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Liver perfusion: an in vitro technique for the study of intracellular protein turnover and its regulation in vivo

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Most mammalian cells lose protein and other cytoplasmic constituents during energy deprivation (Addis *et al.* 1936). Rates of depletion, however, vary widely among different cells and tissues. Liver is particularly responsive and for this reason provides a highly useful model for examining the mechanisms involved. In the rat as much as 25% of its weight (Mortimer *et al.* 1983), 20% of its protein content (Addis *et al.* 1936; Soberon & Sanchez, 1961) and equivalent amounts of RNA (Millward *et al.* 1974; McNurlan *et al.* 1979) are lost during 24 h of starvation without significant decreases in the number of nuclei (Kosterlitz, 1944) or DNA (McNurlan *et al.* 1979). These deficits are promptly regained during refeeding (Conde & Scornik, 1976; Hutson & Mortimore, 1982; Mortimore *et al.* 1983).

In principle, net protein effects of this type could be achieved by alterations in either the general rate of protein synthesis or breakdown, or by a combination of the two. Garlick *et al.* (1973, 1975) and Conde & Scornik (1976) have shown that fractional rates of hepatic protein synthesis, expressed per unit of protein or RNA, are relatively unaffected by starvation and refeeding and have concluded that protein breakdown plays a dominant role in regulating the protein content of liver. More recent investigations of general protein turnover in livers of starved and starved–refed mice (Hutson & Mortimore, 1982; Mortimore *et al.* 1983) have confirmed these observations and provided additional information on absolute rates of protein synthesis and degradation. In these studies, rates of resident protein synthesis per liver decreased during starvation and increased with refeeding, presumably as a result of changes in the content of ribosomal RNA (Conde & Scornik, 1976; McNurlan *et al.* 1979). Absolute rates of breakdown, however, moved independently over a much wider range: they remained consistently higher than synthesis over the course of starvation and, after the start of refeeding, they dropped sharply below synthesis to rates averaging less than 10% of controls. About half the subsequent regrowth of cytoplasmic protein could be attributed to this 'down regulation' of proteolysis; the remainder to the absolute increase in protein synthesis.

A definitive mechanism (or mechanisms) for the breakdown of resident intracellular proteins has not yet been established, but a growing body of evidence supports the notion that protein degradation in hepatocytes is mediated in its final phase by two classes of autophagic activity: (a) overt or macroautophagy, so designated because the vacuoles are comparatively large and easily recognised

(Ericsson, 1969; Mortimore & Schworer, 1977; Pfeifer, 1978), and (b) basal or microautophagy, a less-well-defined process comprising elements of the dense body population (de Duve & Wattiaux, 1966; Novikoff & Shin, 1978; Pfeifer, 1981; Schworer *et al.* 1981; Ahlberg *et al.* 1982; Mortimore *et al.* 1983). Although the two processes are similar in that they both sequester cytoplasmic protein, they differ in their mode of regulation; macroautophagy is actively controlled by the inhibitory action of amino acids and insulin (Mortimore & Pösö, 1983) whereas microautophagy is thought to be passively regulated by intrinsic alterations within the cell (Mortimore *et al.* 1983).

The following report is an account of investigations from our laboratory that were aimed at establishing a quantitative, general relationship between autophagy and intracellular proteolysis in livers of rats and mice. Our decision to use the isolated, perfused organ was based on preliminary evidence that rates of protein synthesis and degradation would not be appreciably altered from those in the intact animal as well as on the need to control the cell's environment and to eliminate interactions with other tissues. This technique also made it possible to shift the liver almost instantaneously from an internal organ to an isolated, perfused tissue simply by altering the source of the vascular inflow. These features made it possible to examine, under controlled conditions, native responses of the degradative system(s) to specific physiological regulators and to assess instantaneous rates of hepatic protein degradation in vivo.

Protein degradation in the perfused rat liver and regulatory effects of amino acids, insulin and glucagon

When livers from normal rats are cyclically perfused in the absence of added amino acids or insulin, protein breakdown normally exceeds protein biosynthesis and products of protein catabolism accumulate in liver water and the perfusion medium. The balance between these two general processes can be monitored conveniently from increases or decreases of an amino acid such as valine which, like leucine and isoleucine, is neither synthesized nor degraded appreciably in liver (Mortimore & Mondon, 1970; Pösö *et al.* 1982*b*). Thus an increase in free valine would indicate a corresponding decrease of valine in peptide linkages and, hence, would provide a moment-to-moment account of net gains or losses of protein.

Although cyclic perfusion has been used advantageously in many early studies of protein degradation in liver (Mortimore & Mondon, 1970; Woodside & Mortimore, 1972; Mortimore *et al.* 1972; Woodside *et al.* 1974; Khairallah & Mortimore, 1976), it is clearly not the procedure of choice for investigating the mechanism of amino acid control of protein breakdown. Because amino acid concentrations are strongly affected by ongoing proteolysis and other metabolic processes, it is difficult or impossible to maintain constant levels in the perfusate and to alter the amino acid composition at will. To obviate these problems, livers from most of the experiments discussed here were initially perfused in the single-pass mode (Mortimore & Schworer, 1977; Schworer *et al.* 1981). After a brief period, usually 40 min, the livers were switched to a second-stage cyclic perfusion with 18

uM-cycloheximide for the determination of proteolysis by a procedure adapted from Khairallah & Mortimore (1976). In the latter study, rates of valine and leucine released from cyclically-perfused rat livers were linear during the 5–15 min interval after the addition of cycloheximide and were shown to provide valid measurements of total proteolysis existing within the livers at the moment cycloheximide was added. Although cycloheximide is known to inhibit proteolysis, the effect is delayed in onset and would not interfere with the measurements (Khairallah & Mortimore, 1976).

