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Research Article

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IN VITRO THYROTROPIN–INDUCED PROTEOLYSIS IN THE THYROID GLAND *

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Numerous studies, both *in vivo* and *in vitro,* have been designed to define the biochemical events influenced by the action of thyrotropin (TSH) on the thyroid gland. *In vivo* experiments have shown that this hormone stimulates growth of the follicular epithelium, trapping of inorganic iodide, biosynthesis of hormonal iodine, turnover of thyroglobulin, and release of inorganic iodide and hormonal iodine. The latter action has received much attention because of its relevance to the concentration of circulating thyroid hormone and because of the promptness of this response *in vivo.* De Robertis demonstrated an increase in intrafollicular proteolytic activity after administration of TSH to rats (1). Keating, Rawson, Peacock, and Evans (2) noted loss of radioiodine from the chick thyroid after TSH administration. Similar results have been observed in other laboratory animals (3, 4). The administration of TSH to euthyroid humans also results in prompt release of radioiodine from the thyroid (5–7), occurring 90 minutes after intramuscular injection in one study (7).

In vitro techniques have permitted investigation in a more rigidly controlled environment. Such observations on the effect of TSH added to thyroid tissue have confirmed the stimulus to the trapping of iodide (8, 9). TSH *in vitro* has also been shown to enhance certain parameters of intermediary metabolism such as carbon-1 oxidation of glucose (10, 11), phospholipid synthesis (12), oxygen consumption, lipogenesis from labeled glucose, incorporation of inositol into phospholipid (13), and the level of triphosphopyridine nucleotide (14). Few studies, however, have demonstrated release of hormonal iodine or inorganic iodide induced by TSH *in vitro.* Clearly, some proteolytic system is required for the release of thyroxin from its peptide linkage in thyroglobulin, and such systems have been isolated (15, 16); however, TSH has failed to influence the proteolytic activity of these isolated systems when added *in vitro.* Histochemical studies, showing increased mobilization and release of follicular colloid, have been taken to indicate an augmented *in vitro* proteolytic response after the addition of TSH (17), and Bottari (18) and El Kabir (19) have demonstrated TSH-induced release of I^{131} from prelabeled guinea-pig thyroids *in vitro.* It is not clear, however, if either of these *in vitro* TSH-induced effects represents proteolytic activity.

The present studies were designed to evaluate directly the effects of TSH on various parameters of proteolysis in the thyroid gland. These are *1*) the *in vitro* release of butanol-soluble I^{131} from prelabeled rat thyroid glands induced by TSH given acutely *in vivo* or added *in vitro,* and *2*) the release of free α-amino nitrogen, tyrosine, and inorganic I^{127} in beef or sheep thyroid slices induced by TSH *in vitro.* Evidence is presented that proteolysis in thyroid slices occurs during incubation at pH 7.4 and that TSH enhances this process.

METHODS AND MATERIALS

Rat experiments. Male rats of the Wistar strain weighing 150 g were used for experiments measuring the release of butanol-extractable I^{131} (BEI^{131}). They were injected intraperitoneally with 10 μc of carrier-free NaI^{131} 48 hours before study. For the *in vivo* experiment, 8 rats were anesthetized with Nembutal, and either 0.2 ml of isotonic NaCl (controls) or 0.2 ml isotonic NaCl containing 0.5 U TSH was injected directly into the femoral vein. After exactly 20 minutes, each animal was exsanguinated via the abdominal aorta. The thyroid gland was removed rapidly by blunt dissection, placed on moist filter paper, and cut into two lobes. These were

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minced with a sharp razor blade, rinsed with several drops of isotonic NaCl, and placed in separate flasks, each containing 2 ml Krebs-Ringer bicarbonate buffer at pH 7.4, with 1.6 mg glucose and 0.1 mg penicillin-streptomycin added. A gas mixture of 95 per cent O_2 and 5 per cent CO_2 was passed through the buffer. The contents of one flask were immediately transferred to a Potter-Elvehjem Teflon homogenizer. The flask containing the contralateral lobe was incubated 3 hours at 37.5° C in a metabolic shaker and then transferred as above for homogenizing. The minced lobes were homogenized with their media for 1 minute. The flasks and homogenizer were washed with several rinses of water. Each homogenate and its rinses were pooled, brought to 4 ml with water, acidified with 1 drop of 10 per cent H_2SO_4, and extracted twice with 5-ml volumes of *n*-butanol. No attempt was made to exclude I^{131} by the usual alkali wash, but the extracts were pooled, brought to a final volume of 13 ml, and counted in a scintillation well detector with a radiation analyzer allowing a background of 8 cpm.

