JOURNAL OF BACTERIOLOGY, Apr. 1976, p. 282-293
Vol. 126, No. 1

Printed in U.S.A.

Glucose Transport in Isolated Prosthecae of *Asticcacaulis biprosthecum*

ROBERT J. LARSON[1] AND JACK L. PATE*

Department of Bacteriology, University of Wisconsin, Madison, Wisconsin 53706

Received for publication 17 December 1975

Active transport of glucose in prosthecae isolated from cells of *Asticcacaulis biprosthecum* was stimulated by the non-physiological electron donor *N,N,N',N'*-tetramethyl-*p*-phenylenediamine dihydrochloride. Glucose uptake was mediated by two transport systems; the apparent K_m of the high-affinity system was 1.8 μM and that of the low-affinity system was 34 μM. Free glucose accumulated within prosthecae at a concentration 60 to 200 times above that present externally, depending on the K_m of the system being observed. The glucose transport system in prosthecae was stereospecific for D-glucose, and neither methyl α-D-glucopyranoside nor 2-deoxyglucose was transported. Uptake of glucose was inhibited by *N*-ethylmaleimide (NEM) and *p*-chloromercuribenzoate (PCMB), and the inhibition by PCMB but not by NEM was reversed by dithiothreitol. Glucose uptake was also inhibited by the uncoupling agents 5-chloro-3-*t*-butyl-2'-nitrosalicylanilide (S-13), 5-chloro-3-(*p*-chlorophenyl)-4'-chlorosalicylanilide (S-6), and carbonyl-cyanide *m*-chlorophenylhydrazone (CCCP) and by the respiratory inhibitor KCN. Efflux of glucose from preloaded prosthecae was induced by PCMB and KCN, but not by S-13 or CCCP. Glucose uptake was not affected by arsenate or an inhibitor of membrane-bound adenosine triphosphatases, *N,N'*-dicyclohexylcarbodiimide. The lack of inhibition by these two compounds, combined with the extremely low levels of adenosine 5'-triphosphate present in prosthecae, indicates that adenosine 5'-triphosphate is not involved in the transport of glucose by prosthecae.

Asticcacaulis biprosthecum is a gram-negative, aerobic, heterotrophic bacterium that undergoes a morphogenetic life cycle characteristic of other bacteria in the caulobacter group (19, 20). Bilateral appendages called prosthecae are produced at one stage of the life cycle. Members of the *Caulobacter* group are often found in aqueous environments with very low concentrations of organic material and, in general, are unable to grow in rich media. The reasons for the inability of *A. biprosthecum* to grow with high concentrations of nutrients have recently been clarified (Larson and Pate, Arch. Microbiol., in press). It has been suggested by Pate and co-workers (18, 19) that the function of prosthecae of *A. biprosthecum* and other caulobacters is to provide increased surface area for the uptake of nutrients, thereby giving these organisms a selective advantage for survival in dilute nutrient environments. In support of this idea, Porter and Pate (21) showed that prosthecae of *A. biprosthecum* possess respiration-linked transport systems for at least four amino acids and one sugar. It was hoped that further studies of sugar transport in prosthecae, specifically glucose transport, would provide additional information regarding the contention that prosthecae represent specialized membrane systems for the transport of nutrients.

The objective of the present study was to characterize the glucose transport system in prosthecae with respect to kinetic parameters and energy requirements. The data indicate that glucose transport in prosthecae is mediated by a respiration-linked active transport system and that other forms of energy, including adenosine 5'-triphosphate (ATP), are not involved.

MATERIALS AND METHODS

Source of reagents. Sodium α-ketoglutarate, sodium phosphoenolpyruvate, reduced nicotinamide adenine dinucleotide (NADH), NADPH, flavine adenine dinucleotide, flavine mononucleotide, methyl α-D-glucopyranoside, carbonyl-cyanide *m*-chlorophenylhydrazone (CCCP), *p*-chloromercuribenzoic acid (PCMB) 2,4-dinitrophenol (DNP), *N*-ethylmaleimide (NEM), dithiothreitol (DTT), tris(hydroxymethyl)aminomethane (Tris), dimethyl sulfoxide,

[1] Present address: Section of Biochemistry, Molecular and Cell Biology, Cornell University, Ithaca, N.Y. 14850.

morpholinepropane sulfonic acid, sodium isocitrate, sodium L-malate, 2-deoxy-D-glucose, D-xylose, D-galactose, β-D-fructose, α-D-glucose, L-glucose, sodium glyoxylate, sodium succinate, sodium pyruvate, sodium fumarate, ATP (sodium salt), sodium D-(+)-malate, and lithium L-(+)-lactate were all obtained from the Sigma Chemical Co., St. Louis, Mo. Glucose-6-phosphate, lithium D-(−)-lactate, oxaloacetate, α-glycerol phosphate, NAD, NADP, and ascorbate were the products of Nutritional Biochemicals Corp., Cleveland, Ohio. D-[U-^{14}C]glucose (281, 287, 297, and 284 mCi/mmol) was obtained from the Amersham/Searle Corp., Arlington Heights, Ill. [^{14}C]ethylene glycol (4.4 mCi/mmol), methyl α-D-[^{14}C]glucopyranoside (177 mCi/mmol), 2-deoxy-D-[^{3}H]glucose (10 Ci/mmol), and L-[^{14}C]glucose (51.6 mCi/mmol) were obtained from New England Nuclear Corp., Boston, Mass. *N,N,N',N'*-tetramethyl-*p*-phenylenediamine dihydrochloride (TMPD) was obtained from the Eastman Kodak Co., Rochester, N.Y. Luciferase was supplied by the DuPont Co., Wilmington, Del., and pump oil no. 702 was supplied by the Dow Corning Corp., Midland, Mich. Versilube F-50 and SF96 (50) were obtained from General Electric, Waterford, N.Y. 5-Chloro-3-*t*-butyl-2'-nitrosalicylanilide (S-13) was a gift from Robert J. Fisher (Boston Biomedical Research Institute, Boston, Mass.); [^{3}H]inulin (591 mCi/mmol), 5-chloro-3-(*p*-chlorophenyl)-4'-chlorosalicylanilide (S-6), and *N,N'*-dicyclohexylcarbodiimide (DCCD) were kindly provided by David L. Nelson (Department of Biochemistry, University of Wisconsin, Madison, Wis.). All other chemicals used were of reagent grade and were obtained from commercial sources.

