Stimulation by Thyrotropin, Long-Acting Thyroid Stimulator, and Dibutyryl 3′,5′-Adenosine Monophosphate of Protein and Ribonucleic Acid Synthesis and Ribonucleic Acid Polymerase Activities in Porcine Thyroid *in Vitro**

P. R. Adiga,† P. V. N. Murthy, and J. M. McKenzie‡

ABSTRACT: The *in vitro* incorporation of [^{3}H]uridine into RNA and [^{3}H]leucine into protein in slices of porcine thyroid was studied. Thyrotropin (10–500 mU/ml of medium), when added with [^{3}H]uridine, inhibited incorporation into RNA, but as little as 10 mU of thyrotropin per ml stimulated incorporation of [^{3}H]orotic acid into RNA. Uridine kinase (EC 2.7.1.48) was found to be inhibited in slices incubated with thyrotropin whereas UMP 5′ nucleotidase (EC 2.1.3.5) was not. Preincubation of slices with thyrotropin (5–50 mU/ml) led to enhanced incorporation of subsequently added [^{3}H]uridine and [^{3}H]leucine. When slices were preincubated with long-acting thyroid stimulator-IgG (2.5 or 5 mg per ml of medium) incorporation of [^{3}H]uridine and [^{3}H]leucine was similarly enhanced, with the smaller concentration being more effective. Without preincubation these stimulatory effects were mimicked by 1 mM dibutyryl 3′,5′-AMP and, to a lesser extent, 1 mM 3′,5′-AMP. AMP and ATP also stimulated [^{3}H]uridine incorporation in this system but only after more prolonged periods of incubation than were required for the other nucleotides. RNA polymerase (EC 2.7.7.6) activity measured in isolated thyroid nuclei had two components, one Mg^{2+}-stimulated and the other requiring Mn^{2+} and high salt content [0.4 M $(NH_4)_2SO_4$]. These activities, and particularly the former, were enhanced if thyroid slices were incubated with thyrotropin (5–100 mU/ml of medium), 2.5 mg or 5.0 mg of long-acting thyroid stimulator-IgG per ml, or 1 mM dibutyryl 3′,5′-AMP, before isolation of the nuclei and measurement of enzyme activities; 1 mM AMP, ADP, or 2′,3′-GMP had no influence. Added directly to the nuclei, thyrotropin, long-acting thyroid stimulator-IgG, and dibutyryl 3′,5′-AMP had no effect on RNA polymerase activities. These data are seen as affording evidence for mediation by 3′,5′-AMP of effects of thyrotropin and long-acting thyroid stimulator on thyroid RNA and protein synthesis, at least in part through an indirect stimulation of nuclear RNA polymerase activities.

It is self-evident that chronic stimulation of the thyroid gland by thyrotropin, such as to produce goiter, involves changes in RNA and protein synthesis, but these need not be primary effects of the tropic hormone. However, rapid (though not necessarily primary) effects on RNA and protein synthesis in the thyroid gland *in vitro*, following addition of thyrotropin, have been described (Hall, 1963; Begg and Munro, 1965; Hall and Tubmen, 1965; Tong, 1965; Shimada and Yasumasu, 1966; Lecocq and Dumont, 1967; Lindsay *et al.*, 1969; Kerkof and Tata, 1969), and there are claims (Begg and Munro, 1965; Shimada and Yasumasu, 1966) for a direct effect of the hormone on RNA synthesis by isolated thyroid cell nuclei. While reports of the effects of LATS[1] on macromolecule synthesis in the thyroid are sparse (Ochi and DeGroot, 1968, 1969), available data indicate that, as with other comparisons that have been made (McKenzie, 1968), influences of LATS on thyroid RNA and protein synthesis are qualitatively similar to those of thyrotropin.

Recent evidence that the action of thyrotropin (Pastan and Wollman, 1967; Pastan and Macchia, 1967; Pastan and Katzen, 1967; Bastomsky and McKenzie, 1967; Gilman and Rall, 1968a,b; Rodesch *et al.*, 1969) and LATS (Kaneko *et al.*, 1970; Levey and Pastan, 1970; Bastomsky and McKenzie, 1968) on several aspects of thyroid function is mediated by 3′,5′-AMP is impressive but almost all of these reports deal with acute metabolic effects that are thought not to depend on fresh protein or RNA synthesis (McKenzie *et al.*, 1968). Although one synthesis-promoting effect of thyrotropin—thyroid phospholipid synthesis as measured by labeling with ^{32}P—was mimicked by dibutyryl 3′,5′-AMP (Pastan and Macchia, 1967), the interpretation, that mediation of this effect of thyrotropin may be by endogenous 3′,5′-AMP, is in dispute (Kerkof and Tata, 1969); indeed, the generalization has been made that mediation of hormone effects by 3′,5′-AMP may be restricted to rapid and "purely metabolic" phenomena, and biosynthetic processes may be otherwise influenced (Kerkof and Tata, 1969).

We have studied the effects of thyrotropin and LATS on macromolecule synthesis in the thyroid and have considered the possible mediation of these effects by 3′,5′-AMP, by examining precursor incorporation into RNA and protein in slices of porcine thyroid *in vitro* and by measuring the influences of thyrotropin, LATS, and 3′,5′-AMP and related nucleotides on these processes and on RNA polymerase activities of subsequently isolated nuclei.

Materials

All nonradioactive nucleotides and [^{3}H]CTP (13.8 Ci/mole) were obtained from Schwarz BioResearch, Inc., Orangeburg,

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† Present address: Department of Biochemistry, Indian Institute of Science, Bangalore 12, Mysore State, India.

‡ Research Associate, Medical Research Council of Canada; author to whom inquiries should be sent.

[1] Abbreviations used are: LATS, long-acting thyroid stimulator; TSH, thyrotropin.

N. Y. [5-^{3}H]Uridine, L-[4,5-^{3}H]leucine, [5-^{3}H]orotic acid, and [^{3}H]UMP were from New England Nuclear Corp. RNase and DNase were crystalline preparations purchased from Worthington Biochemical Corp., as was calf-thymus DNA. Actinomycin D was from Merck, Sharp and Dohme of Canada, Ltd., Montreal, fetal calf serum from Hyland Laboratories, Los Angeles, Calif., and normal human IgG from Immunology, Inc., Glen Ellyn, Ill. Thyrotropin was NIH-TSH-S4. Antiserum to thyrotropin was kindly supplied by Dr. I. R. Hart; it was prepared in this laboratory by injecting rabbits intramuscularly with 1 U of thyrotropin ("Thytropar") in Freund's complete adjuvant every 5 days for 5 doses, and the potency of the antiserum was established by its ability to inhibit thyrotropin in bioassay (McKenzie and Williamson, 1966).

LATS-IgG was prepared from a suitable serum by precipitation with 40% saturated $(NH_4)_2SO_4$ and then chromatography on diethylaminoethyl Sephadex in phosphate buffer, 0.05 M, pH 6.4; IgG, pure by immunoelectrophoresis, was not absorbed to the ion exchanger under these conditions. The preparations of LATS-IgG used in the experiments described in this report were assayed at 3 mg per mouse (McKenzie and Williamson, 1966) and the responses were 600–1000%.

Methods

Preparation of Thyroid Slices and Incubation Conditions. Thyroid glands from freshly slaughtered hogs were obtained from a local abattoir and were carried to the laboratory in ice-cold KRB buffer (pH 7.4) containing 0.4% glucose. After removal of connective tissue, slices of approximately 0.2-mm thickness were prepared using a Stadie-Riggs slicer. Approximately 250–300 mg (fresh wt) of the tissue were incubated at 37° in air in glass vials containing 4 ml of KRB–glucose buffer that included 10% fetal calf serum, appropriate test substances, and, unless otherwise stated, labeled precursors. At specified time intervals equal amounts of tissue were withdrawn, homogenized in cold 0.2 N $HClO_4$, and used for isolation of RNA or protein.

