Artifacts in Studies of Protein Synthesis with Radioactive Amino Acids

INVALIDITY OF ALLEGED STIMULATION OF PROTEIN SYNTHESIS BY THYROXINE IN VITRO IN ABSENCE OF MITOCHONDRIA*

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SUMMARY

Previous studies have demonstrated that thyroid hormones in vitro stimulate microsomal protein synthesis in cell-free systems, but the effect was found to be indirect and dependent on the presence of mitochondria in the incubation system. The mitochondrial requirement has recently been questioned by Carter et al. (1) who claimed to observe direct stimulations by thyroxine in cell-free liver systems lacking mitochondria. The system used by them contained higher Mg2+ concentrations, lower microsomal concentrations, and uniformly labeled L-[14C]valine of very high specific activity. We have examined this effect and confirmed the stimulation of 14C incorporation into protein but found that it bears no relationship to protein synthesis. The effect is not observed with tRNA-[14C]-amino acid as the source of 14C-amino acid. It is not inhibited by cycloheximide, puromycin, or ribonuclease, and is, in fact, enhanced in their presence. It exhibits no dependence on microsomes or ATP and is maximum in their absence. The effect is not diluted out by dilution of the L-[U-14C]valine with unlabeled L-valine. Chromatographic analysis of hydrolysates of the labeled protein reveals that the thyroxine stimulation is on the incorporation not of the [14C]-amino acid but of a radioactive contaminant present in the L-[U-14C]valine. Purification of the [14C]-amino acid by ion exchange chromatography before use reduces or eliminates both the base-line incorporation and the thyroxine effect. Similar spurious incorporation and thyroxine stimulations are observed with L- and D-[1-14C]-valine, but they are almost negligible compared to those observed with L-[U-14C]valine. The thyroxine effect reported by Carter et al. is, therefore, not on protein synthesis but on the energy-independent incorporation of a radioactive contaminant of the L-[U-14C]-amino acid into cell sap protein. It is, in fact, obscured by protein synthesis and was unmasked by them when they reduced protein synthesis by lowering the microsomal concentration and compensated for the reduced 14C incorporation by using L-[U-14C]-amino acid of high specific activity.

Previous studies in this laboratory demonstrated that thyroid hormones added in vitro stimulate protein synthesis in mammalian cell-free microsomal and ribosomal systems under certain conditions (2-6). The stimulation was localized to the transfer of tRNA-bound amino acid into peptide linkage in the interior of the nascent polypeptide chains (2, 3, 6). The effect could not, however, be attributed to a direct action of thyroid hormones on any of the partial reactions of protein synthesis. The stimulation exhibited an absolute dependence on the presence of mitochondria and an oxidizable substrate in the reaction mixture; replacement of these components by an alternative ATP-generating system did not alter the control level of amino acid incorporation into microsomal protein, but stimulation by thyroxine could not then be observed (2, 3, 6, 7). Additional evidence established that the effect on protein synthesis was secondary to and dependent on a prior energy-dependent reaction between the hormone and the mitochondria in the system, which occurred during a short lag immediately preceding the appearance of the stimulation (2, 3, 8). Extensive efforts to find a direct effect of thyroid hormones on protein synthesis in vitro in the absence of mitochondria were uniformly unsuccessful.

Carter et al. (9) recently reported that they could observe stimulations of protein synthesis by thyroxine in vitro in the absence of mitochondria by modifying the reaction system of Sokoloff and Kaufman (2). The modifications consisted of an increased Mg2+ concentration, a markedly reduced microsomal concentration, and the use of low concentrations of uniformly labeled L-[14C]valine of high specific activity instead of the saturating concentrations of [14C]carboxyl-labeled amino acids of low specific activity used by Sokoloff and Kaufman (2).

The results of the present studies demonstrate that the thyroxine effect reported by Carter et al. (9) is not on protein synthesis. It is rather an effect on the incorporation of a radioactive contaminant of the [14C]-amino acid into cell sap protein, uncovered by the lowering of the proportion of microsomal to cell sap protein and the use of uniformly labeled amino acid of high specific activity which is particularly rich in the contaminant. The effect tends, in fact, to be obscured by protein synthesis and is enhanced when 14C incorporation due to protein synthesis is suppressed by omission of microsomes and/or the energy source, addition of protein synthesis inhibitors, or dilution of the L-[U-14C]valine with unlabeled L-valine in the reaction system.

