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# Protein Synthesis in Regenerating Wound Tissue and the Effect of Thyroid Hormone

Cary Bruce Linksy  
*Loyola University Chicago*

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PROTEIN SYNTHESIS IN REGENERATING WOUND TISSUE

AND THE EFFECT OF THYROID HORMONE

by

CARY BRUCE LINSKY

A Dissertation Submitted to the Faculty of the Graduate  
School of Loyola University in Partial Fulfillment  
of the Requirements for the Degree of  
Doctor of Philosophy

June

1971

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## ABSTRACT

Abstract of the dissertation entitled PROTEIN SYNTHESIS IN REGENERATING WOUND TISSUE AND THE EFFECT OF THYROID HORMONE submitted by Cary Bruce Linsky in partial fulfillment of the requirements for the degree of Doctor of Philosophy, June, 1971.

The purpose of this investigation was to obtain information on the rate of protein synthesis in regenerating wound tissue and to explore the feasibility of using the rate of protein synthesis to assess the rate of healing. The most commonly used method for measuring wound healing, tensile strength, has been subject to various interpretations as to its significance. Some investigators presumed tensile strength to correlate with the amount of collagen present in the tissue, but numerous reports have indicated it to be a reflection of many factors. With this in mind, protein synthesis was investigated in regenerating wound tissue as affected by various levels of thyroid hormone, an agent which had previously been shown to affect wound tissue formation.

The action of thyroid hormone on the rate of protein synthesis was studied by following the rate of incorporation of labeled amino acids.  $^3\text{H}$ -proline was used to study the rate of synthesis of regenerating wound tissue in rats 5, 8, and 12 days after wounding, while they were in three different thyroid states (hypo-, eu-, and hyperthyroid). By using this amino acid, it was possible

to differentiate the effect of thyroid hormone on collagen and non-collagenous (cellular) proteins. The rate of incorporation of  $^{35}\text{S}$ -cystine, both by peptide bonds and disulfide bonds, was also studied at the 8th day of regeneration in rats in the three thyroid states.

Results showed that the rate of protein synthesis, based on incorporation of  $^3\text{H}$ -proline, was highest on the 8th day and lower on the 5th and 12th day. This was true for both collagen and non-collagenous proteins. It was suggested that this may be due to a parallel pattern in nuclear RNA (in particular, m-RNA) and/or a changing cell population of the tissue.

The synthesis of the cellular proteins was stimulated but the synthesis of collagen was depressed in the hyperthyroid rats when compared to the euthyroid state. Evidence was cited pointing to an increased catabolism of collagen to account for the depression of its synthesis. This is consistent with the stimulatory effect of the thyroid on cellular protein synthesis and the recent finding of collagenase in granulation tissue.

Incorporation of cystine was highest in the hyperthyroid group, both for that bound by peptide bonds and that bound by disulfide bonds. These results pointed to the possibility that the hyperthyroid animal is stimulated so as to synthesize many enzymes, and also to increase the activity of sulfhydryl-activated enzymes.

The results showed that the rate of protein synthesis could distinguish between different thyroid states. However, further studies with other agents would seem to be in order before warranting the use of protein synthesis as an indicator of the rate of healing.

## BIOGRAPHICAL SKETCH

Cary Bruce Linsky was born in Chicago, Illinois on June 9, 1942. After graduating from Senn High School in 1960, he entered the University of Wisconsin, Madison, Wisconsin, from where he received the degree of Bachelor of Science in June, 1964.

In September of 1964, he entered a program of graduate study in the Department of Anatomy, Loyola University Stritch School of Medicine. In June, 1968, he changed his course of study by entering the Department of Biochemistry and Biophysics at Loyola. He has been a National Institute of Health Trainee since July, 1969.

In June, 1968, he married Anita Steingold.

He is co-author of the following publications:

1. "Influence of Thyroid Hormone on Protein Synthesis in Granulation Tissue", Federation Proceedings 29, 318Abs (1970) (with M. B. Williamson).
2. "Changes in the RNA of Cellular Nuclei during Wound Tissue Regeneration", Proc. Soc. Exp. Biol. Med. 136, 1336-1339 (1971) (with L. G. Wong and M. B. Williamson)

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CHAPTER IINTRODUCTION

The term wound healing is taken to include the processes of reparation by tissues in response to insult such as disease or injury. Though wound healing has been studied in some respects for centuries (e.g., ascorbic acid and its relation to scurvy) (9), it is only in the last half century that the metabolic changes which accompany wound healing have been investigated.

The type of repair which is manifested must of course be dependent on the regenerative capacity of the tissue impaired. The ubiquity of connective tissue and, in turn, of collagen insures their involvement to some varying degree in all wound healing. Thus, one sees a close interrelationship between the metabolism of collagen and wound healing, one that is noted extensively in the literature.

Studies on this relationship have, in the past, and continuing at the present time, looked at the physical and mechanical properties of wound repair. Methods reported for such studies involve mensuration of wound area, extension rates, and tensile strength. Tensile strength has been studied in numerous experiments and has been presumed to be a measure of the amount of collagen present. More recently, reports have cast some doubt on this assumption (37,51).

Biochemical techniques are also now used to follow the course of wound healing. Collagen, due to its unique amino acid composition (18), has lent itself well to amino acid composition studies. In comparison to many other proteins, it is peculiar in its extracellular location, its comparatively low metabolic activity, and its state of aggregation.

Besides collagen, other proteins are synthesized during the course of wound healing. Other important factors involved in wound healing include mucopolysaccharides, lipids, vitamins and endocrine factors. All of these have been reviewed in detail as to their relationships during the course of wound healing (9).

The biochemical approach to studying wound healing has been handicapped by the lack of a quantitatively precise method for measuring the rates of healing. Tensile strength has probably been used most widely as an indicator of the rate of wound healing. Although tensile strength is supposed to be correlated with the amount of collagen and its state of aggregation, actually many constituents, and the interaction of these constituents, influence tensile strength to a significant degree (37).

Another possible approach to a basis for assessing the rate of healing may be by measuring the rate of protein synthesis in the wound tissue. We propose not to develop a method for measuring wound healing but to explore the feasibility of using protein synthesis as a basis for such a method. Since thyroid hormones

have been shown to affect wound tissue formation (44,53), protein synthesis will be studied in regenerating wound tissue of rats exposed to differing levels of thyroid hormone.

## CHAPTER II

### COLLAGEN AND WOUND HEALING

Connective tissue is distributed throughout the body in cartilage, ligaments, bone, blood vessels and skin. It serves as a binding substance in parenchymatous organs such as kidney and muscle. Its mechanical and supportive properties are accomplished by the extracellular, insoluble proteins which are embedded in a ground substance.

Collagen is the extracellular fibrous protein of connective tissues. It is the most abundant protein in the body, comprising about one third of the total protein and about six per cent of the body weight. A well known property of collagen, and the one from which it receives its name (Gr. colla, glue + gen, producing), is its transformation to gelatin upon subjection to boiling water.

The collagen molecule has been shown by carboxymethyl cellulose chromatography to be composed of three polypeptide chains, each approximately 100,000 in molecular weight (5). In many collagens, two of these chains are very similar. This type of chain is called the  $\alpha_1$  subunit. The third chain is the  $\alpha_2$  subunit. Thus, the three subunits form tropocollagen, the name given to the collagen molecule. Dimers are found due to intramolecular cross-links between two of the chains. Thus  $\beta_{11}$

is a dimer of two cross-linked  $\alpha_1$  subunits while  $\beta_{12}$  is a dimer of an  $\alpha_1$  and an  $\alpha_2$  subunit. These  $\beta$  units are believed to be derived after assembly of the molecule and its aggregation into fibrils (58).

There has been some controversy about the subunits of collagen. Due to its high molecular weight and repeating amino acid sequences, various investigators have looked for subunits within the  $\alpha$  chains. Gallop and his workers (22,23) hypothesized that each of the three  $\alpha$  chains is composed of six subunits of three distinct chemical types (A, B, and C), each of about 17,000 molecular weight. Within each strand, these subunits would be in a 3:2:1 ratio. A good part of the evidence for this was that hydroxylamine, a reagent that will cleave ester-like bonds, degrades  $\alpha$  strands into subunits of about 17,000 and 34,000 in molecular weight. Hodge (34) also proposed a subunit for the chains, but in a 5:5:7 ratio for the three chains. Recently, Vuust and Piez (96) studied the biosynthesis of the  $\alpha$  chain of collagen by pulse-labeling in culture. Previous sequencing work by these investigators with cyanogen bromide (an agent which hydrolyzes the peptide bond involving the carboxyl group of methionine) had shown the order of the peptides in the  $\alpha$  chains. Cultures were incubated with radioactive glycine. After extraction and hydrolysis with cyanogen bromide, the peptides were counted and a radioactive gradient was seen, the gradient being steepest after



the shortest pulse. The results indicate that the chains are synthesized as single chains by sequential amino acid addition from the amino terminal end and not as shorter subunits which are later joined.

The amino acid composition of collagen is very unique. Except in the case of some invertebrate collagens, no cysteine or cystine is found, with very small amounts of methionine. This latter fact is the reason cyanogen bromide is used as a hydrolyzing agent for sequence studies. This reagent allows cleavage with high selection and good yield. Since  $\alpha$  chains of collagen have 6-9 residues of methionine per chain, a manageable number of peptides result. Collagen also contains no tryptophane, and the absorbance at 280 nm which is seen is due to the small amount of tyrosine present. Glycine accounts for about one-third of the residues, alanine for about one-ninth. Proline is found in about one-eighth of the residues. What is particularly notable in collagen is the presence of hydroxyproline, and to a much lesser extent, hydroxylysine. Hydroxyproline comprises about 9.3% of the amino acid residues and hydroxylysine less than 0.5%. Piez notes in comparing the two  $\alpha$  chains that  $\alpha_2$  is more basic, in large part due to the higher histidine content. The  $\alpha_2$  chains also contain larger amounts of the amino acids with hydrophobic side chains (valine, leucine, and isoleucine),

but has a lower amount of the imino acids, hydroxyproline and proline (57).

Hydroxyproline and hydroxylysine have a very restricted distribution among natural products. Except for collagen and elastin, no other mammalian tissue has been shown to contain these amino acids. Stetten, in a classical series of experiments (83,84), demonstrated, by the use of labeled proline and hydroxyproline, that the hydroxyproline in collagen arises from proline, while hydroxyproline is not incorporated to a significant extent. The analagous situation has been shown for lysine and hydroxylysine, i.e. hydroxylysine arises from lysine (79,80).

The synthesis of collagen calls for more than just the addition of amino acids; the hydroxylation of proline and lysine at some step is also required. Since Stetten's experiments, much attention and effort have been devoted to finding the substrate for the proline hydroxylation reaction. As recently as 1964 (9), much controversy existed even as to the form that proline was in at the time of hydroxylation. Thus, evidence was offered from various investigators which implied that proline hydroxylation took place at the stage of free proline, prolyl adenylate, prolyl-t-RNA, ribosomal proline peptide, or protein-bound proline.

By 1966, much of this controversy had been settled. Udenfriend, in reviewing the latest work done in the field (95), noted that experimental work using ribonuclease and puromycin strongly

indicated that proline is incorporated into a peptide before it is hydroxylated. Therefore, under certain conditions where hydroxylation does not take place, a polypeptide precursor of collagen, protocollagen, accumulates. This polypeptide is proline-rich and lacks hydroxyproline. Thus, the enzyme which catalyzes the hydroxylation of proline is called both proline hydroxylase and protocollagen hydroxylase. Many consider this enzyme to be involved with lysine hydroxylation in the same manner that it is involved with proline hydroxylation. However, some recent work (28) on a highly purified preparation of this enzyme gave no evidence that lysine was a substrate for the enzyme.

At the present time there is debate on whether the peptide is hydroxylated while still attached to the ribosomes or after it is released. Goldberg and Green, using pulse-labeling, concluded that hydroxylation occurred as the polypeptide was being synthesized on polyribosomes (25). Prockop's group (4,64,65) presented data pointing to the conclusion that hydroxylation takes place after the peptides are released from the ribosomes. By using the antibiotic cycloheximide to prevent the release of nascent chains from ribosomes, they showed that as long as the peptides were attached, hydroxylation did not take place. More recently (52), evidence was given that a significant amount of hydroxylation occurs in nascent chains of collagen during the

process of translation. Though the ratio of hydroxyproline to proline is only 50% of that in completed chains, the authors speculate that this may arise because part of each nascent chain may be buried in the interstices of the ribosome and thus may not be available for enzymatic action.

The source of oxygen for hydroxylation is now known to be atmospheric rather than water (20,60). A direct oxygenase mechanism was confirmed by Fujita et al. (21); they observed after preparing cis and trans 4-tritiated-L-proline that only the tritium trans to the carboxyl group was lost during the synthesis of hydroxyproline in chick embryo.

Ascorbic acid is another factor needed for hydroxylation. As yet no clear mechanism of action has been advanced for it, though its role most probably is linked with another co-factor, ferrous ion. It has also been seen that other enediols can substitute for ascorbic acid. Juva (38) has accumulated data on the importance of ferrous ion in the hydroxylation reaction. If  $\alpha$ - $\alpha'$  dipyridyl, a chelator of ferrous ion, is put into the potential hydroxylating system, synthesis of hydroxyproline is halted. Ferric ions will not replace the ferrous ions. Thus ascorbate may serve in keeping the ferrous ions in their reduced state.

The latest requirement noted for the hydroxylation of proline is  $\alpha$ -ketoglutarate (36). Removal of this keto acid with glutamic dehydrogenase,  $\text{NH}_4^+$ , and NADH leads to inactivation of

of the hydroxylase; related compounds such as oxaloacetate or pyruvate fail to restore hydroxylase activity. Kivirikko et al. (42) showed that the  $\alpha$ -ketoglutarate is not consumed stoichiometrically. However, Rhoads and Udenfriend (62) showed a substrate-dependent and stoichiometric decarboxylation of  $\alpha$ -ketoglutarate coupled to the hydroxylation of peptidyl proline residues. By the use of labeled carbon it was shown that only the C-1 and not the C-5 group is decarboxylated. No reason was given for the difference in conclusions except to attribute it to a shortcoming in methodology or an unknown complexity in the hydroxylating system.

Proline hydroxylase has recently been extensively purified (55,59,63). The enzyme seems to preferentially hydroxylate proline in the sequence Gly-X-Pro where X represents alanine or proline, but not glycine. It was noted that partially purified hydroxylase is sensitive to sulfhydryl reagents such as N-ethylmaleimide and PCMB (59). Olsen et al. (55) have looked at the enzyme under the electron microscope. They saw a ring-like structure which, in one instance, aggregated in groups of four, while in another instance did not aggregate. Evidence has also been given that a free sulfhydryl group is necessary for activity (63).

The fibrogenesis of collagen is accomplished by the formation of covalent interchain bonds. This polymerization imparts

stability to collagen. Much of the current research in collagen is concerned with looking at this cross-linking. As noted before, it can be intramolecular as in the case of the  $\beta$  dimer or it can also be intermolecular, occurring between tropocollagen molecules.

Since collagen contains no cysteine or cystine, disulfide bonds can clearly be ruled out as the source of the cross-linking. Also, since the three chains are probably in parallel and in register (41), whereas the molecules are in a staggered arrangement within the fibril (35), it is likely that the locations of the intermolecular bonds differ from that of the intramolecular bonds. However, since lathyrogens (agents which inhibit cross-linking) affect both types of bonds, the mechanism may be similar. In fact, recent work has shown that intramolecular cross-links may not be a separate entity, but may be part of an intermolecular cross-link (40).

The first step in cross-linking has been shown to be the oxidation of a lysine residue to allysine ( $\alpha$ -amino adipic  $\delta$ -semi-aldehyde). An enzyme catalyzing this reaction, lysyl oxidase, has been found and its properties investigated (77,78); it has been purified 440-fold and shown to have a molecular weight of approximately 170,000. Metal co-factors were seen to be necessary for activity of this enzyme. This is consistent with a previous report which noted that copper deficiency resulted in

an inhibition of cross-linking (10). The step after oxidation is believed to be an aldol condensation of two allysine groups, thus forming a cross-link. Lathyrogens such as BAPN ( $\beta$ -amino-propionitrile) are believed to inhibit the oxidation of lysine in the first step. Penicillamine has also been used to prevent cross-linking of collagen. However, use of this agent yields  $\alpha$  chains with a high aldehyde content. It is believed that this agent acts by tying up the aldehyde group, and thus prevents the aldol condensation (15).

In collagen, which has a highly ordered conformation, steric factors may limit the sequence of reactions forming the aldol product (6). It has been seen that cross-linking takes place near the amino terminus of the molecule in a region which, due to its atypical amino acid sequence, cannot form the collagen helix (39).

Most likely, it is this cross-linking which is responsible for different types of collagen in solution. Thus one finds in the literature terms such as neutral-salt collagen, citrate-soluble collagen, acid-soluble collagen, etc. As one might expect, these terms are operational and do not refer to any specific type of collagen.

In summary, collagen is synthesized through translation of m-RNA on the ribosome. The appropriate proline and lysine residues are hydroxylated either while associated with the ribosome or detached, and perhaps a combination of both.

Cross-linking then occurs, both inter- and intramolecularly, thus leading to the formation of collagen fibrils.

The importance of understanding the chemistry of collagen is realized when one investigates wound healing. Ross et al. (66-71) have extensively studied the fine structure of healing skin wounds in humans, rats, and guinea pigs. The inflammatory response was found to be similar in all three cases. The majority of the cells seen during the first 24 hours are polymorpho-neutrophilic leucocytes and monocytes. The neutrophils migrate through the wound and many of them appear to be lysed during the first 24-48 hours, thus releasing their granules. Most of the phagocytosis seems to be effected by the monocytes which predominate at a later time. They ingest both neutrophilic granules and fibrin. Some have speculated that the degranulation of the neutrophils may stimulate the fibroplasia which ensues.

Fibroblasts, the cells which synthesize collagen, begin to appear about 3 to 4 days after wounding, and can be distinguished by their rough endoplasmic reticulum (67). Their ribosomes appear to exist in aggregates of 20 to 40, which take the form of spirals or long rows and have been presumed to be polyribosomes.

By the use of light and electron microscopic autoradiography, the fate of  $^3\text{H}$ -proline in regenerating wound tissue has been followed (67). From these studies, it was proposed that after amino acids enter the cells, collagen precursors and other



secretory proteins are synthesized on the aggregated ribosomes which are attached to the endoplasmic reticulum. Once the collagen precursors have been aggregated into tropocollagen, they are secreted directly from the cisternae of the endoplasmic reticulum into the extracellular space, either by the formation of vesicles which pinch off the rough endoplasmic reticulum, or through the cisternae which are in direct communication with the surface of the cells. It was further proposed that other proteins move from the rough endoplasmic reticulum to the Golgi complex and then to the extracellular space.

Wound healing has been studied in a variety of different systems (9). Samples take from dermal wounds are often utilized. Studies have also been made on abdominal rectus wounds in dogs and also on divided tendons.

A second major category of systems of wound healing is implantation of foreign bodies. The tissue which grows in response to this is granulation tissue (75). Various agents are used to stimulate this response. Probably the most common material implanted is the polyvinyl sponge. The interstices of the sponge permit ready invasion of the granulation tissue. Schilling has done a great deal of work with the implantation of stainless steel wire mesh cylinders (75). Other methods include injection of agents which cause an inflammatory response (carrageenin, turpentine, etc.) (9,75).

There is some debate on whether tissue obtained by the implantation methods cited above really do mimic wound healing tissue. Many works have pointed to a positive correlation between the experimental granulation tissue and wound healing tissue (9,75). However, Bentley points to data suggesting that even though polyvinyl sponge implants and subcutaneous wire mesh cylinders may serve very well for the study of connective tissue metabolism, they do not necessarily reflect an accurate picture of normal wound healing processes (1).

