JOURNAL OF BACTERIOLOGY, June 1983, p. 1276–1283
0021-9193/83/061276-08$02.00/0

Vol. 154, No. 3

Plasma Membrane Expansion Terminates in *Saccharomyces cerevisiae* Secretion-Defective Mutants While Phospholipid Synthesis Continues

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Received 20 September 1982/Accepted 16 March 1983

Phospholipid synthesis activity and plasma membrane growth have been studied in the *Saccharomyces cerevisiae* temperature-sensitive, secretion-defective mutants isolated by Novick and Schekman (Proc. Natl. Acad. Sci. U.S.A. **76**:1858–1862, 1979; Novick et al., Cell **21**:205–215, 1980). The mutants, *sec1* through *sec23*, do not grow at 37°C and exhibit lower rates of phospholipid synthesis than does the wild-type strain X2180. None of the mutants exhibits a decline in lipid synthesis rapid enough to explain secretion failure. Plasma membrane growth was assessed indirectly by examining the osmotic sensitivity of spheroplasts derived from cultures transferred from 24 to 37°C. Spheroplasts from the normal-growing strain X2180 exhibited a small rapid increase in osmotic sensitivity and stabilized at a more sensitive state. Spheroplasts from the *sec* mutants exposed to the same temperature shift exhibited progressively increasing osmotic sensitivity. Cycloheximide treatment prevented progressive increases in osmotic fragility. These data are compatible with the hypothesis that plasma membrane expansion is restricted in the *sec* mutants. During incubation at 37°C, the accumulation of intracellular materials within the no-longer expanding plasma membrane exerts osmotic stress on the membrane, increasing with time. The gene products defective in Novick and Schekman's *sec* mutants appear to be required for both extracellular protein secretion and plasma membrane growth in yeast cells.

It has been proposed that extracellular protein secretion and plasma membrane growth in eucaryotic cells are accomplished by the same intracellular pathway of organelle membrane traffic (16). Novick and Schekman's *Saccharomyces cerevisiae* temperature-sensitive mutants, which are defective in the export but not the synthesis of secreted enzymes (14, 15), provide an opportunity to determine whether the gene products required for secretory intracellular membrane traffic are also required for plasma membrane growth.

Novick and Schekman's *sec* mutants and the yeast phospholipid synthesis mutants share common cell pathologies, prompting this investigation of membrane phospholipid synthesis in the *sec* mutants. Yeast inositol auxotrophs (2, 10), fatty acid auxotrophs (9), ethanolamine auxotrophs (12), and temperature-sensitive secretion mutants (14, 15) all cease cell surface growth under restrictive growth conditions. Internal metabolism continues within a limited cell volume, and the cells become abnormally dense.

In the present study, we have examined membrane phospholipid synthesis and plasma membrane expansion in secretion-defective mutants. A coordinate failure of secretion and membrane growth in these mutants would support the proposition of Palade (16) that secretion and membrane assembly share a common structural basis in eucaryotes. A deficiency of specific membrane structural lipids, known to block plasma membrane growth (2, 10), could be the molecular basis for curtailed secretory membrane traffic.

MATERIALS AND METHODS

Yeast strains. *S. cerevisiae* haploid strains containing temperature-sensitive *sec1* through *sec23* mutations were obtained from Peter Novick and Randy Schekman. The parental wild-type strain X2180 was originally obtained from the Berkeley Yeast Stock Center. Strain designations and genotypes are given in Table 1. In the text of this report, secretion mutant strains will be referred to simply as "the *sec4* strain," etc.

Growth media. Strains were maintained on YEPD (1% yeast extract, 2% peptone, 2% glucose, 1.5% agar) plates or in liquid YEPD. Cultures were grown at

TABLE 1. *S. cerevisiae* strains

Strain	Genotype
HMSF1	*sec1-1* MATa
HMSF106	*sec2-56* MATa
HMSF68	*sec3-2* MATα
HMSF13	*sec4-2* MATa
HMSF134	*sec5-24* MATa
HMSF136	*sec6-4* MATa
HMSF6	*sec7-1* MATa
HMSF95	*sec8-6* MATα
HMSF271-4B	*sec9-4* MATa
HMSF239-2C	*sec10-2* MATa
HMSF154	*sec11-7* MATa
SF226-1C	*sec12-4* MATa
HMSF163	*sec13-1* MATa
HMSF169	*sec14-3* MATa
HMSF171	*sec15-1* MATa
HMSF174	*sec16-2* MATa
HMSF175	*sec17-1* MATa
HMSF176	*sec18-1* MATa
HMSF178	*sec19-1* MATa
HMSF179	*sec20-1* MATa
HMSF180	*sec21-1* MATa
HMSF183	*sec22-3* MATa
HMSF190	*sec23-1* MATa
X2180	*SEC*$^+$ MATa

24°C, and secretion defects were initiated by transferring 50-ml cultures to a 37°C shaking water bath. Some cultures were treated with cycloheximide (100 μg/ml) added from a 100-fold-concentrated stock solution.

