

Order of Events in the Yeast Secretory Pathway

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Summary

The sequence of posttranslational events in the export of yeast glycoproteins has been determined with the aid of mutants that affect the secretory apparatus. Temperature-sensitive secretory mutants (*sec*) of *S. cerevisiae*, when incubated at a nonpermissive growth temperature (37°C), accumulate intracellular precursor forms of exported glycoproteins, such as invertase, and expand or amplify one or more of three different secretory organelles. Characterization of haploid double-*sec* mutant strains, with regard to the structure of the accumulated invertase and the morphology of the exaggerated organelles, allows assessment of the order in which the gene products are required, the sequence of invertase maturation steps and a pathway of secretory organelles. The transitions from one organelle to the next require energy and *sec* gene products. One of the mutants (*sec7*) accumulates a different organelle depending on the concentration of glucose in the medium. In normal growth medium (2% glucose), a thermally irreversible structure, the Berkeley body, predominates; in low glucose (0.1%), Golgi structures accumulate thermoreversibly. The results are consistent with the following model. Secretory proteins enter the ER, where the initial steps of glycosylation occur. Nine or more *sec* gene products and energy are required to transfer material to a Golgi-like structure, where further glycosylation occurs. Two or more functions and energy are required to package nearly fully glycosylated proteins into vesicles that are then transported into the bud, where they fuse with the plasma membrane in a process that requires at least ten additional gene products and energy.

Introduction

The intracellular pathway followed by secretory proteins in eucaryotic cells has been explored by several techniques. Jamieson and Palade (1967a, 1967b) used autoradiography and cell fractionation to follow pulse-labeled secretory proteins through a series of membrane-bounded organelles. They found that transit of labeled proteins through the pathway was not blocked by cycloheximide, suggesting that ongoing protein synthesis does not force the flow of secretory products (Jamieson and Palade, 1968a). An inhibitor of oxidative phosphorylation, however, did block

movement of labeled proteins from the endoplasmic reticulum (ER) to the cis face of the Golgi apparatus, and from mature zymogen granules to the cell exterior (Schramm, 1967; Jamieson and Palade, 1968b). Other drugs that have a more selective effect on the secretory process, such as the ionophores A23187 and monensin, have been used to define stages in the pathway further (Tartakoff and Vassalli, 1978). Reconstruction in vitro of individual events, such as the import of secretory proteins into the lumen of the ER (Blobel and Dobberstein, 1975), or the transport of a membrane protein from the ER to the Golgi (Fries and Rothman, 1980), offers an analysis of this process at the molecular level.

We have developed a genetic approach to the study of the secretory process in *Saccharomyces cerevisiae*. Secretion in yeast is characterized by low levels of intracellular precursors, few secretory organelles and a short lag time between synthesis and release at the plasma membrane. These factors have hindered analysis of this process. The isolation of a large number of conditionally lethal mutants that thermoreversibly trap secretory proteins at distinct steps in the pathway (Novick and Schekman, 1979; Novick et al., 1980) has enabled us to construct an assembly pathway leading from synthesis to release.

The use of double mutants in ordering the events in a biosynthetic pathway was first described by Mitchell and Houlahan (1946) for the synthesis of adenine in *Neurospora*. Single mutants that block at phenotypically distinguishable stages have been combined pairwise and used to map pathways as complex as the cell division cycle (Hartwell et al., 1974). Jarvik and Botstein (1973) developed a more sophisticated technique for ordering events in the morphogenesis of phage P22. By using reversible and independently applied blocks, they demonstrated that the detection of intermediate structures was not necessary for the assignment of a functional sequence of events. We have used double-mutant analysis and a variation of the Jarvik and Botstein technique to order events in the yeast secretory pathway.

Results

Secretion Is Rapid

External invertase synthesis is derepressed by a decreased supply of glucose. The secreted enzyme remains in the yeast cell wall and can be assayed in a whole-cell suspension (Dodyk and Rothstein, 1964).

The level of external invertase began to rise 10 min after X2180 cells were transferred from YP medium with 5% glucose to YP medium with 0.1% glucose (Figure 1). The specific activity increased at a constant rate for the next 30 min. During this period, inhibition of protein synthesis by cycloheximide allowed the level of external invertase to rise for an additional 5 min as the internal precursor pool was depleted. This

experiment suggested that inhibition of protein synthesis and completion of export require 5 min or less.

Ontogeny of Secretory Organelles

All but one of the mutants that accumulate secretory enzymes also accumulate or exaggerate secretory organelles (Novick et al., 1980). In most cases, only one of three different organelles accumulates in a mutant: ER, toroidal or cup-shaped structures called Berkeley bodies or 80–100 nm vesicles. If these organelles represent stages in the passage of secretory proteins along a linear pathway, a double mutant should accumulate the organelle corresponding to the earliest block.

A number of double *sec* mutants were constructed in which each member, by itself, produced one of the three distinct phenotypes. Single and double mutants were examined by thin-section electron microscopy. Double mutants constructed from *sec18* (ER and some 40–60 nm vesicles), and *sec20* (ER), *sec1* (80–100 nm vesicles) or *sec7* (Berkeley bodies), showed the *sec18* phenotype (Table 1; Figure 2). Mutant alleles of all ten complementation groups that show 80–100 nm vesicle accumulation were combined with *sec7*, and in each case the double mutant accumulated Berkeley bodies. Mutants with more complex phenotypes were also examined in combination with *sec18* and *sec7*. Berkeley bodies and some 80–100 nm vesicles are seen in *sec14*; a double mutant with *sec7* produced Berkeley bodies only. A mixture of 80–100 nm and 40–60 nm vesicles, Berkeley bodies and ER is seen in *sec19*; combination with *sec18* prevented formation of Berkeley bodies and large

