ABSTRACT:

Bestatin, a dipeptide analog, is a potent aminopeptidase inhibitor of bacterial origin. We have previously shown that bestatin inhibits cytosolic exopeptidases in mammalian cells, and results in the accumulation of di- and tripeptide intermediates in cellular protein degradation. Our primary interest is the uptake of bestatin in liver and muscle, 10 min after its intravenous injection into mice. In this short interval, peptide intermediates accumulate linearly in these tissues and permit an estimate of their rates of cellular protein breakdown. Male, CD-1 adult mice received the intravenous injection of 3H-bestatin and 14C-sucrose. The disappearance of 3H-bestatin from the plasma, when normalized by the injected radioactivity, was indistinguishable from that of 14C-sucrose. They both drop rapidly during the first 10 min after the injection, followed by a slower exponential disappearance of 3.4% per min, which extrapolates to an apparent volume of distribution of 25 ml/100 g body weight. In two mice, 3 hr after the injection, the urine contained 77.4% and 79.8% of the injected 14C-sucrose, and 70.9% and 73.9% of the injected 3H-bestatin. Other mice were killed 10 min after the injection of 5 mg of bestatin, and the concentration of 3H-bestatin and 14C-sucrose was determined and subtracted from the total to estimate the cellular uptake of bestatin. Bestatin was taken up readily in the liver (383–452 µg/g), kidneys (175–191 µg/g), and intestine (137–179 µg/g), but much less in red cells (11 µg/g) or skeletal muscle (4.8 µg/g). Bestatin also entered slowly into erythrocytes in vitro (0.3%/min) by a nonsaturable process. It is suggested that bestatin is taken up through transporter-mediated processes in some cells but not others.

Bestatin (Ubenimex), a dipeptide analog, is a potent aminopeptidase inhibitor, first isolated from a culture filtrate of Streptomyces olivoreticuli (1). It has immunoenhancing properties with low toxicity in humans and experimental animals (see refs. 2–4). We became interested in this antibiotic because of our finding that, in mammalian cells, bestatin permits degradation of cellular proteins to di- and tripeptides, but inhibits the further degradation of these peptides to amino acids (5). This effect results in the cytosolic accumulation of intermediates in the breakdown of proteins by either the cytosolic or autophagic pathways, during normal protein turnover, degradation of abnormal proteins, or stimulated degradation of long-lived cellular components under catabolic conditions (6–8). More recently, we proposed that, after intravenous injection of bestatin, the rapid accumulation of peptide intermediates in the liver (9) and skeletal muscle (10) of live mice can be used to estimate instant rates of cellular protein breakdown in these tissues.

When assayed with purified cytosolic exopeptidases, bestatin is effective at nanomolar concentrations (11). The accumulation of peptide intermediates in mammalian cells, however, requires much higher extracellular concentrations. For instance, in reticulocyte lysates, bestatin resulted in accumulation of peptides during degradation of abnormal globin, with concentrations as low as 0.5 µg/ml. By contrast, in intact reticulocytes, it required concentrations in the incubation medium three orders of magnitude higher (5, 6). We assumed that the reticulocyte plasma membrane has a limited permeability to bestatin (6), but until now we had not measured it directly.

Information on cellular uptake of bestatin is limited. Because stimulation of lymphocyte proliferation is thought to result from the interaction of bestatin with enzymes exposed to the surface of cells (12, 13), the primary interest has been to reach effective concentrations of the drug in the extracellular fluid (3). After its intravenous injection into mice, bestatin disappears from the circulation rapidly (3). When given orally, bestatin is absorbed effectively in the intestine, via the H7/dipeptide transport system of the brush border (14, 15). After oral administration to humans, bestatin is largely recovered in the urine after 1 or 2 days (16, 17), most of it unmodified, except for a minor fraction (<5%), which is recovered as the active para-hydroxy-derivative. We only know of one autoradiographic study, one or more hours after the intravenous injection of 3H-bestatin to mice, which reports higher concentrations of silver grains in macrophages, in hepatocytes, in bile duct epithelium, and in the brush border of proximal straight renal tubules (18).

