

Apparent Endocytosis of Fluorescein Isothiocyanate-conjugated Dextran by *Saccharomyces cerevisiae* Reflects Uptake of Low Molecular Weight Impurities, Not Dextran

Robert A. Preston,* Robert F. Murphy,** and Elizabeth W. Jones*

*Department of Biological Sciences, and †Center for Fluorescence Research in Biomedical Sciences, Carnegie Mellon University, Pittsburgh, Pennsylvania 15213

Abstract. Concurrent with Riezman's report (Riezman, H. 1985, *Cell*. 40:1001-1009) that fluid-phase endocytosis of the small molecule Lucifer yellow occurs in the yeast *Saccharomyces cerevisiae*, Makarow (Makarow, M. 1985. *EMBO [Eur. Mol. Biol. Organ.] J.* 4:1861-1866) reported the endocytotic uptake of 70-kD FITC-dextran (FD) and its subsequent compartmentation into the yeast vacuole. Samples of FD synthesized and purified here failed to label yeast vacuoles under conditions that allowed labeling using commercial FD. Chromatography revealed that the commercial FD was heavily contaminated with at least three low molecular weight fluorescent compounds. Dialysis was ineffective for removing the contaminants.

After purification (Sephadex G25, ethanol extraction), commercial FD was incapable of labeling vacuoles. Extracts of cells labeled with partially purified FD contained FITC, not FD, based on Sephadex and thin layer chromatography. In either the presence or absence of unlabeled 70-kD dextran, authentic FITC (10 µg/ml) was an effective labeling agent for vacuoles. The rapid kinetics (0.28 pmol/min per 10⁶ cells at pH 5.5) and the pH dependence of FITC uptake suggest that the mechanism of FITC uptake involves diffusion rather than endocytosis. In view of these results, labeling experiments that use unpurified commercial FD should be interpreted with caution.

SEVERAL lines of evidence suggest that uptake of the small molecule, Lucifer yellow (Riezman, 1985), and macromolecules (Makarow, 1985; Makarow and Nevalainen, 1987) can occur in the yeast *Saccharomyces cerevisiae* by a mechanism that resembles fluid-phase endocytosis. In a potentially related development, recent reports strongly support a role for receptor-mediated endocytosis in the yeast cell's response to α -factor mating pheromone (Jennens and Spatrick, 1986; Chvatchko et al., 1986). Fluid-phase endocytosis should provide a port-of-entry for loading cells with a variety of otherwise impermeant metabolites or tracer molecules. Its occurrence in yeast encourages the application of the powerful genetics of this organism not only for the analysis of endocytotic mechanisms but also for the analysis of phenomena dependent on internalization of impermeant substances.

In the latter of these applications, we have attempted to replicate the reported labeling of yeast vacuoles by high molecular weight, FITC-conjugated dextran (FD)¹ to investigate problems in organelle biogenesis and function (Makarow, 1985; Makarow and Nevalainen, 1987). As will be reported here, we found that the fluorescent staining of yeast

vacuoles by "70-kD FD" results from the uptake of low molecular weight impurities in commercial samples of FD. FITC appears to be the most potent of these impurities, and its uptake most likely reflects simple diffusion and trapping rather than endocytosis.

Our results suggest that attempts to introduce macromolecular materials into whole yeast cells by an endocytotic route may be ill-advised. Also, in view of the potential reactivity of FITC (particularly if concentrated in subcellular organelles) our findings suggest that results obtained using FD of uncertain purity should be interpreted with appropriate caution. In view of these implications, we report here the details of our evidence against the uptake of FD by whole yeast cells.

Materials and Methods

Cells and Media

The reported experiments were done using a diploid strain of *S. cerevisiae* (derivative of S288C), BJ2936, of genotype a/α , *ura 3-52/+*, *leu2/+*, *+trp1*. Cells were grown at 30°C, shaking, in YEPD medium containing 1% yeast extract (Difco Laboratories, Inc., Detroit, MI), 2% Bacto-peptone (Difco Laboratories, Inc.), and 2% glucose (added aseptically after separate sterilization). Log-phase cells were harvested at culture densities of 1-5 × 10⁷ cells/ml and washed twice with PBS (pH 7.5) (Dulbecco and Vogt, 1954) at 24°C in a Sorvall bench-top centrifuge (model GLC II; Dupont

1. *Abbreviations used in this paper:* FD, FITC-conjugated dextran; F-NH₂, fluorescein amide; FTC-NH₂, fluorescein thiocarbonylamide; XRITC, substituted rhodamine isothiocyanate.