In experiments involving preincubation with thyrotropin, slices were incubated in the above manner without labeled precursors for 2 hr at 37°, washed with cold KRB buffer, and resuspended in 4.0 ml of KRB–glucose–10% calf serum containing labeled precursor but without added thyrotropin; incubation was then continued at 37°. Equal amounts of tissue were withdrawn at appropriate time intervals and homogenized in cold 0.2 N $HClO_4$ and the incorporation of the isotope into RNA and protein was estimated.

To determine the specific activity of [^{3}H]uridine incorporated into RNA, the tissue homogenate in cold 0.2 N $HClO_4$ was washed three times with cold $HClO_4$, RNA was solubilized by incubation with 0.5 ml of 0.3 N KOH at 37° for 1 hr, and then the suspension was acidified with 0.3 ml of 1 N $HClO_4$. The tubes were stood in ice for 15 min, the digest was clarified by centrifugation, and a suitable aliquot of the supernatant was used for measuring RNA content by the double wavelength method of Fleck and Begg (1965). A second aliquot was used for radioactivity measurements. The specific activity was expressed as counts per minute of [^{3}H]uridine incorporated per milligram of RNA-P.

[^{3}H]Leucine incorporation into protein was measured in the following way and was expressed per microgram of DNA-P, in view of the experience of Begg *et al.* (1965). After 3 washes of the homogenate with cold 0.2 N $HClO_4$, 0.2 ml of 1.0 M sodium acetate buffer (pH 6.8) was added to the residue and lipids were removed by treatment with methanol–chloroform (2:1, v:v) twice at 45°, and once at room temperature. RNA was solubilized and removed as described above. The residue was dissolved in a known volume of dilute alkali and an aliquot was used for radioactivity determination. Another aliquot was dried under vacuum and digested with concentrated H_2SO_4 and its phosphorus content determined. The specific activity of protein was expressed as counts per minute of [^{3}H]leucine incorporated into protein per microgram of DNA-P.

Assays of Uridine Kinase and UMP 5′-Nucleotidase Activity. For uridine kinase ATP–uridine 5-phosphotransferase (EC 2.7.1.48), the method of Orengo (1969) was followed and for UMP 5′-nucleotidase (EC 3.1.3.5), the method of Canellakis (1957); the enzyme source was the 30,000*g* supernatant of an homogenate of thyroid slices (homogenates were centrifuged at 2° at 15,000*g* for 15 min and the supernatant at 30,000*g* for 30 min). For uridine kinase the reaction mixture was 5 μmoles of Tris-acetate; 168 nmoles of ATP; 420 nmoles of $MgCl_2$; 12.5–50 nmoles of uridine; 5×10^{-4} M dithiothreitol; 0.3–1.5 mg of protein of the enzyme source and 2 μCi of [^{3}H]uridine in a total volume of 75 μl. Incubation was with shaking at 37° and 10- to 20-μl aliquots were withdrawn at 0 time, 15 min, 30 min, and 60 min and applied to strips of diethylaminoethylcellulose paper; 10–15 μg of uridine in solution was previously loaded on the origin. Before the reaction mixture dried on the strips ascending chromatography was started, using 85% ethanol as solvent. The strips were dried, ultraviolet-absorbing areas identified, the origin material, which was the UMP formed, was cut, and ^{3}H measured. In some experiments an alternative method was used for isolating UMP from uridine. The DEAE-paper strips containing the reaction mixture were washed twice for 15 min in 10-ml volumes of 85% ethanol; the strips were blotted and dried and ^{3}H (representing noneluted UMP) was measured.

UMP 5′-nucleotidase reaction mixture was composed of 50 nmoles of UMP, containing 2 μCi of [^{3}H]UMP; 420 nmoles of $MgCl_2$; 5 μmoles of Tris-acetate, pH 7.5; 10^{-4} M dithiothreitol; 0.1–0.4 mg of protein of enzyme source, with or without 168 nmoles of ATP. Postincubation samples were transferred to diethylaminoethylcellulose paper with 10 μg each of UMP and uridine at the origin and processed by ascending chromatography as described above; radioactivity that ascended from the origin represented hydrolyzed UMP.

Isolation of the Whole Nuclei from Thyroid Slices. Initially attempts were made to use a procedure described for the isolation of liver nuclei (Munro *et al.*, 1965) but persistently significant contamination with whole cells resulted. Preparations free of significant whole-cell contamination as judged by light microscopy were, however, obtained by the procedure of Widnell *et al.* (1967) as used for isolation of nuclei from rat uterine tissue. After incubation with suitable concentrations of test materials for specified periods of time, the slices were washed with cold 0.25 M sucrose–0.003 M Mg^{2+} and homogenized with 0.32 M sucrose–0.003 M Mg^{2+} in a Potter-Elvehjem homogenizer equipped with a motor-driven Teflon pestle. The homogenate was clarified by centrifugation at 100*g* for 5 min in a refrigerated centrifuge (International Equipment Co., Model B-20) and a crude nuclear pellet was obtained by centrifugation at 2° at 1500*g* for 15 min. Further purification was achieved by suspending the pellet in 2.2 M sucrose–0.003 M Mg^{2+} and centrifuging at

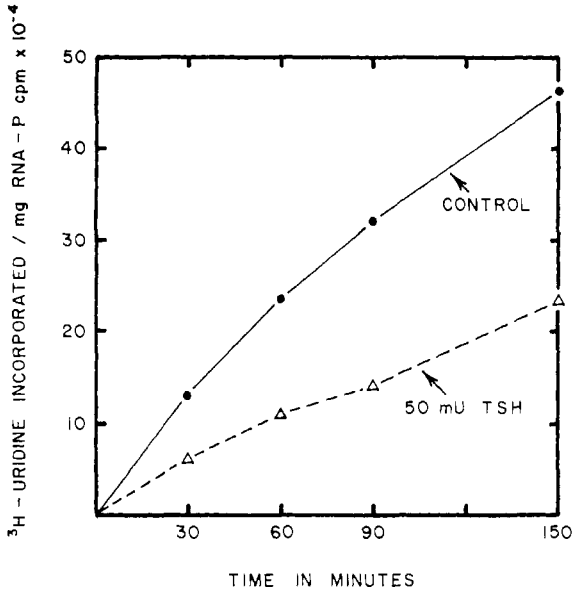

FIGURE 1: Effects of addition of thyrotropin to the incubation medium on the incorporation of [^{3}H]uridine into RNA in thyroid slices. Thyrotropin was added at the start of the incubation along with [^{3}H]uridine. At the times indicated approximately equal amounts of tissue were withdrawn and homogenized in cold 0.2 M $HClO_4$; RNA was extracted and specific activity was determined as detailed under Methods.

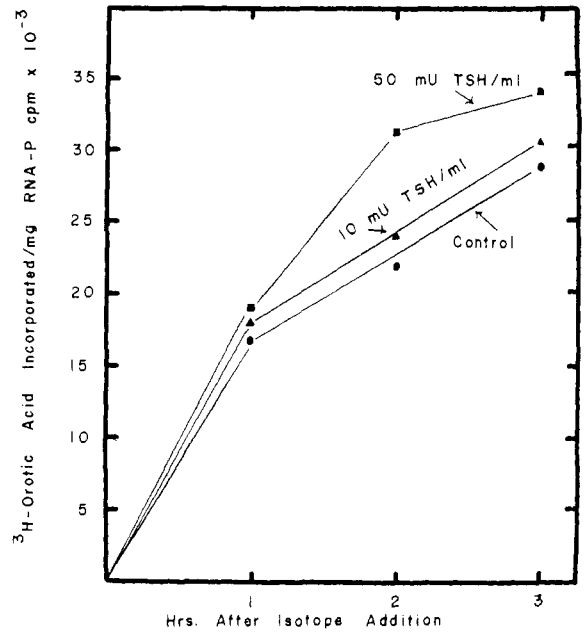

FIGURE 2: [^{3}H]Orotic acid incorporation into RNA in thyroid slices: time course of the effect of 50 mU and 100 mU of thyrotropin per ml of medium. Other details are as in Figure 1.