Bentley came to these conclusions while reviewing the synthesis of mucopolysaccharides in healing wounds (1). These substances, also known as glycosaminoglycans, form a major portion of the ground substance in which collagen is laid down. They are for the most part repeating units of a hexosamine and a uronic acid. The polysaccharides vary in the type of hexosamine and uronic acid present. The general pattern in wound healing seems to be that the less highly charged mucopolysaccharides, such as hyaluronic acid, tend to decrease during the early stages of wound healing and the more highly charged molecules, i.e. the chondroitin sulfates, increase. This increase starts about five days after wounding, a time when collagen synthesis is proceeding at a rapid rate. Different theories have been advanced about the role of mucopolysaccharides. They may take part in a mechanism whereby synthesis of other tissue

components is controlled. Some investigators believe that they determine the size and orientation of the collagen fibers, and still others say their production may be merely a reflection of the degree of differentiation of fibroblasts present in the wound. All of this is speculation and further discussion of the role of mucopolysaccharides is outside the scope of this dissertation.

When assessing the state of the healing wound, tensile strength measurements have often been used. Tensile strength, as noted before, has often been used to correlate with collagen concentration. Dunphy and Jackson (17) have shown especially for rat skin, that this relationship is by no means proportional. Jackson concludes that many more factors other than collagen formation will affect the tensile strength (37). For example, lathyrogens, whose effects on cross-linking have been discussed, will affect tensile strength. Tensile strength may also be affected by the type of ground substance laid down and, of course, by a combination of these and other factors.

Bullough, in discussing epidermal wound healing and its mitotic response during this process, feels that the increased mitotic rate is due to a fall in intracellular chalone concentration (8). A chalone is defined as "an internal secretion produced by a tissue for the purpose of controlling by inhibition mitotic activity of the cells in the tissue" (7). The result

then is an increase in mitosis in all cells which are capable of responding, and an increase in ageing in those which cannot. A few chalones have been extracted, though none purified.

Different hormones have been shown to affect wound healing. By far, cortisone and hydrocortisone have received the most attention in this area. They are generally regarded as inhibitors of wound tissue formation, the result of inhibiting the inflammatory response. Bullough (8) has implicated cortisone and norepinephrine, both known as stress hormones, as potentiators of the epidermal chalone, and thus inhibitors of cell mitoses. It has also been shown that cortisone decreases the amount of collagen in the wound tissue (99). Growth hormone, which stimulates formation of granulation tissue (9) and thyroid hormone have both been looked at for their effects on wound tissue formation (50). The effects of the thyroid hormone will be dealt with in the next section.

The effect of protein nutrition on wound healing has received a great deal of attention. It is generally well accepted that a protein-deficient diet inhibits wound healing. Evidence for this has been summed up in review articles (9,75,78). Localio (48) first noted the effect of methionine on wound healing. By using rats on normal, protein-deficient, and methionine-supplemented protein-deficient diets, he showed that the delayed healing in protein-deficient animals could be shifted

toward normal by methionine. It was later shown that cystine was as efficient as methionine (101-104). This is probably due to the fact that methionine is converted to cystine in the body, but the reverse is not known to occur. Later work, involving use of labeled cystine, showed that the cystine bound into proteins by disulfide bonds only, turned over much more rapidly than that bound into the proteins by peptide bonds (100). These facts have been interpreted to indicate that though collagen does not contain cystine, this amino acid may be required for a mechanism used for collagen formation.

CHAPTER III  
THYROID HORMONES

The hormones derived from the thyroid gland are collectively termed the thyroid hormone (2,94). These hormones include L-thyroxine (3,5,3',5'-L-tetraiodothyronine) and L-triiodothyronine (3,5,3'-L-triiodothyronine). These hormones, also called  $T_4$  and  $T_3$ , are released at appropriate times in response to thyroid stimulating hormone (TSH), a hormone of the pituitary gland.

Thyroid hormone biosynthesis involves several steps. First, there is a mechanism by which the thyroid gland traps most of the serum iodide against a concentration gradient (up to 100:1) (3). This step, believed to be oxygen dependent, can be inhibited by various respiratory inhibitors such as cyanide and azide ions. After the iodide ions are trapped, they are then oxidized by a peroxidase to an active form. It is speculated that this active form may be an iodonium ion.

Once the iodide ion is in its active form, it can be used to iodinate tyrosyl residues. These tyrosyl residues are peptide bound in a large glycoprotein (m.w. 660,000) which is stored in the follicles of the thyroid gland. Recent work has shown that iodination occurs on tyrosyl residues which are usually within a specific type amino acid sequence rather than at random (16).

This iodination yields protein-bound monoiodotyrosine (MIT) and diiodotyrosine (DIT).

The next step in the biosynthesis involves coupling of two DIT residues to produce thyroxine or a DIT and a MIT residue to produce  $T_3$ . In both cases, an alanine residue is extruded during the coupling. This also occurs while at least one of the two residues is bound into thyroglobulin. This results in thyroxine and  $T_3$  which are stored as peptide-linked residues in the thyroglobulin.

When thyroid hormone is called for, through the action of TSH, proteolytic breakdown of the thyroglobulin occurs. This is achieved by thyroglobulin protease, an enzyme secreted by the epithelial cells of the follicle. The free  $T_4$  and  $T_3$  are released into the bloodstream, while any MIT and DIT formed during the process is deiodinated by an iodinase. The latter step is considered to be a conservation of iodine since it prevents its loss from the gland.

After the thyroxine and  $T_3$  diffuse into the blood, they are bound to the following serum proteins; a) a globulin which migrates between the  $\alpha_1$  and  $\alpha_2$  globulins (thyroid-binding globulin, TBG), b) a prealbumin (thyroid binding pre-albumin, TBPA), and c) an albumin (thyroid binding albumin, TBA). TBG is considered to be the major carrier in man, while TBPA has been shown to be the major carrier in rats (14). It has been

noted with both rat and human serum that the binding capacities of these carriers are pH dependent. The binding capacity of TBG decreases and that of TBPA increases with an increase in pH in the range of 7.2 to 8.2 (13).

The catabolism of these hormones involves their deamination to thyropyruvates. These entities undergo decarboxylation to form thyroacetates, compounds which exhibit some hormonal activity. They are then conjugated as glucuronides before they are secreted into the bile. It is probable that specific deiodinases remove the iodine so that it can be conserved.

Various agents can be used to prevent the synthesis of these thyroid hormones. Thiocyanate and perchlorate prevent the uptake of iodide by the thyroid gland. The most commonly used agents for this purpose (thiouracil, thiourea, and their derivatives) prevent the oxidation of the iodide to its active form, thus preventing iodination (29,49).

The effects of thyroid hormones can be noted at different levels. One way is by looking at effects on the whole body (86). Probably the most widely known action of thyroid hormones is their calorogenic effect. Thyroxine and triiodothyronine have the capacity to stimulate the basal metabolic rate (BMR). This stimulation is measured in terms of oxygen consumption, and is also accompanied by changes in the pulse and heart rate. The relationship between BMR and thyroid hormone is a quantitative



one as long as excessive doses of hormone are avoided. In assessing the calorogenic effect, one must take into consideration such modifying factors as species, sex, diet and temperature.

Thyroid hormones exhibit an effect on growth and development. For example, hypothyroidism early in life results in dwarfism. Thyroid hormone is not alone in promoting growth for, as seen in the hypophysectomized rat, it acts synergistically with growth hormone to promote a normal resumption of growth. The cretin, an example of hypothyroidism, shows an impaired mentality, indicating poor development of the central nervous system. Quantitatively, the effect depends on the correct dosage of the hormone. Moderate dosages will increase growth. However, larger dosages will not show this effect and in certain cases, will even suppress growth and development. Of course, the effects will become less pronounced with the advancing age of the animal.

Another manifestation of the thyroid hormone on development is seen with its dynamic effect on amphibian metamorphosis. These hormones will cause precocious metamorphosis, while lack of them, as seen in the thyroidectomized tadpole, will prevent metamorphosis. This last activity lends itself well as the basis for a sensitive assay test for thyroid hormone activity. Cohen and his associates (11) have done extensive work in this area. They have found an increase in activity of the enzymes of

the urea cycle after stimulation of metamorphosis with thyroid hormone. They also note that glutamate dehydrogenase has different physical properties than that found before metamorphosis, including different molecular weights and kinetics, thus pointing to an apparent discontinuity in genetic expression during development.

Thyroid hormones, at the molecular level, affect oxidative phosphorylation (86). If thyroxine is added to mitochondrial preparations, it will uncouple oxidative phosphorylation. However, magnesium ions in the system will exert an antagonistic effect on the thyroxine. As far as the relationship of mitochondrial respiration and oxidative phosphorylation to thyroid hormones is concerned, many diverse and conflicting reports offer no apparent answer (86). Tata has stated that the dosage of thyroid hormone needed to uncouple oxidative phosphorylation is in excess of physiological levels (86), though recently, others have argued with this statement (81).

Thyroid hormones may also affect mitochondrial structure and stability. Administration of thyroxine will cause the mitochondria to swell. As with oxidative phosphorylation, magnesium ions exert an antagonistic effect on the thyroxine. Dinitrophenol does the same. There is also a sensitivity factor involved. Mitochondria from liver are more sensitive to this effect than are mitochondria from other tissues (86). Tata notes again the

high concentrations of hormone needed to elicit this effect. In evaluating the mitochondrial swelling, one must also take care to differentiate between the general effect of thyroid hormones on growth and a specific effect on the mitochondria.

Tata and his associates have looked for a primary site of action for thyroid hormone (85,87-93,97). They saw great significance in the fact that there is a latent period before many of the classical effects of thyroid hormone are seen. For example, the calorogenic effect was rejected as a primary action due to its lengthy latent period. Widnell and Tata showed that thyroid hormone stimulated nuclear RNA polymerase preceding a stimulation of microsomal and mitochondrial amino acid incorporation (97). It was also shown that the in vivo incorporation of amino acids into nuclear protein was accelerated in thyroidectomized rats by administration of thyroxine (87). In a later article, Tata showed that the action of the hormone concerned complex changes in turnover and the structural disposition of the ribosomes (90). It is felt that high ribosomal synthesis may reflect the involvement of a ribosomal precursor for the transport of m-RNA. They also note an increase in microsomal phospholipids, which might account for the more complex endoplasmic reticulum (88,89). Tata feels that protein synthesis may involve a simultaneous control of rates at which cytoplasmic RNA and membranes are generated. For this reason, it is concluded that the site

of action of thyroid hormone is most likely a combination of loci (92). Other reports (54) have shown that thyroxine increases the rate of a rapidly labeled RNA fraction which is similar in base composition to tadpole DNA, thus suggesting m-RNA.

Sokoloff et al. (81) have taken exception to some of the results and conclusions of Tata. The critical point on which the differences arise is whether or not low doses of thyroid hormone can elicit an effect on mitochondria. As noted earlier, Tata could see this effect only with high dosages of thyroid hormone, thus characterizing it as a response to a toxic condition. However, it is noted that he sought the effects on oxidative phosphorylation in the presence of bovine serum albumin and relatively high magnesium concentration, both of which are known to reverse the uncoupling action of thyroid hormones (81). Sokoloff noted that a single dose of triiodothyronine to a euthyroid animal stimulates liver microsomal protein synthesis within less than two hours if mitochondria are present in the assay system. This is followed several hours later by a second delayed increase which is not mitochondrial dependent and is associated with an increase in cytoplasmic, mainly ribosomal, RNA. This secondary phase is felt to correspond to the effects noted by Tata. The mitochondrial-dependent effect is considered closer to the primary chemical action of the hormone, since it occurs without any significant latent period and can be seen in

cell-free systems in vitro. Hoch, in a series of articles, has looked at the relationship between thyroid hormones and mitochondria; some of the work pointed to a primary effect of the hormone on the mitochondria (31,32), while other work pointed to the opposite conclusion (33). Needless to say, the search for a primary site of action of thyroid hormone is still in a state of flux in spite of the progress that has been made.

A small amount of work has been done on the effect of thyroid hormone on tissues involved in wound healing. In wounds, Moltke noted an inhibition of wound healing as indicated by tensile strength measurements (53). However, others (50) have failed to see this effect on tensile strength by thyroxine. It should also be noted that the dosage of thyroxine given in Moltke's experiment exceeded the physiological requirement. The results of work with collagen (43,46) suggest that the rate of collagen synthesis is decreased both in hypothyroidism and hyperthyroidism. Kowalewski (44) noted that hypothyroid rats showed a deficiency in the biosynthesis, and an altered solubility, of collagen.

The effect of thyroid hormone on mucopolysaccharides of connective tissue has also been examined (72-74). It was found that concentrations of hyaluronic acid are increased and that of chondroitin sulfates decreased in hypothyroid rats. Administration of thyroxine reversed these changes. It was believed that

the action of thyroxine in the above case occurs in the latter stages of synthesis or possibly even on a degradative pathway (73).

CHAPTER IV  
THE PROPOSED STUDY

The primary purpose of this work will be to study protein synthesis in wound tissue as it is affected by different levels of thyroid hormone. It will be seen whether it is possible or not to distinguish the effects of thyroid hormone in wound tissue (44,53) by following protein synthesis.

First, the rate of incorporation of tritiated proline will be used to study the rate of protein synthesis. This amino acid is chosen because of its high level in proteins in wound tissue and also for its ability to give rise to hydroxyproline (95), an amino acid which is a convenient marker for collagen. In this way, it should be possible to distinguish differential effects between the synthesis of collagen and that of non-collagenous (cellular) proteins.

Different groups of rats, which have been given standardized wounds, will be used to carry out this study. They will be put into different thyroid states through the use of propylthiouracil and desiccated thyroid powder. The wounded rats will also be given a protein-depletion diet in order to slow down the rate of healing. Tissue samples will then be harvested five, eight, and twelve days after wounding.

To further investigate protein synthesis, the incorporation of labeled cystine will also be studied. This amino acid will only be incorporated into the non-collagenous proteins of the wound tissue (18). An attempt will also be made to distinguish between cysteine which is incorporated by disulfide bonds only and that which is bound into proteins by peptide bonds (100).



CHAPTER VMATERIALS AND METHODS

In all experiments, the regenerating wound tissue was obtained from Sprague-Dawley derived virgin female rats (Abrams Small Stock Breeders, Inc., Chicago). These animals have been shown to tolerate the wounding procedure rather well (101). When they were received, the rats were placed in individual cages. They were given water ad libitum and fed Purina rat chow diet. The rats were used for wounding after their weight had reached  $200 \pm 20$  grams. All rats were received from the supplier at the same time in order to minimize seasonal and population variations, factors which could affect rates of protein synthesis.

After the animals were stabilized, they were wounded by a standardized procedure (100,101,105). The animals were anesthetized with nembutal (sodium pentobarbital); the solution (10 mg/ml) was administered subcutaneously to the rats at the rate of 25 mg per kg body weight. After the animals submitted to the anesthetic (15 to 20 minutes), the hair on the scapular region was clipped and an outline of a four cm diameter circle was traced in the area. The skin of this demarcated area was then excised, and the wound blotted with cotton moistened in 95% ethyl alcohol. Very small amounts of bleeding were seen with this procedure. The animals were then returned to their individual cages. This treatment gave rise to a survival rate of over 99%.

From the time of wounding, the rats were placed on a protein-depletion diet (Nutritional Biochemical Co., Cleveland, Ohio). The diet, by virtue of slowing down the healing process (98), allowed later stages of wound healing to be examined. The rats were given 10 grams/day of the diet, an amount noted to be completely consumed. This diet of course gave rise to symptoms indicative of protein deficiency such as loss of weight and a sparse scraggy coat. Water was still given ad libitum.

To gain a pattern of the course of protein synthesis during wound healing, the tissues were harvested at five, eight, or twelve days after wounding. Thus, one group of sixty animals was harvested after five days, another after eight days, and the third after twelve days. The rats were anesthetized with sodium pentobarbital, as in the wounding procedure. Approximately 15 minutes later, the rats were given 45  $\mu$ Ci subcutaneously of  $5\text{-}^3\text{H-L-proline}$  in one ml of isotonic saline (Schwarz-Mann, Orangeburg, N.Y., lot no. 6902, specific activity = 5.1 Ci/m mole). Then, at intervals of 40, 80, 120, or 180 minutes after the administration of the tritiated proline, the regenerating wound tissue was harvested. This was accomplished firstly by removing the clotted fibrin scab. The exudate from it was wiped from the underlying tissue. The regenerated wound tissue was then removed, taking care not to include any muscle fibers or any outlying tissue such as skin (101, 105). If blood was present on

the wound tissue, it was daubed off at once. The tissue was then immediately frozen in a dry ice sandwich to prevent any further incorporation of the amino acid, and stored in the frozen state until the analyses were performed (105). During the harvesting of the regenerating wound tissue, a piece of liver tissue was also taken for purposes of comparison.

The animals in each of the three groups mentioned above (i.e. five, eight, and twelve days post-wounding) were further subdivided into three more groups by differences in their dietary regimen. The drinking water of all animals was changed to a propylthiouracil solution (1mg/10 ml) seven days prior to the harvesting of the tissue. This antithyroid agent has been shown to inhibit the endogenous production of the thyroid hormone by blocking the activation of iodine (29, 49). Two days later, (i.e. five days prior to the harvesting of the wound tissue), the rats were divided into the three subgroups by the amount of desiccated thyroid powder (3X) (Sigma Chemical Company, St. Louis), which was added to their protein-depletion diet. One group received none, thus giving rise to a hypothyroid group. The second group, which was given 0.67 mg/day of the powder, a physiologically adequate amount (45,76), was labeled the euthyroid group. The third group received 6.67 mg/day, a large excess, thus giving rise to the animals that were labeled hyperthyroid.

In this manner, nine groups of twenty rats each have been created. The five day group included 20 hypothyroid, 20 euthyroid, and 20 hyperthyroid rats. The eight and twelve day groups were subdivided in the same manner. In addition, each of these nine groups include tissue which has been harvested at four different times after the administration of the labeled proline, i.e. after 40, 80, 120, or 180 minutes after injection. All 180 samples of the tissue collected were then analyzed for proline, hydroxyproline, labeled proline, and labeled hydroxyproline.

The protocol for the experiment with  $^{35}\text{S}$  cystine follows much the same pattern as that described for the proline experiment. In this case only tissue taken from rats wounded for eight days was used. The results obtained in the experiments with proline obviated the need for observation of five and twelve day old wound tissue. The eight day group was subdivided into hypothyroid, euthyroid, and hyperthyroid groups as noted above. At harvesting,  $33.3 \mu\text{Ci}$  of L- $^{35}\text{S}$ -cystine was administered (Schwarz-Mann, Orangeburg, N.Y. lot no. Wr 2002, specific activity =  $20.3 \text{ mCi/m mole}$ ). Then 30, 60, 90, or 150 minutes later (100), the tissue was harvested in the manner described above, and then frozen until analysis was undertaken. Though labeled cystine is administered, there is no doubt that amino acid incorporation occurs when it is reduced to cysteine. However, since cysteine

is much less stable than cystine, and also to correlate with previous experimental data, labeled cystine was the amino acid of choice.

Differences between the three groups appeared in the amount of weight lost between the time of wounding and harvesting. Thus the weight loss of the hyperthyroid group was significantly greater than each of the other two groups. The hyperthyroid animals also manifested some differences in behavior; they were much more irritable and jumpy than those in the other two groups.

#### Tissue Sample Analysis for the Studies with Tritiated Proline

Each tissue was individually analyzed. The tissue was weighed on a Roller-Smith balance and then transferred to a 16 x 150 mm pyrex, Potter-Elvehjem homogenizer with 2.0 ml of 5% trichloroacetic acid. The pestle of the homogenizer was attached to a motorized drill press and the tissue homogenized for five minutes. This treatment enabled the denaturation and precipitation of the protein to take place, thus hindering any further metabolism. The homogenate was then transferred into a threaded culture tube along with four washings of 5% trichloroacetic acid to give a total volume of 10 ml. The proteins were then centrifuged down in a desk-top clinical centrifuge (IEC head #1809) at 1800 g for five minutes. The supernatant was then decanted and saved for analysis.

