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**A CONCERTED RESPONSE OF THE ENZYMES OF UREA BIOSYNTHESIS
DURING THYROXINE-INDUCED METAMORPHOSIS OF
RANA CATESBEIANA**

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Running head -

Enzymes of Urea Biosynthesis During Metamorphosis

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SUMMARY

During natural metamorphosis, concomitant, linear increases were found in tadpole liver for the activities of the five enzymes in the ornithine-urea cycle: carbamyl phosphate synthetase-I, ornithine transcarbamylase, argininosuccinate synthetase, argininosuccinate lyase, and arginase. Upon immersion in 2.6×10^{-8} M L-thyroxine for two weeks, the biochemical changes which occur during natural metamorphosis were simulated with an elevation of the same enzymes by factors of 8.0, 3.3, 2.4, 2.3 and 3.0, respectively. The lag period prior to the initial rise of each enzyme activity was about 4 days. On the basis of the rate of change of the leg/tail ratio, the thyroxine-exposed tadpoles had an earlier, steeper rise in the levels of each of the five enzymes than that observed during natural metamorphosis. Argininosuccinate synthetase had the lowest specific activity of the ornithine-urea cycle enzymes, even though the assay was improved by the inclusion of urease in the incubation mixture, yielding higher activities than earlier reported. Plotting one enzyme against each of the other ornithine-urea cycle enzymes gave straight lines with high correlation coefficients and similar slopes for both natural and thyroxine-induced metamorphosis. These results demonstrate the simultaneous, concerted response of both mitochondrial and extra-mitochondrial enzymes in the ornithine-urea cycle enzymes during thyroxine-induced metamorphosis.

The shift from ammonotelism in the tadpole to ureotelism of the frog reflects the biochemical differentiation of the liver during amphibian metamorphosis (1,2). These changes are an integral part of the progressive sequence of the postembryonic, morphological and biochemical changes which permit the tadpole to leave an aquatic environment for a terrestrial existence (1-4). The acquisition of ureotelism is based on the concomitant rise in the liver of the 5 enzymes¹ of the ornithine-urea cycle (1,2):

CP-synthetase-I had been previously shown to undergo the greatest increase in activity of the ornithine-urea cycle enzymes during both natural and thyroxine-induced metamorphosis (2,5). The increase in CP-synthetase-I upon immersion of tadpoles in thyroxine was preceded by a lag period of several days, which was shortened, but not eliminated, by an elevation of the immersion temperature (6), or by injection of thyroxine (7). On the other

hand, immunological and ^{14}C -leucine incorporation studies showed that the de novo synthesis of CP-synthetase-I in the intact tadpole began within 24 hr after the thyroxine exposure (8). Subsequently, the use of pre-metamorphic liver cubes surviving in an antibiotic-amphibian salts medium at 24° for 48 hr permitted the in vitro demonstration of a thyroxine-stimulated increase in the synthesis of CP-synthetase-I (9). This study provided evidence for the formation of an extramitochondrial precursor of CP-synthetase-I and an acceleration of this conversion by thyroxine (9). These findings are not confined to CP-synthetase, since similar approaches have demonstrated the de novo synthesis of glutamate dehydrogenase (EC 1:4.1.3) and the thyroxine-stimulated conversion of its precursor to glutamate dehydrogenase (10). The present experiments were designed to explore the detailed inter-relations of the five sequential enzymes involved in urea biosynthesis during thyroxine-induced metamorphosis.

EXPERIMENTAL PROCEDURES

Experimental Animals - Rana catesbeiana tadpoles and frogs were procured from the Lemberger Company, Oshkosh, Wisconsin. Premetamorphic tadpoles in stages VI to XI in the nomenclature of Taylor and Kollros (11) and metamorphosing tadpoles in Stages XVII to XXI were maintained on a diet of canned spinach twice a week. Stock tadpoles were housed in plastic containers with dechlorinated running tap water at 15°. Ten to 14 days prior to all experiments described herein, the tadpoles were transferred to dechlorinated running tap water at 25°. The role of temperature adaptation in metamorphosis and CP-synthetase responses has been previously documented (6). For the experiments on thyroxine-induced metamorphosis, premetamorphic tadpoles of comparable

size and hind leg length (leg/tail ratio of 0.01-0.04) were transferred to 4 liter plastic containers that were maintained in a 25° constant temperature bath. Water and thyroxine (a final concentration of 2.6×10^{-8} M thyroxine (Sigma) (6)) in these tanks were changed every other day. The tadpoles (both untreated and thyroxine-treated) were not fed during the 2 week experimental period. Mortality was insignificant under these conditions.

For the natural metamorphosis experiments, tadpoles, which were received in three shipments, were fed and maintained as described above at 25°, matched for the same leg/tail ratio, and sacrificed in groups of 3-4 tadpoles. The pooled livers were homogenized, centrifuged and assayed as described. Some tadpoles in advanced stages of metamorphosis were sacrificed soon after arrival and temperature adaptation, whereas others were selected during a 5 week period in the laboratory as metamorphosis progressed to stages of a greater leg/tail ratio.

