Studies on the Mechanisms of Autophagy: Formation of the Autophagic Vacuole

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Abstract. Autophagic vacuoles form within 15 min of perfusing a liver with amino acid-depleted medium. These vacuoles are bound by a "smooth" double membrane and do not contain acid phosphatase activity. In an attempt to identify the membrane source of these vacuoles, I have used morphological techniques combined with immunological probes to localize specific membrane antigens to the limiting membranes of newly formed or nascent autophagic vacuoles. Antibodies to three integral membrane proteins of the plasma membrane (CE9, HA4, and epidermal growth factor receptor) and one of the Golgi apparatus (sialyltransferase) did not label these vacuoles. Internalized epidermal growth factor and its membrane receptor were not found in nascent autophagic vacuoles but were present in lysosome-like degradative autophagic vacuoles. All these results suggested that autophagic vacuoles were not formed from plasma membrane, Golgi apparatus, or endosome constituents. Antisera prepared against integral membrane proteins (14, 25, and 40 kD) of the RER was found to label the inner and outer limiting membranes of almost all nascent autophagic vacuoles. In addition, ribophorin II was identified at the limiting membranes of many nascent autophagic vacuoles. Finally, secretory proteins, rat serum albumin and alpha$_{2u}$-globulin, were localized to the lumen of the RER and to the intramembrane space between the inner and outer membranes of some of these vacuoles. The results were consistent with the formation of autophagic vacuoles from ribosome-free regions of the RER.

CELL growth is governed by a delicate balance between the synthesis and degradation of proteins. Protein degradation may occur by a lysosomal or nonlysosomal mechanism (8, 16, 26, 31). Entry of proteins into lysosomes can occur by fusion of endocytic or autophagic vesicles, by invagination of the lysosomal membrane, or by prp73-mediated transport (5, 8, 10, 12, 26, 29). Although endocytosis may be important in the turnover of plasma membrane proteins (12), lysosomal degradation of endogenous proteins occurs primarily through autophagic mechanisms (26, 32, 38). Autophagy has been implicated in the degradation of normal proteins in response to nutrient deprivation; in the cellular remodeling that occurs during differentiation, metamorphosis, aging, and transformation; in growth of the uterus during gestation and its atrophy after birth; and in the removal of abnormal cellular components that accumulate during cell toxicity or death (6, 26, 27, 45, 46). In addition, the inhibition of autophagy is coincident with a decrease in protein degradation and net cell growth (26, 28, 30, 39).

Autophagy is a general mechanism occurring in many cell types whereby intracellular organelles and cytosol are first sequestered away from the remaining cytoplasm and then degraded within lysosomes. The first recognizable step in this process is the sequestration of a region of cytoplasm by a double-membrane bound vacuole lacking acid phosphatase and aryl sulfatase activities: the nascent autophagic vacuole or autophagosome (AVi)[1] (1, 13, 37, 38). Acid hydrolases are then introduced by fusion of the initial vacuole with preexisting lysosomes or Golgi elements to form a single-membrane bound degradative vacuole or autolysosome (AVd). The loss of the inner membrane is presumably due to hydrolysis or fusion with the outer membrane. The evidence suggests that vacuole formation can be regulated by a variety of physiological (e.g., amino acids and hormones) as well as artificial (e.g., stress) effectors (26, 33). In addition to being the site of regulation, this sequestration event may also dictate those cellular components destined for degradation (8, 20, 23). Therefore, it becomes important to understand the mechanisms and regulation of the formation of the autophagic vacuole.

Despite earlier attempts, the source of the limiting double membrane remains unclear (1, 13, 17, 29, 36). In this report, an immunological approach was used to characterize those protein constituents of the limiting membranes of the newly formed autophagic vacuole. Membrane antigens of plasma membrane, Golgi apparatus, and endosomes were not found in these vacuoles. However, specific membrane proteins of the RER were localized to the limiting membranes of AVis.

1. *Abbreviations used in this paper*: AVd, degradative autophagic vacuole or autolysosome; AVi, nascent autophagic vacuole or autophagosome; TBST, 10 mM Tris, pH 7.2/0.5 M NaCl/0.3% Tween 20.