The protein pellet was defatted in the following way. It was first suspended in 3.0 ml of acetone. Then after centrifugation, the supernatant was discarded. The resulting precipitate was then resuspended in 3.0 ml of a 1:1 acetone-ether mixture, centrifuged, and the supernatant discarded.

The resulting protein pellet was then subjected to hydrolysis. Various types of hydrolyses were carried out, before selecting acid hydrolysis at 120° C for eighteen hours. The hydrolyzing mixture was 1.5 ml of 6 N HCl. Though some of the amino acids (e.g. tryptophane) may be degraded in this procedure, the amino (imino) acids of interest, proline and hydroxyproline, are much more resistant to this type of treatment (18).

The analytical procedures for the determination of proline and hydroxyproline are based on the oxidation of the free imino acids in the hydrolysate, followed by their selective extraction into toluene (56,82). One problem, however, in dealing with biological materials in this procedure is that the hydrolysate frequently contains pigments or humin. These pigments are extractable into toluene and therefore, they will interfere in any colorimetric reaction. For this reason, various means have been tried to remove this pigment. The method used here employed a mixture of activated charcoal and a cation exchange resin (56). The charcoal-resin used was a 1:2 w/w mixture of activated

charcoal and ion exchange resin in the chloride form (Dowex 1 X 8, 200-400 mesh). This mixture was first washed in a sintered glass filter with 6 N HCl and then dried with 95% ethyl alcohol and ether.

The hydrolysate was first made up to a 10.0 ml volume with distilled water. This was in turn transferred to another screw-capped culture tube, and 0.25 gm of the charcoal-resin mixture was added. The tube was then tightly capped and shaken vigorously by hand for one minute, followed by centrifugation at 1800 g for 15 minutes. This treatment resulted in a change from a yellowish-brown solution to a clear aqueous solution.

For the subsequent assays, a 3.0 ml aliquot was removed from the clarified solution. The acidity in this hydrolysate was then neutralized, using one drop of 1% phenolphthalein solution as an indicator. The aliquot was titrated first with 10 N NaOH, until a faint pink tinge was seen, then with 0.1 N HCl, and finally with 0.1 N NaOH.

Studies have shown that the pH used for the oxidation of the imino acids is crucial (56,61,82). It was noted that the reaction proceeds most efficiently at a pH of 8.3 for both imino acids, proline and hydroxyproline. To accomplish this, 0.25 M sodium pyrophosphate buffer, pH 8.3, has been used (56,82). However in this laboratory, and as pointed out by others (47),

at this strength of buffer, crystallization occurred. Therefore, a 0.2 M buffer was used, which proved satisfactory.

After 2.0 ml of the pyrophosphate buffer was added to the neutralized hydrolysate, the solution was ready for the most critical step of the procedure, that of the oxidation of the imino acids. Various oxidants have been used for this step, including sodium hypochlorite, hydrogen peroxide, and sodium hypobromide. Although these oxidants may be adequate if used with precautions, they are relatively unstable and variations arise in their oxidative capacity from one preparation to another. (61).

Chloramine-T is now the most commonly used oxidant in this procedure (61), and is practicable in that it can be obtained in a stable convenient form. However care must be taken in the use of this reagent also. If too little chloramine T is used, some of the imino acids will not be oxidized. However, if too much is added, the amount of the oxidized imino acids will also decrease, due to polymer formation (56,61,82). The need for standardizing the variable amount of amino acids in a hydrolysate then arises. To accomplish this an excess of alanine was added to the mixture, and higher levels of chloramine T were used (61).

With this in mind, a 10% solution of DL-alanine was made up and buffered to pH 8.3. 0.2 ml of the alanine solution was then added to the buffered reaction mixture. Then 1.0 ml of a freshly



prepared 0.25 M chloramine T solution was added, and the reaction mixture was stirred immediately (Vortex Genie Mixer). Exactly 20 minutes later, the oxidation was stopped by adding 0.5 ml of a 2 M sodium thiosulfate solution, and this was again followed by immediate mixing. It was found that results were not precise if the time for oxidation was not strictly adhered to, once again stressing the complexity of this reaction and the need for complete standardization.

After the oxidation, the mixture was again buffered with 1.0 ml of the pyrophosphate buffer. It has been shown that pH 8.3 is satisfactory for the extraction of the proline derivative into toluene. To the newly buffered mixture is added 5.0 ml of toluene. The tube is then tightly capped with a teflon-lined cap. Though extraction has been accomplished by placing the tube in a horizontal shaker unit (61), best results were obtained here with vigorous shaking by hand in a vertically orientated motion, for approximately one minute. The toluene phase then contained the proline oxidative product while the hydroxyproline product remained in the aqueous phase (56,82).

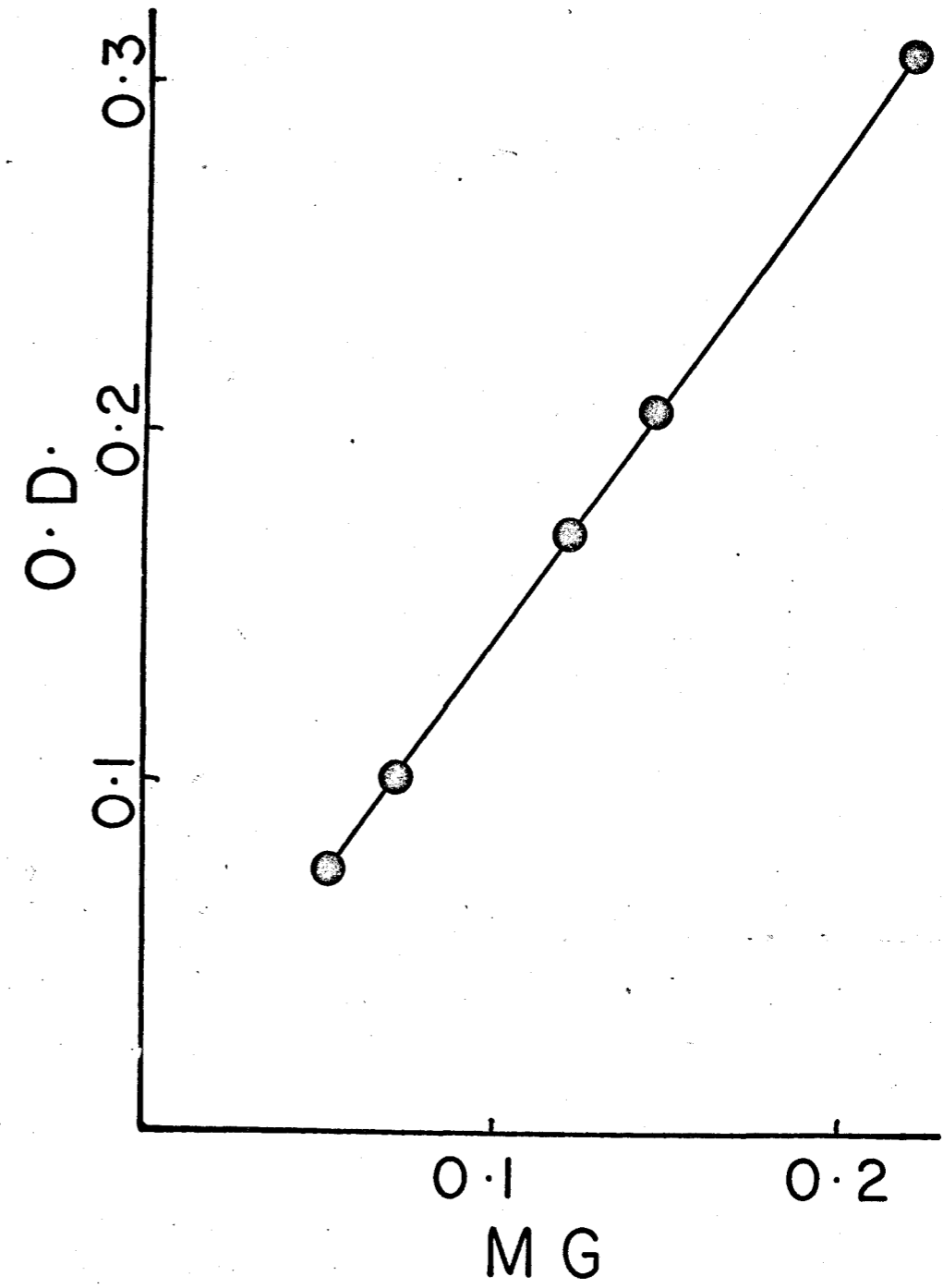
From this toluene layer, two 1.0 ml aliquots were removed. The amount of proline in these aliquots was then determined spectrophotometrically by reaction with a ninhydrin reagent. The ninhydrin reagent was freshly made up by addition of 2 gm of 1,2,3 indanetrione monohydrate to a mixture of 95 ml of glacial

acetic acid and 10 ml of phosphoric acid. Then 2.5 ml of this ninhydrin reagent was reacted with the 1.0 ml aliquots of the toluene layer and heated for one hour (82). Various methods were attempted for heating this mixture, such as 100° C in a drying oven or in a boiling water bath. Maximum color development was obtained with the use of the boiling water bath, but great care had to be taken in tightening the caps on the culture tubes to insure that no steam condensate could enter into the reaction mixture.

After the tubes had cooled sufficiently, the optical density was read at 420 nm in a Beckman DU spectrophotometer equipped with a Beckman power supply. The blank consisted of 1.0 ml of toluene added to 2.5 ml of ninhydrin. All readings were taken in a matched set of Beckman cells. The proline content of the samples was obtained by reference to a standard curve (Fig. 1), which was obtained by analyzing known amounts of proline through the procedure outlined above. Proline was also determined in the presence of a known amount of albumin. The results showed that the albumin always added a constant amount to the optical density reading, pointing to the linearity of this reaction during hydrolysis of proteins.

From the remaining 3 ml of the toluene phase, a 2.0 ml aliquot was pipetted out for measurement of radioactivity. This aliquot was added to a glass counting vial which contained 4.0

Fig. 1: Standard curve for the determination of proline, expressed as optical density at 420 nm against concentration.



ml of a counting solution. The counting solution was prepared by dissolving 4.0 gm of 2,5-diphenyloxazole (PPO), 0.1 gm 1,4-bis-2-(5-phenyloxazolyl) benzene (POPOP) in one liter of toluene. The fact that the radioactivity was already in toluene obviated the use of more complex dioxane-based cocktails, which can often show signs of instability. The radioactivity was measured with a Beckman LS-250 liquid scintillation spectrometer. All samples were counted to a 2 sigma error of 2%. All material showed at least a 10 fold excess over background, and duplicate counts showed only random variation, i.e. within 2%. Radioactivity in the supernatant of the initial trichloroacetic acid homogenate was also determined in the same way.

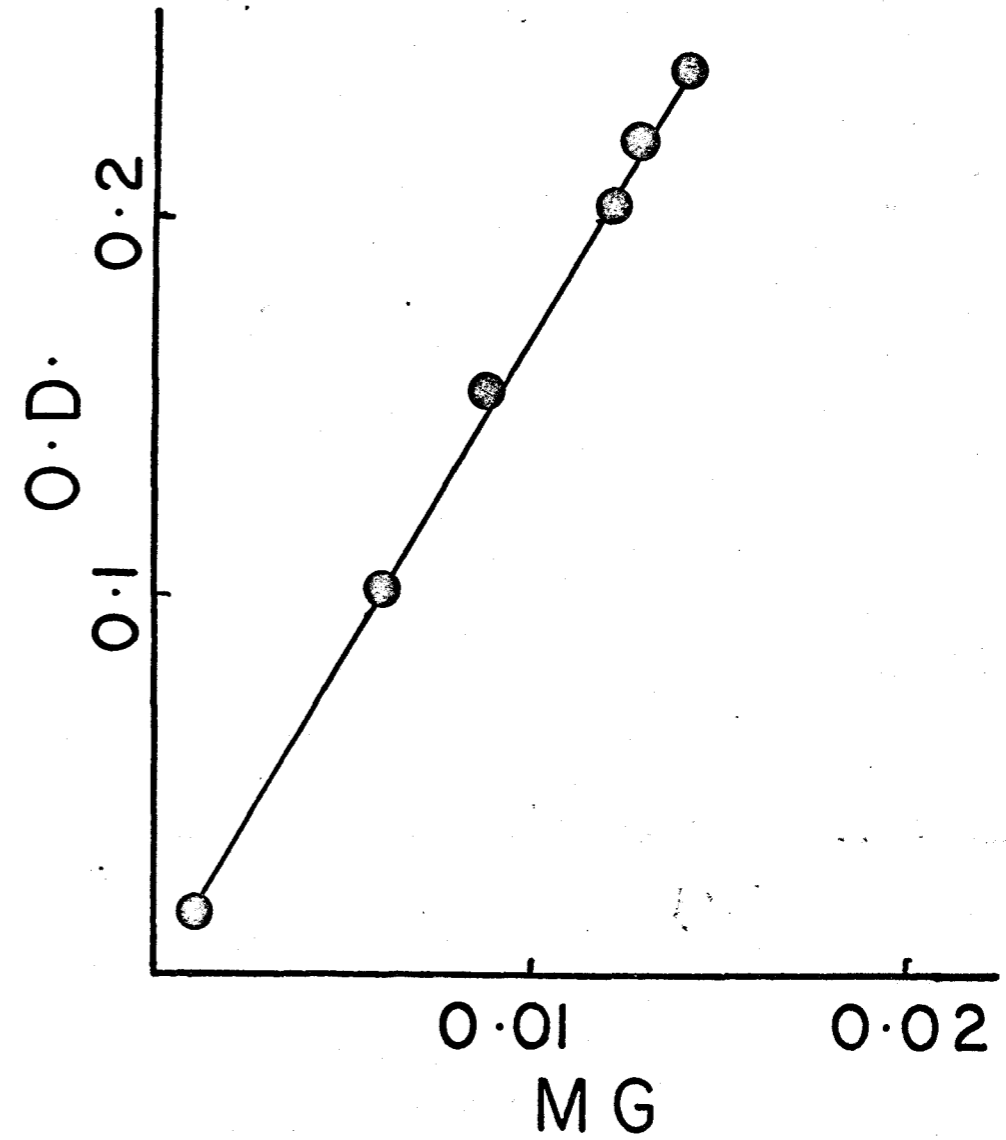
The remainder of the toluene phase was then pipetted off. Further, another 5 ml of toluene was added and then pipetted off to insure against any carryover of proline into the next extraction. The culture tube, now containing only the aqueous phase, was tightly capped and placed in the boiling water bath for 25 minutes. This served to decarboxylate the oxidative product of hydroxyproline, thus enabling it to be extracted into another aliquot of toluene. After the solution was cooled, the hydroxyproline derivative was extracted by adding another 5.0 ml of toluene. The tube was again tightly capped and shaken as before. This phase is referred to as the hydroxyproline assay fraction.

The determination of the amount of hydroxyproline was arrived at by reaction of a portion of this assay fraction with p-dimethylaminobenzaldehyde reagent (Ehrlich's reagent). This reagent is prepared by adding 120 gm of p-dimethylaminobenzaldehyde to 200 ml of 95% ethanol. To this mixture was added another 200 ml of a 95% ethyl alcohol solution containing 27.4 ml of concentrated sulfuric acid. This solution could be stored in the refrigerator for several weeks. The recrystallization which ensued on cooling was readily redissolved on warming of the solution (61).

In the assay for hydroxyproline, 0.2 ml of the assay fraction was made up to 3.0 ml with toluene. To this was added 2.0 ml of p-dimethylaminobenzaldehyde reagent. After waiting 20 minutes for full color development of the chromophore, the optical density was read at 560 nm, again on the Beckman DU spectrophotometer. As with proline, a previously derived standard curve was used to measure the amount of hydroxyproline in the reaction mixture (Fig. 2). The optical density reading should be taken within an hour, for after this time, the intensity of the chromophore slowly starts to wane.

Another 3.0 ml of the hydroxyproline assay fraction was pipetted out and transferred to 4.0 ml of the counting solution in order to measure the amount of radioactivity of the hydroxyproline derivative. All counting was done in the same manner as that for proline.

Fig. 2: Standard curve for the determination of hydroxyproline, expressed as optical density at 560 nm against concentration.



The analytical procedures were also applied to liver tissue. Since this tissue is mainly parenchyma, with very little collagen except that in interlobular septa and blood vessels, it was believed that the effects of different levels of thyroid hormone on protein synthesis in this tissue would serve as another indication of the effects of the hormone on non-collagenous protein synthesis.

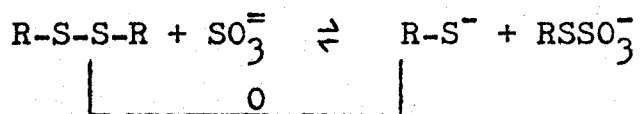
Another experiment was designed to look at the levels of hydroxyproline in the urine in animals of the groups termed euthyroid and hyperthyroid. This was done in animals eight days after wounding. All procedures were the same with the exception of the collection of the urine. After administration of the labeled proline, while the animal was still anesthetized, the urethra was clamped with a hemostat. Then, 180 minutes later, the animal's abdomen was opened and the urine in the bladder collected by puncturing the wall of the organ with a syringe needle and then taking up the urine. The urine was treated analytically the same as the wound tissue, except that there was no homogenization and the time period for hydrolysis was only three hours.

#### Tissue Sample Analysis for the Studies with Labeled Cystine

The wound tissue was first weighed on the Roller-Smith balance. Different media for homogenization were tried for this

tissue. 0.25 M sucrose was used and worked well with liver tissue, but did not allow for a fine enough suspension when used for wound tissue. It was also very difficult to fully homogenize the tissue in sucrose. 5% trichloroacetic acid was finally decided upon as the homogenizing medium. The tissue was homogenized for five minutes in the trichloroacetic acid, the homogenate centrifuged, and the supernatant discarded. The resulting pellet was then suspended in 10.0 ml of 0.1 M sodium carbonate. Aliquots of this fine suspension were next dialyzed against sodium sulfite and sodium carbonate (1.00).

Sodium sulfite has been shown to cleave disulfide bonds in the following reaction:



Sulfitolysis can be used to convert all the half-cystine residues of a protein to the S-sulfocysteine residues. The mercaptans which are formed are then oxidized back to the disulfide which then undergoes sulfitolysis again. Cupric ions are known to accelerate the reoxidation (12,100).

Once all the sulfhydryl and disulfide bonds have been converted to the S-sulfocysteine (thiosulfate) derivatives, it should be possible to separate the derivatives of half-cystine residues which are still peptide bound from those that are not, i.e. those which were originally disulfide bound only. The



smaller molecular weight derivatives can be removed from the solution carrying the protein thiosulfates by the process of dialysis.

A 1.0 ml aliquot therefore was taken from the sodium carbonate suspension and put into dialysis tubing. This was placed in four liters of a 0.03 M sodium sulfite solution, pH 7.8. 1.5 ml of a 0.08 M cupric nitrate solution in 1.0 M  $\text{NH}_4\text{OH}$  was added to the sodium sulfite solution (100). Thus, in this way, both the reaction of the sulfite and the subsequent dialysis of the smaller derivatives take place. The four liter vessel was then placed for four hours on an Eberbach reciprocal shaker, a time sufficient for both the reaction and dialysis to take place (100). Thus left in the tubing is that cysteine which was bound by peptide bonds.

A duplicate 1.0 ml aliquot of the carbonate suspension was dialyzed against four liters of 0.03 M sodium carbonate, pH 7.8 with the addition of the cupric nitrate solution. In this manner, the total amount of radioactivity of the incorporated cystine, i.e. both peptide bound and disulfide bound, is determined. Thus, by using the results from both the sulfite and carbonate dialyses, one can also calculate the relative amount of cysteine bound by disulfide bonds only.

