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Ultrarapid endocytotic uptake of large molecules in *Dunaliella* species

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Summary. This paper describes the uptake of Lucifer Yellow carbohydrazide and fluorescent dextrans labeled with fluorescein isothiocyanate or Sodium Green (molecular masses ranging from 522 to 2×10^6 Da) by *Dunaliella* spp. halotolerant unicellular green algae isolated from salt pools in the Sinai peninsula. The fluorescent dyes were taken up into a set of vesicles around the nucleus and just above the chloroplast. It proved impossible to inhibit uptake of the fluorescent compounds in cells treated with a large variety of metabolic and other inhibitors. Cell labeling was complete within half a minute of addition of fluorescent compounds to the outside medium; efflux was equally rapid. The results are interpreted in terms of an endocytotic process whereby the outside medium, together with any substance dissolved in it, remains within vesicles enclosed within the cell body but cycles rapidly between the plasma membrane and the interior of the cell. The outside medium does not pass across the vesicular membrane, nor enters the cytosol.

Keywords: Algae; *Dunaliella* sp.; Endocytosis; Halophilism.

Abbreviations: LYCH Lucifer Yellow carbohydrazide; FITC fluorescein-5-isothiocyanate; TCA trichloroacetic acid; DMSO dimethylsulfoxide; NEM N-ethyl maleimide; DNP dinitrophenol; CCCP *m*-chlorocarbonyl-cyanide phenylhydrazone; APM amiprophos-methyl.

Introduction

Dunaliella is a genus of green microalgae distributed throughout the world in seas, lakes, and ponds varying from brackish to highly saline. They are distinguished by their ability to grow at high, changeable salt concentrations. Unlike most other plant cells, Dunaliella cells do not have a rigid wall composed of cellulose or of any other inert material. Instead, the cell membrane is exposed directly to the bathing medium and is coated by glycoprotein patches which do not interfere with its mechanical properties (Oliveira et al. 1980). Thus the cells are able to change in volume when there are alterations in outside salt concentration (Trezzi et al. 1965).

Two models have been put forward to explain the mechanism of volume regulation and exceptional halotolerance of Dunaliella cells. The most popular model (Ben-Amotz and Avron 1973) treats the cell as a single compartment enclosed by a plasma membrane highly impermeable to salts and small organic solutes such as glycerol and glucose. According to this model, the "osmotic pressure" of the salt in the outside medium is balanced within the cell by glycerol (Craigie and McLachlan 1964, Ben-Amotz and Avron 1973, Borowitzka and Brown 1974) and the glycerol is distributed uniformly throughout the intracellular compartment where its activity equals the activity of the outside salt solution. Glycerol acts as a "compatible solute"; this means that it does not inhibit the cells' enzymes (Borowitzka and Brown 1974). Thus, according to this model, halotolerance is equivalent to osmoregulation because salt is effectively excluded from the cell and therefore cannot influence it. Results obtained by Ben-Amotz (1974), Bental et al. (1988), Pick et al. (1986), Karni and Avron (1988), and Fisher et al. (1997) have been interpreted in the light of this model.

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However, this model ignores properties unique to *Dunaliella* spp., especially the cells' ability to take up large organic molecules and Na^+, apparently through the plasma membrane. Accordingly, a second model was proposed (M. Ginzburg 1969, Ben-Amotz and Ginzburg 1969, B. Ginzburg 1978, M. Ginzburg and Richman 1985). This model treats the Dunaliella cell as consisting of two major thermodynamic compartments, the outer is bounded by a membrane apparently readily permeable to Na^+ and Cl^-, glycerol, glucose and even inulin (molecular mass 5,500 Da), while the inner compartment, which comprises 50–70% of the cell volume and within which volume regulation is osmotic, is surrounded by typical membranes of low permeability. According to this model, the activity of the Na^+ of the outer compartment is roughly equal to that of the outside medium. The osmotic pressure of the inner compartment is maintained largely by glycerol, which rises and falls with changes in the outside salt concentration.

Strong experimental support for the model was obtained by Ehrenfeld and Cousin (1982, 1984), who, in the course of a study of ion regulation in *D. tertiolecta,* centrifuged cell suspensions in the presence of a marker assumed to be impermeative to the cell membrane, to correct for medium trapped inside cell pellets during centrifugation. The markers used were ^{14}C-sorbitol, and ^{14}C-dextrans with molecular masses of 20 and 70 kDa. The volumes occupied by ^{14}C-sorbitol and ^{14}C-dextran (molecular mass 20 kDa) within cell pellets of equal size were larger than that occupied by ^{14}C-dextran-70,000, leading them to think that the two smaller molecules had actually entered the cells. They were encouraged in this belief by finding that the volume of individual cells, calculated by dividing pellet volume by cell number, after correction for intercellular space by ^{14}C-dextran-70,000, agreed with dimensions found by direct microscopic measurement, whereas calculations of cell volume using corrections made with the two smaller compounds yielded values smaller than those obtained by direct observation. It should be noted that the time given for mixing the sorbitol or dextran with the cell suspension, although not stated, is likely to have been very short.

While experimental results supported the two-compartment model, the main difficulty posed by the model still remained. This difficulty was expressed by M. Ginzburg (1969: p. 376): "The postulation of large pores [in the outer membrane] poses a difficult question in that it is hard to see how small essential molecules such as amino-acids and nucleotides are retained within the cell . . . The answer needs further research."

The present paper demonstrates that endocytotic processes in Dunaliella cells bring about a rapid and extensive uptake of the extracellular milieu. Once in the cells, components of the medium are located in vesicles restricted to a zone surrounding the nucleus. Endocytosis includes various engulfing processes (de Duve 1963) in which a region of the cell membrane becomes invaginated and finally forms a vesicle within, though not forming a part of, the cytosol. It has already been demonstrated in several plants (Tanchak et al. 1984, 1988; O'Driscoll et al. 1993; Roszak and Rambour 1997; D. Miller et al. 1997) and in microalgae with walled cells (Clayton and Ashburner 1994, Domozych and Nimmons 1992, Beech and Wetherbee 1988, O'Neil and La Claire 1988, McFadden et al. 1986). Dunaliella cells are highly suited to perform endocytosis since they have no continuous cell wall and no turgor pressure to inhibit endocytosis. There is, therefore, no barrier preventing large molecules from coming into contact with the plasma membrane. In addition, the presence of clathrin-coated vesicles, putatively involved in endocytosis, has been demonstrated both biochemically and cytologically in *D. salina* (Zhang et al. 1993). We suggest that the present work, demonstrating endocytosis in Dunaliella cells, serves to harmonize the two cell models described above. The one-compartment model posited correctly that the plasma membrane is semipermeable and that Na^+ and Cl^- are largely excluded from the cytosol. However, the fact that the plasma membrane is capable of rapidly forming endocytotic vesicles explains why it could formerly be called "highly permeable". These vesicles account for the detection of large amounts of Na^+ and Cl^- inside the cell, and the movement of large molecules into the cell interior. We emphasize that these substances, though separated from the cytosol by vesicular membranes, are physically contained within the cell.

