METABOLISM OF NICOTINIC ACID AND NICOTINAMIDE IN RATS

By

YU-CHIANG LEE

Bachelor of Science

Taiwan Christian College

Taiwan, China

1959

Submitted to the Faculty of the
Graduate College of the
Oklahoma State University
in partial fulfillment of
the requirements for
the Degree of
DOCTOR OF PHILOSOPHY
May, 1969

OKLAHOMA
STATE UNIVERSITY
LIBRARY

SEP 29 1969

METABOLISM OF NICOTINIC ACID AND

NICOTINAMIDE IN RATS

Thesis Approved:

Robert K. Gholson
Thesis Adviser

George R. Waller

K. E. Ebner

E J Eisenbraun

Eric C. Noller

D. D. Durham
Dean of the Graduate College

724954

ACKNOWLEDGMENTS

The author gratefully acknowledges the continual guidance and advice of his major professor, Dr. R. K. Gholson, during the course of these studies and the preparation of the thesis.

Special thanks are due to Drs. George R. Waller, K. E. Ebner, E. J. Eisenbraun, and E. C. Noller for their suggestion and encouragements.

Sincere appreciation is extended to Dr. Jerry L. Chandler for his advice and correction of the manuscript of this thesis; to Dr. K. Yamaguchi for his suggestions and assistance; to Dr. H. Y. Li and K. Kinneberg for mass spectra facilities.

A very special thanks goes to his wife, Joan, for her help, encouragement, and patience exercised during the years of graduate study.

The author is thankful to Oklahoma State University Biochemistry Department for facilities and financial support during this investigation.

TABLE OF CONTENTS

Chapter		Page
I.	INTRODUCTION	1
II.	LITERATURE REVIEW	4
	Historical	4
	Effect of Nicotinic Acid Deficiency in the Human and Dogs	6
	Preparation and Properties	7
	Biosynthesis of Nicotinic Acid and Nicotinamide	9
	Metabolism of Nicotinic Acid and Nicotinamide	14
	Metabolism of Nicotinamide Adenine Dinucleotide	20
	The Pyridine Nucleotide Cycle	25
	Control of NAD^+ Biosynthesis	28
	Biodegradation of Pyridine Nucleotide . .	30
III.	EXPERIMENTAL	33
	Chemicals	33
	Animals	34
	Methods	35
IV.	RESULTS AND DISCUSSION	43
	Results	43
	Discussion	66
V.	ISOLATION AND IDENTIFICATION OF TWO NEW URINARY NICOTINAMIDE METABOLITES	73
	Isolation of Unknown Metabolite	73
	Identification of Unknown Compound . . .	74
VI.	SUMMARY	106
BIBLIOGRAPHY	. . . .	108

LIST OF TABLES

Table		Page
I.	Descending Paper Chromatography of the Metabolites of Nicotinic Acid and Nicotinamide	45
II.	Radioactivity Distribution Among Urinary Metabolites of Normal and Hypophysectomized Rats Following the Injection of 500 or 5 mg/kg of Nicotinamide-7-^{14}C	65
III.	Radioactivity Distribution Among Urinary Metabolites of Normal and Hypophysectomized Rats Following the Injection of 500 or 5 mg/kg of Nicotinic Acid-7-^{14}C	67
IV.	Recrystallization of Urinary Radioactive Unknown Metabolites of Germ Free Rats With Authentic Carrier	103
V.	A Comparison of the Excretion of Radio-activity in the Urine and Expired CO_2 of Germ Free and Normal Rats 24 Hours After Intraperitoneal Administration of Nicotinic Acid-7-^{14}C and Nicotinamide-7-^{14}C	104
VI.	The Distribution of Radioactivity in the Urinary Metabolites of Germ Free Rats Following the Administration of Nicotinamide-7-^{14}C and Nicotinic Acid-7-^{14}C (Per Cent of Injected Dose)	105

LIST OF FIGURES

Figure		Page
1.	Metabolism of Tryptophan Leading to Formation of Nicotinic Acid and Nicotinamide	12
2.	Biosynthetic Pathways of Nicotinamide Adenine Dinucleotide and the Pyridine Nucleotide Cycle	26
3.	Chromatographic Separation of the Metabolites of Nicotinic Acid in Liver Extracts on a Dowex-1x8 Formate Column	48
4.	Total Radioactivity Recovered in the Liver of Hypophysectomized and Normal Rats After Portal Vein Injection of ^{14}C Labeled Nicotinic Acid and Nicotinamide	51
5.	The Metabolism of Nicotinic Acid in the Liver of Hypophysectomized Rats After Intraportal Injection of Nicotinic Acid-7-^{14}C	53
6.	The Metabolism of Nicotinic Acid in the Liver of Normal Rats After Intraportal Injection of Nicotinic Acid-7-^{14}C	55
7.	The Radioactivity Distribution of Nicotinamide Metabolites in the Liver of Normal and Hypophysectomized Rats After Intraportal Injection of 210 mμ Moles Nicotinamide-^{14}C	59
8.	The Excretion of Total Radioactivity in the Urine of Normal and Hypophysectomized Rats Following the Injection of 500 mg/kg or 5 mg/kg Nicotinic Acid-7-^{14}C	61
9.	The Excretion of Radioactivity in the Urine of Normal and Hypophysectomized Rats Following the Injection of 500 mg or 5 mg of Nicotinamide-7^{14}C	63

Figure Page

10. Isolation of Unknown Compound From the Urine of Nicotinic Acid Injected Rats by Dowex-50-H^+ Column Chromatography 76

11. Mass Spectra of Authentic 6-Hydroxynicotinic Acid and the Compound Isolated From the Urine of Nicotinic Acid Injected Rats 78

12. Proposed Fragmentation Pattern of 6-Hydroxynicotinic Acid Under Electron Impact 80

13. Ultraviolet Absorption Spectra of Authentic 6-Hydroxynicotinic Acid and the Unknown Isolated From the Urine of Nicotinic Acid Injected Rats 83

14. Infrared Spectra of Authentic 6-Hydroxynicotinic Acid and the Unknown Compound Isolated From the Urine of Nicotinic Acid Injected Rats 85

15. Isolation of Unknown Metabolite From the Urine of Nicotinamide Injected Rats 88

16. Dowex-1-Formate Column Chromatographic Separation of Radioactive Peak I From Figure 15 90

17. Ultraviolet Spectra of A, Authentic 6-Hydroxynicotinamide and B, Unknown 92

18. Infrared Spectra of Authentic 6-Hydroxynicotinamide (Upper Spectrum) and the Unknown Compound Isolated From the Urine of Nicotinamide Injected Rats 95

19. Mass Spectra of Authentic 6-Hydroxynicotinamide and the Compound Isolated From the Urine of Nicotinamide Injected Rats 97

20. Proposed Fragmentation Pattern of 6-Hydroxynicotinamide 99

21. Paper Strip Counter Recording of the Descending Paper Chromatogram From a Sample of Germ Free Rat Urine After Dowex-50-H^+ Column Chromatography 102

CHAPTER I

INTRODUCTION

Since the discovery of nicotinamide as one of the hydrolysis products of coenzymes I and II (1, 2) and the finding that nicotinic acid and nicotinamide were active in the cure and prevention of canine blacktongue (3, 4) and human pellagra (5, 6), the metabolism of these compounds has been extensively studied. Five urinary metabolites of niacin have been isolated and identified in mammalian species. These are N^1-methylnicotinamide, nicotinamide-N-oxide, N^1-methyl-2-pyridone-5-carboxamide, N^1-methyl-4-pyridone-3-carboxamide and nicotinuric acid. These same metabolites are formed from both nictonic acid and nicotinamide although the proportion differs with the compound administered and the size of the injected dose.

The biosynthesis of nicotinamide adenine dinucleotide from these vitamins has also been well established (7). In recent years, Greengard and coworkers (8) reported that hypophysectomy before administration of nicotinamide results in an increase of both the degree and duration of the elevated NAD^+ concentration in liver. Further studies have established that the pituitary gland may regulate NAD^+ biosynthesis through its control of the thyroid and

adrenal glands (9). The same workers also reported that nicotinamide deamidase activity in the liver also was increased by hypophysectomy and that the rate of the excretion of injected nicotinamide in hypophysectomized rats was much slower than that in normal rats (10). From these results, they concluded that the increase in nicotinamide deamidase activity was responsible for the increase in the duration of the elevation of NAD^+ concentration which follows the administration of nicotinamide. However, there should be other factors which effect the NAD^+ concentration in hypophysectomized rats, because the injection of nicotinic acid and tryptophan also resulted in an increased of NAD^+ concentration in hypophysectomized rats (11). If the effect of hypophysectomy on NAD^+ bisynthesis is to increase nicotinamide deamidase activity in liver, then there should be no effect on increasing NAD^+ concentration in hypophysectomized rats by injection of nicotinic acid or tryptophan, because nicotinamide deamidase is not involved in the biosynthesis of NAD^+ from these compounds (12).

This dissertation will report investigation of possible factors which may control the NAD^+ concentration in hypophysectomized rats by comparison of the differences in normal and hypophysectomized rats on the excretion of radioactivity in the urine following intraperitoneal injection of different levels of nicotinic acid-7-^{14}C and nicotinamide-7-^{14}C by studying the metabolism of these

radioactive vitamins in the liver at short times after intraportal injection. During the course of these studies, two new urinary metabolites of nicotinic acid and nicotinamide in normal and germ free rats were discovered, isolated, and identified.

CHAPTER II

LITERATURE REVIEW

Historical

Nicotinic acid was first synthesized in 1867 by Huber (13) by oxidizing nicotine with sulfuric acid and potassium dichromate. However, he only knew the compound had the formula $C_5H_5NO_2$ at that time, and he did not recognize it as pyridine carboxylic acid until 1870 (14). The term "nicotinic acid" was first used by Weidel (15), who by the oxidation of nicotine with nitric acid produced a compound which he assumed to have the composition $C_{10}H_8H_2O_3$. Later, Laiblin (16) showed that this compound was pyridine carboxylic acid and it was identical to the compound made by Huber. In 1879, Weidel (15) produced the same compound from β-picoline and, thus, demonstrated that nicotinic acid is pyridine-3-carboxylic acid.

Almost fifty years elapsed before nicotinic acid was isolated from natural products. Suzuki, Shimamura, and Odake (17) isolated the compound from rice bran. Funk (18) isolated nicotinamide from both yeast and rice polishings during the search for the antiberiberi factor, thiamine, but found the compound had no activity in curing pigion beriberi, so its biological role in nutrition was

at first neglected. Willamn (19), in 1917, impressed by the common occurrence of nicotinic acid and the anti-neuritic vitamin in several natural substances, tested nicotinic acid and trigonelline, as well as other pyridine derivatives, for antineuritic potency but found that none of them caused any permanent improvement in polyneuritic fowls. Szymanska and Funk (20) attributed appetite-stimulating and weight-preserving action to nicotinic acid and the amide.

In 1934, Warburg and Christian (1) isolated nicotinamide from coenzyme II and demonstrated its function as a part of this hydrogen transferring coenzyme (21). Shortly thereafter, Euler, Alberts, and Schlenk (2) obtained nicotinamide from coenzyme II, and it was shown that both coenzymes were nicotinamide adenine dinucleotide, but that coenzyme II contained three molecules of phosphoric acid while coenzyme I contained two. Kuhn and Vetter (22) also isolated nicotinamide from heart muscle. These studies stimulated further interest in the possible nutritional significance of nicotinic acid and its amide. In 1936, Funk and Funk (23) found a large food intake and better growth in rats and pigeons on certain diets when given the acid and especially the amide. Frost and Elvehjem (24) also observed a growth stimulus from nicotinic acid when fed with adenylic acid to rats on purified diets. In 1937, Koeln and Elvehjem (25) isolated nicotinic acid and its amide by fractionating a liver extract and showed

these compounds have a marked anti-blacktongue activity in dogs. The activity of nicotinic acid in the treatment of blacktongue was soon verified by a number of workers (3, 4, 25). The beneficial effects of nicotinic acid on pellagrins was reported by Spie _et al_. (5), by Harris (6), and by Smith (27).

Effect of Nicotinic Acid Deficiency in the Human and Dogs

Among the mammalian species, only the dog and human develop syndromes characteristic of a deficiency of nicotinic acid. In other species, this deficiency state is not easily differentiated from other nutritional deficiencies.

In the dog, a deficiency of nicotinic acid results in the syndrome described as canine blacktongue (28). The animal in this state has no appetite for food or water, oral manifestations such as lesions on the inner surfaces of the gums, lips, and cheeks develop, and the tongue assumes a bluish-black color (29). The nerves in the area of these lesions degenerate, and extensive histological changes take place in the spinal cord and other regions of the nervous system (30, 31).

In the human, a deficiency of nicotinic acid is designated as pellagra. The early clinical signs of deficiency include weakness, lassitude, indigestion and anorexia, usually followed in the course of several months

by dermatitis, diarrhea, dementia, and finally death (32). Body lesions are usually found in areas most subject to mechanical irritation or body secretion. The mental symptoms usually include irritability, headaches, sleeplessness, and the loss of memory. Emotional instability is usually an early sign of pellagra, and advanced cases may develop acute delirium. Nicotinamide is preferred over nicotinic acid as therpeutic agent because of better tolerance. It is used in 50 mg oral doses ten times daily for the treatment of the various stages of the human pellagra syndrome and for overcoming toxicity symptoms incurred during sulfonamide therapy.

The specific requirement for nicotinic acid in man has been difficult to assess for many reasons, but a daily dietary intake of less than 7 mg of nicotinic acid is usually associated with pellagra (33). The presently accepted value was calculated on the basis of body weight and caloric intake, and finally by increasing the value by a 50% safety factor. The recommended daily dietary allowance has been set at 18-21 mg equivalent for men, 17 mg for women, and 6-17 mg equivalent for children. Adult dogs and puppies require 200 to 235 µg per kg of body weight per day, respectively (34).

Preparation and Properties

Nicotinic acid was commercially prepared by oxidation of picoline or nicotine with nitric acid, potassium

dichromate, potassium permanganate, etc., as oxidizing agents (13, 15, 35, 36) in earlier times, but now these oxidizing agents have been replaced by air in the vapor phase using a vanadium iron oxide (37). Ciba (38) uses the air oxidation of picoline or quinoline in the vapor phase with vanadium pentaoxide-sulfuric acid catalyst on silica gel to produce nicotinic acid. β-picoline also can be oxidized by means of sulfuric acid in the presence of a selenium compound (39). Nicotinamide was prepared by partial hydrolysis of 3-cyanopyridine (40) with base in the presence of hydrogen peroxide. 3-Cyanopyridine was prepared by treating pyridine with bromine and hydrobromic acid to yield the dark red perbromide; this, when heated with cuprous cyanide at 170°C is converted into 3-cyanopyridine (41).

Nicotinic acid forms white needle-like crystals, of molecular weight 123, which melt at 235-237°C. It is slightly soluble in cold water and ether, but soluble in hot water and alcohol. It behaves as both a base and an acid, forming salts with either inorganic acids or with metals ions. Nicotinic acid also reacts with alkyl halides to form quaternary nitrogen substituted compounds.

Nicotinamide is a white substance, which crystallizes from benzene in needles, m.p. 131°C. It is very soluble in water, 95% alcohol and in glycerol but only slightly soluble in ether. On distillation with phosphorous pentoxide, it yields 3-cyanopyridine and on hydrolysis

with acid or alkali it yields nicotinic acid. On heating in a very dry tube, pryidine is evolved. It can also form N-alkyl compounds of the pyridine nitrogen with alkyl halides.

Biosynthesis of Nicotinic Acid and Nicotinamide

The relationship of nicotinic acid and tryptophan in a number of organisms is well established. In 1945, Krehl (42) observed that tryptophan supported the growth of niacin-deficient rats. Additional studies revealed that addition of tryptophan to the diet resulted in an increase in the excretion of N^1-methylnicotinamide in the urine (43, 44). A number of nutritional studies have shown that tryptophan can replace nicotinic acid in the rat, dog, rabbit, pig, monkey, chick, and in humans (45, 46, 47, 48). Studies with mutant strains of Neurospora crassa (49, 50) and with mammals have shown that the pathway from tryptophan proceeds through kynurenine (51), 3-hydroxy-kynurenine (49), 3-hydroxyanthranilic acid (52, 53, 54), an unstable intermediate, and quinolinic acid (see Figure 1). Further evidence for the metabolic relationship between tryptophan and niacin has come from a study of the metabolism of tryptophan labeled with isotopes at various positions in the molecule (55). The results indicated that position 3 of the indole ring becomes the carboxyl carbon of niacin (55, 56, 57, 58) and that the nitrogen of the indole ring is converted to the pyridine ring

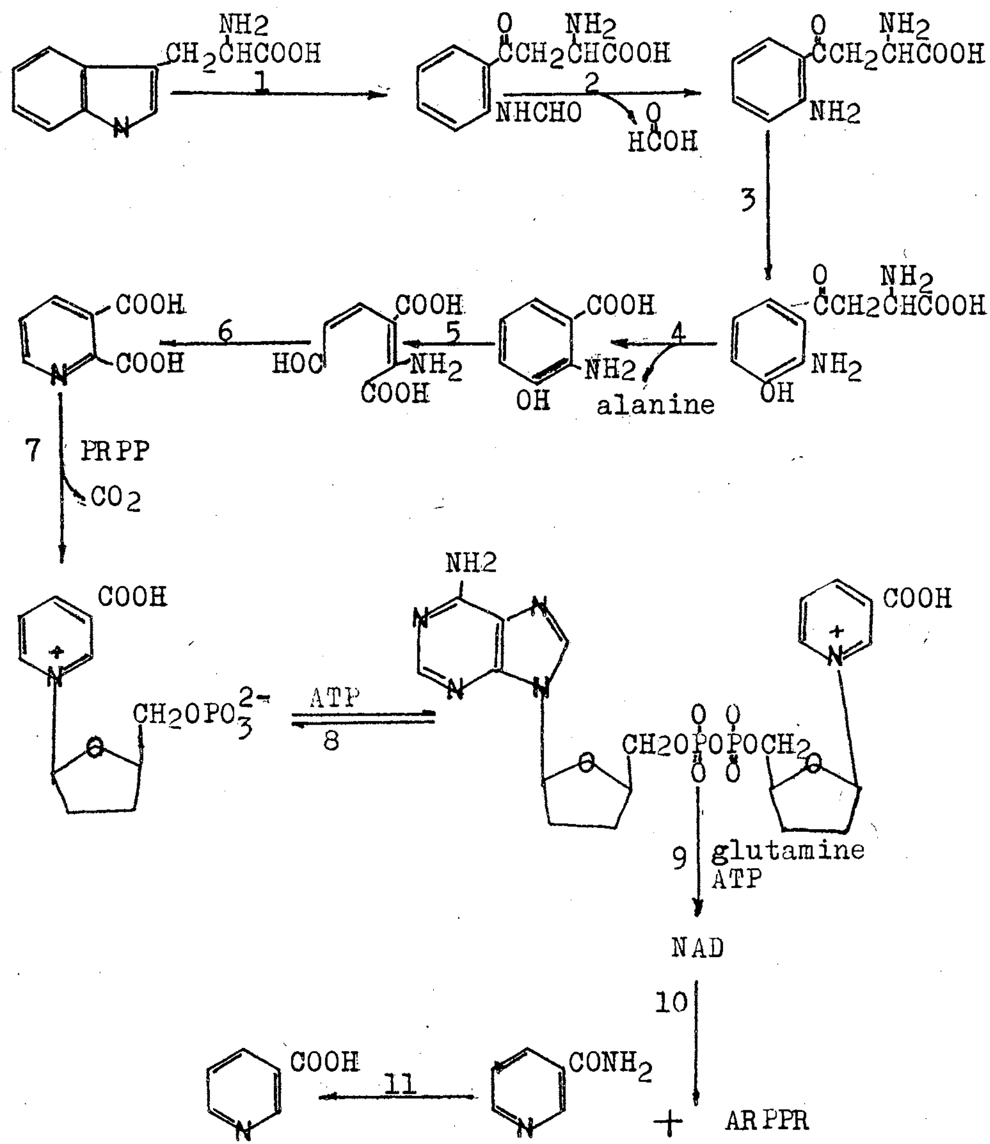
nitrogen of niacin (59, 60). 3-hydroxyanthranilic acid is now well established as an intermediate in the conversion of tryptophan into nicotinic acid. Thus, it can replace tryptophan in promoting the growth of rats (61, 62), but it appears to be converted into quinolinic acid and not nicotinic acid by rat liver slices (60, 63). In 1951, Priest <u>et al</u>. (64) reported that a soluble enzyme system from liver and kidney catalyzes the conversion of 3-hydroxyanthranilic acid to quinolinic acid. Several investigators subsequently showed that the primary product of oxygenation reaction was probably 2-amino-3-acroleyl-fumaric acid and the cyclization of the later resulted in the formation of quinolinic acid (65, 66, 67, 68).

Nishizuka and Hayaishi (69) described an enzyme system from rat liver which converts 3-hydroxyanthranilic acid to nicotinic acid ribonucleotide, when supplemented with 5-phosphoribosyl-1-pyrophosphate. Nicotinic acid ribonucleotide is converted to desamido-NAD in the presence of ATP by pyrophosphorylase, and this compound is then amidated to form NAD^+ in the presence of glutamine and ATP by NAD^+ synthetase (70, 71). The metabolic pathway which leads from tryptophan to the synthesis of NAD^+, which is now known as the "NAD^+ pathway of tryptophan" is shown in Figure 1. NAD^+ can be degradated by diphosphopyridine nucleotidase (NADase, EC 3.2.2.5) to nicotinamide and adenosine diphosphate ribose (72, 73, 74).

Although mammals and <u>Neurospora</u> are able to convert

Figure 1. Metabolism of Tryptophan Leading to Formation of Nicotinic Acid and Nicotinamide

1. Tryptophanpyrolase (EC 1.1.3.12);
2. Kynuenine formylase (EC 3.5.1.9);
3. Kynurenine-3-hydroxylase (EC 1.14.1.2);
4. Kynureninase (EC 3.7.1.3);
5. 3-hydroxyanthranilic acid oxidase (EC 1.13.1.6);
6. Spontaneous;
7. Quinolinate transribosylase;
8. Desamido-NAD pyrophosphorylase (EC 2.7.7.18);
9. NAD^+ synthetase (EC 6.3.5.1);
10. NAD^+ glycohydrolase (EC 3.2.2.5);
11. Nicotinamide deamidase.


NH2
CH2CHCOOH
1
O NH2
CCH2CHCOOH
NHCHO
2
HCOH
NH2
3
4
alanine
OH
COOH
5
HOC
COOH
6
7
PRPP
CO2
CH2OPO3 2-
ATP
8
CH2OPOPOCH2
9
glutamine
ATP
NAD
10
CONH2
11
ARPPR

tryptophan to nicotinic acid via the biosynthetic pathway shown in Figure 1, evidence has accumulated that in bacteria and higher plants the biosynthesis of nicotinic acid is accomplished by a different pathway. Volcani and Snell (75) demonstrated that kynurenine and 3-hydroxyanthranilic acid, both of which are intermediates in the tryptophan pathway, could neither replace nicotinic acid as a growth factor for a member of nicotinic acid requiring species of bacteria nor enhance the growth response of these bacteria to suboptional amounts of nicotinic acid. Stainer and Tsuchida (76) found that Psudomanas species adapted to growth with tryptophan as the sole carbon source lacked the enzyme needed for the oxidation of 3-hydroxyanthranilic acid and, thus, appears to be unable to form nicotinic acid from tryptophan. It has been shown (77, 78, 79) that some microorganism can synthesize nicotinic acid from carbon sources other than tryptophan. Ortega and Brown (80) have shown that nicotinic acid could be synthesized in vivo in E. coli from a 3 carbon compound (glycerol or a glucose metabolite) and a 4 carbon dicarboxylic acid. Other studies with Bacillus subtilis (81) and M. tuberculosis (82) indicated that both aspartate and glycerol could serve as precursors of nicotinic acid in these organisms. Isquith and Moat (78) found that crude extracts of Clostridum butylicum were able to incorporate uniformly ^{14}C labeled aspartate and glycerol in the same manner as did growing cultures.

