

Amino Acid Control of Proteolysis in Perfused Livers of Synchronously Fed Rats

MECHANISM AND SPECIFICITY OF ALANINE CO-REGULATION*

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The primary control of autophagically mediated proteolysis in perfused rat liver is carried out via two alternate mechanisms in response to specific regulatory amino acids. One (L) elicits direct inhibition at low and high plasma levels, but requires a co-regulatory amino acid to express inhibition at normal concentrations. The second (H) is ineffective at normal levels and below, but active at higher concentrations. Because regulation is subject to unpredictable variability with *ad libitum* feeding, we have utilized rats synchronously fed 4 h day⁻¹ to stabilize responses. Proteolytic control is seen to evolve in stages: H appears 12 h after the start of feeding; by 18 h L emerges, alternating with H in a statistically predictable way; with omission of the 24-h feeding, H disappears and L remains constant through 42 h. In both 18- and 42-h rats, alanine, glutamate, and aspartate exhibit similar inhibitory activity when added singly to the regulatory group at normal plasma concentrations. However, since alanine, but not glutamate or aspartate, evokes proteolytic acceleration when it is deleted from a full plasma mixture, alanine appears to be the sole co-regulator. Alanine yields co-regulatory effects with normal plasma leucine (0.2 mM) in 18- and 42-h animals and interacts synergistically with 0.8 mM leucine in 42-h but not in 18-h rats where leucine alone inhibits strongly. Because the inactivation of alanine aminotransferase by aminooxyacetate (determined from the conversion of [¹⁴C]alanine to glucose) does not alter the co-regulatory and synergistic effects of alanine, regulation by alanine must be mediated from a site of recognition before transamination.

Macroautophagy is the principal process by which long-lived proteins are degraded in the hepatocyte, and it can account for much of the net loss of cellular protein that occurs in starvation (1-3). An important feature of the mechanism is that it is subject to rapid regulation by specific regulatory and co-regulatory amino acids (4-7). Results from rat liver perfusion studies (5, 7, 8) and other investigations (9-12) have shown that leucine, glutamine, tyrosine, proline, methionine, histidine, and tryptophan serve as direct inhibitors (or regu-

lators) of proteolysis while the remaining 12 amino acids as a group are ineffective. Although phenylalanine also suppresses, we have excluded it from the regulatory group because it is rapidly converted to tyrosine and is not required for effectiveness of the group (5, 13).

Proteolytic responses to regulatory amino acids are mediated through two alternate control mechanisms. The first (L)¹ evokes direct inhibition at low plasma concentrations, followed by a sharp, zonal loss of effectiveness at normal levels; inhibition is promptly regained at higher levels (6, 7). The zonal loss of inhibition is abolished by 0.5 mM alanine (6, 14), a noninhibitory amino acid believed to act as a co-regulator at normal plasma concentrations (6). Except for differences in magnitude, the multiphasic response curves for the full regulatory group and its three most effective members—leucine, glutamine, and tyrosine—are virtually the same, having inflections at 0.5× and 1.25× normal plasma concentrations in livers of fed rats (7). Similar responses to leucine have recently been reported by Miotto *et al.* (15, 16). The second (H) is less well understood, but is switched on when glucagon is co-administered with regulatory amino acids (7). It is differentiated from the former by the absence of low concentration inhibition.

The mechanism and specificity of the foregoing co-regulation are still unclear. Recent studies with perfused isolated hepatocytes have shown that alanine acts synergistically with leucine at high physiologic concentrations and that the effect can be blocked by aminooxyacetate (17, 18). These findings suggest that alanine might also mediate co-regulation through products of transamination. Because co-regulation is subject to unpredictable variability after *ad libitum* feeding (14), we examined it in more detail after stabilizing the response with the use of synchronously fed rats. The present results show that amino acid control in perfused liver evolves in a reproducible pattern after feeding and that co-regulatory and synergistic effects of alanine in either fed or starved animals are not affected when transamination is inhibited by aminooxyacetate. Alanine must therefore mediate its effects from a site prior to transamination.

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¹ The abbreviations used are: L and H (or low and high) signify two alternate modes or mechanisms of proteolytic control at low and high plasma amino acid concentrations, respectively; with regulatory amino acids, L mediates a multiphasic dose-response (see curves in Figs. 2 and 6); with complete amino acids, its response is the same except for absence of the zonal loss of inhibition. The H mechanism mediates inhibition at high concentrations only with either regulatory or complete plasma amino acids. The number followed by × indicates multiples/fractions of the amino acid concentration in normal plasma.

EXPERIMENTAL PROCEDURES

Animals—Male rats of the Lewis strain (Harlan Sprague-Dawley, Indianapolis, IN) were used as liver donors. They weighed between 120 and 140 g at the time of experiment and were maintained under controlled lighting (off 1900 h, on 0700 h) with water continuously available. Animals used in the experiments of Table I were fed standard laboratory chow *ad libitum* and then starved for 24 h prior to perfusion. The remainder (designated synchronously fed) were given a synthetic diet *ad libitum* from 1600 h to 2000 h or at other times as specified in the text. The diet was essentially the same as that detailed in (19) except for the following modifications: 35% casein (Bioserv, Frenchtown, NJ; 87.6% protein), 37% cornstarch, 15% sucrose, and 8% corn oil. In a few experiments, the casein was decreased to 20% and corn starch increased to 47%. With the 35% casein diet, the animals sustained a small initial weight loss and then grew at about 85% of *ad libitum* controls; they were ready for experimentation by day 11. Rats fed 20% casein grew at approximately 15% of the controls. For the determination of protein degradation, liver protein was labeled *in vivo* by the intraperitoneal injection of L-[U-¹⁴C]valine (Du Pont-New England Nuclear; 4.5 μ Ci in 0.25 ml of 0.85% NaCl), 24 and 18 h before perfusion.

