Biochemical Studies on Amphibian Metamorphosis

I. *The effect of thyroxine on protein synthesis in the tadpole*

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ABSTRACT Thyroxine has been shown to accelerate the synthesis of carbamyl phosphate synthetase in the liver of *Rana catesbeiana*. Stimulation of carbamyl phosphate synthetase synthesis by thyroxine appears to be relatively specific because of the following observations: (1) succinoxidase activity decreased during the time that carbamyl phosphate synthetase increased; (2) liver catalase responded more slowly than carbamyl phosphate synthetase to thyroxine; (3) the ratio of biochemical changes/morphological changes was greatly altered during thyroxine-induced metamorphosis. The relationships between the concentration of thyroxine and (1) temperature; (2) duration of exposure of the tadpole to thyroxine; and (3) the activity of carbamyl phosphate synthetase during the induced synthesis of carbamyl phosphate synthetase by thyroxine are discussed. Chloramphenicol and thiouracil partly counteracted the effect of thyroxine on the synthesis of carbamyl phosphate synthetase.

INTRODUCTION

A characteristic of metamorphosis, in the animal kingdom in general, is that the polymorphic animal expresses itself in such a way that one form replaces another during the normal life of the individual. Amphibian metamorphosis, in particular, represents a preparation for the transition from an aquatic life during the larval stage to the terrestrial habitat in the adult. In preparation for this ecological transition, numerous anatomical and biochemical changes occur. The following biochemical changes have been studied in some detail: (*a*) transition of visual pigment from porphyropsin to rhodopsin (1); (*b*) character of hemoglobin (2); (*c*) plasma proteins (3); and (*d*) ammonotelism to ureotelism (4, 5).

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The discovery of the relationship between the thyroid gland and amphibian metamorphosis by Gudernatsch in 1912 provided an important biochemical development in the study of metamorphosis (6). This observation facilitated research on this phenomenon by shortening the transitory period from the larval stage to the adult under controlled conditions. In the absence of added thyroxine, a *Rana catesbeiana* tadpole may remain in the larval form for 2 to 3 years under certain environmental conditions. The identification of thryoxine as the active agent (7) in the thyroid gland gave further impetus to these studies.

Although the identity of thyroxine as the specific physiological inducing agent of tadpole metamorphosis is still doubted by Barch (8) and others (9), investigations in the past several years have resulted in the discoveries of increased production of urea (4), an increase of arginase activity (9), various phosphatases (10, 11), and all the enzymes in the Krebs-Henseleit urea cycle (5) during the normal metamorphosis of the tadpole.

While there is still uncertainty as to whether thyroxine is *the* normal metamorphosing agent in the tadpole, the study of the relationship between thyroxine and changes in enzymatic activity may be expected to yield useful data on the effect of this hormone on protein synthesis at the molecular level.

Because metamorphosis represents an instance of morphological differentiation, it seemed of interest to study changes which represent examples of biochemical differentiation. The transition of the tadpole from ammonotelism to ureotelism is basically an instance of biochemical differentiation since the fundamental changes involve the synthesis of enzymes of the Krebs-Henseleit urea cycle during metamorphosis (5). One of the enzymes of the cycle, carbamyl phosphate synthetase, has been prepared from frog liver in a highly purified state (12). Preliminary studies in this laboratory revealed a marked increase in carbamyl phosphate synthetase activity after triiodothyronine treatment (13). Because of the key role of this enzyme in urea biosynthesis and the fact that this enzyme is found almost exclusively in the liver, we undertook some experiments to explore the possible relationship between the synthesis of this enzyme (as well as protein synthesis in general) and the effect of thyroxine in the tadpole.

EXPERIMENTAL

Tadpoles[1] of the giant bull frog (*Rana catesbeiana*), in premetamorphic stages (14) weighing 3 to 5 gm., were supplied by North Carolina Biological Supply Co. the year around and maintained in a water bath ranging from a temperature of 21 to 25°C., unless otherwise specified. The water was changed every 2 to 3 days. Canned boiled spinach was fed as the sole food source.

[1] These experiments were carried out from September, 1958, to April, 1959.

For the thyroxine treatment, thyroxine was dissolved in the minimum amount of 1 N NaOH solution and made to the concentration of 1 mg./ml. solution with water. This thyroxine solution was added to water of pH 7.4 to give the desired final concentration.