The release of valine (or leucine) has been shown to consist of two major components: (a) a short-lived fraction $t_{\frac{1}{2}}$ 10–12 min) that is uninfluenced by regulatory agents and thus constant in value, (b) a long-lived fraction representing the breakdown of resident cellular proteins (Schworer *et al.* 1981; Hutson & Mortimore, 1982). The nature of the short-lived component or components is not known, but its breakdown does not appear to involve lysosomes (Neff *et al.* 1979). Endocytosis of plasma proteins may also contribute to the release of valine: however, the quantity involved appeared to be very small in livers of control, fed or starved–refed mice although it increases after 48 h starvation (Hutson & Mortimore, 1982; Hutson *et al.* 1982). Protein degradation rates in Fig. 1 were calculated from the total release of valine, nominally corrected for breakdown of the short-lived component and endocytosis (Schworer *et al.* 1981).

Owing to its large size and numerical superiority, the hepatocyte has by far the greatest aggregate mass of any cell in liver and has been estimated to contain more than 98–99% of the total content of cellular protein (Mortimore *et al.* 1983). Thus from the standpoint of general protein turnover, the perfused liver may be considered to be a reasonably-pure preparation of isolated hepatocytes. Of the total mass of proteins that turn over in liver, more than 99% are contained within the resident, long-lived fraction (Schworer *et al.* 1981). For these reasons then, the foregoing rates of valine release, determined in the presence of cycloheximide and appropriately corrected for the short-lived component, will provide a valid estimate of degradation for the bulk of proteins residing in the hepatocytes of fed or starved–refed animals (Schworer *et al.* 1981; Hutson & Mortimore, 1982).

Amino acids are the prime regulators of protein breakdown in liver and Fig. 1 illustrates the high level of responsiveness of resident protein degradation to varying levels of plasma amino acids perfused in the single-pass mode through normal rat livers (Schworer *et al.* 1981). Minimal (basal) and maximal rates represent 1·5 and 4·5%/h of the total valine residues in liver protein (465 μmol). It is of interest that a zone of high responsiveness was centred in the vicinity of normal plasma concentrations and that nearly full suppression was obtained at four times normal levels, a value that may be considered to lie within physiological limits (Peraino & Harper, 1963; Elwyn *et al.* 1968). These findings provide the basis for a feedback control of proteolysis that could be of importance in free amino acid homeostasis.

Apart from the probability that starvation-induced protein degradation is mediated by macroautophagy (see p. 165), virtually nothing is known of the

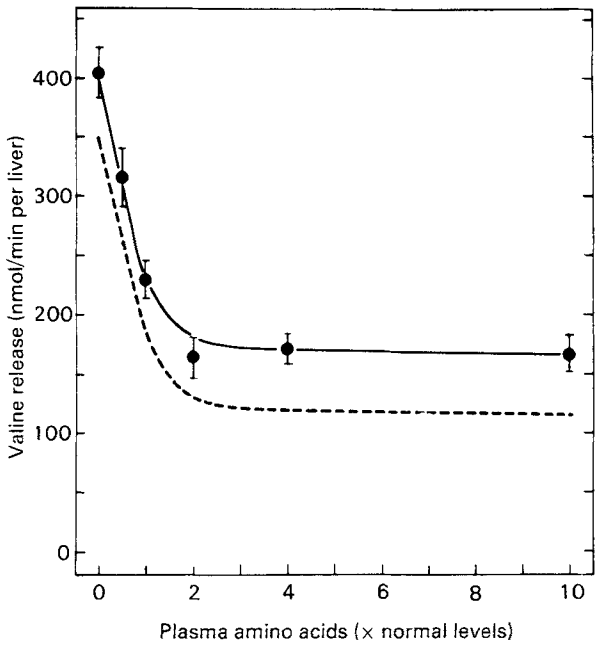

Fig. 1. Effects of varying plasma amino acid concentrations on rates of total (———) and long-lived (– – –) or resident protein degradation. Livers were perfused in the single-pass mode for 40 min; proteolysis was determined from the release of valine in a second-stage perfusion containing cycloheximide. The long-lived curve has been corrected for short-lived release. From Schworer *et al.* (1981).

regulatory mechanisms involved. Glucagon is a potent inducer of autophagy in liver (Ashford & Porter, 1962; Arstila & Trump, 1968) but its effectiveness appears to be strongly modified by amino acids. In a study by Schworer & Mortimore (1979), for example, little additional stimulation was achieved with glucagon when livers were perfused in the absence of external amino acids, and its action on autophagy and proteolysis was completely blocked by plasma amino acids at concentrations as low as four times normal values; the hormone was most effective at normal amino acid levels. The reason for this modulation is not known. The enhancement of proteolysis could be mediated by the intracellular depletion of certain glucogenic amino acids (Schworer & Mortimore, 1979); it is equally possible that other amino acid interactions are involved (Pösö *et al.* 1982*a*).

Insulin is another important regulator of hepatic protein breakdown (Mortimore & Mondon, 1970) and its effects are also influenced by amino acids. However, unlike glucagon, insulin is inhibitory in action and completely effective in the absence of added amino acids (Schworer *et al.* 1981). No further inhibition has been observed in the presence of maximally-effective levels of amino acids (Neely *et al.* 1977; Schworer *et al.* 1981).

