Proc. Natl. Acad. Sci. USA
Vol. 83, pp. 280–284, January 1986
Biochemistry

Variable stoichiometry of phosphate-linked anion exchange in *Streptococcus lactis*: Implications for the mechanism of sugar phosphate transport by bacteria

(antiport/phosphate transport/chemiosmotic transport)

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Communicated by DeWitt Stetten, Jr., September 9, 1985

ABSTRACT **Phosphate/2-deoxyglucose 6-phosphate antiport in *Streptococcus lactis* showed an exchange stoichiometry that varied over a 2-fold range when assay pH was shifted between pH 8.2 and pH 5.2. At pH 7.0 and above, 2 mol of phosphate moved per mol of sugar phosphate; at pH 6.1 the ratio was 1.5:1, while at pH 5.2 the overall stoichiometry fell to 1.1:1. This pattern was not affected by valinomycin in potassium-based media, nor could variable stoichiometry be attributed to altered hydrolysis of the sugar phosphate substrate. In kinetic studies at pH 7.0 or pH 5.2, sugar 6-phosphate was a competitive inhibitor of phosphate transport, indicating operation of a single system. Parallel tests showed that the affinity of antiport for its sugar 6-phosphate substrate was insensitive to pH in this range. Overall, such results suggest a neutral exchange that has specificity for monovalent phosphate but that selects randomly among the available mono- and divalent sugar 6-phosphates. A simple model that shows this behavior suggests a mechanistic role for anion exchange in bacterial transport of sugar phosphate or other organic anions.**

Streptococcus lactis, a Gram-positive anaerobe, can express a membrane transport system whose physiological role is directed to exchanges involving phosphate and/or glucose or mannose 6-phosphates (1). Study of this phosphate/sugar phosphate antiport in membrane vesicles (2) had indicated that such anion exchange could be unbalanced with respect to substrate transfer. For example, a 2 for 1 stoichiometry was observed when heterologous exchange was tested at pH 7, and that finding, with other evidence, pointed to the neutral transfer of two monovalent phosphate anions for a single divalent sugar 6-phosphate. Specificity for monovalent phosphate had been anticipated from the behavior of phosphate self-exchange (1, 3, 4), but the equally important question of selectivity among the various ionic forms of sugar phosphate has remained unexplored.

Work summarized here has examined the roles of monovalent and divalent sugar phosphates by tests of stoichiometry at pH below and above the pK_2 for a sugar 6-phosphate substrate. The results suggest an exchange ratio that depends strikingly on pH, varying between 2 for 1 at pH above, and 1 for 1 (2 for 2) at pH below the pK_2 for sugar phosphate titration. This variable stoichiometry might arise in a natural way if an antiport with monovalent phosphate were always electroneutral, and if the mono- and divalent sugar phosphates were accepted with equal efficiencies. Such observations are of general interest in that they represent the case in which a pH-dependent variable stoichiometry (5–7) is verified by direct measurement of substrate fluxes. But perhaps more important, biochemical and physiological models that accommodate these findings suggest anion exchange as the molecular basis for transport of a variety of anionic materials across bacterial membranes.

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MATERIALS AND METHODS

Most experiments used membrane vesicles prepared by osmotic lysis (2) of *S. lactis* ATCC 7962; cells were grown in broth with 1% galactose to give partial expression of anion exchange (1). Vesicles had internal water of 3–4 μl/mg of protein (2) and were prepared and stored (−80°C) at pH 7 in 50 mM potassium phosphate/10 mM $MgSO_4$/0.25 mM Na_3VO_4 (no vanadate was used in vesicles tested for phosphatase). For each experiment, thawed vesicles were diluted in 125 mM K_2SO_4/20 mM potassium 3-(*N*-morpholino)propanesulfonate (Mops), pH 7.0, centrifuged (2), and resuspended in this solution at 2.5 mg of protein per ml before dilution to 100 μg of protein per ml during assay. Three separate experiments allowed us to estimate that unlabeled phosphate carried into standard assays was 25 ± 4 nmol/ml (SD); this was used to correct $^{32}P_i$ specific activity, as required (2).