Organism, growth conditions, and preparation of cultures. *A. biprosthecum* ATCC 27554 was grown at 30 C either in the dilute peptone-yeast extract (PYE) medium of Poindexter (20) or in the mineral salts-biotin defined medium described by Larson and Pate (Arch. Microbiol., in press). Unless otherwise indicated, uptake experiments were performed on prosthecae harvested from PYE-grown cells. The pH of the medium was adjusted to 7.2. Cells were maintained on refrigerated 0.5% agar deeps on the appropriate media. Liquid cultures were prepared from deeps by inoculating 1 ml of the soft-agar mixture into 100 ml of medium in a 500-ml Erlenmeyer flask. The culture was allowed to remain standing for 24 h, and 2 ml of this culture was then inoculated into another 100 ml of medium. These cells were incubated in static culture for 24 to 36 h and were then transferred to a 4-liter Erlenmeyer flask containing 2 liters of medium. The flask was fitted with a pair of spargers and incubated with aeration for about 24 h. This culture was in turn transferred to a 20-liter carboy containing 15 liters of medium. The carboy was fitted with a pair of spargers and incubated with aeration for 24 h or until the culture reached the late logarithmic phase of growth. Cultures were harvested in a refrigerated Sharples supercentrifuge and suspended in ice-cold 5 mM $MgSO_4 \cdot 7H_2O$. All subsequent centrifugations were done in a Sorvall RC2-B refrigerated centrifuge.

Preparation of isolated prosthecae. Prosthecae were removed from cells and purified by differential centrifugation as described previously by Jordan et al. (9) but with the following modifications. Cells harvested from the carboys were resuspended in 5 mM $MgSO_4 \cdot 7H_2O$ and washed once with 1,200 ml of $MgSO_4 \cdot 7H_2O$. The washed cells were resuspended in 5 mM $MgSO_4 \cdot 7H_2O$ to a final volume of 1,200 ml, and 200-ml aliquots were blended in a Waring blender for 90 s. After blending, the suspension was centrifuged at 4,080 × *g* for 40 min. The pellet was discarded, and the supernatant solution was then centrifuged at 16,300 × *g* for 1 h. The resulting supernatant solution was discarded. The pellet consisted of two layers. The upper layer (prosthecae) was washed from the lower layer (residual cells) with 5 mM $MgSO_4 \cdot 7H_2O$. A few cells were usually suspended with the prosthecae and were removed from the suspension by further cycles of low- and high-speed centrifugation. Prosthecae obtained in this manner were free from whole cells, as observed by phase-contrast microscopy. Isolated prosthecae were suspended in 5 mM $MgSO_4 \cdot 7H_2O$ at a concentration of about 5 mg/ml and frozen in 0.3-ml portions in an acetone-dry ice bath. They were stored in a Kelvinator Ultracold freezer at −72 C. Prosthecae treated in this manner could be stored for at least 6 months with no loss in transport capacity.

Glucose transport assay. The assay for glucose uptake was similar to a method described by Kaback and Milner (13). The basic assay mixture (50-μl final volume) contained 50 mM Tris buffer (pH 7.5), 1 mM $CaCl_2$, 20 mM ascorbate, 2 mM TMPD, an appropriate concentration of ^{14}C- or ^{3}H-labeled substrate, and about 125 μg of prosthecal protein suspended in 5 mM $MgSO_4 \cdot 7H_2O$. Inhibitors and other compounds were included in this mixture depending on the conditions of the experiment. The reaction was started by the addition of [^{14}C]glucose or prosthecae, and the mixture was vigorously aerated with water-saturated oxygen. Samples were incubated at 25 C. The reaction was terminated by diluting the mixture with 5.0 ml of 0.1 M LiCl and rapidly filtering the diluted mixture through a Millipore HAWP (0.45-μm) membrane filter. The reaction tube was immediately washed with an additional 5.0 ml of 0.1 M LiCl and filtered as before, and the filter was then washed with an additional 5.0 ml of LiCl. Dilutions and filtrations were all done within 30 s. Filters were dried thoroughly, placed in 10 ml of liquid scintillation fluid containing (per liter of toluene) 0.1 g of 1,4-bis-2-(5-phenyloxazolyl)-benzene and 4 g of 2,5-diphenyloxazole in glass scintillation vials, and counted at 80 to 90% efficiency in a Packard Tri-Carb liquid scintillation spectrometer, model 3375.

Respiration assay. Reaction mixtures (3.0-ml total volume) were identical to those described for glucose uptake except that [^{14}C]glucose was omitted and the final concentration of prosthecal protein was reduced to allow measurement of oxygen consumption over a period of 5 min. The reaction mixture, containing 500 to 600 μg of prosthecal protein suspended in 5 mM $MgSO_4 \cdot 7H_2O$, was air saturated and

allowed to equilibrate for 2 min at 25 C before the reaction was started by the addition of 0.1 ml of ascorbate-reduced TMPD. Dissolved-oxygen consumption was measured with a Yellow Springs Instrument (Yellow Springs, Ohio) model 53 oxygen meter. Oxygen consumption was linear for 1.5 to 3 min, depending on experimental conditions.

Oxidative phosphorylation. Reaction mixtures (100-μl total volume) contained 1 mM $CaCl_2$, 50 mM Tris (pH 7.5), 2 mM TMPD, 20 mM ascorbate, and from 800 to 900 μg of prosthecal protein. No ^{14}C-labeled substrate was included. The mixture was aerated vigorously with water-saturated oxygen for 1 min and extracted, and the amount of ATP present was determined as described previously (10). Values were converted from milligrams of protein to grams of dry weight based on a prosthecal protein content of 45.2% (9).

Measurement of internal water space. The space inside prosthecae accessible to [^{14}C]ethylene glycol was measured by a modification of the method described by Miovic and Gibson (17). Prosthecae (2-ml volumes) in 5 mM $MgSO_4 \cdot 7H_2O$ were added to duplicate tubes containing either 0.2 ml of 20 mM [^{14}C]ethylene glycol (5 μCi/ml) or 0.2 ml of 8.5 μM [^{3}H]inulin (5 μCi/ml). After 2 and 10 min, triplicate 0.1-ml samples were removed from each tube and layered onto gradients in 0.4-ml centrifuge tubes (Arthur H. Thomas Co., Philadelphia, Pa.). Gradients were prepared with a 1.0-ml syringe and 27-gauge needle and consisted of, from bottom to top, 2 drops of Dow Corning pump oil no. 702, 0.1 ml of 8% sucrose containing 0.5% Triton X-100, and 0.1 ml of silicone oil prepared by mixing 1 g of Versilube F-50 and 1 g of SF96. The gradient tubes were centrifuged at 3/4 maximum speed for 50 min in a Coleman microfuge and immediately frozen in an acetone-dry ice bath. Tubes were cut between the sucrose and silicone layers, placed in 10 ml of Bray scintillation fluid (3), and counted as described above. The space accessible to [^{14}C]ethylene glycol corrected for the intersticial water space occupied by [^{3}H]inulin was calculated using known aliquots of [^{14}C]ethylene glycol and [^{3}H]inulin standards. Due to the low density of prosthecae compared with that of whole cells, relatively long centrifugation times were required and the density of the silicone oil layer had to be decreased. This resulted in significant amounts of suspending fluid going through the silicone oil and sucrose layers, and the [^{14}C]inulin correction factor was instrumental in accounting for this. Washing experiments indicated that the [^{14}C]inulin did not adhere to or enter the prosthecae. Results of control experiments using different concentrations of [^{14}C]ethylene glycol and different incubation times are consistent with a simple equilibration of this compound and not its concentration against a gradient.

