*Review Letter*

# Intracellular protein catabolism: state of the art

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## 1. INTRODUCTION

There are several reasons why intracellular protein catabolism is incompletely understood. The most quoted are the facts that protein catabolism involves multiple mechanisms and does not easily occur in the test tube, at least, to a readily manipulatable degree (cf. protein synthesis). However, a much more fundamental reason is that the multiple systems are intimately connected with all aspects of the life process. Degradation is the alternative for proteins during or immediately after synthesis, during secretion and after reaching their cytomorphological functional sites: in short, protein catabolism (including degradation after protein endocytosis) is primarily a cell biological phenomenon. Extracting the molecular detail of the multiple processes requires multiple biological approaches, providing a literature rich in a mixture of experimental models, designs and interpretations.

In this review we want to consider critically the biological questions, the literature which addresses the questions and the current conundrums: the state of the art. We have taken the excellent 1982 review of Hershko and Ciechanover [1] as representative of knowledge and understanding in 1981 and have predominantly reviewed the literature from 1981 to 85. We have not considered degradative events after endocytosis since they are adequately addressed elsewhere [2]. Those people interested in the views of many workers in the field would do well to read the summaries of presentations at the Vth International Symposium on Protein Catabolism [3].

Hershko's analyses [1] saw intracellular protein catabolism as an extensive, selective set of energy(ATP)-requiring processes which have several functions including elimination of abnormal proteins and provision of amino acids in times of need. Non-lysosomal and lysosomal systems were identified which may act on 'short' and 'long' lived proteins respectively: the latter system being activated in a variety of cellular deprivation states. Finally and importantly, much of the earlier experimentation was seen as phenomenological rather than molecular in nature. The analyses did not consider: (i) the question of cellular architecture, i.e. proteins function in defined sites and yet must interact with the catabolic systems; (ii) that proteins are really degraded with the rate of heterogeneity previously supposed; (iii) the existence of 'special' systems, e.g. in heat shock or regulatory proteases responsive to calcium; (iv) degradation of proteins during secretion; (v) mitochondrial and chloroplast systems.

We will consider intracellular protein catabolism from the point of view of (section 2) the *systems:* (section 2.1) the lysosomal system, (2.2) the secretory degradative system (defined as secretion-coupled degradation), (2.3) non-lysosomal systems, (2.4) ATP-dependent systems in prokaryotes, eukaryotes and energy-transducing organelles, (2.5) calcium-dependent systems; and (section 3) the *substrates:* (3.1) how are the systems organised topographically with respect to

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substrates, (3.2) how selective are the systems, (3.3) how much heterogeneity really exists, (3.4) what are the protein molecular features which determine sequestration into a system and degradative rate.

## 2. THE SYSTEMS

### 2.1. *Lysosomes and degradation*

The basic question concerning these well-characterised organelles is the extent to which they participate in intracellular protein catabolism (cf. extra-lysosomal mechanisms) in each cell type. The studies have generally (but not exclusively) relied on inhibitors, which mostly incompletely inhibit lysosomal function and therefore may give somewhat problematical interpretation. However, initial studies [4–6] led to the so-called two-pathway hypothesis, which proposed that lysosomal and non-lysosomal systems could operate in each cell type. The extent of lysosomal involvement has been estimated to vary considerably depending upon cell type, growth characteristics of the cells and type of proteins studied [short-lived: radiolabelled for minutes (10–30), or long-lived: radiolabelled for hours (16–48)].

Studies in perfused liver have shown a major lysosomal role in protein catabolism, in both the so-called basal (steady state with respect to protein synthesis) and enhanced (e.g. by nutritional deprivation) conditions [7,8]. Restoration of liver protein content on refeeding starved mice is accompanied by an enormous (90%) decrease in degradative rate presumed to be caused by decreased autophagy [8]. A protein subgroup (16–18% of total) with a very short $t_{1/2}$ (10 min) is unaffected by nutritional manipulation in liver [8]. Studies with isolated lysosomes from liver have suggested that both short- and long-lived proteins are degraded by lysosomes with approx. 20% of short-lived proteins (cf. [18]) subjected to extra-lysosomal (cytosol) degradation [9]. These studies with isolated lysosomes illustrate the incompletely inhibitory effect of lysosomotropic agents (which generally increase lysosomal pH) and proteolytic inhibitors (even in combination) on the lysosomal degradation of proteins. The results serve to emphasise the fundamental problem of using incompletely effective inhibitors to study biological processes. The design of these experiments does not permit identification of the so-called short-lived liver proteins although the secretion-coupled degradation (see below) of neosynthesised proteins by a crinophagic mechanism or extensive degradation of neosynthesised membrane proteins [10] may explain the observed large lysosomal contribution to the degradation of short-lived proteins [9].