The 10 rats used for the *in vitro* experiments were killed by a blow to the head. The left lobes of two consecutive animals were pooled and incubated as one of the five control flasks. The right lobes were likewise paired and incubated in 2 ml buffer containing 0.2 U TSH to give 5 control and 5 experimental values. After a 3-hour incubation, the lobes and media were homogenized, acidified, extracted with butanol, and the BEI^{131} counted as described. These extracts were then evaporated in an air-jet to 0.4 ml, and 100 μl of each extract was applied to Whatman no. 1 filter paper together with carrier iodoamino acids. The chromatograms were developed in butanol-dioxane-ammonia (20). To locate the carrier iodoamino acids, the chromatogram was sprayed with diazotized sulfanilic acid and the radioactivity determined by counting 1.0-cm strips in the scintillation well detector.

Experiments with beef and sheep thyroid slices. The beef and sheep thyroid glands were obtained from the local slaughter house and transported to the laboratory on ice, at 5 to 10° C. After adherent tissue was removed, the gland was sliced into 90- to 140-mg slices with a Stadie-Riggs hand microtome. Adjacent sheep thyroid slices were alternated between control and experimental groups, permitting a paired-data statistical analysis of the results. Sheep slices were compared in this manner, since several lobes were used in each experiment. Beef thyroid slices, however, were obtained from a single lobe, allowing statistical comparison of the group means (21). Slices were incubated in 3.0 ml of Krebs-Ringer bicarbonate buffer as previously described. After incubation, the slice and medium were homogenized together, except in the time studies, where the slice and medium were treated separately. Protein was precipitated from the homogenate with 16 per cent trichloroacetic acid (TCA) and the final volume brought to 10 or 15 ml. Care was taken not to allow more than 1 minute from the end of incubation to the time of protein precipitation with TCA. The TCA-soluble fraction was separated by centrifugation and analyzed colorimetrically for free α-amino nitrogen, tyrosine, and inorganic I^{127} as follows. *a*) Free α-amino nitrogen was determined by the Ninhydrin method of Troll and Cannan (22). This method, in contrast to several others, is satisfactory in the presence of 1 to 20 per cent TCA. All standard solutions and reagent blanks were prepared in the same concentration of TCA as the unknowns, since high concentrations of TCA (10 to 20 per cent) tended to give higher blank readings than non-TCA blanks. Also, in order to insure low blank readings, it was found that weekly treatment of the reagents (KCN-pyridine and 80 per cent phenol in absolute alcohol) with Permutit was essential. *b*) Free tyrosine content was quantitated by the 1-nitroso-2-naphthol method of Ceriotti and Spandrio (23). The concentration of TCA used in these experiments in no way interferes with the colorimetric determination. Equimolar quantities of DL-3,5-diiodotyrosine, L-3,5,3′-triiodothyronine,[1] and L-thyroxin (sodium salt) were found not to react in this method. An exception was L-3-monoiodothyrosine, which reacted 70 per cent on an equimolar basis with tyrosine. *c*) Inorganic I^{127} was determined by the ceric ammonium sulfate method of Grossmann and Grossmann (24). All reagent blanks and standard solutions were prepared in the same concentration of TCA as the unknowns. Since it was found that various iodoamino acids contributed, apparently catalytically, to this reaction, they were removed from the TCA-soluble fraction as follows. A 2.0 ml sample of the TCA supernatant fluid was applied to a 6.0×0.8 cm Dowex-50×8 cationic exchange resin column (H^+ ion form, 200–400 mesh) and washed with 5.0 ml deionized water. The first 5.0 ml was collected and quantitated as described above for I^{127}. Control studies on this procedure have indicated a recovery of 95 to 100 per cent of the inorganic I^{127} applied to the column and have also shown that 99 per cent of L-tyrosine-U-C^{14} remains on the column. Furthermore, standard solutions (in 16 per cent TCA) of L-3-monoiodotyrosine, DL-3,5-diiodotyrosine, L-3,5,3′-triiodothyronine, and L-thyroxin were applied to these columns, and analysis of the eluates in each case by the ceric ammonium sulfate method revealed no detectable amounts of these compounds.

The extrathyroidal tissues used in experiments 11 through 16 were handled as described for beef and sheep thyroid slices. In some experiments the glucose remaining in the medium at the end of incubation was quantitated by the glucose oxidase method (25). Protein was first precipitated from a 0.2-ml sample of medium in 3 ml distilled water with 0.4 ml 5 per cent $ZnSO_4$ and 0.4 ml 0.3 N $Ba(OH)_2$. Analyses were then performed on 1.0-ml sample of the supernatant fluid.

