Does Leucine, Leucyl-tRNA, or Some Metabolite of Leucine Regulate Protein Synthesis and Degradation in Skeletal and Cardiac Muscle?*

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A simplified procedure for measuring simultaneously protein synthesis and degradation in isolated rat muscles was used to study whether degradation of leucine is necessary for the stimulation of protein synthesis and the inhibition of proteolysis induced by leucine. In diaphragms, 0.1 mM leucine stimulated protein synthesis significantly without reducing protein degradation. Between 0.2 to 0.5 mM, leucine progressively lowered proteolysis by a greater absolute amount than it stimulated protein synthesis. Over this concentration range, the intracellular content of leucine was proportional to the concentration in the medium. Thus, physiological variations in the blood leucine levels may regulate protein balance in vivo. Unlike leucine, ketone bodies or lactate did not affect protein synthesis or degradation in skeletal muscle, although they do so in cardiac muscle. These effects of leucine were observed in muscles from fed or fasted rats, unlike the inhibition by leucine of glucose oxidation which is specific to the fasted state.

To determine whether leucine catabolism is essential for its effect on protein balance, diaphragm and atrial muscles were incubated with L-cycloserine, which inhibited reversibly the transamination of leucine. Cycloserine prevented the inhibition of proteolysis by leucine, but not that induced with insulin and glucagon. Higher concentrations of leucine overcame this inhibition by cycloserine. In contrast, cycloserine did not interfere with the ability of leucine to stimulate protein synthesis. α-Ketosocaproic acid (0.5 mM), the product of leucine transamination, reduced proteolysis without stimulating protein synthesis. Therefore, some intermediate(s) or product(s) of leucine catabolism inhibits proteolysis, but muscle protein synthesis must be regulated by the intracellular level of leucine or leucyl-tRNA.

The Km for leucine of leucyl-tRNA synthetase was measured in muscle extracts and was found to be only 6 μM, which is well below the intracellular concentration of leucine (100 to 500 μM) under all incubation conditions. In addition, the charging of muscle leucyl- and phenylalanyl-tRNA showed similar behavior, although only leucine affects protein balance. Thus, the stimulation of protein synthesis, and inhibition of proteolysis, by exogenous leucine is probably not caused by increased levels of leucyl-tRNA.

In both animal and bacterial cells, exogenous amino acids appear to promote protein synthesis and inhibit protein breakdown (1). Supplying amino acids to perfused liver (2, 3), perfused heart (4, 5), or incubated skeletal muscle (6) retards the net breakdown of tissue proteins. In skeletal and cardiac muscle, not all plasma amino acids are required for these effects (6-13). The branched chain amino acids together or simply leucine alone (6, 8, 10, 13) stimulate protein synthesis and inhibit protein breakdown in isolated muscles. No other plasma amino acid shows such effects in these tissues. The mechanisms by which the branched chain amino acids exert these effects on muscle protein balance remain undefined.

The acceleration of protein synthesis by these amino acids seems to occur at the level of peptide chain initiation (4). Even less information is available concerning the mechanism by which leucine inhibits proteolysis. This area may have appreciable clinical import because of the reported therapeutic effects of branched chain amino acids and their metabolites in retarding the net loss of tissue proteins in various human diseases. For instance, administration of these amino acids or their α-keto acid derivatives to patients reduces urinary loss of nitrogen (14-16).

Unlike most amino acids, leucine, isoleucine, and valine are catabolized rapidly by skeletal muscle (12, 17). The capacity of this tissue to degrade leucine, isoleucine, and valine increases 2- to 3-fold in rat during fasting (12, 18-20) when utilization of glucose falls and when protein breakdown increases in muscle (21). In fasting, oxidation of leucine, but no other amino acids (22), may provide a significant fraction of the energy of skeletal muscle, and leucine does reduce the use of glucose by this tissue in fasting animals (23). These observations raise the possibility that the ability of leucine to influence protein synthesis and breakdown is somehow related to its rapid oxidation in skeletal and cardiac muscle. It is noteworthy that leucine has less effect on protein turnover in liver (2, 3) which does not rapidly degrade branched chain amino acids (24). Alternatively, the intracellular levels of leucine or leucyl-tRNA may regulate rates of protein turnover in muscle. In bacteria, the intracellular content of aminoacyl-tRNA does control rates of protein breakdown, as well as a number of other growth-regulated processes, including RNA and lipid synthesis (1, 25, 26). In these organisms, the lack of any tRNA species causes the accumulation of the regulator guanosine-3'-diphosphate-5'-diphosphate, which in turn stimulates protein degradation (26, 27). Recent evidence has shown in mammalian cells that levels of aminoacylated tRNA may regulate rates of protein degradation (28), although earlier reports had questioned this possibility (29). Furthermore, it has been proposed that rates of protein synthesis in mammalian...
muscle cells can be influenced by changes in the concentration of aminoacyl-tRNA or the ratio of charged to uncharged tRNA (30, 31).