To measure the effects of bestatin in cellular protein degradation in the liver (9) and skeletal muscle (10) of mice, we collect the tissues 10–15 min after the intravenous injection of the drug. It is during this short interval that peptide intermediates accumulate linearly and permit an estimate of the rates of cellular protein breakdown. Maximal accumulation is observed in liver after injection of 1 mg of bestatin/mouse. For muscle, doses 5 times larger are necessary. In the present study, we add to these injections 3H-bestatin, and 14C-sucrose. We use 14C-sucrose as a nonpermeant marker of the extracellular space accessible to 3H-bestatin, and calculate by difference the cellular uptake of bestatin in the liver, kidney, intestine, red cells, and skeletal muscle.
Materials and Methods

Synthetic bestatin (Ubeneimex), N-[25,3R]-3-aminooxy-2-hydroxy-4-phenylbutanoyl-L-leucine, was a generous gift of Nippon Kayaku Co. (Tokyo, Japan). Tritiated bestatin, labeled on a portion of the 4-(2′3′-H) phenyl moiety (343 μCi/μmol), was purchased from the same company. [14C(U)-]Sucrose (4.95 μCi/μmol) was obtained from DuPont-NEN (Boston, MA). Dulbecco’s phosphate-buffered saline, in powder form, was purchased from Gibco Laboratories (Grand Island, NY).

Mice. Male CD-1 mice (36–38 g) were purchased from Charles River Laboratories (Wilmington, MA). They were kept in a room illuminated from 6 a.m. to 6 p.m., with water and food ad libitum, and fasted for 6 hr (see fig. 1 and table 1) or overnight (see table 2) before the experiment.

Injections. Bestatin was dissolved for injection in phosphate-buffered saline at a concentration of 5 mg/ml. 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 solution was injected into one of these veins at a rate of 100 μl/sec.

Disappearance of 3H-Bestatin and 14C-Sucrose from the Plasma. Before the experiment shown in fig. 1, a 2-mm incision was made near the tip of the tail, along one of the lateral veins. Bleeding was stopped by pressing the wound gently, and resumed for collection by rubbing it open and squeezing the tail, along one of the lateral veins. Bleeding was stopped by pressing the experiment shown in fig. 1, a 2-mm incision was made near the tip of the abdominal aorta (just below the renal arteries), with a solution containing phosphate-buffered saline, 11 mM glucose, 100 mM sucrose, and 10 μM sodium nitroprusside (as a vasodilator agent). The perfusion started 7–8 min after death and continued for 7 min at a rate of 7 ml/min. The muscle groups indicated in the table were dissected separately and acid-extracted as described.

Total and Diffusible 3H-Bestatin in Plasma. The trichloroacetic acid-soluble extract contained all of the plasma 3H and 14C radioactivities. Preliminary experiments, however, suggested that not all the 3H-bestatin in plasma is free to diffuse, possibly because of reversible binding of a fraction of the drug to plasma macromolecules. The diffusible fraction, relative to sucrose, was determined by differential dialysis as follows. We used an ad hoc microcell assembly consisting of two pieces of Plexiglas separated by a dialysis membrane (Spectra/por molecular weight cutout of 12,000–14,000; Spectrum Medical Industries, Inc., Los Angeles, CA). Carved into each half were matching circular cells, 2.3 mm deep and 6.1 mm in diameter, that were accessible from the top through a small channel. Using a microsyringe, 60 μl of plasma was injected into one cell and the same volume of phosphate-buffered saline into the opposite cell. Diffusion through the dialysis membrane was allowed to proceed at room temperature for 1 hr. At this time, the fluids were withdrawn, mixed with 2 volumes of 5% trichloroacetic acid (which precipitated the proteins in the plasma), and centrifuged; 100 μl of the acid-soluble extract was used for scintillation counting. The diffusible fraction was calculated as the H3/14C ratio in the saline side divided by the H3/14C ratio in the plasma side. It was determined in duplicates, which agreed within 1.5% of their mean. We then calculated the plasma concentration of diffusible bestatin as the product of this diffusible fraction and the total bestatin content in plasma. In seven different plasma samples, the diffusible fraction ranged from 60 to 74% of the total bestatin. This range is consistent with the results of bestatin uptake by erythrocytes in vitro; uptake of bestatin from plasma was 61–73% of the uptake from a saline solution (see Results).