Preparation of Liver Extracts - After anesthetization of the tadpoles by immersion in crushed ice, the livers (after removal of the gall bladder) were excised, blotted, weighed and homogenized with 0.1% of CTB¹ (10% W/V) (12) with 6 complete passes of a Potter-Elvehjem type, teflon pestle and a 750 rpm homogenizer. The suspension was poured through two layers of cheesecloth to remove connective tissue. Aliquots were removed for analysis of DNA and total liver protein. The remaining homogenate was centrifuged for 20 min at 15,000 X g at 2° (9). For the CP-synthetase-I assays, the pellet was reextracted with 0.1% CTB (12). The cold supernatant was used for the assay of soluble liver protein, Orn-transcarbamylase, ASA-synthetase, ASA-lyase and arginase.

Chemical Determinations - Citrulline was measured colorimetrically using the diacetylmonoxime (Fisher Scientific Co., St. Louis, Mo.) color reaction at 490 nm as described by Ratner (12 and 13) with a Zeiss PMQII spectrophotometer. Urea was determined by the 1-phenyl-1,2-propanedione-2-oxime (Eastman Kodak Co., Rochester, N.Y.) color reaction at 540 nm absorption (12, 14) with precautions to protect against light and with a standard curve designed to correct for the color production of unreacted citrulline at the end of the incubation period (12, 14). The $\text{FeCl}_3\text{-H}_2\text{SO}_4\text{-H}_3\text{PO}_4$ reagent in these color reactions was prepared as previously described (15). A more sensitive assay method with diacetylmonoxime plus N-phenyl-p-phenylene-diamine (16) was used to measure citrulline in the absence of urea for the CP-synthetase assay, and urea in the absence of citrulline for the ASA-lyase procedure.

The total liver protein was determined with aliquots of the strained liver homogenates by the addition of 1.0 N NaOH and followed by the Lowry et al. (17) colorimetric analysis. The soluble protein in liver (i.e. the protein in the supernatant after centrifugation of the liver homogenates for 20 min at 15,000 X g), was assayed by the same procedure (17). The deoxyribonucleic acid (DNA) in the liver homogenates was extracted twice with 1.0 N HClO_4 at 70° (18, 19) and aliquots of the supernatant were analyzed by the Dische diphenylamine reaction as modified by Burton (20). Preliminary experiments with tadpole liver showed that an initial extraction with cold 1.0 N HClO_4 could be eliminated, but that a second extraction with hot HClO_4 was needed for maximum DNA yield without pentose destruction, and that a third extraction with hot HClO_4 did not yield any further significant amounts of DNA.

Determination of the Enzyme Activity - CP-Synthetase-I was assayed as previously described (21) with citrulline being determined by a diacetylmonoxime plus N-phenyl-p-phenylenediamine color reaction (16). The CP-synthetase determination in whole liver extracts was carried out in the presence and absence of N-acetylglutamate and corrected for the blank values observed in the absence of N-acetylglutamate. Orn-transcarbamylase was estimated by a modification² of an earlier procedure (12) using the diacetylmonoxime color reaction (14) to measure the citrulline formed. The earlier procedure for ASA-lyase (12) was modified by measuring the conversion of 20 μ moles of potassium argininosuccinate (13) (Sigma) to urea (16) in the presence of excess arginase (Sigma) during a 5 min incubation. Arginase was activated in 0.05 M $MnCl_2$ plus maleate buffer, pH 7.2, for 20 min at $54^\circ \pm 1^\circ$ (similar to 22); frog liver extracts required a minimum of 10 min for activation and were stable for up to 30 min at 54° . The arginase assay was performed in 0.1 M L-arginine, pH 9.5, for 10 min at 37° with urea being measured by the p-dimethylaminobenzaldehyde (23) color reaction. All enzyme assays were checked for linearity with graded increases of tadpole and frog liver extracts, and were run in triplicate at 37° with appropriate corrections for blanks. They were performed within 1-2 hr after sacrifice to minimize possible loss of activity, and are reported consistently in units of μ moles of substrate disappeared or product formed/min/g liver wet weight.

