Studies on the Mechanism of Action of 6-Uracil Methyl Sulfone in Mouse Ehrlich Ascites Tumor Cells in Vitro*

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Orotic acid is utilized by many mammalian and bacterial systems for the biosynthesis of the pyrimidines found in ribonucleic (RNA) and deoxyribonucleic (DNA) acids. 6-Uracil methyl sulfone (UMS) is one of several orotic acid analogs which have been synthesized and studied by Greenbaum, Holmes, and Welch (1, 2, 15). Holmes and Welch have shown (4) that UMS non-competitively inhibits the growth of several strains of Lactobacillus bulgaricus in media supplemented with orotic acid, L-dihydroorotate or carbamyl-DL-aspartate. Although this inhibition could not be reversed by uridine-5'-phosphate, a crude preparation of RNA derived from yeast effectively prevented UMS from inhibiting bacterial growth. Holmes (3) has observed that UMS competitively inhibited the conversion of orotate to orotidine-5'-phosphate (O-5-P) by partially purified O-5-P pyrophosphorylase of yeast. Jaffe and Cooper1 have investigated the metabolism, tissue distribution, toxicity, and carcinostatic activity of UMS and have made some biochemical correlations with biological activity.

The present report describes studies of the mechanism of action of UMS and measurements of its inhibitory effects on the utilization of radioactive formate, orotic acid, and thymidine for the biosynthesis of nucleic acid pyrimidines of mouse Ehrlich ascites tumor cells in vitro.

MATERIALS AND METHODS

Preparation of cell suspension.—The Ehrlich ascites tumor was maintained in DBA/2 mice purchased from the Roseoee B. Jackson Memorial Laboratory, Bar Harbor, Maine. Five or 6 days following the intraperitoneal inoculation of mice with tumor cells, ascitic fluid was collected from a suitable number of mice and pooled in a graduated centrifuge tube (40 ml.) which contained 5 ml. of the modification of Chamber's solution described by Totter (14); after centrifugation, the residual packed cells were resuspended in 8 volumes of this solution.

Preparation of solutions.—Sodium formate-C14 and orotic acid-4-C14 had specific activities of 1 μe/μmole. The nucleosides were obtained from Schwarz Laboratories. The orotic acid analog, 6-uracil methyl sulfone (UMS), was supplied by S. Greenbaum2 of this department, 5-hydroxymethyluracil by K. Fink, and thymine riboside by J. Fox. Radioactive thymidine (TdR), labeled in the methyl group with C14, was prepared in this department by T. Ulbricht.*

<table>
<thead>
<tr>
<th>Metabolite</th>
<th>Relative specific activity of DNA-thymine†</th>
</tr>
</thead>
<tbody>
<tr>
<td>None (control)</td>
<td>1.00</td>
</tr>
<tr>
<td>Uracil</td>
<td>1.00</td>
</tr>
<tr>
<td>Uridine</td>
<td>2.30</td>
</tr>
<tr>
<td>Deoxyuridine</td>
<td>2.82</td>
</tr>
<tr>
<td>Orotic acid</td>
<td>2.20</td>
</tr>
</tbody>
</table>

* The reaction mixtures consisted of packed cells (0.25 ml.), sodium formate-C14 (6 μmoles, 5 μc), pig serum (0.1 ml.), metabolite (10 μmoles), and Totter's modified Chamber's solution (14) to 2.6 ml. The incubations were conducted in duplicate in 20-ml beakers in a Duboff metabolic shaker at 37° C. (air); agitation was at 90 cycles per minutes for 4 hours.

† Specific activity of DNA-thymine in the control vessel equated to 1.00.

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tography with an isopropanol-HCl (2 n) system (16). The
individual bases were eluted with water and again subjected to
paper chromatography in a butanol-water system (7). The
concentrations of the individual bases were determined in the
Beckman UV-spectrophotometer; their radioactivity was
measured, following the plating of appropriate aliquots in the
center of a stainless steel planchet in a windowless flow counter.

