The Effect of A-Methopterin on Formate-C\(^{14}\) Incorporation by Mouse Leukemias in Vitro*  

ALBERT D. WILLIAMS, GRANT G. SLATER,† AND RICHARD J. WINZLER  

(Departments of Biological Chemistry, University of Illinois College of Medicine, Chicago, Illinois, and of Biochemistry and Nutrition, University of Southern California, Los Angeles, Calif.)

The 4-amino derivatives of folic acid such as aminopterin and A-methopterin produce temporary remissions in many cases of acute leukemia in children (2, 6) and in some types of experimental rodent leukemia (10, 13). Resistance to the therapeutic effectiveness of folic acid antagonists develops during the course of treatment with these agents (7, 11, 12, 22). Experimental strains of rodent leukemia have been developed which not only are resistant to A-methopterin but are dependent on the administration of the drug for optimal growth (11, 14). Available evidence strongly supports the view that folic acid and its derivatives function in several steps involved in the metabolic interconversion of “one-carbon units,” including the incorporation of such units into purines and amino acids (1, 3, 7—9, 19—21, 25—27).

The present work was undertaken to determine whether the incorporation of formate into purines and amino acids in vitro is differentially affected by folic acid antagonists and whether tissues vary in their susceptibility to the action of these antagonists.

MATERIALS AND METHODS

Tissue and incubation conditions.—Strain DBA/2 mice bearing subcutaneous implants of the L1210-S or the L1210-R lymphomas were used 10 days after implantation. The survival of mice bearing strain L1210-S lymphoma is markedly prolonged by treatment with A-methopterin, whereas survival of mice bearing the resistant variant L1210-R is not changed by A-methopterin therapy (11, 18). Liver tissue was from normal DBA mice. Livers and spleens infiltrated with leukemic cells from Db and Db-FnA mice bearing a transplantable, methylcholanthrene-induced lymphocytic leukemia were used in some of the studies. These tissues were infiltrated to the extent that 80—90 per cent of the cells were leukemic.

Tissue minces were prepared as previously described (31). From 300 to 500 mg. (wet weight) of minced tissue was placed in 25-ml. Erlenmeyer flasks containing 5 ml. of Krebs-Ringer bicarbonate buffer (29) supplemented with 3 mg. of glucose/ml. The tissue minces were incubated at 37° C. in 5 per cent CO\(_2\)-95 per cent O\(_2\) with shaking at 90—100 oscillations/minute for periods up to 6 hours. Potassium formate-C\(^{14}\) (1.6—2.9 \(\mu\)c/microl.) dissolved in buffer, was added to the incubation vessels from a 0.25-ml. syringe and needle at the start of the incubation. Although different amounts of the isotopic formate were used in different experiments, the same quantity of isotope was carefully added to each vessel of a given experiment. The amount and specific activity of formate used for each experiment is indicated in the tables.

Tissue fractionation.—The tissue was fractionated as previously described (25, 31) in order to obtain the trichloroacetic acid-insoluble, lipid-free residue (gross protein) and the lipid fraction. In some experiments, ribonucleic acid was extracted from one portion of the gross protein fraction and hydrolyzed to the free ribonucleotides (30). Amino acids were obtained by acid hydrolysis of another aliquot of the protein fraction (34). The amino acids and the ribonucleotides required extensive purification by several separation procedures in order to achieve radio-purity. A constant specific activity in two or more successive separations of a compound was regarded as a criterion of purity.

In preliminary experiments the individual amino acids were separated on Dowex-50 ion exchange columns (17). Amino acids free from nonamino acid radioactivity were usually obtained by a sequential separation procedure which involved eluting the bands of mixed amino acids from a filter paper ionophoretic separation at pH 1.7 as the first step. Resolution of these mixtures, accomplished by filter paper chromatography with either a butanol-acetic acid-water solvent (15) or a phenol-water solvent (16), yielded compounds free from non-nucleotide radioactivity by filter paper ionophoresis at pH 3.7 (30, 31). Individual ribonucleotides eluted from the paper were further purified by filter paper chromatography (4). The purine nucleotides were then hydrolyzed to the purine bases, which were placed on small Dowex-50 ion exchange columns, washed with water, and eluted with 6 N HCl. The nucleotides and purines were quantitatively determined on

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† Present address: Institute for Medical Research, Cedars of Lebanon Hospital, Los Angeles, Calif.
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the basis of published molar extinction coefficients (8). The ratio of the optical density readings at 400 and 280 mg/ml agreed with published values (30) or with experimentally determined values for pure compounds.

