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## URINARY 5-METHOXYTRYPTAMINE IN PATIENTS WITH RHEUMATIC FEVER\*

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No specific diagnostic test for rheumatic fever has been available to date (1). The tryptophan-nicotinic acid metabolic series was studied exhaustively in the present work. This investigation was based on a previous observation that the tetrahydrofurfuryl ester of nicotinic acid elicited a different response in the skin of patients suffering from active rheumatic fever than in the skin of nonrheumatic patients (2). The reaction was attributed to nicotinic acid, a member of the tryptophan metabolic series.

The present report describes an abnormal product of tryptophan metabolism isolated from the urine of rheumatic subjects.

### MATERIALS AND METHODS

*Studies in human subjects.* Three groups of subjects were used in this study. The first two consisted of both male and female children between 3 and 16 years of age.

Group I consisted of inpatients at the National Children's Cardiac Hospital with inactive rheumatic heart disease,<sup>1</sup> inactive rheumatic fever,<sup>1</sup> or congenital heart disease, and group II, of young subjects from other places who had either active or inactive rheumatic heart disease, or rheumatic fever. These two groups were considered separately because any uniform dietary and environmental effects observable among group I subjects were balanced by dietary and environmental differences among group II subjects. Studies requiring large quantities of urine necessarily excluded group II patients. Group III consisted of adult male patients at the Veterans Administration Hospital, Coral Gables, Florida, with Hodgkin's disease or multiple myeloma.

Normal control subjects were also studied. As controls for groups I and II, urines were collected from local children of comparable ages and sex with no known diseases; as controls for group III, urine was obtained from adult males with no known diseases.

In all subjects, 24- or 48-hour urine samples were collected with toluene as a preservative and were refrigerated as soon as possible after collection. Urine specimens were

analyzed for products in the tryptophan-nicotinic acid metabolic series.

In some instances, "tryptophan loading" tests were done. Five g of L-tryptophan suspended in 200 ml of orange juice was administered orally. The urinary excretion of 5-hydroxyindoleacetic acid and related compounds was measured during the subsequent 24 hours and compared with the amounts excreted over the same interval on the previous day, when only orange juice was fed.

*Chemical analytical methods.* Tryptophan was determined by the *p*-dimethylaminobenzaldehyde method of Spies and Chambers (3). Indoxylsulfate and tryptamine were determined by use of a modified Ehrlich's reagent (4). Kynurenic acid and xanthurenic acid were measured by the fluorometric method of Satoh and Price (5). Kynurenine was converted to *o*-acetophenone, which was measured spectrophotometrically in an ethereal extract (6). Nicotinic acid was determined by the anilinocyanogen bromide method of Banerjee, Ghosh, and Bhattacharya (7). *N*<sup>1</sup>-Methylnicotinamide and the 6-pyridone of *N*<sup>1</sup>-methylnicotinamide were measured fluorometrically with a Farrand photofluorometer as the condensation products with acetone (8, 9). 5-Hydroxytryptamine and 5-hydroxyindoleacetic acid were measured by the fluorometric methods of Udenfriend, Weissbach, and Brodie (10).

The cleavage of methoxy compounds by hydrogen iodide was the basis of a modified Ziesel method (11) for quantitative determination of methoxyl content of certain isolated urinary constituents. After isolation, as described in the next section, quantitative estimations of 5-methoxytryptamine were made by this method.