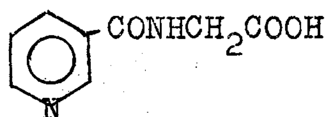
However, upon fractionation of the extract, only aspartate, acetyl-coA and formate gave rise to nicotinic acid. Chandler _et al._ (83) reported that cell free preparations of _E. coli_ are able to convert aspartate-^{14}C and glycerol to quinolinic acid. Degradation of the product formed from uniformly labeled aspartate-^{14}C showed that the ^{14}C was incorporated into carbons 2, 3, 7, and 8 of quinolinate.

Metabolism of Nicotinic Acid and Nicotinamide

Urinary Metabolites of Nicotinic Acid and Nicotinamide

The earliest attempts to study the fate of ingested nicotinic acid and nicotinamide were confined to isolation and identification of various metabolites in different animals species (84-88). In addition to the acid form and amide form of the vitamin, the presence of N'-methylnicotinamide (88, 89), nicotinuric acid (88), nicotinamide-N-oxide (91), N'-methyl-4-pyridone-3-carboxamide (92, 93), N'-methyl-2-pyridone-5-carboxamide (94) have been reported in the urine of various mammalian species, and dinicotinylornithine in the chick (95).

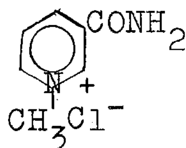
Nicotinuric acid is a major urinary metabolite of nicotinic acid after nicotinic acid is administered to animals. It is formed from nicotinic acid by conjugation with glycine.



This compound constitutes about 60% of tertiary nitrogen derivatives of nicotinic acid excreted by normal rats, but forms a much lower proportion in the case of pantothenic acid deficient rats (94). This suggested that coenzyme-A is involved in the biosynthesis of nicotinuric acid.

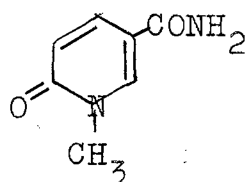
The excretion of N^1-methylnicotinamide in the urine was first noted by Najjar and his coworkers (89, 90). They observed that pellagrins excreted a substance in the urine which fluoresced when treated with strong alkali. This substance, designated as F_1, disappeared when the patients were treated with nicotinic acid and was replaced by another fluorescent substance known as F_2. F_2 is not present in the urine of pellagrins. The administration of nicotinic acid to a normal individual led to a marked increase in the excretion of this second fluorescent substance. They found that in addition to nicotinic acid, nicotinamide, and coramine were able to bring to about this transformation, but not trigonelline, quinolinic acid or pyrazine-carboxylic acid. The excretion of F_1 was also observed in dogs with canine blacktongue; it was replaced by F_2 when dogs were given nicotinic acid (96). Large doses of F_2 were excreted by all animals capable of methylating nicotinamide. In rats, which synthesize

nicotinic acid from tryptophan, F_2 was isolated and shown to be N^1-methylnicotinamide by comparison with the known compound which synthesized by the method of Karrer et al. (97).

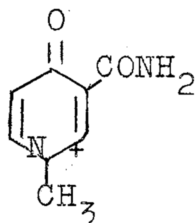


Both compounds formed a picrate, M.P. 189.5°C (96, 97). Nicotinamide was converted into N^1-methylnicotinamide by incubation with rat liver slices, and the extent of the transformation was increased by the addition of methionine (98). Ellinger (99) showed that kidney and muscle did not methylate nicotinamide, the enzyme being present only in liver. Moreover, the enzyme was specific for nicotinamide, and did not methylate nicotinic acid. Liver tissue from rats with a low N^1-methylnicotinamide elimination was only able to convert small amounts of nicotinamide into N^1-methylnicotinamide, the addition of methionine increased the amount formed. An enzyme system, which was partially purified, was isolated by Cantoni (100) and shown to synthesize N^1-methylnicotinamide anaerobically from methionine and nicotinamide in the presence of magnesium ions and an energy, ATP. The enzyme was named nicotinamide methylkinase. The methionine and adenosine triphosphate form S-adenosyl-methionine before the transfer of the methyl group to nicotinamide. N^1-methylnicotinamide

is not the end metabolite of nicotinic acid and nicotinamide, it can be further oxidized *in vivo* to corresponding pyridone. This was demonstrated by Knox and Grossman (93) who isolated an unknown nicotinamide metabolite and identified it as N^1-methyl-6-pyridone-3-carboxylamide (N^1-methyl-2-pyridone-5-carboxamide).



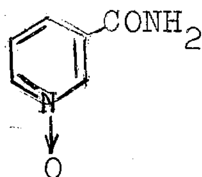
It was biologically synthesized from N^1-methynicotinamide by quinine-oxidizing enzyme of rabbit liver (92, 93) and chemically synthesized by oxidating N^1-methylnicotinamide with alkaline ferricyanide (101). Another nicotinic acid and nicotinamide metabolite also formed by oxidation of N^1-methylnicotinamide and which is present in rat urine in substantial amount has been identified as N^1-methyl-4-pyridone-5-carboxamide (94).



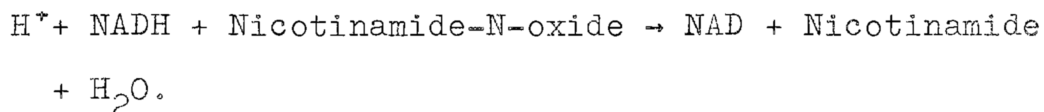
It has since been identified in human urine and monkey urine (94), and in human plasma (102). The enzyme which oxidizes N^1-methylnicotinamide to both N^1-methyl-4-pyridone-3-carboxamide and N^1-methyl-2-pyridone-5-

carboxamide was isolated from hog liver (103). Purification studies of this enzyme have shown that the formation of these two pyridones is catalyzed by the same enzyme (104). This enzyme has similar properties to those which were reported for rabbit liver aldehyde oxidase. The ratio of the 6-pyridone to the 4-pyridone were varied among different animal species, but the ratio was constant for a given species.

Another urinary metabolite of nicotinic acid and nicotinamide is nicotinamide-N-oxide.



This compound was reported by Chaykin et al. (105) in rat liver after injection of nicotinamide. It is also an excretory product of nicotinamide in the mouse (103, 106). The formation of nicotinamide-N-oxide is reversible since it could be reduced to nicotinamide in hog liver (105). The enzyme system which reduced nicotinamide-N-oxide to nicotinamide was isolated from hog liver and purified 280 fold (106). The reduction was dependent on the presence of NADH or a low molecular weight constituent found in boiled liver supernatant. The reaction was as follows:



H^+ + NADH + Nicotinamide-N-oxide → NAD + Nicotinamide + H_2O.

The purified enzyme has the spectrum of a metalloflavoprotein and has xanthine oxidase activity. Milk xanthine oxidase also catalyzed the nicotinamide-N-oxide reduction reaction (107). This evidence led to the proposal that xanthine oxidase is responsible for nicotinamide-N-oxide reaction. The nicotinamide-N-oxide oxygen is transferred to xanthine by xanthine oxidase (108).

Quantitative studies of the excretion of nicotinic acid and nicotinamide metabolites in the urine have demonstrated that the metabolites vary with animal species, age, diet, and the dosage of vitamin. The earlier methods used for estimating trigonelline involved hydrolysis to nicotinic acid with strong alkali, so that in earlier studies N^1-methylnicotinamide was included in the value reported for trigonelline (109). More complete data on the urinary excretion of nicotinamide, nicotinic acid and their metabolites were provided by Elinger _et al_. (87). Although they did not estimate N^1-methyl-2-pyridone-5-carboxamide, they found in contrast to the claims made by many earlier workers that no trigonelline or nicotinuric was excreted by man, rat, cat, guinea pig or rabbit on normal diet. Quantitative studies of the excretion of nicotinic acid and its amide metabolites in the urine of various animal species have been greatly improved by the introduction of chromatographic techniques and the use of radioactive nicotinic acid and nicotinamide tracers.

In the human, Reddi and Kodicek (110) reported that

normal adults excreted 0.5-0.6 mg of N^1-methylnicotinamide and 0.2-0.3 mg of tertiary nicotinyl derivative of nicotinic acid in the urine each 3 hours. When 100 mg of nicotinic acid were given orally to normal adults, 6-15 mg of N^1-methylnicotinamide and 2.8 mg to 5.7 mg nicotinuric acid were excreted in the urine each 3 hours. When 100 mg of nicotinamide were administered orally to normal adults, 7 to 17 mg of N^1-methylnicotinamide and 2.8 to 5.7 mg of nicotinamide were excreted into the urine each 3 hours.

The most complete data on the excretion of urinary metabolites of nicotinic acid and nicotinamide in the rats (111) and mice (103) following the injection of various levels of nicotinic acid and nicotinamide was reported by Greengard _et al_. and Chaykin _et al_., respectively. In addition to injected nicotinic acid and nicotinamide, five urinary metabolites have been reported. These are N^1-methylnicotinamide, nicotinuric acid, nicotinamide-N-oxide, N^1-methyl-2-pyridone-5-carboxamide and N^1-methyl-4-pyridone-3-carboxamide. The percentage of each metabolite in the urine differs with the compound administered, the size of injected dose and species of animal (103, 110, 111).

Metabolism of Nicotiamide Adenine Dinucleotide

Structure Determination

Diphosphopyridine nucleotide (the original name of NAD^+) was isolated from brewer's or baker's yeast by

fractional precipitation followed by chromatography. Its structure was elucidated by degradation. Acid hydrolysis cleaved the substance into one molecule of adenine, two molecules of D-ribose-5-phosphoric acid, and one molecule of nicotinamide (112). Mild alkaline hydrolysis yielded an adenosine-diphosphate ribose compound, and nicotinamide; vigorous alkaline hydrolysis yielded adenosine diphosphate. Enzymatic hydrolysis yielded adenosine and nicotinamide riboside (113). Consequently, the structure proposed for coenzyme I consisted of one molecule of adenosine and one molecule of nicotinamide riboside linked through their 5'-position by pyrophosphate group.

Biosynthesis of Nicotinamide Adenine Dinucleotide

The biosynthesis of NAD^+ from nicotinic acid or its amide was generally accepted at first to proceed from nicotinamide mononucleotide. Later, it was suggested that alternative metabolic pathways might exist for the synthesis of NAD^+ from nicotinic acid or nicotinamide. Now, it appears that a pathway common to many species exists for the biosynthesis of NAD^+ from nicotinic acid mononucleotide via desamido-NAD^+. There are at least two pathways leading to the formation of nicotinic acid mononucleotide; from nicotinic acid and quinolinic acid. The reaction which converts quinolinic acid to nicotinic acid mononucleotide is catalyzed by quinolinate

phosphoribosyltransferase (decarboxylation) in the presence of 5-phosphoribosyl-1-pyrophosphate. Quinolinate phosphoribosyltransferase is a key enzyme in the "NAD$^+$ pathway of tryptophan metabolism" and the "aspartate pathway." The highly purified enzyme was isolated from beef liver (112, 113) and a Psudomonad (114). Gholson *et al.* demonstrated that this enzyme differs from nicotinic acid mononucleotide pyrophosphorylase (nicotinate phosphoribosyl transferase). They also showed that the total activity of the enzyme forming nicotinic acid mononucleotide from quinolinic is almost 20 times that of the enzyme forming this compound from nicotinic acid in acetone powder extract of liver. The pathway for the synthesis of NAD$^+$ from nicotinic acid was proposed by Priess and Handler (70, 71), who established nicotinic acid mononucleotide and desamido-NAD as intermediates. This pathway is known as the Priess-Handler pathway. The enzyme which catalyzes the formation of nicotinic acid was partially purified from erythrocytes and beef liver acetone powder (116). Recently, Smith and Gholson (117) have purified nicotinic acid phosphoribosyltransferase from bovine liver. Kinetic studies of this enzyme indicated ATP is not a required substrate for bovine liver nicotinate phosphoribosyltransferase, as has been reported, but activates the enzyme by lowering the apparent Michaelis constant for both nicotinic acid and 5-phosphoribosyl-1-pyrophosphate by 10 fold. The kinetics of ATP activation indicate that

ATP is binding at only one site on the enzyme. When ATP is present in the reaction, it is cleaved to formed ADP in an amount approximately stoichiometric with nicotinic acid mononucleotide formation. It is suggested that ATP cleavage may be required for the activation of the enzyme.

It has been known for many years that nicotinamide is an adequate form of the vitamin for meeting the general mammalian dietary requirement for the precursors of the nicotinamide coenzymes. In general, nicotinamide is first deamidated by nicotinamide deamidase to nicotinic acid. The later then enters into Priess-Handler pathway for NAD^+ synthesis. Nicotinamide is the predominant form of the vitamin in the blood and other mammalian tissues (118). In contrast to the finding with many nicotinamide utilizing microorganisms, the ability to convert nicotinamide to nicotinic acid is a property possessed by few mammalian cells (119). The question of how mammalian cells lacking nicotinamide deamidase can utilize nicotinamide is a problem of long standing. Even in liver where the deamidase has been found (120), the problem has only been partially resolved. This deamidase has attracted considerable interest because its activity is masked in tissue homogenate by an endogenous inhibitor. Greengard and coworkers (121) have suggested that this phenomenon offers a means for control of NAD^+ biosynthesis. However, there is still some question regarding the physiological role of this enzyme. Its K_m value for nicotinamide

$(4 \times 10^{-2}$ to $2.5 \times 10^{-1}M)$ (11, 119) is rather high in the light of the very low concentration of nicotinamide $(5 \times 10^{-5}$ to $10^{-6}M)$ normally found in mammalian cell (122). This may not be a cause for concern since the quantities of NAD^+ synthesized are ordinary quite small and the enzyme and substrate could be compartmentalized. Another difficulty is that the homogeneous nicotinamide deamidase obtained from rabbit liver microsomes has considerable esterase activity (123). It does show a preference for the pyridine ring in both its amidase and esterase activities, but this does not prove that it functions physiologically as nicotinamide deamidase. There is cause for further evaluation. Hayaishi et al. (124) have recently suggested that nicotinamide administered by intraportal or intraperitoneal injection first passes into the intestine and is deamidated there and then is transferred to the liver for synthesis of NAD^+ through the Priess-Handler pathway.

The discovery by Dietrich et al. (125, 126) of the widespread occurrence of specific nicotinamide ribonucleotide pyrophosphorylase has raised the possibility of yet another pathway by which nicotinamide might be converted to NAD^+. This pathway can permit the utilization of nicotinamide without deamidation. Grunnicke et al. (122) have reported evidence which is compatible with this latter pathway being the predominant one in Ehrlich tumor cells maintained at physiological concentrations of

nicotinamide (5×10^{-6}M), and Greenbaum (127) has also shown that this pathway is predominant in mammary tissue. A summary of the pathways leading to NAD^+ is shown in Figure 2.

Triphosphopyridine nucleotide (TPN^+, coenzyme II) TPN^+ was isolated from hemolyzed horse erythrocytes (21) and its structure was elucidated by degradation (128, 129). Cleavage of TPN^+ by nucleotide phosphotase yielded nicotinamide mononucleotide and a compound established later as adenosine 2'5' diphosphate. Selective hydrolysis of the 5'-phosphate group of the diphosphoadenosine fragment yielded adenosine 2'-phosphate. This structure assignment was based on a comparison of the monophosphate with adenylic acid. TPN^+ is synthesized by enzymic phosphorylation. An enzyme was obtained from yeast that catalyzed the synthesis of the dinucleotide phosphate from dinucleotide by direct phosphorylation with adenosine triphosphate in the presence of magnesium or manganese ion (129).

The Pyridine Nucleotide Cycle

It has been long known that nicotinamide is a degradation product of nicotinamide adenine dinucleotide formed by NAD glycohydrolase (EC 3.2.2.5) and that the part of the nicotinamide formed in this way can be reutilized by conversion to nicotinic acid by nicotinamide deamidase and then converted to NAD^+ _via_ the Priess-Handler pathway

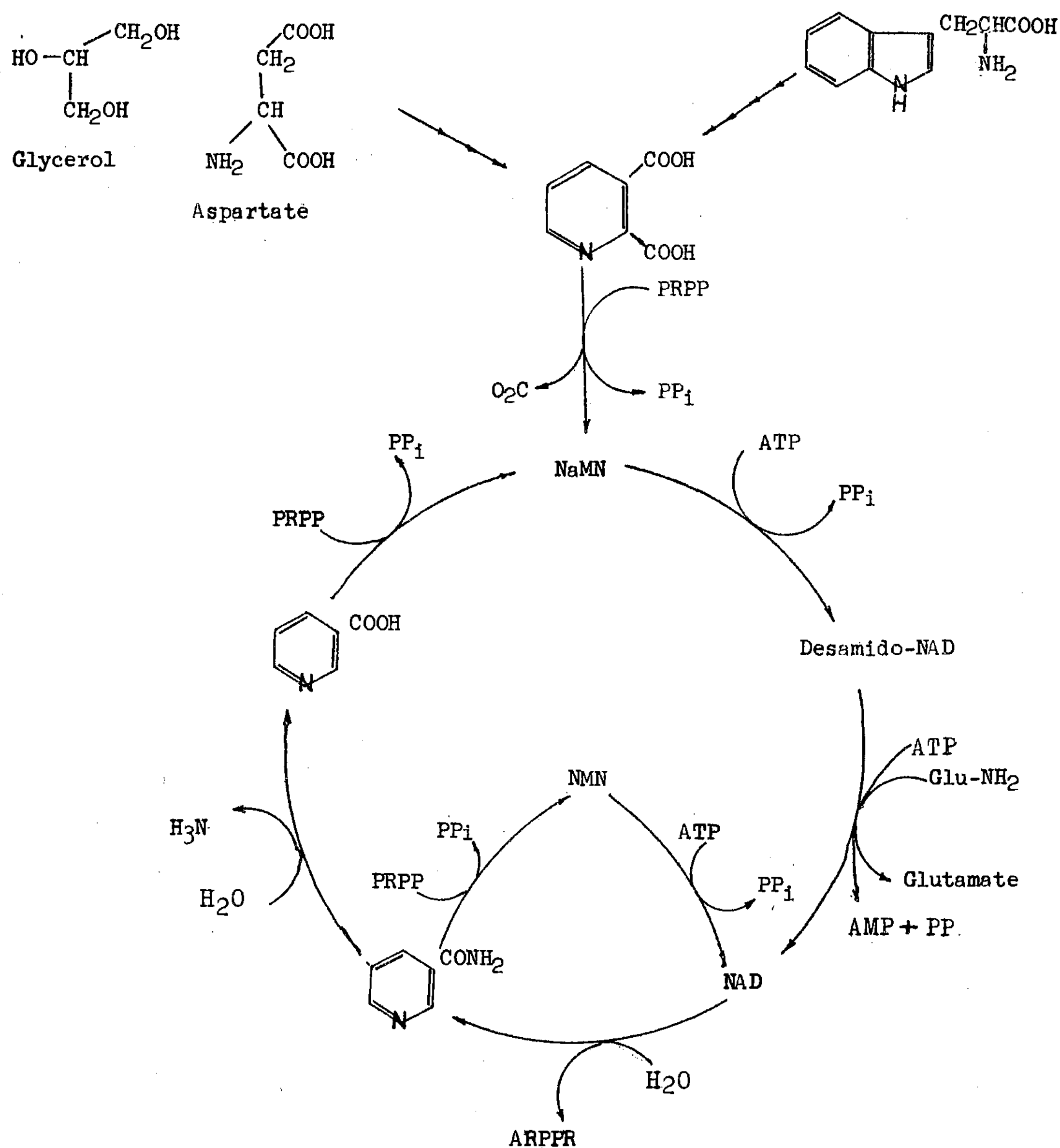


Figure 2. Biosynthetic Pathways of Nicotinamide Adenine Dinucleotide and the Pyridine Nucleotide Cycle

(70). Cyclic schemes for the degradation and resynthesis of NAD^+ have been proposed by Joshi and Handler (130) and Sarma et al. (131). These schemes were postulated on the basis of data indicating that the nicotinyl moiety enters the pathway of NAD^+ biosynthesis de novo at the level of free nicotinic acid. Recently, it has been shown that the nicotinyl moiety enters the pathway as nicotinic acid mononucleotide which is formed from quinolinic acid and 5-phosphoribosyl-1-pyrophosphate (112, 113).

Considering these facts and the following information, Gholson (12) proposed a pyridine nucleotide cycle (Figure 2). This cycle is based on the following evidence. Tryptophan serves as the de novo precursor of quinolinic acid in mammals (61-64) and a number of organisms. In higher plants (79) and some bacterial species, Quinolinic acid is synthesized from glycerol and aspartate (80, 83, 84) by an unknown series of reactions. Once at the levels of NaMN, biosynthesis appears to proceed via the so-called Priess-Handler pathway (70). NAD^+ is cleaved by a number of enzymes, the most common being the glycohydrolase type which splits the NAD^+ molecule to nicotinamide and ARPPR. Nicotinamide released by this cleavage is converted by deamidation to nicotinic acid (11). Other evidence for the existence of this cycle is provided by the observation that when nicotinamide-^{14}C-^{15}N is injected into rats, the carboxyl group is almost undiluted, while there is considerable dilution of amide nitrogen in

hepatic NAD^+ (132). Data consistent with the operation of this cycle in plant have been reported by Waller *et al.* (133).

Control of NAD^+ Biosynthesis

Most cells have the potential for synthesizing NAD^+ by more than one metabolic pathway. Since the nicotinamide coenzyme concentration in these cells is usually remarkably constant, it is most probable that the operation of any given pathway will be regulated with respect to both existing nucleotide levels and the degree of operation of the other pathways. The most likely mechanism for the control of NAD^+ levels will be summarized in the following paragraphs.

It is well known that administration of small quantities of nicotinic acid to mice and rats brings about a rapid increase in the nicotinamide coenzyme levels in the liver. However, the administration of small quantities of nicotinamide does not bring about increased coenzyme synthesis. Hayaishi and coworkers have recently confirmed this phenomenon. This might indicate that NAD^+ biosynthesis from nicotinamide is under restraint of a physiological control mechanism whereas that from nicotinic acid is not. Thus, the data of Hayaishi and coworkers could be interpreted as indicating that the liver cells of an adult mouse only make enough NAD^+ to replace that lost in normal turnover. There are two enzymes which can initiate the

net synthesis NAD^+ from nicotinamide. They are nicotinamide deamidase and nicotinamide ribonucleotide pyrophosphorylase. Greengard and coworkers (11) have found an inhibitor in liver tissue which masks the nicotinamide deamidse activity, and this inhibitor could be removed from deamidase by treatment with bovine serum albumin. This indicated the enzyme, nicotinamide deamidase, may be one control site for the biosynthesis of NAD^+ in vivo.

In 1966, Dietrich et al. (125, 126) showed the nicotinamide pyrophosphorylase activity in the extracts of a number of different cell types only after the removal of an inhibitory material by precipitation with protamine sulfate. Thus, the potential for control of the utilization of nicotiamide is present.

In 1966, Dietrich and Muniz (134) reported that nicotinamide ribonucleotide pyrophosphorylase is subject to feedback control by NAD^+. The levels of nicotinamide deamidase activity in liver do not seem to be affected by the NAD^+ concentration, but are greatly influenced by interplay of hypophyseal adrenal and thyroid hormones in vivo (11, 123). Furthermore, the deamidase is specifically inhibited by thyroxine in vitro (123).

