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SHORT REVIEW

Coupling of Secondary Active Transport with $\Delta\tilde{\mu}_{H^+}$

A. Kotyk[1]

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Abstract

Most nutrients and ions in bacteria, yeasts, algae, and plants are transported uphill at the expense of a gradient of the electrochemical potential of protons $\Delta\tilde{\mu}_{H^+}$ (a type of secondary active transport). Diagnosis of such transports rests on the determination of the transmembrane electrical potential difference $\Delta\psi$ and the difference of pH at the two membrane sides. The behavior of kinetic parameters K_T (the half-saturation constant) and J_{max} (the maximum rate of transport) upon changing driving ion concentrations and electrical potentials may be used to determine the molecular details of the transport reaction. Equilibrium accumulation ratios of driven solutes are expected to be in agreement with the $\Delta\psi$ and ΔpH measured independently, as well as with the Haldane-type expression involving K_T and J_{max}. Different stoichiometries of H^+/solute, as well as intramembrane effects of pH and $\Delta\psi$, may account for some of the observed inconsistencies.

Key Words: Electrical potential; electrochemical potential; pH; secondary active transport; accumulation ratio; coupling stoichiometry.

Introduction

Transport of both ions and nonelectrolytes against a concentration gradient is a major function of most cell and organelle membranes. It has become common usage to distinguish between primary and secondary types of such transport. The primary active transport is, by definition, associated with an exergonic chemical or photochemical reaction, such as hydrolysis of ATP, oxidative processes in the mitochondrial inner membrane and in bacterial membranes, or relaxation of photon-excited pigment molecules of thylakoids and green and purple bacterial membranes. In a broader sense, primary active

[1]Institute of Microbiology, Czechoslovak Academy of Sciences, 142 20 Praha-Krč, Czechoslovakia.

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transport may be considered to include the so-called group-translocation systems where the molecule entering the membrane at the starting side emerges chemically modified at the target side.

The secondary active transport, occasionally called coupled transport, depends for its energy supply on the difference in the electrochemical potential of a cation, such as is generated by one or another type of primary transport. One of the fine treatises describing the classification based on kinetic and thermodynamic principles is that by Heinz (1978).

Among the many ion concentration gradients established through the function of primary ion pumps (H^+, Na^+, K^+, Ca^{2+}, Mn^{2+}, Mg^{2+}, Fe^{3+}, Cl^-, HCO_3^-) only the first two have acquired during evolution the ability to serve as the energy reservoir for driving the secondary active transport of other substances. Furthermore, the role of the H^+ electrochemical potential is most clearly expressed in microoganisms, both prokaryotic and eukaryotic, and plants, while NA^+ is the cation of choice in animal cells. However, Na^+ ions are known to accompany the transport of some substances even in bacteria (e.g., glutamic acid, 2-aminoisobutyric acid, melibiose) and in yeast (one of the phosphate-translocating systems). The evolutionary analogy between bacteria and mitochondria is reflected even here as some solutes are transported across the innser mitochondrial membrane coupled with H^+ ions, but none are known to use Na^+ (Scarpa, 1979).

While both types of secondary active transports (i.e., H^+-driven and Na^+-driven) can be described by the same type of kinetic and thermodynamic equations, the carrier molecules apparently differ from case to case and practically nothing is known about their molecular properties. Our knowledge about the existence of Na^+-driven transport is older than that of the H^+-driven ones (Christensen and Riggs, 1952; Crane *et al.*, 1961). The H^+-dependent transports began to be described after Mitchell's chemiosmotic hypothesis gained momentum (Mitchell, 1973), where, from the very beginning, the full electrochemical (rather than chemical) potential difference was taken into account. The expression for the electrochemical potential difference (PD) of hydrogen ions is properly written

$$\Delta\tilde{\mu}_{H^+} = \tilde{\mu}_{H_{out}^+}{}^0 - \tilde{\mu}_{H_{in}^+}{}^0 - F\Delta\psi - RT \ln (H_{in}^+/H_{out}^+) \text{ [J mol}^{-1}\text{]} \quad (1)$$

where $\Delta\psi$ is the electrical PD (in volts), F is the Faraday constant ($9.649 \cdot 10^4$ C mol^{-1}), R is the gas constant (8.314 J mol^{-1} K^{-1}), and T is the absolute temperature (in Kelvin). The ion- and potential-independent terms $\tilde{\mu}^0$ are taken to be identical at both membrane sides so that one can write

$$\Delta\tilde{\mu}_{H^+} = F\Delta\psi - 2.3RT \log (H_{in}^+/H_{out}^+) \quad (2)$$

