Studies on Fluorinated Pyrimidines

V. Effects on Nucleic Acid Metabolism in Vitro*

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To gain further information on the mechanisms by which the fluorinated pyrimidines and their nucleosides inhibit various processes of nucleic acid biosynthesis, studies similar to those reported in the preceding paper (6) were carried out in vitro in glycolyzing suspensions of the Ehrlich ascites cells system used by LePage for studies of purine biosynthesis (20). In this work the following labeled precursors were used: formate-C14, uracil-2-C14, orotic acid-6-C14, thymidine-6-H3, phosphate-P32, 5-fluorouracil-2-C14, and 5-fluoroorotic-2-C14 acid.

MATERIALS AND METHODS

Ehrlich ascites cells were harvested from female Swiss albino mice 6-7 days after transplantation. The ascites fluid was discarded, and the cells were washed twice with isotonic saline. The cells were dispersed with a loose-fitting homogenizer, and the incubations were carried out in 50-ml. Erlenmeyer flasks, with an equivalent of 0.25 ml. packed volume of cells and 2 /jmoles of the labeled precursor in 12 ml. of Robinson’s medium (92) containing bicarbonate and glucose for 1 hour at 37° C., in an atmosphere of 95 per cent nitrogen and 5 per cent carbon dioxide. All groups of controls and cells treated with various concentrations of the inhibitors were run at least in duplicate. The reactions were terminated by the addition of perchloric acid, the nucleic acid bases isolated, and the specific activities determined as described in the previous paper (6). In some experiments the cold NaCl extraction was omitted.

In one of the incubation experiments with 5-fluoroorotic-2-C14, the acid-soluble fraction was chromatographed as described previously (6). The FUMP fraction was purified and separated from UMP by rechromatography on Dowex-1 formate column, with 0.03 M KOH and 0.02 M NaOH, as described by Cohn (5). Under these conditions, 5-fluoro-2′-deoxyuridine is eluted in the borate system, and, following this, 5-fluorouridine is removed with 0.1 N HCl. The total radioactivities in these fractions were determined with a Packard Tri-Carb liquid scintillation counter. The DNA and RNA were separated and the RNA nucleotides chromatographed as previously described (8).

The permeability studies were carried out as described by Leibman and Heidelberger (19). Following the incubation, the ascites cells were separated by centrifugation and washed twice with Robinson’s medium to give the extracellular fraction, and the cells were then precipitated and extracted with perchloric acid to give the intercellular, acid-soluble fraction. The nucleotide content of the fractions obtained from labeled uracil and 5-fluorouracil was determined by the use of small hand columns of Dowex-1 formate as described by Heidelberger et al. (17). Those fractions obtained from orotic and fluoroorotic acid were separated on the fraction collector (17).

The sources of the labeled compounds have been listed previously (6). All fluorinated pyrimidines were obtained from Dr. Robert Duschinsky of Hoffmann-LaRoche, Inc. (7, 8).

RESULTS

The in vitro rates of incorporation of formate-C14 into DNA thymine in suspensions of Ehrlich ascites cells under aerobic and anaerobic conditions are shown in Chart 1 and confirm the superiority of the anaerobic glycolytic conditions for nucleic acid biosynthesis in these cells as reported by LePage (20). This biosynthetic pathway was investigated first because of the demonstration from in vivo studies that the fluorinated pyrimidines block this conversion (6, 14).

The results of several replicate experiments in which the inhibition of the conversion of formate-C14 into DNA thymine was studied at various concentrations of 5-fluoroorotic acid, 5-fluorouracil, 5-fluorouridine, and 5-fluoro-2′-deoxyuridine are given in Chart 2. There was no preincubation with the inhibitors. At the highest concentration

* Abbreviations used in this paper are as follows:

FO = 5-fluoroorotic acid
FUDR = 5-fluoro-2′-deoxyuridine
FU = 5-fluorouracil
FUMP = 5-fluorouridyl acid
FUR = 5-fluorouridine
used, $0.75 \times 10^{-3} \text{M}$, there was a 65 per cent inhibition by 5-fluoroorotic acid of the conversion of $1.67 \times 10^{-4} \text{M}$ formate-$\text{C}^{14}$ into DNA thymine; and inhibition increased in the order of FO, FU, FUR, and FUDR, with the latter compound demonstrating essentially complete inhibition at a concentration of $10^{-8} \text{M}$.