The complexity of proteolytic regulation by amino acids and hormonal agents underscores the importance of employing a liver cell model whose functioning does not deteriorate appreciably from the general state in vivo. Any significant loss of

performance would raise the disturbing question of whether all or only certain functions were affected. In an earlier study we reported close agreement between direct measurements of resident protein synthesis in the perfused mouse liver and comparable determinations in vivo (Hutson & Mortimore, 1982). An average rate of resident synthesis for the perfused rat liver was calculated to be 0·129 μmol valine/min per 100 g rat, 1·67%/h or 0·40/d (Pösö *et al.* 1982*b*). The fact that this value is equivalent to the average rate of protein degradation at four times normal values (Schworer *et al.* 1981; Pösö *et al.* 1982*b*) suggests that the resident pool of protein in hepatocytes is capable of being brought into balance by levels of amino acids similar to those found in portal vein blood during feeding (Peraino & Harper, 1963; Elwyn *et al.* 1968). This observation together with estimates of protein degradation in the mouse liver in vivo, based on a rapid *in situ* perfusion technique (Hutson & Mortimore, 1982; Hutson *et al.* 1982), indicate that basal protein turnover and responses to deprivation in the perfused liver are not noticeably altered from those in vivo.

Proteolytic and autophagic responses to graded amino acid deprivation in the perfused liver

Our approach to the establishment of a quantitative correlation between autophagy and rates of intracellular protein breakdown was prompted by earlier observations of a close association between macroautophagy and the acceleration of proteolysis when rat livers are perfused with a medium deficient in amino acids or insulin, or both (Neely *et al.* 1974, 1977; Mortimore & Schworer, 1977). Because newly-formed autophagic vacuoles contain well-defined portions of cytoplasm, the aggregate volume of cytoplasm sequestered at any time can readily be determined by electron microscopy combined with stereologic procedures; local differences in protein concentration would be averaged by the large number and variety of vacuoles formed (Mortimore & Schworer, 1977). Rates of cytoplasmic internalization and digestion could be estimated from the steady-state volume of the vacuoles and their turnover, as will be described later (see p. 167).

Macroautophagy is strongly stimulated when livers are perfused in the absence of added amino acids (Mortimore & Schworer, 1977; Schworer *et al.* 1981). The response is immediate in onset, attains a maximum by 20 min and remains constant for periods up to 90 min. The earliest double-walled vacuoles (AVi) appear in areas predominating in rough endoplasmic reticulum where discrete portions of cytoplasm are isolated by smooth-surfaced, double membranes (Fig. 2). After a short lag (7–8 min), AVi are transformed into degradative vacuoles (AVd), followed by rapid digestion of the internalized cytoplasm. This transformation is presumed to occur as the result of fusion of AVi with dense bodies (DB) (Ericsson, 1969; Schworer *et al.* 1981).

The way microautophagic particles (Bi) are formed is less well understood (Fig. 2). Small bits of cytoplasm could be isolated in small double-walled vesicles (b) which simultaneously (a) or subsequently (c) fuse with DB (see Fig. 2). Alternatively, the delimiting DB membrane could invaginate. On average, DB are

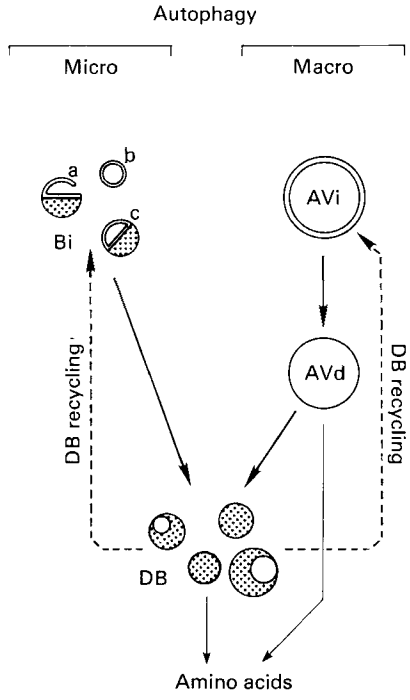

Fig. 2. Scheme depicting known sequestrational mechanisms and routes of lysosomal enzyme flow in macroautophagy and hypothetical mechanisms in microautophagy (for details, see p. 165). AVi, double-walled vacuoles; AVd, degradative vacuoles; Bi, microautophagic particles; DB, dense bodies. From Mortimore & Pösö (1983).

smaller than AVd and can readily be divided into two groups according to whether the profiles contain a sharply demarcated, electron-lucent zone (type A) or not (type R). Because these zones frequently contain glycogen and other cytoplasmic material, type A DB may be considered autophagic.

The addition of a maximally-suppressive load of plasma amino acids causes an immediate cessation in the formation of new AVi (Schworer *et al.* 1981). As shown in Fig. 3, the population of AVi that was induced during 20 min of perfusion without plasma amino acids was rapidly converted to AVd, and the aggregate volume of vacuoles regressed with an apparent first-order rate constant of 0·087/min ($t_{\frac{1}{2}}$ 8 min). Comparable rate-constants have been reported under other experimental conditions (Neely *et al.* 1974; Pfeifer, 1978).

