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Autophagy, Heterophagy, Microautophagy and Crinophagy as the Means for Intracellular Degradation

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Summary. It is generally accepted that the lysosomal compartment plays an important role in the degradation of cellular components.

In this communication we discuss various experimental models which have been used to study mechanisms of intralysosomal degradation and also discuss the evidence obtained in support of the following proposals:

1. The autophagosomes can be isolated into high purity and are the subcellular locus of induced protein degradation.
2. Different membrane components such as proteins and lipids are degraded at different rates inside the lysosomes. Intralysosomal hydrolysis is not the rate limiting step in degradation.
3. Lysosomes take up soluble material in vitro by invagination and pinching off of their membranes (microautophagy).
4. Secretory vesicles can degrade their secretory contents by fusing with the lysosomes.

Key words: Lysosomes – Autophagosomes – Isolation – Proteolysis – Microautophagy – Crinophagy.

Mechanisms of Turnover of Cell Constituents

Membranous and cytosolic constituents of all cells are synthesized and degraded continuously. The regulation and interaction of degradation and synthesis are important mechanisms for achieving tissue growth and occur for example, in normal development and in organ regeneration and involution. Blood amino-acids, as well as glucagon and insulin levels, maintain a steady state by regulating the interaction between synthesis and degradation (Mortimore and Mondon 1970; Mortimore and Schworer 1977; Pfeifer et al. 1978). At the molecular level aminoacyl-tRNA may serve as the final determinant in this process (Scornik et al. 1980). The classical studies of de Duve and others demonstrated that

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lysosomes are the principal subcellular site where acid hydrolysis takes place. Work with perfused liver (Neely et al. 1977) and with isolated cells (Seglen et al. 1979; Dean 1979) has shown that the lysosomal apparatus plays an important role in the degradation of intracellular proteins. By the autophagic pathway subcellular components are sequestered into the lysosomal compartment to be degraded in the autophagosomes. In view of the high proteolytic capacity of lysosomes (Barrett and Heath 1977), it has been suggested that intralysosomal hydrolysis is not the rate-limiting step in this process and therefore does not determine the turnover of proteins. It seems, therefore, that the rate and perhaps the selectivity of the uptake of proteins into lysosomes could function as a regulatory mechanism for protein turnover, rather than the proteolytic rate (Neely et al. 1977).

Hydrolysis of protein and smaller polypeptides also occurs extralysosomally (Ballard 1977). The details of this pathway as well as its subcellular locus have not been fully worked out. It is also unclear at present how intralysosomal and extralysosomal pathways of protein degradation interact, and what is their relative contribution to the net turnover. Estimates of the lysosomal contribution to intracellular degradation during physiologic conditions have been in the range 30–70% depending on the experimental conditions. However, the enhanced proteolysis which occurs as a result of nutrient deprivation and of alterations in homeostasis is predominantly, if not exclusively, a lysosomal process due to increased autophagy.

We have used the induction of autophagy, caused by the microtubule inhibitor vinblastine (VBL) (Hirsimäki et al. 1976), as a means of studying induced proteolysis (Marzella and Glaumann 1980a, b) and have shown that the autophagic vacuole (AV) is the subcellular locus for induced degradation (Marzella et al. 1981). In addition, we have obtained experimental data in support of the hypothesis that lysosomes may be able to internalize their membranes in vitro by a microautophagic mechanism (Marzella et al. 1980a). These recently published results will be summarized briefly. In addition, recent studies on crinophagy, the uptake by lysosomes of marker particles and uptake and degradation of proteins during in vitro incubation will also be discussed. Finally, a model for introducing membranes into lysosomes by means of heterophagy will be discussed.

Induction of Autophagocytosis as a Model to Study Intralysosomal Proteolysis

In the case of VBL-induced autophagy, the appearance of the organelles sequestered into nascent autophagosomes is normal as far as can be judged by electron microscopy (Fig. 1). Moreover, by labeling cellular proteins with ^{14}C-leucine at different intervals before the induction of autophagy, it can be shown that both old and newly synthesized proteins are taken up and degraded by the lysosomes (Table 1). VBL induces proteolysis of proteins labelled for both 48 and 96 h. Even after very short ^{14}C-leucin incorporation interval (2 h) VBL stimulates proteolysis to roughly the same extent (not shown). These observations indicate that VBL-induced autophagic sequestration of cytoplasm is a random process. It follows that cell injury, as defined by Trump and coworkers

