Measurement of muscle protein degradation in live mice by accumulation of bestatin-induced peptides

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Botbol, Violeta, and Oscar A. Scornik. Measurement of muscle protein degradation in live mice by accumulation of bestatin-induced peptides. Am. J. Physiol. 273 (Endocrinol. Metab. 36): E1149–E1157, 1997.—Bestatin, an aminopeptidase inhibitor, permits the degradation of cellular proteins to di- and tripeptides but interferes with the further breakdown of these peptides to amino acids. We propose to measure instant rates of protein degradation in skeletal muscles of intact mice by the accumulation of bestatin-induced intermediates. Muscle protein was labeled by injection of L-[guanidino-14C]arginine; 3 days later, maximum accumulation of intermediates was measured in abdominal wall muscles 10 min after the intravenous injection of 5 mg of bestatin. The peptides were partially purified and hydrolyzed in 6 N HCl, and the intravenous injection of 5 mg of bestatin. The peptides were partially purified and hydrolyzed in 6 N HCl, and the radioactive peptide in peptide-derived arginine was determined, after conversion to 14CO2 by treatment with arginase and urease. The measurement of bestatin-induced intermediates provides a unique tool for studying acute changes in muscle protein turnover in live mice. We observed a 62% increase in muscle protein breakdown after a 16-h fast, which was reversed by refeeding for 3.5 h, and a 38% increase after 3 days of protein depletation.

protein turnover; peptide intermediates

SKELETAL MUSCLE contains almost one-half of the total body protein. The regulation of muscle protein turnover plays a major role in the maintenance of amino acid pools during nutritional deprivation. Studies in isolated and perfused muscle (9, 12, 16, 17) have suggested an important role of protein degradation in the regulation of muscle protein metabolism, but information on intact animals is limited. Whereas instant rates of protein synthesis can be measured in the muscles of intact animals (13, 25, 27), direct measurement of rapid changes in protein breakdown has not been possible until now. In this paper, we propose to measure instant rates of protein degradation in skeletal muscles of live mice by the accumulation of bestatin-induced peptide intermediates. Because measurable accumulation takes only 10 min, it provides a unique tool for the study of very rapid and transient changes in muscle protein turnover, such as those produced by hormones, nutrients, or exercise.

The rate of disappearance of labeled proteins from tissues of intact animals is not a reliable measure of protein degradation. It is affected by reincorporation of the radioactive amino acid, except in liver, where the high activity of the urea cycle provides a unique opportunity to label the metabolically unstable guanidino-C of protein-bound arginine (28). At best, this approach requires prolonged observations, which precludes its use during acute changes. In sustained conditions, muscle protein degradation can be estimated by the balance between synthesis and protein gain (or loss). During short-term effects, however, changes in protein content are too subtle to measure and can only be estimated from net uptake or loss of nonmetabolizable amino acids determined by regional catherization techniques, possible only in humans and large animals (2). The urinary excretion of 3-methylhistidine has also been used as a measure of muscle protein degradation (34). It reflects, however, only the breakdown of contractile elements in muscle, which is much slower than overall muscle protein degradation, and is insensitive to insulin and lysosomal inhibitors (3, 17, 18). It may also derive from 3-methylhistidine-containing proteins in other tissues (21, 22, 31).

The procedure proposed here is based on our finding that bestatin, an aminopeptidase inhibitor, permits the degradation of cellular proteins (by either the cytosolic or the autophagic pathways) to di- and tripeptides but interferes with the further degradation of these peptides to amino acids (4–7). We use the accumulation of bestatin-induced peptides as a measure of protein breakdown (8). Although this principle works well with liver (8, 10, 27), it becomes applicable to muscle only after drastic modifications in both the choice of labeled precursor and the purification of the peptides. We describe here the procedure for skeletal muscle and some of the results obtained in mice under different nutritional conditions.