Fig. 4(*a*) depicts alterations of the fractional cytoplasmic volumes of the major lysosomal components over the full range of amino acid deprivation. Fractional volumes of macroautophagic vacuoles fell strikingly as plasma amino acids were raised, and their formation virtually ceased at four and ten times normal plasma concentrations. The percentage of AVi (52%) and AVd (48%), however, remained constant. Although the total volume of DB did not change, one conspicuous component (type A; often containing sharply-demarcated zones of glycogen, ribosomes and membrane remnants (Mortimore & Schworer, 1977; Schworer

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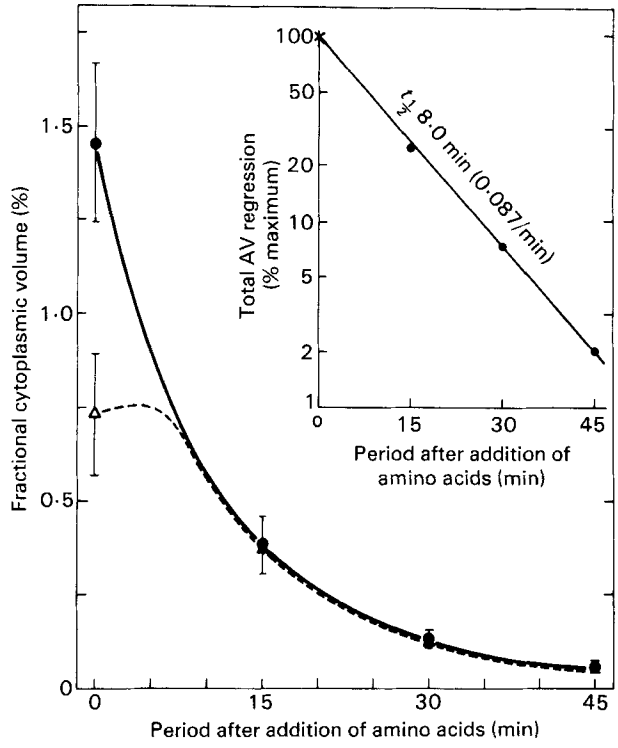

Fig. 3. Regression of autophagy following the addition of amino acids. Livers were perfused in the single-pass mode for 20 min without amino acids, then with a medium containing ten times the normal plasma amino acid levels, and at intervals individual livers were fixed for electron microscopy. A semilogarithmic plot of the aggregate volumetric regression of all autophagic vacuoles (AV) is also shown. (×), Ratio at time zero for double-walled vacuoles (AVi):degradative vacuoles (AVd) of 1:1; because the time required to transform AVi to AVd approximates the half-life of AVd regression from 15 to 45 min, the slope would be expected to pass through 100% at time zero if internalization were immediately stopped following the addition of amino acids. (———), Total AV; (– – –), AVd. From Schworer *et al.* (1981).

et al. 1981)) was more abundant during maximally-stimulated macroautophagy than it was when autophagy was fully suppressed. This suggests that some of these particles are end-products of macroautophagy. It should be emphasized, however, that because type A DB persist despite almost complete inhibition of macroautophagy, they must come from at least two sources (see Fig. 2): (a) macroautophagy and (b) an independent basal sequestrational process (microautophagy). Endocytosis is another possibility but its contribution would be almost negligible (Hutson & Mortimore, 1982).

As shown in Fig. 4(*b*), rates of resident protein degradation correlated well with the aggregate volumes of degradative lysosomal components (AVd + total DB) over the full range of regulation. Such agreement would be expected for the deprivation-accelerated phase of degradation (rates above basal) if macroautophagy were the only proteolytic mechanism. The question was evaluated in more detail (Schworer *et al.* 1981) by computing the fractional turnovers of

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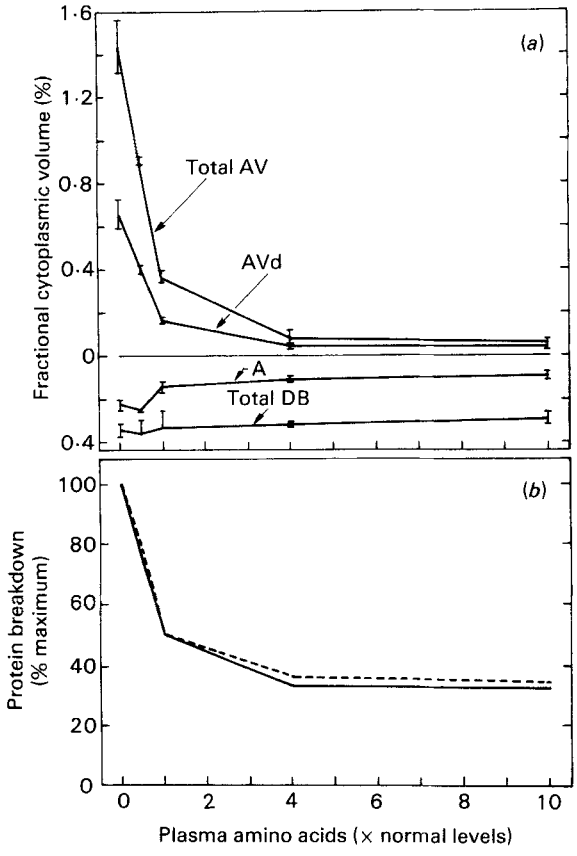

Fig. 4. Effects of varying plasma amino acid concentrations on the degradation of resident proteins and fractional cytoplasmic volumes of lysosomal-vacuolar elements. Single-pass liver perfusions were continued for 40 min; livers were then either fixed for electron microscopy or switched to a cyclic perfusion flask for the determination of proteolysis.

