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Endosomes can undergo an ATP-dependent density increase in the absence of dense lysosomes

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On the basis of evidence that lysosomal enzymes and membrane proteins are present in endosomes, we have previously suggested that the production of lysosomes involves maturation rather than vesicle fusion (Roederer, M., R. Bowser, R. F. Murphy, J. Cell. Physiol. 131, 200–209 (1987)). Since the appearance of endocytosed material in lysosomes is associated with an increase in buoyant density from that of endosomes, a prediction of the model is that endosomes should be capable of undergoing such an increase in vitro. We observe that under appropriate conditions, isolated endosomes containing [^{125}I]EGF can undergo an increase in density in vitro to that of dense lysosomes, mimicking the density change which occurs in vivo. This occurs in the absence of dense lysosomes with which to fuse. The density increase requires ATP and can be efficiently inhibited in vitro by the presence of benzylamine, suggesting that vesicular acidification is required. Since low *p*H has previously been shown to induce formation of a matrix by lysosomal enzymes in vitro (Buckmaster, M. J., A. L. Ferris, B. Storrie, Biochem. J. 249, 921–923 (1988)), we propose that a mechanism by which endosomes and/or lysosomes increase their density is a low *p*H induced aggregation of vesicle contents which decreases the osmotic pressure inside the vesicle. Together with previous data, the results provide highly suggestive evidence that the pathway to lysosomes includes a maturation of the postsorting compartment into what has classically been termed a lysosome.

Introduction

While it has been clear for a number of years that there are several biochemically and biophysically distinct compartments involved in the process of endocytosis, a number of questions remain regarding the mechanisms by which molecules appear in these compartments. This is especially true for the biogenesis of dense lysosomes. It has been proposed that fusion of light vesicles with preexisting dense primary lysosomes mediates the delivery of endocytosed material to dense hydrolytic compartments (for review, see [18, 24]). Recently, the possibility has been raised that the appearance of endocytosed material in dense compartments may involve a maturation event [34, 38].

Recent evidence from in vivo studies supports the concept that fusion of incoming vesicles with preexisting compartments is limited to the first few minutes following endocytosis. For example, 30% of a fluorogenic substrate for cathepsin B which has been endocytosed for 7 min is inaccessible to a chase with a specific inhibitor (leupeptin) [34]. Similarly, after 5 min, FITC-transferrin becomes increasingly inaccessible to quenching by anti-fluorescein antibodies as it is endocytosed [37]. These results suggest that the vesicle fusions observed in vivo are specific to early endocytic vesicles and occur during a brief time window in which preexisting endosomes can fuse with newly formed endocytic vesicles.

Several cell-free systems for assaying fusion of endocytic vesicles have recently been devised [2, 7, 8, 15–17, 22]. These experiments reveal a specific, ATP-dependent fusion process that occurs between "early" compartments. This process probably represents the fusion of newly formed endocytic vesicles with each other and/or with endosomes, which in vivo leads to the formation of new endosomes or the delivery of materials to a preexisting endosome. Gruenberg and Howell [17] and Braell [2] found that maximal competence for vesicle fusion occurred 5 min after the onset of endocytosis, coinciding with the observed delivery of material to endosomes, while fusion between labeled endosomes and lysosomes was not observed in vitro.

A basic premise of many models of lysosome biogenesis is that delivery of endocytosed material to hydrolytic compartments is a relatively late event [18]. This view is

Abbreviations. BSA Bovine serum albumin. — cDMEM Complete Dulbecco's modified Eagle's medium. — EGF Mouse epidermal growth factor. — FITC Fluorescein isothiocyanate. — HB Homogenization buffer. — MB Maturation buffer. — PBS Phosphate-buffered saline. — Tf Diferric human transferrin.