The dialysand remaining after the four hours of dialysis was then counted with the liquid scintillation spectrometer. Due to the fact that this was an aqueous suspension, the toluene fluor

used above was inappropriate. The counting fluor in this case was prepared first by dissolving 6.0 gm PPO in one liter of toluene. To every 100 ml of this solution was added 20 ml of Beckman solubilizing reagent (Bio-Solv BBS-3). The resulting solution was the toluene fluor used in this case. The dialysand was then added to 10.0 ml of the counting solution and radioactivity determined using a  $^{14}\text{C}$  window. This is possible because of the almost identical beta spectra of  $^{14}\text{C}$  and  $^{35}\text{S}$ , both having the same mean energy (0.05 MEV). One property of  $^{35}\text{S}$  that must be taken into account is its half-life of 87 days. For this reason, all  $^{35}\text{S}$  samples were counted within a 48 hour period so differences due to the decay rate were negligible.

CHAPTER VIEXPERIMENTAL RESULTS

The procedures outlined in the previous chapter were designed to follow protein synthesis in regenerating wound tissue while it is under the influence of different levels of thyroid hormone. To accomplish this, the rate of incorporation of labeled proline or labeled cystine was measured.

The experimental design with labeled proline enabled the resulting data to be organized in several ways. First, the incorporation of proline into the total amount of protein could be measured; second, by virtue of proline's conversion to hydroxyproline, the rate of collagen synthesis was ascertained; third, the proline incorporated into non-collagenous (cellular) proteins, could also be obtained. Also, since the proline incorporation was studied at different stages of regeneration, the rates of the above three parameters could be measured to indicate the pattern of protein synthesis as the wound tissue matures.

The incorporation of labeled cystine was studied at only one stage of regeneration. This amino acid, which is not incorporated into collagen, enabled the measurement of the rate of synthesis of cellular proteins. Also, since cystine can be incorporated by peptide or disulfide bonds, the amounts of each type of incorporation could be derived.

### Experiments using $^3\text{H}$ -proline

The amount of radioactive proline in the supernatant of the trichloroacetic acid homogenate was measured by the methods described in the previous section. In this way an understanding of the pool size of the amino acid available for incorporation into proteins could be gained. It was seen that the amount of free proline (i.e. unincorporated) decreases with an increase in the time interval between administration of the label and the harvesting of the tissue (Fig. 3). In this case, the results are shown from animals which had been wounded for five days. All three subgroups (hypothyroid, euthyroid, and hyperthyroid) showed the same rate of decrease. The same pattern was seen in wound tissue of animals which had been wounded for eight and twelve days. This pattern was also seen in liver tissue.

To measure the amount of proline which was incorporated into proteins of the wound tissue, the pellet of the trichloroacetic acid homogenate was analyzed. It was seen that the highest rate of incorporation of proline occurred on the eighth day after wounding (Fig. 4) in the euthyroid rats, with the lowest rate observed on the fifth day. The same relationship was seen to exist in the hypothyroid (Fig. 5) and hyperthyroid (Fig. 6) groups, i.e. a higher rate of activity on the eighth day than on either the fifth or the twelfth day.

Fig. 3: The amount of  $^3\text{H}$ -proline found in the trichloroacetic acid supernatant (free  $^3\text{H}$ -proline) measured in terms of cpm proline per gram of tissue (wet weight) against time after administration of  $45 \mu\text{Ci}$  of  $5\text{-}^3\text{H}\text{-L-proline}$ . The data is obtained from wound tissue harvested five days after wounding. All data points represent the mean of measurements on five separate tissue samples.

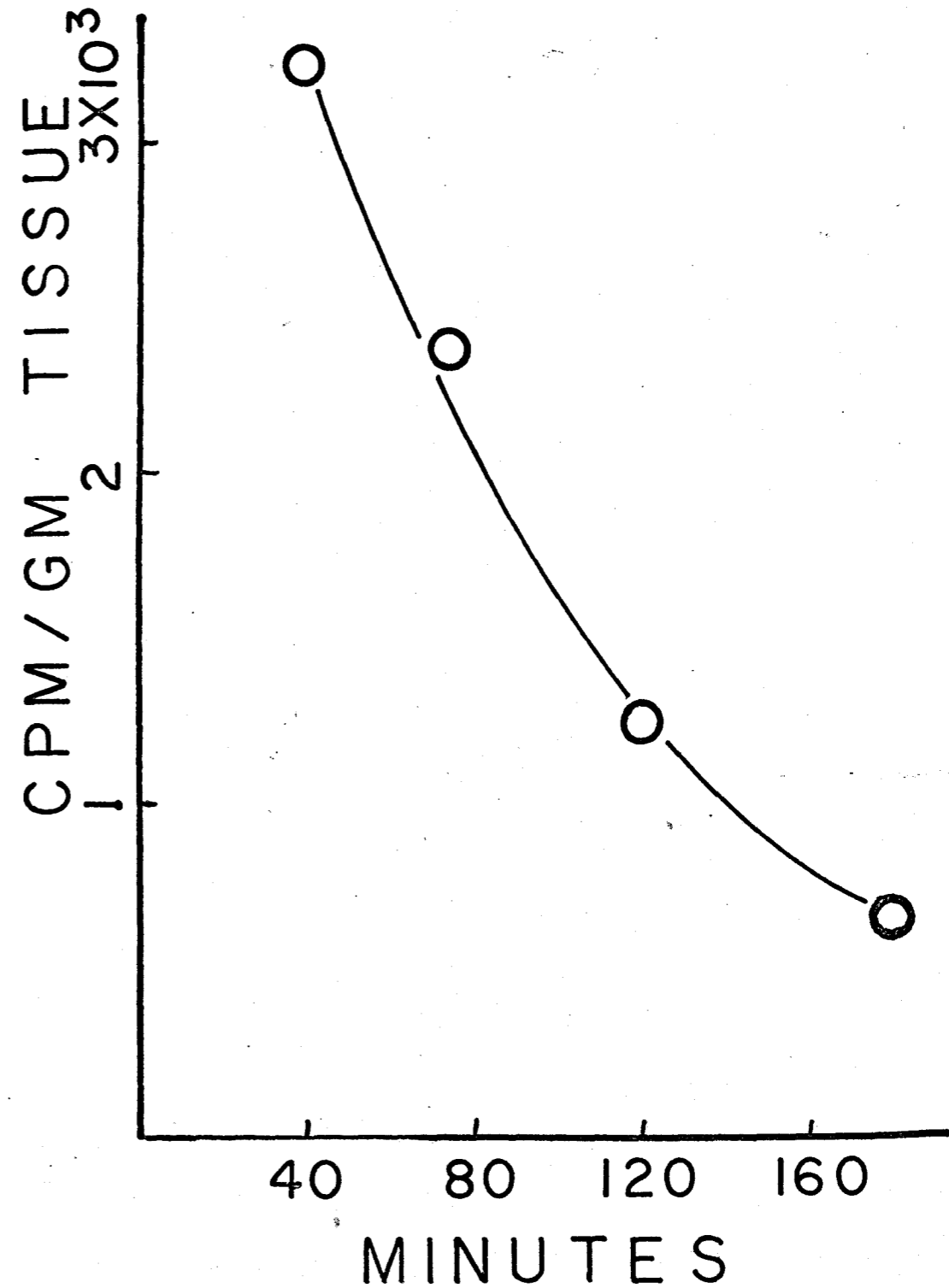


Fig. 4: The rate of synthesis of the total protein in wound tissue of the euthyroid animals measured in terms of cpm proline incorporated per gm of tissue (wet weight) against time after administration of 45  $\mu$ Ci of 5- $^3$ H-L-proline. The data obtained from wound tissue on the 5th day after wounding is indicated by open circles; 8th day, half-solid circles; 12th day, solid circles. All data points represent the mean of measurements on five separate tissue samples. Each data point on the 12th day is significant when compared to that of the other two groups (t test,  $p < 0.05$ ).

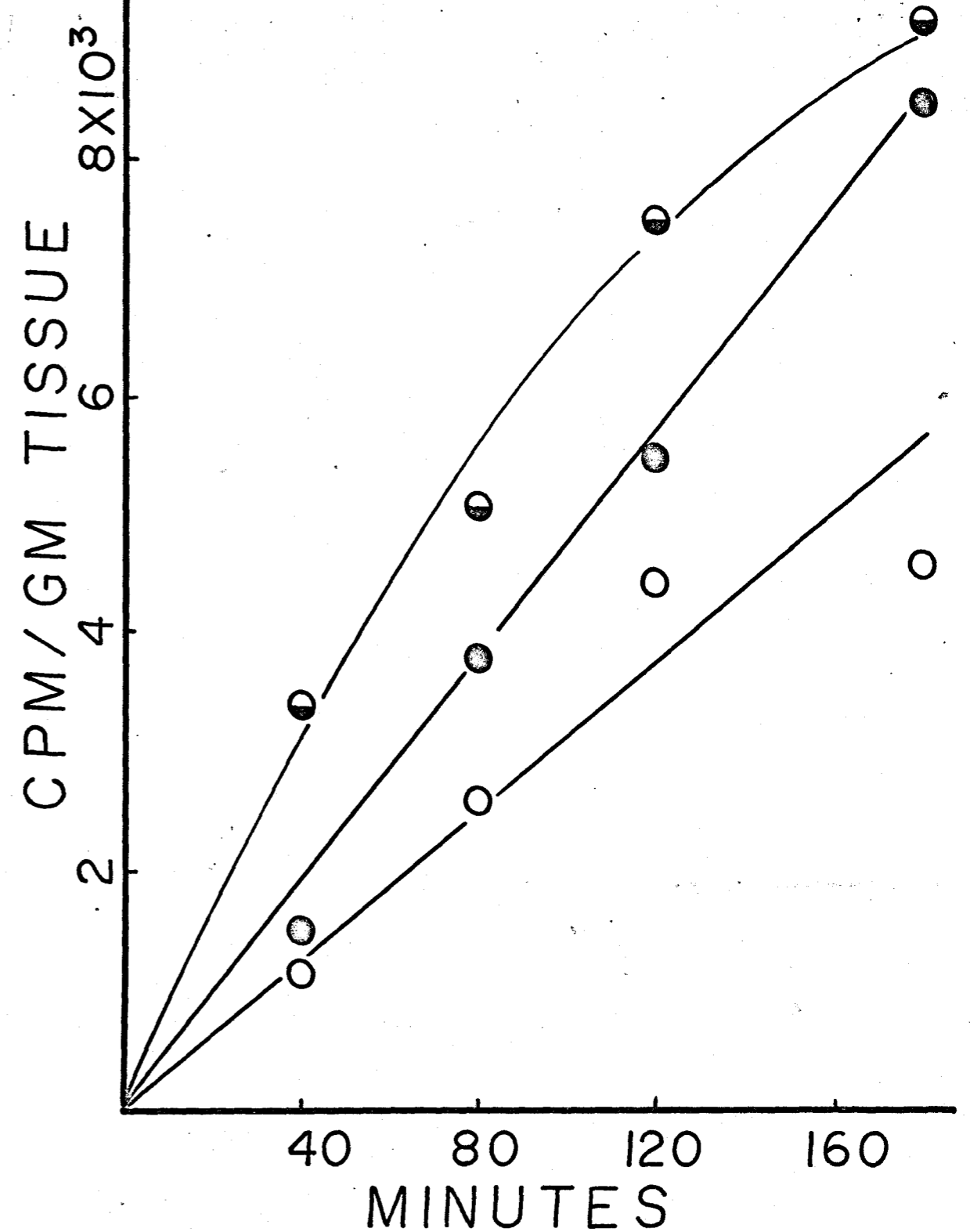


Fig. 5: The rate of synthesis of the total protein in wound tissue of the hypothyroid animals measured in terms of cpm proline incorporated per gm of tissue (wet weight) against time after administration of 45  $\mu$ Ci of 5- $^3$ H-L-proline. The data obtained from wound tissue on the 5th day after wounding is indicated by open circles; 8th day, half-solid circles; 12th day, solid circles. All data points represent the mean of measurements on five separate tissue samples. Each data point on the 12th day (except at 40 minutes) is significant when compared to that of the other two groups (t test,  $p < 0.05$ ).

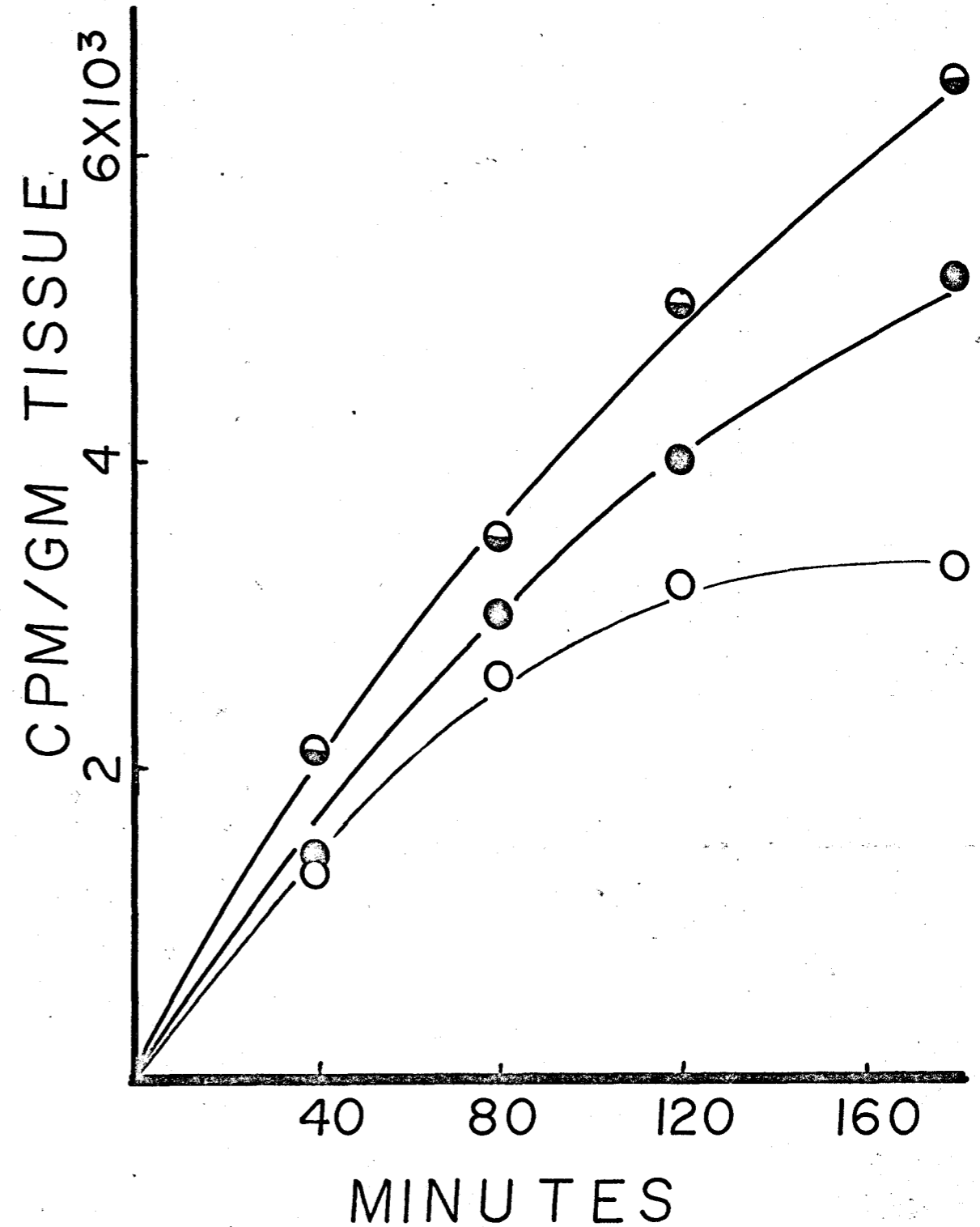
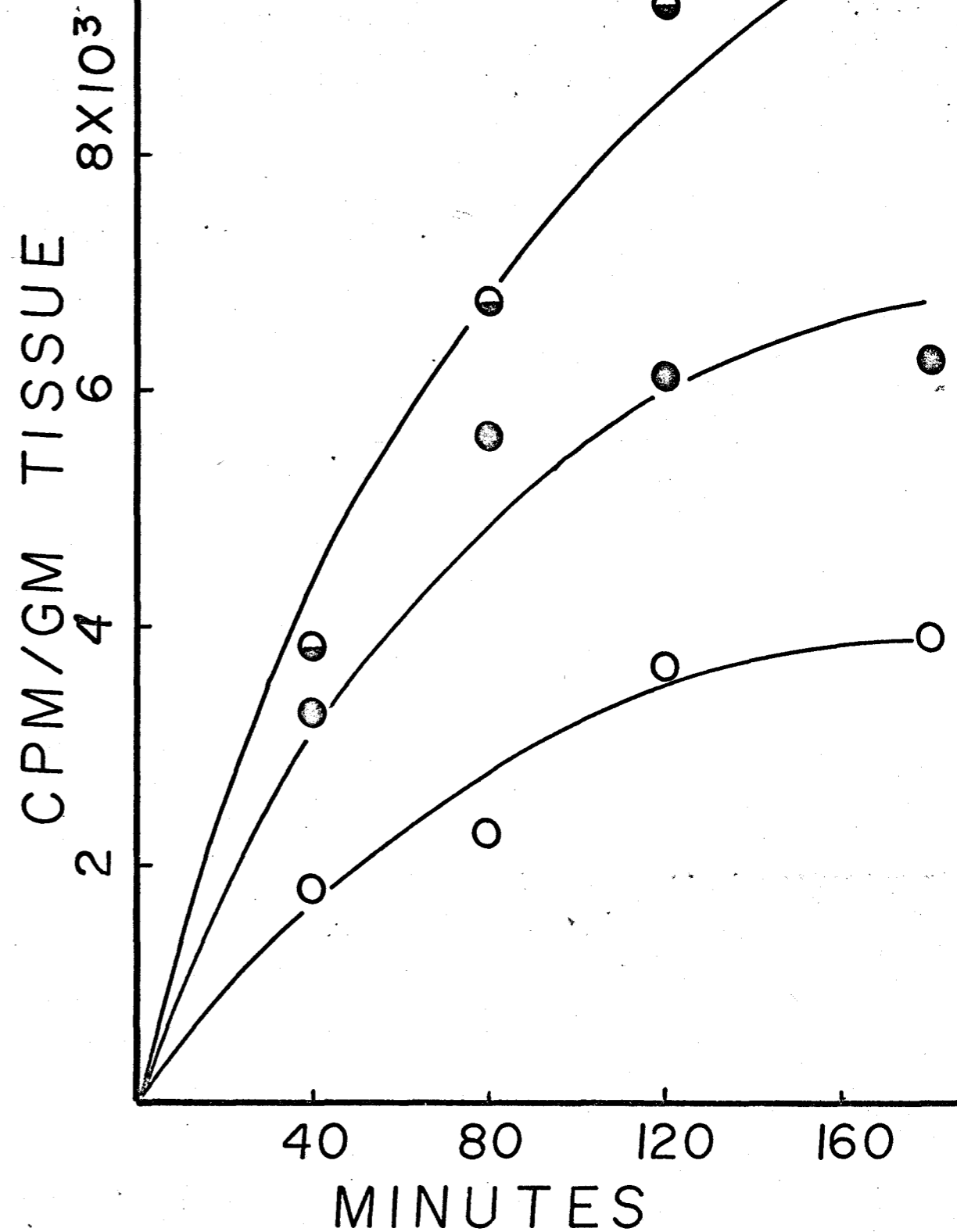


Fig. 6: The rate of synthesis of the total protein in wound tissue of the hyperthyroid animals measured in terms of cpm proline incorporated per gm of tissue (wet weight) against time after administration of 45  $\mu$ Ci of 5- $^3$ H-L-proline. The data obtained from wound tissue on the 5th day after wounding is indicated by open circles; 8th day, half-solid circles; 12th day, solid circles. All data points represent the mean of measurements on five separate tissue samples. Each data point on the 12th day is significant when compared to that of the other two groups (t test,  $p < 0.05$ ).





