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Resolution and reconstitution of anion exchange reactions

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To illustrate the emerging class of anion exchange proteins in bacteria, this article discusses the biochemical and physiological properties of phosphate (Pi)-linked antiporters that accept glucose 6-phosphate (G6P) as their primary substrate. These systems have a bifunctional active site that binds a pair of negative charges, whether presented as a single divalent anion or a pair of monovalent substrates. Exchange stoichiometry therefore moves between the limits of 2:1 and 2:2 according to the ratio of mono- and divalent substrates at either membrane surface. This predicts an interesting reaction sequence *in vivo* because internal pH is more alkaline than external pH; one expects an asymmetric exchange as a pair of monovalent G6P anions moves against a single divalent G6P, and in this way an otherwise futile self-exchange of G6P can result in a net inward flux driven (indirectly) by the pH gradient.

Despite their biochemical complexity, at a molecular level the Pi-linked antiporters resemble other secondary carriers. Indeed, the current listing of nearly two dozen such proteins suggests a structural theme in which the minimal functional unit has two sets of six transmembrane alpha helices separated by a central hydrophilic loop. Presently described examples show that this topology can derive from either a single protein or from pairs of identical subunits. The finding of this common structure makes it possible to begin building more detailed structural models that have more general implications.

Chemiosmotic circuits and secondary carriers

Life at the plasma membrane can be complex, as even the minimal case in figure 1 shows. That simple organization would be appropriate for, say, *Escherichia coli* or *Halobacter halobium* growing in the dark and under anaerobic conditions, or perhaps one of the streptococci or some other anaerobe. In all of these cases we would specify at least one 'primary' ion pump, most often a proton pump whose activity establishes both an electrical gradient (interior negative) and a pH gradient (inside alkaline). In turn, these ion-motive gradients are exploited in the service of solute transport by an accompanying set of 'secondary' carriers. As I will argue, most (possibly all) secondary systems are built in the same way, so that as a class they share important structural and mechanistic features, just as do the differing varieties of the F0F1 or E1E2 ion-motive ATPases (Maloney & Wilson 1985). But for now, I emphasize the diversity among secondary carriers, for it is really this diversity that confers vitality to a living system in its interaction with the environment.

Figure 1 shows five different reactions that we recognize as being mediated by secondary carriers in bacteria (see Maloney (1987) for a recent review). The reaction of *uniport* (1) seems to be of restricted value to the bacterial world and only the uniport of glycerol is clearly described

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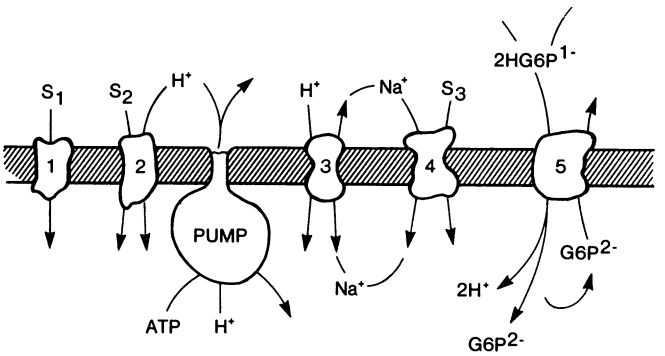

FIGURE 1. Chemiosmotic circuits at the bacterial membrane. Membranes are a collection of pumps and carriers. Those shown here constitute a minimal set, as might be found in anaerobes or in facultative organisms growing under anaerobic conditions. The proton circulation is usually initiated by an F0F1 ATPase and completed by a set of secondary carriers. (From Maloney (1987).)

(Sanno *et al.* 1968). *Symport* on the other hand (2), is both widely distributed (Maloney 1987) and well-studied, particularly as it reflects H^+/solute co-transport (Kaback 1986). The phenomenon of *antiport* is also fundamental finding (Rosen 1986), and in this context Na^+:H^+ exchange has special significance, for its activity supports operation of a Na^+ current ((3) *and* (4)) within the larger and dominant H^+ circulation.

The last example treated by figure 1 (5) may be the most complex of secondary carriers available to bacteria – an *anion exchanger*, which mediates transport of sugar phosphates in Gram-negative (Ambudkar *et al.* 1986*a*; Sonna *et al.* 1988) and Gram-positive cells (Maloney *et al.* 1984; Sonna & Maloney 1988). In such events, the working model proposes a bifunctional carrier that accepts either monovalent or divalent substrates in an overall neutral exchange. One scenario suggests that during glucose 6-phosphate (G6P) transport, a pair of monovalent anions might be accepted at the relatively acidic external surface. Subsequently, in the relatively alkaline cytoplasm, these substrates could be stripped of their protons, generating a pair of divalent anions, of which one could recycle to complete the neutral exchange, leaving the other to serve as a source of carbon or phosphorous.

The available evidence strongly supports the general organization shown in figure 1, and this symposium has as its general focus the biochemical and functional interrelations among these pumps and carriers. Within this setting, this paper reviews and recounts the evidence with regard to the anion exchange reactions noted above to set that work in context.

PHOSPHATE-LINKED ANTIPORT

Anion exchanges of the sort illustrated by figure 1 are called 'phosphate-linked' (Pi-linked) to indicate that they mediate exchanges among both inorganic and organic phosphates. As a result, three distinct reactions might be attributed to any single example: (i) the self-exchange involving phosphate (Pi:Pi exchange); (ii) heterologous exchange of Pi and an organic phosphate (e.g. Pi:G6P antiport); and (iii) an exchange based solely on movements of the organic substrates (cf. figure 1). Each of these reactions is catalysed by the sugar 6-phosphate carriers of *Streptococcus lactis*, *Staphylococcus aureus* and *E. coli*; it is presumed that an equivalent response will characterize most, if not all, members of this general family.

