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A study of protein synthesis in the mouse thyroid gland utilizing tritium labeled leucine

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A STUDY OF PROTEIN SYNTHESIS IN THE MOUSE THYROID GLAND UTILIZING TRITIUM LABELED LEUCINE

by

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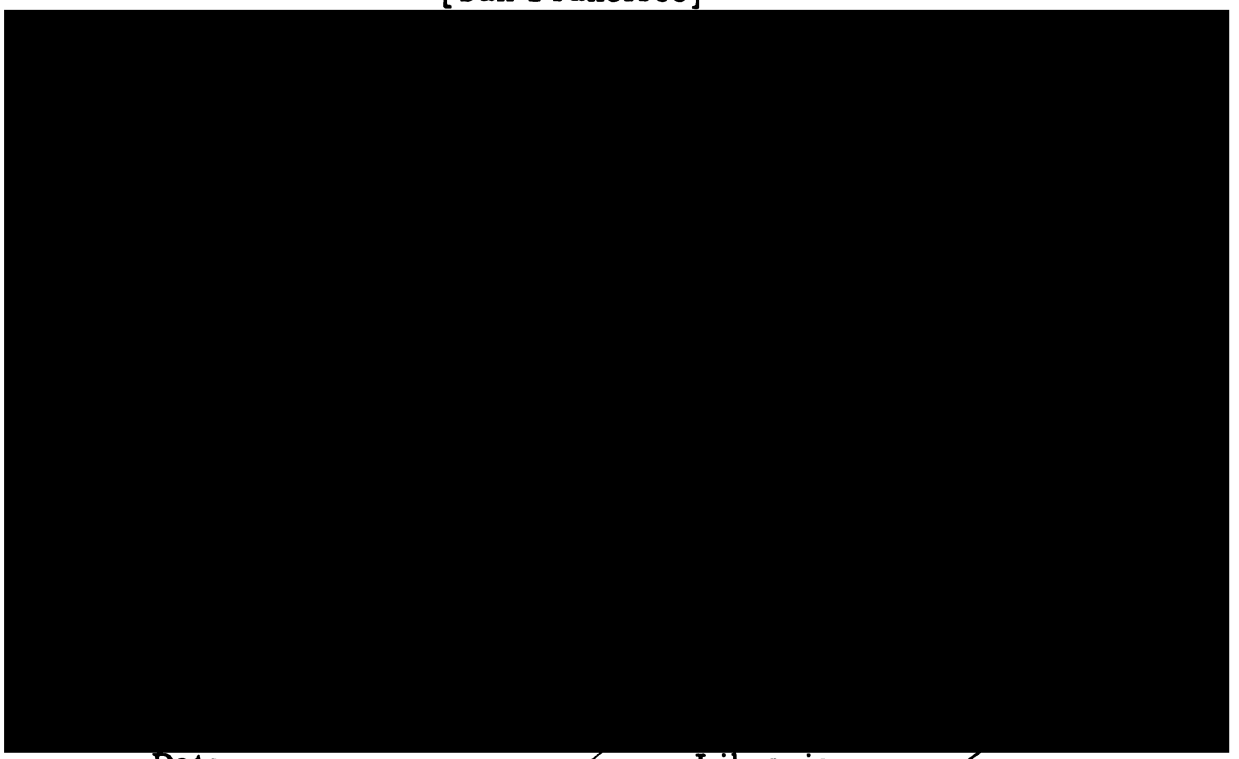

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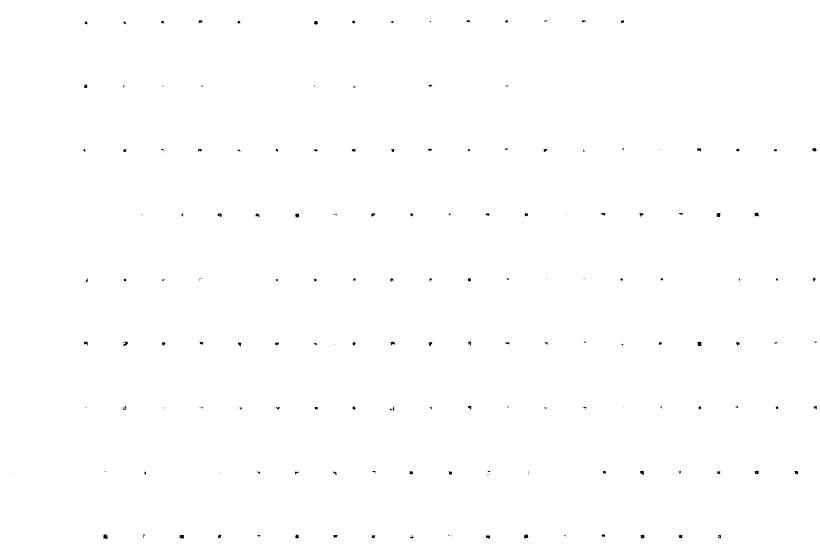


REVIEW

Among the observable effects of thyrotrophin on the thyroid gland are increased rate of release of thyroid hormones, increased trapping of iodide from the circulation, altered distribution of iodine, increased glandular weight, and increased protein synthesis, as well as characteristic changes in the morphology of the follicular cells.

Increased release of thyroid hormones by the thyroid. Thyrotrophin rapidly effects an increased release of thyroid hormone from the thyroid gland which persists for several hours. As early as 1945, Keating et al. demonstrated this by showing that thyrotrophin rapidly released previously accumulated ^{131}I from the thyroid gland. They injected $Na^{131}I$ in young chicks 24 hours before beginning large daily subcutaneous doses of thyrotrophin. The glands were removed for determination of total radioiodine 24, 48 and 72 hours after the first dose of hormone. Three-fourths of the accumulated ^{131}I was released from the thyroid in the first 24 hours. At the end of 72 hours 96 percent of the thyroid ^{131}I had disappeared from the experimental chicks and only half from the controls.

Brown-Grant and his collaborators utilized in vivo counting over the thyroid glands and blood $PB^{131}I$ determinations in rabbits to study the effect of thyrotrophin on the rate of release of thyroid hormone (Brown-Grant et al.,'54; Brown-Grant, '60). Beginning 48 hours after injection of large doses of radioiodine the radioactivity as measured in control animals by daily neck counts declined exponentially for up to 22 days.

