AN IN VITRO SYSTEM FOR STUDYING THE MECHANISM OF ACTION OF THYROID HORMONES

by

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INTRODUCTION

Explanations of how thyroid hormones function were offered even before the chemical identities of the hormones were established. After the isolation of thyroxine from thyroid gland by Kendall (1) in 1914, the properties of the hormone were appreciated. The analogue of thyroxine, 3,5,3'-triiodothyronine (T_3) was isolated from beef thyroid and characterized by Gross and Pitt-Rivers in 1953 (2). Much effort was directed toward the elucidation of the mechanisms of action of thyroid hormones. The number of proposed mechanisms has accumulated over the years, whereas the acceptance of any given explanation has varied according to the extent of understanding about the metabolic activities of the cell at that time. A succession of ideas has not yet answered how the hormones elicit their multiple biological actions.

The main physiological actions of thyroid hormones at whole body level are as follows:

(a) The calorigenic action or capacity to stimulate basal metabolic rate (BMR).

Foster (3) in 1927 observed that tissues from thyroidectomized rats showed a decreased oxygen consumption while those tissues isolated from thyroid-fed animals showed a characteristic high oxygen consumption. Whether or not the calorigenic action is a

direct expression of a fundamental molecular action, the relationship between BMR and thyroid hormones is quantitative if the administration of excessive amounts of the hormones is avoided (4, 5).

(b) Growth and development

Thyroid hormones are indispensable for general body growth and maturation of special tissues, such as bone and the central nervous system of most vertebrates. The well-known dwarfism associated with hypothyroidism at early stages in life is a classical example. Unlike the calorigenic action, the growth promoting effect of the hormones diminishes with the age of the animal.

(c) Anabolic and catabolic effects with respect to protein, lipid, and other body constituents.

It has been known that hypothyroid subjects have a lower capacity to synthesize protein than do euthyroid subjects. The administration of T_3 to hypothyroid subjects restored the low rate of protein synthesis to normal, while administration of the same dose of T_3 to normal subjects caused an apparent lowering of the rate of protein synthesis (6). The lowering of serum cholesterol in hyperthyroidism or after thyroid hormone administration emphasizes the catabolic action of the hormones (7).

Among other cellular and extracellular components whose levels or turnover rates are influenced by thyroid hormones are: carbohydrates, minerals, water, electrolytes, vitamins, and coenzymes.

(d) Influence on physiological functions of organs.

A number of important physiological functions, such as skeletal muscle efficiency, electrical activity of brain, cardiovascular function, spermatogenesis, ovulation, and lactation are directly or indirectly under the control of thyroid activity.

Over the years, thyroid hormone-induced morphological changes in mitochondria have been observed both *in vivo* and *in vitro*. Tapley *et al.* (8) reported that at 10^{-5} M thyroxine caused a rapid swelling of mitochondria. Mitochondria from brain, spleen, and testis of hyperthyroid animals do not swell and do not consume oxygen as fast upon treatment with thyroid hormone, whereas mitochondria from liver, kidney, and muscle tissues do swell and consume more oxygen (9, 10). Administration of thyroxine resulted in an increase in the number of mitochondria as well as of cristae mitochondria (11). Furthermore, mitochondria isolated from thyroxine-treated or hyperthyroid rats have been shown to be more fragile than those from normal rats, and mitochondria from hypothyroid rats were less fragile (12). Thyroxine-induced mitochondria swelling could be reversed by ATP *in vitro* which was recognized as a physiologically reversible phenomenon (13). Thyroxine analogues with biological activities also induced swelling but the relationship between the biological activity and swelling action was not quantitative (9, 12).

Lardy, Martus, and Lipmann reported in 1951 that the addition of thyroxine to isolated liver mitochondria uncoupled oxidative phosphorylation (14-16). Later, Bronk (17, 18) observed that the addition of thyroxine reduced the P:O ratio of mitochondrial particles. Such an inhibitory effect was associated with the inhibition of ATP-^{32}P exchange by thyroxine. They then suggested that the inhibition of the terminal phosphate transferring steps of the phosphorylation provided the basis for some of the action of thyroid hormones (19).

The significance of the effect of thyroxine on the morphology of mitochondria in relation to intracellular processes in vivo largely remains as speculation, since it is an open question whether the uncoupling of oxidative phosphorylation produced in vitro with thyroxine is related to the physiological effects of the hormone in vivo. Suggestions were made that thyroxine exerts its effect on mitochondrial membranes by altering the entry and exit of substances involved in cellular respiratory activity and energy metabolism (20). As yet there is no conclusive evidence for such a regulatory role.

Tipton et al. (21) observed in 1946 that the administration of desiccated thyroid produced an increase in skeletal muscle succin-oxidase activity. Enhancement in the activity of such enzyme was also observed in heart, liver, kidney, and brain of hyperthyroidism

(22-24). Since then activities of various enzymes have been noted to be altered by the *in vivo* administration of thyroid hormones.

DeToit (25) reported in 1952 that the incorporation of radioactive alanine into protein in liver slices was depressed after thyroidectomy and stimulated after the administration of large doses of thyroxine. This suggested that hormones may regulate the quantity of enzyme(s) rather than act on individual enzyme(s) and it became apparent that the regulation of protein and nucleic acid turnover may be an important locus of thyroid hormone action.

Sokoloff and his colleagues undertook to study the effects of thyroid hormones on the incorporation of labeled amino acids into proteins by cell-free preparations (26-33). Their findings were that the rate of amino acid incorporation into protein in cell-free rat liver homogenates was stimulated by L-thyroxine administered *in vivo* or added *in vitro*. Thyroidectomy resulted in a reduction of the incorporation rate (27). The *in vitro* effect of L-thyroxine was dependent on the presence of mitochondria and an oxidizable substrate; their replacement by a creatine phosphate-ATP-generating system eliminated such effect (32).

D-thyroxine, which is physiologically inactive, failed to stimulate amino acid incorporation into protein *in vivo*, but when added directly to cell-free homogenates, it was as potent as the L-isomer in uncoupling oxidative phosphorylation (34, 35), mitochondrial swelling, and amino acid incorporation (27, 32). On the

other hand, the physiologically active analogue, L-T_3 was effective in stimulating the incorporation of amino acid into protein when administered *in vivo*, but was much less effective when added *in vitro* (26).

Sokoloff *et al.* (27-33) then concluded that the stimulation of protein synthesis *in vitro* by thyroxine was localized at the steps involving the transfer of soluble RNA-bound amino acid to microsomal or ribosomal protein in protein biosynthesis. The soluble RNA-amino acid transfer hypothesis was supported by the fact that the calorigenic action of thyroxine was inhibited by puromycin which is believed to inhibit the step of final assembly of new protein at ribosomal level (36).

Since *in vitro* D-thyroxine was just as effective as L-thyroxine, while L-T_3 was less effective in stimulating amino acid incorporation into protein, and no stimulatory effect on protein synthesis was observed when L-thyroxine was added to liver of thyroidectomized rats, it seems unlikely that the soluble RNA-amino acid transfer step in protein synthesis is the primary site of action of the hormone. In addition, the stimulation of protein synthesis by thyroid hormones was noted to occur some time after the administration of the hormone to an intact rat (37).

Tata and his colleagues (38) reported that the rate of incorporation of amino acid into protein by microsomes from livers of thyroidectomized rats was half or one-third that of normal rats. Microsomal RNA content was also lower in the thyroidectomized rats.

Both the capacity to incorporate amino acid into protein and the microsomal RNA:protein ratio returned to normal after a single injection of thyroid hormones. Similar results were observed by Bronk (39).

