Effects of Insulin, Glucose, and Amino Acids on Protein Turnover in Rat Diaphragm*

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RICHARD M. FULKS,† JEANNE B. LI,§ AND ALFRED L. GOLDBERG¶

From the Department of Physiology, Harvard Medical School, Boston, Massachusetts 02115

SUMMARY

A simple method is described for measuring rates of protein synthesis and degradation in isolated rat diaphragm. Muscles incubated in Krebs-Ringer bicarbonate buffer showed a linear rate of synthesis for 3 hours. At the same time, the muscle released tyrosine and ninhydrin-positive material, primarily amino acids, at a linear rate. This release was not a nonspecific leakage of material from the intracellular pools, but reflected net protein degradation. Tyrosine was chosen for studies of protein turnover, since it rapidly equilibrates between intracellular pools and the medium, it can be measured fluorometrically, and it is neither synthesized nor degraded by this tissue. To follow protein degradation independently of synthesis, muscles were incubated in the presence of cycloheximide. Under these conditions, the amount of tyrosine in the intracellular pools was constant, while the muscle released tyrosine at a linear rate. This tyrosine release was used as a measure of degradation.

This preparation was used to study the influence of various factors known to be important for muscle growth on protein synthesis and degradation. Similar effects were obtained with diaphragms of normal and fasted rats although the latter showed decreased synthesis and increased protein degradation. Insulin by itself not only stimulated synthesis but also inhibited protein degradation (even in the presence of cycloheximide). These two effects served to reduce the net release of tyrosine from muscle protein to comparable extents. Effects of insulin on synthesis and degradation were greater when glucose was also present in the medium. Glucose by itself inhibited protein degradation but in the absence of insulin glucose had no significant effect on synthesis. Nevertheless, glucose stimulated incorporation of radioactive tyrosine into protein, but this effect was due to an increased intracellular specific activity. Unlike glucose neither \( \beta \)-hydroxybutyrate or octanoic acid had any demonstrable effects on protein degradation.

The addition of amino acids at plasma concentrations both promoted protein synthesis and inhibited degradation in the diaphragm. Five times normal plasma concentrations of the amino acids had larger effects. The three branched chain amino acids together stimulated synthesis and reduced degradation, while the remaining plasma amino acids did not affect either process significantly. Thus leucine, isoleucine, and valine appear responsible for the effects of plasma amino acids on protein turnover in the muscle. Leucine by itself or isoleucine and valine together, also were able to inhibit protein degradation and promote synthesis.

The protein content of a cell or tissue is determined by the balance between the rates of protein synthesis and degradation. Most investigations of mammalian growth mechanisms have concentrated on the control of protein synthesis. In muscle, for example, over-all rates of synthesis are influenced by a variety of hormones (1), nutritional status (2), and the level of muscular activity (3). However, alterations in rates of protein breakdown may also contribute to the growth of animal and bacterial cells (4). The mean rate of degradation of muscle protein appears to change during compensatory growth (5), denervation atrophy (6), food deprivation (7), muscular dystrophy (8), and hormone treatment (6, 9). The cellular mechanism responsible for these changes in degradative rates or their relationship to the concomitant alterations in rates of protein synthesis are presently unknown.

Investigations of protein degradation have been limited by methodological difficulties. Such studies have generally utilized intact animals and followed the disappearance of radioactively labeled proteins. These experiments are prone to a variety of difficulties and possible artifacts (10, 35). For example, the reported average half-lives of muscle protein from such studies vary over a several fold range (8, 11), presumably because of methodological problems. In this paper we describe a simple method for study of over-all rates of protein synthesis and degradation in isolated skeletal muscle. It has been used here to investigate the influence of insulin, glucose, and amino acids in rat diaphragm.

It has long been known that insulin is essential for normal growth of muscle (1, 3). Insulin can stimulate amino acid transport (12) and protein synthesis (1) in isolated muscle. In addition, this hormone appears to reduce protein degradation in liver...
(13), adipose tissue (14), heart (9), and fibroblasts (15). The present studies have investigated the effects of insulin and glucose on protein degradation in isolated muscle. Glucose by itself has been reported to promote protein synthesis in isolated rat diaphragm (16), although this finding has been questioned. In addition, administration of glucose to a fasted organism (2) sharply reduces the utilization of body protein reserves. Another factor essential for normal growth is an adequate supply of required amino acids. In bacteria (17), cultured animal cells (18), and in perfused liver (19) or heart (20), supply of amino acids can promote protein synthesis or inhibit degradation. Therefore the effects of serum amino acids on protein turnover in muscle were also investigated.

MATERIALS AND METHODS

Chemicals—L-[U-14C]Tyrosine and [14C]inulin were obtained from New England Nuclear Corp. (Boston, Mass.) or Schwartz-Mann (Orangeburg, N. J.). Insulin (Eli Lilly and Co.) was used at a final concentration of 0.1 unit per ml. Higher concentrations of insulin did not have greater effects on protein synthesis or breakdown. Also at higher concentrations, material in the insulin preparation interfered with the determination of tyrosine.

