Disruption of Yeast Membranes by Methylphenidate

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Methylphenidate blocked sorbose uptake and loss by yeast spheroplasts and, at higher concentrations (30 mM), disrupted the spheroplasts. At still higher concentrations (70 mM), methylphenidate also ruptured the membranes of whole yeast cells; sorbose and materials absorbing at 280 nm were lost from the cells, and methylene blue stained them. Intracellular structures were extensively affected, as shown by electron micrographs, and were more sensitive to disruption by methylphenidate than the external membrane. N-ethylmaleimide and Ca\(^{2+}\) enhanced the rupture of external membranes by methylphenidate.

Previous studies have shown that methylphenidate (MP; phenyl-piperidyl-2-acetic acid methyl ester), a drug which has been used clinically as an antidepressant, inhibits the passage of sugars and other solutes across the yeast cell membrane (11). An effect upon the external membrane evidently is responsible for this action. Although the drug has been reported to decrease the cholesterol content of mouse brain (6), little information is available regarding the biochemical reactions in which it may participate.

Additional studies reported below show that the external membranes of yeast are ruptured by high concentrations of MP and that intracellular structures are disrupted even more readily. These effects and an interference with the exit of sorbose from spheroplasts clearly implicate cell membranes as a site of MP action.

MATERIALS AND METHODS

Whole yeast cells. Saccharomyces cerevisiae was grown and handled as described previously (11), the cells being harvested during the exponential phase of growth. Sorbose was utilized for transport measurements because it is not metabolized and enters these cells by facilitated diffusion (3). Potassium phosphate buffers were employed as before, except that 0.06 M sodium maleate buffer was used when solutions of CaCl\(_2\) or MgCl\(_2\) were required. Absorbancy of materials lost from cells was measured at 280 nm, a wavelength at which MP absorption does not interfere as it does at 260 nm (11). Assay of radioactivity was modified, in that membrane filters with collected cells were transferred directly to vials containing 10 ml of Bray’s (2) liquid scintillator, and counts were made in a Packard Tri-Carb instrument. MP-hydrochloride was supplied by Ciba Pharmaceutical Co., Summit, N.J.

Spheroplast preparation. Cells, harvested at a count of 2 \times 10^7/ml, were washed twice with distilled water by suspension and centrifugation and then were resuspended to a concentration of 3 \times 10^7/ml in 0.02 M phosphate buffer (pH 5.6) containing 1.0 M glycine. A 0.2-ml amount of Glusulase (Endo Laboratories, Inc., Garden City, N.Y.), including 0.1% added cysteine, was added to each milliliter of cell suspension, and the mixture was incubated with gentle shaking for 2 hr at 30 C. After incubation, additional buffer-glycine solution was added to dilute the enzyme, and the spheroplasts were removed from the suspension by centrifugation for 3 to 4 min at 3,000 rev/min. (Approximately 10% of the cells did not rupture when diluted 1:10 with water.) Spheroplasts were washed once with buffer-glycine.

Spheroplast uptake and efflux measurements. For uptake measurements, spheroplasts were resuspended, 4 \times 10^7 or 5 \times 10^7/ml, in 5 ml of buffer-glycine containing '\(^4\)C-labeled sorbose and MP as required. They were shaken in a water bath at 30 C during the sampling period. For efflux measurements, spheroplasts were loaded with sorbose during a 90-min period of uptake, chilled in an ice bath and by the addition of ice-cold buffer-glycine, and removed from suspension by centrifugation. They were washed twice with ice-cold buffer-glycine, suspended at zero minute in 30 C buffer-glycine, and shaken in a water bath at 30 C during the sampling period. Samples (0.5 ml) taken at intervals during uptake and efflux measurements were chilled by addition of 10 ml of ice-cold buffer-glycine and centrifuged to remove the spheroplasts which were washed twice with ice-cold buffer-glycine. Spheroplasts were then transferred to vials of scintillation fluid by three rinses with 0.3 ml of water.

Glycine at 1.0 M altered the pH of 0.02 M phosphate buffer solutions; for example, the pH of the generally used buffer was lowered from 7.0 to 6.7. Corrected or adjusted pH values are given in the text.

