Pinocytosis and Intracellular Degradation of Exogenous Protein: Modulation by Amino Acids

JEFFREY M. BESTERMAN,* JUDITH A. AIRHART,‡ ROBERT B. LOW,‡ and D. EUGENE RANNELS*

**Departments of Physiology and Anesthesia, The Milton S. Hershey Medical Center, The Pennsylvania State University, Hershey, Pennsylvania 17033; and ‡Department of Physiology and Biophysics, University of Vermont, College of Medicine, Burlington, Vermont 05405. Dr. Besterman's present address is Department of Molecular Biology, The Wellcome Research Laboratories, Research Triangle Park, North Carolina 27709.*

ABSTRACT Intracellular degradation of exogenous (serum) proteins provides a source of amino acids for cellular protein synthesis. Pinocytosis serves as the mechanism for delivering exogenous protein to the lysosomes, the major site of intracellular degradation of exogenous protein. To determine whether the availability of extracellular free amino acids altered pinocytic function, we incubated monolayers of pulmonary alveolar macrophages with the fluid-phase marker, [^{14}C]sucrose, and we dissected the pinocytic process by kinetic analysis. Additionally, intracellular degradation of endogenous and exogenous protein was monitored by measuring phenylalanine released from the cell monolayers in the presence of cycloheximide. Results revealed that in response to a subphysiological level of essential amino acids or to amino acid deprivation, (*a*) the rate of fluid-phase pinocytosis increased in such a manner as to preferentially increase both delivery to and size of an intracellular compartment believed to be the lysosomes, (*b*) the degradation of exogenously supplied albumin increased, and (*c*) the fraction of phenylalanine derived from degradation of exogenous albumin and reutilized for de novo protein synthesis increased. Thus, modulation of the pinosome-lysosome pathway may represent a homeostatic mechanism sensitive to the availability of extracellular free amino acids.

There are four sources of essential amino acids available for cellular protein synthesis: intracellular free amino acids, amino acids derived from degradation of cellular protein, extracellular free amino acids, and amino acids derived from degradation of extracellular protein. Work in our laboratories (1, 18, 27, 29) and those of others (see references 28 and 34 for review) has demonstrated the intracellular compartmentation of amino acids from these sources. Historically, degradation of serum protein has not been considered a significant source of amino acids for de novo protein synthesis, based almost solely on the work of Eagle and Piez (11). However, new, though indirect, experimental evidence strongly suggests that alveolar macrophages can use exogenous (serum) proteins, specifically albumin, as a source of amino acids for protein synthesis in the presence of physiological (27) and subphysiological (17) levels of extracellular free amino acids. It is also known that serum proteins are internalized by both fluid-phase and adsorptive pinocytosis (12, 13, 32) and are degraded in the lysosomes (10, 12, 14, 25). Therefore, since pinocytosed serum proteins might serve as an alternative source of amino acids (to free extracellular amino acids), we hypothesized that: (*a*) the rate of pinocytosis might be regulated by the availability of extracellular free amino acids, and (*b*) under conditions of amino acid deprivation, intracellular degradation of exogenous protein would increase. We tested these hypotheses using monolayers of alveolar macrophages and employing a kinetic analysis that we have described recently (4).

MATERIALS AND METHODS

Cells: Pulmonary alveolar macrophages were lavaged from lungs of male rabbits (or guinea pigs) with calcium- and magnesium-free phosphate-buffered saline (PBS) as described previously (22). After being washed and counted, the cells were suspended in Dulbecco's PBS supplemented with 5.6 mM glucose, pH 7.4 (PBS_G), plated at 2–4 × 10^6 macrophages per 35-mm Falcon tissue culture dish (Falcon Labware, Oxnard, CA), and allowed to form adherent monolayers for 1 h at 37°C before experiments were begun. The medium used for all experiments was Dulbecco's PBS_G supplemented with amino acids and vitamins, as specified.

HeLa cells were grown in Eagle's minimal essential medium (MEM) supple-

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mented with 10% donor calf serum, 0.075% $NaHCO_3$, and 20 μg kanamycin/ml. Cultures were incubated at 37°C as a closed vessel system.

