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Phosphate/Hexose 6-Phosphate Antiport in *Streptococcus lactis*

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After growth in appropriate media, resting cells of *Streptococcus lactis* 7962 showed a rapid exchange between external and internal pools of inorganic phosphate. This exchange was not found in other strains of *S. lactis* (ML_3, 133, or K_1) or in *Streptococcus faecalis*. Phosphate exchange in *S. lactis* 7962 did not require other anions or cations in the assay medium, nor was phosphate influx affected by the membrane potential and pH gradient formed during glycolysis. Thus, the exchange reaction was independent of known ionic drivers (H^+, Na^+, OH^-, etc.). Experiments testing inhibitions of phosphate entry suggested that alternative substrates for exchange included arsenate, as well as the 6-phosphates of glucose, 2-deoxyglucose, fructose, mannose, or glucosamine, and direct studies with 2-deoxyglucose 6-phosphate verified that resting cells could accumulate this sugar phosphate to levels expected for exchange with internal phosphate. Two other observations supported the idea of an exchange between phosphate and sugar phosphate. First, early addition of the heterologous substrate blocked entry of the test compound, whereas later addition caused efflux of preaccumulated material. Second, expression of phosphate exchange and 2-deoxyglucose 6-phosphate transport varied in parallel. Both activities were found at high levels after growth in medium supplemented with rhamnose or arabinose, at intermediate levels with addition of galactose, and at low levels after growth with glucose, fructose, or mannose. We conclude that these findings describe a novel anion antiporter that mediates the exchange of phosphate (arsenate) and sugar 6-phosphates.

Current descriptions of secondary, ion-linked active transport in bacteria give many examples of the coupling between a driving cation and driven solute (for reviews, see references 4, 15, and 20). If the solute is anionic, or if it carries no net charge, one usually finds that an accumulation is supported by a symport (cotransport) reaction. If, on the other hand, a driven solute is cationic, the coupled event is commonly one of antiport (countertransport, exchange). In keeping with the prominance of H^+ pumps in bacterial systems, H^+ (or its energetic equivalent, OH^-), is most often the driving ion for such reactions, but because primary pumps are also known for Na^+, K^+, and Ca^{2+} (reviewed by P. C. Maloney and T. H. Wilson, Bioscience, in press), one may anticipate alternative, although less extensive, cycles built on these cations as well.

As a general rule, then, one could argue that known secondary porters in bacteria represent a single class in which there is a link with a positively charged "driver." The work reported here describes experiments that suggest the presence of an entirely different type—that of anion antiport. We believe that *Streptococcus lactis* 7962 expresses an antiporter that mediates exchanges among phosphate (or arsenate) and certain hexose 6-phosphates. This leads to the more general conclusion that the circulation of anions, of special importance to eucaryotic cells (1) and their organelles (8), may have physiological relevance to bioenergetic cycles in bacteria.

MATERIALS AND METHODS

Bacterial strain and growth conditions. Most experiments used *S. lactis* 7962, although in a few cases we studied *S. lactis* ML_3, 133, or K_1 (from J. Thompson, National Institute of Dental Research, Bethesda, Md.) and *Streptococcus faecalis* 9790 (provided by F. M. Harold, National Jewish Hospital and Research Center, Denver, Colo.). For routine work, cells were grown to stationary phase in a broth medium with carbohydrate (usually galactose) added to 55 mM (12). Cells were harvested by centrifugation, then twice washed and finally resuspended in a buffer (pH 7) containing 100 mM choline (or potassium) sulfate, 20 mM morpholinepropanesulfonic acid (MOPS), and choline (potassium) hydroxide. Final cell density was about 33 mg (dry weight) per ml, corresponding to 50 μl of cell water per ml (12). Temperature for all experimental work was 23 to 27°C.

Chemicals. *N,N'*-dicyclohexylcarbodiimide was provided by Schwartz/Mann Co., ortho- and metavanadates by Fisher Chemicals, and the anion exchange resin (AG1X2) by Bio-Rad Laboratories. Nigericin was a gift of W. E. Scott (Hoffmann-La Roche, Inc.). Other nonradioactive materials were obtained from Sigma Chemical Co. 2-[1-^{14}C]Deoxy-D-glucose 6-phosphate (52 Ci/mol), $KH_2{}^{32}PO_4$ (200 Ci/mol), and ^{42}KCl (5.8 mCi/mol) were purchased from New England Nuclear Corp.

Assay of phosphate. P_i was measured as outlined by Martin et al. (13), using modifications described earlier (10, 12). Cell P_i was taken as the difference between total phosphate and phosphate in supernatants obtained after a centrifugation through silicone oil (12). Free inorganic phosphate in washed cells represented a pool of 40 to 50 mM, as noted earlier (10, 12).

To determine the fate of $^{32}P_i$ taken up during the resting state (no glucose), cells were placed in 10 ml of 150 mM KCl, 20 mM MOPS-K (pH 7) at 2 mg (dry weight) per ml along with $^{32}P_i$ to 6 μM; 85% of the label was inside cells after a 60-min incubation. Cells were then centrifuged, resuspended in the original volume of buffer, and to extract inorganic but not organic phosphate, two 4-ml samples were added to 2 ml of isobutanol layered on 0.8 ml of 4.25% (wt/vol) ammonium molybdate in 0.85 N sulfuric acid (10). After mixing, 85% of the cellular ^{32}P was found in the organic phase. The same yield was obtained for $^{32}P_i$ alone (with unlabeled phosphate sufficient to match that contributed by cells), whereas recovery of the 2-[1-^{14}C]deoxy-D-glucose 6-phosphate standard was only 0.16%.

