TRYPTOPHAN METABOLITES FOUND IN URINE OF NORMAL AND ENDOTOXIN-POISONED MICE

Thesis for the Degree of M. S.
MICHIGAN STATE UNIVERSITY
KATHERINE MARIE MORRIS
1970

ABSTRACT

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NORMAL AND ENDOTOXIN-POISONED MICE

By

Katherine Marie Morris

A study was undertaken to determine the urinary tryptophan metabolites present in normal and endotoxin-poisoned mice given tryptophan. To accomplish this, normal and endotoxin-poisoned mice were injected with either 5.0×10^{-2} uc of L-tryptophan-1-^{14}C with 20 mg of carrier L-tryptophan or 2.0×10^{-2} uc of D,L-tryptophan (benzene ring-^{14}C) with or without 20 mg of carrier L-tryptophan. In each experiment 92 to 120 mice were injected with 1 cc of the tryptophan solution. At 15 minute intervals for 180 minutes after time of injection, groups of 5 or 10 mice were killed by cervical dislocation and urine was collected. The urinary tryptophan metabolites were separated by thin layer chromatography and autoradiographs of the plates were made. Rf values, fluorescence, and color reactions using Prochazka' or van Urk's reagents were used to tentatively identify the compounds.

Katherine M. Morris

Normal mice given only trace amounts of radioactive tryptophan without carrier, excreted tryptamine, tryptophan, 5-hydroxytryptophan, and two unidentified tryptophan metabolites. When normal mice were given 20 mg of tryptophan with the radioactive tryptophan, they excreted the above five metabolites plus 5-hydroxyindole acetic acid, a small amount of serotonin, and three other unidentified metabolites. Endotoxin-poisoned mice excreted a total of eight radioactive tryptophan metabolites, six of which were the same as in normal mice. These were tryptamine, tryptophan, 5-hydroxyindole acetic acid, 5-hydroxytryptophan, and two unidentified metabolites. Two other distinct radioactive compounds were found in the endotoxin-poisoned mice but were not identified. Endotoxin-poisoned mice showed at least a 45 minute lag before excretion of the metabolites common to normal animals were observed. Fewer metabolites were excreted and these occurred for a shorter period of time than the same compounds did in normal mice.

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By

Katherine Marie Morris

A THESIS

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

MASTER OF SCIENCE

Department of Microbiology
and Public Health

1970

DEDICATION

To my parents and to my major professor.

ACKNOWLEDGMENTS

The author wishes to sincerely thank Dr. Robert J. Moon for his willing guidance, interest, and patience throughout this investigation.

I am also grateful to the staff of the x-ray laboratory at Olin Health Center, Michigan State University, for their assistance and for allowing me to use their facilities.

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INTRODUCTION

It has been observed that endotoxin-poisoned mice die within 8 hours after a delayed but not concurrent injection of L-tryptophan. Death is frequently convulsive in nature and occurs 24 to 36 hours sooner than would be expected among mice given endotoxin alone. While the immediate reasons for the increased sensitivity of endotoxin-poisoned mice to tryptophan are not clear, excess production of toxic metabolites of the amino acid, including serotonin, are thought to play a role. In an effort to determine the validity of this hypothesis, a study of the metabolic fate of tryptophan in normal and endotoxin-poisoned mice has been initiated in our laboratory.

The aim of this project was to determine the distribution of tryptophan metabolites appearing in urine at various time intervals following injection of the amino acid into either normal or endotoxin-poisoned mice. To accomplish this, either D,L-tryptophan (benzene ring-^{14}C (u)) or L-tryptophan-1-^{14}C was injected subcutaneously into either normal mice or mice given endotoxin 10 hours previously. Every 15 minutes for a period of 3 hours, groups of 5 or 10 mice were killed by cervical dislocation and urine was collected. Radioactive tryptophan

metabolites were separated on thin layer chromatographic plates and autoradiographs of the plates were prepared. h-Rf values, fluorescent patterns, and specific color reactions of the radioactive tryptophan metabolites were noted and recorded.

LITERATURE REVIEW

Metabolic breakdown of tryptophan can proceed through several pathways in mammalian systems, yielding a wide variety of end products. The major pathways lead either to the synthesis of nicotinamide adenine dinucleotide (NAD), hereafter referred to as the kynurenine pathway, or to the synthesis of 5-hydroxytryptamine (serotonin), hereafter called the serotonin pathway. These and other pathways of tryptophan catabolism are outlined in Appendix A, Figures A1 and A2.

The Kynurenine Pathway

The kynurenine pathway begins with oxygenation of the indole nucleus of tryptophan to form formylkynurenine. Tryptophan oxygenase is the adaptive liver enzyme which catalyzes this reaction and is commonly thought to be the regulator of the pathway.

Formylkynurenine is subsequently converted to kynurenine by the enzyme kynureninase. Kynurenine may be converted to kynurenic acid or xanthurenic acid or may be further metabolized to NAD or, to either quinolinic acid or carbon dioxide and water.

Hematin is a cofactor for tryptophan oxygenase (12). The activity of this enzyme can be increased by

its substrate tryptophan (41), cortisone (7, 22), and a variety of substrate analogues (41). The activity of the enzyme can be decreased by numerous inhibitors, including endotoxin (27, 41).

The Serotonin Pathway

In the serotonin pathway tryptophan is hydroxylated by tryptophan-5-hydroxylase to form 5-hydroxytryptophan (5HTP). This reaction is the rate limiting step in the biosynthesis of serotonin (25, 45). While the enzyme is found in liver (50), intestinal mucosa cells, and kidney (14), its greatest concentration is in the pineal tissue (32, 35, 52). Serotonin is formed from 5-hydroxytryptophan by the substrate specific enzyme 5-hydroxytryptophan decarboxylase (20). This enzyme is primarily found in mammalian kidney with lesser amounts present in the liver (26) and pineal glad (8, 25, 37). Nerve tissue, sympathetic ganglia, and adrenal medulla also contain substantial 5-hydroxytryptophan decarboxylase activity (20).

The distribution of serotonin among mammalian tissues varies considerably from its sites of synthesis. While the small intestinal mucosa and pineal gland (32) contain the greatest amounts of serotonin in normal animals, the biogenic amine can also be found in lung (51), spleen, and blood platelets (44). Mouse mast tumor cells

also have significant quantities of this active neurohormone (18). Only small amounts of serotonin are found in urine (21).