Liver Perfusion—Livers were perfused *in situ* as described earlier (4, 20, 21), modified for employing previously labeled livers in single-pass perfusions (14). In experiments involving synchronously fed rats, perfusions for proteolytic measurement were carried out at times specified in Fig. 1; the remaining perfusions were performed at 1100 h. The content of cellular protein was stable between 12 and 24 h, although liver weight decreased from 5.0 ± 0.04 to $3.9 \pm 0.07\%$ initial body weight over the same period (Fig. 1). Undoubtedly, most of the loss was glycogen and water.

Where protein degradation was determined, perfusion was begun in the single-pass mode with a medium of specified amino acid composition. In this procedure the outflow from the livers was not returned to the reservoirs as in cyclic (recirculating) perfusions, making it possible to maintain constant amino acid concentrations throughout the runs. At the end of the single-pass phase, flow was switched to a second-stage cyclic perfusion for measurement of the [¹⁴C]valine release.

Perfusion Medium—The perfusion medium, modified slightly from earlier reports (4, 21), comprised the following: Krebs-Ringer bicarbonate buffer, 2% bovine plasma albumin (fraction V, Pentex, ICN Biologicals, Costa Mesa, CA), 10 mM glucose, and freshly washed bovine erythrocytes (27%, v/v). Before its addition to the medium, a concentrated aqueous solution of albumin was dialyzed overnight at 5 °C against 4 liters of glass-distilled water and then passed through a 0.3- μ Millipore filter. Fractions or multiples of normal (1 \times) plasma amino acid concentrations were obtained by adding to the medium appropriate amounts of stock solutions of amino acid mixtures in 0.85% NaCl with pH adjusted to 7.4. Except for the absence of valine, which was omitted to ensure accurate measurement of plasma valine

TABLE I

Screening for co-regulatory activity: proteolytic inhibition by nonregulatory amino acids in the presence of the regulatory group at their zonal peak of ineffectiveness

Animals fed commercial chow *ad libitum* and starved 24 h were perfused with 0.5 \times regulatory amino acids together with the following 1 \times nonregulatory amino acids (see legend to Fig. 2 for concentrations). Values are means \pm S.E. normalized to 100-g body weight before starvation; numbers of experiments in parentheses.

| | Proteolysis | Inhibition | <i>p</i> |
|--------------------------|---|------------|-----------------|
| | nmol Val min ⁻¹ /100-g initial body weight | | |
| 0.5 \times Reg AA (17) | 201 \pm 5.5 | 0 | |
| + 1 \times Ala (7) | 140 \pm 7.0 | 61 | <0.001 |
| + 1 \times Glu (4) | 172 \pm 9.0 | 29 | <0.02 |
| + 1 \times Asp (7) | 177 \pm 7.4 | 24 | <0.02 |
| + 1 \times Arg (5) | 172 \pm 12.7 | 29 | <0.05 |
| + 1 \times Asn (3) | 202 \pm 7.8 | -1 | NS ^a |
| + 1 \times Gly (4) | 209 \pm 4.3 | -8 | NS |
| + 1 \times Cys (4) | 203 \pm 8.3 | -2 | NS |
| + 1 \times Ile (3) | 198 \pm 4.0 | 3 | NS |
| + 1 \times Lys (4) | 200 \pm 7.0 | 1 | NS |
| + 1 \times Ser/Thr (3) | 210 \pm 6.7 | -9 | NS |

^a NS, not significant.

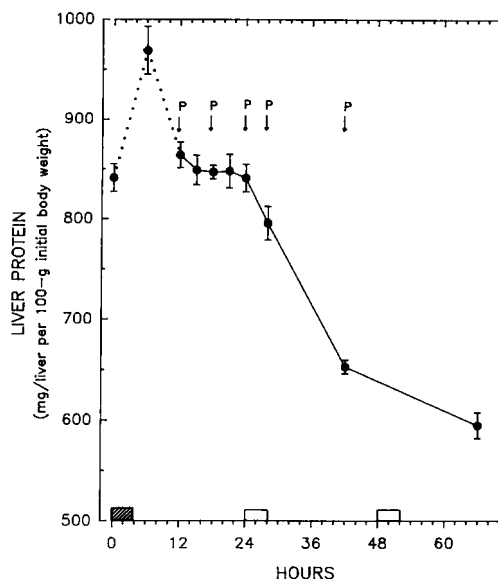


FIG. 1. Alterations in liver protein content during the feeding cycle and starvation in synchronously fed rats. Animals were fed from 1600 to 2000 h (0 = 1600 h). The shaded rectangle depicts feeding; open rectangles, omission of food; P, time of perfusion. The livers were flushed with 30 ml of 0.85% NaCl through the portal vein to remove erythrocytes and plasma proteins. Total liver protein was isolated as described earlier (24), and measured against crystalline albumin standard (29) on the basis of 100-g body weight at 0 h. Each point represents the mean \pm S.E. of 5–11 livers.

specific radioactivity (see under "Determination of Protein Degradation"), the composition of the complete plasma mixture was the same as that employed in previous studies (4–7) and has given identical results (14).

Determination of Protein Degradation—As detailed earlier, long-lived protein degradation was determined in livers previously labeled *in vivo* from the rate of accumulation of [¹⁴C]valine in the presence of cycloheximide (14). This method represents an improvement over our earlier nonisotopic procedure (4, 21) in that proteolytic contributions from the breakdown of short-lived and endocytosed proteins are virtually nil, thus increasing the specificity of measurement. After an initial 40-min single-pass perfusion under experimentally defined conditions, flow was switched to a second-stage cyclic perfusion containing 18 μ M cycloheximide. Following a 45-s washout the medium was recirculated and five samples taken between 5 and 15 min for the measurement of [¹⁴C]valine release. The specific radioactivity of plasma valine was then determined by flushing the liver with fresh medium devoid of valine and continuing perfusion for another 60 min to allow it to rise and level off (14). The latter has been shown to equal that of valine released directly from lysosomes (14).

