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EFFECT OF THYROID HORMONE ON POLYPEPTIDE CHAIN ASSEMBLY KINETICS IN ADULT AND FETAL LIVER PROTEIN SYNTHESIS *IN VIVO*

by

RITA W. MATHEWS

A dissertation submitted to the Graduate Faculty in Biology in partial fulfillment of the requirements for the degree of Doctor of Philosophy, The City University of New York

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Chairman of Examining Committee
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Abstract

EFFECT OF THYROID HORMONE ON POLYPEPTIDE CHAIN ASSEMBLY KINETICS IN ADULT AND FETAL LIVER PROTEIN SYNTHESIS *IN VIVO*

by

Rita W. Mathews

Advisor: Professor Audrey E.V. Haschemeyer

The time constant for assembly and release of polypeptide chains in the process of protein synthesis in fetal and adult rat liver *in vivo* has been determined by a new technique which is independent of most sources of variation and error in amino acid incorporation experiments. In this method the ratio of incorporation of ^{14}C-amino acids into completed soluble proteins to that in total protein (including ribosome-bound chains) is measured as a function of short incubation periods (15 to 105 s) after hepatic portal vein injection in the adult or after umbilical vein injection in the fetus to obtain t_c, the average polypeptide chain assembly time. For normal male Long-Evans rats, ages 2 to 2½ months, t_c in liver *in vivo* is 1.16 ± 0.16 min. Induction of hyperthyroidism by three injections of triiodothyronine (40 μg/100 g body weight) over 72 hours led to a value of t_c = 0.92 ± 0.08 min, representing a 27%

increase in synthetic rate. Surgically thyroidectomized rats showed a markedly longer time for polypeptide chain assembly, t_c = 1.92 ± 0.22 min, corresponding to a 39% depression in protein synthetic output compared with sham operated and normal controls of the same age. Almost complete recovery of protein synthetic rate in thyroidectomized rats was achieved by replacement doses of triiodothyronine (20 µg/100 g body weight) given daily for 2 days prior to assay. The fetal rat at gestational age of 17 to 18 days, the age at which no circulating thyroxine is found in fetal plasma, showed a t_c = 2.28 ± 0.50 min. After the onset of thyroid function at 20 to 22 days, fetal rat liver showed a t_c = 1.63 ± 0.06 min, representing a 29% faster protein synthetic rate than the youngerathreotic fetus. Both of these assembly times are slower than found in normal adult liver.

The results are discussed in relation to the mechanism of control of the protein synthetic pathway, the role of thyroid in development and differentiation, the anabolic action of thyroid hormone, and other methodology which has been used in attempts to estimate protein synthetic capacity.

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I wish to acknowledge the excellent guidance of my advisor, Professor Audrey E.V. Haschemeyer, whose acute scientific mind was a constant inspiration. I wish to thank one whose friendship has sustained me through these last years; and I wish to thank my husband, Sidney, for giving me the opportunity to make it all possible.

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I - INTRODUCTION

Evolution of the thyroid gland - Thyroid hormone is found in all vertebrate phyla while iodoproteins have been detected in both vertebrates and invertebrates. In the invertebrates these iodinated proteins are usually confined to the hard exoskeleton structures or pharyngeal teeth. In these cases, they are probably by-products of what has been called "quinone tanning" (1). Benzoquinones form cross linkages in the molecular structure of the scleroproteins which hardens the cuticle and benzoquinones can iodinate proteins *in vitro* if inorganic iodide is present (2). There is no experimental evidence that these iodinated proteins perform any function in the invertebrates. It would appear that the phylogenetic appearance of a gland or organ capable of concentrating and storing thyroid hormones occurred when the effects of the hormone on metabolism proved of adaptive value.

While the thyroid gland is present in all vertebrates, it is not always paired and encapsulated as it is in the higher mammals. In the most primitive vertebrates, the cyclostomes, there are scattered thyroid follicles embedded in the fibrous tissue in the floor of the pharynx; in the elasmobranches, it is an encapsulated organ lying below the pharynx and located near the point where the afferent branchial arteries leave the ventral aorta; in most teleosts,

the follicles are scattered along the ventral aorta with one exception, the parrot fish; in amphibians and birds, the gland is encapsulated, paired, but widely separated; and in the higher mammals, it is encapsulated, paired and connected by an isthmus that lies across the anterior surface of the trachea at the level of the first pair of pharyngeal pouches of the embryo. It is a derivative of the alimentary tract and first appears as a evagination from the floor of the embryonic pharynx.

The counterpart of the thyroid gland appears in the primitive protochordate, Amphioxus, and since the gland develops from the pharyngeal region in mammals, it is not surprising to find that its possible forerunner, the endostyle, develops from the pharyngeal floor. The endostyle of Amphioxus is open to the pharynx and its mucus-like secretions probably function to trap food particles; however, the endostylar region does contain organically bound iodine and seems to preferentially take up iodine (3) and discharge iodoproteins. The hydrolysis of the iodoprotein to thyroid hormone takes place in the digestive tract.

The connecting link between the endostyle organ of the protochordate and the ductless glands of the higher chordates can be illustrated by the life cycle of the lamprey. The lamprey has a larval stage, the ammocoete, which has an endostyle. However, this endostyle is not open throughout

its length to the pharynx as is the endostyle of Amphioxus, but is closed over until only a small slit-like opening remains, a duct. It discharges through this duct to the pharynx, the iodoprotein being hydrolysed in the intestine. A protease develops prior to metamorphosis in preparation for the ductless thyroid of the adult. When the ammocoete larva metamorphoses, some of the epithelial cells of the endostyle remain and change into the follicles of the thyroid gland. Although the endostyle of the ammocoete collects iodine and produces iodinated proteins, it does not have follicle cells as a true thyroid gland; rather the endostylar epithelium performs this function. Strangely, thyroid hormone has never been shown to cause this metamorphosis (4) while in amphibians it appears the prime trigger.

While the evolution of the gland itself seems straightforward and reasonable, the evolution of the understanding of its functions has proved more elusive. Thyroid hormone has been implicated in many biological phenomena such as calorigenesis, electrolytes and water balance, protein metabolism, fat metabolism, carbohydrate metabolism, reproductive ability, skeletal growth, brain maturation, metamorphosis of amphibian larvae and of teleost fishes, cardiovascular changes, basal metabolic rate and oxygen consumption, hair replacement and molting, thermoregulation, and so forth. Most of these parameters show species differences which further cloud the picture. Although many reports have

appeared on the supposed mechanism of action of thyroid hormone, the literature is contradictory and difficult to evaluate quantitatively. The thyroid gland in humans was first described by Thomas Wharton (5) in 1656, who also gave it its name which means oblong shield. However, he suggested that it was there to "round out and beautify the neck by filling the vacant spaces about the larynx ... particularly in females to whom for this reason a larger gland has been assigned" (6). Although the relationship between the gland and its function was studied in the early 19th century by thyroidectomy in various animals, the subjects usually died of tetany and it was not until 1896 that the separate causes of tetany and hypothyroidism were realized.

In 1896 Bauman showed that the thyroid gland was rich in iodine (7). There had been, previously, some indication that thyroid function was tied to iodine metabolism. The element had been tried therapeutically for a host of human ills and some success attended its use in early goitrogenic patients, though documentation of results was poor. During the period 1850-1876, Chatin (8) determined the comparative iodine content of air, water, soil, and plants in many parts of Europe. He discovered that in those places with a high incidence of goiter, the iodine intake of the inhabitants was low because of the low content of the element in the food, and he found that in those areas of non goiter, the iodine intake of the inhabitants was higher because the content of the element in their food was high. These results led him to

conclude that the lack of iodine was the principal cause of goiter. This finding, however, was not accepted by Chatin's contemporaries. The idea that a simple element was central to a complex disease was not palatable at that time and had to await further growth of human knowledge.

Bauman (9) also showed that most of the iodine was in a protein moiety which, on hydrolysis, yielded what he called "iodothyrin," a substance which proved effective in relieving myxoedema (a human disease characterized by retention of extra-cellular water, contracted blood volume, and slow mental performance caused by hypothyroidism). It was only a matter of time until better separation methods developed and a crystalline product was obtained by Kendall (10) in late 1914. He called it thyroxine.

Before the advent of radioisotopes, early 20th century thyroid research was mostly based on measurement of basal metabolic rate. This assay gave a quantitative approximation of thyroid secretory activity. Anti-thyroid drugs, discovered in 1943, gave a new impetus to thyroid research, and these coupled with radioisotopes, particularly ^{131}I (11) with which thyroid hormone may be traced, have been used to explore thyroid function up to the present time.

History of research - Because there is hardly an organ or tissue that is not affected by thyroid hormone at some stage during an organism's life, it is difficult to pinpoint a simple

mechanism of action. A large literature has developed on the subject, and the following will be limited to those areas most relevant to the present study including development, differentiation, anabolic actions of thyroid hormone, and its relation to the mechanism of control of the protein synthetic pathway.

Most of the biological functions that appear to be regulated by thyroxine show a characteristic lag period, such as amphibian metamorphosis in which thyroxine is obligatory, or increased oxygen consumption that can be evoked by exogenous thyroxine. The lag is also observed in more subtle changes that occur during growth and maturation or in metabolic changes that depend on changing enzyme levels. These observations suggested that synthetic events might be involved in the expression of the hormonal function.

It has long been realized that thyroid hormone profoundly influences development. Study of its role can be dated back to Paracelsus (12) in the early 16th century. In the last 25 years investigations have been carried out on two levels: 1) the observation of gross morphological changes occurring upon ablation of the thyroid gland and the restoration, in most cases, of more normal appearance with therapeutic replacement doses of thyroid hormone, and 2) more recently, since the advent of isotopes and more sophisticated techniques, at the molecular level.

One of the outstanding investigators at the morphological level is R.O. Scow, who made careful and accurate studies in

the late 40's and 50's. By this time it had been established that normal growth is dependent on secretions from both the thyroid and pituitary glands. Scow (13, 14, 15) attempted to elucidate the changes in specific terms, not only of body weight but of skeletal size, sexual maturation, transformation from infantile to adult hair, degree of bone ossification, time course of treatment, histologic changes correlated with X-rays of bone age, muscle growth, and collagen content of both muscle and skin. He obtained clear evidence that 1) thyroidectomy at birth impairs skeletal maturation resulting in a "bone age" of 18 days in a 60 day old thyroidectomized rat; 2) thyroidectomy impairs skeletal muscle growth which results in a subnormal muscle-body weight ratio (the % body weight of the thigh muscle in a normal rat is 4 and in the thyroidectomized rat it is 2); 3) thyroidectomy causes the skin to be very thin, the hair infantile with collagen content and protein below normal, however collagen, protein, and water content of muscle are normal; 4) no change in the maturation of gonads was observed; and 5) epiphyseal ossification centers of skeletal structures (tibia, metacarpals, and caudal vertebrae were examined) are small. In 18 day normal rats the ossification center occupied 25% of the cartilaginous epiphysis, expanding as the animal develops to become 71% of the epiphysis by 61 days. In the thyroidectomized rat at 61 days it occupied only 40% of the epiphysis. Scow examined the effects of growth hormone (STH) on thyroidectomized and

hypohysectomized animals either by itself or with thyroxine. In all cases growth was promoted by growth hormone but thyroxine had an important modulating influence. He concluded that (14) "thyroidectomy markedly retarded growth and differentiation, that growth hormone stimulated growth without differentiation, and that thyroxine stimulated both growth and differentiation."

It was not until the International Conference on Hormones in Development, held in England in September 1968, when far more sophisticated methods had been employed for many years, that Max Hamburgh suggested that normal levels of thyroid hormone may influence development (he was speaking of the central nervous system at the time) by turning off the proliferative phase so that the differentiative phase can begin (16).

Severe thyroid deficiency in early development produces a syndrome known as cretinism, characterized by dwarfism, impaired reflexes, mental retardation and edema. Since a manifestation of cretinism is reduced learning ability, much early work, especially during the 50's and 60's, was done on brain maturation. Hamburgh (17) investigated the state of maturation of both skeletal and nervous systems in newborn and young postnatal rats made hypothyroid by prolonged administration of propylthiouracil (PTU). PTU was fed to the pregnant rat during the last week of gestation and continued until weaning and thereafter fed to the young postnatals. The criterion used to compare the state of neural

maturation in the hypothyroid animals and the normals was the rate of oxygen consumption of the cerebrum and the cerebellum. The criterion for skeletal maturation was the degree of ossification attained at birth of axial and appendicular skeletal structures. Hamburgh also investigated the emergence of the thermoregulatory mechanism; it had been known since 1952 (18) that severely hypothyroid animals die when the temperature is dropped from 26°C to 16°C. Hamburgh's results showed that lack of thyroid hormone in fetal life had no effect on the oxygen consumption of brain tissue at birth nor on the state of ossification at that time. However, there was a marked delay in the emergence of adult cerebellar architecture with the superficial fetal external granular layer being retained as late as 26 to 28 days and continuing to proliferate. However, eventually the normal cerebellar form emerged even in severely hypothyroid animals. Oxygen consumption in the cerebral cortex was lower in hypothyroid animals on day 15 and day 20 postnatally, but when PTU administration was delayed until day 10, oxygen consumption was no longer depressed. Maturation of the thermoregulatory mechanism did not take place in those animals hypothyroid from birth to weaning, but developed in those made hypothyroid at 10 days postnatally.

Hamburgh concluded that lack of thyroid hormone during intrauterine period does not appreciably interfere with fetal maturation with respect to those parameters studied (cerebral metabolism and bone ossification). While deprivation of

thyroxine postnatally leads to permanent defects of the cerebral cortex (19), the thermoregulatory mechanism, and learning ability (20), it merely retards maturation of the cerebellar cortex and the completion of skeletal ossification. The critical period appears to be limited to the first 10 days of post-natal life for PTU treatment for after that time it had little effect on the parameters studied.

Other investigators, however, have obtained different results. Geloso et al (21) found that rat fetuses made hypothyroid by decapitation on day 16 (in utero), showed significant differences from their littermates: at day 21 1) the laminae of the periosteum are thickened, 2) the trabeculae of the endochondrial bones are wider and more anastomosed, 3) cartilage remnants are more marked, and 4) the appearance of the medullary cavity is delayed, the center of diaphysis being still filled with primary bony tissue, and the number of osteoclasts is decreased by about 20%. It would appear from this that the role of thyroid hormone in the role of skeletal development in rat fetuses is confined to differentiation of pre-existent bone. However, fetal decapitation, even by the tightened suture method used in these experiments, must be traumatic for it deprives the fetus of any developing hypothalamic-hypophyseal control over the endocrine glands while at the same time it deprives the fetus of growth hormone which is hypophyseal in origin.

