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Energization of Glucose Transport by *Pseudomonas fluorescens*

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We have measured the capacity of *Pseudomonas fluorescens* to transport the glucose analog 2-deoxy-D-glucose and the amino acids L-alanine and α-aminoisobutyric acid under conditions in which the cells could generate (i) both a membrane proton motive force and high-energy phosphate compounds, (ii) a proton motive force but not high-energy phosphate compounds, and (iii) neither a proton motive force nor high-energy phosphate compounds. This was done by depleting cells of adenosine triphosphate stores by treatment with sodium arsenate and then suspending them in a phosphate-free medium, where they could generate a proton motive force but not phosphate bond energy, or in a phosphate-containing medium, where they could generate both a proton motive force and phosphate bond energy. Inclusion of the proton-conducting ionophore carbonyl cyanide-*m*-chlorophenyl hydrazone under either condition precluded the generation of both a proton motive force and phosphate bond energy. The amino acids L-alanine and α-aminoisobutyric acid were transported independently of phosphate bond energy and required only a proton motive force. 2-Deoxy-D-glucose was transported only under conditions in which phosphate bond energy could be generated. These results are consistent with the findings of others that *Pseudomonas aeruginosa* produces an inducible shock-sensitive glucose-binding protein and conform to the generalization that binding protein-associated transport systems are energized by phosphate bond energy.

Pseudomonas aeruginosa and *Pseudomonas fluorescens* possess an inducible, non-phosphorylative active transport system for glucose and its analogs (3, 5, 10, 11). Evidence has been presented that this transport system is dependent upon a binding protein that is removed from the cell by osmotic shock: Stinnett et al. (18) and Guymon and Eagon (5) showed that membrane vesicles prepared from *P. aeruginosa* which retained the capacity to transport gluconate did not transport glucose. Subsequently, Stinson et al. (19) isolated a glucose-binding protein from osmotic shock fluids derived from this species, which was absent in a glucose transport- and chemotaxis-negative mutant.

By analogy with *Escherichia coli*, it would be expected that such a shock-sensitive, binding protein-associated system would be energized not by a proton motive force, but by phosphate bond energy, possibly ATP (2, 21) or acetyl phosphate (6). This paper presents direct evidence supporting this view.

The experimental design was as follows: resting cells of *P. fluorescens* GC-1, a mutant constitutive for the glucose transport system, were depleted of intracellular ATP stores by incubation with sodium arsenate; they were subsequently suspended in (i) a phosphate-containing medium, where they could presumably regenerate both a proton motive force and ATP by oxidation of intracellular energy stores, and in (ii) a phosphate-free medium, where they could continue to generate a proton motive force, but could not replenish stores of ATP or other high-energy phosphate compounds. The capacity of cells treated under these conditions to transport glucose was determined by measuring uptake of 2-deoxy-D-[U-^{14}C]glucose (2-[U-^{14}C]DOG); the presence of a proton motive force was monitored by the simultaneous determination of the uptake of tritiated α-aminoisobutyric acid (AIBA), a non-metabolizable amino acid whose transport is presumably energized by a proton motive force (17). We show here that the capacity to transport 2-[U-^{14}C]DOG was regained only under conditions in which phosphate bond energy can be regenerated, whereas the amino acid was transported independently of phosphate bond energy.

MATERIALS AND METHODS

Organisms and media. *P. fluorescens* ATCC 13525 and a glucose uptake-constitutive mutant, *P. fluorescens* GC-1, were carried routinely on pseudomonas F agar slants (Difco Laboratories, Detroit, Mich.). The glucose uptake-constitutive mutant was

used in the experiments described here, unless specified otherwise, to minimize variations in glucose permease activity in separate experiments. Cells to be used in uptake experiments were grown on a basal medium containing (in grams per liter of distilled water): K_2HPO_4, 10.5; KH_2PO_4, 19.0; $(NH_4)_2SO_4$, 3.0; $MgSO_4 \cdot 7H_2O$, 0.2; $CaCl_2$, 0.02; $FeSO_4 \cdot 7H_2O$, 0.01; $MnCl_2 \cdot 2H_2O$, 0.01; and $NaMoO_4 \cdot 2H_2O$, 0.001. This medium will be referred to as phosphate-buffered salts (PBS). Carbon sources, D-glucose or sodium succinate as specified, were added separately as sterile solutions to a final concentration of 50 mM. Cultures of 50 ml contained in 250-ml Erlenmeyer flasks were incubated at 30°C in a water bath reciprocal shaker.