EXPERIMENTAL PROCEDURE

Materials. Synthetic bestatin (Ubenimex), N-[(2S,3R)-3-amino-2-hydroxy-4-phenylbutanoyl]-L-leucine, was a generous gift of Nippon Kayaku (Tokyo, Japan). It was dissolved for injection in phosphate-buffered saline at a concentration of 5 mg/ml. Tritiated bestatin, labeled on a portion of the 4-[2-'H]-phenyl moiety (343 µCi/µmol), was purchased from the same company. L-[4,5-'H(N)]leucine (5 mCi/µmol), L-[guanidino-14C]arginine (51.5 µCi/µmol), and [14C(U)]sucrose (4.95 µCi/µmol) were obtained from DuPont NEN (Boston, MA). Dulbecco’s phosphate-buffered saline, in powder form, was purchased from Gibco Laboratories (Grand Island, NY). Unless indicated otherwise in the text, other chemicals were purchased from Sigma Chemical (St. Louis, MO).

Mice. Fully grown, male CD-1 mice, 36–38 g, were purchased from Charles River Laboratories (Wilmington, MA). They were kept in a room illuminated from 6 AM to 6 PM and were fed ad libitum for 5 days a custom-made “4% casein” pellet diet (ICN Biochemicals) containing 4% casein, 0.3% methionine (the limiting amino acid in casein), 55% corn starch, 27% dextrose, 5% corn oil, 5% cellulose fiber (alaphacel) and standard supplements of vitamins and minerals. Protein depletion was established by shifting these animals to a “protein-free diet,” in which the casein and methionine were replaced by starch (27).

Intravenous injections. For intravenous bolus injection, each mouse was placed in a 50-ml conical plastic tube with the end cut out just enough to permit protrusion of the tail.
The tail was immersed in water at 45°C for 1 min or more, until the lateral veins were clearly visible. The solutions were injected into one of these veins at a rate of 100 µl/s with a disposable 1-ml insulin syringe through a 28-gauge 13-mm needle (Micro-fine-IV, Becton-Dickinson, Franklin Lakes, NJ).

Collection of abdominal wall muscles. For the measurements of protein synthesis and degradation we collected abdominal wall muscles. These are abundant, require minimum dissection, contain no bones or discrete tendons, and can be collected very rapidly and clamped frozen with ease (the equally abundant leg muscles require the removal of the leg bones, which takes several minutes). Another advantage of abdominal wall muscles is that, compared with limb muscles, their contraction is not as widely affected by bursts of activity in the unrestrained animal. Finally, as will be described, the abdominal wall muscles are responsive to changes in the nutritional status of the animal. In other experiments, we have found that both the rates of protein synthesis and their response to food are essentially identical in the abdominal wall muscles and the combined leg muscles of the mouse (27).

Each mouse was stunned with a blow to the head and killed by decapitation with large scissors. While the animal was being held, the paws and tail were cut off and the ventral skin was rimed open. The animal was skinned, fat pads still adhering to the abdominal surface were removed, and the skinned carcass was sunk in ice-slush, 30 s after decapitation, for further dissection. The white ventral midline was cut open from the pubic crest to the lower portion of the sternum, and each side was cut along the inguinal, lumbar, and pectoral insertions. Surface water and ice were wiped out of each portion by tapping it down on a paper towel, from which it was picked up by pressing onto it a porcelain pestle previously cooled in dry ice. The frozen muscle was detached, wrapped in aluminum paper, and kept in dry ice until the completion of the experiment. Both sides of the abdominal wall were collected in <1 min (30 s each side). The wall comprised the externus and internus obliquus, transversus and rectus abdominis, and some of the overlapping muscles at the insertions. Both sides combined weighed 1.2–1.4 g.

[3H]bestatin uptake. The measurement of bestatin uptake in muscle was explained in detail elsewhere (26). For these experiments mice were killed by decapitation under ether anesthesia, 10 min after the intravenous injection of 5 mg of bestatin, with 1 µCi of [3H]bestatin and 0.1 µCi of [14C]sucrose. Blood was collected and the plasma separated by centrifugation. We sought to minimize muscle extracellular radioactivity by a short perfusion of the hindquarters through the abdominal aorta with a saline solution (phosphate-buffered saline, 11 mM glucose, 100 mM sucrose, and 10 µM sodium nitroprusside as a vasodilator agent). The hindquarters were favored over the abdominal wall in this experiment, because for the latter the multiple arterial supply makes the perfusion ineffective. Three muscle groups, the muscles of the right and left thighs and both gastrocnemii, were dissected and processed separately. The plasma and the muscle samples were extracted overnight with 2 vol of trichloroacetic acid, and the acid-soluble [14C] and [3H] radioactivities were determined by scintillation counting. Extracellular [3H]bestatin in muscle was calculated from the remaining [14C]sucrose, and the diffusible [3H]bestatin in plasma was determined by differential dialysis (26).