Co., Wilmington, DE; 1,000 g, 3 min). Cells were resuspended in PBS and kept on ice up to 30 min before use in uptake assays, for which the required amount of cells was pelleted in 1.5-ml microfuge tubes (13,000 g, 5 s) and resuspended in the assay medium.

Uptake Assays

Photomicrographic and quantitative FD uptake assays were performed as described by Makarow and Nevalainen (1987) except the usual FD concentration was 100 mg/ml (Makarow, 1985). The assay medium was YEPD containing 20 mM Hepes, pH 7.2, and FD or other substances as specified. Cells were suspended in 0.05–0.20 ml of assay medium at 3×10^8 cells/ml and incubated 30 min at 37°C. Under these assay conditions, we observed that the pH of the medium dropped rapidly during the incubation period, apparently due to fermentative carbon dioxide production. The pH fell below 6.0 within 10 min and stabilized at 5.6 by the end of the 30-min incubation. In specified assays of FITC uptake, pH was held constant by using lower cell concentrations and greater buffering (see Results), but otherwise we allowed the pH to fall as described.

The cells were then harvested and washed using the bottom half (see below) of a 13-mm Swinney filter holder (Millipore Continental Water Systems, Bedford, MA) mounted on a suction flask, as follows. For routine photomicrographic assays, the cells were diluted with 1 ml ice-cold PBS, filtered, washed on the filter with 5 ml cold PBS, resuspended in 5 ml cold PBS, refiltered, washed on the filter with 2 ml cold PBS, and finally resuspended in 0.2–0.8 ml cold PBS (10^8 cells/ml) for microscopy. For quantitative uptake assays, the PBS washing was done at 24°C, and a second resuspension and filtration from 5 ml PBS was included to minimize contamination of cell surfaces by adsorbed fluorescent materials. We emphasize that the filtration apparatus must be kept scrupulously clean by suitable rinsing during cell preparations to avoid contamination by traces of FD—for that reason, filters were held by suction alone, without using the gasket and top half of the Swinney holder (Millipore Continental Water Systems).

After photomicrography of aliquots of labeled cells, the cells were concentrated by centrifugation (13,000 g for 5 s) and resuspension in PBS at 3×10^8 – 3×10^9 cells/ml. Fluorescent substances were then extracted by adding 1/50 vol 10% (wt/vol) SDS, incubating at 24°C for 5 min, centrifuging 13,000 g for 10 min, and removing aliquots of the supernatant for fluorometric and chromatographic analysis. Phase-contrast and fluorescence microscopy revealed no evidence of intact vacuoles or intracellular fluorescence in SDS-extracted cells. A second treatment with 0.2% SDS extracted insignificant amounts of additional fluorescent material from labeled cells.

Fluorometry

Cell extracts and chromatographic fractions were analyzed using a Fluoro IV spectrofluorometer (Gilford Instrument Laboratories, Inc., Oberlin, OH) with 5-nm excitation slit and 10-nm emission slit. Typically, samples were suitably diluted in 20 mM sodium phosphate (pH 8.0) and emission was measured at 520 nm with excitation at 495 nm. Chromatographically purified carboxyfluorescein (Molecular Probes, Eugene, OR) or fluorescein (Sigma Chemical Co., St. Louis, MO) were used as reference standards (these had essentially the same quantum yield).

Thin Layer Chromatography

Extracts, fractions, and fluorescein derivatives were analyzed on Analtech, Inc. (Newark, DE) reverse-phase RPS Uniplates. Development was with solvent system A (20% [vol/vol] methanol, 80% [vol/vol] 10 mM Hepes–NaOH, pH 7.6) or B (35% [vol/vol] methanol, 65% [vol/vol] 10 mM Hepes–NaOH, pH 7.6) as indicated. Where specified, plates containing samples of FD were partially developed in solvent C (80% [vol/vol] methanol, 20% [vol/vol] distilled water), dried, the origin containing insoluble FD cut away, and the contaminants at the solvent front were then resolved by further development in solvents A or B. Developed plates were photographed using a 100-W epifluorescence microscope illuminator (Carl Zeiss, Inc., Thornwood, NY) with 450–490-nm excitation bandpass and with a long-pass 520-nm emission filter on the camera lens.

