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Membrane Changes in Yeast Cells Caused by Sulfhydryl Reagents and Accompanied by a Selective Release of Sugar

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Summary. Iodoacetic acid or N-ethylmaleimide included in cell suspensions during measurements of sorbose exit from yeast cells caused sorbose efflux to occur at a uniform rate in contrast to the usual two-phase exit. Cells pretreated with these agents were still capable of sugar uptake, but the entire efflux now occurred at the usual initial rate. Microscopically, the vacuoles of treated cells were observed to be altered or disrupted. Vacuolar effects occurred before methylene blue was able to penetrate the external cell membrane and stain the cells. Vacuoleless cells also allowed a single rate of sorbose efflux. The selective effect upon intracellular membranes is interpreted as a disruption of the boundaries of an internal sugar compartment with the result that sugar exits from the cell at a rate controlled only by the external membrane.

The exit of sorbose from yeast cells, both fresh [10] and starved [11], has been observed to involve at least two phases. The second phase is a decreased rate of loss which may result in a relatively high retention of sugar even after 3 hr or more. Such exits have also been described for other sugars [5, 10, 11]. Sorbose is normally transported across yeast membranes by a process of facilitated diffusion not accompanied by accumulation [1, 14]. However, cells which have been previously incubated in certain sugars and polyols [11] may take up sorbose in excess of external concentrations. It has been suggested [11] that retention by these incubated cells is explained more simply by binding than by the trapping of sugar within a cellular compartment. Either process could account for more than one rate of exit, but an active uptake would also be required for accumulation into a compartment. Sorbose has been used in transport studies because it is not metabolized [12] but is carried by the same system that transports glucose [1, 14].

The experiments described below show that sorbose is released rapidly upon treatment of fresh cells with compounds as diverse as sulfhydryl rea-

gents and butyl alcohol. These and additional observations indicate that membranes are disrupted in the release of sorbose and, thus, provide support for the interpretation that a cellular compartment affects the normal internal distributions of this sugar. A uniform and rather complete exit of sorbose from cells grown under conditions which prevent vacuole formation suggests that the cell vacuole could be the compartment involved.

Materials and Methods

Organism and Handling

Saccharomyces cerevisiae cells were grown aerobically as described previously [10]. The cells were harvested during exponential growth at a count of 2×10^7 ml, washed twice by centrifugation with distilled water and resuspended in buffer for experimental use. Anaerobically, cells were grown from a high inoculum of mature cells in the yeast extract-glucose medium of Wallace, Huang, and Linnane [15]. Cultures were set up with nitrogen flushing and a trap essentially as described by Wallace et al. [15], and cells were washed and otherwise handled under nitrogen. Potassium phosphate buffer, 0.02 M, containing 2 mM $MgCl_2$ was used in all cases, except for one instance noted (Table). Neutral red, 0.6 mM, and toluidine blue, 1.0 mM, in 0.02 M phosphate buffer, pH 7.0, were used as vital stains for vacuole observations. Cells were exposed for 5 min to 0.2 mM methylene blue in 0.02 M buffer, pH 4.5, to observe dye penetration. Cells were counted in a hemocytometer.

Exit and Radioactivity Measurements

Aerobic cells were incubated in ^{14}C-labeled sugar, usually 0.1 M, in buffer at 30 °C to load them for exit measurements. After uptake they were washed twice by centrifugation with ice-cold water and resuspended, usually to 7 ml (3×10^7/ml), in buffer at 30 °C. Added reagents were included at zero minute, i.e., upon resuspension of the cells. Under anaerobic conditions, cells were loaded in sugar solutions of higher molarity and were washed only once to speed handling; a nitrogen flow was maintained over exit suspensions. (Additional anaerobically grown cells handled in parallel, except that the exit process was carried out in open tubes, i.e., aerobically, showed exit responses similar to the cells handled under nitrogen.) Exit was measured by collecting cells, maintained at 30 °C with shaking, at successive intervals on membrane filters (Millipore Corp., Bedford, Mass.) for radioactivity counts of retained sugar. Samples of suspensions were added to 10 volumes of ice-cold water over the filter and then filtered and washed with 20 volumes of cold water. The filter was removed from the filtering apparatus, attached with stopcock grease to an aluminum planchet and dried in an oven at 50 to 55 °C. Sampling and washing required 30 sec or less, and filters were immediately transferred to planchets. Uptake measurements were made using similar sampling techniques after washed cells had been resuspended in solutions of ^{14}C-labeled sorbose; sulfhydryl reagents were also included at zero minute. Stock sugar solutions were diluted and transferred directly to planchets for drying. Radioactivity on planchets was counted in an automatic gas-flow counter to a statistical error of 5% (usually much less) and corrected for background. Cell material on membrane filters was kept below a dry weight of 0.7 mg, which showed negligible self-absorption.