50,000*g* for 45 min in a Spinco (Model L2-65B) ultracentrifuge equipped with a swinging bucket rotor SW 40. The nuclear pellet so obtained was washed once in the cold with 0.25 M sucrose–0.001 M Mg^{2+}, resuspended in the same solution, and used for RNA polymerase assays.

Assay of RNA Polymerase Activities in Whole Nuclei. Our procedure is similar to that of Hamilton *et al.* (1968) with only minor modifications; 0.2 ml of the nuclear suspension in a total volume of 0.5 ml was used. Two types of reactions were studied; one was Mg^{2+}-dependent and was measured in the absence of $(NH_4)_2SO_4$, while the other was Mn^{2+}-dependent and required 0.4 M $(NH_4)_2SO_4$. For Mg^{2+}-dependent activity measurement, the reaction mixture contained (in 0.5 ml): Tris-acetate buffer (pH 8.5), 50 μmoles; $MgCl_2$, 2.5 μmoles; NaF, 2 μmoles, ATP, GTP, and UTP, 0.5 μmole of each; mercaptoethanol, 10 μmoles; and 0.01 μmole of CTP containing 2 μCi of [^{3}H]CTP. Incubation was at 37° for 15 min, except when the kinetics of [^{3}H]CTP incorporation into RNA was determined. The reaction mixture for Mn^{2+}–$(NH_4)_2SO_4$-activated polymerase assay contained (in 0.5-ml final volume: Tris-acetate (pH 7.5), 50 μmoles; $MnSO_4$, 2 μmoles; saturated $(NH_4)_2SO_4$ solution (pH 7.5), 50 μl; ATP, GTP, and UTP, 0.5 μmole of each; and CTP, containing 2 μCi of [^{3}H]CTP, 0.01 μmole. For the latter reaction CTP was added in a volume of 50 μl after 15 min preincubation at 37° and incubation was continued for another 45 min unless otherwise indicated. Both the reactions were terminated by the addition of 0.4 ml of cold 1 N $HClO_4$.

To determine [^{3}H]CTP incorporation into RNA, we used the procedure described above, including washings with cold 0.2 N $HClO_4$ and treatment with 0.3 N KOH at 37° to solubilize RNA. The neutralized alkaline digest was used directly for radioactivity determinations. The polymerase activities were expressed as counts per minute of [^{3}H]CMP incorporated into RNA per milligram of nuclear DNA.

Radioactivity Measurements. A Packard liquid scintillation spectrometer was used; 0.4 ml of neutralized aliquots of the samples in solution was mixed with 15 ml of the scintillation fluid (equal volumes of toluene and methyl Cellosolve, with 4 g of 2,5-diphenyloxazole and 100 mg of 1,4-bis[2-(5-phenyloxazolyl)]benzene per liter) and counted with an efficiency of approximately 25%.

Chemical Determinations. For estimating the DNA content of the nuclear preparation, the nuclei were washed twice with cold 0.2 N $HClO_4$ to remove sucrose before DNA was hydrolyzed with 0.5 N $HClO_4$ at 70° for 15 min and measured by the method of Burton (1956). The DNA-P in concentrated H_2SO_4 digests from experiments with the thyroid slice system was estimated by a microscale adaptation of the procedure of Morrison (1964).

Results

All data quoted are the means of closely agreeing duplicate or triplicate observations and represent the results of experiments repeated on at least two other occasions. There was variation in the responsiveness of different glands, with an occasional gland being totally unresponsive; however, results within a single experiment were highly consistent.

Effects of Thyrotropin on Incorporation of [^{3}H]Uridine and [^{3}H]Leucine in Thyroid Slices. With the addition of [^{3}H]-uridine and thyrotropin (50 mU/ml) to the medium at the beginning of the period of incubation there was, as shown in Figure 1, marked inhibition of incorporation of the precursor into RNA; this was evident by the first sampling period (30 min) and over 2.5 hr the control preparation had about 100% more incorporation than had the test. Similar data were obtained in 10 replicate experiments using 50 mU/ml of medium. Repeatedly there was dose-related inhibition of incorporation over the range 10–500 mU per ml of medium; the latter concentration resulted in inhibition to 74% of control after 2 hr of incubation. Thyrotropin (1 mU/ml of medium) was ineffective. Addition of theophyl-

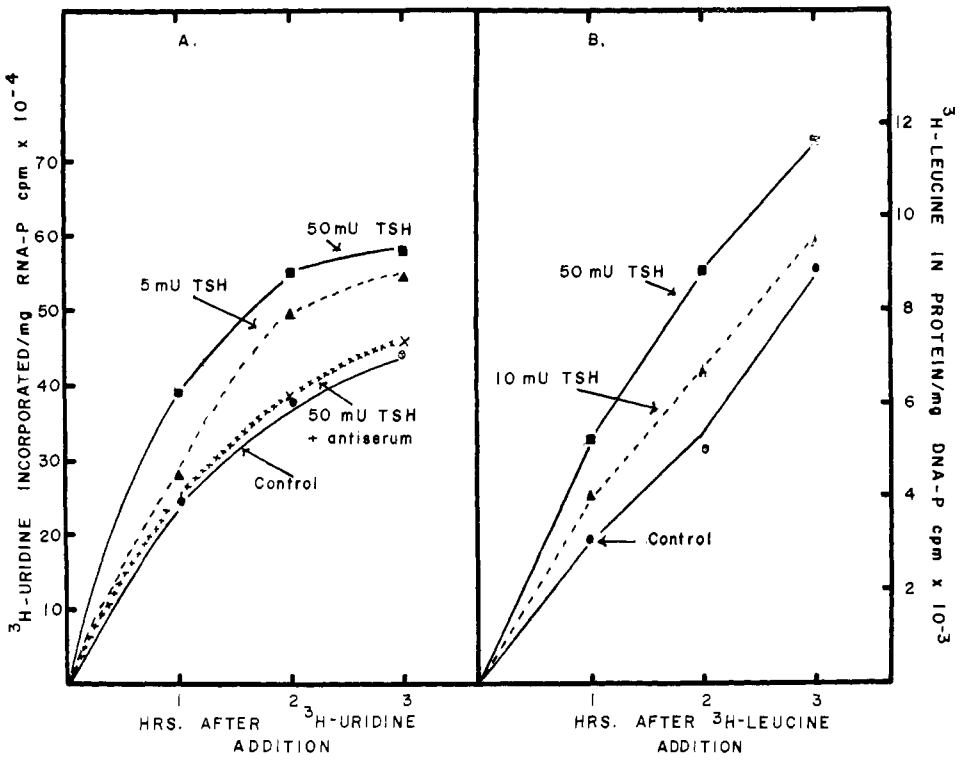

FIGURE 3: Influence of preincubation with thyrotropin on subsequent rates of incorporation of [^{3}H]uridine and [^{3}H]leucine in thyroid slices. For [^{3}H]uridine incorporation, preincubation, with or without thyrotropin, was for 2 hr at 37° and, where indicated, the medium also contained antiserum to thyrotropin, 0.2 ml in the total of 4 ml. After the slices were washed (see Methods), fresh medium was added, containing no thyrotropin but [^{3}H]uridine, 2 μCi/ml. For [^{3}H]leucine incorporation studies, preincubation was for 3 hr before washing of the slices and incubation with fresh medium that contained [^{3}H]leucine. The other details were as described in the legend to Figure 1 and in Methods.

line, 0.5 or 1.0 mM, to the medium that contained 50 mU of thyrotropin per ml had no influence on the inhibition of incorporation. The effects of theophylline by itself are described below.