Pinocytosis, Accumulation, and Exocytosis of [^{14}C]Sucrose: Accumulation of [^{14}C]sucrose into alveolar macrophage monolayers was determined as previously described (4). Briefly, cell monolayers were preincubated with PBS_G supplemented with various concentrations of L-amino acids and vitamins for 1 h at 37°C. Experiments were initiated by adding [^{14}C]sucrose (negligible volume) and concluded by removing media, washing the monolayers with ice-cold PBS_G, and solubilizing the cell layer with 1% SDS. Controls for accumulating radioactivity at zero time and at 4°C were performed routinely.

The kinetics of [^{14}C]sucrose pinocytosis and subsequent exocytosis were determined as previously described (4), including control experiments and methods of data analysis. A brief summary of these procedures is provided in text and in the legend to Table I.

Degradation of Exogenous and Endogenous Protein and Reutilization of Amino Acids: Investigating protein turnover is simplified when the amino acid(s) used to monitor the rates of protein synthesis and degradation is essential and not otherwise metabolized by the cell under study. Previous studies by Woodside and Massaro (33) and Hammer and Rannels (17) have established that phenylalanine is suitable for monitoring protein turnover in pulmonary alveolar macrophages. In the absence of cycloheximide, changes in the concentration of extracellular free phenylalanine reflect the balance between protein synthesis and degradation and shall be referred to as net phenylalanine release. In the presence of cycloheximide (20 μM), changes in the concentration of extracellular free phenylalanine reflect protein degradation alone and shall be referred to as total phenylalanine release. We used this approach to calculate (*a*) the rate of intracellular degradation of both endogenous macrophage proteins and exogenously supplied proteins (i.e., bovine serum albumin [BSA]) and (*b*) the fraction of amino acids (i.e., phenylalanine) derived from degradation of endogenous and exogenous proteins and reutilized for de novo protein synthesis.

Macrophage monolayers were washed free of nonadherent cells and preincubated for 30 min at 37°C with PBS_G containing 2.2 μM phenylalanine, 0 or 20 μM cycloheximide, and either 19 other amino acids at physiological levels (see below) or no additional amino acids (amino acid deprivation). Following the preincubation period the media were removed and replaced with fresh media of identical composition (except with or without 2% BSA) and incubation was continued at 37° for 3 h. It was during this 3-h interval that phenylalanine release into the media was monitored: the media were removed from the monolayers and an aliquot (0.7–1.0 ml) was mixed with 30 μl of 60% sulfosalicylic acid and 50 μl of 1 mM leucyl-alanine. Precipitate was removed by centrifugation at 2,500 rpm for 10 min at 4°C in a clinical centrifuge. The phenylalanine content of the supernatant was determined by ion-exchange chromatography as described previously (31), using the leucyl-alanine as an internal standard. Phenylalanine release was measured against a background of 2.2 μM phenylalanine; this concentration of phenylalanine is the lowest known to provide sufficient substrate for protein synthesis in alveolar macrophages (15).

Radioassay: Radioassay for ^{14}C was performed with the scintillant, Aquasol-2, on a Packard Tri-Carb liquid scintillation spectrometer (model 3320, Packard Instrument Co., Inc., Downers Grove, IL). Quench was monitored by external standard.

Protein Determination: Protein content of solubilized cell monolayers and filtrates were determined according to the method of Lowry et al. (23) using BSA as a standard.

Assay for [^{14}C]Sucrose Binding to BSA: To determine whether [^{14}C]sucrose was bound to BSA under culture conditions, we centrifuged 0.5-ml aliquots of media containing [^{14}C]sucrose, 0 or 2% BSA, and either a complete complement of amino acids or no amino acids at 800 rpm for 10 min at room temperature through conical filters designed to retain macromolecules with molecular weights greater than 25,000 (Amicon Centriflo membrane cones, type CF25; Amicon Corp., Scientific Systems Div., Danvers, MA). Protein determinations on the filtrate indicated that 97–98% of the BSA was retained by the filters. Radioassay indicated that identical amounts of [^{14}C]sucrose were retained on the filters regardless of whether or not 2% BSA was present. Thus, 2% BSA did not bind [^{14}C]sucrose in excess of the 5% precision of this assay.