Although the Pi-linked antiport is of clear interest for the complexity of its biochemical

operation, other features also motivate study in this area. There are, for example, historical concerns. The very first experiments in bacterial transport dealt with phosphate exchange in *Micrococcus pyogenes* (now *S. aureus*) (Mitchell & Moyle 1953; Mitchell 1954) and we now know that this activity reflects a Pi-linked carrier with high specificity for glucose 6-phosphate (Sonna & Maloney 1988). It has been gratifying to see how that early work has been integrated with contemporary studies. On a somewhat larger scale, I should also comment that in eukaryotes both major and minor organelles have invested heavily in anion exchange and we hope that the bacterial systems will serve as useful models for the entire group. This is almost certainly true for the mitochondrial (LaNoue & Schoolwerth 1979) and chloroplast examples (Heldt & Flugge 1986) and probable for such events as dicarboxylate transport across bacteroid membranes (Ronson *et al.* 1984) or sugar nucleotide movements over the Golgi or endoplasmic reticulum membranes (Capasso & Hirschberg 1984).

I concentrate on the main experimental approaches we have used to study Pi-linked antiport in bacteria, with a special emphasis on systems that transport glucose 6-phosphate. To begin, it is useful to summarize the Pi self-exchange reaction as its properties figure importantly in the design of mechanistic models. The second topic deals with techniques that we have developed to analyse antiports in artificial, reconstituted preparations. These methods, which may be valuable to the general field of membrane biochemistry, have confirmed the phenomenon of exchange in a very direct way. I shall also focus on the issue of exchange stoichiometry, for assessment of stoichiometry proves central to the integration of biochemistry with cell physiology. Finally, the review of experimental observations is followed by remarks that use the secondary structure of known anion exchangers to derive some general rules governing the larger class of secondary carriers.

The phosphate self-exchange reaction

The results given in figure 2 show the essential features of the phosphate self-exchange reaction. In that work, we used washed cells of *S. lactis*, suspended in 300 mM KCl buffered

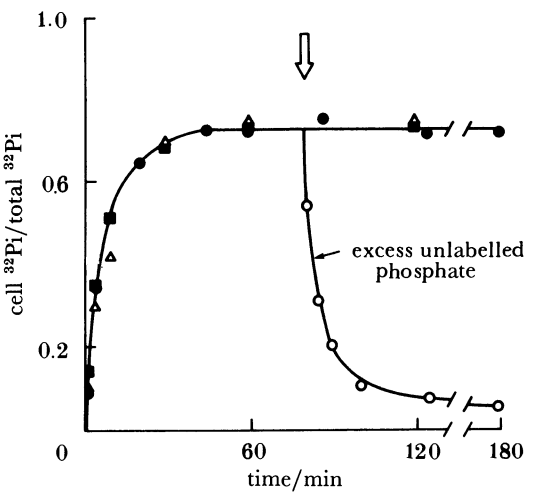

Figure 2. Phosphate self-exchange in *Streptococcus lactis*. Transport was estimated by centrifugations through a silicone oil after addition of 20 μM ^{32}Pi to washed cells in 300 mM KCl, 20 mM MOPS/K (pH 7); (●), control; (■), 10 μM FCCP was present; (△), stock cells had been exposed (30 min) to 1 mM DCCD before assay. At the arrow, part of the control suspension was given 4 mM KPi (From Maloney *et al.* (1984).)

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

at pH 7 with 20 mM MOPS/K. Because they lack internal reserves of metabolizable compounds, such cells are in a resting state, with no residue of the proton-motive gradients characteristic of active growth. As is typical of resting bacterial cells, the internal Pi pool is elevated , to about 50 mM (Maloney 1983), so that addition of 20 μM external ^{32}Pi gave a substantial Pi chemical gradient, directed outward. Samples taken after this addition showed that ^{32}Pi was readily incorporated into, and chased out of, the internal pool of Pi. More importantly, ^{32}Pi transport was unaffected by FCCP, a protonophore (*p*-trifluorocarbonylcyanidemethoxyphenylhydrazone) or by DCCD (N,N′-dicyclohexylcarbodiimide) a covalent inhibitor of the F0F1 ATPase, and these findings seemed contrary to common sense. The cell membrane was 'permeable' to Pi (^{32}Pi moved rapidly into and out of the Pi pool) but how could a 'permeable' membrane sustain a Pi chemical gradient, of roughly 1000-fold, when all sources of metabolic energy were interrupted? This conflict is resolved in a simple way only when ^{32}Pi movement reflects the exchange of internal and external substrate, a suggestion that could be verified for this particular case: despite that most of the added ^{32}Pi was incorporated, the pool of external Pi remained stable at its initial concentration (Maloney *et al.* 1984), documenting a one-for-one exchange of internal and external substrate.

Further work established that phosphate self-exchange has distinctive features that can be used in a diagnostic fashion; (i) the reaction does not distinguish between Pi and AsO_4, yet other inorganic anions are ineffective as either substrates or inhibitors. (ii) Pi self-exchange is not linked to cation movements; it does not result from cation–anion symport. (iii) The overall process is electrically neutral; and this appears to be the case for each of the heterologous reactions (Pi:G6P) considered in this article. (iv) Finally, there is an unusual selectivity that favours monovalent Pi (AsO_4) over the divalent anion. In the analysis of Pi:Pi exchange, this conclusion is based largely on finding that the Michaelis constant (K_t) for ^{32}Pi transport is stable in a pH range (pH 5 to pH 7) enriched for the monovalent anion ($pK_2 = 6.8$), and that K_t increases as pH rises above pH 7 and divalent phosphate appears.

Despite marked kinetic (K_t, V_{max}) discrepancies, there is a striking resemblance between the Pi exchange in *S. lactis* (figure 2 and above) and that originally described in *S. aureus* (Mitchell 1954). In each case, Pi and AsO_4 behave as equivalent substrates, and in each case the reaction favours the monovalent anion. In *S. aureus* such exchange was thought to indicate a system concerned with Pi transport during growth (Mitchell & Moyle 1953), but this idea, although reasonable in its time, is now known to be incorrect. Instead, such Pi:Pi exchange reflects a system normally directed to the transport of organic substrates, in both *S. lactis* and *S. aureus*, a suggestion prompted by the finding that even low levels of certain sugar 6-phosphates markedly inhibited ^{32}Pi:Pi exchange (figure 3) (Maloney *et al.* 1984). Other tests now indicate that such inhibition is a purely competitive interaction (Ambudkar *et al.* 1986*b*) and that sugar phosphates that block ^{32}Pi transport will also act as relatively high affinity substrates during a heterologous exchange (Ambudkar & Maloney 1984). For the sugar phosphate carrier in *S. lactis*, the preferred organic substrates include the 6-phosphates of glucose, 2-deoxyglucose and mannose (Maloney *et al.* 1984: Ambudkar & Maloney 1984), but not the corresponding 1-phosphates. The same is true of *E. coli*, where the *uhp* locus (Kornberg & Smith 1969) directs regulation of hexose phosphate transport by the UhpT protein (Friedrich & Kadner 1987); the apparent transport of glucose 1-phosphate by UhpT (Dietz 1976) is probably due to a contaminating mutase. The range of substrates taken by the UhpT system