It has been known for a long time that the injection of large doses of nicotinamide into rats and mice resulted in large increase in the concentration of hepatic nicotinamide adenine dinucleotide (136). More recently, Greengard and coworkers reported that hypophysectomy before

administration of nicotinamide results in an increase in both the degree and duration of the elevation of the concentration in liver (8). The same results were also reported following the administration of nicotinic acid and tryptophan. Further experiments were carried out to identify the responsible hormones which might be involved in this effect by testing their ability to prevent the increase of hepatic NAD^+ concentration in hypophysectomized rats following the injection of nicotinamide. Greengard _et_ _al_. (9) found that adrenalectomy, thyroidectomy, and to a lesser extent, gonadectomy were each effective in causing NAD^+ level above that in normal rats. The high concentration of NAD^+ in hypophysectomized rats could be greatly reduced by somatotropic hormone (9). Greengard _et_ _al_. (11) also reported that the activity of hepatic nicotinamide deamidase is responsible for the increase in hepatic NAD^+ concentration in hypophysectomized rats following injection of large doses of nicotinamide.

Another possible mechanism for regulation of NAD^+ levels might be provided by NAD^+ nucleotidase (NAD^+ glycohydrolase). Kaplan (137) has postulated that this regulates NAD^+ levels by destroying free NAD^+ but not NAD^+ bound to dehydrogenase. In this way, the level of NAD^+ and NAD^+ required dehydrogenase could be controlled.

Biodegradation of Pyridine Nucleotide

The degradation of nicotinamide adenine dinucleotide

in mammalian liver tissue was observed at a very earlier date. However, the characterization of the responsible enzymes has been carried out only recently. Several NAD^+ degradative enzymes have been reported. The enzymes which degrade NAD^+ to NMN and AMP are NAD^+ pyrophosphatase and NAD^+ pyrophosphorylase (129, 138). NAD^+ pyrophosphatase catalyzes the cleavage of the pyrophosphate bond between the two ribose moieties producing nicotinamide mononucleotide and adenylic acid from NAD^+ and producing nicotinamide mononucleotide and 2'5'-diphosphoadenosine from $NADP^+$. This enzyme has been observed in potatoes (129) and kidney (138). NAD^+ pyrophosphorylase catalyzes the reversible decomposition of NAD^+ to NMN and ATP (73). This enzyme has also been reported (70) to catalyze the reverse reaction yielding NAD^+. The degradative enzyme which splits the bond between the ribose and nitrogen of the pyridine ring is NADase (NAD^+ glycohydrolase). This enzyme also cleaves NMN to nicotinamide and ribose-5-phosphate. This enzyme has been observed in erythrocytes (110, 139) and *Neurospora* (140). The function of NADase in the organism is still unclear (141), however it can release nicotinamide by cleavage of NAD^+ to permit lowering of NAD^+ concentration *in vivo* when that is desirable, so it may play some role in control of NAD^+ levels *in vivo* (137).

In recent years, Mandel *et al*. (147) discovered a nuclear enzyme which polymerizes the ADP-ribose moiety of

NAD^+ with the release of nicotinamide. The polymer is formed of adenosine diphosphate ribose units linked between the C_1 position of the terminal ribose and the C_2 position of the ribose attached to adenine (142). The enzyme activity is exclusively localized in chromatin (143), and the polymer is linked to histone through a covalent bond (144). Bock and coworkers (145) have recently shown that the enzyme which polymerizes adenosine diphosphate-ribose is probably identical to the previously reported nuclear NAD^+ nucleosidase.

CHAPTER III

EXPERIMENTAL

Chemicals

Nicotinic acid-7-^{14}C and nicotinamide-7-^{14}C were purchased from Calibiochem Inc., specific activity 12.4 mc/mmole. They were purified before use by paper chromatography using n-butanol saturated with 3% ammonia solution and n-butanol-acetic acid-water (4:1:2, v/v). Nonradioactive nicotinic acid and nicotinamide were purchased from Nutritional Biochemical Co., nicotinuric acid was purchased from Calibiochem, Inc., 6-hydroxynicotinic acid was purchased from Aldrich Chemical Company. 6-hydroxynicotinamide was prepared by refluxing 11 grams of 6-hydroxynicotinic acid with 10 ml of thionyl chloride in a water bath for one hour. The reaction mixture was slowly poured, with vigorous stirring, into 50 ml of ice-cold concentrated ammonia. The product was collected by filtration and recrystallized twice from hot water, m.p. 285°C. Cellulose powder for preparation of thin layer plates was purchased from Micro Chemical Specialties Co., Berkley, California. All other chemicals were of reagent grade and were obtained from local supply houses.

Dowex-1X8 formate, 200 to 400 mesh, was purchased

from the Bio Rad Company and was regenerated by washing successively with several volumes of 1 N hydrochloric acid, then with 4 N ammonium formate, and then with deionized water until no more chloride ion could be detected coming off of the column by silver nitrate solution. Dowex-50-H^+ form, 200 to 400 mesh, was purchased from Bio Rad Company was regenerated by washing successively with several volumes of 2 N NaOH, then with 6 N HCl solution, and then with deionized water.

Animals

The normal rats used in this study were purchased from Holtzman Company, Madison, Wisconsin. All rats were maintained on a diet of Purina Laboratory Chow and fed _ad libitum_. Hypophysectomized rats were obtained from Hormone Assay Laboratory, Chicago, Illinois. The drinking water of hypophysectomized rats contained 5% glucose. Experiments with hypophysectomized rats were carried out at least one week after surgery. Germ free rat experiments were carried out in the Gnotobiotic Laboratory, Fitzsimons General Hospital, Denver, Colorado, by Nicholas Raica. The urines and wash solution were pooled, adjusted to pH 7.0 and stored frozen until lyophilized, and shipped to Oklahoma State University for analysis.

Methods

Detection of Radioactivity

The radioactivity on paper chromatograms was either determined with a Nuclear-Chicago 4π Actigraph III paper strip counter or the chromatograms were cut into 1 cm lengths and counted in a liquid scintillation spectrometer. Radioactivity in liquid solutions was determined by pipeting 0.1 ml aliquots into 10 ml of scintillation fluid and counting with a Packard Tri-Carb Liquid Scintillation Spectrometer. The scintillation fluid was composed of 600 ml toluene, 400 ml ethanol, 4.0 g of 2,5-diphenyloxazole and 0.2 g of 1,4-bis [2-(5-phenyloxazolyl)] -benzene.

Methods for Study of Nicotinic Acid and Nicotinamide Metabolism in Liver Tissue

Administration of Nicotinic Acid-7-^{14}C and Nicotinamide-7-^{14}C and Extraction of Radioactive Metabolites From the Liver

The weights of the rats used in these studies ranged from 100 to 150 grams. The method used for intraportal injection of nicotinic acid and nicotinamide was adapted from the method described by Hayaishi et al. (124). After laporotomy under slight anesthesia, 0.05 ml of nicotinic

acid-7-^{14}C or nicotinamide-7-^{14}C solution containing 210 mμmole of radioactive compound was injected _via_ the portal vein during a period of 20 seconds. At various time intervals after the termination of injection, namely, 20 seconds, 2, 5, 10, 30, and 60 minutes, the liver was removed, weighed, and homogenized in 20 ml of 0.5% perchloric acid with mortor and pestle at 0°C and centrifuged at 8000 rpm for 20 minutes. The precipitates were washed twice with 10 ml of water, after combining the supernatants, the perchloric acid was neutralized with 6 N KOH solution to pH 6.0, and precipitate of potassium perchlorate removed by centrifugation. The radioactivity in a 0.1 ml aliquot of the supernatant was determined by liquid scintillation counting. The total radioactivity recovered in the liver was calculated from this data.

Analysis of the Metabolites of Nictonic Acid-7-^{14}C and Nicotinamide-7-^{14}C in the Liver Extract

1. Paper chromatography: A 0.2 ml aliquot of liver extract supernatants solution was spotted on a Whatman No. 1 paper strip and descending paper chromatography was carried out 1 M ammonium actate-ethanol (3:7, pH5.0), pyridine-water (2:1, v/v) and isobutyric acid-ammonia-water (66:1.7:33, pH 3.8). After developing and drying, the

radioactivity on the chromatogram was located with a Nuclear-Chicago 4π Actigraph III paper strip counter. Alternatively, the paper strip was cut into 1 cm pieces, placed in a counting vial with 10 ml of scintillation solvent and counted by liquid scintillation spectrometry.

2. Paper electrophoresis: A 0.2 ml aliquot of liver extracts was placed on a Whatman No. 1 paper strip 20 cm from one end and one end of the paper was dipped in buffer (pyridine:acetic acid:water (5.0:3.4:90, v/v) (146). The excess buffer was removed by blotting between two sheets of filter paper. This procedure was repeated to wet the paper at the other end. The paper strip was then placed in an electrophoresis tank in the proper position and 2000 volts was applied through a distance of about 40 cm for 40 minutes. n-Hexane was used as coolant. The paper was removed from the tank, dried, and the radioactivity determined by paper strip counting.

3. Ion exchange chromatography: A portion of the liver extract was placed on a column of Dowex-1x8 formate (2x20 cm, 65 ml). Elution was carried out with increasing

concentrations of formic acid (70) as shown in Figure 5. The flow rate was about 1 ml per minute. Fractions of 10 ml were collected and 0.2 ml aliquots were counted in a liquid scintillation spectrophotometer. The radioactive fractions were pooled and evaporated *in vaco* to 1 ml. A 0.1 ml of this solution was spotted on Whatman No. 1 paper strips with authentic samples and the strips were developed in different solvent systems. Quenching spots were detected under ultraviolet light, and the radioactivity on the paper chromatogram was determined by paper strip counting.

Urinary Metabolites of Nicotinic Acid-7-^{14}C and Nicotinamide-7-^{14}C in Normal and Hypophysectomized Rats

Intraperitoneal Injection of ^{14}C Labeled Nicotinic Acid and Nicotinamide Into Normal and Hypophysectomized Rats and Collection of the Urine

Holtzman strain rats weighing between 250 to 300 grams were injected with 5 mg or 500 mg per kg of nicotinic acid or nicotinamide-7-^{14}C dissolved in 0.9% saline. In all cases, the amount of radioactivity administered

per rat was 5.2 μc. Three rats were placed in a metabolism cage, arranged so that the urine was collected in 150 ml Erlenmyer flask which was immersed in a dry ice bath. At the end of each collection period, the cage was washed with 50 ml of water. The feces from each period was also collected. Aliquots of each urine fraction and washing were counted by liquid scintillation spectrometry. The total radioactivity excreted in each period was calculated from this data.

Analysis of the Metabolites of Nicotinic Acid and Nicotinamide in the Urine of Normal and Hypophysectomized Rats

1. Paper chromatography: A 0.1 ml aliquot of each fraction was applied to Whatman No. 1 paper strips and descending chromatography was carried out in n-butanol saturated with 3% ammonia solution. Radioactive spots on the paper were located with Nuclear-Chicago paper strip counter, and compared with the R_f values of known compounds or with authentic standards chromatographed on the same paper, and located by ultraviolet quenching.
2. Thin layer chromatography: Glass plates of 20x20 and 5x30 centimeters were spread with a 0.5 mm layer of MN 300G cellulose powder by using a commercial spreader apparatus.

The coating material was prepared by blending 15 grams of MN 300 G cellulose powder in 110 ml of deionized distilled water for 5 minutes. The chromatoplates were allowed to air dry for one hour, then activated in an oven at 70-80°C for one hour. The urine samples were applied to plate and developed in two different solvent systems: n-butanol saturated with 3% ammonia solution and n-butanol-acetic acid-water (4:1:2, v/v). After developing and drying, the location of metabolites was determined with a paper strip counter and by quenching under ultraviolet light.

3. Dowex-50-H^+ column chromatography: Ten ml of a water solution of urine solids was placed on a 2.5x20 cm column of Dowex-50-H^+. The column was eluted with water and 10 ml fractions were collected. Radioactivity was determined on 0.1 ml aliquots by liquid scintillation spectrometry and the absorbence at 290 mμ was measured with a Beckman DU spectrophotometer.

4. Dowex-1x8 formate column chromatography: The radioactive fractions obtained from Dowex-50-H^+ chromatography were reduced to dryness on a rotatory evaporator and dissolved in 5 ml

of water. The samples were applied to 2.5x20 cm Dowex-1-formate column. The column was eluted with water, fractions of 10 ml were collected, and the radioactivity and absorbence were measured as above.

5. Infrared spectrometry: Infrared spectra were determined with a Perkin-Elmer 457 Grating Infrared Spectrophotometer in KB_r pellets which were prepared by mixing about 1 μg of unknown, which was isolated from urine by column chromatography and crystallized from hot water, and about 0.5 mg of KBr powder and adding the mixture to a Perkin-Elmer IR Micro Sampling Accessory and compacted with an hydraulic press.

6. Mass spectrometry: Mass spectra were measured using a prototype (146) of the LKB 9000 combination gas chromatography-mass spectrometer instrument (Karolinska Institute, Stockholm, Sweden). The sample in its solid state was introduced directly into the ion source through a direct inlet probe. The ion source temperature was 310°C, ionization current was 65 milliamps, multiplier voltage 2.1 kv, and acceleration voltage 3.5 kv. The mass spectra were computer plotted from

tabular intensity data with a CalComp using a Fortran 11-D program. The mass spectra were reported in terms of relative intensity, the most abundant ion being taken as 100%, and in term of sigma value.

Measurement of Respiratory CO_2

During the collection of urine of rats injected with nicotinamide-7-^{14}C or nicotinic acid-7-^{14}C, the respiratory CO_2 was also collected by connecting the outlet of the metabolic cage to four CO_2 absorption tubes. The first three tubes contained 100 ml of ethanol amine in methylcellosolve (1:2,v/v), (148) and were used for collecting the respiratory CO_2. The final tube contained 50 ml of saturated barium hydroxide which was used as an indicator. The outlet of the final absorption trap was connected to a water aspirator. The CO_2 trapping liquids were combined and 3 ml of this liquid were added to 15 ml of methylcellosolve-toluene (1:2,v/v) which contained 5.5 grams of PPO per liter, for measuring the radioactvity in a liquid scintillation counter.

CHAPTER IV

RESULTS AND DISCUSSION

Results

Paper Chromatographic Separation of Nicotinic Acid and Nicotinamide Metabolites

Paper chromatography has been widely applied for the separation of the metabolites of nicotinic acid and its amide in the urine or in tissue extracts. The most common solvents used for the separation of urinary metabolites are n-butanol saturated with 3% ammonia solution (118, 149) or n-butanol saturated with water (110). These solvents give a good separation of urinary metabolites, except for nicotinuric acid and N^1-methylnicotinamide. However, these two compounds can be easily separated by adding 1 ml of glacial acetic acid to 60 ml of n-butanol saturated with water. In this solvent system, the R_f of N^1-methylnicotinamide is essentially unchanged (R_f 0.08), whereas the R_f of nicotinuric acid increases to 0.5 (118). Hence, radioactive material which chromatographed with R_f 0.04 in the basic solution was eluted with water and applied to a new strip of filter paper for chromatography in the acidic solvent.

The solvent systems, 1 M ammonium acetate-ethanol (3:7, pH 5.0), isobutyric acid-ammonia-water (66:1.7:33, pH 3.6) and pyridine-water are the most common solvents used for separating of the pyridine nucleotides, nicotinic acid mononucleotide, nicotinamide mononucleotide, desamido-NAD and nicotinamide adenine dinucleotide, but the non-nucleotides are all located at R_f 0.65-0.85 in these solvents. These non-nucleotides were further separated by eluting from the paper chromatograms which were developed 1 M ammonium acetate-ethanol at R_f 0.65-0.85, and rechromatographing in n-butanol saturated with 3% ammonia solution. The R_f values of all nicotinic acid metabolites are shown in Table I.

Thin Layer Chromatography and Paper Electrophoresis

MN 300 G cellulose powder thin layer plates were used for the separation of nicotinamide urinary metabolites. This system has the advantage of separating N^1-methylnicotinamide, 6-hydroxynicotinic acid and nicotinuric acid with n-butanol saturated with 3% ammonia solvent. Indeed, the newly discovered metabolite, 6-hydroxynicotinic acid always has the same R_f as nictotinuric acid in both acidic and basic solvent systems during paper chromatography; however, nicotinuric acid can be separated from N^1-methylnicotinamide and 6-hydroxynicotinic acid. The later two compounds can be further separated by

TABLE I

DESCENDING PAPER CHROMATOGRAPHY OF THE METABOLITES OF NICOTINIC ACID AND NICOTINAMIDE

	R_f Value Paper Chromatography				T.L.C.	Mobilities[1] (cm/hr. kv.) Electrophoresis	
	Solvent System					Buffer	
	I	II	III	IV	III	A	B
N^1-methylnicotinamide	0.69	0.81	0.03	0.64	0.08	-8.0	-9.0
Nicotinuric acid	0.66	0.67	0.03	0.64	0.77	5.3	6.2
Nicotinic acid	0.67	0.71	0.15	0.75	0.28	5.7	6.5
Nicotinamide-N-oxide	0.70	0.76	0.25	0.62	0.32	2.0	0.0
2-Pyridone	0.80	0.85	0.41	0.72	0.40	0.0	0.0
4-Pyridone	0.71	0.85	0.49	0.72	0.58	0.0	0.0
Nicotinamide	0.75	0.82	0.68	0.78	0.72	0.0	-0.5
6-Hydroxynicotinic acid	0.75	0.80	0.03	0.75	0.15	5.5	6.2
6-Hydroxynicotinamide	0.78	0.82	0.24	0.75	0.31	0.0	0.0
NaMN	0.21	0.35	0.00	0.30	0.00	---	5.5
NMN	0.25	0.48	0.00	0.30	0.00	---	1.2
Desamido-NAD	0.12	0.30	0.00	0.15	0.00	---	5.2
NAD	0.16	0.43	0.08	0.08	0.00	---	2.3

Solvent:

I. 1 M ammonium acetate-ethanol (3:7, v/v)
II. isobutyric acid-ammonia-water (66:1.7:33, v/v, pH3.8)
III. n-butanol saturated with 3% ammonia solution
IV. n-butanol-acetic acid-water (4:1:2, v/v)

Buffer

A. 0.05 M sodium borate
B. pyridine-acetic acid-water (5.0:3.4:90, v/v, pH 5.0)
1. + and- mark indicate migration toward the anode and cathode respectively.

paper electrophoresis in 0.05 M sodium borate buffer.

Paper electrophoresis was used for the separation of pyridine nucleotides where the buffer was pyridine-acetic acid-water (5.0 3.4:90,v/v, pH 5.0) (146). The R_f values from thin layer chromatography and the mobility on high voltage paper electrophoresis of the nicotinic acid and nicotinamide metabolites are shown in Table I.

Isolation and Identification of Metabolites Formed in the Liver of Nicotinic Acid-7-^{14}C Injected Rats

Samples of 20 ml of liver extract were applied to a Dowex-1-formate form (2.5x20 cm) column, the column was eluted with water followed by increasing concentrations of formic acid (70). The results are shown in Figure 3. Five radioactive peaks were isolated. Each peak was pooled together, concentrated under reduced pressure, and identified by paper chromatography with authentic samples in different solvent systems. These radioactive peaks are in the order of elution, N^1-methylnicotinamide, nicotinamide, nicotinic acid plus NAD^+, nicotinic acid mononucleotide and desamido-NAD.

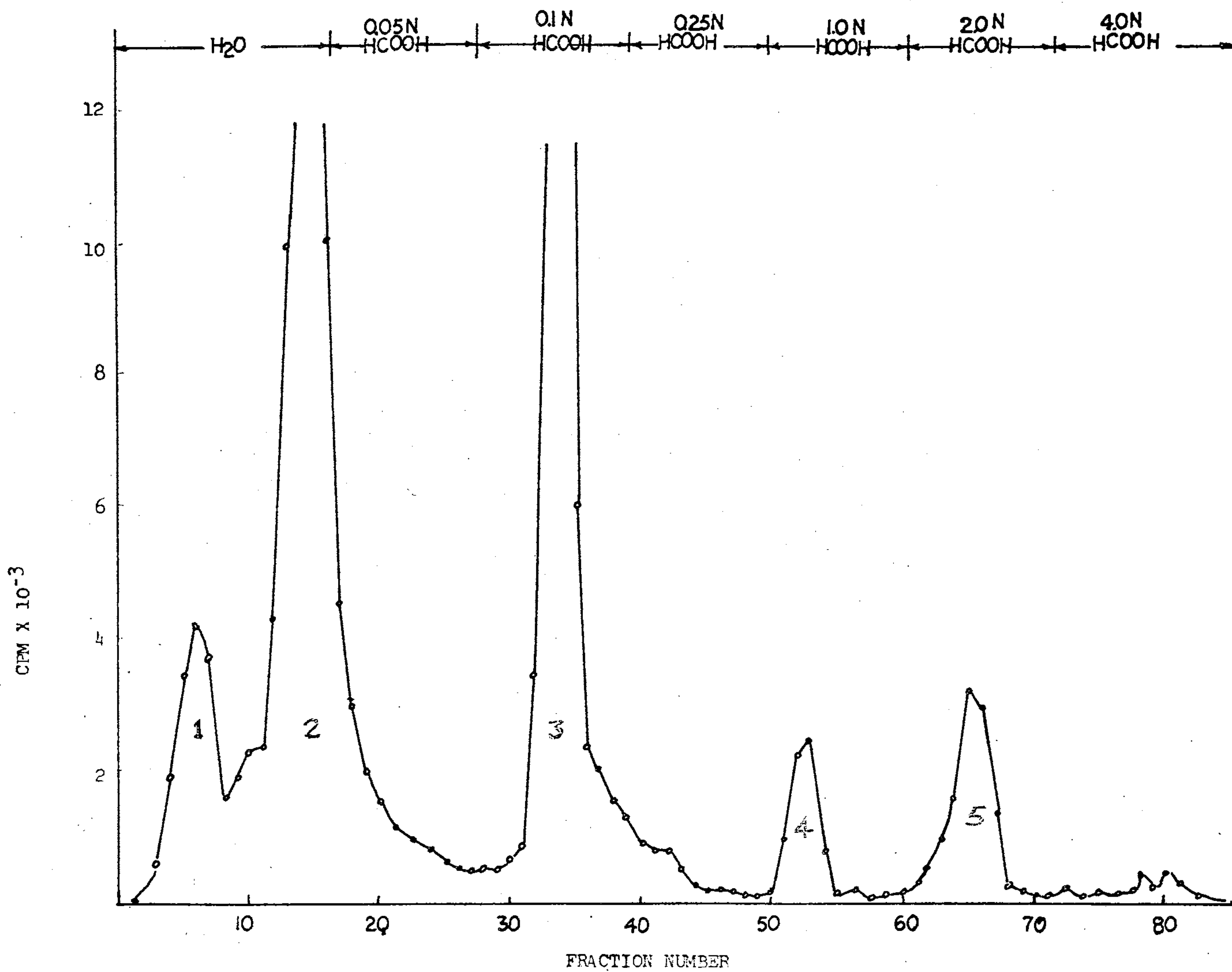
Metabolism of Nicotinic Acid-7-^{14}C and Nicotinamide-7-^{14}C in the Liver of Hypophysectomized and Normal Rats

Each rat in these experiments was injected with

Figure 3. Chromatographic Separation of the Metabolites of Nicotinic Acid in Liver Extracts on a Dowex-1x8 Formate Column

20 ml of liver extract from nicotinic acid injected rats was applied to 2.5x20 cm Dowex-1x8 formate column. The column was eluted with water and then increasing concentrations of formic acid as shown. The metabolites were identified by descending paper chromatography in several different solvent systems with authentic markers.

peak 1 N^1-methylnicotinamide
peak 2 nicotinamide
peak 3 nicotinic acid and NAD^+
peak 4 nicotinic acid mononucleotide
peak 5 desamido-NAD


H2O
0.05N HCOOH
0.1N HCOOH
0.25N HCOOH
1.0N HCOOH
2.0N HCOOH
4.0N HCOOH
12
10
8
6
4
2
1
2
3
4
5
10
20
30
40
50
60
70
80
CPM X 10-3
FRACTION NUMBER

2.0 μc (210 mμ moles) or 1.0 μc (75 mμ moles) of nicotinic acid-7-^{14}C or 2.0 μc (210 mμ moles) of nicotinamide-7-^{14}C into the portal vein. The liver of each rat was homogenized and extracted according to the method of Hayaishi (124). As shown in Figure 4, the total radioactivity remaining in liver was almost constant from 10 to 60 minutes after injection. When comparing the difference between normal and hypophysectomized rats, the total radioactivity recovered from hypophysectomized rats was slightly higher than that from normal rats. The radioactivity recovered in the liver of nicotinic acid injected rats was also higher than that in nicotinamide injected rats.