The total accumulation of free [¹⁴C]valine at each point of sampling was computed as described earlier (4, 20, 21); proteolytic rates then were calculated by least squares regression after correction for the specific radioactivity of released valine. Values were expressed as nanomoles of valine min⁻¹ per liver (100-g body weight; 100-g initial body weight in starved animals).

Analytical Procedures—Valine in perfusate plasma was determined by the chromatographic procedure of Mortimore and Mondon (22); specific radioactivities were measured by analyzing one aliquot of the final extract for valine and a second for radioactivity. Labeled glucose in perfusate plasma (see Table II) was recovered by passing 2.5-ml samples over columns of Dowex 50 and then Dowex 1 to remove labeled cationic and anionic material. Samples with ¹⁴C were counted in Liquescent (National Diagnostics, Inc., Somerville, NJ) with a Beckman LS 7800 liquid scintillation spectrometer; results were corrected by external standards and expressed as disintegrations per min. All data in the figures and tables are stated as means \pm 1 S.E. Differences between means were evaluated by Student's *t* test; values of *p* > 0.05 were considered to be not significant.

RESULTS

Evolution of Proteolytic Regulation in Synchronously Fed Rats—Fig. 2, A and B, depicts proteolytic dose-responses to a

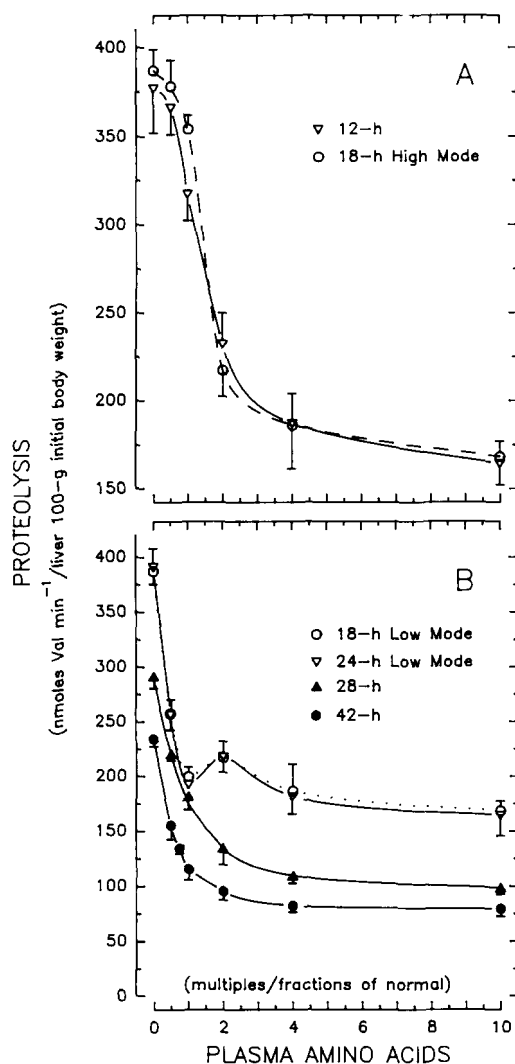


FIG. 2. Changes in the amino acid control of proteolysis during the feeding cycle and starvation in perfused livers of synchronously fed rats. A, comparison of 12- and 18-h high (H) mode dose-responses. B, evolution of amino acid control between 18 and 42 h. Livers were perfused with graded concentrations of a complete plasma amino acid mixture at times shown in Fig. 1, and rates of proteolysis determined as described under "Experimental Procedures." The composition of the 1× complete mixture (Val deleted; see under "Experimental Procedures") was as follows (micromolar): Ala, 475; Arg, 220; Asp, 53; Cys, 34; Glu, 158; Gly, 370; His, 92; Ile, 114; Leu, 204; Lys, 408; Met, 60; Phe, 96; Pro, 437; Ser, 657; Thr, 329; Tyr, 98; Asn, 101; Gln, 716 (14, 21). All rates were normalized to 100-g body weight (initial) at 18 h (1000 h) except those of 12-h rats, which were computed from the animal's weight at the time of perfusion; the resulting error was negligible (2%). The values are means \pm S.E. of 3–20 livers.

complete amino acid mixture in livers perfused at intervals from 12 to 42 h after the start of 35% casein feeding (see Fig. 1). From the observed alterations, regulation evolved in a rather complex way. At 12 h (Fig. 2A) amino acids failed to suppress proteolysis at lower than normal concentrations but inhibited at higher levels (4×) in a manner reminiscent of effects of amino acids in the presence of glucagon (7). However, at both 18 and 24 h (Fig. 2, A and B) two distinct response modes (H and L) were seen: in half of the days in which perfusions were performed, H-type dose-responses, which paralleled the 12-h curve, were evident (Fig. 2A) while in the remaining half, strong inhibition (L mode) was elicited at 0.5× and 1×, although not at 2× and higher (Fig. 2B). An

incidental peak, present in all experiments, was noted at 2×. Since the peak disappeared rapidly with starvation (28 h) and was not seen in livers of rats fed 20% casein (not shown), it was probably related in some way to the level of casein intake. The peak did not interfere significantly with amino acid regulation. Nevertheless, it could have dampened expression of the two populations at 2× since both modes were evident at 2× in the 20% casein-fed rats.

