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# Identification of Autolysosomes Directly Associated with Proteolysis on the Density Gradients in Isolated Rat Hepatocytes[1]

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**Autophagy-related vacuoles, *i.e.*, autophagosomes (AVi), autolysosomes (AVd) and dense bodies (DB), are intracellular organelles within which macroautophagy and bulk proteolysis set out and progress. Separation of these particles in freshly isolated rat hepatocytes, monitored by β-hexosaminidase, a lysosomal marker enzyme, was established by density gradient centrifugation. Percoll density gradients were modified and improved by adding free polyvinylpyrrolidone (PVP, 0.75%) to 60% Percoll, which made it possible to separate AVd (buoyant peak, $d=1.090$) and DB (dense peak, $d=1.131$) effectively. Addition of graded levels of a regulatory amino acid mixture (Reg AA) to hepatocyte incubation not only suppressed proteolysis, but also lead to a shift of vacuolar profiles on the density gradients from the buoyant to the dense region. Alterations in the vacuolar shift and proteolysis were highly proportional over a full range of regulation by Reg AA. Morphometric analysis of autophagic vacuoles by electron microscopy revealed changes in the aggregate volumes of both AVi and AVd by Reg AA, which enabled us to estimate autophagic subpopulation of the buoyant peak on the gradient profile. All the results demonstrate that AVd shifts on the density gradients in proportion to alterations in proteolysis regulated by amino acids, and thus the gradient profile can be used as a measure of macroautophagy; and in addition that AVd actively involved in proteolysis occupies only a part of the buoyant peak on the gradients.**

**Key words: amino acid, autophagy, density gradient, hepatocyte, proteolysis.**

Macroautophagy, a major intracellular mechanism of long-lived protein turnover in a variety of cells, is subject to dynamic regulation by such physiological factors as amino acids, insulin, and glucagon (*1-4*). In particular, amino acids are principal regulators which can control the full range of macroautophagy, and their detailed mode of regulation has been elucidated mostly by use of perfused liver (*3*). In a previous paper we demonstrated that both macroautophagy and the multiphasic control mechanism by amino acids existed in a single population of liver parenchymal cells (*5*). The next step toward a better understanding of this mechanism at the molecular level is to identify the vacuolar apparatus involved in this process.

Among a variety of membrane-limited structures in the cell, autophagy-related organelles consist of remarkably heterogeneous vacuolar particles, *e.g.*, autophagosomes (AVi), amphisomes, late autophagosomes (AVi/d), autolysosomes (AVd), and dense bodies (DB, type A and R, or secondary lysosome), because they are continually sequestering and degrading different types of substrates in a minute-to-minute manner, and are at different stages in their functioning, *i.e.*, sequestration, acidification, fusion, and degradation (*2, 3*). For separation of these particles, numerous density gradient methods have been improved using many kinds of gradient media, *e.g.*, sucrose (*6*), metrizamide (*7*), Nycodenz (*8*), and Percoll (*9*). These gradient media each have unique characteristics for separation of various organelles, but no one method for every purpose has been established. For the present study, which aims to separate subpopulations among autophagy-related vacuoles rather than to purify them from other organelles such as mitochondria, a colloidal silica was chosen. Previous studies (*10, 11*) demonstrated that autophagic vacuoles (AV) induced in the perfused liver could be effectively separated from DB using Ludox, a colloidal silica, mixed with polyvinylpyrrolidone (PVP) , and this has been regarded as the best separation technique of AVd and DB, intermediate and late compartments of autophagy, respectively. We applied this method to freshly isolated rat hepatocytes using Percoll, a more readily available colloidal silica than Ludox.

The present study had the following objectives: (i) to establish an effective method using Percoll density gradient

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Abbreviations: AV, autophagic vacuole; AVi, autophagosome (or initial autophagic vacuole); AVd, autolysosome (or degradative autophagic vacuole); β-hexosaminidase, *N*-acetyl-β-D-glucosaminidase; DB, dense body; M+L fraction, mitochondrial-lysosomal fraction; PVP, polyvinylpyrrolidone; Reg AA, regulatory amino acids plus alanine.

centrifugation to separate AVd and DB in isolated parenchymal hapatocytes, (ii) to clarify the physiological relationship between autophagic proteolysis and the vacuolar profiles on the gradients by using control by amino acids, and (iii) to estimate to what extent these vacuoles, especially AVd, on the gradients are directly associated with ongoing autophagic proteolysis, supported by morphometric findings by electron microscopy.

## EXPERIMENTAL PROCEDURES

*Materials*—Percoll, density marker beads, bovine albumin, and dansyl chloride were purchased from Sigma Chemical. Collagenase was obtained from Boehringer Mannheim. Polyvinylpyrrolidinone (PVP, K=30) was purchased from Kanto Chemical. [*U*-$^{14}$C]Sucrose (sp.act., 4.2 mCi/mmol) was from American Radiolabeled Chemicals, USA.