The distribution of hepatic metabolites following nicotinic acid-7-^{14}C injection greatly varied with the dosage injected, as shown in Figures 5 and 6. A small amount of NaMN and desamido-NAD$^+$ was observed within the first few minutes following injection of both 75 mμ mole and 210 mμ mole per rat of nicotinic acid, while the radioactivity in NAD$^+$ increased with time, reaching a maximum about 10 minutes after injection of 75 mμ moles of nicotinic acid. However, when 210 mμ moles of nicotinic acid were administered, the radioactivity in NAD$^+$ reached a maximum in about 5 minutes and then decreased to two-thirds of the maximum value after 60 minutes. There was no major difference between normal and hypophysectomized rats. The non-nucleotide also appeared within 2 minutes after injection of nicotinic acid. Nicotinamide

Figure 4. Total Radioactivity Recovered in the Liver of Hypophysectomized and Normal Rats After Portal Vein Injection of ^{14}C Labeled Nicotinic Acid and Nicotinamide

Experimental procedures are described in Chapter III. Each value represents the average of three experiments.

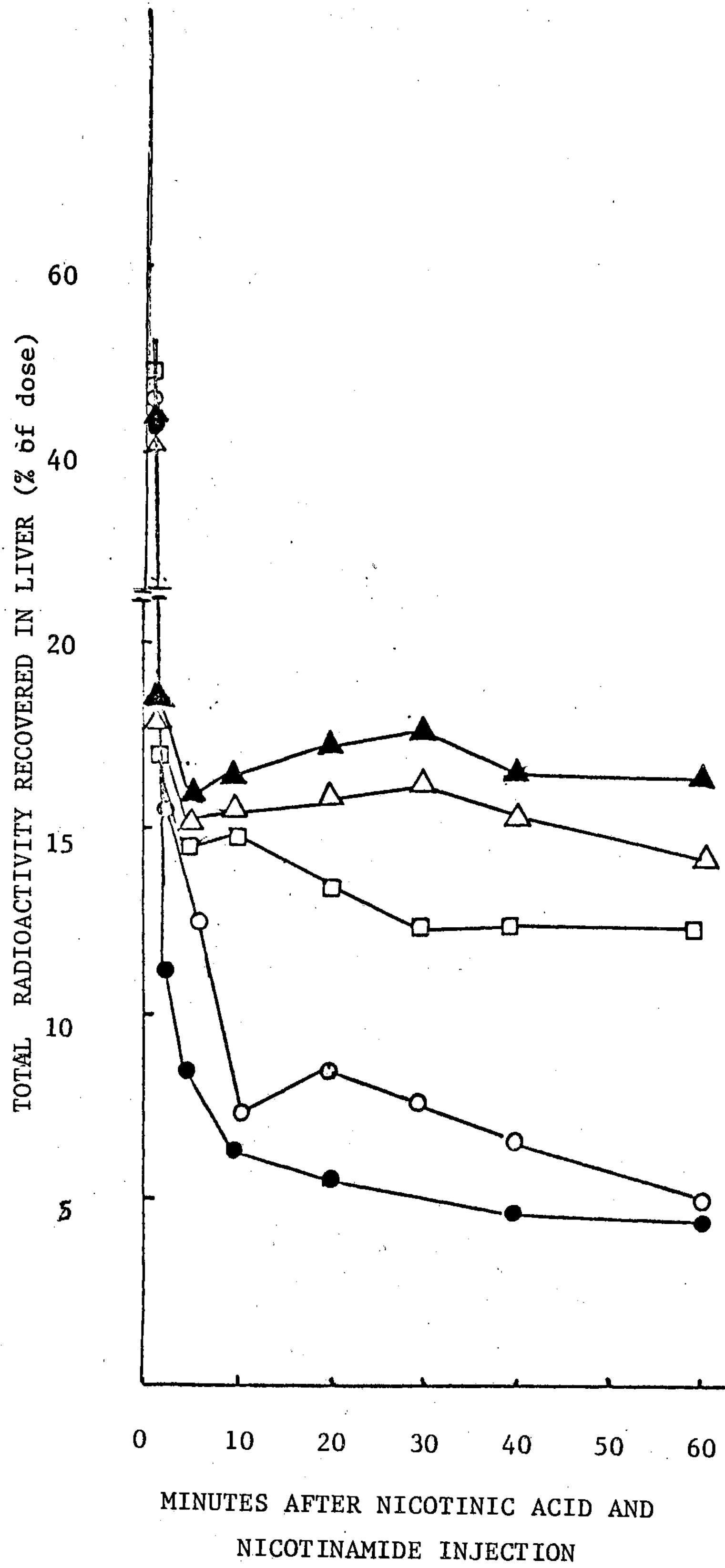
—△—△—, nicotinic acid (210 mμ moles) in normal rats

—▲—▲—, nicotinic acid (210 mμ moles) in hypophysectomized rats

—○—○—, nicotinamide (210 mμ moles) in normal rats

—●—●—, nicotinamide (210 mμ moles) in hypophysectomized rats

—□—□—, nicotinic acid (75 mμ moles) in normal rats



TOTAL RADIOACTIVITY RECOVERED IN LIVER (% of dose)
60
40
20
15
10
5
0
10
20
30
40
50
60
MINUTES AFTER NICOTINIC ACID AND
NICOTINAMIDE INJECTION

Figure 5. The Metabolism of Nicotinic Acid in the Liver of Hypophysectomized Rats After Intraportal Injection of Nicotinic Acid-7-^{14}C (210 mμ moles)

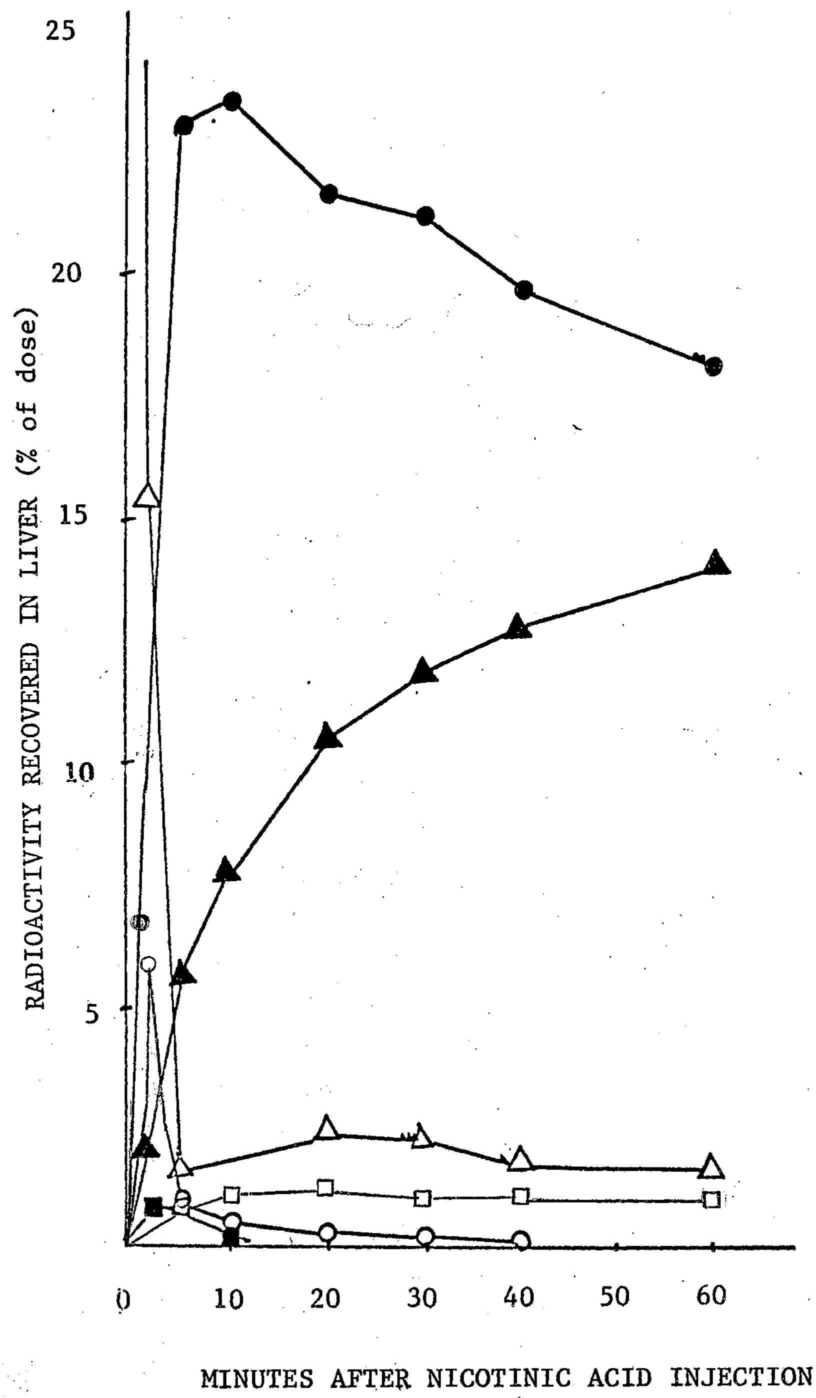
Compounds were separated by descending paper chromatography in the 1 M ammonium acetate-ethanol solvent system. The non-nucleotide metabolites were separated by rechromatography of the sample, which was eluted from the above chromatogram between the range of R_f 0.65-0.85 in the n-butanol saturated with 3% ammonia solvent system. The radioactivity of each peak was measured by cutting the paper chromatogram into 1 cm pieces, and counting by liquid scintillation spectrometry.

—●—●—, NAD; —▲—▲—, nicotinamide;

—■—■—, NaMN; —○—○—, desamido-NAD;

—△—△—, nicotinic acid;

—□—□—, N^1-methylnicotinamide

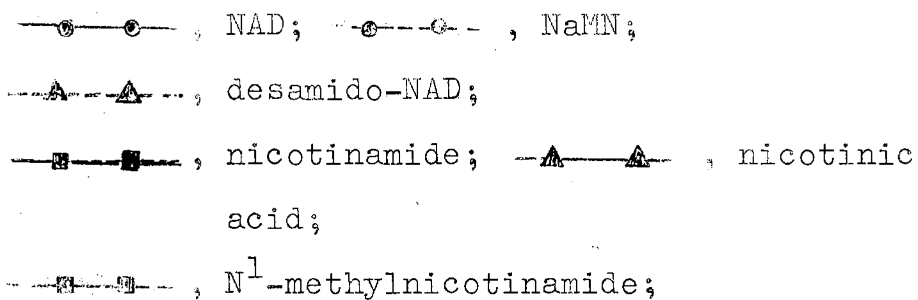


25
20
15
10
5
RADIOACTIVITY RECOVERED IN LIVER (% of dose)
0
10
20
30
40
50
60
MINUTES AFTER NICOTINIC ACID INJECTION

Figure 6. The Metabolism of Nicotinic Acid in the Liver of Normal Rats After Intraportal Injection of Nicotinic Acid-7-^{14}C (210 and 75 mμ moles)

Experimental procedure same as Figure 5.

210 mμ mole dose:



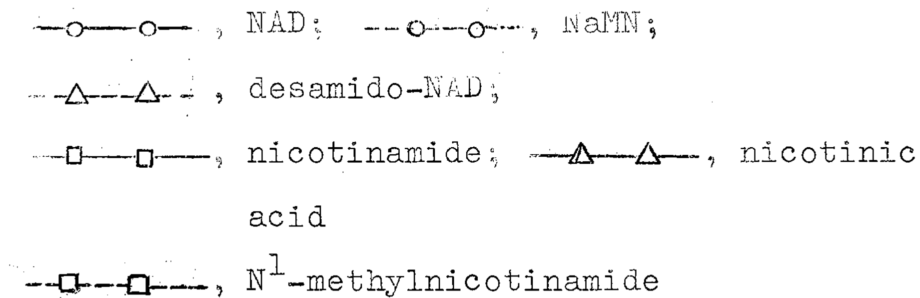
, NAD; , NaMN;

, desamido-NAD;

, nicotinamide; , nicotinic acid;

, N^{1}-methylnicotinamide;

75 mμ mole dose:

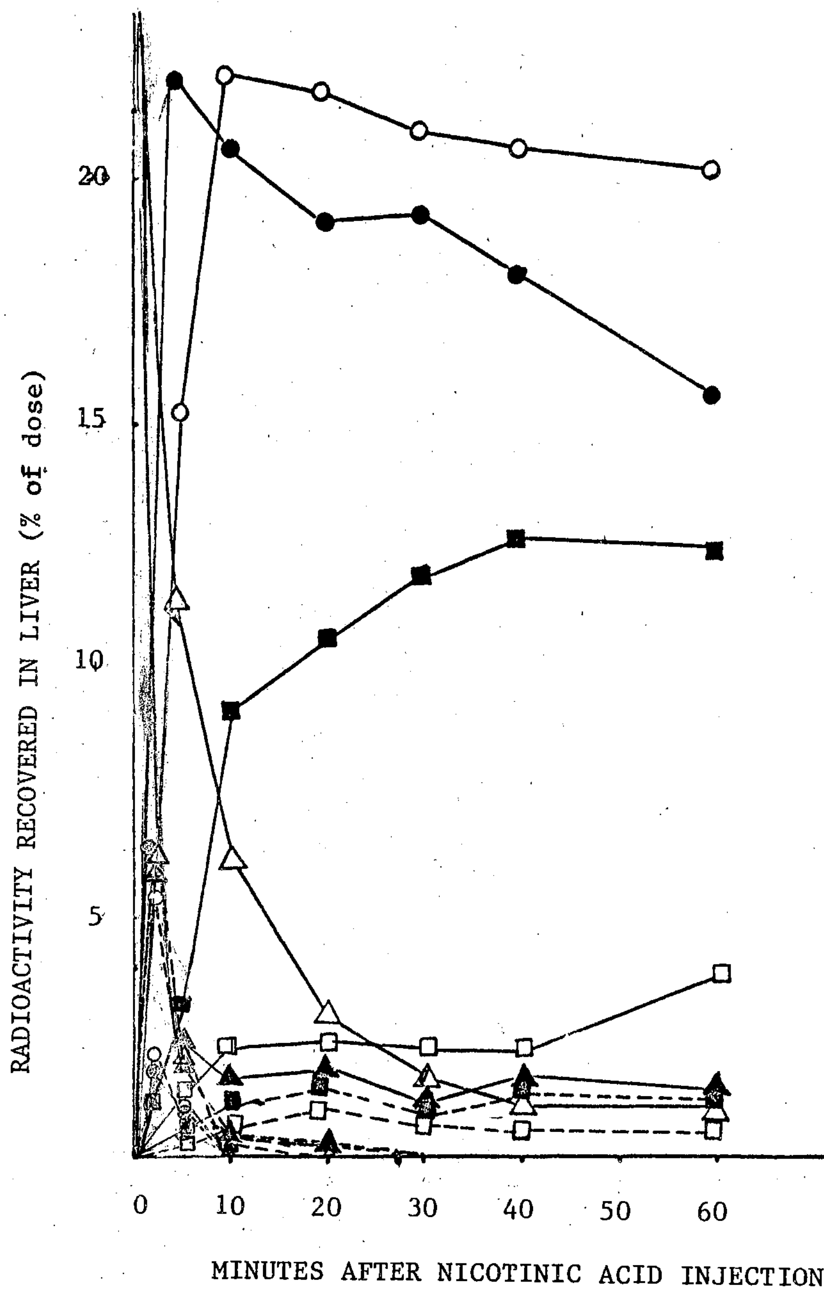


, NAD; , NaMN;

, desamido-NAD;

, nicotinamide; , nicotinic acid

, N^{1}-methylnicotinamide



RADIOACTIVITY RECOVERED IN LIVER (% of dose)
20
15
10
5
0
10
20
30
40
50
60
MINUTES AFTER NICOTINIC ACID INJECTION

constitutes about 80% of these metabolites. After administration of a small dose of nicotinic acid, only a very small amount of nicotinamide (2-5%) was formed within the one hour experimental period. However, when the higher doses of nicotinic acid were administered, the formation of nicotinamide was rapid and constituted up to 15% of the dose, increasing with time as shown in Figures 5 and 6.

The fate of radioactivity in the liver of nicotinamide-7-^{14}C injected rats is shown in Figure 7. In contrast to the results obtained with nicotinic acid, only a small, but definite, incorporation of nicotinamide into NAD^{+}-^{14}C was observed immediately after the injection and no increase of NAD^{+}-^{14}C occurred during the entire experimental period in both normal and hypophysectomized rats. There was no detectable nicotinamide nucleotide intermediates, NaMN, NMN, or desamido-NAD in the liver within 60 minutes after injection of nicotinamide-7-^{14}C. N^{1}-methylnicotinamide and nicotinamide-N-oxide were synthesized within 20 seconds after injection. N^{1}-methylnicotinamide was a major metabolite in the liver a short time after the injection of nicotinamide. The formation of N^{1}-methylnicotinamide reached a maximum in two minutes after injection of nicotinamide in normal rats and constituted about 70% of the total radioactivity in liver, then rapidly disappeared from liver. In hypophysectomized rats, the formation of N^{1}-methylnicotinamide reached a

maximum in five minutes. The rate of excretion of N^1-methylnicotinamide from liver was slower than in normal rats, as shown in Figure 7. There was also present 1-2% of 2- and 4-pyridone in the metabolites in hypophysectomized rats, which were not found in normal rats.

The Metabolites of Nicotinic Acid-7-^{14}C and Nicotinamide-7-^{14}C in the Urine of Normal and Hypophysectomized Rats

It has been reported by Greengard and coworkers (8) that hypophysectomized rats have a higher concentration of hepatic NAD than normal rats following the intraperitoneal injection of nicotinamide. In order to determine the effect of hypophysectomy on nicotinamide metabolism in rats, nicotinamide-7-^{14}C or nicotinic acid-7-^{14}C at either a 5 mg/kg or 500 mg/kg level was intraperitoneally injected into both normal and hypophysectomized rats and both the quantity and the distribution of metabolites in the urine was compared. The results of these experiments are shown in Figure 8. Little difference was observed between normal and hypophysectomized rats when injected with 5 mg/kg of nicotinic acid-7-^{14}C. However, with large doses of nicotinic acid-7-^{14}C (500 mg/kg) normal rats excreted 20-30% more radioactivity in the 0-3 and 3-6 hours time periods.

The total radioactivity excreted in the urine after injection of nicotinamide-7-^{14}C is shown in Figure 9.

Figure 7. The Radioactivity Distribution of Nicotinamide Metabolites in the Liver of Normal and Hypophysectomized Rats After Intraportal Injection of 210 mμ Moles Nicotinamide-^{14}C

Experimental procedure same as Figure 5.

Hypophysectomized rats:

—o—o—, NAD; —△—△—, nicotinamide;

—□—□—, N^1-methylnicotinamide;

--o--o--, nicotinamide-N-oxide

--△--△--, 2 and 4- pyridone;

Normal rats:

—●—●—, NAD; —■—■—, N^1-methylnicotinamide;

—▲—▲—, nicotinamide;

--●--●--, nicotinamide-N-oxide

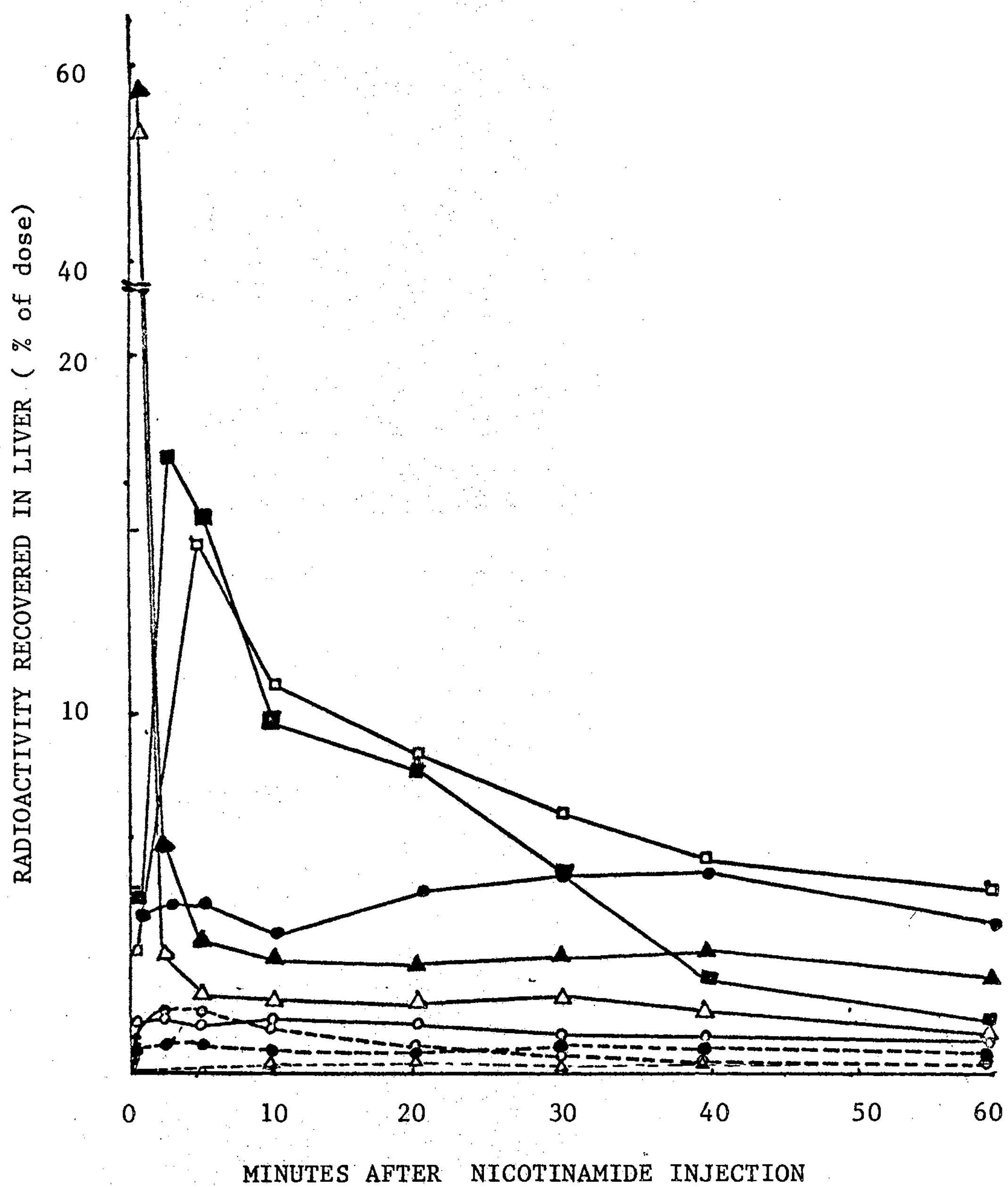

RADIOACTIVITY RECOVERED IN LIVER (% of dose)
60
40
20
10
0
10
20
30
40
50
60
MINUTES AFTER NICOTINAMIDE INJECTION

Figure 8. The Excretion of Total Radioactivity in the Urine of Normal and Hypophysectomized Rats Following the Injection of 500 mg/kg or 5 mg/kg Nicotinic Acid-7-^{14}C

The radioactivity in the urine is expressed as a percentage of the administered dose. Each value is the average of two experiments, each experiment contained pooled urine of three rats.

