Association of Intracellular Low-density Vesicles with Plasma Membranes from *Saccharomyces cerevisiae*NCYC 366

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(Received 24 March 1983; revised 29 April 1983)

Fractionation on sucrose density gradients of spheroplast lysates from *Saccharomyces cerevisiae*NCYC 366 yielded a fraction with a peak density of 1.05 g ml⁻¹, intermediate between that of membranes and intracellular low-density vesicles. Electron microscopy showed the fraction to consist of membranes associated with intracellular vesicles. Evidence for the presence of plasma membranes in the association fraction was obtained by using ¹²⁵I-labelled spheroplasts. The fraction was free from detectable amounts of cytochromes and DNA. Fractionation on sucrose density gradients of incubation mixtures containing isolated crude plasma-membranes and vesicles gave rise to a visible intermediate-density band, which electron microscopy showed to contain membranes associated with vesicles. Evidence for the presence of plasma membranes in the *in vitro* intermediate-density band came from incubating mixtures containing ¹²⁵I-labelled crude plasma-membranes, and evidence for the presence of vesicles came from using ²⁵¹-labelled vesicles. Supplementing incubation mixtures with calcium chloride sharply increased the size of the intermediate-density band. Cycloheximide and methylbenzimidazol-2-yl-carbamate had no effect on its formation. Purified plasma-membranes failed to form an intermediate-density band when incubated with vesicles. Supplementing these incubation mixtures with calcium chloride did not produce an intermediate-density band, but caused extensive association of vesicles with plasma membranes that pelleted in gradients. Isolated vesicles were not osmotically active; their polyphosphate content was 1.61 μmol orthophosphate equivalent (mg vesicle protein)⁻¹. They had diameters in the range 0.45-0.65 μm, as measured on electron micrographs of thin sections. The data reported provide further evidence for a role for intracellular low-density vesicles in envelope growth in *S. cerevisiae*.

INTRODUCTION

Light-microscope observations by Guilliermond *et al.* (1933) established that *Saccharomyces cerevisiae* contains one or more large organelles, termed vacuoles, as well as a larger number of smaller structures which, since they stained with lipid-soluble dyes, were referred to as lipid granules, spherosomes or vesicles (Guilliermond, 1923). The physiological functions of vacuoles and vesicles remained a mystery for many years and are still not fully understood (Schwencke, 1977). Following the refinement of techniques for preparing yeast spheroplasts, fractions rich in intermediate-density band when incubated with vesicles. Supplementing these incubation mixtures with calcium chloride did not produce an intermediate-density band, but caused extensive association of vesicles with plasma membranes that pelleted in gradients. Isolated vesicles were not osmotically active; their polyphosphate content was 1.61 μmol orthophosphate equivalent (mg vesicle protein)⁻¹. They had diameters in the range 0.45-0.65 μm, as measured on electron micrographs of thin sections. The data reported provide further evidence for a role for intracellular low-density vesicles in envelope growth in *S. cerevisiae*.

**INTRODUCTION**

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Transmission electron-microscope studies of thin sections of *S. cerevisiae* by Sentandreu & Northcote (1969) and later Wiemken *et al.* (1970) suggested that vacuoles undergo fission during...
the cell cycle, and that the vesicles produced become concentrated at the neck of the bud. It was inferred that vesicles are involved in envelope growth in *S. cerevisiae* in a manner analogous to that thought to operate in hyphal tips of fungi (Rosenberger, 1979). Further evidence for a role for vesicles in envelope growth in *S. cerevisiae* came from reports that these structures possess β-glucanase and x-mannanase activities, which could be involved in breakdown of wall components before insertion of new wall material (Matile et al., 1971; Cartledge et al., 1977). Vesicles are also reckoned to be involved in chitin synthesis in the bud scar, although their precise role has yet to be clarified (Cabib et al., 1982). Transport and excretion of extracellular enzymes are also thought to be mediated by vesicles (Herrero et al., 1980; Novick et al., 1981). Vesicles are, moreover, known to contain lipids (Clausen et al., 1974; Cartledge et al., 1977; Schaffner & Matile, 1981), some of which are thought to be converted into plasma-membrane phospholipids and sterols.

