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Philip P. Cohen - 1

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Biochemical Differentiation during Amphibian Metamorphosis

The biosynthesis of the mitochondrial enzyme carbamyl phosphate synthetase-I

Philip P. Cohen

The author is Harold C. Bradley Professor of
Physiological Chemistry, University of Wisconsin, Madison, Wisconsin 53706

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Anuran metamorphosis represents a postembryonic period of extensive morphological, cytological and biochemical changes by which the tadpole, adapted to an aquatic life, is transformed into a frog adapted to a terrestrial life. This animal system thus provides an unusual opportunity for study of a number of aspects of differentiation and comparative and developmental biochemistry (1,2). A comprehensive review of several aspects of metamorphosis in vertebrates and invertebrates has appeared recently (3).

While metamorphosis has traditionally been viewed from the standpoint of changes in gross morphology and physiology, biochemical interests in the underlying molecular changes have resulted during the past decade in particular in a considerable literature which deals with the biochemical (e.g., enzymes, plasma proteins, hemoglobins, visual pigments, etc.) changes involved in differentiation and development as aspects of metamorphosis. This literature has for the most part been included in several recent reviews (2,3,4).

The present review will be confined mainly to results from the author's laboratory and thus will be concerned chiefly with the biochemical changes observed in the liver of the amphibian, Rana catesbeiana, during metamorphosis. Stages of development of Rana catesbeiana tadpoles are shown in Fig. 1.

Tadpole liver has been reported to undergo no cell division during metamorphosis (5). From a biochemical standpoint this consideration is of prime importance in that the biochemical changes occurring in essentially a fixed population of cells can be studied without concern with the

additional biochemical factors associated with cell division and mixed populations of new and old cells (2).

I. Studies with Intact Tadpoles

A. Relation of Metamorphosis to Ammonotelism and Ureotelism

Certain species of tadpole excrete ammonia predominantly during their premetamorphic stages but begin to excrete an increasing amount of urea following onset of metamorphosis (6). With the development of suitable assay procedures (7) for the different enzymes involved in urea biosynthesis (see Fig. 2) from bicarbonate and ammonia (8), it was possible to determine the levels of these enzymes at different stages of natural metamorphosis (9). The relationship of urea excretion to levels of enzymes involved in urea biosynthesis is shown in Fig. 3. The rate limiting enzymes appear to be carbamyl phosphate synthetase-I and argininosuccinate synthetase (see Fig. 2, reactions 1 and 3). A further correlation between the enzyme data shown in Fig. 3 and the capacity to synthesize urea from ^{14}C labeled bicarbonate and ammonia has been reported by Brown (10) in studies using liver slices from tadpoles at different stages of development. A direct correlation between the excretion of urea and the level of CP-synthetase-I in liver of tadpoles has been demonstrated by Paik and Cohen (11).

B. Effect of Thyroxine

1. Urea Biosynthesis

Thyroxine has the effect of accelerating not only gross metamorphosis (3,12), but also the induction of the enzymes of

the ornithine-urea cycle which begin to increase ahead of gross morphologic changes in tadpoles exposed to 2.6×10^{-8} M thyroxine (2). Other enzymes which we have studied are shown in Table I. It should be noted that the ratios of enzyme activity (metamorphic/premetamorphic) are approximately the same whether metamorphosis occurs naturally or is thyroxine-induced. It thus appears that thyroxine is acting as an accelerator, or more likely a derepressor. The most dramatic changes in enzyme activities are those involved in the biosynthesis of urea and in particular, CP-synthetase-I (see Fig. 2). Not only are the levels of activity of the ornithine-urea cycle enzymes very high compared to other enzymes (Table I) but in addition, they are induced ahead of most other enzymes. The striking increase in CP-synthetase-I activity ahead of gross morphologic changes is seen in Fig. 4. Because of the unique role of CP-synthetase-I as the initial step in urea biosynthesis and its ready synthesis in response to thyroxine, this enzyme was prepared in pure form from adult frog liver (13). The availability of a pure enzyme permitted the preparation of a highly purified antibody (14) which was employed in demonstrating that the increase in CP-synthetase-I in liver of tadpoles exposed to thyroxine was the result of de novo synthesis (15).

A rather interesting effect of temperature on the induction of CP-synthetase-I in liver of tadpoles exposed to 2.6×10^{-8} M

thyroxine was reported by Paik and Cohen (11). It was observed that 1) the lag period of enzyme induction is shorter at higher temperatures; 2) the lag period is not only prolonged at lower temperatures (15°C), but the enzyme level reaches a plateau at approximately one-half the maximal level and persists; and 3) raising the temperature from 15° to 25° results in a rapid increase in rate of synthesis of CP-synthetase-I with no appreciable lag period. The critical temperature dependence at 15° for synthesis of CP-synthetase-I in the intact animal is reflected in the finding by Tatibana and Cohen (16) in their study of the synthesis of the enzyme in liver slices which revealed a discontinuity of the temperature curve between 15 and 20° with a high temperature quotient for the reaction involving conversion of precursor(s) to active, immunoprecipitable enzyme.

2. Induction of Carbamyl Phosphate Synthetase-I

The lag period preceding the induction of CP-synthetase-I is about 4-6 days after tadpoles are exposed to a thyroxine solution (2.6×10^{-8} M) at 22-25°C (11,17). When tadpoles are exposed to thyroxine for 2 days, the activity of CP-synthetase-I increases slowly but constantly from after the third day (17). Reexposure to thyroxine after 7 days of non-exposure results in a rapid increase of the enzyme activity after a lag period of 2 days (17) (Fig. 5). Reexposure to thyroxine after an

initial exposure for 4 days followed by non-exposure for 6 days resulted in an increase in CP-synthetase-I activity without a significant lag (Fig. 5). These observations suggest that some intracellular mechanism(s) for the induction of CP-synthetase-I has been partially activated during 2 days and more or less fully activated during 4 days of exposure to thyroxine. A similar interpretation of the mode of action of injected triiodothyronine on gross metamorphosis has been proposed by Frieden (4).