These studies described above support the view that

thyroid hormone may play a major role in differentiation. The idea of turning off a proliferative phase and triggering a differentiative one has been approached in many ways. The strong analogy between amphibian metamorphosis and mammalian birth provides additional information on this. Thyroid hormone is obligate for amphibian metamorphosis and for the biochemical changes that come about during this change. The hormone can even trigger metamorphosis prematurely. Although the tadpole has a functional thyroid gland prior to metamorphosis and a functioning hypothalamic-pituitary-axis, thyroxine secretion is kept in check by a minimally active hypothalamus. At the same time low levels of prolactin inhibiting factor lead to high levels of prolactin. Prolactin has been shown to stimulate growth in normal tadpoles and to be antagonistic to thyroxine (22). The control of metamorphosis in anuran tadpoles appears to be under hypothalamic control and is related to the morphological differentiation of the hypothalamus.

The rat fetus, on the other hand, does not have a thyroid gland that secretes thyroid hormone until 18½ days of the gestational period. The fetal rat does have iodoproteins circulating in plasma. Jost (23) isolated an iodinated protein prior to the 18th day of gestation which liberated mono-iodotyrosine after hydrolysis and the amount was a function of the diet of the dam. Geloso (24) pinpointed the onset of thyroid function in rats in a study of thyroidectomized pregnant rats. Radioiodine uptake in fetal thyroids was determined after injection of 150-300 μCi of ^{131}I to the mother 24 hours

before fetal samples were taken. The ratio of the iodide in the thyroid to the iodide in the plasma and in muscle tissue was then measured. In the absence of specific accumulation of ^{131}I by the thyroid gland the ratios between the thyroid gland and the plasma and between the thyroid gland and muscle tissue should be one. The mean ratio from repeated samplings was one prior to the 17th day of gestation. At 17½ days the mean ratio was 12. The 16-day thyroid showed no measurable radioactivity in an acid precipitable protein fraction, but at 17½ days the greater part of the radioactivity (80-84%) was in a protein bound form. It was not possible to detect thyroxine in the plasma of any of the litters of thyroidectomized mothers before 18½ days. It would appear, therefore, that the fetal thyroid starts to take up iodine and bind it organically about the 17th day, while release does not occur until at least 24 hours later.

The question of whether thyroid hormone crosses the placental barrier from mother to fetus has been unclear. The differences in results from one investigator to another appear to be a function of gestational age and dose size. At early stages of gestation plasma PBI (protein bound iodine) and free thyroxine in the fetus are too low for reproducible assay, regardless of the hormone status of the mother (25). When massive doses of thyroxine (100 µg) are injected into the dam some passage of hormone into the late fetus has been reported (26). A more careful study by Geloso (27), however, contradicts this finding. Female rats were given ^{127}I in their food and drinking

water beginning one month prior to breeding. At 17 days of gestation two groups were set up: 1) normal mothers with both normal and thyroidectomized fetuses; and 2) thyroidectomized mothers with normal and thyroidectomized fetuses. Thyroidectomy lowered maternal plasma BEI (butanol extractable iodine) to one-tenth of that of normals after 4 days. Assay of fetal BEI at this time (21st day of gestation) showed no difference between intact fetuses of normal or thyroidectomized mothers. Similarly thyroidectomized fetuses showed BEI values independent of hormonal status of the mother, but only half of that of intact fetuses. It was concluded that the rat fetus does not receive maternal thyroid hormone, and the hormone appears in fetal circulation only when the fetal thyroid is capable of hormone synthesis.

The appearance of thyroid hormone in fetal plasma has been found to be correlated with major changes in the enzyme pattern of the fetal liver (28). This change in liver enzymes also occurs in the premetamorphic tadpole. Clearly, in the transitions of birth in mammals and metamorphosis in amphibia, some systems must change radically. Both organisms must move to an aerobic medium, must change to the metabolism of a different food source, and eliminate nitrogenous wastes differently. The move to aerobic media is also accompanied by greater energy demand which increased oxidative phosphorylation must supply. Fetal tissue is known to be more resistant to anoxia

than is adult tissue (29), probably reflecting the greater use of anaerobic glycolysis. This is borne out by the volume distribution of different cellular compartments of the fetal rat liver tissue three days prior to birth as compared to adult tissue. In Table 1 it can be seen that the fetal nuclei occupy eight times more space than do adult nuclei; mitochondria occupy five times less space in fetal liver than in adult liver. The activity of two key enzymes in the control of glycolysis (phosphofructokinase and pyruvate kinase) is greater in fetal than in adult rat liver. Fetal liver pyruvate kinase is entirely of the M type and cannot be activated by fructose diphosphate. This implies the existence of only one control point in glycolysis and maintenance of a more constant glycolytic flux. Since there is a much lower concentration of mitochondria, the conclusion that fetal liver tissue is better adapted than adult liver to anaerobic conditions is inescapable. The abrupt change to air would necessitate some change in this pattern.

While a direct comparison with the enzyme changes that occur in tadpoles as a result of thyroxine-induced metamorphosis is not possible because, with the exception of the urea cycle, different enzymes have been examined in the two cases, there are many parallels. In the tadpole thyroxine increases the oxygen consumption and provokes a rise in malate dehydrogenase (30); in the rat one finds a rise in flavokinase and FAD pyrophosphorylase around the time of birth and a concomitant rise in flavine adenine dinucleotide which is an important

TABLE 1[a]- Comparative volumes of subcellular compartments of adult and fetal rat liver

Compartment	Volume %	
	Adult	Fetal
Nucleus	8.7 ± 4.2	41.3 ± 10.3
Mitochondria	20.7 ± 6.6	4.4 ± 2.7
Cellsap	30.0 ± 3.6	19.2 ± 4.3
Other structures by difference	40.6	35.1

(a) Adapted from Vergonet, 1970 (29)

factor in the electron transport chain of oxidative phosphorylation (31). These changes coincide with the beginning of thyroxine appearance in the fetal plasma. Another supporting factor is that in adult rats, hypothyroidism is associated with low levels of flavokinase. More direct dependence on thyroxine is exhibited by NADPH dehydrogenase. Greengard and Dewey (32) have evoked premature rise of this enzyme in fetal liver by thyroxine injection. The normal rise begins at three days prior to birth and peaks at the hour of birth. Thyroxine also increases mitochondrial α-glycerophosphate dehydrogenase activity (21). This enzyme is closely related to basal metabolic rate; thyroxine administration in adults evokes a ten-fold rise in activity which parallels a change in liver slice oxygen consumption (33). While the physiological significance of this change has not been established, it has been suggested that its substrate, α-glycerophosphate, acts as a shuttle for transport of hydrogen ions from cytoplasmic reduced nicotinamide-adenine dinucleotide phosphate (NADPH) to the mitochondrial electron transport chain.

While oxygen consumption at birth increases as energy demands rise, the most necessary factor for survival of the mammalian newborn is a ready supply of glucose. This need is taken care of by the storage of glycogen in fetal liver <u>in utero</u>. A rise in glucose-6-phosphatase activity is begun four days prior to birth, peaking at four days after birth

and then decreasing to the adult level. Greengard has again shown that this enzyme can be prematurely evoked by thyroxine (32). Thyroxine also initiates the accumulation of glycogen in tadpole liver and the concomitent rise in glucose-6-phosphatase activity (33a). This enzyme functions in the formation of glucose from glycogen and provides both newly born mammal and metamorphosing tadpole with a supply of glucose during early postnatal or late metamorphic starvation, the latter associated with transformation of the digestive system to that of the adult.

The shift from aquatic to terrestial habitat necessitates the change from ammonotelism to ureotelism both in the tadpole and the mammalian fetus. As can be seen in the urea cycle the arginase synthetase system leading to arginine and thence to urea is the rate limiting step (34).

enzyme: carbamyl phosphate synthetase

reaction: $HCO_3^- + NH_4 + 2\ ATP \longrightarrow H_2NC(=O)\sim P + 2\ ADP + Pi$

enzyme: ornithine transcarbamylase

reaction: $H_2NC(=O)\sim P + ornithine \longrightarrow citrulline + Pi$

enzyme: arginine synthetase

reaction: $citrulline + aspartate + ATP \xrightarrow{Mg^{++}} arginosuccinate + PPi + AMP$

$arginosuccinate \longrightarrow arginine + fumerase$

enzyme: arginase

reaction: $arginine + H_2O \xrightarrow{Mn^{++}} ornithine + urea$

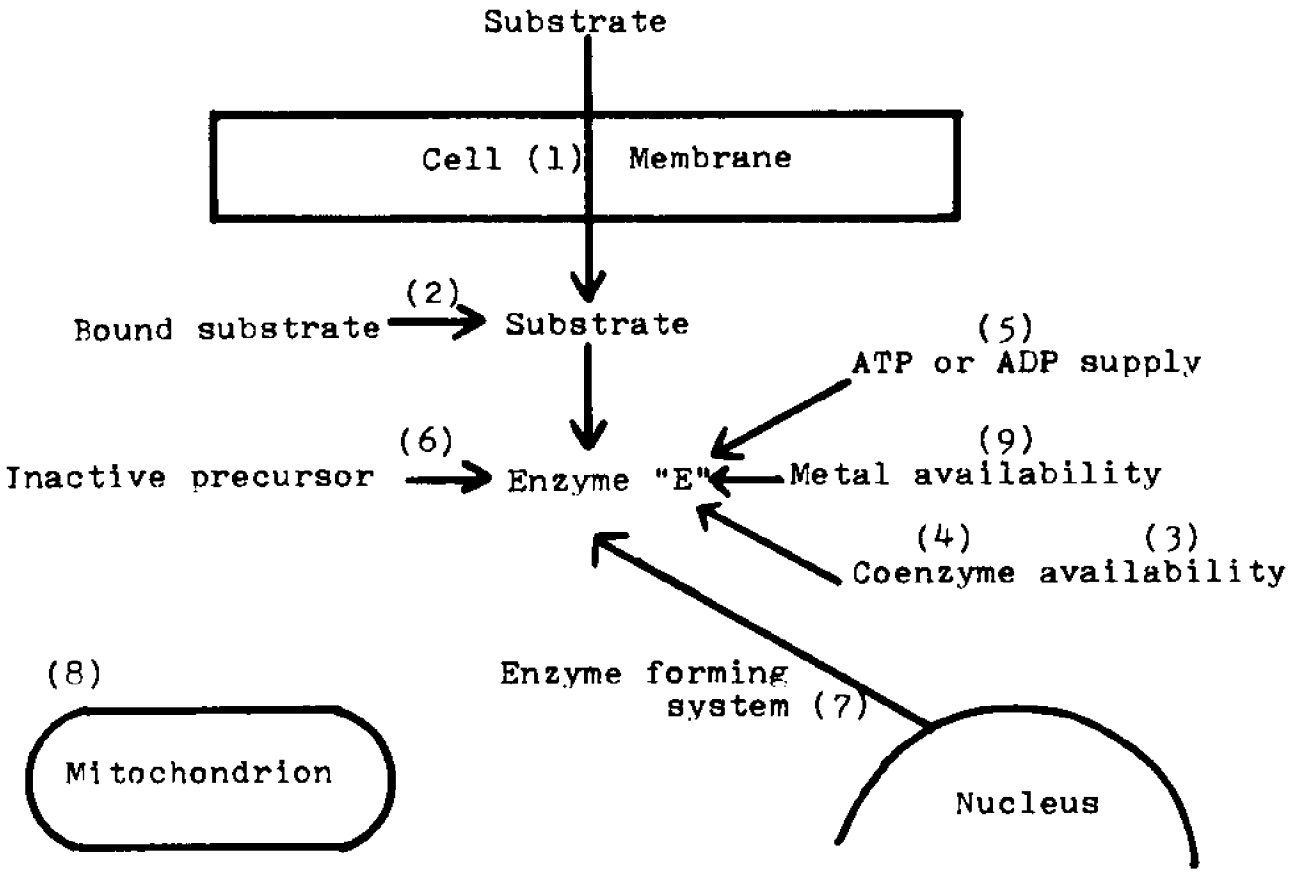
Liver arginase activity during thyroxine induced metamorphosis is even greater than is the normal accumulation showing the direct effect of exogenous hormone. Another enzyme in this cycle, carbamyl phosphate synthetase, was explored by Paik and Cohen (35), who demonstrated increased activity and amount of enzyme concomitant with increased urea production. The time course of these changes demonstrated that biochemical events preceded the morphological responses to exogenous thyroxine.

Similar changes apparently take place in the fetal rat. The fetus has a placental membrane that is closely applied to the maternal blood stream. No difference has been demonstrated in the content of nitrogenous end products in the fetal and maternal serum and these substances probably pass the placenta as NH_4^+ and are metabolized by the mother. Therefore the fetal rat has no need of a urea synthesizing system and therefore has no functional mesonephric kidney nor allantoic cavity (34) which would function as a resevoir for products of mesonephric excretion, but must develop the system prior to need. The activity of the urea cycle enzymes becomes measurable on the 18th day of gestation (34) concomitant with mesonephric kidney function and with plasma thyroxine. Arginase can also be prematurely induced by injection of thyroxine in fetal rat (36). Thyroxine apparently stimulates the formation or activation of these enzymes. New synthesis of these enzymes is likely even though most have usually been quantitated by activity assays. Cytomorphometric measurements of fetal liver

cells show that at the times discussed, parenchymal cells are undergoing a large increase in volume and becoming the principal cell type by mass in liver (44). The enormous change in volume suggests that accumulation of proteins rather than simply changes in activity is occurring. The questions that these studies pose are whether thyroxine is acting directly on the information transfer system (transcription or translation) and whether it acts alone or in a synergistic manner with other hormones.

The earliest ideas of what hormones did at the cellular level was that they modified enzymes directly involved in the observable biological effect. Tepperman and Tepperman (37) considered the problem of primary hormone action at the cellular level by grouping research reports based "on the theory of cellular mechanism of action rather than on hormone identity or classification" (Fig. 1). They assume that "enzyme E represents one or more enzymes which catalyse one or more rate-limiting reactions in a hormone-sensitive cell" and "that cellular activity rises or falls depending on the velocity of the reaction mediated at this step." Nine mechanisms are given for changing reaction velocity; two of these (6 and 7) involve changes in the amount of apoenzyme. These authors recognized the limitations of the <u>in vitro</u> systems when dealing with hormones and warned that striking effects of hormones in such systems could be nothing more than chemical interactions between hormone and a crucial component that might be totally

FIGURE 1[a]- Schematic drawing of possible control points in enzyme regulation.



