process or in terms of the column packing material. Yet a term like "liquid chromatography" is not sufficiently specific. A committee of the American Society for Testing and Materials (ASTM D-20.70.04) and others are working on this problem, and hopefully a solution will be obtained.
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Biochemical Differentiation during Amphibian Metamorphosis

Thyroxine affects liver cytology, transcription, translation, and mitochondrial enzyme level.

Philip P. Cohen

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*). Several aspects of metamorphosis in vertebrates and invertebrates have been reviewed 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 a considerable study of biochemical changes involved in differentiation and development as aspects of metamorphosis (*2–4*).

This review is confined mainly to results from the author's laboratory, particularly 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*).

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 after onset of metamorphosis (*6*). With the development of suitable assay procedures (*7*) for the different enzymes involved in urea biosynthesis (Fig. 2) from bicarbonate and ammonia (*8*), it became possible to determine the activities of these enzymes at different stages of natural

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Table 1. Relative activities of enzymes in liver of premetamorphic, metamorphic, and adult *Rana catesbeiana*.

Enzymes involved in	E.C. No.	Ratio of enzyme activities		
		Metamorphic: premetamorphic		Adult: premetamorphic tadpole
		Thyroxine	Natural	
Urea biosynthesis				
Carbamyl phosphate synthetase-I		14	15	30
Ornithine transcarbamylase	(2.1.3.3)	2	2.5	8
Argininosuccinate synthetase	(6.3.4.5)		15	35
Argininosuccinase	(4.3.2.1)			20
Arginase	(3.5.3.1)		3	30
Dehydrogenation				
Glutamate dehydrogenase	(1.4.1.2)	6	6	10
Lactate dehydrogenase	(1.1.1.27)	0.6	0.6	0.4
Glucose-6-phosphate	(1.1.1.49)	0.8	0.8	0.5
Malate dehydrogenase	(1.1.1.37)		1.2	1.4
Amino acid activation				
20 amino acid-RNA ligases	(6.1.1)	1		2
Transamination				
Glutamic-oxaloacetic transaminase	(2.6.1.1)	2	3	5
Glutamic-pyruvic transaminase	(2.6.1.2)	1	1	0.5
Tyrosine-α-ketoglutaric transaminase	(2.6.1.5)	0.5	0.2	0.2
Ornithine-α-ketoglutaric transaminase	(2.6.1.13)	0.6	0.7	0.5
Nucleic acid metabolism				
Uridine kinase	(2.7.1.48)	1	1	1
Uridine phosphorylase	(2.4.2.3)	2.2	2.3	0.2
Phosphate hydrolysis				
Pyrophosphatase	(3.6.1.1)	1.2		2.2

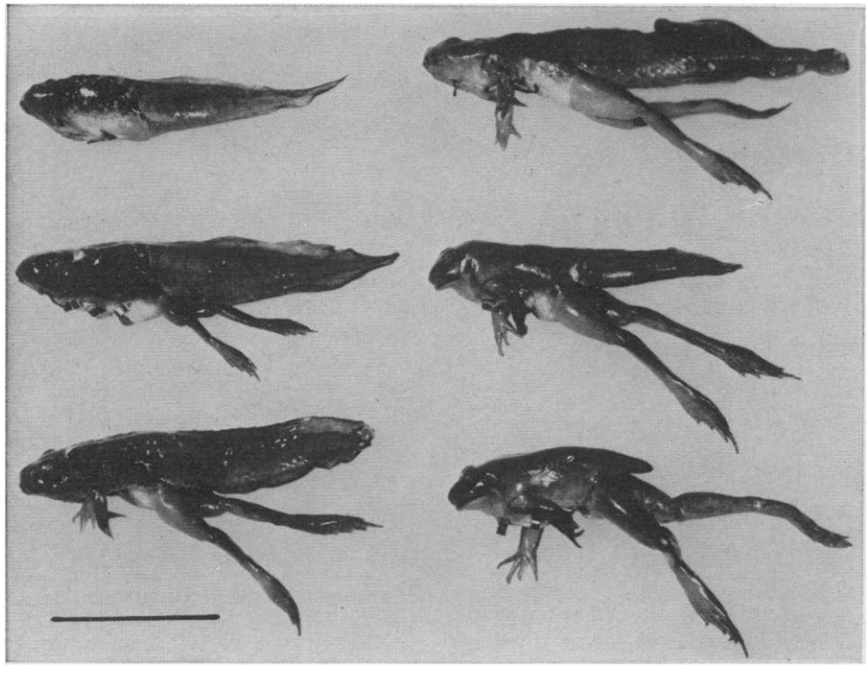

Fig. 1. Stages of development of *Rana catesbeiana* tadpoles. Top to bottom, left to right: Stages X, XVIII, XX, XX 1/2, XXIII, XXIV (*2*). In the limb bud stages, I to V, the length of bud increases from slight elevation to twice the diameter. In the paddle stages, VI to X, there is flattening of limb bud to complete indentation between toes; the fifth toe web is directed to the third toe. The foot stages, which are premetamorphic stages, are designated XI to XVIII. The fifth toe web reaches prehalix, the nasolachrymal duct appears, and the proximal, middle, and distal toe pads appear. In the metamorphic stages, XVIII to XXV, the cloacal tail piece disappears, and the front legs appear. The larval mouth is still present, the labial fringes become complete, and the angle of the mouth tends toward the posterior margin of eyeball. There is a rapid decrease of tail length. The tympanic cartilage ring becomes perceptible. The tail disappears. Scale, 2 cm.

metamorphosis (*9*). The relation of urea excretion to activities of enzymes involved in urea biosynthesis is shown in Fig. 3. The rate-limiting enzymes appear to be carbamyl phosphate synthetase-I (CP-synthetase-I) and argininosuccinate synthetase (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*) who studied liver slices from tadpoles at different stages of development. A direct correlation between the excretion of urea and the activity of CP-synthetase-I in liver of tadpoles has been demonstrated by Paik and Cohen (*11*).

