

Amino Acid Control of Autophagic Sequestration and Protein Degradation in Isolated Rat Hepatocytes

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ABSTRACT Sequestration of the inert cytosolic marker [^{14}C]sucrose by sedimentable organelles was measured in isolated rat hepatocytes made transiently permeable to sucrose by means of electropermeabilization. Lysosomal integrity, protein degradation, autophagic sequestration, and other cellular functions were not significantly impaired by the electric treatment.

Hepatocytes sequestered sucrose at an initial rate of $\sim 10\%/h$, which is threefold higher than the estimated rate of autophagic-lysosomal protein degradation. Almost one-third would appear to represent mitochondrial fluid uptake; the rest was nearly completely and specifically inhibited by 3-methyladenine (3MA) and can be regarded as autophagic sequestration. A complete amino acid mixture was somewhat less inhibitory than 3MA, and partially antagonized the effect of the latter. This paradoxical effect, taken together with the high sequestration rate, may suggest heterogeneity as well as selectivity in autophagic sequestration. There was no detectable recycling of sequestered [^{14}C]sucrose between organelles and cytosol.

Studies of individual amino acids revealed histidine as the most effective sequestration inhibitor. Leucine may have a regulatory function, as indicated by its unique additive/synergistic effect, and a combination of Leu + His was as effective as the complete amino acid mixture. Asparagine inhibited sequestration only 20%, i.e., its very strong effect on overall (long-lived) protein degradation must partially be due to post-sequestration inhibition.

The lysosomal (amine-sensitive) degradation of short-lived protein was incompletely inhibited by 3MA, indicating a contribution from nonautophagic processes like crinophagy and endocytic membrane influx. The ability of an amino acid mixture to specifically antagonize the inhibition of short-lived protein degradation by AsN + GIN (but not by 3MA) may suggest complex amino acid interactions at the level of fusion between lysosomes and other vesicles in addition to the equally complex interactions at the level of autophagic sequestration.

Rat hepatocytes offer a particularly suitable material for the study of intracellular protein degradation. These cells are in positive protein balance in well-fed animals (13) or in a culture medium supplied with insulin and high amino acid concentrations (20, 26), but upon starvation *in vivo* (or amino acid deprivation *in vitro*), the hepatocytes rapidly turn on their autophagic-lysosomal degradation pathway and enter a state of negative protein balance in an attempt to supply the organism (or medium) with amino acids (13, 14, 26, 27, 33). The overall rate of protein degradation may be as high as 4–5%/h, of which the autophagic-lysosomal pathway accounts for $\sim 3\%/h$ on the basis of the extent of inhibition seen with amino acids and lysosome inhibitors (32, 35). The remaining $\sim 1.5\%$ (corresponding to the “basal” degradation generally

observed in well-nourished cultured cells, cf. references 1, 5, 6, 37) presumably reflects the activity of the several nonlysosomal proteolytic systems known to exist (7, 12, 16), although direct evidence for this is yet lacking.

The autophagic rate and hence the protein balance of the isolated hepatocytes can be precisely controlled by adjusting the concentrations of amino acids in the incubating medium. Leucine, histidine, phenylalanine, tyrosine, tryptophan, glutamine, and asparagine are particularly strong suppressors of protein degradation, and various combinations of these, at high concentrations, can effectively substitute for a complete amino acid mixture (33). We have reasoned that the study of single, or a few, individual amino acids may provide a simplified approach to the biochemical mechanisms of amino

acid control, not to be confused with the complex physiology of regulation by the complete set of amino acids *in vivo*.

To investigate the early autophagic-lysosomal mechanisms with biochemical methods, several research groups have recently established procedures for the microinjection of radioactive proteins or inert probes into the cytosol of various cell types (2, 8, 11, 18). In our laboratory we have developed an electropermeabilization technique, whereby [¹⁴C]sucrose is introduced into hepatocyte cytosol with high efficiency (10). The time-dependent transfer of radioactivity from the cytosol to sedimentable vesicles may serve as a convenient bioassay for overall autophagic sequestration. In the present work, we have optimized this technique and used it to study the effects of amino acids on the first step in the autophagic-lysosomal pathway.

MATERIALS AND METHODS

Chemicals: Uniformly labeled [¹⁴C]sucrose (584 Ci/mol; 200 μ Ci/ml; radiochemical purity 99%) was purchased from Amersham International Ltd., Buckinghamshire, England. 3-Methyladenine (6-amino-3-methylpurine; 3MA¹) was from Fluka AG, Buchs, Switzerland; propylamine from Koch-Light, Colnbrook, Berkshire, United Kingdom; metrizamide from Nyegaard & Co, Oslo, Norway; amino acids and other biochemicals from Sigma Chemical Co., St. Louis, MO.

Hepatocyte Preparation: Hepatocytes were prepared from the liver of 16-h starved male Wistar rats, 250–300 g, by collagenase perfusion (28). The purified cells were suspended at a cell concentration of 150–200 mg wet wt/ml in suspension buffer (28) fortified with 20 mM pyruvate, 2 mM MgCl₂, and 10 μ g/ml gentamicin, and maintained at 0°C.

[¹⁴C]Sucrose Loading and Incubation of Cell Suspensions: 10–12 ml of cell suspension was warmed to 37°C and electropermeabilized in a square-bottomed (2 × 2 cm) electrode chamber, using five pulses at 2 kV/cm and 1.2 μ F (10). [¹⁴C]sucrose was added (4 μ Ci/ml) and the cell suspension was cooled to 0°C and maintained at that temperature for 1 h. The suspension was then incubated 30 min at 37°C (for resealing) in two 10-cm petri dishes on a tilting platform (10 rev/min). After washing three times at 0°C in 4 ml of wash buffer (28), the cells were resuspended in fortified suspension buffer at a cell concentration of 100–120 mg/ml.

The sucrose loading efficiency was estimated as the concentration of [¹⁴C]-sucrose in the washed cells (based on a cellular water content of 50% of the wet weight, cf. reference 28) relative to the concentration in the medium, which is expressed in percent.

The cells were incubated as 0.4-ml aliquots (usually 0.3 ml cell suspension + 0.1 ml additives) in 15-ml shaking centrifuge tubes (215 rev/min) at 37°C for up to 2.5 h. For longer incubations, 7 ml of cell suspension (including additives) was incubated in a 10-cm tilting petri dish, and 100 μ -samples were taken serially down to a volume of 3.4 ml. The complete amino acid mixture used in some of the experiments had the composition given in reference 14.

Electrodisruption of the Cells: After incubation, the cells were washed twice in 4 ml ice-cold 10% (wt/vol) sucrose, and the cell pellet was finally suspended in 0.5 ml 10% sucrose. The whole sample was warmed to 37°C, transferred to a 1 × 1-cm-bottomed electrode chamber, and the cells disrupted with a single pulse (2 kV/cm, 0.6 μ F) (10).

Isolation of the Sedimentable Cell Fraction and Determination of Radioactivity: The electrodisrupted cell sample was layered on top of 3 ml ice-cold metrizamide (8%)/sucrose (8%) and centrifuged for 30 min at 7,000 g (5,000 rev/min). The fluid was removed by stepwise washing (with ice-cold wash buffer), the tube walls wiped, and the pellet dissolved in 0.5 ml of 0.1 M NaOH/0.4% (wt/vol) sodium deoxycholate. 8 ml of scintillation fluid (Scint Hei 3; F. Heidenreich, Oslo, Norway) was added, and the radioactivity measured (as disintegrations per minute in a Beckman LS9000 liquid scintillation counter).