That the inhibition of precursor incorporation was related to the use of uridine is indicated in Figure 2 wherein a dose-related stimulation of incorporation of [^{3}H]orotic acid by thyrotropin (10 mU and 50 mU per ml) is graphed; by 3 hr of incubation mean incorporations were 29750, 30500, and 34000 cpm per mg of RNA-P for control, 10 mU per ml, and 50 mU per ml, respectively. Similar data were obtained in 3 subsequent experiments in which 50 mU/ml of medium was used for varying times of incorporation of [^{3}H]uridine; after 1–3 hr increases in incorporation of 18 to 53% were observed. Stimulation by 50 mU/ml was maximal in that 100 mU/ml was consistently less effective. In Figure 3 are shown the results of exposing the thyroid slices to thyrotropin for a period of 2 hr before washing the slices and transferring them to fresh medium containing [^{3}H]uridine and no thyrotropin. (The period of 2 hr for preincubation was chosen because 1 hr resulted in little stimulation and 2.5–3 hr of preincubation was associated with no greater effect.) As indicated, there was enhanced incorporation into RNA with 5 mU/ml, and greater with 50 mU/ml, the maximum increase being, respectively, 29 and 45% after 2 hr; the effect of 50 mU of thyrotropin per ml of medium was entirely prevented by the inclusion of 0.05 ml of antithyrotropin serum per ml of medium. Replacement with thyrotropin-free medium after the preincubation period was necessary in that maintenance of the concentration of thyrotropin throughout the period of [^{3}H]uridine incorporation led to inhibition similar to that illustrated in Figure 1. A probable explanation for the apparent acute inhibition by thyrotropin of [^{3}H]-uridine incorporation into RNA (Figure 1) is indicated in Table I showing an inhibition of uridine kinase in thyroid slices exposed to thyrotropin (*e.g.*, a decrease to 79% with 50 mU/ml); similar observations were repeatedly made with concentrations of thyrotropin ranging from 10 to 100 mU/ml of medium, but there was no consistent dose–response relationship. [Exposure of the slices to thyrotropin for 3 hr before homogenization to prepare the enzyme solution was chosen in view of the experience with the inhibition of [^{3}H]-uridine incorporation (Figure 1); no other time interval was studied.] Dialysis of the enzyme preparation before assay did not affect its potency; increasing the concentration of substrate fourfold did not inhibit kinase activity. Neither theophylline (2 mM) nor dibutyryl 3′,5′-AMP (1 mM) decreased uridine kinase activity (Table I). Addition of thyrotropin (5–50 mU/ml) to the system showed no direct effect on the enzyme activity. There was no influence (not shown) of thyrotropin (25 mU or 50 mU per ml) on UMP 5′-nucleotidase activity, in the presence or absence of ATP.

When thyrotropin and [^{3}H]leucine were added to the medium at the onset of incubation of thyroid slices there was no effect on the incorporation into protein over 3 hr. As illustrated in Figure 3, however, preincubation with 10 mU or 50 mU of thyrotropin per ml of medium, no hormone being present thereafter, resulted in enhanced incorporation over 3 hr of further incubation with [^{3}H]leucine, maximum increases being at 2 hr when there was 33% increase with 10 mU and 76% increase with 50 mU. Similar data were obtained in 4 subsequent experiments in which 10 mU and

TABLE I: Assays of Uridine Kinase Activity in Porcine Thyroid Slices. Influences of Thyrotropin, Theophylline, and Dibutryl 3′,5′-AMP.[a]

(a) The reaction

Enzyme concentration	nmoles of UMP Formed in		
(μg/75 μl)	15 min	30 min	60 min
200	0.03	0.14	0.23
1000	0.26	0.49	1.07

(b) Effects of thyrotropin, theophylline, and dibutyryl 3′,5′-AMP and LATS-IgG. (Enzyme concentration 750 μg per 75 μl).

Test material	nmoles of UMP Formed per mg of Protein per min: % of control (mean ± SD; n = 8)
Thyrotropin	
50 mU/ml	79 ± 7
100 mU/ml	73 ± 10
Theophylline 2 mM	96 ± 17
Dibutyryl 3′,5′-AMP 1 mM	93 ± 7
LATS-IgG 1 mg/ml	94 ± 7
2.5 mg/ml	98 ± 18

[a] Details of the assays are given under Methods. The concentrations of enzymes quoted indicate the total protein in the enzyme source solution, *i.e.*, the 30,000*g* supernatant of an homogenate of thyroid slices (see Methods). Results are from two experiments, each comprised of 4 tubes per test material. The test substances or control solution were added to the slice incubation medium for 180 min before homogenization of the slices.

50 mU per ml of medium were tested; a small further increment in effect was obtained in 2 experiments with 100 mU/ml of medium, but 500 mU was less effective.

Effects of LATS-IgG on Incorporation of [^{3}H]Uridine and [^{3}H]Leucine in Thyroid Slices. Unlike thyrotropin, LATS-IgG did not inhibit incorporation of [^{3}H]uridine in thyroid slices.

TABLE II: The Effect of Dibutyryl 3′,5′-AMP on [^{3}H]Uridine Incorporation into RNA in Porcine Thyroid Slices *in Vitro*.[a]

Concentration of Dibutyryl 3′,5′-AMP (mM)	[^{3}H]Uridine in RNA (cpm × 10^{-4}/mg of RNA-P)	
	2 hr	3 hr
Nil	71.6	118.1
0.5	96.3	158.6
1.0	116.3	172.9
2.0	124.7	210.9
5.0	172.6	215.2

[a] Slices were incubated in 4 ml of buffer with [^{3}H]uridine, 10 μCi per ml, and dibutyryl 3′,5′-AMP in the concentrations shown. Equal amounts of tissue were withdrawn at 2 hr and 3 hr and the specific activity of RNA was determined as described under Methods.

TABLE III: The Effect of Some Nucleotides on [^{3}H]Uridine and [^{3}H]Leucine Incorporation into RNA and Protein in Porcine Thyroid Slices *in Vitro*.[a]

Addition to Medium (1 μmole/ml)	[^{3}H]Uridine in RNA (cpm × 10^{-4}/mg of RNA-P)			[^{3}H]Leucine in Protein (cpm × 10^{-3}/mg of DNA)		
	30 min	90 min	180 min	30 min	90 min	180 min
Nil (Control)	8.6	16.5	16.9	1.4	4.6	7.9
AMP	6.0	14.1	21.3	.7	2.9	5.5
ADP	5.8	17.5	18.7	1.1	1.8	3.3
ATP	6.4	15.7	28.6	1.8	3.6	6.3
2′,3′-GMP	6.2	17.1	18.4	1.2	3.7	8.9
2′,3′-CMP				1.8	4.3	6.4

[a] Slices were incubated in 4 ml of buffer with [^{3}H]uridine or [^{3}H]leucine, 2 μCi per ml, and the various nucleotides. Equal amounts of tissue were withdrawn at 30, 90, and 180 min and the specific activity of RNA and protein was determined as described under Methods. In a repeat experiment the values for stimulation of [^{3}H]uridine incorporation after 180 min were 13.9, 18.7, 17.0, and 21.8 (cpm × 10^{-4}/mg of RNA-P) for control, AMP, ADP, and ATP, respectively.

