Uracil Metabolism in Tadpoles during Thyroxine-induced Metamorphosis*

Nobu Akamatsu,† Raymond H. Lindsay,‡ and Philip P. Cohen

From the Department of Physiological Chemistry, University of Wisconsin, Madison 6, Wisconsin

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Previous work in this laboratory (1) has shown that an increase in carbamyl phosphate synthetase activity in the livers of thyroxine-treated tadpoles is the result of protein synthesis de novo. This induction of the synthesis of a specific enzyme protein by thyroxine was found to be inhibited by chloramphenicol and thiouracil (2). A subsequent survey (3) of several purine and pyrimidine derivatives showed that, of the compounds tested, 2-thiouracil, 2,6-dithiouracil, 2,4-dithiouracil, and 2,4-dihydroxyuridine were potent inhibitors of carbamyl phosphate synthetase activity. While thiourea was also inhibitory, its effect was different from that of the pyrimidine analogues in that it was not inhibitory during the early phases of the induction.

It was postulated that the inhibition by thiouracil and certain related compounds was due to formation of a "faulty ribonucleic acid" which could no longer participate in the synthesis of carbamyl phosphate synthetase. Evidence for the incorporation of thiouracil into ribonucleic acid was presented in support of this hypothesis (3).

Uracil at about the same molarity as thiouracil was able to reverse partially the thiouracil inhibition of synthesis of carbamyl phosphate synthetase. Because uracil seemed to affect the latent period before thyroxine stimulation of carbamyl phosphate synthetase formation was observed, and no direct combination or reaction of thiouracil and thyroxine could be demonstrated, it was suggested that thyroxine might affect uracil metabolism. In this paper additional studies of the influence of pyrimidines on carbamyl phosphate synthetase synthesis in tadpoles have been carried out. The results indicate that uracil, uridine, and orotic acid enhance the stimulating effect of thyroxine. The relationship between uracil metabolism and thyroxine treatment was also examined.

Experimental Procedure

Materials—Tadpoles, Rana catesbeiana, were obtained from the Carolina Biological Supply Company, Elon College, North Carolina, and from Lemberger Company, Oshkosh, Wisconsin. Tadpoles were kept in plastic containers at 22-23° and fed canned spinach. Reagents were added to the water to give final concentrations as indicated in the figures and tables. The solutions were changed every 3 days. Uracil-2-14C and uridine-2-14C were obtained from New England Nuclear Corporation.

Preparation of Extracts—Extracts for assay of uridine phosphorylase and carbamyl phosphate synthetase were obtained by homogenization of three to four tadpole livers in 9 volumes of 0.1% cetyltrimethylammonium bromide solution in a glass homogenizer. The homogenate was centrifuged in the cold (5°) for 10 minutes at 2000 × g. Fat particles were removed from the supernatant solution by decantation through a thin layer of glass wool.

Extracts for assay of uridine kinase were obtained by homogenization of two to three tadpole livers with 9 volumes of 0.05 M Tris buffer, pH 7.4. The homogenate was centrifuged in the cold (5°) for 30 minutes at 9000 × g. The supernatant solution was used without further dilution.

Determinations of Enzyme Activities—Carbamyl phosphate synthetase was assayed by the procedure previously described (4). The reaction mixture had the following composition: 50 μmoles of ammonium bicarbonate; 5 μmoles of ATP; 5 Kmoles of MgSO4; approximately 150 units of partially purified ornithine transcarbamylase (5); and an aliquot of the 0.1% cetyltrimethylammonium bromide extract (6). An aliquot of the 0.1% cetyltrimethylammonium bromide liver extract was incubated with 50 μmoles of uridine and 200 μmoles of phosphate buffer, pH 7.0, in a final volume of 3.0 ml at 37° for 1 hour. The reaction was terminated by the addition of 5.0 ml of 0.5 M HClO4. The amount of citrulline formed was determined spectrophotometrically with diacetylmonoxime. The specific activity of carbamyl phosphate synthetase is defined as the micromoles of citrulline formed per hour per mg of protein under assay conditions.