Hormone preparations and humoral agents. Commercially available lyophilized preparations of thyrotropin (Thytropar), adrenocorticotropin (ACTH), and pituitary gonadotropin (FSH) were used.[2] A highly

[1] Supplied through the courtesy of Smith, Kline & French Laboratories, Philadelphia, Pa.

[2] All purchased from Armour Pharmaceutical Company, Kankakee, Ill.

purified TSH preparation (20 U per mg) was used in experiment 10B.[3] Regular insulin (Iletin),[4] epinephrine hydrochloride,[5] and acetylcholine hydrochloride[6] were purchased from manufacturers. All hormone preparations expressed in units refer to USP units. Inactivated TSH was prepared as follows: commercial lyophilized TSH was treated with 2 ml concentrated sulfuric acid at 0° C for 5 minutes, diluted to 100 ml with cold (4° C) distilled water, and dialyzed against isotonic saline at 4° C for 16 hours. This protein sulfation procedure is a modification of that reported by Reitz, Ferrel, Fraenkel-Conrat, and Olcott (26) and employed by Sonenberg and Money to inactivate TSH (27).

RESULTS

Rat experiments. In the experiment designed to study the release of BEI^{131} from prelabeled rat thyroid glands 20 minutes after the acute intravenous administration of 0.5 U TSH, a small, statistically insignificant increase from the control was observed at the time of sacrifice (Figure 1). Incubation of the contralateral lobe from each control animal for 3 hours resulted in a slightly greater release of BEI^{131} than was seen in the nonincubated lobe. In contrast, incubation of the contralateral lobes from animals given TSH produced a marked increase ($p < 0.01$) in the BEI^{131} over that of the nonincubated lobes. Comparison of the control and TSH group means after incubation revealed a significant increase ($p < 0.01$) in the BEI^{131} of the TSH group. TSH given *in vivo,* then, appeared to enhance the *in vitro* release of BEI^{131} from rat thyroid minces.

In the *in vitro* experiment with prelabeled rat thyroid minces, the addition of 0.1 U TSH per ml incubation medium also produced a significant increase ($p < 0.05$, analysis of group means) in the BEI^{131} when compared with contralateral control lobes after 5 hours of incubation (Figure 1). This difference between control and TSH groups, however, was not so marked as that noted when

[3] Kindly donated by Dr. J. G. Pierce, University of California, Los Angeles, Calif.

[4] Eli Lilly & Company, Indianapolis, Ind.

[5] Parke, Davis & Company, Detroit, Mich.

[6] Merck Sharp & Dohme, Inc., Philadelphia, Pa.

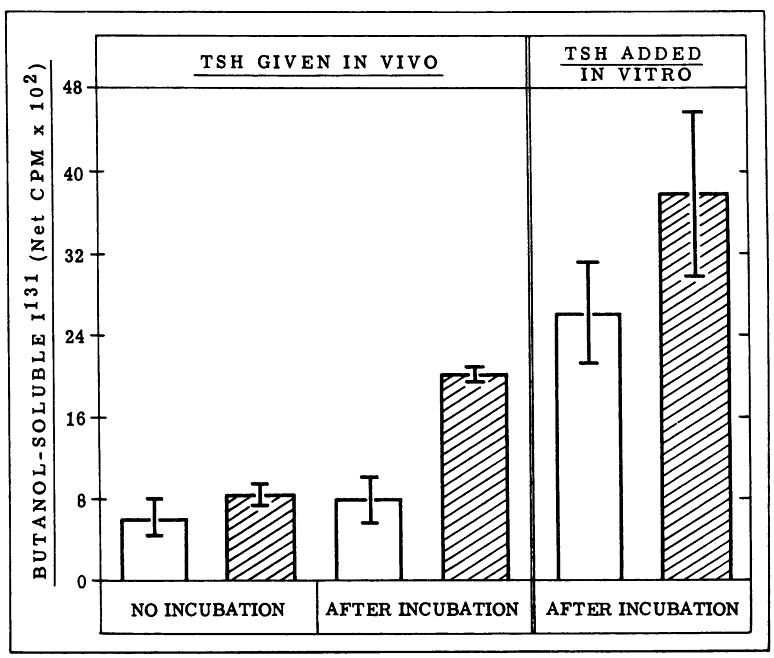

FIG. 1. CONTINUING PROTEOLYSIS *in vitro* AFTER ADMINISTRATION OF THYROTROPIN (TSH) *in vivo* AND PROTEOLYSIS INDUCED BY ADDING TSH *in vitro.* On the left, the butanol-extractable I^{131} (BEI^{131}) of thyroid minces from animals given TSH *in vivo* is compared before and after 3 hours incubation. On the right, the BEI^{131} from minced lobes incubated 5 hours with TSH is compared with the contralateral control lobes. The mean values for saline controls (open bars) and TSH groups (hatched bars) are shown with 1 SD.

