Effects of Thyrotropin on Thyroidal Phospholipid and Adenosine 5'-Triphosphate Metabolism*

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SUMMARY

Thyrotropin stimulates the incorporation of 32P into phospholipids of thyroid slices incubated with 32PO4. To determine whether this effect could be secondary merely to an increase in the specific activity of the terminal (γ) P of ATP, a precursor of phospholipid P, the effect of thyrotropin on calf thyroid slice ATP was measured with an assay which is selective for ATP-γ-32P specific activity. Thyrotropin increases the specific activity of lipid 32P uniformly without altering that of ATP-γ-32P, indicating that it exerts its effect at a step beyond ATP in the sequence of phospholipid biosynthesis. There was no significant effect of thyrotropin on the ATP content of thyroid slices.

It has been known for some time that thyrotropin stimulates the incorporation of 32P into phospholipid of surviving thyroid slices incubated with 32P-orthophosphate (1). The study of this phenomenon is of great interest not only because it may elucidate TSH action but also because of the insights it may furnish into factors controlling phospholipid metabolism. It is not known at what point in the sequence of phosphatide biosynthesis TSH acts. Past experiments (2) have shown that the stimulated uptake of 32P occurs mainly in phosphaticid acid and phosphatidyl inositol when either total or specific activity is measured and the specific activity increase is itself sufficient to account for the increment of total activity, thus excluding a net increase in phosphatide content as the mechanism of the TSH effect. Remaining possibilities are a stimulated turnover of lipid phosphorus or an increase in the specific activity of a phospholipid precursor with no effect on the lipids themselves. The latter mechanism would be trivial from the point of view of phospholipid metabolism.

A key precursor of phosphatide phosphorus is the terminal (γ)P of ATP. This atom could find its way into phosphaticid acid (thence into phosphatidyl inositol) via glycerophosphate (3, 4) or the direct phosphorylation of diglyceride (5). It is theoretically possible that TSH, in altering thyroidal energy requirements, might cause a more rapid turnover of ATP, leading to an increased incorporation of orthophosphate with subsequent increase of the specific activity of the phosphatides. It has been reported (6, 7) that TSH causes no change in the 32P content of the water-soluble phosphates of thyroid slices, but in such studies any effect on ATP-γ-32P might be masked by high activity in other phosphate esters. This study was undertaken, therefore, to examine the effects of TSH on 32P incorporation into thyroidal phosphatides and ATP by a recently developed assay which permits selective measurement of the terminal P of ATP.

MATERIALS AND METHODS

Bovine TSH (2 units per mg) was provided by the Endocrine Study Section of the National Institute of Arthritis and Metabolic Disease. Glycerokinase was the product of Boehringer (Mannheim). 3H-Glyceryl was purchased from New England Nuclear. 32P-Orthophosphate was obtained from Iso-Serve (Cambridge, Massachusetts) and kept in acid solution until used.

Tissue Incubation—Calf thyroids were removed from the animals immediately after slaughter and chilled on ice. The glands were trimmed of fat, and slices of about 0.5 mm thickness were prepared with a Stadie-Riggs slicer. The slices were rinsed in cold 0.9% NaCl for several minutes, lightly blotted, weighed, and incubated at 37° in 2 ml of Krebs-Ringer media with either Tris-Cl buffer in air or Na-bicarbonate buffer in 02-5% CO2 containing 20 μC per ml of 32PO4. The final PO4 concentration of the medium was 1.2 mm, and pH was adjusted to 7.4 at 37°. The slices, free of obvious connective tissue seams, weighed between 50 and 100 mg but in any one experiment were within 10% of each other in weight. Control slices were always from the same lobe as experimental ones.

After incubation with the appropriate substance, the slices were blotted, rinsed briefly in cold NaCl, blotted again, and quickly homogenized in a small glass Dual tissue grinder in cold 2% HClO4 with a volume equivalent to 4 times the slice weight (milliliters per g). The sediment was removed by centrifugation and saved for lipid extraction while a 20-μl aliquot of the clear supernatant fluid was used for assay of ATP content and another aliquot of about 200 μl was taken for determination of specific activity of ATP-γ-32P.

ATP Assay—The assay for ATP and for specific activity of ATP-γ-32P is detailed elsewhere (8) and depends on the transfer...
of the terminal P to $^3$H-glycol of known specific activity by glycerokinase and measurement of the radioactivity of the resultant glycerophosphate. The tritium activity of the glycerophosphate reflects the amount of ATP in the sample while the specific activity of the $^\gamma$-$^3$P can be calculated from the $^3$P:$^3$H ratio.