The assay method for carbamyl phosphate synthetase in the liver of tadpoles was that developed in this laboratory (13, 15). The enzyme, prepared from the livers of two to five tadpoles, was incubated with the reaction mixture for 15 minutes at 38°C. and the reaction was stopped by adding perchloric acid. The amount of citrulline formed was determined colorimetrically with diacetyl monoxime and the enzymatic activity expressed as specific activity. The specific activity is equivalent to micromoles citrulline formed per hour per milligram of protein. Protein was determined by the method of Lowry *et al.* (16)

For the assay of succinoxidase activity, tadpole liver was homogenized in 4 volumes of 0.25 M sucrose solution. Oxygen consumption was determined manometrically in the absence and in the presence of succinate (final concentration, 0.03 M); M/15 phosphate buffer; pH 7.2; 37°C. The amount of oxygen taken up in the 1st hour in the absence of substrate was used for calculation of endogenous respiration, and the difference between the amount of oxygen consumed in the 1st hour in the presence and absence of succinate was used to calculate the activity of succinoxidase. Both values were expressed as Q_{O_2} (microliters of O_2 consumed per hour per milligram dry weight of homogenate).

For estimation of catalase activity, the oxygen evolved in the presence of 0.14 M hydrogen peroxide solution in M/15 phosphate buffer pH 7.2 at 37°C. was determined manometrically. The activity was expressed as microliters of O_2 evolved per minute per milligram protein of homogenate.

5-Fluoroorotic acid, used in these experiments, was obtained through the courtesy of Dr. C. Heidelberger, McArdle Laboratory, University of Wisconsin; chloramphenicol was a gift from Hoffmann-La Roche Inc., New Jersey.

RESULTS

The Concentration of Thyroxine vs. the Increase in the Enzymatic Activity The relationship between the concentration of thyroxine and the increase in the enzymatic activity is shown in Fig. 1. Linearity is observed up to about 0.05 μg. thyroxine/ml. concentration (6.5×10^{-8} M). However, with higher concentration of thyroxine, a progressive increase in the death rate occurred. Thus all the tadpoles exposed to a concentration of 0.5 μg/ml. (6.5×10^{-7} M) (25°C.) died after 1 to 2 days. (Days shown in Fig. 1 indicate the periods of time during which the tadpoles were maintained in the various concentrations of thyroxine.)

A concentration of 0.02 μg./ml. (2.6×10^{-8} M) was chosen as a satisfactory level. At this concentration, enzymatic activity increases at a measurable rate and the mortality is not excessive. This concentration of thyroxine, which was employed in all subsequent experiments, is of the same order of magnitude as that found in human serum (0.04 to 0.08 μg. iodine/ml.) (17).

Kinetics of Carbamyl Phosphate Synthetase Synthesis Fig. 2 shows the relationship between enzymatic activity and time of exposure of the tadpoles to thyroxine. As can be seen from Fig. 2, the shape of the curve is sigmoid. There is a short latent period before the enzymatic activity begins to increase at a logarithmic rate. Under these conditions, the specific activity values of carbamyl phosphate synthetase reach a plateau in 15 to 17 days at a level comparable to that of the adult frog liver (11 to 14). During this period of time the control tadpoles show no increase in enzymatic activity and no obvious morphologic changes.

An actual increase in amount of carbamyl phosphate synthetase during the induction with thyroxine was demonstrated by precipitin studies. The anti-

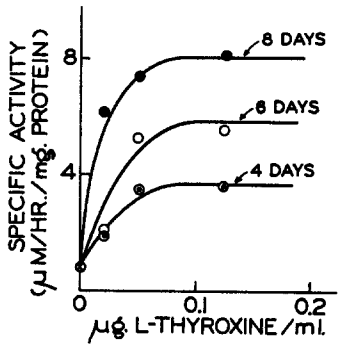

FIGURE 1. Effect of concentration of thyroxine on the increase of carbamyl phosphate synthetase activity. 0.02 μg. per ml. thyroxine (2.6×10^{-8} M). The days shown in the figure represent the period of time during which the tadpoles were exposed to thyroxine.

body employed was prepared by Dr. M. Marshall of this laboratory by immunization of rabbits (data to be published). Purified frog carbamyl phosphate synthetase (12) was employed as antigen.