In hypophysectomized rats:

—□——□— , 500 mg/kg dose

—○——○— , 5 mg/kg dose;

In normal rats:

—■——■— , 500 mg/kg dose

—○——○— 5 mg/kg dose

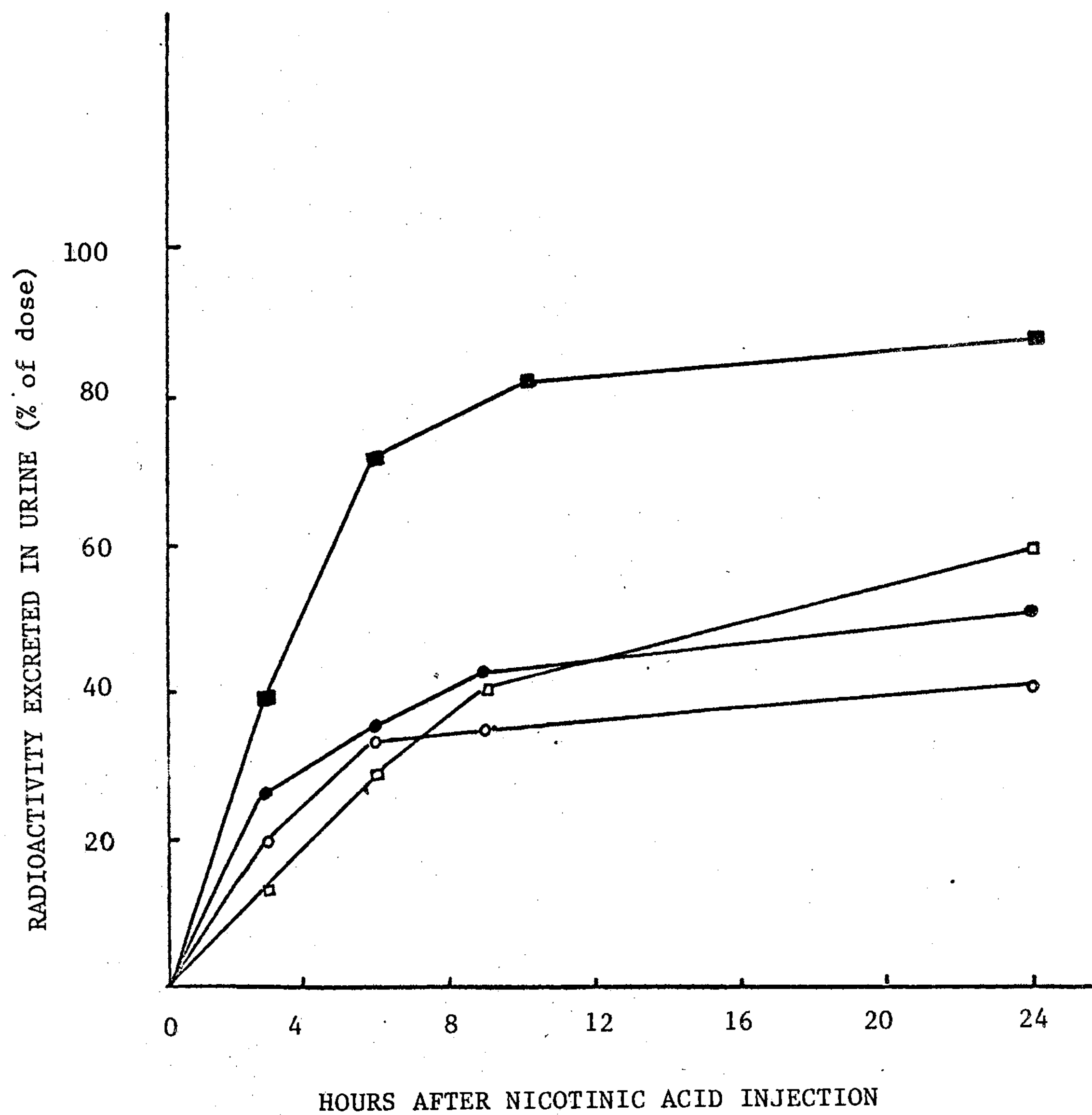

RADIOACTIVITY EXCRETED IN URINE (% of dose)
100
80
60
40
20
0
4
8
12
16
20
24
HOURS AFTER NICOTINIC ACID INJECTION

Figure 9. The Excretion of Radioactivity in the Urine of Normal and Hypophysectomized Rats Following the Injection of 500 mg or 5 mg of Nicotinamide-7^{14}C

The radioactivity in urine is expressed as a percentage of the administered dose. Each value is the average of two experiments, each experiment contained pooled urine of three rats.

In hypophysectomized rats:

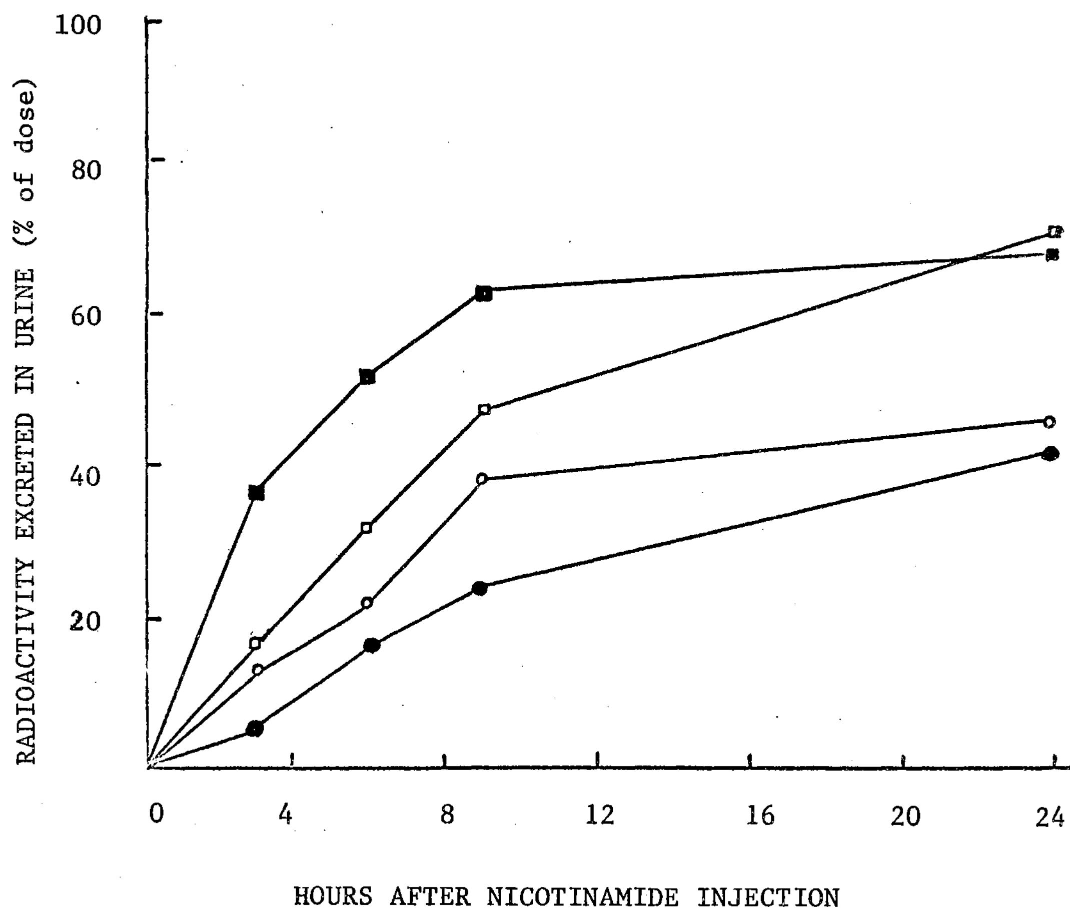
—□—□—, 500 mg/kg dose

—○—○—, 5 mg/kg dose

In normal rats:

- - -■- - -■- - -, 500 mg/kg dose

- - -○- - -○- - -, 5 mg/kg dose


100
80
60
40
20
RADIOACTIVITY EXCRETED IN URINE (% of dose)
0
4
8
12
16
20
24
HOURS AFTER NICOTINAMIDE INJECTION

After the small dose (5 mg/kg), normal rats excreted 37.0% of the administered vitamin, which was three times greater than that excreted by hypophysectomized rats within the first three hour period after injection. Following the loading dose (500 mg/kg), normal rats excreted 70.4% of the radioactivity in the urine, however, hypophysectomized rats only excreted 42.8% of the radioactivity in the urine within 24 hours after injection of the vitamin.

The distribution of radioactivity among the metabolites in the urine of normal and hypophysectomized rats within 24 hours after injection of 5 mg or 500 mg per kg nictonamide is shown in Table II. After small doses, the normal rats excreted more N^1-methylnicotinamide than did the hypophysectomized rats, while the hypophysectomized rats excreted more N^1-methyl-2-pyridone-5-carboxamide and N^1-methyl-4-pyridone-3-carboxamide than the normal rats within 24 hours after injection. In loading experiments, the normal rats excreted 11.7% of N^1-methylnicotinamide in the urine within 24 hours after injection. However, hypophysectomized rats only excreted 2.5% of N^1-methylnicotinamide. Normal rats also excreted more nicotinamide-N-oxide and unchanged nicotinamide than did the hypophysectomized animals within 24 hours after injection. In both normal and hypophysectomized rats, the excretion of nicotinic acid and nicotinuric acid was greatly increased by injection of loading dose (500 mg/kg) of nicotinamide.

TABLE II

RADIOACTIVITY DISTRIBUTION AMONG URINARY METABOLITES OF NORMAL AND HYPOPHYSECTOMIZED RATS FOLLOWING THE INJECTION OF 500 mg/kg OR 5 mg/kg OF NICOTINAMIDE-7-^{14}C

Metabolite	Hypophysectomized Rats					Normal Rats				
	Hours After Injection									
	0-3	3-6	6-9	9-24	0-24	0-3	3-6	6-9	9-24	0-24
	Per Cent of Injected Dose									
Small dose (5 mg/kg)										
N^1-methylnicotinamide	7.2	2.8	6.9	2.2	19.1	24.1	12.6	7.3	3.1	47.1
Nicotinuric acid	0.1	0.6	0.4	0.3	1.4	0.3	0.3	0.3	0.6	1.5
6-Hydroxynicotinic acid	-	-	-	-	0.4	-	-	-	-	0.3
Nicotinic acid	0.6	1.5	0.9	0.5	3.5	1.1	0.4	0.2	0.1	1.8
Nicotinamide-N-oxide	0.2	0.7	1.0	0.6	2.5	0.3	0.3	0.4	0.4	1.4
2 and 4-Pyridone*	1.7	2.3	7.3	3.7	15.0	6.1	1.6	1.4	0.8	9.9
6-Hydroxynicotinamide	-	-	-	-	0.5	-	-	-	-	0.6
Nicotinamide	2.9	0.8	0.9	1.2	5.8	5.1	0.6	1.0	0.1	6.8
Total	12.7	8.7	17.4	8.5	47.3	37.0	15.8	10.6	5.1	68.5
High dose (500 mg/kg)										
N^1-methylnicotinamide	1.2	0.6	0.5	0.3	2.5	1.8	2.0	2.1	5.8	11.7
6-Hydroxynicotinic acid	-	-	-	-	0.6	-	-	-	-	0.4
Nicotinuric acid	-	3.0	1.4	1.7	6.1	1.8	2.5	0.3	3.4	8.0
Nicotinic acid	0.2	1.0	1.8	6.1	9.1	1.5	1.5	1.6	1.5	6.1
Nicotinamide-N-oxide	0.1	0.9	1.0	0.6	2.6	1.1	1.1	1.8	2.8	6.8
2 and 4-Pyridone*	-	0.3	0.4	0.6	1.3	0.3	0.4	1.2	0.5	2.4
6-Hydroxynicotinamide	-	-	-	-	0.7	-	-	-	-	0.8
Nicotinamide	3.8	4.6	3.4	8.1	19.9	9.8	8.2	7.1	9.1	34.2
Total	5.3	10.8	8.7	18.0	42.8	16.4	15.9	14.3	23.4	70.4

*2-pyridone, N-methyl-2-pyridone-5-carboxamide
4-pyridone, N^1-methyl-4-pyridone-3-carboxamide.

Table III shows the radioactivity distribution of nicotinic acid metabolites after the intraperitoneal administration of 5 mg or 500 mg of nicotinic acid-7-^{14}C into normal and hypophysectomized rats. The rate of excretion of nicotinic acid in hypophysectomized rats was reduced from that of normal rats. As in the nicotinamide injected rats, normal rats excreted more N^1-methylnicotinamide in the urine than did the hypophysectomized rats after small doses of nictonic acid-7-^{14}C.

During these studies of the urinary metabolites of nicotinic acid and nicotinamide, small amounts of two unknown compounds which have not previously been reported as mammalian metabolites were discovered. The isolation and identification of these two compounds will be detailed in the following chapter.

Discussion

It is well known that the biosynthesis of nicotinamide adenine dinucleotide from nicotinic acid occurs _via_ nicotinic-acid mononucleotide and desamido-NAD^+ (70). The _in vivo_ experiments reported here which followed the fate of nicotinic acid-7-^{14}C (75 and 210 mμ moles) injected into the portal vein provide supporting evidence for the functioning of this pathway. A rapid conversion of nicotinic acid to nicotinic acid mononucleotide, desamido-NAD^+ and NAD^+ was observed in the liver within one minute

TABLE III

RADIOACTIVITY DISTRIBUTION AMONG URINARY METABOLITES OF NORMAL AND HYPOPHYSECTOMIZED RATS FOLLOWING THE INTRAPEDITIONEAL INJECTION OF 5 mg/kg OR 500 mg/kg OF NICOTINIC ACID-7-^{14}C

Metabolite	Hypophysectomized Rats					Normal Rats				
	Hours After Injection									
	0-3	3-6	6-9	9-24	0-24	0-3	3-6	6-9	9-24	0.24
	Per Cent of Injected Dose									
Small dose (5 mg/kg)										
N^1-methylnicotinamide	1.6	2.1	0.5	1.6	5.7	3.2	1.2	3.1	4.9	14.8
Nicotinuric acid	13.7	6.6	0.7	0.5	21.5	18.0	6.1	1.0	1.5	26.6
6-Hydroxynicotinic acid	-	-	-	-	-	-	-	-	-	1.0
Nicotinic acid	4.5	0.6	0.1	0.2	5.4	4.6	1.3	-	0.4	6.3
Nicotinamide-N-oxide	-	0.9	0.2	0.7	1.8	0.2	0.1	0.2	0.1	0.5
2 and 4-pyridone*	-	0.6	0.2	1.8	2.6	0.1	0.1	0.2	0.1	0.5
Nicotinamide	-	0.8	0.1	0.2	1.1	-	0.5	0.3	0.2	1.0
Total	14.8	12.6	1.8	5.8	41.1	26.1	9.6	6.9	7.8	50.4
High dose (500 mg/kg)										
N^1-methylnicotinamide	0.2	0.3	0.2	1.2	1.9	0.2	0.3	0.3	2.0	2.8
Nicotinuric acid	0.5	1.6	1.6	2.5	6.2	4.8	11.7	2.3	1.1	19.5
6-Hydroxynicotinic acid	-	-	-	-	0.7	-	-	-	-	0.8
Nicotinic acid	11.9	14.1	9.3	13.2	48.2	34.9	20.1	5.6	0.7	60.9
Nicotinamide-N-oxide	-	0.1	0.2	0.7	1.0	0.2	0.3	0.3	1.0	1.8
2 and 4-Pyridone*	0.1	0.5	0.4	0.6	1.6	-	-	0.1	0.1	0.2
6-Hydroxynicotinamide	-	-	-	-	-	-	-	-	-	-
Nicotinamide	0.1	0.1	0.2	0.1	0.5	0.6	0.7	0.1	0.3	1.7
Total	12.9	16.2	12.0	18.2	59.3	39.9	33.2	9.0	5.6	87.7

*2-pyridone, N^1-methyl-2-pyridone-5-carboxamide
4-pyridone, N^1-methyl-4-pyridone-3-carboxamide
Each value is the average of two experiments which contained three rats in each experiment

after injection. The radioactivity in NAD^+ reached a maximum in ten minutes after injection, while radioactive nicotinic acid mononucleotide and desamido-NAD^+ were only observed within a short time after injection. When the same experiment was repeated with nicotinamide-7-^{14}C, the incorporation of radioactivity into NAD^+ was quite small when compared to that of nicotinic acid. No nicotinic acid mononucleotide, desamido-NAD or nicotinamide mononucleotide was detected during the experimental period. All these data are in agreement with the previous report by Hayaishi and his coworkers (124).

These results suggest that the control mechanisms which regulate the biosynthesis of NAD^+ are not present between nicotinic acid and NAD^+ while the biosynthesis of NAD^+ from nicotinamide is under the restraint of a physiological control mechanism. There are two reported reactions which initiate NAD^+ biosynthesis from nicotinamide, namely nicotinamide deamidase and nicotinamide mononucleotide pyrophosphorylase. These two enzyme activities are inhibited by endogenous substances which have been reported by Greengard (11) and Dietrich (125, 126), respectively. The results obtained from intraportal injection of nicotinamide were quite different from those published by Hayaishi's group (35), who reported that nicotinamide injected into the portal vein was rapidly excreted from the liver and stored in gastrointestinal tract, where it was deamidated to nicotinic acid and

reabsorbed into liver cells for NAD biosynthesis. However, the present results obtained by intraportal injection of nicotinamide show that nicotinamide is rapidly methylated to N^1-methylnicotinamide and excreted from the liver. About 80% of the radioactivity recovered in the liver was present in N^1-methylnicotinamide within 2-5 minutes after injection of nicotinamide-7-^{14}C. Similar results were also reported from rat liver perfusion experiments by Henderson and coworker (149). These findings suggest that methylation of nicotinamide in the liver may play a role in the control of nicotinamide concentration. Whether N^1-methylnicotinamide can be further utilized for NAD^+ synthesis or is only excreted in the urine is not yet known.

Another interesting result was the rapid conversion of nicotinic acid into nicotinamide and its derivatives. It is known (7) that nicotinamide can only be formed in mammalian liver from the degradation of NAD^+ or NMN. Thus, the nicotinamide formed in these experiments had to be considered as a degradation product of NAD^+. There are several enzymes which are involved in the degradation of NAD^+. These are NAD^+-pyrophosphorylase, NAD^+-phosphatase, NAD^+-glycohydrolase and ARPPR polymerase. However, the first two enzymes may be tentatively excluded because no NMN can be detected in the liver extracts. The enzymes which probably form nicotinamide from NAD^+ are NAD-glycohydrolase or the nuclear enzyme which polymerizes the

ARPPR moiety of NAD^+ with release of nicotinamide. However, Bock and coworkers (145) have recently reported that the nuclear NAD^+ nucleotidase is probably identical with the enzyme which polymerize the adenosine diphosphate ribose moiety of NAD^+ into an acid insoluble polymer. The formation of nicotinamide increased with increasing dosage of nicotinic acid probably due to the increased activity in the presence of the abnormally high concentration of NAD^+ which is formed from the nicotinic acid in the hepatic tissue within a short time after injection. In order to reduce the NAD^+ concentration in the liver to normal levels, the NADase could degrade the excess NAD^+ in the liver, under normal conditions. Possible mechanisms for increased NADase activity could be either increased NADase synthesis or the NADase activity could be stimulated by high concentrations of NAD^+.

In the urinary studies comparing hypophysectomized and normal rats after injection of 5 mg or 500 mg per kg of nicotinamide or nicotinic acid, the rate of excretion of vitamin in hypophysectomized rats was slower than that in normal rats. Further, the rate of excretion of N^1-methylnicotinamide and nicotinuric acid in the urine of hypophysectomized rats was also slower than in normal rats. The percentage of nicotinic acid excreted in the urine of hypophysectomized rats within 24 hours following the injection of nicotinamide was greater than that in normal rats. This results agrees with the previous report

that hypophysectomized rats have a greater nicotinamide deamidase activity in the liver than do normal rats (156).

Nicotinuric acid and N^1-methylnicotinamide are the metabolic end products of administered nicotinamide and nicotinic acid. N^1-methylnicotinamide can be further oxidized to N^1-methyl-2-pyridone-5-carboxamide and N^1-methyl-4-pyridone-3-carboxamide <u>in vivo</u> and these compounds also excreted in the urine. The small amount and slow rate of the excretion of nicotinuric acid and N^1-methylnicotinamide in hypophysectomized rats followed the injection of nicotinamide and nicotinic acid indicates that the hypophysectomized rats have a reduced capacity to dispose of the administered vitamin. The formation of nicotinamide derivatives in the nicotinic acid injection experiments presumably results from nicotinamide formed by degradation of NAD^+. In normal rats, the nicotinamide formed from degradation of NAD^+ was largely excreted in the urine as N^1-methylnicotinamide (5.7% within 24 hours after injection of nicotinic acid). However, hypophysectomized rats only excreted one-half the amount of N^1-methylnicotinamide which was excreted by normal rats. This provides a possible explanation for the increased hepatic NAD^+ concentration in hypophysectomized rats following the injection of nicotinic acid.

In conclusion, the effect of hypophysectomy on nicotinamide deamidase, the slow rate of excretion of the injected vitamin and the slow rate of methylation of

nicotinamide in hypophysectomized rats provide a possible explanation for the greatly increased magnitude and duration of hepatic NAD^+ concentration following the injection of nicotinamide or nicotinic acid.

Another interesting result in both normal and hypophysectomized rats was the greatly increased excretion of nicotinic acid and nicotinuric acid following the intraperiotoneal injection of a large dose of nicotinamide. It is known that the synthesis of NAD^+ in hepatic tissues is initiated by conversion of nicotinamide to nicotinic acid by nicotinamide deamidase. This hepatic enzyme has been found to have high K_m value (4×10^{-2} to 2.5×10^{-1}M) (11, 119) and its activity in crude extracts is masked by an endogenous inhibitor. The injection of a large dose of nicotinamide may overcome its high K_m value of nicotiamide deamidase in liver or cause the removal of the endogenous inhibitor *in vivo*.

CHAPTER V

ISOLATION AND IDENTIFICATION OF TWO NEW URINARY NICOTINAMIDE METABOLITES

During the course of quantitative studies on the metabolism of nicotinic acid in hypophysectomized rats following the injection of 5 mg per kg of nicotinic acid-7-^{14}C, a previously unreported urinary metabolite of nicotinic acid was observed. This compound had chromatographic behavior similar to nicotinuric acid in n-butanol saturated with 3% ammonia solution and n-butanol-acetic acid (4:1:2,V/V). However, it could be separated from nicotinuric acid on cellulose powder thin layer plates using n-butanol saturated with 3% ammonia solution as a developing solvent. The unknown was tentatively identified as 6-hydroxynicotinic acid by comparison of its migration during electrophoresis and column and paper chromatography with that of authentic 6-hydroxynicotinic acid.

Isolation of Unknown Metabolite

In order to positively identify this unknown compound, three 200 to 300 gram male rats were intraperitoneally injected with 500 mg/kg nicotinic acid-7-^{14}C (50 mg/ml).

The urine was collected for 24 hours after injection. The collected urines were pooled together and evaporated to dryness under reduced pressure at 50°C. The residue was dissolved in 10 ml of water and applied to a 2.5 x 30 cm Dowex-50-H^+ column which was eluted with water. Figure 10 shows the resulting elution pattern. The first radioactive peak contained the unknown and N^1-methyl-2-pyridone-5-carboxamide. This peak was pooled and evaporated to 1 ml under reduced pressure. This solution was then applied to Whatman No. 1 paper and developed by descending chromatography using n-butanol saturated with 3% ammonia as a developing solvent. The unknown was eluted from paper with water and rechromatographed in n-butanol-acetic acid-water. The radioactive peak on the chromatogram was eluted with water and further purified by recrystallizing from hot water.