The glucose uptake-constitutive strain GC-1 was isolated from its wild-type parent by modification of a method used by Parke and Ornston (12) to select a strain of *Pseudomonas putida* constitutive for the uptake of β-ketoadipate. This method consists of repeated sequential transfer of a culture from a noninducing medium to an inducing medium and back to the noninducing medium at early stationary phase; it is based on the rationale that such cultures will become enriched with cells capable of growth on the inducing medium with a minimal period of lag-phase growth. Thus, the parent strain, *P. fluorescens* ATCC 13525, was grown to late exponential phase on 50 mM succinate–PBS, washed and suspended in PBS, and treated with the mutagen ethyl methane sulfonate (0.03 ml of ethyl methane sulfonate per 2.0 ml of cell suspension) for 2 h at 30°C in a shaking water bath. The cells were harvested by centrifugation, washed three times with PBS to remove the mutagen, and used to inoculate tubes containing 10 mM succinate–PBS. These cultures were grown to early stationary phase and were then transferred (0.15 ml) to tubes containing 10 mM glucose–PBS. After growth of these cultures to early stationary phase, they were transferred to fresh tubes of 10 mM succinate–PBS and grown through two transfers. This cycle of transferring at early stationary phase from succinate medium to glucose medium and back to succinate medium was carried out five times. At this point, the culture showed a lag phase of 2 h on 10 mM glucose–PBS, compared with the 4- to 5-h lag phase exhibited by the wild-type parent. Cultures were then plated onto eosin methylene blue agar, made up to contain the following (per liter): peptone, 10 g; K_2HPO_4, 2 g; agar, 13.5 g; eosin Y, 0.4 g; methylene blue, 0.056 g; glucose, 50 mM; and succinate, 50 mM. Presumptive glucose uptake constitutive mutants appeared as black colonies by virtue of their ability to metabolize and produce acid from glucose in the presence of the repressive substrate succinate. These were picked and tested for their capacity to transport 2-DOG (see below). Strain GC-1, selected through this procedure, transported 2-DOG as rapidly when grown on succinate as when grown on glucose, whereas its parent took up this analog only when grown on glucose. It is of interest to note here that GC-1 was not constitutive for enzymes of the Entner-Doudoroff pathway; this is further evidence that the transport system for glucose is regulated separately from enzymes catalyzing its utilization (3, 7).

Inhibitors. Cells were depleted of intracellular ATP stores by treatment with 10 mM sodium arsenate or 1 mM *N,N'*-dicyclohexylcarbodiimide (DCCD) (both obtained from Sigma Chemical Co., St. Louis, Mo.). Cells grown as described above were harvested, washed once with 0.9% NaCl, and then washed and suspended at a cell density of 120 Klett units (0.4 mg [dry weight] per ml) in a glycylglycine-buffered salt solution (GGBS) which contained the following (in grams per liter of 0.02 M glycylglycine, pH 7.4): $MgSO_4 \cdot 7H_2O$, 0.2; $CaCl_2$, 0.02; $FeSO_4 \cdot 7H_2O$, 0.01; $MnCl_2 \cdot 2H_2O$, 0.01; and $NaMoO_4 \cdot 2H_2O$, 0.001. When sodium arsenate was used, it was added at a final concentration of 10 mM to 10 ml of cell suspension held at 30°C in a shaking water bath. When DCCD was used, 0.1 ml of 0.1 M DCCD in 95% ethanol was added to 10 ml of cell suspension held at 30°C in a shaking water bath. A control suspension received the same volume of 95% ethanol. Treatment with DCCD or arsenate was for 60 min, after which the cells were harvested by centrifugation, washed, and suspended in either PBS or GGBS, as indicated in the individual experiments, for determination of uptake capacity as described below.