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

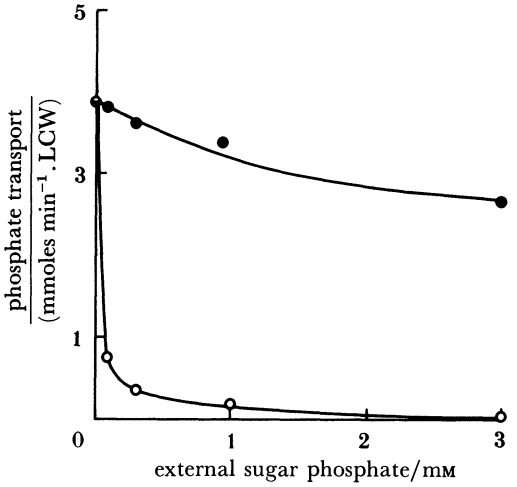

FIGURE 3. Inhibition of phosphate self-exchange by sugar 6-phosphate. Phosphate exchange by *S. lactis* was estimated (figure 2) after 1 min incubations with 60 μM ^{32}Pi. Glucose 1- (●) or 6-phosphates (○) were present at the concentrations shown. (From Maloney *et al.* (1984).)

of *S. aureus* resembles that of *E. coli*, and includes sugar 6-phosphate, at high affinity, and glycerol 3-phosphate (G3P) with low affinity (Sonna & Maloney 1988).

PHOSPHATE:SUGAR PHOSPHATE ANTIPORT: TESTS IN MEMBRANE VESICLES

The idea that Pi and sugar phosphate engage in a mutual exchange was first verified by tests using Pi-loaded membrane vesicles of *S. lactis* (Ambudkar & Maloney 1984), but subsequent work with vesicles of *S. aureus* and *E. coli* (Sonna & Maloney 1988; Sonna *et al.* 1988) has been more convincing of the general mechanism. As well as verifying Pi:G6P antiport, the latter work has excluded the idea that exchange is a partial reaction of a proton-coupled symporter. Although unlikely, this alternative had to be evaluated, as proton-coupled porters can show a substrate exchange that is much more rapid than the reaction of net flux (Wong & Wilson 1970; Kaczorowski & Kaback 1979). To address this issue, we adapted the method of Kaback (1971) to prepare right-side-out membrane vesicles in the absence of Pi. Each vesicle type accumulated Pi (or an amino acid) by proton-coupled mechanisms activated by respiration, but neither vesicle preparation was able to take up glucose 6-phosphate. Rather, sugar phosphate transport was rescued only when an internal pool of Pi had been established by previous activity of the H^+/Pi symport. This dependence on substrates *in trans* is understood in a simple way only if the mechanistic basis of sugar phosphate transport is an anion exchange reaction.

RECONSTITUTION OF THE HOMOLOGOUS AND HETEROLOGOUS EXCHANGE REACTIONS

It is now possible to assess the general features of Pi-linked antiport in an artificial system by using the methods of reconstitution we have developed to analyse the Pi self-exchange

reaction in proteoliposomes. Our general approach is based on three earlier observations. First, we depend heavily on the findings of Baron & Thompson (1975) and Racker and his colleagues (Racker *et al.* 1979) concerning the use of octylglucoside dilution as a convenient technique for reconstitution. Second, we have incorporated the suggestions of Goldin (1977), whose efforts have emphasized the value of purifying and maintaining lipids under reducing conditions. And third, we have exploited the discovery by Newman & Wilson (1980) that when protein is solubilized in the presence of phospholipid there is a significantly increased recovery of activity. To these earlier procedures we now add the use of a special class of protein stabilants, the 'compatible' compounds known as osmolytes (betaine, glycine, proline, various sugars, glycerol and higher polyols, etc.) (Yancey *et al.* 1982).

TABLE 1. OSMOLYTE STABILANTS AND RECONSTITUTION OF Pi-LINKED EXCHANGE

test compound	^{32}Pi transport[a] (nmol per mg protein)
methanol, 20%	27 ± 5
none	36 ± 9
ethylene glycol, 15%	42 ± 16
proline, 12%	306
glycine, 8%	410
glucose, 20%	425
glycerol, 20%	530 ± 98

[a] Steady-state values (±s.d.) for ^{32}Pi transport by proteoliposomes loaded with 100 mM KPi and exposed to 50 μM external substrate. (From Ambudkar & Maloney (1986).)

Table 1 illustrates the striking response obtained when detergent solubilization is performed in the presence of osmolytes at high concentration (Ambudkar & Maloney 1986; Maloney & Ambudkar 1989). When we reconstituted ^{32}Pi:Pi exchange from *S. lactis* by using the traditional method (Newman & Wilson 1980), recovered specific activity (40 nmol ^{32}Pi transported per milligram protein at steady state) was so low as to suggest a significant inactivation during transfer to the artificial system. Such behaviour is typical of many membrane proteins. However, when any of several osmolytes was present at the time of exposure to detergent, the final specific activity increased 10–20-fold, a pronounced elevation that was not confined to Pi-linked exchange, nor to bacterial proteins. Instead, this response is characteristic of many membrane proteins, as broadly summarized by table 2. Most membrane proteins we have tested, including several ion-motive or solute ATPases, various secondary carriers and two enzymic activities, require the use of an osmolyte at high concentration to ensure adequate retention of activity in proteoliposomes. For convenience, we use 20% glycerol as the osmolyte stabilant in the typical case.