3. Nucleic Acids

In view of the fact that the increase in CP-synthetase-I levels in liver of tadpoles exposed to thyroxine is the result of synthesis de novo (15), it became important to know what associated changes were occurring in nucleic acid metabolism.

The pathway for pyrimidine biosynthesis has been studied in the tadpole (18) and more recently in frog eggs (19). While the enzymatic steps in tadpole liver for pyrimidine biosynthesis from simple precursors was established in our earlier studies (18), the problem of how carbamyl phosphate was synthesized in an organ such as the liver of the premetamorphic tadpole, with its relatively low level of CP-synthetase-I, was resolved only after the demonstration of the existence of a glutamine-dependent carbamyl phosphate synthetase (CP-synthetase-II; see reaction 6, Fig. 2) in animal tissues (20). CP-Synthetase-II is present in relatively high concentrations in frog eggs (19).

Unpublished studies (S. J. Lan, H. J. Sallach and P. P. Cohen) indicate that the CP-synthetase-II is also present in premetamorphic and metamorphosing tadpole and frog liver but at a relatively low concentration compared to CP-synthetase-I. CP-Synthetase-II, but not CP-synthetase-I, is present in spleen, kidney and mucosa of stomach and intestine of frog.

Current concepts of differentiation and regulation would suggest that the molecular information needed for the differentiating and regulatory processes would involve at some point transcription of DNA via messenger RNA which in turn would be translated on the ribosomes and thus determine the kind and possibly the amount of enzyme synthesized.

Changes of DNA and total RNA in tadpole liver particularly in relation to thyroxine-induced metamorphosis have been studied (21). We have reported that the amounts of liver DNA-P and RNA-P remain essentially unchanged in thyroxine-treated tadpoles even though the synthesis of CP-synthetase-I increased about 15-fold. However, a significant decrease in rate of incorporation of adenine-8-¹⁴C into RNA, and a decrease in the amount of ATP were observed during thyroxine treatment. The latter effects coincided with the beginning increase in CP-synthetase-I (21).

Early effects of triiodothyronine injection on nucleotide and RNA metabolism in tadpole liver have been reported by Eaton and Frieden (22).

The rate of total RNA synthesis was determined after administration of orotic- ^{14}C -acid at various time intervals after thyroxine treatment (17). The rate of RNA synthesis showed an initial decrease within 24 hours followed by a pronounced increase during the next 48 hours and then a gradual increase in the period from 6 to 15 days (Fig. 6). The turnover of RNA in subcellular fractions of tadpole liver during thyroxine treatment is shown in Fig. 7. The high specific activity of the soluble RNA fraction was maintained during the period of induced metamorphosis. The most striking increase of RNA turnover was seen in the microsomal fraction which had the lowest initial value in the untreated tadpoles. These findings suggest that new ribosomal RNA and s-RNA synthesis occurs as an early response to thyroxine preceding the induction of CP-synthetase-I.

The sedimentation patterns of total RNA from livers of premetamorphic tadpoles before and after exposure to thyroxine were investigated (23). The pattern in the case of premetamorphic tadpoles, pulse-labeled for 2 hours, is shown in Fig. 8a. The major portion of pulse-labeled RNA sediments with a value of 4S. A gradual increase in the labeling of ribosomal RNA is observed with time. However, when similar pulse labeling experiments were carried out with animals treated with thyroxine for 2 days the label appeared in heavier (6-10S) RNA fractions (Fig. 8b). The peak of

specific radioactivity remained constantly in the area between the 6S and 10S fractions over a period of 24 hours after administration of radioactive precursors. Base composition analysis showed that this fraction had a high concentration of UMP in contrast to that of bulk RNA. The ratio of CMP + GMP/total ribonucleotides in this RNA fraction gave values of 45% and 50% in contrast to a value of 65.3% for bulk RNA. These data indicate that the base ratio of the rapidly labeled RNA fraction is similar to that of DNA (DNA-like RNA or D-RNA). DNA prepared from liver of Rana Catesbeiana tadpoles was found to have a value of 46% (23).

These studies of RNA turnover suggest that thyroxine treatment stimulates the synthesis of three major types of RNA, namely s-RNA, t-RNA and D-RNA, prior to the induction of CP-synthetase-I and gross metamorphosis. While the reason(s) for new synthesis of all three types of RNA for the enzyme induction is not clear, the inhibition of both thyroxine-induced RNA synthesis and CP-synthetase I synthesis by actinomycin D suggests that DNA-dependent RNA synthesis is required for the synthesis of CP-synthetase-I (24).

Tata (25) has reported on the formation, distribution and function of ribosomes and microsomal membranes, and RNA fractions during induced amphibian metamorphosis.

4. RNA Polymerase Activity

Since RNA synthesis preceded the induction of the enzymes involved in urea biosynthesis as well as gross metamorphosis, it seemed important to study the effect of thyroxine on RNA synthesis.

The effect of thyroxine treatment of tadpoles on RNA polymerase activity of liver nuclei was investigated (26). As can be seen from Fig. 9, the RNA polymerase activity varied with duration of thyroxine treatment in a manner similar to that of RNA synthesis (see Fig. 6), showing an early peak after 2 days of thyroxine treatment. Since enzyme preparations made from chromatin give similar results, it appears that the observed increase in RNA polymerase activity in vitro is not due to changes in the nuclear membrane as a result of thyroxine treatment.

