Inhibition of Ribosomal Ribonucleic Acid Maturation in Novikoff Hepatoma Cells by 5-Fluorouracil and 5-Fluorouridine*

DAVID S. WILKINSON† AND HENRY C. PITOT

From the Departments of Oncology and Pathology, McArdle Laboratory for Cancer Research, The University of Wisconsin, Madison, Wisconsin 53706

SUMMARY

5-Fluorouracil, at \(10^{-4}\) m concentrations, inhibited ribosomal RNA maturation only slightly in Novikoff hepatoma cells growing in suspension culture. On a molar basis, 5-fluorouridine was a much more potent inhibitor of the maturation process. The difference between the effectiveness of 5-fluorouracil and 5-fluorouridine is probably a result of the greater transport and incorporation of the latter into RNA. The nucleoside analogue strongly inhibited the production of mature 18 S and 28 S ribosomal RNA, while apparently only slightly inhibiting the formation of the initial 45 S ribosomal precursor RNA or the 38 S ribosomal RNA intermediate. Inhibition of 32 S ribosomal precursor RNA production was greater than that of the larger precursor molecules, but less than that of the mature ribosomal RNA molecules. 5-Fluorouridine did not affect the methylation of 45 S ribosomal precursor RNA, indicating that the analogue exerts its inhibitory effect on some other aspect of the ribosomal RNA maturation process.

5-Fluorouracil inhibits ribosomal RNA synthesis in bacteria (1), yeast (2), and rats (3). Recent papers from this laboratory (4, 5) showed that in rat liver 5-fluoroorotic acid, which is a much more efficient precursor of hepatic RNA than is 5-fluorouracil (6), inhibited the formation of mature ribosomal RNA. Although 5-fluoroorotic acid was incorporated into the initial 45 S ribosomal precursor RNA at relatively normal rates, subsequent processing of this precursor molecule into 18 and 28 S ribosomal RNA was significantly inhibited by the analogue (5). Interestingly, the analogue had little effect on the synthesis of messenger-like RNA.

There are numerous detailed reports in the literature dealing with the mechanism of 5-fluorouracil inhibition of ribosome synthesis in bacterial systems (1, 7-14), but the work in mammalian systems which has been published (3-5) does little more than document the inhibition by 5-fluorouracil and 5-fluoroorotic acid. The biosynthetic pathways leading to the formation of mature ribosomal RNA in bacterial cells are rather different from those in mammalian cells (15, 16), and one cannot a priori draw parallels between these two systems concerning the effects of the fluorinated pyrimidines on this process. Since the fluorinated pyrimidines are widely used clinically in humans (17), more should be known about the molecular mechanism of this phenomenon in mammalian cells.

We felt that the investigation of this problem would be most successful in a tissue culture system, where one could more accurately control experimental conditions and manipulate specific parameters. This paper describes the development of an in vitro model for the study of the inhibition of ribosomal RNA synthesis in mammalian cells by fluorinated pyrimidines, using Novikoff rat hepatoma cells which have been adapted to continuous growth in suspension cultures.

MATERIALS AND METHODS

Cells and Media—All experiments were carried out with Novikoff hepatoma cells derived from a parent strain (N1-S1) that has been carried in the laboratory of Dr. Van R. Potter for about 12 years (18). Cells were grown in flasks at 37° under 5% CO\(_2\) and air in a New Brunswick Gyrotory incubator operating at 180 to 190 strokes per min. Cells were grown to near mid-log phase (3 to 5 \(\times\) 10\(^5\) cells per ml) in Swim’s Medium 77 (Grand Island Biological Co.) supplemented with 10% (v/v) calf serum and 0.1% (w/v) Pluronic F-68 (Wyandotte Chemical Co.) and modified to contain 4 mM L-glutamine. This medium has been designated S89 in the literature. Incubation conditions during the isotopic labeling of RNA were the same, except that one or more of the following were added: [6-\(^3\)H]uridine, [5, 6-\(^3\)H]uracil, [methyl-\(^14\)C]methionine, 5-fluorouracil, 5-fluorouridine, thymidine, and adenosine. All radioactive compounds were purchased from New England Nuclear Corp. 5-Fluorouracil was purchased from Sigma and 5-fluorouridine was obtained from the Cancer Chemotherapy National Service Center and as a gift of Dr. Charles Heidelberger. After incubation, cells were transferred to conical centrifuge tubes, chilled in ice, and...
harvested by centrifugation at 1500 rpm for 5 min. Cells were washed once in unlabeled S69 medium. In the case of experiments with a [methyl-¹⁴C]methionine label, cells were collected by centrifugation and resuspended in S69 minus methionine at a concentration of 2.5 × 10⁶ cells per ml before addition of the labeled compounds.

