The Effect of Riboflavin Deficiency upon the Metabolism of Tryptophan by Liver and Kidney Tissue*

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Riboflavin-deficient rats have been observed to excrete considerably more kynurenic acid than normal rats after the ingestion of L-tryptophan, but only slightly more kynurenine and xanthurenic acid (1). Increased excretion of anthranilic acid has also been noted (2, 3). Although the administration of tryptophan by injection also produces an increased excretion of xanthurenic acid in riboflavin deficiency, the injection of kynurenine is reported to yield somewhat less in the deficient than in the normal animal (4).

Riboflavin is a component of the prosthetic groups of D- and L-amino acid oxidase. Its lack might therefore be assumed to alter the production of β-3-indolepyruvic acid, and through such alteration to influence the production and output of other tryptophan metabolites. This hypothesis has been tested by determining the metabolite production after the incubation of liver and kidney slices from riboflavin-deficient and normal animals with L-, Dl-, and D-tryptophan, L- and D-ky

EXPERIMENTAL

Materials—The DL-tryptophan used in these studies was a recrystallized synthetic product.1 The D- and L-tryptophan were prepared through resolution of the brucine salt of acetyl

Incubation-Washed tissue slices, 400 mg. wet weight, were incubated in Warburg vessels for 3 hours at 37° in an atmosphere of O2 in a medium consisting of 2 ml. of Krebs-Henseleit buffer, pH 7.4 (11), or 2 ml. of sodium pyrophosphate buffer, pH 8.2 (12), plus 1 ml. of buffered substrate. Per ml. of the buffered substrate contained 1 mg. of tryptophan (D-, L-, or DL-), kynurenine sulfate (D- or L-), or indolepyruvic acid. After the incubation, the medium was decanted from the tissue and aliquots were used for analysis.

Methods of Analysis—Depending upon the substrate employed and the response to paper chromatography, the media were analyzed for all or several of the following: tryptophan, indole-
pyruvic acid, kynurenine, anthranilic acid, kynurenic acid, and xanthurenic acid.

Indolepyruvic acid gives a color when spotted on paper and sprayed with Ehrlich’s reagent (0.2 per cent solution of p-dimethylaminobenzaldehyde in 2 N HCl in 80 per cent ethanol); with Salkowski’s reagent (0.01 M FeCl₃ in 35 per cent HClO₃); or with diazotized sulfanilic acid (13).

The paper chromatographic technique recommended by Stowe and Thimann (13) for the separation of indole derivatives was first tried without success. When Whatman No. 1 filter paper strips were spotted with 50 to 500 μg. of indolepyruvic acid and developed with isopropanol, water, and ammonia, in an 8:1:1 ratio, no authentic indolepyruvic acid spot could be detected. Marked streaking, indicative of extensive destruction, was observed. Subsequently Bentley et al. (14) recorded a similar experience, but reported that the destruction could be avoided by the use of an acidic solvent. With the solvent of Mason and Berg (15), made acid by the addition of 2 ml. of glacial acetic acid per 100 ml. (16), definite indolepyruvic acid spots were detected. However, subsequent assays by the procedure outlined below showed that, even when the solvent was thus modified, marked degradation (60 to 80 per cent) occurred.

Application of the colorimetric method of Sealock (17) for α-keto acids to the estimation of indolepyruvic acid, whether in eluates or in pure solution, produced an interfering turbidity. This could be avoided by extracting the reaction mixture with ether, evaporating the solvent, and partitioning the residue between equal volumes of ammonium hydroxide solution and chloroform, as directed by Cavallini and Frontali (18). The phenylhydrazones of the keto acids are soluble in the former, the neutral phenylhydrazones and phenylhydrazine in the latter.