When purified preparations of chromatin from tadpole liver nuclei were used as the template for RNA synthesis in the presence of excess E. coli RNA polymerase, the chromatin prepared from liver nuclei of thyroxine-treated animals had a template efficiency 20-50% higher than that of chromatin prepared from untreated animals (Fig. 10) (27). Intrinsic RNA polymerase and RNase activity in both types of chromatin preparations were negligible and no significant difference between them was observed. When both types of chromatin were deproteinized, by the use of CsCl, the isolated DNA's

showed equal template efficiency. Analysis of purified chromatin from control and thyroxine-treated animals revealed no gross changes in chemical composition. Addition of thyroxine to chromatin or nuclear preparations in vitro had no effect. It is thus clear that thyroxine treatment modifies chromatin in vivo in some way to make it a more efficient template for RNA synthesis and that the protein moiety affects template efficiency.

Further evidence that the RNA polymerase activity in nuclei or aggregate enzyme preparations is regulated more by the template efficiency of DNA bound to the preparations than by the catalytic capability of associated proteins comes from studies involving ammonium sulfate. The addition of ammonium sulfate to the RNA polymerase reaction mixtures stimulated RNA synthesis about 3-4 fold (26) (see Fig. 9). The enzyme activity is approximately linear, with respect to the salt concentration, up to 0.4 M, and further increases in salt concentration result in an inhibition. One of the effects of ammonium sulfate addition to isolated nuclei or chromatin preparations is a partial release of histones from the nucleohistone complex into the supernatant. Maximum release of histone is achieved at a concentration of 0.4 M ammonium sulfate (a further increase in the salt concentration has little effect) which is the salt concentration at which maximum stimulatory effect on RNA synthesis was observed. Examination of

the chemical composition of nuclei before and after ammonium sulfate addition showed a noticeable change only in the histone fraction (26). Since isolated RNA polymerase from mammals and microorganisms are inhibited by ammonium sulfate at the concentration used in the present studies, it is reasonable to assume that the effect of ammonium sulfate is on the template, and that RNA polymerase activity is regulated primarily by the template efficiency. Solubilization of tadpole liver RNA polymerase will be necessary for further progress with this aspect of the problem.

5. Stability of Messenger RNA

If actinomycin D is injected into premetamorphic tadpoles before or at the same time as thyroxine, induction of CP-synthetase-I is inhibited (24). If actinomycin is administered 3, 6 or 12 hours after thyroxine injection, there is a decreasing inhibition of induction of synthesis of CP-synthetase-I (24). However, if actinomycin is administered after induction of synthesis of CP-synthetase is fully developed no inhibition of enzyme synthesis is observed (17,24) although RNA synthesis is inhibited (17). The failure of actinomycin to inhibit the induction of synthesis of CP-synthetase-I following thyroxine treatment can be interpreted as follows: a) messenger RNA for CP-synthetase-I is relatively stable, and is formed early in response to thyroxine treatment and once formed no additional new messenger RNA is required; or b) the increase in activity of CP-synthetase-I is the result of conversion of existing enzyme precursor(s) into functional enzyme molecules.

Puromycin, an inhibitor of protein synthesis, has been shown to have no effect on the conversion of CP-synthetase-I precursor molecules into functional enzyme in liver slices (16). However, puromycin injection into tadpoles previously treated with thyroxine inhibited the further increase in CP-synthetase-I activity which occurs in the presence of actinomycin (24). This observation suggests that the increase in enzyme activity in the presence of actinomycin is the result of de novo protein synthesis and not the result of conversion of precursor molecules into functional CP-synthetase-I. On this basis one can assume the persistence of a stable messenger RNA which dictates continued synthesis of CP-synthetase-I.

It has been previously pointed out (see Fig. 5) that continued exposure of tadpoles to thyroxine resulted in a more rapid induction of CP-synthetase-I synthesis than occurs in the case of tadpoles placed in water after previous exposure to thyroxine for 2-4 days (17). If actinomycin D is injected into tadpoles after an initial exposure to thyroxine, a further decrease in synthesis of CP-synthetase-I is observed (24). These observations suggest that thyroxine is in fact required beyond the initial "priming" stage which is assumed to represent the period of synthesis of messenger RNA.

Evidence for an effect of thyroxine at the translational level has been reported by Unsworth and Cohen (28). Microsomal and ribosomal preparations from liver of thyroxine-treated tadpoles showed a rate of incorporation from aminoacyl-t-RNA of the order of 100% greater than similar preparations from untreated tadpoles.

(Fig. 11). Current studies (J. L. Funderburgh and P. P. Cohen, unpublished) with an improved assay system confirm the greater activity of ribosomal preparations from liver of tadpoles treated with thyroxine. The ribosomal preparations from both untreated and thyroxine-treated tadpoles appeared to be identical when examined in the ultracentrifuge and revealed a major peak at 77S with a minor disome peak at 116S. The total ribosomal RNA per gram of liver was found to increase with continued exposure of the tadpoles to thyroxine with a plateau being reached after approximately 7 days at a level of about 1.5 times that found at zero time.

It would thus appear that the effect of thyroxine on induction of CP-synthetase-I involves transcriptional as well as translational events.

C. Cytochemical and Cytological Changes in Liver during Metamorphosis

1) Immunofluorescent Localization of CP-synthetase-I in Liver Sections of Tadpoles and Frog.

With the availability of a highly specific antibody to CP-synthetase-I (14), and the evidence for increasing levels of CP-synthetase-I activity in liver of tadpoles undergoing natural (9) or thyroxine-induced (11) metamorphosis, it seemed of interest to determine whether the increasing levels of CP-synthetase-I would be manifested by use of a fluorescent antibody. The results of such a study (J. W. Campbell, J. W. Anderson and P. P. Cohen, unpublished) are shown in Fig. 12.