Long-lived proteins must have entered their cytomorphological functioning sites before degradation occurs. Lysosomal degradation of such proteins in liver cells is thought to occur by an autophagic process, i.e. cytoplasmic sequestration by an isolating membrane to form a closed vacuole (an autophagosome with a double membrane) followed by fusion with primary or secondary lysosomes (e.g. [11]) to form autophagolysosomes. The initial autophagic process, i.e. formation of autophagosomes, can be inhibited by methylaminopurines [12,13], the most potent of which (3-methyladenine) inhibits hepatic autophagy by at least 60% without affecting the degradation of exogenous proteins [14,15]. [$^{14}$C]Sucrose (indigestible in lysosomes) has been introduced into hepatocytes by electropermeabilization [16] to measure the autophagic rate which is therefore completely inhibited by 3-methyladenine. Interestingly, an unexplained sequestration of [$^{14}$C]sucrose by mitochondria occurs in this type of study which partially accounts for the overestimation of the autophagic rate by this approach. Even after correction for this event the estimated autophagic sequestration rate is approximately double the autophagic degradation rate, perhaps reflecting recycling of sequestered cytoplasm or some selectivity in the process [17]. Clearly, compounds like 3-methyladenine are of great value in distinguishing autophagic degradation from the degradation of proteins obligatorily inserted into intravesicular spaces during exocytosis (see below) and endocytosis.

The degradation of liver proteins has been probed with several other inhibitory or disruptive compounds. Leupeptin, an inhibitor of many proteases, including cathepsins B, H and L [18], causes the initial appearance (30–60 min) of double-membraned autophagosomes and subsequent (3–12 h) appearance of autophagolysosomes in liver after injection into rats [19]. Several

cytosolic enzymes are continuously sequestered non-selectively into autophagolysosomes after leupeptin treatment [20]. Leupeptin causes a marked change in lysosomal density presumably by lysosomal constipation [20]. It has been suggested that leupeptin may offer a good model for elucidating mechanisms related to the formation of autophagic vacuoles in liver [21]. Certainly, hepatocytes in vitro tolerate prolonged exposure to leupeptin which can therefore be used to show convincingly that mitochondrial outer membrane transplanted into hepatocytes accumulates in perinuclear non-lysosomal non-mitochondrial structures from which proteins are donated for degradation at precisely the same rates as from endogenous organelles in vivo, i.e. the proteins in these membranes preserve their degradation rates after deliberate miscompartmentalization [22,23].

The pharmacological disruption of cellular elements does not always lead to simple interpretation. Disruption of hepatocytic microtubular elements by vinblastine causes interference with the autophagic system, probably by preventing autophagosome-lysosome fusion, so that intracellular protein degradation is impaired [24], but the degradation of proteins in isolated lysosome preparations is enhanced [25,26] possibly by in vitro autophagosome-lysosome fusion [24]. Although the functional interaction of elements of the cytoskeleton (microtubules, microfilaments and intermediate filaments) is complex (see below), there is no doubt that properly functioning microtubules are essential for protein degradation in liver parenchymal cells, not only for putative autophagosome-lysosome fusion [24] but also for membrane translocation to perinuclear sites before degradation of incumbent proteins occurs [22,23] and lysosomal translocation to pericanalicular sites in liver parenchymal cells after partial hepatectomy [27].

Low lysosomal pH, which is disrupted by lysosomotropic weak bases (e.g. [28]), is clearly essential for lysosomal function including protein degradation. Lysosomal pH is maintained by an ATP-driven acidification system [29] which is dependent on an ATP-driven electrogenic proton pump [30–32] and is disrupted by carboxylic ionophores in hepatocytes with concomitant almost complete disruption of lysosomal protein degradation [33].

The predominantly lysosomally mediated protein catabolism in hepatocytes must be contrasted with mechanisms operating in other cell types, specifically in growing and non-growing tissue culture cells. Microinjection and other studies in growing tissue culture cells show that autophagy accounts for little of the basal degradation of proteins whereas autophagy can account for most of the enhanced degradation in quiescent (non-growing) cells [34–39].

Finally, the cytoskeletal dependence of the autophagic-lysosomal system must again be emphasised. Not only are microtubules essential for the process as defined by microtubule-dissociating agents (see above) but also lysosomes are reported to be associated specifically with microtubules not intermediate filaments [40] as defined by immunofluorescence studies. Lysosomal membranes are also reported to possess integral membrane proteins which bind actin [41] and may be involved in saltatory lysosomal movements.

