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Control of Hepatic Proteolysis by Leucine and Isovaleryl-L-Carnitine through a Common Locus

EVIDENCE FOR A POSSIBLE MECHANISM OF RECOGNITION AT THE PLASMA MEMBRANE*

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Giovanni Miotto‡, Rina Venerando‡, Kamal K. Khurana§, Noris Siliprandi‡, and Glenn E. Mortimore¶‖

From the ¶Department of Cellular and Molecular Physiology, College of Medicine, The Pennsylvania State University, Hershey, Pennsylvania 17033 and the ‡Dipartimento di Chimica Biologica, Università Degli Studi di Padova, 35121 Padova, Italy

Deprivation-induced proteolysis in the perfused rat liver is controlled through the multiphasic action of 7 regulatory amino acids of which L-leucine plays the dominant role. Recently, isovaleryl-L-carnitine (IVC) was shown to mimic the leucine's effects, suggesting that the two molecules share structural features that are recognized at a common site(s). In this study we find that each evokes identical responses consisting of inhibitory effects at 0.08 and 0.8 mM, separated by a sharp zonal loss of inhibition at 0.15 mM. As monitored by density shifts of β-hexosaminidase in colloidal silica gradients, macroautophagy is suppressed by both. Responses to Leu and IVC at 0.08 and 0.15 mM are stereospecific and require a reactive group at the α-carbon (or equivalent) and a high degree of branched chain specificity. In addition, 0.5 mM Ala coregulates with IVC and Leu by decreasing the zonal loss at 0.15 mM. The fact that the multiphasic responses can be duplicated with equimolar mixtures of Leu + IVC indicates that both react at the same site(s). IVC is readily taken up by a saturable process, but owing to its rapid hydrolysis in the cell, the ratio of internal to external IVC remains low over a 4-fold concentration range. These findings, together with a kinetic analysis of concerted responses to regulatory amino acids, suggest that the recognition sites are at a position in the cell, possibly at the plasma membrane, to react reversibly with plasma amino acids.

It is generally agreed that macroautophagy is the cellular mechanism responsible for deprivation-induced proteolysis in the hepatocyte, a process which serves as a major source of free amino acids for gluconeogenesis and other important pathways *in vivo* (reviewed in Refs. 2–4). It is controlled on a moment-to-moment basis by amino acids and by insulin and glucagon (reviewed in Ref. 4). Amino acids, though, are considered to be the prime regulators, since they can evoke proteolytic responses over the full range of control in the absence of hormones. Of the 20 amino acids that turn over in liver protein, only 7 (or 8) exhibit direct inhibitory activity when added as a group (reviewed in Ref. 4). Of these, leucine is easily the dominant regulator, since it is the single most effective amino acid (5) and is required in amino acid mixtures for maximal inhibition (5, 6).

Except for differences in degree of suppression, proteolytic dose responses to leucine and other regulatory amino acids exhibit similar multiphasic curves consisting of three concentration-dependent components: primary inhibition at half-normal plasma levels, a zonal loss of inhibition within a narrow range at normal concentrations, and secondary inhibition at high physiologic levels (5, 7, 8). The zonal loss can be attributed to the absence of alanine, which is specifically required for expression of inhibition by the regulatory amino acids at normal concentrations (9, 10).

Recently, isovaleryl-L-carnitine (IVC)[1] was reported to mimic leucine's regulatory effects in the perfused rat liver (8). Since leucine is poorly metabolized in liver owing to its low rate of transamination (5, 11, 12), the effects are probably not mediated through products of leucine oxidation but rather by leucine itself at some undefined locus of recognition. If true, leucine and IVC could share structural features that are important in the recognition process. In this study we aimed (i) to determine the extent to which IVC can replace leucine as a regulator, (ii) to determine the cellular location of recognition, and (iii) to integrate the effects of leucine into a general mechanism of amino acid regulation.

EXPERIMENTAL PROCEDURES

Animals—Liver donors were male rats of the Lewis strain (Harlan Sprague-Dawley, Indianapolis, IN) weighing between 120 and 140 g at the time of perfusion. They were maintained under controlled lighting (off 1900 h, on 0700 h) with continuous access to water but with restricted access to food. A synthetic 35% casein diet was available for feeding *ad libitum* between 1600 and 2000 h but was withdrawn for the remainder of the day (10). This regimen, designated synchronous feeding, was employed because it yielded more consistent responses than those obtained after normal *ad libitum* feeding (10). After a 4-day period of adaptation, the animals reestablished growth at approximately 85% of the normal rate and were used for experiment 7–10 days thereafter (10). In all experiments except one group

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§ Present address: Dept. of Pathology, University of Texas Medical School, 6431 Fannin, Houston, TX 77225.

‖ To whom correspondence should be addressed: Dept. of Cellular and Molecular Physiology, College of Medicine, The Pennsylvania State University. Hershey, PA 17033. Tel.: 717-531-8571; Fax: 717-531-7667.

