EXPERIENTIA

Volume 41/No. 9 Pages 1089–1230 15 September 1985

Reviews

Autophagy and lysosomal proteolysis in the liver

by B. Grinde

University of Oslo, Department of Biology, Division of Zoology, P. O. Box 1050, Blindern, 0316 Oslo 3 (Norway)

Summary. Autophagy is defined as any process whereby cellular macromolecules destined for degradation gain access to the lysosomes. A review is presented on the physiological significance, mechanisms and regulation of autophagy in hepatocytes, concentrating on the issue of regulation. The article ends by discussing techniques available for future research.
Key words. Autophagy; hepatocytes; protein degradation; lysosomes.

Introduction

Autophagy may be broadly defined as any process whereby cellular macromolecules destined for degradation gain access to the interior of lysosomes. In the classical theory of autophagy, specialized membrane structures envelope intracellular components. The vacuoles thus formed, called autophagosomes, contain few if any hydrolytic enzymes. The autophagosomes subsequently fuse with primary lysosomes giving rise to autolysosomes, a type of secondary lysosome. Enzymes contained in the lysosome facilitate the breakdown of internalized macromolecules into their subunits (e.g. proteins to amino acids). The subunits escape back to the cytosol where they may be reutilized.

As will become clear, macromolecules en route to degradation may reach the lysosomes by other mechanisms as well. Any vacuole containing macromolecules en route to degradation will be referred to as autophagic vacuoles. The term comprises autophagosomes and any secondary lysosome carrying endogenous (i.e. cellular) macromolecules (excluding secondary lysosomes containing exogenous macromolecules).

All nucleated cells are believed to have lysosomes, that is specialized vacuoles serving the purpose of macromolecule degradation by carrying a variety of hydrolytic enzymes. It seems reasonable to assume that autophagy occurs in most eucaryotic cells, but in some cells (under certain conditions) the process is much more dramatic than in others.

Among mammalian tissues the liver is, for two reasons, particularly well-suited for the investigation of autophagy. First, the technique of collagenase perfusion facilitates the preparation of a large number of rather homogeneous liver parenchymal cells. Second, the liver has an unusual ability to effect rapid and drastic variations in the rate of autophagy.

Much work has recently been carried out to elucidate the mechanism and regulation of autophagic processes in the liver (most experiments being done with liver from rats, either as a perfused organ or as isolated cells). This review concentrates on rat liver, since autophagy is probably best understood in this tissue. Where nothing else is indicated, the data referred to have been obtained from liver preparations, or the type of system employed is considered unimportant in the present context. Most biochemical work on autophagy is related to proteolysis.

The references cited are not meant to be exhaustive, and are chosen to provide convenient leads to current literature rather than to indicate priority. The following review articles carry additional relevant information: on proteolysis[29, 32, 47, 48], on lysosomes[11, 45, 65] and on autophagy[17].

Physiological significance

Supplying substrates

The lysosomes are the only cellular compartment with sufficient enzymes for the complete degradation of all classes of macromolecules. The function of secluding these enzymes inside a vacuole must be to regulate their activity, and create favorable conditions for hydrolytic reactions.

For many cell types, including liver cells, lysosomal activity depends very much on nutritional status[2, 29, 38, 48]. Under 'basal' conditions, i.e. when the cells receive sufficient nutrition, the degradation of proteins, and probably other macromolecules as well, is maintained at a low rate and occurs partly outside the lysosomes. If the cells are transferred to 'step-down' conditions, either by removing amino acids/serum from cells in culture, or by starving the animal, an increase in the degradation is noted. This activated degradation is lysosomal and is believed to be

due to a rather indiscriminate sequestration of cytoplasm.
Supposedly, the purpose of this increase in lysosomal activity is to secure that the cell or organism possesses a mimimal amount of organic substrates (e.g. amino acids).
The liver is involved in regulating the concentration of nutrients in the blood. This may explain why hepatocytes react particularly drastically to nutritional step-down; the rate of proteolysis is reported to vary from nearly 0 to up to 5%/h[36, 47, 69]. At the maximal rate at least 70% of the degradation occurs inside the lysosomes[69].

Regulating the concentration of macromolecules.
It is believed that some degradation occurs in the lysosomes even under conditions where the cells are supposedly receiving sufficient external nutrition[50]. Apparently lysosomes are involved in the down-regulation of greater than optimal concentrations of macromolecules and organelles[17, 52].
Although much of the selective degradation of proteins may take place outside the lysosomes[7, 29, 37], there are probably mechanisms whereby macromolecules are selectively brought into the lysosomes, e.g. protein may interact with the sequestering membrane[9, 17, 75]. There is also evidence indicating that whole organelles can be sequestered discriminately[46, 52].

Other functions
Lysosomes are possibly involved in regulating the secretion of macromolecules. In a process termed *crinophagy*, secretory vesicles fuse with lysosomes, resulting in the degradation of their contents[6]. The process may be considered as a form of autophagy.
For cells to divide and tissue to grow, a positive balance between macromolecule synthesis and degradation is required, implying that the rate of degradation influences tissue development. Preneoplastic hepatocytes have a reduced lysosomal proteolysis, indicating that their higher than normal growth rate is connected with a change in the regulation of autophagy[72].