Gel Chromatography

FD and fluorescein derivatives were analyzed or purified using Sephadex G25 (Pharmacia Fine Chemicals, Inc., Piscataway, NJ). FD was fractionated using a 1.5 cm \times 24 cm column ($V_T = 40$ ml). 18 ml of commercial FD, 10 mg/ml in 20 mM sodium phosphate, pH 8.0, was loaded into the G25 column and allowed to stand for 30 min before resuming elution. Fractions

containing macromolecular material in the void volume were pooled, dialyzed against distilled water, and lyophilized, all under minimal illumination conditions. Fractions (at $4 \times V_T$) containing a major composite impurity peak were pooled, adjusted to pH 3, extracted into ethyl acetate, dried under vacuum, and dissolved in 50% ethanol. Recovery of 495 nm absorbing material in the ethanol extraction was 93%. When used in uptake assays, aliquots of the ethanolic solution of impurities were vacuum-dried before addition to the assay medium. Analytic separations were performed on the same 40-ml column or on a 0.65 cm \times 6 cm ($V_T = 2$ ml) mini-column. Elution buffer contained 20 mM sodium phosphate, pH 8.0.

Microscopy

Cells were examined with phase-contrast and epifluorescence optics using a Zeiss microscope with a 100 \times oil-immersion objective. Fluorescence filters were Zeiss BP450–490 (excitation), FT510 (beam splitter), and LP520 (emission barrier). Cells in PBS (10^8 cells/ml) were immobilized for photography on slides coated with 20 μ l of 1 mg/ml concanavalin A (Sigma Chemical Co.). Photographic exposures were as indicated, using Kodak Tri-X ASA 400 film with film speed pushed to ASA 1600 using Diafine developer (Acufine, Inc., Chicago, IL).

Other Materials and Methods

Concentrations of fluorescein derivatives were determined gravimetrically, by absorbance at 495 nm using $\epsilon_M = 70,000$, or by fluorescence emission at 520 nm as described above. FITC, 4.1-kD FD, and 70-kD FD were purchased from Sigma Chemical Co.; cat. Nos. FD-4, FD-70S). Substituted rhodamine isothiocyanate (XRITC) was from Research Organics, Inc. (Cleveland, OH). 70-kD FITC + XRITC double-labeled dextran (FRD) was synthesized here using the method of de Belder and Granath (1973). 70-kD unlabeled dextran was from Sigma Chemical Co.

Results

Preliminary Experiments

Attempts to label yeast vacuoles using Makarow's (1985) protocol and 70 kD FRD synthesized here were repeatedly unsuccessful. Since we had no difficulties using this FRD (or XRITC-conjugated dextran prepared similarly) as an endocytotic label for mammalian cells (Cain, C. C., and R. F. Murphy, 1986; Cain and Murphy, 1987, manuscript submitted for publication), we supposed that low molecular weight contaminants in commercial FD might account for its effectiveness in labeling yeast cells. This supposition was based on our previous (unpublished) observations of contamination in commercial FD and on the fact that extremely high concentrations of FD (100–200 mg/ml) were required for labeling yeast vacuoles (Makarow, 1985). Preliminary experiments revealed that, in fact, the partial hydrolysis products of FITC, the most likely contaminants in FD, provided fluorescent labeling of yeast vacuoles when tested at concentrations less than 10 μ g/ml (data not shown, but see below). Starting from those observations, we performed a series of experiments to determine the composition of commercial FD and to identify the material that accumulated in the vacuoles of yeast incubated in commercial FD.

Purification of FD

Using the reported protocol for labeling vacuoles with FD (Makarow and Nevalainen, 1987), we found that a commercial sample of FD (Sigma lot #105F–5029) was an effective labeling agent at 100 mg/ml concentration (Fig. 1 *a*). TLC revealed three major fluorescent contaminants in the commercial FD that were virtually absent from our own FRD (Fig. 2, lanes 4, 5, and *II*). We undertook a sequence of purification steps to obtain pure macromolecular FD for en-