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based upon the observations that onset of degradation is a "slow" event and that it can be inhibited by low temperature (i.e., 17 °C) without abrogating uptake [11]. Recent evidence indicates that delivery of at least some "lysosomal" enzymes occurs very early during endocytosis. Diment and Stahl [9] determined that cathepsin D activity was present in a compartment labeled 5 min after endocytosis, while we have established the presence of cathepsin B [34] and acid phosphatase [3] activities within very early endocytic compartments using endocytosed fluorogenic substrates. In addition, we have shown that incubation at 17 °C does not block the hydrolysis of these substrates, demonstrating that at least these enzymes are delivered to endosomes at reduced temperature. In addition, proteolytic processing of endocytosed asialofetuin [1] and β-glucuronidase [13] has been shown to occur in low density, endosomal compartments. Recent evidence presented by Mayorga et al. [23] also indicates that, at least in macrophages, proteolytic activity is delivered to very early endocytic compartments. By lowering intravesicular *p*H in cell homogenates using an ionophore, they demonstrated that mannosylated bovine serum albumin was delivered to a compartment capable of proteolytic digestion within 5 min following receptor-mediated uptake. At earlier times, delivery of proteolytic activity required incubation under conditions which permit vesicle fusion. In either case, the proteolytic activity colocalized with endosomes on Percoll gradients. The results suggest that lysosomal enzymes are delivered before the density increase of endocytosed material in vivo and confirms the crucial role of the mildly acidic endosomal *p*H in regulating enzyme activity [29].

Thus, currently available evidence indicates that at least some lysosomal enzymes are delivered to early (light-density) compartments and that appearance of material within dense lysosomes may reflect a maturation event rather than a process requiring fusion with preexisting dense compartments. A prediction of this hypothesis is that endosomes should be capable of increasing their buoyant density in vitro in the absence of dense lysosomes [34]. We demonstrate here that this process can occur and describe the preliminary characterization of its biochemical requirements. Preliminary reports of this work have been presented [31].[3)]

Materials and methods

Unless noted otherwise, all materials were obtained from Sigma Chemical Co. (St. Louis, MO/USA).

Cell culture

Swiss Albino 3T3 fibroblasts were obtained from American Type Culture Collection, Rockville, MD, and cultured in Dulbecco's modified Eagle's medium supplemented with 10% (v/v) bovine calf serum (Gibco, Grand Island, NY/USA). 100 U/ml of penicillin, 100 μg/ml of streptomycin sulfate, and 2 mM L-glutamine (cDMEM). Cell stocks were maintained at less then 80% confluence.

Preparation of [^{125}I]epidermal growth factor and [^{125}I]transferrin

Mouse epidermal growth factor (Biomeda, Foster City, CA, or Upstate Biotechnology Inc., Lake Placid, NY/USA) and diferric human transferrin (Miles, Naperville, IL/USA) were labeled with ^{125}I (ICN, Irvine, CA/USA) using iodobeads (Pierce Chemical Co., Rockford, II) [20]. The labeled EGF had a specific activity of approximately 10 μCi/μg, and binding was 95% specific at concentrations up to 100 ng/ml. The labeled Tf had a specific activity of approximately 1 μCi/μg, and binding was 99% specific at 4 μg/ml. Aliquots of [^{125}I]EGF were frozen at −20 °C until the day of use. The labeled transferrin was kept at 0 °C.

Labeling conditions

Typically, 8×10^7 cells were used for each experiment. Cells were washed with phosphate-buffered saline (137 mM NaCl, 27 mM KCl, 153 mM Na_2HPO_4, 14.7 mM KH_2PO_4, 0.9 mM $CaCl_2$, 0.5 mM $MgCl_2$, *p*H 7.4) at 0 °C. cDMEM at 17 °C containing 25 ng/ml [^{125}I]EGF or 5 μg/ml [^{125}I]Tf was added, and incubation continued at 17 °C for 1 h. Cells were then washed twice with PBS at 0 °C to stop further endocytic processing and scraped with a rubber policeman into homogenization buffer (250 mM sucrose, 10 mM HEPES, 2 mM EDTA, *p*H 6.8). All further steps were carried out at 0 °C, with the exception of the maturation incubation.

