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MYO-INOSITOL TRANSPORT IN *AEROBACTER AEROGENES*

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SUMMARY

1. *Aerobacter aerogenes* possesses an inducible transport system for *myo*-inositol.

2. The *myo*-inositol transport is an energy requiring process regarding the inhibitions by 2,4-dinitrophenol, azide and cyanide. The process may be accomplished by either active transport or group transfer.

3. The system possesses a V of 12.06 $\pm$ 1.75 nmoles/mg per min and an apparent K_m of 0.24 $\pm$ 0.07 mM.

4. After *myo*-inositol uptake, the following substances have been identified inside the cells: *myo*-inositol (70%), 2-ketoinositol (20%), and inositol phosphate (7%).

5. The hydroxyl group on C-2 of *myo*-inositol does not play a role in the transport.

6. The entrance of *myo*-inositol achieves a maximum at pH 7.

INTRODUCTION

Sugar transport systems in bacteria have been the object of numerous reports, and there have been many proposed models. For cyclitols, however, there are few reports: in yeast *Schizosaccharomyces pombe*[1], in kidney cortex[2,3], in hamster intestine[4] and in Ehrlich ascite cells[5].

Aerobacter aerogenes can use *myo*-inositol as a carbon source[6] and its catabolism has been well studied by Magasanik's group[7–10].

It would be interesting to study the myo-inositol entrance in this bacteria. The present report describes an inducible transport system for *myo*-inositol in *Aerobacter aerogenes*. The principal characteristics of this system are also given.

MATERIALS AND METHODS

Bacteria strain and culture medium

The strain of *Aerobacter aerogenes* 1033 was a generous gift from B. Magasanik. The strain was maintained on solid agar medium containing yeast extract, 0.5%, malt extract broth, 1.5% (Difco Laboratories, Michigan, U.S.A.).

The liquid culture medium contained per l: KH_2PO_4, 12.4 g; $Na_2HPO_4 \cdot 12H_2O$, 5.4 g; NH_4Cl, 1.0 g; $MgSO_4 \cdot 7H_2O$, 0.1 g; $FeSO_4 \cdot 7H_2O$, 50 mg; $CaCl_2$, 10 mg;

$MnSO_4 \cdot H_2O$, 0.2 mg; $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$, 0.64 mg; $CuSO_4$, 0.12 mg; $CoCl_2 \cdot 6H_2O$, 0.2 mg; and $ZnSO_4 \cdot 7H_2O$, 0.22 mg.

In certain cases the carbon source was 1.5 % *myo*-inositol and, in others, 1.5 % glucose (the glucose was separately sterilized).

The cultures were maintained at 30 °C with vigorous stirring for 22 h; the absorbance, measured on a Klett photometer with the No. 54 filter, was between 250 and 300 units. Except when specifically mentioned, the cells were always grown on *myo*-inositol.

Standard uptake assays

After 10 min centrifugation at 2500 × *g*, the cells were resuspended in a *myo*-inositol-less medium and were stirred at 30 °C during 30 min to cause an energy deficiency. This suspension was added to the substrate and incubated during the chosen times. The cells were separated from the incubation medium on Millipore filters AAWP. 04700 (Millipore Corporation, Bedford, Mass., U.S.A.). Then they were immediately washed with 8 ml of *myo*-inositol-less medium. The filtration and the washing of 1-ml samples, containing from 1–3 mg cells (dry weight), lasted less than 5 s. The Millipore filter was placed in a counting vial with 15 ml of scintillation fluid. The uptake is always related to 1 mg cells (dry weight). A sample of the cellular suspension was employed to determine the dry weight.

Chemicals

Radiochemical Center (Amersham, U.K.) supplied *myo*-[2-^{3}H]inositol (0.525 μCi/μmole); *myo*-[$^{14}C_6$]inositol (31.2 μCi/μmole) and D-[$^{14}C_6$]glucose (0.84 μCi/μmole) The specific activities indicated were obtained after dilution with non-labelled material. The analogs and derivatives of cyclitols were a generous gift of Dr T. Posternak, and the other products were purchased from Merck (Darmstadt, Germany) and Fluka (Buchs, Switzerland).