**Determination of radioactivity and expression of results.**—The radioactivity in aliquots of the gross protein acid hydrolysate or the lipid fractions, dried on 1-inch copper planchets, was measured in a windowless flow counter, corrected for background and to a sample thickness of 15 mg/planchet. The results are expressed as counts/minute/mg of starting protein material. Amino acid and ribonucleotide data are corrected to a sample thickness of 1 mg. on 1.89 sq. cm. polyethylene planchets, and are expressed as counts/minute/µM of compound. A sufficient number of counts was observed so that the standard counting error was less than 5 per cent, except for those samples in which there were very few counts.

### TABLE 1

**TIME CURVE FOR THE UPTAKE OF FORMATE-C\(^{14}\) INTO PROTEINS AND LIPIDS OF THE L1210-S LYMPHOMA**

<table>
<thead>
<tr>
<th>Time (hours)</th>
<th>No. incubations</th>
<th>Protein(^{†}) (cpm/mg)</th>
<th>Lipid(^{†}) (cpm/mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>4</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>1</td>
<td>4</td>
<td>3,170 ± 145</td>
<td>411 ± 86</td>
</tr>
<tr>
<td>2</td>
<td>4</td>
<td>9,450 ± 130</td>
<td>907 ± 82</td>
</tr>
<tr>
<td>4</td>
<td>16,900±</td>
<td>1,558±90</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>17,800±1,240</td>
<td>1,558±67</td>
<td></td>
</tr>
</tbody>
</table>

*Incubation in 95 per cent O\(_2\)-5 per cent CO\(_2\) in Krebs-Ringer bicarbonate buffer; 0.1 per cent glucose with 7.06 µc. of C\(^{14}\) in 2.9 µM of potassium formate added at zero time.

\(^{†}\) Specific activity = counts/minute/mg of protein residue.

### RESULTS

The incorporation of formate-C\(^{14}\) into the gross protein or lipid fractions of L1210-S tissue was approximately linear with time for periods up to 4 hours (Table 1). Radioactivity at zero time was insignificant.

The effect of A-methopterin at several concentrations on the incorporation of formate, during a 6-hour period of incubation, into the protein fraction of the L1210-S lymphoma tissue is shown in Table 2. At 0.0033 mg/ml, A-methopterin inhibited the incorporation of formate-C\(^{14}\) into the protein fraction by 75 per cent. An increase of the A-methopterin concentration to nearly 100-fold did not diminish further the uptake of formate C\(^{14}\) into the protein fraction.

In Table 3 are summarized the data obtained when A-methopterin and formate-C\(^{14}\) were incubated for 6 hours with minced L1210-S and L1210-R lymphomas, and for 4 hours with spleen or liver infiltrated with leukemic cells from Db and Db-FnA mice bearing a transplantable lymphocytic leukemia. Also shown in Table 3 are results from incubations of normal DBA mouse liver minces with formate-C\(^{14}\) and A-methopterin. One observation of interest is that the L1210-R lymphoma tissue took up less radioactive formate than did the sensitive L1210-S tissue and was also much less sensitive to A-methopterin. It is also of interest that the spleen and liver infiltrated with leukemic cells were as sensitive to A-methopterin as the L1210-S lymphoma. In contrast to these results, A-methopterin had no effect on the incorporation of formate-C\(^{14}\) into the protein fraction of normal DBA mouse liver. The extent of incorporation of formate-C\(^{14}\) by the L1210 tumors cannot be compared with that in the other tissues (Table 3), since different amounts of isotope, different incubation times, and different counting equipment were used.

To determine more precisely the site of action of A-methopterin on the uptake of formate-C\(^{14}\), its effects on the incorporation of formate-C\(^{14}\) into amino acids and ribonucleotides of the L1210-S lymphoma were studied. Preliminary experiments showed that only two protein-bound amino acids, serine and methionine, contained appreciable amounts of radioactive formate. Consequently, only these two amino acids were extensively purified for further study. Ribonucleic acid, adenine, and guanine had constant specific activities through at least two different separation procedures, while the pyrimidine ribonucleotides contained no radioac-
tivity. The results of these studies are summarized in Table 4. It can be seen that the specific activity of serine was about 2.5 times that of the purines, which in turn was about 3.5 times that of methionine. However, formate-C\textsuperscript{14} incorporation into purines was much more sensitive to A-methopterin than incorporation into serine or methionine.

