Studies on 5-Hydroxyuridine*  

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Bacterial, viral, and tumor growth are inhibited by 5-hydroxyuridine (1–3). The analogue at low concentrations has been reported to inhibit enzyme induction without affecting over-all protein synthesis in Escherichia coli (4). At higher concentrations, 5-hydroxyuridine inhibits both RNA and protein synthesis in E. coli 15 T− (5). Although these observations indicate that the analogue inhibits nucleic acid metabolism, very little information regarding the precise mechanism of these inhibitory effects or the metabolism of the analogue itself has been reported. This study was undertaken to clarify these inhibitory mechanisms of 5-hydroxyuridine. Ehrlich ascites cells were used in most of the experiments because this tumor is a convenient source of cells which are sensitive to 5-hydroxyuridine (6).

The results show that 5-hydroxyuridine undergoes most of the metabolic reactions of uridine in Ehrlich ascites cells, including incorporation into RNA and 5-hydroxyuridine 5'-diphosphate-sugar cofactors. A strong inhibition of orotidylic acid decarboxylase by 5-hydroxyuridine 5'-monophosphate was observed in a soluble enzyme preparation fraction.

EXPERIMENTAL PROCEDURE AND RESULTS  

Materials—Uridine-2-14C was purchased from Schwartz Biochemical Corporation and California Corporation for Biochemical Research. Orotic acid-6-14C and uridylic acid-2-14C were purchased from New England Nuclear Corporation. Valine-1-14C, nucleosides, nucleotides, and ribose 5-phosphate were obtained from California Corporation for Biochemical Research. Phosphoglyceric acid was purchased from Nutritional Biochemicals Corporation.

A hyperdiploid strain of Ehrlich ascites tumor was generously supplied by Dr. Eugene Roberts of City of Hope, Duarte, California and Dr. Ralph McKee of the University of California, Los Angeles.

Synthetic Methods—5-HO-Urd1 was synthesized by the method of Roberts and Visser (7). 5-HO-Urd-2-14C was synthesized by the same procedure and by a modification of the method of Ueda (8). When following the latter procedure it was found that a 4-hour period of heating, rather than 10 hours, did not affect the yield adversely. The yield of product was low unless the bromination step was carried out at a uridine concentration of not less than 100 mg per 3 to 4 ml. Pyridine was added periodically during the heating period to maintain the pH at about 6. 5-HO-Urd-2-14C was purified by chromatography on Dowex 1 (OH−) and paper chromatography in 1-butanol-water-ethanol (50:20:30) for 30 hours. 5-HO-UMP was prepared according to the same modified procedure of Ueda (8), and purified by chromatography on Dowex 1 (formate).

Effect of 5-HO-Urd on Nucleic Acid and Protein Synthesis—Ehrlich ascites cells were grown in the peritoneal cavity of male Swiss white mice purchased from Kurl's Cavia, La Puente, California. The culture was maintained by injecting 0.1 ml of a 7-day-old culture into the peritoneal cavity of 15- to 25-g mice. Cells used for experiments were from 6- to 9-day cultures. The cells were removed from the mice, collected by centrifugation, and washed with 0.9% NaCl. The cells were suspended in Krebs phosphate buffer medium III (9) modified to contain NaCl in place of the sodium salts of the tricarboxylic acid cycle intermediates. A volume of buffer equal in milliliters to the weight of the cells in grams was used to suspend the tumor. Of the cell suspension, 1 ml was added to 3 ml of the buffer which contained 6.7 mg of inosine, 1.0 mg of glucose, 0.5 mg of glutamine, and varying amounts of 5-HO-Urd. The solutions were incubated at 37° for 10 minutes, and then 1 ml of an amino acid solution (10) containing valine-1-14C (1.5 × 10^6 c.p.m.) and orotic acid-6-14C (6 × 10^6 c.p.m.) was added. The incubation was continued for an additional 20 minutes. At the end of the incubation period the reaction mixtures were centrifuged rapidly at 4° and 5 to 6 ml of cold 0.4 N perchloric acid were added to the cells in each tube.