To clarify how leucine regulates protein synthesis and protein degradation in muscle, we measured these processes at various concentrations of leucine and tested whether inhibition of leucine transamination might block its effect on protein turnover. In addition, it appeared important to evaluate how the intracellular content of leucine is influenced by the extracellular concentration of this amino acid and whether the concentration of leucyl-tRNA might be an important regulatory factor under the conditions studied. These studies thus investigated whether overall rates of protein synthesis and of proteolysis are regulated by the same mechanism and whether these effects may differ in skeletal and cardiac muscle.

**EXPERIMENTAL PROCEDURES**

Quarter diaphragms were dissected from male rats (70 to 90 g) obtained from Charles River Laboratories. Atria were excised from larger animals (150 g) and bisected (see Ref. 32). Fasted animals were deprived of food for 48 h prior to killing. After dissection, the tissues were incubated for 30 min and then incubated for an additional 2 h in Krebs-Ringer bicarbonate buffer containing glucose, radioactive precursors, and various additions, as indicated below (6, 17). Most incubations were terminated by removing the tissues and then heating the homogenate in boiling water for 2 min. In experiments in which [1-14C]leucine was collected (see below), the tissues were removed and 0.5 ml of 1.5 M perchloric acid was added to the medium.

The content of the acid-soluble fraction was measured after homogenizing the tissue in 5% trichloroacetic acid, centrifuging, and washing the supernatant pellet once with 3 ml of 5% trichloroacetic acid and once with 3 ml of ethanol:ether (1:1, v/v). Activity in the pellets was measured as described previously (6). For determination of acid-soluble radioactivity and leucine content, the muscles were homogenized in 3 ml of 0.2 M perchloric acid. After centrifugation, 0.4 ml of the supernatant solution was added to 4 ml of scintillation fluid and counted. After adjusting the pH of the acid-soluble fraction to 2.0 with 2.5 mmol/l NaOH, the acid-soluble fraction was precipitated with 3 ml of 5% (w/v) trichloroacetic acid. The precipitates were dissolved in 0.4 ml of Soluene-100 (Packard Instrument Co.). Radioactivity in the pellets was measured as described previously (6). Control incubations in which no RNA or no enzyme extract were added were performed in parallel. The tRNA synthetase activity was expressed as picomoles of amino acid incorporated per A260 unit per min. The K_m was obtained from linear regression analysis of Eadie-Hoffstee plots.

The rate of leucine transamination was determined in muscles isolated from rat liver or muscle and de-acylated as described by Shemyo and Rogers (38) and enriched by the isopropyl alcohol fractionation procedure of Roff et al. (39). The transamination activity was measured by the formation of radiola beled aminoacyl-tRNA. The reaction mixture contained 100 mM Tris-HCl (pH 7.4), 2 mm magnesium acetate, 2 mm diithiothreitol, 4 mm ATP, 4.5 A260 units of rat liver tRNA, and 3 to 5000 pmol of [U-14C]leucine (specific activity 200 mCi/mmol) or [U-14C]phenylalanine (specific activity 240 mCi/mmol) in a total volume of 0.55 ml. A 0.1 ml aliquot of dialyzed muscle extract was added to the reaction mixture, mixed, and incubated at 37 °C for 15 min. Aliquots (0.1 ml) were withdrawn after 3, 6, 10, and 15 min and precipitated with 3 ml of ice-cold 10% trichloroacetic acid. The precipitates were then washed twice with cold 5% trichloroacetic acid containing 10 mM leucine and 10 mM phenylalanine. One mg of bovine serum albumin and 3 A260 units of Escherichia coli tRNA were added as carriers. The precipitates were then dissolved in 0.4 ml of Soluene-100 (Packard Instrument Co.). Radioactivity in the pellets was measured as described previously (6). Control incubations in which no RNA or no enzyme extract were added were performed in parallel. The tRNA synthetase activity was expressed as picomoles of amino acid incorporated per A260 unit per min. The K_m was obtained from linear regression analysis of Eadie-Hoffstee plots.

RESULTS

A Method of Simultaneous Measurement of Protein Synthesis and Degradation—One simple method to measure protein synthesis in isolated muscles is to follow the incorporation of [14C]tyrosine or phenylalanine into tissue proteins (4, 6). When these amino acids or leucine are present in the medium, at 0.5 mM or higher concentrations, their intracellular and extracellular specific activities are similar (40, 41, Table V). Rates of proteolysis in such preparations can be measured most simply by following the release of tyrosine from protein, usually in the presence of cycloheximide, an inhibitor of protein synthesis (6, 42). Since tyrosine is neither synthesized nor degraded in muscle to any significant extent (6, 16), and since the intracellular pool of tyrosine remains constant (6) during such incubations, the appearance of tyrosine in the medium provides an accurate measure of net protein breakdown. Unfortunately, these methods do not permit the simultaneous measurement of both protein synthesis and degradation. In theory, these processes can be determined in the same piece of isolated muscle by measuring simultaneously the rates of protein synthesis and net protein breakdown; the sum of these two measurements must equal the actual rate of proteolysis. The major difficulty with this approach is that only small amounts of tyrosine are liberated from protein during an incubation, while relatively large amounts (0.5 mM) of [14C]tyrosine are added to the media for the measurement of protein synthesis. This problem was overcome in the pres-
ent studies by providing instead [14C]phenylalanine (0.5 mM) and determining its rate of incorporation into muscle protein. These data on rates of protein synthesis (nanomoles of phenylalanine incorporated per mg of tissue for 2 h) were converted to nanomoles of tyrosine per mg for 2 h by using the ratio of [14C]tyrosine to [14C]phenylalanine incorporated into muscle protein.