Time studies on the response of cellular activity to a single injection of thyroid hormones to thyroidectomized rats during the latent period of hormone action by Tata and his associates (37, 38, 40-45) indicated that the sequence of events were: stimulation of the turnover and synthesis of rapidly labeled nuclear RNA, at 3 to 4 hours after the hormone administration; stimulation of RNA polymerase in liver nuclei, at 10 to 12 hours; stimulation of amino acid incorporation into mitochondrial and microsomal protein, and mitochondrial respiration, at 30 to 36 hours. They were, however, unsuccessful in demonstrating the stimulatory effect of thyroid hormones on DNA-dependent RNA polymerase activity when the hormones were added in vitro to isolated nuclei. Also thus far, no reports have appeared which demonstrate a hormonal stimulatory effect on nuclear RNA synthesis in a cell-free system.

Considering the fact that the stimulation of nuclear RNA turnover and DNA-dependent RNA polymerase activity preceded that of cytoplasmic protein synthesis and being well within the latent period of BMR stimulation (which was about 45 hours after thyroid hormone administration), it might be suggested that the site of

action of thyroid hormones may be either in the nucleus or at some steps regulating nucleic acid metabolism. The calorigenic and growth-promoting actions of the hormone may be mediated through the control of protein synthetic activity. Such an hypothesis was supported by the reports which indicated that protein synthesis inhibitors, such as actinomycin D (suppresses m-RNA formation) (46, 47), 5-fluorouracil (acts as an anti-metabolite for RNA synthesis) (36), puromycin (inhibits the final assembling step of new protein at ribosomal level) (36), and starvation (affects turnover of RNA and protein synthesis, etc.) (48), suppress not only the growth-promoting action but also the calorigenic action of the hormone (42). Therefore, a normal function of protein synthetic mechanism is essential for thyroid hormones to elicit their calorigenic and growth-promoting actions.

Early in 1960, Finamore and Frieden (49) had shown that RNA turnover and synthesis in tadpole liver was markedly stimulated by thyroid hormones. Frieden (50) then indicated that thyroid hormone-induced metamorphosis in frogs and toads was accompanied by dramatic changes in the protein and enzymatic constituents of almost every tissue. These changes in protein distribution were accompanied by stimulation of labeled amino acid incorporation into proteins (51, 52). Later Necheles (53) observed that at a concentration of 10^{-7} M, L-thyroxine stimulated purine biosynthesis and the

incorporation of glycine-2-^{14}C into the RNA of rabbit bone marrow slices. More recently Nakagawa *et al*. (54) have shown that thyroid hormones enhanced new ribosomal RNA synthesis in tadpole liver preceding the induction of carbamyl phosphate synthetase as an early response to L-thyroxine treatment. Kim and Cohen (55) also present evidence that thyroid hormones increase the template activity of the chromatin of tadpole liver but the hormones have no effect *in vitro*. These reports strongly suggest that the effect of thyroid hormones on cellular metabolism is mediated through the nucleus of the cell.

Cahilly and Ackerman (56) observed that liver RNA content in the hypothyroid rats was significantly less than that of normal rats and a decreased liver protein synthesis in goitrogen-fed rats was increased by thyroid hormone treatment. They also indicated that the availability of purine nucleotides were somewhat disturbed in hypothyroid rats.

As a consequence of the investigation of the effect of thyroid hormones on the biosynthesis of purine nucleotides, Mah and Ackerman (57) demonstrated that L-thyroxine at 10^{-5} M or L-T_3 at 10^{-7} M when added to the supernatant fraction of rat liver stimulated the incorporation of glycine-1-^{14}C into total purines with a preferential stimulation of the incorporation into adenine. Later, it was shown that the reaction stimulated by thyroid hormones (10^{-9}M of L-T_3 or

10^{-5}M of L-thyroxine) is the conversion of IMP to AMP (58). Their subsequent work indicated that the stimulatory effect on AMP synthesis from IMP by thyroid hormones is through the enhancement of adenylosuccinate synthetase activity. This enzyme catalyzes the reaction: IMP + aspartate + GTP $\xrightleftharpoons{Mg^{++}}$ AMP + fumaric acid + GDP + orthophosphate. This enzyme has been partially purified from the soluble fraction of rat liver (59).

The *in vitro* response of the enzyme to D-thyroxine and other analogues which are physiologically inactive, was well correlated with their *in vivo* effects. Analogues which are physiologically active also stimulated the reaction *in vitro*.

Later, it was observed by Al-Mudhaffar and Ackerman (60) that adenylosuccinate synthetase activity was low in rat brain and testis, and the enzyme isolated from such tissues did not respond to thyroid hormones as did the liver enzyme. Since the respiration of brain and testis tissues from adult animals does not respond to thyroid hormone status (61), the above observation was significant. It is known (62) that thyroidectomy has little or no effect on respiration of rat brain or testis, whereas the respiration of other tissues such as liver, kidney, heart, and skeletal muscle falls rapidly to levels characteristic of the hypothyroid condition.

Since the earlier observation by Mah and Ackerman (57) suggested that thyroid hormones inhibit the synthesis of GMP from IMP, this

pathway was investigated further. IMP-dehydrogenase, which catalyzes the reaction:

$$\text{IMP} + \text{DPN}^{+} \rightleftharpoons \text{xanthine-5'-phosphate} + \text{DPNH} + \text{H}^{+}$$

was partially purified from rat liver (60). It was found that the enzyme activity was inhibited both by L-T_3 at 10^{-9} M and by L-thyroxine at 10^{-7} M. D-Thyroxine showed no inhibitory effect, and other analogues, inactive *in vivo*, also failed to inhibit the reaction, whereas analogues, active *in vivo*, inhibited the reaction.

The present studies were concerned primarily with the investigation of the *in vitro* effect of thyroid hormones on the incorporation of orotic acid-6-^{14}C into nuclear RNA in a cell-free rat liver system with the inclusion of IMP in the incubation medium and the effect of different ratios of ATP/GTP on the incorporation of UTP-^{3}H into nuclear RNA in a purified rat liver nuclei system.

If the sites of action of thyroid hormones are the hormonal control of adenylosuccinate synthetase and IMP-dehydrogenase as proposed by earlier observations (57-60) and localized outside of the nucleus, one should be able to demonstrate a thyroid hormone-induced stimulation of nuclear RNA synthesis *in vitro* by the inclusion of IMP in the incubation medium using rat liver homogenates as enzyme sources. In view of this, an *in vitro* system has been devised.

Since thyroid hormones were observed to stimulate AMP synthesis and inhibit GMP synthesis, it suggested that thyroid hormones maintain a high cellular AMP/GMP ratio. The question then arises, how does a high AMP/GMP ratio produce effects attributable to thyroid hormones? It was easy to visualize that a control of AMP synthesis would regulate the availability of adenine nucleotides for ATP synthesis in the mitochondria, thereby producing other effects as a result of an allosteric effect of adenine nucleotides on certain key enzymes (63). However, the significance of an inhibition of GMP synthesis by the hormone was not clear. An *in vitro* system was needed to investigate this aspect further.

It was then reasoned that if thyroid hormones exert their effect by stimulating AMP synthesis concomitant with an inhibition of GMP synthesis (57-60), or by maintaining a high AMP/GMP ratio, a high ATP/GTP ratio would be one possible result of these effects. Therefore, a high ATP/GTP ratio in the incubation medium should stimulate nuclear RNA synthesis. To test this hypothesis, an *in vitro* system was also devised.

The results demonstrated that thyroid hormones stimulated the nuclear RNA synthesis *in vitro* in cell-free rat liver homogenate system in the presence of IMP. No stimulatory effect was observed when IMP was replaced by AMP and GMP in the system. There was also

no effect by the hormone on the incorporation of UTP-^{3}H into nuclear RNA in isolated nuclei systems. This is evidence that the site of action of the hormone is in the cytoplasm at the reactions concerning the metabolism of IMP to AMP and GMP. It was also observed that a high ATP/GTP ratio stimulated nuclear RNA synthesis and the relative concentrations of the purine nucleotides in the system was the key factor in observing such a stimulatory effect. Therefore, the maintenance of a high cellular adenine nucleotide/guanine nucleotide ratio is a possible consequence of thyroid hormone action in the cell.