Inosine was included in the above incubation mixtures to provide a readily utilisable source of ribose (11) which does not interfere directly with metabolism of pyrimidine nucleosides. In the absence of inosine, incorporation of uracil-2-14C into nucleic acids is greatly stimulated by low concentrations of nucleoside analogues (12). The purine nucleoside was added to minimize any such effect on orotic acid incorporation.

Protein and nucleic acids were isolated by the method of Tyner, Heidelberger, and LePage (13). The nucleic acids were dialyzed against distilled water containing unlabeled orotic acid. The dried protein residue was weighed and hydrolyzed with 12 N HCl at 100° for 8 hours. The hydrolysate was centrifuged and the radioactivity of the supernatant solution was determined.

5-HO-Urd inhibited the incorporation of the labeled precursors
The supernatant solution was collected and stored in the cold. Cell debris and glass powder were extracted twice with buffer and the supernatants added to the first. A total of 10 ml of buffer were used per g of tissue for grinding, transfer, and extraction.

The combined supernatant solutions were centrifuged at 100,000 X g for 1 hour. This supernatant fraction rapidly converts erotic acid-14C or uridine-2-14C to UTP-14C at conditions given in Tables II and III. This soluble enzyme preparation was used for all cell-free inhibition studies. In all cases, the enzyme preparation was used in amounts which gave approximately 50% of the maximum triphosphate yield during the time period used.

**Inhibition of Phosphorylation of Uridine-2-14C and UMP-2-14C**—The effect of 5-HO-Urd on uridine-2-14C phosphorylation was tested (Table II). The neutralized perchloric acid-soluble fractions were analyzed on small columns (16) of Dowex 1 (formate). Three fractions were obtained by elution with: (a) 100 ml of H2O, (b) 30 ml of 0.2 N formic acid, and (c) 100 ml of 6 M HCl.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Radioactivity recovered</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control samples</td>
</tr>
<tr>
<td>----------</td>
<td>-----------------</td>
</tr>
<tr>
<td>UMP</td>
<td>85.4</td>
</tr>
<tr>
<td>UDPX*</td>
<td>3.3</td>
</tr>
<tr>
<td>UDP</td>
<td>3.7</td>
</tr>
<tr>
<td>UTP</td>
<td>6.6</td>
</tr>
</tbody>
</table>

* UDPX, uridine diphosphate-carbohydrate.

**Distribution of radioactivity in uric acid and uridine nucleotides**

The effect of 5-HO-Urd on the incorporation of uric acid-6-14C into acid-soluble nucleotides at conditions given in Fig. 1. Control samples did not contain 5-HO-Urd. The values are percentages of the total radioactivity recovered in the acid-soluble fractions. The values do not total 100% because small amounts of unidentified radioactive components are not included in the table. Orotic acid and UMP are expressed together because the tubes containing these radioactive components were, in one case, inadvertently added to the same flask prior to counting. UMP, however, accounted for 1.5 to 2.0% of the total radioactivity in both control and experimental samples.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Radioactivity recovered</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control samples</td>
</tr>
<tr>
<td>----------</td>
<td>-----------------</td>
</tr>
<tr>
<td>Orotic acid and UMP</td>
<td>85.4</td>
</tr>
<tr>
<td>UDPX*</td>
<td>3.3</td>
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<td>UDP</td>
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<td>6.6</td>
</tr>
</tbody>
</table>

* UDPX, uridine diphosphate-carbohydrate.

**Effect of 5-HO-Urd on uridine-2-14C phosphorylation**

The reaction mixtures contained 10 μmoles of ATP, 25 μmoles of phosphoglyceric acid, 50 μmoles of MgCl2, 3.0 μmoles of uridine-2-14C (20,000 c.p.m.), varying amounts of 5 HO Urd, and 2.5 ml of 100,000 X g supernatant fraction, in a total volume of 5.0 ml. The incubation was continued for 10 minutes and terminated with 1.25 ml of 4.0 N perchloric acid. The chromatographic procedure is described in the text.

