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Glucose transport of *Haloferax volcanii* requires the Na^+-electrochemical potential gradient and inhibitors for the mammalian glucose transporter inhibit the transport

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The uptake of glucose and its non-metabolizing analogues by *Haloferax volcanii*, one of the glucose-utilizing Halobacteria, was examined using intact cells and envelope vesicles. Results obtained were: (1) The transport system is inducible. (2) The uptake requires the gradient of Na^+-electrochemical potential. (3) Inhibitors for mammalian glucose transport also have an effect on this system, implying that the transporters resemble each other. (4) It is suggested that the mobility of the transporter is regulated by the membrane energization.

Introduction

Halobacteria belong to Archaebacteria, which is the third kingdom in addition to the two well-known kingdoms of organisms, i.e., eukaryotes and eubacteria. Woese and co-workers [1,2] as well as other laboratories [3,4] deduced the existence of this third kingdom from comparative studies on partial sequence of the 16S ribosomal RNAs. Archaebacteria show a closer relationship to eukaryotes than to eubacteria [5–7], as concluded by Mukohata [8] et al. in their analysis of the homology of the catalytic and non-catalytic parts of H^+-ATPase. The same conclusion was obtained with the elongation factors, EF-Tu/1a and EF-G/2 [9]. Recently, Woese et al. [10] proposed names for these three kingdoms of Archaea, Bacteria and Eucarya.

Maiden et al. [11] examined the sequences of the arabinose-H^+ and xylose-H^+ transporters of *Escherichia coli* and reported that they are homologous with glucose transport of human hepatoma and rat brain cells. Other investigators [12–14] also reported the sequence conservation among the glucose transporters of lower organisms and mammalian cells.

In this respect, the comparison of transport systems among organisms of the three kingdoms appears an interesting subject. The amino acid transport system of *Halobacterium halobium* has been studied by Lanyi and MacDonald [15,16]. All amino acids are transported by an Na^+-symport system in *H. halobium*. In contrast, they are accumulated usually by protonmotive force (pmf)-driven transport systems in eubacteria [17], except for glutamate [18] and proline [19] transport in *Escherichia coli*. It is well known that amino acids are actively transported into mammalian cells by a symport with Na^+ [20]. The carbohydrate transport system in Halobacteria has not been studied extensively except for the pioneering work of Severina, Pimenov and Plakunov [21–23]. This is due to the fact that any sugar added to the growth medium remained unutilized. Halobacteria have thus been considered a metabolically restricted group of chemoheterotrophs incapable of using carbohydrates who meet their energy requirements by metabolizing amino acids and peptones.

Some Halobacteria, however, are reportedly capable of actively consuming sugars from the medium and using them [21–24]. Here, we examined the properties of the glucose transport system of one of the glucose-using Halobacteria, *Haloferax volcanii*, and found several interesting properties: (1) The transport system is inducible. (2) The uptake requires the gradient of

Abbreviations: CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; 2-DG, 2-deoxy-D-glucose; DMSO, dimethylsulfoxide; 2-DNP, 2-dinitrophenol; Glc, glucose; α-MG, α-methyl-D-glucoside; Mops, 3-(*N*-morpholino)propanesulfonic acid; 3-OMG, 3-*O*-methyl-D-glucose; PCP, pentachlorophenol; PMS, phenazinemethosulfate; PTS, phosphotransferase system.

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Na^+-electrochemical potential, suggesting that the transport occurs not by PTS (phosphotransferase system) but by symport with Na^+. (3) Inhibitors of mammalian glucose transport also affect this system, implying that the transporters somewhat resemble each other, although the kinetic features of the inhibitors are different. (4) It is suggested that the mobility of the transporter is regulated by the membrane energization.

