THE RESPONSE OF RAT THYMUS NUCLEI TO THYROID HORMONES

by

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ABBREVIATIONS

$L\text{-}T_4$	3,5,3',5'-Tetraiodo-L-thyronine
$L\text{-}T_3$	3,4,3'-Triiodo-L-thyronine
BMR	Basal metabolic rate
UTP	Uridine-5'-triphosphate
ATP	Adenosine-5'-triphosphate
CTP	Cytosine-5'-triphosphate
GTP	Guanosine-5'-triphosphate
DNA	deoxyribonucleic acid
RNA	ribonucleic acid
RPM	revolutions per minute
B. W	body weight
DNP	deoxyribonucleoprotein
Tris	Tris(hydroxymethyl)aminomethane
EDTA	Ethylene diamine tetracetate
DOR	2-Deoxyribose
AMP	Adenosine-5'-monophosphate
GMP	Guanosine-5'-monophosphate
IMP	Inosine-5'-monophosphate
GSH	Glutathione
CPM	Counts per minute

INTRODUCTION

The mechanism of action of the naturally occurring and physiologically active thyroid hormones, 3,5,3'-Triiodo-L-thyronine ($L\text{-}T_3$) and 3,5,3',5'-tetraiodo-L-thyronine ($L\text{-}T_4$), has evaded the ingenuity of scientists. However, many of the physiological effects of the hormone have been expounded as well as many of its effects at the molecular level. Still, the specific function of thyroid hormones remains to be explicated.

The biological responses to $L\text{-}T_3$ and $L\text{-}T_4$ are the same. However, $L\text{-}T_4$ is present in blood at higher concentrations than $L\text{-}T_3$ (1), but $L\text{-}T_3$ has a greater initial biological potency than does $L\text{-}T_4$ (2,3). A rat produces about 3 μg of $L\text{-}T_4$ per day per 100 grams of body weight with a half life of the hormone estimated to be 18 hours (4). From this, Lardy and Kent (5) have estimated that the concentration of $L\text{-}T_4$ in the blood of rats to be 5×10^{-8}M. This is considered to be the physiological level of thyroid hormones in the rat.

Many studies of the effects of thyroid hormones have been conducted with non-physiological levels (greater than 50 μg/100 g B.W.) which failed to explain the mechanism of action of the hormone but characterized the toxic effect of the hormone. Also, the mechanism of action of thyroid hormones has been ascribed to its effects on

physiological processes, such as growth and development, BMR, and oxidative phosphorylation. These are secondary effects because these processes have a latent period of several hours after administration of the hormone.

The latent period is the time between the administration of the hormone and the detection of a response. The length of the latent period is dependent upon the amount of the hormone administered and which of the hormones (L-T_3 or L-T_4) is administered (2, 6). A shorter latent period is observed with L-T_3, but the effect is also of shorter duration than that produced by L-T_4 (2). These differences have not been explained but are believed to be related to the relative rates of distribution rather than to the activity of the hormones (7).

The following discussion is devoted to the physiological effects of thyroid hormones. Although these are not the mechanism of action, each must be explained before any hypothesis could be accepted as the mechanism of action of thyroid hormones.

Thyroid hormones have a biphasic effect on growth. If 5-100 μg/100 g body weight of L-T_4 is administered to a hypothyroid rat, there is a continuous increase in the growth rate. However, as the level of L-T_4 is increased from 100 to 2,500 μg/100 g body weight, there is a continuous decrease in the growth rate (8). These results emphasize the necessity of utilizing physiological levels of thyroid hormones if the results are to be interpreted as a physiological response of the hormone.

The necessity of thyroid hormones for normal growth and development is well exemplified in dwarfism of the congenital hypothyroid child or cretin. In cretinism there is a marked retardation of ossification and the body retains infantile characteristics of bone structure. Cretinism is accompanied by severe mental retardation (9). The skeletal abnormalities can be partially corrected if thyroid hormones are administered early but the mental retardation cannot be corrected by thyroid hormone therapy. This demonstrates the necessity of thyroid hormones for brain development in early embryonic growth.

The necessity of thyroid hormones for growth and development is demonstrated by the absolute requirement for thyroid hormones in tadpole metamorphosis (10). Thyroidectomized tadpoles will not metamorphose but may grow to a gigantic size. Such a tadpole will metamorphose when treated with thyroid hormones (11). This is the best known effect of thyroid hormones on cold blooded animals. However, thyroid hormones have been reported to exert an effect on such animals as tunicates, acraniates and fishes (12).

Boothby and his associates (13) set up a technique by which the activity of the thyroid gland may be measured by the BMR. Thus oxygen consumption is used as an indicator of thyroid activity. The BMR is regulated by thyroid hormones. Tata and Shellaberger (7) have

demonstrated that as the amount of L-T_4 injected into a thyroidectomized rat increases from 5 to 2,500 µg/100 g body weight, the BMR will increase from 8 percent to 89 percent over the control. The BMR does not show the biphasic effect as is seen on growth (14), but the rise in BMR exhibits a latent period after hormone administration similar to that observed on growth (2).

Because of the effect of thyroid hormones on BMR, extensive research has been conducted on the effects of thyroid hormones on mitochondria (15, 16, 17, 18, 19). Thyroid hormones lower the P:O ratio of rat liver mitochondria after either *in vivo* or *in vitro* administration, but this response was observed only when a pharmacological level of the hormone had been administered (15, 16). Gustafsson *et al.* (17) did not observe an effect on the P:O ratio or respiratory control index using physiological levels of L-T_4 (18 µg L-T_4/100 g B.W.).

The swelling of liver mitochondria has been observed after treatment of mitochondria with thyroid hormones *in vitro* (18) and after the administration of large doses of hormone to rats (19). However, swelling was not observed in mitochondria from cardiac muscle, skeletal muscle, brain or testes (20). The swelling was observed only after administration of large doses of thyroid hormones. Also, the biologically-inactive D-isomers of T_3 and T_4 produced swelling (21) as well as biologically-inactive analogs of thyroid hormones (22).

Roodyn et al. (23) reported an increase in mitochondrial protein synthesis in vitro after a single injection of L-T_3 to thyroidectomized rats. The stimulation of protein synthesis was followed by an increase in levels of cytochromes a, b, and c. The response observed in vivo could not be demonstrated with L-T_3 was added to a suspension of mitochondria.

Since the effect on mitochondria was observed only after treatment with large non-physiological doses of thyroid hormone and the biological inactive isomers and analogs of thyroid hormones elicited the same responses as the biologically-active hormones, it may be concluded that the effect of thyroid hormones on mitochondria is either a secondary effect of the hormone or the result of its pharmacological effects and are not the mechanism of hormonal action.

Dutoit (24) reported that a single injection of thyroxine into thyroidectomized rats stimulated the incorporation of amino acids into protein of rat liver slices. In subsequent studies, Sokoloff and associates (25, 26, 27, 28, 29) observed a stimulation of the incorporation of amino acids into the protein of rat liver homogenates by thyroid hormone treatment in vitro. He demonstrated that the increased ability to incorporate amino acids into protein was dependent on the presence of the mitochondria, and could not be substituted for by a creatine phosphate-ATP generating system (26).

Pretreatment in vivo or in vitro with L-T_4 stimulated amino acid incorporation into microsomal protein. However, L-T_3 was

effective only *in vivo* (25). Thyroidectomy resulted in a reduction in amino acid incorporation into protein (27). Sokoloff *et al.* (26, 28) proposed that the stimulation of amino acid incorporation into protein by L-T_4 in the cell-free rat liver system was localized at the step involving the transfer of soluble ribonucleic acid bound amino acid to microsomal protein. The action of thyroid hormones on this GTP dependent step in protein synthesis was not altered by varying the levels of GTP, ATP, or GSH (26).

Sokoloff *et al.*(29) observed that a single injection of L-T_3 stimulated liver microsomal protein synthesis within 2 hours. The initial stimulation was followed several hours later by a second increase which is not mitochondrial dependent. From his work, Sokoloff has suggested that the stimulation of protein synthesis *in vivo* consists of two components: (1) an initial, cytoplasmic, mitochondria-dependent stimulation of the existing protein synthesizing apparatus followed by: (2) a secondary, nuclear-mediated, cellular response or adaptation which leads to an increase in the amount of protein-synthesizing components.

Sokoloff and Kaufmans(27) explanation of the effect of thyroid hormone is questionable because the biologically-inactive isomer, D-thyroxine, is as active as L-T_4 in stimulating protein synthesis *in vitro*. Also, L-T_3 is effective when administered *in vivo* but exhibits very low activity when added *in vitro*. Non-physiological levels (60 µg/100 g B.W.) of L-T_4 and L-T_3 were necessary in order to observe a stimulation of protein synthesis (25, 26, 27, 28, 29).

A stimulation of the incorporation of labelled amino acid into rat liver microsomal and mitochondrial protein has been observed 36 hours after the injection of a single dose of L-T_3 (20-26 mg/100 g B. W.) to thyroidectomized rats (23). An increase in the turnover of the nuclear basic protein has also been observed (30) which is paralleled by a stimulation of RNA polymerase activity but not of nuclear RNA (32). From this data, Tata has suggested that the mechanism of thyroid hormone action is the regulation of the synthesis of specific proteins and enzymes.

A study of RNA synthesis by Tata and Widnell (32) indicated that thyroid hormones first stimulated the specific activity of rapidly labelled RNA beginning at 3-4 hours after hormone injection and increasing up to 16 hours. A stimulation of Mg^{++} activated RNA polymerase activity was observed at 10 hours and continued to increase until 42 hours after treatment (31, 32). However, the direct addition of L-T_3 to nuclei isolated from the liver of thyroidectomized rats had no effect on the specific activity of RNA polymerase (32). This result and the observation that RNA polymerase is not stimulated until 10 hours after administration of the hormone suggests that the stimulation of RNA polymerase <u>in vivo</u> does not stem from a direct hormone-enzyme interaction.

Tata and Widnell (32) observed that administration of L-T_3 to rats induced a substantial rise in the amount of cytoplasmic ribosomal RNA and in the microsomal RNA:protein ratio between 24 and

30 hours after treatment (32). Using an in vitro system of kidney epithelial cells, Siegel and Tobias (33, 34) observed an increase in the turnover of nuclear RNA. It may be concluded that thyroid hormone administration accelerates nuclear RNA synthesis and its transport into the cytoplasm.

In an attempt to determine the specific RNA affected by hormone treatment, Wyatt and Tata (35) studied the hybridization capacity of RNA after L-T_3 administration. Hypophysectomized rats treated with L-T_3 and growth hormone stimulated the total incorporation of (^{3}H) orotic acid into liver nuclear RNA as well as the synthesis of hybridizing RNA. The percentage of hybridization of the labelled RNA was invariably decreased after hormone treatment, indicating that the treatment stimulated synthesis of r-RNA more than that of other kinds of RNA. The hormones stimulated the synthesis of readily hybridizable RNA suggesting production of new or more mRNA. However, a greater stimulation of synthesis of non-hybridizing RNA indicates a preferential effect of the hormone on the production of r-RNA.

Wyatt and Tata (35) arrived at these conclusions on the assumption that the high content of ribosomal species in the bulk RNA and the small fraction of their cistrons in the DNA, only negligible proportions of r-RNA and ribosomal precursor RNA species

would hybridize at the low DNA/RNA ratio employed. However, m-RNA species were not present in such excess relative to their cistrons and could hybridize. From these studies Wyatt and Tata have concluded that thyroid hormones effect different kinds of RNA.

Amphibian metamorphosis is a tool for the study of thyroid hormones on development at molecular level. In 1960, Finamore and Frieden (36) observed that thyroid hormones stimulated the incorporation of $H_3{}^{32}PO_4^{=}$ into RNA, DNA, and protein of tadpole liver. Thyroid hormones elevated the production of hybridizing RNA but non-hybridizing RNA was stimulated to an even greater extent, indicating, as in the rat, a production of m-RNA but a greater production of r-RNA (35). Thyroxine stimulated RNA synthesis in tadpole liver 36 hours after treatment. The stimulation was greatest in the microsomal RNA; all RNA synthesis was inhibited by actinomycin (37).

Blatt et al. (38) observed a stimulation of RNA synthesis in a suspension of tadpole liver cells by L-T_3 and L-T_4. Both hormones stimulated RNA synthesis maximally at 10^{-7} M which is in contrast to other reports that L-T_3 is 10-300 times more active than L-T_4. However, the suspension eliminates barriers such as absorption, metabolic alterations and distribution of the hormones.

Cohen and associates have studied the biochemical changes associated with induced tadpole metamorphosis. They observed that thyroid hormones stimulate the de novo synthesis of the enzymes associated with the urea cycle (37, 38, 39, 40, 41). Since the metamorphosis of a tadpole to a frog is one of change from an ammonotelic to a ureotelic animal, one would expect the appearance of urea cycle enzymes. Cohen (42) suggests that a study of the effect of L-T_4 on RNA synthesis is important because RNA synthesis precedes the induction of the enzymes involved in urea biosynthesis.

Cohen et al. demonstrated that the administration of L-T_4 to tadpoles modified chromatin isolated from liver nuclei, into a more efficient template for RNA synthesis. Kim and Cohen states that the modification of the chromatin which increases the template efficiency of the DNA is related to the protein moiety (41). From these observations it may be concluded that a possible mechanism of action of thyroid hormones is at the transcription level. Cohen (42) has authored an excellent review of L-T_4 effects on liver cytology, transcription, translation and mitochondrial enzyme levels in amphibian metamorphosis.

Autoradiography of cultured human kidney epithelial cells (after treatment with L-T_4 labelled with iodine-125) showed that the hormone was concentrated mainly in the nucleus (60-80%). Siegel and Tobias (33) have proposed from this that thyroid hormones act in the nucleus in some manner to regulate genetic expression.

Thyroid hormones have also been implicated in the synthesis of purine nucleotides. Necheles (43) observed that L-T_4 stimulated the incorporation of glycine-1-^{14}C into the adenine of RNA, but it was without effect on adenosine-8-^{14}C incorporation into RNA. He proposed that thyroid hormones control purine nucleotide synthesis.

Mah and Ackerman (44) also observed a stimulation of purine nucleotide synthesis by thyroid hormone in the soluble fraction of rat liver. Thyroid hormones were observed to stimulate the incorporation of glycine-1-^{14}C into AMP while its incorporation into GMP was inhibited (45). Stimulation of glycine-1-^{14}C incorporation into AMP was due to increased adenylosuccinate synthetase activity (46), while the decrease activity of IMP dehydrogenase inhibited incorporation in GMP (47). These results suggested that thyroid hormones regulate nucleotide synthesis at the step where IMP is converted to xanthosine phosphate or adenylosuccinate.

When studied in vivo, thyroid hormones stimulated the incorporation of glycine-1-^{14}C into soluble purine nucleotide. However, the specific activity of GMP from nuclear RNA was greater than that of the AMP (48). The incorporation of glycine-1-^{14}C into GMP and AMP of cytoplasmic RNA in vivo supports the in vitro studies but the effect on nuclear RNA synthesis is in contrast to the in vitro data (49). While this data demonstrates that thyroid hormones have

a definite effect on purine biosynthesis, the specific site of action has not been pinpointed with an in vivo system.

Other hormones have been shown to have effects similar to thyroid hormones on cell metabolism. Dahmus and Bonner (50) demonstrated that the administration of hydrocortisone to adrenolectomized rats increased the template efficiency of liver chromatin for RNA synthesis.

Maurer and Chalkey (51) found estradiol to be concentrated in the nucleus of endometrial cells and bound to the chromatin. Aldosterone (52), cortisol (53), testosterone (54), estrogen (51, 53, 56), and thyrotropin (57) have been shown to bind to chromatin. The binding of the various hormones to chromatin is support for the hypothesis put forth by Karlson (58) in which he suggests that the action of hormones is on the hereditary factor, the gene. This same view has been reiterated by Siegel and Tobias with respect to the site of action of thyroid hormones (33).

Stedman and Stedman (59) developed the hypothesis that the basic proteins (the histones) of the cell nuclei, inhibit gene expression in the cell. Extensive research has been conducted to determine the interrelationship of DNA, RNA, and histone as controls of gene expression.