When 150 to 900 μg. of indolepyruvic acid were added to the media, application of this procedure accounted for 92 to 98 per cent. When media to which indolepyruvic acid or tryptophan had been added before incubation were tested for extraneous α-keto acids by chromatographing the dinitrophenylhydrazones or the diazotizate at 37° for 1 hour (19, 21), but when large amounts were present, the destruction was not complete. Heating with sodium hydroxide and sodium carbonate at 100° for 3 hours (22) destroyed the kynurenine completely, but also produced a partial loss of the tryptophan. Since the use of large amounts of medium seemed desirable, Tabone’s solvent (5 ml. of concentrated H₂SO₄ per l. of 80 per cent ethanol) was tried (21). This produced a wide enough separation of the tryptophan (Rₚ 0.56) and the kynurenine (Rₚ 0.46) to eliminate the overlapping completely in most instances, or to decrease it to the point where the residual kynurenine could readily be destroyed by incubation after diazotization. Interference by indoleacetic acid, which has been produced from the indolepyruvic acid, was ruled out because indoleacetic acid had a relative Rₚ value of 0.8 when chromatographed with Tabone’s solvent.

In determining tryptophan, therefore, 0.2 ml. of medium was spotted on Whatman No. 1 filter paper sheets, along with control strips of 0.07 ml. of L- and D-tryptophan solutions (1 mg. per ml.) Cylinders prepared from the sheets were allowed to stand in air tight vessels for 12 to 14 hours to allow the Tabone solvent to ascend. The spots produced from the L- and D-tryptophan were located by spraying the dried paper with Ehrlich’s reagent. The corresponding unsprayed areas were cut out and clipped into narrow strips for elution with successive 2-ml. portions of freshly prepared 4 per cent trichloroacetic acid solution, followed by centrifugation, until 10 ml. of eluate and washings had been obtained. To these 10 ml., 1 ml. of 0.1 per cent sodium nitrite solution was added. After 3 minutes, the mixture was incubated for 1 hour to destroy any contaminating kynurenine. One ml. of 1 per cent sodium nitrite was then added, followed in 30 minutes by 0.5 ml. of 8 per cent ammonium sulfamate solution, and in 3 more minutes by 1 ml. of 0.1 per cent N-1-naphthylethylendiamine hydrochloride solution. The purple color was assayed 30 minutes later at 500 μm in the Coleman spectrophotometer. The tryptophan content was calculated from a standard calibration curve. The method responded well to 5 μg. of tryptophan in 13.5 ml. of solution.

Kynurenine was estimated by a procedure similar to that described for tryptophan, using 0.2 ml. of medium, but in this instance irrigating for 10 to 12 hours with the acidified solvent of Mason and Berg (15, 16). The control spots produced with 0.05 ml. of L- and D-kynurenine sulfate solutions (1 mg. per ml.) and the kynurenine spots produced from the incubated media were located by their fluorescence under ultraviolet light. The 10 ml. of eluate obtained from each of the kynurenine spots were

Tryptophan was estimated by the diazotization technique previously described (19, 20). Kynurenine, anthranilic acid, and indolepyruvic acid also respond. Upon the addition of the sodium nitrite solution to an admixture, indolepyruvic acid gives a purplish color which persists until the final colorimetric assay. On Whatman No. 1 filter paper strips the acidified Mason and Berg solvent (15, 16) effectively separated the anthranilic and indolepyruvic acids chromatographically from the tryptophan and the kynurenine. The two former substances migrated much more rapidly (Rₚ approximately 0.94) than the two latter (Rₚ approximately 0.44 and 0.42). However, relatively large amounts of tryptophan and kynurenine produced considerable overlapping. The kynurenine could be determined without interference by the tryptophan because it required much less drastic conditions than the latter for diazotization. Moderate amounts of kynurenine could readily be destroyed by incubating the diazotize at 37° for 1 hour (19, 21), but when large amounts were present, the destruction was not complete. Heating with sodium hydroxide and sodium carbonate at 100° for 3 hours (22) destroyed the kynurenine completely, but also produced a partial loss of the tryptophan. Since the use of large amounts of medium seemed desirable, Tabone’s solvent (5 ml. of concentrated H₂SO₄ per l. of 80 per cent ethanol) was tried (21). This produced a wide enough separation of the tryptophan (Rₚ 0.56) and the kynurenine (Rₚ 0.46) to eliminate the overlapping completely in most instances, or to decrease it to the point where the residual kynurenine could readily be destroyed by incubation after diazotization. Interference by indoleacetic acid, which has been produced from the indolepyruvic acid, was ruled out because indoleacetic acid had a relative Rₚ value of 0.8 when chromatographed with Tabone’s solvent.
mixed with 1 ml. of 0.1 per cent sodium nitrite solution. After 3 minutes, excess nitrite was destroyed by the addition of 1 ml. of 4 per cent ammonium sulfamate. Three minutes later 1 ml. of 0.1 per cent N-Naphthylethylenediamine hydrochloride solution was added to develop the color. Readings were made at 580 mµ. Kynurenine values were calculated from the standard kynurenine calibration curve. The sensitivity was approximately the same as for tryptophan.