Acid Phosphatase: Acid phosphatase was assayed according to Barrett (3) using 0.5% Triton X-100 to distinguish between free and total (free plus latent) enzyme activity as previously described (36).

Calculation of Sequestration Rates: The amount of sedimentable radioactivity in electrodisrupted cells was measured at the beginning and end

of an incubation period, generally between 30 and 90 min of incubation, to exclude from the measurement the lag period in the action of added inhibitors. (In one specified instance, incubation from 0 to 60 min was used.) The net accumulation during the measurement period was used to calculate the rate of sequestration. The sequestration has been expressed as percent of the total cell-associated radioactivity at the end of the incubation period (i.e., usually at 90 min) to minimize the error due to cell loss. In time curves, the amount sequestered has been given as percent of the total radioactivity at that time point.

Protein Synthesis: Protein synthesis was measured as the incorporation of [¹⁴C]valine into acid-precipitable material during the first 60 min of incubation. The valine concentration was kept high (10 mM) to prevent isotope dilution (decrease in specific radioactivity) due to valine released through endogenous protein degradation (30). The medium was supplemented with the full complement of amino acids (14, 30) to ensure that the latter did not become rate-limiting for protein synthesis.

Protein Degradation: Protein degradation was measured as the release of [¹⁴C]valine from labeled protein. The medium was supplemented with 15 mM unlabeled valine as a safety precaution against reincorporation, which, however, is usually negligible (35). Measurements were made between 30 and 90 min of incubation to eliminate the effect of the lag phase in the onset of action of the various inhibitors tested.

The degradation of (predominantly) short-lived protein, a protein sub-class that constitutes a minor fraction of the total protein, was measured after labeling the isolated hepatocytes with [¹⁴C]valine for 1 h *in vitro* (0.6 μ Ci/ml), and after extracting most of the intracellular [¹⁴C]valine through three consecutive incubations (each for 10 min at 37°C) (35).

The degradation of long-lived protein, representative of overall protein degradation, was measured after labeling with [¹⁴C]valine for 24 h *in vivo* (50 μ Ci/ml in 1 ml, injected intravenously); in this case the intracellular [¹⁴C]valine pool is in equilibrium with degradation and no additional extraction is necessary. Unless otherwise specified, protein degradation refers to the degradation of long-lived protein.

ATP: ATP was measured in perchloric extracts of the cells by a bioluminescence method (LKB-Wallac). The cells were precipitated with 10% (wt/vol) perchloric acid while incubating at 37°C, to avoid the decline in ATP which generally occurs upon cooling.

RESULTS

Effect of Electropermeabilization on Cellular Functions

The basic properties of our newly developed methodology for the measurement of autophagic sequestration in isolated hepatocytes have recently been described (10). The principle of the method is to introduce an inert probe, [¹⁴C]sucrose, into the cytosol through plasma membrane regions made transiently permeable to sucrose by means of a short series of electric shocks (pulsed high-voltage discharges from a capacitor). The subsequent transfer of sucrose from cytosol to sedimentable vesicles can then be taken as a measure of autophagic sequestration activity.

Optimal conditions for efficient cytosolic [¹⁴C]sucrose loading have been worked out (10), but the effect of electrical treatment on cell functions pertinent to the study of autophagy and protein metabolism has not previously been studied in detail. With the acquisition of electropermeabilization equipment with increased voltage capability, we have re-examined the basic methodological parameters—number of discharges, voltage, capacitance, and incubation at 0°C—on both [¹⁴C]sucrose loading and biological functions such as protein synthesis, protein degradation, and cellular ATP content.

Fig. 1 shows the effect of an increasing number of discharges (pulses) from the capacitor as well as the effect of incubating the cells at 0°C (at which temperature they remain permeable to sucrose, whereas cells incubated at 37°C reseal in 10–15 min). The amount of [¹⁴C]sucrose that can be introduced into the cytosol (Fig. 1A) is considerably increased by a 1-h incu-

¹ Abbreviation used in this paper: 3MA or 3-MeAde, 3-methyladenine (6-amino-3-methylpurine).

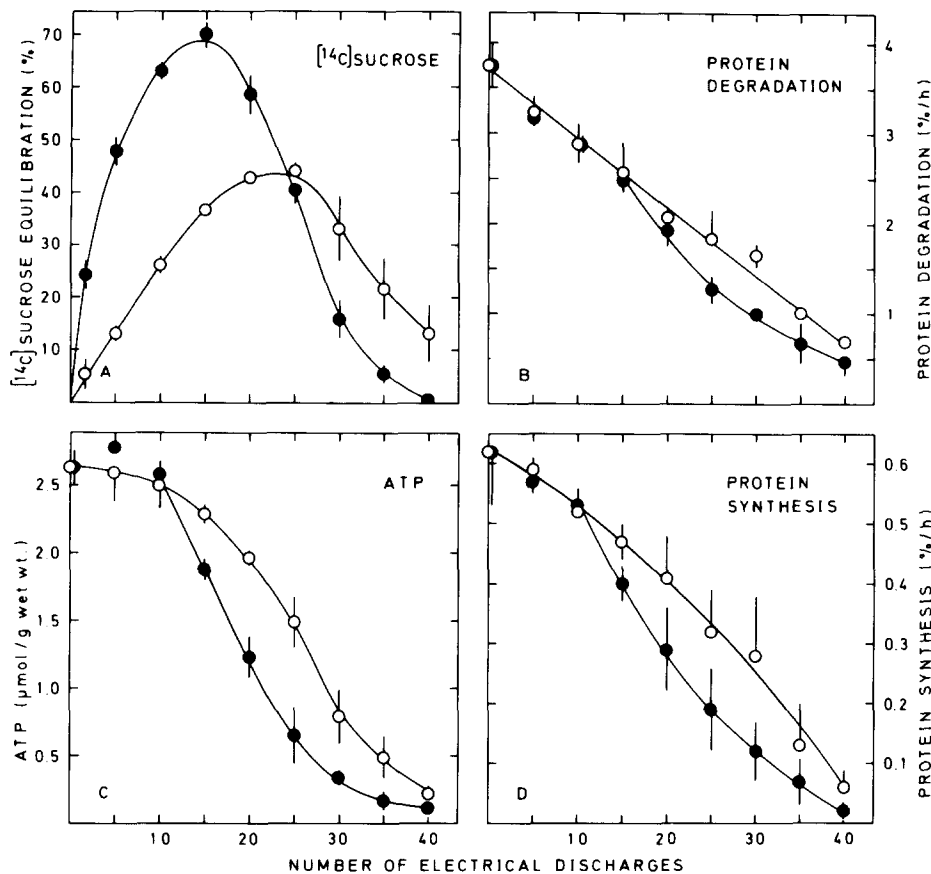


FIGURE 1 Effect of pulse number and incubation at 0°C on [¹⁴C]sucrose loading efficiency and functional properties of electroporabilized-resealed hepatocytes. The cells (1 ml) were permeabilized in the presence of [¹⁴C]sucrose, with the number of pulses indicated at 2 kV/cm and 0.6 μF in a 1 × 1-cm-bottomed electrode chamber; then incubated for 30 min at 37°C directly (○) or after 1 h at 0°C (●). The content of [¹⁴C]sucrose (A) and ATP (C) in the resealed cells was measured immediately after washing, and the rates of protein degradation (B) and synthesis (D) during a subsequent incubation at 37°C. Each point is the mean ± SE of three to seven separate experiments.