EXPERIMENTAL PROCEDURE

Materials

Reagents

Orotic acid-6-^{14}C (specific activity, 4.18 mC per mmole) was purchased from New England Nuclear. Uridine 5'-triphosphate-^{3}H tetralithium (UTP-^{3}H) (specific activity, 2.24 C per mmole), cytidine 5'-triphosphate (CTP), adenosine 5'-monophosphate (AMP), and guanosine 5'-monophosphate (GMP) were obtained from Schwarz. Inosine 5'-monophosphate (IMP), adenosine 5'-triphosphate (ATP), uridine 5'-triphosphate (UTP), and d-ribose-5'-phosphate were purchased from Sigma Chemical Co. Guanosine 5'-triphosphate (GTP) and diphosphopyridine nucleotide (DPN^{+}) were products of Calbiochem. Actinomycin D was purchased from Merck, Sharp and Dohme Research Labs. Deoxyribonuclease 1 (DNase) was obtained from Worthington Biochemical Corp. 2-Mercaptoethanol was purchased from Matheson Coleman and Bell. L-Thyroxine (L-T_4) and 3,5,3'-triiodo-L-thyroxine (L-T_3) were the products of Sigma Chemical Co. L-Thyroxine was recrystallized from sodium carbonate and ethanol by a method described by Harington (64), mp 232-234°, and L-T_3 was recrystallized in boiling 2N HCl as described by Pitt-Rivers *et al.* (61), mp 201-203°.

Animals

Sprague-Dawley male rats, 8-9 weeks of age, were used throughout these studies. Rats were fed a diet containing 1% sulfaguanidine until growth arrest was established. After

the fifth week of goitrogen feeding, such rats gain less than 3 g per week and weigh 110-140 g (65) due to an almost complete cessation of thyroid hormone synthesis.

Methods

Preparation of Homogenates

Homogenates were prepared fresh for each experiment, and the homogenization procedures were performed in a room in which the temperature was maintained at 4^{o}. The rats were killed by a blow on the head and decapitated. Their livers were quickly removed, chilled, weighed, and rinsed once with 0.25 M sucrose containing 1.8 mM $CaCl_2$ and 1 mM 2-mercaptoethanol previously cooled to 0^{o}. Tissues were maintained between 0^{o} and 4^{o} throughout all subsequent operations. After draining off the sucrose solution, the livers were minced first with scissors in a small beaker and then homogenized in 2 ml of 0.25 M sucrose (containing 1.8 mM $CaCl_2$ and 1 mM 2-mercaptoethanol), per g of tissue by means of a motor-driven, loose-fitting, all-glass Potter-Elvehjem homogenizer. The homogenization was continued until no more large pieces of tissue was visible.

Preparation of Nuclei

Nuclei from rat livers were prepared by the method of Blobel and Potter (66) with slight modification according to the nature of the experiment. Seven milliliters of homogenate, prepared as described

above were pipetted into a polyallomer tube that fits the SW 25.1 Spinco rotor. To this was added 14 ml of 2.3 M sucrose in TKM (0.05 M Tris-HCl, pH 7.5, at 25°; 0.025 M KCl; and 0.005 M $MgCl_2$) by means of a syringe with a 13-gauge needle. This was thoroughly mixed with the 0.25 M sucrose homogenate by inversion. The mixture was then underlaid with 7.0 ml of 2.3 M sucrose in TKM with a syringe and a 13-gauge needle. The tip of the needle was placed at the bottom of the tube and the heavy sucrose solution introduced, forcing the lighter homogenate upward. After 2 hours centrifugation at 25,000 rpm in a Spinco SW 25.1 rotor (53,500 x g_{av}) at 0°-4°, the supernatant was decanted. The wall of the tube was wiped dry with tissue paper wrapped around a spatula. The white nuclear pellet was taken up in cold de-ionized water and analyzed for nuclear RNA and DNA, or taken up in 0.25 M sucrose: 1.8 mM $CaCl_2$ and used in the procedures described under incubation System II below.

Incubation

Two different systems were followed according to the nature of the experiment; these will be referred to as Systems I and II.

System I: Incubation was carried out in air in polyallomer centrifuge tubes shaken continuously at 37°. The components of the incubation mixtures and the specific incubation conditions were described in the

legends to the tables. All solutions were prepared in Tris-phosphate buffer (0.05 M, pH 7.4) and were brought to pH 7.0-7.4. L-T_3 or L-thyroxine, when added *in vitro*, was first dissolved in a small volume of 0.01 N NaOH, and then diluted to appropriate concentrations with 0.05 M Tris-phosphate buffer (pH 7.4). The liver homogenates were added last to the reaction mixture. Tubes and solutions were kept in ice between all additions. Incubation was begun after the reaction mixtures had been mixed thoroughly. At the end of incubation, the reaction was terminated by rapid cooling in ice (67). Nuclei were isolated by a modified procedure of Blobel and Potter (66) as follows:

To the terminated reaction mixtures were added 3.0 ml of ice cold 0.417 M sucrose in TKM and mixed. To this was added 14.0 ml of 2.3 M sucrose in TKM and mixed thoroughly by inversion. Finally the mixture was underlaid by 7.0 ml of 2.3 M sucrose in TKM. After centrifugation at 53,000 x g_{av} for 2 hours, the nuclear pellets were taken up in cold de-ionized water and transferred to a 15 ml round bottom polyethylene centrifuge tube and subjected to analysis for nuclear RNA (68) and DNA (69).

System II: Nuclei were first isolated from liver homogenates, as described above with a 1.5-hour centrifugation time. The nuclear pellets from the three centrifuge tubes were pooled and were washed once with 0.25 M sucrose: 1.8 mM $CaCl_2$ and centrifuged at 5,000 rpm in a Servall refrigerated centrifuge to remove excess sucrose. The supernatant was discarded. The nuclear pellets were resuspended in

0.25 M sucrose: 1.8 mM $CaCl_2$ using a Potter-Elvehjem homogenizer and added to the reaction mixture. Incubation was carried out in air in 15 ml round bottom polyethylene centrifuge tubes shaken continuously at 37°. The components of the incubation mixtures and the specific incubation conditions were described in the legends to the tables. All solutions were prepared in Tris-phosphate buffer (0.05 M, pH 7.4): 1.0 mM 2-mercaptoethanol. The nuclei suspensions were added last to the mixture. Tubes and solutions were kept in ice until the reaction was initiated. Incubation was begun after thorough mixing of the reaction mixtures. The reaction was terminated by rapid cooling in ice, and subjected to nuclear RNA preparation (68).

Nuclear RNA

Nuclear RNA was analyzed by the method of Schmidt and Thannhauser with modifications recommended by Munro and Fleck (68). To the isolated nuclear pellet suspension of System 1 or the terminated reaction mixture of System II was added one-half volume of ice-cold 0.6 N perchloric acid. After thorough mixing and standing for 10 minutes in ice, the tube was centrifuged, and the supernatant fraction (acid-soluble) was discarded. The precipitate was washed twice with one volume of ice-cold 0.2 N perchloric acid equal to one-half the original volume. Excess acid was drained off by inverting the tube briefly on the filter paper. To the tissue residue 4 ml of 0.3 N KOH were added and mixed. After incubation for 1 hour at 37°, the

digest was cooled in ice, and the protein and DNA were precipitated by adding 2.5 ml of cold 1.2 N perchloric acid. After 10 minutes standing in ice, the precipitate was separated by centrifugation. One portion of the supernatant was transferred to a scintillation counting vial and counted for radioactivity. The precipitate was washed twice with 1.5 ml of 0.2 N perchloric acid. The remaining supernatant fluid from the first centrifugation and the washings were combined. The ultraviolet absorption of the solution at 260 mμ was measured in a Gilford Optical Density Convertor, Model 220 and a Beckman Spectrophotometer. The concentration of RNA was calculated on the basis that one optical density unit at 260 mμ of the RNA (after alkali hydrolysis) corresponded to 32 μg of RNA per ml for rat liver (68).

