SYMPORT H^+/CARBOHYDRATE TRANSPORT INTO *ACHOLEPLASMA LAIDLAWII* CELLS

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1. Introduction

According to the chemiosmotic theory bacterial transport systems drive their energy from the transmembrane H^+-electrochemical gradient (the protonmotive force) consisting of a pH gradient (ΔpH) and a membrane potential ($\Delta\psi$) [1]. It is well established that the flux of neutral substrates is mediated by H^+ symporters in response to the total protonmotive force [2–4]. We have demonstrated that the aerobic and anaerobic transport of 3-*O*-methyl-D-glucose (3-*O*-MG) into *Acholeplasma laidlawii* cells is sensitive to arsenate, inhibitors of glycolysis, uncouplers and DCCD [5,6]. We describe here the concomitant uptake of H^+ with 3-*O*-MG by *Ach. laidlawii* cells in response to the total protonmotive force. We also demonstrate that an artificially-imposed $\Delta\psi$ and ΔpH, in energy-depleted cells, leads to ATP synthesis and provides energy for the transport of 3-*O*-MG.

2. Methods

The *Acholeplasma laidlawii* strain was grown in tryptose broth and harvested as in [7]. Transport of 3-*O*-[^{3}H]MG (Amersham, England) was measured under the conditions in [5]. To reduce the endogenous energy reserves the cells were incubated with shaking for 2 h at 37°C in 150 mM KCl, 10 mM Tris–HCl, pH 7.5, 10 mM $MgCl_2$ and 1 mM NaF to inhibit glycolysis. Then the cells were harvested and resuspended in the same buffer for 1 h, but KCl was replaced by NaCl.

For H^+ movement the starved cells (5 mg protein) were suspended in a reaction vessel containing: 150 mM NaCl; 10 mM $MgCl_2$; 50 mM NaSCN; 1 mM Tris–maleate, pH 7.0. The cells were allowed to equilibrate for 30 min, while small aliquots of NaOH were added to final pH 7.0. The pH was measured at 22°C using a G 2222C Radiometer glass electrode and research pH meter LPU-01 coupled to the chart recorder KSP-4 with full-scale deflection of 0.1 pH unit.

For valinomycin-stimulated 3-*O*-MG transport or ATP synthesis the concentrated suspension of starved cells was diluted 10-fold into the medium, containing 150 mM NaCl, 10 mM Na-phosphate buffer, pH 7.5, 10 mM $MgCl_2$ and 10 μM 3-*O*-[^{3}H]MG. After addition of valinomycin (final conc. 10 μM) 0.5 ml aliquots were removed at various intervals for the determination of intracellular ATP concentration and intracellular 3-*O*-MG radioactivity. For acid-stimulated 3-*O*-MG transport or ATP synthesis the suspension of starved cells in the same buffer, pH 7.6, was acidified by the addition of HCl, then 0.5 ml aliquots were withdrawn.

ATP concentration was estimated with a firefly lantern luciferine–luciferase assay using a liquid-scintillation counter as in [6].

3. Results and discussion

3.1. *Proton movements associated with the 3-O-MG uptake*

Figure 1 shows that the addition of 3-*O*-MG to the aerobic or anaerobic cell suspension caused an immediate alkalinization of the lightly buffered incubation medium. This result may be interpreted as an effective H^+ uptake into the cells or the extrusion of OH^- ions [2,8–10]. When the cells were incubated without negatively-charged ions (thiocyanate (NaSCN)) the pH increase observed upon 3-*O*-MG addition was smaller than that cells with NaSCN. Only transportable non-metabolizing sugars (3-*O*-MG and 6-deoxy-