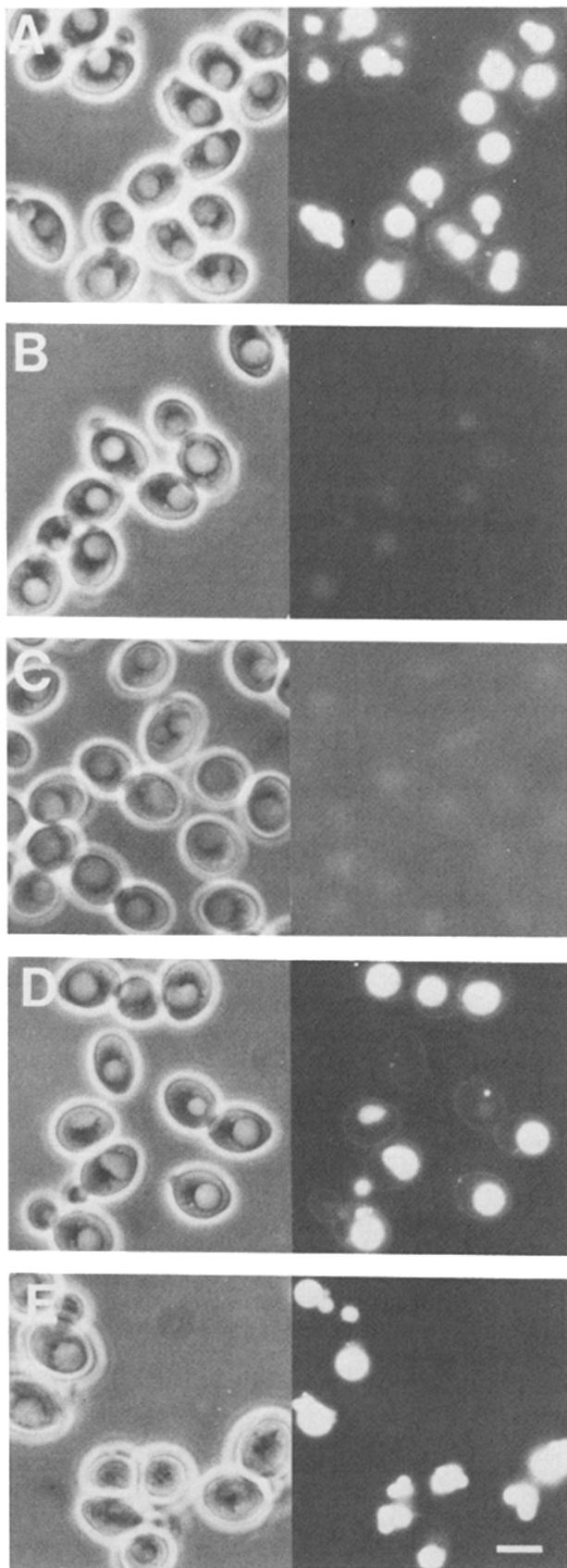


Figure 1. Phase-contrast (*left*) and fluorescence (*right*) photomicrographs of yeast labeled with FD and other substances. Cells were labeled for 30 min at 37°C (see Materials and Methods) with the

doctytic uptake assays. Neither dialysis against water nor dialysis against BSA was effective in removing the various contaminants in FD (Fig. 2, lane 6). Removal of most of the impurities was achieved by serial chromatography on Sephadex G25 (three cycles) followed by two ethanol precipitations (Fig. 2, lanes 8–10; note increased loads). The major impurities in the FD were mostly removed during the first analysis on G25 as two major peaks eluting much later than the macromolecular FD in the void volume. Summarizing the results of Sephadex and TLC analyses of the impurity fractions, we found that 100 mg of the commercial FD contained 4 nmol FITC, 400 nmol of an unidentified degradation product of FITC, named “P” (see below), and 100 nmol of fluorescein amine (F–NH₂) (data not shown).

Loss of Vacuolar Staining using Purified FD

Preparations of FD of two increasing degrees of purity (Fig. 2, lanes 9 and 10) were used in parallel with unpurified FD for labeling yeast cells by the method described (Materials and Methods). The intensity of vacuolar staining was much diminished using the partially purified FDG25(3) (Fig. 1 *b*) and was nearly entirely absent with the most purified preparation of FDEt (Fig. 1 *c*; note exposure times).

Uptake of Contaminants into Vacuoles

If the major low molecular weight contaminants in commercial FD were responsible for the labeling obtained with that material, those contaminants should have been effective labeling agents in the absence of the macromolecular component of FD. We incubated yeast in a standard uptake assay containing material representing the (composite) major contaminant fraction eluted from Sephadex G25 (see above). The concentration of the contaminant was adjusted (using absorbance at 495 nm) to approximate the concentration of these substances in the unpurified FD at 100 mg/ml. As shown (Fig. 1 *d*), the composite contaminant material was essentially as effective as unpurified FD in labeling the vacuole. Similar results were obtained when unlabeled 70-kD dextran (100 mg/ml) was included in the assay mixture along with the contaminant fraction (data not shown).