bation at 0°C, making 15 permeabilizing pulses sufficient to reach a transmembrane [¹⁴C]sucrose equilibration of 70%, which is close to the theoretical maximum (i.e., the accessible cytosolic space as a fraction of total cell volume). However, functional properties such as protein synthesis (Fig. 1B), maintenance of the ATP level (Fig. 1C), and protein degradation (Fig. 1D) apparently deteriorate as a function of the pulse number, and the incubation at 0°C aggravates this tendency when the number of pulses is high. Fortunately the ATP level is completely maintained when <10 pulses are used, despite the low molecular weight of ATP (507, i.e., less than the dye eosin at 648, to which the cells become completely permeable), indicating that molecular properties other than size (e.g., charge or shape) may determine permeation across electrically treated cell membranes. We have chosen to use five pulses as standard treatment, since this allows almost 50% [¹⁴C]sucrose equilibration while the ATP level is maintained and protein synthesis and degradation rates are reduced only by ~10%.

The efficiency of [¹⁴C]sucrose loading increased as a function of the applied voltage (Fig. 2A) up to a maximum around 3 kV (since the distance between the electrodes was 1 cm, the field strength given in kV/cm is numerically identical to the voltage) with the 1-h incubation at 0°C included. However, since the protein degrading ability of the hepatocytes decreased (albeit moderately) as a function of voltage (Fig. 2B), the near-maximal loading efficiency obtained at 2 kV was considered sufficient for all practical purposes. Capacitances of 0.6–1.2 μF (corresponding to pulse durations of 60–120 μs with 1 ml of cell suspension, i.e., 1 cm² electrodes spaced 1 cm apart) provided maximal sucrose loading (Fig. 2C), while having little effect on protein degradation (Fig. 2D). Standard

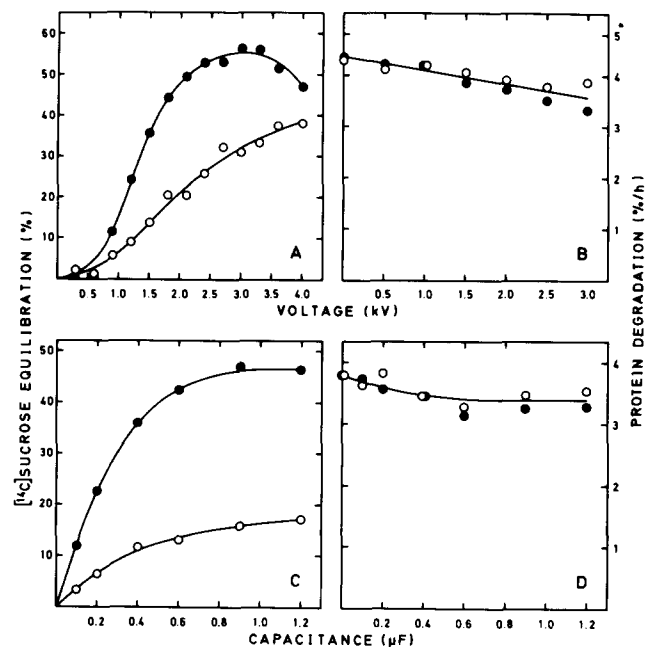


FIGURE 2 Effect of permeabilization voltage and capacitance on [¹⁴C]sucrose loading efficiency and protein-degrading ability of electroporabilized-resealed hepatocytes. The cells (1 ml) were permeabilized in a 1 × 1-cm-bottomed electrode chamber with five pulses at 0.6 μF and the voltage indicated (A, B), or at 2 kV/cm and the capacitance indicated (C, D), then incubated for 30 min at 37°C directly (○) or after 1 h at 0°C (●). The cellular [¹⁴C]sucrose content (A and C) was measured immediately after washing, and the rate of protein degradation (B and D) during a subsequent incubation at 37°C. Each value is the mean of two to three separate experiments.

conditions for [^{14}C]sucrose loading (1 ml of cells) thus include permeabilization with five discharges at 2 kV/cm and 0.6 μF , followed by incubation for 1 h at 0°C and 30 min at 37°C. With a larger volume, e.g., 8 ml of cell suspension, it is necessary to use a discharge chamber with 2 cm^2 electrodes spaced 2 cm apart and a setting of 4 kV and 1.2 μF to obtain the specified pulse intensity (2 kV/cm and 60 μs).

Microscopic examination of the cells showed that cellular integrity was well preserved following permeabilization/re-sealing either with or without the 1-h incubation at 0°C included. During a subsequent 90-min incubation at 37°C, the usual period of sequestration used in the present study, there was at most a 5–10% decrease in general cell viability (i.e., in the percentage of structurally intact cells, as measured by trypan blue staining or loss of [^{14}C]sucrose from the cells). After the sequestration period, the cells were electrically disrupted by a single high voltage pulse (0.5 ml, 2 kV/cm, and 0.6 μF) in electrolyte-free isotonic sucrose (10), a treatment that broke up the plasma membrane (but not intracellular membranes) of ~99.9% of the cells and produced easily sedimentable, trypan blue-stainable cell corpses containing [^{14}C]sucrose sequestered in vesicular structures. Centrifugation of the electrodisrupted cells through a metrizamide cushion (10) removed more than 99% of the cytosolic [^{14}C]sucrose, permitting reliable measurements of sequestered radioactivity.

Integrity of Lysosomes in Sedimented Cell Corpses

Agents such as amino acids (17) and lysosomotropic amines (36) have been shown to alter lysosomal fragility, resulting in variable lysosome rupture e.g., during osmotic stress or assay for lysosomal enzymes. If lysosomal rupture occurred to any significant extent during electrodisruption of the cells and/or gradient isolation of cell corpses, the measurement of sequestered radioactivity could be seriously compromised, and the effects of added inhibitors misinterpreted. However, no loss of lysosomal contents would seem to be incurred by the preparative procedures, since sedimented cell corpses and whole (frozen/thawed) cells contained similar total activities of the lysosomal marker enzyme acid phosphatase according to the assay time curves displayed in Fig. 3. Pretreatment of the cells with 3MA or amino acids did not affect the total amount or the sedimentability of acid phosphatase (results not shown). Furthermore, the initial latency (vesicular containment) of the enzyme was the same (~80%) in autophagically active control hepatocytes and in cells suppressed by amino acids and/or 3MA: the inherent difference in lysosomal fragility was expressed only during, and as a result of, the assay (Fig. 3). Pretreatment with these agents would thus appear to have no effect on the integrity of the lysosomes in the sedimented cell corpses.

No Metabolism of Electroinjected [^{14}C]Sucrose

Thin-layer chromatography of (acid) cell extracts in a system that separated the various mono- and disaccharides showed that there was no detectable metabolic conversion of electroinjected [^{14}C]sucrose during a 2-h incubation of the cells at 37°C (results not shown). The amount of radioactivity sequestered during this period was extracted separately, and shown by chromatography to consist entirely of authentic [^{14}C]sucrose (38). Thus, there seems to be no hepatocytic sucrose metabolism to disturb the sequestration measurements performed with this probe.