A short account of this work has appeared previously (M. Ginzburg et al. 1997).

Material and methods

Organisms used

Most of the work was done on *Dunaliella* sp. var. D13, closely akin to *D. salina,* which was isolated by us some years ago from salt pools in the Sinai peninsula. The cells of this variety are of medium size ($\sim 760 \times 10^{-18}$ m^3) and were green under the conditions of culture used.

Dunaliella salina 1644 (UTEX), C9AA, and E1 were also used. C9AA was isolated from salt pools near the Dead Sea and E1 from saltern pools near Eilat, Israel; both species were isolated by us from single cells and are kept as sterile cultures in our Dunaliella collection. In all examples tested, cells of these isolates behaved in a manner identical to cells of D13.

Methods of culture

Algal suspensions were grown in a growth chamber (Cryo-Fridge; Revco, Asheville, N.C., U.S.A.), maintained at 28 °C during light periods and 20 °C during the dark. Photoperiods consisted of 14 h light and 10 h dark. Light intensity was 25 $\mu mol/m^2 \cdot s$ as measured with a quantum sensor (model LI-189; LICOR Quantum Radiometer/Photometer, Lincoln, Nebr., U.S.A.). These conditions were arranged to keep cell division synchronized during the beginning of the dark period. The culture medium consisted of modified Artificial Seawater Medium (AS100) with an initial pH of 8 (Wayne et al. 1991) and a final NaCl concentration of 1.7 M NaCl.

Microscopic examination of algae

Cells were observed with an Olympus BH-2 microscope equipped with a ×100 (SPLANAPO 100, N.A. 1.4) objective lens and either a ×3.3 or ×5 projection lens connected to a video camera (CCD-72 or CCD-100; Dage MTI, Michigan City, Ind., U.S.A.) and a monitor (PVM-13420; Sony, Ichinomiya, Japan). This microscope was equipped with epifluorescence optics, including a BP490 excitation filter, a DM500 chromatic beam splitter, and a 0515 barrier filter. An additional barrier filter (D605/55; Chroma Technology, Brattleboro, Vt., U.S.A.) was used to suppress chlorophyll autofluorescence. Images were digitized and analyzed with an image processor (Image 1/AT; Universal Imaging, West Chester, Pa., U.S.A.).

Color photographs of cell suspensions containing Lucifer Yellow carbohydrazide (LYCH) were taken after observation through Zeiss Axioplan 2/Axiophot 2 microscopes using the fluorescein isothiocyanate (FITC) filter set. The video camera was an Optronix DEI-470 Peltier-cooled color CCD camera. Prints were printed with a Sony Mavigraph color video printer.

Stock solutions of LYCH (0.2 mg/ml), FITC-dextrans (4 mg/ml), and Sodium Green dextran (4 mg/ml) were prepared in growth medium. Freshly prepared N-propyl-gallate (0.05%, w/v) was used as an antioxidant. Equal volumes of dye, cell suspension, and N-propyl-gallate were mixed prior to examination of cells under the microscope, the time of mixing depending on the experiment. Final dye concentrations were 67 µg/ml for LYCH and 1.3 mg/ml for dextrans. In order to ensure that the addition of dye did not significantly change the osmotic pressure of the solutions surrounding the cells, we measured the osmotic pressure of the various solutions of LYCH and the FITC-dextrans with a freezing-point depression osmometer (Model One-Ten; Fiske Associates, Needham Heights, Mass., U.S.A.). Since the range of our osmometer only goes up to 2000 mOsm/kg, we diluted all the solutions by a factor of two. We then multiplied this value by two to estimate the osmotic pressure of the working solution. Measurements were made in triplicate. The osmotic pressure of the medium with 0.05% (w/v) N-propyl-gallate given as the mean with the standard error was 3792 ± 5 mOsm/kg H_2O. The osmotic pressure of this medium plus LYCH (67 mg/ml), FD4 (1.3 mg/ml), FD10 (1.3 mg/ml), FD20 (1.3 mg/ml), FD40 (1.3 mg/ml), FD70 (1.3 mg/ml), FD150 (1.3 mg/ml), FD250 (1.3 mg/ml), FD500 (1.3 mg/ml), and FD2000 (1.3 mg/ml) was 3794 ± 6, 3796 ± 13, 3799 ± 10, 3798 ± 10, 3807 ± 4, 3837 ± 22, 3803 ± 2, 3802 ± 5, 3794 ± 10 mOsm/kg H_2O respectively. Since the osmotic pressure of these solutions varied by only 1% compared with the control, and none of them was statistically significantly different from the control (by a t-test), we are confident that we are not observing osmocytosis, which is induced by hypertonic media in plant cells (Oparka et al. 1990; Diekmann et al. 1993), but another form of endocytosis (de Duve 1963).

ATP measurements

ATP was measured by the luciferin-luciferase method. Light emission was measured with an FP-750 spectrofluorometer used in the luminescence mode (Jasco, Inc., Easton, Md., U.S.A).

A known number of cells was pelleted by centrifugation in a Coleman micro-centrifuge (6-811; Coleman Instruments, Maywood, Ill., U.S.A.) in plastic tubes and every trace of moisture removed from the tube by careful wiping. The cells were resuspended in 50 µl 5% TCA (w/v) and enough Tris buffer was added to neutralize the TCA. ATP was measured on the supernatant which was separated from the residue by centrifugation in the cold.

A nominal 1 mM solution of ATP was prepared and the true concentration of ATP was calculated from the extinction coefficient to be 0.177 mM after measurements with a Jasco V-530 spectrophotometer. The extinction coefficient of ATP at 259 nm is 15.4 mM (Bock et al. 1956).

Quantitative measurements of dye uptake

A method was devised for the reproducible extraction of fluorescent compounds from cell suspensions to which the compound had been added. The brightness of the extracted fluorescent solutions could be measured by means of the image processor described above and the amount of compound associated with the suspension could therefore be determined. Equal volumes of cell suspension and of fluorescent compound dissolved in 1.7 M NaCl were mixed for various times depending on the experiment. Aliquots of the cell suspension were transferred to 300 µl polyethylene tubes and were centrifuged in a Coleman microcentrifuge, a process which takes only a few seconds. The supernatant (solution A) was removed and stored. The walls of the centrifuge tube were carefully wiped with absorbent tissue and 60 µl fresh medium added to the cell pellet. The cells were dispersed in this medium and allowed to equilibrate after which they were centrifuged again. After centrifugation, the supernatant contained the fluorescent compound derived from the extracellular space between the cells as well as any compound taken up by the cells and liberated into the medium. The brightness of this solution (solution B) could be measured and compared with that of solution A. Care was taken to dilute solution A so that the brightness of the diluted solution was close to that of solution B. At any rate, over the range used, the brightness of a given solution was proportional to the concentration of the fluorescent compound dissolved in it. The brightness of a given solution was determined by pipetting 20 µl of solution onto a microscope slide with two coverslips stuck down on either side of the mid point serving as supports for a third coverslip placed on top of the drop. This occupied a circular area in the middle of the slide. It did not evaporate for periods of up to 6 h and its brightness could therefore be measured at leisure. Measurements remained constant throughout the 6 h period, although, in practice, they were made as soon as the slide was prepared.

