SOME IN VIVO EFFECTS OF TRIIODOTHYRONINE ON PURINE NUCLEOTIDE SYNTHESIS

by

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To
Joyce,
Zeb,
Zelda,
Rags

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To Dr. C. J. Ackerman, I express my sincere appreciation for his advice and guidance throughout the course of these studies.

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L-T_3	3,5,3'-triiodo-L-thyronine
RNA	ribonucleic acid
t-RNA	transfer RNA
DNA	deoxyribonucleic acid
DOR	2-deoxy-D-ribose
BMR	basal metabolic rate
AMP	adenosine-5'-monophosphate
ADP	adenosine-5'-diphosphate
ATP	adenosine-5'-triphosphate
GMP	guanosine-5'-monophosphate
GDP	guanosine-5'-diphosphate
GTP	guanosine-5'-triphosphate
UMP	uridine-5'-monophosphate
UDP	uridine-5'-diphosphate
UTP	uridine-5'-triphosphate
IMP	inosine-5'-triphosphate
NAD^+	nicotinamide adenine dinucleotide
FAD	flavin adenine dinucleotide
NADH	reduced nicotinamide adenine dinucleotide
ppGpp	5' diphosphate guanosine 3' or 2' diphosphate

INTRODUCTION

The primary mechanism through which thyroid hormones exert their effects at the molecular level remains as much a mystery today as it was when Kendall (1) first isolated crystalline thyroxine in 1914. In the intervening fiftyeight years however a vast amount of experimentation directed toward elucidation of such a mechanism has produced a wealth of evidence and theories. Many proposed mechanisms arising from these investigations can be considered improbable as primary molecular mechanisms because the experimental design employed failed to observe one or more of several important criteria inherent in hormone research. Many of the metabolic effects of thyroid hormones, proposed as mechanisms of action, are only demonstrable with extremely high nonphysiological levels of hormone and probably reflect pharmacological or toxic effects. Another factor often overlooked is that thyroid hormones elicit multiple physiological responses and many experiments are not designed to allow for distinction between primary and secondary effects. When all the various effects of thyroid hormones are arranged in chronological order of occurrence following hormone administration, it is readily seen that many metabolic effects such as growth and increased basal metabolic rate are secondary effects occuring as a result of some regulatory effect exerted by the hormone on cellular biosynthetic pathways.

Hormones initiate or regulate almost every aspect of growth and differentiation in higher organisms. Much of the recent research

concerning the effects of thyroid hormones on growth and differentiation is focused on protein synthesis and its control *via* ribonucleic acid metabolism.

The present investigation is concerned with the metabolism of purine nucleotides, precursors of ribonucleic acid, and the effect of thyroid hormones on their synthesis.

Studies of nucleotide metabolism in animal tissues are beset by various problems which include destruction and rapid shifts in cellular levels of the nucleotides during sampling and extraction procedures. It was necessary to develop suitable methods for obtaining and extracting tissue samples in order that the nucleotide distribution and labelling pattern reflect *in vivo* conditions.

The investigations cited in the following literature review are those which form the basis for the presently accepted concepts of the mechanism of action of thyroid hormones and those which concern the present hypothesis--that thyroid hormones influence the synthesis of purine nucleotides. Also included in the literature review is a discussion of pertinent work by various investigators concerning the problems involved in nucleotide extraction and the conditions under which tissue samples are obtained.

LITERATURE REVIEW

Effects of thyroid hormones[1] on growth and differentiation - The most dramatic effect of thyroid hormones on growth and development is the initiation of amphibian metamorphosis. Thyroidectomized tadpoles will not undergo metamorphosis until thyroid hormones are administered (2). That thyroid hormones are necessary for proper growth has been well documented through experiments in which antithyroid drugs have been fed to female animals during pregnancy. The offspring in such cases invariably show signs of cretinism (3). Cretinism, or congenital hypothyroidism, in humans results in severe mental retardation and the body retains infantile characteristics of bone structure. Prompt post-natal treatment with thyroid hormones will partially correct the skeletal abnormalities, but such treatment results in very little improvement in the mental development (4).

An important phenomenon associated with the growth response to thyroid hormones is that the response is biphasic. Small doses of thyroxine or tri-iodothyronine will, after a latent period of three to four days, promote growth, while large doses markedly suppress growth (5,6). In experiments using thyroidectomized rats, Tata (6)

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[1]Throughout this thesis the term "thyroid hormones" refers collectively to the two naturally occuring, biologically active hormones 3,5,3'-triiodo-L-thyronine and 3,5,3',5 -tetraiodo-L-thyronine.

injected varying doses of thyroxine and observed the following results: dose levels up to 40 μg/100 g body weight stimulated growth, while doses above 100 μg/100 g body weight suppressed growth. Physiological plasma levels of thyroxine in normal rats have been estimated by Lardy and Kent (7) and Hasen (8). Both laboratories reported values of 10^{-8} M. Therefore experiments designed to elucidate the role of thyroid hormones should take into consideration the low levels of hormone in normal animals and the effects of large doses of the hormone.

Effects of thyroid hormones on BMR and mitochondria - Thyroid hormones exert a definite stimulatory effect on BMR. Tata (9) has shown that the BMR increases with increasing levels of hormone injected into thyroidectomized rats. While there is no biphasic effect of the hormone on BMR when given in high doses, there is a latent period of 35 to 40 hours, similar to that seen prior to a growth response to the hormone. Some early workers considered the stimulation of BMR to be an initial action of thyroid hormones. However, considering the other responses to the hormone which occur during the latent period prior to a rise in BMR, stimulation of BMR can only be considered a secondary response to some earlier hormonal action (10). Because of the influence of the hormones on BMR much research was concerned with the effect of the hormones on mitochondrial structure and function. The observation that thyroid hormones would lower the P:O ratio in isolated liver mitochondria had been considered particularly significant in the past. This phenomenon was noted in rat liver mitochondria to which the hormone

was added in vitro or with mitochondria isolated from rats to which large, nonphysiological doses of the hormone had been administered (11,12). The lowered P:O ratio was considered to be the result of uncoupling of oxidative phosphorylation by the hormone. Tata (13) however failed to detect any uncoupling of phosphorylation or loosening of respiratory control in mitochondria obtained from thyroidectomized rats after administering physiological levels of thyroxine - an amount which stimulated BMR to normal levels.

Thyroid hormones have also been observed to cause swelling of liver mitochondria, both in vitro (14,15) and in liver isolated from rats made thyrotoxic by chronic administration of large doses of the hormone (15). It is of interest that mitochondria from cardiac muscle, skeletal muscle, testes and brain do not swell under the influence of thyroid hormones (17) and that liver mitochondria exhibit swelling in the presence of the biologically inactive D-isomers of thyroxine and triiodothyronine (16) and biologically inactive analogues of thyroid hormones (18).

Small doses of triiodothyronine administered to thyroidectomized rats will stimulate the incorporation of amino acids into mitochondrial protein (18). This increase in mitochondrial protein synthesis precedes by several hours the stimulation of BMR. There is a 30-80% increase in the levels of cytochromes a, b, and c at ten to fifteen hours after the onset of stimulation of amino acid incorporation (10). This stimulation of protein synthesis

observed in vivo could not be demonstrated when the hormone was added to a suspension of mitochondria. These results led Roodyn and co-workers to conclude that thyroid hormones exert their physiological effects on mitochondrial structure and activity by an increase in the respiratory and phosphorylative unit of the particles as a consequence of an enhanced capacity of the cell to synthesize protein (10). The uncoupling of oxidative phosphorylation and swelling of mitochondria are probably only pharmacological effects of thyroid hormones.

Effects of thyroid hormones on protein synthesis - Dutoit (20) reported in 1952 that thyroidectomy depressed the incorporation of labeled alanine into protein of rat liver slices and that large in vivo doses of thyroxine stimulated the incorporation. Scant notice was given this observation until Sokoloff and coworkers began to study the effects of thyroid hormones on the incorporation of amino acids in protein by cell-free systems (21,22,23,24). These investigators found that thyroid hormones enhanced the capacity of cell-free systems to synthesize protein after either incubation with thyroxine in vitro or thyroxine administration in vivo. These observations have been confirmed in other laboratories (13,18,25,27). Sokoloff and his colleagues concluded that the stimulation, both in vivo and in vitro, was due to the same mechanism. Several facts however argue against such a conclusion. Thyroxine was less effective in stimulating amino acid incorporation into protein in homogenates obtained from

thyroidectomized rats than in homogenates prepared from normal rats. The stimulation of protein synthesis *in vivo* has been shown to be much greater in thyroidectomized rats than in normal rats (19).