Experimental Procedure—Male rats were obtained from the Charles River Breeding Laboratories (Wilmington, Mass.), and were maintained on water and Purina lab chow (Ralston Co., St. Louis). They weighed between 45 and 80 g at killing. In any experiment, groups of rats of similar weights (within 5 g) were used. Fasted animals were deprived of food for about 40 hours prior to the experiment. During this time, they lost approximately 25% of their body weight.

The rate of killing by cervical dislocation. The diaphragms were then removed and placed in Krebs-Ringer bicarbonate buffer (pH 7.4), containing chloramphenicol (0.2 mg per liter) and saturated with a 95% O2-5% CO2 gas mixture. The hemidiaphragms were dissected away from the ribs and cut into two pieces. Each quarter diaphragm was rinsed, blotted, weighed (approximately 30 mg), and placed into a flask containing 5 ml of KRB at 37°C. The flasks were stoppered, re-equilibrated with the gas mixture, and then preincubated for 30 min at 37°C with shaking. Then the muscles were transferred to flasks containing 3 ml of fresh medium, and incubated for 1 to 3 hours.

At the end of incubation 2-ml aliquots of the medium were combined with 0.5 ml of cold 50% trichloroacetic acid, mixed, and centrifuged. The diaphragms were blotted, and homogenized in 1 ml of cold 0.01 M potassium phosphate buffer (pH 7.4); the homogenizing tubes were rinsed with an additional 1.0 ml of the buffer. The homogenate and wash were combined with 0.5 ml of 50% trichloroacetic acid, mixed, and centrifuged. The acid-soluble supernatants were then decanted to obtain a sample of the muscle's amino acid pools. For measurements of radioactivity in protein, the acid-precipitated material was washed twice with 5% trichloroacetic acid and once with ethanol-ether (22). The pellets were dissolved and counted as described previously (22).

Acid-soluble radioactivity in the muscle homogenate and in the medium was determined with 0.4-ml aliquots dissolved in 4 ml of scintillation fluid (23). Radioactivity was measured in a Packard Tri-Carb Counter, and differences in quenching were corrected with an automatic external standard.

The acid-soluble fractions of the medium and muscle homogenates were assayed for ninhydrin-positive material (NPM) by the method of Moore and Stein (24) with leucine as a standard, and for tyrosine according to the fluorometric method of Waalkes and Udenfriend (25). For amino acid analyses, the trichloroacetic acid supernatant was adjusted with 3 volumes of ether; then the samples were lyophilized, dissolved in 0.2 N sodium citrate buffer (pH 2.2), and analyzed on a Beckman model 120C amino acid analyzer.

The ninhydrin space was measured by incubating quarter dis-

1 The abbreviations used are: KRB, Krebs-Ringer bicarbonate buffer; NPM, ninhydrin-positive material; cyclic AMP, adenosine 3':5'-monophosphate; dibutyryl cyclic AMP, 6,6,2,2'-dibutyryl adenosine 3':5'-monophosphate.

The nanomoles of tyrosine incorporated into protein were calculated by dividing the [14C]tyrosine counts in protein by the specific activity of the intracellular tyrosine at the end of incubation. Studies presented elsewhere indicate that this approach provides a valid measure of the amount of protein synthesis (26).

In this paper, "muscle pools" refers to the total amount of NPM or tyrosine in the muscle that is acid-soluble (i.e. muscle pools and extracellular fluid found in both the intracellular space and the inulin space). Data on the effects of insulin or nutrients on breakdown are expressed as differences between the control and treated pieces of the same diaphragm at the end of the incubation. The combined amount of NPM or tyrosine in the muscle pools and the medium from the control piece was subtracted from the combined amount from the treated piece to give the net change in amount released from protein.

Change in net degradation

\[ = (\text{pool} + \text{medium})_{\text{control}} - (\text{pool} + \text{medium})_{\text{treated}} \]

Since the rates of protein synthesis and degradation varied on different days for unknown reasons, all experiments on the effects of hormones and nutrients compared different pieces of the same muscle. In several tables, data have been calculated as per cent changes in rates of synthesis or degradation and combined in order to combine them with data obtained on different days. Differences were evaluated by the method of paired analyses using the one-tailed Student's t test. Values are given as mean ± standard error of the mean from five or more rats.

RESULTS

Measurements of Protein Turnover—The incorporation of [14C]tyrosine into muscle protein was used as a measure of protein synthesis. Initial studies showed that after the 30-min preincubation, the isolated diaphragm (lacking the ribs) incorporated [14C]tyrosine into protein at a linear rate for 3 hours (Fig. 1). During incubation in the presence of 0.1 mM [14C]tyrosine, the specific activity of the [14C]tyrosine within the muscle remained constant (Fig. 1) and the specific activity of the medium was not lowered significantly by the small amount of unlabeled tyrosine released by the tissue. Previous experiments in this laboratory have indicated that tyrosine equilibrates rapidly between the medium and the intracellular fluid and that the free intracellular tyrosine is a valid measure of the precursor pool for protein synthesis (26). Therefore the rate of protein synthesis (i.e. the nanomoles of tyrosine incorporated) was linear after the preincubation (Fig. 1).

