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Effect of 2-Deoxyglucose on Cell Wall Formation in *Saccharomyces cerevisiae* and Its Relation to Cell Growth Inhibition

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The growth inhibition and the lysis of *Saccharomyces cerevisiae* caused by 2-deoxy-D-glucose (2-DG) were shown to be a consequence of unbalanced cellular growth and division. The lysis, but not the repression of growth and osmotic fragility of cells, could be suppressed by the addition of mannitol as an osmotic stabilizer. This result, as well as the morphological changes observed in the cells and changes in the chemical composition of the cell walls, showed that *S. cerevisiae* grown in the presence of 2-DG formed weakened cell walls responsible for the osmotic fragility. Evidence is presented for the first time demonstrating the incorporation of 2-DG into yeast cell wall material. Other data suggest that the inhibition of yeast growth by 2-DG results from an interference of phosphorylated metabolites of 2-DG with metabolic processes of glucose and mannose involved in the synthesis of structural cell wall polysaccharides.

Investigation of the inhibitory effect of 2-deoxy-D-glucose (2-DG) on yeast growth has suggested that this action may be due to an interference of 2-DG metabolites with the synthesis of cell wall polysaccharides (13, 17). This proposal was supported when it was found that 2-DG entered yeast uridine and guanosine nucleotides, forming deoxy analogues of uridine diphosphate glucose and guanosine diphosphate mannose (2, 3), and competitively inhibited the synthesis of cell wall components in regenerating yeast protoplasts (9). The observation that yeast grown in the presence of 2-DG underwent lysis at the sites of new cell wall material synthesis further pointed out that the primary target of 2-DG and its metabolites is directed at cell wall formation (17). Johnson (15) suggested that 2-DG-induced lysis is caused by dissolution of cell wall glucan by glucanases involved in normal growth mechanism. He also postulated that uridine diphosphate-2-deoxy-D-glucose hinders the repair processes of the glucan molecules either by inhibition of glucose insertion or by its own incorporation into glucan cuts.

This paper reports the results of further study of the mechanism of the inhibitory effect of 2-DG on yeast growth. Evidence is presented that the inhibition of polysaccharide synthesis by 2-DG metabolites results in osmotically fragile cells with weakened cell walls, and the molecular basis of this inhibition is discussed.

MATERIALS AND METHODS

Yeast and cultivation. *Saccharomyces cerevisiae* strain CCY 21-4-13 was grown in glucose and lactate medium on a shaker at 28 C. The glucose medium contained 2% glucose, 0.2% $(NH_4)_2SO_4$, and 0.75% brewer's yeast extract prepared by autolysis. In few experiments this medium was osmotically stabilized with 0.7 M mannitol. The lactate medium contained 2% DL-lactic acid (Lachema, Czechoslovakia), 0.2% $(NH_4)_2SO_4$, and 0.75% brewer's yeast extract, all dissolved in 0.02 M KH_2PO_4 and adjusted to *p*H 5.5 with NaOH. 2-DG was sterilized by filtration and added to the above autoclaved media. Growth was followed by measuring the increase in optical density at 420 nm of cell suspensions. The viability of cells was estimated by counting the dead cells after staining with methylene blue. To 0.5 ml of cell suspension in growing medium, 0.1 ml of an 0.05% solution of methylene blue in 0.1 M phosphate buffer (*p*H 5.5) was added. Blue-stained cells, blue-stained buds, and visibly empty cell walls were counted as dead cells.

Preparation of cell walls. Cell walls were prepared from yeast grown in glucose medium in the absence and in the presence of 2-DG (0.2%). After the growth had reached a plateau, the cells were collected by centrifugation, washed with water, and then suspended in 15-g portions (wet cell paste) in 10 ml of distilled water, mixed with 45 to 50 g of glass beads (0.5-mm diameter), and disrupted in a rotory disintegrator for 25 min, which was sufficient for complete rupture of the cells. The preparation of disrupted cells was then diluted with cold distilled water, the glass beads were removed, and the cell walls were isolated by centrifugation at 1,600 × *g* for 10 min. The sedimented cell walls

were further purified by at least 40 washings with cold distilled water followed by centrifugation at 1,600 × *g*. A larger number of washings was required to clean the cell walls of yeast grown in glucose medium with 0.2% 2-DG. These cell walls seemed to be more hydrophobic than the cell walls of control yeast, and they aggregated when suspended in water. The cell walls were examined by light and electron microscopy. The washed cell walls were freeze-dried and chemically analyzed.