In an earlier study (12), ASA-synthetase was determined as arginine synthetase, i.e. the combined action of ASA-synthetase and ASA-lyase for the overall conversion of citrulline to urea in the presence of excess arginase;

its limitations will be observed later in discussion of Fig. 1. The spectrophotometric assay for ASA-synthetase (24, 25) is not applicable to crude tadpole liver extracts containing ATPase, whereas the ASA-synthetase assay based on the measurement of citrulline disappearance (13, 26) is limited to crude preparations free of ASA-lyase or arginase. Since the substance which is most likely to interfere with the colorimetric determination of citrulline in these assays is urea, a simple method for ASA-synthetase in crude liver extracts was developed and is based on following citrulline disappearance in an assay system containing added urease. The ASA-synthetase assay system, modified from (12, 13) with experimental confirmation of each specific change, contained 5 mM L-citrulline, 5 mM L-aspartate (pH 7.5), 5 mM ATP (pH 7.5), 5 mM $MgSO_4$, 100 mM Tris-HCl buffer (pH 7.5), 1.0 unit of urease (0.12 mg protein/tube, Type VI, S.A. of 8300 units/g, Sigma) and liver extract in a final volume of 1.0 ml. After 20 min incubation at 37° for high activity extracts, or 60 min incubation for low activity extracts, the reactions were terminated with 4.0 ml of 0.5 M $HClO_4$ and centrifuged. Aliquots (0.5 ml) of the clear supernatant solutions were analyzed for citrulline (12, 13). The same system treated with $HClO_4$ served at zero time served as a control. The urease activity was checked periodically by measuring the hydrolysis of added urea (a 20-fold excess in a control tube). The rate of citrulline disappearance was calculated to express ASA-synthetase activity in units of μ moles citrulline disappeared/min.

The advantage of the urease addition and the validity of the modified ASA-synthetase assay conditions are revealed in Fig. 1. Upon exaggeration of the usual assay conditions, the urea formation (Δ - Δ in Fig. 1 A, B, C and D)

lagged behind the citrulline disappearance in the presence of urease (●-●), particularly in the tadpole extracts. The addition of urease removed all the urea (▲-▲), and caused steeper, more linear slopes for citrulline disappearance (●-●) than in its absence (○-○) for all 4 graphs. For valid assays, the amount of citrulline disappearance should not exceed 1.0 μ mole, i.e. one-fifth of the total citrulline added (Fig. 1A, B). A linear rate of citrulline disappearance was observed for up to 20 min with frog liver extracts (Fig. 1C), or for up to 60 min for tadpole liver extracts (Fig. 1D). The ASA-synthetase activity was not altered by up to a 10-fold increase in CTB concentration in the assay system. The modified ASA-synthetase assay gave values which were about 3-times greater for frog liver than the earlier assay procedure (12). The modified ASA-synthetase assay should prove useful in assays of other crude liver preparations, particularly for the assessment of liver enzyme levels in human inborn errors of metabolism associated with enzymes of urea biosynthesis, described clinically as cases of hyperammonemia, citrullinuria and arginosuccinic aciduria.

RESULTS

Intracellular Distribution of Ornithine-Urea Cycle Enzymes - Previous studies have indicated that the first 2 enzymes of the ornithine-urea cycle within the mitochondria and arginase is in the cytosol. For the remaining 2 enzymes, earlier evidence indicated that ASA-synthetase and ASA-lyase were also soluble enzymes (12-14, 27). Further direct evidence for this distribution was obtained by use of standard homogenizing conditions and centrifugal procedures (28), the present specific enzyme assays and estimates of recovery (see Table 1, which is typical of several such experiments). An isotonic

mannitol medium was used, since sucrose interferes with the colorimetric determination of citrulline (29). All of the activity in the liver-mannitol-homogenate for ASA-synthetase, ASA-lyase, and arginase was found in the supernatant after centrifugation at 57,000 X g for 60 min. The efficacy of 0.1% CTB for preparation of frog or tadpole liver homogenates and extracts (12) had been previously demonstrated. With the use of the revised enzyme assays and centrifugation at 15,000 X g, one CTP-extraction was now found to remove 95% of the mitochondrial Orn-transcarbamylase, 91% of the cytoplasmic ASA-synthetase, and 100% of the ASA-lyase and arginase activities in the original strained homogenate. Negligible amounts of enzyme activity were found on reextraction with 0.1% CTB of the pellet found after centrifugation at 15,000 X g.

Biochemical Changes in Tadpole Livers During Natural Metamorphosis - In order to provide a basis for the interpretation of the effect of thyroxine exposure, it was necessary initially to define the quantitative relationships of the ornithine-urea cycle enzymes during natural metamorphosis, using the same enzyme assay procedures. Earlier studies indicated that these enzymes increased during natural metamorphosis (5), although these experiments were not as complete as those for CP-synthetase-I (2,6). To provide a sufficient number of tadpoles at various stages of natural metamorphosis, 3 different batches were used for the experiment (Fig. 2). To reduce the known individual variability of animals and to obtain sufficient liver for the multiple assays, the livers from 3-4 tadpoles were pooled, homogenized, and assayed. The leg/tail ratio, which is a sensitive index of metamorphosis, were determined for each animal. Even though appreciable scatter of enzyme values at any