In Sokoloff's cell-free preparation, the stimulation of protein synthesis could only be observed in the presence of mitochondria and a creatine phosphate - ATP generating system failed to replace the mitochondria. He concluded that thyroid hormone interacts with mitochondria to release an unknown substance which stimulates the transfer of amino-acyl t-RNA to microsomal protein (22). However, other investigators have demonstrated an increase in amino acid incorporation into microsomes in the absence of mitochondria (13,25). Two other observations emerging from Sokoloff's work which should be noted are that the biologically inactive D-isomer of thyroxine was as effective as L-thyroxine in stimulating amino acid incorporation in his cell free preparation (21), and the naturally occurring L-triiodothyronine (which is at least as biologically active as L-thyroxine) had no stimulatory effect on his *in vitro* protein synthesizing system (27). Weiss and Sokoloff (28) concluded that the inhibition by puromycin of the thyroxine stimulated increase in BMR was supporting evidence for the amino-acyl t-RNA hypothesis. The following facts cast doubt on the significance of such a conclusion: (a) if protein synthesis is halted by Actinomycin D, 5-flurouracil or starvation, the action of thyroid hormones is blocked (29,30); (b) thyroid hormones stimulate the turnover of nuclear RNA prior to any stimulation

of cytoplasmic amino acid incorporation in protein (31,32). In any event it is difficult to visualize how such a non-specific stimulation of protein synthesis as that proposed by Sokoloff could account for the specific action of thyroid hormones.

Effects of thyroid hormones on nucleic acid synthesis - Tata and his colleagues (29-34) have investigated in detail the effects of thyroidectomy and thyroid hormone administration on the synthesis of rat liver RNA during the physiological latent period of the hormone. Thyroidectomy lowered the DNA-dependent RNA polymerase activity of isolated rat-liver nuclei but in vivo administration of thyroxine or triiodothyronine stimulated the activity of this enzyme. In experiments using [^{14}C] orotic acid, the earliest response to the hormone was a 25 per cent increase in the specific radioactivity of rapidly labeled nuclear RNA three to four hours following hormone administration. At twelve hours the specific activity was doubled. The hormone-stimulated increase in incorporation of orotic acid into RNA was followed by an enhanced total uptake of [^{14}C] orotic acid (acid soluble and acid insoluble) by the liver at sixteen hours after triiodothyronine administration but not at earlier time intervals, suggesting that the increase in uptake may be an adaptation to an accelerated rate of RNA synthesis (30,32). Activity of the Mg^{2+}-activated RNA polymerase of nuclei isolated from thyroidectomized rats was stimulated at ten to twelve hours after hormone injection while the activity of the $Mn^{2+}/(NH_4)_2SO_4$-activated RNA polymerase was

not significantly stimulated until forty-five hours after the hormone was given. Neither of the two RNA polymerase activities could be stimulated *in vitro* by addition of the hormone to isolated nuclei (32). The increase in specific activity of rapidly labeled nuclear RNA prior to any *in vivo* stimulation of RNA polymerase activity and the inability of the hormone *in vitro* to stimulate RNA polymerase activity argues against a direct hormone-polymerase interaction *in vivo*.

That unimpaired RNA synthesis is essential for the full expression of the physiological effects of thyroid hormones has been demonstrated in experiments employing actinomycin D, a potent inhibitor of RNA synthesis (35). Actinomycin D, at levels which completely blocked the thyroid hormone-induced regression of tadpole tails also inhibited completely the hormone-induced stimulation of the incorporation of radioactive uridine into RNA (36). In studies using thyroidectomized rats, actinomycin D blocked completely the stimulation of BMR, growth rate and the rise in RNA polymerase activity following hormone administration without having any effect on the activity of RNA polymerase in control animals (32). While these results do not pinpoint the mechanism or site of action of thyroid hormones, they do provide evidence that RNA synthesis is prerequisite to the ultimate physiological actions of the hormone.

Effects of thyroid hormones on purine synthesis - Necheles (26), using bone marrow slices, observed that thyroid hormones stimulated the incorporation of [2-^{14}C] glycine into adenine of RNA, yet the hormone had no effect on the incorporation of [8-^{14}C] adenosine into RNA. The optimum thyroxine concentration in Necheles' incubation system was 10^{-7}M. Mah and Ackerman (37) demonstrated that when radioactive glycine was incubated with the soluble fraction (100,000 x g supernatant) of rat liver, the incorporation of the label into the purine nucleotides was enhanced by the addition of either thyroxine (at an optimal concentration of 10^{-7}M) or triiodothyronine (at an optimal concentration of 10^{-9}M). When the distribution of the label in the purine nucleotides was determined, it was observed that the concentration and specific activity of the adenine had increased more than had that of guanine. From this it was deduced that the reaction affected by the hormone was the branch point from which IMP is converted to either AMP or GMP. Subsequent studies by Ackerman and colleagues using partially purified enzymes from rat liver showed that adenylosuccinate synthetase (the first enzyme catalyzing the conversion of IMP to AMP) was stimulated by triiodothyronine at an optimal concentration of 10^{-9}M (38,39) and that IMP dehydrogenase (the first enzyme catalyzing conversion of IMP to GMP) was inhibited by triiodothyronine at an optimal concentration of 10^{-9}M (40). These findings suggested that thyroid hormones may regulate RNA synthesis by

altering the availability of RNA precursors. A high cellular AMP/GMP ratio resulting from the stimulation of AMP production from IMP at the expense of GMP could be the mechanism through which thyroid hormones exert their initial effects. Such a control of purine nucleotide synthesis would have far reaching consequences because adenine nucleotides are allosteric regulators of many key enzymes involved in cellular energy metabolism and ATP serves as a substrate for RNA synthesis.

If thyroid hormones play a role in AMP and GMP synthesis as was observed in the in vitro experiments, it would be necessary to observe the same response in the intact animal. In principle, at least, experiments designed to test this response to the hormone are straightforward and involve tracing the incorporation of radiolabeled glycine into the purine nucleotides of intact rats treated with thyroid hormones. However this type of experiment is plagued by technical problems which include rapid changes in metabolite levels during isolation, tedious separation and estimation of metabolites, and the kinetics of precursor uptake and exchange by the tissue under study.

The isolation and quantitative determination of all the nucleotides in an extract of rat liver is tedious and difficult. It was therefore, necessary to develop a procedure for isolation and extraction of nucleotides which would reflect as nearly as possible the actual in vivo conditions at the time of tissue sampling. The

objectives of the work reported in this thesis were to develop a suitable method for nucleotide isolation and to determine the effect of thyroid hormones on purine nucleotide synthesis in vivo.

Several groups of investigators have shown that the anesthesia and delay in freezing of samples with attendant hypoxia (or anoxia) may lead to pronounced depression of tissue levels of labile metabolites resulting in misinterpretation of the results of an experiment (41-44). The ATP/ADP ratio is a sensitive indicator of possibly lowered levels of nucleoside triphosphates and other labile compounds resulting from unphysiological conditions arising during tissue sampling. The level of AMP is reported to be the single most sensitive parameter for tissue anoxia (44). Ischemia produces extremely rapid and reversible changes of the intracellular oxidation state of solid tissues. Chance and coworkers '41) have simultaneously measured fluorescence and metabolite changes during ischemia and recovery from ischemia in rat liver in vivo. They observed rapid and extensive changes in the metabolite pattern of the organ. NADH increased up to 100% and AMP up to 200%, with a ten-fold decrease in the ATP/ADP ratio. Uric acid increased nine-fold. These transitions begin about 5 seconds after the onset of ischemia and reversal begins within 3 seconds after circulation is restored. The chief factor promoting breakdown of ATP seems to be the degree of tissue anoxia developing under anesthesia and before the sample is frozen (decapitation is worse

in this respect than anesthesia). Faupel et al, investigating the effects of anoxia on rat liver, observed that within 30 seconds after the onset of anoxia the level of AMP increased by 700%, ADP increased by 100% and ATP decreased by 36%.

Bucher and Swaffield (43) have pointed out that while the absolute metabolite levels of liver vary according to species, sex and nutritional state, the ratios of these levels remain nearly constant. Faupel has compiled the results of various investigators reporting adenine nucleotide levels and ATP/ADP ratios in rat liver (44). There is reasonable agreement among the reported values for total adenosine phosphates but wide variation in the distribution among mono-, di- and triphosphates. There is a definite upward trend in ATP levels and in the ATP/ADP ratio as analytical and sampling techniques have improved over the years. The highest values in both categories have been reported by Bucher and Swaffield (43), Chance (41), Faupel et al and Hohorst et al. Hohorst and coworkers found that within the first 5 seconds after interrupting circulation to the liver the ATP/ADP ratio fell from 3.3 to less than 2. Their highest value was obtained using light ether anesthesia and freezing the tissue in situ by the method of Wollenberger, Ristau and Schoffa (46). Chance used urethane for anesthesia and the in situ freezing technique of Hohorst et al. Bucher and Swaffield used ether anesthesia supplemented with 100% oxygen and the freezing technique of

Wollenberger et al. These workers reported (43) the result of preliminary experiments under suboptimal conditions wherein no oxygen supplementation was employed and liver samples were first excised and squashed between blocks of carbon dioxide. This procedure requires several seconds longer than freezing in situ and the ATP/ADP ratios obtained after excision of the tissue were in the range of 2.0-2.2, considerably lower than the value of 4.6 obtained by freezing in situ. Thus it is obvious that any experiments attempting to elucidate the effect of thyroid hormones on purine nucleotide metabolism must take into consideration these rapid changes in nucleotide concentration and the method by which the tissue sample is obtained. To alleviate the effects of anoxia and ischemia occuring during tissue isolation it was decided that for these studies, liver samples would be obtained by freezing the liver in situ using ether anesthesia with oxygen supplementation. Little is known, however, about the effects of high oxygen tension on metabolite levels. Lumb (47) has reported that administration of 100% oxygen to rats results in a seven-fold elevation of the level of plasma oxygen. Therefore, it was felt that in order to more accurately reflect normal conditions, the oxygen level during anesthesia should be the same as that in air. An apparatus was designed to maintain light ether anesthesia while providing oxygen at 21%.

