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# Glucagon-induced autophagy and proteolysis in rat liver: Mediation by selective deprivation of intracellular amino acids

(glutamine/gluconeogenesis/amino acid homeostasis/lysosomes)

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**ABSTRACT** **Amino acid deprivation and glucagon are both potent inducers of autophagy and proteolysis in liver. Because glucagon enhanced the metabolic utilization of some amino acids, the catabolic response to both of these stimuli could be achieved by a lowering of intracellular amino acid pools. Alternatively, glucagon could act independently of amino acids. To clarify the mode of hormonal action and also the relationship between the two cellular responses, livers from fed rats were perfused, with and without glucagon, with plasma amino acids over a concentration range of 0 to 10 times normal. Individual amino acids constancy at each level was ensured by perfusion in the single-pass mode. Amino acids alone strongly regulated autophagy and proteolysis in a coordinated fashion; maximal suppression was achieved at twice normal concentration; both effects increased rapidly to maximum at less than normal concentration. Corresponding effects of glucagon, however, could be elicited only at intermediate amino acid levels. None was noted at 4 and 10 times normal; at 0, hormonal stimulation was minimal. The amino acid inhibition was selective because it did not block cyclic AMP production or glycogenolysis. Intracellular pool measurements and systematic alteration of perfusate amino acid composition indicated that the autophagic and proteolytic effects of glucagon are mediated by a hormonally induced depletion of glycine, alanine, glutamate, and glutamine; of these, glutamine alone is the most effective. We conclude that the stimulation of intracellular protein degradation in liver is a manifestation of deprivation-induced autophagy which results from a decrease in certain intracellular glucogenic amino acids, notably glutamine.**

The enhancement of intracellular protein breakdown in response to nutritional deprivation is a basic process that is manifested to varying degrees in most cells (1). In rat liver the response is of sufficient sensitivity and magnitude that it could play an important role in the homeostasis of extracellular amino acids during the postabsorptive period (2, 3). Although the mechanism of this deprivation-induced proteolysis is not completely known, it does seem clear that in cells of isolated, normal rat livers there is a dramatic internalization of cytoplasmic constituents within autophagic vacuoles when extracellular amino acid levels are decreased or insulin is lacking (4, 5). The quantity of cytoplasmic protein that is continuously taken up and digested by lysosomes under these conditions appears to explain adequately the associated increase in total protein breakdown (6).

Cellular catabolism also appears to be enhanced by certain hormones. It is well known that glucagon accelerates glycogen breakdown in liver. This hormone also brings about a net loss of liver protein (7–9). In addition, it is a potent inducer of hepatic autophagy (10–12) and has been reported to decrease some intracellular amino acid pools, an effect attributable largely to the enhanced utilization of amino acids by gluconeogenesis (13). In addressing ourselves to the question of the mechanism of the hormonal stimulation of proteolysis, we considered two possibilities: (*i*) the response results from the depletion of certain critical intracellular amino acid pools and thus would represent a true deprivation reaction, and (*ii*) glucagon, alone or through cyclic AMP, directly stimulates autophagy and proteolysis independently of amino acids. If the former were the case, both autophagic and proteolytic responses to glucagon would be amino acid dependent and thus subject to modulation by extracellular amino acids.

In the present study we measured proteolytic and autophagic responses to glucagon in perfused, fed rat livers that were exposed to various levels of a physiological amino acid mixture. The results support the first possibility strongly and suggest further that most, although not all, of the hormonally induced response is mediated by a decrease in intracellular glutamine.

## MATERIALS AND METHODS

**Animals.** Male rats of the Lewis strain (Microbiological Associates) were used as liver donors. The rats were maintained on regular laboratory chow and water ad lib and weighed 120–140 g at the time of perfusion.

**Liver Perfusion.** Livers were perfused *in situ* by a method described previously (14). The perfusing medium consisted of Krebs–Ringer bicarbonate buffer, 3% bovine plasma albumin (Pentex, Kankakee, IL), 10 mM glucose, and freshly washed bovine erythrocytes (0.27 vol/vol). A concentrated solution of the albumin was passed through a 0.3-$\mu$m filter prior to addition to the buffer. In experiments with glucagon (Lilly Research Laboratories, lot 258-V016-235), a solution of the hormone (9) was infused into the medium entering the liver at a rate equivalent to 10 $\mu$g of glucagon per hr, giving a concentration of 1.5 nM. Additions of amino acids to the perfusing medium were made by using a concentrated solution of amino acids with pH adjusted to 7.4. Details concerning the different amino acid additions are given in the appropriate figure legends. The experimental phase of each perfusion was maintained for 40 min, at a flow rate of 10 ml/min, in the single-pass mode—i.e., the outflow was not returned to the perfusion flask.