*Preparation of Isolated Hepatocytes*—Male Wistar rats meal-fed (14:00-18:00) with a 35% casein diet, weighing about 240 g, were used as hepatocyte donors. Liver parenchymal cells were isolated by the collagenase method (*12*) and preincubated in Krebs-Ringer bicarbonate buffer (pH 7.4) plus 6 mM glucose and 0.5% bovine albumin oxygenated with $O_2$:$CO_2$ (95:5, v/v) gas at 37°C for 45 min. After washing, the cells ($2\times10^6$ cells/ml) were incubated in 3 ml of suspension for 47 min for proteolysis as described previously (*5*), and in 30 ml of suspension in 100-ml flasks for 60 min for gradient study. When necessary, a mixture of six regulatory amino acids and a coregulatory amino acid, Ala, (Reg AA) was added as multiples of their normal portal plasma level as follows ($\mu$M): Leu, 204; Tyr, 98; Pro, 437; Met, 60; His, 92; Trp, 93; plus Ala, 475.

*Percoll-PVP Density Gradients*—After 60 min of incubation, 30 ml of hepatocytes were spun down and resuspended in 4 ml of cold 0.25 M sucrose-1 mM EDTA (pH 7.4), then homogenized with a tight-fitting Dounce homogenizer (100 strokes, Wheaton Science Products, NJ, USA) on ice. The mitochondrial-lysosomal (M+L) fraction was separated by differential centrifugation as follows. First, homogenates were spun at $500\times g$ for 10 min to remove nuclei and unbroken cells. The postnuclear supernatants were spun at $17{,}500\times g$ for 10 min to yield pellets of the M+L fraction. This fraction exhibited 75-80% recovery of total $\beta$-hexosaminidase activity. All the procedures were done at 4°C.

To make an iso-osmotic medium, Percoll was initially mixed with 10% 2.5 M sucrose (density gradient grade, Wako Pure Chemicals) to form "iso-osmotic 100% Percoll" solution. Percoll solutions of various concentrations were then prepared by mixing this 100% Percoll and 0.25 M sucrose and adjusted to pH 7.4 with 0.1 N NaOH. In the experiment to see the effect of PVP addition to Percoll, Percoll was mixed with a PVP solution, solid sucrose was added to make the solution iso-osmotic, and pH was adjusted to 7.4 with 1 N HCl. The PVP used was dialyzed for 3 days against distilled water (30 volumes and 4 changes). A gradient medium (4 ml) was poured into Hitachi 5PA centrifuge tubes (13×50 mm). The M+L fraction (200 $\mu$l) was layered on top, and the tubes were spun at 17,000 rpm ($27{,}500\times g$) at 4°C for 60 min in a Hitachi SCR 18B centrifuge with a Hitachi R20V vertical rotor. The gradients were fractionated into 20 fractions (200 $\mu$l each) numbered from bottom to top as described previously (*13*).

*Measurement of Proteolysis*—Bulk proteolysis was determined by Val release from hepatocytes in the presence of cycloheximide (20 $\mu$M). After incubation of cells with graded levels of amino acids, cycloheximide was added at 30 min. Samples for Val analysis were taken at 37 and 47 min. Val was derivatized with dansyl chloride with L-norvaline as an internal standard (*14*) and separated by RP-HPLC using a Supelcosil LC-18 column (4.6×150 mm) as follows: derivatized samples were eluted at room temperature at a flow rate of 0.8 ml/min with a gradient of eluent A and B, in which eluent B was increased from 40% to 50% until 10 min, maintained at that level until 25 min, then increased to 100%: eluent A, water-acetonitrile (88:12, v/v) containing 0.3% acetic acid and 0.035% triethylamine; eluent B, 100% methanol.

*Analytical Procedures*—Density of the gradients was measured using density marker beads according to the manufacturer's manual. Marker enzymes for organelles were assayed as follows: $\beta$-hexosaminidase (lysosome) according to Peters *et al.* (*15*), cytochrome *c* oxidase (mitochondria) according to Storrie and Madden (*16*). Protein was assayed by Lowry's method (*17*) after removal of Percoll by centrifugation at $100{,}000\times g$ for 30 min. Radioactivity was determined with an Aloka liquid scintillation analyzer LSC-1000: counting efficiency for $^{14}$C was 92%.

*Electron Microscopy*—The hepatocytes incubated with and without an amino acid mixture were prefixed with 2.5% glutaraldehyde, 8% sucrose, and 0.1% $CaCl_2$ in 0.1 M Na cacodylate buffer (pH 7.2) for 1 h, washed 3 times with the buffer, and postfixed with 1% $OsO_4$ and 8% sucrose in the buffer (pH 7.2) at 4°C for 30 min. The samples were dehydrated through a graded series of ethanol and embedded in Epon 812. Thin sections were stained with uranyl acetate for 5 min and lead citrate for 3 min, and examined under a Phillips EM 200 electron microscope. Morphometric analysis was done by point-counting according to Weibel (*18*).