Although protein synthesis occurred at a linear rate, more ninhydrin-positive material (NPM) and tyrosine were present in the combined medium and muscle pools at the end of incubation than at the beginning (Fig. 2). Most of the NPM released into the medium represented amino acids found in muscle protein, and the remainder was ammonia and taurine (Table 1). The composition of the NPM released into the medium differed from that in the intracellular pools; for example, taurine comprised...
about 50% of the NPM in the muscle pools, but only about 5 to 10% of the NPM in the medium. Thus the release of amino acids by the muscle was selective and not a nonspecific leakage from the intracellular pools.

Some of the NPM that appeared in the medium was attributable to a reduction in the NPM within the muscle (Fig. 2). However, the loss of material from intracellular pools could not account for all of the NPM that appeared in the medium. In fact, tyrosine was released into the medium, while no change was observed in the muscle’s content of tyrosine (Fig. 2). Furthermore, the amount of tyrosine released by the muscle in 3 hours was several times larger than the amount of free tyrosine originally present in the tissue (Fig. 2).

Rat diaphragm has been reported neither to synthesize nor degrade tyrosine (27). To verify this point, muscles were incubated in the presence of \[^{14}C\]tyrosine and then samples of the muscle pools and medium were fractionated by thin layer chromatography (butanol-1-glacial acetic acid-H\(_2\)O; 60:15:25). The only measurable band of radioactivity had an \(R_f\) characteristic of L-tyrosine. In addition, when the muscles were incubated with \[^{14}C\]phenylalanine, no \[^{14}C\]tyrosine was measurable in the tissue in accord with previous findings that muscle does not contain phenylalanine hydroxylase (28). Related studies also showed that rat diaphragm does not oxidize \[^{14}C\]tyrosine to \[^{14}C\]CO\(_2\) (29).

Since tyrosine is neither synthesized nor degraded by the diaphragm, the net production of this amino acid must indicate net protein breakdown. The net release of tyrosine and NPM from muscle protein occurred at a linear rate throughout the 3-hour incubation (Fig. 2). During incubation in unsupplemented Krebs-Ringer buffer, the net rate of protein degradation, estimated from the net release of tyrosine, was 2 to 2.5 times greater than the rate of protein synthesis, calculated from the amount of \[^{14}C\]tyrosine incorporated into protein.

Changes in the release of amino acids from cell protein could be due to changes in rates of protein synthesis or breakdown. To facilitate the study of degradation independently of possible changes in protein synthesis, synthesis was blocked by addition of cycloheximide. At a concentration of 0.5 mM, this inhibitor caused a 95% reduction in the incorporation of \[^{14}C\]tyrosine into protein. This degree of inhibition was also observed when insulin or glucose were added in the presence of cycloheximide. When tissues were incubated with cycloheximide, increased amounts of NPM and tyrosine were recovered in the medium, presumably because amino acids destined for protein synthesis were released into the medium. In most experiments, addition of cycloheximide did not alter the amount of NPM or tyrosine recovered in the muscle. In the presence of cycloheximide, the release of tyrosine occurred at a linear rate for 3 hours (Fig. 3), although the net release of NPM from protein was not linear for unknown reasons. Thus, despite the presence of cycloheximide, protein degradation continued at a linear rate.

Effects of Insulin, Glucose, and Amino Acids—Incubation of the diaphragm in the presence of insulin and glucose was found to inhibit the release of amino acids, including tyrosine, from the muscle (Table I). At the same time, the total amount of amino acids in the muscle was reduced by 30 to 40% (Fig. 4). The net release of tyrosine and NPM from muscle protein was also inhibited by the addition of insulin and glucose (Fig. 4). The inhibition was concentration-dependent and was maximal at concentrations of 10 to 20 mM insulin and glucose (Fig. 4). However, the inhibition of protein degradation was not as marked as the inhibition of protein synthesis, and the net rate of protein degradation, estimated from the net release of tyrosine, was only 50 to 60% lower than the rate of protein synthesis, calculated from the amount of \[^{14}C\]tyrosine incorporated into protein. This suggests that the inhibition of protein degradation by insulin and glucose is not as complete as the inhibition of protein synthesis.