**Analytical Methods.** Rates of protein degradation were determined by modification of a method using cycloheximide (15). The procedure was as follows. The experimental phase of the perfusion was followed immediately by an analytical phase in which medium from a second flask, containing 18 $\mu$M cycloheximide, was recirculated through the liver for 15 min. Several perfusate samples were taken during the 5- to 15-min segment of this perfusion for subsequent valine analysis (16). The rate of valine accumulation in the analytical phase of perfusion represents the unidirectional release of valine from liver protein and was used as an index of the rate of protein degradation attained during the single-pass phase of perfusion. Separate control studies utilizing a recirculating-type perfusion

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in both experimental and analytical phases (unpublished data) have shown that this double-flask modification produces results equivalent to the original method (15).

Livers used for analysis of amino acids, cyclic AMP, or glycogen were quickly frozen, by using Wollenberger clamps cooled in liquid nitrogen, immediately after the experimental phase of perfusion. Samples of inflow perfusate were also obtained for analysis of amino acid levels in perfusate plasma. Amino acids were extracted from tissue or plasma with sulfosalicylic acid (17). The extracts were neutralized to pH 7.2–7.5, treated with sodium sulfite to eliminate chromatographic interference by glutathione, and lyophilized. The residues were dissolved in lithium citrate buffer (pH < 2.2), and amino acid levels were determined by ion-exchange chromatography (18) in an amino acid analyzer (Beckman). The method of Steiner *et al.* (19) was used for determining the levels of total cyclic AMP in liver, and liver glycogen content was measured by the method of Carroll *et al.* (20).

**Electron Microscopy.** Preparation of tissue for electron microscopy was as described (5). Fixation of livers involved the introduction of a paraformaldehyde/glutaraldehyde solution (21) directly into the portal vein at the end of the experiment. Ultrastructural observations were quantitated by using standard stereologic procedures (22) to estimate the fractional cytoplasmic volume occupied by lysosomal elements.

## RESULTS

**Effects of Amino Acids and Glucagon on Total Hepatic Protein Degradation.** It is apparent from Fig. 1 that the full range of proteolytic regulation can be achieved independently of glucagon by varying the level of plasma amino acids. Based on the valine content of liver protein (3), minimal and maximal rates of valine release in the presence of cycloheximide represent about 2 and 5% of total liver protein per hr. In the absence of glucagon, proteolysis was maximally suppressed at only twice normal plasma amino acid concentrations, and the zone of greatest responsiveness was around the normal level. These findings provide a basis for a feedback regulation of proteolysis in the intact rat. Because the quantity of valine released in 1 hr at the maximal rate of degradation was more than 8 times greater than the total extracellular valine pool in the animal from which the liver was taken, this response could play a significant role in amino acid homeostasis *in vivo*.

In confirmation of earlier work (9), glucagon clearly stimulated proteolysis but, as shown in Fig. 1, the effects were strongly affected by perfusate amino acids and were virtually abolished at and above the 4X level. We have demonstrated in past experiments that perfusate amino acids do not affect the basal level of tissue cyclic AMP (5), and further studies here showed a similar lack of effect on the response to glucagon. In two groups of five 40-min single-pass perfusions, cyclic AMP was increased from about 1.0 to a mean (±SEM) of 16.0 ± 2.7 nmol/g of liver in the absence of amino acids and to 14.3 ± 1.5 in their presence (at 10X). The 10X addition also failed to block the glycogenolytic effect. In six perfusions, glucagon decreased liver glycogen from a mean of 44.2 ± 3.0 unperfused to 18.7 ± mg/g; untreated controls remained the same (43.7 ± 1.2 mg/g). Thus, the suppressive effect of amino acids cannot be explained by any generalized interference with responses to glucagon or to cyclic AMP.

