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Anion-Exchange Mechanisms in Bacteria

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PUMPS, CARRIERS, AND ION-MOTIVE CYCLES

The functional organization of most membranes centers on the construction of ionic circulations, and in their use of this fundamental strategy, bacteria resemble all other cells. We know, for example, that in the microbial world such chemiosmotic circuits underlie phemomena as different as ATP synthesis, pH regulation, solute transport, and even flagellar rotation. Indeed, it is likely that the most important insight provided by chemiosmotic theory has been to show how such distinct operations can be coordinated by their participation in the ionic currents that pass continually across all membranes.

Because the origins of chemiosmotic theory placed emphasis on the H^+ as a "coupling" ion (44, 74) and because discussions of mammalian systems come with a similar focus on Na^+, considerations of such chemiosmotic organizations often make the unstated assumption that cations alone connect the various reactions in the circuit. While this is probably true in a thermodynamic sense (for the most part, bacteria, plants, fungi, and organelles exploit an H^+ current), one should not overlook the instructive exceptions when anion movement plays an important role. A short while ago, these exceptions would have been limited to such exotic reactions as the light-driven Cl pump, halorhodopsin (93), but recent work shows that the area has been enriched by new examples of an unusual biochemical and physiological complexity. This is perhaps best outlined by showing how a "phosphate-linked" (P_i-linked) exchange responsible for glucose 6-phosphate (G6P) transport might integrate into a chemiosmotic circuit (Fig. 1). Presently, the evidence is most simply interpreted to suggest that G6P accumulation results from the asymmetric exchange of a pair of monovalent anions and a single divalent species. The surprising aspect of this reaction is that its mechanistic basis is not evident at a superficial level. Instead, there is a successful masquerade so that, despite the antiport of anions, a thermodynamic fingerprint would identify a neutral symport of $2H^+$ with divalent $G6P^{2-}$! It is now understandable why earlier classifications had attributed G6P transport to just such a symport reaction (see below). At a minimum, then, an exploration of this and related systems indicates how deciphering authentic mechanisms might not be so easy and therefore suggests that anion exchange may be more frequent than expected. Implicit in this example has been the

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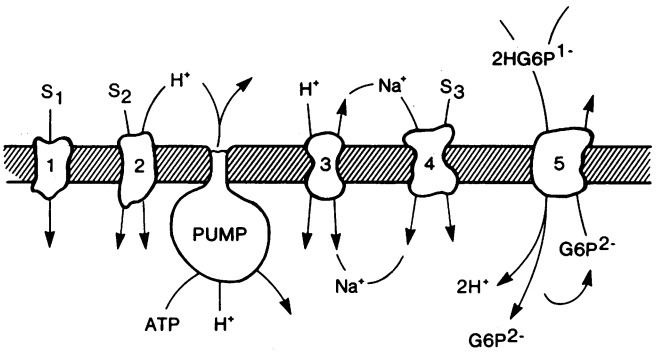

FIG. 1. Chemiosmotic circuits in membrane biology. A minimal organizational plan for a bacterial membrane should include at least one primary H^+ pump and a collection of carriers directly or indirectly linked to the dominant H^+ circulation. Here, the F_0F_1 ATPase is indicated as the sole primary H^+ pump; for an interesting alternative, see Fig. 9. The carriers shown mediate reactions of uniport (no. 1), symport (no. 2 and no. 4), and cation or anion antiport (nos. 3 and 5, respectively). Taken from reference 64 with permission.

idea that participation in an H^+ circulation need not require H^+-linked reactions as such. While correct in principle, this possibility is not commonly encountered. Here, too, anion exchange proves of interest, for bacteria now give us, along with the example of Fig. 1, an exchange reaction that acts as an "indirect" H^+ pump without a molecular coupling to H^+ (6).

The main goal of this article is to review the current evidence concerning bacterial anion exchange, with special attention to cellular, biochemical, and genetic studies that might guide us in an understanding of biochemical mechanisms and with a very particular emphasis on the family of P_i-linked antiport. This very specific focus will allow comment on three kinds of issues. First, our recent work has assigned a new biochemical mechanism and provided additional descriptive information for several systems thought previously to operate in a different way; it is therefore appropriate to summarize these new facts. Second, it is certain that biochemical models of anion exchange will contribute to a more general understanding of the physiological operation of membrane proteins (Fig. 1), and it is feasible that certain general features related to the molecular organization of membrane proteins are more readily apparent in these examples than in others (summarized in reference 65a). Finally, because we anticipate that in the near future anion exchange will continue to be underrepresented among bacterial transporters, the cases described here serve as useful precedents for those to come. To accomplish these aims requires discussion of systems which in many other ways appear to be unrelated, inasmuch as they are found in quite different organisms, as they participate in distinct metabolic pathways, and as they may be subject to diverse forms of genetic regulation. Clearly, all of these areas cannot be treated in the detail they deserve. We do hope, however, that the topics emphasized will offer a perspective that adds positively to the other fields.

PHOSPHATE-LINKED ANION EXCHANGE

Early Studies

Among bacterial anion-exchange systems, the P_i-linked examples are now the best characterized. Each of these accepts one or another organic phosphate, and each, as implied by the name, also accepts P_i, usually at reduced affinity relative to the organic substrate. It is this potential for P_i transport that makes it important to outline the relevant chronology, for there has always been an ambiguity in the distinction between systems designed to move P_i alone and those designed for transport of organic phosphates. Rosenberg (88) has recently presented a detailed account of this history, and only an abbreviated version is given here.

The first adequate description of bacterial transport was made in the early 1950s by Mitchell (72) and Mitchell and Moyle (75). They found that resting cells of *Micrococcus pyogenes* (now *Staphylococcus aureus*) took up external $^{32}P_i$ in exchange for internal phosphate by a reaction that was biased strongly to use of monovalent phosphate. These early studies ended with the reasonable suggestion that such behavior reflected a system used by growing cells to accumulate P_i in the net. Some 10 years later, Harold et al. (45) arrived at notably different conclusions concerning P_i transport by another gram-positive cell, *Streptococcus faecalis*. In that case, no exchange reaction was evident, and when the net flux was characterized, it proved to have an absolute dependence on concurrent metabolism (ATP), with selectivity for divalent phosphate and not the monovalent anion.

The diversity suggested by these contrary findings (*Staphylococcus aureus* versus *Streptococcus faecalis*) was not fully appreciated until the 1970s, when studies of *Escherichia coli* revealed a set of at least four systems, each of which carried $^{32}P_i$ into the cell (12, 89, 90, 99). Two of these systems, now designated Pst and Pit, have turned out to be highly specific for P_i itself, while the remaining two seem to accept P_i as a low-affinity analog of some natural substrate, an organic phosphate. One in the latter group (GlpT) had been described by Lin and his collaborators (46) as a glycerol 3-phosphate (G3P) transport systems, for which P_i was a competitive inhibitor. The other (UhpT), which handles G6P, had been discovered by Winkler (100) and by Pogell et al. (82) and subsequently defined genetically in Kornberg's laboratory (55).

In *E. coli*, the further classification of these four $^{32}P_i$ transport systems has used bioenergetic and structural criteria. Thus, Pst is now known to be a solute ATPase, one whose organization is typical of the many multisubunit complexes that act in conjunction with specific periplasmic binding proteins to accumulate substrate (here, P_i) at the expense of ATP (14, 19). Pit, on the other hand, is a chemiosmotic carrier and probably mediates an electrogenic nH^+/P_i symport (88, 90). Pst and Pit seem to represent the most common solutions to the problem of net P_i transport by bacteria, so that Pst-like energetics accommodates the findings in *Streptococcus faecalis* (45), while Pit-like behavior can account for P_i transport driven by respiration, as found in *M. lysodeikticus* (36), *Paracoccus denitrificans* (18), or *Staphylococcus aureus* (96).

Earlier schemes had considered the remaining $^{32}P_i$ transporters (GlpT and UhpT) to be further examples of nH^+/anion symport, and it is at this point that new findings begin to have their impact, for we now know that both UhpT and GlpT belong in the category of P_i-linked antiport (1, 95). Thus, a simple summary of current evidence would conclude that P_i transport systems come in two varieties. There are those designed for net P_i movement (Pit, Pst, etc.), and there are those which mediate an exchange involving P_i or a phosphorylated substrate (GlpT, UhpT, etc.) or both. With hindsight, we would say that Harold et al. (45) described one

TABLE 1. P_i-linked anion-exchange systems[a]

Cell type	Gene	Primary organic substrate(s)	V_{max} ratio[b]
E. coli	*uhpT*	G6P, M6P	5.5
	glpT	G3P	
Salmonella typhimurium	*pgtP*	PGAs, PEP[c]	
Staphylococcus aureus[d]		G6P, G3P	4.2
Streptococcus lactis[d]		G6P, M6P	4.7

[a] Systems described by four-letter genetic symbols show close sequence homology (27, 37, 40).

[b] Ratio of maximal velocities for homologous (P_i:P_i) and heterologous (P_i:G6P) exchanges (2, 3, 96; unpublished work with UhpT).

[c] PGAs, 2- or 3-Phosphoglycerates; PEP, phosphoenolpyruvate.

[d] These reactions are presumed to reflect the activity of single systems, since transport-negative mutants show neither homologous nor heterologous exchange (96; unpublished work).

of the former (dedicated) category, while Mitchell's discovery (72, 75) belongs to the second (exchange) class.

Diagnostic Features of P_i-Linked Antiport

Pi-linked exchange is found in both gram-positive and gram-negative cells, and the list of well-characterized examples is given by Table 1. These systems were first identified as exchange carriers by work with gram-positive cells, and in important ways those prototypes continue to be the best understood; this is despite the availability of sequence information and the more advanced state of biochemical analysis in *E. coli* and *Salmonella typhimurium*. In particular, the documentation of ionic selectivity is most complete for *Staphylococcus aureus* and *Streptococcus lactis*, and (for technical reasons) determination of exchange stoichiometry is most convincingly done by using the streptococcal system. Unifying biochemical and molecular models (discussed later) have therefore incorporated information from each different system, each according to its strengths. Consequently, it is important to establish first that these various family members differ importantly only in superficial aspects, such as the value of various kinetic constants, and not with regard to fundamental reaction mechanism. The available evidence (outlined subsequently) is entirely consistent with this idea.