Materials and Methods

Bacterium and cell preparation. We first checked various Halobacteria to determine whether or not they transported glucose. Those examined were *Haloarcula hispanicum* (NCIMB 2187), *Haloarcula vallismortis* (NCIMB 2082), *Halobacterium salinarium* (NCIMB 764), *Halococcus morrhuae* (NCIMB 787), *Haloferax gibbonsii* (NCIMB 2188) and *Haloferax volcanii* (NCIMB 2012). They were obtained from the National Collections of Industrial and Marine Bacteria Ltd. (Scotland). Cells were grown at 37 °C to late exponential growth phase. Growth medium was ordinary peptone medium containing 250 g of NaCl, 20 g of $MgSO_4$, 2 g of KCl, 0.2 g of $CaCl_2 \cdot 2H_2O$, 3 g of trisodium citrate $\cdot 2H_2O$, 5 mg of $FeSO_4 \cdot 7H_2O$, 0.19 mg of $MnSO_4 \cdot 7H_2O$ and 10 g of peptone (code L37, Oxoid) per liter supplemented with 0.5% glucose. The pH was adjusted to 6.9 ± 0.1 with NaOH. It is noted that the transport system is induced by glucose. Glucose uptake by various Halobacteria 10 min after the initiation of the experiment was as follows: *H. hispanicum*, 11.8; *H. vallismortis*, 1.9; *H. morrhuae*, 1.8; *H. gibbonsii*, 5.0 and *H. volcanii*, 34.7 nmol/mg protein per 10 min. The concentration of glucose was 100 μM (containing 3 μCi of ^{3}H-labelled compound) and the experimental conditions were as described later. It was confirmed in this experiment that *H. halobium* and *H. salinarium* do not accumulate glucose, and from the findings obtained it was not thought that *H. vallismortis* and *H. morrhuae* have the ability to transport glucose. Therefore, we exclusively used *H. volcanii*, which had shown the largest value.

The bacteria were grown in 10 ml of medium in an L-shaped tube and shaken at 120 rpm. Cells were collected with gentle centrifugation (Hitachi, RPR18-3 rotor, 10 k rpm, $10000 \times g$) at 4 °C and washed three times with 10 ml of a basal salt medium (growth medium minus peptone and glucose). The cells thus obtained were suspended in 2.5 ml of 4.28 M NaCl whose pH was adjusted to 7.0 with 50 mM Mops (3-(N-morpholino)propanesulfonic acid)/NaOH. No decrease in glucose transport activity was observed for at least 12 h if the suspension was stored in ice. The protein concentration was determined by the Lowry method [25] using bovine albumin as a reference.

Chemicals. Mops and phenazinemethosulfate (PMS) were obtained from Dojindo (Kumamoto); radioisotopes of glucose and its analogue from NEM Research Products, (Boston, MA); carbonyl cyanide *m*-chlorophenylhydrazone (CCCP), phloridzin, phloretin, forskolin, cytochalsin B and cytochalsin E from Sigma Chemical (St. Louis, MO). Other chemicals were of analytical grade.

Glucose and its analogue uptake by intact cells. The uptake of glucose or its analogs by intact cells was measured by filtration method using ^{3}H-labelled compounds. 5 ml of an experimental medium was used which contained 4.28 M NaCl; pH was 7.0 with 50 mM of Mops, to which sodium ascorbate (final concentration of 2 mM) and PMS (final concentration of 0.018 mM) had been added. Ascorbate plus PMS was used as an energy source for the uptake. The radioactivity of glucose or glucose analogue was 1–3 μCi in a cuvette. Temperature of the medium was kept at 25 °C by circulating thermostated water around the cuvette.

The uptake experiments were generally started by addition of the cells (0.5 ± 0.1 mg protein as total amounts) to the suspension medium (5.0 ml). The suspension was magnetically stirred to provide sufficient aeration. An aliquot of 200 μl was withdrawn from the suspension at determined time intervals and added into 10 ml of ice-cooled 4.28 M NaCl solution, followed by rapid filtration with a Whatman FG/B filter (Whatman International Ltd., Maidstone, U.K.). The filter was washed twice with 10 ml of ice-cooled 4.28 M NaCl solution, then transferred to a scintillation vial and shaken for 20 min with 2 ml of 10% Triton X-100. Radioactivity was measured with a Packard 460C liquid scintillation counter with a scintillation cocktail of ACS-II (Amersham Corp., Arlington Heights, MA). The binding of chemicals to the filters was corrected by subtracting the counts of control experiments without cells from the sample counts. This correction amounted at most to 7% when the uptake was small, and it was negligible when sufficient uptake by cells was observed. As shown in Fig. 1, the amount of uptake was linear with time up to 7 or 10 min after the onset of experiments and the initial slope was taken as the speed of the uptake.