[1] The abbreviations used are: IVC, isovaleryl-L-carnitine; HPLC, high performance liquid chromatography; AVd, degradative macroautophagic vacuole (autolysosome); AVi, initial macroautophagic vacuole (autophagosome); β-hexosaminidase, *N*-acetyl-β-D-glucosaminidase; MAP, multiple antigen peptide. The number followed by × indicates multiples/fractions of the amino acid concentrations in the standard normal plasma mixture (13).

(Table I), perfusion was carried out 42 h after the start of the last feeding (42-h rats), a period equivalent to ≈18 h of starvation (10). Livers from animals used for experiments in Table I were perfused 18 h after the start of the last feeding (18-h rats) and were similar in responsiveness to normal *ad libitum* fed rats. For the measurement of protein degradation, liver protein was labeled *in vivo* by the intraperitoneal injection of L-[U-^{14}C]valine (Du Pont-New England Nuclear; 4.5 μCi in 0.256 ml of 0.85% NaCl), 24 and 18 h before perfusion.

Liver Perfusion—Livers were isolated and perfused in the single-pass mode as detailed earlier (13) except for a modification which made it possible to determine protein degradation from the release of radiolabeled valine (14). The perfusion medium, modified slightly from earlier reports (13), 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 the albumin was added to the medium, a concentrated aqueous stock solution was dialyzed overnight at 5 °C against 4 liters of glass-distilled water and then filtered through a 0.3-μm Millipore filter. Fractions/multiples of amino acids from the standard plasma mixture (13) were obtained by adding to the medium appropriate amounts in 0.85% NaCl (pH 7.4); acylcarnitine and other additions were prepared similarly. Except for the absence of valine, which was deleted to ensure accurate measurement of plasma valine-specific radioactivity, the composition of the complete plasma mixture was the same as that employed in previous studies (5, 7, 9) and has given similar results (14).

Determination of Protein Degradation—Long-lived protein degradation was determined from the release of labeled valine in livers previously labeled with [U-^{14}C]valine *in vivo* (14). In this isotopic procedure contributions from the breakdown of short-lived and endocytosed proteins are virtually nil (14). In brief, after an initial 40-min single-pass perfusion, during which livers were perfused with various amino acids or test substances (see text), flow was switched to a second-stage cyclic perfusion containing 18 μM cycloheximide. Following a 45-s washout, the medium was recirculated and five samples taken between 5 and 15 min for the measurement of [^{14}C] valine release. The specific radioactivity of plasma valine was then determined (14) after flushing the liver with fresh medium and continuing the perfusion for 60 min to allow the specific radioactivity to rise and level off.

The total accumulation of [^{14}C]valine in perfusate and liver water was computed as described earlier (13); proteolytic rates then were calculated by least squares regression after correction for the foregoing final specific radioactivity of plasma valine. The latter has been shown to equal that of valine released from lysosomes (14). Values were expressed as nanomoles of valine min^{-1}/100 g of body weight or, in the case of 42-h rats, their weight 18 h after the start of the last feeding (initial body weight).

Determination of IVC in Liver and Plasma—At the termination of perfusion liver was quickly frozen with liquid N_2 and powdered. Samples (0.7–0.9 g) were deproteinized in 4 ml of perchloric acid (0.2 N, final). KOH was added to the supernatant to pH 3.0–3.5, and 100 microliters were injected for HPLC analysis as outlined in the legend to Fig. 1. The HPLC eluants were prepared as follows: (*a*) 15 mM KH_2PO_4, 5 mM 1-butanesulfonic acid, and 0.1% triethylamine (pH adjusted to 3.3 with phosphoric acid) and (b) equal volumes of acetonitrile and a solution consisting of 30 mM 1-butanesulfonic acid, 30 mM KH_2PO_4, and 0.2% triethylamine (pH adjusted to 3.3 with phosphoric acid). In the experiments of Fig. 5, IVC uptake was determined from differences in labeled IVC in perfusate plasma, deproteinized with trichloroacetic acid (5%, final). IVC was separated from carnitine and acetylcarnitine by the use of Sep-Pak columns (Waters). The columns were washed with 5 ml each of methanol and distilled water and then loaded with the trichloroacetic acid supernatant samples (0.9 ml). Carnitine and acetylcarnitine were eluted with 5 ml of water; IVC was quantitatively recovered in a second elution with 4 ml of methanol.

Acylcarnitine Derivatives—Isovaleryl-L-carnitine was synthesized by the method of Bøhmer and Bremer (15). The compound had a purity of >95% as determined by mass spectrometry (courtesy of Dr. L. L. Bieber of the Department of Biochemistry, Michigan State University). Additional isovaleryl-L-carnitine was kindly provided by Sigma Tau (Rome) and the Kongo Chemical Co., Ltd. (Tokyo). All other acylcarnitine derivatives were generous gifts of Sigma Tau (Rome). Radiolabeled isovaleryl-L-carnitine was synthesized as above (15) with L-[*methyl*-^{14}C]carnitine hydrochloride (Amersham Corp., 57 mCi/mmol) as the source of label.