Tryptophan Metabolites Found in Urine

Less information is available on the urinary metabolites of the kynurenine pathway than the serotonin pathway. In one study, Benassi et al. (3) identified kynurenine, 3-hydroxykynurenine, N-α-acetylkynurenine, N-α-acetyl-3-hydroxykynurenine, kynurenic acid, xanthurenic acid, xanthurenic acid-8-methylether, anthranilic acid, 3-hydroxyanthranilic acid, and 8-methyl-oxyanthranilic acid from normal human urine. Kynurenine is excreted in trace amounts and after tryptophan loading 3-hydroxykynurenine and 3-hydroxyanthranilic acid have been identified in human urine (43). The glucosiduronate, O-sulfate, and N-α-acetyl derivatives of 3-hydroxykynurenine have also been observed in human urine.

Serotonin may be degraded or conjugated to a wide variety of compounds, many of which can be found in urine. The major catabolic pathway for serotonin is through oxidative deamination by monoamine oxidase to form 5-hydroxyindoleacetaldhyde (13, 39). In humans and many other animals this compound is further oxidized by aldehyde dehydrogenase to 5-hydroxyindole acetic acid (5HIAA), the major excretory product of serotonin.

Herbivores, such as mice, guinea pigs, rabbits, and horses, excrete small amounts (less than 0.3 ug/ml) of 5HIAA in urine. Evidence has suggested that serotonin is broken down by monoamine oxidase to the aldehyde and from there enters pigment formation in herbivores. In dogs, rats, and humans, which are carnivores, 5HIAA concentration in urine ranges from 1.5 to 4.0 ug/ml (42).

Kveder, Iskrie, and Keglevic (33) identified 5-hydroxytryptophol in urine. They also demonstrated that N-acetylserotonin and 5-hydroxytryptophol may be degraded to 5HIAA. When serotonin is injected into humans, 5-hydroxytryptophol and its conjugates account for two per cent of the serotonin injected.

Formation of conjugates through the 5-hydroxy-group is yet another route of metabolism for serotonin and its products. Liver homogenates form serotonin-O-sulfate and this compound has been isolated in urine, especially when monoamine oxidase is inhibited (11). Chadwick also found the O-sulfate derivative of 5HIAA in urine. The O-glucuronide derivatives are very common, with serotonin-O-glucuronide being a major urinary metabolite in humans (1, 25, 39, 53). 5-Hydroxyindole acetic acid forms O-glucuronide conjugates, and in addition may also conjugate with glycine to form 5-hydroxyindoleaceturic acid (38, 39).

Other products of serotonin found in urine are the N-acetyl and N-methyl derivatives. Although N-methylation is uncommon in mammals, Bumpus and Page (10) identified trace amounts of N-methylserotonin in the urine of humans by using chemical separation and then either paper chromatography or bioassay. N-acetylserotonin has also been identified in urine (30, 39). Generally, this compound is O-methylated in the pineal gland to form the pigment melatonin (31) but it may also be catabolized to 5-hydroxyindole acetic acid (33).

Many of the 5-hydroxyindoles isolated in urine were first found in urine of carcinoid patients. A carcinoid is an argentaffin cell tumor with the primary lesion usually appearing in the ileum; lesions may also be found in the stomach and in the pancreas (43). Although it has been shown that the metabolic pathways do not vary between carcinoid and normal tissues, approximately 60 per cent of orally injested tryptophan proceeds to 5-hydroxyindoles in carcinoid patients as compared to approximately one per cent in normal individuals (43). The tumors have been characterized as producing excess amounts of serotonin with the subsequent excretion of increased quantities of 5HIAA in the urine (18, 38). Feldstein (19) reported results contradictory to this thesis.

Effects of Endotoxin on Metabolism

Injection of endotoxin into an experimental animal can elicit dramatic alterations in the normal metabolic or enzymatic homeostasis. For example, 8 hours after injection of 1 LD_{50} of endotoxin, mice are extremely hypoglycemic and liver glycogen is depleted (4). Such mice are unable to convert glucose into liver glycogen but can convert glucose into muscle glycogen. Gluconeogeneogenesis is also inhibited in endotoxin-poisoned mice (5).

Endotoxin can also cause changes in enzymatic homeostasis. Four hours after endotoxin administration, tryptophan oxygenase activity is significantly depressed. Concurrent injection of cortisone and endotoxin maintains the level of tryptophan oxygenase and protects mice from the lethal effects of endotoxin (6, 7). Moon and Berry (41) found that endotoxin-poisoned mice have increased sensitivity to an injection of 20 mg of tryptophan as demonstrated by increased deaths, magnification of hypothermia, and accelerated depletion of blood glucose. All abnormal biological responses were antagonized by the antiserotonin drug, cyproheptadine (41). This effect seemed unique to tryptophan and it was suggested that the increased sensitivity of endotoxin-poisoned mice to tryptophan may be related to a decreased ability to metabolize tryptophan through tryptophan oxygenase and consequent

channeling of the amino acid into alternate pathways including the one to serotonin.

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 (24) demonstrated that a subcutaneous injection of either 0.8 or 1.6 milligram 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. (16) also demonstrated that the precursor of serotonin, 5-hydroxy-tryptophan, and 1-benzyl-2,5-dimethylserotonin (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. This hormone has been shown to be protective against endotoxin (16). In the same group of experiments Des Prez also found that when the monoamine oxidase inhibitor, beta-phenyl isopropyl hydrazine (PIH) was injected prior to endotoxin, it made mice more susceptible to endotoxin. Lasker (34) found similar results. Contrary to this report Ausman et al. (2) and Davis et al. (15) found that monoamine oxidase inhibitors afforded protection to endotoxin. Davis utilized PIH and

1-(2-(benzylcarbamyl)ethyl)-2-isonicotinoyl-hydrazine (Nialamide), which inhibits both monoamine oxidases and diamine oxidases, and N,N-dimethyl-2-phenylcyclo-propyl-amine 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 (40 mg/kg) as compared to 10 mg/kg used by Davis. Davis also found varying results with dose variation.

Summary

It can be seen from the above literature review that very little work has been done on the tryptophan metabolites found in urine of mice but much more has been done with humans. Tryptophan metabolism may be altered by the administration of certain compounds, which inhibit an enzyme in one of the pathways, by endotoxin, which reduces the activity of tryptophan oxygenase, or by syndromes such as carcinoid tumors. All of the above cases may cause alterations in the urinary excretion of the tryptophan metabolites.