Subcellular fractionation

Labeled cells were centrifuged at 1000*g* for 10 min, resuspended in 2 ml HB, and homogenized by 10 strokes of a Potter-Elvehjem homogenizer (Fisher Scientific, Pittsburgh, PA/USA). The homogenates were centrifuged for 6 to 10 min at 1000*g*, to remove unbroken cells and debris. The 2 ml postnuclear supernatants were then mixed with Percoll (Pharmacia, Piscataway, NJ/USA) to a final concentration of 27% in HB (14 ml final volume) and centrifuged for 151 min at 17800*g* or 120 min at 25000*g*, in an SA-600 rotor (DuPont Instruments, Wilmington, DE/USA). This fractionation results in two predominant peaks of endocytic vesicles, with dense lysosomes sedimenting near the bottom and light vesicles towards the top [25]. The gradients were collected from the bottom by pumping. The position of the endosomal peak was determined in separate experiments; fractions 14 to 19 contained the endosomes (Fig. 1b) and were pooled. The positions of light and dense lysosomes were determined by assaying for the presence of β-galactosidase activity using 4-methylumbelliferyl-β-D-galactoside as described [36].

Assay conditions

Usually, 1.0 ml of the pooled fractions (in HB) were mixed with 5.65 ml of maturation buffer (MB, 53 mM NaCl, 53 mM KCl, 1.1 mg/ml BSA, 132 mM sucrose, 1.1 mM EDTA, 5.3 mM Hepes, *p*H 6.8) at 0 °C to which was added 0.35 ml of either water or 100 mM K_2ATP (vanadate free) to yield a final volume of 7 ml (final concentrations: 43 mM NaCl, 43 mM KCl, 142 mM sucrose, 1.14 mg/ml BSA, 1.14 mM EDTA, 5.7 mM Hepes, and either 0 or 5 mM K_2ATP).

Samples were treated as described in Results, then cooled to 0 °C. Each 7 ml sample was mixed with 7 ml of 50% Percoll in HB. Gradients were centrifuged as above. Twenty fractions were again collected for each gradient and counted by liquid scintillation counting using Ecolume scintillation cocktail (ICN, Irvine, CA/USA) for 5 min each. Counting efficiency was greater than 40% (data not shown). Background counts from Percoll in HB (ranging from 3–20% of the peak counts) were subtracted from all samples prior to analysis.

[3)] Fourth International Congress of Cell Biology, Montreal, Quebec/Canada, August 14–19, 1988; American Society for Cell Biology and the American Society for Biochemistry and Molecular Biology Joint Meeting, San Francisco, CA, January 29–February 2, 1989.

Electron microscopy

A postnuclear supernatant from approximately 2×10^8 3T3 cells was prepared and fractionated on a 27% Percoll gradient as described above. β-Galactosidase activity was used to identify peaks. Pooled endosomes (fractions 14–19) were divided in half, and the samples were adjusted to 50 mM NaCl, 50 mM KCl, 1 mg/ml BSA, in HB, *p*H 6.8. One sample was kept on ice and the other was incubated at 37 °C for 150 min in the presence of 5 mM ATP. At the end of the incubation, the samples were placed at 0 °C and fixed for 1 h in 2% glutaraldehyde. After fixation, the samples were centrifuged for 2 h at 100000*g* in a Beckmann SW50.1Ti rotor to remove the Percoll.

The vesicle pellets on top of the hard Percoll pellets were removed with a minimum of supernatant and pelleted in an Eppendorf centrifuge for 40 min. The supernatants were removed, and the vesicle pellets were postfixed in 2% OsO_4 in 0.1 M sodium cacodylate buffer, *p*H 7.1, dehydrated and embedded in Epon/Araldite. The samples were then sectioned, stained with uranyl acetate and lead citrate, and analyzed on a Philips 300 electron microscope.

Results

Endocytic vesicles increase in buoyant density in vitro

A prediction of the maturation model for delivery of endocytosed material to dense compartments is that endosomes should be able to increase their buoyant density in vitro. It has previously been shown that the appearance of endocytosed material in dense compartments in vivo is inhibited at temperatures below 20 °C [10, 11, 21, 40]; therefore, cells were incubated with [^{125}I]EGF at 17 °C for 60 min to label light endocytic compartments. We then examined the ability of these labeled compartments to increase in buoyant density in vitro.