The 20-$\mu$l deproteinized sample is neutralized with 5 $\mu$l of 0.8 M $K_2$CO$_3$ in a total volume of 0.1 ml containing MgCl$_2$, 5 mm; EDTA, 2 mm; mercaptoethanol, 40 mm; Tris-HCl buffer (pH 9.0), 100 mm; glycerokinase, 0.2 $\mu$g; and the $^3$H-glycol substrate, 0.2 mm. After 15 min at room temperature, the reaction is complete and a 10-$\mu$l aliquot is applied with a microsyringe to a cellulose thin layer plate which is developed with a solvent of 55 parts of methanol and 65 parts of 1 M ammonium acetate (pH 8.5, 0.002 M EDTA). The location of the glycerophosphate spot is determined by running a $\alpha$-glycerophosphate marker in a side lane which is sprayed with the Hanes-Isherwood reagent for phosphorus (9) to make the spot visible. Each glycerophosphate band is scraped into a scintillation vial with 2 ml of methanol containing 2.5% (v/v) 4 M HCl and 8 ml of toluene with 0.5% (w/v) 2,5-bis-(2.5-di-tert-butylbenzoxazolyl) thiophene (Packard Instrument). Radioactivity is determined in a scintillation counter set for tritium and $^3$P. The observed tritium activity is compared to that derived from ATP standards to calculate total ATP.

Specific Activity Determination of ATP-$\gamma$-$^3$P.—The ratio of $^3$P to $^3$H obtained in the above assay permits one to calculate the specific activity selectively of the terminal P of ATP if there are no interfering sources of $^3$P. Such sources can be $^2$PO$_4$ from the incubating medium, which runs very close to and may overlap the glycerophosphate band on chromatography, and ATP-$^3$P-glycerophosphate itself, which may be formed during the incubation. To eliminate this interference, the sample is first adsorbed onto charcoal. The deproteinized sample is added without neutralization to 1 mg of activated charcoal (Norit A) suspended in 0.05 ml of 0.01 M HCl-0.05 M H$_2$PO$_4$. The mixture is centrifuged, the supernatant solution is withdrawn, and the charcoal is washed twice with 0.2 ml of 0.01 M HCl. The standard assay system as described above is added directly to the charcoal and allowed to incubate at room temperature for at least 15 min. The charcoal is centrifuged and a 10-$\mu$l sample of the clear solution is withdrawn for chromatography and counting as above. The ratio $^3$P:$^3$H represents the specific activity of the terminal P of ATP, but since recovery from the charcoal is variable, the amount of ATP in the sample cannot be measured by this modification.

The standard deviation of the assay is about 2 to 5% for the ranges of ATP encountered in the present studies. These assays are not specific for ATP since the glycerokinase can utilize the other nucleotide triphosphates as substrates, but this is a second order correction in these experiments since ATP represents the major nucleotide triphosphate in thyroid slices. This was shown in preliminary experiments by concurrent analysis of ATP by the hexokinase method (10). The latter method, which is specific for ATP, gave results about 10% less than the glycerokinase method. Furthermore, when ATP levels were lowered by incubating slices with dinitrophenol, the results obtained with the hexokinase method fell in parallel with those of the other method. As far as measuring the specific activity of the terminal P is concerned, the nonspecificity of the glycerokinase method for ATP is of even less consequence since the terminal P of the various nucleotide triphosphates is in rapid equilibrium and should be of homogeneous activity.

### Lipid Extraction

The precipitate from the HClO$_4$-homogenized thyroid slice was extracted sequentially with 0.5 ml of CH$_2$OH and twice with 1 ml of CHCl$_3$-CH$_2$OH (2:1). Each extract lasted about 5 min, and after centrifugation the supernatant solvent was removed and pooled. To the pooled extracts of each slice were added 1 ml of CHCl$_3$ and 0.9 ml of 0.1 M MgCl$_2$-0.01 M HCl, and, after centrifugation, the upper aqueous phase was withdrawn by suction. The lower phase was washed twice with 2 ml of artificial upper phase as described by Folch, Lees, and Sloane Stanley (11) with the modification that the aqueous component was 0.1 M MgCl$_2$-0.01 M HCl. An aliquot of the washed organic phase was taken for total phosphorus analysis (12), and another aliquot was dried on a planchet for $^3$P counting in a gas flow Geiger system. With appropriate standards, the activity was corrected to the efficiency of the liquid scintillation counter to permit comparison with the specific activity of ATP-$\gamma$-$^3$P.