MATERIALS AND METHODS

Thin Layer Chromatography

Ascending thin layer chromatography was done in a developing chamber (inner measurements of 26.3 cm x 7.0 cm x 26.0 cm) lined on three sides with paper toweling to insure chamber saturation by the solvent. The solvent system was composed of methyl acetate, isopropyl alcohol, and ammonium hydroxide. Methyl acetate, was prepared by refluxing molar quantities of methanol (303 ml) and acetic acid (598 ml) and six ml of concentrated sulfuric acid for 30 minutes. The addition of an 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, allowing the second addition to remain in the solution overnight. After filtration to remove the silicic acid, a second distillation at 57.1 C yielded methyl acetate. Isopropyl alcohol and ammonium hydroxide were purchased commercially. One hundred milliliter of methyl acetate, isopropyl alcohol, and ammonium hydroxide in the proportions of 45:35:20 was prepared fresh daily, added to the tank, and allowed to equilibrate for three hours.

Precoated 20 cm x 20 cm Silica Gel F-254 glass plates, purchased from E. Merck Ag., Brinkman Instruments, Inc., Westbury, New York, were used in all experiments. Preliminary results showed that heat activation of the plates was not necessary. Plates were marked lightly with pencil 3 cm and 13 cm from the bottom. Samples were spotted with micropipettes on the 3 cm line and not less than 2 cm apart or from the edge. A hot air drier was employed between drop application to facilitate rapid drying and to insure a small sample spot. At the 13 cm mark a thin line of silica gel was scraped away to prevent further solvent flow and thus allow a uniform 10 cm run. Developing time usually required 1 to 1-1/2 hours. After the plates were removed from the chamber, they were allowed to dry thoroughly to remove traces of solvents which would interfere with fluorescence of the compounds. Fluorescent compounds were circled lightly with a pencil while viewed under ultraviolet light in a Chromato-Vue Cabinet Model CC-20 (Arthur H. Thomas, Philadelphia, Pennsylvania). hRf-Values were calculated as the distance from the origin to the center of the spot, divided by the distance the solvent traveled (10 cm in this case). Rf-values were expressed as an hRf-value, which was equal to the Rf multiplied by 100. Standard deviations of the hRf-values, as described by Steel and Torrie (49), were calculated for all time periods from the averages of all

groups of 6 or 10 mice containing that individual compound. Although the hRf-values varied slightly, fluorescence and hRf-values in a particular solvent system were characteristic for each compound.

Autoradiography

All manipulations were performed in a dark room using only a safety light. Thin layer chromatography plates were placed in an 8" x 10" (20.3 cm x 25.4 cm) x-ray exposure holder (Eastman Kodak Co., Rochester, New York). Kodak No-Screen-Medical x-ray film (tinted estar safety base) was placed over the plate. Each piece of film was coded by notching the top or right side of it. After 2 weeks exposure at room temperature, the film was developed for 5 minutes at 20 C in Kodak liquid x-ray developer and then fixed in Kodak liquid x-ray fixer for 10 minutes or until clearing of the film occurred. After this, the film was rinsed in water for 15 minutes to remove the fixer, and then drived. A dark spot on the film denoted the presence of a radioactive compound on the thin layer plate.

Collection of Urine

At specified time intervals, mice were removed from their cages, placed on a metal tray, and killed by cervical dislocation. This procedure caused a release

of urine from the bladder onto the tray. Other techniques of collection proved unsuccessful. The urine was then collected in micropipettes. Amounts of urine excreted varied from mouse to mouse and in some cases very little could be collected. From 20 ul to 60 ul of urine was spotted on thin layer chromatography plates depending on the experiment and amount excreted.

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. Thirty milliter aliquots of cells were concentrated by centrifugation at 10,000 - 12,000 x g for 5 minutes utilizing a Sorvall SS-1 table centrifuge. The supernatant was decanted, additional culture was added, and the procedure was repeated until all the culture was concentrated. Following centrifugation, the cells were washed twice with isotonic non-pyrogenic saline (Baxter Laboratories) and finally resuspended in saline to approximately 10 times their original concentration. Cells were heat-killed by exposure to 115 C for 6 minutes. Lack of growth on sub-culture served as proof of sterility. The LD_{50} of this preparation for mice was determined according to the method of Reed and Munch (45).

Spray Reagents

Prochazka's formaldehyde-HCl reagent for detecting indole derivatives (48) was prepared just prior to use by mixing 10 ml of formaldehyde (about 35%), 10 ml 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.

Van Urk's reagent (48), 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.

Chemicals

L-Tryptophan, serotonin creatinine sulfate, 5-hydroxyindole acetic acid, D,L-kynurenine, kynurenic acid, N-acetylserotonin, xanthurenic acid, 5-hydroxytryptophan, indole-3-acetic acid, and quinolinic acid were purchased from Nutritional Biochemicals Co. (Cleveland, Ohio). Tryptamine, indole, 3-indole pyruvic acid, 3-hydroxy- D,L -kynurenine, and glycyl tryptophan were purchased from Sigma Chemical Co. (St. Louis, Mo.).

D,L-Tryptophan (benzene ring-^{14}C), specific activity 52 mc/mM, was purchased from Amersham/Searle (Des Plaines, Illinois). It was diluted to 2.0 x 10^{-2} uc/ml (3.84 x 10^{-7} mM/ml) in 500 ml of sterile non-pyrogenic physiological saline (Baxter Laboratories, Morton Grove, Illinois). Where indicated, 20 mg of unlabeled L-tryptophan was dissolved per milliliter of the radioactive solution immediately before injection. L-Tryptophan-1-^{14}C, specific activity 9.6 mc/mM, was purchased from CALBIOCHEM (Los Angeles, California). This was diluted to 5.0 x 10^{-2} uc/ml (5.2 x 10^{-6} mM/ml) in 100 ml sterile isotonic non-pyrogenic saline. Twenty mg of unlabeled L-tryptophan was dissolved per milliliter in this preparation immediately prior to injection into mice.

Mice

Eighteen to twenty gram female Carworth Farms mice (CF-1), purchased from Carworth, Inc. (Portage, Michigan), were used in all experiments. Purina Laboratory Chow (Ralston Purina Co., St. Louis, Missouri) was available to all mice until 17 hours prior to injection of tryptophan. Water was provided ad libitum. Mice were kept in groups of ten in cages containing wood shavings as litter.