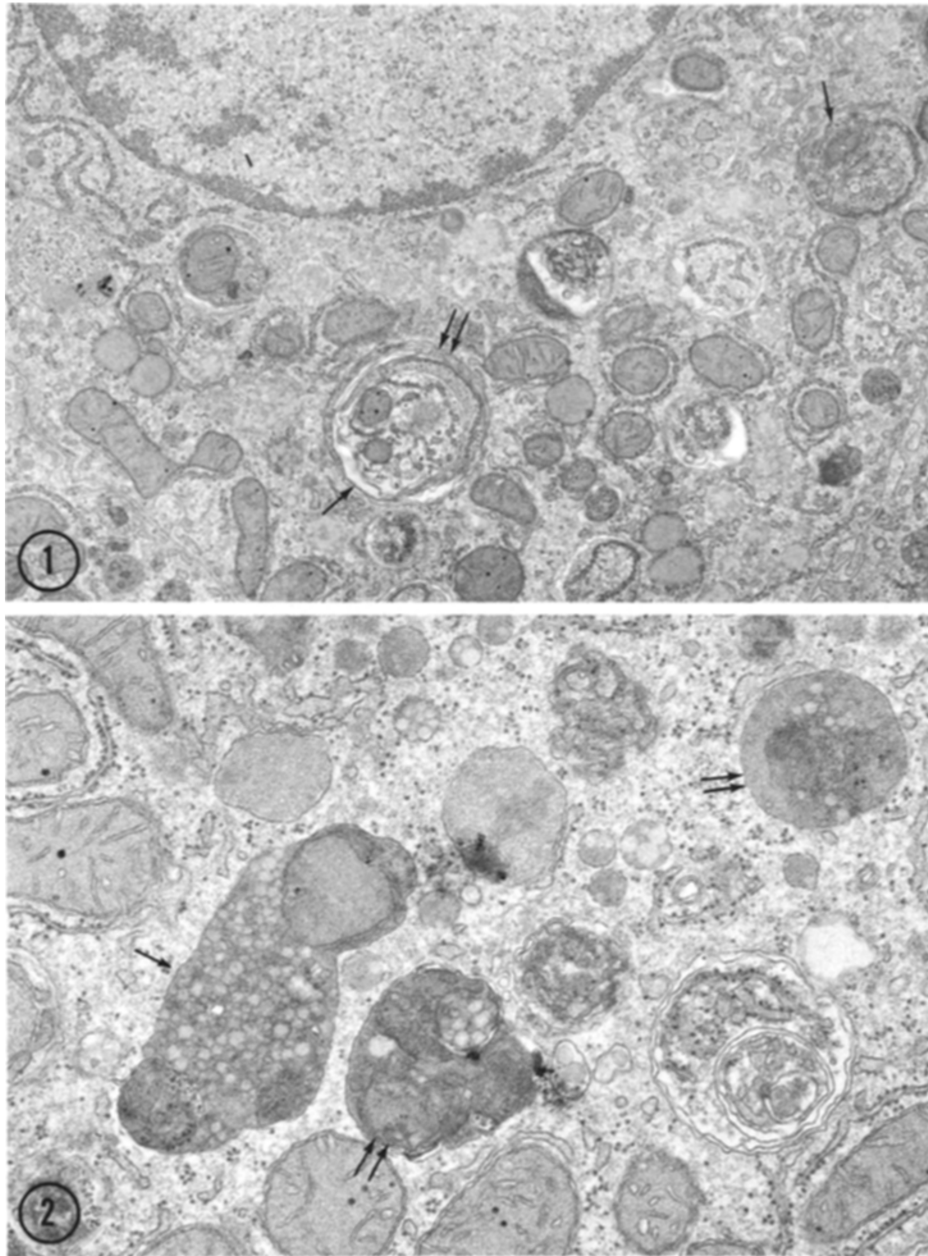


Fig. 1. Ultrastructural appearance of a liver parenchymal cell after vinblastine treatment. The animals received vinblastine (2 mg/100 g) for 16 h before sacrifice. Nascent AVs *(arrow)* are seen containing cytosol, mitochondria, lipid droplets and, in the circumference, ER – like cisternae provide the bounding membranes *(double arrows)*. Autophagosomes with morphological signs of early degradation are also observed. × 11,000

Fig. 2. Crinophagy in a liver parenchymal cell after vinblastine treatment. A recent stage of fusion between AVs and secretory vesicles *(arrow)*. One of the AVs shows a late stage of degradation *(double arrows)*. × 23,000

Table 1. Proteolysis in an ML-fraction from control and VBL treated rats after various time intervals of ^{14}C-leucine labelling

Hours	Hours after ^{14}C-leucine administration			
	48		96	
	Control	VBL	Control	VBL
	TCA-soluble CPM/g of liver			
0.5	350	850	250	510
1	490	1,700	300	1,200
2	820	4,400	490	2,700

All rats were labeled with ^{14}C-leucine intravenously (50 µCi/100 g). Vinblastine treated rats recived intraperitoneal injections of VBL (5 mg/100 g) 3 h before sacrifice. ML fractions were prepared by centrifuging the homogenate (1:5 w/v) at 460 g for 10 min. The supernatant was then sedimented at 5,500 g for 8 min and the pellet resuspended in isotonic sucrose (1:5 w/w). The ML suspension was incubated for 0.5, 1 and 2h at 37° C. Aliquots were sampled at these time points and an equal amount of ice-cold 10% TCA was added and centrifuged 2,500 g for 10 min. The TCA-soluble radioactivity in the supernatant was measured

(Locke and Collins 1980), is not a prerequisite for induction of autophagy and that "old" (since they apparently do not exist) organelles are not sequestered preferentially into developing AVs.

The exact origin of the bounding membranes of the AVs (Hamberg et al. 1977) has not been identified conclusively. In the case of VBL-induced autophagy, electron microscopical analysis suggests that endoplasmic reticulum (ER) cisternae form pockets into which portions of cytoplasm become sequestered by the close apposition and fusion of one or more cisternae (Fig. 1). A vacuole bounded by one or multiple membranes results. Perhaps with the exception of nuclei and plasma membranes, all subcellular components are found sequestered in the nascent AVs. The large majority of AVs have "mixed" contents even though one cytoplasmic component such as ER or mitochondria may predominate. Cytosolic components are found in almost all AVs and are also seen as the only content in some AVs. Whether the quantity and type of organelles present in the AVs reflect the rate of sequestration and/or the rate of degradation of different subcellular organelles is not clear. In the case of phenobarbitalinduced proliferation of smooth endoplasmic reticulum, selective uptake of smooth ER was found after cessation of treatment (Bolender and Weibel 1973). Studies on the decrease in the fractional volume of AVs after the administration of insulin suggest that autophagic degradation is to some extent dependent on the type of organelle sequestered (Pfeifer et al. 1978). In the case of VBL large portions of cytoplasm may be seen sequestered into the AV. Other interesting phenomena are the frequent sequestration of Golgi-derived secretory vesicles into AVs, and the incorporation of secretory vesicles into the lysosomal compartment by fusion (Fig. 2). This latter mechanism is designated crinophagy (Saito and Ogawa 1974; Smith and Farquhar 1966; Halban and Wollheim 1980) and seems to be a way of degrading secretory material when its extracellular export is blocked, as, for example, when VBL is administered (Sandberg et al. 1981).