**RNA Extraction**—Washed cell pellets were suspended by vortexing in buffer containing 10 mM sodium acetate (pH 5.1), 0.14 M NaCl, and 0.01% sodium dextran sulfate (Pharmacia), and sodium dodecyl sulfate was added to 0.3%. An equal volume of phenol phase (phenol-water-m-cresol, 70:20:10 by volume) was added and the mixture was shaken vigorously at 60° for 3 min. After cooling in an ice bath, the phases were separated by centrifugation and the phenol phase removed and discarded. The aqueous phase and interface were extracted again at 60° for 2 min with another equal volume of phenol phase. After cooling and centrifugation, the aqueous phase was transferred to a clean tube and extracted a third time with an equal volume of phenol phase. RNA was precipitated from the final aqueous phase with 2 volumes of 95% ethanol containing 2% potassium acetate at -20°. Precipitated RNA was collected by centrifugation, washed twice in 75% ethanol containing 1% potassium acetate, and dissolved in water at a concentration of 2 to 4 mg per ml.

**Polycrylamide-Agarose Gel Electrophoresis**—Electrophoretic analysis of RNA on composite polyacrylamide-agarose gels was performed as described previously (5). After electrophoresis, gels were scanned at 260 nm in a Gilford model 2400 spectrophotometer equipped with a linear transport module and then cut into 2-mm slices. Gel slices containing both ¹⁴C and ³H label were incubated at 37° overnight. In 0.5 ml of 3 N HCl and assayed for radioactivity in 10 ml of Scintisol.

**Comparison of ³H Uracil and ³H Uridine Incorporation into Total Novikoff RNA**—The inhibition of ribosomal RNA maturation by 5-fluorouracil was much less complete than that observed in rat liver by 5-fluorouracil. This suggests that cell growth in the analogue causes some inhibition of the maturation steps which convert the 45 S precursor to 32 S precursor. The specific radioactivity of the labeled RNA was not affected by 10⁻⁴ M 5-fluorouracil, and the lower doses actually produced a 30 to 40% increase in the specific radioactivity. In these experiments 10⁻⁴ M thymidine had been added to the culture medium to protect the cells from some of the toxic effects associated with 5-fluorouracil’s inhibition of DNA synthesis (18, 19), but elimination of this nucleoside had no effect on the results seen in Fig. 1.
into RNA reflected the cells' differential permeability toward the corresponding natural pyrimidines (20). In those studies, RX-1 after 30 min in the presence of 5-fluorouridine. This uridine incorporation was inhibited by less than 20%. There were definite radioactivity peaks in the 32 S region of the RNA extracted from the analogue-treated culture after 60 min and small amounts of label, representing less than 12% of the radioactivity in the corresponding peaks of the control RNA, were apparent in the 18 and 28 S regions.

When the RNA-labeling interval of the control culture was extended to 90 and 120 min the radioactivity profile gradually shifted to one in which the bulk of the label was found in the mature 18 and 28 S ribosomal RNA peaks. In contrast, the radioactivity profiles of the RNA extracted from the analogue-treated culture after 90 and 120 min changed little from that observed after 60 min, in which the bulk of the label appeared in the 45, 38, and 32 S ribosomal precursor RNA molecules. At these later time points, 5-fluorouridine inhibited [3H]uridine incorporation into mature 18 and 28 S ribosomal RNA by about 90%, whereas it inhibited incorporation into 45 and 38 S ribosomal precursor RNA by only 50%. Incorporation into 32 S RNA was inhibited by about 75%.

Table 1 shows the effect of 5-fluorouridine on the specific radioactivity of the same RNA samples used to obtain the data shown in Fig. 3. One can see that the inhibition of [3H]uridine incorporation into total Novikoff RNA increased with time. The electrophoretograms shown here, and those which allowed the observation of low molecular weight components (like transfer RNA), indicated that this decreased labeling in the presence of 5-fluorouridine was due mostly to decreased incorporation of the labeled precursor into mature 18 and 28 S ribosomal RNA.

Effect of 5-Fluorouridine on Methylation of Ribosomal RNA—Having established that 5-fluorouridine does effectively inhibit ribosomal RNA maturation without initially inhibiting the synthesis of the 45 S ribosomal precursor RNA molecule, we directed our attention to the elucidation of the mechanism of this phenomenon. Since methylation plays such a key role in the maturation of mammalian ribosomal RNA (21-23), and since 5-fluorouracil interferes with transfer RNA methylation in Escherichia coli (24), we examined the effects of 5-fluorouridine on the methylation of ribosomal precursor RNA in Novikoff cells.