To rule out an increase in density by the fusion or aggregation of labeled vesicles with preexisting dense bodies, vesicles were first fractionated on the basis of buoyant density on 27% Percoll gradients. The locations of the dense lysosomal and endosomal fractions were determined in identical gradients examined for density, β-galactosidase activity and for the presence of [^{125}I]Tf or [^{125}I]EGF (Figs. 1a, b). The endosomal fractions which contained [^{125}I]EGF (fractions 14–19) from the initial 27% Percoll gradient (Fig. 1b) were pooled, aliquoted into tubes of MB with or without added ATP, and incubated either at 0 °C or 37 °C for 160 min. Subsequently, the samples incubated at 37 °C were chilled on ice, and all samples were refractionated on Percoll gradients. Upon refractionation, it was observed that the sample incubated with ATP at 37 °C showed a dramatic increase in density such that the radioactive EGF sedimented at the density of dense lysosomes (Fig. 1c). Conversely, the full density increase was inhibited at 0 °C or in the absence of ATP at 37 °C, while samples without ATP which had been kept on ice did not increase in density. A small shift was observed in the sample incubated at 37 °C in the absence of ATP, which may result from residual cellular ATP present during the incubation. To verify that the increase in density was not due to release and aggregation of the label, a parallel sample was incubated with 0.1% Triton X-100 to disrupt vesicles. In the presence of Triton, the radioactivity was evenly distributed throughout the entire gradient (data not shown).