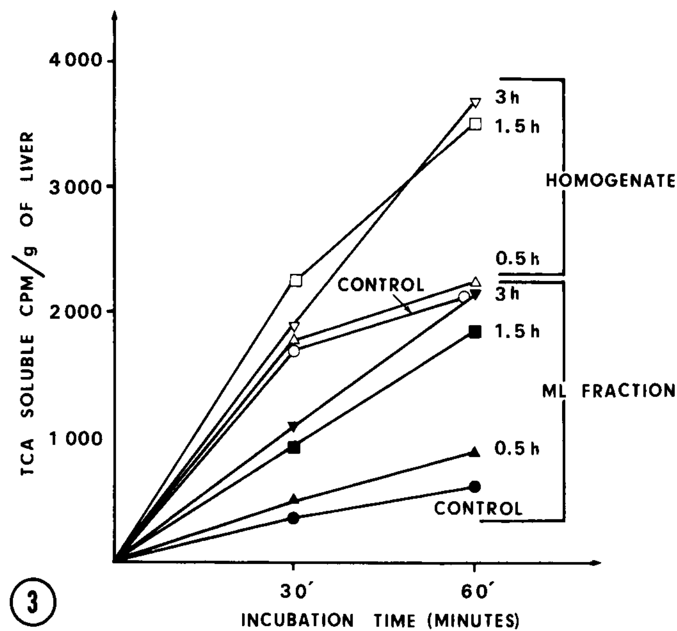


Fig. 3. Time of onset of proteolysis after vinblastine treatment. Rats were labeled 5 h before sacrifice with ^{14}C-leucine. The animals were injected i.p. with vinblastine (5 mg/100 g body weight), except the control, for 0.5, 1.5 and 3 h before the rats were anesthetized and the liver removed. ML-fractions were prepared by centrifuging the homogenate (20%) at 460 g for 10 min and the supernatant at 5,500 g for 8 min. The pellet was resuspended in isotonic sucrose and designated the ML-fraction. Homogenate and ML-suspensions were incubated at 37° C for various time intervals. Aliquots were sampled and an equal amount of 10% TCA was added. After centrifugation at 800 g for 15 min the supernatant was assayed for TCA-soluble radioactivity

As far as can be judged by acid phosphatase histochemistry, nascent AVs lack hydrolytic enzymes and acquire them after fusion with mature AVs (Ericsson 1969) or other secondary lysosomes (Hamberg et al. 1977). Studies by Deter et al. (1967) showed that 45 min after the induction of autophagy by glucagon there was a decrease by half in the number of dense bodies. This supports the notion that a transfer of hydrolytic enzymes occurs from this lysosomal population (dense bodies) to the newly formed autophagosomes.

Following the lag phase required for the formation of AVs and the aquisition of lysosomal enzyme, proteolytic rates in mitochondrial lysosomal fractions begin to rise (Fig. 3). By 1 h following VBL-injection, the biochemical and morphological expressions of autophagy are apparent, and can be summarized as follows. The expansion of the lysosomal compartment in the liver correlates with a two to three-fold increase in proteolysis in the mitochondrial-lysosomal (ML) fraction. There is no concomitant induction of lysosomal enzyme activities, nor is there a significant enrichment of lysosomal enzymes in the ML or microsomal fractions. Morphological analysis of the ML fraction reveals a three-fold expansion in the relative fractional volume AVs and a one-fold increase in the volume of residual bodies. A labilization of the lysosomes is seen during induced autophagy, as determined by loss of sedimentability of acid phosphatase and cathepsin D activities during in vitro incubations. Taken together, these

observations indicate that the increase in proteolysis is correlated with the sequestration of degradable substrate (cytoplasm) into the developing AVs. The increase in the residual body compartment reflects the maturation of AVs which occurs as the degradation of their sequestered contents proceeds. Isolation of ferritin-laden residual bodies from VBL-treated rats (Marzella et al. 1980b) shows that, in the absence of degradable substrate, there is very little measurable proteolysis in this type of lysosome, although they are rich in hydrolytic enzymes.

Isolation of Autophagic Vacuoles

More direct proof that AVs are the subcellular site of induced proteolysis is obtained by subfractionation of the ML fraction in discontinuous Metrizamide gradients (Marzella et al. 1981). This permits the preparation of a fraction which consists of AVs with a purity of around 85–90% (Fig. 4). This fraction is highly enriched in both lysosomal enzymes and proteolytic activity (Table 2). Enzyme markers for other subcellular organelles are also recovered in this AV fraction. EM-analysis shows that this biochemical finding is due to presence of sequestered cell organelles in the AVs and not to contamination. Interestingly, very nascent AVs do not float in the gradient and are not recovered in the isolated AV-fraction. It seems, therefore, that fusion of newly formed AVs with preexisting lysosomes, alteration of the surrounding membrane(s) of the AVs and/or some degree of degradation of the sequestered material is required to alter the density of the AVs and allow their recovery in the less dense fractions of the Metrizamide gradient. The isolated AV fraction is enriched in lipids; ultrastructural analysis shows that lipid-like electron-dense material accumulates in the AVs during the final stages of degradation. Moreover, during incubation of AVs, the release of degradation products following prelabeling with ^{14}C-glycerol is less than occurs with ^{14}C-leucine prelabeling. Taken together, these observations suggest that lipids, and in particular phospholipids, may be degraded and/or their degradation products may permeate more slowly out of the lysosomes when compared with the remnants of protein degradation. This conclusion is supported by studies on intralysosomal degradation using heterophagy of cell organelles as an experimental model (Glaumann et al. 1975; Glaumann and Trump 1975; Glaumann and Marzella 1981).