**Intracellular Amino Acid Pools and Proteolysis.** In the perfused rat liver, glucagon has been shown to decrease some intracellular amino acid levels and increase others, effects attributed to the stimulation of separate processes affecting the

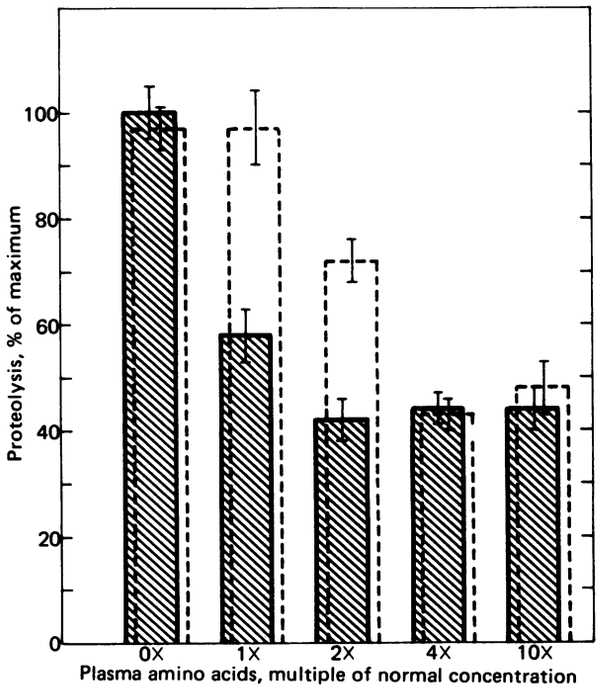


FIG. 1. Effects of amino acids and glucagon on protein degradation. Livers from fed rats were perfused for 40 min in the single-pass mode with (⬚) and without (▨) amino acids and glucagon (1.5 nM). Rate of proteolysis is expressed as percentage of the maximal rate that was achieved when neither amino acids nor glucagon was added to the medium (0.45 μmol of valine per min per liver). Each bar represents the mean ± SEM of 3–11 observations. "Normal" concentration of amino acids (a physiological mixture of 20 amino acids) was as follows (mM): Asp, 0.06; Thr, 0.35; Ser, 0.63; Asn, 0.08; Gln, 0.74; Pro, 0.42; Glu, 0.17; Gly, 0.38; Ala, 0.48; Val, 0.26; Cys, 0.08; Met, 0.06; Ile, 0.12; Leu, 0.21; Tyr, 0.10; Phe, 0.10; Lys, 0.45; His, 0.11; Trp, 0.09; and Arg, 0.22. All 20 amino acids were added at normal levels or multiples of that level except for the 0X condition no amino acids were added to the perfusing medium and residual levels averaged 2.5% of normal concentrations (5).

intracellular utilization and production of amino acids (13). Results of such stimulation in livers perfused at a constant level (1X) of amino acids are depicted in the step curve of Fig. 2. The decrease in glycine, alanine, glutamine, phenylalanine, and glutamate reflects the fact that these amino acids are important carbon sources in gluconeogenesis (23, 24) or, as in the case of phenylalanine, may be substrates for specific metabolic conversions (25). In these instances, intracellular levels are decreased when the hormonal stimulation of utilization exceeds the rate of appearance of new amino acids. Leucine, isoleucine, and valine are not utilized metabolically in liver (15, 16), and their increases over control values provide an internal reference for hormonally stimulated free amino acid inflow or production. Both proteolysis and inward transport are known to be affected (13).

To determine whether one or several of the decreased pools was responsible for the glucagon-enhanced proteolysis, various combinations of the six lowest amino acids in the curve of Fig. 2 were added to the perfusate at concentrations found in the 10X mixture. The remaining amino acids were held at the 1X level and glucagon was present in all runs. This screening experiment allowed us to exclude those amino acids that probably were not involved, but it did not enable us to draw fine distinctions among the effective ones. The results (Fig. 3) eliminated phenylalanine and aspartate from the initial group of six. Of the remaining four, glutamine seemed to be the most ef-