Thyrotrophin injected subcutaneously during the period of exponential decline rapidly effected a deviation from this normal exponential decline. Counts made at one-half to one hour intervals revealed an accelerated decline in thyroidal ^{131}I levels as early as one hour after thyrotrophin injection. The increase in thyroid hormone release rate was shown to persist for ten to 20 hours after small subcutaneous doses (0.24-0.5 mg USP equivalent) of thyrotrophin in hypophysectomized animals. They also showed that the level of circulating thyroid hormone varied with thyroid hormone release rate. Aliquots of blood were taken at daily intervals for $PB^{131}I$ determination. In intact animals the $PB^{131}I$ levels in the blood declined as the thyroid radioiodine release rate declined. The decline in blood $PB^{131}I$ was exponential and had a half life identical to that of the decline of thyroidal radioiodine. However, 24 hours after thyrotrophin administration a marked deviation from the exponential decline was produced. By that time the $PB^{131}I$ levels had risen markedly above the predicted control levels.

Rosenberg et al.('60) confirmed the fact that thyrotrophin effected an increase in release of $PB^{131}I$ from ^{131}I labeled thyroid glands. They measured the difference between $PB^{131}I$ levels in samples of thyroid venous blood and arterial blood (V-A difference) that had been serially drawn from dogs. They compared the V-A differences that were determined before and after intravenous thyrotrophin injection. A relative increase in thyroid venous blood $PB^{131}I$ was detected within 18 to 34 minutes following injection of thyrotrophin. Since

15 minutes or longer was required to draw samples for each measurement, the latency of response could have been less. Their published data indicate that the difference persisted for at least 60 minutes when the experiment terminated. Söderberg ('58) used similar methods in rabbits and found that intravenous thyrotrophin induced a marked V-A difference. This author reports that the effect was detected within five to 30 minutes after injection of thyrotrophin, reaching a peak V-A difference in 30 to 60 minutes. The duration of the effect was not reported.

Increased iodide trapping by the thyroid. Thyrotrophin markedly increases the accumulation or trapping of circulating iodide by the thyroid gland. This increase takes place late and persists for a long period following thyrotrophin administration. The early work of Keating et al. ('45) showed a late and persistent increase in accumulation of circulating ^{131}I by the thyroid following thyrotrophin injections in young chicks. The glands were removed for determination of total radioiodine levels four hours after injection of subcutaneous ^{131}I-iodide. The effect of single large subcutaneous doses of thyrotrophin was studied at daily intervals for four days after injection. The results showed no significant rise of total radioiodine in the thyroids over control values until 48 hours. The maximum increase occurred at four days at which time the experiment was terminated. The thyroidal radioiodine in such an experiment is mostly in the organic form. Wollman ('60) showed that injected ^{131}I-iodide is rapidly bound in organic form in the

unblocked thyroid. Taurog et al. ('51) estimated that about one percent of thyroidal iodine was inorganic. Later, Halmi and Pitt-Rivers ('62) showed that only 0.26 percent of the total iodine was present in the gland in the inorganic form.

Halmi ('64) pointed out that thyrotrophin can alter the thyroid:serum (T/S) ^{131}I-iodide ratio by raising the organic binding rate in the gland. Early changes in iodide transport are therefore obscured in the unblocked gland. When organic binding of iodine is blocked, however, the T/S usually reflects changes in iodide transport.

Studies of changes in the T/S ratio confirm that thyrotrophin administration causes delayed and prolonged increase in iodide trapping. In 1950, Vanderlaan and Greer studied the rise in T/S that occurred in young rats following thyrotrophin injection. In control and experimental young male rats (50 gm body weight) PTU was injected subcutaneously to block organic binding and 30 to 60 minutes later tracer doses of ^{131}I-iodide were injected subcutaneously. One hour after injection of the tracer ^{131}I, aliquots of cardiac blood and the thyroids were taken for T/S determination by radioactive counting. The experimental group also received large subcutaneous doses of thyrotrophin. Stimulation was not seen in the experimental animals until more than six hours after thyrotrophin administration. The maximum increase in T/S occurred in 48 hours and declined to normal values in 72 hours.

In a similar experiment Halmi et al. ('60) showed that thyrotrophin produced a biphasic effect on iodide transport.

An initial fall followed by a prolonged rise of T/S occurred after subcutaneous thyrotrophin injection in hypophysectomized young adult rats. The rats were injected subcutaneously with $Na^{131}I$ 45 minutes after binding was blocked with PTU. The animals were killed one to one and one-half hours after administration of the tracer. The T/S was determined at varying time intervals after injections of 0.9 USP units of thyrotrophin. In hypophysectomized animals the T/S fell initially and then rose rapidly above control values eight to 12 hours after injection of thyrotrophin. The rise persisted for 26 hours, the end of the experiment, at which time the T/S was ten to 20 times control value. In another experiment, when intact rats were used, the initial decline in T/S was prolonged, being most significant after 12 hours at a time when a rise was already apparent in hypophysectomized rats. Only a slight rise over control value for intact animals was seen at the end of 26 hours.

Altered distribution of iodine in the thyroid.

Thyrotrophin alters the distribution of ^{131}I in the thyroid gland. Using chromatographic methods Taurog et al. ('58) compared bound forms of ^{131}I in the thyroid of thyrotrophin-treated hypophysectomized rats to those in hypophysectomized controls. The experimental animals were injected with large subcutaneous doses of thyrotrophin seven hours before sacrifice. The percentages of total ^{131}I present in organic forms were determined three hours after intraperitoneal injections of ^{131}I tracer. They found an increased percentage of labeled thyroglobulin in the stimulated thyroids. There was a significant

increase in the percentage of the total ^{131}I present as thyroxine. A noticeable increase in the ratio DIT-^{131}I/MIT-^{131}I was also shown.

Thyrotrophin also causes a redistribution of iodine between subcellular fractions. Recently Pantic and Ekholm ('63) fractionated cells from thyroids that had been removed from intact guinea pigs one hour after ^{131}I-iodide injection. The thyroids were removed at various time periods from one to 72 hours after single intraperitoneal thyrotrophin injections. Supernatant, microsomal, mitochondrial and nuclear fractions were recovered and total ^{131}I and PB^{131}I of each fraction were determined. They found that the absolute level of ^{131}I in all fractions increased with time after stimulation by thyrotrophin. However, the relative ^{131}I of the different fractions underwent marked changes. The most notable change was an increase in the microsomal/supernatant ^{131}I ratio. This increase was seen by six hours after thyrotrophin injection and reached a maximum by 48 hours. Such a change can be explained by an increase in cell height and decrease in colloid volume known to occur following thyrotrophin injection (Gedda,'60). They reported no significant changes in the PB^{131}I/total ^{131}I ratios of any fraction.