(*a*) Values for double-walled vacuoles (AVi) are represented above zero by the differences between total autophagic vacuoles (AV) and degradative vacuoles (AVd); similarly, values for type R dense bodies (DB) are indicated below zero by differences between DB and type A elements. The fractional volumes are expressed as a percentage of parenchymal cell cytoplasm and exclude nuclei, sinusoidal cells and noncellular spaces. For description of particles, see p. 165.

(*b*) Correlation between rates of resident protein breakdown and the fractional cytoplasmic volumes of degradative lysosomal components; maximal degradation (100%) 351 nmol valine/min per liver (100 g rat). (———), Proteolysis; (– – –) AVd + total DB. From Schworer *et al.* (1981).

cytoplasmic volume at all levels of deprivation using the first-order rate constant of 0·087/min. The values, expressed as %/h, agreed quantitatively with corresponding turnovers of cytoplasmic protein derived from measured rates of resident protein degradation.

Pools of degradable protein in lysosomes

Although the foregoing leaves little doubt that most or all of deprivation-induced proteolysis can be explained by macroautophagy, the mechanism or mechanisms of basal protein degradation are less-well understood. Responses to lysosomotropic

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inhibitors (Ward *et al.* 1979; Neff *et al.* 1979; Grinde & Seglen, 1980) clearly indicate that lysosomes play a role in basal turnover, but because intralysosomal proteolysis is resistant to complete suppression by lysosomotropic agents (see Mortimore, 1982), this pathway has been difficult to quantitate. As an alternative approach, we have assessed the quantity of protein internalized within lysosomes on the theory that ongoing sequestration will create intralysosomal pools, the size of which is directly related to rates of protein degradation.

The method used is simple in principle and takes advantage of the observation that proteins internalized within intact lysosomes appear to be degraded to completion or near-completion during prolonged incubation of isotonic liver homogenates at 37°, pH 6–7 (Mortimore & Ward, 1981). Proteolysis was determined from the total accumulation of free valine. More than 95% of the released amino acid was shown to be generated proteolytically within intact particles whose buoyancy could be altered by previous loading with Triton WR 1339 (Mortimore & Ward, 1981). We concluded from these and other experiments that the degradable protein was located within the lysosomal matrix, and we obtained no evidence that cytosolic proteins had gained access to lysosomal proteases during the course of incubation.

Fig. 5 (*a,b*) shows time-courses of valine release in unfractionated homogenates (pH 7) in states ranging from maximally-induced macroautophagy (glucagon treatment) to near-basal (unperfused fed rats). The quantity of valine released differed widely among the groups, but the time-courses were closely proportional and all levelled off between 120 and 150 min of incubation. Control studies showed that the extent of proteolysis was increased by acidifying the medium and that approximately 30% more valine was released at pH 6 than at pH 7 (Mortimore & Ward, 1981). Although a satisfactory pH optimum could not be established owing to the deleterious effect of low pH on lysosomal latency, other evidence suggested that it would be close to 5 and yield values approximately 43% higher than those at pH 7 (Reijngoud *et al.* 1976).

Table 1 lists corrected values of internalized protein in states ranging from maximal stimulation of macroautophagy to almost complete suppression of macro- and microautophagy in starvation–refeeding, a condition characterized by a dramatic decrease in proteolytic rates (Conde & Scornik, 1976; Hutson & Mortimore, 1982). The apparent turnover (C:A in Table 1) shows that the proportionality between rates of hepatic protein degradation (C) and internalized protein (A) was extremely close over the full proteolytic range, including basal turnover. On the assumption that this degradable pool was the source of valine released under all conditions, C:A would represent the rate-constant of turnover for the pool. One should note that the values varied only slightly from the observed rate-constant of autophagic vacuole regression, 0·087/min.

It is of interest that the shape and characteristic features of the proteolytic release curve in Fig. 5(*b*) remained the same despite large differences in the size of the internalized pool and the proportion of macro- and microautophagic components present. However, rates of proteolysis were consistently lower than

