Stimulation of Purine Synthesis in Vitro in the Calf Thyroid by Thyroid-stimulating Hormone*

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There have been many observations describing morphological and biochemical alterations in the thyroid after administration of thyroid-stimulating hormone. The hormone has been shown to increase oxygen consumption (1-3), incorporation of P32 into phospholipids (4), iodide uptake (5), the formation of iodotyrosines (6, 7), and glucose oxidation (8, 9) by thyroid tissue in vitro.

There have recently been efforts to relate the many effects of the hormone to some fundamental action on a single metabolic site. It has been proposed that the primary action of TSH in the thyroid is to stimulate glucose oxidation via the hexose monophosphate pathway (9). This effect is specific for thyroid tissue and for TSH and can be detected as early as 3 minutes after the addition of physiological amounts of the TSH to the medium (9, 10). The finding that the concentration of triphosphopyridine nucleotide is increased in thyroid slices incubated with TSH (11) suggests that the increased glucose oxidation is secondary to the changed triphosphopyridine nucleotide level. In thyroid homogenates, the concentration of triphosphopyridine nucleotide is a limiting factor in glucose oxidation (12). Evidence has been presented that TSH may increase TPN levels in thyroid homogenate, and this hypothesis has also been postulated (7).

Schussler and Ingbar (7) have recently shown that TPN stimulates the formation of monooiodotyrosine in homogenates of sheep thyroid gland. They have proposed that the energy for the reaction is derived from glucose via TPN-linked dehydrogenations. The stimulating action of TSH on this process can therefore be explained on the basis of its known action on glucose oxidation.

This paper concerns the action of TSH on purine synthesis in the calf thyroid. Incorporation of C14-formate into acid-soluble and ribonucleic acid purines is specifically stimulated by TSH at physiological dose levels. Evidence will be presented relating the action of TSH on purine synthesis to its known effect in stimulating the hexose monophosphate pathway.

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* The abbreviations used are: TSH, thyroid-stimulating hormone; AIC, 5-amino-4-imidazole carboxamide; AIC-ribonucleoside, 5-amino-4-imidazole carboxamide ribonucleoside; RSA, relative specific activity.

EXPERIMENTAL PROCEDURE

Procedure for Isotope Incorporation—One-gram aliquots of thyroid slices were placed in 4 ml of 0.9 % NaCl solution buffered at pH 7.4 with Krebs-Ringer phosphate and incubated in air at 37° for 3 or 6 hours with 2.5 μmoles of sodium formate containing 25 μC of the C14 label, 50 units of penicillin per ml, and 50 μg of streptomycin per ml. In experiments with adenine-8-C14, 1.5 μmoles of the base with 1,657,500 c.p.m. were added to the incubation medium.

Separation of Nucleic Acids—After incubation, the slices were washed with water and frozen overnight. They were then thawed and homogenized in a Potter-Elvehjem glass homogenizer. Protein and nucleic acids were precipitated with cold 2 N perchloric acid, and washed twice with 0.5 N perchloric acid and once with 95 % ethanol. Nucleic acids were separated from the precipitate by a modification of the sodium chloride extraction technique of Davidson and Smellie (13). Sodium nucleates were precipitated with acid-ethanol and washed with 95 % ethanol. The RNA ribonucleotides were separated from DNA by the Schmidt-Thannhauser (14) procedure. DNA was precipitated by 10 N perchloric acid and washed with perchloric acid, ethanol, and ether. The supernatant ribonucleotides were dried under nitrogen.

Separation of Bases—The RNA and DNA fractions were hydrolyzed with concentrated perchloric acid at 100° (15). The bases were isolated by two-dimensional chromatography as described by Wyatt (16) with a descending system of isopropanol-HCl-water followed by an ascending system of n-butanol-water-ammonia. The ultraviolet spots were cut out and sewed to strips of paper, and descending chromatographs were run in distilled water (17). The spots were identified under ultraviolet light (18) and eluted by capillary flow (19) with the use of 0.1 N HCl for adenine and 1.6 N HCl for guanine and hypoxanthine. With the eluate from an adjacent area of paper as a blank, the concentration of bases was estimated in the Beckman model DU spectrophotometer by the differential extinction technique (20).