* A preliminary report of this work was presented at the 56th Annual Meeting of the Federation of American Societies for Experimental Biology, Atlantic City, N. J. 1972 (1).
EXPERIMENTAL PROCEDURES

Materials

Chemicals—AMP and ATP were purchased from P.L. Biochemicals, Inc. Creatine phosphate and creatine kinase (EC 2.7.3.2) were obtained from the Boehringer Mannheim Corp. The sodium salt of 8-14C-valine was purchased from New England Nuclear Corp., Nuchar, Biochemicals Corp. and Calbiochem; in some experiments a mixture of all three preparations which had been reconstituted three times from hot ethanol solutions was used with similar results. L-[U-14C]Valine (specific activity 210, 228, and 248 mCi per mmole), L-[1-14C]valine (specific activity 25.4 and 27.7 mCi per mmole), and D-[1-14C]valine (specific activity 18.1 mCi per mmole) were purchased from New England Nuclear Corp. Lower specific activities of the 14C-valine preparations were obtained by appropriate dilution of the purchased radioactive amino acids with unlabeled D- or L-valine purchased from Calbiochem and Sigma Chemical Co., respectively. Cycloheximide and beef pancreatic ribonuclease A were also obtained from Sigma Chemical Co. Purumycin hydrochloride was purchased from Nutritional Biochemicals Corp. rRNA-D-[1-14C]valine was synthesized as previously described (3), except that the tRNA was yeast tRNA purchased from Miles Laboratories, Inc., the 100,000 x g supernatant fraction of a rat liver homogenate was used as the source of aminoacyl-tRNA synthetase, and a phenol extraction was included in extraction of the tRNA-L-[1-14C]valine from the reaction medium.

Animals—Normal Sprague-Dawley male rats weighing between 100 and 150 g were used in all experiments. The animals were maintained on Purina laboratory chow and water ad libitum but were deprived of food for approximately 17 hours before use.

Methods

Preparation of Cell Fractions—Rat liver homogenates in 5 ml of 0.25 M sucrose per g of fresh liver weight were prepared as previously described (2), except that the homogenates were centrifuged for 15 min at 12,000 x g in a Sorvall RC-2B refrigerated centrifuge to remove the intact cells, cell debris, nuclei, and mitochondria. The supernatant fraction was then centrifuged at 269,000 x g for 2 hours in a Beckman J2-HR ultracentrifuge to separate the microsomes and cell sap. The cell sap was decanted and stored in ice until use. The microsomal pellet was washed with 0.25 M sucrose and then reconstituted by hand homogenization with an all-glass Potter-Elvehjem homogenizer in 3 ml of 0.25 M sucrose per g of original fresh liver weight. The protein concentrations in the cell sap and microsomal suspensions were approximately 10 and 8.5 mg per ml, respectively.

Incubation—Incubations were carried out in air in 25-ml Erlenmeyer flasks or glass stopped bottles in a Dubnoff water bath at 37° at a rate of 92 oscillations per min. The components of the reaction mixtures are described in the legends to the tables and figures. The composition of the basic system was almost identical to the one employed by Carter et al. (9), except that the total volume was raised to 3.4 ml, and the molar concentration of L-[U-14C]valine was increased about 100-fold. The system of Carter et al. (9) contained approximately 2 μM L-[U-14C]valine added at an initial specific activity greater than 200 mCi per mmole. As noted by them and confirmed by measurements in this laboratory, this low concentration contributed less than the endogenous pool of free unlabeled L-valine added with the tissue fractions. Under such conditions the specific activity of the precursor [14C]valine in the reaction mixture increased its progressive decline during the incubation which was responsible for nonlinear kinetics. Furthermore, the declines in the specific activities were not always equivalent in the control and thyroxine-containing flasks, and spurious effects of thyroxine on the incorporation of [14C]valine were observed which were attributable not to effects on protein synthesis but to changes in precursor specific activity. In order to fix the specific activity of the precursor [14C]valine to avoid these spurious effects, we increased the addition of L-[U-14C]valine approximately 100-fold to 0.2 mm, mainly by adding unlabeled L-valine and diluting the specific activity of the original [14C]valine from greater than 200 to approximately 6 mCi per mmole. This change resulted in a more linear time course and a higher rate of total valine incorporation into protein. The effect of thyroxine on 14C incorporation into protein remained, and its percent effect was, in fact, increased.

