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Isolation of Autophagic Vacuoles from Rat Liver: Morphological and Biochemical Characterization

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ABSTRACT The induction of autophagy caused by vinblastine (VBL) has been found to be concomitant with a stimulation of proteolysis in a mitochondrial-lysosomal (ML) fraction from the rat liver (Marzella and Glaumann, 1980, *Lab. Invest.*, 42: 8–17. Marzella and Glaumann, 1980, *Lab. Invest.*, 42:18–27). In this fraction the enhanced proteolysis is associated with a threefold increase in the relative fractional volume of autophagic vacuoles (AVs). In an attempt to isolate the AVs, we subfractionated the ML suspension at different intervals after the induction of autophagy by VBL by centrifugation on a discontinuous Metrizamide gradient ranging from 50% to 15%. The material banding at the 24 to 20% and the 20 to 15% interphases was collected.

Morphological analysis reveals that 3 h after induction of autophagy these fractions consist predominantly (~90%) of intact autophagic vacuoles. These autophagic vacuoles contain cytosol, mitochondria, portions of endoplasmic reticulum, and occasional very low density lipoprotein particles either free or in Golgi apparatus derivatives, in particular secretory granules. The sequestered materials show ultrastructural signs of ongoing degradation. In addition to containing typical autophagic vacuoles, the isolated fractions consist of lysosomes lacking morphologically recognizable cellular components. Contamination from nonlysosomal material is only a few percent as judged from morphometric analysis.

Typical lysosomal "marker" enzymes are enriched 15-fold, whereas the proteolytic activity is enriched 10- to 20-fold in the isolated AV fraction as compared to the homogenate. Initially, the yield of nonlysosomal mitochondrial and microsomal enzyme activities increases in parallel with the induction of autophagy but, later on, decreases with advanced degradation of the sequestered cell organelles. Therefore, in the case of AVs the presence of nonlysosomal marker enzymes cannot be used for calculation of fraction purity, since newly sequestered organelles are enzymatically active. Isolated autophagic vacuoles show proteolytic activity when incubated in vitro. The comparatively high phospholipid/protein ratio (0.5) of the AV fraction suggests that phospholipids are degraded more slowly than proteins. It is concluded that AVs can be isolated into a pure fraction and are the subcellular site of enhanced protein degradation in the rat liver after induction of autophagy.

We have used the stimulation of autophagy as a means of studying mechanisms of sequestration and degradation of intracellular proteins in the lysosomal compartment (36, 37). To this end, vinblastine (VBL) was administered, which depolymerizes microtubules (30, 35) and induces autophagy in the rat liver (6, 37). The induction of autophagy enhances the proteolytic rates of mitochondrial-lysosomal (ML) fractions threefold (36). The stimulation of proteolysis in the ML fractions correlates with a three- to fourfold increase in the fractional volume of autophagic vacuoles (AVs) and residual bodies. In contrast, an isolated lysosome fraction consisting of ferritin-laden lysosomes (corresponding to residual bodies) shows negligible proteolysis (38). This finding indicates that a specific type of lysosome, namely the autophagolysosome, is the organelle where induced proteolysis occurs.

We report here that ML fractions from VBL-treated livers can be used for the isolation of a subcellular fraction consisting of intact AVs. The fraction is pure by morphological criteria and shows high proteolysis in vitro.

The isolation of the autophagic vacuoles makes it possible to

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identify this organelle as the subcellular locus of induced proteolysis. Isolated AVs seem to offer a promising model for further studies on intralysosomal degradation of cytosolic and membranous components. Such studies can be expected to shed some light on one of the main mechanisms for turnover of subcellular organelles. For now, it seems likely that lysosomes play a major role in degradation of intracellular proteins (14).

MATERIALS AND METHODS

Subcellular Fractionation

Fed male albino rats (150–200 g) received vinblastine sulfate (5 mg/100 g of body weight) intraperitoneally 0.5, 1, 3, and 16 h before death. The animals were anesthetized with ether and bled. All subsequent procedures were carried out at 4°C. Livers were minced in 0.3 M sucrose and homogenized (1:2 wt/vol) with four complete strokes of a loose-fitting silicone pestle rotating at 200 rpm in a Potter-Elvehjem homogenizer (clearance 0.16 to 0.24 mm). The homogenate was diluted (1:5 tissue wt/vol) and centrifuged at 460 *g* for 10 min. The pellet was suspended and resedimented once to increase the recovery of lysosomes. The pooled supernatants were then centrifuged at 24,000 *g* for 8 min. The pellet was designated ML fraction and was resuspended (0.33 g/ml) in 50% Metrizamide and 6 ml was placed at the bottom of cellulose nitrate tubes. A discontinuous Metrizamide gradient was constructed by modification of the method described by Wattiaux et al. (50). The layers from bottom to top were as follows: 6 ml of the ML suspension, 10 ml of 26%; 5 ml of 24%; 5 ml of 20%; and 5 ml of 15% Metrizamide (Fig. 1). After centrifugation at 25,000 rpm for 4 h in an SW 27 rotor (Beckman Instruments, Spinco Div., Palo Alto, CA) the fractions banding at the 24%/20% and the 20%/15% Metrizamide interphases were collected by a syringe and assayed biochemically (Fig. 1). The material floating on the 15% layer (fraction 1) and the AV fractions 2 and 3 were examined by electron microscopy.

Mitochondria were prepared by sedimenting the postnuclear supernatant (500 *g* for 10 min) at 7,000 *g* for 8 min. The pellet was washed three times to remove endoplasmic reticulum-(ER) derivatives. Microsomes were prepared by sedimenting the homogenate at 10,000 *g* for 20 min. The supernatant was then centrifuged at 100,000 *g* for 60 min. Plasma membranes were prepared by the method of Coleman et al. (12). Lysosomes from Jectofer [R]-treated rats (1, 25) were isolated on Percoll gradients (38).

Biochemical Assays

Acid phosphatase and cathepsin D were assayed as described by Bowers et al. (9). Aryl sulfatase was measured according to Berthet and de Duve (8). NADPH-cytochrome *c* reductase was determined according to Dallner et al. (13). Glucose-6-phosphatase (G6Pase) was assayed as described by Swanson (48) with 0.05% deoxycholate, and 5'-nucleotidase (AMPase) was measured according to Glaumann and Dallner (24). Succinate cytochrome *c* reductase was determined as described by Ernster (19). Protein was measured by the Lowry method (32) with bovine serum albumin as a standard. Material taken from the Metrizamide gradient was sedimented and resuspended in isotonic sucrose before biochemical and isotopic analyses, since Metrizamide interferes with some of the assays. Phospholipids (PL) (23), triglycerides (TG) (10), and cholesterol (46) were assayed as described in the references.

For measurement of degradation rats were injected intraperitoneally with 50 μCi of DL-1[^{14}C]leucine (59 mCi/mmol) or 50 μCi of [^{14}C]glycerol (53 μCi/mmol)/100 g of body weight before induction of autophagy by VBL. Aliquots from homogenates, ML fractions, and AV fractions were incubated at 37°C. At various time-points, degradation was stopped by the addition of an equivalent volume of cold 10% TCA. The samples were vortexed and centrifuged at 2,500 *g* for 10 min. TCA-soluble radioactivity was measured in a Beckman scintillation spectrophotometer (Beckman Instruments) with Aquasol as scintillation fluid. TCA-soluble radioactivity present in analogous fractions incubated at 4°C served as a blank (27, 36, 39).