Entry of 3H-Bestatin into Red Blood Cells in vitro. The experiments shown in fig. 2 and table 3, 100 μl of whole blood, or a 1:1 suspension of red blood cells in buffer (phosphate-buffered saline, 10 mM glucose, and 50 mM sucrose), was incubated at 37°C with 3H-bestatin, 14C-sucrose, and nonradioactive bestatin as described in the figure legends. At the end of incubation, the extracellular fluid was diluted 100-fold with buffer, and 1 ml of the diluted cell suspension was layered in a 1.5 ml Eppendorf tube, over a cushion of 0.5 ml of 0.5 M sucrose, and centrifuged for 30 sec in a high-speed microcentrifuge (model 3200; Brinkman Instruments, Westbury, NY). The walls were carefully wiped with a cotton-tipped swab, and the red cell pellet lysed with 100 μl of water and transferred to another tube with 0.4 ml of 5% trichloroacetic acid. The acid-soluble extract was used for scintillation counting. Recovery of nonpermeant 14C-sucrose in the pellet (0.14–0.20% of that in the original extracellular fluid) was used to estimate the residual extracellular 3H-bestatin; this was subtracted from the total 3H-bestatin in the pellet to calculate the cellular uptake of the drug.

Scintillation Counting. Samples of the acid-soluble tissue extracts or urines were mixed with 10 ml of scintillation fluid (Liquiscint; National Diagnostics, Atlanta, GA) and counted in separate channels for 3H and 14C in an LKB 1209 Rackbeta Scintillation counter. Samples containing the same solvents were added to each run with the injected solution, as well as a 14C-sucrose standard to determine cross-over in the 3H channel.

Results

Our primary interest in this study is the uptake of bestatin in liver and muscle, 10 min after its intravenous injection. In this short interval, because of inhibition by bestatin of cytosolic exopeptidases, peptide intermediates accumulate linearly in these tissues and permit an estimate of the rates of cellular protein breakdown (9, 10). In the experiments presented herein, we inject bestatin intravenously to mice, together with trace amounts of 3H-bestatin and 14C-sucrose. We use 14C-sucrose as a nonpermeant marker of the extracellular space accessible to 3H-bestatin, and calculate by difference the cellular uptake of bestatin in the liver, kidney, intestine, red cells, and skeletal muscle. We chose labeled sucrose as a marker because its molecular weight (340) is closest to that of bestatin (308). This is significant in tissues, such as skeletal muscle, in which capillaries are lined by a continuous endothelium with tight interendothelial junctions, because in these tissues transcapillary permeability to small solutes are predominantly determined by their molecular size (19). Bestatin is not metabolized; even after 1–2 days, <5% is converted to the bioactive para-hydroxy-derivative (16, 17). We therefore assume that, in the relatively short intervals after its injection, all of the 3H radioactivity...
representative of the same specific radioactivity as in the injected solution.

Bestatin and Sucrose Disappear Rapidly from the Circulation and Are Largely Recovered in the Urine. When injected intravenously to mice, bestatin disappears rapidly from the circulation (3). It has not been clear, however, how much of this rapid disappearance is due to its urinary elimination and how much to its cellular uptake. The following experiments indicate that most of the injected bestatin is rapidly eliminated in the urine. A mouse received the intravenous injection of 1 mg of bestatin in 0.3 ml of phosphate-buffered saline, which also contained $^3$H-bestatin (4.8 $\mu$Ci) and $^{14}$C-sucrose (1.5 $\mu$Ci). At the indicated times, 30–40 $\mu$l of blood was collected in heparinized microhematocrit capillary tubes, and the cells were separated by centrifugation. Plasma $^3$H and $^{14}$C radioactivities were determined in the same 5-$\mu$l aliquot. For comparison, $^3$H and $^{14}$C radioactivities are each normalized for the respective radioactivities of the injected bestatin and sucrose, and expressed as cpm/ml per injected cpm (see Materials and Methods).

Three hours after its intravenous injection, urine contained 77.4% and 79.8% of the injected $^{14}$C-sucrose. We made no attempt to account for the remaining sucrose; after 3 hr, some of it may have diffused into less accessible extracellular compartments (22) and may have been internalized by endocytosis into the vacuolar space of some cells.