RESULTS

Characterization of Tryptophan and Tryptophan Metabolites Utilizing TLC Migration, Fluorescence, and Color and Fluorescent Reactions with Prochazka's and van Urk's Reagents

Tryptophan and numerous tryptophan metabolites were dissolved in either distilled water, ethanol, or normal mouse urine. Twenty ul of the solution containing from 10 to 30 ug of the standard were spotted and dried on a thin layer chromatography plate. The reference solutions were refrigerated. The plates were developed and hRf-values of the compounds measured. Twenty-four hours later the refrigerated solutions were again spotted, developed, and hRf-values measured. This procedure was used to determine if any of these three solvents caused a variation in migration. No significant changes in hRf-values at either time period were observed. hRf-Values and color reactions are listed in Table 1. A comparison with results of other investigators is presented in Appendix B, Tables B1 and B2.

Table 1.--hRf-Values, fluorescence, and color and fluorescence in either Prochazka's formaldehyde-HCl reagent or van Urk's 4-dimethylaminobenzaldehyde reagent.

Compound	hRf*	Fluorescense	Prochazka's Reagent		van Urk's Reagent	
			Visible	Fluorescense	Visible	Fluorescence
Indole	85	yellow tan	pink	green	pink violet	violet
Indole Acetic Acid	32	tan	yellow	green grey	violet	blue
Tryptamine	76	grey	yellow	yellow with blue edge	violet	violet tan
Serotonin	66	tan	yellow tan	yellow tan	grey brown	tan
N-Acetyl Serotonin	74	yellow tan	tan pink	blue	brown	tan brown
5-Hydroxy-Tryptophan	17.5	blue	yellow brown	yellow	grey brown	tan with blue edge
Tryptophan	25	blue	yellow brown	yellow with blue edge	violet	--
5-Hydroxy-Indole Acetic Acid	19	yellow tan	tan	yellow tan	violet	brown
Anthranilic Acid	33	yellow	beige	blue	yellow	light blue
Xanthurenic Acid	48	yellow with blue edge	tan	yellow	--	grey
Kynurenine	40	blue	--	blue	orange tan	blue green
Kynurenic Acid	35	purple	--	purple	green	--
3-Hydroxy-Hynurenine	10	yellow green	dull orange	coral orange	orange tan	yellow green
3-Hydroxy-Pyruvic Acid	23	yellow	light yellow	lemon yellow	blue	--

*hRf-Values in methyl acetate, isopropyl alcohol, and ammonium hydroxide in the proportions of 45:35:20.

Fluorescent Compounds and Indoles in Urine of Normal and Endotoxin-Poisoned Mice

After thin layer chromatographic separation of metabolites in unconcentrated mouse urine from normal and endotoxin-poisoned mice not injected with tryptophan, fluorescence of the metabolites was not intense enough to allow even tentative identification of any compounds. No increase in intensity of fluorescence was observed after spraying with Prochazka's formaldehyde-HCl reagent or van Urk's 4-dimethylaminobenzaldehyde reagent.

Tryptophan Metabolites Found in Urine of Normal Mice Given 20 mg of L-Tryptophan Containing 5.0×10^{-2} uc of L-Tryptophan-1-^{14}C

A subcutaneous injection of 5.0×10^{-2} uc (5.2×10^{-6} mM) of L-tryptophan-1-^{14}C in 20 mg of carrier L-tryptophan was given to 120 mice. The results and tentative identification of the metabolites found in urine are recorded in Table 2. Tryptophan metabolites, which do not contain the terminal carboxy group, would not be evident on the autoradiographs. The following are tryptophan metabolites which could be radioactive and present in urine: tryptophan, 5-hydroxytryptophan, kynurenine, kynurenic acid, quinaldic acid, 3-hydroxykynurenine, xanthurenic acid, 8-hydroxyquinaldic acid, alanine, and conjugates of these compounds. No compound with an hRf value greater than 44.5 was radioactive even though

Table 2.--Tentative identification of radioactive L-tryptophan-1-^{14}C metabolites found in urine of normal mice given 20 mg of unlabeled L-tryptophan as carrier.

Average hRf	Fluorescence	Prochazka Reagent		van Urk's Reagent		Tentative Identification
		Visible	Fluorescence	Visible	Fluorescence	
44.6 (±4.6)*	brown tan	--	tan	--	--	?
32.3 (±2.3)	brown tan yellow	tan (faint)	brown	--	brown	? kyn
26.2 (±1.7)	blue grey	yellow	yellow with blue edge	violet	blue tan	Tryptophan
17.1 (±1.3)	blue	yellow beige	yellow	-?	grey	5 HTP
10.5 (±1.1)	yellow tan	greenish tan	dark yellow	brown	brown tan yellow	? (3 Hkyn)
3.75 (±2.4)	blue	yellow orange	yellow	yellow	yellow tan	?

*Standard deviation calculated from the averages of 12 groups of 10 mice each.

fluorescent spots were observed. This compound was not identified. The compound migrating with an hRf value of 32.3, which may be kynurenine, consistently showed the greatest darkening of the x-ray film, as estimated by visual determination. The compounds with hRf values of 26.2 and 17.1 have been identified as tryptophan and 5-hydroxytryptophan respectively. Table 3 shows the time sequence of occurrence of the metabolites. Most radioactivity, as determined by number and intensity of spots, was found through 105 minutes after tryptophan administration. Tryptophan (hRf of 26.2) occurred only during the first 90 minutes after tryptophan. The two compounds migrating with hRf-values of 44.6 and 17.1 both appeared at 30 and 45 minutes and again at 105 and 120 minutes. The compounds with hRf-values of 32.3 and 10.5 appeared almost continuously throughout the 3 hour period. Visually the spot at 10.5 had a greater intensity of fluorescence, but the amount of radioactivity was not as great. It is known that compounds which have a low hRf, upon chromatographing in a basic solvent system, are acids which contain an additional amino or hydroxyl group or are conjugated (48). Therefore, these compounds at 10.5 and 3.8 may be substituted acids or conjugated compounds.

Table 3.--hRf-Values and time of appearance in urine of radioactive tryptophan metabolites from normal mice given 5.0×10^{-2} uc of L-tryptophan-1-^{14}C containing 20 mg of unlabeled L-tryptophan as carrier.