An expression commonly in use refers to the protonmotive force PMF which is

obtained from the above by dividing by F so that

$$\text{PMF} = -\Delta\psi - (2.3RT/F) \log (H_{in}^+/H_{out}^+) \text{ [V]} \quad (3)$$

Methodology

Estimation of electrochemical PD or, more directly, of the PMF rests on obtaining data of two kinds: (1) difference between intracellular and extracellular pH, and (2) transmembrane difference of the electrical potential.

The difficult determination is of course the intracellular pH (Roos and Boron, 1981). A number of techniques have been in use, including the distribution of weak acids (Waddell and Butler, 1959), nuclear magnetic resonance estimation of the chemical shifts of signals of the ^{31}P atom in dissociable phosphoryl groups (e.g., Navon *et al.*, 1979), and the technique I recommend, viz. the application of fluorescent compounds that show a pH-dependent response to excitation (e.g., guinine, fluorescein, chromotropic acid). From the differential response to excitation at two different wavelengths (reading the emission at a single wavelength), one can directly estimate the ambient pH. As with NMR, the response is independent of the amount of "probe" but, in addition, it gives instantaneous information (Slavík, 1982). Moreover, the first successful pH topography has been achieved using this technique with a resolution to about 0.05 μm^2 (Slavík, 1983).

Membrane potentials are best measured with a suitable microelectrode, a technique widely used in animal and plant cells. However, very few data available from single cells; most information derives from the fungus *Neurospora crassa* (Slayman *et al.*, 1973), and a single valve from the yeast *Endomyces magnusii* (Vacata *et al.*, 1981) and from giant *Escherichia coli* (Felle *et al.*, 1980). The technique used most often with single cells is the measurement of the distribution of lipophilic cations (tetraphenylphosphonium, dibenzyldimethylammonium, tetrabutylammonium) or anions (tetraphenyl borate, phenyl dicarbaundecaborane) which follows the Nernst equation (Grinius *et al.*, 1970):

$$\Delta\psi = -(RT/nF) \ln (\text{ion}_{in}/\text{ion}_{out}) \quad (4)$$

If one makes sure that the compound is not adsorbed to cell constituents and is not itself actively transported (and, of course, if it permeates sufficiently rapidly), this technique yields reliable and reproducible results (e.g., Hauer *et al.*, 1981; Bakker and Harold, 1980). The elegant fluorescent compounds (Waggoner, 1979; Cohen and Salzberg, 1978), such as the cationic cyanines, yield much the same information but here intracellular quenching of fluores-

cence occurs probably due to adsorption (and dimerization?), so that semi-quantitative data can only be obtained.

If the source of energy for uphill transport is to be diagnosed recourse is usually made to two tests:

1. Is the addition of the cotransported substrate to a cell suspension accompanied by a (transient) rise of extracellular pH?
2. Is such addition accompanied by a partial depolarization of the membrane?

If both conditions are met, the conclusion may be drawn that H^+ ions are cotransported with the substrate. However, caution should be exercised in this context. Even if protons do accompany the substrate into the cell, their disappearance from the medium can pass unnoticed because (1) the external solution has a high buffering capacity (Sigler *et al.*, 1980), or (2) the H^+ pump maintaining the low extracellular pH responds rapidly to the pH disturbance caused by adding cotransported substrate and pumps H^+ out more rapidly. The first complication is usually handled by estimating not the actual but the titratable acidity of the medium. The second problem may be circumvented by blocking the pump, either specifically or by depleting its energy reserves (cf. Seaston *et al.*, 1973, using 2-deoxy-D-glucose and antimycin to achieve this in yeast).