Analysis of cell walls. Glucose and mannose were determined essentially as described by Power and Challinor (19). The samples were hydrolyzed in 2 N H_2SO_4 (100 C, 6 hr), and the solution was treated with $BaCO_3$ and passed through a Dowex 50 (H^+) column. Neutralized samples were taken for assay of glucose with the glucose oxidase test (Boehringer and Soehne GmbH, Mannheim, Germany) and for determination of total hexoses by the anthrone method. The mannose content was calculated by subtraction of the glucose value from the hexose value.

Glucosamine was determined in cell wall hydrolysates (4 N HCl, 100 C, 12 hr) by the method of Cessi and Piliego (4).

Phosphorus was determined by using the micromethod of Chen et al. (6).

Total nitrogen was estimated by the method of Dumas. The values of protein nitrogen were obtained by subtracting the glucosamine nitrogen content.

Lipids were determined gravimetrically. Cell walls were first extracted for 24 hr at room temperature with 50 portions of chloroform/methanol (2:1) and then for 24 hr with 50 portions of the same solution containing 1% (v/v) concentrated HCl. Both extracts were combined and evaporated in vacuo. The residue was extracted with absolute ether, and the solution was separated from the insoluble material and evaporated to dryness. The dry etheral extract was weighed and counted as total lipids of cell walls.

For determination of 2-DG, the cell walls were suspended in 0.125 N H_2SO_4 and heated in stoppered tubes at 100 C. After cooling, the mixture was centrifuged and 2-DG was determined in the supernatant fluid by the procedure of Waravdekar and Saslaw (20). Hydrolysis for 10 min was found to be sufficient to obtain the maximum value of 2-DG (*see* Fig. 7). This procedure does not allow the estimation of 2-DG which is in polysaccharide or in oligosaccharide form and substituted at positions 3 or 4. Hydrolysis of cell walls in stronger acids could not be used to give complete hydrolysis, since 2-DG is rapidly decomposed in more acidic media.

Examination of cell walls by electron microscopy. Isolated cell walls (suspended in water) were transferred to Formvar-coated grids, shadowed with gold/palladium (4:6, w/w), and examined with a Tesla electron microscope (type BS242B) and with a JEM-7 electron microscope.

RESULTS

Studies in glucose medium. The effect of three different concentrations of 2-DG on growth and viability of *S. cerevisiae* in 2% glucose medium is shown in Fig. 1. The depression of growth rate and of maximal crop was proportional to the concentration of 2-DG. In the culture with 0.02% 2-DG, the percentage of viable cells decreased only slightly during growing period, and the cell death increased at a time when no further increase in optical density was observed. In the cultures with 0.05 and 0.2% 2-DG, the viability of cells decreased in two different growth phases. The first decrease took place in the early-exponential phase of growth and was not essentially reflected in the growth rate as based on optical density data. The second decrease of viability of population started at stationary phase of growth as in the culture with 0.02% 2-DG. The extent of both lyses was dependent upon 2-DG concentration in the medium. The addition of mannitol as an osmotic stabilizer did not influence the inhibitory effect of 2-DG on growth (Table 1) but remarkably suppressed the lysis (Fig. 2). The cells from cultures containing both2-DG and mannitol were intrinsically as fragile as cells from the culture without mannitol. When such cells from cultures containing both 2-DG and the count of viable cells dropped quickly to approximately the level of those grown with 2-DG in the absence of mannitol.

The morphology of cells grown in the presence of 2-DG was markedly different from that of control yeast cells (Fig. 3). The former appeared to be more variable in size and shape and possessed more spherical forms with small buds, even when the growth had stopped. In the presence of 0.2% 2-DG, the cells grew in large agglomerates. Morphological changes similar to those caused by 2-DG were observed in the cells of regenerated yeast protoplasts (18).

Isolated cell walls from control yeast and from 0.2% 2-DG-inhibited yeast were examined by electron microscopy (Fig. 4 and 5). The walls of control yeast retained the shape of the original cells. They appeared as rigid structures with few if any creases. On the other hand, the walls of yeast grown with 2-DG and prepared in the same manner as control cell walls were more fragmented and possessed numerous creases. However, the main difference observed between cell walls of control and inhibited yeast was the remarkable change in appearance of the bud scars. Anomalously large bud scars occurred on the walls of inhibited yeast, in addition to bud scars of the magnitude of those occurring on the cell walls from control yeast. The anomalous bud scars were also apparent (Fig. 6) on the residues of cell walls extracted as described by Bacon et al. (1).

