**Abstract.** We have developed a purification procedure for the isolation of constitutive post-Golgi secretory vesicles from *Saccharomyces cerevisiae*. Although the post-Golgi stage of the secretion pathway is normally very rapid, we have used a temperature-sensitive secretory mutant, sec 6-4, to greatly expand the population of secretory vesicles. Following invertase as a marker, intact vesicles are enriched 36-fold from the crude lysate. The final preparation contains few contaminants as assessed by morphologic and biochemical examination. Three proteins (110, 40-45, and 18 kD) co-purify with the vesicle marker enzyme invertase. Metabolic labeling experiments indicate that these vesicle-associated proteins are synthesized during the period of vesicle accumulation. They are not apparent in the corresponding fractions from wild-type cells. Analysis of these proteins indicates that the 110-kD protein is a major glycoprotein residing in the vesicle membrane, while the 40-45- and 18-kD proteins are not glycosylated and are firmly associated with the vesicle membrane, each with at least one domain exposed on the cytoplasmic surface.

The process of protein secretion in eukaryotic cells has been studied by a variety of approaches. The secretory pathway was first defined morphologically (Jamieson and Palade, 1967a, b; 1968a, b) in polarized cell types specialized for regulated secretion. Pulse-chase experiments established the pathway of protein transport from the endoplasmic reticulum to the cell surface. Characterization of the biochemical steps involved in the processing of secretory products has demonstrated the localization of these processing events to specific compartments of the secretory machinery (Dunphy and Rothman, 1985; Orci et al., 1985). However, these studies have not revealed the molecular mechanisms underlying protein transport. Efforts to reconstruct portions of the secretory pathway in vitro have resulted in the purification of two components necessary for the targeting of nascent proteins to the endoplasmic reticulum membrane (Walter et al., 1984), and may address the mechanisms of subsequent export steps (Balch et al., 1984). Genetic analysis of the secretory apparatus in the yeast *Saccharomyces cerevisiae* has defined 25 genes whose products are essential for protein transport (Novick and Schekman, 1979; Novick et al., 1980; Ferro-Novick et al., 1984). Temperature-sensitive mutations in these SEC genes block the pathway at various stages. The sec mutants have been used to demonstrate that the components of the secretory pathway in yeast are similar to the machinery found in higher eukaryotes (Novick et al., 1981). Furthermore, the SEC gene products were found to be responsible for the delivery of all secreted proteins to the cell surface and for delivery of membrane proteins and vacuolar enzymes (Novick and Schekman, 1983; Stevens et al., 1982) to their destinations within the cell.

Two major pathways of protein secretion, referred to as regulated and constitutive, have been defined (Palade, 1975; Kelly, 1985). Regulated secretion refers to a pathway in which there is an intracellular accumulation of the secreted proteins in granules. These granules release their contents by exocytosis in response to a stimulus. The half-time of secretion for proteins released in this manner can take several days. Because granules are accumulated to high levels in the absence of a stimulus, they can be purified from unstimulated cells. Secretory granules from a variety of exocrine glands (Cameron et al., 1986), granules from pituitary tumor cells (Gumbiner and Kelly, 1981), and synaptic vesicles from the elasmobranch electric organ (Carlson et al., 1978), and mammalian brain (Nagy et al., 1976) are a few examples. Biochemical characterization of the vesicle membranes has led to the identification of a number of proteins which may play a role in the regulated fusion of the granules with the plasma membrane (Wagner and Kelly, 1979; Hutten et al., 1983; Jahn et al., 1985). It will be important to compare constitutive secretory vesicles with granules from regulated pathways to understand the similarities and differences in these processes. Because constitutive secretory vesicles are not accumulated, biochemical analysis of isolated vesicles has not yet been possible.

Secretion in the yeast *Saccharomyces cerevisiae* is a rapid, constitutive process. For example, the transit time for the glycoprotein invertase from synthesis to release into the periplasmic space is ~3 min (Novick et al., 1981) and does not require an external stimulus. For this reason, components of the secretory machinery are not usually prominent by electron microscopy of wild-type yeast cells. However, the sec mutants have been used to identify the components...
of the secretory pathway (Novick and Schekman, 1979; Novick et al., 1980). These mutants accumulate glycoproteins as well as one of three membrane-bound secretory organelles. Mutations in sec genes result in the accumulation of post-Golgi secretory vesicles when the mutant strains are incubated at the restrictive temperature, 37°C. This phenotype makes it possible not only to visualize but also to isolate constitutive secretory vesicles from these cells. Many of these sec mutants are reversible, and upon return of the cells to the permissive temperature the accumulated vesicles can complete the exocytotic pathway. This implies that the accumulated vesicles are functional organelles.

In many cells secretion is polarized; certain proteins are delivered to a particular portion of the cell surface. Polarized secretion has perhaps been best studied in epithelial cells where the apical and baso-lateral domains are well defined (Rodriguez-Boulan and Pendergast, 1980). In yeast as well, secretion is a polarized event as the release of secreted proteins and the incorporation of newly formed cell surface components are localized to the bud region of the cell. The events that take place in the delivery of a secreted protein to the plasma membrane via vesicular carriers must, therefore, include transport, recognition, and fusion events. Proteins exposed on the cytoplasmic face of the secretory vesicles may play a role in these events. This would allow interaction between the carrier organelles and other elements of the secretory apparatus; e.g., the plasma membrane, the cytoskeleton, or other components of the cytoplasm. To study the mechanisms of transport and fusion, characterization of the vesicles included in these events will be necessary.