Burk and his colleagues have postulated that a number of tumor-inhibitory compounds, including A-methopterin and 6-mercaptopurine, exert their action as inhibitors of glycolysis (2). That this is not the case with the fluorinated pyrimidines is shown in Chart 3, in which the rate of glycolysis, measured as carbon dioxide evolution, is plotted against time for control cells and those treated with the highest concentrations ($0.75 \times 10^{-1} \text{M}$) of the inhibitors used in the current work. There was no significant deviation from the control values, except for a possible minute diminution in the glycolytic rate with 5-fluorouridine. The $Q_{\text{CO}_2}$ was 85.

Since it has been previously demonstrated that both 5-fluorouracil and 5-fluoroorotic acid are converted into the same acid-soluble nucleotides (3), it appeared that the difference in the inhibitory effect of the two compounds in vitro might reflect differences in the degree of permeability of the ascites cells to them or differences in their rates of conversion into nucleotides. That there was indeed a permeability effect is shown in Table 1. In this experiment, equimolar amounts of labeled uracil, orotic acid, 5-fluorouracil, and 5-fluoroorotic acid were incubated with a constant cell and medium volume, under our usual conditions. The cells were centrifuged, washed twice with cold medium, the proteins were precipitated and extracted with 4 per cent perchloric acid, and the amount of radioactivity in the acid-soluble and nucleic acid fractions was measured. The concentration and relative concentrations in the cells were calculated and compared with an equilibrium concentration representing equivalent concentration in the cells and medium as if there were free diffusion. This type of experiment might be subject to criticism on the grounds that the radioactivity measured in the acid-soluble fraction could represent material adsorbed on the cell walls as well as that which has actually entered the cells. That adsorption does not play an important role in these experiments is shown by the fact that the amounts of radioactivity in the nucleic acids parallel the quantities in the acid-soluble fraction. It will be noted that only in the case of 5-fluorouracil was the concentration in the cells as high as that in the medium; in fact, it was almost 2 times as high. In all cases,
the labeled precursors inside the cell were converted into nucleotides and nucleic acids at similar rates. Therefore, the differences in the in vitro metabolism of these compounds are explained on a permeability basis. The greater than tenfold difference in the permeability of the cells to 5-fluorouracil and 5-fluoroorotic acid explains the differences in their inhibitory activities shown in Chart 2. It is of interest to observe the parallel between the cell permeability to the normal and fluorinated pyrimidines. Although the magnitude of the inhibitory effects of the two ribosides appears to be greater than might be attributed to permeability (Chart 2), similar studies will be carried out when the labeled nucleosides become available.

It is well established from the work of Friedkin and Roberts (11), Blakley (1), and Phear and Greenberg (21) that the process leading to the formation of the methyl group of thymine involves the reaction of a one-carbon tetrahydrofolic acid derivative with deoxyuridine to give thymidine, although the reaction probably occurs with the corresponding nucleotides (10). Further evidence for the site of fluorinated pyrimidine inhibition of this process is obtained from the experiment shown in Table 2. The addition of deoxyuridine increased the conversion of labeled formate into DNA thymine, and the inhibition of this reaction produced by 5-fluorouracil was partially reversed by deoxyuridine. The lesser inhibition caused by 5-fluoroorotic acid was reversed by deoxyuridine to such an extent that the thymine specific activity was almost as high as was found with formate and deoxyuridine. The reversal of the inhibitions with deoxyuridine represents further evidence that the fluorinated pyrimidines block the biological formation of the methyl group of thymine.

Because formate is a precursor of nucleic acid purines as well as of the methyl group of thymine, both pathways being catalyzed by tetrahydrofolic acid coenzymes (10–12), the demonstration (Chart 4) that the fluorinated pyrimidines do not significantly inhibit the conversion of formate into nucleic acid purines rules them out as folic acid antagonists, in agreement with the in vivo experiments (6), and establishes their role as specific inhibitors of the metabolic formation of the methyl group of DNA thymine. In this experiment the specific activities of the DNA thymine and mixed nucleic acid adenine in the control cells were 2140 and 420 counts/min/µmole, respectively, which values are in agreement with the data of Smellie et al. (24), Harrington and Lavik (13), and with our own observations (6) that in vivo experiments formate is more efficiently utilized for thymine than for purine biosynthesis, whereas in vitro the relative utilizations are similar.

