A CHROMATOGRAPHIC METHOD FOR THE DETECTION OF TRYPTOPHAN METABOLITES*

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During the course of investigations involving the action of tissue slices on tryptophan, the aqueous medium in which L-kynurenine sulfate had been incubated with kidney slices was found to have acquired a marked bluish violet fluorescence. Subsequent investigation showed that several known metabolites of tryptophan fluoresce when suitably exposed to ultraviolet light. Advantage has been taken of this behavior and of separation by paper chromatography to identify a number of the products formed when tryptophan and certain of its metabolites are incubated with slices of rat liver or rat kidney. This communication describes our observations.

EXPERIMENTAL

Materials—The L-tryptophan and the L-kynurenine employed were from lots whose preparation and purity have been described elsewhere (1). The kynurenic acid was a synthetic product purchased from Hoffmann-La Roche, Inc. It melted at 270°, as compared with published values ranging between 257-278° for kynurenic acid produced in the animal body (2). It behaved like the natural product on chromatographic analysis. The synthetic xanthurenic acid1 melted at 286-288° and gave an intense green color with ferric chloride. Lepkovsky et al. (3) record 288° as the melting point of xanthurenic acid isolated from rat urine. The anthranilic acid was an Eastman Kodak Company product which had been recrystallized twice from water.

The 3-hydroxyanthranilic acid was prepared essentially as directed in references cited by Bray et al. (4). Its melting point of 250-254° agreed well with reported values (4, 5). The α-hydroxy-DL-tryptophan was from a lot synthesized by Kotake, Sakan, and Miwa (6).

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Tissue Preparations—Tissue slices were prepared from the livers and kidneys of healthy well fed albino rats with the aid of a Stadie-Riggs slicer. Prior to weighing, they were kept in covered Petri dishes surrounded with crushed ice. Before use, they were washed with Krebs-Ringer-phosphate solution, pH 7.4.

Incubation—Approximately 800 mg. of liver slices or 400 mg. of kidney slices were placed in each test vessel and covered with 2 ml. of Krebs-Ringer-phosphate buffer, pH 7.4. To this was added 1 ml. of a solution which contained 1 mg. of the test substance in Krebs-Ringer-phosphate solution, adjusted to pH 7.4. The contents were incubated for 3 hours at 37° in an atmosphere of oxygen.

Chromatographic Procedure—5 to 10 μl. aliquots of the media from the Warburg vessels were placed, without deproteinization, in spots approximately 5 cm. from one end of 2.5 X 40 cm. strips of Whatman No. 1 filter paper. The strips were placed in an air-tight vessel and subjected to irrigation by capillary ascent (7) with a solvent mixture consisting of 4 volumes of methanol and 2 volumes each of n-butanol, benzene, and water. After 10 to 12 hours, the strips were removed, dried in air at room temperature, and examined for fluorescence under a Hanovia Inspectolite.

Deproteinization and removal of ions were found unessential; hence they were avoided lest they introduce complications. To compensate for any possible influence of the protein and ionic components in the media (or of other factors) upon the migration, control tests were run simultaneously. In these, the metabolites were mixed with media in which liver or kidney slices had been incubated with the buffer solution alone. In each instance, the same quantity of metabolite was used as in the experimental flask, and the same volume of mixture was employed for chromatography.

Results

The chromatographic patterns obtained in the tests are shown in Fig. 1. The spots are reproduced diagrammatically, because they differed markedly in color and intensity of fluorescence (see Fig. 1, legend), and consequently the photographic reproduction of some of them was poor. In the few instances in which different substances had nearly the same migration rate, divergence in color definitely aided identification.

Strips 1 and 8 show the relative positions taken when kynurenine, α-hydroxytryptophan, kynurenic acid, xanthurenic acid, 3-hydroxyanthranilic acid, and anthranilic acid were added separately to control Warburg flasks in which liver (Strip 1) or kidney slices (Strip 8) had been incubated with the buffer solution alone.

