Role of Wall Phosphomannan in Flocculation of *Saccharomyces cerevisiae*

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SUMMARY

Treatment with 60 % hydrofluoric acid (HF) removed most of the phosphorus and small amounts of mannan, glucan and protein from walls of two non-flocculent strains (NCYC366 and NCYC1004) and two flocculent strains (NCYC1005 and NCYC1063) of *Saccharomyces cerevisiae*. Organisms of all strains showed increased flocculating ability following HF treatment. Flocculation of untreated organisms of NCYC1005 and NCYC1063, and of HF-treated organisms of all four strains, declined appreciably when they were washed in deionized water, with or without EDTA, and the flocculation was measured in deionized water instead of in 0.05 M-sodium acetate containing Ca^{2+} . Treatment with 1,2-epoxypropane also caused a decrease in the flocculating ability of these organisms. Extracting the lipids from organisms of strains NCYC366 and NCYC1004 had no effect on their flocculating ability, but decreased the flocculating ability of organisms of strains NCYC1005 and NCYC1063. pH-electrophoretic mobility curves of untreated and HF-treated organisms confirmed the loss of wall phosphate by HF treatment, and indicated that HF treatment had little effect on the content of protein carboxyl groups in the outer wall layers. Mannose at 0.22 M completely prevented floc formation by organisms of strain NCYC1063; but, even at 0.33 M, it had very little effect on floc formation by HF-treated organisms of strains NCYC366 and NCYC1063. Organisms of all four strains bound fluorescein-conjugated concanavalin A to the same extent after treatment with HF as before, but this treatment led to a greatly diminished binding of fluorescein-conjugated antiserum raised against organisms of strain NCYC366. The results indicate that phosphodiester linkages in yeast-wall mannan are not involved in bridge formation through Ca^{2+} during floc formation and that this arises principally through carboxyl groups.

INTRODUCTION

Flocculation is the term used to describe the property expressed by certain strains of *Saccharomyces cerevisiae* or *S. carlsbergensis* when, at the end of a brewery fermentation, they spontaneously aggregate to form flocs which sediment in the fermentation vessel (for reviews, see Rainbow, 1970; Stewart, 1975). Although the mechanism of flocculation has interested yeast biochemists for many years, the molecular basis of the phenomenon is far from being understood. Two types of aggregation mechanism have been advocated. The first suggests that one or more anionic components in the walls of adjacent cells are linked through salt bridges, and there is general agreement that the bivalent cation involved is Ca^{2+} . Other workers believe that the aggregation mechanism is more complex, and involves not only salt bridges but also other types of secondary interaction between components in yeast walls.

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There is also a division of opinion on the nature of the anionic wall components involved. Harris (1959) postulated intercellular bonding in which bivalent cations link carboxyl groups in adjacent cells. Mill (1964*b*) largely agreed with Harris (1959) but suggested that the floc is stabilized by co-operative hydrogen bonding involving hydroxyl groups on the cell-surface polysaccharide. Recent work by Stewart, Russell & Garrison (1975) furnished additional evidence for the formation of carboxyl- Ca^{2+} complexes in yeast flocs, and they suggested that the carboxyl groups involved are in acidic wall proteins. Lyons & Hough (1970*a, b*, 1971), on the other hand, believe that the anionic groups involved are mainly, if not solely, in the phosphodiester linkages in the wall mannan (Ballou, 1974, 1976). They found that walls of flocculent strains of *S. cerevisiae* contain more phosphorus in the outer layers of the wall than non-flocculent strains, and bind twice as much Ca^{2+} as do walls of non-flocculent strains. Although they found that esterification of carboxyl groups in the wall with 1,2-epoxypropane caused a drop of only 20% in the calcium-binding capacity of the wall, they failed to find a correlation between the amounts of acidic amino-acid residues in the walls and the flocculating ability of organisms from which they were derived. Lyons & Hough (1971) concluded that the capacity of strains of *S. cerevisiae* to flocculate is proportional to the density of phosphate groups in the phosphomannan-protein layer of the wall.

