Transport of a Fluorescent Macromolecule via Endosomes to the Vacuole in Saccharomyces cerevisiae

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Abstract. Fluorescein isothiocyanate–conjugated dextran (FITC–dextran) is internalized by endocytosis into the lysosome-like vacuoles of Saccharomyces cerevisiae (Makarow, M., 1985, EMBO (Eur. Mol. Biol. Organ.) J. 4:1861–1866). Here we show that under energy depletion conditions FITC–dextran accumulated in a cytoplasmic compartment, from which it could be chased to the vacuole when the energy block was removed. The internal pH of the intermediate compartment under energy depletion was determined by fluorometry to be 5.8. The pH could be raised by the lysosomotropic agent ammonium chloride, the protonophore carbonyl cyanide p-trifluoromethoxyphenylhydrazone (CCCP) and the ATPase inhibitors dicyclohexylcarbodiimide (DCCD) and sodium vanadate. The pH of the vacuole was found to be 6.5. It was raised by ammonium chloride, CCCP, and DCCD, but not with sodium vanadate. Efrapetine had no effect on the internal pH of either compartment. By dissecting the endocytic pathway, two portions of the route leading to the vacuole could be studied separately. The internalization of FITC–dextran from the extracellular fluid to the intermediate compartment followed linear kinetics, was independent of energy, and occurred at temperatures of between 15°C and 37°C. Transfer of the marker from the intermediate compartment to the vacuole required energy, took place at temperatures between 19°C and 37°C, and had a half-time of 7 min at 37°C. Transport of the marker from the exterior of the cell to the vacuole did not require acidic pH values in the intermediate compartment or the vacuole. We suggest that the cytoplasmic compartment revealed by FITC–dextran, under energy depletion, represents the equivalent of the endosomes of mammalian cells.

Mammalian cells internalize material from the extracellular milieu by the process of endocytosis (Steinman et al., 1983; Brown et al., 1983; Wall et al., 1980). Many components, notably those that are attached to receptors at the plasma membrane, are endocytosed via coated pits into coated vesicles (Pearse and Bretscher, 1981; Petersen and van Deurs, 1983). The internalized components are soon found in smooth-surfaced endosomes, which have a heterogeneous, vesicular, or tubular appearance (Helenius et al., 1980; van Deurs et al., 1981; Willingham and Pastan, 1980; Matlin et al., 1983; Marsh et al., 1986), and, importantly, an acidic internal pH (Tycko and Maxfield, 1982; Galloway et al., 1983). The endosomal pH of many animal cell types has been found to be ~5.5 (Tycko et al., 1983; Murphy et al., 1984). In this compartment, a number of ligands are detached from their receptors, which enables the ligands to be taken into the lysosomes, while the receptors are recycled to the plasma membrane for reutilization (Gonzales-Noriega et al., 1980; Steer and Ashwell, 1980; Dautry-Varsat et al., 1983; Klausner et al., 1983). The endosomes also serve as the site of penetration for certain enveloped viruses, which release their nucleocapsids to the cytoplasm after fusion of the viral envelope with the endosomal membrane (Helenius et al., 1980; Marsh et al., 1983; Yoshimura and Ohnishi, 1984). The fusion is triggered by the acidic environment within the endosome (White et al., 1980; Helenius, 1984; Doms et al., 1985).

We have recently reported that Saccharomyces cerevisiae cells internalize macromolecules like FITC–dextran and α-amylase by endocytosis into the vacuole, which is the hydrolitic organelle of yeast. Also enveloped viruses were internalized, once the cell wall was removed to expose the plasma membrane (Makarow, 1985a, b). Riezman (1985) has used the organic dye Lucifer yellow to demonstrate endocytosis in S. cerevisiae. He showed that all of the temperature-sensitive transport mutants (Novick et al., 1980), in which secretion was blocked at the secretory vesicle stage, were deficient in endocytosis at the restrictive temperature. Also several of the mutants in which the block in secretion occurs at earlier stages were temperature sensitive for endocytosis. Thus, common gene products appear to be involved both in endocytosis and exocytosis (Riezman, 1985). Here we describe an intracellular compartment involved in endocytosis in S. cerevisiae, which appears to be the equivalent of endosomes of mammalian cells. The internal pH of this compartment and that of the vacuole were determined and shown to be effected by lysosomotropic and proton-dissipating agents as well as by ATPase inhibitors. The kinetics, energy dependency, and temperature dependency of two portions of the endocytic pathway of FITC–dextran, from the exterior of the
cell to the endosomes and from the endosomes to the vacuole, are described.