When carbonyl cyanide-*m*-chlorophenyl hydrazone (CCCP; Sigma Chemical Co.) was used as an inhibitor in uptake determinations, it was added as an ethanolic solution (0.05 ml of 5 mM CCCP per 5 ml of cell suspension) at a final concentration of 50 μM. An equal volume of 95% ethanol was also added to the control suspension.

Uptake measurement. Activity of the D-glucose uptake system was measured by using the non-metabolizable analog 2-DOG. As in *P. aeruginosa* (11), this analog is accumulated without phosphorylation by the *P. fluorescens* strain used in this study at a concentration 25- to 50-fold greater than that of the external medium. It is transported with an apparent K_m of 0.20 mM and is competitively inhibited by D-glucose with a K_i of 7 μM.

Cells treated with the appropriate inhibitor, and controls, as described above, were suspended in the appropriate buffered salt solution at a cell density of 120 Klett units (0.4 mg [dry weight] per ml). Cell suspensions (5 ml) were incubated in a water-bath shaker at 30°C for 15 min to allow temperature equilibration; 2-[*U*-^{14}C]DOG (International Chemical and Nuclear Corp., Irvine, Calif.) was then added at a final concentration of 0.5 mM and a specific radioactivity of 0.2 μCi/μmol. In double-label experiments with amino acids, [*methyl*-^{3}H]AIBA was added at a final concentration of 1 mM and a specific radioactivity of 0.4 μCi/μmol, or L-[2,3-^{3}H]alanine was added at 1 mM and a specific radioactivity of 0.2 μCi/μmol (both from New England Nuclear Corp., Boston, Mass.). Samples of 0.5 ml were removed at appropriate time intervals, filtered rapidly through membrane filters (0.45-μm porosity; Millipore Corp., Bedford, Mass.), and washed immediately with 5 ml of the suspension buffer at room temperature. Sampling, filtration, and washing took no more than 15 s. Washed filters with the cells thereon were transferred to vials containing 10 ml of Aquasol 2 (New England Nuclear Corp.), and the radioactivity was determined with a Packard liquid scintillation spectrometer (Packard Instrument Co., Inc., Downers Grove, Ill.).

Exit measurement. Cells grown on 50 mM succi-

nate–PBS were harvested, washed, and suspended in the appropriate buffered salt solution at a cell density of 0.8 mg (dry weight) per ml. They were then preloaded by incubation with 2-[U-^{14}C]DOG (0.5 mM, 0.2 μCi/μmol) for 15 min at 30°C, collected by centrifugation at 10,000 × g for 10 min, and suspended in prewarmed (30°C) buffered salt solution at a density of 0.4 mg (dry weight) per ml. Samples of 0.5 ml were treated as described above for uptake measurement.

Intracellular ATP measurement. ATP was measured by the luciferin-luciferase method described by Wilson et al. (23). Samples (0.4 ml each) of cell suspensions treated as described below were placed in 0.1 ml of 3 M perchloric acid. After 10 min, the acid extract was neutralized with 0.3 ml of 1 M KOH, and analyses were carried out in triplicate, exactly as described by these authors. Samples were counted for 6 s in a Packard liquid scintillation spectrometer with a window setting of 60 to 65, maximum gain, and the coincidence circuit off. Parallel samples of the cell suspensions were taken at the same times for protein determination. Samples of 1 ml were pipetted into 1 ml of 2 N NaOH and boiled for 15 min to solubilize protein. Determinations were carried out by the method of Lowry et al. (9). Results of ATP measurement are expressed in terms of nanomoles of ATP per milligram of protein.

RESULTS AND DISCUSSION

Figure 1 shows the effect of sodium arsenate, a competitive inhibitor of ATP formation (8), on the uptake of 2-DOG by a resting cell suspension of *P. fluorescens* GC-1 when added at various times during uptake. It is clear that this inhibitor affected both the uptake and the maintenance of a steady-state level of 2-DOG by the cells. However, the effect on uptake was not immediate; when added before a steady-state level of 2-DOG had been reached, uptake of the sugar analog continued for approximately 2 min after addition of the inhibitor, albeit at a reduced rate. Presumably, this continued uptake of 2-DOG in the presence of arsenate took place at the expense of residual high-energy phosphate compounds in the cells.

Klein and Boyer (8) have shown that arsenate depletes *E. coli* cells of ATP and other high-energy phosphate compounds without preventing the formation of an activated membrane state. Arsenate has a similar effect on *P. fluorescens*; Fig. 2 shows the depletion of cellular ATP brought about by incubation of the cell suspension with 10 mM arsenate in GGBS. Thus, the inhibition of 2-DOG accumulation by arsenate can be taken as presumptive evidence that the active transport of 2-DOG is dependent upon phosphate bond energy. However, the possibility that ATP exerts its primary action by generating a proton motive force through the action on an ATPase and that the proton motive force is the crucial determinant of 2-DOG trans-

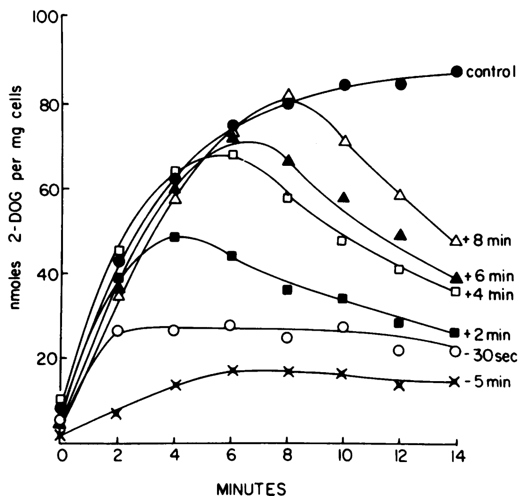

FIG. 1. *Effect of arsenate on uptake of 2-DOG by P. fluorescens GC-1. Cells grown on 50 mM succinate–PBS were washed, suspended in GGBS, and incubated with 0.5 mM 2-[U-^{14}C]DOG (0.2 μCi/μmol). Sodium arsenate (10 mM final concentration) was added at the times indicated.*

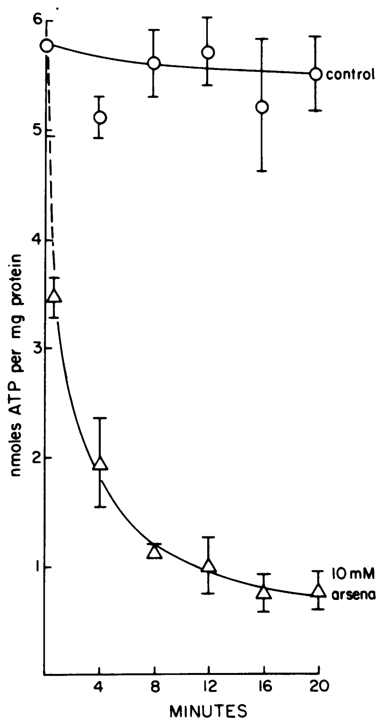

FIG. 2. *Effect of arsenate on cellular ATP pool of P. fluorescens GC-1. Cells grown on 50 mM succinate–PBS were harvested, washed, suspended in GGBS, and incubated at 30°C in the absence (○) or presence (△) of 10 mM sodium arsenate. Error bars indicate standard error in triplicate determinations.*

port cannot be ruled out immediately.