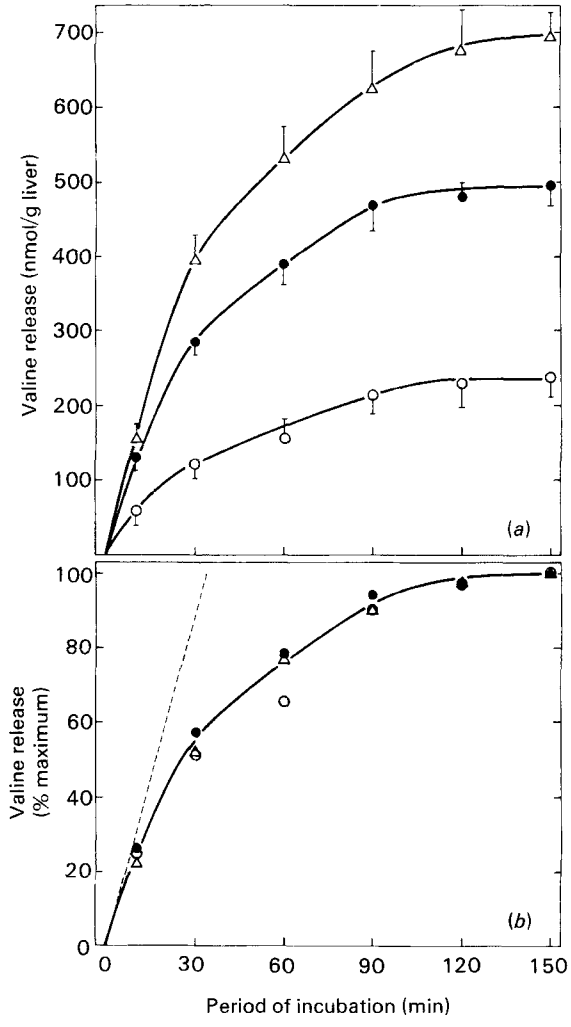

Fig. 5. Time-courses of valine release from homogenate particles of rat livers unperfused (○; n 9) and cyclically perfused for 60 min with (△; n 11) and without (●; n 12) glucagon (10 μg/h). (*a*) Unfractionated homogenates in 0·25M-sucrose+1 mM-EDTA incubated at pH 7, 37°; values represent total release minus the amount released in particle-free supernatant fractions, incubated separately. (*b*) Results shown in (*a*) expressed as a percentage of the maximal value in each group. (– – –) An estimate of initial velocity, expressed relative to total release (2·9% or 0·029/min). From Mortimore & Ward (1981).

values predicted for the intact cell as shown from a comparison of the initial velocity constant in Fig. 5(*b*) (0·029/min) with the turnover constant in Fig. 3 (0·087/min). The reason for this apparent discrepancy is not known, but it seems to affect macro- and microautophagy equally. Metabolic energy is required for optimal rates of resident protein breakdown in the intact cell (Hershko & Tomkins, 1971; Poole & Wibo, 1973) and the decrease could be explained by the lack of ATP. Other factors might include impaired mixing of intralysosomal contents as a result of the absence of particle fusion and other membrane-related activities.

Table 1. *Observed and predicted pools of internalized lysosomal protein and their relation to rates of hepatic protein degradation in various metabolic states*

(Proteolytic states were established under the following conditions: maximal acceleration, stringent amino acid depletion (zero times normal levels) or addition of glucagon; half-maximal acceleration, control cyclic perfusion or single-pass perfusion with 0·5 times plasma amino acids; basal, addition of ten times normal plasma amino acids levels, with or without insulin. Growth suppression of protein breakdown was induced in mice by 48 h of starvation followed by 12 h of refeeding. Observed values (A) were determined as in Fig. 5, corrected by the factor 1·43 (see p. 169). Predicted values (B) were recalculated as in Mortimore *et al.* (1983) from stereologic values in Fig. 4(*a*) and cytoplasmic protein concentrations. From Mortimore & Pösö (1983))

Proteolytic states . . .	Internalized lysosomal protein (mg/g liver)		Hepatic protein degradation (μg/min per g liver)	Apparent turnover (/min)
	(A)	(B)	(C)	(C:A)
Acute regulation				
Maximum acceleration	1·68*	1·72‡	153*	0·091
Half-maximum acceleration	1·20*	1·33‡	102*	0·085
Basal	0·55*	0·57‡	48*	0·087
Growth suppression	0·11†	0·10§	9†§	0·083

*Recalculated from Mortimore & Ward (1981).
†Recalculated from Hutson & Mortimore (1982).
‡Recalculated from Schworer *et al.* (1981).
§Recalculated from Mortimore *et al.* (1983).

Regulation of protein degradation and autophagy during starvation and refeeding

Previous studies have shown that the rate of resident protein breakdown in liver falls rapidly to values near zero when animals are refed after a period of restricted food intake (Conde & Scornik, 1976; Khairallah, 1978; Millward, 1980; Hutson & Mortimore, 1982). Macroautophagy is also suppressed (Pfeifer & Bertling, 1977; Khairallah, 1978) and DB decrease (Khairallah, 1978). Because the pool of degradable protein in lysosomes was recently found to decrease in proportion to protein degradation in starved (48 h), refed (12 h) mice (Hutson & Mortimore, 1982), we considered the possibility that microautophagy as well as macroautophagy is subject to regulation.

Fig. 6 (*a*,*b*) shows characteristic alterations in total liver protein, resident protein synthesis and rates of protein breakdown during starvation and refeeding in the mouse. Synthesis was measured during perfusion as detailed in Hutson & Mortimore (1982); degradation was determined by subtracting the net rates of total protein change (Fig. 6 (*a*)) from rates of synthesis. The observed decrease in protein synthesis with starvation and subsequent restoration during refeeding undoubtedly reflect primary alterations in the total intracellular pool of ribosomal RNA (Conde & Scornik, 1976; McNurlan *et al.* 1979). Rates of resident protein degradation during starvation were consistently greater than those of synthesis and tended to decrease in parallel with the latter (Hutson *et al.* 1982). With