Most of our information concerning P_i-linked exchange comes from studies of four different systems in three cell types: a G6P transporter in *Streptococcus lactis*; a G6P and G3P carrier in *Staphylococcus aureus*; and, in *E. coli*, two separate systems that handle either G6P (UhpT) or G3P (GlpT). All four of these systems appear to share the following properties. (i) Each mediates the self-exchange of P_i (P_i:P_i exchange) (1, 2, 68, 72, 95, 96), a reaction that is probably diagnostic of P_i-linked exchange (see above); the work with *Staphylococcus aureus* (72) and *Streptococcus lactis* (67, 68) suggests as well that this exchange strongly favors monovalent P_i. (ii) Each system catalyzes the heterologous exchange of P_i and some sugar phosphate (P_i:G6P, P_i:G3P, etc.), and in *E. coli* (95) and *Staphylococcus aureus* (96) one can devise direct experiments that exclude the operation of a symport mechanism. (iii) In *Streptococcus lactis* (2, 68), *Staphylococcus aureus* (72), and *E. coli* (UhpT) (95) AsO_4 freely substitutes for P_i during both homologous and heterologous exchange. (iv) In the same cases, the homologous and heterologous reaction velocities are in a characteristic ratio, so that the maximal velocity of P_i:P_i exchange is always faster, by a factor of about 5, than the maximal velocity of the heterologous exchange (Table 1). No one of these systems has been subjected to a complete kinetic analysis, but in *Streptococcus lactis* (4) and *E. coli* (GlpT) (46), the initial studies do suggest a direct competition between P_i and the organic phosphate, as might be anticipated for antiport. (v) An additional kinetic phenomenon (of uncertain significance) is the need for rather high ionic strength (0.3 M KCl) for optimal activity, whether in *Streptococcus lactis* (68), *E. coli* UhpT (28), or the *Salmonella typhimurium* system (PgtP) that transports phosphoglycerates (Table 1) (92); in *Streptococcus lactis* one can rule out a cation-sugar phosphate symport under such conditions (68). (vi) The electrical character of the various reactions deserves special comment. In the best-analyzed cases (*Streptococcus lactis*, *Staphylococcus aureus*, and *E. coli* (UhpT), the evidence favors an electroneutral event for both homologous and heterologous antiport (2, 68, 95, 96). But a reaction of the sort catalyzed by PgtP is also found in chloroplasts, in which the process appears to be electrogenic (30, 32), so that further study, especially of PgtP, is needed before this matter can be considered settled in bacteria. (vii) Finally, sequence homologies among three gram-negative examples (UhpT, GlpT, and PgtP) suggest a common origin and therefore a common mechanism (27, 37, 40).

To be sure, differences among the examples of P_i-linked exchange do exist, but these differences seem to be largely ones of substrate specificity (Table 1). Thus, the Michaelis constant for P_i transport (K_t) is spread over about a 10-fold range (0.25 to 2.3 mM), and while there is an even wider variation in the K_ts for the various sugar phosphates (0.02 to 1.5 mM), K_t for the preferred sugar phosphate is always relatively low, usually about 20 to 50 μM. Clearly, then, the overall balance of observations justifies a gathering of these individual systems into a single family (Table 1); with this conclusion in hand, several further comments are pertinent. First, it is certain that the distribution of P_i-linked exchange is broader than noted here. This is largely due to surveys by Winkler (102) and by Dietz (25) regarding the distribution of hexose phosphate transport. Second, in exploring the wider distribution of such exchangers, a preliminary decision may be afforded by sensitivity to the antibiotic fosfomycin. This toxic analog of phosphoenolpyruvate appears to enter the cell as a result of transport by P_i-linked exchange systems (50, 102).

Phosphate Exchange Reaction

Identification. Each of the systems described in Table 1 can carry out a P_i self-exchange that seems to be evident in intact cells, membrane vesicles, or proteoliposomes. (The P_i exchange accompanying PgtP has been characterized only with intact cells.) Because this event is common to all presently described forms of P_i-linked antiport, it seems appropriate to summarize its most important features by description of a simple experimental protocol. The progress of $^{32}P_i$ transport into washed cells of *Streptococcus lactis* is described by Fig. 2. In the absence of metabolizable substrates, such cells have much reduced levels of ATP, no proton motive force, and, typical of starving cells, an elevated pool of internal P_i (here, 50 mM). Moreover, this internal P_i pool is maintained for long periods, even when outside P_i becomes very low (20 μM in this experiment); for this reason, one expects the cell membrane to be impermeable to P_i. Nevertheless, the experiment of Fig. 2 shows that added $^{32}P_i$ is readily taken up into the internal P_i pool (>85% of internal ^{32}P is found as $^{32}P_i$) and just as readily discharged following a later addition of unlabeled P_i. Access to the

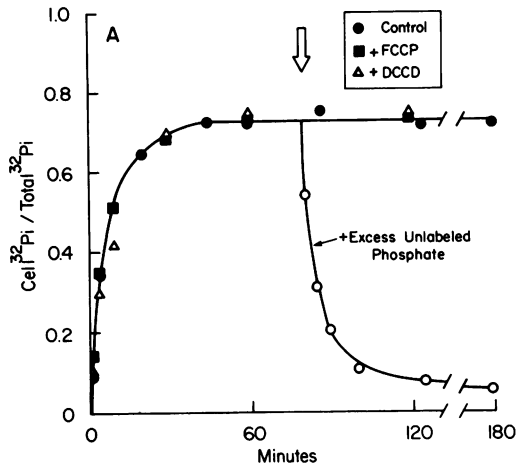

FIG. 2. P_i self-exchange in *Streptococcus lactis*. Washed cells were placed in 0.3 M KCl–20 mM MOPS/K (pH 7) and given 20 μM $^{32}P_i$ to trace P_i self-exchange. Where indicated, cells had been pretreated with 1 mM (*N,N'*-dicyclohexylcarbodiimide (DCCD) or the assay included the protonophore carbonylcyanide *p*-tri-fluoromethoxyphenylhyrazone (FCCP). At the arrow, 5 mM unlabeled P_i was added to a portion of the control suspension. From reference 68 with permission.

internal pool is insensitive to metabolic inhibitors (carbonylcyanide *p*-trifluoromethoxyphenylhydrazone and *N,N'*-dicyclohexylcarbodiimide), so one is left with the paradox of a membrane that retains P_i against a large concentration gradient while at the same time showing a high permeability to $^{32}P_i$, all in the absence of an expenditure of energy. Clearly, only when external $^{32}P_i$ enters in exact exchange for internal P_i is there any simple resolution to this problem. In fact, this self-exchange can be demonstrated directly, simply by showing that internal and external P_i pool sizes remain constant despite the transfer of $^{32}P_i$ (2, 68).

Analysis of P_i:P_i exchange, using intact cells of *Streptococcus lactis*, has proven relatively straightforward because of the ease with which these cells can be maintained in the resting (starving) state, where the reduced availability of ATP limits contributions by Pst-like systems and the absence of a proton motive force diminishes operation of Pit-like activities. (*Streptococcus lactis* seems to resemble *Streptococcus faecalis* or *E. coli* K-10 in the absence of a Pit-like system for P_i transport. But even if present, Pit might not be scored in this kind of assay, for it appears not to mediate a rapid exchange of the kind identified with P_i-linked antiport [68, 95]. This is unexpected, for other symporters [e.g., LacY] can show a vigorous substrate exchange in the absence of a driving ionic gradient [49, 103].) If these other reactions persist, as they do in *E. coli* (which is more difficult to starve), use of the intact cell requires special precautions; for example, we have found that much of this background activity is eliminated in stationary-phase cells grown under nearly anaerobic circumstances.

Defining characteristics. Three relevant aspects of P_i self-exchange are revealed by the study of intact cells of *Streptococcus lactis* and *Staphylococcus aureus* (68, 72). (i) Perhaps most important, such work shows that the reaction accepts monovalent P_i but not divalent P_i. Thus, the apparent K_t for P_i is constant with pH in the acid range, but rises as pH moves above the pK_2 for phosphate (pK_2 = 6.8) and monovalent P_i becomes depleted. If divalent P_i were a substrate, complex explanations for the pH sensitivity of K_t must be invoked, whereas, as it stands now, the true K_t (for monovalent P_i) is pH independent, and pH effects are confined to changes of V_{max}, as is usual in enzymology. The detailed analysis of *Staphylococcus aureus* (72) shows quantitative agreement between this model and exchange kinetics. For *Streptococcus lactis*, this argument is also supported by the findings on exchange stoichiometry (described later).

TABLE 2. Effect of osmolytes on reconstitution of phosphate exchange

Test compound (%)[a]	$^{32}P_i$ transport (nmol/mg of protein)[b]
None	36 ± 9
Proline (12)	305
Sorbitol (20)	350
Glycine (8)	410
Glucose (20)	425
Glycerol (20)	530 ± 98

[a] Octylglucoside extracts of *Streptococcus lactis* membranes were prepared in the presence of the indicated osmolyte stabilants at the stated concentrations.

[b] Proteoliposomes were made by detergent dilution and contained 100 mM KP_i (pH 7) as the internal buffer. After removing external P_i by centrifugation, $^{32}P_i$ was added to 50 μM, and steady-state isotope incorporation was determined 75 min later by membrane filtration. The mean value ± standard deviation is indicated, where appropriate. Data have been selected from the more complete analysis described elsewhere (3, 66).

Less important, but still significant, aspects of the P_i self-exchange include the observations that (i) the reaction has a very small (if any) net flux component (estimated as <0.1%), even for P_i concentration gradients in excess of 1,000-fold (Fig. 2); and (ii) the exchange reaction cannot discriminate between P_i and AsO_4 (2, 68, 72).

Reconstitution. An understanding of P_i:P_i antiport has been useful in two very different ways. On the one hand, its characteristics have strongly influenced the biochemical and molecular models of exchange. In addition, and perhaps of more general value, because of its purely exchange nature and technical accessibility, the reaction has been instrumental to development of a new approach to functional reconstitution (3, 66). Because subsequent work will draw on such experiments of reconstitution, it is best to bring up the topic at this point.

Briefly summarized, by introducing use of an unusual set of protein stabilants at the time of detergent solubilization, our approach to reconstitution has greatly simplified the search for detergent-phospholipid combinations needed to extract membrane proteins without denaturation (3, 66). The comparisons given in Table 2 outline the essential maneuver. If, for example, membrane protein is solubilized by standard techniques, using the detergent octylglucoside (79, 80, 83), the final activity of $^{32}P_i$:P_i exchange in P_i-loaded proteoliposomes is so low as to suggest a substantial denaturation of the antiporter. This is, in fact, the common finding during one's initial attempts at reconstitution, and it is for this reason that one usually screens a large number of solubilizing agents before settling on some workable condition.