given leg/tail ratio is evident in Fig. 2, straight lines and meaningful correlation coefficients were obtained; all had a value of $P < 0.01$. Bearing in mind that the abscissa is a ratio, extrapolation to zero or beyond 2.5 is unwarranted. These graphs show a linear increase in activity of the 4 enzymes shown when plotted against the leg/tail ratio as an index of metamorphic change. These data reveal that the premetamorphic tadpole (Stage XI (11), leg/tail ratio of 0.10) undergoes a 7.7-, 3.2-, 3.6-, and 4.8-fold respective increase in the activity for the enzymes upon attaining Stage XXIV (11) (leg/tail ratio of 2.50). While these tadpoles were adapted to 25° running water, a preliminary experiment with tadpoles held at 15° also showed an increase of ASA-synthetase, ASA-lyase, and arginase between leg/tail ratios of 0.26 and 0.97. The enzyme levels for the small frogs (leg/tail ratio of 3 + 50) adapted to 25°, are shown on the right side of Figs. 2A, B, C and D (0).

Other components of the liver showed slight changes during natural metamorphosis (Fig. 3). The liver weight decreased (Fig. 3A), DNA concentration increased (Fig. 3B), total protein increased slightly (Fig. 3C) and soluble protein decreased slightly (Fig. 3D). These changes probably reflect the decrease in food intake during the period of metamorphic climax. The decrease in soluble liver protein is insufficient to alter the interpretation of expression of enzymatic concentrations on a wet liver weight basis (Fig. 2). The large scatter seen for liver wet weights (Fig. 3A) showed two populations of points. When calculated separately, the tadpoles received in January (Δ) and March (O) showed slight increase in liver weight

per unit change of the leg/tail ratio ($Y = 2.6X + 175$; $r = + (0.02)$), whereas the 30 values for the tadpoles arriving in May (0) had a pronounced negative slope ($Y = -80X + 403$; $r = -0.65$). These differences may relate to the nutritional state of the tadpoles in their natural environment during the different seasons and/or the degree of adaptation to laboratory conditions. For this reason, all of the measurements recorded in Figs. 2 and 3 were evaluated for variation between the three batches of tadpoles, but the decline in liver weights was the only one that showed this lack of homogeneity. The influence of dietary intake in these experiments is yet to be determined.

Adult frog livers were analyzed by the same procedures (Table 1). The female frogs, obtained in April, had a copious supply of eggs, larger liver weights and total protein concentration, and lower DNA concentration. The basis for the differences in liver enzyme concentrations was not apparent. Comparison of the values for the small frogs (Fig. 2, 0) with adult frogs (Table 1) indicates that Orn-transcarbamylase remained about the same, whereas the liver concentration of ASA-synthetase, ASA-lyase and arginase continued to increase toward the adult levels.

Biochemical Changes in Tadpole Livers During Thyroxine-Induced Metamorphosis - The acceleration of metamorphosis by immersion of tadpoles in 2.6×10^{-6} M thyroxine (6) is presented in Fig. 4, which shows one of three such experiments. During the 2 week treatment period, the leg/tail ratio changed from 0.03 to 0.28. Most of the tadpoles had forelimbs emerging by the 10th day and all were protruding at the 14th day. During this time period, the primary factor for change in the leg/tail ratio was the increase in hind leg length. It can also be seen from Fig. 4 that the activities of the ornithine-urea cycle enzymes increased in the liver over that of control, untreated tadpoles: CP-synthetase-1-8.0 fold, Orn-transcarbamylase-

3.3 fold, ASA-synthetase-2.4 fold, ASA-lyase-2.3 fold and arginase-3.0 fold. The lag period prior to the initial rise of the 4 enzymes was 4 days, with Orn-transcarbamylase having a slightly earlier inflection point. The two preliminary experiments with a greater initial leg/tail ratio (means of 0.11 and 0.16) showed a shorter lag period of about 3 days. These observations suggest that thyroxine must trigger certain developmental events in the liver prior to the increase of the enzymes for urea biosynthesis.

Other measurements made during the same experiment (Fig. 5) indicated that while the liver weight falls (Fig. 5A), the concentration of DNA (Fig. 5B), total protein (Fig. 5C), and soluble protein (Fig. 5D) remain unchanged in both the thyroxine-treated and the control groups. The decline in liver weight, also seen in the two preliminary experiments, was probably due to the lack of food. The constancy of the soluble protein level (Fig. 5D) means that calculation of the enzyme assays on the basis of specific activity ($\mu\text{moles/min/mg protein}$), rather than as in Fig. 4 ($\mu\text{moles/min/g wet liver weight}$), would yield the same relative results. The lack of change in liver DNA, total protein, and soluble protein may appear to be a different response from that observed in natural metamorphosis (Fig. 3). However, it should be noted that the following differences in experimental conditions obtain: 1) length of experiment (2 vs. 5 weeks); 2) maximum observed leg/tail ratio (0.28 vs. 2.86); 3) feeding (absent in the experiment represented in Fig. 5 and present in that represented in Fig. 3); and 4) the probability of different levels of endogenous thyroxine. The leg/tail ratio itself depends on 2 variables, which change sequentially, not simultaneously, in early metamorphosis. In other words, there was no detectable decrease in tail length during the short thyroxine-immersion experiments, but tail

shortening and resorption was an integral part of the natural metamorphosis experiment (Fig. 3), particularly after attaining metamorphic climax, i.e. leg/tail ratio of 0.55-0.80, or Taylor and Kollros' stage XVIII-XX (11) (cf. 3).