We determined this ratio in diaphragms incubated with either [14C]tyrosine or [14C]phenylalanine (Table I). The molar incorporation of [14C]tyrosine into protein consistently averaged 77% that of [14C]phenylalanine. Addition of leucine increased the rate of protein synthesis measured with either precursor without altering the ratio of tyrosine to phenylalanine (0.77) incorporated into tissue proteins. This ratio is identical with that calculated from the per cent of tyrosine and phenylalanine found in muscle protein hydrolysates (21, 43-45) and thus the proteins synthesized by the muscles in vitro appear to have a similar average composition to the bulk of tissue protein (Table I). In addition this ratio is the same in muscles from fed, fasted, adrenalectomized, or cortisoltreated rats. The rate of muscle protein degradation in terms of nanomoles of tyrosine can therefore be calculated from measurements of protein synthesis using [14C]phenylalanine and of the net release of tyrosine from tissue proteins: the rate of protein degradation (nanomoles of tyr per mg of tissue for 2 h) = ([incorporation of [14C]phenylalanine into protein] × (0.77)) + [release of tyrosine into the medium].

To validate this procedure, we compared rates obtained in this way with those obtained in the presence of cycloheximide. At 0.5 mM, cycloheximide reduces by at least 95% protein synthesis in muscle (6). Therefore, under these conditions, the rate of net protein breakdown (i.e. net release of tyrosine into the medium) equals approximately the rate of proteolysis. As shown by the net release of tyrosine into the medium (Table II), the diaphragms were in a state of negative nitrogen balance. In these experiments with muscles from fed and fasted animals, the two techniques gave very similar results. This new method was therefore used in most of the subsequent studies. Under these conditions, cycloheximide may have slightly reduced the overall rates of proteolysis as has been reported (1, 41), but this trend was not statistically significant.

In other experimental situations,1,2 we have found a consistent, small decrease in the absolute rate of proteolysis after addition of cycloheximide.

Effects of Branched Chain Amino Acids on Protein Turnover—These amino acids together reduce net protein breakdown in skeletal and cardiac muscle (6-8, 10). Leucine, by itself, decreased this process at concentrations found in vivo (0.5 mM), however, isoleucine and valine together exerted no effect on protein turnover (Table III). Even at 3.0 mM, isoleucine did not affect the net release of tyrosine from muscle protein, even though at these concentrations isoleucine, like leucine, inhibited pyruvate oxidation in diaphragms of fasted rats (Table III). Hence the mechanism(s) by which leucine alters protein turnover seems to differ from that which causes the oxidation of pyruvate to fall.

These results agree with the earlier observation that leucine alone, but neither isoleucine nor valine, can promote protein synthesis in muscle (6, 8, 10). These results disagree, however, with a previous observation (6) in muscles of hypophysectomized animals that isoleucine and valine together could reproduce the regulatory effects of leucine on protein turnover. These discrepant observations cannot be explained readily, but could be related to the different physiological conditions of the animals (i.e. hypophysectomized versus normal). In any case, it seems clear that leucine, but no other plasma amino acid, can reduce net protein breakdown. Leucine also differs from isoleucine and valine in other respects; e.g. at concentrations found in serum, leucine can serve as an energy source for muscle (17, 46) and thus can spare the use of glucose by this tissue in fasting animals (23, 32).

Similar effects of leucine on protein synthesis and degradation were also obtained with diaphragms isolated from rats of 154 ± 6 g. These findings are noteworthy, since one group (11) had reported that leucine only affected protein turnover in perfused muscles from very young rats. The basis of these discrepant results is unclear, although much greater experimental variations are generally obtained with the perfused muscles than with the approach used here.

To determine whether leucine might be a physiological regulator of protein synthesis and degradation in vivo, we measured these processes over the range of leucine concentrations observed in rat plasma (0.1 to 0.5 mM). At low concentrations (0.1 mM), leucine stimulated protein synthesis (p < 0.005) without reducing proteolysis (Table IV). At higher levels (0.2 to 0.5 mM), leucine inhibited proteolysis significantly. Over this latter range of concentrations, the absolute decrease in proteolysis was larger than the absolute increase.