As can be readily seen, there is a progressive increase in amount of fluorescent antibody fixed by liver sections from tadpoles exposed to thyroxine (2.6×10^{-8} M) as compared with untreated, premetamorphic animals. The relative CP-synthetase-I activity correlates very well with the amount of fluorescent antibody fixed. In the case of the premetamorphic tadpole, only a few cells show fluorescence and the intensity of fluorescence is very low. In contrast, in the case of the adult frog, all cells show a very intense fluorescence. At higher magnification, the adult frog liver sections show a particulate character in the distribution of the antibody. This can be correlated with the distribution of the mitochondria which is the intracellular site of localization of CP-synthetase-I (13). During thyroxine-induced metamorphosis, the number of cells which fix the antibody and the amount of antibody fixed is progressively increased. These observations correlate very well with previously reported synthesis de novo of CP-synthetase-I in liver of tadpoles exposed to thyroxine (15) and the finding that CP-synthetase-I may represent as much as 20 per cent of the soluble mitochondrial proteins of frog liver (13).

D. Electronmicrography

While no unique changes are apparent in stained sections of liver during tadpole metamorphosis as seen with the light microscope (5), striking cytologic changes can be observed

with the electronmicroscope.

A systematic study of the ultrastructural changes in liver from tadpoles undergoing metamorphosis, both natural and thyroxine-induced, as well as of premetamorphic and adult frog liver, has been carried out in this laboratory (P. P. Cohen, unpublished). Tata (25,29) has reported electron micrographs of tadpole liver sections from premetamorphic and thyroxine-treated (following injection of triiodothyronine) metamorphic animals.

As can be seen from Fig. 13 liver from tadpoles exposed to thyroxine show an increased development of the endoplasmic reticulum from single random profiles into parallel stacks. During early stages, a close association is observed between a single ER profile and an individual mitochondrion (Fig. 13B). The mean diameter of the cross sections of the mitochondria is significantly increased during the early period of exposure to thyroxine ($1.01 \pm 0.24 \mu$ for mitochondria from premetamorphic animals as compared with a value of 1.70 ± 0.59 for mitochondria from tadpoles exposed to thyroxine for 7 days). Similar, though less striking, changes occur during natural metamorphosis. The mitochondria of the adult frog liver show a very dense matrix as compared with mitochondria from premetamorphic and metamorphic tadpoles and the endoplasmic reticulum profiles appear to have a more random arrangement.

A striking observation is that of an apparent direct association between the endoplasmic reticulum and individual mitochondria (Fig. 13B). The frequency of this finding suggests that a direct pathway exists for transport of precursor(s) of mitochondrial enzymes (probably at the subunit level) from ribosomes on the endoplasmic reticulum to mitochondria, simulating in a sense the relation of the endoplasmic reticulum to that of the golgi apparatus involved in storage and secretion of protein. Continuities between mitochondria and endoplasmic reticulum in the mammalian ovary have been recently reported (30).

The swelling of the mitochondria during early exposure to thyroxine and the marked increase in the density of the mitochondrial matrix seen in the adult frog liver suggest that these events are related in part to osmotic effects associated with the early rapid and relatively specific synthesis of CP-synthetase-I as a response to thyroxine. As has been previously noted, CP-synthetase-I may reach a level of 20 per cent of the soluble protein of frog mitochondria (13). Evidence for the synthesis of precursor(s) of CP-synthetase-I extramitochondrially has been previously reported (16). This enzyme has a molecular weight of 315,000 (13) and thus is not likely to be readily transported across membranes. Current studies (J. R. Strahler and P. P. Cohen, unpublished) of the subunit structure of this

enzyme indicate that there may be 4 identical subunits each made up of 2 different chains. It is also known from work on tadpole liver slices and cubes (see later discussion) that conversion of precursor(s), probably at the subunit level, occurs readily in mitochondria to form functional and immunoprecipitable enzyme (16). Thus the initial swelling of mitochondria from tadpoles exposed to thyroxine could be the result of a rapid synthesis and transport of extra-mitochondrial subunits into the mitochondria. The later decrease in swelling and increase in matrix density (which occur even though exposure to thyroxine is continued) seen in the later stages of metamorphosis and in the adult frog probably reflect the conversion of relatively low molecular weight precursor(s) (estimated to be of the order of 80,000 or possibly smaller subunits of 30,000 and 50,000) to CP-synthetase-I of molecular weight 315,000 and a concentration of up to 20 per cent of the soluble protein.

Of considerable interest is the recent observation (G. E. Shambaugh, G. C. Faith and P. P. Cohen, unpublished) that all of the above electronmicroscopic changes observed in liver sections from tadpoles exposed to thyroxine can be reproduced in vitro by the addition of thyroxine (2.6×10^{-7} M) to cubed liver preparations from premetamorphic tadpoles. The changes were observed only in the systems to which thyroxine was added and occur within 48 hours after the in vitro addition of

thyroxine. Since this system has been shown to be capable of synthesizing CP-synthetase-I under the influence of thyroxine (see below), thyroxine must have as a primary and direct effect that of altering the subcellular structures involved in protein synthesis in addition to its effect on RNA synthesis.

II. Studies with Surviving Liver Preparations

Because of the multiplicity and complexity of the responses of the premetamorphic tadpole exposed to thyroxine at the morphologic as well as biochemical levels, it seemed important to find a system which would permit a more definitive assessment of the role of thyroxine. To determine whether thyroxine per se is responsible for the changes observed in liver cells undergoing biochemical differentiation studies have been carried out with two types of liver preparations, A) surviving suspensions of isolated liver cells and B) surviving cubed liver preparations.