Paper chromatographic techniques used were those outlined by Smith (12). In addition, a polychromatic technique was employed on routine paper chromatograms for the identification of amines and amino acids (13). Fluorescent spots were detected with a mercury vapor arc lamp (Hanovia utility model). Also, urinary indoles and related substances were separated chromatographically and characterized, both as to *R<sub>f</sub>* values and with various color reagents, by the technique of Dalgliesh (14), Rodnight (15), and Armstrong, Shaw, Gortatowski, and Singer (16). After preliminary, tenfold concentration of urine through vacuum distillation and paper chromatographic separation (40  $\mu$ l per application), semi-quantitative measurements of 5-methoxytryptamine were made according to the method of Rodnight (15) by eluting the material from the paper chromatogram with water. The eluate was allowed to react with a modified Ehrlich's reagent (4) sufficiently sensitive to permit recognition

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<sup>1</sup> In this report, "rheumatic heart disease" and "rheumatic fever" are distinguished by the presence or absence of heart involvement, respectively.

of 2  $\mu\text{g}$  or more. The light absorbance was then read at 560  $\mu\mu$  on the Coleman Junior spectrophotometer and compared with a standard curve made with known amounts of 5-methoxytryptamine.

*Procedure for isolation of unknown indole.* Urine specimens were collected from children with rheumatic disease who fasted overnight (group I). A small portion was removed from each sample and frozen for future chromatographic analysis. Repeated morning urine specimens were collected from rheumatic subjects. Whenever 5 L were accumulated, they were processed as described below. A total of 72.24 mg of the unknown indole was recovered from 252 L of urine. The morning urine specimens generally contained over 80% of the total amount of the unknown indole recovered from 24-hour urine samples.

Each sample of pooled urine was acid-hydrolyzed to free that portion of the unknown compound which was excreted in a conjugated form, presumably a sulfate or glucuronide, as described by Kopin, Pare, Axelrod, and Weissbach (17).

One thousand-ml samples of the hydrolyzed urine were passed through columns of Dowex 50. Each column contained a  $1.5 \times 100$ -cm resin bed previously prepared by washing the  $\text{H}^+$  form successively with approximately 1 L each of acetone, water, 2 N NaOH, water, and 2 N HCl. After passage of the urine, each resin column was washed successively with 100 ml of water, 200 ml of 0.1 N sodium acetate, and 100 ml of water. This left the

unknown compound and other aromatic amines on the resin column. The unknown compound was eluted by washing the column with ammonium hydroxide in a series of 20-ml solutions of concentration increasing by increments of 0.1 N from 0.2 N to 1.0 N. The unknown compound could thus be concentrated and isolated from interfering substances.

Samples of 40  $\mu\text{l}$  each were taken at various steps in the procedure to check by paper chromatography the presence or absence of the unknown indole. This indole was followed by noting the presence of a Ninhydrin- and Ehrlich's reagent-positive fluorescent spot having a characteristic  $R_f$  value (see Results) depending on the solvent. Samples were collected from the initial pooled urine after hydrolysis and adjustment to pH 5.4, the initial acidic fraction obtained after passage through the Dowex column, the acetate wash of the Dowex column, and subsequent fractions obtained with each normality of  $\text{NH}_4\text{OH}$  wash in the series. By this procedure, the progress of isolation and purification could be observed.

Twenty-ml fractions of the eluant containing the unknown indole were evaporated to dryness *in vacuo*. The material was resuspended in 2.5 ml of ether, sufficient to dissolve it. Forty 2.5-ml samples were combined for a total of 100 ml, and the entire solution was extracted with 5% hydrochloric acid, 20 ml for three successive washings and 10 ml for each of the next two. The clear, yellow supernatant ether was cooled in ice and made basic with 30% potassium hydroxide. The resulting oil was