Measurement of Radioactivity

Samples containing ^{14}C or ^{3}H were measured in a Nuclear-Chicago Liquid Scintillation Counter. The counting efficiency was 60.3% for orotic acid-6-^{14}C and was 4.3% for UTP-^{3}H. A preset background counts of 38 counts per minute was adopted by the counter and all radioactive measurements were corrected for background. The scintillation fluid contained 4.0 g of 2,5-diphenyloxazole (PPO), 0.05 g of 1,4-bis-2-(4-methyl-5-phenyloxazolyl)benzene (POPOP), and 120.0 g naphthalene in 1.0 liter of p-dioxane. Fifteen milliliters of the scintillation fluid were added directly to 1.0 ml of radioactive ribonucleotide solution.

Miscellaneous Methods

DNA contents of an aliquote of nuclei suspension used per tube were estimated by the method of Webb and Levy (69).

RESULTS

The Effect of Thyroid Hormone In Vitro on Nuclear RNA Synthesis.

In order to demonstrate the effect of thyroid hormone-induced stimulation of nuclear RNA synthesis in vitro, rat liver homogenates were incubated with and without 3,5,3'-triiodo-thyronine (L-T_3) at 10^{-9} M in a medium containing IMP, orotic acid-6-^{14}C, and substrates necessary for the synthesis of AMP and GMP as described in incubation System I under "Methods" and in Table I. Table I illustrates that the addition of 10^{-9} M of L-T_3 in vitro in the presence of IMP markedly increased the incorporation of orotic acid-6-^{14}C into nuclear RNA. The increase was more than 300% over that of the control.

From the results shown in Table II, it can be seen that the stimulatory effect exerted by L-T_3 upon the rate of incorporation of orotic acid-6-^{14}C into nuclear RNA reached a maximum after a 2-hour incubation time under the experimental condition used. A 2-hour incubation time was adopted from hereon unless otherwise stated.

The Effect of Hormone Concentrations In Vitro. In the experiment shown in Table III, the concentration of thyroid hormones added to the system was varied. L-T_3 at 10^{-9} M, was more effective in stimulating orotic acid incorporation into RNA than was 10^{-7} M, and for L-thyroxine, a concentration of 10^{-5} M was more effective than 10^{-7} M. These results parallel the effects of L-T_3 and L-thyroxine on AMP and GMP synthesis. It was observed that a 10^{-9} M of L-T_3

was the optimum concentration for stimulating AMP synthesis and inhibiting GMP synthesis. Stimulation of AMP synthesis by L-thyroxine was optimal when the concentration of the hormone was 10^{-5} M (58). The experiments were handicapped by the fact that the Spinco SW 25.1 rotor, used to isolate the nuclei after incubation, has only 3 cups. Thus, only 3 variables could be studied in each experiment. Attempts to hold additional incubation vessels in ice resulted in a rapid loss of nuclear RNA.

Effect of Inhibitors of RNA Synthesis. Actinomycin D and DNase, well known as potent inhibitors of DNA-dependent RNA polymerase in bacterial and mammalian systems (46, 47), have been reported by Sokoloff et al.(32), Tata and Widnell (37), and Nakagawa et al. (54) to inhibit the synthesis of RNA in both control and thyroxine-treated animals. As can be seen in Table IV, the addition of 26.6 µg of DNase or 30.0 µg of actinomycin D to the incubation systems caused almost complete inhibition of orotic acid-^{14}C incorporation into nuclear RNA, whereas the addition of L-T_3 effectively stimulated the incorporation of orotic acid-^{14}C into nuclear RNA.

Synthesis of Nuclear RNA from AMP and GMP as Affected by Thyroid Hormone In Vitro. In order to further test the hypothesis that the sites of action of thyroid hormones are adenylosuccinate synthetase and IMP-dehydrogenase (or the synthesis of AMP and GMP from IMP) L-T_3

was added to a whole liver homogenate system in which AMP and GMP replaced IMP. As indicated in Table V, there was no stimulatory effect on nuclear RNA synthesis upon the addition of L-T_3 to the incubation medium when AMP and GMP replaced IMP. Thus, the stimulation by thyroid hormones was dependent on the presence of IMP (Tables I-V). These results tended to suggest that the primary hormone action occurred prior to the synthesis of nuclear RNA, or prior to the formation of ATP and GTP in the cytoplasm of the cell. This was in agreement with the hypothesis proposed.

Effects of Purine Nucleoside Triphosphates upon the Incorporation of UTP-^{3}H into Nuclear RNA. Since the results (Tables I-V) demonstrated that nuclear RNA synthesis could be stimulated by thyroid hormones *in vitro*, and if the hormones exerted their effect by stimulating AMP synthesis concomitant with an inhibition of GMP synthesis as suggested (57-60), it seems likely that the hormones would then regulate the quantity of ATP and GTP, which in turn would enhance the synthesis of certain nucleic acids, thus indirectly controlling the biosynthesis of specific enzymic protein(s). If this is correct, it should be possible to simulate the hormonal effect on nuclear RNA synthesis by incubating isolated nuclei with the proper ratio(s) of ATP and GTP. To test this hypothesis, an *in vitro* system referred to as System II under "Methods" was developed. In this system, isolated nuclei were incubated with various ratios of ATP/GTP in the presence of $MgCl_2$ and constant levels of CTP and UTP-^{3}H.

In the first experiment of Table VI, the total purine nucleotide concentration was kept constant at 3.0 µmole/1.5 ml, while the ratio of ATP/GTP was varied. The incorporation of UTP-^{3}H into nuclear RNA was increased with increasing ATP and decreasing GTP concentrations, or with increasing ATP/GTP ratios. When ATP/GTP ratio was 3/1, the stimulatory effect on nuclear RNA synthesis was 50% more than when the ratio was 1/1. As the ratio increased to 5/1, the stimulatory effect was increased to more than 100% that of the ratio of 1/1.

In Experiment II, the concentration of GTP was held constant at 0.45 µmole/1.5 ml while the concentration of ATP was increased. The results indicated that as the ATP concentration increased the incorporation of UTP-^{3}H increased. As the ratio of ATP/GTP varied from 1 to 5, 10, and 20, the stimulatory effect persisted with the greatest stimulation being observed when the ratio was 20/1.

In Experiment III, the concentration of ATP was kept constant (0.3 µmole/1.5 ml) while the GTP concentration was varied from 0.27 to 2.43 µmole/1.5 ml. GTP appeared to inhibit nuclear RNA synthesis, and the inhibitory effect increased with increasing GTP. When the ATP/GTP ratio was kept constant at 10/1 while the concentration of both ATP and GTP were increased at the same time, the stimulatory effect increased as the concentrations of the nucleotides were increased.

The previous observations may be summarized as follows:

(1) At a fixed ATP concentration, the incorporation of UTP-^{3}H into nuclear RNA increased as the GTP concentration decreased and it decreased as the GTP concentration increased.

(2) When the GTP concentration was fixed, the incorporation of UTP-^{3}H increased with increasing ATP concentration.

(3) The concentration of ATP relative to GTP, or the ATP/GTP ratio, was the key factor affecting UTP-^{3}H incorporation into nuclear RNA. As long as the ratio was greater than one, even with high GTP concentration, the stimulatory effect upon the UTP-^{3}H incorporation contributed by ATP would overcome the inhibitory effect exerted by GTP.

(4) When the ATP concentration was lower than that of GTP in the system, a decrease in RNA synthesis was observed. In other words, an ATP/GTP ratio less than one inhibited nuclear RNA synthesis. As can be seen in Experiment III of Table VI, an increase in the GTP concentration from 0.27 to 2.43 µmole resulted in a decrease in the specific activity (cpm per µg nuclear RNA) from 879.1 to 296.6.