The mechanism by which osmolytes exert their positive effect (tables 1 and 2) is not completely understood, but as a working hypothesis we would extend the argument of Timasheff and his colleagues, who have noted that high concentrations of osmolytes confer a thermal resistance to water soluble proteins such as albumin (Gekko & Timasheff 1981; Arakawa & Timasheff 1985). In a three-phase system containing water, protein and an osmolyte (glycerol in this example), there appears to be a preferential interaction of the solvent water and glycerol (osmolyte). As a result, the protein is both under-glycerolated, and (in the three-phase system) over-hydrated. It is this relative excess hydration that then acts as a thermodynamic drive that promotes stabilization of structures that restrict protein–water

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

TABLE 2. OSMOLYTE-MEDIATED RECONSTITUTION[a]

			reference
ATP-driven pumps			
F0F1	*S. lactis*	no	Ambudkar & Maloney (1984)
F0F1	mitochondria	(yes)	D'Souza *et al.* (1987)
F0F1	lysosomes	yes	D'Souza *et al.* (1987)
E1E2 Ca-ATPase	*Streptococcus* sp.	yes	Ambudkar *et al.* (1986*c*)
E1E2 Ca-ATPase	rat (parotid gland)	yes	Ambudkar *et al.* (1988*a*)
solute ATPase	*E. coli* (histidine)	yes	Bishop *et al.* (1989)
secondary carriers			
symport	H/galactose (*S. lactis*)	yes	Maloney & Ambudkar (1989)
anion exchange	Pi-linked	yes	see accompanying text
	oxalate-linked	yes	Anantharam *et al.* (1989)
cation exchange	Arg:Orn (W. Konings)	yes[b]	
membrane-bound enzymes			
G6P-phosphatase	liver microsomes	yes	Ambudkar *et al.* (1988*b*)
phospholipase C′	T-lymphocytes (H. Shin)	yes[c]	

[a] The table lists membrane proteins whose functional reconstitution does (yes) or does not (no) require use of an osmolyte stabilant.

[b] W. N. Konings, personal communication. Department of Microbiology, University of Groningen.

[c] Hyun Shin, personal communication. Department of Biological Chemistry, the Johns Hopkins University School of Medicine.

contacts by internalizing hydrophilic domains and exteriorizing hydrophobic surfaces. Clearly, this scenario has an immediate appeal in the context of reconstitution, for we believe that just such structures are characteristic of membrane pumps and carriers (see below). In an intuitive way then, it is easy to understand why osmolyte-mediated reconstitution might be of special value to membrane biochemistry. We have concluded that introduction of osmolyte stabilants surmounts a significant impediment to the use of reconstitution as an analytic tool in this field.

The analytic use of reconstitution is nicely illustrated by the reconstitution of heterologous Pi:G6P exchange shown in figure 4. For that experiment, we solubilized protein from *E. coli* membranes and used detergent-dilution to prepare proteoliposomes containing varying levels of internal Pi or AsO_4 (Sonna *et al.* 1988). This work showed that sugar phosphate accumulation can be observed in the absence of an external source of energy, and that such transport requires a suitable substrate *in trans*. The experiment also indicates that Pi and AsO_4 serve as equivalent countersubstrates in the exchange with G6P, as might have been predicted from the study of Pi self-exchange in *S. lactis* or *S. aureus* (see above). Equally significant, this experiment suggests it might be important to understand the quantitative aspects of Pi:G6P antiport; although steady-state levels of internal G6P were directly related to the initial load of internal Pi (figure 4, inset), the observed correlation does not agree with the expectation of a simple one-for-one exchange.

THE STOICHIOMETRY OF EXCHANGE

It has been convenient, for technical reasons, to approach measurement of exchange stoichiometry by using Pi-linked antiport as found in *S. lactis*. Consequently, I would re-emphasize that in its fundamental properties, Pi-linked exchange in this cell appears to be identical to that in *S. aureus* and *E. coli*. These systems differ only in the value of various kinetic constants (see below).

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

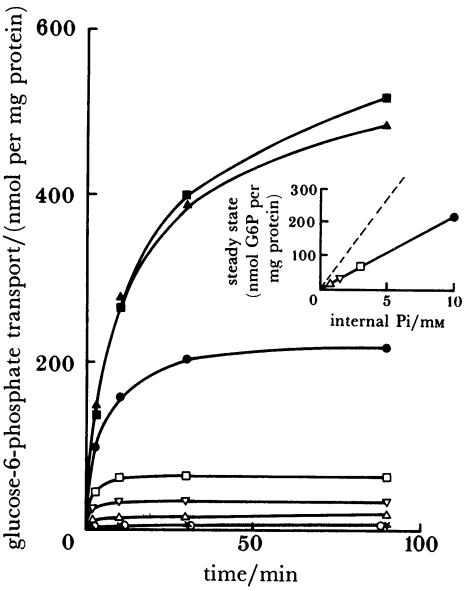

FIGURE 4. Reconstitution of heterologous exchange from *E. coli*. By using mixtures of MOPS/K and KPi or $KAsO_4$ (100 mM total anion), proteoliposomes were made to contain KPi at 0 mM (○), 0.75 mM (△), 1.5 mM (▽), 3 mM (□), 10 mM (●), or 30 mM (■, ★), or $KAsO_4$ at 30 mM (▲). One tube (★) used protein from a UhpT-negative strain; all others had protein from a UhpT overproducing strain. Inset: the solid line correlates steady-state G6P levels with the initial contents of Pi. The dotted line gives the expected correlation for a 1:1 exchange (G6P:Pi). (From Sonna *et al.* (1988).)