TABLE I  
*Urinary tryptophan metabolites excreted by children in group I\**

Metabolite measured	Rheumatic heart disease		Rheumatic fever		Congenital heart disease		Normal subjects	
	No. tested	Excretion	No. tested	Excretion	No. tested	Excretion	No. tested	Excretion
		<i>mg/24 hours</i>		<i>mg/24 hours</i>		<i>mg/24 hours</i>		<i>mg/24 hours</i>
Tryptophan	22	3.8 [.00-6.2]	5	2.2 [.20-6.0]	1	1.5	19	5.2 [.20-8.8]
Indoxylsulfate	22	35. [20.-72.]	5	72. [28.-108.]	1	104.0	19	420. [148.-620.]
Kynurenic acid	22	4.5 [.00-9.4]	5	6.0 [4.2-8.8]	1	4.0	19	5.0 [.00-8.8]
Xanthurenic acid	22	4.2 [.00-12.6]	5	8.2 [4.0-10.2]	1	4.2	19	6.4 [3.2-13.0]
Kynurenine	22	1.9 [.02-4.0]	5	2.4 [.80-4.8]	1	3.1	19	1.8 [.50-3.8]
<i>N</i> <sup>1</sup> -Methylnicotinamide	22	5.0 [3.2-10.0]	5	5.2 [4.2-8.8]	1	5.5	19	10.7 [4.0-12.0]
Nicotinic acid	22	1.5 [.90-3.4]	5	2.1 [1.2-2.5]	1	2.4	19	2.3 [1.8-2.9]
6-Pyridone of <i>N</i> <sup>1</sup> -methyl-nicotinamide	22	3.0 [1.6-4.6]	5	2.4 [1.9-2.9]	1	3.0	19	3.9 [2.4-4.4]
5-Hydroxyindoleacetic acid	22	8.2 [.80-14.5]	5	4.3 [2.4-8.3]	1	5.2	19	8.6 [1.4-13.2]

\* All recorded values are the results of at least three determinations on each subject, and values in brackets are the ranges. Here, "rheumatic heart disease" is taken to involve the heart, whereas "rheumatic fever" is not.