Materials and Methods

Cells and Virus Preparations

A wild-type strain S13 of *S. cerevisiae* (derivative of S288C) was grown in growth medium (YPD-medium) containing 1% yeast extract (Oxoid Ltd., Basingstoke, UK), 2% bacto peptone (Difco, Detroit, MI), and 2% glucose (BDH, UK) at 30°C in a shaker to densities of 20-50 x 10^6 cells/ml, and washed twice with PBS (pH 7.5) before use. For the experiments the growth medium was buffered to pH 7.2 with 20 mM Hepes (Sigma Chemical Co., St. Louis, MO).

Fluorometry

For morphological and fluorometric assays, cells were incubated with 50 mg/ml of FITC-dextran (70S, 0.02 fluorescent units per mole of glucose; Sigma Chemical Co.). Dialysis experiments showed that the preparation contained no detectable amounts of free fluorescein. The standard curve in the insert of Fig. 2 A was constructed essentially as described by Okhuma and Poole (1978). Briefly, the excitation spectra from 400 to 520 nm of 1 µg of FITC-dextran in 1.0 ml of citrate (0.05 M) phosphate (0.1 M) buffers with different pH values, indicated in the insert, were determined using a Hitachi F-3000 fluorescence spectrophotometer, as described by Okhuma and Poole (1978). Emission was measured at 37°C at 540 nm with crossed polarizers. The Raman spectrum of water was substracted before the calculation of the fluorescence intensity ratios at 495 and 430 nm, which were plotted against the respective pH values in the insert in Fig. 2 A. The slits for excitation and emission were 5 and 10 nm, respectively. For quantitation of internalized FITC-dextran, another standard curve was constructed, where the fluorescence intensity at 495 nm of 1 µg/ml of FITC-dextran in 1.0 ml of the different buffers was plotted against the respective pH. This curve was similar to that described by Ohkuma et al. (1982).

Microscopy

A Polvax microscope (Reichert-Jung, Vienna, Austria) equipped with a 100x immersion oil objective, fluorescence filters (exciter filter BP 475-495 and barrier filter BP 520-560) and Nomarski optics was used. The cells were immobilized for photography by using microscopic slides covered with a film of concanavalin A (0.5 mg/ml, Sigma Chemical Co.). Agfapan 400ASA film (Agfa-Gevaert N.V., FRG) was exposed in the case of Nomarski and fluorescence optics for 2 and 45 s, respectively.

ATP Determinations

The ATP concentrations of the yeast cells were determined using the luciferin–luciferase bioluminescence assay kit NRB/Lumit-HS Kit (Lumac Medical Products Division/3M, St. Paul, MN). Samples of 50 µl containing 10^9–10^10 cells were mixed with the nucleotide-releasing agent (NRB reagent). After 1 min, the assay was performed by injecting 100 µl of the luminescent reagent using the Lumac Celltester M 1030 (Lumac Systems Ag, Switzerland). The cell wall did not inhibit the release of the nucleotides by the NRB reagent, since similar values of ATP content were obtained for the NRB reagent, since similar values of ATP content were obtained for the NRB reagent.

Other Methods and Materials

Ammonium chloride, sodium azide, and sodium fluoride were from E. Merck (FRG); sodium vanadate was from BDH Chemicals Ltd., Poole, England; dicyclohexylcarbodiimide (DCCD); 50 mM stock prepared in 70% ethanol) was from Sigma Chemical Co.; and carbonyl cyanide p-trifluoromethoxyphenylhydrazone (CCCP; 10 mM stock prepared in 70% ethanol) was from Boehringer Mannheim GmbH, FRG. Efrapsentin was a kind gift from Dr. R. L. Hamill (Eli Lilly & Co., Indianapolis, IN).

Results

An Intermediate Compartment Mediates Endocytosis of FITC–Dextran to the Vacuole

When *S. cerevisiae* cells were incubated in growth medium with FITC–dextran, the marker was found in the vacuoles, which appeared brightly stained (Fig. 1 A). The vacuole, which may comprise as much as 25% of the volume of the yeast cell (Matle, 1978), can be easily visualized by Nomarski optics (Fig. 1 D). We have described earlier the internalization of FITC–dextran into the yeast vacuole using fluorescence microscopy (Makarow, 1985b).