Separation of Acid-soluble Purines—The purines of the acid-soluble fraction were assayed on activated Norit and eluted with 60 % acetone containing 1 % ammonia (21). The eluate was evaporated to dryness, hydrolyzed with 70 % perchloric acid for 1 hour at 100°, and then separated by chromatography in the same way as the RNA bases.

Separation of Nuclear and Cyttoplasmic RNA—Nuclear and
cytoplasmic RNA were separated by the citric acid fractionation technique described by Smellie et al. (22).

Measurement of Specific Activity—Aliquots of the bases were plated in infinitely thin layers on copper planchets and counted in a Nuclear-Chicago end window gas flow counter. Specific activity was expressed in counts per minute per μmole of the bases and then converted to relative specific activity by the formula

$$RSA = \frac{\text{Specific activity of base}}{	ext{C.p.m. per μmole of radioactive precursor used}} \times 10^6$$

Expression of the results as RSA allows for variation of the specific activity of the radioactive precursor used. All results are expressed as the average of duplicate assays.

Degradation of Adenine—The distribution of C$^{14}$ in the adenine molecule was determined by a modification of the method of Cavalieri et al. (23, 24), the degradation yielding 5-amino-4-imidazole carboxamidine. This was purified by chromatography in isopropanol-HCl-water, and its specific activity was taken to represent that of the C-8 position of the original adenine. The activity of the original C-2 position was calculated by subtracting the C-8 value from the total. When small amounts of labeled adenine were degraded, carrier adenine was added to increase the yield of the carboxamidine.

TSH Preparations—The TSH preparation usually used was purchased from Armour and Company (Thyrotropar). A purified TSH preparation containing 6 to 8 units per mg was the gift of Dr. P. Condliffe.

RESULTS

TSH increased the incorporation of C$^{14}$-formate into RNA-adenine in calf thyroid, had no effect on calf kidney, but repeatedly caused a slight decrease in calf liver (Table I).

An aqueous solution of TSH (10 milliunits per ml) was boiled for 5 minutes. TSH so treated is known to be inactive in the assay procedure described by El Kabir (25). Table II shows that the inactivated TSH had no significant effect on RNA-adenine formation in calf thyroid.

Even at the end of a 6 hour incubation period, there was still evidence of stimulation by TSH, but at this time the difference between control and stimulated slices was less than at earlier periods. This effect was obtained with a purified TSH preparation as well as the commercial TSH (Thyrotropar) usually used.

TSH increased formate incorporation into the acid-soluble adenine and hypoxanthine as well as into RNA-adenine of thyroid slices (Table III).

The effect of graded doses of TSH on RNA-adenine formation is shown in Table IV and on hypoxanthine formation in Table V. A response was observed with amounts of TSH as small as 0.1 milliunit per ml. Bovine serum albumin, 100 μg per flask, was added to prevent loss of small amounts of TSH through adsorption to glassware. It is unlikely that the action of TSH was due to a nonspecific protein effect since bovine serum albumin, 2 mg per flask, had no effect on purine formation.

It was important to exclude the possibility that TSH was acting by stabilizing the pH of the medium, because a fall in pH is known to inhibit the incorporation of C$^{14}$-formate into RNA purines (26). No difference in pH was found in flasks containing 1 unit of TSH after incubation for 3 hours with the usual amounts of buffer, formate, and thyroid slices when compared with control flasks.
The appropriate flasks.

TSH was used in a concentration of 1 unit per flask.

Glucose and TSH ...........
AIC ........................
AIC and glucose. ...........
AIC and TSH ...............
AIC, glucose, and TSH .....
AK-ribonucleoside and TSH
AIC-ribonucleoside. .........