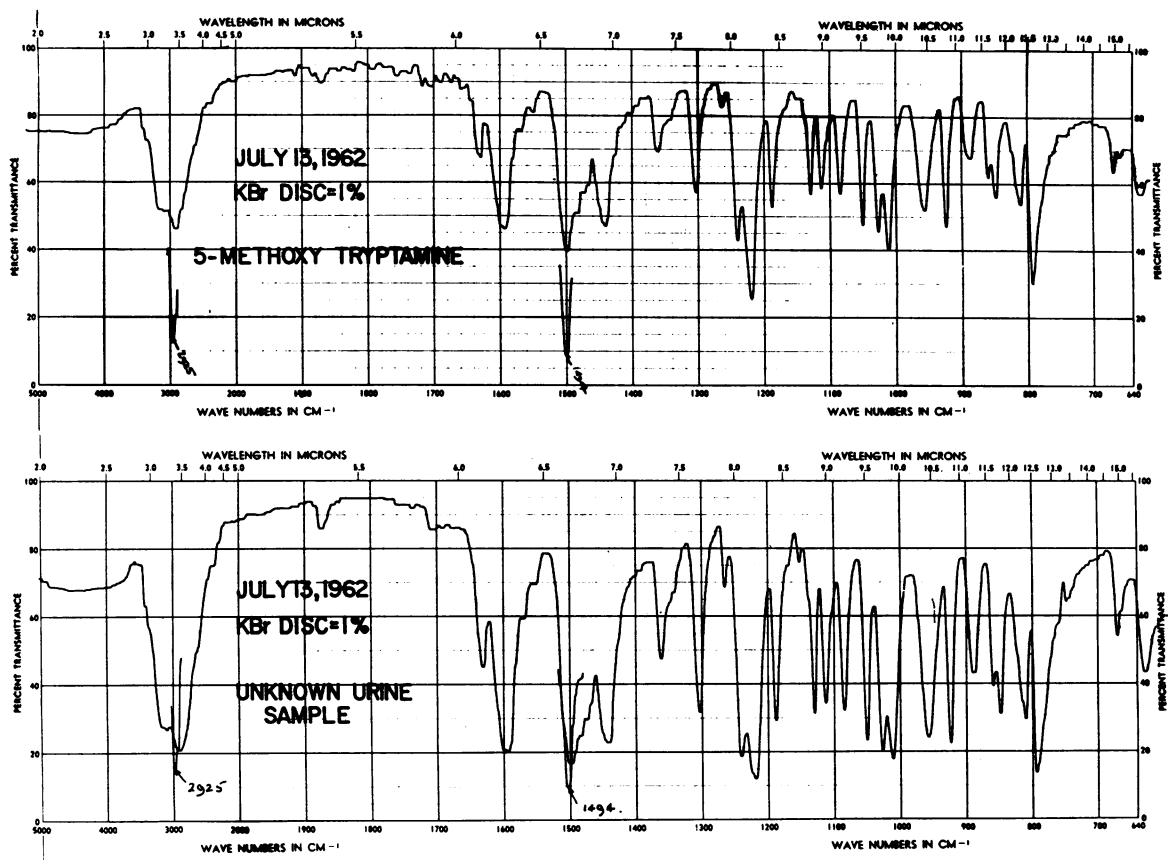


FIG. 1. INFRARED SPECTRA CONFIRMING THE IDENTITY OF THE UNKNOWN INDOLE WITH 5-METHOXYTRYPTAMINE.

extracted three times with ether, 35 ml per washing. The ether extract was washed twice with 20 ml water and then once with 10 ml saturated salt solution. The extract was then dried with sodium sulfate and evaporated under reduced pressure (10 to 15 mm Hg). The resulting material was recrystallized from benzene to yield pale yellow prisms, mp 120 to 121.5° C (uncorrected). Details of the latter portion of this procedure were derived from the work of Szmuszkovicz, Anthony, and Heinzelman (18).

Infrared spectra (in centimeters<sup>-1</sup>) were determined with a Baird recording infrared spectrophotometer with a sodium chloride prism; 0.2-mm cells were used with either benzene, chloroform, or ethyl acetate as the solvent. Additional spectra were obtained by use of a KBr disc.<sup>2</sup> A standard of known 5-methoxytryptamine<sup>3</sup> was studied in the same fashion, for comparison.

The significance of the experimental findings (see Results) was evaluated by probability determinations derived from chi-square and *t* tests of the experimental data (19).

<sup>2</sup> Taken by Dr. Alfred P. Mills, University of Miami, Miami, Fla., and by Dr. James R. Lawson, Fisk University, Nashville, Tenn.

<sup>3</sup> Nutritional Biochemicals Corp., Cleveland, Ohio.

## RESULTS

The quantities of urinary tryptophan metabolites excreted by children with rheumatic disease showed no significant differences ( $p > 0.05$ ) from the amounts excreted by normal children, with the exception of a decrease in the amount of indoxyl-sulfate secreted by children with either rheumatic heart disease or rheumatic fever when compared with that of normal children of comparable age and sex ( $p < .0001$ ) (Table I).

*Unknown indole identification.* At the time of these measurements, one-dimensional paper chromatograms were made of equal portions from each urine sample. In the urines of all the rheumatic children, but in none of the normal, a Ninhydrin- and Ehrlich's reagent-positive fluorescent spot appeared that gave the following  $R_f$  values in different solvents:  $0.76 \pm 3$  in isopropanol: ammonia: water, 20:1:2;  $0.64 \pm 3$  in *n*-butanol: glacial acetic acid: water, 12:3:5;  $0.72 \pm 3$  in *n*-butanol:

TABLE II  
*Effect of 5 g of L-tryptophan on the urinary excretion of 5-hydroxyindoleacetic acid, 5-hydroxytryptamine, and 5-methoxytryptamine\**

Metabolite measured	Subjects (from group I)	No. tested	Before tryptophan	After tryptophan	Average increase
			<i>mg/24 hours</i>	<i>mg/24 hours</i>	<i>mg/24 hours</i>
5-Hydroxyindoleacetic acid	Normal	6	3.43 [1.46-5.40]	6.14 [4.03-8.25]	2.71
	Rheumatic heart disease	9	3.17 [2.26-4.08]	6.05 [4.90-7.20]	2.88
5-Methoxytryptamine	Normal	6	0.00	0.00	0.00
	Rheumatic heart disease	9	0.055 [0.031-0.079]	0.233 [0.178-0.288]	0.178
5-Hydroxytryptamine	Normal	6	0.083 [0.043-0.123]	0.070 [0.038-0.102]	-0.013
	Rheumatic heart disease	9	0.100 [0.048-0.152]	0.094 [0.052-0.136]	-0.006

\* Values are averages  $\pm$  maximal deviations, with the ranges given in brackets.

pyridine: water, 1:1:1; and  $0.35 \pm 3$  in 8% w/v aqueous NaCl: glacial acetic acid, 100:1.