As shown in fig. 1, the disappearance of $^3$H-bestatin from the plasma, when normalized by the injected radioactivity, is indistinguishable from that of $^{14}$C-sucrose. The urinary elimination of $^3$H-bestatin in 3 hr, in the same animals described in the previous paragraph, was, respectively, 70.9% and 73.9%. Most of the injected bestatin seems, therefore, to be eliminated in the urine in the same manner as the impermeant sucrose. There is, however, a difference. The $^3$H/$^{14}$C ratio in the urine was 91.6% and 92.6% of that in the injected solution. Because these were highly radioactive samples and the $^3$H and $^{14}$C radioactivities were measured simultaneously, the $^3$H/$^{14}$C ratios were very accurate (coefficient of variation = 0.4%). The difference between the urine and the injected solution suggests that a minor portion of the injected $^3$H-bestatin was retained in excess of the sucrose. One explanation for bestatin retention could be its entry into the cellular space of some tissues, from which sucrose is excluded. In the remainder of this study, we present measurements of cellular uptake of $^3$H-bestatin in some of the major tissues.

$^3$H-Bestatin Is Taken Up Rapidly by the Liver, Kidney, and Intestine, But Slowly by Erythrocytes and Skeletal Muscle. Two mice received an intravenous injection of bestatin (5 mg) with 1 $\mu$Ci of $^3$H-bestatin and 0.3 $\mu$Ci of $^{14}$C-sucrose. The mice were killed 10 min after the injection. The trichloroacetic acid-soluble $^{14}$C and $^3$H radioactivities were determined in the plasma, red blood cells, skeletal muscles of the abdominal wall, liver, kidneys, and the upper portion of the small intestine. The results are presented in table 1.

In the red blood cell pellet and the liver, extracellular bestatin concentration was calculated from the specific radioactivity of the injected bestatin, the radioactivities of $^3$H-bestatin and $^{14}$C-sucrose in the plasma, and the $^{14}$C-sucrose radioactivity in the tissue. This extracellular $^3$H-bestatin was subtracted from the total to estimate the intracellular $^3$H-bestatin. For instance, in the first mouse in table 1, the radioactivity of $^{14}$C-sucrose in the liver was 17.8% of that in the plasma, a reasonable measure of the extracellular space in liver (24). The plasma contained 183 $\mu$g of bestatin/g, the extracellular bestatin in liver was calculated as 183 $\times$ 0.178 = 33 $\mu$g/g, and the intracellular bestatin was 485–33 = 452 $\mu$g/g. In these tissues, we used total content of bestatin in plasma, because the interstitial fluid is plasma in the red cell pellet, and in liver the fenestrated endothelium of the sinusoidal capillaries is freely permeable to macromolecules (24).

For the other tissues, where the endothelial barrier prevents the escape of plasma macromolecules, we have used, not the total content of bestatin in plasma, but the concentration of free (diffusible) bestatin, relative to that of sucrose, determined by dialysis (see Materials and Methods). In the kidneys, extracellular $^{14}$C-sucrose and the calculated extracellular $^3$H-bestatin were very high, higher than in plasma. This was to be expected, because we have shown in the previous section that bestatin is rapidly eliminated in the urine, and the sample includes concentrated urine in the lumen of the nephrons and the upper urinary tract.

Both the advantages and limitations of these calculations are exemplified in the measurements of bestatin concentration in muscle, where uptake is very low and most of the measured bestatin is extracellular. In the first mouse in table 1, we found 21 $\mu$g of bestatin/g of muscle. The radioactivity of $^{14}$C-sucrose was 16% of that in the plasma, consistent with measurements of sucrose space in muscle in perfused legs (22). If we were to use the total bestatin...
plasma content, we would calculate the extracellular bestatin in muscle as 183 × 0.16 = 29.3 μg/g, which exceeds the amount actually found by 39%. In the second mouse, the result of the same calculation would also exceed the amount found by 40%. When the more appropriate diffusible fraction is used, the calculated extracellular bestatin falls within the measured content. Still, because most of the bestatin contained in the tissue is extracellular, we cannot calculate intracellular bestatin in the muscle. Calculation of intracellular bestatin was not possible (see the text).