Protein determinations. A suspension of prosthecae was mixed with an equal volume of 2 M NaOH and incubated at 80 C for 30 min to solubilize the proteins. The suspension was allowed to cool, and total protein was determined by the method of Lowry et al. (11).

RESULTS

Effect of experimental conditions on glucose uptake. The effect of pH and temperature on the initial rate of accumulation of D-[^{14}C]glucose is shown in Fig. 1 and 2. The optimum pH for glucose transport was 7.5, but significant transport activity was still observed at a pH as low as 6.5 and as high as 9.0. Glucose transport reached an optimum at 25 C and remained constant until temperatures in excess of 35 C were attained.

Time-course graphs indicating the uptake of D-[^{14}C]glucose at two different concentrations of glucose (corresponding to the K_m concentrations of the high- and low-affinity systems discussed below) are shown in Fig. 3. These results were obtained with prosthecae harvested from cells grown in PYE, although similar results were observed for prosthecae harvested from cells grown in defined minimal media with either glucose or xylose as the sole carbon and energy source. Ascorbate-reduced TMPD served as the energy source, and neither ascorbate nor TMPD alone could replace a combination of the two. Uptake was linear for the first 1

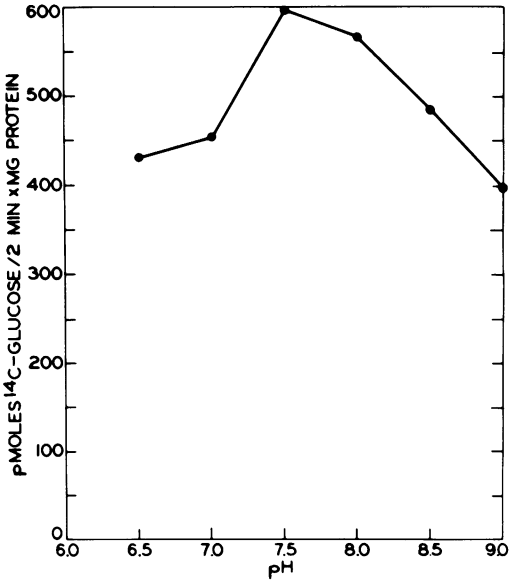

FIG. 1. *Effect of pH on the initial rate of* D-*[^{14}C]glucose uptake by prosthecae in the presence of ascorbate-reduced TMPD. The reaction mixture contained, in a 50-μl total volume: 20 mM ascorbate, 2 mM TMPD, 1 mM $CaCl_2$, 50 mM Tris, 34 μM [U-^{14}C]glucose, and about 125 μg of prosthecal protein suspended in 5 mM $MgSO_4$. The pH of the reaction mixture was varied as shown, and the reaction was allowed to proceed for 2 min at 25 C before it was terminated.*

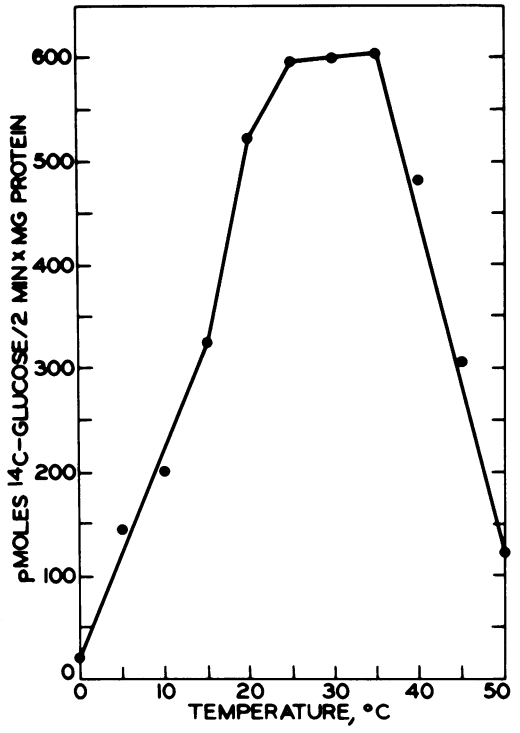

FIG. 2. *Effect of temperature on the initial rate of uptake of [U-^{14}C]glucose by prosthecae in the presence of ascorbate-reduced TMPD. Assay conditions were the same as indicated in the legend of Fig. 1, except that the pH was adjusted to 7.5 and the temperature was varied as indicated. The reaction was terminated after 2 min.*

or 2 min but did not reach a maximum until 10 min. The level of glucose accumulation remained constant for an additional 10 min after the maximum level had been reached provided vigorous aeration with oxygen was maintained. This level of accumulation, based on an internal prosthecal water space of 0.52 ± 0.05 μl/mg of protein, represented a 60- to 200-fold increase in the glucose concentration within prosthecae compared with the external concentration in the assay medium, depending on the K_m of the system being observed.

Both the rate and final extent of glucose uptake, as well as the steady-state level of accumulation, were very oxygen sensitive. Reduced rates of glucose uptake and a steady efflux of substrate after the maximum level of accumulation had been achieved were observed when air was used in place of oxygen. Reduced rates of glucose accumulation were also observed when phosphate buffer was used in place of Tris buffer.

Kinetics of glucose uptake. The effect of D-[^{14}C]glucose concentration on the initial rate of glucose accumulation is shown in Fig. 4. Saturation kinetics were obtained, and the Lineweaver-Burk plot indicates the presence of two systems, each with a different K_m, for the transport of glucose. The apparent K_m and V_{max} for the high-K_m, low-affinity system were 3.4 × 10^{-5} M and 625 pmol/min per mg of prosthecal protein, respectively, whereas the apparent K_m and V_{max} for the low-K_m, high-affinity system were 1.8 × 10^{-6} M and 179 pmol/min per mg of prosthecal protein, respectively. The kinetic constants in these experiments were obtained by using prosthecae harvested from cells grown in PYE. Prosthecae harvested from cells grown in defined minimal media with either glucose of xylose as the sole carbon and energy source also exhibited high- and low-affinity transport systems with apparent Michaelis constants identical to those found in prosthecae harvested from