We have taken advantage of the conditional phenotype of the vesicle-accumulating sec 6-4 mutant to isolate functional secretory vesicles of S. cerevisiae. We have based our purification on the observation that this mutant accumulates a high concentration of vesicles of uniform size. Morphological and biochemical criteria indicate that the preparation contains few contaminating organelles. We have identified three proteins which appear to be specific to the isolated constitutive secretory vesicles and have demonstrated that two of them have domains which reside on the cytoplasmic side of the vesicle membrane.

Materials and Methods

Strains, Media, and Reagents

The Saccharomyces cerevisiae strains used in these experiments were NY17 (a, ura 3-52, sec 6-4) and NY13 (a, ura 3-52). YP medium contained 1% Bacto-yeast extract and 2% Bacto-peptone (Difco Laboratories, Inc., Detroit, MI). Glucose was used as the carbon source at either 2% (rich medium, YPD) or 0.2% (low glucose). To change growth medium cells were pelleted and resuspended in fresh pre-warmed medium. The absorbance of cell suspensions was measured in a 1-cm quartz cuvette at 599 nm in a model No. 25 spectrophotometer (Beckman Instruments, Inc., Palo Alto, CA).

Chemicals for PAGE were obtained from Bio-Rad Laboratories (Richmond, CA). Glucose oxidase and Proteinase K were from Boehringer Mannheim Biochemicals (Indianapolis, IN). Zymolyase 100T was from Miles Scientific (Naperville, IL). Sephacryl S-1000 was obtained from Pharmacia Fine Chemicals (Piscataway, NJ). [35S]Methionine was from Amersham Corp. (Arlington Heights, IL). Spurr embedding medium for preparation of samples for electron microscopy was obtained from Polysciences, Inc. (Warrington, PA). Trypsin type III, trypsin inhibitor type III-O, cytochrome c type III, NADPH, p-nitrophenyl-α-O-mannopyranoside, fluorescamine, o-dianisidine, peroxidase, phenylmethyisalicyl fluoride (PMSF), pyruvate kinase type II, NaBH₄, ATP, phosphonate pyruvate, p-nitrophenol, p-semidine hydrochloride, sorbitol, triethanolamine, EDTA, Triton X-100, Triton X-114, and Lubrol WX were obtained from Sigma Chemical Co. (St. Louis, MO).

Growth of Cells and Fractionation

Cells were grown in 800 ml YPD medium to early exponential phase at 25°C in a shaking water bath. Cells, 1600 OD₅₉₉ units, were pelleted and resuspended in YP + 0.2% glucose warmed to 37°C. Incubation was continued for 2 h and then the cells were pelleted, washed with cold 10 mM NaCl, and resuspended in 15 ml of 10 mM NaCl. An equal volume of spheroplast medium was added such that the buffer contained 1.4 M sorbitol, 50 mM KPi, pH 7.5, 10 mM NaCl, 40 mM β-mercaptoethanol, and 2 mg of zymolyase. The cells were incubated at 37°C for 45 min with occasional gentle mixing. Spheroplasts were pelleted in a table-top clinical centrifuge for 5 min and resuspended in 25 ml of cold 0.8 M sorbitol in 10 mM triethanolamine, 1 mM EDTA, adjusted to pH 7.2, with acetic acid (TEA). The spheroplasts were transferred to a 40-ml Dounce homogenizer (Wheaton Scientific, Millville, NJ) on ice and lysed with 20 strokes of the pestle. Low-speed centrifugation at 10,000 g in an SS-34 rotor (Sorvall, Wilmington, DE) for 15 min yielded Pi, a large pellet containing unlysed cells, cell wall debris, nuclei, and mitochondria. The postmitochondrial supernatant, SI, was then centrifuged at 100,000 g for 1 h in a 70Ti rotor (Beckman Instruments, Inc.) to yield a small clear microsomal pellet, P2, and the supernatant, S2. P2 was resuspended in 600 μl of 0.8 M sorbitol/TEA and loaded onto a 1.5 cm × 90 cm Sephacyr S-1000 gel filtration column at 4°C. Material was eluted in 0.8 M sorbitol/TEA at a flow rate of 9.2 ml/h, and 80 drop fractions (4 ml) were collected. The peak invertase fractions were pooled and pelleted at 100,000 g for 1 h.

Metabolic Labeling, Electrophoresis

Two protocols were followed for radiolabeling the cells. For the prelabel protocol, 10 ml of culture were removed during growth at 25°C and incubated with 0.5 mCi [35S]methionine at 25°C for 2.5 h. The cells were pelleted and resuspended in fresh YPD supplemented with 0.2 mg unlabeled methionine for 0.5 h. The large culture and the labeled culture were then pelleted and shifted to 37°C in low-glucose medium for 2 h and fractionation was carried out as described. For the shift-label protocol, 10 ml of the large culture was removed at the time of the shift to 37°C and incubated with 0.5 mCi [35S]methionine for the duration of the 2-h shift. Fractionation was carried out as described. To label glycoproteins a 10-ml aliquot of the culture was incubated with 1 mCi [3H]mannose during the shift.

For SDS-PAGE, samples were heated for 3-5 min in a 100°C heat block in sample buffer containing 2% SDS and 0.5% 2-mercaptoethanol. Electrophoresis was performed by the Laemmli (1970) procedure on 9, 10, or 12% slab gels. Molecular weight standards were from Sigma Chemical Co. (St. Louis, MO) (SDS-6H). Gels were stained for protein with Coomassie Blue and destained in 10% acetic acid. For fluorography, destained gels were rinsed in 10% MeOH, soaked in 1 M salicylate in 10% MeOH, pH 7.0, and exposed to Kodak X-omat film.

