Formation of Indole-3-acetic Acid and Tryptamine in Animals

A METHOD FOR ESTIMATION OF INDOLE-3-ACETIC ACID IN TISSUES

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The original identification of indole-3-acetic acid as a growth factor for plants was made by Kogl et al. (1) on material isolated from human urine. The presence of IA in human urine has been amply verified by many investigators. Previous measurements of this substance in urine were determined by plant-growth bioassay or paper chromatography, no satisfactory chemical method being available.

To date, an elevated excretion of IA has been reported in two clinical conditions. Armstrong and Robinson (2) reported an increased IA level in the urine of patients with phenylketonuria. Recently, Baron et al. (3) have demonstrated that patients with a hereditary syndrome ("H" disease) consisting of a pellagra-like skin rash accompanied by intermittent cerebellar ataxia, mental deterioration, and renal amino-aciduria, excrete large amounts of this indole acid as well as excess amounts of tryptophan, indole-acetyl-glutamine, and indoxyl sulfate.

From both chemical and physiological standpoints, it is important to determine the intermediates involved in the formation of IA in animals. The conversion of tryptophan to IA might occur through one or more of the following mechanisms: (a) the formation of indole pyruvic acid by transamination followed by decarboxylation, (b) oxidative deamination by L-amino acid oxidase or, (c) decarboxylation to tryptamine followed by oxidative deamination of the amine.

This report describes sensitive and specific chemical methods for the measurement of IA in urine and tissues. Studies on the conversion of tryptophan to IA in vitro and in vivo indicate that whereas transamination is the major route, decarboxylation to tryptamine occurs to a significant extent. Catalysts for both metabolic pathways have been found in tissues and intestinal bacterial flora.

EXPERIMENTAL

Measurements of IA

Reagents—The reagents used were CHCl₃ (reagent grade); xanthodrol reagent, 0.1 per cent xanthodrol in glacial acetic acid which was prepared fresh before each determination; and 5 per cent sodium bisulfite.

IA in Urine—4 ml. of urine in a 40-ml. glass-stoppered centrifuge tube were acidified by the addition of 0.36 ml. of 12 N HCl. 10 ml. of CHCl₃ were added and the tube was shaken for 5 minutes. After centrifugation 9 ml. of the CHCl₃ layer was aspirated and discarded. An aliquot of the buffer was then assayed colorimetrically by the use of a modification of the xanthodrol reaction of Dickman and Crockett (4) as follows: 0.4 ml. of the pH 7.0 buffer extract was transferred to a test tube containing 0.4 ml. of 12 N HCl; 1 ml. of the xanthodrol reagent was added, followed 5 minutes later by 0.5 ml. of the bisulfite reagent. The solution was mixed and the pink color measured within 5 to 10 minutes at 520 mμ in a spectrophotometer. Standard and blank solutions were treated in the same manner. The recovery of 10 to 40 μg. of IA added to urine was in the range of 90 to 100 per cent.

1. Sensitivity. As little as 8 μg. of IA could be detected by this procedure. 20 μg. of IA carried through the entire method gave an optical density of 0.130. Optical density was proportional to IA concentration over the entire range from 8 to 200 μg.

2. Effects of Hydrolysis. Since indoles are stable in alkaline solution, basic hydrolysis was attempted first. Hydrolysis at 100° in 1 N NaOH (final concentration) resulted in a marked increase in color due to acid-extractable indole material. However, indole acids other than IA were formed. Acid hydrolysis also increased the apparent IA value but in this case the increase was shown chromatographically to represent only IA. The effect of acid hydrolysis on IA values in urine is shown in Table I. A 15-minute period of hydrolysis at 100° in 1 N HCl was found to give optimal results. IA in aqueous solutions was decomposed to a slight extent during acid hydrolysis, whereas IA added to urine was not. Addition of a small amount of urine to aqueous solutions of IA prevented its destruction. When acid hydrolysis was used in the studies presented here, the 40-ml. glass-stoppered centrifuge tubes containing 4 ml. of urine and 0.36 ml. of 12 N HCl (see above) were placed in a boiling water bath for 15 minutes. After cooling, the urine was assayed by the method described above. The results obtained by this over-all procedure will be referred to as total IA.