Mechanisms

Origin of the sequestering membrane
Membranes involved in the engulfing of cytoplasm must necessarily have special properties, most probably mediated by specific membrane proteins. From where these membranes originate is a question which has been debated. Areas of the endoplasmatic reticulum or the Golgi region could be directly involved, or the sequestering membranes may be vacuoles budded off from either of these organelles[17].
Lysosomal enzymes are synthesized on the rough endoplasmatic reticulum, and subsequently transported to the smooth endoplasmatic reticulum, and most probably further to the Golgi region before they undergo the final processing and are packed into lysosomes[45]. Autophagosomes derived from these structures may carry some hydrolytic enzymes. It is assumed, however, that for optimal conditions of degradation to occur, autophagosomes need to fuse with lysosomes.
For individual autophagic vacuoles to expand and later to diminish again, it is necessary to envision a traffic of membrane material. A hypothesis by Dean[10] is that membrane components are transported as tiny vesicles.

The internalization of macromolecules
In 'classical' autophagy, the engulfment of cytoplasm gives rise to autophagosomes; vacuoles, supposedly with lysosomal enzymes, that are easily recognized in the electron microscope.
Lysosomes are probably involved in the small-scale engulfment of cytosol, a process referred to as microautophagy[17]. Mortimore et al.[50] have implicated dense bodies (presumably a form of primary lysosomes) in the process of microautophagy. It should be mentioned that both primary and secondary lysosomes may participate in large-scale sequestration as well (lysosomal autophagy).
Macromolecules belonging to the plasma membrane can reach the lysosomes by the invagination of this membrane to form endosomes, vacuoles that subsequently fuse with lysosomes. This process is normally connected with endocytosis, and does not necessarily imply the degradation of plasma membrane components, since many components are known to be recycled[76]. Studies on the turnover of proteins belonging to the plasma membrane suggest, however, that the formation of endosomes is a major mechanism for the turnover of these proteins[14]. Conceivably some endosomes could be formed, not for the purpose of endocytosis, but for the purpose of plasma membrane turnover.
Similarly, while crinophagy has been connected with the regulation of secretion, the fusion of vesicles deriving from the Golgi or endoplasmatic reticulum with lysosomes could play a role in the turnover of proteins belonging to these structures.
Whichever the pathway, uptake is most probably the rate-limiting step in the lysosomal degradation of macromolecules. The short half-life of autophagic vacuoles, approximately 10 min[48, 52], and the close correlation between changes in proteolysis and the number of autophagic vacuoles[43, 64] are indications for this.

Factors required
The most obvious requirement for degradation is the presence of *hydrolytic enzymes*. The role of these enzymes can be studied with inhibitors. More or less specific inhibitors of the main lysosomal proteinases have been developed, some of them acting selectively on lysosomal proteolysis[23, 65, 77], some apparently on both lysosomal and nonlysosomal proteolysis[26]. Inhibitors of thiol proteinases are particularly effective in blocking lysosomal proteolysis.
Most lysosomal enzymes are optimally active under acidic conditions[5]. The elevated H^+*-concentration* inside the lysosomes is sustained partly by a Donnan equilibrium, i.e. a high concentration of negatively-charged macromolecules inside the lysosomes causes a retention of positive ions[11]. An ATP-driven proton pump in the lysosomal membrane most probably decreases the pH beyond the Donnan equilibrium, down to about pH 5[51]. Experiments with lysosomotropic weak bases[73], and ionophores that cause the breakdown of pH-gradients[19],

indicate that a low pH is indeed necessary for degradation to take place in the lysosomes.

Lysosomal degradation requires *energy*, presumably in the form of ATP[33,69]. Energy is probably utilized in two processes: the sustaining of a low pH[62], and the mechanisms leading to the internalization of cytoplasm into lysosomes[61]. Once inside the lysosomes, protein seems to be degraded without the expenditure of further ATP[1,28].

It has been debated to what extent autophagy requires an on-going *protein synthesis*[2]. Inhibitors of protein synthesis such as cycloheximide tend to diminish lysosomal activity relatively rapidly[42]. As amino acids are known to inhibit lysosomal degradation[64], a likely explanation of the short-term effect of cycloheximide is that a block in protein synthesis causes an accumulation of amino acids. It is reasonable to assume that lysosomal activity in hepatocytes may be sustained for some time without requiring newly-synthesized protein. Upon the removal of amino acids a maximal autophagic response is observed within 20–30 min[43,48]. Such a short lag-period further indicates that the cells contain reserves of the macromolecules necessary for autophagy to occur.

Autophagy can hardly be envisioned as going on without organizing filaments. Three types of cytoskeletal proteins have been suggested to be involved in the formation of autophagic vacuoles:

Microtubuli are probably required for the processing of autophagosomes, i.e. their fusion with lysosomes, without being involved in the formation of autophagosomes[44].

Microfilaments are investigated using the inhibitor cytochalasin B. In some types of cells this drug seems to influence autophagy, in others not[3,31]. Cytochalasin did not appreciably decrease the degradation of endogenous protein in hepatocytes (Grinde, unpublished data), but it did inhibit the degradation of endocytosed protein[40].

Intermediate filaments are represented in the hepatocytes as keratins, and are, by the process of elimination, possible candidates for organizing filaments responsible for autophagic sequestration. However, a lack of suitable inhibitors makes the evaluation of their involvement difficult. Vanadate has been reported to interfere with intermediate filaments in baby hamster kidney cells[80], which could help to explain vanadate's strong inhibition of lysosomal proteolysis in hepatocytes, although other explanations for its effect have been suggested[65,66].

