THE EFFECT OF THYROXINE ON PROTEIN BIOSYNTHESIS AND RIBONUCLEIC ACID METABOLISM IN THE RAT

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INTRODUCTION

Through the years our knowledge of the thyroid gland and its function has become more and more refined. Today the major physiological actions of the thyroid hormones are well established. However, the mechanism by which the hormones bring about these actions is still unknown, even though the work done to date on this problem is voluminous. Since many investigators have accepted the idea that "the" action of the thyroid hormones is to control metabolic rate, much data has accumulated on possible mechanisms involved in the stimulation of metabolism, while little work has been done on the observed thyroid hormone influence on growth, metamorphosis, and body fluids (as in myxoedema). Many reasons may be given to explain this unbalanced or one-sided approach, but perhaps the most important is the relatively parallel elucidation of the reactions, enzymes, and substrates involved in oxidative phosphorylation which, of course, is directly tied in with the basal metabolic rate (BMR). Since the results to date have been largely unsuccessful in producing a concept of thyroid hormone action which is not only universally acceptable but is broad enough in scope so as to explain the variety of thyroid hormone induced physiological effects, the present study was undertaken as a new approach to the problem. It must, of course, be conceded that this new approach has been made possible, as had the previous thyroid hormone work, by the developments in other areas of biochemical research; in particular, the more recent developments in protein biosynthesis.

The following literature review is a compilation of those works most often cited in the current literature and which have, through time, welded together to form the backbone of the more popular current concepts of the mechanism of action ofthyroid hormones. However, greater emphasis is necessarily placed on those works which lend themselves in support of the present hypothesis--a regulating or controlling influence of thyroid hormones on protein biosynthesis.

LITERATURE REVIEW

In 1927 Foster (1) reported that tissues isolated from mice fed dessicated thyroid were characterized by increased oxygen consumption. Tissues from thyroidectomized rats showed a decreased oxygen consumption. Later, Green (2) proposed the theory that hormones in general produce their effects by participating in certain enzyme systems as co-enzymes or as specific enzyme inhibitors. These two factors were, most likely, foremost in the thinking of those men who did much of the work involving the concept of a direct action of thyroid hormones on the respiratory enzymes.

In 1946 Tipton et al.(3) reported that the administration of dessicated thyroid produced an increase in skeletal muscle succinoxidase activity. Maley (4) later found an increased succinoxidase activity in isolated liver mitochondria from hyperthyroid rats. The succinoxidase system's activity, of course, represents the sum total of the activity of succinic dehydrogenase, cytochrome c, cytochrome oxidase, and the electron carriers. After observing the thyroid hormone effect on the system as a whole, it then appealed to various workers to study the effect on the individual components of the system.

Thyroxine administration was shown by Barker (5) to result in higher liver succinic dehydrogenase activity. Lardy (6) reported that the increased succinic dehydrogenase activity in hyperthyroid rat livers is accompanied by an increase in mitochondrial nitrogen concentration. It was similarly shown that the levels of cytochrome c

(7) and cytochrome oxidase (4) were lowered by thyroidectomy and increased by thyroxine administration.

Although many of the attempts to reproduce the *in vivo* effects of thyroid hormones with *in vitro* systems have failed, a relative amount of success has been had with the succinoxidase system. Gemmill (8) reported that the *in vitro* addition of thyroxine stimulated the succinoxidase system of rat heart mitochondria. This observation has been confirmed by many workers (9, 10, 11). Wolff and Ball (9) suggested that the increased rate of succinate oxidation observed with thyroxine administration was the result of the specific inhibition of the oxidation of malate to oxaloacetate. The latter is a strong inhibitor of succinic dehydrogenase. They then demonstrated that the addition of thyroxine to rat heart mitochondria inhibited the formation of oxaloacetate from malate while it had no effect on the conversion of fumarate to malate. This effect was confirmed by Barker (10). However, Barker concluded that the stimulatory action of thyroxine on succinoxidase *in vivo* cannot be accounted for simply by an inhibition of malate oxidation because . . . ."an unphysiological situation would be created by the accumulation of malate derived from the oxidation of succinate."

The observation by Loomis and Lipmann in 1948 (12) that dinitrophenol (DNP), which has the ability, like thyroxine, to raise the BMR, could uncouple phosphorylation from respiration, led to the possibility that the thyroid hormones control mitochondrial oxidative

phosphorylation. Indeed, it was soon shown by Martius and Hess (13) that mitochondria from the livers of rats administered large doses of thyroxine were characterized by a lowered phosphorylating efficiency. Again, as with the succinoxidase system, the *in vivo* effect of thyroxine has been demonstrated with *in vitro* systems. Soon Maley and Lardy (14) reported that the range for thyroxine's uncoupling effect *in vitro* is quite narrow. They observed no uncoupling to total uncoupling between 5×10^{-5} and 5×10^{-4} M thyroxine. More recently, the above work has been the subject of much criticism because of the pharmacological levels of the hormones used in producing the uncoupling effect (15, 16). It has even been reported that mitochondria from livers of rats injected with 30 μg quantities of triiodothyronine showed an increased capacity in the electron transport phosphorylation system and *no* uncoupling (17).