Identification of Internalized Fluorescent Label

Cells were labeled in FD using the quantitative uptake assay (Materials and Methods) with 100 mg/ml of partially purified FDG25(1) or FDG25(3). We used partially purified FD to avoid results that might reflect unusual contamination levels in a particular batch of commercial FD. After extensive washing to minimize surface-bound FD, the cells were extracted with SDS and the fluorescent extracts analyzed by Sephadex G25 and thin layer chromatography. The G25 analysis (Fig. 3) revealed that most of the extracted fluorescence

following: A, unpurified commercial FD; B, FD purified on Sephadex G25 (see Fig. 2, lane 9); C, FD purified by ethanol precipitation after Sephadex G25 (see Fig. 2, lane 10); D, the FITC-containing impurity fraction from G25 analysis of commercial FD (included at a concentration giving an absorbance of 30 at 495 nm); E, 10 µg/ml FITC. Exposure times for the fluorescence micrographs were as follows: A, 2 s; B, 2 s; C, 12 s; D, 1 s; and E, 1/4 s. Bar, 5 µm.

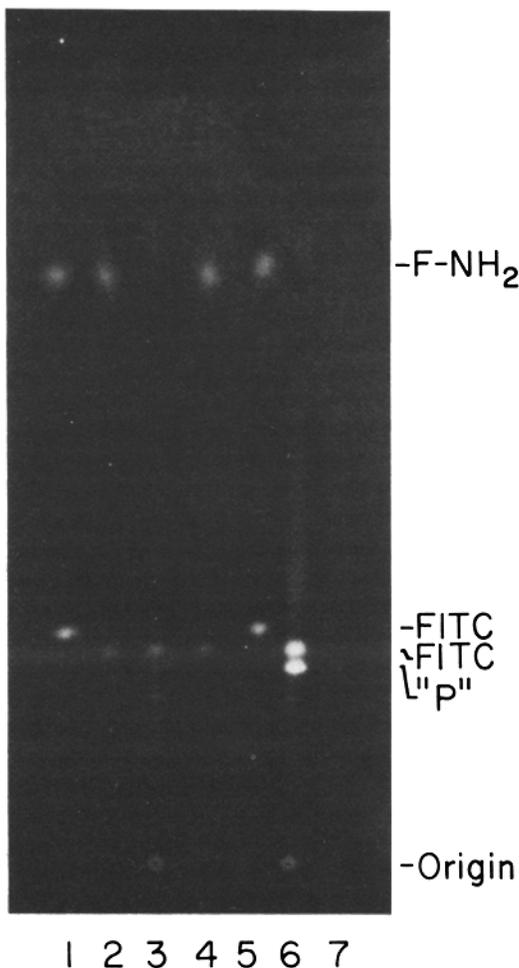


Figure 4. Thin layer chromatogram of extracts of yeast labeled in FD. SDS extracts of cells labeled in FD were developed partially in solvent C (see Materials and Methods), the section of the plate containing the origins was cut away, and the remainder of the plate further developed in solvent A. The sections of the plate were then photographed together with an exposure time of 30 s (see Materials and Methods). Samples in lane 1, 2 μ l of a reference mixture containing 0.2 pmol FITC and 2 pmol F-NH₂ dissolved in water; lane 2, the same reference mixture dissolved in an SDS extract of unlabeled cells; lane 3, 2 μ l of an extract of 5×10^6 cells labeled in 100 mg/ml FDG25(1); lane 4, same as lane 2; lane 5, same as lane 1; lane 6, 2 μ l of an extract of 5×10^6 cells labeled in unpurified, commercial FD; lane 7, 2 μ l of an extract of 5×10^6 unlabeled cells. Note that cell extracts at the concentrations used here contain interfering material(s) that slightly decrease the mobility of FITC. Re-analysis of spots eluted from the TLC plate supports identification of the extracted material as FITC (data not shown).

dex G25 void volumes for the uptake assays above (Fig. 3) could represent a substantial weight of material, if it were assumed that the fluorescence in the G25 void volume actually represented 70-kD dextran conjugated with 0.01 mol FITC per mol of glucose residues (such being the specifications of the FD used for labeling). We did not further analyze the material in the G25 void volume and, given the extremely low recovery of this material, we would not assume that it shared any property of the starting material beyond a molecular weight sufficient for exclusion from Sephadex G25. If the material in the G25 void volume were in fact 70-kD FD, it