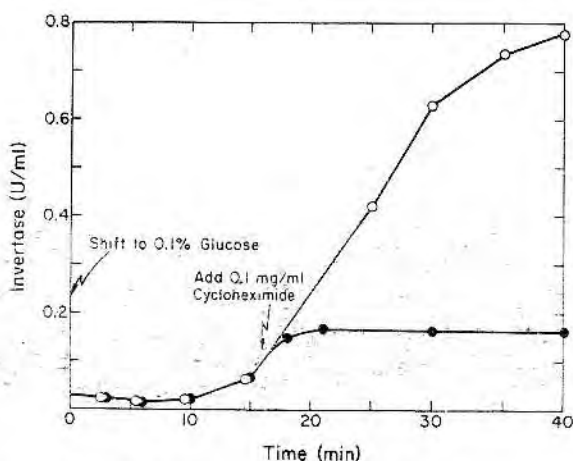


Figure 1. Transit Time of Invertase to the Cell Surface

X2180-1A cells were grown overnight in YP medium with 5% glucose. Cells ($20 A_{500}$ U) were sedimented in a clinical centrifuge, resuspended in 10 ml of YP medium with 0.1% glucose and incubated at 37°C. After 16 min, cycloheximide (final concentration 0.1 mg/ml) was added to a portion of the culture (●—●). At timed intervals 0.5 ml aliquots were chilled, sedimented in a clinical centrifuge and resuspended in 0.5 ml of 10 mM NaN_3 at 0°C.

vesicles, whereas a double mutant with *sec7* eliminated only the large vesicles.

Maturation of Invertase

The *sec* mutants have been divided into two classes based on the extent of glycosylation of the accumulated invertase (Esmon et al., 1981). Mutants that accumulate ER produce a form of invertase that has a shorter, possibly core, oligosaccharide. The other mutants accumulate invertase that has the core and outer-chain oligosaccharide. These two forms can be distinguished by electrophoresis on SDS-polyacrylamide gels. The immature invertase migrates with an apparent molecular weight of 79 to 83 kd; the mature form migrates diffusely with an apparent molecular weight of 100 to 140 kd. Double mutants were used to evaluate the influence on invertase maturation.

Single and double *sec* mutants (*sec1*, *sec18*; *sec7*, *sec18*) were grown in minimal medium and transferred to 37°C under invertase derepressing conditions. Cells were labeled for 1 hr with $^{35}\text{S}\text{O}_4^{2-}$ and converted to spheroplasts with lyticase (Scott and Schekman, 1980), and extracts were prepared by detergent lysis. Labeled invertase was precipitated in a two-stage reaction with antibody and fixed *Staphylococcus A* cells. Immunoprecipitates were dissolved in SDS and were subjected to electrophoresis on polyacrylamide gels. Double mutants that included *sec18* showed that this phenotype was epistatic to the *sec1* and *sec7* form of invertase (Figure 3). In this experiment, the

Table 1. Double-*sec*-Mutant Phenotypes^a

Single-mutant phenotype	Single-Mutant Phenotype	Double-Mutant Phenotype with	
		<i>sec7-1</i>	<i>sec18-1</i>
		Bbs	ER and sv
<i>sec1-1</i>	ves	Bbs	ER and sv
<i>sec2-56</i>	ves and Bbs	Bbs	
<i>sec3-2</i>	ves	Bbs	
<i>sec4-2</i>	ves	Bbs	
<i>sec5-24</i>	ves	Bbs	
<i>sec6-4</i>	ves	Bbs	
<i>sec7-1</i>	Bbs		ER and sv
<i>sec8-1</i>	ves	Bbs	
<i>sec9-4</i>	ves and Bbs	Bbs	
<i>sec10-2</i>	ves	Bbs	
<i>sec14-3</i>	Bbs and ves	Bbs	
<i>sec15-1</i>	ves	Bbs	
<i>sec19-1</i>	ER and Bbs and ves and sv	Bbs and ER and sv	ER and sv
<i>sec20-1</i>	ER		ER and sv

^a sv: small vesicles (40–60 nm). ves: vesicles (80–100 nm). Bbs: Berkeley bodies.