<table>
<thead>
<tr>
<th>5-HO-Urd</th>
<th>Phosphorylated uridine-2-14C</th>
</tr>
</thead>
<tbody>
<tr>
<td>μmoles</td>
<td>μmoles</td>
</tr>
<tr>
<td>0</td>
<td>1.74</td>
</tr>
<tr>
<td>30</td>
<td>1.55</td>
</tr>
<tr>
<td>60</td>
<td>1.56</td>
</tr>
<tr>
<td>90</td>
<td>1.46</td>
</tr>
</tbody>
</table>

**Preparation of Supernatant Fraction**—The cells were placed in tared centrifuge tubes, centrifuged to remove ascitic fluid, washed with 0.9% NaCl and weighed. They were transferred with a small volume of buffer to a mortar embedded in ice. The buffer was composed of 250 ml of 0.25 M sucrose, 30 ml of 0.2 M Tris-HCl, pH 8.0 and 10 ml of 0.15 M KCl. Glass powder (325 mesh) was used to grind the cells.

After the cells had been thoroughly ground (15 to 20 minutes) the homogenate was centrifuged at 4000 r.p.m. for 10 minutes in an International refrigerated centrifuge, model PR-2 at 0°.
Since the inhibitory effect of 5-HO-Urd on incorporation of erotic acid to uridine nucleotides. Preliminary experiments indicated that 5-HO-UMP strongly inhibits conversion of uracil and uridine; the third fraction contained phosphorylated uridine derivatives. The results (Table II) demonstrate that uridine phosphorylation is not appreciably inhibited even at very high inhibitor-to-substrate ratios. Similar experiments were performed to test the effect of 5-HO-UMP on conversion of uracil and uridine; and neutralized with KOH. Inorganic pyrophosphate, when added at the start of the 2-hour incubation period and the isolated nucleic acids were dialyzed against unlabeled 5-HO-Urd. The nucleic acids were hydrolyzed in 0.1 N NaOH at 37° for 18 to 22 hours (13). DNA, precipitated by adjusting the pH to 1 with HCl, was not radioactive. The 2',3'-ribonucleotides were separated by column chromatography on Dowex 1 (formate) with a concentration gradient of formic acid. The radioactive peak eluted immediately after 2',3'-UMP was lyophilized and rechromatographed with synthetically prepared 2',3'-5-HO-UMP. The elution pattern of this chromatography as shown in Fig. 3 positively identifies this radioactive constituent of RNA as 2',3'-5-HO-UMP.

A very small amount of radioactivity was always found in UMP and CMP of the RNA hydrolysate (Fig. 3) even though uridine-6-14C was rigorously excluded as a contaminant of 5-HO-Urd.

### Table III

**Effect of 5-HO-UMP on conversion of erotic acid-6-14C to orotic acid and uridine nucleotides**

<table>
<thead>
<tr>
<th>Molar ratio of 5-HO-UMP to erotic acid</th>
<th>Radioactivity recovered as %</th>
<th>Orotic acid</th>
<th>OMP</th>
<th>UMP</th>
<th>UDP</th>
<th>UTP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td></td>
<td>21.7</td>
<td>1.8</td>
<td>7.1</td>
<td>10.2</td>
<td>59.2</td>
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<tr>
<td>0.1</td>
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<td>10.4</td>
<td>5.2</td>
<td>6.2</td>
<td>39.5</td>
</tr>
<tr>
<td>0.5</td>
<td></td>
<td>45.1</td>
<td>23.3</td>
<td>8.5</td>
<td>3.2</td>
<td>26.9</td>
</tr>
<tr>
<td>5.0</td>
<td></td>
<td>61</td>
<td>33</td>
<td>0</td>
<td>0</td>
<td>4</td>
</tr>
</tbody>
</table>