Assays of transport. Washed cells were placed at 1 to 2 mg

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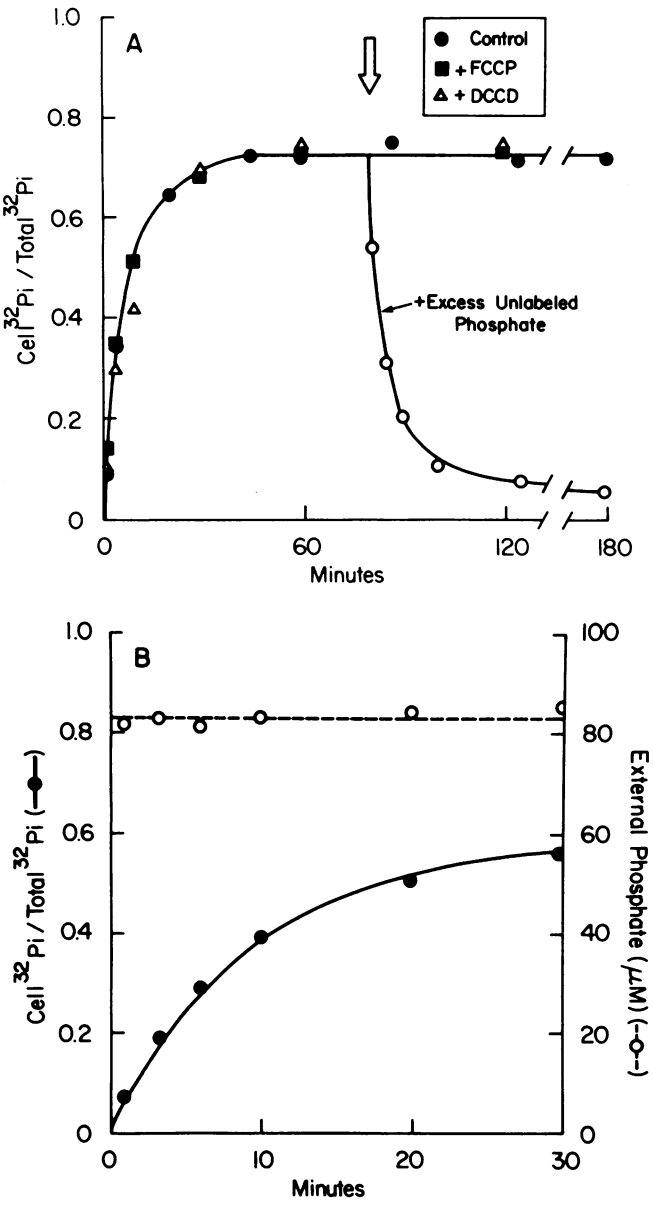

FIG. 1. Phosphate transport by *S. lactis* 7962. (A) Cells were suspended (2 mg [dry weight] per ml) in 300 mM KCl–20 mM MOPS-K at pH 7. After $^{32}P_i$ was added to 20 μM, transport was estimated by centrifugations through silicone oil. Symbols: ●, control cells; ■, cells treated with 10 μM FCCP; △, stock cells exposed for 30 min to 1 mM DCCD (*N,N'*-dicyclohexylcarbodiimide) before dilution and assay. At the arrow, part (○) of the control suspension received 4 mM unlabeled phosphate. (B) Cells (1.2 mg [dry weight] per ml) were treated as in (A) although labeled phosphate was 70 μM. Pellets gave estimates of phosphate incorporation; supernatants were used for chemical assays of total phosphate.

(dry weight) per ml in 200 to 300 mM KCl or 125 mM K_2SO_4, buffered at pH 7 by 20 mM MOPS-K. After addition of a labeled substrate, cells were separated from the medium by either centrifugation through silicone oil or a filtration step (12). Radioactivity in pellets or on filters was then measured with a Nuclear Chicago Mark II liquid scintillation counter. Most experiments used $^{32}P_i$ incorporation to study anion exchange, and for those studies $^{32}P_i$ transport was linear with cell density (0.6 to 3 mg [dry weight] per ml for centrifugation; 0.1 to 1.5 mg [dry weight] per ml for filtration). Despite the 10-s lag before separation of cells and medium, the less expensive centrifugation method was used most often, since this work did not usually require sampling before a 1-min incubation.

RESULTS

Phosphate exchange. The experiment illustrated in Fig. 1A shows that added $^{32}P_i$ was readily incorporated by washed cells, at a rate and to an extent unaffected by the protonophore, *p*-trifulorocarbonylcyanidemethoxyphenylhydrazone (FCCP), or by pretreatment with *N,N'*-dicyclohexylcarbodiimide, an inhibitor of the membrane-bound proton-translocating ATPase, BF_0F_1. This experiment also suggested that the incorporated phosphate remained as free phosphate, since cell ^{32}P was rapidly chased by a late addition of unlabeled phosphate; other work (see above) verified that most of the cellular label (>85%) was free inorganic phosphate. Because washed cells of *S. lactis* are metabolically inactive, with low levels of ATP and with no membrane potential or pH gradient (10, 12), such findings seemed best explained by a simple exchange of internal and external phosphate. Results compatible with this idea are described by Fig. 1B, which shows that external phosphate remained at a constant level, despite eventual transfer of most of the isotope into cells. Moreover, if one assumed a simple exchange between internal and external compartments of constant size, those measurements of external P_i and steady state $^{32}P_i$ incorporation predicted a cell phosphate pool equal in capacity (55 mM) to that determined by chemical assay (see above). Taken together, these data describe a system that mediates the equimolar exchange of free phosphate between the internal and external compartments. The work outlined below was directed to studies of this exchange reaction in the intact cell.