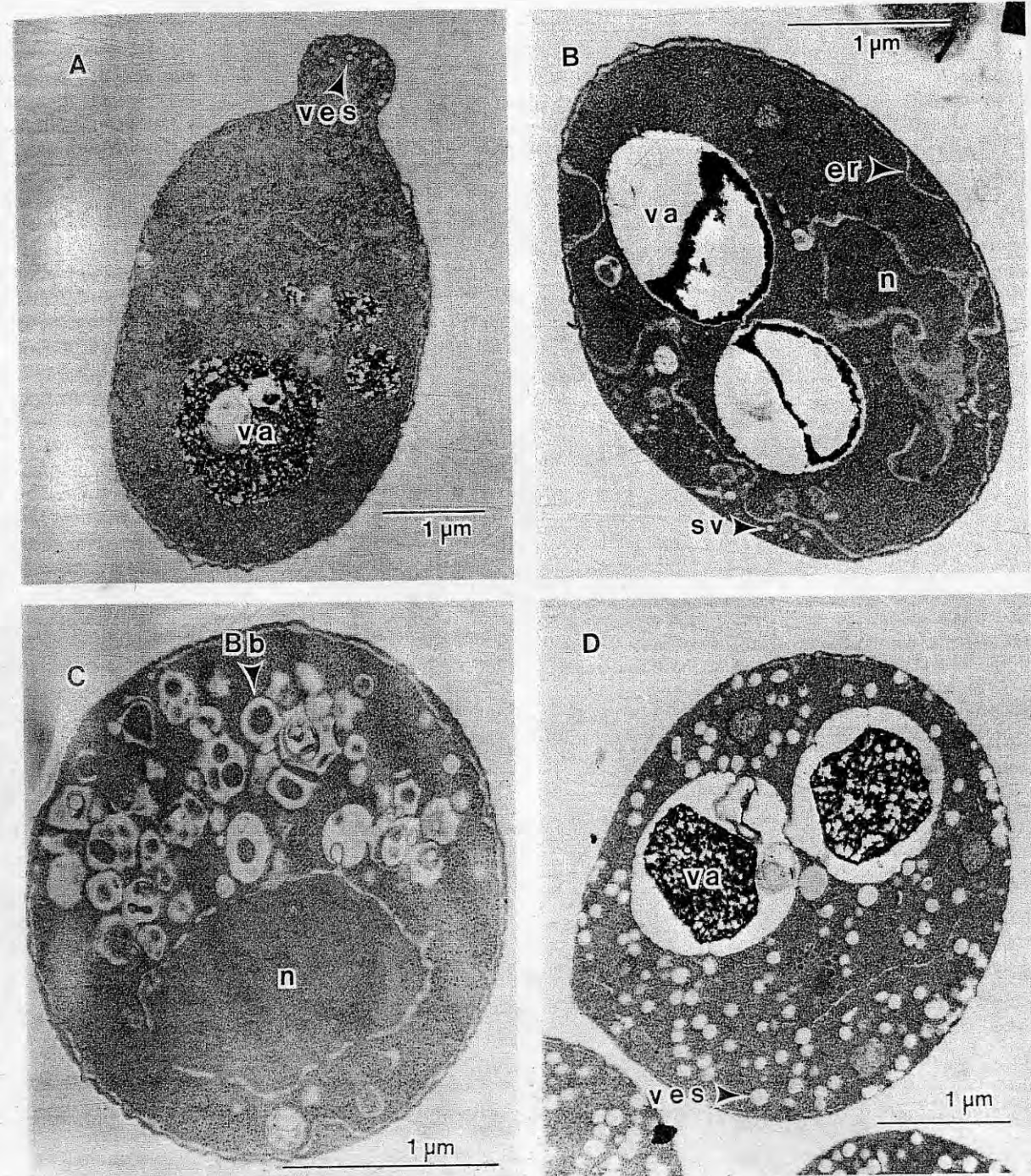


Figure 2. Thin-Section Electron Micrographs of Cells Grown in YPD Medium

(A) HMSF 183 (*sec22-1*) grown at the permissive temperature (25°C). (B) SF 230-1 (*sec1-1, sec18-1*) grown for 2 hr at 37°C. (C) SF 250-1 (*sec7-1, sec15-1*) grown for 2 hr at 37°C. (D) HMSF 147 (*sec10-2*) grown for 2 hr at 37°C. n: nucleus. va: vacuole. er: endoplasmic reticulum. sv: small vesicles. Bb: Berkeley body. ves: vesicles.

three closely spaced bands of invertase produced in ER-blocked mutants (Esmon et al., 1981) were not resolved clearly. Control experiments showed that the preimmune serum did not precipitate invertase from extracts of *sec1* cells.

Energy Poisons and Reversibility

Many of the *sec* mutants are thermoreversible; upon return to the permissive temperature (25°C) the accumulated invertase is secreted whether or not protein synthesis is allowed to continue (Novick et al., 1980).

sec *sec* *sec* *sec* *sec*
1 7 18 1,18 7,18



Figure 3. Immunoprecipitation of Invertase Accumulated in the Single and Double Mutants

(Lane 1) *sec1* (HMSF 1); (lane 2) *sec7* (HMSF 6); (lane 3) *sec18* (HMSF 176); (lane 4) *sec1, sec18* (SF 230-1); (lane 5) *sec7, sec18* (SF 231-1).

This situation allowed a screening of potential secretion-inhibiting drugs without regard to their effect on protein synthesis. The most dramatic effect on the reversibility of the *sec* mutants was seen with drugs, such as dinitrophenol (DNP), that uncouple oxidative phosphorylation and electron transport. In the experiment shown in Table 2, invertase was allowed to accumulate at 37°C in various *sec* mutants and, after the addition of cycloheximide, cells were transferred to minimal medium at 25°C in the presence or absence of DNP and with or without glucose. Secretion of accumulated invertase and cellular ATP levels was measured during a 2 hr incubation. With glucose and no DNP, the mutants secreted varying amounts of invertase, and ATP levels were normal. In the absence of glucose, 0.1 mM DNP blocked secretion and caused the ATP level to decline by a factor of at least 10. In the presence of glucose (0.5%) the effect of DNP on secretion was diminished, and ATP levels were maintained at near normal. Sodium azide and cyanide *m*-chlorophenylhydrazine also blocked secretion of accumulated invertase when *sec*-mutant cells were returned to 25°C in the absence of glucose (data not shown).

Other reagents were screened for their effect on secretion and ATP levels in an effort to find non-energy-related blocks. The calcium ionophore A23187 blocks secretion in certain mammalian cells

Table 2. Secretion of Accumulated Invertase Requires Energy^a

Strain	100 μ M DNP, No Glucose		100 μ M DNP and 0.5% Glucose		0.5% Glucose	
	Invertase Secreted ^b	ATP ^c	Invertase Secreted ^b	ATP ^c	Invertase Secreted ^b	ATP ^c
HMSF 1 (<i>sec1-1</i>)	0.003	1.2	0.093	8.7	0.124	12.7
HMSF 106 (<i>sec2-56</i>)	0.004	1.0	0.034	8.9	0.081	11.1
HMSF 134 (<i>sec5-24</i>)	0.004	0.8	0.086	9.7	0.055	12.4
HMSF 136 (<i>sec6-4</i>)	0.000	0.6	0.332	11.5	0.277	12.2
HMSF 6 (<i>sec7-1</i>)	0.003	0.2	0.480	13.0	0.063	17.4
HMSF 143 (<i>sec9-4</i>)	0.000	0.7	0.303	12.1	0.215	12.0
SF 226-K (<i>sec12-4</i>)	0.004	1.2	0.203	9.9	0.641	14.5
HMSF 189 (<i>sec14-3</i>)	0.016	0.8	0.333	10.0	0.286	11.4
HMSF 174 (<i>sec16-2</i>)	0.010	2.3	0.441	10.9	0.196	14.6
HMSF 176 (<i>sec18-1</i>)	0.000	0.8	0.566	9.0	0.550	11.8
HMSF 178 (<i>sec19-1</i>)	0.000	0.8	0.086	8.7	0.363	11.6
HMSF 179 (<i>sec20-1</i>)	0.054	1.2	0.523	11.2	0.525	12.8
HMSF 183 (<i>sec22-3</i>)	0.015	1.3	0.516	14.9	0.494	13.4
HMSF 190 (<i>sec23-1</i>)	0.011	0.8	0.377	17.4	0.370	12.9
X2180-1A		3.0		17.2		12.5

^a Cultures were grown overnight in minimal medium with 5% glucose. Cells (7 A₆₀₀ U) were sedimented in a clinical centrifuge, resuspended in 3.5 ml of minimal medium with 0.1% glucose and incubated at 37°C for 1 hr. Derepressed cells were sedimented, washed once with minimal medium containing 0.1 mg/ml cycloheximide and no glucose and resuspended in 3.5 ml of the same medium. A 0.5 ml sample was transferred to 0°C, and 1 ml aliquots were put into tubes containing 0.1 mM DNP and 0.5% glucose, or 0.5% glucose alone, and incubated at 25°C. After 1 hr, a 0.5 ml sample was removed for measurement of ATP. One hour later the remaining cells were chilled, centrifuged and resuspended in an equal volume of 10 mM sodium azide at 0°C.

^b Values given are units of invertase per milligram of dry weight secreted during the final incubation at 25°C.

^c Values given are nanomoles per milligram of dry weight.

(Tartakoff and Vasalli, 1978). We found that secretion of invertase was blocked by A23187 when X2180 cells were incubated in minimal medium without divalent cations (data not shown). Unfortunately, conditions that inhibited secretion also lowered ATP levels. Other reagents that appeared to block secretion but also interfered with energy metabolism were: tosyl-sulfonyl fluoride, diamide and *n*-ethylmaleimide. In no case did these drugs allow invertase accumulation in wild-type cells. Monensin and valinomycin had no effect on invertase production or secretion.

Glucose Interferes at a Golgi Block

Two mutants, *sec7* and *sec16*, secreted more accumulated invertase when cells were returned to 25°C and incubated with glucose and DNP than with glucose alone (Table 2). This surprising result was investigated more thoroughly with *sec7*. Invertase was depressed at 37°C for 1 hr and cells were transferred to minimal medium containing cycloheximide and increasing amounts of glucose with or without DNP. After 2 hr at 25°C, cells were collected and assayed for external invertase (Figure 4A) and ATP (Figure 4B). Secretion and ATP levels were low when no glucose or DNP was present. With low glucose (0.01%–0.05%), ATP levels rose to normal, and invertase was secreted optimally. At 0.01% glucose, for example, 61% of the accumulated invertase was secreted. At higher levels of glucose, in the absence of DNP, secretion was inhibited; glucose inhibition of reversibility was not seen with the other *sec* mutants. Treatment with high glucose produced an irreversible block. When *sec7* cells were returned to 25°C with 2% glucose for 2 hr, subsequent incubation in 0.02% glucose did not allow secretion of accumulated invertase (data not shown).

DNP blocked the glucose effect on *sec7*. At low glucose (0%–0.02%), DNP inhibited secretion of accumulated invertase. At higher glucose (0.1%–0.5%), DNP stimulated secretion as much as tenfold.

Glucose also had a dramatic effect on the shape of the organelle accumulated in *sec7* at 37°C. Our previous electron microscope thin-section analysis was performed on *sec*-mutant strains grown in YP medium with 2% glucose (Novick et al., 1980). These conditions were not compatible with *sec7* reversibility. Thin-section analysis was repeated with *sec7* cells shifted to YP medium with 0.1% glucose at 37°C for 2 hr. In contrast to the Berkeley bodies seen in 2% glucose (Figure 2C), 0.1% glucose allows the accumulation of stacked membrane-bounded lamellae that strongly resemble Golgi bodies (Figure 5). Golgi bodies, and not Berkeley bodies, are seen when the *sec1 sec7* double mutant is incubated at 37°C in YP medium with 0.1% glucose.

Energy and *sec* Products Required Concurrently

Inhibition of reversible secretion by energy poisons implies an energy requirement for export that is at or

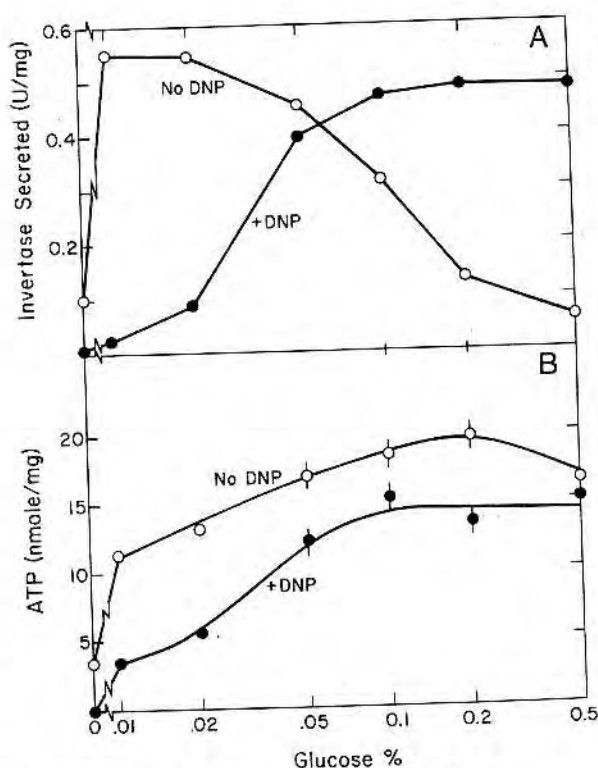


Figure 4. Effect of Glucose on the Reversibility of *sec7* Cells
HMSF 6 (*sec7-1*) cells were grown overnight in minimal medium with 5% glucose at 25°C. Cells (50 A_{600} U) were harvested by filtration, resuspended in 25 ml of minimal medium with 0.1% glucose and incubated at 37°C for 1 hr. The cells were collected, washed twice with minimal medium containing 0.1 mg/ml cycloheximide and no glucose and resuspended in 10 ml of the same medium. A 1 ml sample was transferred to 0°C, and 1 ml aliquots were added to tubes containing the indicated final concentrations of glucose and 0.1 mM DNP. After 1 hr at 25°C a 0.5 ml sample was removed for measurement of ATP (B). One hour later the cells were chilled, centrifuged and resuspended in an equal volume of 10 mM sodium azide at 0°C, and invertase activity was measured (A).

after the block imposed by the defective *sec* gene product. The simple alternatives of concurrent or sequential requirements for a *sec* product and energy were tested in two-stage incubations where each block was imposed separately. Invertase was allowed to accumulate for 1 hr at 37°C and cells were transferred to minimal medium without glucose and with DNP. After 2 hr at 25°C, cells were transferred to fresh minimal medium with glucose and without DNP, and aliquots were further incubated for 2 hr at 25°C and 37°C. Cells were collected and assayed for external invertase and ATP. If the *sec*-product- and energy-requiring steps were sequentially executed, the invertase accumulated at 37°C would proceed past the temperature-sensitive block during the 25°C incubation. Upon release of the energy block, invertase secretion would occur at 25°C or 37°C. This test was performed with seven strains selected for their restrictive and thermoreversible effect on secretion. The results in Table 3 demonstrate that accumulated

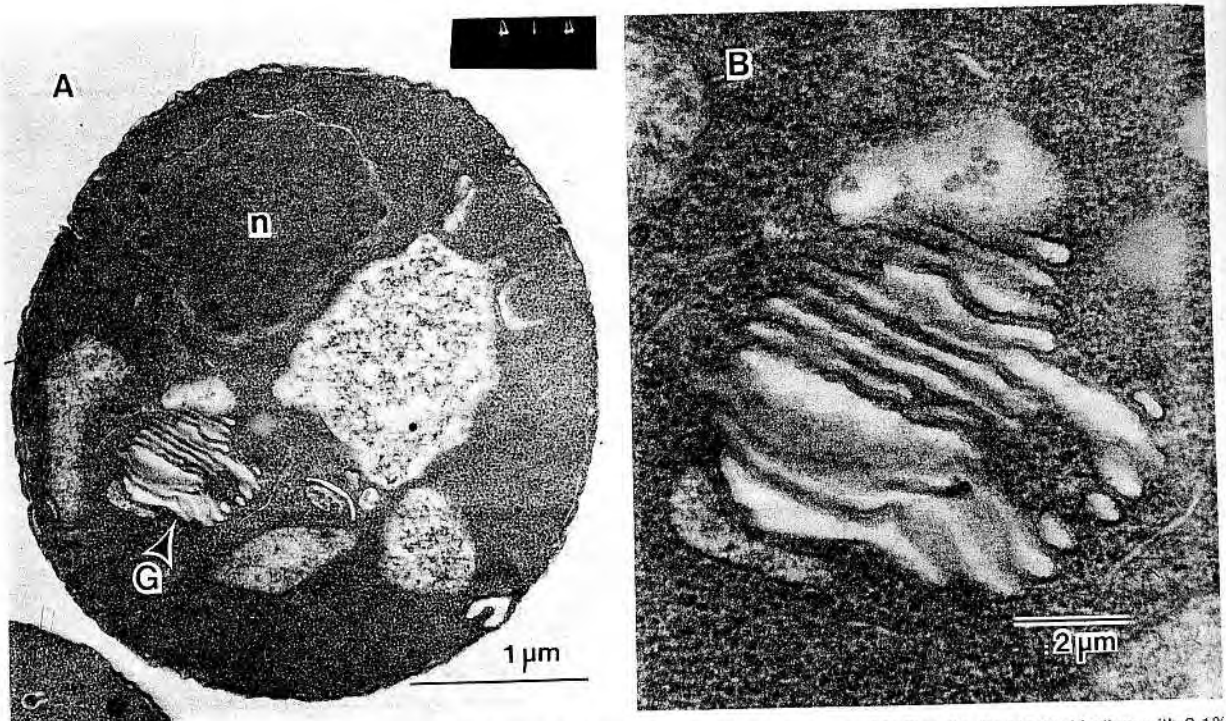


Figure 5. Thin-Section Electron Micrographs of HMSF 6 (*sec7-1*) Cells Grown in YPD Medium at 25°C, Then Shifted to YP Medium with 0.1% Glucose at 37°C for 2 hr

(B) Same cell as in (A) at higher magnification. n: nucleus. G: Golgi.

invertase did not proceed beyond the temperature-sensitive step in any of the mutants. In each case the cells recovered from the energy block, since ATP levels were normal after incubation at 25°C and 37°C.