In Chart 4 are also shown the results of experiments with tritium-labeled thymidine and phosphate-P32. In neither of these cases was any significant inhibition of incorporation produced by the highest concentration (0.75 × 10^{-3}M) of any of the fluorinated pyrimidines or nucleosides. When an equivalent amount of thymine-2-C14 was used as the precursor, no detectable radioactivity was found in the DNA thymine. In the in vivo ex-
Experiments FU and FO produced an inhibition of $^{32}$P incorporation into DNA and a stimulation of the conversion of thymidine into DNA thymine (6). The differences between these experiments probably reflect the more complex situation in the intact animal, in which drug distribution and metabolism play an important role, and where 12 hours elapsed between the injection of the labeled precursors and the sacrifice of the mice, as compared with the 1-hour in vitro incubation.

The effects of fluorinated pyrimidines on the conversion of labeled uracil and orotic acid into RNA uracil, DNA thymine, and mixed nucleic acid cytosine were also investigated in the in vitro system. At the end of the incubation, the medium was separated from the cells as described previously, and the radioactivity in the acid-soluble fraction was determined. As shown in Table 3, the unexpected finding was made that 5-fluorouridine at the highest concentration used greatly stimulated the entrance of labeled uracil and orotic acid into the cells. 5-Fluorouracil had less effect, and FO and FUDR did not affect this process. Consequently, the specific activities of the nucleic acid pyrimidines obtained from the 5-fluorouridine incubations, shown in Chart 5, were corrected for the increased amount of precursors in the cells.

As shown in Chart 5, the effects of the inhibitory compounds on the incorporation into RNA uracil and mixed nucleic acid cytosine of both labeled uracil and orotic acid were quite similar. Insufficient radioactivity was obtained in DNA thymine from orotic acid incubations for accurate measurement. It is evident from the experiments with both precursors that the incorporation into RNA uracil was not inhibited by 5-fluoro-$2'$-deoxyuridine. The other drugs inhibited in increasing order of activity from FO to FU to FUR. The fact that 5-fluorouracil was a better inhibitor of both precursors than 5-fluoroorotic acid results from its greater entry into the cells (Table 1). Essentially complete inhibition of this metabolic process was obtained with the highest concentrations of FU and FUR.

There was no appreciable effect by any of the fluorinated pyrimidines on the conversion of uracil or orotic acid into mixed nucleic acid cytosine, a process which occurs to only a small extent, as shown by the specific activities of the untreated controls given in parentheses in Chart 5. The cytosine specific activities were also corrected for the increased entry of uracil and orotic acid into the cells produced by 5-fluorouridine. If this correction had not been made, there would have appeared to be a large stimulation by FUR of the incorporation of both precursors into cytosine. This is taken as further evidence for the necessity of correcting for permeability effects in this type of experiment. These specific activities also demonstrate that, in Ehrlich ascites cells in vitro, uracil is a much more efficient precursor of RNA uracil and DNA thymine than orotic acid, a finding in accord with the trends previously observed in vitro with the Flexner-Jobling carcinoma (17).

In contrast to the lack of effect of the 5-fluoro-$2'$-deoxyuridine on the conversion of labeled uracil into RNA uracil, this compound powerfully inhibited the conversion of uracil into DNA thymine. Moreover, FU, FO acid, and FUR all in-

### TABLE 3

<table>
<thead>
<tr>
<th>COMPOUND</th>
<th>MOLARITY</th>
<th>PER CENT OF CONTROLS</th>
<th>URACIL-$6$-C$_{14}$</th>
<th>OROTIC-$6$-C$_{14}$ ACID</th>
</tr>
</thead>
<tbody>
<tr>
<td>Controls</td>
<td>100*</td>
<td>100†</td>
<td></td>
<td></td>
</tr>
<tr>
<td>FU</td>
<td>0.75X10$^{-4}$</td>
<td>118</td>
<td>151</td>
<td></td>
</tr>
<tr>
<td>FO</td>
<td>0.75X10$^{-4}$</td>
<td>98</td>
<td>111</td>
<td></td>
</tr>
<tr>
<td>FUR</td>
<td>0.75X10$^{-4}$</td>
<td>98</td>
<td>111</td>
<td></td>
</tr>
<tr>
<td>FUDR</td>
<td>0.75X10$^{-4}$</td>
<td>98</td>
<td>111</td>
<td></td>
</tr>
<tr>
<td>$\ast$</td>
<td>0.75X10$^{-6}$</td>
<td>90</td>
<td>102</td>
<td></td>
</tr>
<tr>
<td>$\ast$</td>
<td>0.75X10$^{-7}$</td>
<td>89</td>
<td>101</td>
<td></td>
</tr>
<tr>
<td>$\ast$</td>
<td>0.75X10$^{-8}$</td>
<td>105</td>
<td>100</td>
<td></td>
</tr>
</tbody>
</table>
| Molarity of labeled precursors = 1.65 $\times$ 10$^{-4}$.
| Total counts/min in acid-soluble = 49,000.
| Total counts/min in acid-soluble = 7,960. |
hibited this process to a greater extent than they inhibited incorporation of uracil into RNA uracil.