An approach to this question was made as follows: after depletion of their high-energy phosphate compounds by arsenate treatment, the cells were washed free of arsenate and suspended in either (i) a phosphate-free GGBS solution, where they would be expected to continue to generate a proton motive force across the cytoplasmic membrane through the oxidation of intracellular metabolites, but would not be able to resynthesize high-energy phosphate compounds, or (ii) a PBS solution, where they would be expected to be capable both of generating a proton motive force and of synthesizing ATP by oxidative phosphorylation. We then determined the capacity of cells treated in these two ways to transport 2-[*U*-^{14}C]DOG and a tritiated amino acid, the latter of which is presumably actively transported by a proton motive force (17). The results of such an experiment are shown in Fig. 3. Arsenate-treated cells that were suspended in phosphate-free GGBS remained strongly impaired in their capacity to take up 2-DOG, relative to control cells that were not treated with arsenate, whereas these same cells showed essentially no decrease in their ability to take up [^{3}H]AIBA (Fig. 3A). In contrast, arsenate-treated cells that were suspended in PBS for 15 min regained most of their capacity to transport 2-DOG, while retaining their ability to take up AIBA (Fig. 3B). Inclusion of the proton-conducting ionophore CCCP in either medium, which would inhibit both the generation of a proton motive force and the synthesis of ATP, since *P. aeruginosa* is a nonfermenting strict aerobe and can generate ATP only by oxidative phosphorylation, abolished the uptake of both 2-DOG and AIBA (Fig. 3B).

The capacity of *P. fluorescens* to concentrate AIBA was quantitatively less than its ability to concentrate 2-DOG (note the difference in scale); nevertheless, the steady-state levels of AIBA reached correspond to a 10-fold concentration over the extracellular level used in these experiments. When [^{3}H]AIBA was replaced with L-[^{3}H]alanine, entirely similar results were obtained (data not shown).

The capacity to take up 2-DOG correlated closely with the ATP levels measured in cells that were treated identically as the cells used in

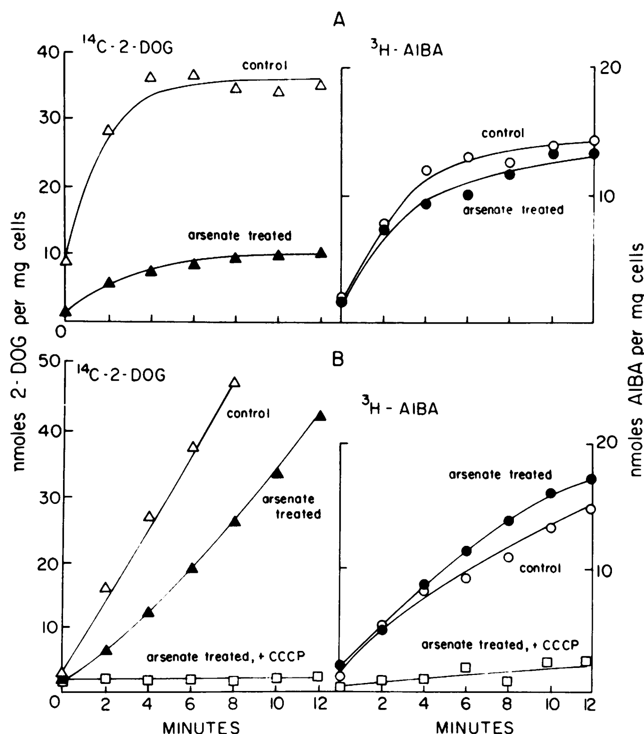

FIG. 3. *Effect of suspension medium on uptake of 2-DOG and AIBA by arsenate-treated P. fluorescens GC-1. Cells grown on 50 mM succinate-PBS were preincubated in the absence (control) or presence of 10 mM sodium arsenate for 1 h, harvested, washed, and incubated in GGBS (A) or PBS (B) for 15 min before the addition of 2-[U-^{14}C]DOG (0.5 mM, 0.2 µCi/µmol) and [methyl-^{3}H]AIBA (1 mM, 0.4 µCi/µmol). CCCP (50 µM) was added to the suspension medium as indicated.*

the experiment described above. Table 1 shows that arsenate treatment drastically lowered the ATP pool; washing, resuspension, and incubation in phosphate-free GGBS did not allow restoration of a significant ATP pool, whereas cells suspended and incubated in PBS recovered more than 50% of their original ATP level.