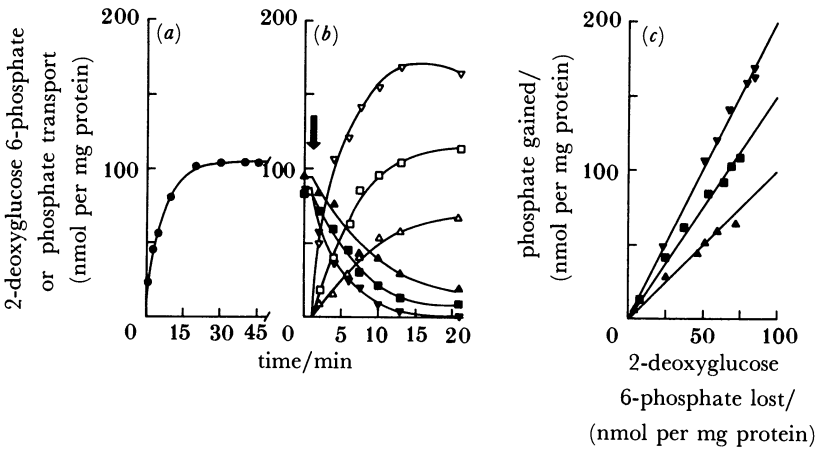

FIGURE 5. The stoichiometry of phosphate:sugar phosphate antiport. (*a*) Membrane vesicles loaded with 50 mM KPi were suspended in 125 mM K_2SO_4/20 mM MOPS/K (pH 7) and exposed to 0.18 mM [^{14}C]2DG6P, with 0.25 mM Na_3VO_4 present to inhibit phosphatase(s). (*b*) After 45 min, vesicles were isolated by centrifugation, resuspended in a small volume and distributed to tubes with assay buffer at pH 7 (▽, ▼), pH 6.1 (□, ■), or pH 5.2 (△, ▲). After 10 min, 3 mM ^{32}Pi 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 as in (*b*). The lines have slopes of 1 (pH 5.2), 1.5 (pH 6.1) and 2 (pH 7). (From Ambudkar *et al.* (1986).)

The work outlined by figure 5 illustrates the strategy used to estimate stoichiometry during heterologous exchange. In addition, the work shows that this exchange ratio is profoundly influenced by pH. In the initial phase of the experiment in question (figure 5*a*), vesicles prepared to contain 50 mM KPi were allowed to accumulate 2-deoxyglucose 6-phosphate

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

(2DG6P) at pH 7. After a steady state had been reached, vesicles were collected by centrifugation and redistributed to three different tubes by using buffers at pH 7, at pH 6.1 (the pK_2 for sugar phosphate) and at pH 5.2. After sampling to verify retention of 2DG6P and after a further 10 min incubation to allow pH equilibration, the second phase of the experiment was initiated by addition of excess ^{32}Pi (figure 5*b*). Stoichiometry could then be determined by matching ^{32}Pi gained with [^{14}C]2DG6P lost. Those comparisons (figure 5*c*) establish two crucial facts. (i) That Pi and 2DG6P exchange in a strict stoichiometric ratio, whatever the pH; and (ii) that stoichiometry varies with pH, moving from its high value of 2:1 at pH 7 (confirming early work by Ambudkar & Maloney 1984) to its low value of 1:1 at pH 5.2.

Biochemical and cellular models

A productive interpretation of 'variable stoichiometry' (figure 5) is based, in part, on the attributes of Pi self-exchange. For that reaction, analysis in both *S. lactis* and *S. aureus* had shown that Pi participates only as the monovalent anion (e.g. the ratio K_t/V_{max} is expected to be at least 10-fold higher for divalent Pi than for monovalent Pi). If this is true during heterologous exchange, then two-for-one antiport at pH 7 could be understood in a simple way as the neutral exchange of 2 monovalent Pi anions for a single, divalent sugar phosphate. And because charge-carrying ionophores have no effect on either the kinetics or stoichiometry of Pi:G6P exchange (Ambudkar & Maloney 1984; Ambudkar *et al.* 1986*b*), we suggest that this macroscopic 2:1 ratio reflects the molecular properties of a bifunctional carrier that effects neutral exchange by the binding of either a pair of monoanions or a single divalent substrate (figure 6*a*) – a kind of biological version of the (cation) ionophore A23187, which uses a pair of carboxylate oxygens to bind either two protons or a single calcium or magnesium ion.

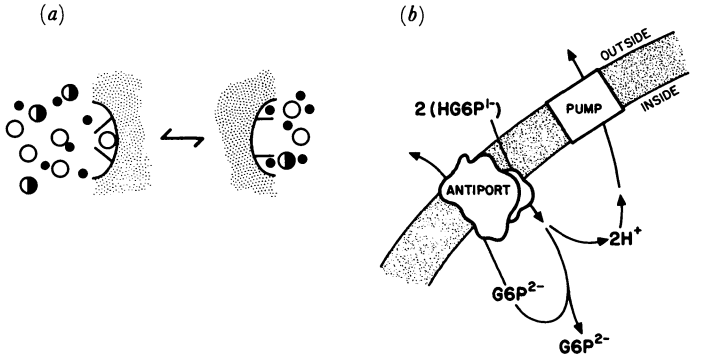

Figure 6. Biochemical and cellular models of Pi-linked exchange. (*a*) A general model for neutral antiport involving three substrates; monovalent phosphate (●) and mono- (◐) or divalent (○) glucose 6-phosphate. Re-orientation requires occupancy at both binding sites, by either a pair of mono-anions or a single divalent substrate. In the situation shown, the bifunctional protein binds either a single divalent substrate (left) or a pair of monovalent substrates (right). (*b*) If G6P is a preferred substrate (table 3), the model predicts that in the presence of a pH gradient (alkaline inside) the net reaction is an asymmetric exchange, as two monovalent G6P anions move in exchange for a single divalent G6P. (From Ambudkar *et al.* (1986*b*) and Maloney *et al.* (1986).)

With this working model at hand, it is possible to give a reasoned account of the pH dependence of stoichiometry by supposing only that the mono-anion binding sites can accept monovalent sugar phosphate as well as monovalent Pi. Indeed, there is support for this view, as the K_t for sugar phosphate transport (and the K_i for sugar phosphate inhibition of Pi

Table 3. Kinetic parameters for phosphate-linked exchanges

(The table gives Michaelis constants for transport of the indicated substrate by Pi-loaded vesicles or proteoliposomes. From Ambudkar & Maloney (1984, 1986) (*S. lactis*), Sonna & Maloney (1988) (*S. aureus*) and P. C. Maloney, unpublished work (*E. coli*).)

test substrate	K_t (μM)
2DG6P	
S. lactis	26
S. aureus	27
E. coli	40
Pi	
S. lactis	250
S. aureus	1500
E. coli	>2200

exchange) is not much different at pH 5 and pH 7 (Ambudkar *et al.* 1986*b*). This implies that monovalent sugar phosphate (pH 5) is as acceptable as divalent sugar phosphate (pH 7). Thus both kinetic and stoichiometric data suggest a mechanism fundamentally based in the exchange of suitable monovalent or divalent substrates. And in the elementary case, one would imagine the carrier selecting randomly among its substrates so that at any pH, the macroscopic stoichiometry results from a mixture of molecular exchanges that are either 2:2 (mono:mono), 2:1 (mono:di) or 1:1 (di:di). Accordingly, the pH dependency of stoichiometry is attributed entirely to pH effects on one of the substrates and not to specific effects on the carrier protein. This interpretation offers a new perspective as to the origins of variable stoichiometry (see Ramos & Kaback 1977; Konings & Booth 1980).