On the other hand, proton-driven substrate transport could be simulated in both types of test by a completely different scheme. Supposing that the primary proton pump draws its energy from splitting ATP (as it indeed does) and that the solute under investigation is transported by an ATP-driven mechanism, after addition of the solute to the suspension it will compete for ATP with the proton pump and hence will at least temporarily cause a decrease of the outward flow of protons so that both an alkalinization of the external medium and a membrane depolarization will be observed (Kotyk, 1979). A truly unequivocal refutation of such possibility was provided in the case of transport of amino sugars in the yeast *Rhodotorula gracilis* (Niemietz *et al.*, 1981) which is driven by the membrane potential but not by the pH difference because of their own positive charge. Their addition causes a membrane depolarization but not a change in pH (in fact, a drop of pH_{out} was recorded due to exchange of H^+ for amino sugar to maintain electroneutrality).

Kinetics and Its Predictive Value

The H^+-driven transport is always found to be saturable (finite K_T and J_{max} were determined). Wherever tested, structurally related compounds competed for such transport, and countertransport of such compounds was

observed. All these properties, shared with mediated diffusion systems, are in accord with the function of a carrier protein in the membrane, in the sense that it faces alternately one or the other side of the membrane and its binding site cannot be accessible simultaneously from both sides (cf. Stein and Honig, 1977). (It is not necessary that the complexed carrier be so restricted—even if it is accessible from both sides the essential features of carrier transport, such as countertransport, are preserved.)

In its unabridged form, the operation of such a H^+-driven carrier system can be represented as in Fig. 1. In this scheme the individual partial reactions are assumed to be in virtual equilibrium so that dissociation constants (K_H, K_{HS}, K_S, K_{SH}) can be used to describe the relative concentrations of each of the partners. (Parts of this scheme were thoroughly analyzed, best perhaps by Heinz *et al.*, 1972, and by Geck and Heinz, 1976.)

The system may in fact operate without rapid equilibration. In that case, each partial step in both directions of the scheme should be described by a separate rate constant. Initial rate expressions can be derived for all possible situations; most of them can be found in Kotyk and Janáček (1977) and in Kotyk (1979).

Depending on the section of Fig. 1 and on whether the individual reactions are in rapid equilibrium or not, the basic transport parameters K_T (the half-saturation constant) and J_{max} (the maximum initial rate of transport) will be differently influenced by the driving ion at each of the sides. Likewise, the membrane potential will play its diagnostic role, especially with respect to K_T. The effects of the driving ion concentration at both sides of the membrane and of the membrane potential will differ quantiatively and in some cases even qualitatively if the carrier itself has a charge; only a negative charge is expected to bind the driving cation and bring the complex to neutrality. All these effects are listed in Table I, with the exception of fully steady-state (i.e., nonrapid equilibrium) paths. In such cases, an interesting situation arises if the carrier can combine randomly with either H^+ or with S first, regardless of whether k_{CH} and/or k_{CS} are zero. The initial rate formula then contains squared terms in substrate concentration even in the numerator and, if one of the paths of CHS formation (say, C + H + S) is preferred over the other, nonlinear reciprocal plots may occur. For an example from intestinal glucose transport (with Na^+ as driving ion) see Crane and Dorando (1980). It is of interest that as the H^+ concentration in the external medium rises, the apparent negative cooperativity should turn to positive cooperativity and hence from a convex to a concave Lineweaver–Burk plot.

There is finally a situation not charted in Table I, namely that the carrier does not redistribute itself at the two membrane sides according to the offered substrate and H^+ concentrations but that its amount is fixed at each side of the membrane (van den Broek, 1982).