1.25 mM ammonium formate. The first two fractions contained uracil and uridine; the third fraction contained phosphorylated uridine derivatives. The results (Table II) demonstrate that uridine phosphorylation is not appreciably inhibited even at very high inhibitor to substrate ratios. Similar experiments were performed to test the effect of 5-HO-UMP on conversion of uracil and uridine; and neutralized with KOH. Inorganic pyrophosphate, when added at the start of the 2-hour incubation period and the isolated nucleic acids were dialyzed against unlabeled 5-HO-Urd. The nucleic acids were hydrolyzed in 0.1 N NaOH at 37° for 18 to 22 hours (13). DNA, precipitated by adjusting the pH to 1 with HCl, was not radioactive. The 2',3'-ribonucleotides were separated by column chromatography on Dowex 1 (formate) with a concentration gradient of formic acid. The radioactive peak eluted immediately after 2',3'-UMP was lyophilized and rechromatographed with synthetically prepared 2',3'-5-HO-UMP. The elution pattern of this chromatography as shown in Fig. 3 positively identifies this radioactive constituent of RNA as 2',3'-5-HO-UMP.

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It was assumed that the radioactive peak, tentatively identified as 5-HO-UDP-sugars, was formed directly from 5-HO-UTP and sugar phosphates. More positive identification was attempted by testing the effectiveness of inorganic pyrophosphate on reversal of this reaction. 5-Hydroxyuridine diphosphate-carbohydrate peaks from several chromatographic runs were pooled and passed through Dowex 50 (H+) to remove ammonium ions. After lyophilization, the material was taken up in water and neutralized with KOH. Inorganic pyrophosphate, when incubated with this material, stimulated its conversion to 5-HO-UDP and 5-HO-UTP as compared to the control without pyrophosphate (Table IV). Any 5-HO-UTP formed during the incubation would not be expected to remain as such since ATP was not present in the reaction mixtures. The increased amount of 5-HO-UDP plus 5-HO-UTP may be considered evidence for the stimulation of 5-HO-UTP formation by pyrophosphate. From these data, and the presumptive evidence provided by the position at which the 5-HO-Urd compound is eluted from resin columns, it may be concluded that this compound is a 5-hydroxy derivative of UDP-sugar cofactors.

Incorporation of 5-HO-Urd-2-14C into RNA of Ehrlich Ascites Cells—Since 5-HO-Urd is converted to 5-HO-UTP in Ehrlich ascites cells (Fig. 2) it was of interest to determine whether or not the analogue is incorporated into RNA. The experimental approach was the same as that previously described except that 5-HO-Urd-2-14C (12 pmol, 0.4 × 10^6 c.p.m. per pmole) replaced labeled erotic acid and valine. All compounds were added at the start of the 4-hour incubation period and the isolated nucleic acids were dialyzed against unlabeled 5-HO-Urd. The nucleic acids were hydrolyzed in 0.1 N NaOH at 37° for 18 to 22 hours (13). DNA, precipitated by adjusting the pH to 1 with HCl was not radioactive. The 2',3'-ribonucleotides were separated by column chromatography on Dowex 1 (formate) with a concentration gradient of formic acid. The radioactive peak eluted immediately after 2',3'-UMP was lyophilized and rechromatographed with synthetically prepared 2',3'-5-HO-UMP. The elution pattern of this chromatography as shown in Fig. 3 positively identifies this radioactive constituent of RNA as 2',3'-5-HO-UMP.

A very small amount of radioactivity was always found in UMP and CMP of the RNA hydrolysate (Fig. 3) even though uridine-6-14C was rigorously excluded as a contaminant of 5-HO-Urd.
Ehrlich ascites cells were incubated with 5-HO-Urd-2,14C for 1, 1.0, 2.0, 4.0, and 6.0 hours as described in the text. The perchloric acid-soluble fractions from these incubations were combined and chromatographed on Dowex 1 (formate) (14). 5-HO-UDPX, 5-hydroxyuridine diphosphate-carbohydrate.