Heterophagy as a Model in the Study of Intralysosomal Degradation

In spite of the fact that the various models of induced or retarded autophagy have contributed significantly to our understanding of the bulk degradation of cell organelles, these models do not permit the study of sequestration of only a single type of cell organelle, e.g. mitochondria into the lysosomal apparatus. In searching for such a model we were attracted by the Kupffer cell, due to its large capacity to phagocytose and degrade foreign particles. This design was set up as a means to mimic autophagocytosis as follows. Isotopically prelabelled subcellular organelles were isolated from the livers of inbred rats and subsequently injected intravenously into a series of rats (Glaumann et al. 1975). By this approach we have studied the manner in which different cellular membranes are phagocytosed and digested inside the lyosomes of Kupffer cells,

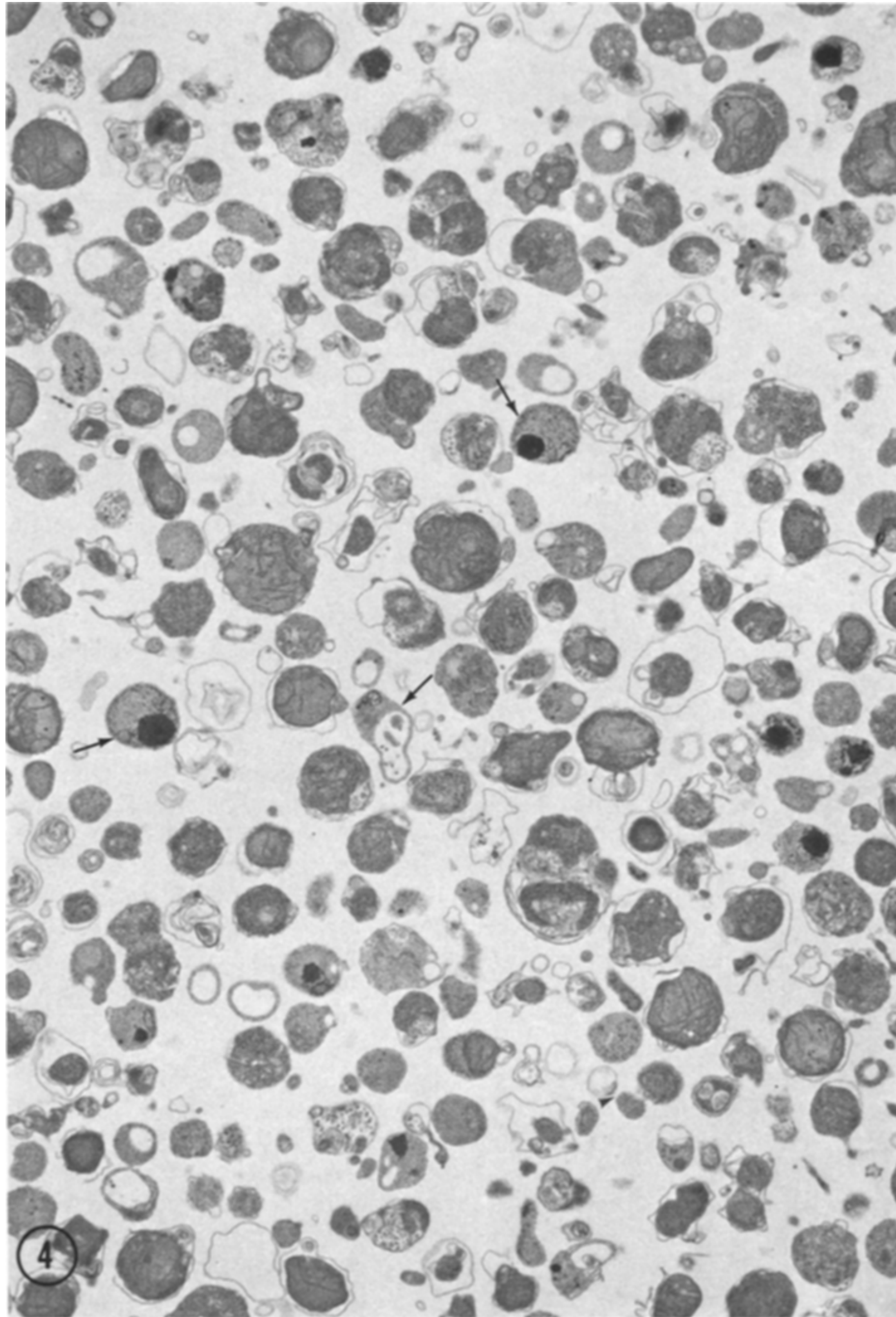


Fig. 4. Ultrastructural appearance of the isolated AVs. The rats received 5 mg of vinblastine/100 g bodyweight 3 h before decapitation. An ML-fraction was prepared as described in Fig. 3 and subfractionated on a discontinuous Metrizamide gradient as described in detail by Marzella et al. (1981). The AV fraction collected from the gradient was processed for electron microscopy. Note the different contents in the AVs including cytosol, mitochondria and ER membranes. All sequestered cell organelles display some loss of ultrastructural details as a morphological sign of ongoing degradation. There are no very nascent AVs, since these forms do not float in the gradient. Some "ordinary" secondary lysosomes are seen in the fraction *(arrow)*. × 13,000.

Table 2. Proteolysis in an isolated AV-fraction at various time points after induction of autophagy

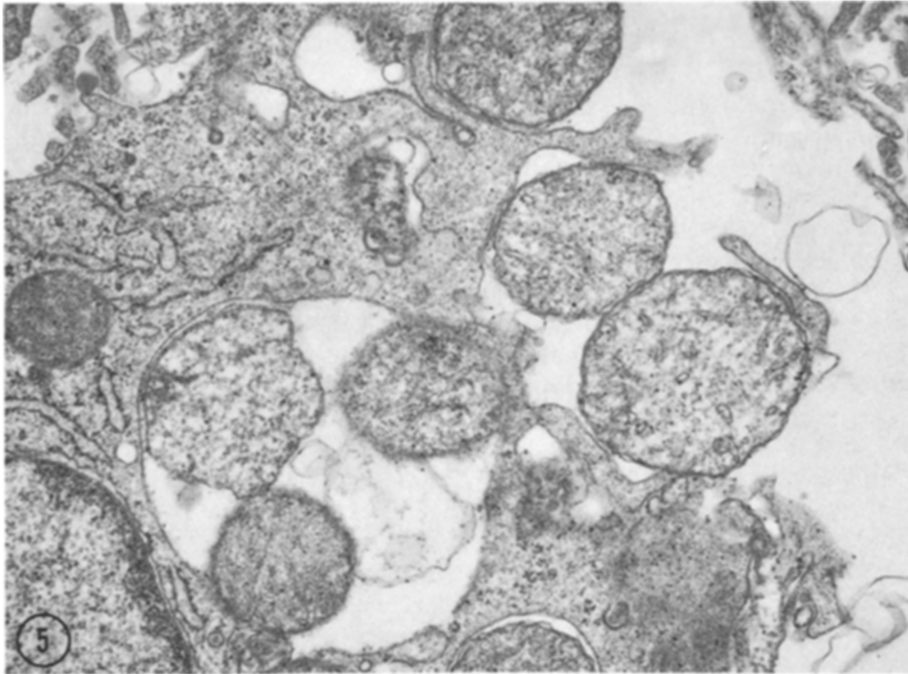
Incubation time min	% recovery of proteolysis		
	30 min VBL	60 min VBL	180 min VBL
15	2	16	11
30	2.5	18	24
60	4.9	31	35