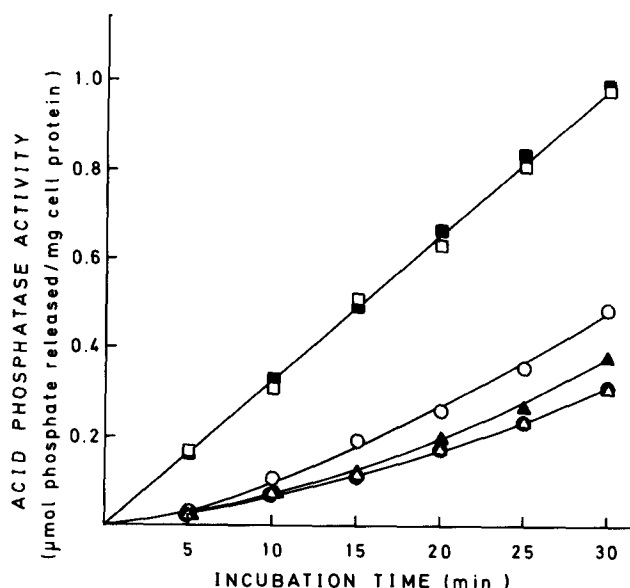


FIGURE 3 Lysosomal integrity in cell corpses as measured by acid phosphatase latency. Acid phosphatase was measured in whole cells (■) or cell corpses (□) in the presence of 0.5% Triton X-100 to obtain the total enzyme activity, or in cell corpses in the absence of Triton (other symbols) to obtain nonlatent (i.e., extralysosomal) enzyme activity. All cells were incubated for 90 min with or without amino acids/3MA before electrodisruption and assay. ○, control (no addition); ●, amino acids (1 \times); △, 3MA (10 mM); ▲, amino acids + 3MA. Each value is the mean of three parallel cell samples from one representative experiment.

Stable Localization of the Sequestered Material

A basic premise of the present methodology is that the electroinjected [^{14}C]sucrose is retained by the cells, i.e., that it does not leak out through the plasma membrane. However, the incubation of hepatocytes in shaking suspensions usually results in some cell damage due to mechanical stress, with the loss of some [^{14}C]sucrose and other cytosolic components as a consequence. The extent of damage depends on the overall cell quality, which varies slightly, primarily as a function of the purity of the collagenase batch used for cell preparation. In the series of experiments presented in Fig. 4, electroinjected [^{14}C]sucrose was lost from the resealed hepatocytes at a rate comparable with the rate of cell death under these conditions. Between 30 and 90 min, a time period commonly employed in our sequestration experiments, the [^{14}C]sucrose loss averaged $6.4 \pm 0.6\%$ (mean \pm SE of six experiments). Since dead cells do not contribute to sequestration, this loss was corrected for by expressing the amount of sequestered [^{14}C]sucrose as percent of the total cellular radioactivity content at the time of measurement. The loss of cell-associated sucrose was not significantly affected by any of the treatments used in the present investigation (e.g., amino acids or 3MA).

To determine whether [^{14}C]sucrose, once sequestered, would remain stably located in the sequestering (vesicular) compartment or whether it would recycle back to the cytosol, a double permeabilization experiment was performed. After having allowed hepatocytes to sequester electroinjected [^{14}C]sucrose for 2 h at 37°C, the cells were subjected to a second electropermeabilization and left at 0°C for 2 h to extract (through leakage) cytosolic radioactivity. The cells were then resealed and incubated for another 2 h at 37°C, to see if the sequestered [^{14}C]sucrose would redistribute to the cytosol. The sequestering ability of the cells during this second period,

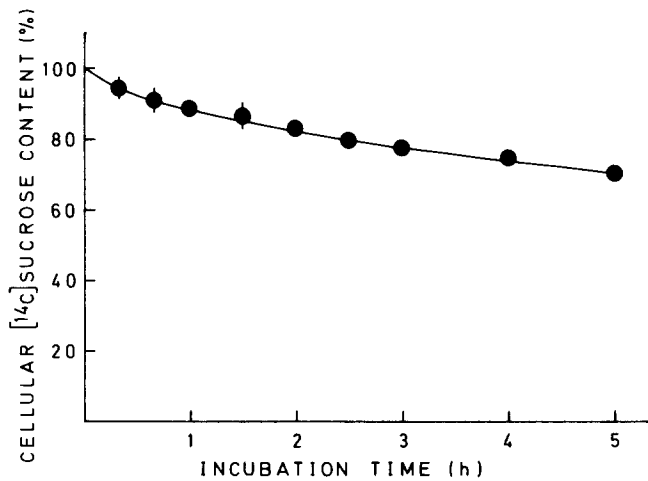


FIGURE 4 Loss of [¹⁴C]sucrose from electro-loaded hepatocytes during extended incubation. The cells were loaded with [¹⁴C]sucrose and resealed according to standard procedures; then incubated at 37°C for the length of time indicated. Data from experiments using shaking tubes (up to 2 h) and tilting petri dishes have been combined; no difference between the two types of incubation was observed. The sucrose content is given as percent of the value at time zero. Each point is the mean ± SE of two to seven experiments; some of the standard errors are concealed by the symbols.

as measured in separate experiments, was found to be normal (results not shown).

As shown in Fig. 5, both the sequestered and the remaining nonsequestered radioactivity (each contributing about 50% of the total) was lost from the cells at the same rate as the enzyme lactate dehydrogenase, indicating that the [¹⁴C]sucrose loss from both compartments could be accounted for entirely by cell death. The pool of nonsequestered, cytosolic [¹⁴C]sucrose had been depleted by ~80% as a result of the second permeabilization, so the measurable rate of sequestration of radioactivity would have to be reduced by at least this amount. Since there was still no detectable release of sequestered [¹⁴C]sucrose back to the cytosol, any steady-state rate of recycling could be no greater than one-fifth of the sequestration rate. If part of the remaining cytosol radioactivity were less accessible to sequestration than the radioactivity extracted, the rate of recycling could be lower still.

The experiment thus indicates that [¹⁴C]sucrose recycling, if it occurs at all, is not of a sufficient magnitude to significantly influence the kinetics of [¹⁴C]sucrose sequestration as measured in our short-term studies.

Effect of an Amino Acid Mixture and 3-Methyladenine on Sequestration

Electroinjected [¹⁴C]sucrose was sequestered continuously during a 5-h incubation of the hepatocytes (Fig. 6, control). The initially high sequestration rate ($8.9 \pm 0.3\%/h$ between 30 and 90 min, mean ± SE of six experiments) gradually declined, partially due to depletion of sequestrable cytosolic sucrose, partially due to an increasing inhibition of autophagy by proteolysis-derived amino acids (24, 33).