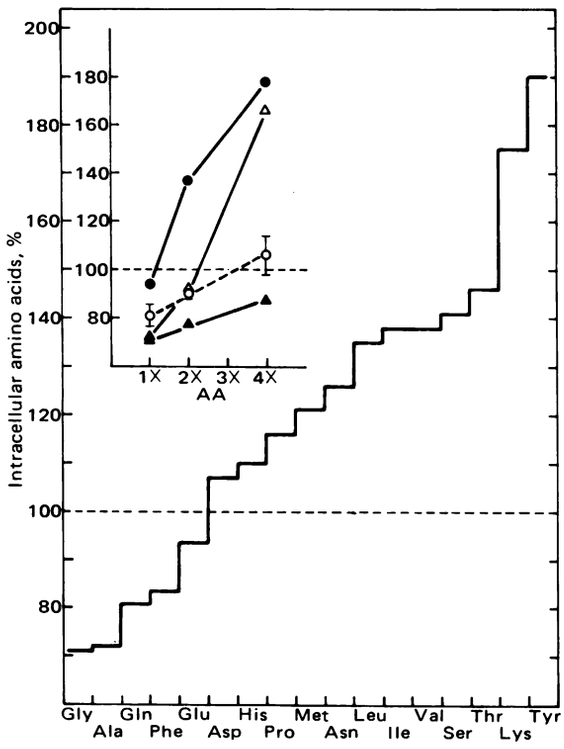


FIG. 2. Effects of glucagon and perfusate amino acid levels on intracellular amino acid concentrations. Intracellular amino acid values (—) after 40 min of liver perfusion with glucagon and 1× plasma amino acids (same experimental conditions as in Fig. 1) are indicated by the step curve as percentages of concentrations obtained with the 1× mixture alone, which were (μmol/ml of intracellular water): Asp, 1.89; Thr, 1.16; Ser, 3.44; Asn, 0.16; Gln, 8.31; Pro, 0.33; Glu, 11.67; Gly, 5.13; Ala, 3.67; Val, 0.44; Met, 0.22; Ile, 0.22; Leu, 0.46; Tyr, 0.13; Phe, 0.11; Lys, 0.52; and His, 1.19. Values are means from seven livers. Broken line, with 1× amino acid mixture alone. (*Inset*) Broken line again depicts 1× intracellular amino acid levels without glucagon. The curves represent intracellular concentrations of amino acids (●, Glu; △, Ala; ○, Gln; ▲, Gly) after perfusate amino acids were increased in multiples of the 1× level; glucagon was present in all experiments. The values shown are means (±SEM) of four to seven observations.

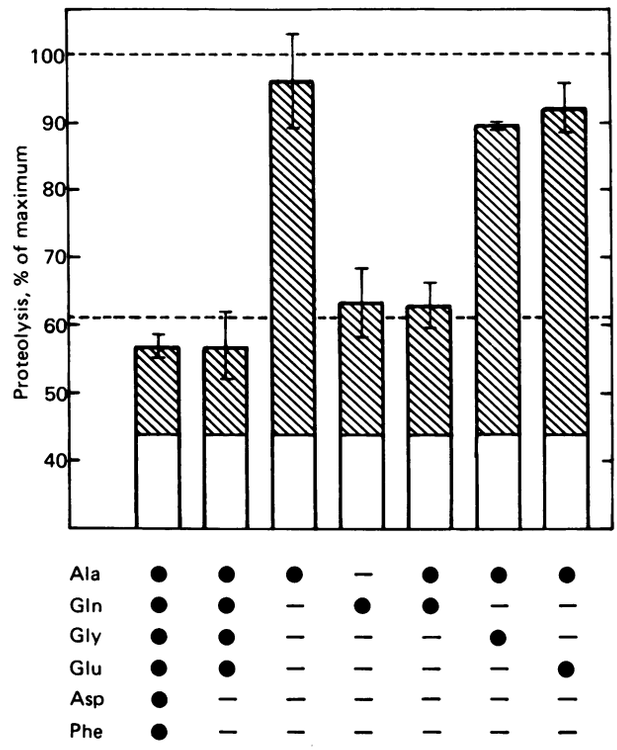


FIG. 3. Effects of different amino acid mixtures on rates of proteolysis. Conditions of perfusion were as in Fig. 1. The rate of proteolysis in the presence of normal levels of amino acids is indicated by the lower broken line. Maximal stimulation of proteolysis was achieved in the presence of 1× amino acids by the addition of glucagon (upper broken line). The unshaded bars depict maximal suppression of proteolysis achieved by additions of amino acids at 4× and greater (Fig. 1). Values for the remaining experimental conditions in the figure (hatched bars) are depicted as percentages of the maximal rate. All 20 amino acids were present in each condition: ●, amino acid present at 10×; —, present at 1×; in all conditions the remaining acids of the 20 amino acid mixture were present at 1× concentrations. Glucagon was also present in each condition. Each bar represents the mean ± SEM of 2–13 observations.

fective and, surprisingly, alanine had little activity. Because the combination of six amino acids was not as effective in blocking the glucagon response as was the complete mixture (Fig. 1), it is likely that several amino acids are required for complete suppression.