Results

Sorbose Exit

Fig. 1A shows that the exit of sorbose from these yeast cells is not affected by the usual handling procedures. That is, exit curves exhibited the same two-phase pattern for uptake times of 30, 90, or 180 min. The patterns were also similar when cells were suspended in buffer before uptake, and when uptake, exit, and uptake again occurred before exit was measured (and upon exit into water [11]). Although exit patterns were similar, total efflux was greater following the shorter uptake periods. This difference in efflux could reflect a slow fill rate for the normally slow exiting fraction, or a change in the permeability of an intracellular compartment membrane.

Fig. 1 shows also that when iodoacetic acid (IAA) or N-ethylmaleimide (NEM) was added to exit suspensions at appropriate concentrations, the

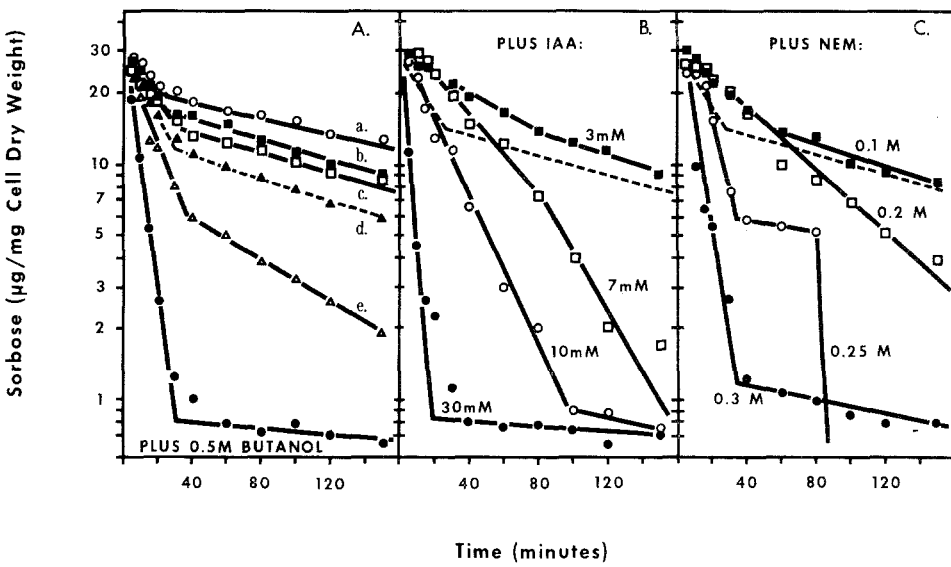

Fig. 1. Exit of ^{14}C-L-sorbose (uniformly labeled) from yeast cells. A. Exit into 0.02 M phosphate buffer, pH 7.0, at 30 °C. Before exit, cells were suspended: (a) in 0.1 M ^{14}C-sorbose, pH 5.6, for 180 min for sugar uptake; (b) in sorbose solution for 90 min, transferred to allow exit for 60 min, and retransferred for an additional 90 min of uptake; (c) in buffer only for 90 min, then in sorbose solution for 90 min; (d) in sorbose for 90 min (standard condition for other control curves shown as dashed lines without plotted points); and (e) in 0.2 M ^{14}C-sorbose for 30 min. The bottom curve was obtained from an exit suspension which included 0.5 M butyl alcohol. B, C. IAA and NEM were included in exit suspensions at the indicated concentrations; cells were exposed at zero minute when they were resuspended in the exit solutions. Both uptake and exit were at pH 4.5. For all experiments, sorbose content was calculated from the specific label, cell weight, and radioactivity count for the individual experiment; label ranged from 10 to 40 counts/min per µg sorbose

