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Hydrogen-Mediated Mannose Uptake in *Azotobacter vinelandii*†

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***Azotobacter vinelandii* can grow mixotrophically with H_2 plus mannose under N_2-fixing conditions (T. Y. Wong and R. J. Maier, J. Bacteriol. 163:528–533, 1985). Mixotrophically grown cultures incubated in H_2 transported mannose with a V_{max} fourfold greater than that observed for cultures incubated in argon, but H_2 did not change the apparent K_m for mannose. Respiratory inhibitors, such as potassium cyanide, hydroxylamine, and *p*-chloromercuribenzoic acid, as well as the proton conductor carbonyl cyanide *m*-chlorophenylhydrazone inhibited mannose uptake. We suggest that one of the roles of H_2 in mixotrophic metabolism is to supply energy that facilitates mannose transport.**

Azotobacter vinelandii can grow mixotrophically, with H_2 as the inorganic energy source and mannose as the carbon source (16). Both the growth rate and the cell yield are dependent on the amounts of both substrates, H_2 and mannose. Hydrogen does not significantly stimulate the growth of *A. vinelandii* with other carbon sources, and the mannose-dependent mixotrophic growth occurs only when the cultures are fixing nitrogen (16). In ammonium-containing medium, mannose-mediated growth of *A. vinelandii* is not dependent on added H_2 (16). In *Azotobacter chroococcum*, H_2 oxidation via hydrogenase is also beneficial for the growth of the organism under N_2-fixing but not NH_4^+-utilizing conditions (1). It has been proposed that under C-limiting conditions, hydrogenase is crucial for the initiation of diazotrophic growth (1). Hydrogen uptake is known to be able to support oxidative phosphorylation in *A. vinelandii* (8), and H_2 oxidation results in electron flow ultimately to O_2 (8, 10) through a cytochrome-dependent respiratory chain (8, 15).

^{14}C-sugar uptake experiments on mixotrophically grown cultures (16) suggested that H_2 plays a role in mannose transport; the mannose uptake rate when cells were incubated with H_2 was two- to threefold greater than that when cells were incubated with argon. In this report we demonstrate that H_2 has the ability to provide energy for mannose transport and that H_2 provided to cultures causes a significant increase in the V_{max} for mannose transport in *A. vinelandii*.

Mannose uptake experiments were performed similarly to those previously described (16). All experiments used *A. vinelandii* CA (provided by Paul Bishop, North Carolina State University, Raleigh), which is the same as strain OP (4). Strain OP was utilized previously for H_2-dependent mixotrophic-growth experiments (16). Cells were grown in Burk medium (6) with H_2 and 2% mannose as described previously (16), harvested at a concentration of 4×10^8 to 6×10^8 cells per ml, and then washed three times with 50 mM potassium phosphate buffer (pH 7.5). The cells were suspended in carbon-free Burk medium to about 1.5×10^8 cells per ml, and the culture was then incubated at 30°C for approximately 2 h with gentle stirring to deplete the endogenous substrate (16). Samples (2.5 ml) were removed into 70-ml serum vials. The vials were stoppered tightly, and the appropriate gas (H_2 or Ar) was added. At 1 min after addition of 5 cm^3 of H_2 or argon, [^{14}C]mannose was injected to initiate the 1-min assay. The [^{14}C]mannose (D-[U-^{14}C]mannose) (Amersham Corp., Arlington Heights, Ill.) had a specific activity of 280 mCi/mmol. Unless otherwise indicated, each reaction vial received 0.5 mM mannose at a specific activity of 0.5 μCi/μmol. The vials were placed on a rotary shaker at 300 cycles per min during the 1-min assay. For the kinetic experiments in which the substrate concentration was varied, the lower concentrations tested (0.05, 0.10, 0.20, 0.30, and 0.40 mM) contained 2.0 μCi/μmol, and the other concentrations (0.6, 0.8, 1.0, 1.5, and 2.0 mM) had 0.5 μCi/μmol in the reaction vial. After 1 min at 30°C with shaking, a 2.0-ml sample was removed and four 0.5-ml portions from this sample were rapidly placed on a filter unit (12-sample filtering manifold; Millipore Corp., Bedford, Mass.) under vacuum. The filters (pore size, 0.22 μm; GSWP 02400; Millipore) were immediately washed with two 10-ml volumes of ice-cold 50 mM potassium phosphate (pH 7.5). Filters were allowed to air dry and placed into 6-ml scintillation vials, and then 5 ml of Aquasol-2 (New England Nuclear Corp., Boston, Mass.) was added and radioactivity was counted in an LKB model 1218 scintillation spectrometer. Background counts from filters receiving the [^{14}C] mannose solution but without cells were subtracted from all experimental values, and control experiments with boiled cells demonstrated negligible incorporated radioactivity. The viable cell number was determined by plate counts of appropriate dilutions, and protein amounts were determined from a standard curve of *A. vinelandii* viable cell number versus protein concentration (15). For the inhibitor studies the amount of mannose taken up in 1 min by cells with Ar plus the inhibitor was subtracted from the rate with H_2 plus the inhibitor. The difference is called the H_2-dependent rate (Table 1). The [^{14}C]mannose was added 1 min after the injection of H_2 or Ar, and the samples were removed 1 min after the addition of mannose. For the study of mannose uptake with time (Fig. 1), 10 ml of cells at 2×10^8 viable cells per ml was added to 250-ml prescription bottles. The bottles were sealed with a serum stopper, the appropriate gas (20 cm^3 of H_2 or Ar) was added, and then 0.5 mM mannose at 1.0 μCi/μmol was added to initiate the assay.