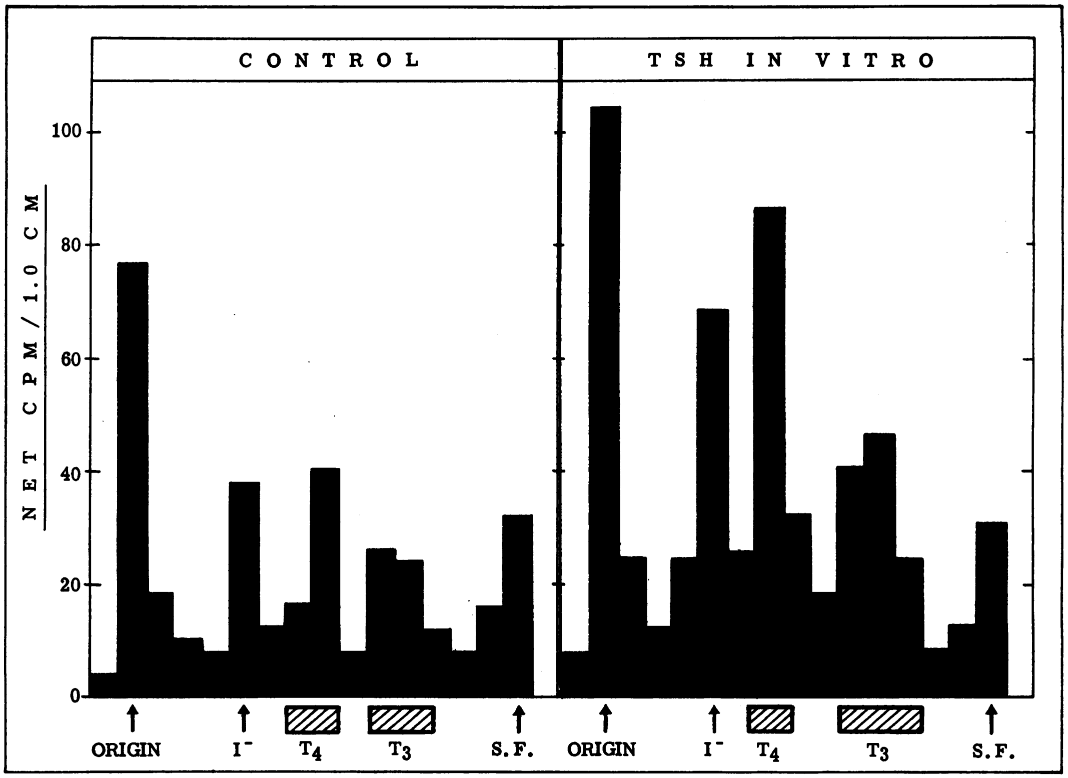

FIG. 2. CHROMATOGRAMS OF BUTANOL EXTRACTS FROM PAIRED, PRELABELED, RAT THYROID MINCES INCUBATED WITH AND WITHOUT THYROTROPIN (TSH) IN THE MEDIUM. Bars indicate the I^{131} activity in each centimeter of the chromatogram, and locations of iodide, thyroxin (T_4), and triiodothyronine (T_3) are shown below. The solvent front is indicated as S.F.

TSH was given *in vivo* before incubation. Chromatograms of the butanol extracts from the *in vitro* experiment demonstrated increases in labeled thyroxin, triiodothyronine, and iodide in the TSH-supplemented flasks. A characteristic pair of these chromatograms is shown in Figure 2. Although inadequate separation of mono- and diiodotyrosine was obtained, the total radioactivity of this locus, which appears near the origin, was increased in the TSH group. Thus, the addition of TSH *in vitro* stimulated the release of hormonal and other iodoamino acids as well as of inorganic iodide.

Experiments with beef and sheep thyroid slices. Table I summarizes the data obtained from a series of experiments on beef and sheep thyroid slices. Addition of TSH (0.1 U per ml incubation medium) produced statistically significant increases in free α-amino nitrogen, tyrosine, and inorganic I^{127} when compared to control groups. Mean values for the free α-amino nitrogen in the TSH groups were always greater than control group means; however, these differences did not achieve statistical significance in three of the ten experiments. The free tyrosine differences between control and TSH groups were more apparent, achieving statistical significance in all experiments in which tyrosine was measured. The TSH-induced increase in inorganic iodide was the most marked of the three parameters measured ($p < 0.001$). The quantitative differences in these constituents, however, should be noted. On an equimolar basis, the concentrations of free tyrosine and α-amino nitrogen were approximately 50 and 1,000 times larger, respectively, then the concentration of inorganic iodide. The average TSH-induced increase expressed as a percentage of change from control was 22 per cent for α-amino nitrogen, 37 per cent for tyrosine, and 175 per cent for iodide.