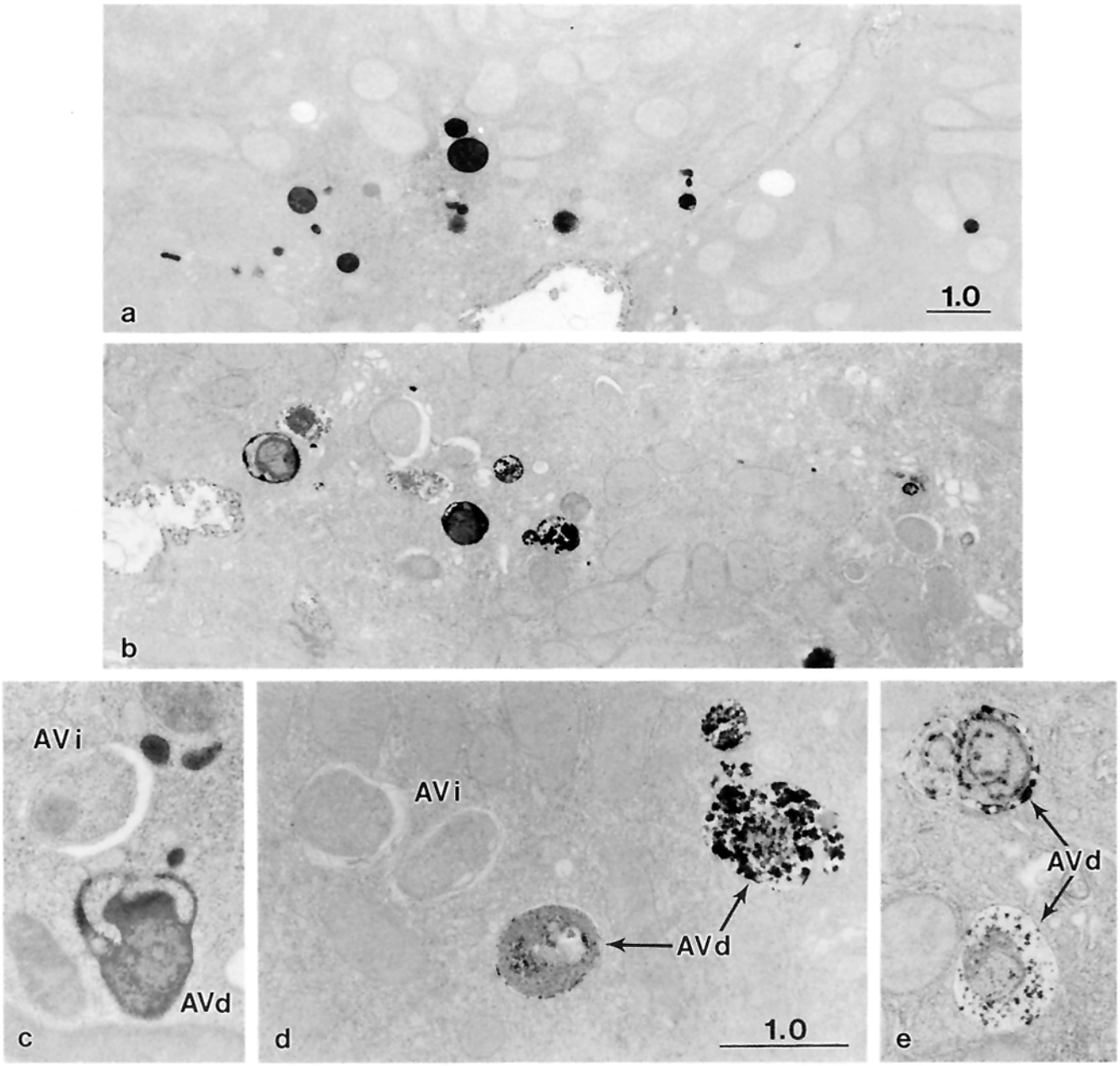

Figure 1. Morphological characterization of autophagic vacuoles. Livers were perfused for 90 min with media enriched with amino acids and containing insulin (*a*) or depleted of amino acids (*b–e*), fixed with 2% glutaraldehyde/2% paraformaldehyde/0.1 M cacodylate, pH 7.4, and processed for acid phosphatase cytochemistry. The omission of amino acids from the perfusion medium resulted in the accumulation of numerous autophagic vacuoles (*b*). These vacuoles were routinely found to contain mitochondria, peroxisomes, and cytoplasmic components, e.g., ribosomes. However, vacuoles containing recognizable RER were not visualized. Despite their diverse morphological appearance, these vacuoles have been classified morphologically and cytochemically into two groups: (*1*) nascent autophagic vacuoles or autophagosomes (*AVi*) and (*2*) degradative autophagic vacuoles or autolysosomes (*AVd*) (see text). Bar, 1.0 μm.

Materials and Methods

Materials

Most reagents were purchased from Sigma Chemical Co. (St. Louis, MO), from Bio-Rad Laboratories (New York, NY), or from Fisher Scientific (Springfield, NJ). Sprague-Dawley rats were purchased from Charles River Breeding Laboratories (Wilmington, MA) or University of Florida Breeding Facility (Gainesville, FL). New Zealand white female rabbits were obtained from Bunnyville Farms, Littleton, PA. Rabbit anti-rat serum albumin was purchased from Cooper Biomedical (Malvern, PA); Freund's adjuvant from Difco Laboratories Inc. (Detroit, MI); LRGold resin from Polysciences, Inc. (Warrington, PA); and horseradish peroxidase-conjugated sheep anti-mouse Fab and horseradish peroxidase-conjugated sheep anti-rabbit Fab from Pasteur Productions (Marnes La Coquette, France). Epidermal growth factor was conjugated to horseradish peroxidase as previously described (10). Protein A (Pharmacia Inc., Piscataway, NJ) was iodinated using the chloramine T method (12).

Preparation and Characterization of Antibodies

Antibodies recognizing plasma membrane proteins, CE9, HA4, and EGF receptor, have been previously characterized (12, 19). Antiserum to α2,6 Gal β1, 4 GlcNAc sialyltransferase was prepared by Dr. G. Hart (Johns Hop-

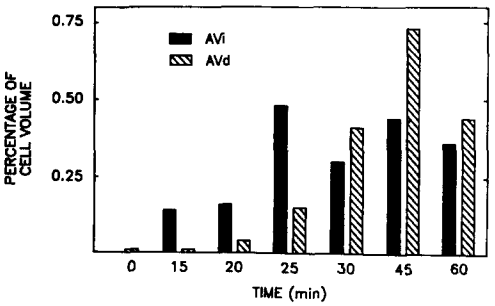

Figure 2. Morphometric quantitation of the autophagic response. Livers from previously fasted rats were excised and perfused with media enriched with amino acids and containing insulin (14 nm). At 90 min, the media was switched to one depleted of amino acids and containing glucagon (86 nm). The perfusion was continued for selected periods of time, the liver fixed by perfusion and processed for acid phosphatase cytochemistry. The relative volumes of nascent and degradative autophagic vacuoles were measured and expressed as a percentage of cell volume as described in Materials and Methods.

kins University, Baltimore, MD) against an antigen purified from rat liver. On Western blots, this antiserum recognized a single protein band corresponding to the 47-kD sialyltransferase (data not shown).

Rough microsomes were prepared from a postmitochondrial supernatant obtained from rat liver homogenates as previously described (22). This fraction contained ~33% of the NADH cytochrome C reductase and 60–70% of the RNA and ribophorin II present in the postmitochondrial supernatant (see Fig. 5). Morphologically, >90% of the vesicle profiles present in this fraction had ribosomes bound to their outer surfaces. Minor contamination by lysosomes, mitochondria, and a few smooth vesicles was also observed. The vesicles were stripped of ribosomes and content by carbonate lysis (14). The stripped rough microsomes (50 μg) were emulsified in Freund's complete adjuvant and injected into the lymph nodes of a rabbit (24). A regimen of boost injections was performed that included 10–50 μg of antigen in either PBS or Freund's incomplete adjuvant injected intradermally (at 6 wk after initial immunization), intramuscularly (at 6.5 wk), or intravenously (at 7–8 wk). On Western blots, the antiserum recognized seven to eight proteins of the rough microsomes and three lysosomal proteins. Antibodies specific for the RER were purified on a column containing stripped rough microsomes bound to Sepharose 4B (12).

Antisera against ribophorin II purified from canine liver rough microsomes was prepared by Dr. D. Meyer (18). In addition, ribophorin II was isolated from rat liver rough microsomes by affinity chromatography on a column of anti-ribophorin II mouse Ig conjugated to Sepharose 4B and further purified by preparative SDS-PAGE (3). The protein band was cut from the gel and used to immunize a rabbit. Antisera to both canine and rat ribophorins recognized only the 65-kD ribophorin II on Western blots.

Two proteins of 16 and 29 kD were isolated from rat liver homogenates using procedures for the purification of cathepsin B (2). Rabbits were immunized by intradermal injections of the proteins emulsified in Freund's adjuvant. The antiserum produced at 4–30 wk after a single boost recognized exclusively a 16-kD protein. NH_2-terminal amino acid sequencing of the immunoprecipitated 16-kD protein showed it to be $alpha_{2u}$-globulin, a secretory protein.