Furthermore, C^{14}-labeled leucine incorporation into the enzyme *in vivo* demonstrated that the rate of *de novo* synthesis of enzyme protein was greatly accelerated in thyroxine-treated tadpoles (Metzenberg *et al.*, data to be published).

The Effect of Temperature on Enzyme Synthesis As shown in Fig. 3, three different temperatures (30, 25, and 15°C.) were tested on the thyroxine-induced enzyme synthesis. At 30°C., the synthesis of the enzyme proceeds most rapidly, but the mortality rate was so high that the experiment had to be discontinued.

In this experiment, an interesting phenomenon was observed in the group of tadpoles maintained at 15°C. As can be seen in Fig. 3, there is a prolonged latent period and the enzyme increases at a slower rate than at 25°C. However, the enzyme activity reaches a plateau at about half the maximum level attained at 25°C. When these animals are brought to the higher temperature, the increase is resumed without delay.

While a number of possible explanations may be offered for the findings, additional experiments, now in progress, are necessary for proper interpretation.

The Dose of Thyroxine In order to decide how long the tadpoles must be treated with thyroxine solution to achieve maximum carbamyl phosphate synthetase activity, the animals were treated in the solution for the periods of time indicated in Fig. 4, washed, and kept thereafter in water. As seen from Fig. 4, a certain minimal dose is required for initiation of enzyme synthesis. The final level of the enzyme is also determined by the dose. These data suggest that a certain level of thyroxine must be maintained continuously in order to achieve maximum activity.

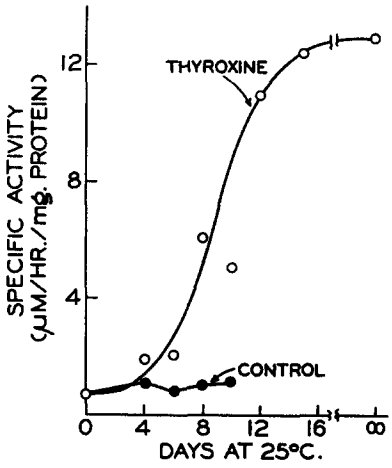

FIGURE 2. Kinetics of carbamyl phosphate synthetase synthesis induced by 2.6×10^{-8} M thyroxine at 25°C. Infinitesimal sign represents the level found in the adult bull frog.

The Effect of pH of the Medium An earlier report showed that an acidic medium accelerates and an alkaline medium retards the thyroxine-induced metamorphosis of tadpoles (18). Furthermore, a considerable change in blood pH was reported to occur during metamorphosis (19). Since carbamyl phosphate synthetase functions as part of the mechanism to eliminate ammonia and thus control the alkali reserve, the synthesis of the enzyme was examined in tadpoles maintained in water adjusted to pH 4.5, 6.5, 7.4, and 8.5. The water was changed daily. It was observed that in each case, there was a tendency for the pH to change in the direction of 7.4.

It was observed that there was no significant difference in the rate of synthesis of the enzyme, in spite of the differences in pH of the medium. However, there was, indeed, an acceleration of morphological changes in acidic medium. This apparent discrepancy may arise from the fact that certain biochemical changes precede morphological ones in the presence of thyroxine and that the synthesis of carbamyl phosphate synthetase is not intimately related to the morphological changes which occur.

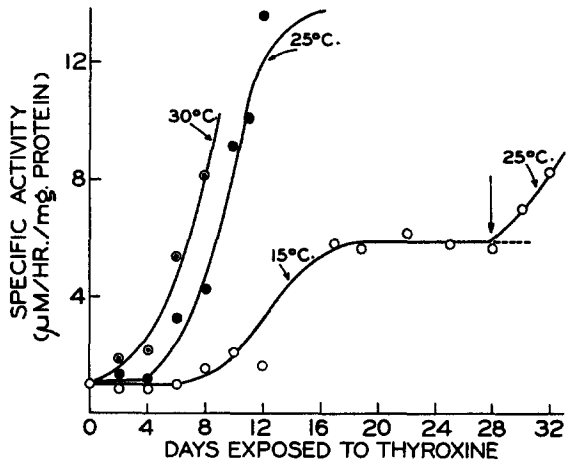

FIGURE 3. Effect of temperature on the rate of increase in carbamyl phosphate synthetase activity. The straight arrow indicates the time at which the tadpoles, previously maintained at 15°C., were brought to 25°C.