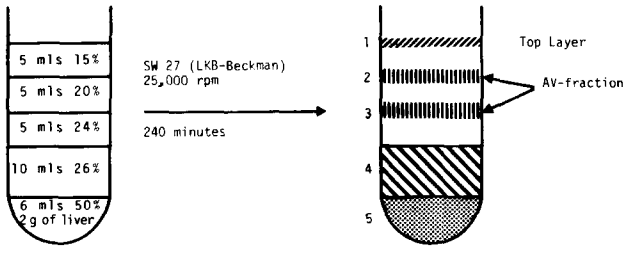

FIGURE 1 Schematic representation of the discontinuous Metrizamide gradient for isolation of autophagosomes. For details, see text.

Electron Microscopy

Livers were perfusion-fixed with glutaraldehyde as described before (26). 1-mm cubes of liver tissue were fixed in 3% glutaraldehyde in 0.1 M cacodylate (pH 7.4) containing 0.1 M sucrose for 24 h at 4°C. 1 ml of the subcellular fractions containing ~1 mg of protein was fixed with 1 ml of glutaraldehyde and placed on the bottom of a 10-ml centrifuge tube and pelleted at 35,000 *g* for 60 min. After an overnight rinse in 0.1 M cacodylate buffer (pH 7.4) containing 0.1 M sucrose, the pellets were cut through the entire depth. The resulting strip was either embedded as such or cut in the transverse plane into two or three pieces. The specimens were postfixed in 1% OsO_4 in 0.1 M *s*-collidine buffer (pH 7.4) for 1 h and dehydrated in a series of graded alcohols and propylene oxide and embedded in Epon 812 epoxy resin. 1% uranyl acetate was added to the final alcohol solution to obtain en bloc staining. Ultrathin sections were cut on diamond knives and stained with lead citrate. Sections were analyzed from top, middle, and bottom parts of the pellet. The specimens were examined and photographed in a Jeol 100 C microscope.

For morphological analyses of the AV fractions, randomly obtained electron micrographs at a standard magnification of 8,000 were examined. The relative fractional volume of AVs and the relative fractional volume of their sequestered contents were determined by point counting with a grid of 4 cm^2. Fifteen electron micrographs from different experiments were examined.

RESULTS

Electron Microscopy

AVs IN LIVER AND ML FRACTION: The ultrastructural appearance of the rat liver 3 h after the induction of autophagy with VBL can be seen in Fig. 2. All cellular components with the exception of morphologically recognizable nuclei and plasma membranes can be found sequestered in the AVs. Nascent AVs seem to lack hydrolytic enzymes (18), and the sequestered cell organelles appear ultrastructurally intact (Fig. 2). However, some of the AVs appear to have acquired hydrolytic enzymes because the organelles sequestered into these AVs show loss of distinct ultrastructural features as a sign of ongoing degradation.

By measuring degradation of radiolabeled cytoplasmic constituents by homogenates and ML fractions obtained from these VBL-treated animals, it can be shown that both autophagy and proteolysis increase concomitantly (36, 37). In addition to containing mitochondria the ML fraction contains intact autophagic vacuoles which have sequestered mitochondria, portions of ER, cytosol (glycogen, free ribosomes, and "ground substance") as well as very low density lipoprotein (VLDL) particles (Fig. 3).

ISOLATED AUTOPHAGIC VACUOLES: It seems reasonable to assume that the induced proteolysis measured in the heterogeneous ML fraction is due to the increased fractional volume of AV caused by VBL (37). To identify more precisely the subcellular site of induced degradation, attempts were made to purify the AVs in sucrose, Percoll and Metrizamide gradients using both isopycnic and rate differential centrifugation. Satisfactory results were obtained only with a discontinuous Metrizamide gradient (Fig. 1). As can be seen in Fig. 4, 3 h after VBL treatment the material banding at the interphase of 24%/20% and of 20%/15% consists predominantly of AVs, identifiable by the presence of single or occasionally multiple limiting membranes and also by the presence of sequestered cellular components in various stages of degradation. In Fig. 5, high-magnification views from this fraction are presented in an attempt to reconstruct the progressive alterations of the sur-

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rounding membranes and the contents. An electron-lucent space separates the limiting membrane(s) and the sequestered components. As degradation proceeds, only the outermost limiting membrane of the AV is preserved and becomes apposed to the material undergoing degradation. The AV finally assumes the appearance of a residual body with ultrastructurally nonrecognizable contents.

Morphometric analyses show that 90% of this fraction consists of AVs and ~8% of the remaining profiles were classified as other lysosomal forms, because they were bounded by a single membrane and contained maorphologically nonrecognizable components. As far as nonlysosomal contaminants are concerned, no mitochondria, very few Golgi apparatus or ER derivatives were present (Table I). The contents of the AVs were determined by point counting (Table I). 3 h after the induction of autophagy, the ER is the most frequently identifiable sequestered component (31%). Mitochondria are also relatively frequently seen inside the AVs (25%). Free ribosomes and glycogen particles are present more infrequently (9%). The uptake of cytosolic "ground substance" could not be quanti-