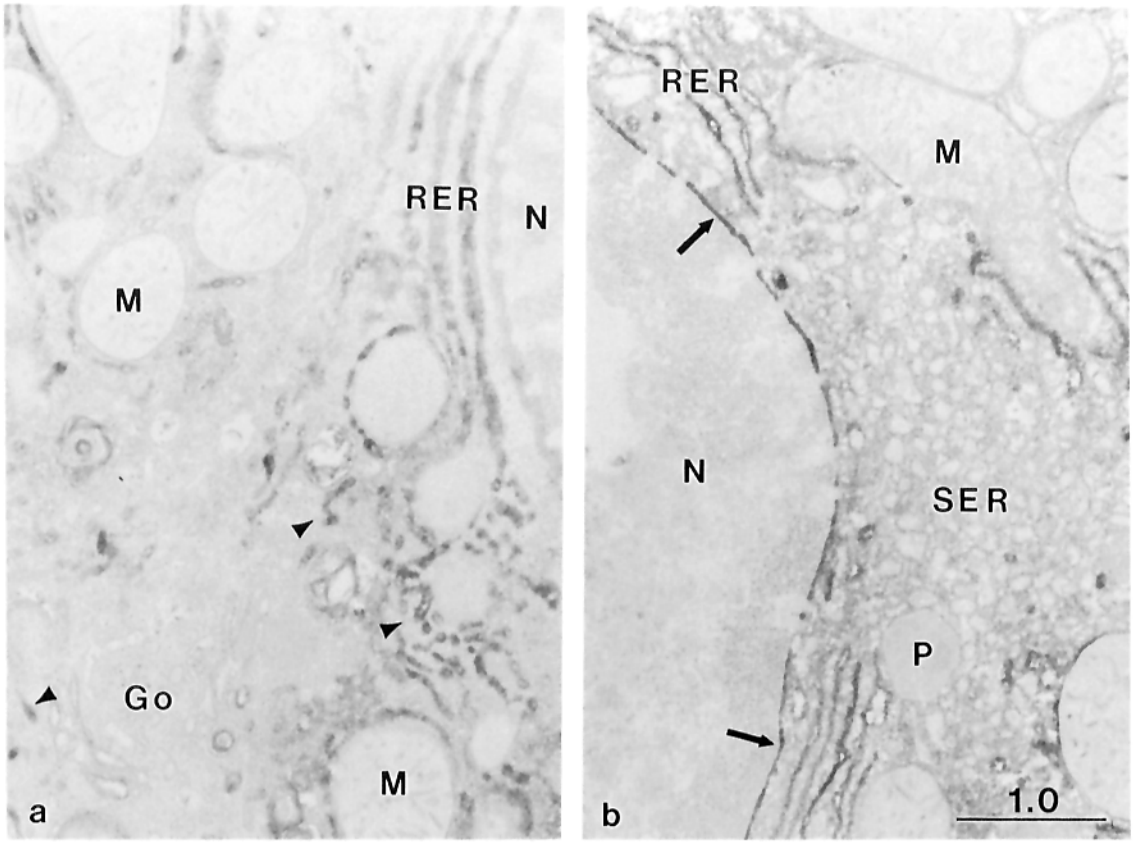

Figure 3. Immunostaining of liver tissue with affinity-purified anti-ER antibodies. Livers from untreated or phenobarbital-treated (five daily intraperitoneal injections of 10 mg/100 g body wt) rats were perfused for 90 min with an amino acid–enriched media containing insulin, then fixed, and processed for immunoperoxidase cytochemistry. This antibody routinely labeled the nuclear envelope (*arrows*), rough endoplasmic reticulum (*RER*), and smooth vesicles/tubules in close association with the RER (*arrowheads*) in both untreated (*a*) and phenobarbital-treated (*b*) livers. Golgi apparatus (*Go*), mitochondria (*M*), peroxisomes (*P*), and phenobarbital-induced smooth endoplasmic reticulum (*SER*) were devoid of reaction product. *N*, nucleus. Bar, 1.0 μm.

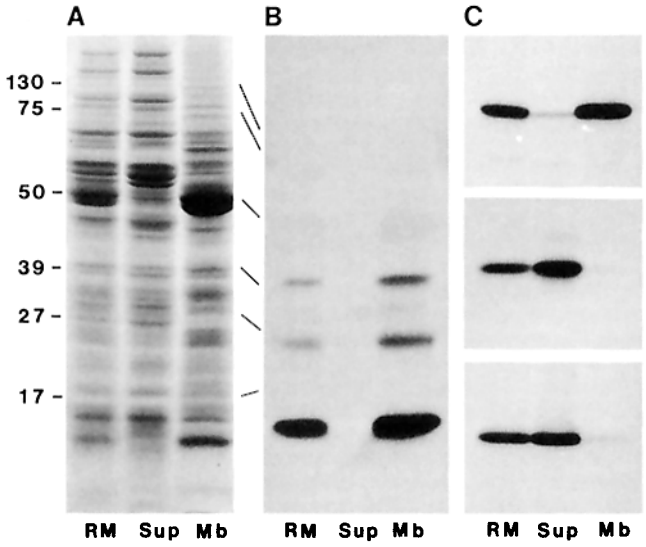

Figure 4. Characterization of affinity-purified anti-RER antibodies. Livers from previously fasted rats were saline perfused and homogenized. A postmitochondrial supernatant fraction was obtained and subfractionated on a discontinuous sucrose gradient (22). The rough microsomes (*RM*) were recovered, treated with 0.1 M sodium carbonate on ice, and the membranes (*Mb*) separated from ribosomes and lumenal proteins (*Sup*) by ultracentrifugation (14). All fractions (50 μg protein) were then analyzed on SDS-PAGE by Coomassie staining (*A*) and Western blotting. (*B*) Affinity-purified anti-RER and (*C*) anti-ribophorin II (*top*, anti-alpha$_{2u}$-globulin (*center*), and anti-rat serum albumin (*bottom*).

Liver Perfusion

Livers were excised from anesthetized rats previously fasted for 18–24 h and perfused cyclically with media designed to either inhibit or stimulate hepatic autophagy. The Hepes-buffered media was prepared as previously described, oxygenated by passing 100% O_2 through the gas-permeable fibers of the oxygen exchange reservoir, and maintained at 35°C by a circulator bath (11). Perfusate containing insulin (14 nM) and amino acids at levels equivalent to four times that present in serum effectively inhibited autophagy (38). The autophagic response was stimulated by perfusing the liver with media depleted of amino acids and containing glucagon (86 nM).

Cytochemistry

Epidermal Growth Factor Conjugated to Horseradish Peroxidase. An isolated liver was perfused with media depleted of amino acids to ensure an optimal autophagic response. EGF conjugated to horseradish peroxidase was then pulsed into a liver for 25 min (10, 11). Liver was fixed by perfusion with 1% glutaraldehyde/0.1 M sodium cacodylate, pH 7.4, and the ligand localized at the ultrastructural level as previously described (11).

Acid Phosphatase. Livers were fixed by perfusion with 2% glutaraldehyde/2% paraformaldehyde/2 mM $CaCl_2$/0.1 M sodium cacodylate, pH 7.4, cut into small blocks, and sectioned into 75-μm-thick pieces using a Smith-Farquhar Tissue Sectioner. Acid phosphatase activity was localized using cytidine 5'-monophosphate and cerium chloride as described by Robinson (35).

Immunoperoxidase and Immunogold. Livers were fixed by perfusion then postfixed for 4 h with 2% paraformaldehyde/0.75 M lysine/0.01 M sodium periodate/0.037 M sodium phosphate, pH 7.4, and processed for immunoperoxidase cytochemistry according to Brown and Farquhar (4) or embedded in LRGold as follows. The liver was cut into small cubes (1 mm^3) and sequentially washed with 0.1 M $NaPO_4$ pH 7.4, 50 mM NH4Cl in PBS (0.1 M NaCl/0.05 M $NaPO_4$, pH 7.4), and 0.1 M $NaPO_4$ pH 7.4. The tissue was then dehydrated at −20°C with increasing concentrations of ethanol washes, infiltrated with LRGold monomer plus initiator, and polymerized at −20°C for 24 h under an ultraviolet lamp as suggested by the manufacturer (Data Sheet No. 305A; Polysciences Inc., Warrington, PA). Thin sectioning of the embedded tissue was done on an Ultracut E microtome (Cambridge Inst., Deerfield, IL) and the sections placed ontc nickel grids (Ernest F. Fullam, Inc., Latham, NY). Antibody incubations were done at 22°C in humidified chambers directly on the sections following the procedures of Titus and Becker (44). Briefly, the sections were quenched with 50 mM NH_2Cl in PBS and rinsed with 10 mM Tris, pH 7.2/0.5 M NaCl/0.3% Tween-20 (TBST) containing 1% BSA. The sections were then incubated with affinity-purified anti-RER Ig (34 μg/ml TBST/BSA) overnight at room temperature, washed with TBST/BSA, incubated with protein A-gold 5 nm (1:30 dilution in TBST/BSA) for an additional 3 h, and again washed with TBST/BSA then TBST. Finally, the sections were rinsed with distilled water and counter stained with uranyl acetate and lead citrate (34).

