Effect of Uridine on de Novo Pyrimidine Biosynthesis in Rat Hepatoma Cells in Culture*

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SUMMARY

Some of the factors responsible for the control of pyrimidine nucleotide biosynthesis in intact cells have been investigated using rat hepatoma cells grown in continuous culture. The addition of uridine (0.5 mM) to culture medium causes a decrease of approximately 70% in the rate of de novo pyrimidine nucleotide biosynthesis, as determined by incorporation of $^{14}$CO$_2$. Maximum inhibition of $^{14}$CO$_2$ incorporation into pyrimidine nucleotides occurs within 60 min of addition of the uridine. After the uridine has been removed, the rate of $^{14}$CO$_2$ incorporation recovers to normal levels with 8 hours. This recovery can be prevented by the addition of actinomycin D or cycloheximide to the medium. Uridine causes a decrease of approximately 70% in the level of activity of orotate phosphoribosyltransferase; it does not significantly affect the activity of carbamyl-P synthetase, aspartate transcarbamylase, dihydroorotase, orotidine decarboxylase, or uridine kinase when these enzyme activities are measured in cell extracts. The decreased level of activity of orotate phosphoribosyltransferase is not due to a direct effect of uridine on the enzyme. The inhibition can be reversed by removal of uridine from the culture medium, but only if transcription and translation are not interfered with by the addition of actinomycin D or cycloheximide. Measurement of enzymes of the de novo pyrimidine biosynthetic pathway (except for dihydroorotase dehydrogenase) under optimal conditions suggests that orotate phosphoribosyltransferase is rate-limiting in the biosynthesis of uridylic acid. The parallel effects of addition and removal of uridine on $^{14}$CO$_2$ incorporation into pyrimidine nucleotides and on orotate phosphoribosyltransferase activity support this suggestion.

In vitro studies concerning the enzymes of the de novo pyrimidine biosynthetic pathway in mammalian cells indicate that pyrimidine nucleotide biosynthesis can be regulated by end-products. For example, the glutamine-dependent carbamyl-P synthetase is inhibited by IMP (1-4) and orotidine decarboxylase (EC 4.1.3.23) is inhibited by UMP and CMP (5-8). In addition, the synthetase is activated by PP-ribose-P (4, 9), an intermediate required for conversion of orotate to orotidylate.

The inhibition of carbamyl-P synthetase appears to be allosteric (3), whereas inhibition of orotidyl decarboxylase is not (8), suggesting that carbamyl-P synthetase is likely to be a critical site of control of de novo pyrimidine biosynthesis in mammalian cells. In addition, Ito and Uchino found that there was an increase in the level of this enzyme after stimulation of human lymphocytes with phytohemagglutinin (10) and that the activities of carbamyl-P synthetase and aspartate transcarbamylase (EC 2.1.3.2) were also rapidly elevated after stimulation of cells from mouse salivary glands with isoproterenol (11). Orotate phosphoribosyltransferase (EC 2.4.2.10), however, also may be a potential site of control of pyrimidine nucleotide biosynthesis since this enzyme appears to be rate-limiting in Ehrlich's ascites cells (12) and since this enzyme utilizes PP-ribose-P which is a common intermediate required for purine and pyrimidine nucleotide biosynthesis.

Little is known about the factors responsible for control of synthesis of enzymes required for pyrimidine biosynthesis in mammalian cells. In more primitive eukaryotes, such as yeast and Neurospora, the synthesis of the glutamine-requiring carbamyl-P synthetase and of aspartate transcarbamylase is repressed by the addition of uracil to the growth medium (13, 14).

EXPERIMENTAL PROCEDURE

Materials—Ba$^{14}$CO$_3$ (60 mCi per mmole), [$^{14}$C]carbamyl-P (16.9 mCi per mmole), [carbozy-14C]orotic acid (10.2 mCi per mmole), [carbozy-14C]orotidyl acid (21 mCi per mmole), [2-14C]uridine (56.7 mCi per mmole), and [6-1H]uridine (2.5 Ci per mmole) were purchased from New England Nuclear Corporation. Na$_4$CO$_3$ was prepared as previously described (16). [14C]carbamyl-P was purified as described by Adair and Jones (17) to remove an unacceptable quantity of radioactive impurity. The purified [14C]carbamyl-P was stored in small aliquots in a liquid N$_2$ freezer. Hepes, 2-(N-morpholino)ethanesulfonic acid, Tris, N-tris(hydroxymethyl)methylglycine, N-ureidoisocitric acid, uridine, adenosine, and pyrimidine nucleotides used as standards in chromatography were obtained from Calbiochem. PP-ribose-P, ATP, GTP, glutamine, l-aspartic acid, and cycloheximide were purchased from Sigma Chemical Company. Actinomycin D was obtained from the Department of Chemistry, Stanford University.