1 M. E. Tischler and A. L. Goldberg, unpublished observations.
2 A. Kunin and A. L. Goldberg, unpublished observations.
Control of Muscle Protein Turnover by Leucine

**Table III**

Effect of branched chain amino acids on net protein breakdown and pyruvate oxidation in diaphragms of fasted rats

<table>
<thead>
<tr>
<th>Branched chain amino acid</th>
<th>Concentration</th>
<th>Net protein breakdown</th>
<th>Inhibition of net protein breakdown</th>
<th>Inhibition of pyruvate oxidation*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>mM</td>
<td>nmol/mg tissue/2 h</td>
<td>%</td>
<td>%</td>
</tr>
<tr>
<td><strong>Experiment 1</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>None</td>
<td></td>
<td>0.339 ± 0.015</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Leucine</td>
<td>0.5</td>
<td>0.198 ± 0.022</td>
<td>41 ± 5b</td>
<td>26-30°</td>
</tr>
<tr>
<td>Isoleucine, valine</td>
<td>1.0 (each)</td>
<td>0.300 ± 0.017</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td><strong>Experiment 2</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>None</td>
<td></td>
<td>0.603 ± 0.026</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Leucine</td>
<td>1.2</td>
<td>0.639 ± 0.005</td>
<td>0</td>
<td>13°</td>
</tr>
<tr>
<td>Isoleucine</td>
<td>3.0</td>
<td>0.622 ± 0.009</td>
<td>0</td>
<td>30°</td>
</tr>
</tbody>
</table>

* Significant effect of leucine addition: p < 0.005.

in protein synthesis. However, in the control muscle incubated only with glucose, protein degradation occurred more rapidly than did protein synthesis so that the per cent stimulation of synthesis induced by leucine was larger than the per cent inhibition of proteolysis.

The intracellular leucine concentration in the incubated diaphragm was found to be 75 μM in the absence of extracellular leucine. When the concentration of leucine in the medium was increased from 0 to 1.0 mM, the intracellular content increased in parallel (Fig. 1). Thus, under these conditions, changes in intracellular leucine content correlated with the reduction in protein catabolism and the enhancement of synthesis. It is noteworthy that over the range of leucine concentrations found in the plasma (0.15 to 0.5 mM) (47-49), leucine can both stimulate protein synthesis and inhibit protein degradation. In addition, the rate of leucine oxidation by skeletal muscle increases continually over the same extracellular concentration range (17).

Is Catabolism of Leucine Required For Its Effect on Protein Turnover? In order to test this possibility, we attempted to identify an agent that would inhibit leucine aminotransferase (the initial step in the pathway for leucine degradation) without affecting the incorporation of leucine into protein, its uptake by the tissue or rates of protein degradation. We found that L-cycloserine, a known transaminase inhibitor (50), could block leucine degradation in this selective manner. The effect of cycloserine was tested by incubating diaphragms with [1-14C]leucine and measuring the production of 14CO2 and α-[1-14C]ketoisocaproic acid (i.e. total transamination of [1-14C]leucine equals the sum of the recovered 14CO2 and α-[1-14C]ketoisocaproic acid released). At 5 mM, cycloserine reduced by 50% the rate of transamination of 0.5 mM leucine (Table V) and decreased this rate by greater than 95% at a leucine concentration of 0.1 mM.

Previous studies showed that leucine inhibits the oxidation of glucose in muscles of fasted rats and that this effect can be blocked if leucine transamination is inhibited by addition of aminooxyacetic acid (23). To examine further the capacity of cycloserine to reduce leucine transamination, we incubated diaphragms with [U-14C]glucose and unlabeled leucine in the absence or presence of cycloserine. In accord with these previous findings, addition of cycloserine reversed the inhibition by leucine of 14CO2 production from [U-14C]glucose (data not shown).

It is possible that cycloserine, in part, decreased leucine degradation by inhibiting its uptake by the tissue and thereby decreasing the intracellular leucine content. To test this pos-

**Table IV**

Comparison of effects of various concentrations of leucine on protein synthesis and degradation

Diaphragms were incubated with glucose (5 mM) and [U-14C]phenylalanine (0.5 mM; 0.1 mCi/mmol) in the absence or presence of varied concentrations of leucine. Rates of protein synthesis and degradation were determined as in Table II and data are presented as the changes due to leucine addition. The number in parentheses is the number of animals used for the study.

<table>
<thead>
<tr>
<th>Leucine</th>
<th>Protein synthesis</th>
<th>Protein degradation</th>
</tr>
</thead>
<tbody>
<tr>
<td>mM</td>
<td>Absolute increase</td>
<td>Stimulation</td>
</tr>
<tr>
<td></td>
<td>nmol/mg tissue/2 h</td>
<td>%</td>
</tr>
<tr>
<td></td>
<td>Absolute decrease</td>
<td>Inhibition</td>
</tr>
<tr>
<td></td>
<td>nmol/mg tissue/2 h</td>
<td>%</td>
</tr>
<tr>
<td>0.1</td>
<td>+0.008 ± 0.007</td>
<td>+0.2 ± 3</td>
</tr>
<tr>
<td>0.2</td>
<td>+0.016 ± 0.002</td>
<td>+0.1 ± 9</td>
</tr>
<tr>
<td>0.25</td>
<td>ND</td>
<td>0</td>
</tr>
<tr>
<td>0.5</td>
<td>+0.033 ± 0.004</td>
<td>+0.4 ± 12</td>
</tr>
</tbody>
</table>

a Significant effect of leucine addition: p < 0.005.
b p < 0.001.
c ND is not determined.