Johns (60) described a method for the isolation of the five histone fractions: f_1, f_{2a}, f_{2b}, f_3, from calf thymus. Optical rotary dispersion (61) and infrared (62) studies indicates that about two-thirds of the histone is in the α helix form. The surface of the DNA molecule has α helical grooves, into which one of the α helix of histones would fit. This is in agreement with x-ray diffraction patterns which demonstrate that the double helix configuration is mostly preserved in the nucleoprotein.

Studies of the interaction of histones and DNA have shown very slight signs of specificity. Each histone fraction is capable of precipitating the whole of DNA. The only difference being that DNA is precipitated by f_1 at about one-half the concentration required of other fractions (63).

It has been demonstrated that histones decrease the ability of DNA to act as a template for DNA dependent RNA synthesis (64, 65, 66, 67, 68, 69). Allfrey et al. (67) proposed that the repressor activity of histones is controlled by their degree of acetylation. They have supported this view by demonstrating that the inhibition of RNA synthesis by arginine-rich histone in vitro was reduced by small degrees of acetylation. Pogo et al. (70) observed a stimulation of acetylation of histones by phytohaemagglutinin which also stimulates RNA synthesis. Phosphorylation is also a possible control mechanism for the control of histone in that the degree of phosphorylation of serine in histones may control to what extent the histone is bound to the DNA (71).

Although the mechanism of action of thyroid hormones has not been elucidated, Tata (72) has summarized the sequence of events that probably occur after the administration of a single dose of L-T_3 as the stimulation of:1) the synthesis of rapidly-labelled nuclear RNA, 2) Mg^{++} activated DNA-dependent RNA polymerase in nuclei, 3) ribosomal RNA of nuclei, 4) mitochondrial increase in amino acid incorporation into protein, 5) cytochrome oxidase/mg mitochondrial proteins, 6) microsomal NADPH-cytochrome c reductase, 7) BMR, 8) liver weight. Kim and Cohen (41) observed an increase of the template efficiency of tadpole chromatin following the administration of thyroid hormone. Seigel and Tobias (33) have suggested that thyroid hormones exert their effects by action on the nucleus and specifically on gene expression.

Since the first response to thyroid hormones appears to be in the nucleus and many of the other effects of the hormone are directly on the nucleus, this study was undertaken to find the biochemical effects induced by thyroid hormones on rat thymus nuclei. The thymus was selected as the organ to study because it has been observed that the administration of thyroid hormone to thyroid hormone deficient rats had a dramatic effect on the size of the thymus (73). This study attempts to elucidate the effect of L-T_3 on the nuclear protein, RNA, and DNA of rat thymus nuclei.

MATERIALS AND METHODS

Male weanling Sprague-Dawley derived rats were fed a complete diet containing 1 percent sulfaguanidine (73) for 5 weeks prior to sacrifice. 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.

Glycine-1-^{14}C (116 mc/mM), phenylalanine-UL-^{14}C (369 mc/mM), UTP-5-^{3}H (29 c/mM), and alanine-1-^{14}C (136 mc/mM) were purchased from New England Nuclear Corporation, Boston, Massachusetts. UTP, ATP, CTP, GTP, and 2-deoxyribose were purchased from Sigma Chemical Company, St. Louis, Missouri and were used without further purification. Triiodo-L-thyronine (L-T_3) was purchased from Calbiochem, Los Angeles, California and was recrystallized from boiling 2 N HCl (mp 201-203°) (74). Histone, Type II-A from calf thymus, was used as a protein standard and was purchased from Sigma Chemical Company, St. Louis, Missouri.

RNA was purchased from Pabst Laboratories, Milwaukee, Wisconsin, and used as a standard without further purification. RNA polymerase was purchased from Miles Laboratories, Inc., Kankakee, Illinois. Acrylamide, N, N' methylenebisacrylamide, and N, N, N', N', tetramethylethylenediamine were purchased from Eastman Organic Chemicals, Rochester, New York. Ammonium persulfate was purchased from Baker Chemical Company, Phillipsburg, New Jersey. Escherichia coli, Type B, DNA was a gift from Dr. J. L. Johnson of the Anaerobic Laboratory, V.P.I., Blacksburg, Virginia.

Male Sprague-Dawley rats were injected intraperitoneally using 0.1 M sodium phosphate buffer at pH 7.0 as the vehicle for treatment. L-T_3 was administered at a level of 15 μg/rat. The radioactive amino acids were injected at a level of 15 μc/100 g B.W., 15 minutes prior to sacrificing. The rats were sacrificed by a sharp blow on the head followed immediately by decapitation. The thymus was removed and placed in sucrose: TKM buffer, (0.25 M sucrose, 0.02 M Tris-HCl, pH 7.4, 0.25 M KCl, and 0.05 M $MgCl_2$) at 4°. All subsequent steps were performed at 4°.

Isolation of Nuclei

The thymus was homogenized in sucrose:TKM buffer using a Sorvall Omnimixer at 4800 rpm for 20 seconds. The final homogenate was filtered through two layers of cheese cloth and nuclei were isolated from the filtrate by the method of Blobel and Potter (75).

The isolated nuclei were washed in 0.9 percent NaCl in the Sorvall Omnimixer at 16,000 rpm for one minute. The washing was repeated three times at 8,000 rpm for 30 seconds, and the pellet was recovered each time by centrifugation at 5,000 x g. The nuclei were suspended in 10 ml of 0.9 percent NaCl by homogenization of 8,000 rpm for 30 seconds and aliquots were taken for the determination of DNA, total protein, whole histone and histone fractionation.

Determination of Radioactivity

All radioactive samples were evaporated to dryness at reduced pressure over phosphorus pentaoxide. The residue was dissolved in

1 ml of hyamine hydroxide and 15 ml of POPOP (.4 g/100 ml, 1,4 bis 2-(4-methyl-5-phenylorazolyl)-Benzene), PPO (5 mg/100 ml, 2,5-diphenyloxazole) in toluene was used as the scintillation cocktail. Radioactivity was determined with a Packard Tri-carb scintillation spectrometer Model 3310.

Determination of Protein and DNA

Protein was determined by the method of Lowry et al. (76). DNA was determined by the method of Schmid et al. (77) with modifications by Short et al. (78).

Isolation and Determination of Whole Histones

The nuclei were recovered from the 0.9 percent NaCl by centrifugation at 10,000 x g for 15 minutes. Whole histones were extracted from the nuclei by washing nuclei 3 times with 10 ml of 0.25 N HCl and homogenizing each time at 16,000 rpm for 30 second. The suspension was centrifuged at 10,000 x g for 15 minutes each time and the supernatants were pooled. The pooled supernatants were filtered through a medium sintered glass filter after which the filtrate was added to 5 volumes of acetone and allowed to stand for 5 hours. The precipitate was collected by centrifugation, washed 3 times by centrifugation with acetone and dried under reduced pressure. The protein was then dissolved in water; protein was determined by the method of Lowry et al. (76) and radioactivity was determined as previously described.

Isolation and Determination of Histone Fraction

Histone fractions were isolated from nuclei by the method of Johns (60). The procedure (Fig. 4) was scaled down to the small quantity of starting material used in these studies. The fractions were dried under vacuum and the residues were dissolved in water. Protein was determined by the method of Lowry et al. (76) and radioactivity was determined as previously described.

Amino Acid Composition of Histone Fractions

The histone fractions were dissolved in 6 N HCl and hydrolyzed under nitrogen in sealed ampules for 24 hours at 100°. The hydrolysates were evaporated to dryness with a rotary evaporator. The residues were dissolved in a sodium citrate buffer at pH 2.2 and the amino acid composition of each fraction was determined with a Beckman amino acid analyzer, Model 120-B.

Determination of RNA:DNA Ratios

Isolated nuclei were suspended in 0.2 N perchloric acid and allowed to stand for 15 minutes. The nuclei were recovered by centrifugation in a RC-2 Sorvall centrifuge at 3,000 x g for 10 minutes. The pellet was suspended in 0.40 ml of 0.32 N KOH and incubated at 37° for one hour. The solution was cooled and brought to 0.2 N with 10 N perchloric acid. The solution was cooled for 15 minutes and the pellet collected by centrifugation. The pellet was washed twice with 2 ml of 0.2 N trichloroacetic acid and the supernatant was collected each time and pooled. The pooled supernatants

were brought to 10 ml with water and read at 260 mμ. An absorbance of 1.0 at 260 mμ was taken to equal 32.0 μg of RNA per ml (79).

The pellet was washed 3 times with 4 ml of 0.32 N KOH and the supernatants were pooled. The supernatant fractions were neutralized, made to volume, and the DNA content determined.

Disc Gel Electrophoresis of Histones

Histones were separated on polyacrylamide gels by the method of Johns (80) with modifications by Benson and Ruark (81). The gel solution contained equal volumes of 1)acrylamide (40% w/v), N. N-methylene bisacrylamide (0.6% w/v); 2) N, N, N', N' tetramethyl-ethylene diamine in 4.6 N acetic acid; 3) ammonium persulfate (1% w/v); 4) water. The solutions were stored in actinic red flasks at 4° until ready for use. Ammonium persulfate was stable for one week at which time a fresh solution was prepared. The solutions were allowed to equilibrate to room temperature before mixing to prevent the formation of air bubbles in the gel during polymerization. The gel solution was pipetted into standard disc electrophoresis sample tubes (5 x 63 mm) to a height of about 50 mm. The gel solution was overlaid with water and the tubes were placed in a water bath at 37° for polymerization which occurred in 30 minutes. The polymerized gels were placed in a Canalco Model 66 disc electrophoresis apparatus and equilibrated for one hour with 1 N acetic acid adjusted to pH 2.0 with HCl using a Canalco Model 300-B power supply at 5 ma per tube.

The histone fractions were dissolved in 1 M sucrose, 2 μM acetic acid to a final concentration of 1.6-2 mg/ml. The sample was applied to the upper surface of the equilibrated gel using an Eppendorf micropipette (5 μl). The sample was overlaid with fresh buffer, placed back in the Canalco apparatus previously described, and subjected to a constant current of 4 ma per tube for 30 minutes. The gels were removed from the tubes by rimming the tube with a syringe and needle filled with water, placed in 1 percent (w/v) Naphthol blue black in 14 percent (v/v) acetic acid for at least 12 hours. After staining, the gels were destained electrophoretically using the previously mentioned power supply and bath assembly with 10 ma per tube and 7 percent (v/v) acetic acid as the buffer. After destaining the gels were stored in 7 percent acetic acid.

Densitometer tracings were obtained using a Gilford spectrophotometer Model 240 adapted for linear density determinations.

Determination of DNA / Dry Weight

Isolated nuclei were washed 5 times with 0.9 percent NaCl. The nuclei were suspended in 10 ml of 0.9 percent NaCl, and an aliquot was taken for DNA analysis and another aliquot was dried in a tared container in a circulating air oven at 90° until a constant weight was obtained.

Isolation and determination of deoxyribonucleoprotein

Deoxyribonucleoprotein (DNP) was isolated by the method of Commerford et al. (82). The DNP was brought to volume in water and aliquots were taken for protein and DNA analysis as previously described.

Isolation and characterization of rat thymus chromatin and DNA

Chromatin was isolated from rat thymus by the method of Marushige and Bonner (83). The chromatin was deproteinized by the method of Huang and Bonner (84) using 4 M CsCl and centrifuging at 35,000 rpm for 40 hours in a Spinco SW-40 head. The chromatin or DNA was dialyzed against 0.01 M Tris over night to remove sucrose and CsCl, respectively. The T_m of the chromatin or DNA was determined using a Gilford spectrophotometer Model 2400. The T_m was standardized against E. coli, type II DNA in 1 x SSC which had a T_m of 90.5°. The spectra for the chromatin and DNA were determined using a Beckman DB spectrophotometer. The DNA, RNA and protein were determined by the methods of Schmid et al. (77), Webb (85), and Lowry (76), respectively.

The template efficiency of the chromatin and DNA was determined by a modification of the method of Marushige and Bonner (83). The complete incubation mixture contained in 0.25 ml: 10 μmoles Tris buffer (pH 8.0), 1 μmole $MgCl_2$, 0.25 μmoles $MnCl_2$, 3 μmoles β-mercaptoethanol, 0.1 μmoles each of ATP, GTP, CTP, UTP, 2.5 μc UTP-5-^{3}H, DNA or chromatin, and 5 units of RNA polymerase for

chromatin, and 15 units of RNA polymerase for DNA. Incubation was carried out at 37° for 20 minutes. A blank containing no RNA polymerase was run in each group to correct the endogenous RNA synthesis. A 0.1 ml aliquot was removed at 10 and 20 minutes, and immediately air dried on paper discs. The discs were prepared for counting by the method of Mans and Novelli (86). The discs were immersed in 5 percent trichloroacetic acid: 0.002 M pyrophosphate, and washed for 20 minutes with constant stirring. This was repeated once in the cold and twice at room temperature. The discs were then washed in 1:1 ethanol:ether for 15 minutes and then in ether for 15 minutes, then air dried. Each disc was placed in a glass-counting vial and 5 ml of scintillation mixture was added.

RESULTS

In preliminary cytological studies of rat thymus nuclei a comparison of nuclei obtained from growth-arrested sulfaguanidine-fed rats with nuclei obtained from rats which had received 15 μg of L-T_3 showed that thyroid hormones increase the number of nucleoli per nucleus. This could not be substantiated statistically, neither was it possible to determine if these were new nucleoli or were newly-revealed nucleoli due to a change in the constituents of the nucleus. Siegel and Tobias (33) have also presented evidence that thyroid hormones effected an increase in the number of nucleoli per nucleus of kidney epithelium cells. However, these studies did indicate that further investigation of the nucleus was warranted. With hopes of returning later to the microscopic studies, the results reported here are the biochemical observations on the *in vivo* effect of thyroid hormones on rat thymus nuclei.

Reports from other laboratories have shown that the ratio of DNA:dry weight of somatic tissue is constant (87, 88). Tata and Widnell reported (32) that the ratio of DNA:dry weight of thyroidectomized rat liver was not affected by thyroid hormones. Since the studies reported here were conducted on isolated thymus nuclei, it was necessary to determine the effect of thyroid hormones on the DNA per gram of dry weight of thymus nuclei. The results summarized in Table 1 show that the ratio of deoxyribose:gram dry weight of thymus nuclei remains constant up to 16 hours after L-T_3 administration.

Previous reports have shown that there is no change in the amount of nuclear RNA in the liver of thyroidectomized rats after treatment with L-T_4 (32). In agreement with these results, the data in Table 2 shows that the level of RNA in rat thymus nuclei was constant up to 16 hours after the administration of a single dose of L-T_3.

The effects of thyroid hormone on the synthetic rate of the total protein of isolated nuclei is summarized in Figure 1. The incorporation of labelled amino acid into the total protein of the nucleus increased 50 percent over the control in 4 hours after L-T_3 administration and remained constant up to 16 hours. These results suggested that thyroid hormones affect the protein moiety of the nucleus which, coupled with the hypothesis of Stedman and Stedman (39) that histone regulates gene activity, suggested the possibility that thyroid hormones exert some effect on the genetic machinery.

A study of the effect of thyroid hormone (L-T_3) on deoxyribonucleoprotein (DNP) indicated that the amount of protein associated with the DNA was increased 4 hours after the administration of L-T_3 (Figure 2). In two separate experiments, the protein:DNA ratio was increased 10 and 17 percent in 4 hours and then remained constant through 8 and 16 hours after L-T_3 administration (Figure 2). The effect of thyroid hormone on the protein:DNA ratio followed the same pattern as was observed on the total nuclear protein (Figure 1).

Since histones are an important component of deoxyribonucleoprotein, the effect of L-T_3 on the histone fraction of nuclear protein was studied. Three ^{14}C-labelled amino acids--alanine, phenylalanine, and glycine were used separately in studies on their rates of incorporation into nuclear histones. The maximum incorporation of label was observed to occur 4 hours after treatment with L-T_3 (Figure 3). The incorporation decreased 8 and 16 hours after the administration of L-T_3, but only with glycine did the incorporation drop below the control value (Figure 3). These results demonstrated a stimulatory effect of L-T_3 on the incorporation of amino acids into histones.