Kinetics of phosphate exchange. When initial rates of exchange were examined with substrate between 25 μM and 1.8 mM, phosphate entry showed a Michaelis constant (K_t) of 0.3 mM and a maximal velocity (V_{max}) of 26 mmol/min per liter of cell water (Fig. 2). Other work used phosphate as high as 30 mM, with similar results, but lower levels were not usually explored (later chemical assay showed residual extracellular phosphate of 5 to 15 μM at the working cell density). In seven separate experiments during the course of this work, K_t was 0.31 to 0.72 mM, with a mean value of 0.41 ± 0.07 mM (± standard error of the mean); V_{max} for these galactose-grown cells was 22 ± 4 mmol/min per liter of cell water (range of 15 to 27 mmol/min per liter of cell water).

Cation and anion requirements for phosphate exchange. No evidence was found for a coupled transport of phosphate and inorganic cations. Initial rates and final levels of $^{32}P_i$ incorporation were the same for assay media having 200 to 300 mM choline chloride, KCl, or NaCl. Similarly, there was no effect of EDTA (potassium salt), $MgCl_2$, or $CaCl_2$ (each 10 mM, in 300 mM KCl), and among ionophores tested (10 μM FCCP, 1 μM nigericin or gramicidin G, 1 to 5 μM valinomycin), only valinomycin had a significant effect. In that case, the rate was elevated 1.4- to 1.8-fold (four trials, as in Fig. 2) by an increased V_{max}. This was not due to the capacity of the ionophore to conduct charge (as K^+), since stimulations were not found with either FCCP or gramicidin G. Accordingly, a possible potassium-phosphate coupling was tested by studies of $^{42}K^+$ transport with cells suspended in 200 mM choline chloride. That work excluded any significant potassium-phosphate linkage, since for potassium added at 0.1 to 30 mM, $^{42}K^+$ entry showed a K_t of 0.3 mM and a V_{max} of 0.24

mmol/min per liter of cell water, in the presence *or* absence of 3 mM phosphate (data not shown; the positive control gave sevenfold stimulation of $^{42}K^+$ entry with 8 nM valinomycin).

Less extensive trials of anion substitutions showed that phosphate exchange was largely indifferent to other anions. Exchange rates were not changed when the usual buffer anion ($MOPS^-$) was replaced by other organic buffers; KNO_3 replaced KCl without effect; only 30 to 50% inhibition was found when sulfate replaced chloride. Indeed, the rate of exchange was only 50 to 60% reduced with no added salt. Overall, such findings suggest that phosphate exchange occurs without a coupling to common inorganic cations or anions.

A possible coupling to H^+ or OH^- was assessed by assays of phosphate entry before and after addition of glucose, using a competitive inhibitor (2-deoxyglucose 6-phosphate; see below) to identify phosphate influx by exchange. This indirect test showed that whereas net $^{32}P_i$ entry rates nearly doubled after onset of glycolysis, $^{32}P_i$ entry attributable to the exchange reaction remained constant (Table 1). The finding that the rate of exchange was not affected by the membrane potential or pH gradient developed during glycolysis (10) aruges against a coupling with H^+ (OH^-).

pH sensitivity of phosphate exchange. To examine the pH sensitivity of the exchange reaction, cells were first preincubated with 300 mM KCl and 5 μM valinomycin so that the inside and outside pH were closely matched (11) (tests at pH 5.4, 6.3, and 6.9 showed no change of the internal phosphate pool). When exchange was later studied with subsaturating phosphate (Fig. 3A), its activity appeared maximal near pH 7, with a sharp decrease at acid pH values still within the physiological range for *S. lactis*. In other work the effect of pH on kinetic parameters was determined

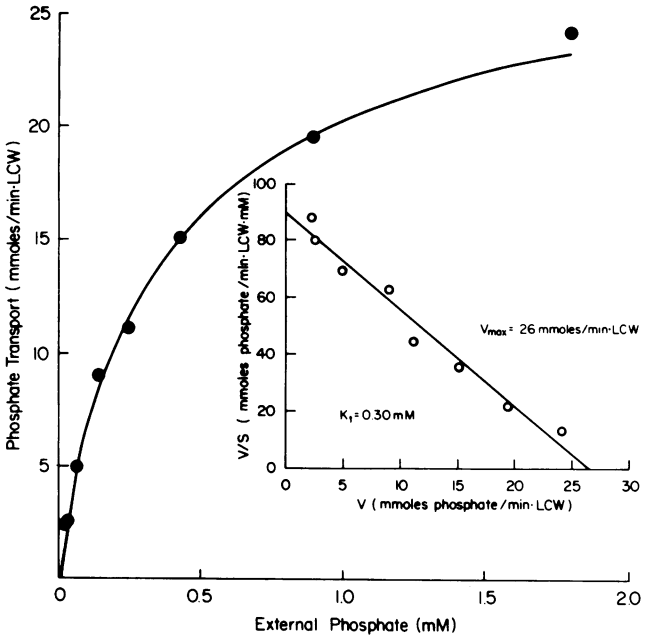

FIG. 2. Kinetics of phosphate exchange. Rates of phosphate exchange were estimated by 1-min incubations with $^{32}P_i$ (25 μM to 1.8 mM) for cells treated as described in the legend to Fig. 1A. The inset shows the data presented according to Eadie and Hofstee. LCW, Liters of cell water.

TABLE 1. Phosphate incorporation before and after onset of glycolysis[a]

Additions to assay	Phosphate incorporation (mmol/20 s per liter of cell water)	
	No glucose	Glucose added
None	0.58 ± 0.05	1.08 ± 0.13
2-Deoxyglucose 6-phosphate	0.022 ± 0.004	0.53 ± 0.05
Difference	0.56	0.55

[a] Initial rates of phosphate incorporation were measured before and 20 min after initiation of glycolysis with 20 mM glucose. Phosphate transport was measured (filtration) after a 20-s incubation with 10 μM $^{32}P_i$, with and without added 0.25 mM 2-deoxyglucose 6-phosphate; assay conditions were as described in the legend to Fig. 1A. Mean values ± standard error of the mean are given for three separate experiments.

at selected points between pH 7.2 and 5.6. Those data (Fig. 3B) showed that K_t was relatively stable with pH. However, V_{max} was strongly pH sensitive and fell 40-fold between pH 7.2 and pH 5.6, as H^+ concentration rose by this same factor.