The *inset* of Fig. 2 shows the effects of plasma amino acids on intracellular glycine, alanine, glutamine, and glutamate levels during glucagon treatment. All pools increased with amino acid additions, but the increases varied widely among the different amino acids. It is of interest that only glutamine reached its intracellular control value at a plasma level (3×) corresponding to that which, by interpolation, would have resulted in a rate of proteolysis equivalent to the control rate (see Fig. 1). In these experiments the control levels are the rates of proteolysis and values of intracellular amino acids achieved at 1× plasma amino acids without glucagon.

**Lysosomal Alterations.** Effects of varying additions of amino acids, with and without glucagon, on autophagy are given in Fig. 4 and in Table 1. These findings substantiate previous observations that both amino acid deprivation (5) and glucagon (10–12) are potent stimulators of cellular autophagy in rat hepatocytes, but we now report also that lysosomal responses to glucagon, like the aforementioned proteolytic effects, are strongly inhibited by plasma amino acids as well. Fig. 4*A* depicts the enhancement of autophagy that is seen when livers are perfused with glucagon in the absence of amino acids. Qualitatively and quantitatively, the autophagic changes are similar to those induced by amino acid deprivation alone (Table 1 and ref. 5) and thus largely reflect the latter condition. The effectiveness of amino acids in suppressing autophagy in the presence of glucagon is clearly illustrated in Fig. 4*B*.

As with the proteolytic effects shown in Fig. 1, the most pronounced autophagic response to glucagon was obtained at normal plasma amino acid concentrations; there were virtually no effects at 4× and higher (Table 1). In the absence of added amino acids, glucagon appeared to elicit a small, additional enhancement of autophagy that was not reflected in proteolytic rates (see Fig. 1). This discrepancy was not seen when the comparison was made with vacuoles in which some digestion of contents was evident (autolysosomes), suggesting that the development of the latter from earlier forms (autophagosomes) could be rate-limiting in proteolysis. This might be so, for example, if the total intracellular pool of lysosomal protease remained constant in these experiments (11) in the face of a large increase in the internalization of cytoplasmic protein.

The fractional cytoplasmic volumes of dense lysosomes, including glycogen-containing elements, remained about the same in all experiments (Table 1).