Phospholipid labeling. Rates of phospholipid synthesis were determined by pulse-labeling with $^{32}P_i$ (carrier-free; New England Nuclear Corp.). Strains were grown overnight in YEPD at 24°C to a cell density of no more than 2×10^7 cells per ml. Cultures were diluted or concentrated to 10^7 cells per ml in fresh YEPD. A 5-ml sample was removed for labeling at 24°C, and the remaining culture was placed at 37°C. Immediately, and after 15, 30, 45, 60, and 90 min, 5-ml samples were removed for labeling. Samples were mixed with 100 μCi of $^{32}P_i$ and incubated for 15 min at 24°C for the initial sample and at 37°C for all samples taken from the warmed culture. After labeling, cells were harvested by centrifugation and suspended in 5 ml of ice-cold 5% trichloroacetic acid. The cells were placed on ice for 1 h and then harvested by centrifugation. Cells were extracted with 1.0 ml of ethanol-water-diethyl ether-pyridine (15:15:5:1) at 60°C for 20 min (18). After cooling, cell debris was sedimented by centrifugation and extracts were drawn off. Extracts were subjected to a modified Folch wash (7) by adding 0.5 ml of water and 5 ml of chloroform-methanol (2:1). After mixing well, layer separation was enhanced by centrifugation, and the lower organic phase was withdrawn. The final extract was dried under air (passed through a charcoal and cotton filter) and spotted onto chromatography paper. Papers were dried Whatman SG81 paper, dipped in 2% EDTA, pH 7.0. Two-dimensional resolution of lipids was obtained in the first dimension with chloroform–methanol–30% ammonium hydroxide–water (66:27:3:0.9) and in the second dimension with chloroform-methanol-glacial acetic acid-water (32:4:5:1) (17, 19). Chromatograms were placed with Kodak SB-5 X-ray film overnight, and the radioactive spots were cut out and counted in a scintillation counter.

Continuous $^{32}P_i$ labeling was used to indicate the total cellular phospholipid composition in experimental cultures. Fresh cultures were started from overnight cultures, with 5×10^5 cells per ml in YEPD. $^{32}P_i$ (2 μCi/ml) was added, and the culture was incubated at 24°C for 10 to 20 h. Constant specific activity was assumed after 5.5 generations (3) when the cultures reached an optical density of 100 in a Klett spectrophotometer (3×10^7 cells per ml). Cultures were then shifted to 37°C and incubated for 90 min. Cells were harvested by centrifugation, and lipids were extracted, separated, and analyzed as described above.

Spheroplast osmotic sensitivity. Samples of 5 to 7 ml (6×10^7 cells) were removed from experimental cultures and washed with distilled water. Cell walls were removed by digestion for 15 min at 24°C in 3 ml of 1.5 M glycerol–100 mM thioglycolate–50 mM Tris-hydrochloride (pH 7.5) with 0.5 mg of Zymolyase 5000 (Kirin Breweries) per ml. A series of mixtures providing less and less osmotic support than that provided by the enzyme digestion mixture was prepared by diluting buffered glycerol-thioglycolate with water. For example, samples identified as "0.8" were 0.3-ml portions of the spheroplast digestion suspension placed in 1.5 ml of 80% 1.5 M glycerol–100 mM thioglycolate–50 mM Tris-hydrochloride (pH 7.5)–20% water. The proportion of spheroplasts remaining intact after 20 min at 24°C in each of these osmotic dilutions was determined by one of two methods. Intact spheroplasts were counted in a hemacytometer, or the optical density at 600 nm was measured (1). Data obtained by either method yielded nearly identical estimations of the proportion of spheroplasts remaining intact.

Cell viability. Viable cells were enumerated by plating suitable dilutions onto YEPD plates, incubating for 3 days at 24°C, and counting colonies. Colonies were replica plated to a fresh YEPD plate and incubated at 37°C to determine which colonies were temperature sensitive. The effects of osmotic supplementation on whole-cell growth and death were examined with two different types of medium supplements. Cultures were supplemented with salts at concentrations equal to normal yeast intracellular ion concentrations (5) (262 mM KCl, 14.5 mM $MgSO_4$, 5.5 mM NaCl, and 9.6 mM $CaCl_2$), or they were supplemented with 0.6 M sucrose, which offers osmotic support similar to the mixed salts, but without the specific ions. Cultures that had osmotic support were grown in YEPD medium with the supplements, diluted in the same medium, and plated on similarly supplemented agar plates. Auxotrophy for lipid precursors that are not present in YEPD medium was examined by replica plating colonies to YEPD plates supplemented with fatty acids (1.5% Tween 40, 1.5% Tween 80, 1 mM myristic acid, and 1 mM oleic acid) or ethanolamine (1 mM from a neutralized stock solution added to plates 1 h before use).