The fluorescence of anthranilic acid and its 3-hydroxy derivative is well known (4, 8). Kynurenine sulfate shows no fluorescence in aqueous solu-
tion, but does fluoresce in organic solvents and when dried on filter paper. The kynurenic acid spot shows little fluorescence when the dried chromatogram is first viewed under the Inspectolite, but becomes fluorescent after an initial exposure of several minutes. The xanthurenic acid spot is especially brilliant.

Tryptophan is not detectable by fluorescence, but its position can, of course, be located with ninhydrin or the p-dimethylaminobenzaldehyde.

![Fig. 1. Chromatograms of fluorescent metabolites produced by the action of slices of rat liver and kidney on L-tryptophan and related intermediates. For details as to their preparation see the text. Strips 1 and 8 are composites of the several chromatograms of control mixtures prepared by adding each of the test substances separately to media incubated with slices of liver (1) or kidney (8), but without added substrate. The other strips represent tests of media after incubation of the substance indicated at the bottom of the strip with liver (Strips 2 to 7) or with kidney (Strips 9 to 14) slices. Chromatograms of media in which 3-hydroxyanthranilic acid had been incubated with liver or kidney slices were free of fluorescing spots; hence they are not reproduced.

In ultraviolet light the anthranilic acid (Spot AA) and 3-hydroxyanthranilic acid (Spot HA) appeared as violet spots. Xanthurenic acid (Spot XA) was a brilliant light blue, kynurenic acid (Spot KA) a brilliant yellowish green, and α-hydroxytryptophan (Spot HT) and kynurenine (Spot K) a dull, washed out blue, with a lavender tinge. The substances responsible for Spots XA-1, XA-2, XA-3, HT-1, and AA-1 were not identified. The color of Spot XA-1 was bluish to violet, of Spot XA-2 grayish lavender, and of Spot XA-3 somewhat more violet than of Spot XA-2. Spot HT-1 gave a fainter and somewhat grayer color than Spot HT. The color of Spot AA-1 was similar to that of Spot AA.
Several metabolites of tryptophan also respond to these reagents. Where it can be applied, fluorescence seems to be the more sensitive method of detection.

The patterns produced on chromatographing media in which α-hydroxytryptophan (Strips 3 and 10), kynurenine acid (Strips 5 and 12), or anthranilic acid (Strips 7 and 14) had been incubated indicate that the fluorescing metabolites produced by the kidney were the same as those formed by the liver. Incubation with either tissue caused the complete disappearance of 3-hydroxyanthranilic acid and produced no fluorescing derivatives. The blank chromatograms obtained are not reproduced in Fig. 1. The rapid metabolism of 3-hydroxyanthranilic acid by rat liver has been noted previously (8, 10–12). Kynurenic acid appeared to be relatively resistant to destruction by either liver or kidney tissue. Anthranilic acid was partially converted into a second unidentified fluorescing substance, but colorimetric analysis of the media3 indicated that the amount converted must have been very small. When α-hydroxytryptophan was added to incubated control media and chromatographed, it showed only one spot (HT). The substance responsible for the spot (HT-1) produced by its incubation with liver or kidney slices has not been identified.

The chromatograms show that slices of rat liver were able to convert L-tryptophan (Strip 2) to kynurenine, kynurenic acid, and anthranilic acid, and to transform kynurenine (Strip 4) into kynurenic acid and anthranilic acid. Under the conditions employed, kidney slices produced no fluorescing metabolites from L-tryptophan (Strip 9). They metabolized L-kynurenine (Strip 11) about twice as rapidly per gm. of wet weight3 as did liver slices. They appeared to produce as much kynurenic acid, but no anthranilic acid could be detected. Two other fluorescing substances, as yet unidentified, were also formed. These occupied the same relative positions and showed the same fluorescences as the two products produced from xanthurenic acid (Strip 6) by liver slices. One of the products (XA-3), but not the other (XA-2), was produced from xanthurenic acid by kidney slices (Strip 13), along with a third substance (XA-1) which occupied a position close to xanthurenic acid on the strip.