Since a high ATP/GTP ratio does indeed enhance RNA synthesis, it seems likely that the stimulation of RNA synthesis by thyroid hormones is due to the fact that the hormones stimulate AMP synthesis, thereby maintaining a high cellular adenine nucleotide/guanine nucleotide ratio.

The time studies, as shown in Table VII, indicated that the specific activity of nuclear RNA increased up to 60 minutes incubation. After 75 minutes incubation the specific activity of the RNA was approximately one-half of that observed after 30 minutes of incubation.

Effects of Ribonucleoside Triphosphates, Actinomycin D, and DNase on Nuclear RNA Synthesis. As can be seen in Table VIII, the addition of 10 µg of actinomycin D or DNase to the system resulted in a marked inhibition of UTP-^{3}H incorporation into nuclear RNA. Also the incorporation was dependent on the presence of the three nucleoside triphosphates; namely, ATP, GTP, and CTP.

In order to further test the hypothesis that the enhancement of nuclear RNA synthesis by thyroid hormones is the result of the stimulation of AMP synthesis and the maintenance of a high ATP/GTP ratio, L-T_3 (10^{-9} M) was added to systems containing ATP/GTP ratios of 1 and 10. An increase in RNA synthesis was again observed in a system with an ATP/GTP ratio of 10 as compared to a ratio of 1, and there was no additional stimulation of nuclear RNA synthesis upon the addition of L-T_3.

In view of these results, it may be concluded that under the experimental conditions described, the nuclear RNA synthesized was a new synthesis of DNA-dependent RNA and it was the result of the stimulatory effect enhanced by the high ATP to GTP ratio in the system.

Since no additional stimulatory effect on nuclear RNA synthesis was induced by L-T_3 when added at 10^{-9} M to the complete system regardless of the relative concentrations between ATP and GTP, it was concluded that thyroid hormones are ineffective in promoting nuclear RNA synthesis at the nuclear level, or the site of action of the hormones is not in the nucleus nor is it concerned with the transport of the nucleotides into the nucleus. This observation supports the hypothesis that the site of action of thyroid hormones is in the cytoplasm and the results summarized in Table V suggested that the hormone exerts its effect prior to the synthesis of AMP and GMP.

The experimental data shown in Table VIII suggested that L-T_3 stimulated UTP-^{3}H incorporation when the ATP/GTP ratio was 1. This was tested again and the results are summarized in Table IX. UTP-^{3}H was incorporated into RNA to the same extent whether L-T_3 was present or absent. L-T_3 induced no stimulatory effect when incubated with isolated nuclei. These results further strengthened the observations and interpretations made in Table VIII, i.e., the primary sites of action of the hormones, are adenylosuccinate synthetase and IMP-dehydrogenase in cytoplasm. The possibility of the nucleus as a primary target site of the hormone action could be excluded.

When the concentrations of ATP and GTP were varied from 30 to 300 µmole/1.5 ml while holding the ATP/GTP ratio at 1, the rate of RNA synthesis apparently was not affected. Again, it was the

ATP/GTP ratio but not the concentrations of the individual purine nucleotide itself playing the key role in regulating the synthesis of RNA (Tables VI and IX).

DISCUSSION

An *in vitro* system for studying the effect of thyroid hormones on RNA synthesis has been devised. This investigation provides evidence that thyroid hormones added to cell-free rat liver homogenates in the presence of IMP stimulated nuclear RNA synthesis. It was demonstrated (Tables I-V) that the rate of incorporation of orotic acid-6-^{14}C into nuclear RNA was promoted by thyroid hormones. No stimulation was observed if IMP was replaced by AMP and GMP, thus the hormonal effect on RNA synthesis was IMP dependent. A biphasic effect of the hormoneswas suggested by the observation that L-T_3 at 10^{-9} M was more effective in stimulating RNA synthesis than was 10^{-7} M, and 10^{-5} M of L-thyroxine was more effective than 10^{-7} M (Table III). These observations correlate well with the biphasic effects of the hormones on AMP and GMP synthesis noted by others (57-60).

The incorporation of UTP-^{3}H into nuclear RNA was enhanced when the ATP/GTP ratio in the incubation medium was greater than one (Tables VI-IX). It was observed that the relative concentrations of the purine nucleotides had a profound effect on RNA synthesis. Therefore, the stimulation of RNA synthesis by thyroid hormones may be a consequence of the hormonal stimulation of AMP synthesis and the inhibition of GMP synthesis (57, 60). A high cellular ATP/GTP ratio would be one result of these effects, since the ATP and GTP levels are dependent on the AMP and GMP levels. Thyroid hormones had no

effect on the incorporation of UTP-^{3}H into RNA which is evidence that the site of action of the hormones is not in the nucleus nor is it concerned with the transport of the nucleotides into the nucleus. The site of action is in the cytoplasm, i.e., the conversion of IMP to AMP and GMP.

Since a high cellular ATP/GTP ratio as a consequence of thyroid hormone action stimulated the nuclear RNA synthesis and such a stimulation was markedly inhibited both by actinomycin D and DNase (Tables IV and VIII), it was reasoned that the hormone-induced nuclear RNA synthesis is DNA-dependent.

The *in vivo* responses of nucleic acid synthesis to thyroid hormones have been well documented, such as, an increase in the synthesis and turnover of rapidly labeled nuclear RNA, an increase in the DNA-dependent RNA polymerase activity after the *in vivo* administration of L-T_3 to thyroidectomized rats by Tata and Widnell (41, 44), an increase in RNA turnover following L-thyroxine treatment in tadpole liver by Finamore and Frieden (49), and a thyroxine-induced synthesis of a different kind of RNA in tadpole by Eaton, Cory, and Frieden (76). Kim and Cohen (55) also demonstrated that the administration of L-thyroxine to tadpoles resulted in a modification of chromatin to a more efficient template for RNA synthesis. However, attempts to demonstrate a hormone-induced stimulation of RNA synthesis *in vitro* were not successful. Widnell and Tata (41, 44) were unable to

demonstrate any stimulatory effect of the hormones on RNA polymerase activity *in vitro*. Kim and Cohen (55) also reported that the pre-incubation of both intact nuclei and isolated chromatin with L-thyroxine *in vitro* did not improve the template efficiency of chromatin for RNA synthesis.

The successful demonstration of an *in vitro* stimulation of nuclear RNA synthesis by thyroid hormones in this investigation resulted from the inclusion of IMP in the incubation medium along with other necessary substrates and cofactors for RNA synthesis. That the metabolism of IMP is the site of action of thyroid hormones stems from the observations made by Mah and Ackerman (57, 58) and Al-Mudhaffar and Ackerman (60) in which it was observed that thyroid hormones stimulated the incorporation of glycine-1-^{14}C into total purine with a preferential incorporation of the label into adenine and that the hormones stimulated adenylosuccinate synthetase activity. Also, GMP synthesis from IMP was inhibited by the hormones due to an inhibition of IMP-dehydrogenase activity.

Because of the low effective concentration of the hormones on adenylosuccinate synthetase and IMP-dehydrogenase, the high correlation of the effect of analogues with their *in vivo* responses and the importance of the purine nucleotides to RNA synthesis and energy metabolism, these two enzymes were seriously considered as the sites of thyroid hormone action, or the primary action of the hormones is the control of the conversion of IMP to AMP and GMP.

Also, it was observed by Necheles (53) that L-thyroxine at 10^{-7} M stimulated purine biosynthesis and this suggested that thyroid hormones may not act directly on the nucleic acids themselves but rather by controlling the availability of purine nucleotides for RNA synthesis.