Previous results indicated that cultures incubated with H_2

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TABLE 1. Effect of respiratory inhibitors on H_2-mediated O_2 uptake and mannose uptake rates[a]

Inhibitor	Concn (mM)	Uptake rate (% of uninhibited)	
		Oxygen[b]	Mannose[c]
KCN	0.02	81 ± 5	88 ± 18
	0.10	72 ± 5	46 ± 7
	0.70	5 ± 0.3	1.5 ± 0.3
Hydroxylamine	0.10	133 ± 7	104 ± 4
	1.0	105 ± 7	104 ± 4
	5.0	55 ± 13	84 ± 6
	20.0	42 ± 6	43 ± 9
Antimycin A	0.05	104 ± 6	107 ± 3
	0.5	95 ± 8	99 ± 4
	10.0	51 ± 5	50 ± 10
pCMPS	0.05	84 ± 9	110 ± 13
	0.5	70 ± 13	81 ± 4
	2.0	38 ± 5	67 ± 5
CCCP	0.01	89 ± 10	75 ± 2
	0.1	113 ± 17	41 ± 3
HQNO	0.5	97 ± 6	93 ± 6
	5.0	79 ± 5	61 ± 9
pCMB	0.01	45 ± 9	80 ± 5
	0.05	28 ± 5	33 ± 3
	0.5	6 ± 3	16 ± 4
Chlorpromazine	0.1	84 ± 7	ND
	1.0	89 ± 5	ND
	5.0	79 ± 10	ND

[a] Cultures were grown with H_2 and mannose, the cells were harvested by centrifugation, and the pellet was washed three times and suspended in carbon-free Burk medium (see text). After depletion of endogenous substrate, samples were incubated with the indicated inhibitor for 1 h, and then O_2 or mannose uptake in the presence of H_2 was determined (see text). [^{14}C]mannose (0.5 mM at 0.5 μCi/μmol) was added 1 min after the addition of Ar or H_2 to initiate the 1-min mannose uptake assay. Abbreviations: CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; HQNO, 2-*n*-heptyl-4-hydroxyquinoline-*N*-oxide; pCMB, *p*-chloromercuribenzoic acid; pCMPS, *p*-chloromercuriphenylsulfonate; ND, not done.

[b] Values are means ± standard deviations for five determinations; 100% activity (uninhibited rate) was 26.5 ± 2.6 nmol of O_2 per min per 10^8 cells.

[c] Values are means ± standard deviations for four samples; 100% activity (uninhibited rate) was 0.15 ± 0.02 nmol of mannose per min per 10^8 cells. The 100% activity value is the mean ± standard deviation for eight filtered samples.

transport mannose at a rate about twofold greater than that of cultures incubated with argon (16). In this study, we repeated those experiments and also performed similar ones with a larger gas phase during the 1-min mannose uptake assay (see above). A typical mannose uptake experiment over a 2-min period is shown in Fig. 1. It is clear that the culture with H_2 was better able to transport mannose than the argon-supplied culture was. This difference is clear even in the first 30 s of assay (Fig. 1). Exchange experiments with cultures that were provided with cold (nonradioactive) mannose after they took up radioactive mannose indicated that most of the mannose taken up in 1 min was not exchangeable with cold mannose. Therefore, it was evident that the mannose taken up from the medium was either tightly associated with cellular components or metabolized, rather than remaining loosely bound to the cell surface. By use of large vials (70 ml) and small culture volumes (2.5 ml) with rapid shaking during the assay (conditions used for all the