dpM/mg tissue for 2 h

**Table V**

Effect of L-cycloserine on [1-14C]leucine metabolism

Diaphragms were incubated with [1-14C]leucine (0.5 mM; 0.2 mCi/ mmol) and glucose (5 mM) in the absence or presence of L-cycloserine (5 mM). The rate of leucine transamination, the amount of radioactivity incorporated into protein and into the acid-soluble intracellular pool, and leucine content of this pool were measured as described under "Experimental Procedures." The amounts of intracellular (acid-soluble) 14C and leucine were obtained by subtracting from the total tissue measurements the amounts in the extracellular (i.e. insulin) space (0.35 μM/mg of tissue). The specific activity of leucine in the medium (135 dpm/nmol) was not significantly different from that in the tissue.

<table>
<thead>
<tr>
<th>Additions</th>
<th>Leucine transamination</th>
<th>[1-14C] recovered in</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Protein</td>
<td>Soluble pools</td>
</tr>
<tr>
<td></td>
<td>nmol/mg tissue/2 h</td>
<td>dpm/mg tissue/2 h</td>
</tr>
</tbody>
</table>

None | 3.7 ± 0.2 | 34 ± 1 | 41 ± 2 | 0.30 ± 0.04 | 137 ± 23 |
Cycloserine | 1.9 ± 0.1 | 34 ± 1 | 52 ± 2 | 0.35 ± 0.04 | 149 ± 21 |
Difference | -1.8 ± 0.1 | <0.001 | +1 ± 1 | +0.05 ± 0.02 | 12 ± 8 |

a NS, not significant.
sibility, we determined the rates of [1-14C]leucine in diaphragms incubated with cycloserine. Although the total uptake of leucine (i.e. sum of transamination, incorporation into protein, and accumulation in the tissue) fell from 4.3 to 2.5 nmol/mg of tissue for 2 h in the presence of cycloserine, this difference could be accounted for by the lower rate of leucine transamination. In the presence of cycloserine, accumulation of both 14C radioactivity and leucine in the absolute muscle pool increased without changing the specific activity of leucine. This result suggested that this transaminase inhibitor does not block the transport of leucine into the cell. Cycloserine also did not affect the incorporation of [1-14C]leucine into muscle protein (Table V). In other experiments, this agent also did not alter rates of synthesis or degradation of protein in diaphragms or atria (Tables VI and VII). Therefore, based on the criteria outlined above, cycloserine seemed to be a suitable inhibitor of leucine transamination.

In the absence or presence of cycloserine, 0.5 mM leucine increased the rate of protein synthesis by a similar per cent (Table VI). Even at low concentrations of leucine (0.1 mM), where cycloserine inhibits leucine transamination by greater than 95%, leucine stimulated protein synthesis to a similar extent (19). While cycloserine by itself also had no effect on proteolysis, without affecting proteolysis itself, cycloserine also reversed the inhibitory effect of leucine on protein degradation (Fig. 2). To test whether cycloserine may act by interfering with the overall regulation of protein turnover, we examined whether this agent altered the ability of glucose and insulin to stimulate protein synthesis.

### TABLE VI

<table>
<thead>
<tr>
<th>Leucine</th>
<th>Cycloserine</th>
<th>Protein synthesis</th>
<th>Protein degradation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>nmo1 Tyr/mg tissue/2 h</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Experiment 1a</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>None</td>
<td>-</td>
<td>0.088 ± 0.004</td>
<td>0.80 ± 0.02</td>
</tr>
<tr>
<td>0.5 mM</td>
<td>-</td>
<td>0.117 ± 0.004</td>
<td>0.58 ± 0.03</td>
</tr>
<tr>
<td>Per cent change</td>
<td>+</td>
<td>+32 ± 6</td>
<td>-28 ± 2'</td>
</tr>
<tr>
<td>None</td>
<td>+</td>
<td>0.085 ± 0.004</td>
<td>0.81 ± 0.02</td>
</tr>
<tr>
<td>0.5 mM</td>
<td>+</td>
<td>0.107 ± 0.005</td>
<td>0.82 ± 0.03</td>
</tr>
<tr>
<td>Per cent change</td>
<td>+</td>
<td>+26 ± 9'</td>
<td>0</td>
</tr>
<tr>
<td>Experiment 2</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.1 mM</td>
<td>-</td>
<td>0.088 ± 0.005</td>
<td>0.56 ± 0.02</td>
</tr>
<tr>
<td>0.5 mM</td>
<td>-</td>
<td>0.120 ± 0.004</td>
<td>0.57 ± 0.02</td>
</tr>
<tr>
<td>0.1 mM</td>
<td>+</td>
<td>0.117 ± 0.006</td>
<td>0.56 ± 0.03</td>
</tr>
</tbody>
</table>

a The data shown in Experiment 1 have also been presented in a recent symposium (61).
b Significant effect of leucine addition: p < 0.02.
c p < 0.001.

