization of ricinine as the sole carbon source

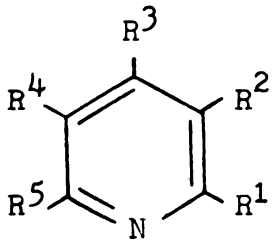

OCH3
CN
2 H2O
NH3
COOH
O
N
CH3
Ricinine
Ricinine acid
- C9 - ?
+
OH
+ CO2
COOH COOH
+
C7 - ?
+ N - ?


- C2O2 - C6O2
two β-decarboxylations
oxidation
3C=O
3COOH
cellular components
+
some C3O2

THE MASS SPECTROMETRY OF PYRIDINE COMPOUNDS

INTRODUCTION

The mass spectral fragmentation patterns of many homocyclic aromatic acids, amides, and nitriles have been well documented and cited in various books on mass spectrometry. A survey of the literature revealed only scant knowledge concerning the effects of inserting a hetero-atom into the basic carbon ring structure of these types of aromatic compounds. Our laboratory's interests in the chemistry and biochemistry of pyridine compounds prompted the investigation presented in this paper. The major emphasis was placed on structural determination from the mass fragmentation patterns of mono- and di-carboxylic acid pyridines and the effects of amidation, hydroxylation and dehydration on the spectra. The following compounds were examined:



		R^1	R^2	R^3	R^4	R^5
I	(isonicotinic acid)	H	H	COOH	H	H
II	(nicotinic acid)	H	COOH	H	H	H
III	(picolinic acid)	COOH	H	H	H	H
IV	(cichomeronic acid)	H	COOH	COOH	H	H
V	(quinolinic acid)	COOH	COOH	H	H	H
VI	(dipicolinic acid)	COOH	H	H	H	COOH
VII		COOH	COOH	OH	H	H
VIII	(nicotinamide)	H	$CONH_2$	H	H	H
IX		COOH	$CONH_2$	H	H	H
X		$COOCH_3$	$CONH_2$	H	H	H
XI	(quinolinimide)	O=C ——	N(H)C=O	H	H	H
XII	(nicotinonitrile)	H	CN	H	H	H
XIII		COOH	CN	H	H	H

MATERIALS AND METHODS

All organic chemicals were purchased from Aldrich Chemical Co. except for VII, IX, XI and XIII. These four compounds were prepared according to procedures described in the Methods section of Chapter I.

The spectra were obtained with the assistance of Mr. Jack Harten and Dr. C. C. Sweeley, Department of Biochemistry using a direct probe with an LKB Gas Chromatograph-Mass Spectrometer 9000. The ion source temperature was 290° and the electron bean energy was 70 eV. High resolution spectrometry was courtesy of Upjohn Company.

RESULTS AND DISCUSSION

Monocarboxylic Acids of Pyridine

The spectrum of I as seen in Figure 20A has the major fragmentation ions falling into four main mass groups. The strong mass (parent) ion at m/e 123 is due to the known high resonance stabilizing effects of an aromatic ring on an ion under the conditions of mass spectrometry (74a, 75). The fragment group at m/e 105 to 106 is most easily accounted for by a loss of H_2O and -OH from the acid; the loss of H_2O being the more dominant peak. This dominance is in contrast to that found for benzoic acid in which the [M-OH] group is greater (75). Through a loss of the carboxyl group from the parent ion, the "naked" pyridine ring is obtained. The m/e 77 to 79 group contained this type of fragment. With I, it is evident that the complete loss of COOH is the dominant reaction in this group. The fourth group of peaks found in the area m/e 50 to 52 is most readily explained by an opening of the "naked" ring and subsequent loss of HCN. HCN loss from a pyridine type nucleus has been well documented (74b, 76-78). It should be noted that there is no direct loss of HCN from the parent ion. Absolute interpretation of the fragmentation sequences was hampered by a lack of metastables, but due to the simplicity of the spectrum the following two compet-