Stabilants of the sort required by this new method may be found among the compounds known as "osmolytes," the compatible solutes accumulated by bacteria and animal cells in response to elevated external osmolality (105). These osmolytes include a number of amino acids, sugars, and polyols, many (all?) of which confer a general thermal stability to water-soluble proteins (9, 38). Data presented in Table 2 show a related effect during reconstitution of membrane protein. Thus, when detergent solubilization occurs in

the presence of an osmolyte, there is a markedly increased recovery (nearly 100%) of P_i exchange activity. This response is not confined to P_i-linked antiport, nor is it restricted to bacterial transport proteins. Instead, this is the general pattern found for many different membrane proteins, both procaryote and eucaryote (references 65a and 66 give other examples). The origin of this effect is not yet clear but appears to reflect that, at high concentrations, osmolytes establish conditions which favor structures whose externalized surfaces are relatively hydrophobic. This may be beneficial for several reasons. Many believe that the structure of most carrier proteins includes just this kind of disposition of surfaces (hydrophobic exterior, hydrophilic interior), and if so, we might expect to stabilize the native conformation of membrane proteins by use of such agents (see references 3, 38, and 66 for discussion). In addition, by encouraging a relative hydrophobicity, these solubilizing conditions may promote retention of any essential lipids; such lipids, if present, probably serve a general structural role, since eucaryote systems found on microsomal, lysosomal, or plasma membranes may be adequately recovered by using only the phospholipid from *E. coli* (see reference 66).

Whatever the operative mechanism, osmolyte-mediated reconstitution has removed a significant impediment to the biochemical analysis of anion exchange in bacteria, and we hope it will have a similar impact on the more general field. In this connection, a reasonable extension of principle suggests that osmolytes could be used as suitable hydrophilic substituents for detergents that might solubilize membrane protein with minimal denaturation. As it happens, a number of the detergents commonly used during solubilization and reconstitution incorporate this strategy, including the alkyl derivatives of (thio)glucose, maltose, glucamides, and betaine; digitonin; and certain of the detergents derived from bile acids (see reference 78 for detergent structures). If such detergents are effective for the same reasons that osmolytes promote functional reconstitution, then attempts to use appropriate derivatives of other osmolytes might prove especially instructive. Biology may even have anticipated this action, for the lipids of gram-positive cells are often modified by sugars or amino acids (39), and one might speculate that such modification acts to stabilize integral membrane protein during its insertion or to accelerate the renaturation of secreted proteins after their export. In fact, during solubilization and reconstitution of P_i-linked exchange, phospholipids extracted from the gram-positive *Streptococcus lactis* are more effective than those from gram-negative *E. coli* (3), and preliminary work (unpublished data) with the gram-positive *Staphylococcus aureus* suggests that functional reconstitution from this cell is less dependent on use of osmolytes than is reconstitution from *E. coli*. These anecdotal observations await a coherent set of experiments to address such questions.

Phosphate Exchange and Sugar Phosphate Transport

Identification of sugar phosphates as substrates. The finding of a P_i self-exchange in *Staphylococcus aureus* led to speculation that the reaction found in resting cells was modified to provide a mechanism for net P_i import during growth (72, 75). But this proposition was much less likely in *Streptococcus lactis*, in which the rate of exchange was far in excess of that needed to supply phosphorous to the cell (68). For that case, it was instead more sensible to imagine that $^{32}P_i$:P_i exchange reflected a reaction directed to the transport of something else, presumably a phosphorylated compound.

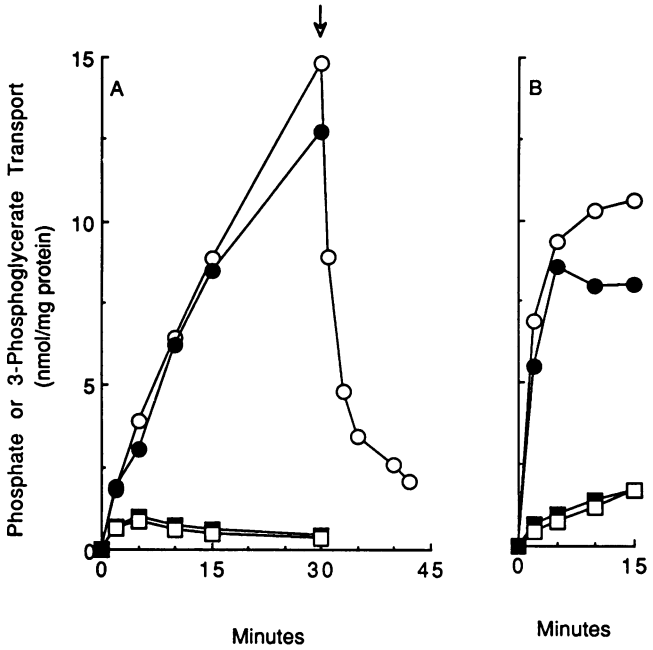

FIG. 3. P_i self-exchange mediated by PgtP activity in *Salmonella typhimurium*. A GlpT-negative (fosfomycin-resistant) derivative of *S. typhimurium* LT-2 (ATCC 23564) was grown to stationary phase in a capped tube, using minimal medium with 0.5% 3-phosphoglycerate as a source of carbon. Washed cells were placed in a MOPS-buffered medium at pH 7 (68) and given either 100 μM $^{32}P_i$ (A) or 50 μM 3-[^{14}C]phosphoglycerate (B). Symbols: (○), control with no further additions; (●), with 3 mM G3P; (□), with 3 mM 3-phosphoglycerate; (■), with 3 mM phosphoenolpyruvate. At the arrow, 30 mM KP_i was added to elicit a P_i exchange (Varadhachary and Maloney, unpublished data).

The initial tests of this idea involved examining the inhibition of $^{32}P_i$:P_i exchange by putative alternate substrates, and this approach soon identified a small group of sugar 6-phosphates whose presence at relatively low concentration gave a substantial block of $^{32}P_i$ entry during assays of exchange (68). To outline this approach, the experiment of Fig. 3 shows the behavior of PgtP in a GlpT-negative derivative of *Salmonella typhimurium*. In this case, induced cells were given $^{32}P_i$ or 3-[^{14}C]phosphoglycerate in the presence of unlabeled competitors. Incorporation of the labeled materials was blocked only by PgtP substrates (2- or 3-phosphoglycerate and phosphoenolpyruvate) and not by G3P, a sugar phosphate handled by the independent system, GlpT.

Verification that sugar phosphate inhibitors of P_i self-exchange are authentic substrates of an exchange reaction has also required tests in subcellular preparations that allow an experimental control over *cis* and *trans* compartments. Thus, it was important to show directly that sugar phosphate was taken up by membrane vesicles or proteoliposomes, without provision of metabolic energy (respiration, etc.) and that this depended on use of a suitable *trans* substrate. This kind of evidence is illustrated by the experiment of Fig. 4, which describes the reconstitution of UhpT function from *E. coli* (95). In that case, incorporation of sugar phosphate into P_i-loaded proteoliposomes was correlated directly with the initial load of internal P_i. In the same experiment, it was also possible to document the equivalence of P_i and AsO_4 in a gram-negative system, since proteoliposomes loaded with 30 mM KP_i or 30 mM $KAsO_4$ gave identical responses. Finally, note that the two negative controls in these trials establish

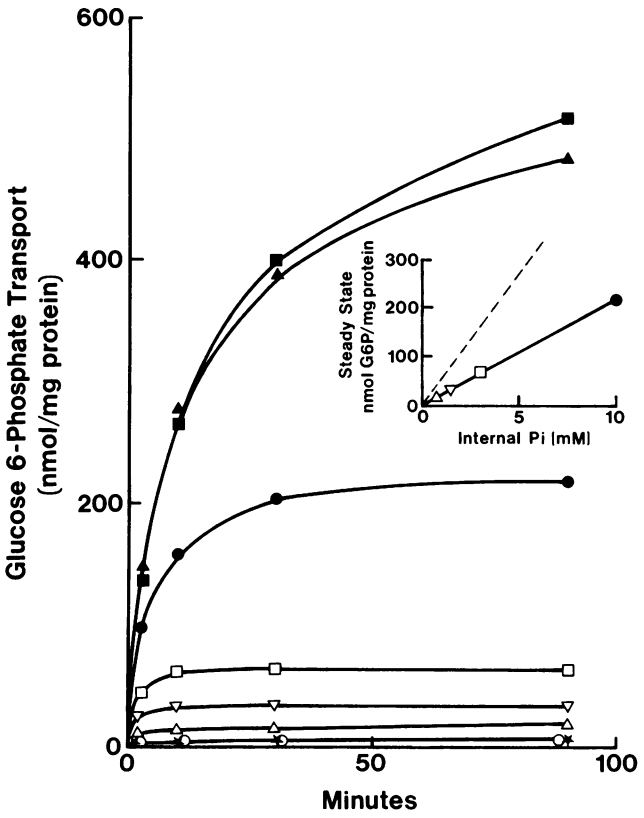

FIG. 4. Reconstitution of phosphate:sugar phosphate antiport in *E. coli*. Membrane protein from a UhpT overproducer was reconstituted into proteoliposomes containing 0 (○), 0.75 (△), 1.5 (▽), 3 (□), 10 (●), and 30 mM KP_i (■) or 30 mM $KAsO_4$ (▲); 30 mM internal KP_i (★) was used for reconstitution of protein from an otherwise isogenic strain with a transposon insertion into *uhpT*. In all cases, MOPS/K was also present to bring total internal buffer (pH 7) to 100 mM. After loading by detergent dilution, proteoliposomes were centrifuged and suspended in an assay buffer with 75 mM K_2SO_4, 20 mM MOPS/K (pH 7), and 2.5 mM $MgSO_4$. Transport was estimated by membrane filtration after addition of 35 μM [^{14}C]G6P. (Inset) The observed steady-state levels of G6P accumulation are correlated with the initial concentration of internal KP_i. The linear correlation indicates a bulk exchange stoichiometry of 2.5:1 (Pi: G6P); the dotted line gives the relationship expected of a 1:1 exchange stoichiometry. From reference 95 with permission.