Inhibitors of CCCP, 2-dinitrophenol (2-DNP), pentachlorophenol (PCP), phloridzin, phloretin, forskolin, cytochalasin B and cytochalasin E were added as dimethylsulfoxide (DMSO) solution. Even though 0.4% of DMSO was added, we observed no effect of this addition.

Substrates such as 2-deoxy-D-glucose (2-DG), 3-*O*-methyl-D-glucose (3-OMG), α-methyl-D-glucoside (α-MG), L-glucose, D-galactose, D-fructose, D-sorbitol and D-lactose were added as an aqueous solution.

Preparation of envelope vesicles and transport experiments. Envelope vesicles from *H. volcanii* were prepared by essentially the same method as that used for

H. halobium developed by MacDonald and Lanyi and their colleagues [15]. The contents of the vesicles were changed to the desired salt of 3 M composition by the osmotic-dilution method. Procedures of transport experiments were the same as those for intact cells. The transport activity remained unchanged for at least one month provided the vesicles were kept cool at 4 °C.

Measurements of efflux from the cells. Efflux from a cell was measured using 3-OMG that had not been metabolized by the cell. The 3-OMG-loaded cells were prepared by incubation in a suspension medium containing sodium ascorbate (2 mM), PMS (0.018 mM) and [^{3}H]3-OMG (53 μM, 2 μCi). The cells were spun down with a microcentrifuge (Kubota KM-15200, Tokyo) and washed twice. Experiments were begun by addition of the loaded cells into the suspension medium containing neither sugar, tracer nor energy source.

Results and Discussion

Glucose transport is inducible

Fig. 1 shows 2-DG uptake under various conditions. Bacterium grown in the presence of 0.5% of glucose (Glc) accumulated this glucose analogue. On the other hand, if no Glc was present in the medium, no uptake was observed, indicating that the glucose transport system of this bacterium is inducible. This figure also shows that the addition of uncouplers to the experimental medium inhibited the uptake, meaning that this transport system is energy-dependent.

Kinetics of glucose transporter

Kinetic analysis of this glucose transporter showed that $K_t = 30.9$ μM, $V_{max} = 4.61$ nmol/min per mg protein for Glc and $K_t = 34.6$ μM, $V_{max} = 2.78$ nmol/min per mg protein for 2-DG. For respective sugars, the data are well fitted by a single kinetic equation (data not shown), suggesting that there may be only one glucose transport system in this bacterium.

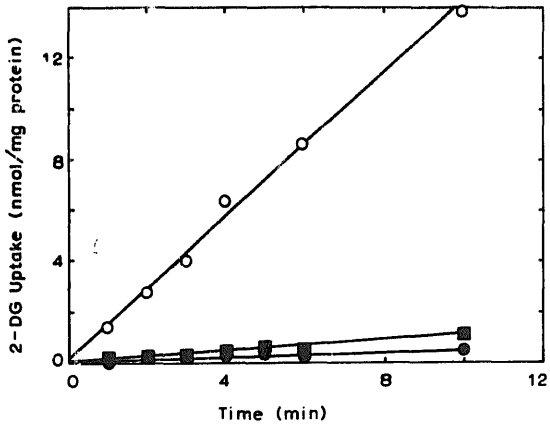

Fig. 1. The glucose transporter is inducible and is inhibited in the presence of CCCP. ○, Time-course of 2-DG uptake by cells grown in the medium containing 0.5% glucose: ●,uptake by cells grown in the absence of 0.5% glucose: ■, effect of 10 μM CCCP on the 2-DG uptake by cells grown in the presence of glucose. The experimental medium was 5.0 ml in volume containing 4.28 M NaCl buffered with 50 mM Mops (pH 7.0), 2 mM Na ascorbate, 0.018 mM PMS and 35 μM (1 μCi) [^{3}H]2-DG. The cell density was 0.10±0.02 mg protein/ml. The temperature was 25 °C.