Phosphate/sugar phosphate exchange was measured with $KH_2{}^{32}PO_4$ (200–1000 Ci/mol; 1 Ci = 37 GBq) and 2-deoxy-D-[1-^{14}C]glucose 6-phosphate (55 Ci/mol) from New England Nuclear. Washed vesicles were usually placed directly in assay buffer having 100 mM KCl, 60 mM Na_2SO_4, 0.25 mM Na_3VO_4, and 0.5–1 μM valinomycin, with KOH and 20 mM Mops (to pH 7.0) or 20 mM 3-(*N*-morpholino)ethanesulfonic acid (pH 6.1, 5.2). Typically, vesicles were loaded with sugar phosphate by an exchange with internal phosphate at the desired pH. This took place during a 45-min incubation (23°C) with ^{14}C-labeled 2-deoxyglucose 6-phosphate at 0.18 mM. Excess (3 mM) $^{32}P_i$ was then added, and over the next 30 min samples were taken to estimate net substrate fluxes by double-label counting of washed Millipore filters (2). Other experimental procedures are described elsewhere (1, 2).

RESULTS

Exchange at Various pH Values. Fig. 1 describes an experiment in which exchange stoichiometry was tested at various pH values during the efflux of sugar phosphate. Vesicles were first loaded with labeled 2-deoxyglucose 6-phosphate by an exchange with preexisting internal phosphate (2). Because sugar phosphatase action had been blocked by vanadate (ref. 2; see below) the steady-state incorporation of 105 nmol of sugar phosphate per mg of protein (Fig. 1*A*) represented about a 100-fold accumulation of substrate above its original medium concentration. The vesicles, loaded with sugar phosphate, were then reisolated by centrifugation, resuspended, and redistributed into three tubes, having assay buffer at pH 7.0, pH 6.1, or pH 5.2.

Abbreviation: Mops, 3-(*N*-morpholino)propanesulfonic acid.

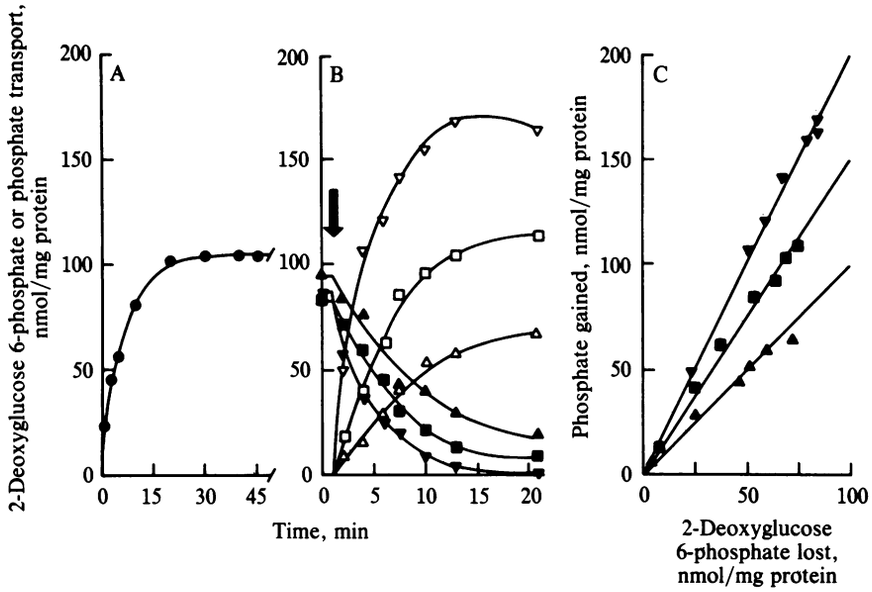

FIG. 1. Effect of pH on phosphate/2-deoxyglucose 6-phosphate exchange. (*A*) Sugar phosphate accumulation was measured for vesicles suspended at 23°C in 125 mM K_2SO_4/20 mM K Mops, pH 7/0.18 mM 2-deoxy[^{14}C]glucose 6-phosphate/0.25 mM Na_3VO_4. (*B*) After 45 min, vesicles were spun (145,000 × *g* for 45 min; 4°C), resuspended in a small volume without sugar phosphate (82% recovery of protein), and distributed to tubes having assay buffer (23°C) at pH 7.0 (▽, ▼), pH 6.1 (□, ■), or pH 5.2 (△, ▲). Each tube was tested for sugar phosphate content 10 min later, after which 3 mM $^{32}P_i$ was added (arrow), and further samples taken to evaluate ^{32}P gained (open symbols) and ^{14}C lost (closed symbols). (*C*) Phosphate gained is correlated with sugar phosphate lost; symbols are as for *B*. The lines have slopes of 1 (pH 5.2), 1.5 (pH 6.1), or 2 (pH 7.0).