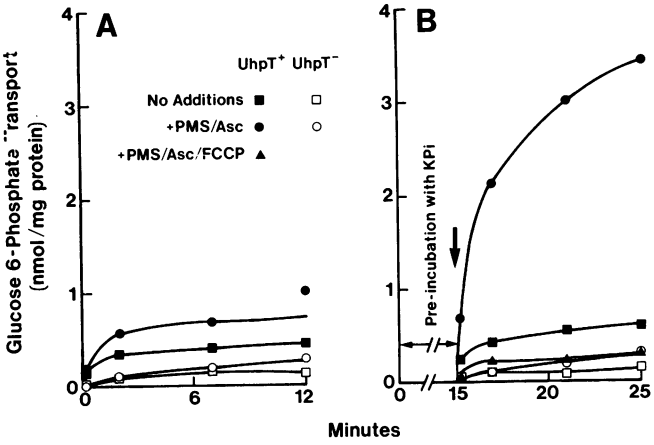

FIG. 5. Evidence that G6P transport by *E. coli* requires a suitable countersubstrate. Right-side-out membrane vesicles of UhpT-positive and UhpT-negative cells (legend to Fig. 4) were prepared to contain 30 mM MOPS/K–25 mM K_2SO_4 (pH 7). For assay of sugar phosphate transport, vesicles were placed in 20 mM MES (morpholineethanesulfonic acid)/K (pH 6)–125 mM K_2SO_4, along with 0.25 mM Na_3VO_4 to inhibit residual phosphatase(s), and, when indicated, 0.12 mM phenazine methosulfate plus 33 mM ascorbate (PMS/Asc) to establish a proton motive force, using respiration. The protonophore carbonylcyanide *p*-trifluoromethoxyphenylhydrazone (FCCP) was used at 5 μM, where indicated. (A) Sugar phosphate transport was initiated by addition of 0.1 mM [^{14}C]G6P at time zero. (B) In a parallel experiment, addition of [^{14}C]G6P was delayed until vesicles had been incubated with 0.1 mM KP_i for 15 min. Under these conditions, respiration drives P_i accumulation to at least 4 nmol/mg of protein in vesicles of either UhpT-positive or UhpT-negative strains; such P_i accumulation can be blocked by FCCP. From reference 95 with permission.

both a biochemical and a genetic specificity: substrate accumulation was not found for MOPS (morpholinepropanesulfonic acid)-loaded particles (as expected of an exchange reaction); nor was sugar phosphate taken up by P_i-loaded proteoliposomes made with protein from a UhpT-negative strain.

Exclusion of a symport mechanism. Work that centers on quantitative aspects of P_i-linked exchange (Fig. 4; see below) confirms that antiport can occur, but this does not constitute proof of a biochemical structure that mediates antiport as an exclusive reaction mechanism. In particular, it has been most important to address the possible operation of nH^+/G6P symport. This is more than just a formal consideration, since we know that such proteins as the H^+/lactose symporter show a substrate exchange which is much faster than the net reaction (49, 103). (This tendency is exaggerated in certain LacY mutants [20, 21], so much so that they might be viewed as sugar:sugar antiporters. This raises an interesting point. If antiport and symport can be related through the change of a single amino acid residue, is it truly convincing to use sequence information alone to infer reaction mechanism (cf. discussion of Table 1)? Or is it only that symport, antiport, and uniport are as closely related structurally as they are kinetically [73]?) For this reason, one could imagine that UhpT and related systems are actually ion-coupled symporters which simply exchange their various substrates (P_i, G6P, etc.), absent an ion-motive force. This incidental exchange reaction is very different from the idea of a mechanistic antiport. Evidence bearing on this issue comes from work with *Staphylococcus aureus* and *E. coli* membrane vesicles prepared in the absence of P_i. In *E. coli*, such MOPS-loaded vesicles, from either wild-type or UhpT-negative cells, accumulate substrates by H^+-coupled systems when allowed to respire; judged by the sustained gradients of P_i or proline, these oxidation reactions generate a cation-motive force near 140 mV (95). Nevertheless, such vesicles fail to take up sugar phosphate (Fig. 5A). Instead, appearance of UhpT function requires a preincubation to establish a pool of internal P_i; only then is there a restoration of the activity found in cells (Fig. 5B). This finding, that UhpT has a strict requirement for some substrate in *trans*, excludes operation of a traditional form of symport and argues rather strongly in favor of the idea of antiport.

Substrate Specificity of P_i-Linked Exchanges

Experiments of the sort detailed in the preceding paragraphs have allowed us to conclude that P_i-linked exchange systems (Table 1) are alike in that they each operate by an antiport mechanism. These antiporters differ, however, in

TABLE 3. Substrates of P_i-linked anion exchange[a]

Substrate	System K_t or K_{iapp} (μM)			
	Staphylococcus aureus	*Streptococcus lactis*	*E. coli* UhpT	*E. coli* GlpT
Inorganic				
P_i	*2,300 ± 400*	*250 ± 40*	*1,210 ± 100*	1,000[b]
AsO_4	1,500	300 ± 30	1,200 ± 175	
Organic				
2dG6P		*26 ± 4*	50 ± 7 (+)[c]	
G6P	*27 ± 2*	20 ± 3	130 ± 16 (+)	
M6P		23 ± 7	91 (+)	
F6P		150 ± 7	140 (+)	
$GluNH_26P$		420 ± 80	760 (+)	
R5P		850	(+)[d]	
αMeG6P		740 ± 30	(+)	
F1P		1,160 ± 60	(+)	
Gal6P		>2,000	>4,000	
G1P		>2,000	>4,000 (+)	
G3P	*1,300 ± 190*	>2,000	>4,000 (+)	
PEP		>2,000		

[a] Unless otherwise indicated, kinetic parameters were measured by using P_i-loaded membrane vesicles or proteoliposomes; mean values ± standard errors are given, when appropriate. The data come from measurements of K_t (in italics) or of the apparent K_i, K_{iapp}. K_{iapp} is the concentration giving 50% inhibition of $^{32}P_i$:P_i exchange when $^{32}P_i$ is low relative to its own K_t (0.1 to 0.25 K_t). K_{iapp} is 80 to 90% of true K_i (or K_t), given simple competitive interaction (4, 46, 68). Values for the sugar phosphate transport systems of *Staphylococcus aureus* and *Streptococcus lactis* are from earlier work (2, 3, 68, 95, 96), as are data for GlpT (1); information on UhpT in *E. coli* was collected for this publication. Abbreviations not previously specified: 2dG6P, 2-deoxyglucose 6-phosphate; F6P, fructose-6-phosphate; $GluNH_26P$, glucosamine 6-phosphate; R5P, ribose 5-phosphate; αMeG6P, α-methylglucoside 6-phosphate; F1P, fructose 1-phosphate; Gal6P, galactose 6-phosphate; G1P, glucose 1-phosphate.

[b] Calculated from earlier work (1) assuming a 5:1 ratio of maximal velocities for homologous and heterologous exchanges (Table 1).

[c] Previously reported as a substrate (+) of UhpT (25).

[d] Arabinose 5-phosphate is reported as a UhpT substrate (25); ribose and arabinose 5-phosphates differ only in the orientations of –H and –OH about C-2.

their choice of primary substrate. Table 3 summarizes these differences as revealed by kinetic tests designed to avoid certain technical difficulties. For a few cases, this analysis suggests some revision to earlier conclusions.

Technical concerns. In constructing this kind of summary, three general problems are encountered. Information from the earlier literature sometimes comes from experiments with a cell or vesicular system in which external P_i had been present, and in such cases quantitative information is made less certain by the likely competition between P_i and the organic phosphate. For example, reports of the Michaelis constant for G6P transport by UhpT have varied over a nearly 10-fold range, largely due to the different levels of P_i in the assay mixtures (29). A separate issue arises for *E. coli* and *Salmonella typhimurium*, since these gram-negative cells have periplasmic enzymes (phosphatases, isomerases, and mutases) that pose a particular problem. These enzymes are often quite active, and if they remain associated with membrane vesicles, one can overestimate the breadth of acceptable substrates. This appears to be the case for glucose 1-phosphate (G1P), which has been considered a substrate for UhpT in *E. coli* on the basis of work with both cells and membrane vesicles (25). In our hands, too, tests in vesicles make it appear that G1P is a substrate, for its addition to $^{32}P_i$-loaded vesicles causes immediate loss of internal label. However, this effect is not reproduced in proteoliposomes, where G1P proves to be a poor substrate, if one at all. The same appears true for G3P, which has also been reported to be a substrate of UhpT (25), but which does not behave so in proteoliposomes (Table 3). Rather than invoke strain differences, we suggest that it is more likely that, in cells or vesicles (and especially vesicles from *E. coli* K-10 or K-12 strains), the adventitious presence of periplasmic enzymes may allow conversion of precursors into authentic substrates. Fortunately, in some cells these problems are not as serious. The streptococci appear to lack surface isomerases and mutases, and possess phosphatases which are easily blocked by *ortho*-vanadate (2, 68), without effect on P_i-linked exchange. As a result, there is an added confidence in the qualitative and quantitative conclusions derived from studies of a cell like *Streptococcus lactis* (Table 3). Even so, interconversion of things as metabolically reactive as sugar phosphates should be anticipated as a general problem.

Lastly, it has been convenient to use P_i-loaded cells, vesicles, or proteoliposomes to identify substrates of P_i-linked exchange. This avoids the time-consuming (at times impossible) alternative of loading each suspected substrate in *trans* so that a completely homologous exchange can be tested. Convenient though it is, this strategy may introduce unexpected problems if P_i and the test substrate have very different affinities for the process. This is often true (Table 3), in which case the kinetics of heterologous exchange can become complex. As an exercise, imagine the progress of UhpT-mediated G6P transport by *Staphylococcus aureus* into proteoliposomes loaded with 10 mM P_i. Because of the 100-fold difference in the K_t for P_i and G6P, the incorporation of labeled G6P will begin to slow after only a modest accumulation of internal G6P as this high-affinity substrate competes with the low-affinity internal P_i for exit. When internal G6P has reached only 1 mM, the rate of its subsequent net entry will have been reduced to 10% of its initial value, reflecting the new partitioning of carriers among productive (P_i:G6P) and futile (G6P:G6P) reaction modes. Viewed from the perspective of internal P_i, there is a rapid release of a small portion of the internal pool, followed by a very much slower loss of the remainder. (This does not consider the further complexity required to account for the effects of the different maximal velocities associated with the different reaction modes.) This, in turn, shows how difficult it might be to screen substrates by the release of $^{32}P_i$ brought in previously by exchange; at similar concentrations, low-affinity substrates may give a more prompt and effective chase than high-affinity substrates (see the example in reference 96). For all of these reasons, it has turned out to be most sensible to rank test substrates by how well they inhibit $^{32}P_i$ entry rather than by how well they provoke an efflux. When further direct studies are warranted, one uses very high (100 mM) internal P_i and restricts analysis to the earliest possible times.