3. Specificity. As shown in Fig. 1, the distribution of the extracted urinary IA between CHCl₃ and aqueous solutions of various pH values compared well with that of synthetic IA. Further identification was obtained by paper chromatography. 50- to 100-ml. urine samples, both hydrolyzed and unhydrolyzed, were extracted by the procedure outlined above, and aliquots of the final buffer were chromatographed on paper. Unhydrolyzed urine and acid-hydrolyzed urine gave only one positive xanthodrol and Ehrlich spot in the three solvent systems tested (Table II). This was identical with IA. Although
indole lactic acid is also extractable by this procedure, it was not detected in extracts of normal urine subjected to paper chromatography. Tryptophan (3 mg.), 5-hydroxyindoleacetic acid (1 mg.), and indole (0.1 mg.) were not extracted in this procedure.

IA Formed Enzymatically—Precise measurement of the amounts of IA formed from tryptophan by tissue enzymes required a more sensitive procedure than the colorimetric method used for urine. Increased sensitivity was obtained by the use of a fluorometric assay following protein precipitation and extraction. Each incubation mixture (3.5 ml.) was transferred to a plastic centrifuge tube containing an equal volume of water. Proteins were precipitated by the addition of 1 ml. of 10 per cent zinc sulfate and 0.5 ml. of 1 N NaOH. After thorough mixing, the tubes were centrifuged at high speed (8,000 × g) using an angle head centrifuge. An aliquot portion of the protein-free filtrate containing 1.0 to 10.0 μg. of IA was transferred to a 40-ml. glass-stoppered centrifuge tube and acidified by the addition of 0.3 ml. of 6 N HCl. 15 ml. of CHCl₃ were added and the tube was shaken for 1 to 2 minutes. After centrifugation the aqueous layer was removed and discarded. The CHCl₃ phase was washed by shaking with 10 ml. of 0.1 N HCl, thus removing any tryptophan that had been extracted into the CHCl₃. 10 ml. of the CHCl₃ were then transferred to another 40-ml. glass-stoppered shaking tube containing 1.5 ml. of 0.5 M phosphate buffer, pH 7.0. After thorough shaking and centrifugation, the CHCl₃ layer was discarded. The aqueous phase was transferred to a cuvette and the fluorescence determined in an Aminco-Bowman spectrophotofluorometer (activation wave length, 285 mp; fluorescence wave length, 365 mp). Enzyme blanks, standards, and reagent blanks were all treated in the same manner. IA added to tissue was recovered quantitatively.

Because of the small amounts of IA formed and the fact that tryptophan has similar fluorescence characteristics, it was important to determine to what extent tryptophan was extracted through the procedure. Extraction of 3 mg. of tryptophan showed that less than 0.3 μg. of the amino acid appeared in the final extract.

**Urinary Excretion of IA**

24-hour collections of urine were obtained from a group of hospitalized young adult normal volunteers and from a large number of patients with various clinical conditions. The IA values are summarized in Table III. There was considerable individual variation in the excretion of IA. However, the levels in the same individual, based on the daily assay of IA in three normal subjects for a 2-week period, were fairly constant from day to day. Five instances of elevated IA excretion were found in this study. Two patients with phenylketonuria were found to have high excretions of CHCl₃-extractable indole acids (20 to 33 mg. per day) which chromatographically were found to be primarily indole lactic acid. In the other conditions in which high values were found, chromatography showed only IA to be present.

To evaluate the role of intestinal bacteria in the production of IA, neomycin (0.5 gm. every 6 hours) in combination with...
urinary 5-hydroxyindoleacetic acid in man (6). Tryptophan could be accounted for by the increased excretion of IA. It has been shown that as much as 100 mg. per kg. of tryptophan are required to produce a significant increase in IA over control levels of about 200 mg. per day (Fig. 2). The inability to eliminate IA from normal urine completely or to reduce elevated levels to the normal range with orally administered antibiotics suggested that tissue degradation of tryptophan must also be considered as an important source of urinary IA.