Furthermore, there was no effect of 1 or 2.5 mg of LATS-IgG (Table I) per ml of medium on uridine kinase and 5′-UMP nucleotidase, in two experiments wherein this was examined. Consequently studies of [^{3}H]uridine and [^{3}H]leucine incorporation as influenced by LATS-IgG were carried out with the continued presence of the thyroid stimulator in the medium. After 1 hr of preincubation of slices with LATS-IgG there was enhanced incorporation of [^{3}H]uridine into RNA (Figure 4A) but 2.5 mg/ml had a greater effect than had 5.0 mg of LATS-IgG per ml of medium; with 2 hr of labeling the percentage increases were 122% with the lesser dose and 49% with the greater. With [^{3}H]leucine incorporation LATS-IgG was stimulatory also, although effects were less striking than with [^{3}H]uridine, but again 5.0 mg/ml was less potent than 2.5 mg/ml (Figure 4b). Similar biphasic data were obtained with two other preparations of LATS-IgG, 2.5 mg/ml having a greater effect on both [^{3}H]uridine and [^{3}H]leucine incorporation.

Effects of 3′,5′-AMP, Its Dibutyryl Ester, and Theophylline on Incorporation of [^{3}H]Uridine and [^{3}H]Leucine. Without preincubation the nucleotides in millimolar concentration led to enhanced incorporation of [^{3}H]uridine into RNA over 3 hr of measurement with maximum effects at 2 hr, namely 41% with 3′,5′-AMP and 62% with dibutyryl 3′,5′-AMP (Figure 5A). When [^{3}H]leucine incorporation was studied (Figure 5B), 1 hr of preincubation of the slices with the nucleotides resulted in no significant effect with 1 mM 3′,5′-AMP, but the dibutyryl ester increased incorporation by 53–54% at 3 and 5 hr of labeling; the effect of dibutyryl 3′,5′-AMP was delayed if the [^{3}H]leucine was present from the beginning of the incubation. The dose–response relationship of dibutyryl 3′,5′-AMP and incorporation of [^{3}H]-uridine is shown in Table II; there was a progressive effect from 0.5 mM to 5.0 mM, the highest concentration tested In Table III the influences of AMP, ADP, ATP, 2′,3′-CTP,

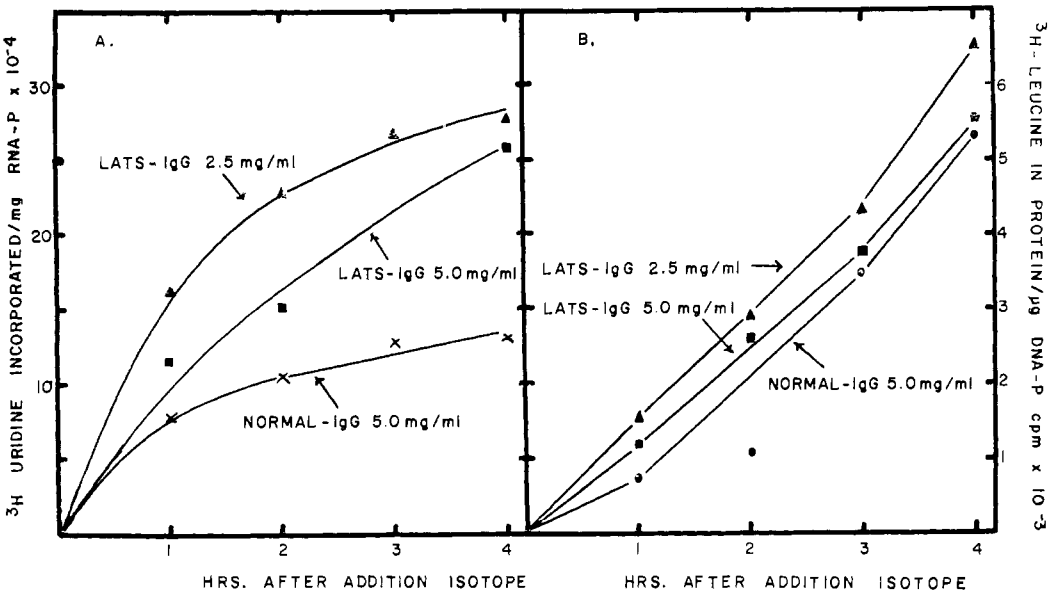

FIGURE 4: Influence of LATS-IgG on the time course of incorporation of [^{3}H]uridine and [^{3}H]leucine in thyroid slices. Experimental conditions were similar to those described with Figure 1 except that control vials contained normal human IgG and test vials LATS-IgG, from the start of the incubations. [^{3}H]Uridine was added after 1 hr and [^{3}H]leucine after 2 hr. Other details are given under Methods. (A) [^{3}H]Uridine incorporation into RNA; (B) [^{3}H]leucine incorporation into protein.

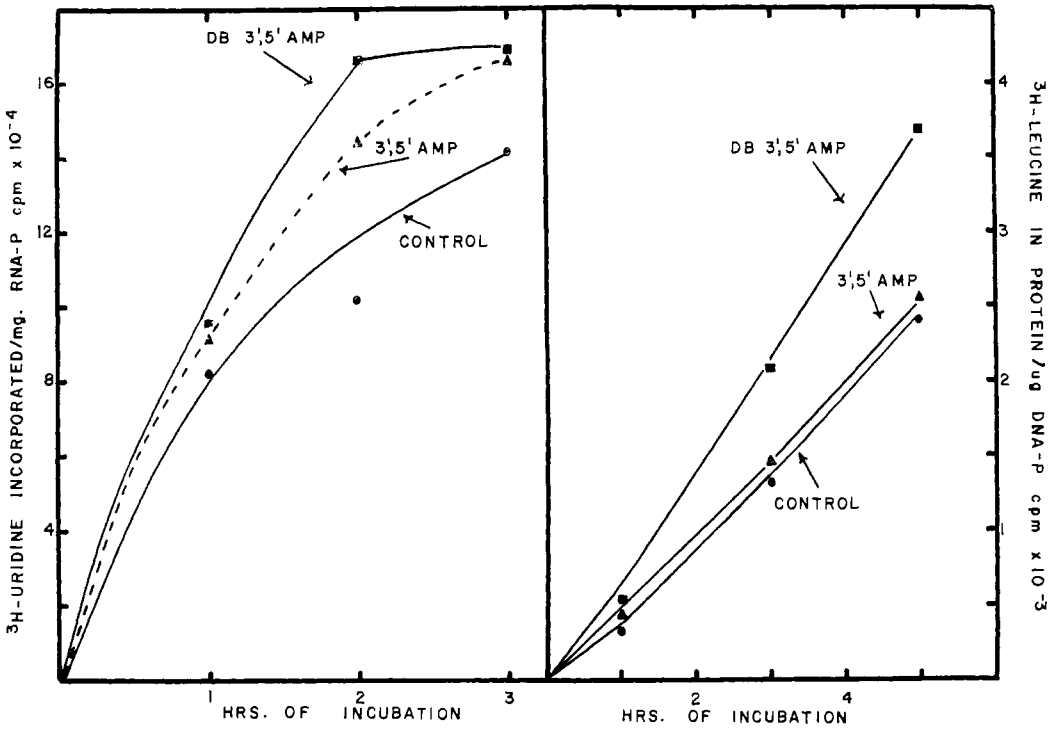

FIGURE 5: Effects of 3',5'-AMP and dibutyryl 3',5'-AMP on the time course of incorporation of [^{3}H]uridine and [^{3}H]leucine in thyroid slices. [^{3}H]Uridine was added at the beginning of the incubation and [^{3}H]leucine after 1 hr. Other details were as given with Figure 1 and in Methods. (A) [^{3}H]Uridine incorporation into RNA; (B) [^{3}H]leucine incorporation into protein.

and 2',3'-GMP (all 1 mM) on [^{3}H]uridine and [^{3}H]leucine incorporation are listed. For [^{3}H]uridine incorporation, until 90 min of incubation, maximum increase was, with ADP, 6%; however, by 3 hr all nucleotides had caused some increase in incorporation, with the greatest being a 69% increase associated with ATP. On the other hand, there was little stimulatory effect of the nucleotides on incorporation of [^{3}H]leucine over 3 hr of incubation; the most pronounced influence was inhibition by AMP and ADP, to a maximum of 58% by ADP in 3 hr. Similar testing of the effect of theophylline on [^{3}H]uridine incorporation showed no consistent effect of 2 and 5 mM over 3 hr of observation; there was stimulation (*e.g.*, 70% over control after 1.5 hr and 60% after 3 hr) with 10 mM concentration.