It appeared that the transport of FITC–dextran to the vacuole could be interrupted by energy depletion. When *S. cerevisiae* cells were pretreated for 15 min with sodium fluoride in the absence of glucose, and then incubated under the same conditions with FITC–dextran, the marker was still internalized, but it did not stain the vacuoles; instead, it accumulated unevenly in the cytoplasm (Fig. 1, B and E). We therefore assumed that FITC–dextran was accumulated in a membrane-enclosed compartment, although the resolution at the light microscopic level did not reveal distinct organelles. This staining pattern was seen in ~80% of the cells. When the cells displaying the cytoplasmic staining (Fig. 1 B) were washed to remove the extracellular FITC–dextran and subsequently incubated in growth medium (supplied with glucose and lacking sodium fluoride), the marker disappeared from the cytoplasm and accumulated in the vacuoles (Fig. 1, C and F). The chase of FITC–dextran to the vacuole was efficient, since the cytoplasmic staining was seen only in 10% of the cells after 30 min (Fig. 1).

Determination of the Internal pH of the Intermediate Compartment and the Vacuole

The fluorescence intensity and the excitation spectrum of fluorescein are dependent on pH. This has been exploited to determine the internal pH of lysosomes and endosomes in mammalian cells after endocytosis of fluorescein-conjugated macromolecules into these organelles (Ohkuma and Poole, 1978; van Renswoude et al., 1982; Tycko et al., 1983). Since FITC–dextran could be accumulated in the vacuole or the intermediate compartment of *S. cerevisiae*, depending on the conditions, the internal pH of these compartments could be measured. *S. cerevisiae* cells were incubated with FITC–dextran under the energy depletion (Fig. 1 B) or under normal conditions (Fig. 1 A), and subjected to the determination of the excitation spectra of the internalized dye (Fig. 2). The fluorescence intensity ratio 495 nm/450 nm was 3.78 under the energy depletion conditions. The respective pH value for the intermediate compartment, as read from the standard curve in the insert of Fig. 2 A, was 5.8 (the average of eight determinations was 5.8; range 5.6–6.1). The fluorescence ratio for the vacuolar marker was 5.22, corresponding to a pH value of 6.5 (Fig. 2 B) (the average of eight determinations was 6.5, range 6.2–6.7). Similar results as presented in Figs. 1 and 2 were obtained when sodium azide (10 mM) was used instead of sodium fluoride. It has to be noted that the fluorescence intensity at 495 nm at pH 6.5 is ~2.5 times higher than at pH 5.8 (Ohkuma et al., 1982). This is why the cytoplasmic staining in Fig. 1 B appears weaker than after the chase to the vacuole in Fig. 1 C. The amounts of FITC–dextran that...
Figure 1. Transport of FITC-dextran via a cytoplasmic compartment to the vacuole. In A, cells (1.5 x 10⁶) were incubated for 30 min at 37°C with FITC-dextran (50 mg/ml) in growth medium (50 μl). The cells were then washed four times with cold PBS and viewed through fluorescence filters. The Nomarski image (D) of the same cells revealed that the fluorescent areas coincided with the vacuoles of the cells and that almost all cells displayed a stained vacuole. In B and C, the cells were incubated similarly with FITC-dextran, except that the incubation medium was PBS containing 50 mM NaF, and that the cells were preincubated in this medium for 15 min at 37°C before addition of the marker. The cells were viewed after the washes through fluorescence filters (B) and Nomarski optics (E). In B, 80% of the cells showed cytoplasmic staining (arrow 1), 7% showed vacuolar staining (arrow 2), and in 13%, both compartments were stained (arrow 3). In C, the cells were incubated further after the washes for 30 min at 37°C in growth medium lacking the marker, washed twice, and viewed through fluorescence filters (C) or Nomarski optics (F). In C, 10% of the cells displayed cytoplasmic fluorescence, 79% vacuolar fluorescence, and in 11% both compartments were stained. For the calculations of the subcellular distributions of FITC-dextran, 500 cells were counted in each case. Bar, 5 μm.