RESULTS

Rates of phospholipid synthesis. The rate of total cellular phospholipid synthesis increased in all *sec* mutant strains, similar to the increase in the wild-type strain after raising the growth

temperature from 24 to 37°C. None of the *sec* mutants exhibited immediate decreases in the rates of total phospholipid synthesis. Table 2 shows the changes in phospholipid synthesis detected in the *sec* mutants and in the wild-type X2180 strain. In the wild-type strain, the rate of total phospholipid synthesis increased immediately when the culture was warmed to 37°C and continued to increase as the cells grew. All *sec* mutant strains showed a rapid increase in the rates of phospholipid synthesis when shifted to the nonpermissive higher growth temperature. After the initial increase in phospholipid synthesis, most of the *sec* mutants exhibited decreasing synthesis. The 23 *sec* mutants presented a full spectrum of alterations in phospholipid synthesis, ranging from a rapid and drastic decrease in synthesis after the initial increase, in *sec20*, to a continually increasing rate of synthesis, greater than in the growing wild-type strain, in *sec8*. In all experiments, culture samples were plated and grown at 37°C to determine the presence of *SEC*$^+$ revertants. In no case reported did the culture contain more than 0.5% revertants contributing to the metabolic events studied.

Pulse-labeling of each major phospholipid species is shown in Fig. 1. The data presented were obtained from the wild-type strain X2180, from the *sec13* strain, which yielded a pattern typical of most of the *sec* mutants, and from the *sec20* and *sec21* strains, which showed the most severe alterations in phospholipid synthesis. The wild-type strain X2180 exhibited several distinct alterations in phospholipid synthesis during h 1 after warming to 37°C. Phosphatidylinositol and phosphatidic acid synthesis at 37°C increased steadily, in parallel with cell growth. Transient alterations in aminophospholipid synthesis were evident. Phosphatidylserine and phosphatidylethanolamine synthesis increased immediately when the culture was warmed and then remained constant, in the face of continued cell growth, for nearly 1 h before resuming increasing synthesis rates to match cell growth. The deficit in phosphatidylserine and phosphatidylethanolamine labeling appeared in increased phosphatidylcholine labeling.

The *sec8* and *sec15* strains exhibited phospholipid synthesis patterns similar to those shown in Fig. 1 for X2180 during the first 90 min at 37°C. All but three of the remaining *sec* mutants exhibited changes in phospholipid synthesis similar to those shown for *sec13*. The typical changes seen included (i) an initial increase in the synthesis of each phospholipid species; (ii) an early decline in phosphatidylserine and phosphatidylethanolamine labeling, similar to the wild-type labeling pattern, except that synthesis of these two phospholipids did not resume; and (iii) eventual failure of phosphatidylinositol and phosphatidylcholine synthesis. Phosphatidylinositol and phosphatidylcholine synthesis rates were lower at the 30- to 45-min pulse-labeling point than they were at the 15- to 30-min point, as shown for *sec13*, in all mutants in this group, except for five mutants that showed increasing synthesis of these two phospholipids until the 45- to 60-min pulse-labeling point: *sec1*, *sec11*, *sec17*, *sec18*, and *sec19*.

TABLE 2. Relative rates of phospholipid synthesis

Strain	Relative rate of phospholipid synthesis[a]					
	0 min[b]	15 min	30 min	45 min	60 min	90 min
sec1	2.62	3.59	4.15	4.25	3.16	2.08
sec2	1.79	1.63	1.40	0.81	0.82	0.40
sec3	1.18	1.96	1.67	1.19	0.77	0.85
sec4	1.99	1.90	1.46	1.88	1.14	0.56
sec5	1.01	2.18	1.68	1.50	1.25	1.06
sec6	2.30	3.41	3.54	3.42	2.97	1.38
sec7	0.98	2.68	2.06	1.59	1.52	1.03
sec8	1.60	4.23	3.43	2.73	3.94	5.54
sec9	1.69	2.94	2.44	1.56	1.12	0.93
sec10	1.65	1.86	1.45	1.51	1.05	0.54
sec11	1.52	1.82	2.02	1.80	1.58	0.96
sec12	2.05	1.90	1.78	1.44	1.29	0.81
sec13	1.63	2.14	1.31	1.19	1.06	0.73
sec14	1.56	1.85	0.93	0.61	0.66	0.52
sec15	1.11	1.75	1.26	1.75	1.79	2.05
sec16	1.00	1.83	2.80	2.06	2.09	1.07
sec17	1.64	2.30	2.02	2.93	1.83	1.72
sec18	1.71	2.02	2.33	2.03	1.51	1.52
sec19	2.29	1.54	3.22	3.02	1.42	1.52
sec20	1.56	1.05	0.74	0.59	0.51	0.27
sec21	1.84	1.16	0.90	1.27	0.82	0.72
sec22	1.03	1.42	1.28	1.15	0.78	0.76
sec23	1.33	1.73	1.45	1.34	1.02	0.87
X2180	1.45	2.33	2.29	2.66	3.08	4.56

[a] Ratios of total phospholipid labeled at 37°C pulse-labeling points to phospholipid labeled in initial 24°C culture samples.