To determine whether thyroid hormones altered the synthesis and/or cellular levels of free purine nucleotides, hypothyroid rats were

injected with [1-^{14}C] glycine and triiodothyronine. Control animals received only radioactive glycine. At appropriate times following injection animals were anesthetized and liver samples were obtained for subsequent separation and determination of nucleotide levels and label distribution.

EXPERIMENTAL PROCEDURE

Important Details

Preparation of animals - Male weanling Sprague-Dawley derived rats were purchased from Flow Laboratories, Inc., Dublin, Va. and were fed a complete diet containing 1.2% sulfaguanidine for 5 weeks prior to sacrifice (48). The rats weighed 110-130 grams and gained less than 3 grams during the fifth week. Such rats are considered to be thyroid hormone deficient. All experiments were initiated at 8:00 p.m. to take into consideration diurnal variations in metabolic activity. Hypothyroid rats were denied food at 4:00 p.m., 4 hours prior to injection of glycine or hormone. Labeled glycine was injected intraperitoneally into the first rat in a group at 8:00 p.m. and subsequent rats were injected at eight minute intervals to allow time for anesthesia and tissue isolation. [1-^{14}C] glycine dissolved in 0.13 M sodium phosphate buffer at pH 7.4 was administered at a dose level of 10 μCi per 100 g body weight. Rats receiving thyroid hormone were injected intraperitoneally with 15 μg of 3,5,3'-triiodo-L-thyronine in a volume of 0.3 ml of isotonic saline. Control groups received saline only.

Anesthesia and isolation of tissue - At 7 minutes prior to sacrifice, rats were lightly anesthetized with ether by placing them in a chromatography jar containing a wad of cotton wool saturated with ether. As soon as the rat was limp (usually less than 1 minute), it was removed from the jar and placed in the plastic gas bag of the

anesthesia apparatus. The apparatus depicted in Figure 1 was a modification of the oxygen supplementation system of Bucher and Swaffield (43) and provided a constant flow of breathing air containing 4.5% ether while maintaining the oxygen level at 21 $\pm$ 1%. After a rat was placed in the gas bag, a 3 to 4 minute equilibration period was allowed to permit blood and tissue oxygen levels to return to normal. The peritoneal cavity was then opened to expose the liver, one lobe of which was lifted for access. Other tissues were teased aside and the liver was then squashed between metal plates attached to tongs that had been precooled in liquid nitrogen according to Wollenberger, et al (46).

All tissue not immediately frozen and compressed to wafer thinness was trimmed away. The liver sample was then placed in a porcelain mortar which was also precooled in liquid nitrogen.

Extraction of acid soluble nucleotides - Pooled frozen liver samples from 5 to 8 rats in a group were ground to a fine powder in the precooled mortar and stirred into 8 volumes of ice-cold 0.2 M perchloric acid and allowed to thaw in contact with the acid during homogenization at half maximal speed in a Sorvall Omnimixer at 4°. All subsequent procedures were carried out at 4°. The homogenates were centrifuged at 27,000 x g for 10 minutes in a Sorvall RC2B refrigerated centrifuge to eliminate acid-insoluble components. The supernatant was decanted and the pellets were washed by resuspension in 10 ml of 0.2 M perchloric acid and centrifugation. The pellet was

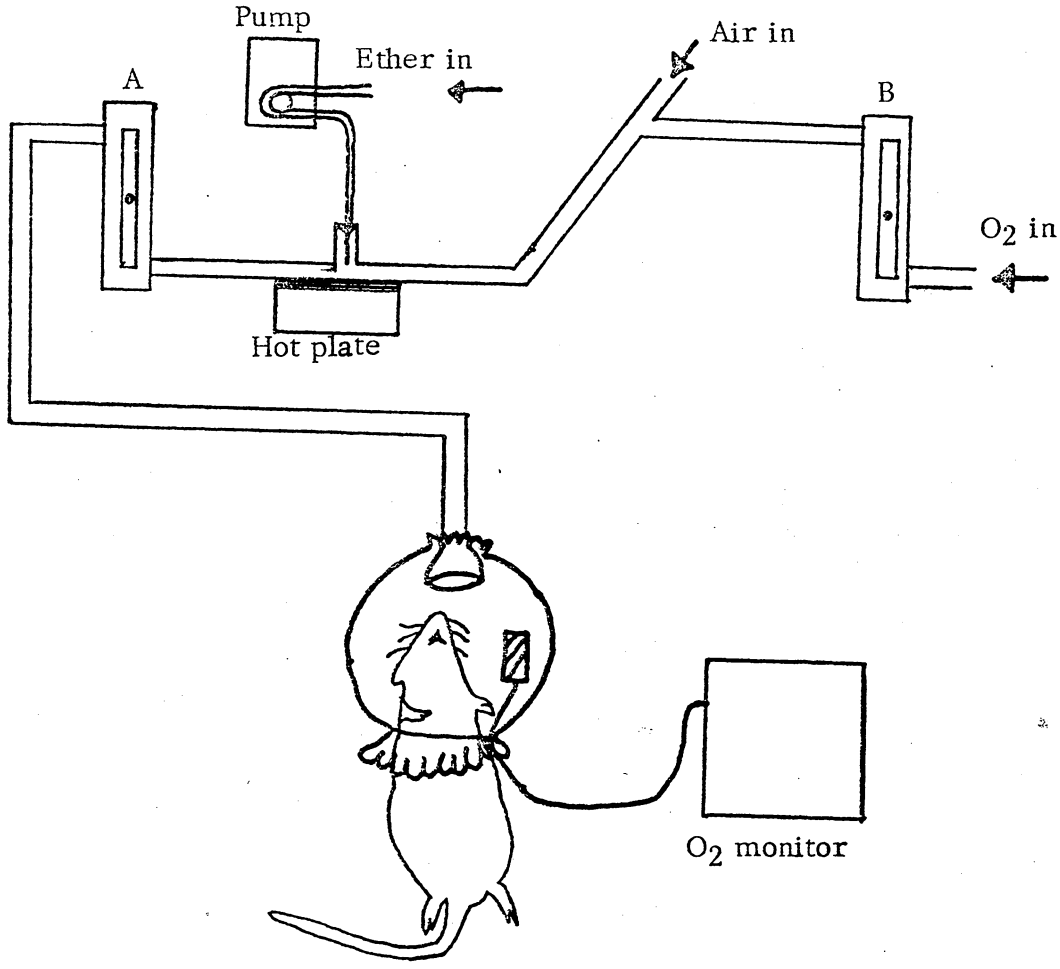

Figure 1. Apparatus for maintaining ether anesthesia with controlled oxygen levels.

Ether is introduced into the air line from a reservoir by means of a peristaltic pump (Buchler Dekastaltic). The ether is pumped through a 27 gage syringe needle inserted into a pyrex glass tee which rests on an electric hot plate. In operation breathing air at the rate of approximately 280 ml per minute passes through flowmeter A and ether is introduced until the oxygen level, monitored by the sensor placed in the gas bag, is reduced to 20%. This results in a gas mixture containing approximately 4.7% ether. Oxygen, passing through flowmeter B, is then bled into the air line at the point marked C to bring the oxygen level of the mixture to 21%. The plastic bag is tied around the rat with a rubber band and the flowing gas inflates the bag which is continuously flushed as gas escapes beneath the rubber band. Oxygen is continuously monitored with a Beckman Model 777 Laboratory Oxygen Analyzer. Gas flows are controlled by Gilmont Calibrated Flowmeters with micrometer valves.

saved for DNA analysis. The washes were combined with the original supernatants and the pH was adjusted to neutrality with 7 M KOH. The $KClO_4$ precipitate was removed by centrifugation at 12,000 x g for 10 minutes, leaving the acid soluble components in the clear supernatant. The neutralized extracts were either chromatographed immediately or frozen at -20° for later use.

DNA analysis of acid extracted pellets - The perchloric acid washed precipitate was extracted twice by resuspension in 30 ml of 0.5 M perchloric acid at 70° for 15 minutes with constant stirring and centrifugation at 27,000 x g for 10 minutes. The combined extracts were diluted to a suitable volume with 0.5 M perchloric acid and aliquots were taken for DNA determination by the method of Burton (49) as modified by Giles and Meyers (50).

RNA extraction and estimation - RNA was extracted from the perchloric acid washed pellets by alkaline digestion and its concentration was estimated spectrophotometrically, correcting for any protein contamination by the two-wavelength method of Munro and Fleck (51).