RESULTS

Effect of orotic acid and uracil derivatives on the
utilization of formate-C$^{14}$ for DNA-thymine bio-
synthesis.—Orotic acid increased the utilization
of radioactive formate for the synthesis in vitro of
the methyl group of DNA-thymine of mouse
Ehrlich ascites tumor cells (Table 1). The magni-
tude of the increase was equivalent to that ob-
served previously with uridine (UR) and de-
TABLE 2

| EFFECT OF OROTIC ACID, URIDINE, AND |
| URACIL ON THE CONVERSION OF FOR- |
| MATE-C$^{14}$ TO CARBON DIOXIDE* |

<table>
<thead>
<tr>
<th>Metabolite</th>
<th>Specific activity of BaCO$_3$ (counts/min/mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None (control)</td>
<td>63,000</td>
</tr>
<tr>
<td>Orotic acid</td>
<td>62,000</td>
</tr>
<tr>
<td>Uridine</td>
<td>62,700</td>
</tr>
<tr>
<td>Uracil</td>
<td>55,000</td>
</tr>
</tbody>
</table>

* Details of the incubation mixture are
given in Table 1, with the modification that
the incubation was performed in Warburg
vessels with alkali in the center well.

oxoyuridine (UdR) (10, 11). Uracil, in contrast to
its nucleoside, exerted no effect under the condi-
tions used for the incubation. These compounds do
not exert their effect by merely depressing for-
mate-C$^{14}$ degradation, since the amount of carbon
dioxide formed from formate-C$^{14}$, when these
tumor cells were incubated in the presence or
absence of uracil, uridine, or orotic acid (Table 2),
was essentially the same.

It had been shown previously that uridine-C$^{14}$
was utilized for the biosynthesis of DNA-thymine
by mouse Ehrlich ascites cells and that it probably
exerted its effect on formate utilization by in-
creasing the formate acceptor pool (9). Radioac-
tive orotic acid was also utilized for the synthesis
of DNA pyrimidines of Ehrlich ascites cells under
our conditions of incubation, and, in agreement
with the results of Lagerkvist and Reichard (5), a
much greater incorporation of orotic acid was observed into DNA-thymine than into DNA-
cytosine (about 4 times as much).
Inhibition of formate utilization by UMS and its
reversal.—The effect of UMS on the utilization
of formate-C$^{14}$ for the biosynthesis of the methyl
portion of DNA-thymine is shown in Table 3. An
inhibition was observed of over 80 per cent in the
utilization of formate-C$^{14}$ for the biosynthesis of
DNA-thymine, and this inhibition could not be
reversed by orotic acid when the molar ratio of
orotic acid to UMS was as high as 88 to one. In an
two attempt to elucidate the site of inhibition by UMS,
compounds were investigated which were known
to exert a stimulatory effect on formate utilization
for DNA-thymine biosynthesis of Ehrlich ascites
tumor cells. These compounds and their effects are
shown in Table 4. In the absence of an inhibitor,
it has been shown that orotic acid, UR, and UdR
doubled, and CR and CdR tripled or quadrupled
(9–11), the observed specific activity of DNA-
thymine when these cells were incubated with
radioactive formate in vitro. In the present study
none of these compounds reversed the inhibition

TABLE 3

| EFFECT OF 6-URACIL METHYL SULFONE ON THE UTILIZATION OF FORMATE-C$^{14}$ FOR THE BIOSYNTHESIS OF THE METHYL GROUP OF DNA-THYMINE BY MOUSE EHRLICH ASCITES CELLS in Vitro* |

<table>
<thead>
<tr>
<th>Concentration of 6-uracil methyl sulfone (µmoles/ml)</th>
<th>Specific activity of DNA-thymine (counts/min/µmole)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>260</td>
</tr>
<tr>
<td>0.3</td>
<td>360</td>
</tr>
<tr>
<td>1.0</td>
<td>200</td>
</tr>
<tr>
<td>3.0</td>
<td>100</td>
</tr>
</tbody>
</table>

* Details of the incubation mixtures are
given in Table 1, with the modification of
addition of the indicated concentrations of
UMS and elimination of pyrimidine metab-
olite.