The preceding results directed attention to the possibility that thyroid hormones may affect the synthesis of one or more of the histone fractions. Before a study of the effects of thyroid hormone on the isolated fractions could be conducted, it was necessary to characterize each of the histone fractions of rat thymus. The four fractions isolated by the method of Johns (60) / Figure 4, were analyzed for their amino acid content (Table 3) and behavior on polyacrylamide gels (Figure 5). The amino acid content and behavior on polyacrylamide gels indicated that the fractions isolated from rat thymus had characteristics similar to those isolated from calf thymus, and the identification system used by Johns (60) / (f_1, f_{2a}, f_{2b}, f_3) will be used here to identify the histone fractions.

The effect of L-T_3 on the incorporation of glycine-1-^{14}C into the four histone fractions at 15 minutes, 30 minutes, one hour, and four hours after hormone administration was studied. In all four fractions the incorporation was lower than the control 15 minutes after the administration of L-T_3. Thirty minutes after the administration of L-T_3 the incorporation increased but did not return to the level of the control. The incorporation into all fractions continued to rise one hour after L-T_3 administration. The incorporation into fractions f_1, f_{2a}, and f_3 were above the control four hours after L-T_3 administration but f_{2b} was 9 percent below the control (Figure 6).

Since glycine is utilized in numerous pathways, the experiments were repeated with alanine-1-^{14}C. Figures 7 to 10 summarize the data from two separate experiments in which the incorporation of alanine-1-^{14}C into the four histone fractions was followed. Fraction f_1 was stimulated 57 percent over the control four hours after the administration of L-T_3. Eight and 16 hours after the injection of a single dose of L-T_3 the incorporation of alanine-1-^{14}C into fraction f_1 was increased 39 and 31 percent, respectively, over the control (Figure 7). The incorporation into fraction f_{2a} was increased 37 and 43 percent over the control 4 and 8 hours after the administration of L-T_3 (Figure 8). Four, 8, and 16 hours after the administration of L-T_3 the incorporation into fraction f_{2b} was increased 33, 66, and 22 percent over the control (Figure 9). The incorporation into fraction f_3 was increased 12, 23,

and 14 percent over the control 4, 8, and 16 hours after the administration of L-T_3 (Figure 10). The incorporation of alanine-1-^{14}C into all four histone fractions was stimulated by L-T_3.

Since each fraction is composed of more than one protein, the histone fractions from the control and from the group that had received L-T_3 four hours previously were compared on polyacrylamide gel. The four fractions (f_1, Figure 11; f_{2a}, Figure 12; f_{2b}, Figure 13, f_3, Figure 14) were observed visually and with a densitometer, but no difference was detected between the control and L-T_3 treated groups. This indicates that L-T_3 stimulated the turnover of histone fractions and did not stimulate the synthesis of new polypeptides.

Chromatin was studied to determine what effect L-T_3 has on its protein and RNA components and if L-T_3 altered the template efficiency of DNA. The effects of L-T_3 on the RNA bound to chromatin was studied 2, 4, and 8 hours after the administration of the hormone (Figure 15). The amount of bound RNA was not effected 2 hours after the administration of L-T_3. However, in two experiments, the average level of bound RNA was increased 50 percent over the control 4 hours after the administration of L-T_3 (Figure 15). Eight hours after the administration of L-T_3 the bound RNA was 10 percent above the control.

The protein moiety of chromatin was studied 2, 4, and 8 hours after the administration of a single dose of L-T_3 (Figure 16). The protein moiety of chromatin was not affected 2 hours after administration

of L-T_3. Four hours after the administration of L-T_3 the protein moiety was increased 40 percent over the control. The protein moiety was 13 percent above the control 8 hours after hormone administration (Figure 16). When DNP was isolated by the method of Commerford et al. (82) (Figure 2), the biphasic effect of L-T_3 was not observed as seen here (Figure 16).

Chromatin and DNA were examined in the ultraviolet region of the spectrum. Thyroid hormone did not affect the spectra of either DNA or chromatin at any time studied. Typical spectras for DNA and chromatin are shown in Figure 17.

The effect of L-T_3 treatment on the T_m of chromatin and DNA was determined. The T_m of DNA did not change 2, 4, or 8 hours after the administration of L-T_3 (Figure 18, Curve A). The chromatin of growth-arrested sulfaguanidine-fed rat thymus had a T_m of 79° (Figure 18, Curve A). The T_m of the chromatin was 2° higher than the control 2, 4, and 8 hours after the administration of L-T_3 (Figure 18, Curve C). The T_m of DNA was not changed by L-T_3 administration at varying times, but the T_m of the chromatin was increased 2° above the control.

The template efficiency of chromatin was slightly decreased (-16%) 2 hours after the administration of L-T_3. Four hours after the administration of L-T_3 a dramatic increase (171%) in the template efficiency was observed. The template efficiency of chromatin was increased 45 percent over the control 8 hours after the administration of L-T_3 (Figure 19).

The DNA was obtained by deproteinization of chromatin with CsCl and was analyzed for template efficiency. Two, 4, and 8 hours after the administration of L-T_3 the template efficiency of DNA was no different than the control (Table 4). These results suggest that L-T_3 is producing its effect on the RNA and protein moieties of chromatin and has no effect on the DNA as shown by T_m and template efficiency.

DISCUSSION

With the "birth" of metabolic control, scientists have turned their investigation from the effects of thyroid hormones on gross physiological processes to their effects on protein metabolism and RNA synthesis. The attempt has been to elucidate the site of thyroid hormone action which should in turn explain the mechanism of action. The approach has been to determine the earliest response to thyroid hormones on the assumption that this would be at or near the site of action. Subsequent effects would only be a consequence of the initial response.

The effect of L-T_3 and L-T_4 on protein synthesis has been studied extensively (30, 41, 26, 27, 28, 29). However, much of the work is of doubtful significance since non-physiological doses and prolonged periods of treatment probably masked the initial effect of the hormone. According to Tata (30) protein synthesis in the nucleus is not stimulated until 10 hours after the administration of a single dose of L-T_3 to thyroidectomized rats. The hormone increased the incorporation of labelled amino acids into total and basic nuclear protein of rat liver. The specific activity of the protein increased with time up to 43 hours, which was the longest period studied. Only a slight increase was observed in the protein:DNA ratio 45 hours after the administration of L-T_3. Kim and Cohen (41) studied the effects of L-T_4 on the protein moiety of liver chromatin from tadpoles after 11 days of hormone treatment. They concluded that no significant change occurred in the protein:DNA ratio, in spite

of the fact that their data showed a 36 percent increase in the residual protein:DNA ratio in two separate experiments.

In the studies reported herein, thyroid hormones do have an effect on the protein of isolated nuclei. The incorporation of alanine-1-^{14}C into total nuclear protein of rat thymus nuclei (Figure 1), was increased 42 percent over the control 4 hours after the administration of L-T_3. Further investigation revealed that L-T_3 stimulated the incorporation of alanine-1-^{14}C into the whole histones (Figure 3). The incorporation of glycine and phenylalanine exhibited a pattern similar to that of alanine, which indicates that the increased incorporation was not due to the effect of the hormone on a specific amino acid pool.

The incorporation of alanine-1-^{14}C into histone fractions; f_1, f_{2a}, f_{2b}, f_3, was increased maximally either 4 or 8 hours after L-T_3 administration (Figures 7, 8, 9, 10). The increased turnover rate was accompanied by an increase in the protein:DNA ratio (Figure 16) of the isolated chromatin.

Two different procedures were used for the isolation of chromatin and the response to L-T_3 appeared to be different. A comparison of Figures 2 and 16 reveals that the protein:DNA ratio of chromatin isolated by the method of Marushige and Bonner (83), Figure 16, exhibited a biphasic response with a maximum at 4 hours after the administration of the hormone. When chromatin was

isolated by the method of Commerford et al. (82), Figure 2, the protein:DNA ratio was approximately 0.1 of that observed in Figure 16, and the biphasic response was not observed. Instead, the protein:DNA ratio rose to 3.28 mg protein/mg DNA within 4 hours and remained at approximately the same level through 16 hours. It appears that a protein fraction has been removed during isolation by the method of Commerford et al. (82), and the rate of synthesis of this fraction increases up to about 4 hours and then decreases. Further investigation of this fraction appears to be in order.

The two methods of isolation differ in that the method of Commerford et al. (82) involved extraction of the nuclear pellet with 0.05 M $NaHCO_3$; 0.1 M NaCl buffer (μ = .15) followed by solubilization of the chromatin in the insoluble pellet with water while the method of Marushige and Bonner (83) involved extraction with 0.05 M Tris buffer (μ = .05) followed by solubilization of the chromatin in 0.01 M Tris.

Tata (30) observed a continual increase in the incorporation of labelled amino acids into basic protein after L-T_3 treatment. In the studies reported here thyroid hormones produced a biphasic effect on the basic protein of rat thymus nuclei. The differences in the system of Tata (30) as contrasted to those reported here might account for the different results. Tata (30) used liver from thyroidectomized rats which had been injected with amino acids two minutes before sacrificing, and he isolated nuclei and protein by a different

procedure than reported here. The results reported here were obtained from thymus glands of growth-arrested sulfaguanidine-fed rats which were injected with amino acids 15 minutes prior to sacrificing.

It has been suggested that thyroid hormones control the synthesis of RNA. However, the nuclear RNA:DNA ratio of thyroidectomized rat liver is not changed by L-T_3 (32) and the ratio of RNA:DNA in thymus gland of growth-arrested sulfaguanidine-fed ratsremains constant as seen in Table 2. Nevertheless, the incorporation of orotic acid into nuclear RNA is stimulated 3.3 hours after L-T_3 administration (32). Wyatt and Tata (35) using hybridization techniques, have demonstrated that L-T_3 stimulates the synthesis of a new or more m-RNA and r-RNA. These results suggest that the turnover rate increased with no accumulation of nuclear RNA synthesis.

A stimulation of RNA synthesis suggested to Widnell and Tata (89) that thyroid hormones may be stimulating RNA polymerase directly. However, a thorough investigation by Tata revealed that isolated rat liver RNA polymerase did not respond to thyroid hormones and that the activity of RNA polymerase did not increase until 16 hours after the administration of L-T_3 to intact rats or 12 hours after the increase in RNA synthesis was observed. Tata did not offer an explanation for this, but the data presented in this report provides an explanation for the stimulation of RNA synthesis which

is not mediated through RNA polymerase. A stimulation of RNA synthesis may occur by other means; namely, a modification of the chromatin.

Kim and Cohen (41) reported that L-T_4 had no effect on the ratio of RNA:DNA of isolated tadpole liver chromatin. However, their data shows a 17 and 60 percent increase over the control after prolonged treatment of tadpoles with L-T_4. The ratio of RNA:DNA of rat thymus chromatin was increased 40 percent over the control 4 hours after administration of L-T_3 (Figure 15). This increase in bound RNA indicates that at the time the animals were sacrificed the synthesis of RNA was increased.

Goldberg and Atchley (90) reported that L-T4 had no effect on the T_m of DNA nor was the T_m of rat thymus DNA affected by L-T_3 (Figure 18). However, 2, 4, and 8 hours after the administration of L-T3 the T_m of chromatin of rat thymus was increased 2° over the control (Figure 18). This change in T_m of the chromatin was observed as early as 2 hours after the administration of L-T_3 and again indicates a marked change in the composition or structure of chromatin in response to L-T_3.

Further evidence of a modification of chromatin by L-T_3 was obtained by a study of the template efficiency of rat thymus chromatin. Template efficiency of rat thymus chromatin was increased 171 percent over the control 4 hours after the administration of L-T_3 (Figure 19),

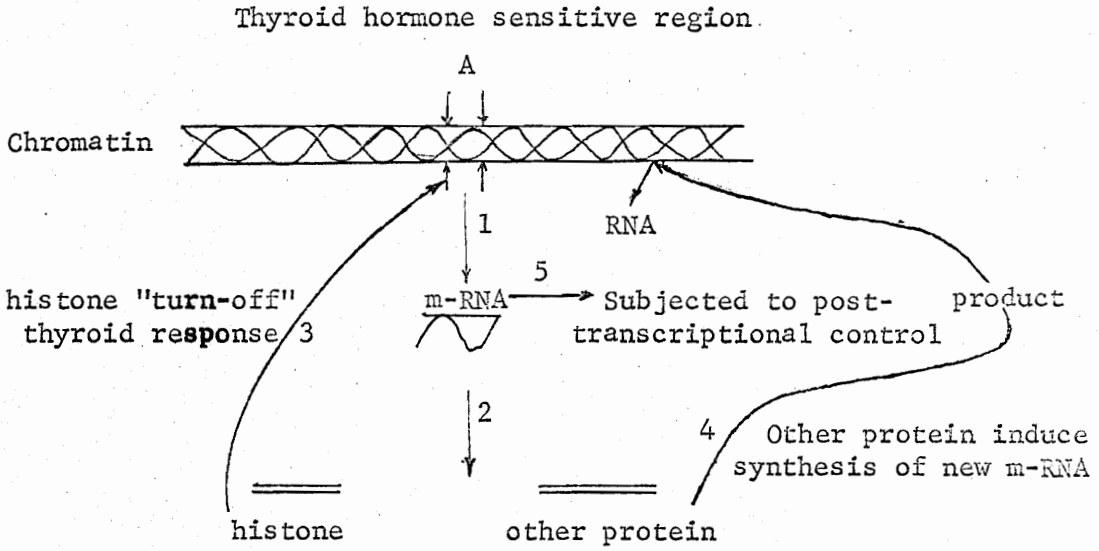
and it is significant that the template efficiency of DNA was not affected by L-T_3 treatment, Table 4. This is supported by the work of Kim and Cohen (41) who had observed an increase in template efficiency of tadpole liver chromatin after exposure of tadpoles to L-T_4 for 11 days.

The previous data and the data of Kim and Cohen (41) suggests that the site of action of thyroid hormone is on the chromatin. However, the mechanism by which L-T_3 affects the protein moiety of chromatin, increasing its template efficiency, has not been elucidated. While the details of the change in the chromatin have not yet been elucidated, the following hypothesis is proposed as the mechanism of action of thyroid hormones.

The response of the nucleus to thyroid hormones is very rapid, probably within 30 minutes after L-T_3 injection. The drop in the specific activity of histone fractions (Figure 6) could be the initial effect of L-T_3 on chromatin. It is during this initial effect of L-T_3 on chromation that the m-RNA is synthesized for processes that occur later.

These studies suggested that the initial effect of L-T_3 is to promote the synthesis of a stable m-RNA which codes for specific proteins which elicits those responses associated with thyroid hormone. The m-RNA is not easily degraded and is translated or may function as an inducer. It is possible that the m-RNA induced by thyroid hormone acts on chromatin as an inducer for the transcription of other m-RNA.

The results observed in these experiments are viewed as a stimulation of the synthesis of m-RNA which codes for the synthesis of histones and other specific protein. The synthesis of histones "turns off" the process of transcription. This build-up of histones and "turning off" occurs at approximately 4 hours after hormone administration and produces the biphasic effect that is observed in all these experiments. This is illustrated schematically as follows:



1. Thyroid hormones "react" with a thyroid hormone sensitive area of chromatin which results in the synthesis of m-RNA.
2. The m-RNA is translated producing histone and other protein.
3. The histone "turn-off" the response to L-T_3.
4. Other proteins function as inducers of m-RNA at sites other than A.
5. The m-RNA is subject to post-transcriptional control.

The rate of synthesis and the protein:DNA ratio of chromatin reach a maximum 4 hours after administration of L-T_3 indicating that the synthesis of histone and its binding to DNA is decreasing with longer treatment. This suggests that the message for histone synthesis has been "turned off" and is directed back to the control.

As the m-RNA for synthesis of histone is being translated, other proteins are being synthesized and act as inducers of other parts of the genome. There is a continuous induction of new m-RNA, which when translated produces new protein. These protein act as inducers or repressors of the chromatin and control the rate of synthesis of RNA.

The fact that thyroid hormones do not effect all tissue may be due to the impermeability of the cell membrane to thyroid hormones or to the acidic chromosomal protein. The acidic protein, which have been postulated as being responsible for organ-specific restriction of DNA transcription (91), represses the initial stimulation of thyroid hormones on the DNA.

In an elegant study of the effects of steroid hormone on metabolic control, Tompkins (93) has demonstrated post-transcriptional hormonal control of m-RNA. Other workers have shown that insulin (92)

and hydrocortisone (50) increase the template efficiency of chromatin. It is suggested that hormones in general, may have as their site of action the protein moiety of chromatin. The hormones act as an inducer or repressor for transcription of their specific m-RNA. The translation of this m-RNA into protein produces the physiological characteristics ascribed to that hormone.