[b] Pulse-labeling period: time at 37°C that 15-min pulse began.

Three exceptional mutants—*sec2*, *sec20*, and *sec21*—showed an initial increase in the synthesis of each phospholipid species, but did not show continued increases in phosphatidylinositol and phosphatidylcholine synthesis. Data from *sec20* and *sec21* are shown in Fig. 1. During the first 30 to 45 min at 37°C, labeling of each phospholipid species declined. However, only the declines in phosphatidic acid, phosphatidylinositol, and phosphatidylcholine labeling differ from the wild-type pattern. The *sec2* strain exhibited a phospholipid synthesis pattern similar to *sec20*, with less overall loss of synthesis, as is indicated by the total phospholipid data in Table 2.

Phospholipid composition. Cellular phospholipid composition, as opposed to rates of synthesis, was examined by steady-state ^{32}P labeling.

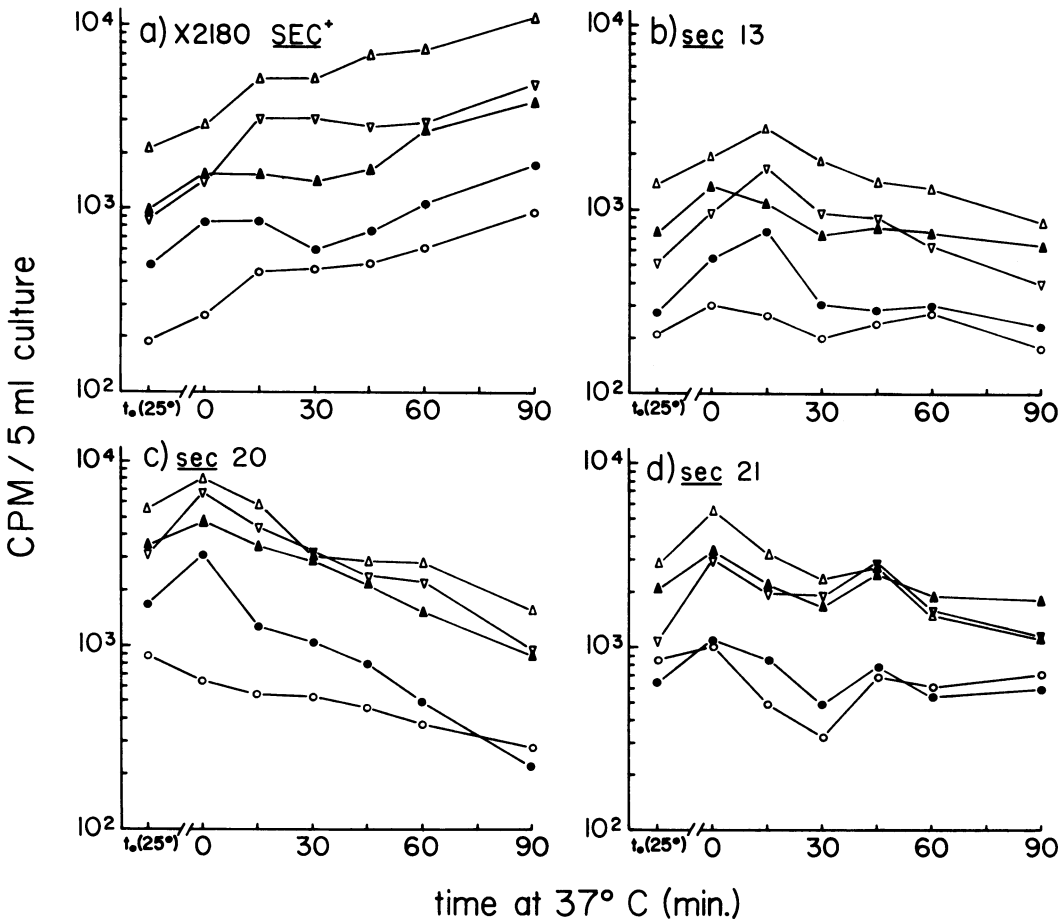

FIG. 1. Rates of phospholipid synthesis. ^{32}P pulse-labeling of each phospholipid species. Phospholipids indicated are: △, phosphatidylinositol; ▽, phosphatidylcholine; ●, phosphatidylethanolamine; ▲, phosphatidylserine; and ○, phosphatidic acid.