TABLE I

Inhibition of various sugars on glucose uptake by H. volcanii cells

The measuring medium contained 4.28 M NaCl, 50 mM Mops (pH 7.0), 2 mM sodium ascorbate, 0.018 mM PMS, 30 μM Glc and 1 μCi [^{3}H]Glc. The cell density was 0.1 mg protein/ml. The value of % uptake indicates the per cent of inhibition of glucose uptake due to the presence of various sugars (150 μM). None (control) means a condition in which only glucose was present without any other sugars. Temperature was 25 °C

Sugar	% Uptake
None (control)	100
2-Deoxy-D-glucose	37
3-*O*-Methyl-D-glucose	24
α-Methyl-D-glucose	96
L-Glucose	98
D-Galactose	100
D-Fructose	100
D-Sorbitol	113
D-Lactose	124

Effects of other sugars on the glucose transporter

Effects of other sugars on the rate of glucose uptake are listed in Table I. Here, we used intact cells. The concentration of glucose was 30 μM (almost equal to the K_t value) and that of other sugars was three-times higher than glucose, i.e., 150 μM. The fact that L-glucose, D-galactose, D-fructose, D-sorbitol and D-lactose did not affect the uptake rate of glucose reflects the high specificity of the substrate. Non-metabolizable glucose-analogues such as 2-DG and 3-OMG strongly inhibited the glucose transport, while α-methyl-D-glucoside (α-MG) did not inhibit it. It is very interesting that although α-MG is a substrate of PTS in *E. coli* [26], α-MG does not inhibit this system. Moreover, 3-OMG which is not a substrate of PTS does inhibit this system. This may suggest that the glucose transport system in this bacterium is different from that of *E. coli* or other ordinary bacteria. Although the glucose system of lactic acid bacteria [27] has both a H^+-symporter system and PTS, for almost all bacteria glucose is accumulated by PTS. It can therefore be suggested that the glucose transport system of this bacterium is not PTS (see below).

The present glucose transport system is not PTS but requires the gradient of Na^+ electrochemical potential

If we assume the intracellular volume to be 1.5 μl/mg protein, the intracellular concentration of 2-DG after 10 min of the experiment is calculated as 10 mM, about 300-fold the extracellular concentration. This

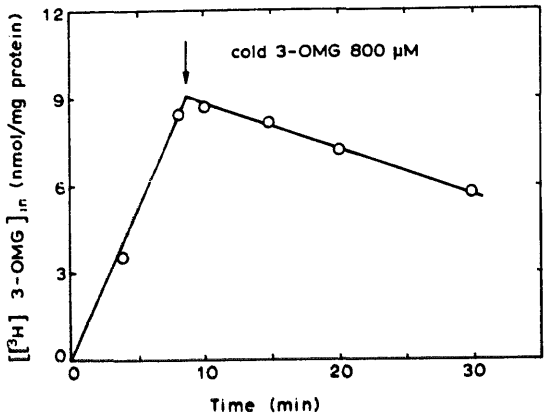

Fig. 2. Addition of non-labelled sugar leads to efflux of the sugar accumulated previously. The experimental medium and the conditions were the same as described in Fig. 1. 9 min after the start of experiments, 800 μM of cold (non-labelled) 3-OMG was added. The temperature was 25 °C. The efflux of ^{3}H-labelled 3-OMG was induced, implying that the transport system is not PTS but carrier-mediated.

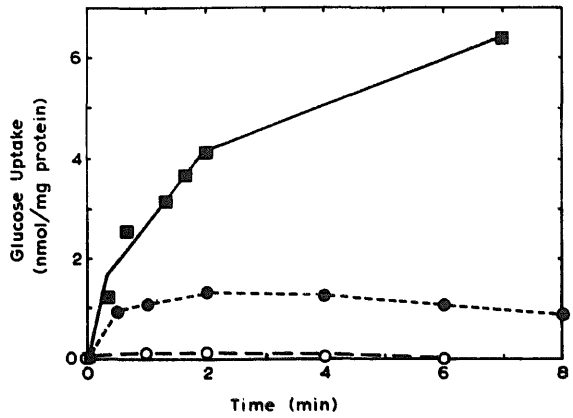

Fig. 3. Glucose uptake by envelope vesicles derived from *Haloferax volcanii* whose inside ionic composition was 3.0 M KCl. ■, External ionic composition was 3.0 M NaCl plus respiratory substrate; ●, external composition was 3.0 M NaCl without respiratory substrate; ○, external composition was 3.0 M KCl. Respiratory substrate was 2 mM Na ascorbate plus 0.018 mM PMS. All media were buffered with 50 mM Mops (pH 7.0). Vesicle concentration was 0.1 mg protein/ml. The concentration of glucose was 30 μM and its radioactivity was 2 μCi. The temperature was 25 °C. The presence of external Na^+ induces the uptake. Respiration increases both rate and extent of the uptake and sustains the uptake.

confirms that this transport system is not a facilitate transport. As shown in Fig. 1, 10 μM CCCP almost completely inhibited the transport, meaning that the system is supplied with energy.