Samples taken 10 min later showed only minor (16–22%) loss of this substrate, despite the substantial concentration gradient (>1000-fold after resuspension). Nevertheless, sugar phosphate was released promptly on addition of the heterologous substrate, $^{32}P_i$ (Fig. 1*B*). Other work has shown that the inorganic and organic phosphates remain chemically separate during their exchange (2), so that the stoichiometry of antiport could be directly measured by comparison of the respective unidirectional fluxes.

The behavior of the different samples in this experiment indicated that varying the pH had two kinds of effects. On the one hand, as pH was lowered there was marked inhibition of the absolute rates of both sugar phosphate and phosphate movements. More important, however, the relative rates were changed, and a purely kinetic effect, which might have been anticipated (1, 3, 4), was superimposed on a systematic change of the exchange ratio. That conclusion was evident in the correlation of phosphate gained and sugar phosphate lost, as shown by Fig. 1*C*. Those data confirm the earlier finding of a 2 for 1 exchange at pH 7.0 (ref. 2) and now document that as pH falls, exchange stoichiometry undergoes a striking shift, from 2:1 at pH 7.0 to about 1:1 (2:2) at pH 5.2.

Sugar Phosphatase Activity. Such a changing stoichiometry (Fig. 1*C*) might be explained if the assay conditions had not adequately controlled for the presence of sugar phosphatase(s). Indeed, the streptococci have considerable sugar phosphatase capacity (8), and a large part of this is retained by vesicles of *S. lactis*, which can split 2-deoxyglucose 6-phosphate at 3–5 nmol/min per mg of protein at pH 7.0 (2). Earlier work had suggested orthovanadate as an appropriate inhibitor (as in Fig. 1), and to examine this general approach, the effectiveness of a vanadate block was tested at several different pH values (Fig. 2). Net sugar phosphatase activity was maximal at pH 6, but even there the standard level of 0.25 mM vanadate was sufficient to give >98% inhibition. At no pH was residual activity (0.02–0.1 nmol/min per mg of protein) sufficient to have biased stoichiometry by more than 7% of its assigned value (Fig. 1).

Bidirectional Exchange and the Effect of Ionophores. The next series of experiments confirmed the basic observation with a simplified protocol in which fluxes were estimated without centrifugation to remove external material after preloading (Fig. 3). The additional studies also included an experiment in which measurements were made when the direction of anion exchange was reversed, and that particular

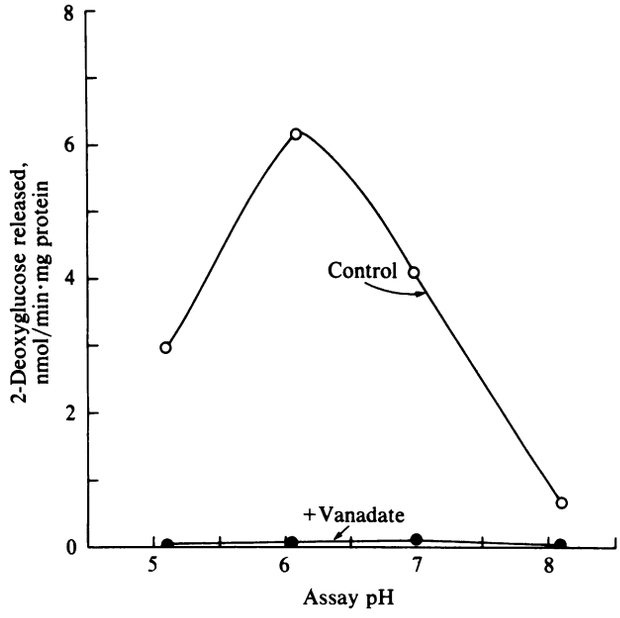

FIG. 2. Sugar phosphatase activity. Vesicles were incubated with 2-deoxy[^{14}C]glucose 6-phosphate at the specified pH, for assay conditions as described for Fig. 1*B*, and with KOH and 20 mM 1,3-bis[tris(hydroxymethyl)methylamino]propane as buffer at pH 8.2. Sugar released was measured at timed intervals by passing quenched aliquots over an anion-exchange column as described (2). ●, 0.25 mM Na_3VO_4 inside and outside vesicles; ○, no vanadate.