Studies of the chemical composition of cell walls from control and inhibited yeast showed

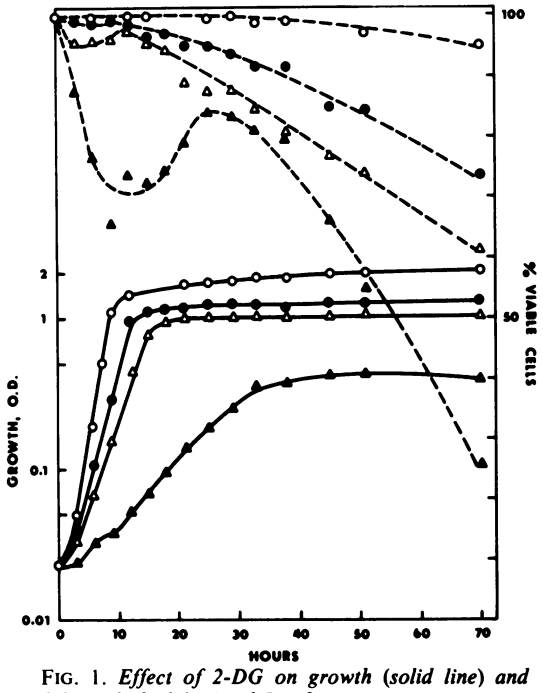

FIG. 1. *Effect of 2-DG on growth (solid line) and viability (dashed line) of Saccharomyces cerevisiae in 2% glucose medium. Control (○); 0.02% 2-DG (●); 0.05% 2-DG (△); 0.2% 2-DG (▲). Cells pregrown on glucose were used as inoculum.*

TABLE 1. *Effect of 2-DG and mannitol (0.7 M) on doubling time of Saccharomyces cerevisiae grown in 2% glucose medium*

Addition of 2-DG (%)	Mannitol absent (min)	Mannitol present (min)
None	85	75
0.02	115	115
0.05	140	155
0.2	430	360

that 2-DG causes marked changes in the amount of some cell wall components (Table 2). Cell walls from *S. cerevisiae* grown in the presence of 0.2% 2-DG contain approximately the same amount of glucose but less mannose (about one-third) than the normal cell walls. The walls of 2-DG-inhibited yeast also contain more glucosamine, protein, phosphorus, and lipid than normal cell walls.

Treatment of isolated yeast cell walls (grown with 0.2% 2-DG) with 0.125 N H_2SO_4 at 100 C liberated free 2-DG (Fig. 7). The content of unsubstituted 2-DG in the cell walls is listed in Table 2. 2-DG liberated from the cell walls upon mild acid hydrolysis was further identified by paper chromatography.

Studies in lactate medium. The lactate medium used did not directly supply the yeast with glucose in an amount required for the synthesis of structural polysaccharides. The organism must produce the glucose for polysaccharide biosynthesis by gluconeogenesis. When 2-DG was added to such a medium at concentrations 10 times lower than those affecting the growth in glucose medium, the growth was greatly influenced (Fig. 8). The growth inhibition was accompanied by a loss in viability of cells, during exponential phase, proportional to the concentration of 2-DG. An extensive lysis occurred at 0.005% 2-DG and resulted in a temporary reduction of the rate of the initially exponential growth. Later the lysis stopped, and the growth rate rose again without further mortality (Fig. 8). The addition of 0.7 M mannitol to lactate medium containing 0.005% 2-DG reduced the extent of lysis during the exponential phase (Fig. 9). Consequently, the initial growth rate was not so markedly and durably altered as it was in the culture without mannitol.

At 0.01% 2-DG, the growth of *S. cerevisiae* ceased after 20 hr, owing to almost complete lysis of the population (Fig. 10). About 1 to 3% of viable cells were estimated between 40 and

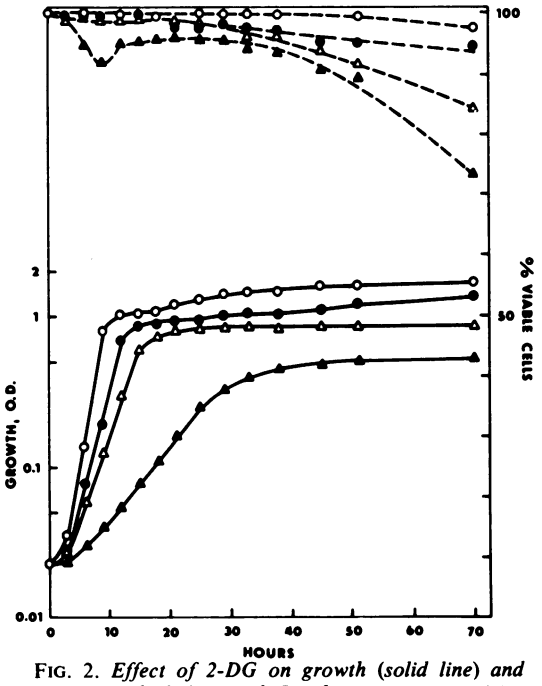

FIG. 2. *Effect of 2-DG on growth (solid line) and viability (dashed line) of Saccharomyces cerevisiae grown in 2% glucose medium osmotically stabilized with 0.7 M mannitol. Control (○); 0.02% 2-DG (●); 0.05% 2-DG (△); 0.2% 2-DG (▲). Cells pregrown on glucose were used as inoculum.*