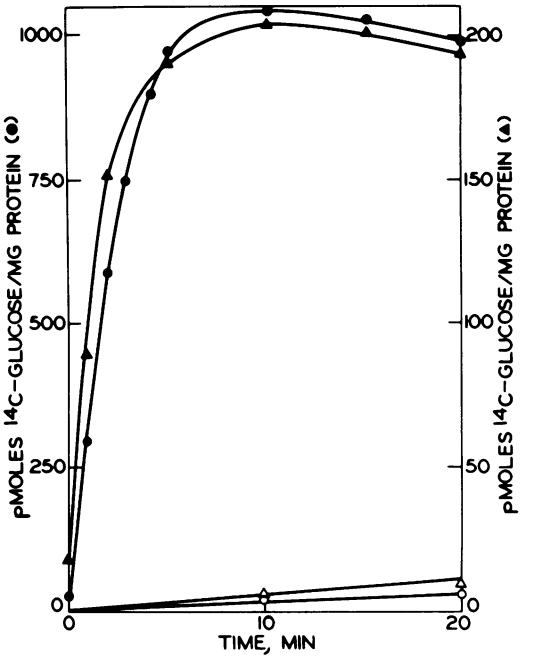

FIG. 3. *Uptake of D-[^{14}C]glucose by prosthecae in the presence of ascorbate-reduced TMPD. The reaction mixture contained, in a 50-μl total volume: 20 mM ascorbate, 2 mM TMPD, 1 mM $CaCl_2$, 50 mM Tris, and about 125 μg of prosthecal protein suspended in 5 mM $MgSO_4$. The pH of the reaction mixture was 7.5, and assays were performed at 25 C. The concentration of [^{14}C]glucose was 1.8 μM (▲) or 34 μM (●). A second set of reaction mixtures without ascorbate or TMPD was incubated simultaneously with the first set (△, ○). The reaction was allowed to proceed for the time indicated and was then terminated.*

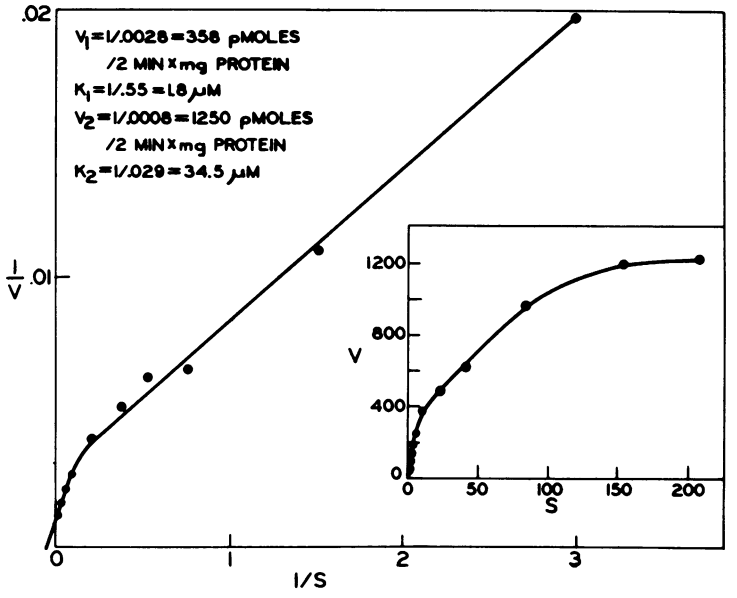

FIG. 4. *Effect of [^{14}C]glucose concentration on the initial rate of [^{14}C]glucose uptake by prosthecae. Assay conditions were the same as indicated in the legend for Fig. 3, except that the concentration of [^{14}C]glucose was varied from 0.33 to 212 μM and the reaction was terminated after 2 min. Reciprocals presented on the Lineweaver-Burk plot (large plot) were taken from the data shown in inset. V, Velocity given as picomoles per 2 min × mg of prosthecal protein. S, Micromolar concentration of glucose.*

PYE-grown cells. However, prosthecae harvested from glucose- or xylose-grown cells exhibited somewhat reduced maximum velocities compared to prosthecae from PYE-grown cells, even though the rates of respiration were the same in all cases (data not shown).

The initial rate of uptake of labeled glucose analogues and the effect of unlabeled analogues on the initial rate of accumulation of D-[^{14}C]glucose are shown in Table 1. Neither methyl α-D-[^{14}C]glucopyranoside, L-[^{14}C]glucose, nor 2-[^{3}H]deoxyglucose was transported to any significant extent. Unlabeled forms of L-glucose and methyl α-D-glucopyranoside at concentrations as high as 3,400 μM (100 K_m) did not appreciably decrease the initial rate of uptake of [^{14}C]glucose. High concentrations of unlabeled 2-deoxyglucose inhibited the rate of glucose uptake about 50%. Other monosaccharides used by *A. biprosthecum* as sole carbon and energy sources had various effects on transport of D-[^{14}C]glucose (Table 2). Good carbon sources such as D-xylose and D-galactose inhibited both systems involved in glucose transport, whereas D-fructose, a relatively poor carbon source compared with glucose, had little effect on glucose uptake.

Reversibility of glucose uptake. The effect of temperature on the exchange reaction between labeled D-glucose in preloaded prosthecae and unlabeled exogenous D-glucose after a steady-state level of accumulation had been reached is shown in Fig. 5. At 25 C, a rapid decrease in the internal concentration of D-[^{14}C]glucose was observed after the addition of 1 mM D-[^{12}C]glucose. A smaller decrease was observed when the assay mixtures were incubated at 0 C. The exchange reaction was very sensitive to temperature, and exchange assays performed at 8 C or higher exhibited curves similar to 25 C controls. The exchange between labeled and unlabeled D-glucose was also significantly affected by CCCP (Fig. 6). The amount of exchange in prosthecae incubated with CCCP was less than one-half of the amount observed in controls incubated without CCCP.

Energetics of glucose uptake. The effect of a number of potential energy sources on glucose transport in prosthecae is shown in Table 3. Slight stimulation of transport was observed when NADH and NADPH were used with flavine mononucleotide and flavine adenine dinucleotide, respectively. No other potential electron donors stimulated glucose transport significantly in either prosthecae harvested from PYE-grown cells or prosthecae harvested from cells grown in defined media with glucose as the sole carbon and energy source.

The effects of anoxia and various inhibitors and energy poisons on respiration and on the

TABLE 1. *Uptake of L-glucose and glucose analogues and the effect of these compounds on the initial rate of glucose accumulation in prosthecae*[a]

Analogue	Concn (μM)	% Inhibition of uptake of [^{14}C]glucose[b]	% Uptake of labeled analogue[c]
L-Glucose	34	<1	2
	340	<1	10
	3,400	5	
Methyl α-D-glucopyranoside	34	<1	2
	340	7	8
	3,400	6	
2-Deoxyglucose	34	<1	<1
	340	43	8
	3,400	55	

[a] The reaction mixture contained, in a 50-μl total volume, 20 mM ascorbate, 2 mM TMPD, 1 mM $CaCl_2$, 34 μM [^{14}C]glucose, the appropriate concentration of labeled or unlabeled test compound, and about 125 μg of prosthecal protein suspended in 5 mM $MgSO_4$. The reaction was allowed to proceed for 2 min and was then terminated.