Using a non-metabolizing glucose analogue, 3-OMG, an experiment was conducted on the efflux of previously accumulated ^{3}H-labeled 3-OMG upon addition of a high concentration of unlabelled 3-OMG; the results are plotted in Fig. 2. Since 3-OMG inhibits the glucose transport system (Table 1), 3-OMG uptake may occur by the glucose transporter. If sugar is transported by PTS, addition of external sugar does not necessarily cause the efflux of the substrate. The observation of the efflux shown in Fig. 2 thus implies that this transport system may not be PTS.

Fig. 3 shows the uptake of Glc by envelope vesicles that are free from intracellular proteins. The accumulation of Glc by 3 M KCl-loaded vesicles was observed when the ionic composition of the outside of the vesicles was 3 M NaCl. Together with the fact shown in Fig. 4, we infer that the driving force of this accumulation is an electrochemical potential gradient of Na^+, suggesting that this system is a symport (co-transport) with Na^+. The addition of sodium ascorbate (2 mM) plus PMS (0.018 mM) led to a large and sustained uptake. This may have been caused both by the expulsion of inflowing Na^+ by Na^+/H^+ antiporter and by the generation of interior negative membrane potential; they are energized by the respiration.

The effect of the membrane potential on the glucose uptake was examined. To this end, we used vesicles imposing no ionic gradient across the membrane; Fig. 4 showed results obtained. Without respiration substrates, we were unable to observe uptake (data not shown). The experimental condition shown in (B) was that in which 3 M NaCl-loaded vesicles were suspended in 3 M NaCl, and we were able to find the uptake. Respiration supports the transport, showing that the membrane potential (interior negative) can serve as the driving force. The condition indicated by (A) was that both the inside and outside of vesicles were 1.5 M KCl plus 1.5 M NaCl. The uptake rate was initially small, but the rate increased gradually. A simi-

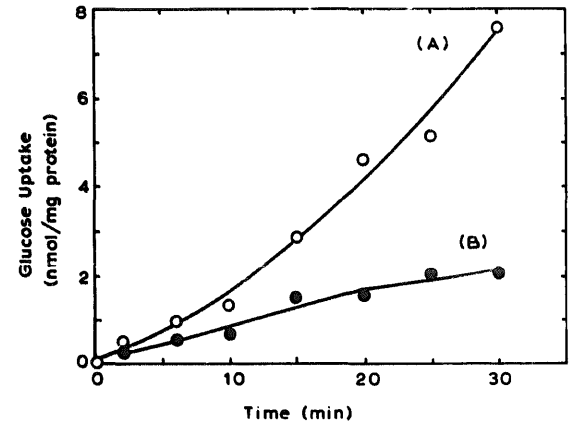

Fig. 4. Effects of membrane potential on glucose uptake. (A) The ionic compositions of both inside and outside were 1.5 M KCl plus 1.5 M NaCl. (B) Both inside and outside were 3.0 M NaCl. For each, 2 mM Na ascorbate plus 0.018 mM PMS was added as a respiratory substrate. These were the final concentrations. Media were buffered with 50 mM Mops (pH, 7.0). Vesicle concentration was 0.1 mg protein/ml. The concentration of glucose was 30 μM and its radioactivity was 2 μCi. The temperature was 25 ° C. Generation of the membrane potential by ascorbate plus PMS induces the uptake. In (A), the uptake rate was increased after about 15 min; this may have been due to the Na^+-gradient created by Na^+/H^+ antiporter. These findings support that the present transport system is Na^+-driven.

