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# Penetration of Mannitol into the Intracellular Space of *Chlorella sorokiniana*[1]

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## ABSTRACT

**It was found that in the alga, *Chlorella sorokiniana*, mannitol penetrated the plasmalemma (normally thought to be impermeable to mannitol) into the intracellular space. The rate of penetration is exponential and relatively slow, having a half-time of 6 to 12 minutes and requiring over 60 minutes for complete penetration. This penetration was demonstrated both by the Millipore filtration of cells incubated with $^{14}$C-mannitol and by centrifugation of the cells through a silicon oil layer after incubation with tritiated water and $^{14}$C-mannitol. Further, mannitol caused an inhibition of both autotrophic (on $CO_2$) and heterotrophic (on glucose) growth. A low rate of mannitol metabolism was demonstrated, although this rate could not support heterotrophic growth.**

Mannitol and sorbitol are often used to estimate the free space (that volume which is between the outside surface of the wall and the plasmalemma) of algal cells (10) or the amount of trapped supernatant during pelleting of cells and organelles (5). Other researchers have used high concentrations of sorbitol (1, 9) or mannitol (4) to change the osmotic potential outside plant cells and, thus, to affect the internal water potential of the plant cell.

In the above studies it was assumed that mannitol did not and could not penetrate the cell since if it did, it could neither counterbalance the internal water potential nor be used to measure free space reliably. In our studies of ozone-induced $K^+$ loss from *Chlorella sorokiniana* (2), mannitol was used to alter the external water potential. However, we obtained some anomalous results which could not be explained if the membrane were truly impermeable to mannitol. In this paper the slow penetration of mannitol into *C. sorokiniana* has been demonstrated by two separate methods.

## MATERIALS AND METHODS

*C. sorokiniana* (high temperature strain 07-11-05) was grown autotrophically as previously described (2) at 38 C with 5% $CO_2$ in air. The light was provided by a bank of 40 fluorescent lamps (intensity at about 8 mw/cm$^2$ as measured by a Yellow Springs Instrument radiometer). Cells were grown heterotrophically on a 1% glucose supplement to the autotrophic medium in the dark under air at 38 C. The growth was monitored by the optical density of the cells, measured in the growth tubes at 550 nm using a Coleman junior spectrophotometer, model 6C (path length, 2.5 cm). This procedure measures light scattering by the cells and is nearly proportional to the cell concentration.

The cells were incubated in various media with $^{14}$C-mannitol or $^{14}$C-inulin as tracers (exact concentrations are noted in figures and tables). At various times, aliquots were subjected to either Millipore filtration or microfuge pelleting to separate cells and supernatant. When Millipore filtration was used, the sample (0.5 ml) was placed on a 0.22-$\mu$m (pore diameter) Millipore filter and vacuum was applied to the lower surface of the filter to remove the supernatant. The cells were then washed with 10 volumes of unlabeled medium and the Millipore + cells were counted in a scintillation cocktail (90 mg POPOP, 4 g PPO/l of toluene, which was mixed with Triton X-100 and water at ratio of 2:1:0.3, v/v) on a Beckman LS-100 scintillation counter. The microfuge pelleting was similar to the technique described by Gaensslen and McCarty (5) and used a Beckman microfuge. To the sample, 1.38 $\mu$Ci/ml of $^3$H-$H_2O$ was added to act as a marker for the water space. The top layer in the microfuge tube was 100 $\mu$l of the cell sample (optimum concentration was $4 \times 10^8$ cell/ml), the organic separation phase was 100 $\mu$l of Dowex silicon fluid (No.200) and the lower phase was 50 $\mu$l of 1 M sucrose. The lower phase often was placed on an impenetrable 50-$\mu$l cushion of 70% sucrose to facilitate the removal of the final pellet. After a 15-sec centrifugation (about 50,000$g$) to pellet the cells, a sample of the top phase (20 $\mu$l, supernatant only) and the full bottom phase were counted as described above. The amount of mannitol in the pellet was used to correct the total amount of $H_2O$ in the pellet to calculate the water space of the cells (called mannitol-excluded space). When $^{14}$C-inulin was used as a tracer for the supernatant carried into the pellet, the calculated water space was called the inulin-excluded space.

