IDENTIFICATION AND QUANTITATION OF TRYPTOPHAN METABOLITES IN URINE OF NORMAL AND ENDOTOXIN - POISONED MICE

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ABSTRACT

IDENTIFICATION AND QUANTITATION OF TRYPTOPHAN METABOLITES IN URINE OF NORMAL AND ENDOTOXIN-POISONED MICE

By

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Twenty tryptophan metabolites from urine of normal and endotoxin-poisoned mice given D,L-tryptophan (benzene ring-^{14}C) with or without tryptophan load have been separated by thin layer and DEAE-cellulose chromatography and tentatively identified by fluorescence and color reactions and mass spectral analyses. Of these, eleven are metabolities of the kynurenine pathway and nine are metabolites of the serotonin pathway. Injection of 5-hydroxytryptamine-3'-^{14}C instead of labeled tryptophan revealed four additional serotonin metabolites, three of which could be identified. Eleven of the major kynurenine and serotonin pathway metabolites were quantitated. Significantly less tryptophan and its metabolites were excreted by endotoxin-poisoned mice. The decreased excretion of radioactivity was accompanied by significant decreases in the amount of kynurenine metabolites and increases in the serotonin metabolites excreted. After one hour in normal mice

with load 43.4% of the metabolites are from the kynurenine pathway and 8.4% from the serotonin pathway. By contrast, in endotoxin-poisoned mice, 30.4% are from the kynurenine pathway and 17.5% from the serotonin pathway. Similar results are seen in mice given only the labeled amino acid. These results strongly suggest that a shift in tryptophan metabolism occurs in vivo in endotoxin-poisoned animals with resultant increases in the production of serotonin pathway metabolites and decreases in kynurenine pathway metabolites. The implications of these data with respect to the hyper-reactivity of endotoxin-poisoned mice to tryptophan are discussed.

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Katherine Marie Morris

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TABLE OF CONTENTS

Tab

1

2

3

4

5

6

7

8.

9.

LIST OF TABLES

Table | Page

LIST OF FIGURES

Figure Page

INTRODUCTION

Endotoxin-poisoned mice frequently die in convulsions within 8 hours after a delayed but not concurrent injection of tryptophan load (1 mg/gm body weight). Death occurs 24 to 36 hours sooner than in mice given endotoxin alone (11,102,104). While the immediate reasons for the increased hyper-reactivity of endotoxin-poisoned mice to tryptophan have not been established, such responses may be due to the increased production in vivo of potentially toxic tryptophan metabolites such as serotonin. Supporting this latter hypothesis is the fact that pretreatment of endotoxin-poisoned mice with cyproheptadine, an anti-serotonin drug, protects against the enchanced lethality of a delayed injection of tryptophan (104). Further, cyproheptadine protects against serotonin induced hypothermia and the tryptophan induced hypothermia in endotoxin-poisoned mice housed at 15°C. (103).

Depression of the activity of the adaptive liver enzyme tryptophan oxygenase has been the primary enzymatic lesion in tryptophan metabolism described to date in endotoxemia (1,11,104). While it is known that tryptophan oxygenase is depressed, no definitive evidence exists as to whether

there is a correlation between the depressed enzyme activity as monitored *in vitro* and altered tryptophan metabolism *in vivo*.

Funneling of tryptophan from one pathway to another due to changes in tryptophan oxygenase activity has been suggested by Curzon and Green (30) who showed that increased tryptophan oxygenase activity in stessed rats correlated with decreased brain serotonin. Further, Schmike (138) and Sourkes (150) have independently shown that ∂-methyl-tryptophan-mediated increases in tryptophan oxygenase correlated with decreases in blood levels of tryptophan and subsequent decreases in brain serotonin and 5-hydroxyindoleacetic acid levels as well.

In an effort to provide more quantitative data on whether funneling of tryptophan into the serotonin pathway occurs in endotoxin-poisoned animals, the primary objective of the present study has been to determine whether or not there are significant increases in serotonin pathway metabolites in the urine of endotoxin-poisoned compared to normal mice. Studies have been done in animals without and with tryptophan load, the former to monitor tryptophan metabolism at dose levels well below quantities which produce hyper-reactivity and the latter to compare these data with changes in the mice given a potentially lethal dose of the amino acid. Monitoring of urinary tryptophan metabolites as a method of estimating *in vivo* enzyme activity

has been used previously (3,144) and it is believed that the accurate quantitative monitoring of urinary tryptophan metabolites should lead to an accurate description of the *in vivo* catabolism of this amino acid.

To accomplish our objective certain specific goals had to be reached. Techniques had to be devised which would not only allow us to study the wide variety and diverse nature of tryptophan metabolites but would also be sensitive enough to detect the minute quantities of tryptophan metabolites excreted in a single mouse urine sample. Thin layer chromatography (including fluorescence and color reactions), column chromatography, and gas chromatography-mass spectrometry were chosen as methods for this study. Initially Rf values, fluorescence, color reactions, and mass spectral data were established for standard tryptophan metabolites. The use of radioactive D,L-tryptophan (benzene ring-^{14}C) and 5-hydroxytryptamine-3'-^{14}C provided markers in the tryptophan metabolites excreted in urine. From autoradiographs of thin layer plates, individual metabolites could be estimated, Rf values measured, and when concentrations of metabolites were great enough, fluorescence and color reactions determined. Elution profiles from DEAE-cellulose chromatography and gas chromatographic-mass spectral analyses aided in identification of unknown tryptophan metabolites scraped and eluted from thin layer

chromatographic plates. From a combination of these data tentative identification of the unknown urinary tryptophan metabolites could be made. To quantitate the individual metabolites, the location of isotope on the thin layer chromatographic plate was determined by autoradiography and individual spots were scraped and counted by liquid scintillation spectrometry. By comparing the relative quantities of isotope found in the products of the serotonin and kynurenine pathways, one could then determine whether there was a shift in metabolism from the kynurenine pathway in endotoxin-poisoned mice and evaluate the effect of a tryptophan load on the excretion of these metabolites.

LITERATURE REVIEW

PART I

TRYPTOPHAN METABOLISM

Introduction

Metabolism of tryptophan by mammalian systems can proceed through several pathways, yielding numerically more metabolites than any other amino acid. The major pathways primarily lead either to the synthesis of nicotinamide adenine dinucleotide (NAD) and glutaryl CoA, hereafter referred to as the kynurenine pathway, or to the synthesis of 5-hydroxytryptamine (serotonin), hereafter referred to as the serotonin pathway. A third pathway produces tryptamine and metabolites similar in structure to those of the serotonin pathway. The enzymes of this latter pathway are found both in intestinal flora and mammalian systems. Several minor branch pathways are also found throughout the primary pathways. A comprehensive review of tryptophan metabolism has been compiled by Meister (99). These pathways for tryptophan metabolism are presumably regulated not only by substrate availability (43,45,102) but also

by adaptive enzyme activity (44,45,83) and can be disrupted by effectors such as endotoxin (61,102). The purpose of the first two parts of the present review is to familiarize the reader with tryptophan metabolism and endotoxin, and to present the relationship between these two diverse subjects. The third part of the review will summarize some of the techniques utilized to isolate and identify tryptophan metabolites.

The Kynurenine Pathway

The initial reaction in the kynurenine pathway is cleavage of the indole nucleus of tryptophan between C2 and C3 with the addition of molecular oxygen to form formylkynurenine (Figure 1). Tryptophan oxygenase (L-tryptophan:oxygen oxidoreductase, EC 1.13.1.12), is the adaptive liver enzyme which catalyzes this reaction. Hematin is a cofactor for tryptophan oxygenase (25,42). The activity of this enzyme can be increased by its substrate tryptophan (103,104), glucocorticoids (12,43,62,83,86), and a variety of substrate analogues (104,150). A decrease in the activity of this enzyme can be caused by numerous inhibitors (102,146) including endotixin (61,104).

Formylkynurenine is converted to kynurenine by the enzyme kynurenine formylase found in the mammalian liver (84). Kynurenine is metabolized primarily to 3-hydroxyxykynurenine by kynurenine-3-hydroxylase and requires NADPH

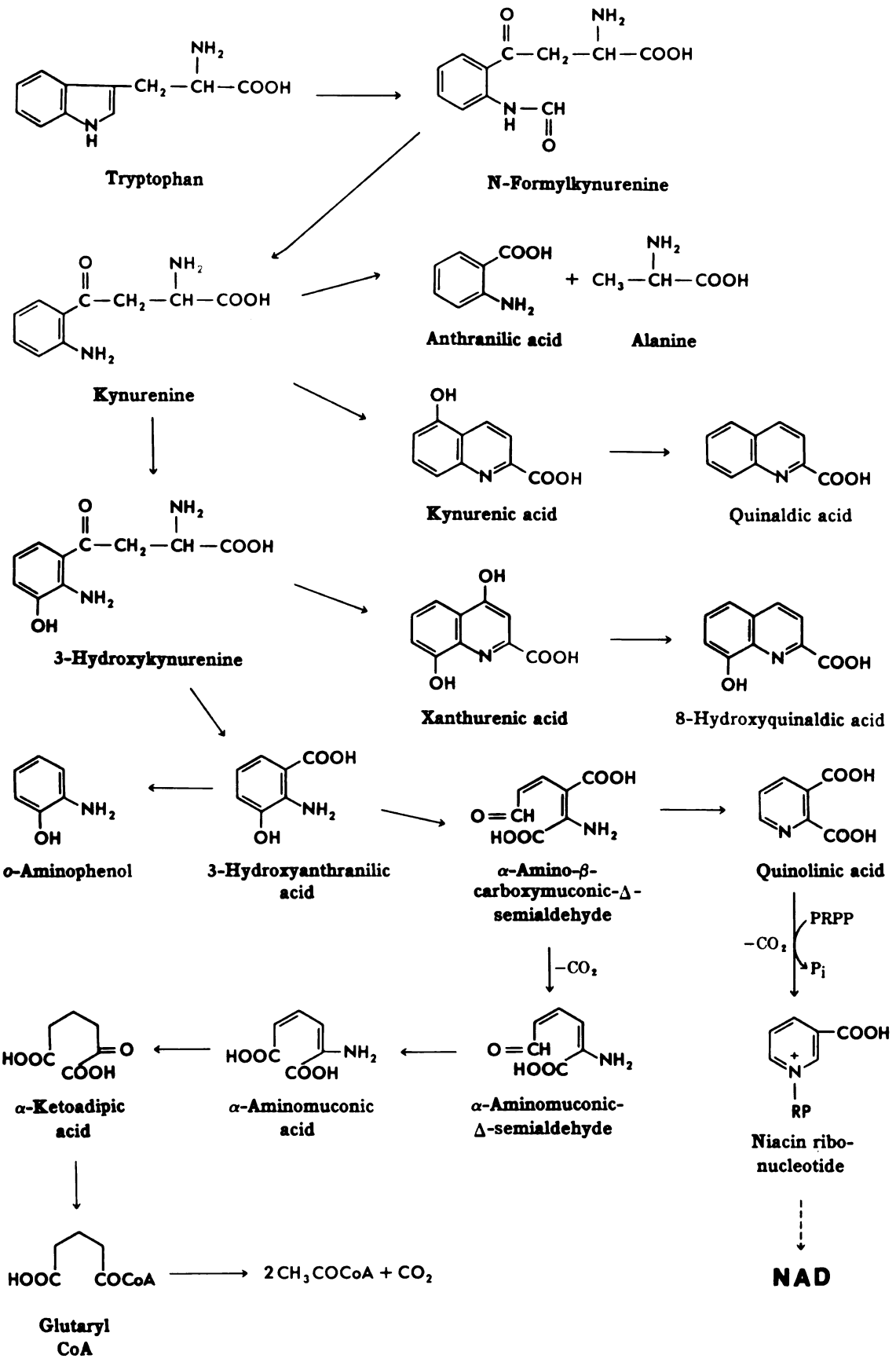


Figure 1.--The Kynurenine Pathway.

and molecular oxygen. The cleavage of alanine from both kynurenine and 3-hydroxykynurenine yielding anthranilic acid and 3-hydroxyanthranilic acid respectively, is catalyzed by the enzyme kynureninase, a pyridoxal phosphate dependent enzyme. Similarly, both kynurenine and 3-hydroxykynurenine may be converted to their keto acids by kynurenine transaminase (160). The keto acids undergo spontaneous ring closure to form kynurenic acid and xanthurenic acid which may then be converted to quinaldic acid and 8-hydroxyquinaldic acid.

3-Hydroxyanthranilic acid may also be converted to the end product o-aminophenol or to α-amino-β-carboxymucconic-Σ-semialdehyde (2-acroleyl-3-aminofumaric acid). The latter reaction is catalyzed by the enzyme 3-hydroxyanthranilic acid oxidase which is found in the liver and kidney (77,112). The semialdehyde is the branch point between the pathways which lead to either NAD or glutaryl CoA. Spontaneous ring closure produces quinolinic acid which is converted in one step to quinolinic acid ribonucleotide. This compound is decarboxylated to nicotinic acid ribonucleotide. It condenses with ATP and is aminated with glutamine to synthesize NAD. Nicotinic acid is not a substrate for these reaction (79).

Glutaryl CoA is synthesized by the decarboxylation of α-amino-β-carboxymuconic-δ-semialdehyde by the liver

enzyme picolinic carboxylase to yield α-aminomuconic-δ-seimaldehyde. This compound is oxidized to α-aminomuconic acid (γ-oxalocrotonic acid) by α-hydroxymuconic-Σ-semialdehyde dehydrogenase and required NAD. The acid is reductively deaminated to α-ketoadipic acid in an NADH requiring reaction. α-Ketoadipic acid is oxidatively decarboxylated to glutaryl CoA by the α-ketoglutarate dehydrogenase complex.

The Serotonin Pathway

In the serotonin pathway, tryptophan is hydroxylated by tryptophan-5-hydroxylase to form 5-hydroxytryptophan (Figure 2). This reaction is the rate limiting step in the biosynthesis of 5-hydroxytryptamine (serotonin) (58). Tryptophan may also be hydroxylated in the 5 position by phenylalanine hydroxylase (127). These enzymes are found in liver (158), intestinal mucosa cells, and kidney (119), but the specific tryptophan-5-hydroxylase is found in highest concentration in the pineal body (82,90,162). Serotonin (5HT) is formed from 5-hydroxytryptophan (5HTP) by the substrate specific enzyme, 5HTP decarboxylase (49). This enzyme is found in the mammalian kidney, with lesser amounts present in the liver (60) and pineal body (13,58, 93). Nerve tissue, sympathetic ganglia, and adrenal medulla also contain substantial 5HTP-decarboxylase activity (49).

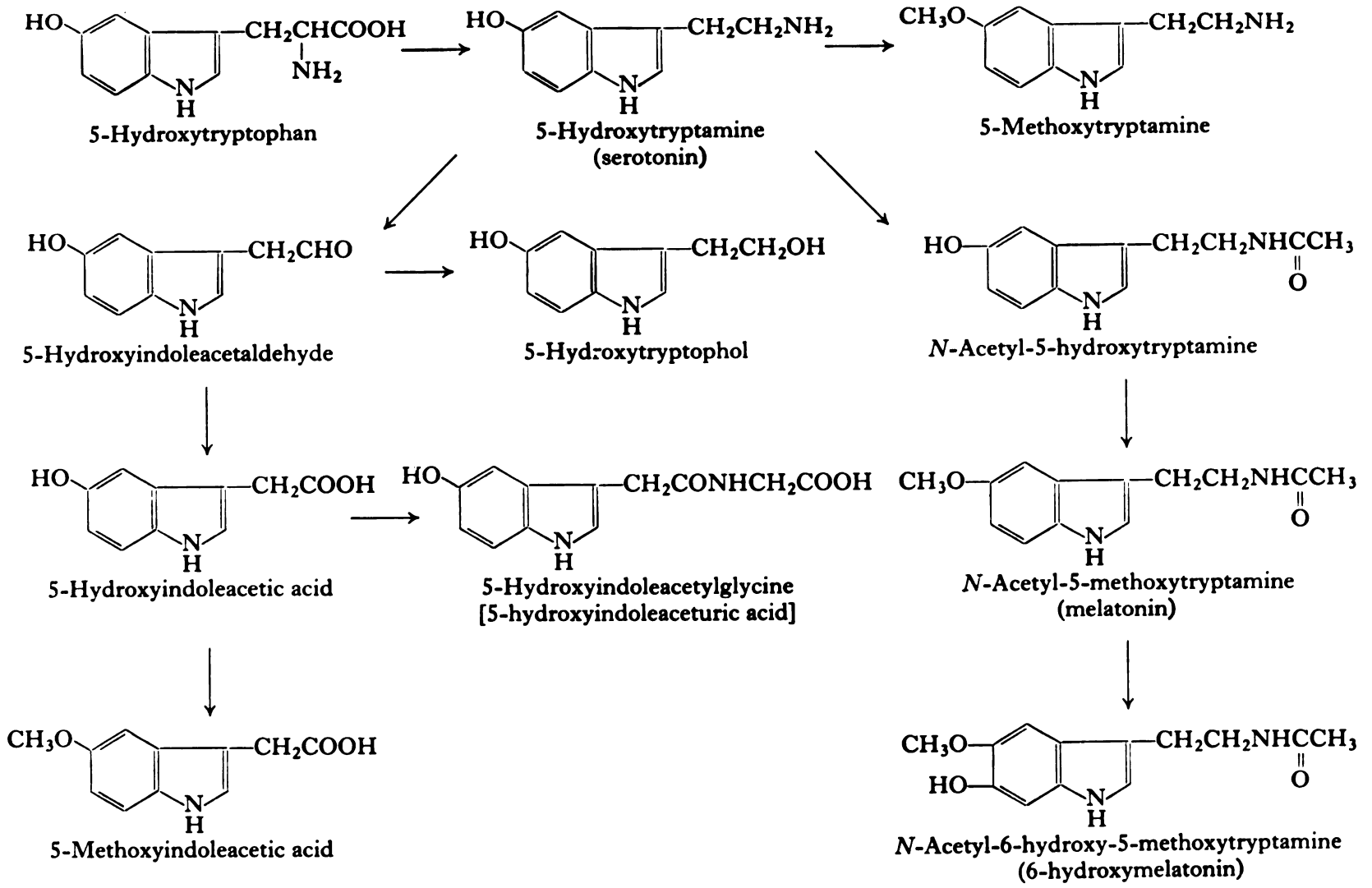


Figure 2.--The Serotonin Pathway.

Aromatic amino acid decarboxylase has a very low affinity for tryptophan and does not play a major role in its decarboxylation (99).

Serotonin may be catabolized to 5-hydroxytryptophal (5-hydroxyindoleacetaldehyde) by monoamine oxidase. This compound is oxidized to 5-hydroxyindoleacetic acid (5HIAA) by aldehyde dehydrogenase. Monoamine oxidase can also form the alcohol, 5-hydroxytryptophol, from 5HT (99). Two important amines found in the brain, bufetonin and melatonin, are synthesized by N-methylation of N'-methylserotonin and N'-acetylserotonin, respectively. Most of the serotonin metabolites may also exist as the 5-methoxy, O-sulfate, and O-glucuronide conjugates (87).

Minor Pathways

In the third pathway, tryptophan is decarboxylated to tryptamine by the liver enzyme, aromatic amino acid decarboxylase (Figure 3). This enzyme has a very low affinity for tryptophan. Tryptamine, which has no specific role in mammals, may then be oxidized by monoamine oxidase to the aldehyde which is acted upon by aldehyde oxidase to form indoleacetic acid. The major route for the synthesis of indoleacetic acid, though, is by transamination of tryptophan to produce indolepyruvic acid which is oxidatively decarboxylated to yield the acid. Enzymes present in bacteria and yeast also catalyze these same reactions (99).

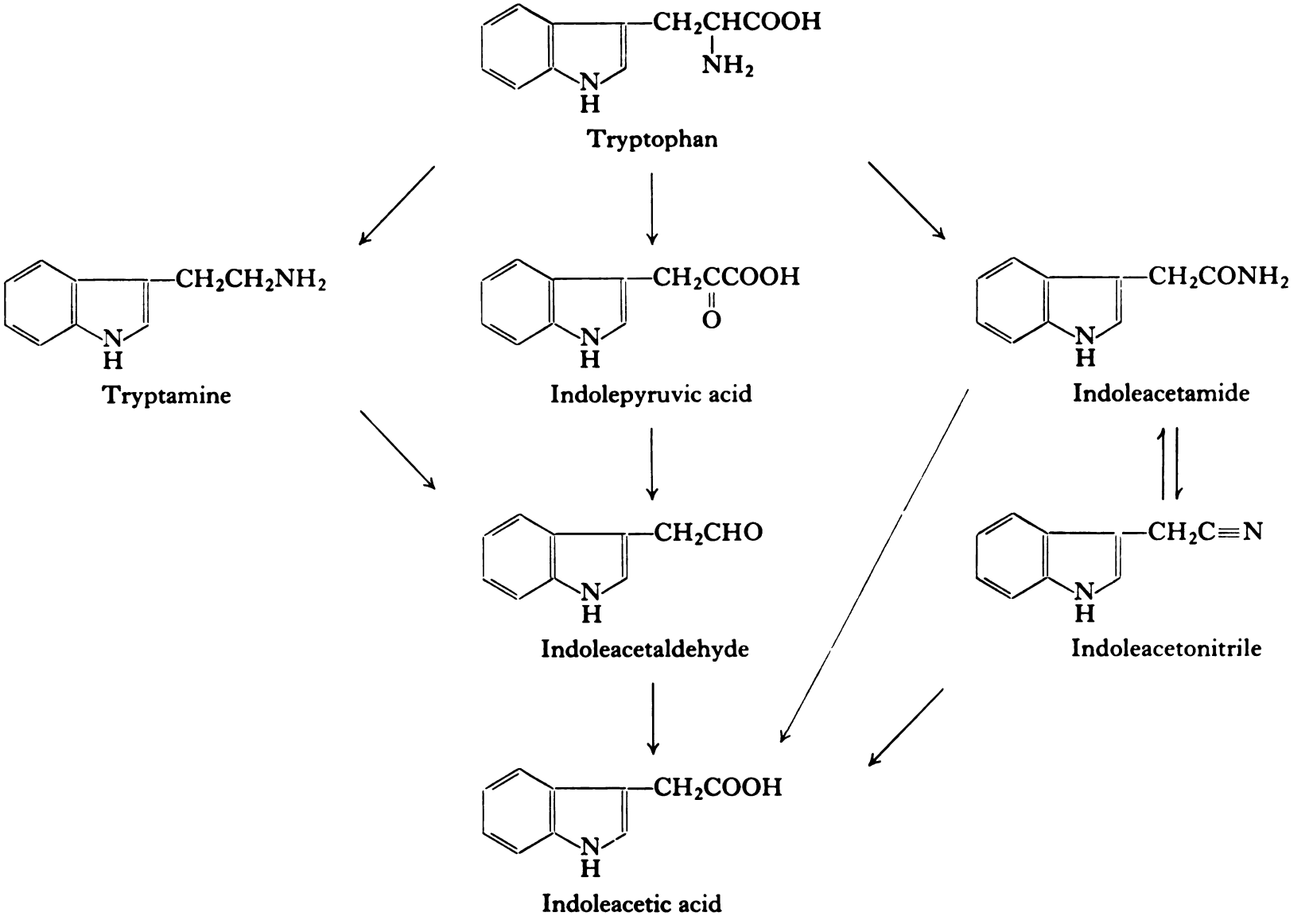


Figure 3.--The Tryptamine Pathway.

Many of the tryptophan metabolites synthesized within the major pathways can take alternate metabolic routes. In the mouse kynurenine can also be hydroxylated in the 5 position and then decarboxylated to 5-hydroxykynuramine (104). The other hydroxykynurenine, 3-hydroxykynurenine, can be converted to 4,8-dihydroxyquinolinic acid, instead of xanthurenic acid, by mouse liver homogenates. This occurs by the decarboxylation of 3-hydroxykynurenine and cyclization to form 3-hydroxykynuramine (94).

Many bacteria and yeast also contain the enzyme tryptophan 2,3-dioxygenase but the metabolic pathways are not the same as in mammals. The microorganisms produce kynurenine which is degraded to either anthranilic acid, 3-hydroxykynurenine, or kynurenic acid. Both anthranilic acid and kynurenic acid are end products in mammals but are the starting point for tryptophan metabolism in bacteria. The end products in bacteria and yeast are nicotinic acid, succinic acid, and acetic acid, and α-ketoglutarate, oxaloacetate, and ammonia (99). Anthranilic acid may be utilized in the synthesis of tryptophan in bacteria (99).

Tryptophan Metabolites Found in Urine

The variety of the kynurenine metabolites isolated from urine is almost equal to the variety of techniques and pathological conditions utilized to obtain them. There are fewer studies on urinary kynurenine metabolites from

normal subjects than abnormal. However, in one study, Benassi et al. (8) identified kynurenine, 3-hydroxy-kynurenine, kynurenic acid, xanthurenic acid, xanthurenic acid-8-methyl ether, anthranilic acid, 3-hydroxyanthranilic acid, and 8-methyl-oxyanthranilic acid from normal human urine.

The effects of a loading dose of tryptophan on tryptophan metabolism have been studied in normal and pathological conditions. Hankes and coworkers (65,66,67) studied the effects on tryptophan metabolism of loading doses of tryptophan and other kynurenine metabolites utilizing D, L-tryptophan-2-C^{14} as a tracer. They monitored kynurenic acid, xanthurenic acid, 3-hydroxykynurenine, kynurenine, anthranilic acid, quinolinic acid, nicotinic acid, and N-methylnicotinamide. Only xanthurenic and kynurenic acids were reported isotopically. In all three studies they measured total radioactivity found in urine and carbon dioxide. These values are not as valid as possible because they utilized tryptophan labeled on the side chain. The label is cleaved from tryptophan as alanine in the formation of both anthranilic acid and 3-hydroxyanthranilic acid. These are both early events in the kynurenine pathway. Therefore, they are more accurately monitoring alanine metabolism rather than tryptophan metabolism. An interesting observation to come from one of their studies

(67) was that a loading dose of 3-hydroxyanthranilic acid caused a decrease in CO_2 respired and urinary output of quinolinic acid. Tryptophan oxygenase was not blocked because the quantity of kynurenine, xanthurenic acid, and kynurenic acid excreted remained the same. They hypothesized that 3-hydroxyanthranilic acid oxidase may have been blocked due to excess substrate.