DISCUSSION

Data on the specific sites in which metabolism of L-tryptophan occurs in the intact rat are lacking. This is true also of the rabbit, in the blood of which kynurenine is reported to accumulate after large doses of L-tryptophan (13).

In the dog, hepatectomy markedly decreases the amount of kynurenic acid excreted after tryptophan is injected subcutaneously and perfusion through the liver converts kynurenine into kynurenic acid (14). The

3Mason, M., and Berg, C. P., unpublished data.
capacity of slices from rat liver, but not of those from rat kidney, to form kynurenine and kynurenic acid from L-tryptophan is in harmony with these observations. However, the rapid metabolism of L-kynurenine by kidney slices seems to indicate that, at least in the rat, the kidney may participate together with the liver in the production of kynurenic acid from kynurenine.

Some years ago Kotake (15) postulated that α-hydroxytryptophan might function as an intermediate in the conversion of tryptophan to kynurenine. This assumption was supported by the finding of Butenandt, Weidel, and Becker (16) that α-hydroxytryptophan promotes the development of eye pigment in insect mutants, though somewhat less readily than does kynurenine. The chromatographic evidence reported here seems to indicate that slices of neither the liver nor the kidney of the rat can convert α-hydroxytryptophan to kynurenine. We have been advised⁴ that Sakan and Hayaishi (17) and Kikkawa⁵ have also obtained negative evidence as to the intermediate character of α-hydroxytryptophan, the former with bacteria, the latter with silkworm larvae.

Anthranilic acid has been reported to be formed in relatively large amounts by homogenates of cat liver and by the intact cat (18). Our data show that it is produced by rat liver, but probably not by rat kidney. If 3-hydroxyanthranilic acid is formed during the incubation of tryptophan or kynurenine with liver or kidney slices, it must be rapidly degraded into non-fluorescing substances. In certain insects 3-hydroxykynurenine has been reported to be an intermediate metabolite of tryptophan (19). No evidence was obtained for its formation or accumulation, but since none of the product was available for comparative studies, the possibility that it could have escaped detection cannot be discounted.

The metabolism of xanthurenic acid by the liver and kidney slices used in our tests was too slow to justify the assumption that either tissue readily destroyed it. Reid et al. (20) have reported that it is destroyed in the rat receiving pyridoxine. It is possible that it undergoes metabolism more rapidly in the liver or kidney of the intact animal or that some other tissue participates in its degradation.

SUMMARY

A technique for the study of tryptophan metabolism is described which involves the chromatographic separation and identification of several fluorescent metabolites. The technique has been used to determine comparatively the capacities of slices of rat liver and slices of rat kidney to metabolize tryptophan.

The data indicate that the liver of the normal rat is able to convert L-

⁴ Personal communication from Dr. O. Hayaishi.
⁵ Kikkawa, H., personal communication to Dr. O. Hayaishi.
tryptophan into kynurenine, kynurenic acid, and, to a small extent, anthranilic acid. It converts kynurenine into kynurenic acid and anthranilic acid. By contrast, the kidney of the normal rat is apparently unable to produce any of these or any other fluorescent metabolites from L-tryptophan. However, it metabolizes L-kynurenine even more rapidly than does the liver. Instead of anthranilic acid, it produces two other fluorescent metabolites which appear to be related to xanthurenic acid.

Xanthurenic acid and anthranilic acid were metabolized to a limited extent by slices of both the liver and the kidney, as judged by the formation of new but unidentified fluorescing substances. Kynurenic acid was apparently not attacked by either to any marked degree. Both tissues acted rapidly on 3-hydroxyanthranilic acid, but produced no fluorescent metabolites.

Incubation of α-hydroxy-DL-tryptophan with slices of either liver or kidney yielded small amounts of apparently the same fluorescing substance. The chromatographic pattern produced by the liver slices was quite distinct from the pattern which they produced from L-tryptophan or L-kynurenine, thus indicating that α-hydroxytryptophan may not be an intermediate in the conversion of L-tryptophan to kynurenine and kynurenic acid in the rat.

BIBLIOGRAPHY

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