Both amino acids and the purine 3-methyladenine (3MA) are well-established inhibitors of autophagic-lysosomal protein degradation, and are believed to exert their effect at the sequestration step as indicated by their suppression of the cellular autophagosome content (14, 27, 31). Fig. 6 shows that 3MA as well as an amino acid mixture indeed did inhibit the

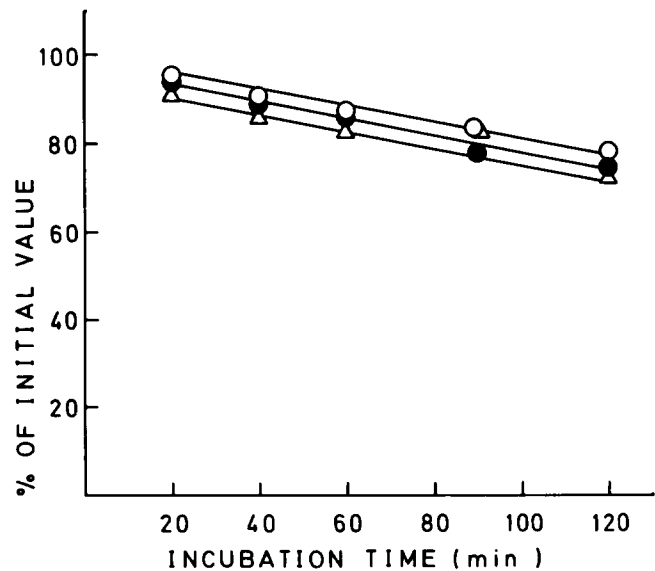


FIGURE 5 Equal stability of sequestered radioactivity, total cellular radioactivity, and cellular LDH content. Hepatocytes were first electropermeabilized/[¹⁴C]sucrose-loaded/resealed and allowed to sequester [¹⁴C]sucrose for 2 h, then electropermeabilized a second time and left at 0°C for 2 h to allow most of the cytosolic [¹⁴C]sucrose to leak out. The cells were then incubated at 37°C for the length of time indicated, electrodisrupted, and the total cell-associated radioactivity (○) and LDH (△) as well as the radioactivity sequestered in cell corpses (●) was measured. Each value is the mean of three parallel cell samples from one representative experiment.

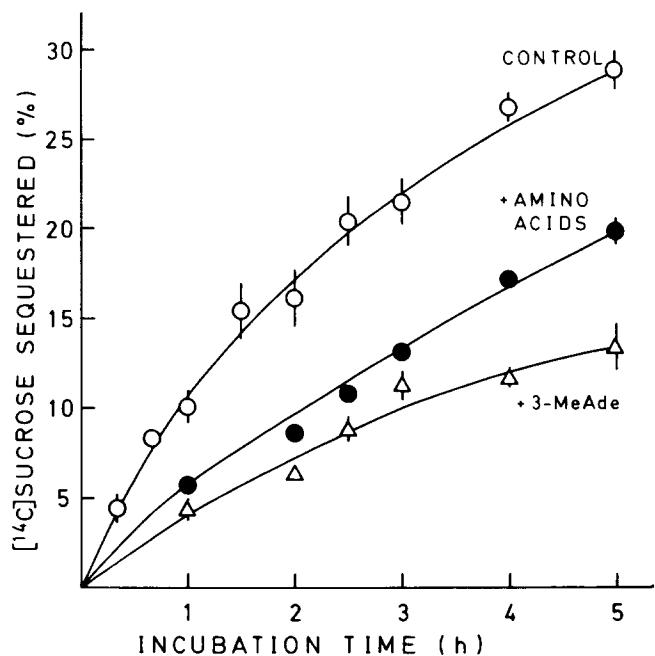


FIGURE 6 Time course of [¹⁴C]sucrose sequestration: effects of an amino acid mixture and 3-methyladenine. Hepatocytes were electro-loaded with [¹⁴C]sucrose, and incubated for the length of time indicated without additions (○), with a complete amino acid mixture (●), or with 10 mM 3MA (△). The net increase in the sedimentable radioactivity content of electrodisrupted cells was measured and expressed as percent of the total cellular radioactivity at the time of measurement. Each value is the mean ± SE of two to five separate experiments; some of the standard errors are concealed by the symbols.

sequestration of electroinjected [14 C]sucrose. Both agents required a certain time lag before maximal inhibition was reached (the inhibition by 10 mM 3MA between 0 and 30 min was only $33 \pm 3\%$, and the inhibition by amino acids only $22 \pm 4\%$; means \pm SE of eight experiments); quantitative effects are therefore best measured, between 30 and 90 min. The effects of these inhibitors on protein degradation have previously been found to be rapidly reversible (14, 31).

Dose-response curves (Fig. 7) revealed increasing inhibition with increasing concentrations, near-maximal effects being obtained at 20 mM 3MA (80% inhibition) and at a total amino acid concentration of 25 mM (50% inhibition), corresponding to the "1 \times " strength (14) ordinarily used, e.g., in Fig. 6. The amino acid effect was not additive to that of 3MA; on the contrary, the amino acid mixture paradoxically reduced the effect of the purine somewhat (Table I). The complete amino acid mixture also failed to add to the effect of the potent amino acid couple Leu + His (see below), i.e., both 3MA and Leu + His appear to be capable of effectively substituting for the amino acid mixture.

The amino acid inhibition of protein degradation has previously been found to be demonstrable even in the presence of cycloheximide, i.e., it is independent of protein synthesis (15). [14 C]Sucrose sequestration, on the other hand, was inhibited by cycloheximide alone to such an extent (40%) as to make analysis of the amino acid effect difficult (Table I). An additional inhibition was observed both with the amino acid mixture and with Leu + His, but the effects were too small to be statistically significant at the 95% confidence level. The 3MA-antagonistic effect of the amino acid mixture, however, was clearly significant even in the presence of cycloheximide

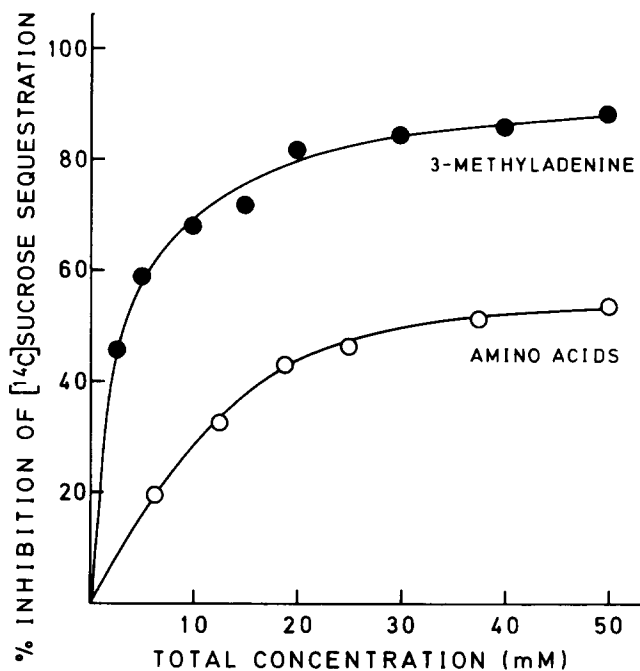


FIGURE 7 Dose-response characteristics of the inhibition of [14 C]-sucrose sequestration by an amino acid mixture and 3-methyladenine. [14 C]sucrose-loaded hepatocytes were incubated at 37°C, and the rate of sequestration measured between 30 and 90 min in the presence of various total concentrations of an amino acid mixture (○) or 3-methyladenine (●). The amino acid mixture has a total concentration of \sim 25 mM at the 1 \times level generally used (14). Each point is the mean of two separate experiments, the range averaging \pm 3%.

($P < 0.005$), and hence apparently independent of protein synthesis.

Effects of Individual Amino Acids

A limited number of amino acids (leucine, histidine, phenylalanine, tyrosine, tryptophan, glutamine, and asparagine) have been found to be particularly active in the inhibition of hepatocytic protein degradation (22, 32, 33). Several mechanisms have been indicated, including a general inhibition of sequestration as well as inhibition (particularly by high concentrations of asparagine and glutamine) at a postsequestrational step (32), and, furthermore, a special function for leucine (22, 33).