Protease Protection Experiments

Vesicles were isolated from shift-labeled cells. The pooled vesicles were pelleted at 100,000 g and resuspended in 1 ml 0.8 M sorbitol/TEA. Samples containing 100-200 μg of protein in a total volume of 35 μl were incubated for 1 h on ice with either trypsin or proteinase K in the presence and absence of Triton X-100. Trypsin concentrations ranged from 2 to 250 μg/ml. Proteinase K concentrations ranged from 0.5 to 20 μg/ml. Triton X-100 was used at 1, 0.1, and in the experiments shown, 0.05%. Trypsin inhibitor, 1 μl of 10 mg/ml, or PMSF, 1 μl of 10 mM, were added on ice and samples were boiled in sample buffer.

To monitor the integrity of the vesicles during protease treatment, the accessibility of invertase to sucrose was used as an assay for lysis of the vesicles. Reactions were prepared exactly as those described above for analysis by SDS-PAGE. After 1 h the reactions were stopped with inhibitor. Aliquots were then taken to measure invertase activity. For each sample invertase was measured in 50 mM NaAc, pH 5.1, with 0.8 M sorbitol for osmotic support in the presence and absence of 0.1% Triton X-100. The invertase activity was measured with Triton X-100 in the buffer represents the total activity in the vesicle sample. The ratio of the total activity measured (lysed) to the activity measured without detergent in the buffer (intact), indicates the proportion of the vesicles which are intact or impermeable to sucrose. By determining the ratio with and without protease treatment we can determine the percent.
Purification of Vesicles from sec6-4

Grow cells at 25°, in 2% glucose

→ Shift to 37°, in 0.2% glucose

→ Spheroplast in 4M sorbitol

→ Lyse cells in 8M sorbitol, dounce

→ Low speed spin, 10K G, 10 min.

Load on Sephacryl S-1000 Column

→ Assay for invertase and Pool Peak Fractions

Figure 1. Outline of the isolation procedure for obtaining constitutive secretory vesicles from sec 6-4 cells.

We have used the secretory protein invertase to follow the age of vesicles that remain intact during protease treatment. Total invertase activity is not affected by the concentrations of trypsin or proteinase K used in these experiments.

Extractions of Vesicles

Shift-labeled cells were fractionated as described and the vesicle fractions were pooled from the column. The pool was split into 16 aliquots containing 200 μg protein and the vesicles were pelleted at 100,000 g for 1 h in a 50Ti rotor (Beckman Instruments, Inc.). Each pellet was resuspended in 200 μl of one of the following buffers: TEA (no osmotic support), 1 M NaCl in 0.8 M sorbitol/TEA, 1% Triton X-100 in 0.8 M sorbitol/TEA, 100 mM Na2CO3, pH 11.5, or 6 M urea in 0.3 M sorbitol/TEA. After incubation on ice for 30–60 min, samples were centrifuged for 1 h at 100,000 g. Supernatants were saved and pellets were resuspended in 0.8 M sorbitol/TEA. All samples were then boiled in sample buffer. For phase-separation in Triton X-114, the initial pellet was resuspended in 50 μl of 0.8 M sorbitol/TEA and 50 μl of 2X Triton X-114 buffer was added such that the final buffer contained 1% Triton X-114, 150 mM NaCl, 0.8 M sorbitol/TEA. After 2 min on ice the sample was placed at 30°C for 1 min. The sample was then diluted with 100 μl of water and spun for 1 min in a microfuge to separate the detergent and the aqueous phases. The aqueous phase was removed and spun at 100,000 g, and the resulting pellet resuspended in 200 μl of 0.8 M sorbitol/TEA. 0.8 M sorbitol/TEA was added to the detergent phase to bring the volume up to 200 μl.

Protein and Enzyme Assays

Protein was measured using fluorescamine as described by Udenfriend et al. (1972), with BSA as standard. Invertase was assayed for 3 min at 37°C as described by Goldberg et al. (1975) except as described in the protease protection experiments. Vanadate-sensitive plasma membrane ATPase was assayed according to the method described by Bowman and Slayman (1979). The assay for α-mannosidase followed the method of Talusini et al. (1977) with the modifications that 0.8 mM p-nitrophenyl-α-mannopyranoside was used and the pH of the buffer was 6.5, based on the assay described by Opheim (1978). The method described by Kreibich et al. (1973) was used to assay NADPH cytochrome c reductase. Cytochrome c oxidase was assayed based on the method of Mason et al. (1973).

Electron Microscopy

Thin-section analysis of intact cells was performed as follows. Liquid cultures were grown to early log phase in YPD, then shifted to YP + 0.2% glucose media pre-warmed to 37°C. After 2 h, ~5 × 10⁸ cells were harvested by filtration onto nitrocellulose, washed on the filter with 10 ml of 0.1 M cacodylate, pH 6.8, and immediately resuspended in 8 ml of a solution containing 0.1 M cacodylate and 3% glutaraldehyde. After a 2-h fixation at 25°C the cells were collected by centrifugation in a clinical centrifuge and washed twice with 50 mM potassium phosphate, pH 7.5, and resuspended in 2 ml of phosphate buffer containing 0.125 mg of Zymolyase 100T. After digestion of the cell wall for 40 min at 30°C, the cells were washed twice in cacodylate buffer and resuspended in cacodylate buffer containing 2% OsO4 at 0°C. After a 1-h incubation the cells were washed twice in cacodylate buffer and resuspended in cacodylate buffer containing 2% OsO4 at 0°C. After a 1-h incubation the cells were washed three times in H2O then incubated in 2% aqueous uranyl acetate. The cells were washed twice with H2O and then formed into agar blocks. The blocks were dehydrated in ethanol and embedded in Spurr media. Thin sections were cut, stained with lead citrate and UrAc, and viewed in an electron microscope at 80 kV.

Thin-section analysis of membrane fractions was performed as follows. An aliquot of the pooled peak fractions from the Sephacryl column was fixed by addition of 0.15 M cacodylate, pH 7.2, 1% paraformaldehyde, and 0.8% glutaraldehyde (final concentrations). After 1 h in suspension at 4°C, the vesicles were pelleted at 100,000 g for 1 h. The pellet was allowed to fix in situ for 30 min and then it was washed three times with cacodylate buffer. The pellet was treated with 1% OsO4 in cacodylate buffer at 4°C for 1 h, washed three times with 0.15 M NaCl, and stained with 0.5% MgUrAc in 0.15 M NaCl. After washing the pellet three times in 0.15 M NaCl, it was dehydrated in graded acetone and embedded in Spurr media. Thin sections were cut, stained with lead citrate and UrAc, and viewed in an electron microscope at 80 kV.

Results and Discussion

Isolation of Secretory Vesicles

To study the properties of constitutive secretory vesicles, we have devised an isolation procedure using a vesicle accumulating secretory mutant, sec 6-4. We have chosen this mutant because it has essentially wild-type properties at 25°C, yet transport of all known surface proteins is tightly blocked at 37°C (Novick al., 1980). This mutant is also reversible in the absence of continued protein synthesis. One half of the accumulated invertase can be released by exocytosis if the culture is returned to the permissive temperature. While sec 1-1 has been characterized in somewhat greater detail it is not quite as reversible as sec 6-4 (Novick et al., 1980).

We have used the secretory protein invertase to follow the purification of the vesicles. This enzyme is under hexose repression. After a shift from repressing media (2% glucose) to derepressing media (0.2% glucose) synthesis of invertase...
begins within 15 min. In sec 6-4 cells at 37°C the invertase accumulates intracellularly, compartmentalized in secretory vesicles. Invertase can therefore be used as a biochemical marker for intact vesicles throughout the purification protocol.

The fractionation procedure was designed to separate intact vesicles from other organelles (Fig. 1). An 800-ml culture of NY17 (sec 6-4) is grown at 25°C in YPD to early logarithmic phase. The cells are then transferred to YP medium containing 0.2% glucose at 37°C to simultaneously derepress invertase synthesis and impose the secretory block. After 2 h the cells are pelleted and washed in cold 10 mM NaN₃. The cell walls are removed enzymatically in buffer containing 1.4 M sorbitol to protect the spheroplasts from hypotonic lysis. We have followed the lysis procedure developed by Makarow (1985) for the isolation of intact yeast vacuoles. Makarow has demonstrated that spheroplasts will lyse at sorbitol concentrations as high as 0.8 M if the buffer contains triethanolamine, and that this high concentration of sorbitol stabilizes the osmotically sensitive vacuoles. Based on these observations, we resuspended the spheroplasts in ice-cold TEA containing 0.8 M sorbitol. After homogenization with 20 strokes of a Dounce homogenizer, the cell homogenate is centrifuged at 10,000 g for 10 min to remove unlysed cells, cell wall debris, nuclei, and mitochondria. The supernatant from this centrifugation (S₁) is spun at 100,000 g

Figure 2. Electron micrographs of intact cells and of purified constitutive secretory vesicles. (a–c) Wild-type (NY13) and sec 6-4 (NY17) cells were shifted to 37°C in YP + 0.2% glucose for 2 h, then fixed for electron microscopy. (a) Wild-type cells. Arrows point to secretory vesicles. Bar, 500 nm. (b) sec 6-4 cells (NY17) at the same magnification as in a. (c) Portion of a sec6-4 cell in b, at higher magnification. Bar, 100 nm. (d) Representative micrograph of the vesicle pool from the Sephacryl column shown at the same magnification as the micrograph in c.
Fraction Protein Invertase activity purification

| Fraction | Protein (mg) | Lysate | P1 | S1 | P2 | S2 | Pool |
|----------|-------------|--------|----|----|----|----|------|
| Lysate   | 724         | 1070   | 1.48 |
| P1       | 483         | 545    | 1.13 |
| S1       | 310         | 660    | 2.13 |
| P2       | 17.5        | 409    | 23.4 |
| S2       | 335         | 230    | 0.69 |
| Pool     | 3.05        | 164    | 53.8 |

Abbreviations are as described in Materials and Methods.

Table I. Invertase Distribution during Vesicle Purification

| Fraction | Specific activity | Fold purification |
|----------|-------------------|-------------------|
| Lysate   | 1.813             | 100               |
| P1       | 100               | 28                |
| S1       | 100               | 57                |
| P2       | 100               | 69                |
| S2       | 100               | 34                |
| Pool     | 100               | 15                |

Table II. Distribution of Marker Enzymes in Cell Fractionation of sec 6-4

| Fraction | Invertase* | PM ATPase† | NADPH cyt c red§ | Cyt c oxidase‖ | α-Mannosidase¶ |
|----------|------------|------------|------------------|---------------|---------------|
| Lysate   | 1.813      | 100        | 28               | 3.5           | 100           |
| P1       | 661        | 36         | 16               | 2.4           | 69            |
| S1       | 1,115      | 62         | 9.5              | 0.90          | 26            |
| P2       | 640        | 35         | 4.1              | 0.28          | 8.0           |
| S2       | 370        | 20         | 5.8              | 0.33          | 9.4           |
| POOL     | 320        | 18         | 1.1              | 0.019         | 3.9           |

Abbreviations for fractions are as described in Materials and Methods.