Effect of Oral Doses of L-tryptophan on Urinary Excretion of IA and 5-Hydroxyindoleacetic Acid—It was reported previously (5) that the urinary excretion of 5-hydroxyindoleacetic acid in dogs was not affected appreciably by the administration of fairly large amounts of tryptophan. The effects of oral doses of L-tryptophan (20 mg. per kg.) on IA and 5-hydroxyindoleacetic acid excretion in man are shown in Fig. 3. The slight effect of tryptophan on 5-hydroxyindoleacetic acid excretion observed in these studies agrees with the earlier results in dogs (5). The excretion of IA, on the other hand, was increased markedly for a period of several hours after the administration of tryptophan. Nevertheless, only a small percentage (about 0.1 per cent) of the administered tryptophan could be accounted for by the increased excretion of IA. It has been shown that as much as 100 mg. per kg. of tryptophan are required to produce a significant increase in urinary 5-hydroxyindoleacetic acid in man (6).

Conversion of Tryptophan to IA in Vitro

The results of the experiments in vivo suggested that conversion of tryptophan to IA was brought about through the action of a mixture of both tissue and bacterial enzymes. The following experiments were carried out to explore these suggestions.

Animal Tissues—Enzyme incubations were carried out in 20-ml. beakers at 37° in a Dubnoff metabolic incubator. The incubation mixture had a final volume of 3.5 ml., containing enzyme, substrate, buffer, and water. Specific details are given in the appropriate tables. The high speed supernatant fraction from various tissues (1 part of tissue homogenized with 4 parts of water and centrifuged at 80,000 X g) was tested for activity (Table IV). Guinea pig kidney and liver extracts catalyzed IA formation most actively and many other tissues were also active. Guinea pig liver was used as the source of enzyme for further studies. A preliminary 6- to 10-fold purification was obtained by fractional precipitation with ammonium sulfate. The protein fraction, precipitated between 35 and 50 per cent of ammonium sulfate saturation, contained the major portion of enzymatic activity. The optimal pH for IA formation was 8.0 and maximal activity was obtained when 3 mg. of L-tryptophan were present in the standard incubation mixture (final molar concentration 4.2 X 10-5). As shown in Figs. 4 and 5, IA formation was proportional to enzyme concentration and time of incubation under the conditions that obtained.

It seemed probable that indole pyruvic acid was the inter-
mediate in IA formation. Although chromatography of incubation extracts showed only the presence of IA, the lability of indole pyruvic acid would very likely have prevented its detection. The following findings are consistent with a transamination mechanism for the formation of most of the IA shown in Table IV. When α-ketoglutarate (1 mg.) or pyridoxal phosphate (25 μg.) were added, they each enhanced IA formation by 20 to 30 per cent. The pyridoxal phosphate antagonists, L-penicillamine (1 × 10⁻³ M) and semicarbazide (2 × 10⁻² M), inhibited IA formation 50 and 100 per cent, respectively. Furthermore, tryptophan conversion to IA was not appreciably diminished by anaerobic conditions. An interesting observation was that isopropyl isonicotinyl hydrazine (iproniazid), which has been considered a fairly specific monoamine oxidase inhibitor, completely abolished IA formation when added in vitro (10⁻³ M) or when previously administered by injection into guinea pigs (150 mg. per kg.). Although smaller amounts of tryptamine were also formed by these tissues (see below), the complete inhibition of IA formation by iproniazid suggested that this drug is also an inhibitor of tryptophan transamination in animal tissues.

Some evidence for decarboxylation of tryptophan to tryptamine in animal tissues has been presented previously (7). However, this has never been verified and studies in this laboratory have, in the past, failed to detect such a reaction (8). However, since some IA could conceivably be formed through such a route, additional experiments were carried out to determine whether this actually occurred. Tryptophan-2-C¹⁴ (170,000 c.p.m.) (plus 3 mg. of L-tryptophan) was incubated for 3 hours with kidney homogenate (1 gm. of tissue) from rats pretreated with iproniazid (150 mg. per kg.). The incubation flask also contained 1 mg. of nonisotopic tryptamine, 50 μg. of pyridoxal phosphate, and 0.05 M phosphate buffer, pH 7.4, to a total volume of 7 ml. At the end of the incubation the contents of the flask were transferred to a 60-ml. glass-stoppered bottle containing 2 ml. of 1 N NaOH and 20 ml. of benzene. After shaking and centrifuging, the benzene was transferred to another tube and washed three times with equal volumes of 0.1 N NaOH to remove traces of tryptophan. The benzene layer was then shaken with 2 ml. of 0.1 N HCl. The acid extract was evaporated to dryness, taken up in about 0.3 ml. of methanol and transferred as a 2 inch band to Whatman No. 1 paper for chromatography. When the chromatogram was developed with n-propanol-1 N NH₃ (5:1), one indole band appeared, as shown by spraying a segment of the chromatogram with p-dimethylaminobenzaldehyde. This was identical with the tryptamine controls in color and RF. When the tryptamine carrier was eluted, it was found to contain 870 c.p.m. per μmole representing a total of 5400 c.p.m. formed in the 3 hour incubation. That this radioactivity was really associated with the tryptamine was shown by treating the carrier with excess purified monoamine oxidase, aldehyde