Although we are still far from the "mechanism" of thyroxine's action, we are at the point where we can state the hypothesis widely accepted as describing the "function" of thyroxine. That is, the control of the basal metabolic rate via the balancing of the rate of oxidation of various substrates with energy transfer. This concept, stated in a variety of ways, has found itself in most all of the more popular current textbooks. However, in addition to the criticisms and inconsistencies pointed out above, this entire concept is presently being considered by some workers to be representative solely of a secondary effect attributable to the thyroid hormone's action on some removed but energetically related metabolic process. These ideas will be discussed later in this review.

In addition to the above enzymes, many other enzymes and enzyme systems have been shown to be affected by thyroid hormones. For example, adenosine triphosphatase (4), glucose-6-phosphate dehydrogenase (18), hexokinase (19), amylase (20), acid phosphatase (21), alkaline phosphatase (22), tyramine oxidase (23), show increased activity under hyperthyroid conditions while DOPA-decarboxylase (23), betaine-hemocysteine transmethylase (24), alanine-glutamic transaminase (25), and others show decreased activity. It must also be pointed out here that hypothyroidism has not always reflected the converse of the hyperthyroid condition with respect to various enzyme's activity. For example, liver glucose-6-phosphate dehydrogenase activity shows no change from the normal under hypothyroid conditions (18). The stimulatory effect of thyroid hormones on adenosine triphosphatase activity has also been cited as additional evidence for their uncoupling effect (26). Since the enzymes listed above are involved in such diversified reactions as oxidation, hydrolysis, phosphorylation, dephosphorylation, transamination, and so on, no coherent or comprehensive theory as to the mechanism of the thyroid hormone's action has been put forward.

Another effect of thyroid hormones which has received considerable attention is that on mitochondrial membrane permeability. In 1956 Tapley (27) reported that thyroxine and Ca^{++} caused mitochondrial swelling while other uncouplers of oxidation phosphorylation such as 2,4-dinitrophenol, dicoumarol, and pentachlorophenol had no effect. He also observed that agents which antagonized the

thyroxine-induced uncoupling of oxidative phosphorylation such as Mg^{++}, Mn^{++}, and EDTA also antagonized the mitochondrial swelling caused by thyroxine. He then felt that the uncoupling of oxidative phosphorylation in mitochondria by thyroxine was not due to a direct interaction of thyroxine with any of the various enzymes involved in oxidative phosphorylation, but rather due to the hormone's effect on the mitochondrial structure--in particular, on the membrane permeability. The suggestion was then made that thyroxine exerted its controlling effects on those enzymes indirectly by regulating the entry and exit of substances involved in respiratory activity and energy transfer. Although this explanation could be readily used to justify the inconsistencies observed when various workers investigated the hormone's effect on the individual enzymes involved in respiratory activity and energy transfer, it has not escaped being the subject of inconsistency itself. For instance, mitochondrial swelling has been observed with 10^{-8} M thyroxine (28) while the uncoupling of oxidative phosphorylation has been reported to be confined to a thyroxine range of 5×10^{-5} to 5×10^{-4} M (14). It has also been reported by Park et al. (29) that thyroxine and triiodothyronine can uncouple oxidative phosphorylation in sub-mitochondrial particles where, of course, the mitochondrial membrane permeability is no factor.

Since none of the work to date on the thyroid hormones has led to either a conclusive or comprehensive concept of the hormones' action, a new approach was initiated based on the following points. It is considered possible that the thyroid hormones might influence oxygen

consumption indirectly via the control of some process which requires energy, rather than produces energy. More clearly, the increased oxygen consumption observed upon thyroid hormone treatment results from increased energy production which itself results from increased energy utilization by some metabolic process stimulated by the thyroid hormones. The metabolic process thought most likely is protein synthesis. As far back as 1929 Kendall (20) discussed the possibility that protein metabolism was controlled by the action of the thyroid gland. This idea was supported by the fact that many of the clinical symptoms of thyroid disease involved disturbances in nitrogen metabolism. Also in support of this are the observations, previously indicated (page 7), by other workers that thyroid hormone treatment results in the appearance of increased quantities of mitochondrial nitrogen (6), cytochrome c (7) and cytochrome oxidase (4). More recently Paik et al. (31) have demonstrated increased liver levels of carbamyl phosphate synthetase upon thyroxine pretreatment of tadpoles. DuToit reported in 1952 (32) that thyroxine pretreatment in vivo resulted in a stimulation of the incorporation of radiolabeled alanine into protein by rat liver slices. Sokoloff et al., (33) reported that the in vitro addition of thyroxine to a cell-free liver system from normal and thyroidectomized rats stimulated amino acid incorporation into protein. This, however, could not be duplicated by Stein and Gross (34). Thus, with the exception of the latter contradiction, there exists data giving credence to the thesis that thyroid hormones may be involved in protein biosynthesis. The question to be investigated is: how are the thyroid hormones involved in protein biosynthesis?