The distinctiveness of the foregoing H and L modes at 18 h is clearly seen in Fig. 3 where proteolytic rates with 0.5× and 1× complete amino acids from individual perfusions were subtracted from the corresponding 0× value on each day and plotted as pair differences. No overlap between responses was evident, and each pair difference in a given population was >3 S.D. from the opposing mean. Because of the constant 1:1 distribution between H and L, the modes must have alternated randomly on a day-to-day basis. Similar findings were obtained in 24-h rats (not shown). In several of these experiments the period of feeding was changed from 1600–2000 h to 1000–1400 h to eliminate evening perfusions. This had no discernable effect on the frequency distribution of H and L or on proteolytic rates.

With omission of the 24-h feeding, the liver responded rapidly with a sustained loss of intracellular protein (Fig. 1), an effect attributable in large part to the maintenance of rates of long-lived protein degradation at values higher than synthesis (1, 23, 24). The small peak at 2× as well as the two-population phenomenon disappeared 4 h later (28 h). The responses at 28 and 42 h were comparable to the L response curves at 18 and 24 h (Fig. 2B) except for substantial decreases in the absolute rate of protein degradation.

On the other hand, in the 12-h period before feeding where the content of liver protein was stable the only observed alteration in proteolytic regulation was a change in the frequency distribution of H and L. At 12 h, for example, only H

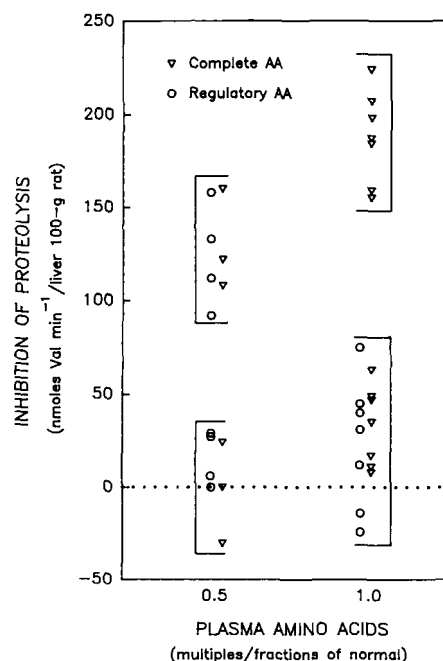


FIG. 3. Two populations of inhibitory responses to amino acids in perfused livers of 18-h synchronously fed rats. The effects shown represent pair differences between individual proteolytic rates obtained under the specified conditions and the corresponding 0× rates on that day; each response within brackets was obtained on a different day. All pair differences within a high or low group are >3 S.D. from the mean of the opposing group.

was evident while at 18 and 24 h L emerged as an alternative mechanism. Given the unphysiologically high proteolytic rate of the H mode at $1 \times$ in Fig. 2A, it is possible that L was the dominant form *in vivo* and that interconversion between the two modes was brought about hormonally. The reasonableness of this suggestion is illustrated in Fig. 4, which shows that insulin in 12-h rats is capable of shifting regulation from H to L, giving responses comparable to the L mode of 18-h animals.

Multiphasic Responses to Regulatory Amino Acids—In 18-h rats, regulatory amino acids at $0.5 \times$ elicited the same two proteolytic responses that were observed with the complete amino acid mixture (Figs. 3 and 5). However, in keeping with the expected zonal loss of proteolytic inhibition (6, 7), only a single high response was evoked at $1 \times$. Our failure to see two populations at $2 \times$ was also noted with the complete mixture (see Fig. 2, A and B, and related text). The regulatory amino acid dose-responses in Fig. 5, utilizing the low population at $0.5 \times$, revealed a typical multiphasic curve (6, 7) that is clearly independent of the incidental peak at $2 \times$. Moreover, differences between responses to the regulatory and complete amino acid mixtures (L mode) describe a curve that is remarkably similar to the original co-regulatory effect of alanine in livers of *ad libitum* fed animals (6). No insensitivity to alanine, as reported earlier in fed animals (14), was seen.

The effect of overnight starvation (42-h group, Fig. 1) on responses to regulatory amino acids is shown in Fig. 6. Note that the points of inflection at amino acid concentrations corresponding to the initial inhibition and the zonal loss of inhibition were identical for leucine, glutamine, and the full regulatory mixture. As observed earlier, the inflections moved to lower concentrations with starvation (14). In the present experiments, the values were approximately 60% of the control, a slightly smaller shift than that noted previously (14) where the period of hepatic deprivation was longer (24 instead of 18 h). Since suppressive effects of the two strongest regu-

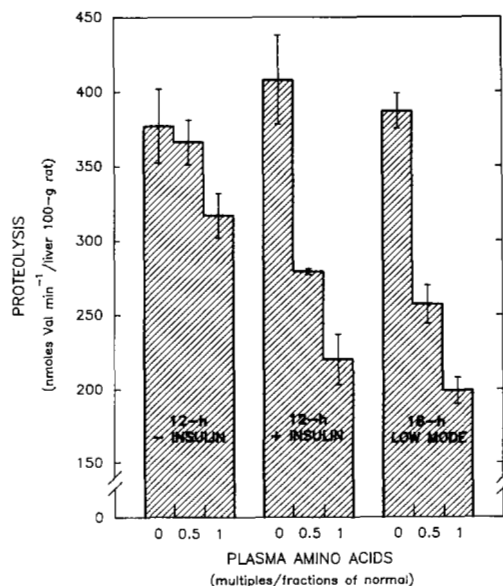


FIG. 4. Conversion of H to L mode by insulin. Livers from 12-h rats were perfused with $0 \times$, $0.5 \times$, and $1 \times$ normal levels of a complete plasma mixture (see legend to Fig. 2) in the presence or absence of insulin infused at $2.4 \mu\text{g h}^{-1}$; L responses (no insulin) in 18-h rats are shown for comparison. Before its addition, crystalline zinc pork insulin (lot 258-25J-120, Lilly Research Laboratories) was first dissolved in dilute HCl, pH 2.6, and then added to a solution of 0.5% albumin in bicarbonate buffer. The results in each group are means \pm S.E. of three to five experiments.