Figure 2. Determination of the internal pH of the cytoplasmic compartment and the vacuole. (A) S. cerevisiae cells (1.5 x 10⁶) were incubated for 10 min at 37°C in PBS containing 50 mM NaF (solid and dashed lines). The cells were pelleted and resuspended in 50 μl of prewarmed PBS–NaF containing FITC–dextran, and incubated for 30 min at 37°C. The cells were then washed four times with cold PBS and subsequently three times with cold PBS containing 0.5 M NaCl, and resuspended in 500 μl of PBS. (Dashed line) All solutions contained 0.1 M NiCl₂. (Dotted line) Cells treated similarly as described (solid line), but all incubations were performed at 0°C to control for surface-bound marker. Samples of 100 μl of the final cell suspensions were diluted with 900 μl of PBS to carry out the determination of the excitation spectra as described in Materials and Methods. In B, the preincubations and the incubation with FITC–dextran were performed in YPD-medium. The fluorescence intensities at 450 nm and 495 nm of the surface-control samples (dotted line) in A and B were subtracted from the corresponding values of the two other samples before the calculation of their fluorescence intensity ratios 495 nm/450 nm. The pH values corresponding to the intensity ratios were read from the curve in the insert (see Materials and Methods). The amount of FITC–dextran bound to the cell surface (dotted line) was ≤2% of that internalized into the cells (solid and dashed lines). The excitation spectrum of cells not treated with FITC–dextran was subtracted from all displayed spectra (relative fluorescence intensity at 450 nm and 495 nm was 0.09 U).

were accumulated into the yeast cells are shown in Table I. Under the energy depletion conditions ~1.3–1.7 times more of the marker could be found in the cells than after incubation in growth medium (see below).

To differentiate the cytoplasmic staining due to what we as-


**Table I. Quantitation of Internalization at 37°C**

<table>
<thead>
<tr>
<th>Conditions</th>
<th>Amount of FITC-dextran (ng/10^6 cells)</th>
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<tbody>
<tr>
<td>YPD-medium</td>
<td>2.8 (2.3-3.4)*</td>
</tr>
<tr>
<td>YPD-medium + NH₄Cl</td>
<td>3.4 (3.3-3.5)*</td>
</tr>
<tr>
<td>PBS + 50 mM NaF</td>
<td>4.5 (2.9-5.8)*</td>
</tr>
<tr>
<td>PBS + 50 mM NaF + 0.1 M NH₄Cl</td>
<td>6.0 (5.5-6.5)*</td>
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* S. cerevisiae cells were incubated at 37°C for 30 min with 50 mg/ml of FITC-dextran (1.5 × 10^6 cells/50 μl) in YPD-medium or in PBS containing 50 mM NaF. The amounts of internalized marker were determined by fluorometry as in Fig. 2.

† The ranges in parentheses represent the averages of eight determinations.

The ranges in parentheses represent the averages of two determinations.

Assume to be a membrane-enclosed compartment (Fig. 1B) from that due to leakiness of the plasma membrane to FITC-dextran, heat-permeabilized cells were exposed to the marker. A bright uniform staining covering even the vacuole could be detected (Makarow, 1986). The pH sensed by FITC-dextran was 6.7-7.0. After an incubation of 30 min at 37°C or at 0°C, 7.5 or 3 times more FITC-dextran, respectively, was detected in the cells, than presented in Table I for intact cells after a 30-min incubation at 37°C under energy depletion (not shown).

**Effect of Ammonium Chloride, CCCP, and ATPase Inhibitors on the Intraorganellar pH Values**

Weak bases like ammonium chloride accumulate in lysosomes and endosomes of mammalian cells as a result of protonation, and thus raise their internal pH (de Duve et al., 1974; Ohkuma and Poole, 1981; Maxfield, 1982). When FITC-dextran was allowed to accumulate into the vacuole or into the intermediate compartment of *S. cerevisiae* in the presence of 0.1 M ammonium chloride, similar staining, except somewhat brighter, as shown in Fig. 1, A and B, respectively, was observed. Fluorometric assays showed that under these conditions the pH of the intermediate compartment was 6.5 (Fig. 2A) and that of the vacuole was 7.2 (Fig. 2B). With 0.05 and 0.2 M ammonium chloride, similar results were obtained. Ammonium chloride appeared to effect somewhat the amount of FITC-dextran taken up by the cells. In its presence, 1.2-1.4 times more FITC-dextran was accumulated into both compartments, as compared to the controls (Table I). The effect of ammonium chloride on the internal pH of the intermediate compartment and the vacuole was fast, occurring within 1 min at 37°C (Fig. 3A and C). After removal of the drug, the original pH values of both compartments were recovered within 1-2 min (Fig. 3B and D). When FITC-dextran was internalized into the intermediate compartment in the presence of 0.1 M NH₄Cl and the energy block was then removed, the marker was chased into the vacuole similarly in the presence and absence of the drug, as visualized by fluorescence microscopy (not shown).