Methylene blue staining. Samples containing cells and spheroplasts were diluted (5 \times 10^7/ml) with a solution of methylene blue in 0.02 M buffer (including 1.0 M glycine with spheroplasts), pH 4.5, and incubated for 5

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RESULTS

Uptake and loss of sorbose by spheroplasts. To differentiate an effect on the external cell membrane from one on the cell wall (11), yeast spheroplasts, presumably having little wall material, were utilized to measure the effects of MP on sorbose uptake and efflux. MP at 10 mM inhibited sorbose uptake (Fig. 1A). With 30 mM MP, a brief period of uptake was followed by a sharp decrease in spheroplast sorbose content. The decrease indicated either that membranes had become permeable and sorbose was lost during the washing procedure or that the spheroplasts had been entirely disrupted. Because MP action is pH-sensitive (11), the effect on uptake was observed at several pH levels. At pH 7.0, the results were similar to those at pH 6.7 (Fig. 1A); at pH 5.6, 10 mM MP was ineffective and 30 mM MP reduced uptake about 50%.

Sorbose was lost rapidly from spheroplasts incubated with 30 mM MP (Fig. 1B), but efflux was reduced by 10 mM MP as it was from whole yeast cells (11). The rapid loss again indicated cell disruption or membrane leakage.

Rupture and staining of spheroplasts. Staining of yeast cells by methylene blue has been used as a measure of increased membrane permeability (10); normal living cells do not stain at low concentrations of the dye (8). Table 1 lists the number of intact spheroplasts and the percentage which stained with methylene blue at various times during suspension in solutions of MP. Staining evidently preceded complete spheroplast rupture. MP at 20 mM caused increasing numbers of spheroplasts to rupture during the period of observation so that at 90 min only 45% of the original number remained; approximately one-third of these stained with methylene blue. MP at 10 mM caused less than 20% of the spheroplasts to rupture or to stain during the 90-min period, and 30 mM MP caused most of them to rupture. Clearly the sharp inflexion in the uptake curve and the rapid loss during exit measurements are correlated with membrane rupture.

Loss of constituents from whole cells. The rupture of spheroplasts suggested that MP may also disrupt the membranes of whole cells. If this occurs, previously observed decreases in sorbose uptake by whole cells treated with 30 mM MP (11) may have combined two effects: a block in sorbose passage and a loss from cells with ruptured membranes unable to retain the sugar during the washing procedure. Losses of materials that absorb at 280 nm increased as cells were incubated in higher concentrations of MP (Fig. 2). A reduced loss, compared with the control, from yeast treated with 10 mM MP confirms an earlier measurement demonstrating this effect (11). Incubation with MP also increased the number of cells stained by methylene blue (Table 2), although higher concentrations were required than caused spheroplasts to stain. The loss of cell materials establishes that increased permeability or membrane rupture occurred only at higher concentrations of MP.

Sorbose uptake and loss by whole cells. Because 10 mM MP markedly inhibited carbon dioxide production, concentrations above 30 mM were not studied.

TABLE 1. Microscope counts of total and methylene blue-stained spheroplasts after methylphenidate (MP) treatment

<table>
<thead>
<tr>
<th>MP concn. (mM)</th>
<th>Total spheroplasts* at 15 min</th>
<th>Total spheroplasts* at 30 min</th>
<th>Total spheroplasts* at 60 min</th>
<th>Total spheroplasts* at 90 min</th>
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<tbody>
<tr>
<td>0</td>
<td>101</td>
<td>100</td>
<td>98</td>
<td>104</td>
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<td>45</td>
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<tr>
<td>30</td>
<td>69</td>
<td>53</td>
<td>32</td>
<td>12</td>
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* Values are averages of six separate experiments, except for treatments with 5 and 30 mM MP which are averages of three and two values, respectively.

* Per cent of zero minute count.
FIG. 2. Effect of MP on the loss of materials from whole yeast cells. Yeast was resuspended (6 × 10^8 cells/ml) at 0 min in 0.02 M phosphate buffer (pH 7.0) containing 0.1 M sorbose and indicated amounts of MP and shaken in a water bath at 30 C. Samples were taken at the plotted time intervals; the cells were centrifuged from suspension, and the optical density (OD) of the supernatant fluid was measured at 280 nm against a buffer-sugar reference.