When 5-fluorouracil-2-C\(^14\) was incubated with glycolyzing suspensions of ascites cells, the results obtained, as shown in Table 4, were similar to those found in vivo. The purified FUMP fraction from a chromatogram of the acid-soluble fraction (3) was dephosphorylated by an intestinal phosphatase, and the nucleoside fraction was separated on a Dowex-1 chloride column with borate buffer (3) in the presence of 5-fluoro-2'-deoxyuridine and 5-fluorouridine carriers. Clean peaks were obtained, and it was found that 11 per cent of the radioactivity in the FUMP peak was present as 5-fluoro-2'-deoxyuridine monophosphate. The location of the phosphate group has not been established, but it may be inferred by analogy to other deoxyribonucleotides to be in the S'-position. Thus, the in vitro production from 5-fluorouracil of its deoxyribonucleotide has been demonstrated. The experiment also showed that the 5-fluorouracil of its deoxyribonucleotide has been demonstrated. The experiment also showed that the 5-fluorouracil is incorporated into RNA but not DNA in vitro, as well as in vivo (3), and that all the isotope in the RNA was accounted for as FUMP. Thus, there is no defluorination at the pyrimidine level. The amount incorporated appears to be of the same order of magnitude as one would calculate for in vivo utilization for a comparable period of time.

**DISCUSSION**

In this and in the two preceding papers (3, 6) evidence has been presented to show that the fluorinated pyrimidines exert three biochemical effects: (a) inhibition of the methylation reaction leading to the formation of thymidine or thymidylic acid, (b) inhibition of the conversion of uracil or orotic acid into RNA uracil, and (c) incorporation into RNA to produce an unnatural or “fraudulent” ribonucleic acid.

In order to summarize the metabolic reactions, the scheme shown in Chart 6 has been constructed on the basis of knowledge available in the literature and the biochemistry of the fluorinated pyrimidines studied here. Because these drugs have little or no effect on the formation of nucleic acid cytosine, the amino pyrimidines are excluded from the diagram. The similarities between the normal precursors and the fluorinated analogs are stressed. Uracil is shown as a precursor of uridine-5'-monophosphate (UMP) through uridine (UR) as the intermediate. UMP is also formed from orotic acid, with orotidine monophosphate (OMP) as the probable intermediate. Uridine monophosphate (UMP) can, possibly at the diphosphate level, be incorporated into RNA.

**Table 4**

**In Vitro Metabolism of 5-Fluorouracil-2-C\(^14\)**

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Total counts/min</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acid-soluble FUMP</td>
<td>450,000</td>
</tr>
<tr>
<td>FUR from FUMP</td>
<td>355,000</td>
</tr>
<tr>
<td>FUDR from FUMP</td>
<td>49,800</td>
</tr>
<tr>
<td>DNA</td>
<td>300</td>
</tr>
<tr>
<td>RNA</td>
<td>220,000</td>
</tr>
<tr>
<td>FUMP from RNA</td>
<td>200,000</td>
</tr>
</tbody>
</table>

**Chart 5.** The effects of fluorinated pyrimidines on the conversion of uracil-2-C\(^14\) and orotic-6-C\(^14\) acid into RNA uracil, DNA thymine, and mixed nucleic acid cytosine. Per cent of control specific activities against molarity of inhibitors. The figures in parentheses are the specific activities of the control bases in counts/min/\(\mu\)mole.