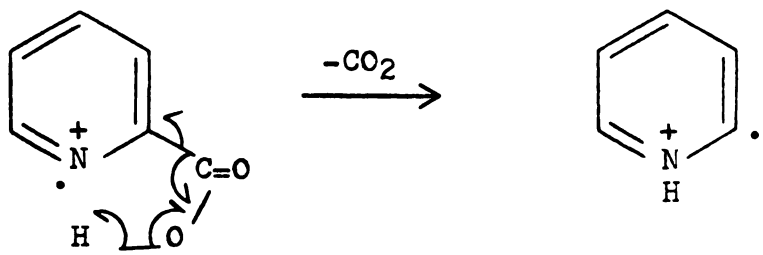
ing reactions can be proposed:

$$M^+ \text{ (m/e 123)} \xrightarrow{-H_2O} \text{m/e 105}$$

$$M^+ \text{ (m/e 123)} \xrightarrow{-CO_2H} \text{m/e 78} \xrightarrow{-HCN} \text{m/e 51}$$

With only very small variations in the relative abundance of a few peaks, the spectrum of nicotinic acid (II) is almost identical to that if I (Figure 20B). Thus, the transition patterns found under mass spectral conditions for both β- and γ-pyridine acids are the same and cannot be used to readily differentiate between the location of the two acid groups on the ring.

The most distinguishing feature of the spectrum of the α-pyridine acid (III) is the extremely low amount of parent ion (Figure 20C). The mass ion, found at M-44, is best explained through a hydrogen rearrangement loss of the carboxyl group.



m/e 123 m/e 79

Similar rearrangements have been noted for α-methylpyridine (76). The retention of the hydrogen with carboxyl loss demonstrates that not only [M-H_2O] as found in the spectra of I and II, but also [M-OH] is a competing rather than a sequential reaction toward carboxyl loss. This is in

contrast to that of benzoic acid (74c). The major ion in the m/e 50 to 52 group is 27 mass units less than the dominant ion in the m/e 77 to 79 group. Even without the aid of metastables, the most important transition from parent ion to "naked" ring to ring splitting loss of HCN is evident. As with I and II, α-pyridine acid shows no sign of HCN loss until the ring's substitutes are removed. The major differences in the relative abundance in fragmentation patterns of I and II and III allow for the differentiation between α and β or γ pyridine monocarboxylic acids.

Pyridine Dicarboxylic Acids

The addition of a second carboxylic acid group to the pyridine ring resulted in complications of the mass spectrum (Figure 20D). The parent ion of IV, though small, is easily detectable at m/e 167 (Figure 20D). This phenomenon could easily have been due to the analogous situation with phthalic acid in which inlet dehydration occurred (75). In support of this conclusion is the similarity between the spectrum of 2,3-pyridinedicarboxylic anhydride (79) and that of IV which amply justifies the following transitions:

$$M^+ \text{ (m/e 167)} \xrightarrow{-H_2O} \text{m/e 149} \xrightarrow{-CO_2} \text{m/e 105}$$

$$\xrightarrow{-CO} \text{m/e 77} \xrightarrow{-HCN} \text{m/e 50 .}$$

In addition, there is a 20 per cent mass unit at [M-CO_2] (m/e 123). As with the monocarboxylic acids of pyridine, there is no evidence for ring opening with HCN removal until all of the ring substitutes have been removed.

The presence of one of the carboxylic groups of the diacid pyridine α to the ring nitrogen as in V completely changes the relative contribution of the mass fragments (Figure 20E). The parent ion is almost nonexistent. A direct dehydration reaction from the ortho carboxyl groups contributes very little. The most prominent mass is M-44 which by analogy to the α-pyridine acid (III) is a result of a hydrogen rearrangement from the α-carboxyl group to the ring nitrogen. It should be noted that with ortho carboxylic acids (V), there is no [M-COOH], whereas with the α-mono acid (III) the M-45 due to a loss of COOH is 38 per cent. This phenomenon demonstrates the high degree of steric hinderance caused by the addition of a second acid group ortho to the α-carboxyl. In addition, it shows that the position of the hindered α-acid group is such that there is maximum interaction between the ring nitrogen and the -OH hydrogen. The m/e 123 fragment tends to lose water to give m/e 105 similar to I and II. The ions in the m/e 77 to 79 group are almost equal in intensity which, without metastables, result in too much uncertainty to attempt to account accurately for all the

transitions leading to their formation. The mass group (m/e 50 to 52) resulting from a loss of HCN from the "naked" ring exhibited a similar degree of complication. There is no direct loss of HCN from any substituted pyridine peaks.