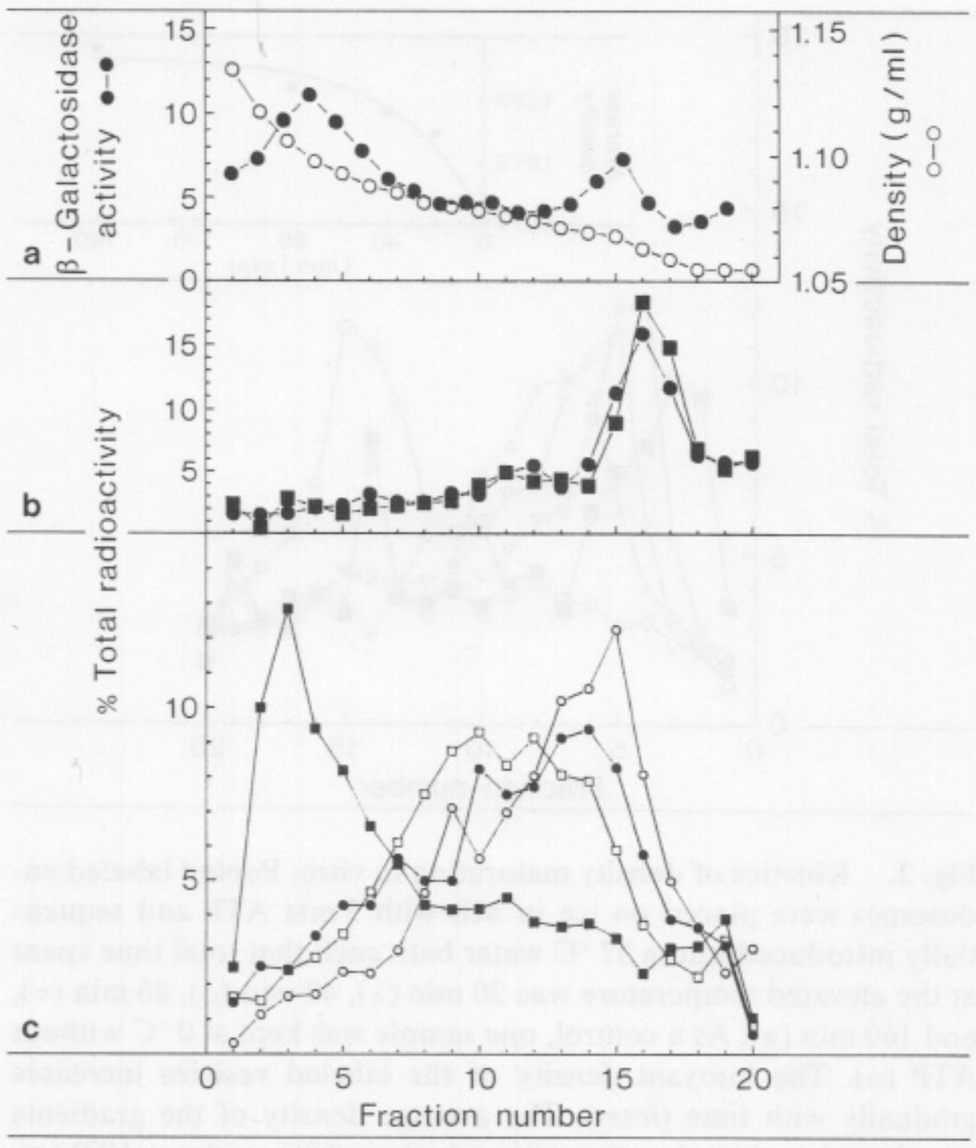

Fig. 1. Endosome maturation in vitro. Postnuclear supernatants were prepared and fractionated on 27% Percoll gradients. To determine the location of dense lysosomes, the density profile and β-galactosidase activity (**a**) were determined for each fraction, while the location of endosomes was determined by labeling cells in medium containing either [^{125}I]EGF (●) or [^{125}I]transferrin (■) for 1 h at 17 °C (**b**). To assay maturation in vitro, fractions containing [^{125}I]EGF were pooled, mixed with MB, and incubated at 0 °C (*open symbols*) or 37 °C (*filled symbols*) in the presence (□, ■) or absence (○, ●) of 5 mM K_2ATP for 165 min (**c**). After incubation, samples were refractionated on Percoll gradients. The density increase is clearly ATP-dependent and inhibited at low temperature. These results are representative of four separate experiments.

Kinetics of density increase in vitro

To measure the kinetics of the density increase, aliquots of pooled vesicle fractions were incubated at 37 °C with ATP for various periods. As seen in Figure 2, intermediate periods of incubation resulted in intermediate density upon refractionation. By non-linear least squares fitting of the average density, the density maturation has a $t_{1/2}$ of 23 min (Fig. 2, *inset*). The observed kinetics are consistent with those observed for the appearance of endocytosed material in dense compartments in vivo [27].

Inhibition of density increase by benzylamine

To determine if vesicle acidification is required for the density increase, we measured the effect of 1 mM benzylamine. Benzylamine was used since this weak base neutralizes proton gradients without accumulating within the