### RESULTS

The observed control values of ATP content in incubated thyroid slices were quite variable from animal to animal. The usual level was about 0.5 $\mu$ mole per g (wet weight), and the range was from 0.33 to 1.0 $\mu$ mole per g. Within this range, levels were independent of the type of incubating medium, the presence or absence of glucose, and the time of incubation between 15 and 90 min. However, a limited number of observations on slices from the same gland showed that 0.01 M glucose increased the ATP content by about 10%. The specific activity of the terminal P of the ATP after 1 hour of incubation was typically about 15 to 25% of the initial specific activity of the $^3$P-orthophosphate. The specific activity of the phospholipids at the same time was 0.5 to 1% of that of the terminal P of ATP. These values were independent of incubating medium or glucose content. As is to be

### Table I

<table>
<thead>
<tr>
<th>Time</th>
<th>Conditions</th>
<th>Number of slice pairs</th>
<th>ATP content</th>
<th>ATP-$\gamma$-$^3$P specific activity</th>
<th>Lind.$^3$P specific activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>min</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>15</td>
<td>KRB, glucose</td>
<td>4</td>
<td>92 (79-103)</td>
<td>99 (88-105)</td>
<td>161 (140-184)</td>
</tr>
<tr>
<td>30</td>
<td>KRB, glucose</td>
<td>8</td>
<td>103 (91-108)</td>
<td>90 (81-108)</td>
<td>171 (129-235)</td>
</tr>
<tr>
<td>60</td>
<td>KRB, glucose</td>
<td>3</td>
<td>89 (68-92)</td>
<td>100 (92-100)</td>
<td>255 (224-250)</td>
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<tr>
<td>60</td>
<td>KRB, glucose</td>
<td>7</td>
<td>109 (95-120)</td>
<td>88 (85-90)</td>
<td>200 (172-230)</td>
</tr>
<tr>
<td>60</td>
<td>KRB, glucose</td>
<td>3</td>
<td>108 (92-119)</td>
<td>94 (84-108)</td>
<td>171 (145-184)</td>
</tr>
<tr>
<td>60</td>
<td>KRT, glucose</td>
<td>8</td>
<td>106 (82-110)</td>
<td>96 (90-114)</td>
<td>187 (142-300)</td>
</tr>
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</table>
expected, however, the specific activities were time-dependent and were increasing up to at least 2 hours of incubation. Table I presents the effect of TSH on ATP levels and 32P incorporation into ATP and phospholipids of thyroid slices incubated with [32P]. In either Krebs-Ringer bicarbonate or Krebs-Ringer Tris buffer, with or without glucose, there was no significant effect of TSH on ATP content or specific activity of ATP-γ-32P while a uniform increase of phospholipid specific activity occurred. In these experiments, and all others reported here, the total lipid phosphorus per milligram (wet weight) was not significantly different in control and experimental slices (values constant within 10%) so that an increase in specific activity also represents an increase in total activity of lipid-32P in the slice.

Table II lists the effect of varying concentrations of 2,4-dinitrophenol on thyroid slices. The dinitrophenol decreased ATP levels and the specific activities of the ATP-γ-32P and phospholipids. However, the ratio of lipid to ATP specific activities remained relatively constant, demonstrating the essentially linear dependence of phospholipid activity on that of ATP. Other experiments with higher concentrations of dinitrophenol do, however, show a fall in this ratio as ATP production is more severely depressed. Experiment B also presents data on the effect of dinitrophenol on TSH action. The effect of TSH in increasing the ratio of lipid to ATP specific activities persists although to a diminishing extent as ATP production is depressed.