<table>
<thead>
<tr>
<th>Drug</th>
<th>Tissue fraction</th>
<th>RSA of hypoxanthine</th>
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<tbody>
<tr>
<td>Control</td>
<td></td>
<td>7,993</td>
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<tr>
<td>TSH</td>
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<td>14,092</td>
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<td>TSH</td>
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<td>8,543</td>
</tr>
<tr>
<td>TSH</td>
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<td>11,348</td>
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<tr>
<td>TSH</td>
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<td>10,795</td>
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<tr>
<td>TSH</td>
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<td>8,288</td>
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Table VI indicates that TSH increased the incorporation of C14-formate into both nuclear and cytoplasmic RNA-adenine.

TSH consistently stimulated the incorporation of formate into RNA and acid-soluble adenine, but the effect on RNA and acid-soluble guanine was variable. In some experiments, guanine formation was increased by an amount similar to that of adenine, but in others no effect could be observed. Incorporation of formate into RNA-cytosine and uracil was unaffected by TSH. The specific activity of thymine, the only DNA base into which significant incorporation of formate could be observed, was unaffected by TSH.

In an effort to determine the mechanism of action of TSH, glucose and the purine ring precursor, 5-amino-4-imidazole carboxamide and its ribonucleoside, were added to the incubation medium (Table VII). Glucose, 5 μmoles per ml, increased the incorporation of formate into RNA- and acid-soluble adenine to the same extent as 0.2 unit per ml of TSH. A combination of glucose and TSH had a greater effect than did either alone. A much greater incorporation of C14-formate into RNA-adenine was observed in the presence of AIC. When glucose was added as well as AIC, labeling of RNA-adenine was doubled. Likewise, TSH, in the presence of AIC, doubled RNA-adenine formation. A combination of glucose, TSH, and AIC had an even greater effect. AIC-ribonucleoside had the same effect as AIC with glucose or AIC with TSH. In the presence of AIC-ribonucleoside, TSH caused a moderate increase in purine formation.

The effect of TSH on the incorporation of adenine-8-C14 into RNA purines appears in Table VIII. A definite stimulation was observed for both adenine and guanine.

**DISCUSSION**

The increased incorporation of C14 formate into RNA adenine produced by TSH in the calf thyroid reflects the increased specific activity of the acid-soluble adenine pool. Therefore, TSH appears to increase the formation of adenine nucleotides. The specificity of the effect of TSH on thyroid purine synthesis is indicated by the fact that the hormone had no stimulating action on liver and kidney.

The amount of TSH required to increase purine synthesis is close to the mean physiological value. Estimations of the amount of TSH in human plasma have varied, but a figure of 0.5 milliunit per ml is widely accepted (27). The main gradient for the action of TSH on purine synthesis was found between 0.1 and 4 milliunits per ml. When higher concentrations of TSH were used, a plateau was reached from 4 milliunits per ml up to 400 milliunits per ml. This range of dose response is not unlike that observed by Dumont (10) for the effect of TSH on C14O2 production from glucose-1-C14 in sheep thyroid slices. He found the main gradient of action to lie between 1 and 10 milliunits per ml and a plateau to be reached at concentrations of TSH higher than 12 to 20 milliunits per ml. Field et al. (9) failed to demonstrate increased glucose oxidation by TSH in calf thyroid slices below 50 milliunits per ml, but this may have been due to loss of smaller amounts of TSH through adsorption on glassware. In these experiments and in those of Dumont (10), bovine serum albumin was added to prevent this loss.

The heterogeneity of nuclear RNA has now been established (28). Separation of the nuclei by the usual citric acid technique (29), or by neutral phosphate buffer (28), removes a fraction of RNA with lower specific activity. This fraction has an electrophoretic mobility and base composition similar to cytoplasmic...
RNA and kinetic studies in nonproliferating tissues suggest that it may serve as a precursor for cytoplasmic RNA (28). The results reported here refer only to the residual, high specific activity fraction of nuclear RNA. TSH increased the incorporation of C\textsubscript{4}-formate into this fraction as well as into cytoplasmic and whole RNA.