Many of the enzymes of the kynurenine pathway require pyridoxyl phosphate. This fact has been utilized to study tryptophan metabolism in both humans and rats with a Vitamin B_6 deficiency. Yess et al. (170) found that there was an increase of kynurenine, 3-hydroxykynurenine, xanthurenic acid, and kynurenic acid in the urine of pyridoxine deficient humans. 3-Hydroxykynurenine was found to be the most sensitive indicator of Vitamin B_6 depletion. In 1952, Dalgliesch (32) did an extensive study on the relationship between pyridoxin and tryptophan metabolism in rats. Unlike the previous authors (170) who utilized a fluorometric assay for determining the tryptophan metabolites, Dalgliesch separated the individual urinary tryptophan metabolites by paper chromatography. Utilizing fluorescence and different spray reagents, he determined that 3-hydroxykynurenine was also excreted as its glucuronide, sulfate, and acetyl conjugates. The acetyl derivative of kynurenine was also present. These

results indicate that the animal cannot metabolize these compounds and is therefore detoxifying them so that they may be excreted. Rothstein and Greenberg (134) found variations in the conjugates of xanthurenic acid excreted in pyridoxine deficient animals. Rats excreted xanthurenic acid alone and as the mono- and diglucuronides with a serine attached in an amide linkage. Rabbits, however, excreted one O-sulfate conjugate linked to serine.

Musajo et al. (106) found that normal human urine contains only very slight amounts of kynurenic and xanthurenic acids. In patients with neoplastic diseases, urinary kynurenine pathway metabolites are not altered. On the contrary, patients with hemoblastic diseases show an increase in kynurenine, 3-hydroxykynurenine, α-N-acetylkynurenine, α-N-acetyl-3-hyroxykynurenine, and 3-hydroxykynurenine-O-glucuronide. In another type of cancer, bilharzial bladder cancer, Khalfallah and Abul-Fadl (80) found that urinary excretion of 3-hydroxyanthranilic acid was increased about eight times, anthranilic acid about six times, 5-hydroxyindoleacetic acid about four times and kynurenic acid about two times. Kynurenic and xanthurenic acid excretion decreased about 50%. These results indicate a general increase in activity in the major route of both pathways of tryptophan metabolism. Along similar lines Watanabe and coworkers (162, 163) have found that 3-hydroxyanthranilic acid and

3-hydroxykynurenine are carcinogenic and cause bladder cancer. The O-sulfate and O-glucuronide conjugates of these compounds may be cleaved in the kidney to produce the toxic metabolites. Recently, Gailani et al. (50) observed increased kynurenine and 3-hydroxykynurenine levels in pre- and post-operative patients with bladder cancer.

Enzyme regulation by drugs, hormones, and substrate can be determined by monitoring variations in urinary excretion of tryptophan metabolites. For example, to determine if kynurenine kydroxylase required a reduced pteridine cofactor, an aromatic pteridine which would inhibit the reaction if the cofactor was necessary, was given to rats. Quantitative changes in kynurenine and 3-hydroxykynurenine were measured. It was demonstrated that the aromatic pteridine caused an enhancement of tryptophan oxygenase activity and a concomitant rise in kynurenine excretion but 3-hydroxykynurenine could not even be detected in the urine of treated animals. These results showed that the enzyme did require the reduced pteridine for activity. In another study concerned with enzyme levels, Altman and Greengard (3) showed that the increase in the in vitro activity of tryptophan oxygenase caused by either hydroxortisone or tryptophan administration could be correlated with increased urinary kynurenine excretion. Shaw and Feigin (144) found that, under the stress of a bacterial infection, glucocorticoids elevated tryptophan

oxygenase and urinary excretion of kynurenic acid and xanthurenic acid were increased. During endotoxic shock, however, tryptophan oxygenase was decreased as were the levels of kynurenic acid and xanthurenic acid excreted in the urine.

The metabolism of metabolites within the kynurenine pathway has been studied too. Kaihara and Price (78) demonstrated that two thirds of an oral dose of kynurenic and xanthurenic acids was dehydroxylated by the rabbit. After subcutaneous injection, only 2-10% of the quinoline derivatives were dehydroxylated. They were unable to determine if dehydrogenation of kynurenic and xanthurenic acids occurred in the tissues or in the gut by the bacterial flora. Either oral or subcutaneous administration of quinaldic acid resulted in over 90% recovery of unchanged quinaldic acid in the urine.

Serotonin may be degraded or conjugated to a wide variety of compounds, many of which can be isolated from urine. The major catabolic pathway for serotonin is through oxidative deamination by monoamine oxidase to form 5-hydroxyindoleacetaldehyde (5-hydroxytryptophal) 27,98). This compound may be further oxidized by the enzyme aldehyde dehydrogenase to yield 5-hydroxyindoleacetic acid (5HIAA), the major excretory product of serotonin in humans and many other animals (5,68). In dogs, rats, and humans, which are carnivores, 5HIAA concentration in urine ranges from 1.5 to 4.0

ug/ml (108). However, herbivores, such as mice, guinea pigs, rabbits, and horses, excrete only small amounts (less than 0.3 ug/ml) of 5HIAA in urine (42,108). Instead of producing the acid, herbivores form the alcohol, 5-hydroxytryptophol (5HTOH). Evidence suggests that 5HTOH may enter pigment formation (108).

Kveder, Iskrie, and Keglevic (87) identified 5HTOH in human urine. When serotonin is administered to humans, 5HTOH and its conjugates account for two percent of the serotonin injected. They also identified the glucuronide of 5HTOH in the urine of rats and showed that 60-70% of 5HTOH was being oxidized to 5HIAA or conjugated.

In a very detailed and complete study on serotonin metabolism in normal and cirrhotic rats, Pentikainen, Mekki, and Mustala (121) identified 5HT and its glucuronide, 5-hydroxyindoleacetaldehyde-O-sulfate, and the O-sulfate and O-glucuronide derivates of 5HTOH and 5HIAA. They found that in normal rats given ^{14}C-5HT intravenously, 5HIAA was initially the predominant metabolite but its relative amount steadily decreased with time while 5HT-O-glucuronide continually increased. If these rats were cirrhotic, they produced significantly more 5-HT-O-glucuronide and 5HIAA-O-glucuronide than other metabolites throughout the whole experiment.

N-Acetylserotonin has been described as a major metabolite by some workers (98) but further investigation has

shown that although it is present in urine it is not a major metabolite (87). Kveder et al. (64) found that the compound identified as N-acetylserotonin by McIsaac and Page (98) was composed primarily of the glucuronide of 5HTOH. The two compounds had similar chromatographic properties under the conditions utilized by McIsaac and Page (98).

Delvigs et al. (36) studied the metabolic fate of 5-methoxytryptophol, which is localized in the pineal gland. This compound is rapidly metabolized; with an average of 91% of the administered activity appearing in urine within 24 hours. As much as 65% of the activity in one hour and 92% in 2 hours was found in the urine of some animals. The major metabolite excreted was 5-methoxyindoleacetic acid (5MIAA) representing 93% of the administered 5-methoxytryptophol.

Formation of conjugates through the 5-hydroxyl group is yet another route of metabolism for serotonin and its products. This is a common method of detoxification for these compounds. Liver homogenates form serotonin-O-sulfate (20, 133) and this compound has been isolated from urine, especially when monoamine exidase is inhibited (20). Chadwick (20) also found the O-sulfate derivatives of 5HIAA, in urine. The O-sulfate derivatives of 5-hydroxyindoleacetaldehyde, 5HIAA, and 5HTOH have also been described (121, 122). The O-glucuronide derivatives are very common, with

serotonin-O-glucuronide being a major urinary metabolite in rats and humans (2,58,97,98,121,122,165). 5-Hydroxyindoleacetic acid and 5-hydroxytryptophol form O-glucuronide conjugates (97, 98,121,122) and, in addition, 5HIAA may also combine with glycine to form 5-hydroxyindoleaceturic acid (97,98).

Other products of serotonin found in urine are the N-methyl and N-acetyl derivatives. Although N-methylation is uncommon in mammals, Bumpus and Page (15) identified trace amounts of N-methylserotonin in human urine. Trace amounts of N-acetylserotonin have also been identified in urine (68, 98). Generally, this compound is O-methylated in the pineal body to form the pigment melatonin (82) but it may also be catabolized to 5HIAA (87).

Many of the 5-hydroxyindoles were first isolated from urine of carcinoid patients. A carcinoid is an argentaffin cell tumor with the primary lesion usually appearing in the ileum but lesions also may be found in the stomach and pancreas (119). Although it has been shown that the metabolic pathways of tryptophan do not vary between carcinoid and normal tissues, approximately 60% of orally ingested tryptophan proceeds to 5-hydroxyindoles in carcinoid patients as compared to approximately one percent in normal individuals (119). The tumors have been characterized as producing excess amounts of serotonin with the subsequent

excretion of increased quantities of 5HIAA in the urine (41, 98). Feldstein (47) has reported results contradictory to this thesis.

The Influence of Tryptophan and its Metabolites on Enzyme Levels

Tryptophan increases the activity of tryptophan 2, 3-dioxygenase by converting the inactive apoenzyme to the active holoenzyme. Substrate activation is accomplished by the binding of tryptophan at both the allosteric and catalytic sites (14). This binding also exerts a stabilizing effect on tryptophan oxygenase and prevents degradation both *in vitro* (26,43,108) and *in vivo* (138,139,140,146). Recent evidence suggests that in addition to inhibiting degradation, tryptophan also stimulates mRNA synthesis by stimulating adrenocortical hormonal secretion (146).

Unlike tryptophan which elevates tryptophan oxygenase levels for 8-12 hours, α-methyltryptophan elevates the enzyme for at least a week. This compound binds to the enzyme and stabilizes it but cannot be metabolized. Due to the increased metabolism of available tryptophan, thus limiting substrate availability, there is decreased protein synthesis (138, 150). Along similar lines it has been shown that tryptophan plays a special role in the regulation of polyribosome aggregation and protein synthesis in the liver (147). Brain 5HT nad 5HIAA are also decreased after administration

of α-methyltryptophan. This deficiency is due to decreased blood levels of tryptophan and thus less substrate availability. Although tryptophan oxygenase cannot metabolize α-methyltryptophan, tryptophan hydroxylase and 5-hydroxytryptophan decarboxylase can, and forms α-methyl-5-hydroxytryptophan and α-methyl-5-hydroxytryptamine. However, monoamine oxidase cannot convert the α-methylamine to the acid (120).

Other tryptophan metabolites also effect the activity of tryptophan oxygenase. Cho-Chung and Pitot (24) demonstrated that the highly active form of tryptophan oxygenase is sensitive to inhibition by its end product NADPH. Kinetic analysis of the inhibition indicates that it is a type of allosteric inhibition. In a somewhat contradictory report, Powanda and Wannemacher (123) reported that NAD synthesis from tryptophan was regulated by the substrate availability rather than variations in tryptophan oxygenase activity. They found that allopurinol given simultaneously with tryptophan decreased tryptophan oxygenase activity but did not reduce the increase in NAD concentration. Hydrocortisone and α-methyltryptophan, which increase tryptophan oxygenase activity, did not increase NAD levels.

Tagliamonte and co-workers (155) have stated that the concentration of tryptophan in the brain is a more important control mechanism than the concentrations of

tryptophan hydroxylase. Another interpretation of their data might suggest that the rate of serotonin synthesis could control the concentration of tryptophan in the brain.

Foster et al. (48) described a paradoxical effect of tryptophan on the gluconeogenic enzyme phosphopyruvate carboxylase (phosphoenolpyruvate carboxykinase - PEPCK). Exogenously administered tryptophan enhanced PEPCK activity in vitro but depressed it in the intact rat (48,159). Quinolinate, a catabolite of the kynurenine pathway, appears to be the metabolite primarily responsible for the in vivo inhibition of the enzyme. The mechanism of inhibition is not known but may be partially due to the metal chelator properties of quinolinate (150). Other metabolites of tryptophan, such as xanthurenic acid, reduce PEPCK activity to some extent (150, 159).

PART II

ENDOTOXIN

Introduction

Classically, the term "endotoxin" was introduced because of the view that this material was an internal constituent of gram-negative bacteria and that disruption of cells was essential for its release. It is now known that, not only is endotoxin concentrated at the cell surface, but that intact young cells release substantial amounts of

endotoxin into the medium (29) making the term endotoxin a misnomer. The primary chemical composition of endotoxin makes it a lipopolysaccharide, suggesting the name lipopolysaccharide toxin, but this name does not cover the toxic extracts from heptoseless mutants which do not appear to contain any true polysaccharide. Milner, Rudbach, and Ribi (101) suggest the term endotoxic phospholipopolysaccharide and give the following somewhat detailed definition.

> Endotoxic phospholipopolysaccharides (endotoxins) are found principally at or near the cell surfaces of gram-negative bacteria. As extracted by common procedures, they are macromolecular aggregates of subunits, united in various forms by hydrophobic bonds, incorporating both the major somatic antigens of the bacteria and a large number of toxic and other host-reactive properties that are described collectively as "endotoxic." All are stable to boiling in neutral water. The probability of correctly identifying a substance as an endotoxin increases rapidly with the number of typical host responses that are demonstrated. Of particular value in this regard are the production, in suitable animals, of characteristic biphasic fever, lethal shock after a latent period, the Sanarelli-Shwartzman reactions, hemorrhagic necrosis of transplantable tumors, leukopenia followed by leukocytosis, enhancement of the antigenicity of proteins, and nonspecific resistance to infection or to damage by irradiation. Basic to many of these pathophysiological effects of endotoxin is an injury to the cardiovascular system, by means not yet fully understood, which alters or disrupts normal function.

From this definition one can succinctly see the diversity of manifestations elicited by this molecule.

Biochemistry

Biochemically, endotoxin is composed of three regions: the O-specific chain, the basal core polysaccharide,

and lipid A (Figure 4). The lipopolysaccharide may be extracted from the cell by a number of methods, the most common being the phenol water method (166,167) and the aqueous ether method (128,129,130).

Over thirty aldoses have been identified in O-antigens of gram-negative bacteria. Another important sugar, 3-deoxy-D-mannooctulosonic acid (KDO), has been regularly isolated from lipopolysaccharides. This sugar forms a stable glycosidic linkage with heptose. Most lipopolysaccharides contain five or more sugar constituents. Glucosamine, heptose, KDO, glactose, and glucose are, however, the only ones commonly encountered and represent the units of the core polysaccharide (region II, Figure 4). The exact structures and linkages of the sugars have been elaborately worked out by the combination of gas chromatography with mass spectrometry [for a review, see Luderitz et al. (91)].

Lipid A is a complex structure containing glycosidically linked glucosamine units. The hydroxyl groups of glucosamine are substituted by long-chain fatty acids, such as lauric, myristic, palmitic, and β-hydroxymyristic acids. β-Hydroxymyristic acid is also bound to the amino groups of the glucosamine units. KDO is linked directly to lipid A (91).

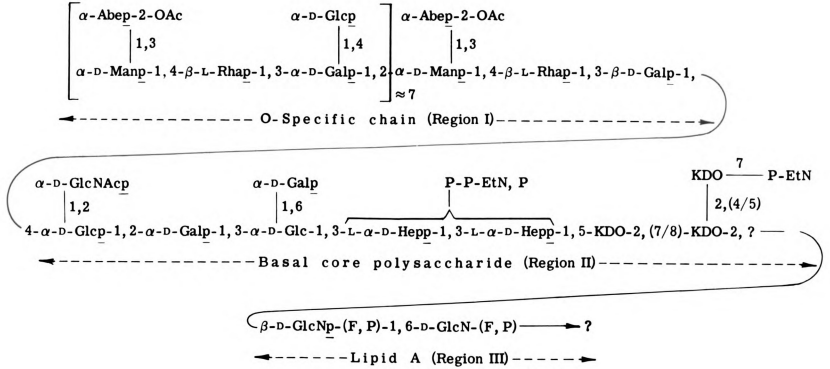


Figure 4.--The Proposed Structure of the Lipopolysaccharide of Salmonella typhimurium (91).

Localization and Detoxification

To gain a clearer understanding of where endotoxin localizes in the host, studies have been undertaken utilizing ^{14}C, ^{32}P, ^{51}Cr, and ^{131}I labeled endotoxins. Noyes et al. (117) found that ^{14}C-labeled endotoxin persists in the blood and localized in the liver. It appears to be sequestered in phagocytes of the reticuloendothelial system. Willerson et al. (169) found that intravenously administered ^{14}C-labeled Salmonella enteritidis endotoxin was recovered predominantly from the nuclear and mitochondrial fractions prepared from livers and spleens of mice and rats. Mice intravenously inoculated with endotoxin labeled with ^{51}Cr (18), ^{32}P (76), or ^{131}I (144) retained a major portion of the endotoxin in the liver over several hours to several days. Immediately after intravenous injection Carey (18) found endotoxin in the buffy coat of blood samples. By immunofluorescence, Rubenstein et al. (137) detected endotoxin in patchy distribution throughout the walls of the peripheral vascular system. Golub, Groschel, and Nowotny (56) found that detoxified endotoxin is not taken up by the liver and spleen and remains in circulation for a relatively long time.

Although there is some controversy, both humoral and cell-mediated mechanisms of the host are probably inseparably involved in the detoxification of endotoxin. Proposing a humoral mechanism for detoxification, several authors (63,141,142, 148) have presented evidence for two serum alpha globulins

which have the nonspecific, carboxylic type of esterase activity. The first is a heat-stable alpha lipoprotein which leads to degradation or disaggregation of the large molecules of endotoxin. The exposed toxic particles are then degraded by a heat-labile alpha globulin. The reactions are inhibited by physiological concentrations of serum Ca^{++} but Skarnes (148) found a gradual drop in Ca^{++} levels with time in post-endotoxin serum which coincided with an increase in the enzyme levels and detoxification.

Rutenberg (137) found that peritoneal macrophages combined with immune serum detoxified E. coli 011 endotoxin more effectively than macrophages in normal serum. Chedid and coworkers (21,22) found that toxic ^{51}Cr-labeled endotoxin was present in plasma six hours after injection and smooth strains of salmonellae are not removed as rapidly as rough strains. Opsinizing antibodies directed toward the R antigen, which must be exposed, may be necessary for detoxification to occur. They also observed that only non-toxic degraded endotoxin can pass the kidney and occur in urine. Rowley (135) suggests that the decisive event in the destruction of bacteria is phagocytosis and its efficiency is dependent upon the presence of specific antibody which may be free or bound to the cell.

Molecular Aspects of Biological Activity

In the introduction to Bacterial Endotoxins, Ivan Bennett, Jr., sums up the expansive variety of biological

effects of endotoxin with the statement: "an investigator in almost any biological field is likely to obtain a 'positive' result if he tried endotoxin in the experimental system he is using" (10). A summary of the major biological effects of endotoxin is presented in Table 1.

It is assumed that the elicitation of the diverse endotoxin reactions do not involve the entire lipopolysaccharide molecule but are localized in certain active sites. These active sites are formed by specific steric arrangements of certain functional groups of the structure (116). The polysaccharide moiety is responsible for O-antigenic specificity and has never been shown to be involved in the toxic manifestations. Glycolipids extracted from rough mutants, and thus lacking the polysaccharide moiety, are fully toxic. Lipid A moieties, some of which are composed of only KDO and lipid A, appear to contain the active sites and are responsible for the potent endotoxic activity (68,100, 156). When KDO is removed from the glycolipid by mild acid hydrolysis, the free Lipid A has a lower activity. However, this appears to be due to its insolubility in water as glycolipids treated with agents that chemically modify the KDO (130) or free Lipid A combined with water soluble bovine serum albumin (51) are fully toxic. These results suggest that the polysaccharide portion and the KDO of the glycolipid act as water-solubilizing carriers for the insoluble Lipid A which represents the biologically active center of the molecule.

Table 1.--Characteristic Endotoxic Reactions (116).

Pyrogenicity
Release of endogenous pyrogen
Immunogenicity
Adjuvant effect and inhibition of antibody production
Effect on properdin or natural antibody levels
Leukopenia and leukocytosis
Protection against irradiation
Effect on RES
Development of tolerance
Enhancement of nonspecific resistance
Mobilization of interferon
Changes in blood clotting
Metabolic changes
Endocrinological changes
Release and sensitization to histamine
Vascular effects
Sanarelli-Shwartzman phenomenon
Cytotoxicity
Abortion
Tumor-necrotizing effect
Interaction with complement
Shock and lethality

The Effects of Endotoxin on Tryptophan Metabolism

Tryptophan oxygenase is depressed in endotoxin-poisoned mice (1,11,12,104). This enzyme catalyzes the first reaction in the pathway leading to the formation of pyridine nucleotides. Its depression implies a block in the biosynthesis of these compounds, the consequence of which could be biologically significant in endotoxin-poisoning. This concept is consistent with evidence that administration of the end products NAD or nicotinamide concurrent with endotoxin protects mice from endotoxin lethality (11).

The adrenocortical hormone, cortisone, is known to elevate tryptophan oxygenase (44,45,83). When cortisone is given concurrently with endotoxin, mice are protected against endotoxin lethality (12,52,86,104) and their level of total oxidized pyridine nucleotides is maintained (12). The protective role of cortisone and its elevation of tryptophan oxygenase are dependent upon the time of administration of cortisone relative to the administration of endotoxin (11,12). If 5 mg of cortisone is given more than one hour after 1 LD_{50} of endotoxin, it fails to maintain the tryptophan oxygenase activity at control levels and to protect mice from endotoxin lethality. Corresponding to these results, Shaw and Fegin (143) found that children having gram-negative infections had increased kynurenic and xanthurenic acid excretion and postulated that this was due to stimulated

adrenocortical function whereas those suffering from severe endotoxic shock had decreased excretion of the acids, possibly due to inhibited tryptophan oxygenase activity.

Tryptophan elevated tryptophan oxygenase activity in normal mice (43,45,104). When tryptophan is given concurrently with 1 LD_{50} of endotoxin, it fails to maintain tryptophan oxygenase levels and does not protect against lethality (11,104).

Elevation of tryptophan oxygenase activity with α-methyltryptophan does not decrease, nor does depression with 5-hydroxytryptophan increase, protection from endotoxin lethality (11,104). These results imply that tryptophan oxygenase per se is not directly related to survival of endotoxin-poisoned mice.

When endotoxin-poisoned mice are given 20 mg of L-tryptophan at a time when tryptophan oxygenase is depressed, they frequently die in convulsions within 8 hours. Death occurs 24 to 36 hours sooner than in mice given endotoxin alone (11,102,104). This hyper-reactivity to tryptophan may be due to the altered enzymatic and metabolic homeostasis in endotoxin-poisoned animals which results in the disruption of the normal balance of tryptophan metabolites.

Prior treatment of endotoxin-poisoned mice with cyproheptadine, an antiserotonin drug, protected mice from the convulsive death (104). This result suggests that

tryptophan is funneled in excess into serotonin production. Further indications of the excess conversion of tryptophan to serotonin synthesis are observed in the protection afforded by cyproheptadine against hypothermia produced in mice kept at 15°C and given serotonin alone, or against hypothermia produced by tryptophan in endotoxin-poisoned mice (103).

If serotonin is important in the altered response of endotoxin-poisoned mice to tryptophan, injection of serotonin might be expected to cause enhancement of toxicity. Conflicting data on this subject have been obtained. Gordon and Lipton (57) demonstrated that a subcutaneous injection of either 0.8 or 1.6 milligrams of serotonin per killogram of body weight reduced mortality in mice given 24 or 32 mg/kg of endotoxin intraperitoneally 30 minutes after serotonin. Des Prez et al. (37) also demonstrated that the precursor of serotonin, 5-hydroxytryptophan, and 1-benzyl-2,5-dimethyl-serotonin (BAS), an antimetabolite of serotonin, protected mice from the toxicity of endotoxin. It is believed that protection from endotoxin-poisoning by serotonin may be mediated by the adrenal corticoids, since serotonin administration increases ACTH output. In the same group of experiments Des Prez (37) also found that when the monoamine oxidase inhibitor, β-phenylisopropyl hydrazine (PIH) was injected prior to endotoxin, it made mice more susceptible to endotoxin. Lasker (88) found similar results. Contrary to these reports

Susman et al. (7) and Davis et al.(35) found that monoamine oxidase inhibitors afforded protection to endotoxin. David utilized PIH and 1-(2-(benzylcarbamyl) ether)-2-isonicotinoyl-hydrazine (nialamide), which inhibits both monoamine oxidases, and N,N-dimethyl-2-phenylcyclopropylcyclopropylamine hydrochloride (S.K.F. 556), which is specific for monoamine oxidases. The probable reason for the different results using the same inhibitor (PIH) was that Des Prez used a larger dose (40mg/kg) as compared to 10 mg/kg used by Davis. Davis also found varying results with dose variations.

In addition to increased serotonin synthesis, there is also an increase in serotonin released from platelets after endotoxin administration (38,39). Recently, Nomura and coworkers (113,114,115) isolated and purified a prothrombin-like protein from plasma which causes the release of 5-hydroxytryptamine from platelets. This factor appears to induce a change in the permeability of the platelet membrane, thereby facilitating the entry of calcium ions into platelets which in turn release 5HT from the storage granule.

The implications of funneling of tryptophan from one pathway to another are demonstrated by the work of Curzon and Green (30). When tryptophan oxygenase was increased in stressed rats, there was a decrease in brain serotonin due to the flow of tryptophan into the kynurenine pathway. Along similar lines, Schmike (140) and Sourkes (150) have independently shown that elevation of tryptophan oxygenase by

administration of α-methyltryptophan to rats causes a decrease in blood levels of tryptophan with a consequent decrease in brain serotonin and 5HIAA levels. These results suggest that although tryptophan oxygenase itself probably does not influence survival of mice given endotoxin, the funneling of tryptophan into other pathways and the production of potentially toxic matabolites may be a factor in convulsive death observed in endotoxin-poisoned mice given a loading dose of tryptophan.