Effect of Thyroxine

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 $2.6 \times 10^{-8}M$ thyroxine (*2*). Other enzymes which we have studied are shown in Table 1. It should be noted that the metamorphic:premetamorphic ratios of enzyme activities (activities determined during metamorphosis divided by activities during premetamorphosis) are approximately the same whether metamorphosis occurs naturally or is induced by thyroxine. It thus appears that thyroxine is acting as an accelerator, or more likely a depressor. The most dramatic changes in enzyme activities are those involved in the biosynthesis of urea and, in particular, CP-synthetase-I (Fig. 2). Not only are the activities of the enzymes in the ornithine-urea cycle very high compared with those of other enzymes (Table 1) but in addition they are induced ahead of most other enzymes. The striking increase in CP-synthetase-I activity taking place 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 used in demonstrating that the increase in CP-synthetase-I in the liver of tadpoles exposed to thyroxine was

the result of synthesis de novo (*15*).

A rather interesting effect of temperature on the induction of CP-synthetase-I in liver of tadpoles exposed to $2.6 \times 10^{-8}M$ thyroxine was reported by Paik and Cohen (*11*). It was observed that (i) the lag period of enzyme induction is shorter at higher temperatures; (ii) the lag period is not only prolonged at lower temperatures (15°C) but also the enzyme activity reaches a plateau at approximately one-half the maximum and persists; and (iii) raising the temperature from 15° to 25°C results in a rapid increase in the rate of synthesis of CP-synthetase-I with no appreciable lag period. The critical temperature dependence at 15°C 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 that there was discontinuity of the temperature curve between 15° and 20°C with a high temperature quotient for the reaction involving conversion of precursor (or precursors) to active, immunoprecipitable enzyme.

The lag period preceding the induction of CP-synthetase-I is about 4 to 6 days after tadpoles are exposed to a thyroxine solution ($2.6 \times 10^{-8}M$) at 22° to 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 nonexposure 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 nonexposure 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 (or mechanisms) 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*).

In view of the fact that the increase in CP-synthetase-I activities 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 were 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 CP-synthetase-I activity, 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*). This enzyme is present in relatively high concentrations in frog eggs (*19*). More recent studies have shown that the CP-synthetase-II is also present in premetamorphic and metamorphosing tadpole livers and in frog liver, but at a relatively low concentration compared with CP-synthetase-I (*21*). The enzyme CP-synthetase-II, but not CP-synthetase-I, is present in spleen and kidney and in the mucosa of the stomach and intestine of the 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 by 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 (*22*). The amounts of liver DNA and RNA (estimated on the basis of phosphorus content of

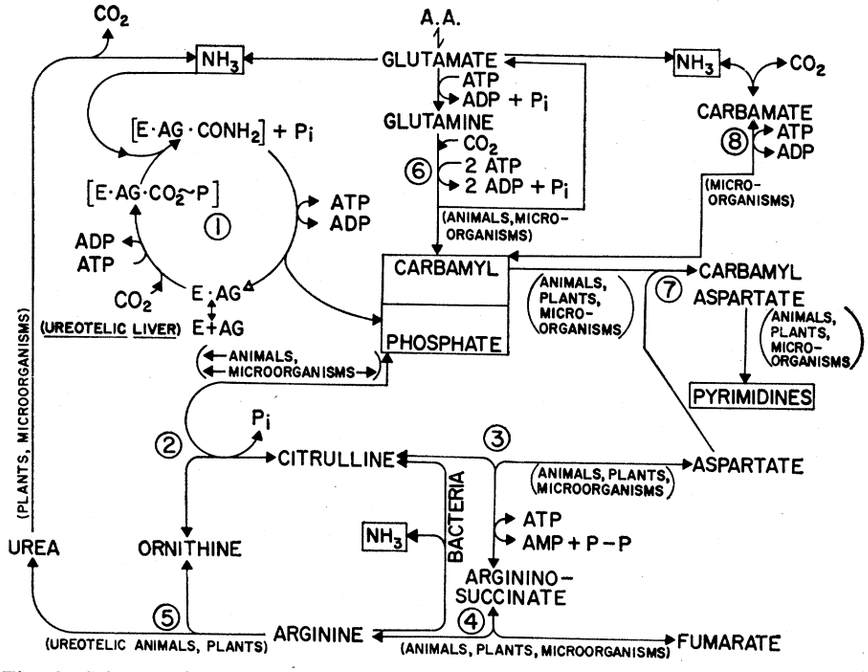

Fig. 2. Scheme of reactions generating and utilizing carbamyl phosphate. Enzymes catalyzing the reactions are (1) carbamyl phosphate synthetase-I (*48*); (2) ornithine transcarbamylase; (3) argininosuccinate synthetase; (4) argininosuccinase; (5) arginase [reactions (1) through (5) constitute the ornithine-urea cycle]; (6) carbamyl phosphate synthetase-II; (7) aspartate transcarbamylase; and (8) carbamate kinase. The two 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. This carbamyl phosphate is used for synthesis of citrulline by means of the mitochondrial enzyme, ornithine transcarbamylase. The upper compartment represents the carbamyl phosphate generated by the enzyme CP-synthetase-II which is extramitochondrial in the liver of all animals. This carbamyl phosphate is used 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. The enzyme carbamate kinase (E.C. 2.7.2.7) 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. The symbols used in the reaction scheme 1 are AG, *N*-acetylglutamate; E·AG, enzyme-*N*-acetylglutamate complex; [E·AG·$CO_2 \sim$ P], postulated intermediate enzyme complex; [E·AG·$CONH_2$], postulated intermediate enzyme complex; P_i, inorganic phosphate.

the nucleic acid fractions) 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 [8-^{14}C]adenine into RNA and a decrease in the amount of adenosine triphosphate were observed during thyroxine treatment. The latter effects coincided with the beginning increase in CP-synthetase-I (*22*). Early effects of triiodothyronine injection on nucleotide and RNA metabolism in tadpole liver have been reported by Eaton and Frieden (*23*).