Preparation of free purines for analysis of total pools - In order to determine the effect of triiodothyronine on the distribution of labeled glycine throughout the total purine pools, the individual purines were isolated after hydrolysis of the acid soluble extract of rat liver. Rats were injected with [1-^{14}C] glycine and hormone

as described above. Preparation of the acid extract and analysis of the DNA content of the acid extracted pellet were performed as described above. Sufficient concentrated $HClO_4$ was added to each acid soluble extract to bring the acid concentration to 1 normal and the extracts were heated in a boiling water bath for one hour. This method yields purines as free bases but the pyrimidines remain as nucleosides or nucleotides (52,53). After hydrolysis, the extracts were adjusted to pH 1.1 with concentrated NaOH and the free purines were precipitated by adding 1 ml of 1M $AgNO_3$ per five ml of hydrolysate. This preparation was chilled overnight at 4^o and centrifuged at 12,100 x g for 10 minutes. The supernatant was discarded and the precipitate was washed with 2 ml of 0.1 N H_2SO_4 at 55^o and centrifuged at 12,100 x g for 10 minutes. The supernatant was discarded and purines were recovered by extraction 3 times with 2 ml portions of 2 M HCl followed by centrifugation at 12,000 x g for 10 minutes. The supernatants were combined and the pH was adjusted to 1.2 by dilution with deionized water. This preparation was chromatographed on a Dowex-50-H column or frozen at -20^o for chromatography later.

Chromatography of purines by cation exchange - The purine samples prepared as described above were added to a 1.5 x 18 cm Dowex-50-H^+ column. The column was washed with several bed volumes of water. Gradient elution of the purine bases was accomplished by use of an apparatus consisting of a separatory funnel containing

1.5 M HCl which acts as a reservoir, and an erlenmeyer flask, containing initially 525 ml of 0.1 M HCl, which serves as a mixing chamber. The HCl in the reservoir flowed by gravity into the mixing flask where mixing was achieved by means of a magnetic stirrer. A volume of 3 to 4 ml of eluent was maintained above the surface of the resin bed. The flow rate of the eluate was adjusted to 1 ml per minute. The column eluate was monitored at 254 nm with a Buchler Uviscan ultraviolet monitor. Ten ml fractions were collected with an automatic fraction collector. Fractions making up the same peak were pooled and made up to a known volume for spectral analysis and quantitation. Suitable aliquots were evaporated to dryness in counting vials, the residue was dissolved in 1 ml of distilled water and 10 ml of liquid scintillation solution was added. Radioactivity was determined in a Beckman Model LS-133 liquid scintillation spectrometer.

Separation of acid soluble nucleotides by anion exchange chromatography - Initial separation of nucleotides was accomplished using a modification of the triethylammonium acetate system of gradient elution described by Caldwell (54). Flow rate of the eluate was 60 ml per hour and the eluate was monitored at 254 nm with a LKB Uvicord ultraviolet absorptiometer. The eluate was collected with an automatic fraction collector set to collect 10 ml fractions. Recoveries of individual nucleotides were 87% for AMP and 94% for GMP.

The procedure developed by Caldwell utilizes a 1 x 90 cm bed of DEAE-Sephadex A-25-Ac^- and elution is achieved by a series of concave ionic strength gradients at pH 4.7. Preliminary experiments showed that increasing the bed length from 90 to 150 cm resulted in a better separation of NAD^+ from several unidentified components which are eluted immediately before NAD^+. Also, uric acid, which according to Caldwell, elutes in the same peak as NAD^+ could not be detected either spectrally or by thin layer chromatography after 25-fold concentration of the fractions making up the NAD^+ peak from a sample of in situ frozen liver. Addition of authentic uric acid to an acid soluble extract of liver before chromatography resulted in spectral distortion of the fractions making up the NAD^+ peak.

By increasing the column temperature from ambient to 45° it was possible to completely separate NAD^+ from uric acid, however, this procedure caused the unidentified components which eluted ahead of NAD^+ at room temperature to elute in the same peak as NAD^+. It was necesssary, therefore, to chromatograph initially at room temperature and to rechromatograph the fractions containing NAD^+ and uric acid at 45° on an identical column. Because of the extremely small amount of endogenous uric acid found in frozen liver samples and because uric acid has an absorption minimum at 254 nm, the wavelength monitored, it was necessary to add a measured amount of unlabeled uric acid as carrier in order to detect its presence in the column eluate.

AMP and IMP were eluted in the same peak, and no separation of these could be obtained in spite of many attempts which included variation in bed length, column temperature or pH. It was found, however, that separation could be achieved by rechromatography on DEAE-Sephadex A-25-Cl^-. Fractions making up the AMP/IMP peak were pooled, diluted to 3 volumes with deionized water and the pH was adjusted to neutrality with concentrated NH_4OH. The sample was then added to a 1.5 x 20 cm column of DEAE-Sephadex A-25-Cl^-. The column was washed with several bed volumes of water and AMP and IMP were eluted sequentially by stepwise addition of 10 mM and 50 mM HCl. The amounts of IMP recovered from in situ frozen liver samples were so small that it was necessary to add authentic, unlabeled IMP as carrier in order to detect its presence in the column eluates. Recovery of AMP was 95%.

Fractions making up the same peak from either the 150 cm acetate form column or the 20 cm chloride form column were pooled, frozen and lyophilized in a Virtis lyophilizer. After lyophilization, the nucleotides were dissolved in a known volume of water and quantitated spectrophotometrically in a Beckman DU spectrophotometer fitted with a Gilford Model 220 Optical Density Converter. Radioactivity was determined by pipetting 1 ml aliquots into 10 ml of liquid scintillation solution and counting in a Beckman Model LS-133 liquid scintillation spectrometer.

Identification of eluted materials - Purines eluted from the cation exchange column and nucleotides eluted from the anion exchange columns were identified on the basis of the following criteria: (a) ultraviolet absorption spectra; (b) coincident elution from the column with authentic samples added to the purine or nucleotide preparations; and (c) co-chromatography with authentic compounds on microcrystalline cellulose thin layer plates (for purines) or polyethyleneimine cellulose plates (for nucleotides) in at least two solvent systems according to Randerath and Randerath (55).

Supportive Data

Materials - [1-^{14}C] glycine (15 mCi/mmole and Aquasol xylene based liquid scintillation solution were purchased from New England Nuclear Corporation, Boston, Massachusetts. Guanine and GTP were purchased from P-L Biochemicals Incorporated, Milwaukee, Wisconsin. Adenine, hypoxanthine, uric acid, AMP, ATP, CMP and actinomycin D were purchased from Schwarz-Mann, Orangeburg, New York. ADP, GDP, UDP, CDP, UTP, CTP, 2-deoxy-D-ribose and 3,5,3'-triiodo-L-thyronine were purchased from Sigma Chemical Company, St. Louis, Missouri. Triiodothyronine was recrystallized from boiling 2 N HCl (melting point 201-203°) (56). Xanthine, UMP, GMP, IMP, and NAD^+ were purchased from Calbiochem, Los Angeles, California. The cation exchange

resin Dowex-50-H^+ was obtained from Bio-RAD Laboratories, Richmond, California. The anion exchanger, DEAE-Sephadex A-25 was purchased from Pharmacia Fine Chemicals, Incorporated, Piscataway, New Jersey. Prepared microcrystalline cellulose thin layer chromatography plates were obtained from Quantum Industries, Hanover, New Jersey. Prepared polyethyleneimine cellulose thin layer chromatography plates were purchased from Schleicher and Schuell, Incorporated, Keene, New Hampshire. Triethylamine was purchased from J. T. Baker Chemical Company, Phillipsburg, New Jersey and purified by refluxing for 3 hours with 2, 4-diaminophenol dihydrochloride (3 g/liter), followed by distillation at ambient pressure (57). This purification procedure did not lower the ultraviolet absorption of the triethylamine. However, dilute aqueous buffers prepared from such purified material showed negligible absorption. Diphenylamine for use as a color reagent for the estimation of DNA was purchased from Fisher Scientific Company, Fair Lawn, New Jersey and purified by steam distillation. Other chemicals were obtained from commercial suppliers and were of reagent grade.

RESULTS AND DISCUSSION

Results obtained from in vitro studies on the soluble fraction of rat liver showed that low levels (10^{-9} M) of triiodothyronine stimulated the synthesis of AMP while inhibiting the synthesis of GMP. The results of the in vivo studies reported in this thesis provide evidence for a thyroid hormone stimulation of AMP synthesis. The results do not provide clear cut evidence of an inhibition of GMP synthesis, but rather, they indicate that guanine nucleotides follow a more complex metabolic pattern than can be elucidated from this study.

As a first approach to this problem, only the total adenine and guanine in the acid soluble extract of frozen liver samples were determined because of the relatively simple analytical techniques. Figure 2 represents the elution curves of the purines adenine, guanine and hypoxanthine eluted from a Dowex-50-H^+ column illustrating the comparatively small amount of hypoxanthine that is present, part of which is derived from IMP. Parenthetically, this can be compared with Figure 3 which represents the elution pattern of these purines obtained from the acid soluble fraction of liver obtained from rats killed so that anoxia occurred. The relatively large quantity of hypoxanthine indicates a shift in the metabolic pathways that could lead to a misinterpretation of results.