Substrate specificity for exchange. The likely substrate specificity for exchange was identified by studying inhibitions of phosphate entry. For example, in the experiment outlined in Fig. 4, the response to glucose 1- or 6-phosphate was examined. Glucose 1-phosphate itself had no effect, since its action as an inhibitor was attributable to contaminating free phosphate (0.7%). By contrast, glucose 6-phosphate (0.3% free phosphate) was highly effective in blocking exchange, and this seemed due to primary rather than secondary interactions. Certainly, the strength of inhibition at low levels of glucose 6-phosphate argues that influx of $^{32}P_i$ was not blocked by unlabeled phosphate generated by external phosphatase activity. Also, the inhibition by 0.3 mM glucose 6-phosphate was not relieved by a 10-fold excess of several compounds (see Table 2) whose presence should have reduced sugar phosphate hydrolysis (e.g., orthovanadate [9]).

Table 2 summarizes these results, along with data from a survey that examined many potential inhibitors of phosphate transport. When at 50-fold excess over phosphate itself, most test substrates proved, at best, to be weak inhibitors of exchange (the ratio of velocities, v_t/v_c, was more than 0.3), and only certain sugar 6-phosphates or arsenate gave substantial inhibitions ($v_t/v_c \leqq 0.12$). In two cases we also performed kinetic studies (data not shown). The work in which both arsenate and phosphate were varied clearly showed a simple competitive inhibition of phosphate transport by arsenate, with a K_i of 0.34 ± 0.03 mM (three experiments). Tests of glucose 6-phosphate inhibition was less extensive, but the linear Dixon plots under standard conditions (as shown in Fig. 4) were also compatible with a simple competitive inhibition (K_i = 0.02 ± 0.004 mM; three experiments).

Sulfhydral reactivity. The rate and extent of phosphate exchange were not affected by 30-min incubations with the following sulfhydral reactive compounds: 1 mM 5,5′-dithiobis-(2-nitrobenzoic acid) or methylmethanethiosulfonate and 10 mM *N*-ethylmaleimide or iodoacetic acid. Three other agents, however, were quite effective inhibitors, even at low concentrations—5-min treatments gave 50% reduced initial (60 s) rates with 15 μM mersalyl acid, 45 μM *p*-hydroxymercuribenzenesulfonic acid (*p*CMBS), and 45 μM *p*-hydroxymercuribenzoate (*p*CMB). These blocks were readily re-

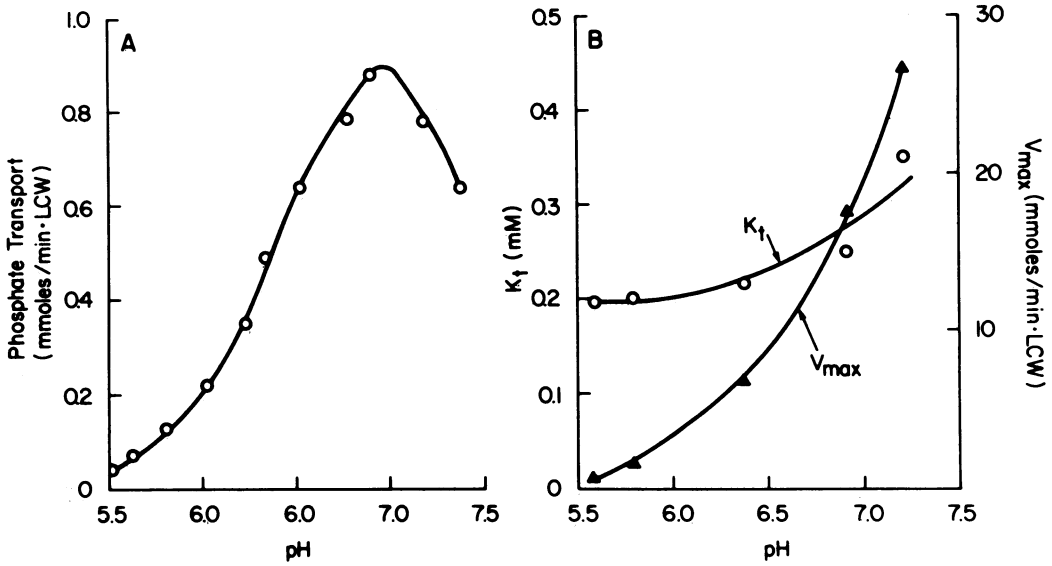

FIG. 3. pH sensitivity of phosphate exchange. (A) Cells (as described in the legend to Fig. 1A) were treated with 5 μM valinomycin before the pH was adjusted with HCl or KOH. Portions were then placed with 20 μM labeled phosphate for 1 min to estimate exchange. (B) Cells were treated as above, except that transport was measured at 70, 250, and 600 μM phosphate; kinetic parameters were evaluated as in Fig. 2 (inset). LCW, Liters of cell water.

versed by dithiothreitol (Fig. 5). Thus, after its suppression by 0.25 mM *p*CMB, phosphate exchange was recovered at the control level after addition of dithiothreitol. That experiment also shows that substrate entry and exit were each blocked by *p*CMB, since its addition after completion of isotope exchange prevented the phosphate efflux normally elicited by arsenate.