1 The abbreviations used are: Hepes, N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid; HTU, hepatoma tissue culture.
from Merck and Company, Inc. All other chemicals were reagent grade.

**Tissue Culture**—Cloned HTC cells were kindly provided by D. W. Martin (Department of Medicine and Biochemistry, University of California, San Francisco), and were grown in suspension culture in Swinn 77 medium (Grand Island Biological Company, Grand Island, N.Y.) containing 10% fetal calf serum and 10% to 5 ml of 35 inactivated fetal calf serum. Tetracycline (100 μg per liter) was added to the culture medium. Cell suspensions maintained at densities between 3 and 7 × 10⁴ cells per ml were used for all experiments.

**Enzyme Assays**—At appropriate times 50 ml of cells were removed from the suspension cultures and rapidly cooled in ice baths. Cells were sedimented at 300 × g for 10 min at 4º and washed once with a phosphate-buffered saline solution (8.2 g per liter of NaCl, 0.2 g per liter of KCl, 2.16 g per liter of Na₂HPO₄, 0.2 g per liter of KH₂PO₄, pH 7.4). The washed cells were finally suspended in 1 ml of a sonicating solution devised to stabilize enzymes of the de novo pyrimidine pathway (5 mM ATP, 5.5 mM MgSO₄, 30 mM phosphate buffer (pH 7.4), 1 mM dithiothreitol, and 0.25 M sucrose). Cell suspensions were disrupted by sonicating for 10 s (two times, 5 s each at 4º, using a sonicator (Branson Ultrasonic Co.) set at 30% output. Disrupted cell suspensions were centrifuged at 10,000 × g for 10 min, at 4º, and the supernatant solution was used for enzyme assays and protein determinations. Each of the enzyme assays described below were carried out under conditions such that the supernatant enzyme concentration was 100 μg per ml of the dihydroorotase assay, for which 0.3 ml of supernatant was used.

The protein concentration of the enzyme fraction was in excess of 5 mg per ml. In all enzyme assays less than 5% of the limiting substrate was utilized.

**Carbamyl-P Synthetase**—The activity of this enzyme was determined using the radioactive assay of Levine and Krebsheimer (18). The incubation solution contained 50 mM sodium-Hepes buffer (pH 7.1), 100 mM KCl, 10.5 mM MgSO₄, 7.5 mM ATP, 7.5% (v/v) dimethylsulfoxide, 2.5% (v/v) glycerol, 1 mM glutamine, and 4.0 mM [¹⁴C]bicarbonate in a total volume of 0.8 ml. The incubation was for 15 min at 37º, the total sample was counted in 10 ml of Aquasol (New England Nuclear Co.).

**Aspartate Transcarbamylase**—The activity of this enzyme was determined by the method of Bethell et al. (19) using [¹⁴C]carbamyl-P. The incubation was carried out in 50 mM sodium-Hepes (pH 7.4), 5 mM potassium aspartate (pH 7.4), and 0.4 mM [¹⁴C]carbamyl-P in a total volume of 0.5 ml. The incubation was for 5 min at 37º, and the reaction was stopped by the addition of 0.5 ml of 0.5 M HClO₄. The samples were heated in a boiling water bath for 5 min, at 95º, and after which from carbamyl-P was removed with crushed Dry Ice. The total sample was counted in 10 ml of Aquasol (New England Nuclear Co.).

**Dihydroorotase**—The activity of this enzyme was measured by converting dihydroorotate to carbamyl-aspartate, which was determined colorimetrically by the method of Prescott and Jones (20). The incubation was carried out in 100 mM Tris-HCl (pH 9.0) and 0.5 mM dihydroorotate in a total volume of 1.0 ml. The incubation was for 30 min at 37º, and the reaction was stopped by the addition of 0.2 ml of 4 N HClO₄.