It is of interest to note that the effect of thyroid hormones on adenylosuccinate synthetase and IMP-dehydrogenase is biphasic. It was observed (58) that the stimulation of AMP synthesis or the activity of adenylosuccinate synthetase was maximal with 10^{-9} M of L-T_3 or 10^{-5} M of L-thyroxine. As the concentration of the hormones increased, the stimulatory effect decreased. It was also observed that the maximal inhibitory effect of the hormones on IMP-dehydrogenase occurred with 10^{-7} M of L-thyroxine or 10^{-9} M of L-T_3, and as the concentration of these hormones increased, the inhibitory effect decreased (60). The biphasic effects of the hormones have also been noted by other workers. Necheles (53) observed a peak stimulatory effect on protein synthesis when L-thyroxine at 10^{-7} M was added to rabbit bone marrow slices. A slight variation in hormone concentration from 10^{-7} M caused a drastic decrease in the stimulatory effect. Malic dehydrogenase was inhibited by 10^{-5} M and stimulated by 10^{-6} M of L-thyroxine (71). As observed by Sugisawa (72), L-thyroxine at 7×10^{-7} M stimulated liver succinic dehydrogenase, but 1.4×10^{-6} M inhibited this enzyme. L-thyroxine and L-T_3 stimulated ascorbic acid oxidase at low concentration and

inhibited it at high concentration (73). It is known that small doses of thyroid hormones promote body growth whereas large doses will arrest growth or even cause a substantial weight loss (74, 75). Similarly, the anabolic effects of the hormones on protein and lipid metabolism and on glycolysis at near physiological level can be reversed with larger or pharmacological doses (76). These observations tend to suggest that the biphasic effect which has been observed in both intact animals and isolated tissue systems is one of the characteristics of thyroid hormone effects.

It is not clear at this point why thyroid hormones should inhibit GMP synthesis. It may imply a role for guanine nucleotide as an inhibitor of some key enzymes or as a repressor. It is also possible that it may have some physico-chemical relationship to the stability of histone-DNA complex. Further investigation on this aspect is needed. From the results of Tables VI-IX, no definite conclusion could be reached as to whether GTP function as an inhibitor whose effect was overcome by ATP, since the inhibition of nuclear RNA synthesis by GTP could be demonstrated only when the level of GTP concentration was equal to that of the ATP, regardless of the concentrations of each individual purine nucleotide, no inhibitory effect by the GTP could be demonstrated (Table IX), and when the GTP concentration was less than that of the ATP, a stimulation of RNA synthesis was observed (Table VI). One might reason that an increase in nuclear RNA synthesis by a high ATP/GTP ratio was merely due to an increase

in the supply of energy by ATP to the system. This does not appear to be the case, since the results in Tables VI and IX indicated that an increase in ATP concentration did not result in an increase in RNA synthesis as long as the ATP/GTP ratio was one. It is more likely that a high cellular ATP/GTP ratio as a result of thyroid hormone action enhances the synthesis of a specific RNA whose production is favored.

It is clear that nucleic acids play an important role in protein biosynthesis, and the role of thyroid hormones in controlling purine nucleotide metabolism (57-60) and in regulating nucleic acid metabolism (37, 49, 53, 54, 70) have been demonstrated. These accumulated results lead us to postulate the following working hypothesis: The sites of action of thyroid hormones are adenylosuccinate synthetase and IMP-dehydrogenase which result in a stimulation of AMP synthesis concomitant with an inhibition of GMP synthesis. The net effect is the maintenance of a high adenine nucleotide/guanine nucleotide ratio. A high ATP/GTP ratio enhances the synthesis of a specific RNA which promotes the synthesis of specific enzymic protein and consequently leads to a specific biological effect which results in a characteristic physiological action. A high ATP/GTP ratio produced by thyroid hormones functions as a mechanism for enhancing the transcription of DNA to m-RNA and the translation of the m-RNA to specific protein synthesis. Also, a control of adenine nucleotide synthesis would indirectly affect

other metabolic pathways by virtue of the fact that these nucleotides are allosteric effectors of a number of key enzymes in glycolysis and tricarboxylic acid cycle (63).

Thyroid hormones maintain a high ATP/GTP ratio which may, in turn, direct the synthesis of a specific RNA rich in adenine nucleotide content. The possibility that a high ATP/GTP ratio is a mechanism for directing the synthesis of a specific RNA is strengthened by the observation of Eaton, Cory, and Frieden (70). They reported a 2-3 fold increase in AMP/GMP labeling pattern of liver RNA from adenine-8-^{14}C following a 2-day exposure of tadpole to L-T_3, and concluded that a different kind of RNA has been synthesized.

Adenine rich nucleic acids have been isolated from different tissues. Hadjivassiliou and Brawerman (77) have isolated a nucleic acid from rat liver with low guanine and cytosine but high adenine. From Hela cells, Salzman, Shatkin, and Sebring (78) isolated a m-RNA with a relatively high AMP content as compared to GMP. The synthesis of such a RNA molecule would be affected if the supply of AMP was limited as would occur in hypothyroid rats. A rapid reappearance of such a molecule would be expected after hormone administration.

If the function of thyroid hormones is to maintain a supply of adenine nucleotide for the synthesis of specific RNA(s) as proposed, it may be expected that the activities of certain enzymes would be controlled indirectly by the hormones through their control of purine nucleotide synthesis. For example, Tatibana and Cohen (79)

have demonstrated an induction of the de novo synthesis of carbamyl phosphate synthetase in tadpole liver by thyroid hormones. The synthesis of this enzyme may have followed the synthesis of a m-RNA rich in adenine.

As pointed out by Tata and his collaborators, the overall increase in cytoplasmic protein synthesizing capacity and the stimulation of BMR after "acute" administration of thyroid hormones were proceeded by several hours by an enhanced turnover of nuclear RNA and a rise in the DNA-dependent RNA polymerase activity of nucleus (37, 38). These observations, along with our present findings, suggested that genetic expression in the nucleus prior to the formation of cytoplasmic protein may be an important locus of thyroid hormone action. Regulation of nucleic and protein synthesis may not be the only mechanism of thyroid hormone function, but the problems deserve to be explored further.

As reported by Kim and Cohen (55), administration of L-thyroxine to tadpoles caused the modification of chromatin prepared from liver nuclei, to a more efficient template for RNA synthesis. The addition of $(NH_4)_2SO_4$ to nuclei caused a dissociation of about 15-20 percent of nuclear histone associated with chromatin and resulted in an increased RNA synthesis. When chromatin prepared from both

thyroxine-treated and control tadpoles were deproteinized with CsCl, the deproteinized DNA showed equal template efficiency with a four-fold increase in RNA synthesis. They suggested that the molecule involved in repression of genetic activity is protein in nature.

Frenster (83) suggested that nuclear polyanions, RNA and protein may be involved in the regulation of genetic expression by their association with the nuclear histone fraction. There is no evidence as yet of any direct hormone-histone interaction. Recently, it has been shown by Hurlbert et al. (80) that histones are involved in control of RNA synthesis in nucleoli by selective suppression of DNA templates other than the template for ribosomal RNA, and since a high ATP/GTP enhances nuclear RNA synthesis, it could be the result of a direct involvement of ATP and GTP in genetic expression by their association with nuclear histone. Instead of the possible direct interaction of thyroid hormones with histone, the hormone may modify the protein moiety associated with chromatin via ATP and GTP. These nucleotides may exert their effects independently on the same target site but in opposite directions. ATP, for instance, may increase the template efficiency of chromatin for RNA synthesis through deproteinzation or derepression, while GTP prevents the derepression of a repressor and the transcription of DNA to m-RNA. The net result of these effects will depend on the relative importance of the individual effect in the system and the relative concentrations of these purine nucleotides present in the system for such function.

It was discovered in this study that the relative concentrations of purine nucleotides had a profound effect on RNA synthesis. It may suggest a control mechanism involving both purine and pyrimidine nucleotides. For example, by controlling the relative levels of these nucleotides, other metabolic pathways could be affected.

It will be worthwhile to investigate and to characterize different RNA molecules in thyroid hormone deficient and normal rats. Characterization of different RNA molecules synthesized as a consequence of thyroid hormone action may provide evidence for the synthesis of a specific RNA as suggested by Eaton et al. (70). For example, an increase in the synthesis of a m-RNA rich in A + U as a result of thyroid hormone action, would indicate a selective control of the synthesis of a specific m-RNA by the hormone, and explain the specificity of biological action of the hormones.