Heterologous exchange. Two sorts of experiments have reinforced the idea that effective inhibitors of phosphate exchange (Table 2; $v_i/v_c \leqq 0.12$) might be alternate substrates for the reaction. For example, in the indirect test shown by Fig. 5, addition of arsenate caused a partial efflux of $^{32}P_i$. In that experiment, if both phosphate and arsenate were substrates for exchange, expansion of the external pool with arsenate should have lowered the apparent steady state internal $^{32}P_i$ from 20 to about 3 mmol/liter of cell water. Since this prediction was closely followed (a final $^{32}P_i$ level of 5 mmol/liter of cell water), we concluded that both phosphate and arsenate could serve as substrates for exchange. Similarly, it was shown that 2-deoxyglucose 6-phosphate "chased" internal $^{32}P_i$ (2-deoxyglucose did not), although in that case the quantitative analysis was not useful, due to cellular phosphatase(s) (see below).

Direct evidence that sugar 6-phosphate participated in exchange came from studies of the nonmetabolizable analogue 2-deoxyglucose 6-phosphate. Although without concurrent metabolism *S. lactis* lacks the usual sources of energy associated with solute accumulation, one does expect an incorporation of sugar phosphate if phosphate/sugar 6-phosphate exchange occurs. Figure 6 shows that, indeed, there was substantial (50-fold) accumulation of 2-deoxyglucose 6-phosphate by washed cells. Moreover, substrate entry was blocked by an early addition of phosphate, whereas a delayed addition caused efflux of preaccumulated material. Thus, phosphate affected sugar 6-phosphate transport in the same way that sugar 6-phosphate influenced the movements of phosphate.

Other work established that 2-deoxyglucose 6-phosphate accumulation was unaffected by free 2-deoxyglucose (3 to 20 mM) or orthovanadate (0.2 to 2 mM), and those findings have ruled out significant contributions by (i) extracellular phosphatase(s), (ii) sugar (re)entry and phosphorylation driven by phosphoenolpyruvate (27, 30), or (iii) sugar phosphate:sugar transphosphorylation (21, 26) after either intracellular or extracellular hydrolysis of the sugar 6-phosphate. And although extensive studies of the kinetics of transport were not performed, one experiment did confirm that, as expected, K_t for 2-deoxyglucose 6-phosphate entry was about 20 μM. Finally, 2-deoxyglucose 6-phosphate move-

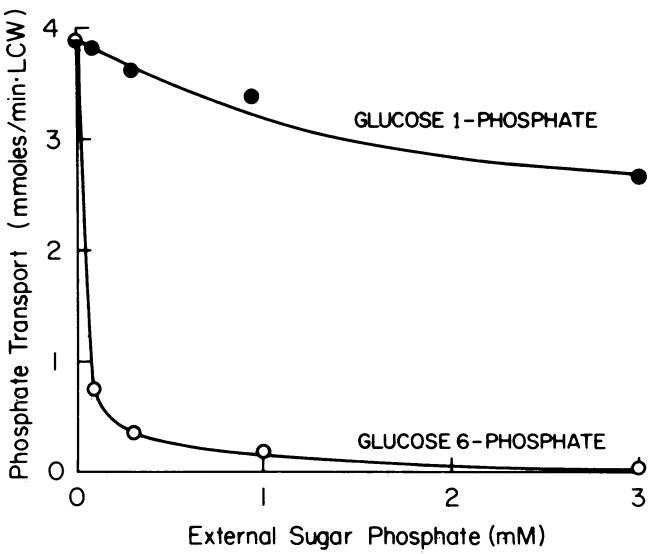

FIG. 4. The effect of sugar phosphate on phosphate entry. Rates of entry were estimated (Fig. 1B) after 1-min incubations with 60 μM labeled phosphate plus indicated levels of α-glucose 1-phosphate or glucose 6-phosphate. LCW, Liters of cell water.

ment was found to be unresponsive to *N,N'*-dicyclohexylcarbodiimide, FCCP, valinomycin, or *N*-ethylmaleimide, but sensitive to *p*CMB, *p*CMBS, and mersalyl acid. Clearly, each of these findings is compatible with the idea that the same carrier mediates phosphate/phosphate or phosphate/sugar 6-phosphate exchange.

We studied transport of 2-deoxyglucose 6-phosphate because it is not further metabolized (24, 29), unlike other candidate sugar phosphates (Table 2). Even so, 2-deoxyglucose 6-phosphate was subject to a net hydrolysis, presumably by the internal sugar 6-phosphatase described in other strains of *S. lactis* (28, 30). For the same conditions used to study sugar 6-phosphate transport (Fig. 6), we measured free sugar released by subjecting the total reaction mixture to anion exchange chromatography after cell rupture with 7% *n*-butanol in the presence of 0.5% Triton X-100 and 0.5 mM orthovanadate. With or without 20 mM unlabeled 2-deoxyglucose, the appearance of the labeled free sugar was linear with time (5 to 60 min). An intracellular location for phosphatase(s) was inferred, since 0.5 mM *p*CMBS (impermeant) was an effective inhibitor, yet 0.25 mM vanadate was not. Most important, the initial rate of entry of 2-deoxyglucose 6-phosphate was only three times faster than its steady rate of hydrolysis (means of 0.84 and 0.28 mol/min per liter of cell water, respectively), and this made it difficult to derive quantitative conclusions regarding the heterologous exchange. Nevertheless, two observations seem relevant. First, despite its generation within the cell, free sugar was only a minor fraction (≃5%) of the intracellular label, as judged by chromatography of material extracted from cells washed on filters. Second, the high-capacity phosphate pool of washed cells was sufficient, in principle, to support the accumulation of sugar 6-phosphate (typically, 6 to 10 mM [Fig. 6]).