Autophagy probably requires a minimal concentration of cytosolic Ca^{2+} [20], but this does not imply that Ca^{2+} is involved in the regulation of autophagy. In muscle cells an elevation of cytosolic Ca^{2+} is believed to induce prostaglandin synthesis, which again stimulates lysosomal proteolysis[58]. Prostaglandins E_2 and $F_{2\alpha}$, however, have no appreciable effect on proteolysis in hepatocytes (Grinde, unpublished data).

The various mechanisms of autophagy and possible factors required are outlined in figure 1.

Regulation

What is written about the physiological significance and mechanism of autophagy in the liver, is to a great extent relevant for other types of cells as well. When it comes to the regulation of autophagy, the data indicate more variation.

There are at least three possible ways whereby cells can influence which and how many macromolecules are to be degraded in the lysosomes:

One, cytosolic modifications of proteins, including limited proteolysis, may regulate their chance of being sequestered[7,16]. Very little is known about such processes and to what extent they influence autophagy. It is possible to recover the activity of certain cytosolic enzymes in lysosomes isolated from cells given inhibitors of lysosomal proteinases[28,41], indicating that these enzymes have not been drastically changed prior to sequestration.

Two, coarse modulation of lysosomal activity may be brought about by changing the concentrations of components necessary for the autophagic machinery (e.g. hydrolytic enzymes, relevant membrane proteins and contractile elements)[4,45]. This would be a relatively slow regulation.

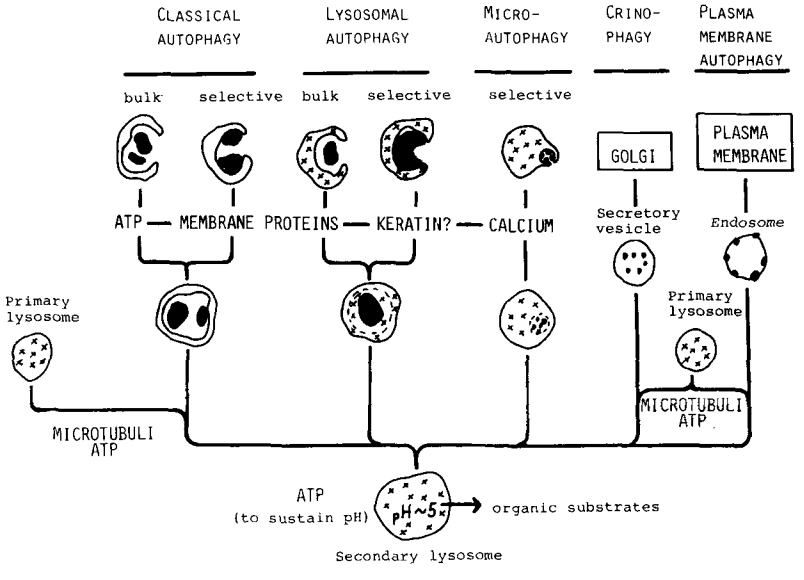

Figure 1. Mechanisms of autophagy and required factors. Autophagy is in this article defined as any mechanism whereby cellular macromolecules destined for degradation reach the interior of lysosomes. Postulated mechanisms and factors involved in liver autophagy are depicted schematically. × indicates hydrolytic enzymes.

Three, supposedly independent of the previous two mechanisms, the hepatocytes are capable of rapid and drastic variations in bulk autophagic sequestration[43,50]. The regulation of this rapid change will be discussed in more detail.

Hormones

Various hormones, depending on cell type, have been implicated in adjusting the level of autophagy[32,45]. In the liver, glucagon causes a stimulation and insulin an inhibition[34,49]. However, glucagon enhances the metabolic utilization of some amino acids[63], and insulin stimulates the uptake of some amino acids[53]. This indicates that both hormones influence autophagy by changing the intracellular amino acid pools.

The in vivo stimulation or inhibition of liver autophagy observed when the animal is starved or refed could be due either to these hormones or to changes in the blood levels of amino acids. In a recent report Mortimore et al.[50] indicate that macroautophagy is more readily turned on or off than microautophagy.

Glucocorticoids have been reported to stimulate hepatic proteolysis, but it is suggested that this effect is due to an increase in protein synthesis, e.g. in the synthesis of membrane proteins required for autophagy[35]. Similarly, thyroxine-induced protein degradation in liver may be due to an increase in the concentration of lysosomal enzymes[12].

Amino acids

A mixture of amino acids rapidly blocks most of the lysosomal degradation of endogenous proteins, without affecting the (lysosomal) degradation of a protein (asialofetuin) taken up by endocytosis[48,70]. Morphometric examinations of electron micrographs reveal a concomitant reduction in the number and volume of autophagic vacuoles[43,64]. Amino acids are assumed to be physiological regulators, which implies a feed-back mechanism: amino acids released by the lysosomes decrease lysosomal activity. It should be noted that the amino acid concentrations employed in these investigations (on in vitro systems) tend to be much higher than normal plasma concentrations.

Maximal inhibition is achieved with a complete mixture, but certain amino acids have an appreciable effect alone, notably: leucine, tryptophan, histidine, asparagine, glutamine, phenylalanine, tyrosine and methionine[24,56,57,70,74]. The effect of phenylalanine is probably due to its conversion to tyrosine[56].