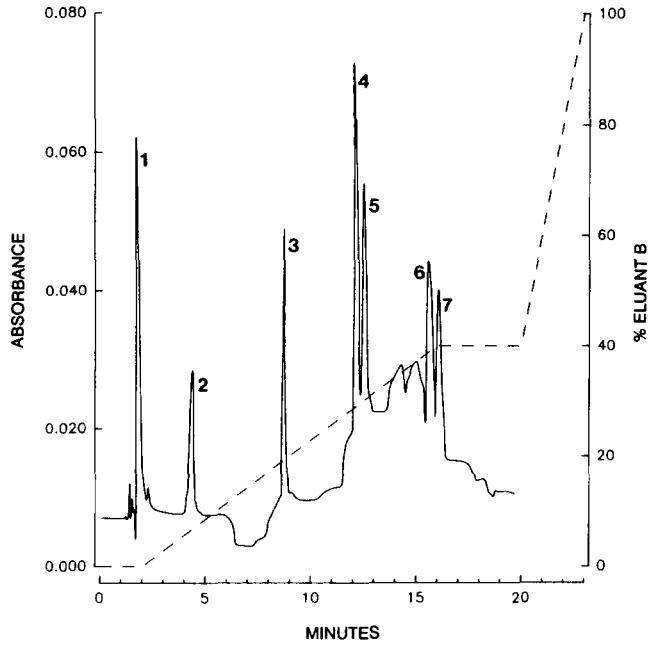


FIG. 1. **HPLC separation of carnitine and acylcarnitine standards on a Rainin Microsorb C-18 column (4.6× 100 mm, 3 μm).** The above peaks in the digitized chromatogram are: *1*, L-carnitine; *2*, acetyl-L-carnitine; *3*, propionyl-L-carnitine; *4*, isobutyryl-L-carnitine; *5*, butyryl-L-carnitine; *6*, α-methylbutyryl-L-carnitine; and *7*, isovaleryl-L-carnitine. Some impurities are noted between *peaks 5* and *6*. The compositions of eluants A and B are given under "Experimental Procedures," and the gradient itself (percentage of eluant B) is shown in the figure. Flow was maintained at 0.8 ml/min for 20 min. At this time it was increased over a 3-min period to 1.4 ml/min and then maintained until 31 min; the column was reequilibrated at 0.8 ml/min.

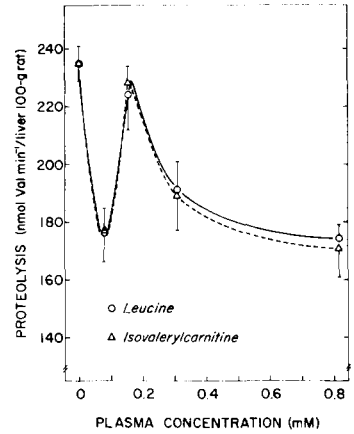


FIG. 2. **Proteolytic dose responses to L-leucine and IVC.** Livers from 42-h rats, previously labeled with [U-^{14}C]valine, were perfused in the single-pass mode for the determination of long-lived protein degradation as described under "Experimental Procedures." The sharp zonal loss of inhibitory effectiveness at 0.15 mM is a characteristic feature of the multiphasic response to regulatory amino acids (7, 10, 14). With the exception of a few additional data, the leucine results were part of an earlier study (10); the IVC experiments were carried out in parallel with the leucine controls. The results are means ± S.E. of 4–33 experiments.

RESULTS AND DISCUSSION

Proteolytic Dose Responses to L-Leucine and IVC—As first described by Miotto *et al.* (8) and confirmed in Fig. 2, proteolytic responses to IVC in the perfused rat liver closely mimic the multiphasic effects of leucine over the physiological range of leucine concentration (5, 7). It should be pointed out that

the sharp zonal loss of effectiveness at 153 μM (0.75×) and the preceding inhibition or decrease from the maximum at 77 μM (0.375×) are both lower than corresponding values in the fed rat (5, 7), a shift in proteolytic responsiveness associated with starvation (10, 14). It has been shown previously that isovalerate and L-carnitine are not inhibitory alone or when added together (8); hence, the action of IVC is mediated through itself, not its components.

One important difference between the leucine response curve in Fig. 2 and earlier findings (5, 7) deserves consideration. We have observed recently that the degree of inhibition at high physiological concentrations (816 μM, 4×) is decreased by approximately 50% after 24 h of starvation (14). Because the relative inhibition at 77 μM is not altered, both the low and high concentration responses are nearly equal in magnitude. The cause of this curious disturbance is not known but, as has been demonstrated earlier, inhibitory effectiveness can be restored by the coadditon of alanine, which acts synergistically with leucine at higher physiological levels. Leucine/alanine synergism has been observed in the perfused rat liver (10, 14) and in perifused hepatocytes from starved rats (16, 17) but not in livers from fed animals where leucine's inhibitory effectiveness is fully expressed (10). In this respect it differs from the coregulatory effect of alanine (see below), which is manifested in both fed and fasted states (9, 10, 14).