When glucose was added to the incubation medium, an increase in the incorporation of formate into both RNA and acid-soluble adenine could be observed. A much greater increase of formate incorporation by glucose has been reported in the Ehrlich ascites cell (30, 31). Two theories have been put forward to explain the action of glucose on purine synthesis in the ascites cell. Harrington (30) has suggested that glucose acts as a source of ribose necessary for nucleotide biosynthesis. The principal evidence for this view is that low concentrations of cytidine and uridine (0.5 mm), which can liberate ribose 1-phosphate by the action of nucleoside phosphorylase, produce the same effect as much higher concentrations of glucose. Henderson and LePage (32), who have shown that the effects of glucose are abolished by iodosaccharate (alone or with dinitrophenol), conclude that the principal role of glucose is as an energy supply. Thomson et al. (33) have provided evidence that supports Harrington’s theory. When the ascites cell is incubated aerobically in vitro, the cells have a high content of ATP and this is not increased by glucose or uridine. Therefore, the increase in purine biosynthesis produced by these compounds is unlikely to be due to their acting as sources of energy. Free ribose and ribose 5-phosphate are ineffective in stimulating purine biosynthesis even in large amounts (30), but it is possible that cells lack ribokinase and are impermeable to phosphorylated sugars. In pigeon liver homogenates in which cell barriers do not play any part, ribose 5-phosphate does not stimulate purine synthesis (34).

Glucose oxidation via the hexose monophosphate pathway is the principal source of nucleic acid ribose. Ribose 5-phosphate is formed from ribulose 5-phosphate in the oxidative steps. A reversal of the transketolase-transaldolase sequence also provides ribose 5-phosphate from xylulose 5-phosphate by a nonoxidative mechanism. Isotope studies (35–37) have indicated that both mechanisms contribute to nucleic acid ribose in mammalian tissues, but the quantitative importance of the two mechanisms seems to differ in different species. In rat liver, the nonoxidative route is more important (35, 36), whereas in human tissue, the oxidative route preponderates (38).

In calf (8) and sheep (39) thyroid slices, there is good evidence for an active hexose monophosphate pathway. When slices are incubated in vitro with C\textsubscript{4} labeled glucose, a ratio of greater than unity is observed when C\textsubscript{4}O\textsubscript{2} derived from glucose-1-C\textsubscript{4} is compared to C\textsubscript{4}O\textsubscript{2} from glucose-6-C\textsubscript{4}. Even allowing for the difficulties involved in using differentially labeled glucose to estimate the relative proportions of glucose metabolized via the Embden-Meyerhof pathway and the hexose monophosphate pathway (40), the results reported in thyroid tissue indicate that the hexose monophosphate pathway is operative in this tissue.

It was found that TSH preferentially increased the production of C\textsubscript{4}O\textsubscript{2} from glucose-1-C\textsubscript{4} compared with that from glucose-6-C\textsubscript{4} (9, 10). Therefore, TSH seems to increase glucose oxidation via the hexose monophosphate pathway and in this way is able to make greater supplies of ribose available for purine biosynthesis. This mechanism could well explain why glucose and TSH both cause a similar increase in purine formation in the calf thyroid. It also provides an explanation for the increased effect of TSH in the presence of glucose, more substrate being available for ribose production.