(a) after Tepperman and Tepperman (37)

irrelevant to *in vivo* action. Thyroxine studies proved particularly difficult to evaluate in relation to what seemed to go on physiologically and they postulated that the effect of thyroxine is collaborative, particularly with epinephrine or norepinephrine. Evidence on the administration of sympatholytic blocking agents that prevented the usual rise in oxygen consumption following thyroxine administration was given.

The multiple effects of thyroxine can be separated into two main categories, that of growth and development and that of metabolic change. In both cases, changes in protein metabolism has been implicated. The primary effects still remain obscure. The advent of the cell free protein synthesizing system made available the chance perhaps to localize the action of thyroid hormone, for it was known as early as 1950 (38) that liver slices of thyrotoxic animals accelerated incorporation of labelled amino acid into protein. Growth requires protein accumulation; enzyme induction and metabolic change could either be caused by increased synthesis or decreased degredation. Lee et al (39) found that rats fed dessicated thyroid glands oxidized α-glycerophosphate up to five times the rate of the controls, this peak being reached at ten days. The increase could be detected as early as twelve hours after thyroid feeding. When thyroid hormone (1 mg thyroxine) was injected subcutaneously, the rate of oxidation was 20% above normal after 24 hours; triiodothyronine (T3) at 0.2 mg elevated the rate to 200% above normal. When

ethionine was given to block protein synthesis thyroid hormone had little effect on the α-glycerophosphate activity and ethionine fed to the control group did not significantly lower enzyme activity. This result implied new synthesis of α-glycerophosphate dehydrogenase (GPD) and not just enhanced activity. Measurement was by reduction of oxidized nicotinamide-adenine dinucleotide (NAD) by GPD from sub-mitochondrial particles and was corroborated by changes in oxygen consumption.

In 1962 Stein and Gross (40) reported a study of amino acid incorporation by normal and thyroidectomized animals. The microsomal system, supplemented with 100,000 x g supernatant and an energy system with 0.05 μmole of ^{14}C leucine, was incubated 40 minutes at 37°C. Preparation from thyroidectomized animals incorporated 85% less protein than that of normal controls. Protein synthetic activity, however, was extremely low compared to later determination of *in vivo* rates (reported in this thesis).

The two laboratories that have done the most *in vitro* work are those of J.R. Tata and L. Sokoloff. No agreement has been obtained on what is the first manifestation of thyroxine administration. Tata (41) proposes that thyroxine acts on the nucleus to produce an increase in ribosomal ribonucleic acid (RNA) which upon migrating to the cytoplasm causes increased cytoplasmic protein synthesis after a 42-hour delay. Tata summarized the effects of a single dose of T3 (15-22 μg/100 g body weight) to thyroidectomized rats as follows: the first

phenomenon seen is an increase in the specific activity of rapidly labeled nuclear RNA beginning at 3-4 hours after hormone administration and reaching a peak 300% elevation at 16 hours. At the same time a stimulation in magnesium activated RNA polymerase (that polymerase that transcribes ribosomes) is detected with activity continuing to rise until 42 hours. A 50-70% increase in the amount of cytoplasmic rRNA occurs at 20 to 30 hours. According to Tata, ribosomal content of thyroidectomized animals is 40 to 50% lower than in normals. Increased ribosomal content was not noted in normal animals given triiodothyronine. No change in the content of nuclear RNA was observed. These results suffer from serious statistical uncertainty; only two animals were averaged for each determination. A 150% rise in protein synthetic activity of microsomal preparations was reported. No effect on RNP protein synthesis stimulated by a synthetic messenger (Poly U) was found (RNP being that fraction that sediments through 60% sucrose solutions; according to Tata this is the membrane-bound portion of the ribosomes). This was interpreted in terms of newly formed messenger RNA and stronger binding of RNP particles to membrane in hormone treated animals. Activity of these preparations was poor, and little characterization of the <u>in vitro</u> system (time and temperature dependence, optimal concentrations of components, etc.) was reported.

Sokoloff (42) has proposed a cytoplasmic mechanism involving a mitochondria-dependent thyroid hormone stimulation of ribosomal protein synthesis that is independent of any new RNA synthesis. This effect has essentially no latent period and occurs before changes in nuclear RNA metabolism. An additional delayed effect was associated with a rise in the RNA content of the microsomes. This mechanism is based on studies of low level amino acid incorporation by crude cell-free systems from liver. A significant rise in the "rate of amino acid incorporation into microsomal protein" was claimed to be measurable in vitro two hours after a single dose of T3 (60 µg/100 g body weight). Mitochondria were required in the assay system for observation of the effect. Despite the claim of a rate change no time dependence in the assay was reported, the incubation time for all preparation being 25 minutes. A direct effect of thyroid hormone on amino acid incorporation in vitro was also reported. Incorporation was elevated by addition of T3 in the presence of mitochondria. Without mitochondria, preparations showed no increase in incorporation until the latent period (after in vivo hormone injection) is over and a rise in RNA content is noted. None of these papers that make up the bulk of the work that has been done on the action of thyroxine on protein synthesis has dealt with the actual rate of protein synthesis in vivo. This would have to be based on a system that took into account

the determination of a linear relationship between incorporation levels and reaction time. Nor has any careful evaluation of the validity of crude cell-free systems for assessment of protein synthetic activity been done.

Fetal liver protein synthesis - Growth of fetal liver presents a very different aspect than that found in adult tissue. Adult tissue shows an approximate balance between synthesis and degradation with a resultant slow (or zero) rate of growth, depending on the species. In contrast, the fetal liver is growing rapidly and protein is accumulating at all times. Winick and Noble (43) showed these quantitative changes in the DNA, RNA and protein of fetal rat liver. Beginning at ten days after conception and continuing well into the postnatal period, liver growth is characterized by rapid cell division and an increase in total DNA. Total organ protein increases until maturity; during early prenatal growth it is proportional to the increase in DNA. In late fetal life protein increases more rapidly than DNA, during the period of enormous growth in parenchymal cell size (from a cell volume of $1.370\ \mu^3 \times 10^{-3}$ at 15.2 days of gestation to $7.01\ \mu^3 \times 10^{-3}$ at 21.6 days) (44). Despite the obvious developmental significance in studies of adult and fetal tissue and the important similarities between fetal and neoplastic tissue, careful search of the literature reveals few papers concerned with fetal protein synthesis. The earliest paper on the subject is by Zamecnik (45) who was

investigating peptide bond synthesis in normal and neoplastic rat liver tissue and included fetal liver because of its great growth potential. The incorporation was done with liver slices incubated in a Krebs-Ringer-Phosphate medium for 2-3½ hours in presence of 10,000 cpms of ^{14}C labeled DL-alanine. The paper reports the total incorporation on ^{14}C into a $BaCl_2$ precipitate of the homogenized liver. It shows that pooled slices from 15 day old fetuses incorporated 179 cpms while adult slices incorporated 38 cpms.

Another early paper that deals with embryonic amino acid incorporation in comparison to adult was that of Burraston and Pollak in 1961 (46). The incorporation studies were done on liver slices perfused in a medium containing radioactive amino acids. Fetal slices were incubated 15 minutes; in order to get a measurable amount of incorporation, adult slices were incubated 60 minutes. The results show that fetal liver incorporated more radioactivity (cpm/mg protein) than the adult tissue in the four fractions studied. While overall incorporation is greater in fetal tissue, this may not reflect changed rates of synthesis but rather slower turnover in growing tissue.

The only other papers that are concerned with "rates" of fetal protein synthesis are two by Grahn (47, 48) dealing with the role of histamine in 18-20 day fetal rats and in adults. Amino acid incorporation of fetal tissue in minced liver preparations greatly exceeded that of adult tissue. The time course of incorporation for fetal minced liver and in fetal

isolated hepatocytes at long incubation times (an hour or more) was reported. Values at these times may reflect accumulation of radioactive protein rather than protein synthetic rate. No correlation between the high rate of histamine formation in fetal liver and the protein synthesis, measured in this way, was found.

Present work - The purpose of this thesis is to clarify changes in protein synthetic rates associated with thyroid hormone in adults *in vivo* and in fetal rats *in vivo*. The adult was studied in artificially induced hyperthyroidism (by triiodothyronine injection) in relation to normal, sham-operated, and hormone-supplemented thyroidectomized animals. The fetal rat was investigated in its natural athreotic state at 17 days of gestation and at 21 days, after onset of fetal thyroid function. In order to carry out these studies, a rapid kinetic method has been developed for the measurement of polypeptide chain synthesis time in liver *in vivo* (49). Because the method uses individually normalized data, it is possible to obtain reliable kinetics even though each time point is provided by a different animal. The method was first used to study environmentally induced changes in fish metabolism and has now been adapted for use in the rat and applied to the investigation of thyroid hormone action *in vivo*. Part of this thesis has been published (50).

II - THEORETICAL

There has been considerable need for a theoretical and experimental approach to the determination of protein synthetic rates *in vivo*. MacDonald and Gibbs (51) set up general differential equations for protein synthesis taking into account initiation, termination, and a pattern of non-uniform polymerization rates. The computer-generated results, however, do not lend themselves to evaluation of actual biological data. The authors concluded that "it is unlikely that an accurate complete curve can be obtainable experimentally *in vivo*."

In 1969 Haschemeyer (49) proposed that a simple translational model could be developed by inspection which would yield average polypeptide chain assembly time from data readily obtained in different individual animals. The model has been used to interpret incorporation of radioactive amino acids into toadfish liver fractions as a function of time after hepatic portal vein injection. Those results suggested that the increase in liver protein synthesis produced by cold acclimation is due to a more rapid rate of addition of amino acid residues to the growing polypeptide chains.

In the Haschemeyer model it is assumed that polypeptide chain growth proceeds uniformly [from the amino terminal to the carboxyl terminal as shown by Dintzis (52)] and that ribosomes are evenly distributed on the messenger RNA, as found for reticulocyte polyribosomes by Hunt, Hunter and

Monro (53), and that ribosomes carry chains of varying lengths.

Figure 2 illustrates the reasoning behind the development of the equations presented by Haschemeyer (49) that permit determination of an average polypeptide assembly time in liver from *in vivo* amino acid incorporation experiments. The upper drawing represents a polyribosome engaged in the synthesis of a polypeptide chain of n residues. The ribosomes are evenly distributed along the messenger such that the average chain length per ribosome is n/2 and if chain growth proceeds at a relatively constant rate, as indicated in the hemoglobin studies (52, 53), all chain lengths up to n are equally probable. Now if the following experiment is to be done: At t = 0 an injection of radioactive amino acids is made in such a way as to rapidly establish a specific activity A in the intracellular amino acid pool. It will be assumed that this free amino acid pool is equilibrated with the direct precursor aminoacyl-tRNA pool at a rate which is fast compared with subsequent steps in protein synthesis. (For the study of liver synthetic rates the injection is made into the hepatic portal vein in adults and into the extra-abdominal umbilical vein in the fetus to assure rapid uptake of amino acids by the liver.) If the tissue is homogenized and fractionated after a time t_c that exactly corresponds to the average assembly time for a complete chain of n residues, a distribution of radioactivity as illustrated in the lower part of Figure 2 will be found. All chains present at t = 0 will have been completed with radioactive amino acids and released, yielding

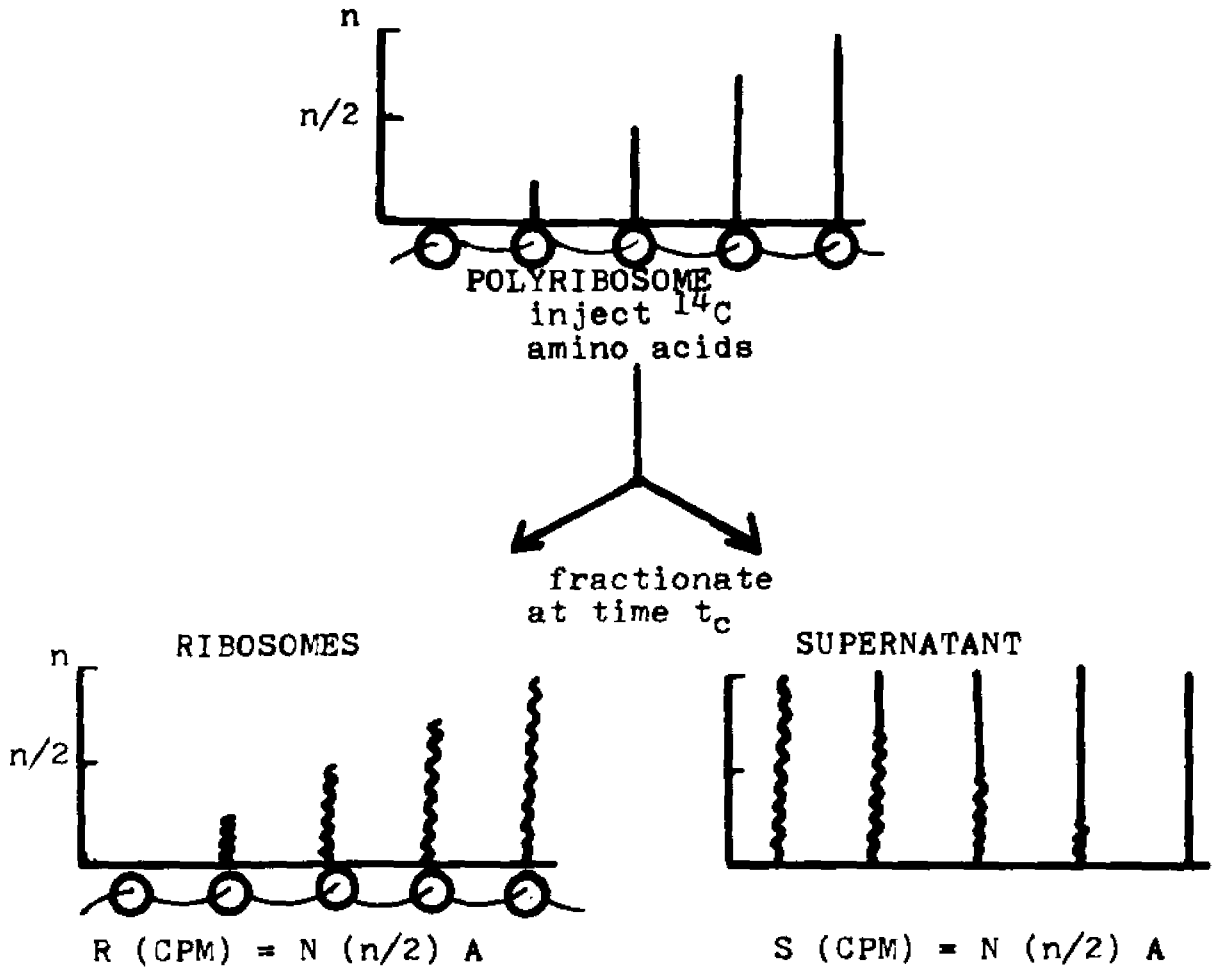


FIGURE 2 - Determination of polypeptide chain assembly time t_c. Schematic drawing to illustrate theory.

a soluble (completed) protein radioactivity S = N(n/2)A, where N refers to the total number of functional ribosomes (in the drawing a single polyribosome of five ribosomes is shown). The ribosome fraction now contains radioactively labeled polypeptide chains of average length (n/2) per ribosome. Thus, at time t_c radioactivity measured in the ribosomal fraction R is also N(n/2)A. Total incorporation T in the system is the sum of the two fractions (ribosomes + completed protein) and is simply NnA. The fraction S/T is therefore 0.5 at time t_c, this result being independent of ribosome number and pool specific activity. The same reasoning can be used to determine the complete time dependence of S/T in units of t_c. The resulting theoretical curve is shown in Figure 3. The equations for S/T are:

$$S/T = t/2t_c \quad \text{for } 0 \leq t \leq t_c$$

and

$$S/T = 1 - t_c/2t \quad \text{for } t \geq t_c$$

If all the proteins synthesized are assumed to have a similar content of the radioactive amino acids measured in the S and T fractions, then the resulting t_c will be a weight average over chains of different lengths.