## RESULTS

*Separation of Autophagy-Related Vacuoles by Percoll-PVP Density Gradients*—The optimum conditions for density gradient centrifugation, *e.g.*, time and speed, were chosen previously (*13*). As shown in Fig. 1 (top panels), a wide range of Percoll concentration (30-60%) gave various $\beta$-hexosaminidase profiles with two peaks on the gradients. With an increase in Percoll concentration, the major peak shifted from the high density (dense) to the low density (buoyant) region. This was probably due to changes in the density pattern accompanying the increase in the initial medium density. However, compared with a Ludox-PVP mixture (*10, 19*), separation of these two fractions by Percoll alone was not sufficient. Therefore, we added free PVP to Percoll as in Ludox gradients. When PVP was added (Fig. 1, lower panels), separation of these two peaks was markedly improved, showing a clear bimodal distribution. Since addition of PVP did not influence the density (data not shown), it seems likely that free PVP helps separate and stabilize various types of particles by affecting other factors than density, *e.g.*, particle size, viscosity, *etc.* To obtain a clear bimodality comparable to that in Ludox-PVP gradients (*19*), the separation conditions were examined in more

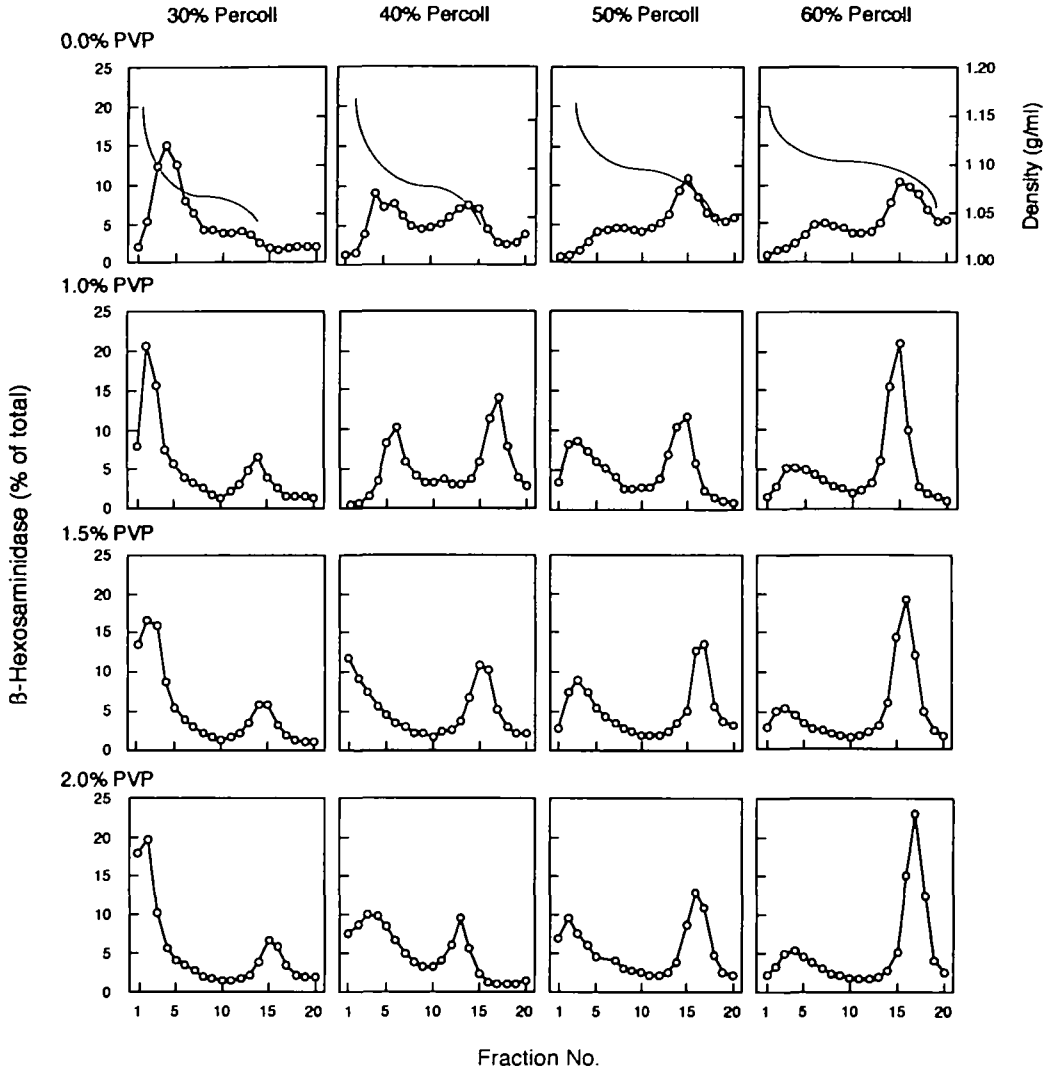


Fig. 1. **Effects of Percoll and PVP concentrations on density gradient profiles of β-hexosaminidase in the mitochondrial-lysosomal (M+L) fraction from isolated rat hepatocytes.** Samples were layered on the gradient medium and centrifuged as described in "EXPERIMENTAL PROCEDURES." Because PVP addition had no influence, density (thin solid curve) is presented only in the top panels.