It goes without saying that the effects listed in Table I are fully

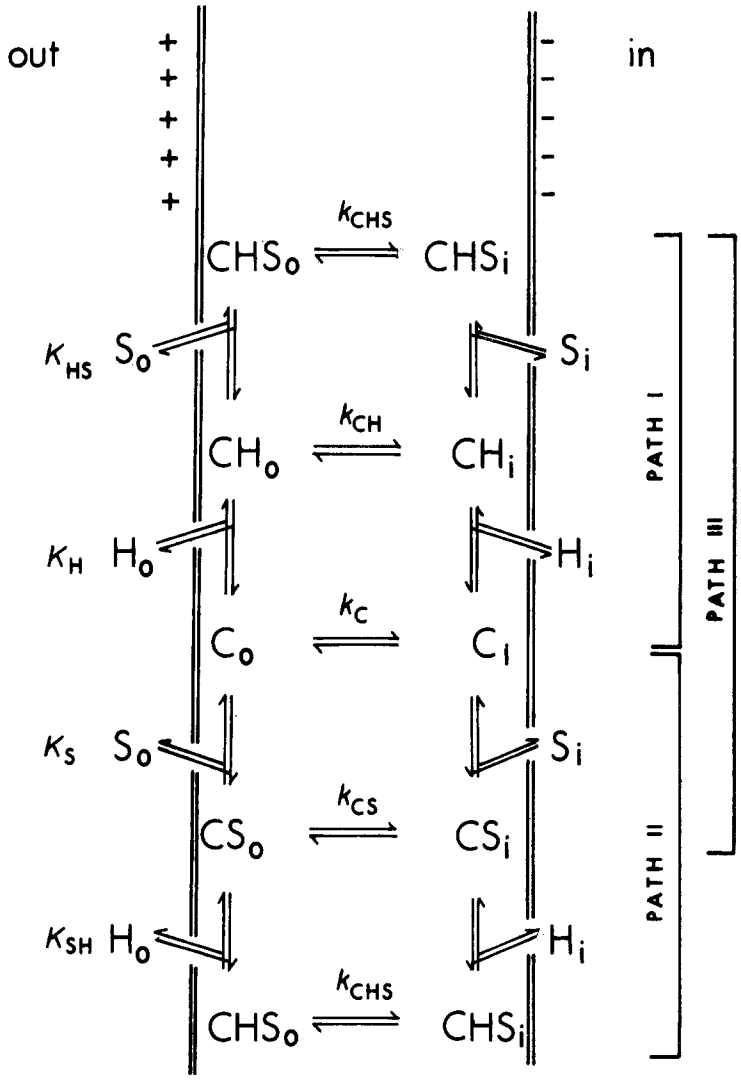

Fig. 1. A mobile carrier model of H^+-driven transport. K_H, etc., are the dissociation constants of the individual steps ($K_H = C \cdot H^+/CH$, etc); k_C, etc., are the first-order rate constants of translocation. The membrane potential causes the charged carrier species (here CH and CHS) to move with greater probability toward the inside of the membrane where the potential is relatively negative, the mobilities being affected as follows: $k_{CH} = k_{CH}\, e^{-F\Delta\psi/2RT}$, $k_{CH} = k_{CH} e^{F\Delta\psi/2RT}$.

Table I. Effects of the Driving Ion Concentration at the Starting Side (H_{out}^+) and at the Target Side (H_{in}^+) and of the Membrane Potential $\Delta\psi$ on the Maximum Rate of Transport (J_{max}) and the Half-Saturation Constant (K_T)[a]

	Increase H_{out}^+		Increase H_{in}^+		Increase $\Delta\psi$	
Model sequence	J_{max}	K_T	J_{max}	K_T	J_{max}	K_T
Equilibrium						
Path I	0 (0)	− − (− −)	− − (− −)	− (+ +)	+ + (+ +)	− (− +)
path I, $k_{CH} = 0$	0 (0)	− − (− −)	− − (− −)	− (+ +)	+ + (+ +)	− − (− +)
path II	+ (+ +)	− − (− −)	0 (0)	0 (0)	+ + (+ +)	− − (− +)
path II, $k_{CS} = 0$	+ + (+ +)	− − (− −)	0 (0)	0 (0)	+ + (+ +)	− − (− +)
path III	+ (+)	− − (−)	− − (−)	− (−)	+ + (+ +)	− − (+)
path III, $k_{CH} = 0$	+ (+ +)	− − (− −)	− − (− −)	− (− −)	+ + (+ +)	− − (− −)
path III, $k_{CS} = 0$	+ + (+ +)	− − (− −)	− − (−)	− (+)	+ + (+ +)	− − (− −)
path III, $k_{CH} = k_{CS} = 0$	+ + (+ +)	− − (− −)	− − (− −)	− (−)	+ + (+ +)	− − (− −)
Steady state						
Path I, $k_{CH} = 0$	+ + (+ +)	− − (− −)	− − (− −)	+ + (−)	+ + (+ +)	− (+)
Path II, $k_{CS} = 0$	+ + (+ +)	− − (− −)	− − (− −)	− (− −)	+ + (+ +)	+ (− −)