Purification of peptides. Peptide intermediates were measured 3 days after subcutaneous injection of 20 µCi of L-[guanidino-14C]arginine, in trace amounts. For the measurement of bestatin-induced peptides, mice were killed 10 min after the intravenous injection of 5 mg of bestatin, and the abdominal wall muscles were collected as we have described. The following procedure (Fig. 1) has given satisfactorily low background and good recovery of bestatin-induced peptides. The reasons for the modifications to our earlier procedure (8) are explained in Discussion.

Both halves of the abdominal wall muscle, kept frozen until the completion of the experiment (up to 1 h), were weighed, transferred to a 15 × 150-mm glass tube with 7 vol of ice-cold 10% trichloroacetic acid, and extracted overnight in the refrigerator: (grinding the frozen tissue to a powder in a mortar with dry ice did not improve the extraction of peptides). The supernatant was saved for the purification of peptides. The extracted tissue was further processed for the determination of protein radioactivity. Free arginine in the acid extract, 4.8 ml (representing 0.6 g of tissue), was decarboxylated by ninhydrin treatment (0.8 ml, 90 mg/ml ethanol, 4 h at 90°C, followed by a drop of 30% H2O2 for 1 min before cooling in tap water to destroy ninhydrin). The purpose of ninhydrin treatment in the present procedure is to convert arginine to its aldehyde derivative (14), insensitive to the specific enzymatic hydrolysis by arginase. Lipids, trichloroacetic acid, and colored substances were extracted three times with 1 vol of ether. The aqueous phase was filtered through glass wool and percolated through a 30 × 7.4-mm column of AG50-X2-H+ . The fraction was then rinsed with 5 ml of 10 mM urea (to facilitate elution of [14C]urea, the major radioactive contaminant in the original extract), followed twice by 5 ml of water, and the peptides were eluted with 5 ml of 1 N NH4OH and dried in vacuo in a Speed-vac evaporator. The dry residue was redisolved in 0.4 ml of 6 N HCl and hydrolyzed in vacuo in a sealed ampoule at 107°C for 16 h. The acid was again evaporated, and the residue was dissolved in 0.5 ml water.

Arginine derived from the peptide hydrolysate was purified as follows (28). Dry AG1-X2-bicarbonate (0.1 g or more) was added until pH was greater than 4 (pH paper, range 3–5.5). The fluid and suspended resin (and 2 rinses of 0.5 ml H2O) were then percolated in a column (7.4 mm diameter) through a 3-mm layer of AG1X2-OH . Brown color produced during hydrolysis was retained by the resin. The volume of the collected clear solution was ~1.6 ml.

In the next step, the guanidino-[14C] of arginine was converted (as described in detail elsewhere (26)) to [14C]urea, 400 U/ml H2O) and was incubated for 30 min at 37°C. Before hydrolysis of the peptide-derived arginine, to eliminate any residual [14C]urea, the tube received 1 drop of freshly prepared urease (Sigma Chemical U1–500, from jack beans, 400 U/ml H2O) and was incubated for 30 min at 37°C. We then added 4 drops of 1 N HCl (to eliminate excess alkali, as shown by the indicator), and 3 drops of freshly prepared 1 N sodium bicarbonate. After vigorous elimination of the resulting CO2 in a Vortex mixer, the indicator turned pink (pH 8.0–8.3). Before hydrolysis of the peptide-derived arginine, to eliminate any residual [14C]urea, the tube received 1 drop of freshly prepared urease (Sigma Chemical U1–500, from jack beans, 400 U/ml H2O) and was incubated for 30 min at 37°C. We then added 4 drops of 1 N HCl, incubated for 1 h at 90°C to inactivate the enzyme, and brought the pH up again with 4 drops of 1 N sodium bicarbonate. Tubes were cooled in tap water, received 1 drop of arginase (Sigma Chemical A-8013, from bovine liver, 200 U/ml in 25 ml Mn-maleic buffer, pH 7.3, stored frozen), and were incubated for 2 h at 37°C, with the addition of 1 drop of 10 mM arginine midway into the incubation (to ensure complete hydrolysis of the radioactive substrate). Finally, we added 1 drop of 50 mM EDTA, pH 7.3 (which removed Mn2+, necessary for arginase activity but inhibitory to urease).