The size distribution of the cells was measured by the Coulter Counter, as previously described (7). In addition to the volume given by the peak of the distribution (peak volume), the median volume – or that volume for which half of the number of the cells in the sample are below and half are above – could be determined by integration of the total cell distribution (7).

Metabolically released $^{14}CO_2$ was measured in a closed system. Pressurized air (10 ml m$^{-1}$; flow rate) flowed through a well shaken flask containing the cells and then into a 1-ml phenethylamine solution in an open scintillation vial. The cells ($2 \times 10^9$ cells) were incubated in 3 ml of 25 mM tris-Cl, 1 mM $CaCl_2$ (pH 8.3) at 38 C in the dark. At zero time, radioactive glucose 1-$^{14}$C (10 mM, 1 $\mu$Ci) or U-$^{14}$C-mannitol (50 mM; [1 $\mu$Ci]) was injected into the vessel containing the cells. As the injected compound was metabolized, the $^{14}CO_2$, which was produced by the cells during a 5-min interval, was trapped by 1 ml of phenethylamine in the scintillation vial (efficiency 100%). The phenethylamine solution was then counted as described above.

The radiochemicals were purchased from New England Nuclear Corp.

## RESULTS AND DISCUSSION

The determination of the free space is usually measured by the $^{14}$C-mannitol which is included within the cell (10). Chimiklis and Heath (2) found that added mannitol changed the apparent

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size of the cells, as measured by the Coulter Counter. They proposed that the free space was being increased by the added mannitol.

In order to determine the stability of the free space change (with external osmoticum), a time course of mannitol uptake by *Chlorella* was performed with results as shown in Table I. No change in the amount of mannitol taken up by the cells with time of incubation was expected, if mannitol did not penetrate into the protoplasts (that portion of the cell within the plasmalemma). As seen in Table I, this is, unfortunately, not the case.

The variation of the data on mannitol uptake in part can be ascribed to experimental variability especially during the washing. Sites within the cell wall might bind mannitol especially tight at low mannitol concentrations, making it difficult to wash away (there is little mannitol binding to the Millipore filter). The zero time value of the data is usually variable and is not to be taken to be quantitative. The uptake can be expressed by subtracting the zero time point. Figure 1 shows the kinetics of mannitol uptake at two widely differing concentrations of mannitol (4 $\mu$M and 0.5 M). The kinetic data are exponential (Fig. 1, C and D) with half-times of about 6 to 12 min. The apparent final concentration of mannitol taken up (the extrapolated value from the curves) is in excess of the concentration added. Calculated from the known volume of the cells (Table II or ref. 2), the internal concentrations of mannitol are approximately 4 M for an external concentration of 0.5 M and 31 $\mu$M for an external concentration of 4.1 $\mu$M. Further from these data, the permeability coefficient, calculated for the initial rate of mannitol uptake, is $7.4 \times 10^{-7}$ cm $sec^{-1}$ for an external mannitol concentration of 4 $\mu$M and $3.6 \times 10^{-7}$ cm $sec^{-1}$ for an external mannitol concentration of 0.5 M. It is not important for this paper to determine whether the variation in the permeability coefficient is real. It is enough to state that the uptake of mannitol is real, has normal passive-like kinetics, and leads to an apparent accumulation of mannitol over the external concentration.

With a double label ($^{14}$C-inulin or mannitol and $^{3}$H-water), the water space of *Chlorella* can be measured by the microfuge techniques (5) using mannitol or inulin to correct for the trapped supernatant. The measured cell water volume using mannitol is much lower than either the peak volume of the distribution or the median volume, as measured by the Coulter Counter (Table II). On the other hand, the cell water space measured with the large polymer, inulin, is the same as or slightly higher than the