There was not a similar correlation between the ATP pool and the capacity to take up AIBA. This indicates further the independence of AIBA uptake of ATP.

It was important to establish that glycylglycine was not toxic in some way that would prevent 2-DOG uptake after arsenate treatment or would prevent ATP synthesis, apart from a phosphate deficiency. Therefore, experiments were carried out in which phosphate was added back to the GGBS suspension medium used to suspend the cells after arsenate treatment. The addition of 20 mM phosphate to the GGBS suspension medium allowed arsenate-treated cells to regain the capacity to transport 2-DOG, presumably because of the ability to synthesize ATP in the presence of added phosphate.

The ATPase inhibitor DCCD was slower to exert an inhibitory effect on 2-DOG uptake than was arsenate. Figure 4 shows that DCCD-treated cells showed no significant decrease in 2-DOG uptake after 10 min; there was a 50% inhibition after 20 min and an 80% inhibition after 1 h. This decay in 2-DOG uptake capacity paralleled the relatively slow depletion of the cellular ATP pool (Table 1). Moreover, unlike

TABLE 1. *Effect of arsenate and DCCD treatment on cellular ATP pool*

Treatment	ATP pool (nmol/mg of protein)
Arsenate	
None[a]	5.7 ± 0.7
None, 20 min	5.4 ± 0.3
None, 60 min	4.5 ± 0.1
10 mM arsenate, 20 min	0.7 ± 0.2
10 mM arsenate, 60 min	0.5 ± 0.2
10 mM arsenate, 60 min; washed and suspended in GGBS for 20 min	1.1 ± 0.2
10 mM arsenate, 60 min; washed and suspended in PBS for 20 min	3.2 ± 0.5
DCCD	
None	5.6 ± 0.1
None, 20 min	6.2 ± 0.5
None, 60 min	4.5 ± 0.2
1 mM DCCD, 20 min	4.1 ± 0.5
1 mM DCCD, 60 min	1.0 ± 0.2
1 mM DCCD, 60 min; washed and suspended in PBS for 20 min	1.5 ± 0.2

[a] Cells grown on 50 mM succinate-PBS were harvested, washed, and suspended in GGBS.

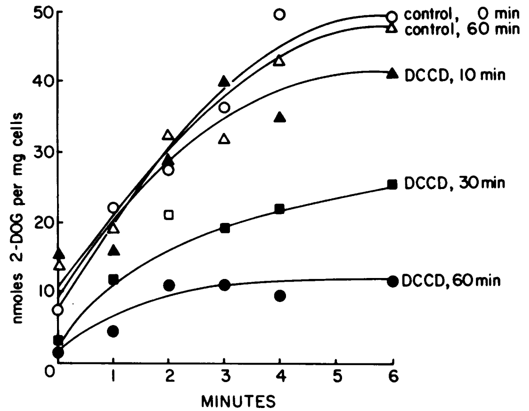

FIG. 4. *Effect of DCCD on 2-DOG uptake by P. fluorescens GC-1. Cells grown on 50 mM succinate-PBS were washed, suspended in GGBS, and incubated with no further addition (○, zero time; △, 60 min) or with 1 mM DCCD for 10 min (▲), 30 min (■), or 60 min (●) before the addition of 2-[U-^{14}C]DOG (0.5 mM, 0.2 μCi/μmol).*

arsenate-treated cells, there was neither a replenishment of the ATP pool (Table 1) nor a recovery in the capacity to transport 2-DOG when the cells were washed and suspended in PBS (Fig. 5). Apparently, DCCD did not penetrate rapidly to the membrane-bound ATPase, but once it bound to the ATPase, it could not be washed away readily. However, DCCD-treated cells were unimpaired in their capacity to take up AIBA, whether they were suspended in the absence of DCCD or in its continuous presence (Fig. 5). These data show further that the transport of this amino acid is independent of cellular ATP. As before, CCCP abolished AIBA transport, indicating the requirement of a proton motive force for the transport of this amino acid. Identical results were obtained with L-alanine (data not shown).