The spectrum of VI again exhibits a complete domination by the fragment resulting from the rearrangement loss of COOH (Figure 20F). There is almost no parent, [M-OH] or [M-H_2O] ion. The remainder of the fragmentation pattern, with the exception of some intensities, closely resembles that of V. The dominant ion in the m/e 50 to 52 group (m/e 51) is best explained for both V and VI as having originated from a "naked" group ion which had retained a rearrangement hydrogen (m/e 78-HCN) rather than from the dominant m/e 77 ion in that group. It is of interest with VI that for the m/e 50 fragment to form by m/e 77-HCN, a hydrogen which was not originally adjacent to the ring nitrogen would have to have been used to form the HCN. Such types of hydrogen scrambling has been previously observed (80). VI did not have m/e 79 as the dominant "naked" group ion which would have had to result from a double hydrogen rearrangement double decarboxylation. This would be expected if most of the hydrogen which rearranged with the first carboxyl loss remained on the ring nitrogen blocking it from participation in further hydrogen rearrangements.

The addition of an oxygen (VII) to a dicarboxylic pyridine (V) to form the 4-pyridone does not greatly alter the basic fragmentation pattern previously observed for a pyridine diacid with an α-carboxyl, but only shifts the peaks by one oxygen mass unit (Figure 20G). The major differences are a decrease in the intensities of the m/e group due to "naked" ring loss of HCN because of the competing CO loss (77) from this same group and an intense unidentified peak at m/e 44.

Thus, with mass spectrometry one has a powerful tool to determine the presence of a carboxyl group adjacent to the pyridine ring nitrogen; however, it cannot readily determine the number of these groups.

Cyano- and Amido-Pyridine Compounds

Because of the interesting behavior of an α-carboxylic pyridine group in mass spectrometry, it was decided to extend the study to the effects of this acid group on ring substitutes other than acids and visa-versa.

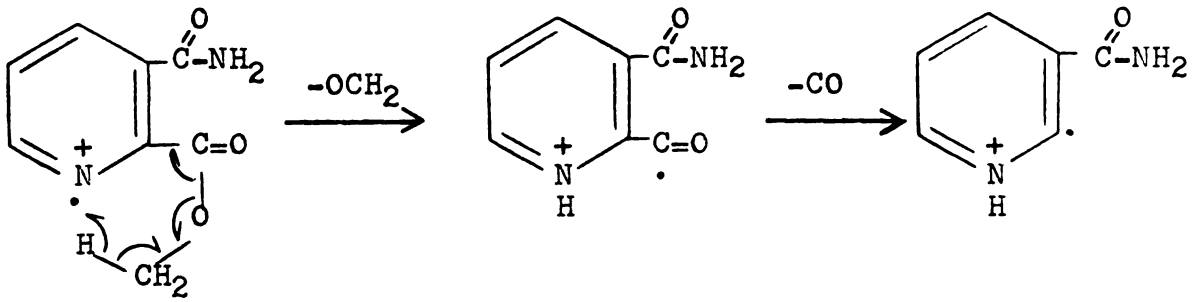
The fragmentation pattern of the biologically important compound nicotinamide (VIII) (Figure 20H) containes four major peak groupings quite similar to the corresponding acid. There is very little [M-H_2O] as found under certain dehydrating condition with benzamide (81a). By analogy with the mass spectrum of benzamide (81b) and with the addition of pyridine ring cleavage the following dominant transitions are evident:

M^+ (m/e 122) $\xrightarrow{-NH_2}$ m/e 106 $\xrightarrow{-CO}$ m/e 78 $\xrightarrow{-HCN}$ m/e 51.

Unlike benzamide, the mass ion is the unsubstituted ring (m/e 78) rather than the fragment due to amine radical lost.