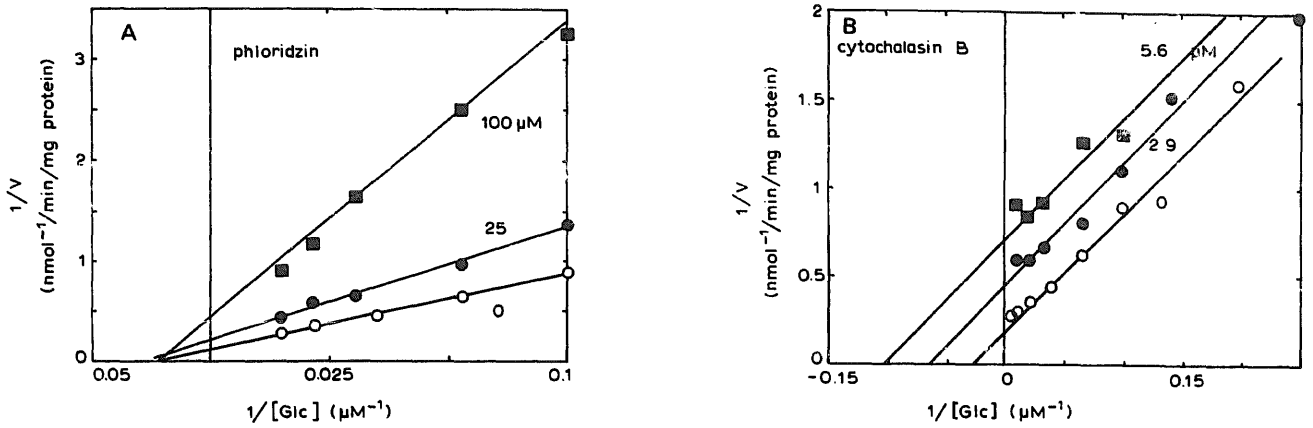

Fig. 5. Phloridzin (A) and cytochalasin B (B) inhibit the glucose transport system of *H. volcanii*. These inhibitors are those for a glucose transport system for mammalian cells. Experimental conditions were the same as in Fig. 1 except for varying concentrations of glucose. The concentration of inhibitors used is shown.

lar phenomenon was reported for the uptake of amino acids by *Halobacterium halobium* [15]. Respiration generates the membrane potential and drives the Na^+/H^+ antiporter, which leads to the formation of the Na^+-concentration gradient during the experiments. This may be the reason for the accelerated uptake shown in (A). These observations reveal that Glc/Na^+ symporter is electrogenic. This means that the stoichiometry of the symport system may be 1:1, although further study on this stoichiometry is necessary.

Inhibitors of glucose transporter

Table II summarizes the effect of phloridzin, phloretin, forskolin and cytochalasin B on 2-DG uptake. They act as inhibitors for glucose transporters in *mammalian cells*. Cytochalasin E is not an inhibitor, but was used as a control having similar chemical structure as cytochalasin B. It is noted that cytochalasin B inhibits the transport, while cytochalasin E does not. As far as we know, these inhibitors of glucose transporters of animal cells have not been reported to have an effect on the glucose transport of ordinary bacteria (eubacteria). The facts that glucose transport of this bacterium requires the Na^+-electrochemical gradient and that such inhibitors as listed in Table II exert an effect suggest that the glucose transport of this bacterium somehow resembles that of animal cells. Of interest in this regard is a paper of Cairns et al. [28] reporting that galP D-galactose transport protein of *E. coli* is photoaffinity labelled with cytochalsin B.

TABLE II

Effects of various inhibitors on 2-DG uptake by H. volcanii cells

Measuring medium was the same as that shown in the legend to Table I. The cell density was 0.1 mg protein/ml. The uptake of 2-DG (35 μM) was measured with 1 μCi of [^{3}H]2-DG. The values of % uptake indicate the ratio of velocity to that in the absence of inhibitors.

Inhibitor	Concen.	% Uptake
None		100
Phloridzin	0.1 mM	18
	2	0
Phloretin	1 μM	11
	10	0
Forskolin	1 μM	47
	10	15
Cytochalasin B	2 μM	68
	15	5
Cytochalasin E	8 μM	98

Fig. 5 shows the kinetic analysis of the effect of phloridzin and cytochalasin B on the glucose uptake. This figure shows that phloridzin is a non-competitive inhibitor and cytochalasin B is an uncompetitive inhibitor. It is well known that there are two types of mammalian glucose transporters [29]: one is a Na^+/D-Glc symporter which is present in the brush border membranes of intestinal and renal epithelia, and the other is facilitate passive transport system which distribute in various cell types and the basolateral membranes of intestinal and renal epithelia. The former has affinity for D-glucose, D-galactose, α-MG and 3-OMG, and inhibited by phloridzin in a competitive manner. The latter is inhibited by cytochalasin B, the manner of which is markedly different from that of the present cell [30]. The glucose transport system of this bacterium resembles partially the symporter and partially the facilitate passive transporter of the mammalian cells.