If energy is required for transit between each of the three major stages in the pathway (ER to Golgi; Golgi to vesicles; vesicles to cell surface), then an organelle accumulated at 37°C should not be converted to the succeeding structure when cells are returned to 25°C in an energy-deprived state. This prediction was tested with a representative of each class (ER, *sec18*; Golgi, *sec7*; 80–100 nm vesicles, *sec6*). Cells were transferred to minimal medium with 0.1% glucose and incubated for 1.5 hr at 37°C. Aliquots were withdrawn and prepared for electron microscopy, and the rest were transferred to fresh minimal medium containing cycloheximide (0.1 mg/ml) and no glucose. Each sample was divided; one group received glucose (0.5% for *sec6* and *sec18*, 0.1% and 2% for *sec7*), and the other DNP (0.1 mM). Samples were further incubated for 3 hr at 25°C and prepared for electron microscopy (data not shown).

In each case, structures that accumulated at 37°C remained unchanged during subsequent incubation at 25°C in the presence of DNP. However, all structures diminished or declined in abundance during incubation at 25°C in minimal medium with glucose (0.5% for *sec6* and *sec18*; 0.1% for *sec7*). The only exception was *sec7*. Golgi bodies that accumulated at 37°C

in 0.1% glucose gave way to Berkeley bodies during continued incubation at 25°C in 2% glucose.

Discussion

We have established, in broad form, the sequence of events required for maturation and export of external invertase in yeast. Invertase secretion is normally rapid (transit time ≤ 5 min), making detection of intermediates somewhat difficult. Secretory mutants that reversibly trap the enzyme at distinct steps in the pathway have greatly facilitated the analysis. Our results support a model of secretion in yeast that is strikingly similar to that observed in mammalian cells (Figure 6). The morphology of the secretory organelles, the division of glycosylation steps between the ER and Golgi and the location of energy-requiring steps are directly comparable to those seen in specialized exocrine cells. This pathway is a major one in the cell, since bud growth and the export of at least two other cell-surface proteins are blocked in the *sec* mutants (Novick et al., 1980).

The use of double-mutant analysis to establish the order of gene product function, and the sequence of intermediates, assumes that both gene products act in the same pathway. If the gene products operate in independent pathways that contribute to the same process, the phenotypes of both single mutants would appear in the double mutant. This is not seen in the

Table 3. Energy and Functioning *sec* Gene Products Are Required Concurrently^a

Strain	1 hr, 37°C, 0.1% glucose		2 hr, 25°C, DNP, no glucose		2 hr, 25°C, 0.5% glucose		2 hr, 37°C, 0.5% glucose	
	Invertase Secreted (25°C) ^b	ATP (25°C) ^c	Invertase Secreted (37°C) ^b	ATP (37°C) ^c	Invertase Secreted (25°C) ^b	ATP (25°C) ^c	Invertase Secreted (37°C) ^b	ATP (37°C) ^c
HMSF 1 (<i>sec1-1</i>)	0.132	8.5	0.006	14.0				
HMSF 136 (<i>sec6-4</i>)	0.288	8.7	0.026	11.9				
HMSF 143 (<i>sec9-4</i>)	0.276	10.4	0.024	12.9				
SF 226-1C (<i>sec12-4</i>)	0.533	8.5	0.052	14.9				
HMSF 176 (<i>sec18-1</i>)	0.491	10.1	0.025	12.5				
HMSF 178 (<i>sec19-1</i>)	0.348	9.6	0.027	12.0				
HMSF 183 (<i>sec22-3</i>)	0.552	8.0	0.145	14.1				

^a Cultures were grown overnight in minimal medium with 5% glucose at 25°C. Cells (7 A₈₀₀ U) were sedimented in a clinical centrifuge, resuspended in minimal medium with 0.1% glucose and incubated for 1 hr at 37°C. Derepressed cells were sedimented, washed once with minimal medium containing 0.1% mg/ml cycloheximide and no glucose and resuspended in 3.5 ml of the same medium. A 0.5 ml sample was transferred to 0°C and DNP (0.1 mM final concentration) was added to the remainder of the culture. After 1 hr at 25°C a 0.5 ml sample was removed for measurement of ATP. One hour later a 0.5 ml sample was transferred to 0°C and the remainder of the culture was centrifuged. Cells were resuspended in 2 ml of minimal medium containing 0.1 mg/ml cycloheximide and 0.5% glucose; 1 ml was incubated at 25°C, and 1 ml was incubated at 37°C. After 1 hr a 0.5 ml sample was removed for measurement of ATP; 1 hr later the cells were chilled, centrifuged and resuspended in an equal volume of 10 mM sodium azide, and invertase activity was measured.

^b Units of invertase secreted per milligram of dry weight during the final incubation at 25°C or 37°C in the presence of 0.5% glucose and no DNP.

^c Nanomoles of ATP per milligram of dry weight during the final incubation at 25°C or 37°C.

double *sec* mutants; in cytological and invertase structural analyses the double *sec* mutants display the phenotype of one parent only. Both methods of analysis indicate that the ER-accumulating mutants are blocked before the Golgi and 100 nm vesicle stage in the pathway.