Table 3 shows the final phospholipid composition in each *sec* mutant after 90 min of incubation at 37°C. Also shown are X2180 compositions at 24°C and after 90 min at 37°C. Minor changes in the phospholipid composition of X2180 accompanied the change in growth temperature. Phosphatidylserine levels declined somewhat, balanced by increases in the other negatively charged lipids: phosphatidylinositol, cardiolipin, and phosphatidic acid. However, variability in phospholipid composition detected in different parallel cultures was nearly as great as the average changes observed. With this variation in mind, the *sec* mutants do not appear to differ from X2180 in total phospholipid composition, except with regard to phosphatidylcholine. Many, but not all, mutants contain higher levels of phosphatidylcholine than do similarly treated X2180 cells.

Spheroplast osmotic sensitivity. Osmotic sensitivity of spheroplasts prepared from normal and mutant cells that had been cultured at 24 or 37°C indicated that the *sec* mutants became progressively more sensitive at the restrictive temperature. The normal X2180 strain exhibited a distinct change in osmotic sensitivity when warmed to 37°C (Fig. 2). Growth at the higher temperature rapidly induced normal cells to change to a new stable state that is reflected by altered osmotic sensitivity. All of the *sec* mutants showed a pattern of progressively increasing osmotic sensitivity, similar to the pattern shown for *sec19* in Fig. 2. After 30 min at 37°C, spheroplasts of each *sec* mutant strain were more osmotically sensitive than spheroplasts derived from cells of the same strain grown at 24°C. However, the mutant strains did not exhibit as large an increase in osmotic sensitivity as did the X2180 strain within the first 30 min at 37°C. The very rapid shift to a new, more

TABLE 3. Final phospholipid composition[a]

Temp (°C)	Strain	% Total phospholipid[b]					
		PI	PS	PC	PE	CL	PA
37	*sec1*	25.1	10.4	37.4	20.4	3.7	3.0
37	*sec2*	32.2	10.6	36.1	17.6	2.3	1.4
37	*sec3*	28.5	11.3	35.6	18.7	5.1	0.8
37	*sec4*	34.1	9.4	35.5	16.2	3.9	0.9
37	*sec5*	25.4	12.4	39.6	18.4	3.1	1.1
37	*sec6*	21.8	11.0	39.9	21.7	2.8	2.8
37	*sec7*	25.1	11.9	36.5	21.6	4.9	1.0
37	*sec8*	27.3	13.9	32.4	22.3	3.2	0.9
37	*sec9*	29.8	8.6	39.3	18.0	2.8	1.5
37	*sec10*	33.6	5.9	31.6	23.1	4.3	1.5
37	*sec11*	28.5	11.6	37.7	19.0	1.9	1.1
37	*sec12*	25.8	6.2	39.0	23.7	4.0	1.4
37	*sec13*	29.1	7.5	35.5	22.1	4.4	1.5
37	*sec14*	24.3	11.0	43.4	14.6	4.4	2.4
37	*sec15*	25.4	12.2	39.2	17.0	5.5	0.8
37	*sec16*	33.9	9.3	29.5	22.4	3.6	1.3
37	*sec17*	31.8	8.9	35.2	19.6	3.4	1.1
37	*sec18*	30.8	6.3	35.5	23.2	3.3	0.9
37	*sec19*	26.2	9.5	37.0	22.2	3.9	1.2
37	*sec20*	26.0	6.0	38.2	25.8	2.4	1.6
37	*sec21*	26.9	10.6	38.2	19.9	3.4	1.0
37	*sec22*	25.0	8.7	35.5	22.8	7.1	1.8
37	*sec23*	27.5	7.2	37.8	21.5	4.1	2.0
37	X2180	30.5	9.4	34.2	19.5	5.6	0.9
37	X2180	25.9	10.4	37.4	19.5	5.0	1.7
37	X2180	30.4	9.9	33.3	18.5	5.3	2.7
37	X2180	32.5	8.3	34.0	17.4	5.4	2.3
24	X2180	26.9	10.3	35.0	22.1	4.0	1.8
24	X2180	24.1	14.4	36.6	19.3	3.8	1.9
24	X2180	31.2	11.0	35.2	17.1	3.5	1.9

[a] Phospholipids were labeled to a constant specific activity before transfer to 37°C.

[b] PI, Phosphatidylinositol; PS, phosphatidylserine; PC, phosphatidylcholine, PE, phosphatidylethanolamine; CL, cardiolipin (not detected in pulse-labeling); PA, phosphatidic acid.

sensitive stable state did not occur in any of the *sec* mutants. With time at 37°C, mutant spheroplasts exhibited a regular progression of increasing osmotic sensitivity, exceeding the temperature-induced change characteristic of normal cells. After 90 min at 37°C, most of the *sec* strains had become much more osmotically sensitive than normal cells and became even more so by 2 h at 37°C (Table 4).