The theory described above permits the determination of chain assembly rates from <u>in vivo</u> incorporation data, independent of the variable A and the unknown quantities N and <u>n</u>. The important feature of the model is that S/T is linear from t = 0 until the time that one round of synthesis

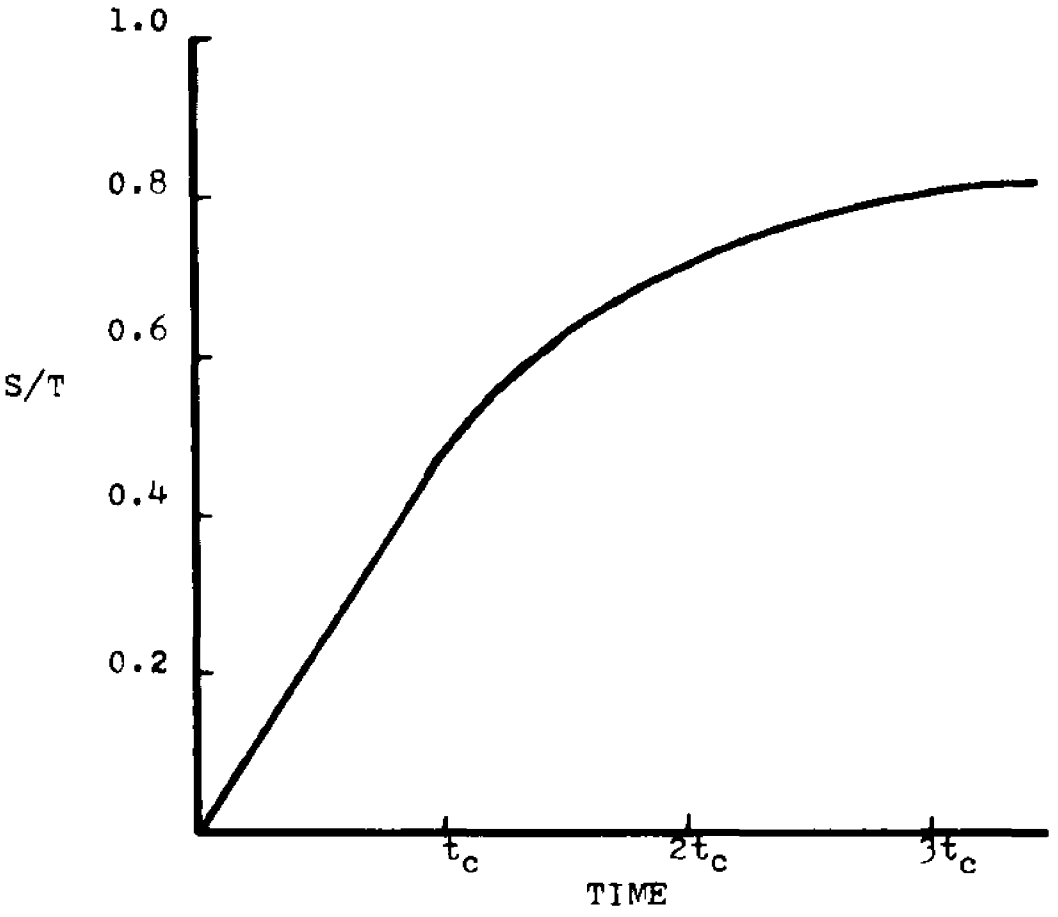


FIGURE 3 - Theoretical curve for the dependence of S/T on incubation time in units of t_c (the average assembly time for polypeptide chains) determined for the proposed model in which addition of amino acid residues is rate-limiting.

has been completed and thus t_c, reflecting chain elongation and release time, is obtained. Chain initiation rate does not influence this result, but will affect total protein synthesis (absolute values of S and T).

Clearly values of T can readily be determined from the total liver incorporation; determination of S depends on a suitable fractionation scheme (described in Experimental). It will be shown that data obtained in this way with the hepatic portal vein injection route in adults and the umbilical vein in fetuses can be fitted to the theoretical curve for S/T to yield values of t_c for various physiological states.

III - EXPERIMENTAL

Animals - For the adult experiments, male Long-Evans rats, bred from stock kept at Hunter College, were used throughout. Thyroidectomized rats were fed an iodine-deficient diet, TD-70283 (General Biochemicals); cages were warmed at one end with an electric heater. All others received Purina laboratory chow and were maintained at normal building temperature and outdoor light cycles (Fall 1971 to Spring 1972). Tap water was given ad libitum. Experimental animals were matched with controls either from the same litter or from other litters born within two days. Animals were sacrificed at 10:00 to 11:00 A.M. after overnight fasting.

Fetal rats - Five female Long-Evans rats of the same stock as the males were placed at 100 days of age in cages with two adult male rats. After suitable intervals (17-22 days of gestation) the pregnant females were anesthetized and a small incision made through the abdominal wall to reveal the uterine horns and to give access to the fetuses. Fetal age was determined by crown-rump length according to the standard curve established by Gonzalez (54).

Thyroparathyroidectomy - Thyroparathyroidectomy was performed under ether anesthesia at 30 days of age as follows. A ventral midline incision is made over the thyroid area and the sub-maxillary glands are retracted to the sides. The sternohyoid

muscle is parted in the midline by two micro forceps and retracted by means of two 3-pronged skin retractors to expose the thyroid gland. The recurrent nerve must be located in order to avoid damaging it. The superior and inferior thyroid arteries are visualized by lifting the edges of the thyroid gland with a micro probe. Cautery is applied to each artery by passing a low voltage over a resistance wire (nicron) from a 6 volt filament transformer. After cauterization, the thyroid is gently released from its attachments at one lobe and then the other until only the isthmus remains attached. The gland can then be lifted off in its entirety by peeling from one lobe across the isthmus to the other. Only the exposed trachea and the recurrent nerve remain in the vicinity. The incision was closed with clips. One group of control animals received sham operations consisting of incision, retraction of muscle, and closure.

3,3', 5 -L-Triiodothyronine (T3) obtained from Calbiochem was dissolved in a minimal volume of 0.05 N NaOH (approximately 0.2 ml per mg) and diluted to 0.5 mg per ml with 0.9% NaCl solution. T3 treated normal rats received a subcutaneous injection of T3 (40 µg/100 g of body weight) at 10:00 A.M. daily for three days prior to the day of the experiment. Thyroidectomized rats on replacement therapy received 20 µg/100 g body weight daily for two days prior to use, a dose suitable to restore enzyme levels to the euthyroid state (55). Controls for this group received NaCl injections at the same times.

Serum thyroid hormone levels were determined using the T4 by column test (Bio-Rad Laboratories) as a criterion of thyroid status (Figure 4). Effectiveness of thyroidectomy was tested by uptake of $Na^{131}I$ (25 μCi/100 g body weight given by intraperitoneal injection). Radioactivity over the regions of thyroid, adbomen, and bladder was determined at times from 3 hours to 3 days after injection using a Picker 1 inch x 1 inch scintillation detector and Cliniscaler adjusted for optimal response to ^{131}I (300 k.e.v.) (Table 3).

Determination of polypeptide chain assembly time in liver in the adult rats was carried out by a modification of a former procedure (49). Under ether anesthesia, the abdomen of the rat was opened by a 6 cm incision along the ventral midline. A hemostat was clamped across the midline just below the sternum to control bleeding, after which a transverse incision of about 2 cm was made. The hepatic portal vein was exposed to view by gently shifting the intestines within the abdominal cavity, and an injection of 5 μCi of L-U-^{14}C-amino acid mixture of L-[U^{14}C] leucine (New England Nuclear) in 0.1 ml of buffered NaCl solution of the adults was made over a period of 10 ± 2 sec, using a 28 gauge needle. The needle was kept in the vein to prevent bleeding. After the desired period for incorporation, ranging from 15 to 105 sec, the liver was quickly excised and homogenzied in 10 ml cold medium A (0.25 M sucrose, 0.05 M Tris, pH 7.4, 0.025 M KCl,

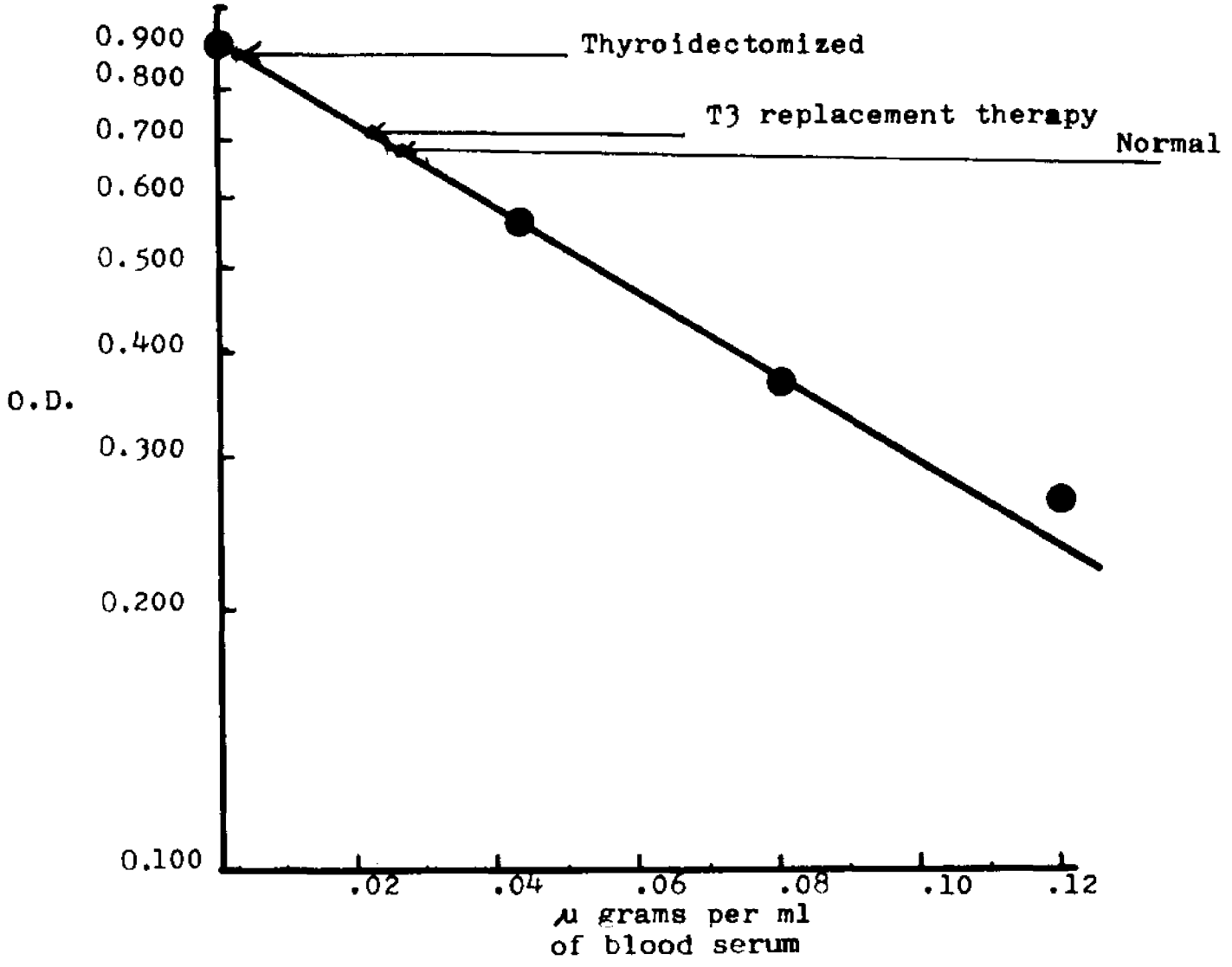


FIGURE 4 - T^4 by column test. Wavelength is set to read blank at .900 O.D. units. Quantitated by ceric-arsenious oxidation-reduction reaction in presence of iodide.

TABLE 2 - Radioactivity (in cpms) over the thyroid, abdomen, and bladder regions in thyroidectomized and normal male rat from 3 hours to 3 days after injection of 37.5 uCi of ^{131}I intraperaneally.