[a]Plus signs indicate an increase, minus signs a decrease of the parameter. Minus followed by plus stands for a concave dependence. Zero means no effect. The first columns refer to a neutral carrier, the ones in parentheses to a negatively charged carrier.

symmetrical, i.e., if the cell interior is the starting side, the K_T and J_{max} values refer to efflux from cells rather than to influx.

In contrast to Na^+-driven transport, the H^+-driven one possesses a feature that may complicate the above analysis. Like all protein-mediated reactions, they are pH-dependent in the catalytic sense of the word—a vast majority of them function optimally at an intermediate pH value, corresponding to a half-protonated state of the "active site" so that J_{max} decreases while K_T increases in both acid and alkaline direction (Kotyk and Horák, 1983). This protonation is different from that involved in the H^+-driven transports, and its analysis shows that it does not affect the thermodynamics of the reaction (see next section), but it can—and apparently does—unfavorably affect the behavior demonstrated in Table I, particularly at the acid end of the pH range.

Thermodynamics and Its Corrective Value

As in enzyme kinetics, it is the equilibrium state of the reaction which represents a thermodynamic quantity. In H^+-driven transport, as in any active transport, the equilibrium distribution ratio of the driven solute (K_{eq}) must reflect the driving potential such that

$$n\Delta\tilde{\mu}_{H^+} = -\Delta\mu_{solute} \tag{5}$$

If one proton accompanies the solute on the carrier (as assumed in the previous kinetic treatment), $n = 1$, but this is not necessarily the case. Going back to Eq. (2) we can write that the maximum accumulation ratio (S_{in}/S_{out}) of an H^+-driven solute S will obey the following equation:

$$RT \ln (S_{in}/S_{out}) = -(n + z)F\Delta\psi - nRT \ln (H_{in}^+/H_{out}^+) \tag{6a}$$

and thus

$$S_{in}/S_{out} = (H_{out}^+/H_{in}^+)^n e^{-(n+z)F\Delta\psi/RT} \tag{6b}$$

where n is the number of cotransported protons and z the number of positive charges on the substrate. Figure 2 is instructive as it shows (a) the great influence a two-proton cotransport has on accumulation, and (b) the relatively small effect of low PMF and the pronounced effect in the range above 100 mV where a minor error in the estimation of PMF can lead to grossly erroneous predictions of accumulation ratios.

The accumulations ratios shown need not to attained for two, conceptually different, reasons: (1) if the solute under investigation can also be transported in the form of CS (see Fig. 1), this will in fact represent a leak which, at high concentrations of S, will gain in importance and will cause the