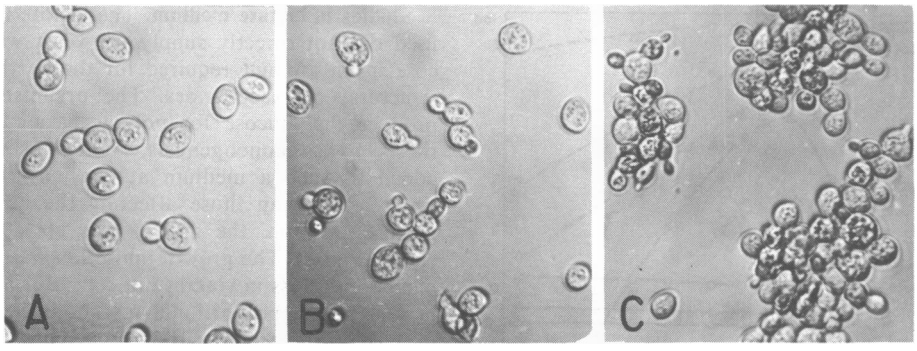

FIG. 3. *Effect of 2-DG on the cell morphology of Saccharomyces cerevisiae grown in 2% glucose medium. A, Control cells; B, cells grown in the presence of 0.05% 2-DG; C, cells grown in the presence of 0.2% 2-DG. ×600.*

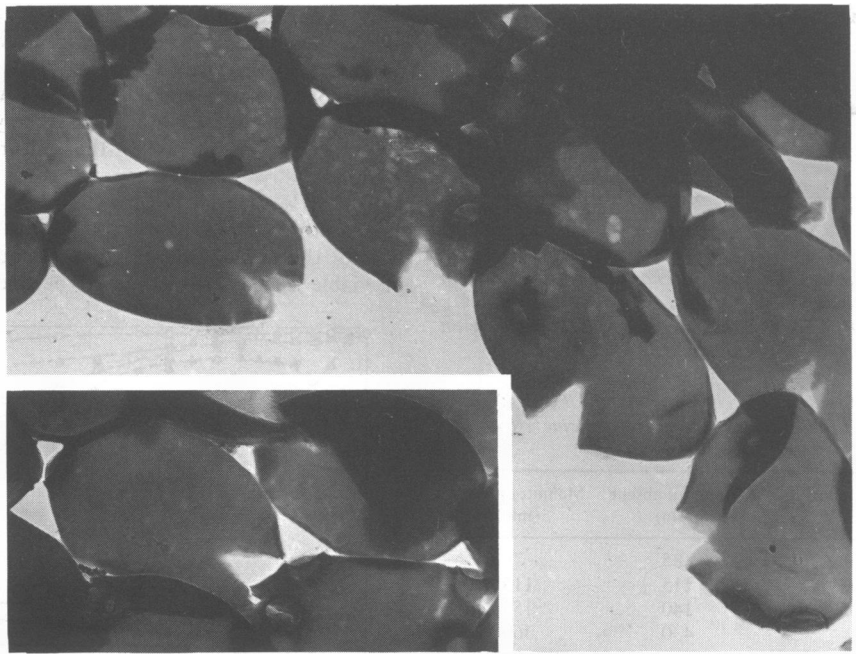

FIG. 4. *Electron micrographs of cell walls of Saccharomyces cerevisiae grown in 2% glucose medium in the absence of 2-DG. ×3,800.*

100 hr of the experiment. After about 100 hr, the surviving cells started to multiply in a typical exponential pattern (Fig. 10, growth curve with optical density of the dead population subtracted). The level of 2-DG in the medium at this point was not much lower than at the beginning of the experiment. This indicated that the growth following lysis can be attributed to yeast cells with an altered biochemistry resulting in less sensitivity to 2-DG. When the cells from this culture at the 169th hr (arrow, Fig. 10) were transferred to fresh lactate medium supplied with 0.01% 2-DG, they grew at the same rate as parental 2-DG-grown cells, and they also did not undergo lysis during the exponential phase. Their growth was accompanied by continued uptake of 2-DG from the medium. Less than one-third 2-DG was estimated in the medium when growth ceased. The biochemical basis of this behavior of *S. cerevisiae* grown in lactate medium in the presence of 2-DG has not been determined.

At 0.05% 2-DG, the growth of *S. cerevisiae* in lactate medium was blocked for more than one week. A portion of the inoculum still survived, since the growth of the culture resumed after addition of glucose, fructose, and mannose. The rate of the resumed growth and the final crop after addition of glucose was strictly dependent

on the concentration at which it was added to the lactate medium (Fig. 11).