Collagen becomes the predominant protein as the wound tissue matures (27). The rate of formation of this protein was determined from the  $^3\text{H}$ -hydroxyproline content of the tissue at different intervals after administration of the  $^3\text{H}$ -proline. From the  $^3\text{H}$ -hydroxyproline content and by utilizing the well characterized amino acid composition of collagen (18,58), the theoretical amount of  $^3\text{H}$ -proline which was bound into collagen was calculated. It was assumed that the rate and degree of hydroxylation was the same for the  $^3\text{H}$ -proline as for the unlabeled proline. The difference between the  $^3\text{H}$ -proline in collagen and the total  $^3\text{H}$ -proline in the tissue may be assigned to the cellular (non-collagenous) proteins. Fig. 7 shows the rate of synthesis of cellular proteins in wound tissue of the euthyroid animals at the three stages of regeneration that were studied. The changes in the rate of synthesis of the cellular proteins follow a pattern similar to that seen in Figures 4-6, i.e. the highest rate is seen on the eighth day. As was the case with total protein synthesis, the hyperthyroid and hypothyroid groups show the same relationship as does the euthyroid group.

The rate of formation of  $^3\text{H}$ -hydroxyproline, resulting from hydroxylation of peptide-bound  $^3\text{H}$ -proline (95), was used to measure the rate of collagen synthesis. Fig. 8 shows the  $^3\text{H}$ -hydroxyproline content of tissue from euthyroid rats plotted against time after the administration of  $^3\text{H}$ -proline. It can be

Fig. 7: The rate of synthesis of cellular proteins in wound tissue of euthyroid rats presented as cpm proline incorporated per gm of tissue (wet weight) against time after administration of 45  $\mu$ Ci of 5- $^3$ H-L-proline. The data obtained from wound tissue on the 5th day after wounding is indicated by open circles; 8th day, half-solid circles; 12th day, solid circles. All data points represent the mean of measurements on five separate tissue samples. Each data point on the 12th day is significant when compared to that of the other two groups (t test,  $p < 0.05$ ).

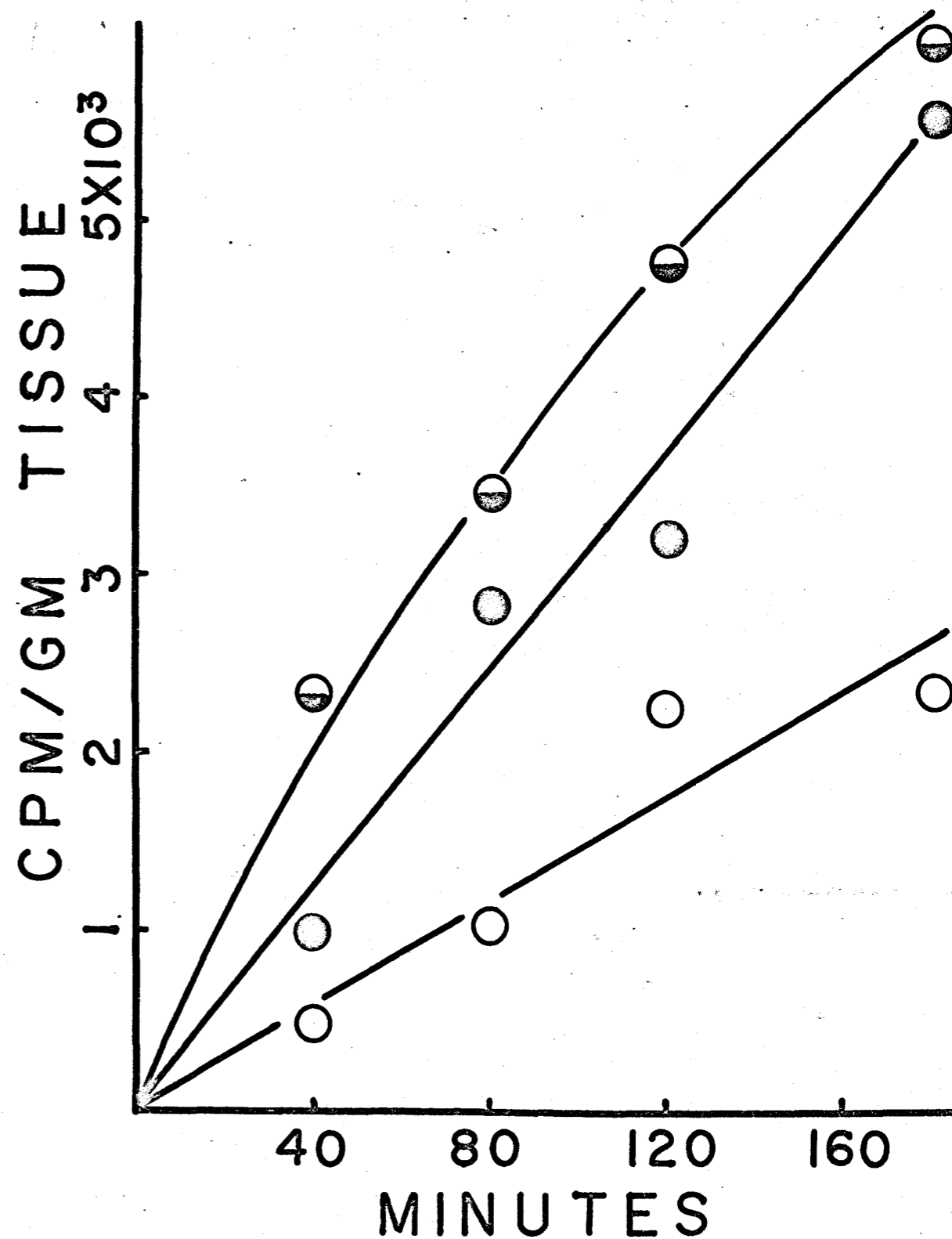
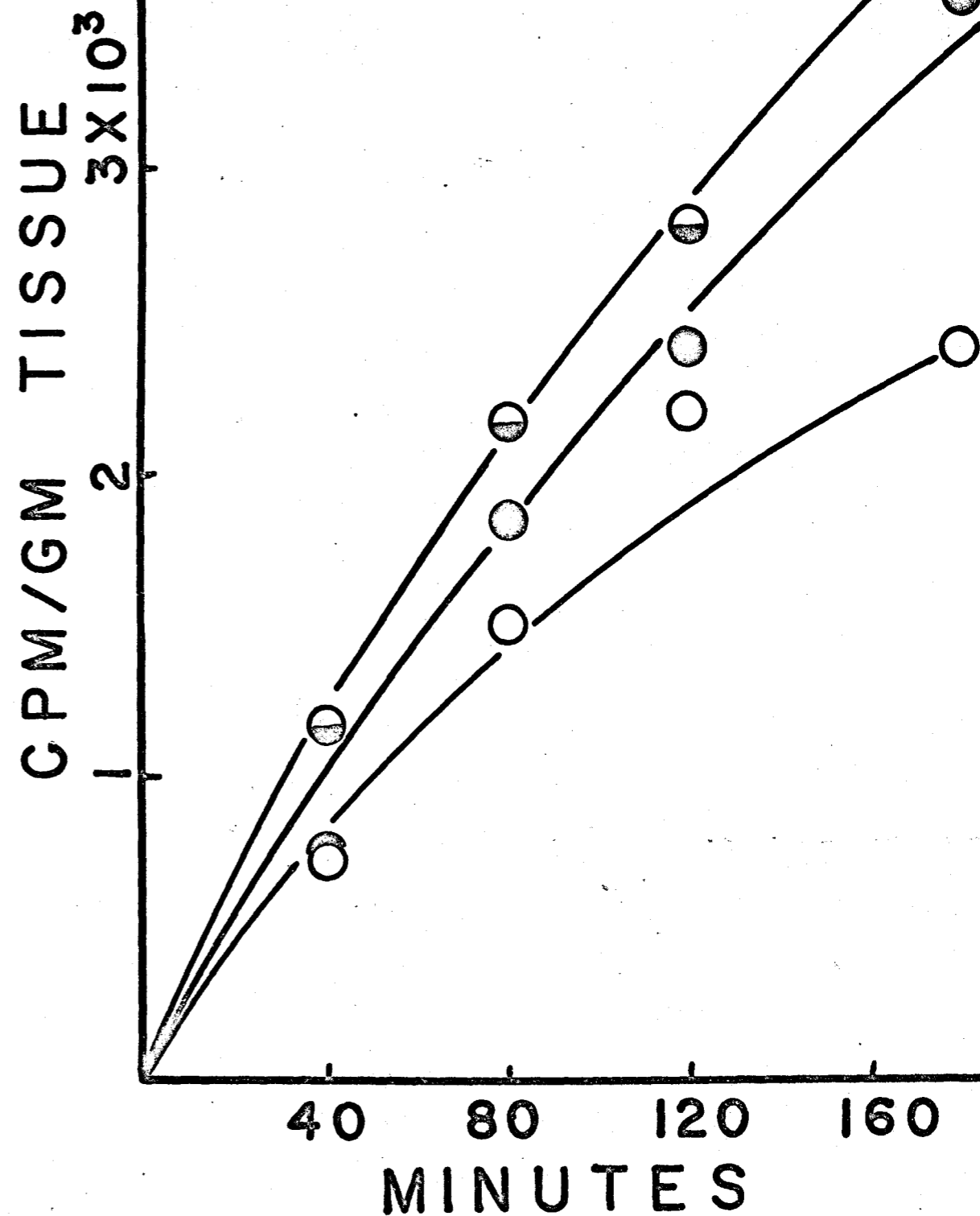


Fig. 8: The rate of formation of collagen in wound tissue of euthyroid rats shown in terms of cpm of  $^3\text{H}$ -hydroxyproline found per gm of tissue (wet weight) plotted against time after administration of  $45 \mu\text{Ci}$  of  $5\text{-}^3\text{H}\text{-L}$ -proline. The data obtained from wound tissue on the 5th day after wounding is indicated by open circles; 8th day, half-solid circles; 12th day, solid circles. All data points represent the mean of measurements on five separate tissue samples. Each data point on the eighth day is significantly greater than those of the other two groups (t test,  $p < 0.05$ ).



seen that the rate increases up to the eighth day after wounding and, as was the case with the cellular proteins, decreases significantly thereafter. Figs. 9 and 10 show that this relationship holds true for the hypothyroid and hyperthyroid groups also.

By looking at each of the three groups individually (5, 8, or 12 days after wounding), it is possible to subdivide them into the three different thyroid states. Starting with animals who had been wounded for 8 days, the rate of incorporation of proline into total proteins of the wound tissue was measured. Fig. 11 shows that the greatest incorporation occurred in the hyperthyroid group and the lowest in the hypothyroid group. This same general pattern was also seen in animals which had been wounded for five and for twelve days before the tissue was harvested, though in these instances, differences between the hyperthyroid and euthyroid groups were not as evident.

When the rate of incorporation of the proline into the cellular (non-collagenous) proteins on the 8th day was measured, it was found that in this instance also, the highest rate of incorporation was observed in the animals in the hyperthyroid group and the lowest in the hypothyroid group (Fig. 12). This same relationship could also be noted in tissue obtained from the five and twelve day groups.

To see whether the premises used for deriving the level of cellular proteins were warranted, and also to check on the

Fig. 9: The rate of formation of collagen in wound tissue of hypothyroid rats shown in terms of cpm of  $^3\text{H}$ -hydroxyproline found per gm of tissue (wet weight) plotted against time after administration of  $45 \mu\text{Ci}$  of  $5\text{-}^3\text{H}\text{-L}$ -proline. The data obtained from wound tissue on the 5th day after wounding is indicated by open circles; 8th day, half-solid circles; 12th day, solid circles. All data points represent the mean of measurements on five separate tissue samples. Each data point on the eighth day is significantly greater than those of the other two groups (t test,  $p < 0.05$ ).

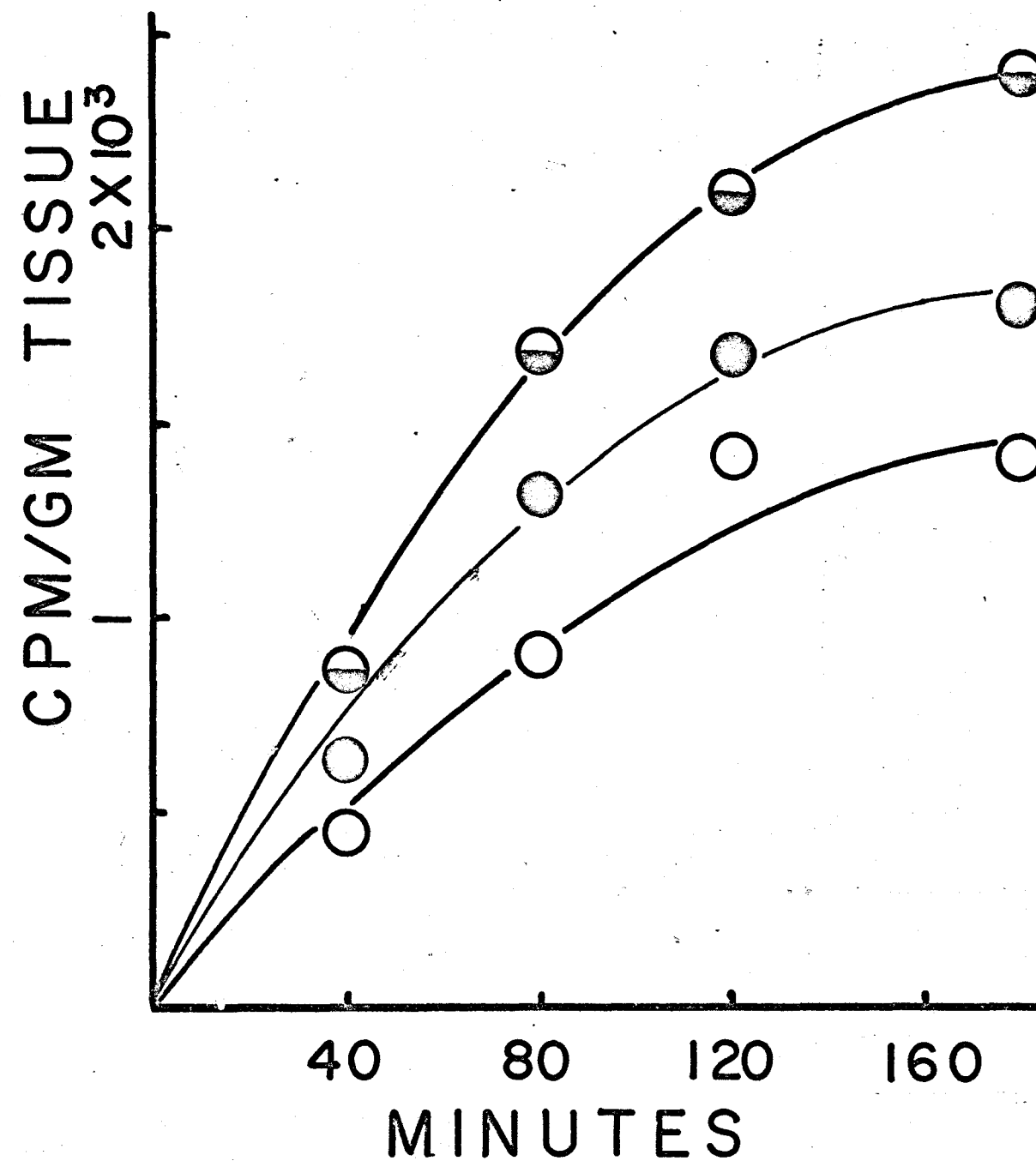


Fig. 10: The rate of formation of collagen in wound tissue of hyperthyroid rats shown in terms of cpm of  $^3\text{H}$ -hydroxyproline found per gm of tissue (wet weight) plotted against time after administration of  $45\ \mu\text{Ci}$  of  $5\text{-}^3\text{H}\text{-L}$ -proline. The numerals by each curve identify the age of the wound tissue in days. All data points represent the mean of measurements on five separate tissue samples. Each data point on the eighth day is significantly greater than those of the other two groups (t test,  $p < 0.05$ ).

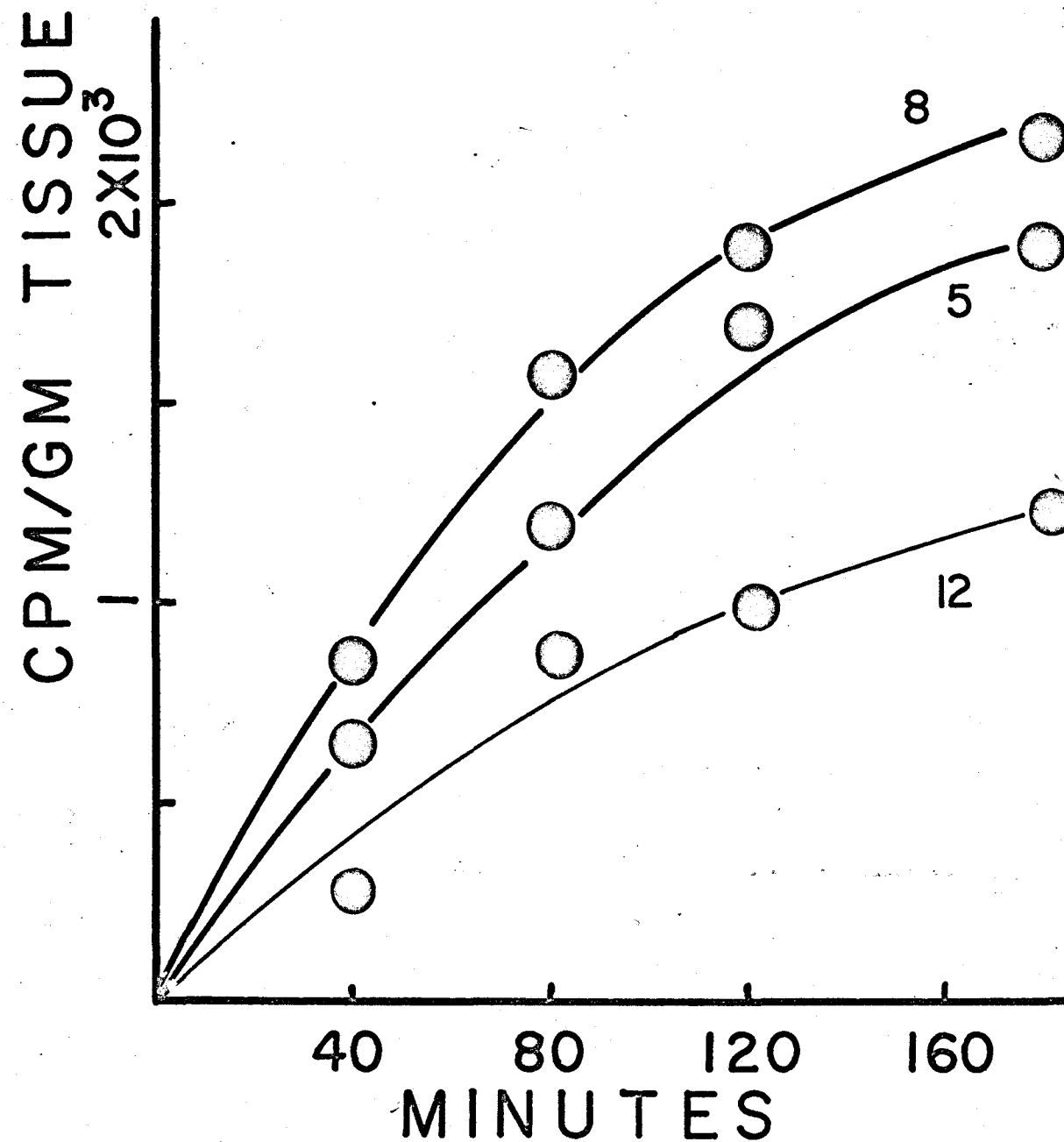


Fig. 11: The rate of synthesis of the total protein in wound tissue harvested eight days after wounding measured in terms of cpm proline incorporated per gm of tissue (wet weight) against time after administration of  $45 \mu\text{Ci}$  of  $5\text{-}^3\text{H-L-proline}$ . The data obtained from the hypothyroid group is indicated by open circles; the euthyroid group, half-solid circles; the hyperthyroid group, solid circles. All data points represent the mean of measurements on five separate tissue samples. Each data point of the euthyroid group is significant when compared to those of the other two groups (t test,  $p < 0.05$ ).