FIG. 2. Comparison of the rates of leucine degradation and the inhibition of proteolysis by leucine in the presence of cycloserine. Diaphragms from fasted rats were incubated with various concentrations of [1-14C]leucine (0 to 8 mM; 0.2 mCi/mmol) in the presence of glucose (5 mM), cycloheximide (0.5 mM), and cycloserine (5 mM). The maximal inhibition of protein degradation was determined in diaphragms incubated with glucose (5 mM) and cycloheximide (0.5 mM) in the absence or presence of 8 mM leucine. Data are expressed as production of 14CO2 and as per cent of the maximal inhibition by 8 mM leucine. In the absence of leucine, the rate of protein degradation was 0.56 ± 0.02 nmol of tyrosine/mg of muscle/2 h.
proteinsynthesis or inhibit protein breakdown (Table VIII). Addition of cycloserine did not alter these effects of glucose and insulin.

**Influence of Other Metabolites on Protein Turnover**—The above results suggest strongly that leucine, or possibly leucyl-tRNA, promotes directly protein synthesis, while some intermediate or side product of leucine breakdown is responsible for the inhibitory effect on protein degradation in muscle. Therefore, α-ketoisocaproic acid, the product of leucine transamination, should by itself reduce proteolysis but should not stimulate protein synthesis unless its addition causes accumulation of intracellular leucine. To test this possibility, we compared rates of protein synthesis and degradation in diaphragms incubated with leucine or α-ketoisocaproic acid (Table IX). In experiment 1, leucine decreased protein degradation and enhanced protein synthesis. α-Ketoisocaproic acid, like leucine, reduced proteolysis but failed to promote protein synthesis. In another experiment where α-ketoisocaproic acid decreased protein degradation, α-hydroxyisocaproic acid had no influence on this process.

**TABLE VIII**
Effect of insulin and glucose on protein synthesis and degradation in diaphragms in the absence or presence of cycloserine

<table>
<thead>
<tr>
<th>Addition</th>
<th>Protein synthesis</th>
<th>Protein degradation</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>0.063 ± 0.003</td>
<td>0.70 ± 0.04</td>
</tr>
<tr>
<td>Glucose + insulin</td>
<td>+68 ± 9</td>
<td>-23 ± 4</td>
</tr>
<tr>
<td>Glucose + insulin + cycloserine</td>
<td>+71 ± 10^a</td>
<td>-20 ± 4</td>
</tr>
</tbody>
</table>

* Significant effect of glucose and insulin p < 0.005.

**TABLE IX**
Effects of leucine, α-ketoisocaproic acid, ketone bodies, and lactate on protein synthesis and degradation in rat diaphragm

In experiment 1, diaphragms were incubated in the presence of glucose (5 mM) and a mixture of the plasma amino acids at concentrations found in the rat (47), except for leucine, isoleucine, valine, and tyrosine. In experiment 2, the diaphragms were incubated with glucose and the designated substrates. 1C-labeled phenylalanine was also included in the medium and rates of protein synthesis and degradation were measured as in Table II and are expressed as nanomoles of tyrosine per mg of tissue for 2 h. Additions of acids were made as the Na salts.

**FIG. 3.** Leucyl- or phenylalanyl-tRNA synthetase activity in muscle extracts. Extracts of muscle were prepared, dialyzed, and assayed as described under “Experimental Procedures.” The K_m values were obtained through linear regression analysis of the experimental data.

Charging of Leucyl-tRNA in Muscle Extracts—The finding in Tables VI and VII suggests that the intracellular levels of either leucine or leucyl-tRNA, but not some metabolite generated by leucine catabolism, can regulate the rate of protein synthesis in muscle. To determine whether the supply of leucyl-tRNA could be the important factor regulating protein synthesis, we tested whether the rate of charging of leucyl-tRNA might vary with the changes in extracellular leucine concentrations that affect protein synthesis (Table IV). We therefore measured the K_m of the leucyl-tRNA synthetase in muscle extracts and compared it with the K_m of phenylalanyl-tRNA synthetase, since the supply of phenylalanine does not alter protein synthesis in muscle (6). The K_m of leucyl-tRNA synthetase determined in extracts from skeletal muscle using partially purified muscle tRNA as an acceptor was 6 μM. A similar K_m (2 μM) was obtained for phenylalanyl-tRNA synthetase in muscle extracts (Fig. 3). Similar values were also obtained using tRNA extracted from muscle, liver, or E. coli.