All rats were labeled with ^{14}C-leucine. Vinblastine (5 mg/100 g bodyweight) was injected intraperitoneally at 30, 60 and 180 min before sacrifice. The homogenate (20%) was centrifuged at 460 g for 10 min, the supernatant was run at 24,000 g for 8 min and the pellet resuspended in 50% Metrizamide (0.33 g/ml). 6 ml were placed at the bottom of a cellulose nitrate tube and following layers were 10 ml of 26%; 5 ml of 24%; 5 ml of 20%; 5 ml of 15% Metrizamide. After centrifugation at 25,000 rpm for 4 h in an SW 27 rotor (LKB-Beckman) the autophagosomes were recovered in the 24%/20% and 20%/15% interphases. Homogenate and AV-fraction were incubated in vitro for 15, 30 and 60 min at 37° C. The reaction was stopped by adding an equal amount of 10% TCA. TCA soluble radioactivity was measured in the supernatant after centrifugation. The activity released from the AV fraction during the incubation is expressed as percent of the TCA-soluble radioactivity released by the homogenate. Blanks were incubated at 4° C

and have calculated the degradation rates of different membrane constituents such as proteins, glycoproteins and various lipids. In this sense, the study of a heterophagic model may have some advantages over induced autophagy, because the degradation of each membrane constituent or cell organelle can be studied separately. As with biological membranes, injected reconstituted membranes (liposomes) are rapidly taken up and degraded by Kupffer cell lysosomes (Glaumann and Marzella 1981). Liposomes therefore seem to be useful in the study of intralysosomal degradation, since this approach makes it possible to study a uniform membrane, the size, charge, chemical and physical composistion of which can be easily altered (Ingelman-Sundberg and Glaumann 1980). The process of phagocytosis and intralysosomal digestion can be divided into four phases, namely *attachment, engulfment, intralysosomal degradation* and *residual body phase*.

Attachment Phase. Only a few minutes after administration, the injected organelles are present in the liver sinusoids and attached almost exclusively to the surface of Kupffer cells, and rarely to other sinusoidal cells or hepatocytes (Fig. 5). A gap of 200–300 Å is always present between the adsorbed organelle and the surface of the Kupffer cells. It seems likely that this gap corresponds to the glycocalyx covering the outer surface of the plasma membrane (Pfeifer 1970).

Engulfment Phase. The engulfment phase is completed within a few minutes. In this process the Kupffer cell plasma membrane invaginates into deep folds and extends into flap-like processes (Fig. 5). These flaps cup around the cell organelles and eventually fuse to form a vacuole into which the attached organelles are trapped. As the vacuoles containing engulfed cell organelles are translocated into the cells, an increasing number of organelles were seen within them.



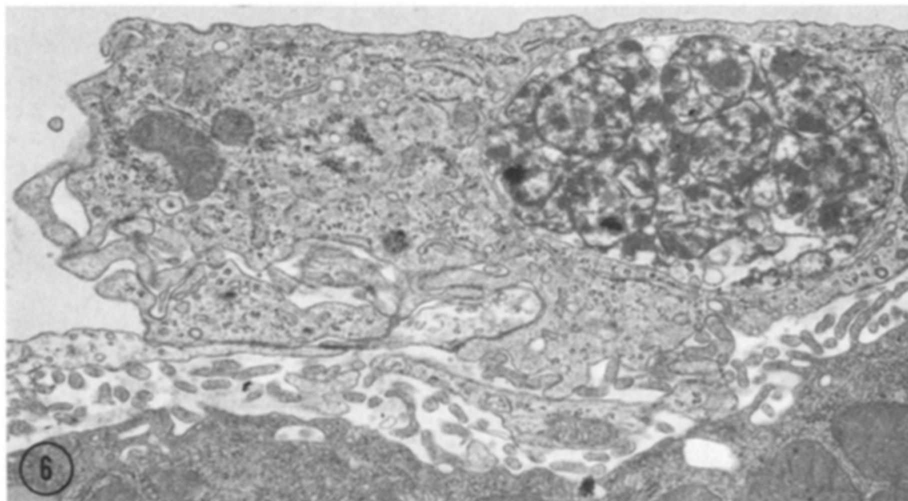


Fig. 5. Uptake of intravenously injected mitochondria by a Kupffer cell. Three mitochondria are trapped in a developing endocytic vacuole, and two mitochondria are in the process of being engulfed by flap-like extensions of the plasma membranes. ×25,000

Fig. 6. Phagocytosed mitochondria 30 min after intravenous injection. Note the presence of flocculent material representing denatured protein and calcium. The outer mitochondrial membranes and cristae are partially ruptured. ×17,000

This phenomenon may be explained by fusion of incoming phagosomes or emptying of several phagocytic invaginations into one large phagosome.

Degradation and Residual Body Stages. As early as 10 to 30 min after injection, the phagocytosed organelles show morphological evidence of degradation (Fig. 6). By 8 to 24 h, depending on the amount and organelle injected, degradation is morphologically completed and organelles are converted to non-recognizable debris. A gradual accumulation of round electron-dense bodies inside the phagolysosome occurs in the final stage of degradation (Fig. 7).