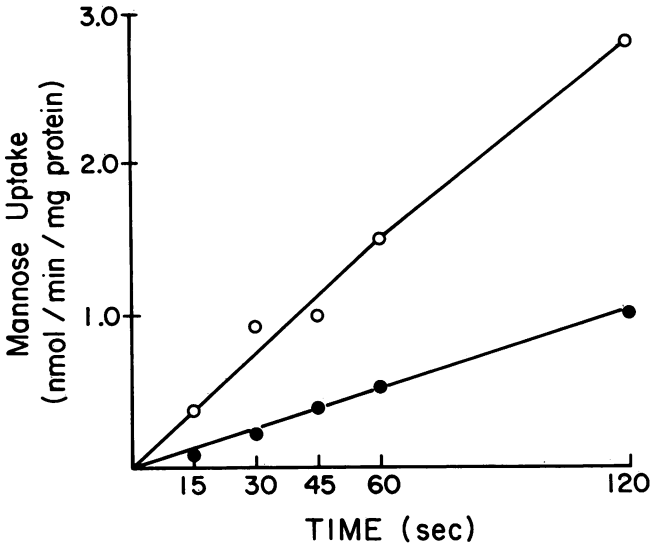

FIG. 1. Mannose uptake in the presence of H_2 (○) or Ar (●). Cells grown in Burk medium with mannose plus H_2 were harvested and treated as described in the text, and 10 ml of cells was added to a 250-ml prescription bottle. The bottle was sealed with a serum stopper, the appropriate gas (20 cm^3 of H_2 or Ar) was added, and then a [^{14}C]mannose solution was injected to initiate the assay (see text). Samples were removed from the sealed bottle by use of an N_2-sparged syringe. Three 0.5-ml samples were filtered for each indicated time point.

1-min assays), we observed up to a fivefold greater amount of mannose uptake in 1 min by cultures incubated in H_2 rather than in Ar (data not shown). The above-described conditions were designed to maximize the respiratory ability of the cells by ensuring the availability of ample dissolved O_2 to the cells in the sealed vial.

We determined the mannose uptake rate in 1 min at various substrate concentrations to learn more about the nature of H_2-supported mannose uptake. A plot of uptake versus substrate concentration (Fig. 2) revealed that the system showed Michaelis-Menten kinetics. A double-reciprocal plot (Fig. 2) of the same data indicated that the apparent K_m for mannose of the cells incubated in H_2 was similar to that of Ar-incubated cells. In that experiment both cultures had a K_m of 0.30 to 0.36 mM. However, the V_{max} was significantly different, i.e., 0.6 nmol/min per mg of protein for the Ar-incubated cultures and 2.5 nmol/min per mg of protein for the cultures in H_2. Other experiments gave similar results, with the V_{max} of the cultures in H_2 being three to five times greater than that of the cultures in Ar.

Inhibitor studies were done to examine the possibility that H_2 supplied energy for mannose uptake. Inhibitors that have previously been shown to be effective at inhibiting *A. vinelandii* respiratory activity (3, 9) were used. Initially, inhibitor concentrations that significantly inhibited O_2 uptake in the presence of H_2 were found (Table 1). However, some of the inhibitors (such as chlorpromazine and 2-*n*-heptyl-4-hydroxyquinoline-*N*-oxide) did not significantly inhibit respiratory activity and probably did not penetrate the cell wall very effectively. The inhibitor concentrations that were effective at inhibiting H_2-dependent O_2 uptake were then tested for their effect on H_2-mediated mannose uptake. At the concentrations that were effective at inhibiting respiration, most of the respiratory inhibitors also significantly inhibited mannose uptake (Table 1). The best inhibitors of