THYROXINE AND PROTEIN SYNTHESIS

Abstract. The *in vivo* administration of thyroxine stimulated the incorporation of leucine 1-C^{14} into protein by a cell-free liver system of microsomes and soluble proteins from both normal and hypothyroid rats. However, the *in vitro* additions of the hormone to the cell-free systems was found to produce a stimulatory effect only in that derived from the normal. The effect of thyroxine on protein synthesis appears to be indirect. Both the microsomal and the 105,000 x g supernatant fractions obtained from hypothyroid rat liver were shown to be defective with respect to their ability to synthesize protein.

In 1929 Kendall (1) discussed the view once held that protein metabolism was controlled by the action of the thyroid gland. This idea was supported by the fact that many of the clinical symptoms of thyroid disease involved disturbances in nitrogen metabolism. The development of radiochemical methods and cell-free systems, by which protein synthesis may be studied, has renewed interest in the possibility that thyroid hormones may be involved, either directly or indirectly in protein metabolism.

DuToit reported in 1952 (2) that thyroxine pretreatment *in vivo* resulted in a stimulation of the incorporation of radiolabeled alanine into protein by rat liver slices. Since then other workers

have presented data corroborating this effect of thyroxine and of triiodothyronine as well (3, 4, 5). However, reports on the effect produced by the *in vitro* addition of thyroid hormones have been less consistent.

Sokoloff *et al.* (6, 7) reported that the *in vitro* addition of thyroxine to a cell-free liver system from normal and thyroidectomized rats stimulated amino acid incorporation into protein and that the stimulation was dependent on the presence of mitochondria and an oxidizable substrate. Moreover, the effect was entirely localized in the reaction involving transfer of the soluble-RNA-amino acid complex to the microsomes. Stein and Gross (4) reported that the *in vitro* addition of triiodothyronine had no effect on amino acid incorporation by a cell-free system from the livers of thyroidectomized rats. Hanson *et al.* (8) similarly have presented data indicating that the *in vitro* addition of thyroxine did not affect incorporation of amino acids by kidney cortex slices from thyroidectomized rats when incubated at 37° C for as long as 9 hours. However, they found that pre-incubation of the tissue slices with thyroxine at 5° for 2 days, followed by incubation at 37°, resulted in a three-fold increase in the amount of L-proline incorporated. They concluded that their results indicated the effect of thyroxine was not stimulatory but rather one of maintainence.

In spite of the divergence of opinion that presently exists as to the mode of action of the thyroid hormones, the possibility of a "more than casual" relationship to protein synthesis remains attractive. Indeed, Tata et al. (5) observed that an increase in the rate of incorporation of amino acids into microsomal protein occurred before the rate of glutamate oxidation was stimulated when triiodothyronine was administered to thyroidectomized rats. They suggested that the observed changes in oxidative capacity may be an adaptation to increased energy demands. Even more recently Bronk (9) reported that triiodothyronine administration to thyroidectomized rats increased the total capacity of the electron transport phosphorylation system when different substrates were tested and, concomitantly, stimulated the incorporation of amino acids into the protein of isolated mitochondria. The present study provides further data substantiating the involvement of thyroid hormones with protein synthesis.

Weanling male Sprague-Dawley rats were fed a complete diet containing 1% sulfaguanidine until growth arrest was established (10). Controls consisted of rats which were fed the same diet without sulfaguanidine. All rats were starved for 18 hours before each experiment with the goitrogen-fed group being supplied with sulfaguanidine-saturated water to prevent resumption of thyroid hormone synthesis. The rats were decapitated, the livers quickly excised, weighed and homogenized in 2.5 volumes (w/v) of 0.02 M potassium phosphate buffer with 0.35 M sucrose, 0.165 M KCl, and 0.035 M $KHCO_3$

at a final pH of 7.4. Homogenization was performed at 0-2° in an all-glass Potter-Elvehjem tissue homogenizer for a maximum of 45 seconds. Nuclei, cell debris, and mitochondria were removed by centrifugation at 15,000 x g for 15 minutes in a Servall RC-2 refrigerated centrifuge. The quantity of the resulting supernatant added to each of the experimental incubation tubes was 0.7 ml.

In the recombination experiments the microsomal and supernatant fractions from both control and goitrogen-fed rats were prepared as follows: nuclei, cell debris, and mitochondria were removed as described. The 15,000 x g supernatant was then centrifuged at 105,000 x g for 60 minutes in a Spinco Model L ultracentrifuge, the supernatant containing soluble enzymes and transfer RNA was removed, and the microsomal pellet rinses, resuspended to original volume in 0.25 M sucrose, and recentrifuged at 105,000 x g for 60 minutes. The microsomal pellet was then resuspended in buffer such that 0.2 ml of the final suspension contained the amount of microsomal material calculated to be in 0.7 ml of the 15,000 x g supernatant. Each incubation tube then received 0.2 ml of the microsomal suspension along with 0.7 ml of the first 105,000 x g supernatant in the four possible combinations. Incubations were carried out as described in the title of Table 1.