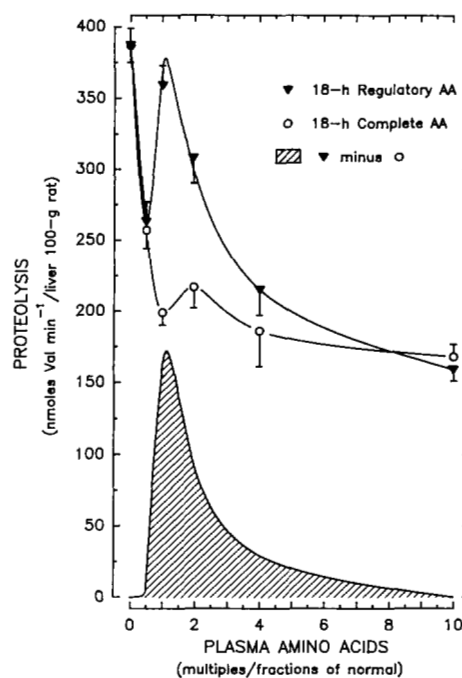


FIG. 5. Proteolytic dose-responses to complete and regulatory amino acids in perfused livers of 18-h rats. Values in the top two curves are from the L mode (see Figs. 2 and 3). The shaded curve depicts mean differences between them, and represents the mean co-regulatory effect of alanine (see text). Points are means \pm S.E. of 4-17 perfusions.

latory amino acids, leucine and glutamine, were previously found to be strongly additive at $4 \times$ (5), we evaluated their combined responses over the full range of plasma concentrations. The results in Fig. 6 show that their concerted effects can duplicate all responses of the regulatory group in 42-h rats.

Specificity and Mechanism of Co-regulation—In an earlier study we demonstrated that alanine, which exhibits little or no direct inhibition (6, 14, 17, 18), appears to be required for expression of proteolytic inhibition by the regulatory amino acids at normal plasma concentrations (6). The possibility that this co-regulatory effect is mediated by amino acid products of alanine transamination was raised by Meijer's group from results with perfused hepatocytes (17, 18). In experiments designed to identify co-regulatory amino acids from among the 12 nonregulatory amino acids (Table I), we found that $1 \times$ alanine, glutamate, aspartate, and arginine each decreased proteolytic rates when added to $0.5 \times$ regulatory amino acids, a level associated with the zonal loss of inhibition in livers from 24-h starved rats (14). None of the remaining noninhibitory amino acids was effective. Valine was not tested because it is not a constituent of the amino acid mixture and is known to have no co-regulatory activity (14).

These effects were confirmed in experiments with 18- and 42-h synchronously fed rats (Fig. 7). To avoid interference from the two-population phenomenon in the 18-h group, all responses on given days were evaluated as pair differences by subtracting them from corresponding $0 \times$ rates (see Fig. 7). In relative terms, effects were similar in both groups of animals. Alanine and aspartate proved to be the most inhibitory, and each decreased rates of proteolysis to values obtained with the respective complete amino acid mixtures. Glutamate was slightly less effective. Arginine was marginally active.

To determine whether the inhibition by alanine, aspartate, or glutamate was mediated through amino acid products of transamination, as suggested by Caro *et al.* (18), selected

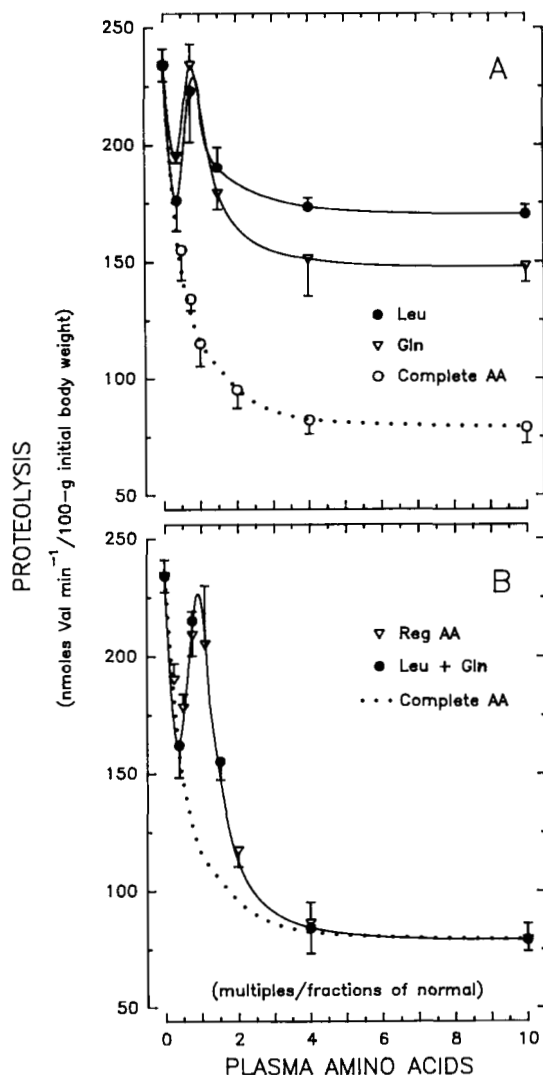


FIG. 6. Proteolytic dose-responses to leucine, glutamine, and a complete amino acid mixture (A) and leucine + glutamine and the regulatory group (Leu, Gln, Tyr, Pro, Met, His, and Trp) (B) in perfused livers of 42-h rats. See legend to Fig. 2 for absolute amino acid concentrations. Plotted values are means \pm S.E. of 3-18 experiments normalized to 100-g body weight (initial) at 18 h (1000 h).

experiments from the 42-h group in Fig. 7 were carried out in the presence and absence of 0.5 mM aminooxyacetate. From results in Table II, it is apparent that aminooxyacetate failed to modify the inhibition produced by alanine, aspartate, or glutamate. The degree of enzyme inactivation produced by 0.5 mM aminooxyacetate (99%) was evaluated from its effect on the conversion of [U-¹⁴C]alanine to labeled glucose under conditions similar to those in Fig. 7 (legend to Table II). Since aminooxyacetate is a known inhibitor of aspartate as well as alanine aminotransferase, these results exclude amino acid interconversion by transamination as a mediator of the proteolytic inhibition.