Because these technical concerns significantly affect the interpretation of experiments aimed at defining substrate specificity, Table 3 collects information from studies in which these potential difficulties have been circumvented, insofar as possible. For the most part, this has required a cell, vesicle, or proteoliposome preparation with high (50 to 100 mM) internal P_i and low (ca. 100 μM) external substrate, where metabolic interconversion has been limited, and where possible kinetic problems have been avoided, to the extent feasible. In addition, these data (Table 3) emphasize quantitative measures, such as Michaelis constants for substrate transport (K_t) or inhibition constants (K_i) determined during $^{32}P_i$:P_i exchange. When these criteria have over-

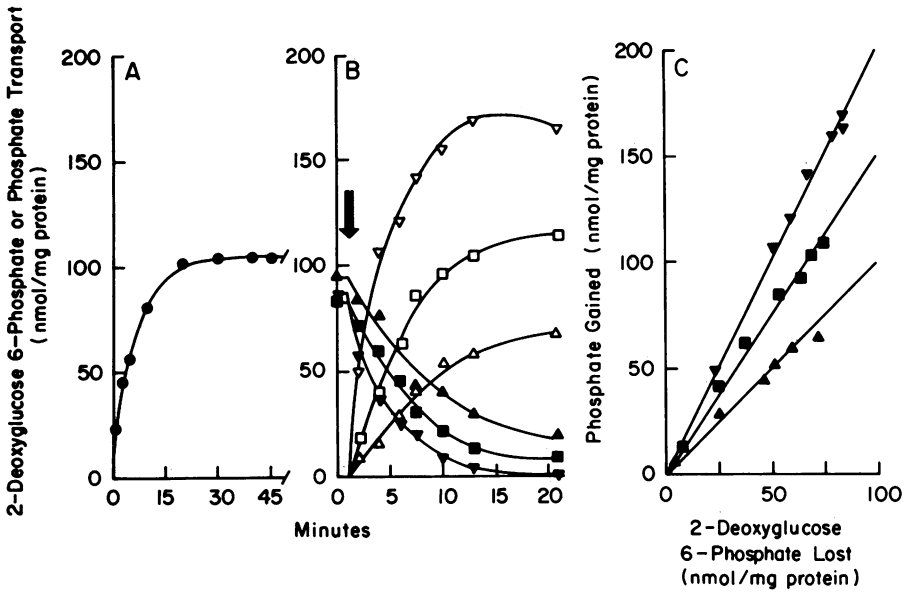

FIG. 6. Stoichiometry of P_i-linked exchange in *Streptococcus lactis*. (A) Transport of [^{14}C]2dG6P was measured for vesicles loaded with 50 mM KP_i (pH 7) and suspended in 125 mM K_2SO_4–20 mM MOPS/K (pH 7)–0.25 mM Na_3VO_4. (B) After 45 min, vesicles were centrifuged, suspended without sugar phosphate (82% recovery of protein), and redistributed to tubes having buffers at pH 7 (▼, ▽), pH 6.1 (■, □), and pH 5.2 (▲, △); in the latter two instances, MES/K replaced MOPS/K. Each tube was tested for sugar phosphate content 10 min later, after which 3 mM $^{32}P_i$ was added (arrow). Subsequently, samples were taken to evaluate ^{32}P gained (open symbols) and ^{14}C lost (closed symbols). (C) Phosphate gained is correlated with sugar phosphate lost, using symbols as in panel B. The lines have slopes of 1.1 (pH 5.2), 1.5 (pH 6.1), and 2 (pH 7). From reference 4.

lapped, there is excellent agreement (2, 4, 96), but one should be aware that coarser tests could give very different impressions of substrate specificity. For example, because the usual bacteriological media use carbohydrates at saturating concentrations (5 to 40 mM), various sugar phosphates may support identical growth rates while differing in their kinetic behavior by orders of magnitude. The more quantitative comparisons were preferred for Table 3.

Substrate specificities. The summary of Table 3 gives data for well-characterized P_i-linked exchange systems. As a group, these systems share P_i and AsO_4 as substrates, but otherwise each protein tends to respond to a distinctive set of organic phosphates. It is evident that the best-characterized examples are the *Streptococcus lactis* and *E. coli* systems, each of which handles sugar 6-phosphates. Clearly, while these two differ in their reactivity to P_i (AsO_4), they have much in common in their responses to other substrates. For each there is a preference for G6P and M6P, exclusion of the corresponding sugar 1-phosphates (see above), and a bias in favor of the six-membered pyranose ring. Moreover, neither system responds to galactose 6-phosphate (Gal6P) (see references 25 and 68), indicating that the —OH group at C-4 should lie below the plane of the ring (as in G6P and M6P), and in both instances groups at the C-2 position are not strictly controlled (M6P, G6P, and 2-deoxyglucose 6-phosphate [2dG6P] differ in this respect). This latter impression is reinforced if one proposes that glucosamine 6-phosphate ($GluNH_26P$) is accepted with its amino group in the neutral ($-NH_2$) rather than charged ($-NH_3^+$) form; if so, the true K_i of the amino sugar phosphate is comparable to that for G6P or M6P.

Studies of substrate specificity have revealed a closely similar rank order of substrate preference for the *Streptococcus lactis* and *E. coli* systems that transport G6P, and in these same cases there is a curious relationship between anion exchange and the phosphotransferase system. As noted after analysis of the streptococcal system (2, 68), substrates of exchange are at the same time products of enzyme $II^{mannose}$, the main constitutive element of the phosphotransferase system. This may be an unimportant coincidental finding; it may express an underlying biochemical similarity or even a biological attempt to balance the effects of irreversible (phosphotransferase) and reversible (anion-exchange) mechanisms.

Stoichiometry of P_i-Linked Exchange

While the preceding material has been largely descriptive, the present discussion will focus closely on two special aspects of the antiport reaction: the stoichiometry of exchange and the striking effect of pH on this process. These topics have become important to the construction of biochemical and molecular models.

For technical reasons, it has proven most sensible to rely on studies of antiport in *Streptococcus lactis* for measurements of stoichiometry. This is because the K_t for P_i is relatively low in this organism (Table 3), so that in this system it is easier to observe and to measure an extensive net heterologous exchange. Such experiments are usually performed in two steps (Fig. 6) (4). In the example shown, P_i-loaded membrane vesicles were first allowed to accumulate sugar phosphate at pH 7 (Fig. 6A) until steady state was reached. At that point, vesicles were collected by centrifugation, suspended in substrate-free buffer, and then distributed to three different tubes, using buffers at pH 7, pH 6.1 (the pK_2 for sugar 6-phosphate), and pH 5.2. After further incubation (10 min) to allow pH equilibration, and after a sampling to verify retention of 2dG6P, the second part of the experiment began with addition of excess $^{32}P_i$ (Fig. 6B); stoichiometry was then determined by matching $^{32}P_i$ gained

TABLE 4. Effect of pH on kinetic constants for heterologous exchange[a]

pH	V_{max} (nmol/min per mg of protein)	K_t (μM)	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[b]	8.2 ± 1.2

[a] Values are mean ± standard deviation of three or four experiments, using *Streptococcus lactis* membrane vesicles loaded with 50 mM KP_i. The apparent K_i for 2dG6P was measured at 75 μM external $^{32}P_i$, using 2.5 to 100 μM inhibitor; uninhibited $^{32}P_i$ transport was 55 ± 2.6 (pH 7) or 6 ± 0.5 (pH 5.2) nmol/min per mg of protein. Given purely competitive interactions, the true K_i for sugar phosphate would by 77% of the value given.

[b] Includes one trial with vesicles made from cells fully induced for the antiport reaction. In these vesicles, maximal velocity of 2dG6P transport was 97 (pH 7) or 12 (pH 5.2) nmol/min per mg of protein. Data are taken from reference 4.

with [^{14}C]2dG6P lost. Those comparisons (Fig. 6C) establish that, at any given pH, P_i and 2dG6P move against each other at some characteristic ratio and that this ratio is the same at all times during exchange. Thus, for the usual conditions of assay (pH 7), this stoichiometry reflects $2P_i$ moving in exchange for a single G6P. Earlier work (2) had also found this value and had shown that stoichiometry was independent of the direction of substrate fluxes. More significant, however, it is now apparent that pH has prominent effects that deserve a close look.

Exchange is affected in two important ways by variation of assay pH. First, acidification slows the reaction, as might be expected. This turns out to be entirely a velocity effect, and as pH falls from pH 7 to 5.2, the V_{max} of P_i:2dG6P exchange is reduced 10-fold, with little change in the K_t for 2dG6P or in the K_i for sugar phosphate inhibition of P_i:P_i exchange (Table 4). Moreover, this depressive effect of low pH is a general one, since it affects to the same degree the heterologous (P_i:2dG6P) and homologous (P_i:P_i) reactions (Table 4 and footnote). Accordingly, the pH independence of sugar phosphate K_t (and K_i), in a range that spans the pK_2, implies that monovalent and divalent sugar phosphate are equally effective as substrates. This finding is important and should be considered in light of the specificity for the monovalent P_i anion as inferred by analysis of P_i self-exchange. The second event associated with changes in pH is perhaps the more intriguing: pH has a direct impact on stoichiometry itself, and the exchange ratio moves from an upper limit of 2:1 (P_i2dG6P) at pH 7 to a lower limit of 1:1 at pH 5.2. This, too, is an observation that must be accommodated by any successful mechanistic model.

A Biochemical Model for Anion Exchange

To this point, there has been an emphasis on the recitation of facts and on a straightforward interpretation of such information. The goal of the present discussion is to present an explicit model that organizes these facts into a context that might guide new experiments.

A simple biochemical model. Fig. 7 shows a biochemical model that incorporates the findings related to ionic selectivity and substrate specificity of exchange. The model proposes that the carrier protein has a bifunctional active site which accepts either two monovalent anions (Fig. 7A, right) or a single divalent species (Fig. 7A, left), but not both, as a prerequisite to transport. Perhaps two positively charged groups on the protein must pair with negative charges on the substrate(s) before movement over the membrane occurs. At the least, by requiring that a single catalytic surface bind the same number of charges as either mono- or divalent anions, one ensures an electroneutral exchange which acts with simple Ping-Pong kinetics as the protein samples at random the *cis* and *trans* compartments. Given a preference for monovalent over divalent P_i (as observed during P_i:P_i exchange), this model naturally accommodates the finding of a 2:1 exchange stoichiometry (P_i:G6P) at pH 7, since at this pH the high-affinity sugar phosphate is mostly available as a divalent substrate. It would not be an oversimplification to view this version of P_i-linked exchange as a biological analog of the ionophore, A23187, an ionophore which uses two carboxylate anions to bind either a pair of H^+ or a single Ca^{2+} (Mg^{2+}) during a 2:1 (H:Ca) neutral antiport.