After microinjection proteins can be segregated to become tightly associated with the vimentin-rich intermediate filament fraction in 3T3-L1 cells [42] before subsequent slow lysosomal degradation [36]. Although much work in recent years has identified and quantitated the extent of lysosomally mediated autophagy in different cell types, it has not determined how proteins enter the lysosomal system other than to describe morphologically the exploitation of smooth endoplasmic reticulum membranes in the initial events (e.g. [21]). A key unresolved question concerns whether cytosolic and membrane proteins accumulate at some 'nucleation' site (cytoskeletally directed?) before the autophagic processing begins [42].

The importance of organisation in intracellular space for normal protein degradation is well illustrated by studies of membrane transplantation [22,23] and cytochalasin B enucleation (microfilament disruption) [43–45]. In the latter, enucleation [46,47] or centrifugal steps used in enucleation [44] are sufficient to prevent the degradation of short-lived proteins [43,44] and short- and long-lived proteins [45]. Clearly, disruption of intracellular organisation and/or transcriptional events is essential for protein catabolism. These events deserve much further study.

2.2. *Secretion-coupled degradation*

The degradation of secretory proteins en route from the Golgi apparatus to the cell surface is a well-established phenomenon [50]. The phenomenon may be viewed in relation to precursor polypeptide processing, whereby for example in a secretory granule precathepsin B may process proinsulin and then itself be converted to cathepsin B during the later senescence of the insulin granule [51], presumably entering the general secondary lysosome compartment by vesicular fusion. Alternatively, a prelysosomal vesicle (containing cathepsin D) may fuse with procollagen containing secretory granules before secretion [52]. The proportion of collagen degraded is conformation-dependent [53,54]. Complete degradation may occur in the endoplasmic reticulum [1,55] or between Golgi and the cell surface by lysosomotropic-sensitive pathways [56]. The process is selective, partially degrading fibrinogen, but not transferrin during secretion in hepatocytes [57] and IgM $\mu_s$ chains and not $\mu_m$ chains in Daudi cells [58]. Predictably, glycosylation has a role in modulating degradation affecting lysosomal enzyme precursors and enzymes themselves in the endoplasmic reticulum and lysosome, respectively [55]. In mammary lobulo alveolar cells secretion-coupled degradation [56] is transcriptionally and cell-shape related [59], being inversely related to secretory rate (Mayer, unpublished). Secretion-coupled degradation offers a major post-translational route of controlling net protein secretion as well as precursor processing. Clearly, alternative routes may exist for lysosomal enzyme precursors or enzymes to enter secretory vesicles, e.g. direct packaging or vesicular fusion. Whatever the route, protein conjugate structure and dwell-time in the secretory labyrinth must determine the fate of the protein.

2.3. *Non-lysosomal degradation*

There are several aspects of this system which need consideration (see section 2.5) but direct evidence for the existence of non-lysosomal systems will be first evaluated (cf. indirect evidence for non-lysosomal systems based on lysosomotropic agents, see section 2.1). Microinjected $^{125}$I-labelled bovine serum albumin has provided the most clear-cut evidence for a cytosolic degradation system since its degradation ($t_{1/2}$ approx. 20 h) is largely unaffected by lysosomotropic agents [46,48], the majority of the degradation products of [$^{14}$C]sucrose-bovine serum albumin accumulate in the cytosol [47], its degradation rate is unaffected by serum withdrawal [48] and the protein remains in the digitonin-extractable cytosolic fraction throughout the course of degradation [42]. Exhaustive methylation of the protein does not prevent non-lysosomal degradation in HTC cells [49]. Therefore one or more cytosolic degradation systems exist in eukaryotic cells. It is worth emphasising here (see section 2.4) that prokaryotes obviously have no lysosomes and therefore must have extra-lysosomal degradative systems. In the course of evolution special adaptations of these systems may therefore be expected in eukaryotic cells.

2.4. *ATP-dependent extralysosome sytems*

2.4.1. Prokaryotic ATP-dependent protein systems

Following the discovery that abnormal proteins of *E. coli* were degraded in an ATP-dependent fashion [60] a soluble ATP-dependent protease was identified and termed protease La [61]. Mutant strains of *E. coli* known as lon (capR) mutants exhibit reduced proteolysis of nonsense [62], missense [63] and normal proteins [64] and the purified lon gene product was found to be a protease dependent on ATP hydrolysis [65]. Protease La and the lon gene product were found to have the same sensitivity to inhibitors, identical molecular masses and La was absent from lon$^-$ mutants suggesting that La and the lon gene product were the same protein [66].