Assay for Cathepsin D: Cathespin D activity in cell lysates was assayed by modifying method III described by Barrett (2). Adherent cell layers were removed from culture vessels and solubilized in 0.2% Triton X-100, so as to determine total cathepsin D activity. Lysates were assayed at pH 3.0 for 30 min at 37°C. Enzymatic activities were normalized per milligram of cell lysate protein. Inhibition of activity by pepstatin A (final concentration 71 μg/ml) was used as an index of the specificity of the assay for cathepsin D (2, 8). Samples and blanks were assayed in triplicate. All reagents were kindly provided by Dr. Balvin H. Chua, Hershey Medical Center, Hershey, PA

Materials: The following materials were obtained from the indicated sources: male guinea pigs, Canadian Breeders Laboratory, St. Constant, Quebec, Canada; male New Zealand white rabbits, Charles River Breeding Laboratories, Wilmington, MA; Eagle's basal medium (BME) amino acid solution (50×) without glutamine, MEM amino acid solution (50×) without glutamine, MEM vitamin solution (100×), Gibco Laboratories, Grand Island, NY; fraction five BSA (Pentex), Miles Laboratories Inc., Research Products Div., Elkhart, IN; [U-^{14}C]sucrose, specific radioactivity 477 mCi/mmol, Amersham Corp., Arlington Heights, IL. All other chemicals were reagent grade.

TABLE I

Effects of Availability of Extracellular Essential Amino Acids on the Characteristics of Intracellular Compartments and Fluxes Involved in Fluid-Phase Pinocytosis

	Amino acid concentration in media		
	* Physiological	‡ Subphysiological	None
Half-life of compartment 1 (*minutes*)	5	5	5
Half-life of compartment 2 (*minutes*)	174	167	170
Size of compartment 1 (nl/10^6 cells)	37	36	35
Size of compartment 2 (nl/10^6 cells)	716	977	931
K_3 (nl/min·10^6 cells)	5.2	5.1	4.9
$K_4 = K_2$ (nl/min·10^6 cells)	2.9	4.0	3.8
K_{influx} (i.e., K_1) = K_{efflux} (i.e., $K_3 + K_4$) (nl/min·10^6 cells)	8.1	9.1	8.7

Monolayers of guinea pig alveolar macrophages were incubated for 20 min at 37°C with media containing chromatographically-repurified [^{14}C]sucrose and varying levels of the essential amino acids (see below). This incubation period was chosen because previous work indicated that it was optimal for data analysis (4). At the end of incubation the medium was removed, the monolayers were rapidly washed six times with ice-cold PBS_G, the dishes quickly drained onto absorbant paper, and the monolayers immediately reincubated at 37°C to monitor the release of [^{14}C]sucrose from the cells. The reincubation media were identical to those used during incubation except that isotopically-labeled sucrose was omitted. At intervals over the next 3 h the reincubation media were collected and replaced with fresh media. The monolayers were finally solubilized in 1% SDS. Both media and cell fractions were assayed for radioactivity. Data analysis for exocytic kinetics subsequent to pinocytic loading was performed as described in the text and reference 4. Compartments 1 and 2 and rate constants, *K*'s, are defined in both Fig. 2 and the text. The mean of duplicate determinations from a single experiment are presented. The error in determining fractional rate constants (half-lives) by curve peeling was 5–10%. Qualitatively similar observations have been made in three additional experiments. The half-life of compartment 1 ranged from 4 to 6 min whereas the half-life of compartment 2 ranged from 150 to 175 min.

* Physiological (micromolar): Val, 140; Leu, 100; Ile, 60; Phe, 60; Met, 40; Thr, 120; Lys, 280; Arg, 220; His, 100.

‡ Subphysiological (micromolar): Val, 70; Leu, 50; Ile, 30; Phe, 30; Met, 20; Thr, 60; Lys, 140; Arg, 110; His, 50.