Concomitant Urea Production A parallel increase in the production of urea with the increase in carbamyl phosphate synthetase activity was observed (Fig. 5). These data are in good agreement with the findings of Munro, who employed thyroid extract (4).

Relationship of Carbamyl Phosphate Synthetase Synthesis and Morphological Changes Having established conditions under which the synthesis of carbamyl

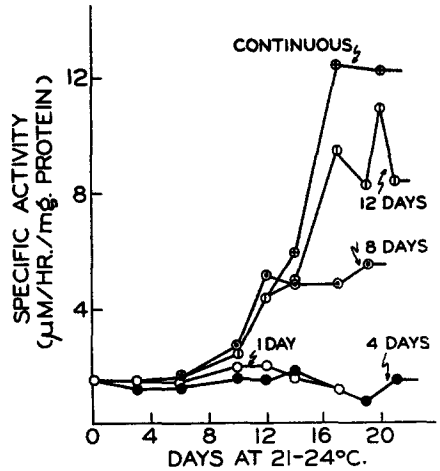

FIGURE 4. Effect of the dose of thyroxine on the rate of carbamyl phosphate synthetase synthesis. The number of days the tadpoles were exposed to thyroxine is indicated for each series.

phosphate synthetase is accelerated by thyroxine, we felt it would be of interest to examine the degree of correlation between gross morphological changes and synthesis of carbamyl phosphate synthetase. In order to explore this question, we plotted specific activity of this enzyme during normal and

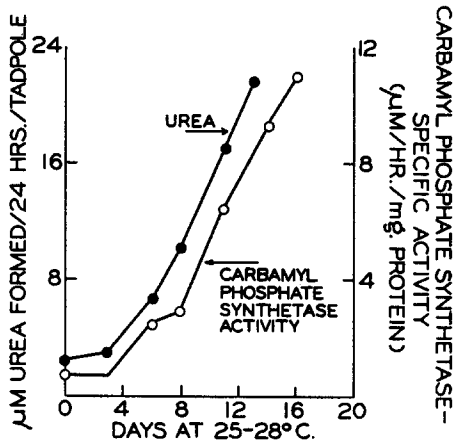

FIGURE 5. Relationship between the synthesis of carbamyl phosphate synthetase and urea production. Each point represents the average value from six tadpoles.

thyroxine-induced metamorphosis *vs.* the ratio of leg length/tail length. The latter was employed as the index of morphological change and was found to be readily correlated (5) with the criteria used by Taylor and Kollros (14).

As seen from Fig. 6, a striking difference is observed in the levels of carbamyl phosphate synthetase between the normal and thyroxine-treated animals. If

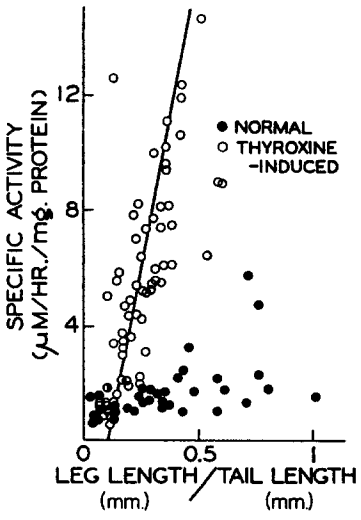

FIGURE 6. Plot of specific activity of carbamyl phosphate synthetase *vs.* the ratio of hind leg length (millimeters) and tail length (millimeters) during normal and thyroxine-induced metamorphosis. Open and closed circles represent thyroxine-induced and normal metamorphosis, respectively.

the rate of synthesis of carbamyl phosphate synthetase is taken as an arbitrary index of biochemical response, it is evident that the biochemical changes precede the morphological response to exogenous thyroxine. Similar findings have been made by Barch (8) and Dolphin and Freiden in the case of arginase (9).