The rate of total RNA synthesis was determined after administration of [^{14}C]orotic acid at 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 (or transfer) RNA fraction was maintained during the period of induced metamorphosis. The most striking increase of RNA turnover was seen in the microsomal fraction that had the lowest initial value in the untreated tadpoles. These findings suggest that new synthesis of ribosomal and soluble RNA occurs as an early response to thyroxine treatment 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 (*24*). The pattern in the case of premetamorphic tadpoles, labeled with [^{14}C]orotic acid for 2 hours, is shown in Fig. 8a. The major portion of the labeled RNA sediments with a value of 4*S*. A gradual increase in the labeling of ribosomal RNA is observed with time. However, in similar experiments with animals treated with thyroxine for 2 days, the ^{14}C appeared in heavier (6 to 10*S*) RNA fractions (Fig. 8b). The peak of specific radioactivity remained constantly between the 6*S* and 10*S* fractions over a period of 24 hours after administration of radioactive precursors. Analysis of the base composition showed that this fraction had a high concentration of uridine monophosphate (UMP) in contrast to that of bulk RNA. The ratio of the sum of cytidine monophosphate (CMP) and guanidine monophosphate (GMP) to the total ribonucleotides in this RNA fraction gave values of 45 and 50 percent in contrast to a value of 65.3 percent 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 messenger RNA). The base ratio in the DNA prepared from liver of *Rana catesbeiana* tadpoles was 46 percent (*24*).

These studies of RNA turnover suggest that thyroxine treatment stimulates the synthesis of three major types of RNA, namely ribosomal RNA, soluble or transfer RNA, and DNA-like or messenger RNA, before the induction of CP-synthetase-I and gross metamorphosis. While the reason 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 (*25*). Tata (*26*) has reported on the formation, distribution, and function of ribosomes and microsomal membranes and of RNA fractions during induced amphibian metamorphosis.

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 (*27*). As is shown in 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 treatment with thyroxine.

Purified preparations of chromatin of liver nuclei from tadpoles treated with thyroxine used as the template for RNA synthesis in the presence of excess *Escherichia coli* RNA polymerase had a template efficiency 20 to 50 percent higher than that of chromatin prepared from untreated animals (Fig. 10) (*28*). Intrinsic RNA polymerase and ribonuclease 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

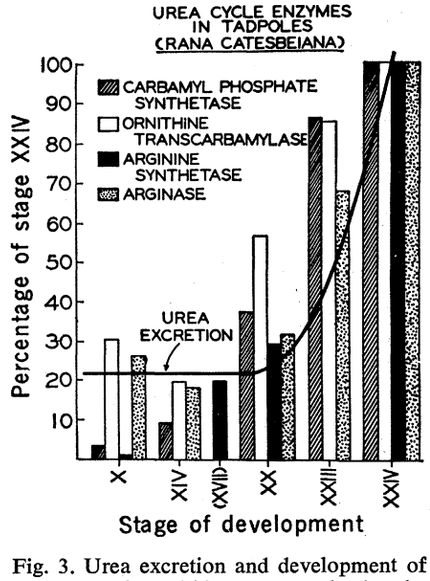

Fig. 3. Urea excretion and development of enzymes of ornithine-urea cycle in the metamorphosing tadpole (*2*). Arginine synthetase represents the overall synthesis of arginine from citrulline and aspartate and involves argininosuccinate synthetase (see reaction 3, Fig. 2) and argininosuccinase (see reaction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

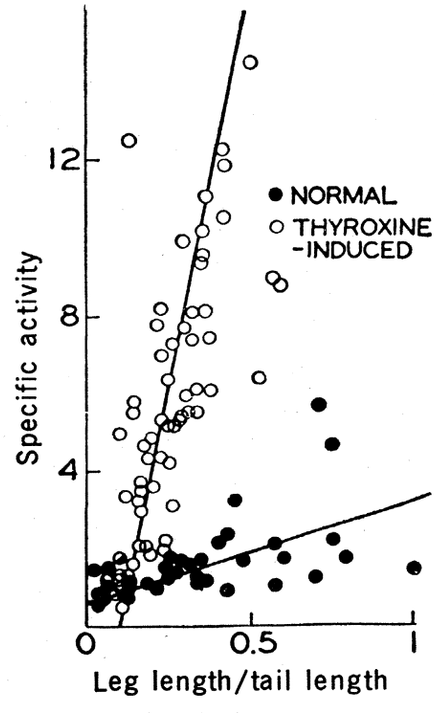

Fig. 4. Carbamyl phosphate synthetase activity as related to metamorphosis of *Rana catesbeiana*. Specific activity (micromoles per hour per milligram of protein) of CP-synthetase-I is plotted against the ratio of hind leg length and tail length during normal and thyroxine-induced metamorphosis (*2*).

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, making 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 catalytic capability of associated proteins comes from studies in which ammonium sulfate was added to the RNA polymerase reaction mixtures. Such mixtures stimulated RNA synthesis about three- to fourfold (*27*) (Fig. 9). The increase in enzyme activity is approximately linear with increases in the salt concentration up to 0.4*M*, and further increases in salt concentration result in an inhibition of activity. One of the effects of addition of ammonium sulfate 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 with 0.4*M* ammonium sulfate (further increase in concentration has little effect), and this is the concentration at which maximum stimulatory effect on RNA synthesis was observed.

The chemical composition of nuclei before and after ammonium sulfate addition was noticeably changed only in the histone fraction (*27*). Since isolated RNA polymerase from mammals and microorganisms is inhibited by ammonium sulfate at the concentration used in our 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.

If actinomycin D is injected into premetamorphic tadpoles before or at the same time that thyroxine is administered, induction of CP-synthetase-I is inhibited (*25*). 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 (*25*). However, if actinomycin is administered after induction of synthesis of CP-synthetase is fully developed, enzyme synthesis is not inhibited (*17*, *28*) although RNA synthesis is inhibited (*17*). The failure of actinomycin to inhibit the induction of synthesis of CP-synthetase-I after thyroxine treatment can be interpreted as follows. (i) 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. (ii) The increase in activity of CP-synthetase-I is the result of conversion of existing enzyme precursor into functional enzyme molecules.

Puromycin, an inhibitor of protein synthesis, has no effect on the conversion of CP-synthetase-I precursor molecules into functional enzyme in liver slices (*16*). However, injection of puromycin into tadpoles previously treated with thyroxine inhibited the further increase in CP-synthetase-I activity, which occurs in the presence of actinomycin (*25*). This observation suggests that the increase in enzyme activity in the presence of actinomycin is the result of protein synthesis de novo 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.