The concentration relationship between nuclear RNA and DNA as a consequence of thyroid hormone action and a comparison with that of the normal rats may be investigated. If thyroid hormones are to stimulate nuclear RNA synthesis without affecting the DNA content in the process, an increase in the nuclear RNA/DNA ratio in the hormone-treated animals will be expected.

By using a m-RNA resulting from the action of thyroid hormones as a template, a particular protein can be synthesized in vitro. By

analyzing the amino acid composition and pattern of the synthesized protein molecule and comparing them with known enzymic protein molecules, it may provide direct evidence for the hormonal control of the synthesis of a specific protein as proposed. The sequence of events of thyroid hormone action on protein synthesis and the site of action may be clarified.

If we are to assume that the AMP/GMP ratio regulates the ATP/GTP ratio, an increase in the AMP/GMP ratio in a cell-free liver homogenate may be expected to produce a similar stimulatory effect on nuclear RNA synthesis as was observed in an isolated nuclei system with an increasing ATP/GTP ratio.

It will be of great importance to determine the distribution of the nucleotides in rat tissues in different thyroid states and to compare them with the distribution of nucleotides in normal rats, and to establish the level and pattern of free nucleotides in various subcellular fractions since they are fundamental structural units of nucleic acids, function as a source of energy, and are allosteric regulators of specific enzymes in glycolysis and the tricarboxylic acid cycle (63).

The immediate experiments to be followed along this line of study will be the in vitro study of nuclear RNA synthesis in subfractions of nuclei.

Whatever the site of action of thyroid hormones is, our hypothesis is compatible with the physiological role of the hormones in growth and development, basal metabolic rate, anabolic effects on protein, lipid, carbohydrate, and other body constituents as well as on mitochondrial respiration. This occurs by way of selective control of nucleic acid and protein synthesis by regulating the synthesis of purine nucleotides.

SUMMARY

An *in vitro* system for studying the effect of thyroid hormones on nuclear RNA synthesis was devised. This investigation provides evidence that thyroid hormones added to cell-free rat liver homogenates in the presence of IMP and necessary substrates stimulated the incorporation of orotic acid-6-^{14}C into nuclear RNA. In order to observe the stimulatory effect exerted by the hormones, it was necessary to include IMP into the medium as a precursor of purine nucleotides. Thus, the hormone-induced stimulation of RNA synthesis was IMP-dependent. It was observed that L-T_3 at 10^{-9} M was more effective in stimulating nuclear RNA synthesis than was 10^{-7} M, and 10^{-5} M of L-thyroxine was more effective than 10^{-7} M.

If thyroid hormones exert their effects by stimulating AMP synthesis concomitant with an inhibition of GMP synthesis, the maintenance of a high ATP/GTP ratio would be one result of these effects. When an isolated nuclei system was incubated with various ratios of ATP/GTP, it was found that the incorporation of UTP-^{3}H into nuclear RNA was enhanced when the ATP/GTP ratio was greater than one as compared to that when the ratio was one, and it was inhibited when the ratio was less than one. The relative concentrations of the purine nucleotides was observed to have a profound effect on nuclear RNA synthesis. DNase and actinomycin D markedly inhibited the stimulatory effect exerted by a high ATP/GTP ratio.

Thyroid hormones had no effect on the incorporation of UTP-^{3}H into nuclear RNA which is evidence that the site of action of the hormones is not in the nucleus nor is it concerned with the transport of the nucleotides into the nucleus. The site of action of thyroid hormones is in the cytoplasm, i.e., the conversion of IMP to AMP and to GMP.

The accumulated results lead us to postulate the following hypothesis: The sites of action of thyroid hormones are adenylosuccinate synthetase and IMP-dehydrogenase which result in a stimulation of AMP synthesis concomitant with an inhibition of GMP synthesis. The net effect is to maintain a high adenine nucleotide/guanine nucleotide ratio. It was postulated that a high ATP/GTP ratio enhances the synthesis of a specific RNA which promotes the synthesis of a specific enzymic protein and consequently leads to a specific biological effect and a characteristic physiological action.

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APPENDICES

TABLE I

Effect of L-triiodothyronine *in vitro* on orotic acid-6-^{14}C incorporation into nuclear RNA

Complete system (in μmoles): Tris-phosphate buffer, pH 7.4, 100; DPN^+, 20; IMP, 80; GTP, 4; ATP, 4; d-ribose-5'-phosphate, 80; L-aspartic acid, 80; L-glutamine, 160; succinic acid, 40; Na_2HPO_4, 40; $MgCl_2$, 20; orotic acid-6-^{14}C (specific activity, 4.18 mC per mmole), 0.299 (1,676,606 cpm); and 2.0 ml of whole liver homogenates equivalent to the yield from 0.67 g of fresh liver, prepared as described under "Methods." The final concentration of L-T_3 when added was 10^{-9} M. The final volume was 4.0 ml. Incubation time at 37° was 2 hours. The reaction was terminated by rapid cooling in ice. Nuclear RNA was estimated by a modified Schmidt-Thannhauser procedure (as described under "Methods"). The concentration and the radioactivity of nuclear RNA were determined after alkaline hydrolysis. DNA content was measured.

System	Specific Activity			L-T_3 effect %
	mμmole orotic acid incorp. per μg RNA	cpm per μg RNA	cpm per μg DNA	Stimulation based on cpm per μg RNA
Complete	0.007	39	22	0
Complete + L-T_3	0.033	182	163	+ 363

TABLE II

Time course of the L-triiodothyronine effect on orotic acid-6-^{14}C incorporation into nuclear RNA

Contents of the reaction mixtures and incubation conditions were the same as those described in Table I. Incubation time was as indicated in the table. The final concentration of L-T_3 was 10^{-9} M.

System	Incubation time (hour)	Specific Activity		
		mμmole orotic acid incorp. per μg RNA	cpm per μg RNA	cpm per μg DNA
Complete + L-T_3, 10^{-9} M	1	0.182	1025	162
	2	1.038	5840	898
	3	0.105	591	79

TABLE III

Effects of various concentrations of L-triiodothyronine and L-thyroxine added *in vitro* on the incorporation of orotic acid-6-^{14}C into nuclear RNA

The components of the reaction mixtures and incubation conditions were identical to those described in Table I. Experimental procedures were described under "Methods." Incubated 2 hours at 37°. The final concentration of L-T_3 and L-thyroxine were as indicated in the table.

System	Specific Activity			orotic acid incorp. %	Thyroid hormone effect % Stimulation based on cpm per µg RNA
	mµmole orotic acid incorp. per µg RNA	cpm per µg RNA	cpm per µg DNA		
Experiment I					
Complete	0.14	794	60	4.2	0
Complete + L-T_3, 10^{-9}M	0.28	1595	427	12.0	+ 101
Complete + L-T_3, 10^{-7}M	0.20	1126	325	8.4	+ 42
Experiment II					
Complete	0.53	2976	---	22.0	0
Complete + L-T_4, 10^{-7}M	0.69	3853	---	37.7	+ 30
Complete + L-T_4, 10^{-5}M	0.99	5568	---	40.7	+ 87

TABLE IV

Effects of DNase and Actinomycin D on nuclear RNA synthesis

The composition of the reaction mixture was identical to those described in Table I, except that L-T_3 was replaced by 26.6 µg of DNase or 30.0 µg of Actinomycin D in the specified tubes as indicated in the table. The inhibitors were dissolved separately in Tris-phosphate buffer (pH 7.4, 0.05 M) and added. The final concentration of L-T_3 when added was 10^{-9} M. Incubated at 37^{o} for 2 hours.