3 hours after	Normal	Thyroidectomized
thyroid region	2540	730
abdominal region	2840	3000
bladder region	920	770
24 hours after		
thyroid region	5450	450
abdominal region	700	699
bladder region	747	422
48 hours after		
thyroid region	6140	480
abdominal region	343	718
bladder region	298	471
72 hours after		
thyroid region	5540	384
abdominal region	370	919
bladder region	205	397

0.01 M $MgCl_2$) in a Sorval Omnimixer for 90 sec at half speed. The time elapsed from excision to the beginning of homogenization was 15 sec ± 3 sec. Determination of liver weight and dilution of the homogenate to four times liver weight followed. Homogenization and all subsequent procedures were carried out in the cold (about 4°C). In the previously described procedure (49) it was found that significant losses of radioactive microsomal protein occurred in the first centrifugation [for removal of nuclei (800 x g)], and this step was omitted. Membrane dissolution by sodium deoxycholate (at 1.5%) was carried out directly in the total homogenate. The previous step of glygogen separation also was omitted; the amount of glycogen present in the overnight-fasted rats was not sufficient to interfere with ribosome isolation. Homogenates were kept on ice until all the rats in the experiment (usually eight) had been finished. Previous studies on <u>in vitro</u> protein synthesis indicated that no further synthesis would occur under these conditions. The total homogenate (T), after addition of sodium deoxycholate, was centrifuged for 3 hours at 100,000 x g in a Spinco Model L2 at 4°C. The supernatant (S) was drawn off. The clear ribosome pellets were freed of the upper layers of sediment (cell debris) by thorough rinsing with cold medium A and suspended in about 1.5 ml of the medium to obtain fraction R. (Fraction R incorporation + fraction S incorporation equaled total incorporation (T), demonstrating that the sediment contained no newly synthesized polypeptide chains.) All fractions (T,S,R) were analysed for ^{14}C incorporation into protein by a filter paper disc method (56). Aliquots of 100 µl were

used, and the discs were washed first in cold 10% trichloroacetic acid (TCA) (two times), then in hot 10% TCA (90°C) to remove any radioactive amino acids from tRNA, four times in cold perchloric acid (5%), then in cold 95% ethanol, and finally in ether. Free radioactivity (FR), i.e., acid soluble, in the homogenates was determined by removing a 0.5 ml aliquot from the total homogenate and precipitating this with an equal amount of 10% TCA. The resulting precipitate was spun down and an aliquot of the clear supernatant (100 µl) was counted in 10 ml Brays solution. Aliquots of the total homogenate were analyzed for protein and total nucleic acids with a value of 30 for A_{260} of 1 mg/ml acid hydrolysed nucleic acids. DNA was determined with diphenylamine (57). Counting efficiencies were 47% for ^{14}C on filter paper discs in toluene based scintillation fluid and 43% for free radioactivity determination in Brays solution, using a Packard scintillation spectrometer. Relative activity due to self-absorbtion by protein was 0.90 for T and R fractions and 0.95 for S fractions. Statistical analysis was done by standard $\underline{t}$ test.

Determination of polypeptide chain assembly time in the fetal liver was done in much the same manner with a few modifications necessary to the fetal system. Under ether anesthesia, a small incision (2 cm) is made in the abdominal wall of the pregnant female. The pregnant female was placed in a 37°C physiological saline bath (head elevated and in an

ether cone) so that the water level came to within a centimeter of the incision. One uterine horn at a time is externalized, the right one first, to determine which fetus lies nearest the ovary. The first fetus used is the one that lies next to the right ovary and henceforth will be called #1. The rest of the uterine horn is replaced in the abdominal cavity so as to create the least possible disturbance to the remaining fetuses until their turn for the experiment. The uterine wall is opened with an iris scissors and fetus #1 is carefully extruded and immediately wrapped in gauze. Part of the gauze is kept dipped in the surrounding water bath so that there is no temperature change in the fetus while it is being worked on. The amniotic sac is pierced at the level of the umbilical cord, but the sac is not removed. The extra abdominal umbilical vein is easily visualized by the color of the oxygenated blood. The vein is gently lifted with a micro forceps and a 32 gauge needle is inserted and 0.025 ml of ^{14}C leucine solution is injected over a period of 10 sec. The solution can be seen to flow into the cord and thence into the liver, the fetal skin being transparent. The needle is removed and the cord is gently massaged to maintain blood flow. Bleeding is minimal. After the proper incubation time, ranging from 15 sec to 60 sec, the fetus is removed and the liver quickly excised and placed in a Dounce homogenizer with 0.4 ml cold medium A. Time elapsed from excision to first stroke of the pestle was 20 - 30 sec $\pm$ 3 sec. Two strokes of the A pestle (loose fitting) is sufficient to homogenize the liver and

terminate synthetic activity. The next fetus is extruded and the operation repeated. When all the fetuses from one horn have been used, the left horn is externalized and the fetus closest to the left ovary is used. All preparations are kept on ice during the entire operation. After the last fetus is used, the dam is sacrificed by etherization.

The fetal homogenates were weighed and liver weight determined, followed by dilution to four times the wet weight of the liver. Depending on the fetal size, the final homogenates ranged from 0.4 ml to 1.2 ml. It was found that the large quantity of DNA in fetal liver interfered with sampling for T and S fractions. A method was developed using adult liver of known T and S activity to rid the homogenate of DNA. For this purpose deoxyribonuclease (EC 3. 1. 4.5) was added to the homogenates at the concentration of 1 mg/ml and the homogenate was incubated for 5 minutes on ice. Sodium deoxycholate was then added to a final concentration of 0.5% to achieve membrane dissolution without loss of nascent polypeptide chains from the ribosomes. The concentration required for adult liver (1.5%) caused total release of nascent chains from fetal ribosomes. After addition of deoxycholate, the T sample was taken and the remaining homogenate was centrifuged for 3 hours at 100,000 x g. S and R samples and FR samples were prepared in the same manner as described for the adult. In each fetal experiment the livers of several fetuses were pooled and homogenized

(without deoxyribonuclease) for analysis of protein, total nucleic acids, DNA, RNA, and nuclear RNA. Nuclei were prepared according to a modification of the method of Bresnick et al (58). The fetal livers were homogenized in 2.3 M sucrose containing 3.3. mM $CaCl_2$ (1:12, w/v). The homogenate was then centrifuged at 40,000 x g for 60 minutes. The crude nuclei were washed in 1 M sucrose containing 1 mM $CaCl_2$ and centrifuged at 900 x g for 10 minutes. The nuclear pellet was resuspended in medium A and layered over 1 M sucrose-1 mM $CaCl_2$ and centrifuged at 1000 x g for 10 minutes. This last step was repeated after suspending in 1 M sucrose-1 mM $CaCl_2$. Hydrolysis in 5% TCA was used to determine total nucleic acids; hydrolysis in 5% PCA followed by diphenylamine assay was used to determine DNA content. RNA content was obtained from the difference. The same procedure was used for the adult livers. Counting efficiencies in this experimental series were 59% for ^{14}C on the filter paper discs in toluene based scintillation fluid and 73% for free radioactivity determination in Bray's solution, using a Packard scintillation spectrometer. Relative activity due to self absorbtion by protein was .90 for T and R fractions and .95 for S fractions. Statistical analysis was done by the standard $\underline{t}$ test.

IV - RESULTS - ADULT

It has been previously shown (49) that following a rapid pulse injection of radioactive amino acids, the proportion of the isotope incorporated into total (T) protein which can be recovered as soluble completed protein (S) follows a simple time course which depends upon the average time t_c for polypeptide chain assembly as shown in equation 1 ($S/T = t/2t_c$) (page 31). Measurement of the quantities S and T for a series of time points will yield t_c directly from the slope of a plot of S/T versus t. Although the measured values of S and T for a given t vary widely among individuals, the ratio, which is independent of precursor pool specific activity and of ribosome initiation frequency, has been found to be highly reproducible. For studies in liver, injection via the hepatic portal vein is necessary to achieve a high level of precursor uptake and to establish a realistic zero time point. Uptake is optimized by a short period of starvation prior to the experiment. With standardization of injection and liver preparation times (time of excision to homogenization was 15 sec $\pm$ 3 sec) reproducible experimental data could be obtained for incorporation periods as short as 15 sec. Free radioactivity measurement indicated that liver amino acid pool specific activity was essentially constant throughout the time period studies, as required for use of equation 1.

t_c results for euthyroid, thyroidectomized, hyperthyroid, and thyroxine-replaced animals - Figure 5 presents the averaged data for euthyroid control rats, including untreated, saline-injected and sham-operated groups. The slope of the regression line yielded a value of t_c = 70 sec, when evaluated according to equation 1. This represents the average synthesis time for all protein synthesis in liver measurable within the total experimental time of about 1.5 min. Previous estimates of synthesis time for plasma proteins in rat liver (59) are in accord with this result. The effect of triiodothyronine treatment for 3 days before experiment is illustrated in Figure 6. The hormone dosage schedule was similar to that found to produce two-fold increases in hepatic enzymes sensitive to thyroid hormone levels (55). The results indicate an elevation in protein synthetic rate of 27%, significant at $P < 0.001$.

Animals surgically thyroidectomized at one month of age showed body weight 20% below that of normal litter mates at the time of sacrifice (2-2½ months). Circulating thyroid hormone levels were 0.2 to 0.6 mg/100 compared with 2 to 3 mg/100 for the controls. The ratio of ^{131}I radioactivity over the thyroid region to that over the bladder region was 1.0 ± 0.3 at 3 to 72 hours after radioiodide injection, compared with a ratio of 25 for the unoperated controls as shown in Table 3. The lower line of Figure 7 (designated -T3) presents the protein synthesis data for the thyroidectomized rats.

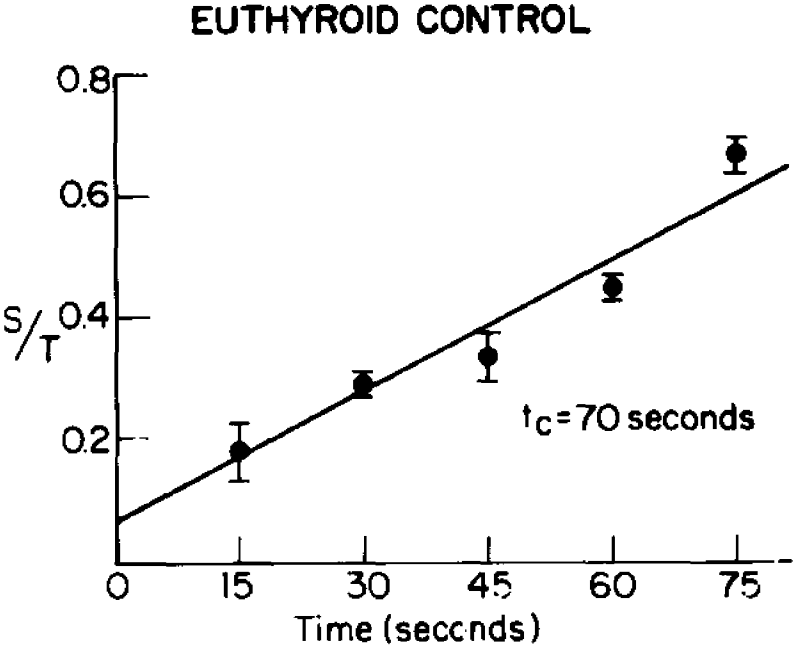


FIGURE 5 - Determination of polypeptide chain assembly time t_c for normal rat liver in vivo. Data for 4 animals per point have been averaged (Long-Evans, male, age 2-2½ months). Vertical bars indicate standard error.

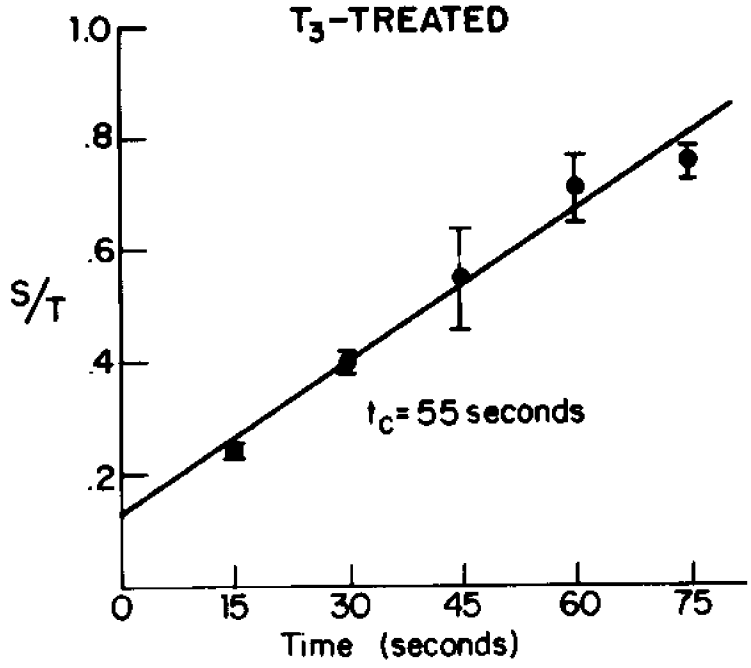


FIGURE 6 - Effect of excess triiodothyronine treatment (40 μg/100 g body weight daily for 3 days) on polypeptide chain assembly rate in rat liver *in vivo*.

TABLE 2 - Radioactivity (in cpms) over the thyroid, abdomen, and bladder regions in thyroidectomized and normal male rat from 3 hours to 3 days after injection of 37.5 μCi of ^{131}I intraperaneally.

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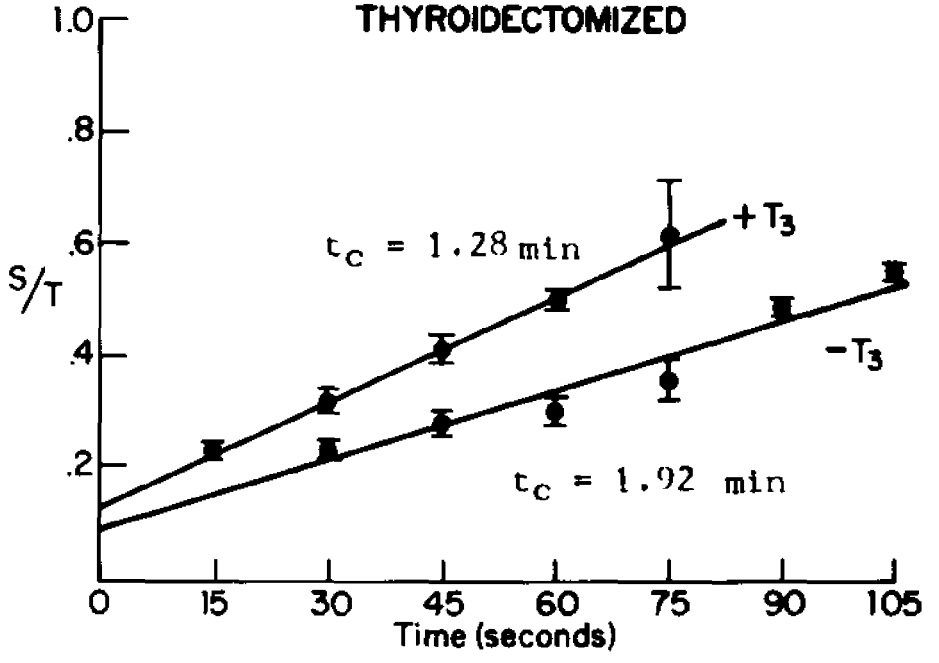


FIGURE 7 - Polypeptide chain assembly rate in surgically thyroidectomized rat with (+T3) and without (-T3) hormone replacement by triiodothyronine injections.