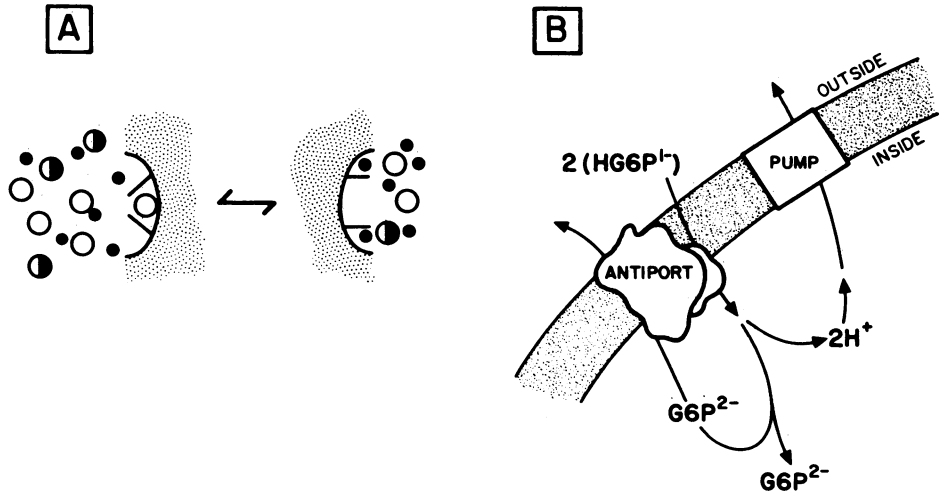

FIG. 7. Biochemical and cellular models of P_i-linked exchange. (A) The diagram shows a carrier with a bifunctional active site that requires occupancy by two negative charges before reorientation; this requirement is satisfied by binding of a pair of monoanions (right) or a single divalent anion (left). The reorientation step (left ↔ right) is presumed to be rate limiting. The model predicts an exchange stoichiometry that reflects the averaged result of molecular exchanges that are either 2:1 or 2:2; the latter exchange is evident, macroscopically, as a 1:1 event. Symbols: (●) monovalent P_i; (◑) monovalent sugar phosphate; (○) divalent sugar phosphate. (B) A consequence of the model in panel A is that net sugar phosphate accumulation can occur in the presence of a pH gradient. From references 4 and 67 with permission.

This biochemical model (Fig. 7A) prompts two specific questions concerning the substrate specificity of the monovalent and divalent binding surfaces. Does the divalent binding site accept divalent phosphate in addition to divalent sugar phosphate? And do the monovalent binding sites accept monovalent sugar phosphate anions as well as monovalent P_i? The experimental answers to these questions have relied on tests of exchange stoichiometry when variations of assay pH are used to enrich for one or the other of these ionic species. For example, when stoichiometry is measured at pH 8, at which both phosphate and sugar phosphate are mainly divalent, one expects that stoichiometry should move from 2:1 to 1:1 if the divalent binding site accepts both the inorganic and organic substrates as divalent anions. Since stoichiometry was 2:1, even at pH 8 (4), we have concluded that divalent P_i is not a substrate of the antiport reaction. (Such statements really mean that the ratio K_t/V_{max} is at least 10-fold higher for divalent P_i than for monovalent P_i.) The answer to the second question is arrived at in much the same way: the possible role of monovalent sugar phosphate is inferred by using results obtained as pH is lowered past the pK_2 so as to enrich for the monovalent anion. Thus, if the monovalent binding sites accept both inorganic and organic substrates, one predicts (again) a fall in exchange ratio from 2:1 at pH 7 to a low of 2:2 (= 1:1) at pH 5, as pairs of monoanions move against each other. Now, in anticipation of this argument, the relevant experiment had been presented earlier, with the interesting conclusion that the antiporter accepts both mono- and divalent sugar phosphate (Fig. 6 and text). In the present setting, then, we take this to mean that the monovalent binding sites (Fig. 7A) can accept either inorganic or organic phosphates, albeit with somewhat different preferences (Table 3).

To summarize, then, a detailed look at the biochemical model (Fig. 7A) distinguishes two kinds of substrates: monovalent forms (monovalent P_i, sugar phosphate, etc.) that exchange in the ratio of 2:2; and divalent forms (sugar phosphates alone) that exchange at 1:1. Macroscopic stoichiometry must consequently be a mixture of these elementary events, each weighted according to the relative abundance of acceptable substrates in the *cis* and *trans* compartments and the relative strengths of their interaction with the carrier. Accordingly, it might be expected that pH alters exchange stoichiometry, since the pK_2 for sugar 6-phosphates is within a physiological range. This means, of course, that responsibility for such "variable" stoichiometry is given to the substrate and that the protein catalyst plays no instructive role. This differs from the usual interpretations (54, 84).

Immediate consequences of the biochemical model. Although the details of the model of Fig. 7A derive largely from experiments with *Streptococcus lactis*, the general features of this idea are consistent with observations in *E. coli* (Fig. 4 and text), and in a satisfying way they can be used to reinterpret otherwise paradoxical findings in this latter cell type. For example, in *E. coli*, G6P accumulation is mediated by the UhpT protein, and while we know that UhpT operates to exchange anions (Fig. 5 and text), it should be acknowledged that earlier and careful studies by Essenberg and Kornberg (28) have implicated a symport (nH^+/G6P) mechanism; indeed, much evidence points to symport as an appropriate functional view for this system (28, 101). Happily, it is now possible to reconcile these and other conflicts simply by extending the biochemical model (Fig. 7A) to examine its physiological consequences (Fig. 7B).

From previous discussion, one might presume that heterologous antiport (e.g., P_i:G6P) is the preferred reaction in vivo, since the homologous exchanges (P_i:P_i, G6P:G6P, etc.) do not (normally) lead to net fluxes. But in bacteria, the cytoplasm is usually more alkaline than the periplasm, and this allows the otherwise futile self-exchange of sugar phosphate to be put to good use. Thus, the biochemical model predicts a cycle (Fig. 7B) in which net G6P accumulation arises from the exchange of two monovalent anions and a single divalent species, yielding the net import of $2H^+$ and $1G6P^{2-}$. Phenotypically, this would appear as a symport with protons, resolving any conflict between thermodynamics (symport) and mechanism (antiport).

The perspective accompanying this physiological model (Fig. 7B) allows one to understand still another observation in *E. coli*. It is known that mutants which lack Pit and Pst (the usual P_i transport systems) can nevertheless survive when G6P or G3P is used in the growth medium. But this observation is puzzling if UhpT or GlpT operates only to give heterologous exchange ($2P_i$:G6P or $2P_i$:2G6P), since these reactions do not provide for net P_i entry. On the other hand, a asymmetric self-exchange using sugar phosphate accommodates these findings in a straightforward way by allowing either UhpT or GlpT to serve as the port of net entry of P_i.

Physiological Interactions

It is evident that the biochemical model (Fig. 7A) carries with it implications for understanding exchange in other settings. For these more general issues, the idea of an asymmetric self-exchange using sugar phosphate has been especially valuable (Fig. 7B), for it avoids an overreliance on the heterologous reaction as the only mode of operation. But neither should there be too rigid an application of the idea of sugar phosphate self-exchange. Rather, the aim of that earlier discussion was to show only that the biochemical model specifies a credible approach to understanding events on some larger scale. Only by achieving some appropriate balance of all possible reaction modes will one understand the biology associated with P_i-linked exchange. Three examples illustrate this point.

Titrating carbon against phosphorus. The picture of exchange given by Fig. 7B is realistic, but hypothetical. Even if it does occur (as is likely), it is only one of several exchange modes that must operate if G6P is to provide both carbon and phosphorus. This must be so, because bacteria (*E. coli*) have a carbon/phosphorus ratio of about 40:1 (mol:mol) (62). Clearly, sugar phosphates bring with them too little carbon and too much phosphorus, so that the maintenance of a physiological C/P ratio during growth on something like G6P requires the presence of a pathway(s) to remove excess phosphorus. One might imagine that this is accomplished by the coordination of a regulatory mechanism(s) provoked by fluctuations of metabolic pools. While this may happen, it is simpler to consider first only the predicted properties of P_i-linked exchange (Fig. 7). For example, one can readily see that mass action will specify within the population of antiporters some equilibrium mixture of (i) exchanges that bring both carbon and phosphate into the cell ($2[HG6P^{1-}]$:$[G6P^{2-}]$, inward/outward); (ii) antiport reactions that are neutral for P_i balance ($2[HG6P^{1-}]$:$2[H_2PO_4{}^{1-}]$); and (iii) those which extrude P_i in the net (as in $G6P^{2-}$:$2[H_2PO_4{}^{1-}]$). Therefore, the desired C/P balance could be achieved if the first two exchange modes occur in the ratio (*a*/*b*) of about 6:1 (C/P = 42:1). Or one could suggest a mixture of the first and last reactions, in a ratio (*a*/*c*) of 7:5

(C/P = 36:1) or 11:8 (C/P = 38:1). Or perhaps all three reaction modes are found in some suitable distribution; and so on. At the least, we conclude that the physiology of these matters must be complex, even without suggesting that regulation could influence the allocation of carriers among these reaction types or the activity of other systems. If such regulation does exist (and it probably does), it is likely an indirect one that centers on control of periplasmic or cytosolic pH and the relative sizes of the inorganic and organic phosphate pools, so that the appropriate balance of exchange modes becomes determined by mass action.

P_i balance. The balancing of carbon and phosphorus during growth on G6P or G3P illustrates a P_i "shock" in which there is a need to eliminate excess material and, to satisfy that demand, it was enough to use statistical arguments based on the properties of P_i-linked exchange. This style of reasoning has added value in that it also rationalizes several other instances of apparent P_i shock, particularly those concerning G3P. If growth of G3P is mediated by P_i-linked exchange (GlpT), we may presume, as before, that some distribution of reaction modes exists and that the ensemble activity of GlpT antiporters effectively reduces the net C/P ratio of incorporated material to a suitable value. What if *E. coli* instead transports G3P by the solute ATPase, UgpT (94)? If so, the cellular C/P ratio could not be adjusted as described for GlpT, because UgpT is irreversible in a biological setting: as an ATPase, it lacks the flexibility to operate as a pathway for both G3P influx and P_i efflux, unlike GlpT. For this reason, we might now understand why cells thrive when G3P enters via GlpT, but fail to grow when G3P enters through UgpT; the latter cells can use G3P as a source of phosphorus but require a second pathway to provide for carbon entry (94); presumably, this avoids a toxic C/P ratio.