Although the data within each group of observations agreed closely, considerable variation was noted from experiment to experiment (Table

I). The tyrosine values, however, were more consistent than either the α-amino nitrogen or iodide results. The magnitude of the TSH-induced response also varied among experiments. For example, in experiment 2S the average increase in the α-amino nitrogen was only 6 per cent. In experiment 1S the increase was 50 per cent. The time of incubation in the experiments shown in Table I ranged from 3.5 to 5 hours, but this appeared to have no consistent relationship to the magnitude of the hormone response.

The relationship of the time of incubation to the TSH-induced increase in free α-amino nitrogen is shown in more detail in Figure 3. In these studies, the slice and incubation medium were analyzed separately for free α-amino nitrogen and the percentage of increase from zero time plotted. A continuing rise in the medium free α-amino nitrogen was noted throughout incubation. A less marked rise was found in the slices. In both studies, TSH produced a significant increase ($p < 0.05$) in the slice free α-amino nitrogen when compared to the control after 30 minutes of incubation. This effect was not yet apparent in the medium. After 3 hours of incubation, a significant TSH-induced increase was noted in both the slice and medium ($p < 0.05$). At 6 hours, the slice difference was not apparent, although the medium still contained a significantly greater amount of free α-amino nitrogen ($p < 0.05$). The mean values for free α-amino nitrogen in the thyroid slice and in the incubation medium at the end of incubation were as follows. In the study on the right, the control slices averaged 1.14 ± 0.11 and the TSH slices, 1.26 ± 0.16 μmoles per 100 mg wet weight. The incubation medium in the control and TSH groups averaged 1.53 ± 0.09 and 2.07 ± 0.21 μmoles per 100 mg, respectively. These time studies indicate an initial TSH-induced increase in proteolysis, first evident in the slice, and followed by a continuing accumulation of free α-amino nitrogen in the medium.

Evidence to support the viability of the slices in these experiments was obtained by measuring the

TABLE I

*The effects of commercial and purified thyrotropin (TSH) on parameters of proteolysis in thyroid slices**

Experiment	No. of observations per group	Hours of incubation	Control	Thyrotropin	p
			μmoles/100 mg wet wt		
A. Free α-amino nitrogen					
1S	6	4.0	1.86 ± .20	2.79 ± .57	<.02
2S	6	4.0	2.69 ± .24	2.85 ± .32	NS
3S	6	3.0	3.63 ± .52	3.91 ± .56	NS
4S	5	3.5	4.32 ± .50	5.05 ± .41	<.001
5S	5	4.0	3.36 ± .13	4.26 ± .12	<.005
6S	4	5.0	3.46 ± .44	3.95 ± .37	<.001
7B	6	5.0	3.08 ± .37	4.57 ± .71	<.005
8B	6	5.0	2.43 ± .43	2.70 ± .42	NS
9B	6	5.0	3.05 ± .23	3.54 ± .17	<.005
10B	5	4.5	3.49 ± .29	4.20 ± .29	<.005
B. Free tyrosine					
1S	6	4.0	0.108	0.175	pooled samples
3S	6	3.0	0.141 ± .020	0.181 ± .028	<.001
4S	5	3.5	0.130 ± .021	0.181 ± .022	<.001
5S	5	4.0	0.131 ± .017	0.181 ± .023	<.001
10B	5	4.5	0.199 ± .010	0.233 ± .015	<.02
C. Iodide[127]					
7B	6	5.0	3.23 ± .55	6.06 ± .31	<.001
8B	6	5.0	4.72 ± 1.26	9.37 ± 1.50	<.001
9B	6	5.0	2.52 ± .71	6.93 ± .87	<.001
10B	5	4.5	2.28 ± .13	9.97 ± .49	<.001

* S and B after the experiment number denote sheep and beef slices, respectively. The concentration of commercial TSH used in experiments 1 through 9 was 0.1 U per ml incubation medium. In experiment 10B, 0.001 U per ml of the purified TSH preparation was used. Means with 1 SD are shown. The differences between the control and experimental groups were considered NS if the probability did not reach the 5 per cent level. Paired-data statistical analysis was used in the sheep thyroid experiments, but comparison of group means in the beef experiments (see Methods).