Another radioactive peak, containing 40 to 80% of the total radioactivity, was eluted just before 2',3'-GMP. It was suspected that this radioactive compound might be a breakdown product of 2',3'-5-HO-UMP formed during alkaline hydrolysis of RNA. This tentative conclusion was strengthened by experiments in which it was found that 5-HO-Urd or 2',3'-5-HO-UMP slowly lost their characteristic spectra upon treatment with alkali at conditions comparable to those used in the hydrolysis of RNA. In addition, it was found that treatment of 2',3'-5-HO-UMP with alkali produced a compound which gave a positive test for carbamyl groups with reagents used in a modification (18) of the method of Koritz and Cohen (19). To obtain further information on the identity of the labeled compound, unlabeled 2',3'-5-HO-UMP was treated with alkali and chromatographed on Dowex 1 (formate) together with pooled radioactive peaks containing the unknown compound from RNA hydrolysates. The radioactivity and color yield of material eluted from the column were eluted from the column at nearly identical positions (Fig. 4). The lack of complete correspondence may be due to different origins of the radioactive and nonradioactive materials. The identical nature of the material from the two sources was verified by paper chromatography. The radioactive peak containing the carbamyl compound was lyophilized, spotted on Whatman No. 3 MM paper and the chromatogram developed with 60% ethanol, 0.3 N in acetic acid. The paper was run through a strip counter; the radioactive area and adjacent areas were cut out and eluted. Only the area containing radioactivity gave a positive carbamyl test. It may be concluded that the radioactive peak present in the RNA hydrolysate is a breakdown product of 2',3'-5-HO-UMP formed during alkaline degradation, and most likely is an analogue of β-N-ribosyl-

![Figure 2. Ion exchange chromatography of the acid-soluble fraction of Ehrlich ascites cells incubated with 5-HO-Urd-2,14C.](image)

**Table IV**

<table>
<thead>
<tr>
<th>Incubation of presumed 5-HO-UDP-sugar with inorganic pyrophosphate</th>
</tr>
</thead>
<tbody>
<tr>
<td>To each of two flasks were added 10 ml (50,000 c.p.m.) of the presumed 5-HO-UDP-sugar and 10 ml of 100,000 x g supernatant. To one flask, 15 μmoles of potassium pyrophosphate were added. The incubation was carried out for 5 minutes at 37°. The reaction was halted by addition of 5 ml of 4.0 N perchloric acid. The neutralized acid-soluble fractions were chromatographed on Dowex 1 (formate) by an ammonium formate, pH 4.4, gradient. Unlabeled UDP and UTP were added to the column to facilitate identification of the labeled peaks as they were eluted.</td>
</tr>
<tr>
<td>Compound</td>
</tr>
<tr>
<td>------------------------</td>
</tr>
<tr>
<td>5-HO-UDPX</td>
</tr>
<tr>
<td>5-HO-UDP</td>
</tr>
<tr>
<td>5-HO-UTP</td>
</tr>
</tbody>
</table>

*5-HO-UDPX, 5-hydroxyuridine diphosphate-carbohydrate.*
Identification of 2',3'-5-HO-UMP-2-14C from RNA hydrolysates. The alkaline hydrolysate of RNA isolated from Ehrlich ascites cells incubated in the presence of 5-HO-Urd-2-14C had previously been chromatographed on Dowex 1. The radioactive peak immediately following 2',3'-UMP was collected, lyophilized, and rechromatographed on a 1- × 30-cm column of Dowex 1 (formate). Unlabeled synthetic 2',3'-5-HO-UMP was added to the column. Mixing flask: 1 liter of water initially; reservoir, 9.0 N formic acid. ---, counts per minute per tube.

50

Comparison of Metabolism of 5-HO-Urd-2-14C and Uridine-2-14C by Ehrlich Ascites Cells—Since 5-HO-Urd is converted to nucleotides and RNA in the same manner as uridine, quantitative differences in the metabolism of the two compounds were studied. Ehrlich ascites cells were incubated separately in the presence of 5-HO-Urd-2-14C or uridine-2-14C for 4 hours as described in the experiments on incorporation of 5-HO-Urd-2-14C into RNA. The extent of incorporation of each radioactive nucleoside into their respective nucleotides (14) and into nucleic acids (13) was determined. The results are shown in Table V. A maximum 2-fold difference in concentration is found in the respective acid-soluble compounds, while the amount of uridine-2-14C incorporated into nucleic acids is 27 times that of 5-HO-Urd-2-14C.