Serine protease inhibitors were found to inhibit both proteolysis and ATP hydrolysis by La. The ATPase inhibitor vanadate also inhibited both activities suggesting that they occurred in a linked fashion [67]. Protein substrates stimulate ATP hydrolysis by La and proteolytic activity in the presence of ATP is stimulated by DNA [68].

Protease La has been implicated in the response of *E. coli* to various stress situations including heat shock and is one of 17 known proteins produced during the heat shock response [69–71], which is controlled by the htpR-encoded protein, a sigma factor required for transcription of heat shock genes [72]. The effect of various inducers of the

heat shock response on the transcription of the lon gene has been studied using a lon-lacZ operon fusion gene where increased transcription led to increased $\beta$-galactosidase activity. Amino acid analogues, puromycin and the presence in cells of multicopy plasmids carrying the gene for human plasminogen activator all served to increase lon transcription. Similarly, cells with decreased translational fidelity showed increased lon transcription [73]. It would seem therefore that the lon gene product-protease La is involved in the rapid degradation of abnormal proteins including cloned foreign gene products.

Protease La is also involved in the response of *E. coli* to ultraviolet irradiation. $Lon^-$ mutants showed decreased ability to recover from ultraviolet irradiation as cells failed to divide. The sulA gene product is produced after irradiation and is thought to inhibit cell septation. When sulA is cloned into $lon^-$ mutants the half-life of the product is much longer (19 min) than in $lon^+$ cells (1.2 min) [74]. Thus, protease La may be involved in the regulation of important cell processes by controlling the concentration of critical proteins. A similar role for a protease-like activity has been seen with recA and the SOS response in *E. coli* (see [75] for a review).

So far the ATP-dependent protease La has been implicated in the degradation of abnormal proteins in *E. coli* and its role, if any, in the degradation of normal proteins is not known.

2.4.2. Eukaryotic ATP-dependent degradation

The clearest example of non-lysosomal ATP-dependent proteolysis in eukaryotic systems has been found in the reticulocyte where the breakdown of endogenous protein was blocked when ATP synthesis was prevented [76]. This observation has led to extensive study of the ATP-dependent proteolytic system in the reticulocyte.

A cell-free system from rabbit reticulocytes capable of ATP-dependent proteolysis was established [77] which was clearly soluble and non-lysosomal. Fractionation of reticulocyte lysate yielded two fractions (I and II) both of which were required for ATP-dependent proteolysis [78,79]. The factor in fraction I required for proteolysis was found to be a heat-stable polypeptide of 9 kDa [78] and was subsequently found to be identical with ubiquitin, a polypeptide found in many species and tissues which can form conjugates with histone H2A [80]. Conjugation of ubiquitin with protein substrates is via the carboxyl glycine ([81]; for a review of this system up to 1983, see [82]) as with H2A and can lead to the formation of conjugates with ubiquitin attached to more than one lysine [83] per substrate molecule and with poly-ubiquitins attached to the same lysine [84]. It had been thought that the $\epsilon$-amino group of lysine in substrate proteins is the site of ubiquitin attachment but recent work suggests that the N-terminal amino group will also accept ubiquitin and may in fact be necessary for maximum ubiquitin-mediated proteolysis [85]. Conjugates of ubiquitin and $^{125}$I-labelled lysozyme are degraded by reticulocyte extracts free of ubiquitin-conjugating activity in an ATP-dependent fashion whereas $^{125}$I-labelled lysozyme is not, indicating that ubiquitin conjugates are the substrates for this ATP-dependent mechanism and that both conjugate formation and degradation are ATP-dependent [86]. The conjugation of ubiquitin to substrate proteins takes place via the formation of an adenylated ubiquitin catalysed by a ubiquitin-activating enzyme (E1) and subsequently the formation of an E1-ubiquitin thiol ester bond. The process results in the hydrolysis of ATP [87,88]. The transfer of ubiquitin to substrate proteins is catalyzed by enzyme E3 which transfers the ubiquitin moiety from another intermediate (E2-S-Ub) [89]. A total of 5 E2 enzymes have been found so far with at least 4 being capable of transferring ubiquitin to small amines, H2A and cytochrome *c* in the absence of E3 [90].