Continued exposure of tadpoles to thyroxine (Fig. 5) resulted in a more rapid induction of CP-synthetase-I synthesis than occurs in tadpoles placed in water after previous exposure to thyroxine for 2 to 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 (*25*). 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 on translation has been reported by Unsworth and Cohen (*29*). Microsomal and ribosomal preparations from liver of thyroxine-treated tadpoles showed a rate of incorporation from aminoacyl-tRNA of the order of 100 percent greater than that of similar preparations from untreated tadpoles (Fig. 11). Current studies (*30*) with an improved assay system confirm the greater activity of ribosomal preparations from liver of tadpoles treated with thyroxine. When examined in the ultracentrifuge the ribosomal preparations from both untreated and thyroxine-treated tadpoles appeared to be identical and revealed a major peak at 77*S* with a minor disome peak at 116*S*.

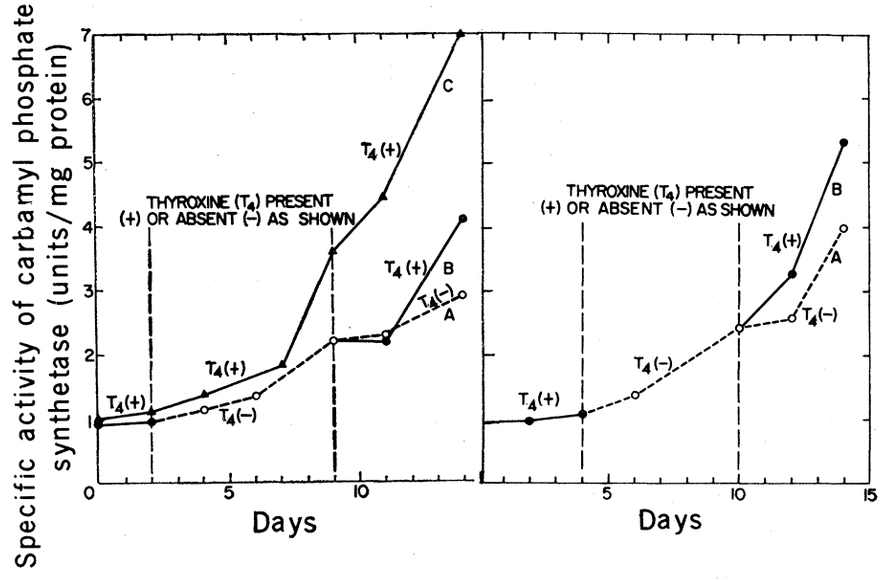

Fig. 5. Effect of time of exposure to thyroxine on induction of carbamyl phosphate synthetase. (Left) Tadpoles were kept in thyroxine (T_4) solution ($2.6 \times 10^{-8}M$) for 2 days at 22° ± 1°C. They were then washed thoroughly and placed in water for the next 7 days. On the day 9 after initial treatment with thyroxine, a group of tadpoles was again exposed to thyroxine (curve B); another group was kept in water (curve A). Curve C shows the induction curve of CP-synthetase activity in a group of tadpoles continuously exposed to thyroxine. (Right) Tadpoles were maintained in thyroxine ($2.6 \times 10^{-8}M$) for 4 days at 22° ± 1°C, washed, and then kept in water for 6 days. On the day 10 after the initial treatment with thyroxine, one group was again exposed to thyroxine (curve B); a second group was maintained in water (curve A). In both cases, livers from two tadpoles were pooled and assayed for CP-synthetase activity (*17*).

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, the amount being about 1.5 times that found at zero time.

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

With the availability of a highly specific antibody to CP-synthetase-I (*14*) and with the evidence for increasing CP-synthetase-I activity in liver of tadpoles undergoing natural (*9*) or thyroxine-induced (*11*) metamorphosis, it seemed of interest to determine whether the increased amount of CP-synthetase-I would be manifested by use of a fluorescent antibody. The results of such a study (*31*) 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 ($2.6 \times 10^{-8}M$) 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, all liver cells of the adult frog 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 that fix the antibody and the amount of antibody fixed is progressively increased. These observations correlate very well with 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 percent of the soluble mitochondrial proteins of frog liver (*13*).

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 electron microscope.

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 (*32*). Tata (*26*, *33*) has reported electron micrographs of tadpole liver sections from premetamorphic and thyroxine-treated (after injection of triiodothyronine) metamorphic animals.

Liver from tadpoles exposed to thyroxine show an increased development of the endoplasmic reticulum from single random profiles into parallel stacks (Fig. 13). During early stages, a close association is observed between a single endoplasmic reticulum 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 ± 0.24 micrometers for mitochondria from premetamorphic animals as compared with 1.70 ± 0.59 micrometers 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 compared with those from premetamorphic and metamorphic tadpoles, and the endoplasmic reticulum profiles appear to have a more random arrangement.

The frequent occurrence of an apparent direct association between the endoplasmic reticulum and individual mitochondria (Fig. 13B) suggests that there is a direct pathway for transport of precursor of mitochondrial enzymes (probably at the subunit level) from ribosomes on the endoplasmic reticulum

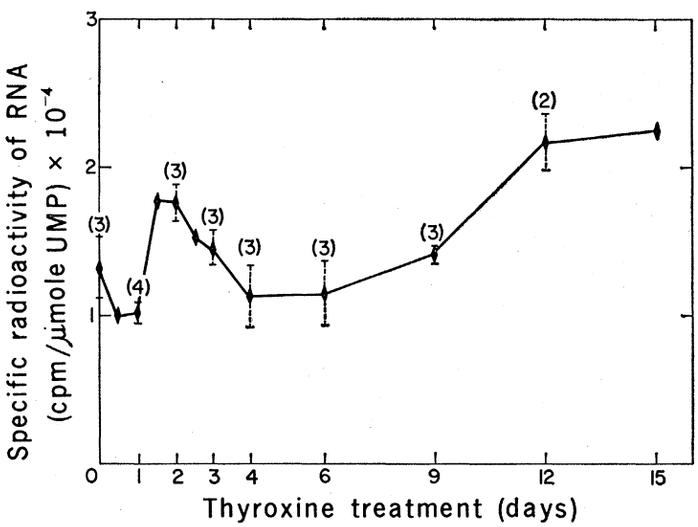

Fig. 6 (left). Rate of RNA synthesis during metamorphosis induced by thyroxine. Tadpoles were treated with thyroxine ($2.6 \times 10^{-8}M$) at 22°C and, at the times indicated, received [6-^{14}C]orotic acid intraperitoneally (1 μc per gram of body weight). The specific activity is represented as the number of counts per minute (*cpm*) per micromole 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 (*17*).