**Orotidinyl Decarboxylase**—The activity of this enzyme was determined by measuring the release of [¹⁴C]CO₂ from [carboxy-¹⁴C]-orotate, as described by Appel (8). In preliminary experiments it was determined that purification of yeast orotidinyl decarboxylase (Sigma Chemical Co.) was not required to convert [¹⁴C]orotic acid derived from [¹⁴C]orotate to uridylc acid plus [¹⁴C]CO₂ since the activity of endogenous orotidinyl decarboxylase was present in excess of that of orotidase isorotatytransferase under the conditions used to assay the transferase. Incubations were performed in 25 mM Tris-HCl buffer (pH 7.4), 6 mM MgCl₂, 0.125 mM PP-ribose-P, and 0.06 mM [¹⁴C]orotic acid in a total volume of 2.0 ml. The assays were carried out for 15 min at 37º. The reaction was stopped by injection of 0.2 ml of 4 N HClO₄ into the sealed vessels, which were left to shake for 1 hour at 37º. ³⁵Cl₂ released in the reaction was trapped in plastic center wells containing 0.2 ml of hyamine hydroxide (1 N in methanol), and was weighed or transferred to a toluene-based scintillation fluid (4.8 g of 2,5-diphenyloxazole (PPO) and 0.5 g of 1,4-bis[2-(4-methyl-5-phenyloxazol)benzene (POPOP) per liter).
incorporation of \[^{14}\text{C}\] uridine into pyrimidine nucleotides, RNA, and DNA

Cells were subcultured into fresh 100-ml batches of medium in suspension culture flasks at a density of \(3 \times 10^6\) cells per ml and grown for 16 hours at 37°C. To each of three cultures was added \[^{14}\text{C}\] uridine (10 \(\mu\) Ci; 50 amoles); cultures were harvested 1, 8, and 24 hours after the addition of uridine. Incorporation of \[^{14}\text{C}\] uridine into the acid soluble pyrimidine nucleotide pool, RNA, and DNA was measured as described under "Experimental Procedure." The addition of uridine beyond a concentration of 0.5 mM had within approximately 30 to 60 min of addition of the uridine. Since the maximum inhibition of \(^{14}\text{CO}_2\) incorporation occurred effect of uridine on de novo pyrimidine biosynthesis was rapid, pyrimidine nucleotide to 30% of its original value (Fig. I). The concentration of 0.5 mM, reduced the incorporation of \(^{14}\text{CO}_2\) into addition of uridine to suspension cultures of HTC cells, to a final return in the rate of de novo pyrimidine biosynthesis following uridine incorporation into pyrimidine nucleotides, RNA, and DNA. Even after 24 hours, only 4.4% of the uridine had been transported into the cells. Chromatographic analysis of the free nucleotide fraction showed that between 60 and 80% of the radioactive label was in UTP; CTP and TTP were the next most heavily labeled nucleotides.

Effect of Uridine on de Novo Pyrimidine Biosynthesis—The addition of uridine to suspension cultures of HTC cells, to a final concentration of 0.5 mM, reduced the incorporation of \(^{14}\text{CO}_2\) into pyrimidine nucleotide to 30% of its original value (Fig. 1). The effect of uridine on de novo pyrimidine biosynthesis was rapid, since the maximum inhibition of \(^{14}\text{CO}_2\) incorporation occurred within approximately 30 to 60 min of addition of the uridine. The addition of uridine beyond a concentration of 0.5 mM had no additional effect on \(^{14}\text{CO}_2\) incorporation.

Effect of Removal of Uridine on de Novo Pyrimidine Biosynthesis—Removal of uridine from the culture medium resulted in a return in the rate of de novo pyrimidine biosynthesis to normal values within 8 hours (Fig. 2). In these reversal experiments, cells were resuspended in preconditioned medium obtained by culturing cells at identical cell densities in uridine-free medium for 16 hours before use. Addition of actinomycin D (0.1 \(\mu\) g per ml), or cycloheximide (2 \(\times\) 10\(^{-4}\) M), to recovery cultures prevented the return of the rate of \(^{14}\text{CO}_2\) incorporation into pyrimidine nucleotides to control levels. These results suggest that RNA synthesis and protein synthesis are required for the resumption of de novo pyrimidine biosynthesis following uridine inhibition.