[b] Percent inhibition of initial rate of uptake of D-[^{14}C]glucose by the ^{12}C-labeled analogue. The rate of accumulation in control samples without unlabeled analogues was 580 pmol/2 min × mg of prosthecal protein.

[c] Percentage of initial rate of uptake of labeled analogue compared with rate of uptake of D-[^{14}C]glucose, which was 595 pmol/2 min × mg of prosthecal protein.

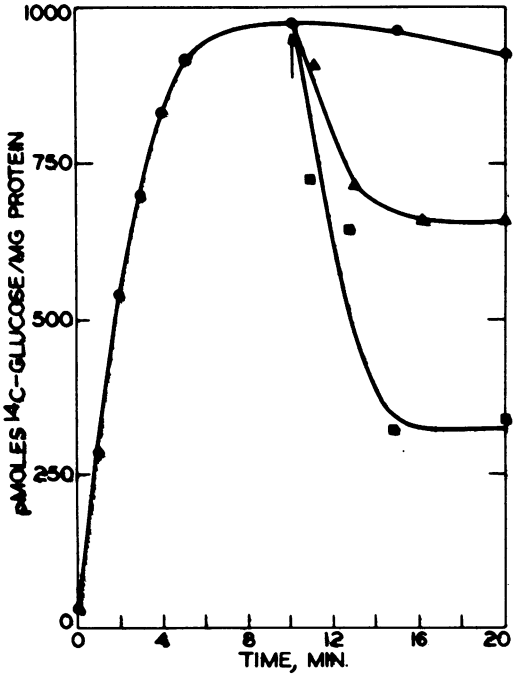

FIG. 5. *Effect of temperature on efflux of [^{14}C]glucose from preloaded prosthecae in the presence of exogenous [^{12}C]glucose. Assay conditions were identical to those listed in the legend for Fig. 3, except that the reaction was allowed to proceed for 10 min before 1 mM [^{12}C]glucose was added (indicated by arrow). The reaction mixtures were then shifted to 0 C (▲) or allowed to remain incubating at 25 C (■). Control samples (●) received no additions and were incubated at 25 C. The concentration of [^{14}C]glucose used to preload prosthecae was 34 μM.*

TABLE 2. *Effect of ^{12}C-labeled monosaccharides on the initial rate of [^{14}C]glucose uptake in prosthecae*[a]

^{12}C-labeled monosaccharide	Concn (μM)	% Uptake of [^{14}C]glucose[b]	
		34[c]	1.8[c]
D-Glucose	34	70	
	340	25	
	3,400	3	<1
D-Xylose	34	66	
	340	41	
	3,400	27	28
D-Galactose	34	55	
	340	45	
	3,400	24	14
D-Fructose	34	105	—[d]
	340	93	
	3,400	92	

[a] Assay conditions were identical to those listed in footnote *a* of Table 1, except that ^{12}C-labeled monosaccharides were included in the reaction mixture as indicated and the concentration of [^{14}C]glucose was either 34 or 1.8 μM.

[b] Percent uptake of [^{14}C]glucose in the presence of ^{12}C-labeled monosaccharide compared with the rate of uptake in control samples without ^{12}C-labeled monosaccharide. The initial rates of [^{14}C]glucose uptake in control samples at 34 μM [^{14}C]glucose and 1.8 μM [^{14}C]glucose were 639 and 197 pmol/2 min × mg of prosthecal protein, respectively.

[c] [^{14}C]glucose concentration (micromolar).

[d] Not done.

initial rate of glucose uptake in prosthecae are shown in Table 4. Little uptake of glucose was observed in prosthecae incubated without oxygen or without an energy source. The endogenous rate of respiration of prosthecae in the absence of ascorbate-reduced TMPD was less than 1% of controls incubated with this energy source. Uncoupling agents including S-13, S-6, and CCCP inhibited glucose transport significantly while substantially increasing respiration. Agents known to affect reactions involving sulfhydryl groups (PCMB and NEM) inhibited glucose transport but had no effect on respiration. The inhibition by PCMB was reversed about 50% by adding DTT to the reaction mixture (data not shown). Inhibition by NEM was not reversible by DTT, and both transport

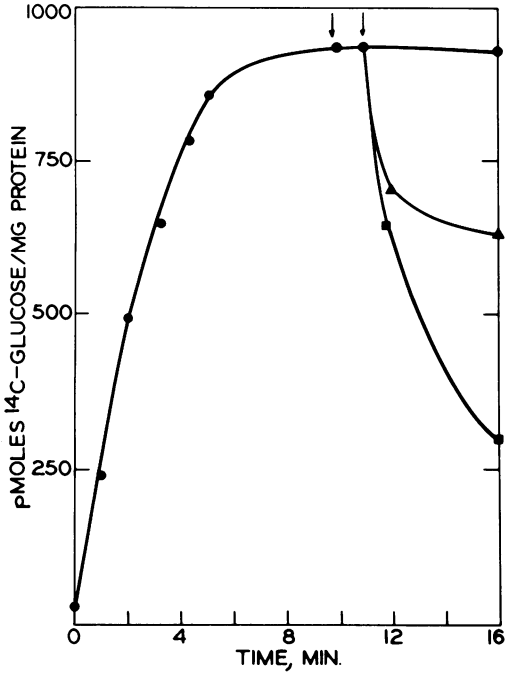

FIG. 6. *Effect of CCCP on efflux of [^{14}C]glucose from preloaded prosthecae in the presence of exogenous [^{12}C]glucose. Assay conditions were identical to those listed in the legend for Fig. 3, except that the reaction was allowed to proceed for 10 min before 100 μM CCCP (▲) was added (indicated by first arrow). A second set of samples (■) received an equal volume of double-distilled water. The reaction mixtures were then incubated for 1 min before 1 mM [^{12}C]glucose was added (indicated by second arrow). The concentration of [^{14}C]glucose used to preload prosthecae was 34 μM.*

systems were affected to the same extent. An effective inhibitor of membrane-bound adenosine triphosphatases (ATPases) (DCCD), had no effect on respiration or transport in prosthecae. Arsenate, which acts by competitively inhibiting ATP formation, had no effect on respiration or transport at concentrations as high as 10 mM. Transport of glucose was inhibited at 50 mM arsenate but respiratory activity increased as well, indicating that uncoupling effects might be responsible for the observed inhibition. Cyanide, which blocks respiration at the level of cytochrome *a* in most bacteria, inhibited both respiration and transport in prosthecae. The apparent ability of prosthecae to accumulate glucose in the absence of respiration (at 5, 10, and 20 mM KCN) was probably