**Chart 6.** Scheme representing nucleic acid uracil and thymine biosynthesis, metabolism of fluorinated pyrimidines, and the probable site of their inhibitory action. The explanation of the abbreviations is given in the text.
uracil. The analogous reactions have been shown to take place with 5-fluorouracil (FU) and 5-fluoroorotic acid (FO) to form 5-fluorouridine monophosphate (FUMP), which undoubtedly is also formed from 5-fluorouridine (FUR). Uridylic acid (UMP) is known to be converted into deoxyuridylic acid (DUMP) by a reaction which is probably not reversible. DUMP reacts with a 1-carbon tetrahydrofolic acid derivative (TFA-C1) to form thymidylic acid (TMP), which may then be incorporated into DNA thymine, most probably with thymidine triphosphate as the intermediate (18). In analogy, 5-fluorouracil has been demonstrated to be converted into 5-fluoro-2′-deoxyuridine monophosphate (DFUMP), which is presumably also formed from 5-fluoro-2′-deoxyuridine (FUDR).

Our knowledge of the mechanism of the reaction leading to the formation of the methyl group springs from the work of Friedkin and Roberts (11), who demonstrated the production of DNA thymine from uracil deoxyriboside in minced embryo tissue and bone marrow cells, and the stimulation of the process with citrovorum factor and its inhibition by A-methopterin. Phear and Greenberg (21) studied the methylation of deoxyuridine with formaldehyde in supernatants of Dowex-treated homogenates of thymus gland. They found thymidyl acid (TMP) to be the product and established the cofactor requirements of tetrahydrofolic acid, Mg++, ATP, and DPNH. Friedkin has reported (10) that in fractionated cell-free extracts of E. coli deoxyuridine monophosphate is the substance methylated. On the other hand, Blakley has stated that with high-speed supernatants of thymus glands, deoxyuridine was methylated by serine-3-C14 at higher rates than was DUMP (1). Thus, there appears to be a divergence of opinion as to whether the primary substrate is the nucleoside, deoxyuridine, or the nucleotide, DUMP. We have chosen the latter alternative for the purposes of our diagram (Chart 6).

The evidence for the site of fluorinated pyrimidine inhibition at this methylation reaction stems from the findings, both in vivo and in vitro, that FO, FU, FUR, and FUDR, increasing in activity in this order, inhibit the conversion of labeled formate into DNA thymine, whereas thymidine-14C incorporation into DNA thymine is not inhibited in vitro and is stimulated in vivo. The lack of effect on the incorporation of formate into purines, in agreement with the results obtained in vivo, rules out the possibility that these compounds act as folic acid antagonists. The reversal of the inhibition of the fluorinated pyrimidines by 5-fluorouracil lends strong support to the assignment of the inhibitory block at this locus. It is interesting to note that the inhibitory effect of FU on the methylation of pyrimidines reported here is in agreement with the original observations of Scheiner et al. (23) that FU blocks the methylation of pyrimidines in the bacterium L. leichmannii. It is also reasonable to assign this locus of action on the basis of structural considerations. Because of the location of the fluorine atom on the pyrimidine 5-carbon to which the methyl group becomes attached, it is reasonable to suppose that the fluorinated antimetabolite blocks the attachment of the methyl group to the pyrimidine ring. However, it must be admitted that with the data now at hand one cannot exclude the possibility that the drugs block the conversion of UMP to DUMP. This possibility could be eliminated by the demonstration that FUDR would block the conversion of labeled deoxyuridine to DNA thymine in this system.1

We have found both in vivo and in vitro that 5-fluoro-2′-deoxyuridine is by far the most potent inhibitor of the conversion of formate into DNA thymine of the series investigated. Although differences in permeability of the cell walls to these inhibitors can explain the tenfold difference in effect between 5-fluoroorotic acid and 5-fluorouracil shown in Chart 2, the 104-fold difference between 5-fluorouracil and its deoxyriboside cannot be attributed to permeability. Therefore, it can be concluded that FUDR is the compound most closely related to the true blocking agent, which is probably 5-fluoro-2′-deoxyuridine monophosphate (DFUMP), a compound we have shown to be formed in vitro from 5-fluorouracil and to be present in the acid-soluble fraction of Ehrlich ascites cells during the course of the inhibition. The final proof of the site of the block must await the demonstration of inhibition of the reaction by DFUMP in a highly purified system such as that of Friedkin (10).