DISCUSSION

It is not surprising that A-methopterin should inhibit the incorporation of formate-C\textsuperscript{14} into purines or into serine and methionine, since similar observations have been made in other systems (26, 27) and since the role of folic acid derivatives in purine and serine biosynthesis is well known (1, 8, 9, 18, 21). It is interesting, however, to note that formate incorporation by liver tissue was insensitive to high A-methopterin concentrations.

No definite conclusions can be drawn as to whether the resistance of the L1210-R lymphoma to therapy by folic acid antagonists in vivo is related to the reduced sensitivity of this tumor to the effect of A-methopterin on formate-C\textsuperscript{14} incorporation in vitro. A more complete dose-response curve of the resistant strain might help to define the relationships of A-methopterin sensitivity in vitro and in vivo.

In previous studies with L1210-S and L1210-R lymphomas, it has been shown that A-methopterin did not inhibit the incorporation of P\textsubscript{32}O\textsubscript{4} into the protein or ribonucleotides (31). Since the present studies show a marked inhibition of formate-C\textsuperscript{14} uptake by the ribonucleotides, it is clear that the purine and phosphate groups of ribonucleic acid turn over independently. A rough comparison between the turnover rates of purine and of phosphate in L1210-S tissue may be made by comparing the specific activities of the purines relative to that of the formate pool and of the ribonucleotide phosphate with the inorganic phosphate pool. In the latter case, the relative specific activity has been determined directly (31).

### TABLE 3

**EFFECT OF A-METHOPTERIN ON FORMATE-C\textsuperscript{14} INCORPORATION INTO THE PROTEIN OF NORMAL MOUSE LIVER AND VARIOUS LEUKEMIC MOUSE TISSUES**

<table>
<thead>
<tr>
<th>Tissue</th>
<th>A-methopterin</th>
<th>No. incubations</th>
<th>Specific activity</th>
<th>Per cent inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal liver from DBA mice†</td>
<td>-</td>
<td>8</td>
<td>870±50</td>
<td>0±7.0</td>
</tr>
<tr>
<td>L1210-S lymphoma in DBA mice§</td>
<td>+</td>
<td>8</td>
<td>990±51</td>
<td>0±7.0</td>
</tr>
<tr>
<td>L1210-R lymphoma in DBA mice§</td>
<td>+</td>
<td>8</td>
<td>9,120±885</td>
<td>0±7.0</td>
</tr>
<tr>
<td>Infiltrated spleen from leu-</td>
<td>+</td>
<td>8</td>
<td>465±55</td>
<td>78.8±3.5</td>
</tr>
<tr>
<td>kemic Db mice§</td>
<td>+</td>
<td>8</td>
<td>530±115</td>
<td>75.8±5.8</td>
</tr>
<tr>
<td>Infiltrated liver from leu-</td>
<td>+</td>
<td>8</td>
<td>955±114</td>
<td>75.8±5.8</td>
</tr>
<tr>
<td>kemic Db-FnA mice§</td>
<td>+</td>
<td>8</td>
<td>455±138</td>
<td>75.8±5.8</td>
</tr>
<tr>
<td>Infiltrated spleen from leu-</td>
<td>+</td>
<td>8</td>
<td>910±150</td>
<td>75.8±5.8</td>
</tr>
<tr>
<td>kemic Db-FnA mice§</td>
<td>+</td>
<td>8</td>
<td>1,990±94</td>
<td>75.8±5.8</td>
</tr>
</tbody>
</table>

† Specific activities of the tumors from DBA mice cannot be compared directly with the other tissues in this table owing to the different experimental conditions described in the text.