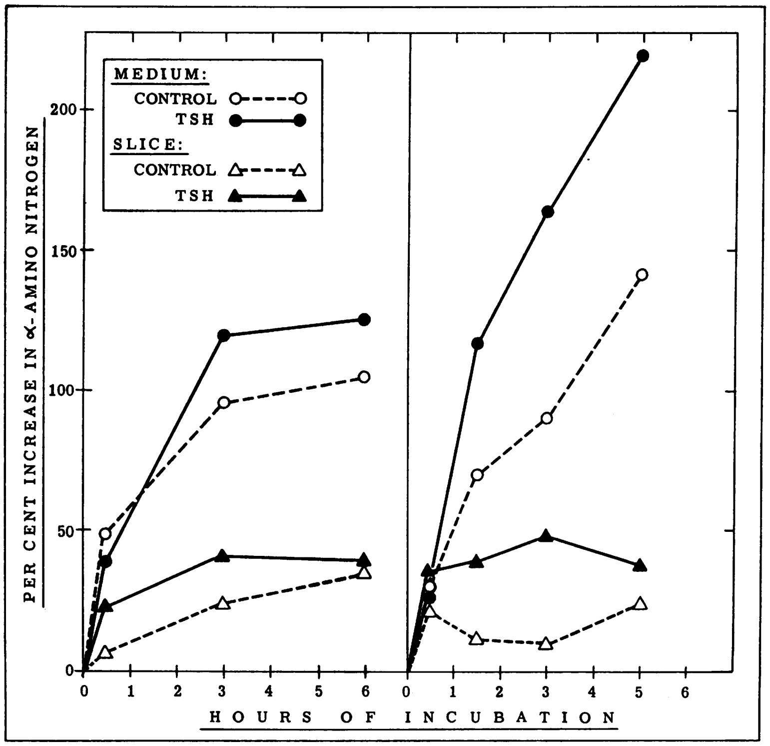

FIG. 3. TIME STUDIES ON THE PERCENTAGE INCREASE IN THE FREE α-AMINO NITROGEN OF SHEEP THYROID SLICES AND THEIR INCUBATION MEDIA WITH AND WITHOUT THE ADDITION OF THYROTROPIN (TSH). In the study shown on the left, 0.01 U TSH per ml medium was added and each point represents the mean of six determinations; in the study on the right, 0.1 U TSH per ml medium was used and group means were computed from four determinations.

disappearance of glucose from the medium. In the first time study, glucose continued to disappear from the medium in a linear fashion throughout incubation. When expressed in μmoles per 100 mg slice weight, glucose uptake in control groups averaged 2.76 ± .64 μmoles and in TSH supplemented groups, 3.44 ±.52 μmoles after 6 hours of incubation.

Several studies were designed to test the specificity of TSH-induced proteolysis in this *in vitro* system. In experiment 10B, a purified TSH preparation[3] (20 U per mg) was used (Table I). At a concentration of 0.001 U per ml incubation medium, this TSH preparation produced statistically significant increases in all three parameters of proteolysis when compared with control. Increases were observed of 20 per cent in free α-amino nitrogen, 17 per cent in free tyrosine, and 338 per cent in inorganic iodide.

Inactivated TSH is compared to control and

TABLE II

*The effect of inactivated thyrotropin (TSH) on parameters of proteolysis in beef thyroid slices**

Experimental group	No. of observations per group	Free α-amino nitrogen		Inorganic I^{127}	
		μmoles/100 mg wet wt	p	μmoles $\times 10^{-3}$/ 100 mg wet wt	p
Control	6	3.79 ± .14		1.74 ± .35	
TSH	6	4.18 ± .16	<.02	5.46 ± .78	<.001
TSH^{I}	6	3.81 ± .31	NS	1.64 ± .32	NS

* Incubation was 4.5 hours. The concentration of untreated and inactivated TSH was 0.001 U per ml incubation medium. Means with 1 SD are shown. Statistics are presented as in Table I.

TABLE III

*The effects of other hormones and humoral agents on parameters of proteolysis in beef thyroid slices**

Experimental group	Hormone preparation: μg/ml incubation medium	Hormone preparation: U/ml incubation medium	Free α-amino nitrogen: μmoles/100 mg wet wt	Free α-amino nitrogen: p	Iodide[127]: μmoles ×10⁻³/100 mg wet wt	Iodide[127]: p
Control			1.78 ± .21		3.60 ± .91	
TSH	0.6	0.001	2.31 ± .10	.001	10.52 ± 2.44	<.001
FSH	33.0	0.031	2.15 ± .31	.02	11.61 ± 2.19	<.001
ACTH	33.0	0.083	1.89 ± .18	NS	4.72 ± .61	NS
Insulin	1.3	0.033	1.90 ± .25	NS	3.18 ± .84	NS
Acetylcholine	5.1		1.86 ± .27	NS	3.86 ± .67	NS
Epinephrine	27.5		2.04 ± .26	NS	2.15 ± .33	<.01

* There were 5 slices per group; incubation was 4.5 hours. Means with 1 SD are shown. Statistics are presented as in Table I. Abbreviations: TSH = thyrotropin, FSH = pituitary gonadotropin, and ACTH = adrenocorticotropin.

untreated TSH in Table II. In the flasks supplemented with the sulfated TSH, no increase from control was observed in free α-amino nitrogen or inorganic iodide. In this same experiment, untreated TSH produced the expected increase in these parameters.