The involvement of ubiquitin in a putative cycle for the degradation of proteins requires the regeneration of ubiquitin from the products of proteolysis of ubiquitin-protein conjugates. Recently, an enzyme that will cleave small amines – including lysine – from the carboxyl terminus of ubiquitin has been discovered which would serve this purpose [91]. Removal of ATP from incubations of reticulocyte extracts containing ubiquitin-lysozyme conjugates prevents proteolysis of conjugates and results in the release of free ubiquitin, suggesting the presence of an isopeptidase similar to that found in rat liver [92]. The presence of these activities permits the proposition of the scheme shown in fig.1. However, to date almost nothing is known about the reticulocyte enzyme(s) actually responsible for the degradation of ubiquitin-protein complexes.

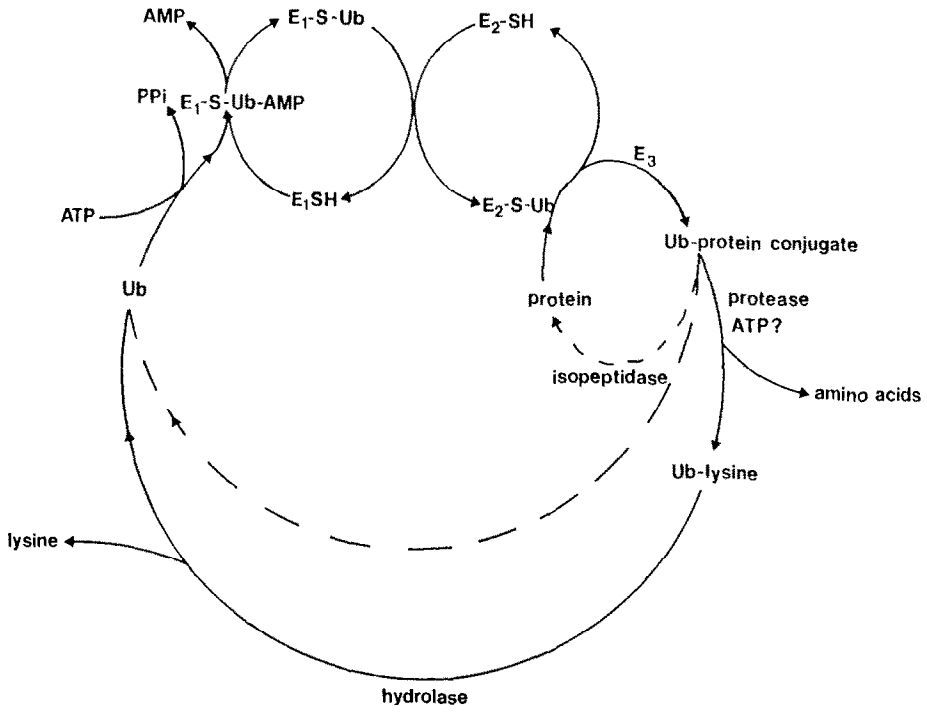


Fig.1. Proposed ubiquitin (Ub)-mediated proteolysis in reticulocytes (adapted from [82]).

An alternative explanation for the stimulatory effects of ATP and ubiquitin in proteolysis by reticulocyte extracts to the one described above has been put forward [93]. A proteolytic activity could be obtained from reticulocytes after removal of an ammonium sulphate precipitable fraction which exhibited rates of ATP-independent proteolysis similar to the complete system which includes both ATP and ubiquitin. Addition of the precipitated fraction led to an inhibition of proteolysis which was relieved by ATP and ubiquitin. It was suggested that reticulocytes contain an inhibitor (ammonium sulphate precipitable) which is inactivated in the presence of ATP and ubiquitin. This could occur through the formation of ubiquitin-inhibitor conjugates or ubiquitin-substrate conjugates which prevent inhibitor binding [3].

The same workers found proteolytic activity in erythrocytes which is masked by an ATP- and ubiquitin-repressible labile inhibitor.

2.4.2.1. *Physiological role of the ubiquitin-ATP proteolytic system.* While a great deal has been learned about the ubiquitin-mediated proteolytic system in reticulocytes, its physiological role remains unclear in both reticulocytes and other cells. Most investigators studying the system have employed exogenous protein substrates, e.g. bovine serum albumin (BSA), lysozyme, casein. However, it has been found that while BSA radioiodinated using chloramine-T is a good substrate for the reticulocyte system, enzymically iodinated BSA is a poor substrate [94] suggesting that oxidative damage makes BSA more susceptible to proteolysis by this system. However, the effect of denaturation on the proteolysis of BSA by this system is not clear [95,96]. Reticulocytes incubated with amino-acid analogues show greater formation of protein-ubiquitin conjugates, suggesting that abnormally synthesized proteins may be more rapidly degraded by this system [97]. It is certainly the case that a large range of normal intracellular proteins are not readily degraded by the reticulocyte system [98].