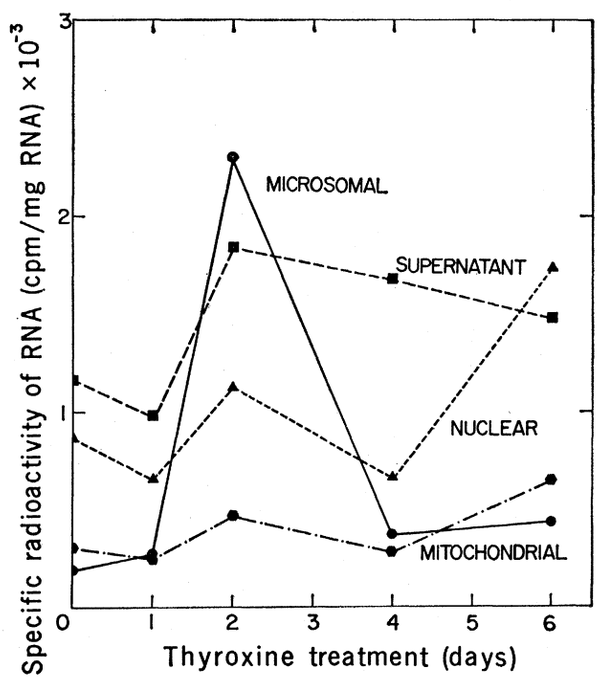

Fig. 7 (right). Specific radioactivity of RNA from various cell fractions of tadpole liver during metamorphosis induced by thyroxine. Tadpoles were exposed to thyroxine ($2.6 \times 10^{-8}M$) and received [6-^{14}C]orotic acid intraperitoneally (1 μc per gram of body weight). The tadpoles were killed 2 hours after administration of the orotic acid (*17*).

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 reported (*34*).

The swelling of the mitochondria during their 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 in a response to thyroxine. As noted, CP-synthetase-I may constitute as much as 20 percent of the soluble protein of frog mitochondria (*13*). Evidence for the synthesis of precursor protein (or proteins) of CP-synthetase-I extramitochondrially has been reported (*16*). This enzyme has a molecular weight of 315,000 (*13*), and thus it is not likely to be readily transported across membranes. Studies (*35*) of the subunit structure of this enzyme indicate that there may be four identical subunits each made up of two different chains. It is also known from work on tadpole liver slices and cubes that conversion of precursor, probably at the subunit level, occurs readily in mitochondria to form functional and immunoprecipitable enzyme (*16*). Thus the early swelling of mitochondria from tadpoles exposed to thyroxine could be an osmotic effect resulting from the rapid synthesis and transport of extramitochondrial subunits into the mitochondria. The later decrease in swelling and increase in matrix density (which occur even though exposure to thyroxine is continued) both in the later stages of metamorphosis and in the adult frog probably reflect the conversion of precursors of relatively low molecular weight (estimated to be units of the order of 80,000 or possibly smaller subunits of 30,000 and 50,000) to high molecular weight CP-synthetase-I (315,000).

Of considerable interest is the observation that all of the above changes, observed by electron microscopy in liver sections from tadpoles exposed to thyroxine can be reproduced in vitro by the addition of thyroxine ($2.6 \times 10^{-7}M$) to preparations of cubed liver from premetamorphic tadpoles. The changes were observed only in the systems to which thyroxine was added and occur within 48 hours after the addition in vitro of thyroxine. Since this system can synthesize CP-synthetase-I under the influence of thyroxine, thyroxine must, as a primary and direct effect, alter the subcellular structures involved in protein synthesis in addition to affecting RNA synthesis.

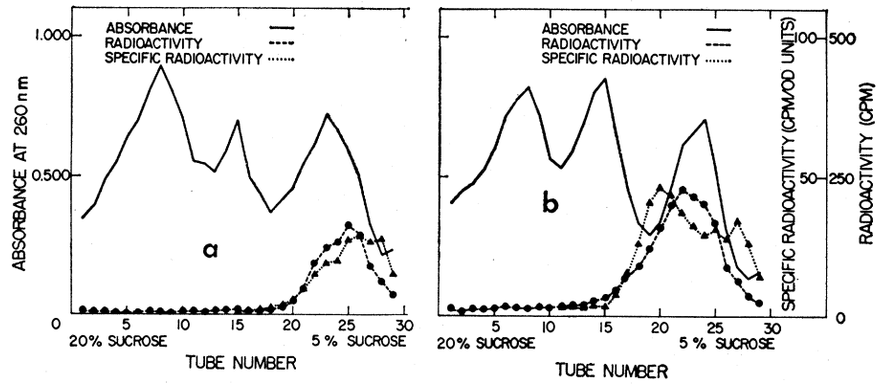

Fig. 8. Pattern of RNA synthesis in liver from (a) untreated tadpoles and (b) tadpoles exposed to thyroxine ($2.6 \times 10^{-8}M$) for 2 days. Tadpoles received [6-^{14}C]orotic acid intraperitoneally (1 μc per gram of body weight). Zonal centrifugation patterns show RNA 2 hours after radioactive orotic acid was administered. *CPM*, counts per minute; *OD*, optical density (*24*).

Studies with Surviving Liver Preparations

Because of the multiplicity and complexity of the biochemical and morphological responses of the premetamorphic tadpole exposed to thyroxine, 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, two types of liver preparations have been studied: suspensions of isolated liver cells and cubed-liver preparations.