It is generally agreed that in mammalian systems there is no turnover of DNA, and hence incorporation of labeled precursors into DNA must reflect a net synthesis. Although there is good incorporation of labeled formate into DNA thymine in these cell suspensions in vitro, during the 1-hour incubation a net increase of DNA cannot be detected; and the possibility exists that the methylation reaction may take place to some extent independently of DNA synthesis. That this possibility does not invalidate the results that we have obtained and the conclusions that we have

1 Note added in proof: This demonstration has now been made (Harbers and Heidelberger, unpublished).
drawn is demonstrated by the complete parallel observed between the in vitro experiments and the in vivo dose-response curves when the quantitative effects of the series of fluorinated pyrimidines were compared. Thus, regardless of whether net DNA synthesis occurs in the in vitro experiments, the results are the same as in the whole animal experiments where the biosynthesis of DNA is known to take place.

The pronounced inhibition produced by these drugs in vivo on the incorporation of labeled orotic acid and uracil into DNA thymine and in vitro on the conversion of uracil into DNA thymine is most probably also explained on the basis of inhibition of the methylation reaction.

After our work, demonstrating that fluorinated pyrimidines inhibit the conversion of labeled uracil and orotic acid into RNA uracil both in vivo and in vitro, had been completed, a "Letter to the Editor" appeared by Eidnoff et al. (9) in which they confirmed our findings on the inhibition by 5-fluorouracil of the incorporation of labeled orotic acid into nucleic acid thymine, uracil, and cytosine, using as their system slices of human tumors, maintained by transplantation in x-irradiated rats.

The question arises whether the inhibition of the incorporation of uracil and orotic acid into RNA uracil results from a general backing up of metabolism, in a sort of mass action effect, produced by the block of the methylation reaction, or whether blocks at additional sites are produced by these drugs. If there are additional blocks, their metabolic sites become of importance. The first alternative is excluded by the fact that 5-fluorouridine, although the most potent inhibitor of the methylation reaction, has no effect whatever on the conversion of both uracil and orotic acid into RNA uracil in vitro. Therefore, there must be additional block(s). The possibilities are: (a) inhibition by the fluoropyrimidine bases of the conversion of uracil and orotic acid into acid-soluble uridine nucleotides, (b) inhibition of the same process by FUMP, and (c) inhibition by FUMP of the incorporation of uridine nucleotides into RNA. The fact that 5-fluorouracil is a more potent inhibitor of the conversion of orotic acid into RNA uracil than is 5-fluoroorotic acid might be cited as evidence that the inhibition is not produced by the pyrimidine bases, since 5-fluoroorotic acid is structurally more closely related to orotic acid than is 5-fluorouracil. However, the difference is more probably explained by the permeability differences of the cells to the two inhibitors. Similarly, the greater inhibitory effectiveness of 5-fluorouridine than of the bases suggests that the inhibition is at the nucleotide level. However, until studies are carried out on labeled 5-fluorouridine the contribution of permeability to these results cannot be assessed.

Some light is shed upon this problem by the studies of Stone and Potter (25). They measured the effects of a number of pyrimidine analogs on the conversion of labeled orotic acid into orotidyl acid and uridine nucleotides in a high-speed supernatant from rat liver homogenate, using phosphoglyceric acid as the source of high-energy phosphate. 5-Fluorouracil exerted no effect on this system. However, 5-fluoroorotic acid produced a profound inhibition of the conversion of orotic acid into uridine nucleotides, and no orotidyl acid was found to be present. The findings show that, in their system, 5-fluoroorotic acid itself is most probably the blocking agent, although some evidence for drug metabolism was inferred. Current experiments on ascites cells and their high-speed supernatant fractions are designed to clarify this matter.