entire sorbose efflux occurred at a uniform rate that was similar to or faster than the initial rate. Butyl alcohol also produced rapid efflux at a 0.5 M concentration (Fig. 1 A), but not at 0.4 M. Other experiments showed that NEM acted in the same manner at pH 4.5 or 7.0, but that IAA was much less effective at pH 7.0 (indicated pH values are those of the 0.02 M potassium phosphate buffer used; the actual pH was 3 to 3.5 when effective amounts of IAA were included in the pH 4.5 buffer). At low concentrations, IAA and NEM increased the retention of sorbose or reduced the initial rate of exit. Mannitol, a nonmetabolized sugar alcohol, retained in an even greater proportion than sorbose by these yeast cells [11], also was released when IAA or butyl alcohol was present (data not shown).

Methylene Blue Staining

Butyl alcohol was added to these experiments because it is known to disrupt yeast and other cell membranes [9]. Thus, the rapid release of sorbose when butyl alcohol was present supported the hypothesis that the sugar effluxes observed are the result of cell lysis or rapid washing from cells with broken membranes. The exit patterns observed in the presence of increasing concentrations of IAA and NEM indicated a more selective effect upon cell membranes and possibly an effect which depends upon the time of exposure. To evaluate these possibilities, cell counts to measure lysis and microscopic observations of methylene blue staining were made. Methylene blue does not stain living yeast cells but it is taken up immediately by "dead" cells [8], presumably because the external cell membrane no longer functions adequately to prevent dye penetration. The Table confirms that butyl alcohol indeed caused staining or external membrane rupture, and that IAA and NEM produced a similar effect which depended both upon concentration and upon the time of exposure. Total cell numbers were not reduced. Leakage of cell materials measured spectrophotometrically at 260 nm could not be used as an additional method of measuring membrane injury with IAA and NEM because these compounds absorb at this wavelength. With 0.4 M butyl alcohol present, however, leakage at 60 min was only 10% of that of the large loss which occurred immediately with 0.5 M butyl alcohol; at 120 min it was less than 20% (average of three determinations).

Vacuolar Changes

At concentrations above 1 mM, IAA disturbed internal cell arrangements. These effects are obvious under the microscope, although the details are not easily described. A pronounced change occurs in the cell vacuole

Table. *Methylene blue staining of cells and cell persistence*[a]

Treatment	Stained cells (% of total)						Total cells (% of avg count)
	15	30	45	60	90	120 min	120
Buffer, pH 4.5	—	0[b]	—	1	1	1	103
pH 3.0[c]	—	1	—	1	3	3	100
IAA, 3 mM	0	0	1	2	2	3	102
5 mM	0	0	1	2	4	11	106
7 mM	1	2	2	7	44	80	102
9 mM	1	2	15	32	66	85	98
12 mM	15	26	52	78	95	99	100
NEM, 0.1 M	—	2	—	5	6	7	102
0.2 M	6	15	16	15	25	68	98
0.3 M	16	24	100	100	100	100	96
Butyl alcohol,							
0.4 M	1	2	2	1	4	7	97
0.5 M	60	70	100	100	100	100	102

[a] Cells were allowed to take up sorbose as in exit experiments; they were then washed and resuspended in 0.02 M phosphate buffer, pH 4.5, containing the indicated agent, at 30 °C and successively sampled for counts.

[b] Values are averages of three or more determinations. A few stained cells usually occurred in suspensions given a rounded zero value.

[c] 0.2 M potassium citrate buffer.

which appears to lose its integrity and shape. The changes occur shortly after exposure to IAA, and, depending upon the IAA concentration, cells may or may not be stained by methylene blue. With 3 and 5 mM IAA, internal changes were readily apparent, but only a few cells were stained by methylene blue even after 120 min (Table). Similar changes were induced by NEM at higher concentrations. Thus, it seemed possible that IAA or NEM may affect the boundary or membrane of an internal sugar compartment, or may affect the binding of sugar selectively, as compared with its effect upon the external cell membrane, so that retained sugar is released for normal exit from the cell proper through the intact external membrane.

