Incorporation of Uracil-2-$^14$C into the Nucleic Acids of Neurospora crassa*  

K. P. Chakraborty† and Hubert S. Loring  

From the Department of Chemistry, Stanford University, Stanford, California  

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The pyrimidine-deficient mutant (1298) of Neurospora crassa, utilizes orotic acid, uracil, uridine, or cytidine for growth, but neither cytosine nor thymine (1). It seems likely, therefore, that this mutant can convert orotic acid and uracil to the pyrimidine bases of ribonucleic acid and deoxyribonucleic acid, and can utilize the nucleoside directly for ribonucleic acid biosynthesis and for conversion to deoxycytidine and thymidine of deoxyribonucleic acid. As the latter two nucleosides have not been studied before as possible growth supplements in pyrimidine-deficient Neurospora, it has not been determined previously whether these compounds might also function as sources of pyrimidine components for RNA, or for DNA, or for both types of nucleic acids. Recently, from experiments with uniformly labeled $^14$C-cytidine in the pyrimidine-deficient Neurospora mutant 36601, McNutt (2) has demonstrated that this nucleoside is incorporated into RNA and is also converted to uridine, deoxyctydine, and thymidine. A surprising result, however, was that the specific activities of the nucleosides isolated from the recovered RNA and DNA were only about 70% of that of the starting cytidine. It was concluded that this mutant has a considerable capacity for synthesizing the pyrimidine nucleosides, even though provided with an outside pyrimidine source.  

By studying the incorporation of uracil-2-$^14$C into the nucleic acids of the mutant 1298, it seemed possible to determine whether uracil utilization was the only pathway of pyrimidine biosynthesis in this organism, or whether some other pathway might also be involved. In the latter event the pyrimidine components of RNA and DNA would be expected to show decreased specific activities as compared to that of the initial uracil-2-$^14$C. Similarly, if deoxycytidine or thymidine were utilized for DNA synthesis only, it should be possible to label RNA specifically with uracil-2-$^14$C.  

In the present paper we have studied the availability of deoxouridine, deoxycytidine, and thymidine for growth of mutant 1298, as well as the supplementary growth effects of the above nucleosides and of uridine and cytidine, in the presence of a minimal amount of uracil. The utilization of uracil-2-$^14$C for the formation of uracil, cytosine, thymine, adenine, and guanine, of Neurospora nucleic acids was investigated both in the mutant and in the wild strain, and also in the mutant in the presence of uridine, deoxyuridine and thymidine.  

EXPERIMENTAL PROCEDURE  

Uridine, cytidine, deoxyuridine, deoxycytidine, and thymidine were purchased from the California Corporation for Biochemical Research, Los Angeles, California. Urea-$^14$C of 1 mc of specific activity per mmole was obtained from Research Specialties Company, Berkeley, California.  

Synthesis of Uracil-2-$^14$C—The synthesis of uracil-2-$^14$C by the method of Davidson and Baudisch (3) has been reported by Mandel and Brown (4). When this procedure was applied to small quantities of urea, e.g. 1 to 2 mmoles, no crystalline uracil could be recovered from the resulting sulfuric acid solution. By bringing the reaction mixture to about pH 5 with Dowex 2 bicarbonate, it was possible to prepare twice-crystallized uracil-2-$^14$C of 1 mc of specific activity per mmole in a yield of 46% of the theory from 2 mmoles of urea-$^14$C. The modified procedure used was as follows.  

Fuming sulfuric acid (2 ml, 20% SO$_3$), was added gradually to a mixture of finely powdered urea-$^14$C and malic acid (120 mg, 2 mmoles, and 320 mg, 2.4 mmoles, respectively, dried over CaCl$_2$ for 2 days at room temperature), in a small test tube. The mixture was stirred mechanically for about 10 minutes. The temperature of the bath was allowed to rise slowly and maintained at 80° for 45 minutes and finally at 100° for 10 minutes. The resulting strongly acidic solution was brought to about pH 5 by treatment with Dowex 2 bicarbonate, and the solution concentrated under reduced pressure to a volume of 3 to 4 ml. After standing overnight in the refrigerator, the crystalline uracil, which separated, was filtered, recrystallized from a minimal volume of water with alcohol, and washed with a small quantity of alcohol and ether. The dried uracil 2 $^14$C, which was snow-white in color, decomposed at 335°, and a mixture with known uracil showed no depression of the decomposition point. The ultraviolet absorption spectrum and behavior on paper chromatography were typical of uracil.  