Column chromatography

Columns of 1.5 ml Dowex 50WX8, a cation-exchange resin, equilibrated with medium were prepared in Pasteur pipettes according to the method of Pick et al. (1986). Suspensions of Dunaliella cells with LYCH were passed through the column and eluted with medium free of LYCH at a rate of 6.4 ml/min. 175 µl samples were collected and the cells examined for the presence of fluorescent vesicles.

Chemicals used

LYCH (molecular mass 522 Da) and FITC-dextrans were obtained from Sigma. Sodium Green dextran was obtained from Molecular Probes, Inc. Prior to use, FD4 and FD2000 were tested for free FITC by passing solutions down a column of Sephadex G25. The colored solutions passed down the column in a single band: no free FITC was detected (data not shown). Before and after a 10 min exposure to Dunaliella cells, the homogeneity of the entire series of FITC-dextrans and of Sodium Green dextran was confirmed by subjecting 4 µg samples of the dextrans to thin-layer chromatography on silica plates (Whatman TLC plates with fluorescent indicator; Whatman International Ltd., Maidstone, England) with various concentrations of ethanol from 30 to 95% (data not shown). In view of the possibility of hydrolysis (M. Ginzburg and Richman 1985), FD2000 (molecular mass 2×10^6 Da) was incubated in the presence of cells for 30 min and tested for breakdown by chromatography on a silica plate. No hydrolysis was found (data not shown).

Results

Qualitative observations with LYCH

Immediately after cells of *Dunaliella* sp. were exposed to a solution of LYCH, the LYCH appeared in a collection of bright yellow vesicles in the anterior region of the cell (Fig. 1). In order to observe better the precise location of the vesicles in these rapidly swimming and spiralling cells, we arrested their movement by reducing the total volume of liquid on the microscope slide to 7 µl. The cells then looked slightly flattened which accounts for their rounded appearance in Fig. 1 B. In side-view the fluorescent vesicles are seen to form a hollow truncated cone in the anterior part of the cell between the plasma membrane and the nucleus. The average diameter of the vesicles is 40 µm. The bright yellow color is due to LYCH since Dunaliella cells untreated with LYCH showed no yellow autofluorescence in the anterior part of the cell. Probenecid (Cunningham et al. 1981) does not affect uptake of LYCH in *Dunaliella* sp., indicating that in this cell LYCH enters by endocytosis and not by means of an anion transporter (Oparka et al. 1991).

In order to see whether changes in the salt concentration affected endocytosis, LYCH-stained cells grown in 1.7 M NaCl were transferred to either 1.2 M NaCl plus LYCH or 5 M NaCl plus LYCH (Table 1). On hypoosmotic treatment, the cells increased in size by 10%, as determined by measurements of median-sectional area, and simultaneously the area taken up by the endocytotic-exocytotic vesicles decreased by

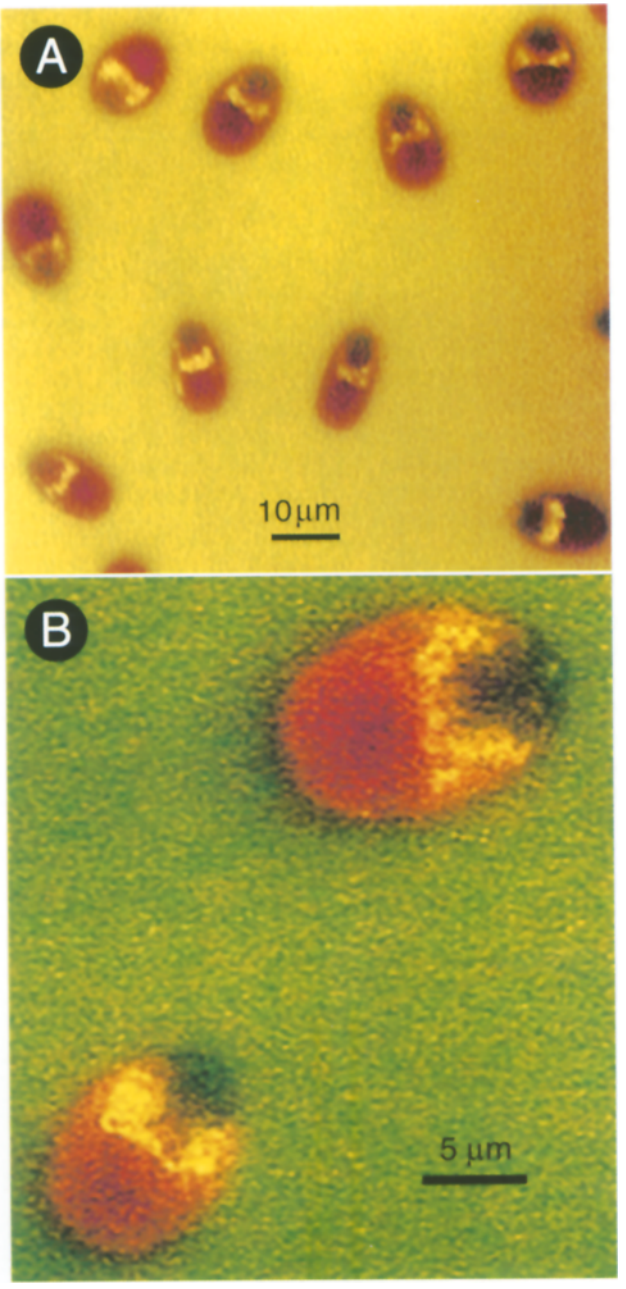

Fig. 1 A, B. Dunaliella cells with 100 µg of LYCH per ml and without N-propyl-gallate. The yellow-green color of the background is due to LYCH in process of quenching (unquenched LYCH is bright yellow). The bright yellow coloration within each cell is due to LYCH within the cell. Note that the bright yellow equatorial band in **A** appears as a section through a hollow truncated cone in **B.** The red color in the region of the chloroplast is due to autofluorescence of chlorophyll. Bar: A, 10 µm; B, 5 µm

Table 1. Comparison of median-sectional areas of Dunaliella cells and endocytotic and exocytotic vesicles within the cells in growth culture and hypo- or hyperosmotic medium

Concentration of NaCl (M)	Cell area (arb. units)[a]	Total vesicular area	
		arb. units[a]	% of cell area
1.7	41220 ± 4403	9992 ± 978	24.2 ± 1.6
1.2	45292 ± 2981	8754 ± 758	19.3 ± 1.4
5	26133 ± 1210	8030 ± 639	30.7 ± 1.8

[a] Areas expressed in arbitrary units (see Material and methods)

12%. While these differences were not statistically significant ($P = 0.48$ and 0.32, respectively), the proportion of the cell taken up by the vesicles decreased from 24.2 ± 1.6% ($n = 20$) to 19.3 ± 1.4% ($n = 20$) in a statistically significant manner. When the cells were challenged with hyperosmotic medium, the cells decreased in size by 58% ($P = 0.0034$) as determined by measurements of median-sectional area, and simultaneously the area taken up by the vesicles also decreased by 20% ($P = 0.10$). Following hyperosmotic treatment, the proportion of the cells taken up by the vesicles increased to 30.7 ± 1.8% ($P = 0.043$). We would like to stress that the measurements of median-sectional areas are estimates only of total cell and total vesicle volumes because Dunaliella cells change in shape when challenged with hypoosmotic and hyperosmotic stresses, and the true vesicle volume depends on vesicle shape and degree of packing. However, with this caveat in mind, the data indicate that a portion of the endocytotic-exocytotic vesicles may function as a source and sink of plasma membrane during hypoosmotic and hyperosmotic stresses, respectively, and in fact may be identical to the vesicles seen by Maeda and Thompson (1986). Confirmation of this tentative conclusion will depend on the development of specific markers that will allow identifying the endocytotic-exocytotic membranes and following their movements to and from the plasma membrane.