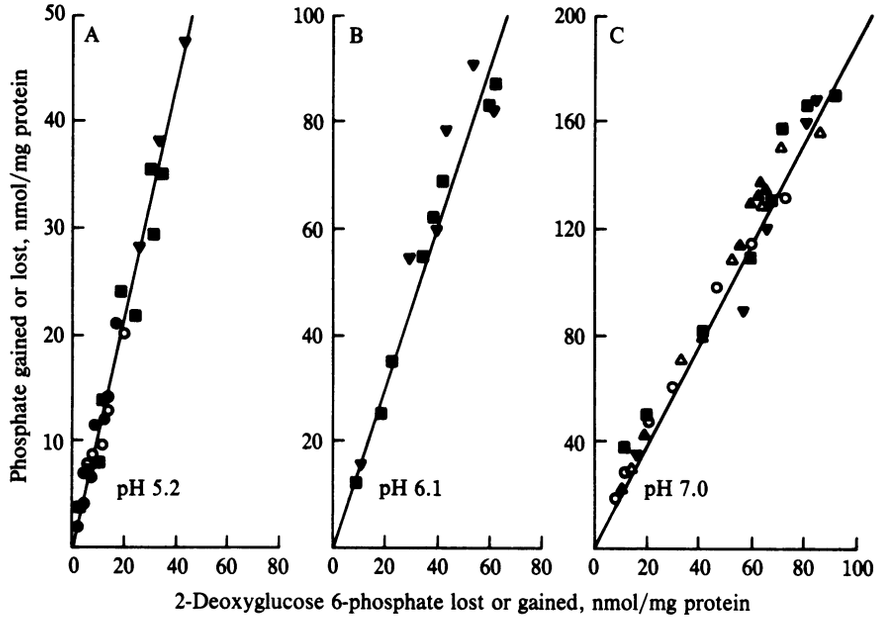

FIG. 3. Phosphate and sugar phosphate exchanges at various pH values. Vesicles were prelabeled with sugar phosphate (●, ■, △) in assay buffer (Fig. 1) or with 80 μM external $^{32}P_i$ (○); in the last case valinomycin was left out, and KCl and Na_2SO_4 were replaced by 125 mM K_2SO_4. Duplicate samples were taken at 45 min to establish steady-state contents before reversal of exchange by the heterologous substrate (3 mM $^{32}P_i$ or 0.18 mM 2-deoxy[^{14}C]glucose 6-phosphate, respectively). In another experiment (▼) the isotopes were added to separate tubes (2). Samples were taken to correlate the phosphate and sugar phosphate exchanges at pH 5.2 (*A*), pH 6.1 (*B*), and pH 7.0 (*C*). The lines have slopes of 1.1 (*A*), 1.5 (*B*), and 1.89 (*C*), reflecting the average charge on sugar phosphate (see Fig. 5 and accompanying text).

trial has supported more than one conclusion. For example, because those data also showed variable stoichiometry (Fig. 3, open circles), it seemed unlikely that the other results were seriously affected by a vesicle lysis causing an underestimate of stoichiometry at acid pH. Such lysis should have been detected as the corresponding overestimate when exchange was reversed in direction. That same trial was also one in which valinomycin had been omitted, and since neither the rate (not shown) nor the extent of exchange was changed from when the ionophore was present, we concluded that heterologous antiport operates as an electroneutral event throughout the pH range studied. This was confirmed by two other experiments, which specifically tested possible effects of valinomycin and the protonophore carbonylcyanide *p*-trifluoromethoxyphenylhydrazone. No rate elevation or depression was noted at pH 7.0 or 5.2, with either ionophore at 1 μM (range of response, 90% to 120% of control).