The present paper reports evidence for the existence in randomly dividing populations of *S. cerevisiae*NCYC 366 of plasma membrane/intracellular vesicle associations, and for *in vitro* formation of such associations following incubation of isolated plasma-membrane preparations with low-density vesicles. Two types of plasma-membrane preparation were used in the *in vitro* experiments to examine the effect which the degree of processing in their isolation had on their ability to fuse with low-density vesicles. *Saccharomyces cerevisiae*NCYC 366 was used because of its ready susceptibility to the action of wall-degrading enzymes (Pringle et al., 1979).

METHODS

Experimental cultures. *Saccharomyces cerevisiae*NCYC 366 was maintained on slopes of malt extract/yeast extract/glucose/peptone/agar medium (Wickerham, 1951). The yeast was grown aerobically at 30°C in 1 litre cultures as described by Cartledge et al. (1977). Growth was followed by measuring absorbance at 600 nm (A<sub>600</sub>), measurements being related to dry weight by a standard curve. Organisms were harvested from late exponential-phase cultures (0.24-0.26 mg dry wt ml<sup>-1</sup>) by filtration through a membrane filter (0.45 μm pore size; 50 mm diam.; Millipore).

Isolation of plasma membrane and vesicles. Spheroplasts were prepared by a modification of the methods of Alterthum & Rose (1973) and Cartledge et al. (1977). Organisms were suspended at 10 mg dry wt ml<sup>-1</sup> in buffered sorbitol (1:2 M, containing 10 mm-imidazole and 10 mM-MgCl<sub>2</sub>, adjusted to pH 7.2 with HCl) and Zymolase 60000 [0.1 mg (6 mg dry wt organisms)<sup>-1</sup>] and incubated at 30°C with reciprocal shaking (120 r.p.m.). Spheroplast formation was monitored by measuring the A<sub>600</sub> value when portions (0:1 ml) of the suspension were diluted into either 2:9 ml buffered sorbitol or 2:9 ml water. Spheroplast formation was judged to be complete after 1 h incubation (Pringle et al., 1979). All subsequent operations were carried out in an ice–water mixture or in a centrifuge at 4°C. Spheroplasts from 240–260 mg dry wt organisms were washed gently three times with buffered sorbitol, and suspended in 9 ml buffered mannitol (0:3 M, containing 50 mM-Tris, adjusted to pH 7:2 with HCl). Lysis of spheroplasts was effected by a combination of osmotic lysis and gentle mechanical disruption by incubating the spheroplast suspension for at least 20 min in an ice–water mixture, and then subjecting the suspension to six strokes of a Teflon-glass hand homogenizer (0:1 mm clearance). The lysate from 240–260 mg dry wt organisms was made up to 10 ml with buffered mannitol. This was supplemented with 3 ml 62.5% (w/v) sucrose in buffered mannitol, and on to it was layered in a 40 ml centrifuge tube a discontinuous gradient of sucrose made up successively with portions (2 ml) of 7, 6, 5, 4, 3, 2 and 1% (w/v) sucrose in buffered mannitol. The gradient was overlaid with 2 ml buffered mannitol, and the tube centrifuged for 1 h at 4°C at 11000 g (r<sub>av</sub> 9-7 cm) in a 3 × 40 ml swing-out rotor with an MSE Superspeed 50 centrifuge. A mixture of intracellular vesicles accumulated in the top 2 ml of the gradient, and was removed with a hypodermic syringe. Electron microscopy (Fig. 1b) showed this fraction to be largely free from other cytoplasmic particulate material, and it was used without further treatment. A few vacuoles were present in the preparations. On electron micrographs of thin sections, vesicles measured 0:45–0:65 μm diam. (in a sample of 115 objects). They differed in the extent to which they were stained during preparation for electron microscopy (Fig. 1b). Since vesicles are difficult to count routinely in the light microscope, a unit of vesicles was defined as a population which when contained in 1 ml buffered mannitol gave an A<sub>eoe</sub> value of 1-0. Yields of vesicles were 51:0 ± 3:6 units (g dry wt organisms)<sup>-1</sup> (six determinations) from populations harvested at a density of 0:24–0:26 mg dry wt ml<sup>-1</sup>. The protein content of vesicles was 0:90 ± 0:10 mg (100 units)<sup>-1</sup> (seven determinations).