Certain *sec* gene products may perform a function early in the pathway that is not required until a late step. Yeast mutants have been described that block steps in the cell division cycle, but that have early execution points (Hartwell et al., 1974). If such a function cannot be executed at the late step, a mutant of this sort would not be thermoreversible. Perhaps coincidentally, the *sec* mutants that are <20% thermoreversible are blocked late in the pathway—that is, they accumulate 100 nm vesicles (*sec3*, *sec4*, *sec5*, *sec8* and *sec10*; Novick et al., 1980). Alternatively, these mutant proteins may not regain function upon return to the permissive temperature.

Clathrin-coated vesicles have been implicated in the movement of proteins from the ER to the Golgi in

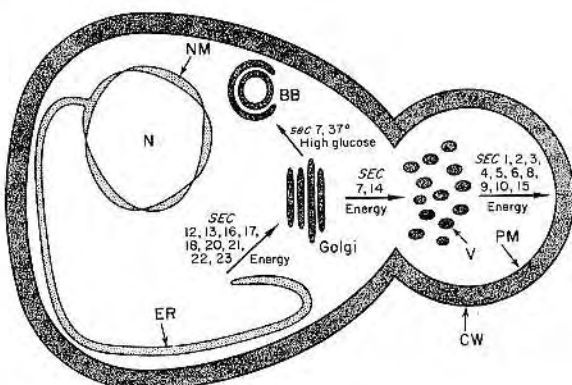


Figure 6. Yeast Secretory Pathway

N: nucleus. NM: nuclear membrane. ER: endoplasmic reticulum. SEC: wild-type gene product. sec: mutant gene product. V: vesicle. PM: plasma membrane. CW: cell wall. BB: Berkeley body.

mammalian cells (Rothman and Fine, 1980). The 100 nm vesicles seen in mutants such as *sec1* are not candidates for an ER-to-Golgi transporter; all mutants of this type are blocked after the Golgi stage. Three mutants accumulate small (40–60 nm) vesicles in addition to ER (*sec17*, *sec18* and *sec21*). A double mutant of *sec20* (ER only) and *sec18* (ER and small vesicles) has the *sec18* phenotype. This result is inconsistent with the possibility that these small vesicles mediate transfer of material from the ER to the Golgi. Perhaps small vesicles arise from organelle fragmentation in some, but not all, of the ER-accumulating mutants. If vesicles play a role in ER-to-Golgi movement in yeast, either no mutant in our collection affects this step or vesicles of this sort cannot accumulate.

We have established (Esmon et al., 1981) that outer-chain oligosaccharides are present on invertase and bulk mannan produced in all but the ER-accumulating *sec* mutants. The Golgi body is the first in the sequence of organelles described here that contains highly glycosylated secretory proteins. Our interpretation is that outer-chain oligosaccharides are added to glycoproteins in the Golgi, although this could occur at a transitional stage between the ER and Golgi. The former view has ample support in mammalian exocrine cells.

Energy requirements in a eucaryotic secretory pathway have been known for some time. Jamieson and Palade (1968b) described an energy requirement for transport of amylase between the ER and Golgi in the pancreatic acinar cell, and Schramm (1967) showed the same requirement for exocytosis of secretory granule contents in the parotid gland. Likewise, in the yeast secretory process, none of the *sec* mutants completes the export of accumulated invertase when ATP levels are dropped by DNP treatment. The relief of DNP inhibition by glucose argues against a drug effect unrelated to energy. This analysis demonstrates

an energy requirement at or after the last of the sec-mediated steps.

Although cells inhibited by DNP at 25°C recover when the drug is removed, secretion of invertase accumulated before the DNP treatment remains thermosensitive. This result suggests, for the seven mutants where this test was possible, that the sec-dependent steps can be executed only when energy is available. Alternatively, each sec gene product may act in multiple events punctuated by separate energy-requiring reactions. Organelles accumulated in *sec18* (ER and 40–60 nm vesicles), *sec7* (Golgi) and *sec6* (100 nm vesicles) are neither diminished nor converted to the succeeding organelle when cells are returned to 25°C in the presence of DNP. Thus energy is required at least three times during transit of secretory proteins from the ER to the cell surface.

The behavior of *sec7* deserves special comment. This mutant, when incubated at 37°C in YP medium with 2% glucose, produces unique structures called Berkeley bodies (Novick et al., 1980). Unlike the other sec mutants, secretion of invertase accumulated in *sec7* is stimulated tenfold by DNP when cells are returned to 25°C in medium containing 0.5% glucose (Figure 4). In the absence of DNP, reversible secretion is inhibited by glucose concentrations above 0.05%, although the ATP level is not affected significantly. The glucose effect can be overcome by simultaneous treatment with DNP. In the absence of the drug, glucose inhibition is irreversible; transfer at 25°C from 2% to 0.02% glucose does not restore secretion of accumulated invertase. It should be noted that high glucose concentration has no effect on the secretory process in normal cells at 37°C, or in *sec*-mutant cells at 25°C (Novick et al., 1980).

In addition to an effect on reversible secretion in *sec7*, glucose causes a morphological change in the organelle that accumulates at 37°C. In YP medium with 0.1% glucose, the mutant accumulates stacks of membrane-bounded discs that closely resemble Golgi bodies. These stacks disappear when cells are returned to 25°C in 0.1% glucose, but seem to be converted to Berkeley bodies when incubated in 2% glucose. It may be pertinent that Berkeley bodies are distributed throughout the cytoplasm, whereas the Golgi form usually appears as a stack of discs (compare Figures 2C and 5). High glucose concentration may influence the ability of Golgi discs to form an ordered array when movement beyond this step in the secretory pathway is blocked.