Cultures were incubated with 10^-4 M [3H]uridine and 10^-4 M [methyl-14C]methionine in the presence or absence of 10^-4 M 5-fluorouridine. Fig. 4 shows the results of the electrophoretic analysis of total cellular RNA extracted 30 and 60 min after the addition of the labeled precursors. The inhibition of methyl-14C incorporation into ribosomal RNA is not unlike the inhibition of [3H]uridine incorporation. However, 5-fluorouridine treatment decreased the (3H dpm) : (14C dpm) ratio in the 45 S ribosomal precursor molecule from 5.72 to 4.24 after 30 min and from 7.91 to 5.90 after 60 min. This result implies that in the presence of the analogue methyl-14C is incorporated relatively more frequently than is [3H]uridine into the pool of 45 S molecules. The most likely explanation of these data is that 5-fluorouridine has some inhibitory effect on 45 S synthesis during a 30- or 60-min label, thus decreasing [3H]uridine incorporation, but has little or no effect on the methylation of 45 S molecules synthesized either before or after its addition. The data of Table II, which were obtained from the same RNA samples used to obtain the data of Fig. 4, show directly that the incorporation

![Graph](image-url)
FIG. 3. Polyacrylamide-agarose gel electrophoresis of total Novikoff cell RNA labeled by [3H]uridine for various time intervals in the presence and absence of 5-fluorouridine. A culture containing $3.1 \times 10^6$ cells per ml was divided into two 100-ml portions. To the control culture (A through D) were added $10^{-4} \text{M}$ [3H]uridine (0.5 µCi per ml) and $10^{-4} \text{M}$ thymidine. The experimental culture (E through H) contained the same additives plus $10^{-4} \text{M}$ 5-fluorouridine. A 25-ml aliquot was removed from each culture after 30 min (A and E), 60 min (B and F), 90 min (C and G), and 120 min (D and H). RNA extraction and electrophoresis were the same as described in the legend of Fig. 1.

TABLE I

<table>
<thead>
<tr>
<th>Incubation time</th>
<th>Control</th>
<th>$10^{-4} \text{M} \text{FUrd}$</th>
<th>Control dpm/A%O</th>
<th>%</th>
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<tbody>
<tr>
<td>min</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>30</td>
<td>1288</td>
<td>647</td>
<td>50.3</td>
<td></td>
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<tr>
<td>60</td>
<td>2360</td>
<td>1088</td>
<td>46.1</td>
<td></td>
</tr>
<tr>
<td>90</td>
<td>3745</td>
<td>1430</td>
<td>39.2</td>
<td></td>
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<tr>
<td>120</td>
<td>4690</td>
<td>1695</td>
<td>38.1</td>
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</table>
TABLE II

Differential effect of 5-Fluorouridine on labeling of total Novikoff cell RNA by [methyl-14C]methionine and [3H]uridine

<table>
<thead>
<tr>
<th>Incubation time</th>
<th>5-FUrdl</th>
<th>3H</th>
<th>14C</th>
<th>Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>(min)</td>
<td>M</td>
<td>dpm/Acu</td>
<td>%</td>
<td>%</td>
</tr>
<tr>
<td>30</td>
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<td>66</td>
</tr>
<tr>
<td>60</td>
<td>0</td>
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<td>1390</td>
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Discussion

5-Fluorouridine inhibits the maturation of ribosomal RNA in Novikoff hepatoma cells grown in suspension culture in a manner which is similar to the inhibition of this process in rat liver by 5-fluoroorotic acid (5). 5-Fluorouracil is much less effective than 5-fluorouridine, on a molar basis, as an inhibitor. This is probably a consequence of the relatively greater transport and incorporation into RNA of 5-fluorouracil in Novikoff cells, and may explain some of the toxicity differences between 5-fluorouracil and 5-fluorouridine in these cells (19).

As was the case with 5-fluoroorotic acid inhibition of ribosomal RNA maturation in vitro, 5-fluorouridine appeared to have only limited effects in Novikoff cells on either the synthesis of the initial 45 S ribosomal RNA precursor or the formation of the 38 S intermediate molecule, whereas subsequent maturation steps were strongly inhibited by the analogue.