Recently a technique has been developed for measuring the sequestration process biochemically[18]. Sucrose does not penetrate biological membranes and this molecule, labeled with ^{14}C, is therefore employed as a fluid-phase marker. It is introduced into the cytosol by electroshock 'injection'; i.e. by subjecting the cells to intense electric fields of brief duration, it is possible to create either reversible or permanent holes in the plasma membrane[18,82]. The subsequent transfer of sucrose to sedimentable intracellular vesicles is taken as an estimate of sequestration.

Additional information can be achieved by injecting lactose instead of sucrose. Lactose is hydrolyzed by lysosomal enzymes, and the monosaccharides thus formed escape from the lysosomes. Agents that block the fusion of autophagosomes with lysosomes will cause the accumulation of sedimentable lactose; agents that block the process of sequestration will decrease the accumulation of sedimentable sucrose. Some caution is needed when considering results obtained by this method. The rate of sequestration found is two to three times as high as the calculated rate of lysosomal proteolysis[72], and half of the sequestered sucrose appears in the mitochondria, not in autophagic vacuoles[79].

The method has been employed to study the effect of amino acids[68,72]. A complete mixture causes a 50% inhibition of sucrose sequestration, while among the individual amino acids tested, histidine is most active.

Leucine may play a special role among the amino acids. Alone, its inhibition of proteolysis and sucrose sequestration is not so substantial, in the range 15–25%, but in both cases it seems to be the one amino acid most indispensable for a maximal effect[71,72].

Recently Pøsø and Mortimore[55] have demonstrated the involvement of alanine. This amino acid is not inhibitory alone; however, when it is removed from a complete amino acid mixture at physiological concentrations, the effect of the mixture is drastically reduced. Alanine may act as a coregulator, possibly as a component of a regulatory link between energy demands and protein degradation.

At least some lysosomal proteolysis is probably not regulated by amino acids[42,48]. Seglen et al.[72] have suggested a lysosomal pathway for the degradation of short-lived proteins; a pathway that is not inhibited by an amino acid mixture, while it is sensitive to high concentrations of asparagine.

It seems reasonable to assume that amino acids influence lysosomal activity via several mechanisms. Knowledge of these mechanisms is still sparse.

Beyond amino acids

It is a reasonable hypothesis that amino acids act by influencing the concentration of other regulatory factors in the cell. This could help to explain why the effect of amino acids tends to vary considerably between experiments, and why relatively high concentrations are required to achieve optimal inhibition.

One possibility is that regulation involves metabolites of the amino acids. In muscle tissues only the branched chain amino acids, particularly leucine, are active in decreasing proteolysis, and apparently leucine acts via its transamination product 2-oxoisocaproate[8].

Most amino acids tend to be metabolized by way of transamination. The transamination inhibitor aminooxyacetate does not, however, influence the effect of amino acids on hepatic proteolysis[53], and it seems clear that leucine does not act via 2-oxoisocaproate in hepatocytes[25,56].

While transamination products are probably not involved in the regulation of liver autophagy, the tryptophan metabolite kynurenine might be[22]. This substance selectively inhibits the lysosomal degradation of cellular protein by approximately 40%. An electron microscopic examination of kynurenine-treated cells revealed a drastic reduction in secondary lysosomes, but only an insig-

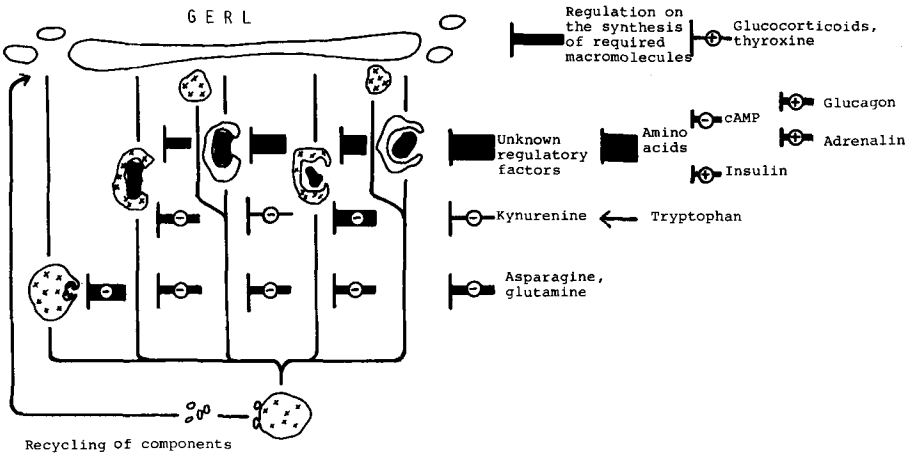

Figure 2. Regulation of autophagy. A proposed scheme for the regulation of liver autophagy is depicted. The thickness of the bars indicates a tentative evaluation of the importance of the regulatory influence. The regulation of crinophagy and plasma membrane autophagy may be independent of the suggested regulators, and is not shown. GERL stands for Golgi/endoplasmatic reticulum/lysosome-structures.

nificant decrease in autophagosomes. An effect on the processing of autophagosomes could be envisaged. However, secondary lysosomes may be formed by lysosomal autophagy as well as via classical autophagy. The action of kynurenine might be explained by a selective inhibition of the lysosomal type of sequestration.