RESULTS

Effect of Amino Acids on [^{14}C]-Sucrose Accumulation

Two approaches were used to determine whether the availability of extracellular essential amino acids altered pinocytic function. First, the time course of macrophage accumulation of [^{14}C]sucrose was determined in three different media, one free of amino acids and two containing essential amino acids at levels frequently employed in cell culture. Fig. 1 illustrates that, whereas there was little difference in the response to the two amino acid mixes, omitting amino acids increased net accumulation of [^{14}C]sucrose ~40% without noticeably changing the time course of accumulation. These results may explain why fluid-phase pinocytic rates reported by others for macro-

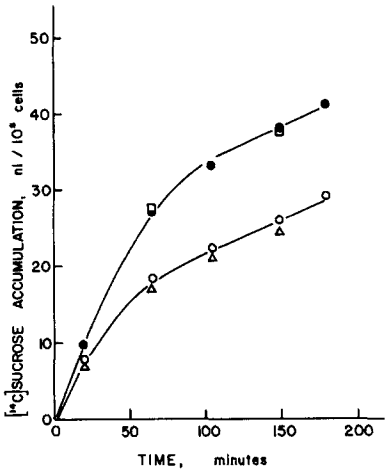

FIGURE 1 Kinetics of accumulation of [^{14}C]-sucrose by cultured rabbit pulmonary alveolar macrophages in the presence of various levels of essential amino acids. Cell monolayers were incubated for specific periods at 37°C with media containing [^{14}C]sucrose. ●, PBS_G alone; □, PBS_G plus vitamin mix identical to that found in BME or MEM; △, PBS_G plus amino acid mix identical to that in BME (micromolar): Val, 200; Leu, 198; Ile, 198; Phe, 100; Met, 50; Thr, 202; Lys, 200; Arg, 107; His, 52; Cys, 50; Tyr, 100; Trp, 20; ○, PBS_G plus amino acid mix identical to that in MEM (micromolar): Val, 400; Leu, 400; Ile, 400; Phe, 200; Met 100; Thr, 400; Lys, 500; Arg, 600; His, 201, Cys, 100; Tyr, 200; Trp, 50. Details of cell culture, experimental protocol, and determination of cell-accumulated radioactivity were described in Materials and Methods. The amount of [^{14}]sucrose accumulated with time is expressed as the volume of culture medium (nanoliters), the substrate of which has been accumulated by 10^5 cells. Controls for accumulation of [^{14}C]sucrose in zero time have been subtracted. Values represent the mean of four to six determinations, with 1 SD never exceeding 10% of the mean.

phages cultured in complete media (21, 26, 30) were consistently lower than the rates observed for macrophages cultured in media containing no or very low levels of amino acids (3). As another indication that the lower pinocytic rates reported for macrophages cultured in complete media could be attributed to the presence of physiological levels of essential amino acids, it was found that adding a complete vitamin mix to amino acid-free medium did not result in the lower pinocytic rate observed in the presence of essential amino acids (Fig. 1). That nonlinear accumulation was observed in all cases was consistent with our previous findings (4) and those of others (30) for fluid-phase markers in macrophages, an observation we have analyzed in detail recently (4).

Effect of Amino Acids on Kinetics of [^{14}C]-Sucrose Exocytosis

The approach described above for quantitation of fluid-phase pinocytosis provided information concerning only the net accumulation of [^{14}C]sucrose, which reflects the balance between endocytosis and exocytosis (4). However, a kinetic analysis of exocytosis of accumulated pinocytic marker ([4]; summarized in the legend to Table I) allowed for a more detailed examination of the pinocytic process through analysis of the intracellular compartments involved and of the flux of pinocytic marker through these compartments. We have previously established that in the presence of a physiological level of amino acids, the process of fluid-phase pinocytosis and subsequent exocytosis requires at least two intracellular compartments in series, one small compartment turning over rapidly (probably corresponding to pinosomes), and the other, apparently larger compartment turning over slowly (probably corresponding to lysosomes) (4). It was within the context of this two-compartment model (Fig. 2) that the effect of extracellular amino acid depletion on pinocytic function was analyzed.

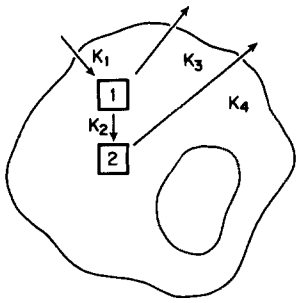

FIGURE 2 Sequential model depicting the kinetically defined compartments and fluxes involved in fluid-phase pinocytosis and exocytosis in alveolar macrophages. *K*'s are absolute rate constants. Reproduced from the Journal of Cell Biology, 1981, 96:716-727, by copyright permission of The Rockefeller University Press.