It was previously reported (6, cf. 2) that the increase of CP-synthetase, when plotted against leg/tail ratio, was faster for thyroxine-treated tadpoles than for tadpoles undergoing natural metamorphosis. Similar observations have been reported for arginase (20). However, data for the levels of all the enzymes in urea biosynthesis from livers of tadpoles exposed to thyroxine have not been previously reported in relation to the leg/tail ratio. The data in Fig. 6 (solid lines) portray linear relationships for these increases in enzyme activity with high correlation coefficients; the slopes are the same as those shown in Fig. 2 (dash line) but with a different scale for the abscissa. Comparison of the solid lines in Figs. 2 and 6 (or the solid line with the dash line in Fig. 2 or 6) show that the tadpoles immersed in thyroxine had a more rapid increase in activity (3.4-fold for Orn-transcarbamylase, 5.2-fold for ASA-synthetase, 5.0-fold for ASA-lyase and 4.1-fold for arginase) than those undergoing natural metamorphosis.

Concerted Response of the Urea Cycle Enzymes - The earlier graphs show that the increased activities for the ornithine-urea cycle enzymes were time dependent in both thyroxine-stimulated metamorphosis (Figs. 4 and 6) and natural metamorphosis (Fig. 2). By plotting the activity of one enzyme against each of the other 3 enzymes, the relative rates of increase of enzyme activity can be assessed. Fig. 7 shows the incremental increase of ASA-synthetase, ASA-lyase and arginase when plotted against Orn-transcarbamylase for natural (Figs. 7A, B, C) and for thyroxine-induced metamor-

phosis (Figs. 7A', B', C'). The relation of the levels of Orn-trans-carbamylase vs. CP-synthetase activity during thyroxine treatment was calculated ($Y = 4.00X + 9.92$, $r = +0.98$), but is not shown in Figs. 7A', B', C'. The data represented in Figs. 7A', B', C' have higher correlation coefficients than the data shown in Figs. 7A, B, C primarily because the former represent data from one batch of tadpoles and are averages of 5 separate enzyme determinations. Conversion of these correlation coefficients to probability values indicates that the relationships in all 6 graphs have statistical levels of significance greater than 0.01 (31). However the paramount observation from all parts of Fig. 7 is that the removal of the time factor leads to essentially similar slopes in the thyroxine-induced and natural metamorphosis (Figs. 7A vs. 7A', 7B vs. 7B' and 7C vs. 7C'). The significance and limitations of this interpretation of a concerted response in enzyme activities must be considered from the viewpoint of relative rates of enzyme synthesis and degradation.

DISCUSSION

The rise in the liver of the ornithine-urea cycle enzymes during metamorphosis is a part of the overall changes in the structural proteins and enzymes not only in the liver and tail, but also in the other organs and tissues (3,4). All of these time-dependent processes are to some degree expressions of the genetic regulation of metamorphosis under the influence of thyroxine. In addition, the data in Figs. 2 and 6 represent further evidence on the action of thyroxine in accelerating the development of the enzymes for urea biosynthesis. The interpretation of these results is limited by the lack of information on the rates of synthesis and degradation of all of the enzymes of the ornithine-urea cycle under the conditions of thyroxine stimulation. Although such information is available for CP-synthetase-I on an in vivo (8) and in vitro basis (9) and for Orn-transcarbamylase on an in vitro basis², similar data are lacking for the remaining enzymes, i.e. the three extramitochondrial enzymes. Whatever the effects of thyroxine on the rates of enzyme synthesis and degradation, the ultimate response is a coordinated pattern of increase in activity for the ornithine-urea cycle enzymes (Fig. 2 and 6). Indeed, the paramount evidence for a concerted response is the straight line graphs of one enzyme activity plotted against a second, third and fourth enzyme activity (Fig. 7). These linear relationships reveal a similarity to the phenomena of coordinate repression and induction (32), as observed in the microbial biosynthesis of histidine (33), leucine, isoleucine-valine, tryptophan, and pyrimidines (32,34), or the bacterial catabolism of lactose, galactose and arabinose (32).

Previous investigators have observed linear relationships when the following enzyme activities were plotted against the leg/tail ratios as

shown in amphibian liver arginase (30), tadpole liver CP-synthetase (6), and lysosomal enzymes in the tail of tadpoles (35,36). A correlation was reported to exist between the activities found for CP-synthetase, Orn-transcarbamylase and arginine synthetase in the livers of different vertebrates with different nitrogen excretory patterns (37, Fig. 5).