Another implication of these findings is that thyroxine accelerates the synthesis of certain proteins selectively. This statement is further strengthened by the concomitant decrease in succinoxidase activity and the delayed

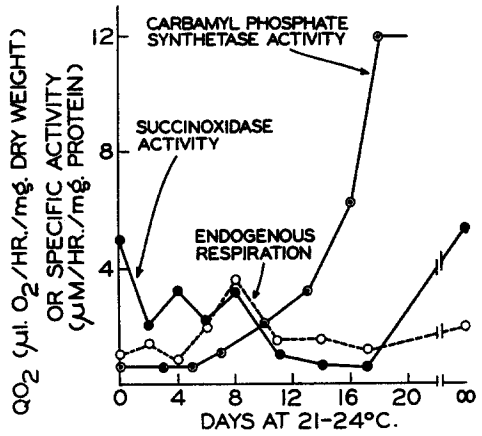

FIGURE 7. Relationship between carbamyl phosphate synthetase activity, succinoxidase activity, and endogenous respiration during thyroxine-induced metamorphosis. The activity of carbamyl phosphate synthetase is expressed as specific activity; succinoxidase activity and endogenous respiration are expressed as Q_{O_2} (microliters O_2 uptake per hour per milligram dry weight).

initiation of the increase in liver catalase activity. The latter findings will be discussed in greater detail below.

The Role of the Tail during the Thyroxine-Accelerated Synthesis of the Enzyme Removal of up to 60 per cent (length) of the tadpole's tail had no significant effect on the rate of synthesis of carbamyl phosphate synthetase.

Effect of Thyroxine on Succinoxidase Activity and Endogenous Respiration Tadpole liver homogenates oxidized succinate at a relatively low rate; glucose and pyruvate were not oxidized under the same conditions. Fig. 7 shows the relationship between the succinoxidase activity, endogenous respiration, and carbamyl phosphate synthetase activity during thyroxine-induced metamorphosis.

The first noticeable change after thyroxine treatment is a decrease in succinoxidase activity. In contrast, the endogenous respiration increases. When carbamyl phosphate synthetase increased about twofold the succin-

oxidase activity decreased considerably, and endogenous respiration reached a maximum. However, succinoxidase and endogenous respiration attain a minimum when carbamyl phosphate synthetase has reached maximum activity. Examination of the succinoxidase activity in the liver of the bull frog showed that the level of this enzyme is about the same as that of the tadpole liver prior to thyroxine treatment.

The significance of an increase or decrease in enzyme activity *in vivo* is often difficult to interpret. However, the above observation suggests that the

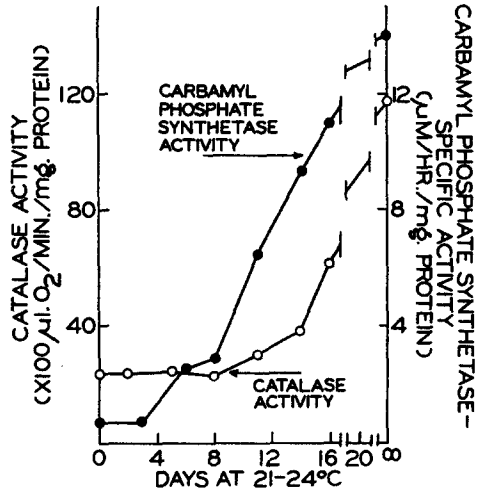

FIGURE 8. Liver catalase activity during thyroxine-induced metamorphosis as compared with carbamyl phosphate synthetase activity. Infinitesimal sign represents the levels found in the adult bull frog.

decrease in succinoxidase activity is indeed *due to a decrease in the enzyme protein itself.* Since starvation for as long as 2 months does not decrease either carbamyl phosphate synthetase or succinoxidase activities in the liver of the bull frog (unpublished studies), this observation is quite striking.

Since both succinoxidase and carbamyl phosphate synthetase are mitochondrial enzymes, the decrease in succinoxidase activity indicates that the increase in the activity of carbamyl phosphate synthetase is not merely the result of an increase in the number of mitochondria.

Liver Catalase vs. Carbamyl Phosphate Synthetase Catalase in livers of tumor-bearing animals decreases dramatically (20) and this enzyme can be formed adaptively by yeast (21) as a response to aeration.