The combined data of Fig. 3 also gave firm quantitative estimates of stoichiometry at the different pH values tested. Average stoichiometry at pH 5.2 was 1.11 mol of phosphate moved per mol of sugar phosphate. At pH 6.1, mean stoichiometry was 1.51; at pH 7.0 the exchange ratio was 2.0. In a separate experiment, exchange was followed at pH 7.0 and 8.2 (as for Fig. 1), and in that case, phosphate/sugar phosphate stoichiometries were 2.09 and 1.98, respectively. This last trial was of special interest, since the continued finding of a 2 for 1 exchange, even at alkaline pH, strengthened the earlier conclusion (1, 2) that divalent inorganic phosphate plays a minor role (if any) during antiport.

Kinetic Behavior. Other experiments have examined the kinetics of interaction of phosphate and sugar 6-phosphate. It seemed especially important to establish this relationship at pH 7.0, since the 2:1 stoichiometry might reflect phosphate transfer by more than one pathway. Evidence verifying a single system is presented in Fig. 4, which shows glucose 6-phosphate as a purely competitive inhibitor of all $^{32}P_i$ flux at pH 7.0. A less extensive study at lower pH (Table 1) suggests that sugar phosphate substrates act competitively for all conditions.

It has already been reported that a lowered pH reduces the maximal velocity of phosphate self-exchange without marked effect on substrate affinity, a result that was interpreted as support for the idea of specificity for monovalent phosphate (1, 3). Data in Table 1 extend this analysis to the heterologous exchange, using 2-deoxyglucose 6-phosphate as substrate. It

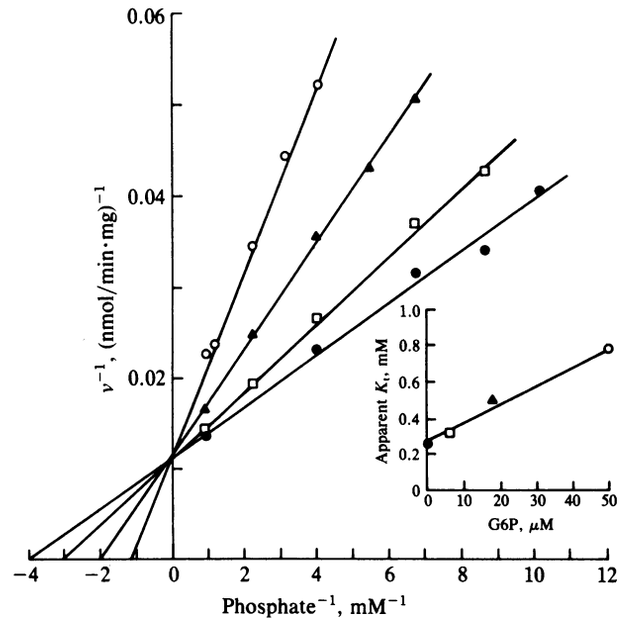

FIG. 4. Inhibition of phosphate exchange by glucose 6-phosphate. Vesicles were suspended at pH 7.0 in 125 mM K_2SO_4/20 mM K Mops, with $^{32}P_i$ added to the concentrations shown. Rates of transport were estimated at 23°C by triplicate 1-min incubations with the stated levels of glucose 6-phosphate. (*Inset*) Plot of apparent K_t values as a function of glucose 6-phosphate concentration. Derived kinetic parameters are consistent with other work (maximal velocity for $^{32}P_i$ transport, 86 nmol/min per mg of protein; K_t for phosphate, 250 μM; K_i for glucose 6-phosphate, 22 μM) (ref. 2; Table 1).

Table 1. Kinetic parameters for 2-deoxyglucose 6-phosphate during anion exchange

pH	V_{max}, nmol/min per mg of protein	K_t, μM	Apparent K_i, μM
7.0	22.8 ± 1.2	12.5 ± 1.0	17.0 ± 3.0
5.2	2.0 ± 0.3	5.7 ± 2.0*	8.2 ± 1.2

Values are mean ± SD of three or four experiments as in Fig. 3 (closed symbols), with incubations for 1 min (pH 7.0) or 5 min (pH 5.2) to estimate initial rate. Apparent K_i represents the concentration of 2-deoxyglucose 6-phosphate giving 50% inhibition of phosphate exchange when the latter was tested as described (2), using 75 μM $^{32}P_i$ substrate and 2.5–100 μM inhibitor. Uninhibited $^{32}P_i$ transport was 55 ± 2.6 (pH 7.0) or 6 ± 0.5 (pH 5.2) nmol/min per mg of protein. Given a purely competitive interaction, and the phosphate K_t as in Fig. 4, the true K_i for sugar phosphate would be 77% of the apparent K_i.