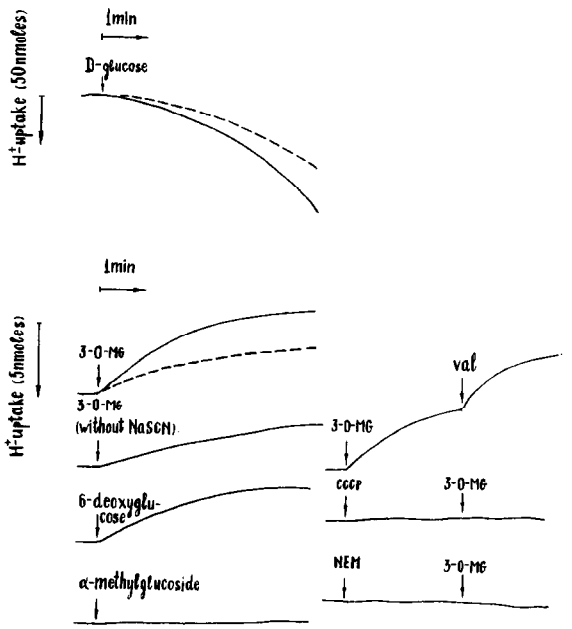

Fig.1. Extracellular pH changes on the addition of carbohydrates to non-metabolizing *A. laidlawii* cells. Final concentrations: carbohydrates, 1 mM; CCCP, 10 μM; valinomycin, 1 μM; *N*-ethylmaleimide (NEM), 1 mM. Dashed lines indicate pH changes in *A. laidlawii* PTL-S strain, defective in glucose and 3-*O*-MG transport system.

glucose) gave the pH change, and not sugars such as α-methylglucoside and 2-deoxyglucose, which are not transported by *Ach. laidlawii* cells. Metabolized carbohydrates, such as D-glucose, induce a rapid acidification of the external medium due to the exit of lactic acid [11]. Uncoupler CCCP prevents the 3-*O*-MG-induced pH shifts. Valinomycin slightly increases H^+ uptake. H^+ uptake is apparently mediated by the physiological 3-*O*-MG carrier, since this uptake was inhibited by SH-reagent NEM (fig.1). PTL-S mutant cells which are deficient in 3-*O*-MG and glucose transport [7] take up some H^+ as well, but not as efficiently as did the cells of wild strain. The stoichiometry calculated [2,9,19] on the basis of the magnitudes of the pH change and of the 3-*O*-[^{3}H]MG uptake was approx. 1 H^+/3-*O*-MG molecule transported. The same results were obtained in anaerobic conditions. It should be noted that the addition of 3-*O*-MG to *Ach. laidlawii* cells resulted in a depolarization of the plasma membrane observed by ANS^- fluorescence (unpublished data). Thus, our observations are consistent with the notion that carbohydrate is driven by H^+ symport mechanism [1].

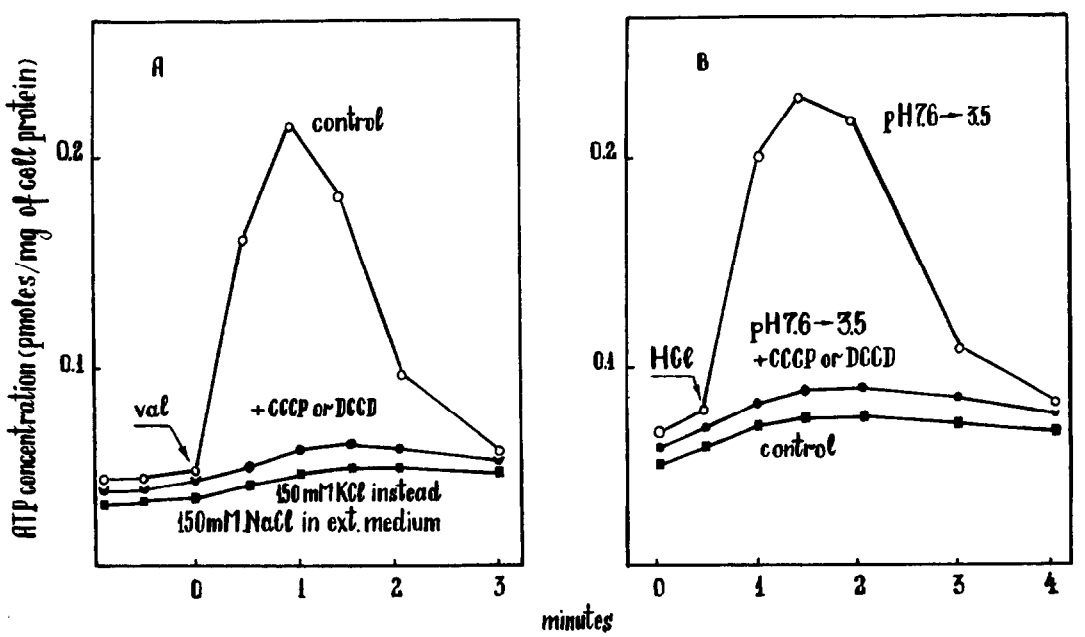

Fig.2. ATP synthesis in energy-starved *A. laidlawii* cells driven by Δψ (A) and ΔpH (B). The cells were exposed for 10 min to 1 mM DCCD or 10 μM CCCP or ethanol. After this pretreatment, valinomycin (A) or HCL (B) were added as indicated by arrows.