Isolation of the unknown indole was accomplished as described in the preceding section. Reactions of the compound with Ehrlich's reagent, and with Ninhydrin coupled with the modified Zeisel procedure, indicated an indoleamine with a single methoxy group. The only compound of this type having the  $R_f$  values above on paper chromatography and a melting point in the immediate vicinity of 120 to 121.5° C is 5-methoxytryptamine. Further confirmation was obtained from the infrared spectrum (Figure 1).

Commercially prepared 5-methoxytryptamine<sup>3</sup> gave the same  $R_f$ , color reactions, and mp values, and the same infrared spectrum as those obtained above with the unknown indole. When authentic

5-methoxytryptamine was subjected to the isolation procedure described above, there was 70 to 78% recovery.

No evidence, either chemical or chromatographic, could be found for the presence of 5-methoxyindoleacetic acid, or any other methoxyindole in the urine from children with rheumatic disease.

*Tryptophan loading tests.* The results of tryptophan loading tests indicate that the excretion of 5-hydroxyindoleacetic acid and 5-hydroxytryptamine are normal in children with rheumatic heart disease. There is, however, a significant increase in the amount of 5-methoxytryptamine excreted (Table II).

*Quantitative estimates of 5-methoxytryptamine and indoxylsulfate in various disease states.* No significant differences were noted in the excretion

TABLE III  
*Daily urinary excretion of indoxylsulfate and 5-methoxytryptamine\**

Subjects†	No. tested	5-Methoxytryptamine	Indoxylsulfate
		<i>mg/24 hours</i>	<i>mg/24 hours</i>
Rheumatic heart disease, active	2	0.100 [0.070-0.130]	40 [36-44]
Rheumatic heart disease, inactive	10	0.112 [0.040-0.210]	54 [28-72]
Rheumatic fever, active	4	0.120 [0.090-0.150]	50 [32-64]
Rheumatic fever, inactive	5	0.140 [0.070-0.180]	44 [24-60]
Hodgkin's disease (group III)	3	0.00	220 [180-250]
Multiple myeloma (group III)	1	0.00	180
Normal adults	8	0.00	200 [140-250]
Normal adult	1	0.040 [0.020-0.080]‡	170 [135-205]‡

\* Semiquantitative results were obtained by estimating spot intensities on paper chromatograms, either by visual inspection (accuracy  $\pm 25\%$ ) or by photometric determination of spot density (accuracy  $\pm 15\%$ ) (14). Ranges are given in brackets. Here, "rheumatic heart disease" is taken to involve the heart, whereas "rheumatic fever" is not.

† Rheumatic subjects included 7 rheumatic heart disease and 4 rheumatic fever patients in group I and all 10 subjects from group II.

‡ Results of 4 separate chromatographic analyses.

of 5-methoxytryptamine in children with rheumatic heart disease and rheumatic fever, whether active or inactive (Table III). The amount of indoxylsulfate excretion, however, is significantly lower than that excreted by normal children (Table I). The excretion of these compounds in 3 patients with Hodgkin's disease and 2 with multiple myeloma was normal. One normal adult, among 33 nonrheumatic subjects, including 20 normal children, 8 normal adults, 3 adults with Hodgkin's disease, and 2 with multiple myeloma, has been found with measurable amounts of urinary 5-methoxytryptamine. The excretion of indoxylsulfate is apparently normal in this person.