In view of the numerous possibilities that exist for amino acid interaction in biological systems, the evaluation of a potential amino acid regulator must include responses to its removal from a physiological mixture as well as responses to its addition. In Fig. 8, the deletion of alanine from a normal plasma mixture (1 \times for the 18-h animals; 0.75 \times for 42-h rats) accelerated proteolysis to near maximal rates, whereas the omission of aspartate, glutamate, or aspartate plus glutamate

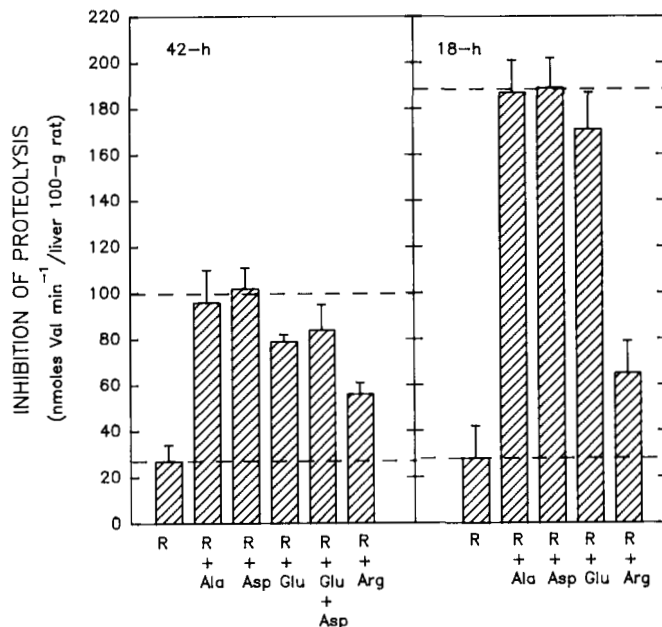


FIG. 7. Proteolytic inhibition by alanine, aspartate, and glutamate in the presence of regulatory amino acids (R) at normal plasma concentrations (peak of inhibitory ineffectiveness). Left panel, livers from 42-h rats perfused with 0.75 \times amino acids. Right panel, livers from 18-h rats perfused with 1 \times amino acids. Amino acid concentrations are given in legend to Fig. 2. Proteolytic rates were normalized to 100-g body weight at 18 h. The lower broken lines represent inhibition by R alone; upper lines depict inhibition produced by the complete amino acid mixture. The effects in each group represent means \pm S.E. of three to seven paired differences (decreases from corresponding 0 \times rates). In the 18-h group, differences in all groups averaged 27 ± 3 in half of the experiments and were excluded. This value, which is very close to the inhibition obtained with R, was predicted from the 1:1 distribution between the H and L modes in 18-h livers (Figs. 2 and 3).

TABLE II

Failure of aminooxyacetate to alter proteolytic inhibition by alanine, aspartate, and glutamate in the presence of regulatory amino acids (Reg)

Livers from 42-h rats were perfused as in Fig. 7 (left panel) with and without 0.5 mM aminooxyacetate (AOA); amino acids were added at 0.75 \times (peak of regulatory ineffectiveness); see legend to Fig. 2 for absolute concentrations. Values are means \pm S.E. of 3-13 experiments normalized to 100-g body weight at 18 h (1000 h). The extent to which AOA inhibited transamination under these conditions was assessed from its effect on alanine gluconeogenesis. After 30 min of 0 \times perfusion with and without 0.5 mM AOA, 0.375 mM L-[U-¹⁴C] alanine was added and two effluent samples taken between 38 to 40 min for the determination of glucose production, the latter computed from the known specific radioactivity of plasma alanine. In four parallel perfusions, glucose formation without AOA was 107 and 112 nmol min⁻¹/g liver; the same with AOA was 1.7 and 0.8.

| | Proteolysis | |
|-------------------------|--|--------------|
| | - AOA | + AOA |
| | nmol Val min ⁻¹ /100-g 18-h body weight | |
| 0 \times | 234 \pm 19 | 236 \pm 11 |
| 0.75 \times Reg | 207 \pm 8 | 205 \pm 15 |
| 0.75 \times Reg + Ala | 138 \pm 14 | 137 \pm 6 |
| 0.75 \times Reg + Asp | 132 \pm 9 | 136 \pm 8 |
| 0.75 \times Reg + Glu | 155 \pm 4 | 149 \pm 11 |

in 42-h animals evoked no increases. The effect of arginine was not tested. Owing to the need for additional controls in evaluating deletion effects in the presence of the foregoing dual populations, experiments with 18-h rats were limited to alanine.