CP-synthetase (2,27) and Orn-transcarbamylase (2,38) are known to be localized in mitochondria. Arginase has long been recognized as being present in the cytosol (27). Although the overall conversion of citrulline to arginine has long been known to occur in the soluble fraction of rat liver homogenates (39), rigorous subcellular distribution studies of ASA-synthetase and ASA-lyase have not been reported. The present studies show that ASA-synthetase and ASA-lyase can be fully recovered in the supernatant after high speed centrifugation of frog liver-isotonic mannitol homogenates. This difference of enzyme localization within the cell separates the first 2 enzymes of urea biosynthesis from the latter 3, creating a situation which must require a continuous transport of ornithine into the mitochondria, or a shuttle exchange of ornithine and citrulline. While the details of how this distribution affects the rate of enzyme synthesis, the rate of degradation and the flow of metabolites through the cycle have yet to be unravelled, it is pertinent to point out that the 2 mitochondrial enzymes, CP-synthetase and Orn-transcarbamylase, had the greatest increase in activity following thyroxine immersion of the tadpoles, 8- and 3.3-fold respectively (Fig. 4). Furthermore as previously noted (1,5,22), ASA-synthetase remains the rate-limiting enzyme (Fig. 4 mainly, but see also Figs. 2 and 6, and Table II), even though the modifications of the assay gave a 3-fold increase in activity for adult frog liver.

While this study has focused on the biochemical development of amphibian liver, earlier studies demonstrated the increased activity of all 5 ornithine-urea cycle enzymes during the gestation of rat, pig and human fetuses, and then further increases during the neonatal period (40,41). A species difference in pattern was detected, since the fetal pig had an earlier (as per cent gestation) rise of the enzymes than the fetal rats (40). These findings in the rat and human have been confirmed and extended (42,43,44,45). The injection of thyroxine, but not hydrocortisone, enhanced the prenatal level of arginase in rat liver, while the 5-8 day old rat responded in a reverse manner to these hormonal stimuli (46). Thus, at least one of the ornithine-cycle enzymes is influenced by thyroid hormone during fetal development in the mammal.

Other experimental conditions have also demonstrated that the activities of the 5 enzymes for urea biosynthesis rise or fall as a group. Schimke (22, Fig. 1) found a linear relationship for all 5 enzymes of rat liver when plotted against the protein concentration of the diet. This relationship has been confirmed (47), and has also been extended to the monkey (48). Other conditions that caused a parallel increase of the urea biosynthesis enzymes in rats were starvation (49) and administration of corticosteroids (50,51). By contrast, those factors that caused decreases in enzyme levels were a protein-free diet (49), adrenalectomy (50,51), and administration of growth hormone (51). However arginine-free diets in rats, or low arginine concentrations in the medium for several mammalian cell cultures, produced no change or a decrease in arginase respectively, while ASA-synthetase and ASA-lyase increased 5-fold (50,52). By contrast, high arginine concentrations in the cell medium produced elevated levels of arginase and lower levels

of the other 2 enzymes (52). More complex responses were found for rats subjected to CCl_4 administration, alloxan diabetes and glucagon administration (47,53,54). To go beyond measurement of enzyme activities, Schinke (55) showed that the rate of de novo synthesis of arginase was enhanced for rats on high protein diets, whereas the high level of arginase during starvation was demonstrated to be related to a decreased rate of arginase degradation. The significance of these competing rates has been reviewed (56). Thus while these 5 enzymes in the livers of the amphibian and mammals respond with changes in the same direction for some administered agents, it would appear that other regulatory factors in addition to thyroxine are operating in mammals.

Exposure to thyroxine results in an earlier increase in the activities of the enzymes of urea biosynthesis in tadpole liver than the changes seen in natural metamorphosis (see comparison of the 2 slopes in Figs. 2 and 6). The time of initial enzyme response to thyroxine was found to depend on the age of the tadpoles (i.e. initial leg/tail ratio) (cf. Fig. 4 and Ref. 3). The apparent specificity of the hormonal stimulus on the urea biosynthetic enzymes was demonstrated earlier, when cortisol, insulin, growth hormone, epinephrine, and cyclic 3', 5'-AMP failed to promote the de novo formation of CP-synthetase-I under conditions where thyroxine and triiodothyronine enhanced the in vitro synthesis of this enzyme in liver cube preparations from premetamorphic tadpoles (9). Morphological studies have elucidated other key features of the action of thyroxine - i.e. the differences in specificity and mode of response of target tissues, and the variation in time of onset of sensitivity, threshold for response and rate of tissue response (57, cf. also 3,6). Interactions in the hypothalamus-pituitary-thyroid axis are also involved in the metamorphic process (3).