Increased weight of the thyroid. Hypertrophy and increase in weight of the thyroid gland are well known effects of thyrotrophin. In 1945 Keating et al. showed that thyroid wet weight increased rapidly following single large thyrotrophin doses in chicks. Gedda ('60) demonstrated a rise in the wet weight of

the thyroid glands in two hours, increasing up to 24 hours, after subcutaneous thyrotrophin was injected in guinea pigs. The increase in dry weight during the first 24 hours was slower than that of the wet weight. The amount of blood, as determined from total thyroidal iron measurements, increased rapidly during the first two hours and during this period accounted for about 50 percent of the increase in fluids. Later increases in fluid could not be accounted for by increased blood. A slight increase in mitosis was seen only after 12 hours.

Increased protein synthesis in the thyroid. Few reports are found in the literature directly pertaining to the effect of acute administration of thyrotrophin on protein synthesis in the thyroid gland. No studies on the intact animal have been reported. It has been shown by studies in vitro, however, that thyrotrophin increases the incorporation of ^{14}C labeled amino acids into thyroid protein (Raghupathy et al.,'63). Surviving guinea pig thyroid slices were incubated for two hours in medium containing ^{14}C labeled amino acids. The incorporation of ^{14}C into protein in thyroids that had been removed from thyrotrophin injected animals was compared to that from uninjected control animals. In one experiment the thyroids were removed 20 hours after the first of three intraperitoneal injections of thyrotrophin spaced seven hours apart. There was an increased incorporation of all seven amino acids used in the experiment. A 60 to 110 percent increase in specific activity of thyroid protein over control values was reported. Another experiment was designed to determine the

effect of duration of action of thyrotrophin. Three equally spaced doses of thyrotrophin were injected in each animal. The animals were killed ten, 15, and 20 hours after the first injection. The most pronounced response to thyrotrophin occurred after 20 hours. The response in the ten hour group was inconsistent. In another experiment thyroid slices removed from hypophysectomized rats showed a reduced ability to incorporate ^{14}C labeled amino acid. The slices were removed one to seven days after hypophysectomy. The results showed that a progressive decline of incorporation of amino acid occurred for seven days after hypophysectomy. No data were presented in this paper to show the effect of thyrotrophin on the level of unincorporated amino acid in the gland.

The addition of thyrotrophin in vitro has not been shown to have an effect on amino acid incorporation (Raghupathy et al.,'64). Sheep thyroid slices and dispersed cell monolayers were incubated for two hours in the presence of different concentrations of thyrotrophin. No increase in incorporation of leucine-^{14}C from the incubation medium was found.

Uptake of amino acids by the thyroid. No studies in vivo could be found pertaining to the effect of thyrotrophin on uptake of amino acids by the thyroid gland. There is conflicting evidence on the effect of thyrotrophin in vitro on uptake of amino acid by thyroid gland slices. Raghupathy et al. ('64) incubated sheep thyroid slices in a medium containing alpha-aminoisobutyric acid-^{14}C (AIB-^{14}C). This amino acid is actively transported into cells, but is not incorporated into

proteins. No enhancement of uptake of AIB-^{14}C occurred when thyrotrophin was added to the mixture. However, Debons and Pittman ('62) previously reported that the uptake of AIB-^{14}C was enhanced when bovine and dog thyroid slices were incubated for one hour in the presence of thyrotrophin.

It is known that thyrotrophin increases the RNA concentration in thyroid follicular cells. This effect may be related to increased protein synthesis. A sharp increase in total thyroidal RNA levels occurred in hypophysectomized rats that had received four intramuscular injections of thyrotrophin in 24 hours (Fiala et al.,'57). Only a slight increase in thyroid weight, number of cells and DNA occurred. These data support the conclusion that thyrotrophin rapidly increases the amount of intracellular RNA. Another study showed that chronic stimulation produces a marked rise in RNA and a lesser rise in DNA (Matovinovic and Vickery,'59). In intact guinea pigs thyrotrophin was injected daily for 14 days or PTU was fed for three months to produce prolonged high levels of circulating thyrotrophin. The greater rise in RNA was attributed to a rise in RNA/cell, while the lesser DNA change reflected an increase in number of cells.

Changes in follicular cell morphology. Changes in thyroid cellular morphology are known to occur rapidly following acute thyrotrophin administration. These changes include increase in cellular height, extension of apical pseudopodia, appearance of apical colloid droplets and an increase in golgi vesicles. Keating et al. in 1945 described a rapid increase in cell height

in chicks after a single large subcutaneous injection of thyrotrophin. The height increased for 48 hours before beginning to decline. After 96 hours the cell height was still markedly augmented. This change paralleled changes in wet weight of the gland. Nadler et al. ('62), in a more recent study, showed extension of apical pseudopodia containing colloid droplets in as little as eight minutes after large intraperitoneal doses of trophic hormone in rats. The droplets were counted at frequent time intervals up to six hours after stimulation. The droplets reached a peak number in the pseudopodia in eight to 12 minutes and in the cell itself in two to four hours. When the apex and base of the cells were compared they found that the droplets increased faster in the apex in the first 30 minutes after which there was a faster increase in the basal droplets. These findings were interpreted as evidence that the colloid droplets were derived from the follicular colloid. Wissig ('63) has utilized the electron microscope to study changes in cell morphology one and two hours after intraperitoneal thyrotrophin injections in rats. In these early time periods he has noted extension of pseudopodia and increased numbers of apical colloid droplets. He reported an increase at the same time in number of golgi vesicles and associated small membrane bound droplets. By 12 hours after thyrotrophin injection the cellular changes had subsided.

Origin of colloid droplets. The relationship of the cytological changes to protein synthesis has not been unequivocally defined. The morphologic analogy between the exocrine secretory

apparatus in the pancreas and the changes seen in the thyroid following stimulation has supported the hypothesis that colloid droplets are secretory in nature (Wissig,'64). The assertion that colloid is pinched off by the cell (Williams, '41) and that the class of large colloid droplets that arises in the apices of the follicular cells soon after thyrotrophin stimulation represents absorption from the lumen rather than synthesis of thyroglobulin has received recent experimental support (Nadler et al.,'60; Wollman and Spicer,'63; Nadler et al.,'64; Sheldon et al.,'64; Wollman et al.,'64). Radioautographic studies showed that follicular colloid previously labeled with, for example, ^{131}I (Wollman and Spicer,'63) or ^{125}I (Sheldon et al,'64) moved from the lumen into large colloid droplets after thyrotrophin stimulation.