After removing the low-density vesicle fraction, the remainder of the gradient was discarded, leaving a pellet containing membranes. The surface of the pellet was washed with buffered mannitol, and the pellet suspended in 10 ml buffered mannitol. The membrane suspension was then homogenized (four to six strokes of a Teflon-glass
hand homogenizer, 0.1 mm clearance), made up to 30 ml with buffered mannitol, and incubated for at least 20 min in an ice-water mixture. The suspension was supplemented with 9 ml 62% (w/v) sucrose in buffered mannitol and centrifuged for 30 min at 11 000 g in a 3 x 40 ml swing-out rotor at 4°C. Vesicles released from the membrane fraction accumulated at the top of the centrifuge tube. The pellet, termed the crude plasma-membrane preparation, was suspended by homogenization in 2 ml buffered mannitol and used as soon as possible and without storage (Fig. 1a).

Purified plasma-membrane preparations were obtained by a modification of the sucrose density gradient procedure of Hossack (1975). A suspension (2 ml) of crude plasma-membrane preparation from 240–260 mg dry

Fig. 1. Electron micrographs of thin sections through preparations of (a) crude plasma-membrane, (b) intracellular vesicles (a single vacuole is visible in the bottom left-hand corner of the micrograph), (c) in vivo and (d) in vitro crude plasma-membrane-vesicle associations from S.cerevisiae NCYC 366. The bar markers represent 0.5 μm.
wt organisms was diluted to 4 ml with buffered mannitol and layered on top of a discontinuous gradient of sucrose solutions. The gradient was made up of 5 ml each of 62 and 50% (w/v) sucrose in buffered mannitol, followed by 3 ml each of 45, 40, 35, 30, 25, 20 and 10% (w/v) sucrose in buffered mannitol. Visible bands which formed in the gradients after centrifugation for 90 min at 4°C and 24000 g (rs, 9.7 cm) in a 3 × 40 ml swing-out rotor were removed with a hypodermic syringe. After removing the remaining supernatant liquid, the pellet was resuspended in 35 ml buffered mannitol. The pellet obtained after centrifuging this suspension for 15 min at 11000 g was resuspended in a small volume (2 ml) of buffered mannitol and used as soon as possible and without storage.

**Labelling of spheroplasts and vesicles.** Spheroplasts from 500 mg dry wt organisms were washed three times with buffered sorbitol and suspended in 48 ml of the same buffer. The suspension was supplemented with 0.7 mg lactoperoxidase and 25 μCi Na125I [16.7 mCi (618 MBq)] in buffered sorbitol and the suspension made up to 50 ml with the same buffer. Iodination was carried out at room temperature by adding H2O2 to a final concentration of 8 μM. Equal amounts of 0.5 mM-H2O2 in buffered sorbitol were added to the suspension at 1 min intervals up to 5 min. Labelled spheroplasts were then washed four times with buffered sorbitol, and crude plasma-membrane preparations isolated as already described. Vesicles, isolated from sucrose gradients as already described above, were labelled by diluting a population (10-0 units) to 98 ml in buffered mannitol, supplementing with 0.7 mg lactoperoxidase and 25 mCi Na125I in buffered mannitol and making the suspension up to 100 ml with the same buffer. Iodination was carried out as already described for membranes. The suspension was then made 5% (w/v) with respect to sucrose and centrifuged for 1 h at 11000 g and 4°C. Vesicle suspensions collected from the top of the centrifuge tubes with a hypodermic syringe were diluted to 50 ml with buffered sorbitol. The mixture was supplemented with 0.1 mM-KI and incubated at 4°C for 1 h. It was then made 5% (w/v) with respect to sucrose and the vesicles harvested by centrifugation. The suspension of vesicles was made up to 10 ml with buffered mannitol, supplemented with 3 ml 62% (w/v) sucrose in buffered mannitol, and the vesicles collected after centrifugation on a discontinuous 0–7% (w/v) sucrose gradient as previously described.