Macrophage monolayers were allowed to pinocytose [^{14}C]-sucrose for 20 min and the exocytosis of this marker back into isotope-free medium was expressed as the percentage of [^{14}C]-sucrose remaining inside the cell as a function of reincubation time. The resultant curves were dissected into their exponential components by "curve peeling"; the slope of each component process was taken as a measure of that component's half-life. The *y*-intercepts obtained by back extrapolation to time zero were taken as a measure of the distribution of the [^{14}C]sucrose among the component processes (4). Results indicated that pinocytosis and subsequent exocytosis involved at least two intracellular compartments (processes) under all three media conditions tested (Table I). These results were consistent with previous findings (4) and with the working model shown in Fig. 2. However, the sizes of these two compartments and the associated intercompartmental fluxes were clearly affected in a prescribed way (Table I). Specifically, the size of compartment 1 appeared essentially unaffected by varying the extracellular amino acid concentration, whereas the size of compartment 2 increased 30–37% in the absence of a physiological level of essential amino acids. Moreover, although the overall uptake rate (K_1) increased only slightly (7–12%), this increase was transmitted in toto to K_2, effectively increasing the flux from compartment 1 to compartment 2 by 31–38%. Hence, the increase in fluid-phase pinocytosis in response to reducing the concentration of extracellular amino acids was selective and of double-design, increasing both the size of compartment 2 (presumptive lysosomes) and the delivery of extracellular material from compartment 1 (presumptive pinosomes) to compartment 2. These observations suggested that amino acid deprivation may result in increased delivery of exogenous protein to the lysosomes.

Effect of Amino Acids on the Release of Phenylalanine from both Endogenous and Exogenous Protein Sources

To test the hypothesis that degradation of exogenous protein is stimulated under conditions of amino acid deprivation, we measured changes in the extracellular concentration of phenylalanine in the presence and absence of a physiological level of free amino acids (Table II). In the absence of albumin, total phenylalanine release (plus cycloheximide) was unaffected by amino acid deprivation (2.24 ± 0.25 at 1× vs 2.66 ± 0.30 at 0×). Thus, endogenous protein degradation appeared insensitive to an acute depletion in the concentration of free extracellular amino acids. In addition, the fraction of phenylalanine derived from degradation of endogenous protein and reutilized for protein synthesis could be calculated from Table II. In the presence of a physiological level of amino acids (1×), total phenylalanine release (degradation) occurred at a rate of 2.24

TABLE II

Effect of Availability of Extracellular Free Amino Acids on Phenylalanine Release from Endogenous and Exogenous Proteins

Incubation conditions				
Extracellular amino acid concentration	2% albumin	20 μM cycloheximide	Phenylalanine release	Phenylalanine release from 2% albumin
			(nmol/3 h · 10^6 cells)	*(nmol/3 h · 10^6 cells)*
0 ×	−	−	1.45 ± 0.22	
0 ×	+	−	4.18 ± 0.26	2.73
0 ×	−	+	2.66 ± 0.30	
0 ×	+	+	7.37 ± 0.48	4.71
1 ×	−	−	1.16 ± 0.18	
1 ×	+	−	3.46 ± 0.27	2.30
1 ×	−	+	2.24 ± 0.25	
1 ×	+	+	4.72 ± 0.32	2.48

Monolayers of rabbit alveolar macrophages were incubated for 3 h at 37°C with Dulbecco's phosphate-buffered saline containing 5.6 mM glucose, 2.2 μM phenylalanine, and additional components as specified: in the absence (0 ×) or presence (1 ×) of a physiological level of 19 amino acids (see below); ± 2% albumin; ± 20 μM cycloheximide. The phenylalanine content of the media was assayed before (initial) and after (final) the 3-h exposure to the cell monolayer and the data are expressed as the net (final minus initial) release of phenylalanine during this 3-h period (normalized to 10^6 macrophages). Phenylalanine released from albumin degradation was calculated as the difference between phenylalanine release in the absence (−) and presence (+) of 2% albumin. Values represent the mean ± SEM of three to four experiments; each experiment was performed in duplicate.