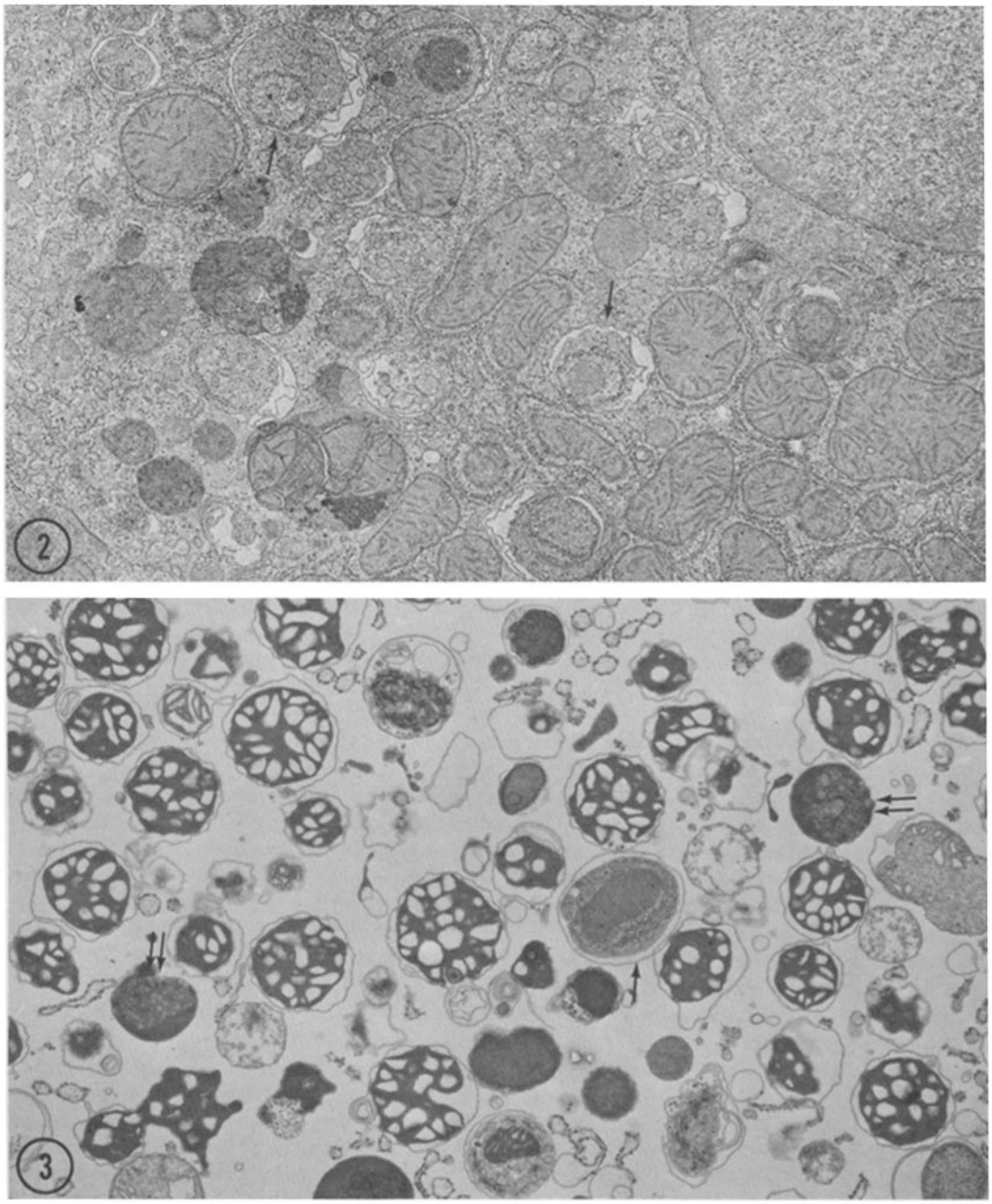

FIGURE 2 Ultrastructural appearance of liver parenchymal cell after the induction of autophagy. 5 mg of VBL/100 g of body weight was administered 3 h before sacrifice. Nascent autophagosomes (arrows) containing cytosol, portions of ER, and secretory vesicles are seen in this cell. × 12,000.

FIGURE 3 Ultrastructural appearance of ML fraction after the induction of autophagy. VBL was administered as indicated in Fig. 2, and an ML fraction was prepared as described in Materials and Methods. The ML fraction after VBL treatment is typically enriched (three- to fourfold over control) in AVs (38). Note the presence of nascent AVs (arrow) and autophagosomes containing VLDL-like particles (double arrows). × 12,000.

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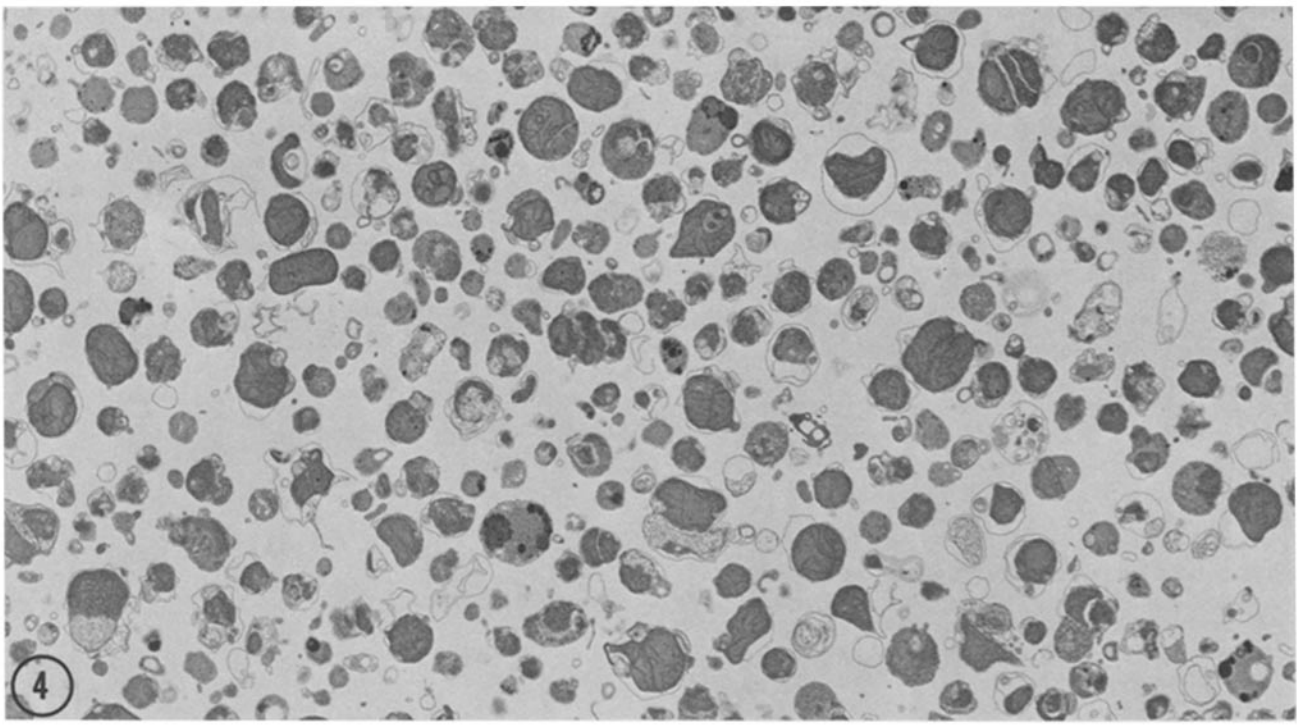

FIGURE 4 Low-magnification micrograph of the isolated AV fraction. 5 mg of VBL/100 g of body weight was administered 3 h before sacrifice, and an ML suspension was subfractionated on a discontinuous Metrizamide gradient. The material banding at the 24%/20% and at the 20%/15% interphase was pooled and processed for electron microscopy as described in Materials and Methods. Note the heterogeneity in size, shape, and content of the AVs as well as the good preservation of ultrastructure. × 8,000.

tated by morphological analyses for obvious reasons. It is likely therefore that the value for the cytosol is underestimated. Finally, Golgi apparatus components and peroxisomes are seen very rarely.

The material floating on the 15% Metrizamide layer (Fig. 6) was also examined by electron microscopy. In this fraction the AVs appear to contain more lipidlike inclusions as well as myelin figures and cytosolic ground substance. Large profiles of cytoplasm surrounded by one limiting membrane are at times seen in this fraction. We do not know at present whether these profiles are true lysosomal forms of profiles which have been created during homogenization by the shearing and subsequent sealing of membrane sheets (for example, plasma membranes). Occasional Golgi apparatus components and smooth or rough surfaced vesicles are also seen extralysosomally.

By isolating the AV fraction at different time-points after the induction of autophagy by VBL, we tried to determine whether the AVs showed proteolysis and lipolysis, and we searched for biochemical and morphological differences between autophagosomes. This approach was motivated by our previous finding that there is a relationship between intervals of VBL pretreatment and advancement of degradation of AVs (36).