Two mice received an intravenous injection of bestatin (5 mg) with 1 μCi of 3H-bestatin and 0.3 μCi of 14C-sucrose. The mice were killed 10 min after injection, blood was collected, and red blood cells and plasma were separated by centrifugation. These and the indicated tissues were extracted overnight with 2 volumes of trichloroacetic acid, and the acid-soluble 14C and 3H radioactivities were determined by scintillation counting. Extracellular 3H-bestatin was calculated from the apparent volume of distribution of 14C-sucrose (expressed herein as per cent of plasma 14C-sucrose) and the plasma content of bestatin (from its 3H-radioactivity and the specific radioactivity of the injected 3H-bestatin). In this calculation, total plasma bestatin content was used for the red cell pellet and liver. For the kidney and intestine, the extracellular 3H-bestatin was calculated using the plasma concentration of diffusible 3H-bestatin, determined by differential dialysis (see Materials and Methods). In either case, extracellular 3H-bestatin was subtracted from the total to estimate the intracellular 3H-bestatin. In the kidneys, extracellular 14C-sucrose (and 3H-bestatin) includes concentrated urine in the urinary tract. In the muscle, calculation of intracellular 3H-bestatin was not possible (see the text).

### Materials and Methods

#### Uptake of Bestatin into Erythrocytes Is Slow and Nonsaturable.

The experiments previously described show a large difference in the uptake of bestatin, which is high in the liver, kidneys, and intestine, but much lower in red cells or skeletal muscle. This could indicate the entry of bestatin through transporter-mediated processes in some cells, but not others (see Discussion). The slower entry of bestatin into erythrocytes and skeletal muscle cells may depend entirely on passive diffusion across the plasma membrane. Red blood cells, because of their simple structure, the limited number of transporters in their plasma membrane, and the facility with which we can study them in suspension, lent themselves to additional experiments that reproduced in vitro the slow uptake of bestatin and suggested a nonsaturable mechanism for it.

As shown in fig. 2, when added to fresh whole blood at a concen-
at 10 min of incubation, when the cells contained 3% of the original escape from the cells. In a second experiment (also shown in fig. 2), linear rate of 0.3%/min. Once taken up, bestatin is just as slow to calculate the extracellular contamination of the pellet and the intracellular concentration of 100 mCi, 14C-sucrose (0.075 mCi), and nonradioactive bestatin at the initial extracellular concentrations indicated in the second column. At the end of incubation, extracellular fluid was diluted 100-fold by pipetting 20 µl of the incubated suspension in 1 ml of buffer), and the cells were centrifuged for 30 sec through a 0.5 M sucrose cushion. In the second experiment (triangles), an identical mixture was incubated for 10 min, at which time it was diluted by addition of 5 ml of prewarmed buffer and incubated further. One-milliliter aliquots were withdrawn 1, 3, 8, and 18 min after the dilution, and the cells were centrifuged as previously described. Recovery of nonpermeant 14C-sucrose was used to calculate the extracellular contamination of the pellet and the intracellular 3H-bestatin radioactivity (see Materials and Methods).

Entry of 3H-bestatin into erythrocytes seems to be nonsaturable. This is shown in the experiments presented in table 3. In the first experiment, 3H-bestatin was added to fresh blood at plasma concentrations of 1, 10, or 100 µg/ml. If a saturable transporter was involved, we expected the relative uptake to decrease as the concentration became larger. This was not found. If anything, with a 100-fold higher concentration, bestatin uptake increased somewhat from 2.1 to 3.0% in 10 min. This experiment was not ideal, because as we have seen in the previous section, not all of the bestatin in plasma is free to diffuse, as measured by its rate of dialysis relative to sucrose. If this is due to interactions of bestatin with macromolecular components in plasma, the diffusible fraction could decrease at lower concentrations. For this reason, the experiment was repeated with a suspension of red blood cells in a saline solution. This time, the results at all concentrations were identical. It is noteworthy that red cell uptake of bestatin from plasma, at an initial concentration of 100 µg/ml (3.0% in 10 min in the first experiment in table 3, and 2.9 and 2.5% in the experiments in fig. 2), was 61–73% of the uptake from a saline solution (4.1% in 10 min, in the second experiment in table 3). This agrees with the range of 60–74% found for the diffusible fraction of bestatin in plasma, when determined by differential dialysis (see Materials and Methods).

**Fig. 2.** Time course of entry of 3H-bestatin into red blood cells.

Two separate experiments are shown. In the first (circles), 100 µl of whole blood was incubated at 37°C with 3H-bestatin (0.24 µCi), 14C-sucrose (0.1 µCi), and nonradioactive bestatin (0.1 mg/ml of plasma). At the indicated times, the extracellular fluid was diluted 100-fold by pipetting 20 µl of the incubated suspension in 1 ml of buffer (phosphate-buffered saline, 10 mM glucose, and 50 mM sucrose), and the cells were centrifuged for 30 sec through a 0.5 M sucrose cushion. In the second experiment (triangles), an identical mixture was incubated for 10 min, at which time it was diluted by addition of 5 ml of prewarmed buffer and incubated further. One-milliliter aliquots were withdrawn 1, 3, 8, and 18 min after the dilution, and the cells were centrifuged as previously described. Recovery of nonpermeant 14C-sucrose was used to calculate the extracellular contamination of the pellet and the intracellular 3H-bestatin radioactivity (see Materials and Methods).