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**Table IV**  
**IA formation in various tissues**

<table>
<thead>
<tr>
<th>Tissue</th>
<th>IA formed μg/gm. tissue/hr.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Guinea pig liver</td>
<td>94.8</td>
</tr>
<tr>
<td>Guinea pig kidney</td>
<td>297.0</td>
</tr>
<tr>
<td>Rabbit brain</td>
<td>13.6</td>
</tr>
<tr>
<td>Rabbit kidney</td>
<td>30.0</td>
</tr>
<tr>
<td>Rabbit lung</td>
<td>8.4</td>
</tr>
<tr>
<td>Rabbit liver</td>
<td>29.4</td>
</tr>
<tr>
<td>Rat kidney</td>
<td>33.0</td>
</tr>
<tr>
<td>Rat liver</td>
<td>20.4</td>
</tr>
</tbody>
</table>

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The incubation mixture contained 1 ml. of guinea pig liver high speed supernatant (equivalent to 333 mg. of wet tissue), 0.5 ml. of 0.5 M phosphate buffer, pH 8.0, 3 mg. of L-tryptophan, and H₂O to a total volume of 3.5 ml.

**Fig. 4.** IA formation as a function of time. The incubation mixture contained 1 ml. of guinea pig liver high speed supernatant (equivalent to 333 mg. of wet tissue), 0.5 ml. of 0.5 M phosphate buffer, pH 8.0, 3 mg. of L-tryptophan, and H₂O to a total volume of 3.5 ml.

**Fig. 5.** IA formation as a function of enzyme concentration. The incubation beakers contained guinea pig liver high speed supernatant (as in Fig. 4); 0.5 ml. of 0.5 M phosphate buffer, pH 8.0; 3 mg. of L-tryptophan; and H₂O to a total volume of 3.5 ml. Incubation was for 1 hour.
dehydrogenase, and diposphopyridine nucleotide as described previously (9). It was shown that for each equivalent of diposphopyridine nucleotide reduced, 1 equivalent of IA was formed. The isolated IA was found to have approximately the same specific activity as the original carrier tryptamine. The finding of tryptophan decarboxylation catalyzed by mammalian tissue was surprising enough but the extent of the conversion in this experiment (approximately) 75 pg. of tryptamine in 3 hours) indicated that nonisotopic procedures should be adequate for its detection. It should be pointed out that 900 Mg. of IA would have been formed and that tryptamine formation accounts for only a small percentage of the total IA formed in any tissue. When nonradioactive tryptophan was incubated with kidney homogenates from Marsilid-pretreated guinea pigs (150 mg. per kg.), tryptamine was indeed found. The amine was again isolated by the procedure described above and characterized by chromatography in several solvent systems. It appears, however, that even in the presence of iproniazid (and in the absence of oxygen) tryptamine is further metabolized. For this reason the amine does not accumulate to any large extent in nonisotopic studies.

Studies with Fecal Bacteria—Mixed fecal bacteria were obtained from the patient with idiopathic sprue who had a high IA excretion (Table III). Inocula were prepared first by subculturing stool samples in tubes containing several milliliters of medium (2 per cent tryptone, 1 per cent glucose, 0.5 per cent yeast extract). The inocula were then transferred to 1 l. of the same medium and incubated for 16 hours at room temperature. The cells were harvested by centrifugation, and washed twice with 300 ml. of 0.1 m phosphate buffer, pH 6.0, after which they were suspended in 40 ml. of the same buffer. Each milliliter of suspension contained approximately 18 mg. of dried cells. Portions of this suspension were incubated with tryptophan and tryptamine for 60 hours. The results are shown in Table V. It is evident that the fecal bacteria form IA. The marked stimulation by α-ketoglutarate is indicative of transamination. The transaminating activity of a number of fecal bacteria has been known for some time (10). The bacterial transamination was not inhibited by iproniazid.