The present paper reports findings which allow an assessment to be made of the relative contributions from acidic amino-acid residues and phosphodiester linkages in the flocculation of *S. cerevisiae*. A technique was used which excises from the wall mannan phosphodiester linkages and mannose residues distal to the phosphodiester linkages. The technique, in which organisms and walls are treated with hydrofluoric acid (HF), has previously been used to excise phosphodiester linkages from other polymers including teichoic acids (Glaser & Burger, 1964; Archibald, Baddiley & Shaikat, 1968) and bacterial phospholipids (Shaw & Stead, 1974), as well as from mannan extracted from *S. cerevisiae* (Cawley, Harrington & Letters, 1972). The reaction catalysed by HF may be represented as:

METHODS

Organisms. Four strains of *Saccharomyces cerevisiae*, NCYC366, NCYC1004, NCYC1005 and NCYC1063, were used in this study. Stock cultures were maintained at 4 °C on slopes containing (% w/v): 0.3 malt extract, 0.3 yeast extract, 0.5 mycological peptone, 1.0 glucose, and 2.0 agar (MYGP medium, pH 4.5; Wickerham, 1951).

Experimental cultures. Organisms were grown batchwise in 1 l portions of MYGP medium in 2 l round flat-bottomed flasks. Batches of medium were inoculated with a portion from a 24 h culture in MYGP medium containing approximately 10.0 mg dry wt organisms. Cultures were incubated at 30 °C with stirring, as described by Patching & Rose (1970). Organisms were harvested from stationary-phase cultures, containing 1.3 to 1.5 mg dry wt ml^{-1} , by centrifuging at 0 °C at 2000 *g*. They were washed twice with distilled water and, unless otherwise indicated, freeze-dried and stored at 4 °C over silica gel in a vacuum desiccator.

Measurement of flocculation. The flocculating ability of organisms was measured by a modification of the methods used by Mill (1964*a*) and Greenshields *et al.* (1972). Organisms were suspended at approximately 4 mg dry wt ml⁻¹ in either 0.05 M-sodium acetate buffer pH 4.5 containing 0.1 % (w/v) CaCl₂ or in deionized water adjusted to the same pH. A portion (3 ml) of this suspension was placed in a cuvette (1 cm path length), which was then inverted two or three times to bring the organisms into uniform suspension. Using a Pye-Unicam SP1800 spectrophotometer with an automatic recorder, the extinction of the suspension at 600 nm was measured over a period of 5 to 10 min against an appropriate blank. Extinction values were converted to μg dry wt ml⁻¹ and a graph was plotted of dry wt ml⁻¹ against time. Sedimentation rates were calculated by measuring the maximum slope of the curve, and are expressed as μg dry wt ml⁻¹ min⁻¹.

Preparation of walls. Yeast walls were prepared by shaking a suspension of organisms (200 mg dry wt in 20 ml 0.1 M-KH₂PO₄) with glass beads (30 g; 0.17 to 0.18 mm diam.; Glasperlen, from Braun, Melsungen, West Germany) for 2 to 3 min in a Braun homogenizer, while cooling the glass homogenizer bottle with liquid CO₂. Walls were separated from the suspension of disrupted organisms by the method of McMurrough & Rose (1967), and washed 10 times with ice-cold water. The absence of intact organisms from the wall preparations was established by microscopic examination of methylene blue-stained preparations (Chattaway, Holmes & Barlow, 1968). Walls were freeze-dried and stored at 4 °C over silica gel in a vacuum desiccator.

Treatment of organisms and walls with HF. Freeze-dried organisms or walls (100 mg) were incubated with 58 to 62 % (v/v) HF (1 ml) at 0 °C in a polythene tube for 3 to 5 h. The tube was then cooled to about -60 °C in a bath containing solid CO₂ and ethanol, and a calculated quantity of 2 M-KOH was added to largely neutralize the HF. The mixture was then allowed to warm slowly to 0 °C and the pH value was adjusted to 7.0 with solid K₂CO₃. The organisms or walls were separated by centrifuging and washed at least five times with water. Washed organisms or walls were freeze-dried and stored at 4 °C over silica gel in a vacuum desiccator.

Analysis of walls. Glucan and mannan contents of walls were determined by the method of McMurrough & Rose (1967) which is a modified sulphuric acid-carbazole differential extinction method. Total nitrogen content of walls was determined by the micro-Kjeldahl technique, using a colorimetric method (Fraser & Russell, 1969) to determine the NH₄⁺ content of the micro-Kjeldahl digest. The protein content was calculated by multiplying the value for the nitrogen content by 6.3. The method of Chen, Toribara & Warner (1956) was used to determine the phosphorus content of walls.