### TABLE 4

**EFFECT OF A-METHOPTERIN ON FORMATE-C\textsuperscript{14} INTO PROTEIN-BOUND SERINE AND METHIONINE AND INTO THE ADENINE AND GUANINE OF THE RIBONUCLEIC ACID OF L1210-S LYMPHOMA MINCES**

<table>
<thead>
<tr>
<th></th>
<th>Experiment 1*</th>
<th>Experiment 2†</th>
<th>Experiment 3‡</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serine</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>14,000</td>
<td>29,910±1,140</td>
<td>29,180±2,100</td>
</tr>
<tr>
<td>+A-Opterin</td>
<td>3,600±61</td>
<td>12,070±1,390</td>
<td>7,510±2,080</td>
</tr>
<tr>
<td>Methionine</td>
<td>4,670±66</td>
<td>3,990±400</td>
<td>3,340±244</td>
</tr>
<tr>
<td>Control</td>
<td>900±44</td>
<td>1,310±350</td>
<td>1,040±605</td>
</tr>
<tr>
<td>+A-Opterin</td>
<td>4,840±800</td>
<td>3,810±90</td>
<td>78.9±1.9</td>
</tr>
</tbody>
</table>

* Incubated 4 hours in the presence or absence of 100 μg A-methopterin and 0.8 μg (1 μM) formate-C\textsuperscript{14}/ml.

† Incubated 6 hours in the presence or absence of 200 μg A-methopterin and 2.85 μg (0.97 μM) formate-C\textsuperscript{14}/ml.
It is interesting to consider possible explanations for the observation that very small amounts of A-methopterin have a maximal effect on the incorporation of formate-C\(^{14}\), although much greater amounts do not further inhibit incorporation. Two plausible explanations present themselves. It is possible that formate incorporation occurs by two pathways, one sensitive to and one resistant to the folic acid antagonists. In the L1210 tumors, where the cells are predominantly of one type, this explanation would appear to have some merit. Another possibility, however, is that there are mixed populations of cells, some of which are completely inhibited by the antagonists and some of which are completely resistant. The present data cannot be used to distinguish between these possibilities. It is unlikely that folic acid-like impurities in the A-methopterin are responsible for the formate incorporation in the presence of the antagonist, since, in human leukocytes at least, folic acid does not counteract the inhibitory effects of A-methopterin on in vitro formate-C\(^{14}\) incorporation, and citrovorum factor will do so only at high concentrations (to be published). Nichol (18) has found that the rate of synthesis of citrovorum factor from folic acid is not significantly different in the L1210-sensitive and L1210-dependent lymphomas, or in line I (sensitive) and line I/A (resistant) mouse leukemia. In the latter case it was found that citrovorum factor synthesis in the line I/A strain was somewhat less sensitive to the folic acid antagonists than strain I. Synthesis in both strains could be almost completely inhibited, however.

The data in Table 4 indicate that the incorporation of formate-C\(^{14}\) into serine and methionine is less sensitive to A-methopterin than is incorporation into purines. The significance of this observation is obscure, since folic acid is presumed to be involved in the transfer of the one-carbon unit to both of these amino acids. The strong inhibitory action of A-methopterin observed in this work supplies further evidence for the participation of a folic acid derivative as a co-factor for the metabolism of formate.

**SUMMARY**

The in vitro incorporation of formate-C\(^{14}\) into the protein and lipids of several leukemic mouse tissues and of normal mouse liver has been studied. The folic acid analog, A-methopterin, has been shown to inhibit by 72 per cent the incorporation of formate-C\(^{14}\) into the protein of leukemic cells sensitive in vivo to A-methopterin. A-methopterin inhibited by 40 per cent the incorporation of the isotope into the proteins of a lymphoma resistant in vivo to A-methopterin therapy. There was no significant effect of A-methopterin on the incorporation of formate-C\(^{14}\) into the gross protein fraction of normal mouse liver.

The protein residue from the L1210-S lymphoma incubated in the presence and absence of A-methopterin was further fractionated to isolated pure methionine, serine, adenine, and guanine. Serine had the highest specific activity, with the purines having about 40 per cent as much activity as serine, and methionine about one-third of the activity of the purines. The inhibitory effect of A-methopterin on the incorporation of formate-C\(^{14}\) into ribonucleic acid purines was significantly greater than its effect on incorporation into serine and methionine.

**ACKNOWLEDGMENTS**

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1. This value is calculated from the data in Table 4, Experiment 1, where formate with a specific activity of 4.06 \(\mu\)c/\(\mu\)M was used. With the counting procedures employed, 1 \(\mu\)c = 1 \(\times\) 10\(^4\) c.p.m.
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