Uridine phosphorylase was assayed spectrophotometrically (6). An aliquot of the 0.1% cetyltrimethylammonium bromide liver extract was incubated with 50 μmoles of uridine and 200 μmoles of phosphate buffer, pH 7.0, in a final volume of 3.0 ml at 37° for 1 hour. The reaction was terminated by the addition of 5.0 ml of 0.5 M HClO4. After cooling in an ice bath for 1 hour, the solution was centrifuged and 1.0 ml of 4 N KOH was added to a 4.0-ml aliquot of the supernatant solution. Absorbance at 300 μm was measured against a blank uridine solution to which HClO4 was added before incubation. It was not necessary to correct for an enzyme blank since the absorbance at 300 μm did not change during incubation. Under these conditions uracil had a molar extinction coefficient of 1.52 × 104. The specific activity of uridine phosphorylase is defined as the micromoles of uracil formed per hour per mg of protein under assay conditions.

Uridine kinase was assayed according to a modification of the
method of Canellakis (7). The reaction was carried out in conical test tubes in a following composition: 20 μmoles of uridine-2-14C (18,000 to 20,000 c.p.m. per μmole); 40 μmoles of ATP, pH 7.4; 100 μmoles of Tris buffer, pH 7.4; 50 μmoles of MgCl2; and an aliquot of the 0.05 M Tris liver extract, 0.2 ml, containing from 0.6 to 0.9 mg of protein in a final volume of 1.0 ml. A control which contained no enzyme and a control to which enzyme was added after addition of HClO4 were run simultaneously. Incubation was carried out at 37° for a period of 2 hours. The reaction was stopped by the addition of 0.11 ml of 4.0 N HClO4. After centrifugation the precipitate was washed twice with 0.5 N HClO4. The combined supernatant solutions were neutralized with KOH and centrifuged to remove the precipitate. The precipitate was washed twice with cold water, and the combined supernatant solutions used to charge a Dowex 1-formate column 2.0 to 2.5 cm in length and 1 cm in diameter. Two 5-ml portions of 0.2 N formic acid, which eluted both uracil and uridine, were added to the column. The column was further treated with two 5-ml portions of a mixture of 4.0 N formic acid and 1.0 N ammonium formate, which eluted the uridine nucleotides (7). The 10 ml of eluate were collected in a volumetric flask and 0.5-ml aliquots were plated on stainless steel planchets. The samples were dried in an oven at 150-170° overnight, and the radioactivity was measured in a low background gas flow detector with a Micromil end window. The specific activity of uridine kinase is defined as the micromoles of uridine nucleotides formed in 2 hours per mg of protein under assay conditions.

Protein was determined by the method of Lowry et al. (8).

Tadpole Liver Slices—Slices of tadpole liver were prepared according to the method of Deutsch as outlined in Umbreit, Burris, and Stauffer (9) and incubated in Warburg flasks with 3.0 ml of Krebs-Ringer-phosphate medium containing 0.3 μmole of uracil-2-14C with a specific activity of 1.3 × 107 c.p.m. per μmole. The center well contained 0.15 ml of 20% KOH. Incubation was carried out at 37° for 90 minutes in an atmosphere of air with constant shaking.

Preparation and Determination of Specific Activities of RNA Mononucleotides—At the end of the incubation period, 0.33 ml of 5.0 N HClO4 was tipped from the side arm of the Warburg flasks into the main compartment. The flasks were shaken for an additional 15 minutes to permit collection of 14CO2. The liver slices were removed, homogenized in a glass homogenizer, and centrifuged for 10 minutes. The precipitate was washed twice with 0.5 N HClO4. The rest of the procedure for the preparation of RNA was essentially that of Davidson and Smellie (10). The RNA was hydrolyzed in 1 to 2 ml of 1 N KOH for 18 hours at 37°. The alkaline hydrolysate was acidified to pH 1 to 2 with HClO4 and the precipitate, containing DNA, protein, and potassium perchlorate, was removed by centrifugation. The supernatant solution and two cold water washes of the precipitate were neutralized with KOH and centrifuged to remove the remaining potassium perchlorate. The supernatant solution was applied to a column, 0.5 × 20 cm, of Dowex 1-2X (formate form, 200 to 400 mesh), and the individual nucleotides were eluted with a gradient of formic acid. The mixing vessel contained 25 ml of water, and the reservoir, 45 ml of 0.5 N formic acid, with subsequent addition of 105 ml of 4.0 N formic acid.

Aliquots of eluates of RNA nucleotides, 3.0 ml each, were collected by an automatic fraction collector, and the optical densities were measured at 260 nm. After measurement of absorbance, 0.5-ml aliquots of each fraction were pipetted onto planchets and air-dried, and counts of radioactivity were determined with a low background gas flow counter with a Micromil end window.