Effect of Uridine on Activity of Enzymes Required for Pyrimidine Nucleotide Biosynthesis—Carbamyl-P synthetase, aspartate transcarbamylase, dihydroorotase, orotate phosphoribosyltransferase, orotidine decarboxylase, and uridine kinase activities were measured at varying protein concentrations. The carbamyl-P synthetase, orotate phosphoribosyltransferase, and orotidin de-
Fig. 3 (left). Effect of protein concentration on carbamyl-P synthetase activity. Enzyme was assayed under the conditions described under "Experimental Procedure," using varying amounts of HTC cell extract.

Fig. 4 (center and right). Effect of protein concentration on orotate phosphoribosyltransferase and orotidine decarboxylase activity. Enzymes were assayed under the conditions described under "Experimental Procedure," using varying amounts of HTC cell extract. Curve on the left gives the activity of orotate phosphoribosyltransferase against protein concentration; curve on the right gives the activity of orotidine decarboxylase against protein concentration.

**TABLE II**

**Effect of uridine on activity of pyrimidine biosynthetic enzymes**

A suspension culture of HTC cells was grown in fresh medium for 24 hours until the cell density was $3.4 \times 10^6$ cells per ml. The culture was then split into six 50-ml subcultures. Uridine was added to three cultures to a final concentration of 0.5 mM; the other three cultures were controls. All six cultures were grown in suspension for an additional 16 hours at 37°C. One hour before harvesting, fresh uridine (0.5 mM) was added to the uridine cultures. Cells were harvested and prepared for enzyme assays as described under "Experimental Procedure." The same cell extract was used to determine all six enzyme activities. The results represent the mean ± S.E.

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Enzyme activity</th>
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<tbody>
<tr>
<td></td>
<td>Control</td>
</tr>
<tr>
<td></td>
<td>nmoles/min/mg protein</td>
</tr>
<tr>
<td>Carbamyl-P synthetase</td>
<td>0.373 ± 0.007</td>
</tr>
<tr>
<td>Aspartate transcarbamylase</td>
<td>9.520 ± 0.330</td>
</tr>
<tr>
<td>Dihydroorotase</td>
<td>0.400 ± 0.081</td>
</tr>
<tr>
<td>Orotate phosphoribosyltransferase</td>
<td>0.090 ± 0.013</td>
</tr>
<tr>
<td>Orotidine decarboxylase</td>
<td>1.240 ± 0.130</td>
</tr>
<tr>
<td>Uridine kinase</td>
<td>0.967 ± 0.024</td>
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HTC cells (Fig. 5). As was the case with $^{14}$CO$_2$ incorporation into pyrimidine nucleosides, the maximum inhibition was obtained within 30 min of addition of uridine, and the degree of inhibition was approximately the same. Mixing of enzyme extract from uridine cultures with extract from control cultures showed that there were no inhibitory substances present in the extract from uridine cultures; between 95 and 106% of expected activity was recovered in mixing experiments.

**Effect of Removal of Uridine on Activity of Orotate Phosphoribosyltransferase**—Removal of uridine from culture medium resulted in a return of orotate phosphoribosyltransferase activity to that obtained in control cultures (Fig. 6).

This increased activity does not appear to be due to the removal of a pyrimidine compound which inhibits the activity of orotate phosphoribosyltransferase since uridine, UMP, and UTP, when added to the assay mixture (to a final concentration of 1 mM), did not inhibit the activity of orotate phosphoribosyltransferase. This is in agreement with the findings of Hatfield and Wynaarden (30) who find that, although UMP will inhibit the phosphorylation of xanthine or uracil, it produces less than 10% inhibition of orotate phosphoribosyltransferase at 2 mM concentration.