Analytical Procedures

Assays. NADH cytochrome C reductase activity was assayed by measuring the production of reduced cytochrome C (21). Chemical determinations of RNA and protein were done using orcinol and Lowry reagents, respectively (40, 43).

Western Blotting. Identification and quantitation of antigens immobilized on nitrocellulose was done as previously described (12). Briefly, proteins were separated on 10% SDS-polyacrylamide gels and transferred to nitrocellulose. Antigens were then identified by incubating the nitrocellulose with primary antibody followed by ^{125}I-protein A. The radiolabeled marker was then visualized by exposure of X-OMAT film and the density of the exposed silver grains quantified using a Zeineh Soft-Laser Densitometer (Biomed Instruments Inc., Fullerton, CA).

Morphometry. Morphometric quantitation of autophagic vacuoles was done by point counting lattice intersections (47) or by using a digitizing tablet interfaced with Sigmascan software (Jandel Scientific, Corte Madera, CA). For each experiment, 15–20 electron micrographs of individual cells from one or two areas of the liver were analyzed and expressed as a percentage of total cell volume.

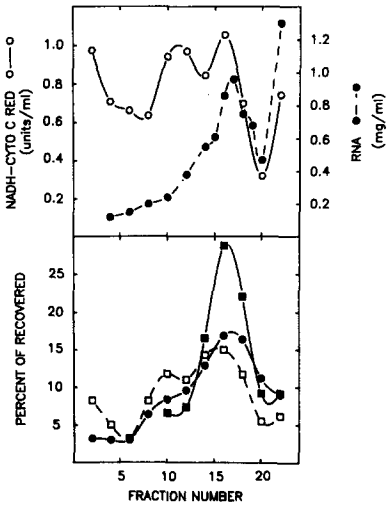

Figure 5. Subcellular distribution of the 14-, 25-, and 40-kD proteins. Liver was homogenized and the postmitochondrial supernatant fractionated on a discontinuous sucrose gradient (22). The distribution of the 14-, 25-, and 40-kD membrane proteins across the gradient was examined with respect to RNA, NADH cytochrome C reductase, and ribophorin II. By using the antibodies prepared here, RER membrane proteins and ribophorin II were quantified on Western blots and the results expressed as the percentage of that recovered from the gradient. NADH cytochrome C reductase displayed a bimodal distribution representing smooth (fractions 10–12) and rough (fractions 16–18) endoplasmic reticulum. Two proteins, 25 and 40 kD (■), comigrated with RNA, ribophorin II (●), and a portion of the NADH cytochrome C reductase activity. The third protein, 14 kD (□), had a unique distribution that overlapped both peaks of reductase activity.

Results

Morphological Characterization of the Autophagic Response

It has been reported that nutrient deprivation combined with glucagon administration stimulates the autophagic response in many cell types (7, 26, 33, 42). Livers from fasted rats were perfused with an amino acid–enriched medium containing insulin for 90 min to eliminate the presence of those autophagic vacuoles that had formed in vivo. The perfusate was then switched to one lacking amino acids and containing glucagon. At 30 min, two types of vacuoles could be distinguished using morphological criteria (36, 37). AVis were limited by a double or multiple membrane and contained intact cytoplasmic organelles. The outer and inner membranes were usually separated by a distinct electron-lucent area (Fig. 1). Of the 231 vacuoles counted only 4% contained acid phosphatase activity as detected cytochemically. AVds were bound by a single membrane and contained cytoplasmic organelles in various stages of degeneration. These organelles contain acid phosphatase activity, β-glucuronidase, cathepsin L and cathepsin D and therefore, were secondary lysosomes (9). Comparable to results presented by Mortimore and co-workers (37, 38), AVis were identified within 15 min after perfusing the liver with media lacking amino acids (Fig. 2). Soon afterwards, AVds were visualized. The fractional volumes of AVi and AVd increased until an apparent steady state was reached at 30–60 min, whereby the fractional volumes of both vacuole populations remain the same.

Characterization of Antibodies Specific for RER Proteins

Affinity-purified antibodies to integral membrane proteins of the RER were prepared as described in Materials and Methods. These antibodies labeled only the RER, the nuclear envelope, and small vesicles in close association with the RER (Fig. 3). In livers from phenobarbital-treated rats antibody labeling was restricted to the RER, while large areas of smooth endoplasmic reticulum contained no label (Fig. 3). The affinity-purified antibodies recognized three RER proteins, 14, 25, and 40 kD, on Western blots (Fig. 4). When RER membrane proteins (e.g., ribophorin II) were separated from ribosomes and lumenal proteins (e.g., rat serum albumin and alpha$_{2u}$-globulin), all three proteins were quantitatively recovered with the membrane fraction (Fig. 4). Two of the proteins, 25 and 40 kD, colocalized exclu-