Efflux mobility of glucose-carrier

As shown in Fig. 2, the glucose and glucose analogues accumulated are released by the addition of a high concentration of cold analogues. To examine this further, efflux was measured. As described in the experimental section, intact cells containing hot 3-OMG inside were suspended in a medium without any sugars, isotopes or energy source. If we assume that the intracellular volume of cells is 1.5 μl of mg protein, the

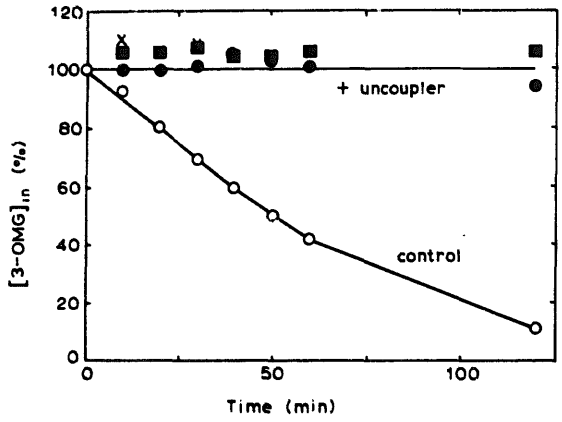

Fig. 6. Uncoupler inhibits the efflux. The cells (1.0±0.1 mg protein) into which [^{3}H]3-OMG was previously accumulated were suspended in a medium without sugar or isotopes, and then the remaining radioactivity was measured. The value of 100% of the ordinate represents the level of the accumulated [^{3}H]3-OMG before the start of the efflux experiment. Uncouplers used were: ■, PCP 6 μM; ●, CCCP 10 μM; ×, DNP 2 μM. The experimental medium was 4.28 M NaCl buffered at pH 7.0 with 50 mM Mops. The temperature was 25°C.

3-OMG concentration in the cell was 30 mM. Residual isotopes within cells were counted after the suspension. Fig. 6 shows that the presence of various uncouplers inhibits the efflux. Since various uncouplers are effective, the specific interaction between an uncoupler and the translocator can be ruled out.

One kinetic feature of active transport is the difference in carrier affinity for substrates between inside and outside [31]. This difference is generated by the metabolic energy and the active transport system becomes the facilitate transport system when the energy supply is stopped. The efflux rate, therefore, should become faster in the presence of uncoupler. In this respect, the result shown in Fig. 6 is very interesting. Although further studies on the mechanism are necessary, the physiological implication may be that the translocator is 'locked' under the condition of de-energization, which prevents the loss of accumulated sugars. There is a report that there exists a threshold of the Na^+/H^+ antiporter of *Halobacterium halobium* (120 mV of interior negative membrane potential); only when the potential exceeds this value does the antiporter begin to work [32]. The inhibition of uncouplers to the efflux was not observed in envelope vesicles. The reason is not yet clear, but it may suggest the presence of regulatory components inside cells. Further study is necessary.

Concluding remarks

In bacteria, glucose is usually taken up by the phosphotransferase system (PTS) and in mammalian cells, either by a symport system with Na^+ or by a facilitate system. Glucose transport in heterofermentative strains of Lactobacilli [27] and in Cyanobacterium [32] is considered to occur by a pmf-driven glucose permease system. It is noted that the glucose transport of *H. volcanii* requires the gradient of Na^+-electrochemical potential. Although detailed further study is necessary, this suggests that the present system is a symporter with Na^+. In addition, it is unique that the inhibitors of the glucose transporter in mammalian cells work for the glucose system of this bacterium. There are two kinds of glucose transport system in mammalian cells, and the inhibitors for these two systems work for the present system. This suggests that the present system resembles partially both systems. Recent publications [11–14] reveal that the sequences of transporters of bacteria are homologous with each other and with the glucose transporters of human hepatoma and rat brain. Therefore, determination of the amino acid sequences of this transporter will provide information on the molecular evolution of glucose transporter and molecular insight on the transport mechanism.

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