Synthesis and Polymerization of 5-HO-UDP and 5-Br-UDP and Effect of Poly-5-HO-UMP and Poly-5-bromouridylate on Amino Acid Incorporation—5-HO-UDP was synthesized by a modification of the method of Ueda (8) for 5-HO-UMP synthesis. 5-Br-UDP was isolated as one of the products of the reaction and separated by column chromatography on Dowex 1 (formate). Polynucleotide phosphorylase from Micrococcus lysodeikticus was purified through the acetone fractionation step and used for the preparation of poly-5-HO-UMP and poly-5-bromouridylic acid by the method of Beers (21). Protein-free polymers were isolated with s20 values of 1.8 and 2.0 for poly-5-HO-UMP and poly-5-bromouridylic acid, respectively. These polymers were tested for their ability to stimulate amino acid incorporation into acid-insoluble material according to the procedure of Nirenberg and Matthaei (22). Results similar to those recently reported by Grunberg-Manago and Michelson were obtained.
TABLE V
Comparison of metabolic fate of 5-HO-Urd-2-14C and uridine-2-14C in Ehrlich ascites cells

Labeled uridine or 5-HO-Urd (12 μmoles per flask) of the same specific activity were incubated separately with Ehrlich ascites cells for 4 hours as described in the text. The acid-soluble phosphorylated derivatives of uridine and 5-HO-Urd were, in each case, separated by gradient elution on Dowex 1 (formate) (14). The total nucleic acids were isolated by the method of Tyner et al. (13) and dialyzed against uridine or 5-HO-Urd. The values represent the amount of material (calculated from the specific activity of the nucleotides) recovered in the acid-soluble fraction and total nucleic acids expressed on a per flask basis. The acid-soluble fractions from two flasks containing uridine-2-14C were combined and analyzed. Acid-soluble fractions from five flasks containing 5-HO-Urd were combined and analyzed. The amount of radioactive nucleoside incorporated into nucleic acids was determined separately for each flask and the results averaged.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Concentration</th>
<th>Ratio of uridine-2-14C to 5-HO-Urd-2-14C*</th>
</tr>
</thead>
<tbody>
<tr>
<td>UDPX†</td>
<td>0.45</td>
<td>0.37</td>
</tr>
<tr>
<td>UDP</td>
<td>0.13</td>
<td>0.19</td>
</tr>
<tr>
<td>UTP</td>
<td>0.48</td>
<td>0.27</td>
</tr>
<tr>
<td>Nucleic acid</td>
<td>0.40</td>
<td>0.015</td>
</tr>
</tbody>
</table>

* The amount of uridine-2-14C converted to a given compound divided by the amount of 5-HO-Urd-2-14C converted to the analogous compound.
† UDPX, uridine diphosphate-carbohydrate.
† 5-HO-UDPX, 5-hydroxyuridine diphosphate-carbohydrate.

(23). Details of these experiments will be reported in a later communication.

DISCUSSION

As is the case in bacteria (5), 5-HO-Urd inhibits RNA and protein synthesis in Ehrlich ascites cells (Fig. 1). In contrast to the results obtained with E. coli 15 T−, the effect of the analogue on RNA synthesis in Ehrlich ascites cells is much more pronounced than the effect on protein synthesis. This is consistent with the more rapid turnover of messenger RNA in bacterial than in mammalian cells. In bacteria the half-life of messenger RNA is 2 to 8 minutes (24, 25), and interference with RNA synthesis is reflected in an almost immediate decrease in protein synthesis (5, 26). Messenger RNA of some mammalian cells may be assumed to have a much longer half-life since inhibition of RNA synthesis in these cells has little or no effect on protein synthesis for an hour or more (27). It is not surprising, therefore, that although 5-HO-Urd greatly inhibits RNA synthesis, incorporation of label into protein continues relatively unaffected during the 30-minute incubation period.