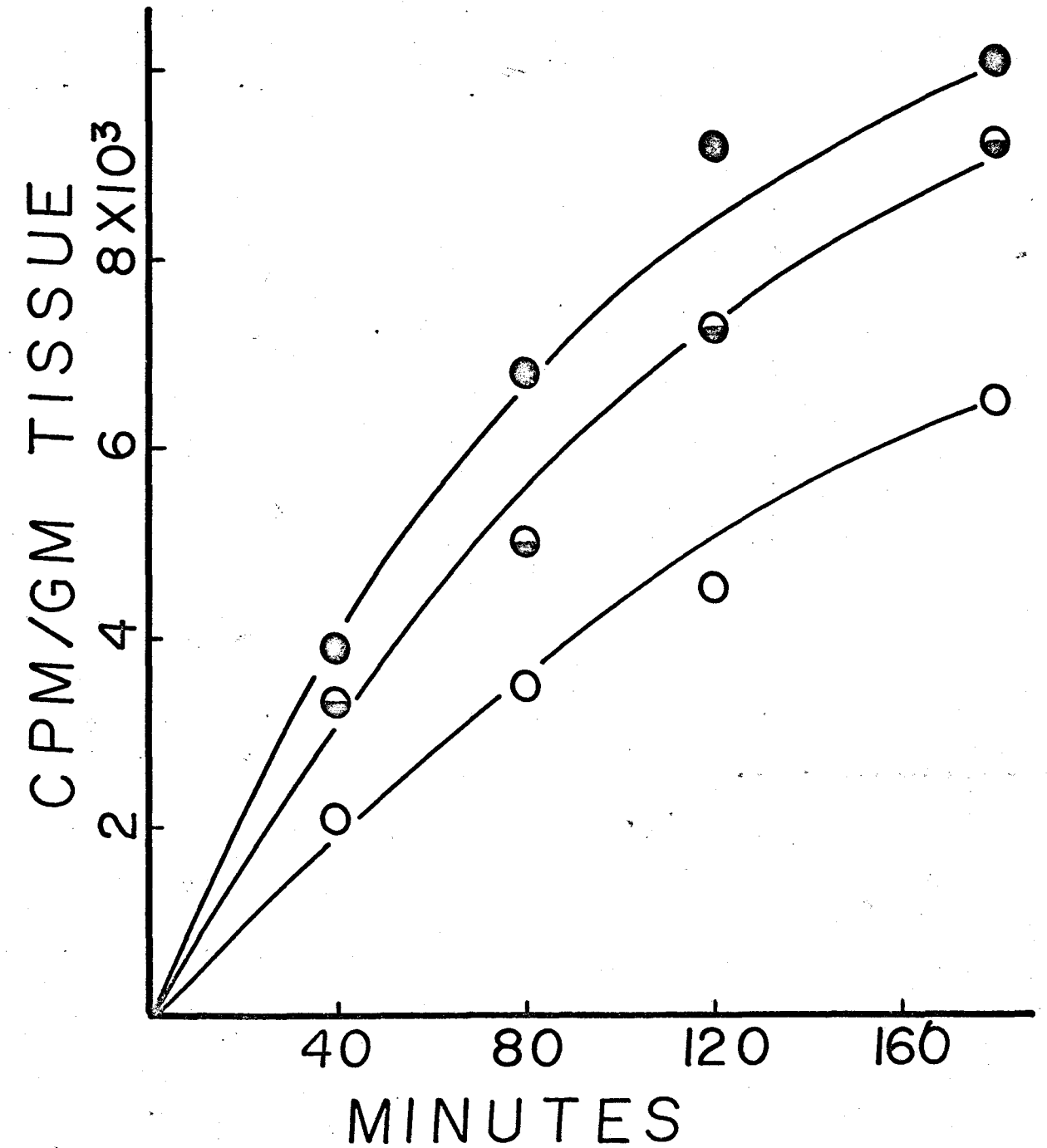
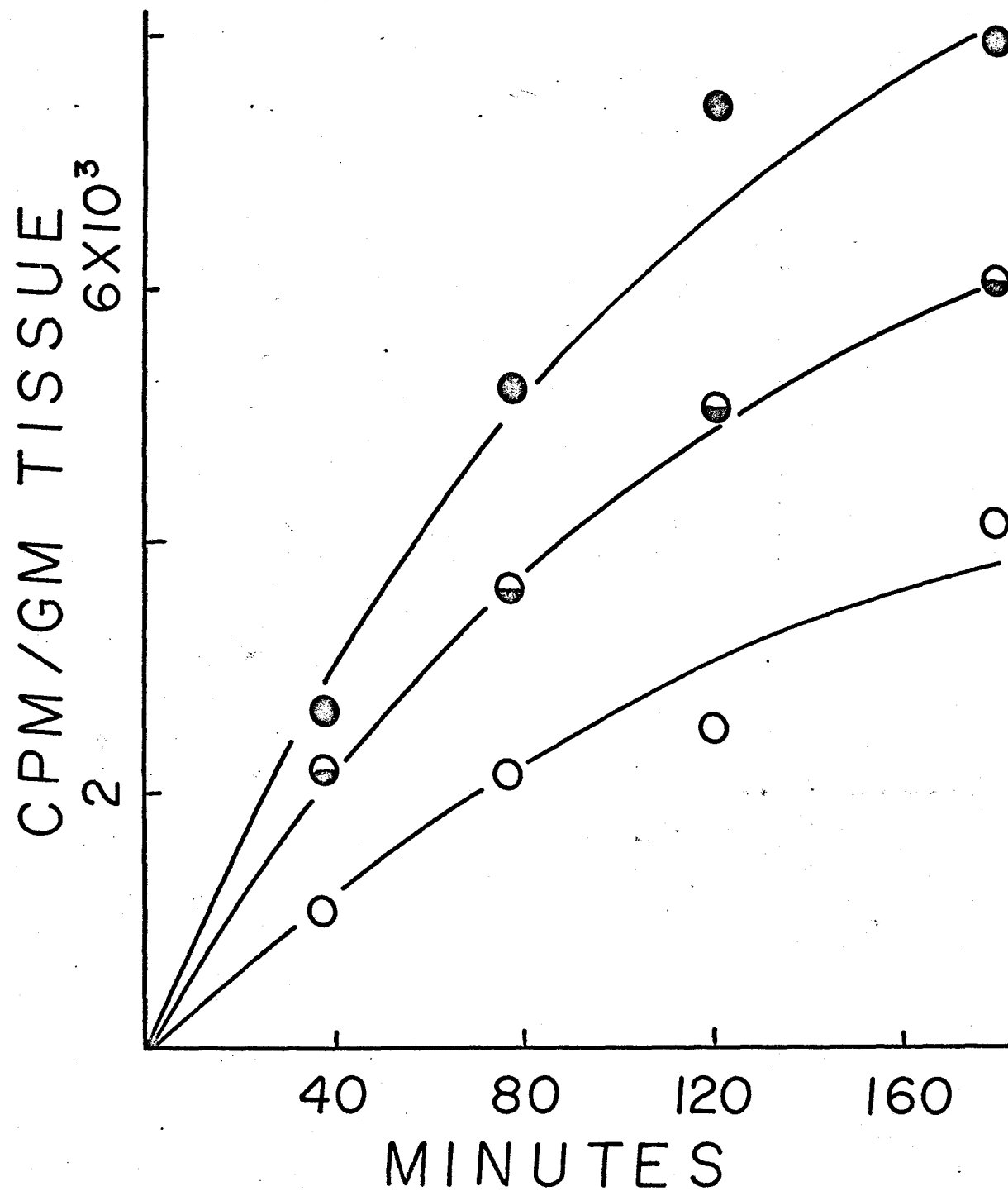


Fig. 12: The rate of synthesis of cellular proteins in wound tissue harvested eight days after wounding measured in terms of cpm proline incorporated per gm of tissue (wet weight) against time after administration of  $45 \mu\text{Ci}$  of  $5\text{-}^3\text{H-L-proline}$ . The data obtained from the hypothyroid group is indicated by open circles; the euthyroid group, half-solid circles; the hyperthyroid group, solid circles; All data points represent the mean of measurements on five separate tissue samples. Each data point of the euthyroid group is significant when compared to those of the other two groups (t test,  $p < 0.05$ ).





relationship shown in Fig. 12, the rate of incorporation of  $^3\text{H}$ -proline into the proteins of liver tissue was examined. This tissue upon analysis yielded negligible amounts of hydroxyproline and was thus considered to be essentially non-collagenous (cellular) in nature. When this tissue was examined, the highest rate of incorporation was observed in the hyperthyroid group and the lowest in the hypothyroid group (Fig. 13). One can also see by comparing the rates of incorporation of proline into liver and wound tissue in the wounded animal that the rate of incorporation into the liver proceeds at a much faster rate than that of the wound tissue (Fig. 14). Liver is, of course, a very metabolically active tissue.

The rate of synthesis of  $^3\text{H}$ -hydroxyproline was then measured in each of the three groups. Using the eight day animals as an example, a change was seen in the patterns noted above. The highest rate of synthesis of  $^3\text{H}$ -hydroxyproline was noted in the euthyroid group; in the hypothyroid and hyperthyroid groups, the rate of synthesis was substantially lower (Fig. 15). It is believed that the almost identical rates of the hypothyroid and hyperthyroid groups is coincidental. Fig. 16 bears this out in the five day group and also shows the euthyroid group with the highest rate of the three groups. The pattern exhibited by the twelve day animals is similar to that of the five day group, showing the highest rate of synthesis for the euthyroid group, with lower rates for the hypothyroid and hyperthyroid groups.

Fig. 13: The rate of synthesis of the protein in liver tissue harvested eight days after wounding measured in terms of cpm proline incorporated per mg total proline against time after administration of  $45 \mu\text{Ci}$  of  $5\text{-}^3\text{H-L}$ -proline. The data obtained from the hypothyroid group is indicated by open circles; the euthyroid group, half-solid circles; the hyperthyroid group, solid circles. All data points represent the mean of measurements on five separate tissue samples. Each data point of the euthyroid group is significant when compared to those of the other two groups (t test,  $p < 0.05$ ).

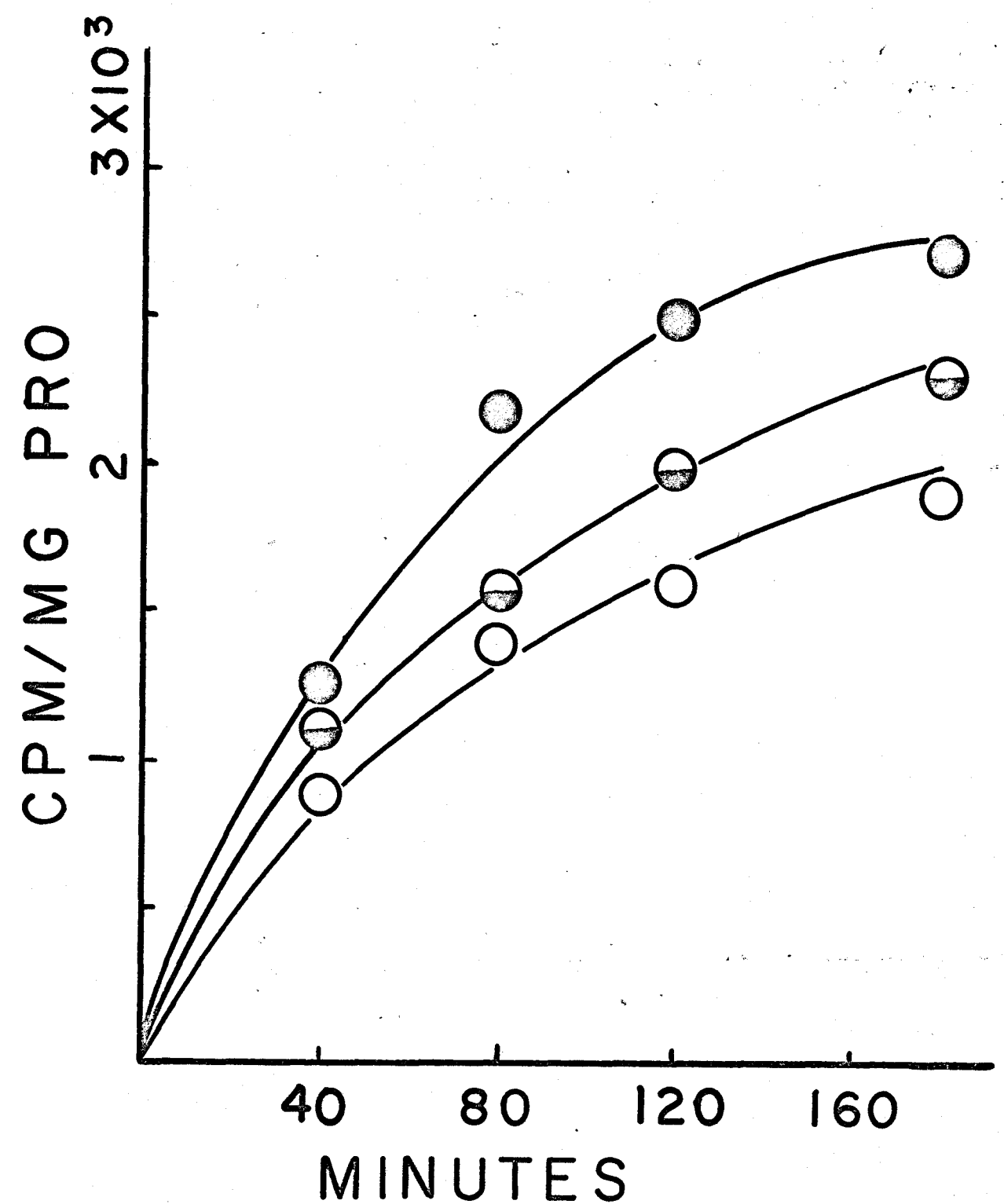


Fig. 14: The rate of synthesis of cellular proteins in wound and liver tissue of euthyroid animals harvested eight days after wounding, measured in terms of cpm proline incorporated per gm of tissue (wet weight) against time after administration of  $45 \mu\text{Ci}$  of  $5\text{-}^3\text{H-L-proline}$ . The data obtained from wound tissue is indicated by open circles; the liver tissue, solid circles. All data points represent the mean of measurements on five separate tissue samples. Each data point of the liver tissue is significant when compared to that of the wound tissue (t test,  $p < 0.05$ ).

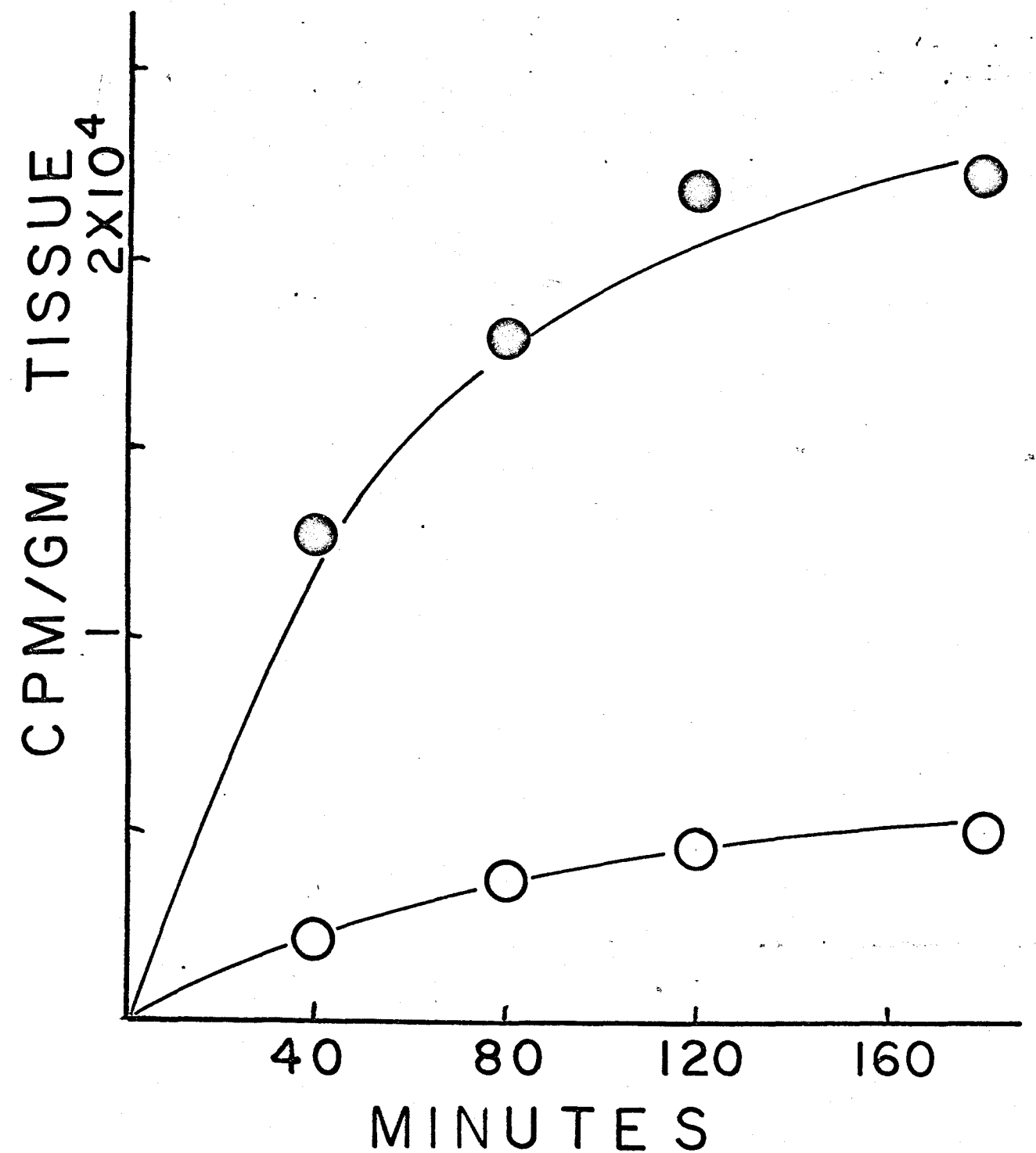


Fig. 15: The rate of formation of collagen in wound tissue harvested eight days after wounding shown in terms of  $^3\text{H}$ -hydroxyproline found per gm of tissue (wet weight) plotted against time after administration of  $45 \mu\text{Ci}$  of  $5\text{-}^3\text{H}\text{-L-proline}$ . The data obtained from the hypothyroid group is indicated by open circles; the euthyroid group, half-solid circles; the hyperthyroid group, solid circles. All data points represent the mean of measurements on five separate tissue samples. Each data point of the euthyroid group is significantly greater than those of the other two groups (t test,  $p < 0.05$ ).