30 MIN OF VBL-PRETREATMENT: The material banding at 24%/20% and 20%/15% is presented in Fig. 7. This fraction consists of typical lysosomal dense bodies with an electron-lucent halo between the membrane and contents. Many dense bodies are vacuolated and in ring forms. These lysosomes correspond to the lysosomal fraction isolated by Wattiaux et al. (50). In addition, short-time VBL-pretreatment resulted in the formation of AVs containing cytosol but no mitochondria.

60 MIN OF VBL-PRETREATMENT: By this time-point the fraction consisted of typical AVs containing sequestered portions of ER, mitochondria, in addition to cytosol (not shown). Both nascent AVs and AVs showing various stages of degradation of their sequestered material were present.

16 h OF VBL-PRETREATMENT: By long exposure of VBL the AVs were found to contain preferentially amorphous material, indicative of advanced stages of degradation (Fig. 8).

In Vitro Degradation in Autophagic Vacuoles

LABELING STUDIES: After labeling with [^{14}C]leucine, autophagy was induced with VBL and proteolysis was measured in the homogenate and along the gradient as the release of TCA-soluble radioactivity during in vitro incubation (27, 36, 39). As can be seen in Table II, 3 h of VBL treatment increases the net proteolytic activity in the homogenate by 50–60%. The total proteolytic activity was increased by 80–100% in the AV fractions (2+3) and by 40% in fractions 4 and 5. The enrichment of proteolysis over the homogenate (TCA-soluble activity per mg protein) was 10- to 20-fold in the AV fractions and twofold in fractions 4 and 5. VBL does not significantly alter the enrichment factor for proteolysis in any of the fractions. The radioactivity in the TCA-supernatants were only a minor fraction in comparison to the precipitable activity. In the control rat, proteolytic rates were not fully linear with time as were those after VBL treatment.

The release of TCA-soluble radioactivity from isolated AVs was followed after [^{14}C]glycerol labeling. In Fig. 9, the rats were pretreated with VBL for 60 min. The proportion of soluble radioactivity is small in the AV fraction as compared to leucine labeling described above.

MORPHOLOGICAL CORRELATION: The appearance of the AV fraction following 60 min of in vitro incubation is presented in Fig. 10. As can be seen, the limiting membranes of the AVs are relatively intact. However, the sequestered

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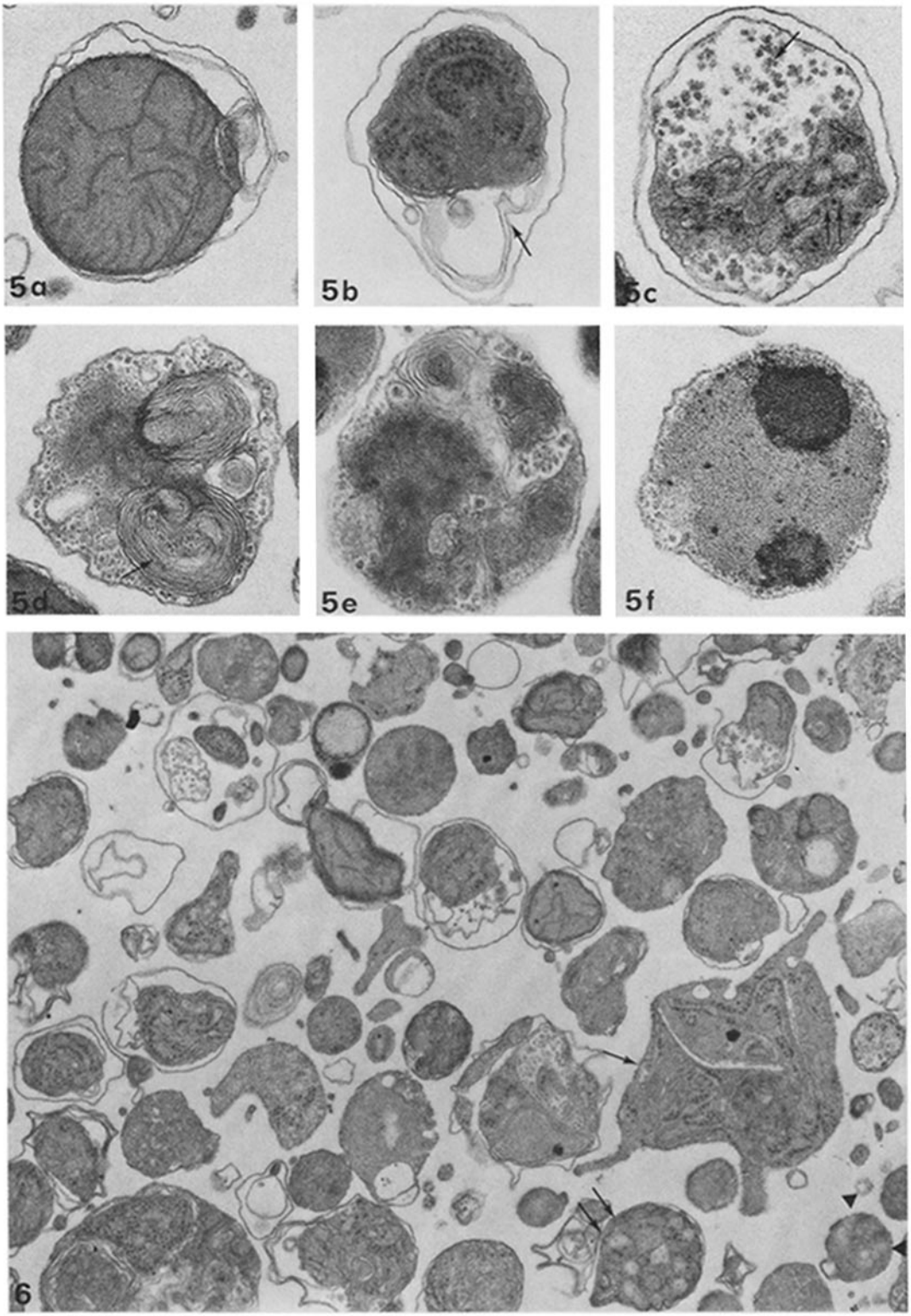

FIGURE 5 Ultrastructural appearance of AVs at various stages of degradation. High-magnification views from the AV fraction chosen to demonstrate sequestered contents and stages of degradation. Almost all the sequestered organelles show some loss of ultrastructural details and increased electron density (*a–c*). A prominent electron-lucent space separates the sequestered material from the limiting membranes in the earlier stages (arrow) (*b*). Glycogen particles (arrow) and ribosomes (double arrows) are seen in the same AV (*c*). Older AVs are surrounded by only one limiting membrane which appears more closely apposed to the material being degraded (*d–f*). At this stage, myelin figures (arrow) and dense deposits are often seen (*d*). *f* shows the final stage of the degradation process which results in the formation of a typical dense body. 3 h of VBL pretreatment. *a*, × 35,000; *b*, × 40,000; *c*, × 47,000; *d*, × 43,000; *e*, × 34,000; and *f*, × 54,000.

FIGURE 6 Ultrastructural appearance of the material recovered at the top of the Metrizamide gradient (top layer). 5 mg of VBL/100 g of body weight was administered 3 h before sacrifice. Note the presence of large membrane profiles containing portions of cytoplasm (arrow). Lipidlike densities are frequently seen in the AVs found in this fraction (double arrows). Vesicles containing VLDL-like particles are also seen in this fraction (arrowheads). × 22,000.