The results summarized in Table 1 show that as early as one hour after the administration of 15 µg of triiodothyronine (4 hours

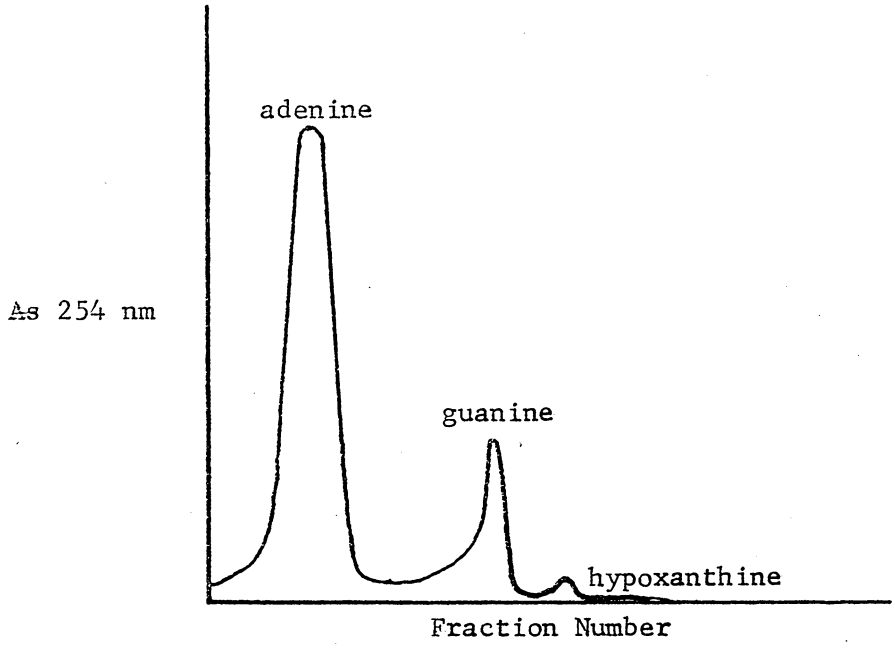

Figure 2. Chromatographic elution profile of purines isolated from the acid soluble extract of rat liver obtained by in situ freezing.

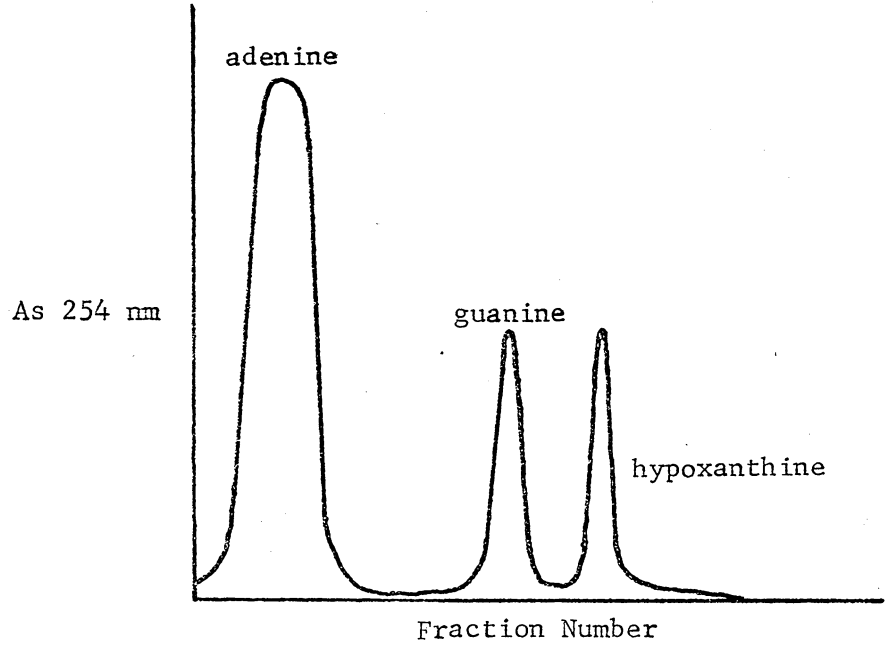

Figure 3. Chromatographic elution profile of purines isolated from the acid soluble extract of rat liver obtained under conditions of anoxia.

TABLE I

Effect of triiodothyronine on the specific activity, size, and total radioactivity distributed between the hepatic adenine and guanine pools of hypothyroid rats

Purine	L-T_3[a] hrs	μmoles[b] / mg DOR	Specific Activities: cpm / μmole	% change from control	cpm / mg DOR	% change from control	Total cpm in purine	Total cpm in adenine / Total cpm in guanine
Adenine	none	9.42	273	----	2580	----	3986	4.41
Guanine	none	0.75	771	----	583	----	902	
Adenine	1	9.8	305	+12	2980	+15	3074	4.64
Guanine	1	0.74	868	+13	643	+10	662	
Adenine	2	9.35	590	+115	5520	+114	7683	6.82
Guanine	2	0.72	1126	+46	810	+39	1126	
Adenine	4	8.42	426	+56	3590	+39	2909	8.92
Guanine	4	0.78	516	-33	403	-31	326	

[a]All animals were injected with 10 μCi of [1-^{14}C] glycine per 100 g body weight 4 hours prior to sacrifice. Three of the groups were also injected with 15 μg L-T_3 (triiodothyronine) at 1,2 and 4 hours, respectively, prior to sacrifice.

[b]DOR = 2-deoxy-D-ribose.

after the administration of labeled glycine) an increase in specific activity of adenine was detectable. This increased from 12% over the control value at 1 hour to 115% at 2 hours. At four hours, the specific activity had declined to 56% over controls. The specific activity of guanine was greater than that of the adenine (evident throughout these experiments) but it increased to 46% above the control at 2 hours and then decreased to -33% of the control level at 4 hours. Of greater interest, is the distribution of radioactivity between the guanine and adenine. Comparison of the total radioactivity recovered in the purines cannot be compared between groups because of the different sizes of the pooled samples that were obtained from each group. However, the ratio of counts in adenine and guanine provides information as to how the hormone affected the metabolic pathway leading to both adenine and guanine. The ratio of cpm in adenine to that in guanine increased from 4.41 in the controls to 8.92 four hours after the hormone was administered. This supports the hypothesis that the hormone favors the synthesis of adenine.

Triiodothyronine stimulated the incorporation of 1-^{14}C glycine into AMP (Table II) but the incorporation into GMP appears to be inhibited. The specific activity of the AMP increased from 42%, 1.5 hours after hormone administration to 128% at 3 hours and radioactivity incorporated into the AMP pool (cpm/mg deoxyribose) increased from 38% at 1.5 hours to 110% at 3 hours. While the

TABLE II

Effect of triiodothyronine on the specific activity, size and total radioactivity distributed among the hepatic AMP, GMP and NAD^+ pools of hypothyroid rats

Nucleotide	L-T_3,[a] hrs	μmoles[b] mg DOR	Specific Activities: cpm/μmole	% change from control	cpm/mg DOR	% change from control	Total cpm in nucleotide	Total cpm in AMP / Total cpm in GMP
AMP	none	1.02	505	----	517	----	1,120	0.12
GMP	none	0.19	22,700	----	4,400	----	9,550	
NAD^+	none	1.04	4,700	----	4,900	----	10,640	
AMP	1.5	1.00	715	+42	715	+38	1,710	0.19
GMP	1.5	0.21	17,750	-22	3,790	-14	9,000	
NAD^+	1.5	1.23	16,500	+250	20,400	+320	48,500	
AMP	3	0.95	1,150	+128	1,085	+110	2,120	0.23
GMP	3	0.29	16,100	-29	4,760	+8	9,240	
NAD^+	3	1.54	22,300	+375	34,400	+600	67,000	

[a]All animals were injected with 10 μCi of [1-^{14}C]glycine per 100 g body weight 3 hours prior to sacrifice. Two of the groups were also injected with 15 μg L-T_3(triiodothyronine) at 1.5 and 3 hours, respectively, prior to sacrifice.

[b]DOR = 2-deoxy-D-ribose.

specific activity of the GMP was 16 to 40 times greater than that of AMP, the effect of the hormone was to decrease the specific activity of this compound from -22% at 1.5 hours after hormone administration, to -29% at 3 hours. This decrease in specific activity may be due to an increase in the pool size of GMP (umoles/mg deoxyribose) from some unlabeled source. Calculation of the radioactivity in the GMP pool per mg deoxyribose suggests that this is so, since triiodothyronine caused this to decrease to -14% in 1.5 hours and to +8% in 3 hours. Again the total radioactivity in the pools cannot be compared between groups but the ratio of cpm in AMP to that in GMP obtained from the same samples increased from 0.12 in the control to almost double this value (0.23) in 3 hours.

The results in Table III were obtained from rats which had been injected simultaneously with triiodothyronine and labeled glycine, 1.5 hours prior to sacrifice. Again the hormone stimulated the incorporation of the label into AMP (31%) and into NAD^+ (87%). Unfortunately the GMP sample was lost during chromatography as well as a portion of the NAD^+ fraction.