*Includes one trial with vesicles in which high expression of antiport (1) allowed rate estimates at 1 min for each pH. In these vesicles maximal velocity of sugar phosphate transport was 97 (pH 7.0) or 12 (pH 5.2) nmol/min per mg of protein.

is clear that, for this reaction as well, lowered pH has its major impact on the velocity term rather than the affinity term. Maximal velocity fell by a factor of 10 as assay pH shifted from pH 7.0 to pH 5.2, while the Michaelis constant for sugar phosphate transport (K_t) was largely unchanged. These trials also examined the sugar phosphate inhibition of phosphate self-exchange (an activity which also fell by a factor of 10 as pH was lowered) to derive the appropriate inhibition constant (K_i) from the linear Dixon plots (2). In assays at both pH 7.0 and pH 5.2, K_i for sugar phosphate was similar to its measured K_t. Therefore, by two criteria (K_t and K_i), the assay pH had only minor (2-fold) effects on the affinity of antiport for a sugar 6-phosphate substrate. Since these findings were made 0.9 pH units above and below the pK_2 for sugar 6-phosphate titration (pH 6.1), the simplest interpretation is that selectivity during heterologous exchange favors neither monovalent nor divalent sugar phosphate.

DISCUSSION

A variable stoichiometry appears to be an intrinsic attribute of phosphate-linked exchanges in *S. lactis*, and this unusual finding is relevant to several areas of study. That stoichiometry might change with pH in bacterial systems has been suggested before (5–7) but always on the basis of comparisons of driving forces or of their kinetic effects and not from measurement of the respective net fluxes. In this regard, then, the data collected here provide a clear and direct test to reinforce the general finding. At the same time, it must be noted that the interpretation we offer differs importantly from earlier explanations.

On a phenomenological level, our results are understood if the heterologous exchange operates with the following constraints: (*i*) a specificity for monovalent phosphate; (*ii*) a random choice among available mono- and divalent forms of sugar 6-phosphate; and (*iii*) an electroneutral exchange in all modes of operation. Overall stoichiometry would then represent a mixture of transfers, either 2:1 or 1:1 (2:2) at the molecular level, whose frequencies are determined by the degree of titration of sugar phosphate. Such considerations predict that exchange ratios should reflect the average charge on sugar phosphate, and for 2-deoxyglucose 6-phosphate (pK_2 = 6.1), one expects net stoichiometries (phosphate/sugar phosphate) of 1.89:1 (pH 7.0), 1.50:1 (pH 6.1), and 1.11:1 (pH 5.2). The experimental data agree well with these expectations, as illustrated by Fig. 3, where the solid lines give this theoretical correlation.

At a biochemical level, the behavior of such phosphate-linked antiport is compatible with a simple scheme (Fig. 5*A*) in which binding sites are so structured that two of them, each capable of accepting monovalent substrate, contribute to the binding of a single divalent sugar phosphate (but not, in this case, divalent phosphate). Occupancy by the divalent substrate would preclude binding of any monovalent form. Antiport would occur after relatively slow reorientations to either membrane surface, as the protein (re)associates with substrate(s) according to the usual kinetic rules (9, 10). In this case, a rate reduction at low pH is pictured as common to all forms of the protein, as suggested by the finding that homologous and heterologous exchanges are each slowed by a factor of 10 as pH is shifted from 7.0 to 5.2 (Table 1). Accordingly, the pH dependency of stoichiometry is attributed entirely to pH effects on one of the substrates and not to specific effects on the protein.