detail (Fig. 2). A mixture of 60% Percoll and 0.75% PVP was finally chosen, in which the profile consisted of a major buoyant peak ($d=1.090$) and a minor dense peak ($d=1.131$), quite similar to the profile obtained previously (*19*). However, this β-hexosaminidase profile obtained from isolated hepatocytes was quite different from that in liver perfusion study (*10, 11, 20*). In order to test if this was the result of a difference in gradient media or lysosomal population in these two preparations, they were directly compared under the same gradient conditions (Fig. 3). The profiles showed a remarkable contrast. The liver tissue exhibited a large peak in the dense region just as in Ludox gradients (*10, 11*), whereas hepatocytes exhibited a smaller peak in the dense region and a much larger peak in the buoyant region. Therefore, the difference in the profiles is due not to the gradient medium, but to autophagic/lysosomal particles in the preparation.

Since the mode of sample application on the gradients affected the profile of lysosomal particles in liver perfusion study (*10*), the responses of particles from parenchymal cells were similarly tested. As shown in Fig. 4A, when the M+L fraction was dispersed through the gradient medium before centrifugation, a small portion of vacuolar particles in the buoyant peak moved down to the dense region, which was the same response as that in the liver (*10, 11, 20*). The behaviour of soluble, non-particulate materials in these two modes was monitored by $^{14}$C-sucrose (Fig. 4B), which was uniformly distributed by the dispersion mode, but was diffused from top to bottom by the layering mode. The enzyme shift was small but distinct, and was specific to lysosomes. It was not seen with mitochondria (Fig. 4C) or total protein (Fig. 4D), or with endosomes and Golgi vesicles (*19, 21*). Although the amounts of the M+L fraction from the liver tissue applied to the gradient affected the β-hexosaminidase profile significantly (*10*), this was not the case with the M+L fraction from hepatocytes (1 to 4 mg protein equivalents per gradient, data not shown). Furthermore, cell numbers for homogenization (3 *vs.* $20\times10^6$ cells/ml), and resuspension for making M+L fraction with homogenizer or by pipetting were confirmed

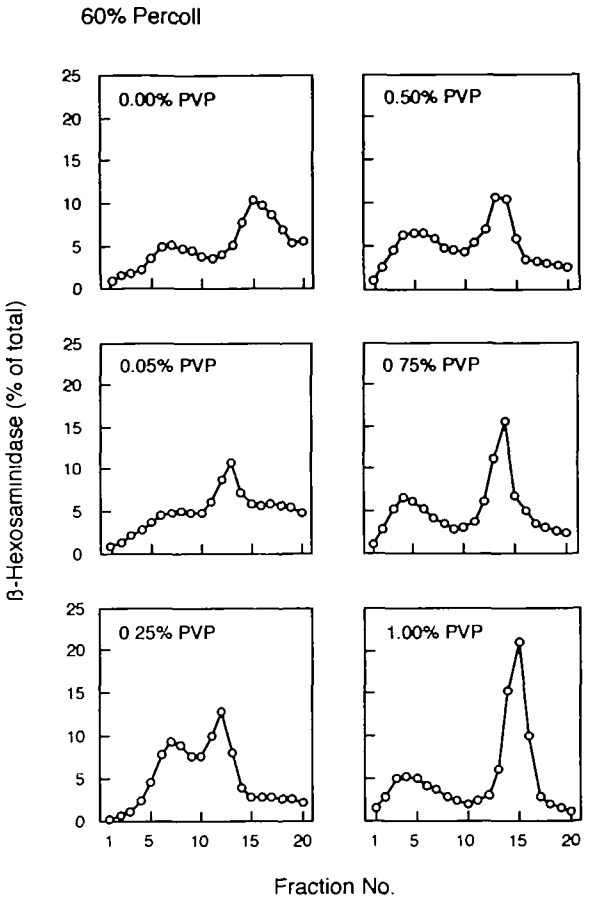


Fig. 2. **Further examination of PVP effect on 60% Percoll gradient profiles of β-hexosaminidase.**

not to affect the profile (data not shown).

*Effect of Amino Acids on Proteolysis and the Gradients*—Since amino acids control not only proteolysis but also lysosomal profiles on the gradients in perfused liver (*20*), the responses of isolated hepatocytes were similarly tested. Proteolytic response of hepatocytes to graded levels of Reg AA was measured (Fig. 5). Although a regulatory amino acid mixture usually showed multiphasic inhibition (*5, 22*), Reg AA exhibited a suppression curve with a single component like a complete amino acid mixture. This was because Ala, a coregulator, was added to a regulatory mixture to eliminate the unique response that occurs at its low concentration (*5*). Tenfold Reg AA fully suppressed proteolysis down to 43% of 0 × control.