In the final step, [14C]urea (the product of arginase in the previous incubation) was converted by urease to [14CO2], which was trapped in an alkaline solution. This required a closed
**Fig. 1.** Schematic comparison of purification of peptides in liver and muscle. For a full description of the procedure for liver, see Ref. 8. Overall purification recovery for liver was 88% (SD/mean = 0.015) and for muscle, 47% (SD/mean = 0.038). TCA, trichloroacetic acid; 1 d and 3 d, 1 and 3 days. (See EXPERIMENTAL PROEDURE.)

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A system consisting of an ad hoc polyethylene disk (61 mm in diameter, 12 mm high) fit snugly in the bottom of a 125-ml polycarbonate screw-cap straight-side jar (Nalgene 2116-0125, Nalge, Rochester, NY). A hole, 27.5 mm wide, in the center of the disk housed a 20-ml plastic scintillation vial containing 1 ml of the trapping solution, 2-methylaminoethanol-water (1:5). Incubations were carried out in a moat (11 mm wide, 9 mm deep) in the periphery of the disk. The moat was first carved with a drill all around to a depth of 6.5 mm and then to the full depth of 9 mm in two unequal sectors (the major sector 5 times longer than the minor one) separated by lips (2.5 mm high, 4 mm thick). The incubation mixture was transferred to the major sector of the moat, and the minor sector received two drops of 2 M sulfuric acid. To start the incubation, two drops of the urease solution were added to the incubation mixture, and the jar was capped immediately. The solution was moved away from the minor sector by tilting the jar, mixed by gentle rocking, incubated at room temperature for 2 h, and finally mixed with the sulfuric acid by tilting and rotating the jar (successful mixing was visualized by the change in indicator color). The resulting $^{14}$CO$_2$ was allowed to diffuse into the trapping solution overnight, and 10 ml of scintillation fluid were added to the vial for counting.

For best comparison, all samples from a single experiment were processed together. It took a single operator 1 wk to process all 60 samples from the four different conditions shown in Fig. 3.

Recovery of peptides. Peptides were prepared by proteolytic digestion of arginine-labeled protein. The protein was obtained from muscles of a mouse injected intravenously 30 min before with 100 µCi of L-[guanidino-$^{14}$C]arginine. The abdominal and leg muscles were ground in dry ice, and a protein powder was prepared by extractions with hot trichloroacetic acid and organic solvents, as described in Protein radioactivity. The powder was suspended (10 mg/ml) in 0.1 M sodium bicarbonate, with 0.05% sodium azide, and incubated at 37°C with trypsin and chymotrypsin (0.1 mg/ml each). Fresh enzymes were added 24 h later, and the incubation continued for another day. The solution was dialyzed twice against 5 vol of water (Spectra/por mol wt cutout 12,000–14,000). The dialyzate was percolated through a 30 $^{3}$7.4-mm column of AG50-X2-H$^{+}$, and the peptides were eluted with 5 ml of 1 N NH$_4$OH and dried in vacuo. Free arginine was eliminated by treatment with arginase and urease, as described in the previous section. The peptides recovered after this treatment were used to determine their recovery when added to nonradioactive muscle acid extracts and carried through various steps of the purification procedure. Overall purification recovery in eight samples was 47% (SD/mean = 0.038). Recovery at other steps is described in Fig. 1. The prolonged acid ninhydrin treatment, necessary to remove any trace of free arginine, resulted in a loss of 40% of the peptide-derived arginine. We do not know whether the loss was due to deamination of NH$_2$-terminal arginine or to other secondary reactions. For comparison with the procedure for liver (Fig. 1), [1-$^{14}$C]arginine-labeled peptides were prepared in the same manner, from the liver of a mouse injected with 90 µCi of [1-$^{14}$C]ornithine. Carboxy-labeled arginine, ornithine, and citrulline were eliminated by acid-ninhydrin treatment before the AG50 step. The peptides were added to nonradioac-
tive extracts and purified through the procedure previously described for liver (8). Recovery in six samples was 88% (SD/mean = 0.015).