The morphological changes of *S. cerevisiae* grown in lactate medium with 2-DG were similar to those observed in glucose medium. The cells were, however, much smaller, and the agglomerates of round-shaped cells possessed more bush-like forms. This was particularly true for cells which started growing after extensive lysis. Properties of the cell walls of lactate-grown yeast were not investigated.

DISCUSSION

Spherical forms of cells grown in the presence

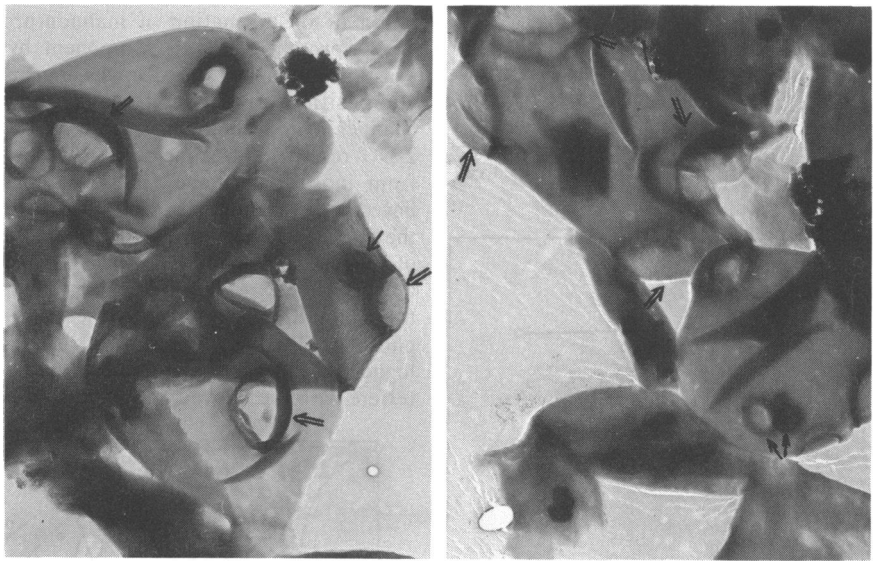

FIG. 5. *Electron micrographs of the cell walls of Saccharomyces cerevisiae grown in 2% glucose medium in the presence of 0.2% 2-DG. Normal bud scars (single-shafted arrow) as well as large bud scars can be seen (double-shafted arrow). Note the granular appearance of the large bud scars.* ×*3,800.*

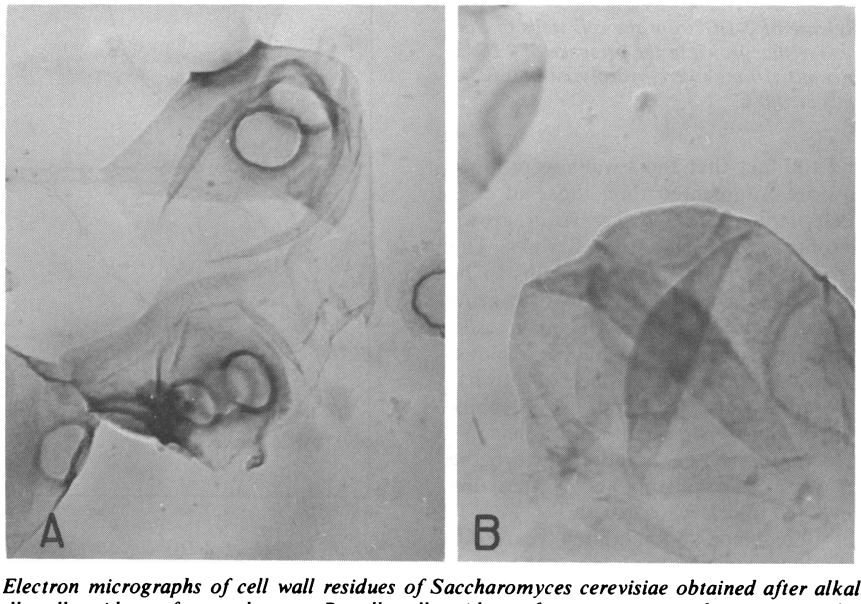

FIG. 6. *Electron micrographs of cell wall residues of Saccharomyces cerevisiae obtained after alkaline extraction. A, Cell wall residues of control yeast; B, cell wall residues of yeast grown in the presence of 0.2% 2-DG.* ×*5,800.*

TABLE 2. *Chemical composition of cell walls of normal yeast and with 0.2% 2-DG-grown yeast*[a]

Component	Content[a] in cell walls from	
	Control yeast	Yeast grown with 0.2% 2-DG
Glucose	52	50
Mannose	27	17
Glucosamine	0.65	2.5
Protein-nitrogen	2.05	3.8
Phosphorus	0.2	0.4
Lipid	2.5	4.1
2-Deoxy-D-glucose		0.9

[a] Values are expressed as per cent of lyophilized cell walls.