The reactions were initiated with the addition of the amino acid and terminated by precipitation of the protein with 0.1 ml of 70% perchloric acid added to the reaction mixture. Zero time controls in which the reaction was terminated immediately after the addition of the [14C]valine were included in all experiments.

Purification and Assay of Specific Activity of Protein—The precipitated protein was purified by the method of Siewekeins (10), plated on tared, aluminum-ringed filter paper discs, reweighed to obtain the weight of the protein sample, and assayed for radioactivity in a Tracerlab Omni-Guard thin window, gas-flow Geiger-Mueller counter with an efficiency of about 20%. Sufficient counts were collected to achieve a coefficient of variation of less than 2%. All counting rates were corrected for background, self-absorption, and zero time controls.

Chromatographic Analysis of 14C-labeled Protein—Purified 14C-labeled protein samples which had been plated and assayed for specific activity were scraped off the filter papers, and samples of approximately 1 mg were transferred to glass vials. One milliliter of 5 N HCl (constant boiling mixture) was added to each vial, and the vials were flushed with nitrogen, washed, and dried. The protein was then hydrolyzed by heating the vials at 110° for 19 hours. The hydrolysate was evaporated to dryness in vacuo and redissolved in 0.5 ml of water. Portions of the solutions of the hydrolysates were spotted in 1-inch streaks on Whatman No. 31 E/T filter paper, and the chromatograms were developed by descending chromatography in either butanol-acetic acid-water (4:1:1, v/v/v) or 80% aqueous phenol. Paired samples of protein hydrolysate from control and thyroxine-containing flasks, samples of the precursor L-[U-14C]valine solution used in the experiment, and an unlabeled L-valine standard were chromatographed in parallel on the same chromatographic sheet. The developed chromatograms were cut into strips and scanned for radioactivity in a Packard model 7201 radiochromatogram scanner; the standard was stained with ninhydrin to identify the position of L-valine.

Miscellaneous Procedures—Protein contents of the cell fractions were measured by the method of Lowry et al. (11) with bovine serum albumin as the standard.

RESULTS

In addition to the absence of mitochondria, Carter et al. (9) introduced three modifications into the system used by Sokoloff and Kaufman (2, 3); an increased Mg2+ concentration, a reduced concentration of microsomes, and the use of low molar concentrations of L-[U-14C]valine of high specific activity rather than saturating concentrations of carboxyl-labeled amino acid of low specific activity or of tRNA-14C amino acid. The influence of each of these modifications was examined individually. The effects of the difference in 14C valine concentrations are discussed under "Methods"; it was found not to be responsible for the different results obtained by the two groups.

Influence of Mg2+ Concentration—The system of Sokoloff and Kaufman (2) contained 3 mM Mg2+ which was suboptimal for the rate of protein synthesis but optimal for the mitochondria-dependent, thyroxine stimulation of microsomal protein synthesis which they were studying; higher concentrations of Mg2+ inhibited this effect (12). At this Mg2+ concentration they observed no thyroxine stimulation of protein synthesis in the absence of mitochondria (2, 3, 6, 7). Carter et al. (9) also found little if any effects of thyroxine in their system with 3 mM Mg2+ and required 5.9 mM or higher to observe significant effects. The results in Fig. 1 confirm that in their system both the rate of incorporation into protein and the magnitude of the thyroxine effect increase with higher Mg2+ concentrations but establish that the Mg2+ concentration is not the critical variable responsible for the difference in the results obtained with the two systems. Even with Mg2+ concentrations as low or lower than
0.2 ml of 0.01 concentration was 6.5 °C was 30 min. That used by Sokoloff and Kaufman (2) thyroxine effects, all presented no data concentration used by Eokoloff and Kaufman (2, 3, 7). Hey and standardized at a microsomal concentration of about 0.5 mg results as microsomal protein specific activity, a quantity calculated in the absence of added microsomes and apparently assumed no the reaction mixture and the per cent stimulation by thyroxine C Carter et al. (9) contained 5.9 mM Mg++, this concentration was used in all subsequent experiments in the present studies. Inasmuch as the standard system of Carter et al. (9) contained 5.9 mM Mg++, this concentration was used in all subsequent experiments in the present studies.