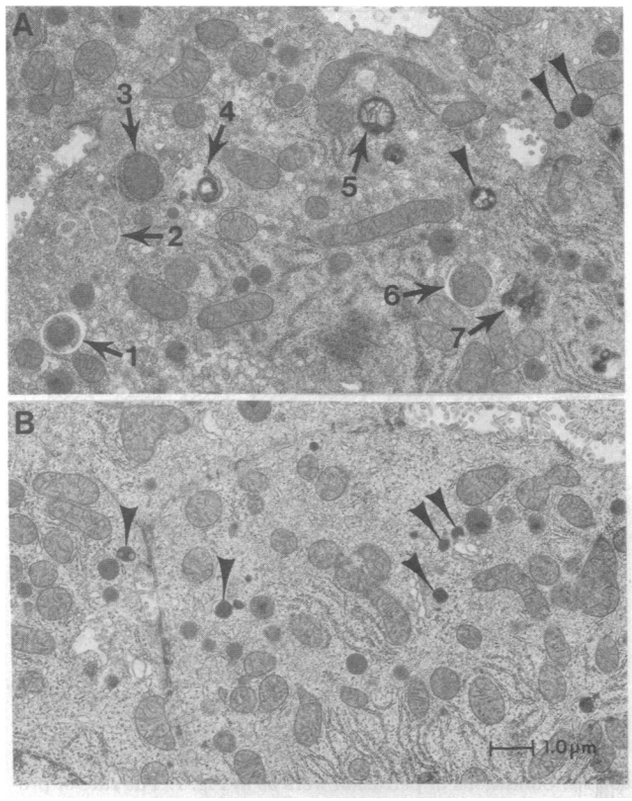


FIG. 4. Electron micrographs showing lysosome morphology in hepatocytes in which proteolysis had been maximally stimulated (*A*) or maximally suppressed (*B*). (*A*) From a liver perfused with medium containing 0× amino acids and glucagon. We classified lysosomes on a morphological basis as being either dense lysosomes or autophagic vacuoles. The dense lysosome class is comprised of (*i*) residual bodies whose entire contents are electron opaque and (*ii*) lysosomes that contain, in addition to a sharply demarcated zone of electron opacity, a zone of granularity indicating the presence of glycogen. The latter type of dense lysosome was previously designated type A autophagic vacuoles (5) and is similar to the glycogen-containing residual bodies of Novikoff and Shin (26). Dense lysosomes are indicated in the micrograph by plain arrowheads. Autophagic vacuoles, whose formation is enhanced under these conditions, contain organelles or portions of cytoplasmic matrix that may appear normal or partially digested. The more conspicuous vacuoles in the micrograph are indicated by arrows numbered 1 through 7. (*B*) From a liver perfused with medium containing 4× amino acids and glucagon. Dense lysosomes (plain arrowheads) are the predominant lysosomal component.

## DISCUSSION

The evident association in this study between increases in rates of proteolysis and volumetric alterations of lysosomal elements supports the growing belief that there is a functional, probably causal, relationship between these two cellular variables. Previous studies have shown that agents such as leupeptin, pepstatin, chloroquine, and ammonia that inhibit lysosomal proteolysis also suppress intracellular protein degradation in several mammalian cells and tissues (31–37). Moreover, we have shown that lysosomal proteolysis in rat liver homogenates correlates closely with rates of protein degradation in intact livers perfused under various states of deprivation and supplementation (3, 38). Based on recent measurements of intralysosomal protein content in liver (3, 6), we believe it is likely that overall protein degradation associated with insulin or amino acid deprivation or both is determined largely by the rate of internalization of cytoplasmic protein within vacuolar components of the lysosomal system.

Table 1. Effects of amino acids and glucagon on the fractional cytoplasmic volumes of lysosomal elements

| Experimental conditions | | Fractional cytoplasmic volume of lysosomal components, % | | |
|---|---|---|---|---|
| AA level | Glucagon | Dense lysosomes | Autophagic vacuoles | Autolysosomes |
| 0× | − | 0.35 ± 0.03 | 1.44 ± 0.13 | 0.66 ± 0.07 |
| | + | 0.49 ± 0.07 | 1.66 ± 0.18 | 0.63 ± 0.08 |
| 1× | − | 0.23 ± 0.03 | 0.29 ± 0.01 | 0.09 ± 0.01 |
| | + | 0.38 ± 0.04 | 1.06 ± 0.15 | 0.46 ± 0.04 |
| 4× | − | 0.32 ± 0.01 | 0.08 ± 0.04 | 0.04 ± 0.02 |
| | + | 0.31 ± 0.05 | 0.20 ± 0.02 | 0.14 ± 0.004 |
| 10× | − | 0.29 ± 0.05 | 0.06 ± 0.01 | 0.04 ± 0.02 |
| | + | 0.30 ± 0.05 | 0.09 ± 0.01 | 0.06 ± 0.01 |

The experiments were identical in design to those in Fig. 1. Dense lysosomes include predominantly electron-dense components such as residual bodies and dense glycogen-containing elements commonly seen in livers of fed rats (4, 5). Autophagic vacuoles are as classically defined. Traditionally, these have been divided into autophagosomes and autolysosomes (27): the former probably represent early forms because their content appears normal and they reputedly lack acid hydrolase activity (28); the latter show cytochemical evidence of enzyme activity (28) and contain cytoplasmic material in various stages of digestion (see vacuoles 4, 5, and 7 in Fig. 4). It is believed by some that autolysosomes are derived by fusion of autophagosomes with enzyme-containing primary or secondary lysosomes (11, 28), but studies by Novikoff (29) and Marty (30) support alternative views. Regardless of the mode of formation, however, the notion that there are distinct functional groups of autophagic vacuoles is a reasonable and useful hypothesis. In the present study we have identified autolysosomes morphologically because we have found (unpublished observations) that acid phosphatase reaction product generally overlies these profiles but not other types of autophagic elements. Random blocks of tissue were taken from the left and median lobes; each was trimmed prior to thin sectioning so that predominantly midzonal cells of the hepatic lobules were samples. An average of six micrographs (final magnification, ×6800) from each block was evaluated stereologically.