P_i-linked exchange is, understandably, considered a mechanism for the inward transport of carbon and phosphorus. However, the reversible nature of the system suggests that it might also be used to accumulate P_i under some conditions of P_i starvation. This could be accomplished by bringing in a pair of monovalent P_i anions at the expense of a divalent organic phosphate (e.g., $2P_i$:G3P or $2P_i$:G6P). Could this help explain the curious finding that GlpT is often constitutively expressed, especially in *E. coli* K-10? K-10 strains can otherwise rely only on a solute ATPase (Pst) for P_i accumulation (88), and this system has the double disadvantage of being both irreversible (see above) and P_i repressible. By exporting divalent G3P in exchange for a pair of monovalent P_i anions, constitutivity on the part of GlpT would overcome each of these deficits. (In a metabolic sense, P_i starvation can also be induced by overindulgence in sugars transported by the phosphotransferase system. In that case, to lower the cellular level of sugar phosphate and to replete the P_i pool, one might also suggest the export of divalent organic phosphate in exchange for the accumulation of two monovalent phosphate anions. It has been suggested [68] that, for some gram-positive cells, this may be an alternative to "inducer expulsion" [85, 86].) Superficially, the metabolic cost of this maneuver seems excessive, since the cell loses 1 mol of glycerol for each mol of P_i gained. On further thought, this lifestyle is not so extravagant, for either of two reasons. Glycerol is highly permeant, and once excreted it has a reasonable probability of recapture by phosphorylation after free diffusion from the periplasm back into the cell. To the extent that this occurs, the cost of P_i transport is limited to that of (re)phosphorylation, a respectable 1ATP/P_i. As discussed by Berg (13), the probability of this recapture is reasonable even for a single cell sitting alone in an infinite medium. In a population of some size (10^7/ml or greater), recapture becomes more efficient still, as the infinite medium is replaced by neighboring cells (65). In that case, because only glycerol at the fringes is subject to permanent loss, the expense of P_i transport can approach its overall lower limit of 1ATP/P_i (see reference 65 for further comments on such population effects). It is also worth noting that this same lower limit is reached when the carbon source is available in excess and that, in both of these instances, P_i transport can be seen as driven by a circulation of carbohydrate.

P_i circulations. In discussion of the responses to P_i excess of P_i starvation, priority was given to circumstances that emphasized operation of P_i-linked exchange in isolation. Of course, these antiporters do interact with other membrane reactions, and among possible chemiosmotic couplings, the most likely concerns an anion (P_i) circulation whose net effect is to mediate sugar transport. Thus, when nH^+/P_i symport operates in parallel with G6P:$2P_i$ antiport, there is an accumulation of glucose with no effect on the P_i pool, and the physiological reaction is nH^+/glucose symport. Whether this cycle contributes significantly to sugar movement would depend largely on P_i and G6P concentrations relative to their affinities for the carrier, and for this reason it would seem more likely to occur in the streptococci, for which the K_t for P_i is much lower than in other examples (Table 3). Nevertheless, since most bacteria accumulate high internal P_i under conditions of nutrient depletion and in the stationary phase, this reaction cycle might become realistic in other cases.

A MOLECULAR MODEL OF MEMBRANE TRANSPORT

Discussion has so far centered on the biochemical and cellular aspects of P_i-linked exchange, but it should not be overlooked that these antiport systems may both illustrate and contribute to our understanding of the general structure of secondary carriers.

A Likely Topology for P_i-Linked Exchange

The amino acid sequences of UhpT and GlpT (27, 37) are punctuated by segments, each of about 20 to 25 residues, whose overtly hydrophobic character could account for as many as 12 α-helical columns which might traverse the membrane. When this assumption is incorporated into a prediction of secondary structure (27, 37; R. Kadner, personal communication), one is struck by the fact that these putative hydrophobic columns are organized as two clusters of six columns each, with a intervening cytoplasmic loop (Fig. 8), suggesting a dimer as the organizing unit of overall structure. This now seems to be true of many transporting systems (see below), but more important, in the immediate context of P_i-linked exchange it is now possible to imagine a structural basis for the bifunctional reaction discussed earlier.

This topological model (Fig. 8), particularly its assertion with regard to membrane and extramembrane domains, is largely derived from an analysis of "hydropathy" (59). While one may have reservations about too literal an interpretation of such techniques (61), this initial guess merits serious attention. For example, in this specific case such a structure is consistent with the limited data from PhoA and LacZ fusion studies (42). Certainly, for membrane proteins of known structure, this reasoning correctly identifies membrane-spanning regions (26, 59), and in cases in which

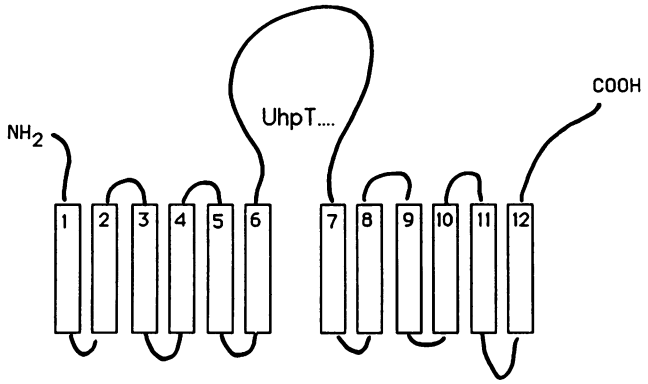

FIG. 8. Molecular model for membrane transport carriers. The amino acid sequences of many carrier proteins (see Maloney, in press), including UhpT (37) and GlpT (27) of *E. coli*, suggest the arrangement shown here. The 12 parallel rectangles indicate presumed α-helices that span the membrane from the cytoplasm (top) to the periplasm (bottom). The solid line indicates extramembranal protein. From reference 65a, with permission.

structure is unknown (e.g., LacY) biophysical measurements indicate an appropriately high degree of α-helicity (33). Perhaps most provocative is the finding that this general argument, when applied to a large number of membrane transport proteins, generates a surprisingly consistent result, as described below.

General Topology of Membrane Carriers

A remarkable feature of P_i-linked topology is that, at the resolution given by Fig. 8, it resembles any one of nearly two dozen other secondary carriers (summarized in reference 65a); indeed, this is true of all carriers of known sequence. In each case, hydropathy algorithms implicate an integral membrane protein, usually with 12 transmembrane segments (presumptive α-helical structures) per functional unit and often (but not always) a cytoplasmic loop that divides the larger structure into domains of roughly equal size. Carriers that follow this general plan come from both procaryote and eucaryote worlds and fall into one of two groups. The majority seem to be structured as shown for P_i-linked exchange (Fig. 8), with 12 (usually) transmembrane segments and a cytoplasmic domain of significant size. Judging from the behavior of LacY, the first of these carriers to be analyzed in this way (33), these systems should function as monomers (23). Our unpublished work with UhpT also points to a functional monomer, since the solubilized and monomeric protein shows a substrate-protectable inactivation by a lysine-reactive agent (V. Anantharam and P. C. Maloney, unpublished data). The very interesting minority is represented by the four examples so far available from the study of mitochondria and chloroplasts. Remarkably, these examples display six (three cases [7, 91]) or seven (one instance [31]) transmembrane columns, and in two cases the evidence suggests that these proteins function as homodimers (32, 52, 53). The general rule, then, appears to be that a full complement of 12 transmembrane helical elements is required for biological activity, and if so, this idea would satisfactorily resolve a long-standing discrepancy in the field. Klingenberg's laboratory, which was among the first to consider such issues, has for some time believed that the ADP:ATP exchange carrier of mitochondria operates as a homodimer (see references 7, 52, and 53), and this has tended to support the general feeling that oligometric structures, particularly homodimers, may be characteristic of membrane transport. Such a view, which derives from considerations of subunit packing in water-soluble proteins, rests on the idea that a substrate diffusion pathway perpendicular to the plane of the membrane could be most readily understood as the space entrapped between associating subunits floating in the membrane (51, 58). This pathway is expected to arise in a natural way because identical subunits are unlikely to have complementary surfaces that allow a tight packing without an internalized space. It has been difficult to reconcile these ideas with recent findings that monomeric structures can have full activity (23, 41), but if these monomers themselves have significant substructure, so as to act as a functional dimer (oligomer), experimental observations once again are in accord with such theoretical arguments. The impact of this unexpected occurrence is considerable. Among other things, it reemphasizes that one might understand membrane protein structure by using the principles revealed by the study of hydrophilic proteins; it reinforces the importance of symmetry, encourages the search for isofunctional regions within single membrane proteins, and rationalizes the finding that multisubunit complexes in one organism have their functional and mechanistic equivalents in the larger monomeric forms of other systems (see below).

While the summary outline above does not reveal the detailed architecture of carrier systems, the consistency of their apparent organization is strong, so strong as to argue that it reflects the general solution to the control of substrate translocation, and probably not just for these secondary carriers, it appears. Thus, the presence of 12 transmembrane segments overlain by a large cytoplasmic (and nucleotide-binding) domain(s) is found also for bacterial binding protein-dependent solute ATPases (5, 17, 47) and this rhythm is repeated in their eucaryote counterparts, the multiple drug resistance factors (22, 43). In animal cells, this family now also includes several putative transport proteins, such as the yeast STE6 protein (70), a mammalian adenyl cyclase (57), and CFTR, a protein likely involved in maturation of mammalian Cl channels (87). In several of these cases (both procaryote and eucaryote), one can cite examples in which definitive (or nearly so) evidence suggests that the functional unit arises by dimerization, either physical or genetic (5, 17, 22, 43).

It is important to note that such an organizational plan (Fig. 8) is most evident among solute transporters, for only in those instances has the genetic analysis been supplemented with biochemical evidence sufficient to define the minimal functional unit (monomer, dimer). Even so, the E_1E_2 ion-motive ATPases may also follow this general plan (see reference 63). Channels, too, can be considered in this context, but on a somewhat larger scale, since even those whose sequences predict proteins with six transmembrane segments (11, 35, 76) appear to operate as a tetramer or pentamer (76).

A General Model

Such considerations suggest that a minimal model based on P_i-linked exchange might rationalize observations in its immediate area and also contribute productively to an ongoing discussion about the structure of many transporting proteins. It is apparent that this general field is advanced to the point where such structural paradigms could be useful, and in that effort a simple set of models is described here.

An even number of transmembrane α-helices (to total 12)

is assumed, so that N and C termini are on the same surface (presumably facing the cytoplasm). As well, pseudo-twofold symmetry is specified so as to accommodate either two identical subunits (as in the mitochondrial examples) or two related domains (as in the bacterial carriers and other cases). Within these boundries, three simple structures can be visualized—those in which two, four, or six α-helices lie equidistant from a central point (65a). The first of these (two core helices) is equivalent to that proposed by Aquila et al. (7) for the mitochondrial ATP:ADP antiporter; the other limiting case (six core helices) more closely resembles the usual artist's rendition in which a ring of hydrophobic columns penetrates the membrane. For each of these possible structures, the two, four, or six core helices are seen as the more amphipathic, lying internal to the remaining and more hydrophobic ones, so that together they exclude phospholipid and delineate an inner space to act as a diffusion path for solute movement. Access to (egress from) this pathway would then be determined by the sympathetic and cooperative articulation of core helices with the overlying cytoplasmic loop(s), whose bulk would further limit inadvertent exposure to the aqueous cytoplasm. Thus, both membrane and cytoplasmic domains could act in a structural capacity to isolate the diffusion pathway from lipid and water, and in a catalytic fashion to regulate passage through and access to this pathway, respectively.

