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 on the presence of mitochondria in the system, which was observed with L-[U-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 [U-14C]valine with unlabeled L-valine. Chromatographic analysis of hydrolysates of the labeled protein reveals that the thyroxine stimulation is on the incorporation of the 14C-amino acid but of a radio-active 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 (9, 3, 8). Extensive efforts to find a direct effect of thyroxine 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.
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 L-thyroxine was obtained from Sigma Chemical Co., N,N-Diisopentylamine from Sigma Chemical Co., and the Biochemicals Corp., and Calbiochem; in some experiments a mixture of all three preparations which had been recrystallized three times from hot ethanol solutions was used with similar results. L-[U-14C]Valine (specific activity 210, 225, and 248 mCi per mmole), L-[1-14C]valine (specific activity 25.4 and 27.7 mCi per mmole), and L-[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 L- or D-valine purchased from Calbiochem and Sigma Chemical Co., respectively. Cylclobeximide and beef pancreatic ribonuclease A were also obtained from Sigma Chemical Co. Purinomycin hydrochloride was purchased from Nutritional Biochemicals Corp. tRNA-L-[U-14C]valine was synthesized as previously described (3), except that the tRNA was yeast tRNA purchased from Miles Laboratories, Inc., the 100,000 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-[U-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 of fresh liver weight were prepared as previously described (2). The homogenates were centrifuged for 15 min at 12,000 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 g for 2 hours in a Beckman L2-65B ultracentrifuge to separate the microsomes and cell sap. The cell sap was decanted and stored in ice until use. The microsomal pellet was resuspended with 0.25 m sucrose and then resuspended by hand homogenization with an all-glass Potter-Elvehjem homogenizer in 3 ml of 0.25 m sucrose per gram 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—The reactions were initiated with the addition of the amino acid mixture marked by guest on November 11, 2017 http://www.jbc.org/ Downloaded from

This change resulted in a more linear time course and a higher rate of total valine incorporation into protein. The effect of thyroxine on incorporation into protein remained, and its per cent 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 Siekevits (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 8 N HCl (constant boiling mixture) was added to each vial, and the vials were flushed with nitrogen, sealed, and heated on a sand bath for 2 hours in a Beckman L2-65B ultracentrifuge to separate the microsomes, and cell sap. The cell sap was decanted and stored in ice until use. The microsomal pellet was resuspended with 0.25 m sucrose and then resuspended by hand homogenization with an all-glass Potter-Elvehjem homogenizer in 3 ml of 0.25 m sucrose per gram 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.

Influence of Mg++ Concentration—The system of Pokoloff and Kaufman (2) contained 3 mM Mg++ which was suboptimal for the mitochondrial-dependent, thyroxine stimulation of microsomal protein synthesis which they were studying; higher concentrations of Mg++ inhibited this effect (12). At this Mg++ concentration they observed no thyroid 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 Mg++ 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 Mg++ concentrations but establish that the Mg++ concentration is not the critical variable responsible for the difference in the results obtained with the two systems. Even with Mg++ concentrations as low as or lower than
that used by Sokoloff and Kaufman (2) thyroid effects, although reduced, were still apparent in the system of Carter et al. (9). Inasmuch as the standard system of Carter et al. (9) contained 5.9 mM Mg^{2+}, 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 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 increase 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 incorporated directly into cell sap protein varied with the batch of 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-
Lack of requirement of microsomes and energy source for thyroxine stimulation of \(^{14}C\) incorporation into protein from \(L-[U-^{14}C]\)valine solution

The composition of the complete system was the same as that in the experiment in Fig. 1, except that the MgCl\(_2\) 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 Mg\(^{2+}\) and replaced by an equivalent amount of water. The thyroxine concentration was 6.5 \(\times\) 10\(^{-4}\) M. Incubation time at 37\(^\circ\) was 90 min.

**Table I**

<table>
<thead>
<tr>
<th>Reaction system</th>
<th>[^{14}C] incorporation into total flask protein</th>
<th>Thyroxine effect</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Cpm/mg/protein</td>
<td>Thyroxine effect</td>
</tr>
<tr>
<td></td>
<td>Control</td>
<td>+Thyroxine</td>
</tr>
<tr>
<td>Complete</td>
<td>1852</td>
<td>2065</td>
</tr>
<tr>
<td>Minus microsomes</td>
<td>775</td>
<td>948</td>
</tr>
<tr>
<td>Minus ATP-generating system</td>
<td>782</td>
<td>907</td>
</tr>
<tr>
<td>Minus microsomes, minus ATP-</td>
<td>300</td>
<td>475</td>
</tr>
<tr>
<td>generating system</td>
<td></td>
<td></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 \(^{14}C\) 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-[U-\(^{14}C\)]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 \(^{14}C\)-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 \(L-[U-^{14}C]\)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 \(^{14}C\) incorporation into protein from the free \(L-[U-^{14}C]\)valine was enhanced, but essentially no incorporation of radioactivity into protein from tRNA-\(L-[U-^{14}C]\)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 \(^{14}C\) 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 \(^{14}C\) 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 \(^{14}C\) 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 but is unmasked or enhanced by inhibition or elimination of any protein synthesis occurring in the reaction mixture.