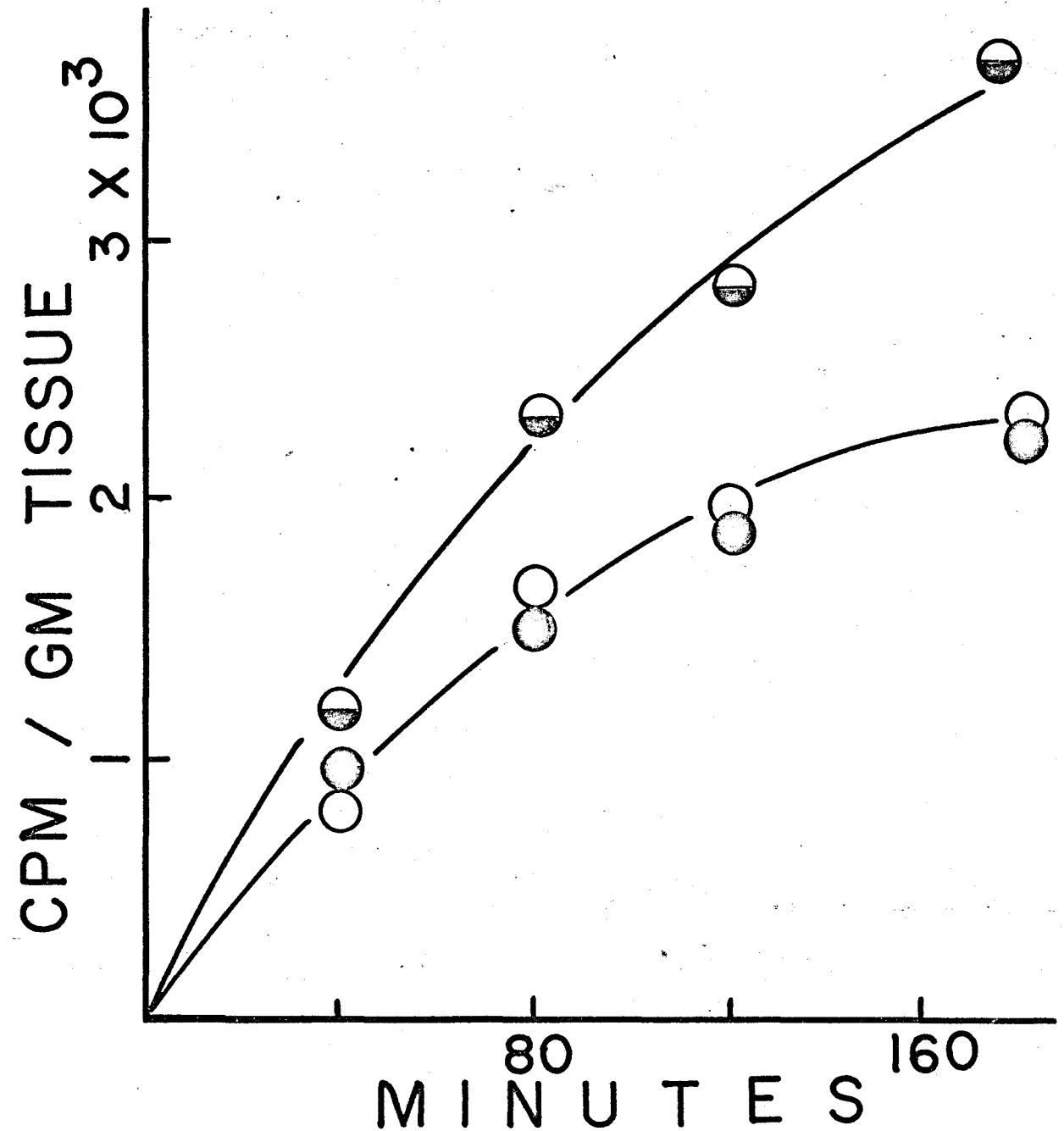
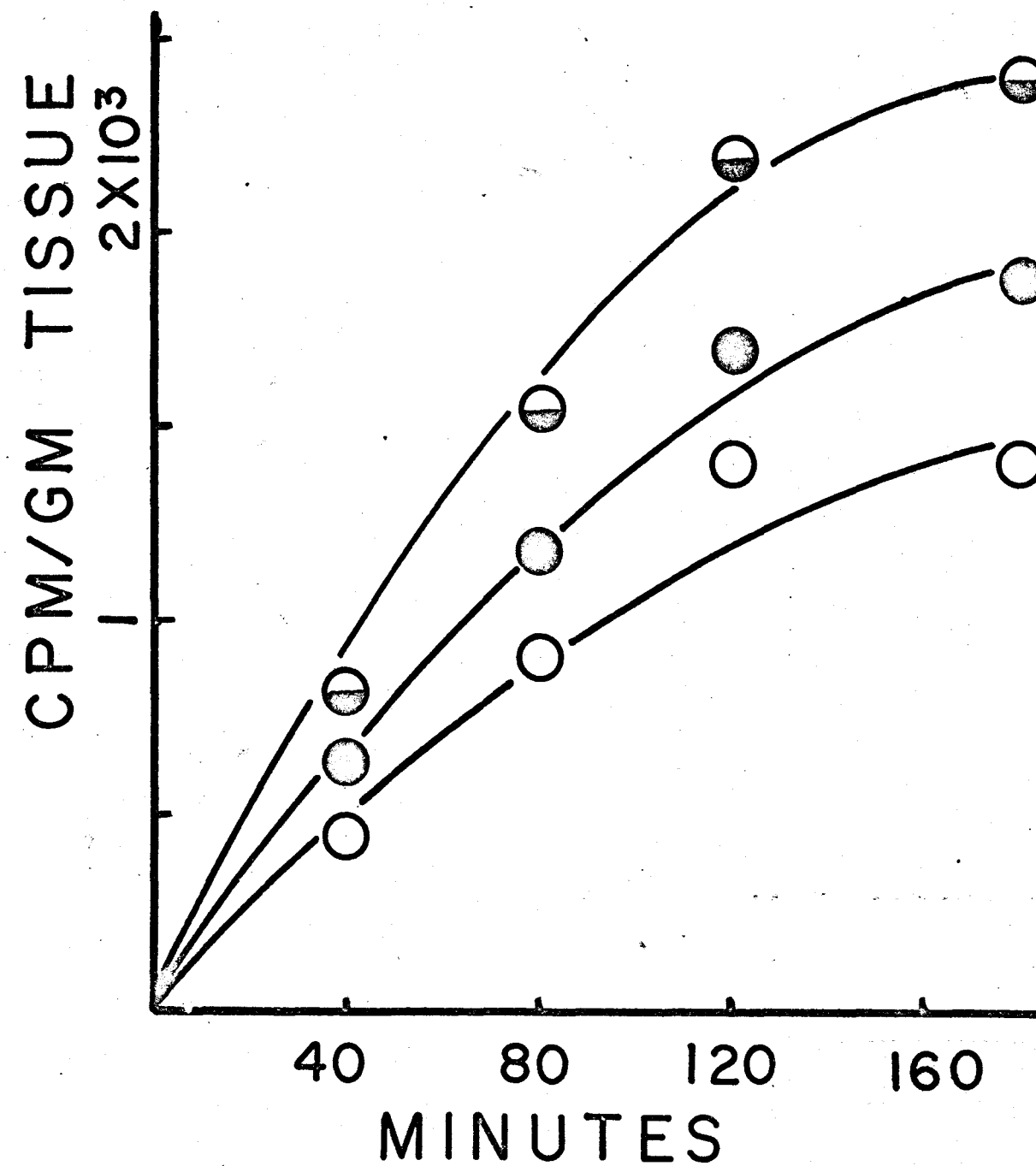


Fig. 16: The rate of formation of collagen in wound tissue harvested five days after wounding shown in terms of  $^3\text{H}$ -hydroxyproline found per gm of tissue (wet weight) plotted against time after administration of  $45 \mu\text{Ci}$  of  $5\text{-}^3\text{H-L-proline}$ . The data obtained from the hypothyroid group is indicated by open circles; the euthyroid group, half-solid circles; the hyperthyroid group, solid circles. All data points represent the mean of measurements on five separate tissue samples. Each data point of the euthyroid group is significantly greater than those of the other two groups (t test,  $p < 0.05$ ).



An attempt was then made to find out why the hyperthyroid animal, which exhibits a higher rate of synthesis of the cellular proteins than the euthyroid animal (Fig. 12), shows a decreased rate of collagen synthesis as compared to the euthyroid animal (Figs. 15-16). It was thought that by analyzing the urine for labeled proline and hydroxyproline, insight might be gained into any differences in catabolism between the two thyroid states. Table I shows that no differences were seen between the two states in unwounded animals. However, in the wounded animals, an increase in specific activity of hydroxyproline was seen in the hyperthyroid group as compared to the euthyroid group. The hyperthyroid group also excreted a larger amount of labeled proline during the three hour period of the experiment.

The total amount of proline and hydroxyproline in the tissue harvested at various stages after wounding and from different thyroid states is expressed in Table II. It is noted that the amount of each amino acid increases as the wound tissue matures. However, the hydroxyproline content on any of the three given days is higher in the euthyroid than in the other two groups. This is consistent with the data shown above which indicated that the euthyroid animals exhibited an optimal rate of hydroxyproline synthesis.

TABLE I

EXCRETION OF  $^3\text{H}$ -HYDROXYPROLINE AND  $^3\text{H}$ -PROLINE  
BY WOUNDED AND NORMAL RATS

		$\frac{^3\text{H-Hyp}}{\text{Mg. Hyp}}$	$\frac{^3\text{H-Pro}}{3 \text{ Hours}}$
Wounded	Euthyroid	2450 $\pm$ 600	855 $\pm$ 226
	Hyperthyroid	4850 $\pm$ 690	1663 $\pm$ 153
Normal	Euthyroid	2780 $\pm$ 470	1339 $\pm$ 460
	Hyperthyroid	2680 $\pm$ 400	1511 $\pm$ 350

All data represent the mean ( $\pm$  S.D.) of measurements on six separate samples.

TABLE II

EFFECT OF THYROID HORMONE ON PROLINE AND  
HYDROXYPROLINE CONTENT OF GRANULATION TISSUE

Days After Wounding		PROLINE	HYDROXYPROLINE
		Mg/Gm Tissue	
5	Hypothyroid	8.19 ± 1.02	1.53 ± 0.38
	Euthyroid	8.25 ± 1.03	2.76 ± 0.49
	Hyperthyroid	8.34 ± 1.71	2.16 ± 0.44
8	Hypothyroid	11.93 ± 1.58	4.00 ± 0.80
	Euthyroid	13.43 ± 2.21	5.62 ± 1.05
	Hyperthyroid	12.55 ± 1.49	3.65 ± 0.51
12	Hypothyroid	14.57 ± 1.87	8.13 ± 1.71
	Euthyroid	21.28 ± 3.18	12.00 ± 2.20
	Hyperthyroid	11.12 ± 1.49	8.40 ± 2.20

All data represent the mean ( $\pm$  S.D.) of measurements on twenty separate tissue samples. The hydroxyproline content of the euthyroid group is significantly greater than that of the other groups on all three days (t test,  $p < 0.05$ ).



### Experiments using $^{35}\text{S}$ -cystine

The data gathered from the experiments using  $^3\text{H}$ -proline showed that the relationship between the three thyroid states in regenerating wound tissue was largely independent of the three ages (five, eight, or twelve days) at which the tissue was studied. This weighed against studying all three ages of regeneration with  $^{35}\text{S}$ -cystine and thus only wound tissue taken eight days after wounding was used for this set of experiments.

When the rate of incorporation of  $^{35}\text{S}$ -cystine by peptide bonds, as represented by dialysis against sodium sulfite, is measured, it is seen that the hyperthyroid group exhibits the highest rate and the hypothyroid group the lowest rate (Fig. 17). Since cystine is not incorporated into collagen, Fig. 17 also represents the rate of incorporation of the amino acid into cellular proteins. The pattern seen is the same as that noted with the incorporation of proline.

When the amount of  $^{35}\text{S}$ -cystine bound by disulfide bonds only is measured by taking the difference in counts between the sodium carbonate and sodium sulfite dialyses, a quite different pattern emerges. Here all three groups seem to reach a peak sixty to ninety minutes after injection of the label (Fig. 18). The hyperthyroid group is distinguished from the other two groups by reaching a higher peak and attaining it at an earlier time.

Fig. 17: Rate of formation of wound tissue proteins on the eighth day after wounding, measured in terms of cpm of  $^{35}\text{S}$  per gm of tissue (wet weight) incorporated by peptide bonds against time after administration of  $33.3 \mu\text{Ci}$  of  $^{35}\text{S}$ -L-cystine. The data obtained from the hypothyroid group is indicated by open circles; the euthyroid group, half-solid circles; the hyperthyroid group, solid circles. All data points represent the mean of measurements on five separate tissue samples. Each data point of the euthyroid group is significant when compared to those of the other two groups (t test,  $p < 0.05$ ).

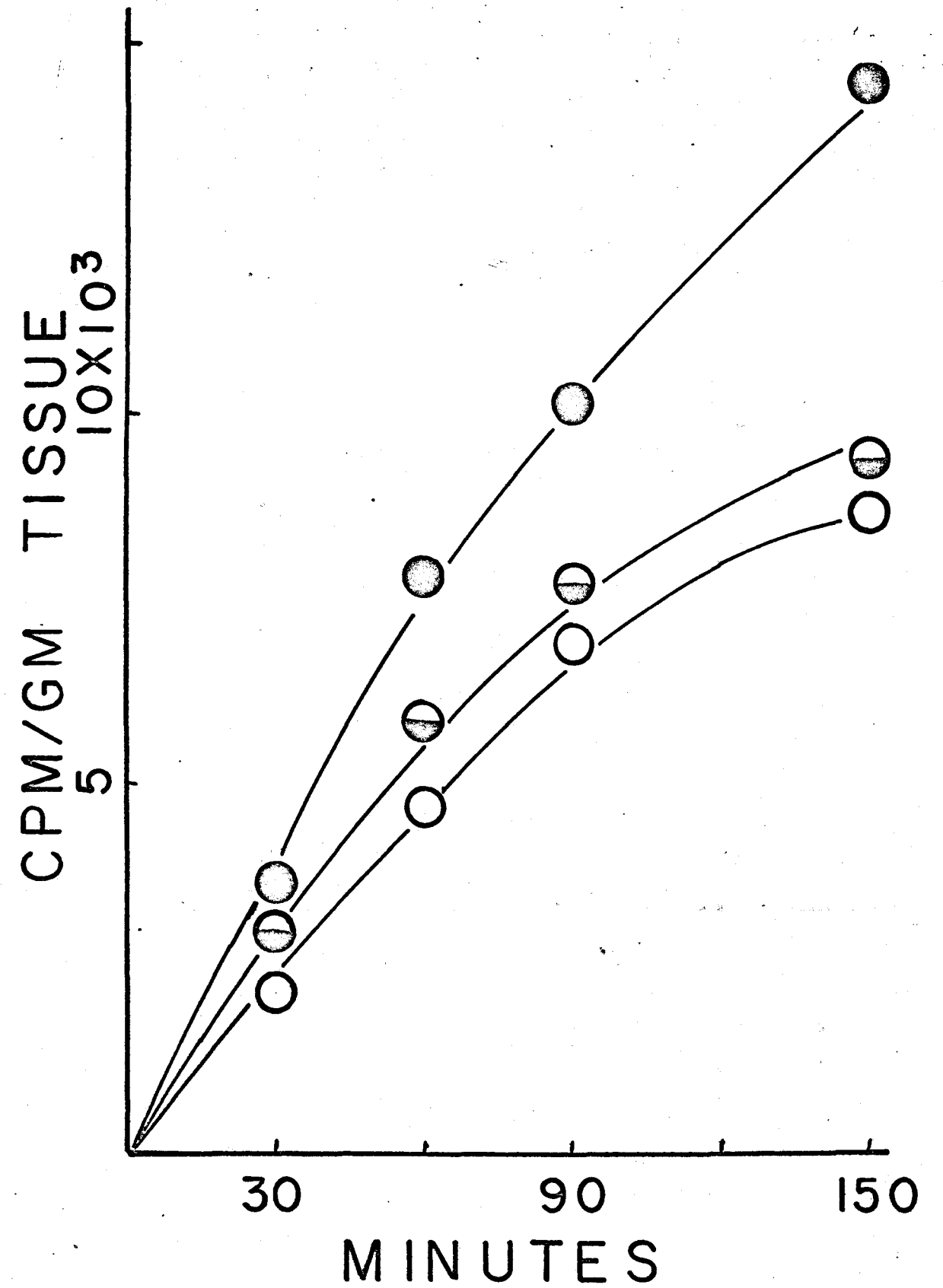
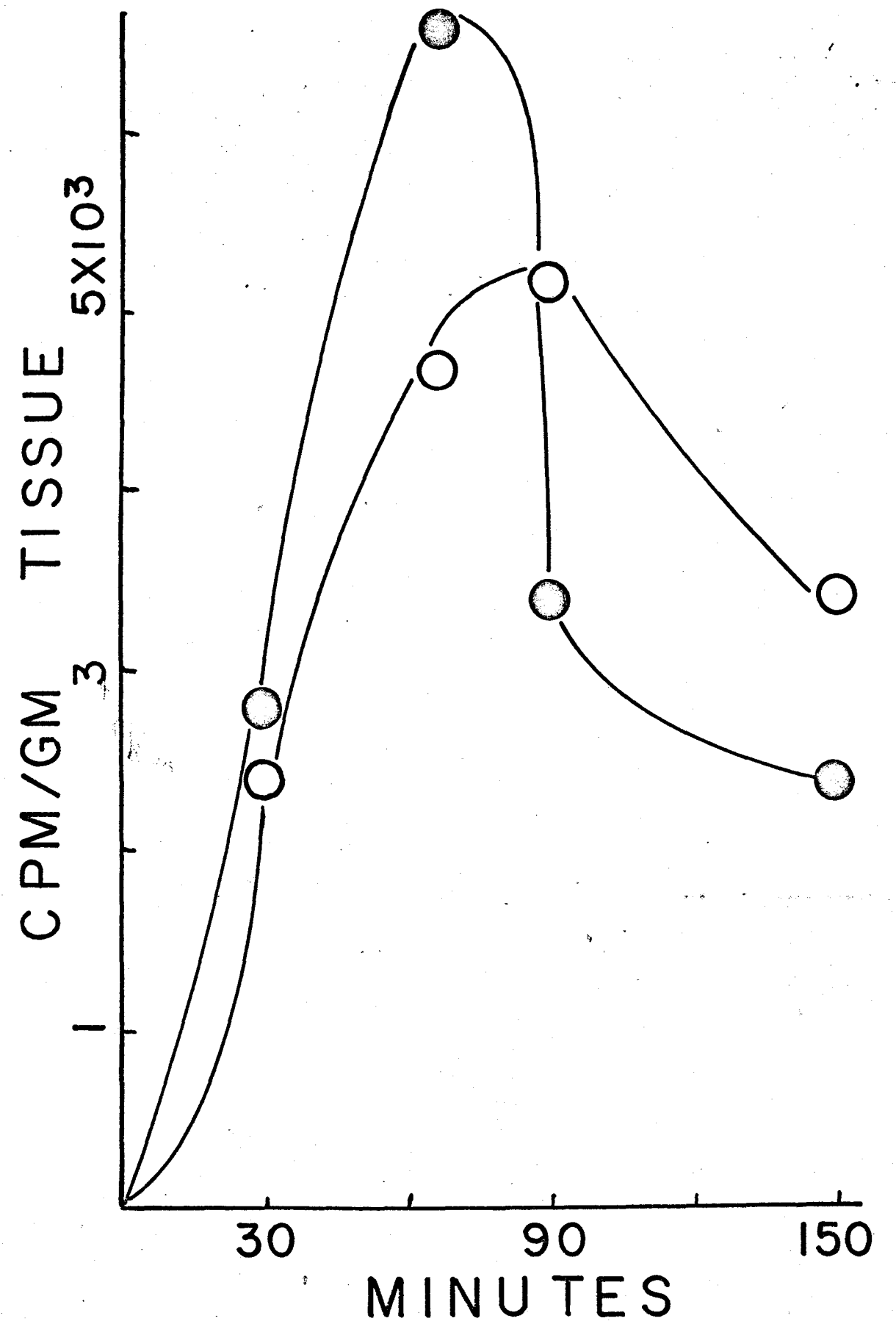


Fig. 18: Rate of turnover of cystine associated with wound tissue proteins by disulfide bonds only, on eighth day after wounding, measured in terms of cpm of  $^{35}\text{S}$  per gm tissue (wet weight) against time after administration of  $33.3 \mu\text{Ci}$  of  $^{35}\text{S}$ -L-cystine. The data obtained from the hypothyroid and euthyroid groups (almost identical) is indicated by open circles; the hyperthyroid group, solid circles. All data points represent the mean of measurements on five separate tissue samples. Each data point of the hyperthyroid group is significant when compared to those of the other two groups (t test,  $p < 0.05$ ).