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 (*36*). Addition of thy-

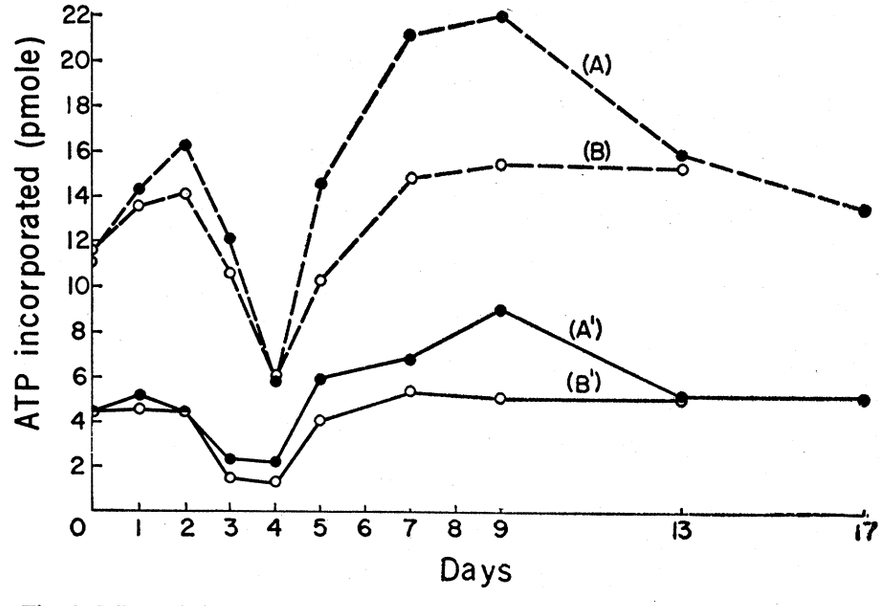

Fig. 9. Effect of thyroxine treatment of tadpoles on RNA polymerase of liver nuclei. RNA polymerase from thyroxine-treated animals was assayed in the presence (curve A) and absence (curve A′) of ammonium sulfate. The enzyme activities of control animals (curves B and B′) were assayed in the same manner (*27*).

roxine ($10^{-7}M$) in vitro 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*S* and 10*S* with a base composition similar to DNA of this species. Triiodothyronine had a similar effect at the same concentration as thyroxine, an indication that in this system of suspended cells, these congeners behave identically. While the cell suspensions responded to thyroxine with a stimulation of RNA synthesis, including a fraction with a base ratio (sum of cytosine plus guanine divided by the sum of total bases) typical of DNA, thyroxine had no effect on the 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.

Our lack of success in exploiting the isolated thyroxine-treated liver cell suspensions for information on mitochondrial enzyme synthesis led us to examine the technique developed by Wicks (*37*) for study of enzyme synthesis in suspensions of fetal rat liver for use with tadpole liver (*38*).

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 can survive in an appropriate medium and under appropriate conditions for 48 hours with cytologic integrity and biosynthetic capacity.

These preparations have been shown (*39*) to maintain an essentially constant uptake of oxygen 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 activity of CP-synthetase-I in these preparations. Thus the complex of enzymes representing the ornithine-urea cycle (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 $2.6 \times 10^{-9}M$). Other agents including a number of

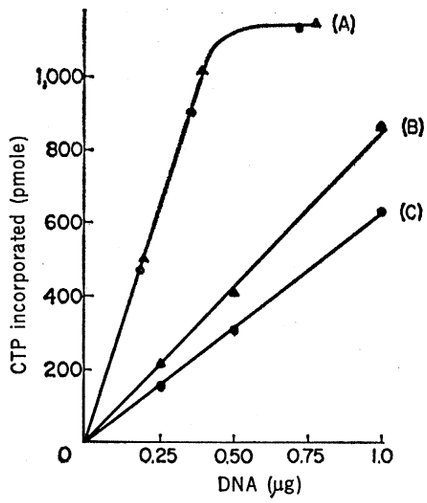

Fig. 10. RNA synthesis in the presence of chromatins and DNA from thyroxine-treated and control tadpole liver. Curve A represents RNA synthesis primed with deproteinized DNA. Curve B, values with chromatin from treated tadpole liver; curve C, values with control chromatin (no thyroxine) (*28*).

hormones, adenosine 3′5′-monophosphate (cyclic AMP), tadpole serum, and frog serum had no effect when added in vitro. Thyroxine added in vitro stimulated the rate of CP-synthestase-I synthesis in liver preparations from premetamorphic and metamorphosing tadpoles and the frog. In liver cubes CP-synthetase-I synthesis was specifically enhanced relative to that of the other soluble mitochrondial proteins at thyroxine concentrations of $2.6 \times 10^{-8}M$ and $2.6 \times 10^{-9}M$ and in liver cubes

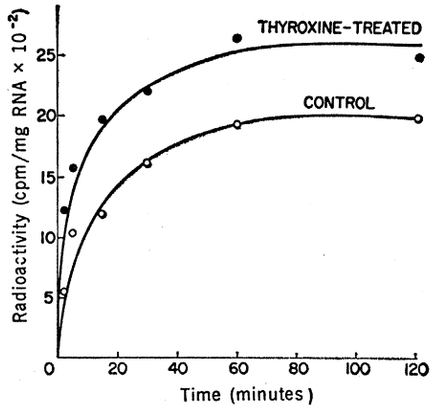

Fig. 11. Time course of incorporation of amino acids from [^{14}C]aminoacyl-tRNA into ribosomes prepared from the livers of both treated and control tadpoles. Tadpoles were kept at 25°C for 20 days, then placed in thyroxine solution ($2.6 \times 10^{-8}M$). Tadpoles were killed after 6 days (*29*) and ribosomes were prepared.

from tadpoles previously treated with thiouracil and from those previously treated by immersion in $2.6 \times 10^{-7}M$ thyroxine (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 [^{3}H]leucine in vivo were incubated in unlabeled leucine in vitro, an increase in labeled immunoprecipitable CP-synthetase-I was observed in the presence of actinomycin or puromycin, an indication of an effect on the conversion of nonimmunoprecipitable precursor to labeled immunoprecipitable enzyme. The mechanism underlying the observed increase in conversion of precursor was 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 amount of CP-synthetase-I suggested that enhancement of CP-synthetase-I levels in vitro was related largely to conversion of precursor to immunoprecipitable enzyme. This concept is consistent with the observation of an increase in total amount of enzyme 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 breakdown of enzyme or precursor (or both). (The half-life of newly synthesized CP-synthetase-I was of the order of 54 hours in vivo and 70 hours in vitro in the liver-cube system.) Thyroxine stimulates synthesis of precursor protein and possibly conversion to active enzyme, as well as degradation. Because the lag phase observed during induction of synthesis of CP-synthetase-I and of enzyme activity in tadpoles exposed to thyroxine in vivo was not observed in the liver-cube preparations, the existence of a repressor mechanism in vivo is postulated that is not operative in vitro. The nature of the repressor (or repressors) remains unknown.