Average hRf	Time (Minutes)										
	30	45	60	75	90	105	120	135	150	165	180
44.6	+	+				+	+				
32.3	+	+	+	+	+	+		+	+	+	
26.2	+	+	+	+	+						
17.1	+	+				+	+				
10.5	+	+	+	+	+			+	+	+	
3.8	+						+				

+ indicates presence of the compound.

Tryptophan Metabolites Found in Urine of Normal and Endotoxin-Poisoned Mice Given 2.0 x 10^{-2} uc of D,L-Tryptophan (Benzene Ring-^{14}C)

At intervals of 1, 2, and 3 hours, mice, given a subcutaneous injection containing 7.84 x 10^{-5} mg (2.0 x 10^{-2} uc) of D,L-tryptophan (benzene ring-^{14}C), were killed by cervical dislocation and urine was collected and processed as above. Although there were a total of five radioactive tryptophan metabolites excreted at 1, 2, and 3 hours after injection of tryptophan, no individual mouse excreted more than 3 radioactive compounds (Table 4). The compounds which may be tentatively identified are tryptamine (hRf of 76.0), tryptophan (hRf of 25.0), and 5-hydroxytryptophan (hRf of 19.2). Those compounds with hRf values equal to 45.5 and 10.0 could not be identified. Tryptophan consistently produced the darkest and largest spot on the autoradiograph. In general, intensity of the spots decreased with increasing time after injection.

An unexpected result occurred in the mice injected with endotoxin 10 hours previous to the radioactive tryptophan. No radioactivity was detectable in urine collected at either 1, 2, or 3 hours after tryptophan on plates exposed to x-ray film for 2 weeks.

Table 4.--Tentative identification of radioactive tryptophan metabolites found in urine of normal mice given 7.84×10^{-5} mg (2.0×10^{-2} uc) of D,L-tryptophan (benzene ring-^{14}C).

Average hRf	Fluorescence	Prochazka Reagent		van Urk's Reagent		Tentative Identification
		Visible	Fluorescence	Visible	Fluorescence	
76.0	yellow	yellow	yellow with blue	yellow tan	blue green	Tryptamine
45.5	brown tan	green	dark yellow tan	--	--	?
25.0	blue grey	yellow	yellow with blue	blue tan	brown	Tryptophan
19.0	blue	tan beige	yellow	grey	-?	5HTP
10.0	yellow	yellow	dark yellow brown	brown tan yellow	brown	?

Tryptophan Metabolites Found in Urine of Normal and Endotoxin-Poisoned Mice Given 20 mg of L-Tryptophan Containing 2.0×10^{-2} uc of L-Tryptophan (Benzene Ring-^{14}C)

A detailed description of the radioactive tryptophan metabolites found in normal mice given labeled plus carrier tryptophan is shown in Table 5. These mice excreted 9 radioactive tryptophan metabolites as compared to only 5 labeled metabolites found in normal mice not given carrier tryptophan. Tryptophan (hRf of 27.9) and 5-hydroxytryptophan (hRf of 16.3) were excreted by these mice. The compound with an hRf-value of 22.6, which was not radioactive in the tryptophan-1-^{14}C experiment, has been tentatively identified as 5-hydroxyindole acetic acid. Compounds having hRf values of 33.7, 10.6 and 4.3 were also observed. Of these, the compound with the hRf of 10.6 may be either 3-hydroxykynurenine, a conjugate of it, or another conjugated compound. The compound with an hRf of 81.0 was observed and tentatively identified as tryptamine. Serotonin was also detected, having an hRf value of 67.5. As seen in Table 6, the compounds which have the higher hRf-values are generally found in the early time periods after tryptophan injection. Tryptamine (hRf of 81.0 was observed through 60 minutes and not again until 180 minutes. While serotonin (hRf of 67.5) was seen only at 30 and 45 minutes. The compound with an hRf of 44.0 was seen through 60 minutes and then

Table 5.--Tentative identification of radioactive D,L-tryptophan (benzene ring-^{14}C) found in urine of normal mice given 20 mg of unlabeled L-tryptophan as carrier.

Average hRf	Fluorescence	Prochazka Reagent		van Urk's Reagent		Tentative Identification
		Visible	Fluorescence	Visible	Fluorescence	
81 (±2.4)*	tan	tan	blue	--	grey	Tryptamine
67.5 (±0.2)	tan	tan	tan	tan	--	Serotonin
44 (±4.2)	brown tan	--	tan	--	--	?
33.7 (±0.3)	brown tan yellow	tan	brown	--	brown	?
27.9 (±1.7)	blue grey	yellow	yellow with blue	violet	--	Tryptophan
22.6 (±1.1)	yellow tan green	yellow	yellow with blue	brown	--	5HIAA
16.3 (±1.3)	blue	tan	yellow	tan	blue	5HTP
10.6 (±1.2)	yellow tan	orange yellow	dark yellow tan	orange	tan	? (3Hkyn)
4.3 (±2.1)	blue	tan	yellow	tan with blue	white blue	?

*Standard deviation calculated from averages of 12 groups of 6 mice each.

Table 6.--hRf-Values and time of appearance in urine of radioactive tryptophan metabolites from normal mice given 2.0 x 10^{-2} uc of D,L-tryptophan (benzene ring-^{14}C) with 20 mg of unlabeled L-tryptophan as carrier.

Average hRf	15	30	45	60	75	90	105	120	135	150	165	180
81	+	+	+	+								+
67.5		+	+									
44	+	+	+	+			+	+				
33.7		+	+		+	+	+					
27.9	+	+	+	+	+	+	+				+	+
22.6	+		+	+			+	+	+	+	+	+
16.3	+	+	+		+	+	+	+	+			
10.6	+	+	+	+	+	+	+	+	+	+	+	+
4.25			+			+	+	+				

+ indicates presence of the compound.

from 105 to 120 minutes. The metabolites having an hRf of 33.7 appeared at 30 and 45 minutes and from 75 to 105 minutes and then later at 165 to 180 minutes. Tryptophan (hRf of 27.9) was excreted continuously throughout the 3 hour period with 120 minutes being the only time it did not appear. The compound tentatively identified as 5-hydroxyindole acetic acid (hRf of 22.6) appeared frequently, being seen at 15, 45 through 60, and then from 105 through 180 minutes. Also appearing regularly was 5-hydroxytryptophan (hRf of 16.3), which was seen from 15 to 45 minutes and 75 to 135 minutes. The only metabolite occurring throughout the 3 hour period was again the compound with the hRf of 10.6.