Determination of radioactivity

The radioactivity was measured on a Beckman LS 250 liquid scintillation counter. The scintillation fluid was composed of 100 g naphthalene and 5 g PPO per l of dioxane. The lipid radioactivity was measured in 0.4 % toluene–Omnifluor (NEN Chemicals GmbH, Dreieichenhain, Germany). The chromatogram scannings were recorded on a Radio-chromatogram scanner Actigraph III (Nuclear Chicago).

Chromatography and electrophoresis

Chromatographic analyses of water-soluble products were carried out on Whatman No. 1 paper in the following solvents: (a) *n*-butanol–acetic acid–water (4:1:5, by vol., upper phase), (b) water-saturated phenol, (c) 2-propanol–NH_4OH–water (7:1:2, by vol.).

The lipids were chromatographed on Whatman No. SG 81 in the following system: (d) chloroform–methanol–water (65:25:4, by vol.).

Electrophoreses were accomplished on Whatman No. 1 paper in a 0.075 M sodium tetraborate buffer, 30 V/cm.

RESULTS

Induction of myo-inositol transport

In Fig. 1, the influence on *myo*-inositol uptake, using either glucose or *myo*-inositol as carbon source, is given. These uptakes were measured to know if the transport systems were inductible or constitutive. Since both substrates are metabolized, a short influx time of 30 s was chosen.

The cells, grown on glucose, are incapable of *myo*-inositol uptake, regardless of its concentration, but the cells grown on the cyclitol show an uptake of both substrates.

The induction time for the *myo*-inositol transport system is shown on Fig. 2. Glucose grown cells were used. The cells were resuspended in mineral medium (carbon-free) to starve them. Two carbon starvation times, 5 and 30 min, were chosen. The uptake was compared to cells identically treated except for the addition of 100 μg/ml chloramphenicol to inhibit protein synthesis. At zero time, the cells were added to labelled *myo*-inositol and incubated at 30 °C with stirring. Samples were taken as

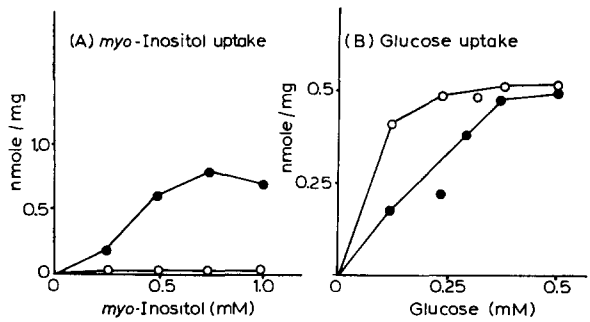


Fig. 1. Glucose and *myo*-inositol uptake as a function of their concentrations on cells grown either on *myo*-inositol (●—●) or on glucose (○—○). (A) *myo*-Inositol uptake; (B) Glucose uptake. Sample volume, 1.0 ml. Cellular concentration, 1.5 mg/ml (dry wt).

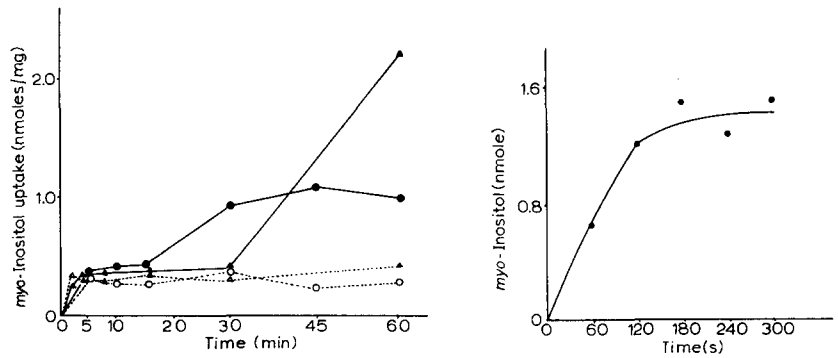


Fig. 2. *myo*-Inositol uptake by glucose grown cells: ▲—▲, 5 min starvation; △···△, 5 min starvation with chloramphenicol; ●—●, 30 min starvation; ○···○, 30 min starvation with chloramphenicol. *myo*-Inositol concentration, 40 μg/ml. Sample volume, 1.0 ml. Cellular concentration, 2.5 mg/ml (dry wt).