14CO2 Determinations—The KOH from the center well of the Warburg flasks in the incubation experiments with tissue slices was quantitatively transferred to a 10-ml volumetric flask. The barium carbonate method outlined by Hedrick and Sallach (11) was used for the determination of 14CO2.

RESULTS

Effects of Pyrimidines on Thyroxine-Stimulation of Carbamyl Phosphate Synthetase Formation—In order to clarify further the uracil-thiouracil competition on carbamyl phosphate synthetase synthesis in thyroxine-treated tadpoles (3), experiments were carried out to determine the effects of uracil and uridine in the absence of thiouracil (Fig. 1). Neither uracil nor uridine has any effect on carbamyl phosphate synthetase in the absence of thyroxine during 13 days of treatment. However, in the presence of thyroxine both compounds enhanced the thyroxine stimulation of carbamyl phosphate synthetase synthesis, uridine was the more effective, but a pronounced synergistic action of both compounds appears on the 7th day of thyroxine treatment. It should also be noted that neither compound affected the latent period preceding the initiation of enzyme synthesis. This is in contrast to the results seen when thyroxine, thiouracil, and uracil are all present (3). Orotic acid is as effective as uracil and uridine in enhancing the thyroxine stimulation of carbamyl phosphate synthetase synthesis, while cytosine and adenosine appear to have no influence on the formation of the enzyme (Fig. 2). These findings indicate that only those compounds directly involved in uridine nucleotide biosynthesis effectively influence the thyroxine stimulation.

![Graphs](http://www.jbc.org/)
Uridine Phosphorylase Activity

In order to investigate the influence of thyroxine on uracil metabolism, it was necessary to determine the effects of the hormone on enzymes involved in uracil anabolism and catabolism. The conversion of uracil to uridine is catalyzed by the enzyme uridine phosphorylase. Optimal conditions of pH and concentrations of substrate, phosphate, and enzyme for the assay of the tadpole enzyme were determined. It was found that the requirements were similar to those for the enzyme from rat liver (7) and purified Ehrlich ascites tumor (12). However, the pH optimum was 7.0 compared to 8.0 and 8.2, respectively, for the enzyme from rat liver and ascites tumor. The activity of the tadpole liver uridine phosphorylase, like that from the other sources, was phosphate-dependent.

Thyroxine treatment of tadpoles resulted in a marked increase in uridine phosphorylase activity (Fig. 3). The thyroxine stimulation of enzyme activity, after a lag period of 2 days, was greatest after 3 to 4 days of treatment and then decreased until the 6th day, after which it remained relatively constant. Thus the latent period for uridine phosphorylase response to thyroxine is somewhat shorter than that of carbamyl phosphate synthetase (9).

From Fig. 4 it is evident that uridine phosphorylase activity increases during normal metamorphosis in the tadpole (13) and reaches a peak between Stages XVIII (onset of metamorphosis) and XX (appearance of front legs). It was previously observed that carbamyl phosphate synthetase, ornithine trans-carbamylase, and arginase activities increased at logarithmic rates during these stages (14). The time course for uridine phosphorylase differs, however, since after reaching a peak in Stages XVIII to XX the activity decreased in the later stages of metamorphosis and in the adult frog to levels below those seen in the premetamorphic stages XIV to XVI.

Uridine Kinase Activity—The enzyme uridine kinase, which converts uridine to UMP, was assayed in tadpole liver during normal metamorphosis. Thyroxine concentration was $2.6 \times 10^{-8}$ m. Each determination represents a pooled liver sample from three to four tadpoles. Thyroxine-treated, $\circ \cdots \circ$; untreated, $\bullet \cdots \bullet$.

![Fig. 3. Uridine phosphorylase activity during thyroxine-induced metamorphosis. Thyroxine concentration was $2.6 \times 10^{-8}$ m. Each determination represents a pooled liver sample from three to four tadpoles. Thyroxine-treated, $\circ \cdots \circ$; untreated, $\bullet \cdots \bullet$.](http://www.jbc.org)

Fig. 3. Uridine phosphorylase activity during thyroxine-induced metamorphosis. Thyroxine concentration was $2.6 \times 10^{-8}$ m. Each determination represents a pooled liver sample from three to four tadpoles. Thyroxine-treated, $\circ \cdots \circ$; untreated, $\bullet \cdots \bullet$.