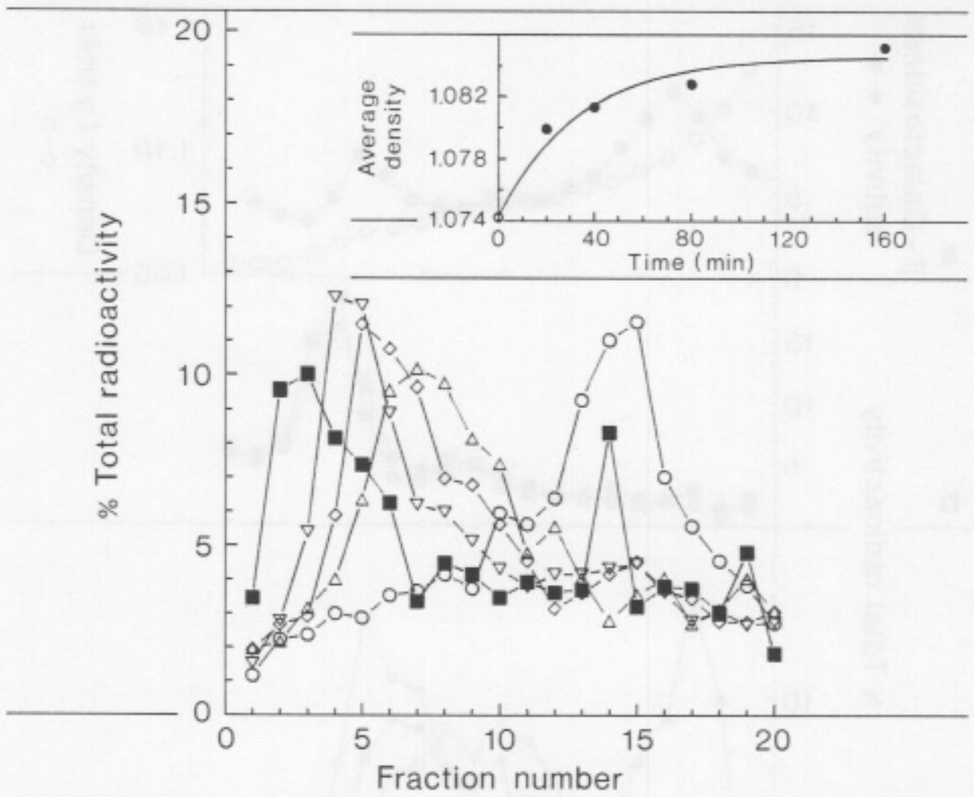

Fig. 2. Kinetics of density maturation in vitro. Pooled labeled endosomes were placed on ice in MB with 5 mM ATP and sequentially introduced into a 37 °C water bath such that total time spent at the elevated temperature was 20 min (△), 40 min (◇), 80 min (▽), and 160 min (■). As a control, one sample was kept at 0 °C without ATP (○). The buoyant density of the labeled vesicles increases gradually with time (*inset*). The average density of the gradients are plotted against duration of incubation; 0 °C without ATP (○), 37 °C with ATP (●). Also shown is the non-linear least squares exponential fit of the average density versus time. Results are representative of three separate experiments.

acidic compartment [5, 32, 33] and therefore, should not alter the density of the compartment directly. As shown in Figure 3, benzylamine significantly inhibits the ATP-dependent increase in density, indicating that acidification of endosomes is involved in the density increase.

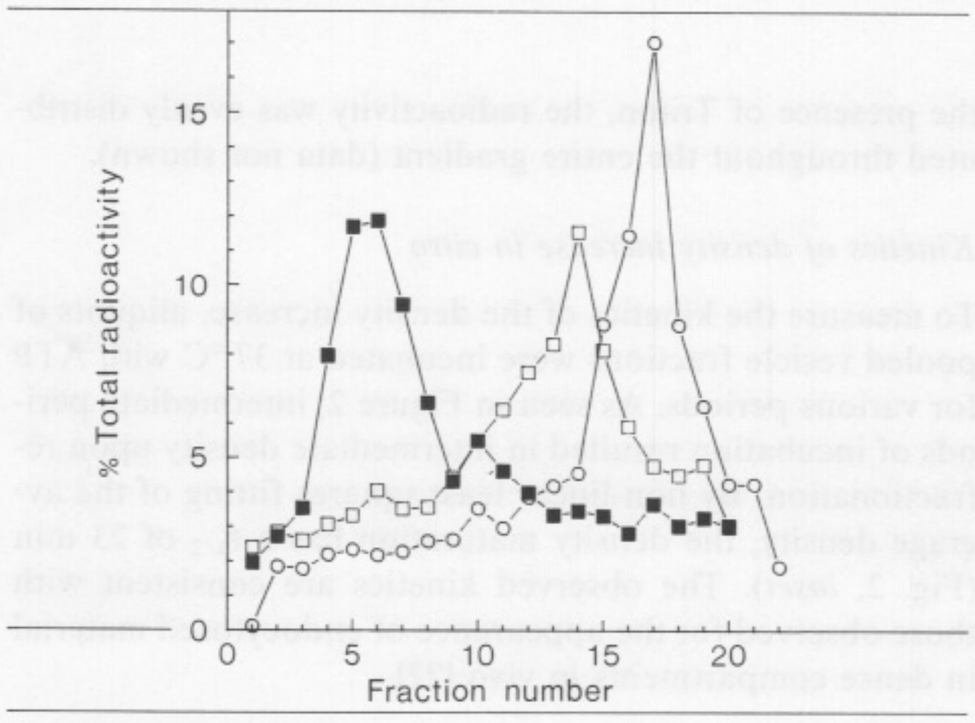

Fig. 3. Inhibition of in vitro density maturation by benzylamine. Pooled endosomes were incubated in MB for 80 min at 37 °C with ATP and 1 mM benzylamine (□), at 37 °C with ATP (■), or 0 °C without ATP (○). The addition of benzylamine clearly inhibits the density maturation. Results are representative of three separate experiments.