In order to quantify the rate of endocytosis, we rapidly mixed the dye and cell suspension on a microscope slide. The time needed for uptake is less than 26 s. To test dye efflux, cells treated with LYCH were centrifuged down and resuspended in medium without the dye. The cells themselves lost the dye even after the shortest time we were able to reach, a matter of seconds.

When cell suspensions containing LYCH (Fig. 2 A) were passed down columns of Dowex resin, it took

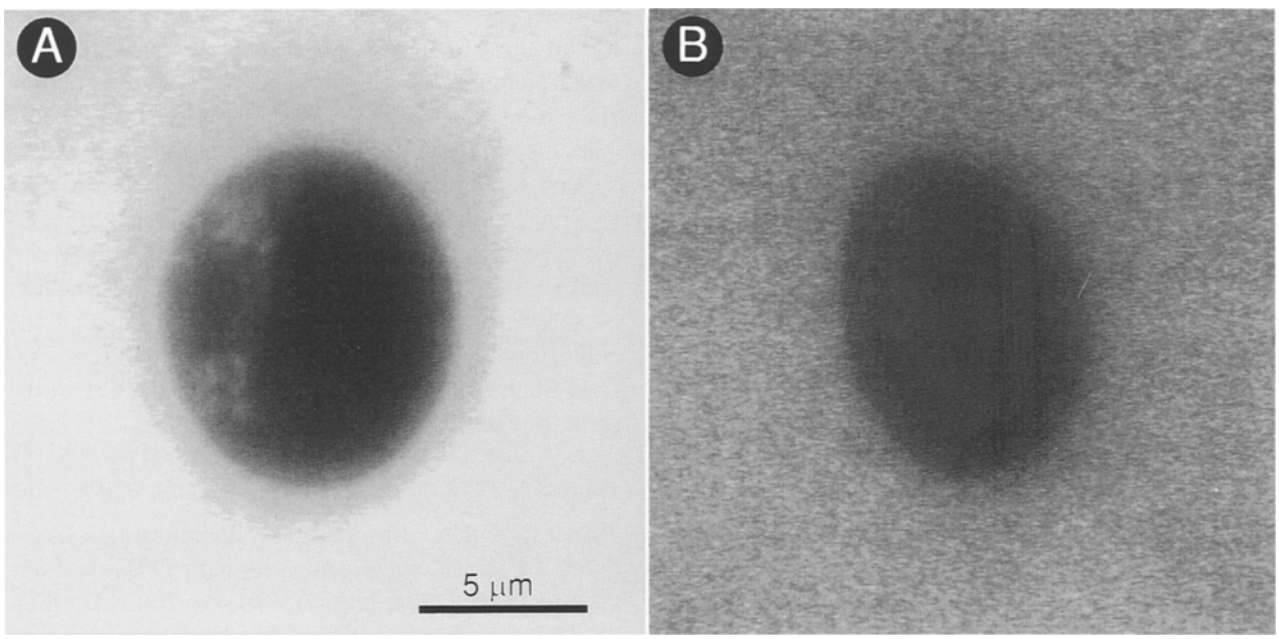

Fig. 2. Dunaliella cells with 66 µg of LYCH per ml and N-propyl-gallate before (**A**) and after (**B**) passage through a column of Dowex 50WX8 resin. Time of elution: 18 s. **A** and **B** were not digitally image processed in an identical manner. When **B** was processed in an identical manner to **A**, the cell appeared black on a black background

Table 2. Inhibitors tested for effect on uptake of LYCH in Dunaliella cells

Compound used	Concentration (μM)
Respiratory inhibitors	
iodoacetate	3000
sodium fluoride	10,000
sodium azide	3000
sodium cyanide	3000
SHAM[a]	3000
sodium dithionite	saturated solution
quinacrine	1000
chloroquine	1000
dinitrophenol	1000–3000
CCCP[b]	16–160
Photosynthetic inhibitor	
DCMU[c]	3000
Ionophores and channel blockers	
monensin	10
nigericin	10
DCCD[d]	1000
probenecid	5000
Cytoskeletal inhibitors	
cytochalasin D	10
APM	10–40
cytochalasin D + APM	10 + 10
sodium vanadate	25–2000
N-ethylmaleimide	1000
Other inhibitors	
caffeine	500–50,000
cycloheximide	350

[a] Salicylhydroxamic acid
[b] *m*-Chlorocarbonyl-cyanide phenylhydrazone
[c] 3-(3,4-dichlorophenol)-1,1-dimethylurea
[d] Dicyclohexylcarbodiimide

Table 3. Effect of metabolic inhibitors on ATP levels in *Dunaliella* sp.

Treatment (no. of replicates)	Concentration of ATP (μM)[a]
Control (8)	229 ± 19
0.13% DMSO (3)	293 ± 18
3 mM sodium azide (3)	169 ± 0.58
3 mM dinitrophenol[b] (3)	45 ± 10[c]
3 mM sodium cyanide (6)	234 ± 10
30% glycerol (3)	159 ± 33
0.16 μM CCCP[b] (3)	159 ± 9[d]

Inhibitors were used for about 1 h prior to extraction.
[a] Calculated by assuming that the ATP was contained within the cytosol and enclosed organelles, i.e., ATP occupied 70% of total cell volume
[b] Dissolved in DMSO
[c] Significantly different from DMSO control at 0.01 level
[d] Significantly different from DMSO control at 0.034 level

18 s to elute cells free of LYCH (Fig. 2 B); such cells were entire and swam normally. LYCH was eluted from the column in later fractions. The experiment demonstrates the extreme rapidity by which Dunaliella cells loaded with a fluorescent dye can be made to lose the dye when washed with a dye-free medium. Thus, the rapid exchange that takes place between the endocytotic-exocytotic compartments and the external solution must be taken into consideration when measuring the ion content of cells with the column technique (Pick et al. 1986).