Much work remains to be done in order to elucidate the details of the response of chromatin to thyroid hormones, but certain aspects of this problem deserve attention: 1) Earlier time studies of the effect of L-T_3 on chromatin, 2) A study of the effects of actinomycin and puromycin on the L-T_3 response, 3) The physical changes of chromatin induced by L-T_3, 4) Obtain an <u>in vitro</u> system that would respond to L-T_3.

SUMMARY

Preliminary cytological studies suggested that L-T_3 modified the structure of rat thymus nuclei. This led to studies which attempted to determine the nature of the changes which took place, specifically with respect to the effects of L-T_3 on the protein, RNA and DNA components of the nuclei.

The administration of 15 μg of L-T_3 to growth-arrested sulfaguanidine-fed rats stimulated the incorporation of labelled amino acid into the total nuclear protein and into the histone fraction of thymus nuclei.

The incorporation of alanine-1-^{14}C into total nuclear protein was increased 4 hours after the administration of a single dose of L-T_3 and remained constant through 8 and 16 hours. However, a biphasic response was observed when the incorporation of labelled amino acids into histones was followed with time. The incorporation into the whole histone fraction exhibited maxima at 4 hours and then declined at 8 and 16 hours after the administration of L-T_3.

The individual histone fractions followed the same pattern although maximal incorporation of label occurred at different time periods for the fractions. The incorporation of label into fraction f_1 reached a maxima at 4 hours while the maxima occurred at 8 hours for fractions f_{2a}, f_{2b}, and f_3.

No difference was observed on polyacrylamide gel when the histone fraction from control rat thymus was compared with histone obtained from thymus of L-T_3-treated rats.

Quantitative analysis of the protein and RNA components of thymus chromatin revealed that the response to L-T_3 was also biphasic with maxima at 4 hours. These changes in protein and RNA were reflected in the changes of the T_m value of the chromatin. The administration of L-T_3 resulted in a 2^o increase in the T_m value of the chromatin as early as 2 hours. In contrast, the T_m value of naked DNA was not affected by L-T_3.

Further evidence for a modification of the chromatin was obtained by a study of the template efficiency. The template efficiency of naked DNA was not affected by L-T_3, but a dramatic increase (171%) was observed in the template efficiency of chromatin 4 hours after the administration of L-T_3, which dropped to 45 percent at 8 hours.

These results suggest that L-T_3 has a direct effect on rat thymus chromatin which enhances its potential as a template for RNA synthesis. A hypothetical mechanism for the action of thyroid hormones is presented.

VITA

The author was born in Deltaville, Virginia on April 11, 1941. He is the son of Mary Ruark and the late Rufus Ruark. He received his primary and secondary education in the Middlesex County School System. In 1959 he entered Lynchburg College, graduating in 1963 with a Bachelor of Science degree in Biology and Chemistry. From July 1963 until July 1964 he worked for Dr. J. C. Forbes as a laboratory technician in the Department of Biochemistry at the Medical College of Virginia. In July 1964 he entered Virginia Polytechnic Institute receiving his Master of Science degree in 1966 and that same year began working on his Doctor of Philosophy degree, the requirements for which were completed in November 1970.

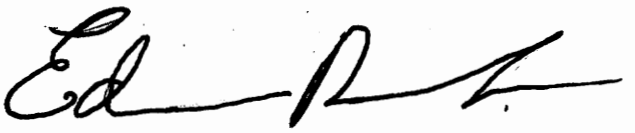


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Table 1

The Effect of Triiodothyronine ($L\text{-}T_3$) on the DNA of Rat Thymus Nuclei *

Time after Hormone Administration (hrs.)	mg of deoxyribose/gm dry weight of thymus nuclei
Control	16.0
2	15.9
4	15.9
8	16.1
16	16.0

*Triiodothyronine (15 μg) was injected intraperitoneally and the rats were sacrificed at the times indicated. Each point is the result obtained from the pooled thymus nuclei of 5 growth-arrested sulfaguanidine-fed rats.

Table 2

The Effect of Triiodothyronine (L-T_3) on the RNA of Rat Thymus Nuclei*

Time after Hormone Administration (hrs.)	mg RNA/mg deoxyribose
Control	.105
4	.110
8	.098
16	.110

*Triiodothyronine (15 μg) was injected intraperitoneally and the rats were sacrificed at the times indicated. Each point is the result obtained from the pooled thymus nuclei of 5 growth-arrested sulfaguanidine-fed rats.

Table 3

Composition of Histone Fractions Obtained from Rat Thymus Nuclei*

Amino Acids	Fractions			
	f_1	f_{2a}	f_{2b}	f_3
Lysine	23.00	9.64	11.92	8.12
Histidine	.20	2.09	2.00	1.67
Arginine	1.95	9.34	6.52	7.29
Ammonia	7.96	8.71	10.13	15.30
Aspartic acid	2.78	4.84	5.18	4.86
Threonine	5.17	4.40	5.18	5.02
Serine	5.66	2.59	5.33	3.40
Glutamic acid	4.44	7.68	8.97	10.11
Proline	8.33	3.11	4.82	4.73
Glycine	6.49	10.21	6.85	7.13
Alanine	21.57	9.59	10.68	10.79
Half-cystine	---	---	---	Trace
Valine	5.12	6.16	6.35	4.78
Methionine	---	5.29	.90	.57
Isoleucine	1.22	4.44	4.57	4.36
Leucine	3.81	8.27	6.58	7.97
Tyrosine	.54	2.19	2.25	1.65
Phenylalanine	.78	1.44	1.78	2.27

*The fractions were isolated by the method of Johns (Figure 4). The amino acids are expressed as moles/100 moles of the total amino acids. No corrections have been made for hydrolytic losses.

Table 4

The Effect of Triiodothyronine on the Template Efficiency of Chromatin and DNA of Rat Thymus *

Time of Hormone Treatment (hrs.)	Chromatin		DNA	
	cpm ^{3}H-UTP/µg DOR	% Change from Control	cpm ^{3}H-UTP/µg DOR	% Change from Control
Control	31		4760	
2	26	-16%	4900	+ 3%
4	84	+171%	4670	- 2%
8	45	+ 45%	5200	+11%

*Triiodothyronine (15µg) was injected intraperitoneally and the rats were sacrificed at the times indicated. Each point is the result obtained from the pooled thymus nuclei of 5 growth-arrested sulfaguanidine-fed rats.

The complete incubation mixture contained in 0.25 ml: 10 µmoles Tris buffer (pH 8.0), 1 µmole $MgCl_2$, 0.25 µmoles $MnCl_2$, 3 µmoles β-mercapto-ethanol, 0.1 µmoles each of ATP, GTP, CTP, UTP, 2.5 µc UTP-5-^{3}H, DNA or chromatin and 15 units of RNA polymerase for DNA and 5 units for chromatin. Incubation was carried out at 37°. A 0.1 ml aliquot was removed at 10 minutes and prepared for counting by the method of Mans and Novelli (86). DOR = 2 droxyribose.

Figure 1. The effect of triiodothyronine (L-T_3) on the incorporation of alanine-1-^{14}C into total nuclear protein of rat thymus nuclei.

Triiodothyronine (15 μg) was injected intraperitoneally and the animals were sacrificed at the times indicated. Alanine-1-^{14}C (15 μc) was injected 15 minutes before the animals were sacrificed. Each point is the result obtained from pooled thymus nuclei of 5 growth-arrested sulfaguanidine-fed rats.

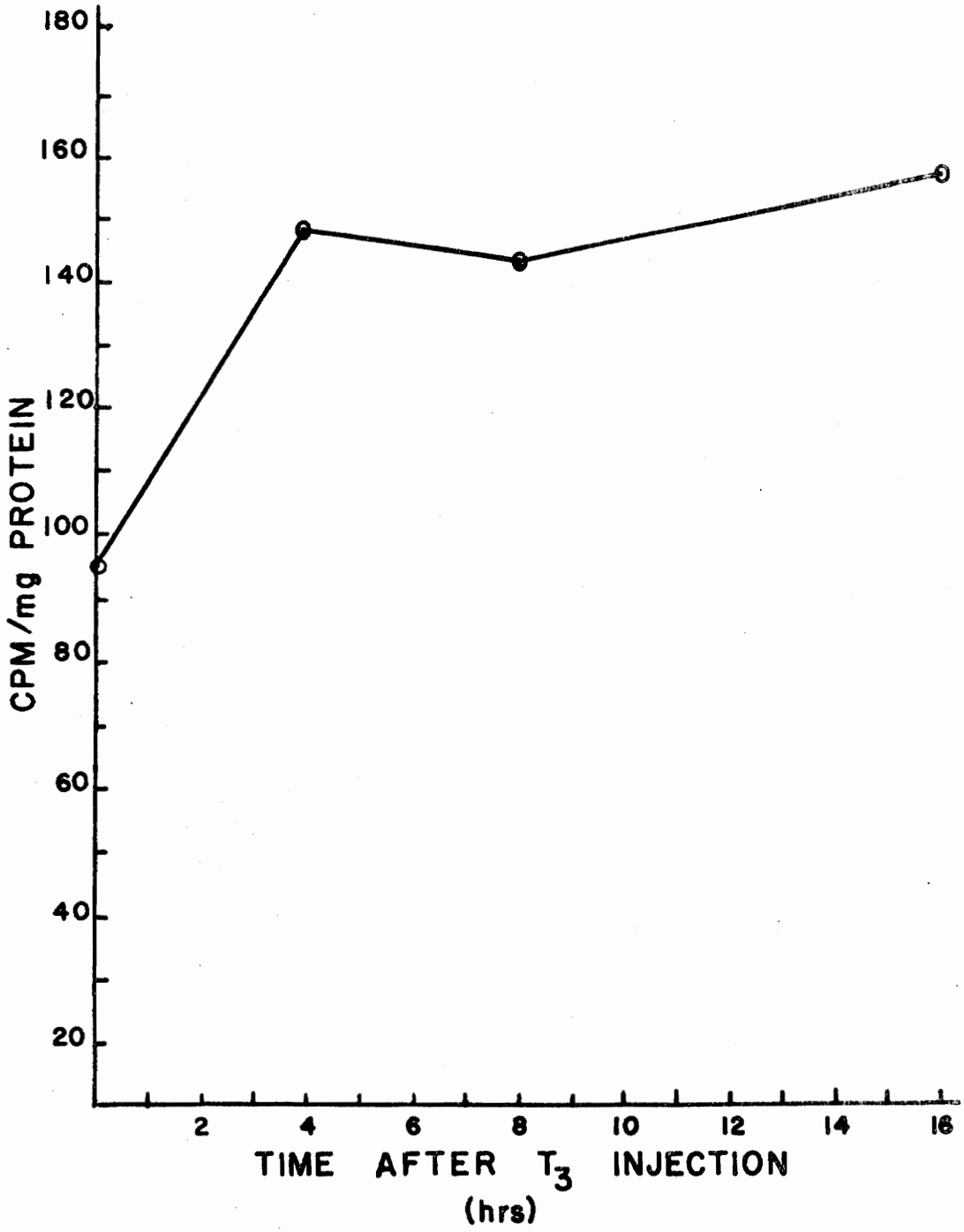

180
160
140
120
100
80
60
40
20
CPM/mg PROTEIN
2
4
6
8
10
12
14
16
TIME AFTER T_3 INJECTION
(hrs)

Figure 2. The effect of triiodothyronine (L-T_3) on Deoxyribonucleoprotein (DNP) of rat thymus nuclei.

Triiodothyronine (15 μg) was injected intraperitoneally and the rats were sacrificed at the times indicated. Each point is the result obtained from the pooled thymus nuclei of 5 growth-arrested sulfaguanidine-fed rats. DOR = 2-Deoxyribose.

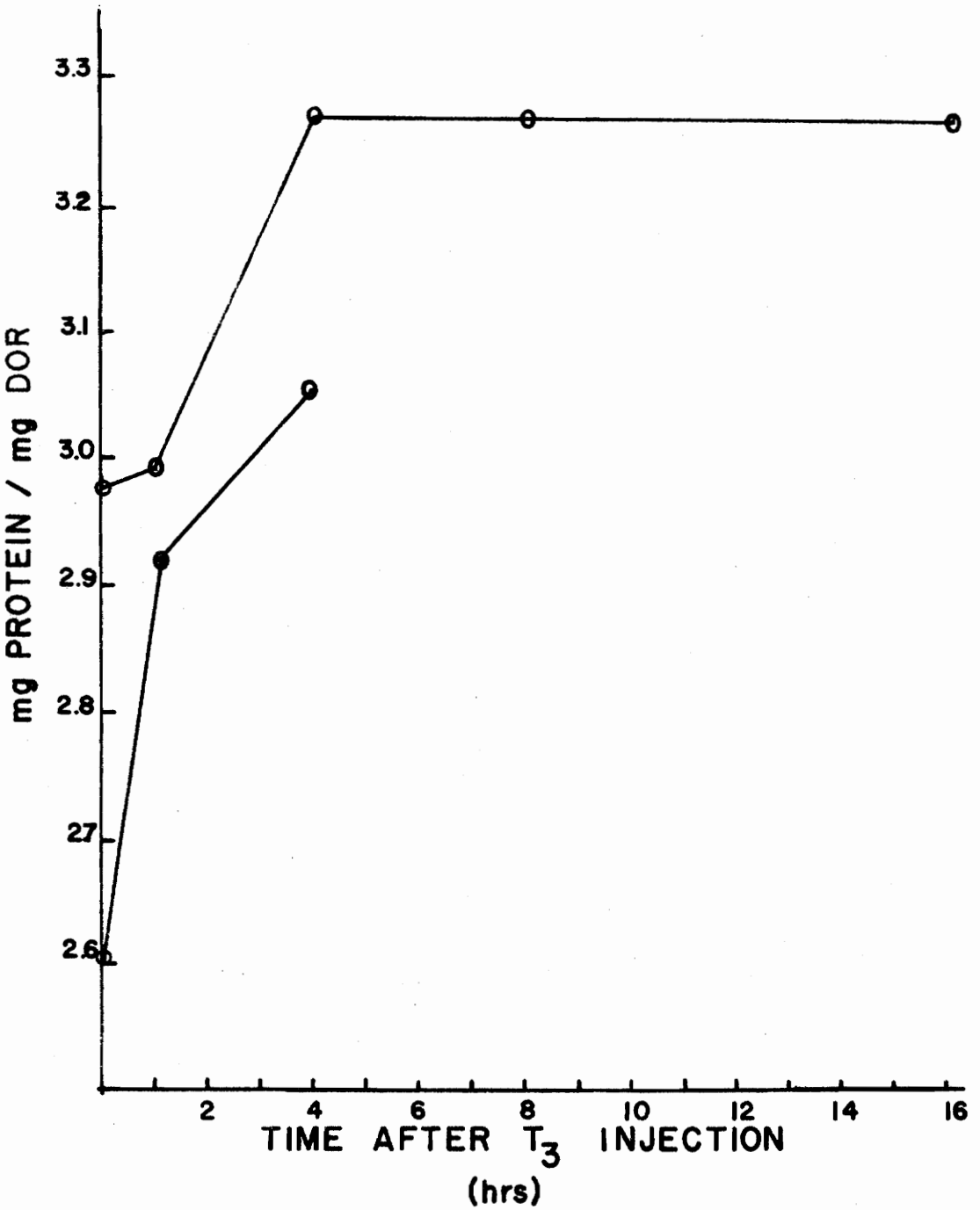

3.3
3.2
3.1
3.0
2.9
2.8
2.7
2.6
mg PROTEIN / mg DOR
2
4
6
8
10
12
14
16
TIME AFTER T_3 INJECTION
(hrs)

Figure 3. The effect of triiodothyronine (L-T_3) on the incorporation of labelled amino acids into whole histones of rat thymus nuclei.

Triiodothyronine (15 μg) was injected intraperitoneally and the animals were sacrificed at the times indicated. Labelled amino acids (15 μc) were injected 15 minutes before the animals were sacrificed. Each point is the result obtained from pooled thymus nuclei of 5 growth-arrested sulfaguanidine-fed rats.