The experiments summarized in Tables IV and V were conducted at the same time to determine whether or not actinomycin D would prevent the stimulatory effect of the hormone on AMP synthesis. The results in Table IV, which represents that part of the experiment in which actinomycin D was not administered, show that triiodothyronine increased the specific activity of all the nucleotides, (except GMP)

TABLE III

Effect of triiodo-thyronine on the specific activity and cellular levels of hepatic AMP and NAD^+ from hypothyroid rats

Nucleotide	L-T_3, hrs [a]	μmoles [b] / mg DOR	Specific activity, cpm/μmole	% change from control
AMP	none	1.17	524	---
NAD^+	none	1.15	1030	---
AMP	1.5	0.87	685	+31
NAD^+	1.5	----[c]	1920	+87

[a] Both groups of animals received 10 μCi [1-^{14}C] glycine per 100 g body weight 1.5 hours prior to sacrifice. One group was also injected with 15 μg of L-T_3 (triiodothyronine) 1.5 hours prior to sacrifice.

[b] DOR = 2-deoxy-D-ribose

[c] A part of the NAD^+ from the hormone-treated group was lost following chromatography, therefore calculation of pool size was impossible. Also, all of the GMP from the control group was lost.

TABLE IV

Effect of triiodothyronine on the specific activities and levels of nucleotides and RNA of hypothyroid rat liver

Nucleotide	L-T_3,[a] hrs	μmoles mg DOR	Specific Activities: cpm μmole	% change from control	cpm mg DOR	% change from control	Total cpm[b] recovered in nucleotide
AMP	none	1.13	1,155	---	1,310	---	744
	1.5	1.10	1,565	+36	1,730	+32	904
GMP	none	0.20	4,330	---	945	---	492
	1.5	0.37	4,350	0	1,610	+80	844
NAD^+	none	1.26	1,720	---	2,170	---	1,236
	1.5	1.50	2,980	+73	4,470	+106	2,344
ADP	none	3.73	1,480	---	6,030	---	3,136
	1.5	3.46	1,950	+32	6,780	+12	3,556
GDP	none	1.37	2,340	---	3,200	---	1,828
	1.5	0.79	2,920	+25	2,330	-28	1,224
RNA[c]	none	744[d]	66.7[e]	---	49,500	---	28,300
	1.5	730	82.1	+23	59,000	+19	30,960

[a]Both groups of animals received 10 μCi [1-^{14}C]glycine per 100 g body weight 1.5 hours prior to sacrifice. One group also received 15 μg L-T_3(triiodothyronine) per rat 1.5 hours prior to sacrifice.

[b]The ratio, total cpm in AMP: total cpm in GMP was 1.51 for the controls. For the hormone treated rats, this ratio was 1.07.

[c]Total cellular RNA estimated as described in Experimental Procedure.

[d]RNA levels given are μg RNA-phosphorus/mg deoxyribose.

[e]RNA specific activity in this column is in cpm/μg RNA phosphorus.

TABLE V

Effect of triiodothyronine on the specific activities and cellular levels of nucleotides and RNA isolated from hypothyroid rats after pretreatment with actinomycin D [a]

Nucleotide	L-T_3 hrs	μmoles mg DOR	Specific Activities: cpm/μmole	% change from control	cpm mg DOR	% change from control	Total cpm recovered [b] in nucleotide
AMP	none	0.95	1,370	---	1,300	---	992
	1.5	1.30	1,745	+27	2,268	+75	2,268
GMP	none	0.25	4,970	---	1,220	---	928
	1.5	0.30	5,240	+5	1,572	+29	1,572
NAD^+	none	2.6	3,200	---	10,900	---	8,336
	1.5	2.18	5,440	+70	11,860	+8	11,860
ADP	none	3.57	1,645	---	6,240	---	4,476
	1.5	3.44	2,398	+46	8,240	+32	8,240
GDP	none	1.06	3,290	---	3,500	---	2,672
	1.5	0.85	5,500	+67	4,660	+33	4,660
RNA	none	670 [d]	59.0 [e]	---	39,500	---	30,080
	1.5	695	72.5	+23	50,280	+27	50,280

[a] In this experiment both groups of animals were injected intraperitoneally with actinomycin D in two doses of 8 μg/100 g body weight at 22.5 and 5.5 hours prior to sacrifice. Details of glycine and hormone administration were exactly as described in Table IV.

[b] The ratio, total cpm in AMP:total cpm in GMP was 1.07 for controls. For the hormone treated rats, this ratio was 1.44.

[c] RNA isolated as described in Experimental Procedure..

[d] RNA levels are μg RNA-phosphorus/mg deoxyribose.

[e] RNA specific activity in this column is in cpm/μg RNA phosphorus.

and that of RNA. Calculation of the distribution of the radioactivity in each nucleotide either as cpm/mg deoxyribose or total cpm recovered in each pool reveals anomalous behaviour in the GMP and GDP pools. The hormone appeared to effect an increase in the size of the GMP pool (from 0.20 to 0.37 umole/mg deoxyribose) and an increase in total amount of radioactivity in the GMP pool (from 945 to 1610 cpm/mg deoxyribose). The radioactivity incorporated into the AMP pool relative to that in the GMP pool appeared to decrease from 1.51 to 1.07 in response to the hormone (see footnote, Table IV). An error in the analysis of deoxyribose appears to be ruled out because such an error would be reflected in the calculations for the other nucleotides. The GDP pool appeared to decrease in size accompanied by a 25% increase in its specific activity and when the radioactivity in the GDP pool was calculated as cpm/mg deoxyribose, the hormone appeared to effect a 28% decrease in the incorporation of label.

The effect of triiodothyronine on guanine nucleotide synthesis is apparently not direct and the results observed may be dependent on when the labeled precursor and hormone were administered. In Table I, the hormone appeared to inhibit the incorporation of glycine into guanine more than 2 hours after the administration of glycine. In Table II, glycine was administered 3 hours prior to sacrifice and an apparent inhibition of GMP synthesis occurred. Compartmentation of the IMP to AMP pathway may explain the results. The effect of

hormone on the IMP to AMP pathway may be direct and rapid while the effect of the hormone on IMP to GMP may be delayed and modified by other changes in metabolism that have already taken place.

By far the greatest changes in specific activity occurred in the NAD^+ pools. When hormone was administered simultaneously with the tracer 1.5 hours prior to sacrifice, the specific activity was increased by as much as 87% (Table III). After 3 hours of hormone treatment the specific activity was almost 4 times that of control animals (Table II). In comparing the 250% increase in the specific activity of NAD^+ shown in Table II with the increases of 87 and 73% observed after 1.5 hours in Tables III and IV, it should be noted that the experiments are not strictly comparable. In the instance where a 250% increase was observed, glycine was administered 1.5 hours prior to hormone. In the experiments described in Tables III and IV tracer and hormone were administered simultaneously. The difference in magnitude of change in specific activity may be due to the relative timing of administration of hormone and precursor.

Inspection of Tables III-VI reveals that in each of these experiments the specific activity of NAD^+ was higher than that of AMP at any point in time. Additionally, the specific activity of ADP in the experiment described in Table IV was higher than that of AMP. This apparently anomalous situation may be explained by the concept of cellular compartmentation. Bucher and Swaffield (43) have

TABLE VI

Effect of actinomycin D on the specific activity and levels of nucleotides and RNA of hypothyroid rat liver[a]

Nucleotide	treatment[b]	μmoles mg DOR	Specific Activities cpm μmole	% change from control	cpm mg DOR	% change from control	Total cpm recovered in nucleotide
AMP	none	1.13	1,155	---	1,310	---	744
	act. D	0.95	1,370	+18	1,300	0	992
GMP	none	0.20	4,330	---	945	---	492
	act. D	0.25	4,970	+15	1,220	+28	928
NAD^+	none	1.26	1,720	---	2,170	---	1,236
	act. D	2.6	3,200	+86	10,900	+400	8,336
ADP	none	3.73	1,480	---	6,030	---	3,136
	act. D	3.57	1,645	+11	6,240	+3	4,476
GDP	none	1.37	2,340	---	3,200	---	1,828
	act. D	1.06	3,290	+40	3,500	+9	2,672
RNA	none	744	66.7	---	49,500	---	28,300
	act. D	670	59.0	-11	39,500	-20	30,080

[a]Data in this table is from the experiments described in Tables IV and V.
[b]act. D = actinomycin D

observed a parallel phenomenon in studies involving incorporation of [^{14}C] orotic acid into the pyrimidine nucleotides of normal and regenerating rat liver. Using pulse labeling of as little as 1 minute duration they found that the specific activity of UTP was higher than that of UDP which was in turn higher than that of UMP. They postulated a subdivision of the nucleotide pools within the cell with some pools being labeled more rapidly than others and, incomplete equilibration occurring between these compartments within the times studied. Plagemann (58) has found evidence for two distinct pools of both ATP and UTP in Novikoff rat hepatoma cells. His studies indicated that the pools which are most rapidly labeled are the ones which serve as precursors for RNA synthesis.