To determine the source of energy for endocytosis in *Dunaliella* spp., we treated cells for 2 h with compounds known to inhibit respiratory processes, photosynthesis, and oxidation-reduction reactions before we visually inspected them under the microscope (Table 2). None of the agents was found to inhibit endocytosis even though at least two of them bring about a fall in cell ATP (Table 3) and some of them inhibit swimming. The lack of effect of CCCP, azide and especially cyanide on ATP production seems surprising although metabolism in *Dunaliella* spp. is known to be hard to inhibit (A. Zamir pers. commun.), perhaps because the impermeability of the plasma membrane acts as a barrier to the entry of inhibitors. Endocytosis in *Dunaliella* spp. was not inhibited by agents that interfere with the cytoskeleton in these cells (Ginzburg et al. unpubl. results); these agents certainly did penetrate the membrane since they affected cell shape.

The metabolic processes of Dunaliella cells were slowed down by immersion in a bucket of ice or in ice to which solid NaCl had been added to bring the temperature down to –7 °C. After 2 h the cells were examined for their ability to take up LYCH. Care was taken to adjust the microscope beforehand so that the cells stood only for a few seconds at room temperature. Such cells were found to take up LYCH in the same way as cells at room temperature.

Uptake of FITC-labeled dextrans by Dunaliella cells

To see if Dunaliella cells take up molecules larger than LYCH, we challenged cells with FITC-dextrans varying in molecular mass from 4.4 to 2000 kDa (Fig. 3). These dextrans were found to have been taken up by the cells into endocytotic vesicles in a manner similar to LYCH. Cells challenged with unlabeled dextrans showed no autofluorescence in the vesicular

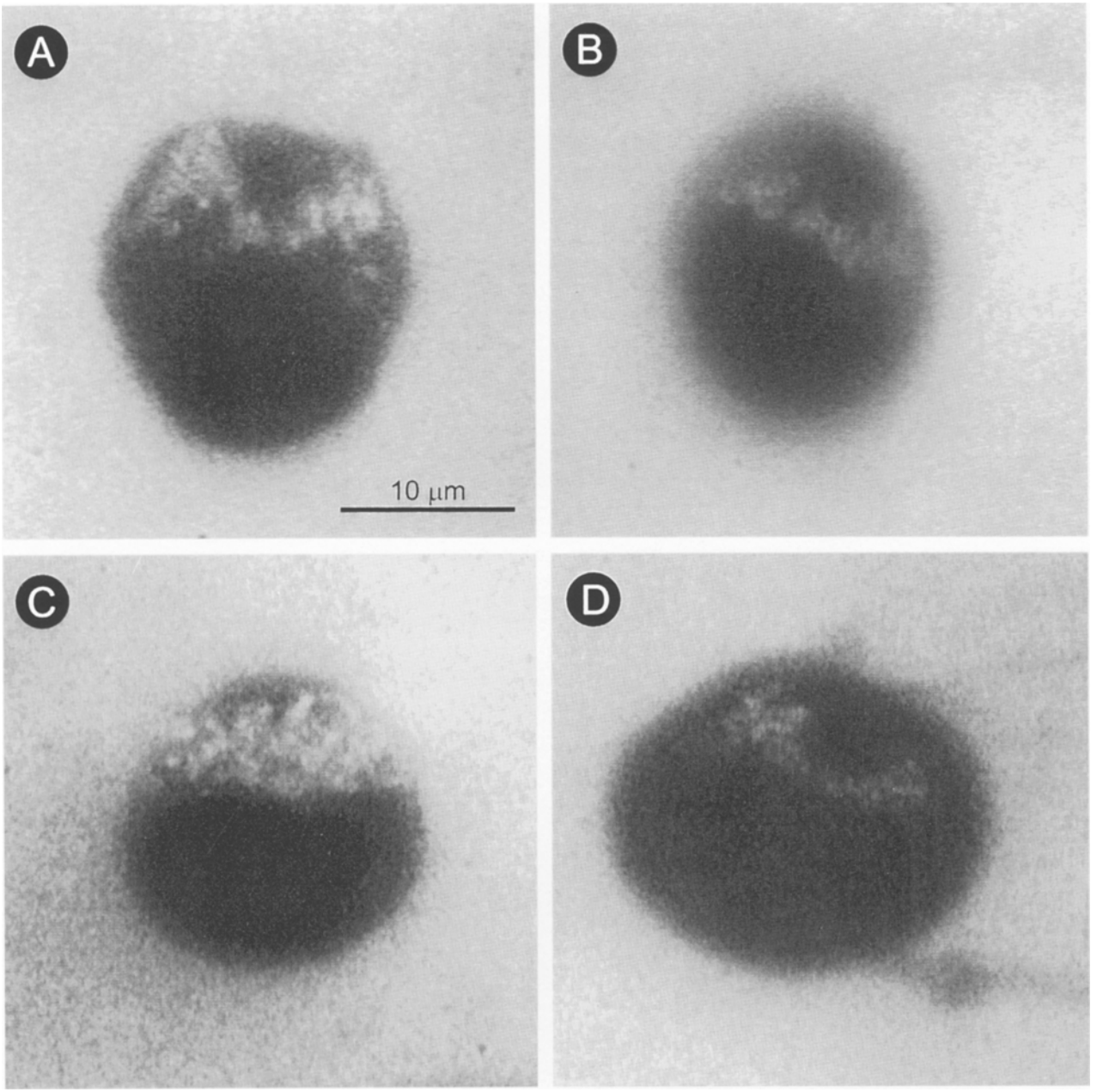

Fig. 3. Dunaliella cells with 2 mg of FITC-dextrans per ml, molecular masses: **A** 4.4 kDa, **B** and **C** 38.9 kDa, **D** 2000 kDa, showing presence of dye in the anterior region of the cell

region. The vesicles in the anterior part of the cell can be seen with differential interference optics (Fig. 4). The outline of the chloroplast can also be discerned with the pyrenoid within.

Quantitative fluorometric measurements

The brightness of the vesicular region occupied by FITC-dextrans (FD4 through FD2000) was 82.3 ± 5.8% (n = 90) of the background, suggesting that the concentrations of dye within the vesicles and in the outside medium are similar.

A major subject of concern was the time needed for uptake of dextrans by the cells. The development of a quantitative method for determining the content of a given fluorescent compound within cell pellets made it possible to determine maximum times of entry and washout. Figure 5 shows that for FD4 these times are shorter than 29 s for uptake and 15 s for washout.