In contrast, the inhibition of protein degradation by amino acids was more complete than the inhibition of protein synthesis. When the diaphragm was incubated with L-tyrosine, the net release of tyrosine and NPM from muscle protein was inhibited by 80 to 90% (Fig. 5). The inhibition was concentration-dependent and was maximal at concentrations of 10 to 20 mM L-tyrosine (Fig. 5). However, the inhibition of protein degradation was not as marked as the inhibition of protein synthesis, and the net rate of protein degradation, estimated from the net release of tyrosine, was only 50 to 60% lower than the rate of protein synthesis, calculated from the amount of \[^{14}C\]tyrosine incorporated into protein. This suggests that the inhibition of protein degradation by amino acids is not as complete as the inhibition of protein synthesis.
The inhibition of net amino acid release from protein by insulin and glucose could represent a direct effect of insulin or could be secondary to insulin's promotion of glucose transport (12). To resolve this question, the actions of insulin and glucose were examined separately. Addition of insulin alone inhibited net proteolysis, as indicated by reduced amounts of ninhydrin-positive material lost from protein (Table II). This decreased release of amino acids could have resulted from the well known (1) stimulation of protein synthesis by insulin (see below). However, in preliminary studies (3), the stimulation of tyrosine incorporation by insulin appeared insufficient to account for the net reduction in tyrosine release from tissue protein. This finding suggested that insulin also inhibited protein degradation in the muscle.

To test this possibility, muscle was incubated in the presence of cycloheximide with or without insulin. Although protein synthesis was blocked almost completely, insulin still reduced the amounts of NPM and tyrosine released from muscle protein (Tables II and III). This small, but significant, change indicates that insulin by itself can inhibit protein degradation independently of any effect on synthesis. Addition of glucose (10 to 22 mM) by itself to the incubation medium also reduced the amount of NPM and tyrosine released from muscle protein both in the presence and absence of cycloheximide (Tables II and III). Thus glucose, like insulin, can inhibit protein degradation. Similar effects were observed in tissues from fed and fasted animals (Table III). In the latter, the average rate of proteinolysis was significantly enhanced over the rate in tissues from fed rats.

Insulin, by itself, consistently stimulated protein synthesis (Table III). Similar effects were found in diaphragms from fasted rats, in which the basal rate of synthesis was significantly lower than in tissues from fed animals (Table III). By contrast, in most experiments, glucose seemed to stimulate protein synthesis slightly (Table III), but we were never able to demonstrate a significant effect of glucose on synthesis with tissues from fed or fasted rats (p > 0.05).

When insulin and glucose were present in the medium at the same time, they caused larger decreases in the release of NPM (Table II) and tyrosine (Tables II and III) from protein than did either agent alone (p < 0.01). Since these results were obtained in both the presence and absence of cycloheximide, insulin and glucose appeared to have additive effects on protein degradation. No evidence for synergistic effects was obtained. Interestingly, addition of glucose to the medium in the presence of insulin consistently augmented tyrosine incorporation (Table III), although glucose by itself had no significant effect on protein synthesis.

The absence of any effect on synthesis appears to contradict previous observations (16), where increased incorporation of labeled amino acid was reported upon incubation with glucose. In our experiments, addition of glucose also stimulated the incorporation of radioactivity (counts per min) into protein, particularly when low concentrations of tyrosine were present in the medium (Table IV). At the same time, however, glucose increased the intracellular specific activity of [14C]tyrosine to an

**TABLE I**

| Amino acid | In muscle | Released into medium | Net change |
|------------|-----------|----------------------|------------|
|            | Control + Insulin + glucose | Control + Insulin + glucose |
| Alanine    | 0.08      | 0.26                 | 1.39       | 2.34       | +1.13 |
| Arginine   | Trace     |                      | Trace      | 1.21       | -0.75 |
| Aspartic acid | 0.36   | 0.20                 | 0.83       | 0.58       | -0.41 |
| Glutamic acid | 0.03  | 0.01                 | 1.15       | 0.79       | -0.48 |
| Glycine    | 0.25      | 0.24                 | 2.86       | 1.77       | -1.10 |
| Histidine  | 0.05      | 0.06                 | 0.47       | 0.56       | -0.10 |
| Isoeicline | 0.03      | 0.01                 | 0.72       | 0.38       | -0.36 |
| Leucine    | 0.04      | 0.01                 | 1.45       | 0.84       | -0.64 |
| Lysine     | 0.20      | 0.13                 | 2.79       | 1.77       | -1.00 |
| Phenylalanine | 0.04  | 0.01                 | 1.26       | 0.75       | -0.54 |
| Proline    | 0.15      | 0.09                 | 1.12       | 0.69       | -0.49 |
| Serine     | 0.37      | 0.30                 | 6.58       | 3.65       | -3.00 |
| Threonine  | 0.11      | 0.07                 | 2.57       | 1.19       | -1.42 |
| Tyrosine   | 0.04      | 0.02                 | 1.06       | 0.71       | -0.37 |
| Valine     | 0.04      | Trace                | 1.21       | 0.58       | -0.67 |
| Trace amino acids | 0.15   | 0.13                 | 1.34       | 0.21       | -1.15 |
| Total amino acids | 2.53   | 2.03                 | 28.42      | 17.56      | -11.36 |
| Taurine    | 2.07      | 1.89                 | 2.37       | 2.25       | -0.30 |

acids within the tissue also decreased (Table I). Thus addition of insulin and glucose reduced the net output of all amino acids, with the exception of alanine. The magnitude of this reduction varied for the different amino acids, presumably because of differences in the amino acid composition of proteins (30) and in the metabolism of the various amino acids. The anomalous increase in alanine in response to insulin and glucose is in accord with previous reports that muscle synthesizes large amounts of this amino acid from glucose (31, 32). These findings are consistent with the well documented effects of insulin on protein balance in muscle in vivo (1).