Inhibition of Macroautophagy by Leucine and IVC—Because macroautophagy is the cellular process that is largely responsible for the regulated phase of long-lived protein degradation in liver (2–4), the proteolytic inhibition induced by IVC (Fig. 2) would very likely be associated with a corresponding suppression of macroautophagic vacuole formation. Nevertheless, in view of the importance of IVC as a probe for investigating the regulatory mechanism of leucine, it seemed desirable to compare the inhibitory effectiveness of IVC and leucine on vacuole production. The rate of AVd formation in liver can be assessed from the density shift of β-hexosaminidase activity on colloidal silica gradients that results from the prior fusion of AVi with secondary lysosomes (18–20). The shift represents the redistribution of marker enzyme from gradient fractions of higher density to more buoyant fractions containing macroautophagic vacuoles and has been shown to correlate directly with AVd formation; with inhibition the direction is reversed (18).

From results in Table I it can be seen that the maximal induction of macroautophagy by stringent amino acid deletion (10× plasma amino acids *versus* 0×) caused a loss of marker enzyme in the dense fractions that amounted to about 13% of the total β-hexosaminidase activity on the gradient. By comparison, separate 102 μM additions of leucine and IVC each resulted in shifts of nearly 4%, effects that represent 30–31% of maximally induced autophagy. It is of interest that these suppressive effects, which were observed in livers of fed rats (see legend to Table I), equalled earlier low concentration proteolytic responses to leucine in fed rat livers when values were expressed as a percentage of maximally induced proteolysis (5, 7).

Structural Requirements for the Recognition of Leucine and IVC—Spatial modeling of leucine and IVC by the ball-and-stick method revealed a surprisingly close fit between the positively charged groups and the branched chain in the two molecules (not shown). The only major difference appeared to reside in a 3-carbon span between the quaternary amine and the carboxyl group of IVC (Fig. 3) which extended the latter beyond its position in leucine. The present findings together with earlier evidence (5, 7) suggest the existence of at least two requirements for low concentration inhibition: (i) the ability of the end group opposite the carboxyl terminus to attach to the recognition site and (ii) specificity of the branched chain itself. In the case of IVC and leucine, the end group has a positive charge, and both compounds are fully active (Table II). Substituting the α-amine in leucine with a keto group abolishes low concentration activity without loss of high concentration inhibition (5, 7); analogous, although less pronounced, results have been obtained with chloro replacement (Table II, Ref. 7). On the other hand, the α-hydroxyl analogue of leucine is as effective as leucine in evoking the full multiphasic response (7). This could be explained by the ability of the hydroxyl group to donate

TABLE I

Inhibitory effects of L-leucine and isovaleryl-L-carnitine on hepatic macroautophagic vacuole formation as determined by density shifts of β-hexosaminidase in colloidal silica gradients

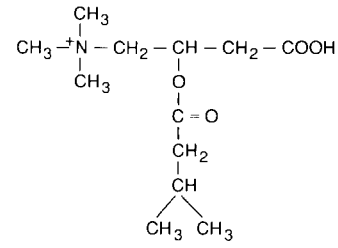
Livers from 18-h rats were perfused for 40 min in the single-pass mode with 100 μM (0.5 ×) L-leucine or 100 μM IVC. They were homogenized in 0.25 M sucrose, 1 mM EDTA, and lysosome-enriched mitochondrial + lysosomal fractions prepared from them were then separated on colloidal silica-povidone density gradients (18). The degree of macroautophagic inhibition was determined from the redistribution of β-hexosaminidase activity in autophagic vacuoles (located in the buoyant regions of the gradient) to dense lysosome fractions 1–8, the direct result of diminished AVi formation (18, 19); 18-h rather than 42-h rats were employed because of better separation between vacuoles and dense bodies. Each group of experiments was carried out on a separate day and livers were paired as shown below to eliminate day-to-day differences (10). The 10× complete amino acid (AA) mixture is known to suppress autophagy by 95–97% (4). Values are means ± S.E. of four to eight pair differences.

Treatment pairs	Shift of enzyme activity from buoyant to dense fractions 1–8	Inhibition of autophagy
	% total gradient activity	%
10× complete AA *minus* 0×	12.97 ± 1.11[a]	100
100 μM leucine *minus* 0×	3.93 ± 0.84[b]	30
100 μM IVC *minus* 0×	3.99 ± 0.97[c]	31

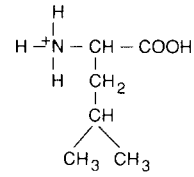
[a] Probability that mean pair differences are different from zero is $p < 0.001$.

[b] Probability that mean pair differences are different from zero is $p < 0.01$.

[c] Probability that mean pair differences are different from zero is $p < 0.05$.



Isovaleryl - L - Carnitine



L-Leucine

FIG. 3. **Comparison of L-leucine and IVC.** See text for discussion.

TABLE II

Low concentration inhibition of hepatic proteolysis by leucine, leucine analogues, and acylcarnitine derivatives

The experimental conditions were the same as in Fig. 2; where indicated, values are means ± S.E. of 3–33 experiments. Inhibition denotes the effect of the additions.