Units of activity for each enzyme are expressed as follows.

* Invertase: pmol glucose produced per minute per fraction.
† PM ATPase: pmol phosphate produced per minute per fraction.
§ NADPH cytochrome c reductase: pmol cytochrome c reduced per minute per fraction.
‖ Cyt c oxidase: pmol cytochrome c oxidized per minute per fraction.
¶ α-Mannosidase: pmol p-nitrophenyl-α-d-mannopyranoside hydrolyzed per minute per fraction.

Table M. Invertase Distribution during Vesicle Purification

| Fraction | Protein (mg) | Lysate | P1 | S1 | P2 | S2 | Pool |
|----------|-------------|--------|----|----|----|----|------|
| Lysate   | 724         | 1070   | 1.48 |
| P1       | 483         | 545    | 1.13 |
| S1       | 310         | 660    | 2.13 |
| P2       | 17.5        | 409    | 23.4 |
| S2       | 335         | 230    | 0.69 |
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| S2       | 370        | 20         | 5.8              | 0.33          | 9.4           |
| POOL     | 320        | 18         | 1.1              | 0.019         | 3.9           |

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‖ Cyt c oxidase: pmol cytochrome c oxidized per minute per fraction.
¶ α-Mannosidase: pmol p-nitrophenyl-α-d-mannopyranoside hydrolyzed per minute per fraction.

To characterize the composition of the constitutive secretory vesicles we need to obtain a pure vesicle population. We have used morphological and biochemical criteria to demonstrate that we have successfully enriched for secretory vesicles in our preparations while minimizing contaminants.

To assess the effectiveness of the fractionation, the enriched vesicle pool was examined by thin-section electron microscopy. The peak invertase fractions from the column were pooled, and an aliquot was removed for electron microscopic study. The pool sample was fixed in suspension on ice for 1 h in cacodylate/paraformaldehyde/glutaraldehyde and the vesicles were then pelleted at 100,000 g for 1 h. After osmium impregnation and staining with uranyl acetate, the pellets were embedded in Spurr and sectioned. Examination of the micrograph in Fig. 2 d reveals that the pool contains a population of mostly closed vesicles of homogeneous size. The vesicles measure 95-120 nm in diameter and are bounded by a continuous bilayer. Some appear to contain an amorphous content while others seem to be devoid of content. When scanning a wide field at low power, a few membrane-enclosed particles are seen which are similar in size to the majority of the vesicles, but seem to have a more electron-dense content (not shown). We believe that the electron-dense structures are contaminating particles which co-purify with vesicles by virtue of their similar size. We do not know the nature of this contaminant, yet it appears to be a low percentage of the total material of the peak column fractions.

Clathrin-coated vesicles have been identified in yeast (Mueller and Branton, 1984), and the clathrin heavy chain gene has been cloned and disrupted (Payne and Schekman, 1985). Although disruption of the CHC1 gene is lethal in some strain backgrounds (Elizabeth Jones, personal communication), in other backgrounds this mutation is not lethal and in these cases invertase secretion is not blocked (Payne and Schekman, 1985). Vesicles bearing a nonclathrin coat have been implicated as bulk protein carriers through the Golgi apparatus (Orci et al., 1986). In yeast, we find that post-Golgi secretory vesicles have neither a clathrin nor a nonclathrin coat by thin-section analysis (Fig. 2 d). Negative staining (not shown) also indicates that the purified vesicles are smooth.

Because different enzyme activities are associated with specific cellular organelles, the concentration of various organelles in the cell fractions can be determined biochemically. The results of marker enzyme assays from a typical
fractionation are shown in Table II. The markers are as follows: cytochrome c oxidase is a marker for the inner membrane of the mitochondria (Mason et al., 1973); NADPH cytochrome c reductase marks the endoplasmic reticulum (Kubota et al., 1977); the membrane-bound enzyme α-mannosidase marks the vacuole (Opheim, 1978); vanadate-sensitive Mg$^{2+}$-ATPase is an integral membrane protein of the plasma membrane (Willsky, 1979); and the soluble transported enzyme invertase is used as the vesicle marker. Of the membrane marker, vanadate-sensitive ATPase, was present in S1 as 34% of the total activity and in P2 as 15% of the total activity. After column fractionation, there was still 3.9% of the plasma membrane activity associated with the pool. However, the possibility exists that the ATPase activity in the pool may represent newly synthesized enzyme which is on the vesicles in transit to the plasma membrane. This question is addressed below.