TABLE 4

| REVERSAL OF THE INHIBITION BY 6-URACIL METHYL SULFONE OF THE UTILIZATION OF FORMATE-C$^{14}$ FOR THE BIOSYNTHESIS OF DNA-THYMI-
| NINE OF MOUSE EHRLICH ASCITES TUMOR CELLS in Vitro* |
|------------------------------------------------------|-----------------------------------------------------|
| Reversal agent (10 µmoles) | Relative specific activity of DNA-thymine† |
| None                        | 0.18                                                |
| Orotic acid                 | 0.33                                                |
| Uridine                     | 0.48                                                |
| Cytidine                    | 0.52                                                |
| Deoxyuridine                | 0.68                                                |
| Deoxycytidine               | 1.10                                                |
| RNA (0.3 mg.)               | 0.10                                                |
| RNA (0.5 mg.)               | 0.10                                                |

* Details of incubation mixtures are
given in Table 1. The concentration of
UMS in all vessels was 0.3 µmole.
† Specific activity of DNA-thymine
in control vessels containing no UMS
(in which the specific activity was about
5.5 times that in the presence of UMS)
was equated to 1.00.
completely, although CdR, the most efficient reversal agent investigated, did increase by sixfold the utilization of formate-C\(^{14}\) for DNA biosynthesis. However, deoxycoformycin, in the presence of UMS, at a molar ratio of CdR to UMS of 33 to 1, was not able to raise the level of formate utilization beyond that observed in the absence of inhibitor and of nucleoside. Although UdR caused a fourfold increase in the utilization of formate-C\(^{14}\), this effect was less than that observed with CdR; however, in the absence of the inhibitor, the effect of UdR on formate utilization for DNA-thymine biosynthesis was also less than that of CdR.

Of greater significance is the disparity between the effect of the ribonucleosides and the corresponding deoxyribosides in overcoming the effects of UMS on the incorporation of formate-C\(^{14}\) into DNA-thymine, in that the deoxyribosides were significantly more active than the corresponding ribosides. This is in marked contrast to the close similarity of the effects of the corresponding nucleosides in the absence of the inhibitor. The inhibition of \(L\). \(\text{bulgaricus}\) 09 by UMS had been shown by Holmes and Welch (4) to be prevented by partially purified yeast RNA, but not by highly purified RNA; however, with Ehrlich ascites cells such an effect could not be obtained with their preparations of RNA.

**Effect of UMS on orotic acid utilization.**—Since CdR was the most efficient compound investigated in preventing the inhibition of formate utilization by UMS (Table 4), the site of major inhibition in this system would appear to be removed from reactions concerned with the formation of pyrimidine ribosides. Therefore, the effect of UMS on the utilization of radioactive orotic acid was investigated; the results are shown in Table 5. No significant inhibition of the utilization of orotic acid for the biosynthesis of RNA-pyrimidines was observed. In three experiments the specific activity of DNA-cytosine in the absence of UMS was about 30 per cent greater than that observed in the inhibited cells. Most striking, however, was the marked inhibition in the formation of thymine. The specific activity of the control DNA-thymine was 19-63 times greater than that derived from the UMS-inhibited cells. Whether the inhibition occurred prior to, or after, the methylation of the precursor of TdR was investigated in the following experiment.

**Effect of UMS on thymidine utilization.**—The effect of UMS on the utilization of \(C^{14}\)-methyl labeled thymidine\(^{4}\) for the biosynthesis of DNA-thymine of Ehrlich ascites tumor cells in vitro was investigated; the results are shown in Table 6. No significant inhibition was observed when the molar ratio of UMS to thymidine was as high as 1,000 to 1.

**Effect of miscellaneous compounds on DNA biosynthesis.**—Compounds tested which exerted no effect on the utilization of formate-C\(^{14}\) for the biosynthesis of DNA-thymine by Ehrlich ascites tumor cells in vitro included thymine, uracil, cytosine, 5-methyl cytosine, and 5-hydroxymethyl cytosine; presumably, their inactivity reflected the fact that the corresponding nucleosides could not be formed from them in adequate concentrations under the conditions used. 6-Azauracil and 6-aza thymine, analogs of uracil and thymine, respectively, in which an N-atom replaced —CH= in position 6, also failed to exert any effect; however, the deoxyriboside of azathymine, azathymidine, had been shown previously to be a very effective inhibitor of the utilization of formate for DNA-thymine biosynthesis (10, 11). Thymine riboside probably cannot be converted to the corresponding deoxyriboside, under these conditions, since it