Even after the mitochondria and microsomes were apparently extensively degraded, the lyosomes remained swollen. It is likely that degradation products temporarily accumulate within the lysosomes. The intralysosomal osmolarity thus increases and enhanced hydration ensues. Eventually, the degradation products are presumed to diffuse out of the lysosomes into the cell sap. Morphologically, this loss corresponds to shrinkage of the lysosomes (Glaumann et al. 1979). Degradation of mitochondria, microsomes and plasma membranes gives rise to essentially similar types of residual bodies, containing electron-opaque droplets. That these droplets are derivatives of membrane lipids is most likely, since when lipid-depleted microsomes were injected the lipid droplets were much less frequently seen (Fig. 8). Available evidence indicates (Fowler and de Duve 1969) that these lipid-like residues consist partially of phosphodiesters which are not further hydrolyzed by lysosomes, but are finally degraded in the cell sap perhaps by an alkaline lipase (De Beer et al. 1979), or form intralysosomal lipofuscin-like materials. *In summary* it is obvious that cell organelles are rapidly degraded in lysosomes and that residual bodies arise from partially digested cell organelles.

Isotope Prelabeling of Injected Organelles. The vascular clearance of injected isotopically prelabeled organelles is rapid and 50–60% of the injected material is recovered in the liver; of this, 75% was recovered in the ML-fraction (Glaumann and Marzella 1981). When the liver is excluded from the circulation (by applying ligatures around the portal vein and the hepatic artery), vascular clearance is considerably longer, demonstrating that the Kupffer cells are the main eliminators of endogenous cell debris.

The heterophagy model also seems useful in evaluating the capacity of lysosomes to degrade various membrane components in vivo. Isotopically prelabeled subcellular organelles were injected into a series of rats and the livers removed for measurements of TCA non-soluble (sedimentable) activity in a crude lysosomal fraction. Table 3 gives the results for microsomal proteins labeled with ^{14}C-leucine and ^{55}Fe. When experiments were performed with prelabeled phospholipids, the degradation rates varied depending on which isotope was used. For ^{32}P-labeled PLP the $t_{1/2}$ was 4 h and for ^{14}C-glycerol labeling the corresponding value was 6 h, as far as can be calculated from the decay curves (Table 2). Labeling the membranes with ^{14}C-mevalonate gave a $t_{1/2}$ for cholesterol of 9 h, whereas when cholesterol esters were labeled with ^{14}C-palmitate, the decay curve corresponded to a $t_{1/2}$ for the fatty acid of approximately 3 h.

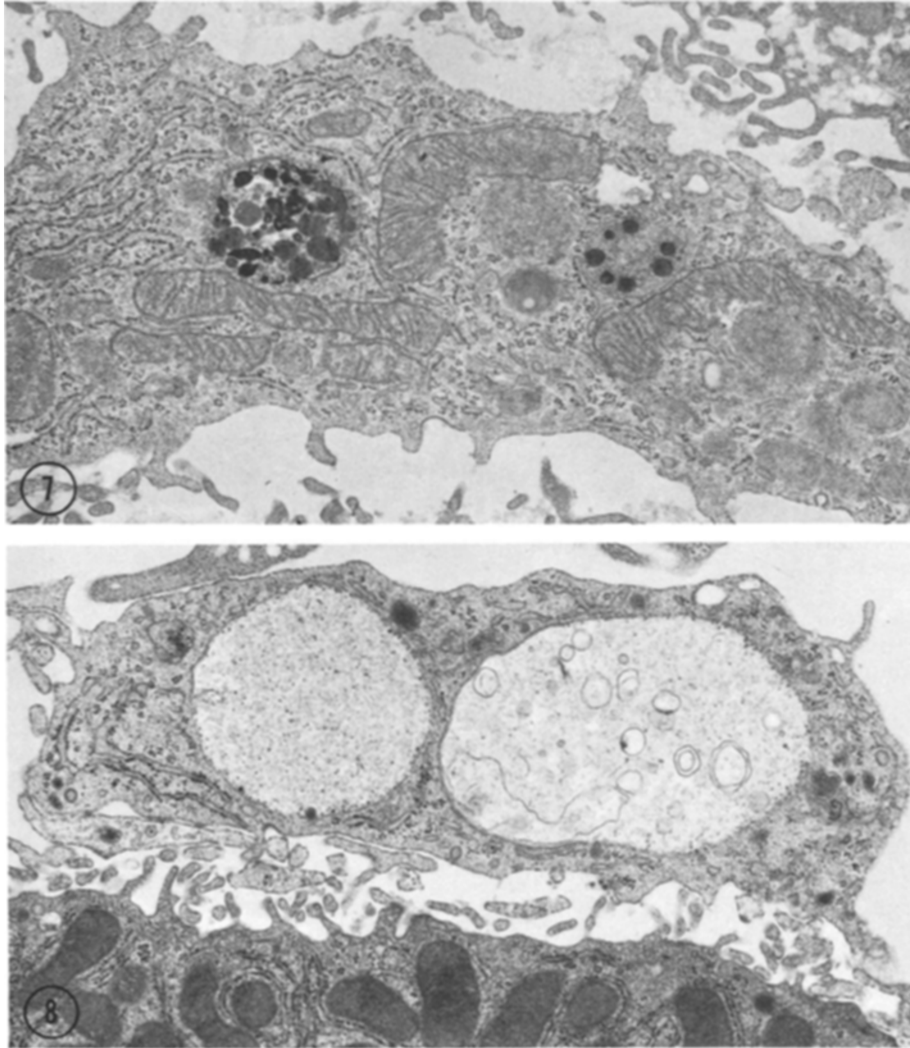


Fig. 7. 24 h after intravenous injection of mitochondria. The Kupffer cell lysosomes are laden with lipid-like droplets as a sign of incomplete digestion of membrane lipids. × 16,000

Fig. 8. Appearance of Kupffer cell lysosomes following injection of lipid depleted mitochondria. Note large electron lucent lysosomes lacking lipid like material. Compare with Fig. 7. × 14,000