Studies of RNA Polymerase of Isolated Thyroid Nuclei. Two components of RNA polymerase activity—one Mg^{2+}-

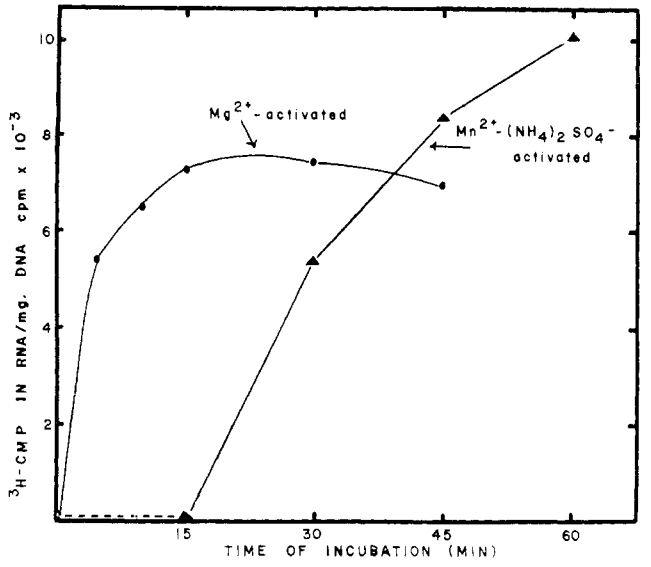

FIGURE 6: Time course of incorporation of [^{3}H]CMP (from [^{3}H]-CTP) into RNA by two RNA polymerase reactions in nuclei isolated from thyroid slices. Purified nuclei were incubated for various times with Mg^{2+} or Mn^{2+}–0.4 M $(NH_4)_2SO_4$ in the reaction mixture; for Mg^{2+}-dependent activity [^{3}H]CTP was present at the start of the incubation but for the other, the labeled precursor was added after 15 min of incubation (Hamilton *et al.*, 1968). Other details are given in the text.

activated and the other Mn^{2+}-activated, $(NH_4)_2SO_4$-dependent—were present in isolated thyroid nuclei and the time course of the activities as incorporation of [^{3}H]CMP into RNA are shown in Figure 6. A maximum activity was reached in 15 min with the Mg^{2+}-activated component but, with the other, incorporation was progressive over 45 min of labeling. Both activities were found (Table IV) to depend on the presence of ATP, GTP, and UTP and the Mg^{2+}-activated component was enhanced about one-half by 20 mM mercaptoethanol (which had no effect on the other activity, so was not included in that reaction mixture). Preincubation of the nuclei with actinomycin D or DNase reduced activities by 80–90% and addition of RNase prohibited accumulation of product (Table IV).

TABLE IV: Characteristics of RNA Polymerase Activities from Porcine Thyroid Nuclei.[a]

Addition to Medium	Activity (% control)	
	Mg^{2+}-Stimulated	Mn^{2+}–$(NH_4)_2SO_4$-Stimulated
Nil (Control)	100	100
Actinomycin D (10 μg/ml)[b]	8	18
Omit ATP, GTP, UTP	6	8
DNase (10 μg/ml)[b]	18	11
Omit mercaptoethanol	61	
RNase (15 μg/ml)	3	3

[a] Conditions for the assay of RNA polymerase activities are described under Methods. Activities are expressed as per cent control, *i.e.*, that obtained under standard assay conditions. [b] Preincubation for 15 min before addition of [^{3}H]CTP.

TABLE V: The Influence of Preincubation of Porcine Thyroid Slices with Thyrotropin, Dibutyryl 3′,5′-AMP, and Some Other Purine Nucleotides on the RNA Polymerase Activities of Subsequently Isolated Nuclei.[a]

Addition to Slice Incubation Medium (per ml)	RNA Polymerase Activities (% control)					
	Mg^{2+}-Stimulated Experiment			Mn^{2+}–$(NH_4)_2SO_4$-Stimulated Experiment		
	1	2	3	1	2	3
Thyrotropin						
5 mU	143	148		129	134	
25 mU	157	203	123	130	122	125
50 mU	146	144	134	123	128	110
100 mU	145	159		111	135	
Dibutyryl						
0.5 μmole	95	115		120	121	
0.8 μmole		139			152	
1.0 μmole	118	108	149	157	132	142
1.3 μmoles	121					
2.0 μmoles		100			130	
AMP (1 μmole)	104			103		
ADP (1 μmole)	97			98		
2′,3′-GMP (1 μmole)	92			90		

[a] Approximately 5-g slices (equal amounts of tissue pooled from several glands) were incubated with shaking at 37° for 2 hr with 8.0 ml of KRB buffer (pH 7.4) containing 0.4% glucose and 10% fetal calf serum and other additions as listed. Slices were washed with cold 0.25 M sucrose–0.003 M Mg^{2+} and homogenized in cold 0.32 M sucrose–0.003 M Mg^{2+} and the nuclei isolated as described under Methods. The two RNA polymerase activities were assayed as described under Methods, 15 min of incubation for the Mg^{2+}-dependent activity and 45 min for the Mn^{2+}-dependent. Enzyme activities were calculated as [^{3}H]CMP (from [^{3}H]CTP) incorporated per milligram of DNA and expressed as a per cent of the corresponding control preparation. In studying the effect of thyrotropin, the solvent (1% human serum albumin) was the control addition; for the various nucleotides, 100% was the enzyme activity when there was no addition to the reaction mixture.

Influences of Thyrotropin, LATS-IgG, and Dibutyryl 3′,5′-AMP on Thyroid RNA Polymerase Activities. When added directly to the preparation of nuclei, thyrotropin (1–100 mU/ml of medium), LATS-IgG (2.5 and 5 mg/ml), or 3′,5′-AMP (1 mM final concentration) failed to stimulate incorporation of [^{3}H]CMP into RNA.

As shown in Table V, however, preincubation of thyroid slices with these agents (3′,5′-AMP as its dibutyryl ester) resulted in stimulation, relative to control preparations, of RNA polymerase activities in subsequently isolated nuclei. (A 2-hr period of preincubation was chosen on the basis of experience with stimulation by thyrotropin of incorporation of [^{3}H]uridine into RNA.) In the data from the three experiments listed there was no dose–response relationship of

thyrotropin over 5–100 mU per ml of medium; the Mg^{2+}-stimulated activity (increased an average of 51%) was consistently more markedly affected than the other (average 25% increase). Similar data were obtained with 4 additional experiments, 3 using 25 mU/ml and 1 with 50 mU/ml of medium. The effect of dibutyryl 3′,5′-cyclic AMP was studied at 0.5–2.0 mM concentration; enhancement of the Mn^{2+}–$(NH_4)_2SO_4$-dependent activity was the more marked over this range. In three experiments with 0.8–1.3 mM concentration of the nucleotide the average increase of the Mg^{2+}-stimulated activity was 27% and the increase in the Mn^{2+}–$(NH_4)_2SO_4$-dependent activity was 52%. Other purine nucleotides, AMP, ADP, and 2′,3′-GMP, had no stimulatory effect when present in the slice-incubation medium. LATS-IgG was tested only once, at 2.5 mg/ml of preincubation medium. There was an increase in both RNA polymerase activities, the Mg^{2+}-activated component being enhanced by 74% and the other by 43%; comparisons in this instance were made with enzyme activities measured after the addition of similar quantities of normal human IgG to the medium used for incubation of the slices.