#### DISCUSSION

This report is the first demonstration of 5-methoxytryptamine in the urines of rheumatic subjects; in fact, the substance has never been observed as a naturally occurring product in mammals, although it has been postulated.

Until recently, the *o*-methylation of catecholamines (20) but not of hydroxyindoles has been assumed to occur naturally in mammals. Erspamer (21) and Kveder and McIsaac (22) have demonstrated that feeding 5-methoxytryptamine to rats resulted in the excretion of 5-methoxyindoleacetic acid in the urine. The recent work of Lerner, Case, and Takahashi (23) on the isolation and metabolism of melatonin and 5-methoxyindoleacetic acid from mammalian tissue points to the natural occurrence of *o*-methylation of hydroxyindoles. These investigators believe that melatonin is formed and metabolized by acetylation of serotonin to *n*-acetylserotonin, which in turn is *o*-methylated to melatonin. Kopin and his associates (17) have shown that melatonin is completely metabolized in the body; the major pathway involves 6-hydroxylation, followed by conjugation mainly with sulfate, with only traces of administered melatonin going to form 5-methoxyindoleacetic acid. An *o*-methyltransferase (24) has been isolated from mammalian tissues that utilized 5-hydroxyindoleacetic acid as well as serotonin as substrates, but at a slow rate. The present concept of 5-methoxytryptamine metabolism is represented in Figure 2.

Our observations of excretion of no 5-methoxyindole compounds other than 5-methoxytryptamine

lead to the conclusion that rheumatic subjects are unable to form 5-methoxyindoleacetic acid from either 5-hydroxyindoleacetic acid or 5-methoxytryptamine.

Since Erspamer (21) and Kveder and McIsaac (22) have demonstrated the rapid conversion of 5-methoxytryptamine to 5-methoxyindoleacetic acid in the normal animal, the metabolic abnormality observed in the present study may be due to disfunction of a specific monoamine oxidase involved in this transformation. At the same time, increased *o*-methyltransferase activity may account for increased conversion of serotonin to 5-methoxytryptamine without apparent increased 5-hydroxyindoleacetic acid formation. The formation and metabolism of melatonin per se do not appear to be directly involved with these metabolic changes, but their relationship, if any, to melatonin is being explored further.

The general toxicity of 5-methoxytryptamine has been well demonstrated in cats, dogs, rats, sheep, calves, and rabbits (25, 26). To prevent the accumulation of the toxic amine, the oxidation of 5-methoxytryptamine to 5-methoxyindoleacetic acid should proceed rapidly. Further studies are essential to determine the presence or absence of this compound in the blood of rheumatic subjects.

Altered excretion of indoxylsulfate occurs under various circumstances and therefore provides no diagnostic help in rheumatic fever. A close relationship exists between the intestinal flora and indoxylsulfate excretion (27). Since rheumatic fever patients usually receive periodic doses of antibiotics, the intestinal flora should decrease, resulting in a decrease in the production and excretion of indoxylsulfate, which may account for the lower observed values. All 27 of the rheumatic subjects whose urines were checked for indoxylsulfate received sulfadiazine (9 patients) or penicillin (18 patients). The altered intestinal flora, possibly lowering the indoxylsulfate level, probably does not account for the 5-methoxytryptamine found in the urine. If this substance were absorbed from the intestinal tract, its urinary excretion would be anticipated as 5-methoxyindoleacetic acid, as described by Erspamer (21) and Kveder and McIsaac (22).