**Chemical Nature of Thyroxine-stimulated \(^{14}C\) 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 \(^{14}C\)-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 \(L-[U-^{14}C]\)valine as much as 20-fold had little if any effect on the incorporation of \(^{14}C\) into protein in the absence of microsomes. In the presence of microsomes \(^{14}C\) incorporation was inhibited almost down to levels observed in the absence of microsomes. The absolute increase 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 \(^{14}C\) incorporation in the presence of microsomes, which reflects the dilution of \(L-[U-^{14}C]\)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 \(^{14}C\) or the change caused by thyroxine in the absence of microsomes further suggests that the thyroxine effect is not on \(L-[U-^{14}C]\)valine incorporation.
Table III

Effects of inhibitors of protein synthesis on thyroxine stimulation of 14C incorporation into protein from L-[U-14C]valine

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 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 × 10^-5 M. Incubation time at 37° was 30 min.

<table>
<thead>
<tr>
<th>Exper. Reaction System</th>
<th>Inhibitor</th>
<th>14C Incorporation into Total Flask Protein</th>
<th>Thyroxine Effect</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Control</td>
<td>+Thyroxine</td>
</tr>
<tr>
<td></td>
<td></td>
<td>cpm/flask</td>
<td></td>
</tr>
<tr>
<td>A</td>
<td>Complete</td>
<td>None</td>
<td>1057</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.5 mM Cycloheximide</td>
<td>663</td>
</tr>
<tr>
<td></td>
<td>Minus microsomes</td>
<td>None</td>
<td>341</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.5 mM Cycloheximide</td>
<td>416</td>
</tr>
<tr>
<td>B</td>
<td>Complete</td>
<td>None</td>
<td>679</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.3 mM Puromycin</td>
<td>358</td>
</tr>
<tr>
<td></td>
<td>Minus microsomes</td>
<td>None</td>
<td>132</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.3 mM Puromycin</td>
<td>95</td>
</tr>
<tr>
<td>C</td>
<td>Complete</td>
<td>None</td>
<td>1501</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Ribonuclease A (59 μg/ml)</td>
<td>667</td>
</tr>
<tr>
<td></td>
<td>Minus microsomes</td>
<td>None</td>
<td>217</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Ribonuclease A (59 μg/ml)</td>
<td>235</td>
</tr>
</tbody>
</table>

Fig. 3. Effects of dilution of the L-[U-14C]valine with unlabeled L-valine on the thyroxine stimulation of 14C incorporation into protein in the presence and absence of microsomes. When no unlabeled L-valine was added, the assay conditions for the complete system were the same as those 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 3.2 mg and 2.7 mg per flask, respectively. When microsomes were omitted, an equal volume of 0.25 M sucrose was added for replacement. L-[U-14C]Valine (specific activity 6.4 mCi per mmole) was added to a final concentration of 0.22 mM. When added, the quantities of unlabeled L-[U-14C]valine to either 0.59 or 0.31 mCi per mmole. Incubation time at 37° was 30 min.

Final confirmation that it is not L-[U-14C]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-[U-14C]valine added to the reaction mixtures (Fig. 4). More than 99% of the radioactivity in the L-[U-14C]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-[U-14C]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 14C incorporated into the protein during incubation.

Purification of the L-[U-14C]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-[U-14C]valine into protein but of a radioactive contaminant in the 14C-amino acid solution.