The efficacy of the column can be appreciated by assaying marker enzyme activities across the column fractions. Elution profiles for some of these enzymes are shown in Fig. 3. The vesicle marker invertase elutes as a single major peak reflecting the size homogeneity of the vesicles seen in the electron micrograph. A small peak of invertase activity eluting after the major peak is typically seen, which may represent soluble invertase released from lysed vesicles. When the vacuolar marker α-mannosidase was assayed in the fractionation procedure, 7.3% of the total activity was found in P2, but the column profile indicates that the vacuolar activity was separated into two peaks which elute on either side of the invertase peak. The plasma membrane ATPase activity elutes in two peaks, one before the invertase peak and one which coelutes with the vesicle fraction. To test the possibility that this activity resides with the vesicles rather than on contaminating plasma membrane vesicles, a comparable high-speed pellet from wild-type cells was prepared and fractionated on the column. Wild-type cells do not accumulate vesicles (see Fig. 2a) or secretory enzymes; therefore it is not relevant to assay invertase activity during fractionation. However, membranes from other organelles should be comparable in abundance to those from sec 6–4 cells since the wild-type strain is essentially isogenic to the sec 6–4 strain and was grown, shifted, and lysed exactly as was the mutant strain. The ATPase profile for wild-type cells indicates a peak of activity eluting in the same location as the first peak from a sec 6–4 preparation. The peak of ATPase activity seen coeluting with invertase during fractionation of sec 6–4 cells is absent in the corresponding position in the elution profile of wild-type cells. This suggests that the second ATPase peak in the mutant preparation represents enzyme on secretory vesicles which is in transit to the plasma membrane rather than contaminating pieces of plasma membrane. The profile of ATPase activity from sec 6–4 cells also shows a low level of activity trailing into the late fractions. This trailing activity is not seen in the profile from wild-type cells. This difference is reproducible and may result from more extensive fragmentation of the plasma membrane from the mutant cells.

This comparison of mutant and wild-type fractionation also supports our conclusion that the vesicle fraction is largely pure. The final panel of Fig. 3 shows a protein elution profile for the two strains of cells. There is a protein peak which cofractionates with invertase in the sec 6–4 preparation which is conspicuously absent in wild type. If we assume that the proteins present in the pooled fractions of wild-type cells are not components of secretory vesicles and that these proteins are also present in the same abundance in the fractions from sec 6–4 cells, but as contaminants, then we can conclude that the vesicle preparation is ~81% pure. These assumptions are supported by the analysis of the constituent proteins as described below.

**Identification of Vesicle Proteins**

To assess the protein composition of the column fractions, aliquots were boiled in SDS and the proteins were separated by SDS-PAGE. Fig. 4 is a Coomassie-stained gel of aliquots of column fractions from a sec 6–4 cell fractionation. When the invertase elution profile for that preparation is compared to the protein elution profile it is apparent that there are three bands which cofractionate with invertase. When the Coomassie-stained protein profile obtained from a wild-type fractionation (Fig. 5) is compared to the profile from a sec 6–4 fractionation, it is apparent that the bands which cofractionate with invertase in the sec 6–4 preparation are not present in the corresponding fractions from the wild-type preparation. In contrast, the side fractions from sec 6–4 and wild-type cells exhibit very similar protein profiles. We have assigned apparent molecular masses of 110, 40–45, and 18 kD to the proteins which cofractionate with invertase in the sec 6–4 profile. These bands are only present in fractions which contain invertase and are not apparent in other fractions from...
this column. The three bands that cofractionate with invertase and are not apparent in wild type will be referred to as vesicle proteins.

The conditional phenotype of the sec 6-4 mutant makes it possible to determine the time of synthesis of the vesicle proteins relative to the time of vesicle formation. We would like to use this information to distinguish between two possibilities regarding the association of these proteins with secretory vesicles. The first possibility is that the vesicle proteins are synthesized as soluble proteins which then associate reversibly with the vesicles. This type of behavior might be expected for a vesicle coat protein which undergoes multiple rounds of assembly and disassembly. The second possibility is that these proteins associate irreversibly with the vesicles soon after synthesis. We can address the first possibility by labeling cells with [35S]methionine during growth at 25°C and then chasing in unlabeled medium during the shift to the restrictive temperature, 37°C. If the vesicle proteins associate slowly or reversibly we should be able to pre-label them at permissive temperature, and then observe association with the vesicles accumulated during the chase period at the restrictive temperature. When fractionated, the labeled vesicle proteins would be enriched over other labeled proteins to the same degree as they appear to be enriched by Coomassie staining. If, however, these proteins associate with the vesicles quickly and irreversibly, then the labeled vesicle proteins would be relatively less prominent by labeling than by Coomassie staining. As can be seen in Fig. 6 A, the latter possibility held true. The relative labeling intensity of the three vesicle proteins was less than the relative amounts of those proteins detected by Coomassie staining (see Fig. 4). This would suggest that these proteins are either unstable or that they associate with vesicles quickly after synthesis.

A second labeling protocol was used to confirm this hypothesis. Cells were grown at 25°C without label and then shifted to 37°C with [3S]methionine in the medium. Newly synthesized (labeled) proteins which associate quickly with the newly formed secretory vesicles should be highly enriched in the purified vesicle preparation. As can be seen in Fig. 6 B, the 110-, 40–45-, and 18-kD bands are greatly enhanced by this labeling protocol. In the fractions eluting with the peak of invertase activity they are the major labeled bands. These results are not consistent with the presence of a large soluble pool of protein that can associate slowly with new vesicles. Rather, it suggests that these proteins are synthesized as the vesicles are formed. The proteins could be either passengers in transit to the cell surface or elements of the secretory machinery that associate quickly and irreversibly with the vesicles. To try to address these possibilities we have analyzed these proteins further.

To investigate the glycosylation state of these proteins, sec 6-4 cells were incubated with [3H]mannose for 1 h during the shift to 37°C and low glucose. Fig. 7 shows that in the high-speed pellet (P2), neither the 40–45- nor the 18-kD proteins incorporate detectable mannose. The 110-kD band does incorporate mannose, thus suggesting that it is a glycopro-
Figure 5. Coomassie-stained protein pattern for column fractions of a wild-type cell fractionation. Wild-type cells were fractionated and the high-speed pellet run on the Sephacryl S-1000 column. Aliquots (140 μl) of each column fraction were diluted with sample buffer (60 μl) and heated at 100°C for 5 min, and 75-μl aliquots of each sample were analyzed. P2 is an aliquot of the high-speed pellet at approximately the same dilution as the column fractions. The Coomassie Blue-stained gel is shown. The fractions in the profile which would correspond to the peak invertase fractions in sec 6-4 are marked as POOL.