**Transmission electron microscopy.** Vesicles were fixed by mixing in a glass conical centrifuge tube with an equal volume of 4% (w/v) osmium tetroxide in water, and leaving for 60 min at 4°C with occasional agitation. All other samples were fixed by similarly mixing with an equal volume of 2% (w/v) osmium tetroxide in 50 mM-sodium cacodylate and incubating as described for vesicles. The tube was then centrifuged at 2500 g for 5 min. The supernatant was discarded and the pellet resuspended gently in 50 mM-sodium cacodylate. The pellet was washed three times and resuspended in 2% (w/v) agar or, when preparing vesicles, agarose (Sigma). This suspension was taken into a Pasteur pipette and extruded, as it set, on to a glass plate. The set agar containing the sample was cut into small sections. Vesicle samples were stained with 1% (w/v) uranyl acetate at 20°C for 1 h in the dark. All samples were then dehydrated in the following acetone (v/v) sequence: 10 min 15%, 10 min 50%, 10 min 90% and three times 15 min 100%. The samples were transferred into Taab EM resin (Taab Laboratories Equipment, Reading, Berks., U.K.) and rotated for 24 h. They were then transferred to Taab capsules, fresh resin was added, and they were labelled and polymerized at 60°C for 3 d. Gold sections were cut on a Reichert OMU3 ultramicrotome (Reichert-Jung U.K., Slough, U.K.). The sections were stained for 10 min in 70% (v/v) ethanol saturated with uranyl acetate, then for 15 min in Reynolds (1963) lead citrate. The sections were viewed on a JEOL 100 CX transmission electron microscope (JEOL U.K., Colindale, London, U.K.). All photographs were taken on Kodak 4489 film.

**Analytical methods.** Sucrose concentrations were measured with a Bellingham & Stanley refractometer (Bellingham & Stanley, Tunbridge Wells, Kent, U.K.). Activity of 125I was measured with an LKB Ultragamma counter, DNA contents were measured as described by Setaro & Morley (1977) and cytochromes by the method of Stewart (1975). Membrane and vesicle preparations were diluted sixfold with 14.4% (w/v) sodium deoxycholate (Maddy & Spooner, 1970) before protein was estimated by the Lowry method using bovine serum albumin as a standard. The polyphosphate content of vesicles was measured by determining the orthophosphate content in a suspension (made 0.5 mM with H2SO4) before and after boiling for 7 min (Dürr et al., 1979). Orthophosphate was assayed by the method of Marsh (1959). Polyphosphate contents are expressed as orthophosphate equivalent. Calcium was determined in the presence of 0.2% (w/v) SrCl2 in a Pye-Unicam atomic absorption spectrophotometer.

**Chemicals.** All chemicals used were Analar or of the highest purity available commercially. Lactoperoxidase [from milk; 60–80 units (mg protein)-1] was purchased from Sigma. Zymolyase 60000 was obtained from the Kirin Brewery Co., Takasake, Gunma Prefecture, Japan. Radioactively-labelled compounds were from Amersham. K. Gull provided us with methylbenzimidazol-2-yl-carbamate.

**RESULTS**

**Isolation of in vivo plasma membrane-vesicle associations**

When the process for isolating crude plasma-membranes from 250 mg dry wt organisms was scaled up to at least 500 mg dry wt organisms, a turbid band appeared as an intermediate-density
fraction on the gradient used to separate crude plasma-membrane and vesicles from spheroplast lysates. Electron microscopy showed that the intermediate-density band was composed of membranes associated with intracellular vesicles (Fig. 1c). Vesicles in these associations appeared uniformly lightly stained. The yield of this fraction, when corrected for contamination with non-particulate protein (that which was not retained by a 0-2 μm pore-size membrane filter), was 3 mg protein equivalent (g dry wt organisms)⁻¹ (average of two determinations). Evidence for the presence of plasma membranes in the intermediate-density band was obtained by isolating associations from ¹²⁵I-labelled spheroplasts by the standard procedure. The association band, when centrifuged on a modified second sucrose gradient, revealed a corresponding concentration of protein and ¹²⁵I activity (Fig. 2). This fraction contained 11% of the ratio of ¹²⁵I:protein found in purified plasma-membranes. The association fraction appeared as a broad peak with a maximum at a sucrose density of 1.05 g ml⁻¹. The associations, after recentrifugation in a sucrose density gradient (Fig. 2), did not contain detectable amounts of cytochromes [less than 0.3 × 10⁻⁶ mmol cytochrome b equivalent (mg protein)⁻¹] or DNA [less than 0.25 μg (mg protein)⁻¹].