The recovery of orotate phosphoribosyltransferase activity following the removal of uridine from cultures indicates the involvement of transcription and translation since the addition of actinomycin D (0.1 µg per ml) and cycloheximide ($2 \times 10^{-4}$ M) completely prevented the recovery of enzyme activity (Fig. 6).
Experiments carried out with HTC cells grown in continuous culture have shown that the de novo pyrimidine biosynthetic pathway is subject to regulation by pyrimidine metabolites. Previous reports have also indicated that the biosynthesis of pyrimidine nucleotides can be inhibited by addition of uridine to mammalian cells grown in tissue culture (29, 31), although in these studies the site at which the uridine metabolites had their effect was not elucidated. In HTC cells uridine affects the incorporation of labeled bicarbonate into pyrimidine nucleotides and the orotate phosphoribosyltransferase in an identical fashion. Even the recovery from uridine inhibition, and the effects of cycloheximide and actinomycin D on this recovery, showed the same patterns for pyrimidine biosynthesis and activity of orotate phosphoribosyltransferase. In addition to this parallelism, the site at which the uridine metabolites had their effect is subject to regulation by pyrimidine metabolites.

The effect of uridine on orotate phosphoribosyltransferase does not exclude the possibility that pyrimidine metabolites may be important in the regulation of pyrimidine nucleotides at other points in the pathway. For example, the increased level of UTP may cause an inhibition of the glutamine-dependent carbamyl-P synthetase (1–4). Experiments with Novikoff rat hepatoma cells (29) show that uridine is rapidly phosphorylated to UTP and that uptake of uridine results in a maximum uridine nucleotide pool of 15 pmol/10^6 cells, representing an intracellular concentration of about 6 mM. Such a concentration of UTP would result in very marked inhibition of carbamyl-P synthetase in HTC cells, but this inhibition would not be detected in vitro since dilution of cell extract in the assay would result in a UTP concentration of no greater than 0.05 mM.

It is equally conceivable that uridine may inhibit pyrimidine nucleotide biosynthesis by affecting the intracellular concentration of PP-ribose-P. Addition of adenine to cultures of E. coli resulted in a marked decrease in the de novo synthesis of pyrimidine nucleotides, due to the effect of adenine on lowering PP-ribose-P levels (33). Addition of adenine to HTC cells also rapidly inhibited cell growth (15). Although adenine inhibited de novo purine nucleotide biosynthesis, Martin and Owen (15) could not find the site of action of adenine. More recently, Ishii and Green (32) found that adenine is toxic to mammalian cells by inhibiting de novo pyrimidine biosynthesis. The adenine apparently results in a block of pyrimidine biosynthesis at the level of orotate phosphoribosyltransferase, since orotate accumulated in cultures containing adenine. It is quite conceivable that orotate accumulates due to a reduced availability of PP-ribose-P, although levels of this metabolite were not measured. If, in the present work, the decreased pyrimidine biosynthesis was due to a decrease in the level of PP-ribose-P, the synthesis of carbamyl-P may also have been affected since the glutamine-dependent carbamyl-P synthetase is activated by PP-ribose-P (4, 9, 12). Obviously, the actual site at which uridine affects the biosynthesis of pyrimidine nucleotides will depend on the reaction which is rate-limiting. Thus, although a number of alternative mechanisms of action can be postulated, it is most likely that uridine inhibits pyrimidine biosynthesis by affecting the level of activity of orotate phosphoribosyltransferase.

The effect of uridine on orotate phosphoribosyltransferase was extremely rapid and the reversal of this inhibition could be blocked by actinomycin D and cycloheximide, suggesting that transcription and translation are involved in the control mechanism. Although one interpretation may be that a metabolite of uridine represses the synthesis of orotate phosphoribosyltransferase activity, the rapidity of the effect of uridine suggests that some other mechanism is involved. For example, addition of uridine may result in a covalent modification of orotate phosphoribosyltransferase analogous to the modification of glycogen phosphorylase or glutamine synthetase (34).

Addition of uridine only affected the level of activity of orotate phosphoribosyltransferase, not orotidine decarboxylase. This may be surprising, since many experiments suggest that the two enzyme activities exist as a complex in mammalian (8, 12, 30, 35). However, the two enzyme activities may be separated by electrophoresis (35), indicating that the two enzyme activities do not exist as a single protein.

The existence of multifunctional complexes of carbamyl-P synthetase, aspartate carbamylase (16, 36) and dihydroorotase (37), as well as the complex mentioned above, makes it extremely difficult to predict what the relative enzyme activities are in intact mammalian cells. However, the data presented here suggest that orotate phosphoribosyltransferase is a principal site of control in the biosynthesis of pyrimidine nucleotides.
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