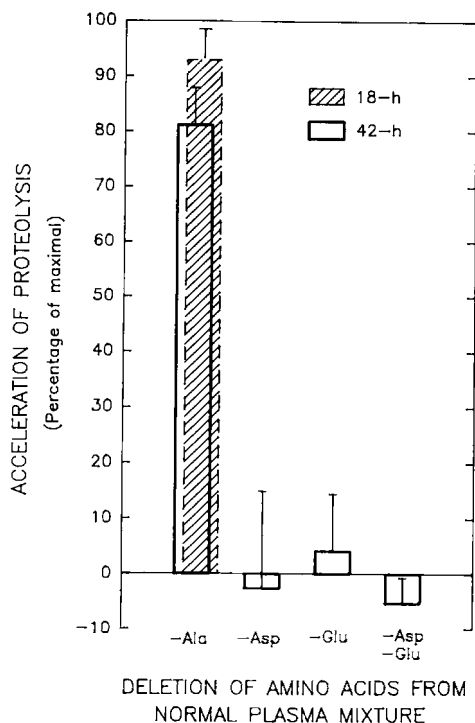


FIG. 8. Effect on proteolysis of deleting specific amino acids from a normal plasma mixture. Livers from 18- and 42-h rats were perfused with their respective normal plasma mixtures (1× or 0.75×) from which amino acid deletions were made. See legend to Fig. 2 for amino acid concentrations. The effect of the deletion on proteolysis is expressed as a percentage of the increase from the mean rate with the complete mixture at 1× or 0.75× to that obtained with the corresponding regulatory mixture; in 18- and 42-h animals these maximal increases were 160 and 73 nmol Val min⁻¹/100-g rat, respectively. With 18-h animals, responses were determined only in livers expressing the L mode. The latter was ascertained from rates of proteolysis with a 1× complete mixture on the same day. Results in each condition are means ± S.E. of three to four experiments.

The striking accelerating effect of alanine deletion means that glutamate, aspartate, as well as arginine possess no inhibitory effectiveness in the presence of the complete amino acid mixture. This was confirmed with the two dicarboxylic acids. The mechanism of this phenomenon is not understood, although it has been shown that the group of 12 nonregulatory (noninhibitory) amino acids, which includes the above four, exhibits no suppressive activity (5). Moreover, alanine evokes little or no direct inhibition by itself (6, 14, 18), a conclusion we confirmed for conditions of the present study (Table III). Because alanine does not mediate its effect through transamination, co-regulation appears to be a specific function of the amino acid *per se*.

Leucine and Leucine-Alanine Interactions—Although leucine plays a dominant role in the control of deprivation-induced autophagy and is required for maximal inhibition (5, 10), its effectiveness varies widely, ranging from near zero to about half of the overall proteolytic rate (5, 10, 12, 15). Since the degree of inhibition might be affected by food intake, we compared responses in synchronously fed and starved animals to see whether a significant difference could be obtained under controlled conditions. As shown in Table III, this appeared to be the case. In 18-h rats, 0.8 mM leucine (4×) inhibited deprivation-induced proteolysis by 89% while in 42-h animals the effect (39%) was less than half as large. No suppression was observed with alanine alone in either fed or starved rats although it evoked co-regulatory effects with 0.2 mM leucine in 18-h rats and with 0.15 mM in the 42-h group. The

TABLE III
Interactions between leucine and alanine
in control of hepatic proteolysis
Values are means ± S.E. of 3–20 experiments. Induced, increases above rates (basal) with the 4× complete mixture (see Fig. 2).

| | Proteolysis | | |
|--------------------------------------|--|---------|------------|
| | Total | Induced | Inhibition |
| | nmol Val min ⁻¹ /100-g initial body weight | | % |
| 18-h Group | | | |
| 0× | 387 ± 12 | 201 | 0 |
| 0.2 mM Leu | 374 ± 22 | 188 | 6 |
| 0.8 mM Leu | 209 ± 26 | 23 | 89 |
| 0.5 mM Ala | 384 ± 30 | 198 | 1 |
| 1.0 mM Ala | 374 ± 10 | 189 | 7 |
| 2.0 mM Ala | 379 ± 28 | 193 | 4 |
| 0.2 mM Leu + 0.5 mM Ala | 259 ± 18 | 73 | 64 |
| 0.8 mM Leu + 2.0 mM Ala | 210 ± 12 | 24 | 88 |
| 42-h Group | | | |
| 0× | 234 ± 7 | 152 | 0 |
| 0.15 mM Leu | 224 ± 22 | 142 | 7 |
| 0.8 mM Leu | 174 ± 5 | 92 | 39 |
| 0.5 mM Ala | 240 ± 19 | 158 | -4 |
| 1.0 mM Ala | 226 ± 21 | 141 | 5 |
| 2.0 mM Ala | 238 ± 20 | 156 | -3 |
| 0.15 mM Leu + 0.5 mM Ala | 149 ± 5 | 67 | 56 |
| 0.15 mM Leu + 2.0 mM Ala | 148 ± 3 | 66 | 57 |
| 0.8 mM Leu + 2.0 mM Ala ^a | 111 ± 4 ^a | 29 | 81 |

^a Same + 0.5 mM aminooxyacetate, 110 ± 13 (four experiments).

latter effect was maximal at normal levels of plasma alanine (0.5 mM).

It is of further interest that 2 mM alanine acted synergistically with 0.8 mM leucine in starved (42-h), but not in fed (18-h) animals. The effect doubled the inhibition in the starved group (39 versus 81%), yielding a total response (81%) that compared closely with suppression by leucine alone in fed animals (89%). As with co-regulation, 0.5 mM aminooxyacetate failed to block the synergism, indicating that it too is mediated at a step before alanine transamination.

DISCUSSION

The use of synchronous feeding has made it possible to examine in detail the sequence of changes in the amino acid control of long-lived protein degradation in liver. It should be noted that the content of liver protein was remarkably constant during the latter half of the feeding cycle, and except for a transient rise and fall during the first 12 h (Fig. 1), no cellular protein was lost unless food was withheld. Despite random day-to-day switching between the H and L modes of regulation, their average distribution of 1:1 remained highly reproducible (Fig. 3 and legend to Fig. 7), and amino acid control was more stable over extended periods of time than it was in *ad libitum* fed rats (14).