Measurement of electrophoretic mobility. The electrophoretic mobility of organisms at different pH values was measured using a Cytopherometer (Zeiss, Oberkochen, West Germany) and a modification of the method of Somers & Fisher (1967). Movement was timed over 80 μm in both directions (current reversal). Each mobility value was the mean of at least 20 observations. Mobilities of organisms were measured using suspensions (10 ml) containing approximately 0.5 mg dry wt (10⁷ organisms) ml⁻¹. The buffers used (Gittens & James, 1963) were NaCl/HCl (pH 1.5 to 2.0) or NaCl/sodium acetate/sodium barbiturate/HCl (pH 3.0 to 9.0), and had an ionic strength of 0.05 or 0.005. The conductivity of each buffer was measured using a Griffin conductivity bridge (Griffin & George, Wembley, Middlesex).

Staining with fluorescein-conjugated antibody. Antiserum against *S. cerevisiae* NCYC366 was prepared by injecting healthy adult rabbits (approx. 2.5 to 3.0 kg wt) with organisms grown in Sabouraud dextrose broth at 30 °C and harvested in the stationary phase of

growth, as described by Klaushofer & Dorfworth (1970). Immunofluorescent staining of organisms was carried out using the sandwich technique described by Richards & Cowland (1967); stained smears were examined using a Leitz Orthoplan microscope (Leitz, Wetzlar, West Germany) fitted with a fluorescence vertical illuminator.

Staining with fluorescein-conjugated concanavalin A. Fluorescein was conjugated to concanavalin A by the method of Tkacz, Cybulska & Lampen (1971), and the conjugate was purified using a column of Sephadex G-75 (Pharmacia). Strains of *S. cerevisiae* were stained with the fluorescein-conjugated concanavalin A using the method of Tkacz *et al.* (1971). Stained smears were examined microscopically in the same manner as preparations stained with fluorescein-conjugated antibody.

Lipid extraction. Lipids were extracted from organisms by the method of Hunter & Rose (1972). After extraction, organisms were washed in distilled water.

Chemicals. All chemicals, except those listed below, were of Analar grade, obtained from BDH. Hydrofluoric acid (58 to 62 %, w/v) was from Fisons Scientific Apparatus, Loughborough, Leicestershire. All ingredients of MYGP medium except malt extract were obtained from Oxoid. Malt extract was purchased from Munton & Fison, Stowmarket, Suffolk, concanavalin A from Pharmacia, and fluorescein isothiocyanate from Calbiochem. Fluorescein-conjugated anti-rabbit (sheep) immunoglobulin was supplied by Wellcome Reagents, Beckenham, Kent.

RESULTS

Effect of HF on wall composition

Walls from the strains of *S. cerevisiae* used in this study differed slightly in their contents of glucan, mannan, protein and phosphorus (Table 1). These components accounted for 84 to 91 % of the dry weight of all walls, the remainder presumably being lipid and glucosamine residues, neither of which was determined. Treating walls with HF led to the loss of 7 to 10 % of the dry weight. About half of this loss can be attributed to the disappearance of a large proportion of the phosphorus from the walls, together with some mannan and a little glucan. Walls of strain NCYC1063 also lost some protein during treatment with HF.

Flocculation of organisms before and after treatment with HF

Sedimentation rates were routinely measured on freeze-dried organisms, since both freshly-harvested and freeze-dried organisms formed flocs and sedimented at the same rate. Organisms of strains NCYC366 and NCYC1004 sedimented much more slowly than those of strains NCYC1005 and NCYC1063 (Table 2), which is in general agreement with the findings of Lyons & Hough (1970*b*). Treatment with HF increased the sedimentation rates of organisms of all four strains, but proportionately more so with strains NCYC366 and NCYC1004. When the rates of floc sedimentation of untreated organisms of strains NCYC1005 and NCYC1063, and of HF-treated organisms of all four strains, were measured in deionized water after washing in the same, they were appreciably lower, and with untreated organisms of strains NCYC1005 and NCYC1063 were of the order obtained with untreated and unwashed organisms of strains NCYC366 and NCYC1004. The reduction in the rates was even greater when HF-treated organisms were washed in 10 mM-EDTA before measuring the rates in deionized water (Table 2). Organisms which had been deflocculated by washing in deionized water or EDTA regained the ability to form flocs when CaCl_2 (0.1 %, w/v) was included in the deionized water in which the rate was measured. Treating organisms with the esterifying agent 1,2-epoxypropane (Mill, 1964*b*; Lyons & Hough, 1970*b*) lowered the rate of floc sedimentation of all four strains after they had been treated with HF. This 1,2-epoxypropane

Table 1. *Changes in composition of walls of Saccharomyces cerevisiae caused by treatment with hydrofluoric acid*

Values quoted are mean values $\pm 95\%$ confidence limits. Analyses for HF-treated walls give the amounts of each component present in the residue remaining after HF treatment of 100 mg dry wt walls.