	Proteolysis[a]	Inhibition
	nmol Val min⁻¹/liver	
Amino acids/analogues		
None	235 ± 6	0
4× complete AA mixture	82 ± 7[b]	153
77 μM L-leucine	176 ± 10[b]	59
153 μM L-leucine	225 ± 13	10
77 μM D-leucine	234 ± 7	1
77 μM L-α-chloroisocaproate	240 ± 13	−5
77 μM L-norleucine	170 ± 12[b]	65
153 μM L-norleucine	237 ± 18	−2
Acylcarnitine derivatives		
77 μM isovaleryl-L-carnitine	177 ± 7[b]	58
153 μM isovaleryl-L-carnitine	229 ± 7	6
77 μM isovaleryl-D-carnitine	235 ± 9	0
77 μM acetyl-L-carnitine	223 ± 12	12
77 μM propionyl-L-carnitine	233 ± 15	2
77 μM butyryl-L-carnitine	242 ± 10	−7
77 μM isobutyryl-L-carnitine	242 ± 12	−7
77 μM α-Methylbutyryl-L-carnitine	225 ± 15	10

[a] 100-g initial body weight.
[b] $p < 0.001$ *versus* no additions.

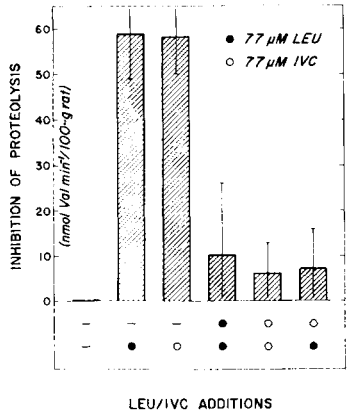


FIG. 4. **Additivity of low concentration proteolytic responses to L-leucine and IVC, expressed as the mean decrease in rate (inhibition) from the maximum (no additions) in paired experiments.** Each *circle* denotes a 77 μM concentration unit of leucine or IVC, added singly or in combination during perfusion; with *two circles*, the total concentration is 153 μM. In other respects the experimental conditions were identical to those described in the legend to Fig. 2. The *bars* represent means ± S.E. of four to six perfusions.

TABLE III

Effectiveness of alanine in restoring the zonal loss of proteolytic inhibition by leucine and IVC

The experimental conditions were the same as those in Fig. 2 and Table II. The control values for leucine and leucine + alanine were reported earlier in a study on alanine coregulation (10); the IVC data were obtained at that time in parallel experiments. Where indicated, values are means ± S.E. of 3–33 experiments. Inhibition denotes the effect of amino acid additions.

Additions	Proteolysis[a]	Inhibition
	nmol Val min⁻¹/liver	
None	235 ± 6	0
153 μM Leu	225 ± 13	10
153 μM IVC	229 ± 7	6
500 μM Ala	229 ± 12	6
500 μM Ala + 153 μM Leu	151 ± 5[b]	84
500 μM Ala + 153 μM IVC	189 ± 1[b,c]	46

[a] 100-g initial body weight.
[b] $p < 0.001$ versus no additions.
[c] $p < 0.01$ *verus* Ala + Leu.

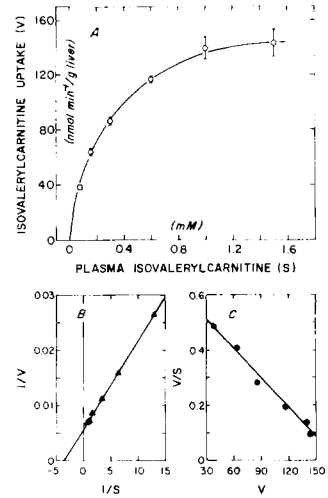


FIG. 5. **Kinetics of IVC uptake by the perfused rat liver.** Uptake at the IVC concentrations shown was measured from the difference in labeled IVC (isovaleryl-L-[*methyl*-^{14}C]carnitine) across the liver during single-pass perfusion (see "Experimental Procedures"), multiplied by the plasma flow rate. The process leveled off by 5–10 min, but perfusion was continued for 30–60 min; IVC did not equilibrate with erythrocyte water. *A*, linear plot of IVC uptake *versus* concentration; values are means ± S.E. of three to eight experiments. *B*, double-reciprocal plot of the data in *A*. *C*, single-reciprocal plot of the data in *A*.

hydrogen bonds, a feature not found in the keto and chloro substitutions.

With regard to the specificity of the side chain, Table II lists low concentrations responses to several amino acids and acylcarnitine derivatives, tested at the effective concentration of leucine in Fig. 2. The results conveniently fall into three general groups. First, responses to leucine and IVC were elicitable only with the L-isomers, indicating a high degree of sterospecificity. Second, no effects were obtained with acylcarnitines derived from decarboxylation products of isoleucine (α-methylbutyryl-L-carnitine) or valine (isobutyryl-L-carnitine); in addition, none of the short chain derivatives were effective. The results were not unexpected since isoleucine and valine exhibit no regulatory activity (5, 6) and concur with earlier findings of Miotto *et al.* (8). The fact that L-norleucine mimics L-leucine's low concentration responses suggests that the structural requirement for the branched chain is not absolute. A similar conclusion was reached by Grinde and Seglen (21) who obtained inhibition in isolated hepatocytes with several leucine analogues including norleucine and D-leucine. Their effects, however, were measured at 10 mM levels and cannot be compared with the low concentration responses in this study.