When FITC-dextran was allowed to accumulate into the intermediate compartment under energy depletion, and the protonophore CCCP was then added, the pH sensed by the marker was raised within 1-2 min at 37°C (Fig. 4A). The pH of the vacuoles was raised within 2 min after the addition of the drug (Fig. 4B). The original pH of the vacuole (pH 6.5) of control cells was maintained at 37°C (Figs. 3C and 4B). However, the original pH of the intermediate compartment (pH 5.8) of control cells was raised gradually to 6.05 in 10 min at 37°C (Figs. 3A and 4A). This may reflect the dissipation of the proton gradient across the membrane of the intermediate compartment, due to depletion of ATP (see below).

The plasma membrane and the vacuole membrane of *S. cerevisiae* contain proton-translocating ATPases which have been isolated and assayed in vitro for sensitivity to different ATPase inhibitors (Willsky, 1979; Uchida et al., 1985). We studied the effect of DCCD (100 μM), vanadate (10 μM), and efrapeptin (10 μg/ml) on the pH of the intermediate compartment and the vacuole in vivo (Fig. 4). DCCD raised the pH of both compartments, but efrapeptin, even in a concentration of 100 μg/ml, had no effect. Vanadate raised the endosomal pH, though more slowly than DCCD. However, even 100 μM vanadate had no effect on the vacuolar pH. Fluorescence microscopy showed that CCCP, DCCD, vanadate, or efrapeptin did not inhibit the internalization of FITC-dextran to the intermediate compartment under...
Figure 4. Effect of CCCP and ATPase inhibitors on the pH of the intermediate compartment and the vacuole. Cells were incubated with FITC-dextran under the energy depletion (A) or normal (B) conditions as in Fig. 2. The cells were subjected to determination of the pH of the intermediate compartment (A) or the vacuole (B) as before. Then, CCCP (open circles), DCCD (solid squares), sodium vanadate (open squares), or efrapeptin (open triangles) were added to the final concentrations of 100 nM, 50 nM, 10 nM, and 10 μg/ml, respectively, to the cell suspensions in the cuvette. The fluorescence ratios 495 nm/450 nm were monitored with 1-min intervals, and the respective pH values were plotted against incubation time at 37°C. (Solid circles) No drugs were added.

energy depletion. The transport to the vacuole took place also in their presence, provided that the cells were supplied with glucose. These results were confirmed by quantitative analysis by fluorometry (data not shown).

Characterization of the Dissected Endocytic Pathway

The intermediate compartment described above mediates the transport of internalized FITC-dextran to the vacuole. It has an acidic content, pH 5.8, which can be raised by NH₄Cl, CCCP, and ATPase inhibitors. These properties are similar to those of endosomes of mammalian cells (Maxfield, 1982; Tycko et al., 1983; Galloway et al., 1983). We take this as sufficient evidence that the intermediate compartment of S. cerevisiae identified under the energy depletion conditions represented endosomes. Thus, from here on the compartment stained by FITC-dextran under energy depletion will be referred to as endosomes.

Kinetics and Energy Requirements. The energy requirements of the endocytic pathway were studied in more detail. In the energy depletion experiments described above, the cells were preincubated for 15 min at 37°C with sodium fluoride before the addition of FITC-dextran. After the preincubation, the ATP level of the cells had dropped to ~3% of normal. In spite of this, FITC-dextran was internalized by linear kinetics for at least 30 min at 37°C (Fig. 5 A). When the marker was allowed to be internalized under conditions where the ATP level remained normal, uptake also followed linear kinetics, but the rate of accumulation was slower (Fig. 5 B; see also Table I). The inhibition of transfer of FITC-dextran from the exterior of the cell to the vacuole appeared to occur concomitantly with the depletion of ATP. During the first 5 min of incubation under energy depletion, when the ATP level was in the process of dropping (Fig. 5 A), the vacuoles were stained in about half of the cells. Later only the endosomes were stained in most of the cells like in Fig. 1 B.