The reaction was terminated with 10% trichloroacetic acid and the protein isolated and purified by the procedure

of Siekevitz (11). The samples were then dried in a paraffin-coated dessicator over concentrated H_2SO_4. A 1-3 mg portion of each dried sample was weighed with a Cahn electrobalance, quantitatively transferred to a scintillation counting vial and dissolved in 1.0 ml of M hydroxide of hyamine in methanol by recapping the vials and heating at 60° for approximately one hour. To each vial was added 15 ml of scintillator fluid containing 0.4% PPO and 0.01% POPOP in toluene. Counting was then carried out in a Packard Tri-Carb scintillation spectrometer using a window setting of 10-100 v at 800 volts.

It can be seen in Table 1 that the feeding of a diet containing sulfaguanidine at the 1.0% level results in a highly significant depression in the rate of incorporation of leucine-1-C^{14} into protein by a cell-free liver system of microsomes and soluble proteins. The administration of thyroxine <u>in vivo</u> to both the control group and the goitrogen-fed group resulted in a stimulation in the rate of incorporation of both groups. These effects are similar to those obtained by workers using normal and thyroidectomized animals (2,3,4,5) and would, therefore, suggest that thyroid hormones are involved directly or indirectly in some phase of protein synthesis. However, the response of the two groups to the <u>in vitro</u> addition of thyroxine proved to be quite different. Here the system derived from the goitrogen-fed group showed no thyroxine effect while the control system showed a positive response or was stimulated upon the addition of thyroxine. The average percentage increase in incorporated label by the control group upon the <u>in vitro</u> addition of thyroxine was

49.42% for a total of seven experiments. However, the standard error was calculated to be ± 24.22% indicating a positive, although highly erratic in magnitude, response in all experiments. At this point our efforts were concentrated on attempts at stabilizing this response. Several factors or conditions were tested which included: various levels of thyroxine, $MgCl_2$, KCl, GTP, and GSH, preincubation with thyroxine and/or $MgCl_2$ at 0-5°, aerobic and anaerobic incubation, perfusion of the liver with saline or sucrose prior to homogenization, amino acid supplementation of the crude homogenate, and the consideration of rat diet, and age of the rat. Although all these attempts failed with respect to their primary objective, several points of interest were discovered. The level of thyroxine most effective in producing a stimulatory response was 2.5×10^{-5} M and the response to thyroxine was found to be K^+ dependent (Figure 1). However, the thyroxine stimulatory effect could be completely eliminated by Mg^{++} levels equal to, or in excess of 0.01 M as also reported by Sokoloff and Kaufman (3).

Our experiments have demonstrated that the thyroxine-induced stimulation of amino acid incorporation into protein is not dependent on the presence of the mitochondria. However, the complete lack of a stimulatory effect upon the addition of thyroxine to a hypothyroid system, as similarly reported by Stein and Gross (4), has led us to believe that the effect of thyroxine on protein synthesis is one of an indirect nature. This contention is supported by the observation that

triiodothyronine additions *in vitro* had a smaller stimulatory effect on the incorporation of amino acids into protein than did thyroxine, while D-thyroxine (physiologically inactive) was as effective as L-thyroxine (6). These considerations led us to believe that the positive response of the system obtained from normal rat liver indicates that some factor or factors, necessary for the mediation of the hormone effect, is missing in the hypothyroid system.

Table 2 shows the results of our preliminary attempts to localize the defective fraction(s) in the hypothyroid system. Microsomes and 105,000 x g supernatant were prepared from the livers of both the normal and hypothyroid groups as described. Incubations were then carried out with the four possible combinations. It may be seen that both the microsomal and the 105,000 x g supernatant fractions are defective in the livers of the hypothyroid animals with respect to their ability to incorporate leucine-1-C^{14} into protein. However, the supernatant fraction appears to be more limiting than the microsomal fraction. The cause of these observations is currently being investigated.

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Table 1. The effect of thyroxine on leucine-1-C^{14} incorporation into protein. Each reaction tube contained 0.7 ml of the liver homogenate 15,000 x g supernatant, 20 μM phosphocreatine, 3 μM $MgCl_2$, 1 μM ATP, 0.25 μM GTP, and 3 x 10^6 dpm DL-leucine-1-C^{14} (16.9 μc/μM). In experiments testing the effect of the *in vitro* addition of thyroxine, Na-L-thyroxine was dissolved in minimal amounts of 0.01 N NaOH and was added to the reaction tube to make a final concentration of 2.5 x 10^{-5}M. Equivalent amounts of NaOH were added to the control tubes. Final incubation volume was 1.0 ml and incubation time at 37° was 30 minutes. For the *in vivo* experiments Na-L-thyroxine was dissolved in 0.01 N NaOH and taken up in 0.9% NaCl. Injections were administered daily for four days at a level of 2.5 μg thyroxine/100 gms body weight. Controls were paired with experimental animals according to weight.