TABLE 3. *Effect of various energy sources on the initial rate of uptake of [^{14}C]glucose in prosthecae*[a]

Energy source	Glucose uptake[b]				
	No coenzyme	FAD	FMN	NAD	NADP
Succinate	11	13	11	13	12
L-Malate	10	9	10	10	14
Fumarate	9	9	8	9	8
Isocitrate	5	6	6	6	7
D-Malate	6	5	7	6	7
Oxaloacetate	8	9	7	8	10
α-Ketoglutarate	9	7	9	11	11
Glyoxylate	12	15	15	14	13
Phosphoenolpyruvate	9	6	8	8	9
D-Lactate	13	11	12	12	21
Acetate	12	13	15	13	13
ATP	6	8	7	7	6
Citrate	11	9	12	9	15
Glucose-6-phosphate	4	6	4	4	8
L-Lactate	5	5	15	6	5
α-Glycerolphosphate	5	5	6	8	7
Pyruvate	11	12	12	12	14
NADH	8	8	21	—[c]	—
NADPH	8	25	8	—	—
Ascorbate/TMPD	585	602	591	610	596

[a] The reaction mixtures contained, in a 50-μl total volume; 50 mM Tris, 1 mM $CaCl_2$, 50 μM coenzyme (replaced by water where appropriate), 34 μM [^{14}C]glucose, and about 125 μg of prosthecal protein suspended in 5 mM $MgSO_4$. The concentration of all energy sources was 20 mM, except for NADH and NADPH, which were 2 mM, and ascorbate and TMPD, which were 20 and 2 mM, respectively. The pH of the reaction mixtures was 7.5, and assays were performed at 25 C.

[b] Initial rate of [^{14}C]glucose uptake measured in terms of picomoles per 2 min × mg of prosthecal protein. FAD, Flavin adenine dinucleotide; FMN, flavin mononucleotide.

[c] Not done.

TABLE 4. *Effect of inhibitors and uncoupling agents on the initial rate of uptake of [^{14}C]glucose in prosthecae*[a]

Compound	Concn (μM)	nmol of inhibitor/ mg of protein[b]	% Uptake[c]	Concn (μM)	nmol of inhibitor/ mg of protein[b]	% Respiration[d]
$-O_2$, + N_2	–[e]	–	5	–	–	–
–Ascorbate –TMPD	–	–	1	–	–	<1
S-13	1	0.4	43	0.067	0.43	
	10	4	4	0.67	4.3	425
S-6	1	0.4	95	0.067	0.43	
	10	4	8	0.67	4.3	331
CCCP	1	0.4	76	0.067	0.43	
	5	2	48	0.34	2.15	
	10	4	23	0.67	4.3	
	100	40	6	6.7	43	220
DNP	100	40	99	6.7	43	
	1,000	400	56	67	430	160
	2,500	1,000	43	170	1,075	
PCMB	100	40	20	6.7	43	
	500	200	9	34	215	102
	5,000	2,000	5	340	2,150	
NEM	1,000	400	77	67	430	100
	10,000	4,000	53	670	4,300	
KCN	5,000	2,000	40	340	2,150	<1
	10,000	4,000	21	670	4,300	<1
	20,000	8,000	10	1,340	8,600	<1
	40,000	16,000	2	2,680	17,200	<1
As	10,000	4,000	101	670	4,300	99
	50,000	20,000	64	3,400	21,500	137
DCCD	100	40	98	6.7	43	105

[a] Assay conditions were identical to those listed in footnote *a* of Table 1, except that the appropriate concentration of inhibition or uncoupler was included where indicated.

[b] Prosthecal protein.

[c] Percent rate of uptake of [^{14}C]glucose in the presence of inhibitor compared with the rate of controls without inhibitor, which varied from 580 pmol/2 min × mg of prosthecal protein to 616 pmol per 2 min × mg of prosthecal protein. Three or more samples were taken at each concentration of inhibitor.

[d] Percent rate of respiration in the presence of inhibitor compared with the rate of control samples without inhibitor, which was 6.5 μg of O_2/min × mg of prosthecal protein. All rates of respiration were corrected for autooxidation of TMPD by subtacting the rate of oxygen consumption in reaction mixtures containing no prosthecae. Values for the rate of respiration were obtained at the concentration of inhibitor indicated. Three or more samples were taken for each value listed, and the results shown are similar to those obtained in other experiments.

[e] Not done.

due to differences in the method of aeration in transport versus respiratory reaction mixtures since no transport was observed in a nitrogen atmosphere.

The effects of various inhibitors and uncoupling agents on efflux of [^{14}C]glucose from preloaded prosthecae are shown in Fig. 7. The sulfhydryl reagent PCMB caused a rapid efflux of glucose from prosthecae, whereas CCCP, an uncoupling agent, had no effect on the steady-state level of glucose accumulation. Another uncoupling agent, S-13, also had a neglible effect on efflux of glucose from prosthecae. Cyanide, at a concentration of 40 mM, however, caused a rapid efflux of glucose from prosthecae after a short lag period.

When respiration in prosthecae was stimulated by ascorbate-reduced TMPD, a 19-fold de-

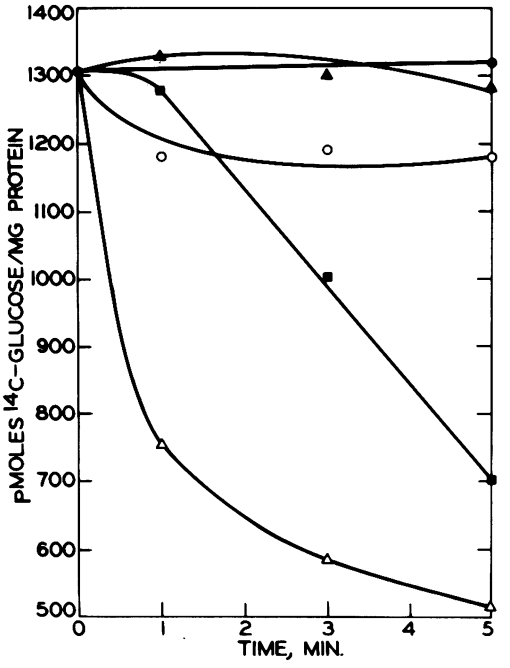

FIG. 7. *Effects of inhibitors and uncoupling agents on efflux of [14C]glucose from prosthecae in the absence of exogenous [12C]glucose. Assay conditions were identical to those listed in the legend for Fig. 3, except that the reaction was allowed to proceed for 10 min (zero time) before the addition of 100 μM CCCP (▲), 500 μM PCMB (△), 10 μM S-13 (○), or 40 mM KCN (■). Control samples (●) received no additions. The concentration of [14C]glucose used to preload prosthecae was 34 μM.*

crease in the endogenous levels of ATP in prosthecae was observed (Table 5). This decrease was partially sensitive to arsenate but totally unaffected by DCCD.