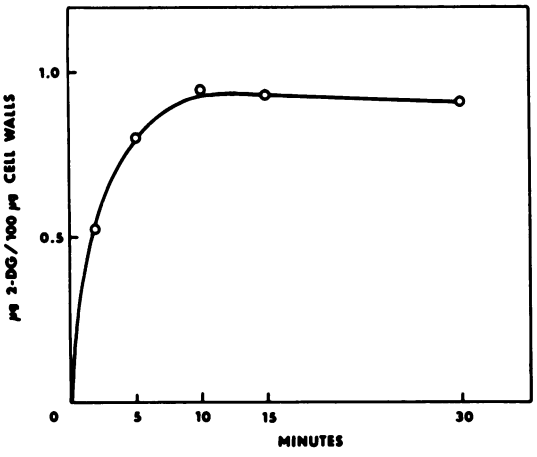

FIG. 7. *Release of 2-DG from the cell walls of Saccharomyces cerevisiae grown in the presence of 0.2% 2-DG. The cell walls (10 mg) were hydrolyzed in 0.125 N H_2SO_4 (10 ml) at 100 C.*

of 2-DG and the fact that their walls were more folded and more fragmented than those of control yeast cells indicate that *S. cerevisiae* grown with 2-DG synthesizes weakened cell walls. The process of the cellular division also seems to be influenced by 2-DG, since the cells tend to grow in agglomerates. This effect of 2-DG may result from an unbalanced synthesis of bud scar plugs, which is suggested by the occurrence of anomalously large bud scars on cell walls from inhibited yeast. This fact is consistent with Johnson's proposal (15) that 2-DG metabolites disturb the balance between glucan splitting and synthesizing processes involved in cell wall growth.

Cell walls of control and inhibited yeast differed also in macroscopic appearance. The cell walls from inhibited yeast were found more hydrophobic than the control cell walls. This property, apparently a result of an increased lipid content (Table 2), made the purification of such cell walls more tedious. There is a possibility that the hydrophobic properties of the cell walls are related to agglomerate formation.

Addition of 2-DG to growing medium results in changes in the chemical composition of the yeast cell walls. The reduced content of mannose in these cell walls is consistent with the results of Farkaš et al. (9). They observed a stronger inhibition of the formation of mannan-protein than glucan-protein cell wall component by 2-DG in yeast protoplasts. The changes in carbohydrate composition of the cell walls and even the changes in morphology of the cells produced by 2-DG resemble, in some respects, those resulting from inositol deficiency (5, 19). Inositol is believed to be an important yeast factor involved in the interconversion of phosphorylated derivatives of glucose and mannose which are indirect precursors of cell wall glucan and mannan (7). In this connection, it is reasonable to assume that phosphorylated metabolites of 2-DG, as analogues of glucose and mannose metabolites, interfere with the above interconversion.

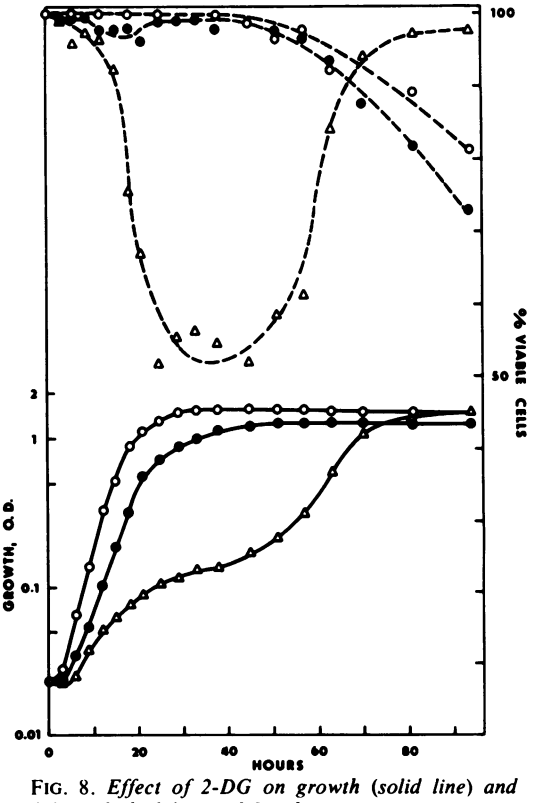

FIG. 8. *Effect of 2-DG on growth (solid line) and viability (dashed line) of Saccharomyces cerevisiae in lactate medium. Control (○); 0.002% 2-DG (●); 0.005% 2-DG (△). Cells pregrown on lactate were used as inoculum.*