Component	Weight of component (mg) in wall							
	NCYC366		NCYC1004		NCYC1005		NCYC1063	
Glucan	Untreated	HF-treated	Untreated	HF-treated	Untreated	HF-treated	Untreated	HF-treated
	35.0	33.9	36.4	35.9	40.8	38.8	46.9	44.7
	± 4.4	± 5.1	± 4.6	± 3.9	± 4.6	± 4.6	± 4.5	± 4.2
Mannan	45.0	42.0	44.5	41.1	44.2	41.2	31.1	28.3
Protein	7.0 ± 0.3	6.9 ± 0.3	6.3 ± 0.3	6.0 ± 0.3	7.6 ± 0.2	7.5 ± 0.2	12.1 ± 0.3	10.0 ± 0.3
Phosphorus	1.20 ± 0.01	0.10 ± 0.01	0.40 ± 0.2	0.08 ± 0.01	0.50 ± 0.06	0.08 ± 0.01	0.70 ± 0.01	0.10 ± 0.01
Weight of wall sample	100	91	100	91	100	93	100	90

Table 2. *Effect of various secondary treatments on flocculation of strains of Saccharomyces cerevisiae before and after treatment with hydrofluoric acid*

Sedimentation rates quoted are mean values $\pm 95\%$ confidence limits.

Secondary treatment	Flocculation measured in	Sedimentation rates ($\mu\text{g dry wt ml}^{-1} \text{ min}^{-1}$)							
		NCYC366		NCYC1004		NCYC1005		NCYC1063	
None		Untreated	HF-treated	Untreated	HF-treated	Untreated	HF-treated	Untreated	HF-treated
	Sodium acetate	165 ± 12	1846 ± 26	120 ± 7	1666 ± 11	1764 ± 11	3000 ± 21	1200 ± 13	2181 ± 12
	(0.05 M) containing CaCl_2 (0.1 % w/v)	—	600 ± 22	—	400 ± 8	100 ± 6	420 ± 11	100 ± 5	360 ± 7
Deionized water	EDTA (10 mM)	—	150 ± 6	—	128 ± 9	—	90 ± 7	—	120 ± 6
Deionized water	I,2-Epoxypropane (5 % w/v)	—	1153 ± 38	—	705 ± 7	880 ± 11	705 ± 7	675 ± 7	461 ± 6
	Sodium acetate	—	—	—	—	—	—	—	—
	(0.05 M) containing CaCl_2 (0.1 % w/v)	—	—	—	—	—	—	—	—
KF (11 % w/v) for 5 h	Sodium acetate	151 ± 7	—	124 ± 5	—	925 ± 23	—	1065 ± 25	—
	(0.05 M) containing CaCl_2 (0.1 % w/v)	—	—	—	—	—	—	—	—
Lipid extraction	Sodium acetate	148 ± 9	—	132 ± 8	—	540 ± 17	—	200 ± 11	—
	(0.05 M) containing CaCl_2 (0.1 % w/v)	—	—	—	—	—	—	—	—

Fig. 1. Effect of mannose on the sedimentation rates of untreated and HF-treated organisms of *Saccharomyces cerevisiae*: ●, Untreated organisms of strain NCYC1063; ○, HF-treated organisms of strain NCYC1063; and ▽, HF-treated organisms of strain NCYC366.

Fig. 2. pH-mobility curves of untreated and HF-treated organisms of *Saccharomyces cerevisiae*: (a) NCYC366; (b) NCYC1004; (c) NCYC1005; and (d) NCYC1063. Mobilities of (○, ●) untreated organisms, and (△, ▲) HF-treated organisms were measured in buffers with ionic strengths of (○, △) 0.005, and (●, ▲) 0.05. Mobility values quoted are the means of at least 20 observations. The standard error of the means was less than 2%.

treatment also lowered the sedimentation rates of untreated organisms of strains NCYC1005 and NCYC1063; the effect on untreated organisms of strains NCYC366 and NCYC1004 was such that the rates were too slow to be measured accurately.