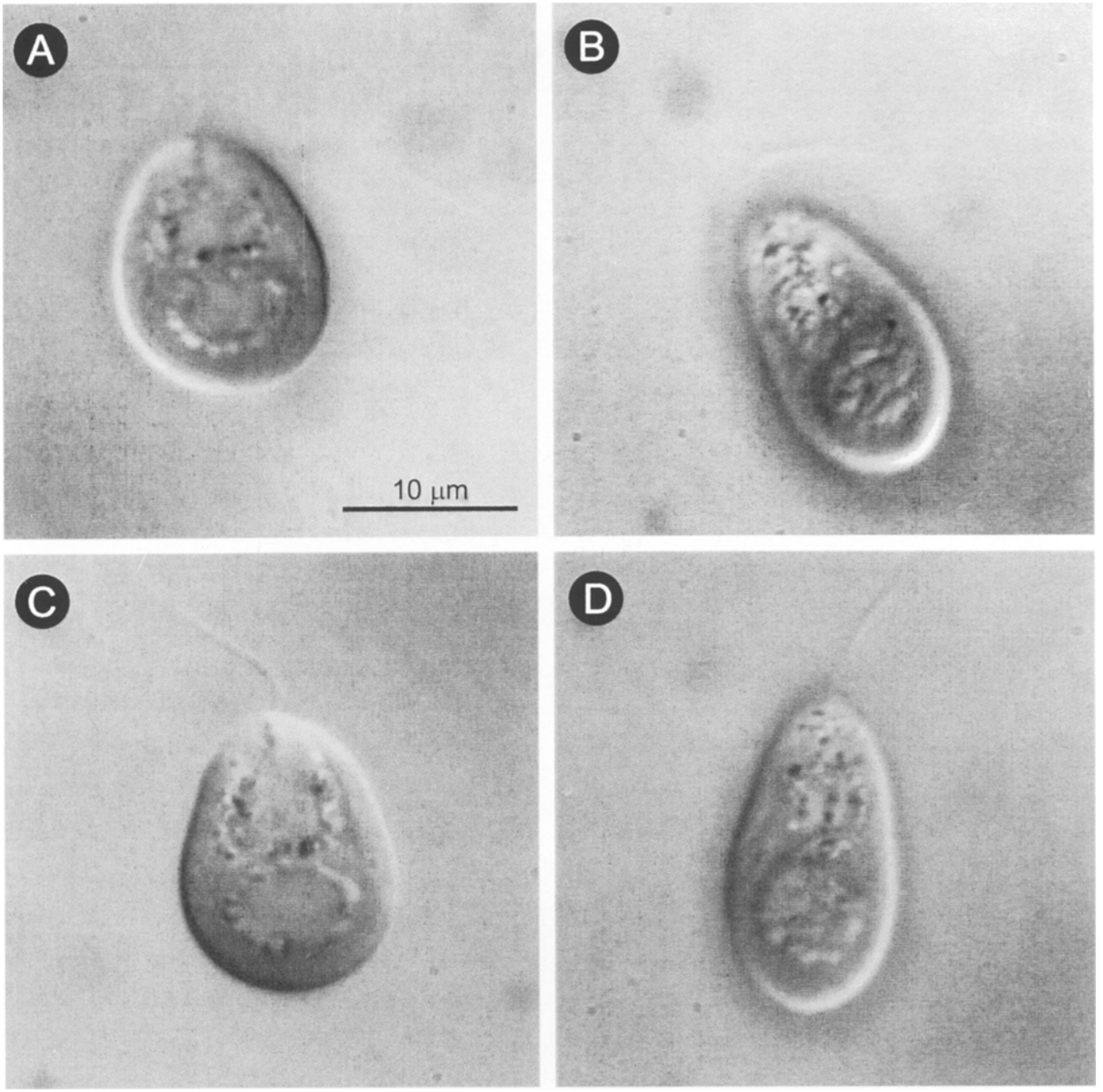

Fig. 4 A–D. Dunaliella cells seen by differential interference optics. **A** and **C** Optical sections through the middle of the cells. **B** and **D** Optical sections taken close to the cell boundary

We were interested to know whether every dextran would be taken up to the same extent, regardless of size of molecule, so we measured uptake of the series of dextrans. 2–5 min was allowed for uptake in every case and Fig. 6 shows that the amount of dextran taken up in this time period falls with increase in molecular mass. It is evident that much of the dextran, especially at the higher end of the scale, must be in the intercellular space though it is not possible to determine how much.

Uptake of dextrans for longer periods varying from 15 to 20 min was tested optically and quantified with a digital image processor (autofluorescence of chlorophyll was suppressed with a filter). The advantage of this method is that measurements are made directly on individual cells; there is no need for a correction for intercellular fluid. The proportion of the cell area occupied by fluorescent material varied between 24.4 and 33.4% of total cell area and does not differ significantly with molecular mass of the FITC-dextran

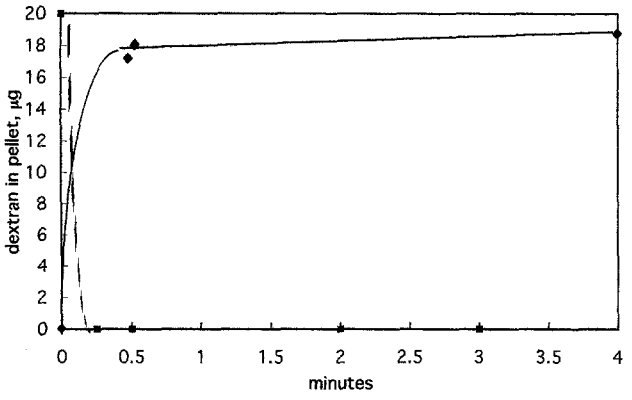

Fig. 5. Rates of uptake and washout of 2 mg FITC-dextran (molecular mass 4.4 kDa) per ml by Dunaliella cells. Uptake (◆) was determined at different times after addition of the FITC-dextran to the cell suspension as the amount of FITC-dextran recovered from pellets of centrifuged cells. The washout curve (■) was obtained from cells treated with FITC-dextran for 50 min after resuspension in fresh medium. Each point is the mean of two determinations

(Table 4). Thus, the larger ones are taken up by Dunaliella cells though more slowly than smaller ones: it takes less than 29 s for uptake of FD4 and ca. 15 min for uptake of FD2000. The increase in time with size of molecule may be due to the slower diffusion of larger molecules through the medium.

Uptake of Sodium Green dextran by Dunaliella cells

Evidence that the endocytotic vesicles contain Na^+ was obtained by adding to a cell suspension Sodium Green dextran, an indicator that fluoresces after binding to Na^+ (Fig. 7): fluorescent vesicles were found in the same cell region as LYCH and fluorescent dextrans. 29.9 ± 1.1% (n = 30) of the total cell area was occupied by fluorescent vesicles containing Sodium Green dextran (cf. vesicular area obtained with FITC-dextrans in Table 4). The brightness of the vesicles containing the dextran was 73.4 ± 3.2% of the background.