![Fig. 4. Relationship between stage of tadpole development (13) and specific activity of A, uridine phosphorylase ($\circ$ and $\bullet$), and B, uridine kinase ($\triangle$). Open and closed circles of Curve A represent data from different batches of tadpoles.](http://www.jbc.org)

Fig. 4. Relationship between stage of tadpole development (13) and specific activity of $A$, uridine phosphorylase ($\circ$ and $\bullet$), and $B$, uridine kinase ($\triangle$). Open and closed circles of Curve A represent data from different batches of tadpoles.

Uracil Incorporation into RNA—Uracil incorporation into RNA was investigated in liver slices to determine whether the utilization of uracil for RNA synthesis could be demonstrated and whether thyroxine treatment had any effect on RNA synthesis. Radioactivity in the acid-soluble fraction after incubation with uracil-2-14C was found in uridine, UMP, UDP, UTP, and an unidentified compound which was probably a uridine diphosphate conjugate similar to that seen by Reichard and Sköld (6) and by Hurlbert and Potter (15) in rat liver. Analyses

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of the RNA showed incorporation of radioactivity into CMP and UMP with no activity in either of the purine nucleotides. The radioactivity in UMP was approximately 4 times that in CMP. The effect of thyroxine on uracil incorporation into RNA (Table I) indicates that such treatment, in vivo, for a period of 10 days had no significant effect on uracil incorporation into UMP of tadpole liver RNA in vitro.

The availability of uracil for RNA synthesis must depend in part upon other reactions competing for uracil. Stimulation of uracil degradation by thyroxine could, therefore, mask an increase in uracil incorporation into RNA. Uracil-2-14C degradation is conveniently followed by measurements of 14CO2 formation (16). A study of the influence of thyroxine treatment of tadpoles on the ability of liver slices to oxidize uracil (Table I) shows that, while uracil is oxidized to a considerable extent by tadpole liver, thyroxine administration appears to have no effect on the extent of degradation. The results presented in Table I suggest that the activities of the key enzymes which are involved in uracil incorporation into RNA and in uracil degradation are not significantly affected by thyroxine treatment. However, this conclusion is made with the reservation that an alteration in RNA synthesis in vivo would not necessarily be reflected in uracil incorporation in vitro.

DISCUSSION

The demonstration that addition of uracil, uridine, and orotic acid stimulated the synthesis of carbamyl phosphate synthetase in thyroxine-treated tadpoles, while cytosine and adenosine had no effect, provides evidence for a relative deficiency of uridine nucleotides. The data make it clear that thyroxine stimulation during metamorphosis affects pyrimidine requirements. Supplementation by exogenous precursors for uridine nucleotide synthesis during thyroxine stimulation would permit an increase in RNA template formation and thus permit an enhancement of carbamyl phosphate synthetase synthesis. It should be noted that during metamorphosis resorption of the tail cartilage and connective tissues occurs, with the subsequent formation of new cartilage, bone, connective tissue, etc., for the emerging frog. The key role of uridine diphosphate glucose and other uridine diphosphate conjugates in the formation of components of cartilage, mucopolysaccharides, and connective tissue is well established (17-19). Although the identity of a specific "deficient" uridine nucleotide is not known, the very nature of the metamorphic transition from tadpole to frog, especially when accelerated by thyroxine, would be expected to increase the demand for uridine diphosphate conjugates and related nucleotides. However, the effect of thyroxine may be on several uridine nucleotide pools of which the pool of uridine diphosphate conjugates is only one. In the absence of thyroxine stimulation the tadpole presumably would be capable of meeting its uridine requirements because of a slower rate of metamorphosis, and thus exogenously administered pyrimidines would be expected to have no effect on carbamyl phosphate synthetase formation.

Although cytidine has been shown to be a precursor of UMP in RNA of chick embryo cells (20) and of rat liver (21), cytosine did not enhance carbamyl phosphate synthetase synthesis in the tadpole during thyroxine treatment. It appears that if a uridine nucleotide deficiency does exist, the rate of conversion of cytosine to uridine nucleotides is not rapid enough to overcome the deficit.

In view of the evidence for increased pyrimidine requirements during accelerated metamorphosis, the thiouracil inhibition of carbamyl phosphate synthetase synthesis reported earlier (3) may be the result chiefly of a thiouracil effect on the availability of uridine nucleotides. It has been shown by Strominger and Friedkin (22) that mammalian liver is capable of forming thiourea riboside and thiouracil deoxyriboside. Thiouracil has been reported to inhibit the induction of liver tumors in rats fed 2-acetylaminofluorene (23). The addition of uracil overcome the inhibiting action of thiouracil in these experiments as it did in the case of the tadpole (3). The competition between thiouracil and uracil suggests that thiouracil may alter uracil metabolism either by competition for RNA synthesis or for synthesis of an essential uridine nucleotide. Actually, thiouracil may be producing in effect a deficiency of uridine nucleotides in the tadpole by acting as a ribose "trap" (24) or by competition with other pyrimidines for uridine nucleotide synthesis.