Influence of Microsomal Concentration—Carter et al. (9) noted an inverse relationship between the content of microsomes in the reaction mixture and the per cent stimulation by thyroxine and standardized at a microsomal concentration of about 0.5 mg of microsomal protein per ml, approximately one-third of the concentration used by Sokoloff and Kaufman (2, 3, 7). They presented no data on ¹⁴C incorporation into soluble protein in the absence of added microsomes and apparently assumed no labeling of soluble protein inasmuch as they reported their results as microsomal protein specific activity, a quantity calculated by dividing the incorporation into total protein by the microsomal protein content per flask. The influence of microsomal concentration was, therefore, examined (Fig. 2). No assumption was made as to the degree of labeling of cell sap or microsomal protein, and incorporation rates were compared on the basis of total incorporation into total protein per flask. The incorporation rates were directly proportional to the microsomal content, both in the absence and presence of thyroxine, but there was significant incorporation of radioactivity into protein in the complete absence of microsomes (Fig. 2). Thyroxine enhanced the incorporation across the entire range of microsomal concentrations from zero to the maximum added. The absolute difference caused by thyroxine was essentially the same at all concentrations of microsomes; the per cent stimulation by thyroxine, therefore, increased with decreasing microsomal concentration and was, in fact, maximal in the complete absence of microsomes. The incorporation into protein in the absence of added microsomes did not appear to reflect contamination of the cell sap by microsomal or ribosomal elements; similar results were obtained with cell sap prepared by centrifugation at 100,000 × g for 1 hour or 269,000 × g for 1 to 2 hours. The fractional part of the total ¹⁴C incorporation into protein per flask corresponded directly to the ¹⁴C incorporation into protein from L-[U-¹⁴C]valine and the microsomal concentration. With 0.5 mg of microsomal protein per ml, the standard concentration used by Carter et al. (9), labeling of cell sap protein represented 10 to 40% of total incorporation per flask.

These results demonstrate that the thyroxine effect reported by Carter et al. (9) is not dependent on the presence of microsomes and suggest that it does not represent an effect on protein synthesis. Indeed, enhancing the rate of protein synthesis by increasing the concentration of microsomes tends to dilute it out. The use of at least 3-fold higher concentrations of microsomes in their reaction mixture is probably one of the reasons why Sokoloff and Kaufman (2) failed to observe this effect of thyroxine in their system.

Lack of Energy Requirement for Thyroxine Effect—Carter et al. (9) observed the greatest thyroxine effects in their system when the sole energy source was the initial amount of ATP without any ATP-regenerating system included in the reaction mixture. Under these conditions the ATP concentration was rapidly depleted, and the amount of amino acid incorporation into protein was very much lower than that observed in the complete system (9). In view of the preceding evidence that the thyroxine effect was not on protein synthesis, the possibility was considered that the thyroxine effect was not on an energy-dependent process and that a deficiency in the energy source enhanced the per cent effect by inhibiting thyroxine-insensitive but energy-dependent ¹⁴C incorporation due to protein synthesis. The results in Table I show that this is indeed the case. Omission of either microsomes or the ATP-generating system (i.e. creatine phosphate, creatine kinase, and adenine nucleotide) reduced ¹⁴C incorporation into protein to the same degree, and omission of both reduced incorporation even further. The absolute increase pro
The composition of the complete system was the same as that in the experiment in Fig. 1, except that the MgCl₂ concentration was 5.9 mM, and the contents of cell sap and microsomal protein were 2.8 and 2.4 mg per flask, respectively. When microsomes were omitted, the microsomal addition was replaced by an equal volume of 0.25 M sucrose. The ATP-generating system was deleted by omission of creatine phosphate, creatine kinase, AMP, and Mg²⁺ and replaced by an equivalent amount of water. The thyroxine concentration was 0.5 × 10⁻⁴ M. Incubation time at 37° was 30 min.