PART III

METHODS FOR SEPARATION AND IDENTIFICATION OF TRYPTOPHAN METABOLITES

Paper Chromatography

Classically, one dimensional ascending paper chromatography has been the method of choice for the separation of urinary tryptophan metabolites. The solvent system most widely utilized to separate metabolites of the kynurenine pathway is composed of butanol, acetic acid, and water in various combinations (54,55,80,106,133,134). For better resolution of the metabolites Dalgliesch (33) utilized descending paper chromatography with butanol, acetic acid, and water to separate a wide variety of tryptophan metabolites. Of the several other solvent systems utilized, methanol, butanol, benzene, and water in the proportion 2:1:1:1 was preferred (134).

A wide variety of solvent systems have also been used to separate the serotonin metabolites. Butanol, acetic acid, and water (4:1:5) and several other organic solvent systems have been utilized to separate 5HT, 5HIAA, 5HTOH, and a few other serotonin metabolites (87,97,98). Pentikainen and coworkers (121,122) used another combination of the butanol system along with butanol, pyridine, and water (1:1:1) to isolate nine different 5HT metabolites. Rodnight (132) separated urinary indoles by column chromatography and then used paper chromatographic separation in a variety of solvents for identification. Urinary indoles extracted under alkaline and acidic conditions have also been separated by two dimensional chromatography with isopropanol, ammonium hydroxide, and water (8:1:1) followed by butanol, acetic acid and water (4:1:5) (151).

Thin Layer Chromatography

During the last decade thin layer chromatography has become an excellent analytical tool for the rapid separation and detection of minute amounts of biogenic amines and other tryptophan metabolites. Diamenstein and Ehrhart (40) found that either methyl acetate, isopropyl alcohol, and ammonium hydroxide, 45:35:20, or chloroform, methanol, and acetic acid, 90:5:5, were good solvent systems to separate a variety of biologically relevant tryptophan metabolites by one or two dimensional thin layer chromatography. Cotte

et al. (28) and Stahl and Kaldeway (153) used the methyl acetate, isopropanol, and ammonium hydroxide solvent system but used chloroform and acetic acid (95:5) for the second direction runs. They found this method was a rapid, sensitive way of detecting metabolites of the kynurenine and serotonin pathways.

Aures et al. (6) developed a microanalysis of tissue amines utilizing a wide variety of solvent systems for thin layer chromatography and then o-phthaldehyde as a spray reagent for the detection of the compounds. Hill et al. (69) also used fluorescent techniques after development on thin layer chromatography with chloroform, methanol, acetic acid solvent system (75:20:5) and removal from the gel to detect xanthurenic acid, kynurenic acid, and kynurenine.

Column Chromatography

Separation of tryptophan metabolites by column chromatography presents an interesting problem. The metabolites are all similar in molecular weight and are either similar in molecular weight and are either similar in chemical nature such as many of the serotonin metabolites or diverse as are some of the kynurenine metabolites.

Burtis and Warren (16) utilized a high pressure anion exchange chromatographic system to separate more than 100 UV-absorbing urinary metabolites. Of these, nine were tentatively identified as tryptophan metabolites. Due to some technical

problems this same group of workers (17) prefractionated the human urine samples on Sephadex G-10 and thus simplified the anion-exchange chromatograms. Nishino et al. (111) also used ion exchange chromatography and separated 5HTP, 5HT, and 5HIAA on Dowex 1 (acetate form). Chen and Gholson (23) recently reported the fractionation of a variety of tryptophan metabolites on DEAE-cellulose (amine and formate forms).

Gas Chromatography

The use of gas chromatography to separate and identify tryptophan metabolites as well as other biologically important amine and acids has been hampered by the relative insolubility of the kynurenine metabolites in the solvents used for derivatization and by the formation of multiple derivatives. Problems have also encompassed partial or complete decomposition of the derivatives, trailing of peaks and incomplete separation of closely related substances. However, several studies have been reported. Urinary acids contain several tryptophan metabolites which have been isolated by various techniques (34,71,73,74,75). Dalgliesch et al. (34) prepared the methyl esters, trimethylsilyl esters and ethers, and their combinations of many acids of both the kynurenine and serotonin pathways found in urine and separated them on a 6 ft. x 3.5 mm glass U-shaped column containing F-60 coated on Gas Chrom P, 80/100 mesh. The Hornings

and their coworkers (72,73,74,75) also studied a wide variety of urinary metabolites and the different methods of derivatizing and chromatographing them. Maruyama and Takemori (96) described a method for separating 5-hydroxyindoleacetic acid and 5-hydroxytryptamine from biological samples and chtomatographing them on 3% OV-17. Altschuler and Gold (4) chromatographed the metabolites of the kynurenine pathway on 2% OV-17 using N,O-bis-trimethylsilylacetamide (BSA) as a silylating agent.

MATERIALS AND METHODS

Chemicals and Radioisotopes

N-Acetylserotonin, 5-hydroxyindoleacetic acid, 5-hydroxytryptamine creatinine sulfate, indole-3-acetic acid, kynurenic acid, D,L-kynurenine, N-methylnicotinamide, nicotinamide, nicotinic acid, quinolinic acid, and xanthurenic acid were purchased from Nutritional Biochemicals Co. (Cleveland, OH). Bufetonin, 3-hydroxy-D,L-kynurenine, D,L-5-hydroxytryptophan, L-5-hydroxytryptophan, indole, and tryptamine were purchased from Sigma Chemical Co. (St. Louis, MO). o-Aminophenol, anthranilic acid, 3-hydroxyanthranilic acid, and tryptophan were purchased from Aldrich Chemical Co., Inc., (Milwaukee, WI). 5-hydroxytryptophol, 5-methoxyindoleacetic acid, 5-methoxyserotonin, and 5-methoxytryptophol were purchased from Regis Chemical Co. (Chicago, IL). Quinaldic acid was purchased from Eastman Organic Chemicals (Rochester, NY).

D,L-Tryptophan (benzene ring-^{14}C), specific activity 95 mCi/mM, and 5-hydroxytryptamine-3'-^{14}C creatinine sulfate, specific activity 55 mCi/mM, were purchased in 50 μCi aliquots from Amersham/Serle (Des Plaines, IL). Each was diluted to 0.5 μCi/ml (1.1 x 10^6 dpm/ml) in 100 ml of sterile nonpyrogenic physiological saline (Baxter Laboratories, Morton

Grove, IL). The radioactive tryptophan and serotonin were frozen in dry ice, lyophilized, and stored at -70°C to reduce degradation. They were reconstituted to original volume in deionized distilled water (to maintain the proper salt concentration) just prior to use. For tryptophan load, studies, 20 mg of unlabeled L-tryptophan was dissolved per milliliter of the radioactive solution just prior to injection. All injections of the radioactive tryptophan and serotonin with and without load were given intraperitoneally to the mice at 0900.

Mice

Eighteen to 20 gram female CF-1 mice (Carworth Farms, Portage, MI) were used throughout the study. They were housed six per cage with wood chips as bedding. Purina Laboratory chow (Ralston Purina Co., St. Louis, MO) and water were provided ad libitum. All mice were starved for 17 hours prior to tryptophan injection (seven hours prior to endotoxin).

Endotoxin

Heat killed cells of Salmonella typhimurium, strain SR-11, served as the source of endotoxin in all experiments. Cultures were grown for 18 hours in brain heart infusion broth to a concentration of approximately 10^9 cells per milliliter and havested by continuous flow centrifugation in a Sorvall RC-2B refrigerated centrifuge. Following centrifugation, the cells were washed twice with isotonic non-pyrogenic saline (Baxter Laboratories, Morton Grove, IL) and

finally resuspended in saline to approximately ten times their original concentration. Cells were heat-killed by exposure to 115°C for six minutes. Lack of growth on subculture served as proof of sterility. The LD_{50} of this preparation for mice was determined according to the method of Reed and Muench (126). The endotoxin was diluted with nonpyrogenic physiological saline so that 1 LD_{50} (approximately 4×10^9 heat-killed organisms) was contained in 0.5 ml. Endotoxin was administered to mice intraperitoneally 10 hours prior to administration of radioactive tryptophan or serotonin (at 2300).

Collection and Quantitation of Total Radioactivity in Urine

To determine the total amount of radioactivity excreted in urine, each mouse was placed in a 500 ml Erlenmeyer flask which had a 9.0 cm Whatman #1 filter paper disc stapled to the underside of a circular piece of aluminum screen. Upon completion of the experiment, the filter paper was removed from the screen, dried, cut into six pieces and placed in scintillation vials containing 20 ml of a scintillation cocktail consisting of 4 g of PPO (2,5-diphenyloxazole) and 50 mg of POPOP (1,4-bis[2-(5-phenyloxazolyl)]benzene) in 1 liter of toluene. To determine counts per minute (cpm), each sample was counted for 10 minutes in a Packard 2420 liquid scintillation spectrometer and the background counts

subtracted. A counting efficiency of 62% for filter paper samples was used for conversion of cpm to disintegrations per minute (dpm).

Collection of Urine for Identification of Tryptophan Metabolites

Immediately after mice were injected with the isotope they were placed in a metabolism cage with a screen bottom which allowed the urine to be collected in a test tube. At the end of the experiment each mouse was removed from the cage and killed by cervical dislocation. Any urine excreted at this time onto a metal tray was collected with a micropipette and added to the tube. If the urine was cloudy or contained solid material, it was centrifuged for 5 minutes at 4000 rpm in a Phillips-Drucker Model 708 combination centrifuge (Astoria, OR). Depending on the volume of urine excreted, duplicate samples of 5, 10, or 20 µl were spotted on strips of filter paper and counted by liquid scintillation spectrometry in a Packard Model 2420 Tri-Carb spectrometer.

The urine sample was treated in one of several ways. Generally, 40 to 60 µl of urine from an individual mouse were spotted directly onto thin layer plates for two dimensional thin layer chromatography. For DEAE-cellulose chromatography, urine from 10 mice was pooled, the volume measured, and duplicate 5 µl samples spotted on filter paper for liquid scintillation spectrometry (to determine the cpm/ul and the total cpm/

sample). The remaining urine was frozen in dry ice and kept at -70°C until it was loaded onto the column.

Thin Layer Chromatography

Ascending two dimensional thin layer chromatography was done in multiplate developing chambers (E. Merck Ag, Brinkmann Instruments, Inc., Westbury, NY) lined on two sides with paper towels. The solvent system for the first development was composed of methyl acetate, isopropyl alcohol, and ammonium hydroxide in the proportion of 45:35:20. Methyl acetate was prepared by refluxing molar quantities of methanol (303 ml) and glacial acetic acid (598 ml) and 12 ml of concentrated sulfuric acid for 30 minutes. The addition of slight excess of acetic acid and distillation of the resulting methyl acetate from the reaction mixture facilitated the formation of the product. Further removal of any water was accomplished by two additions of 12 grams of silicic acid. A second distillation at 57.1°C yielded methyl acetate. Isopropyl alcohol and ammonium hydroxide were purchased commercially.

The second solvent system was composed on n-butyl alcohol, glacial acetic acid, and deionized distilled water in the proportion of 12:3:5. n-Butanol was redistilled and Linde Type 3A (1/16") molecular sieves (Matheson, Coleman, and Bell, Norwood, OH) added to the storage container. One hundred and fifty milliliters of the two solvent systems were

prepared fresh daily and added to individual tanks from which the solvents of the previous day had been just removed. The tanks were covered tightly using vacuum grease on the lid and allowed to equilibrate for at least one hour. This procedure has been shown to give optimum tank saturation and resolution (89).

The two solvent systems were chosen after testing several combinations of different solvents. Opienska-Blauth (118) reported instances where tryptophan metabolites were decomposed in preparation for chromatography and in development, but Lipton et al. (89) and Benassi et al. (9) have reported that with proper procedures degradation was eliminated. This was the experience in this laboratory.

Precoated 20 cm x 20 cm Silica Gel F-254 glass plates, purchased from E. Merck Ag., Brinkmann Instruments, Inc., Westbury, NY, were used in all experiments. Preliminary results showed that heat activation of the plates was not necessary. Plates were marked lightly with a soft lead pencil 2 cm and 17 cm from the bottom and on one side to give a two dimensional run of 15 cm x 15 cm. Samples were spotted with "microcaps" lambda pipettes (A. H. Thomas Co., Philadelphia, PA) as one spot on the 2 cm point at the left side of the plate. A warm air drier was employed between drop application to facilitate drying and to insure a small sample spot. Developing time usually required 3 to 3 1/2 hours for

the first run. After the plates were removed from the chamber, they were allowed to dry thoroughly to remove all traces of solvents and then turned 90° and placed in the second solvent system which required 6 to 6 1/2 hours for development. After removal from the second solvent, the plates were again dried thoroughly. All operations were performed in a hood.

One dimensional ascending thin layer chromatography utilizing the two solvents previously discussed was done on reference standards and eluates from the DEAE-cellulose column. For these runs plates were marked lightly with a soft lead pencil 2 cm and 17 cm from the bottom. Samples were spotted on the 2 cm line at least 1.75 cm from the edge. One dimensional runs of 5 to 20 μg of each standard compound were made at least ten times in each solvent system. One milligram of each reference standard was dissolved in either distilled water, ethanol, or pyridine and 5 to 20 ul spotted and developed. Each compound was also dissolved in normal mouse urine and chromatographed. Fluorescent compounds were circled lightly with a pencil while viewed under "long wavelength" (356 Å) ultraviolet light in a Chromato-Vue Cabinet Model CC-20 (A. H. Thomas, Philadelphia, PA). Rf-values were calculated at the distance from the origin to the center of the spot divided by the distance the solvent traveled.

Autoradiography

All manipulations were performed in a dark room using a safety light. Thin layer chromatographic plates

were placed in an 8" x 10" (20.3 cm x 25.4 cm) x-ray exposure holder (Eastman Kodak Co., Rochester, NY). One 5" x 7" Kodak No-Screen-Medical x-ray film (tinted estar safety base) was placed over the plate near the bottom and another half piece (2 1/2" x 7") was placed above this to cover the whole plate. Spots of radioactive material had been put on the edges of the plate and their location circled with a pencil to determine the exact position of the film on the plate. After 5 to 7 days exposure at room temperature, the film was developed for 5 minutes at 20°C or until spots were observed on the film in Kodak liquid x-ray developer and then fixed in Kodak liquid x-ray fixer for 8 minutes or until clearing of the film occurred. After this, the film was rinsed in water for 10 minutes to remove the fixer, and then dried. The two pieces of film were taped together. A dark spot denoted the presence of a radioactive compound on the thin layer plate.

Spray Reagents

Prochazka's formaldehyde-HCl reagent for detecting indole derivatives was prepared just prior to use by mixing 10 ml of formaldehyde (about 35%), 10 ml of pure HCl, 25%, and 20 ml of ethanol.

The reagent was sprayed onto the thin layer plates, which were then heated to 100°C for 5 minutes producing yellow, orange, and greenish fluorescent colors (152).

Van Urk's reagent (152), 4-dimethylaminobenzaldehyde, was a second reagent used for detection of indole derivatives. One gram of 4-dimethylaminobenzaldehyde was dissolved in 50 ml of HCl and then 50 ml of ethanol was added. The thin layer plates were heated to 60°C for 5 minutes and then sprayed exhaustively until they became transparent. After the plates were dried in air, fluorescent and visible colors of the compounds were recorded.

Ninhydrin (152) was used to detect primary and secondary amines. It was prepared by dissolving 0.3 g of ninhydrin in 100 ml of n-butanol and mixing this with 3 ml of glacial acetic acid. This solution was sprayed onto the thin layer plates and heated to 60°C for 30 minutes or 110°C for 10 minutes.

1,3-Dihydroxynaphthalene (Naphthoresorcinol)-trichloroacetic acid (152) was used to detect glucuronic acid residues present in conjugates. This reagent was prepared by dissolving 0.2 g of naphthoresorcinol in 100 ml of ethanol and mixing it with an equal volume of an aqueons 20% solution of trichloroacetic acid. The plates were sprayed and heated to 70-80°C for 10 to 15 minutes in a humid atmosphere (water bath).

Column Chromatography

DEAE-cellulose, Cellex-D (Bio-Rad Laboratories, Rockville Centre, NY), was prepared by a slight modification

of the method of Chen and Gholson (23). Twenty grams of resin were added with stirring to 400 ml of 0.5 N HCl in a 1 liter beaker and stirred for 10 minutes. After sitting for 30 minutes, the supernatant was decanted or filtered through a Buchner funnel. The resin was washed extensively (8 to 10 times; 2.5 to 3.0 l) with deionized distilled water until the supernatant was pH 4 with pH paper. All water used was deionized and distilled. The resin was poured into a 2 liter beaker containing approximately 1 liter of 0.5 N NaOH (5 times the volume of swollen exchanger), stirred gently for 10 minutes and allowed to sit for 30 minutes. The supernatant was decanted or filtered and the resin again washed with water (approximately 2 liters) until the pH was 10 with pH paper.

One liter of 1 M sodium formate was added to the resin, allowed to sit for 1 hour and filtered. Another liter of 1 M sodium formate was added and after 45 minutes, filtered, and washed 4 times with water to remove the sodium. The resin should be almost white at this point.

The resin was the put into an aspirator bottle and stirred. A Chromaflex 1.2 cm x 30 cm column (Kontes Glass Co., Franklin Park, IL) was filled with 0.001 M triethylamine-formate buffer and the column stopcock was opened. Nitrogen pressure (7 lb/sq in) was applied to the top of the mixing chamber forcing the resin into the column as the buffer flowed out. The column was packed to a height of 10 cm. It was

placed in a 4°C room and equilibrated with 0.001 M triethylamine-formate pH 4.0. Standard solutions or urine samples were loaded carefully onto the column and 80 ml eluted in 2 ml samples with 0.001 M triethylamine-formate. A gradient was then set up by placing 250 ml of 0.001 M triethylamine-formate buffer (pH 4.0) in a cylindrical mixing chamber that was connected by tubing to a reservoir of equal dimensions containing 250 ml of 0.1 M triethylamine-formate buffer (pH 4.0). Fractions of 4 ml were collected. All samples were frozen in dry ice, stored at -70°C if necessary, and lyophilized in a Refrigeration for Science lyophilizer (Island Park, NY). They were reconstitut to 0.1 ml with deionized distilled water. Five microliters from each tube was spotted on filter paper and counted by liquid scintillation spectrometry. The remaining sample was used for thin layer chromatography or gas chromatography-mass spectrometry.

Preparation of Methyl Esters for Gas Chromatography-Mass Spectrometry

Initially ethereal diazomethane made from N-nitro-N-nitroso-N-methylguanidine (NMNG) was used for methylation; but due to the insolubility in both ether and methanol, plus the hazardous nature of diazomethane and NMNG, methyl esters were prepared using 2,2-dimethoxypropane. One dram vials with teflon lined screw caps containing 100 µg of a reference standard, lyophilized or dissolved in 100 µl of methanol, or

an unknown urine sample were used throughout derivatization. Unknowns were isolated by drawing a vacuum through the small end of a Pasteur pipette containing a small piece of glass wool and removing the gel from the thin layer plate where a spot was observed on an autoradiograph. Working in a hood 100 µl of an aqueous HCl (36%) solution and then 2 ml of 2, 2-dimethoxypropane (Fisher Chemical Co., Detroit, MI) were added directly to the reference sample or run through the Pasteur pipette with the unknown and mixed slightly. Darkening of the solution occurred with sitting. After 19 hours the sample was removed from the vial with a Pasteur pipette and placed in a 5 or 10 ml pear-shaped flask (Ace Glassware, Vineland, NJ). The solution was evaporated to dryness in a rotary evaporator over a 50°C water bath to insure removal of the HCl. The sample was reconstituted in 50 µl of pyridine, transferred to a pyridine rinsed one dram vial, and recapped tightly for storage at 4°C.

Preparation of Trimethylsilyl (TMSi) Ethers or Esters for Gas Chromatography-Mass Spectrometry

Twenty five to 50 microliters of Regisil® (bis-(trimethylsilyl)trifluoroacetamide) (Regis Chemical Co., Chicago, IL) was added to the vial containing the methyl esters or to unknown or standard compounds (prepared as stated in the previous section) containing 25 µl of pyridine, recapped tightly, and allowed to sit at room temperature or

heated at 84°C for 10 minutes. The first is preferred as heating of the samples may cause degradation of the derivatives. The samples should be kept dry as TMSi esters and ethers hydrolyze easily. The pyridine in the samples did not interfere with derivative formation or gas chromatographic-mass spectral analyses. Two microliters of the methyl ester-trimethylsilyl ethers (ME-TMSi) or trimethylsilyl (TMSi) esters and ethers of the standards and variable amounts of the unknowns were injected directly into the gas chromatograph-mass spectrometer.

Gas Chromatography-Mass Spectrometry

Mass spectrometry was performed at 100, 130, 150, or 200°C on a combined gas-chromatograph-mass spectrometer LKB 9000 equipped with a column (6' x 3 mm) of either 3% SE-30 or 2.5% SP-2401 on Supelcoport 100/120 (Supelco Inc., Bellefort, PA). The runs were made with helium as the carrier gas, an ionizing electron energy of 70 ev, the molecular separator at 230°C and the ion source at 290°C. The spectra were recorded as bar graphs by means of an on-line computer system (154).

Quantitation of Urinary Tryptophan Metabolites

After thin layer plates were developed and autoradiographs made, the Rf values of the radioactive spots were measured and the spots carefully scraped from the silica gel layer with a small spatula, collected on the spatula by means

of a small paint brush and placed in scintillation vials. The sample was crushed to a fine powder with a spatula and 10 ml of toluene, PPO, and POPOP scintillation fluid was added to 5 ml of ethyleneglycolmonomethylether:ethanolamine solution in the vial. The samples were counted in a Packard Model 2420 Tri-Carb liquid scintillation spectrometer and dpm were determined by the calculated efficiency of 62% for these samples. The percent of total dpm recovered from the urine was calculated for each spot on the chromatogram. Several plates, with the spots observed by autoradiography removed, were sectioned into 150, 1 cm squares, scraped, and counted to determine the quantity of residual radioactivity on the plate. No more than 1% of the original isotope remained on the plate.

Statistical Methods

Since the absolute quantities of individual metabolites varied extensively among the plates as a direct consequence of the amount of radioactivity spotted, all data were calculated as a function of a percentage of the total radioactivity recovered. The amount of total radioactivity recovered was consistently over 99%. The statistical differences between individual metabolites were determined by applying the White Rank Test (168) to the individual percentage calculated. For statistical comparison of the total kynurenine or serotonin pathway metabolites excreted, the relative

percentages of total pathway metabolites were obtained by adding the percentages of individual metabolites from each given pathway of a single mouse.

RESULTS

Rf Values, Fluorescence, and Color Reactions of Standard Tryptophan Metabolites

Tryptophan and 24 of its metabolites were each dissolved in an appropriate solvent (either distilled water, ethanol, or pyridine) to a concentration of 1 mg/ml. Compounds were also dissolved in mouse urine. Five, 10, or 20 micrograms of each compound were spotted on thin layer plates. The plates were developed in either the butanol, acetic acid, and water solvent system or the methyl acetate, isopropanol, ammonium hydroxide solvent system. Rf values were calculated and are reported in Table 2. The fluorescence and color reactions with Prochazka's formaldehyde-HCl reagent and van Urk's 4-dimethylaminobenzaldehyde reagent are presented in Table 3. No significant change in Rf values or color reactions were observed after refrigeration of the standard solutions at $4^{o}C$ for 24 hours or after dissolving them in urine. Similar results of other investigators are shown in Appendix A, Tables A1, A2, and A3.

DEAE-Cellulose Chromatography of Standard Tryptophan Metabolites

One hundred micrograms of each of 22 standard tryptophan metabolites were dissolved in 0.001 M Triethylamine-formate

TABLE 2.--Rf values of standard tryptophan metabolites in two solvent systems.

METABOLITE	SOLVENT	Rf[1]	Rf[2]
Tryptophan	H_2O	0.27[3]	0.45
5-Hydroxytryptophan	H_2O	0.21	0.37
5-Hydroxytryptamine	H_2O	0.63	0.47
5-Hydroxytryptophol	H_2O	0.84	0.75
5-Hydroxyindoleacetic Acid	EtOH	0.27	0.73
N-Acetylserotonin	H_2O	0.82	0.69
5-Methoxytryptamine	H_2O	0.66	0.50
5-Methoxytryptophol	H_2O	0.87	0.73
5-Methoxyindoleacetic Acid	EtOH	0.28	0.75
Bufetonin	H_2O	0.81	0.33
Tryptamine	H_2O	0.77	0.58
Indoleacetic Acid	EtOH	0.30	0.75
Indole	EtOH	0.94	0.79
Kynurenine	Pyr	0.27	0.38
3-Hydroxykynurenine	Pyr	0.23	0.43
Anthranilic Acid	EtOH	0.39	0.77
3-Hydroxyanthranilic Acid	Pyr	0.22	0.84
Kynurenic Acid	Pyr	0.37	0.43
Xanthurenic Acid	Pyr	0.43	0.47
Quinaldic Acid	Pyr	0.46	0.55
Quinolinic Acid	EtOH	0.03	0.03
o-Aminophenol	EtOH	0.80	0.67
Nicotinamide	EtOH	0.76	0.58
N-Methylnicotinamide	EtOH	0.85	0.54
Nicotinic Acid	EtOH	0.37	0.47

1 - Methyl Acetate, Isopropyl Alcohol, Ammonium Hydroxide (45:35:20)

2 - Butyl Alcohol, Acetic Acid, Water (12:3:5)

3 - All values are averages of at least 25 individual runs with deviations no greater than $\pm$0.05 cm.

TABLE 3.--Characteristics of standard tryptophan metabolites with respect to fluorescence, color reactions with van Urk's reagent, and fluorescence and color reactions with Prochazka's reagent.