**Discussion**

The immunoenhancing effects of bestatin are attributed to its interaction with enzymes exposed in the surface of some of the cellular components of the immune system (12, 13). Because of this, the primary interest has been to reach effective concentrations of the drug in the extracellular fluid (3). Our purpose is different. We are interested in the uptake of bestatin in the liver and muscle, within 10 min after its intravenous injection. In this short interval, because of inhibition by bestatin of cytosolic exopeptidases, peptide intermediates accumulate linearly in these tissues and permit an estimate of instant rates of cellular protein breakdown (9, 10). This is an important tool in studies of protein metabolism, because there is no other way at present to estimate rates of protein degradation in the tissues of live animals in such a short time.

The experiments reported in this study indicate that, after a single intravenous injection, most of the drug is rapidly eliminated in the urine in the same manner as the impermeant sucrose, with a half-life in the extracellular fluid of ~20 min. Whereas the drug is available to cells, there is a large difference in the uptake of bestatin, which seems to readily enter the liver, kidneys, and intestine, but much less so in red cells or skeletal muscle. This could indicate the entry of bestatin through transporter-mediated processes in some cells but not others, and has implications both in the targeting of the drug to specific tissues and in anticipating the potential toxicity of high doses.

We calculate the cellular uptake by difference (i.e. the total minus the calculated extracellular bestatin). It could be argued that the excess bestatin has not entered the cells, but is bound to extracellular peptidases on their surface. This is the mechanism proposed for the effects of bestatin in some of the cells of the immune system (12, 13). It has been proposed that tissue distribution of bestatin is closely related to the distribution of aminopeptidases, particularly the abundant leucine aminopeptidase (18, 25). This is conceivable in studies (18) in which 3H-bestatin was detected by autoradiography, one or more hours after the injection of a dose of the drug 14-fold smaller than that used in the

**TABLE 3**

<table>
<thead>
<tr>
<th>Bestatin Concentration</th>
<th>% of initial extracellular concentration</th>
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<tbody>
<tr>
<td>Whole blood</td>
<td>1</td>
</tr>
<tr>
<td>10</td>
<td>2.1</td>
</tr>
<tr>
<td>100</td>
<td>3.0</td>
</tr>
<tr>
<td>Red blood cells, 1:1 in buffer</td>
<td>1</td>
</tr>
<tr>
<td>10</td>
<td>4.3</td>
</tr>
<tr>
<td>100</td>
<td>4.1</td>
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</table>

One hundred microliters of whole blood, or a 1:1 suspension of red blood cells in buffer (phosphate-buffered saline, 10 mM glucose, and 50 mM sucrose), was incubated at 37°C for 10 min with 3H-bestatin (0.24 µCi), 14C-sucrose (0.075 µCi), and nonradioactive bestatin at the initial extracellular concentrations indicated in the second column. At the end of incubation, extracellular fluid was diluted 100-fold (20 µl of the incubated suspension in 1 ml of buffer), and the cells were centrifuged for 30 sec through a 0.5 M sucrose cushion. Recovery of nonpermeant 14C-sucrose was used to estimate the residual extracellular contamination of the pellet, which was subtracted from the total to calculate the intracellular 3H-bestatin radioactivity (see Materials and Methods).
Because of its hydrophobic side chains, it may enter cells by partitioning across the plasma membrane. Bestatin is an analog of the dipeptide phenylalanyl-leucine. Of entry, the lack of saturation, and the paucity of plasma membrane transporters, the intravenous injection, enough bestatin found its way into the intracellular bestatin/g of tissue or 12 μg/g (0.1 mg/g), and its hexameric molecular weight (360,000) (26), we calculate that a maximum of six molecules of bestatin bound per hexamer (27) would represent ~0.5 μg of bestatin/g of liver; the concentrations actually found were three orders of magnitude larger. The slow, linear, nonsaturable uptake of bestatin into erythrocytes (fig. 2, table 3) is also consistent with entry into the cells, rather than binding to their surface.