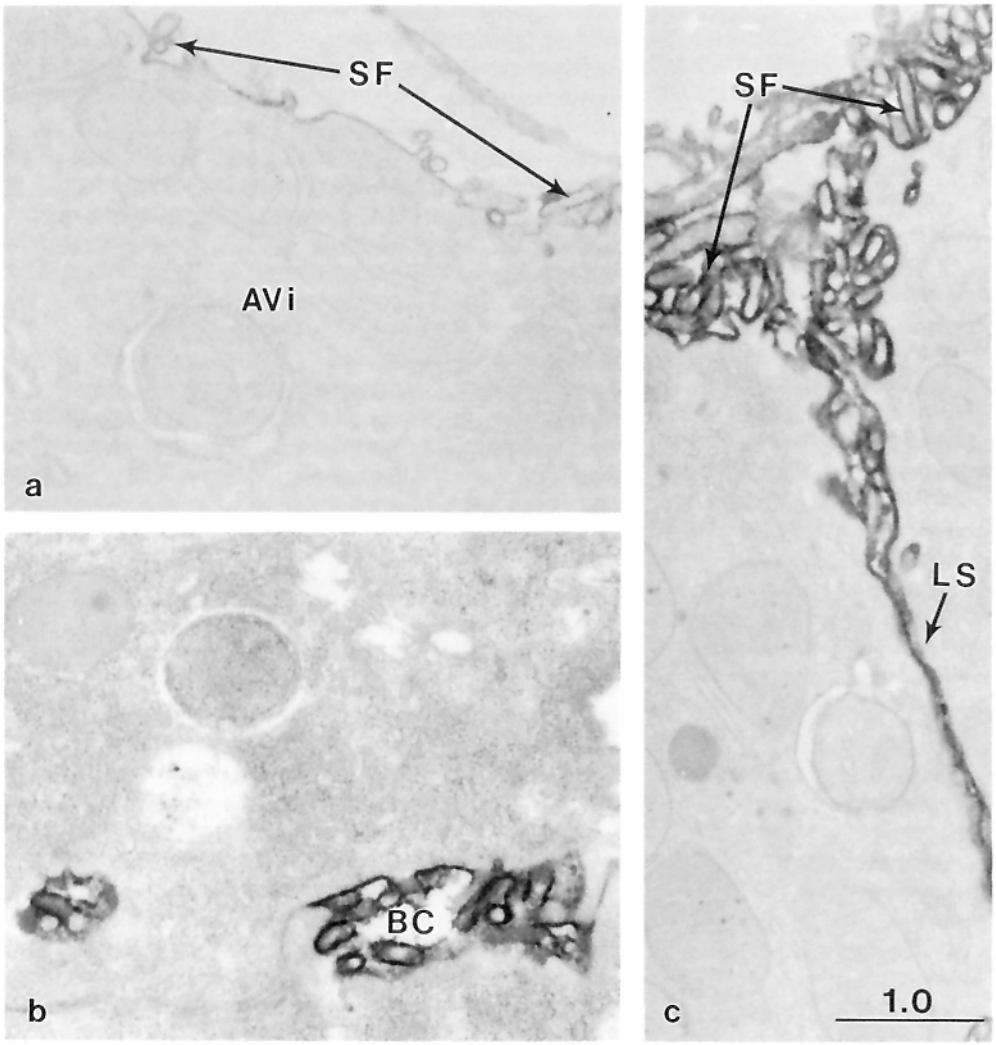

Figure 6. Immunolocalization of plasma membrane antigens. Livers were excised and perfused for 90 min with a medium supplemented with amino acids and insulin. Afterwards, the medium was changed to one depleted of amino acids and containing glucagon, and the liver perfused for an additional 25 min. The liver was fixed by perfusion and processed for immunoperoxidase cytochemistry as described in Materials and Methods. Plasma membrane antigens, CE9 (*a*), HA4 (*b*), and EGF receptor (*c*), were identified at the cell surface but absent from autophagic vacuoles (*AVi*). *SF*, sinusoidal front; *LS*, lateral surface; and *BC*, bile canalicular surface. Bar, 1.0 μm.

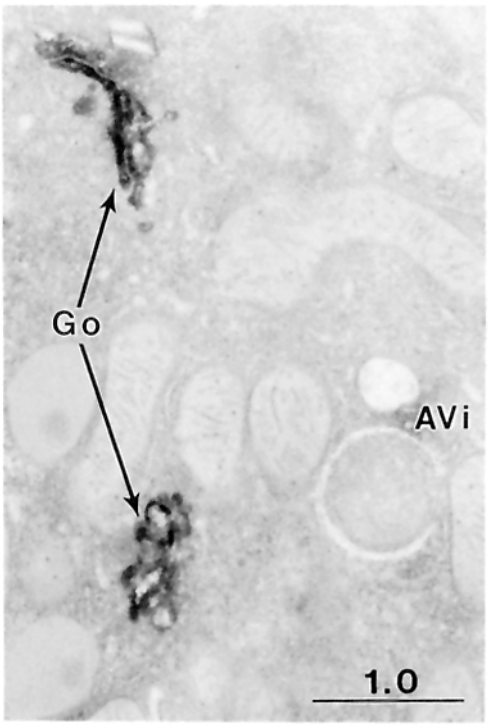

Figure 7. Immunolocalization of sialyltransferase. A liver was perfused as described in Fig. 6 and processed for immunoperoxidase cytochemistry using an antiserum to sialytransferase. The reaction product was restricted to two and sometimes three cisternia of the Golgi apparatus (*Go*). The absence of reaction product indicated that sialytransferase was not present in autophagic vacuoles (*AVi*). Bar, 1.0 μm.

sively with ribophorin II and RNA (e.g., ribosomes) on sucrose gradients (Fig. 5). However, the third protein, 14 kD, was present in vesicles of two different densities. The low density vesicles (d <1.2 g/cc) comigrated with a low density peak of NADH cytochrome C reductase activity (e.g., smooth or ribosome-free endoplasmic reticulum) while the high density vesicles (d >1.2 g/cc) distributed with the 25- and 40-kD proteins, ribophorin II, RNA, and a high density peak of NADH cytochrome C reductase (e.g., RER) (Fig. 5). The combined morphological and biochemical data indicated that the 25- and 40-kD membrane proteins were localized exclusively to the RER, while the 14-kD protein was distributed between RER and smooth vesicles closely associated with the RER.

Absence of Golgi Membrane and Plasma Membrane Antigens from Nascent Autophagic Vacuoles

At 25–30 min of perfusion with nutrient-deprived media, livers were fixed and processed for immunoperoxidase cytochemistry (4). The plasma membrane antigens, CE9, HA9, and EGF receptor, were not present in AVis (Fig. 6). Furthermore, sialyltransferase was not detected in these vacuoles (Fig. 7) and a second Golgi membrane protein, the mannose-6-phosphate receptor, was absent from a majority of the autophagic vacuoles (9). The above results clearly show that autophagic vacuoles were not formed from the plasma membrane or Golgi elements.

Interrelationship between Endocytic and Autophagic Pathways

Studies have shown endocytosed proteins present in autophagic vacuoles suggesting a possible role for endosomes in the formation of these vacuoles (7, 12, 15). To establish whether autophagic vacuoles were formed from endosomes, I examined the distribution of endocytosed EGF and its membrane receptor within AVis and AVds. EGF conjugated to horseradish peroxidase was restricted to endosomes and lysosomes, including AVds (Fig. 8, *a* and *b*). Leupeptin inhibition of proteolysis resulted in an accumulation of the EGF receptor within AVds (Fig. 8 *c*). However, neither internalized EGF nor its receptor were found in AVis.

Immunological Identification of RER Proteins to Nascent Autophagic Vacuoles

Membrane Proteins. Both anti-RER antibodies and anti-ribophorin II antiserum labeled autophagic vacuoles (Fig. 9). Of those vacuoles identified as an AVi, 44% were labeled with the anti-ribophorin II antibody and 79% with the anti-RER antibodies (Table I). The reaction product was present in the vacuolar lumen of degradative vacuoles (Fig. 9 *b*) but restricted to the electron-lucent area between the two limiting membranes of the AVi. In comparison, the reaction product distributed within the lumenal area of the RER. By using immunogold methodology, RER antigens were identified at the outer and inner membranes of AVis (Fig. 10). These results were consistent with the concept that the electron lucent region between the two limiting membranes of the AVi and the lumen of the RER were equivalent and therefore, both membranes originated from the RER.

Matrix Proteins. If indeed the region between the inner and outer membranes of the nascent autophagic vacuole was equivalent to the RER lumen, then one might predict RER lumenal proteins to be present in this region. To test this, the distribution of two secretory proteins, rat serum albumin and alpha$_{2u}$-globulin, in liver tissue fixed at 25 min after onset of the autophagic response was examined (Fig. 11). Both proteins were found throughout the secretory pathway (e.g., RER and Golgi apparatus). In addition, these proteins were localized to the region between the inner and outer membranes of the AVi. Rat serum albumin was present in 30% of these vacuoles while 67% contained alpha$_{2u}$-globulin (Table I).