Identification of Unknown Compound

This new metabolite crystallized as white needles and had a melting point of 304°C with decomposition. The mass spectrum of the unknown compound (Figure 11, upper spectrum) was almost identical with that 6-hydroxynicotinic acid (lower spectrum). Both compounds have a molecular ion of m/e 139 and fragment ions of m/e 122, 111, 94, 66, 41, 39, and 28. The postulated route of formation of these ions is given in Figure 12. The molecular ion, m/e 139, loses an OH to form ion m/e 122, further loss of

Figure 10. Isolation of Unknown Compound From the Urine of Nicotinic Acid Injected Rats by Dowex-50-H^+ Column Chromatography

Ten ml of a water solution of urine was placed on a 2.5 x 20 cm column of Dowex-50-H^+. The column was eluted with water and 10 ml fractions were collected. Radioactivity was determined on 0.1 ml aliquots by liquid scintillation counting and the absorbance at 290 mμ measured with a Beckman DU Spectrophotometer. The radioactive peak contained a mixture of N'-methyl-2-pyridone-5-carboxamide and the unknown compound.

———— A_{290}, - - - - - - Radioactivity

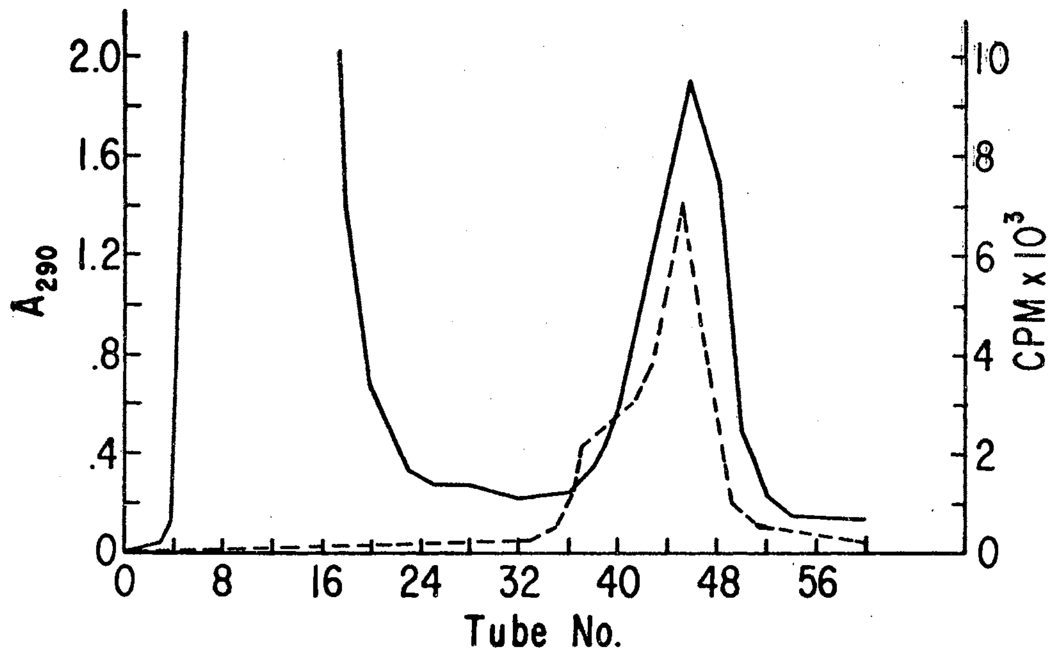
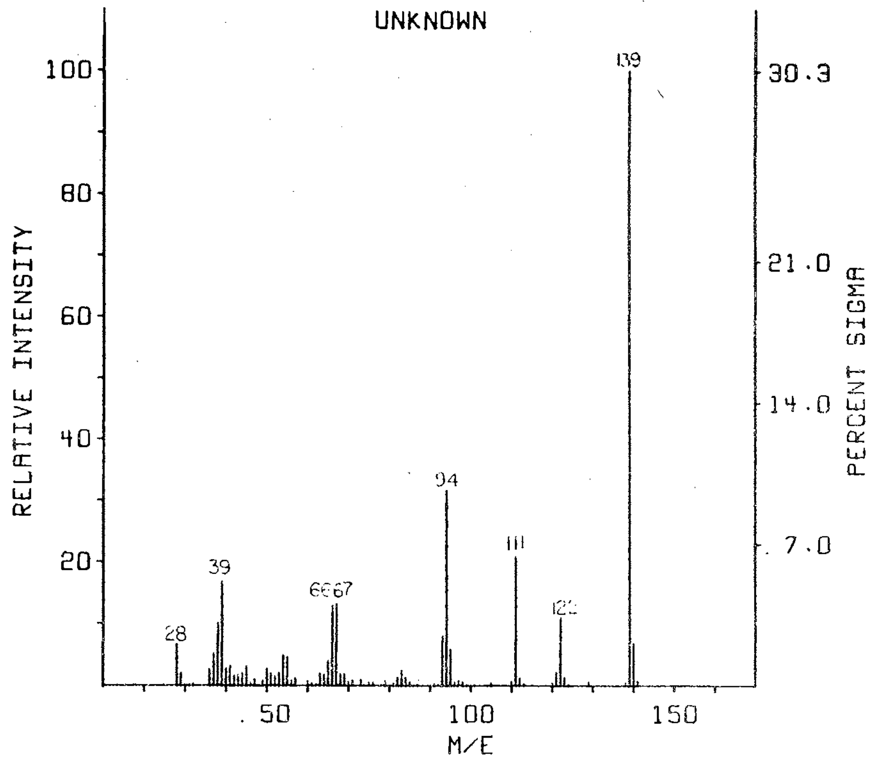

2.0
1.6
1.2
.8
.4
0
A_{290}
10
8
6
4
2
0
CPM x 10^3
0
8
16
24
32
40
48
56
Tube No.

Figure 11. Mass Spectra of Authentic 6-Hydroxynicotinic Acid and the Compound Isolated From the Urine of Nicotinic Acid Injected Rats

Mass spectra were determined using the prototype (146) of the LKB-9000 gas chromatograph-mass spectrometer using the direct inlet probe. The ion source temperature was 70 eV, and acceleration voltage was 3.5 volts. The mass spectra were computer-plotted from tabular intensity data with a Calcom 565 plotter driven by an IBM 1620 computer using a Fortran II-D program.


UNKNOWN
RELATIVE INTENSITY
PERCENT SIGMA
M/E
139
94
111
39
6667
28
100
80
60
40
20
50
100
150
30.3
21.0
14.0
7.0

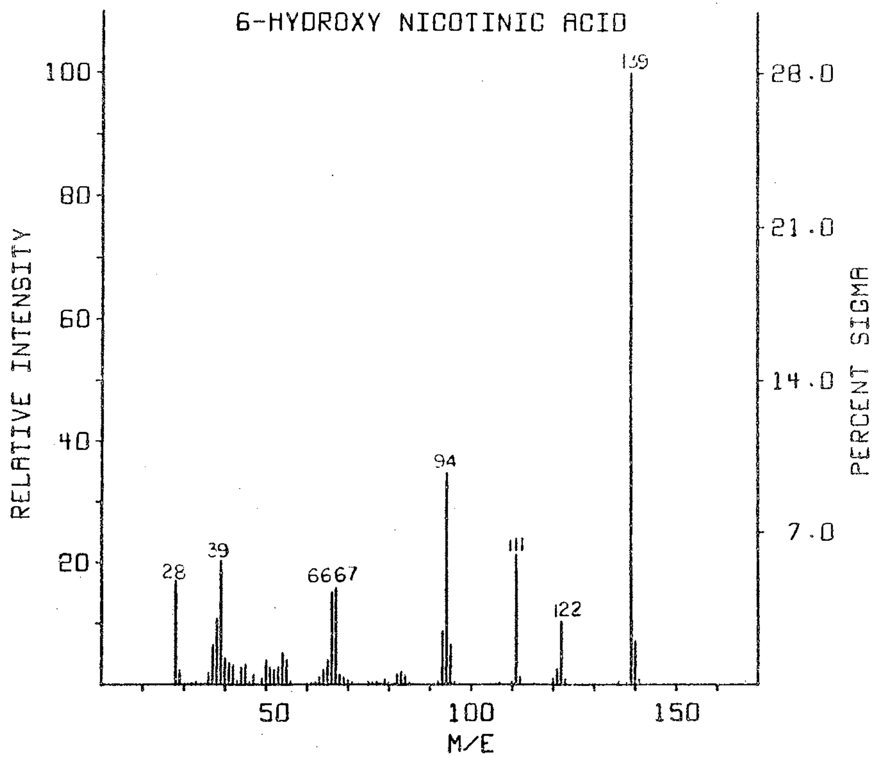
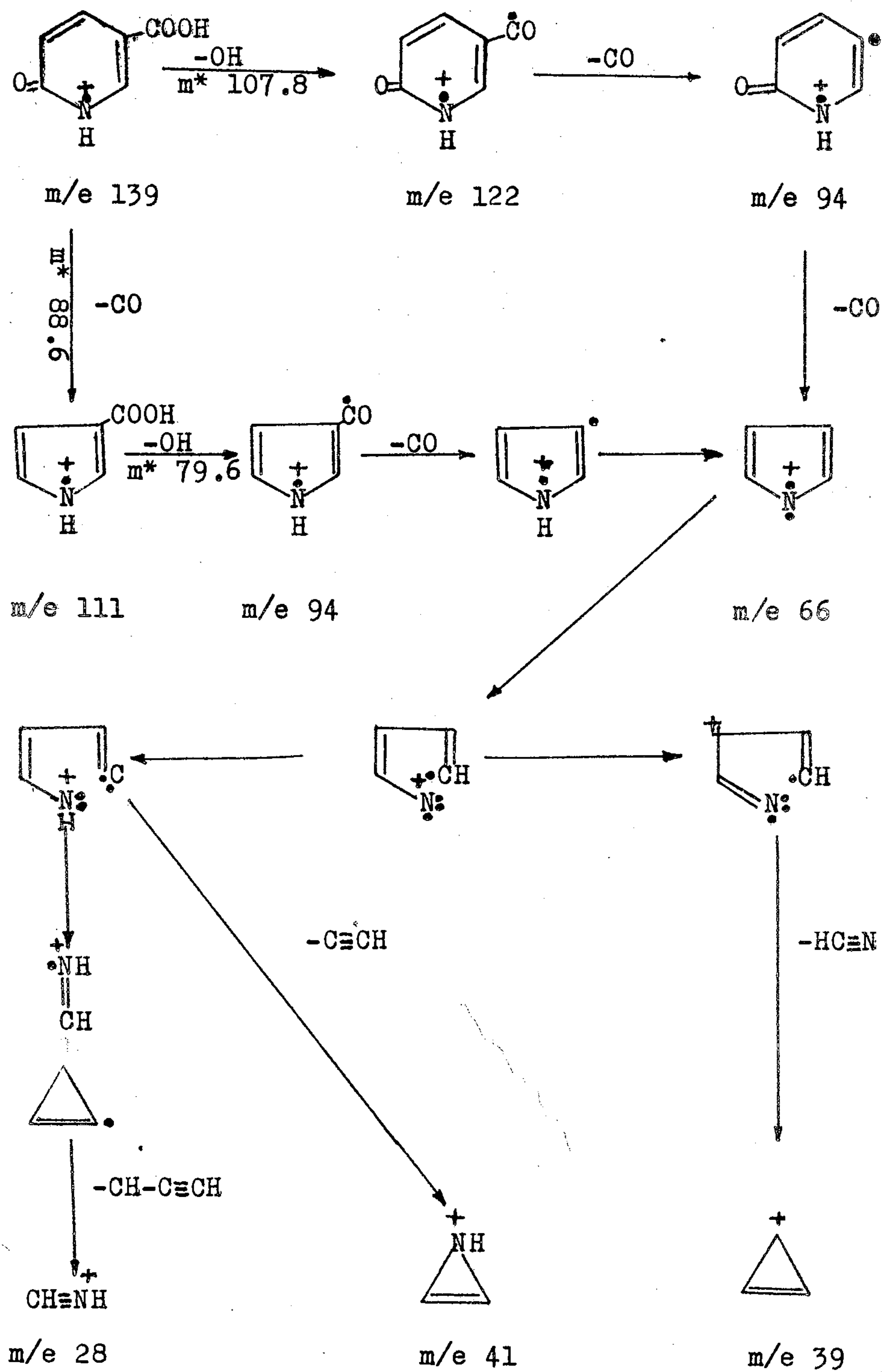

6-HYDROXY NICOTINIC ACID
RELATIVE INTENSITY
PERCENT SIGMA
M/E
139
94
111
122
39
6667
28
100
80
60
40
20
50
100
150
28.0
21.0
14.0
7.0

Figure 12. Proposed Fragmentation Pattern of 6-Hydroxynicotinic Acid Under Electron Impact

M* metastable ion



COOH
-OH
m* 107.8
m/e 139
CO
-CO
m/e 122
m/e 94
m* 88.6
-CO
-CO
COOH
-OH
m* 79.6
CO
-CO
m/e 111
m/e 94
m/e 66
CH
CH
-C≡CH
-HC≡N
NH
CH
-CH-C≡CH
CH≡NH
NH
m/e 28
m/e 41
m/e 39

CO and CO at the 6 position of the pyridine ring form ions of m/e 94 and m/e 66. The ion m/e 66 could also be formed by successive loss of CO at the 6-position of the pyridine ring to form ion m/e 111, OH to form ion m/e 94 and CO to form ion m/e 66. The ion m/e 66 can be further cleaved at the C-N bond, and then could either lose H-C≡N to form an ion m/e 39 or lose -C≡H to form an ion m/e 41 or rearrange to ion $\overset{NH}{\underset{\triangle}{C}}H$ and then lose C-C≡CH to form an ion m/e 28.

The ultraviolet spectra of the unknown compound and authentic 6-hydroxynicotinic acid in water, 1 N HCl and 1 N NaOH solution are given in Figure 13. Both compounds have two identical maximum UV absorption peaks at 296 m and 254 mμ in the water solution. The absorption maxima at 254 mμ and 295 mμ of both compounds are shifted to 258 mμ when measured in 1 N HCl, and are shifted to 265 mμ when measured in 1 N NaOH solution. Figure 14 shows the infrared absorption spectra of unknown (upper spectrum) and 6-hydroxynicotinic acid in KBr pellet. Both compounds have identical very broad bonded OH stretching absorption in the region of 3300 to 2500 cm^{-1} (3.0-4.0 μ), and strong -CO absorption at 1420 cm^{-1} (7.0 μ) and 1160 cm^{-1} (8.0 μ).

In summary, these methods confirmed the identification of the unknown as 6-hydroxynicotinic acid.

The finding of urinary 6-hydroxynicotinic acid suggested that the corresponding 6-hydroxy derivative might also be formed from nicotinamide in the rat. In fact,

Figure 13. Ultraviolet Absorption Spectra of Authentic 6-Hydroxynicotinic Acid and the Unknown Isolated From the Urine of Nicotinic Acid Injected Rats

These spectra were measured using a Beckman D.B. Spectrophotometer. Compound dissolved in water ———— ,

in 1 N HCl - - - - - - - , in 1 N NaOH —.—.—

A, authentic 6-hydroxynicotinic acid;

B, unknown compound.

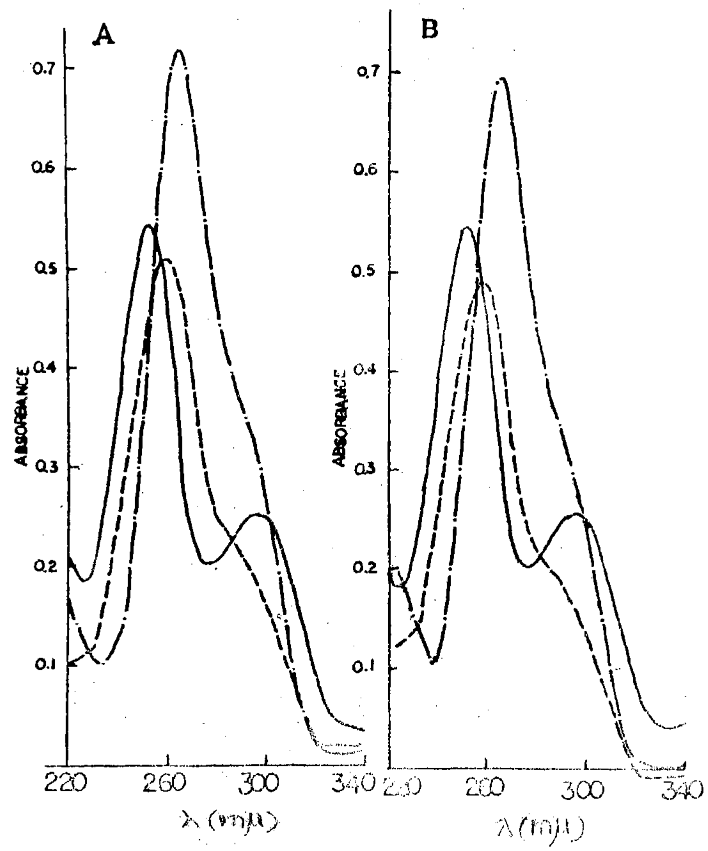
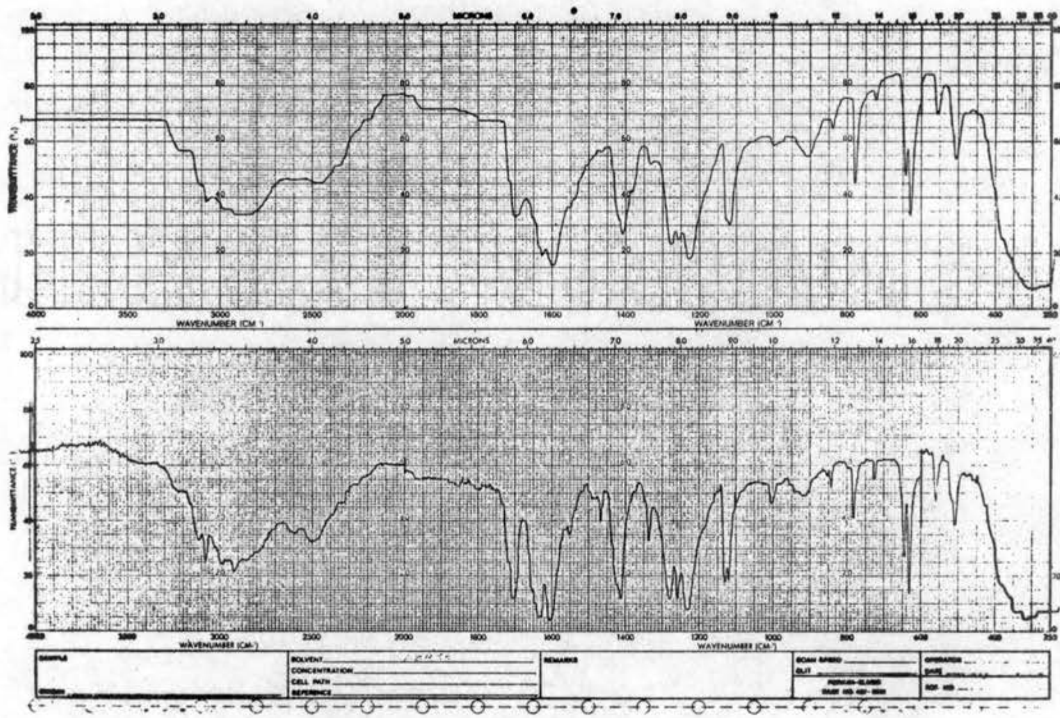

A
B
0.7
0.6
0.5
0.4
0.3
0.2
0.1
ABSORBANCE
220
260
300
340
λ (mμ)

Figure 14. Infrared Spectra of Authentic 6-Hydroxynicotinic Acid and the Unknown Compound Isolated From the Urine of Nicotinic Acid Injected Rats

The spectra were determined with a Perkin-Elmer 457 Grating Infrared Spectrophotometer in a KBr pellet prepared with a Microsampling Kit. 6-Hydroxynicotinic Acid (lower spectrum); Unknown Compound (upper spectrum).



thin layer chromatography and high voltage paper electrophoresis of urine from rats injected with 500 mg/kg nicotinamide-7-^{14}C revealed a new compound which was isolated from pooled 24 hours urines of three rats by ion exchange chromatography. Figure 15 shows the elution pattern obtained when this urine was applied to a Dowex-50-H^{+} column and eluted with water. The first radioactive peak contained a mixture of unknown and N^{1}-methyl-2-pyridone-5-Carboxamide. Further chromatography of this peak of Dowex-1-formate using water as the eluting solvent produced two peaks (Figure 16). Peak I contained the pyridone contaminated with a small amount of the unknown and peak II contained the unknown contaminated with a small amount of pyridone. The pyridone was separated by paper chromatography in n-butanol saturated with 3% ammonia. The unknown was eluted from the paper and recrystallized from hot water.

Figure 17 shows a comparison of the ultraviolet absorption spectra of authentic 6-hydroxynicotinamide (A) and isolated material in distilled water, 1 N HCl and 1 N NaOH solution. Both compounds have two absorption maxima at 295 mμ and 260 mμ in water and in 1 MN HCl solution, however, in 1 N NaOH solution both peaks were shifted to 270 mμ and the extinction coefficient is increased.

The infrared spectra of 6-hydroxynicotinamide (upper spectrum) and isolated compound (lower spectrum) are shown

Figure 15. Isolation of Unknown Metabolite From the Urine of Nicotinamide Injected Rats

Ten ml of a water solution of urine solids was placed on a 2.5 x 20 cm column of Dowex-50 H^+. The column was eluted with water and 10 ml fractions were collected. Radioactivity was determined on 0.1 ml aliquots by liquid scintillation counting and the absorbance at 290 mu measured with a Beckman DU spectrophotometer. Radioactive peak I contained a mixture of N^1-methyl-2-pyridone-5-carboxamide and the unknown compound. Tubes 34-43 were pooled and concentrated to 15 ml under vacuum.

——— A_{290} ------- radioactivity

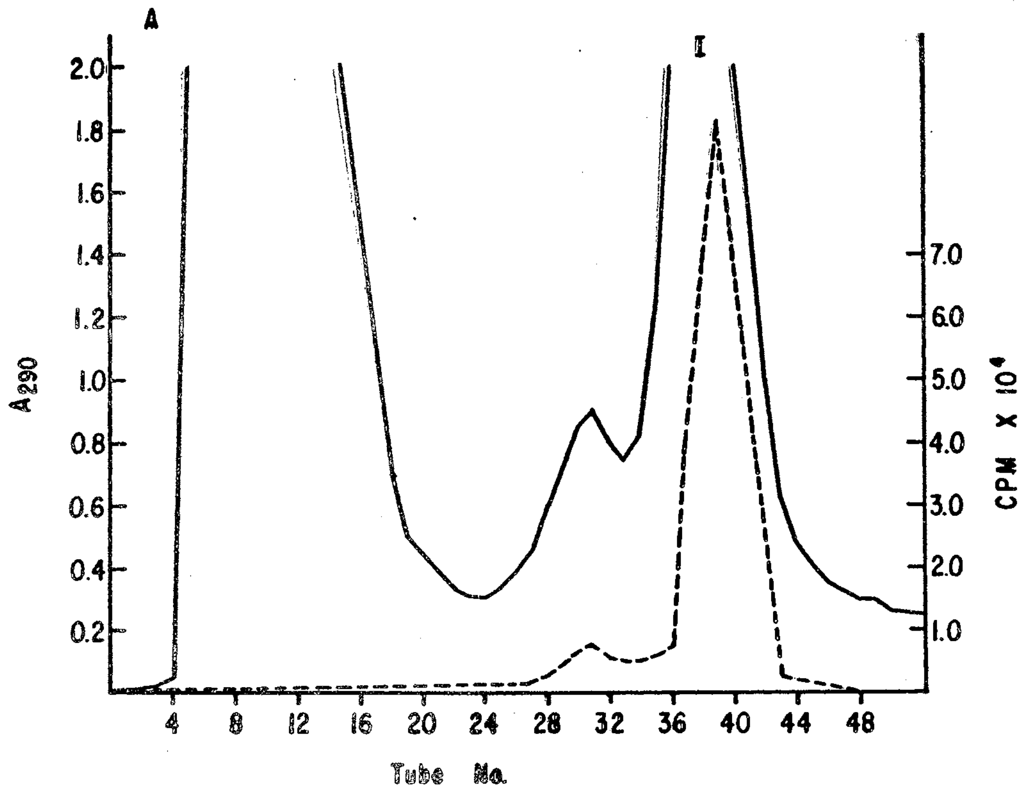

A
I
2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
A_{290}
7.0
6.0
5.0
4.0
3.0
2.0
1.0
CPM X 10^4
4
8
12
16
20
24
28
32
36
40
44
48
Tube No.