3.2. *ATP synthesis driven by a protonmotive force*

Membrane-bound Ca^{2+}, Mg^{2+}-stimulated ATPase in microorganisms plays a central role in the generation of protonmotive force [1]. However, artificially-imposed $\Delta\psi$ and ΔpH, which permits H^+ entry to the cell via ATPase, can drive ATP synthesis or active nonelectrolyte transport [12–16]. When *Ach. laidlawii* cells were suspended in a K^+-free medium, $\Delta\psi$ (negative inside) could be artificially generated by the addition of the K^+-ionophore valinomycin (unpublished data). This $\Delta\psi$-induced diffusion may be registered by the rapid drop in the intensity of ANS^- fluorescence. Indeed, the addition of valinomycin to the K^+-loaded starved cells resulted in the short synthesis of ATP (fig.2A, table 1). The maximal level of ATP observed was comparable to the level of ATP found in glycolyzing cells [6]. The optimum pH for synthesis was found to be between 6.5 and 7.7. The nonability of nigericin, which permits K^+-efflux by electroneutral exchange for H^+, to drive ATP synthesis demonstrates the necessity of an electrogenic efflux of K^+. When K^+ was added instead of Na^+ to the external medium, ATP synthesis was eliminated, thus demonstrating a requirement for a K^+-gradient across the membrane. Addition of uncoupler, CCCP, or ATPase inhibitor, DCCD, prevents valinomycin-driven ATP synthesis. Arsenate considerably inhibits synthesis due to competition with the phosphate. A glycolysis inhibitor such as azide does not influence ATP synthesis.

When HCl was added to the starved cells, to impose a large transmembrane pH gradient (alkaline inside), a temporary increase in the intracellular concentration of ATP was observed (fig.2B). The rate of increase in the ATP level after the pH jump was comparable with the rate of ATP synthesis generated by $\Delta\psi$. When the cells were pretreated with CCCP or DCCD pH-driven ATP synthesis did not occur. This increase in the ATP level was not observed when the external medium was pH 7.6. Hence, these data demonstrate the involvement of $\Delta\psi$ and ΔpH in ATP synthesis generated in the absence of cellular metabolism.

Table 1
Effects of inhibitors on valinomycin-induced synthesis of ATP in K^+-loaded *A. laidlawii* cells

Additions	Conc. (mM)	Intracellular ATP (pmol/mg cell protein)
None	–	49
Valinomycin	0.01	103
	0.03	296
Nigericin	0.02	14
Valinomycin	0.03	
+ NaF	10.0	309
Valinomycin	0.03	
+ CCCP	0.01	51
Valinomycin	0.03	
+ DCCD	1.0	86
Valinomycin	0.03	
+ DCCD	0.1	93
Valinomycin	0.03	
+ Arsenate	10.0	152

The cells were preincubated with inhibitors for 10 min at 37°C and ATP synthesis was assayed as in section 2. In the case of arsenate, Na-phosphate buffer was replaced by Na-arsenate buffer

3.3. *3-O-MG transport in response to a protonmotive force*

To explore the role of $\Delta\psi$ and ΔpH in active 3-*O*-MG transport in the absence of exogenous substrates, we returned to the procedure illustrated in fig.3. It shows that the addition of valinomycin to K^+-loaded cells (fig.3A) induces accumulation of 3-*O*-MG. In the absence of antibiotics the uptake is insignificant. Nigericin does not stimulate transport. Valinomycin-induced uptake was unaffected by DCCD, which inhibites 3-*O*-MG transport in non-starved *Ach. laidlawii* cells [5].

This suggests that in starved cells ATPase is not involved in 3-*O*-MG transport when $\Delta\psi$ or ΔpH is artificially induced. Accumulation was a function of the gradient of K^+ concentrations across the membrane: when K^+ concentrations inside and outside the cell were approximately the same no 3-*O*-MG accumulation occured. 3-*O*-MG accumulation was completely abolished by CCCP and by NEM (data not shown).