Other workers (28, 29) have cautioned that measurements of urinary constituents require rigid dietary and environmental controls. These

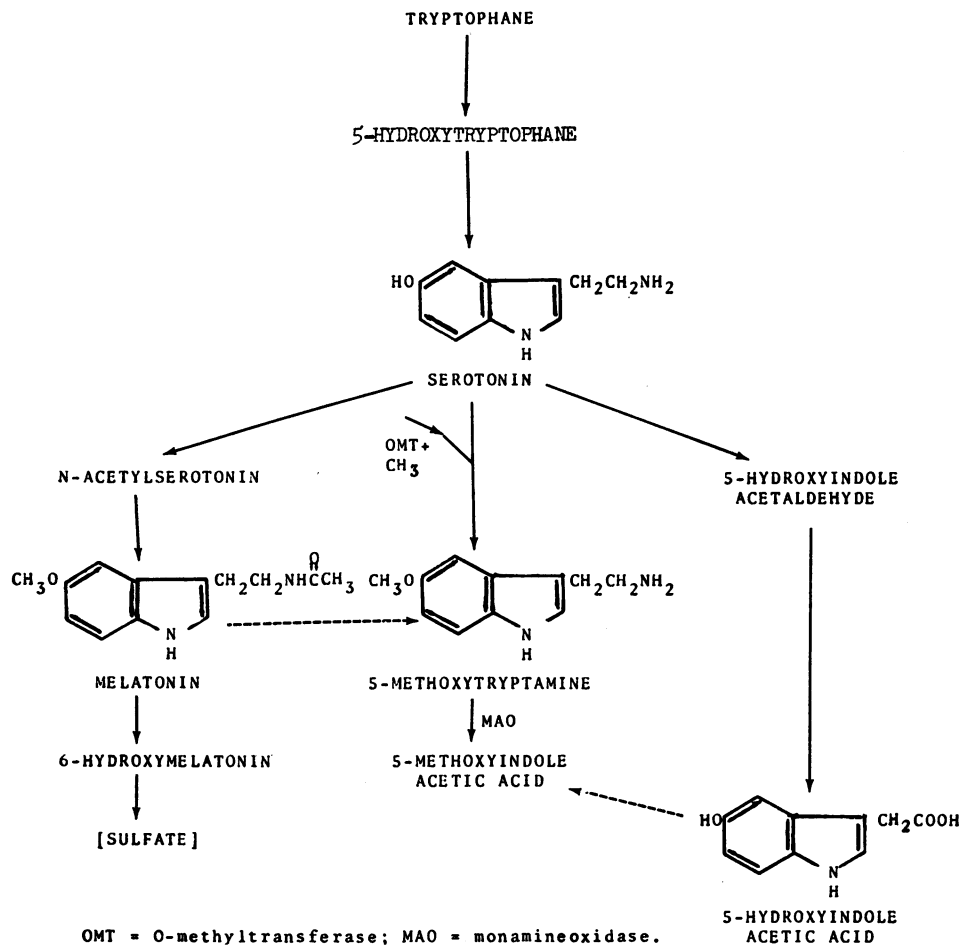


FIG. 2. THE PRESENT CONCEPT OF 5-METHOXYTRYPTAMINE METABOLISM. Adapted from Kveder and McIsaac (22).

controls were not attempted in the present study. The presence or absence of urinary 5-methoxytryptamine did not appear to be affected qualitatively by these extraneous environmental factors, although quantitative effects may be possible as demonstrated by the increases that occurred after administration of large amounts of L-tryptophan.

The diagnosis of rheumatic fever in patients suspected of suffering from this disease may be facilitated by the identification of 5-methoxytryptamine in the urine. Further study is in progress to determine the specificity of this finding and, if warranted, to develop a simple technic for its observation.

#### SUMMARY

A previously unreported excretory product, 5-methoxytryptamine, has been found in the urine

of 48 rheumatic subjects and in the urine of only one out of 33 nonrheumatic subjects. This product quadrupled in quantity on tryptophan loading. Details of the isolation procedure and identification tests used are presented. In children with rheumatic heart disease and rheumatic fever, the urinary excretion of a number of tryptophan-nicotinic acid metabolites was normal, with the exception of a reduced indoxylsulfate excretion. The significance and metabolic implications of these findings are reviewed. The possible value of identification of 5-methoxytryptamine in the urine, as an aid to the diagnosis of rheumatic fever, is discussed.

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