TABLE 2. Inhibition of phosphate exchange[a]

v_i/v_c	Test substrate
0.02 ± 0.01	Glucose 6-phosphate
0.02 ± 0.01	2-Deoxyglucose 6-phosphate
0.03 ± 0.02	Mannose 6-phosphate
0.09 ± 0.03	Fructose 6-phosphate
0.11 ± 0.01	Arsenate
0.12 ± 0.03	Glucosamine 6-phosphate
0.33	Ribose 5-phosphate
0.37 ± 0.08	Fructose 1-phosphate
0.38 ± 0.08	Phenylphosphate[b]
0.43 ± 0.05	Acetylphosphate[b]
0.56 ± 0.06	Galactose 6-phosphate
0.62	Phenyldichlorophosphate
0.72	Sorbitol 6-phosphate[b], phosphoenolpyruvate[b], malate
0.74	Pyrophosphate, β-glycerophosphate
0.79 ± 0.08	α-Glucose 1-phosphate[b]
0.80 − 1.21	β-Glucose 1-phosphate, galactose 1-phosphate[b], mannose 1-phosphate[b], xylose 1-phosphate[b], α-lactose 1-phosphate[b], α-glucuronic acid 1-phosphate, glucose 6-sulfate[b], 6-phosphogluconate[b], fructose 1,6-bisphosphate, (+)2-phosphoglycerate, (−)3-phosphoglycerate, α-methylglucoside, α-methylmannoside, 2-deoxyglucose, phosphotyrosine[b], *o*-phospho-L-serine[b], phosphorylcholine, trimetaphosphate, tripolyphosphate, monomethylphosphate, AMP, ADP, ATP, cAMP, ortho- and metavanadate[b], fluoride[b]

[a] In 11 experiments (see Fig. 4) rates of phosphate exchange were estimated with (v_i) and without (v_c) test compound at 3 mM (50 mM fluoride). Mean ± standard error of the mean is given if three or more independent trials were available. Sugars or derivatives were of the D configuration; Na^+ or K^+ salts were used.

[b] At 3 mM (50 mM fluoride) this compound had no effect on inhibition by 0.3 mM glucose 6-phosphate.

Variable expression of exchange. The apparent substrate specificity for exchange (Table 2) includes sugar 6-phosphates that would appear as intracellular products of phosphotransferase action, especially by the enzyme II^{man} of *S. lactis* (21, 25, 27). Clearly, it was important to describe the correlation between these two activities, and for this reason phosphate exchange was tested for cells grown in the presence of several carbohydrates. The experiment shown in Fig. 7 showed severe reduction of anion exchange after growth in broth with added fructose, glucose, or mannose, although chemical assays gave phosphate pools of 40 to 50 mM capacity for each cell type. By contrast, after growth with rhamnose or arabinose, the reaction was so rapid that it could not be accurately tracked. Overall, initial rates varied about 200-fold, with cells grown as usual (galactose) at a position intermediate between full expression and repression.

Studies of cells with elevated or reduced expression (grown with arabinose or glucose, respectively) showed (i) that phosphate exchange was always inhibitable by glucose 6-phosphate or 2-deoxyglucose 6-phosphate (as shown in Fig. 4), (ii) that *p*CMB inhibitions of both phosphate and 2-deoxyglucose 6-phosphate transport were reversible by dithiothreitol (as shown in Fig. 5), and (iii) that initial rates of both phosphate and 2-deoxyglucose 6-phosphate transport were elevated, or reduced, compared with measurements made with cells grown in galactose (Fig. 6). Thus, an inverse relationship between the expression of anion exchange and growth conditions that recruit phosphotransferase activity makes it unlikely there is a direct link between the two events.

Further evidence separating anion exchange and phosphotransferase activities came from studies with other strains of *S. lactis* (ML_3, 133, K_1) and with *S. faecalis* 9790. That work gave no indication of phosphate exchange in those strains, even for conditions that yielded maximal activity with *S. lactis* 7962 (Fig. 7). Although a phosphotransferase system is common to all of these strains, *S. lactis* 7962 is alone in expressing anion exchange.

DISCUSSION

Our results lead us to conclude that *S. lactis* 7962 has a novel anion antiport that catalyzes exchanges among phosphate, arsenate, and certain hexose(amine) 6-phosphates. This conclusion is supported both by the direct demonstrations of anion exchange and by arguments showing that known pathways fail to account for the data. It seems clear that the pattern of phosphate transport outlined here has little or no correspondence to presently described systems that handle phosphate. The rapid self-exchange that requires no metabolic energy (Fig. 1) rules out any straightforward interpretations invoking systems directed to the net accumulation of substrate linked to ATP or other "phosphate-bond" energy donors (5, 6, 22, 32). Nor is it likely that homologous or heterologous exchange reflects some ion coupled system simply acting in the absence of a driving ion gradient: (i) no ion dependency was revealed by substitution trials; (ii) addition of a protonophore did not accelerate decay of the