As some of the effects following treatment of yeasts with HF may have been caused by retention of F^- in the envelope layers of organisms, untreated organisms were suspended in 11% (w/v) KF for 5 h, and their rates of floc sedimentation were then measured. This treatment, however, had no measurable effect on the rates for organisms of any of the strains except NCYC1005, for which the rate was reduced to about half of that for organisms that had not been suspended in KF solution (Table 2).

Since the phosphodiester group in phospholipids is susceptible to the action of HF (Shaw & Stead, 1974) and yeast walls may contain small amounts of phospholipid, the effect of lipid extraction on the rates of floc sedimentation was examined. Lipid-depleted organisms of strains NCYC366 and NCYC1004 formed flocs and sedimented at the same rate as untreated organisms, but the rate at which organisms of the more flocculent strains sedimented after lipid extraction was appreciably reduced (Table 2).

Mannose is very effective in preventing floc formation by *S. cerevisiae* when included in the suspending buffer (Eddy, 1955). When this sugar was included in the buffer at 0.22 M, it virtually prevented floc formation by the flocculent strain NCYC1063 (Fig. 1). However, the presence of mannose up to 0.33 M in the buffer had little retarding effect on the sedimentation rates of HF-treated organisms of strains NCYC1063 or NCYC366 (Fig. 1). Data on the effect of mannose on the flocculation of untreated organisms of strain NCYC366 are not included, since in the presence of mannose their sedimentation rate was too slow to permit accurate measurement.

pH-mobility curves of untreated and HF-treated organisms

Figure 2 shows the effect of pH value on the electrophoretic mobility, in buffers of ionic strength 0.05 or 0.005, of untreated and HF-treated organisms of all four strains of *S. cerevisiae*. Mobility at pH 4.0, which is attributable to ionization of phosphodiester groups (Eddy & Rudin, 1958), was greatly decreased in HF-treated organisms, and with organisms of strain NCYC1004 in buffer with an ionic strength of 0.05 and of strain NCYC1005 at both ionic strengths, mobility at this pH value was almost completely absent. The mobility attributable to protein in the wall surface, a measure of which is the difference between the mobilities at pH 7.0 and 3.0 (Eddy & Rudin, 1958), was also reduced by HF treatment, but not nearly to the same extent as that for which phosphodiester linkages are responsible. Mobilities in buffers of ionic strength 0.05 and 0.005 have been attributed to groups which lie at depths of 1.4 and 4.2 nm, respectively, from the wall surface (Fisher, 1975). Organisms of strains NCYC1004 and NCYC1005 generated appreciably fewer ionogenic groups in the deeper surface shell of the wall, whereas organisms of strain NCYC1063 and to some extent of NCYC366 had approximately the same mobility at different pH values irrespective of the ionic strength of the buffer.

Binding of fluorescein-conjugated concanavalin A and antibody by untreated and HF-treated organisms

Organisms of all four strains of *S. cerevisiae* bound fluorescein-conjugated concanavalin A to the same extent after treatment with HF as before. However, binding of fluorescein-conjugated antiserum raised against *S. cerevisiae* NCYC366 was appreciably less in all four strains after HF treatment (scored as 1+ compared with untreated organisms at 4+).

DISCUSSION

Treatment of walls with HF led to the removal of 80 to 90 % of the wall phosphorus which it is assumed is located mainly, if not exclusively, in phosphodiester linkages in the outer chains of the wall mannan. Losses of other wall components following HF treatment, with the exception of the protein from walls of strain NCYC1063, were very small and, with glucan, probably within the limits of error of the analytical method. The consistently larger decrease in the mannan content is probably attributable to the loss of mannose and manno-biosyl residues that were located distal to the phosphodiester linkages in the mannan side chains. The loss of protein from walls of strain NCYC1063 could mean that, in this strain, the mannan has a somewhat different structure from the polymer in other strains of the species, with protein being associated in some way with the mannan side-chains. That staining with fluorescein-conjugated concanavalin A, which binds specifically to mannan in the wall (Tkacz *et al.*, 1971), was not altered by treating any of the organisms with HF confirms that the bulk of the α -linkages in the mannan are not affected by the treatment. However, HF-treated organisms were less able to bind fluorescein-conjugated antibody raised against strain NCYC366; this is almost certainly due to loss of terminal side-chain α -D-mannopyranosyl phosphate units which are known to be immunological determinants in strains of *S. cerevisiae* (Raschke & Ballou, 1971).