Table I. Mannitol uptake by Chlorella cells in tris-medium

| Mannitol concentration | Mannitol uptake | | |
|---|---|---|---|
| Time | 0.01 M | 0.1 M | 0.6 M |
| Min | ($10^{-15}$ mol/cell) | | |
| 0 | 0.90 | 0.18 | 1.68 |
| 10 | 0.37 | 1.21 | 1.46 |
| 20 | 2.5 | 1.02 | 3.07 |
| 30 | 2.1 | 1.22 | 3.18 |

The cells ($10^8$ cells/ml) were suspended in tris-Cl (25 mM), $CaCl_2$ (1mM) at pH 9. Mannitol tracer ($^{14}$C) was added (0.8$\mu$Ci/ml) with unlabeled mannitol to give the indicated concentration. At various times a 200$\mu$l aliquot was removed (out of 5 ml initial volume), filtered, washed as in Methods, and counted.

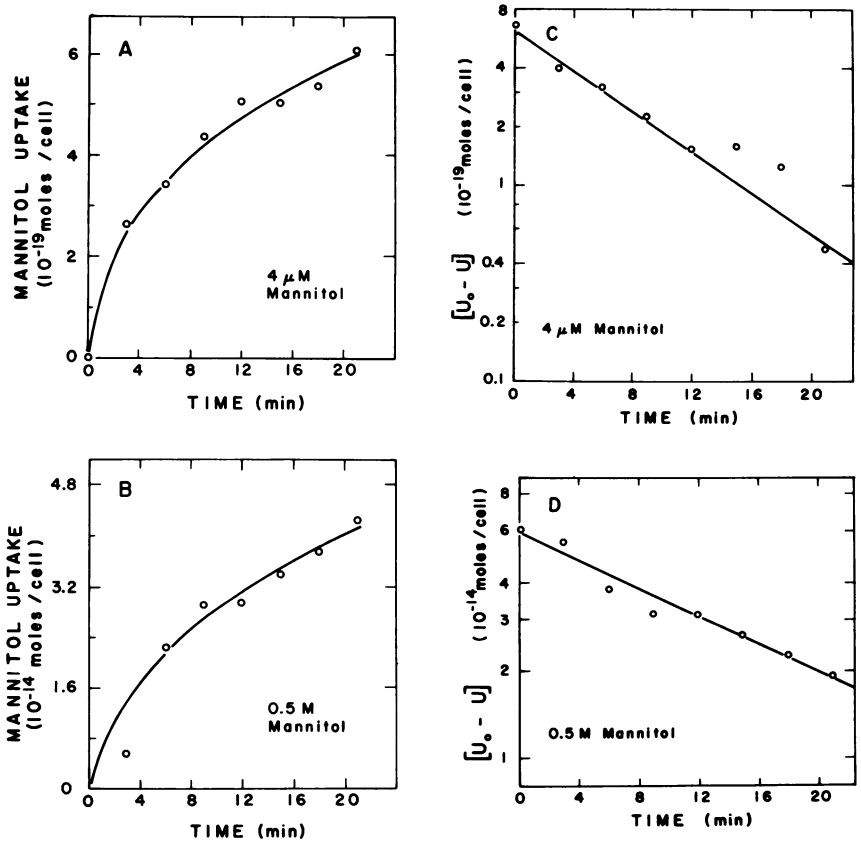


FIG. 1. Kinetics of mannitol uptake by *Chlorella*. *C. sorokiniana* were grown and washed as described under "Materials and Methods." Uptake experiments were run in 5 mM K-phosphate buffer at pH 6.3, 0.5 mM $CaCl_2$, 0.5 mM $MgCl_2$ with $10^8$ cells/ml and either 4.1 $\mu$M mannitol (0.087 $\mu$Ci/ml) or 0.5 M mannitol (1.20 $\mu$Ci/ml) at 38 C. Sampling and counting were carried out as described under "Materials and Methods." A: Linear kinetics for 4 $\mu$M mannitol; B: linear kinetics for 0.5 M mannitol; C: semilog plot of A. Least squares fit is $6.1 \times 10^{-19}$ moles/cell $(1 - \exp[-0.106\ t \cdot min^{-1}])$ with a correlation coefficient of 0.966; D: semilog plot of B. Least squares fit is $5.8 \times 10^{-14}$ moles/cell $(1 - \exp[-9.054 \cdot min^{-1}])$ with a correlation coefficient of 0.981. Time ($t$) in C and D is in min. U-uptake and $U_0$ is maximum uptake.