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TABLE I

Morphological Analysis of Isolated Autophagosomal Fraction 3 h after the Induction of Autophagy

	Profiles outside the autophagosomes	Profiles inside the autophagosomes
	%	
Nonidentifiable	0	27
Mitochondria	0	25
Lysosomes	8	0
Endoplasmic reticulum	<1	31
Golgi apparatus	<1	1
Cytosol (ribosomes and glycogen particles)	<1	9
Peroxisomes	<1	0

Fraction purity 90%. The relative fractional volume of cellular components in the AV fraction was determined by point counting as described in Materials and Methods.

organelles show more advanced stages of degradation such as amorphous material when compared to the nonincubated fraction (Figs. 4 and 5).

Marker Enzymes

The AV fraction 1 h after the induction of autophagy was also characterized by assay of typical "marker" enzymes. Table III shows that cathepsin D is enriched approximately 15-fold in the AV fraction as compared to the homogenate. A much higher enrichment of cathepsin D is obtained in a purified lysosomal fraction from iron-treated rats (1, 2, 25). In Fig. 11, it can be seen that there is some increase in the recovery of protein following longer exposure times of VBL. Up to 60 min after VBL, mitochondrial and ER marker enzymes also increase in the AV fraction. Since few mitochondrial and ER profiles were seen to contaminate the AV fraction, the enzyme activities are most likely due to the sequestration of these organelles into the AV. By 180 min after the administration of

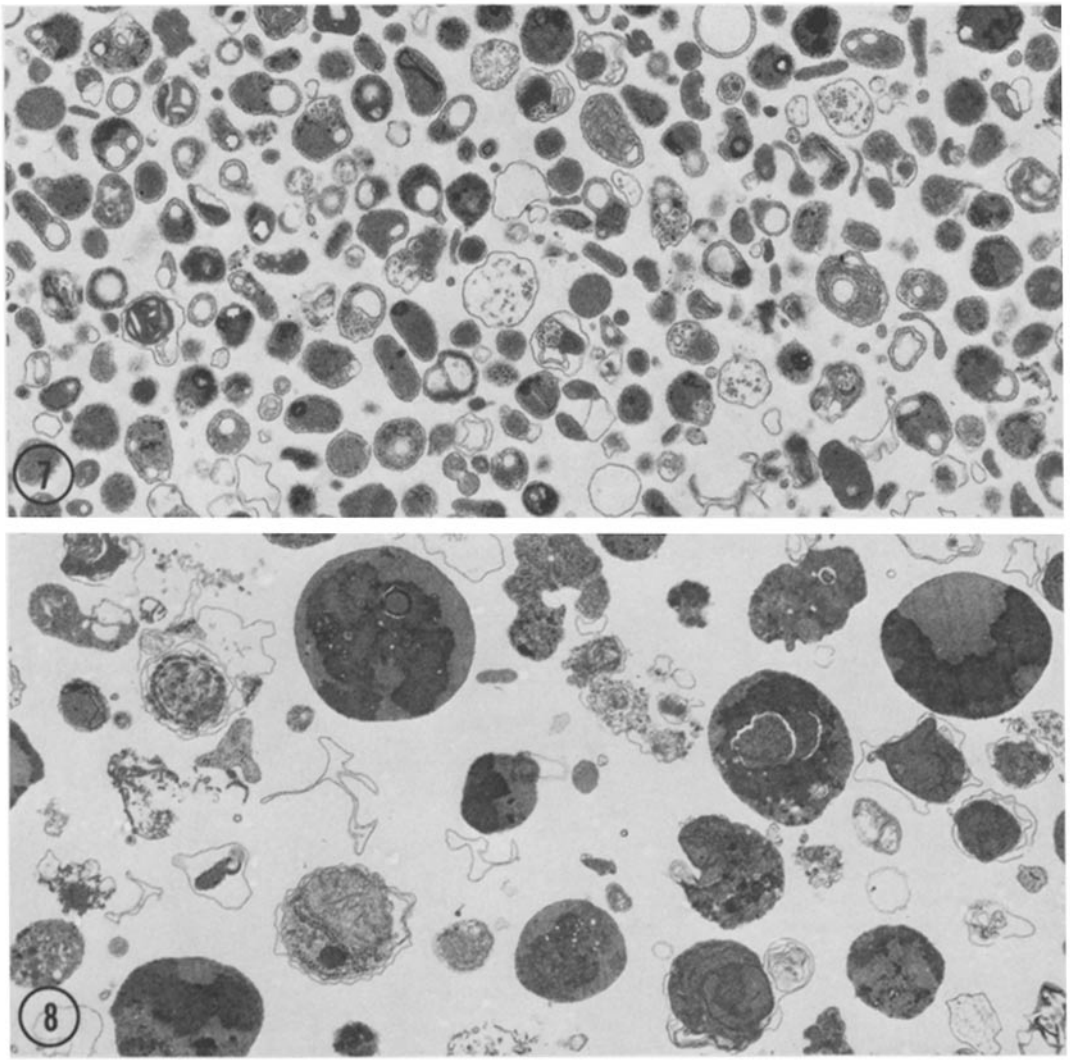

FIGURE 7 Ultrastructural appearance of the AV 30 min after the induction of autophagy. By this early time-point, the AV compartment has not expanded remarkably. The AVs seen contain almost exclusively cytosolic components and in particular glycogen as an indication of microautophagy. The predominant component of this fraction is typical dense bodies of heterogeneous shape and size corresponding to the lysosomal fraction isolated by Wattiaux et al. (50). × 18,000.

FIGURE 8 Ultrastructural appearance of the AV fraction 16 h after the induction of autophagy. By this time-point, the number of AVs containing amorphous, degraded material increases. × 14,000.

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TABLE II

Proteolysis in Liver Homogenate and along the Gradient of Control and VBL-treated Rats

Fraction	Control				180 min of VBL exposure			
	TCA-soluble		TCA-pellet		TCA-soluble		TCA-pellet	
	30 min	60 min	30 min	60 min	30 min	60 min	30 min	60 min
Homogenate								
Dpm/g of liver	4,175	5,415	908,020	887,610	4,766	7,695	1,036,000	984,000
Dpm/mg protein	16	22 (1)	3,661	3,579	22	35 (1)	4,863	4,617
Metrizamide gradient								
Fraction 1								
Dpm/g of liver	139	141	1,683	1,635	120	160	3,119	2,834
Dpm/mg protein	198	201 (9)	2,405	2,335	171	229 (7)	4,455	4,048
Fraction 2								
Dpm/g of liver	169	227	2,150	1,924	260	448	6,664	6,578
Dpm/mg protein	376	504 (23)	4,777	4,276	553	953 (27)	14,179	13,995
Fraction 3								
Dpm/g of liver	328	405	3,796	3,752	472	724	12,461	12,116
Dpm/mg protein	410	506 (23)	4,745	4,690	278	426 (12)	7,330	7,127
Fraction 4+5								
Dpm/g of liver	1,426	1,805	93,855	90,736	1,638	2,581	86,888	85,472
Dpm/mg protein	41	52 (2)	2,681	2,592	47	74 (2)	2,482	2,442
Recovery of proteolysis in gradient fractions	49%	48%			52%	51%		

Animals were prelabeled with DL-[^{14}C]leucine (50 μCi/100 g). 16 h later the treated rats received 5 mg vinblastine/100 g. 180 min later the rats were killed, the livers homogenized, and an ML fraction was prepared and layered on the Metrizamide gradient. Fractions (Fig. 1) were collected and incubated at 37°C. TCA-soluble and -insoluble (TCA-pellet) radioactivity was measured. The numbers in parentheses denote enrichment (homogenate = 1) and recovery (homogenate = 100%) of proteolysis. Means of two experiments.