Pretreatment with Sulfhydryl Reagents

If such a selective internal effect occurs, appropriately treated cells should be able to transport sorbose into the cell normally through the unaffected external membrane and should allow complete exit at a uniform rate back through this membrane. Such a demonstration would functionally isolate an effect of the added agent upon internal membranes. In the above experi-

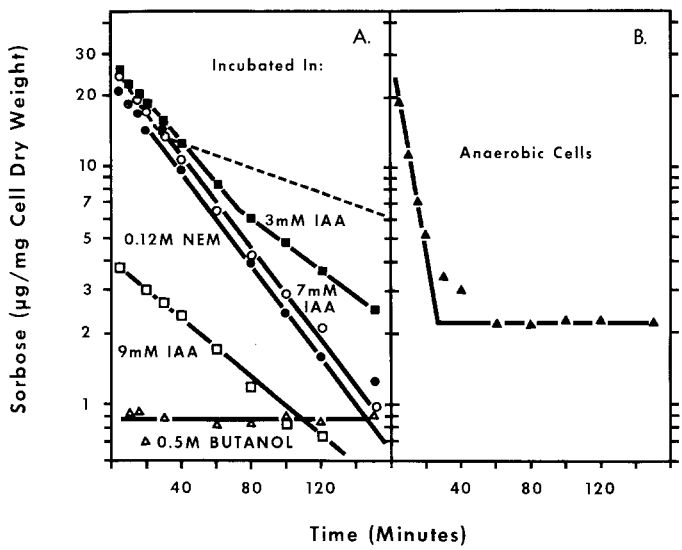

Fig. 2. A. Sorbose exit from cells incubated at 30 °C in solutions of the indicated agents for 40 min before ^{14}C-sorbose uptake and the succeeding exit measurements. The control, with cells incubated in buffer, is shown by the dashed line. Incubation, uptake, and exit were at pH 4.5. B. Cells were grown and handled anaerobically; uptake was in 0.2 M ^{14}C-sorbose, pH 5.6, for 30 min; exit was at pH 7.0

ments, internal effects were not clearly distinguishable from effects upon external membranes. Fig. 2A shows exit curves for cells which were suspended for 40 min in buffer solutions of butyl alcohol, IAA, or NEM before they were allowed to take up sorbose. The cells were washed with water after this treatment and then resuspended in the uptake solution. Butyl alcohol, by disrupting external cell membranes during the pretreatment period, produced cells that were unable to take up and hold sorbose. However, cells treated with either NEM or IAA at appropriate concentrations allowed normal sorbose uptake (zero-minute content, Fig. 2A), and exit was complete and occurred at a uniform rate rather than in two phases. Clearly the effect upon sugar retention, and thus upon internal structure, was separated from an effect which disrupts external membranes.

When external membranes are disrupted during pretreatment, total sugar uptake is reduced proportionately as with butyl alcohol or with 9 mM IAA (Fig. 2A); i.e., total uptake is proportional to the intact cells contained in the suspension and is reduced by an amount equivalent to the proportion of cells with disrupted external membranes. Disruption may continue after the period of pretreatment due to retained IAA or to a weakened membrane unable to withstand osmotic or other stresses encountered

in the uptake and exit procedures. Thus, less than 10% of the cells treated with 7 mM IAA were stained with methylene blue at the end of the 40-min pretreatment, but more than 50% were stained by the time exit measurements were completed. With 5 mM IAA, staining increased only slightly (remaining at less than 15% throughout the experiment), but at 9 mM, 95% or more of the cells were stained at the end of exit measurements. With 0.12 M NEM, less than 5% of the cells stained at any time during the experiment. (These percentage values are based on averages of three or more separate experiments.)