Table II. Water space of Chlorella

| | Exclusion space | Coulter Counter Size: Peak | Coulter Counter Size: Mean | Ratio of Space/Mean |
|---|---|---|---|---|
| | | $10^{-15}$ l/cell | | % |
| Mannitol | | | | |
| 1. | 6.9 ± 3.1 (6) | 11.2 ± 2.2 | 15.0 ± 3.2 | 46 |
| 2. | 5.0 ± 1.0 (9) | 9.8 ± 2.4 | 14.4 ± 3.1 | 35 |
| Inulin | | | | |
| 1. | 16.8 ± 0.8 (9) | 11.9 ± 0.5 | 17.7 ± 1.8 | 95 |
| 2. | 24.2 ± 1.3 (9) | 10.9 ± 0.7 | 18.9 ± 2.1 | 128 |

The water space (either mannitol - or inulin-exclusion space) was calculated as in Methods. The incubation was carried out for 60 min in 10 mM tris-Cl, 1 mM $CaCl_2$ pH 8. The concentration of mannitol was 35 μM (0.69 μCi/ml) and the concentration of inulin was 10 μM (0.17 μCi/ml). The standard deviation is given with the number of experiments in brackets. The Coulter Counter size was calculated as in Methods. The standard deviation for the Coulter Counter size is the deviation of the least-squares fit of the peak or mean volume at various amplification settings (see ref. 7).

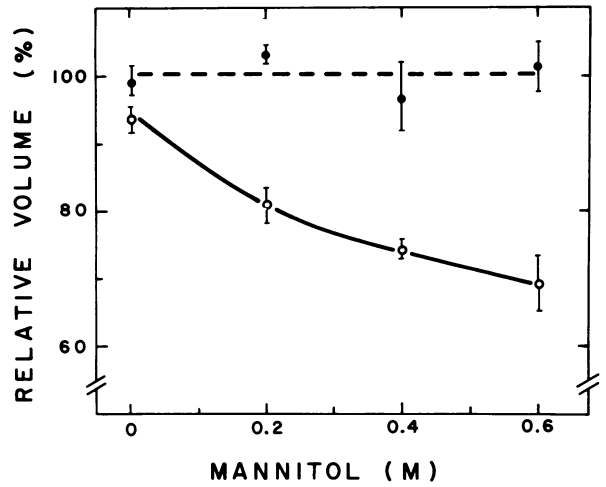


FIG. 2. Volume of *Chlorella* cells upon suspension in mannitol. Relative volume of the cells was measured by the Coulter Counter in a suspension medium of 3.3 mM K-phosphate buffer (pH 6.3), 0.33 mM $CaCl_2$, 0.33 mM $MgSO_4$, and 26 mM choline chloride. The initial volume of the cells upon being suspended in mannitol (within 1-2 min) is shown as (○) and (——). The volume of the cells upon being suspended in mannitol for 90 min is shown by (●) and (·····). The bars represent one standard deviation as determined as in Table I. Note the offset on the ordinate.