A. Studies with Suspensions of Isolated Liver Cells

A technique has recently been developed in this laboratory for the preparation of isolated cells from tadpole liver which are capable of surviving in an appropriate medium for at least 7 days without cell division or significant loss of cells (31). In vitro addition of thyroxine (10^{-7} M) results in RNA synthesis with a pattern typical of that observed in the liver from intact animals exposed to thyroxine, including a fraction of RNA in the region between 4 and 10S with a base composition similar to DNA of this species. Triiodothyronine had a similar effect at the same

concentration as thyroxine suggesting that at the cellular level in this system these congeners behave identically. While the cell suspensions responded to thyroxine with a stimulation of RNA synthesis, including a fraction with a ratio of C + G/total bases typical of DNA, no effect of thyroxine was observed on incorporation of amino acids into protein or specific enzymes. Examination of the cell suspensions with the electron microscope revealed that the cytoplasmic organelles (particularly mitochondria) had undergone degeneration which may account for the failure to observe synthesis of protein and in particular the mitochondrial enzyme CP-synthetase-I.

B. Suspensions of Cubed Liver Preparations

1) Carbaryl Phosphate Synthetase

Our lack of success in exploiting the isolated liver cell suspensions for information on mitochondrial enzyme synthesis under the influence of thyroxine led us to examine the technique developed by Wicks (32) for study of enzyme synthesis in fetal rat liver suspensions, for use with tadpole liver (33).

The surviving cubed liver preparations from tadpoles have yielded some basic information on the role of thyroxine and other regulatory factors involved in the synthesis and maintenance of the enzyme CP-synthetase-I (and glutamate dehydrogenase). Cubed liver preparations are capable of surviving in an appropriate medium and under appropriate

conditions for 48 hours with cytologic integrity and biosynthetic capacity.

These preparations have been shown (G. E. Shambaugh, III, and P. P. Cohen, unpublished) to maintain an essentially constant O_2 -uptake for 48 hours. Further, the capacity to synthesize urea from ^{14}C -bicarbonate and ammonia remains intact and the rate of ^{14}C -urea biosynthesis correlates directly with the level of CP-synthetase-I activity in these preparations. Thus the complex of enzymes representing the ornithine-urea cycle (see Fig. 2) remains functional in this system.

An enhancement of CP-synthetase-I activity was seen in cubed liver preparations from premetamorphic tadpoles after incubation in vitro with L-thyroxine (2.6×10^{-8} or 2.6×10^{-9} M). Other additives, including a number of hormones, cyclic 3', 5' AMP, tadpole serum and frog serum had no effect when added in vitro. Thyroxine added in vitro stimulated the rate of CP-synthetase-I synthesis in liver preparations from premetamorphic and metamorphosing tadpoles and the frog. CP-Synthetase-I synthesis in liver cubes was specifically enhanced relative to the synthesis of the other soluble mitochondrial proteins at thyroxine concentrations of 2.6×10^{-8} M and 2.6×10^{-9} M and in liver cubes from both thiouracil-pretreated tadpoles and from tadpoles pretreated by immersion in thyroxine 2.6×10^{-7} M (Fig. 14). Actinomycin D and

puromycin added in vitro at zero time inhibited de novo synthesis of CP-synthetase-I. When liver cubes prepared from tadpoles injected with leucine-³H in vivo were incubated in cold leucine in vitro, an increase in labeled immunoprecipitable CP-synthetase-I was observed in the presence of actinomycin or puromycin, suggesting an effect on the conversion of nonimmunoprecipitable precursor(s) to labeled immunoprecipitable enzyme. The mechanism underlying the observed increase in conversion of precursor(s) was shown to be due to an inhibition of degradation of CP-synthetase-I by puromycin and actinomycin. Thyroxine added in vitro resulted in an increase in precursor conversion. The small contribution of de novo synthesis in vitro to the total pool of CP-synthetase-I suggested that enhancement of CP-synthetase-I levels in vitro was related largely to conversion of precursor(s) to immunoprecipitable enzyme. This concept is consistent with the observation of an increase in total enzyme levels when liver cubes were incubated in the presence of actinomycin or puromycin. The regulation of CP-synthetase-I levels in the tadpole and frog appears to be determined by the relative rates of precursor synthesis, precursor conversion to enzyme, and enzyme and/or precursor breakdown. (The half-disappearance time of newly synthesized CP-synthetase-I was determined to be of the order of 54 hours in vivo

and 70 hours in vitro in the liver cube system.) Thyroxine stimulates synthesis of precursor(s) and possibly conversion to active enzyme, as well as degradation. The lag phase observed during induction of CP-synthetase-I synthesis and activity in tadpoles exposed to thyroxine in vivo was not observed in the liver cube preparations, suggesting the existence of a repressor mechanism in vivo that was not operative in vitro. The nature of the repressor(s) remains unknown.

2. RNA Changes in Liver Cube Preparations

Studies still in progress (S. J. Lan and P. P. Cohen, unpublished) have shown that thyroxine added in vitro to preparations of liver cubes from premetamorphic tadpoles resulted in a marked enhancement of RNA synthesis as compared with controls to which no thyroxine has been added.

3. Glutamate Dehydrogenase Synthesis in Liver Cube Preparations

Earlier studies from this laboratory reported that glutamate dehydrogenase, partially purified from premetamorphic tadpole liver, had different physical, kinetic and substrate specificity properties (34) than the crystalline enzyme prepared from frog liver (35). Crystalline glutamate dehydrogenase has recently been prepared from liver of premetamorphic tadpoles (36) and compared with the crystalline enzyme from frog liver. The enzymes differ in important respects kinetically and with respect to substrate specificity.