### Table I

<table>
<thead>
<tr>
<th>Reaction system</th>
<th>[¹⁴C] incorporation into total flask protein</th>
<th>Thyroxine effect</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>cpm/flask</td>
<td>Per cent</td>
</tr>
<tr>
<td>Control</td>
<td>1852</td>
<td>+213 +12</td>
</tr>
<tr>
<td>Minus microsomes</td>
<td>775</td>
<td>+173 +22</td>
</tr>
<tr>
<td>Minus ATP-generating system</td>
<td>782</td>
<td>+175 +22</td>
</tr>
<tr>
<td>Minus microsomes, minus ATP-</td>
<td></td>
<td></td>
</tr>
<tr>
<td>generating system</td>
<td>300</td>
<td>+166 +54</td>
</tr>
</tbody>
</table>

Produced by thyroxine, however, remained relatively unaffected, and the per cent stimulation was enhanced as the base-line incorporation was reduced. These results demonstrate that the thyroxine effect on [¹⁴C] incorporation into protein in the system of Carter et al. (9) does not involve any energy-dependent process and is magnified when protein synthesis is suppressed by removal of microsomes, deletion of the energy source, or both.

**Lack of Thyroxine Effect with (tRNA-L-[¹⁴C]Valine)—**

The stimulation of protein synthesis by thyroxine in vitro described by Sokoloff and Kaufman (2, 3) exhibited an absolute dependence on the presence of mitochondria in the reaction medium and occurred with either free or tRNA-bound ¹⁴C-amino acids. The thyroxine effect described by Carter et al. (9) in the absence of mitochondria was observed only with free and not with tRNA-bound L-[¹⁴C]Valine, even in the presence of a complete protein-synthesizing system (Table II). When the microsomes and energy source were omitted from the reaction mixture, the thyroxine stimulation of [¹⁴C] incorporation into protein from the free L-[¹⁴C]Valine was enhanced, but essentially no incorporation of radioactivity into protein from tRNA-[¹⁴C]Valine was observed. These results also indicate that the thyroxine effect described by Carter et al. (9) is not on protein synthesis.

**Effects of Inhibitors of Protein Synthesis—**
The addition of inhibitors of microsomal protein synthesis, puromycin, cycloheximide, or ribonuclease, had essentially the same effects as reducing the microsomal concentration (Table III). In the presence of microsomes these agents reduced [¹⁴C] incorporation into protein, but the absolute change due to thyroxine was not affected, and the per cent stimulation by thyroxine was, therefore, enhanced. In the absence of microsomes the inhibitors had negligible effects on both [¹⁴C] incorporation into protein and the magnitude of the thyroxine effect. Indeed, the absolute change produced by thyroxine remained essentially the same whether or not microsomes and/or the inhibitors of protein synthesis were present. These results add further evidence that the thyroxine stimulation of [¹⁴C] incorporation in the system of Carter et al. (9) is not an effect on protein synthesis; the effect tends rather to be obscured by the presence of protein synthesis and is unmasked or enhanced by inhibition or elimination of any protein synthesis occurring in the reaction mixture.

**Chemical Nature of Thyroxine-stimulated [¹⁴C] Incorporated into Protein—**
The findings that the thyroxine effect exhibited none of the dependencies of protein synthesis and was unaffected or even enhanced by conditions which inhibited protein synthesis raised the question whether it was the incorporation of the [¹⁴C]-amino acid that was stimulated. Results of experiments of the type illustrated in Fig. 3 indicated that it was not. Addition of unlabeled L-valine to dilute the specific activity of the precursor L-[¹⁴C]Valine as much as 20-fold had little if any effect on the incorporation of [¹⁴C] into protein in the absence of microsomes. In the presence of microsomes, [¹⁴C] incorporation was inhibited almost down to levels observed in the absence of microsomes. The absolute increases in incorporation produced by thyroxine were only slightly if at all affected both in the presence and absence of microsomes, and the per cent stimulation in the presence of microsomes was, therefore, enhanced by the dilution with L-valine almost to the magnitude observed in the absence of microsomes. The inhibition of [¹⁴C] incorporation in the presence of microsomes, which reflects the dilution of L-[¹⁴C]Valine incorporation due to protein synthesis, without a proportionate reduction in the change produced by thyroxine, indicates that the thyroxine effect is not on protein synthesis. The fact that dilution with L-valine did not reduce the base-line incorporation of [¹⁴C] or the change caused by thyroxine in the absence of microsomes further suggests that the thyroxine effect is not on L-[¹⁴C]Valine incorporation.