METABOLITE	FLUORESCENCE[1]	VAN URK'S	PROCHAZKA'S	
			VISIBLE	FLUORS.
Tryptophan	- b g[2]	b g→gr	y	y
5-Hydroxytryptophan	- y t	b g	- g br	b gr br
5-Hydroxytryptamine	t br	b g	- br	dk y t
5-Hydroxytryptophol	br ro	dk b t ro	t o	t ro
5-Hydroxyindoleacetic Acid	pi t	br b	y be	br
N-Acetylserotonin	- t	b gr g	br	dk y
5-Methoxytryptamine	- y t	b	t br	t br
5-Methoxytryptophol	- y t	b	t br	t br
5-Methoxyindoleacetic Acid	- t	b	t br	t br
Bufetonin	- t	b g→b	y br	y br
Tryptamine	- y t	b g	y	br y o
Indoleacetic Acid	y t	b	y	br y
Indole	y t	ro	b g	ro v
Kynurenine	b	y o	y pi	y
3-Hydroxykynurenine	ro o	y o	dk o br	o ru
Anthranilic Acid	p b	y	pale b br	be y
3-Hydroxyanthranilic Acid	br v b	o y	dk y b	o y
Kynurenic Acid	p with gr	-	-	p
Xanthurenic Acid	y	-	-	-
Quinaldic Acid	-	-	-	-
Quinolinic Acid	- br	-	-	-
o-Aminophenol	y o	ru	dk o br	dk ro br
Nicotinamide	-	-	-	-
N-Methylnicotinamide	-	-	-	-
Nicotinic Acid	- y	-	-	-

[1]All characteristics represent data collected from at least 25 individual runs.

[2]Abbreviations: b=blue, be=beige, br=brown, dk=dark, g=grey, gr=green, o=orange, p=purple, pi=pink, ro=rose, ru=rust, t=tan, v=violet, y=yellow, -=colorless.

buffer (1 mg/ml) and chromatographed on a DEAE-cellulose column. After lyophilization and reconstitution, each fraction was run on one dimensional thin layer chromatography in both solvent systems and Rf values calculated. These values combined with fluorescence and color reactions were used to determine in what fraction a given compound was eluted (Table 4). The neutral and basic compounds were recovered first while the acids appeared in later fractions.

Gas Chromatography-Mass Spectrometry of ME-TMSi and TMSi Derivatives of Standard Tryptophan Metabolites

Gas chromatography-mass spectrometry proved helpful in characterization of tryptophan metabolites in this study. Most of the ME-TMSi derivatives of the tryptophan metabolites gave multiple peaks with similar retension times on gas chromatographic analysis. The indole derivatives (tryptophan, 5-hydroxytryptophan, 5-hydroxytryptamine, N-acetylserotonin, 5-methoxytryptamine, 5-hydroxytryptophol, 5-methoxytryptophol, 5-hydroxyindoleacetic acid, 5-methoxyindoleacetic acid, tryptamine, and indole acetic acid) and the kynurenine metabolites (kynurenine, 3-hydroxykynurenine, 3-hydroxyanthranilic acid, xanthurenic acid, kynurenic acid, quinolinic acid, and quinaldic acid) also gave almost identical mass spectra. A major peak with mass 131, whose structure was not elucidated, was consistently observed in analysis of these compounds. Several other peaks with masses of 285, 270, 257, or 258, 217, 185,

TABLE 4.--Elution pattern of standard tryptophan metabolites from a DEAE-cellulose (formate form) column.

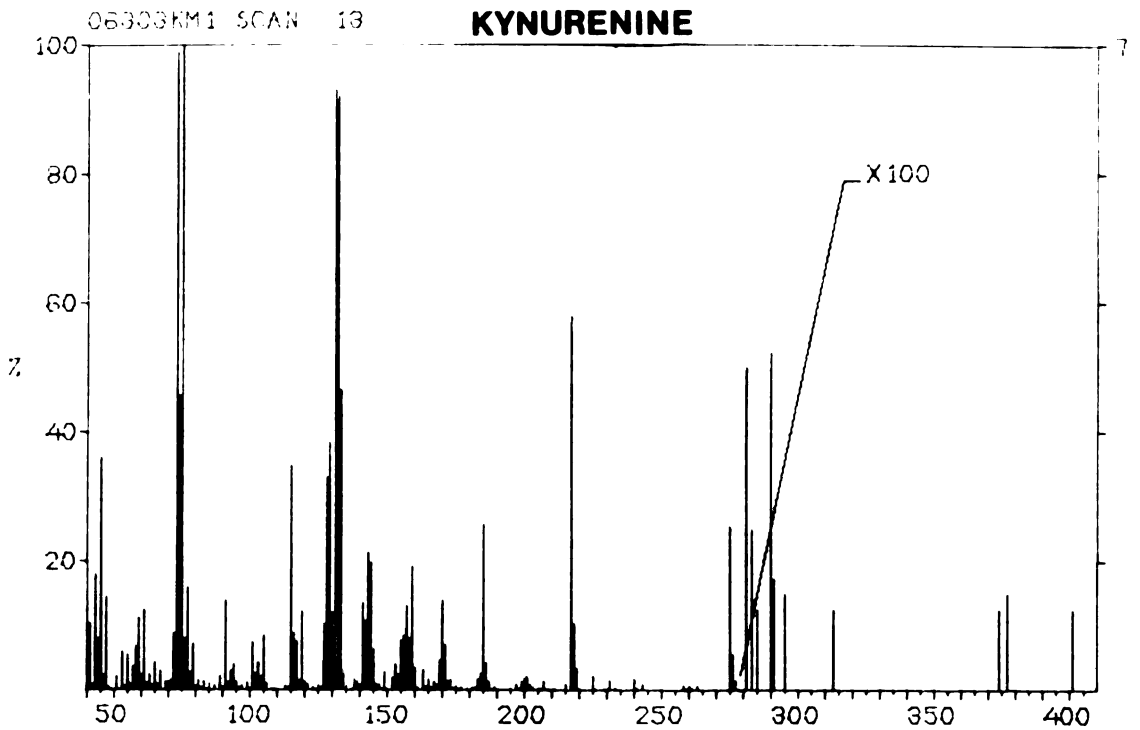
METABOLITE	DEAE-ELUANT
5-Methoxytryptophol	5 - 8[a]
o-Aminophenol	5 - 12
Tryptamine	5 - 10
5-Hydroxytryptamine	5 - 12
Bufetonin	5 - 12
Kynurenine	5 - 12
Tryptophan	8 - 16
5-Hydroxytryptophan	8 - 16
Nicotinamide	9 - 12
N-Acetylserotonin	9 - 17
5-Hydroxytryptophol	10 - 12
N-Methylnicotinamide	17 - 21
Anthranilic Acid	19 - 46
3-Hydroxykynurenine	40 - 55
3-Hydroxyanthranilic Acid	43 - 61
Indoleacetic Acid	48 - 70
5-Hydroxyindoleacetic Acid	40 - 89
Quinolinic Acid	51 - 130
5-Methoxyindoleacetic Acid	62 - 68
Kynurenic Acid	81 - 105
Xanthurenic Acid	110 - 130
Quinaldic Acid	110 - 130

[a]Average of two individual DEAE-cellulose column runs.

and 170 were also regularly observed. Although these spectra were similar, small variations occurred which allowed general conclusions about basic structure to be drawn. For example, only kynurenine, 3-hydroxykynurenine, 3-hydroxyanthranilic acid, and xanthurenic acid contained a peak at mass 105, and only xanthurenic acid had a major peak at 240. The spectra of the ME-TMSi derivatives obtained from kynurenine and 5-hydroxytryptamine are shown in Figure 5. Those obtained from the ME-TMSi derivatives of 3-hydroxykynurenine, kynurenic acid, and xanthurenic acid, and 5-hydroxytryptophol are shown in Appendix B, Figure B1. Spectra of the terminal metabolites of the kynurenine patheay: i.e., o-aminophenol, anthranilic acid, nicotininc acid, nicotinamide, and N-methylnicotinamide showed that methyl esters of these compounds were not formed and only silylation occurred. These compounds either did not have a carboxylic acid group or were insoluble in the reagents. These spectra are shown in Figure 6.

Preparation of TMSi derivatives was simple and rapid but care has to be taken in manipulation of the derivatized samples as they hydrolyzed easily to give multiple gas chromatography peaks. The spectra obtained from these derivatized standards generally gave a molecular ion and predictable breakdown ions. Spectra of the TMSi derivatives obtained from kynurenic acid, anthranilic acid, N-methylnicotinamide, and 5-hydroxyindoleacetic acid are shown in Figure 7.

Figure 5.--Mass Spectra Obtained from the ME-TMSI Derivatives of Kynurenine and 5-Hydroxytryptamine Demonstrating the Similarities in Spectra.


06303KM1 SCAN 13
KYNURENINE
100
80
60
40
20
%
X100
50
100
150
200
250
300
350
400

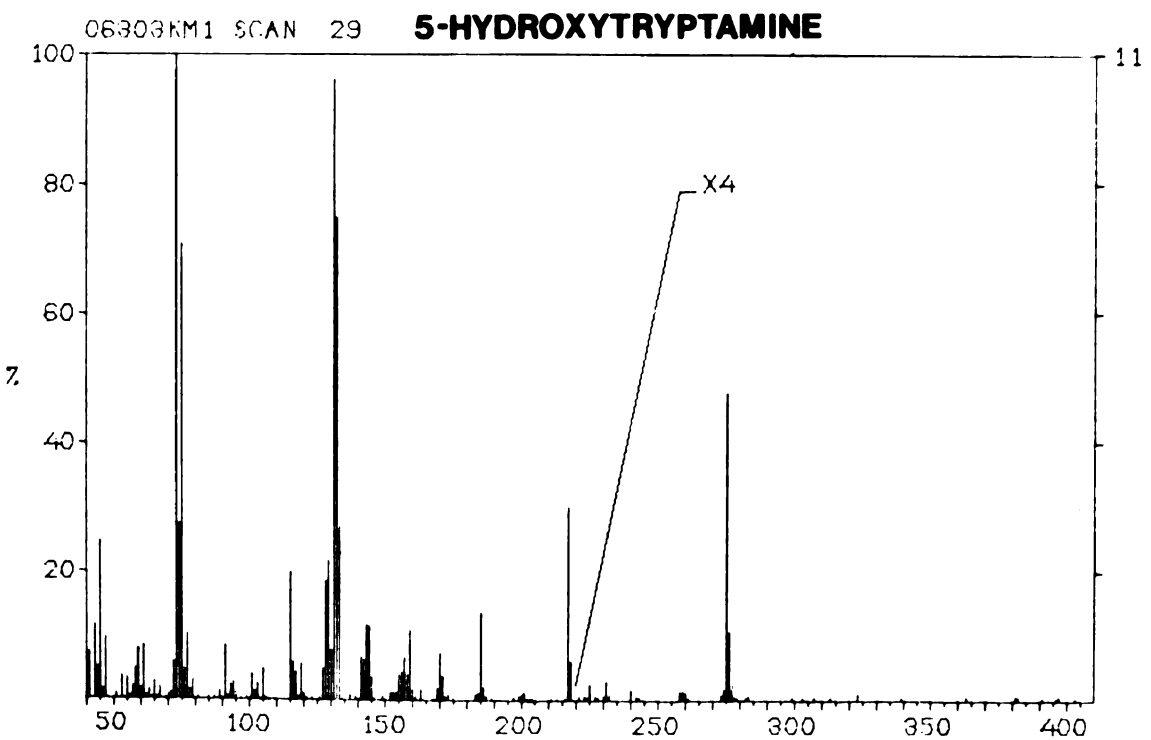
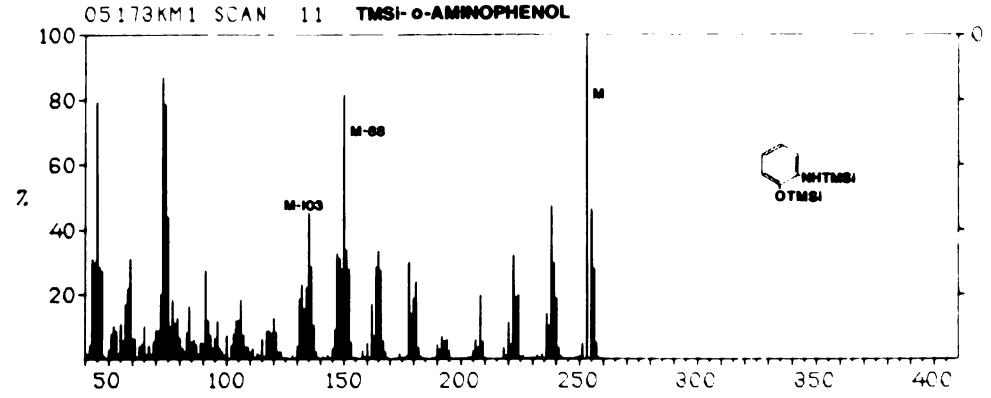
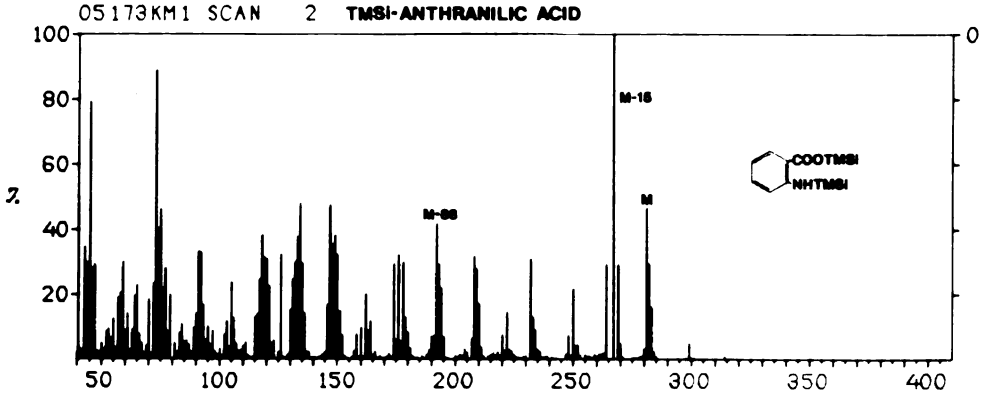
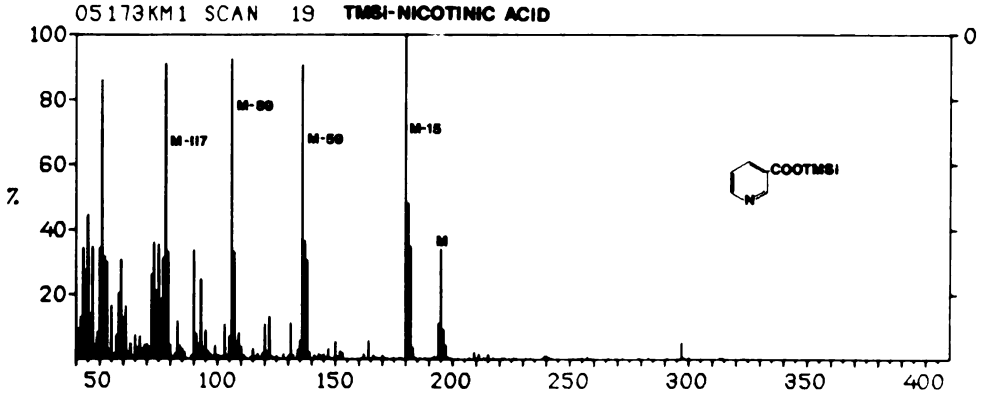

06303KM1 SCAN 29
5-HYDROXYTRYPTAMINE
100
80
60
40
20
%
X4
50
100
150
200
250
300
350
400

Figure 6.--Mass Spectra Obtained from o-Aminophenol, Anthranilic Acid, Nicotinic Acid, Nicotinamide, and N-Methylnicotinamide Prepared as ME-TMSi Derivatives But Due to Structure or Insolubility Forming Only the TMSi Ethers or Esters.


05173KM1 SCAN 11 TMSI-o-AMINOPHENOL
100
80
60
40
20
%
0
M
M-88
M-103
NHTMSI
OTMSI
50
100
150
200
250
300
350
400


05173KM1 SCAN 2 TMSI-ANTHRANILIC ACID
100
80
60
40
20
%
0
M-15
M
M-88
COOTMSI
NHTMSI
50
100
150
200
250
300
350
400


05173KM1 SCAN 19 TMSI-NICOTINIC ACID
100
80
60
40
20
%
0
M-117
M-88
M-88
M-15
M
COOTMSI
N
50
100
150
200
250
300
350
400

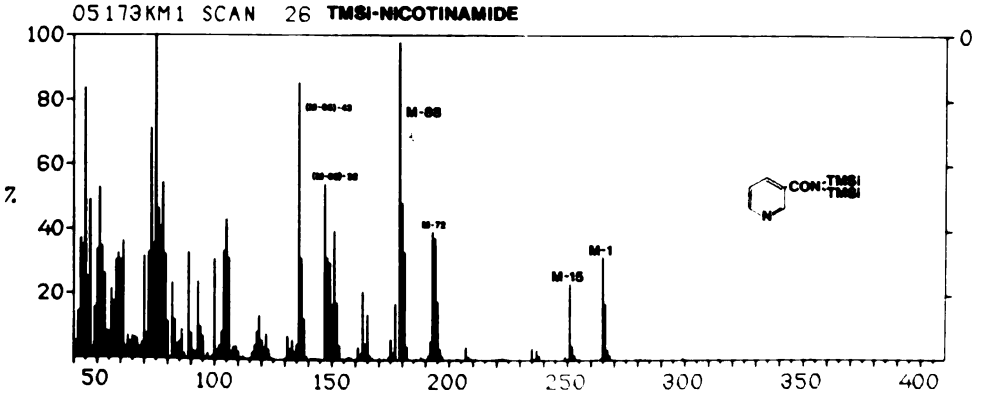
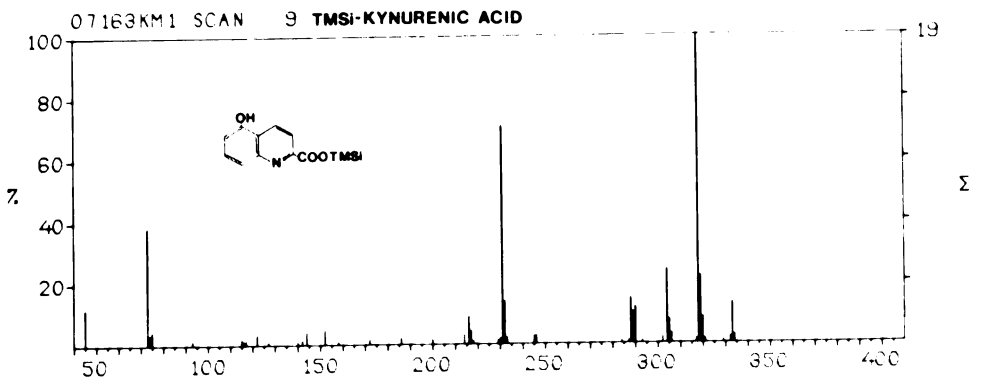
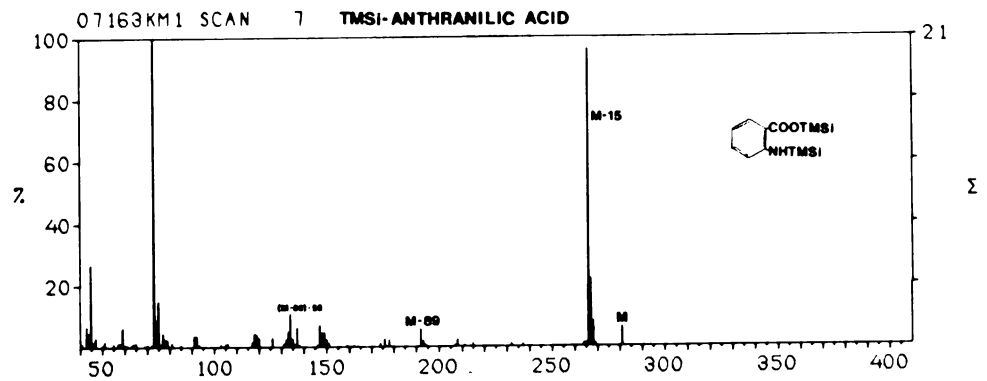
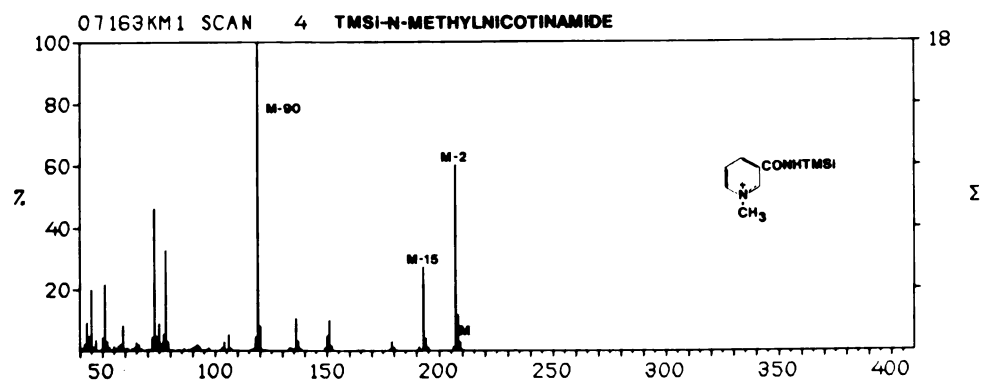
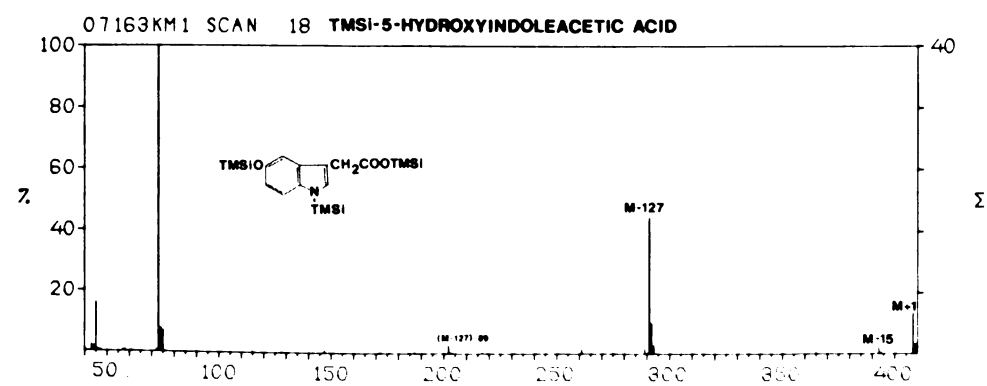

05173KM1 SCAN 26 TMSI-NICOTINAMIDE
100
80
60
40
20
%
0
M-88
M-72
M-15
M-1
CON
TMSI
TMSI
N
50
100
150
200
250
300
350
400

Figure 7.--Mass Spectra Obtained from the TMSi Derivatives of Kynurenic Acid, Anthranilic Acid, N-Methylnicotinamide and 5-Hydroxyindoleacetic Acid.


07163KM1 SCAN 9 TMSi-KYNURENIC ACID
100
80
60
40
20
%
OH
N
COOTMSi
50
100
150
200
250
300
350
400
19
Σ


07163KM1 SCAN 7 TMSi-ANTHRANILIC ACID
100
80
60
40
20
%
M-15
COOTMSi
NHTMSi
M-89
M
50
100
150
200
250
300
350
400
21
Σ


07163KM1 SCAN 4 TMSi-N-METHYLNICOTINAMIDE
100
80
60
40
20
%
M-90
M-2
M-15
M
CONHTMSi
N
CH3
50
100
150
200
250
300
350
400
18
Σ


07163KM1 SCAN 18 TMSi-5-HYDROXYINDOLEACETIC ACID
100
80
60
40
20
%
TMSiO
CH2COOTMSi
N
TMSi
M-127
M+1
M-15
50
100
150
200
250
300
350
400
40
Σ

Thin Layer and DEAE-Cellulose Chromatography of Tryptophan Metabolites in Urine of Normal and Endotoxin-Poisoned Mice

Autoradiograms of two dimensional thin layer plates revealed between 8 and 25 different tryptophan metabolites in the urine of normal and endotoxin-poisoned mice given D, L-tryptophan (benzene ring-^{14}C) with and without a tryptophan load. A representative autoradiogram is shown in Figure 8. When normal and endotoxin-poisoned mice were given 5-hydroxy-tryptamine-3'-^{14}C with and without load, eight to 12 metabolites were isolated. A representative autoradiogram from a two dimensional development of urine from these mice is shown in Figure 9.

Pooled urine from ten normal or endotoxin-poisoned mice given labeled tryptophan with or without load was chromatographed on DEAE-cellulose. Samples were lyophilized and reconstituted in 100 µl of deionized distilled water. Five microliters of each sample were spotted on filter paper and counted by liquid scintillation spectrometry. Radioactive profiles obtained from normal and endotoxin-poisoned mice given labeled tryptophan without load are shown in Figure 10. Profiles obtained from mice with load were essentially the same. To determine the metabolites recovered, individual fractions containing more than 100 cpm were spotted on two different thin layer plates, developed in the butanol, acetic acid, and water and in the methyl acetate, isopropanol, and ammonium hydroxide solvent systems, autoradiographed, and Rf values determined.

Figure 8.--A Representative Autoradiogram of a Two Dimensional Thin Layer Development of a Urine Sample from an Individual Mouse Given D,L-Tryptophan (Benzene Ring-^{14}C).