Fig. 3. Time-course uptake of *myo*-inositol. *myo*-Inositol, 40 μg/ml. Carbon starvation, 80 min. Sample volume, 1.0 ml. Cellular concentration, 1.8 mg/ml (dry wt).

indicated. Within 5 min, the cells reached a constant level of radioactivity. In untreated cells, induction proceeds after a time lapse depending on starvation. The chloramphenicol treatment inhibits the induction process.

Time-course uptake of myo-inositol

Fig. 3 shows a typical time curve for *myo*-inositol uptake. It levels off after an initial 2-min period of linearity. Since *myo*-inositol is rapidly metabolized, being the carbon source, it was not necessary to conduct the experiment further.

We have observed differences in uptake rates within short periods of time. This is the reason for simultaneous pipetting, filtering and washing all the samples on a filtration rack, except in time course experiments.

Uptake kinetics

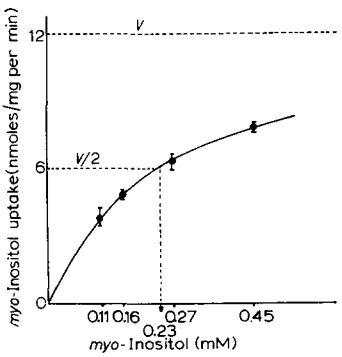
The apparent K_m and V determinations were calculated using the method of Wilkinson[11], which is a statistical adjustment of the values on a hyperbole. This method was considered more precise than the traditional Lineweaver–Burk. The uptake experiments were all conducted at 30 °C during 30 s with stirring. The values shown in Fig. 4 were then calculated on an IBM 1620 computer to obtain an apparent K_m of 0.24 ($\pm$ 0.07) $\cdot 10^{-3}$ M and a V of 12.06 $\pm$ 1.75 nmoles/mg per min.

pH dependence

Fig. 5 shows the pH dependence of *myo*-inositol transport. The cells have been starved the usual 30 min. Incubations were performed by adding the cell suspension to labelled *myo*-inositol solutions containing calculated amounts of NaOH or HCl to get desired pH. Samples were removed after 30 s and filtered. The remaining incubation medium was used to determine the exact pH.

Effects of uncouplers

Uncouplers of oxidative phosphorylation were introduced in *myo*-inositol uptake experiments. Table I shows two types of experiments. In Part A, the uncouplers and *myo*-inositol were added simultaneously; the uptake was determined in



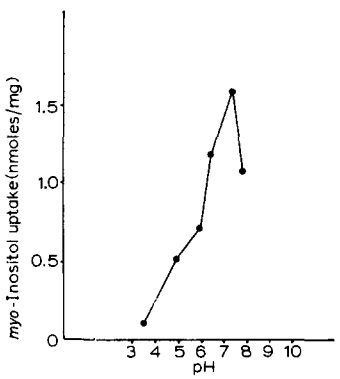


Fig. 4. *myo*-Inositol uptake as a function of substrate concentration. Sample volume, 1.0 ml. Cellular concentration, 1.3 mg/ml (dry wt).

Fig. 5. pH dependence. Sample volume, 1.0 ml. Cellular concentration, 2.0 mg/ml (dry wt).

time course experiment. In Part B, the cells were preincubated for varying periods with inhibitors before inositol was added, and the influx was measured under 30 s standard conditions.

All three substances showed various degrees of inhibition. Dinitrophenol exhibited more activity than azide. The delayed action of cyanide contrasted with the immediate inhibition of the others. From these results it is possible to deduce that *myo*-inositol transport system is energy-dependent.

Effect of various cyclitols

Results presented in Table II show the influx inhibition by cyclitols. Labelled *myo*-inositol concentration was 50 μg/ml, and the tested substances concentration was 100-fold higher. The degree of inhibition of these substances is related to their affinity to the transporting system.