Next we studied the kinetics of the chase of FITC-dextran from the endosomes to the vacuole. The dye was allowed to accumulate into the endosomes for 30 min at 37°C, as in Fig. 1 B. Extracellular FITC-dextran was then removed and the chase of the marker to the vacuole was followed at 37°C for 15 min. Fig. 6 A, panel b, shows that after 5 min most cells exhibited both vacuolar and endosomal staining. After 15 min, most of the marker had shifted to the vacuoles (Fig. 6 A, panel c). The finding that the pH values of the endosome and the vacuole were different was used to follow by fluorometry the kinetics of the chase to the vacuole. During the chase period, samples of cells were withdrawn at 2-min intervals for the determination of the environmental pH of the FITC-dextran. The pH started to rise from the initial value of 6.0 and reached the plateau (pH 6.55) after ~14 min (Fig. 6 B) indicating that now most of the marker had reached the vacuoles (as demonstrated visually in Fig. 6 A, panel c). Fig.
Figure 6. Kinetics and energy dependency of the transfer of FITC-dextran from the intermediate compartment to the vacuole. (A) Cells were incubated under the energy depletion conditions with FITC-dextran and washed free of extracellular marker as before and photographed through fluorescence filters immediately (a) or after a chase of 5 (b) or 15 (c) min at 37°C in growth medium. The fraction of cells displaying staining mainly in the cytoplasm, in the cytoplasm as well as in the vacuole, or mainly in the vacuole were 80, 13, and 7% in a; 20, 63, and 17% in b; and 15, 9, and 76% in c, respectively (500 cells counted). Similar data was obtained when the chase was performed in PBS supplemented with 2% glucose. Bar, 5 μm. In B, the chase was performed in PBS with 2% glucose. Samples were withdrawn after the indicated periods of time, resuspended in cold PBS, pelleted, and subjected to the determination of the pH of the intracellular FITC-dextran as in Fig. 2. The pH values are plotted against the chase time. In C, the concentration of ATP of the cells was determined during the chase at 37°C. It is expressed as nmol of ATP per mg of dry weight of cells.

Figure 7. Temperature dependency of internalization of FITC-dextran. Cells were incubated under the energy depletion conditions with FITC-dextran (1.5 x 10⁶ cells/50 μl) for 30 min at the indicated temperatures. The amounts of the internalized marker were determined by fluorometry, expressed per 10⁶ cells, and plotted against the incubation temperature.

6 C shows that the normal ATP concentration was recovered in 30 min after the energy block was removed. After 14 min, when the pH value of 6.5 for FITC-dextran had been reached, the ATP level was ~60% of normal.

In control experiments, FITC-dextran was allowed to be internalized directly into the vacuoles under normal conditions as in Fig. 1 A. External dye was removed and the incubation was continued in growth medium at 37°C. During 30 min, no change in the staining of the vacuoles was observed by fluorescence microscopy. At the same time, the intravacuolar pH remained in the range of 6.4–6.6 (not shown).

Temperature Dependency. The temperature dependency of internalization into the endosomes was studied by pretreatment of the cells with sodium fluoride as before followed by incubation under energy depletion with FITC-dextran at different temperatures. The amounts of the internalized marker were determined by fluorometry. It appears that the threshold temperature for uptake was ~15°C (Fig. 7). The amount of FITC-dextran internalized at 15°C was ~9% of that taken up at 37°C.

To study the temperature dependency of the traffic from the endosomes to the vacuole, cells were allowed to accumulate FITC-dextran into the endosomes at 37°C as before, washed, and then incubated for 30 min in growth medium at different temperatures. At 0°C, virtually no shift of the marker to the vacuole could be detected (Fig. 8 A, panel a). At 15°C, most of the marker still resided in the endosomes (Fig. 8 A, panel b). Between 19° and 35°C, FITC-dextran had been shifted to the vacuole as efficiently as at 37°C (Fig. 8 A, panel c). The pH of the FITC-dextran was determined under the above experimental conditions by fluorometry. At 0°C, the original pH value of 5.9 was obtained. A sharp increase of the pH was observed at 19°C (Fig. 8 B) indicating that the marker was in the vacuole (cf. Fig. 8 A, panel c). ATP determinations of the respective samples revealed that at 15° and 19°C, 32 and 40%, of the normal levels, respectively, had been reached (Fig. 8 C). Since transport of the marker from the endosomes to the vacuole occurred efficiently at 19°C, it appeared that it was the low temperature and not the low level of ATP which inhibited the transport at 15°C. To confirm this, longer incubations at 15°C were performed to increase the level of ATP. After 60 min at 15°C, the ATP concentration reached a plateau, which was 65% of the control level obtained in 30 min at 37°C (Fig. 6 C). However, FITC-dextran was still largely retained in the endosomes, like in Fig. 8 A, panel b.