REPRESENTATIVE AUTORADIOGRAM OF URINE
FROM MICE GIVEN D,L-TRYPTOPHAN(BENZENE RING-^{14}C)

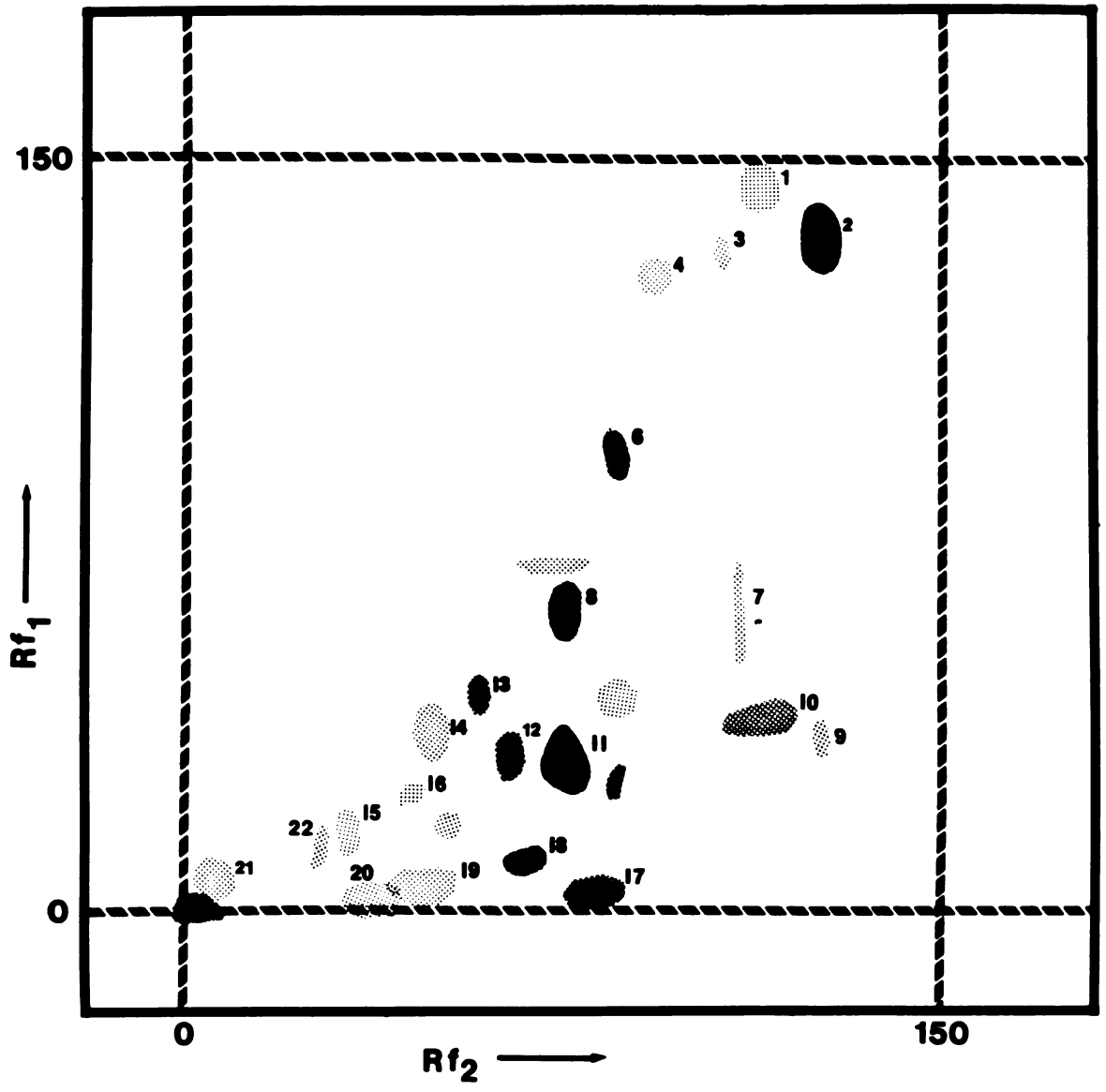


Figure 9.--A Representative Autoradiogram of a Two Dimensional Thin Layer Development of a Urine Sample from an Individual Mouse Given 5-Hydroxytryptamine-3'-^{14}C.

REPRESENTATIVE AUTORADIOGRAM OF URINE FROM MICE GIVEN 5-HYDROXYTRYPTAMINE-3'-^{14}C

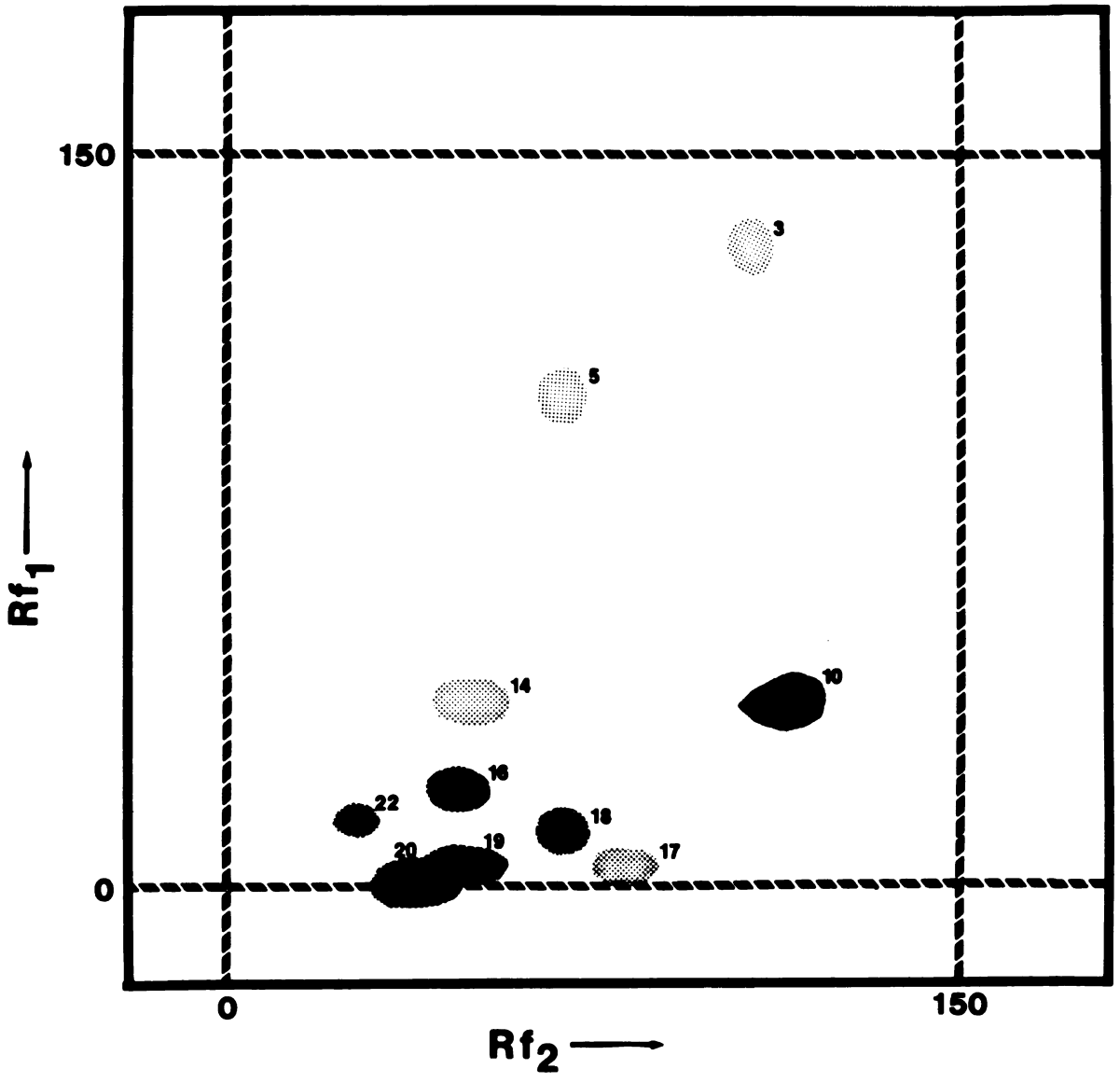
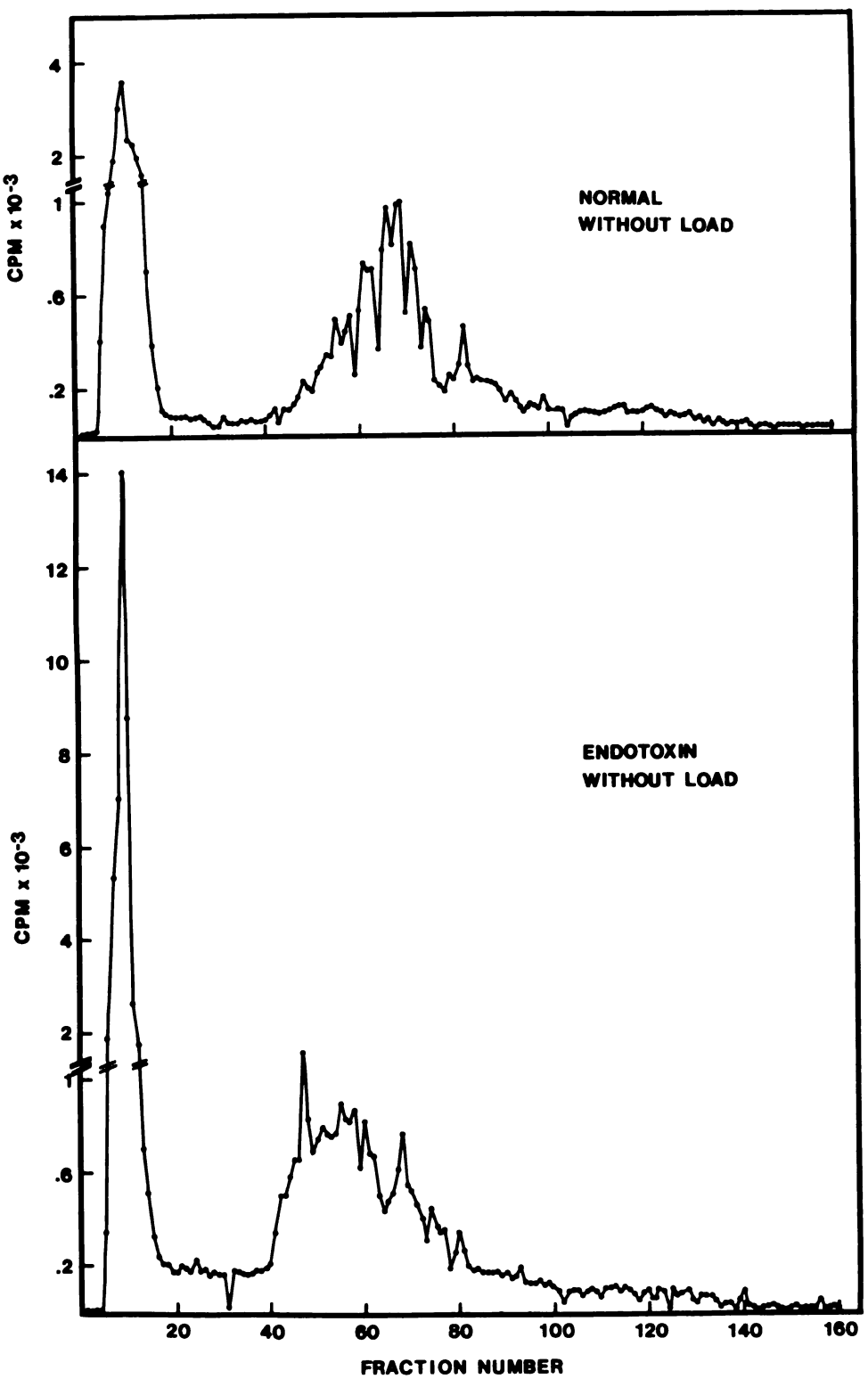


Figure 10.--Radioactive Profiles of a Pooled Urine Sample from Ten Normal or Endotoxin-Poisoned Mice Given D,L-Tryptophan (Benzene Ring-^{14}C) Chromatographed on a DEAE-Cellulose (Formate Form) Column.

DEAE-CELLULOSE CHROMATOGRAPHY OF URINE FROM MICE

GIVEN D,L-TRYPTOPHAN(BENZENE RING-C^{14})



The same procedures were followed for the urine of normal and endotoxin-poisoned animals given 5-hydroxytryptamine -3'-^{14}C with and without load. The radioactive profiles from normal and endotoxin-poisoned mice without load are shown in Figure 11 and with load in Figure 12.

Gas Chromatography-Mass Spectrometry of ME-TMSi and TMSi Derivatives of Tryptophan and Serotonin Metabolites from Urine of Normal and Endotoxin-Poisoned Mice

Mass spectra of unconjugated urinary tryptophan or serotonin metabolites reisolated from thin layer plates gave similar results to the ME-TMSi derivatives of known standards. Metabolites tentatively identified as conjugates had strong 79 and 128,129 peaks but no mass 131 peak. Several of the serotonin metabolites also thought to be conjugates had the 79, and 128, 129 mass spectral pattern.

The spectra of the TMSi derivatives of the unknown urinary tryptophan and serotonin metabolites gave two major patterns and did not resemble the spectra of the standards. One group had major peaks at 137, 159, 185, 193, 215, 237, 257, and 331 and the other at 129, 157, 185, 259, 329, 343, and 396 (Appendix B, Figure B2).

On analysis of the spectra obtained from the compound tentatively identified as 5-hydroxytryptamine, the mass peaks of the TMSi derivatives of 5-hydroxytryptamine: 393 = M + 1, 291 = (M + 1) - 102, 276 = (M - 116) - 43, and 219 = (M-116) - 43,

Figure 11.--Radioactive Profiles of a Pooled Urine Sample from Ten Normal or Endotoxin-Poisoned Mice Given 5-Hydroxytryptamine-3'^{14}C Without Load Chromatographed on a DEAE-Cellulose (Formate Form) Column.

DEAE-CELLULOSE CHROMATOGRAPHY OF URINE FROM MICE GIVEN 5-HYDROXYTRYPTAMINE-3'-C^{14}

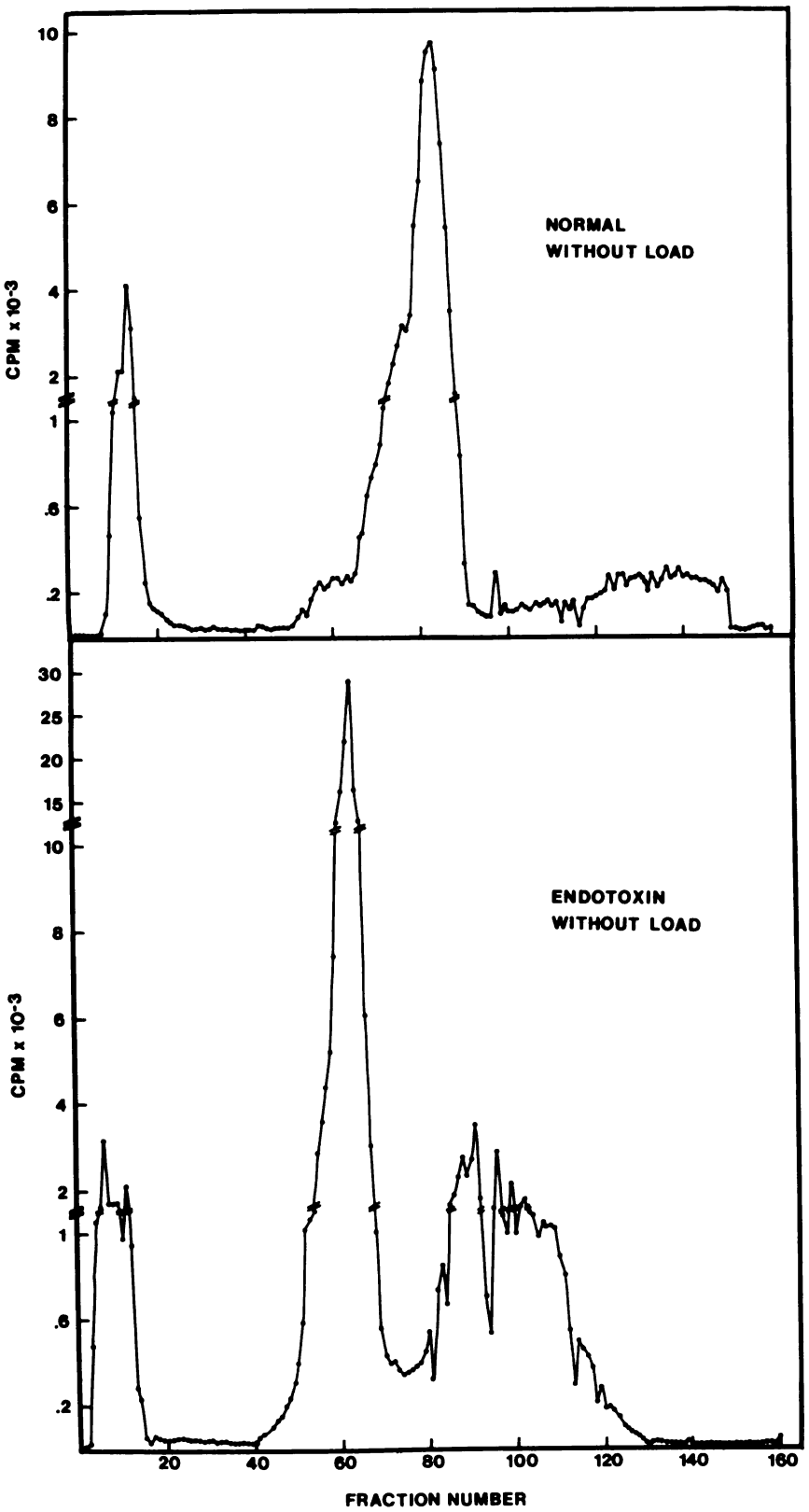
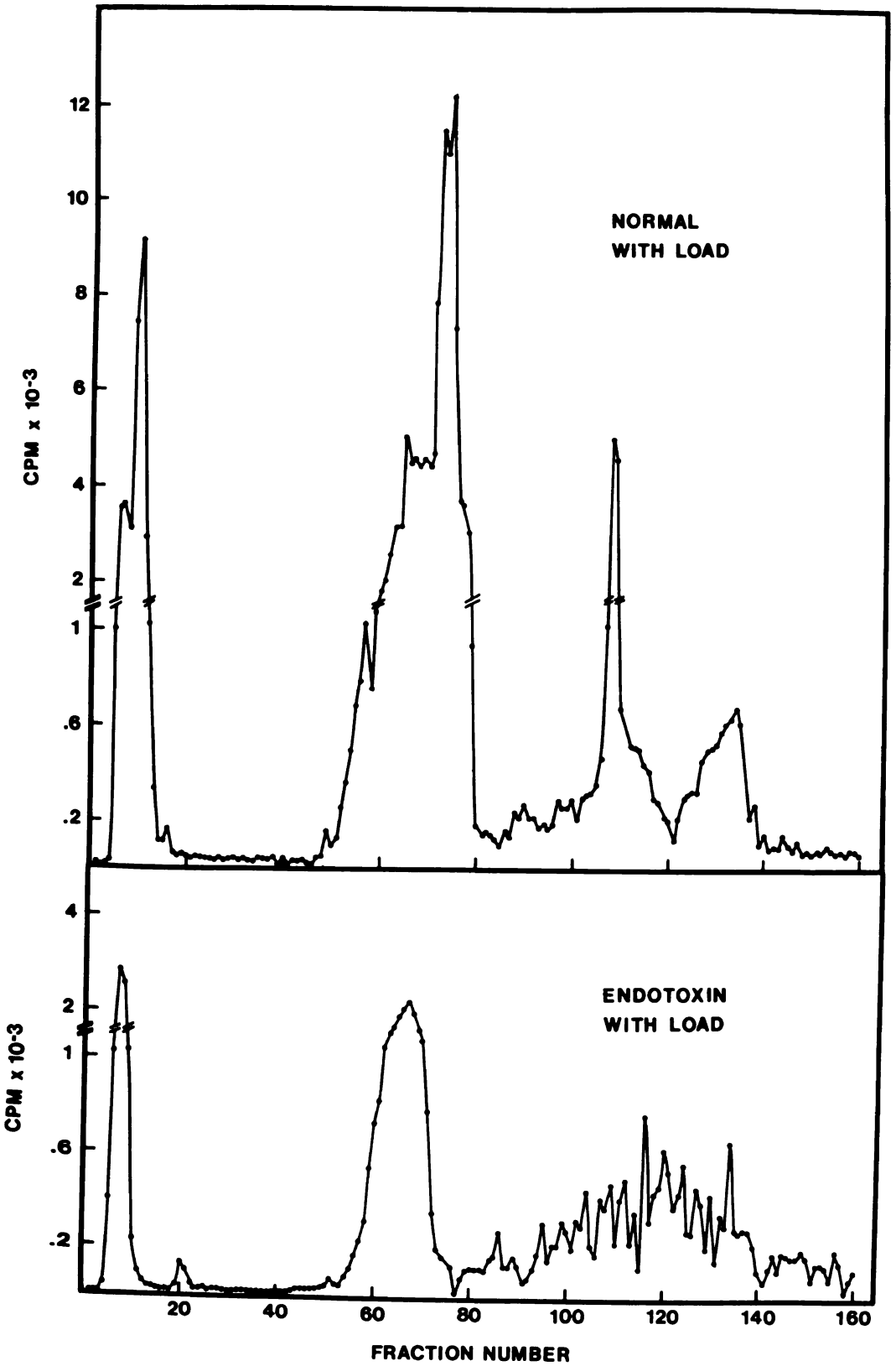


Figure 12.--Radioactive Profiles of a Pooled Urine Sample from Ten Normal or Endotoxin-Poisoned Mice Given 5-Hydroxytryptamine-3'^{14}C With Load Chromatographed on a DEAE-Cellulose Column (Formate Form).

DEAE-CELLULOSE CHROMATOGRAPHY OF URINE FROM MICE GIVEN 5-HYDROXYTRYPTAMINE-3^{1}-C^{14}

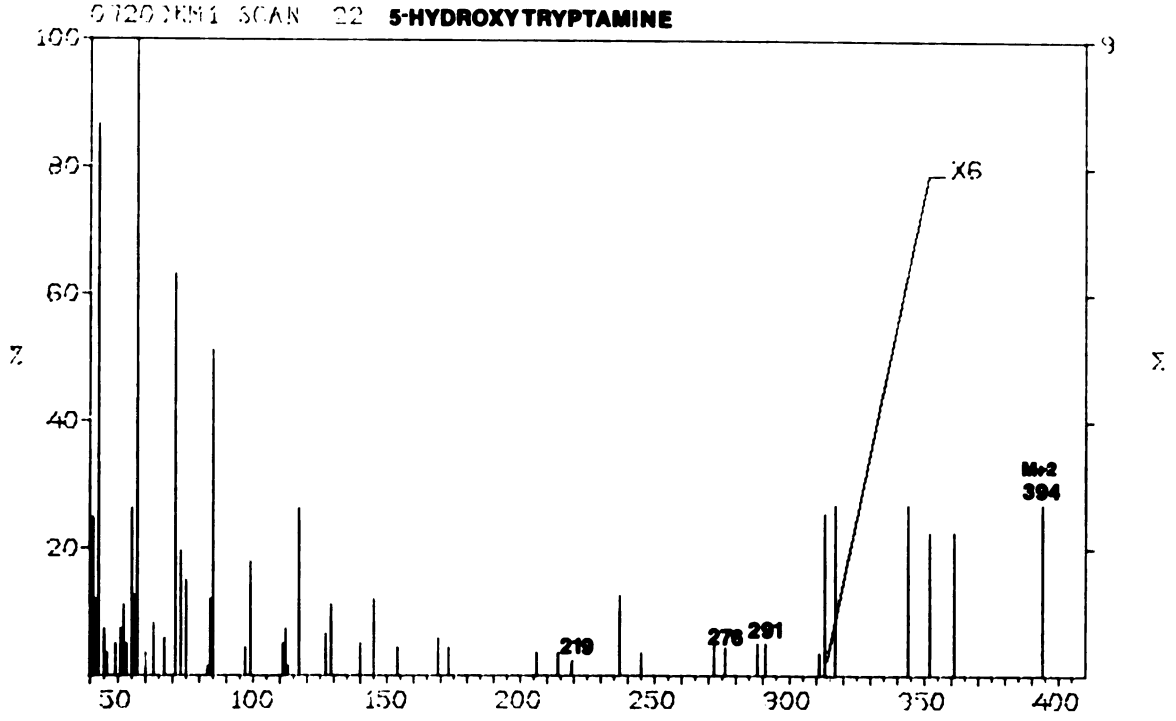


were present but in smaller proportions than other peaks found in most of these spectra (Figure 13, top spectra). The mass peaks identified as belonging to serotonin did not appear in a spectra from the same run which did contain the other peaks observed in the spectra with 5-hydroxytryptamine (Figure 13, bottom spectra). Assuming this was a problem in concentration, urine from normal mice given 5-hydroxytryptamine-3'^{14}C was spiked with 100 µg of 5HIAA and 5HTOH and chromatographed on a DEAE-cellulose column. The effluents from several fractions were lyophilized and TMSi derivatives formed. The spectra of the sample were screened and of them all, two serotonin metabolites were observed. They were tentatively identified as 5-hydroxyindoleacetic acid with one TMSi group and 5-hydroxytryptophol with two TMSi groups (Figure 14).

Tentative Identification of Kynurenine Pathway Metabolites Isolated from Urine of Normal and Endotoxin-Poisoned Mice Given D,L-Tryptophan (Benzene Ring-^{14}C)

Eleven kynurenine metabolites were selected for identification. The data obtained from thin layer and DEAE-cellulose chromatography, fluorescence and color reactions along with their tentative identification is contained in Table 5. Kynurenine, 3-hydroxykynurenine, kynurenic acid, and xanthurenic acid were tentatively identified by comparison of fluorescence, color reactions, mass spectra, and Rf values with known authenic standards.

Figure 13.--Mass Spectra Obtained from TMSi-5-Hydroxy-tryptamine and Background.