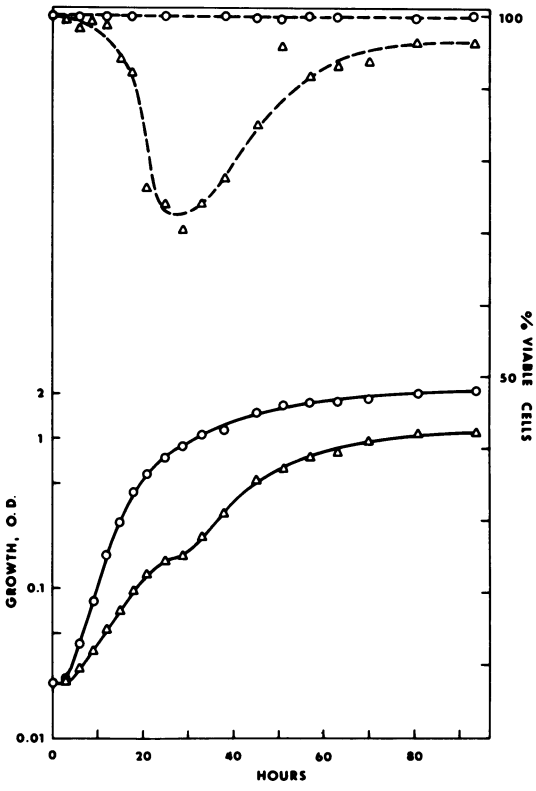

FIG. 9. *Effect of 2-DG on growth (solid line) and viability (dashed line) of Saccharomyces cerevisiae in lactate medium osmotically stabilized with 0.7 M mannitol. Control (○); 0.005% 2-DG (△). Cells pregrown on lactate were used as inoculum.*

Some of the changes which occurred in the cell wall composition of yeast grown in the presence of 2-DG, e.g., the increased content of glucosamine and lesser content of mannose, were reported in the walls of regenerated protoplasts (8, 12). In general, the changes in chemical composition of cell walls caused by 2-DG are, from the qualitative point of view, similar to those produced by other factors which result in the synthesis of abberant cell wall.

We also observed, as reported by other authors (13, 15–17), a strong lethal effect of 2-DG on growing cells. This effect was microscopically characterized as lysis (15). The correlation of growth and viability of *S. cerevisiae* grown in glucose and lactate medium, as presented in this paper, showed that 2-DG causes cell lysis during the exponential phase and, in addition, accelerates the cell mortality after the final stage of growth has been reached. The extent of both exponential-phase lysis and stationary-phase lysis appears to be dependent upon the ratio 2-DG/glucose to which the cells are exposed. The fact that mannitol can effectively suppress both kinds of 2-DG-induced lysis but not the inhibition of growth and the formation of osmotically fragile cells suggests that the lysis of *S. cerevisiae* grown in the presence of 2-DG is due to osmotic fragility of cells. These results also indicate that the inhibition of yeast growth by 2-DG is a consequence of an inhibition of continued cell wall biosynthesis. This effect, at higher 2-DG concentration, leads to formation of weakened cell walls which cannot sustain the turgor pressure of the cytoplasm and thus results in osmotic lysis. The sites of such lysis may correspond to regions of glucan synthesis in cell walls as reported by Johnson (15). To establish whether the 2-DG-induced lysis of *S. cerevisiae* is associated with dissolution of cell wall glucan, as it is with *Schizosaccharomyces pombe* (16), needs further investigation. However, the effect of mannitol on 2-DG-induced lysis of *S. cerevisiae* appears to differ from the effect of sorbitol on dissolution of *S. pombe* glucan in the presence of 2-DG (16).

The lysis observed in stationary phase of growth (in some extent also in cultures without