In the liver, previous autoradiographic studies have shown uptake of bestatin primarily in hepatocytes (18). Although we know at this time of no specific transport mechanism that can account for this uptake, the sinusoidal domain of the hepatocyte plasma membrane is the most likely site of entry. The uptake of bestatin is reversible. In another experiment (not shown), a mouse injected and studied in the same manner as those in table 1 was killed 1 hr after the injection. Intracellular bestatin in liver was found to be 16 μg/g, or 20-fold less than in the livers of mice after 10 min.

The uptake of bestatin in kidneys and intestine indicate that the liver is not unique in this respect. Because of the complex and heterogeneous structure of these organs, however, the interpretation of these results is less clear. Autoradiographic measurements, 1 hr after the intravenous injection of bestatin, have shown higher density of silver grains in the brush border of proximal straight renal tubules (18). It suggests that bestatin may enter renal cells from the lumen of the nephron, through which most of the drug is eliminated from the body, rather than the sinusoidal domain. Bestatin is a substrate for a H+/coupled peptide transporter in the renal brush border (29). This transporter (PEPT2) has been recently cloned and shown to be distinct from the similar transporter in the intestinal brush border (PEPT1) (30). The autoradiographic study previously mentioned did not include intestine. We do not know how much of the drug was in the muscular wall or in the intestinal epithelium. Bestatin has been shown to enter enterocyte epithelium from the lumen by H+/coupled active transport via the PEPT1 system in the brush border, but not the amino acid transport systems (14, 15). It seems, however, unlikely that within 10 min after its intravenous injection, enough bestatin found its way into the intestinal lumen, from which other small molecules, such as sucrose, are excluded (21).

By contrast with the liver, kidney, or intestine, the slower entry of bestatin into erythrocytes and skeletal muscle cells may depend entirely on passive diffusion across the plasma membrane. The low rate of entry, the lack of saturation, and the paucity of plasma membrane transporters in the erythrocyte membrane make this very likely in red cells. Bestatin is an analog of the dipeptide phenylalanyl-leucine. Because of its hydrophobic side chains, it may enter cells by partitioning across the lipid bilayer of the plasma membrane. Ten minutes after the intravenous injection of 5 mg of bestatin, we find in the muscle 4.8 μg of intracellular bestatin/g of tissue or 12 μg/ml of intracellular water. This is almost 100-fold less than in liver, but it still is 10-fold higher than the concentrations of bestatin found to produce accumulation of peptide intermediates in reticulocyte lysates (6). This explains why, although with great inefficiency, the intravenous injection of a large dose of bestatin can produce intracellular accumulation of peptide intermediates in skeletal muscle (9).

In addition to its uses as an aminopeptidase inhibitor, bestatin has proven to be valuable as a nonhydrolyzable probe for the H+/coupled peptide transporters PEPT1 and PEPT2, in the brush border of the intestine and kidney (14, 15, 29). These transporters permit the efficient intestinal absorption of di- and tripeptides resulting from the digestion of dietary proteins, and the reabsorption of peptides from the glomerular filtrate (31, 32). They are also involved in the absorption and retention of many peptidomimetic drugs, including cephalosporins, penicillins, and inhibitors of angiotensin-converting enzyme (32). PEPT1 and PEPT2 are, so far, the only well characterized mammalian peptide transporters, but not the only ones. We have shown that di- and tripeptides resulting from the breakdown of proteins by lysosomal enzymes are released from autophagic organelles to the cytosol, where they are degraded to amino acids by bestatin-sensitive exopeptidases (7, 8). More recent studies have shown the selective release by lysosomes, in vitro, of endocytosed di- and tripeptide probes (33). There is also a transporter in the endoplasmic reticulum that mediates the translocation of oligopeptides for antigen presentation (34). These oligopeptides are, however, larger than the di- and tripeptides discussed herein. The ready uptake of bestatin by the liver, shown in this study, suggests the existence of a novel peptide transporter in the sinusoidal domain of the hepatocyte plasma membrane. It must be different from PEPT1 and PEPT2, because Northern blots using specific cDNA probes failed to reveal mRNAs for either of these transporters in liver (30, 35). Radioactive bestatin should be a useful probe for the identification and study of this transporter in hepatocytes, either freshly isolated or in primary cultures.

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