Because the requirements for low concentration recognition appear to be more stringent than those at the high end, one must regard the former as having greater relevance for physiological regulation. It should be pointed out that inhibitory responses generated by leucine's low concentration site (and by similar sites for the other regulatory amino acids) are not limited to test concentrations, since inhibition at higher plasma levels is fully expressed in the presence of alanine, a specific coregulator (9, 10). Under these conditions the inhibition progresses steadily with increasing plasma amino acids without the zonal peak seen in Fig. 2.

Additivity between Effects of Leucine and IVC; Alanine

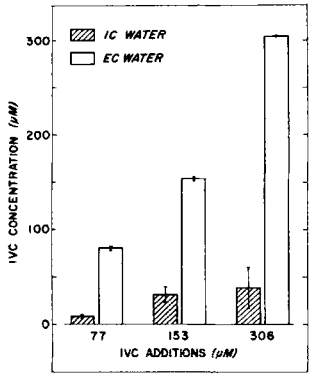


FIG. 6. **Intracellular/extracellular distribution of IVC.** Livers were perfused as in Fig. 5 with labeled IVC at the three concentrations shown. At the termination of perfusion IVC was determined in liver water and plasma as described under "Experimental Procedures." Since IVC is excluded from erythrocytes, the latter volume of ≈0.05 ml/g (13) was subtracted from the nominal extracellular (*EC*) space of 0.25, giving a value of 0.2 ml/g;. intracellular (*IC*) water was taken as 0.47 ml/g (13). Each *bar* depicts the mean ± S.E. of three experiments.

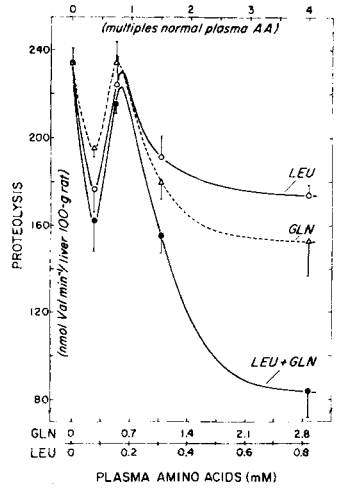


FIG. 7. **Proteolytic dose responses to leucine, glutamine, and leucine + glutamine.** The experiments were carried out as described in Fig. 2 and, with the exception of a few additional data, were part of an earlier study (10). In that study dose responses to leucine + glutamine were identical to those of the full group of regulatory amino acids. The molar values for leucine and glutamine on the *abscissa* (*bottom*) correspond to fractions/multiples of their concentrations in a complete amino acid mixture (*top*) which we have employed as a standard for normal plasma (13). Plotted values are means ± S.E. of 3–33 experiments, normalized to 100 g of initial body weight.

Coregulation—Although it is probable that the same recognition sites are utilized by both leucine and IVC in producing the zonal peak in Fig. 2, one cannot exclude the possibility that the observed effects were achieved through separate mechanisms. The question was tested by adding equal concentrations (77 μM) of leucine and IVC to the perfusate and measuring the proteolytic response. If the first assumption were correct, sites mediating the inhibition and its reversal would be randomly occupied by leucine and IVC. Hence, inhibition would be lost as the response shifted into the zonal peak. If the second were true, proteolytic rates would simply reflect the initial inhibition. The findings in Fig. 4 clearly support the first hypothesis. They show that rates of proteolysis with the mixture are equivalent to those obtained with 153 μM leucine or IVC and suggest that the low concentration

TABLE IV

Low and high concentration proteolytic inhibition by leucine and glutamine in comparison with responses to a complete amino acid mixture

The experimental conditions were the same as those in Figs. 2 and 7 from which data in lines 1, 3, and 5 were taken. Values for Leu + Ala and the complete amino acid (AA) mixture were from a recent study (10) carried out in parallel with the present experiments. The apparent relative K_m for these two conditions were taken from Fig. 8; corresponding values for Gln and Leu + Gln were computed as in Fig. 8 from the numbers shown in the table. Inhibition represents the decrease from maximal rates of proteolysis in the respective experiments (234-235 nmol of Val min^{-1}/liver (100-g rat).

Additions	Proteolytic inhibition[a] A 0.375×	Proteolytic inhibition[a] B 4.0×	A/B	Apparent relative K_m
	nmol Val min⁻¹/liver			
Leu	59	61	0.97	
Leu + 2 mM Ala		123	0.48[b]	0.50
Gln	40	82	0.49	0.49
Leu + Gln	72	150	0.48	0.50
Complete AA mixture	74	153	0.48	0.50

[a] 100-g initial body weight.

[b] The value for 0.375× was taken to be 59; Ala has no regulatory effect at this concentration (9, 10).

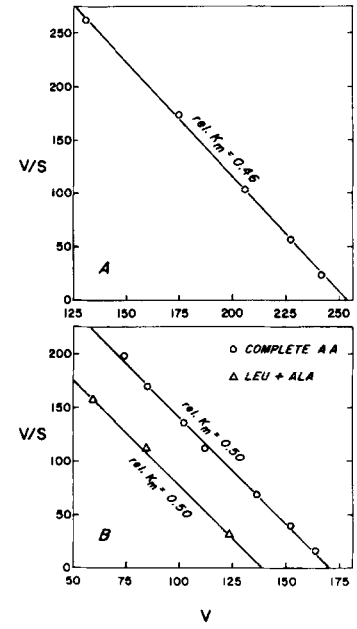


FIG. 8. ***V/S versus V* plots of proteolytic dose-responses to the standard complete amino acid mixture (13) and to leucine + alanine.** *V* represents proteolytic inhibition, expressed as nanomoles of valine min^{-1}/liver (100 g of initial body weight); *S* denotes fractions/multiples of the complete amino acid mixture or of any designated amino acid in the mixture. In this plot (as opposed to the reverse, *V* over *V/S*), V_{max} is conveniently shown as the *V*-intercept; the computed values for K_m and V_{max} were virtually the same when the axes were reversed. The *apparent* K_m values in these plots are also *relative* (*rel.*) in that they are based on fractions/multiples of the molar values in the standard plasma mixture. See Table IV and text for further discussion. *A*, responses of livers from normal fed rats to the complete amino acid mixture (Fig. 1 in Ref. 14). *B*, responses of livers from 42-h rats to the same complete mixture (10) and to leucine + alanine (data from Tables III and IV).

sites of recognition do not discriminate between the two.

As mentioned above, alanine is required for expression of proteolytic inhibition by the regulatory amino acids within a narrow range of concentrations centered at normal plasma

levels (9, 10). This coregulatory effect is specific for alanine, since it is the only amino acid whose deletion from a normal plasma mixture evokes a near-maximal acceleration of proteolysis; alanine alone is not directly inhibitory (9, 10). Previous experiments have shown that 0.5 mM alanine (1 ×) abolishes the zonal loss of inhibition by leucine (10), and it is reasonable to ask whether it interacts with IVC as well. Results in Table III demonstrate that the zonal peak at 153 μM IVC is significantly decreased in the presence of alanine, although to a lesser degree than that observed with leucine. The reason for this difference is not known. Because alanine's coregulatory effect requires an additional set of interactions (probably allosteric in nature), it is possible that they may not accommodate IVC as well as leucine.

Hepatic Uptake of IVC and Its Intracellular/Extracellular Distribution—As depicted in Fig. 5*A*, the hepatic uptake of IVC (labeled with [*methyl*-^{14}C]carnitine) is saturable and exhibits linear Michaelis-Menten kinetics when the data are expressed either as a double-reciprocal plot (Fig. 5*B*) or as *V/S versus V* (Fig. 5*C*); the apparent K_m and the V_{max} were found to be 270 μM and 170 nmol min^{-1}/g, respectively. Although the nature of the IVC transporter has not been fully characterized (22), the linearity in Fig. 5*C* eliminates passive diffusion as a component of the uptake mechanism.

In agreement with Hokland (22), IVC was rapidly hydrolyzed after uptake and the label distributed among other acyl derivatives. In the single-pass perfusion experiments of Fig. 6 in which labeled IVC was added at 80, 156, and 304 μM concentrations, 66–77% of the intracellular label was associated with free carnitine, 14–18% with acetylcarnitine, and less than 1% with IVC. It is evident from Fig. 6 that actual concentrations of intracellular IVC were not affected to the same extent, although they were decreased by as much as 90% in relation to extracellular values.

Because leucine and IVC appear to mediate their low concentration effects through the same regulatory sites, the low intracellular/extracellular ratio for IVC when compared with the ratio for leucine could have a bearing on potential cellular locations of the sites. Values for intracellular leucine, like those for valine, approximate extracellular levels when corrected for compartmentalized lysosomal pools (23).[2] Thus at 77 μM (the lowest effective external concentration for leucine and IVC), the quantity of intracellular leucine would be as much as 10-fold greater than that of IVC; similar differences would be found at higher plasma levels. Because the findings of Fig. 4 require the presence of equal concentrations of leucine or IVC at the sites of recognition, one possibility is the plasma membrane. On the other hand, a cytoplasmic location cannot be excluded. Proposals based on the latter, though, would be more complex in that a mechanism of some undetermined type would be required to transport the regulatory amino acids (and IVC) at their external concentrations to cytoplasmic sites of vacuole formation.

The present pool data are insufficient to differentiate clearly between these possibilities. Recent findings, however, indicate that the question of cellular location can be approached in another way with the use of specific amino acid derivatives (25).[3] Prompted by evidence that the carboxyl group of leucine is not required for biological activity (25), we coupled leucine via its carboxyl group to MAP, the multiple antigen peptide (24) to form a ≈1900-Da molecule with 8 isopeptide residues of leucine.[3] MAP-Leu proved to be as effective as leucine in suppressing proteolysis in the isolated hepatocyte. At the highest concentration (molar residue equivalent, 0.8 mM), electron microscopy revealed that the volume density of autophagic vacuoles was decreased in parallel with proteolytic rates. As expected, MAP-Ile was inactive. Because of its bulk, it is unlikely that MAP-Leu was transported into the cytoplasm during the short term incubations. Thus its inhibition may have been mediated from the cell surface. These findings indicate the feasibility of synthesizing specific noninternalizable probes to obtain new information on both the function and location of the recognition sites.