	Mean Counts/min/mg protein	
	Control	+ Thyroxine
Thyroxine administration *in vivo* (2 experiments)		
Normal rat	389	532
Hypothyroid rat	24	147
Thyroxine administration *in vitro* (7 experiments)		
Normal rat	345	442
Hypothyroid rat	23	22

Figure 1. Effect of K^+ concentration on amino acid incorporation into protein by a cell-free rat liver system of microsomes and soluble proteins upon the <u>in vitro</u> addition of Na-L-thyroxine (2.5×10^{-5} M). Potassium ion was added as KCl to the reaction tubes containing the microsomal system suspended in 0.02 M Na_2HPO_4 buffer with 0.35 M sucrose and 0.035 M $NaHCO_3$ at a final pH of 7.4. Ionic concentration was maintained constant by the addition of NaCl. Magnesium ion, radio-leucine and an ATP-generating system were added as described in the title of Table 1.

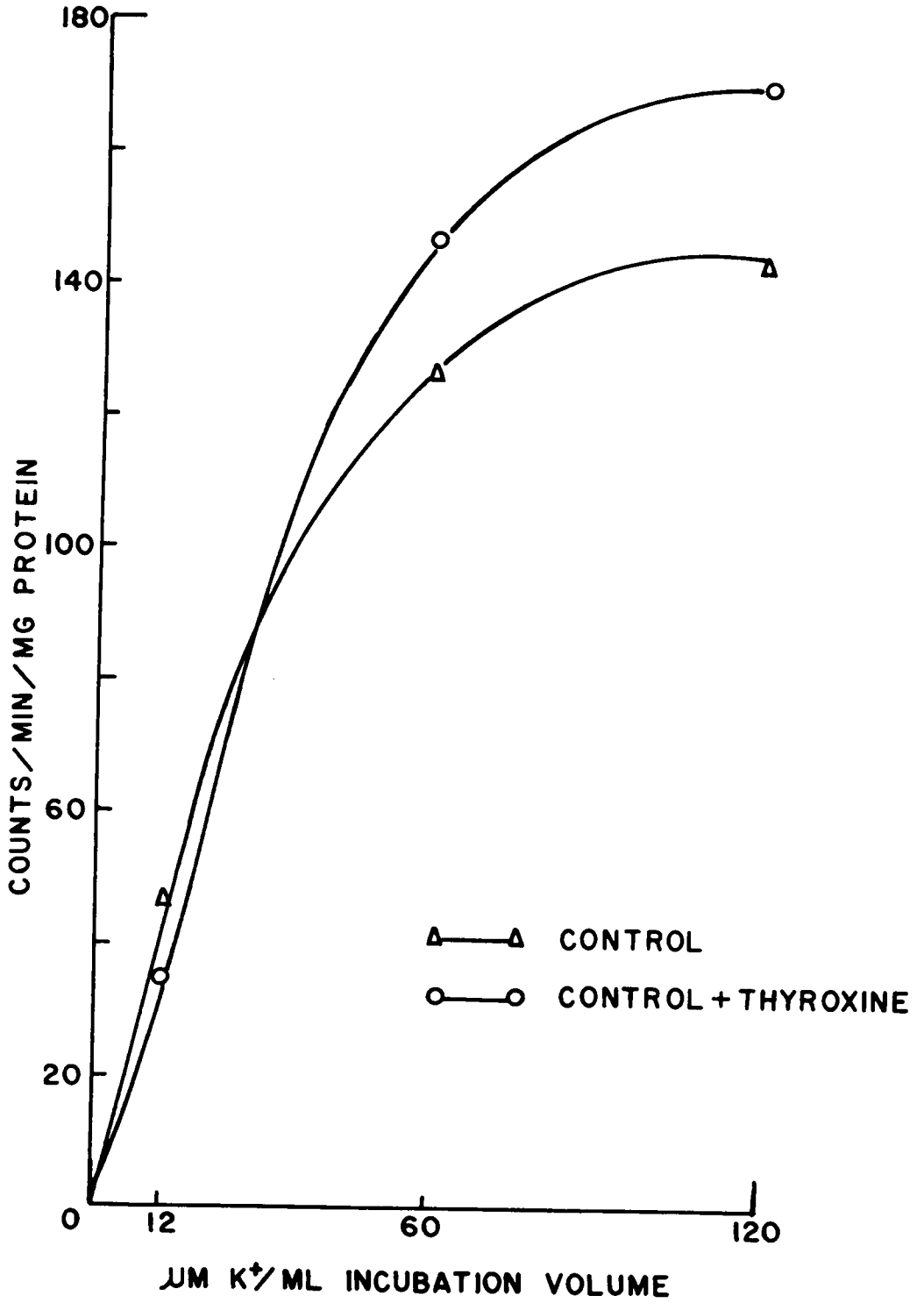
180
140
100
60
20
0
12
60
120
COUNTS/MIN/MG PROTEIN
μM K+/ML INCUBATION VOLUME
CONTROL
CONTROL + THYROXINE

Table 2. Localization of defective fraction(s) in amino acid incorporating activity of a cell-free liver system from hypothyroid rats. Each reaction tube contained 0.7 ml of the liver homogenate 105,000 x g supernatant and 0.2 ml of the microsomal suspension prepared as described in the text. Co-factors were added and incubation was carried out as described in the title of Table 1.