While these considerations would indicate that thyroxine either directly or indirectly initiates- or induces cellular changes which must involve transcriptional and translational events (2), it is clear that a concerted response occurs in the liver involving two mitochondrial and three extra-mitochondrial enzymes. Available evidence indicates that both CP-synthetase (9) and Orn-transcarbamylase² are converted to active enzymes in mitochondria from precursors formed extra-mitochondrially. While it is thus probable that thyroxine is affecting transcription and translation at the nuclear and extra-mitochondrial ribosomal levels, the means by which the levels of mitochondrial enzyme activity are regulated in concert with the levels of the extramitochondrial enzyme levels remains to be determined.

The "concerted" response of five enzymes concerned with a specific function, viz. urea biosynthesis and thus ureotelism, occurs in essentially a fixed population of hepatocytes in the case of the liver. While cytologic changes are seen (2) there is no evidence of gross or histological differentiation during metamorphosis. Thus the liver cells can be considered to be undergoing biochemical differentiation. The striking morphological changes which occur during metamorphosis (natural and thyroxine-induced) must of course mean that even more extensive biochemical differentiation occurs in the cells of the resorbing tail, developing limb buds, etc., in a concerted fashion.

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FOOTNOTES FOR TITLE PAGE

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FOOTNOTE FOR TYPED PAGE FIVE

1. The abbreviations used are: CP-synthetase-I for N-acetyl-L-glutamate-dependent carbamyl phosphate synthetase; Orn-transcarbamylase for carbamoyl-phosphate: L-ornithine carbamolytransferase (EC 2.1.3.3); ASA-synthetase for L-citrulline:L-aspartate ligase (AMP), argininosuccinate synthetase (EC 6.3.4.5); ASA-lyase for L-argininosuccinate arginine-lyase (EC 4.3.2.1); arginase for L-arginine ureohydrolase (EC 3.5.2.1); CTB for cetyltrimethyl-ammonium bromide, and T_4 for thyroxine. The reader is referred to a review (2), for the distinction between CP-synthetase-I and II.

FOOTNOTE FOR TYPED PAGE NINE

2. Reddy, M. K. and Cohen, P. P., manuscript in preparation.

TABLE 1

Subcellular localization of ornithine-urea cycle enzymes in frog liver

An adult frog liver was homogenized (10% W/V) in 0.1% CTB and in isotonic 0.25 M mannitol. The homogenates were centrifuged at 2° at the g values shown.

Medium for homogenate	Component assayed	Activity (μmoles/min/g initial liver)			
		Strained homogenate	Supernatant after 10 min at 700 X g	Supernatant after 10 min at 5000 X g	Supernatant after 60 min at 57,000 X g
0.25 M Mannitol	Protein(mg)	170.	138.	110.	75.
	ASA-synthetase	1.60 ^a	1.10	1.22	0.97
	ASA-lyase	5.12	5.60	5.61	5.61
	Arginase	760.	620.	640.	740.
0.1% CTB	Protein(mg)	210.	95.	90.	87.
	ASA-synthetase	2.12	2.37	2.75	2.85
	ASA-lyase	5.52	5.92	6.00	6.16
	Arginase	920.	960.	1000.	1080.

Footnote

^aThis one value was atypically high; in another experiment, values of 0.80, 0.90, 1.00 and 1.00 were obtained.

TABLE II

Levels of protein, DNA and ornithine-urea
cycle enzymes in frog liver (R. catesbeiana)

Five adult frogs of each sex were sacrificed for analysis of the
liver as described under experimental procedures.

Parameter measured	Male frogs	Female frogs
Body weight, g	440 $\pm$ 38	699 $\pm$ 168 ^a
Liver weight, g	13 $\pm$ 3	18 $\pm$ 5 ^b
Total protein conc., mg/g	131 $\pm$ 7	153 $\pm$ 19 ^b
Soluble protein conc. mg/g	87 $\pm$ 21	77 $\pm$ 12
DNA conc., mg/g	3.9 $\pm$ 0.7	2.7 $\pm$ 0.4 ^b
Orn-transcarbamylase, units/g	75.0 $\pm$ 13.7	89.3 $\pm$ 31.2
ASA-synthetase, units/g	2.68 $\pm$ 0.36	2.03 $\pm$ 0.43 ^b
ASA-lyase, units/g	7.14 $\pm$ 1.30	6.82 $\pm$ 1.18
Arginase, units/g	406 $\pm$ 63	529 $\pm$ 137

Footnotes

a. $P < 0.05$.

b. $P < 0.01$.

Fig. 1 Validity of the assay conditions for ASA-synthetase with R. catesbeiana liver extracts. In Figs. A and C, an extract of adult bullfrog liver (supernatant of a 10% W/V 0.1% CTB homogenate, 0.88 mg protein/0.1 ml) was used as the source of the enzyme in a test for the validity of the 20 min incubation time. In Figs. B and D, a similar extract of tadpole liver (leg/tail ratio of 2.0, 0.97 mg/0.1 ml) was employed in order to ascertain the linearity in a 60 min incubation assay at 37°. Using the same scale, the apparent citrulline disappearance, represented as the solid lines, (i.e. the diacetylmoxime color reaction at 490 nm) and the corrected urea formation, represented as the dash lines, (i.e., the 1-phenyl-1,2-propanedione-2-oxime color reaction at 540 nm) are plotted on the ordinate for the mean of duplicate analyses for each point. The values for the assay systems with added urease (1.67 mg/tube) are represented by the solid circles or solid triangles, and those without added urease by open circles or open triangles.