However, the experiments described herein were carried out with a continuous labeling protocol, and at least two different inhibition models are consistent with the data. On the one hand, 5-fluorouridine could inhibit maturation without affecting 45 S precursor synthesis, a high turnover rate of the substituted molecule accounting for the lack of precursor accumulation in the face of analogue inhibition. On the other hand, both synthesis and maturation of the 45 S molecule could be blocked in such a way as to allow "apparently" normal levels of [3H]uridine incorporation during the various labeling periods. The authors favor the former possibility for two reasons. Firstly, it is rather clear from the data presented that 45 S ribosomal precursor RNA synthesis is almost normal during the initial 30 min. Secondly, the incorporation of 5-fluorouridine into the 45 S molecule will change the physicochemical properties of this molecule, quite possibly increasing its lability within the cell and, consequently, increasing its turnover rate. Future experiments, using pulse labels at various times after the addition of the analogue should differentiate between these two possibilities.
8-Azaguanine inhibited [H]cytidine incorporation into cytoplasmic ribosomal RNA by 80% in L cells, which had been chased with excess unlabeled cytidine for 4 hours after a 30-min exposure to the radioactive nucleoside (20). 8-Azaguanine caused some accumulation of radioactivity within the nucleolus, and sedimentation analysis of nucleolar RNA showed that the 45 S component was transformed into the 32 and 18 S components, but that there was little conversion of the 32 S intermediate to mature 28 S ribosomal RNA. Camptothecin, a plant alkaloid, also blocked the conversion of 32 to 28 S ribosomal RNA, while allowing the conversion of 43 to 32 S RNA (26).

However, camptothecin is not a nucleic acid derivative and its mechanism of action may well differ from that of the analogues. 5-Fluorouridine caused no apparent accumulation of radioactivity in the ribosomal precursors of Novikoff cells, and appeared to block the maturation process before the formation of the 32 and 18 S molecules.

Toyocamycin, an adenosine analogue, completely inhibited 28 and 18 S ribosomal RNA synthesis in L cells growing in suspension culture (27). This analogue permitted the synthesis of 45 S ribosomal precursor RNA, which accumulated in the nucleolus. It was postulated that the toyocamycin-substituted 45 S ribosomal RNA precursor had altered physicochemical characteristics which prohibited its maturation. This could be the cause for the maturation inhibition of analogue-substituted 45 S molecules synthesized in the presence of 5-fluorouridine in Novikoff cells. 5-Fluorouridine at 10^{-4} M concentrations did not inhibit 28 and 18 S ribosomal RNA production as completely as toyocamycin.

Cordycepin (3',deoxyadenosine) caused an accumulation in Hepa cells of partially completed 45 S molecules, from which 18 S ribosomal RNA, but not 32 S ribosomal precursor RNA, could be cleaved (28). Cordycepin and another 3',deoxy analogue of adenosine, 3'-amino-3'-deoxyadenosine, also inhibited cytoplasmic ribosomal RNA labeling in Ehrlich ascites cells in vitro (29).

These analogues inhibit RNA synthesis as a result of their incorporation into polyribonucleotides, thereby producing chains which cannot support normal polymerization because they lack free 3'-hydroxyl end groups. The ribosomal RNA molecules, both precursor and mature, produced in the presence of 5-fluorouridine appear (within the limits of the electrophoretic analysis) to have normal molecular weights, suggesting that the mechanism of inhibition of ribosomal RNA maturation by the analogue does not depend on premature chain termination.

5-Fluorouracil treatment of E. coli resulted in the accumulation of abnormal ribosomal particles containing nascent 23 and 16 S ribosomal RNA, with up to 70% replacement of uracil (8-12). 5-Fluorouracil was incorporated into normal ribosomes after removal of the analogue from the medium, but only after degradation of the highly substituted nascent ribosomal RNA and reutilization of the analogue at a lower frequent rate. Although this mechanism was discussed as a possible explanation for the eventual appearance, after extended labeling periods with 5-fluoro2',4'-chloroic acid, of radioactivity in mature rat liver ribosomal RNA, it seems an unlikely explanation of the formation of small amounts of mature ribosomal RNA in Novikoff cells, since the concentration of 5-fluorouridine was held at 10^{-4} M for the duration of the experiment.

Methylation of the 45 S precursor is important in ribosomal RNA maturation, but this process is apparently unaffected by 5-fluorouridine. The maturation of ribosomal RNA also involves the formation of pseudouridylate and ribosylthymidylate residues. The incorporation of 5-fluorouridine into the high molecular weight precursor molecules, by virtue of the fluorine substitution at the critical position 5 in the pyrimidine ring, could inhibit these steps. Investigation of this possibility may lead to a better understanding of the role of minor bases in ribosomal RNA synthesis, structure, and function.

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