*Formation of in vitro associations between crude plasma-membranes and vesicles*

Preliminary experiments revealed that incubation of crude plasma-membranes and vesicles in buffered mannitol for several hours led to formation of a fraction that separated as a visible intermediate-density band when the incubation mixture was fractionated on the gradient used to separate membranes and vesicles from spheroplast lysates (Fig. 1d). Unlike populations of

![Graph](image-url)
isolated vesicles, those in associations appeared uniformly lightly stained. An assay procedure for formation of the intermediate-density band was developed based on incubating fixed amounts of crude plasma-membranes with different amounts of vesicles. Preliminary experiments indicated that the optimum temperature for formation of the band was around 35 °C, and that formation was prevented at temperatures below 20 °C and above 40 °C. There was rapid formation of the band as the content of vesicles in an incubation mixture containing crude plasma-membranes (2.5 mg protein) was increased up to 5 units, with a less rapid increase as the content was raised further to 20 units (Fig. 3). Monitoring utilization of vesicles revealed a similar pattern (Fig. 3) except that a greater proportion of vesicles appeared to be associated with membranes than was indicated by the absorbance value of the intermediate-density band. Over a period of 11 h, there was a linear increase in formation of bands at density exceeding 6% (w/v) sucrose (intermediate-density band; Fig. 4) but, after 4 h incubation, bands also appeared at a lower density (low-density band; Fig. 4). As both density bands were formed, there was a linear decrease in size of the membrane pellet as judged by protein content (Fig. 4). The standard assay procedure adopted for measuring in vitro formation of plasma membrane/vesicle associations was as described in the legend to Fig. 4, with an incubation time of 4 h.

Electron microscopy of the intermediate-density band showed it to be made up of membrane/vesicle associations free from other particulate cytoplasmic constituents (Fig. 1d). Associations were free also from chemically detectable amounts of cytochromes and DNA. Further evidence for the presence of plasma membranes in the intermediate-density band came from experiments in which 125I-labelled crude plasma-membrane preparations were included in incubation mixtures. Analysis of fractions from the gradient used to fractionate the incubation mixture

![Graph](image-url)

**Fig. 3.** Effect of vesicle concentration on formation of in vitro associations between crude plasma-membranes and vesicles from *S. cerevisiae* NCYC 366. Incubation mixtures (2.5 ml) consisted of buffered mannitol (0.3 M, pH 7.2) containing crude plasma-membranes (2.5 mg protein) and up to 20 units of vesicles. Mixtures were incubated with gentle shaking (60 r.p.m.) at 35 °C for 5 h. Associations and residual vesicles were then separated from the mixture using the gradient employed to separate membranes and vesicles from spheroplast lysates (see Methods). After centrifugation, residual vesicles were removed from the top of the gradient, and the number of units in the fraction measured. The turbid intermediate-density band, containing plasma membrane/vesicle associations, was removed with a hypodermic syringe. The size of the association band was determined by multiplying the \(A_{600} \times \text{vol. in ml}\), a correction being made for non-specific absorbance measured in the corresponding band obtained after fractionation of a control mixture lacking vesicles. The number of vesicle units associated with membranes was calculated as the difference between the number of vesicle units included in the incubation mixture and the number of residual vesicle units. The data presented are for a typical separation which was repeated with minor modifications with five different incubation mixtures. ○, Size of the association band; ●, number of vesicle units utilized.
Plasma membrane/vesicle associations in yeast

Fig. 4. Time-course of formation of in vitro associations between crude plasma-membranes and vesicles from S. cerevisiae NCYC 366. The composition of the incubation mixture was as described in the legend to Fig. 3; 10 units (90 μg protein) of vesicles were included in each mixture, but omitted from control mixtures. Association bands and residual vesicles were separated from mixtures using the gradient employed to separate membranes and vesicles from spheroplast lysates (see Methods). (a) O, Size of the intermediate-density association band (measured as A_{400} x vol. in ml); □, size of the lower-density association band (A_{400} x vol. in ml); O, number of vesicle units utilized. In (b), protein contents are indicated for the intermediate-density association band (■), and the lower-density association band (▲). Also shown is the decline in the protein content of the membrane pellet (△). The data presented are the results of typical experiments which were repeated at least twice.

showed that peak activity of 125I roughly coincided with peaks in A_{400} value and protein concentration (Fig. 5). However, there was appreciable 125I activity on either side of the peak. The residual vesicle band contained 10% of the total 125I activity. On a protein basis, the intermediate-density band contained 25% of the 125I activity present in plasma membranes. Vesicles were also labelled with 125I in order to determine their fate during incubation with crude plasma-membranes (Fig. 6). A peak of label roughly coincided with peaks of absorbance and protein concentration in the middle of the gradient. Label was also associated with the membrane pellet.