Discussion

To evaluate the regulatory mechanisms of autophagy, it first becomes necessary to examine the events of autophagic vacuole formation. Therefore, I have set out to identify the preexisting source of the autophagic vacuolar membrane in order to evaluate the mechanism of vacuole formation. Earlier studies relying on morphological observations or cytochemical localization of specific enzymes have not been conclusive. Golgi apparatus, endoplasmic reticulum, endosomes, and a unique membrane structure termed a phagophore have all been implicated as possible sources of the autophagic vacuole membrane (1, 13, 17, 29, 41). I have used an approach taking advantage of the the specificity and sensitivity of immunological probes to identify specific membrane antigens to the limiting membranes of AVis. The

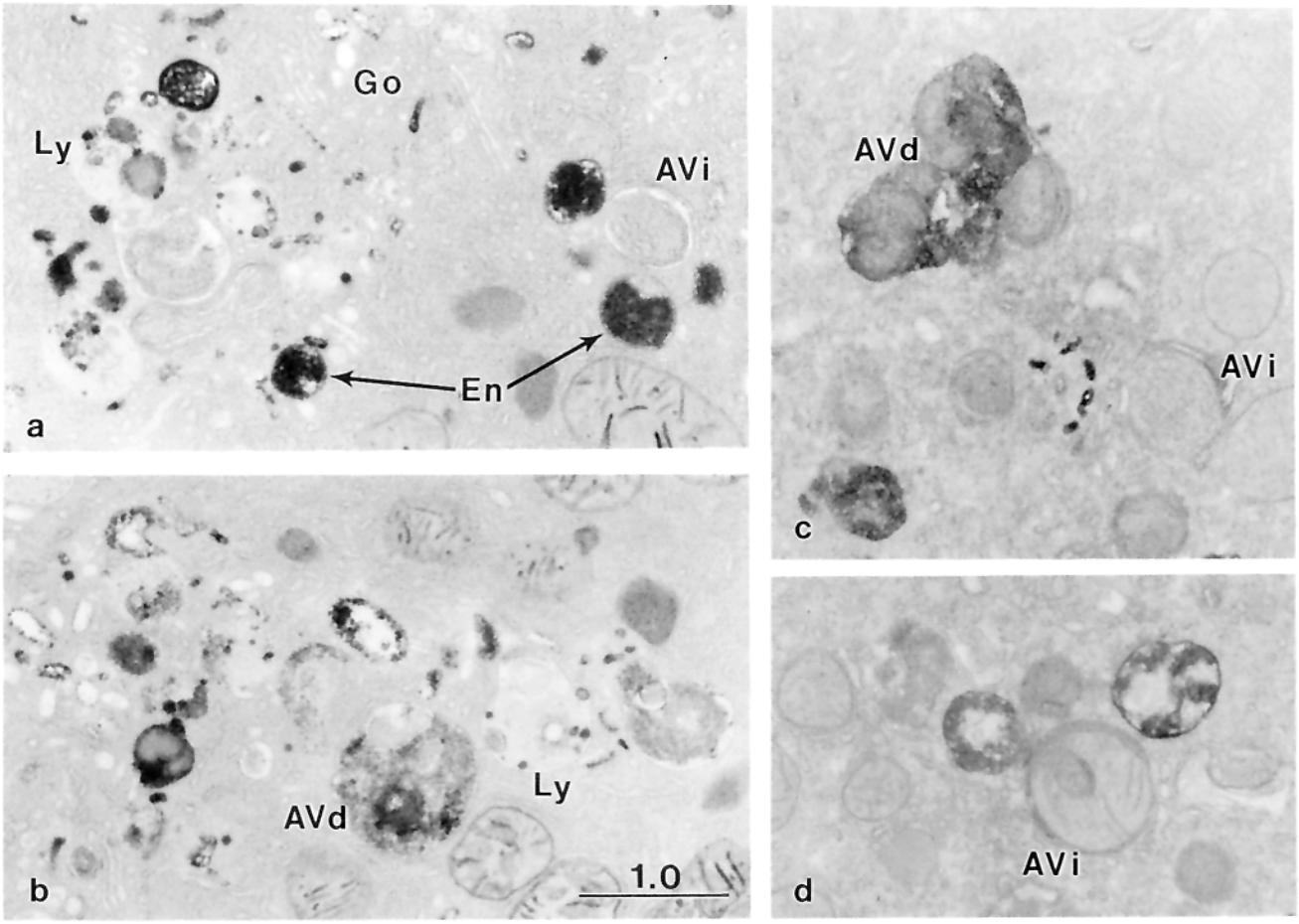

Figure 8. Localization of internalized EGF (*a* and *b*) and EGF receptor (*c* and *d*). A liver was exposed to EGF conjugated to horseradish peroxidase for 25 min and the ligand visualized as described in Materials and Methods. In a separate experiment, a liver was excised from a rat previously fasted for 24 h and perfused in the absence of amino acids for 30 min. The liver was then perfused with EGF in the presence of leupeptin for 120 min, fixed by perfusion, and processed for immunoperoxidase cytochemistry. EGF and its receptor were localized exclusively to endosomes (*En*), some lysosomes (*Ly*), and degradative autophagic vacuoles (*AVd*). However, these proteins were not detected in nascent autophagic vacuoles (*AVi*). Bar, 1.0 μm.

findings are consistent with the formation of autophagic vacuoles from ribosome-depleted areas of the RER.

Of three plasma membrane antigens examined, all were absent from the AVi membranes. In addition, the limiting membranes of AVis did not contain sialyltransferase suggesting that these vacuoles are not originating from Golgi cisternae. These results are consistent with the observed absence of thiamine pyrophosphatase (plasma membrane and Golgi apparatus) and adenosine triphosphatase (plasma membrane) from these vacuoles (1, 13). It has been shown that EGF and its receptor are internalized into endosomes and eventually degraded within lysosomes and AVds (12). Internalized thorotrast follows the same fate (8). However, neither EGF nor its receptor are detected in bona fide AVis. These results suggest that the prelysosomal compartments of the endocytic (i.e., endosomes) and autophagic (i.e., AVi) pathways exist independently but that these compartments deliver their contents to the same degradative lysosome. Gordon and Seglen have reported biochemical evidence suggesting that endosomes fuse with a prelysosomal form of autophagic vacuole (15). However, their interpretation was based on a presumption without morphological evidence that asparagine inhibits lysosome fusion with autophagic vacuoles but not fusion of endosome with these vacuoles.

Glucose-6-phosphatase and inosine-diphosphatase (membrane proteins localized to the endoplasmic reticulum) activities have been observed in autophagic vacuoles but the existence of these enzymes to the limiting membrane was inconclusive (1, 13, 17, 29, 36). The data presented here clearly show that some RER membrane proteins are present in both inner and outer limiting membranes of AVis. However, whether all or only one of the three antigens recognized by the anti-RER antibody are present has yet to be determined. The apparent absence of ribophorin II from 50% of the AVis was consistent with the inability to label all the RER within a particular section of a cell using this antibody. Such variable labeling seen with the anti-ribophorin II antibody may be due to antibody penetration into fixed tissue, inaccessibility of the antibody to the antigen (e.g., antibody recognition site being blocked by RER proteins binding to the lumenal tail of ribophorin II), or RER heterogeneity. Results with other antibodies (Figs. 9 and 11) and lectins (data not