As described in Table 7, endotoxin-poisoned mice given labeled plus carrier tryptophan excreted eight radioactive tryptophan metabolites. Of the compounds excreted, there were six which were common to normal mice given tryptophan. These were tryptophan (hRf of 27.4), 5-hydroxyindole acetic acid (hRf of 23.6), 5-hydroxytryptophan (hRf of 16.1), the compound believed to be tryptamine (hRf of 82.9), and the compounds migrating with hRf values of 32.2 and 9.8. There were two compounds (hRf-values of 53.1 and 38.8) not seen in normal mice given tryptophan and 3 (hRf values of 67.5, 44.0, and 4.3) which appeared in normal mice but not endotoxin-poisoned mice. The time sequence of appearance of the radioactive metabolites is

Table 7.--Tentative identification of radioactive D,L-tryptophan (benzene ring-^{14}C) metabolites found in urine of endotoxin-poisoned mice given 20 mg of unlabeled L-tryptophan as carrier.

Average hRf	Fluorescence	Prochazka Reagent		van Urk's Reagent		Tentative Identification
		Visible	Fluorescence	Visible	Fluorescence	
82.9 (±3.2)*	tan	tan pink	pale light yellow green	green	--	Tryptamine
53.1 (±3.3)	lt. green with brown center	green tan	green blue	yellow	pink yellow tan	?
38.8 (±1.9)	tan yellow	tan	tan	tan	tan	Kyn (?)
32.2 (±1.3)	tan yellow green	tan	yellow tan	yellow brown	yellow tan	?
27.4 (±0.7)	blue with yellow green	yellow	dark yellow with green blue	violet	--	Tryptophan
23.6 (±1.2)	blue	yellow	yellow with blue	tan	blue	5HIAA
16.1 (±1.9)	blue	brown green	grey	tan orange	tan blue	5HTP
9.8 (±1.1)	yellow	pink yellow	dark yellow	blue grey	tan	? 3HKyn

*Standard deviation, calculated from averages of all time periods.

shown in Table 8. The compounds with the higher hRf values were again detected during the earlier time periods. Tryptamine (hRf of 82.9) was seen from 45 through 90 minutes and the unidentified compound with an hRf of 53.1 was observed from 30 to 90 minutes and once again at 120 minutes. The compound with an hRf of 38.8 only was detected twice, once at 60 minutes and again at 165 minutes. Appearing at all times throughout the 3 hour period except at 75 minutes, was the compound with an hRf of 32.2 Tryptophan was seen at 45 through 90 minutes and again at 135 and 165 minutes, while 5-hydroxy-indole acetic acid was observed at 30 and 45 minutes and again from 75 to 150 minutes. Observed during the middle of the time period was 5-hydroxytryptophan, which occurred at 60 to 90 minutes and 105 to 150 minutes. Again appearing at all times except 30 minutes was the compound at an hRf of 9.8.

Table 8.--hRf-Values and time of appearance in urine of radioactive tryptophan metabolites from endotoxin-poisoned mice given 2.0×10^{-2} uc of D,L-tryptophan (benzene ring-^{14}C) containing 20 mg of unlabeled L-tryptophan as carrier.

Average hRf	30	45	60	75	90	105	120	135	150	165	180
82.9		+*	+	+	+						
53.1	+	+	+	+	+		+				
38.8			+							+	
32.2	+	+	+		+	+	+	+	+	+	+
27.4		+	+	+				+		+	
23.6	+	+		+	+	+	+	+	+		
16.1			+	+		+	+	+			
9.8		+	+	+		+	+	+	+	+	+

*+indicates presence of metabolite.

DISCUSSION

Throughout these studies, hRf-values of individual compounds varied slightly. As long as the color reactions were the same and the hRf-values did not vary significantly, a compound was called identical. The results obtained with standard compounds in these experiments were in close agreement to those reported by other investigators using the same solvent system (17, 46).

In determining which tryptophan metabolites were present in urine of normal and endotoxin-poisoned mice, it was necessary to consider not only the 40 known metabolites but also many tryptophan metabolites which have been recognized but not identified (43, 47).

When normal unconcentrated mouse urine was separated chromatographically, no tentative identification of any fluorescent compounds could be made. After injecting 2.0×10^{-2} uc (1.1×10^{-3} mg) of L-tryptophan (benzene ring-^{14}C, five radioactive spots were observed on the autoradiographs. Of these, tryptamine, tryptophan, and 5-hydroxytryptophan have been tentatively identified, but the other two have not been resolved. Upon injecting 20 mg of L-tryptophan with the 2.0×10^{-2} uc of L-tryptophan (benzene ring-^{14}C), four additional tryptophan

metabolites were observed in the urine. Serotonin and 5-hydroxyindole acetic acid have been tentatively identified but the other two having hRf-values of 33.7 and 4.3, have not been identified.

By utilizing either L-tryptophan-1-^{14}C or D,L-tryptophan (benzene ring-^{14}C) with 20 mg of carrier L-tryptophan, a comparison of the radioactive metabolites excreted in the mouse urine was made. This provided another aid in identification of the compounds. Those compounds, which appeared in both groups, had hRf values of 44.0, 33.7, 27.9, 16.3,and 10.6. Of these, tryptophan and 5-hydroxytryptophan have been identified. All of these metabolites have retained the terminal carbon atom of the carboxyl group. Those, which were not radioactive as shown by the autoradiographs and thus had lost the carboxy carbon, were tryptamine, serotonin, 5-hydroxyindole acetic acid, and the unidentified compound migrating with an hRf of 4.3.

By comparing Table 5 with Table 7, it can be seen that normal and endotoxin-poisoned mice excreted six common tryptophan metabolites. Of these, tryptophan and 5-hydroxytryptophan have been identified conclusively, tryptamine and 5-hydroxyindole acetic acid have been tentatively identified, and the two others remain to be resolved. The variation of the urinary tryptophan metabolites between the normal and endotoxin-poisoned mice

tends to indicate that tryptophan has been metabolized through different pathways or that the amount of tryptophan entering a specific pathway may have been altered.