Fig. 8 shows the relationship between the increased activity of catalase and of carbamyl phosphate synthetase. Up to the time that the activity of carbamyl phosphate synthetase has increased about fourfold, the catalase activity re-

TABLE I

EFFECT OF DIFFERENT SUBSTANCES ON CARBAMYL PHOSPHATE SYNTHETASE SYNTHESIS INDUCED BY THYROXINE

Agents added to thyroxine solution*	Final concentration	Effect
5-Fluoroorotic acid	2.9×10^{-4} M	None
Dinitrophenol	2.7×10^{-7} M	None
Arginine	1.0×10^{-4} M	None
Chloramphenicol	6.2×10^{-4} M	Inhibits
Thiouracil	3.5×10^{-3} M	Inhibits

* Thyroxine was maintained at 0.02 μg. per ml. concentration (2.6×10^{-8} M).

mains unchanged. After this interval, the activity of catalase begins to increase and reaches a plateau at the same time as does carbamyl phosphate synthetase. This level is about five times that which is observed in the premetamorphic tadpole.

The Effect of Some Chemical Agents on the Synthesis of Carbamyl Phosphate Synthetase Induced by Thyroxine Several chemical agents and antibiotics were studied in the hope that they would aid in the interpretation of the mechanism of thyroxine action on protein synthesis. However, significant results were observed only with chloramphenicol and thiouracil. Further studies of the effect of these agents on protein synthesis in this system are promising and are in progress.

Table I lists the agents employed, the concentrations used, and the effects observed. 5-fluoroorotic acid has been found to inhibit the biosynthesis of

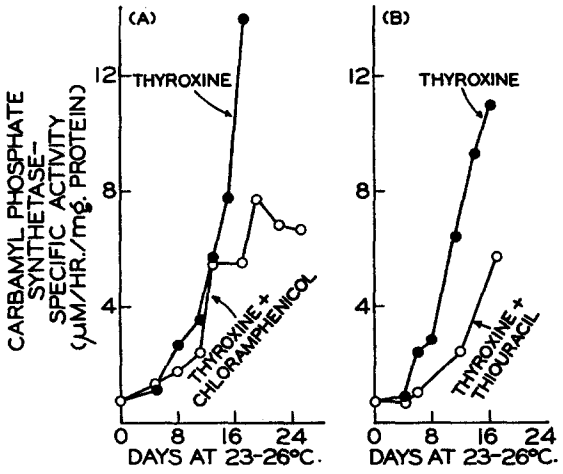

FIGURE 9. Effect of chloramphenicol and thiouracil on carbamyl phosphate synthetase synthesis induced by thyroxine. (A) thyroxine + chloramphenicol, (B) thyroxine + thiouracil. The concentrations used are listed in Table I.

RNA and DNA in the rat (22). However, this compound has no effect on protein synthesis induced by thyroxine. P^{32} incorporation experiments showed that 5-fluoroorotic acid does not inhibit the turnover of either RNA or DNA in tadpole liver. There is a similarity of action between thyroxine and dinitrophenol in that both agents increase oxidation and uncouple oxidative phosphorylation. Dinitrophenol has been reported previously to inhibit metamorphosis of the tadpole (23). However, dinitrophenol had no effect on the synthesis of carbamyl phosphate synthetase at the concentration reported in Table I. Thus again, a difference is observed in the response of morphological as compared with biochemical changes.

As seen in Table I, chloramphenicol and thiouracil inhibit protein synthesis induced by thyroxine. However, the mode of action appears to be quite different in the two cases since the effect of the chloramphenicol becomes apparent at a later stage of development while that of thiouracil is apparent from the start. The results are shown in Fig. 9A and B. Chloramphenicol has been shown to inhibit amino acid incorporation into bacterial protein (24). It is of some interest to compare the temperature effect (15°C.-Fig. 3) and the effect of chloramphenicol on carbamyl phosphate synthetase. A striking similarity is seen in the two effects. The possibility exists that a common enzymatic step is involved which is blocked in the one case by the low temperature (15°C.) and in the other by chloramphenicol.

The effect of thiouracil (Fig. 9B), which is used as an antithyroid agent and is considered to interfere with the synthesis of thyroxine, on carbamyl phosphate synthetase synthesis is striking. A satisfactory explanation for this effect is not possible with the experimental data available. Further studies of this effect are under way.

DISCUSSION

The biological significance of an increase in activity of any enzyme *in vivo* is apt to be difficult to interpret. The increase may be caused by an increase in enzyme protein, activation of a precursor, destruction of an inhibitor, changes in permeability, etc.