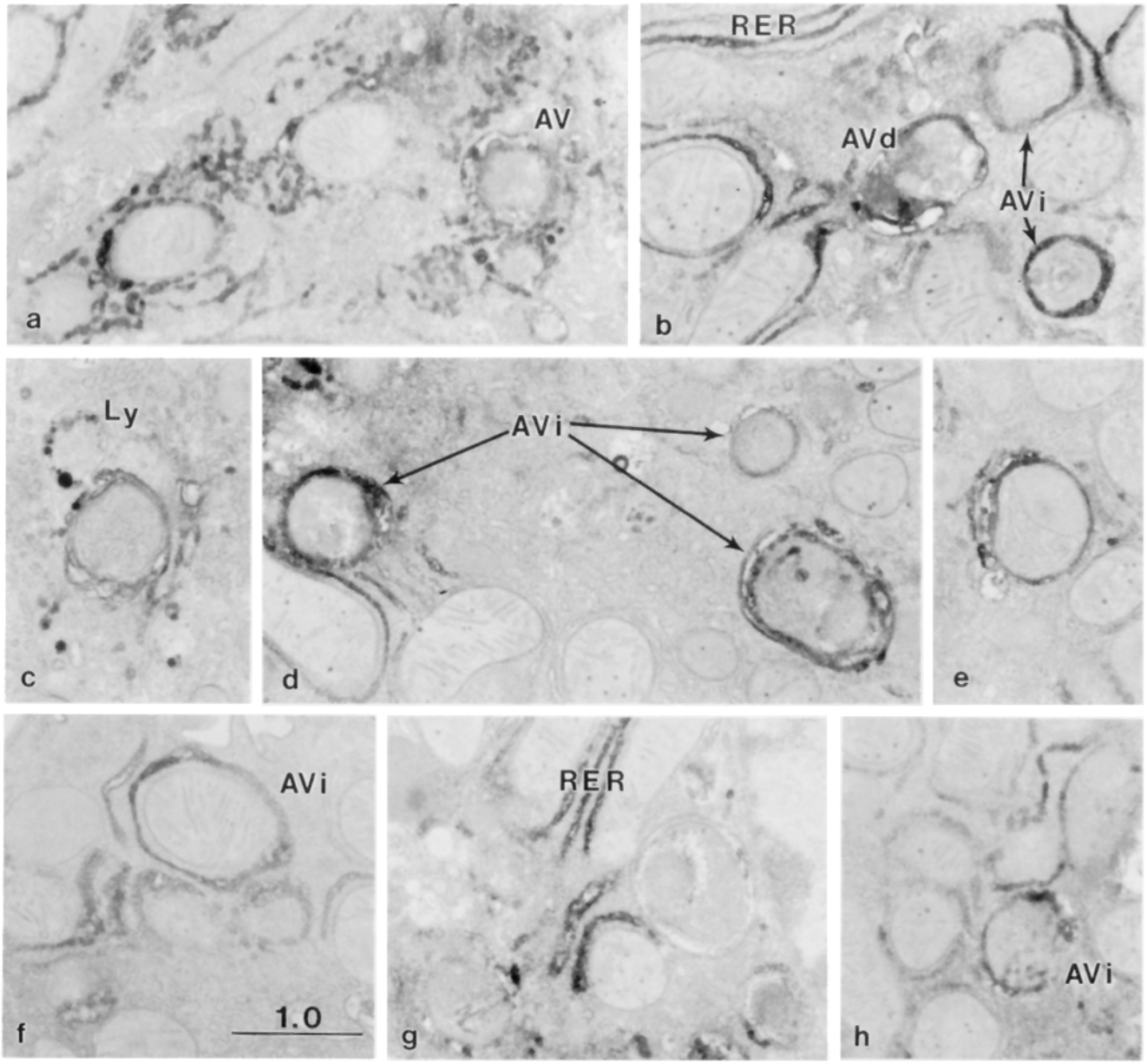

Figure 9. Immunoperoxidase localization of RER membrane proteins to autophagic vacuoles. Livers were perfused under conditions described in legend to Fig. 6, formaldehyde fixed, and processed for immunoperoxidase cytochemistry as described in Materials and Methods. Affinity-purified anti-RER (*a–e*) labeled the rough endoplasmic reticulum (*RER*) and both nascent (*AVi*) and degradative (*AVd*) autophagic vacuoles. Lysosomes (*Ly*) were not labeled. The reaction product was deposited between the inner and outer membranes of the AVi and distributed within the lumen of the AVd. Occasionally, tubules and vesicles containing reaction product were observed in close association with an autophagic vacuole (*AV*). Anti-ribophorin II antiserum (*f–h*) labeled the rough endoplasmic reticulum (*RER*) and AVis. Some autophagic vacuoles were not labeled by either antibody (*g*). Bar, 1.0 μm.

shown), which label the RER, suggest that antibody penetration was not a contributing factor. Due to the unavailability of a specific antibody, the contribution, if any, of smooth endoplasmic reticulum to vacuole formation can not be assessed at this time.

Although the results suggest that the limiting membranes of a majority of AVis contain RER proteins, qualitative studies have shown that the membranes of vacuoles that morphologically resemble AVis differ from that of the RER. For example, the ability of vacuole membranes to bind cationized ferritin is greater than that seen for the RER (36). In addition, imidazole-buffered OsO_4 staining indicates that the content of unsaturated fatty acids is greater in membranes of autophagic vacuoles than that seen for RER membranes (33). Combined, the data suggest that the membranes of these vacuoles originate from the RER and soon become altered after vacuole formation. Such changes in membrane characteristics may be due to fusion with other organelles. Indeed, I have found that the lysosomal glycoprotein, lgp120, is localized to the outer membrane of 50% of morphologically defined AVis (9).

The presence of RER proteins at the inner and outer mem-

Table I. Quantitation of Nascent Autophagic Vacuoles Containing Proteins of the Rough Endoplasmic Reticulum

Antibody	Total AVi profiles counted	Percent of AVi labeled positive
Membrane proteins		
Anti-RER (14, 25, 40 kD)	216	79
Anti-ribophorin II	209	44
Lumenal proteins		
Anti-rat serum albumin	60	35
Anti-alpha$_{2u}$-globulin	46	67

Livers were perfused with an amino acid-enriched medium containing insulin for 90 min and then switched to a medium depleted of amino acids containing glucagon for an additional 25 min. The livers were fixed and processed for immunoperoxidase cytochemistry (4). Blocks (two to three) of embedded tissue from triplicate experiments were cut and AVis identified using morphologic criteria (see text and Figs. 9 and 11). Those AVis that were positively labeled were quantified and tabulated as a percentage of the total number of AVis counted.

branes of the AVi suggests that both membranes originated from the RER. The distribution of the reaction product suggests that the space between the inner and outer membrane is equivalent to the lumen of the RER. This was substantiated by the presence of secretory proteins within this space (Fig. 11). From these results, a mechanism for vacuole formation from the RER was formulated. Upon a stimulus, a "sheet" of ribosome-free RER invaginates in much the same manner as that seen at the plasma membrane during phagocytosis. It is possible that this invagination is either allowed to occur or initiated by alterations in the cytoskeleton (17, 29, 33). The resulting nascent vacuole maintains a double membrane until the inner membrane is presumably degraded. The multiple or myelin membrane structures sometimes seen limiting autophagic vacuoles may originate from the ongoing lipid metabolism associated with the RER.

Although results presented here demonstrate that autophagic vacuoles are formed from the RER, the fate (e.g., recycling vs. degradation) of the membrane proteins has not yet been evaluated. The disappearance of the inner membrane has been presumed to be due to proteolysis. By using leupeptin to inhibit proteolysis, cytochrome P-450 and NADPH-cytochrome P-450 reductase, membrane proteins of the endoplasmic reticulum, and EGF receptor have been localized to degradative autophagic vacuoles, the presumed site of proteolysis (12, 25). In addition, cytosolic enzyme activities have been measured in autophagic vacuoles purified from livers previously treated with leupeptin (20). However, in preliminary studies, RER membrane antigens were absent from a majority of the degradative vacuoles visualized in livers previously treated with leupeptin, suggesting the possibility that these proteins may be recycled. Indeed, membrane antigens of the RER were localized to vesicles and tubules in close proximity to autophagic vacuoles (Fig. 9, *a* and *c*). Whether or not these vesicles are involved in the delivery (degradation) or removal (recycling) or membrane proteins to or from the autophagic vacuole has yet to be determined. Studies are ongoing to evaluate the fate of these membrane proteins.