The cells possibly contain one or several ultimate regulators of autophagy; substances which interact specifically with the involved membranes or contractile elements, and either stimulate or block the process. Conceivably, such effectors could be similar in many cell types, whereas stimuli leading to their production or destruction would be more tissue-specific.

3-Methyladenine blocks most lysosomal proteolysis, decreases the level of autophagic vacuoles even more than amino acids, and causes an 80% inhibition of sucrose sequestration[67,72]; i.e. it acts as a very powerful sequestration inhibitor. It was originally reported not to influence the degradation of asialo-fetuin[67]; Winkler and Segal, however, report an effect on the degradation of another endocytosed protein, presumably due to an inhibition of the fusion of endosomes with lysosomes[81].

Benzyl alcohol, which is known as a membrane-perturbing agent, has similar effects to 3-methyladenine[21,78]. Both these substances probably interact, more or less specifically, with the membranes involved in autophagy. The substances are most probably not physiological effectors, but the real regulators could have structural similarities. The suggested regulators of autophagy are indicated schematically in figure 2.

Research on autophagy

Compared to processes that are susceptible to biochemical analysis in completely defined cell-free systems, autophagy is very poorly understood. The difficulty of establishing good assays for autophagy has been one of the problems in this field of research. Some possible assays do, however, exist. One is measuring the increased proteolysis after transferring various cell cultures to conditions of nutritional 'step-down'[2,29]. Another is measuring the lysosomotropic weak base-sensitive proteolysis in hepatocytes[65]. In both cases the lysosomal activity may be underestimated.

The possibility of relating biochemical data to morphological changes is an advantage in this field of research. The probable existence of autophagic vacuoles too small to be recognized should, however, be borne in mind. Another difficulty in interpreting morphological data is to distinguish between effects on the formation, vs on the processing of autophagic vacuoles.

More recently some new and more refined biochemical techniques have been developed. The microinjection of labeled proteins is a promising approach for investigating the selectivity of autophagy[13,15,37,59,60]. At least in some cases the microinjected proteins are degraded lysosomally. The injection of fluid-phase markers such as dextran or sucrose provides information about bulk autophagy[18,27].

All peroxisomal enzymes have similar half-lives, indicating that this organelle is degraded exclusively via autophagy[54]. It is possible to induce peroxisomes to a high level[30]. The subsequent descrease in the activity of peroxisomal enzymes might be convenient assay for an organelle type of autophagy.

A good assay is probably not sufficient to investigate the postulated ultimate regulatory factors. Such factors would be expected to exhibit a rapid turnover as autophagy in hepatocytes can be started or stopped within 20–30 min by removing or adding amino acids[43]. The factors would, furthermore, not be expected to penetrate the plasma membrane.

A possible way of addressing the question of ultimate regulators is to incubate cells with an open plasma membrane, but intact internal structures. A number of feasible means for achieving such a system exist, electroshock[82] being a particularly promising method. Electroshock has been employed with some success in a comparable investigation of secretion in adrenal medullary cells[39].

1 Ahlberg, J., Henell, F., and Glaumann, H., Proteolysis in isolated autophagic vacuoles from rat liver. Exp. Cell Res. *142* (1982) 373–383.

2 Amenta, J.S., and Brocher, S.C., Mechanisms of protein turnover in cultured cells. Life Sci. *28* (1981) 1195–1208.

3 Amenta, J.S., Sargus, M.J., and Baccino, F.M., Effect of microtubular or translational inhibitors on general cell protein degradation. Biochem. J. *168* (1977) 223–227.

4 Baccino, F.M., Fiszer-Szafarz, B., Messina, M., Nadal, C., Barrera, G., DeMurillo, A.G., and Tessitore, L., Lysosomal hydrolases in liver growth. Biol. Cell *46* (1982) 21–28.

5 Barrett, A.J., and Heath, M.F., Lysosomal enzymes, in: Lysosomes a Laboratory Handbook, pp. 19–195. Ed. J.T. Dingle. North-Holland Publ. Co., Amsterdam 1977.

6 Bienkowski, R.S., Intracellular degradation of newly synthesized secretory proteins. Biochem. J. *214* (1983) 1–10.

7 Bigelow, S., Hough, R., and Rechsteiner, M., The selective degradation of injected proteins occurs principally in the cytosol rather than in lysosomes. Cell *25* (1981) 83–93.

8 Chua, B., Siehl, D.L., and Morgan, H.E., Effect of leucine and metabolites of branched chain amino acids on protein turnover in heart. J. biol. Chem. *254* (1979) 8358–8362.

9 Dean, R.T., Concerning a possible mechanism for selective capture of cytoplasmic proteins by lysosomes. Biochem. biophys. Res. Commun. *67* (1975) 604–609.

10 Dean, R.T., Lysosomes and membrane recycling. Biochem. J. *168* (1977) 603–605.

11 Dean, R.T., Barrett, A.J., Lysosomes. Essays Biochem. *12* (1976) 1–40.

12 DeMartino, G.N., and Goldberg, A.L., Thyroid hormones control lysosomal enzyme activities in liver and skeletal muscle. Proc. natn. Acad. Sci. USA *75* (1978) 1369–1373.

13 Docherty, P.A., and Aronson, N.N., Injection of proteins into primary rat hepatocytes by erythrocyte-mediated techniques. Biochem. biophys. Res. Commun. *109* (1982) 527–532.