Estimation of metabolized myo-inositol

To be able to identify metabolites of *myo*-inositol, bulk experiments were conducted employing a 10-fold increase of the standard assay procedure at 30 s. There was an additional 15-s delay between the end of the washing and the extraction, thus causing a total metabolizing time of 45 s. Three different types of extraction were employed to identify the metabolites.

After a cold 6 % trichloroacetic acid extraction, the trichloroacetic acid was removed with ether. The aqueous phase was concentrated under vacuum and chromatographed with Solvent a and b, using *myo*-inositol and 2-keto-*myo*-inositol as migration standards.

60 % ethanol extraction was used to identify the presence of inositol phosphate. After concentration, chromatographic separation in Solvent c was carried out, using

TABLE I

INHIBITION IN THE PRESENCE OF INHIBITORS AT VARIOUS CONCENTRATIONS

myo-Inositol concentration was 45 μg/ml. Cellular concentration, 1.5 mg (dry wt). (A) Simultaneous addition of *myo*-inositol and inhibitor; (B) various preincubations with inhibitor.

Inhibitor	*Inhibition* (%)						
	A			*B*			
	Influx (*min*)	*Concentration*		*Preincubation* (*min*)	*Concentration*		
		10^{-3} *M*	10^{-4} *M*		10^{-3} *M*	10^{-4} *M*	10^{-5} *M*
2,4-Dinitrophenol	0.5	84	66	2	91	—	17
	2	85	65	4	79	45	44
	4	92	66	8	88	—	25
	6	90	63				
NaN_3	1	4.5	22	2	—	13	—
				4	—	30	—
				8	—	44	—
KCN				4	—	0	—
				8	—	25	—
				15	—	60	—

TABLE II

INHIBITION OF *myo*-INOSITOL TRANSPORT

Sample volume, 1.0 ml. Cellular concentration, 2.5 mg/ml (dry wt). Incubation, 30 s at 30 °C.

Compound	*Inhibition* (%)
scyllo-Inositol	97.5
2-Ketoinositol	82
Isomytilitol (2-*C*-methyl-*myo*-inositol)	32
2-Methylene oxide-1,3,5/4,6-pentahydroxycyclohexane	0

TABLE III

DISTRIBUTION OF THE RECOVERED RADIOACTIVITY AFTER UPTAKE

Sample volume, 1.0 ml. Cellular concentration, 2.0 mg/ml (dry wt). Incubation as indicated under results.

Substance	%
myo-Inositol	70
2-Ketoinositol	20
myo-Inositol phosphate	7
Lipid fraction	3

myo-inositol and *myo*-inositol-2-*P* as standards. The migrations referred to *myo*-inositol were 0.12 for *myo*-inositol-2-*P*, and 0.10 for the unknown product. Electrophoresis was accomplished with *myo*-inositol-2-*P* as standard.

The fraction of *myo*-inositol incorporated in lipids was determined by extraction with chloroform–methanol (160:70, w/v) mixture and was chromatographed with Solvent d. Two radioactive spots of equal intensity with R_F 0.72 and 0.83 observed. In this system free *myo*-inositol stayed at the origin.

Radioactivity on the chromatograms was measured by cutting and dipping into scintillation fluid. From the radioactivity measurements, the percentage of metabolites was computed (Table III).

DISCUSSION

Aerobacter aerogenes Strain 1033 can utilize either glucose or *myo*-inositol as carbon source. The cyclitol degradative pathway in this particular bacteria has been well studied by Magasanik's group[7–10] on a mutant, derived from the Strain 1033. The first product in the catabolic pathway, which is catalyzed by constitutive enzymes in this mutant, is the 2-ketoinositol. It is formed by dehydrogenation at the 2-position, and this reaction only occurs on the free cyclitol. This differs from the sugars' catabolic reactions where the degradations occur on their phosphoryl derivatives.

Aerobacter aerogenes possesses an inducible *myo*-inositol transport system in contrast to that for glucose, which is constitutive (Fig. 1). The glucose-grown cells cannot transport *myo*-inositol without a contact time with this cyclitol, during which induction takes place (Fig. 2).