Figure 6. Labeling of proteins with [35S]methionine. Aliquots, 10 ml of an 800-ml culture of NY17, were labeled with 0.5 mCi of [35S]methionine either before or during the 37°C shift. The aliquots were then combined with the larger cultures and the cells were collected and fractionated. (A) Pre-label. The trace culture was labeled for 2.5 h in YPD at 25°C, then pelleted and resuspended in fresh YPD supplemented with unlabeled methionine. Incubation at 25°C continued for 0.5 h and then both cultures were shifted to 37°C for 2 h in YP + 0.2% glucose. After fractionation, aliquots of each column fraction were removed, combined with sample buffer, and boiled for 5 min. Aliquots of 40 μl were then analyzed by SDS-PAGE and the gel was fluorographed. (B) Shift-label. Incubation of the trace culture began at the time of the shift to 37°C and low glucose. After fractionation 140-μl aliquots of each column fraction were removed, diluted with 60 μl sample buffer and boiled. Aliquots of 40 μl were separated by SDS-PAGE and the gel was fluorographed.
dication of the proportion of vesicles which are intact. The invertase activities measured in this experiment are shown in Table III. For samples not treated with protease, the ratio of invertase activity measured with 0.1% Triton X-100 in the buffer to invertase activity measured in buffer without detergent was 3:1. This ratio implies that either 1/3 of the vesicles are lysed within a given population or that the vesicles are somewhat permeable to sucrose. After trypsin treatment at 50 μg/ml or proteinase K treatment at 20 μg/ml (the highest concentrations of protease used in this experiment), this ratio was 2.8:1 and 2.9:1, respectively. Invertase itself is extremely resistant to protease as indicated by the fact that the total activity measured in each sample was not altered by the presence of protease. Therefore, we conclude that protease treatment does not significantly affect vesicle integrity.

Havmg established that the vesicles were largely intact during protease digestion, the samples were separated by SDS-PAGE and the gel was fluorographed. Examination of the fluorogram in Fig. 8 demonstrates that both the 40–45- and the 18-kD proteins have at least one domain that is accessible to trypsin in the absence of detergent. This implies that a portion of each protein resides on the cytoplasmic surface of the vesicle membrane. At 5 μg/ml trypsin the 40–45-kD band is reduced in molecular mass by 3 kD and incubation with trypsin at 20 or 50 μg/ml trypsin results in a further reduction for a total loss of 7 kD. The 18 kD-band shifts at a trypsin concentration of 2 μg/ml generating a second band at 16 kD in addition to the 18 kD-band. At 5 μg/ml and above, only the 16 kD-band is seen. The resistant fragments could be re-pelleted at 100,000 g, indicating that they are still associated with the vesicles. The 110-kD protein was not sensitive to trypsin at any concentration (up to 250 μg/ml; not shown) even in the presence of detergent.

The same experiments done with proteinase K instead of trypsin revealed additional information (Fig. 9). The 110-kD protein was sensitive to high concentrations of proteinase K, but only in the presence of Triton X-100, which would sug-

### Table III. Assay for the Integrity of the Vesicle Membrane after Protease Treatment

| Protease* | Triton X-100 | Invertase activity | Ratio† |
|-----------|--------------|--------------------|-------|
| µg/ml     | µg/ml        | U/ml               |       |
|           | Lysed        | Lysed              |       |
| Lysed:intact                     | 3:0:1       |
|           | 1.2:1        | 2.8:1              |
| Prot K 20 + 69.6 80.7 1.2:1     | 2.9:1       |
| Prot K 20 - 20.7 62.2 3.0:1     | 1.2:1       |
| Isolated vesicles were incubated in the presence and absence of trypsin and proteinase K at concentrations of up to 50 and 20 μg/ml, respectively. Invertase was only measured for the samples listed in the table, which represent the highest concentrations of protease used. Appropriate volumes of 0.8 M sorbitol/TEA were added to samples lacking protease or detergent to keep all volumes equivalent.
| Isolated vesicles were treated with protease in the presence and absence of Triton X-100, used at 0.05% in the experiments shown in this paper.
| Invertase measured in 50 mM NaAc, pH 5.1, with 0.8 M sorbitol for osmotic support, represents enzyme in an intact vesicle preparation which is accessible to sucrose.
| Total invertase activity measured by including 0.1% Triton X-100 in the assay buffer to lyse the vesicles.
| The ratio of total invertase activity to invertase measured in the absence of detergent indicates the proportion of vesicles which are lysed or permeable to sucrose. Therefore, at most, one-third of the vesicles are lysed.

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Figure 8. Trypsin sensitivity of vesicle proteins. A pool of shift-labeled vesicles was pelleted and resuspended in 1 ml of 0.8 M sorbitol/TEA. Aliquots of 25 μl containing 100–200 μg of protein were incubated on ice with 0–50 μg/ml of Trypsin. 10X trypsin stocks were made in 0.8 M sorbitol/TEA. Samples incubated with trypsin in the presence of detergent were first incubated with 0.05% Triton X-100 (also made as a 10X stock in 0.8 M sorbitol/TEA). To control for proteolysis by contaminating proteases two samples were incubated with trypsin in the presence of 100 μg/ml trypsin inhibitor, one each in the presence and absence of Triton X-100. After a 1-h incubation, trypsin inhibitor, 1 μl of 10 mg/ml, was added to all samples. Samples were then diluted with sample buffer (15 μl) and boiled for 5 min. Aliquots of each sample were run on two different percentage gels. The top gel is a 9% gel to cleanly separate the 40–45 kD-protein and its proteolyzed forms. The bottom panel is a portion of a 12% gel which clearly shows the 18-kD band.