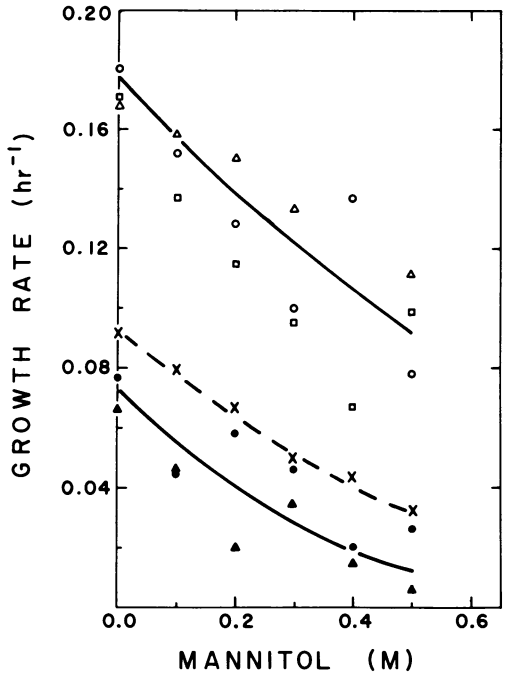


FIG. 3. Rate of growth of *Chlorella* in the presence of mannitol. The growth rate was based upon the change in light absorption ($A$) of the culture with the rate coefficient ($k$) being defined by $A = A_o \times 10^{+kt}$ where $t$ = time after inoculation. Culturing techniques are described under "Materials and Methods." The autotrophic growth rate (○△□) was determined between 0 and 3 hr of culture (○), 3 and 6 hr (△), and 4 and 8 hr on the second cycle of growth (□) after dilution with fresh media. The heterotrophic growth rate (■▲ and ×) was determined between 0 and 7 hr of culture (●) and 7 and 24 hr (▲). (×) represents a culture of cells, which was induced by added glucose (24 hr of growth in the dark on glucose without added mannitol).

median. Inulin should be too large a molecule (mol. wt. = 5000) to pass through the membrane or cell wall and, from these measurements, apparently does not. In addition, the conditions of this experiment were such that the volume outside the the wall must have been minimal (since the concentrations of the added molecules were nearly zero). The incubation time was long enough for a complete penetration of mannitol to occur.

As a further test of the penetration, the Coulter Counter volume of the *Chlorella* cells was determined initially and after 90 min of incubation in several concentrations of mannitol (Fig. 2). Initially, cell volume was less with the higher concentrations of mannitol. However, after long term incubation, the volume was constant—independent of external mannitol concentration. The slight increase in volume may represent a small amount of growth over the 90-min period (see Fig. 3 for growth rate). The explanation is clear. Mannitol does not penetrate the cell at first and so the water potential outside induces a net water flow out of the cell and the volume decreases. As mannitol penetrates, water moves back into the cell and the volume returns to normal.

More mannitol accumulates within the cell than predicted by the assumption that the inside and outside mannitol concentrations are in equilibrium. Therefore, either that assumption is false, which is unlikely, or the excess amount ($1.9 \pm 0.2 \times 10^{-20}$ moles/cell for 13 μM external mannitol) represents sites on the cell wall which bind mannitol as previously postulated for the zero time value (Table I).

Obviously, another likely explanation for this apparent accumulation is that this amount of mannitol is in a metabolically altered form within the cell. In fact, some radioactive $CO_2$ can be shown to be released upon feeding $^{14}$C-mannitol to cells ($2.7 \pm 0.2 \times 10^{-19}$ moles-cell$^{-1}$ m$^{-1}$), though the rate was less than 1% that of glucose-supported $CO_2$ release ($3.4 \pm 1.4 \times 10^{-17}$ moles cell$^{-1}$ m$^{-1}$). This rate could be due to some radioactive mannose

contaminant of the mannitol, although neither mannose nor mannitol supports cell growth either in the light or dark (ref. 6 and unpublished data). Mannitol, in fact, suppressed the long term autotrophic and heterotrophic growth of cells nearly equally (Fig. 3). Since mannitol penetrates the cells (Fig. 2 and Table II), suppression of growth is probably not due to an external osmotic stress but to an alteration of the metabolism of or to an elevated, internal osmotic pressure within the *Chlorella*.

## CONCLUSIONS

It is clear that the use of mannitol as an external osmoticum for plant cells is unreliable unless it is shown that there is indeed no penetration. The suppression of growth by mannitol in these *Chlorella* (Fig. 3) cannot be attributed to simple external osmotic pressure or to a simple reduction in internal water potential, since altered water potentials do not occur for long incubations (Fig. 2). The results show a decline in growth rate over day's growth period, even though complete penetration of mannitol takes less than 1 hr (Fig. 1).

Larger molecules should be used as impenetrable, osmotically active compounds, and even then, the lack of penetration should be shown. Mannitol is trustworthy in *Chlorella* cells as an osmotic agent only during the first few min after addition (Fig. 2 and ref. 2).

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