Cycloheximide rescue. Cycloheximide treatment substantially prevented the progressive increase in spheroplast osmotic sensitivity of the *sec1* mutant strain (other mutants were not examined). Cycloheximide treatment itself, at 24°C, induced a rapid small increase in spheroplast osmotic sensitivity. After 1 h of cycloheximide treatment at 24°C, cell growth, monitored spectrophotometrically, had fully halted, and the induced osmotic increase was completed. The cycloheximide-treated *sec1* culture, warmed to 37°C, exhibited a further small increase in spheroplast osmotic fragility (Fig. 3). The small osmotic shift was similar in magnitude to the shift characteristic of normal warmed cells (Fig. 2). Cycloheximide-treated *sec1* cells at 37°C did not exhibit the progressively greater spheroplast osmotic sensitivity characteristic of all of the other *sec* mutants in the absence of the drug.

Cell viability. As has been shown for the *sec1* mutant (15), most of the 23 different *sec* mutants die at the restrictive growth temperature. Most mutant strains stopped growing immediately when warmed to 37°C, remained fully viable for 2 to 6 h, and then began to die logarithmically, leaving fewer than 5% of the cells alive after 24 h. Three mutants began logarithmic death immediately after warming to 37°C: *sec2*, *sec3*, and *sec23*. Two mutants suffered less death than

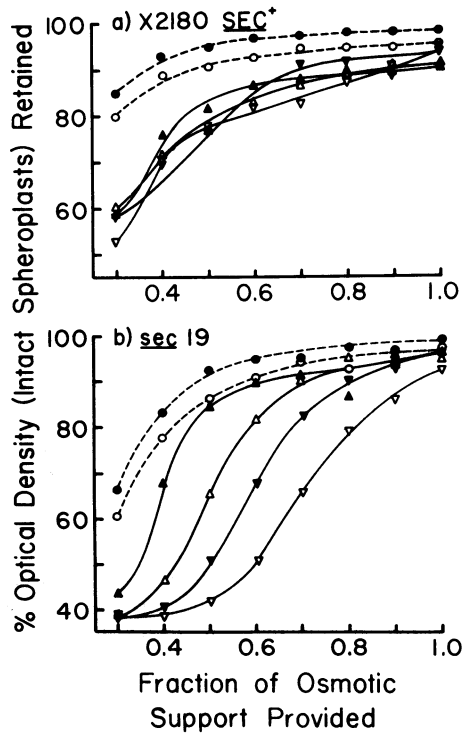

FIG. 2. Spheroplast osmotic sensitivity. Cell walls were stripped from cells grown at 24°C (● and ○, duplicates) and from cells grown at 37°C for 30 min (▲), 60 min (△), 90 min (▼), and 120 min (▽). The proportion of spheroplasts still intact after 20 min in dilutions of the spheroplast digestion mixture is shown. The digestion mixture (= 1.0 osmotic support) was buffered 1.5 M glycerol and 0.1 M sodium thioglycolate.

TABLE 4. Spheroplast osmotic sensitivity

Strain	Spheroplast osmotic sensitivity[a] at:		
	C 24°C[b]	37°C at 90 min	37°C at 2 h
X2180	92	76	76
sec1	84	20	16
sec2	73	48	25
sec3	80	50	33
sec4	83	58	48
sec5	91	63	58
sec6	93	75	69
sec7	88	60	47
sec8	88	74	65
sec9	80	61	44
sec10	84	55	47
sec11	76	33	26
sec12	64	17	14
sec13	74	29	30
sec14	73	28	16
sec15	78	47	35
sec16	88	46	36
sec17	90	70	54
sec18	76	27	31
sec19	86	51	42
sec20	82	63	50
sec21	90	51	52
sec22	88	51	43
sec23	90	63	46

[a] The percentage of spheroplasts maintained intact by half the osmotic support given in the cell wall digestion mixture.

[b] Lowest of two independent determinations.

other strains: *sec11* cultures did not begin to lose viability until 10 h at 37°C, and *sec14* began to die after 8 h but retained more than 30% viability after 24 h.

Cell viability was not affected by the addition of osmotic supplements or phospholipid precursors, with one exception. The *sec10-2* allele studied here appears to cause an osmotic-remedial alteration in its gene product, a common situation among temperature-sensitive mutants (8). In the presence of salts or 0.6 M sucrose, the *sec10-2* mutant strain grew normally at 37°C. None of the *sec* mutants grew or retained viability better at 37°C when supplemented with fatty acids or ethanolamine.