With regard to P_i-linked antiporters, this model now suggests alternative interpretations to recent work. For example, implicit in the argument given above was the idea that the minimal functional unit would be the monomer. Nevertheless, GlpT and LacY mutants can display a negative dominance of the sort often associated with oligomeric structures (60, 71), and in animal cell systems there are periodic reports of higher-order forms of one or another secondary carrier. Factors unrelated to transport probably explain the dominant LacY phenotype (104), but in principle one could imagine such behavior arising if during or after assembly there is interaction between, say, transmembrane segments of one unit and those of its neighbor, so as to affect the final shape of both. This sort of effect should be most pronounced for overexpressed mutant proteins or when the experimental system has one or several carriers at rather high concentration. In these cases, therefore, perhaps it would be worth exploring conditions of reconstitution where otherwise inactive material is distributed at relatively low protein density so as to maximize the independence of each monomer; it is often found that specific activities in reconstituted systems are higher as the protein/lipid ratio decreases (see comments in reference 3). This kind of argument might also presume a relatively low specificity for the interaction of structural helices with their normal partners, and there is therefore the interesting prediction that one could exchange these external helices among different carriers with an unexpected retention of function.

To conclude, it is worth commenting on the value of such explicit models. They are meant to be used in two ways. This field is at a point at which structural studies are both necessary and feasible, and these models provide targets for new experiments. Just as important, because they act as convenient summaries of present knowledge, these models serve to highlight exceptional cases that might need another look, perhaps from a different perspective. The most striking example in the latter category is, of course, the F_0F_1 ATPase, whose F_0 sector has on the order of 24 (!) transmembrane segments, whether in its procaryotic (34) or eucaryotic (8, 69) versions. Does this suggest that F_0F_1 catalyzes two separate transport reactions? Or is this structural evidence that F_0 is built along lines of a channel (76) rather than a carrier?

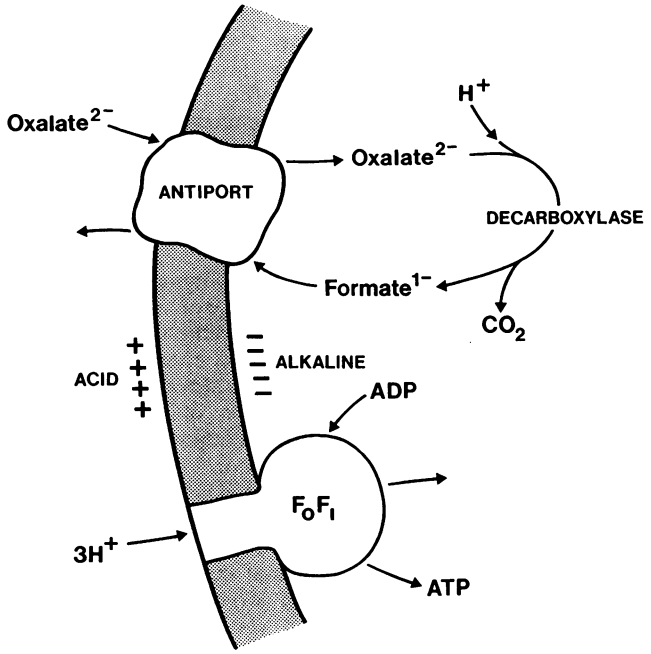

FIG. 9. Generation of a proton motive force in *O. formigenes*. Generation of a proton motive force is based on an electrogenic oxalate^{2-}:formate^{1-} exchange. A membrane potential (negative inside) is maintained by electrogenic exchange, while a pH gradient (alkaline inside) is generated by consumption of protons during oxalate decarboxylation. The overall cycle (influx → decarboxylation → efflux) constitutes an H^+ pump with a stoichiometry of $1H^+$/turnover. From reference 6.

CARBOXYLATE-LINKED EXCHANGE

The family of P_i-linked exchange lends itself to discussions of how anion exchange might contribute during various secondary transport events. By contrast, recent work (6) has identified the first member of a new family, one termed "carboxylate linked," whose behavior should be viewed in relation to the operation of ion pumps. The prototypical example is found in *Oxalobacter formigenes*, an anaerobe that exploits oxalate transport and decarboxylation to sustain membrane energetics (10). By using osmolyte-mediated reconstitution (Table 2), it has been possible to study such oxalate transport in proteoliposomes, and this approach has identified an oxalate self-exchange that is rapid, electrically neutral, and unresponsive to ionophores. Because formate is the product of oxalate decarboxylation in *O. formigenes* ($^-OOC\text{-}COO^- + H^+ \rightarrow HCOO^- + CO_2$), the discovery of an oxalate self-exchange quite naturally led to tests of oxalate:formate antiport. That work has verified the presence of the heterologous reaction and has demonstrated its electrogenic character by showing that exchange can be accelerated or retarded over a 50-fold range by an imposed membrane potential. These data are most easily understood as arising from the antiport of divalent oxalate against monovalent formate, and the implied 1:1 (oxalate:formate) exchange has an unexpected impact on how we view membrane biology in this cell.

The work described above suggests that *O. formigenes*

sustains a proton motive force, not by action of a primary H^+ pump (the usual mechanism), but by operation of a secondary anion exchange (Fig. 9). Thus, electrogenic oxalate:formate antiport would underlie generation of membrane potential, while proton consumption during the decarboxylation reaction would support formation of a pH gradient. Taken together, these separate events (oxalate $^{2-}$ influx $\rightarrow$ oxalate decarboxylation $\rightarrow$ formate^{1-} efflux) constitute an indirect H^+ pump with a net stoichiometry of $1H^+$ extruded per turnover.

This indirect H^+ pump (Fig. 9) is of interest for both theoretical and practical reasons. For example, this model makes an explicit account of the transduction of chemical (scalar) into electrochemical (vectorial) energy, all without invoking a coupling to H^+ movement at a molecular level. More practically, these data raise the possibility that antiport contributes to energy conservation in other anion-degrading systems. If so, the formulation, anion^{n-}:$(n - 1)OH^-$, is probably more relevant as a general scheme for exchange, since use of OH^- as a countersubstrate spares carbon for biosynthesis. In the latter context, there is an interesting special limit when $n = 1$. That limit described a monocarboxylate uniport or channel, of which there is a likely example in the anaerobic decarboxylation of acetate to yield CO_2 plus methane in methanogenic bacteria (106). Verification of an archaebacterial uniport or channel would certainly affect how we view the origins of anion exchange and perhaps the beginnings of membrane transport as well. This prospect is made all the more exciting by the extraordinarily high velocity found for oxalate transport in *O. formigenes*. In the crude reconstituted preparation, this anion exchange moves substrates more rapidly (V_{max} of 100 μmol/min per mg of protein) than any purified carrier yet studied!

BROADER IMPLICATIONS

It is unfortunate that, for reasons of economy, this brief review has neglected several additional examples of bacterial anion exchange. In particular, the work of Winkler, who has made a special study of ADP:ATP exchange (AtpX) in *Rickettsia prowazekii* (56), has not received adequate discussion. Such nucleotide antiport will be of significance for what it may teach us concerning those mechanisms exploited by intracellular parasites and symbionts. With successful solubilization of the AtpX protein (81), one can anticipate that here, too, there will be an appropriate partnership between biochemical and genetic studies. To complement this documented example, it is clear that one should examine more explicitly the possibility of nucleotide exchange in organisms such as *Bdellovibrio* sp. or *Mycoplasma* sp. (77). As for examples yet to come, common sense suggests anion exchange as the basis for dicarboxylate transport by intracellular forms of *Rhizobium meliloti*, *Bradyrhizobium japonicum* (16), or *Frankia* sp. (97). Surely most metabolite traffic across such symbiont membranes will reflect this sort of exchange. On a more speculative note, one might point to citrate transport by *Bacillus subtilis*. This is presently understood as H^+,Mg^{2+}/citrate cotransport (15, 98), but in keeping with the model described for G6P movements (Fig. 7), one can imagine a satisfactory alternative in the form of a neutral exchange between two Mg citrate monoanions and a single divalent citrate; it should be possible to explore this idea in reconstituted proteoliposomes. Finally, we had earlier suggested that cyclic AMP extrusion might operate by an exchange mechanism (65a), and although this is a reasonable idea, preliminary studies in this direction are not encouraging.

Because this review has focused so closely on a limited set of reactions, it might be worth noting what has been learned of more general value from these model systems, in addition to the technical issues noted earlier (reconstitution, etc.). For example, in the arena of cell biology, the lessons we have learned from attempts to define substrate specificity (Table 3 and text) suggest that the gram-negative periplasm is an hospitable and attractive metabolic compartment, one in which various mutases, isomerases, phosphatases, etc., all at rather high concentration, increase the likelihood of an effective metabolite "channeling." It remains to be seen whether this will be the result of mass action and the laws of diffusion, or whether there is some specific association between soluble enzymes and membrane proteins which act as their metabolic partners. As to a better understanding of biochemical issues, it seems that anion exchange will contribute importantly to our appreciation of diversity in the microbial world. This may be of particular concern in dealing with microbial degradations and fermentations, since these events often involve utilization of organic anions. Thus, the finding of an indirect ion-motive pump in *O. formigenes* (Fig. 9) may be more instructive than now recognized. Finally, at the level of molecular biology, we may finally be at the point at which structure-function relationships can take on real meaning. Thus, there is the exciting possibility that a physical structure with some validity on theoretical grounds (Fig. 8 and text) is echoed in the attributes of a biochemical reaction (e.g., exchange stoichiometry; paired P_i binding sites, etc.).

Anion exchange is presently underrepresented in the bacterial world, but as judged from its eucaryote counterparts, it is perhaps the most frequent transport event to occur at a cell or organelle membrane. Indeed, some time ago, in an essay entitled "On the Importance of Being Ionized," Bernard Davis noted that the organic material within cells is mostly anionic, whether it as an intermediate or end product of cellular transformation (24). Given this, it is not unexpected that anion transport should be a constant theme in all of biology. Continued study of bacteria should add important new examples to reinforce this view, and we hope this will reveal still other ways of understanding the cell biology, biochemistry, and molecular biology of membrane transport.

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