These suggestions are embodied in the biochemical model of figure 6*a*, a model that is successful in two different ways. On the one hand the idea carries with it clear and testable predictions concerning the number of Pi or sugar-phosphate binding sites, their pH dependence and their likely cooperativity; these topics should make for interesting future studies. The model also accommodates certain cellular phenomena that might otherwise be disturbing. Take, for example the data given by table 3. That table lists the Michaelis constants for the transport of 2DG6P or Pi by Pi-linked antiport in *S. lactis*, *S. aureus* and *E. coli*. Note that in each case the organic substrate is preferred, by factors of 10 or 100. This disparity raises the unexpected question of whether Pi itself plays any necessary role during the physiological reaction; studies of Pi transport have been essential to the analysis of exchange. But to what degree is Pi involved in the 'natural' reaction? It appears likely that Pi:G6P exchange is appropriate to *S. lactis*. But it would seem arguable whether Pi:G6P antiport is the dominant reaction during active growth of *E. coli* or *S. aureus*, when the free Pi pool is presumed low (Maloney 1983) and when sugar phosphate is plentiful. A satisfactory alternative, arising directly from the biochemical model (figure 6*a*), invokes the exchange of a single divalent sugar phosphate (internal) for two sugar phosphate mono-anions (external) to give *net* entry of $2H^+$ and $1G6P^{2-}$ (figure 6*b*). More over, given a simple kinetic mechanism, in which the carrier selects randomly among acceptable substrates at either surface, the asymmetric self-exchange of the high-affinity G6P is clearly favoured as the bacterial cytoplasm is (usually) alkaline with respect to the periplasm.

For two other reasons, the asymmetric self-exchange of G6P is worth keeping as an interim model. In *E. coli*, G6P transport by the UhpT protein can fulfill all cellular demands for Pi. Yet this cannot be true if sugar phosphate moves inward by either G6P:2Pi or 2G6P:2Pi

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

antiport; the former reaction leads to a loss of Pi, whereas the latter has no effect on overall Pi balance. On the other hand, the self-exchange by using G6P (figure 6*b*) can accommodate the net influx of Pi (as would an occasional *export* of G6P in exchange for 2Pi). Sugar phosphate self-exchange also reconciles an apparent conflict between mechanism (anion exchange) and thermodynamics. Careful work by Essenberg & Kornberg (1975) demonstrated that G6P entry into *E. coli* was accompanied by proton uptake: G6P transport is proton-coupled, at least phenotypically. Again, this might be expected by the model in figure 6*b*. Experiments that infer stoichiometry by matching changes of external pH with substrate transport should indicate a stoichiometry near $1H^+/G6P^{2-}$ (as observed), as the efflux of unlabelled internal $G6P^{2-}$ goes unnoticed. Moreover, in a formal sense the thermodynamics of the overall reaction (figure 6*b*) represents $2H^+/G6P^{2-}$ symport.

The snapshot of anion exchange given by figure 6*b* is feasible in principle, although of unproven significance. But even if realistic, it is surely only one of several exchange modes required if G6P (or any other organic phosphate) is to serve as a source of both carbon and phosphorous. Bacteria have a carbon to phosphorous ratio of about 40:1 (mol:mol) (Luria 1960), so that sugar phosphates provide far too little carbon and far too much phosphorous. Almost certainly then, during growth on G6P (*E. coli*, etc.) a manageable C:P ratio is achieved by some mixture of exchanges that bring carbon inward (e.g. $2[HG6P^{1-}]:[G6P^{2-}]$, in:out) and extrude excess Pi ($G6P^{2-}:2[H_2PO_4{}^{1-}]$). A similarly poised ratio of exchange modes is presumed to occur during growth on G3P, where the C:P balance is even more at risk (see below). It is unclear whether regulation can influence the poise and distribution of these reaction modes or whether this equilibrium is set by mass action alone.

Families of anion exchange

Pi-linked antiport describes a family of anion exchange processes in bacteria (table 4). Within this family, perhaps the best described members are those with preference of G6P; this group would include the *E. coli* UhpT protein and its equivalents in *S. lactis* and *S. aureus*. *E. coli* also houses GlpT, encoded by *glpT*, which functions as Pi-linked antiport on the basis of both direct tests (Ambudkar *et al.* 1986*a*) and sequence homology with UhpT (Eiglmeier *et al.* 1987). For several reasons, operation of GlpT is relevant to the preceding comments regarding

Table 4. Families of anion exchange

(Known (+) and suspected (?) examples of anion exchange in bacteria. Systems described by four letter genetic symbols are of known sequence. Among those, UhpT, GlpT and PgtP show close homology. See text for other details.)

family	substrate(s)	*gene*	
Pi-linked			
E. coli	G6P	*uhpT*	+
	G3P	*glpT*	+
	cAMP	—	?
S. typhimurium	PGA, PEP	*pgtP*	+
S. aureus	G6P, G3P	—	+
S. lactis	G6P	—	+
R. prowazekii	ATP, ADP	*atpX*	+
carboxylate-linked			
O. formigenes	oxalate	—	+
B. subtilis	Mg/Citrate	—	?