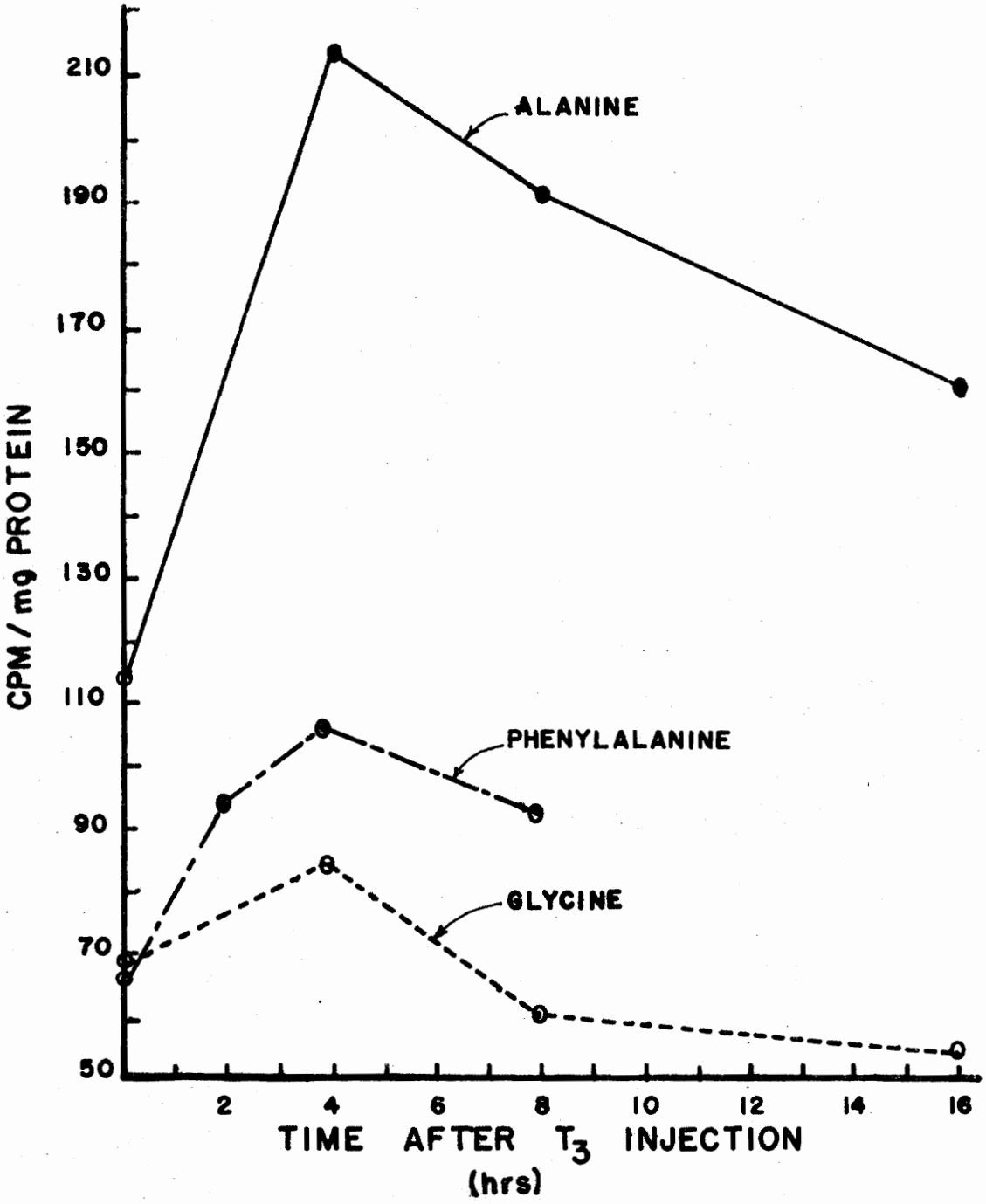

210
190
170
150
130
110
90
70
50
CPM / mg PROTEIN
ALANINE
PHENYLALANINE
GLYCINE
2
4
6
8
10
12
14
16
TIME AFTER T_3 INJECTION
(hrs)

Figure 4. Summary of the method for extraction of histone fractions from rat thymus nuclei.

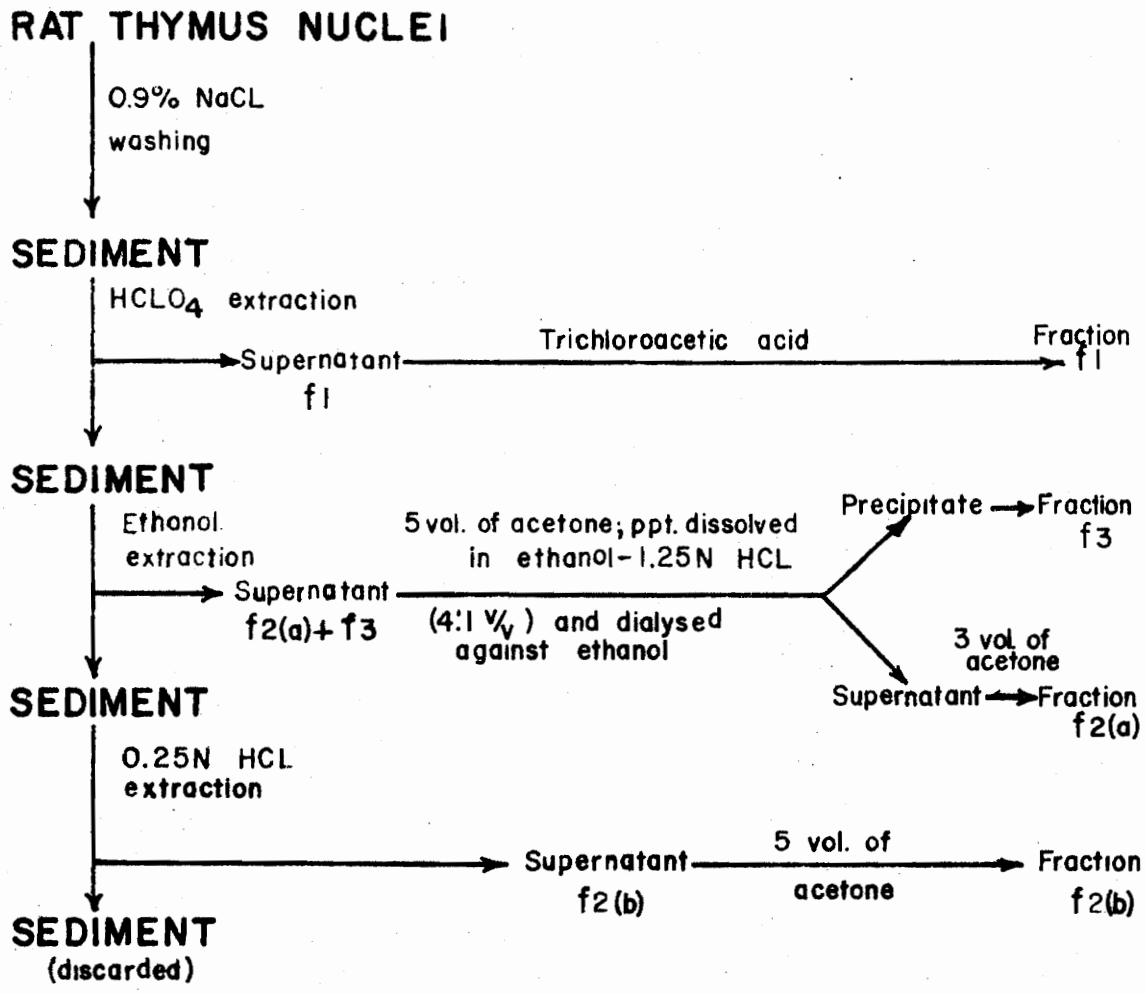

RAT THYMUS NUCLEI
0.9% NaCL
washing
SEDIMENT
HCLO4 extraction
Supernatant
f1
Trichloroacetic acid
Fraction
f1
SEDIMENT
Ethanol
extraction
Supernatant
f2(a)+f3
5 vol. of acetone; ppt. dissolved
in ethanol-1.25N HCL
(4:1 v/v) and dialysed
against ethanol
Precipitate
Fraction
f3
3 vol. of
acetone
Supernatant
Fraction
f2(a)
SEDIMENT
0.25N HCL
extraction
Supernatant
f2(b)
5 vol. of
acetone
Fraction
f2(b)
SEDIMENT
(discarded)

Figure 5. Separation of histone fractions on polyacrylamide gel.

The histone fractions were obtained from pooled thymus nuclei of growth-arrested sulfaguanidine-fed rats.

a. f_1

b. f_{2a}

c. f_{2b}

d. f_3

e. whole histone

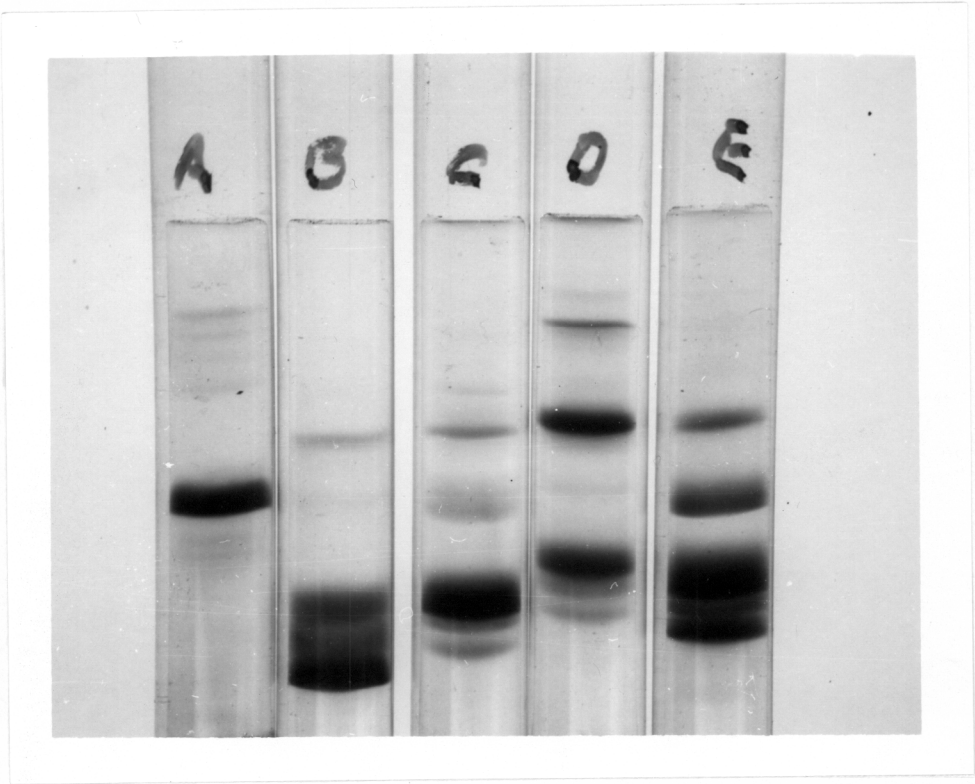

A
B
C
D
E

Figure 6. The effect of triiodothyronine (L-T_3) on the incorporation of glycine-1-^{14}C into four histone fractions of rat thymus nuclei.

Triiodothyronine (15 μg) was injected intraperitoneally and the animals were sacrificed at the times indicated. Glycine-1-^{14}C (15 μc) was injected 15 minutes before the animals were sacrificed. Each point is the result obtained from pooled thymus nuclei of 5 growth-arrested sulfaguanidine-fed rats. The fractions were isolated by the method of Johns (60), Figure 4.

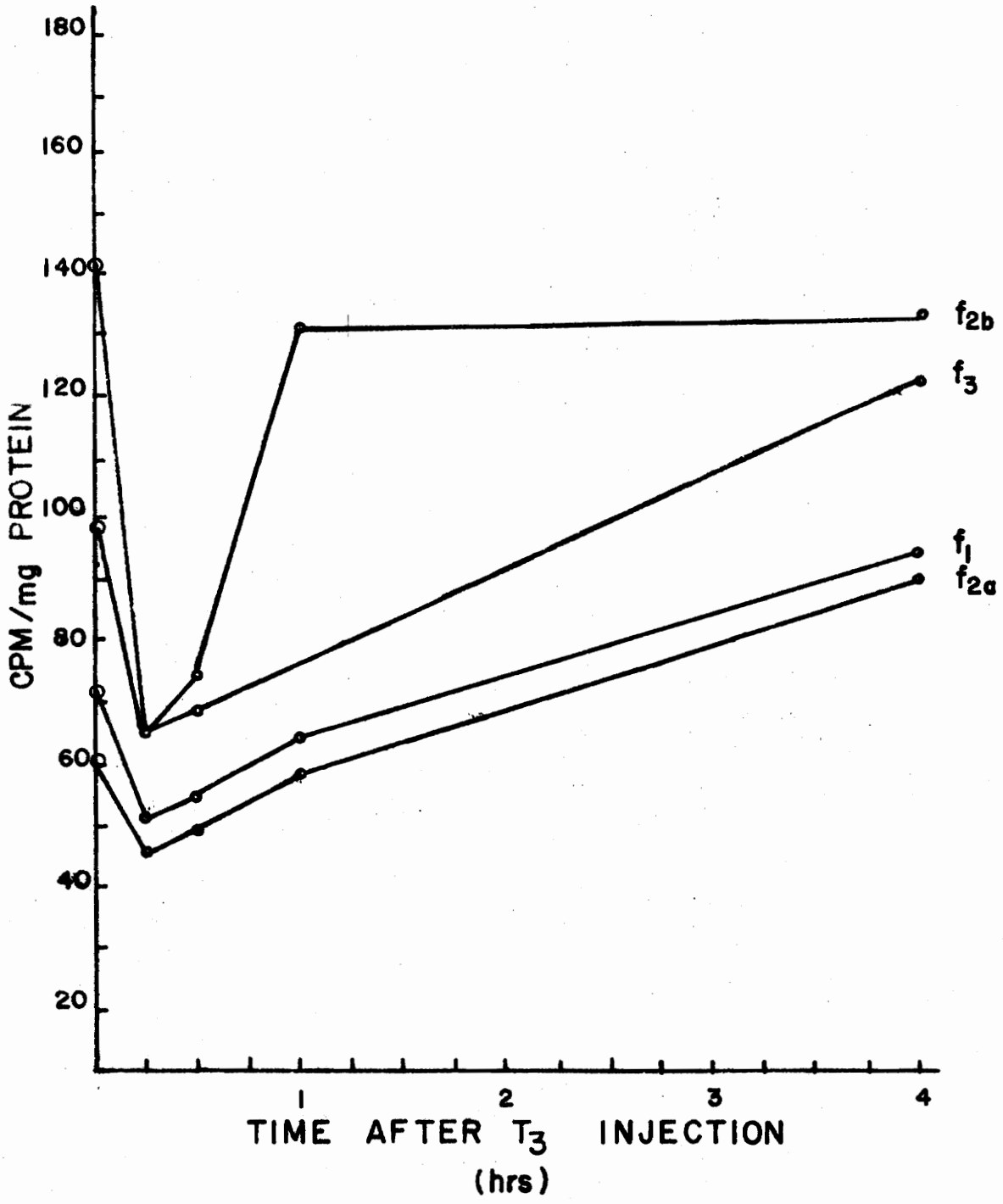

180
160
140
120
100
80
60
40
20
CPM/mg PROTEIN
1
2
3
4
TIME AFTER T3 INJECTION
(hrs)
f2b
f3
f1
f2a

Figure 7. The effect of triiodothyronine (L-T_3) on the incorporation of alanine-1-^{14}C into the histone fraction of f_1 of rat thymus nuclei.

Triiodothyronine (15 μg) was injected intraperitoneally at times indicated. Alanine-1-^{14}C was injected 15 minutes before the animals were sacrificed. Fraction f_1 was isolated by the method of Johns (60) Figure 4. The broken line is the average of 2 experiments.