The effects of five degradation-inhibitory amino acids, alone and in combination with each of the other four, on [14 C]sucrose sequestration are shown in Table II. All the amino acids were tested at the standard concentrations of 10 and 20 mM, which were found to give near-maximal inhibition of protein degradation in previous dose-response studies (32, 33). Although some inhibition could also be seen at low, physiological concentrations, it is not unreasonable that superphysiological concentrations of individual amino acids are needed to produce effects comparable with that of the complete amino acid mixture (which contains a total of \sim 25 mM amino acids). Individual effects (at 20 mM) ranged from $<20\%$ (leucine) to $>40\%$ inhibition (glutamine and histidine). Strikingly, asparagine produced only a 20% inhibition, despite its ability to almost completely suppress the autophagic-lysosomal pathway of protein degradation at the concentration used (32). This observation provides strong indirect support for an additional, i.e., postsequestrational, effect of asparagine.

Amino acids that were combined two and two at 10 mM each (Table II) had, in general, additive effects, but usually not beyond the level of inhibition obtained with the most active partner alone at 20 mM. A notable exception was leucine, which produced a 60% inhibition in combination with either glutamine or histidine; an effect that even exceeded that obtained with a complete amino acid mixture (cf. Table I). The special regulatory interaction between leucine and other amino acids previously noted (22, 33) may thus be exerted at the level of sequestration. Combinations with 20 mM histidine (the most effective amino acid) were not significantly more inhibitory than with 10 mM histidine (Table II).

None of the individual amino acids tested were additive to 3MA, and, unlike the complete mixture, they did not antagonize the purine effect (Table II, last column).

The combinations Leu + His and Leu + GIN were equally effective according to Table II. Since histidine, unlike glutamine, is not thought to act postsequestrationally (32), the Leu + His couple would seem to be preferable as a specific means of inhibiting sequestration (other than 3MA). 10 mM His was apparently both a necessary and sufficient concentration, whereas near-maximal Leu-effects were obtained even at 2 mM (Fig. 8A). Routinely, 10 mM of each is used to ensure a consistent maximal effect.

Effect of Amino Acids and 3-Methyladenine on Protein Degradation

A complete mixture of 20 amino acids, at concentrations optimal for inhibition of protein degradation, inhibits overall protein degradation in hepatocytes by \sim 70% (14). When tested individually, leucine, histidine, and phenylalanine in-

TABLE I
Effect of an Amino Acid Mixture, 3-Methyladenine and Cycloheximide on [¹⁴C]Sucrose Sequestration

	Percent inhibition of [¹⁴ C]sucrose sequestration			
	None	+ Amino acids	+ Cycloheximide	+ Amino acids + Cycloheximide
Series 1 (0–60 min)				
Control	—	30.6 ± 5.6 (7)	41.1 ± 3.3 (7)	51.0 ± 6.4 (7)*
3MA (20 mM)	76.6 ± 2.3 (7)	58.3 ± 5.1 (7)*	74.6 ± 2.7 (7)	49.6 ± 5.9 (7) [§]
Leu + His (10 + 10 mM)	38.3 ± 5.1 (7)	39.1 ± 6.0 (7)	56.3 ± 6.5 (7)	48.6 ± 6.1 (7)
Series 2 (30–90 min)				
Control	—	50.5 ± 2.6 (14)	39.3 ± 3.8 (3)	—
3MA (20 mM)	80.9 ± 2.2 (8)	74.0 ± 2.0 (4) [†]	—	—

[¹⁴C]sucrose-loaded, resealed hepatocytes were incubated at 37°C. Inhibitors were added at the beginning of incubation, while sequestration was measured either between 0 and 60 min (series 1) or between 30 and 90 min (series 2). The 30–90-min measurement period corrects for the lag phase in the action of amino acids; the inhibitory effect of the latter is therefore found to be greater in the second series. Control rates of sequestration were 10.1 ± 0.2%/h (7) between 0 and 60 min, and 7.5 ± 0.3 (18) between 30 and 90 min. The total concentration of the amino acid mixture (14) was ~25 mM; the concentration of cycloheximide was 1 mM. The percent inhibition (relative to control) was determined in each experiment; values given are means ± SE of the number of experiments indicated in parentheses. Statistical significance was estimated by the *t*-test.

* *P* < 0.05 vs. amino acids alone.

† *P* < 0.01 vs. 3MA alone.

§ *P* < 0.005 vs. 3MA + cycloheximide.

† *P* < 0.05 vs. 3MA alone.

TABLE II
Effect of Individual Amino Acids on [¹⁴C]Sucrose Sequestration

	Percent inhibition of [¹⁴ C]Sucrose Sequestration					
	Leu (10 mM)	Phe (10 mM)	AsN (10 mM)	GIN (10 mM)	His (10 mM)	3MA (20 mM)
Alone	13.9 ± 2.4 (12)	15.9 ± 5.9 (8)	23.2 ± 3.2 (12)	25.5 ± 3.3 (12)	38.6 ± 3.2 (12)	82 (2)
+ 10 mM Leu	<i>16.3 ± 1.2 (4)</i>	39.5 ± 3.0 (4)	37.0 ± 4.4 (6)	62.2 ± 2.0 (6)*	61.3 ± 3.2 (6) [‡]	82 (2)
+ 10 mM Phe	39.5 ± 3.0 (4) [§]	<i>31.8 ± 3.5 (4)</i>	31.5 ± 2.0 (4)	39.3 ± 7.6 (4)	46.5 ± 3.9 (4)	76 (1)
+ 10 mM AsN	37.0 ± 4.4 (6) [§]	31.5 ± 2.0 (4)	<i>19.8 ± 6.3 (4)</i>	44.2 ± 1.4 (6)	44.3 ± 3.4 (6)	83 (2)
+ 10 mM GIN	62.2 ± 2.0 (6) [§]	39.3 ± 7.6 (4)	44.2 ± 1.4 (6)	<i>42.5 ± 7.1 (4)</i>	45.3 ± 3.7 (6)	80 (2)
+ 10 mM His	61.3 ± 3.2 (6) [§]	46.5 ± 3.9 (4)	44.3 ± 3.4 (6)	45.3 ± 3.7 (6)	<i>44.0 ± 2.9 (4)</i>	82 (2)
+ 20 mM His	58.4 ± 1.7 (5) [§]	50.2 ± 2.8 (5)	47.2 ± 3.2 (5)	51.0 ± 2.3 (5)	—	—

The sequestration of [¹⁴C]sucrose between 30 and 90 min of incubation was measured in the presence of individual amino acids at 10 or 20 mM, combinations of two amino acids at 10 mM each, or 10 mM amino acid + 20 mM 3-methyladenine or His. Each value is the mean ± SE of the number of experiments given in parentheses. Statistical significance was estimated by the *t*-test. To facilitate column reading, all heterotypic combinations are entered twice. The effect of each amino acid at 20 mM (the basis for statistical evaluation of additive effects) is shown in italics. The control rate of sequestration was 7.4 ± 0.2%/h (30).

* *P* < 0.05 vs. 20 mM GIN.

† *P* < 0.005 vs. 20 mM His.

‡ *P* < 0.005 vs. 20 mM Leu.