Influence of Species of [14C]Valine on Incorporation of 14C into Cell Sap Protein and on Thyroxine Effect—The energy-independent incorporation of 14C into cell sap protein and the magnitude of the thyroxine effects varied considerably with the batch of L-[U-14C]Valine. Some 14C incorporation and detectable thyroxine effects were also observed with both the L. and D. isomers of carboxyl-labeled [14C]valine, but when compared at concentrations and specific activities adjusted to be equal, these were only
Fig. 4. Paper chromatographic analysis of cell sap protein labeled with $^{14}$C by incubation with L-[U-$^{14}$C]valine in presence and absence of $6.5 \times 10^{-4}$ M L-thyroxine. The incubation conditions were the same as those in the experiment in Fig. 1, except that the MgCl$_2$ 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-$^{14}$C]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 planchets, 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

Effects of purification of L-[U-$^{14}$C]valine preparation by ion exchange chromatography on the thyroxine stimulation of $^{14}$C incorporation into cell sap protein

The assay conditions were the same as those in the experiment in Fig. 1, except that the MgCl$_2$ 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 the creatine phosphate, creatine kinase, and AMP and replaced by an equivalent volume of water, and the cell sap protein content was 4.4 mg per flask. The untreated L-[U-$^{14}$C]valine had a specific activity of 6.4 mCi per mmole and was added to the reaction mixture to a final concentration of 0.22 mM. A portion of the L-[U-$^{14}$C]valine solution was purified on Dowex-1 and Dowex-50 by the method of Hirs et al. (13), adjusted to the same concentration of radioactivity as in the untreated solution of L-[U-$^{14}$C]valine, and added to the equivalent final concentration in flasks assayed simultaneously with those which received the unpurified L-[U-$^{14}$C]valine. The thyroxine concentration was $6.5 \times 10^{-4}$ M. Incubation time at 37° was 30 min.

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>Protein specific activity</th>
<th>Thymoxine effect</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control +L-Thyroxine</td>
<td>Δcpm/mg protein</td>
</tr>
<tr>
<td>Untreated L-[U-$^{14}$C]valine</td>
<td>98</td>
<td>+68</td>
</tr>
<tr>
<td>Dowex-treated L-[U-$^{14}$C]valine</td>
<td>5</td>
<td>+2</td>
</tr>
</tbody>
</table>

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

The very low, almost negligible rates of incorporation of $^{14}$C into cell sap protein observed with L-[1-$^{14}$C]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 $^{14}$C-amino acids, as well as higher concentrations of microsomes than Carter et al. (9).

### Table V

Comparative effects of thyroxine on $^{14}$C incorporation into cell sap protein from various species of $^{14}$C-labeled valine

The assay conditions were the same as those described in Table IV. The original specific activities of the $^{14}$C-amino acid preparations used in these experiments were as follows: L-[U-$^{14}$C]valine, 248 mCi per mmole; L-[l-$^{14}$C]valine, 25 to 28 mCi per mmole; and d-[l-$^{14}$C]valine, 18 mCi per mmole. All were diluted with unlabeled valine to the same specific activity of 6.4 mCi per mmole and added to the reaction mixtures to a final $[^{14}$C]valine concentration of 0.22 mM. The numbers in parentheses represent the number of experiments in which at least two of the species of labeled amino acids were assayed in parallel flasks. The thyroxine concentration was $6.5 \times 10^{-4}$ M. The values presented are the means ± standard errors of the number of experiments indicated. The mean ± standard error of the content of cell sap protein per flask was 4.5 ± 0.1 mg. Per cent represents the mean of the individual per cent effects, not the per cent difference between the means.

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>Protein specific activity</th>
<th>Thyroxine effect</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control +Thyroxine</td>
<td>Δcpm/mg protein</td>
</tr>
<tr>
<td>L-[U-$^{14}$C]Valine (4)</td>
<td>58 ± 6</td>
<td>110 ± 9</td>
</tr>
<tr>
<td>L-[l-$^{14}$C]Valine (6)</td>
<td>3.7 ± 0.2</td>
<td>5.1 ± 0.4</td>
</tr>
<tr>
<td>L-[d-$^{14}$C]Valine (7)</td>
<td>17 ± 1</td>
<td>21 ± 2</td>
</tr>
</tbody>
</table>

### DISCUSSION

The present studies confirm the report of Carter et al. (9) that thyroxine in vitro stimulates the incorporation of $^{14}$C from $^{14}$C-
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 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 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 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 14C-amino acid. It cannot be diluted out by dilution of the precursor 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 14C incorporation, demonstrates that thyroxine enhances the incorporation not of the 14C-amino acid but of a radioactive contaminant present in the 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-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 14C-amino acid into peptide linkage in the interior of the polypeptide chain (2, 9). Finally, it should be emphasized that in their reaction system Sokoloff and Kaufman (2) did not observe any thyroxine stimulation of 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 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 with the highest rates of artificial 14C incorporation into soluble protein. The representation of protein synthesis in the 14C incorporation is, therefore, reduced, and the artificial incorporation is magnified and becomes apparent. 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 14C incorporation into soluble protein. Under these circumstances the rate of 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 a higher 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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REFERENCES
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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