CHAPTER VIIDISCUSSION

The design of the set of experiments using labeled proline allows for organizing the data with one of two factors held constant while varying the other. Data were first analyzed by looking at the regenerating wound tissue at different stages of regeneration while the amount of thyroid hormone was kept constant. When this was done, the relationship between the three stages studied was generally independent of the amount of thyroid material given to the animal. This has been illustrated in Figs. 4-6 and Figs. 8-10.

When Figs. 4-10 are examined, it is noted that the peak period of protein synthesis occurs about the eighth day. Using the euthyroid animals as an example, this peak at eight days occurs for total protein synthesis (Fig. 4), cellular (non-collagenous) protein (Fig. 7), and the synthesis of collagen (Fig. 8). This same relationship is also seen in the hypothyroid animals (Figs. 5 and 9), and in the hyperthyroid rats (Figs. 6 and 10).

The changing rate of protein synthesis observed as the regenerating wound tissue develops may be due to changes in relative metabolic activity of the cells. Independent work done in this laboratory has shown that the amount of nuclear RNA per mg

of DNA appears to be correlated with the rate of protein synthesis, i.e. there is an increase from the fifth day to the eighth day after wounding followed by a decline on the twelfth day (106,107). These fluctuations in the amount of nuclear RNA have been shown to be due largely to changes in the DNA-like RNA content of the nucleus (106). This type of RNA is considered to be mainly messenger RNA (m-RNA) (24). It then follows that the rate of protein synthesis in the regenerating wound tissue is related to the nuclear m-RNA content of the cells.

The amount of DNA per gm of tissue, an indication of the number of cells present, can be calculated from previous reports (27,105). When this is done, it is seen that the DNA increased up to eight days after wounding and remained practically constant thereafter. Thus, one can see that the apparent changes in the rate of protein synthesis in regenerating wound tissue may be attributed both to changes in the number of cells and alterations in the amount of m-RNA that is found in the nuclei of these cells.

The work of Lampiaho and Kulonen on the synthesis of protein in experimental granulation tissue in vitro (implanted cellulose sponges) using labeled proline has shown that the maximal rate of collagen synthesis occurs about three weeks after implantation (47). The highest level of DNA and RNA in this tissue appeared at about the same time. The results of our experiments also

indicate that the highest level of DNA and RNA in the wound tissue coincides with the time of maximal protein synthesis (both cellular proteins and collagen). These differences between the work reported here (in vivo) and the in vitro studies may be due to the differences in the rate of development between the two systems.

As was mentioned earlier, wound healing can be studied in various ways. In our work, wound tissue was made available by excision of skin. Others have used implantation of sponges to create an artificial dead space which is then invaded by fibroblasts, leucocytes, and vascular buds to produce granulation tissue (9). In still other experiments, agents which create an inflammatory response (e.g., carageenin, turpentine) have been used. Though the qualitative processes of regeneration may be closely related in these various methods, one must take extreme care when comparing them on the basis of time of development. Thus, it is significant that both the work reported in this dissertation and that of Lampiaho and Kulonen (47) show that the highest levels of DNA and RNA coincide with the time of maximal protein synthesis, even though the time when these peak levels occur is radically different.

The second objective of the set of experiments using  $^3\text{H}$ -proline was to assess the effect of different levels of thyroid hormone on protein synthesis at specific stages of regeneration.

Thus by giving three different levels of thyroid hormone to produce states approximating hypothyroidism, euthyroidism, and hyperthyroidism, and by keeping the time of tissue regeneration constant (either five, eight, or twelve days after wounding), the data can be organized to show the effect of thyroid hormones. Figs. 11-16 illustrate this type of organization.

Since the eighth day had been shown to have a higher rate of synthesis than the fifth and twelfth days, it has been selected as the model for all three days. When the rate of incorporation of  $^3\text{H}$ -proline into all proteins of the wound tissue was measured, it was seen that the highest rates of incorporation belonged to the hyperthyroid group (Fig. 11). This was also true for incorporation of proline into the cellular proteins of the tissue (Fig. 12). Liver tissue, considered to be essentially non-collagenous, showed the same pattern of rate of incorporation (Fig. 13). As previously noted, and shown in Fig. 14, incorporation of the amino acid is of a much greater magnitude in liver tissue than in the wound tissue. However, the rate of synthesis of hydroxyproline, and hence collagen, did not follow the pattern seen in Figs. 11-13. Here (Fig. 15) the euthyroid animal had the greatest rate while the other two groups exhibited similar, but significantly lower rates than the euthyroid group. This similarity between the hypothyroid and hyperthyroid groups was not seen in the five day group (Fig. 16).

The question then arises, why did the thyroid hormone act in one manner with the rate of incorporation of proline into most proteins (i.e. an increase in rate with an increased amount of hormone), but in a different manner with collagen? Collagen seemed to exhibit what has been called the biphasic effect (86) with thyroid hormone. This term refers to the fact that thyroid hormone can exhibit an optimal level and either a deficiency or excess of this level will move a given parameter in a similar direction, in this case, a lower rate of protein synthesis.

To investigate a phase of this differential response to the thyroid hormone with respect to proline incorporation in collagen and non-collagenous proteins, the excretion rate of labeled hydroxyproline was followed in hyperthyroid and euthyroid animals. Table I showed that the specific activity in normal (unwounded) animals is quite similar. However, when wounded animals are examined, the specific activity of the hydroxyproline is significantly higher in hyperthyroid animals than in the euthyroid group. These data point to an increased rate of formation of collagen in hyperthyroid rats, but it is believed that an even greater increase in catabolism brings about the decreased rate noted in Figs. 15-16. This catabolism could be due to an increase in collagenase or in other enzymes that might exhibit collagenase-like activity.

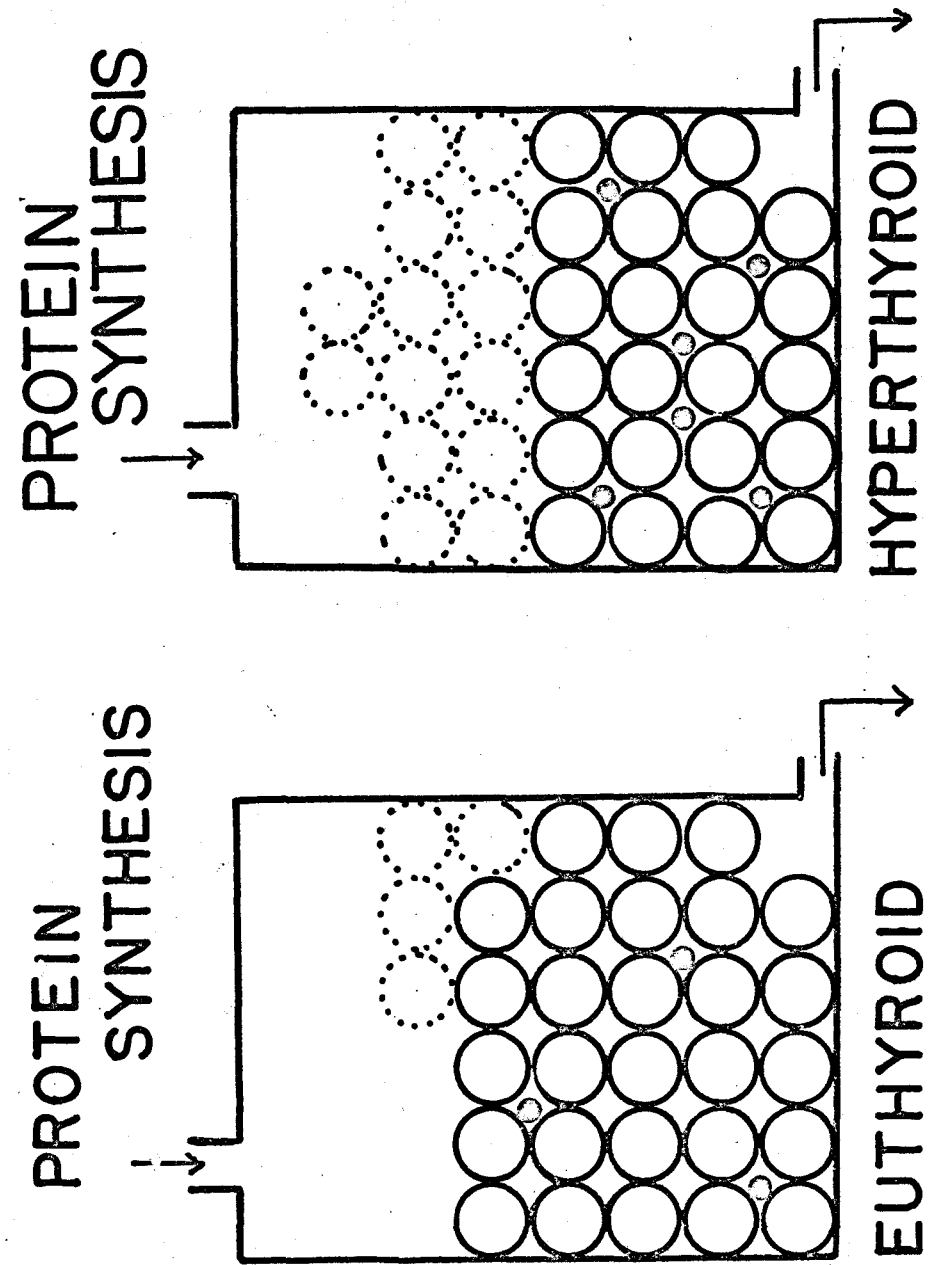
Until recently, reports of collagenase in mammalian systems were relatively rare (30). However, Gross and his coworkers



have been able to demonstrate collagenase activity in various systems including mammalian wound tissue (19,26). It has been concluded that collagenase is found regularly in the epidermis and in much higher amounts in wounded tissue. It appears firstly in the new mesenchymal elements of the healing wounds, though at this time the specific function of the enzyme is not elucidated.

This recent clarification on the presence of collagenase in regenerating wound tissue has enabled the postulation of a stimulatory effect of the thyroid hormone either on the synthesis of the enzyme or an activation of it. This would be consistent with its general effects on most non-collagenous proteins. Fig. 19 summarizes this explanation for the finding that an excess of thyroid hormone inhibits the formation of collagen, a phenomenon first seen by Moltke (53) and verified in this experiment. In Fig. 19, collagen is represented by the larger circles. The non-collagenous proteins (including of course collagenase) are represented by the smaller black dots. The thyroid hormone is seen to stimulate both the synthesis of collagen and non-collagenous proteins. However, due to the increased amount of collagenase present, there is a greater breakdown of the collagen itself (represented by the large dashed circles). Thus the paradox arises that thyroid hormone is stimulating protein synthesis while at the same time decreasing the amount of collagen present, and in turn inhibiting the rate of wound healing (53).

Fig. 19: A postulated scheme to explain the decreased synthesis of collagen in regenerating wound tissue of hyperthyroid animals. The large circles represent collagen, while the smaller black dots represent non-collagenous proteins. Breakdown of collagen is represented by the large dashed circles.



As noted in the previous chapter, the effects of different levels of thyroid hormone on proline incorporation were similar on each of the three days that the regenerating wound tissue was studied. Since the purpose of this study was to investigate the effect of different levels of thyroid hormone on protein synthesis in regenerating wound tissue it was decided that in light of the observations with proline, to study more than one stage of regeneration would not have a readily apparent value. With this in mind, the experiments with labeled cystine were performed only on regenerating wound tissue harvested from animals eight days after wounding.

When  $^{35}\text{S}$ -cystine is incorporated into proteins by peptide bonds, the pattern observed is again very similar to that seen with the proline incorporation experiments (Fig. 17). The hyperthyroid animal has the highest rate and the hypothyroid the lowest. Of course, this actually represents non-collagenous proteins since neither cysteine or cystine is found in collagen (18). The amount of  $^{35}\text{S}$ -cystine incorporated by disulfide bonds only is illustrated in Fig. 18. It is to be noted that all three groups reach their peak in sixty to ninety minutes after injection of the label, though the hyperthyroid group reaches a higher peak and attains it at an earlier time than the other two groups. Disulfide bound cysteine has been shown previously to turn over at a more rapid rate than the peptide bound cysteine (100).

It has been noted that the cysteine residues bound by disulfide bonds only may serve to activate various enzymes by alternate oxidation and reduction of the cysteine residues, and that this parameter gives an indication of the amount of cysteine in the tissue being utilized for metabolic purposes as opposed to those residues which are peptide bound and are involved with the structural make-up of the protein (100). The significance of the involvement of thyroid hormone on the disulfide bound cysteine turnover can only be speculated upon, but it does seem that an excess of the hormone promotes a faster turnover of this metabolically important factor. This may indicate that sulfhydryl activated enzymes in the hyperthyroid animals are being utilized at a faster rate than the other two groups (99). Thus, not only is the amount of these cellular proteins increased in the hyperthyroid group, but their activity may also be increased.

CHAPTER VIIISUMMARY AND CONCLUSIONS

Female rats were wounded and their regenerating wound tissue collected five, eight, or twelve days after wounding. The rats were further subdivided into three different thyroid states (hypothyroid, euthyroid, or hyperthyroid) by the use of propylthiouracil and desiccated thyroid powder. At short intervals prior to harvesting the wound tissue, 45  $\mu\text{Ci}$  of tritiated proline was given.

Another group of wounded rats was also subdivided into the above three thyroid states. On the eighth day after wounding, the tissue was harvested. In this case however, the animals received 33.3  $\mu\text{Ci}$  of  $^{35}\text{S}$ -cystine at short intervals prior to the collection of the wound tissue.

By the use of labeled proline, it was found that the rate of incorporation of proline into the proteins of regenerating wound tissue was higher on the eighth day than either the fifth or twelfth day. This was also true for cellular (non-collagenous) proteins and collagen. It is believed that this relationship may be due to a parallel of nuclear RNA, in particular m-RNA and/or a changing number of cells in the tissue.

Total protein synthesis and cellular protein synthesis was seen to be highest in the hyperthyroid group and lowest in the

hypothyroid group. However, the synthesis of collagen was highest in the euthyroid group and lower in the hyperthyroid and hypothyroid groups. Evidence is put forth indicating an increased catabolism of collagen in the hyperthyroid animal, perhaps due to an increase in collagenase.

The incorporation of  $^{35}\text{S}$ -cystine by peptide bonds was seen to be highest in the hyperthyroid animals and lowest in the hypothyroid group. The turnover rate of the cystine bound by disulfide bonds only also seems more rapid for the hyperthyroid rat. It is believed that this last parameter may be indicative of an increase in activity of sulfhydryl-activated enzymes in the hyperthyroid animal.

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APPENDIX

TABLE III  
RATE OF INCORPORATION OF  $^3\text{H}$ -PROLINE INTO  
PROTEINS OF REGENERATING WOUND TISSUE

Days After Wounding	Time (minutes)	Hypothyroid   Euthyroid   Hyperthyroid (cpm per gram of tissue)		
5	40	1336 ± 196	1119 ± 105	1829 ± 218
	80	2631 ± 500	2629 ± 320	2286 ± 350
	120	3173 ± 318	4450 ± 298	3668 ± 277
	180	3299 ± 323	4593 ± 359	3966 ± 185
8	40	2101 ± 219	3413 ± 205	3920 ± 125
	80	3523 ± 413	5091 ± 584	6804 ± 491
	120	4565 ± 143	7542 ± 870	9331 ± 856
	180	6552 ± 534	9223 ± 411	10216 ± 752
12	40	1385 ± 340	1511 ± 99	3361 ± 230
	80	2995 ± 299	3784 ± 758	5636 ± 317
	120	4041 ± 424	5500 ± 648	6104 ± 702
	180	5610 ± 560	8506 ± 459	6360 ± 559

All data represent the mean ( $\pm$  S.D.) of measurements on five separate tissue samples. Portions of the above table are represented in Figures 4-6 and Figure 11.

TABLE IV  
RATE OF FORMATION OF  $^3\text{H}$ -HYDROXYPROLINE

Days After Wounding	Time (minutes)	Hypothyroid   Euthyroid   Hyperthyroid (cpm per gram of tissue)		
5	40	451 $\pm$ 37	791 $\pm$ 43	649 $\pm$ 67
	80	909 $\pm$ 120	1547 $\pm$ 104	1185 $\pm$ 73
	120	1448 $\pm$ 131	2211 $\pm$ 169	1697 $\pm$ 62
	180	1384 $\pm$ 133	2446 $\pm$ 150	1855 $\pm$ 129
8	40	867 $\pm$ 122	1171 $\pm$ 130	864 $\pm$ 122
	80	1689 $\pm$ 302	2174 $\pm$ 116	1564 $\pm$ 188
	120	2052 $\pm$ 232	2792 $\pm$ 140	1888 $\pm$ 138
	180	2380 $\pm$ 283	3873 $\pm$ 206	2161 $\pm$ 108
12	40	529 $\pm$ 67	796 $\pm$ 39	229 $\pm$ 67
	80	1217 $\pm$ 185	1839 $\pm$ 196	931 $\pm$ 149
	120	1531 $\pm$ 149	2316 $\pm$ 195	1017 $\pm$ 185
	180	1547 $\pm$ 275	3486 $\pm$ 174	1147 $\pm$ 275

All data represent the mean ( $\pm$  S.D.) of measurements on five separate tissue samples. Portions of the above table are represented in Figures 8-10 and Figures 15-16.



TABLE V

RATE OF INCORPORATION OF  $^3\text{H}$ -PROLINE INTO  
PROTEINS OF LIVER IN WOUNDED RATS

Time (minutes)	Hypothyroid (cpm per mg of proline)	Euthyroid (cpm per mg of proline)	Hyperthyroid (cpm per mg of proline)
40	850 $\pm$ 94	1132 $\pm$ 74	1270 $\pm$ 65
80	1360 $\pm$ 128	1550 $\pm$ 98	2219 $\pm$ 172
120	1631 $\pm$ 162	2056 $\pm$ 138	2498 $\pm$ 174
180	1899 $\pm$ 125	2308 $\pm$ 238	2763 $\pm$ 153

All data represent the mean ( $\pm$  S.D.) of measurements on five separate tissue samples. The above table is represented in Figure 13.

TABLE VI

RATE OF INCORPORATION OF  $^{35}\text{S}$ -CYSTINE INTO  
PROTEINS OF REGENERATING WOUND TISSUE

	Time (minutes)	Hypothyroid (cpm per gram of tissue)	Euthyroid (cpm per gram of tissue)	Hyperthyroid (cpm per gram of tissue)
Peptide- Bound	30	2449 $\pm$ 222	3436 $\pm$ 316	4036 $\pm$ 139
	60	4886 $\pm$ 271	5931 $\pm$ 345	7871 $\pm$ 219
	90	7005 $\pm$ 141	7659 $\pm$ 218	10130 $\pm$ 345
	150	8893 $\pm$ 198	9341 $\pm$ 167	14632 $\pm$ 561
Disulfide- Bound	30	2493 $\pm$ 139	2516 $\pm$ 147	2843 $\pm$ 113
	60	4820 $\pm$ 259	4890 $\pm$ 323	6679 $\pm$ 521
	90	5231 $\pm$ 287	5246 $\pm$ 246	3430 $\pm$ 316
	150	3473 $\pm$ 119	3419 $\pm$ 180	2406 $\pm$ 98

All data represent the mean ( $\pm$  S.D.) of measurements on five separate tissue samples. The above table is represented in Figures 17-18.

APPROVAL SHEET

The dissertation submitted by Cary Bruce Linsky has been read and approved by a committee from the faculty of the Graduate School.

The final copies have been examined by the director of the dissertation and the signature which appears below verifies the fact that any necessary changes have been incorporated, and that the dissertation is now given final approval with reference to content, form, and mechanical accuracy.

The dissertation is therefore accepted in partial fulfillment of the requirements for the Degree of Doctor of Philosophy.

May 18, 1971  
Date

Maurice J. P. Heuser  
Signature of Advisor