The demonstration that the mitochondria containing stroma of reticulocytes is rapidly degraded by the reticulocyte-ubiquitin system has led some authors to propose that the system is involved in

the maturational loss of reticulocyte organelles [99]. In support of this it has been found that the ATP-dependent ubiquitin system is almost completely lost in mature erythrocytes [100]. Similarly, the capacity of reticulocytes to degrade puromycin peptides and analogue-containing proteins is also lost [108]. Erythroid cells from donors with $\beta$-thalassaemia showed rapid degradation of the excess $\alpha$-chains of haemoglobin which was ATP-dependent but it is not known if ubiquitin is involved [101]. It would seem therefore that several possibilities for a cell-specific role for the ubiquitin system in reticulocytes may exist. It must be emphasised at this point that cyanogen bromide peptides are degraded in reticulocyte lysates by an ATP-independent system: these substrates may be the best model substrates to study this system [107].

2.4.2.2. *Other cell types.* If the ubiquitin-ATP system is concerned with reticulocyte-specific processes then it may not be active in other cell types. In fact it has proven difficult to demonstrate ubiquitin-mediated proteolysis in other cell types until recently. It appears that there exists in rabbit liver a ubiquitin-degrading activity and only when this is inhibited can ubiquitin-mediated proteolysis be demonstrated [102].

In an attempt to demonstrate ubiquitin proteolysis in intact cells microinjection techniques have been employed. $^{125}$I-labelled ubiquitin when microinjected into HeLa cells was found to conjugate mainly with histone H2A and few other proteins [103]. Treatment of erythrocytes, into which $^{125}$I-labelled ubiquitin had been loaded with phenylhydrazine to denature the haemoglobin, led to much greater conjugate formation in recipient cells [104]. However, this does not tell us if ubiquitin conjugates are formed with recipient cell proteins. Microinjection has also been employed to introduce labelled substrate proteins into culture cells. BSA and lysozyme were degraded rapidly after microinjection into HeLa cells in a lysosome-independent and ATP-dependent fashion [49]. However, when the substrates were exhaustively methylated to block free amino groups the degradation of the proteins was unaltered after microinjection but was drastically reduced when these proteins were exposed to the reticulocyte system. These experiments seem to demonstrate the presence of an ATP-dependent non-lysosomal proteolytic system in cultured cells but do not seem to show conclusively that ubiquitin is involved.

An alternative approach has been to develop cell mutants lacking ubiquitin-conjugating activity which fail to conjugate ubiquitin to histone H2A [105]. These cells are unable to degrade proteins that have been labelled with radioactive methionine for very short times, i.e. newly synthesized proteins, in both the presence and absence of amino-acid analogues [106]. No data were shown for any effect on the turnover of proteins labelled for longer periods. It may be that the system is active only on newly synthesized proteins which are in fact subject to extensive rapid hydrolysis [116,167]. Interestingly, it has been shown that protein turnover in both reticulocyte lysates and CHO cells is sensitive to the level of charged tRNA which suggests a link between the protein-synthesizing machinery of the cell and ubiquitin-mediated proteolysis [168,169]. Finally, it has been noted that heat shock induces the synthesis of ubiquitin mRNA in chick fibroblasts, which may serve to allow the degradation of increased amounts of abnormal protein [170].

2.4.3. Energy-transducing organelles

ATP-dependent proteolytic systems have been demonstrated in both mitochondria [110–113] and chloroplasts [114–116]. In mitochondria an ATP-dependent matrix enzyme may be essential for ensuring the balance between products of nuclear and mitochondrial genomes [113,117] by degrading excess polypeptides. The role of the ATP-dependent enzyme on the outside of the inner mitochondrial membrane is less clear [110,111]. In chloroplasts proteolytic systems degrade the imported small subunit of ribulose-1,5-bisphosphate carboxylase [114] when the large subunit is depleted. Newly synthesised proteins are degraded by an ATP-dependent proteolytic system [116]. Chloroplast degradation is also light-sensitive and can be coupled to electron transport rather than phosphorylation [115]. Although a proteolytic role in controlling assembly of products of the two genomes is important and attractive, there is as yet no real substance for understanding the degradation of individual proteins in these supramacromolecular structures (see section 3).