In the present study, however, the increase of carbamyl phosphate synthetase as a result of thyroxine treatment is known to be the result of *de novo* protein synthesis on the basis of immunological and C^{14}-labeled leucine incorporation studies. As far as we are aware, the observations reported in this paper represent the first instance in which an actual relationship between synthesis of a specific protein molecule and thyroxine has been demonstrated.

A relationship between the thyroid gland and protein synthesis has been suggested since the early observation that hypothyroidism in young mammals

results in cretinism. By using liver slices from the rats treated with thyroxine *in vivo*, Lipmann and DuToit observed an increased incorporation of radioactive alanine (25). More recently, Sokoloff and Kaufman demonstrated that thyroxine pretreatment *in vivo* and thyroxine *in vitro* could increase the rate of C^{14}-labeled DL-leucine incorporation into the mitochondrial fraction (26).

However, the fact that the accelerating effect of thyroxine on protein synthesis does not affect all enzymes, but rather involves some degree of specificity, is seen from the following observations in the present study: (*a*) succinoxidase decreased while carbamyl phosphate synthetase increased; (*b*) liver catalase activity responded to exogenous thyroxine more slowly than carbamyl phosphate synthetase; (*c*) the combined effects of thyroxine and chloramphenicol, and exogenous thyroxine and thiouracil inhibited significantly the synthesis of carbamyl phosphate synthetase, but not morphological changes; and (*d*) the relationship between the increase in carbamyl phosphate synthetase activity and leg length/tail length was greatly altered during the thyroxine-induced metamorphosis. It should be emphasized that the increase in carbamyl phosphate synthetase activity occurred before any noticeable changes in the alimentary tract which has been reported by Swingle to be the first to be affected by thyroid administration (27).

The observation that the enzymatic changes under study preceded the morphological response to exogenous thyroxine probably explains the apparent discrepancy found in the acceleration of morphological metamorphosis by acidic media. The observation that acidic media could accelerate morphological changes, but not carbamyl phosphate synthetase synthesis, suggests that the factors operating in the tadpole for the synthesis of the enzymes of the urea cycle are not intimately correlated with the mechanism leading to the development of gross morphological changes.

The decrease in succinoxidase activity is evidence against the hypothesis that the increase of carbamyl phosphate synthetase is the result of an increase in the number of mitochondria during metamorphosis. However, this possibility is not excluded and more direct studies in this regard are now in progress.

The increase in carbamyl phosphate synthetase, which occurred in the absence of exogenous food as well as in the absence of approximately 60 per cent of the tail, indicates that some preexisting source of protein is converted for synthesis of this enzyme. The decrease in succinoxidase activity, ahead of the increase in carbamyl phosphate synthetase synthesis, might be interpreted to mean that some protein component(s) of the former system is being utilized for the synthesis of the latter.

The uncoupling effect of thyroxine on oxidative phosphorylation (28) as well as the swelling action on the mitochondrial membrane (29) cannot offer a complete explanation for the physiological mechanism of this hormone, because of the following considerations: (*a*) rather high unphysiological

concentrations of thyroxine are required; (*b*) both D- and L-thyroxine are active (26) for uncoupling oxidative phosphorylation. Furthermore, in the present experiments the uncoupling agent, dinitrophenol, does not accelerate the synthesis of carbamyl phosphate synthetase as did thyroxine. In addition to the above evidence, Sokoloff and Kaufman observed that with thyroxine concentrations between 1×10^{-7} to 1×10^{-4} M increasing, graded effects occurred in the incorporation of radioactive amino acid (26). However, at 1×10^{-3} M, the effect of the uncoupling of oxidative phosphorylation superseded, and a marked inhibition of amino acid incorporation occurred, indicating a qualitatively different phenomenon.

As early as 1919, Kendall pointed out that the acceleration of tadpole metamorphosis and the increase in basal metabolism (calorigenic effect) were two different phenomena in that the acetyl derivative of thyroxine did not affect the metabolic rate in the patient, but had about the same effect on metamorphosis of the tadpole as thyroxine (30). This difference has also been observed by other investigators (31).

There is thus to date no completely satisfactory hypothesis to account for the action of thyroxine *in vivo*. Since the ability of thyroxine to stimulate the synthesis of a specific, enzymatically functional protein was demonstrated *in vivo* in this series of experiments, it will be of interest to test for this effect *in vitro*. The combined use of chloramphenicol and other blocking agents may be of help in elucidating this problem.

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