Anthranilic acid also produces spots on paper which can be located by their fluorescence in ultraviolet light (15). The same chromatograms were used for estimating anthranilic acid as had been used for kynurenine. Conditions for the elution of the anthranilic acid spots, the diazotization, and the color development were the same. The amount of anthranilic acid present was approximately the same as for tryptophan.

The interference noted in direct tests of the mixtures for anthranilic acid was complete enough to avoid appreciable interference.

Neither the solvent of Tabone (22) nor the acidified solvent of Mason and Berg (15, 16) will effectively separate indolepyruvic acid chromatographically from anthranilic acid. When present in appreciable amount, indolepyruvic acid increases the intensity of color developed in the assay method for anthranilic acid. Eluates of sections of control chromatograms of mixtures of indolepyruvic acid and anthranilic acid prepared with the acidified Mason and Berg solvent, which permits marked destruction of the indolepyruvic acid during the irrigation, failed to show the interference noted in direct tests of the mixtures for anthranilic acid. Evidently the destruction of the indolepyruvic acid is complete enough to avoid appreciable interference.

Xanthurenic acid was estimated by deproteinizing 1 to 2 ml. of the medium with 4 per cent trichloroacetic acid solution, and by adding 0.1 ml. of 0.425 per cent FeNH₄(SO₄)₂ and 2 ml. of 1 N sodium bicarbonate solution, essentially as described by Glazer et al. (23). After half an hour the green color was measured at 620 mµ in the Coleman spectrophotometer and the concentration was computed from comparisons with a calibration curve prepared with standard solutions of xanthurenic acid. The method is sensitive to 15 µg in 8 ml. Neither tryptophan nor any of the other metabolites studied respond, nor do media incubated without added substrate.

Kynurenine acid in 20 per cent trichloroacetic acid solution absorbs strongly at 312 mµ (20), xanthurenic acid less strongly. Indolepyruvic acid interferes, but can be eliminated by the use of paper chromatograms developed with the acidified solvent of Mason and Berg, with which it shows an Rf value of 0.94 versus 0.58 and 0.56 for kynurenine acid and xanthurenic acid, respectively.

The same chromatographic technique was applied as for kynurenine and anthranilic acid, but 0.5 ml. of medium was applied to the paper and elution of the fluorescing spot was effected with a 20 per cent solution of trichloroacetic acid instead of 4 per cent. Kynurenine acid is relatively insoluble in the latter. Optical densities of the eluate were measured at 312 mµ in the Beckman DU spectrophotometer and the kynurenine acid content was calculated from a curve prepared with standard kynurenine acid solutions. Corrections based on the calculated interference of the xanthurenic acid present in the media were applied. The test was sensitive to 2 µg of kynurenine acid in 10 ml. of solution.

RESULTS AND DISCUSSION

Tables I and II present summaries of the data obtained when liver and kidney slices from normal (Table I) and riboflavin-deficient (Table II) rats were incubated with tryptophan and with kynurenine. More kynurenine accumulated when DL- or L-tryptophan was incubated with liver slices from the riboflavin-deficient rat than when normal liver was employed. The same was true of kynurenine and anthranilic acid, whether the substrate was DL- or L-tryptophan or L-kynurenine. Too little xanthurenic acid was produced by normal liver or kidney to estimate, but fairly large amounts accumulated when DL- or L-tryptophan or L-kynurenine was incubated with these tissues from the riboflavin-deficient animal. Kidney tissue produced kynurenine acid from both D- and L-kynurenine, but not from tryptophan. The amount which accumulated upon incubation with L-kynurenine was much larger with kidney from the riboflavin-deficient animal. Neither type of liver slice produced

| TABLE I |

Production of metabolites upon incubation of tryptophan and kynurenine with liver and kidney slices from normal rats