In the presence of glucose and TSH, ribose formation is presumably maximal and the limiting factor in purine biosynthesis will then be purine ring production. When the purine ring precursor AIC was added, a marked stimulation of purine synthesis occurred and ribose formation became a limiting factor. Addition of glucose by increasing the availability of ribose allowed a further increase of purine synthesis. TSH, by accelerating oxidation of glucose already present in the slices, also increased purine synthesis to the same extent as the combination of glucose and AIC. The ribonucleoside was much more effective than free aminomimidazole carboxamide. The magnitude of stimulation produced by AIC-ribonucleoside in comparison to AIC suggests that the addition of ribose in available form was important for purine synthesis de novo. As shown in Table VI, the combination of AIC and glucose or AIC and TSH appeared to have a similar effect to AIC-ribonucleoside. This strongly suggests that both glucose and TSH act by increasing the supply of available ribose. In the presence of AIC-ribonucleoside, TSH was able to increase purine synthesis still further, possibly by provision of additional ribose and hence, PP-ribose-P which, under the influence of nucleotide pyrophosphorylases (41), could react with free bases to form nucleotides.

Further evidence for the ability of TSH to stimulate nucleotide formation from free bases is seen in the increased incorporation of adenine-S-C\textsubscript{4} into RNA purines in the presence of TSH. Similarly, glucose in the Ehrlich ascites cell is capable of increasing the incorporation of adenine-8-C\textsubscript{4} into RNA purines (30). This is still a further example of the similarity in action of TSH and glucose in stimulating nucleotide formation.

In comparing the effect of glucose and TSH on purine formation in the thyroid with the action of glucose on the Ehrlich ascites cell in vitro, one major discrepancy must be explained. Glucose causes a 13-fold increase in purine formation in the ascites cell (30), whereas glucose and TSH never produce more than a 2-fold increase in purine formation in the thyroid. The reason for the discrepancy probably lies in the different capacity of the two tissues to synthesize purines in vivo under conditions in vitro. Purine synthesis is relatively inactive in the ascites cell in vitro, and only 10% of the C\textsubscript{4}-formate is incorporated into the C-8 position of adenine; the C-8 label representing purine formed by the full de novo pathway (31, 33). Thomson et al. (33) suggest that incorporation of formate in the carcinoma in vitro is chiefly into existing AIC-ribonucleotides to form purine nucleotides, or to labeling of existing nucleotides by transformation (32).

The calf thyroid shows a striking difference in the incorporation of formate in vitro. An average of 43% (40, 42, and 47% in three separate experiments) of the label was found in the C-8 position of RNA adenine, indicating extensive utilization of the full de novo pathway. A similar pattern of purine synthesis was reported in rabbit bone marrow in vitro (32), in which 39% of the isotope was incorporated into the C-8 position of adenine.

In the Ehrlich ascites cell, the availability of glucose, and therefore of its product ribose-5-phosphate, seems to be the limiting factor in the utilization of the full de novo path of purine synthesis in vitro, since glucose, as well as stimulating purine synthesis, also increases the C-8 label from 10% to 30% (31). Purine synthesis in vitro in the ascites cell in the presence of glucose is then similar to that found in vivo.
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The calf thyroid in vitro has much more active purine synthesis than the ascites cell. This might be related to the active hexose monophosphate pathway that exists in thyroid tissue and makes ribose available in adequate amounts for purine synthesis. Under these circumstances, the effect of further supplies of ribose by the action of TSH or by adding glucose would be limited by the ability of the tissue for purine ring formation.

The present observations afford a link between glucose catabolism and purine synthesis in the thyroid. Like the formation of iodotyrosine (7), the regulation of purine synthesis in the thyroid is mediated by stimulation of glucose oxidation via the hexose monophosphate pathway. This is further evidence that the diverse actions of TSH on the thyroid may result from its action on a single metabolic site.

**SUMMARY**

Thyroid-stimulating hormone (TSH) is capable of increasing the incorporation of C4-formate into purines in calf thyroid slices in vitro. This effect seems to be specific, since the hormone has no stimulant effect on purine synthesis in liver and kidney slices. Amounts of TSH in the physiological range are effective in stimulating purine formation. Both glucose and TSH cause a 16-fold increase in purine formation in the presence of AIC, an increment similar to that produced by AIC-ribonucleoside. It seems likely that the action of TSH on purine synthesis in the thyroid is mediated by stimulation of glucose oxidation via the hexose monophosphate pathway, thereby increasing the supply of available ribose.

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