Experimental Procedures

Strains, Growth Conditions and Materials

S. cerevisiae *sec*-mutant strains were described previously (Novick et al., 1980; Esmon et al., 1981). Standard genetic techniques were used to construct the haploid double *sec* mutants listed in Table 1 and Figures 2 and 3. In each relevant experiment, the double-*sec*-mutant cultures were checked by complementation against each parent allele to ensure the retention of both alleles.

YPD medium contained 1% Bacto-Yeast Extract, 2% Bacto-Pep-tone and 2% glucose; YP medium was the same with different levels of glucose. Wickerham's minimal medium (Wickerham, 1946) was used with different levels of glucose. Liquid cultures were grown in flasks or tubes with agitation, and the experiments were initiated with exponentially growing cells from stock cultures at an A_{600} of 1–5. The absorbance of cell suspensions was measured in a 1 cm quartz cuvette at 600 nm in a Zeiss PMQ11 spectrophotometer; 1 A_{600} U corresponds to 0.15 mg dry weight.

Other reagents were obtained as indicated: glucose oxidase, o-dianisidine, peroxidase, cycloheximide, phenylmethylsulfonyl fluoride, tosyl lysine chloromethyl ketone and firefly lantern extract were from Sigma; glutaraldehyde, osmium tetroxide and Spurr embedding medium were from Polysciences; DNP was from Mann Research Laboratories. IgG sorb (fixed *Staphylococcus aureus* Cowan I cells) was from the Enzyme Center, Boston, Mass. The IgG fraction of invertase antibody was the same preparation described by Esmon et al. (1981). Lyticase is a yeast lytic enzyme preparation useful in spheroplast formation (Scott and Schekman, 1980). Fraction II (30,000 U/mg; 1 U will lyse 0.2 A_{600} U of logarithmic-phase *S. cerevisiae* in 30 min at 30°C) was used.

Assays and Electron Microscopy

External invertase was assayed at 37°C as described by Goldstein and Lampen (1975); units of activity are micromoles of glucose released per min. Internal invertase activity was determined by assaying spheroplast lysates, prepared as previously described (Novick and Schekman, 1979).

Cellular ATP was extracted and measured by modifications of other procedures (Weibel et al., 1974; Kimmich et al., 1975). Culture aliquots (0.5 ml) were mixed with 0.125 ml of 35% HClO₄ at 0°C. The samples were freeze-thawed three times in a dry ice-ethanol bath, and neutralized with 10 N KOH. The cells and KClO₄ precipitate were centrifuged and the supernatant solution was diluted 1:10 with 20 mM imidazole-HCl (pH 8.0) and set aside for assay. A crude luciferin-luciferase solution was prepared by mixing 5 ml water and 50 mg lyophilized firefly lanterns. After 2 hr at 0°C the insoluble material was removed by centrifugation. The assay mix contained 10–50 μ l of the dilute cell extract in 0.9 ml of 20 mM imidazole-HCl (pH 8.0), and the reaction was initiated by addition of the luciferin-luciferase solution (0.1 ml). After 20 sec the assay vials were counted for 30 sec in a Searle Delta 300 liquid scintillation counter set on the tritium channel.

Thin-section electron microscopy was performed on samples prepared by the method of Byers and Goetsch (1975).

Immunoprecipitation of Invertase

Cells were grown to early exponential phase at 25°C in minimal medium containing 0.1 mM ammonium sulfate and 5% glucose. An aliquot (6 A_{600} U) was centrifuged and cells were resuspended in 3 ml of fresh minimal medium containing 0.05 mM ammonium sulfate, 0.1% glucose and 300 μ l ³⁵S₂O₄²⁻. After incubation at 37°C for 1 hr, cells were sedimented, washed with 10 mM sodium azide and resuspended in 0.4 ml of spheroplasting medium (1.4 M sorbitol, 23 mM potassium phosphate [pH 7.5], 2 mM MgCl₂, 10 mM sodium azide, 40 mM 2-mercaptoethanol and 50 U lyticase per A_{600} unit of cells). Spheroplasts formed during a 30 min incubation at 30°C were sedimented at 3000 \times g for 10 min and the pellet was dissolved in 0.2 ml of 1% Triton X-100. The sample was diluted with an equal volume of 2 \times phosphate-buffered saline (phosphate-buffered saline: 0.02 M NaCl, 12.5 mM potassium phosphate [pH 7.6]) containing 2 mM each of phenylmethylsulfonyl fluoride and tosyl lysine chloromethyl ketone. The lysate was centrifuged at 12,000 \times g for 10 min; the supernatant solution was removed and sedimented again at 100,000 \times g for 90 min; and the final soluble fraction was mixed with 5 μ l anti-invertase IgG in an Eppendorf centrifuge tube. Immunoprecipitation mixtures were incubated at 0°C for 16–18 hr. Fixed *Staphylococcus A* cells in a 10% suspension (w/v) were washed twice with equal volumes of phosphate-buffered saline containing Triton X-100 (0.5%) and bovine serum albumin (1 mg/ml), and 150 μ l were added to the immunoprecipitate. The mixture was kept at 0°C for 30 min and centrifuged for

1 min in a Beckman microfuge B, and the pellet was washed twice with phosphate-buffered saline-Triton-bovine serum albumin buffer. The precipitate was transferred to a new Eppendorf tube, washed twice more and mixed with 50 μ l of sample buffer (45 mM Tris-Cl [pH 6.8], 9% glycerol, 1.7% SDS, 1% 2-mercaptoethanol and 0.01% bromophenol blue). After heating in a boiling water bath for 3 min, samples were subjected to electrophoresis on SDS-polyacrylamide (8.25%) slab gels, stained for protein and treated with Enhance according to the procedure described by New England Nuclear. Dried gels were allowed to expose Kodak X-Omat R film XR-5 for 1-2 days.

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