14 Doyle, D., and Baumann, H., Turnover of the plasma membrane of mammalian cells. Life Sci. *24* (1979) 951–966.

15 Evans, P.J., and Mayer, R.J., Degradation of transplanted mitochondrial proteins by hepatocyte monolayers. Biochem. J. *216* (1983) 151–161.

16 Freikopf-Cassel, A., and Kulka, R.G., Regulation of the degradation of ^{125}I-labelled glutamine synthetase introduced into cultured hepatoma cells by erythrocyte ghost-mediated injection. FEBS Lett. *128* (1981) 63–66.

17 Glaumann, H., Ericsson, J.L.E., and Marzella, L., Mechanisms of intralysosomal degradation with special references to autophagocytosis and heterophagocytosis of cell organelles. Int. Rev. Cyt. *73* (1981) 149–182.

18 Gordon, P.B., and Seglen, P.O., Autophagic sequestration of ^{14}C-sucrose, introduced into rat hepatocytes by reversible electro-permeabilization. Exp. Cell Res. *142* (1982) 1–14.

19 Grinde, B., Effect of carboxylic ionophores on lysosomal protein degradation in rat hepatocytes. Exp. Cell Res. *149* (1983) 27–35.

20 Grinde, B., Role of calcium for protein turnover in isolated rat hepatocytes. Biochem. J. *216* (1983) 529–536.

21 Grinde, B., Inhibition of autophagy by benzyl alcohol. Biochim. biophys. Acta *800* (1984) 140–144.

22 Grinde, B., Effect of amino acid metabolites on lysosomal protein degradation. A regulatory role for kynurenine? Eur. J. Biochem. *145* (1984) 623–627.

23 Grinde, B., and Seglen, P.O., Differential effects of proteinase inhibitors and amines on the lysosomal and non-lysosomal pathways of protein degradation in isolated rat hepatocytes. Biochim. biophys. Acta *632* (1980) 73–86.

24 Grinde, B., and Seglen, P.O., Leucine inhibition of autophagic vacuole formation in isolated rat hepatocytes. Exp. Cell Res. *134* (1981) 33–39.

25 Grinde, B., and Seglen, P.O., Effects of amino acid analogues on protein degradation in isolated rat hepatocytes. Biochim. biophys. Acta *676* (1981) 43–50.

26 Grinde, B., Galpin, I.J., Wilby, A.H., and Beynon, R.J., Inhibition of hepatic protein degradation by synthetic analogues of chymostatin. J. biol. Chem. *258* (1983) 10821–10823.

27 Hendil, K.B., Autophagy of metabolically inert substances injected into fibroblasts in culture. Exp. Cell Res. *135* (1981) 157–166.

28 Henell, F., and Glaumann, H., Effect of leupeptin on the autophagic vacuolar system of rat hepatocytes. Correlation between ultrastructure and degradation of membrane and cytosolic proteins. Lab. Invest. *51* (1984) 46–56.

29 Hershko, A., and Ciechanover, A., Mechanisms of intracellular protein breakdown. A. Rev. Biochem. *51* (1982) 335–364.

30 Hikai, K., and Ogawa, K., Appearance of giant peroxisomes in mouse hepatocytes treated with a hypolipidemic drug. Acta histochem. cytochem. *6* (1973) 193–206.

31 Hirsimaki, Y., and Hirsimaki, P., Vinblastine-induced autophagocytosis: The effect of disorganization of microfilaments by cytochalasin B. Exp. molec. Path. *40* (1984) 61–69.

32 Holzer, H., and Heinrich, P.C., Control of proteolysis. A. Rev. Biochem. *49* (1980) 63–91.

33 Hopgood, M.F., Clark, M.G., and Ballard, F.J., Inhibition of protein degradation in isolated rat hepatocytes. Biochem. J. *164* (1977) 399–407.

34 Hopgood, M.F., Clark, M.G., and Ballard, F.J., Protein degradation in hepatocytes monolayers. Effects of glucagon, adenosine 3′:5′-cyclic monophosphate and insulin. Biochem. J. *186* (1980) 71–79.

35 Hopgood, M.F., Clark, M.G., and Ballard, F.J., Stimulation by glucocorticoids of protein degradation in hepatocyte monolayers. Biochem. J. *196* (1981) 33–40.

36 Hutson, N.J., and Mortimore, G.E., Suppression of cytoplasmic protein uptake by lysosomes as the mechanism of protein regain in livers of starved-refed mice. J. biol. Chem. *257* (1982) 9548–9554.

37 Katznelson, R., and Kulka, R.G., Degradation of microinjected methylated and unmethylated proteins in hepatoma tissue culture cells. J. biol. Chem. *258* (1983) 9597–9600.

38 Kneckt, E., Hernandez-Yago, J., and Grisolia, S., Regulation of lysosomal autophagy in transformed and non-transformed mouse fibroblasts under several growth conditions. Exp. Cell Res. *154* (1984) 224–232.

39 Knight, D.E., and Baker, P.E., Calcium-dependence of catecholamine release from bovine adrenal medullary cells after exposure to intense electric fields. J. Membrane Biol. *68* (1982) 107–140.

40 Kolset, S.O., Tolleshaug, H., and Berg, T., The effects of colchicine and cytochalasin B on uptake and degradation of asialo-glycoproteins in isolated rat hepatocytes. Exp. Cell Res. *122* (1979) 159–167.