Growth of N. crassa—The composition of the basal medium, unless otherwise stated, was the same as that described by Horowitz and Beadle (5). The stock cultures of the wild strain and of the mutant 1298 were maintained on agar slants, with a supplement in the latter case of 5 mg of uracil per 10 ml of basal medium (1). Growth response of the various compounds and mixtures employed was determined as previously described (6).
from average dry weight of washed mycelium in triplicate experiments after incubation of the culture in 25 ml of medium at 30° for 72 hours.

Growth Effects of Deoxyuridine, Deoxycytidine, and Thymidine—The ability of the compounds mentioned as possible growth supplements for the mutant was determined by using a culture medium containing 3 mg of uracil per 10 ml of medium. At this concentration of uracil small amounts of growth, e.g., 10 to 15 mg of dried mycelium, were found in a 3-day period in the absence of other growth-promoting substances. The concentration of the nucleosides ranged from 0.05 to 1 mg per 10 ml of medium, and in order to provide a basis for comparison, the mutant was grown on the same concentrations of uridine and cytidine in the absence of uracil. The average mycelial weights found in triplicate experiments at the concentrations studied are summarized in Table I.

The stimulatory effects of uridine, cytidine, and deoxyuridine on growth in the presence of 3.0 mg of uracil per 10 ml of medium are similar, and maximal growth is obtained with 0.2 mg of each supplement. From a comparison of the amount of growth found on uridine and cytidine in the absence of uracil, and the failure of deoxyuridine and deoxycytidine alone to allow growth as mentioned before, it is clear that the supplementary effect of these nucleosides is not just an additive one. Deoxycytidine, even at a level at which maximal growth is found with uridine or cytidine, appears to have less stimulatory effect than the other nucleosides, and thymidine shows no enhancement of growth.

Incorporation of Uracil-2-{C}^{14} into Nucleic Acids of Neurospora Mutant 1298 and of the Wild Type, 74A—In these experiments the Neurospora was grown in Fernbach flasks containing 400 ml of culture medium (6), supplemented with 200 mg of uracil-2-{C}^{14} (10 to 20 μc per mmole) in the case of the mutant and with 4 mg for the wild strain. The flasks were inoculated in each case with 2 to 3 ml of dilute conidial suspension and incubated for 3 days at 30°. At the uracil concentration mentioned for the mutant strain maximal or nearly maximal growth corresponding to about 500 mg of dried mycelium was obtained. The wild strain grows normally on the basal medium in the absence of uracil and no additional growth was found in the presence of uracil in amounts from 0.1 to 5.0 mg per 10 ml of basal medium.

At the end of the growth period the culture solution was decanted and the mycelial mat washed with ice-cold physiological salt solution made 0.01 m with sodium citrate. It was ground (7), and freed from acid-soluble components by washing at the centrifuge with 5% trichloroacetic acid and with alcohol and ether. The RNA and DNA in the dried mycelium were converted into acid-soluble components by two successive treatments with 5 ml of 0.5 N HClO₄ at 90° for 1 hour, and the soluble fraction was converted to the free purine and pyrimidine bases by further heating at 100° for 1 hour after concentration of the HClO₄ solution to 6 to 7 ml. Perchlorate ion was removed as the potassium salt, and the purine and pyrimidine bases in aliquots of the resulting solution were separated and purified by paper chromatography in aqueous butanol (8), isopropanol-HCl (9) and ammoniacal butanol (10), in the order mentioned. The

### Table I

<table>
<thead>
<tr>
<th>Milligrams of nucleosides used per 10 ml of medium</th>
<th>Supplements used</th>
<th>Uridine</th>
<th>Cytidine</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.05</td>
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<td>9</td>
<td>0</td>
</tr>
<tr>
<td>0.1</td>
<td>65</td>
<td>38</td>
<td>11</td>
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<td>0.2</td>
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<tr>
<td>0.5</td>
<td>70</td>
<td>54</td>
<td>12</td>
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<tr>
<td>1.0</td>
<td>76</td>
<td>51</td>
<td>12</td>
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</table>