NAD^+ pyrophosphorylase (E.C. 2.7.7.18), the enzyme responsible for the incorporation of ATP into NAD^+ is located in the nucleus of rat liver cells '59). Tata and Widnell found (32) that the amount of ATP in the nuclei of thyroidectomized rats is less than 2 nmoles per gram of liver. Comparison of this value with a conservative value of 2000 nmoles for total cellular ATP per gram of liver reveals that the fraction within the nucleus is considerably less than 1% of the total. Hurlbert and Hardy (60) have found a similar situation in nuclei of normal rats. Less than 2% of the total cellular adenine nucleotides are located within the nucleus. If this nuclear ATP pool was labeled more rapidly than the bulk of the total ATP, one would expect to see

a greater increase in the specific activity of the NAD^+ when compared with the total cellular pool of adenine at any time before complete equilibration had occurred. It is of interest that Tata and Widnell (32) saw no effect on the specific activity of NAD^+ pyrophosphorylase after administration of triiodothyronine to thyroidectomized rats and that no difference was observed between the specific activity of the enzyme from normal rats and that from thyroidectomized rats.

The greater specific activity of NAD^+ and AMP at three hours (Table II) than those seen in the specific activity of the total adenine pool (Table I) at any point in time is also suggestive of compartmentation, with some pools being labeled more rapidly than others. The specific activity of the highly labeled components would be decreased as a result of dilution by less active components when the total adenosine phosphate pool is subjected to acid hydrolysis and the specific activity is calculated per unit of adenine (as in Table I).

An effect of triiodothyronine on the size of the free glycine pool of the liver could account for the increased specific activity of the total purine pools. O'brien (61), however, could detect no significant changes in the size of the free glycine pool of rat liver for up to 8 hours following injection of triiodothyronine. That the hormone could affect the turnover rate of the free glycine pool without affecting its size is not excluded by the results of O'brien's work, however, it is difficult to visualize how such an effect on the

turnover rate of the glycine pool could account for the distinct changes in the specific activity of the individual nucleotides observed in the present study. Similar findings were reported by Edmonds and Lepage (62) who observed that the specific activity of the guanine nucleotides were greater than those of the adenine nucleotides in Flexner-Jobling carcinoma after in vivo administration of [^{14}C] glycine to tumour-bearing rats. The greater specific activity of GMP than that of AMP in every experiment conducted raises the possibility that IMP, a common precursor of these compounds, may reside in two pools having different turnover rates.

When rats were pretreated with actinomycin D at a dose level which has been shown to completely inhibit the growth response and stimulation of BMR due to thyroid hormones (32), the stimulatory effect of triiodothyronine on the specific activity of the soluble nucleotides was still apparent (Table V). Table V shows that after actinomycin D pretreatment the hormone caused about the same increases in the specific activity of AMP and NAD^+ as those seen in the experiments of Tables III and IV. In addition, the percentage increases in the specific activity of ADP and GDP observed after actinomycin D treatment (Table V) were greater than those produced by the hormone alone. In fact, the increase in GDP was more than twice as great as that produced by hormone alone. The increase in specific activity of the RNA was the same as that due to hormone in Table IV. The AMP pool was expanded

after pretreatment with actinomycin D and subsequent hormone injection. The distribution of radioactivity between the AMP and GMP pools was enhanced in favor of the AMP by the hormone as is indicated by the ratio of total cpm in AMP/total cpm in GMP (footnote, Table V) which increased from 1.07 to 1.44. While the hormone reduced the NAD^+ pool somewhat the animals pretreated with actinomycin D still had 40% more NAD^+ than any other hormone treated group.

Actinomycin D treatment also resulted in a greater total activity in the nucleotides and a greater specific activity in each. Harbers and Muller (63) have noted a similar situation in studies of RNA synthesis in Erlich ascites cells. At concentrations of actinomycin D which inhibited RNA synthesis, the formation of labeled nucleotides from [8-^{14}C] guanine was not affected. On the contrary, actinomycin D stimulated the incorporation of the guanine by the cells and as the concentration of actinomycin D was increased to a level which completely blocked incorporation of the label into RNA, there was a concomitant increase in the total radioactivity of the acid soluble fraction, the majority of which could be accounted for in GMP and other purine di- and triphosphates. Other workers have seen no effect on the incorporation of [^{14}C] adenine into ATP in isolated rat diaphragm exposed to levels of actinomycin D which completely suppressed the incorporation of the label into RNA (64).

Table VI shows the effect of actinomycin D alone on the labeling pattern and specific activity of the nucleotides. The effect of the drug imitates that of the hormone inasmuch as the specific activity of the NAD^+ registered the greatest increase and the specific activities of ADP and AMP were lower than that of NAD^+. Actinomycin D produced greater increases in the specific activity of GDP than did the hormone.

It is interesting that under the influence of the hormone alone (Table IV) or actinomycin D alone (Table VI), the specific activity of GMP was greater than that of GDP. Administration of hormone following pretreatment with actinomycin D reversed this situation (Table V) resulting in a slightly higher specific activity of GDP than GMP. Actinomycin D alone resulted in a slight decrease in both the level and specific activity of the RNA.

Table VII, calculated from the data in Tables IV and V, shows the effect of actinomycin D on the hormone treated animals. The specific activity of the NAD^+ was increased to an extent comparable to that produced by the hormone alone. The specific activity of GDP was actually greater than that of NAD^+ in this instance, although the total activity in NAD^+ still exceeded that of any other nucleotide. The small decreases in level and specific activity of RNA were similar to the effect of actinomycin D on the control animals.

The increases in specific activity of the RNA produced by the hormone in these experiments is probably due to terminal adenosine

TABLE VII

Effect of actinomycin D on the specific activities and levels of hepatic nucleotides and RNA from triiodothyronine treated hypothyroid rats[a]

Nucleotide	treatment[b]	μmoles mg DOR	Specific Activities cpm μmole	% change from control	cpm mg DOR	% change from control	Total cpm recovered in nucleotide
AMP	Hormone	1.10	1,565	---	1,730	---	904
	Hormone + act. D	1.30	1,745	+12	2,268	+31	2,268
GMP	Hormone	0.37	4,350	---	1,610	---	844
	Hormone + act. D	0.30	5,240	+20	1,572	-2	1,572
NAD^+	Hormone	1.5	2,980	---	4,470	---	2,344
	Hormone + act. D	2.18	5,440	+80	11,860	+165	11,860
ADP	Hormone	3.46	1,950	---	6,780	---	3,556
	Hormone + act. D	3.44	2,398	+23	8,240	+7	8,240
GDP	Hormone	0.79	2,920	---	2,330	---	1,224
	Hormone + act. D	0.85	5,500	+88	4,660	+100	4,660
RNA	Hormone	730	82.1	---	59,000	---	30,960
	Hormone + act. D	695	72.5	-12	50,280	-15	50,280

[a]Data in this table is from the experiments described in Tables IV and V.
[b]act. D = actinomycin D

turnover of t-RNA. Merits (65) has shown that administration of as much as 800 μg of actinomycin D per 100 g body weight to rats prior to administration of labeled phosphate did not prevent the rapid and extensive labeling of the terminal adenylic acid of t-RNA.

Table VIII, calculated from the data in Tables IV and V, shows the combined effects of triiodothyronine and actinomycin D on the specific activities and pool sizes of the nucleotides. The similarity between the labeling patterns and changes in specific activity with those produced by the hormone alone are apparent. The largest change in specific activity occurred in the NAD^+. The increase in specific activity of AMP was significantly greater than that seen in the GMP. The specific activity of NAD^+ was greater than ADP which was greater than AMP. The chief differences in the combined effects and those caused by the hormone alone seemed to be the effect of actinomycin D to stimulate the specific activity of the GDP pool and to increase the size of the NAD^+ pool.

Mandel, et al, in studies of cellular levels of NAD^+ in various tissues in different experimental states (66,67) found that the level of NAD^+ is depressed below normal in tissues in which RNA snythesis is proceeding at above normal rates. In regenerating rat liver and ascitic hepatomas, the level of NAD^+ is lower than that of normal liver cells (66). These workers postulated that there may be a competion between NAD^+ synthesis and RNA synthesis for available

TABLE VIII

Effect of actinomycin D and triiodothyronine on the specific activities and levels of nucleotides and RNA of hypothyroid rat liver[a]

Nucleotide	treatment[b]	µmoles / mg DOR	Specific Activities: cpm / µmole	% change from control	Specific Activities: cpm / mg DOR	% change from control	Total cpm recovered in nucleotide
AMP	none	1.13	1,155	---	1,310	---	744
	L-T_3 + act. D	1.30	1,745	+51	2,268	+73	2,268
GMP	none	0.20	4,330	---	945	---	492
	L-T_3 + act. D	0.30	5,240	+21	1,572	+66	1,572
NAD^+	none	1.26	1,720	---	2,170	---	1,236
	L-T_3 + act. D	2.18	5,440	+215	11,860	+450	11,860
ADP	none	3.73	1,480	---	6,030	---	3,136
	L-T_3 + act. D	3.44	2,398	+62	8,240	+37	8,240
GDP	none	1.37	2,340	---	3,200	---	1,828
	L-T_3 + act. D	0.85	5,500	+135	4,660	+46	4,660
RNA	none	744	66.7	---	49,500	---	28,300
	L-T_3 + act. D	695	72.5	+9	50,280	+2	50,280

[a]Data in this table is from the experiments described in Tables IV and V.
[b]act. D = actinomycin D

nuclear ATP. Using rat kidney undergoing compensatory hypertrophy, these workers were able to demonstrate a direct competitive effect between the amount of RNA synthesized and the amount of induced synthesis of NAD^+ following injection of nicotinamide (67). If such a relationship existed in the liver of rats, then blockade of RNA synthesis by actinomycin D might allow an increase in the snythesis and level of NAD^+.