The effect of other hormones and humoral agents is compared to control and TSH in Table III. FSH was the only substance tested other than TSH that produced an increase greater than control in free α-amino nitrogen and inorganic iodide. This effect was comparable to the TSH response. The TSH concentration used in this experiment was 0.6 μg (0.001 U) per ml incubation medium; the FSH concentration was 33 μg (0.031 U) per ml. ACTH, insulin, acetylcholine, and epinephrine did not stimulate proteolysis. Although the addition of epinephrine produced a small decrease in inorganic iodide, no significant change from control was observed in free α-amino nitrogen.

In Table IV, the effect of TSH on proteolysis in extrathyroidal tissue is compared with that in the thyroid. In these studies, slices of rat and sheep liver or kidney, and slices of rat diaphragm and testis were compared with the thyroid from the same species. TSH did not significantly increase the free α-amino nitrogen of these extrathyroidal tissues when compared to control; however, the expected rise in thyroidal free α-amino nitrogen was observed.

DISCUSSION

In the present studies, one of the parameters measuring TSH-induced proteolysis was the increase in the BEI[131] from prelabeled rat thyroid glands. Since the iodination of the labeled iodo-

TABLE IV

*The effect of thyrotropin (TSH) on the free α-amino nitrogen of extrathyroidal tissue**

Experiment	Tissue	No. of observations per group	Hours of incubation	Control	TSG	p
				μmoles/100 mg wet wt		
11S	Thyroid	5	2.5	4.32 ± .50	5.05 ± .41	<.005
	Liver	5	2.5	6.93 ± .45	7.12 ± .28	NS
12S	Thyroid	5	3.0	2.50 + .33	2.87 ± .36	<.001
	Liver	5	3.0	5.01 ± .34	5.03 ± .24	NS
	Kidney	5	3.0	5.23 ± .32	5.53 ± .24	NS
13R	Thyroid	1	4.0	1.56	2.05	
	Diaphragm	3	4.0	7.19 ± .29	6.82 ± .56	NS
	Testis	4	4.0	3.34 ± .22	3.58 ± .18	NS
	Liver	5	4.0	6.06 ± .17	5.91 ± .20	NS
	Kidney	5	4.0	8.31 ± .51	8.62 ± .59	NS

* S and R after the experiment numbers denote sheep and rat slices, respectively. The concentration of commercial TSH was used 0.1 U per ml incubation medium. The rat thyroid data in experiment 13R are expressed in μmoles per 10 mg wet weight. One lobe was incubated as control, the other as TSH. Means with 1 SD are shown. Statistics are presented as in Table I.

amino acids found in these butanol extracts occurs after the tyrosyl residues are incorporated into the thyroglobulin molecule (28, 29), their presence in the free, or loosely-bound state, or both, must presumably result from proteolytic release. The increase in BEI^{131} during *in vitro* incubation after both the administration of TSH *in vivo* and its addition *in vitro* was therefore attributed to enhanced proteolytic activity. Furthermore, the release of BEI^{131} during 3 hours of *in vitro* incubation of thyroid glands removed 20 minutes after TSH administration *in vivo* is compatible with the rapid release of hormonal iodine that occurs *in vivo* after TSH. Studies of a similar design have recently been reported; in these, however, TSH failed to affect proteolysis whether given *in vivo* before sacrifice or added *in vitro* (30). No clear explanation can presently be offered for this discrepancy.

The increase in free α-amino nitrogen and tyrosine have been employed by others in assaying the activity of isolated proteolytic enzyme fractions from the thyroid (15, 31). These purified enzyme studies have characteristically shown optimal proteolytic activity at a low pH, but the *in vitro* addition of TSH to these enzyme preparations has failed to influence substrate proteolysis. It has been shown, however, that after administration of TSH to rats for 5 days, the *in vitro* activity of an isolated proteinase (pH 3.5) and peptidase (pH 5.0) was increased (31).