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 (*40*).

Glutamate dehydrogenase, partially purified from premetamorphic tadpole liver, had physical, kinetic, and substrate specificity properties (*41*) different

from those of the crystalline enzyme prepared from frog liver (*42*). Crystalline glutamate dehydrogenase has recently been prepared from liver of premetamorphic tadpoles (*43*) 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 yet been possible (because of the limited amount of tadpole enzyme available) to be certain of the exact 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 (*44*). Enzyme activity increases slightly during incubation in vitro of liver cubes from premetamorphic tadpoles. In the presence of thyroxine ($2.6 \times 10^{-8}M$ to $2.6 \times 10^{-10}M$) or triiodothyropropionate ($2.6 \times 10^{-9}M$ to $2.6 \times 10^{-11}M$), the mean increase in the specific activity in vitro was twice that of the control during a 48-hour incubation period. Thyroxine stimulated de novo synthesis of immunoprecipitable glutamate dehydrogenase as well as of 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 $2.6 \times 10^{-8}M$. 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 into immunoprecipitable enzyme, observed in vitro in the absence of any additives to

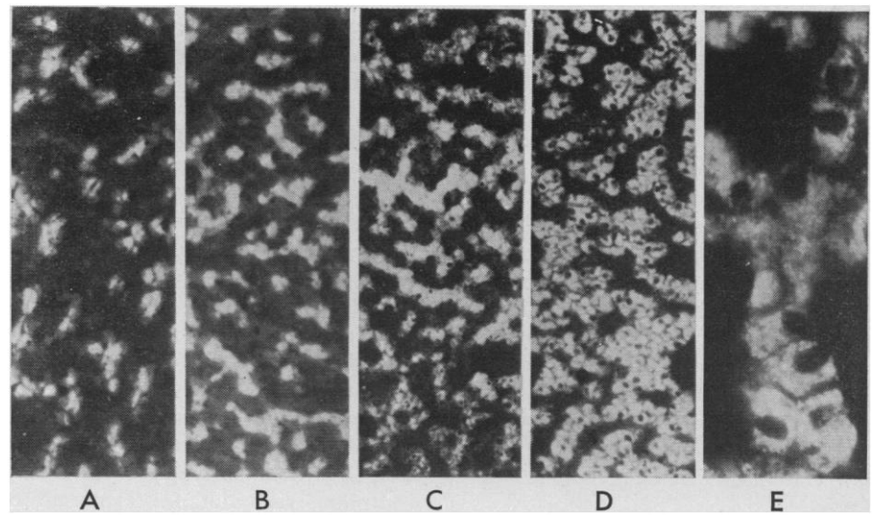

Fig. 12. Fixation of antibody (made fluorescent with fluorescein isothyocyanate) by CP-synthetase-I in liver sections from tadpoles and frog. (A) Liver from premetamorphic tadpole; CP-synthetase-I assay represented as 1 on a relative scale; magnification × 92; (B) liver from tadpole exposed to thyroxine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

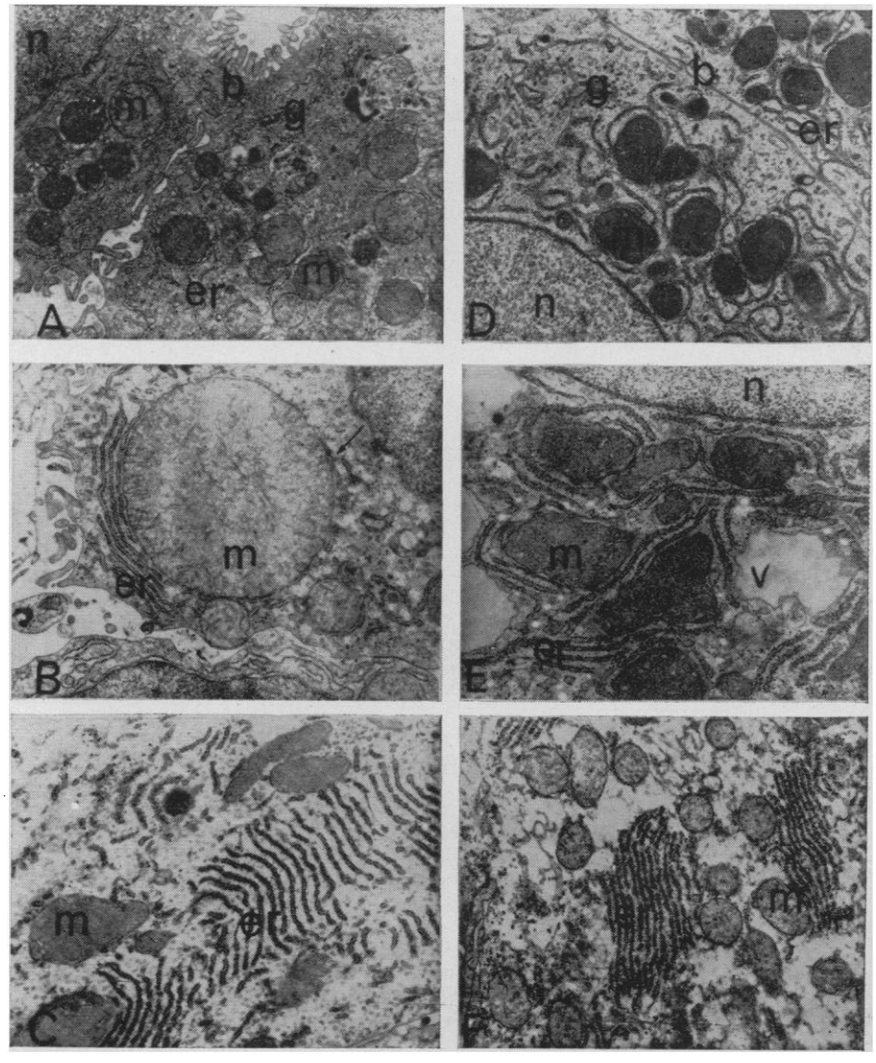

Fig. 13. Electronmicrographs of sections of liver from (A) premetamorphic tadpole; (B) tadpole exposed to thyroxine ($2.6 \times 10^{-8}M$) for 7 days; (C) tadpole exposed to thyroxine for 10 days; (D) adult frog; (E) tadpole during natural metamorphosis at stage XVI; (F) tadpole during natural metamorphosis at stage XIX. Magnification is × 12,600 except in (E) in which it 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.

the incubation system, was stimulated in the presence of thyroxine and was increased in the presence of actinomycin and puromycin as well. The half-life of labeled immunoprecipitable glutamate dehydrogenase was 21.2 ± 1.7 hours in vitro, and 22.8 hours in vivo. Actinomycin and puromycin inhibited breakdown of immunoprecipitable glutamate dehydrogenase. The observation 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 indicates that these factors determine the basic mechanism of regulation of this enzyme in this animal.