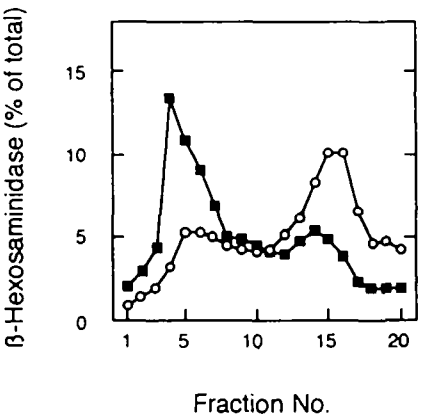


Fig. 3. **Distribution of β-hexosaminidase on Percoll-PVP gradients.** Comparison of liver tissue (■) and isolated hepatocytes (○). The M+L fractions from both preparations were centrifuged in the same run.

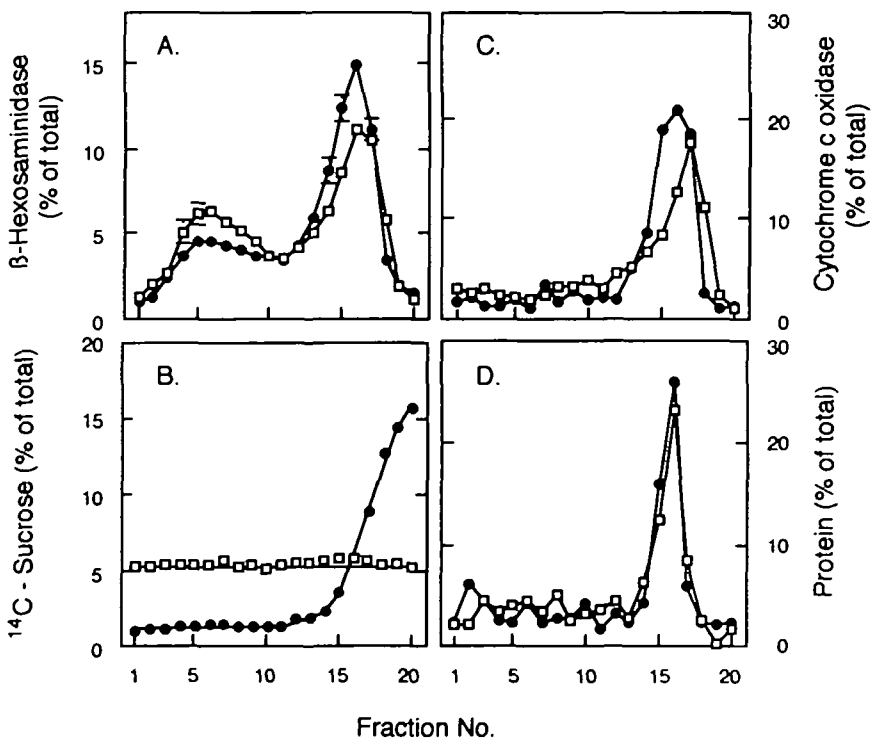


Fig. 4. **Shift effect of autophagic/lysosomal particles on Percoll-PVP gradients by layering (●) and dispersion (□) modes.** The M+L fraction was applied on the gradient medium in two modes, and after centrifugation several markers were assayed: β-hexosaminidase (A), $^{14}$C-sucrose (B), cytochrome *c* oxidase (C), and protein (D).

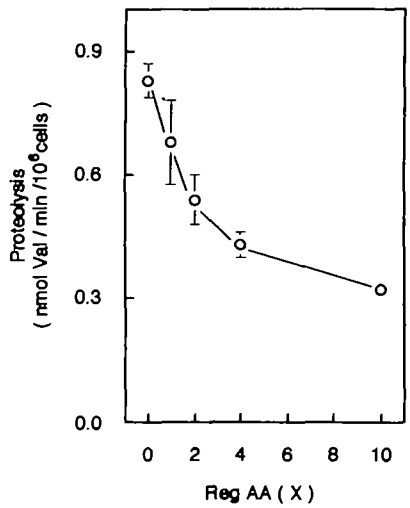


Fig. 5. **Effect of graded Reg AA levels on proteolysis in isolated hepatocytes.** Bulk proteolysis was measured using Val release with cycloheximide as described in "EXPERIMENTAL PROCEDURES." Data were means±SE ($n$=9-21).