Discussion

Effects of thyrotropin on the *in vitro* incorporation of various radioactive precursors into thyroid RNA have been described in several reports (Hall, 1963; Begg and Munro, 1965; Hall and Tubmen, 1965; Shimada and Yasumasu, 1966; Lecocq and Dumont, 1967; Kerkof and Tata, 1969) but our preparation, labeled uridine and porcine thyroid slices, seems not to have been used previously. The nature of the precursor and the species of thyroid are probably important variables, and ought to restrict generalization of results. Lindsay and his colleagues (1969), comparing bovine, porcine, and canine thyroid slices and using [^{14}C]orotic acid, showed a species variability in the synthesis of pyrimidine nucleotides and incorporation of label into RNA; they reported an enhanced specific activity of RNA formed in porcine thyroid slices in medium to which 1 U of thyrotropin was added so that our observing similar results with [^{3}H]-orotic acid and 10–50 mU of thyrotropin per ml of medium was not unexpected. In preparations in which thyrotropin and labeled precursor were added to thyroid slices simultaneously, we found stimulation of incorporation of labeled orotic acid, but not uridine, into RNA. This indicated that uridine kinase was possibly the site at which the inhibitory effect of thyrotropin occurred and, in fact, rapid inhibition of this enzyme activity by thyrotropin was observed. The inhibition was not materially affected either by increasing the concentration of uridine as substrate or by dialyzing the preparation before enzyme assay; consequently the apparent inhibition of enzyme activity was not due to alteration of the size of the uridine pool. We did not investigate the mechanism of inhibition further beyond establishing that there was no rapid hydrolyzing of the product, UMP, in similar experimental circumstances. Since thyrotropin rapidly stimulates synthesis of pyrimidine nucleotides in the thyroid (Lindsay *et al.*, 1969), inhibition of the kinase by UTP or CTP (Orengo, 1969) is a possible explanation. Theophylline neither enhanced nor mimicked thyrotropin in inhibiting the incorporation of [^{3}H]uridine; dibutyryl 3′,5′-AMP had no influence on uridine kinase activity. It is probable therefore that this effect of thyrotropin is not mediated by 3′,5′-AMP, making the reaction akin in this respect to stimulation in bovine thyroid slices of glucose carbon-1 oxidation by similar concentrations of thyrotropin (Gilman and Rall, 1968b).

We found that addition of [^{3}H]uridine to the medium after the removal of thyrotropin (washing of the slices and use of fresh medium with no thyrotropin were necessary for reproducibility of results) was associated with enhanced incorporation of the nucleotide into RNA, and this effect was prevented by inclusion of antithyrotropin serum with the thyrotropin. In similar experimental preparations there was enhanced incorporation of [^{3}H]leucine into protein. These data are thus in line with the experience of others regarding *in vitro* stimulation by thyrotropin of precursor incorporation into thyroid protein (Tong, 1965) and RNA (Hall, 1963; Begg and Munro, 1965; Hall and Tubmen, 1965; Shimada and Yasumasu, 1966; Lecocq and Dumont, 1967; Kerkof and Tata, 1969). They do not, of themselves, prove stimulation of RNA and protein synthesis since, among other possibilities, the data might reflect increased specific activity of the precursor pool which is being labeled, as occurred, in part, with ^{32}P incorporation (Kerkof and Tata, 1969). That they do, indeed, reflect stimulation of synthesis is likely from the facts that incorporation of precursors of both RNA and protein was stimulated and that nuclear RNA polymerase activities also were increased; stimulation of this enzyme would be expected to lead to enhanced synthesis of specific RNA and protein molecules.

The actions of thyrotropin on precursor incorporation into RNA and protein and on nuclear RNA polymerase activities were largely duplicated by LATS-IgG and 3′,5′-AMP or its dibutyryl ester. The results with LATS-IgG constitute further evidence of identity of actions of thyrotropin and LATS on the thyroid, there being no major qualitative differences so far recognized (McKenzie, 1968). Our data from *in vitro* studies complement recent reports of stimulation by thyrotropin and LATS of ^{32}P and [^{3}H]uridine incorporation into RNA (Ochi and DeGroot, 1968) and [^{3}H]leucine into protein (Ochi and DeGroot, 1969) in *in vivo* studies using mice. Lesser stimulation (of [^{3}H]uridine and [^{3}H]leucine incorporations), by the larger concentration of LATS-IgG, was similar to lesser stimulation found with larger doses of thyrotropin and is reminiscent of actual inhibition observed with higher levels of thyrotropin-releasing factor that influences precursor incorporation *in vitro* into RNA and protein in pituitary slices (McKenzie *et al.*, 1970). Shimada and Yasumasu (1966) also reported lesser stimulation with increased doses of thyrotropin when they studied ^{32}P incorporation into RNA in porcine thyroid slices.

The specificity of the action of dibutyryl 3′,5′-AMP on nuclear RNA polymerase activities was supported by there being no effect of 1 mM AMP, ADP, or 2′,3′-GMP. On the other hand, AMP and ATP clearly had enhancing effects on precursor incorporation into RNA (but not into protein) in thyroid slices. The effects, however, were seen only with 3 hr of incubation, unlike the responses to dibutyryl 3′,5′-AMP that occurred earlier. It may be that, as has been suggested in other studies (Bastomsky and McKenzie, 1967; Kowal and Fiedler, 1969), such stimulatory nucleotides enter into metabolic pathways leading to increased concentrations of 3′,5′-AMP; the relative delay in their effects would be compatible with this speculation. Gilman and Rall (1968a) reported that the concentration of 3′,5′-AMP in slices of bovine thyroid was not influenced by 5′-AMP or ATP; however, these nucleotides were added only at 0.1 mM concentration for a maximum of 15 min of incubation in their system.

The characteristics of the nuclear RNA polymerase activities of the thyroid are like those reported for other tissues, for instance the enzyme of uterine nuclei (Hamilton *et al.*, 1968). Similar stimulation of the RNA polymerase in nuclei of target tissues has been reported for thyroid hormone, growth hormone, and testosterone acting on rat liver (Widnell and Tata, 1966), estrogen on rat uterus (Hamilton *et al.*, 1968), and thyrotropin-releasing factor on adenohypophysis (McKenzie *et al.*, 1970). As noted in the Results section, stimulation was obtained only with preincubation of the slices before isolation of the nuclei and not with direct addition of stimulating agents to the nuclei; we conclude from this that if 3′,5′-AMP is a mediator of stimulation of RNA polymerase activities by thyrotropin and LATS-IgG, the action of the nucleotide is not directly on the nuclear enzyme complex. Our failure to obtain a direct effect of thyrotropin on isolated nuclei is in contradistinction to results reported by two other groups (Begg and Munro, 1965; Shimada and Yasumasu, 1966). Begg and Munro (1965) described enhanced labeling of RNA when thyrotropin was added to isolated thyroid nuclei, but this was with [^{14}C]adenine as substrate and did not require added nucleotide triphosphates so that clearly more than RNA polymerase was involved in the reaction; they did, however, find a similar effect using [^{32}P]-UTP in the presence of unlabeled triphosphates. In the other report (Shimada and Yasumasu, 1966) increased labeling of RNA was described with thyrotropin added to thyroid nuclei and using ^{32}P as NaH_2PO_4 or [^{3}H]uridine as precursors so that, again, more than RNA polymerase was involved. Early in these studies we used the procedure of Begg and Munro (1965) for isolation of nuclei except that the final pellet was achieved by centrifugation at 50,000*g* whereas they used 30,000*g;* with [^{3}H]uridine as precursor we consistently found that addition of thyrotropin decreased incorporation of ^{3}H into RNA (as occurred with the thyroid slice experiments, Figure 1). We considered this was related to the contamination of the nuclei with whole cells that we were unable to avoid.