### Table II

<table>
<thead>
<tr>
<th>Amino acid source</th>
<th>Reaction system</th>
<th>[¹⁴C] incorporation into total flask protein</th>
<th>Thyroxine effect</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>cpm/flask</td>
<td>Per cent</td>
<td></td>
</tr>
<tr>
<td>L-[¹⁴C]Valine</td>
<td>Complete</td>
<td>966</td>
<td>+94 +10</td>
</tr>
<tr>
<td></td>
<td>Minus microsomes, minus ATP-</td>
<td>195</td>
<td>-100 +64</td>
</tr>
<tr>
<td></td>
<td>generating system</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>tRNA-L-[¹⁴C]Valine</td>
<td>483</td>
<td>+16 +3</td>
</tr>
<tr>
<td></td>
<td>Complete</td>
<td>448</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Minus microsomes, minus ATP-</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>generating system</td>
<td>0*</td>
<td>0</td>
</tr>
</tbody>
</table>

* Less than 1 cpm difference from zero time controls which were 1 to 2 cpm per mg of protein.
paper chromatographic analyses of acid hydrolysates of labeled into protein which is stimulated by thyroxine was obtained by protein in the presence and absence of microsomes. When no respect. When microsomes were omitted, an equal volume of cell sap protein (Fig. 4). The scans for radioactivity revealed of cell sap and microsomal protein were 3.2 mg and 2.7 mg per flask, except that the MgCl\textsubscript{2} concentration was 5.9 mM, and the contents

Influence of Species of \textsuperscript{14}C VaZine on Incorporation of \textsuperscript{14}C into Cell Sap Protein and on Thyroxine Effect—The energy-independent incorporation of \textsuperscript{14}C into cell sap protein and the magnitude of the thyroxine effects varied considerably with the batch of L-[\textsuperscript{U-14}C]valine. More than 90% reductions in base-line incorporation of radioactivity into cell sap protein and a marked attenuation of the thyroxine effect (Table IV).

Final confirmation that it is not L-[\textsuperscript{U-14}C]valine incorporation into protein which is stimulated by thyroxine was obtained by paper chromatographic analyses of acid hydrolysates of labeled cell sap protein (Fig. 4). The scans for radioactivity revealed low levels of radioactivity distributed diffusely throughout the chromatograms of the hydrolysates of control and experimental samples but no peaks at the positions of L-valine (Fig. 4). There were no discrete peaks in the chromatograms of the control samples, but in the thyroxine-treated samples there was one peak of radioactivity in one solvent system and three in the other corresponding to far slower moving materials than L-valine. Corresponding peaks were observed in the chromatograms of the solution of L-[\textsuperscript{U-14}C]valine added to the reaction mixtures (Fig. 4). More than 99% of the radioactivity in the L-[\textsuperscript{U-14}C]valine solution was confined to the L-valine peak, but there was clear evidence of a variety of radioactive contaminants. Although small in comparison with that of the L-[\textsuperscript{U-14}C]valine, the peaks corresponding to those seen in the protein hydrolysates from the thyroxine-containing flasks were more than sufficient to account for all of the relatively small amount of \textsuperscript{14}C incorporated into the protein during incubation.

Purification of the L-[\textsuperscript{U-14}C]valine by a modification of the procedure of Hirs et al. (13, 14) prior to use resulted in greater than 90% reductions in base-line incorporation of radioactivity into cell sap protein and a marked attenuation of the thyroxine effect (Table IV).