As depicted in Fig. 6A, Reg AA induced changes in the gradient profile: it progressively decreased the buoyant peak and increased the dense peak. Since total $\beta$-hexosaminidase activity in the M+L fraction was not changed by Reg AA after 60 min of incubation, these changes in the profile seemed to reflect alterations in the subpopulation of autophagic/lysosomal particles including this marker enzyme, *i.e.*, AVd and DB, along with their formation, maturation, and recycling. The fraction subject to change from 0× to 10× Reg AA was about 15% of total activity on the gradients, which was supported by liver perfusion study: a similar range (12-23%) was obtained (*10, 20*). The gradient shift of autophagic/lysosomal particles was calculated as the elevation in the buoyant peak by amino acid deprivation based on 10× Reg AA level, and the results are plotted in Fig. 6B. The correlation of the accelerated proteolysis and the gradient shift of autophagic/lysosomal particles is plotted in Fig. 7, which shows a highly linear relationship between these two measurements.

*Electron Microscopy of Autophagic Vacuoles*—To evaluate the contribution of autophagic vacuoles to the buoyant peak in the profile, we measured the aggregate volumes of autophagic vacuoles in isolated hepatocytes. Since a 10× plasma level of amino acids completely suppressed macroautophagy in perfused liver (*23*), the effect of amino acids was also examined in isolated hepatocytes. The difference in the effects of amino acids between perfused liver and hepatocytes has not yet been fully clarified. The amino acids effect acted more slowly in hepatocytes than in perfused liver (Goto, M. *et al.*, manuscript in preparation), but their suppressive effect on AVi and AVd was nearly complete after 60 min (Fig. 8). Morphometric analysis revealed that AVi and AVd were suppressed to 21% and 31%, respectively, after 10× Reg AA treatment for 60 min (Table I). This implies that the majority of AV particles were lost in the M+L fraction of 10× Reg AA treated hepatocytes, and the buoyant peak at 10× consists of $\beta$-hexosaminidase-containing components other than AVd.

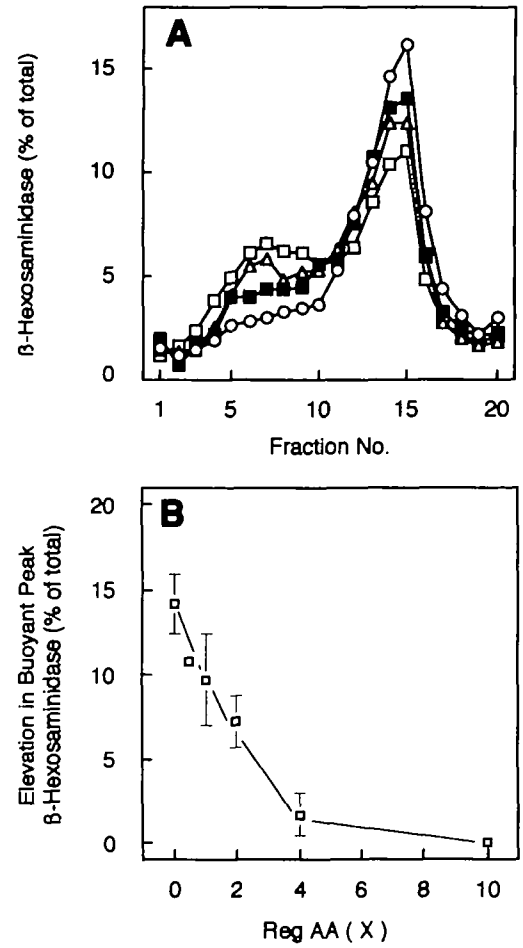


Fig. 6. **(A) Effect of graded Reg AA levels on $\beta$-hexosaminidase profiles on Percoll-PVP gradients.** Freshly isolated hepatocytes were incubated for 60 min with Reg AA at 0 (○), 2 (■), 4 (△), and 10× (□) level. The M+L fraction was layered on the gradient medium and analysed as described in "EXPERIMENTAL PROCEDURES." **(B) Gradient shift of autophagic/lysosomal particles by Reg AA.** The data were calculated as elevation of $\beta$-hexosaminidase in the buoyant peak by amino acid deprivation above 10× level, and plotted dose-dependently. Data were means±SE ($n$=4-6), except 0.5× ($n$=2).

## DISCUSSION

Since a report by Pertoft *et al.* (*24*), separation of heterogeneous lysosomes has been tried using colloidal silica density gradients. Rome *et al.* improved separation by employing a mixture of Ludox, a colloidal silica, and PVP (*25*). Surmacz *et al.* were the first to apply this method successfully to autophagy-related particles (*10, 11*). Since then, the colloidal silica has been found most effective for separating two lysosomal populations, AV and secondary lysosomes, compared with other gradient media, *e.g.*, metrizamide or Nycodenz. However, Ludox has several disadvantages, such as instability at physiological pH, possible toxicity toward cells, and the difficulty of removing it from samples (*26*). Thus, in the present study, Percoll, a more readily available colloidal silica that is chemically coated with PVP, was tested as an alternative gradient medium. Percoll is a stable and modified colloidal silica

without free PVP and toxicity. However, since Percoll alone did not show such good separation (Fig. 1) as a mixture of Ludox-PVP in the liver tissue (*10*), we added free PVP to Percoll. This modification resulted in highly reproducible gradients very similar to Ludox-PVP gradients, although the compositions of the two gradient media differed considerably (Ludox-PVP: 8.04-4.0% *vs.* Percoll-PVP: 60-0.75%). In general, lysosomal profiles from isolated hepatocytes showed much clearer separation (or bimodality) than liver tissue, which made it easy to distinguish AV from DB.