To establish that the properties of the glucose and the amino acid transport systems described here are not a peculiarity of the glucose transport-constitutive mutant, an experiment identical to that described in the legend to Fig. 3 was carried out with the parental strain, *P. fluorescens* ATCC 13525, grown on 50 mM glucose-basal salts to induce the glucose transport system. Essentially identical results were obtained (data not shown).

Silhavy et al. (16) have emphasized the importance of considering the exit reaction, particularly in systems that may be mediated by binding proteins. There is evidence that different carriers, which are energized differently, function in entry and exit reactions; moreover, exit may be profoundly stimulated by a high-energy state (4, 22), so that measurements of net uptake

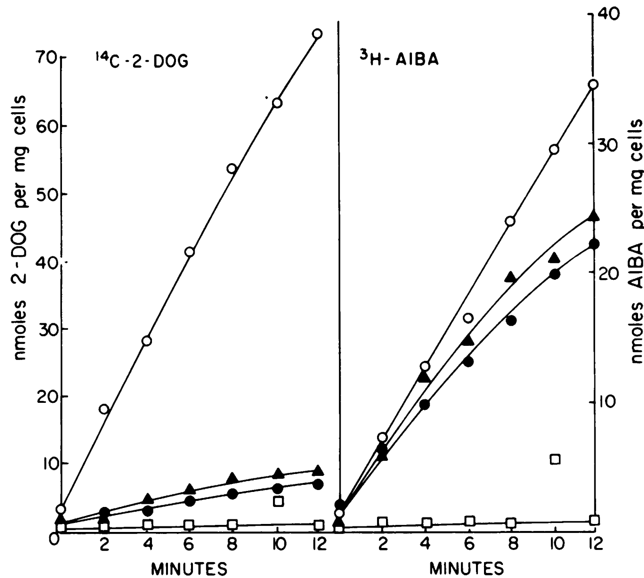

FIG. 5. *Uptake of 2-DOG and AIBA by DCCD-treated P. fluorescens GC-1. Cells grown on 50 mM succinate-PBS were preincubated in the absence (○) or presence (▲, ●, □) of 1 mM DCCD for 1 h, harvested, washed, and incubated in PBS with no further treatment (○, ▲) or in the presence of 1 mM DCCD (●) or 50 μM CCCP (□) for 15 min before the addition of 2-[U-^{14}C]DOG (0.5 mM, 0.2 μCi/μmol) and [methyl-^{3}H]AIBA (1 mM, 0.4 μCi/μmol).*

and accumulation may be affected in consequence. Therefore, it became important to study the exit of 2-DOG under the energetic conditions prevailing in the uptake experiments described above. Figure 6 shows that the exit rate from control cells (which represent the highest energy state of cells used in the previous experiments, that is, cells washed and suspended in PBS or GGBS without an external energy source) was not great ($t_{1/2}$ = 26 min). Since exit is a first-order reaction and is therefore linearly dependent upon internal concentration, its contribution to the measured net uptake rate would not be manifest until the internal concentration was high. Thus, the measurements of uptake rate at early times in the experiments described above would not be severely affected by this exit rate. The presence of CCCP or arsenate increased the exit rate somewhat ($t_{1/2}$ = 10 min). It is of interest, however, that the addition of a rapidly utilized external energy source (malate) greatly stimulated the exit rate ($t_{1/2}$ = 2.5 min) and that this stimulation was prevented by CCCP, but not by arsenate. The uncoupler 2,4-dinitrophenol (5 mM) also inhibited the accelerated exit caused by malate (data not shown). Thus, this system appears to be similar to the maltose transport system of *E. coli*, in which exit is promoted by a proton motive force (4). This acceleration of exit of 2-DOG, and therefore probably of glucose, from *P. fluorescens* by a high proton motive force derived from the oxidation of malate may have important consequences for the regulation of glucose uptake and accumulation by alternate, preferred energy sources and may be the basis for the observed inhibition of the uptake of glucose analogs by organic acids in *P. aeruginosa* (10). This aspect deserves more study.