The inhibition of net amino acid release from protein by insulin and glucose could represent a direct effect of insulin or could be secondary to insulin's promotion of glucose transport (12). To resolve this question, the actions of insulin and glucose were examined separately. Addition of insulin alone inhibited net proteolysis, as indicated by reduced amounts of ninhydrin-positive material lost from protein (Table II). This decreased release of amino acids could have resulted from the well known (1) stimulation of protein synthesis by insulin (see below). However, in preliminary studies (3), the stimulation of tyrosine incorporation by insulin appeared insufficient to account for the net reduction in tyrosine release from tissue protein. This finding suggested that insulin also inhibited protein degradation in the muscle.

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The absence of any effect on synthesis appears to contradict previous observations (16), where increased incorporation of labeled amino acid was reported upon incubation with glucose. In our experiments, addition of glucose also stimulated the incorporation of radioactivity (counts per min) into protein, particularly when low concentrations of tyrosine were present in the medium (Table IV). At the same time, however, glucose increased the intracellular specific activity of [14C]tyrosine to an
Release of amino acids from muscle in presence or absence of cycloheximide

Quarter diaphragms were preincubated for 30 min, and then transferred to a second flask containing the same medium, 0.1 unit per ml of insulin, 10 mM glucose, or both insulin and glucose and incubated for 2 or 3 hours. In some experiments 0.5 mM cycloheximide was present. In Experiment I diaphragms were from fed rats, while those in Experiment II were from fasted rats. The nanomoles of ninhydrin-positive material or tyrosine released per mg of muscle per hour are given for the control tissues incubated in KRB. The effects of insulin and glucose are presented as the differences between the treated piece and the control piece from the same diaphragm. Values are means ± S.E. The number of muscles tested are given in parentheses, and p values were calculated from paired pieces of diaphragm.

| Experiment | Changes upon addition of Insulin | Changes upon addition of Glucose | Changes upon addition of + Insulin + glucose |
|------------|---------------------------------|---------------------------------|---------------------------------|
| Control    |                                 |                                 |                                 |
|           |                                 |                                 |                                 |
| Experiment I |                                 |                                 |                                 |
| + Cycloheximide | -2.1 ± 0.4 (24)* | -0.6 ± 0.2 (16)* | -3.2 ± 0.3 (20)* |
| - Cycloheximide | -0.8 ± 0.3 (18)* | -0.9 ± 0.4 (12)* | -2.5 ± 0.6 (16)* |
| Experiment II |                                 |                                 |                                 |
| + Cycloheximide | -0.024 ± 0.009a | -0.023 ± 0.006a | -0.045 ± 0.003a |

* p < 0.01.
+ p < 0.025.

Effects of insulin and glucose on protein synthesis and degradation

Pieces of diaphragm from fed or 2-day-fasted rats were incubated as described in Table II. Tyrosine incorporation into protein was measured for 2.5 to 3.5 hours, and tyrosine release from protein in the presence of 0.5 mM cycloheximide was measured for 2 to 3 hours. The length of the incubations varied in different experiments. Insulin (0.1 unit per ml) and glucose (10 or 22 mM) were added as indicated. Effects of insulin and glucose are expressed as per cent changes from a control piece of the same diaphragm incubated in the absence of insulin and glucose. p values were calculated for paired pieces of muscle. Positive numbers reflect stimulation of synthesis while negative numbers reflect inhibition of degradation.

| Animal | Incubation condition | Tyrosine incorporated into protein | Tyrosine released from protein |
|--------|----------------------|----------------------------------|-------------------------------|
|        |                      | nnol NPM/hr/mg muscle            | % change                      |
| Fed    | No additions         | 0.145 ± 0.014                    | 0.290 ± 0.020                 |
|        |                      | 0.065 ± 0.005a                   | 0.398 ± 0.014a                |
| Fasted | No additions         | +57 ± 16.1 (7)b                  | -16 ± 4.5 (17)b               |
|        | + Insulin            | +54 ± 7.8 (26)a                  | -15 ± 4.3 (13)a               |
|        | + Glucose (22 mM)    | +15 ± 9.0 (7)                    | -10 ± 2.3 (14)a               |
| Fed    | + Glucose (10 mM)    | +71 ± 18.0 (6)a                  | -19 ± 1.9 (6)d                |
|        | + Insulin + glucose (10 mM) | +91 ± 13.3 (8)a | -19 ± 1.9 (6)d              |

* p < 0.003.
+ p < 0.05.
Quarter diaphragms were preincubated for 30 min in the presence or absence of nonradioactive tyrosine and then transferred to the same media for 3 hours incubation. [14C]Tyrosine (0.015 μCi per ml) was added to all flasks. Glucose was used at 22 mM. The nanomoles of tyrosine incorporated into protein were calculated for each piece of muscle in the usual fashion. The anomalously high amount of incorporation in the presence of the very low concentration of tyrosine in the medium (0.02 μM) probably resulted from an inability to determine accurately the extracellular specific activity (26). The extracellular specific activity was assumed to resemble that in the medium, which is probably incorrect under these conditions (26). If instead, the extracellular specific activity was assumed to resemble the intracellular one, the calculated tyrosine incorporation would be 0.387 ± 0.048 (control) and 0.396 ± 0.030 (+ glucose).