### Table 2. Microscope counts of total and methylene blue-stained whole yeast cells after methylphenoxyanilide (MP) treatment

<table>
<thead>
<tr>
<th>MP concn. (mM)</th>
<th>No. staineda (%)</th>
<th>Total cells at 90 minb</th>
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<tr>
<td></td>
<td>30 min</td>
<td>60 min</td>
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<td>74</td>
</tr>
<tr>
<td>90</td>
<td>74</td>
<td>94</td>
</tr>
</tbody>
</table>

a Values are averages of four separate measurements, except for treatments with 70 and 90 mM MP which are averages of two measurements.
b Per cent of initial count.

employed in previous measurements of sugar transport across yeast membranes (11). But higher concentrations ruptured the membranes of whole cells as demonstrated by experiments similar to those with spheroplasts (Fig. 3). MP at 100 mM acted slowly enough so that some sorbose was taken up before cell membranes ruptured and the sorbose was lost (Fig. 3A). Why the rate of uptake was greater than that for cells incubated in 50 mM MP is not apparent; membrane injury sufficient to allow leakage into the cell (4) but not to increase loss during washing could explain this result. The 50 mM concentration reduced the rate of uptake immediately, presumably by partly blocking sorbose entry; rupture of external membranes was not a major factor in the reduced total uptake until after about 90 min, as shown by the exit curve (Fig. 3B). Exit from cells incubated with 100 mM MP evidently consisted of a total loss from cells with ruptured external membranes; the increased rate of loss is shown by the steeper initial slope of the exit curve, combined with some usual efflux, indicated by the fact that exit still occurred in two phases.

Treatment of yeast with iodoacetic acid (1AA) or N-ethylmaleimide (NEM) before sorbose uptake eliminates the slower second phase of sorbose efflux, apparently by selectively affecting intracellular membranes which form a compartment for this sugar (11). Because the yeast takes up sorbose, a functional external membrane must
survive the pretreatment; rates of exit, then, should not confound normal efflux with losses from ruptured cells, as evidently occurs in some experiments (Fig. 3). However, pretreatment with MP for 40 or 60 min only partially eliminated the second phase of efflux (Fig. 4), indicating that a sorbose restrictive mechanism persisted in these cells, or in some of them. Pretreatment for 90 min was more effective, but it also increased the initial rate of exit.

**Intracellular effects of MP.** Electron micrographs (Fig. 5) show marked internal changes in yeast treated with MP. MP (50 mM) caused severe disruption of intracellular membranes with an accompanying thickening or agglomeration of membrane fragments, and 30 mM MP initiated similar changes. MP (10 mM) affected vacuolar membranes as evidenced by breaks and by increased amounts of debris within the vacuole; mitochondria were distorted and showed membrane disruptions, and nuclear membranes assumed a more symmetrical shape and appeared to thicken. Although some internal changes may be mediated by effects on the external membrane, the extensive changes indicate that MP entered the cell. Mitochondria are large and few in number in these cells grown in medium with a high glucose concentration.

**Modified MP effects.** MP and NEM together did not block sorbose uptake more effectively than they do individually, but they did cause more rapid disruption of cell membranes (Fig. 6A), an effect which was apparent also in an increased rate of sorbose loss (not shown). Ca\(^{2+}\) has been observed to stabilize yeast membranes against disruption by butyl alcohol (7). With MP, no evident effect occurred at low concentrations, but at a concentration of 50 mM (Fig. 6B) the cell membrane was ruptured more rapidly when CaCl\(_2\) was present. Similarly, disruption was speeded when CaCl\(_2\) was included with NEM. MgCl\(_2\) (100 mM) produced results (not shown) similar to those with 20 mM CaCl\(_2\). CaCl\(_2\) alone had no effect on sorbose uptake (not shown).

**DISCUSSION**

That an inhibition of sorbose uptake by yeast spheroplasts is due to an effect by MP on the cell membrane rather than on the wall is not unequivocal. Wall material may be retained by spheroplasts (1, 5) although this may not always be the case (9). However, the breakdown of sugar-restraining structures, evidenced by sharp decreases in sorbose content during uptake experiments and rapid losses during efflux measurements, and the disruption of spheroplasts show that membranes were affected and ruptured. The large pores of the normal yeast wall (13) cannot retain sorbose without the cell membrane.

Less sorbose was taken up by spheroplasts than by whole cells [compare Fig. 1 and 3; whole cells averaged 0.25 mg (dry weight)/10⁷ cells]. Several factors may be involved in this difference, the most significant one probably being a breakdown and loss of cells during handling. A few cell counts, taken before and after washing samples for radioactivity measurements, showed as much as a 25% decrease in number. Additionally, a greater apparent rate of exit from spheroplasts than from whole cells may have allowed some sorbose loss during washing, and 1.0 mM glycine may have had an osmotic or other effect. Because the amount of uptake was not of primary concern in these studies, the difference was not further evaluated.