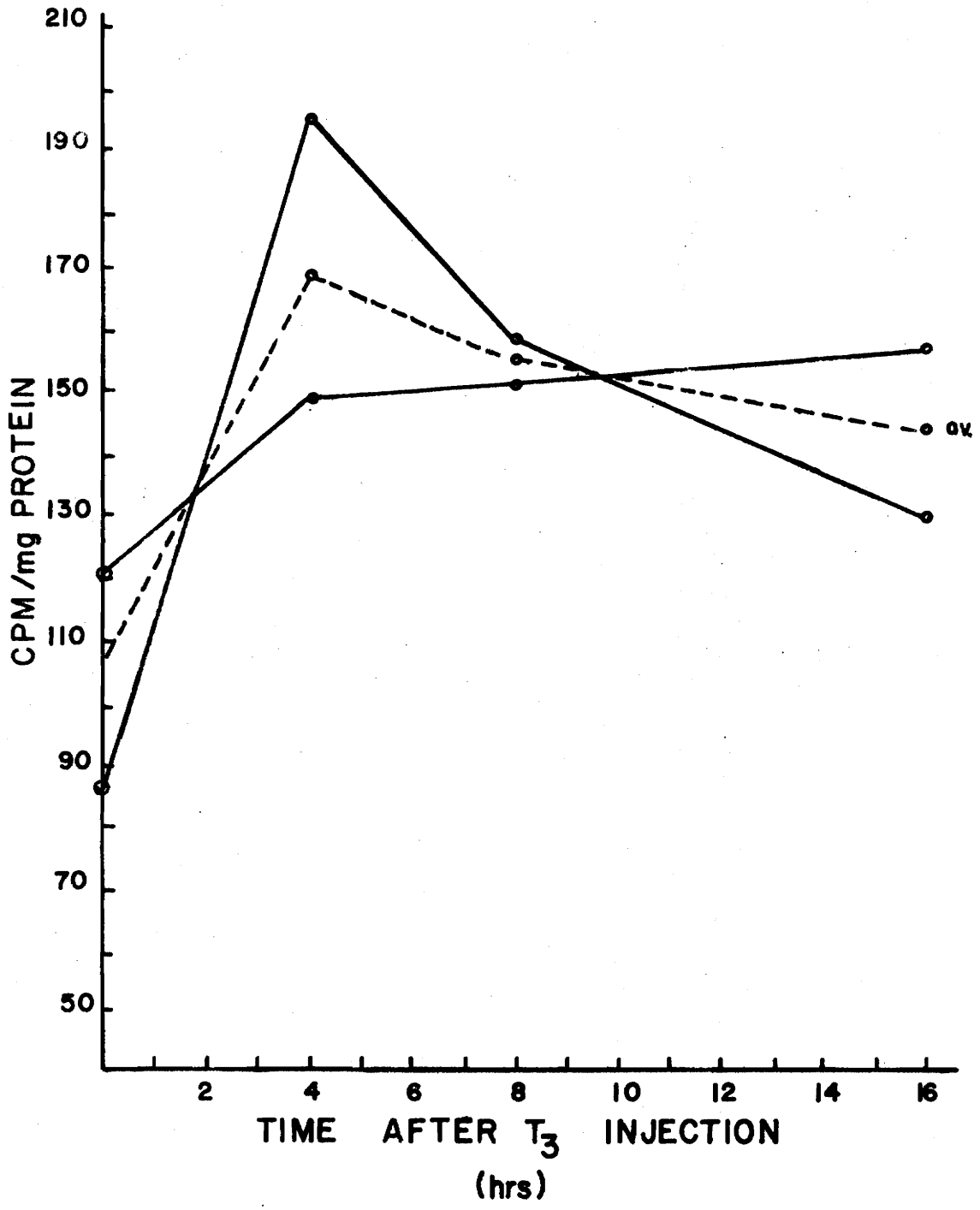

210
190
170
150
130
110
90
70
50
CPM/mg PROTEIN
ov.
2
4
6
8
10
12
14
16
TIME AFTER T_3 INJECTION
(hrs)

Figure 8. The effect of triiodothyronine (L-T_3) on the incorporation of alanine-1-^{14}C into the histone fraction f_{2a} of rat thymus nuclei.

Triiodothyronine (15 μg) was injected intraperitoneally at times indicated. Alanine-1-^{14}C was injected 15 minutes before the animals were sacrificed. Fraction f_{2a} was isolated by the method of Johns (60) Figure 4. The broken line is the average of 2 experiments.

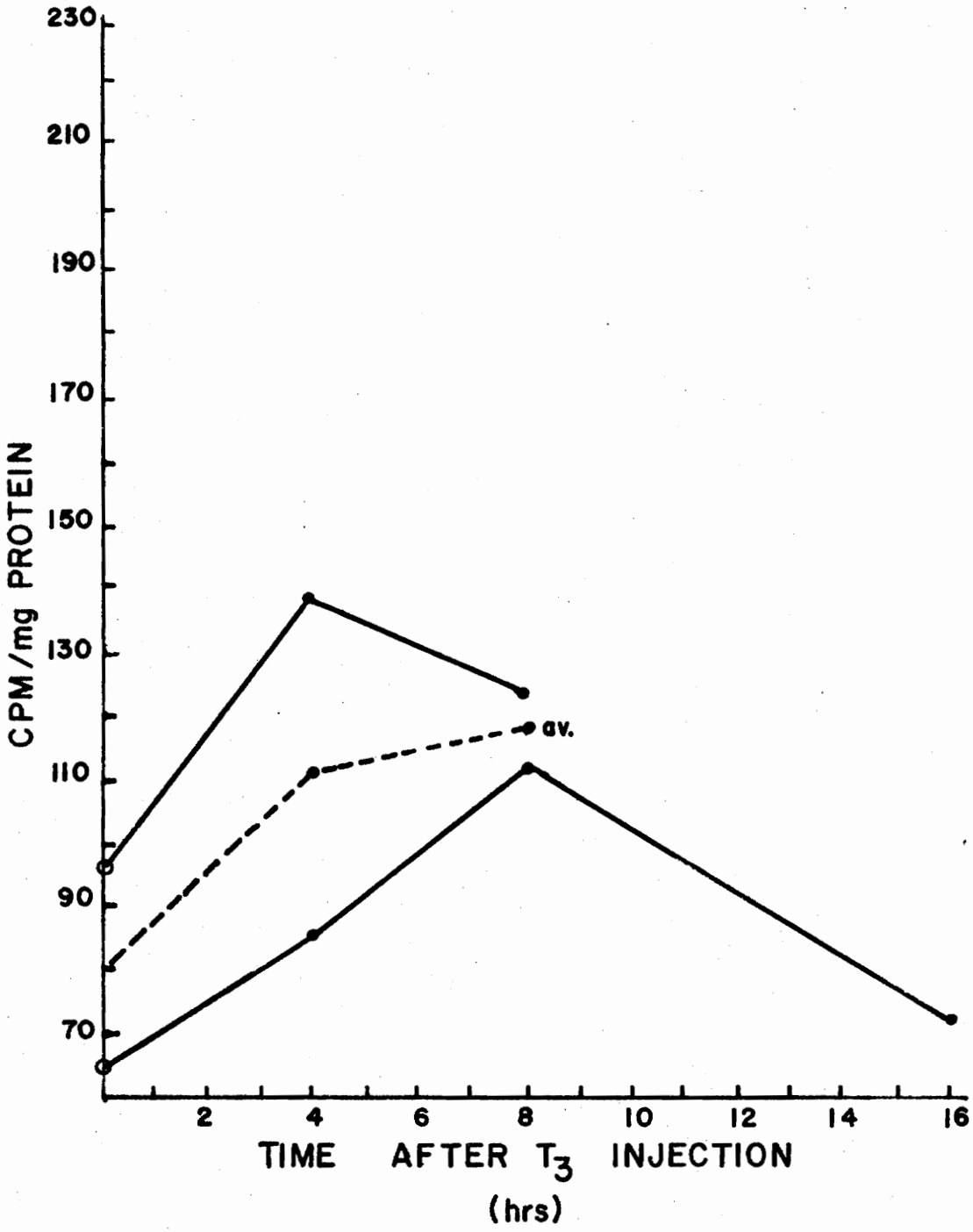

230
210
190
170
150
130
110
90
70
CPM/mg PROTEIN
av.
2
4
6
8
10
12
14
16
TIME AFTER T_3 INJECTION
(hrs)

Figure 9. The effect of triiodothyronine (L-T_3) on the incorporation of alanine-1-^{14}C into histone fraction f_{2b}.

Triiodothyronine (15 μg) was injected intraperitoneally at times indicated. Alanine-1-^{14}C was injected 15 minutes before the animals were sacrificed. Fraction f_{2b} was isolated by the method of Johns (60) Figure 4. The broken line is the average of 2 experiments.

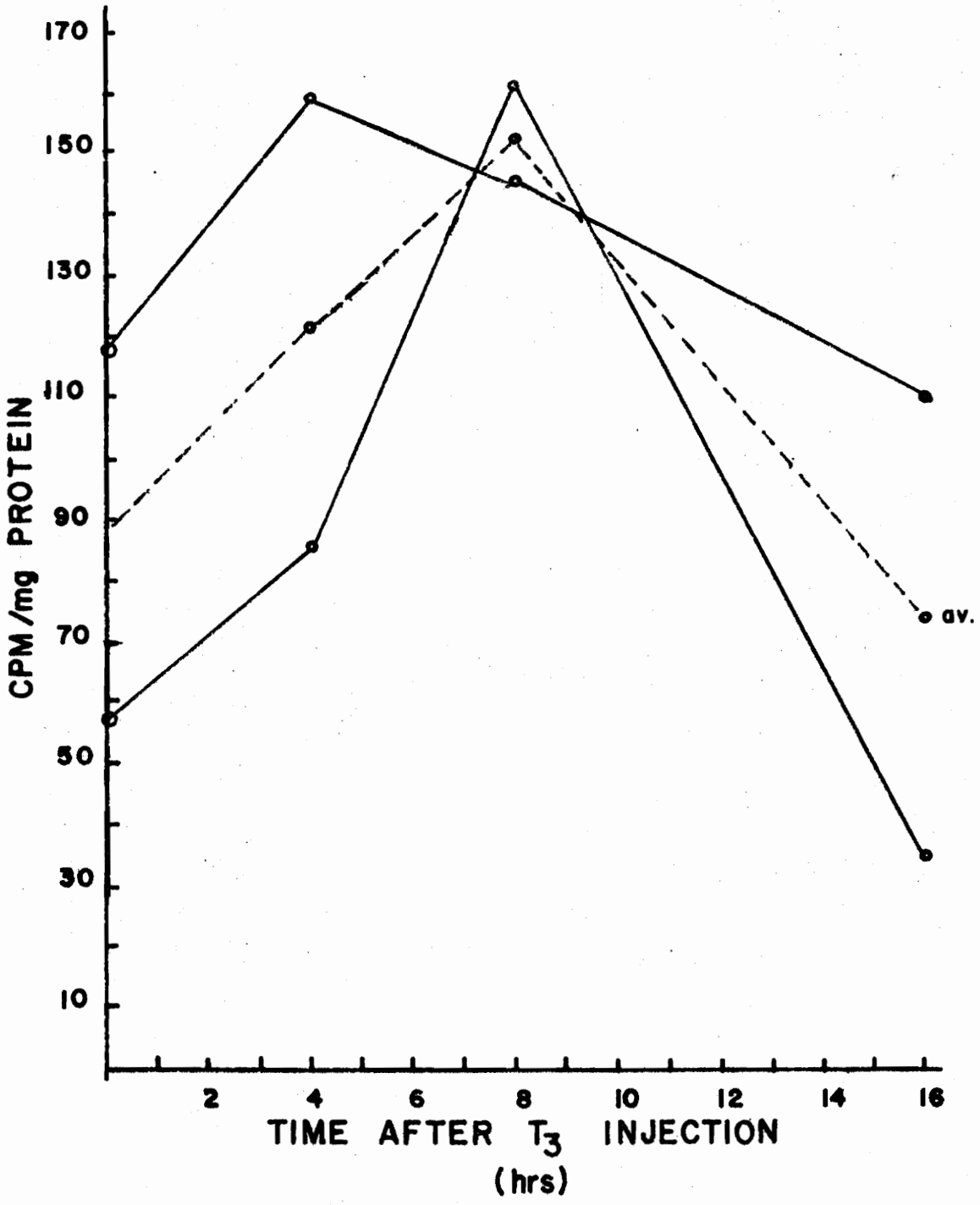

170
150
130
110
90
70
50
30
10
CPM/mg PROTEIN
av.
2
4
6
8
10
12
14
16
TIME AFTER T_3 INJECTION
(hrs)

Figure 10. The effect of triiodothyronine (L-T_3) on the incorporation of alanine-1-^{14}C into histone fraction f_3.

Triiodothyronine (15 μg) was injected intraperitoneally at times indicated. Alanine-1-^{14}C was injected 15 minutes before the animals were sacrificed. Fraction f_3 was isolated by the method of Johns (60) Figure 4. The broken line is the average of 2 experiments.

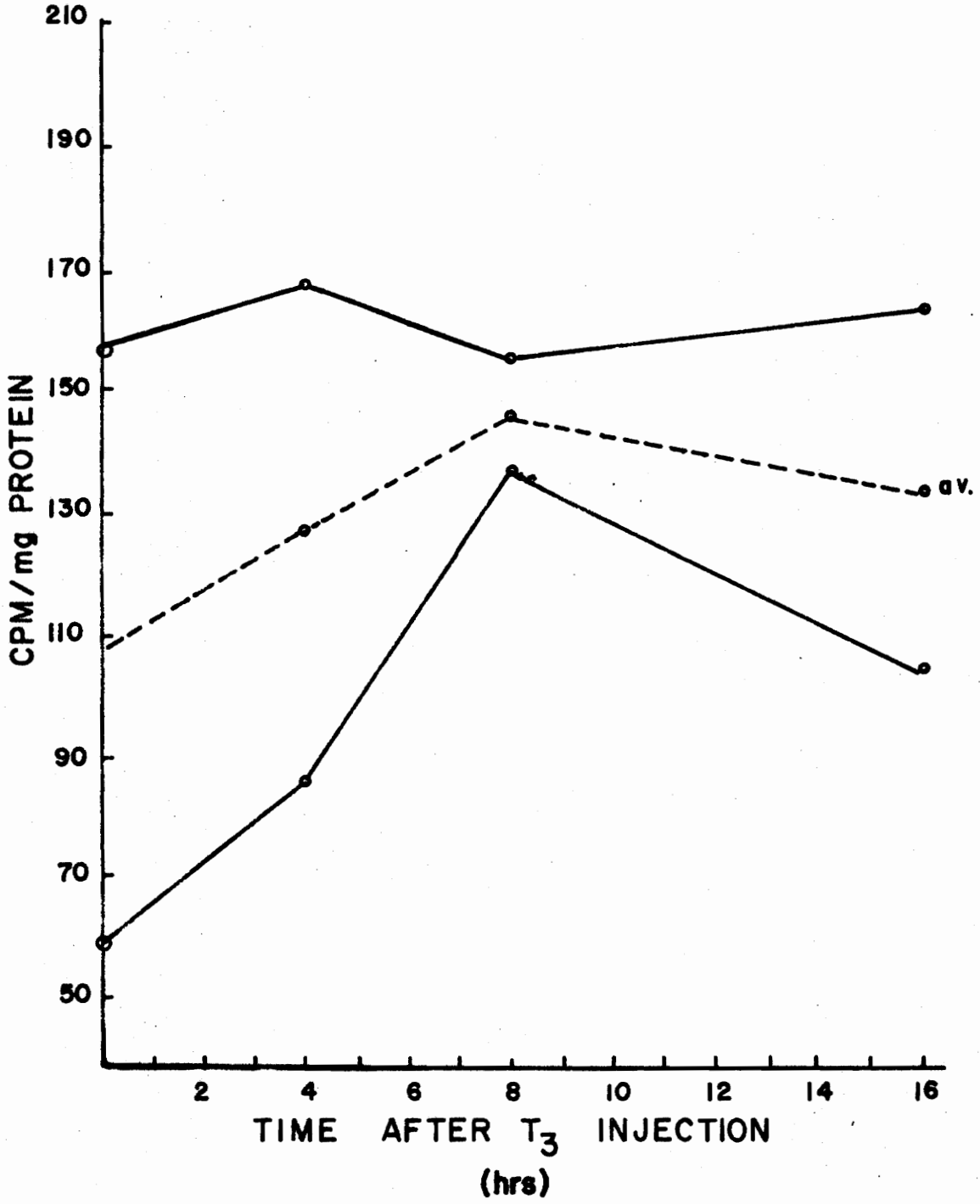

210
190
170
150
130
110
90
70
50
CPM/mg PROTEIN
a.v.
2
4
6
8
10
12
14
16
TIME AFTER T_3 INJECTION
(hrs)

Figure 11. Comparison of histone fraction f_1 on polyacrylamide gel.