Early morphologic and histochemical changes associated with thyrotrophin administration in rats were interpreted by Wollman and Spicer ('63) and Wollman et al. ('64) as evidence of the resorptive nature of the large droplets. They found that pseudopodia, which rapidly extended into the luminal colloid, appeared to engulf colloid droplets. The colloid droplets came to be closely associated with dense granules that contained acid phosphatase. Subsequently the enzyme concentration in the droplets increased as the droplets decreased in size and moved to the base of the cells. This sequence suggested that hydrolysis of thyroglobulin and release of thyroid hormones may occur in the large droplets.

Nadler et al. ('64) in an electron microscope

radioautographic study of protein synthesis in the unstimulated thyroid found that protein bound radioactive label moved through the cell and into the follicular lumen in the absence of large colloid droplets. Leucine-^{3}H was injected intravenously in young rats and the movement of the label through the thyroid cells was determined by grain counts at various time intervals. The label appeared in ten minutes over ribosome-studded membranes of the ergastoplasm. By one hour grains were found in the cysternae of the ergastoplasm and over small vesicles in the golgi region. At three and one-half hours more grains were found over small vesicles in the apical region and over the colloid. It, therefore, appears that protein synthesis and secretion into the follicular lumen occurs in the absence of colloid droplets.

Relation of thyroglobulin synthesis and iodination. The synthesis of thyroglobulin and its iodination are generally believed to be two independent processes. Current thinking is that uniodinated thyroglobulin is secreted into the follicular lumen by the thyroid epithelial cell. After iodide is transported into the cell iodination of tyrosine to MIT and DIT occurs by some oxidative process within the thyroglobulin molecule in the lumen. Several recent studies have corroborated this concept. For example, a material identified as thyroglobulin by the fluorescent antibody technique was found in the fetal rat thyroid cell before iodine concentration occurred (Feldman et al.,'61). The different effect of thyrotrophin in vitro with respect to iodination and protein

synthesis further suggests that the two reactions are independent. When thyrotrophin was added to cultures of trypsinized thyroid cells the incorporation of ^{131}I into thyroxine and iodinated tyrosines was increased (Tong,'64), but no such *in vitro* stimulating effect was seen on protein synthesis (Raghupathy *et al.*,'64). A clear cut separation between iodination and protein synthesis was shown in a recent study (Maloof *et al.*,'64). Thiourea or puromycin was administered to rats 30 minutes before labeled iodide or amino acid. Thiourea completely inhibited the iodination reaction without affecting the incorporation of the amino acid. On the other hand, puromycin had no effect on iodination while inhibiting amino acid incorporation.

The following study was undertaken to determine the effect of acute thyrotrophin administration on amino acid uptake and amino acid incorporation into protein in the thyroid gland of the intact mouse. These two parameters were determined at a series of time intervals after single intravenous injections of thyrotrophin. ^{3}H-labeled dl-leucine was injected 30 minutes before sacrifice to measure amino acid uptake and incorporation. The glands were removed and homogenized and the homogenate was divided into three aliquots. One aliquot was analyzed for protein by the Lowry method. Another aliquot was dessicated and the radioactivity of the residue was counted. The protein in the third aliquot was precipitated with TCA and the radioactivity of the precipitate was counted. The uptake and the incorporation of amino acid by the thyroid were computed from these radioactivity

measurements. The uptake and incorporation of amino acid in animals that had been injected intravenously with large doses of thyrotrophin 30 minutes, four hours and 20 hours before sacrifice were compared with saline injected controls. The latency of effect of the hormone was determined and compared with the known latencies of other effects.

MATERIALS AND METHODS

Forty-four male C3H mice,[1] nine to 13 weeks of age, were maintained for at least ten days on a low iodine diet.[2] The animals were placed into three groups designated as 30-minute, four-hour, and 20-hour groups. Each group consisted of control and experimental animals.

Experiments were carried out over a period of several weeks utilizing four to six mice on any one day. It was not always possible to run an experimental animal and its control on the same day. Experimental mice were injected with thyrotrophin[3] (1 USP unit) in 0.05 ml of saline. Controls were injected with an equal volume of saline. Both control and experimental animals were injected with 25, 50, or 100 uc of carrier free dl-leucine-1,4-^{3}H in 0.05 ml of saline 30 minutes before sacrifice.

Thyrotrophin and saline as well as the labeled amino acid were given by intravenous injection into the external jugular vein under anesthesia. The animals were anesthetized by intraperitoneal pentobarbital (50 mg/gm of body weight) (Pilgrim and DeOme,'55) about 30 minutes before each injection. The animals were starved overnight prior to injection of the labeled amino acid. Thyroid specimens were collected after the mice were sacrificed by exsanguination. Sacrifice was carried out between 11:00 a.m. and 2:00 p.m. to minimize

1. Obtained from Simonson Laboratories, Gilroy, Calif. and from the Cancer Research and Genetics Laboratory of the Department of Zoology, University of California, Berkeley, Calif.
2. Modified McCollum Diet I obtained from Simonson Laboratories, Gilroy, Calif.
3. Thyropar; Armour.

diurnal variation. The thyroids were quickly removed after sacrifice, washed briefly in cold 0.7 percent aqueous carrier leucine solution, and dissected free of adherent fat and connective tissue. The glands from each mouse were homogenized in 2 ml of cold carrier leucine solution in a ground glass tissue grinder.[1] The homogenate was transferred to a centrifuge tube and stored in crushed ice. The tissue grinder was rinsed with another 2 ml of carrier leucine solution and this solution was added to the stored homogenate.