### Table 5

<table>
<thead>
<tr>
<th>Concentration of UMS (µmoles)</th>
<th>Specific activity of isolated pyrimidines</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>DNA thymine (counts/min/µmole)</td>
</tr>
<tr>
<td></td>
<td>RNA uracil (counts/µmole)</td>
</tr>
<tr>
<td></td>
<td>RNA cytosine (counts/µmole)</td>
</tr>
<tr>
<td>0.1</td>
<td>10.0</td>
</tr>
<tr>
<td>1.0</td>
<td>8.450</td>
</tr>
<tr>
<td>10.0</td>
<td>7.640</td>
</tr>
<tr>
<td>100.0</td>
<td>6.825</td>
</tr>
</tbody>
</table>

*Orotic acid-4-C\(^{14}\) (8.0 µmoles 0.9 µc.)

† Details of the incubation conditions are described in Table 1 with the appropriate changes indicated.

### Table 6

<table>
<thead>
<tr>
<th>Experiment no.</th>
<th>Thymidine-C(^{14}) (µmoles)</th>
<th>UMS (µmoles)</th>
<th>Specific activity of DNA-thymine (counts/min/µmole)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.5</td>
<td>10.0</td>
<td>8.640</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.05†</td>
<td>7.630</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.01†</td>
<td>6.330</td>
</tr>
</tbody>
</table>

* Details of the incubation conditions are described in Table 1 with the appropriate changes indicated above.

† Specific activity of thymidine was 1.5 µc/µmole.

‡ Specific activity of thymidine was 5.0 µc/µmole.
had no effect on the utilization of radioactive formate for the biosynthesis of DNA, whereas thymine deoxyriboside had been shown previously to reduce markedly the utilization of formate-C14 in this system (10, 11).

DISCUSSION

That several sites of inhibition by UMS may be involved was indicated by Holmes and Welch (4), and the present report contains additional information concerning an anabolic reaction which is inhibited. Holmes (3) clearly demonstrated that the conversion of orotate to orotidine-5'-phosphate was competitively inhibited by UMS. However, other sites may be more critically involved in the Ehrlich ascites tumor cell system, since there was no inhibition in the utilization of orotic acid-4-C14 for the biosynthesis of RNA-uracil or -cytosine. The conversion of radioactive orotic acid to DNA-thymine was inhibited by more than 95 per cent, whereas the incorporation into DNA-cytosine was depressed to a much less marked degree (25 per cent). In view of the inability of UMS to inhibit the utilization of radioactive thymidine at a molar inhibition index of 1,000 to 1, it would appear that a major site of inhibition is at a stage in the conversion of the precursor of DNA thymine to a methyl-containing compound.

Of particular importance is the failure of the pyrimidine ribosides to be as effective as the corresponding pyrimidine deoxyribosides in reversing the inhibitory effect of UMS in Ehrlich ascites cells. This would imply either an inhibition in the conversion of the ribose moiety into deoxyribose or in an essential alteration of the ribonucleoside (phosphorylation?) prior to its conversion to a deoxypentose-containing compound.

The inability of the crude RNA preparation to reverse the inhibition in Ehrlich ascites cells in contrast to L. bulgaricus 09 cells possibly may be explained by a lack of appropriate enzymes in Ehrlich ascites cells which may be required for partial degradation of the polymer to appropriate utilizable units. Of pertinence are the observations of Greenbaum (4), who found that inhibition of the growth of L. bulgaricus 09 by UMS could be reversed by high levels of various nucleosides, of which the most efficient was cytosine deoxyriboside. Recent studies by Cooper (4) have demonstrated that UMS undergoes very rapid non-enzymatic conversion to other derivatives, which may explain, in part, why several sites of inhibition have been observed.

REFERENCES

7. MARKHAM, R., and SMITH, J. D. Chromatographic Studies of Nucleic Acids. I. A Technique for the Identification and


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