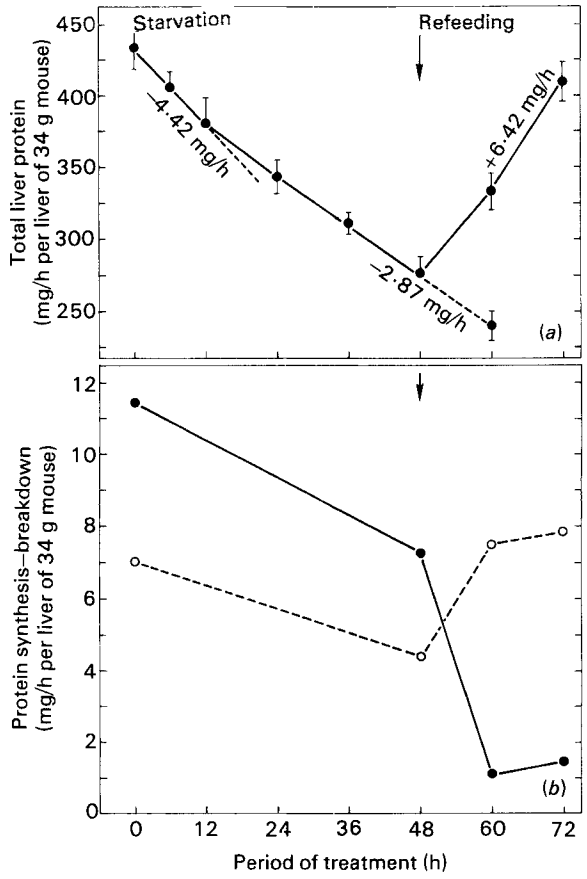

Fig. 6. Alterations in (*a*) the content of liver protein and (*b*) rates of protein synthesis (– – –) and degradation (————) during starvation and refeeding in the mouse. Net rates of liver protein loss at 0 and 48 h were calculated by least squares regression from the first three and last four points; rates of gain at 12 and 24 h of refeeding represent the mean rate between 60 and 72 h. Mean (with SE) rates of resident protein synthesis were 3·73 (0·26), 3·96 (0·20), 3·56 (0·21) and 3·91 (0·16) mg/h per g liver respectively, in fed, 48 h starved, 12 h refed and 24 h refed mice (four mice/group). Rates per liver were obtained by multiplying these values by the following corresponding average liver weights for the same groups: 1·88, 1·11, 2·11 and 2·01. From Mortimore *et al.* (1983).

refeeding, however, breakdown fell dramatically to rates about 10% of that of control (fed) animals (Hutson & Mortimore, 1982), a response that appears to be an important determinant in hepatic cytoplasmic growth (Conde & Scornik, 1976; Hutson & Mortimore, 1982).

Macroautophagy, calculated as the absolute cytoplasmic volume of AVd, remained about the same over 48 h of starvation but fell more than 90% during refeeding (Table 2); the striking increase in fractional volumes of macroautophagic vacuoles in 48-h starved mice shown in Table 2 was thus the direct consequence of a reduction in hepatocyte volume. Absolute volumes of total DB diminished moderately over the course of starvation–refeeding (Table 2). Of more interest was

the fact that the type A particle (the putative marker for microautophagy) decreased to a greater extent than did the remainder of the particles (type R). This shift in the distribution of the two classes of particles suggests that microautophagy was selectively reduced. The electron-lucent zones in the type A DB did not become smaller (see Fig. 2) (Mortimore *et al.* 1983).

In predicting the quantities of protein sequestered by DB during starvation and refeeding, we considered two possibilities for computing the hypothetical protein space within the DB population. The first was the observed volume of DB itself. The second, termed DBp, was the volume of DB in control (fed) animals, multiplied by the relative volume of type A particles in each state. We reasoned that if type A lysosomes are valid markers for micro-autophagy, the hypothetical space should decrease in direct proportion to the fall in type A DB shown in Table 2. Calculated values of DBp are given in Fig. 7. They differ from the absolute volumes of DB in Table 2 only in starved and refed states where the numbers reflect the apparent shift from type A to type R.

Quantitative predictions of internalized protein, based on absolute volumes of DBp + AVd and estimates of cytoplasmic protein concentration proved to be remarkably similar to observed values of degradable, intralysosomal protein (Mortimore *et al.* 1983). (Note the values for 48 h starved, 12 h refed mice given in Table 2).

Having tested the predictability of internalized protein, we extended our calculations to proteolytic rates. For this we assumed that the turnover constant of

Table 2. *Lysosomal volumes in hepatocytes of fed, starved, and starved–refed mice*

(Mean values with their standard errors for three to five livers; values in parentheses are expressed as a percentage of control values. Fractional cytoplasmic volumes were converted to absolute volumes by multiplying them by the fractional parenchymal volumes of hepatocyte cytoplasm in livers fixed for electron microscopy and by corresponding parenchymal volumes. From Mortimore *et al.* (1983))

	Fed		Starved 48-h		Refed 12-h		Refed 24-h	
	Mean	SE	Mean	SE	Mean	SE	Mean	SE
Fractional volumes (ml $\times 10^{-3}$/ml cytoplasm)								
AVi + AVd	1·55	0·58	3·01	0·62	0·10*†††	0·09	0·13*††	0·07
Type A DB	1·18	0·26	0·98	0·27	0·22**†	0·03	0·30*	0·15
Type R DB	3·18	0·99	4·94	0·82	1·80‡	0·35	1·38‡	0·43
Absolute volumes (ml $\times 10^{-3}$/liver of 34 g mouse)								
AVd	0·839	(100)	0·927	(110)	0·060	(7)	0·075	(9)
Total DB	4·916	(100)	3·797	(77)	2·528	(51)	2·024	(41)
Type A DB	1·331	(100)	0·629	(47)	0·275	(21)	0·361	(27)
Type R DB	3·585	(100)	3·168	(88)	2·253	(63)	1·663	(46)

AVi, double-walled vacuoles; AVd, degradative vacuoles; DB, dense bodies.

Mean values were significantly different from those for fed mice: *$P<0.05$, **$P<0.01$.

Mean values were significantly different from those for starved mice: †$P<0.05$, ‡$P<0.02$, ††$P<0.01$, †††$P<0.005$.