Determination of total protein. 0.2 ml aliquots of homogenate were transferred to serum tubes. After 0.1 ml of 2N NaOH was added to each aliquot, the serum tubes were sealed and agitated for one hour at 37°C. to dissolve the suspended tissue. The resulting solution was analyzed by the method of Lowry ('51). The results were read on a Beckman DU spectrophotometer against a bovine albumin standard.[2]

Determination of radioactivity. 1.0 ml aliquots of each homogenate were used for determination of total gland radioactivity. The aliquots were transferred to 15 ml test tubes and dessicated under vacuum at 60°C. In order to measure the radioactivity incorporated into protein an equal volume of 20 percent TCA was added to the remaining homogenate and the mixture was centrifuged for 15 minutes. Then the precipitated protein was successively resuspended by ultrasonic vibration and washed in five percent cold TCA, 95 percent ethanol, and ether. The dessicated aliquots of total homogenate and the

1. Microchemical Specialties Co., Berkeley, Calif.
2. Armour.

samples of washed TCA precipitate were ultrasonically suspended in 0.5 ml of hyamine[1] and dissolved by heating for 60 minutes at 60°C. Radioactivity was counted in a Packard Tri-Carb liquid scintillation counter. Quenching was determined by internal standards. In order to evaluate the effect of thyrotrophin injection on amino acid incorporation in non-thyroid tissue diaphragm specimens were collected from some animals in each experimental and control group and processed in the same manner as the thyroid glands.

Thirty-minute group. After the external jugular veins were surgically exposed under anesthesia, thyrotrophin or vehicle and dl-leucine-1,4-^{3}H were injected simultaneously 30 minutes before sacrifice.

Four-hour group. Thyrotrophin or vehicle were injected into the external jugular veins of anesthetized mice four hours before sacrifice. One hour before sacrifice the animals remained lightly anesthetized and were given a reduced dose of pentobarbital. Thirty minutes before sacrifice the labeled amino acid was injected intravenously.

Twenty-hour group. Under anesthesia thyrotrophin or vehicle was injected 20 hours before sacrifice. One hour before sacrifice the animals were anesthetized for the second time. Thirty minutes before sacrifice the veins were re-exposed and the labeled amino acid was injected.

1. Hydroxide of Hyamine 10-X, 1 M in methanol; Packard Instrument Co., Downers Grove, Ill.

RESULTS

Total uptake of the label in the thyroid. The specific activity (dpm/mg of thyroid protein) of the thyroid tissue was determined for each animal. This radioactivity includes both incorporated and unincorporated label. The mean value for each control and experimental group is reported in Table 1. According to these figures there was no consistent difference between the control and experimental animals. In the 30-minute group the experimental results were lower than the control results, while this finding was reversed in the 20-hour group. No difference was seen in the four-hour group. The data were tested by analysis of the variance to determine the significance of the small differences that were noted. When analyzed this way there was no significant difference between control and experimental results within time groups or between time groups.

When the results obtained on one day were compared with those obtained on another a daily variation of specific activities was noted. This daily variation appeared to be independent of the controlled experimental variables since both control and experimental results showed the same trend on a given day. For example, on some days all values were high while on others they were low. It was thought that the total amount of radioactive amino acid that was injected on each day, though obstensibly the same, may have varied, perhaps as a result of variation in the methods of preparing solutions for injection.

To minimize the effect of daily variation on the comparison

of control and experimental figures for specific activity in each time period the data were rearranged and reported in Table 2. To obtain the figures in the table the mean of control and experimental results for each time group obtained on the same day were calculated and reported as single scores. This treatment of the data permits a comparison between control and experimental results within each time group. A trend was noted indicating that the uptake of the label in the thyroids was increased after thyrotrophin injection. This trend is substantiated by the finding that in all three time periods the results in the experimental groups were higher than in the comparable control groups. Analysis of the variance showed, however, that the experimental results were not significantly different from the control results.

Incorporation of the label into protein. The percentage of the total radioactivity of the thyroid glands that was incorporated into protein was computed for each animal. The mean score of the results for each experimental and control group is reported in Table 1. These percentage figures are independent of variation in dosage of labeled amino acid. There was a marked increase in incorporation in the thyrotrophin injected animals as compared to the controls in the 30-minute group. In the four-hour group the increase of experimental over control results is even greater. Both the control and experimental figures, however, are lower than the comparable figures in the 30-minute group. There was only a slight increase in incorporation in the experimental 20-hour group over control values. The control results for this group

were slightly higher than the 30-minute control results and much higher than those for the four-hour group.

The results were tested by analysis of the variance. The results of this analysis are shown in Table 3. There was a probability at the 99 percent level that the injection of thyrotrophin increased the incorporation of amino acid under the conditions of this experiment. The t test was used to test the probability that the difference between the experimental and control results within each time group was caused by thyrotrophin administration under the conditions of this experiment. This test revealed a probability at the 90 percent level in the 30-minute group that thyrotrophin injection increased the incorporation of amino acid. The probability was 95 percent for the four-hour group. However, the difference between the control and the experimental results in the 20-hour group was not significant.

The mean increase in incorporation of each time group brought about by thyrotrophin, expressed as percentage increase over control values, is shown in Figure 1. The increase was 35.8 percent in the 30-minute group, 67.9 percent in the four-hour group and 14.2 percent in the 20-hour group.

The percent of the total radioactivity that was incorporated into TCA-precipitated protein in diaphragm control tissue was determined in 29 mice (Table 1). There was a much lower incorporation than that observed in the thyroids. No pattern of change was seen as a result of thyrotrophin administration.

DISCUSSION

Fate of labeled amino acid. When injected intravenously in mice practically all l-leucine-1-^{14}C was shown to disappear from the blood in ten minutes (Borsook et al.,'50). In guinea pigs, after intravenous injection of dl-leucine-4,5-^{3}H, most of the label was available in the blood as a short pulse of three to four minutes duration (Caro and Palade,'64). A small portion, mostly in the form of d-leucine, was still present in the blood after 15 minutes. The relative persistence in the circulation of the d-leucine can be explained by the known slower rate of transport across cell membranes of d-isomers as compared to l-isomers of amino acids (Oxender, '64).

After mice were injected intravenously with l-leucine-1-^{14}C the fate of the label in the viscera was analyzed and found to be present mostly as free amino acid or protein (Borsook et al.,'50). After intravenous injection of dl-leucine-4,5-^{3}H in guinea pigs the label appeared to enter a TCA-soluble intracellular pool in the pancreas from which it was rapidly incorporated into protein (Caro and Palade, '64). The amount and distribution of radioactivity in the gland was measured at short time intervals up to one hour after the label was injected. Total radioactivity rose very quickly in the first four to five minutes and reached 90 percent of the 60 minute value 20 minutes after the label was injected. Administration of very large chaser doses of cold leucine three minutes after the injection of the radioactive material stopped the uptake of additional label by the gland.