Polypeptide chain assembly time was 1.92 minutes, representing a 39% depression in synthetic rate compared to euthyroid controls ($P < 0.001$). Replacement doses of thyroid hormone led to virtually complete recovery of protein synthetic rate (+T3 curve in Figure 7). Normal values for circulating thyroid hormone were found in this group as can be seen in Figure 4.

The regression lines of Figures 5 to 7 intersect the abscissa in the range of -12 to -20 sec. This additional time of synthesis coincides approximately with the time elapsed during injection and liver excision, which is not included in the incorporation time as plotted.

Figure 8 presents the averaged data for total incorporation of ^{14}C amino acids into liver protein, normalized for each animal by dividing protein bound radioactivity per unit of liver homogenate by measured free radioactivity. Although this presentation of the data eliminates variation due to differences in radioisotope injections, a considerable amount of individual variation remains which does not appear in the S/T ratios. It is very likely related to differences in precursor pool specific activity. The results for total incorporation rate generally confirm the more precise assembly time determination, i.e., hyperthyroid animals show increased incorporation of amino acids into protein as predicted by the shorter assembly time, whereas thyroidectomized animals have a lower incorporation rate consistent with a longer assembly time. Hormone treated thyroparathyroidectomized

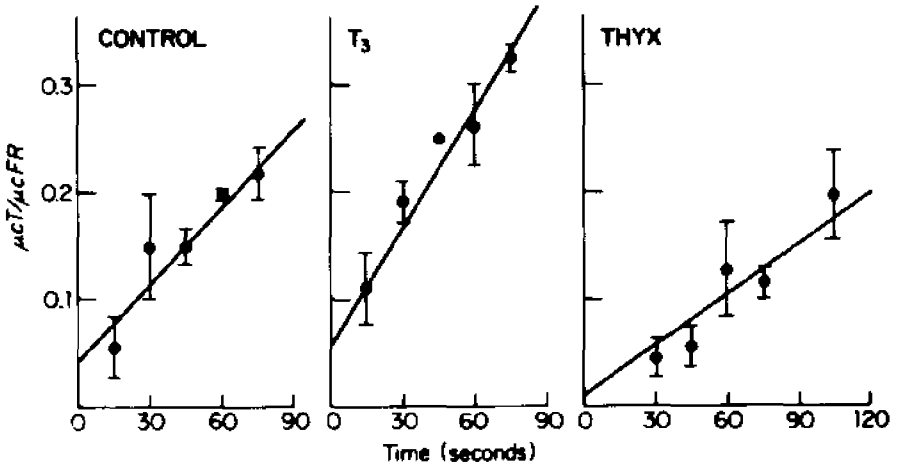


FIGURE 8 - Total amino acid incorporation rates in livers of normal, T3-treated and thyroidectomized rats. Total protein incorporation (microcuries of T) for each animal has been normalized with the value for free (acid-soluble) radioactivity (microcuries of FR) recovered in the liver to correct for variations in injection and amino acid uptake by the liver. Results have been averaged and are shown with standard errors.

animals gave results statistically indistinguishable from normal controls.

A summary of the results for the four experimental groups is presented in Table 4. The recovery of labeled amino acids as free (acid soluble) radioactivity in liver following the hepatic portal vein injection was about 50% of the injected dose. Values of the polypeptide chain assembly time t_c and the net incorporation rate normalized by free radioactivity are presented with their standard errors. Chemical analyses, which were not significantly different among the four groups, were as follows: DNA, 3.0 ± 0.6 mg per g of liver; RNA, 7.3 ± 1.0 mg per g; protein, 185 ± 11 mg per g.

Estimation of net liver protein synthetic rate from t_c - If all liver ribosomes are presumed to be active in protein synthesis, the measured average chain assembly time t_c can be used together with an estimate of the average chain size (50,000 daltons) to calculate the synthetic output of the tissue. Ribosomal RNA content in rat liver is about 6 mg per g, thus ribosome concentration is given by:

$$\text{ribosome concentration} = \frac{6 \text{ mg ribosomal RNA/g}}{2{,}000{,}000 \text{ daltons RNA/ribosome}}$$

$$= 3 \text{ nanomoles/g}$$

Using the value of t_c = 1.16 min for normal rats and assuming that all ribosomes are engaged in protein synthesis, one obtains the following chain synthesis rate:

TABLE 3 - Collected protein synthetic rate data obtained for rat liver *in vivo*

Group	No. of animals	Average body weight	Average liver/ body	Free radio-activity	t_c	Incorporation rate
		g	%	μCi/g liver	min	μCiT/μCiF R/min
Controls	17	180 ± 40	3.6 ± 0.3	0.43 ± 0.13	1.16 ± 0.16	0.15 ± 0.06
T3 - treated	15	182 ± 20	3.6 ± 0.3	0.42 ± 0.27	0.92 ± 0.08	0.23 ± 0.05
ThyX[a]	16	143 ± 14	3.7 ± 0.9	0.41 ± 0.21	1.92 ± 0.22	0.09 ± 0.06
ThyX + T3	10	156 ± 45	2.9 ± 0.2	0.36 ± 0.17	1.28 ± 0.15	0.14 ± 0.06

[a] ThyX, thyroparathyroidectomy

$$\text{chain synthesis rate} = \frac{3 \text{ nanomoles/g}}{1.16 \text{ min}}$$

$$= 2.6 \text{ nanomoles/g/min}$$

If the average chain has a molecular weight of 50,000 daltons, protein snythetic rate per g of liver per hour is:

$$(2.6 \text{ nanomoles/g/min})(50{,}000 \text{ daltons})(60 \text{ min/hour})$$

$$= 7.8 \text{ mg protein/g liver/hour}$$

This in turn yields 29 mg/100 g body weight/hour:

$$(7.8 \text{ mg/g liver/hour})(0.037)(100)$$

$$= 29 \text{ mg/100 g body weight/hour}$$

where 0.037 is the liver/body weight ratio found experimentally.

Peters and Peters (60) obtained a result comparable to that above for net protein synthesis (28.3 mg/100 g body weight/hour) in Wistar rats of similar age. Incorporation of radioactive leucine into liver protein was determined for a 16 minute labelling period *in vivo* and the rate of synthesis calculated from the following equation:

$$F = \frac{60(m_{16})\ L}{a\int_0^{\blacksquare} L_t^* \, dt}$$

where F = rate of liver protein synthesis in mg/g liver/hour

m_{16} = dpm in total liver protein/g liver at 16 min

L = free leucine concentration as mg/g liver

a = (.0875) mg leucine/ mg liver protein

L_t^* = dpm in free leucine/g liver at time t

60 = conversion factor from min to hours

The interval of 0 to 14 was chosen since is requires about 2 minutes for synthesis of an albumin molecule. The integral was determined graphically by plotting free ^{14}C leucine in dpm/g liver on the ordinate versus time and determining the area under the curve. The agreement on net protein synthesis between the net incorporation result of Peters and Peters and our calculation from t_c tends to validate our assumption, i.e., that all ribosomes are continuously functional producing polypeptide chains of average size.

If 80% of the synthetic production is directed toward intracellular proteins, as found in fish liver (61) and suggested by data for rat (60), then the average half time for turnover of intracellular liver protein may be calculated as follows:

Synthetic rate:

$$\left(\frac{dP}{dt}\right)_S = (7.8 \text{ mg/g liver/hour})(80\%) = 6.24 \text{ mg/g liver/hour}$$

Turnover rate:

$$-\left(\frac{dP}{dt}\right)_T = kP$$

where P = 185 mg/g liver = protein content of normal liver.

If no protein accumulation occurs, then

$$k = \frac{6.24 \text{ mg/g liver/hour}}{185 \text{ mg/g}} = 0.034 \text{ hour}^{-1}$$

and

$$t_{\frac{1}{2}} = \frac{\ln 2}{k} = \frac{0.694}{0.034} = 20 \text{ hours}$$

If turnover is neglected, synthesis equivalent to total liver protein based on $(dP/dt)_S$ above is achieved in about 30 hours:

$$\frac{185 \text{ mg/g liver}}{6.24 \text{ mg/g liver/hour}} = 30 \text{ hours}$$

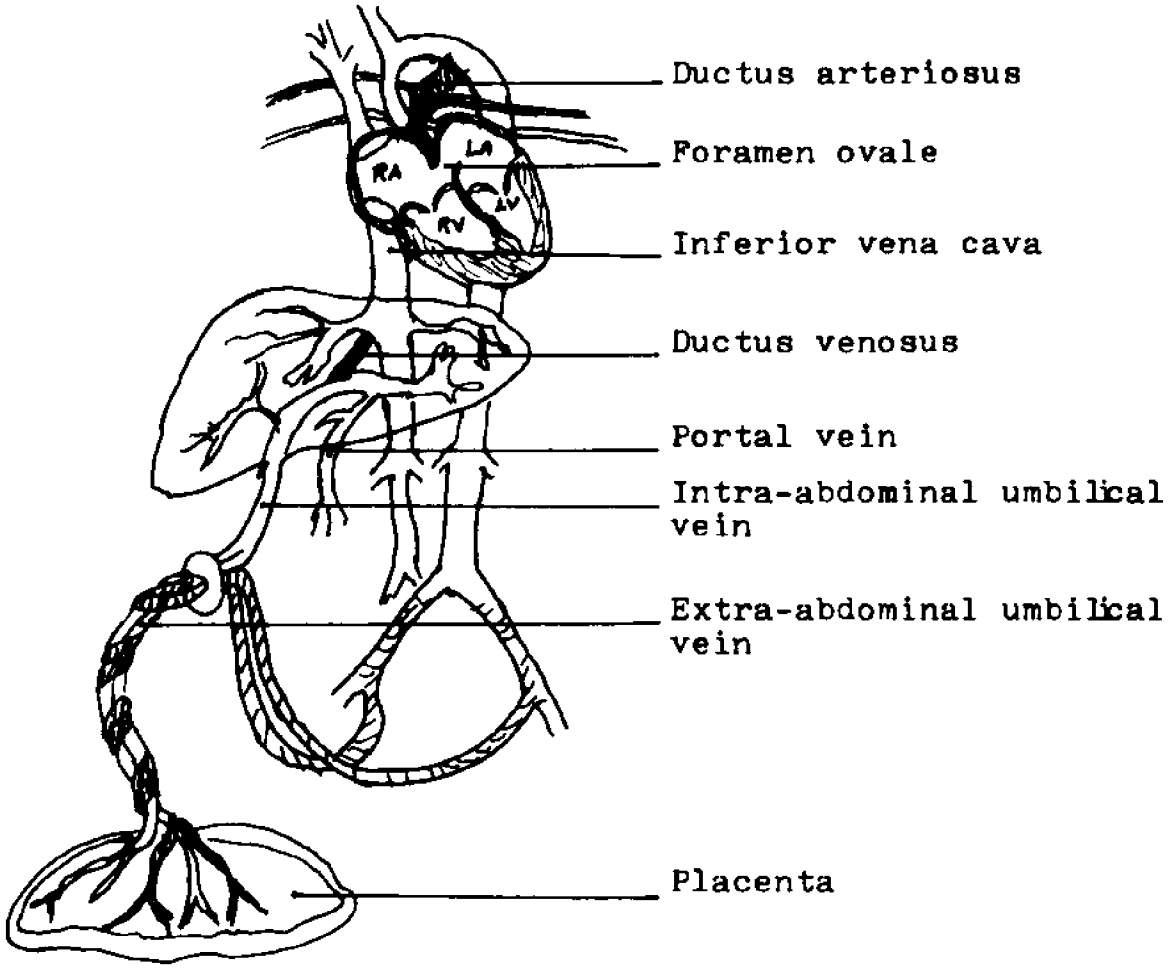
The effect on liver protein synthetic output due to thyroidectomy or excess hormone treatment can similarly be calculated from the polypeptide chain assembly time constants, with the assumption that no change in porportion of active ribosomes has occurred. The value of t_c = 1.92 min in the thyroidectomized group yields a protein synthetic output of 4.8 mg per g of liver per hour, or 17.8 mg per 100 g body weight per hour. Animals treated with excess T3 (t_c = 0.92 min) would produce 10 mg of protein per g of liver per hour, or

37 mg per 100 g body weight per hour, compared with euthyroid normals at 29 mg per 100 g body weight per hour. A similar shift-up in protein synthesis occurs with cold acclimation in fish, another process in which thyroid hormone is implicated (49).

V - RESULTS - FETAL

Since the studies in the adult liver depended on a fast route of injection of the radioactive amino acids to achieve a high level of precursor uptake and to establish a realistic zero time point, it was necessary to devise some way in which the precursor could be injected directly into the fetal liver. The extra-abdominal umbilical vein gave such a route. The rat umbilicus consists of four blood vessels, two minor ones and two major ones, as shown in Figure 9 (62). The two incoming vessels probably converge before entering the fetal liver. Isotope solution injected by this route can be seen moving along the cord, entering the intra-abdominal portion of the vein and thence in an anterior direction into the liver at approximately the point where the adult portal vein will be. Data from this method are reproducible and can be obtained for periods as short as 15 sec. Circulation time is very fast in the fetus. The fetal heart beats much faster than the adult heart and the fetal circulatory system is proportionately shorter, having three major vasuclar shunts (66). The first of these shunts is the ductus venosus. The umbilical vein enters the liver and

FIGURE 9[a]- Schematic representation of fetal circulation



(a) adapted from Biology of Gestation (62)

branches, one branch carrying the most arterial of the fetal blood goes to the liver sinusoids, the other goes directly to the ductus venosus and makes a direct link between the umbilical vein and the inferior vena cava. The next shunt is the foramen ovale and blood coming from the inferior vena cava probably passes directly through this shunt into the left atrium and left ventricle providing heart and head with a direct supply of oxygenated blood. The blood that reaches the left atrium through the foramen ovale and that which comes from the pulmonary veins is ejected into the left ventricle and thence into the ascending aorta. Part of this blood supplies the head and upper anterior extremities but the rest continues to the descending aorta. The third shunt is the ductus arteriosus. This shunt keeps the arterial flow to the lungs at a minimum, only a small fraction of the blood from the pulmonary artery circulates across the lungs, the major portion is shunted back to the aorta via the ductus arteriosis and thence back down through the descending aorta from whence it returns to the placenta.