Protein radioactivity. In the protein degradation protocol, after acid extraction of the peptides, we were left with two halves of abdominal wall muscle. The acid treatment hardened the tissue, which permitted successive extractions without need for centrifugation. One of the two portions was transferred to a preweighed 13 × 100-mm glass tube, extracted with hot trichloroacetic acid (90°C, 30 min) and ethanol-ether-chloroform (2:2:1), dried overnight at 90°C, and suspended in 10% N-lauroylsarcosine sodium salt in 1 M bicarbonate (5 ml/g fresh tissue). The tissue was fully dissolved only after prolonged incubation (up to 2 days) with the detergent at 90°C. The evaporated water was replaced, and a 0.2-ml aliquot was mixed with 0.8 ml 0.125 M HCl and 10 ml of scintillation fluid for counting. The same procedure was followed in the protein synthesis protocol, except that the acid-hardened tissue was first soaked twice for 1 h each time in 50 ml H2O to help remove excess radioactive leucine, and an additional extraction with acetone was added at the end to eliminate residual water. The resulting protein was measured by weight and dissolved at a concentration of 50 mg per milligram of the alkaline detergent solution.

Protein synthesis. Protein synthesis was determined by the incorporation into protein of radioactive leucine, after its injection in massive amounts, as described in the companion article (27).

Scintillation counting. Samples were mixed with 10 ml of scintillation fluid (Liquiscint, National Diagnostics, Atlanta, GA) and counted in an LKB 1209 Rackbeta Scintillation Counter. In each run, internal standards were added to blanks under the same conditions to determine counting efficiency as well as channel crossover when both isotopes were counted in the same sample.

RESULTS

For the degradation of constitutive long-lived muscle proteins, we injected 20 µCi of L-[guanidino-14C]arginine subcutaneously and allowed for a 3-day chase. All the experiments include a group of control mice in which bestatin was omitted, and the resulting background peptide radioactivity was subtracted from values in the experimental group.

Dose response and time course of peptide accumulation. The accumulation of peptide intermediates best represents rates of protein degradation if their accumulation is linear with time and the injected dose of bestatin is sufficient for maximum accumulation of peptides. These conditions were met 10 min after the intravenous injection of 5 mg per mouse, as shown in Fig. 2. In these experiments we used fasted animals, in which degradation is fastest and bestatin least effective (see DISCUSSION). The dose selected was twice as large as that necessary for maximal accumulation.

Effects of a meal on muscle uptake of [3H]bestatin. Uptake of bestatin in muscle is much slower than in liver and probably results from passive diffusion across the plasma membrane (26). We were concerned that circulatory changes, produced by an abundant meal, could divert blood away from skeletal muscle enough to reduce the supply of bestatin to muscle cells. We decided to measure this directly; each animal received an injection of 5 mg of bestatin, with [3H]bestatin and [14C]sucrose (as a nonpermeant extracellular marker), and was killed 10 min later. At this time, extracellular [3H]bestatin far exceeded the [3H]bestatin taken up by muscle cells. Bestatin uptake could be measured only after most of the extracellular drug was eliminated by a brief perfusion. Because abdominal muscles receive their blood from different arterial sources, they did not lend themselves to efficient perfusion. For this reason, we turned in this particular experiment to leg muscles, which could be effectively perfused through the abdomi-
nal aorta. The procedure is briefly described in EXPERIMENTAL PROCEDURE and discussed in detail elsewhere (26). The results of this experiment are presented in Table 1. Intracellular bestatin concentration was found to be significantly lower (80%) in the leg muscles of refed mice. This was not due, however, to a less efficient uptake of bestatin by muscle. Rather, the difference was entirely attributable to a similarly lower concentration of diffusible bestatin in plasma (78%). This difference was also apparent with the nonpermeant [14C]sucrose, which was lower in refed animals as well (69%). In the experiments of this study, we compensated for these effects by injecting into fasted animals 80% as much bestatin as that injected into the fed ones.

Conversion of radioactive intermediates to rates of degradation. Although values of synthesis or degradation obtained by our procedures are useful measurements of relative changes, they cannot be compared with each other directly. Protein synthesis is measured as micromoles of leucine per unit time and can be converted to milligram protein per day; protein degradation is obtained as a fraction of the labeled proteins degraded per unit time. If the proteins were uniformly labeled, this value could be converted directly into degradation per unit time. If the proteins were uniformly labeled, this value could be converted directly into milligram protein per day. The labeling protocol intends to approximate this condition by eliminating the most rapidly labeled proteins by a prolonged chase, but some proteins (notably collagen, and possibly contractile proteins in muscle) are metabolically very stable and may participate very little in this turnover. The measured rate of breakdown of long-lived labeled proteins is proportional to total degradation but does not measure it directly. To calculate actual protein degradation, we must calibrate the measured rates by balance, where sustained conditions permit it.