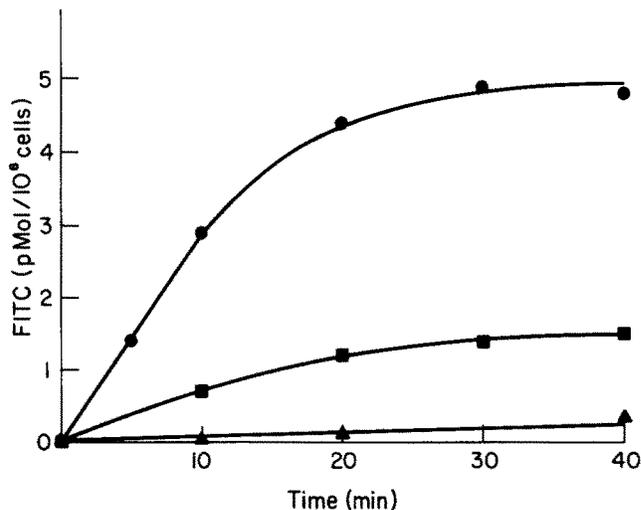


Figure 5. Kinetics of FITC uptake by yeast. 10 ml of PBS-washed cells (3×10^7 /ml) were incubated in YEPD + 50 mM sodium citrate + 10 μ g/ml FITC in a rotary shaker. At the indicated times, 1-ml samples were withdrawn, washed, and extracted with SDS as described for quantitative uptake assays (see Materials and Methods). Fluorescence of suitable dilutions of the extracts was assayed (495 nm excitation, 520 nm emission) using 200 nM fluorescein as a reference standard. Extracts of unlabeled control cultures had negligible fluorescence at equivalent dilutions. Cells were incubated at 37°C, pH 5.5 (●); 37°C, pH 6.0 (■); 0°C, pH 5.5 (▲). Measurements of the pH of the cultures taken after 40 min incubation showed less than 0.05 pH unit differences from the initial pH values.

can be estimated from our results (Figs. 2–4) that the material extracted from yeast cells labeled in partially purified FD showed a greater than 20,000-fold enrichment for FITC vs. FD, relative to the molar ratio of these compounds in the assay medium. That enrichment for FITC, in itself, would be very suggestive evidence for the specificity of the internalization of FITC rather than 70-kD FD in these uptake assays. The fact that extracts of cells incubated in partially purified FD at 0°C contained an amount of “FD” similar to that obtained at 37°C (Fig. 3) strongly suggests that this “FD” is superficial rather than internalized, since fluid-phase endocytosis does not occur in yeast at 0°C (Riezman, 1985). Even if all the void volume fluorescence were internalized 70-kD FD, the amount of that material observed in these experiments (Fig. 3) was 70 pg/10⁶ cells, for the incubation in FDG25(3), which is only 2% of the amount of “internalized FD” reported by Makarow and Nevalainen (1987) using the same uptake assay.

Uptake of FITC into Vacuoles

The major contaminants in commercial FD were identified (see above) as FITC and two degradation products of FITC, F-NH₂ and an unidentified product, substance “P.” Neither degradation product detectably accumulated in vacuoles when they were tested in the photomicrographic uptake assay at 10 μ g/ml concentration (data not shown).² However, 10

2. Product P was found to be the major breakdown product of FITC in aqueous solution at pH 6 after several days incubation at 24°C. Attempts to characterize it were abandoned when FITC was found to be a more potent label-