Source		Mean
Microsomes	105,000 x g Supernatant	Counts/min/mg protein
Normal rat	Normal rat	87.5
Hypothyroid rat	Hypothyroid rat	18.0
Hypothyroid rat	Normal rat	76.0
Normal rat	Hypothyroid rat	22.5

THYROXINE AND THE METABOLISM OF NUCLEIC ACIDS AND NUCLEOTIDES IN RAT LIVER

Abstract. Hypothyroidism in rats results in decreased levels of liver ribonucleic acid. However, ribonucleic base composition remains essentially unchanged. Thyroxine administration to normal and hypothyroid rats effects an increase in liver levels only in the latter. Phosphorus-32 incorporation data indicate no change in rate of synthesis upon thyroxine treatment of hypothyroid rats although shifts in purine P^{32} incorporation were observed. A possible influence of thyroxine on guanosine monophosphate (GMP) synthesis is considered.

Previous work in this laboratory has provided evidence for the decreased rate of liver protein biosynthesis observed in hypothyroidism (1). We also reported that the microsomal and the 105,000 x g supernatant fractions from the livers of hypothyroid rats were defective or deficient with respect to their ability to carry out protein synthesis. In view of these observations, it appeared worthwhile to investigate the metabolism of ribonucleic acid (RNA) which is now accepted as an essential component in the overall scheme of protein biosynthesis.

Weanling male Sprague-Dawley rats were fed a complete diet containing 1% of the goitrogen sulfaguanidine until growth arrest was established (1). All rats were starved for 18 hours before each experiment and controls, fed the basal diet without sulfaguanidine, were paired with treated rats according to weight. The livers were weighed, and homogenized for 30 seconds in 25 ml of physiological saline with a

Virtis tissue homogenizer. All operations were conducted at 0-2°. RNA was isolated (2) and measured spectrophotometrically at 260 and 280 mμ in a Beckman DU spectrophotometer and then analyzed for phosphorus (3).

Table 1 shows the results of the RNA analysis of the liver homogenate 15,000 x g supernatant. The RNA content of this fraction from the hypothyroid rat proved to be lower than that of the normal. This observation is similar to that reported by Guggenheim et al. (4) working with whole liver from thiouracil-fed and thyroidectomized rats. This RNA pool responded to a single injection of 2.5 μg of L-thyroxine per 100 gms body weight within 72 hours, though only in the hypothyroid rat. There was essentially no change in the RNA content of this fraction upon thyroxine administration to the normal rat. Furthermore, it was observed, upon the subsequent fractionation of this RNA pool into the microsomal and soluble RNA fractions (Table 2), that both fractions are quantitatively deficient in the hypothyroid rat liver. However, thyroxine pretreatment of the hypothyroid rat resulted in a preferential increase in the concentration of microsomal RNA, although an increase in total liver-soluble RNA was apparent.

In Table 3 the composition of the liver soluble and microsomal RNA fractions from normal and hypothyroid rats are compared. The composition of the respective fractions remained essentially constant despite the differences in total RNA between the normal and hypothyroid livers.

Paik et al. (5) similarly reported no change in RNA composition when they treated tadpoles (Rana catesbeiana) with 2.6 x 10^{-8} M thyroxine. However, they found no change in liver RNA-phosphorus content but at the same time observed an increase in the rate of protein synthesis. They suggested that thyroxine caused a re-arrangement and perhaps a re-distribution of RNA molecules among the different cellular compartments and functional sites. Their adenine-8-C^{14} incorporation data did indeed indicate a decreased rate of RNA turnover upon thyroxine treatment. These results are similar to those obtained by us upon thyroxine treatment of normal rats, but as seen above, differ with those obtained from hypothyroid rats where thyroxine treatment effected an increase in liver RNA.

It can be seen in Table 4 that there was little change in the total amount of P^{32} incorporated into RNA after thyroxine treatment of hypothyroid rats. This proved to be true for both the soluble and microsomal RNA fractions and would seem to indicate no change in the overall rate of RNA synthesis. However, upon the chromatographic separation of the 4 major nucleotides of these two RNA fractions, it became apparent that thyroxine effected shifts in the rate of P^{32} incorporated into the different nucleotides. The most significant differences were with the purines with AMP showing a decrease in the amount of P^{32} incorporated (as Paik et al. (5) also reported using adenine-8-C^{14}) while GMP showed an increase in incorporated P^{32} after

thyroxine treatment. Only small differences were observed with the pyrimidine-incorporated P^{32}. These trends were most prominent in the microsomal RNA nucleotides. It thus appears as though thyroxine treatment brings about a shift in purine-monophosphate synthesis with GMP being synthesized at the expense of AMP. Janney and Isaacson (6) showed that thyroidectomy resulted in a decreased urinary purine excretion and that hyperthyroidism was characterized by an increased urinary purine excretion. However, Chilson and Sacks (7) reported that hyperthyroidism in guinea pigs did not cause any appreciable change in the liver content of adenine-purines ATP, ADP, and AMP. It is, therefore, possible that thyroxine preferentially stimulates the synthesis of the purine GMP and that the decreased liver RNA content of hypothyroid rats is a result of the presence of only limiting amounts of GMP, thereby restricting RNA synthesis. Since the turnover rate of messenger and soluble RNA is greater than that of insoluble RNA, their demand for the available GMP at any particular moment would necessarily be greater than that of insoluble RNA. Thus, it is possible to explain the seemingly preferential stimulation of microsomal RNA synthesis upon thyroxine treatment of the hypothyroid rats (Table 2) rather as an "accumulation" of microsomal RNA which is made possible by a greater availability of GMP. This is corroborated by the fact that the specific activity of the RNA-bound GMP increases and that of the RNA-bound AMP decreases, while

the total amount of P^{32} incorporated into RNA remains constant after thyroxine treatment. Further experimentation is in progress to test this possibility.