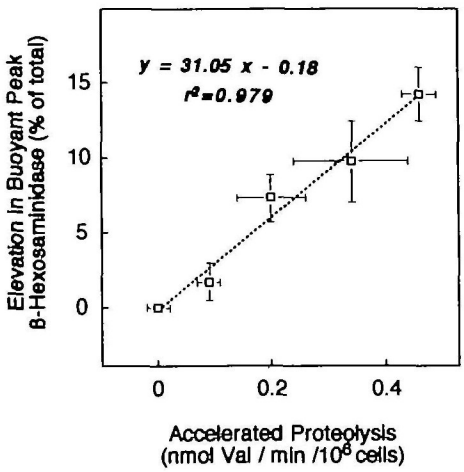


Fig. 7. **Correlation between accelerated proteolysis and the gradient shift of autophagic/lysosomal particles.** Accelerated proteolysis was calculated from Fig. 5 as the increase above 10× level. Gradient shift data were from Fig. 6B.

The difference between liver tissue and hepatocytes as vacuole donors deserves mention. In intact liver tissues, non-parenchymal cells, *e.g.*, Kupffer cells and endothelial cells, account for only 6.3% of total liver volume, but their lysosomes occupy as much as 43% of total liver lysosomes (*27*). Accordingly, it is conceivable that the greater part of the dense peak on the gradient from the liver tissue (Fig. 3) may be due to lysosomes of non-parenchymal origin. These lysosomes from non-parenchymal cells are regarded as contributing mostly to heterophagy or phagocytosis (*28*), and it is not known whether they are substantially involved in autophagy or not. On the other hand, isolated hepatocytes prepared by the present method mainly consist of parenchymal cells and include only 1-2% non-parenchymal cells (*12*). Therefore, it is highly probable that both peaks

TABLE I. **Effect of Reg AA on aggregate volume of autophagic vacuoles in isolated hepatocytes.**[a]

| | AVi | AVd | Total |
|---|---|---|---|
| | (% of cytoplasmic volume) | | |
| 0× | 1.37±0.22[b] (100)[c] | 2.09±0.14 (100) | 3.46±0.30 (100) |
| 10× Reg AA | 0.29±0.09 (21) | 0.64±0.17 (31) | 0.92±0.19 (27) |

[a]Hepatocytes were incubated for 60 min with or without amino acids, and their electron micrographs (Fig. 8) were subjected to morphometric analysis according to Weibel (*18*). [b]Data are means±SE ($n$ = 12). [c]Numbers in parentheses are expressed as percentages of 0×.