41 Kominami, E., Hashida, S., Khairallah, E.A., and Katunuma, N., Sequestration of cytoplasmic enzymes in an autophagic vacuole-lysosomal system induced by injection of leupeptin. J. biol. Chem. *258* (1983) 6093–6100.

42 Kovács, A.L., and Seglen, P.O., Inhibition of hepatocytic protein degradation by methylaminopurines and inhibitors of protein synthesis. Biochim. biophys. Acta *676* (1981) 213–220.

43 Kovács, A.L., Grinde, B., and Seglen, P.O., Inhibition of autophagic vacuole formation and protein degradation by amino acids in isolated hepatocytes. Exp. Cell Res. *133* (1981) 431–436.

44 Kovács, A.L., Reith, A., and Seglen, P.O., Accumulation of autophagosomes after inhibition of hepatocytic protein degradation by vinblastine, leupeptine or a lysosomotropic amine. Exp. Cell Res. *137* (1982) 191–201.

45 Marzella, L., and Glaumann, H., Biogenesis, translocation, and function of lysosomal enzymes. Int. Rev. exp. Path. *25* (1983) 239–278.

46 Matsushita, M., Saito, T., Keino, H., Kuruhara, Y., Abe, S., and Ogawa, K., Autophagy of peroxisomes in hepatic parenchymal cells. Acta histochem. cytochem. *15* (1982) 277–283.

47 Millward, D.J., Protein degradation in muscle and liver. Compreh. Biochem. *19B* (1980) 153–232.

48 Mortimore, G.E., Mechanisms of cellular protein catabolism. Nutr. Rev. *40* (1982) 1–12.

49 Mortimore, G.E., and Ward, W.F., Behaviour of the lysosomal system during organ perfusion. An inquiry into the mechanism of hepatic proteolysis, in: Lysosomes in Biology and Pathology, vol. 5, pp. 157–184. Eds J.T. Dingle and R.T. Dean. North-Holland Publ. Co., Amsterdam 1976.

50 Mortimore, G.E., Hutson, N.J., and Surmacz, C.A., Quantitative correlation between proteolysis and macro- and microautophagy in mouse hepatocytes during starvation and refeeding. Proc. natn. Acad. Sci. USA *80* (1983) 2179–2183.

51 Ohkuma, S., Moriyama, Y., and Takano, T., Identification and characterization of a protein pump on lysosomes by fluorescein isothiocyanate-dextran fluorescence. Proc. natn. Acad. Sci. USA *79* (1982) 2758–2762.

52 Pfeifer, U., Werde, E., and Bergeest, H., Inhibition by insulin of the formation of autophagic vacuoles in rat liver. J. Cell Biol. *78* (1978) 152–167.

53 Poli, A., Gordon, P.B., Schwarze, P.E., Grinde, B., and Seglen, P.O., Effects of insulin and anchorage on hepatocytic protein metabolism and amino acid transport. J. Cell Sci. *48* (1981) 1–18.

54 Poole, B., Leighton, F., and DeDuve, C., The synthesis and turnover of rat liver peroxisomes. J. Cell Biol. *41* (1969) 536–546.

55 Pøsø, A.R., and Mortimore, G.E., Novel requirement for alanine in the amino acid control of deprivation-induced protein degradation in liver. Proc. natn. Acad. Sci. USA *81* (1984) 4270–4274.

56 Pøsø, A.R., Wert, J.J., and Mortimore, G.E., Multifunctional control by amino acids of deprivation-inducted proteolysis in liver. Role of leucine. J. biol. Chem. *257* (1982) 12114–12120.