The absence of sharp peaks when incubation mixtures containing labelled crude plasma-membranes or vesicles were fractionated by density-gradient centrifugation suggested that plasma membrane/vesicle associations were located both above and below the visible band on the gradient. This was confirmed when fractions from above and below the visible band were examined in the electron microscope. Small fragments of membrane were associated with vesicles in the residual vesicle fraction, and some vesicles were present in the membrane pellet.

Supplementing incubation mixtures with calcium chloride, up to 2.5 mM, sharply increased the size of the intermediate-density band coincident with a rise in the number of vesicle units consumed (Fig. 7). This was accompanied by a small increase in the size of the pellet. A further rise in Ca^{2+} concentration led to increased consumption of vesicles, and to a decrease in the size of the intermediate-density band and an increase in the size of the pellet (Fig. 7).

Replacing Tris buffer with imidazole-HCl (10 mM, pH 7.2), while not altering the yield of crude plasma-membranes and vesicles from spheroplasts, decreased the extent to which these membranes and vesicles associated in the standard assay procedure. Moreover, use of imidazole buffer drastically decreased the stimulatory effect of Ca^{2+} on formation of associations between the membranes and vesicles. Including cycloheximide [2 μg (mg membrane protein)^{-1}] or methylbenzimidazol-2-yl-carbamate [0.15 ml of a saturated solution in DMSO (5 mg membrane protein)^{-1}] in incubation mixtures had no effect on formation of associations between crude plasma-membranes and vesicles.
Fig. 5. Fractionation of an incubation mixture containing $^{125}$I-labelled crude plasma-membrane preparation and vesicles from *S. cerevisiae*NCYC 366. Incubation mixtures (3.5 ml) consisted of buffered mannitol (0.3 M, pH 7.2) containing iodinated crude plasma-membrane preparation (5.0 mg protein, 6.0 x 10$^4$ c.p.m.) (a) with, or (b) without, 15 units of vesicles. After the mixture was incubated with gentle shaking (60 r.p.m.) at 35 °C for 4 h, it was fractionated using the gradient employed to separate membranes and vesicles from spheroplast lysates (see Methods), and fractions (2 ml) were collected with a hypodermic syringe fitted with a cannula needle. The data presented are for typical fractionations which were repeated with two further experimental and control incubation mixtures. $\bigodot$, $A_{600}$ value of fractions; $\bullet$, $^{125}$I activity; $\square$, protein content of fractions. Horizontal lines indicate sucrose concentration.

Fig. 6. Fractionation of an incubation mixture with the composition described in the legend for Fig. 5 except that vesicles (12 units, 14.9 x 10$^4$ c.p.m.) and not crude plasma-membranes (5.0 mg protein) were labelled with $^{125}$I. $\bigodot$, $A_{600}$ values of fractions; $\bullet$, $^{125}$I activity; $\square$, protein content of fractions. Horizontal lines indicate sucrose concentration. Data in (a) are for a complete incubation mixture, those in (b) for a mixture lacking membranes. The data presented are for typical fractions which were repeated with two further experimental and control incubation mixtures.

Formation of in vitro associations from purified plasma-membranes

Initial attempts to produce a visible intermediate-density band on sucrose density gradients following incubation of purified plasma-membranes with vesicles failed. Vesicles and plasma membranes were recovered from gradients in amounts similar to those recovered when these organelles were incubated on their own. Supplementing incubation mixtures with CaCl$_2$ (10 mM) produced a barely visible intermediate-density band, but led to a large decrease in the recovery of vesicles (Table 1); MgCl$_2$ at the same concentration had a somewhat smaller effect. EGTA failed to elicit both formation of a visible intermediate-density band and removal of
Plasma membrand vesicle associations in yeast

Fig. 7. Effect of calcium ion concentration on formation of in vitro associations between crude plasma-membranes and vesicles from *S. cerevisiae*NCYC 366. Incubation mixtures (2-5 ml) consisted of buffered mannitol (0-3 M, pH 7-2) containing crude plasma-membrane preparation (2-5 mg protein) and vesicles (5 units). Mixtures were supplemented with CaCl₂ at the concentrations indicated, and formation of plasma membrane-vesicle associations followed as indicated in the legend to Fig. 3; the incubation time was 4 h. ○, Size of the association band (A₄₅₀ × vol. in ml); ●, number of vesicle units utilized; □, size of the membrane pellet (A₄₅₀ × vol. in ml).