The time necessary for the formation of the induced system can be measured. It varies as a function of the carbon starvation time (Fig. 2). After a deficiency of 5 min, the cells need about 30 min to develop the transport system whereas 15 min is sufficient after a 30-min starvation.

Induction is started by the small amount of *myo*-inositol which reaches a constant level very rapidly, probably by simple diffusion. In the case of prolonged starvation, the cells have expended the biggest portion of their reserve energy and quickly develop the new transport system. The chloramphenicol inhibition of induction nicely shows that the transport proteins are synthesized *de novo* (Fig. 2). The *myo*-inositol uptake during the induction process depends on the energetic status of the cells.

For most of the experiments, the starvation time of the carbon source was 30 min because a more reproductible *myo*-inositol uptake was observed under this condition, especially with cells from different cultures. Also the internal residual concentration of cyclitol can be considered as negligible. The influx measurements were done simultaneously to prevent errors due to the variable energetic states of the cells during the experiment.

The uptake curve as a function of time (Fig. 3) shows a plateau after 3 min, preceded by a quasilinear phase. The normal uptake time was chosen at 30 s to be in the linear portion of the curve. This was also done to prevent a too large metabolism of the transported *myo*-inositol, so that the radioactivity represents the transport process.

The entrance kinetics are characteristic of the Michaelis–Menten type. The calculation method of Wilkinson[11] permitted the determinations of the V of 12.06 $\pm$ 1.75 nmoles/mg per min, and the apparent K_m (given by half the V concentration) of 0.24 ($\pm$ 0.07)$\cdot 10^{-3}$ M. The apparent K_m does not vary in appreciable amounts with the starvation time, but the V varies slightly.

The *myo*-inositol entrance varies considerably as a function of the pH with a maximum around 7. This pH dependence is different from observations in bacteria for leucine[12] or for galactose-binding protein[13], but it is comparable to that noted for *myo*-inositol[1] and sugar[14] in yeast.

The transport systems, utilizing a "carrier", are classified in three groups: carrier-mediated diffusion, active transport, and group transfer. The first group can be excluded because the *myo*-inositol penetrates into the cell against a concentration gradient. After 30 s, the internal cyclitol concentration is 2- to 4-fold higher than the external concentration. The internal concentration has been calculated on the basis that the dry weight represents 20 % of the cell weight and that all of the internal volume is accessible.

The *myo*-inositol transport is energy-requiring, which is demonstrated by inhibition with oxydative phosphorylation uncouplers (Table I). The entrance must then be accomplished by either active transport or group transfer.

A translocation coupled with phosphorylation as described by Roseman[15] and Simoni *et al.*[16], appears of little probability in the case of *myo*-inositol transfer. The internal concentration of free *myo*-inositol is very high compared to *myo*-inositol phosphate (Table III); however, the cyclitol utilization in the catabolic reactions can only be accomplished after dephosphorylation. Such a system is energetically favored in the case of sugars because they are catabolized as their phosphoryl deriva-

tives. The free *myo*-inositol percentage at 30 s, indicated in Table III, represents a minimal value because in the bulk experiment, during the 15 additional s, a part of *myo*-inositol has already metabolized.

This assumption is also supported by the fact that the phosphoenolpyruvate-dependent phosphotransferase system, using the technique described by Tanaka and Lin[17], failed to demonstrate the formation of *myo*-inositol phosphate. The presence of *myo*-inositol phosphate could be attributed either to a phosphorylation not related to transport, or to a degradation of some phosphoinositides.

Since 2-ketoinositol and *scyllo*-inositol are strong inhibitors (Table III), the axial hydroxyl on Position 2 of *myo*-inositol does not have an influence on the attachment on the transfer system. The weak inhibition by isomytilitol and the absence of action with epoxide (Table III) can explain the absence of attachment on the transport's active site because of the presence of either the methyl group or the epoxide group; however, in *Schizosaccharomyces pombe*[1], isomytilitol has an affinity for the carrier which is similar to that of *myo*-inositol. The weak inhibition by *myo*-inositol phosphate can be due to the phosphate group volume and also to an anionic charge effect.

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