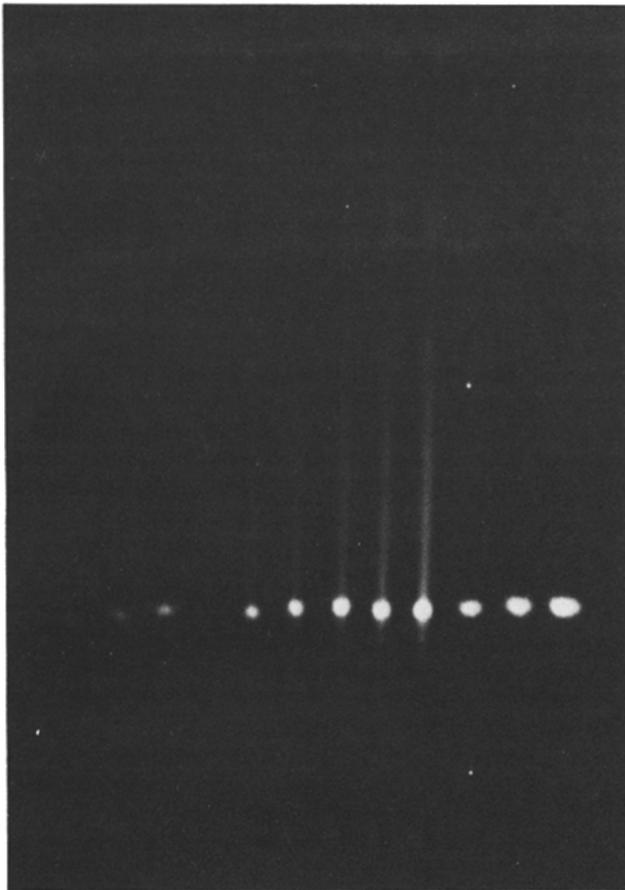


Figure 6. Thin layer chromatogram of extracts of yeast labeled with FITC. SDS extracts from the time course for FITC uptake at pH 5.5, 37°C (Fig. 5) were chromatographed as described (legend to Fig. 2), except the photographic exposure time was 30 s. 1 μ l of authentic FITC at various dilutions or 1 μ l of extracts from the indicated time points (equivalent to material from 0.3×10^6 cells) were chromatographed. Lanes 1–3, 50, 100, and 200 fmol FITC; lanes 4–9, cell extracts from 0, 5, 10, 20, 30, and 40 min time points; lanes 10–12, 0.5, 1, and 2 pmol FITC. The mobility of FITC was unaffected by extract components with the low extract concentrations used here (see Fig. 4).

μ g/ml FITC was a potent fluorescent vacuole stain under the same conditions (Fig. 1 e). Preliminary experiments suggested that FITC and impure FD both labeled vacuoles more efficiently under acidic conditions. This was confirmed, for FITC, by comparing the kinetics and extent of uptake of FITC at pH 5.5 and pH 6.0, using relatively low cell concentrations and well-buffered media (Fig. 5). The initial rate of uptake of FITC, under these conditions, was 2.8 pmol/min per 10^7 cells at pH 5.5, 4.2-fold higher than the initial rate at pH 6.0 (0.67 pmol/min per 10^7 cells). Uptake of FITC was much diminished at 0°C (Fig. 5), but it was still sufficient to provide vacuole-specific labeling that was easily detectable by fluorescence microscopy (data not shown). Both

ing agent for vacuoles. The presence of P in extracts of cells labeled in commercial FD (Fig. 4, lane 6) suggests that P at high concentration does label yeast vacuoles.

Sephadex G25 (Fig. 3) and thin layer chromatography (Fig. 6) of extracts of cells labeled with FITC showed that internalized FITC remained substantially unchanged after uptake and extraction.

Discussion

The results reported here provide three lines of evidence against the endocytotic uptake of macromolecular FD in yeast. First, both demonstrably pure FRD synthesized here and FD purified from commercial material failed to give significant labeling of yeast vacuoles (Fig. 1 c). Second, extracts of cells labeled in partially purified FD contained 84–90% of their fluorescence in low molecular weight material that co-chromatographed with FITC on G25 and TLC (Figs. 3 and 4). Third, the FITC and/or substance “P” contamination levels in commercial FD, or even in partially purified FD, were sufficient to account for the vacuole-specific labeling (Fig. 1, d and e) and the fluorescence of cell extracts (Figs. 3 and 6) observed with cells incubated in impure FD.

The simplest interpretation of these results is that essentially all vacuolar labeling by contaminated FD is due to FITC and substance P, but more complicated explanations are not ruled out by our data. We presume that the macromolecular component extracted from cells labeled with partially purified FD (Fig. 3) reflects FD (or fluorescent macromolecular impurities in FD) superficially adsorbed to cell walls rather than internalized material, but other interpretations could be made. For example, it seems possible that an intermediate molecular weight FD fraction in the Sephadex G25 void volume ($M_r > 5,000$) could be taken up by endocytosis and, perhaps, partially hydrolyzed to FITC within the yeast vacuole. Lack of labeling using ethanol-precipitated FD (Fig. 2) could be explained if the hypothetical intermediate-weight fraction had been removed by ethanol precipitation but not by Sephadex chromatography. Evidence against this hypothesis was found in the fact that commercial 4.1-kD FD (which was not contaminated with FITC) was also incapable of detectably labeling yeast vacuoles, whether or not it was ethanol precipitated (data not shown).