System	Specific Activity	
	mµmole orotic acid incorporated per µg RNA	cpm per µg RNA
Complete + L-T_3	0.35	1952.90
Complete + DNase	0.0	0.17
Complete + Actinomycin D	0.0	0.10

TABLE V

The effect of L-triiodothyronine on the incorporation of orotic acid-^{14}C into RNA when IMP is replaced by AMP and GMP

Complete system (in µmoles): IMP, 2; DPN^+, 2; ATP, 0.1; GTP, 0.1; d-ribose-5'-phosphate, 2; L-aspartic acid, 2; L-glutamine, 4; succinic acid, 40; $MgCl_2$, 20; Na_2HPO_4, 40; Tris-phosphate buffer, pH 7.4, 100; orotic acid-6-^{14}C , 0.299 (1,676,606 cpm). When AMP and GMP were added in place of IMP as indicated in the table, the concentration was 1.0 µmole each. The final concentration of L-T_3 was 10^{-9} M when added. Incubated at 37° for 2 hours. The experimental conditions were as described under Table I.

System	Specific Activity		orotic acid incorporated %
	mµmole orotic acid incorp. per µg RNA	cpm per µg RNA	
Complete	0.0003	1.8	0.0063
Complete, - IMP + AMP and GMP	0.0004	2.3	0.0076
Complete, - IMP + AMP, GMP, and L-T_3	0.0004	2.0	0.0066

TABLE VI

Effects of ATP and GTP upon the incorporation of UTP-^{3}H into nuclear RNA

The contents of the reaction mixture were (in μmoles): ATP and GTP, as indicated in the table; CTP and UTP, 0.03 each in Experiment I and III, or 0.06 each in Experiment II; UTP-^{3}H, 0.00223 (476,865 cpm); $MgCl_2$, 7.5; KCl, 1.5 in Experiment II; and Tris-phosphate buffers, pH 7.4, 25; 2-mercaptoethanol, 0.5; and 1.0 ml of the nuclear suspension containing approximately 900 μg, 845 μg, and 531 μg of DNA in Experiments I, II, and III, respectively, in a total volume of 1.5 ml. Incubated in air with shaking for 1 hour at 37°. The reaction was terminated by rapid cooling in ice. RNA was determined as described in the text.

TABLE VI

Addition (μmole/1.5 ml) ATP	GTP	ATP/GTP	Specific Activity mμmole UTP incorporated per μg RNA	cpm per μg RNA	UTP incorporated %
Experiment I					
1.50	1.50	1/1	0.014	201	14.0
2.25	0.75	3/1	0.022	323	21.3
2.50	0.50	5/1	0.033	489	34.7
Experiment II					
0.45	0.45	1/1	0.0055	42	2.99
2.25	0.45	5/1	0.0083	64	3.64
4.50	0.45	10/1	0.1504	1153	68.00
9.00	0.45	20/1	0.1751	1342	73.70
Experiment III					
0.30	0.27	1/0.9	0.060	879	34.7
0.30	0.81	1/2.7	0.041	608	21.0
0.30	2.43	1/8.1	0.020	297	10.9
0.30	0.03	10/1	0.088	1299	42.7
0.90	0.09	10/1	0.083	1228	46.6
2.70	0.27	10/1	0.086	1275	50.0
8.10	0.81	10/1	0,116	1714	56.0
24.3	2.43	10/1	0.118	1752	64.1

TABLE VII

Time studies of UTP-^{3}H incorporation into nuclear RNA

The components of the reaction mixture were (in μ moles): ATP, 0.3; GTP, 0.03, CTP, 0.03; UTP, 0.03; UTP-^{3}H, 0.00223 (476,865 cpm); $MgCl_2$, 7.5; Tris-phosphate buffer, pH 7.4, 25; 2-mercaptoethanol, 0.5; and 1.0 ml of nuclear suspension containing approximately 385 μ g of DNA. The ATP/GTP ratio was 10/1. Incubation time was as indicated in the table. Incubation conditions and experimental procedures were the same as those described in Table VI.

Incubation time (minute)	Specific Activity		UTP incorporated %
	mμmole UTP incorporated per μg RNA	cpm per μg RNA	
30	0.0004	5.0	0.14
45	0.0010	12.3	0.38
60	0.0014	17.3	0.45
75	0.0002	2.6	0.08

TABLE VIII

Effects of L-triiodothyronine, ribonucleoside triphosphates, DNase and Actinomycin D on the incorporation of UTP-^{3}H into nuclear RNA

The composition of the reaction mixture was the same as that described in Table VII. ATP concentrations of 0.3 and 0.03 µmole were used. In addition, 10 µg DNase, 10µg Actinomycin, or 10^{-9} M L-T_3 was added as indicated in the table. Nuclei equivalent to 359 µg DNA were added to each tube. Incubation conditions and procedures were identical to those described in Table VI.

System	mµmole UTP incorporated per µg RNA	cpm per µ g RNA	UTP incorporated %
ATP/GTP (0.3 µ mole/0.03 µ mole = 10/1)			
Complete	0.0895	1324	25.0
ATP omitted	0.0001	2	0.03
GTP omitted	0.0006	9	0.16
CTP omitted	0.0004	6	0.10
Complete + Actinomycin D	0.0006	10	0.17
Complete + DNase	0.0003	4	0.06
Complete + L-T_3	0.0847	1253	24.4
ATP/GTP (0.03 µ mole/0.03 µmole = 1/1)			
Complete	0.0496	733	12.3
Complete + L-T_3	0.0667	987	15.0

TABLE IX

L-Triiodothyronine effect *in vitro* on DNA-dependent nuclear RNA synthesis in nucleus

The components of the reaction mixture were identical to those described in Table VII. The purine nucleotides were (in µmole): ATP, 0.3 or 0.03; and GTP, 0.3 or 0.03. The ATP/GTP ratio was 1/1. The final concentration of L-T_3 was 10^{-9} M, when added. Approximately 835 g DNA as nuclei were added in 1.0 ml to each tube. Incubation conditions and procedures were the same as those described in Table VI.

System	Addition ATP/GTP (µ mole)	Specific Activity: mµmole UTP incorporated per µ g RNA	Specific Activity: cmp per µ g RNA	UTP incorporated %
Complete	0.3 / 0.3	0.013	194.1	8.0
Complete + L-T_3	0.3 / 0.3	0.014	205.7	6.9
Complete	0.03/0.03	0.016	225.7	7.7
Complete + L-T_3	0.03/0.03	0.015	215.4	8.3

AN IN VITRO SYSTEM FOR STUDYING THE MECHANISM OF ACTION OF THYROID HORMONES

Ruei-Choo Chen Lo

ABSTRACT

The effect of thyroid hormones on nuclear RNA synthesis was studied in vitro. Results indicated that thyroid hormones added to cell-free rat liver homogenates, in the presence of IMP as a precursor of purine nucleotides and necessary substrates, stimulated orotic acid-6-^{14}C incorporation into nuclear RNA. The stimulatory effect was observed with 10^{-9} M triiodo-L-thyronine (L-T_3) and with 10^{-5} M thyroxine.

Thyroid hormones had no effect on the incorporation of UTP-^{3}H into nuclear RNA which is evidence that the primary site of action of the hormones is in the cytoplasm, i.e., the conversion of IMP to AMP and to GMP. The net effect is to maintain a high adenine nucleotide/guanine nucleotide ratio. The incorporation of UTP-^{3}H into nuclear RNA was enhanced when the ATP/GTP ratio was greater than one as compared to that when the ratio was one, and it was inhibited when the ratio was less than one. The relative concentrations of the purine nucleotides had a profound effect on nuclear RNA synthesis. DNase and actinomycin D inhibited nuclear RNA synthesis induced by a high ATP/GTP ratio.

Based on these results, it was proposed that thyroid hormones regulate the synthesis of AMP and GMP from IMP and maintain a high adenine nucleotide/guanine nucleotide ratio which enhances the synthesis of a specific RNA.

The significance of the maintenance of a high adenine nucleotide/guanine nucleotide ratio and the consequences of the synthesis of a specific RNA were discussed.