### Table II

<table>
<thead>
<tr>
<th>Counts per minute per μmole X 10^{-6}</th>
<th>Uracil</th>
<th>Cytosine</th>
<th>Thymine</th>
<th>Adenine</th>
<th>Guanine</th>
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</thead>
<tbody>
<tr>
<td><strong>Experiment 1</strong></td>
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<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>a</td>
<td>328</td>
<td>285</td>
<td>322</td>
<td>3</td>
<td>30</td>
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<tr>
<td>b</td>
<td>298</td>
<td>278</td>
<td>295</td>
<td>46</td>
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<tr>
<td>c</td>
<td>241</td>
<td>246</td>
<td>347</td>
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<td></td>
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<tr>
<td><strong>Experiment 2</strong></td>
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<tr>
<td>b</td>
<td>472</td>
<td>492</td>
<td>447</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Mean relative specific activity</strong></td>
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<td>86</td>
<td>99</td>
<td>5</td>
<td>5</td>
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<td><strong>Experiment 3</strong></td>
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<td></td>
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<tr>
<td>a</td>
<td>564</td>
<td>139</td>
<td>135</td>
<td>141</td>
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<tr>
<td>b</td>
<td>160</td>
<td>124</td>
<td>128</td>
<td></td>
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<td>c</td>
<td>131</td>
<td>140</td>
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<tr>
<td><strong>Mean relative specific activity</strong></td>
<td>26</td>
<td>24</td>
<td>23</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*Relative specific activity = c.p.m. per μmole of recovered base / c.p.m. per μmole of uracil used X 100.
final products, which were eluted with water, gave characteristic absorption spectra in 0.1 N HCl. Their respective concentrations were measured by absorption spectrophotometry (11), and their radioactivity as counts per micromole was determined in comparison with that of the uracil employed by counting on aluminum planchets in a Geiger-Müller gas flow counter to a standard error of ±1%. The amount of uracil removed from the medium during mold growth was only a small percentage of that present originally for both the mutant and the wild strain. No attempt was made in any of the experiments to recover the purine and pyrimidine bases quantitatively.

The results of the incorporation of uracil-2-C\textsuperscript{14} at two levels of radioactivity into the nucleic acids of mutant 1298 and of a comparable experiment with the wild strain are summarized in Table II. Although the specific activities of the recovered uracil and cytosine were somewhat low in the first experiment with the mutant strain, as compared to the uracil used, it is clear from the second experiment and the averages of all of the results found that the uracil, cytosine, and thymine of the nucleic acids have an activity per mole comparable to that of the uracil used. The mutant, accordingly, must utilize uracil per se for incorporation into RNA and DNA as a precursor for RNA- and DNA-pyrimidine biosynthesis in both cases.

Samples of guanine and adenine isolated from the nucleic acids in both types of Neurospora and examined for radioactivity showed an insignificant utilization of carbon 2 of uracil for purine biosynthesis.

The incorporation of uracil-2-C\textsuperscript{14} into nucleic acids of mutant strain in presence of uridine, deoxyuridine, and thymidine—

The incorporation of uracil-2-C\textsuperscript{14} into the nucleic acids of the mutant strain was studied in the presence of relatively small and relatively large amounts of uridine, e.g., 0.05 mg and 1.0 mg, respectively, per 10 ml of medium containing 3 mg of uracil. Under the former condition, as shown in Table I, a large stimulatory effect on growth was found, whereas in the latter case maximal growth was obtained even in the absence of uracil. Amounts of deoxyuridine and of thymidine corresponding to 1 mg per 10 ml of medium containing 3 mg of uracil were also used, and in addition one experiment with thymidine was performed in which 5 mg of uracil were present per 10 ml of medium. The method of culture, hydrolysis of the nucleic acids, fractionation of the bases, and determination of their specific activities were the same as previously described. The average results of triplicate experiments for uridine and deoxyuridine, and of duplicate experiments for thymidine, are summarized in Table III.

The results of the experiments with both uridine and uracil present in the medium show a definite incorporation of uracil-2-C\textsuperscript{14} into the three pyrimidine components of the nucleic acids under the conditions studied. The extent of incorporation of uracil at a uridine concentration of 0.05 mg per 10 ml is probably significantly less than in the experiments in which uracil-2-C\textsuperscript{14} was the only growth supplement present, although the differences in the case of cytosine are relatively small. At the higher uridine concentration, e.g., 1.0 mg per 10 ml of medium and a molar ratio to uracil of 1 to 6.6, the results are more striking as the specific activities of the recovered pyrimidine bases decreases to about 30% of that of the starting uracil, representing an incorporation of approximately 3 molecules of uridine for every 1 of uracil. The specific activities of the recovered uracil, cytosine and thymine are apparently comparable to one another at each uridine concentration.

The specific activity of thymine is quite appreciably lowered in the presence of deoxyuridine, whereas that of uracil and of cytosine, within the limits of experimental error, are comparable to each other and to that of the uracil used. The lowering of the specific activity of thymine indicates that deoxyuridine is utilized for DNA-pyrimidine biosynthesis, but not to any significant extent for the synthesis of the RNA-pyrimidine components. These results thus provide an explanation for the failure of deoxyuridine alone to support growth in contrast to its supplementary growth effect when provided together with uracil.