DISCUSSION

The studies on phospholipid synthesis and spheroplast osmotic sensitivity presented in this report support two conclusions about Novick and Schekman's 23 *sec* mutants. None of these secretion-defective mutants possesses membrane phospholipid defects that could be the cause of secretion failure. All of the mutants develop progressively greater spheroplast osmotic sensitivity, which can be interpreted to mean that plasma membrane growth fails at the same time that secretion fails.

Membrane phospholipids. All 23 of the *sec* mutants exhibited an initial increase in the rate of phospholipid synthesis equal to or greater than the increase characteristic of normal yeast cells when warmed from 24 to 37°C. Each phospholipid species was synthesized faster in warmed mutant cultures, with a single exception. The *sec20* mutant exhibited a consistent slight decline in the rate of phosphatidic acid labeling after warming to 37°C. Initial increased synthesis rates for the other phospholipid species, all derived from phosphatidic acid (Fig. 1), argue against a true defect in phosphatidic acid production in *sec20*. Increased synthesis of phospholipids in all 23 *sec* mutants indicates that the mutants do not possess temperature-sensi-

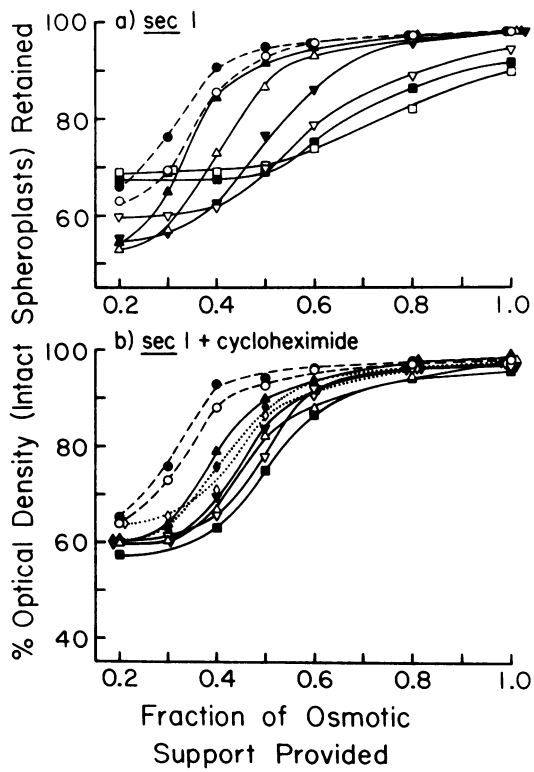

FIG. 3. Prevention of osmotic sensitivity by cycloheximide. Osmotic sensitivity was determined as described in the legend to Fig. 2, with spheroplasts prepared from *sec1* mutant cells grown with (b) or without (a) cycloheximide. Samples were taken from cells grown at 24°C (● and ○, duplicates), from cells grown at 24°C with cycloheximide (◆ and ◇, duplicates during 1-h treatment), and from untreated or treated cells cultured at 37°C for 30 min (▲), 60 min (△), 90 min (▼), 120 min (▽), 150 min (■), and 180 min (□).

tive lesions in membrane lipid biosynthesis enzymes.

After 30 min or more at 37°C, phospholipid synthesis declined in most of the *sec* mutants. In most cases it is clear that lipid biosynthesis declines only after secretory intracellular membrane traffic is completely blocked. Table 5 indicates the time, after warming to 37°C, when secretion fails (14), compared with the time when phospholipid synthesis first begins to decline. Most of the *sec* mutants continued vigorous phospholipid synthesis after secretion was blocked. In five mutants, phospholipid synthesis began to decline immediately after the secretory block took effect: *sec2*, *sec4*, *sec12*, *sec13*, and *sec20*. Only two mutants, *sec11* and *sec21*, showed reduced lipid synthesis before secretion completely failed. The two exceptions are unlikely candidates for a hypothesis that phospholipid deficiency blocks secretion, for the following reasons.

The *sec11* mutant rapidly accumulates internal invertase, but does not show a straightforward block in extracellular invertase secretion (14). Massive intracellular invertase accumulation within h 1 at 37°C (14) indicates that the secretory anomaly in *sec11* has been in effect for some time before a decline in phospholipid synthesis is detected. The *sec21* mutant exhibits declining rates of phospholipid synthesis before the time at which we estimate that secretion is fully blocked. The decline in phospholipid synthesis (Fig. 1) is not impressive. In yeast mutants known to possess specific lipid biosynthetis defects, a rapid 10-fold drop in the rate of lipid synthesis precedes other detectable cellular abnormalities (9, 10). Phospholipid synthesis in *sec21* declines gradually, taking a full 1 h to drop as low as the rate of synthesis in *sec21* cells at 24°C. This decline is not distinct enough to readily explain secretion failure. The *sec21* mutants accumulate distended endoplasmic reticulum at 37°C (14). Postreticulum secretory activity may continue after the *sec21* defect is fully established. Lipid biosynthetis enzymes, located at the endoplasmic reticulum, may respond with diminishing activity before secretion finally halts.