Thereafter the total radioactivity within the gland did not decline but the incorporated/unincorporated ratio of label rose rapidly until at 60 minutes after labeling 73 percent of the radioactivity was present in protein. It can be concluded from these results that the labeled leucine was actively transported into cells and that during the period of the experiment little if any label, either incorporated or unincorporated, was lost from the cells.

After slices of sheep thyroid were incubated for 2 hours in a medium containing uniformly labeled l-leucine most of the label in the tissue was in the form of free amino acid or protein (Raghupathy et al.,'64). Almost 99 percent of the incorporated label was present in l-leucine. When the newly labeled slices were incubated for one hour in non-labeled medium the radioactivity of the protein did not decline. On the basis of the findings in these earlier studies of the pancreas and thyroid the major portion of the radioactive label in the thyroid homogenates examined in the current study is considered to be present in free amino acids and protein. Since the earlier studies showed that all label that is taken up is retained by the tissues, at least for short time intervals, it seems justifiable to let the radioactivity detected in the homogenates of the thyroid gland removed a short time after administration of label serve as a measure of the amount of labeled amino acid taken up by the cells.

Effect of thyrotrophin on uptake of amino acid by the thyroid gland. This study revealed no clear effect of thyrotrophin on the total amount of amino acid taken up by

the thyroid gland. However, when the data were analyzed to minimize the effect of daily variation on the results, there was a trend toward an increase in specific activity of the total homogenate in the mice injected with thyrotrophin above that of the controls in each time period. This trend may indicate that thyrotrophin did in fact cause an augmented uptake of the label.

It should be emphasized that specific activity is based on both amount of radioactive label and amount of protein in the gland. Increase in specific activity could result from a depletion of protein as well as increased uptake of label. It is conceivable that in this study the trend towards specific activity increase arose by virtue of loss of thyroglobulin, especially in the early time periods after administration of thyrotrophin. This possibility is hard to evaluate since there have been no studies specifically designed to show the early effects of thyrotrophin on total protein levels in the thyroid gland.

Effect of thyrotrophin on protein synthesis by the thyroid gland. The percent incorporation into protein, i.e., the proportion of the total label in the thyroid that was incorporated into protein, is independent of variation in the total uptake of dl-leucine-1,4-^{3}H by the gland that resulted from differences in dosage and body weight among the animals. This figure can therefore be used as a comparative measure of rate of protein synthesis.

After administration of thyrotrophin, incorporation of

label increased about 35 percent in the first 30 minutes. This result indicates a rapid onset of stimulation and a pronounced rise of protein synthesis during this period. The stimulating effect of thyrotrophin was doubled at four hours, when incorporation increased almost 70 percent. However, at 20 hours after thyrotrophin injection, little stimulation was apparent.

Identification of labeled protein. Although the identity of the labeled proteins was not determined in this study, one may draw some logical inferences as to their identity. Among the apparent possibilities are thyroglobulin and thyroglobulin precursors, as well as structural proteins used for replication, growth or replacement. After tyrosine-^{14}C was injected into normal rats, a major portion of the labeled protein in the thyroid gland was found by electrophoresis to be identical to thyroglobulin (Maloof et al.,'64). In addition, radioautographic studies utilizing leucine-^{3}H in rats have shown most of the label to be located in structures that are presumed to be associated with synthesis and release of thyroglobulin (Nadler et al.,'64). Similarly, in the exocrine pancreas the rate of synthesis of exportable protein was higher than that of other protein (Siekevitz and Palade,'60). The rare labeling of thyroid DNA by thymidine-^{3}H (Seed and Goldberg,'63) and the failure of acute stimulation with thyrotrophin to produce a significant rise in thyroid DNA (Fiala et al.,'57) suggest that structural protein necessary for cell replication is not a significant part of the labeled protein in this study. On the

basis of these prior findings, it is assumed that a major portion of the protein synthesis that was measured in this study is of thyroglobulin or its precursors.

Effect of thyroid hormones. The possibility that the experimental findings resulted from increased levels of circulating thyroid hormones must be considered. The thyroid gland, as judged by iodine uptake and release, is not a target of its own hormones (Halmi and Stuelke,'56) and the effect of thyroid hormones in protein metabolism is generally considered to be catabolic (Pitt-Rivers and Tata,'59). However, thyroxine has recently been shown to increase incorporation of amino acid by the liver, kidney and heart, but not by the spleen, testis of brain (Michels et al.,'63). Since in this study incorporation of amino acid by the diaphragm was not increased after thyrotrophin injection, thyroid hormones appeared to have no effect on protein synthesis under the conditions of this experiment.

Control values. It is of interest that among the experimental animals incorporation of label, as measured in terms of percent of incorporation, showed no significant variation at any of the time periods following thyrotrophin injection. The differences are apparent only when experimental animals are compared to control animals in each time group and, therefore, appear to reflect differences in the latter. The effect of anesthesia and surgical procedures may explain these differences. Barbiturates are known to directly affect thyroid function, as judged by short term response, by inhibiting

organic binding of iodine (Wase and Foster,'56). It is conceivable that they have the same inhibiting effect on protein synthesis. The differences in control values may also have resulted from the effect of general anesthesia on secretion of endogenous thyrotrophin. Study of the rate of release of thyroid hormone in cats and rats showed that general anesthesia markedly slowed thyrotrophin secretion after two or three hours and thereafter completely arrested it (Söderberg,'58; Purves, '64). The arrest was ascribed to reduction of the 'hypothalamic' effects which are concerned with control of thyrotrophin synthesis and release, as well as threshold of pituitary response to blood levels of thyroid hormone. In addition, various hormonal and neural factors which are known to influence markedly the sensitivity of the thyroid gland to circulating thyrotrophin may have effected the response of the gland in this study. ACTH, catecholamines, acetylcholine and strong nociceptive stimuli (Söderberg,'58) as well as severe surgical stress (Brown-Grant,'56) have been found to produce such effects. Some of these effects are apparently related to alterations in blood flow through the gland: those factors which increase vasodilation tend to increase the sensitivity to thyrotrophin and vice versa (Söderberg,'58). The low control values in the four hour group may reflect a combination of suppression of endogenous secretion of thyrotrophin resulting from general anesthesia and a direct depression of protein synthesis by pentobarbital. High incorporation values for the 20 hour controls might be attributed to a rise in

endogenous secretion of thyrotrophin resulting from a prolonged depression of the level of thyroid hormone in the blood brought about by the initial experimental procedures.