In the experiments with thymidine in a medium supplemented with 5 mg of uracil per 10 ml, the specific activities of uracil, cytosine, and thymine are close to 100% that of the initial uracil. These results indicate, in agreement with the growth studies, that thymidine is not utilized for DNA synthesis. The low specific activity of the cytosine at a uracil level of 3 mg per 10 ml of medium is probably explained by the small yield of mycelium produced under these conditions and the recovery of a less pure product.

In each of the above-mentioned experiments one or two su-

<p>| Table III | Relative incorporation of uracil-2-C\textsuperscript{14} (3 mg per 10 ml) into the uracil, cytosine and thymine of the nucleic acids of Neurospora mutant 1298 in the presence of uridine, deoxyuridine, and thymidine |
|---|---|---|---|---|
| Uracil concentration of medium, 3 mg per 10 ml. | Specific Activity in c.p.m per micromole \times 10\textsuperscript{4} | Pyrimidine bases of nucleic acids | Relative specific activity\textsuperscript{a} |</p>
<table>
<thead>
<tr>
<th>Milligrams of supplement per 10 ml of medium</th>
<th>Uracil used</th>
<th>Uracil</th>
<th>Cytosine</th>
<th>Thymine</th>
<th>Uracil</th>
<th>Cytosine</th>
<th>Thymine</th>
</tr>
</thead>
<tbody>
<tr>
<td>Uridine, 0.05\textsuperscript{b}</td>
<td>605</td>
<td>443</td>
<td>485</td>
<td>427</td>
<td>73</td>
<td>80</td>
<td>71</td>
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<td>Uridine, 1.0\textsuperscript{b}</td>
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<td>225</td>
<td>218</td>
<td>248</td>
<td>29</td>
<td>28</td>
<td>32</td>
</tr>
<tr>
<td>Deoxyuridine, 1.0\textsuperscript{b}</td>
<td>675</td>
<td>554</td>
<td>595</td>
<td>287</td>
<td>82</td>
<td>88</td>
<td>43</td>
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<tr>
<td>Thymidine, 1.0\textsuperscript{c}</td>
<td>458</td>
<td>411</td>
<td>314</td>
<td>392</td>
<td>89</td>
<td>69</td>
<td>70</td>
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<tr>
<td>Thymidine, 1.0\textsuperscript{d}</td>
<td>458</td>
<td>408</td>
<td>428</td>
<td>403</td>
<td>89</td>
<td>94</td>
<td>88</td>
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</tbody>
</table>

\textsuperscript{a} See Table II.

\textsuperscript{b} The numbers shown are the averages of experiments performed in triplicate.

\textsuperscript{c} The numbers shown are the averages of experiments performed in duplicate.

\textsuperscript{d} In this case 1.0 mg of thymidine was used as a supplement to medium containing 5.0 mg of uracil per 10 ml of solution.
DISCUSSION

The incorporation of uracil-2-C\textsuperscript{14} with unchanged specific activity into the uracil, cytosine, and thymine of the nucleic acids of the Neurospora mutant 1298 proves the utilization of this compound for both RNA- and DNA-pyrimidine biosynthesis in this organism. The results also show that uracil utilization is the only pathway of RNA- and DNA-pyrimidine biosynthesis in this mutant in the presence of uracil, and are similar, therefore, to the utilization of uracil-2-C\textsuperscript{14} by the pyrimidine-deficient Escherichia coli mutant 63-86 (12). In both organisms, and possibly contrary to the Neurospora mutant 36601 studied by McNutt (2), it appears that no alternative pathway of pyrimidine biosynthesis is available when uracil is used as the growth supplement. As cytidine rather than uracil was used as the pyrimidine supplement by McNutt, the differences found for the two Neurospora mutants may be explained by differences in nucleic acid biosynthesis when different precursors are provided, or alternatively by specific metabolic differences between the two mutants.

Because uracil-2-C\textsuperscript{14} is also incorporated into the nucleic acids of the wild strain when it is present in the medium, it is clear that both "salvage" and de novo pathways of pyrimidine biosynthesis (13) are present in the wild strain and that the metabolic block in the present mutant involves synthesis de novo. It seems probable, by analogy with E. coli and some lactic acid bacteria, that uracil may be utilized either by direct synthesis of uridine 5'-phosphate through the action of a uracil-phosphoribosyl-pyrophosphate pyrophosphorylase, or by conversion to uridine by a uridine phosphorylase in the presence of ribose 1-phosphate, followed by phosphorylation (13-16).