Fig. 2 Ornithine-urea cycle enzymes during natural metamorphosis of R. catesbeiana. The pooled livers from 3-4 tadpoles with the same leg/tail ratio were analyzed for the above 4 enzymes, whose activities on the ordinate were consistently expressed in units of μ moles of substrate disappeared or product formed/min/g liver. The data in Fig. 2A represent 44 determinations and those in Figs. 2B, C and D, 59, all of which were included in the respective formula for the linear regression curves and linear correlation coefficients (r). The right side of each graph shows the mean values ($\bar{0}$) with the standard deviations for 6 froglets from the same shipment with a leg/tail ratio of 3 to 50. The dash line, labelled T_4 , is derived from the data in Fig. 6 and is discussed later in the text.

Fig. 3 Liver changes during natural metamorphosis of R. catesbeiana. The same tadpole livers and extracts as described in Fig. 2 were analyzed for total liver wet weight, DNA concentration, total liver protein concentration, and CTB-soluble protein concentration (i.e. protein concentration of the supernatant after centrifugation). The data of lines A and D represent 59 assays, whereas those of B and C represent 44 assays which were used in the calculation of the linear regression equations and linear correlation coefficients (r) shown. Fig. A is discussed further in the Results Section.

Fig. 4 Ornithine-urea cycle enzymes during thyroxine-induced metamorphosis of R. catesbeiana. A single shipment of 500 tadpoles with an initial leg/tail ratio of 0.03 ± 0.01 were divided into control (open circles) and thyroxine-treated groups (closed circles). At the indicated time intervals, 15 tadpoles were removed and divided into 5 groups of 3 tadpoles each. The supernatant extracts of the pooled homogenized livers were assayed for the 5 indicated enzymes, whose mean activities and standard deviations are expressed on the ordinate in units of $\mu\text{moles substrate utilized or product formed/min/g liver}$.

Fig. 5 Liver changes during thyroxine-induced metamorphosis of R. catesbeiana. The same 5 pooled livers, described in Fig. 4, were used for the analysis of total liver wet weight, DNA concentration, total liver protein concentration, and CTB-soluble protein concentration (i.e. protein concentration of the supernatant after centrifugation). The mean values, linear regression equations and the linear correlation coefficients (r) for 5 livers from control animals (open circles) and 10 livers from thyroxine treated animals (closed circles) are shown in each graph.

Fig. 6 Relation of the leg/tail ratio to the activities of the enzymes of urea biosynthesis during thyroxine-stimulated metamorphosis of R. catesbeiana. The data from the experiment in Fig. 4 were plotted with the leg/tail ratio on each abscissa and activity of one of the 5 liver enzymes on each ordinate. All of the data, whether from the control, non-thyroxine treated tadpoles (open circles) or from the thyroxine-immersed tadpoles (closed circles), were employed for the calculation of the statistical values. The dash lines, labelled M, are the comparable lines for natural metamorphosis from Fig. 2.

Fig. 7 Concerted responses of the enzymes of urea biosynthesis during metamorphosis of R. catesbeiana. The data from Fig. 2 for the tadpoles undergoing natural metamorphosis were plotted with the activity of Orn-trans-carbamylase as the ordinate against each of the other 3 enzymes on the abscissa (Figs. 7A, 7B and 7C). The data from Fig. 4 for tadpoles undergoing thyroxine-stimulated metamorphosis were plotted similarly in Figs. 7A', 7B' and 7C'.

APPARENT CITRULLINE DISAPPEARANCE, or
CORRECTED UREA FORMATION ($\mu\text{moles/tube}$)

VOLUME OF EXTRACT (ml)

TIME OF INCUBATION (hr)

1. Validity of the assay conditions for ASA-synthetase with *R. catesbeiana* liver extracts.

Fig. 2. Ornithine-urea cycle enzymes during natural metamorphosis of *R. catesbeiana*.

LEG/TAIL RATIO

Fig.3. Liver changes during natural metamorphosis of *R. catesbeiana*.

DAYS OF TREATMENT

4. Ornithine-urea cycle enzymes during thyroxine-induced metamorphosis of *R. catesbeiana*.

DAYS OF TREATMENT

Fig. 5. Liver changes during thyroxine-induced metamorphosis of *R. catesbeiana*.

Fig. 6. Relation of the leg/tail ratio to the activities of the enzymes of urea biosynthesis during thyroxine-stimulated metamorphosis of *R. catesbeiana*.

Fig. 7. Concerted response of the enzymes of urea biosynthesis during metamorphosis of *R. catesbeiana*.