### Table II

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Concentration of 2,4-dinitrophenol</th>
<th>ATP Activity of ATP-γ-32P</th>
<th>Lipid-32P Activity</th>
<th>Specific activity ratios, lipid-32P to ATP-γ-32P</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
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<td></td>
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<tr>
<td>A</td>
<td></td>
<td></td>
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<tr>
<td></td>
<td>0.0 pmol/g</td>
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<td>17.6</td>
<td>2.34 x 10^-3</td>
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<td>0.2 pmol/g</td>
<td>1906</td>
<td>19.8</td>
<td>6.3 x 10^-3</td>
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<tr>
<td></td>
<td>0.4 pmol/g</td>
<td>1335</td>
<td>19.7</td>
<td>6.6 x 10^-3</td>
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<tr>
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<td>0.6 pmol/g</td>
<td>1068</td>
<td>18.0</td>
<td>2.1 x 10^-3</td>
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<tr>
<td>B</td>
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<td></td>
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<tr>
<td></td>
<td>0.8 pmol/g</td>
<td>1265</td>
<td>17.7</td>
<td>8.8 x 10^-3</td>
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<td></td>
<td>1.2 pmol/g</td>
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<td>17.9</td>
<td>1.1 x 10^-3</td>
</tr>
<tr>
<td></td>
<td>1.5 pmol/g</td>
<td>1838</td>
<td>18.1</td>
<td>2.5 x 10^-3</td>
</tr>
</tbody>
</table>

### Discussion

In these experiments, the specific activity of the phospholipid 32P should be a linear reflection of that of its distant precursor, ATP-γ-32P, if the intervening biosynthetic rates and the total lipid phosphorus levels remained constant. Such effect is illustrated by the findings that 2,4-dinitrophenol, although depressing ATP levels and specific activity, did not significantly change the ratio of lipid-32P to ATP-γ-32P specific activities. If the stimulating action of TSH on lipid incorporation of 32P were mediated solely by an effect on ATP, an approximate doubling of ATP-γ-32P specific activity would be required. Such changes would have been easily measured in these experiments. Observations at various time intervals showed that ATP specific activity was continuously increasing during any experiment so that any early peak and decline could not have occurred. Thus, the findings that TSH uniformly increases lipid-32P specific activity without altering that of ATP-γ-32P indicates that TSH must exert its effect beyond ATP in the sequence of phospholipid biosynthesis. In a more trivial sense, however, TSH action is dependent on ATP for, as the experiments with dinitrophenol have shown, diminished ATP levels depress the magnitude of the TSH response. This might be due either to an insufficiency of metabolic energy which might be required for the effect or merely to a limitation of precursor ATP.

Acknowledgment—I am indebted to Miss Laila Karlsson for technical assistance.

### References


2 Cyclic adenosine 3',5'-monophosphate has been postulated as a mediator of TSH action (13), but no effect of this compound on thyroid phospholipid 32P incorporation has been demonstrated. However, the N,N'-O-dibutylryl derivative of cyclic AMP has been reported to increase lipid 32P uptake (13) possibly because it is more resistant to hydrolysis by tissue phosphodiesterases than is cyclic AMP (14). Accordingly, we measured the effect of both of these compounds on thyroid phosphorous metabolism. Contrary to the previous report, we could not demonstrate any effect of the dibutyryl derivative at levels of 0.06 to 0.8 mM on the specific activity of the phospholipids although a variety of experimental conditions, including those of the previous report, were tried. Incubations in Krebs-Ringer bicarbonate, with or without glucose, and for time intervals of 15 to 180 min showed no effect of the dibutylryl cyclic AMP on phospholipid 32P, ATP levels, or ATP-γ-32P activity. Incubation of slices in Krebs-Ringer Tris with glucose at 37° for 1 hour and 20 hours, at pH 7 or 7.4, in an attempt to elute possible interfering substances or partially damaged cell membranes to increase their permeability, did not reveal any effects in subsequent incubation with dibutylryl cyclic AMP although these slices were still responsive to TSH. To ascertain that our cyclic AMP and the dibutylryl derivative were biologically active, their effect on the transmural potential of rabbit ileum (15) was measured (studies kindly performed by Dr. M. Field) and all batches used were indeed active. Furthermore, to exclude the possibility that dibutylryl cyclic AMP was inactivated during the incubation, the incubation medium itself was assayed by the above method after incubation with thyroid slices. Media with the dibutyl compound retained their activity while control media with equimolar amounts of AMP or with TSH showed no activity. Cyclic AMP at levels of 0.6 mM had no effect on phospholipid-32P, but it did increase ATP levels (up to 200% of control) without altering ATP-γ-32P specific activity. The effect on ATP levels, however, was shared by AMP and to a lesser extent by ADP and thus may merely reflect an increase in available substrate for ATP synthesis. These experiments offer no evidence that cyclic AMP is a mediator of TSH with respect to its action on phospholipids.
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