There did not appear to be any correlation between the contents of wall phosphorus and the sedimentation rates of untreated organisms of all four strains. Lyons & Hough (1970*b*) found that the outer layers of walls of strains NCYC1005 and NCYC1063 contained more phosphorus than the corresponding layers from the walls of non-flocculent organisms of strain NCYC1004. However, our electrophoretic mobility measurements at pH 4.0, which give a measure of the density of phosphate groups in the outer layers of the wall to a depth of 4.2 nm with buffer of ionic strength 0.005 (Eddy & Rudin, 1958; Fisher, 1975), did not indicate any correlation between the phosphate contents of the outer layers of walls and the flocculation rate of organisms. Nor could the mobility attributable to protein (the mobility at pH 3.0 subtracted from that at pH 7.0; Eddy & Rudin, 1958) be correlated with sedimentation rates.

The increased flocculation rates shown by HF-treated organisms were rather surprising since removal of phosphodiester linkages from the wall mannan was expected, at least, not to increase the rate, and possibly to lower it. HF-treated organisms showed a very large decrease in mobility due to phosphate, and in some strains this mobility was completely absent. However, the decrease in the mobility attributable to protein in all strains was very much smaller. There was no indication with any of the strains that new ionogenic groups were exposed following HF treatment. While these findings may indicate that phosphodiester linkages in the wall mannan are not involved in cross-bridge formation during flocculation of *S. cerevisiae*, we still need to identify those anionic groups in the wall which may be involved in the cross-linking process, and to resolve why removing wall phosphomannan increases flocculation.

Our evidence suggests that carboxyl groups in the wall protein are involved in cross-linkages with Ca^{2+} . The diminished ability of treated and untreated organisms to form flocs after they had been washed in deionized water or 10 mM-EDTA, and the capacity of Ca^{2+} to restore floc-forming ability, suggest that Ca^{2+} ions are involved in bridge formation in both HF-treated and untreated organisms. The decrease in flocculation of untreated flocculent organisms and of HF-treated flocculent and non-flocculent organisms after exposure to the esterifying agent 1,2-epoxypropane is evidence that the ionic groups involved are

probably carboxyl groups in the wall protein. That treatment with the esterifying agent caused only a partial loss of floc-forming ability can be explained by the incomplete nature of the esterification process, as reported by Gittens & James (1963). The decrease in flocculation following extraction of lipids could well be due to removal from the wall of hydrophobic proteins along with lipids—proteins that, despite their overall hydrophobic nature, may well contain a sufficiently large proportion of free carboxyl groups to play an important role in bridge formation.

The increased rate of sedimentation of HF-treated organisms is not caused by retention of F^- in the wall following HF treatment as exposing untreated organisms to KF solution had no effect on flocculation, and indeed lowered it with organisms of strain NCYC1005. The most plausible explanation of the increased flocculation in HF-treated organisms is that it results from removal of the repulsive force created by the negative charges in the phosphodiester linkages which, in untreated organisms, help to keep organisms apart. Extending this hypothesis, we suggest that, in untreated flocculent organisms, there is a competition between the repulsive forces generated by the anionic groups (carboxyl as well as phosphodiester groups) and the attractive forces that arise when conditions are conducive for Ca^{2+} bridge formation between carboxyl groups in the wall proteins. When the attractive forces overcome the repulsive forces and a floc begins to form, the attraction of one cell to another is probably intensified by hydrogen bonding between mannose residues which lie distal to the phosphodiester on mannan side-chains in adjacent cells. Evidence for this last suggestion comes from the discovery that, whereas floc formation by untreated organisms is prevented by mannose, floc formation by HF-treated organisms of both NCYC1063 and NCYC366, which lack mannose residues distal to the excised phosphodiester linkages, is not prevented by mannose. Ballou (1974, 1976) has isolated a mutant of *S. cerevisiae* x2180 which lacks the ability to add mannose phosphate side-chains to the wall mannan. The availability of this mutant and of its parent appeared to offer a system with which to confirm our findings with untreated and HF-treated organisms. Unfortunately, the rate at which these haploid organisms sedimented was too low to permit accurate measurements to be made.

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