A striking feature of the decay curves is a marked heterogeneity. In addition, the curves are not strictly exponential, but show multiple slopes, the first of which is always faster than the following ones. This is in contrast with the decay curves described for the degradation of a single protein in macrophages, namely denatured albumin (Edelson and Cohn 1974). One likely explanation for this heterogeneity is dissimilar rates of degradation of different membrane proteins. Garlick et al. (1976) have also reported complex curves for the decay

Table 3. Summary of measured half-lives of phagocytosed microsomal membrane components in Kupffer cell lysosomes

Component	Isotope	Disappearance curve during interval 0–48 h	Shortest $t_{1/2}$ h
Proteins			
microsomes	C^{14}-leucine	Triphasic	0.8–1.5
microsomes	C^{14}-leucine + leucine load	Triphasic	0.7–1.3
microsomes	C^{14}-leucine + cycloheximide [a]	Triphasic	0.8–1.2
mitochondria	C^{14}-leucine	Biphasic	1.5
ribosomes	C^{14}-leucine	Biphasic	1.5
Hemeproteins	Fe^{55}	Biphasic	20
Phospholipids	P^{32}	Biphasic	4
Phospholipids	C^{14}-glycerol	Biphasic	6
Phospholipids	C^{14}-glycerol + glycerol load	Biphasic	5–6
Phospholipids	C^{14}-phosphatidylcholine [b]	Monophasic	4
Cholesterol	C^{14}-mevalonate/C^{14}-cholesterol [c]	Monophasic	9
Cholesterol esters	Cholesteryl-C^{14}-palmitate	Biphasic	3
Glycoproteins	C^{14}-glucosamine	Biphasic	2
Glycoproteins	C^{14}-galactosamine	Biphasic	1.5
Ribosomes	C^{14}-orotic acid	Triphasic	0.5

[a] Cycloheximide was given 10 min before injection of microsomes
[b] Data from reconstituted microsomal membranes
[c] These experiments were performed with microsomes labeled in vitro by incubation

of label in a mixture of liver proteins. Unlike decay curves for homogenous proteins, the curve did not fit a single exponential, but a good fit was obtained with three enponentials. Reutilization of leucine is another possible explanation, although experiments with cycloheximide and dilution with cold leucine do not alter the decay curves significantly, as could be expected if reutilization was extensive (Glaumann and Marzella 1981).

A similar conclusion can be drawn for the intralysosomal degradation of phospholipids. When one specific phosphatide was followed, the decay curve was monophasic, in contrast with the finding with total microsomal phospholipids (Table 3). This supports the notion that individual phospholipids are degraded at different rates. The shorter half-life for ^{32}P-labeled PLP compared with glycerol, suggests that all the ester linkages of the phospholipid molecule are not split simultaneously. Since glycerol is the "backbone" of the PLP-molecule, it can be expected that ^{14}C-glycerol prelabeled PLP would show the longest half-life, as was indeed the case. Other possible explanations for the non-exponential appearance of the decay curves, e.g. isotope reutilization and exchange reactions have been discussed in detail elsewhere (Glaumann and Marzella 1981).

Crinophagy and Microautophagy as Means for Intracellular Degradation

Recently we have obtained biochemical data which supports and complements the morphological observations (Fig. 2) of the occurrence of crinophagy (Sand-

Table 4. Proteolysis during in vitro incubation of lysosomes with isotopically labeled proteins

Minutes	TCA-soluble CMP/g of liver	
	^{125}I-Insulin	^{14}C-Metheamoglobin
15	850	5,100
30	1,690	10,200
60	2,900	12,100

Lysosomes were isolated according to Wattiaux et al. (1978). 0.1 μCi ^{14}C-metheamoglobin or 0.1 μCi ^{125}I-insulin was added to the lysosomal suspension before incubation, which was preformed in 0.3 M sucrose at 37° C for 0, 15, 30 and 60 min. The reaction was stopped by adding TCA. The suspension was pelleted at 45,000 g for 10 min and TCA-soluble radioactivity was measured in the supernatant

berg et al., unpublished observations) as an additional pathway for intracellular proteolysis. This mechanism, known to occur in endocrine gland cells, designates fusion between secretory granules and lysosomes, and serves to degrade secretory material when exocytosis is inhibited as, for example, following vinblastine treatment. It is evident that autophagy and crinophagy are of physiologic significance.

Another pathway for the uptake of cytoplasmic material into lysosomes has been proposed on the basis of morphological observations (Marzella et al. 1980a). This mechanism, termed microautophagy (De Duve and Wattiaux 1966; Saito and Ogawa 1974), involves the uptake of soluble or membrane-bound material directly into the lysosome by a process of invagination and pinching off of the lysosomal membranes. This results in an intralysosomal vesicle followed by either degradation or opening up and release of the contents to the lysosomal milieu. Perhaps the vesicle thereafter recycles to the lysosomal membrane (Dean 1977). Experiments along this line have been hampered previously by the lack of methods to isolate purified normal lysosomes. We have investigated this question with the use of isolated secondary lysosomes (Wattiaux et al. 1978), autophagosomes (Marzella et al. 1981) and ferritin-laden residual bodies (Marzella et al. 1980b).

To this end, experiments were performed with isolated lysosomes incubated with ^{14}C-methemoglobin or ^{125}I-insulin. By this approach we found that there was an increase in TCA-soluble degradation products with time (Table 4). However, the results could, in fact reflect extralysosomal hydrolysis due to leakage of hydrolytic enzymes into the incubation medium. When checking this possibility, we found that the lysosomes displayed a marked stability during incubation as judged from the distribution of acid phosphatase and cathepsin D activities in the supernatant following incubation. Furthermore, lysosomes were also incubated with a labeled protein together with an inhibitor of cathepsin D, namely chloroquine, which also increases the intralysosomal pH. Incubation with chloroquine revealed a 50% decrease in degradation products in the supernatant and a parallel increase of radioactivity in the lysosomal pellet indicating ongoing uptake but inhibited proteolysis.