1 × amino acids (micromolar): Asp, 14; Asn, 68; Glu, 120; Gln, 652; Ala, 326; Val, 188; Leu, 166; Ile, 108; Ser, 247; Thr, 203; Met, 54; Cys, 100; Pro, 108; Tyr, 69; Trp, 100; His, 52; Lys, 380; Arg, 119; and Gly, 336 (5).

nmol/3 h · 10^6 cells, whereas net phenylalanine release (degradation minus synthesis) occurred at a rate of 1.16 nmol/3 h · 10^6 cells. Therefore, phenylalanine was recycled into the protein synthetic pathway at a rate of 1.08 nmol/3 h · 10^6 cells (2.24 − 1.16), which represents reutilization of 48% of the phenylalanine derived from degradation of endogenous protein. Likewise, under conditions of amino acid deprivation (0×), 46% of the phenylalanine derived from degradation of endogenous proteins was reutilized for protein synthesis: (2.66 − 1.45)/2.66. Thus, reutilization of phenylalanine derived from degradation of endogenous protein was unaffected by amino acid deprivation.

In the presence of albumin, total phenylalanine release (plus cycloheximide) attributable to albumin degradation (phenylalanine release in presence of albumin minus phenylalanine release in absence of albumin) was increased 90% in the absence of amino acids (Table II; 2.48 at 1× vs. 4.71 at 0×). Thus, unlike degradation of endogenous protein, intracellular degradation of exogenous protein appeared sensitive to amino acid deprivation. Furthermore, whereas only 7% of the phenylalanine derived from degradation of albumin was reutilized for protein synthesis in the presence of a physiological level of amino acids, the reutilization efficiency increased to 42% in the absence of amino acids: (2.48 − 2.30)/2.48 = 7% at 1×; (4.71 − 2.73)/4.71 = 42% at 0×.

Having established increased degradation of albumin by macrophages cultured in medium deficient in amino acids, we next determined whether the lack of amino acids could have increased degradation of albumin via a direct elevation in the activity of lysosomal proteases, specifically cathepsin D. Table III indicates that (*a*) total cathepsin D activity in macrophage lysates (assayed in the presence of detergent) was independent

TABLE III

Total Cathepsin D Activity of HeLa Cells and Alveolar Macrophages

Cell type	Micrograms hemoglobin degraded/milligram cell protein·h
HeLa cells	127 ± 7
Macrophages (freshly isolated)	754 ± 15
Macrophages (cultured in the presence of amino acids)	935 ± 56
Macrophages (cultured in the absence of amino acids)	883 ± 35

Assay of total cathepsin D activity in cell lysates and culture of HeLa cells were performed as described in Materials and Methods. Rabbit alveolar macrophages were obtained by pulmonary lavage and allowed to adhere to culture dishes for 1 h at 37°C and either immediately lysed (designated freshly isolated) or cultured for an additional 3 h at 37°C in the presence or absence of amino acids at physiological levels and then lysed. Adding pepstatin A inhibited enzymatic activities by 85–90%. Values represent the mean ± SEM of three determinations.

of whether the macrophages had been cultured in the absence or presence of amino acids, and (*b*) the total activity of cathepsin D is 6–7 times greater in macrophages than in HeLa cells. The comparatively low level of cathepsin D in HeLa cells offers one possible explanation why Eagle and Piez (11) were unable to demonstrate use of exogenous proteins as a source of amino acids for protein synthesis in HeLa cells (see Discussion).

Effect of BSA and Cycloheximide on Kinetics of [^{14}C]Sucrose Exocytosis

The above observations indicated that increased degradation of BSA occurred in the absence of extracellular amino acids, as was predicted from increased delivery of [^{14}C]sucrose to a compartment believed to be the lysosomes. However, 20 μM cycloheximide was present to measure degradation of BSA, whereas the [^{14}C]sucrose kinetic studies were performed in the absence of both BSA and cycloheximide. Therefore, we assessed whether the presence of 20 μM cycloheximide and 2% BSA could, in and of themselves, alter [^{14}C]sucrose kinetics in such a way to account for the increased degradation of BSA under amino acid-deprived conditions. Irrespective of the absence or presence of extracellular amino acids, (*a*) cycloheximide caused compartment 2 to decrease in size by ~40% and to turn over slightly faster, and (*b*) BSA caused compartment 2 to increase in size by ~30% and to turn over slightly slower. Neither cycloheximide nor BSA appeared to affect the size or rate of turnover of compartment 1. The effects of cycloheximide and BSA together were additive, resulting in little or no net effect on the steady state kinetics of [^{14}C]sucrose pinocytosis and subsequent exocytosis. In addition, [^{14}C]sucrose did not to bind to BSA (see Materials and Methods). Thus, in the presence of both cycloheximide and BSA, amino acids modulated pinocytic pathway traffic.