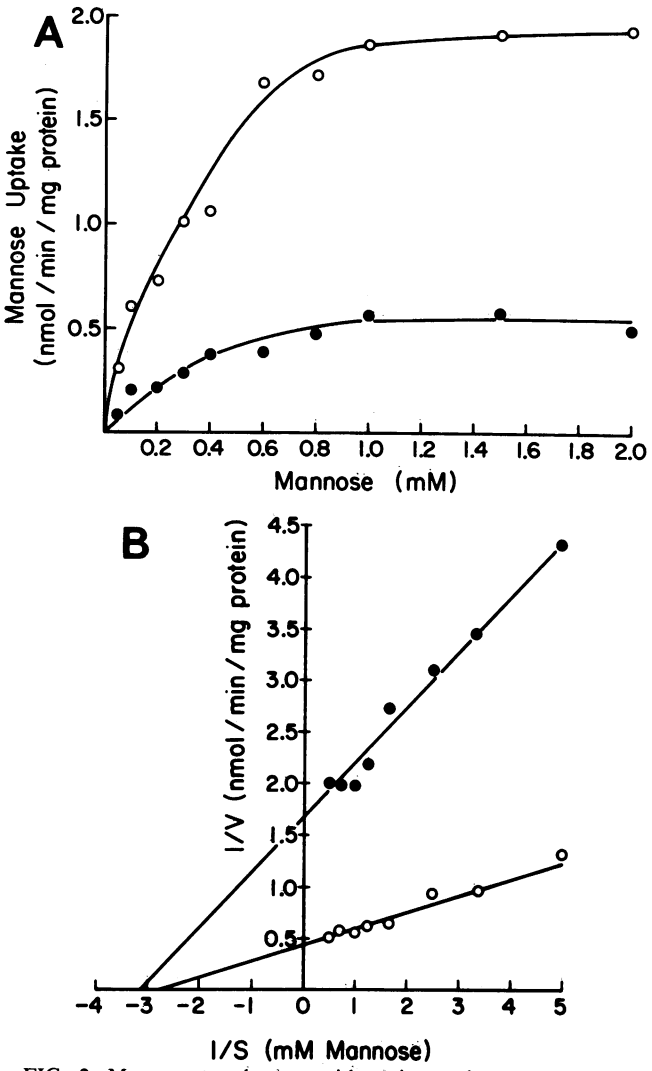

FIG. 2. Mannose uptake rate with various substrate concentrations in the presence of H_2 (○) or Ar (●). The mannose uptake in 1 min was determined at 10 different mannose concentrations as described in the text. (A) Plot of uptake rate versus substrate concentration. (B) Double-reciprocal plot with 8 of the 10 substrate concentrations used; the two lowest substrate concentrations (0.05 and 0.1 mM) were not plotted.

respiratory activity, KCN and *p*-chloromercuribenzoic acid, were also the most effective at inhibiting H_2-mediated mannose uptake. The uncoupler carbonyl cyanide *m*-chlorophenylhydrazone, which may have stimulated O_2 uptake slightly, also inhibited H_2-dependent mannose uptake. The results indicate that energy provided by H_2 oxidation can support mannose uptake. A glucose transport system in *A. vinelandii* membranes that depends primarily on malate oxidation for energy has been described (2, 3). The glucose uptake was also inhibited by respiratory inhibitors (2, 3).

The roles of H_2-oxidizing hydrogenases in bacterial physiology are not well understood at the molecular level (5). The oxidation of H_2 certainly has the potential to produce a proton gradient or a membrane potential that allows energy transduction (11). Useful work, such as transport, is thought to be coupled to the energetics of the bacterial membrane by several different mechanisms (7); the molecular nature of H_2-mediated mannose transport in *A. vinelandii* cannot yet be predicted.

H_2 oxidation in *A. vinelandii* can be coupled to oxidative phosphorylation (8), and H_2 oxidation is also believed to be involved in enhancing respiratory protection of nitrogenase (13). Our results indicate an additional role for H_2 oxidation in sugar (mannose) transport, and this must be one role for H_2 in mixotrophic metabolism. It is possible that H_2 oxidation plays such a role in other H_2-metabolizing bacteria. For example, Pankhania et al. (12) observed slow growth of lactate-limited *Desulfovibrio vulgaris* in a gas phase of N_2-CO_2 but rapid growth when the gas phase was changed to H_2-CO_2. However, on the basis of other experiments they suggested that the effect of H_2 was not to serve as an energy source for growth but perhaps had some other role in facilitating lactate-dependent growth. In another bacterium, H_2 oxidation confers increased nickel transport ability; constitutive strains of *Bradyrhizobium japonicum* exhibiting hydrogenase activity had a V_{max} for nickel that was more than fourfold greater than that of the wild type not expressing hydrogenase (14). Other roles for H_2 in facilitating transport processes in microorganisms seem possible. The use of membrane vesicles of *A. vinelandii* that have the capacity to transport mannose in a H_2-dependent manner would be most useful for further studies of the mechanisms involved.

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