Concerted Effects of Regulatory Amino Acids in the Control of Proteolysis—The possibility that the locus of leucine recognition is at the plasma membrane has important implications concerning the location of other amino acid regulatory sites. Because leucine, glutamine, and tyrosine individually and the regulatory amino acids as a group all evoke multiphasic responses at identical fractions of their molar concentrations in normal plasma (7), it is possible that they act in a concerted manner through sites in proximity to each other. Inhibitory effects of leucine and glutamine have been shown to be additive in rats fed commercial pellets *ad libitum* (5), and in synchronously fed rats these 2 amino acids together evoke multiphasic dose responses equivalent to those of the complete group of 7 (10). Thus the amino acid control of proteolysis can be reduced to two direct regulators with coregulatory/synergistic assistance from alanine.

Proteolytic dose responses to leucine and glutamine, both singly and combined, are displayed in Fig. 7. Because the concentration of glutamine in the standard plasma mixture is almost exactly 3.5 times that of leucine, the values on the abscissa were normalized so that each point depicts the molar concentration relative to its level in normal plasma. In line with comments in the above paragraph, it will be noted that the major inflections in each curve, namely, the low concentration inhibition and the sharp zonal loss of inhibition, occur at similar normalized plasma levels, 0.375 and 0.75 × (see scale at top of Fig. 7). We have previously shown that dose response curves for the regulatory group and for leucine + glutamine are superimposable (10) and except for the presence of a zonal peak closely match those of the complete amino acid mixture (compare lines 4 and 5 in Table IV). Also note in Table IV that the magnitude of proteolytic inhibition at 0.375× relative to that at 4 × is the same with the complete amino acid mixture and each of the conditions of Fig. 7 except when leucine was given by itself. It will be recalled from the discussion of Fig. 2 that starvation induces an unexplained decrease in leucine's high concentration effectiveness (10) which, as seen in Table IV, is restored by alanine (10, 14, 16, 17). Owing to the strongly concerted effect of leucine and glutamine at the 4× level, it appears that glutamine also restores leucine's effectiveness at 4×, a finding in agreement with Caro *et al.* (17).

As a coregulator, alanine is specifically required for expression of proteolytic inhibition by leucine, glutamine, and the regulatory group at normal plasma concentrations (9, 10). Thus in the presence of alanine, the zonal peak is lost, and individual responses to added leucine (Tables III and IV, graph not shown) and to glutamine[2] become proportional to those of the complete mixture over the physiological range of plasma amino acids. These findings suggest that recognition sites for glutamine as well as for leucine are directly accessible to plasma amino acids and that proteolytic inhibition is determined by reversible reactions between the sites and the amino acids. If so, one should obtain a linear inverse relationship between proteolytic inhibition (V) and normalized amino

[2] G. E. Mortimore, unpublished data.

[3] G. Miotto, O. Marin, R. Venerando, N. Siliprandi, and G. E. Mortimore, manuscript in preparation.

acid concentrations (S) utilizing conventional Michaelis-Menten kinetics.

Indeed, this proved to be the case when dose responses to the complete amino acid mixture from the experiments in Table IV and a previous study (14) were graphed either as V/S over V (Fig. 8, *A* and *B*) or as a double-reciprocal plot (not shown). Both were highly linear and gave apparent relative K_m values that were close to 0.5 normal plasma concentrations. A corresponding plot for leucine + alanine from data in Tables III and IV (Fig. 8*B*) yielded the same relative value ($K_m = 0.50$) as that computed from experiments with complete amino acids ($K_m = 0.50$); preliminary estimates of the apparent relative K_m for glutamine and leucine + glutamine, based on two points, were 0.49 and 0.50 (Table IV). This overall agreement in relative K_m suggests that, in molar terms, the apparent values for the 2 strongest regulatory amino acids (leucine and glutamine) in their reactions with regulatory sites are close to 0.5 of their concentrations in the standard plasma amino acid mixture. The agreement is very likely not fortuitous but an essential feature of a mechanism that is regulated by more than one amino acid.

Because the concerted responses to leucine and glutamine (or to the complete mixture) in Table IV are not additive but, instead, proportional to the individual responses, the recognition sites themselves must interact in a way that maintains, with increasing amino acid concentrations, a proportional inhibitory signal to loci of vacuole formation. This could be achieved by conformational alterations of subunits if the sites existed together within a plasma membrane complex. On the other hand, we cannot fully exclude the notion that the interaction occurs distally at points of vacuole formation. This alternative seems remote, though, since each amino acid recognition site then would require an independent cytoplasmic signal and for this reason add an unreasonable degree of complexity to the regulation.

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G Miotto, R Venerando, K K Khurana, N Siliprandi and G E Mortimore

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