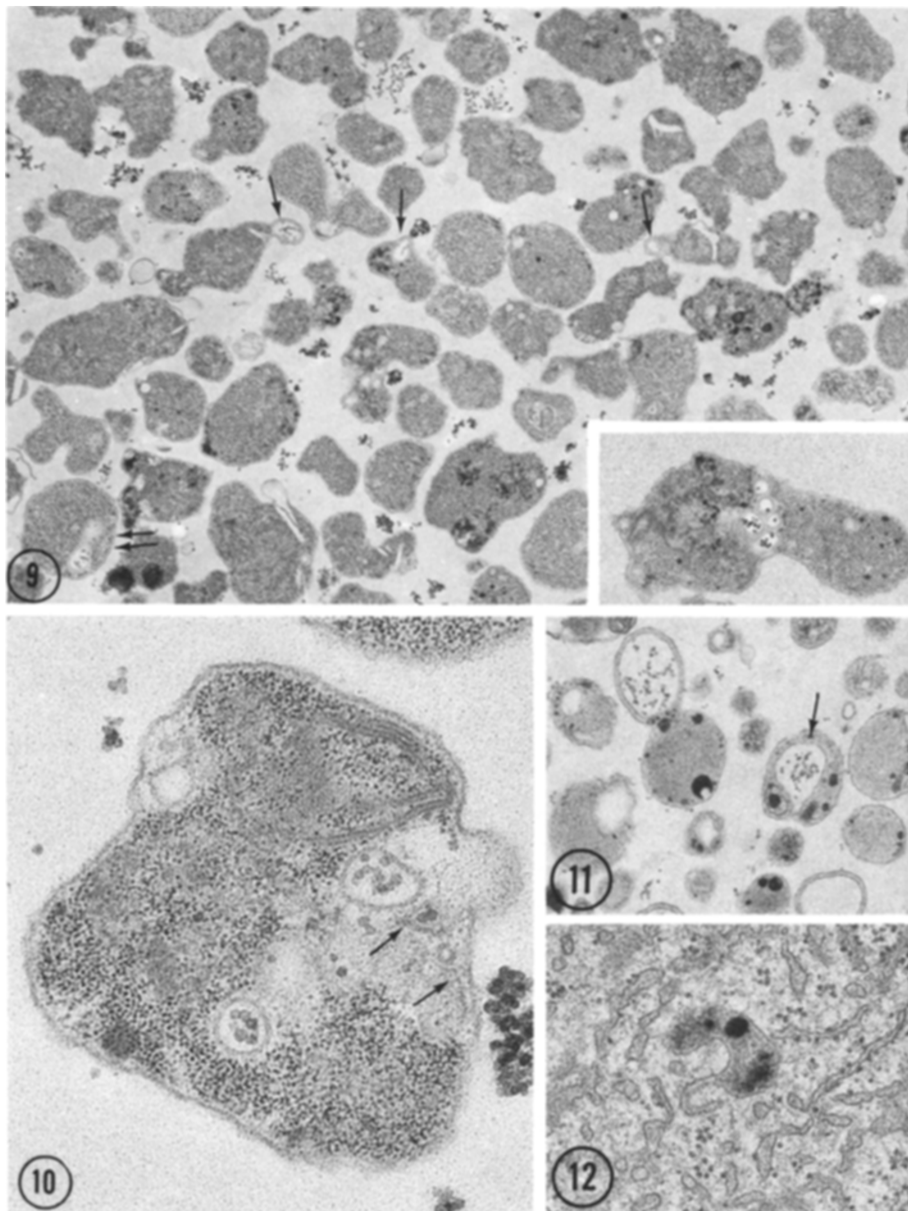


Fig. 9. Isolated iron laden lysosomes. The animals were injected with Jectofer and isolated on a Percoll gradient (Marzella et al. 1980a). The pellet was resuspended in 0.3 M sucrose and incubated for 30 min with 2 ml of Percoll. Some of the lysosomes display vesicles, some of which contain Percoll particles *(arrow)*. In one lysosome two Percoll-containing vesicles seem to have fused *(double arrows)*. ×15,000. *Inset.* A lysosome containing six vesicles with Percoll particles. ×20,000

Fig. 10. High magnification of an iron-laden lysosome incubated with Percoll in vitro. In the lysosomal matrix there are intact vesicles two of which contain Percoll particles. The membranes of the other vesicles seem to be partially degraded *(arrow)*. ×64,000

Fig. 11. Isolated lysosomes incubated with Percoll in vitro. The lysosomes were prepared according to Wattiaux et al. (1978), and were incubated in isotonic sucrose containing Percoll particles for 60 min at 37° C. A cup-shaped lysosome is seen to embrace Percoll particles *(arrow)*. ×22,000

Fig. 12. Detail from liver parenchymal cell. A secondary lysosome with an invagination and flap like extension into the cytosol *(arrow)* giving the impression of initiated microautophagy. ×30,000

Different types of lysosomes (iron-laden resiudal bodies, autophagosomes and "Wattiaux" lysosomes) were incubated with isotopically labeled Percoll particles (Marzella et al. 1980a). After extensive washing procedures, radioactivity was measured in the pellet and the uptake was found to be temperature dependent and a function of incubation time. Morphological studies with electron-dense particles (Percoll) to visualize the process indicated that these particles are internalized into the lysosomes by the formation of vesicles (Figs. 9–11). Although it is recognized that electron microscopy gives a static representation of dynamic events, Percoll particles appeared to be taken up via the formation of deep cupping of the lysosomal membrane as well as by protrusions of the membrane. In what may represent the next step, these membrane flaps and invaginations became closely apposed and eventually fused, trapping incubation medium and suspended Percoll particles (Fig. 11). No significant binding of the Percoll to the lysosomal membranes was seen. Profiles interpreted as breaking up of the intralysosomal vesicles, were observed and lysosomal profiles containing Percoll free in the matrix were also seen (Fig. 10). Similar invaginations and flaplike processes are also occasionally present in vivo (Fig. 12).

Taken together, it seems that crinophagy and microautophagy, in addition to autophagy, are important mechanisms for the intracellular degradation of secretory material and cytosolic compounds, respectively.

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