As shown previously (1) and confirmed in the present experiments with the mutant strain, uridine and cytidine are much more efficiently utilized for growth than is uracil. Similarly, in the presence of small amounts of nonradioactive uridine together with uracil-2-C\textsuperscript{14}, the radioactivities of the recovered uracil, cytosine, and thymine are greatly reduced but are comparable to each other. A common intermediate, probably uridine or its nucleotide, is thus indicated for both RNA- and DNA-pyrimidine biosynthesis in the mutant strain. As a similar uniform distribution of radioactivity was found for the wild type Neurospora and the mutant 36601, it seems likely that the same common intermediate is involved in these strains of Neurospora and possibly in all strains.

Deoxyuridine and deoxyctydine, unlike the corresponding ribonucleosides, fail to support growth of the mutant, but it is clear from the experiments with the two compounds in the presence of a minimal quantity of uracil that both are utilized for growth under these conditions. A logical interpretation of these results is that both deoxy compounds are available for DNA but not for RNA biosynthesis. This conclusion is confirmed by the relative specific activities of the recovered uracil and thymine when both deoxyuridine and uracil-2-C\textsuperscript{14} are present as growth supplements. The recovered thymine under these conditions, as shown in Table III, contains only about 42% of the radioactivity of the original uracil-2-C\textsuperscript{14}, as compared to about 82% for the recovered uracil. The utilization of deoxyuridine for thymine biosynthesis in Neurospora accordingly is in agreement with previous studies with suspensions of chick embryo (17), with the whole rat (18), with rat (19) or rabbit (20) thymus homogenates, with mouse Ehrlich ascites tumor and rabbit bone marrow (21), and with extracts of E. coli (22). If the conversion of uridine (or a corresponding nucleotide) to DNA-thymine occurs by reduction to deoxyuridine (or a corresponding nucleotide), followed by methylation, it is clear that the first reaction is largely an irreversible one.

In the experiments with uracil-2-C\textsuperscript{14} and deoxyuridine, the specific activity of the recovered cytosine is comparable to that of the recovered uracil rather than of the recovered thymine. Since DNA and RNA were not separated before hydrolysis to the free bases, the relative specific activities of the DNA- and RNA-cytosine are not known but would necessarily depend on the relative amounts of the two nucleic acids in Neurospora mycelium. Determination of RNA- and DNA-phosphorus in dried mycelium by the Schmidt and Thannhauser procedure (23) gave values of 0.5 and 0.06%, respectively (24). It is apparent, therefore, that about 8 times as much of the recovered cytosine was formed from RNA as from DNA, and accordingly would be more characteristic of RNA- rather than DNA-cytosine.

The failure of thymidine either to increase growth in the presence of uracil or to decrease the radioactivity of the thymine recovered from the mutant grown in the presence of uracil-2-C\textsuperscript{14} and thymidine shows that this nucleoside is not available for DNA biosynthesis and consequently that methylation does not occur at the nucleotide level in thymine biosynthesis in this organism. A similar conclusion with respect to thymidine utilization has also been reported in E. coli B (25). However, the direct incorporation of thymidine into DNA-thymine has been reported in the intact rat (18, 26), in rat liver homogenate (27), in embryonic tissue (28), in bone marrow and isolated thymus nuclei (29), and in E. coli (30-32). As a specific kinase appears to be involved in thymidine utilization in E. coli (13), it seems that the comparable enzyme is lacking in Neurospora mutants 1298 and in E. coli B.

SUMMARY

A modification of the method of Davidson and Baudisch is described for the synthesis of millimolar quantities of uracil-2-C\textsuperscript{14} with the same specific activity as that of the urea used.

Although deoxyuridine and deoxyctydine do not support growth of the Neurospora mutant 1298 when either is provided as the sole pyrimidine supplement, each compound stimulates growth in the presence of a minimal quantity of uracil. Thymidine per se neither supports growth nor produces enhancement of growth in the presence of uracil.

The results of the incorporation of uracil-2-C\textsuperscript{14} into the nucleic acids of the Neurospora mutant 1298 prove that this compound can serve as a precursor of all the common pyrimidine components of the nucleic acids. Uridine decreases the incorporation of uracil uniformly into the pyrimidine components and this incorporation is unaffected by thymidine. Deoxyuridine, on the contrary, decreases incorporation of uracil into the deoxyribonucleic acid-thymine only.

The partial incorporation of uracil-2-C\textsuperscript{14} into the nucleic acids of the wild strain, 74A, shows that both de novo and "salvage" pathways of pyrimidine biosynthesis are available in this case. It seems clear that it is pyrimidine biosynthesis de novo that is blocked in the mutant.
REFERENCES

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