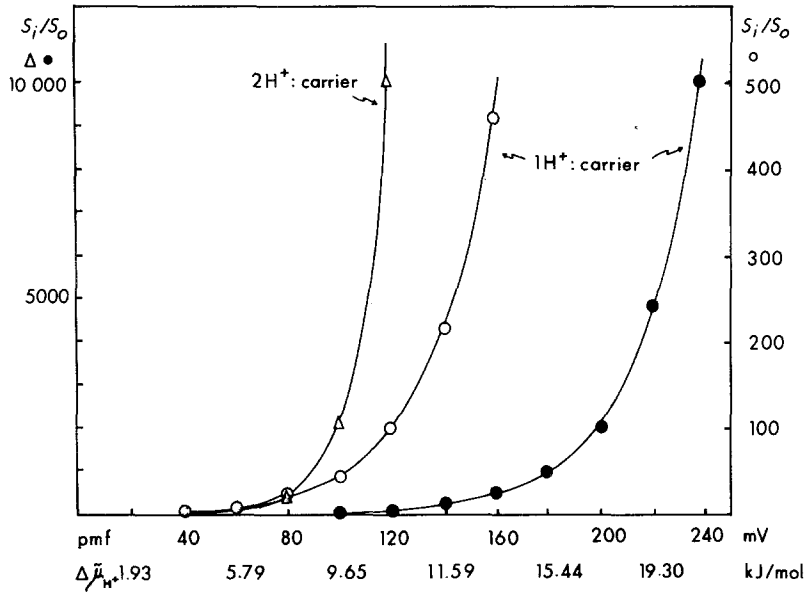

Fig. 2. Maximum accumulation ratios of an H^+-driven neutral solute, dependent on the electrochemical potential difference (in kJ mol^{-1}) or on the protonmotive force (in millivolts) for obligatory coupling where either one or two H^+ ions are cotransported with one substrate molecule.

S_{in}/S_{out} ratio to tend toward unity (for the earliest such findings, see Heinz and Mariani, 1957; (2) even in a tightly coupled secondary active transport the accumulation ratio will decrease with increasing S_{out}, occasionally below unity (Kotyk and Michaljaničová, 1977), so that no leak can be present. The decrease is apparently due to inadequate availability of the source of energy, i.e., H^+ ions in this case (Kotyk and Stružinský, 1977).

It can be demonstrated that in all of the H^+-driven transport systems with a 1:1 stoichiometry, the equilibrium distribution of the driven solute K_{eq} is equal to a Haldane-type combination of kinetic constants, viz.

$$K_{eq} = S_{in}/S_{out} = \overrightarrow{J_{max}}\overleftarrow{K_T}/\overleftarrow{J_{max}}\overrightarrow{K_T} \tag{7}$$

More often than not his clear-cut condition imposed on the attainable accumulation ratio is not met, which might argue for the operation of several parallel systems and/or a higher stoichiometry of H^+ to solute.

It might appear from Eq. (6) that in a given cell all the H^+-driven solutes should attain the same accumulation ratio which is governed solely by the electrochemical PD. This should indeed be true for the tightly coupled systems with no movement of the CS complex. However, if differences in the accumulation of related H^+-driven solutes are found (e.g., Aldermann and

Höfer, 1981), it means that either full path II or path III of Fig. 1 or one of the steady-state paths of Fig. 2 must operate since the accumulation ratios of solute transported by such mechanisms contain dissociation or rate constants which are substrate-dependent. This thus excludes path I or simplified paths of all types from consideration.

Some Observed H^+-Driven Transport Systems and Their Heuristic Value

Authoritative reviews of H^+-driven transport have appeared during recent years (Harold, 1976; Hamilton, 1977; Eddy, 1978; West, 1980), and I will mention here only some more recent papers and those that have raised points of special interest.

H^+/substrate stoichiometry is one of such points. Usually a 1:1 ratio has been reported for neutral substrates (Serrano, 1977, for maltose in yeast, and Seaston *et al.*, 1973, for various amino acids in yeast). However, deviations in both directions can be found. Ratios lower than 1 are apparently due to slipping through the carrier when the CS complex is also mobile, or to the operation of a parallel system not driven by H^+ ions (Hauer and Höfer, 1982; van den Broek and van Steveninck, 1980). Ratios higher than 1 can be due to various reasons. There may be an intrinsic 2:1 binding to the carrier, such as with glycine, methionine, phenylalanine, and even lysine in yeast (Seaston *et al.*, 1973). It is a pity that the accumulation ratio predicted on the basis of this stoichiometry cannot be verified because amino acid transport in yeast is virtually unidirectional and not freely reversible (Kotyk and Říhová, 1972). Other, well documented, cases are two protons with one glutamate in *Staphylococcus aureus* (Mitchell *et al.*, 1979), three protons with one sulfate (Roomans *et al.*, 1979), and two protons with one phosphate on one of the carriers (Cockburn *et al.*, 1975) in baker's yeast.