07203KM1 SCAN 22 5-HYDROXYTRYPTAMINE
100
80
60
40
20
%
X6
M+2
394
219
278
291
50
100
150
200
250
300
350
400
Σ

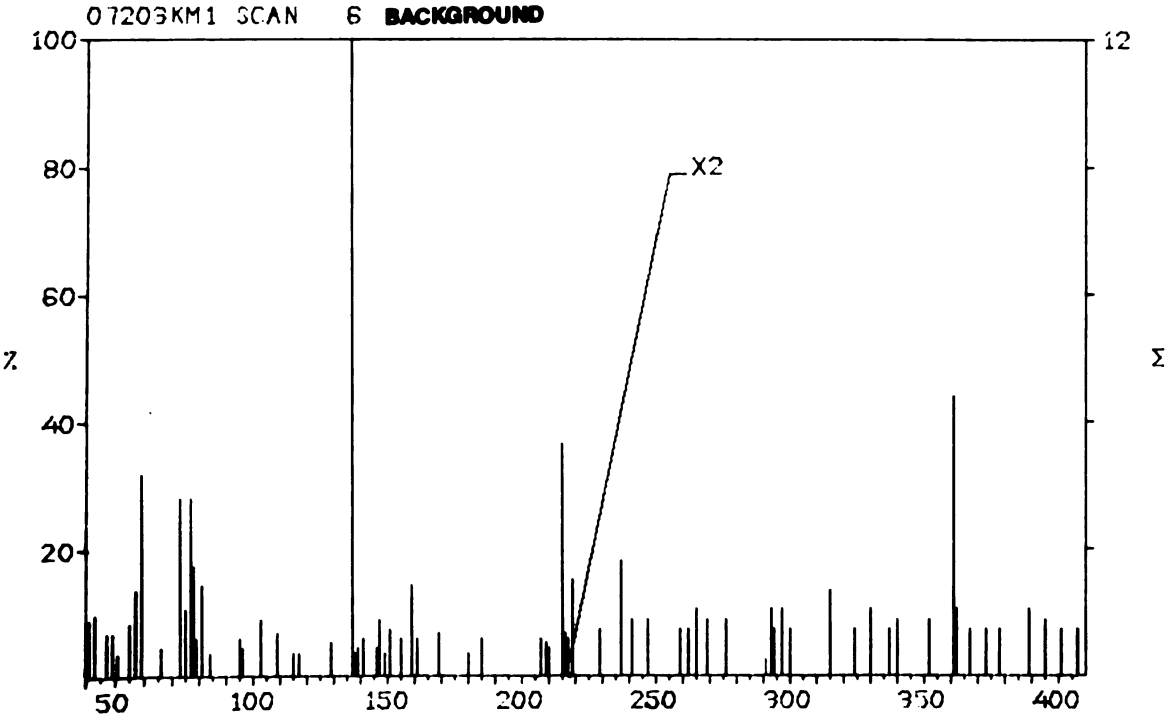
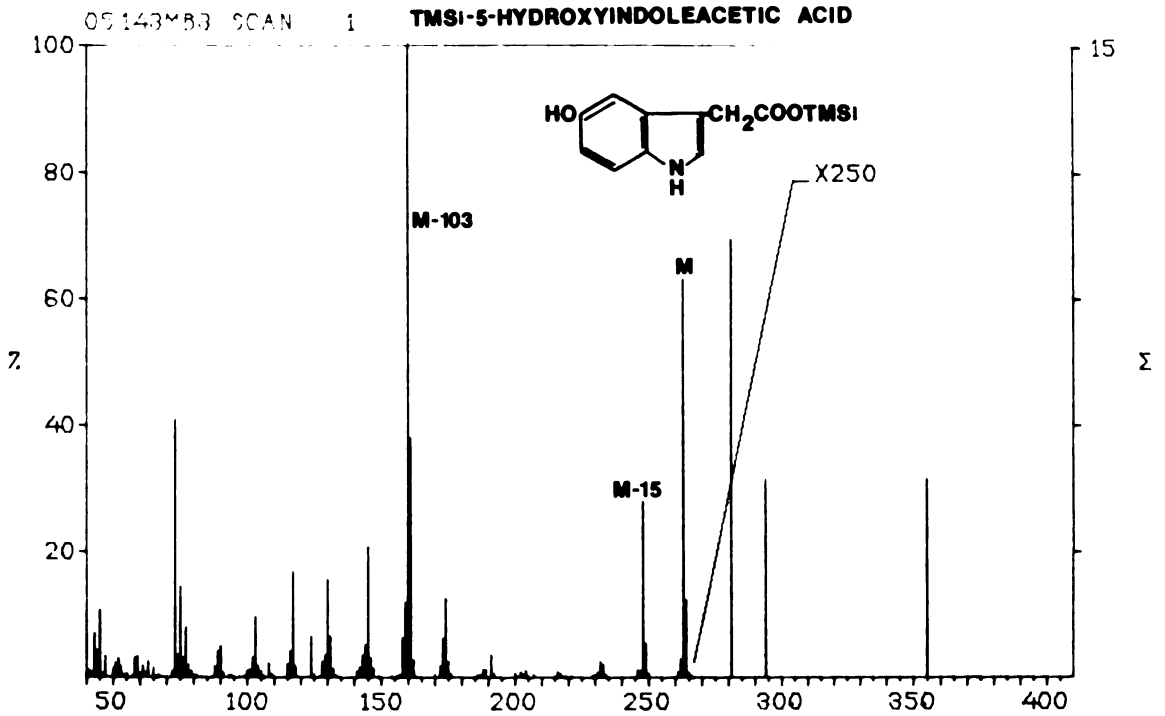

07203KM1 SCAN 6 BACKGROUND
100
80
60
40
20
%
X2
50
100
150
200
250
300
350
400
Σ

Figure 14.--Mass Spectra of TMSi-5-Hydroxyindoleacetic Acid and TMSi-5-Hydroxytryptophol.


05143MB3 SCAN 1
TMSi-5-HYDROXYINDOLEACETIC ACID
HO
CH2COOTMSi
N
H
X250
M-103
M
M-15
%
Σ
100
80
60
40
20
15
50
100
150
200
250
300
350
400

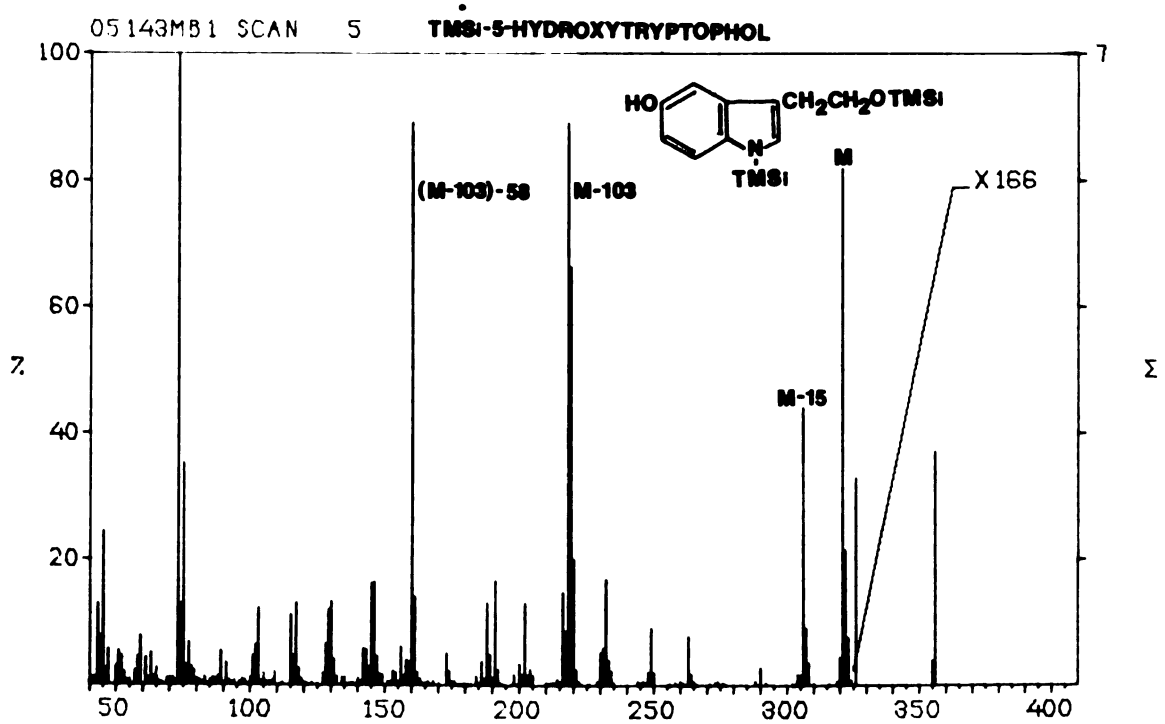

05143MB1 SCAN 5
TMSi-5-HYDROXYTRYPTOPHOL
HO
CH2CH2OTMSi
N
TMSi
X166
(M-103)-58
M-103
M
M-15
%
Σ
100
80
60
40
20
7
50
100
150
200
250
300
350
400

TABLE 5.--Tentative Identification and Characterization of Kynurenine Pathway Metabolites in Urine of Normal and Endotoxin-Poisoned Mice Given D,L-Tryptophan (Benzene Ring-^{14}C).

No.[5]	TENTATIVE IDENTIFICATION	Rf[1]	Rf[2]	DEAE-ELUANT	FLUORESCENCE	PROCHAZKA'S VISIBLE	PROCHAZKA'S FLUOR.	VAN URK'S	NINHYDRIN
13	Kynurenine	0.24[3]	0.32	8 - 13	b[4]	g	g	y t	pi
12	3-Hydroxykynurenine	0.20	0.43	39 - 45	y o	o br	o ru	y o	pi
8	Kynurenic Acid	0.38	0.50	79 - 100	p with gr	-	p	-	-
6	Xanthurenic Acid	0.59	0.57	89 - 130	y	-	-	-	-
15	Kynurenine-O-Sulfate	0.15	0.32	61 - 82	b	g	g	- y	*
2	N-Acetylkynurenine	0.90	0.84	7 - 17	y t	-	- g	- y	y pi
9	3-Hydroxyanthranilic Acid	0.23	0.84	58 - 74	y	*	*	*	*
7	Anthranilic Acid	0.39	0.73	34 - 48	y	*	*	*	*
4	N-Methylnicotinamide	0.84	0.62	8 - 15	-	*	*	*	*
1	o-Aminophenol	0.95	0.76	8 - 14	y	y 0	v o	ro	*
21	Qunolinic Acid	0.03	0.03	103 - 130	- b v	-	-	-	-
11	Tryptophan	0.24	0.50	8 - 16	- b g	y	y t	b gr	pi

[1]Methyl Acetate, Isopropyl Alcohol, Ammonium Hydroxide (45:35:20)

[2]Butyl Alcohol, Acetic Acid, Water (12:3:5)

[3]Average Rf values calculated from at least 25 individual mouse urine samples.

[4]Abbreviations: b=blue, g=grey, o=orange, p=purple, pi=pink, ro=rose, ru=rust, t=tan, v=violet, y=yellow, -=colorless

[5]Number corresponding to representative autoradiograph

*Concentration of metabolite below limits of detection

The tentative identification of kynurenine-O-sulfate was based on its Rf value in the butanol, acetic acid, and water solvent system (32,106), its fluorescence and color reactions. It also gave a yellow color when sprayed with naphthoresorcinol indicating a conjugate. The mass spectrum of the ME-TMSi derivative gave major peaks at 79, 128, and 129 indicative of conjugation (Appendix B, Figure B3).

The other kynurenine metabolite was less easily identified. The fluorescence and color reactions indicated an unconjugated, non-indole. The high Rf values in both solvent systems indicated it could contain an alcohol function or an acetyl group. The standard compounds with Rf values in the range of this unknown were o-aminophenol, N-methylnicotinamide, 5-hydroxytryptophol, and indole. 5-Hydroxytryptophol and indole were eliminated as they gave positive (blue and rose tan) reactions with van Urk's reagent. 5-Hydroxytryptophol was often isolated from the same sample several times. The mass spectra of this unknown (Appendix B, Figure B3) and negative reaction with naphthoresorcinol excluded a conjugate. Based on this information plus that of other workers (8,32,106) this compound was identified as N-acetylkynurenine.

Five other kynurenine metabolites were also periodically observed but they appeared in concentrations too low to give reliable results with the spray reagents and thus reproducible identification. Anthranilic and 3-hydroxyanthranilic

acid were observed and were tentatively identified by comparison to reference standards. Quinolinic acid, N-methylnicotinamide, and o-aminophenol also appeared sporadically. Quinolinic acid, due to its low Rf values, merged with the residual radioactivity at the origin and hence was not always easy to identify. Tryptophan was also regularly identified and is included with these metabolites for identification.

Tentative Identification of Serotonin Pathway Metabolites Isolated from Urine of Normal and Endotoxin-Poisoned Mice Given 5-Hydroxytryptamine-3'-^{14}C or D,L-Tryptophan (Benzene Ring-^{14}C).

To aid in identification of serotonin metabolites in urine from mice given the labeled tryptophan, normal and endotoxin-poisoned mice were injected with 5-hydroxytryptamine-3'-^{14}C creatinine sulfate with and without a tryptophan load. Two dimensional thin layer chromatography and column chromatography revealed ten different serotonin metabolites of which nine could be tentatively identified (Table 6). With this information as a guideline, six serotonin metabolites could be regularly identified in the urine of mice given D,L-tryptophan (benzene ring-^{14}C) with two other appearing sporadically.

5-Hydroxytryptophol, 5-hydroxyindoleacetic acid, and 5-hydroxytryptamine were tentatively identified by comparing their characteristic Rf values, fluorescence, color reactions,

TABLE 6.--Tentative Identification, Rf Values in Two Solvent Systems, and Elution Pattern from DEAE-cellulose of Serotonin Pathway Metabolites in Urine of Normal and Endotoxin-Poisoned Mice Given D,L-Tryptophan (Benzene Ring-^{14}C).

No.[4]	TENTATIVE IDENTIFICATION	Rf[1]	Rf[2]	DEAE-ELUATE
5	5-Hydroxytryptamine	0.67[3]	0.45	5 - 16
10	5-Hydroxyindoleacetic Acid	0.25	0.76	60 - 85
3	5-Hydroxytryptophol	0.88	0.78	5 - 8
18	5HT-O-Glucuronide	0.08	0.45	8 - 16
20	5HTOH-O-Glucuronide	0.01	0.26	80 - 95
19	5HIAA-O-Glucuronide	0.02	0.30	103 - 150
22	5HIAA-O-Sulfate	0.07	0.18	82 - 150
16	5HTOH-O-Sulfate	0.13	0.31	8 - 16
14	5HT-O-Sulfate	0.25	0.33	5 - 12
17	?	0.02	0.53	43 - 67

[1]Methyl Acetate, Isopropyl Alcohol, Ammonium Hydroxide (45:35:20).

[2]Butyl Alcohol, Acetic Acid, Water (12:3:5).

[3]Average Rf values calculated from at least 25 individual mouse urine samples.

[4]Corresponds to metabolite number on the representative autoradiograph.

and mass spectra (Appendix B, Figure B4) to known standards. 5-Hydroxytryptamine was only observed in the urine of mice given labeled 5-hydroxytryptamine.

Six metabolites were tentatively identified by comparing their Rf values to those obtained by Pentikainen (122), Tyce (157) and Dalgliesch (32). They were identified as 5-hydroxytryptamine-O-glucuronide, 5-hydroxytryptophol-O-glucuronide, 5-hydroxyindoleacetic acid-O-glucuronide, 5-hydroxytryptamine-O-sulfate, 5-hydroxytryptophol-O-sulfate, and 5-hydroxyindoleacetic acid-O-sulfate.

The compounds tentatively identified as the glucuronides of 5-hydroxytryptamine, 5-hydroxytryptophol, and 5-hydroxyindoleacetic acid were analyzed by gas chromatography-mass spectrometry and found to give the 79, 128 and 129 mass peaks of conjugates instead of the major 131 peak (Appendix B, Figure B5). 5-Hydroxytryptamine-O-glucuronide was present in concentrations high enough to give a positive naphthoresorcinol reaction.

The sulfate conjugates of the three major metabolites were tentatively identified by comparison of their Rf values with those reported by other workers (32,122,157). The mass spectra (Appendix B, Figure B4) gave the characteristic mass peaks for conjugates.

Quantity of Radioactivity Recovered from Urine of Normal and Endotoxin-Poisoned Mice Given D,L-Tryptophan (Benzene Ring-^{14}C) With and Without Load.

Within one hour after intraperitoneal injection of labeled tryptophan without load, normal mice excreted 21.6% of the label in their urine (Table 7). The amount gradually increased to a total of 29.2% after 6 hours. Endotoxin-poisoned animals excreted only 6.0% of the label in the first hour. At 6 hours they had excreted 17.0% of the radioactivity in their urine. All decreases were statistically significant ($p<0.01$). The dpm/ul did not vary significantly between the normal and endotoxin-poisoned mice without load at any time period except at three hours.

The variations in total urinary excretion observed between normal and endotoxin-poisoned mice with load were significant at one ($p<0.001$) but not at six hours (Table 8).

The presence of tryptophan load did not statistically alter the total quantity of label excreted in either normal or endotoxin-poisoned mice (Table 8 vs Table 7) but dpm/ul did vary at one point where statistically less label ($p<0.01$) label was excreted by normal mice without load than with load (Table 8 vs Table 7, 1 hour).

TABLE 7.--Quantity of Radioactivity Recovered from Urine of Normal and Endotoxin-Poisoned Mice Given D,L-Tryptophan (Benzene Rine-^{14}C) Without Load.

TIME (HR)	NORMAL[a]		ENDOTOXIN	
	TOTAL DPM (%)	DPM/µl ± S.D.	TOTAL DPM (%)	DPM/µl ± S.D.
1	238,990** (21.6)	661.5 ± 296.2	66,412 (6.0)	675.1 ± 298.6
2	261,105** (23.7)	493.7 ± 140.5	108,487 (9.9)	738.4 ± 362.0
3	298,807** (27.2)	351.1* ± 5.0	132,650 (12.1)	530.6 ± 97.9
6	321,243** (29.2)	486.2 ± 152.6	187,505 (17.0)	579.0 ± 137.6

[a]All values represent averages of data from at least 5 mice.

*$p<0.05$

**$p<0.01$

TABL

TIME (HR)	
1	
2	
3	
6	
5 mi	

TABLE 8.--Quantity of Radioactivity Recovered from Urine of Normal and Endotoxin-Poisoned Mice Given D,L-Tryptophan (Benzene Ring-^{14}C) With Load.

TIME (HR)	NORMAL[a] TOTAL DPM (%)	NORMAL[a] DPM/µl ± S.D.	ENDOTOXIN TOTAL DPM (%)	ENDOTOXIN DPM/µl ± S.D.
1	250,695*** (22.8)	1681.5** ± 415.8	104,464 (9.5)	665.8 ± 240.0
2	310,442** (28.2)	598.6 ± 107.0	153,707 (14.0)	574.8 ± 149.4
3	313,440*** (28.5)	473.5 ± 158.3	168,071 (15.3)	370.8 ± 89.3
6	276,566 (25.1)	453.0 ± 60.2	210,219 (19.1)	711.5 ± 271.8

[a]All values represent averages of data from at least 5 mice.

*p<0.05

**p<0.01

***p<0.001

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Quantitation of Tryptophan Metabolites in Urine of Normal and Endotoxin-Poisoned Mice Given D,L-Tryptophan (Benzene Ring-^{14}C) With and Without Load

To determine if quantitative variations occurred among the distribution of tryptophan metabolites among the major pathways *in vivo* in normal and endotoxin-poisoned mice with and without tryptophan load, individual radioactive metabolites were scraped from two dimensional thin layer plates and the quantity of isotope determined by liquid scintillation spectrometry. Preliminary studies showed that scraping all radioactive spots observed on autoradiograms removed 99% of the radioactivity from the thin layer plate. Therefore, the total radioactivity recovered (assumed to be 100%) per urine sample was calculated for each metabolite. The per cent radioactivity recovered as metabolites of either the kynurenine or the serotonin pathways is summarized in Table 9 with load and Table 10 without load.

By one hour after tryptophan excretion of kynurenine pathway metabolites was significantly ($p<0.01$) decreased in endotoxin-poisoned mice compared to normal mice (13.0% with load or 9.1% without load). Throughout the six hour time period, kynurenine pathway excretion was significantly reduced ($p<0.01$) except at 3 and 6 hours without load and 6 hours with load. The overall percentage of metabolites accounted for as kynurenine metabolites also decreased with time in all groups

TABLE 9.--Relative Percentage of Kynurenine or Serotonin Pathway Metabolites in Urine of Normal and Endotoxin-Poisoned Mice Given D,L-Tryptophan (Benzene Ring-^{14}C) With Load.

TIME (HR)	KYNURENINE		SEROTONIN	
	NORMAL[a]	ENDOTOXIN	NORMAL	ENDOTOXIN
1	43.4 ± 2.5**	30.4 ± 3.4	8.4 ± 1.0**	17.5 ± 4.1
2	38.3 ± 3.9**	32.1 ± 3.2	13.2 ± 4.5	20.7 ± 1.5
3	40.8 ± 8.0**	29.5 ± 3.6	10.1 ± 2.6**	24.2 ± 3.2
6	31.4 ± 5.7	24.6 ± 6.8	9.5 ± 1.3**	19.3 ± 0.6

**$p<0.01$

[a]All values represent averages of data from at least 5 mice.

TABLE 10.--Relative Percentage of Kynurenine or Serotonin Pathway Metabolites in Urine of Normal and Endotoxin-Poisoned Mice Given D,L-Tryptophan (Benzene Rine-^{14}C) Without Load.

TIME (HR)	KYNURENINE		SEROTONIN	
	NORMAL[a]	ENDOTOXIN	NORMAL	ENDOTOXIN
1	45.2 ± 6.7**	36.1 ± 3.3	13.2 ± 1.9**	17.9 ± 2.7
2	45.7 ± 4.3**	33.1 ± 7.2	16.1 ± 2.8*	21.1 ± 4.7
3	41.0 ± 8.5	34.0 ± 2.3	15.8 ± 1.4**	24.0 ± 5.4
6	35.3 ± 11.3	24.2 ± 5.4	14.0 ± 0.7**	20.7 ± 3.2

*$p<0.05$

**$p<0.01$

[a]All values represent averages of data from at least 5 mice.

of mice. No significant differences in excretion patterns between animals with and without tryptophan load were observed.

Excretion of serotonin metabolites was significantly increased in endotoxin-poisoned animals at all periods tested except at two hours with load. By one hour endotoxin-poisoned mice with load excreted 9.1% ($p<0.01$) more serotonin metabolites than normal animals. After three hours endotoxin-poisoned animals with load had excreted 14.1% ($p<0.01$) more serotonin metabolites than normal animals and after six hours serotonin pathway metabolites still predominated ($p<0.01$) with load. Without load endotoxin-poisoned mice excreted 6.7% ($p<0.01$) more serotonin metabolites than normal mice. The increased excretion of serotonin pathway metabolites is also significant at 2 ($p<0.05$), 3 ($p<0.01$), and 6 ($p<0.01$) hours.

Quantitative Enumeration of Individual Metabolites of the Kynurenine and Serotonin Pathways in Normal and Endotoxin-Poisoned Mice Given D, L-Tryptophan (Benzene Ring-^{14}C)

The quantitative variations in individual metabolites of the kynurenine and serotonin pathways between normal and endotoxin-poisoned mice with and without a tryptophan load at one hour are shown in Table 11. At one hour with load only kynurenic acid was significantly reduced ($p<0.05$) in endotoxin-poisoned compared with normal animals. Without load in normal mice kynurenine ($p<0.05$) and N-acetylkynurenine ($p<0.05$) were significantly reduced. Endotoxin-poisoned mice

TABLE 11.--Relative Percentage of Individual Kynurenine and Serotonin Pathway Metabolites in Urine of Normal and Endotoxin-Poisoned Mice Given D,L-Tryptophan (Benzene Ring-^{14}C) After One Hour.

METABOLITE	WITH LOAD		WITHOUT LOAD	
	NORMAL[a]	ENDOTOXIN	NORMAL	ENDOTOXIN
Kynurenine	3.2 ± 0.9[b]	1.3 ± 1.3	5.1 ± 1.0*	1.4 ± 0.4
3-Hydroxykynurenine	6.7 ± 2.4	5.5 ± 1.9	6.9 ± 2.5	4.4 ± 0.9
Kynurenic Acid	14.8 ± 4.1*	6.8 ± 2.3	11.7 ± 6.2	2.8 ± 1.9
Xanthurenic Acid	9.7 ± 2.2	6.9 ± 3.4	11.5 ± 6.9	2.6 ± 0.9
Kynurenine-O-Sulfate	1.0 ± 0.2	1.2 ± 1.0	1.8 ± 1.6	1.7 ± 0.5
N-Acetylkynurenine	7.6 ± 2.4	8.8 ± 4.9	8.8 ± 3.0*	21.7 ± 3.6
Total Kynurenine Metabolites	43.4 ± 2.5**	30.4 ± 3.4	45.2 ± 6.7**	36.1 ± 3.3
5-Hydroxyindoleacetic Acid	3.5 ± 1.5*	12.1 ± 4.7	4.3 ± 2.9	8.5 ± 3.3
5HT-O-Glucuronide	3.1 ± 1.1	4.1 ± 1.6	4.3 ± 1.2	4.5 ± 1.4
5HTOH- & 5HIAA-O-Glucuronide	1.1 ± 0.6	2.5 ± 0.4	4.9 ± 1.1	4.4 ± 1.1
5-Hydroxytryptophol	0.9 ± 0.5	0.7 ± 0.9	0.0 ± 0.0	0.6 ± 0.9
Total Serotonin Metabolites	8.4 ± 1.0**	17.5 ± 4.1	13.2 ± 1.9**	17.9 ± 2.7
Tryptophan	32.9 ± 3.5	37.6 ± 11.9	21.0 ± 8.2	14.8 ± 4.9
Other Metabolites	16.4 ± 5.0	17.1 ± 3.4	17.7 ± 7.5	29.5 ± 6.1
Total Metabolites	101.1 ± 3.0	102.6 ± 5.7	97.1 ± 6.1	98.3 ± 4.3

[a]All values represent averages of data from between 4 and 7 mice

[b]Average percentage of radioactivity recovered ± standard deviation

*$p<0.05$

**$p<0.01$

with load excreted a significant amount more 5-hydroxyindole-acetic acid than did normal mice ($p<0.05$).