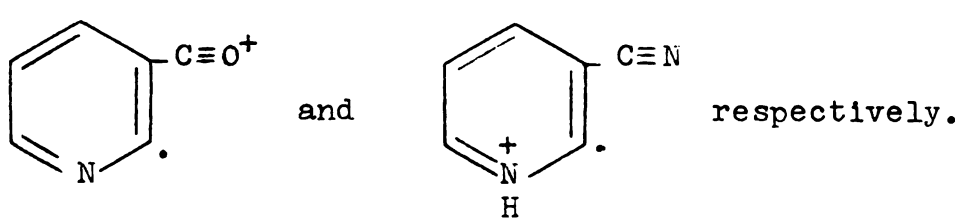
The addition of an α-carboxyl group to VIII to give IX results in a spectrum with almost no parent ion (Figure 20I); a phenomenon previously found with other α-carboxylic pyridines. The mass ion at M-44 was shown by high resolution mass spectrometry to be $C_6H_6N_2O$ resulting from a loss of CO_2 rather than $CONH_2$ from the parent ion. The presence of an adjacent amide group does not alter the hydrogen rearrangement during the loss of the α-carboxyl group. As with V, the lack of metastables renders the accurate interpretation of the transitions involved in the formation of the multiple peaks at m/e 105 to 106 and m/e 77 to 79 impossible. The fragment at m/e 78 was C_5H_4N, thus, as in the spectra of pyridine acids, it consists of the "naked" ring. It is of interest that the m/e 50 to 52 group is very unexplainably weak in intensity.

X (Figure 20J), the methylated form of IX, again exhibits the hydrogen rearrangement phenomenon under the conditions of the mass spectrometer. High resolution determinations showed m/e 150 to be $C_7H_6N_2O_2$ ([M-OCH_2]) and m/e 122 to be $C_6H_6N_2O$ ([M-$COOCH_2$]), both of which can be readily explained through the familiar McLafferty rearrangement.



m/e 180 m/e 150 m/e 122

As with other α-acids, the mass ion is a result of the hydrogen rearrangement loss of the complete carboxyl group. The greater stability of the parent ion of the ester of IX allows sufficient time for some loss of an amine radical (m/e 164) and an amide (m/e 136). The fragment at m/e 149, shown to be $C_7H_5N_2O_2$, is obviously due to a simple loss of OCH_3 (75). The abundant ion at m/e 105 was found to be composed of C_6H_3NO and $C_6H_5N_2$ in a ratio of 2 to 1. These ions can be represented as follows:



In both IX and X a significant amount of m/e 105, best explained by m/e 122-NH_3 , is observed. This ion is insignificant in VIII. Thus, there is the strong possibility that the amide nitrogens also actively participate in the hydrogen rearrangement loss of the α-carboxylic acid and ester. This conclusion is further supported by the

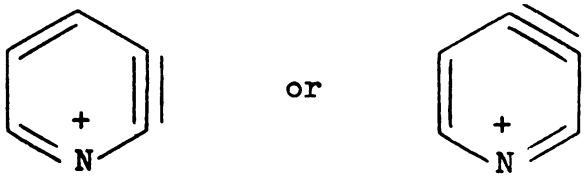
relatively large abundance of m/e 77 in both IX and X as compared to VIII due to a complete loss (including carboxyl and amide group hydrogens) of all ring substitutes.

The dehydration form of IX, XI, as seen in Figure 20K exhibits the strongest parent ion of any of the α-substituted compounds. This stabilizing effect was even more significant than that observed for the dehydrated form of V (79) or with phthalic acid (75). By analogy to these two compounds and the fragmentation of other imides (82-84) the transitions are easily accountable.

$$M^+ \text{ (m/e 148)} \xrightarrow{-CHNO} \text{m/e 105} \xrightarrow{-CO} \text{m/e 77} \xrightarrow{-HCN} \text{m/e 50}$$

With the side group hydrogen removed from proximity of the ring nitrogen, the contribution from hydrogen rearrangement (m/e 78) is insignificant.

Figure 20L showing the spectrum of XII shows that the mass and parent ion are the same (m/e 104). The largest peak in the "naked" ring group is m/e 77 resulting from M-HCN . Since none of the previously discussed pyridine compounds lose HCN directly from the parent ion, one must conclude that the HCN initially lost by XII gives the ion.



Benzonitrile (74d) and pyrolized benzamide (81a) also exhibit these types of spectra. Further loss of HCN from ring opening accounts for the dominant peak in the m/e 50 to 52 group.

The presence of the nitrile ortho to the α-carboxylic acid in XIII stabilized the parent ion (Figure 20M). There is some direct loss of -CN and HCN but as with the other α-pyridine acid, the mass peak at m/e 104 is the result of a hydrogen rearrangement loss of the carboxyl group. Loss of HCN from this group gives m/e 77 and the ring splitting of another HCN accounts for m/e 50.