Uptake

Direct measurements of the effects of IAA upon sorbose uptake show that the rate of uptake was slowed by each concentration of IAA employed (Fig. 3A). At concentrations above 3 mM, the curve describing uptake inflects sharply after a period of time as the measured sorbose content of the cells decreases. Considering the data on membrane rupture (Table 1), this decrease occurs as external cell membranes are ruptured and sorbose is lost from the cells on the membrane filter at the time of washing. With 5 mM IAA, rupture and loss occurred after 90 min of exposure. With 30 mM IAA,

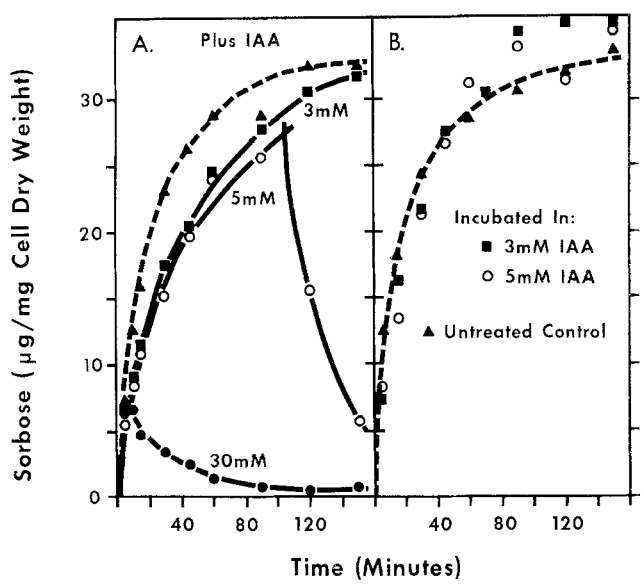

Fig. 3. Uptake of ^{14}C-L-sorbose (uniformly labeled) by yeast cells suspended in 0.02 M phosphate buffer, pH 4.5, containing 0.1 M sorbose. Dashed lines represent control cells. A. Uptake with indicated concentrations of IAA included at zero minute. B. Uptake by cells previously incubated for 40 min (pretreated) at 30 °C in buffer or in buffer-IAA solutions; after incubation, cells were washed twice and resuspended in sorbose solution for measurements of uptake

rupture occurred almost immediately; such cells, like those exposed to 0.5 M butyl alcohol (Fig. 2A), were not able to take up sorbose. NEM affected sorbose uptake similarly; at 0.1 M, the rate of uptake was slowed; at 0.3 M, rupture was almost immediate (not shown). Pretreatment with IAA did not reduce total sorbose uptake (Fig. 3B). Washing after pretreatment evidently relieved the usual inhibition, except for a slight reduction in the initial rate of uptake. The tendency for pretreated cells to take up more sorbose than untreated cells (*see* also Fig. 3B) cannot be explained at this time, although the immediate availability of a normally slow-filling or restricted compartment must be considered; cells pretreated with 0.12 M NEM also tended to show an increased uptake.

Anaerobically Grown Cells

Disruption of the cell vacuole as observed in IAA-treated cells raised the possibility that this vacuole functions as the sorbose compartment in question and is responsible for the retention of sugar. Such a hypothesis would be supported if it could be shown that sorbose exits at one rate from cells without vacuoles. To test this possibility, yeast cells were grown anaerobically, essentially as described by Wallace et al. [15], so that they would be vacuoleless. Neutral red and toluidine blue stains and an electron microscope preparation confirmed the lack of usual vacuoles, although small vacuoles were frequently present. Fig. 2B shows that efflux from these cells occurred at a constant rate. However, many cell membranes and organelles may be variously affected by this type of anaerobic culture [2, 15]. Thus the boundary of some other sugar compartment may have been affected; observed differences from aerobic cells include a greater rate of sugar exit and the staining of more cells (approximately 20%) by methylene blue. Because the slower second component of the exit curve was eliminated, sugar binding obviously did not significantly affect exit.