We have reported earlier that perfused livers of normal rats fed *ad libitum* are subject to unpredictable periods of altered regulation manifested by sharply elevated proteolytic rates at normal plasma levels of amino acids (14, 21). This alteration, which was attributed to a 90% decrease in alanine sensitivity, is reminiscent of the H mode of regulation at 1× plasma concentrations. Rates at 0.5×, though, are not increased, and the underlying response to regulatory amino acids is of the L (multiphasic) type (14). The cause of the insensitivity is unknown. It could, as suggested earlier (14), result from some change in the pattern of feeding. The fact that it failed to appear in synchronously fed rats tends to exclude the possibility that it is an essential step in the evolution of amino acid control.

The H mode of regulation, which was seen in all of the 12-

h rats and approximately half of the 18- and 24-h animals, closely resembles proteolytic responses to amino acids in the presence of glucagon in *ad libitum* fed rats (7). Its expression here is especially notable because the full response was produced with amino acids alone. Similar curves, though, have been obtained with the α -keto acids of leucine (5, 7) and tyrosine (25). The fact that insulin is capable of eliciting the L mode in 12-h rats is of interest physiologically since it appears to place insulin's effect in direct opposition to that of glucagon—not unlike the control of hepatic glycogenolysis. As far as we are aware, the putative interaction between these two agents in regulating macroautophagy has not been assessed.

Little is known of the factors responsible for the spontaneous conversion between the H and L modes. In 18-h rats, for example, one or the other mode predominates on any given day, but on a day-to-day basis their predicted occurrence is strictly random, averaging 1:1 over time. Thus the conversion is reversible and involves most of the population. Taken together, the evidence suggests that it is integrated as a daily event within the feeding cycle. On the other hand, when starvation is induced by withholding food at 24 h, interconversion is abolished, and L becomes the dominant mode. Regarding the observation that animals on a given day manifest the same regulatory mode, it is relevant to point out that macroautophagic responses in a group of animals move together on any given day (26) and that proteolytic rates at 0 \times , which vary widely between days, exhibit less variability among animals on the same day (26). Although this "day effect" phenomenon does not provide a molecular explanation for the conversion, it does emphasize the dynamic aspects of macroautophagic regulation, and underscores the likelihood that the reproducible 1:1 distribution in 18-h rats is actively maintained.

A unique feature of the multiphasic dose-response to regulatory amino acids is the sharp loss of proteolytic inhibition that occurs within a narrow zone centered at normal amino acid concentrations (6, 7). The effect was attributed to a lack of alanine, which is believed to play a specific co-regulatory role in expressing inhibition by the regulatory amino acids at normal levels (6). However, in subsequent studies with perfused hepatocytes Meijer and co-workers (17, 18) have shown that an analogous synergistic effect between alanine and leucine at high physiological concentrations can be blocked by aminooxyacetate, and have concluded that the effect was mediated by metabolites of alanine such as glutamate or aspartate. It is known that pyruvate and lactate are not effective substitutes for alanine in co-regulation (6, 14) or in synergism with leucine.² Thus the failure of aminooxyacetate to alter these effects in the present investigation must mean that alanine does not act metabolically in the perfused liver, but instead through some site of recognition prior to its transamination; leucine is believed to act similarly (5, 7). We can offer no explanation for the discrepancy in aminooxyacetate effectiveness between the two preparations except that the putative site may have been inactive in the perfused hepatocytes.

Although the co-regulatory and synergistic effects of alanine have similar features, it is probable that separate mechanisms are involved. In addition to differences in amino acid concentration requirements, the two effects can be dissociated. In Table III, for example, synergism was absent in 18-h animals but present in the 42-h group, whereas co-regulation was expressed in both. Interestingly, the response to 0.8 mM leucine in fed (18-h) rats, as a percentage of the total depri-

vation response, was twice that of the starved (42-h) group, suggesting that the synergistic effect may be a consequence of a more fundamental alteration in leucine effectiveness.

The conclusion that alanine plays a specific role in co-regulation is strongly supported by results of the deletion experiments. That the removal of alanine from a normal plasma amino acid mixture accelerates proteolysis to near maximal rates in these and earlier experiments (6) means that alanine is an effective inhibitor in the presence of plasma amino acids while little or no inhibition is offered by the remaining amino acids. By the same reasoning, the lack of acceleration after removal of glutamate and aspartate, each of which exhibits strong inhibitory activity with the regulatory mixture but none in the presence of nonregulatory (noninhibitory) amino acids (5), very likely signifies that their inhibition is nulled by other amino acids. Low cellular permeability would not seem to be a probable factor since there is convincing evidence that glutamate and aspartate are both rapidly transported in liver (27, 28). Loss of inhibitory activity in the presence of other amino acids is not unusual: tyrosine is ineffective in the presence of leucine or glutamine (5), and asparagine, which is inhibitory alone (12), has no overt activity when added to the noninhibitory group (5).

The subcellular location and characterization of the amino acid recognition site(s) are challenging questions which must be answered before regulation is fully understood. In view of the complexity of amino acid control as illustrated by leucine-alanine interactions, it is reasonable to suppose that they exist close together, possibly as subunits of a large complex. Recent studies, reported in preliminary form (15, 16), have shown that isovaleryl-L-carnitine, which is structurally analogous to leucine, mimics leucine's multiphasic dose-response exactly and acts synergistically with alanine. Evidence that leucine and its analogue bind to the same site was also obtained (16). Because of the rapid catabolism of isovaleryl-carnitine, its apparent intracellular concentration at plasma levels equivalent to leucine is indistinguishable from zero.³ Thus responses to this agent as well as to leucine should reflect external concentrations, a conclusion consistent with a locus of recognition on or close to the plasma membrane.

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