Balance measurement may be complicated by superimposed diurnal food-related fluctuations in the protein content of the tissue and its rates of protein synthesis and degradation. In these cases, calculation of balance are still possible but require the average of several measurements of synthesis and degradation throughout the day. Fortunately, in our current work (27) we are interested in two conditions in which this is not necessary.

The first condition is that of fully grown mice, adapted for 5 days to a synthetic diet containing 4% casein supplemented with 0.3% methionine. This diet provides a slight excess over the minimum obligatory N loss (27) and is sufficient to maintain the weight of the animals unchanged for periods of 2 wk. We observed that these mice tended to space the intake of food throughout the day, and we found that diurnal variations in the rates of muscle protein synthesis were minimal. In one experiment, we measured protein synthesis in three groups of seven mice each, at 8 AM, 4 PM, and midnight. They were, respectively, 100 ± 3, 97 ± 3, and 104 ± 2% of their average. In this condition, where balance was zero and there were no daily fluctuations in turnover, we considered that the fractional rate of degradation was equal to the fractional rate of synthesis, 4.4% per day (Fig. 3). In the same condition, 10 min after injection of bestatin (as in Fig. 2), we found 0.096 ± 0.012 peptide disintegrations·min⁻¹·dpm⁻¹, 1,000 protein dpm⁻¹ above control (see values for these and the other conditions in Fig. 3 legend). Once corrected for the 47% of the purification recovery (see EXPERIMENTAL PROCEDURE) this translates to 2.94% of the radioactive protein per day. This rate of breakdown of radioactive long-lived proteins is 0.67 of the actual degradation rate, 4.4% per day. In all the other calculations in Fig. 3, we used 0.67 as a conversion factor; relative rates of peptide accumulation were divided by 0.67 to estimate absolute rates of protein breakdown.

The second sustained condition is the 3-day protein depletion shown by the fourth set of columns in Fig. 3. In this group, compared with the 4% casein controls, muscle protein synthesis was 48% lower and degradation 38% faster. Using the same calibration factor, we calculate that the fractional rate of degradation exceeds that of synthesis by 3.3% per day, which agrees closely with the actual loss of 3.0% per day estimated by repeated measurements of abdominal wall muscle protein from 2 to 8 days after the shift to the protein-free diet (27). The agreement between the estimated balance and the actual protein loss confirmed the calibration factor and gave us confidence in the calculations.

Effects of fasting and refeeding. Whereas in the sustained conditions just described protein breakdown can be measured by balance, that is not possible in acute changes. It is here that our procedure becomes uniquely useful. To illustrate this point, we demon-
stratge in Fig. 3 that the accumulation of bestatin-induced peptides permits the direct measurement of the acute effect of a meal on muscle protein breakdown. The second set of columns in Fig. 3 shows that, after an overnight fast, protein synthesis decreased by 53% and protein degradation increased by 62%. These changes were reversed by refeeding for 3.5 h, as shown in the third set of columns. The difference between rates of synthesis and degradation indicates an estimated loss of muscle protein of 5.1% per day after fasting for 16 h, fully suppressed after refeeding.