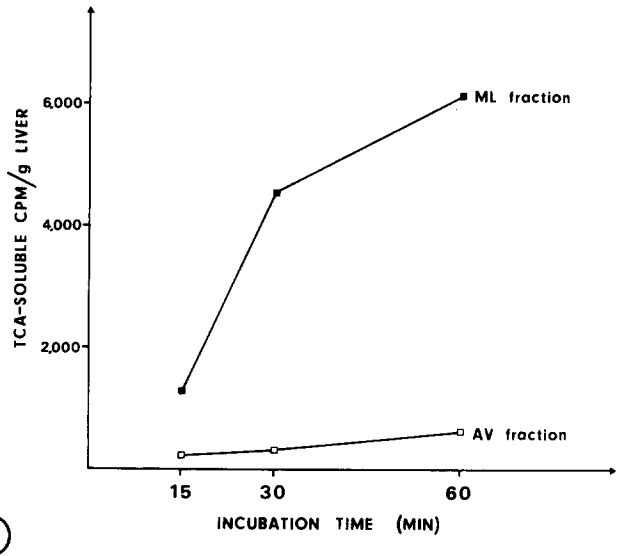

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FIGURE 9 In vitro release of TCA-soluble radioactivity from [^{14}C] glycerol-labeled autophagosomes. Rats received 50 μCi [^{14}C]-glycerol/100 g of body weight intraperitoneally 60 min before the induction of autophagy. The release of TCA-soluble radioactivity during in vitro incubation was determined in both the ML fraction and the isolated AVs. Blanks were incubated at 4°C. VBL exposure, 120 min.

VBL, some decrease in the activities of the nonlysosomal markers was seen (e.g., G6Pase and succinate cytochrome *c* reductase) indicative of ongoing proteolysis and inactivation of the enzymes.

As can be seen in Table IV, vinblastine like glucagon (17) does not alter the total activity of acid hydrolases in the liver homogenate. Furthermore there was little or no change in the ML fraction despite the increased presence of AV in this fraction (37) after induced autophagy. The specific and total activities of acid phosphatase and cathepsin D along the gradient are also shown in Table IV. The only obvious change occurred in the lower part of the gradient, with 100% increase in the recovery of both cathepsin D and acid phosphatase activity in fractions 4 and 5 together by 60 and 180 min of VBL exposure. As to the AV fractions (fractions 2 and 3), the total recovery was not significantly altered for either acid phosphatase or cathepsin D. Consequently, since the protein recovery in fractions 2 and 3 increased by 50%, the specific activities of the lysosomal enzymes decreased to the same extent. There was some over-recovery (30%) in total lysosomal enzyme activities in the gradient as compared to the original material placed in the gradient. Most of this could be accounted for by an over-recovery in protein.

Protein and Lipid Content

It was finally of some interest to determine the PL/protein, TG/PL and cholesterol/PL ratios of the isolated AVs. This could provide some clue as to the origin of the membranes contained in the AVs and might give some information on the relative rates of degradation of these membrane constituents. As can be seen from Table V, the AV fraction is enriched in PL and TG compared to the ML fraction. The high PL/protein ratio may possibly result from a relatively slow rate of degradation of membrane phospholipids compared to protein. The high TG-content may reflect sequestration of cytosolic TG and of secretory granules containing VLDLs as well as crinophagy (fusion between VLDL-filled secretory vesicles and AVs) which was demonstrated to occur after the administration of VBL (38).

DISCUSSION

All subcellular components such as protein have been shown to undergo random replacement (22, 42). The different molec-

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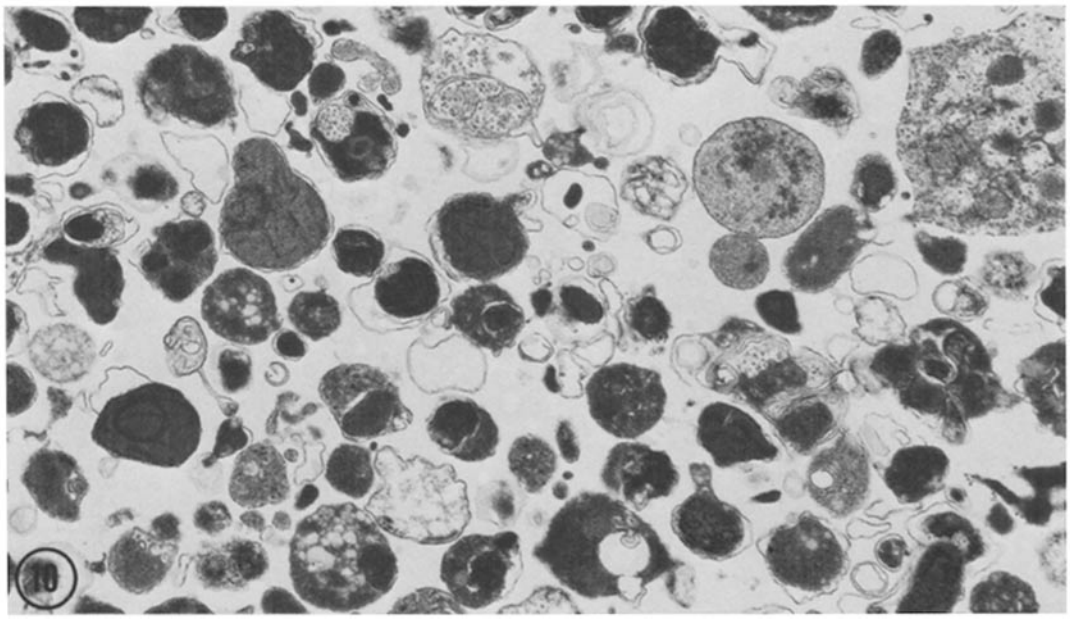

FIGURE 10 Ultrastructural appearance of isolated autophagic vacuoles after in vitro incubation for 60 min. Autophagic vacuoles isolated 3 h after the induction of autophagy were incubated in 0.3 M sucrose for 60 min. Note the relatively good preservation of AV ultrastructure. The sequestered contents appear more amorphous and electron-dense when compared to the unincubated AV fraction (Figs. 4 and 5). × 18,000.

TABLE III

Distribution of Protein and Marker Enzymes in Isolated Subcellular Fractions

	Protein*	Succinate cytochrome *c*‡ reductase	Cathepsin D§	AMPase‖	G6Pase‖	NADPH-cytochrome *c*‡ reductase
Homogenate	208	95	0.0074 (100%)	1.8	1.2	29
ML fraction	34	260	0.0232 (51%)	1.0	1.7	17
Microsomes	19	20	0.0071	1.1	4.9	130
Plasma membranes	1.7	—	—	18	0.6	—
Ferritin-laden lysosomes	0.67	—	0.25	3.8	0.35	0
AVs	1.7	98	0.112 (10%)	4.6	1.12	0

Animals were treated with 5 mg of vinblastine for 1 h before subcellular fractionation. The ML fraction was pelleted at 24,000 *g* for 8 min. The numbers in parentheses designate enzyme recovery related to the homogenate as 100%.