Ehrlich ascites cells and cell-free enzyme preparations metabolize 5-HO-Urd to phosphorylated derivatives (Fig. 2, Table V) which are analogous to those formed from uridine and fluorouracil (28, 29). However, 5-HO-UMP has only a slight inhibitory effect on the conversion of UMP to UTP, whereas conversion of uracil-3-14C to UTP is inhibited by the nucleotide analogue accompanied by an accumulation of orotidylate acid. At a concentration one-tenth that of uracil acid, 5-HO-UMP inhibits the conversion of orotic acid to uridine nucleotides by 35%. At 5 times the uracil acid concentration, 5-HO-UMP inhibits conversion to uridine nucleotides by 95% (Table III). These data show that orotidylate acid decarboxylase is the only enzyme which is highly sensitive to 5-HO-UMP in the conversion of uracil acid to UTP. Orotic acid-3-14C is also utilized to a lesser extent in the presence of 5-HO-UMP than in its absence (Table III). This cannot be interpreted as a direct inhibitory effect on orotidylate acid pyrophosphorylase. The observed accumulation of orotidylate acid would be expected to retard orotic acid utilization since the equilibrium constant for the reaction is 0.12 (30). The conditions at which this inhibitory effect was demonstrated approximate those used by Pasternak and Handschumacher (31) who reported that 6-azauridylic acid inhibits decarboxylation of OMP. In a supernatant fraction of several tumors, azauridylic acid inhibited uracil acid conversion to uridine nucleotides at analogue to uracil acid ratios similar to those observed in these studies. Orotidylate acid decarboxylase is also inhibited by UMP, although the quantity required is much greater (32-34) than the amount required for inhibition by azauridylic acid or 5-HO-UMP.

The steady state concentrations of uracil acid and OMP are very small as compared to other nucleoside phosphates as evidenced by the fact that neither compound has been isolated from cells to which uracil acid was not added exogenously (33). Even in cell-free incubations to which uracil acid is added as a substrate, the amount of OMP present in the nucleotide pool is very small (Table V). Thus, it would be expected that azauridylic acid inhibits decarboxylation of OMP. In a supernatant fraction of several tumors, azauridylic acid inhibited uracil acid conversion to uridine nucleotides at analogue to uracil acid ratios similar to those observed in these studies. Orotidylate acid decarboxylase is also inhibited by UMP, although the quantity required is much greater (32-34) than the amount required for inhibition by azauridylic acid or 5-HO-UMP.

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It is interesting to note that Pasternak and Handschumacher (31) in their studies on azauridine inhibition of mouse tumors did not report accumulation of OMP or a significantly lowered utilization or uracil acid (Table I). These experiments, however, were not designed to test the effectiveness of 5-HO-UMP as an inhibitor of orotidylate decarboxylase. In particular, the method of gradient elution (14) used in the analysis of the acid-soluble fraction does not adequately separate OMP from UDP and a small accumulation of OMP would pass unnoticed (34). In addition, some tumor extracts contain phosphatases which rapidly degrade OMP to orotidine (31). In the gradient elution used, orotidine is not well separated from uracil acid. Thus, inhibition of orotidylate decarboxylase by 5-HO-UMP might not be detected by analysis with the formic acid-ammonium formate gradient method. It is interesting to note that Pasternak and Handschumacher (31) in their studies on azauridine inhibition of mouse tumors did not report accumulation of OMP, but rather an accumulation of orotidine. In mice, azauridine is metabolized only to its monophosphate which acts as an inhibitor of orotidylate decarboxylase (31). This raises another point which is important in assessing the inhibition produced by 5-HO-Urd. Azauridine is metabolized only to its monophosphate in animal cells and thus acts only on orotidylate decarboxylase, whereas 5-HO-Urd is metabolized to other phosphorylated derivatives (Fig. 3, Table V). Experiments in which 5-HO-Urd was incubated with ascites cells for different periods of time have shown that the concentration of 5-HO-UMP is much higher after 30 minutes of incubation than it is after 4 hours when comparatively small concentrations of 5-HO-UMP are found. After longer incuba-

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tion periods most of the analogue in the acid-soluble fraction is found as 5-IIO-UTP and as 5-IIO UDP-sugars (Table V). In view of these findings, the reaction most influenced by 5-HO-Urd might vary depending on the length of exposure to the analogue. Initially, when the concentration of 5-HO-UMP is relatively high, orotidyl acid decarboxylase is probably the major site of inhibition. Later, other sites of inhibition might be more important.