1. Control growth-arrested sulfaguanidine-fed rat.
2. Four hours after the administration of L-T_3 (15 μg).

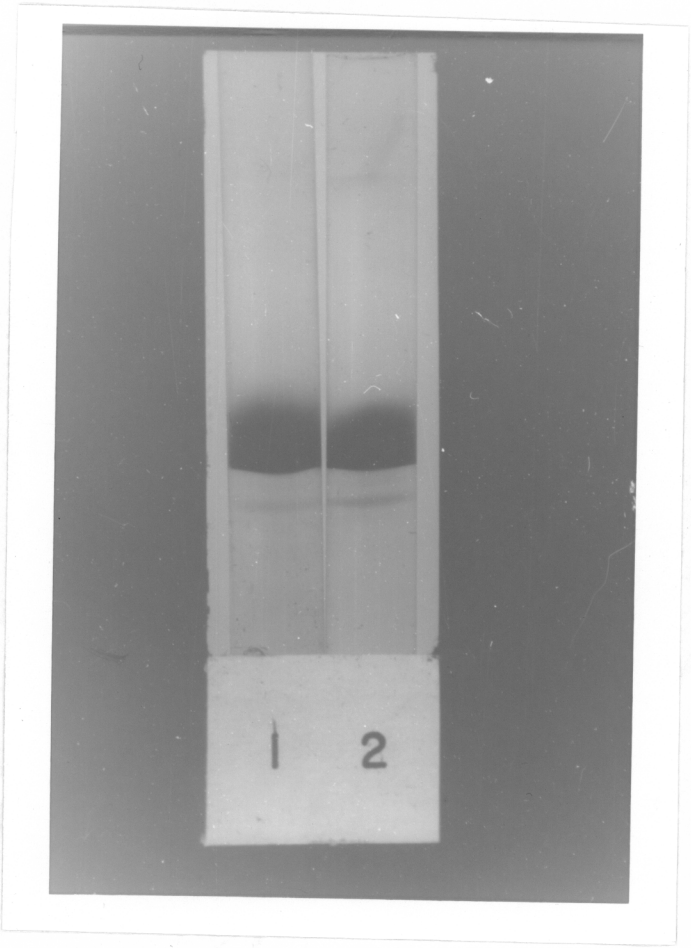

1 2

Figure 12. Comparison of histone fraction f_{2a} on polyacrylamide gel.

1. Control growth-arrested sulfaguanidine-fed rats.
2. Four hours after the administration of L-T_3 (15 μg).

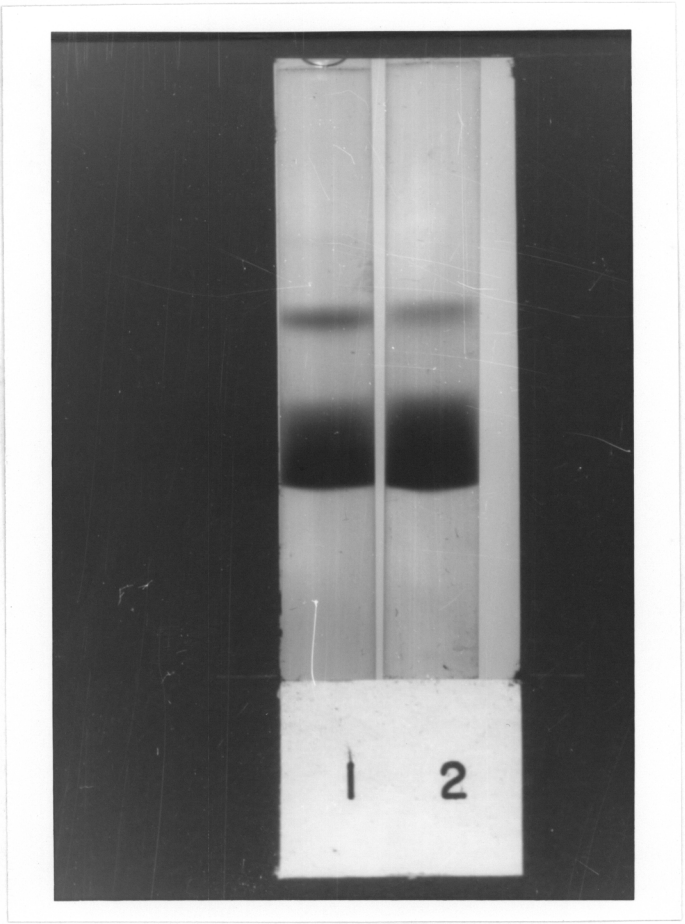

1
2

Figure 13. Comparison of histone fraction f_{2b} on polyacrylamide gel.

1. Control growth-arrested sulfaguanidine-fed rats.
2. Four hours after the administration of L-T_3 (15 μg).

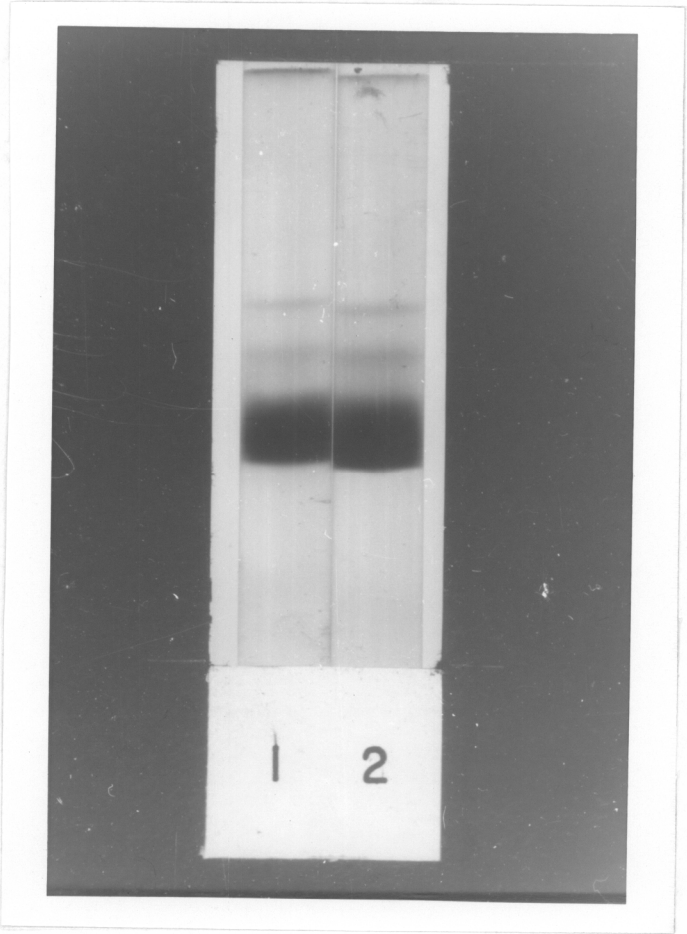

1
2

Figure 14. Comparison of histone fraction f_3 on polyacrylamide gel.

1. Control growth-arrested sulfaguanidine-fed rats.
2. Four hours after the administration of L-T_3 (15 μg).

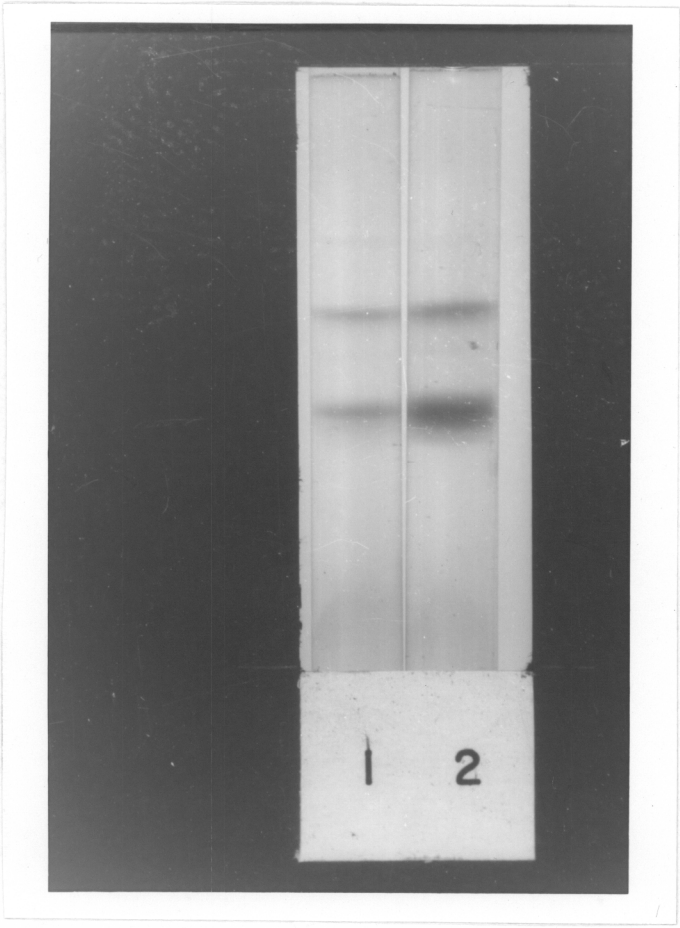

1
2

Figure 15. The effect of triiodothyronine on bound RNA of rat thymus chromatin.

Triiodothyronine (15 μg) was injected intraperitoneally and the rats were sacrificed at the times indicated. Each point is the result obtained from the pooled thymus nuclei of 5 growth-arrested sulfaguanidine-fed rats. DOR = 2-Deoxyribose.

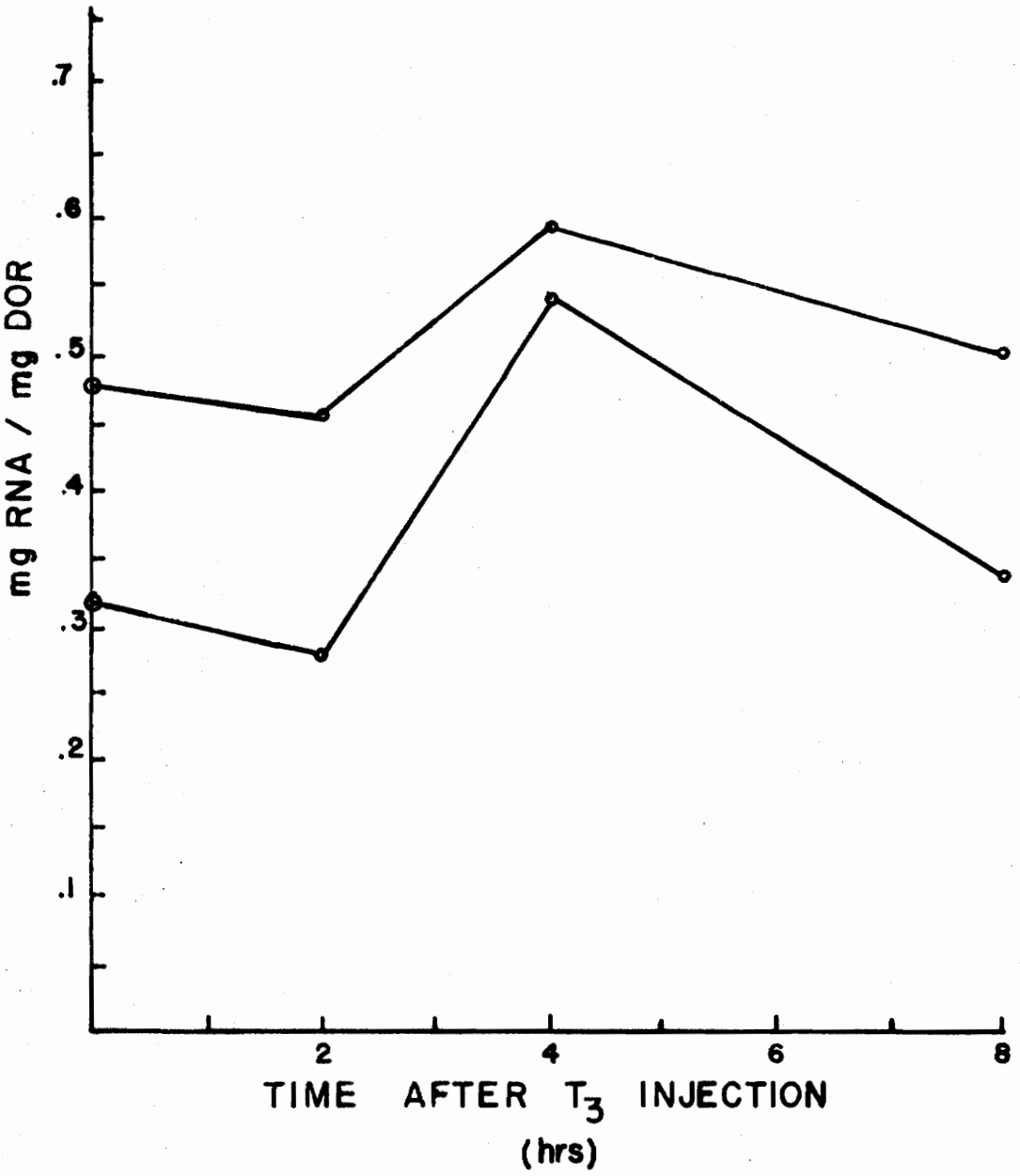

mg RNA / mg DOR
.7
.6
.5
.4
.3
.2
.1
2
4
6
8
TIME AFTER T_3 INJECTION
(hrs)

Figure 16. The effect of triiodothyronine (L-T_3) on protein of rat thymus chromatin.

Triiodothyronine (15 μg) was injected intraperitoneally and the rats were sacrificed at the times indicated. Each point is the result obtained from the pooled thymus nuclei of 5 growth-arrested sulfaguanidine-fed rats. Compare results with Figure 2. DOR = 2-Deoxyribose.

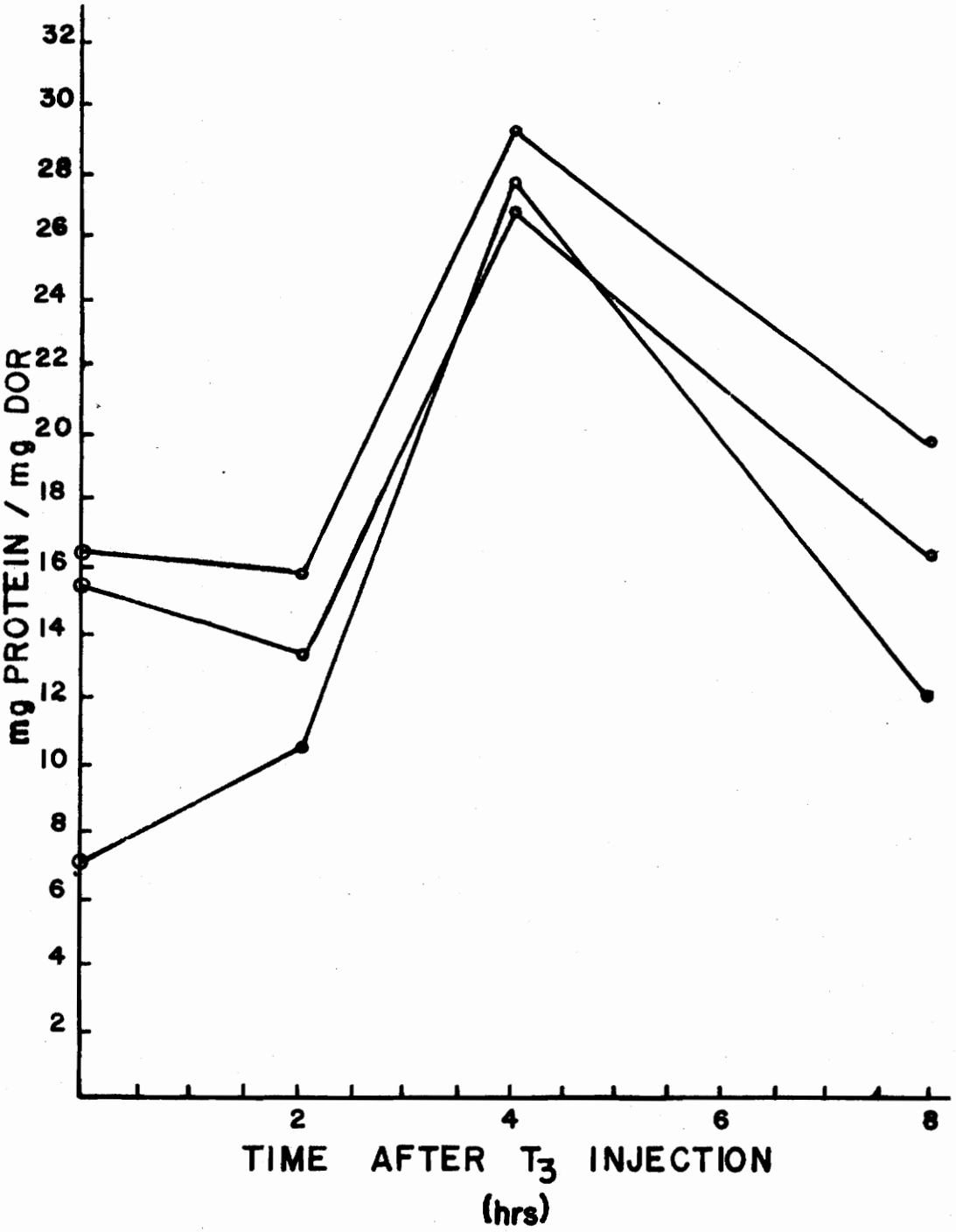

mg PROTEIN / mg DOR
32
30
28
26
24
22
20
18
16
14
12
10
8
6
4
2
2
4
6
8
TIME AFTER T_3 INJECTION
(hrs)

Figure 17. The absorption spectra of rat thymus chromatin and DNA.