Rivlin and Langdon (68) have investigated the relationship between thyroid status and hepatic levels of FAD (Flavin adenine dinucleotide) in rats. They found that hypothyroid rats had significantly smaller amounts of FAD than euthyroid rats and that administration of thyroxine to normal rats did not increase the FAD level above normal. They also found that the flavokinase activity of hypothyroid rats was only half that of normal rats and the enzyme activity in hyperthyroid rats was even greater than that in normal rats. Administration of 10 μg of actinomycin D/100 g body weight for 4 days resulted in no loss of the thyroxine-induced increase in flavokinase activity in the hyperthyroid rat. Instead, a further increase in enzyme activity had occured. Actinomycin D treatment also increased the enzyme activity in normal animals.

Mah and Ackerman (37) found that addition of triiodothyronine or thyroxine to the soluble (100,000 x g supernatant) of rat liver stimulated the incorporation of labeled glycine into adenine nucleotides and decreased the rate of incorporation into guanine nucleotides.

Subsequent studies employing partially purified enzymes from the liver of hypothyroid rats indicated that the hormones stimulated the activity of adenylosuccinate synthetase, the first enzyme in the pathway leading from IMP to AMP, and inhibited IMP dehydrogenase, the first enzyme in the pathway leading from IMP to GMP (38,39). These findings led to the conclusion that thyroid hormones may regulate RNA synthesis by altering the availability of RNA precursors. Hormonal control in vivo over this branch point in the pathway of de novo synthesis of AMP and GMP (and by extension, ATP and GTP) would affect nearly all metabolic processes.

The results reported herein provide evidence that triiodothyronine does indeed stimulate the synthesis of AMP from glycine and that this occurs prior to any event reported previously using levels of hormone lower than those used by other workers. Tata and Widnell (32) using 25 μg of triiodothyronine observed a stimulation of the incorporation of orotic acid into nuclear RNA only after 3 hours. This was followed after 8 to 10 hours by an increase in the specific activity of RNA polymerase. Elegant studies by Tata and Widnell (32) failed to show that the hormone stimulated this enzyme directly.

The substantial increases in the specific activity of NAD^+ resulting from hormone administration supports the concept of hormonal stimulation of AMP synthesis. The greater specific activity of NAD^+ than the total adenine observed here supports the conclusions

of other workers that independent nucleotide pools exist, having different turnover rates, and among which equilibration is slow. That the percentage increase in the specific activity of the NAD^+ pool was much greater than the percentage increase in activity of total adenine suggests that one effect of the hormone is to determine the cellular site of utilization of the newly synthesized AMP molecules. The high specific activity of NAD^+ implicates the nucleus.

The effect of the hormone on GMP synthesis and its subsequent metabolism is not clear but more experiments directed at this problem are desirable. More detailed information on the rate of formation and distribution of other nucleotides (ATP, GTP) are needed.

Other approaches which would define more clearly the mechanism responsible for the stimulation of adenine nucleotide synthesis should include the following:

(a) experiments employing shorter time intervals of hormone action (30-45 minutes) followed by isolation of AMP on the assumption that the earliest detectable response to the hormone represents the mechanism of action.

(b) comparison of the incorporation of radiolabeled adenine with that of labeled hypoxanthine into AMP. If the site of action of the hormone is the conversion of IMP to AMP, then administration of hormone would not stimulate the conversion of adenine into AMP, but would stimulate the conversion of hypoxanthine into AMP.

(c) short term labeling experiments to determine the effect of the hormone on the intracellular distribution of de novo synthesized AMP and the hormonal effect on turnover rates of nuclear and total cellular ATP.

ADDENDUM

Pertinent to a discussion of the effects of thyroid hormones on purine nucleotide synthesis is the discovery of ppGpp (5' diphosphate guanosine 3' or 2' diphosphate) by Cashel and Gallant (69). This unusual nucleotide has been shown to appear in certain strains of bacteria under conditions in which RNA synthesis is inhibited (70). In addition, there is an inverse relationship between the cellular levels of ppGpp and GTP. Under conditions in which ppGpp accumulates, the GTP pool declines rapidly (71) and ATP levels also fall (70).

If such a relationship between RNA synthesis and purine ribonucleoside triphosphate levels existed in higher organisms one might reasonably suspect the existence of similar polyphosphorylated nucleotides in animal tissues during conditions of inhibited or depressed RNA synthesis. Thyroidectomized rats have markedly reduced levels of RNA and their rate of RNA synthesis is depressed below normal (32). Since we had observed that triiodothyronine administration to hypothyroid rats resulted in a stimulation of the rate of purine nucleotide synthesis within 1-2 hours (a response which precedes the onset of increased RNA synthesis reported by Tata (32)), it was tempting to speculate that ppGpp could be present in such rats and that control of its level and synthesis was a function of thyroid hormones. To investigate this possibility, some effort was expended in an attempt to isolate any unusual polyphosphorylated nucleotides from the liver of hypothyroid rats and hypothyroid rats given triiodothyronine.

The procedure employed was to label nucleotides *in vivo* with either [1-^{14}C] glycine or $^{32}PO_4^{-3}$, isolate the liver by freezing *in situ* and prepare an extract suitable for chromatography on columns or thin layer plates and subsequent autoradiography. No evidence of any unusual nucleotide, purine or pyrimidine, was found in any of the rat liver examined. This finding corroborates the results of investigations by Tompkins[1] who has been unable to detect ppGpp or any similar compound in rats subjected to a wide variety of experimental and nutritional stresses.

[1]Dr. G. M. Tompkins, personal communication.

SUMMARY

The synthesis in vivo of hepatic purine nucleotides was stimulated by administration of triiodothyronine to hypothyroid rats. When the total cellular pools of adenine and guanine were isolated following injection of [1-^{14}C]glycine and triiodothyronine the specific activity of both pools was increased as early as one hour following hormone treatment. The synthesis of the adenine nucleotides was stimulated more than that of the guanine nucleotides.

Administration of triiodothyronine resulted in a stimulation of the synthesis and turnover of AMP and NAD^+ within 1.5 hours. The magnitude of the stimulation observed was dependent upon the timing of injection of hormone and labeled precursor. These results support the hypothesis that thyroid hormones stimulate the synthesis of adenine nucleotides and confirm the findings of earlier, in vitro studies in this laboratory. The effect of the hormone on GMP synthesis was not obvious. At times the hormone appeared to inhibit GMP synthesis and in other cases it appeared to have no effect. The observed results varied with the timing of administration of hormone and precursor.

Treatment of hypothyroid rats with actinomycin D resulted in an increase in the specific activities of the purine nucleotides. In some respects actinomycin D produced changes similar to those caused by the hormone. Actinomycin D enhanced the effect of the hormone in stimulating the synthesis

of AMP and NAD^+.

To prevent rapid shifts in the levels of the nucleotides which occur during anoxia, an apparatus was designed which would maintain ether anesthesia while providing adequate oxygen to the animals during tissue sampling.

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SOME IN VIVO EFFECTS OF TRIIODOTHYRONINE ON PURINE NUCLEOTIDE SYNTHESIS

by

Richard F. Zimmerman

(ABSTRACT)

The in vivo effects of triiodothyronine on the synthesis of hepatic purine nucleotides was investigated in sulfaguanidine-fed hypothyroid rats.

To prevent the rapid destruction of nucleotides caused by anoxia and ischemia an apparatus was designed to maintain ether anesthesia while providing normal levels of oxygen. Liver samples were obtained from anesthetized rats by freezing in situ.

Administration of 15 μg of triiodothyronine to hypothyroid rats weighing 110-120 g resulted in stimulation of the incorporation of [1-^{14}C]glycine into the total soluble adenine and guanine nucleotide pools as early as 1 hour following injection. The increase in the specific activity of adenine was greater than that of guanine for up to 4 hours. No increases were detected in the levels of total adenine or guanine.

Hormone treatment stimulated the rate of incorporation of glycine into AMP and NAD^+ within 1.5 hours. The magnitude of the observed increases in specific activity of AMP and NAD^+ were dependent upon the relative timing of administration of hormone and glycine. The greatest increase in specific activity was observed in NAD^+.

The effect of the hormone on the synthesis of GMP was variable and dependent upon relative timing of administration of hormone and glycine. At the shortest times studied there was no apparent effect of the hormone on the specific activity of GMP. At longer times of hormone treatment a moderate decrease in specific activity was observed in the GMP.

Treatment of rats with actinomycin D prior to hormone injection did not prevent the stimulation of synthesis due to triiodothyronine. Actinomycin D alone stimulated the incorporation of the precursor into the soluble nucleotides and magnified the effects of the hormone in increasing the specific activities of AMP and NAD^+.