It is possible that the commercial FD used for these experiments has an atypically high level of impurities. However, of two additional lots of 70-kD FD obtained from the same source as that used for the experiments reported here, one was equally contaminated with FITC and the other, while considerably more pure, still contained sufficient FITC to provide intensely fluorescent vacuoles in yeast. Similar impure samples of FD were obtained from a second supplier. In any case, our labeling (Fig. 1 b) and cell extracts (Figs. 3 and 4) were obtained using FD of considerably higher purity than commercial FD, so those results are certainly not dependent on the use of atypically impure FD.³

3. At a typical level of contamination of roughly 40 pmol FITC per mg of FD, endocytotic labeling protocols that contain a typical 1 mg/ml FD concentration would also contain 0.04 μ M FITC. That amount of FITC would most likely be insignificant in an endocytotic labeling experiment, particularly if the labeling is done at neutral pH. On the other hand, FITC is a reactive chemical, it inhibits various ATPases at micromolar levels (Pick and Bassilian, 1981), and it appears to become concentrated by some mechanism, at least in yeast vacuoles. These considerations argue against routine reliance on commercial FD of uncertain purity, even at 1 mg/ml concentrations.

There are at least six mechanisms by which FITC might enter yeast cells: fluid-phase endocytosis, adsorption, or covalent bonding to surface macromolecules followed by endocytosis, active or facilitated transport, or simple diffusion across the plasmalemma. We will not discuss the relative merits or evidence concerning most of these possible mechanisms, since it is not our purpose to analyze details of the uptake of a highly reactive labeling agent of doubtful cytochemical use. We point out, however, that the observed rate of uptake of FITC (10 $\mu\text{g/ml}$, pH 5.5, 37°C; see Fig. 5) of 0.28 pmol/min per 10^6 cells would correspond to a rate of fluid-phase endocytosis of 140 $\mu\text{l/mg}$ cell protein/h. This rate would be 3,000-fold higher than that observed for the endocytotic uptake of Lucifer yellow in yeast (Riezman, 1985), and would be roughly equivalent to 140% of the total cell volume per hour. On the other hand, we observed an uptake rate at pH 6.0 that was 24% of the rate at pH 5.5 (Fig. 5). At pH 6.0, there is 30% as much of the neutral form of FITC as at pH 5.5.⁴ The similarity of these percentages suggests that the relatively lipophilic, neutral form of FITC is rate-limiting for uptake, and this would be consistent with entry by simple diffusion across the plasma membrane. (The inhibition of uptake of FITC at low temperature [Fig. 5] need not imply an endocytotic uptake mechanism, since changes in membrane permeability or energy-dependent processes could affect diffusional or other mechanisms as well.) While the mechanism of entry of FITC into yeast vacuoles remains unknown, it seems clear that its rate of uptake exceeds reasonable expectations for a fluid phase endocytotic route.

We conclude that it is highly unlikely that fluid-phase endocytosis of macromolecular FD occurs in yeast. Certainly there appears to be no good evidence for such an occurrence. On the contrary, results obtained using commercial FD can be accounted for by non-endocytotic uptake of low molecular weight impurities in the FD, including both FITC and an uncharacterized degradation product of FITC. In view of these findings, the use of impure FD in yeast cytology poses two specific problems. First, unlike the case for FD, which is a well-known marker for endosomal and lysosomal compartments in animal cells, no similar information is available concerning the likely distribution of FITC (and product "P") in cells or organelles. Although FITC is clearly capable of labeling the yeast vacuole, the mechanism of such labeling is unclear, and there is no a priori reason to believe that label-

4. The 30% figure is based on calculations that use the pK_a 's of fluorescein as an approximation for those of FITC (Martin and Lindqvist, 1975). For the 10 $\mu\text{g/ml}$ FITC (26 μM) used here, the calculated concentrations of neutral FITC at pH 5.5 and 6.0 are 1.8 μM and 0.5 μM , respectively.

ing patterns observed with FITC bear any relation to endosomal transport. Second, although FD fluorescence has been a valuable tool for measuring intracellular pH in animal cells (Ohkuma and Poole, 1978), this technique is applicable only with the provisos that the intracellular labeling agent is the same as the material used to generate in vitro pH-fluorescence standard curves, and that the fluorescent probe is known to be relatively immune to spectral perturbations in vivo. Neither of these conditions would be met in using impure FD as a labeling agent. For these reasons, previously reported yeast endosomal and vacuolar pH measurements (Makarow and Nevalainen, 1987) obtained with FD of unknown purity as a probe are of uncertain significance.

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