Discussion

We have recently shown that Saccharomyces cerevisiae...
spheroplasts and cells with intact cell walls internalize macromolecules, applied to their growth medium, by a process similar to that of endocytosis of mammalian cells. Yeast spheroplasts internalized radiolabeled vesicular stomatitis virus and Semliki Forest virus particles in a time- and temperature-dependent process. The internalized viruses were found in association with cytoplasmic membranes, which were distinct from the vacuole, as revealed by organelle fractionation (Makarow, 1985a). Bacterial α-amyrase (M, 54,800) was internalized both by spheroplasts and intact cells of *S. cerevisiae* with kinetics that showed features of adsorptive endocytosis (Makarow, 1985b). Most of the internalized enzyme was found in the vacuole as shown by organelle fractionation (Makarow, 1986). The fluorescent marker FITC–dextran (M, 70,000) was readily transported to the vacuole of intact yeast cells in a time- and temperature-dependent way. Here we have extended the characterization of the endocytic process in *S. cerevisiae*. We chose FITC–dextran for these experiments for the following reasons. (a) It is evidently internalized solely by fluid phase uptake; (b) it can be visualized directly by fluorescence microscopy of intact yeast cells; (c) the fluorescence can be quantitated by fluorometry; and (d) the fluorescence spectrum is pH dependent, thus allowing the determination of the pH of the microenvironment of the marker (Ohkuma and Poole, 1978).

The basic observation that internalization was not inhibited by depletion of ATP to 1–3% of the normal level, whereas the transport to the vacuole was strictly dependent on ATP, made it possible to study an intermediate compartment in the process of endocytosis. In some cases, a partial energy independence of endocytosis has been documented for higher eukaryotes (Marsh and Helenius, 1980; Ukkonen et al., 1982; Clarke and Weigel, 1985). Coated vesicles and clathrin have been found in *S. cerevisiae* (Mueller and Branton, 1984; Payne and Schekman, 1985), suggesting that similar mechanisms may operate in endocytosis in yeast as shown for higher eukaryotes. This would also help to understand the fact that internalization takes place against turgor pressure. When the turgor pressure of the yeast cells is relieved by incubating them with FITC–dextran in the presence of 1.2 M sorbitol, similar uptake of the marker occurred as in the absence of sorbitol (Makarow, 1986; and our unpublished results). However, the recent report by Payne and Schekman (1985) that an *S. cerevisiae* mutant from which the clathrin gene has been deleted is viable and capable of exocytosis should be taken as a warning for too wide generalizations. It will be interesting to see whether the above mutant is capable of endocytosis.

The intermediate compartment into which FITC–dextran was internalized during energy depletion could be directly visualized by fluorescence microscopy. An uneven cytoplasmic peripheral staining was seen (Fig. 1B). Due to the small size of the yeast cell and the limits of the resolution of the light microscope, distinct organelles or even clear vesicles could not be seen with certainty. Staining of the cytoplasmic compartment was temperature dependent with a threshold of ~15°C. Thus, we assumed that FITC–dextran was contained in membrane-enclosed structures, a conclusion supported by a variety of other indirect evidence as discussed below.

The pH of this compartment could be measured by fluorometry. It was within the range of 5.6–6.1, somewhat higher than that reported for animal cell endosomes (Tycko and Maxfield, 1982; Tycko et al., 1983; Murphy et al., 1984). Since the pH of the intermediate compartment was measured after depletion of the ATP pool below 3% of the normal level (Fig. 5), we have to take this pH range as an estimate for the upper limit. If we assume that the pH of this compartment is maintained by an ATPase-driven proton pump as suggested for animal cell endosomes (Galloway et al., 1983), the low...
ATP level might limit the activity of the enzyme. To overcome this inherent possibility of error, we conducted the following experiment. FITC–dextran was internalized at 37°C in the absence of energy; glucose was then supplied and the ATP pool was allowed to recover at 15°C. At this temperature, the transport of the marker to the vacuole was largely inhibited (Fig. 8). The pH of FITC–dextran was now close to 6, which was within the range determined in the absence of ATP. Apparently, the pH was maintained for some time after the depletion of the ATP pool. Longer incubation at 37°C in the absence of ATP seemed to result in the rise of the pH of this intermediate compartment (Figs. 3 and 4). An immediate rise of the pH was observed with ammonium chloride (a lysosomotropic agent) and CCCP (a proton-dissipating drug), as expected for acidic compartments (Maxfield, 1982). The above results show that the intermediate compartment stained with internalized FITC–dextran displayed properties of endosomes of animal cells, and therefore we regard this compartment as endosomes of S. cerevisiae.