The most logical interpretation of all of these data is that the transport of L-alanine and AIBA is energized by a proton motive force, whereas the active transport of 2-DOG and, by analogy, glucose requires phosphate bond energy, probably ATP, that would be derived from the proton motive force via oxidative phosphorylation. These experiments do not rule out the possibility that a proton motive force is required in addition to phosphate bond energy to transport glucose or its analogs, as has been suggested by Plate (13) for binding protein-associated systems in *E. coli*. Also, these experiments do not positively identify ATP as the crucial high-energy phosphate compound; acetyl phosphate may play a crucial role, as has been shown for *E. coli* by Hong et al. (6). Acetyl phosphate pools would be expected to decay during arsenate treatment and to require the presence of phosphate for regeneration during the metabolism of stored intracellular metabolites, such as polybetahydroxybutyric acid. However, acetyl phosphate formation should take place independently of a

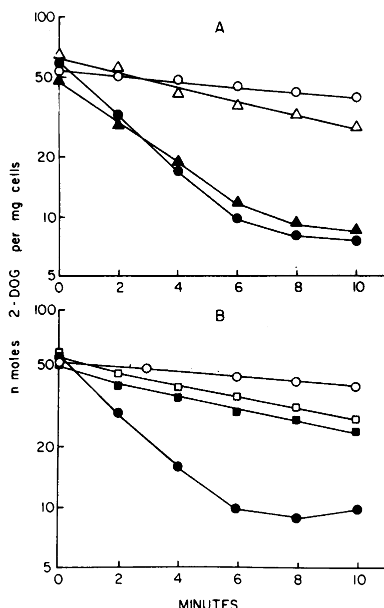

FIG. 6. *Exit of 2-DOG from P. fluorescens GC-1. Cells preloaded with 2-[U-^{14}C]DOG as described in the text were suspended in GGBS (A) or PBS (B) with no further addition (○) or with 10 mM sodium arsenate (△), 50 μM CCCP (□), 50 mM sodium malate (●), 50 mM sodium malate plus 10 mM sodium arsenate (▲), or 50 mM sodium malate plus 50 μM CCCP (■).*

proton motive force or the action of ATPase; thus, it would be expected that acetyl phosphate might be generated under the conditions of the experiment shown in Fig. 5 where DCCD-treated cells were suspended in a phosphate-buffered medium; such cells did not regain the ability to transport 2-DOG. Nevertheless, clarification of a possible role of acetyl phosphate must await experiments with mutants impaired in their capacity to form this compound, similar to those carried out by Hong et al. (6) with *E. coli.*

The generalization that the glucose transport system of pseudomonads is energized by phosphate bond energy fits well with findings of Stinson et al. (19) that this sugar is transported by *P. aeruginosa* via a shock-sensitive binding protein, and with the observation that membrane vesicles derived from *P. aeruginosa* that retain the capacity to transport gluconate do not transport glucose (5, 18). Thus, the pseudomonads appear to conform to the generalization first made by Berger and Heppel (2) and Wilson (21) for *E. coli* that shock-sensitive, binding protein-associated transport systems that are not retained by membrane vesicles are dependent upon phosphate bond energy.

It is of interest that *Pseudomonas* species appear to have evolved different modes of energization of inducible transport systems for different sugars: glucose uptake is energized by phosphate bond energy, fructose is transported via a phosphoenolpyruvate-dependent phosphotransferase system (1, 14, 15, 20), and gluconate uptake is apparently powered by a proton motive force. The physiological advantage of such diversity is not immediately apparent. The advantages may be related to the regulation of these different systems. This aspect is being studied further.

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