* mg/g liver.

‡ mmoles reduced cytochrome *c*/min/mg protein.

§ μmoles of tyrosine/released/min/mg protein.

‖ μmoles of P_i relased/20 min/mg protein.

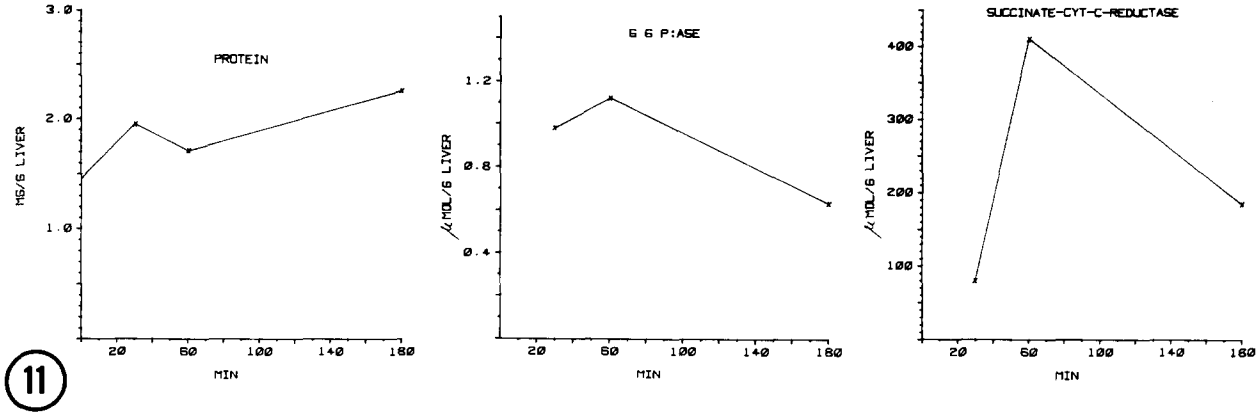

FIGURE 11 Recovery of protein and marker enzymes in the isolated AV fractions as a function of time after the induction of autophagy. 30, 60, and 180 min on the abscissa denote time of VBL-pretreatment before fractionation. For further details, see Materials and Methods. Succinate-cytochrome *c* reductase: μmoles reduced cytochrome *c*/min/g liver. G6Pase: μmoles of P_i released/20 min/g liver.

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TABLE IV

Distribution of Protein, Acid Phosphatase, and Cathepsin D in Homogenate, ML Fraction, and along the Gradient

	Protein				Acid phosphatase				Cathepsin D			
	C	30 min	60 min	180 min	C	30 min	60 min	180 min	C	30 min	60 min	180 min
	mg/g of liver				*μmol P_i released/min/mg protein*				*μmol tyrosine released/min/mg protein*			
Homogenate	230	235	208	213	2.6	2.5	2.6	3.0	0.006	0.0055	0.0074	0.0075
					(598)	(588)	(541)	(639)	(1.4)	(1.3)	(1.5)	(1.6)
ML fraction	36	35	34	40	7.4	7.5	7.9	7.4	0.021	0.020	0.028	0.025
					(266)	(263)	(269)	(296)	(0.76)	(0.70)	(0.95)	(1.00)
Gradient												
1 = Top Layer	0.91	0.89	0.73	0.79	18.7	16.4	11.9	16.0	0.044	0.042	0.042	0.042
					(17.0)	(14.6)	(8.7)	(12.6)	(0.040)	(0.037)	(0.031)	(0.033)
2 (AV fraction)	0.51	0.67	0.55	0.43	71.8	58.9	26.5	28.9	0.16	0.14	0.067	0.059
					(36.6)	(39.5)	(14.6)	(12.4)	(0.082)	(0.093)	(0.037)	(0.025)
3 (AV fraction)	0.95	1.28	1.16	1.83	92.4	55.4	80.6	54.6	0.17	0.11	0.13	0.11
					(87.8)	(70.9)	(93.5)	(99.9)	(0.16)	(0.14)	(0.15)	(0.20)
4	14.5	12.5	17.6	19.5	6.1	8.0	8.5	7.7	0.015	0.013	0.024	0.027
					(88.5)	(100)	(150)	(150)	(0.22)	(0.16)	(0.42)	(0.53)
5	21.3	26.3	20.2	20.6	4.0	3.5	4.8	7.7	0.010	0.0096	0.018	0.017
					(85.2)	(92.1)	(97.0)	(159)	(0.21)	(0.25)	(0.36)	(0.35)
Recovery in fraction 1–5	38.2	41.6	40.2	43.2	315	317	364	434	0.71	0.68	1.00	1.14

The rats were pretreated with VBL at 30, 60, and 180 min before death. Homogenate, ML fraction and gradient fractions, numbered as in Fig. 1, were prepared as described in Materials and Methods. The values in parentheses are the total activity per gram of liver. Means of two experiments. *C*, control.

TABLE V

Protein, PLP, Cholesterol, and Triglyceride Content in Isolated AV Fraction

	Protein*		PLP*		Cholesterol*		TG*	PLP/protein		Cholesterol/PLP		TG/PLP
	1 h	3 h	1 h	3 h	1 h	3 h	3 h	1 h	3 h	1 h	3 h	3 h
Homogenate	222	231	29.8	26.3	1.82	1.88	4.31	0.13	0.11	0.061	0.071	0.16
ML fraction	40	42	12	16	0.38	0.58	0.29	0.30	0.38	0.032	0.036	0.018
AV fraction	1.90	2.20	1.12	1.18	0.084	0.12	0.25	0.60	0.54	0.075	0.100	0.21

The rats were given 5 mg of Vinblastine/100 g of body weight 1 or 3 h before death.
* mg/g liver.

ular constituents, i.e., lipids and enzymes, of the same membrane turn over at different rates (31). In addition, many membrane components turn over by exchange reactions not associated with degradation (51). In the liver, the steady state turnover can be affected by alterations in homeostasis, such as occur during amino acid infusion (41) and insulin (40, 43, 49) or glucagon (5) administration.

Mortimore and co-workers (41) have presented evidence which suggests that the lysosomal compartment is the site where induced degradation takes place. Seglen et al. (47) and Dean (15) using cultured cells have shown that the lysosomes play an important role in modulating both physiologic degradation and the induced degradation which occurs during pathological conditions. Heterophagy of cell organelles (26–28) is an additional experimental model of studying intralysosomal degradation of membranes. The classical studies of de Duve and co-workers and others (3, 4, 11, 20, 29, 33, 34, 44) have proven the capacity of the lysosomes to degrade nearly all biological substrates in vitro.

An extralysosomal pathway of degradation has also been proposed (7). The presence of some extralysosomal hydrolases, nonexponential decay curves for a mixture of liver proteins (22), the different half-lives of closely related membrane constituents, and a general correlation betweena protein structure and degradation rates represent the main evidence for a nonlysosomal site of degradation. On the other hand, recent data indicate that different proteins are degraded at different rates when introduced into the lysosomal apparatus (28).