When the ATP pool was allowed to recover after removal of sodium fluoride and supplying glucose to the medium, FITC–dextran was transferred with a half-time of 7 min into the yeast vacuole. This transfer had a threshold temperature of 19°C, which is about the same as for the fusion of animal cell endosomes with the lysosomes (Dunn et al., 1980; Marsh et al., 1983; Pesonen et al., 1984). The transport of FITC–dextran from the endosomes to the vacuole must involve fusion reactions of endosomal and vacuolar membranes in order to enable the transport of the content. The energy dependence of this process was similar to those identified in vitro for the transport of the G protein of vesicular stomatitis virus within the Golgi complex (Wattenberg et al., 1986; Dunphy et al., 1986), for the fusion between endocytic vesicles (Davey et al., 1985), and for the transport of yeast invertase from the endoplasmic reticulum to the Golgi body (Haselbeck and Schekman, 1986).

An interesting observation was made when the total amount of internalized FITC–dextran was determined by fluorometry in the endosomes (under energy depletion) and in the vacuole (in the presence of a full ATP pool) (Fig. 5). About 30% less of the marker was transported directly to the vacuole than taken into the endosomes during the same time. One explanation would be recycling of part of the endosomal content back to the medium. In macrophages, about one-fourth of the internalized fluid is recycled back to the medium shortly after uptake (Besterman et al., 1981). It appears that receptor-mediated endocytosis of asialoglycoprotein in rat hepatocytes was unaffected by depletion of cellular ATP to <1% of control level. However, continuous internalization of the ligand was stopped, since the depletion of ATP inhibited the recycling of the receptor back to the plasma membrane (Clarke and Weigel, 1985).

The vacuole of yeast is a digestive organelle with storage functions. It contains a variety of hydrolytic enzymes, the pH optima of which vary from acidic to alkaline (Wiemken et al., 1979). The membrane of the vacuole contains a proton-translocating Mg2+-ATPase which generates a potential across the membrane of isolated vacuole vesicles and acidifies their interior (Kakinuma et al., 1981). Since the internal pH of the vacuole in vivo has not been known, the role of the H+-translocating enzyme for the intravacuolar pH has remained unclear. Using FITC–dextran as a probe, we have here determined the intravacuolar pH to be mildly acidic, pH 6.5 (Fig. 2). This is a considerably higher value than that (pH 4.7–4.8) found for the lysosomes of mammalian cells (Ohkuma and Poole, 1978). The pH of the vacuole, like that of the endosomes, could be raised rapidly by ammonium chloride and CCCP. Previously it has been reported that another lysosomotropic agent, chloroquine, is accumulated into the vacuole (Lenz and Holzer, 1984).

The ATPase of the vacuole membrane has been purified and shown to be inhibited by DCCD, but not with sodium vanadate or efrapeptin, which inhibit the plasma membrane Mg2+-ATPase of S. cerevisiae and the F1, F0-type mitochondrial ATPase, respectively (Uchida et al., 1985; Willsky, 1979). We tested the effect of these drugs on the pH of the vacuole and the endosomes. Only DCCD raised the intravacuolar pH (Fig. 4). This result strongly suggests that the Mg2+-ATPase is at least in part responsible for the maintenance of the mildly acidic pH of the vacuole of S. cerevisiae. The endosomes of mammalian cells are thought to contain a proton pump, which would have a role in the acidification of this compartment. DCCD (100 μM), but not efrapeptin or sodium vanadate, inhibited acidification of the mammalian endosomes in vitro (Galloway et al., 1983). Since we show here that DCCD, but not efrapeptin, raised the endosomal pH in vivo of S. cerevisiae (Fig. 4), the yeast endosomes may also contain a proton-translocating ATPase. Interestingly, this activity would differ from the vacuolar Mg2+-ATPase of yeast, since the endosomal pH could be affected also by sodium vanadate. However, these observations have to be verified by in vitro experiments using isolated endosomal vesicles.

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