2.5. *Calcium-dependent proteolytic systems*

Calcium-dependent proteases and their inhibitors have been discovered in a variety of cell types [118–123]. The calcium sensitivity of many acute intracellular regulatory processes may suggest regulatory roles for calcium-dependent proteases. The currently most intriguing systems are proposed to relate to the cytoskeleton. A calcium-activated neutral protease was isolated from Ehrlich cells with a high but limited proteolytic activity for the N-termini of vimentin, desmin and a 60 kDa cytokeratin [124,125]. The protease was found to be ubiquitous in nature and its action prevents formation of intermediate filaments [125]. It was originally proposed that the calcium-activated protease was involved in vimentin turnover [124] but intriguingly vimentin may turn over very slowly, if at all, in Ehrlich cells [126]. In astrocytes, filaments containing vimentin and glial fibrillary acidic protein are degraded into fragments by a calcium-dependent proteolytic system giving rise to some detergent-soluble fragments as well as insoluble aggregates [125,127] which may be disulphide-linked. Several related calcium-dependent proteases can be purified from brain which may mediate cytoskeletal protein breakdown [128]. Neurofilament breakdown into detergent-insoluble fragments can be mediated by the calcium-dependent protease [129]. The occurrence of aggregates [125,127,128] after partial degradation of cytoskeletal elements may be important for cataract development in lens, which contains a vimentin-specific calcium-activated protease as well as high-molecular-mass aggregates containing crystallins, actin and vimentin [130]. From a putative regulatory viewpoint it is fascinating that calcium-activated proteases purify with associated cAMP-dependent and -independent protein kinases which may involve interactions with inhibitors of the proteases [131].

These fascinating studies need to be accompanied by definitive proof that intracellular cytoskeletal fragmentation is dependent on calcium-activated proteases. However, in view of the tight association of some microinjected proteins with the vimentin intermediate filaments of fibroblasts [42] before lysosomal degradation, it is likely that intermediate filaments in each cell type might act as nucleation sites for proteins destined to be degraded lysosomally after partial calcium-dependent filament fragmentation and aggregation.

The binding of haemoglobin to the cytosolic face of the erythrocyte membrane (membrane cytoskeleton related?) appears necessary for procalpain activation and regulated degradation [109].

Finally, a word on calcium and muscle. Preliminary results suggested that calcium-stimulated muscle protein catabolism may involve prostaglandins and be mediated lysosomally [132,133]. However, recently [134] it has been shown that non-lysosomal leupeptin and E-64-sensitive proteases are responsible for calcium-dependent proteolysis in muscle. Since calcium may also control autophagy in hepatocytes [135] much further work needs to be carried out to clarify the role of calcium in these processes.

## 3. THE SUBSTRATES

3.1. *Proteins in functional topographic sites*

Intracellular protein catabolism obviously needs not only the systems but also the substrates. Full understanding of protein catabolism in eukaryotic cells means that the routing of individual proteins from functional sites to degradative sites will need to be understood. Attention must be paid in all models to the fact that proteins function in sites as diverse as nucleus and plasma membrane and yet must gain access to the proteolytic systems. Non-selective autophagy will not suffice for nuclear protein catabolism or differential turnover of plasma membrane receptors and membrane cytoskeleton. This concept is enshrined in the view of a protein turnover cycle in eukaryotic cells whereby proteins not only enter their functioning sites during organelle biogenesis but exit for the retrograde destructive arm of the cycle [136]. Impedance of either the orthrograde or retrograde arms of such a cycle would result in cellular inclusions (see section 2.5) including, e.g. neurofibrillary tangles in Alzheimer's disease, which could occur in specific neurones with concomitant memory malfunction [137].

Equally important for the overall view is recognition that intracellular proteins function in topographic sites equivalent to the cytosolic space and lysosomal lumen, e.g. in secretion-coupled degradation. It is clear that proteins in lysosomal lumenal equivalents are lumenally degraded

whereas proteins enjoying cytosolic topographical location may be degraded cytosolically [42,46–48] or lysosomally [34–36,42,46–48]. The latter route begs the unresolved question of how proteins gain access to the lysosomal lumen, e.g. by a microautophagic route [138].

### 3.2. *Selectivity and heterogeneity*

Attitudes to the topographical question rely on an appreciation of not only the number of degradative systems (see section 2) but also the selectivity of the systems which determines the heterogeneity of degradation rates. Current opinion would agree that proteins with extremely fast degradation ($t_{1/2} < 15$ min) (e.g. [8]) exist (related to the infidelity and abnormality of protein synthesis) and proteins, often regulatory enzymes, with fast degradation rates (e.g. [140–142]) clearly exist. The degree of heterogeneity of degradation of all other intracellular proteins may logically necessitate the determination of rates for 50–100000 proteins per cell. In earlier reviews [143–145] published degradation rates, often determined by imprecise methodology, were quoted as supporting a total heterogeneity of degradation rates. However, two-dimensional isoelectric focussing – SDS-polyacrylamide gel electrophoretic analyses of proteins in liver mitochondrial subcompartments [146] and mammary cytosol [147] – indicate that proteins in biosynthetically defined cytomorphological sites are degraded in small numbers of families or populations (e.g. <4) where the rates in each population are similar if not identical. This view is supported by rate frequency analysis of degradation rates of proteins in hepatoma plasma membrane [148] and mitochondria [149,150]. It is also supported by the fact that proteins in transplanted mitochondrial outer membrane are degraded at strict alternative rates in a variety of tissue culture cells [139,151] and not with a great heterogeneity of rates. Therefore, a strictly limited degree of heterogeneity of degradation rates may be found in each cell type for long-lived proteins, simplifying the currently incompletely understood problem of selectivity considerably.