The physiological model of Fig. 5*B* depicts antiport in the absence of pH or electrical gradients, and even this simplest case prompts instructive comparisons with other exchanges. For example, phosphate/triose phosphate antiport is a major event at the chloroplast envelope membrane (11), and mitochondria show an important phosphate/dicarboxylate (malate, malonate) exchange (12). It seems probable, therefore, that antiport in *S. lactis* belongs to a broadly distributed class that relies on anion exchange as the molecular basis of transport. With this in mind, it would seem appropriate to examine other bacterial systems, most especially (but not exclusively) those that transport phosphorylated compounds. *sn*-Glycerol 3-phosphate transport (*glpT*) in *Escherichia coli* surely belongs to this class, since the most recent work reveals both phosphate self-exchange and a tightly coupled, heterologous antiport (13). Other likely examples from *E. coli* would include *uhpT*, which directs sugar phosphate transport (14), and possibly the system that mediates extrusion of cyclic nucleotide (15). In *Salmonella typhimurium* the most interesting candidate transports phospho*enol*pyruvate (16), while in *Staphylococcus aureus* it already seems clear that phosphate self-exchange (3, 4) reflects a generalized sugar phosphate antiport (unpublished data).

In bacteria, several of these putative anion exchanges, including those mediated by the *glpT* and *uhpT* gene prod-

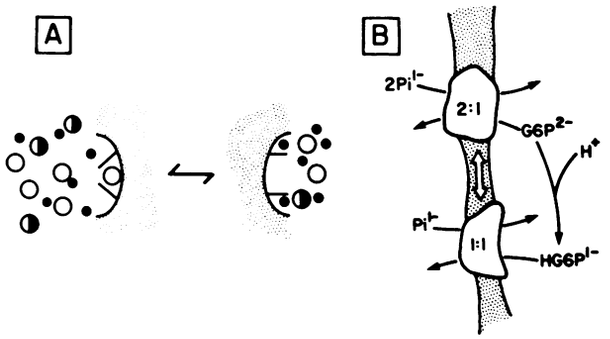

FIG. 5. Models of anion exchange. (*A*) A general model for antiport involving three substrates—monovalent phosphate (•) and mono- (◑) or divalent (○) glucose 6-phosphates. Reorientation, presumed rate-limiting at all pH values, requires simultaneous occupancy of both sites. In the situation shown, the binding is to divalent (left) or to two monovalent (right) substrates. (*B*) During heterologous exchange, the model predicts overall stoichiometry that reflects the average result of molecular exchanges that are either 1:2 or 2:2 (shown here as 1:1, for convenience). The frequency of each transfer type would be determined by sugar 6-phosphate titration. Average exchange stoichiometry would be pH dependent as indicated by solid lines in Fig. 3. G6P, glucose 6-phosphate.

ucts, have been placed in the category of H^+/anion symport (17), and while this need not conflict with an added requirement for exchange, it should be appreciated that tests of H^+ coupling are often indirect. In this setting the model of Fig. 5*A* has special appeal, since if a pH gradient were present, one expects a kind of "bootstrap" response overall, in which one divalent sugar phosphate moves in an exchange for two monovalent sugar phosphates. Such a system would behave physiologically, and in some experiments, as $2H^+$/anion symport, especially if affinity for the organic substrate were much higher than for inorganic phosphate, as appears to be the case in the *glpT* and *uhpT* systems (13, 14, 18). It may be noted, however, that in the simple model of Fig. 5 the role of any protonmotive gradient would be purely thermodynamic and incidental to substrate (de)protonation. The crucial mechanistic event would be anion exchange.

Among the bacterial examples cited, only antiport in *S. lactis* is well characterized in regard to stoichiometry and selectivity for its ionic substrates (refs. 1 and 2; this work), although similar properties might be expected of *Staphylococcus aureus*, since there, too, phosphate self-exchange builds on the monoanion (3, 4). Further tests are now required to establish the extent of anion exchange as a general mechanism in bacteria and to outline the relative importance of the inorganic and organic substrates in any specific instance. In this connection as well the scheme of Fig. 5 may be useful, since it readily extends to include divalent anion exchange (19), or even cases of electrogenic antiport (20, 21). This general idea offers a convenient biochemical and physiological mechanism with which to integrate anionic and H^+ circulations.

This work was supported by Grant GM24195 from the Public Health Service.

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