The observed K_m values agree with published data for liver (53) or yeast (54) leucyl-tRNA synthetase. The value for charging of leucyl-tRNA synthetase (6 μM) is well below the intracellular leucine concentration (75 to 100 μM) over the range of extracellular leucine concentrations (100 to 500 μM)
that stimulate protein synthesis (Table IV and Fig. 1). Thus, it appears unlikely that the stimulation of protein synthesis by exogenous leucine involves changes in the intracellular levels of charged tRNA.3

**DISCUSSION**

The modified procedure presented here for simultaneously measuring rates of protein breakdown and synthesis offers several distinct advantages over previous methods (42). (a) This procedure eliminates systematic errors that may be caused by use of inhibitors of protein synthesis (1, 25, 26). Although no such effects were observed in Table II, it is well established that such inhibitors can also reduce proteolysis under certain conditions (e.g. nutritionally poor conditions). In fact, in similar studies using muscles from adrenalectomized or fasted animals, rates of protein breakdown appeared higher when measured by the present approach than when determined using cycloheximide.1,4 (b) These simultaneous measurements do not require prior labeling of muscle protein with radioactive isotopes, and therefore, the assays are relatively inexpensive to perform. (c) The incorporation of [14C]phenylalanine into protein and the accumulation of tyrosine in the medium can be measured by simple, sensitive, and highly reliable techniques. For most measurements, the standard error obtained for rates of protein turnover were less than 5% of the mean values (x = 6). (d) Since rates of protein synthesis and proteolysis are determined in the same piece of isolated muscle, this method allows the study of multiple experimental conditions on tissues from a single animal, and thus offers an important advantage for the statistical testing of results.

The fundamental assumption of this approach is that the ratio of tyrosine to phenylalanine incorporated into protein is constant. While this assumption appears valid for tissues incubated in the presence or absence of leucine (Table I) and in muscles of fasted, adrenalectomized, or cortisol-treated animals, its applicability to other experimental situations must also be established. For example, use of this method in comparisons of protein metabolism in normal, hormone-treated, or hormone-depleted (e.g. diabetic) animals would require evidence that the overall ratio of tyrosine and phenylalanine incorporation into protein did not differ in the two situations. It is theoretically possible that dramatic changes in the patterns of protein synthesized under such conditions could alter this ratio.

**Mechanisms of Leucine’s Effect on Protein Synthesis—**
The stimulation of protein synthesis by leucine and the inhibition of protein degradation seem to occur through different mechanisms, as shown in experiments with cycloserine and by the different dose-response curves (Table IV). The present results with cycloserine and α-ketosocaproic acid suggest that the stimulation of protein synthesis is signaled by changes in intracellular levels of leucine, itself, or leucyl-tRNA, and not by some products generated by leucine catabolism. In both skeletal and atrial muscle, cycloserine blocked leucine transamination without altering leucine’s effect on protein synthesis (Tables V to VII). In addition, α-ketosocaproic acid, the product of leucine transamination, did not affect the rate of protein synthesis in skeletal muscle (Table IX). Base and Weigand (9) also reported that in diaphragm, this compound did not enhance the incorporation of [14C]tyrosine into protein, under conditions where leucine increased this process by 36%. In cardiac muscle, unlike skeletal muscle, α-ketosocaproic acid promotes protein synthesis (10) possibly because of a higher rate of transamination to leucine in that tissue.

Since the stimulation of protein synthesis by leucine is independent of leucine transamination, the response seems to be signaled by an accumulation of leucine, itself, or of leucyl-tRNA. It appears, however, unlikely that the changes in leucyl-tRNA content regulate protein synthesis in skeletal muscle.3 The Km of leucyl-tRNA synthetase is well below the intracellular concentration of leucine under all conditions studied, by about 10- to 100-fold. Although these cell-free studies attempted to mimic intracellular conditions, we cannot definitely exclude the possibility that the extent of charging of leucyl-tRNA in vivo does not differ from that measured here.

**Mechanisms of Leucine’s Effect on Protein Degradation—**
Although transamination of leucine is not essential for its inhibitory effect on protein synthesis, it is essential for the inhibitory effect on proteolysis. In both skeletal and atrial muscle, cycloserine blocked leucine’s effect on protein breakdown (Tables V to VII) in a dose-dependent fashion. The degree of inhibition of this effect correlated closely with the inhibition of leucine degradation (Fig. 2). Furthermore, α-ketosocaproic acid, the product of leucine transamination, also reduced proteolysis (Table IX). In contrast to our results, Buse and Weigand (9) failed to show an effect of α-ketosocaproic acid on the release of tyrosine into the medium measured in the presence of cycloheximide. However, in their study, 0.5 mM leucine decreased proteolysis in skeletal muscle by only 4% compared to the 24 to 28% reduction observed in the present experiments (Tables IV, VI, and VIII). In the perfused heart, α-ketosocaproic acid has been reported recently also to decrease protein degradation (10). It is noteworthy that in human patients, the administration of α-ketosocaproic acid appears more effective than leucine in improving body nitrogen balance (16). Thus, in skeletal and cardiac muscle, rates of protein degradation, as well as glucose metabolism in fasting (12, 23, 32) appear to be regulated by intermediates or products of leucine catabolism.