<table>
<thead>
<tr>
<th>No. of rats</th>
<th>Substrate*</th>
<th>Liver slice tests</th>
<th>Kidney slice tests</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Substrate recovered</td>
<td>Metabolites found</td>
</tr>
<tr>
<td></td>
<td></td>
<td>IPA</td>
<td>KA</td>
</tr>
<tr>
<td></td>
<td></td>
<td>µg</td>
<td>µg</td>
</tr>
<tr>
<td>5</td>
<td>A-L</td>
<td>527 ± 62f</td>
<td>40 ± 5</td>
</tr>
<tr>
<td>5</td>
<td>A-L</td>
<td>205 ± 22</td>
<td>48 ± 9</td>
</tr>
<tr>
<td>5</td>
<td>A-L</td>
<td>239 ± 20</td>
<td>72 ± 15</td>
</tr>
<tr>
<td>5</td>
<td>D-K</td>
<td>202 ± 21</td>
<td>0</td>
</tr>
<tr>
<td>5</td>
<td>D-K</td>
<td>206 ± 21</td>
<td>0</td>
</tr>
<tr>
<td>5</td>
<td>D-K</td>
<td>336 ± 21</td>
<td>0</td>
</tr>
<tr>
<td>5</td>
<td>D-K</td>
<td>336 ± 21</td>
<td>0</td>
</tr>
</tbody>
</table>

* In each test 1000 µg, of substrate in 3 ml. of Krebs-Henseleit buffer, pH 74, were incubated with 400 mg of tissue slices for 3 hours in an atmosphere of O₂. The K used as substrate was the hydrated sulfate; the 1000 µg. were equivalent to 643 µg of K. Recoveries and assays are recorded as K.
† No XA production was observed by either liver or kidney slices from the normal, well-fed rat, with any of the substrates. Kidney slices failed to produce K from T, or to yield AA from any of the substrates.
‡ ± = Standard deviation of the mean.
Table II

Production of metabolites upon incubation of tryptophan and kynurenine with liver and kidney slices from riboflavin-deficient rats

<table>
<thead>
<tr>
<th>No. of rats</th>
<th>Substrate*</th>
<th>Liver slice tests†</th>
<th>Metabolites found</th>
<th>Substrate recovered</th>
<th>Kidney slice tests†</th>
<th>Metabolites found</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>IPA</td>
<td>KA</td>
<td>XA</td>
<td>AA</td>
</tr>
<tr>
<td>10</td>
<td>d-T</td>
<td>322 ± 23†</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>17</td>
<td>DL-T</td>
<td>240 ± 31</td>
<td>109 ± 20</td>
<td>70 ± 16</td>
<td>58 ± 29</td>
<td>89 ± 12</td>
</tr>
<tr>
<td>11</td>
<td>L-T</td>
<td>205 ± 63</td>
<td>201 ± 17</td>
<td>108 ± 27</td>
<td>108 ± 21</td>
<td>124 ± 20</td>
</tr>
<tr>
<td>8</td>
<td>d-K</td>
<td>470 ± 46</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>L-K</td>
<td>177 ± 39</td>
<td>74 ± 31</td>
<td>96 ± 22</td>
<td>185 ± 46</td>
<td>202 ± 45</td>
</tr>
</tbody>
</table>

* For abbreviations used and amounts of substrate and conditions of tests, see Table I.
† Liver slices failed to produce IPA in measurable amounts from T. Kidney slices failed to produce K from T or to yield AA from any substrate.
‡ ± = Standard deviation of the mean.

either kynurenine acid or anthranilic acid from d-kynurenine, but the normal liver slice formed a small amount of kynurenine acid from d-tryptophan.