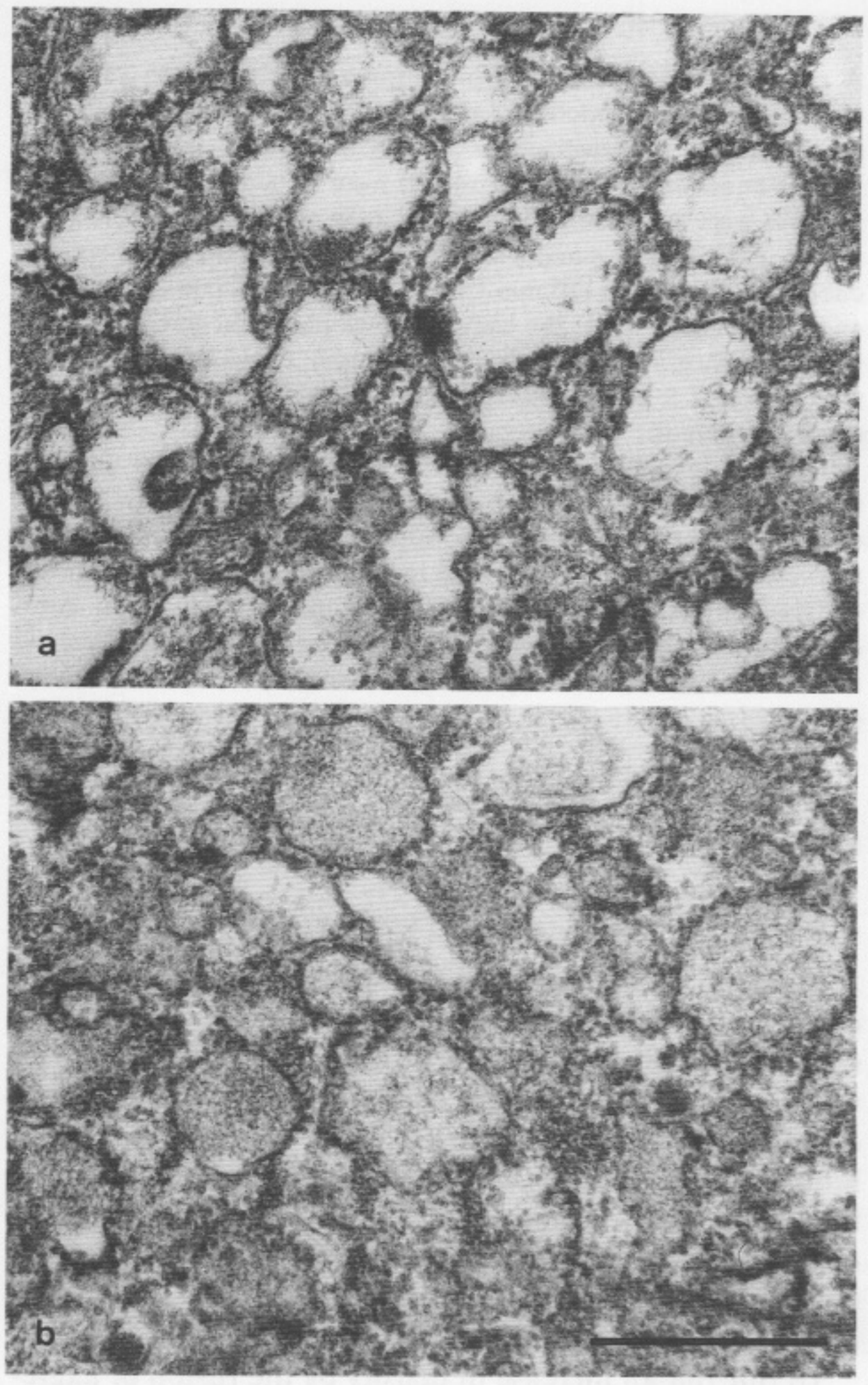

Fig. 4. Morphological analysis of vesicles matured in vitro. Pooled endosomes were divided into two fractions. One remained on ice (**a**) while the other (**b**) was incubated at 37 °C for 150 min with ATP. — Bar 0.5 μm.