Of greater interest was the finding of tryptamine formation. Here, as in the case of tissues, decarboxylation of tryptophan by fecal bacteria had been reported (11) but never verified. The tryptamine formed by bacteria was identified by its extractability into organic solvents from alkali and reextraction into acid (see above), its characteristic colors with p-dimethylamino-benzaldehyde and xanthohydrol, and by its Rp values on paper chromatograms using propanol-1: NH₃ (5:1) and n-butanol-1: HCl. The mixed bacteria did not metabolize tryptamine. This would suggest that tryptamine formed by intestinal flora may be absorbed from the gut.

**DISCUSSION**

The development of a simple chemical method for the assay of IA in urine has made possible a study of the urinary excretion of this metabolite in normal and pathological states in man. The daily excretion of IA varied considerably even among normal individuals. The finding of elevated levels in five patients with apparently unrelated diseases requires explanation. A possible common denominator could be alterations in the production of IA by intestinal bacteria, although there were apparent symptoms related to the intestine in only one case. The finding of an IA value of 9.4 mg. per day in the patient whose intestinal tract had been almost entirely removed indicates that significant amounts of IA are formed in human tissues. IA excretion may prove to be a simple and sensitive index of alterations in tryptophan metabolism. Although the significance of such alterations is not known, they may reflect not only an altered bacterial flora in the gut but also an altered tryptophan metabolism in the tissues.

Recent observations by Mirsky et al. (12) have shown that IA is an inhibitor of "insulnase." One might expect either spontaneous hypoglycemia or decreased insulin tolerance in a patient excreting large amounts of IA. None of the patients with high IA was hypoglycemic and one was actually a diabetic. Insulin tolerance tests in one patient when his IA excretion was 50 mg. per day did not significantly from controls.

It is apparent from the present studies that metabolic conversions of tryptophan take place in the intact animal as illustrated in Diagram 1. Both pathways are demonstrable in the animal tissues as well as in the fecal bacteria. Although the bulk of IA probably arises from transamination, the smaller amounts arising through decarboxylation deserve serious consideration since tryptamine is a potent pharmacological agent. These findings are consistent with the recent report of tryptamine in human urine (13). Further studies on the nature of the tryptophan decarboxylase in mammalian tissues and in human fecal bacteria are in progress.

**TABLE V**

<table>
<thead>
<tr>
<th>Flask No.</th>
<th>Contents</th>
<th>IA found</th>
<th>Tryptamine found</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>200 mg. L-tryptophan</td>
<td>0.25</td>
<td>0.18</td>
</tr>
<tr>
<td>2</td>
<td>200 mg. L-tryptophan + 50 mg. α-ketoglutarate</td>
<td>5.20</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>200 mg. L-tryptophan + 35 mg. iproniazid</td>
<td>0.24</td>
<td>0.16</td>
</tr>
<tr>
<td>4</td>
<td>50 mg. tryptamine</td>
<td>0.00</td>
<td>5.00</td>
</tr>
</tbody>
</table>

* Both indoleacetic and indolepyruvic acids are measured in this assay.

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A method for the quantitative measurement of tryptamine in tissues based on solvent extraction followed by spectrophotometric assay was used. The details of this procedure will be described in a future publication.
SUMMARY

A simple chemical method for the determination of indole-3-acetic acid in urine and tissues is described. The daily excretion of this acid in man is generally in the range of 5 to 18 mg. per day and may be as high as 200 mg. per day in certain pathological states. The excretion of indole-3-acetic acid was increased markedly by tryptophan loading. Most of this acid which is formed by mammalian tissues in vitro and by human fecal bacteria, arises through transamination involving α-ketoglutarate and pyridoxal phosphate. Mammalian kidney and liver, and human fecal bacteria were both found to be capable of decarboxylating tryptophan to yield tryptamine.

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REFERENCES

Formation of Indole-3-acetic Acid and Tryptamine in Animals: A METHOD FOR ESTIMATION OF INDOLE-3-ACETIC ACID IN TISSUES
Herbert Weissbach, William King, Albert Sjoerdsma and Sidney Udenfriend

J. Biol. Chem. 1959, 234:81-86.

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