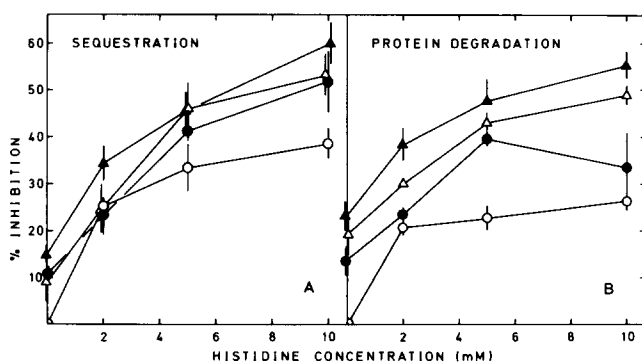


FIGURE 8 Concerted inhibition of [¹⁴C]sucrose sequestration (A) and protein degradation (B) by leucine and histidine. Both processes were measured in hepatocytes between 30 and 90 min of incubation at 37°C in the presence of histidine at the concentration indicated, either without leucine (○) or with 2 (●), 5 (△), or 10 (▲) mM leucine. The total inhibition relative to an amino acid-free control is given; each value is the mean ± SE of three to five experiments.

hibit maximally 25–35%, whereas glutamine and asparagine may be as effective as the complete mixture (32).

The sequestration-inhibitory amino acid couple Leu + His inhibited protein degradation more effectively than either Leu or His alone (Fig. 8B). As was the case with sequestration, maximal inhibition (55%) was obtained with a combination of 10 mM of each.

The degradation of short-lived protein, selectively measured by using a short (1 h) period of radioactive labeling of the protein, is less sensitive to inhibition by an amino acid mixture than is the degradation of long-lived protein (32). Table III compares the effects of individual amino acids as well as 3MA on the degradation of the two protein classes. A lysosomotropic amine, propylamine, is used to define the fraction of degradation accountable for by the lysosomal (acidic vacuolar) system, i.e., ~70% for long-lived protein and 45% for short-lived protein. None of the other treatments were significantly additive to the inhibition by propylamine (results not shown), implying that amino acids and 3MA inhibit lysosomal protein degradation only.

TABLE III
Effect of Amino Acids, 3-Methyladenine, and Propylamine on the Degradation of Long-lived and Short-lived Protein

mM	Percent inhibition of protein degradation	
	Long-lived protein (24-h labeled)	Short-lived protein (1-h labeled)
Leu (10)	26.1 ± 2.3 (11)	6.2 ± 2.7 (5)
Phe (10)	31.2 ± 1.9 (5)	15.4 ± 3.9 (5)
His (10)	35.4 ± 5.2 (9)	19.4 ± 1.9 (5)
AsN (10)	65.4 ± 1.9 (10)	33.6 ± 3.3 (5)
GIN (10)	67.8 ± 4.1 (5)	39.6 ± 2.7 (5)
AsN + GIN (5 + 5)	71.7 ± 5.7 (3)	40.8 ± 2.5 (5)
Leu + His (10 + 10)	55.4 ± 2.9 (5)	24.0 ± 7.0 (2)
Amino acid mix (~25)	69.3 ± 3.1 (6)	21.8 ± 1.4 (5)*
3-Methyladenine (10)	69.1 ± 3.0 (7)	34.4 ± 2.1 (5)*
Propylamine (10)	70.4 ± 2.3 (9)	46.4 ± 2.5 (5)

Hepatocytes were labeled with [¹⁴C]valine for either 24 h in vivo or 1 h in vitro. Protein degradation was measured between 30 and 90 min of incubation, and the percent inhibition by agents added at time zero recorded. Each value is the mean ± SE of the number of experiments given in parentheses. Control degradation rates were 4.10 ± 0.21%/h (17) for long-lived protein and 8.42 ± 0.24%/h (8) for short-lived protein. Statistical significance was estimated by the t-test.

* $P < 0.001$ vs. (AsN + GIN) or propylamine.

* $P < 0.01$ vs. propylamine.

Table III shows that the degradation of long-lived protein was inhibited to approximately the same extent by Asn + GIN, the amino acid mixture, 3MA, and propylamine, indicating that a single, homogeneous autophagic-lysosomal pathway is responsible for the degradation of this protein class. The degradation of short-lived protein, on the other hand, was less inhibited by 3MA and the amino acid mixture than by propylamine, suggesting a greater heterogeneity in the mechanisms of degradation of these proteins. The difference between long-lived and short-lived protein was particularly striking in the case of inhibition by leucine and the amino acid mixture: whereas these two treatments were, respectively, 37 and 98% as effective as propylamine in inhibiting the degradation of long-lived protein, the corresponding figures for short-lived protein were only 13 and 47% (as calculated from Table III). Although the amino acid mixture contains both AsN and GIN at 5 mM each, the mixture curiously enough had only one-half of the inhibitory activity of the AsN + GIN couple alone (Table III). In other words, the other amino acids in the mixture antagonized the effect of AsN + GIN on the degradation of short-lived protein.

DISCUSSION

Electropermeabilization and Cell Function

The use of electroinjected [¹⁴C]sucrose as a probe in the study of intracellular digestion is a novel approach that would seem to warrant particularly careful consideration of the methodological aspects involved. The electrical punching of functional holes in the plasma membrane, large enough to allow the exchange of ions and metabolites of small molecular weight, might be expected to cause considerable disturbance of cellular function. However, functional recovery is apparently rapid once the plasma membrane has resealed, since such complex and energy-dependent processes as protein synthesis and protein degradation were only marginally af-

ected, and since no detectable loss of ATP occurred under the conditions of electropermeabilization used in the present work.

The high rate of [¹⁴C]sucrose sequestration was independent of the number of electropermeabilizing pulses used, and could even be observed after nonelectric sucrose injection procedures. The red cell ghost-microinjection technique (8, 11, 39) gave a sequestration rate that was 70% of the rate measured after electropermeabilization (Myklebost, O., and P. O. Seglen, unpublished results), and another nonelectric method recently developed in our laboratory gave a rate identical to that obtained with electropermeabilization (our unpublished results). We can therefore disregard the possibility of the high sequestration rate being an artifact of the electropermeabilization treatment. The large (tenfold) difference in sucrose-sequestering activity between our hepatocytes and e.g., the red cell ghost-injected fibroblasts studied by Hendil (11) must therefore be due to intrinsic differences between the cell types and/or incubation conditions rather than to differences in methodology.

Methodological Aspects of Sequestration Measurements

The calculation of sequestration rates is based on the premise that after plasma membrane resealing, [¹⁴C]sucrose does not leak out of intact cells; in particularly good cell preparations, no sucrose leakage was in fact detectable during a 2-h incubation. Assuming that dead cells do not contribute sequestered material (their exposed, vesicular membranes are unlikely to persist during incubation of the cell suspensions at 37°C, and most of the damaged cells are removed by the sucrose washes prior to electrodisruption anyway), we are presently correcting for variable cell death by always expressing the amount of sequestered sucrose as percent of the total cellular radioactivity (in viable cells) at that particular time point, and calculating the sequestration rate as the net change between two time points.