**Table IV**

**Effect of tyrosine and glucose on [14C]tyrosine incorporation into protein**

| Experiment I | Medium tyrosine | Final intracellular specific activity [14C]tyrosine | Incorporation into protein (per hr per mg muscle) |
|--------------|-----------------|-----------------------------------------------|-----------------------------------------------|
|              | Initial | Final | cpm/mmol | cpm | nmol |
| Control      | 0.02    | 8.3 ± 0.7 | 165 ± 7 | 26.3 ± 2.5 | 0.22 ± 0.03 |
| + Glucose    | 0.02    | 6.6 ± 0.5 | 202 ± 15 | 32.3 ± 3.0 | 0.23 ± 0.02 |
| Difference   |         |         | +36 ± 13 | +5.9 ± 2.3 | +0.01 ± 0.01 |
| Experiment II|         |         |         |       |      |
| Control      | 40      | 49 ± 1   | 137 ± 7  | 11.1 ± 0.9 | 0.11 ± 0.01 |
| + Glucose    | 40      | 45 ± 1   | 157 ± 12 | 11.9 ± 1.1 | 0.10 ± 0.01 |
| Difference   |         |         | +20 ± 16 | +0.8 ± 0.5 | -0.01 ± 0.01 |

* p < 0.025.

**Table V**

**Effects of oxidizable substrates on protein degradation**

Quarter diaphragms from six fed rats were incubated for 1.5 hours, after a 30-min preincubation, in the presence of cycloheximide (0.5 mM). β-Hydroxybutyrate (0.5 mM), octanoic acid (1 mM), or glucose (10 mM) were added as indicated. Average value for the control tissue is given along with the average percent change in treated pieces from the same diaphragm. Significance was determined by paired analysis.

| Incubation condition | Tyrosine released from protein |
|----------------------|--------------------------------|
|                      | [μmol/hr/mg muscle] |
| Control              | 0.253 ± 0.015 |
| + β-Hydroxybutyrate  | −5 ± 3.4 |
| + Octanoate          | −4 ± 2.4 |
| + Glucose            | −12 ± 5.6 (p < 0.05) |

DISCUSSION

These experiments with skeletal muscle indicate that supply of nutrients and hormones can rapidly alter rates of protein degradation independently of any effect they may have on protein synthesis (Tables II through VII). These studies utilized relatively simple procedures that are applicable to other incubated tissues and that offer significant advantages for investigations of average rates of protein turnover. Most studies in this area have followed the disappearance of labeled proteins in vivo; this latter approach is more expensive than that employed here and is subject to potential artifacts (10, 11, 35).

The present experiments used diaphragms from young (50 to 80 g) rats, because the muscles are thin and permit rapid diffusion of nutrients. In such growing animals, the diaphragm must be accumulating protein, but upon incubation in KRB, the muscle released amino acids into the medium. These effects could not be accounted for simply by the leakage of materials from the intracellular pools (Table I), but instead reflected net protein catabolism. During the incubation, degradation of muscle proteins occurred at 2 to 2.5 times the rate of synthesis (Figs. 1 and
feedback mechanism (17). In fact, in related studies, we have also
protein degradation (18, 19, 37) probably through some indirect
adding the rate of protein synthesis measured simultaneously.
One potential difficulty with this approach is that under certain
conditions, inhibitors of protein synthesis can reduce intracellular
ing net tyrosine release in the absence of cycloheximide and
obtained by the alternative, more laborious procedure of measur-
approach yielded data that were far more reproducible than those
studies avoided this problem by following degradation in the
presence of cycloheximide. In addition to its simplicity, this
Conditions that influence rates of incorporation can thus give
the impression of altering rates of degradation. The present
studies of protein turnover in muscle as does phenylalanine in
cardiac muscle (20) and valine in perfused liver (13).
release of tyrosine, which is neither synthesized nor degraded in
specific amino acids (but not NPM) remained con-
stant (Fig. 2). Thus tyrosine offers similar advantages for
studies of protein turnover in muscle as does phenylalanine in
muscle. Release of tyrosine from protein (unlike NPM) oc-
curred at a linear rate under all conditions, while the intracellular
concentration of this amino acid (but not NPM) remained con-
stant in KRB containing 0.5 mM cycloheximide and 10 mM glucose. Leucine, isoleucine, and valine or all
other amino acids found in plasma (34) were added as indicated,
at 5 times plasma levels, except tyrosine which was used
at 0.01 mM (in order that the release of tyrosine could be deter-
mined). For measurements of protein synthesis, pieces of dia-
phragm from six rats were incubated for 2 hours in KRB containing [14C]tyrosine (0.35 mM). Experiment I used normal
rate, and Experiment II used hypophysectomized animals (65
to 80 g), 3 weeks after removal of the pituitary. Average values
for the control pieces are given in addition to the per cent changes
observed in treated pieces from the same diaphragm. Signifi-
cance was determined by paired analysis.