Plasma membrane growth. Each of the 23 *sec* mutants develops progressively increasing spheroplast osmotic fragility at the restrictive growth temperature. We interpret osmotic sensitivity as an indicator that plasma membrane growth is restricted. Support for this interpretation is provided by ruling out other membrane changes known to make yeast spheroplasts more fragile and by finding that cycloheximide blocks osmotic fragility development. The phospholipid composition found in the *sec* mutants (Table 3) falls within the normal, somewhat flexible, range.

TABLE 5. Correlation of phospholipid synthesis and secretion failures

Mutant(s)	% Invertase secretion[a]	Time (min) secretion fails[b]	Time (min) phospholipid synthesis fails[c]
sec1, *sec3*, *sec5*, *sec7*, *sec8*, *sec9*, *sec10*, *sec14*, *sec16*, *sec18*, *sec19*, *sec22*, and *sec23*	20 or less	10–15	30 or more
sec6, *sec15*, and *sec17*	50	30	45 or more
sec2, *sec4*, *sec12*, and *sec20*	20	15	15
sec13	50	30	30
sec11	100	60	45
sec21	75	45	15

[a] Calculated from the data of Novick et al. (14). Percentage of the wild-type amount of invertase secreted in 60 min at 37°C.

[b] Simplistic extrapolation: percent secretion × 60 min.

[c] The earliest pulse-labeling point where total phospholipid synthesis is lower than the previous point (data in Table 2).

Substantial increases in phosphatidylcholine and decreases in phosphatidylethanolamine make yeast spheroplasts more fragile (11), but are not evident as a possible cause of fragility in the *sec* mutants. Changes in lipid fatty acid composition and desaturation are the basis for the increased fragility of yeast cells grown at warmer temperatures (6). We have documented such an increase in warmed normal cells (X2180, shown in Fig. 2), but find that it is rapidly completed. By contrast, the *sec* mutants become progressively more fragile than can be explained by phospholipid adaptations to the growth temperature. Increased susceptibility to cell wall digestion does not explain progressively increasing osmotic sensitivity in the *sec* mutants. Spheroplasts that were offered very low osmotic support lysed to the same extent in samples from most cultures (see the lowest points shown in Fig. 2). The warmed *sec* mutants rapidly become more readily digested, as do normal cells, but further changes in digestibility are not evident. Some mutants, such as *sec1* (Fig. 3), become more resistant to cell wall digestion with time at the restrictive temperature.

Progressively increasng osmotic sensitivity in starved yeast inositol auxotrophs has been attributed to continuing internal metabolism within a restricted plasma membrane (2, 10). If the plasma membrane cannot expand, internal metabolite accumulation exerts greater and greater

osmotic stress on the membrane. Osmotic stress produced from within is prevented by cycloheximide (2). Cycloheximide substantially prevents progressively increasing osmotic fragility in the *sec1* mutant (Fig. 3), lending support to the interpretation that fragility reflects restricted plasma membrane growth.

Coordinate failure of secretion and plasma membrane growth. The *sec* mutants all exhibit progressively increasng osmotic sensitivity that exceeds the "adaptive" shift in fragility normal for warmed cells. Because the adaptive shift obscures events during the first 30 min at 37°C, we cannot detect plasma membrane growth earlier. After 30 min at 37°C, plasma membrane growth appears to be restricted in all of the *sec* mutants. Coordinate failure of secretion and plasma membrane growth during h 1 at the restrictive temperature is compatible with the hypothesis of Novick et al. (14) and Novick and Schekman (15) that the yeast secretory pathway is responsible for both extracellular protein secretion and plasma membrane assembly. The *sec* mutants exhibit several features shared by yeast inositol auxotrophs whose plasma membranes stop growing. Both sets of mutants become very dense with accumulated material inside a restricted cell volume and eventually die due to cytoplasmic congestion (2, 10, 14, 15). Secretion-defective mutants that do not become dense and do not die have not been sought in yeast cells, although nonlethal secretory mutants are known in *Paramecium* cells (4, 13). The available sets of yeast secretory mutants indicate that at least 23 gene products are required simultaneously for both secretory intracellular membrane traffic and plasma membrane assembly.

ACKNOWLEDGMENTS

We are indebted to Randy Schekman and Peter Novick for providing us with their *sec* mutants and advice in proceeding with this project.

This work was supported by American Heart Association grant 81-679, the University of California Cancer Research Coordinating Committee, the University of California Academic Senate, and a University of California Vice Chancellor's Junior Faculty Fellowship and undergraduate research grants.

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