Sequestration would be expected to follow first-order kinetics, with a progressive decrease in the amount of available nonsequestered sucrose, in which case real rates would be underestimated by ~10% (the average amount already sequestered during the standard measurement period from 30 to 90 min). However, since we do not have any reliable information about long-term sequestration kinetics under constant conditions (without rate alterations due to medium conditioning etc.), we have chosen not to make any corrections for the presumably nonavailable sucrose. A similar consideration applies to the "background," i.e., the radioactivity sedimentable at time zero (measured to be ~5% of the total cell-associated radioactivity at that time). This radioactivity would be the sum of [¹⁴C]sucrose sequestered and endocytosed during the resealing period, plus the small amount of sucrose (<1%) adsorbed to or trapped by sedimenting cellular structures.

A major contribution to nonlinear sequestration kinetics is the conditioning of the incubation medium by the cells: when starting with an amino acid-free medium, the gradual accumulation of proteolysis-derived amino acids will cause an increasing suppression of autophagic sequestration (24, 33), hence the decline in the control rate e.g., in Fig. 6. Conversely, when starting with high amino acid levels in the medium, the autophagic rate will initially (after a lag reflecting amino acid uptake) be low, and may then increase as amino acids are

being consumed. The near-constant sequestration rate seen with amino acids in Fig. 6 may represent a balance between amino acid consumption (rate increase) and the decrease in nonsequestered sucrose (apparent rate decrease); with 3MA (which is not consumed) the measured rate will decrease due to the latter factor.

The use of the novel electrodisruption technique as a means of breaking up cells does not appear to introduce any methodological problems, since the latency and yield of the lysosomal marker enzyme acid phosphatase as well as of sequestered [^{14}C]sucrose in the sedimented cell corpses is better than in lysosomes isolated by conventional (Dounce) homogenization. The lysosomes are virtually quantitatively recovered in the cell corpses, and neither the yield nor their structural integrity (initial latency) is affected by pretreatment of the cells with sequestration-inhibitory agents like amino acids and 3MA.

Control Mechanisms and Pathways of Sequestration and Protein Degradation

Sucrose is well established as an inert fluid-phase marker e.g., for studies of endocytosis (4), and the sequestration of intracellular [^{14}C]sucrose can therefore be regarded as a measure of fluid sequestration. In classical autophagy, i.e., the enclosure of a large region of cytoplasm within a membrane-bounded vacuole (autophagosome) (9, 19), cytosolic fluid, and protein should be sequestered at the same rate, whereas the observed rate of hepatocytic [^{14}C]sucrose sequestration is almost three times higher than the estimated rate of autophagic protein degradation (and even twice the over-all protein degradation rate) (35). This rate discrepancy could mean either that a major fraction of the sequestered proteins escapes degradation and is recycled back to the cytosol, or that there is a selectivity in the sequestration mechanism(s), favoring fluid uptake. Although recycling of sucrose seems unlikely on the basis of the present results, we cannot rule out that some recycling of protein may occur. As for selective fluid sequestration, it was recently discovered that a significant part (25–30% under the conditions used in the present work) of the hepatocytic [^{14}C]sucrose sequestration could be ascribed to mitochondrial uptake (38). The latter has been found to be restricted to molecules of low molecular weight (Tolleshaug, H. and P. O. Seglen, unpublished results), and may represent an intracellular fluid filtration process, unrelated to protein degradation. The mitochondrial sucrose/fluid uptake would not seem to be osmoregulatory, since the overall [^{14}C]sucrose sequestration was unaffected by medium osmolality within the nontoxic range 150–320 mosM.

The discovery of mitochondrial sequestration simplifies interpretation of the inhibitor effects considerably. The 3MA-resistant sequestration is entirely mitochondrial; it is virtually unaffected by 10 mM 3MA (and moderately inhibited at higher concentrations), whereas autophagic sequestration is practically completely blocked by 10 mM 3MA (our unpublished results). However, despite this effective and specific inhibition, subtraction of the mitochondrial sequestration still leaves us with an autophagic sequestration rate that is almost twice as high as the estimated rate of autophagic-lysosomal protein degradation, suggesting additional heterogeneity and/or selectivity in sequestration mechanism(s). Although the bulk of the nonmitochondrially sequestered [^{14}C]sucrose appears to be localized in the lysosomes (38), it could conceiv-

ably be brought there along several different pathways. There is some evidence for selective autophagy of long-lived, small basic proteins in fibroblasts (2), and electron microscopic studies of several cell types have revealed invaginations of the lysosomal membrane which could be interpreted as direct lysosomal “microautophagy” of cytosol. Internalization of solid particles by isolated lysosomes may be a reflection of such microautophagic capacity (9).

The amino acids, be it a complete mixture or the Leu + His couple, are not as effective sequestration inhibitors as 3MA, but this alone is insufficient as evidence for heterogeneity. The amino acid mixture has no effect on mitochondrial sequestration; both the inhibitory effect and the 3MA-antagonistic (stimulatory) effect appear to be exerted on autophagic sequestration. Whether the amino acid paradox reflects antagonistic effects of different individual amino acids on a common sequestration pathway, opposite effects of the amino acid mixture on different pathways (e.g., macro- and microautophagy), or other, less specific effects (e.g., on 3MA pharmacokinetics), remains to be shown. Since neither the degradation of long-lived (31) nor short-lived (25) [^{14}C]valine-labeled protein is detectably subject to 3MA-antagonistic amino acid stimulation, this effect on autophagic sequestration may have very limited (if any) functional significance. Other paradoxical protein degradation-stimulatory effects of amino acids are apparently unrelated to sequestration, being either nonlysosomal (25) or postsequestrational (see below).

3MA inhibits both autophagic sequestration and the lysosomal (propylamine-sensitive) degradation of long-lived protein virtually completely; long-lived protein would therefore seem to be degraded by a single, autophagic-lysosomal pathway. The degradation of short-lived protein, on the other hand, is significantly less inhibited by 3MA than by propylamine, indicating the participation of a nonautophagic lysosomal mechanism. Crinophagy, i.e., the fusion of secretory vesicles with lysosomes (9) could be one contributory process; fusion of endocytic vesicles with lysosomes (and subsequent degradation of short-lived membrane-associated proteins) could be another. If the secretory vesicle-lysosome fusion (like the autophagosome-lysosome fusion) is inhibited by AsN and GIN (32), it can be imagined that the paradoxical (AsN + GIN)-antagonistic stimulation of short-lived protein degradation by the amino acid mixture may also be exerted at the level of fusion.

The degradation of short-lived protein is strongly inhibited by AsN + GIN, an effect attributed both to inhibition of sequestration as demonstrated in the present work, and to inhibition of autophagosome-lysosome fusion as indicated by the maintenance of high autophagosome levels (32). An amino acid mixture antagonized this effect of AsN + GIN without antagonizing the effect of 3MA on degradation of short-lived protein, i.e., the amino acid stimulation would seem to be nonsequestrational. If it is imagined that the crinophagic fusion between secretory vesicles and lysosomes is inhibited by AsN + GIN, in analogy with the inhibition of autophagosome-lysosome fusion, the antagonistic effect of the amino acid mixture could conceivably be exerted at the level of fusion. In the perfused liver, certain noninhibitory amino acids were found to similarly antagonize the protein degradation-inhibitory effect of glutamine (21, 22).

It has been suggested that part of the degradation-inhibitory effect of glutamine could be due to its deamination to the lysosome inhibitor ammonia (21), a process known to take

place in hepatocytes under certain conditions (29). A similar deamination may participate in the effect of asparagine; however, the lack of detectable lysosome swelling and the unaffectedness of asialofetuin degradation (33) would seem to speak against a major effect at the level of lysosomal proteolysis.

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