**Association of the Vesicle Proteins with the Membrane**

A variety of extractions can be used to assess the membrane association of a protein. Soluble content proteins can be released if the vesicles are lysed by reducing the osmotic support. High salt/EDTA buffers have been used to strip extrinsic proteins and ribosomes from membrane preparations (Walter and Blobel, 1980). Proteins associated peripherally with a membrane through protein–protein interactions have been solubilized with urea (Steck and Yu, 1973) or high pH (11.3) (Howell and Palade, 1982). Integral membrane proteins are often solubilized with non-ionic detergents such as Triton X-100 or Triton X-114. Because the cloud point of Triton X-114 is about 20°C, separation of the detergent and aqueous phases after solubilization at 0°C has allowed many integral membrane proteins which partition into the detergent phase to be effectively separated from hydrophilic proteins (Bordier, 1981).

Shift-labeled vesicles were isolated and the pool was divided into 16 aliquots, each containing 200 μg of protein.

| Band   | No sorbitol* | 1 M NaCl | 6 M Urea | Na₂CO₃ | 1% Triton X-100 | 1% Triton X-114 |
|--------|--------------|----------|----------|--------|-----------------|-----------------|
| 110 kD | P            | P        | S        | S      | S               | S               |
| 40–45 kD | P          | P        | P        | P      | P               | P               |
| 18 kD  | P            | P        | P        | P      | P               | P               |

* Extraction conditions are described in Materials and Methods.

† S and P refer to the presence of the band in the 100,000 g supernatant or pellet after treatment with the various buffers.

‡ After Triton X-114 phase separation, the aqueous phase was centrifuged at 100,000 g.

Table IV. Behavior of Vesicle Proteins after Extractions

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The vesicles were pelleted at 100,000 g and resuspended in one of a variety of buffers. After treatment, the samples were again centrifuged at 100,000 g to separate soluble and insoluble fractions. Each sample was then boiled in sample buffer for 5 min and separated by SDS-PAGE, and the resulting gel was fluorographed. When pertinent, invertase was measured in the supernatant and pellet to determine if the vesicles were pelleted as intact entities or if they had been lysed.

The results of various extraction procedures are summarized in Table IV. In each case S and P refer to the presence of the protein in the 100,000 g supernatant or pellet fraction, respectively. Content proteins were released by resuspending the vesicles in TEA buffer without sorbitol for osmotic support. Invertase and the 110-kD protein were both found in the supernatant. The 40-45 kD- and the 18-kD proteins were found in the pellet. To strip the vesicles of extrinsic proteins, they were resuspended in 0.8 M sorbitol/TEA with 1 M NaCl. With this buffer, invertase and the 110-, 40-45- and 18-kD proteins all were found in the pellet. In attempts to solubilize peripherally associated proteins, the vesicles were resuspended in 6 M urea in TEA with 0.3 M sorbitol, or 100 mM Na₂CO₃, pH 11.5. In both cases, the 110-kD protein was released into the supernatant, but both the 40-45- and the 18-kD proteins pelleted. To solubilize membrane proteins, the vesicles were resuspended in 1% Triton X-100 in 0.8 M sorbitol/TEA. The 110-kD protein was released into the supernatant, but the 40-45- and 18-kD proteins were still pelletable. Solubilization of the vesicles with 1% Triton X-114 followed by phase separation caused all three proteins to partition in the aqueous phase. After centrifugation of the aqueous phase, the 110-kD protein was in the supernatant and the 40-45- and the 18-kD proteins were in the pellet.

This set of results allows us to conclude that the 110-kD protein is a transported, soluble glycoprotein. It is labeled with [³H]mannose during the shift when transport vesicles are made; it is protected from protease digestion in the absence of detergent; and it is released from the vesicles upon lysis. The association of the 40-45- and the 18-kD proteins with the vesicles is more difficult to assess. Both must have a domain which resides on the cytoplasmic side of the vesicle membrane because each is reduced in molecular weight upon treatment with protease in the absence of detergent. They must also be tightly associated with the membrane, perhaps as Triton X-100-insoluble integral membrane proteins, or as a peripheral aggregate of substantial size to remain pelletable in each of the cases described. Thin-section analysis of the Triton-insoluble pellet shows small open membrane sheets (not shown) which suggests that the detergent does not completely extract the lipid bilayer. Therefore, this observation does not rule out either possibility. Further analysis of the vesicles and the plasma membrane constituents will be necessary to resolve this issue.

Conclusion

We have taken advantage of the conditional phenotype of the vesicle accumulating sec 6-4 mutant to isolate constitutive secretory vesicles of *S. cerevisiae*. The purification scheme yields morphologically uniform, intact vesicles with a low level of contaminating membrane. We have identified three
proteins which appear to be specific to the vesicle population and have begun to characterize them biochemically. Two of these proteins contain domains that are on the cytoplasmic side of the vesicle membrane. This implies that these two proteins could interact with the plasma membrane, the cytoskeleton, or components of the cytoplasm. The genetic flexibility of yeast will enable us to address the role of these two proteins on the secretory pathway.

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