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Table 1. The effect of thyroxine on rat liver RNA content. Both normal and goitrogen-fed rats were given a single subcutaneous injection of 2.5 μg Na-L-thyroxine per 100 gms body weight 72 hours before sacrifice. The hormone was dissolved in minimal amounts of 0.01 N NaOH and taken up in physiological saline. After homogenization the nuclei, cell debris, and mitochondria were removed by centrifugation at 15,000 x g for 15 min. RNA content of the resulting supernatant was determined as described in the text. Results are representative of four such experiments.

Treatment	mg 15,000 x g supernatant RNA/ gm wet liver
Normal	7.56 ± 0.40[1]
Normal + 2.5 μg thyroxine	7.76 ± 0.36
Hypothyroid	2.92 ± 0.03
Hypothyroid + 2.5 μg thyroxine	3.57 ± 0.15

[1] mean ± standard error.

Table 2. The effect of hypothyroidism and thyroxine pre-treatment on rat liver soluble and microsomal RNA content. Microsomes and the 105,000 x g supernatant fraction were separated as previously described (1). Rats receiving the hormone treatment were given subcutaneous injections of 2.5 μg Na-L-thyroxine/100 gms body weight daily for six days before the experiment. Each value represents the mean of four animals.

	Normal	Hypothyroid	Hypothyroid + thyroxine
Microsomal RNA			
mg/gm wet liver	6.90	2.19	2.84
mg total/liver	29.28	6.82	13.23
Soluble RNA			
mg/gm wet liver	2.11	1.05	1.02
mg total/liver	8.96	3.27	4.74

Table 3. Nucleotide composition of soluble and microsomal RNA in the livers of normal and hypothyroid rats. Nucleotides were separated by 2-dimensional chromatography on Whatman No. 3 MM paper. Samples of the respective RNA alkaline hydrolyzates were spotted directly. Separations were run at room temperature. The solvents used were (I) isobutyric acid/H_2O/conc. NH_4OH (66/33/1) and (II) M ammonium acetate pH 7.5/95% ethanol (3/7). Spots were detected with ultraviolet light, cut out, and eluted with M potassium phosphate buffer pH 7.0 for 18 hours at 37°. Calculation of the nucleotide concentration in the eluates were based on optical density and published extinction data at the respective absorption maxima. Standard nucleotide samples indicated 96-100% recovery with this method. All samples were run at least in duplicate.

Source	Mole %			
	AMP	CMP	GMP	UMP
Soluble RNA				
Normal rat	16.2	31.9	28.1	23.9
Hypothyroid rat	16.3	32.4	27.7	23.6
Microsomal RNA				
Normal rat	16.3	32.5	29.7	21.5
Hypothyroid rat	17.1	32.9	29.4	20.6

Table 4. Effect of thyroxine pre-treatment on P^{32} incorporation into liver RNA by hypothyroid rats. Hypothyroid rats were administered Na-L-thyroxine as described in the title of Table 2. Each rat received 10 μc $NaH_2P^{32}O_4$/100 gms body weight intraperitoneally 2 hours before sacrifice. Nucleotides were separated, eluted, and quantified as previously described (Table 3). Aliquots of the eluates were transferred to scintillation counting vials, evaporated to dryness, dissolved in hyamine and counted with a Packard Tri-Carb scintillation spectrometer.

Source	% Total counts/min P^{32} incorporated into RNA nucleotides/gm liver				Total RNA-P^{32}
	AMP	CMP	GMP	UMP	(cpm/gm liver)
Soluble RNA					
Hypothyroid rat	**25.4**	**24.5**	**25.4**	**24.7**	**2157**
Hypothyroid rat + thyroxine	23.4	26.4	26.1	24.1	2031
Microsomal RNA					
Hypothyroid rat	24.5	23.8	30.8	20.9	4215
Hypothyroid rat + thyroxine	20.5	24.0	36.5	19.0	4394

SUMMARY AND CONCLUSIONS

A decrease in liver protein biosynthesis has been demonstrated in weanling male rats made hypothyroid by the feeding of a diet 1% in sulfaguanidine. It was found that thyroid hormone treatment increased the amount of radio-labeled amino acid incorporated into liver proteins of both normal and hypothyroid rats. However, the *in vitro* addition of thyroid hormones to the cell-free liver systems was observed to have a stimulatory effect, though quite erratic in magnitude, only on the normal system. This observation was investigated further but remains unexplained at this time.