In previous studies (3, 25) it was observed that thyroxine inhibited the rate of incorporation of adenine-8-14C and thiouracil-2-14C into tadpole liver RNA. The findings in the present study, which indicate that thyroxine causes a relative deficiency of pyrimidine nucleotides (or precursors), suggest an alternative explanation for the earlier observations. In the absence or decreased availability of pyrimidine nucleotides or precursors, there will of necessity be a slower rate of RNA synthesis. While it was previously suggested (3) that the primary effect of thiouracil as an inhibitor of carbamyl phosphate synthetase was the result of incorporation of thiouracil into RNA to form a "faulty" RNA, it seems equally likely, in the light of the data in the present paper, that thiouracil is serving to interfere with the synthesis of uridine nucleotides needed for RNA synthesis. Although evidence for incorporation of thiouracil-2-14C and thiouracil-35S into RNA was reported (3), the uncertainties of the extent of conversion of thiouracil-2-14C to uracil-2-14C and the nonspecific distribution of thiouracil-35S in the isolated nucleotide fractions from RNA make it necessary to obtain more compelling experimental evidence before the hypothesis of a "faulty" RNA can be invoked.

Thyroxine was found to stimulate uridine phosphorylase activity, but not uridine kinase, in the metamorphosing tadpole. The specific activity of uridine phosphorylase was approximately

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* Per cent of initial uracil.
twice that of uridine kinase, on an hourly basis, in the assay system employed. If these activities represent the relative activities of the two enzymes in tadpole liver, uridine kinase would be rate-limiting in the conversion of uracil to UMP. A similar observation was made by Reichard and Sköld (6), who considered uridine kinase to be rate-limiting in rat liver, which is also characterized by relatively low uridine phosphorylase and uridine kinase activities and by relatively high catabolic activity. In these circumstances the increase in uridine phosphorylase activity observed in tadpole liver would not be expected to result in an increase either in uracil incorporation or in RNA synthesis. The real significance of the increase in uridine phosphorylase activity is yet to be established, but it may represent a more efficient utilization of this pathway for the “salvage” of uridine.

It has been shown by Sköld (26) that there is a correlation only in some cases between the activities of uridine phosphorylase and uridine kinase and the growth rate of the tissue examined. Regenerating rat liver exhibited an increase in activities of both enzymes, with a decrease nearly to the initial level 44 hours after partial hepatectomy. Furthermore, it has been shown by Stevens and Stocken (27) that uridine phosphorylase increases slightly in regenerating rat liver on the basis of the supernatant protein content but changes very little on the basis of DNA content. It was demonstrated, however, that a strikingly higher uridine phosphorylase activity was present in young adult rats as compared to fetal and newborn animals. It has also been noted1 that activity of this enzyme in older adult rats (400 to 500 g) was only a fraction of that seen in young adults, while little change in uridine kinase activity was observed. In addition, it was found that the activity of uridine phosphorylase in calf liver was considerably higher than that in the adult animal, while uridine kinase activity was about the same in both. It appears, therefore, that while both uridine phosphorylase and uridine kinase may vary with changes in the growth rate of tissues, this is not obligatory, and that changes in uridine phosphorylase, which are not necessarily correlated with tissue growth, may occur and are not necessarily associated with corresponding changes in uridine kinase activity.

SUMMARY
Carbamyl phosphate synthetase formation in liver taken from tadpoles treated with thyroxine was enhanced by the addition of uracil, uridine, or orotic acid, while cytosine and adenosine had no effect. The synthesis of this enzyme was not influenced by uracil, uridine, or orotic acid in untreated animals. The data indicate that there is a relative pyrimidine deficiency during thyroxine-induced metamorphosis.

After thyroxine treatment in vivo, uridine phosphorylase activity of liver increased. A similar increase was observed during normal metamorphosis, with a subsequent decrease in the later stages of development and in the adult frog. Uridine kinase activity remained essentially unchanged during normal and thyroxine-induced metamorphosis.

REFERENCES

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