While apparent differences in molecular weights can be demonstrated, it has not been possible as yet (because of the limited amount of tadpole enzyme available) to be certain as to the exact molecular weight of tadpole glutamate dehydrogenase.

The availability of a viable system of cubed liver preparations has led us to investigate the factors involved in biosynthesis and regulation of glutamate dehydrogenase in tadpole and frog liver (37).

A small rise in enzyme activity was observed during incubation in vitro of liver cubes from premetamorphic tadpoles. In the presence of thyroxine (2.6×10^{-8} M to 2.6×10^{-10} M) or triiodothyropropionate (2.6×10^{-9} M to 2.6×10^{-11} M), the mean increase in the specific activity in vitro was twice that of the control over a 48 hour incubation period. Thyroxine stimulated de novo synthesis of immunoprecipitable glutamate dehydrogenase as well as soluble mitochondrial proteins. A specific stimulation of glutamate dehydrogenase synthesis relative to soluble mitochondrial protein synthesis in vitro was observed at a thyroxine concentration of 2.6×10^{-8} M. Higher concentrations of thyroxine resulted in a specific effect only in liver cubes prepared from tadpoles previously treated with thiourea.

De novo synthesis of glutamate dehydrogenase was inhibited by puromycin and actinomycin but the total enzyme activity in vitro was increased in the presence of these antibiotics. A continuous conversion of labeled nonimmunoprecipitable precursor(s) into immunoprecipitable enzyme, observed in vitro in the absence of any additives to the incubation system, was stimulated in the presence of thyroxine, and was increased in the presence of actinomycin and puromycin as well. The half-disappearance time of labeled immunoprecipitable glutamate dehydrogenase was 21.2 ± 1.7 hours in vitro, and 22.8 hours in vivo. Actinomycin and puromycin were found to inhibit breakdown of immunoprecipitable glutamate dehydrogenase. The observations of de novo synthesis, precursor conversion and a rapid turnover of glutamate dehydrogenase in vitro in liver cubes from premetamorphic and metamorphosing tadpoles as well as in the adult frog indicate that these factors determine the basic mechanism of regulation of this enzyme in this animal.

Discussion and Summary

In this review, attention has been focused on the enzyme CP-synthetase-I as a "marker" in the series of events which occur in liver of the tadpole during metamorphosis under the influence of thyroxine. While it is recognized that apparently related events may in fact be independent events and that apparently independent events may be related events, the documentation provided in this review supports the validity of the use of CP-synthetase-I

as a "marker". The fact that no significant cell division occurs in the liver of the intact tadpole exposed to thyroxine, or in the in vitro preparations in which comparable effects are observed when thyroxine is added to the in vitro system, gives assurance that the changes being observed represent differentiation in a fixed population of cells. This fact alone would make it highly probable that the changes observed in the induction of enzyme synthesis under the influence of thyroxine and the changes observed in transcriptional and translational events are related and not independent. While many other changes are occurring in the liver involving other enzymes, plasma protein synthesis, etc. (see (4)), the choice of CP-synthetase-I as a "marker" is based on the following observations: 1) induction of synthesis precedes changes in gross morphology; 2) CP-synthetase-I may reach a concentration representing 20% of the soluble protein of mitochondria; and 3) in the intact animal induction of CP-synthetase-I has a shorter lag period than induction of synthesis of adult hemoglobin and plasma albumin. As a matter of fact shorter lag periods are seen only in the case of the enzymes involved in initiation of tail resorption (25,29). The ability to reproduce many of the effects relating to CP-synthetase-I observed in liver of the intact tadpole exposed to thyroxine in surviving liver preparations by the direct addition of thyroxine in vitro lends support to the validity of this approach. An interesting opportunity is now at hand, with the availability of a surviving cubed liver preparation, to put to test the thesis that the response of the enzymes of the ornithine-urea cycle to thyroxine is a concerted one. Reference has been made to unpublished studies which show that the

biosynthesis of urea in the surviving cubed liver system increases in relation to the level of CP-synthetase-I. This enzyme has been shown to be synthesized de novo under the influence of thyroxine in vivo (15) and in vitro (33). A useful antibody has recently been produced which will permit the simultaneous study of the effect of thyroxine on the induction of ornithine transcarbamylase (unpublished studies, I. K. Reddy, G. E. Shambaugh, III, M. Marshall and P. P. Cohen). Thus it will be possible to determine whether the initial two mitochondrial enzymes concerned with urea biosynthesis (see reactions 1 and 2, Fig. 2) respond in concert or independently in their synthesis. An in vitro effect of thyroxine on ornithine transcarbamylase activity in tadpole liver organ cultures has recently been reported (38).

Crucial experiments to test the validity of the approach under discussion will emerge from studies in progress dealing with, 1) isolation of a specific messenger RNA, 2) determination of the nature of the polypeptides synthesized by ribosomes from liver of animals exposed to thyroxine (or from the cubed liver preparation) and 3) the structural relation of the polypeptides synthesized by ribosomes to the subunits of purified CP-synthetase-I.

In addition to the promise these studies offer for understanding biochemical differentiation, the system provides a unique opportunity for study of an animal system which can provide information on 1) how a soluble mitochondrial enzyme is synthesized at the subunit level extramitochondrially and the transport of these subunits to the mitochondria for enzyme assembly; 2) how the hormone thyroxine exerts its effect(s) at the molecular level; 3) what kind of regulation exists with respect to a functioning biosynthetic pathway involving a series of 5 enzymes and

4) the nature of the regulators (repressors, derepressors, etc.) which operate at the transcriptional and translational levels.