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

maintenance of an appropriate C:P ratio during growth on sugar phosphate. *E. coli* has two different systems, UgpT and GlpT, that transport G3P into *E. coli*, but G3P provides both carbon and phosphorous only if it moves inward via GlpT. Absent GlpT, UgpT can satisfy cellular demands for phosphorous, but not for carbon; a second pathway must provide for carbon entry before growth occurs (Schweizer *et al.* 1982). Because UgpT resembles other binding protein-dependent systems, one presumes it is ATP-driven (Berger 1973) (table 3), implying that UgpT is irreversible in a biological setting and unable to operate as a pathway for both G3P influx and Pi efflux. On the other hand, by analogy with UhpT, neutral antiport by GlpT should be able to arrive at the necessary balance of inward:outward exchanges to give both carbon entry (2G3P:G3P) and Pi loss (G3P:2Pi). It may also be recalled that reversible Pi-linked antiport might be used to accumulate Pi (e.g. 2Pi:G3P or 2Pi:G6P) (Maloney *et al.* 1984), and this may help to explain the curious finding that GlpT is often constitutively expressed, especially in K10 strains. Such strains otherwise depend on a binding-protein dependent system (Pst) that has a double disadvantage of being irreversible (see above) and Pi-repressible; constitutivity on the part of GlpT could overcome each of these deficits.

PgtP of *Salmonella typhimurium* (Saier *et al.* 1975) also appears to operate by anion exchange, insofar as can be judged by the high degree of sequence homology of *pgtP* and both *uhpT* and *glpT* genes (Goldrick *et al.* 1988). The PgtP protein also merits notice as a functional counterpart to the electrogenic antiport catalysing Pi:triose phosphate exchange at the chloroplast envelope membrane (Flugge & Heldt (1986). *Rickettsia prowazekii* harbours still another example of bacterial anion exchange (Winkler 1976), and although the adenine nucleotide exchange protein has no obvious homology with other examples listed here (Krause *et al.* 1985; H. Winkler, personal communication), its functional properties warrant inclusion in this list.

To such documented examples, I suggest that we add cases of special interest and in this context the transport of cAMP is most appealing. That cAMP is released by *E. coli* (and by most cells) is not questioned, yet the mechanism of release is uncertain. In light of the earlier discussion, it is entirely reasonable to suggest that cyclic nucleotide metabolism is influenced directly by Pi-linked antiport. At the least, the simple idea of Pi:cAMP exchange is readily testable.

To conclude this brief review of anion exchange, I would note that recent work (Anantharam *et al.* 1989) identifies a new family of antiport, a 'carboxylate-linked' exchange, in *Oxalobacter formigenes*, an anaerobe that exploits oxalate transport and metabolism to sustain a proton-motive force. Current evidence suggests an entry of divalent oxalate in exchange for monovalent formate (the product of oxalate metabolism) in an electrogenic antiport that underlies generation of membrane potential. A pH gradient is formed secondarily, following consumption of a proton during intracellular decarboxylation of oxalate to formate ($^{-}OOC{-}COO^{-} + H^{+} \rightarrow HCOO^{-} + CO_2$). Taken together, the cycle of oxalate^{2-} influx, oxalate decarboxylation, and formate^{1-} efflux constitutes an 'indirect' H^{+} pump with a net stoichiometry of $1H^{+}$ extruded per turnover.

As a final speculation, one might propose the Mg–citrate cotransporter of *B. subtilis* (Willeke *et al.* 1973) as an honorary member of this carboxylate-linked family, despite its prior classification as a proton-coupled system (Bergsma & Konings 1983) and its formal position as a Mg symporter. My suggestion is based largely on the feasibility of a neutral exchange mechanism (2[MgCitrate^{1-}]:[HCitrate^{2-}]) that incorporates the principles outlined earlier

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

(figure 6) and on the argument that materials that participate in metabolic activity (G3P, G6P, PGAs, cAMP, citrate etc.) are more likely to be handled by antiport than by symport. In the resting or starving state, symporters run the risk of substrate efflux by reversal. Antiport mechanisms allow efflux only if there is concomitant influx.

A structural paradigm – the rule of 12

I have discussed anion exchange with a focus on the biochemical and cellular aspects of function. But I believe that these antiport systems serve equally well as a base for arguments that speak to a new consensus for the structure of secondary carriers (perhaps all membrane transporters), a contemporary casting of the 'carrier hypothesis'.

The linear amino acid sequence of UhpT and GlpT proteins (Friedrich & Kadner 1987, Eiglmeier *et al.* 1987) suggests the secondary structure shown by figure 7 (top). This topology

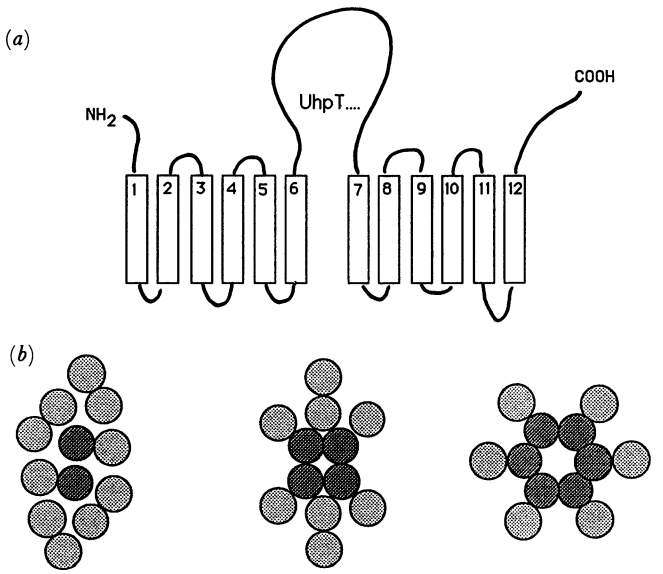

Figure 7. Structural themes in membrane transport. (*a*) A coarse view of the UhpT and GlpT secondary structure (R. Kadner, personal communication; Gott & Boos 1988). (*b*) Three arrangements of the 12 transmembrane helices of secondary carriers; cross-sectional view. Amphipathic helices are shaded; see also Aquila *et al.* (1987).)

derives largely from hydropathy analysis but because this inference is also supported by PhoA and LacZ fusions (R. Kadner, personal communication; Gott & Boos 1988), even this preliminary guess deserves respect. These two proteins (UphT, GlpT) are very hydrophobic, as are all membrane carriers, with hydrophobic or amphipathic stretches that might accommodate 12 α-helical transmembrane segments. These hydrophobic columns appear as two clusters of six, separated by a cytoplasmic loop, suggesting a dimer as the unit of structure. At the very least, this arrangement makes it possible to imagine there is a structural basis to the bifunctional biochemical reaction discussed earlier. Dimerization is an emerging theme in membrane biology, whether as a traditional complex, a dimer of two separable units, or in the fashion shown by UhpT, a genetic dimer (see also P. Henderson's comments, this symposium).