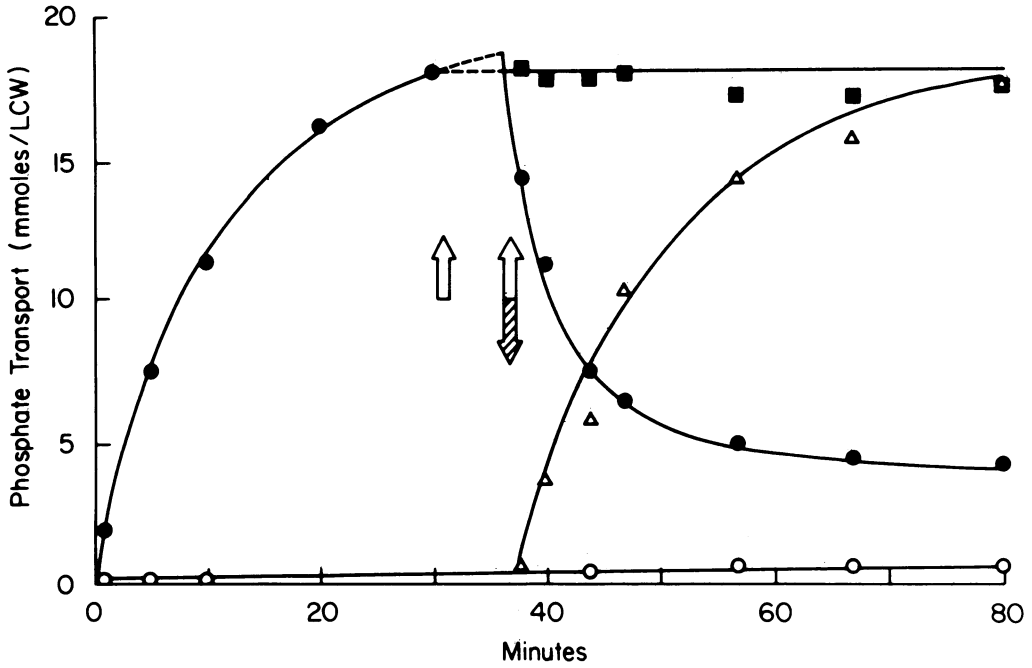

FIG. 5. Reversible inhibition of anion exchange by *p*CMB. Cells (1.2 mg [dry weight] per ml) were in 125 mM K_2SO_4–20 mM MOPS-K at pH 7. To one tube (○), 0.25 mM *p*CMB was added 7 min before 60 μM phosphate, and at the cross-hatched arrow, part of that suspension received 1 mM dithiothreitol (△). A parallel tube received phosphate at zero time, with (■) or without (●) later addition of 0.25 mM *p*CMB (first arrow); 1 mM arsenate was then added to both the *p*CMB-treated and untreated portions (second arrow). Transport was assayed by filtration. As in the later figures, the ordinate scale shows incorporation of extracellular material without regard to any preexisting intracellular pool. LCW, Liters of cell water.

substantial chemical gradients that could be established (Fig. 1 and 6); (iii) reaction rate was not affected by the membrane potential or pH gradient present during glycolysis (Table 1).

Such observations do not preclude phosphate exchange as a partial activity of some known system, and in this regard there might be several candidates. In *Staphylococcus aureus*, for example, phosphate movement has been described as either a net transfer or an exchange, depending on the availability of metabolic energy (16, 17). But in *S. lactis* 7962 these modes appear to reflect entirely different systems, since the increment to $^{32}P_i$ entry during glycolysis was clearly not by way of the exchange reaction (Table 1). Instead, that increase most likely represented phosphate influx as described for *S. faecalis* (5, 6), where an ATP dependency is found. Also, phosphate exchange of the kind observed in *S. aureus* (16), but not *S. lactis* 7962 appears inhibitable by vanadate. In *Escherichia coli* a phosphate exchange (K_t ~10 mM) has been attributed to operation of the Pit system (H^+/phosphate symport) (23), and were it not for the striking sensitivity we find to sugar 6-phosphates, our results might have been similarly interpreted, despite marked quantitative differences. (It may be worth reexamining the earlier findings with *E. coli*.) Finally, one could suggest that one or more parts of the phosphotransferase system (21, 25) participate in phosphate or sugar 6-phosphate movements. This idea would seem especially attractive, since the products of enzyme II^{man} activity in *Salmonella typhimurium*, *E. coli*, and *S. lactis* (21, 25, 27) are precisely those hexose(amine) 6-phosphates that effectively compete with phosphate during exchange (Table 2). Nevertheless, the pertinent observations argue against any simple conclusion along these lines. Thus, exchange is repressed by growth with sugars (glucose, fructose, mannose) whose presence is expected to elevate enzyme II^{man} (Fig. 7). Moreover, the exchange reaction is sensitive to *p*CMB, yet enzyme II^{man} in *S. lactis* is thought not to be (27). And last,

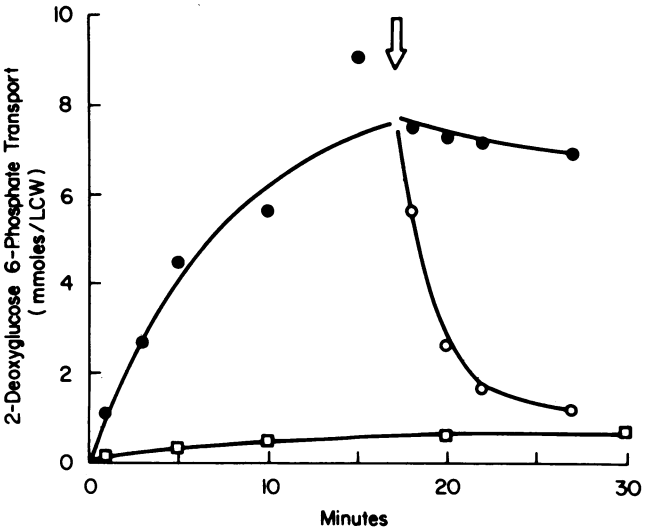

FIG. 6. Phosphate and sugar phosphate exchange. Cells were suspended (as described in the legend to Fig. 5), along with 0.2 mM sodium orthovanadate to block extracellular phosphatase(s). 2-Deoxyglucose 6-phosphate was added to 0.18 mM, with (□) or without (●) 30 mM potassium phosphate. At 16 min the latter suspension was divided into portions receiving 30 mM potassium phosphate (○) or 20 mM potassium sulfate (●). Transport was measured by filtration. LCW, Liters of cell water.

anion exchange is not affected by simultaneous free sugar entry via group translocation (Tables 1 and 2). It is clear, then, that if phosphotransferase elements were involved, they must be so in a previously unrecognized way. Could some component of enzyme II^{man} act, alternately, in group translocation or anion exchange? Although this sort of explanation cannot yet be eliminated, we prefer the simpler alternative that assigns anion exchange to an independent system.