At two hours kynurenic acid was significantly reduced ($p<0.05$) between normal and endotoxin-poisoned mice with load and kynurenine was again significantly reduced ($p<0.01$) without load (Table 12). N-Acetylkynurenine was significantly increased in endotoxin-poisoned mice without load ($p<0.05$). Interestingly, 5-hydroxytryptamine-O-glucuronide was significantly reduced in endotoxin-poisoned mice with load. The glucuronide conjugates of 5-hydroxytryptophol and 5-hydroxyindole-acetic acid, however, were increased significantly ($p<0.05$) in endotoxin-poisoned mice without load.

After three hours no statistically significant differences in the kynurenine metabolites between normal and endotoxin-poisoned mice with or without load were detected (Table 13). However, among the serotonin metabolites, 5-hydroxyindoleacetic acid was significantly increased ($p<0.05$) in endotoxin-poisoned mice both with and without load. 5-Hydroxytrgptophol-and 5-hydroxyindoleacetic acid-glucuronides were also increased significantly ($p<0.05$) in endotoxin-poisoned mice with load.

At six hours kynurenine and kynurenine-O-sulfate were reduced ($p<0.01$ and $p<0.05$, respectively) in endotoxin-poisoned mice with load and xanthurenic acid and 3-hydroxykynurenine in the poisoned animals without load. Once again 5-hydroxyindoleacetic acid was increased significantly ($p<0.05$) in

TABLE 12.--Relative Percentage of Individual Kynurenine and Serotonin Pathway Metabolites in Urine of Normal and Endotoxin-Poisoned Mice Given D,L-Tryptophan (Benzene Ring-^{14}C) After Two Hours.

METABOLITE	WITH LOAD		WITHOUT LOAD	
	NORMAL[a]	ENDOTOXIN	NORMAL	ENDOTOXIN
Kynurenine	1.9 ± 1.6[b]	2.2 ± 2.2	5.1 ± 1.1**	0.1 ± 0.2
3-Hydroxykynurenine	5.9 ± 1.4	3.8 ± 1.1	6.6 ± 1.9	5.8 ± 1.7
Kynurenic Acid	13.3 ± 3.3*	4.7 ± 2.1	7.0 ± 1.6	7.8 ± 2.4
Xanthurenic Acid	13.4 ± 6.9	6.5 ± 2.2	7.9 ± 3.2	5.0 ± 2.3
Kynurenine-O-Sulfate	1.1 ± 1.1	1.2 ± 0.6	3.2 ± 2.3	2.1 ± 2.5
N-Acetylkynurenine	8.8 ± 1.9	11.6 ± 3.5	19.1 ± 2.0*	11.7 ± 3.6
Total Kynurenine Metabolites	38.3 ± 3.9**	30.4 ± 3.4	45.7 ± 4.3**	33.1 ± 7.2
5-Hydroxyindoleacetic Acid	7.3 ± 3.6	12.3 ± 1.4	5.7 ± 2.4	9.2 ± 2.6
5-HT-O-Gluucuronide	4.0 ± 0.9*	2.9 ± 0.4	5.1 ± 1.2	5.9 ± 1.5
5HTOH- & 5HIAA-O-Glucuronide	3.7 ± 1.6	3.3 ± 1.4	4.5 ± 1.5*	6.4 ± 1.8
5-Hydroxytryptophol	1.3 ± 1.1	1.6 ± 0.7	0.0 ± 0.0	0.2 ± 0.4
Total Serotonin Metabolites	13.2 ± 4.5	20.7 ± 1.5	16.1 ± 2.8*	21.1 ± 4.7
Tryptophan	36.1 ± 7.8	32.9 ± 3.3	20.3 ± 7.3	17.7 ± 4.0
Other Metabolites	12.6 ± 3.5	13.3 ± 1.7	20.2 ± 3.8	20.2 ± 10.6
Total Metabolites	100.2 ± 4.0	97.3 ± 7.9	102.3 ± 4.6	92.1 ± 6.6

[a]All values represent averages of data from between 4 and 7 mice.

[b]Average percentage of radioactivity recovered ± standard deviation

*$p<0.05$

**$p<0.01$

TABLE 13.--Relative Percentage of Indidivual Kynurenine and Serotonin Pathway Metabolites in Urine of Normal and Endotoxin-Poisoned Mice Given D,L-Tryptophan (Benzene Ring-^{14}C) After Three Hours.

METABOLITE	WITH LOAD		WITHOUT LOAD	
	NORMAL[a]	ENDOTOXIN	NORMAL	ENDOTOXIN
Kynurenine	4.3 ± 2.2[b]	3.1 ± 2.4	5.6 ± 1.6	2.3 ± 1.1
3-Hydroxykynurenine	4.4 ± 4.3	5.8 ± 1.7	8.3 ± 3.3	7.0 ± 1.9
Kynurenic Acid	10.9 ± 4.3	9.0 ± 3.6	6.9 ± 2.1	7.1 ± 5.0
Xanthurenic Acid	8.4 ± 4.9	4.8 ± 1.3	5.3 ± 1.3	6.9 ± 0.9
Kynurenine-O-Sulfate	3.2 ± 1.9	0.6 ± 0.7	2.3 ± 1.5	0.8 ± 1.0
N-Acetylkynurenine	8.8 ± 2.1	7.6 ± 3.9	14.3 ± 5.4	12.4 ± 3.2
Total Kynurenine Metabolites	40.8 ± 8.0**	29.5 ± 3.6	41.0 ± 8.5	34.0 ± 2.3
5-Hydroxyindoleacetic Acid	3.2 ± 1.4*	12.4 ± 3.3	7.6 ± 1.3*	14.7 ± 5.1
5HT-O-Glucuronide	3.6 ± 1.5	5.5 ± 1.3	5.2 ± 1.6	4.8 ± 1.6
5HTOH- & 5HIAA-O-Glucuronide	4.1 ± 2.3*	8.4 ± 1.2	3.8 ± 1.0	3.8 ± 1.0
5-Hydroxytryptophol	0.4 ± 0.6	1.2 ± 1.1	0.6 ± 1.0	1.9 ± 1.2
Total Serotonin Metabolites	10.1 ± 2.6**	24.2 ± 3.3	15.8 ± 1.4**	24.0 ± 5.4
Tryptophan	22.6 ± 9.2	29.5 ± 4.9	19.0 ± 7.6	14.1 ± 2.6
Other Metabolites	26.2 ± 6.8	14.8 ± 2.8	18.8 ± 5.2	27.6 ± 7.1
Total Metabolites	99.7 ± 6.7	98.0 ± 3.7	94.6 ± 5.7	99.7 ± 4.4

[a] All values represent averages of data from between 4 and 7 mice

[b] Average percentage of radioactivity recovered ± standard deviation

* $p<0.05$

** $p<0.01$

endotoxin-poisoned animals with load as was 5-hydroxytryptamine-O-glucuronide ($p<0.01$) (Table 14).

In mice given tryptophan load between 26 and 35% of the radioactivity was excreted as tryptophan at all time points tested. In mice not given load, approximately 17% of the label was excreted as tryptophan (Tables 11,12,13,14).

TABLE 14.--Relative Percentage of Individual Kynurenine and Serotonin Pathway Metabolites in Urine of Normal and Endotoxin-Poisoned Mice Given D,L-Tryptophan (Benzene Ring-^{14}C) After Six Hours.

METABOLITE	WITH LOAD		WITHOUT LOAD	
	NORMAL[a]	ENDOTOXIN	NORMAL	ENDOTOXIN
Kynurenine	2.5 ± 0.6*[b]	0.7 ± 0.8	3.1 ± 2.3	2.6 ± 0.3
3-Hydroxykynurenine	2.5 ± 0.7	3.1 ± 1.8	7.9 ± 1.4**	3.1 ± 0.4
Kynurenic Acid	7.5 ± 2.5	6.6 ± 3.0	8.3 ± 1.2	14.9 ± 1.5
Xanthurenic Acid	9.9 ± 4.8	6.1 ± 3.1	6.3 ± 2.8*	1.9 ± 0.7
Kynurenine-O-Sulfate	1.7 ± 0.5*	0.0 ± 0.0	0.5 ± 0.8	0.2 ± 0.2
N-Acetylkynurenine	7.3 ± 3.0	9.9 ± 3.5	9.7 ± 3.7	12.3 ± 4.5
Total Kynurenine Metabolites	31.4 ± 5.7**	24.6 ± 6.8	35.3 ± 11.3	24.2 ± 5.4
5-Hydroxyindoleacetic Acid	4.3 ± 1.7*	9.2 ± 1.8	6.0 ± 1.5*	13.4 ± 4.1
5HT-O-Glucuronide	2.8 ± 0.7**	7.3 ± 2.4	3.9 ± 0.7	3.9 ± 1.2
5HTOH- & 5HIAA-O-Glucuronide	3.5 ± 0.5*	3.1 ± 0.5	4.4 ± 1.0	3.2 ± 0.7
5-Hydroxytryptophol	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.1 ± 0.1
Total Serotonin Metabolites	9.5 ± 1.3**	19.3 ± 0.6	14.0 ± 0.7**	20.7 ± 3.2
Tryptophan	36.6 ± 11.6	33.2 ± 12.2	27.7 ± 13.3	34.1 ± 13.4
Other Metabolites	18.6 ± 8.7	18.4 ± 7.1	15.4 ± 7.1	26.1 ± 8.4
Total Metabolites	96.1 ± 6.8	95.5 ± 6.7	92.4 ± 8.1	105.1 ± 7.6

[a] All values represent averages of data from between 4 and 7 mice

[b] Average percentage of radioactivity recovered ± standard deviation

* $p<0.05$

** $p<0.01$

DISCUSSION

In determining which tryptophan metabolites were present in urine of normal and endotoxin-poisoned mice, it was necessary to consider not only the 50 or more known metabolites of tryptophan but also the numerous tryptophan metabolites which have been recognized but not identified (119,152). This problem is further complicated by the fact that there are over 200 "common" acids in urine. Of these only one (5-hydroxyindoleacetic acid) or sometimes two (5-hydroxyindoleacetic acid and indoleacetic acid) of the 18 known acid metabolites of tryptophan appear in quantities large enough to be included in the "common" urinary acids (16,17,34). Hence, tryptophan labeled with ^{14}C in the benzene ring provided an invaluable tool for the accomplishment of our primary objective. Due to the position of the label, it was retained in the metabolites of the serotonin and tryptamine pathways and all but the terminal reactions of the kynurenine pathway. In addition, 5-hydroxytryptamine-3'-^{14}C provided a useful tool, particularly in identifying the serotonin pathway metabolites. The various isotopes allowed us to identify and quantify specific metabolites even though they were present in only nanogram amounts.

A major emphasis in this project has been placed on devising techniques which would establish distinctive characteristics for each individual metabolite or group of metabolites in the kynurenine and serotonin pathways and thereby aid in identification. Thin layer chromatography proved a simple, rapid and reliable method for the separation of tryptophan metabolites. Although Rf values of compounds obtained from a given biological sample may vary slightly from individual standards or other biological samples, their relative position usually remains constant as does their fluorescence and color reactions. Throughout the experiments great care had to be exercised in handling the radioactive tracers and urine samples since many tryptophan metabolites are readily degraded.

Elution patterns from DEAE-Cellulose columns are characteristic (23). Being able to separate groups of tryptophan metabolites based on ionic charge, provided a means of categorizing the metabolites into subgroups which could then be more easily identified on thin layer chromatography.

Gas chromatography combined with mass spectrometry also proved useful in confirming the identity of certain unknown tryptophan and serotonin metabolites. Several papers have been published on the derivatization of the serotonin metabolites for gas chromatography-mass spectrometry (19,34,

75,96,110). Such information is less available on the kynurenine metabolites (4,111). Multiple peaks on gas chromatography have been described for kynurenine metabolites derivatized with trimethylsilyl groups. The serotonin pathway metabolites readily formed TMSi derivatives as did a few of the kynurenine metabolites. These conjugates hydrolyze within a short time and therefore must be analyzed by gas chromatography or gas chromatography-mass spectrometry within 24 hours. Spectra of several TMSi derivatives of authenic compounds were obtained (Figures 6,7,13,14). Preliminary studies showed considerable discrepancies between the tentative identification of unknown compounds and reference mass spectra. Further investigation showed that this discrepancy was not due to the method but rather to the concentration of the unknown metabolites available for study. When urine from normal mice given 5-hydroxytryptamine-3'^{14}C was spiked with 5-hydroxytryptophol and 5-hydroxyindoleacetic acid, the spectra resembled the reference standards (Figure 14). The unlabeled material co-migrated with the label on thin layer plates.

The ME-TMSi derivatives are more stable than the TMSi derivatives. The most common method for the preparation of methyl esters (diazomethane) is not applicable to many of the tryptophan metabolites because of the insolubility of these compounds in methanol and ether; hence, 2,2-dimethoxypropane was used for methylation. Following preparation of the ME-TMSi derivatives, multiple gas chromatography peaks were

obtained and mass spectral analysis demonstrated that the majority of the authenic as well as unknown tryptophan metabolites formed similar ions with a major peak at 131 (Figure 5 and Appendix B, Figure B1). Although the structure of this compound has not been elucidated, the mass matches that of a quinone type structure commonly produced in the oxidation of these indoles (30,95). The kynurenine metabolites which formed the 131 peak in the gas chromatograph had structures which could form the quinone-like structure upon rearrangement.

Several conclusions drawn from the spectra of unknowns were particularly valuable in identification. For example, the compounds tentatively identified as conjugates gave different major ion peaks at 79, 128, and 129 (Appendix B, Figure B5) than their parent compounds (Appendix B, Figure B1). Further, the spectra showed that the major metabolites of urine from mice given labeled tryptophan were the unconjugated metabolites from either the initial reactions of the kynurenine pathway (kynurenine, 3-hydroxykynurenine, kynurenic acid, xanthurenic acid, and 3-hydroxyanthranilic acid) or the primary serotonin pathway (5-hydroxytryptamine, 5-hydroxytryptophol, and 5-hydroxyindoleacetic acid). The interpretation of spectra coincided with the results from Rf values, fluorescence, color reactions, and DEAE-cellulose chromatography elution patterns. The metabolites of the kynurenine pathway (nicotinamide, nicotinic acid,

N-methylnicotinamide, o-aminophenol, and anthranilic acid), which formed only TMSi esters and not methyl esters, were not major metabolites. Although anthranilic acid, N-methylnicotinamide, and o-aminophenol were observed in mouse urine, their concentrations were well below those where gas chromatography-mass spectrometry would be feasible.

Eleven kynurenine metabolites were isolated from urine of normal and endotoxin-poisoned mice given 0.5 μCi of D,L-tryptophan (benzene ring-^{14}C) with and without tryptophan load. Kynurenine, 3-hydroxykynurenine, kynurenic acid, xanthurenic acid, kynurenine-O-sulfate, and N-acetylkynurenine were the major kynurenine pathway metabolites. Anthranilic acid, 3-hydroxyanthranilic acid, N-methylnicotinamide, o-aminophenol, and quinolinic acid also were tentatively identified but they appeared infrequently.

Six serotonin metabolites were isolated from urine of mice given the labeled tryptophan. They were tentatively identified as 5-hydroxyindoleacetic acid, 5-hydroxytryptophol, 5-hydroxytryptamine-O-glucuronide, and 5-hydroxyindoleacetic acid-O-sulfate. When mice were given 5-hydroxytryptamine-3'-^{14}C, four additional metabolites were observed. These were 5-hydroxytryptamine, 5-hydroxytryptophol-O-sulfate, 5-hydroxytryptamine-O-sulfate, and an unidentified metabolite.

When endotoxin-poisoned mice were given benzene ring-labeled tryptophan without load, they excreted significantly

less tryptophan and its metabolites than normal mice throughout the six hour time period (Table 7). Tryptophan load did not significantly alter the quantity of label appearing in urine, except after one hour in normal mice despite the fact that 20,000 times as much tryptophan was administered (Table 8). Interestingly, the amount of label per unit volume was similar in both normal and endotoxin-poisoned mice with and without load except again at 1 hour in normal mice (Tables 7 and 8). These data suggest a passive rather than active mechanism for the filtration of tryptophan metabolites. The decrease observed in urine of endotoxin-poisoned mice is probably due to the decreased blood flow to the kidneys due to vascular shock (70,107).

The decreased excretion of radioactivity in endotoxin-poisoned mice was accompanied by significant decreases in the amount of kynurenine metabolites and increases in serotonin metabolites excreted (Table 9 and 10). These results suggest a shift in metabolism from the kynurenine to the serotonin pathway in endotoxin-poisoned mice. These results may be due to the well-established depression of tryptophan oxygenase in endotoxin-poisoned mice (1,11,104) or to the pooling of blood in the intestine, a major site of serotonin synthesis (70,119). Further studies, perhaps using isolated perfused organ systems rather than whole animals, should aid considerably in determining whether metabolic or physiologic alterations are ultimately

responsible for the observed *in vivo* alterations in tryptophan metabolism in poisoned mice.

Analysis of individual metabolites showed that there were significant decreases in all of the kynurenine metabolites evaluated (kynurenine, 3-hydroxykynurenine, kynurenic acid, xanthurenic acid, and kynurenine-O-sulfate) except N-acetylkynurenine. These data do not suggest the location of any specific enzymatic lesions along the kynurenine pathway. The increases in N-acetylkynurenine in endotoxin-poisoned mice cannot be explained at present. Although tryptophan oxygenase levels are depressed in endotoxin-poisoned mice, the enzyme is not completely inhibited. It is possible, though only suggested until enzyme levels are measured, that there may be a depression of an enzyme, possible kynurenine hydroxylase, so that metabolite flow beyond that point is sharply curtailed. Such an occurrence could account for the increase in the production of N-acetylkynurenine. Such a hypothesis would also anticipate an increase in both anthranilic acid and kynurenic acid. The former was not concentrated enough to monitor reliably, and the latter was not increased.

Endotoxin-poisoned mice consistently excreted significantly more 5-hydroxyindoleacetic acid than normal mice. At two, three, and six hours, the glucuronide conjugates of the serotonin metabolites were also significantly increased in endotoxin-poisoned animals. Glucuronides are known to be

detoxification products in mammals (2,58,98,121). The data show that there was a significant shift in the flow of tryptophan from the kynurenine to the serotonin pathway. 5-Hydroxyindoleacetic acid, which has been used as an indicator for metabolic changes in several diseases (41,98,119), seemed to be a valid marker for endotoxemia as well.

5-Hydroxytryptophol appeared as only a minor metabolite in our studies. This data is in direct conflict with a report by Nakai (108) suggesting that 5-hydroxytryptophol and not 5-hydroxyindoleacetic acid was the major serotonin metabolite of herbivores. While 5-hydroxyindoleacetic acid was the major metabolite isolated, the glucuronide of 5-hydroxytryptophol was also present in significant amounts. This may have been the metabolite observed by Nakai (108). Pentikainen (121) also reported that although 5-hydroxytryptophol is a metabolite of serotonin, it is not excreted in appreciable quantities in urine of rats.

Although hyper-reactivity to endotoxin occurrs only with a tryptophan load, no differences were noted in the quantity of radioactivity excreted in individual metabolites or pathways among normal or endotoxin-poisoned mice with and without load. It must be remembered however, that there was 20,000 times more unlabeled tryptophan given to mice with load so that the per cent of radioactivity represents a much higher concentration of metabolites both excreted and remaining *in vivo*.

The high excretion rate of unmetabolized tryptophan (Tables 11-14) was not expected. In animals given tryptophan load, some tryptophan may be excreted but greater than 30% seems excessive. In animals given the isotope alone, 17% of the label was excreted as tryptophan after 6 hours. It is possible that the D-isomer of the label was being selectively excreted. Although mice have the enzymes to convert D-tryptophan to L-tryptophan (64), there may have been no need to do so, especially in experiments with load. This problem should not alter the interpretation of our data on pathway flow since evaluation of the relative distribution of label among pathways only took into account the amount of metabolite which entered the pathway. If the D-isomer is being selectively excreted however, such an occurrence could lead to slight misinterpretations on the significance of data describing the distribution of labeled tryptophan among the organs and tissues of normal and endotoxin-poisoned mice (Moon et al., unpublished data). Clarification of this issue must await further investigation.

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LIST OF REFERENCES

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APPENDICES

APPENDIX A

TABLE A1.--Rf Values and Color Reactions of "Simple" Indole Derivatives (145).

Compound	Rf*	Color with van Urk's Reagent	Color with Prochazka's Reagent	Fluorescence in Prochazka Reagent
Indole	0.84	dark red to violet	pale green	green
Indole-3-aldehyde	0.86	pink	orange with 2,4-dinitrophenyl hydrazine reagent	
Indole-3-acetaldehyde	0.86	reddish brown	yellow with 2,4-dinitrophenyl hydrazine reagent	
Indole-3-acetic acid	0.31	blue, tinge of violet	yellow	yellow with green border
5HIAA	0.19	blue to violet	pale yellow to beige	deep violet
Tryptamine	0.77	blue-green	yellow	yellow with blue border
Serotonin	0.65	grey	yellow	brown
D,L-Tryptophan	0.23	blue-green	yellow	yellow with blue border
D,L-5HTP	0.14	blue-grey	pale yellow to beige	yellow
Anthranilic Acid	0.33	becomes intensive yellow	pale beige	blue, turning brown

*Rf Values in methyl acetate, isopropyl alcohol, and ammonium hydroxide, 45:35:20.

TABLE A2.--Rf Values and Detection of Tryptophan Metabolites Utilizing 4-Dimethylaminobenzaldehyde Reagent (119).

Substance	Rf*	Detection with p-Dimethylaminobenzaldehyde Reagent	
		Fluorescence	Color
Tryptophan	0.25	-	Violet
Indole	0.90	Blue	Violet
Indican	0.61	Brown	Brown
D,L-Kynurenine	0.32	Green-blue	Yellow-brown
3-Hydroxy-Kynurenine	0.16	Yellow green	Orange
Kynurenic Acid	0.45	Green after 12 hours	-
Xanthurenic Acid	0.45	Grey	-
Anthranilic Acid	0.45	Light blue	Yellow
3-Hydroxy-Anthranilic Acid	0.31	Light blue	Yellow

*Rf Values in methyl acetate, isopropyl alcohol, and ammonium hydroxide, 45:35:20.

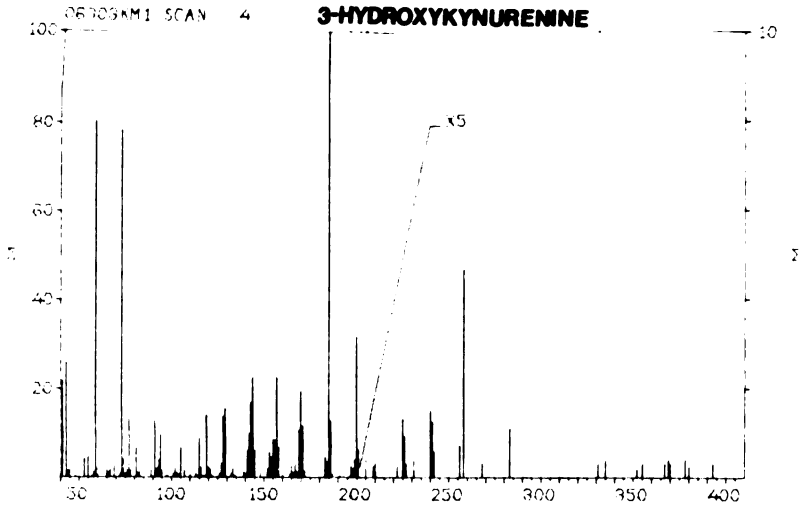
TABLE A3.--Rf Values of Serotonin Metabolites and Their Variations (121).

Compound	Rf[a]
5-Hydroxytryptamine Glucuronide	0.13 (0.11 - 0.16)
5-Hydroxyindoleaceturic Acid Sulfate	0.15 (0.13 - 0.17)
5-Hydroxyindoleacetic Acid Glucuronide	0.28 (0.23 - 0.32)
5-Hydroxytryptophol Glucuronide	0.28 (0.23 - 0.33)
5-Hydroxyindoleacetic Acid Sulfate	0.35 (0.31 - 0.41)
5-Hydroxytryptamine	0.37 (0.35 - 0.44)
5-Hydroxyindoleacetic Acid	0.75 (0.66 - 0.81)
5-Hydroxytryptophol	0.78 (0.66 - 0.88)
5-Hydroxytryptophol Sulfate	0.27 (0.24 - 0.32)

[a]Developed in butanol, acetic acid, and water, 12:3:5.