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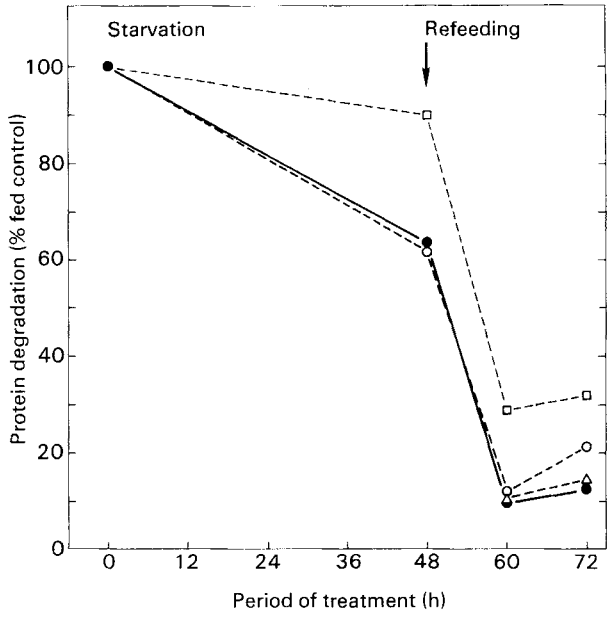

Fig. 7. Correlation between predicted and observed rates of hepatic protein degradation during starvation and refeeding. Observed rates were taken from Fig. 6*b*. Volumes of total dense bodies (DB) are given in Table 2; volumes of DB in control (fed) animals, multiplied by the relative volume of type A particles in each state (DBp) for fed, 48 h starved, and 12 and 24 h starved–refed mice were calculated to be: 4·916, 2·319, 1·016, and 1·334 ml × 10^{-3}/liver (34 g mouse) respectively (see p. 173). The observed control (fed) rate (100%) was 11·4 mg/h per 34 g mouse; corresponding predicted rates were 11·0 (maximum), 10·5 (average), and 10·0 (minimum). From Mortimore *et al.* (1983). (□), Degradative autophagic vacuoles (AVd) + total DB; (○) AVd + DBp; (△), AVd + DBp (based on protein in 48 h livers); (●), observed values.

the sequestered protein is the same in all autophagic components, 0·087/min or 5·22/h; the basis for this assumption was discussed earlier in connection with values in Fig. 5(*a*,*b*) and Table 1. Fig. 7 (and legend) show close relative and absolute agreement between observed rates of resident protein degradation and values predicted from AVd + DBp, estimates of cytoplasmic protein concentration, and the first-order rate-constant; the small discrepancy at 72 h has been discussed elsewhere (Mortimore *et al.* 1983). By contrast, substituting DB as an estimator of the microautophagic protein space gave predictions that deviated significantly upward from the observed results.

The fact that macroautophagic activity did not accelerate during starvation is in general agreement with observations of Pfeifer (1973) and can be attributed in part to the maintenance of relatively-normal plasma amino acid concentrations. Its sharp suppression with refeeding then would be the expected response to increased levels of insulin and amino acids. However, reasons for the decrease in microautophagy are not as apparent. Ahlberg *et al.* (1982) have observed that lysosomes in vitro continuously take up and degrade proteins from the medium, presumably by a process analogous to volume endocytosis. On the other hand,

Novikoff & Shin (1978) have called attention to the probable role of smooth endoplasmic reticulum (ER) in isolating small bits of cytoplasm. In support of this, we have encountered type A DB most frequently in areas filled with dilated, smooth ER. Because the quantity of smooth ER per cell decreases appreciably in starvation (Cardell, 1977), one might expect to see a progressive decline in microautophagic activity if indeed there is a connection between smooth ER and this process.

The continued decrease in microautophagic activity with refeeding is equally obscure. The most striking difference between refed animals and the other groups was an apparent redistribution of type A particles. They were not found at the periphery of the zones of glycogen as we typically see them, but instead were situated almost exclusively in areas that had the general appearance of cytoplasm in starvation, i.e. glycogen was sparse and the smooth ER vesiculated (Mortimore *et al.* 1983). It seems entirely possible that newly-synthesized cytoplasm, which is heavily overlaid with glycogen in livers of starved–refed animals, is transiently resistant to autophagic attack. If so, this would suggest that a large fraction of the total suppression of proteolysis during cytoplasmic regrowth is a consequence of the intracellular remodelling that directly follows the resynthesis of cytoplasmic components.

Conclusion

Livers of rats and mice can be perfused *in situ* with no noticeable impairment in their ability to synthesize and degrade intracellular proteins. This feature has made it possible to obtain information on the mechanism(s) of general protein turnover in vitro that can be applied directly to the interpretation of degradative events in vivo. Our findings indicate that cytoplasmic protein in hepatocytes is continuously internalized and degraded by two lysosomal-vacuolar processes, (a) macroautophagy and (b) a microautophagic function involving DB. The first is actively regulated by amino acids, insulin and glucagon; the second is also alterable, but responses are slow and apparently passive in nature as suggested from results in starvation and refeeding. The total quantity of protein sequestered in lysosomes under a variety of metabolic conditions in vitro and in vivo was assessed and quantitative agreement was obtained between two independent determinations. Agreement was equally close when the foregoing results were used to compare observed and predicted rates of hepatic protein breakdown over 95% of the full range. These findings indicate that sequestration is the initial and probable rate-limiting step in intracellular protein degradation both in the isolated, perfused liver in vitro and the liver in vivo.

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