Another cause of an apparent 2:1 ratio may lie in a much increased dissociation constant of the carrier–solute–H^+ complex with respect to H^+ at the intracellular face of the membrane so that H^+ ions are released while substrate returns to the starting side (1-deoxy-D-glucose in *Chlorella vulgaris;* Grüneberg and Komor, 1976). Variable H^+/lactose ratios (1:1 to 2:1) have been much discussed (e.g., ten Brink *et al.*, 1981), but no reasonable mechanism has emerged to explain how a carrier would bind more protons with decreasing proton concentration.

Another interesting point is the charge-compensating process which accompanies the electrogenic inflow of protons as they drive a substrate into the cell. Generally, it is K^+ ions which move out of the cell both in bacteria and in yeast, the outflow being nearly stoichiometric with H^+ (cf. Eddy, 1978; Hauer *et al.*, 1981; Hauer and Höfer, 1982). However, it should be observed that no evidence exists that this compensating movement takes place on the

same carrier—the "antiport" is merely an electrophoretic movement through whatever channels are available.

It was observed in *Chlorella* (Komor and Tanner, 1976), *Saccharomyces* (de la Peña *et al.*, 1982), *Halobacterium* (Michel and Oesterhelt, 1976, and giant *Escherichia coli* (Felle *et al.*, 1980), but not in inverted *Escherichia coli* vesicles and in *Staphylococcus aureus* (Mitchell *et al.*, 1979), that $\Delta\psi$ increases with increasing pH_{out}. This increase may have purely thermodynamic reasons. If it is assumed that the energy required to establish $\Delta\tilde{\mu}_{H^+}$ is constant under given conditions and if the capacity of the H^+ pump is substantially greater than that of H^+ leaks, any artificial change of ΔpH will be reflected in a "mirror" change of $\Delta\psi$ (Spanswick, 1982). A decrease in $\Delta\psi$ may follow even the increase in ΔpH produced physiologically by the proton pump after addition of a metabolic substrate such as glucose to a yeast cell suspension (Kotyk *et al.*, 1982a).

If $\Delta\tilde{\mu}_{H^+}$ is the source of energy for different solute transports in a given cell, one would expect a mutual inhibition between such transports—it may be of a partially competitive nature. Unfortunately, no systematic data are available on this, the only case published along these lines being the countertransport of proline stimulated by lactose efflux from *E. coli* (Kaczorowski and Kaback, 1979).

Although the consensus now is that a great many transport systems are indeed driven by $\Delta\tilde{\mu}_{H^+}$, the agreement is not universal that the measured values of $\Delta\psi$ and ΔpH are reliable for predicting quantitatively the accumulation ratios of H^+-driven substrates. Nothing is known about the events taking place on the carrier molecule or even where such a molecule is exactly located. There are indications (e.g., Schwab and Komor, 1978) that changes in the carrier conformation are brought about by the membrane potential, resulting in different K_T values, and there is now increasing evidence that it is the intramembrane pH generated by the proton pump which is seen by the secondary transport processes, rather than the pH_{out} measured with electrodes: (1) there are often accumulation ratios exceeding the thermodynamic predictions (ten Brink *et al.*, 1981; Kotyk *et al.*, 1982b), (2) the plot of the pH dependence of the accumulation ratio is often bell-shaped (Kotyk, 1980), the optimum pH coinciding with the optimum of the H^+-extruding ATPase (Goffeau and Slayman, 1981), (3) the temperature dependence of this ratio is quite different from that of $\Delta\tilde{\mu}_{H^+}$ and exceeds the predicted maximum values particularly at low temperatures (Kotyk and Horák, 1983). A disjunction of the overall potential difference from its effect on accumulation was recently indicated by the finding in *Rhodotorula glutinis* that at pH 7.5 (when $\Delta pH = 0$) 0.1 M KCl depolarized the membrane from 110 to 30 mV but the accumulation ratio of nonmetabolizable monosaccharides remained high at 80:1.

It is to be hoped that in the near future it will be possible to isolate and

reconstitute in defined systems both the $\Delta\tilde{\mu}_{H^+}$-generating device and the $\Delta\tilde{\mu}_{H^+}$-employing carrier and to define the extent to which we are entitled to use macroscopically determined components of $\Delta\mu_{H^+}$ for predicting its involvement in a given proton-driven transport system.

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