Table 1. Effect of divalent cations and EGTA on recovery of vesicles from incubation mixtures containing purified plasma-membranes and vesicles from *Saccharomyces cerevisiae*NCYC 366

<table>
<thead>
<tr>
<th>Gradient fraction</th>
<th>Vesicles (A₄₅₀ × vol. in ml)</th>
<th>Plasma membranes (A₄₅₀ × vol. in ml)</th>
<th>Plasma membrane + vesicles supplemented with:</th>
</tr>
</thead>
<tbody>
<tr>
<td>VESICLE</td>
<td>7-97</td>
<td>0-03</td>
<td>Nothing, EGTA, Ca²⁺, Mg²⁺</td>
</tr>
<tr>
<td>PELLET</td>
<td>0-0</td>
<td>6-54</td>
<td></td>
</tr>
</tbody>
</table>

Purified plasma-membranes and vesicles were obtained as described in Methods. Incubation mixtures (2-0 ml), which consisted of buffered mannitol (0-3 M, pH 7-2) containing purified plasma-membrane (1-0 mg protein) and vesicles (10-0 units), were supplemented as indicated, at 10 mM. Mixtures were incubated with gentle shaking (60 r.p.m.) at 35 °C for 4 h. Incubation mixtures were then fractionated using the gradient employed to separate membranes and vesicles from spheroplast lysates (see Methods). Vesicles were removed from the top of the gradient, and the number of vesicle units measured. The fraction corresponding to the intermediate-density band, where visible, was removed with a hypodermic syringe, and the absorbance contribution included with that of the pellet. The size of the pellet was determined by suspending it in a known volume of buffered mannitol, and measuring its A₄₅₀ value after appropriate dilution. The values quoted are averages of three separate experiments. It was also established that 10 mM is an optimum concentration for the effect of the supplements.

vesicles (Table 1). On a protein basis, vesicles contained somewhat more Ca²⁺ [5-3 μg (mg protein)⁻¹; two determinations] than purified plasma-membranes [2-5 μg Ca²⁺ (mg protein)⁻¹; two determinations]; the value for crude plasma-membranes was 0-57 μg Ca²⁺ (mg protein)⁻¹.

Properties of vesicles

When populations of vesicles, after removal from a sucrose density gradient, were diluted 20-fold into either buffered mannitol or water, the absorbance of the diluted suspensions differed by only 15%. Further dilution into buffered mannitol of each of these diluted suspensions gave suspensions with identical absorbance values. The polyphosphate content of vesicles was 1-61 ± 0-62 μmol orthophosphate equivalent (mg vesicle protein)⁻¹ (five determinations).
DISCUSSION

A close association between vesicles and plasma membrane at the neck of budding *S. cerevisiae* (Sentandreu & Northcote, 1969; Wiemken et al., 1970) is recognized as a vectorially important process in envelope growth in this and other eukaryotic (Rosenberger, 1979) microorganisms. However, the present paper is the first to report isolation from a eukaryotic microorganism of a plasma membrane/intracellular vesicle association. The *in vitro* associations isolated are not thought to be artefacts formed during the isolation procedure. They were obtained from spheroplast lysates by a procedure in which the temperature was not allowed to rise above 4 °C; studies on *in vitro* plasma membrane/vesicle associations showed that these are not formed in visible amounts in 4 h at temperatures below 20 °C. Moreover, there would appear to be strong bonding between the plasma membrane and vesicles in *in vivo* associations, since they remained intact even after being submitted to two strong centrifugal forces during isolation. Nevertheless, it is unlikely that the *in vivo* associations isolated are identical with those present in intact organisms, since some damage, no matter how slight, must have been incurred during isolation, especially when vesicles were in the early stages of associating with plasma membrane.

Several workers have isolated vacuoles (Indge, 1968a; Schwencke, 1977) and vesicles (Clausen et al., 1974; Schaffner & Matile, 1981) from *S. cerevisiae*. However, few of these workers have commented on the possibility that their preparations contained both types of subcellular organelle, and only Cartledge et al. (1977) fractionated populations of subcellular organelles into size classes. The vesicles isolated in the present study would appear to be the same as those in the unfractionated preparation isolated by Cartledge et al. (1977), and were probably identical with the spherosomes described by Schaffner & Matile (1981), mainly on the basis of their similarity in size (approximately 0.5-0.7 μm diam.). Electron micrographs indicated that vesicle preparations contained a small proportion of vacuoles, and also (for the first time) that they did not stain uniformly and therefore may be heterogeneous in structure. Further evidence that vesicle populations contained few vacuoles came from the discovery that they were only slightly osmotically sensitive (Cartledge et al., 1977) and had a low content of polyphosphate. The polyphosphate content of vesicles isolated in the present study was 1-61 μmol orthophosphate equivalent (mg protein)⁻¹ compared with an approximate value of 50 μmol (mg protein)⁻¹ as calculated from the data reported by Dürr et al. (1979).