DISCUSSION

Purified prosthecae isolated from cells of *A. biprosthecum* possess a respiration-linked transport system for D-glucose. The system is temperature, pH, and energy dependent and accumulates glucose within prosthecae at a level many times above that present in the external medium. The accumulated [14C]glucose can rapidly exchange with exogenously added unlabeled glucose, and Porter and Pate (21) showed, using paper chromatography, that over 90% of the [14C]glucose taken up can be recovered as the free sugar. These results indicate that glucose uptake in prosthecae is catalyzed by a carrier-mediated, active transport system.

Kinetic data indicate that two systems are involved in the transport of glucose in prosthecae, although the possibility of negative cooperativity cannot be ruled out. The apparent K_m of the low-affinity system (34 μM) is similar to values obtained with membrane vesicles prepared from other aerobic bacteria (1). The apparent K_m of the high-affinity system (1.8 μM), however, is lower than previously reported values in membrane vesicles (12, 16) and is probably a reflection of the adaptation of *A. biprosthecum* for survival in very dilute environments. Glucose transport appeared to be constitutive in prosthecae harvested from cells grown in either PYE or in defined media with glucose or xylose as the sole carbon source. High- and low-affinity transport systems were present in prosthecae from cells grown under all three conditions, and the apparent affinity constants of both systems were the same irrespective of growth substrate.

The glucose transport systems in prosthecae were stereospecific for D-glucose, and analogues of glucose (methyl α-D-glucoside, 2-deoxyglucose) were not transported. These results are similar to those obtained by Barnes (1), who found that membrane vesicles prepared from cells of *Azotobacter vinelandii* grown with D-glucose did not accumulate significant amounts of either methyl α-D-glucopyranoside or 3-*O*-methyl D-glucoside. Guymon and Eagon (4) also showed that the glucose transport system of *Pseudomonas aeruginosa* has a 1,000-fold lower affinity for methyl α-D-glucoside than for glucose and does not accumulate significant amounts of this analogue. Rest and Robertson (23) reported that alpha- or beta-methyl gluco-

TABLE 5. *Utilization of ATP in prosthecae in the presence of ascorbate-reduced TMPD*[a]

Addition	ATP (μmol/g of dry weight)[b]
None	8.4×10^{-3}
Ascorbate-TMPD[c]	4.4×10^{-4}
Ascorbate-TMPD + 10 mM As	3.4×10^{-3}
Ascorbate-TMPD + 100 μM DCCD	4.0×10^{-4}

[a] The basic assay mixture contained in a 100-μl total volume: 50 mM Tris, 1 mM $CaCl_2$, 800 to 900 μg of prosthecal protein suspended in 5 mM $MgSO_4$, and the appropriate compounds listed.

[b] Results shown are averages of duplicate samples, each of which was assayed in triplicate. The luminescense biometer was standardized against an ATP standard solution of 10^8 fg/ml which was made up in the same reaction mixture and run concurrently with the samples. The results shown are from a single experiment but are typical of those from several experiments.

[c] Concentrations were 20 mM ascorbate and 2 mM TMPD. Assays were performed at room temperature.

sides were not transported by the glucose transport system of *Brucella abortus*, although 2-deoxyglucose was accumulated by this organism. In prosthecae, 2-deoxyglucose did affect the initial rate of glucose uptake, apparently by competing with glucose at a binding site. The ability of 2-deoxyglucose to inhibit glucose transport in other bacteria has been reported elsewhere (4, 23). Glucose transport in prosthecae was also inhibited by D-xylose, and D-galactose, which are D-aldoses, but not by D-fructose, a D-ketose, presumably due to steric requirements of the binding site. Galactose has been found to inhibit glucose uptake in cells of *P. aeruginosa* (4) and *B. abortus* (23). Since glucose transport is usually mediated by a phosphotransferase system in bacteria (2), parallel studies comparing the effects of monosaccharides on active transport of glucose in membrane vesicles are lacking. However, Kewar et al. (16) showed that [^{14}C]galactose uptake in membrane vesicles prepared from *Escherichia coli* ML-3 was inhibited to the same extent by unlabeled forms of both glucose and galactose. The effects of D-xylose on glucose transport in cells or membrane vesicles have not been studied.

In prosthecae, both systems involved in glucose transport were inhibited by galactose and xylose. This is in contrast to results obtained elsewhere (7, 25) in which one transport system (usually the high-affinity system) is specific for the primary substrate whereas the other system (usually the low-affinity system) can accommodate other substrates. We have shown that *A. biprosthecum* is unable to grow with high concentrations of nutrients (Larson and Pate, Arch. Microbiol., in press), reflecting its extreme adaptation for survival in environments with low concentrations of organic material. The ability of both glucose transport systems in prosthecae to bind other substrates besides glucose may reflect this specialization for dilute habitats, allowing the transport of a number of substrates to be mediated by a few highly efficient transport systems.

Respiratory activity was found to be a necessary, but not sufficient, condition for glucose transport in prosthecae. Active transport was blocked by respiratory inhibitors (KCN and anoxia), but uncouplers of oxidative phosphorylation (S-13, S-6, and CCCP) effectively inhibited transport while substantially increasing respiration. The effects of S-13, an uncoupler that acts stoichiometrically at the level of cytochrome oxidase in mitochondria (28), were especially dramatic in prosthecae since transport was almost completely inhibited while the rate of respiration increased over 400%. The effects of S-13 and CCCP on efflux of glucose from preloaded prosthecae (in the absence of exogenous unlabeled glucose) should also be mentioned. Only a slight efflux of glucose from preloaded prosthecae was caused by S-13, and CCCP had no effect on the steady-state level of glucose accumulation. The lack of efflux in the presence of CCCP is in sharp contrast to results of other studies with membrane vesicles (1, 12, 14) in which rapid efflux of substrate was observed after the addition of CCCP. However, lack of solute efflux in the presence of an energy poison has been reported. Kaback (12) showed that oxamate (a competitive inhibitor of lactic dehydrogenase) did not cause efflux of lactose from preloaded membrane vesicles of *E. coli*, despite the fact that both D-lactate oxidation and lactose uptake were almost completely inhibited. Similar results were also observed in membrane vesicles prepared from cells of *Staphylococcus aureus* grown on glucose (15, 26). Efflux of serine from preloaded vesicles was not observed in the presence of oxalate, and efflux induced by DNP, respiratory inhibitors, or anoxia was inhibited by oxalate. These findings have been used to substantiate the theory that the redox state of the respiratory chain "carrier" at the site of energy coupling determines solute flux and do not wholly apply to the effect of CCCP on efflux of glucose from prosthecae since CCCP does not affect respiration. However, they do indicate that the lack of solute efflux in prosthecae in the presence of an energy poison is not an isolated phenomenon. One major difference between prosthecae and membrane vesicles of *S. aureus*, which concerns the exchange of internal and external solute, should be mentioned. Oxalate did not interfere with serine exchange in vesicles, whereas glucose exchange in prostheacae was inhibited by CCCP. The glucose exchange reaction in prosthecae was sensitive to temperature, indicating that exit of glucose was mediated by a carrier-dependent system and not by simple diffusion.