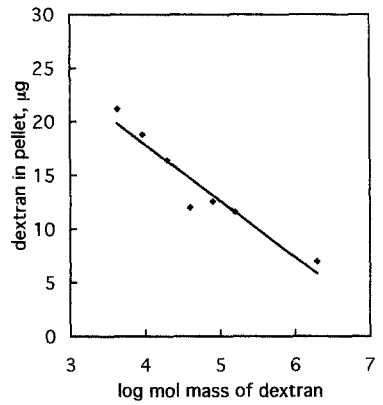

Fig. 6. Recovery of a graded series of FITC-dextrans (2 mg/ml) varying in molecular mass from 4.4 to 2000 kDa from pellets of centrifuged Dunaliella cells. 2–5 min were allowed for mixing of the compound with the cells before centrifugation. The volume of packed cells in the centrifuged pellet was the product of average cell volume and cell number and equalled 10 µl. Uptake expressed in microgram of dextran. Each point is the mean of 2–4 determinations

Table 4. Percentage of cell area occupied by fluorescent material after addition of FITC-dextran to Dunaliella cell suspensions

Molecular mass of dextran (kDa)	% vesicles of cell area[a]
4	28.0 ± 1.1
10	24.4 ± 2.6
20	33.4 ± 3.8
40	30.5 ± 1.0
70	26.5 ± 1.9
150	24.7 ± 1.5
250	24.8 ± 6.0
500	29.1 ± 1.0
2000	27.8 ± 1.0

[a] Ratio of fluorescence of total cell to that of background, both corrected for the fluorescence of the chloroplast. Values are averages with SE of measurements on 10 cells

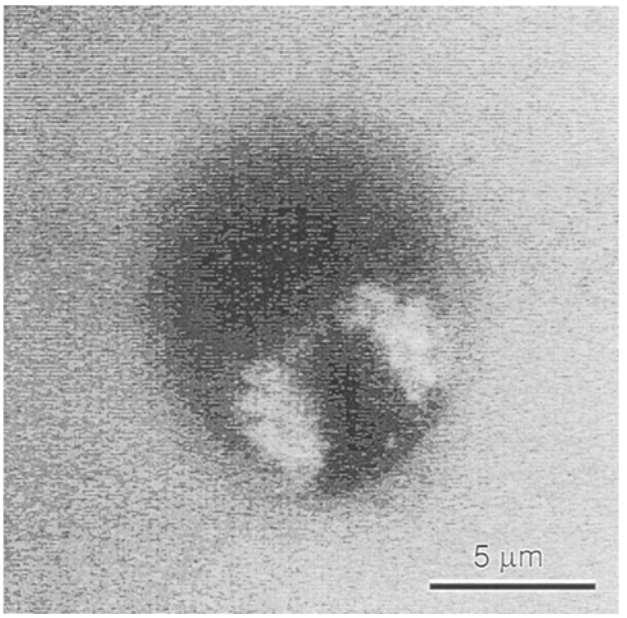

Fig. 7. Dunaliella cells containing Sodium Green dextran. Compare with Figs. 1 and 3 to see that fluorescence is limited to same area of cell. Note that in this preparation, concentrations of Na^+ cannot be determined with this dye, the K_d of which is 6.0 mM, since the Na^+ of the medium is 1.7 M

Table 5. Time parameters in calculations of rates of endocytosis

System measured	Type of cell	Time parameter used by original author	Reference
Volume of fluid pinocytosed	*Acanthamoeba*	25% of cell volume/h	Bowers and Olsewski 1972
	L cells; many cultured cells	25% of cell volume/3–6 h	Steinman et al. 1976
	mouse peritoneal M p	25% of cell volume/h	Steinman et al. 1976
Filling of pinocytosis vacuoles	macrophages	25% of cell volume/180 s	Steinman et al. 1976
Membrane internalization	L cell fibroblasts	120 min/cell surface[a]	Steinman et al. 1976
	macrophages	33 min/cell surface[a]	Steinman et al. 1976
	elongating pollen tubes	30–80% of produced membrane recycled	Picton and Steer 1983
Clathrin-mediated endocytosis	BHK cells	$\tau = 33$ min[b]	Marsh and Helenius 1980
	chromaffin-coated vesicles	$\tau = 15$ min	Patzak et al. 1_{987}
	nerve cells	τ = several tens of seconds	T. Miller and Heuser 1984
Retrieval of exocytosed membrane	mast cells	$\tau = 3$ h	Thilo 1985
	chromaffin cells	$\tau = 4$ min	von Grafenstein et al. 1986
	pituitory cells	$\tau = 4$ s	Thomas et al. 1994

[a] Time needed for recycling of entire cell surface

[b] Time coefficient τ is a measure of rate of endocytosis and depends on the experimental conditions and the kinetic model. For details, see von Grafenstein et al. (1986)

Discussion

Dunaliella cells constitutively take up and release large fluorescent molecules to the external medium by a process of fluid-phase endocytosis and exocytosis. This conclusion follows from the observation that fluorescent dyes taken up by the cells are not distributed evenly throughout the cell, but are limited to vesicles. Uptake is entirely nonspecific and nonconcentrating.

Endocytosis is traditionally considered to be a slow process (Watts and Marsh 1992). This is illustrated in Table 5 which shows that the time needed to recycle the cell surface often takes several hours. On the other hand, studies with several cell types, including synaptic vesicles of the goldfish retinal bipolar cell, have consistently revealed a relatively rapid process with a time constant of a few seconds (Betz and Angleson 1998). Endocytosis in Dunaliella cells belongs to this latter group of rapidly endocytosing cells, since the time needed to internalize the entire cell membrane is less than 4 s (Table 6).

Cell volume is calculated on the assumption that the cell is a prolate ellipsoid with major axis $2A$ and minor axis $2B$. The proportion of cell volume occupied by vesicles can be estimated from the percentage of cell area occupied by vesicles (Table 4) by modeling the vesicular region as a hollow truncated cone with height a and width of each side b: area of vesicular region in median section, $A_v = 2ab/2$; area of the cell in median section, $A_c = \pi AB$; volume of the vesicular region, $V_v = \pi ab^2$; volume of the cell, $V_c = (4/3)\,\pi \cdot AB^2$; $V_v / V_c = 3\pi\, ab^2 / (4\pi\, AB^2) = [0.75ab / (AB)]\,(b/B)$. Since $A_v / A_c = ab / \pi AB = 0.3$, then $ab/AB = 0.3\pi$. Hence, $V_v / V_c = 0.75 \cdot 0.3\pi(b/B) = 0.70b/B$. When $b/B = 0.5$, then $V_v / V_c = 0.35$. Therefore, provided that $b/B = 0.5$, the ratio of A_v to A_c is approximately equal to V_v / V_c.