The chromatin and DNA were solubilized in 0.01 M Tris buffer at pH 8.0

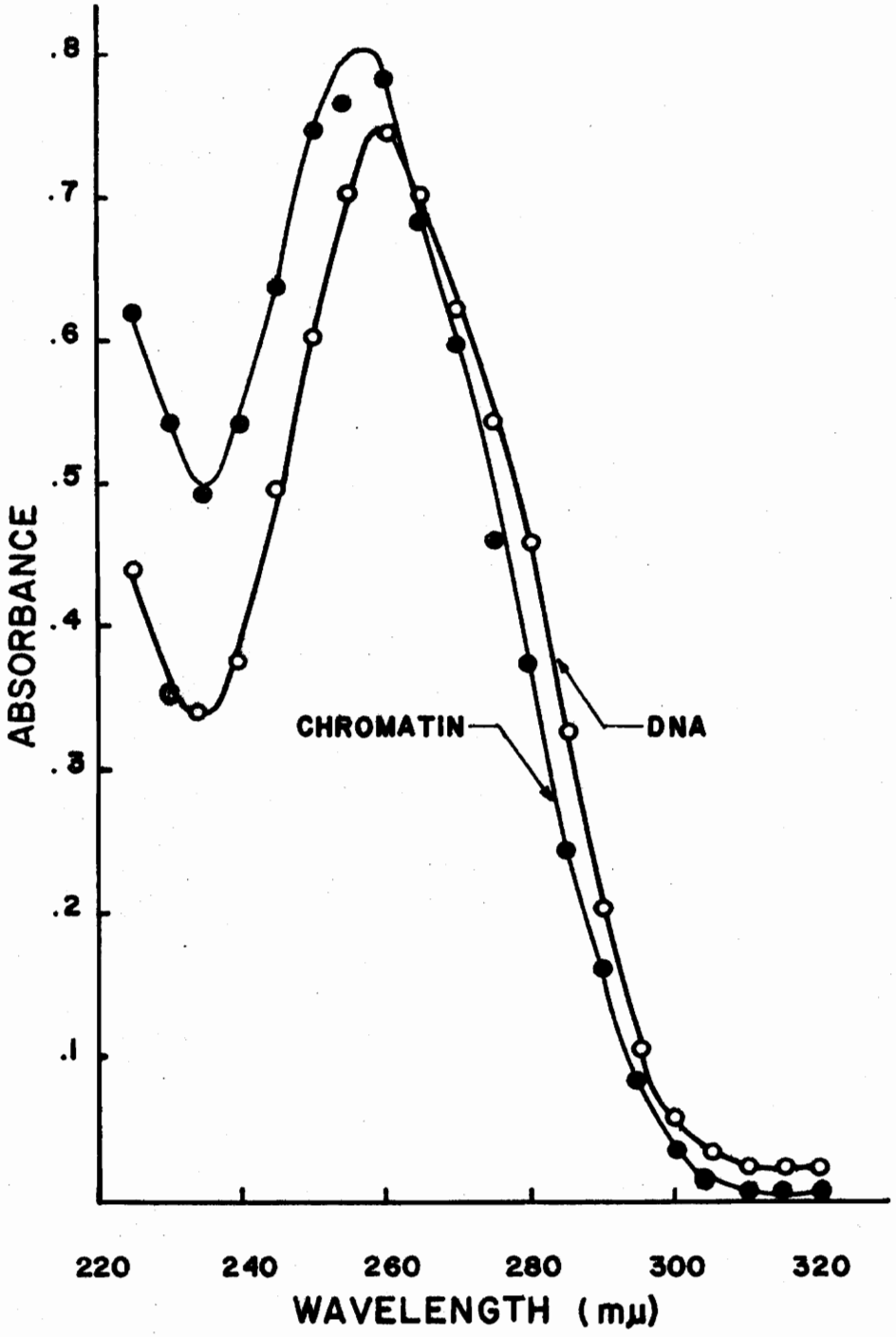

.8
.7
.6
.5
.4
.3
.2
.1
ABSORBANCE
CHROMATIN
DNA
220
240
260
280
300
320
WAVELENGTH (mμ)

Figure 18. The Effect of triiodothyronine (L-T_3) on the T_m of DNA and chromatin of rat thymus.

Triiodothyronine (15 μg) was injected 2, 4, and 8 hours before the animals were sacrificed. The T_m was determined using 0.01 M Tris buffer at pH 8.0.

A. DNA after chromatin had been deproteinized with CsCl.
B. Chromatin from growth-arrested sulfaguanidine-fed rats.
C. T_m after the administration of L-T_3. The T_m values of chromatin 2, 4, and 8 hours after the administration of L-T_3 were identical.

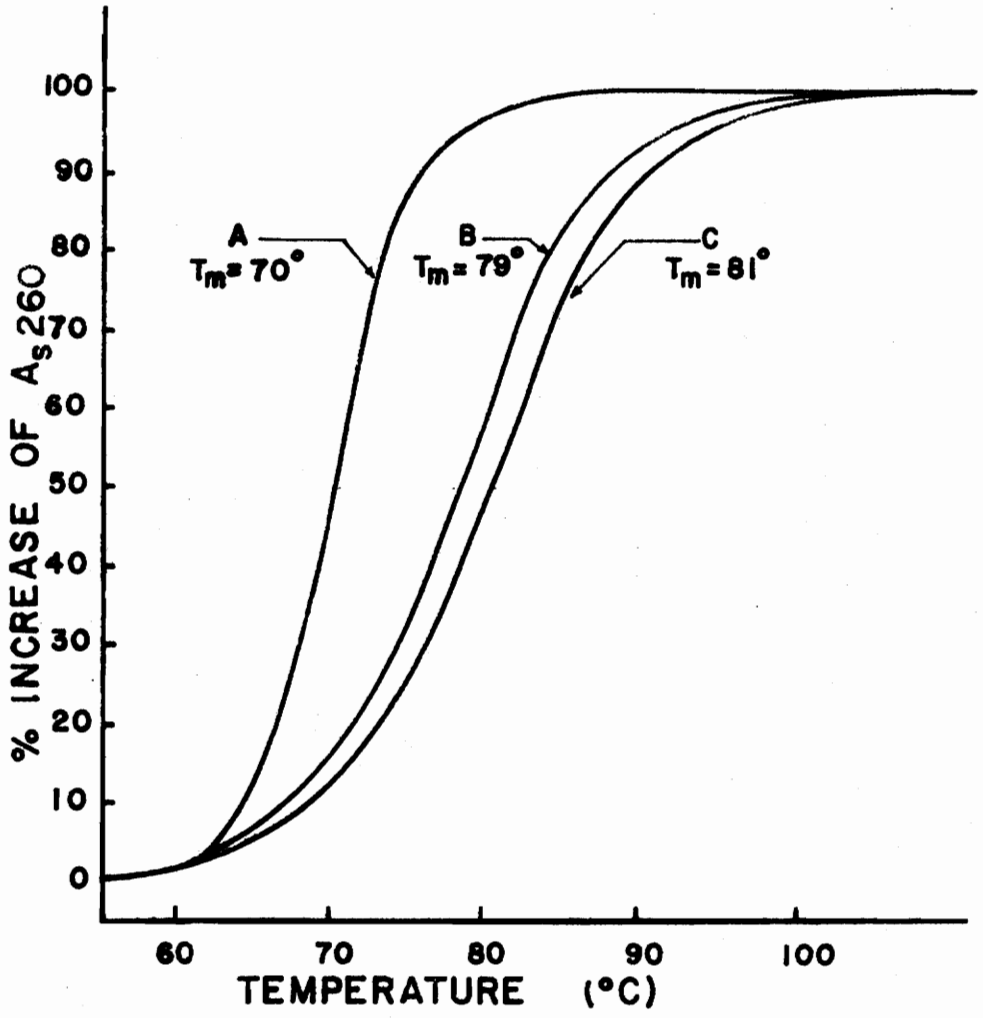
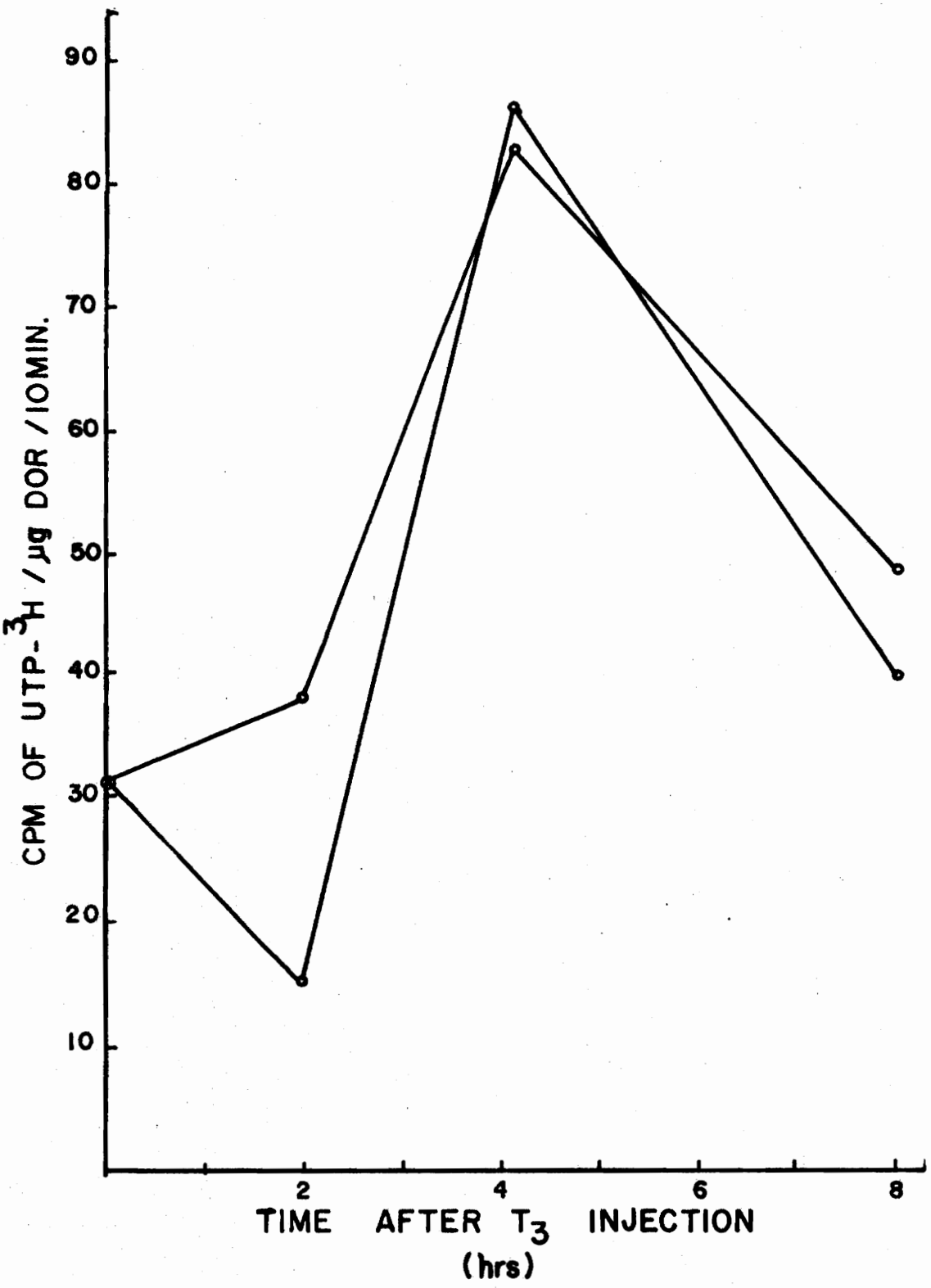

100
90
80
70
60
50
40
30
20
10
0
% INCREASE OF A_{s260}
A
T_m= 70°
B
T_m= 79°
C
T_m= 81°
60
70
80
90
100
TEMPERATURE (°C)

Figure 19. The effect of triiodothyronine (L-T_3) on the template efficiency of rat thymus chromatin.

Triiodothyronine (15 μg) was injected intraperitoneally and the rats were sacrificed at the times indicated. Each point is the result obtained from the pooled thymus nuclei of 5 growth-arrested sulfaguanidine-fed rats.

The complete incubation mixture contained in 0.25 ml: 10 μmoles Tris buffer (pH 8.0), 1 μmole $MgCl_2$, 0.25 μmoles $MnCl_2$, 3 μmoles β-mercaptoethanol, 0.1 μmoles each of ATP, GTP, CTP, UTP, 2.5 μc UTP-5-^{3}H, chromatin and 5 units of RNA polymerase. Incubation was carried out at 37°. A 0.1 ml aliquot was removed at 10 minutes and prepared for counting by the method of Mans and Novelli (86). DOR = 2-deoxyribose.


90
80
70
60
50
40
30
20
10
CPM OF UTP-^{3}H /µg DOR /10MIN.
2
4
6
8
TIME AFTER T_3 INJECTION
(hrs)

THE RESPONSE OF RAT THYMUS NUCLEI THYROID HORMONES

Edwin W. Ruark

Abstract

The effect of triiodothyronine ($L\text{-}T_3$) on the protein, RNA and DNA fractions of the thymus nuclei of growth-arrested sulfaguanidine-fed rats has been studied.

A single dose of 15 μg of $L\text{-}T_3$ stimulated the incorporation of ^{14}C-labelled amino acids into total nuclear protein, whole histone and histone fractions. The incorporation of the labelled amino acid into nuclear protein was increased 40 percent over the control 4 hours after the administration of $L\text{-}T_3$. The incorporation of alanine-1-^{14}C into the total histone fraction reached a maximum 4 hours after the administration of $L\text{-}T_3$ and then decreased 8 and 16 hours after the administration of $L\text{-}T_3$. The incorporation of alanine-1-^{14}C into histone fraction f_1 reached a maximum at 4 hours after the administration of $L\text{-}T_3$ while the incorporation into fractions f_{2a}, f_{2b} and f_3 did not reach a maximum until 8 hours after the administration of $L\text{-}T_3$.

Time course studies showed that the protein and RNA moieties of thymus chromatin of rats injected with a single dose of $L\text{-}T_3$ was increased to a maximum at 4 hours. The T_m of chromatin was increased 2^o by $L\text{-}T_3$ administration as early as 2 hours. However, the T_m of DNA was not affected.

Four hours after the administration of L-T_3 the template efficiency increased 171 percent. The template efficiency of DNA wasnot affected by L-T_3.

These results demonstrate that thyroid hormones modify the chromatin in such a way that the template efficiency is increased. An hypothesis for the mechanism of thyroid hormone action was presented.