The conversion of UTP to CTP is evidently not affected by 5-HO-Urd nucleotides. In a single experiment, 5-HO-Urd-2-¹⁴C containing uridine-2-¹⁴C (0.4%) was incubated with Ehrlich ascites cells in the usual manner. RNA from these cells contained a significant amount of radioactive cytidine.

When the metabolism of uridine and 5-IIO-Urd are quantitatively compared (Table IV), only a single component shows a striking difference in the amount formed from the respective precursors during the time period studied. The amount of uridine-2-¹⁴C incorporated into RNA is 27 times greater than that from 5-HO-Urd-2-¹⁴C. Since the synthesis of RNA presumably involves not only enzymatic specificity but also requires that a base pair be formed with a base from the DNA or RNA primer, the observed differences may be due to a relative inability of 5-HO-UTP to form hydrogen-bonded base pairs with adenine. This explanation is consistent with the observation that substitution of groups with negative inductive effects at position 5 of the uracil moiety apparently does alter the base pairing characteristies of the molecule (23, 35). Recently Grunberg-Manago and Michelson reported that various polyuridylic acid analogues, including poly-5-HO-UMP, stimulate phenylalanine incorporation into a product precipitable by hot trichloroacetic acid (23). Some of these polynucleotide analogues, including poly-5-HO-UMP, stimulated the incorporation of amino acids other than phenylalanine indicating that 5 substituted uracil derivatives can indeed form base pairs which are not characteristic of the uracil moiety. This suggests the possibility that 5-HO-Urd when incorporated into messenger RNA may produce altered proteins as has been observed with 5-fluorouracil (35-38).

Since phosphorylated derivatives of 5-HO-Urd are formed to about the same extent as those of uridine (Table V), several other potential sites of inhibition exist. These include the formation and function of uridine dipho-sugars, formation of deoxyribonucleotides, and RNA polymerase. The present data give no information regarding the effect of 5-HO-Urd derivatives on these reactions.

**SUMMARY**

Inhibitory effects of 5-hydroxyuridine and 5-hydroxyuridylic acid were studied in Ehrlich ascites cells and cell-free homogenates. In addition, 5-hydroxyuridine-2-¹⁴C metabolism was compared with the metabolism of uridine-2-¹⁴C.

Incorporation of orotic acid-6-¹⁴C into RNA and valine-1-¹⁴C into protein of Ehrlich ascites cells incubated in vitro was followed as a function of 5-HO-Urd concentration. 5-HO-Urd inhibited orotic acid incorporation into RNA to a maximum of 70% and to a lesser extent inhibited valine incorporation into protein. Ion exchange chromatography of the acid-soluble nucleotides from the control and the 5-HO-Urd-exposed cells showed no significant differences in the distribution of radioactive nucleotides.

5-Hydroxyuridine 5'-monophosphate was found to be a potent inhibitor of orotidyl acid decarboxylase. The formation of uridine nucleotides from orotic acid was almost completely prevented by the presence of 5-IIO-UMP. Other reactions involved in the conversion of orotic acid to uridine triphosphate were not affected by 5-HO-Urd or its phosphorylated derivatives.

5-HO-Urd-2-¹⁴C was synthesized and its metabolism followed in Ehrlich ascites cells. Evidence was obtained for the formation of 5-hydroxy derivatives of uridine monophosphate, uridine diphosphate, uridine triphosphate, and uridine diphosphatesugars. Radioinactive 2',3'-5-HO-UMP and its breakdown product containing a carbamyl group were identified in the alkaline hydrolysate of RNA isolated from these cells.

Ehrlich ascites cells were incubated in the presence of equal amounts of either uridine-2-¹⁴C or 5-HO-Urd-2-¹⁴C to determine quantitative differences in the metabolic fate of the two compounds. Differences in the amounts of uridine-2-¹⁴C and 5-HO-Urd-2-¹⁴C in the respective acid-soluble nucleotides were no greater than 2-fold. The amount of uridine-2-¹⁴C incorporated into RNA, however, was 27 times greater than that of 5-HO-Urd-2-¹⁴C.

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