Discussion and Summary

Attention has been focused on 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. The fact that no significant cell division occurs in the liver of the intact tadpole exposed to thyroxine, or in the preparations in vitro 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, and the like (*4*), the choice of CP-synthetase-I as a "marker" is based on the following observations. (i) Induction of synthesis precedes changes in gross morphology. (ii) CP-synthetase-I may reach a concentration representing 20 percent of the soluble protein of mitochondria. (iii) 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 (*26, 33*). 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. Unpublished studies show that the biosynthesis of urea in the surviving cubed-liver system increases in relation to the activity of CP-synthetase-I. This enzyme is synthesized de novo under the influence of thyroxine in vivo (*15*) and in vitro (*38*). A useful antibody has been produced that permits the simultaneous study of the effect of thyroxine on the induction of ornithine transcarbamylase (*45*). 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 effect in vitro of thyroxine on ornithine transcarbamylase activity in tadpole liver organ cultures has been reported (*46*).

Crucial experiments to test the validity of the approach under discussion will emerge from our studies dealing with (i) isolation of a specific messenger RNA, (ii) determination of the nature of the polypeptides synthesized by ribosomes from liver of animals exposed to thyroxine (or from the cubed-liver preparation), and (iii) 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 (i) how a soluble mitochondrial enzyme is synthesized at the subunit level extramitochondrially and the transport of these subunits to the mitochondria for enzyme assembly; (ii) how the hormone thyroxine exerts its effect at the molecular level; (iii) what kind of regulation exists with respect to a functioning biosynthetic pathway involving a series of five enzymes; and (iv) the nature of the regulators (such as repressors and derepressors) which operate during transcription and translation.

A further comment about glutamate dehydrogenase is in order. The crystalline enzymes isolated from frog liver (*42*) and premetamorphic tadpole liver (*43*) have different kinetic and substrate specificity properties as well as molecular weights. The synthesis of what ap-

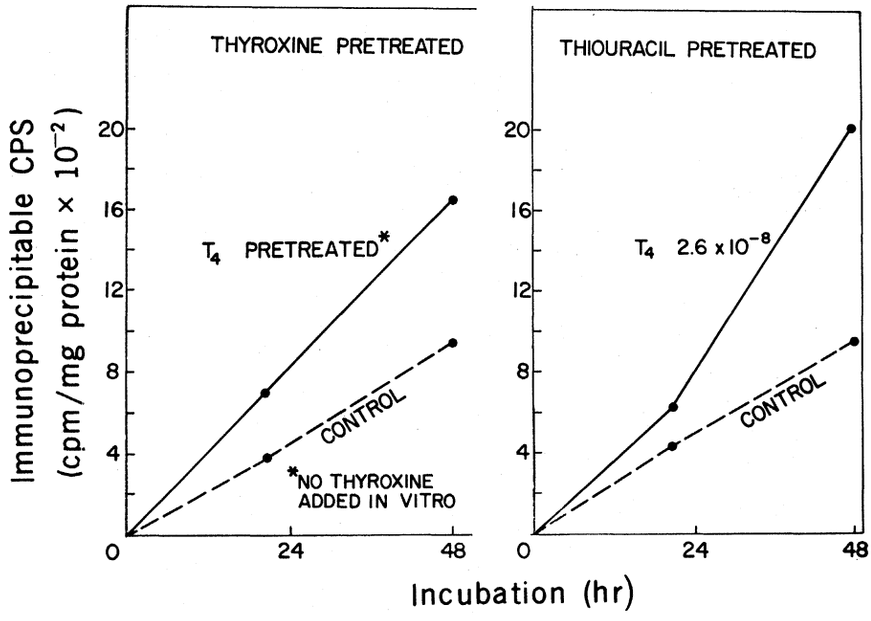

Fig. 14. Effect of thyroxine on synthesis of CP-synthetase-I in liver-cube preparations. Left side of figure shows effect of prior treatment of tadpoles ($2.6 \times 10^{-8}M$ of thyroxine for 48 hours at 24°C). The control group was held at 24°C for 48 hours in water. Right side of figure shows effect of prior treatment of tadpoles with thiouracil ($3.5 \times 10^{-3}M$) for 72 hours. Preparations of cubed liver were then made, and one portion was incubated in the absence of thyroxine (control) and the other in the presence of thyroxine ($2.6 \times 10^{-8}M$). The preparations of cubed liver were incubated in the presence of [^{3}H]leucine. After incubation for 24 and 48 hours, mitochondrial extracts were prepared and treated with antibody to CP-synthetase-I. The ordinate represents specific radioactivity of immunoprecipitable CP-synthetase-I (*CPS*) per milligram of soluble mitochondrial protein (*38*).

pears 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 occurring 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 (*47*).

The apparent discontinuity in genetic expression during development in the case of glutamate dehydrogenase and the hemoglobins indicates the need for studies concerning earlier stages of embryogenesis, namely from the fertilized ovum to the early tadpole stage. With the availability of crystalline glutamate dehydrogenase preparations from tadpole and frog liver, and of antibodies to 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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48. Carbamyl phosphate synthetase-I and carbamyl phosphate synthetase-II have not been assigned systematic names and do not appear in the Report of the Commission on Enzymes of the International Union of Biochemistry, 1961 (Pergamon Press, 1961). Because the mechanisms for the reactions catalyzed by these enzymes are yet to be established unequivocally, it may be premature to assign systematic names. A discussion of possible systematic names for these two enzymes appears as a footnote in the paper by McKinley, Anderson, and Jones (*49*).
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