Figure 10 shows the averaged data for fetuses of 20 to 22 days. The slope of the regression line yielded a value of $t_c = 1.63 \pm 0.06$ min. There have been no previous estimates of synthesis time in the fetal rat to my knowledge and rather than being faster than the adult, as had been supposed (46, 47, 48), it is 29% slower than the normal euthyroid rat of 2 to 2½ months of age.

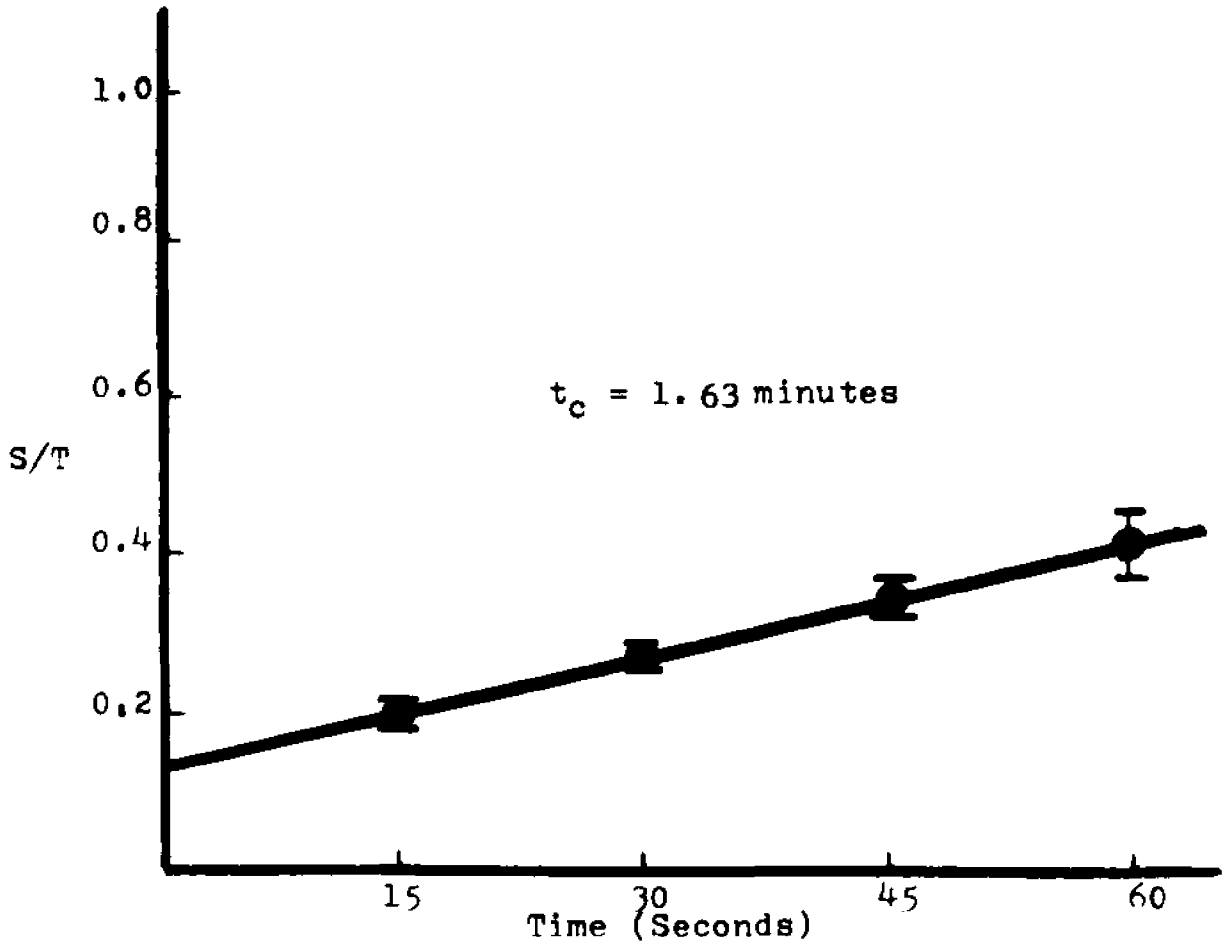


FIGURE 10 - Determination of polypeptide chain assembly time t_c for 20 to 22 days of gestation in fetal rat. Data for 4 fetuses per point have been averaged. Vertical bars indicate standard error.

Figure 11 shows the averaged data for fetuses of 17 to 18 days old. This age precedes the time at which circulating thyroxine appears (60). The slope of the regression line shows a value of $t_c = 2.28 \pm 0.50$ or 29% slower than the fetus with thyroxine circulating in its plasma. These results when tested by standard t test against the later fetal rat are significant at $P < 0.001$.

The regression lines of Figure 10 and Figure 11 intersect the abscissa at about 20 to 30 seconds which coincides approximately with the time needed for fetal decapitation and surgical removal of the liver and is not included in the incorporation time as plotted. For times longer than 60 sec, the plot of S/T versus t showed departure from linearity, even before the expected change of shape at S/T = 0.5. The reason for this is not yet clear.

Figure 12 represents the averaged fetal data for total incorporation of ^{14}C leucine into liver protein again normalized for each animal by dividing protein bound radioactivity per unit of liver homogenate by measured free radioactivity as was done for the adult data. As in the adult, while the presentation tends to eliminate variation due to differences in radioisotope injection, there is considerable variation between individuals which is not reflected in the S/T ratios and again is probably related to differences in precursor pool specific activity. The

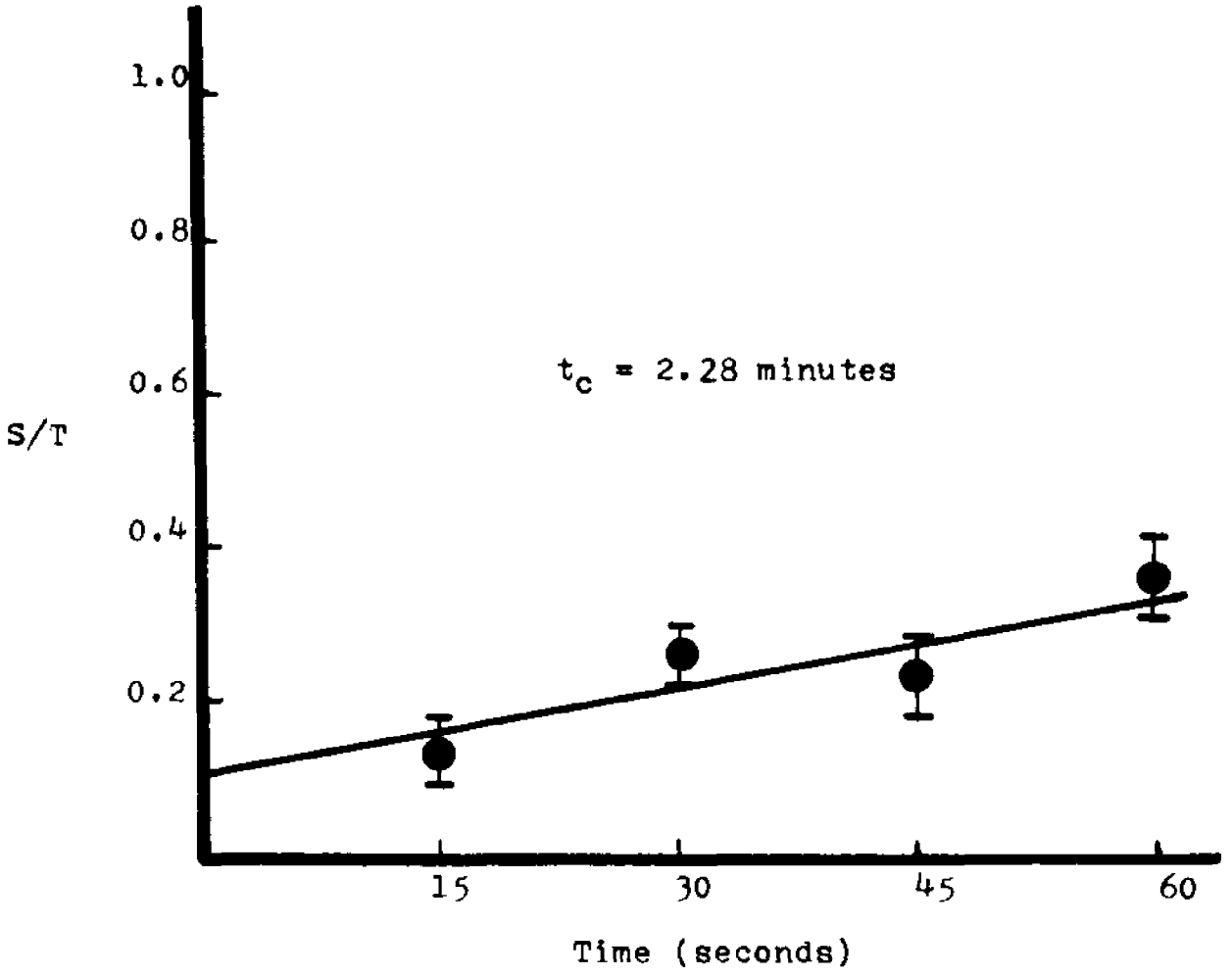


FIGURE 11 - Determination of polypeptide chain assembly time t_c for 17 to 18 days of gestation in fetal rat. Data for 4 fetuses per point have been averaged. Vertical bars indicate standard error.

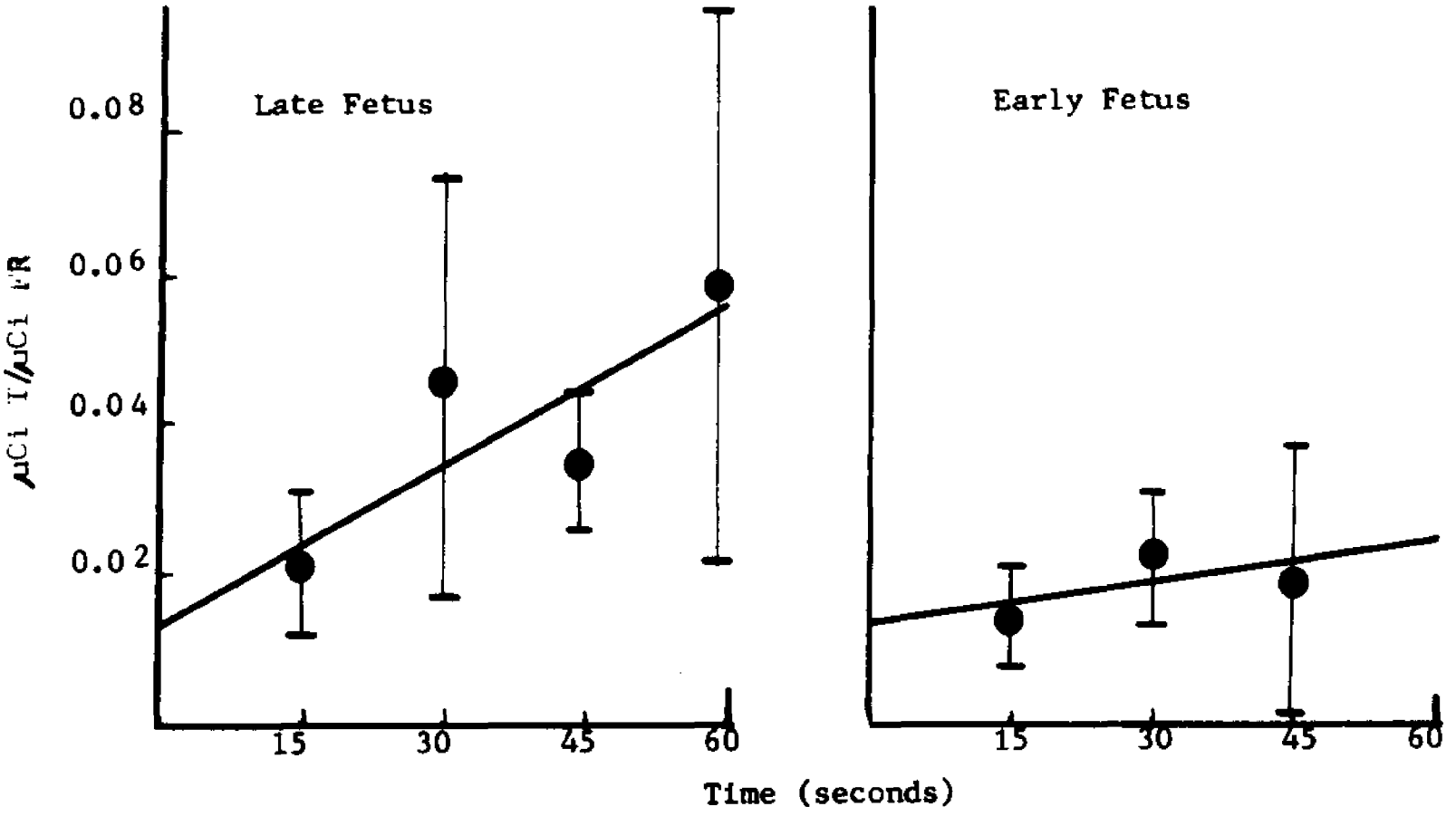


FIGURE 12 - Total amino acid incorporation rates in livers of early and late fetuses. Total protein incorporation (µCi of T) for each animal has been normalized with the value for free (acid soluble) radioactivity (µCi of FR) recovered in the liver to correct for variations in injection and amino acid uptake by the liver. Results have been averaged and are shown with standard errors.

results for total incorporation rate (0.042 μCi T/μCi FR/min for the early fetus and 0.07 μCi T/μCi FR/min for the late fetus) confirm the more precise assembly time determination. The fetal animals with circulating thyroxine show a 29% increase in synthetic rate based on t_c (polypeptide chain assembly time) over the early athreotic fetuses and an increase of 40% in total incorporation rate.