In *E. coli* (26, 27), unlike in muscle, amino acids suppress overall protein degradation by increasing the intracellular content of charged tRNA. The lack of involvement of tRNA in leucine’s effect in muscle does not eliminate other possible regulatory effects of charged tRNA on protein catabolism. In fact, in fibroblasts as in *E. coli* (26, 27), pharmacological inhibitors and mutants that reduce amino acid charging can stimulate overall proteolysis (28).

In mammalian cells other than muscle, exogenous amino acids are also known to affect protein balance (2, 9). It remains to be established in these non-muscle cells whether the amino acids themselves or some product of their degradation influence protein turnover. Leucine affects protein breakdown in skeletal and cardiac muscle but not in adipose tissue (55) or in liver (2, 9). In the liver, the inhibitory effect of plasma amino acids on tissue proteolysis (2, 3, 56) results from a different group of amino acids, all of which are rapidly catabolized in that tissue but not in muscle (24).

It remains unclear what intermediate or side product of leucine degradation actually causes the reduction of proteolysis in muscle following addition of leucine. The metabolite affecting protein degradation seems to differ from that responsible for the inhibition of pyruvate oxidation even though both effects require leucine transamination. In muscles from fed rats, 0.2 mM leucine reduced proteolysis (Table IV), but even 3-fold greater concentrations of leucine did not inhibit pyruvate oxidation (23). In addition, isoleucine even at 3 mM concentrations failed to decrease proteolysis although such
high concentrations of isoleucine did mimic the ability of leucine (0.5 mm) to inhibit the oxidation of pyruvate by muscles from fasted rats (23; Table III). It seems unlikely that in skeletal muscle, changes in acetyl-CoA, due to leucine and α-ketosaccharic acid addition, are responsible for leucine's effect on proteolysis (Table IV, 55). Although glucose also inhibits this process in diaphragm (6), pyruvate addition produced no change in the rate of protein degradation. It is possible that these inhibitory effects of leucine, α-ketosaccharic acid and glucose may be mediated through changes in the oxidation-reduction state, as suggested in a recent report (55). The inability of other substrates (e.g. lactate, ketone bodies) to retard protein degradation in skeletal muscle (Table IX) may be explained by their ineffectiveness in altering the NAD-couple (55). In contrast, lactate (51), acetoacetate (10), and acetate (10) all reduce protein breakdown in perfused hearts perhaps because they may alter the oxidation-reduction state in this tissue. Data in support of this idea has been found recently. 

Alternatively, leucine or glucose may possibly raise in some specific fashion the ATP content or energy charge of the cell. In E. coli, a modest decrease in ATP production leads to increased breakdown of cell proteins (58) even though some ATP is required for proteolysis to occur (11). Therefore, supplying metabolized substrates to bacteria in nutritionally poor conditions reduces overall protein breakdown by raising ATP. Possibly similar effects occur in mammalian muscles and other cells. In any case, future studies must focus on whether these various oxidizable substrates lower protein degradation by common mechanisms and what specific intermediates in leucine degradation influences this process.

Physiological Significance of Leucine's Effects on Protein Turnover—In diaphragms, at all concentrations of leucine tested (0.1 to 0.5 mm), the per cent stimulation of protein synthesis appeared greater than the per cent inhibition of proteolysis (Table IV). However, under these particular in vitro conditions, the absolute rate of protein degradation is significantly larger than the rate of protein synthesis (6). Consequently, the absolute magnitude of leucine's effect on proteolysis exceeded that on protein synthesis (Table IV). In vivo, in muscles of well fed animals, rates of protein synthesis generally are equal to or greater than rates of proteolysis. It is impossible to predict whether, under such conditions, the magnitude of the stimulation of protein synthesis by leucine is larger or less than the absolute decrease in protein breakdown. In either case, following ingestion of protein (59), the levels of leucine in human plasma rise (60) sufficiently to induce either an enhancement of protein synthesis or a decrease in overall protein catabolism. Thus, the rise in blood leucine may serve to facilitate the disposal of ingested amino acids in muscle protein.

By contrast, in fasting there is a marked loss of muscle weight, which results in large part from the lack of insulin and serves to provide the organism with amino acids for gluconeogenesis (59). Yet during fasting, blood levels of leucine also rise. For example, in rats after 6 days of food deprivation and in humans fasted for 2 days (48, 49), plasma levels of leucine rise approximately 2-fold, which is sufficient to promote protein synthesis and reduce proteolysis in the isolated muscles (Table IV). The physiological significance of such effects in a fasted organisms are not obvious. Possibly during fasting, this increase in plasma leucine may retard the continuous breakdown of essential muscle tissue (which if unchecked could perhaps become deleterious to the organism). Studies in intact fed and fasted organisms should be able to test these possibilities and thus clarify the physiological importance of leucine's regulatory effects.

Acknowledgments—We wish to thank Dr. Tae-Wen Chang and Nicolina Fedele for their assistance in obtaining some of the data presented here. We are grateful to Pearl Lu for her invaluable technical assistance in these experiments.

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Does leucine, leucyl-tRNA, or some metabolite of leucine regulate protein synthesis and degradation in skeletal and cardiac muscle?

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