The results obtained with L-tryptophan correlate reasonably well with the reported increases in urinary output of kynurenine, xanthurenic acid, and kynurenic acid (1, 3) and in the urinary excretion of anthranilic acid (2, 3) in the riboflavin-deficient rat after the administration of L-tryptophan. The greater production of xanthurenic acid from L-kynurenine in tissue slices from the riboflavin-deficient rat fails to agree with the small excretion reported to occur in the deficient animal after the injection of L-kynurenine (4). Failure of the normal liver or kidney slice to produce xanthurenic acid from L-tryptophan and L-kynurenine is in accord with previous observations (15). If xanthurenic acid derivatives were produced in our incubation mixtures from L-kynurenine (15), they did not respond to the colorimetric assay for xanthurenic acid described above. They identified the more brilliant chromatographic spot which Mason and Berg had mistakenly associated with free xanthurenic acid as the somewhat more rapidly migrating 8-methyl ether of xanthurenic acid (24).

The normal liver slice yielded considerably more indolepyruvic acid (about 80 per cent more) from L-tryptophan than from d-tryptophan. None was found in analogous tests with liver slices from the riboflavin-deficient rat. The accumulation of indolepyruvic acid in media which had been incubated with tissue from the riboflavin-deficient rat fails to agree with the small production persisted in kidney slices from rats that were riboflavin-deficient, the accumulation was only a third or fourth as great.

The formation of indolepyruvic acid from L-tryptophan could presumably have been effected either by transaminase or by the riboflavin phosphate-containing L-amino acid oxidase. More active preparations of L-amino acid oxidase have been obtained from rat kidney than from rat liver (25). Although more flavin adenine dinucleotide is present per unit weight in rat liver than in rat kidney (26), the d amino acid oxidase activity of the former is only an eighth as great, or even less (27). No other enzyme has been found to convert D-amino acids to the corresponding α-keto acids in animal tissue.

Table III shows that the capacity of liver and kidney slices from the riboflavin-deficient rat to produce indolepyruvic acid could be restored, apparently fully, by the addition of riboflavin to the media.

Table IV presents evidence that indolepyruvic acid undergoes appreciable conversion to tryptophan upon incubation with either the liver or the kidney slice, and that incubation with the former also yields kynurenic acid. The riboflavin deficiency did not enhance significantly either the tryptophan or the kynurenine accumulation, but considerably less indolepyruvic acid was recovered from the media incubated with tissue from the riboflavin-deficient animal. The data substantiate the assumption that indolepyruvic acid can be utilized for growth in the rat (28, 9) fed no tryptophan because it is converted into L-tryptophan; hence also the assumption that d-tryptophan becomes available for growth (29, 30) by inversion through indolepyruvic acid. Evidence that rat kidney does convert d-tryptophan to L-tryptophan has been presented by Kotake and Goto (31).

Inversion of d-tryptophan by the normal liver slice could conceivably account also for its conversion to kynurenine (cf. Table I). Failure to detect kynurenine and anthranilic acid may reflect a smaller sensitivity of the tests for these pro-

Table III

Influence of riboflavin upon conversion of d-, DL-, and L-tryptophan to indolepyruvic acid by liver and kidney slices from riboflavin-deficient rats

<table>
<thead>
<tr>
<th>No. of rats</th>
<th>Tryptophan (1000 /a.)</th>
<th>Indolepyruvic acid production</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Liver slices*</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Alone</td>
</tr>
<tr>
<td></td>
<td></td>
<td>µg.</td>
</tr>
<tr>
<td>5</td>
<td>d</td>
<td>0</td>
</tr>
<tr>
<td>5</td>
<td>DL</td>
<td>0</td>
</tr>
<tr>
<td>5</td>
<td>L</td>
<td>0</td>
</tr>
</tbody>
</table>

* 1000 µg. of substrate in 3 ml. of Krebs-Henseleit buffer at pH 7.4, incubated with 400 mg. of tissue slices for 3 hours at 37° in an O2 atmosphere.
† ± = Standard deviation of the mean.
ducts, but, more likely, differences in relative rates of the various reactions involved in the conversion of n-tryptophan to indolepyruvic acid, to L-tryptophan, to L-kynurenine, and thence to kynurenic acid or anthranilic acid. Weak spots for kynurenine were detected on the chromatograms in a few instances, in two of which 4 µg. were measured. In four tests, definite anthranilic acid spots were revealed by ultraviolet fluorescence, but the quantities present were too small for estimation.