**DISCUSSION**

Purification of peptides. The procedure proposed here is based on our finding that bestatin, an aminopeptidase inhibitor, permits the degradation of cellular proteins (by either the cytosolic or the autophagic pathways) to di- and tripeptides but interferes with the further degradation of these peptides to amino acids (4–7). We use the accumulation of bestatin-induced peptides as a measure of protein breakdown. This principle works well with liver (8, 10, 27), but it becomes applicable to muscle only after drastic modifications in both the choice of labeled precursor and the purification of the peptides. In fully grown mice, muscle protein turnover (and hence the accumulation of intermediates) is an order of magnitude slower than in liver (27). Our major challenge was to devise a practicable purification protocol that lowers the background enough to permit an acceptable signal-to-noise ratio. With isolated hepatocytes and the livers of live mice, we have previously found that arginine-labeled peptides are less likely to escape from the cells (7, 8). For the preferential labeling of liver protein, we use as precursor carboxy-labeled ornithine, which is converted to carboxy-labeled arginine by the hepatic urea cycle (8). At present, carboxy-labeled arginine is not available commercially. Here, we use [guanidino-14C]arginine, which is incorporated into protein more efficiently in muscle than in liver, where the label is lost by its rapid metabolic turnover in the urea cycle (28). Its disadvantages are that some of the label ends up in the guanidino-C of creatine in muscle, and that, because we no longer use here a carboxy-labeled precursor (as with liver), we cannot eliminate the radioactivity in free arginine or creatine by the acid-ninhydrin decarboxylation. Thus, in muscle, the purification scheme used for liver (8) results in a highly radioactive mixture of decarboxylated arginine and creatine that masks the small amount of peptides present. To separate the peptide-bound arginine from the other contaminants, the peptides are then hydrolyzed in 6 N HCl, and the peptide-derived [guanidino-14C]arginine is subjected to enzymatic treatment with arginase (which yields radioactive urea) and urease (which decomposes urea to ammonia and radioactive carbon dioxide). The 14CO2 is captured and counted. To better appreciate the changes introduced here, this procedure is compared in Fig. 1 with that previously described for liver. With the new procedure, muscle from mice not injected with bestatin exhibit values about fivefold lower than in liver, which permits the detection of the much smaller accumulation of peptide intermediates in muscle.

Conversion of accumulated intermediates to rates of degradation. At present, the only alternative measure of muscle protein degradation in intact animals is by balance (see introductory comments). This is possible only in sustained conditions, where changes in protein content can be measured reliably over a long period of time. The experiments presented in this and the companion article (27) include two such conditions. We calibrated the rate of degradation against the rate of
synthesis in zero growth conditions (4% casein diet). Using the same calibration factor, we calculated that the fractional rate of degradation exceeds that of synthesis by 3.3% per day, which agrees closely with the actual protein loss of 3.0% per day (27).

For the acute changes of fasting and refeeding (Fig. 3) balance is not possible; changes in protein content are too subtle to measure. We have shown before, in livers of intact mice as well as isolated hepatocytes and cultured cells, that acute changes in the rate of protein degradation and nutritional and hormonal conditions of the cells do not affect their sensitivity to bestatin (7, 8). In this study, we minimized the uncertainty by using an excess of the drug (Fig. 2). Ideally, we should have a dose-response curve for each situation. Given the work-intensive nature of this measurement, we limited ourselves to fasted animals, where the rate of protein degradation is highest, on the basis of the following reasoning. The accumulation of intermediates depends on three factors. 1) The first factor is the intracellular concentration of bestatin. A meal had no effect on bestatin uptake (Table 1), other than a somewhat lower extracellular concentration, which was corrected by injecting a correspondingly lower dose. Because after their intravenous injection [14C]sucrose and most of the [3H]bestatin are distributed in the extracellular fluid and eliminated in the urine (26), the higher plasma concentrations of bestatin and sucrose in fasted mice could be due to a contraction of the extracellular space, a decreased glomerular filtration, or both. This is probable, because the fasted animals had been deprived not only of dietary fuels but electrolytes as well. 2) The second factor is the concentration of exopeptidases. When the fact that muscle turns over only a small fraction of its protein per day is considered, short-term changes in levels of cytosolic exopeptidases are unlikely. 3) The third factor is the concentration of peptides. The level of accumulated peptides is important, because bestatin inhibits exopeptidases competitively with their substrates (11, 29). We reasoned that if a 5-mg amount of bestatin was twice as large as necessary for maximum accumulation of intermediates in fasted animals, where the rate of protein degradation is highest, it was more than sufficient in fed animals. For other muscle groups, in which concentration of peptidases may be different, or in other conditions, in which the rate of degradation is faster (such as prolonged starvation or the more rapidly turning over muscles of growing animals), the dose-response curve should be repeated.