Discussion

The experiments reported here show that certain concentrations of IAA and NEM selectively affect internal cell structures and not the external cell membrane. Disruption of the vacuole was readily observed. It is hypothesized, in interpreting the additional data, that this selective internal action makes permeable an intracellular sugar compartment which normally accounts for the slow phase of sorbose exit. It is considered also that exit occurs from the compartment characterized by the slower rate through the compartment exhibiting the faster rate, a compartment bounded by the external

cell membrane which exerts final control on the rate. The observed single-phase efflux with appropriate concentrations of IAA or NEM provides direct evidence for these hypotheses. Thus, exit at a uniform rate was possible when internal membranes were disrupted. The rate of loss was increased as external membranes were ruptured at still higher concentrations. Most decisively, pretreatment with these agents resulted in an intact, functioning external membrane which allowed normal uptake and allowed efflux to occur at a uniform rate similar to that of the usual fast phase. No indication of a compartmented non-uniform internal distribution of sugar remained. When the external membrane was affected by pretreatment with higher concentrations of IAA or NEM, sorbose uptake did not occur, or was proportional to the number of cells with uninjured external membranes. The reduced size of the slow-exiting sorbose pool, observed after a 30-min uptake period (Fig. 1 A, line e), indicates a slower filling rate consistent with the slower rate of exit. However, the relatively faster rate of exit from this smaller pool appears to reflect a change in the bounding membrane of the compartment. Such a change could develop during the period of uptake, but this aspect of the data requires further study.

Several authors [3, 7] have considered the influence which vacuolar membranes can have upon solute movement into, out of, and within plant cells. Yeast cells have been somewhat neglected in this respect, but reports of differences between external and vacuolar membranes exist [4, 13], and some consideration has been given to the vacuole as a sugar compartment [5]. The evidence given above that the vacuole may function as the compartment responsible for the slow phase of sugar exit is by no means unequivocal. If mitochondria functioned as a sugar compartment, their reported absence in anaerobically grown cells [14] would also be consistent with a uniform rate of sorbose efflux. But other studies describe promitochondria, at least, as occurring in anaerobic cells [2]. Obviously, altered capacities for sugar uptake could characterize these organelles in anaerobic as compared to aerobic cells, but, even so, the available space in mitochondria would seem to be inadequate for the amount of sugar retained [11]. Several sugar compartments may exist [5, 11]; clearly, more precise accounts of sugar distribution and localization are needed.

By definition, sulfhydryl reagents affect sulfhydryl groups and the bonds in which they participate, both of which occur in cell membranes. However, the specific manner in which these reagents affected membranes in the above experiments is still entirely unclear. Other effects, perhaps not even sulfhydryl reactions, may be primary at high rather than low concentrations. External cell membranes were ruptured at relatively high concentrations.

At lower concentrations (3 mM IAA; 0.1 M NEM), both the rate of sorbose uptake (Fig. 3A) and the initial rate of sorbose efflux (Fig. 1B, C) were decreased. This reduced rate of transport appears to be functionally similar to an effect produced by methylphenidate, presumably upon the external cell membrane [10]. As the concentration of the sulfhydryl reagents is increased (Fig. 1B, C), increased rates of initial efflux occur, evidently because external membranes are ruptured in increasing numbers of exposed cells. Thus, the reaction which causes a reduced rate of transport ostensibly occurs in the external cell membrane, but could be either an additional independent effect or a part of the change which leads to membrane rupture. At still lower concentrations, IAA has been used commonly in transport experiments to poison glycolysis [6, 14].

These results contribute toward knowing if sugar binding is a factor in determining sorbose exit patterns. With the above cells which equilibrate internal with external sorbose at a diffusion equilibrium level, an intracellular compartment evidently accounts for the observed second phase of exit. In measurements with fucose involving another aspect of yeast cell sugar distributions, Kotyk [5] observed similar patterns of exit which he ascribes to a compartmented arrangement. Moreover, sonication of the above cells releases sorbose in parallel with a decrease in viability (W.A. Maxwell, *unpublished*). Because the release is measured by collecting cell material on a membrane filter with a pore size of 0.45 μ, sorbose, if it is bound at all, is obviously not bound to large cell structures or organelles. A unique sugar binding which occurs at low concentrations [6] presumably would not be significant at the higher concentrations used in the above experiments. This binding is reported to depend upon cellular integrity and is not observed when the cell is broken open. On the other hand, when sorbose is taken up in excess of external concentrations, as it is by cells which have been preincubated with sugars [11], evidently either active accumulation or binding is involved.

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