The summary of the fetal results appears in Table 5. The recovery of labeled amino acid as free (acid soluble) radioactivity in the fetal liver following injection via the extra abdominal umbilical vein was about 50% of the injected dose, as in adult rat. Although the fetal livers weigh less than a gram all results are expressed per gram of liver so as to directly compare the figures with the adult results. Values of the polypeptide chain assembly time t_c and the net incorporation rate normalized by free radioactivity are presented with their standard errors. Chemical analyses for the early group were as follows: DNA 4.86 ± 0.9 mg/g liver; RNA, 8.96 ± 1.6 mg/g liver of which 65% was sedimentable at 100,000 x g for 3 hours; RNA/DNA, 1.8; protein, 64.66 ± 4.87 mg/g liver; liver weight, 121 mg ± 28 mg. The late fetal analyses were: DNA, 3.73 ± 0.79 mg/g liver; RNA, 10.79 ± 2.2 (65% of this was also sedimentable at 100,000 x g for 3 hours; RNA/DNA, 2.9; protein, 84.45 ± 7.45 mg/g liver; liver weight 309 mg ± 38 mg. The increase in RNA and protein relative to DNA reflects cell growth in late fetal life.

TABLE 4 - Collected protein synthetic rate data obtained for fetal rat liver in vivo

Group	No. of animals	Average body weight	Average liver/body	Free radio-activity	t_c	Incorporation rate
		g	%	μCi/g liver	min	μCiT/μCiFR/min
Early fetal 17-18 days gestation Average age 17.9 ± 0.25 days	16	0.9 ± 0.1	13.0 ± 3	0.40 ± 0.2	2.28 ± 0.50	0.042 ± 0.03
Late fetal 20-22 days gestation Average age 21.29 ± 0.38 days	16	4.2 ± 0.9	7.5 ± 0.9	0.23 ± 0.1	1.63 ± 0.06	0.07 ± 0.05

Estimation of net liver protein synthesis - Using the same method as used for the adult rats, the value of t_c = 2.28 min for the early fetal rat yields protein synthetic rate of 3.9 mg protein per gram of liver per hour. The late fetal rat with a t_c of 1.63 showed a protein synthetic rate of 6.6 mg protein per gram of liver per hour. Comparing these figures with the adult rats shows that the fetal rats' protein synthesis per gram of liver is much less than the normal adult (7.8 mg/g liver/hour). However, as can be seen in Table 4, the liver to body weight ratios are much higher in the fetal than in the adult (13-7.5% as compared to 3.7%) which makes the liver contribution to total synthetic activity in the body approximately the same in both groups.

VI - DISCUSSION

This study establishes a correlation between the kinetics of protein synthesis in liver of the adult and fetal rat *in vivo* and thyroid status. The present technique eliminates many of the shortcomings of previous studies on protein synthesis in relation to hormonal action. The experiments are carried out *in vivo*, under conditions as close to physiological as possible. The general agreement between polypeptide chain assembly time in normal adult rats (anesthetized) and estimates for liver-synthesized plasma proteins obtained in rats awakened from light anesthesia (59) suggests that anesthesia probably did not significantly influence the results. Thyroidectomy of the young rat was carried out by an established surgical procedure, and its effectiveness was checked by two methods. For induction of hyperthyroidism in normal rats relatively low doses of triiodothyronine were used in order to minimize effects unrelated to the physiological action of the hormone. Although similar enzymic responses have been noted at both high (500 µg per rat) and low levels (20 µg per rat) (6), the uncertainty associated with the use of pharmacologic amounts of thyroid hormones is recognized (64).

Fetuses were kept warm and moist by a saline water bath at 37°C. This method was an attempt to maintain as closely as possible the conditions of intra-uterine life and to

reduce effects of trauma on protein synthetic rates. By using the fetal rat, the effect of newly circulating thyroxine, which has been shown to induce the formation of a cluster of enzymes (28), on the rate of polypeptide chain assembly time was investigated in a system that was as little perturbed as possible.

An important factor in measurement of protein synthesis in vivo by isotope methods is the determination of a linear relationship between incorporation levels and reaction time. This is facilitated in the measurement of t_c because, by use of a ratio of incorporation levels (S/T), much of the individual variation inherent in assays of total incorporation levels (T) alone is eliminated. Such variation may obscure rate changes actually occurring. For example, a careful study of the effect of triiodothyronine on leucine incorporation by tadpole liver in vivo failed to reveal a statistically significant pattern over most of the time period studied (65).

Relation to studies in vitro - The present in vivo method for study of protein synthetic rate also does not suffer the shortcomings of in vitro systems where, due to the complexity of the reaction being studied, preparation artifacts may play an inordinately large role. For example, results for leucine incorporation into protein of crude liver microsomal preparations from thyroidectomized rats indicated a drop in protein synthetic capacity to 15% of control levels (40). The present

data do not support such a drastic effect of thyroidectomy upon protein synthesis in the liver. In another study, changes in microsomal incorporating activity found upon hormone treatment of thyroidectomized rats disappeared or were reversed by 18 to 24 hours starvation of the animals (64). Even in assays of individual components of the protein synthetic system, under conditions of saturation or optimization with respect to all other components capable of influencing reaction rate (67), the possibility remains that the behavior of the system *in vitro* does not adequately reflect the *in vivo* state. The enormous differences in protein synthesis claimed to occur in fetal systems as compared to adult systems (46, 47, 48) are not warranted by the results of this paper. In fact rather than fetal protein synthetic rates being higher than the adult as has been supposed, they are slightly slower.

The physiological significance of direct thyroxine effects on crude amino acid incorporating systems *in vitro* (42, 68) has previously been questioned (64). Present results indicate that liver protein synthetic rate *in vivo* is 100 to 1000 times that of such preparations, based on comparable amounts of components. The present results indicate an *in vivo* synthetic rate of 7.8 mg/g liver/hour. In terms of leucine incorporation alone, this represents a rate of leucine utilization of 6.1 μm leucine/g liver/hour [9% of liver

protein by weight is leucine (66)]. In order to determine the efficiency of a typical *in vitro* system prepared from rat liver, we have used the data of Sokoloff and Kaufmann (68). Reaction mixtures of 1.7 ml were prepared containing ribosomes and mitochondria corresponding to 200 mg of liver and cell sap corresponding to 30 mg of liver. ^{14}C-L-leucine (5.4 μCi/μm) was added to a concentration of 0.4 μm/1.7 ml. Adjusting for endogenous leucine introduced with the cell sap, one finds a final leucine specific activity of 5.2 μCi/μm.

$$\mu\text{m endogenous leucine in 1.7 ml reaction mixture} = (0.2\ \mu\text{m/g liver})\left(\frac{0.25\ \text{g liver}}{\text{ml cell sap}}\right)(.15\ \text{ml cell sap})$$

The incorporation of L-leucine during a 25 minute incubation period was 130 cpm/mg protein (corrected for self-absorbtion). After adjustment for counting efficienty (33%) total incorporation is 390 dpm/mg protein or 1.75×10^{-4} μCi/mg protein. From the specific activity of the leucine as calculated above (5.2 μCi/μm), the amount of leucine incorporated per mg of protein is

$$\frac{1.75 \times 10^{-4}\ \mu\text{Ci/mg protein}}{5.2\ \mu\text{Ci}/\mu\text{m}} = 3.4 \times 10^{-5}\ \mu\text{m/mg protein/25 min}$$

If we assume that incorporation was linear during the 25-minute period, the rate of incorporation is

$$1.4 \times 10^{-6}\ \mu\text{m/mg protein/min}$$

Although the authors did not indicate the amount of protein in the reaction mixture, this can be calculated from the components utilized. Our own studies have indicated the following distribution of protein in rat liver:

Total protein	180 mg/g (wet weight) liver	
↓ 800 x g, 10 min		
Postnuclear supernatant protein	151 mg/g	(84%)
↓ 100,000 x g, 90 min		
Post-mitochondrial and ribosomal supernatant protein	117 mg/g	(65%)
Microsomal and mitochondrial protein	34 mg/g	

Thus, we may calculate for the Sokoloff and Kaufmann system containing mitochondria and ribosomes from 0.2 g liver and cell sap from 0.03 g liver that the protein content will be 6.8 mg + 3.5 mg = 10.3 mg.

We then calculate for incorporation per sample:

$$(1.4 \times 10^{-6}\ \mu m\ leu/mg\ protein/min)(10.3\ mg\ protein)(\frac{60\ min}{hour})$$

$$= 8.4 \times 10^{-4}\ \mu m\ leu/sample/hour$$

The sample contained the ribosomes from 0.2 g of liver, thus we may convert to the equivalent of 1 g of liver as follows:

$$\frac{8.4 \times 10^{-4}\ \mu m\ leu/sample/hour}{0.2\ g\ liver/sample}$$

$$= 4.2 \times 10^{-3}\ \mu m\ leu/g\ liver/hour$$

This result may be compared with the leucine incorporation rate *in vivo* of 6.1 μm leu/g liver/hour. The ratio of *in vivo* to *in vitro* incorporation is

$$6.1/4.2 \times 10^{-3} = 1.5 \times 10^{3}$$

Thus, liver ribosomes *in vitro* have a protein synthetic activity less than one-thousandth of their activity *in vivo*. Data of other investigators (41,64) show similarly poor activity levels in *in vitro* protein synthetic systems, when compared to the *in vivo* results reported here and by Peters and Peters (60).

Control of protein synthesis in vivo - Equation 1 was derived on the basis of a model of protein synthesis in which chain elongation was taken to be rate controlling (49), changes in t_c thus reflecting changes in elongation rate. The present results in the adult and fetal rat liver indicate that one or more reactions in chain growth are subject to change in rate depending on thyroid hormone level in the animal. Comparable findings in fish liver in connection with metabolic adaptation to cold have been related to alteration in elongation factor I activity (67), an enzyme currently under study in our laboratory.

Another factor which can influence protein synthetic output is the proportion of ribosomes engaged in synthesis (or initiation frequency). A change in this parameter would not influence the time course of S/T or the resulting t_c, but would affect total incorporation rate (T/FR per minute)

independently of changes associated with t_c. The results of Tables 1 and 5, however, show that the relative values of T/FR per minute for the various groups fall within the range predicted by the observed changes in t_c, provided that endogenous amino acid pools are the same in all groups compared. Among the adult rats, an increase in t_c from 1.16 min to 1.92 min with thyroidectomy would cause a 39% drop in protein production per unit time, if all other factors were equal. The observed decrease in incorporation rate was 40%. The hyperthyroid rats show a decrease in t_c from 1.16 in the controls to 0.92 min. This would cause an increase in protein production of 20%. Net incorporation rate was increased 34%. Because of individual variation in the latter quantity, these results are viewed as not significantly different.

The fetal rats show the same correlation between protein production and the observed incorporation rate. The late fetus has a t_c of 1.63 min and the early fetus one of 2.28 min. This represents an increase of 29% in protein production per active ribosome. The increase in incorporation rate was 40%, a figure which would reflect in part increased ribosome concentration. The results in both cases suggest that the principal effect of thyroxine on protein synthesis is in the control of elongation rate. However, the possibility that chain release mechanisms may be rate-limiting (and hence modulated by thyroid status) cannot be completely excluded by the model used for determination of t_c (49).

The quantitative effect of thyroxine on polypeptide chain assembly rate was similar in the two systems studied. In the fetus, the onset of circulating thyroxine is accompanied by an approximate 30% elevation in assembly rate. In the adult, comparing thyroidectomized and euthyroid animals, one finds a 40% rate increase associated with normal thyroxine levels. An additional 28% increase occurs with hyperthyroidism.

Thyroxine induced protein synthesis and growth - The net protein synthetic rates calculated under Results indicate that synthesis in the livers of the adult euthyroid rats (normals and hormone-supplemented thyroidectomized animals) exceeds that of thyroidectomized rats by 270 mg/100 g body weight per day, of which 80% may be assumed to represent intracellular protein (61) yielding 216 mg of liver protein per 100 g of body weight per day. Growth data for comparable groups at the same age (70, 71) show a difference of about 3 g/100 g per day in body weight gain associated with thyroid hormones. Thus if the liver represents 3.7% of the body weight then liver growth would be 0.111 g per 100 g body weight per day:

$$(3 \text{ g}/100 \text{ g body weight/day})\ (0.037) = 0.111 \text{ g liver}/100 \text{ g body weight/day}$$

Protein content of the liver is 20%, thus the weight gain

in terms of liver protein is:

(0.111 g)(0.2) = 22 mg liver protein/100 g body weight/day

Hence about 10% of the thyroxine induced synthetic activity is associated with protein accumulation under normal growth conditions. This represents normal growth rate, since the thyroidectomized animals in the growth study cited showed no growth in the period studied.

Net protein synthetic rates calculated for the fetal rats indicate that protein synthesis in the livers of those fetuses that have circulating thyroxine is about twice that of the early fetuses (6.6 mg/ g liver/hour compared to 3.9 mg/ g liver/hour). On the basis of Williamson's (72) data, fetal liver growth rate is about 40 mg of liver per day at age 17-18 days; liver size is about 170 mg at this age. From the protein content (6.5%) growth rate corresponds to a protein accumulation rate of 2.6 mg liver protein per day:

(40 mg liver)(0.065 mg protein/mg liver) = 2.6 mg liver protein/day

Synthetic rate for intracellular protein is given by

(3.9 mg/g liver/hr (0.17 g liver)(0.80)(24)

=12.7 mg intracellular protein synthesized per day

assuming 20% of total synthesis is for export. Thus 20% of synthesis is associated with accumulation. For the late fetus liver growth is 129 mg per day or

(129 mg liver)(.085 mg protein/mg liver) = 11 mg liver protein/day

Synthetic rate for the late fetuses, calculated as above, is

(6.6 mg/g liver/hr)(0.24 g liver)(0.80)(24)

= 30.5 mg intracellular liver protein synthesized per day.

Thus at this stage, approximately 37 % of protein synthesis leads to protein accumulation. In contrast only 10% is so used in the adult. Changes in turnover rates are to be expected under circumstances of generalized changes in protein synthetic activity and are reflected by these results.

The relation of the observed changes in polypeptide chain assembly rate to the site of action of triiodothyronine or thyroxine cannot yet be ascertained. Prior work (49,50) tends to implicate an alteration in elongation factor I activity. Further studies on the time course following hormone administration may help to distinguish whether these changes represent a primary or secondary effect.

Part of this thesis was published as Effect of Thyroid Hormone on Polypeptide Chain Assembly Kinetics in Liver Protein Synthesis in Vivo by Rita W. Mathews, Arnold Oronsky, and Audrey E.V. Haschemeyer. It appeared in The Journal of Biological Chemistry, Volume 248, February 25, 1973, pp. 1329-1333.

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