Not only were there differences in the compounds excreted, but the time of appearance of certain metabolites varied among the groups of mice. Radioactive tryptophan was excreted only through 90 minutes when L-tryptophan-1-^{14}C was administered, but it was seen at every time period except 120 minutes when D,L-tryptophan (benzene ring ^{14}C) was used. This discrepancy may possibly be explained by the excretion of D-tryptophan by the mice.

In general, there was a lag of at least 45 minutes in the excretion of tryptamine, tryptophan, and the compound with an hRf of 9.8 and a 60 minute lag for 5-hydroxytryptophan in endotoxin-poisoned mice; whereas normal mice excreted these products from the beginning of the time period. Tryptophan and 5-hydroxytryptophan also did not appear as regularly in endotoxin-poisoned mice and they ceased being detected sooner than in normal mice. The compound with the hRf-value of 32.2, as yet unidentified, was the major metabolite in endotoxin-poisoned mice but appeared only spasmodically in normal mice. Although 5-hydroxyindole acetic acid appeared in both normal and endotoxin-poisoned mice, the spots, as determined by visual detection, on the autoradiographs were small and

at times hard to detect. It appeared in the center half of the time period in endotoxin-poisoned mice and in the last half in normal mice. A possible explanation of the low amounts of 5-hydroxyindole acetic acid excreted may be that herbivores, such as guinea pigs, rabbits, and mice, excrete less than 0.3 ug of 5HIAA/ml of urine. In the experimental conditions utilized, only 6×10^{-3} to 1.8×10^{-2} ug of 5HIAA would theoretically be present. It follows that you would expect so little that it would not be detected by the methods employed.

The compound which migrated with an hRf of approximately 10 in all groups of mice could not be conclusively identified. It had the same Rf as 3-hydroxykynurenine but the color reactions and fluorescence with the spray reagents were not the same as 3-hydroxykynurenine. Even though it is known that conjugates and substituted acids migrate only slightly (producing low Rf values) in the solvent system utilized, a tentative identification could not be made.

In all groups of mice, residual radioactivity was observed at the origin of the sample spot after development of the thin layer plate. No tryptophan metabolite known to this author does not migrate in the solvent system used. Therefore, no identification of this compound was made.

Overall, endotoxin-poisoning produced a variation in the tryptophan metabolites excreted following tryptophan injection in mice. Further, the time sequence of their appearance, as compared to normal mice given tryptophan, was altered.

SUMMARY

Normal and endotoxin-poisoned mice given 20 mg of L-tryptophan, both excreted tryptophan, 5-hydroxytryptophan, 5-hydroxyindole acetic acid, tryptamine, and two unidentified tryptophan metabolites, one of which may be 3-hydroxykynurenine. Normal mice given tryptophan also excreted a small amount of serotonin, and two other unidentified tryptophan metabolites. Endotoxin-poisoned mice given tryptophan excreted two other tryptophan metabolites besides the six common to both groups. Not only did administration of endotoxin cause a variation in the excretion of tryptophan metabolites, but there was also variation in the time of appearance in urine of the metabolites. Endotoxin caused a lag of about 45 minutes after tryptophan administration before the common metabolites were excreted.

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LITERATURE CITED

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APPENDICES

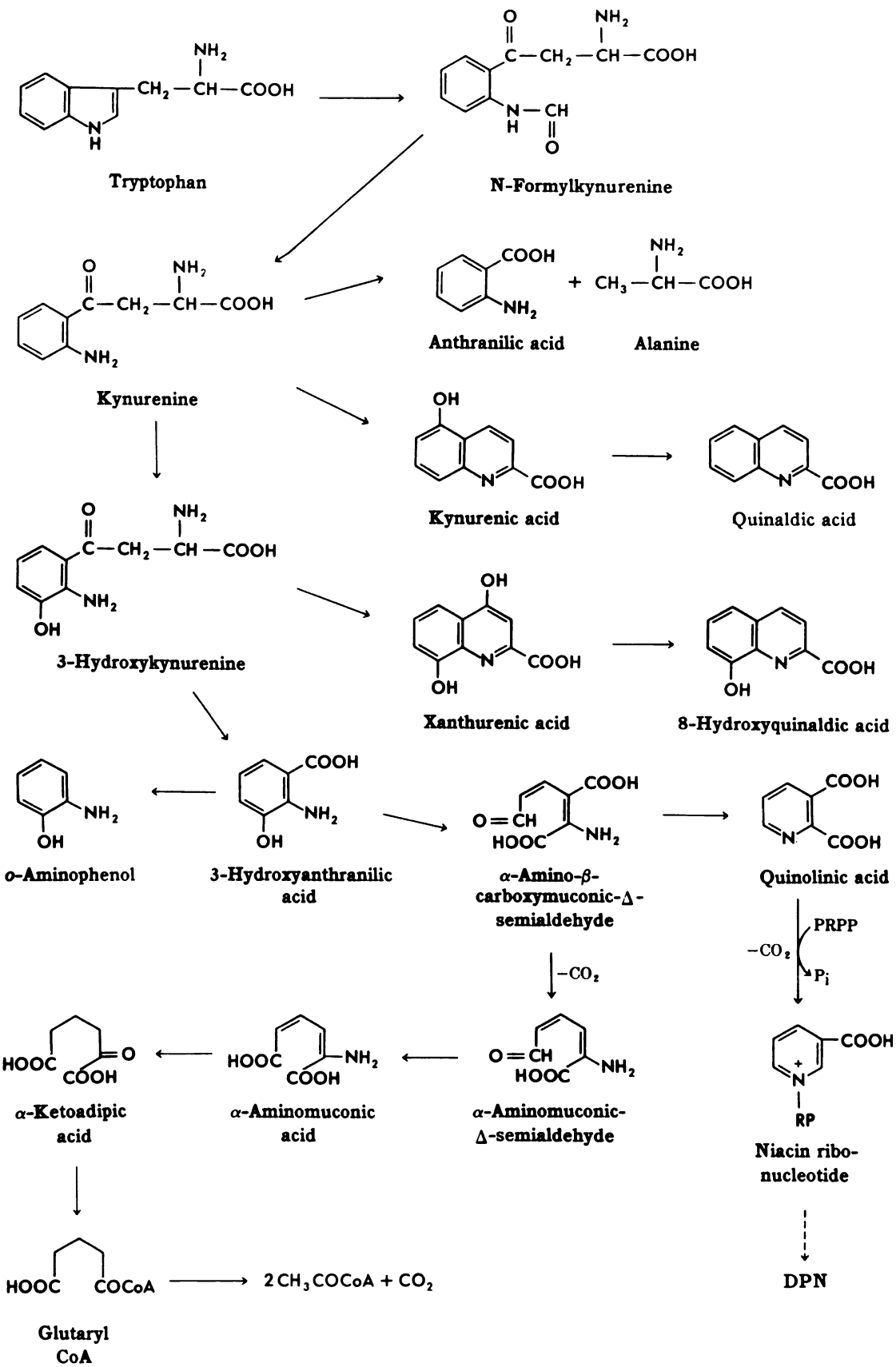

Figure A1.--The kynurenine pathway.