The present studies have shown that the *in vitro* addition of TSH produced an increase in the free α-amino nitrogen and tyrosine of surviving thyroid slices and their incubation media at pH 7.4. This effect was apparent in the slice after 30 minutes of incubation. These studies show that TSH is capable of producing activation of thyroidal proteolytic enzymes when added *in vitro*.

The increase in inorganic iodide produced by TSH has been well demonstrated *in vivo* (32–34) and was consistently observed in these studies after the addition of TSH *in vitro*. Although this parameter is not a direct measure of proteolysis, it may be an indirect one. The thyroid and several extrathyroidal tissues are known to contain a microsomal, TPNH-dependent deiodinase for which mono- and diiodotyrosine are specific substrates (35, 36). The iodothyronines apparently are not deiodinated by this enzyme (37). Therefore, the observed increase in inorganic iodide might result from *1*) an activation of proteolysis producing an increase in free mono- and diiodotyrosine, leading to an increase in iodide, or *2*) activation of deiodinase. A third possibility is that both enzyme systems may be independently influenced by TSH. Michel states that TSH *in vivo* enhances thyroidal deiodinase (37); however, such an effect was not observed during a 24-hour incubation *in vitro* (38). The data presented here do not allow discrimination of these possibilities, and clarification of this TSH-induced response must await further *in vitro* study. The chromatographic data presented in Figure 1, however, suggest an increase in labeled iodotyrosines^{131} after the addition of TSH to prelabeled rat thyroid minces.

The specificity of TSH-induced proteolysis was examined in several different experimental situations. Since a contaminating protease has been reported in purified pituitary hormone preparations (39), 0.6 mg per ml Thytropar was incubated 6 hours in the presence of two different protein substrates, albumin and thyroglobulin,[7] in Krebs-Ringer bicarbonate buffer at pH 7.4. No significant proteolysis was detected with either substrate. In addition, a highly purified TSH preparation, probably devoid of a significant amount of contaminating proteolytic activity, produced increased thyroid proteolysis at a concentration of 0.001 U (0.05 μg) per ml incubation medium. If this TSH preparation contained proteolytic activity, the amount added would be negligibly small.

Further support indicating that this *in vitro* proteolytic response was a specific effect of TSH on its target organ was obtained from the data demonstrating the failure of TSH to increase the free α-amino nitrogen of extrathyroidal tissues.

Reaction of TSH with sulfuric acid in the cold produced inactivation of the hormone when added to this *in vitro* system. Inactivation of TSH by this procedure has also been observed in studies *in vivo* (27). Although the physiological significance of these observations is presently unclear, the data do demonstrate that *in vitro* thyroid proteolysis is not activated by the sulfate derivative of

[7] Hog thyroglobulin obtained by $(NH_4)_2SO_4$ fractionation (40).

TSH. It is of interest in this regard that not all protein hormones are inactivated by this treatment. Reaction of insulin with sulfuric acid to give insulin sulfate does not destroy biological activity (26).

The effect of other hormones and humoral agents was also evaluated, since several of these preparations have been reported to produce metabolic effects in the thyroid gland *in vivo*, or *in vitro*, or both. Insulin has been shown to enhance glucose uptake comparable to that of TSH *in vitro* (11). Epinephrine, norepinephrine, and acetylcholine have been found to increase the release of radioiodine from the thyroid *in vivo* (41, 42), and recently it was demonstrated that these agents increase thyroidal glucose metabolism *in vitro*, similarly to TSH (43, 44). In these studies, insulin, epinephrine, and acetylcholine had no stimulatory effect on proteolysis. Since the *in vitro* concentrations of these preparations were comparable with those used in previous studies, this further emphasizes the TSH-specificity of this response. The FSH preparation did produce an increase in proteolysis, but this effect was attributed to contaminating TSH, since 33 μg of the lyophilized FSH preparation contained 0.002 U TSH (45). This concentration of TSH was consistently found to increase proteolysis in beef thyroid slices.

SUMMARY

The effect of thyrotropin (TSH) on proteolysis in thyroid slices was studied *in vitro* at a physiologic pH. After the prior administration of TSH *in vivo* or its addition *in vitro*, an increased release of butanol-extractable iodine^{131} from prelabeled rat thyroid minces was observed during *in vitro* incubation. An increase in thyroxin and triiodothyronine, as well as iodotyrosine and iodide, was demonstrated in these extracts.

TSH-induced proteolysis was also studied in beef and sheep thyroid slices. In these experiments free α-amino nitrogen, tyrosine, and inorganic iodide^{127} were measured as indices of proteolytic activity. TSH produced increases in all three parameters during *in vitro* incubation. This increased proteolysis was further shown to be a highly specific, TSH-induced effect on its target organ.

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