In *Dunaliella* spp. the rates of endo- and exocytosis for molecules with a mass equal or less than 4000 Da, as measured by volume flow, are at least one order of magnitude faster than rates in other eucaryotic cells

Table 6. Calculation of rate of endocytosis in *Dunaliella* sp., assuming that 30% of the cell is occupied by endocytosed fluid and that this fluid is within spherical vesicles

Parameter	Value
Diameter of vesicle, $2r$	$400 \cdot 10^{-9}$ m
Volume of vesicle, $(4/3)\,\pi r^3 = V_v$	$3.4 \cdot 10^{-20}$ m^3
Surface area of vesicle, $4\pi r^2 = S_v$	$5.0 \cdot 10^{-6}$ m^2
Major cell axis, A	$5.5 \cdot 10^{-6}$ m
Minor cell axis, B	$6.0 \cdot 10^{-6}$ m
Cell volume, $(4/3)\,\pi AB^2 = V_c$	$7.6 \cdot 10^{-16}$ m^3
Surface area of cell (= surface area of plasma membrane), $4\pi AB = S_c$	$4.6 \cdot 10^{-10}$ m^2
Number of vesicles, $n = 0.3\ V_c/V_v$	$6.7 \cdot 10^3$
Total surface area of vesicles, nS_v	$3.4 \cdot 10^{-9}$ m^2
$(nS_v)/S_c$	7.7
Time to fill endocytotic vesicles	~30 s
Time to internalize entire cell membrane, $30/(nS_v/S_c)$	3.9 s

Table 7. Endocytotic volume flow into various types of cells

Cell type	Cell volume (10^{-18} m^3)	Cell surface area (10^{-12} m^2)	Volume flow (m/s)[a]	Reference
Yeast	100	100	$8.3 \cdot 10^{-13}$	Lenhard et al. 1992
Human fibroblast	1765	2100	$2 \cdot 10^{-12}$	van Deurs et al. 1989
Dictyostelium discoideum	500	314	$7 \cdot 10^{-10}$	Lenhard et al. 1992
Macrophage	385	825	$2.5 \cdot 10^{-9}$	Steinman et al. 1976
Dunaliella sp. var. D13	760	460	$1.7 \cdot 10^{-8}$	this work

[a] Volume flow was calculated from data on percentage of cell volume taken up by endocytotic vesicles, cell surface area, and time constants presented in the references cited. Volume flow is defined as volume of fluid (m^3) to cross one unit of cell surface area (m^2) per unit of time (s)

Table 8. Dimensions of types of endocytotic vesicles measured on thin sections by electron microscopy

Type of vesicle	Source	Diameter (10^{-9} m)	Reference
Coated vesicles; partially coated reticulum and dictyosomes	soybean protoplast	50–70	Tanchak et al. 1984, 1988
Coated vesicles	various plant cells	84–91	Coleman et al. 1987
	Lobelia root cells	~100	Samuels and Bisapultra 1990
	soybean protoplasts	~100	Fowke and Tanchak 1988
	various plant cells	72–96	Emons and Traas 1986
	Dunaliella salina	70–100	Zhang et al. 1993
Pinocytosis primary vesicle	*Acanthamoeba*	250	Bowers et al. 1981
Primary endocytosis vesicle	macrophages and L-cells	150	Steinman et al. 1976
Clathrin-coated vesicles	human fibroblasts	100	van Deurs et al. 1989
Nonclathrin-coated vesicles (a)	smooth muscle cells, fibroblasts, endothelial cells	50–100	van Deurs et al. 1989
Nonclathrin-coated (b)	Vero cells, Hep-2 cells, A431 cells	150–300	van Deurs et al. 1989
"Slow" endocytosis vesicles	BHK cells	~100	Thomas et al. 1994
"Fast" endocytotic vesicles	rat melantrophs	800	Thomas et al. 1994

(Table 7). The high rates of endocytotic volume flow in *Dunaliella* spp. may be a consequence of the high specific rate of internalization per vesicle, the lack of a thick extracellular matrix and of turgor pressure, the large volume fraction of vesicles per cell (25–30% of cell volume as compared to 1–10% in other cells) and/or the relatively large size of the endo- and exocytotic vesicles (400 nm diameter as compared to 100 nm in other cells, see Tables 6 and 8).

On the other hand, these very fast rates are limited to LYCH and the smaller dextrans; we found that FD2000 took several minutes to enter the cells. The difference in behavior between the smaller and larger molecules accounts for the fact that previous workers did not find larger dextrans to be taken up by Dunaliella cells (Ehrenfeld and Cousin 1982, M. Ginzburg and Richman 1985); these workers centrifuged cell suspensions after mixing their dextran markers with cell suspensions for not more than 2 min, thus avoiding the slow endocytotic uptake of large molecules.

It is unclear whether the vesicles once inside the cell remain independent, fuse with other vesicles to form an endosomal reticulum involved in membrane recycling (Hopkins et al. 1990) or interact with other internal membrane systems in the cell such as the trans-Golgi network (Tanchak et al. 1984, Fowke et al. 1991, Mukherjee et al. 1997, Low and Chandra 1994, Prescianotto-Baschong and Riezman 1998). Given the rapid rates of endo- and exocytosis, we favor the first, or first two of these possibilities for the majority of vesicles at least.

Vesicle formation involved in membrane trafficking typically requires chemical energy coupled to dephosphorylation of ATP and GTP. In an attempt to search for the processes that provide the energy necessary for vesicle formation, we treated the cells with a wide variety of inhibitors, yet, in all cases we were unable to inhibit endocytosis, which was not stopped even in cells cooled to –7 °C. Thus, either endocytosis

requires little energy or is energized by some unusual source.

In this paper we present a new model that synthesizes the two antithetical models concerning halotolerance in *Dunaliella* spp. The discovery of the rapid constitutive processes of endocytosis and exocytosis in the Dunaliella cell solves a long-standing controversy concerning the structure and composition of this cell. The one-compartment model treated halotolerance as an osmotic problem and considered the plasma membrane to be of the usual semipermeable type. Several authors (e.g., Ben-Amotz and Avron 1973, Pick et al. 1986) have equated the cytosol with the cell and have not taken the 25–30% of the cell volume occupied by endocytotic vesicles into account. We consider the cell Na^+ measurements of those authors to characterize the cytosol and nonendo- and nonexocytotic organelles rather than the entire cell. On the other hand, the two-compartment model, based on static and dynamic measurements of total cell glycerol, Na^+, K^+, and Cl^-, assumed that the plasma membrane was unusually permeable to salts and glycerol, although it was also assumed that typically semipermeable membranes enclosed the organelles. Neither model took the possibility of endo- or exocytosis into account. We suggest that the so-called outer compartment described by the two-compartment model can be accounted for by the endo- and exocytotic vesicles. The high cell Na^+ concentrations found by several workers (summarized in M. Ginzburg 1987) can be accounted for by NaCl from the outside medium trapped within endocytotic vesicles.

Is endocytosis related to the extraordinary capacities for salt regulation and osmoregulation found in *Dunaliella* spp.? Certainly the endo- and exocytotic membranes serve as a ready source and sink of plasma membrane for cells challenged by hypoosmotic and hyperosmotic stresses (Table 1) (Maeda and Thompson 1986). However, the amount of vesicular membrane present is far greater than is needed to account for any increase in surface area that has ever been observed to occur after a hypoosmotic stress. It is therefore possible that the vesicles have some additional function. Cells living in high concentrations of salt (up to several molar) may also benefit from membrane systems through which there is a continuous flow of large volumes of highly concentrated NaCl solutions. We suggest that the enormous membrane area created by the system could serve to synthesize ATP by using the large differences in Na^+ or Cl^- existing across the cell membrane: molar concentrations in the outside medium and cytosolic Na^+ kept to a low millimolar concentration by light-driven ion pumps. Reversible Na^+-ATPases "form a large group of enzymes found in all kingdoms of living organisms" (Skulachev 1994). Na^+-dependent ATP synthesis may help to mitigate the stress experienced by cells living in highly saline environments.

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