### 3.3. *Protein structure and degradation*

A preoccupation of researchers in the late sixties and seventies was to establish the so-called correlations between protein structure and degradation rate. These correlations purported to show that proteins were degraded faster because they were larger, more acidic or more hydrophobic. These correlations were claimed for proteins in many subcellular compartments, including the cytosol [152–157]. Correlation of such general physicochemical properties with specific individual degradation rates seems inherently unlikely. Subsequently, many reports contradicted the correlations for all subcellular fractions [147,148,158–161] and also refuted protein unfolding or denaturation as indicators of degradation rate [162,163]. Indeed, microinjection studies have shown that removal of a short (20 amino acid) N-terminal portion from a protein can prevent lysosomal degradation on serum withdrawal [35]. Furthermore, the specificity of the sensitive sterol control of HMG-CoA reductase degradation in endoplasmic reticulum [140] is abolished with the N-terminal truncated product of a truncated transfected reductase gene [141,142], again showing the exquisite nature of the signal sequences for protein degradation. Finally, in a recent report on $\alpha$- and $\beta$-spectrin precursor degradation it was noticed that gross physical criteria are too general to account for the high degree of specificity displayed by two proteolytic mechanisms (lysosomal and non-lysosomal) which distinguish these two closely related precursor polypeptides [164].

Protein modification as a signal for degradation (cf. the ubiquitin system) has been a recurring concept in trying to unravel the intricacies of protein degradation. A popular hypothesis involves oxidative modification [165] or conjugation with biological disulphides [166]. Perhaps readers of this review will feel that the earlier correlations should be laid to rest so that the elegant new cellular and molecular biological techniques [22,142,143] can be used to resolve the macromolecular recognition features which determine degradation rate.

## 4. THE CURRENT STATE OF THE ART

Fig.2 attempts to rationalize available data for eukaryotes as a protein turnover cycle [136] in which both the synthesis and degradation of proteins can be accommodated. The orthograde arm

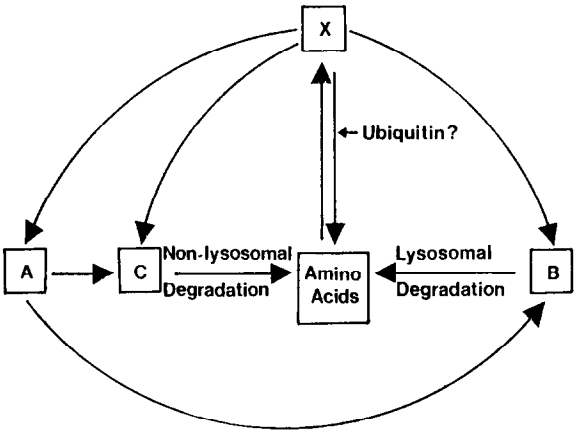


Fig.2. Protein turnover cycle. X, newly synthesized protein; A, protein functional site; C, cytosol; B, sequestration site for autophagic pathway.

of the cycle involves translocation of proteins either to functional organellar sites (A) or cytosolic spaces (C) from a pool of newly synthesized proteins (X). The retrograde processes are multiple with newly synthesized proteins (including abnormal proteins) produced on free polysomes subject to a degree of rapid ATP-dependent (ubiquitin?) degradation and those produced on membrane-bound polysomes subject to topographically distinct rapid lumnal (endoplasmic reticulum or lysosomal) degradation. Proteins may leave their functional sites (A), e.g. the nucleus (Fernig and Mayer, unpublished), for degradation by cytosolic or lysosomal mechanisms at much slower rates. Transfer to lysosomes involves collection at some site (B) for donation into the autophagic system. In prokaryotes and energy-transducing organelles the amino acid→X, X→C, C→amino acids steps are presumably operative. Each mechanism may well be ATP-dependent. The model cannot be exclusive and new data from the many biological approaches reviewed here will determine if it stands the test of time.

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