The intracellular changes observed in electron micrographs add another dimension to MP effects and provide a basis for explaining a reduced...
oxygen uptake by cells supplied with ethyl alcohol (11), assuming that MP did not block alcohol entry. Even low concentrations of MP affected mitochondrial structure, an effect which clearly might be expected to influence oxygen use. An apparent greater reduction in oxygen uptake than in carbon dioxide output with glucose as substrate (11), also, is not inconsistent with MP effects upon mitochondria. Possible involvement of intracellular damage in the inhibition of glycolysis must be considered in designing future studies.

It is apparent from these experiments that staining with methylene blue, which indicates nonviable cells (8), is not always a measure of increased mitochondrial permeability. Staining occurred in MP-treated cells which did not lose sorbose or which lost it at a later time (compare Table 2 and Fig. 3), showing that external membranes functioned to retain sorbose although the cells were nonviable. The intracellular damage observed in electron micrographs offers a ready reason for the loss of viability. Staining and sugar loss were closely correlated in IAA-treated cells (10), possibly because nonviable cells were produced by first rupturing external membranes. Evidence that IAA disrupts the vacuolar membrane more effectively than the external membrane (10) does not preclude (although it does not reinforce) this possibility. That nonviable cells produced by X-irradiation may not stain with methylene blue (12) emphasizes the need to correlate stain responses with other evidence for specific cellular changes.

MP was not as selective as IAA in eliminating the second phase of sorbose efflux. If this slower efflux is due to an internal compartment, possibly the cell vacuole (10), the extensive intracellular disruptions caused by MP should eliminate it and allow exit at a single rate through the unaffected
external membrane. Two-phase efflux after pretreatment with MP (Fig. 4) indicates most probably that part of the yeast population, not clearly specifiable by electron micrographs, resisted MP action. The 40- and 60-min curves of Fig. 4 could be accounted for if 35 and 10%, respectively, of the cells retained a second phase of efflux similar to that of control yeast. The increase in methylene blue staining which occurred between 30 and 90 min of MP treatment (Table 2) is consistent with a decreasing number of cells which resist internal disruption and, therefore, with steeper second slopes of efflux. The small decreases in total sorbose uptake by pretreated cells (zero minute ordinate values, Fig. 4) confirm that external membranes were ruptured in only a small part of the yeast population (see also the exit curve of Fig. 3). Thus, these results are consistent with a difference in the manner in which MP and IAA affect cell viability as suggested above.

The accumulated data indicate that MP affects membranes in a general way by reacting with one or more constituents as pointed out previously (11). The inhibition of carbon dioxide production by low concentrations of MP was not entirely reversible, and different transport systems were inhibited (11); at higher concentrations, cells lost materials which absorb at 280 nm, cells were stained with methylene blue and lost sorbose, and spheroplasts were lysed. By way of comparison, polyene antibiotics, for example, cause graded damage to yeast membranes ranging from specific effects, i.e., largely reversible, by N-acetyl-candidin to more general, nonreparable damage by filipin (4). Various proteins also may affect yeast in a general way, producing leaky external membranes and disorganizing internal structures (13). MP effects are not specific in the sense of being reversible, but selectivity occurs. Thus, inhibition of sugar entry and exit and a decrease in the loss of materials absorbing at 280 nm were initiated separately from membrane rupture and affected solute movement oppositely. The intracellular changes seen in electron micrographs also occurred independently of the rupture of external membranes. Moreover, because cells pretreated with MP take up almost as much sorbose as untreated cells (Fig. 4) but glycolytic inhibition can be reversed only about 50% (11), the block in transport may be separable from effects on internal structures and functions. Other measurements could supply further details for this general picture; they are perhaps best accomplished in additional specific studies with MP.

The nature of the reaction(s) in which MP participates is still unclear. The fact that MP and NEM at low concentrations together produced a synergistic increase in membrane rupture sug-

\[ \text{Fig. 6. Effects of MP combined with NEM and with Ca}^{2+} \text{ on sorbose uptake by whole yeast cells. Uptake measurements were made as described for Fig. 3. Control cells were resuspended at 0 min in buffer-sorbose solutions; other cells were resuspended in buffer-sorbose plus the indicated additions.} \]

ACKNOWLEDGMENTS

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LITERATURE CITED

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