*In vitro* associations formed between isolated plasma-membranes and vesicles seemed to vary considerably in composition, since they banded extensively on either side of the visible band isolated from density gradients. However, the bulk of the *in vitro* associations banded at the same sucrose density as the *in vivo* fraction. Formation of *in vitro* associations appeared to be a physiological process in view of its temperature dependence, while protein synthesis would not appear to be necessary for their formation. Our inability to detect differences in the staining properties of vesicles in both *in vivo* and *in vitro* associations might indicate that stainable material is lost from vesicles after they become associated with plasma membrane.

Assuming that fusion of plasma membranes with vesicles is involved in envelope growth in *S. cerevisiae*, it is appropriate to consider the manner in which this process is spatially directed. One possibility is that vesicles are directed towards the sites of envelope growth by cytoplasmic microtubules, for Byers & Goetsch (1975) showed that in *S. cerevisiae* these microtubules radiate from the spindle pole body complex towards the site of bud emergence. A role for microtubular material attached to plasma membranes or vesicles in formation of *in vitro* associations cannot be excluded. Methylbenzimidazol-2-yl-carbamate (Quinlan et al., 1980), which was without effect on formation of *in vitro* associations, as well as related benzimidazole carbamates (Hoebke et al., 1976), owe their inhibitory effect on growth of *S. cerevisiae* to their ability to inhibit microtubule formation rather than to depolymerize stable microtubules.

Calcium ions have been shown to be necessary for fusion of a wide variety of biological membranes (Papahadjopoulos et al., 1979), and specifically for fusion of secretory vesicles with animal-cell plasma membranes (Gratzl et al., 1980). It was not therefore surprising to discover that Ca²⁺ stimulated formation of associations between crude plasma-membrane preparations and vesicles. The ability of these subcellular organelles to form associations in suspensions not
supplemented with Ca\(^{2+}\) was presumably attributable to the presence of this ion, possibly together with synergistic ions such as Mg\(^{2+}\) (Gratziel et al., 1980), in membranes and/or vesicles. While concentrations up to 5 mM-Ca\(^{2+}\) caused an increase in the size of the intermediate-density band, higher concentrations tended to induce formation of membrane/vesicle associations of greater density than those which accumulated in the intermediate-density band, and sufficiently dense to accumulate in the membrane-containing pellet. Failure to demonstrate formation of membrane/vesicle associations when purified plasma-membranes and vesicles were incubated appears at first sight to be attributable to loss of Ca\(^{2+}\) and possibly other cations in the additional processing of crude plasma-membrane preparations required to obtain purified membranes. This view is supported by the finding of a threefold decrease in the protein:Ca\(^{2+}\) ratio in purified as compared with crude plasma-membranes, and the very low ratio in vesicles. There is also the possibility that other components required for formation of associations may have been lost from preparations of purified plasma-membranes. Endorsement for this view comes from the discovery that suspensions of purified plasma-membrane preparations and vesicles, when supplemented with Ca\(^{2+}\) or Mg\(^{2+}\), failed to produce associations that banded as an intermediate-density band. However, there is evidence that purified plasma-membranes became associated with vesicles in a manner that gave rise to associations with a density such that they sedimented with the membrane-containing pellet. Such an alteration in the properties of plasma membrane/vesicle associations might result from loss from plasma membranes of proteins that regulate the extent to which vesicles become associated with them.

We thank the Science and Engineering Research Council (U.K.) for supporting D. S. T., under grant GR/A/41564, and for providing funds to purchase electron microscopes. We are grateful to the Leverhulme Trust for a fellowship awarded to P. A. H. We also acknowledge preliminary research carried out on this topic by Adelina A. Goodall, supported by S.E.R.C. research grant B/RG/73016. Valuable advice and technical assistance were provided in electron microscopy by John Forsdyke, Kate Powell and Doreen Crellin. W. J. D. Whish kindly helped with DNA analyses.

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