DISCUSSION

Correct interpretation of pinocytic studies requires understanding the relationship between the rate of intracellular accumulation of a pinocytic marker and the actual rate of pinocytosis (4). Using the rate of intracellular accumulation of [^{14}C]sucrose as an index of pinocytic activity, we observed a 40–45% increase in accumulation in the absence of extracellular amino acids (Fig. 1), which is consistent with our hypothesis that amino acid deprivation would stimulate fluid-phase pinocytosis. How-

ever, because accumulation of [^{14}C]sucrose reflects the balance between pinocytosis and exocytosis in alveolar macrophages (4), the cellular response to amino acid deprivation was examined more closely. The increase in intracellular sucrose accumulation was due to a redistribution in intracellular pathway traffic: a small increase in the pinocytic rate, K_1, was transmitted entirely to K_2, resulting in a 31–38% increase in delivery to the lysosomal compartment. Thus, sucrose accumulation was satisfactory only as a qualitative measure of pinocytic function, in that it indicated an increased pinocytic activity in response to amino acid deprivation, but neither did it accurately determine the magnitude of the increase in pinocytic rate, nor did it indicate how the change in accumulation was achieved.

The kinetics of [^{14}C]sucrose exocytosis predicted that in the absence of extracellular amino acids, protein degradation would increase due to increased delivery of pinosomes to the lysosomal compartment and subsequent degradation of their exogenous protein content. By measuring changes in the extracellular concentration of phenylalanine, we found that during amino acid deprivation total protein degradation (endogenous plus exogenous) was increased 56% (7.37 at 0× versus 4.72 at 1×; Table 2). This increase resulted from a combination of invariant degradation of endogenous protein (2.66 ± 0.30 at 0× vs. 2.24 ± 0.25 at 1×; Table 2), and a 90% increase in intracellular degradation of exogenous protein (4.91 at 0× vs. 2.48 at 1×; Table II).

In the presence of albumin, increased release of phenylalanine was attributed to the intracellular degradation of albumin. This premise was based on previous studies that showed that albumin did not alter degradation of endogenous macrophage proteins, that phenylalanine release from albumin did not occur in cell-free conditioned medium, and that the intracellular pool of free phenylalanine was of stable and insignificant size compared with the size of the extracellular phenylalanine pool (17).

The mechanism(s) responsible for altering fluid-phase pinocytic pathway kinetics in the absence of extracellular amino acids is not known. In this regard, it might be postulated that the additional amino acids, released as a result of increased intracellular degradation of BSA in the absence of extracellular amino acids, could function to alter pinocytic pathway traffic in the same way as do extracellular amino acids, e.g., decrease the size of compartment 2. Results indicate, however, that the presence of 2% BSA increases the apparent size of compartment 2, irrespective of whether extracellular amino acids are absent or present.

Reutilization of phenylalanine derived from intracellular degradation of endogenous and exogenous protein was calculated from measurements of phenylalanine release in the absence and presence of cycloheximide. At 20 μM, cycloheximide inhibits protein synthesis in alveolar macrophages by 95–97% (data not shown; [16]). Unfortunately, however, 20 μM cycloheximide also inhibits protein degradation by 15–20% (data not shown; [9, 19]), resulting in an underestimation of the true rate of phenylalanine reutilization. Thus, the reutilization rates calculated from the studies presented here must be considered apparent, although recalculated rates corrected for the inhibitory effect of cycloheximide on protein degradation result in the same conclusions using slightly greater reutilization rates.

We postulate that the increased degradation of BSA observed in the absence of extracellular amino acids may result from increased delivery of pinocytic material to the lysosomal compartment. There are, however, alternate explanations that warrant consideration. If cycloheximide and BSA caused a disproportionate increase in pinocytic flux to the lysosomal compartment in the absence of amino acids as compared with in the presence of amino acids, increased degradation of BSA in the absence of amino acids might result. The present findings are not consistent with this possibility. Alternatively, freshly isolated macrophages cultured for a few hours in the absence of amino acids may have increased levels of lysosomal proteases. However, Table III indicates that, at least for cathepsin D, total activity was identical for macrophages incubated in the presence or absence of extracellular amino acids.