The greater accumulation of indolepyruvic acid in the tryptophan tests with normal liver was inadequate to account fully for the smaller accumulation of the other metabolites noted in these media. The possibility of metabolism by other routes is, of course, not precluded. The considerably larger accumulation of indolepyruvic acid also in L-tryptophan media incubated with kidney slices from the normal as compared with the riboflavin-deficient rat leads one to suggest that its production in the normal intact rat may simply impair the efficiency of conversion of L-tryptophan to kynurenine, kynurenic acid, and anthranilic acid; may promote metabolism by routes available only to indolepyruvic acid; or may lead to the elimination of indolepyruvic acid in the urine.

In no instance did the metabolites produced and the substrates recovered in the experiments recorded in any of the tables fully account for the substrates employed. Some destruction of substrate could be shown to occur during incubation in the absence of the tissue slices, but this did not exceed 10 per cent, hence was far too small to account for the incomplete recovery.

Mason observed that, in a nitrogen atmosphere, homogenates of normal kidney catalyzed the conversion of L-kynurenine to kynurenic acid, presumably by transamination, but yielded very much less kynurenic acid from n-kynurenine under analogous conditions (32). Conversion of n-kynurenine to o-amino-benzoic acid by n-amino acid oxidase might well have accounted for the greater production of kynurenic acid by the kidney slice in our tests in which an oxygen atmosphere was employed. The yield of kynurenic acid from n-kynurenine was not measurable with liver tissue (Table I) in which n-amino acid oxidase was much less active, nor was it increased appreciably with kidney from the riboflavin-deficient rat (Table II). Analogous tests with L-kynurenine showed an appreciable accumulation of kynurenic acid, even with liver tissue, and an approximately doubled yield with liver and kidney from the riboflavin-deficient animal.

The optimal pH for n-amino acid oxidase has been reported to be 8.2 to 8.4 (22), but at pH 8.4 flavin adenine dinucleotide undergoes more rapid degradation than at pH 7.3 (33). In a series of tests made with normal kidney slices at pH 8.2, in which sodium pyrophosphate was used as the buffer (12) but all other conditions were the same as in the studies recorded in Table I, the indolepyruvic acid found after incubation with tissue from 6 rats was only 460 ± 35 µg. with the n-tryptophan, 196 ± 28 µg. with DL-tryptophan, and 32 ± 15 µg. with L-tryptophan. Corresponding figures with kidney from 9 riboflavin-deficient rats were also lower: 102 ± 31, 68 ± 22, and 0 µg., respectively.

**SUMMARY**

The influence of riboflavin deficiency upon the metabolism of tryptophan was studied by comparing metabolite accumulation in media incubated at pH 7.4 with liver and kidney slices from the deficient rat with the accumulation when tissues from the normal animal were employed.

Indolepyruvic acid accumulation was considerably less in the tests with liver and kidney slices from the riboflavin-deficient rat. Addition of riboflavin to the media increased the indolepyruvic acid production, but the deficiency exerted little, if any, influence upon the capacities of the liver and kidney slices to convert indolepyruvic acid to tryptophan, as judged by the accumulation of the latter in the media.

The accumulation of kynurenine, kynurenic acid, and anthranilic acid was greater in the L-tryptophan media incubated with liver slices from the riboflavin-deficient animal. The deficiency stimulated the production of xanthurenic acid by both the liver and the kidney slices from L-tryptophan and L-kynurenine. It increased the production of kynurenic acid and anthranilic acid from L-kynurenine.

No kynurenic acid or anthranilic acid was produced by either the normal or deficient liver from n-kynurenine. A small amount of kynurenic acid was formed in the normal liver from n-tryptophan, possibly after its initial inversion. No kynurenic acid was produced from tryptophan by either the normal or the deficient kidney slice. It was produced by both from n- and l-kynurenine. Riboflavin deficiency greatly increased its production by the kidney slice from L-kynurenine, but only slightly, if at all, significantly its production from D-kynurenine.

Correlation of the results obtained in these tests with the urinary output of tryptophan metabolites in the intact riboflavin-deficient animal is discussed.
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The Effect of Riboflavin Deficiency upon the Metabolism of Tryptophan by Liver and Kidney Tissue
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