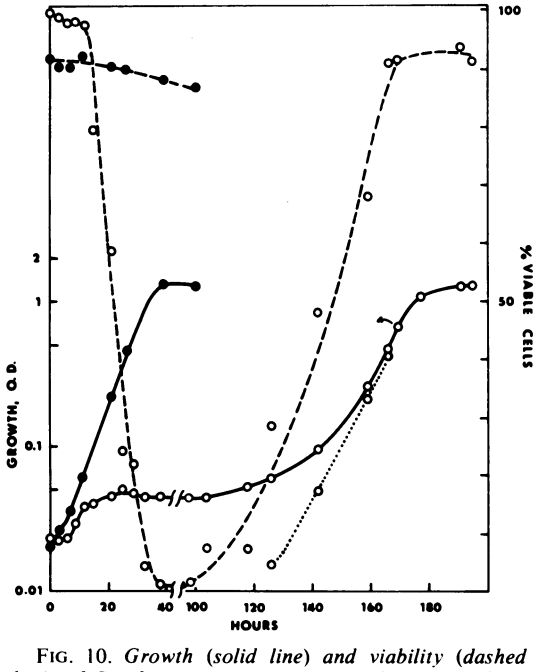

FIG. 10. *Growth (solid line) and viability (dashed line) of Saccharomyces cerevisiae in lactate medium containing 0.01% 2-DG. Cells pregrown on lactate* (○). *(Dotted line represents growth of surviving cells after lysis; optical density of the dead population at 100th hr was subtracted from the values measured after this time); cells pregrown in lactate medium with 0.01% 2-DG transferred at 169th hr to fresh medium, indicated by arrow,* (●).

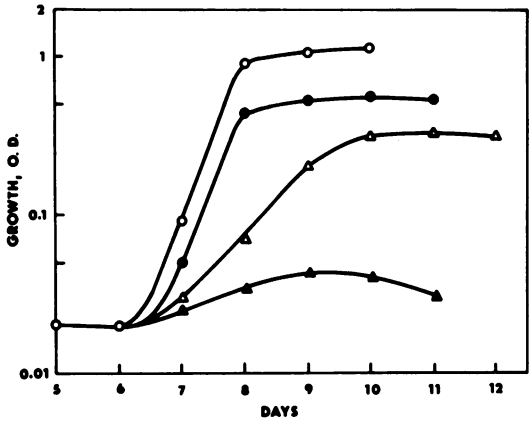

FIG. 11. *Initiation of the growth of Saccharomyces cerevisiae in lactate medium containing 0.05% 2-DG with glucose added 6 days after inoculation (arrow). Concentrations of glucose added: 2% (○), 1% (●), 0.5% (△), 0.2% (▲). Cells pregrown on lactate were used as inoculum.*

2-DG) also appears to be of osmotic nature, in that it is diminished by addition of mannitol.

Based on the results obtained by growing yeast in lactate medium, interference with transport of fermentable sugars by 2-DG as the mechanism of growth inhibition appears to be secondary or negligible. If *S. cerevisiae* is grown in the absence of glucose or any other fermentable sugar, the cells are extremely sensitive to 2-DG but adaptable to resist the toxic effect of 2-DG. In the presence of 0.01% 2-DG, almost the entire culture lysed when the optical density of the inoculum was doubled. The cells which survived the exponential-phase lysis in this experiment started, after some time, to grow at a reduced rate compared to the control, but they were resistent to 2-DG-induced lysis. The biochemical basis of this resistance will be of interest to study, particularly because of a strong uptake of 2-DG from medium by these cells during growth. An *S. cerevisiae* mutant resistant to 2-DG has been isolated by Heredia and Sols (14), and its resistance was found to be associated with its increased ability to hydrolyze 2-DG-6-phosphate.

S. cerevisiae cells which survived in lactate medium containing 0.05% 2-DG were not able to grow for more than a week without addition of exogenous hexose. We assume that this behavior might result from a repression of the synthesis of inducible enzymes of gluconeogenesis by 2-DG metabolites which may substitute the corresponding metabolites of glucose in metabolic control. Such action of 2-DG-6-P was reported by Gancedo et al. (11).

It may be concluded, therefore, that the phosphorylated metabolites of 2-DG (2-DG-6-phosphate, 2-DG-1-phosphate, 2-DG-1,6-diphosphate, uridine diphosphate-2-DG, and guanosine diphosphate-2-DG) apparently interfere with those processes of yeast metabolism in which the corresponding metabolites of glucose and mannose take part or are formed. If this conclusion is correct, the processes most affected by 2-DG metabolites should be those leading to the synthesis of cell wall polysaccharides, including the formation of hexose-phosphates from smaller carbon substrates. The plausibility of such a mechanism for the inhibition of yeast growth by 2-DG is strengthened by our recent observation (*unpublished data*) that guanosine diphosphate-2-DG competitively inhibits the incorporation of radioactivity from guanosine diphosphate-^{14}C-mannose into particulate mannan-synthesizing enzyme system prepared from *S. cerevisiae*. The interference of 2-DG metabolites in cell wall polysaccharide synthesis is further demonstrated by its incorporation into cell wall material. Further support for this mechanism of the growth inhibition by 2-DG may be found in the recent work of Farkaš et al. (10). They demonstrated that 2-DG, present in media at concentrations not affecting the protein synthesis in yeast protoplasts, considerably inhibited the secretion of the mannan-protein cell wall component by protoplasts into the medium.

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