The use of the mass spectrometer for the detection of the presence of a carboxyl group alpha to the pyridine nitrogen is effective even when the carboxyl group is esterified or in the presence of an ortho amido or nitrile group. In addition, this study indicated the possibility of some interesting hydrogen transfers involving the carboxyl substitute and the nitrogen of the ortho group which await a more detailed study with stable isotopes.

Figure 20. The mass spectra of the following compounds

A. I (isonicotinic acid)

B. II (nicotinic acid)

C. III (picolinic acid)

D. IV (cinchomeronic acid)

E. V (quinolinic acid)

F. VI (dipicolinic acid)

G. VII

H. VIII (nicotinamide)

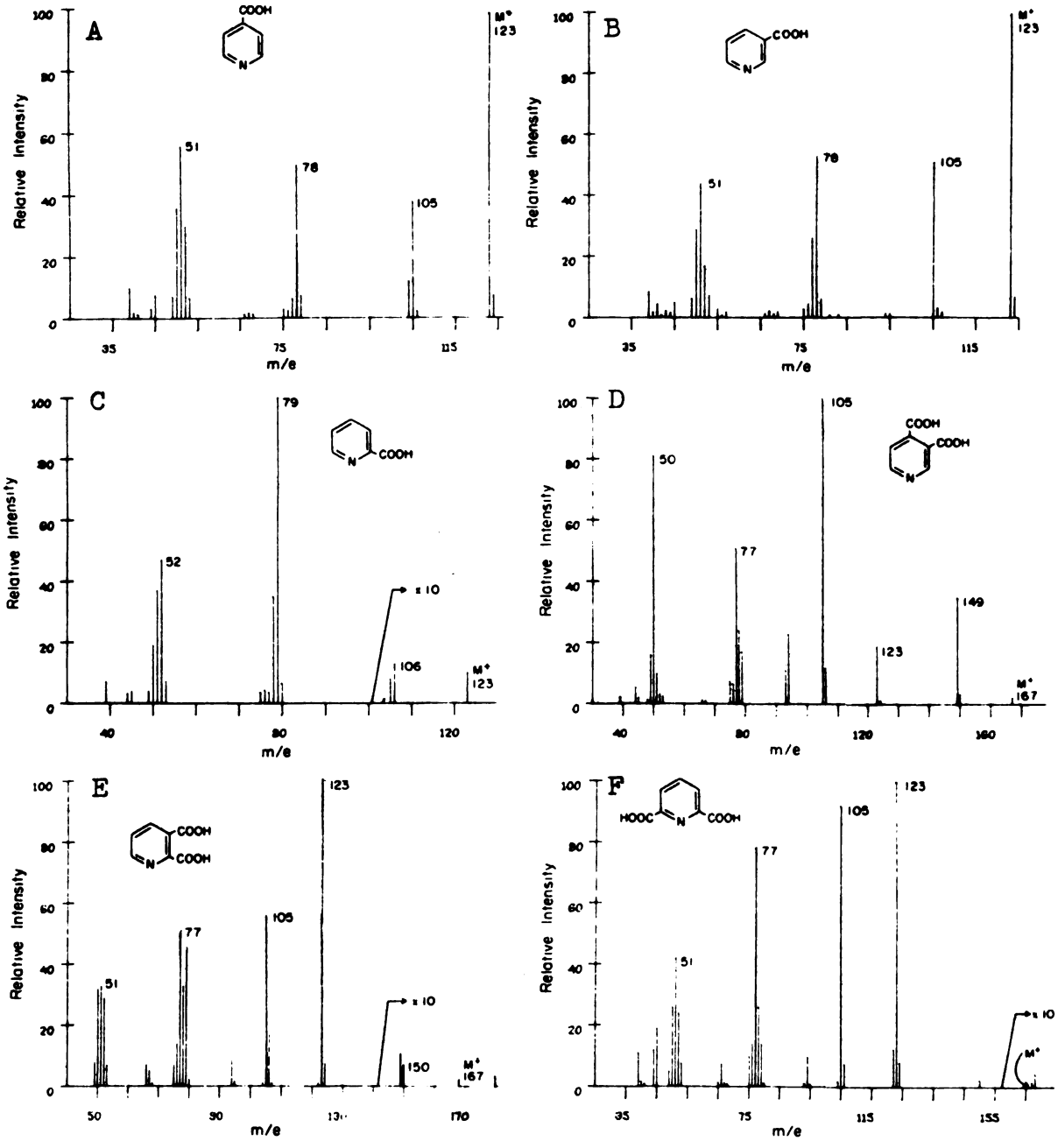
I. IX

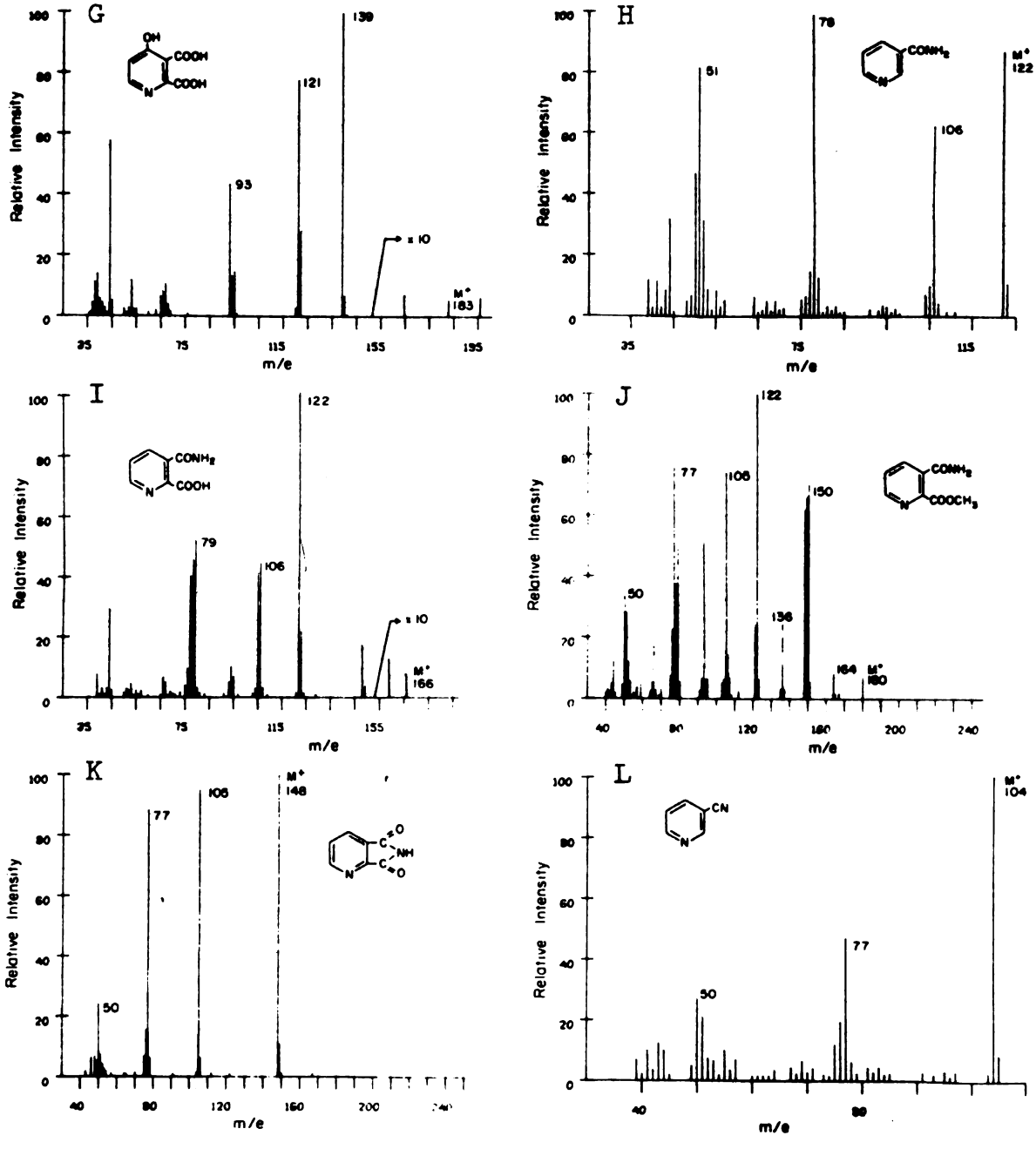
J. X

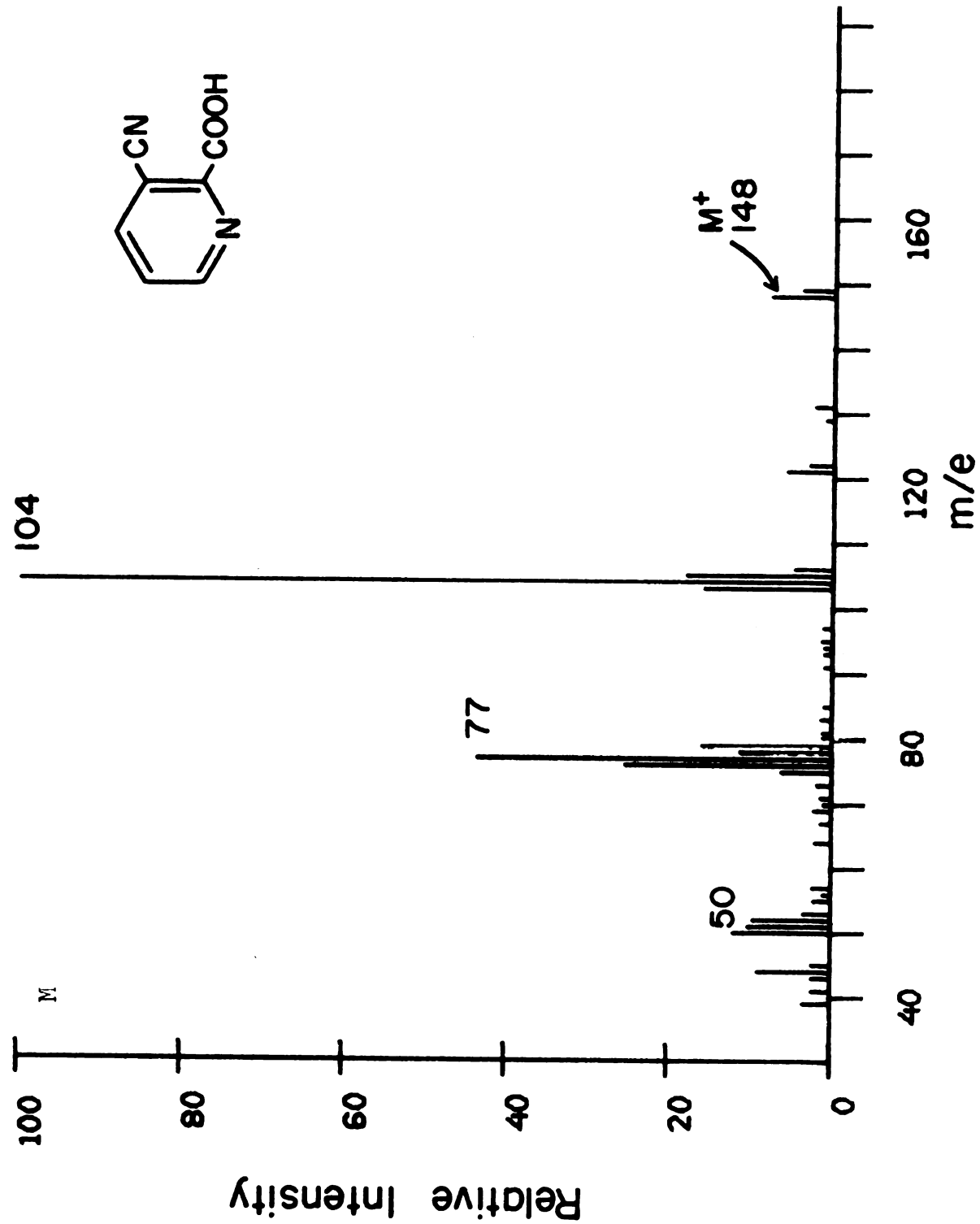
K. XI (quinolinimide)

L. XII (nicotinonitrile)

M. XIII


A
B
C
D
E
F
Relative Intensity
m/e
M+ 123
M+ 167
x 10


G
OH
COOH
COOH
N
139
121
93
x 10
M+
183
Relative Intensity
m/e
H
78
51
CONH2
M+
122
106
I
CONH2
COOH
122
79
106
x 10
M+
166
J
CONH2
COOCH3
122
77
106
150
50
136
164
M+
180
K
M+
148
77
106
O
C
NH
C
O
50
L
CN
M+
104
77
50


Relative Intensity
100
80
60
40
20
0
M
104
77
50
40
80
120
160
m/e
M+
148
CN
COOH
N

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