Mechanism of action of thyrotrophin. The fundamental mechanism or mechanisms by which thyrotrophin acts on the thyroid follicular cell to produce its several effects are not known. Recently some information has been obtained concerning the mode of action of other hormones on their target tissues. Some hormones have been shown to induce synthesis of enzymes to produce their physiologic effect. It is presumed they must first induce synthesis of messenger RNA which then serves as a template for enzyme synthesis. Generally speaking, hormones that act in this way produce their effect only after lengthy latencies. Cortisone *in vivo* has been shown to induce synthesis of hepatic tyrosine-alpha-ketoglutarate transaminase (Greengard *et al.*,'63) and tryptophan pyrrolase (Feigelson *et al.*,'62). Aldosterone appears to regulate active Na^+ transport in toad bladders *in vitro* by induction of enzymes involved in oxidation of pyruvate (Edelman *et al.*,'63). Estradiol (Ui and Mueller,'63; Noteboom and Gorski,'63) and testosterone (Liao and Williams-Ashman,'62) appear to stimulate protein synthesis in target organs by inducing synthesis of messenger RNA. Intraperitoneal injection of estradiol increased both RNA polymerase and nuclear RNA in rat uterus after two hours but had no effect on amino acid incorporation until four hours (Noteboom and Gorski,'63).

Other hormones appear to have more rapid and presumably more direct effects on physiological processes. Vasopressin

directly effects the permeability of certain epithelial membranes to water, possibly by a mechanism involving activation of cyclic AMP (Orloff and Handler,'61; Brown et al., '63). Insulin is known to facilitate the entry of glucose and other structurally related sugars into cells, presumably by an effect on the cell membrane, and can act directly to stimulate the incorporation of amino acids into protein in liver by a mechanism dependent on glucose (Penhos and Krahl,'62). There is also evidence that insulin can increase the uptake of several structurally related amino acids by a mechanism independent of its effect on glucose entry (Wool and Krahl,'59; Akedo et al.,'62).

The observable effects of thyrotrophin on the thyroid gland exhibit both short and long latencies. Augmented rate of release of hormone, changes in cell morphology, increase in wet weight of the gland, increase of its blood volume, and depressed iodide uptake take place very early after administration of thyrotrophin, while increased iodide uptake is a much more delayed effect.

It is clear from the findings in the 30-minute group that there is little latency in the onset of the stimulating effect of thyrotrophin on protein synthesis. The two-fold greater increase over control values seen at the end of four hours as compared to 30 minutes is consistent with a rapid early rise of protein synthesis to a maximum level following thyrotrophin stimulation.

The varied latencies may be related to fundamental

differences in the mechanism by which thyrotrophic hormone induces each effect or may simply reflect temporal differences between direct and indirect effects. Freinkel ('64) has extensively discussed the early changes in thyroidal energy metabolism induced by thyrotrophin and has pointed out that such responses produce an early pattern of change which includes realignment of cytostructure, an enhanced flux of substrate, and alteration in the pathway of substrate disposition. The observable effects of thyrotrophin reviewed in this paper may be the indirect results of such metabolic changes. Since stimulation of iodide uptake is the only one of the effects of thyrotrophin that exhibits a long latency, it is conceivable that stimulation of the iodide "pump" depends on a mechanism that directly or indirectly requires increased synthesis of enzymes.

The rapid stimulation of protein synthesis in the thyroid following thyrotrophin injection suggests that the hormone directly activated some late stage of the synthetic process and in this regard resembles that group of hormones previously cited that appear to act on cell membranes to increase their physiologic activity. It has recently been shown in slices of lamb thyroid that synthesis of thyroglobulin was not sensitive to the action of actinomycin and therefore is apparently not dependent on new RNA synthesis. However synthesis of another smaller fraction of protein was relatively sensitive to actinomycin. Actinomycin was added to the medium and incorporation of labeled leucine continued at a normal rate for one

hour and at a reduced but fairly level rate for at least an additional 20 hours. When the slices were pre-incubated in medium containing high levels of actinomycin for 21 hours the labeling of thyroglobulin during the subsequent five hours was not significantly different from the controls which were pre-incubated without actinomycin. This study suggests that under acute conditions the supply of messenger RNA is not the limiting factor in the synthesis of thyroglobulin and that stimulation of thyroglobulin synthesis does not depend upon de novo synthesis of nucleic acid or enzymes.

Persistence of response to thyrotrophin. On the one hand some changes induced in the thyroid gland by acute administration of thyrotrophin, in particular those concerned with cellular morphology, i.e., increased numbers of colloid droplets, small vesicles and golgi vesicles, as well as the appearance of apical pseudopodia, are of only a few hours duration. This is also true of increased blood flow. On the other hand, increases in hormone release, iodide uptake, wet and dry weight, and cell height may persist for several days. In this study, the results indicate a persistence of a high level of protein synthesis for at least four hours. This prolonged response could be due to either persistence of the effect once initiated or to persistence of high levels of circulating thyrotrophin. Very high thyrotrophin doses were used in this study and, based on estimates of physiologic levels and the half life of exogenous thyrotrophin in the blood or rats (Bakke and Lawrence,'62; Bakke,'63), high circulating levels of thyrotrophin could persist up to time of

the injection of radioactive label in the four-hour group. It is not clear from the results that the effect of thyrotrophin injection persisted for 20 hours. Only a trend toward increase over control values was seen at the end of that period. However, it should be noted that the incorporation rate in the experimental animals in this group was not statistically different from that in the 30-minute and four-hour experimental animals. Purves ('64) has indicated that high doses of thyrotrophin give a hormone release response in the thyroid which reaches a limiting plateau. This fact suggests the possibility that a maximum stimulation of protein synthesis occurred very early and persisted for at least 20 hours after thyrotrophin injection.

TABLE 1

Effect of intravenous TSH on the uptake and incorporation of dl-leucine-1,4-^{3}H into protein of mouse thyroid gland and diaphragm.