Even under optimal conditions, the intracellular accumulation of degradation intermediates may be less than complete for at least two reasons. First, a portion of the peptides may be degraded by bestatin-insensitive exopeptidases. In a cell-free reticulocyte system, we have previously shown that bestatin produces accumulation of not more than 80% of degradation intermediates (5). Of the best-known mammalian cytosolic exopeptidases, three (leucyl, alanyl, and arginyl aminopeptidases) are very sensitive to inhibition by bestatin, but two (aspartyl and prolyl aminopeptidases) are not (19). Second, we have shown before, in incubated hepatocytes, that 20% of the arginine-labeled peptides escape the cells (7). On the basis of these numbers, maximal accumulation of peptides may be no larger than 64% of degradation (80% accumulation × 80% retained by the cell). In livers of starved animals, we have also shown that the recovered peptides (uncorrected for purification recovery) represented 55% of the disappearing arginine-labeled proteins (8). When it is corrected for a recovery of 88% (Fig. 1), we can now recalculate that earlier value to represent 62% of the degradation products, which compares well with the maximum 64% calculated above. Thus, it seems likely that maximal inhibition by bestatin results in the intracellular accumulation of a subset of peptide intermediates. This subset may not include those with proline and acidic amino acids in the NH₂-terminal position, and the most hydrophobic ones, escaping the cells. Given the variety of proteins being degraded, and our previous observations in liver and cultured cells that acute stimulation of proteolysis did not affect the accumulation of intermediates (7), it seems unlikely that the average composition of di- and tripeptides will be significantly different during acute changes. Until we accumulate more experience with this procedure, however, conversion to absolute rates should be restricted to experiments including a sustained condition, where recalibration by balance is possible.

Fasting, refeeding, and protein depletion. The changes shown here in muscle protein synthesis after fasting and refeeding are consistent with previous studies in rats (20). Measurements of urinary excretion of 3-methylhistidine in young rats have also shown a stimulation of the degradation of actomyosin by starvation. Urinary 3-methylhistidine-to-creatinine ratios increased by 50% 8 h after food deprivation and up to threefold after 2 days of starvation, and the increase was reversed by refeeding (24, 32).

Ours is the first direct evidence for increased muscle protein degradation during protein depletion. Previous studies in rats revealed a progressive decrease in 3-methylhistidine excretion during protein restriction (15, 23, 30). There are, however, two major differences in the design of the previous experiments and that of ours. First, 3-methylhistidine excretion reflects only the breakdown of contractile elements in muscle, which is much slower than overall muscle protein degradation and is insensitive to insulin and lysosomal inhibitors (3, 17, 18). A second important difference is that their subjects were very young, rapidly growing rats. This is significant because muscle protein turnover is much faster in young animals, probably because growth of this highly structured tissue requires extensive remodeling (33). Muscle growth was delayed (30) or reversed (15) during protein deprivation. Thus, in young rats, dietary restrictions were likely to produce decreases in muscle protein turnover because they stopped muscle growth. The resulting slowdown of actomyosin breakdown could have masked any increase produced by the catabolic condition itself. Our success in measuring increased muscle protein breakdown in protein depriva-
tion is facilitated by the choice of fully grown mice, adapted to a diet with a low but adequate protein content. In these nongrowing, balanced conditions, muscle protein turnover is at its lowest, and increased muscle breakdown after protein deprivation may be more easily revealed. Resolution of these issues will require further investigation.

It is noteworthy that the combined abdominal wall muscles, which we chose for practical reasons (see EXPERIMENTAL PROCEDURE), respond well to these nutritional changes. This is important because, at least as measured by protein synthesis, selected muscle groups can exhibit responses of different intensity; muscles containing higher proportion of oxidative slow-twitch fibers are the least responsive (1). Muscles in which one type of fiber predominates are, however, relatively rare. Also, we have found that both the rates of protein synthesis and their response to food are essentially identical in the abdominal wall muscles and the combined leg muscles of the mouse (27). One of the advantages of abdominal wall muscles, compared with limb muscles, is that their contractile activity is less likely to vary widely in the unrestricted animal. For studies of effects of contraction (for instance in an exercise wheel), the limb muscles may prove more appropriate.

Whereas measurement of protein degradation by the accumulation of bestatin-induced intermediates may prove useful in comparing sustained conditions, it is in the study of acute changes, such as those produced by nutrients, hormones, or exercise, where its value becomes unique. This is exemplified by the effects of a single meal on protein turnover, shown in the refed group in Fig. 3. Because we measure the accumulation of peptides in just 10 min, we expect that the procedure presented in this article will contribute a significant tool in the study of rapid and transient changes in muscle protein metabolism in intact animals.

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