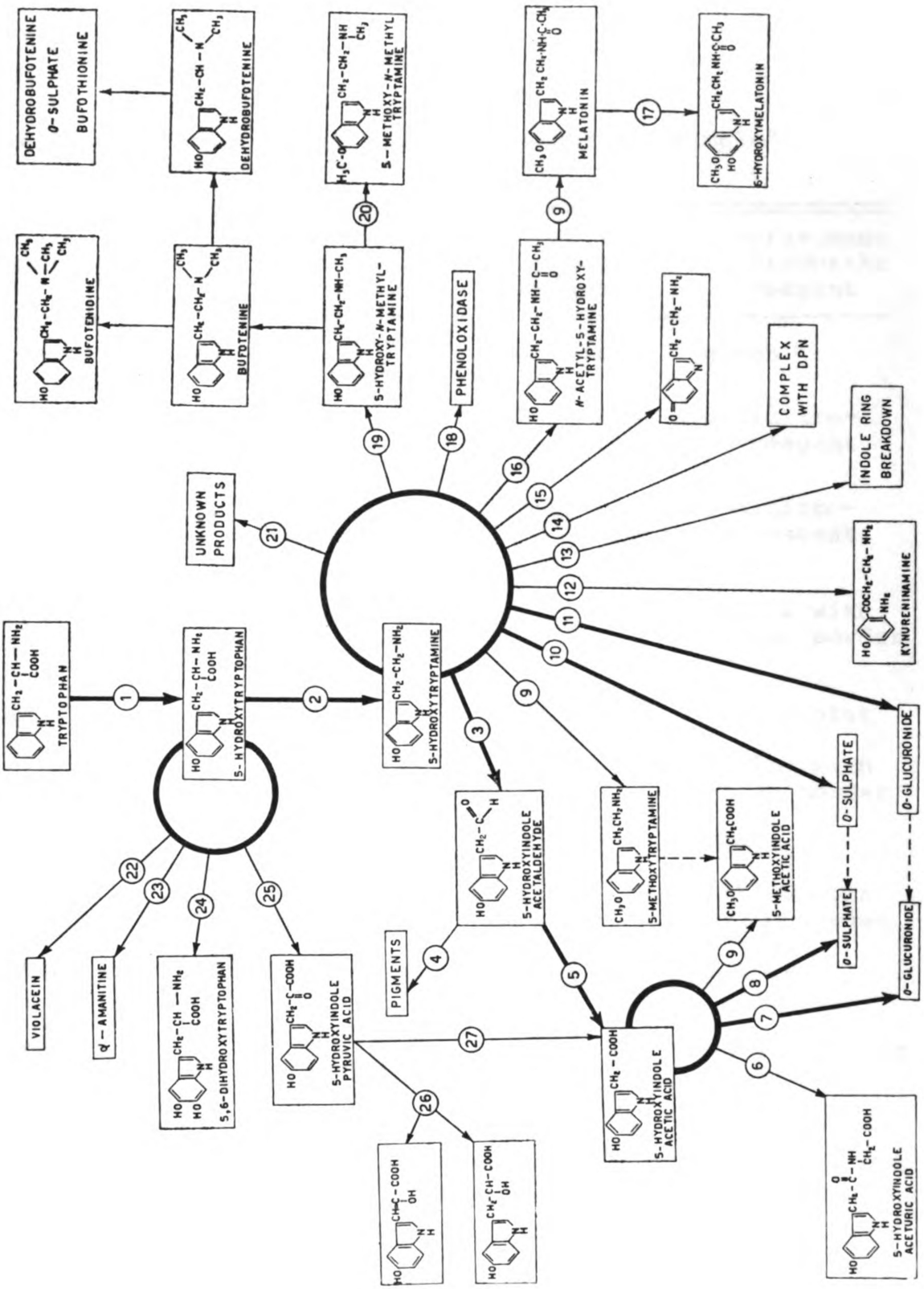

Figure A2.--The serotonin pathway.

APPENDIX B

Table B1.--hRf-Values and color reactions of "simple" indole derivatives (46).

Compound	hRf*	Color with van Urk's Reagent	Color with Prochazka's Reagent	Fluorescence in Prochazka Reagent
Indole	84	dark red to violet	pale green	green
Indole-3-aldehyde	81	pink	orange with 2,4-dinitrophenyl hydrazine reagent	
Indole-3-acetaldehyde	86	reddish brown	yellow with 2,4-dinitrophenyl hydrazine reagent	
Indole-3-acetic acid	31	blue, tinge of violet	yellow	yellow with green border
5HIAA	19	blue to violet	pale yellow to beige	deep violet
Tryptamine	77	blue-green	yellow	yellow with blue border
Serotonin	65	grey	yellow	brown
D,L-Tryptophan	23	blue-green	yellow	yellow with blue border
D,L-5HTP	14	blue-grey	pale yellow to beige	yellow
Anthranilic Acid	33	becomes intensive yellow	pale beige	blue, turning brown

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

Table B2.--hRf-Values and detection of tryptophan metabolites utilizing p-dimethylaminobenzaldehyde reagent (43).

Substance	hRf*	Detection with p-Dimethylaminobenzaldehyde Reagent	
		Fluorescence	Color
Tryptophan	25	-	Violet
Indole	90	Blue	Violet
Indicane	61	Brown	Brown
D,L-Kynurenine	32	Green-blue	Yellow-brown
3-Hydroxy-Kynurenine	16	Yellow green	Orange
Kynurenic Acid	45	Green after 12 hours	-
Xanthurenic Acid	45	Grey	-
Anthranilic Acid	45	Light blue	Yellow
3-Hydroxy-Anthranilic Acid	31	Light blue	Yellow

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