Figure 16. Dowex-1-Formate Column Chromatographic Separation of Radioactive Peak I From Figure 15

The solution obtained from Figure 15 was applied to a 2.5 x 20 cm Dowex-1 formate column. The column was eluted with water, 10 ml fractions were collected and the radioactivity and absorbency measured as above. Peak I: N'-methyl-2-pyridone-5-carboxamide contaiminated with a small amount of the unknown compound. Peak II: The unknown contaminated with a small amount of pyridone.

- - - - - - - - : radioactivity

———————— : A_{290}

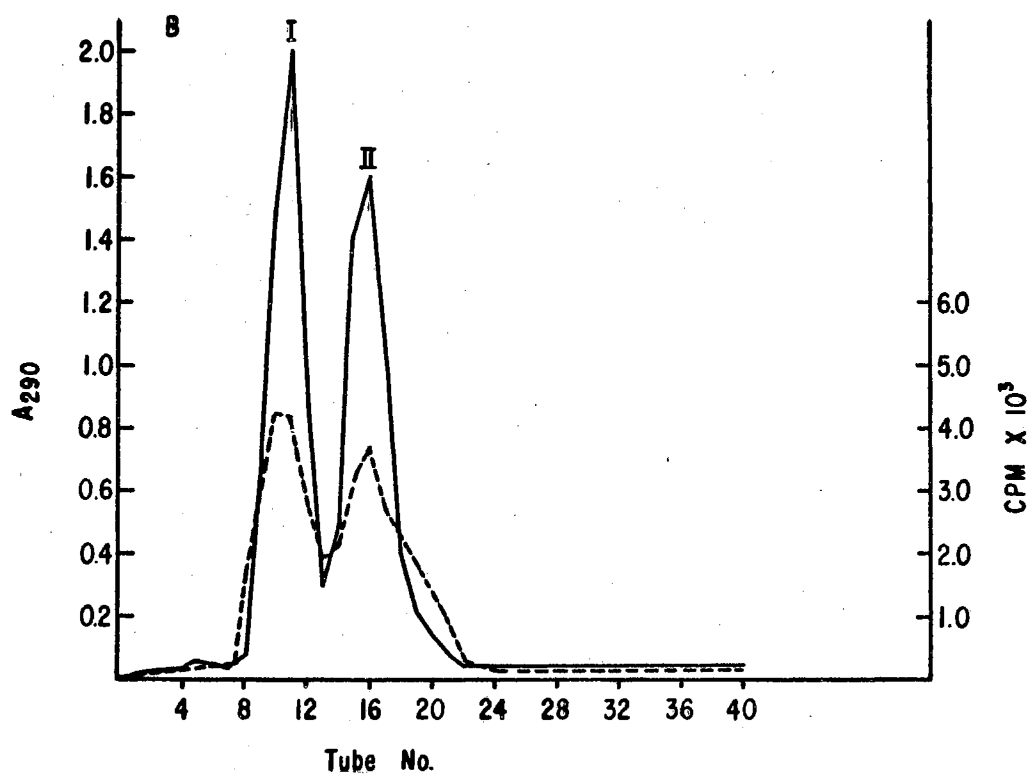
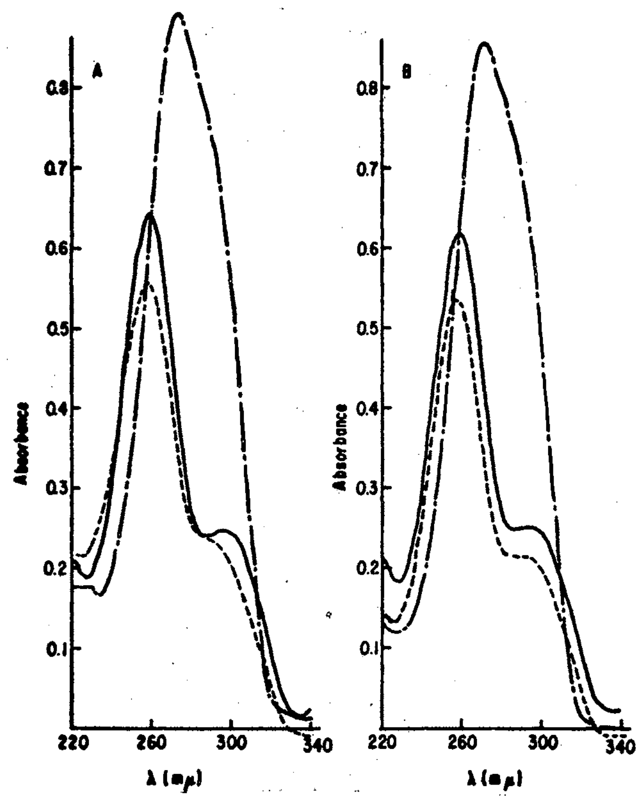

B
I
II
2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
A_{290}
6.0
5.0
4.0
3.0
2.0
1.0
CPM X 10^3
4 8 12 16 20 24 28 32 36 40
Tube No.

Figure 17. Ultraviolet Spectra of A, Authentic 6-Hydroxynicotinamide and B, Unknown

The material isolated from the urine of nicotinamide injected rats; measured using a Bechman D.B. spectrophotomer. Compound dissolved in water ________,
in 1 N HCl - - - - - - ; in 1 N NaOH -.-.-.-


A
B
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
Absorbance
220
260
300
340
λ (mμ)

in Figure 18. The strong absorption at 3300-3400 cm^{-1} indicates the presence of $-NH_2$ or -OH group, the strong absorption between 1600-1700 cm^{-1} indicates a -CO group. Figure 19 shows the mass spectra of 6-hydroxynicotinamide (upper spectrum) and the unknown. Both compounds have a molecular ion of m/e 138 and fragment ion of m/e 122, 110, 94, 66, 41, 39, and 28. The proposed fragmentation pattern of these compounds is shown in Figure 20. The parent peak is 138. Loss of $-NH_2$ to gives B. (m/e 122). Further loss of -CO and CO from the 6-position of the pyridine ring would result in ion C (m/e 94) and ion D (m/e 66). Ion D also could be formed by successive loss of -CO (at the 6 position of the pyridine ring) to form ion D (m/e 66). Ion D can be further cleaved at the C-N bond to form ion G which can either lose H-C≡N to form ion K (m/e 39) or C ≡ CH to form ion H (m/e 41) or rearrange to ion I followed by loss of Ċ - C ≡ C to form ion J (m/e 28).

It is known that 6-hydroxynicotinic acid is a bacterial metabolite of nicotinic acid (150). In order to determine whether the 6-hydroxy derivatives of nicotinic acid and amide are formed by the intestinal flora in the rats' tissues, germ free rats were used. Following injection with 500 mg per kg or 5 mg per kg of nicotinic acid or nicotinamide into germ free rats, the urines were subjected to Dowex-50 chromatography. The first radioactive peak eluted with water was chromatographed from the urine of rats injected high doses of nicotinamide. Two

Figure 18. Infrared Spectra of Authentic 6-Hydroxynicotinamide (Upper Spectrum) and the Unknown Compound Isolated From the Urine of Nicotinamide Injected Rats.

Spectra were determined with a Perkin-Elmer 457 Grating Infrared Spectrophotometer in a KBr pellet prepared with a Microsampling Kit.

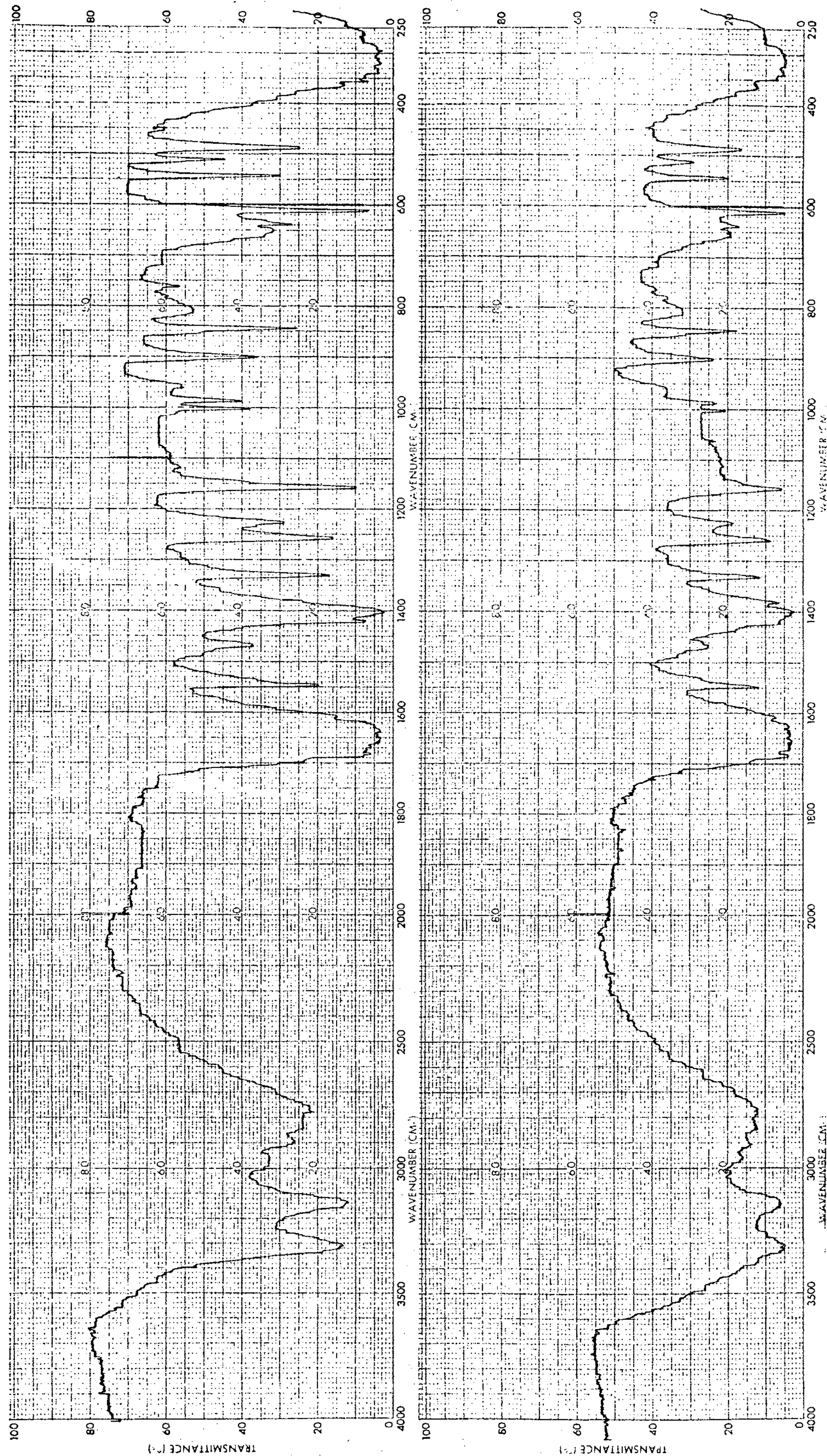
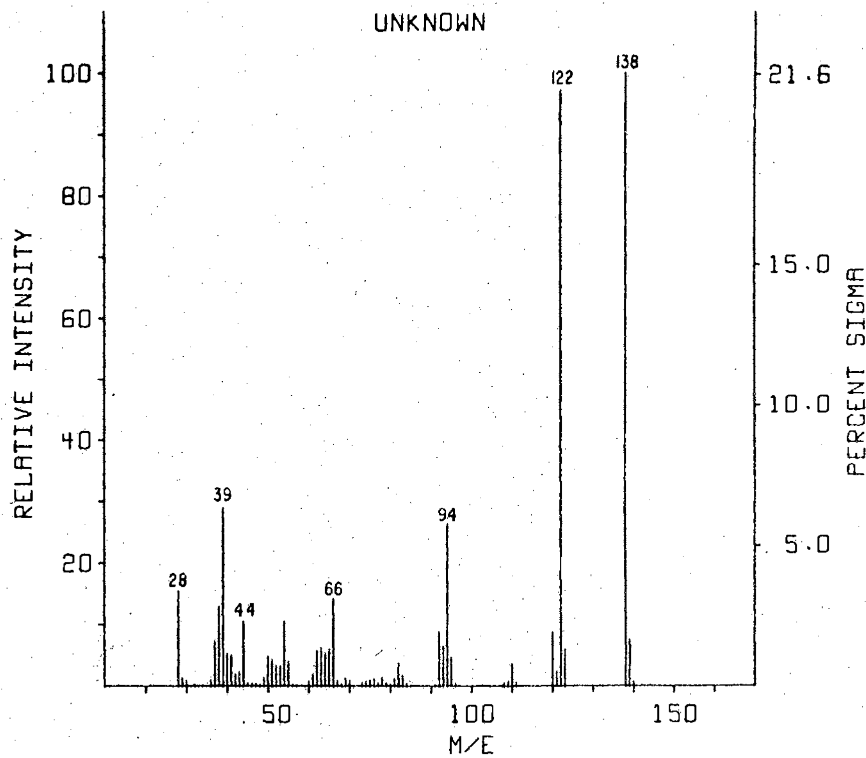

WAVENUMBER (CM⁻¹)
TRANSMITTANCE
4000 3500 3000 2500 2000 1800 1600 1400 1200 1000 800 600 400 250
0 20 40 60 80 100

Figure 19. Mass Spectra of Authentic 6-Hydroxynicotinamide and the Compound Isolated From the Urine of Nicotinamide Injected Rats

Mass spectra were determined using the prototype (146) of the LKB-9000 gas chromatograph-mass spectrometer using the direct inlet probe. The ion source temperature was 310°C, ionization current was 65 μamps, electron energy was 70 eV. and acceleration voltage was 3.5 volts. The mass spectra were computer-plotted from tabular intensity data with a CalCom 565 plotter driven by an IBM 1620 computer using a Fortran II-D program.


UNKNOWN
RELATIVE INTENSITY
PERCENT SIGMA
M/E
100
80
60
40
20
21.6
15.0
10.0
5.0
50
100
150
28
39
44
66
94
122
138

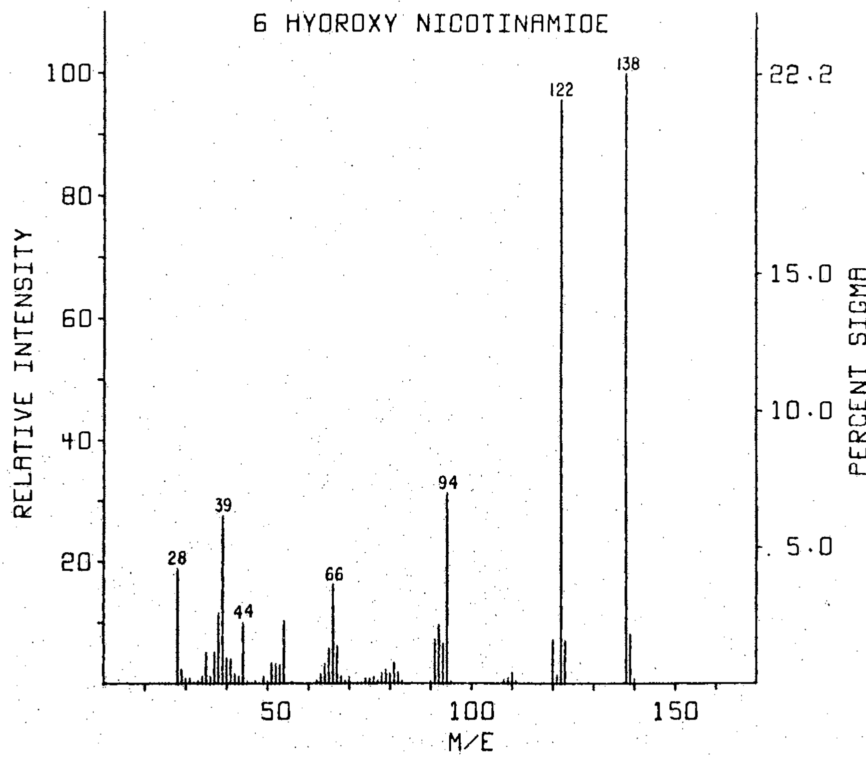
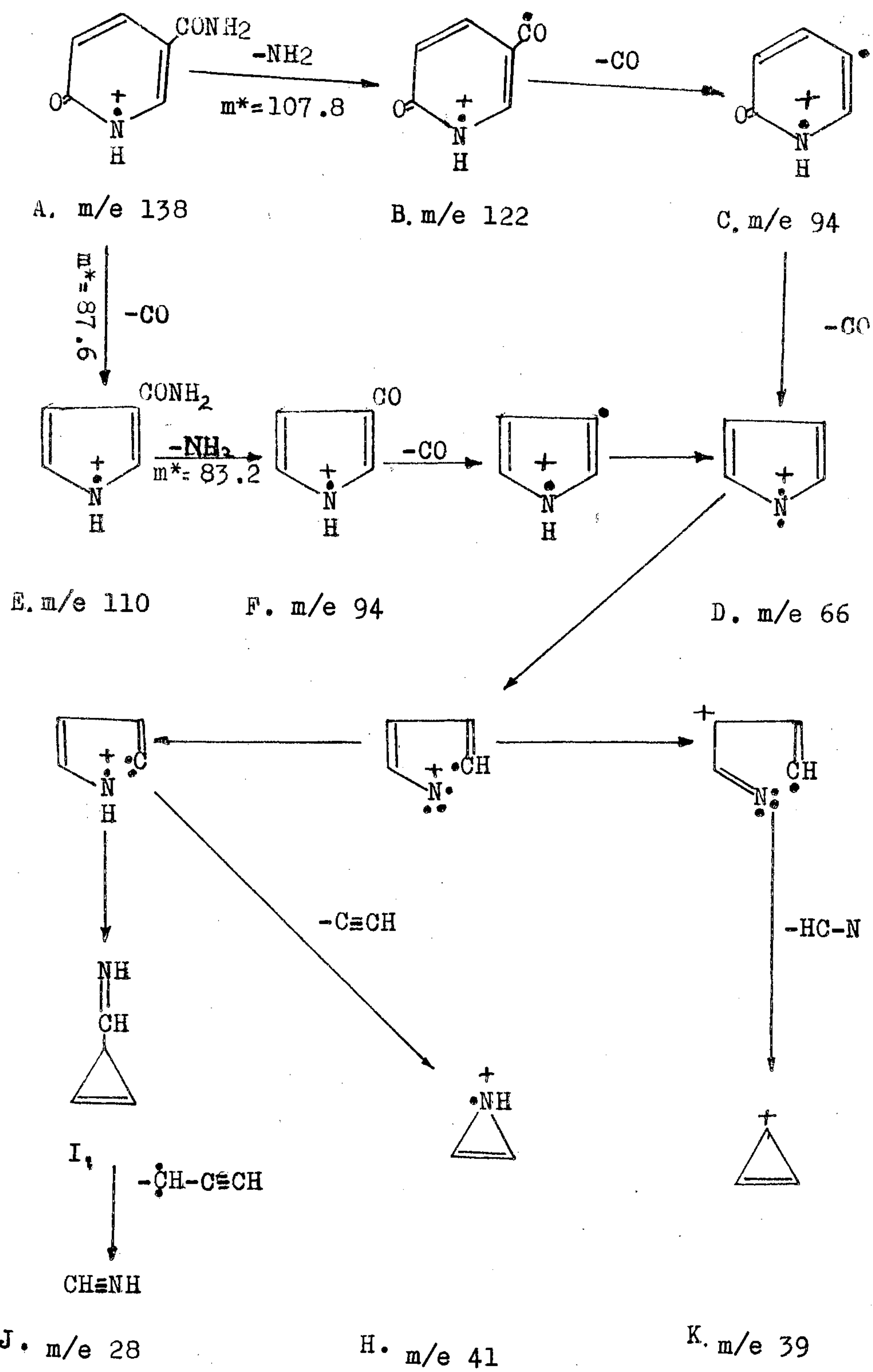

6 HYDROXY NICOTINAMIDE
RELATIVE INTENSITY
PERCENT SIGMA
M/E
100
80
60
40
20
22.2
15.0
10.0
5.0
50
100
150
28
39
44
66
94
122
138

Figure 20. Proposed Fragmentation Pattern of 6-Hydroxynicotinamide

M^* = Metaslable ion.

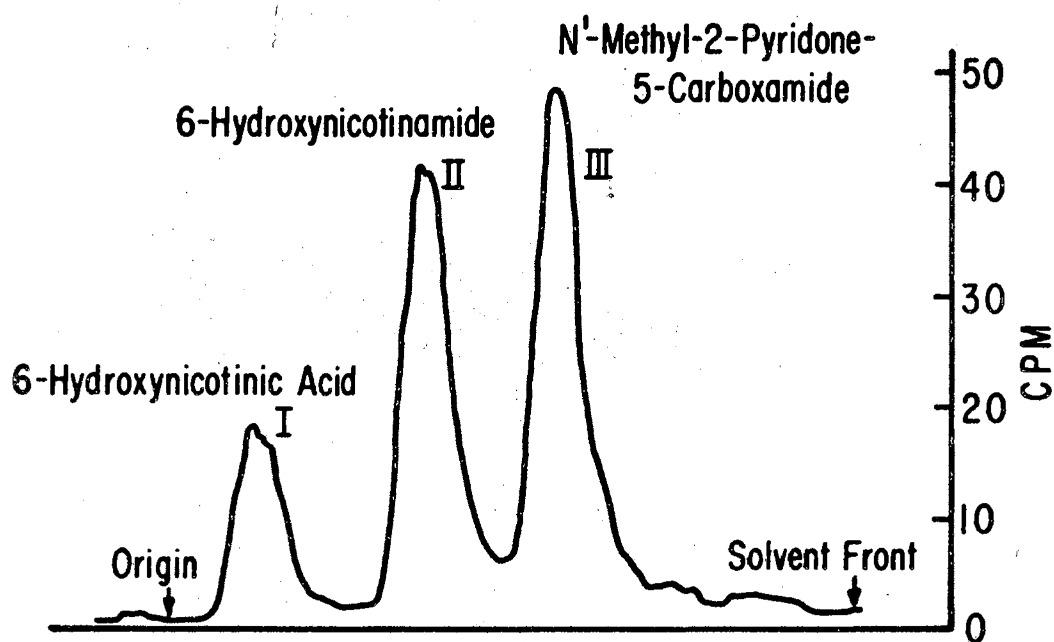

CONH2
-NH2
m*=107.8
CO
-CO
A. m/e 138
B. m/e 122
C. m/e 94
m*= 87.6
-CO
-CO
CONH2
-NH2
m*= 83.2
CO
-CO
E. m/e 110
F. m/e 94
D. m/e 66
CH
CH
-C≡CH
-HC-N
NH
CH
NH
I.
-CH-C≡CH
CH≡NH
J. m/e 28
H. m/e 41
K. m/e 39

radioactive peaks were observed. The first peak was 6-hydroxynicotinamide. In order to further establish the identity of these compounds, they were eluted with water from the paper, 40 mg and 20 mg of carrier, respectively, were added and the compounds were recrystallized five times. As shown in Table IV, after the second recrystallization, the specific activity of both compounds remained essentially constant. Therefore, 6-hydroxynicotinamide and 6-hydroxynicotinic acid are products of metabolism in rat tissue and are not products of intestinal flora.