Another biochemical effect described in an accompanying paper (3) is the incorporation of 5-fluorouracil and 5-fluoroorotic acid into RNA in various tissues. The most pressing theoretical question now remaining is to determine the relationship between these various biochemical effects and the inhibition of tumor growth produced by the fluorinated pyrimidines. Let us consider in more detail the case of the Ehrlich ascites tumor, for which most biochemical and biological information is available. The survival time of mice bearing this tumor is prolonged to a greater extent by 5-fluorouridine and 5-fluoro-2'-deoxyuridine than by 5-fluorouracil (15). Since FUDR does not inhibit RNA biosynthesis, that pathway is excluded as the site of tumor-inhibitory mechanism. It appears, on the basis of these experiments, that FUDR is not converted into FUR, and hence cannot be incorporated into RNA, thus excluding "fraudulent" RNA as a mechanism of action of this compound. The incorporation of FUDR into DNA seems to be excluded, since 5-fluorouracil, which has been shown to be converted into DFUMP, is not incorporated into DNA. These conclusions will be investigated further when labeled FUDR becomes available. Inhibition of glycolysis is not caused by these compounds, and hence this is excluded from the tumor-inhibitory mechanism. Therefore, it seems clear that if any of the biochemical studies thus far carried out relate to the tumor-inhibitory properties of 5-fluoro-2'-deoxyuridine, only the inhibition of the methylation reaction can qualify. It is of interest that this is the site of reaction that Cohen and
Barner have considered, on the basis of their experiments on "thymineless deaths" of bacteria, to be desirable in a tumor-inhibitory drug (4). One fact, however, is inconsistent with the hypothesis that tumor growth inhibition in the Ehrlich ascites tumor is produced by the inhibition of the formation of the methyl group of thymine. It has been shown that, on an equivalent dosage level, 5-fluorouridine is more effective than 5-fluoro-2'-deoxyuridine in prolonging the lives of mice afflicted with this tumor (15), whereas the former drug is less effective in blocking the methylation reaction. This finding may result from differences in the metabolic distribution and excretion of these two compounds, and further work will be required before this discrepancy can be shown either to be explained or else to invalidate the conclusion. If inhibition of the methylation reaction explains the carcinostatic effect of FUDR, the possibility exists that the other fluorinated pyrimidines may well act by other mechanisms, even in the Ehrlich tumor, and considerable work must be carried out to elucidate this matter. The situation, already complex enough, is further complicated by the fact (15) that the carcinostatic properties vary among the series of fluorinated pyrimidines from tumor to tumor. Biochemical studies are currently being carried out in other tumors and in resistant tumors in an attempt to unravel this problem.

Although the determination of the exact biochemical mechanism of tumor-inhibitory compounds has not been attained, except possibly in the case of the antifolics and azaserine, it is nevertheless of theoretical as well as practical importance to discover how and why drugs act upon tumors. One of the great unsolved problems in cancer chemotherapy today is why most human tumors do not respond to compounds active against transplanted tumors in animals. The goal of understanding the mechanism of action of tumor-inhibitory compounds is elusive, but with the amount of information now available about these fluorinated pyrimidines further investigation might lead to its attainment with this series.

SUMMARY

1. A study of the effects of fluorinated pyrimidines on nucleic acid biosynthesis has been carried out in suspensions of Ehrlich ascites cells incubated anaerobically with various labeled substrates. The drugs did not inhibit glycolysis.

2. The conversion of formate-C14 into DNA thymine was inhibited in increasing order of potency by 5-fluoroorotic acid, 5-fluorouracil, 5-fluorouridine, and 5-fluoro-2'-deoxyuridine. This inhibition by 5-fluorouracil and 5-fluoroorotic acid was reversed by deoxyuridine. However, formate incorporation into nucleic acid adenine was not inhibited.

3. In studies of permeability it was found that 5-fluorouracil was taken up by the ascites cells to a greater extent than were uracil, orotic acid, and fluoroorotic acid. 5-Fluorouridine caused an increased cellular uptake of uracil and orotic acid.

4. The fluorinated pyrimidines did not affect the conversion of thymidine-6-H3 into DNA thymine, nor the incorporation of phosphate-P32 into DNA or RNA in these cell suspensions.

5. The fluorinated pyrimidines inhibited the metabolic conversion of uracil-2-C14 and orotic-6-C14 into DNA thymine and, with the exception of 5-fluoro-2'-deoxyuridine, inhibited the incorporation of the same precursors into RNA uracil. The metabolic transformation of uracil and orotic acid into nucleic acid cytosine was not greatly affected by the drugs.

6. 5-Fluorouracil-2-C14 was converted into 5-fluoro-2'-deoxyuridine monophosphate and was also incorporated as such into RNA in these cell suspensions.

7. It is concluded that these fluorinated pyrimidines inhibit the metabolic methylation of deoxyuridine monophosphate to thymidine monophosphate. With the exception of 5-fluoro-2'-deoxyuridine, the compounds inhibit the conversion of pyrimidines into RNA uracil. The exact metabolic locus of this block has not yet been determined. These biochemical observations have been discussed in the light of the tumor-inhibitory properties of these drugs.

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