57 Pøsø, A. R., Schworer, C. M., and Mortimore, G. E., Acceleration of proteolysis in perfused rat liver by deletion of glucogenic amino acids: regulatory role of glutamine. Biochem. biophys. Res. Commun. *107* (1982) 1433–1439.
58 Rodemann, H. P., and Goldberg, A. L., Arachidonic acid, prostaglandin E_2 and $F_{2\alpha}$ influence rates of protein turnover in skeletal and cardiac muscle. J. biol. Chem. *257* (1982) 1632–1638.
59 Rote, K. V., and Rechsteiner, M., Degradation of microinjected proteins: effect of lysosomotropic agents and inhibitors of autophagy. J. Cell Physiol. *116* (1983) 103–110.
60 Russel, S. M., Amenta, J. S., and Mayer, R. J., Degradation of proteins in rat liver mitochondrial outer membrane transplanted into different cell types. Biochem. J. *220* (1984) 489–498.
61 Sakai, M., and Ogawa, K., Energy-dependent lysosomal wrapping mechanism (LWM) during autophagolysosome formation. Histochemistry *76* (1982) 479–488.
62 Schneider, D. L., ATP-dependent acidification of intact and disrupted lysosomes: evidence for an ATP-driven proton pump. J. biol. Chem. *256* (1981) 3858–3864.
63 Schworer, C. M., and Mortimore, G. E., Glucagon-induced autophagy and proteolysis in liver: Mediation by selective deprivation of intracellular amino acids. Proc. natn. Acad. Sci. USA *76* (1979) 3169–3173.
64 Schworer, C. M., Shiffer, K. A., and Mortimore, G. E., Quantitative relationship between autophagy and proteolysis during graded amino acid deprivation in perfused rat liver. J. biol. Chem. *256* (1981) 7652–7658.
65 Seglen, P. O., Inhibitors of lysosomal functions. Meth. Enzym. *96* (1983) 737–764.
66 Seglen, P. O., and Gordon, P. B., Vanadate inhibits protein degradation in isolated rat hepatocytes. J. biol. Chem. *256* (1981) 7699–7701.
67 Seglen, P. O., and Gordon, P. B., 3-Methyladenine: A specific inhibitor of autophagic/lysosomal protein degradation in isolated rat hepatocytes. Proc. natn. Acad. Sci. USA *79* (1982) 1889–1892.
68 Seglen, P. O., and Gordon, P. B., Amino acid control of autophagic sequestration and protein degradation in isolated rat hepatocytes. J. Cell Biol. *99* (1984) 435–444.
69 Seglen, P. O., Grinde, B., and Solheim, A. E., Inhibition of the lysosomal pathway of protein degradation in isolated rat hepatocytes by-ammonia, methylamine, chloroquine and leupeptin. Eur. J. Biochem. *95* (1979) 215–225.
70 Seglen, P. O., Gordon, P. B., and Poli, A., Amino acid inhibition of the autophagic/lysosomal pathway of protein degradation in isolated rat hepatocytes. Biochim. biophys. Acta *630* (1980) 103–118.
71 Seglen, P. O., Gordon, P. B., Grinde, B., Solheim, A., Kovács, A. L., and Poli, A., Inhibitors and pathways of hepatocytic protein degradation. Acta biol. med. germ. *40* (1981) 1587–1598.
72 Seglen, P. O., Gordon, P. B., and Schwarze, P. E., Autophagy and protein degradation in rat hepatocytes, in: Isolation, Characterization and Use of Hepatocytes, pp. 153–163. Eds R. A. Harris and N. W. Cornell. Elsevier Publ. Co., Amsterdam 1983.
73 Solheim, A. E., and Seglen, P. O., Structural and physical changes in lysosomes from isolated rat hepatocytes treated with methylamine. Biochim. biophys. Acta *763* (1983) 284–291.
74 Sommercorn, J. M., and Swick, R. W., Protein degradation in primary monolayer cultures of adult rat hepatocytes. J. biol. Chem. *256* (1981) 4816–4821.
75 Stacey, D. W., and Alfrey, V. G., Evidence for the autophagy of microinjected proteins in Hela cells. J. Cell Biol. *75* (1977) 807–817.
76 Steinman, R. M., Mellman, I. S., Muller, W. A., and Cohn, Z. A., Endocytosis and the recycling of plasma membrane. J. Cell Biol. *96* (1983) 1–27.
77 Tanaka, K., Ikegaki, N., and Ichihara, A. Effects of leupeptin and pepstatin on protein turnover in adult rat hepatocytes in primary culture. Archs Biochem. Biophys. *208* (1981) 296–304.
78 Tolleshaug, H., and Berg, T., Evidence for the selective inhibition of fusion between endocytic vesicles and lysosomes by benzyl alcohol. Biochem. Pharmac. *31* (1982) 593–595.
79 Tolleshaug, H., Gordon, P. B., Solheim, A. E., and Seglen, P. O., Trapping of electro-injected ^{14}C-sucrose by hepatocyte mitochondria: a mechanism for cellular autofiltration? Biochem. biophys. Res. Commun. *119* (1984) 955-961.
80 Wang, E., and Choppin, P. W., Effect of vanadate on intracellular distribution and function of 10-nm filaments. Proc. natn. Acad. Sci. USA *78* (1981) 2363–2367.
81 Winkler, J. R., and Segal, H. L., Inhibition by swainsonine of the degradation of endocytosed glycoproteins in isolated rat liver parenchymal cells. J. biol. Chem. *259* (1984) 1958–1962.
82 Zimmermann, U., Electric field-mediated fusion and related electrical phenomena. Biochim. biophys. Acta *694* (1982) 227–277.

0014-4754/85/091089-07$1.50 + 0.20/0

'Waldsterben': Our Dying Forests – Part III

Forest dieback: Extent of damages and control strategies

by W. Bach

Center for Applied Climatology and Environmental Studies, Department of Geography, University of Münster, Robert-Koch-Str. 26, D-4400 Münster (Federal Republic of Germany)

Summary. This paper starts from the generally accepted premise that forest dieback is a complex phenomenon caused by multiple stresses that are exerted by a host of contributing factors. It is argued that the rapidly proceeding forest dieback, as documented in the damage inventories, is in itself enough reason to warrant action. Therefore, at this late stage, this paper emphasizes the pressing need to introduce active control measures which will reduce pollution emission through more efficient use of fossil fuels, through abatement techniques at the source, and through substitution with less polluting sources.
Key words. Forest dieback; Waldsterben; damage inventory; efficient energy use; abatement techniques; pollution source substitution.

Can we prove the causes of forest dieback?

At first glance the argument that control measures cannot be taken before the causes of forest dieback have been clarified beyond doubt seems enticing. Before this can be achieved, so the reasoning goes, more research will be required to establish a clear linear cause and effect relationship between forest dieback and its likely causes. At a second glance this line of argumentation becomes invalid, since, in principle, such an unequivocal cause and effect relation is not possible in complex and multi-dimensional systems, such as a forest ecosystem. Forest dieback is not the result of a mono-causal but rather of a complex disease caused by multiple stresses[60,69]. The extent to which in each case a specific stress may contribute to damaging a certain tree species at a specified site, could only be verified with sufficient scientific exactitude, if it were possible to carry out, under controlled (laboratory)