A further comment about glutamate dehydrogenase is in order. The crystalline enzymes isolated from frog liver (35) and premetamorphic tadpole liver (36) have different kinetic and substrate specificity properties as well as molecular weights. The synthesis of what appears to be a new enzyme (it is not yet certain whether the frog and tadpole enzymes differ in their basic subunits or whether they are made up of the same subunits but in different proportions) during metamorphosis of the tadpole to the frog with each enzyme serving what appears to be the same metabolic role, indicates that biochemical differentiation from the stage of the fertilized ovum to that of the tadpole involves a different or modified genetic expression than that which occurs in the biochemical differentiation of the tadpole to the adult frog. A similar situation has been reported for the hemoglobins of the tadpole and frog (39).

The apparent discontinuity in genetic expression during development in the case of glutamate dehydrogenase and the hemoglobins indicates the need for studies in earlier stages of embryogenesis, i.e. from the fertilized ovum to the early tadpole stage. With the availability of crystalline glutamate dehydrogenase preparations from tadpole and frog liver, and antibodies for CP-synthetase-I and ornithine transcarbamylase, experiments have been initiated to gain information on the induction of these enzymes at earlier stages of embryogenesis.

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8. Urea biosynthesis refers to the formation of urea from carbon dioxide and ammonia. The formation of urea from arginine by the action of arginase is only one step in the biosynthetic pathway and does not in itself represent biosynthesis of urea. In the same sense, ureotelism is used in this paper to refer to an animal capable of

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TABLE I
Relative Activities of Enzymes in Liver of Premetamorphic,
Metamorphic, and Adult R. catesbeiana (See Ref. 2)

Enzymes	Metamorphic: premetamorphic		Adult: premeta- morphic ^c
	Thyroxine ^a	Natural ^b	
Urea biosynthesis			
Carbamyl phosphate synthetase	14	15	30
Ornithine transcarbamylase	2	2.5	8
Argininosuccinate synthetase	—	15	35
Argininosuccinase	—	—	20
Arginase	—	3	30
Dehydrogenases			
Glutamate	6	6	10
Lactate	0.6	0.6	0.4
Glucose-6-PO ₄	0.8	0.8	0.5
Malate	—	1.2	1.4
Amino acid activation			
20-Amino acids	1	—	2
Transaminases			
Glutamate-oxalacetate	2	3	5
Glutamate-pyruvate	1	1	0.5
Tyrosine- α -ketoglutarate	0.5	0.2	0.2
Ornithine- α -ketoglutarate	0.6	0.7	0.5
Nucleic acid metabolism			
Uridine kinase	1	1	1
Uridine phosphorylase	2.2	2.3	0.2
Phosphatase			
Pyrophosphatase	1.2	—	2.2

^aRatio of enzyme activities of metamorphic to premetamorphic stages in thyroxine-induced metamorphosis.

^bRatio of enzyme activities of metamorphic to premetamorphic stages in natural metamorphosis.

^cRatio of enzyme activities of adult frog to premetamorphic tadpoles.

Fig. 1. Stages of development of Rana catesbeiana tadpoles. Top to bottom, left to right: Stages X, XVIII, XX, XX 1/2, XXIII, XXIV. (See Ref. 2).

General designation	Stage number	Synopsis of characteristics
Limb bud stages	I-V	Length of bud increases from slight elevation to 2 X diameter
Paddle stages	VI-X	Flattening of limb bud to complete indentation between toes; 5 th toe web directed to 3 rd toe
Foot stages (premetamorphic stages)	XI-XVII	5 th toe web reaches prehalix; nasolachrymal duct appears; proximal, middle, and distal toe pads appear
Metamorphic stages	XVIII-XXV	Cloacal tail-piece disappears; front legs appear; larval mouth still present; labial fringes complete, angle of mouth tends toward posterior margin of eyeball, rapid decrease of tail length; tympanic cartilage ring perceptible; tail absent

Fig. 2. Scheme of reactions generating and utilizing carbamyl phosphate.

Enzymatic reactions are 1) carbamyl phosphate synthetase-I (AG represents α -acetylglutamate); 2) ornithine transcarbamylase; 3) argininosuccinate synthetase; 4) argininosuccinase; 5) arginase [reactions 1) through 5) constitute the ornithine-urea cycle]; 6) carbamyl phosphate synthetase-II; 7) aspartate transcarbamylase; and 8) carbamate kinase.

The 2 compartments of carbamyl phosphate shown are intended to represent the situation in liver of ureotelic animals. The lower compartment is the pool of carbamyl phosphate synthesized by the mitochondrial enzyme CP-synthetase-I. Carbamyl phosphate from this pool is utilized for synthesis of citrulline by means of the mitochondrial enzyme, ornithine transcarbamylase. The upper compartment represents the pool of carbamyl phosphate generated by the enzyme CP-synthetase II which is extramitochondrial in the liver of all animals. Carbamyl phosphate from this pool is utilized for the synthesis of carbamyl aspartate, a precursor of pyrimidines. The biosynthesis of arginine (and urea) from citrulline occurs extramitochondrially in liver of ureotelic animals. It should be noted that the enzyme carbamate kinase catalyzes a reversible reaction (in contrast to CP-synthetases I and II) and serves primarily as a pathway for generation of ATP from arginine and citrulline in certain microorganisms rather than for synthesis of carbamyl phosphate.

Fig. 3. Urea excretion and development of enzymes of ornithine-urea cycle in the metamorphosing tadpole (see Ref. 2). Arginine synthetase represents the overall synthesis of arginine from citrulline and aspartate and involves the 2 enzymes argininosuccinate synthetase (see Reaction 3, Fig. 2) and argininosuccinase (see Reaction 4, Fig. 2).

Fig. 4. Carbamyl phosphate synthetase activity as related to metamorphosis of Rana catesbeiana. Plot of specific activity of CP-synthetase-I vs. the ratio of hind leg length and tail length during normal and thyroxine-induced metamorphosis (see Ref. 2).