### Table VI

| Animal       | Incubation condition | Tyrosine incorporated into protein | Tyrosine released from protein |
|--------------|----------------------|----------------------------------|-------------------------------|
| Fed, No additions |                       | 0.145 ± 0.014                   | 0.295 ± 0.056                 |
| Fasted, No additions |                   | 0.065 ± 0.005                   | 0.398 ± 0.014                 |
| Fed, + Amino acids, 1X |                   | +23 ± 9.0 (6)*                   | -19 ± 5.2 (6)*                 |
| Fed, + Amino acids, 5X |                   | +23 ± 11.5 (6)*                  | -25 ± 5.6 (6)*                 |
| Fasted, + Amino acids, 1X |                 | +83 ± 12.3 (6)*                  | -11 ± 5.1 (8)*                 |
| Fasted, + Amino acids, 5X |                 | +122 ± 8.2 (6)*                  | -25 ± 4.9 (10)*                |
| Fed, + Insulin + amino acids, 1X |            | +85 ± 24.2 (5)*                  | -17 ± 4.1 (6)*                 |
| Fasted, + Insulin + amino acids, 1X |         | +136 ± 58.6 (6)*                 | -15 ± 5.8 (6)*                 |

* p < 0.025.
† p < 0.05.
‡ p < 0.005.

#### Table VII

| Incubation condition | Tyrosine released from protein | Tyrosine incorporated into protein |
|----------------------|-------------------------------|----------------------------------|
| I. Control           | 0.175 ± 0.007                 | 0.116 ± 0.006                    |
| + Leucine, isoleucine, valine | -18 ± 6.9*              | +24 ± 5.9*                       |
| + Other amino acids  | 13 ± 13.1                     | +23 ± 6.6                        |
| + All plasma amino acids | -26 ± 2.9*                | +23 ± 11.5*                      |

#### Table VII

| Incubation condition | Tyrosine released from protein | Tyrosine incorporated into protein |
|----------------------|-------------------------------|----------------------------------|
| II. Control          | 0.164 ± 0.013                 | 0.113 ± 0.003                    |
| + Leucine, isoleucine, valine | -28 ± 7.5*              | +47 ± 5.1*                       |
| + Leucine            | -25 ± 11.6*                   | +35 ± 8.2*                       |
| + Isoleucine, valine | -22 ± 8.3*                    | +16 ± 5.8*                       |

* p < 0.05.
† p < 0.005.

Effects of amino acids and insulin on protein synthesis and degradation

Pieces of diaphragm were incubated as in Table III. Amino acids were added at plasma concentrations (34), or 5 times plasma concentrations without or with insulin (0.1 unit per ml). Five times (5X) plasma concentrations had a greater effect than 1 time (1X) (p < 0.01).

For measurements of protein degradation, pieces of diaphragm from six fed rats were preincubated for 30 min, transferred, and then incubated for 2 hours in KRB containing 0.5 mM cycloheximide and 10 mM glucose. Leucine, isoleucine, and valine or all other amino acids found in plasma (34) were added as indicated, at 5 times plasma levels, except tyrosine which was used at 0.01 mM (in order that the release of tyrosine could be determined). For measurements of protein synthesis, pieces of diaphragm from six rats were incubated for 2 or 3 hours in KRB containing [14C]tyrosine (0.35 mM). Experiment I used normal rate, and Experiment II used hypophysectomized animals (65 to 80 g), 3 weeks after removal of the pituitary. Average values for the control pieces are given in addition to the per cent changes observed in treated pieces from the same diaphragm. Significance was determined by paired analysis.
mean half-life calculated from these data and from the measured tyrosine content of diaphragm proteins is approximately 6 days. This figure is indeed similar to the value of 6 to 8 days measured by Millward (11) in intact rats. In addition, the factors found to reduce proteolysis in the presence of cycloheximide had similar effects on net protein degradation measured without this drug. Similarly our unpublished studies of muscles from fasted or dystrophic animals showed changes in protein degradation that correlate with data obtained by isotopic methods in intact animals.*