We postulated that during amino acid deprivation, increased pinocytosis provides an increased alternate source of amino acids (derived from degradation of exogenous protein) for protein synthesis. Consistent with this postulate, Hammer and Rannels (17) reported that in the absence of extracellular amino acids protein synthesis decreased by ~30% and the presence of exogenous albumin (2%) could restore the synthetic rate to that measured in the presence of plasma levels of amino acids. On the basis of the rates of albumin degradation and protein synthesis determined by Hammer and Rannels (17), we calculate that only 36% of the phenylalanine derived from degraded albumin was required to restore the protein synthetic rate to normal. This value is in excellent agreement with our empirically determined rate of utilization for protein synthesis of phenylalanine derived from degradation of exogenous albumin (42%, see Results).

The findings reported here and those reported by Hammer and Rannels (17) are consistent in many, but not all, aspects. For example, Hammer and Rannels (17) used suspended alveolar macrophages and reported that the cells were in nitrogen balance in the presence of a physiological level of amino acids. We found monolayers of alveolar macrophages in negative nitrogen balance under similar conditions. Whether the origin of these differences results from comparing suspended with adherent cell function or whether these differences reflect variability inherent to the source of the cells (27) is not known.

The present findings, along with those of Hammer and Rannels (17), and Rannels et al. (27), provide strong experimental evidence that intracellular degradation of exogenous protein by pulmonary alveolar macrophages can provide a source of amino acids for protein synthesis. In contrast, Eagle and Piez (11) using HeLa cells concluded that degradation of exogenous protein was not a significant source of amino acids for de novo protein synthesis. There are at least three explanations for these differences. (*a*) The apparent rate of pinocytosis by HeLa cells is approximately 20 times lower than that of alveolar macrophages (6, 20, 21). (*b*) Compared with macrophages, HeLa cells have very low levels of lysosomal proteases (i.e., cathepsin D, Table III). (*c*) The experiments with HeLa cells were performed in culture medium containing either a complete supply of amino acids at supraphysiological concentrations or, likewise, omitting six amino acids (five essentials). We have demonstrated that macrophages cultured in medium containing physiological levels of amino acids utilize only 7% of the phenylalanine derived from degradation of exogenous protein for de novo protein synthesis. Whether omitting five essential amino acids would produce results analogous to those in the absence of all amino acids is not known.

The question remains as to whether the contribution of amino acids from degradation of exogenous protein is significant in the presence of a physiological level of exogenous

protein (0.6% compared with the 2% used in the studies presented here). In this regard, Airhart et al. (1) and Rannels et al. (27) have shown that in the presence of 10% serum (≈0.6% exogenous protein) and a physiological level of free amino acids a significant fraction of the amino acid used to charge tRNA does not come from the extracellular pool. Furthermore, raising the concentration of extracellular amino acids could bring the specific activity of amino acyl-tRNA to that of the extracellular pool in the absence of serum, but not in the presence of serum (27). Thus, it appears that 10% serum may be capable of providing a significant source of amino acids for macrophage protein synthesis. Additionally, Hammer and Rannels (17) have shown that degradation of exogenously supplied BSA is linear over a range of concentrations from 0.02 to 6.0%. Calculations based on those findings, in relation to those presented here, predict that under conditions of amino acid deprivation 0.6% exogenous protein would provide 83% of the amino acids (i.e., phenylalanine) necessary to maintain a normal rate of protein synthesis by alveolar macrophages. Therefore, even at a physiological concentration of exogenous protein, it seems likely that pinocytosis coupled to intracellular hydrolysis of exogenous protein could provide a significant source of amino acids for protein synthesis, especially during periods of reduced availability of extracellular free amino acids. Whether this pathway is common to cell types other than alveolar macrophages remains to be determined.

Lastly, recent evidence from two different receptor-mediated systems indicates that amino acids may determine the intracellular pathway followed subsequent to internalization of ligand (7, 24). Thus, amino acid modulation of intracellular pathway traffic may be a characteristic of pinocytosis in general.

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