Anion exchange is rare, and its description in *S. lactis* 7962 gives the first example in a free living procaryote. Other unambiguous cases (that is, with no uncertainty regarding OH^- antiport and H^+ symport) occur only at the eucaryote plasma membrane (where chloride exchanges with biocarbonate [1, 24] or organic anions [3, 7]), in eucaryote parasites (the rickettsial adenine nucleotide exchange [33]), and in eucaryote organelles (mitochondria, chloroplasts [2, 8, 14]). These last examples are of special interest to the present discussion, since the organelles, just as *S. lactis* 7962, show the antiport of phosphate and organic anions—malate or malonate in mitochondria (8, 14) and triose phosphates in chloroplasts (2). These substrate specificities are not shared by the bacterial antiporter (Table 2), but the three phosphate-linked exchanges do have common features. Each has a marked sensitivity to mercurials, but not maleimides, and each mediates an electroneutral exchange. In our work, this latter conclusion follows from the lack of effect by charge carrying ionophores (FCCP, valinomycin) on the heterologous reaction and lack of effect of membrane potential on homologous exchange (Table 1). (The modest stimulation of phosphate exchange by valinomycin is not understood.)

Differences among these phosphate shuttles are most apparent with regard to substrate preference. The choice of organic counteranion seems unique to each system, and our work suggests that the ionic form of phosphate may also differ. In the chloroplast, for example, it is thought that the divalent anion, HPO_4^{2-}, is the substrate (2), but this seems unlikely in the bacterial case, since depression of activity at acid pH was accounted for by changes in V_{max}, not K_t (Fig. 3); exactly the opposite is found with chloroplasts (2). The simple interpretation of our work would suggest that monovalent phosphate, $H_2PO_4^{1-}$, is preferred in bacteria, and that protonation of some group with pK_a near 5 can inactivate the carrier. Further studies are needed before the role (if any) of divalent phosphate is understood for the bacterial reaction. That information, of course, is crucial to understanding quantitative aspects of the reaction. Both the stoichiometry of exchange and its electrical character depend importantly upon which ionic forms of phosphate and organic phosphate are acceptable substrates. Thus, the suggestion that $H_2PO_4^{1-}$ can exchange with 2-deoxyglucose 6-phosphate in an electrically neutral reaction makes the interesting prediction of a 2:1 stoichiometry for antiport at pH 7, since divalent sugar 6-phosphate is the dominant ionic form near neutrality. This question could not be addressed in these studies, because an intracellular phosphatase was present, but our newer work with membrane vesicles (in preparation) is in full agreement with that expectation. Other studies, too, must be pursued in such simpler systems, especially the kinetic analysis of phosphate/sugar phosphate exchange.

The physiological role of the anion exchange reaction in *S. lactis* 7962 is not yet clear, but we suggest two possibilities. First, that it may serve to capture sugar 6-phosphates from the medium after more readily utilized free sugars become depleted. The high internal P_i accompanying a resting state would then rescue cells from stationary phase by driving

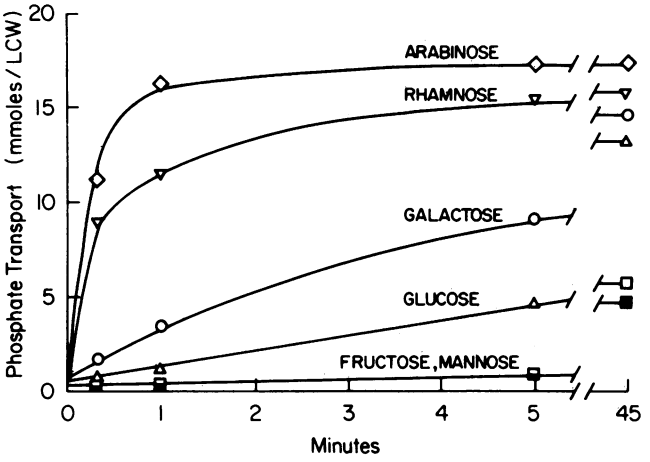

FIG. 7. Variable expression of phosphate exchange. Washed stationary-phase cells were placed in fresh broth media with the indicated sugars at 55 mM for 5 h at 35°C (40- to 80-fold mass increases). Cells were harvested, and phosphate exchange by washed cells was measured by centrifugation (other conditions as described in Fig. 5). For cells grown with arabinose, rhamnose, or galactose, $^{32}P_i$ incorporation after 45 min corresponded to equilibration with an internal P_i pool of 35 to 42 mM. LCW, Liters of cell water.

accumulation of sugar phosphate, and subsequently, together with energy-dependent phosphate import, a continued sugar phosphate entry could be based on the circulation of P_i. Second, one can imagine function that depends on the reverse reaction, since modulation of metabolite levels could exploit antiport in the same way that regulation might be linked to intracellular phosphatase (28); both reactions elevate the P_i pool at the expense of sugar phosphate. In either circumstance *S. lactis* 7962 presents an unusual case in which transmembrane anion movement appears independent of a normally dominant cation (H^+) flux. We also note that the substrate specificity of this antiporter places it in a special position with regard to more general aspects of bacterial transport. For example, given a 2:1 stoichiometry (see above), the anion exchange in *S. lactis* 7962 could perform the same net task attributed in other streptococci to sequential operation of an intracellular phosphatase and an enzyme II-mediated extrusion of free sugar (18, 19, 28). This striking parallel between anion exchange and inducer expulsion does suggest that phosphate/sugar 6-phosphate antiport might share features with two important mechanisms of sugar transfer in bacteria-phosphotransferase group translocation and cation-linked sugar symport. Perhaps evolutionary and biochemical relationships connecting these classes will be revealed by study of this intermediate example.

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