When ΔpH was created across the membrane by reducing external medium from pH 7.6–4.0, a transient accumulation of 3-*O*-MG was observed (fig.3B). Control cells incubated at pH 7.6 showed no accumulation. The addition of H^+ conductor CCCP completely blocked the accumulation induced by ΔpH. A similar observation has been made in other microorganisms [12–15,17].

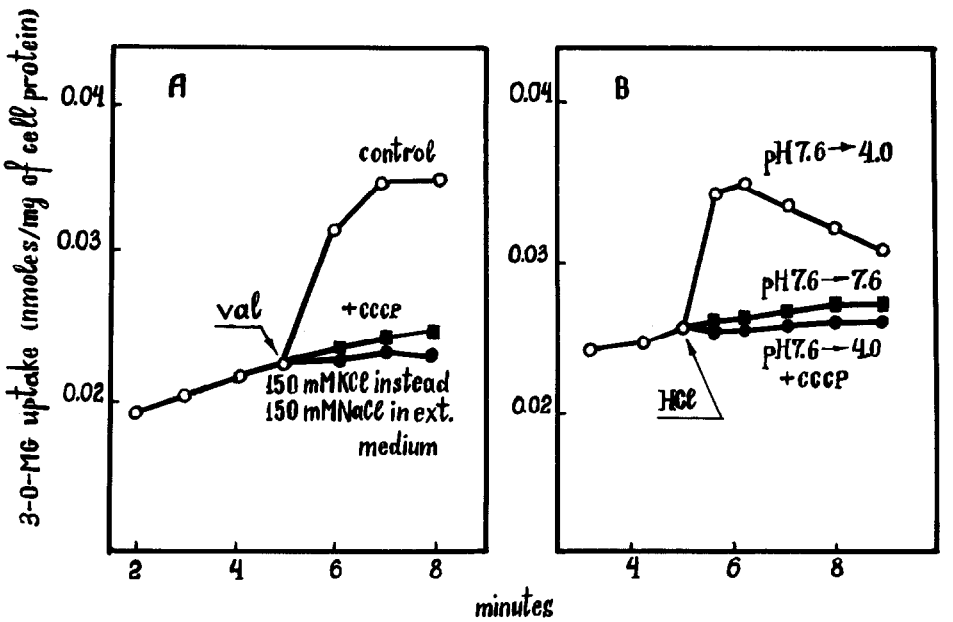

Fig.3. Uptake of 3-*O*-MG by energy-starved *A. laidlawii* cells in response to $\Delta\psi$ (A) and ΔpH (B). Conditions were as in fig.2.

Thus, these data unequivocally point to an inwardly-directed protonmotive force which drives 3-*O*-MG transport. Hence one would expect ionophores which carry univalent cations to inhibit sugar transport [18,19]. Indeed, the lipid-soluble cation DDA^+ (but not the anion TPB^-) which should discharge $\Delta\psi$ inhibited the metabolic accumulation of 3-*O*-MG. Nigericin, or combination of valinomycin with CCCP which mediates an electrical-neutral exchange of K^+ with H^+, dissipates a ΔpH (under low external K^+) and strongly inhibits 3-*O*-MG transport. Ammonium chloride which also collapses ΔpH shows the same effect.

Table 2
Effect of ionophores and lipid-soluble permeant ions on 3-*O*-MG transport by *A. laidlawii* cells

Additions	Conc. (mM)	Transport activity (% control)
Valinomycin	0.001	90
Valinomycin + CCCP	0.001 0.001	32
Nigericin	0.01	23
N,N-dibenzyldimethyl ammonium chloride (DDA^+)	10.0	35
Tetraphenylboron (TPB^-)	0.1	107
NH_4Cl	10.0	30

All of the inhibitors were preincubated with the cells 10 min at 37°C. The uptake of 3-*O*-MG (10 μM) was measured after 20 min of incubation with the cells

These observations are consistent with the following sequence of events:

(i) Hydrolysis of glycolytically-formed ATP leads to extrusion of H^+ via the membrane-bound ATPase in energy-rich cells.
(ii) Creation of $\Delta\psi$ estimated from the distribution of K^+ in the presence of valinomycin [11].
(iii) Creation of ΔpH (estimated from the transmembrane distribution of the methylamine (unpublished data)).
(iv) 3-*O*-MG uptake in turn directly coupled to H^+ entry via the the sugar carrier.

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