It was also seen that the hypothyroid rats had significantly less liver RNA than the normal rats. Thyroxine pre-treatment was shown to increase the liver RNA content only of the hypothyroid rat. Fractionation revealed that the increase in RNA after thyroxine treatment could be accounted for mainly by an accumulation of microsomal RNA. Base ratio analysis of the soluble- and microsomal-RNA fractions indicated no differences when the hypothyroid rats were compared to the normal.

Phosphorus-32 incorporation data showed that there was no change in the rate of RNA synthesis after thyroxine administration to hypothyroid rats. However, this conclusion became questionable when it was found that thyroxine effected shifts in the amount of P^{32} incorporated into the different nucleotides. The most significant

differences were with the purines with AMP showing a decrease in the amount of P^{32} incorporated while GMP showed an increase in incorporated P^{32} after thyroxine treatment. This trend was most prominent in the microsomal RNA nucleotides.

These results suggest that thyroxine affects protein synthesis indirectly through an effect on RNA metabolism. However, the nature of the latter effect remains conjecture until further work is carried out. It appears though that thyroxine may have some directing influence on purine-monophosphate synthesis with a preferential stimulatory effect on GMP synthesis. It would thus be possible to explain the decreased RNA levels observed in hypothyroidism as due to the presence of only limiting quantities of GMP for RNA synthesis. Consequently, upon thyroxine treatment more GMP becomes available and more RNA is synthesized. It must be emphasized that this does not imply a change in the rate of RNA synthesis. The foregoing results show that microsomal RNA builds up while there is no change in the amount of P^{32} incorporated after thyroxine treatment. Also, since messenger- and soluble- have a faster turnover rate than does microsomal-RNA, they would necessarily have a greater demand for the available GMP (thereby restricting microsomal RNA synthesis in a hypothyroid condition). Thus, it follows that in a given period of time thyroxine's effect would appear most pronounced on microsomal RNA which merely "accumulates" because of its lower turnover rate.

By controlling nucleotide synthesis the thyroid hormones may alter the balance of free nucleotides available for RNA synthesis and thereby favor the synthesis of particular RNA molecules and thus the synthesis of particular enzymes or other proteins. It has already been pointed out that some enzymes do indeed appear in larger quantities after thyroid hormone treatment. This may also be the means by which other hormones carry out their function. Quite recently Wool and Munro (35) postulated that RNA synthesis is the molecular site of the action of insulin in promoting the synthesis of a series of specific proteins which (might) condition the several anabolic actions of the hormone. A similar line of thought may also be found in the literature pertaining to the male hormone testosterone. Since much of the phenomena of RNA metabolism and protein biosynthesis has been elucidated only within the last few years, this may explain our relative lack of knowledge of the action of the various hormones--though we have known of their existence and importance for many years.

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APPENDIX

The following is a list of the chemicals used which were purchased from the respective companies in the most pure form available. Adenosine triphosphate (ATP), quanosine triphosphate (GTP), and phosphocreatine from Sigma Chemical Corporation; Na-L-thyroxine from Mann Research Laboratories; adenosine monophosphate (AMP), cytidine monophosphate (CMP), quanosine monophosphate (GMP), and uridine monophosphate (UMP) chromatography standards from Pabst Laboratories; isobutyric acid from Fisher Chemical Company; PPO (2,5-diphenyloxazole), POPOP (p-bis ((2 (5-phenyloxazolyl)))-benzene), and hydroxide of hyamine 10-x from Packard Instrument Company; scintillation grade toluene from Fisher Scientific Co; sulfaguanidine from American Cyanamid Co.; and DL-leucine-1-C^{14} and $NaH_2P^{32}O_4$ from New England Nuclear Corp.

ABSTRACT OF DISSERTATION

Submitted in Candidacy for the Degree of

Doctor of Philosophy

in

Biochemistry and Nutrition

THE EFFECT OF THYROXINE ON PROTEIN BIOSYNTHESIS AND RIBONUCLEIC ACID METABOLISM IN THE RAT

by

Glenn M. Cahilly, Jr.

Thyroxine was observed to stimulate the incorporation of radiolabeled amino acids into protein of cell-free liver systems from treated normal and hypothyroid rats. However, the *in vitro* addition of the hormone only had a stimulatory effect, though quite erratic in magnitude, on the normal system. This difference in response has not been explained.

The hypothyroid rat was also characterized by lowered liver ribonucleic acid levels. It was found that thyroxine pretreatment resulted in increased ribonucleic acid levels only in the hypothyroid rat with the greater increase occurring in microsomal ribonucleic acid. No change was observed in the ribonucleic acid base ratios of the hypothyroid rat when compared to those of the normal. However, thyroxine pretreatment of the hypothyroid rat did alter the rate of incorporation of radio phosphorus into the various nucleotides, although there was little difference in the total amount of incorporated label.

Phosphorus-32 appeared to be incorporated into guanosine-monophosphate at the expense of adenosine monophosphate. The suggestion was made that thyroxine effects protein synthesis through ribonucleic acid metabolism by regulating the rate of guanosine-monophosphate synthesis.