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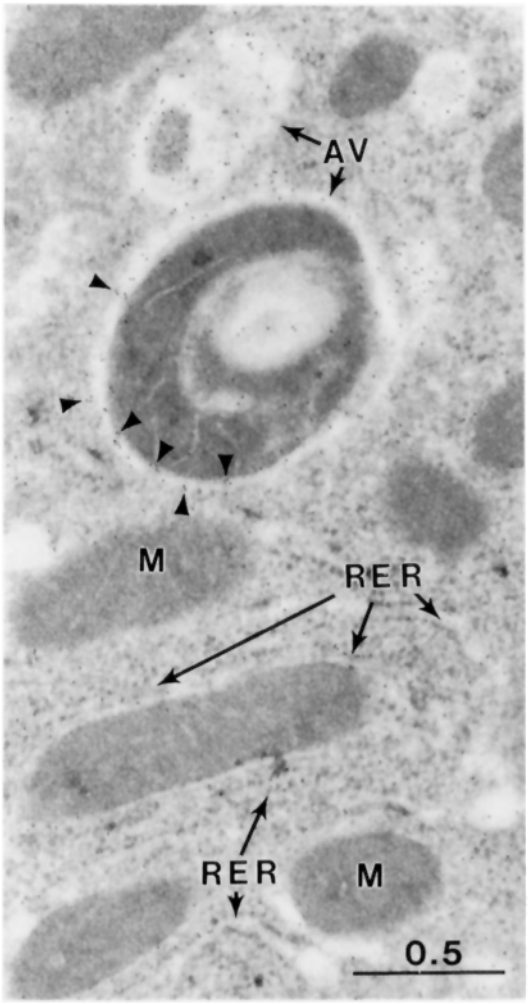

Figure 10. Immunogold localization of RER membrane proteins to autophagic vacuoles. Livers were perfused under conditions described in legend to Fig. 6, formaldehyde fixed, and processed for immunogold cytochemistry as described in Materials and Methods. Labeling of mitochondria (*M*), plasma membrane, nuclei, and peroxisomes was negligible. Profiles of rough endoplasmic reticulum (*RER*) were routinely labeled. Immunogold labeling was observed at both inner and outer membranes of nascent autophagic vacuoles (*arrowheads*). The relative densities of gold particles over mitochondria, RER, and autophagic vacuoles (*AV*) were 0.13, 3.09, and 2.18 gold particles per surface area, respectively. Bar, 0.5 μm.

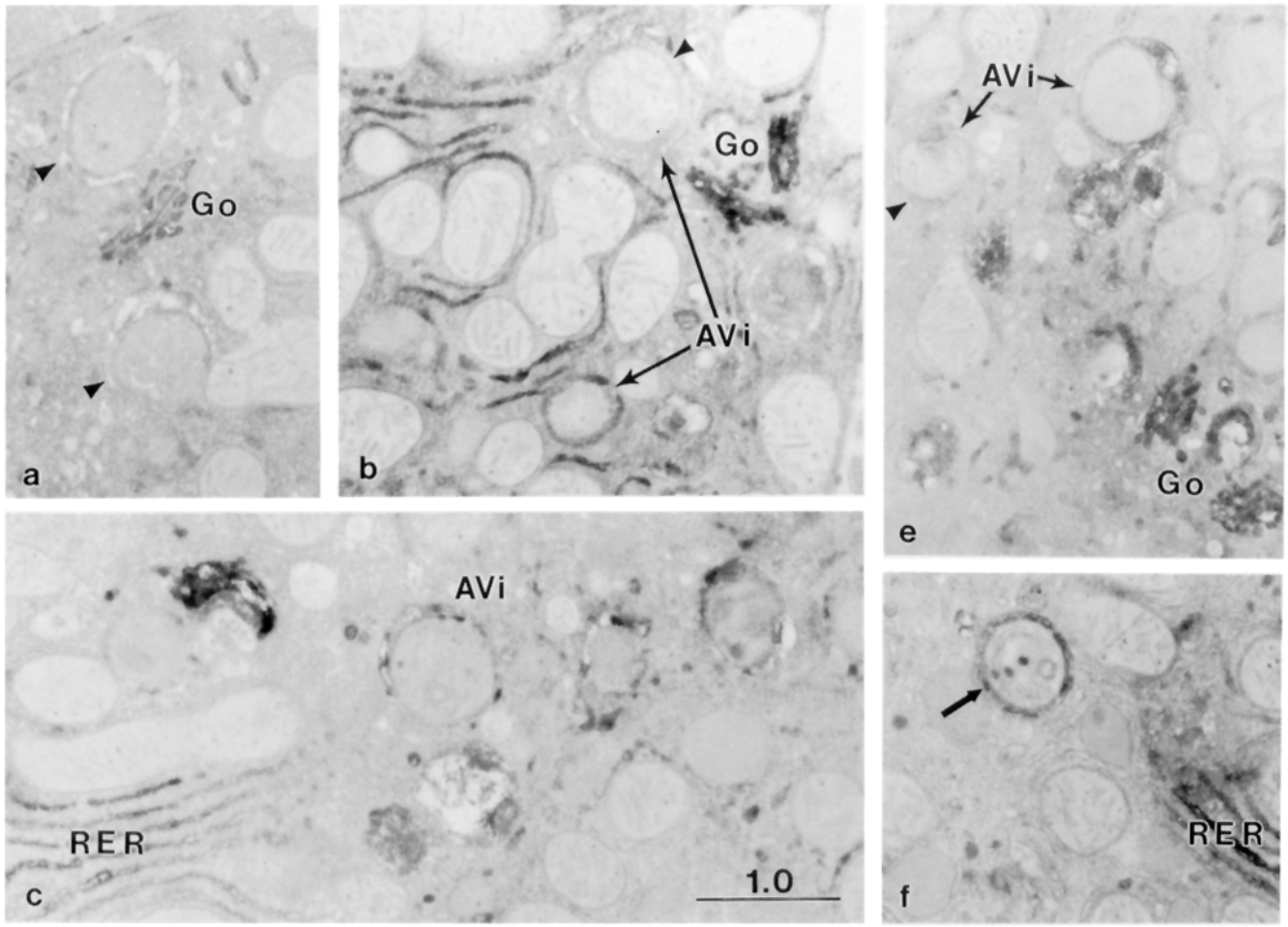

Figure 11. Immunoperoxidase localization of secretory proteins to autophagic vacuoles. Autophagy was induced by perfusing the liver with nutrient-depleted media as described in Fig. 6. The liver was then fixed by perfusion and processed for immunoperoxidase cytochemistry as described in Materials and Methods. Rat serum albumin (*a–c*) and alpha$_{2u}$-globulin (*d* and *e*) were identified in rough endoplasmic reticulum (*RER*), Golgi apparatus (*Go*), and some nascent autophagic vacuoles (*AVi*). Autophagic vacuoles not containing these proteins were also observed (*arrowheads*). When present, these proteins were localized to the lumenal area between the inner and outer membranes (*arrow*). Bar, 1.0 μm.

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