		Thyroid gland							Diaphragm	
					Specific activity					
Group		# of mice	Body wt. (gm)*	Protein (mg)*	Homogenate* (10^{-3} dpm/mg protein)	TCA precipitate* (10^{-3} dpm/mg protein)	Incorporation (%)*†	Incorporation increase (%)**	# of mice	Incorporation (%)*
30-Min.	C	5	19	.297	37.4±11.4	8.2±2.6	21.8± 3.8		4	7.4
	TSH	7	22.7	.350	35.1± 6.9	10.4±3.0	29.6± 6.1	35.8	5	7.6
4-Hr.	C	5	24	.386	39.0± 7.8	6.5±3.7	15.9± 6.7		5	6.1
	TSH	9	25.3	.369	39.3± 9.2	10.0±2.9	26.7±10.2	67.9	6	8.7
20-Hr.	C	8	24	.362	40.0±13.2	9.9±2.7	25.3± 9.4		4	7.2
	TSH	9	23.1	.342	44.7±13.2	12.5±3.8	28.9± 7.4	14.2	5	7.0

* Group means; standard deviation indicated in some cases.
† Percent of total homogenate radioactivity incorporated into TCA precipitate.
** Percent increase of experimental mean incorporation over control mean incorporation.

TABLE 2

Effect of intravenous TSH on uptake and incorporation of dl-leucine-1,4-^{3}H by the mouse thyroid gland.

Group		Number of mice	Number of daily means*	Specific activity: Homogenate (10^{-3} dpm/mg protein)**	Specific activity: TCA Precipitate (10^{-3} dpm/mg protein)**
30-Min.	C	2	2	30.0± 0.4	5.8±1.8
	TSH	6	2	34.3± 4.5	9.8±0.5
4-Hr.	C	5	3	39.4± 7.6	6.2±1.2
	TSH	8	3	42.4± 5.0	9.3±2.3
20-Hr.	C	8	4	41.3± 8.9	9.9±2.3
	TSH	9	4	43.7±12.4	12.2±3.3

* Days when results were obtained on both experimental and control animals in a group.

** Group means derived from daily mean figures; standard deviation indicated.

TABLE 3

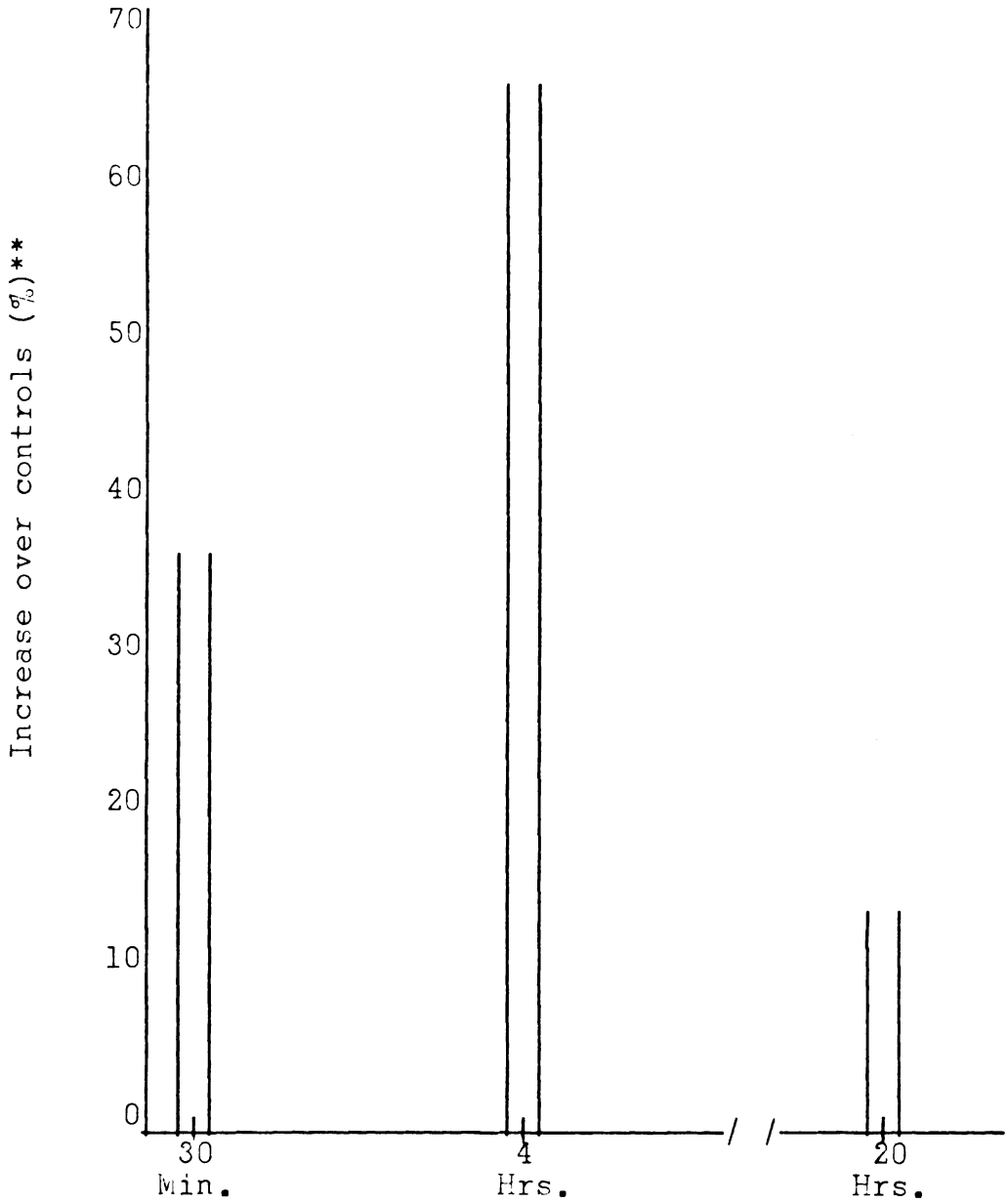
Analysis of variance of percent of incorporation of dl-leucine-1,4-^{3}H into protein of the mouse thyroid.

Source of variation	Sum of squares	Degrees of freedom	Mean square	F ratio
Between time group means	246.54	2	123.27	2.35**
Between control and experimental group means	553.91	1	553.91	10.56*
Interaction	101.64	2	50.82	.97**
Within groups	1992.29	38	52.43	

* $P < .01$
** $P > 0.1$

FIGURE 1

Increase in incorporation of dl-leucine-1,4-^{3}H into thyroid protein of mice following intravenous injection of TSH (1 USP unit).



** Percent increase of experimental mean incorporation over control mean incorporation.

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