Data shown are percentages of the total cytoplasmic volume occupied by a given component. Each value represents the mean ± SEM of three to seven livers except that at the 4× level only two observations in each condition were made (in these instances, results are shown as the mean ± half the difference between the observations).

It should be emphasized that the above structure–function relationship explains those regulatory effects that result in net losses of intracellular protein. The process or processes responsible for generating free amino acids under basal or steady-state conditions are more obscure. The possibility that the latter is nonlysosomal was raised earlier from the failure of lysosomal inhibitors to suppress proteolysis in isolated cells incubated in enriched media (32, 34, 35). However, the applicability of these findings to perfused mammalian tissues has recently been questioned (36), and the results of preliminary studies utilizing other approaches suggest that the population of electron-dense heterolysosomes normally found in fed rat livers could make an appreciable contribution to basal proteolysis (3, 4, 6).

Virtually nothing is known of the mechanism(s) by which autophagy is initiated or regulated. However, it is evident from the micrographs examined in this study that both amino acids and glucagon affect the initial steps leading to autophagic vacuole formation. We considered the idea that the inhibition of proteolysis by amino acids is mediated in part by the liberation of ammonia, a known suppressant of hepatic proteolysis (33, 39), but we rejected it when we failed to decrease gluca-

gon-induced autophagy with ammonium chloride at an effective proteolysis-inhibiting level, 5 mM (unpublished observation). We now realize that this notion has little relevance to our studies because ammonia is highly diffusible and would not have accumulated intracellularly in the single-pass perfusions.

Regarding the mechanism of action of glucagon, our results are fully in accord with the view that the enhancement of autophagy is mediated by intracellular amino acid depletion, an effect resulting from primary actions of the hormone on gluconeogenesis, amino acid transport, and metabolic interconversions (13). Although we cannot totally exclude the possibility that some aspect of the process is directly stimulated independently of amino acids, as has been suggested (40), the fact that the catabolic responses to glucagon are blocked by relatively small, physiological (41) concentrations of amino acids argues strongly against it. From this, we interpret the augmented responsiveness to glucagon in the absence of perfusate amino acids in Table 1 as evidence of further amino acid pool depletion rather than of direct stimulation. It is of interest that glucagon inhibits protein degradation in the perfused rat heart (42). Thus, if any direct hormonal mechanism does exist, its basic nature must differ widely from tissue to tissue.

In concluding that the catabolic responses to glucagon and nutrient deprivation are both mediated by the decrease in intracellular amino acids, we may ask whether the mediatory role of glutamine is common to both or limited to the glucagon effect. There appears to be agreement that deprivation-induced proteolysis in liver is the result of deficiencies of methionine, phenylalanine, tryptophan, and possibly proline which arise in the course of normal amino acid metabolism (2, 23). However, the fact that glutamine as well as glutamate and alanine attain high levels under these conditions (2) indicates that (*i*) glucogenic amino acids *per se* are not implicated in the simple deprivation response and (*ii*) overall proteolytic regulation involves more than one site of inhibition. It would thus appear that loss of amino acid inhibition at any site is sufficient to trigger an autophagic response in the presence of adequate concentration of the remaining amino acids.

The intracellular amino acid deficiency induced by glucagon in the presence of normal concentrations of plasma amino acids (see Fig. 2) would be more representative of conditions *in vivo* than depletion developing spontaneously in the absence of added amino acids (2). Because glutamine is nearly as effective as alanine as a glucogenic substrate in rat liver (23, 24), one may assume that its intracellular decrease with glucagon, or in other conditions stimulating gluconeogenesis (24), reflects its enhanced utilization as a glucogenic substrate. But, regardless of the pathways affected, its depletion could provide an important link between the demand for glucogenic substrate and an immediate supply of utilizable amino acids in liver protein.

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