Fig. 5. Effect of time of exposure to thyroxine on induction of carbamyl phosphate synthetase. In the experiments represented on left, tadpoles were kept in thyroxine (T_4) solution (2.6×10^{-8} M) for 2 days at $22^\circ \pm 1^\circ$. They were then washed thoroughly and placed in water for the subsequent 7 days. On the 9th day following initial treatment with thyroxine, a group of tadpoles was again exposed to thyroxine (Curve B), while another group was kept in water (Curve A). Curve C shows the induction curve of carbamyl phosphate synthetase activity in a group of tadpoles continuously exposed to thyroxine. In the experiments represented on right, tadpoles were maintained in thyroxine solution (2.6×10^{-8} M) for 4 days at $22^\circ \pm 1^\circ$ washed, and then kept in water for a period of 6 days. On the 10th day following the initial treatment with thyroxine, one group was again exposed to thyroxine (Curve B), while a second group was maintained in water (Curve A). In both cases, livers from two tadpoles were pooled and assayed for carbamyl phosphate synthetase activity.

Fig. 6. Rate of RNA synthesis during metamorphosis induced by thyroxine. Tadpoles were treated with thyroxine (2.6×10^{-8} M) at 22° and, at the times indicated, received orotic acid-6- ^{14}C intraperitoneally ($1 \mu\text{C}$ per g body weight). The specific activity is represented as counts per min per μmole of UMP. *Vertical broken lines* represent the standard deviation, and the *numbers in parentheses* indicate the number of groups (three tadpoles in each group) used for each experiment. For experimental details, see Ref. (17).

Fig. 7. Specific radioactivity of RNA from various cell fractions of tadpole liver during metamorphosis induced by thyroxine. Tadpoles were exposed to thyroxine (2.6×10^{-8} M) and received orotic acid-6- ^{14}C intraperitoneally (1 μC per g of body weight). The tadpoles were killed 2 hours after administration of the orotic acid. For experimental details, see Ref. (17).

Fig. 8. Pattern of RNA synthesis in liver from untreated tadpoles, a, and from tadpoles exposed to thyroxine (2.6×10^{-8} M) for 2 days, b. Tadpoles received orotic acid-6- ^{14}C intraperitoneally per g of body weight. Zonal centrifugation patterns of RNA are shown 2 hours after administration of radioactive orotic acid. For experimental details, see Ref. (23).

Fig. 9. Effect of thyroxine treatment of tadpoles on RNA polymerase of liver nuclei. RNA polymerase from thyroxine-treated animals was assayed in the presence (Curve A) and absence (Curve A') of ammonium sulfate. The enzyme activity of control animals (Curves B and B') was assayed in the same manner. For experimental details, see Ref. (26).

Fig. 10. RNA synthesis in the presence of chromatin and DNA from thyroxine-treated and control tadpole liver. Curve A represents RNA synthesis primed with deproteinized DNA. Curve B, Δ — Δ values with chromatin from thyroxine-treated tadpole liver, Curve C, $\bullet$ --- $\bullet$ values with control chromatin (no thyroxine). For experimental details, see Ref. (27).

Figure 11. Time course of incorporation of amino acids from [^{14}C]aminoacyl-tRNA into ribosomes prepared from the livers of both thyroxine-treated and control tadpoles. Tadpoles were kept at 25° for 20 days, then placed in 2.6×10^{-8} M thyroxine solution. Tadpoles were killed after 6 days and ribosomes prepared. For experimental details, see Ref. (28).

Fig. 12. Fixation of antibody (made fluorescent with fluorescein isothiocyanate) by CP-synthetase-I in liver sections from tadpoles and frog. A) liver from premetamorphic tadpole; CP-synthetase-I assay represented as 1; magnification, X 92; B) liver from tadpole exposed to thyroxine (2.6×10^{-8} M) for 3 days; relative CP-synthetase-I activity, 1.5; magnification X 92; C) liver from tadpole exposed to thyroxine for 6 days; relative CP-synthetase-I activity, 8; magnification X 92; D) liver from adult frog; relative CP-synthetase activity, 12; magnification factor X 120; E) same as D, magnification factor X 850.

Fig. 13. Electronmicrographs of sections of liver from A) premetamorphic tadpole; B) tadpole exposed to thyroxine (2.6×10^{-8} M) for 7 days; C) tadpole exposed to thyroxine for 10 days; D) adult frog; E) tadpole during natural metamorphosis at stage XVI; F) tadpole during natural metamorphosis at stage XIX. Magnification is $\times 12,600$ except in case of E) which is $17,600$. b, bile canaliculus; er, endoplasmic reticulum; g, Golgi apparatus; m, mitochondrion; n, nucleus; v, vacuole. Arrow in B points to mitochondrion with an er connection as discussed in text.

Fig. 14. Effect of thyroxine on synthesis of CP-synthetase-I in liver cube preparations. Left side of figure shows effect of prior treatment of tadpoles (2.6×10^{-8} M for 48 hours at 24°). The control group was held at 24° for 48 hours in water. Right side of figure shows effect of prior treatment of tadpoles with thiouracil (3.5×10^{-3} M) for 72 hours.

Cubed liver preparations were then made and one portion was incubated in the absence of thyroxine (control) and the other in the presence of thyroxine (2.6×10^{-8} M). The cubed liver preparations were incubated in the presence of leucine- ^3H . After 24 and 48 hours incubation, mitochondrial extracts were prepared and treated with antibody to CP-synthetase-I. The ordinate represents specific radioactivity of immunoprecipitable CP-synthetase-I (CPM) per mg of soluble mitochondrial protein. For details, see Ref. 33.