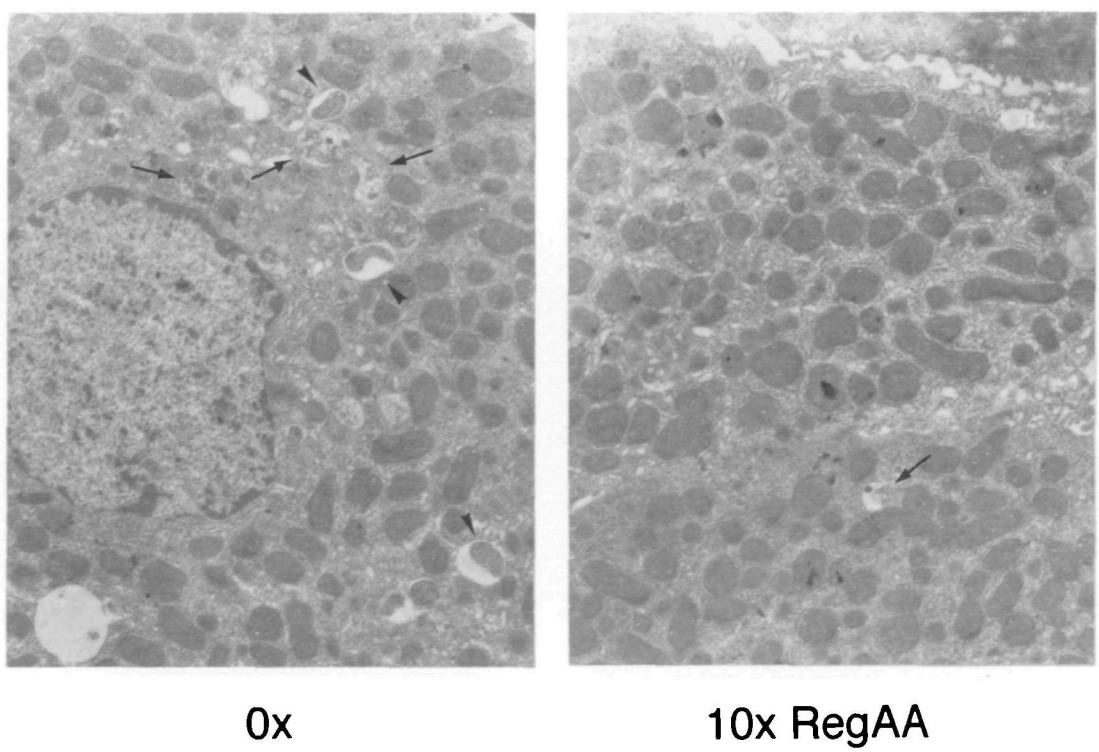


Fig. 8. **Electron micrograph of autophagic vacuoles in isolated hepatocytes.** Isolated cells were incubated for 60 min with 0× (left) and 10× Reg AA (right), and fixed and processed for electron micrography as described in "EXPERIMENTAL PROCEDURES." Typical autophagic vacuoles, AVi (arrowheads) and AVd (arrows), are mainly seen with 0×. Magnification: ×5,000.

on the gradients from hepatocyte preparation are derived only from parenchymal cells which are active in autophagy.

The two gradient peaks, dense and buoyant, were characterized in the previous papers for the case of perfused liver (*10, 11*). The dense peak consists mostly of DB or secondary lysosomes, while the buoyant peak includes AV as well as mitochondria, ER, peroxisomes, Golgi bodies, *etc.* The characteristics of the corresponding peaks from isolated hepatocytes seem to be the same, based on their biochemical data in Fig. 4 and Ref. *19*. Thus, if AV is required in a purer form, another technique to separate AV from other organelles, *e.g.*, Nycodenz gradients, is necessary in combination with this method.

In interpreting the data of density gradient profiles, an important question is whether all the particles including lysosomal enzymes on the gradients are involved in proteolysis or not, and if not, to what extent they are actually involved in the process. The linear relationship obtained in Fig. 7 strongly supports the notion that a subpopulation of the buoyant peak regulatable by amino acids corresponds to the vacuoles actively involved in proteolysis. This subpopulation involved in macroautophagic pathway accounts for only 15% of total $\beta$-hexosaminidase activity in the M+L fraction, or at most 22% [15% divided by the volume of AVd suppressed by 10× Reg AA (1-0.31), from Table I]. On the other hand, although the aggregate volume of AVd was largely suppressed at 10× Reg AA from EM study (Table I), there still remains a substantial peak in the buoyant region, implying that there exist some kinds of vacuoles or membraneous particles including $\beta$-hexosaminidase other than AVd, that are not related to proteolysis. Likely candidates in the buoyant region include GERL, the acid-phosphatase containing network which was originally described by Novikoff (*29*), or otherwise endosome, Golgi network, ER, and plasma membrane. However, it is not known how much these membraneous organelles include lysosomal enzymes. $\beta$-Hexosaminidase is not necessarily present only as typical spherical lysosomes. Recently, unusual elongated lysosomes (or nematolysosomes) were reported in rat hepatocytes (*30*), which may occupy 20-40% of total lysosome population; and although their function remains unclear, it is probably unrelated to autophagic proteolysis (Araki, N., personal communication). It should be noted that, of the total membraneous compartments including $\beta$-hexosaminidase, only a portion is actively involved in autophagic function.

In the present study, AVi could not be detected on the density gradients, because $\beta$-hexosaminidase is not present in this initial vacuole. However, in that it is a major target of autophagic regulation by a number of physiological factors, it is important to detect, identify and purify it for characterization. Originally AVi was defined morphologically and is easy to identify by electron microscopy. But it is not easy to detect biochemically, because it has no good marker enzymes located on its membrane or in the matrix. At the moment, several techniques have been developed, *e.g.*, to monitor *de novo* vacuole formation with residualizing probes such as $^{125}$I-labeled tyramine cellobiitol (*19*), $^{14}$C-sucrose or $^{3}$H-raffinose (*31*) after permeabilization, or to measure sequestration of cytoplasmic proteins like LDH by AVi after inhibition of subsequent processes such as proteolysis (leupeptin) or fusion with lysosomes (vinblastin) (*32*). To accumulate AVi more specifically, we are trying to inhibit the acidification step by bafilomycin $A_1$, an inhibitor of vacuolar $H^+$-ATPase (Goto *et al.*, manuscript in preparation).

In summary, we have demonstrated that autophagy-related vacuoles from isolated hepatocytes can be sharply separated by use of modified Percoll-PVP density gradients. Both buoyant and dense particle fractions on the gradients shift in proportion to proteolytic changes which are regulated by amino acids, and thus the gradient profiles can be used as a measure of autophagic proteolysis. In addition, it was proved that the AVd that is actually involved in proteolysis occupies only a part of the buoyant peak on the gradients.

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