3′,5′-AMP is now accepted as a mediator of many actions of thyrotropin and LATS on the thyroid; these actions are largely restricted to events not obviously involving biosynthetic mechanisms and, indeed, the generalization has been offered (Kerkof and Tata, 1969) that 3′,5′-AMP is unlikely to mediate the actions of growth and developmental hormones. However, since the effects we observed of thyrotropin and LATS on RNA and protein incorporation of precursors and on RNA polymerase activities were duplicated by 3′,5′-AMP or the dibutyryl analog, and since the concentration of 3′,5′-AMP is known to be enhanced in the thyroid gland stimulated by thyrotropin (Gilman and Rall, 1968a), or by LATS (Kaneko *et al.*, 1970; Levey and Pastan, 1970), it is reasonable to speculate that these biosynthetic phenomena in the thyroid may be influenced through the mediation of endogenous 3′,5′-AMP. With similar data we came to a parallel conclusion regarding the actions of thyrotropin-releasing factor on the pituitary (McKenzie *et al.*, 1970). That the cyclic nucleotide can mediate biosynthetic processes is also supported by other data; for instance, Grand and Gross (1969) reported almost doubling of *in vitro* incorporation of [^{14}C]L-amino acids into protein by slices of rat parotid gland as an effect of 2 mM dibutyryl 3′,5′-cyclic AMP, and, in like vein, Sharma and Talwar (1970) reported *in vitro* stimulation by 3′,5′-AMP, and its dibutyryl derivative, of RNA and protein incorporation of radioactive precursors by rat uterine horns.

Recently enhancement by the nucleotide of the protein-synthesizing capacity of thyroid polysomes *in vitro* was reported (Lissitzky *et al.*, 1969); furthermore, we have observed (Adiga *et al.*, 1971) stimulation of protein synthesis by polysomes of bovine pituitaries and recognize that in addition, or as an alternative, to stimulation of RNA polymerase, this is another means by which 3′,5′-AMP might enhance protein synthesis.

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Stimulation by Adenosine 3′,5′-Cyclic Monophosphate of Protein Synthesis by Adenohypophyseal Polyribosomes*

P. R. Adiga,† P. V. N. Murthy, and J. M. McKenzie‡

ABSTRACT: Addition of dibutyryl 3′,5′-cyclic AMP to slices of bovine pituitary stimulated incorporation of [^{3}H]leucine into protein, whether or not actinomycin D was present; therefore the influence of 3′,5′-cyclic AMP on protein synthesis by bovine pituitary polysomes was studied. If the cyclic nucleotide was added to the complete protein-synthesizing system (including pH 5.0 enzyme), stimulation of [^{3}H]leucine incorporation occurred only with pH 5.0 enzyme from rat liver; there was no stimulation when homologous enzyme, *i.e.*, from bovine pituitary, was used. Addition of 3′,5′-cyclic AMP to the polysomes, before addition of pH 5.0 enzyme, resulted in stimulation of protein synthesis with either source of enzyme, but stimulation was facilitated to a greater degree, over the range 0.5–2 mM 3′,5′-cyclic AMP, when rat liver was the source. The stimulation of protein synthesis was prevented by the addition of cycloheximide. With rat liver pH 5.0 enzyme the product of hydrolysis of 3′,5′-cyclic AMP was mainly 5′-AMP whereas with pituitary pH 5.0 enzyme there was also dephosphorylation and deamination resulting in production of hypoxanthine and other bases. However, using either source of pH 5.0 enzyme and the complete protein-synthesizing system (*i.e.*, including an ATP-regenerating mechanism) most of the ^{3}H from hydrolysis of [^{3}H]3′,5′-cyclic AMP was incorporated into ATP. The data are seen as compatible with a stimulation by 3′,5′-cyclic AMP of translation by pituitary polysomes; the significance of the importance of the source of pH 5.0 enzyme used in the system is obscure.

The mediation of many hormonal effects on target tissues by 3′,5′-cyclic AMP has been increasingly recognized in recent years (Robison *et al.*, 1968). The great majority of the effects are characterized as rapid "purely metabolic" (Sutherland *et al.*, 1967) actions, such as increase in the rate of glycogenolysis as an effect of epinephrine on liver (Sutherland and Robison, 1966) or release of stored hormone, exemplified by release of thyroid hormone by thyrotropin (Bastomsky and McKenzie, 1967) or of thyrotropin by thyrotropin-releasing factor (Wilber *et al.*, 1969). However, there is some evidence that 3′,5′-cyclic AMP may mediate other hormonal effects involving biosynthetic mechanisms, such as thyrotropin-enhanced phospholipid synthesis in the thyroid (Pastan and Macchia, 1967), and we have observed stimulatory effects of dibutyryl 3′,5′-cyclic AMP *in vitro* on RNA and protein incorporation of labeled precursors in thyroid (Adiga *et al.*, 1971) and pituitary slices (McKenzie *et al.*, 1970). In investigating the mechanism of the phenomenon we recognized a stimulation of RNA polymerase activities of subsequently isolated nuclei when dibutyryl 3′,5′-cyclic AMP was added to pituitary slices (McKenzie *et al.*, 1970). However, as reported here, the stimulation by dibutyryl 3′,5′-cyclic AMP of [^{3}H]leucine incorporation into protein in pituitary slices still occurred to some extent in the presence of actinomycin D. Consequently we turned to study the influence of 3′,5′-cyclic AMP on protein synthesis in a bovine pituitary cell-free system (Adiga *et al.*, 1966, 1968) that did not involve RNA synthesis and hence was not affected by actinomycin D.

Experimental Section

Materials. L-[^{3}H]Leucine, 5 Ci/mmole, was purchased from New England Nuclear Corp. Pyruvate kinase, phosphoenol pyruvate (trisodium salt), and 3′,5′-cyclic AMP were obtained from Sigma Chemical Co., dibutyryl 3′,5′-cyclic AMP and [^{3}H]3′,5′-cyclic AMP, 1.4 Ci/mmole, from Schwarz BioResearch, and [^{32}P]3′,5′-cyclic AMP, 6.1 Ci/mmole, from International Chemical & Nuclear. Nonradioactive AMP, ADP, ATP, adenine, adenosine, xanthine, and inosine were all from Nutritional Biochemical Corp. The source of other biochemicals used was previously described (Adiga *et al.*, 1966, 1968).

Preparation of the Constituents of the Anterior Pituitary Cell-Free System. Fresh bovine pituitary glands were obtained from a local abatoir. The processing of the glands and the methods of preparing a polysome-enriched suspension, pH 5.0 enzyme and supernatant fractions, and reaction mixture that contained energy sources, [^{12}C]amino acid mixture, and cofactors were previously described in detail (Adiga *et al.*, 1966, 1968). Rat liver pH 5.0 enzyme and super-

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† Present address: Department of Biochemistry, Indian Institute of Science, Bangalore, India.
‡ Medical Research Associate, Medical Research Council of Canada. To whom correspondence should be addressed.