APPENDIX B

Figure B1.--Mass Spectra Obtained from the ME-TMSi Derivatives of 3-Hydroxykynurenine, Kynurenic Acid, Xanthurenic Acid, and 5-Hydroxytryptophol, Demonstrating the Similarities in Spectra Obtained.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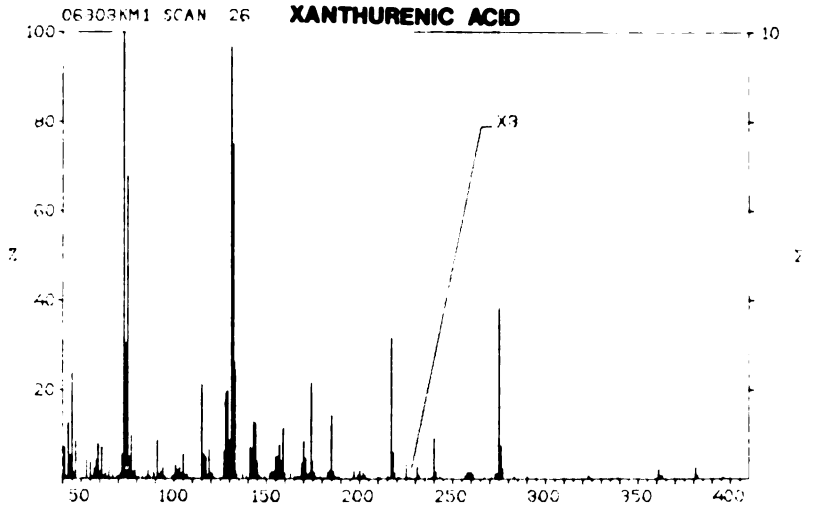
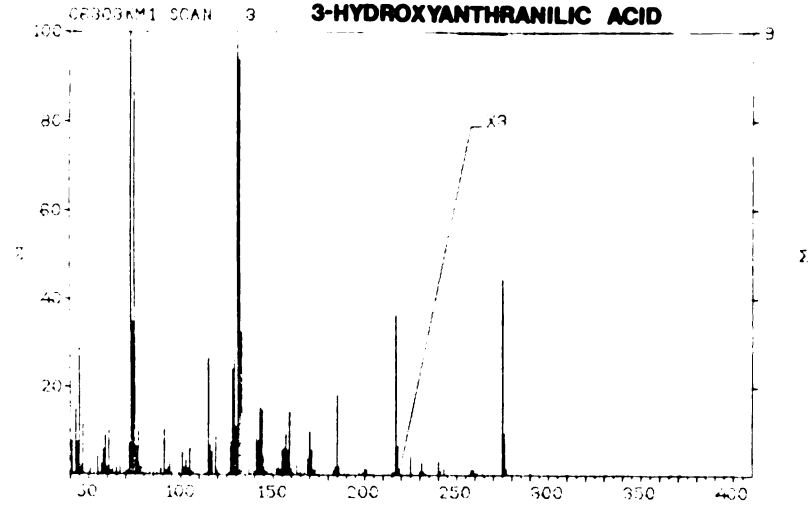
X5


XANTHURENIC ACID
X3


3-HYDROXYANTHRANILIC ACID
X3

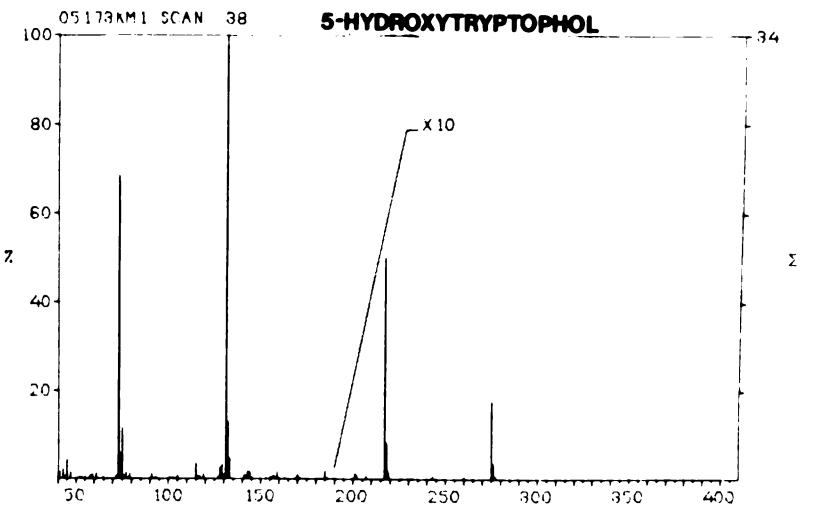
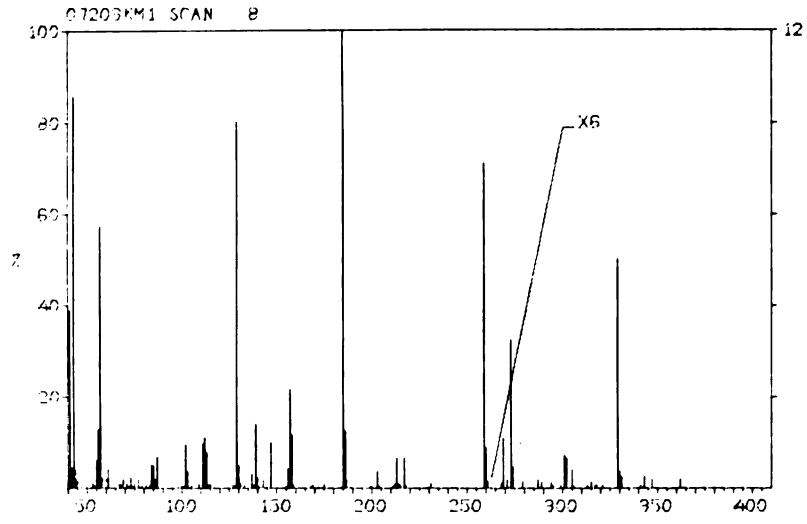
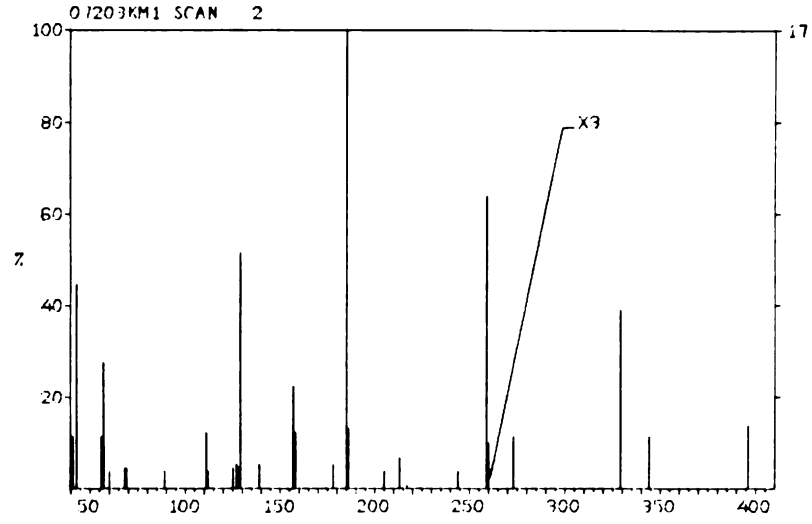
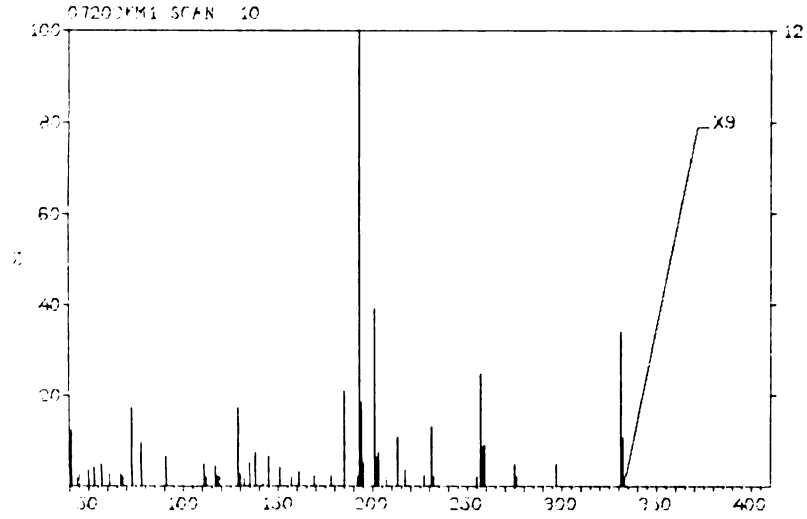

5-HYDROXYTRYPTOPHOL
X10

Figure B2.--Major Patterns Obtained from Mass Spectra of Unknown Tryptophan and Serotonin Metabolites from Urine of Mice Given D,L-Tryptophan (Benzene Ring-^{14}C) or 5-Hydroxytryptamine-3'^{14}C Prepared as the TMSi Derivatives.


0720BKM1 SCAN 8
X6
%
100
80
60
40
20
50
100
150
200
250
300
350
400
12


0720BKM1 SCAN 2
X3
%
100
80
60
40
20
50
100
150
200
250
300
350
400
17


0720BKM1 SCAN 10
X9
%
100
80
60
40
20
50
100
150
200
250
300
350
400
12

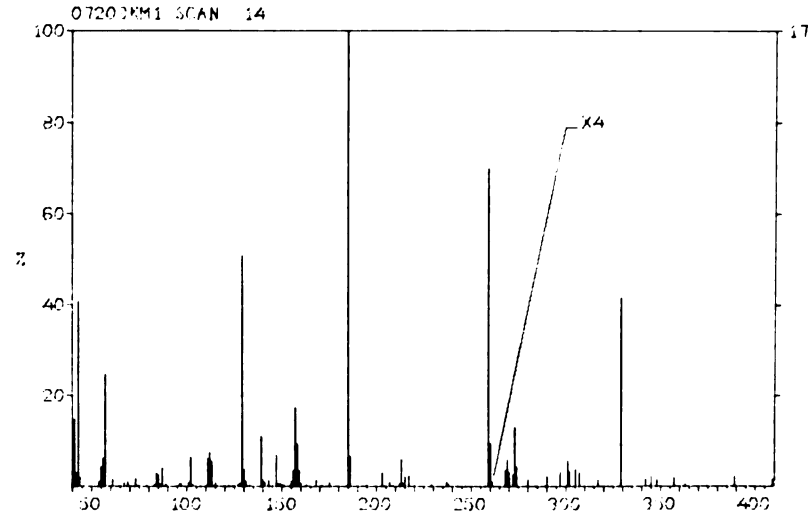
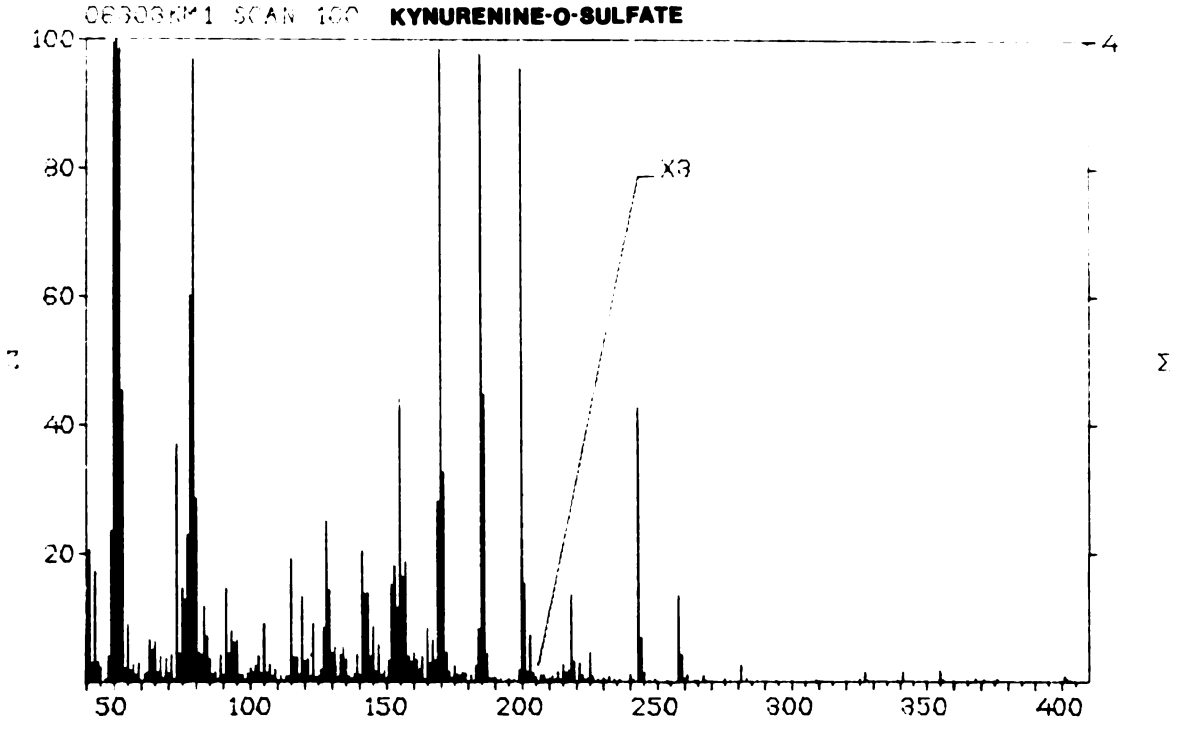

0720BKM1 SCAN 14
X4
%
100
80
60
40
20
50
100
150
200
250
300
350
400
17

Figure B3.--Mass Spectra Obtained from ME-TMSi Derivatives of Kynurenine-O-Sulfate and N-Acetylkynurenine.


KYNURENINE-O-SULFATE
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80
60
40
20
X3
50
100
150
200
250
300
350
400
Σ

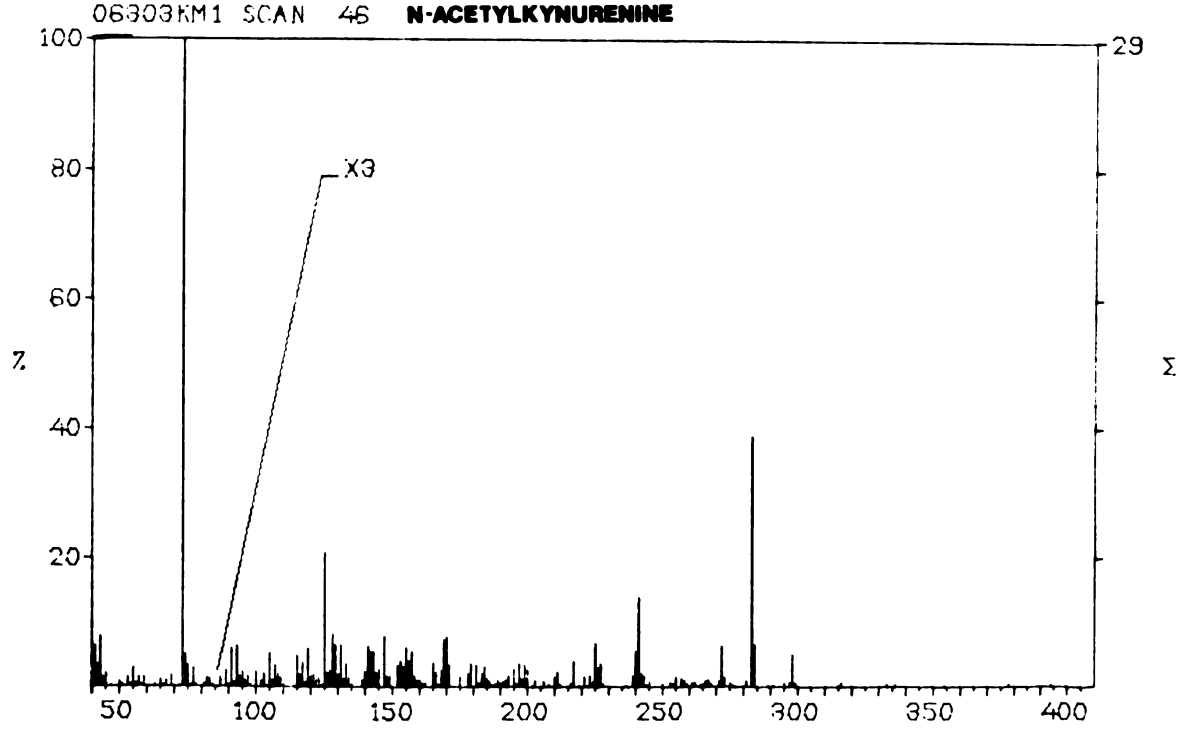
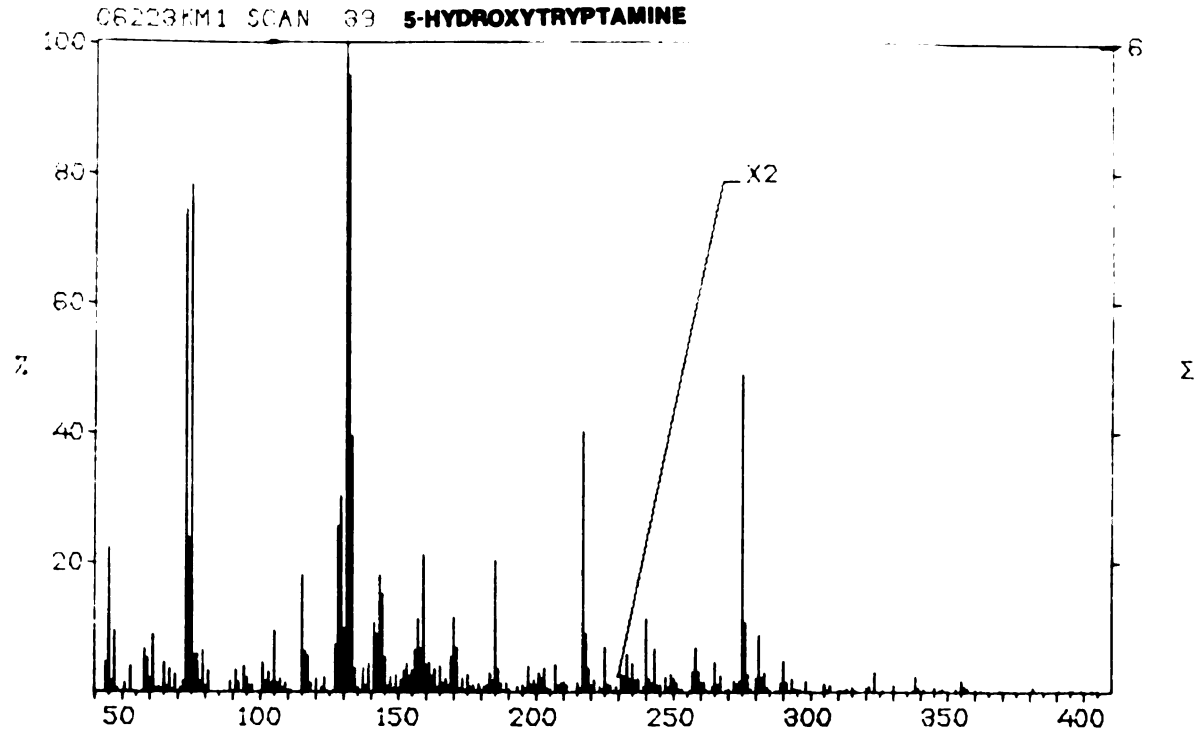

06303KM1 SCAN 46
N-ACETYLKYNURENINE
100
80
60
40
20
X3
50
100
150
200
250
300
350
400
Σ

Figure B4.--Mass Spectra Obtained from ME-TMSi Derivatives of 5-Hydroxytryptamine and 5-Hydroxyindoleacetic Acid.


06223KM1 SCAN 39 5-HYDROXYTRYPTAMINE
100
80
60
40
20
%
X2
6
Σ
50
100
150
200
250
300
350
400

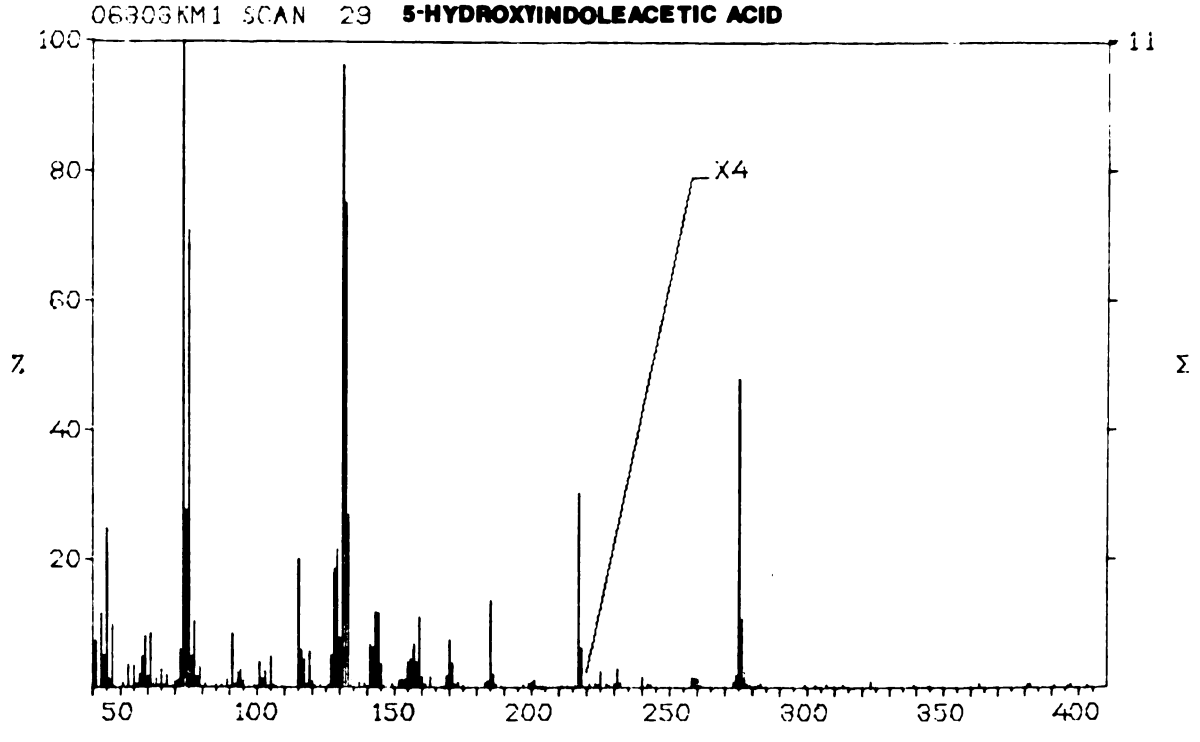
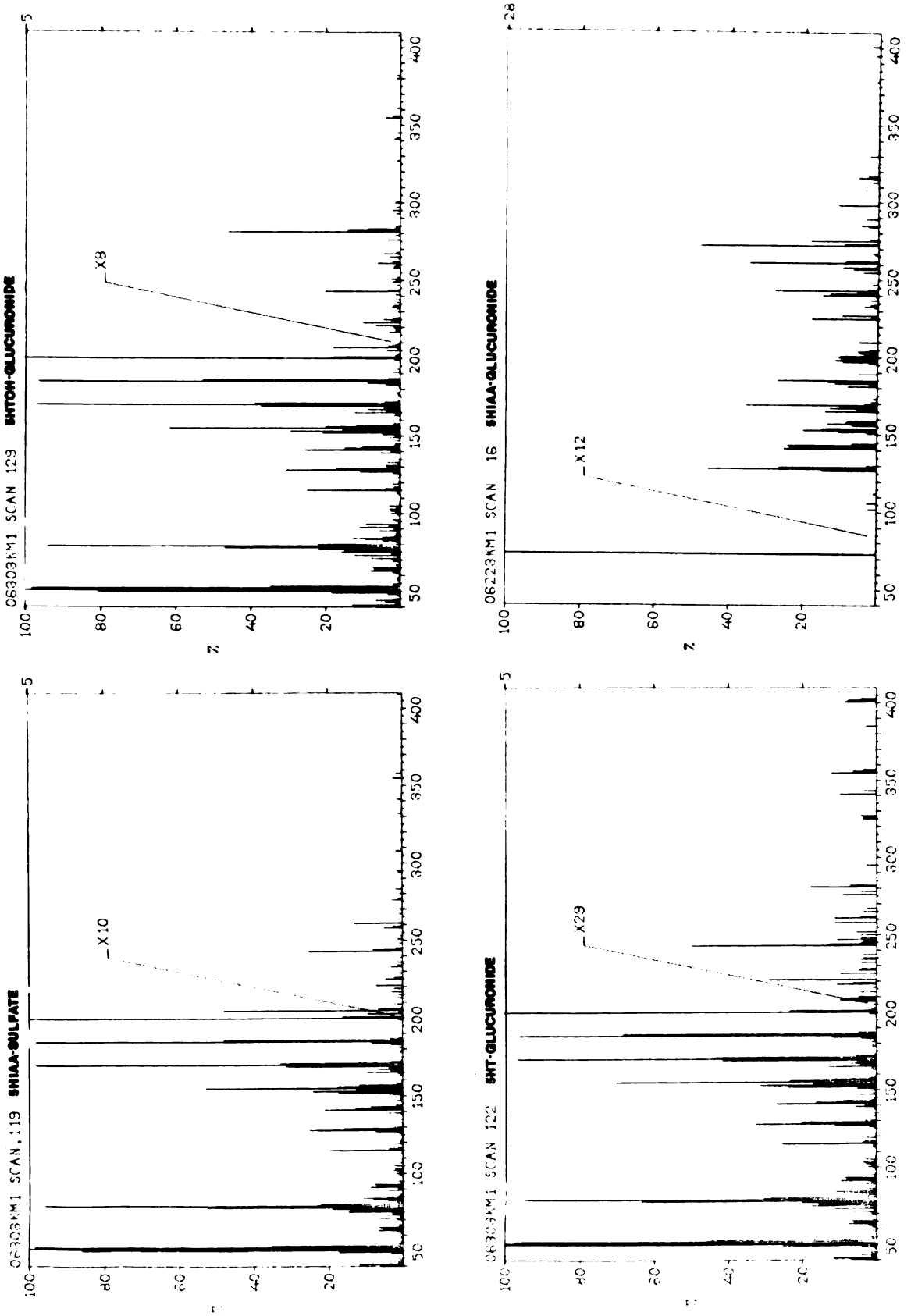

06303KM1 SCAN 29 5-HYDROXYINDOLEACETIC ACID
100
80
60
40
20
%
X4
11
Σ
50
100
150
200
250
300
350
400

Figure B5.--Mass Spectra Obtained from ME-TMSi Derivatives of 5-Hydroxyindoleacetic Acid-O-Glucuronide, 5-Hydroxytryptophol-O-Glucuronide, 5-Hydroxytryptamine-O-Glucuronide, and 5-Hydroxyindoleacetic Acid-O-Sulfate.


06303KM1 SCAN 129 5HTOH-GLUCURONIDE
X9
06303KM1 SCAN 119 5HIAA-SULFATE
X10
06223KM1 SCAN 16 5HIAA-GLUCURONIDE
X12
06303KM1 SCAN 122 5HT-GLUCURONIDE
X29
m/z