Morphological analysis of vesicles after maturation

Associated with the increased density observed in vivo of late endocytic compartments compared to early compartments is an accumulation of electron-dense material within the lumen of the dense vesicles. To determine if such a morphological change is associated with the density increase in vitro, we analyzed isolated vesicles by electron microscopy. Samples were prepared as in Figure 1 without refractionation on the second Percoll gradient, and then fixed and prepared for electron microscopy. As shown in Figure 4, the sample incubated at 0 °C without ATP (Fig. 4a) contained electron-lucent vesicles. After density maturation (Fig. 4b) the vesicles were filled with electron-dense material. These electron-dense structures are essentially absent from the endosomal starting material (Fig. 4a).

Discussion

In view of the biochemical evidence which suggests that the transport of endocytosed material from light to dense compartments may involve a maturation step [29, 38], we developed an assay to specifically test for the ability of endocytically labeled vesicles to increase their buoyant density in vitro. In order to rule out fusion with preexisting dense bodies, vesicles were first isolated by fractionation on Percoll density gradients. We have shown that endosomes isolated from 27% Percoll gradients can be induced to increase their buoyant density in vitro. This process requires ATP, is inhibited at 0 °C, has a half-time of 23 min, and is inhibited by benzylamine (which inhibits endosome acidification). We have also shown that under the conditions used here, isolated vesicles change in appearance from hollow compartments to compartments filled with electron-dense material.

During the increase in density of endocytosed material in vivo, there is a slow acidification within the compartments from *p*H 6 to *p*H 5 [19, 26, 30, 34, 35]. It has also been established that delivery of materials to mature (dense) lysosomes is inhibited both by temperatures in the range of 17 °C to 20 °C [10, 11, 21, 40] and by treatment of cells with ionophores [27, 39]. We have previously shown that vesicular acidification to values below 6 is inhibited at reduced temperatures [34]. Recent results from in vivo and in vitro studies indicate that the Na^+/K^+-ATPase acts to limit the acidification of early, but not late, endocytic compartments [6, 12]. Exclusion of the Na^+/K^+-ATPase from late endosomes is presumed to allow acidification to lysosomal levels. Removal of the Na^+/K^+-ATPase from endosomes may be inhibited by a loss of membrane fluidity at temperatures below 20 °C, accounting for the observed inhibition of acidification and the lack of delivery of endocytosed ligand to dense lysosomes at reduced temperature. Therefore, a possible explanation for both the temperature block and the inhibition by ionophores is that the density increase is coupled to vesicular acidification to *p*H values below 6. The fact that agents which inhibit intravesicular acidification also inhibit appearance of material in dense lysosomes both in vivo and in vitro suggests that the process we described here occurs in living cells.

Buckmaster et al. [4] demonstrated that under specific conditions (low *p*H, low ionic strength), lysosomal enzymes will form a matrix independent of membranes. The formation of such a complex inside a light vesicle would be expected to decrease the osmotic strength inside the vesicle (due to a reduction in the number of particles), causing an outward flow of water and an increase in buoyant density. We have shown that, under appropriate conditions, endocytic vesicles can increase their buoyant density in vitro. We propose that the formation of a matrix within these vesicles is the underlying cause for the increase in density.

While our results indicate that structures with the characteristics of dense lysosomes can be formed from endosomes without the need for fusion with preexisting dense compartments, they do not rule out the possibility of other types of fusion or fission events. For example, vesicles involved in mannose 6-phosphate receptor recycling may arise by fission [14], or lysosomes may fuse with other lysosomes. Such fusions have been observed in vivo both by measuring the amount of endocytosed FITC-dextran per vesicle [28] and by detecting fusion of lysosomes containing invertase with other lysosomes containing sucrose [14]. Fusion of endosomes with existing dense compartments may be more frequent in cell types containing large numbers of residual bodies (such as confluent fibroblasts [36]). However, the results described here strongly support the possibility that new dense lysosomes may arise by a process linked to progressive acidification.

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