These results demonstrate that the thyroxine effect reported by Carter et al. (9) is not on the incorporation of the L-[\textsuperscript{U-14}C]valine into protein but of a radioactive contaminant in the \textsuperscript{14}C-amino acid solution.

In Table III, the composition of the complete system was the same as that in the experiment in Fig. 1, except that the MgCl\textsubscript{2} concentration was 5.9 mM, and the contents of cell sap and microsomal protein per flask were as follows: A, 4.6 and 1.5 mg, respectively; B, 2.5 and 2.6 mg, respectively; C, 3.0 and 0.8 mg, respectively. When the microsomes were deleted, they were replaced by an equal volume of 0.25 M sucrose. The concentrations of the inhibitors were as indicated. The thyroxine concentration was 6.5 \times 10^{-5} M. Incubation time at 37° was 30 min.
Fig. 4. Paper chromatographic analysis of cell sap protein labeled with 14C by incubation with L-[U-14C]valine in presence and absence of 6.5 \times 10^{-4} \text{ M} L\text{-thyr}oxine. The incubation conditions were the same as those in the experiment in Fig. 1, except that the MgCl₂ concentration was 5.9 mM, the microsomes were omitted and replaced by an equal volume of 0.25 M sucrose, the ATP-generating system was deleted by omission of creatine phosphate, creatine kinase, and AMP and replaced by an equivalent volume of water, the specific activity of the added L-[U-14C]valine was 17.2 mCi per mmole, and the content of cell sap protein was 4.8 mg per flask. Incubation time at 37° was 30 min. The labeled protein was precipitated, purified, plated, and assayed for specific activity, and a thyroxine stimulation of 71% was confirmed. The plated protein was then scraped off the filter paper planchet, hydrolyzed, chromatographed on Whatman No. 31 E/T paper, and scanned for radioactivity as described under “Methods.” The solvent systems were: A, 1-butanol–acetic acid–water (4:1:1, v/v/v); B, 80% aqueous phenol.

TABLE IV  

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>Protein specific activity</th>
<th>Thyroxine effect</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>+T-Thyroxine</td>
<td>Δcpm/mg protein</td>
</tr>
<tr>
<td>Untreated L-[U-14C]valine</td>
<td>99</td>
<td>27</td>
</tr>
<tr>
<td>Dowex-treated L-[U-14C]valine</td>
<td>5</td>
<td>7</td>
</tr>
</tbody>
</table>

small fractions of those observed with L-[U-14C]valine (Table V). Similar effects are observed with other species of 14C-amino acids, such as [14C]leucine and [14C]cystine.

The very low, almost negligible rates of incorporation of 14C into cell sap protein observed with L-[1-14C]valine explain in part why this effect of thyroxine was insignificant and undetectable in the system of Sokoloff and Kaufman (2). They used carboxyl-labeled or tRNA-bound 14C-amino acids, as well as higher concentrations of microsomes than Carter et al. (9).

DISCUSSION

The present studies confirm the report of Carter et al. (9) that thyroxine in vitro stimulates the incorporation of 14C from [14C]-
amino acid solutions into protein in cell-free liver systems lacking mitochondria but demonstrate that, contrary to their conclusion, the thyroxine effect is not on protein synthesis. The thyroxine effect is, in fact, obscured by U-14C-amino acid incorporation due to protein synthesis and is unmasked and magnified by conditions which minimize labeling of protein due to protein synthesis. Removal of microsomes and/or ATP or the addition of protein synthesis inhibitors in the reaction mixture, all procedures which block protein synthesis, have no effects on the absolute change in U-14C incorporation into protein produced by thyroxine and increase the per cent stimulation by eliminating the contribution of protein synthesis to the base line rate of U-14C incorporation. Indeed, the maximum thyroxine effects are seen in reaction mixtures which contain only the 269,000 x g supernatant fraction of liver homogenates and no source of ATP. The thyroxine effect does not even appear to be on the incorporation of U-14C-amino acid. It cannot be diluted out by dilution of the precursor U-14C-amino acid with unlabeled amino acid of the same species, and chromatographic analysis of hydrolysates of the labeled cell sap protein, which contains essentially all of the thyroxine-stimulated U-14C incorporation, demonstrates that thyroxine enhances the incorporation not of the U-14C-amino acid but of a radioactive contaminant present in the U-14C-amino acid preparation.