Table V shows the comparison of the excretion of ^{14}C in urine and expired $^{14}CO_2$ from germ free and normal rats treated with nicotinic acid-7-^{14}C and nicotinamide-7-^{14}C. Table VI shows the distribution of radioactivity among the urinary metabolites following the injection 5 mg or 500 mg per kg of nicotinamide-7-^{14}C or nicotinic acid-7-^{14}C into germ free rats. These data from germ free rats are similar to those obtained from normal rats, with the exception that in germ free rats there was no detectable radioactive carbon dioxide from injected nicotinamide and nicotinic acid-7-^{14}C. This suggests that the formation of radioactive carbon dioxide from injected nicotinamide and nicotinic acid-7-^{14}C is due to the metabolism of the intestinal flora and not to metabolism by the rats tissue.

Figure 21. Paper Strip Counter Recording of the Descending Paper Chromatogram From a Sample of Germ Free Rat Urine After Dowex-50-H Column Chromatography

The Paper Chromatogram was developed in n-butanol saturated with 3% ammonia solution. The radioactive peak was identified by comparison with an authentic sample on the same chromatogram.



N'-Methyl-2-Pyridone-
5-Carboxamide
6-Hydroxynicotinamide
II
III
6-Hydroxynicotinic Acid
I
Origin
Solvent Front
50
40
30
20
10
0
CPM

TABLE IV

RECRYSTALLIZATION OF URINARY RADIOACTIVE UNKNOWN METABOLITES OF GERM FREE RATS WITH AUTHENTIC CARRIER

Peak Number	Carrier	Specific Activity Following Recrystallization (CPM/mg)			
		2nd	3rd	4th	5th
I	6-Hydroxynicotinic acid	107	119	119	102
II	6-Hydroxynicotinamide	165	136	149	155

TABLE V

A COMPARISON OF THE EXCRETION OF RADIOACTIVITY IN THE URINE AND EXPIRED CO_2 OF GERM FREE AND NORMAL RATS 24 HOURS AFTER INTRAPERITONEAL ADMINISTRATION OF NICOTINIC ACID-7-^{14}C AND NICOTINAMIDE-7-^{14}C

Type of Rat	Treatment	Radioactivity Excreted in the Urine (% of dose)	Expired Radioactive CO_2 (% of dose)
	Nicotinamide		
germ free	500 mg/kg	76.5	0.0
germ free	5 mg/kg	54.3	0.0
normal	500 mg/kg	70.4	2.1
normal	5 mg/kg	68.5	7.7
	Nicotinic Acid		
germ free	500 mg/kg	76.9	0.0
germ free	5 mg/kg	50.3	0.0
normal	500 mg/kg	87.7	1.2
normal	5 mg/kg	50.4	4.7

TABLE VI

THE DISTRIBUTION OF RADIOACTIVITY IN THE URINARY METABOLITES OF GERM FREE RATS FOLLOWING THE ADMINISTRATION OF NICOTINAMIDE-7-^{14}C AND NICOTINIC ACID-7-^{14}C (PER CENT OF INJECTED DOSE)*

Compound	nicotinic acid 500 mg/kg	nicotinic acid 5 mg/kg	nicotinamide 500 mg/kg	nicotinamide 5 mg/kg
N^1-methylnicotinamide	1.4	8.4	8.3	45.1
6-Hydroxynicotinic acid	0.6	1.0	0.4	0.4
Nicotinuric acid	17.3	24.9	9.8	0.5
Nicotinic acid	56.8	9.9	15.0	0.5
Nicotinamide-N-oxide	2.6	1.3	8.0	3.2
6-Hydroxynicotinamide	trace	0.2	0.6	0.5
N'-methyl-2-pyridone-5-carboxamide	0.2	1.0	0.6	4.5
N'-methyl-4-pyridone-3-carboxamide	trace	0.6	0.9	2.0
Nicotinamide	1.6	0.7	38.2	5.9

*Each figure in this table is the average of two rats.

CHAPTER VI

SUMMARY

Nicotinic acid is rapidly incorporated into NAD^+ within a short time after intraportal injection of nicotinic acid into normal and hypophysectomized rats. The newly formed NAD^+ is also rapidly degraded probably by NADase into nicotinamide. The formation of nicotinamide increases with an increasing dose of injected nicotinic acid. This indicates that NADase probably plays a key role in control of the concentration of NAD^+ in liver. In the experiment in which nicotinamide was injected, the nicotinamide was rapidly incorporated into N^1-methylnicotinamide immediately after injection and only a small amount of NAD^+ was formed within 60 minutes in hypophysectomized and normal rats.

The rate of excretion of nicotinic acid and nicotinamide in the urine followed the intraperitoneal injection of nicotinamide and nicotinic acid in hypophysectomized rats is slower than that in normal rats. Hypophysectomized also excrete smaller amounts of N^1-methynicotinamide and nicotinuric acid within 24 hours after injection of nicotinic acid and nicotinamide than do normal rats. This finding provides a possible explanation for the increase

in the magnitude and duration of the elevation in rat liver NAD following the administration of nicotinamide and nicotinic acid to hypophysectomized rats. The excretion of nicotinic acid in the urine was greatly increased by injection of a large dose of nicotinamide. Two new urinary nicotinamide metabolites have been isolated and identified by spectrometric methods as 6-hydroxynicotinic acid and 6-hydroxynicotinamide. Studies with germ free rats indicate that these new metabolites are formed by tissue metabolism and not by metabolism of the intestinal flora. The formation of respiratory $^{14}CO_2$ following intraperitoneal injection of nicotinamide-7-^{14}C and nicotinic acid-7-^{14}C into normal rats which was previously reported is not due to the metabolism of rat tissue but to the metabolism of intestinal flora.

BIBLIOGRAPHY

1. Warburg, O., and W. Christian. Biochem. Z., 274, 112 (1934) 275, 464 (1935).

2. Fuler, H. V., H. Albers, and F. Shlenk. Z. Physiol. Chem., 240, 113 (1936).

3. Dann, W. J. Science, 86, 616 (1937).

4. Street, H. R., and G. R. Cowgill. Proc. Soc. Expl. Biol., 37, 547 (1937).

5. Spices, T. D., C. Cooper, and M. A. Blankerhorn. J. Amer. Med. Assoc., 110, 622 (1938).

6. Harris, L. J. Chem. and Ind., 56, 1134 (1937).

7. Kaplan, N. O. in Metabolic Pathways (edited by Greenberg, D. M.), 2, 627. New York: Academic Press, 1961.

8. Greengard, P., G. P. Quinn, and M. B. Reid. J. Biol. Chem., 239, 1887 (1964).

9. Greengard, P., G. P. Quinn, and M. B. Reid. J. Biol. Chem., 240, 486 (1965).

10. Greengard, P., B. Petrack, and H. Kalisky. J. Biol. Chem., 242, 152 (1967).

11. Petrack, B., P. Greengard, A. Craston, and H. Karlinsky. Biochem. Biophys. Res. Comm., 13, 478 (1963).

12. Gholson, R. K. Nature, 212, 933 (1966).

13. Huber, C. Ann. Chem. Pharm., 141, 271 (1867).

14. Huber, C. Ber., 3, 849 (1870).

15. Weidel, H. Ann. Chem. Pharm., 165, 328 (1873).

16. Laiblin, R. Ber., 10, 2136 (1877).

17. Suzuki, U., T. Shimura, and S. Odake. Biochem. Z., 43, 89 (1912).

18. Funk, C. J. Physiol., 46, 173 (1913).

19. Williams, R. R. J. Biol. Chem., 29, 495 (1917).

20. Szymanska, R. M., and Funk, C.. Chem. Zelle. Gewebe, 13, 44 (1926).

21. Warburg, O., W. Christian, and W. Griese. Biochem. Z., 282, 157 (1935).

22. Kuhn, R., and H. Vetter. Ber., 68 (1935).

23. Funk, C., and I. C. Funk. J. Biol. Chem., 119, XXXV (1937).

24. Frost, D. V., and C. A. Elvehjem. J. Biol. Chem., 121, 255 (1937).

25. Koehn, C. J., and C. A. Elvehjem. J. Nutrition, 11, 67 (1936).

26. Sebrell, W. H., R. H. Onstat, H. F. Franser, and F. S. Daft. J. Nutrition, 16, 355 (1938).

27. Smith, D. T., J. M. Ruffin, and S. G. Smith. J. Amer. Med. Asso., 109, 2054 (1937).

28. Goldbert, J., and G. A. Wheller. Public Health Report (U S), 43, 172 (1928).

29. Denton, J. Amer. J. Pathol., 4, 341 (1928).

30. Jensenius, H., and F. Norgaard. Acta. Pathol. Microbiol. Scan., 19, 433 (1942).

31. Lillie, R. D. Natl. Inst. Health. Bull., 162, 13 (1933).

32. Joliffe, N., K. M. Bowman, L. A. Rosenblum, and H. D. Fein. J. Amer. Med. Assoc., 114, 307 (1940).

33. Recommended Dietary Allowance, Natl Acad. Sci. Natl. Res. Council Publ., Washington, D. C., 1958, p. 13.

34. Kodicek, E. Lancet, 1, 380 (1942). Schaeffer, A. E., J. M. McKibbin, and C. A. Elvehjem. J. Biol. Chem., 25, 341 (1943).

35. Laiblin, R. Annalen, 196, 135 (1879).

36. Weidel, H., and Leibbrandt. Ber. 50, 385 (1917).

37. Reilly Tar and Chemical Corp. B.P. 563273.

38. Ciba B.P. 831561.

39. Allied Chemical and Dye Corp. U.S.P. 243600.

40. McElvain, S. M., and M. A. Goese. J. Amer. Chem. Soc., 63, 2283 (1941).

41. Koeppe, R., and R. Hall. Biochem. Biophys. Acta., 22, 544 (1956).

42. Krehl, W. A., L. J. Teply, P. S. Sarma, and C. A. Elvehjem. Science, 101, 489 (1945).

43. Rosen, F., J. W. Huff, and W. A. Perlzweig. J. Biol. Chem., 163, 343 (1946).

44. Singal, S. A., A. P. Briggs, V. P. Sydensticken, and J. M. Littlejohn. J. Biol. Chem., 166, 573 (1946).

45. Luecke, R. W., W. N. McMillen, F. Thorp, and C. Tull. J. Nutrition, 36, 417 (1948).

46. Singal, S. A., V. P. Syndentricker, and J. M. Littlejohn. J. Nutrition, 176, 1051 (1948).

47. Sarett, H. P., and G. A. Goldsmith. J. Biol. Chem. 177, 461 (1949).

48. Henderson, L. M., G. B. Ramasrma, and B. C. Johnson. J. Biol. Chem., 166, 573 (1946).

49. Beadle, G. W., H. K. Mitchell, and J. F. Nyc. Proc. Natl. Acad. Sci. U. S., 33, 155 (1952).

50. Partridge, C. W. H., D. M. Bonner, and C. Yanofsky. J. Biol. Chem. 194, 269 (1952).

51. Wilson, R. G., and L. M. Henderson. Fed. Proc., 20, 448 (1961).

52. Mitchell, H. K., and J. F. Nyc. Proc. Natl. Acd. Sci. U. S., 34, 11 (1948).

53. Haskins, F. A., and H. K. Mitchell. Proc. Natl. Acad. Sci. U. S., 35, 500 (1949).

54. Bonner, D. M. Proc. Natl. Acad. Sci. U. S., 34, 5 (1948).

55. Heidelberg, C., E. P. Abraham, and S. Lepkovsky. J. Biol. Chem., 175, 471 (1948).

56. Heidberg, C. J. Biol. Chem., 176, 1461 (1949).

57. Heidberg, C. J. Biol. Chem., 179, 139 (1949).

58. Sadan, D. R., and D. M. Greenberg. Arch. Biochem., 25, 323 (1950).

59. Joshi, J. G., and P. Handler. J. Biol. Chem., 237, 929 (1962).

60. Schayer, R. W., G. L. Foster, and D. Shemin. Federation Proc., 8, 248 (1949).

61. Mitchell, H. K., J. F. Nyc, and R. D. Owen. J. Biol. Chem., 175, 433 (1948).

62. Krehl, W. A., D. Bonner, and C. Yanofsky. J. Nutrition, 41, 159 (1950).

63. Schweiger, B. S. J. Biol. Chem., 178, 707 (1949).

64. Priest, R. E., A. H. Bokman, and B. S. Schweigert. Proc. Soc. Expt. Biol. Med., 78, 477 (1951).

65. Bokman, A. H., and B. S. Schweigert. Arch. Biochem. and Biophys., 33, 270 (1951).

66. Wiss, Z. Natureforsch., 9B, 740 (1954).

67. Miyake, A., A. H. Bokman, and B. S. Schweigert. J. Biol. Chem., 211, 391 (1954).

68. Long, C. L., H. N. Hill, I. M. Weinstock, and L. M. Henderson. J. Biol. Chem., 211, 405 (1954).

69. Nishizuka, V., and O. Hayaishi. J. Biol. Chem., 238, 3369 (1963).

70. Piress, J., and P. Handler. J. Biol. Chem., 233, 488 (1958).

71. Priess, J., and P. Handler. J. Biol. Chem., 233, 493 (1958).

72. Handler, P., and J. R. Klein. J. Biol. Chem., 143, 49 (1942).

73. Handler, P., and J. R. Klein. J. Biol. Chem., 144, 453 (1942).

74. Ito, E., and J. L. Strminger. J. Biol. Chem., 237, 2696 (1962).

75. Snell, E. E., and L. D. Wright. J. Biol. Chem., 139, 675 (1941).

76. Stainer, R. Y., and M. Tsuchida. Bacterial., 58, 45 (1949).

77. Yanofsky, C. J. Bacterial., 68, 577 (1954).

78. Isquith, A. J., and A. G. Moat. Biochem. Biophys. Res. Commun., 22, 55 (1966).

79. Hadwiger, L. A., S. E. Badiei, G. R. Waller, and R. K. Gholson. Biochem. Biosphys. Res. Commun., 13, 466 (1963)

80. Ortega, M. V., and G. M. Brown. J. Biol. Chem., 235, 2939 (1960).

81. Albertson, J. N., and A. G. Moat. J. Bacterial., 89, 540 (1965).

82. Ahmad, F., and A. G. Moat. Biochem. Biophys. Res. Commun., 22, 565 (1966).

83. Chandler, J. L. R., N. Ogasawa, R. K. Gholson, and A. J. Andreolli. Federation Abstract, 25, 217 (1966).

84. Ackerman, D. Z. Biol., 59, 17 (1912).

85. Komori, Y., and Y. Sendju. J. Biochem., Tokyo, 6, 163 (1926).

86. Perlzueig, W. A., F. Rosen, I. G. Leader, S. Hunter, and P. B. Pearson. Federation Proc., 8, 236 (1949).

87. Ellinger, P., and W. A. Perweig. Biochem. J., 44, 77 (1949).

88. Huff, J. W., and W. A. Perweig. J. Biol. Chem., 142, 401 (1942).

89. Najjar, V. A., and L. E. Holt. Proc. Soc. Exp. Biol. Med., 44, 386 (1940).

90. Najjar, V. A., and L. E. Holt. Science, 93, 20 (1941).

91. Bonavita, V., S. A. Narrod, and N. O. Kaplan. J. Biol. Chem., 236, 936 (1961).

92. Knox, W. E., and W. L. Grossman. J. Biol. Chem., 166, 391 (1946).

93. Knox, W. E., and W. L. Grossman. J. Biol. Chem., 168, 363 (1947).

94. Wu-chang, M. L., and B. C. Johnson. J. Biol. Chem., 236, 2096 (1961).

95. Dann, W. L., and J. W. Huff. J. Biol. Chem., 169, 47 (1947).

96. Najjar, V. A., A. J. Stein, and C. V. Kabler. J. Clin. Inves., 21, 26 (1936).

97. Karrer, P., G. Schwartzenbach, F. Benz, and V. V. Solmssen. Helv. Chim. Acta., 19, 826 (1936).

98. Perlzweig, W. A., M. L. C. Bernheim, and F. Bernheim. J. Biol. Chem., 150, 401 (1943).

99. Ellinger, P. Biochem. J., 42, 175 (1951).

100. Cantoni, G. L. J. Biol. Chem., 189, 203 (1948).

101. Pullman, M. E., and S. P. Colowick. J. Biol. Chem., 206, 121 (1961).

102. Abelson, D., A. Boyle, and H. Seligson. J. Biol. Chem., 238, 717 (1963).

103. Chaykin, S., M. Dagani, L. Johnson, and M. Samli. J. Biol. Chem., 240, 932 (1965).

104. Felsted, R. L., and S. Chaykin. J. Biol. Chem., 242, 1274 (1966).

105. Chaykin, S., and K. Bloch. Biochem. et Biophys. Acta., 31, 213 (1959).

106. Murray, K. N., and S. Chaykin. J. Biol. Chem., 241, 2029 (1966).

107. Murray, K. N., and S. Chaykin. J. Biol. Chem., 241, 3468 (1966).

108. Murray, K. N., J. G. Watson, and S. Chaykin. J. Biol. Chem., 241, 4798 (1966).

109. Huff, J. H., and W. A. Perzweig. Science, 97, 538 (1953).

110. Hoffman, E. C. G., and S. Rapoport. Biochem. Z., 329, 437 (1957).

111. Petrack, B., P. Greengard, and H. Kalisky. J. Biol. Chem., 241, 2367 (1965).

112. Schlenk, F. J. Biol. Chem., 146, 619 (1942).

113. Schlenk, F. Arch. Biochem., 3, 93 (1943).

114. Andrealli, A. J., M. Ikeda, V. Nishizuka, and O. Hayaishi. Biochem. Biophys. Res. Commun., 12, 92 (1963).

115. Gholson, R. K., I. Ueda, N. Ogasawada, and L. M. Henderson. J. Biol. Chem., 239, 1208 (1964).

116. Imsande, J., and P. Handler. J. Biol. Chem., 236, 525 (1961).

117. Smith, L. D., and R. K. Gholson. J. Biol. Chem., 244, 68 (1969).

118. Chaykin, S., M. Dagani, L. Johnson, M. Sami, and J. Bettili. Biochem. Biophy. Acta., 100, 351 (1965).

119. Kirchner, J., J. G. Watson, and S. Chaykin. J. Biol. Chem., 241, 953 (1966).

120. Petrack, B., P. Greengard, A. Craston, H. J. Kalisky. Biochem. Biophys. Res. Commun., 13, 472 (1963).

121. Petrack, B., P. Greengard, A. Craston, and F. Sheppy. J. Biol. Chem., 240, 1725 (1965).

122. Grunicke, H., M. Liersch, M. Hinz, B. Puschendorf, E. Richter, and H. Holtzer. Biochem. Biophys. Acta., 121, 228 (1966).

123. Su, S., and S. Chaykin. Federation Proc., 25, 280 (1966).

124. Ijichi, H., A. Ichiyama, and O. Hayaishi. J. Biol. Chem., 241, 3701 (1966).

125. Dietrich, L. S., L. Fuller, I. L. Yero, and L. Martinz. J. Biol. Chem., 241, 347 (1965).

126. Dietrich, L. S., I. L. Yero, and L. Martinez. Nature, 208, 347 (1965).

127. Greenbaum, A. L., and S. Pinder. Biochem. J., 107, 63 (1968).

128. Kornberg, A., and W. E. Pricer. J. Biol. Chem., 186, 557 (1968).

129. Kornberg, A., and W. E. Pricer. J. Biol. Chem., 182 763 (1950).

130. Joshi, J. G., and P. Handler. J. Biol. Chem., 237, 929 (1962).

131. Sarma, D. S. R., S. Rajalakshmi, and P. S. Sarma. Biochem. Biophys. Res. Commun., 6, 389 (1961).

132. Narrod, S. A., V. Bovita, E. R. Ehrenfeld, and N. O. Kaplan. J. Biol. Chem., 236, 931 (1961).

133. Waller, G. R., K. S. Yang, R. K. Gholson, and L. A. Hadwiger. J. Biol. Chem., 241, 4411 (1966).

134. Dietrich, L. S., and O. Muniz. Federation Proc., 25, 747 (1954).

135. Kaplan, N. O., A. Goldin, S. R. Humphreys, M. M. Ciotti, and F. E. Stalzenback. J. Biol. Chem., 219, 287 (1956).

136. Kaplan, N. O. J. Vitamol., 14, 103 (1968).

137. Kornberg, A., and O. Linberg. J. Biol. Chem., 176, 5665 (1948).

138. Hoffman, E. C. G., and S. Rapoport. Biochem. Biophys. Acta., 18, 296 (1956).

139. Nason, A., N. O. Kaplan, S. P. Colowick, and M. M. Ciotti. J. Amer. Chem. Soc., 75, 3292 (1953); J. Biol. Chem., 209, 467 (1954).

140. Rafer, G. W., S. P. Colowick. J. Biol. Chem., 209, 467 (1954).

141. Chambon, P., J. D. Weill, J. Doly, M. T. Strosser, and P. Mandel. Biochem. Biophys. Res. Commun., 25, 638 (1966).

142. Ueda, K., R. H. Reeder, T. Honjo, Y. Nishizuka, and O. Hayaishi. Biochem. Biophys. Res. Commun., 31, 379 (1968).

143. Nishizuka, Y., K. Ueda, T. Honjo, and O. Hayaishi. J. Biol. Chem., 243, 3765 (1968).

144. Bock, K. W., V. Gang, H. P. Beer, R. Kronau, and H. Grunicke. Eur. J. Biochem., 4, 357 (1968).

145. Ryle, A. R., F. Sanger, L. F. Smith, and R. Kitai. Biochem. J., 60, 541 (1955).

146. Waller, G. R. Proc. Okla. Acad. Sci., 47, 000 (1967). (in press).

147. Jeffay, H., and Alvarez. *J. Anal. Chem.*, *33*, 612 (1961).

148. Leifer, E., L. J. Roth, D. S. Hogness, and M. H. Carson. *J. Biol. Chem.*, *190*, 595 (1951).

149. Hagino, Y., S. J. Lan, C. Y. Ng, and L. M. Henderson. *J. Biol. Chem.*, *243*, 4980 (1968).

150. Tsai, L., I. Paston, and E. R. Stadtman. *J. Biol. Chem.*, *241*, 1807 (1966).

VITA

Yu-Chiang Lee

Candidate for the Degree of

Doctor of Philosophy

Thesis: METABOLISM OF NICOTINIC ACID AND NICOTINAMIDE IN RATS

Major Field: Chemistry

Biographical:

Personal Data: Born on August 11, 1935, in Hsin-Chu, Taiwan, Republic of China, the son of Ah-Fan and Tao-Mei Lee.

Education: Attended grade school in Kwan-se Primary School Kwan-se, Hsin-chu, Taiwan, China; Graduated from Hsin-chu Technical Professional School, June, 1954; received Bachlor of Science degree from Taiwan Christian College, Chung-Li, Taiwan, Republic of China, with a major in Chemistry in August, 1959; completed the requirements for the Doctor of Philosophy degree in May, 1969.

Professional Experience: Research Chemist, 1959-1960; Union Industrial Research Institute, Hsin-chu, Taiwan, China; Teaching Assistant, 1960-1963, Taiwan Christian College; research assistant, Oklahoma State University, 1965-1969.

Professional Organizations: The American Chemical Society, Society of Sigma Xi, Phi Lambda Upsilon.