Here, by using the expansion of autophagosomal compartment induced by VBL (6, 36, 38), we have isolated AVs, taking advantage of the method of Wattiaux et al. (50) for the isolation of lysosomes in a Metrizamide gradient. The AVs formed shortly (30 min) after the induction of autophagy contained for the most part glycogen interspersed with what appeared to be cytosolic ground substance and rarely ER derivatives or mitochondria. By 60 min, all recognizable cellular compartments were found sequestered in the AVs.

The purity of the AV fraction is ~90%, with only a few percent contamination with nonlysosomal organelles. The remainder of the fraction consists of other types of lysosomes similar to those isolated by the Wattiaux method (50). In agreement with morphological analysis, by 30 and 60 min, following the induction of autophagy, both ER and mitochondrial enzymes were recovered in increasing amounts in the AV fraction. Later on, with more advanced stages of degradation, the activities of nonlysosomal markers such as G6Pase and succinate cytochrome *c* reductase seemed to decline in the AV fraction. A crude correlation can be said to exist between the preservation of the ultrastructure of the sequestered organelles and the presence of associated enzymatic activities.

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At first sight the enrichment of hydrolytic enzymes in the AV fraction may appear somewhat low when compared to isolated lysosomes (2, 25, 50). However, since the AV contains various cell organelles and cytosol and thus an "excess" of substrate protein, the enrichment factor for lysosomal enzymes is not a reliable criterion for calculation of fraction purity. The morphological analysis should be considered the most useful tool available to estimate purity of AV preparations. The isolation of autophagic vacuoles and the demonstration that these organelles manifest autoproteolysis permits the identification of the AVs as the subcellular site of induced protein degradation.

Mechanism of Induced Protein Degradation by Autophagy

The only apparent mechanism for the formation of AVs in the VBL-treated liver, which can be detected morphologically, seems to be through the formation of pockets of ER cisternae which fuse and sequester portions of cytoplasm (6, 18, 37). As far as we can infer from acid phosphatase histochemistry, these nascent AVs lack hydrolytic enzymes and acquire them by fusion with mature AVs or other secondary lysosomes (38). VBL caused some increase in total lysosomal enzyme activities recovered in the lower positions (fractions 4+5) by 60 and 180 min of exposure. There was no significant increase in the total activities of either acid phosphatase, aryl sulfatase, or cathepsin D after VBL treatment (36). The study by Deter et al. (17) demonstrated that, for each AV formed, two or three dense bodies disappeared from the ML fraction. According to their interpretation, preexisting lysosomes (preferentially the smaller ones) fuse with AVs formed by other membrane structures such as the ER. Similarly, Ward et al. (49) described a translocation of hydrolytic enzymes within various forms of lysosomes after insulin and amino acid treatment of livers during perfusion in vitro.

In our case the losses of activity of acid phosphatase and cathepsin D in the top layer are too small to account for the observed increases in these activities in fractions 4 and 5. The lysosomal activities that appear in both the AV fractions and fractions 4 and 5 may come from ER sequestered into AVs or from other yet unidentified sources. Therefore, at present we have no satisfactory explanation for the increase in lysosomal enzyme activities in the lower part of the gradient after VBL treatment.

Interestingly, the very nascent AVs (Figs. 2 and 3) are seldom recovered in the AV fractions (Fig. 4). Therefore fusion with lysosomes and partial degradation of the sequestered material seem to be a prerequisite for decreasing the density of the AV so that only the more "mature" AVs equilibrate as a purified fraction in the upper part (fractions 2 and 3) of the Metrizamide gradient. Most AVs showed positive reaction products when incubated for demonstration of acid phosphatase, which also supports the notion that they have fused with pre-existing lysosomes (unpublished observations). Alterations in the density of the sequestered membranes due to different rates of permeation of degradation products out of the AV could possibly explain this shift in density between nascent and mature AVs. Another alternative could be that proteins in general are degraded faster inside the AV than are lipids (28). Additionally, as the AVs mature the permeability of the surrounding membrane may change. Microsomal membranes (ER) are relatively permeable to Metrizamide (789 mol wt) (21), whereas lysosomes are impermeable to compounds with a molecular weight of 400 or greater (45). This may suggest that the permeability of the bounding membrane of the nascent AVs is similar to that of the ER membranes. Nascent AVs would thus be relatively permeable to Metrizamide and consequently show a higher density in such a gradient medium in comparison with mature AVs.

As discussed in a previous article (36), the increase in degradation measured in the ML fraction after VBL treatment is due to an enrichment in degradable substrate (cytoplasm) inside the lysosomal compartment without any significant increase of lysosomal enzyme activities. These findings are in agreement with the studies of Deter and co-workers on glucagon-induced autophagy (16, 17). There the increase in the AVs was paralleled by a decrease in dense bodies. In our study, by the time-points selected, an expansion of both the autophagic vacuolar and residual body volume has occurred in the liver and in the ML fractions (37, 38). Since there is no increase in lysosomal enzymes in VBL-treated liver, this indicates that these residual bodies represent in part the end stage of the degradation inside the AV.

Incubation of the isolated AVs shows that they possess high proteolysis in vitro with an enrichment factor of 12 to 27 over the homogenate. By 180 min of VBL exposure, ~50% of the proteolysis in the homogenate is recovered in the gradient. VBL caused a 100% increase in proteolysis in the AV fractions (2 and 3) and a 50% in the lower part of the gradient. Interestingly, in the control rat, fractions 2 and 3 also exhibited relatively high rates of proteolysis in spite of lack of morphological signs of true AVs. However, fractions 2 and 3 from control rat contain various types of secondary lysosomes which apparently also possess proteolytic activity. The enrichment factor for proteolytic activity in control lysosomes and AVs after VBL was approximately similar. The total proteolytic activity was, however, higher after VBL treatment. As was discussed above, calculations of enrichment for an AV fraction which contains sequestered organelles is not meaningful when compared with control.

As to whether or not the relative fractional volume of the contents of the isolated AVs reflects the frequency of sequestration and/or the rate of degradation of the various cell organelles in vivo, no definite conclusion can be drawn at present. We are now studying this question by altering the relative fractional volume of cell organelles before the induction of autophagy.

During in vitro incubation, some release of TCA-soluble radioactivity was seen in the AVs labeled with [^{14}C]glycerol. Both the recovery and the absolute amount of this "lipolytic" activity were less than that seen in the AVs labeled with [^{14}C]leucine. Moreover the AV fraction was enriched in phospholipids compared to other cell organelles (23), and electron microscopy showed an increase in lipidlike deposits at later time-points after the induction of autophagy. This suggests that phospholipids may be degraded relatively slowly inside the AV (28).

In conclusion, AVs can be isolated in adequate purity and with good preservation of ultrastructure. The AV is the subcellular locus of induced protein degradation. The hydrolytic enzymes of the AVs are derived at least in part from preexisting lysosomes. Further characterization of the isolated AVs may add more insight into the mechanisms of sequestration and degradation of cellular components and it may help to clarify the exact role of autophagy in the turnover of cell organelles and cytosol.

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