The question arises as to whether there is any relationship between this effect first observed by Carter et al. (9) and the mitochondria-dependent stimulation of microsomal protein synthesis previously reported by Sokoloff and Kaufman (2). Comparison of the properties of the two effects indicates that there is none. In contrast to the effect of Carter et al. (9), the effect of Sokoloff and Kaufman (2) is absolutely dependent on the presence of mitochondria in the reaction mixture (2, 3, 6-8); it is dependent on the presence of an ATP-generating system (2, 8); it is dependent on Mg2+ but is inhibited at higher concentrations of Mg2+ (12); it is blocked by inhibitors of microsomal protein synthesis and is ribonuclease-sensitive (7); it is observed with tRNA-U-14C-amino acid as the amino acid source and has, in fact, been localized to the transfer of tRNA-bound amino acid to microsomal protein (3); and it has been demonstrated by analysis of the labeled protein to represent a stimulation of the incorporation of the precursor U-14C-amino acid into peptide linkage in the interior of the polypeptide chain (2, 6). Finally, it should be emphasized that in their reaction system Sokoloff and Kaufman (2) did not observe any thyroxine stimulation of U-14C incorporation into protein in the absence of mitochondria like that described by Carter et al. (9).

The effect studied by Carter et al. (9) is, therefore, qualitatively different. The focus on a different effect is the result of the ostensibly minimal but actually major changes introduced into the reaction system by them. The key changes were the reduction in the content of microsomes and the use of uniformly labeled U-[14C]valine of high specific activity. The lowering of the microsomal protein content results in both a reduced rate of protein synthesis and a reduced proportion of microsomal to cell sap protein in the final labeled protein assayed for specific activity. Of all the species of [14C]valine studied, L-[U-14C]valine is associated by far a tiny fraction of the added radioactivity is recovered with mitochondria in the reaction mixture (2, 3, 6-8); it is dependent on Mg2+ but is inhibited at higher concentrations of Mg2+ (12); it is blocked by inhibitors of microsomal protein synthesis and is ribonuclease-sensitive (7); it is observed with tRNA-U-14C-amino acid as the amino acid source and has, in fact, been localized to the transfer of tRNA-bound amino acid to microsomal protein (3); and it has been demonstrated by analysis of the labeled protein to represent a stimulation of the incorporation of the precursor U-14C-amino acid into peptide linkage in the interior of the polypeptide chain (2, 6). Finally, it should be emphasized that in their reaction system Sokoloff and Kaufman (2) did not observe any thyroxine stimulation of U-14C incorporation into protein in the absence of mitochondria like that described by Carter et al. (9).

In contrast, in the system of Sokoloff and Kaufman (2) the higher content of microsomes increases the rate of true protein synthesis, and the use of carboxyl-labeled L-[14C]valine is associated with relatively little U-14C incorporation into soluble protein. Under these circumstances the rate of U-14C incorporation into protein due to protein synthesis is so predominant that the thyroxine effect of Carter et al. (9) becomes negligible and unobserved.

The results of the present studies re-emphasize a caveat which is unfortunately too often ignored in this present era of widespread use of radioisotopes in studies of biochemical processes. The ease of measurement which radioisotopic techniques provide does not obviate the need to establish, often by laborious means, that the radioactive reactants and products are indeed those of the chemical reactions thought to be under study. There is particular danger in experiments with slow chemical reactions in which only a tiny fraction of the added radioactivity is recovered in the radioactive products. The danger is amplified by the tendency in such circumstances to enhance the sensitivity of the measurements by the use of uniformly labeled substrates of the highest specific activity available. These compounds are most likely to contain traces of radioactive contaminants which can lead to high blanks and spurious radiochemical interconversions and thus obscure and confound the reaction under study (15). Such problems have been previously noted with radioactive amino acids in studies of protein synthesis (14, 16), but they exist as well with other classes of radioactive substrates and chemical processes (15). Proper awareness of this danger and experimental design to control for it may prevent misinterpretations of the type clarified by the present studies.

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