The rate of glucose uptake in prosthecae was inhibited by PCMB, and this inhibition was reversed about 50% by DTT, indicating that sensitive SH groups are involved in active transport of glucose. Since respiratory activity was not affected (Table 4), the sensitive SH group cannot be part of the respiratory chain and must be localized in a carrier protein or within some membrane protein involved in energizing the transport process. PCMB also caused efflux of glucose from preloaded prosthecae (in the absence of exogenous glucose), which suggests that the sensitive SH groups is

not in a carrier protein. These results are again in contrast to results obtained with membrane vesicles where PCMB did not cause efflux and actually inhibited efflux induced by CCCP and other uncoupling agents (12). In membrane vesicles prepared from *E. coli*, PCMB and NEM have been shown to inhibit electron transfer (i.e., D-lactate oxidation), although neither lactic dehydrogenase nor the cytochrome system was affected. This was interpreted to mean that the SH group affected by sulfhydryl reagents resides between the primary dehydrogenase and the cytochromes, i.e., in the respiratory chain "carrier" itself. This general idea is not applicable to prosthecae since respiratory activity was not affected by PCMB. However, inhibition of glucose transport was partially relieved in prosthecae by DTT, which is similar to results obtained with membrane vesicles and which indicates that the sensitive SH group is relatively accessible. It is possible that the sulfhydryl groups resides in an "energized intermediate" located between the cytochrome chain and the carrier involved in the energization of transport. However, the possibility that efflux of glucose is mediated by a different carrier than that involved in uptake cannot be discounted. Alternatively, the permeability characteristics of prosthecal membranes might be affected by PCMB, leading to an efflux of preloaded substrate and an apparent inhibition of uptake, even though the transport system itself is otherwise functional. Efflux of substrate in the presence of a sulfhydryl reagent has been noted elsewhere. Short and Kaback (Abstr. Annu. Meet. Am. Soc. Microbiol. 1973, P302, p. 191) found that SH reagents induce efflux of amino acids from membrane vesicles of *S. aureus* when α-glycerol phosphate is used to preload the vesicles. *p*-Chloromercuribenzenesulfonate also induces efflux of substrate in reconstituted membrane vesicles prepared from a D-lactate dehydrogenase mutant of *E. coli* 308-225 (22).

Although CCCP has been used in a variety of transport studies, the mode of action of this uncoupling agent is still unclear. It is generally thought to function as a lipid-soluble proton conductor that inhibits active transport by dissipating the pH gradient (2). In this connection, inhibition of transport by CCCP has been shown to be pH dependent, reflecting the pK of the uncoupler (8). Moreover, the effectiveness of CCCP and other agents as uncouplers correlates well with their ability to conduct protons in artificial membranes (6). However, Kaback et al. (14) showed that CCCP exerts its effects on D-lactate-driven transport in membrane vesicles simply as a sulfhydryl reagent (other uncouplers [DNP] have been shown to inhibit transport by affecting the temperature stability of the membrane [2]). In prosthecae, it is unlikely that CCCP affects glucose transport as a sulfhydryl reagent since it does not induce efflux, whereas another classical sulfhydryl reagent, PCMB, does cause efflux of glucose from preloaded prosthecae.

No physiological electron donors were found which would drive glucose transport in prosthecae. These results are in agreement with those obtained by Porter and Pate (21), who showed that the failure of certain potential electron donors to drive glucose and proline transport in prosthecae is related to the inability of these energy sources to enter prosthecae rather than to the absence of enzymes for their oxidation.

Romano et al. (24) found that glucose phosphotransferase systems are absent from strictly aerobic bacteria, although the presence of other phosphotransferase systems in strict aerobes has been reported (27). A number of lines of evidence indicate that ATP and other high-energy phosphate compounds are not required for glucose transport in prosthecae. Exogenously added ATP and phosphoenolpyruvate did not stimulate transport, although this could have been due to the inability of these compounds to enter prosthecae. Arsenate, which inhibits the formation of high-energy phosphate bonds, had no effect on transport at concentrations up to 10 mM, and DCCD, an effective inhibitor of membrane-bound ATPases did not inhibit glucose uptake. Prosthecae did not synthesize ATP in the presence of ascorbate-reduced TMPD, and the endogenous levels of ATP in prosthecae were extremely low, less than 1% of typical values in whole cells (5). These last results are different from those reported by Porter and Pate (21), who showed that prosthecae do synthesize ATP in the presence of ascorbate-reduced phenazine methosulfate. The observed differences might be due to the different energy sources used.

Previous studies have shown that cells of *A. biprosthecum* grown in defined media with relatively low concentrations of nutrients produced much longer prosthecae than do cells grown in complex PYE medium (Larson and Pate, Arch. Microbiol., in press). This behavior, along with the data presented in this study, supports the contention that prosthecae represent specialized organelles, which provide increased surface area for the transport of nutrients from dilute environments. Prosthecae of *A. biprosthecum* possess a stereospecific transport system with a high affinity for glucose, one

of the few good substrates for growth of this organism. Glucose accumulates within prosthecae at a concentration over 200 times above that present in the external medium. This concentrative ability of prosthecae, combined with the high affinity of the transport system for glucose, would provide definite advantage for survival in dilute environments. In addition, the high concentration of glucose attained in prosthecae would facilitate the diffusion of this solute from prosthecae into the cell proper. Porter and Pate (21) have already discussed some of the more important aspects of solute movement from prosthecae to cell and vice versa. It is apparent from these findings that prosthecae provide cells of *A. biprosthecum* with a very effective extended membrane system for the transport of nutrients into the cell, enabling this organism to successfully compete with other organisms in their dilute natural habitats.

ACKNOWLEDGMENTS

This research was supported by the College of Agricultural and Life Sciences, University of Wisconsin, Madison, and Public Health Service grants 1-R01-GM 19859 and 5-T01-GM 00686-10-MIC from the National Institute of General Medical Sciences.

Special thanks go to David Nelson, who provided much helpful information during the course of this work. The technical assistance of Joanne Glaser in the preparation of the manuscript is also gratefully acknowledged.

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