Since the incubated muscles studied here were in negative nitrogen balance, the factors found to influence protein turnover may not do so in the intact organism. However, the factors that promoted synthesis and inhibited degradation in the incubated diaphragm have long been recognized as important for muscle growth in vivo. Insulin is essential for normal growth of muscle, and in fasting or diabetic organisms (1, 31), where muscle wasting occurs, administration of insulin decreases the net release of amino acids from muscle (38). This effect is similar to that seen with incubated diaphragms and has generally been believed to result from a stimulation of protein synthesis (1) and amino acids transport (1, 38). In addition, insulin can inhibit the degradation of protein in muscle (Tables IV to VI), as it also does in liver (13), heart (20), adipose tissue (14), and mouse fibroblasts (15). These various actions of insulin should be additive in promoting the accumulation of protein in muscle. In the incubated diaphragm, the percentage change in degradation (15 to 20%) was actually severalfold smaller than the percentage stimulation of synthesis (30 to 50%); however, since degradation occurred 2 to 3 times more rapidly than synthesis in the untreated tissues, these two actions of insulin had comparable effects on net amino acid release from the tissue.

Since the inhibition of degradation, like the stimulation of synthesis, was demonstrable in un-supplemented KRB buffer, these effects of insulin also can not be secondary to the hormone's stimulation of glucose or amino acid transport. Presumably the various effects of insulin are dependent on some single common intracellular regulatory factor. It has been suggested that insulin may exert its "pleiotropic effects" by lowering intracellular cyclic AMP; however, related experiments with the incubated diaphragm have failed to show a stimulation of proteolysis by cyclic AMP or dibutyryl cyclic AMP.

In the fasting organism, supply of carbohydrates spares the utilization of body nitrogen reserves, which are primarily in muscle protein (2). These effects have been attributed to the release of insulin in response to increased glucose levels; however, the present experiments indicate that glucose supply may also act on muscle directly to inhibit the mobilization of tissue proteins for gluconeogenesis. Glucose by itself reduced protein degradation in diaphragms of fed and fasted rats. Since β-hydroxybutyrate and octanoate, which can also serve as major energy substrates for the diaphragm, did not reduce protein degradation (Table V), glucose is probably not serving simply as a source of ATP. In repeated experiments, glucose by itself was not found to stimulate protein synthesis in muscles of fed or fasted rats, although glucose did so when added in the presence of insulin. Thus glucose and insulin had synergistic actions in stimulating synthesis and additive effects in reducing proteolysis (Table III).

The experiments with glucose (Table IV) documented possible dangers inherent in using data on amino acid incorporation as a measure of protein synthesis. By inhibiting degradation, glucose increased intracellular specific activity of labeled precursors and thus caused increased incorporation of radioactivity into protein. This effect, without corrections for precursors' specific activity, would give the impression of enhanced protein synthesis. Such complications can be minimized, if the labeled precursors are present in the incubation medium in high concentrations (at least plasma levels), such that release of nonradioactive amino acids from tissue proteins has less influence on intracellular and extracellular specific activities. Interestingly, previous studies on the rat diaphragm have disagreed as to whether glucose can promote amino acid incorporation (1, 16). Those studies reporting increased incorporation of labeled amino acids (16) used only trace amounts of precursor and thus their findings probably resulted from the reduced degradation.

Although the addition of serum amino acids to the incubation medium increased rates of protein synthesis and decreased degradation in a dose-dependent fashion (Table VI), the branched chain amino acids alone appeared responsible for these effects (Table VII). Supply of all other amino acids together was ineffective against either process. The circulating levels of leucine, isoleucine, and valine vary under different physiological conditions (e.g. fasting) and thus may influence protein turnover in muscle in vivo. It is interesting in this regard that the branched chain amino acids have also been shown to increase production of alanine in muscle and thereby influence the amount of gluconeogenic precursors reaching the liver (32).

Unlike other required amino acids, leucine, isoleucine, and valine are degraded in skeletal muscle at rates comparable to their rates of incorporation into protein (29, 39). It is thus possible that the rapid oxidation of these compounds, which increases in fasting (4) and diabetes (32), may limit the supply of precursors for protein synthesis. In the perfused liver, plasma amino acids have also been found to influence net protein turnover, although the specific amino acids responsible for these effects are not leucine, isoleucine, or valine. Tryptophan appears especially important in controlling hepatic protein synthesis, while proline, methionine, tryptophan, and phenylalanine appear critical in retarding protein degradation (19). These amino acids are all rapidly metabolized in liver, but not muscle, and liver does not degrade leucine, isoleucine, or valine to a significant extent. Thus those amino acids capable of regulating protein turnover in different organs vary in a manner that may be related to their rates of degradation.

It is presently unclear whether the branched chain amino acids themselves or some metabolite is involved in regulating the degradative process in muscle. In bacteria, lack of a required amino acid also leads to increased protein degradation, although this effect appears to result from lack of the corresponding amino acyl-tRNA rather than lack of the amino acid (17). Insufficient supply of any specific charged tRNA prevents synthesis, increases degradation, and causes a variety of other growth-inhibitory changes, such as decreased RNA synthesis (4). Possibly analogous control mechanisms regulate protein catabolism in muscle and other mammalian tissues.

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