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

But the provocative feature of UphT and GlpT topology is that at this level of resolution, these proteins resemble *all* other secondary carriers. One could be forgiven for mistaking this topological map for any one of at least 15 others; in each case, hydropathy algorithms point to a highly hydrophobic protein with 12 (usually) helical transmembrane segments, often with a cytoplasmic loop that divides the larger structure into two domains of roughly equal size. Systems that obey this rule now include both prokaryote and eukaryote carriers, as follows; (brackets enclose the number of putative transmembrane helices; isoforms are excluded). *Symporters*: H^+/arabinose (12) (Maiden *et al.* 1988); H^+/xylose (12) (Davis & Henderson 1987); H^+/lactose (12) (Foster *et al.* 1983); $Na^+(H^+)$/proline (12) (Yamamoto & Anraku 1988); Na^+/glucose (12) (Hediger *et al.* 1987); H^+/citrate (12) (Maiden *et al.* 1987); Na^+/melibiose (12) (Botfield & Wilson, personal communication; Botfield & Wilson 1988). *Antiporters*:HCO_3:Cl (12) (Kopito & Lodish 1985); Pi:G6P (12) (Friedrich & Kadner 1987); Pi:G3P (12) (Eiglmeier *et al.* 1987); ATP:ADP antiport (*R. Prowazekii*) (12) (H. Winkler, personal communication; Krause *et al.* 1985); Na^+:H^+ (10) (Karpel *et al.* 1988); transposon or plasmid-enclosed exchangers (cf. Maiden *et al.* 1988). *Uniporters*: glucose (12) (Mueckler *et al.* 1985); arginine (10) (Ahmad & Bussey 1988).

This conformity surely shows us the structural paradigm for such membrane proteins, and in support of this view one might examine similar carriers in the major organelles. Mitochondria provide three relevant examples; adenine nucleotide exchange, the brown fat uncoupler protein, and an H^+/Pi symporter. Each of these cases gives an apparent contradiction to the rule as hydropathy analysis implicates six transmembrane helices (Aquila *et al.* 1987; Runswick *et al.* 1987). But it is thought that mitochondrial carriers may operate as physical dimers (Klingenberg *et al.* 1980; Aquila *et al.* 1987) whereas the bacterial (and other) carriers are presumed to function as monomers (Costello *et al.* 1987), so that concordance with the rule is preserved even on this rather large scale. The single example available from chloroplasts also appears to follow the rule (more or less) and Pi:triose phosphate exchange is mediated by a homodimer (Flugge & Heldt 1986) whose subunit has seven hydrophobic segments (Flugge *et al.* 1989). Overall the sequences of nearly two dozen secondary carriers follow the 'rule of 12' and it will be of considerable interest to keep track of these correlations as other information becomes accessible. If verified, this rule may have considerable impact, as it reconciles present experiment with early thoughts on the structure of membrane transporters. For example, Klingenberg (1979) and Kyte (1980) argued that such proteins might often be dimeric (oligomeric), with a transmembrane diffusion pathway identifiable as the internal space defined during association of identical and therefore non-complementary subunits. Although supported by work with mitochondrial exchangers, which operate as dimers, this idea was contradicted by the finding that other carriers have a fully active monomer. But perhaps we should resurrect these early models, if as suggested here, the substructure of these active monomers enables them to generate a kind of 'internal' dimer.

The hydrophobic columns shown in figure 7 constitute the most striking topological feature we can now associate with membrane transport proteins. How might these helical segments be arranged? At the very least, we might begin to model with two simple restraints in mind; (i) that the N- and C-termini lie on the same side of the membrane and (ii) that there be a pseudo-two-fold symmetry to accommodate two identical or related monomers (too many structures are feasible if we also consider an odd number of helices and non-identical monomers). Within these boundaries are three structures that seem realistic (figure 7, bottom); those in which two,

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

four or six helices lie equidistant from some central point (see also, Aquila *et al.* (1987)). In each case one imagines external helices as the more hydrophobic, internal ones as the more hydrophilic, and that the former appose the latter so as to exclude phospholipid and delineate an inner core that acts as a diffusion path for solute movement. Access to (egress from) this pathway would then be determined as the core helices articulate in sympathy with the overlying cytoplasmic loop(s), whose considerable bulk could limit inadvertent exposure. The naive view would assign the biochemistry of translocation to the enclosing transmembrane helices, reserving issues of selectivity and specificity to the superficial loop(s).

Whatever the authentic architectural plan, it is surely a common phenomenon, one which represents Nature's solution to the control of substrate translocation. This rule is replicated in significant ways by other membrane transport proteins. The finding of 10–12 putative transmembrane helices is a recurrent motif arising on analysis of both the binding-protein dependent solute ATPases and their eukaryote homologues, the multiple drug resistance factors (see Chen *et al.* (1986*a*, *b*) and therein). And even the E1E2 Ca^{++}-motive (MacLennan *et al.* 1985) and Na^{+}, K^{+}-motive ($\alpha+\beta$ subunits) (Shull *et al.* 1985) ATPases seem to follow this general plan. In such instances too, one can find cases in which the helical clusters arise by either physical or genetic dimerization (cf. Chen *et al.* 1986*a*, *b*).

This overall argument is strong on correlation, weak on supporting fact, but none the less a workable hypothesis. Perhaps most important, the possibility that the core helices (figure 7) play a functional as well as structural role suggests there is real significance to the hierarchy of hydrophobicity we infer for transmembrane columns. Some (the outer ones) should be clearly hydrophobic, even by strict criteria, but a few of the others will be ambiguous and may be missed by stringent algorithms. In consequence, we might underestimate membrane topology if we are too careful, missing (core) helices that may be the more interesting ones. Perhaps it is worth introducing a little 'slip' into the analysis of these regions or concentrating efforts on those few examples (UhpT, etc.) where the global structure seems evident even now.

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PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

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PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES