Biochimica et Biophysica Acta, 604 (1980) 91–126

BBA 85205

ENERGY COUPLING IN SECONDARY ACTIVE TRANSPORT

IAN C. WEST

Department of Biochemistry, Ridley Building, University of Newcastle upon Tyne, Newcastle upon Tyne, NE1 7RU (U.K.)

(Received September 6th, 1979)

Contents

I. Introduction

The term active transport is usually used to refer to the transport of a solute across a biological membrane from one solution to another where its electrochemical potential is higher. No spontaneous processes operate in a manner that leads to an overall increase in

Abbreviations: V, velocity constant of a reaction following Michaelis-Menten kinetics (velocity at infinite substrate concentration); K_M, concentration constant for a reaction following Michaelis-Menten kinetics (concentration giving half maximum velocity); K_M^{ent}, K_M for influx; K_M^{eff}, K_M for efflux; K_D, dissociation constant; Δ, value of a quantity (e.g., pH) in inner phase minus that in outer phase; pH_0, pH_i, pH of outer and inner phase, respectively; μ_i, chemical potential of component i; $\tilde{\mu}_i$, electrochemical potential of component i; ψ, electrical potential; ΔP, proton-motive force; $\Delta\tilde{\mu}_{H^+}/F$, proton-motive force; z, number (and sign) of charges on an ion; Z, 2.3 RT/F; P_x, translocation probability of unloaded carrier; P_c, translocation probability of carrier-substrate complex; R, gas constant; T, absolute temperature; F, Faraday constant; $t_{1/2}$, time for quantity to reach half its value; pK_a, $\log_{10}$ of acid dissociation constant.

free energy, so this uphill transport of solute must be part of a process in which other particles move down their free energy gradient. In the context of active transport two types of free energy yielding process have been distinguished: (a) those in which chemical changes occur, and covalent bonds are broken and reformed; (b) those in which a solute is translocated from one solution to another down its electrochemical potential gradient, breaking only non-covalent bonds. This latter is called 'secondary active transport' because such processes depend ultimately on a process of the former type ('primary active transport') to establish the gradient of the driving solute. The distinction has proved useful even though at a certain level of analysis it turns out that there is no formal difference between the two [1,2]. If the two solutes flow in the same direction we can speak of symport or co-transport, if they flow in opposite directions we can speak of antiport or counter-transport. The membrane component responsible for the coupled translocation can be called a symporter or an antiporter, respectively.

What then is energy coupling? Weber [3] has made the provocative, indeed startling, statement that 'every chemical compound generated in metabolism is the result of a reaction which runs towards thermodynamic equilibrium with complete independence of any other reaction occurring at the same time'. This statement may be misleading and its derivation obscure; Weber may even be wrong *, but he certainly warns us of the danger of drawing two parallel arrows to represent the fluxes of the two solutes A and B and then saying that they interact or are coupled energetically [4]. The fallacy is in thinking of the fluxes as two processes, when they are only two facets of one process. What we are looking for is the pathway; the set of intermediate states involved in binding solute A and solute B on one side of the membrane and their eventual release on the other side. We want to know the free energy of those intermediate states and the rate constants for the transitions between them. In other words we want to know the intrinsic binding constants between substrates and carrier, the order of binding, the amount of physical movement of the carrier-substrate complex, the effect of an electric field on the binding constants and on the transitions, both of the complex and the empty carrier.

With the experimentally observable facts known it ought to be possible to work out the rest theoretically; to make a model which can explain how the transport V or K_M is affected by co-ion concentration, or by membrane potential, how partial diffusion barriers can distort kinetic parameters. This is not the easiest part of the process and may be unduly neglected by some workers. This is unfortunate. A theoretical model is often necessary in order to interpret the experimental data and to draw from them the information we seek. The measurement of V and K_M data is useless if we do not know the effects of diffusion barriers, electric fields, leak pathways, etc., on the measurements.

Of course there are several other interesting questions relating to the energetics of secondary active transport besides the mechanistic details. There are questions such as: what substances are accumulated, in what tissues, with what co-ions or counterions, whether the ion gradients are adequate, whether there are other types of free energy yielding reaction that can influence the uptake of solute either by interacting with the secondary active transport carrier itself or by operating a parallel primary active transport process. Then there is the question of the physiological regulation of transport, either to

---

* Consider a liposome, containing dilute H_2SO_4, suspended in a dilute solution of triphenylmethylphosphonium chloride. The cation is lipid-soluble and penetrates the liposome to equilibrium. On adding a protonophore, H^+ flows out but this establishes a diffusion potential, and the equilibrium distribution of the cation is displaced. It flows into the liposome.

modify the extent of transport or possibly to vary the efficiency of coupling. Sometimes an active transport process is essential for subsequent and wider physiological processes. Thus, lactose active transport is essential for induction of the lactose operon, though not for growth on lactose (where facilitated diffusion is adequate) [5]. Similarly, the active transport of neurotransmitters will have wide ranging consequences, as will the specific inhibition of that active transport by drugs.

The subject area of this review has been covered by several authors in the last two years, and no attempt will be made to repeat their conclusions. This review should therefore be read in conjunction with the excellent papers of Crane [6], Eddy [7], and Rosen and Kashket [8]. Crane's review is outstanding for historical perspective and the clarity with which he presents the fundamental model that covers equally the microbial and the vertebrate systems. Eddy restricts himself here to microbial systems, and his review is particularly valuable for his knowledge of the eucaryotic microorganism. He asks penetrating questions and uncovers neglected issues. Rosen and Kashket's review is limited to bacteria but not to secondary active transport. Their review succeeds in being comprehensive while retaining a sense of progress. This last review is in a book on Bacterial Transport, edited by Rosen, which provides an excellent summary of that field. There is another useful book, the proceedings of a symposium, edited by Oxender and Fox which covers a wide field from mammalian to bacterial transport [9]. Though the papers describe original research they were written for a general, non-specialist, audience. Most of the papers have also been published separately in the Journal of Supramolecular Structure, and in those cases I give both references.

II. Isolation, purification and reconstitution of transport systems

The last two years have seen great progress in the development of techniques for the isolation of membrane proteins and the reconstitution of facilitated diffusion and secondary active transport systems in functionally active form in phospholipid vesicles. (Primary active transport systems have always been easier to purify by conventional enzymological techniques because they catalyse covalent reactions that can be followed even in a disrupted, one-phase system.) There seems to be a widespread feeling that the isolation, purification and reconstitution of carrier systems is the ultimate goal of the research worker in the transport field, that the mechanism of active transport will be revealed in the process. I am sure this is not the case and I incline much more to Crane's view [6] that the ultimate description of the process of coupled-transport will entail a kinetic description of a set of transitions between identifiable transitional states. Nevertheless, isolation and the demonstration that the isolated protein is still capable of catalysing transport when re-inserted into a phospholipid membrane is an important achievement and a first step towards a great many interesting experiments concerning carrier function [10]. I shall therefore refer briefly to recent advances in this area, trying only to identify the main types of method used in these studies.

One of the first problems in carrier isolation is that of identifying the carrier protein. This can be done by reconstituting transport activity in artificial phospholipid vesicles following the basic methods pioneered by Racker and co-workers [9,10]. This approach has been successfully applied to secondary coupled systems by several authors (Table I).

Other workers have successfully identified substrate binding proteins in membranes. This is obviously a weaker criterion than the identification of a protein capable of substrate transport, and in any case it requires the availability of a radioactive substrate with

TABLE I

COUPLED TRANSPORT SYSTEMS RECONSTITUTED IN PHOSPHOLIPID VESICLES

Organism (tissue)	Transport system	Ref.
Rat brain	L-glutamate	47
Ascites cells	amino acids	218, 219
Rat renal tubule	Na^+-glucose	220
Bacillus subtilis	alanine ($?H^+$)	221
Thermophilic bacterium	alanine	9, 222
E. coli	H^+-proline	223
Mycobacterium phlei	proline ($?H^+$ $?Na^+$)	135, 136

a low dissociation constant (K_D, which is not necessarily the same thing as a low K_M for transport -- see section VII). Thus, a radioactive substrate at 1 mM will half-saturate a carrier when the K_D is 1 mM, and thus cannot reveal the presence of a carrier unless the carrier is present at 2 mM or greater. Such concentrations of carrier protein are usually unattainable at the beginning of a purification procedure. In vitro binding of thiodigalactoside to the β-galactoside carrier of *Escherichia coli* has been reported by Kennedy et al. [11], and Belaich et al. [12]. But the latter authors say that binding is uninhibited by Triton X-100 concentrations of up to 1%, whereas Kennedy found complete abolition of binding by Triton, and this is confirmed in a recent paper [13]. Anraku's group [14] have been able to study the pH dependence of proline binding to a presumptive H^+-proline symporter from *E. coli* membranes.

To mistake transport for binding is a classic error [15] though in other contexts this error is still being made (see section VIII). In the present context it does not matter, for a protein can be identified in vitro as well by transporting as by binding substrate.

Another approach to the identification of carrier proteins, which is finding fairly general application, is the classical approach of Fox and Kennedy [16] using a tight binding substrate or substrate analogue to protect the active site of the carrier while reactive groups on the rest of the membrane are reacted covalently with, e.g., nonradioactive *N*-ethylmaleimide. The removal of the substrate reveals the now uniquely reactive groups of the active site which had been protected, and which can now be reacted with radioactive reagent. Yet another approach which could find fairly wide application is that introduced by Stein [17–19] and used successfully by others on the β-galactoside transport system of *E. coli* [20,21]. In this technique, induced bacteria are fed ^3H-labelled amino acids and uninduced bacteria are fed ^{14}C-labelled amino acid. The bacterial cultures are mixed, membranes are prepared and fractionated. Fractions containing a raised $^3H/^{14}C$ ratio contain proteins specified by the induced genes. Care must be taken not to let unequal quenching of e.g., ^3H radiation, interfere with the measurements of isotope ratio.

Most purification procedures use cholate or deoxycholate as detergents for these achieve complete separation of peptides (rather than merely the formation of multimolecular micelles, as is observed with Triton X-100), but do not cause extensive and perhaps irreversible denaturation (as does sodium dodecyl sulphate). Deoxycholate and cholate are also diffusable, whereas the very low critical micellar concentration of Triton X-100 ($3 \cdot 10^{-4}$ M [22]) makes it very slow to dialyse away.

Altendorf et al. [23,24] reported the solubilization of the *E. coli* β-galactoside carrier

in high concentrations (90%, v/v) of aprotic organic solvents (e.g. hexamethyl phosphoric triamide) [9]. However, the degree of molecular association in such a solvent has not been assessed and techniques for further purification (such as ion-exchange chromatography) require development. Amanuma et al. [223] used acidic *n*-butanol for the solubilization of the proline carrier of *E. coli*, but the above remarks apply here also.

The most exciting advance in this field is the recent work from the laboratories of Overath and Müller-Hill [21]. An *E. coli* plasmid has been constructed containing the gene for the *E. coli* β-galactoside carrier and scarcely any additional *E. coli* DNA. Bacteria containing multiple copies of the plasmid, when induced for the lac operon, produce up to ten times the normal amount of membrane located lactose carrier. It is unambiguously identifiable on polyacrylamide electrophoresis gels stained with Coomassie blue, and can be located by the Kennedy technique and quantitated by the Stein technique. This seems to me to be a most promising development with a potentially very wide field of application.

Three recent papers on the sequencing of bacteriorhodopsin will enormously open up the difficult field of membrane research by providing new standard techniques applicable to membrane proteins [25–27].

III. New organisms and new tissues

A feature of transport research in the last two years is that coupled transport processes have been reported in a variety of novel situations. For example sucrose appears to be pumped into phloem cells by symport with H^+ [28–33], glucose and α-methyl glucoside uptake in the cyanobacterium (blue-green alga) *Plectomena borganum* is dependent on the proton motive force and is probably H^+-symport (Raboy and Padan [34]). Kikutu and Hoshi [35] discuss the role of Na^+ in the transport of *p*-aminohippurate by newt kidney. Joseph et al. [36], have studied sodium-amino acid symport in hepatocytes, and Lever [37] reports sodium phosphate symport in plasma membrane vesicles from mouse fibroblasts (but see Simonsen and Cornelius [38], for phosphate distribution in ascites cells). Benderoff et al. [39] report electrogenic Na^+-dependent glycine transport in sheep reticulocytes, and Hofer and colleagues [40,41] report electrogenic H^+-sugar symport in the yeast *Rhodotorula gracilis*, though this finding is disputed by Kotyk [42]. Ferrichrome transport in membrane vesicles of *E. coli* was very sensitive to uncoupler and valinomycin suggesting ion ($?H^+$) symport [43]. These observations fit so well into our current picture of secondary active transport that they could almost be said to have been expected.

I put in a slightly different category and discuss at slightly greater length the reports indicating coupled transport in synaptosomes, synaptic vesicles, platelets, mast cells and chromaffin granules, because it seems to me that these studies have fascinating implications for the study of neural and hormonal function and the interaction of drugs with these functions. It seems that the termination of neural transmission involves two steps in the sequestration of neurotransmitter. In the first step neurotransmitter is taken up from the area of the synaptic cleft by a high-affinity, Na^+-dependent, transport system at the plasmalemma of the nerve cell. In the second step the intracellular transmitter is sequestered inside sub-cellular vesicles in the nerve terminal, again by a secondary active transport process (Maron et al. [44]). (See Fig. 1.) The considerable similarities between the uptake of α-aminobutyric acid and L-glatamic acid by synaptosomes from brain [45–48], of 5-hydroxytryptamine by brain slices, synaptosomes from brain and nervous tissue, and

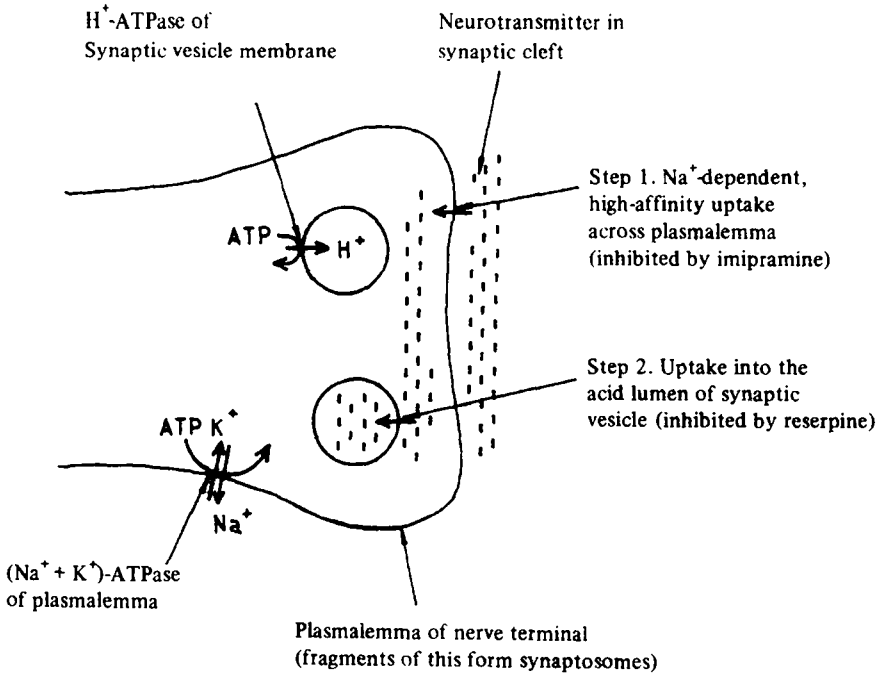


Fig. 1. Diagrammatic representation of nerve terminal showing processes involved in the re-uptake of neurotransmitter.

by platelets [49–51] suggests that the step 1, mentioned above, is common to these experimental systems. Likewise there is great similarity between amine uptake into chromaffin granules of adrenal medulla [52–54] into 5-hydroxytryptamine-granules in platelets, histamine-granules in mast cells and the synaptic vesicles prepared from nervous tissues [44,55], suggesting that a process like the above mentioned step 2 is common to these experimental systems. Both these processes are discussed further in the next section.

IV. Which ions are involved as co-substrate?

An ion species may influence solute uptake without necessarily being a co-substrate for the transport process. In order to establish involvement in symport or antiport it must be shown that the ion is translocated [56] in a manner stoichiometric [57] with the translocation of solute. It is also important to show whether the two fluxes are coupled by the porter and not merely by forces maintaining electroneutrality or osmotic equilibrium [58]. In order to show further that the ion coupling has a role in active transport it must be shown that the free energy available from the ion translocation is adequate to account for the solute translocation [59].

Two years ago the picture that had emerged as to which co-ions were involved in coupled transport was broadly as follows. In bacterial cells and bacterioid organelles (chloroplasts and mitochondria) the primary active transport systems (involving covalent bond changes and group translocation) are proton translocating; the proton-translocating respiratory chain and the proton-translocating ATPase. In these cells and organelles coupled transport involved protons. (Of course, it involved Na^+ as well in the case of the

Na^+/H^+ antiport, etc.) In animal cells the primary pump is the $(Na^+ + K^+)$-ATPase, electrogenic and exchanging 3 Na^+ for 2 K^+. In these cells coupled processes involved Na^+ (and probably not K^+; but see later). In algae, fungi and higher plants the plasmalemma is equipped with an electrogenic proton-translocating ATPase. The reasons for such a pump are somewhat obscure but are discussed most interestingly by Raven and Smith [52] who argue that the primary function might be pH regulation. In any event, coupled transport systems involving protons have been described for algae, fungi and higher plants.

Already in 1977 there were exceptions to these broad generalizations, but in the last two years research has highlighted some further notable exceptions.

By analogy with the porter system of mitochondria [61], Mitchell [62] suggested that the apparent H^+-lactose symport of *E. coli* [63] might really be a Na^+-lactose symport coupled to respiration by a Na^+/H^+ antiport. That possibility was carefully excluded for the lactose carrier of *E. coli* [59] but seems to pertain for thiomethylgalactoside uptake by the melibiose porter in the closely related *Salmonella typhimurium* [63,64], and for the analogous system (melibiose porter or thiomethylgalactoside II porter) of *E. coli* [65]. The *Salmonella* system has been confirmed in vesicles [66]. The natural milieu for these enteric bacteria is no doubt rich in sodium ions (120–150 mM) and, provided that the Na^+/H^+ antiporter is sufficiently active, the chemical gradient of Na^+ ($\Delta\mu_{Na^+}$) should approach the magnitude of the pH gradient (ΔpH or $\Delta\mu_{H^+}$) and the total sodium motive force ($\Delta\tilde{\mu}_{Na^+}$) will approach the total proton motive force ($\Delta\tilde{\mu}_{H^+}$). But why is Na^+ best for melibiose and H^+ best for other sugars?

Another process which has long been thought to involve Na^+ is the uptake of glutamate by *E. coli* [67–70]. This is now fairly well established as a symport of Na^+ with glutamate for the *E. coli* B strain, but there is controversy as to the K-12 strain and ignorance about ML and other strains. Perhaps the Na^+ requirement was evolved here as a secondary step to increase the positive charge on the translocated complex and hence cause the glutamate to be more strongly accumulated (Fig. 2a). I would like to dwell briefly on this example. The most convincing evidence for symport with Na^+ comes from the work of Tsuchiya et al. [70] who demonstrated glutamate uptake driven by $\Delta\tilde{\mu}_{Na^+}$ artificially established in the following way (Fig. 2b). With equal Na^+ concentrations on each side of the membrane a membrane potential (H^+ diffusion potential) was formed by adding a protonphore and alkali. The proton motive force was therefore small, and in any case directed outwards. Note, however, that, while the proton motive force is small, the pH gradient is large, is opposite to and approximately equivalent to the electric field. The 40-fold accumulation of glutamate must indicate that its uptake is electrogenic, being coupled to 2 Na^+ and no H^+. Much less convincing is an experiment of MacDonald et al. [69] in which glutamate uptake into membrane vesicles in the presence of respiration is shown to be unaffected by nigericin (an artificial K^+/H^+ antiporter) but abolished by monensin. Now the classical action of monensin is as an artificial Na^+/H^+ antiporter [71] and it has been shown to repair the deficiency in a mutant strain probably lacking the normal Na^+/H^+ antiporter [72]. In the above experiment monensin might be expected not to decrease but to increase $\Delta\tilde{\mu}_{Na^+}$ at the expense of $\Delta\tilde{\mu}_{H^+}$, i.e., to expel Na^+ and acidify the lumen (Fig. 2c). Why did it abolish glutamate uptake? Perhaps because of that acidification.

The relatively early reports of a Na^+ requirement for sugar and amino acid uptake by a marine Pseudomonad [73,77] seemed intelligible in terms of the relatively high Na^+ concentration in sea water (0.47 M). A much more extreme, and more recently investigated example of this type is exhibited by *Halobacterium halobium*. This bacterium survives the

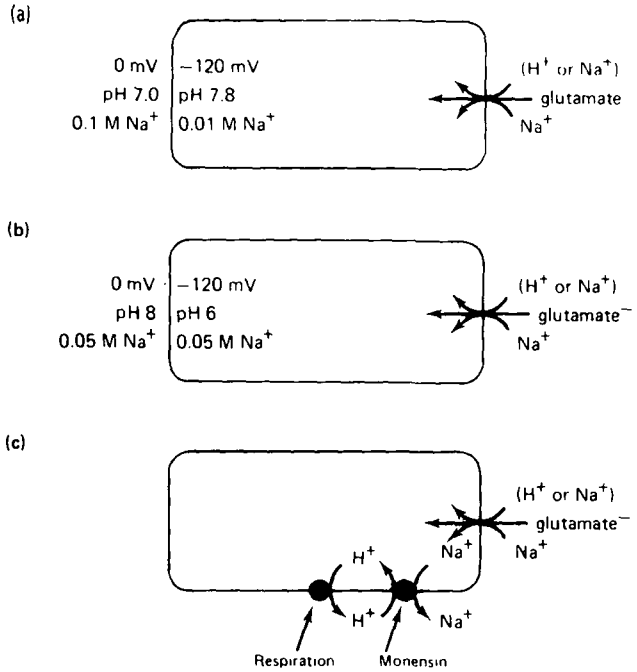


Fig. 2. (a) Suggested scheme for glutamate uptake by *E. coli* B. (b) Glutamate uptake driven by an electric field when the proton motive force is absent and the pH gradient is directed outwards. (c) Expected effect of monensin on glutamate uptake.

relative anaerobiosis in drying-out salt pans by synthesising a rather specialized, light-driven, proton pump called bacteriorhodopsin. Here, the primary energy source is in the form of a proton motive force as in more typical bacteria, but the ambient Na^+ concentration of 2–3 M provides special problems and opportunities. One of these (discussed in another section) is the question of the H^+ stoichiometry of the Na^+ efflux pump. Another is the enormous capacity of the energy store represented by the cation gradients. Oesterheldt [78] has emphasised that in whole cells the high internal K^+ level (3 M) and high K^+ permeability of the outer membrane allow a large diffusion potential to form (negative inside) which is capable of sustaining ATP synthesis for the 10 h or so of darkness, during which the H^+ flux is not available from the light-driven proton pump. No doubt there is a similar amount of energy stored in the inwardly directed Na^+ gradient which could be made available by a reversal of the $Na^+/2\ H^+$ antiport. Indeed, a sodium/proton antiport would probably be necessary in any case to prevent acidification of the cytoplasm. Lanyi [79–81] found no K^+ diffusion potential in his vesicle system and regards the Na^+ gradient as the sole component of the osmotic free energy store. In fact, Lanyi and colleagues found that vesicles loaded with KCl and placed in NaCl in the dark have essentially no membrane potential. They must conclude that not only is there no K^+ diffusion potential, but that either the electrogenic $Na^+/2\ H^+$ antiporter is irreversible or it changes its stoichiometry. It may be that there is a radical difference between whole cells and membrane vesicles.

A third point about this organism is that the amino acid symports seem to be coupled to the Na^+ gradient [79]. The complexities concerning the stoichiometries of these symports will be discussed in section VI. One additional interesting point here is the recent report of a light-driven Na^+-pump [82].

Is the large outwardly directed K^+ electrochemical potential gradient in *Halobacterium* used directly in secondary active transport? A requirement for internal K^+ has been reported, though only for Ca^{2+}/Na^+ antiport [83]. K^+, though required, does not appear to contribute to the driving force. This was inferred from the lack of effect of the $\Delta\tilde{\mu}_{K^+}$ on the rate of Ca^{2+} accumulation driven by a constant, outwardly directed $\Delta\tilde{\mu}_{Na^+}$. (In general there is only a somewhat dubious and non-linear relationship between driving force and rate of transport, as discussed in section VII, but a more or less linear relationship between Ca^{2+} transport and $\Delta\tilde{\mu}_{Na^+}$ was demonstrated experimentally in this case.) This requirement for K^+ which is shown also by several H^+–anion symport systems may be because K^+ is necessary to prevent cellular pH from becoming too acidic, as discussed previously [7,84,85], and in section V.

Is the outwardly directed K^+ potential gradient used in any coupled transport process? One is tempted to say probably not, as maintaining internal K^+ seems to be one of the main preoccupations of nearly every type of living cell. Eddy, at one time, believed that the K^+ efflux accompanying Na^+-glycine symport into ascites cells and amino acid uptake by yeast was mediated by the respective uptake carriers [86,87] but he now favours the view that K^+ efflux is a passive leak via another route [7]. Murer and Hopfer [88] have shown that valinomycin is necessary in order for the $\Delta\tilde{\mu}_{K^+}$ of brush border vesicles to influence Na^+-glucose symport, showing that the interaction here also is indirect. On the other hand, $\Delta\tilde{\mu}_{K^+}$ has recently been implicated in neurotransmitter uptake, where a clear K^+/amine antiport is indicated, as mentioned below.

There is another possible effect of internal K^+, that was suggested by Crane [6] and interpreted by Eddy [7], whereby internal K^+ enhances coupling of solute to the Na^+ flux without itself actually being transported out of the cell.

Lithium is occasionally encountered as an ion that can replace Na^+, e.g. in the Na^+/H^+ antiport of *E. coli* [65,89,132]. I imagine that Li^+ has little physiological function in contributing to the normal thermodynamics of active transport. The case of the Na^+/H^+ antiport of bacteria (and bacteroid organelles) is somewhat different in that Li^+ may indeed be a normal substrate. The antiport may be required to pump Li^+ out of the cell or lumen, acting like the bilge pump of an incompletely waterproof ship (see Mitchell [90]). Li^+ has been reported to stimulate proline transport in *E. coli* [91] though it is not clear why.

Geck and colleagues [92] have recently postulated a proton pump at the plasma membrane of ascites cells which would function to maintain the alkaline pH of the cytoplasm in the face of the small (–40 to - 60 mV) membrane potential (inside negative relative to the medium). This proton pump need not be a primary active transport system but could be Na^+/H^+ antiport or Cl^-/OH^- antiport. However, there is no evidence for either of these systems, though the Cl^-/OH^- antiport has been postulated before in another context (Heinz et al. [9,93]). Heinz and colleagues have also postulated an electroneutral K^+/Cl^- symport in ascites cells, which they describe as weakly active, but consuming no noticeable energy. It is not clear exactly what this means. Does it mean that KCl moves into the cells against its electrochemical potential gradient (in which case something more than symport must be involved), or merely that the movement of KCl is blocked by metabolic inhibitors? One thing seems clear about these cells which is that they lack a K^+/H^+ antiport, for that would electroneutrally lower the internal pH and not raise it. Such an antiport has nevertheless also been postulated by Heinz [9,93].

Proton translocating ATPase (H^+-ATPase) activity is, or course, found in animal tissues in specialized situations. Thus, ATPase of chromaffin granules and gastric cells appears to

be proton translocating [52,94] (see below). However, there have been two recent reports indicating that the uptake of amino acid across the plasmalemma of trypanosomes may be coupled to H^+ inflow rather than Na^+ inflow, and that would represent an interesting comparison with the otherwise all-pervading Na^+-coupled plasmalemma porters of animal cells. Midgley [95] has found that transport of α-aminoisobutyric acid into *Crithidia fasciculata* shows no specific Na^+ or K^+ requirement, is ouabain-insensitive and even when uptake is being supported by anaerobic metabolism it is inhibited by 2,4-dinitrophenol, dicyclohexylcarbodiimide and azide. Similarly, Voorheis [96] has investigated the pH sensitivity of threonine uptake into *Trypanosoma brucei* and shown it to be compatible with H^+ symport, a rather tenuous extension of the argument.

Vidaver et al. [97,98] suggested that Cl^- may take part in the formation of the active (quinternary) complex in the case of glycine uptake into pigeon erythrocytes (carrier + 2 Na^+ + Cl^- + amino acid). They certainly demonstrate that the rate of glycine uptake is determined by the anion present. Specificity is broad and there is little obvious correlation between the stimulatory anions and their chemistry; Cl^- is most stimulatory followed by NO_3^-, SCN^-, I^-, F^- with SO_4^{2-} and $CH_3SO_3^-$ supporting essentially no uptake on that carrier. If there were an electrogenic uniport for Cl^- the series would, in fact, correlate quite well with the ability of the ions to diffuse passively across the membrane electrogenically, and thus serve as counterions for an electrogenic 2 Na^+/amino acid symport. The evidence that the anion travels on the amino acid carrier is weak. It is unlikely that Cl^- contributes to the energetics of transport for such an energetic contribution would require that Cl^- itself be pumped away from equilibrium. Because Cl^- concentration affects K_M for glycine but not V, Vidaver suggested that Cl^- must bind to the carrier before the binding of amino acid (see section VII, Kinetic Models).

Chloride has also been postulated to be an essential component of the translocated complex in the case of γ-aminobutyric acid and L-glutamic acid uptake into rat brain synaptosomes (Kanner [45,46]) and the uptake of 5-hydroxytryptamine into platelets (Rudnick [49,50]).

These 'biogenic amine' uptake systems show a further interesting feature in common (not reported for the avian erythrocyte) in that an outwardly directed potassium concentration gradient seems to contribute directly to the energetics of uptake [45,46,50]. Rudnick and Nelson [50] have shown that 5-hydroxytryptamine uptake appears to be electroneutral, and thus rule out the possibility (previously favoured) that the K^+ gradient stimulated uptake by developing a diffusion potential, negative inside. Valinomycin, added to the potassium-loaded platelet suspension produced a membrane potential of −50 mV (relative to the medium) but did not significantly stimulate 5-hydroxytrypta-

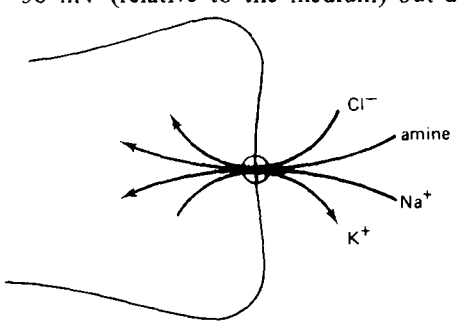


Fig. 3. Fluxes possibly coupled to the transport of biogenic amine across the plasmalemma; step 1 of Fig. 1.

mine uptake. Thus, these 'biogenic amine' transport systems represent the first cases where the outwardly directed K^+ gradient appears to be used energetically for solute uptake (see Fig. 3). However, care should be taken to test the Crane and Eddy effect referred to above.

Subsequent to the uptake of neurotransmitter across the plasma membrane, by the high-affinity, Na^+-dependent process described above, the neurotransmitter (or other active amine) is sequestered in granules or vesicles (as discussed more fully in section III). Studies of the energetics of that second step suggest that it involves electroneutral uniport followed by trapping of the protonated amine in the acidic lumen of the vesicle (pH 5.5 in the case of the chromaffin granule of adrenal medulla (Johnson and Scarpa [99]). The pH gradient is maintained by a Mg^{2+}-dependent ATPase associated with chromaffin granules (Casey et al. [52], Flatmark and Ingebretsen [100]) and synaptic vesicles (Toll and Howard [55]). It is not clear whether there is in each tissue a specific carrier, for the same eventual distribution of amine can be achieved in phospholiposomes, across which a pH gradient is maintained, by simple diffusion of the uncharged form, followed by trapping in the positively charged, protonated form (Nichols and Deamer [101]). However, sensitivity of uptake to the specific inhibitor, reserpine, indicates the involvement of a specific carrier in most cases. Then again, it is not clear what translocation reaction the carrier catalyses as $H^+/(amine)^+$ antiport, OH^-–$(amine)^+$ symport and uniport of the uncharged amine would all show the same dependence on the pH gradient.

V. Alternative sources of free energy

This review explicitly excludes a consideration of the bioenergetics of primary active transport systems, i.e. the well known ion-translocating ATPases (2 Ca^{2+}-ATPase of sarcoplasmic reticulum, $(Na^+ + K^+)$-ATPase of animal plasma membranes, H^+-ATPases of plants, bacteria and bacterioid organelles). However, there are some active transport systems in bacteria and mammalian tissues where it seemed natural to expect the mode of energy coupling to be by ion-symport or ion-antiport, but where it has turned out that another source of free energy is used instead. In this section, I shall consider recent work on these alternative systems.

There are two ion transport systems in bacteria, recently reported to be driven by direct hydrolysis of ATP; Ca^{2+}-efflux in *Streptococcus faecalis* [102] and the so-called Kdp system for potassium uptake in *E. coli* [103]. The Kdp system in *E. coli* is a high affinity system, presumably to allow K^+ uptake at very low external K^+ concentrations [104]. It is tempting to conclude that the reason that K^+ uptake by this sytem uses ATP hydrolysis (releasing about 60 kJ per mol ATP), rather than simply the membrane potential (180 mV is equivalent to 17.2 kJ per gramion K^+ transported) is because with extracellular K^+ concentrations below 10^{-4} M, the membrane potential does not provide enough energy to achieve the required internal K^+ concentration (0.1 M). The Ca^{2+}-ATPase of *S. faecalis* can be understood in a different way. With a membrane potential of between −150 and −180 mV [105] calcium must be continuously pumped out of the cell or it would gradually accumulate to between 10^5 and 10^6 times the extracellular Ca^{2+} concentration. (Calcium extrusion is characteristic of essentially all living cells.) But, as *S. faecalis* has no functional respiratory chain, its primary source of energy for transport is ATP hydrolysis and not the proton motive force as in aerobic bacteria (Fig. 4).

A very interesting and somewhat similar story is beginning to emerge concerning carbohydrate and amino acid transport processes in bacteria. Berger [106,107] suggested

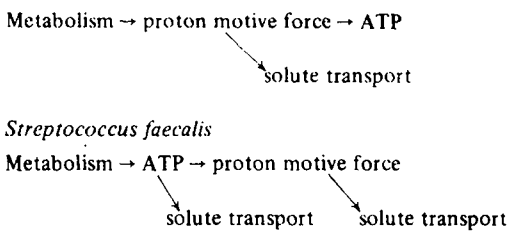


Fig. 4. Sources of free energy for transport in bacteria.

that, while the transport of certain amino acids into *E. coli* was by proton symport (e.g., glycine, proline, serine, phenylalanine and cysteine), the transport of certain other amino acids (e.g., glutamine, methionine, diaminopimelate, histidine, ornithine and arginine) depended much more directly on the presence in the cell of ATP or some phosphorylated derivative, let us call it X ~ P, and did not require for accumulation the proton motive force (pmf or ΔP of Mitchell [90]). Certain carbohydrates and ions also seemed to be X ~ P dependent and ΔP independent. (See Rosen and Kashket [8] for a recent discussion of this subject.) Some reports indicate that both X ~ P and ΔP are required simultaneously (Rhoads and Epstein [104,108]) or that a particular system can use either source of energy (Rosen [109]). Other reports are contradictory: thus Plate et al. [110] concluded, from the inhibitory effects of cholicin K in an ATPase-negative mutant, that glutamine and proline transport were both dependent on ΔP. Singh and Bragg [9,111] have investigated the uptake of proline, glutamine, galactose and β-methylgalactoside in a cytochrome-deficient strain of *Salmonella typhimurium*, where the $\Delta\tilde{\mu}_{H^+}$ must be formed via ATP hydrolysis and not from respiration. They found that the accumulation of all four substrates was inhibited by uncouplers and by dicyclohexylcarbodiimide and azide (inhibitors of the H^+-ATPase). There was no differential sensitivity of the supposedly proton-coupled proline uptake to uncouplers, while dicyclohexylcarbodiimide, which blocks the H^+-ATPase but does not noticeably lower cellular ATP levels, did inhibit the supposedly X ~ P dependent systems.

Several of the criteria frequently used to distinguish the two modes of energy coupling are of doubtful reliability and the results are seldom clear-cut. Thus, proton inflow driven by substrate inflow into non-metabolizing cells, though often observed in H^+-symport systems [56–59,143,144] would not be expected in the case of a high affinity carrier-substrate combination because the carrier would soon become saturated with substrate on both sides of the membrane [112]. Several uncouplers undoubtedly react with sulphydryl groups, such as the essential cysteine residue in the arabinose-binding protein (Quiocho et al. [9,113], Daruwalla [114]). Uncoupler, while undoubtedly lowering ΔP may also cause some lowering of ATP levels. Dicyclohexylcarbodiimide is not highly specific and could react with other proteins besides the BF_0 component of the H^+-translocating ATPase. One might ask whether arsenate lowers the $\Delta\tilde{\mu}_{H^+}$; it might be expected to. There is thus considerable uncertainty surrounding the distinction between the proton coupled and the X ~ P driven uptake systems.

The identity of X ~ P has until very recently [125] remained obscure. It has not yet been shown, except in the case of the Kdp potassium system, that there is a stoichiometric hydrolysis of a phosphoryl compound with each molecule of transported substrate; it could be that ATP is required for regulatory reasons, not energetic ones (Lieberman et al. [115]). Alternatively, it could be that ATP is used stoichiometrically, but the

membrane potential (or the entire proton motive force) has a regulatory function (as suggested by Rhoads and Epstein [104]). Indeed, the adenyl cyclase has recently been shown to be influenced by $\Delta\tilde{\mu}_{H^+}$, both in bacteria and in fungi (Peterkofsky et al. [116]; Trevillyan et al. [117]; see Ref. 118 for a discussion of regulation).

Recent work by Hogg [9,119] and by Henderson and collaborators [114,120] has brought some clarification of these issues, suggesting a framework in which they can be understood. Arabinose is transported into *E. coli* by two transport systems called AraE and AraF. The former is a proton symporter and has a K_M of 125 μM, the latter is typical of the X ~ P dependent systems, has a K_M of 8.3 μM, involves a periplasmic binding protein and does not appear to catalyse H^+-arabinose symport. When bacteria were grown on adequately high arabinose concentrations the efficiency of growth (growth yield per g carbohydrate substrate) and growth rate were both higher in strains carrying only the AraE system than in strains carrying only the AraF system. So, under these conditions the proton symport system was more rapid and more efficient. The story for galactose is very similar. There are two systems analogous with the two arabinose systems and indeed mapping very close to them, called GalP and MglP. GalP is a low affinity, H^+-galactose symport system, MglP is a high affinity, X ~ P-dependent, binding protein dependent system. It has been observed (Hunter, cited in Ref. 114) that in a carbon-limited chemostat the maintenance energy is higher for cells possessing only the proton-symport system (GalP) than for cells possessing only the X ~ P or binding protein system (MglP). That is to say, at low carbohydrate concentrations H^+–symport is less efficient, and at high concentration it is more efficient than the X ~ P system.

There appear to be at least two transport systems for lysine in *E. coli* [8]. It seems possible that the existence of multiple transport systems for many of the amino acids, as is already indicated for several carbohydrates, could explain some of the ambiguous and conflicting results obtained when trying to determine the nature of energy-coupling for a particular substrate.

The role of the binding proteins is still somewhat obscure [121 123]. The point of view of Wilson [122] is that the binding protein with its high substrate affinity successfully scavenges the rare substrate molecules, but would be unable to release them again rapidly enough for the metabolic needs of the cell without some energetic contribution from the second, membrane-bound, element of the uptake system, which must therefore cause a conformational change in the protein-substrate complex. Silhavy et al. [123] also conclude that the binding protein is not simply a modifier of the affinity of the membrane-bound component, for without the binding protein there is probably no uptake, even at high substrate concentration (see, however, Robbins et al. [124]; the problem in this work may be to construct bacterial strains which lack the other carrier systems and components completely.)

The identity of X ~ P, which appears to be ATP for the two ion transport systems cited above, may now be known for the amino acid systems also. Hong and coworkers [125] have recently shown that acetyl phosphate may be required for the uptake of glutamine, histidine and methionine by *E. coli*. In mutants blocked in phosphotransacetylase, and thus requiring ATP synthesis for acetyl phosphate formation (via acetate kinase, see Fig. 5), the blocking of ATP formation (by metabolic inhibitors) blocked active transport; but in phosphotransacetylase-positive strains it did not. Thus acetylphosphate, rather than ATP, is indicated as the energy source for glutamine transport. The acetylphosphate levels were confirmed to be as expected, by direct assay.

Hong and coworkers [115,126–128] have postulated a somewhat mysterious energy

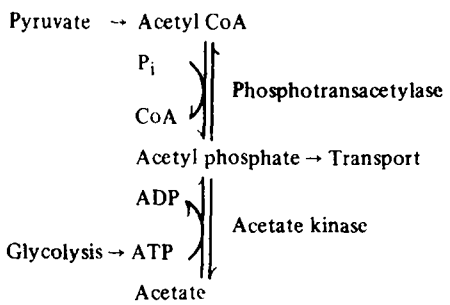
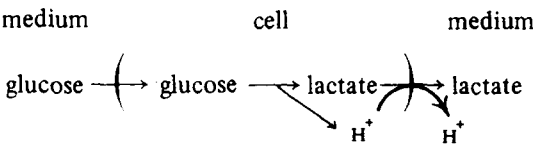


Fig. 5. Two pathways for the formation of acetyl phosphate in *E. coli*.

coupling factor, thought at one time to be involved in both proton-coupled active transport and in the X ~ P-dependent active transport systems. However, it is now found not to be involved in the latter system [125]. These energy coupling factor mutants have not yet been tested in other laboratories and this work is unconfirmed.

An alternative source of free energy for coupled transport, again in bacteria, has been suggested by Michels et al. [129]. They suggest that, in the absence of both respiration and H^+-ATPase activity, a proton motive force can still be generated by the proton-coupled excretion of lactate as metabolic end product of glucose fermentation. However, the products of the metabolism of electroneutral glucose must themselves be electroneutral, viz. lactic acid and not lactate, thus:



Protons are both generated inside and removed from the cells. Only with an H^+/lactate translocation stoichiometry of greater than 1.0 will a proton motive force be established. The driving force is the lactate concentration gradient and so it is very sensitive to external lactate concentration. To establish a large proton motive force from a small lactate gradient the authors postulate a variable stoichiometry of H^+ per lactate which falls from 2 towards 1 as the external lactate concentration rises. There is at present little evidence in favour of the scheme.

Several authors have suggested that, for the uptake of anions, proton coupled solute transport may be merely a means of preserving electroneutrality and not of energizing active transport. As the pH gradient in many bacteria seldom exceeds 1 pH unit [8] the accumulation of a singly charged anion by electroneutral H^+ symport could not exceed 10-fold. Mandel and Krulwich [130] point out that in *Arthrobacter pyridinolis* D-gluconate is transported electroneutrally with a proton but is immediately phosphorylated; it is 6-phosphogluconate which accumulates.

In cases like this the desired electroneutrality of transport may produce an undesired acidification of the cell interior. Proton extrusion (followed or accompanied by alkali cation uptake) may then be necessary, and here again not for energetic reasons but to regulate internal pH [85,131]. Uncoupling agents may inhibit uptake but not because the $\Delta\tilde{\mu}_{H^+}$ is required for accumulation.

Brodie and coworkers [132,133] have described a novel system for amino acid accumulation in membrane vesicles of *Mycobacterium phlei*. They report that accumulation of glutamate, glutamine and proline occurs on adding Cu^{2+} to the suspension but that, while the uptake of proline requires Na^+ (or Li^+) also to be present, the uptake of glutamate and glutamine was Na^+-independent [132]. Increasing Na^+ concentration increased V for proline uptake but did not affect K_M [133].

Brodie and colleagues [132,133] believe that the driving force for amino acid accumulation could be a small pH gradient, detected with dimethyloxazolidine dione, which forms on adding Cu^{2+}, but which is directed outwards, acid inside. They suggest that Cu^{2+} oxidizes thiol groups, shown to be present and to be oxidized stoichiometrically with Cu^{2+} reduction, and that the released protons are responsible for the 0.7 unit pH gradient which forms. However, I do not think that they have adequately ruled out the possibility of Cu^{2+}-amino acid complex formation inside the vesicles. Brodie et al. point out that the accumulation of leucine, histidine, etc., is not driven by Cu^{2+} though these amino acids also form complexes. But neither are those latter amino acids actively transported by respiring vesicles. Only proline, glutamate and glutamine are accumulated by Brodie's preparations of respiring vesicles [132], and only these accumulate with Cu^{2+}.

Brodie and colleagues [134,135] have succeeded in extracting and reconstituting the proline carrier in liposomes, formed from soya bean phospholipids. Proline uptake was demonstrated in two rather different ways. In one procedure they loaded the vesicles with K^+, added proline to the medium and initiated uptake with the simultaneous addition of NaCl and valinomycin. The success of this now classical experiment suggests electrogenic ion symport, either with Na^+ or conceivably with H^+ [135]. The other procedure was to reconstitute vesicles containing ferricyanide, suspend them in a medium containing NaCl and ascorbate and initiate uptake by adding benzoquinone. This system generates H^+ inside the vesicle and a pH gradient outwards. The addition of Na^+ and proline caused a surprisingly rapid discharge of the pH gradient, and uncoupler caused a slower release of protons [134]. I suppose the outwardly directed pH gradient, in this and the earlier experiment, could cause a proton diffusion potential, negative inside, which could lead to the uptake of Na^+-proline. The crucial experiment would then be to see if uncoupler stimulated proline uptake by facilitating the outward diffusion of H^+. Nevertheless, I suspect that a better explanation of these results will be found.

The adequacy of the Na^+ electrochemical potential gradient to account for amino acid accumulation in ascites cells has often been debated [93,136]. Even allowing for the misestimates of intracellular Na^+ concentrations, the efficiency of coupling and the electrogenicity of $(Na^+ + K^+)$-ATPase, some workers feel there is still a need for a supplementary form of energy input [137]. It has been suggested that the NADH dehydrogenase activity found in plasma membrane fractions [138–140] might be coupled to amino acid uptake [141,142]. What has been demonstrated is a stimulation by pyruvate or reduced phenazinemethosulphate of amino acid uptake into ascites cells that had been depleted of ATP (and ion gradients). This stimulation was sensitive to ouabain, but instead of concluding that the $(Na^+ + K^+)$-ATPase was involved, these authors suggested ouabain might have wider actions than was previously suspected.

When amino acid uptake was driven by reduced phenazinemethosulphate it was not sensitive to rotenone, but when it was driven by pyruvate uptake it was sensitive to rotenone [141]. Christensen et al. suggested that mitochondria might transmit free energy to the plasma membrane in a form other than ATP. However, in their latest paper [140] the redox activity they investigate is a rotenone-insensitive NADH-ferricyanide oxidoreduc-

tase. The natural oxidant for this enzyme is unclear: it is not oxygen and the activity with cytochrome *c* or cytochrome b_5 is very slow. There is thus considerable uncertainty and several contradictions concerning this proposal. Is the $(Na^+ + K^+)$-ATPase really not involved, though uptake is inhibited by ouabain? Is the plasma membrane NADH dehydrogenase relevant if it is rotenone-insensitive while uptake is rotenone-sensitive? On the other hand, this plasmalemma NADH dehydrogenase requires further investigation; it lacks an oxidant and it lacks a role. Could it be involved in pH regulation? (See section IV.)

VI. Stoichiometries

The gradient hypothesis places no restrictions on stoichiometries other than that the free energy from the down-hill components should be at least equal to that required to drive the uphill components of the coupled transport process. In many cases the simplest possible stoichiometry, 1 co-ion/1 substrate, appears to be sufficient [57].

There are some interesting exceptions. There are anion symports where the carrier–substrate–co-ion complex only acquires a net positive charge when two or more co-ions are bound. Only with a 2/1 stoichiometry will the effect of the membrane potential be obtained (see section IV). Lagarde [143] finds 2 H^+/1 2-oxo-3-deoxygluconate in *E. coli*. Mitchell et al. [144] find that glutamate transport by *Staph. aureus* is electrogenic and the H^+/glutamate stoichiometry is between 1.5 and 2.0. (The earlier work of Niven and Hamilton [145] was apparently invalid, as discussed by West [84] and Eddy [146], who actually predicted that a stoichiometry of 2 would be found.) Glutamate transport in *E. coli* vesicles responds to the full proton electrochemical potential gradient indicating two positive charges per glutamate (perhaps 1 H^+ and 1 Na^+; see section IV) [147].

Eddy et al. [148] have reported for various strains of the yeast genus *Saccharomyces* that, while proline, methionine, arginine, canavanine, lysine and some carbohydrates were taken up each with a single proton, other amino acids such as glycine were taken up by the 'general amino acid permease' with 2 protons. More recently they have shown that the glutamate ion is taken up with about 3 protons (2 net positive charges) while phosphate (if one extrapolates their data to infinitely small pulses) is taken up with 3.3 protons and 2.4 net positive charges. It is perhaps surprising that they have not observed a glutamate concentration gradient (in/out) greater than 70 [146].

In the cases of glutamate and phosphate the H^+ stoichiometry was found to vary with the amount of added substrate and with the time lapse between the addition of metabolic inhibitors and the addition of substrate. This is a bit disconcerting, because unexplained. I find the suggestion of a physiologically variable stoichiometry unconvincing, and would look at experimental snags for an explanation.

There is a similar type of non-unitary stoichiometry in the case of the cation/cation antiports. It is most probable that in mesophilic bacteria these are electroneutral. Thus, in West and Mitchell [58] the pH excursion induced by adding Na^+ was of essentially the same speed in the presence and absence of thiocyanate (admittedly, a weak argument). However, in the case of the halophilic bacterium, *H. halobium*, the concentration gradient against which Na^+ has to be pumped greatly excedes the pH gradient. There is evidence that the stoichiometry here is 2 H^+/Na^+ [81].

Kaback and coworkers, when they turned their attention to the symport model for the lactose transport system of *E. coli*, introduced several new techniques and concepts. Thus, they argued (correctly) that at equilibrium the free energy required to transport the

substrate up its concentration gradient should be exactly balanced by the free energy available from transporting the co-ion (here H^+) down its electrochemical potential gradient. They proceeded to let galactosides accumulate in respiring membrane vesicles until they would accumulate no more, assumed that this represented equilibration with the $\Delta\tilde{\mu}_{H^+}$ which they then measured. The measured $\Delta\tilde{\mu}_{H^+}$ in these vesicles was equal to $-\Delta\tilde{\mu}_{lactose}$, when the external pH was 5.5 but fell with rising pH so that Ramos and Kaback [149] concluded that at pH 7.5 two protons must be involved in the translocation of each lactose. Schuldiner and Fishkes [150] came to a similar conclusion regarding the Na^+/H^+ antiport of *E. coli*. Rottenberg proposed a model [151] elaborating this idea of pH dependent stoichiometries.

However, that is not the case with whole cells. Booth et al. [152] have actually measured initial rates of H^+ entry and lactose entry between pH 6.5 and 7.7 (the limit of reliable measurement) and found a stoichiometry of 1 : 1 (cf. West and Mitchell [57]). Furthermore, they have measured $\Delta\tilde{\mu}_{H^+}$ and $\Delta\tilde{\mu}_{lactose}$ over the pH range 5.9–8.7 and found that at no point was the $\Delta\tilde{\mu}_{H^+}$ less than $\Delta\tilde{\mu}_{lactose}$. In fact, at all pH values it was greater, indicating that the lactose accumulation never came into equilibrium with the proton motive force. It should be mentioned that Booth and Hamilton made two important points about the measurement of $\Delta\tilde{\mu}_{H^+}$; the cytoplasmic, not the cytoplasmic plus periplasmic volume must be used in calculating pH_i, and methylamine may be unsuitable as an indicator of the pH_i as it may not equilibrate (perhaps because of a methylamine porter [153]).

Zilberstein and colleagues, in a convincing paper [154], make these same points. In whole cells $\Delta\tilde{\mu}_{H^+}$ is adequate from pH 6 to 8 for a stoichiometry of 1, which they also measure directly over the same range.

Booth and colleagues [152] have gone on to emphasise in a useful and timely way the difference between a true equilibrium and a kinetic steady-state. In any system with appreciable leaks, equilibrium will be impossible and the equations of thermodynamics will be inapplicable.

There have been other reports of varying stoichiometry. A particularly complex example is afforded by the work of Lanyi on amino acid transport systems of *H. halobium*. Here, as in the work of Kaback and coworkers, the argument rests entirely on accumulation ratios and the assumption that the steady-state is a quasi-equilibrium. When membrane vesicles derived from this bacterium are loaded with K^+ and suspended in Na^+ in the dark, Lanyi [79] argues that there is no membrane potential, only a Na^+ concentration gradient. The evidence for this crucial proposition is 'not shown'. It must mean, as pointed out in section IV, that there is no appreciable K^+-diffusion potential. It was also pointed out that Oesterhelt et al. [78], working with whole cells as opposed to a vesicle preparation, report an appreciable K^+-permeability and a large K^+ diffusion potential under these conditions which is capable of driving ATP synthesis. Returning to Lanyi's experiment and accepting for the time being that he is establishing a purely chemical Na^+ potential gradient, the results are anomalous. At low Na^+ gradients (1- 3-fold) aspartate appears to travel with 4 Na^+ and serine with 2 Na^+, though with higher Na^+ gradients this relationship is not maintained and accumulation eventually plateaus.

Amino acid accumulation in these vesicles can also be driven by the light-dependent proton pump under conditions where Lanyi believes there is essentially no Na^+ concentration gradient. By monitoring the accumulation of lipophilic cations Lanyi has correlated the accumulation of amino acids with the membrane potential and concluded that aspartate must travel with 2 Na^+ and serine (uncharged at neutral pH) with one. Lanyi thus

postulates an amino acid/Na^+ stoichiometry which depends on whether the Na^+ electrochemical potential gradient is electrical or chemical.

In a later paper [80], Lanyi goes on to explain these stoichiometries in terms of an electrically operated switch, in some respects analogous with the models which I have just mentioned of Ramos and Kaback [149] and Rottenberg [181] where the stoichiometries were pH dependent. Thus, for the symport of aspartate, 4 Na^+ were found to be necessary with small membrane potentials (and in chemically-driven accumulation), but only 2 Na^+ as the negative membrane potential increased. Similarly, for serine the figures were 2 Na^+ and 1 Na^+ at small and large membrane potentials, respectively.

While such a model appears to account for the somewhat strange data, I would add that I find these papers extremely obscure and much of the argument somewhat circumstantial. A systematic failure to measure the electric potential correctly might also account for the data.

There is another type of variable stoichiometry in the literature which I think is open to question. Collins et al. [155] reported the isolation of mutants of *E. coli* from a chemostat where alanine was the limiting substrate. These mutants showed an increased displacement of H^+ into the cells on adding alanine, from which it was concluded that the mutants had a 2- or 4-fold H^+/alanine stoichiometry. Now, there are classic papers (Horuichi et al. [156,157]) on the isolation of bacteria with gene duplications from chemostats with limiting lactose in which there were 2- or 4-fold activity of β-galactosidase. In spite of the original papers saying the contrary, it is easy to show that permease activity is also increased (West, I.C., unpublished data; Booth, I.R., unpublished data). If we bear in mind the concept of kinetic steady-states it is clear that the above method of adducing co-ion stoichiometry (albeit introduced by West [56]) is invalid. It seems likely that the greater rate of H^+–alanine inflow results in an increased steady-state displacement of the pH trace, without there being any need to invoke increased H^+/alanine stoichiometry.

In summary, co-ion stoichiometries greater than one are frequently found; particularly when such a stoichiometry is necessary to confer a net charge on the translocated complex (or a net charge difference between the completely unloaded carrier and the completely loaded carrier–substrate–co-ion complex) so that the resulting distribution ratio may be influenced by the membrane potential. On the other hand, variable stoichiometries are harder to visualize, and however convenient they might be for organisms possessing such features, the evidence for them is still dubious.

VII. Kinetic models

The trouble with kinetic models is that, to be general and valid, they have to be complex: the subject is inherently complex. There are numerous analyses of carrier transport, though relatively few which deal at all fully with coupled transport [162,163,168]. However, these earlier treatments did not consider the question of the electric field because it was believed that chemical and electrochemical activities would be interchangeable in kinetics, as chemical and electrochemical potentials are interchangeable in thermodynamics. This turns out not to be the case and it is now appreciated that a kinetic model of symport or antiport must consider the charge on the various translocated intermediates. There are several authors who have considered the effect of the electric field in a cursory way or for a simplified or specialized model [158–160]. Vidaver [98] has elaborated his earlier model for the 2 Na^+–glycine transport system of pigeon erythrocytes [161] by consider-

ing the effect of an electric field on those translocation steps perpendicular to the plane of the membrane, but it remains a specialized model. There are two groups who have developed a general analysis of the effects of the electric field on the kinetics of coupled transport; those of Stein [162,163] and Heinz (164–166].

The usual way [98,158,163] of introducing the electric field into kinetic equations is to consider the equilibrium condition described by the Nernst equation:

$$[A^+]_1 \underset{\psi_1}{\overset{}{\rightleftharpoons}} [A^+]_2 \quad \psi_2$$

$$\Delta\psi = \psi_2 - \psi_1 = \frac{2.3RT}{zF} \log \frac{[A_1^+]}{[A_2^+]}$$

At 30°C, where $2.3\ RT/F = 60$ mV, a potential of such a size will sustain a concentration gradient of 10-fold. Now, it is clear that flux of A^+ from left to right under the influence purely of the chemical gradient would be ten times the flux from right to left, and it is argued therefore that the electric field must produce an equal but opposite effect. Therefore, influenced purely by the electric field, the flux of A^+ from right to left must be ten times the flux from left to right. Without either the field or the concentration difference the flux rates would be equal and be accelerated or retarded by the field by a factor of $\sqrt{10}$, respectively. This simple argument applies to the equilibrium condition, with no net flux. For diffusion of charged particles in a homogeneous membrane and in a constant field the integrated Goldman-Hodgkin-Katz equation applies, to allow for the altered diffusion due to the altered concentration which the electric field produces [167]. Geck and Heinz [164,165] point out that a simplification can be made in this rather more complicated equation if one postulates a considerable, but symmetrical, barrier to diffusion so that translocation is a rare event, determined by the Boltzmann energy of the diffusing species. This assumption allows the simplification of the flux equation to a form identical with that derived from the simple equilibrium argument.

Another standard procedure found in all the papers considering the electric field is to assume that reactions parallel to the surface of the membrane will be unaffected by the field, while reactions perpendicular to the membrane will be affected by the full extent of the field, $\exp(-z F \Delta\psi/2 RT)$ where $\Delta\psi$ is the membrane potential as measured between the two aqueous phases. In deciding which reactions are parallel and which are perpendicular to the membrane, authors always use the standard ferry-boat type of model (Fig. 6), but are always careful to say that the more modern gated-pore type model would give an identical kinetic analysis. This seems doubtful, for in a gated-pore model the translocation or conformational change which effectively changes the accessibility of binding sites from face 1 to face 2 need involve a movement through a very few ångströms, which may, in any case, be in the plane of the membrane (Fig. 6). The electric field effects in such a model will, of course, be confined to effects on the concentration of charged substrates (see the 'proton-well' concept discussed in the next section, and illustrated in Fig. 6).

In their kinetic analysis Heinz and colleagues [164,166,168] make the simplifying assumption that binding reactions are rapid with respect to translocation reactions. This sounds like the transport equivalent of a Michaelis-Menten equilibrium treatment of enzyme kinetics which is generally regarded as of considerably narrower applicability than the steady-state treatments, but they justify the assumption by saying that without

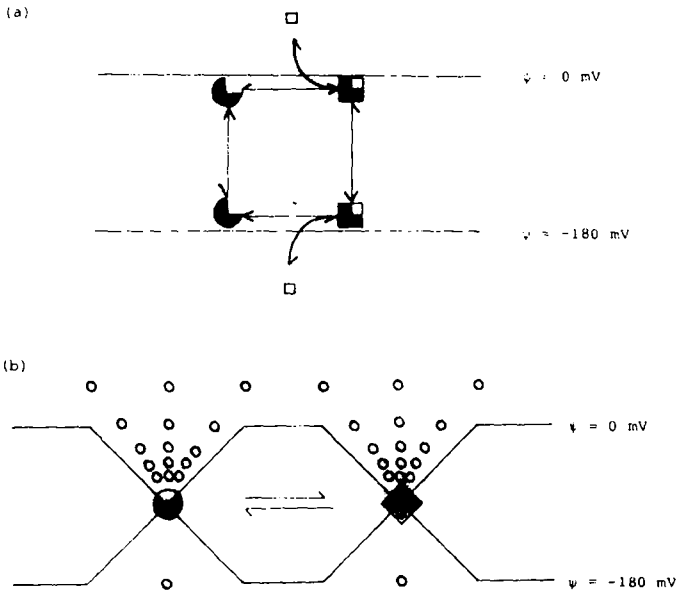


Fig. 6. Types of mobile carrier. (a) Extreme form of the shuttling of ferry-boat carrier, showing a conformational change on binding substrate (or substrates). (b) Extreme form of the gated-pore type of carrier again showing a conformational change on binding substrate. See also the 'proton-well' effect whereby charged substrates may be concentrated by the electric field. There need be no movement of the carrier along the axis of the field.

it energetically coupled transport is impossible in any case. However, only two of the translocation reactions have to be slow (or absent) in order to obtain energetic coupling, and those are the translocations of the binary complexes, i.e., the carrier with the co-ion but not the substrate, or with the substrate but not the co-ion. (There are several experimental reports indicating slow or zero translocation of the binary complexes [161,169, 170].) It seems intuitively obvious that, by analogy with enzyme kinetics, if the reactions following the substrate binding step are of comparable speed then the measured K_M parameters are complex functions of the binding equilibria and the translocation rate constants. The assumption of binding equilibrium may be an unjustified oversimplification.

Heinz and colleagues analyse two basic types of coupled transport which they call the 'affinity type' and the 'velocity type', though they admit that most experimental cases lie between these two extremes. The terms 'affinity type' and 'velocity type' have perhaps become somewhat confusing as the analysis developed. 'Velocity type' does not mean that the effects of Na^+ are confined strictly to V, but only that in constructing the model Geck and Heinz have allowed Na^+ to affect only a translocation and not a binding step. The 'affinity type' is likewise a model builder's concept. This problem becomes even worse when Geck and Heinz consider the effect of the electric field, for it turns out that in the cases that they have been calling pure 'affinity type' models the maximum velocity can be affected by the electric field. Similarly, in what they called a pure 'velocity type' model, the electric field can either raise or lower the apparent K_M, depending on whether the loaded or the unloaded carrier respectively bears zero net charge, while the V can, in the latter case, be scarcely affected.

The results of the Geck and Heinz analysis can be most clearly summarized by the

diagrams in Fig. 7 (taken from Heinz and Geck [165]). This powerful and valuable analysis seems to tell us that we can still infer rather little about the nature of the underlying kinetic model from steady-state kinetic experiments in which we vary substrate concen-

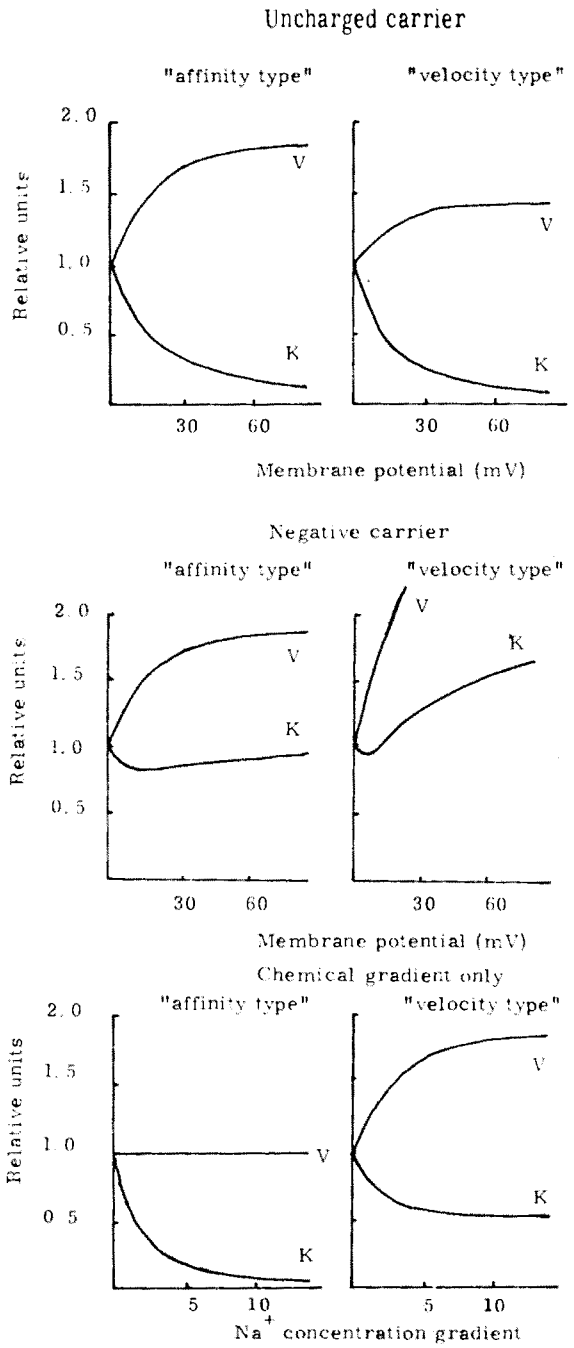


Fig. 7. Predicted effects of membrane potential on the kinetic parameters for various carrier models. Taken, with permission, from Ref. 165.

tration, co-ion concentrations, and membrane potential. Deduction can be made with more confidence about the charge on the unloaded carrier. (See the next section.)

Heinz has performed an interesting excercise in comparing the traditional 'law of mass action' approach with the more controversial and esoteric 'non-equilibrium thermodynamic' approach [165,166]. It is interesting to see over how small a range the phenomenological coefficients of the thermodynamic approach remain constant; in the least favourable case for less than one log unit of concentration gradient.

The approach of Stein and colleagues is somewhat different. The most lucid introductory exposition of their steady-state kinetic analysis of coupled transport is in the paper by Stein and Honig [1], though much analysis is dealt with more rigorously elsewhere [162,163]. In this paper they consider the essential basis of energy coupling in active transport and show that there is no formal difference between primary and secondary active transport. The simplifying assumption with which these authors start is that the translocation rate constants of the carrier loaded with substrate only, or with co-ion only, are both zero (see above for justification). They analyse the resulting models by the standard enzymological techniques pioneered by King and Altman [171], using the approach of Britten [172]. They have not yet considered the effects of an electric field on coupled transport, but Stein and colleagues [162,163] have considered the effects of an electric field on a simple carrier. They have also considered the effects of unstirred layers [162].

Several useful insights are discussed in the paper of Stein and Honig [1]; I shall briefly mention some of them. In real experimental systems which inevitably contain leaks, the rate of pumping is an important consideration. An active transport system in which the co-substrate affects only the transport velocities could, in principle, achieve a high ratio of intracellular to extracellular substrate but it is likely to be an inefficient pump for the following reason. If the affinity of the carrier is inappropriate it will either fail to load at the low external concentration or fail to unload at the high internal concentration. Thus, efficient pumping is likely to show different substrate affinities at the two faces.

Stein and Honig [1] formulate quite neatly the relationship between the ratio of the affinities for substrate on the two sides of the membrane and the affinities for counter substrate on the two sides. (I presume there is an equivalent equation for co-substrate affinities.) This ratio is again related to the difference in standard free energy of the unloaded carrier between the two sides, and the affinity ratio for all other substrates that can be transported by the carrier. That is an intriguing insight. The unloaded carrier may have different free energy on the two faces of the membrane either because of changes to the carrier on translocation, or because of an asymmetrical environment provided by the membrane. Let me consider an extension of this argument not actually spelt out by the authors. Suppose, for a symport system, that the unloaded carrier were negatively charged, so that the protonated (or Na^+ complexed) and substrate-loaded carrier is uncharged. Suppose, in addition, that an asymmetry in the membrane means that the empty carrier is at lower free energy on the inside. In the absence of a membrane potential the measured affinity on the outside will be reduced because of the tendency of unloaded carrier to revert to the inside. With a membrane potential (inside negative) there will be a considerable change in free energy of the unloaded carrier; it will now prefer the outer face. This, according to Stein's analysis, will generate a lower apparent affinity for substrate binding at the inside and raise the affinity at the outside. One problem here is that this result is not in accord with the Geck-Heinz analysis (see Fig. 7) where such a K_M effect is characteristic of a neutral rather than a negative carrier.

Also discussed by Stein and Honig [1] is the co-transport case where there is no differ-

ence in the free energies of the unloaded carrier (E) at the two membrane faces (face 1 and face 2). In this case "all the difference in affinity found for the driven substrate will arise from the intrinsic difference between E_1 and E_2 for binding of the driving substrate. That side at which the driving substrate binds with lower intrinsic affinity will have a higher affinity for the driven substrate .... In the case of osmotic-osmotic co-transport, the product of the affinities for the two substrates is constant at the two faces of the membrane ..." . I shall return to these points in the next section.

This analysis of Stein also emphasizes what is now well known to students of facilitated diffusion, but does not seem to be so widely appreciated yet among those studying the more complex systems of coupled transport. The translocation rate constants may be asymmetric for the inwards and outwards transitions, and they may be, and usually are, different for loaded and unloaded carrier. It matters crucially whether the carrier returns from side 2 to side 1 loaded or empty. The measured affinity constant (K_M), i.e., the concentration giving half maximum flux, is different for exchange and for net flow. It is worth stressing that in transport kinetics (unlike enzyme kinetics) the return of the empty carrier is an important reaction in net transport and that the kinetic parameters obtained in an equilibrium exchange experiment will (in general) be different from those obtained under infinite-trans or zero-trans conditions (i.e., conditions of saturating or zero substrate concentration on that side of the membrane towards which the measured flux is occurring [162]). Hopfer [173] has formulated and applied an interesting analysis of Na^+–glucose symport using an integrated rate equation. The model is less general than those discussed above but raises some points of general interest. Hopfer argues (though without showing a rigorous analysis) that if Na^+–glucose symport is assayed under conditions of flux there will be a tendency for the inflowing Na^+ to reduce both the Na^+ concentration gradient and the membrane potential. This effect, Hopfer maintains, can lead to a systematic underestimation of both K_M and V. Indeed, if the re-extrusion of Na^+ is sluggish, the experiment may unwittingly be performed with zero $\Delta\tilde{\mu}_{Na^+}$; V can appear Na^+-independent because there is, in fact, no $\Delta\tilde{\mu}_{Na^+}$ gradient, and K_M may appear Na^+ dependent. This effect should be less pronounced in whole cells (where Na^+ extrusion may be quite rapid) than in membrane vesicles or reconstituted systems, but even in whole cells the effect may be appreciable. Geck et al. [174] demonstrated biphasic amino acid uptake into ascites cells, presumably the result of inflowing Na^+ (or amino acid!) on the driving force for subsequent uptake (Fig. 8).

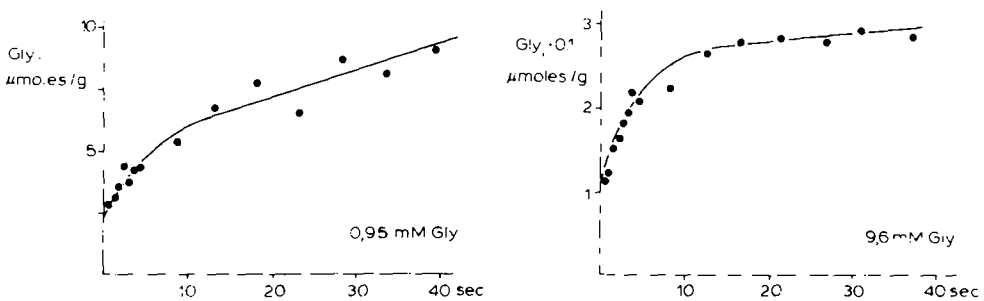


Fig. 8. Glycine uptake during the first minute of incubation. The cells were centrifuged through a layer of medium containing ^{14}C-labelled glycine, the thickness of which determined the incubation time. Note the biphasic time-course, particularly at the higher glycine concentration. Taken, with permission, from Ref. 174.

Hopfer therefore evolved a different kinetic assay for measuring the effect of $\Delta\bar{\mu}_{Na^+}$ on K_M and V in isolated brush-border vesicles. He established glucose and Na^+ equilibrium in the absence of a membrane potential and then measured the time-course of the exponential [^{14}C]glucose influx on adding tracer glucose to the medium. The $t_{1/2}$ of that influx is a simple function of the glucose concentration from which V and K_M can be determined. Hopfer also considered the effect of a heterogeneous population of vesicles and how this could be allowed for, provided one only wanted relative V values and the effects of Na^+ on those and did not need to know the absolute values. He also included the contribution of a glucose leak pathway in his analysis.

VIII. Recent experimental investigations of coupling mechanisms

In this section, I shall discuss several examples of co-transport systems where interesting information on the coupling mechanism has been published in the last year.

The very thorough kinetic analysis by Geck and Heinz, discussed above, has been applied to results obtained in Heinz's laboratory on the Na^+-amino acid transport into ascites cells [165,175]. When the membrane potential was systematically increased by adding increasing amounts of the K^+ ionophore, valinomycin, to a suspension of cells in a low-potassium medium, the standard kinetic parameters of α-aminoisobutyrate uptake were modified as follows. Increased membrane potential caused a decreased apparent K_M and an unaltered V. A plot of V/K_M against membrane potential therefore increased with increasing potential. According to their theoretical model, discussed in the previous section, these results indicate unequivocally that the charge on the unloaded carrier is zero, so that the ternary complex bears a positive charge. Heinz and Geck [165] point out that it is much more difficult to infer from these results whether the carrier behaves more like their 'velocity type' or 'affinity type' model. However, as the absence of any appreciable effect of electric field on V is, paradoxically, a feature of their 'velocity type' model they tentatively conclude that the ascites amino acid carrier is of the former type. It is perhaps worth remarking once again that Geck and Heinz use the terms 'velocity type' and 'affinity type' to describe two alternative hypothesis used in constructing their models, viz. that the co-ion influences the rate of a translocation reaction, or a binding reaction, respectively. It is thus paradoxical but not self-contradictory to find that in the 'velocity-type' model the effect of co-ion is largely seen in changes of the apparent K_M.

The major paper from Vidaver's group [98] on amino acid uptake in pigeon erythrocytes approaches the same problem, that of the charge on the unloaded carrier and again by investigating the effect of electric field on the kinetic parameters, but the kinetic analysis is different and worth considering in a little detail. Earlier work from this group suggested a model [161] in which 2 Na^+ and possibly even a Cl^- are required to bind to the carrier before amino acid is bound. Na^+ affects only the K_M parameter. The experimental system was as follows. Erythrocytes were haemolysed and resealed to contain diluted haemoglobin and anions (and cations) of choice and were then incubated in saline. By haemolysing and resealing in a chloride medium a very small Donnan ratio ($[Cl^-]_o/[Cl^-]_i = 1.1$) could be obtained, while resealing in medium containing the non-permeating toluenedisulphonate anion produced a Donnan potential or chloride diffusion potential, again measured as a chloride ratio, of 3.6 ($-2.3\ RT/F \log 3.6 = -33$ mV relative to the medium). Unfortunately, this potential affected the intracellular pH, and the effects of the pH change had to be corrected for, but when that was done it appeared that the Donnan potential induced an increase in the V of glycine entry of 70% and opposite

changes in the two components of K_M for glycine of about 50%. These changes in the kinetic constants were matched with considerable precision by predictions from the model assuming symmetry of the binding constants between inside and outside, and for the case where there was a single net negative charge on the unloaded carrier. For the net inflow cycle with such a model the exit of unloaded carrier is the only step sensitive to the electric field, but of course the rate constant for that step enters at several points into the overall kinetic equation.

There are several drawbacks about this experiment. The Donnan potential achieved is very small, and the kinetic consequences are even smaller. The data appear very scattered. The pH effects are large and complex, requiring the assumption of three ionizing groups of pK_a 6.2, 6.8 and 7.9, the first and last sensitive to the pH of the intracellular milieu, the second responding to extracellular pH. Several other corrections were necessary, such as for re-exit of absorbed glycine, differences in the efficiency of resealing in the different media, and so on. However, accepting the data as sound, the result is that increased membrane potential increased V for glycine entry, while the K_M moved considerably less, if at all. According to both the Vidaver analysis [98] and the rather more general Geck and Heinz analysis [164,165] this is consistent with the unloaded carrier carrying a single negative charge. This Na^+- glycine symport of pigeon erythrocytes, which differs in several respects from the Na^+–amino acid symport of ascites cells would therefore seem to differ also in the charge on the unloaded carrier: negative in the pigeon erythrocytes, neutral in the ascites cell. Eddy [159] concluded on the basis of a different argument that the glycine carrier in a yeast was neutral. Rottenberg's model (section VI) was based on the hypothesis that the translocated complex is always neutral. This appears not always to be the case.

The Na^+ -glucose transport in isolated brush-border vesicles has recently been studied by Hopfer [9,173] and general aspects of his novel kinetic analysis have been discussed in the previous section. Using Hopfer's integrated rate equation on the data from equilibrium exchange experiments, a K_M value of 14 mM was obtained for glucose and it was independent of Na^+ concentration between 1 and 100 mM. Over the same range of Na^+ concentration the V value increased 15-fold. Phlorizin inhibited with a K_I of around 2 μM independently of $[Na^+]$, both in equilibrium exchange experiments and during glucose uptake. Hopfer comments that the several reports [176- 180] of Na^+-independent V and Na^+-dependent K_M in similar vesicle experiments are likely to be in error, as discussed in the previous section. Similarly, Hopfer argues that the very low K_M values reported for some vesicle preparations (80 μM) are more likely to be wrong than is the much higher value of 10.8 mM obtained in renal microperfusion experiments in vivo [181]. Against this it must be remembered that Hopfer's measurements were made under conditions of no net flux and no membrane potential, and that the kinetic parameters under these conditions may not be applicable to conditions of net flux. It is interesting to note that this question is still being debated ten years after the major review of Schultz and Curran [182].

Tannenbaum et al. [9,183] report that high affinity phlorizin binding to brush-border membrane vesicles is dependent on $\Delta\mu_{Na^+}$, an effect not found by Hopfer [173].

The glucose transport system of *Chlorella vulgaris* has been discussed in several papers by Komor and coworkers [184- 188]. The inducible glucose carrier catalyses H^+–glucose symport and can accumulate non-metabolizable analogues to more than 1000-fold. Proton conducting uncouplers inhibit not only sugar accumulation, but cause an unexpected inhibition of the efflux of previously accumulated sugar and, even more unexpectedly,

they inhibit equilibrium exchange. (These effects are not generally found in bacteria but are found in fungi; see Refs. 7 and 189 for a review.) Variation of the external pH titrates the carrier from a high affinity form (K_M = 0.3 mM) at acid pH to a low affinity form (K_M = 50 mM) at alkaline pH; the pK_a for this titration is 6.85. The V of sugar uptake is not affected by external pH in the range pH 6.0–8.8. The stoichiometry of H^+ translocated to sugar translocated falls from 1 to 0 with increasing pH, again showing a pK_a at 6.9. Sugar uptake is electrogenic.

The pH effect on K_M could explain an accumulation of 100-fold, provided there is an adequate ΔpH, which normally there is not. It is clear that both the electrical and chemical parts of the proton motive force must be involved in the very high accumulations mentioned above. Schwab and Komor [190] have recently investigated the role of the electric field. They found no effect of electric field on the V of transport, measured at an external pH of 6.0, but they found a shifting of the whole titration curve mentioned above (Fig. 9). On increasing the membrane potential from -75 mV (relative to the medium) to -135 mV, the apparent pK_a moved from pH 7.01 to 7.54 (i.e., protonation was facilitated by the membrane potential). They explained the effects in terms of a 'proton well', a concept invented by Mitchell [191–194]. The 'proton well' is a proton conducting invagination of the membrane such that protons are attracted inwards by the electric field to the bottom of the well; $\Delta\psi$ is thus converted to ΔpH. In these experiments the manipulation of the membrane potential ($\Delta\psi$) was achieved by adding potassium citrate to depolarize the membrane, presumably by forming an inwardly directed K^+ diffusion potential. The possible effects of citrate on the internal pH were not discussed, but would have been important (see below). This 'proton well' mechanism could explain how accumulations of up to 100-fold could be observed even under conditions where the measured ΔpH was inadequate, but I do not see how accumulations greater than 100-fold could be generated by the system as described so far, i.e., assuming that the internal K_M for exit responds exactly as does the outward K_M for entry.

Komor et al. [195] have recently reinvestigated the inhibition of efflux and equilibrium exchange caused by uncouplers. Uncouplers cause a rapid lowering of the membrane potential ($\Delta\psi$) from -130 to -90 mV, the remaining potential presumably being a Donnan potential or potassium diffusion potential. Now, with adequate uncoupler, the pro-

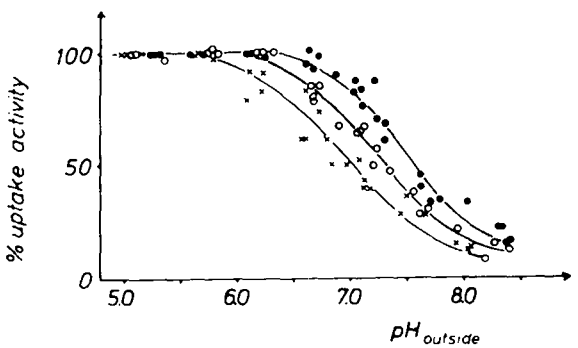


Fig. 9. The effect of pH on 6-deoxyglucose influx into *Chlorella vulgaris* at three different membrane potentials (relative to the medium). Taken, with permission, from Ref. 190. •——•, -135 mV; ○– –○, -105 mV; x——x, -75 mV.

ton motive force (ΔP) will fall from around 200 mV to zero.

$$\Delta P = \Delta\psi \quad Z\Delta pH = 0$$

where $Z = 2.3\,RT/F = 60$ mV at these temperatures [194].

Consequently, the pH gradient, originally about 1 unit alkaline inside, must now form the other way round (inside 1.5 pH units acid relative to the medium) to balance the Donnan potential. Komor et al. [195] have confirmed that uncoupler causes considerable acidification (their measurements at a pH_0 of 6.0 indicate a fall of pH_i from 7.1 to 6.1, but dimethyloxazolidine dione with a pK_a of 6.32 becomes a very insensitive indicator of internal pH below pH 6. It seems to me possible that pH_i did in fact go considerably more acid, as expected from the above calculation). They showed further that, as the internal pH fell from 7 to 6, sugar uptake fell to near zero, and was dependent on pH_i, not ΔpH.

Remember that uncoupler blocks exchange as well as efflux and net uptake in this organism. If one assumes symmetry between carrier behaviour on the inside and outside faces of the membrane that would not be expected because an external pH of 6 is optimal for net inflow. (The exactly symmetrical experiment, viz. net efflux, has not been reported.) It is instructive to refer back to the discussion of Stein's paper where it was predicted that asymmetry would, in general, be found, and that on the inside face of the membrane where affinity for the sugar should be low the affinity for the proton would be high. Nevertheless, the problem remains: at pH_i of 6 and below, something occurs to block efflux in a way which has not been observed for inflow at any pH_0. Komor et al. [195] suggest that a protonation (or indeed several protonations) of the carrier may be occurring in a reaction designed by evolution to protect the cell against excessive acidification, though I do not find this totally convincing.

Another coupled transport system which I would like to consider is the H^+-lactose symport in *E. coli*. Three recent papers have stated that the carrier system is qualitatively symmetrical [196–198]. Inside-out vesicles and right-side-out vesicles can be prepared and both will accumulate β-galactosides if presented with a $\Delta\tilde{\mu}_{H^+}$ directed inwards. However, it is not claimed that the kinetic parameters (K_M and V) of the carrier are quantitatively symmetrical.

The working model which has guided much fruitful research during the last decade has been that of Winkler and Wilson [199] propounded before Mitchell's suggestion [200] of proton-coupled co-transport had been properly considered and evaluated [56,201]. Winkler and Wilson demonstrated an 'energy'-dependent raising of K_M^{eff} by 100-fold. There was essentially no effect of metabolism on K_M^{ent} or on V^{eff} (Table II). (V for entry was lowered approx. 10-fold, but Wilson argued that the entry V data were particularly dubious in poisoned cells as true initial rates could not be obtained (cf. Hopfer above). When entry was measured into poisoned cells preloaded with lactose it was found that the entry V was restored to the uninhibited unloaded value.)

These results were more or less corroborated ten years later by Lancaster et al. [202], who were more careful to maintain adequate aeration but who used an inhibitor which achieved only partial abolition of lactose accumulation (2000-fold gradient reduced to 100-fold). Once again inhibitor lowered K_M^{eff} markedly but did not significantly alter K_M^{ent} (Table III).

A recent report from Overath's laboratory [13] examines the affinity parameter of membrane vesicles prepared in the manner of Kaback from cells containing 10–15-times as much of the lactose carrier protein as is present in normal, fully induced, membranes (see section II). Lactose transport into these vesicles driven by the H^+-translocating respi-

TABLE II

KINETIC PARAMETERS OF β-GALACTOSIDE TRANSPORT IN *E. COLI*

Data of Winkler and Wilson [199]. Assays involved either the uptake of ^{14}C-labelled sugar or monitoring of in vivo hydrolysis. Inhibited assays contained 1 mM CH_2ICOO^- plus 30 mM N_3^-.

Substrate (and assay)	Entry		Exit	
	K_M (mM)	V (μmol/ min per g wet wt.)	K_M (mM)	V (μmol/ min per g wet wt.)
Lactose (^{14}C assay)				
Uninhibited	0.6	40	>16	1.8
Inhibited	0.9	3.4	0.7	1.2
2-Nitrophenylgalactoside (^{14}C assay)				
Uninhibited	0.5	15	>20	Dubious
Inhibited	0.5	1.0	1.8	0.9
2-Nitrophenylgalactoside (in vivo hydrolysis assay)				
Uninhibited	0.9	70		
Inhibited	1.5	19		

ratory chain shows a K_M of 0.050 mM, one order of magnitude lower than the values found in whole cells, and a factor of four lower than the value of Barnes and Kaback [203] for vesicles. (Is this the Hopfer effect again?) Similar concentrations of lactose reduced the uptake of other galactosides to 50% (K_I = 0.08–0.09 mM). However, when the dissociation constant for lactose from the carrier was measured in non-respiring vesicles in either of the following two ways a markedly higher value was obtained. Flow dialysis was used to detect the binding constant of one galactoside substrate to the carrier.

TABLE III

KINETIC PARAMETERS OF β-GALACTOSIDE TRANSPORT IN *E. COLI*

Data of Lancaster et al. [202] with the units transformed to those of Table II, assuming 2.7 ml cell H_2O per g dry wt. [199] and 228 mg dry wt. per g wet wt. [224]. Inhibited assays contained 10 mM KCN.

Substrate (and assay)	Entry		Exit	
	K_M (mM)	V (μmol/ min per g wet wt.)	K_M (mM)	V (μmol/ min per g wet wt.)
Lactose (^{14}C assay)				
Uninhibited	0.26	16.6	∞	∞
Inhibited	0.3–0.6	3.6	60	5.2

This binding was then inhibited by different levels of lactose. Similarly, though less quantitatively, the covalent reaction of *N*-ethylmaleimide with a cysteine residue in the carrier could be inhibited, but only by very high concentrations of lactose. Under these circumstances, i.e., with non-respiring membrane vesicles, the K_D for lactose was in the range of 10–18 mM, far higher than that observed with whole cells whether in the presence or absence of inhibitors of metabolism, and even more divergent from the very low K_M for transport into respiring vesicles. The results with another substrate, thiodigalactoside, were in marked contrast; the dissociation constant K_D measured in binding experiments was identical with the transport K_M (50 μM).

Wright et al. proposed the following model. Unloaded carrier (X) reorientates across the membrane at frequency P_x; at the outside it binds substrate and proton with a dissociation constant K_D (the order of binding is not specified). The complex XSH^+ reorientates at a frequency P_c. They suggest that protonation of the carrier at the outer face as a result of the proton motive force causes an increased affinity for sugar. Added to this effect they suggest a further lowering of the transport K_M due to the greater mobility of the complex relative to unloaded carrier ($P_c > P_x$). If P_c is considerably greater than P_x, then at maximum inflow rate (V) a large proportion of the carrier will be in the free form but on the inside of the membrane, struggling to get back out. As [S] is lowered towards the concentration achieving half V (i.e. K_M), less of the carrier is in this futile position, more is on the outer side of the membrane and thus active, so [S] has to be lowered even further to compensate. The measured K_M is therefore considerably lower than K_D determined without net transport. Note that for the lactose carrier in whole cells it was estimated many years ago that P_c was approx. 3-fold greater than P_x for lactose, but was less than P_x when the substrate was thiodigalactoside [204]. Kaczorowiski and Kaback [170] have recenlty shown that exchange of lactose is approx. ten times faster than net efflux in membrane vesicle preparations, i.e., P_c may be ten times P_x. Note also that the pH dependence of the binding of thiodigalactoside to membrane-bound carrier has been measured. Binding was optimal at pH 6 and fell to 43% at pH 8 (Kennedy et al. [11]). These two effects could account for up to 20-fold change in K_M. And how would a membrane potential promote protonation at the exterior face of the membrane? One must have recourse again to Mitchell's 'proton well'.

Another immediately striking feature of Wright's paper is that in their vesicles it is K_M^{ent} which is lowered by metabolism, while the two earlier papers on whole cells found metabolism to affect K_M^{eff} and to affect it in the opposite direction. Wright also remarks that V for transport of thiodigalactoside and lactose are similar, while that also is not true in whole cells [205]. One must also remark that if the striking difference between K_M and K_D reported in Wright's paper is fundamental to the mechanism of active transport, then it would follow that lactose be accumulated and thiodigalactoside not, whereas the latter can be accumulated to very large ratios [205]. Nevertheless, the reported very high K_D for lactose is in accord with the failure of lactose to block *N*-ethylmaleimide reaction, an observation made on azide-treated whole cells as well as on vesicles. It is also interesting that lactose does protect against *N*-ethylmaleimide if azide is omitted, as pointed out by Kepes [206].

In a recent paper from Kaback's laboratory [170] the Winkler and Wilson experiment has been repeated using vesicles instead of whole cells, and using an artificially imposed pH gradient or membrane potential instead of metabolism to generate the proton motive force. The results were different. A proton motive force directed inwards lowered V of exit (V^{eff}) but did not affect K_M^{eff}; directed outwards the proton motive force raised V^{eff}

but again did not affect K_M^{eff}. These authors also measured the kinetics of lactose entry by facilitated diffusion in the absence of metabolism or imposed gradients. V was the same as for lactose efflux under the same conditions, but the K_M was much higher (19 mM for entry, 2 mM for efflux). This high value compares with the results of Wright et al. [13] and is 100 times higher than their values for lactose uptake driven by respiration [170, 203].

There are several problems raised by this new work. Are vesicles significantly different from whole cells? Are there systematic errors which affect vesicles worse than whole cells, as postulated by Hopfer? Is there a significant difference between the two bacterial strains used, bearing in mind that the genetic material in Wright's strain was on a plasmid? Do unstirred layers cause artifacts in whole cells (see Ref. 204).

It is interesting to look at these results in the light of the earlier discussion of kinetic models. The striking new observation is that the K_M for facilitated passive entry of lactose is 100-fold higher than for active entry, is 10-fold higher than the K_M for passive exit, and is the same as K_D measured with essentially no transport in passive vesicles. This would fit the qualitative predictions of Stein's model if one postulates that in passive cells or vesicles the internal K_M is lower than the external K_M due to an inherent free energy difference for the unloaded carrier in those two locations, but that in the presence of an electric field the carrier (assuming it carries a net negative charge) moves over to the outside and the relative affinities are reversed. However, the different behaviour of lactose and thiodigalactoside is harder to explain. The observed difference in the relative rates of transport of these two substrates, the observed differences in efficiency in exchange-diffusion experiments together with Wright's suggestion that K_M can be influenced by the ratio P_c/P_x may go some way towards explaining the phenomena.

The simplest model for symport postulates that the two binary complexes do not reorientate or do so very much more slowly than the empty carrier and the ternary complex. The 'uncoupled' mutants of Wilson [207–209] could fail to accumulate galactosides either because they carry the proton both inwards and outwards (in which case the binary complex of carrier and proton can now reorientate) or they carry substrate without proton (in which case the other binary carrier-substrate complex is mobile) (Fig. 3). A recent paper by Kaczorowski and Kaback [170] investigates the efflux of [^{14}C]lactose from vesicles into media of varied pH. As expected, protonation of the unloaded carrier at the outer face prevented reorientation and thus slowed net efflux. When lactose was present outside at a concentration sufficient to saturate the carrier, the efflux of label did not depend on the rate-limiting re-entry of unloaded carrier; it was approx. ten times faster under these conditions and was pH insensitive. This result is corroborated by some experiments I did in 1975, which showed that acidic media slowed thiomethylgalactoside efflux from whole cells uncoupled with a proton-conducting ionophore. The inhibition was markedly less in the 'uncoupled' mutant of Wilson (Fig. 10), suggesting that the binary complex which is mobile in this mutant is that of the protonated carrier.

In several papers [210–216] over the last five years Kaback has reported experiments using dansylated derivatives of amino-alkylgalactosides, emphasizing that these compounds bound to, but were not transported by, the lactose carrier. It is now clear that these dansylated galactosides are transported [217]. This crucially affects Kaback's arguments about the number of carrier molecules per mg membrane protein, and the question of whether the unloaded carrier is charged or neutral. It would also undermine Kaback's conclusion that $\Delta\tilde{\mu}_{H^+}$ is required for galactoside binding though independent evidence on the difference between K_M and K_D now supports that conclusion to some extent [13].

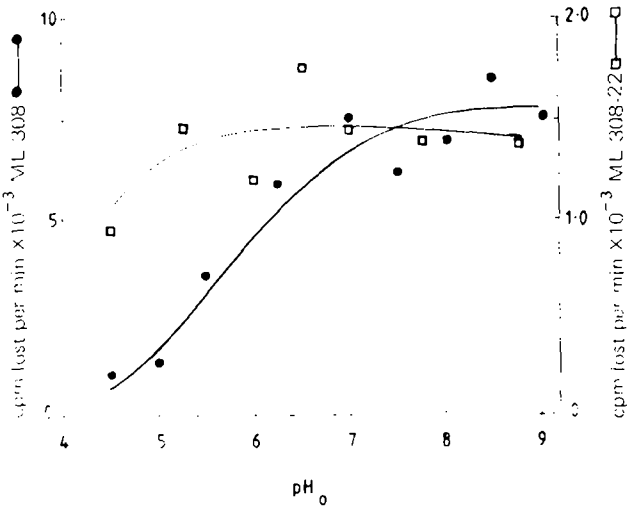


Fig. 10. Effect of pH on [^{14}C]thiomethylgalactoside efflux from *E. coli*. Cells were loaded by incubating them at pH 6.4, at 20°C, for 30 min in medium containing 0.16 mM [^{14}C]thiomethylgalactoside (3.12 Ci/mol). The suspension was diluted 60-fold into medium of the indicated pH containing uncoupler (5 μM carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone) and samples filtered at intervals during the next 120 s.

IX. Concluding remarks

There are several cases in this review where it is clear that the behaviour of membrane vesicles is different from that of whole cells or tissues [79–81, 147,149,150,173,210 - 216] and that in vesicles special care must be taken to ensure valid measurements.

Kinetic theory seems to be lagging behind the experimental collection of data, in that the present tentative and partial analyses already indicate that some of the existing data could be logical consequences of simple transport mechanisms. On the other hand, theoretical papers tend to be too abstruse.

It still seems valuable for microbiologists to keep abreast of the vertebrate literature and vice versa, with the botanists keeping an eye on both.

Acknowledgments

I am grateful for the opportunity of seeing papers not yet published sent to me by I.R. Booth, P. Overath and P.J.F. Henderson. I am grateful to all colleagues for their views and their published papers. My work in 1975 was supported by the Medical Research Council.

References

1 Stein, W.D. and Honig, B. (1977) Mol. Cell. Biochem. 15, 27–44
2 Heinz, E. and Geck, P. (1978) in Membrane Transport Processes (Hoffman, J.F., ed.), Vol. 1, pp. 13–30, Raven Press, New York
3 Weber, G. (1974) Ann. N.Y. Acad. Sci. 227, 486–496
4 Christensen, H.N. (1976) J. Theor. Biol. 57, 419–431
5 Wilson, T.H., Kashket, E.R. and Kusch, M. (1972) in The Molecular Basis of Biological Transport (Woessner, J.F. and Huijing, F., eds.), Vol. 3, pp. 219–247, Academic Press, New York

6 Crane, R.K. (1977) Rev. Physiol. Biochem. Pharmacol. 78, 99–159
7 Eddy, A.A. (1978) in Current Topics in Membrane Transports (Bronner, F. and Kleinzeller, A., eds.), Vol. 10, pp. 279–360, Academic Press, London
8 Rosen, B.P. and Kashket, E.R. (1978) in Bacterial Transport (Rosen, B.P., ed.), pp. 559–620, Marcel Dekker, Basel
9 Oxender, D. and Fox, C.F. (1978) Molecular Aspects of Membrane Transport, Alan R. Liss, New York
10 Racker, E. (1977) J. Supramol. Struct. 6, 215–228
11 Kennedy, E.P., Rumley, M.K. and Armstrong, J.B. (1974) J. Biol. Chem. 249, 33–37
12 Belaich, A., Simonpietri, P. and Belaich, J.P. (1978) Biochem. Biophys. Res. Commun. 82, 847–852
13 Wright, J.K., Teather, R.M. and Overath, P. (1979) in Functional and Molecular Aspects of Biomembrane Transport (Klingenberg, E.M., Palmieri, F. and Quagliariello, E., eds.), pp. 239–248, Elsevier/North-Holland Biomedical Press, Amsterdam
14 Amanuma, H., Itoh, J. and Anraku, Y. (1977) FEBS Lett. 78, 173–176
15 Le Fevre, P.G. and Masiak, S.J. (1970) J. Membrane Biol. 3, 387–399
16 Fox, C.F. and Kennedy, E.P. (1965) Proc. Natl. Acad. Sci. U.S.A. 54, 891–899
17 Kolber, A.R. and Stein, W.D. (1966) Nature 209, 691–694
18 Kolber, A.R. and Stein, W.D. (1967) Curr. Mod. Biol. 1, 244–248
19 Homewood, C.A., Smith, L.R. and Stein, W.D. (1970) in Fundamental Concepts in Drug Receptor Interactions (Danielli, J.F., Moran, J.F. and Triggle, D.J., eds.), pp. 117–203, Academic Press, London
20 Jones, T.H.D. and Kennedy, E.P. (1969) J. Biol. Chem. 244, 5981–5987
21 Teather, R.M., Müller-Hill, B., Abrutsch, U., Aichele, G. and Overath, P. (1978) Mol. Gen. Genet. 159, 239–248
22 Makino, S., Reynolds, J.A. and Tanford, C. (1973) J. Biol. Chem. 248, 4926–4932
23 Altendorf, K., Müller, C.R. and Sandermann, H. (1977) Eur. J. Biochem. 73, 545–551
24 Altendorf, K., Lukas, M., Kohl, B., Müller, C.R. and Sandermann, H. (1977) J. Supramol. Struct. 6, 229–238
25 Gerber, G.E., Anderegg, R.J., Herlihy, W.C., Gray, C.P., Biermann, K. and Khorana, H.G. (1979) Proc. Natl. Acad. Sci. U.S.A. 76, 227–231
26 Ovchinnikov, Y.A., Abdulaev, N.G., Feigina, M.Y., Kiselev, A.V. and Lobanov, N.A. (1979) FEBS Lett. 100, 219–224
27 Khorana, H.G., Gerber, G.E., Herlihy, W.C., Gray, C.P., Anderegg, R.J., Nihei, K. and Biermann, K. (1979) Proc. Natl. Acad. Sci. U.S.A. 76, 5046–5050
28 Komor, E., Rotter, M. and Tanner, W. (1977) Plant. Sci. Lett. 9, 153–162
29 Racusem, R.H. and Galston, A.W. (1977) Planta 135, 57–62
30 Malek, F. and Baker, D.A. (1977) Planta 135, 297–299
31 Komor, E. (1977) Planta 137, 119–131
32 Hutchings, V.M. (1978) Planta 138, 229–235
33 Hutchings, V.M. (1978) Planta 138, 236–241
34 Raboy, B. and Padan, E. (1978) J. Biol. Chem. 253, 3287–3291
35 Kikutu, Y. and Hoshi, T. (1979) Biochim. Biophys. Acta 553, 404–416
36 Joseph, S.K., Bradford, N.M. and McGivan, J.D. (1978) Biochem. J. 176, 827–836
37 Lever, J.E. (1978) J. Biol. Chem. 253, 2081–2084
38 Simonsen, L.O. and Cornelius, F. (1978) Biochim. Biophys. Acta 511, 213–223
39 Benderoff, S., Johnstone, R.M. and Blostein, R. (1978) Can. J. Biochem. 56, 545–551
40 Höfer, M. and Misra, P.F. (1978) Biochem. J. 172, 15–22
41 Hauer, R. and Höfer, M. (1978) J. Membrane Biol. 43, 335–349
42 Kotyk, A. and Michaljaničová, D. (1979) J. Gen. Microbiol. 110, 323–332
43 Negrin, R.S. and Neilands, J.B. (1978) J. Biol. Chem. 253, 2339–2342
44 Maron, R., Kanner, B.I. and Schuldiner, S. (1979) FEBS Lett. 98, 237–240
45 Kanner, B.I. (1978) Biochemistry 17, 1207–1212
46 Kanner, B.I. and Sharon, I. (1978) Biochemistry 17, 3949–3953
47 Kanner, B.I. and Sharon, I. (1978) FEBS Lett. 94, 245–248
48 Kanner, B.I. (1978) FEBS Lett. 89, 47–50
49 Rudnick, G. (1977) J. Biol. Chem. 252, 2170–2174

50 Rudnick, G. and Nelson, P.J. (1978) Biochemistry 17, 4739–4742
51 Sneddon, J. (1973) Proc. Neurobiol. 1, 151–198
52 Casey, R.P., Njus, D., Radda, G.K. and Sehr, P.A. (1977) Biochemistry 16, 972–977
53 Njus, D. and Radda, G.K. (1978) Biochim. Biophys. Acta 463, 219–244
54 Johnson, R.G., Carlson, N.J. and Scarpa, A. (1978) J. Biol. Chem. 253, 1512–1521
55 Toll, L. and Howard, B.D. (1978) Biochemistry 17, 2517–2523
56 West, I.C. (1970) Biochem. Biophys. Res. Commun. 41, 655–661
57 West, I.C. and Mitchell, P. (1973) Biochem. J. 132, 587–592
58 West, I.C. and Mitchell, P. (1974) Biochem. J. 144, 87–90
59 West, I.C. and Mitchell, P. (1972) J. Bioenerg. 3, 445–462
60 Raven, J.A. and Smith, F.A. (1976) J. Theor. Biol. 57, 301–312
61 Klingenberg, M. (1970) Esssays Biochem. 6, 119–159
62 Mitchell, P. (1973) J. Bioenerg. 4, 63–91
63 Stock, J. and Roseman, S. (1971) Biochem. Biophys. Res. Commun. 44, 132–138
64 Van Thienen, G.M., Postma, P.W. and van Dam, K. (1978) Biochim. Biophys. Acta 513, 395–400
65 Tsuchiya, T., Lopilato, J. and Wilson, T.H. (1978) J. Membrane Biol. 42, 45–59
66 Tokuda, H. and Kaback, H.R. (1977) Biochemistry 16, 2130–2136
67 Halpern, Y.S., Barash, H., Dover, S. and Druck, K. (1973) J. Bacteriol. 114, 53–58
68 Miner, K.M. and Frank, L. (1974) J. Bacteriol. 117, 1093–1098
69 MacDonald, R.E., Lanyi, J.K. and Greene, R.V. (1977) Proc. Natl. Acad. Sci. U.S.A. 74, 3167–3170
70 Tsuchiya, T., Hasan, S.M. and Raven, J.J. (1977) J. Bacteriol. 131, 848–853
71 Henderson, P.J.F., McGivan, J.D. and Chappell, J.B. (1969) Biochem. J. 111, 521–535
72 Harold, F.M. and Papineau, D. (1972) J. Membrane Biol. 8, 45–62
73 Drapeau, G.R. and MacLeod, R.A. (1963) Biochem. Biophys. Res. Commun. 12, 111–115
74 Drapeau, G.R., Matula, T.I. and MacLeod, R.A. (1966) J. Bacteriol. 92, 63–71
75 Thompson, J. and MacLeod, R.A. (1971) J. Biol. Chem. 246, 4066–4074
76 Thompson, J. and MacLeod, R.A. (1973) J. Biol. Chem. 248, 7106–7111
77 Thompson, J. and MacLeod, R.A. (1974) J. Bacteriol. 120, 598–603
78 Wagner, G., Hartmann, R. and Oesterhelt, D. (1978) Eur. J. Biochem. 89, 169–179
79 Lanyi, J.K. (1978) Biochemistry 17, 3011–3018
80 Lanyi, J.K. and Silverman, M.P. (1979) J. Biol. Chem. 254, 4750–4755
81 Lanyi, J.K. (1978) in Energetics and Structure of Halophilic Microorganisms (Caplan, S.R. and Ginzburg, M., eds.), pp. 415–426, Elsevier, Amsterdam
82 Lindley, E.V. and MacDonald, R.E. (1979) Biochem. Biophys. Res. Commun. 88, 491–499
83 Belliveau, J.W. and Lanyi, J.K. (1978) Arch. Biochem. Biophys. 186, 98–105
84 West, I.C. (1974) Biochem. Soc. Trans. 2, 800–803
85 Harold, F.M. (1977) in Current Topics in Bioenergetics (Sanadi, D.R., ed.), Vol. 6, pp. 83–149, Academic Press, London
86 Eddy, A.A. (1968) Biochem. J. 108, 195–206
87 Eddy, A.A. and Nowacki, J.A. (1971) Biochem. J. 122, 701–711
88 Murer, H. and Hopfer, U. (1974) Proc. Natl. Acad. Sci. U.S.A. 71, 484–488
89 Beck, J.C. and Rosen, B.P. (1979) Arch. Biochem. Biophys. 194, 208–214
90 Mitchell, P. (1970) Symp. Soc. Gen. Microbiol. 20, 121–166
91 Kayama, Y. and Kawasaki, T. (1976) J. Bacteriol. 128, 157–164
92 Geck, P., Pietrzyk, C., Heinz, E. and Pfeiffer, B. (1978) Acta Physiol. Scand. 103, Special Suppl. Gastric Ion Transport (Öbrink, K.J. and Flemström, G., eds.), pp. 363–372
93 Heinz, E., Geck, P., Pietrzyk, C., Burckhardt, G. and Pfeiffer, B. (1977) J. Supramol. Struct. 6, 125–133
94 Lee, J., Simpson, G. and Scholes, P. (1974) Biochem. Biophys. Res. Commun. 60, 825–832
95 Midgley, M. (1978) Biochem. J. 174, 191–201
96 Voorheis, H.P. (1977) Biochem. J. 164, 15–25
97 Imler, J.R. and Vidaver, G.A. (1972) Biochim. Biophys. Acta 288, 153–165
98 Vidaver, G.A., Shepherd, S.L., Lagow, J.B. and Wiechelman, K.J. (1976) Biochim. Biophys. Acta 443, 494–514
99 Johnson, R.G. and Scarpa, A. (1976) J. Biol. Chem. 251, 2189–2191
100 Flatmark, T. and Ingebretsen, O.C. (1977) FEBS Lett. 78, 53–56

101 Nichols, J.W. and Deamer, D.W. (1976) Biochim. Biophys. Acta 455, 269–271
102 Kobayashi, H., van Brunt, J. and Harold, F.M. (1978) J. Biol. Chem. 253, 2085–2092
103 Wieczorek, L. and Altendorf, K. (1979) FEBS Lett. 98, 233–236
104 Rhoads, D.B. and Epstein, W. (1977) J. Biol. Chem. 252, 1394–1401
105 Harold, F.M. and Papineau, D.J. (1972) J. Membrane Biol. 8, 27–44
106 Berger, E.A. (1973) Proc. Natl. Acad. Sci. U.S.A. 70, 1514–1518
107 Berger, E.A. and Heppel, L.A. (1974) J. Biol. Chem. 249, 7747–7755
108 Rhoads, D.B. and Epstein, W. (1978) J. Gen. Physiol. 72, 283–295
109 Rosen, B.P. (1976) Fed. Proc. 35, 77
110 Plate, C.A., Suit, J.L., Jetten, A.M. and Luria, S.E. (1974) J. Biol. Chem. 249, 6138–6143
111 Singh, A.P. and Bragg, P.D. (1977) J. Supramol. Struct. 6, 389–398
112 Ussing, H.H. (1949) Physiol. Rev. 29, 127–155
113 Quiocho, F.A., Gilliland, G.L., Miller, D.M. and Newcomer, M.E. (1977) J. Supramol. Struct. 6, 503–518
114 Daruwalla, K. (1979) Ph.D. Thesis, Cambridge
115 Lieberman, M.A., Simon, M. and Hong, J. (1977) J. Biol. Chem. 252, 4056–4067
116 Peterkofsky, A. and Gazdar, C. (1979) Proc. Natl. Acad. Sci. U.S.A. 76, 1099–1103
117 Trevillyan, J.M. and Pall, M.L. (1979) J. Bacteriol. 138, 397–403
118 Saier, M.H. and Moczyldowski, E.G. (1978) in Bacterial Transport (Rosen, B.P., ed.), pp. 103–125, Dekker, New York
119 Hogg, R.W. (1977) J. Supramolec. Structure 6, 411–417
120 Henderson, P.J.F., Giddens, R.A. and Jones-Mortimer, M.C. (1977) Biochem. J. 162, 309–320
121 Wilson, D.B. and Smith, J.B. (1978) in Bacterial Transport (Rosen, B.P., ed.), pp. 495–557, Marcel Dekker, Basel
122 Wilson, D.B. (1978) Annu. Rev. Biochem. 47, 933–965
123 Silhavy, T.J., Ferenci, T. and Boos, W. (1978) in Bacterial Transport (Rosen, B.P., ed.), pp. 127–169, Marcel Dekker, Basel
124 Robbins, A.R., Guzman, R. and Rotman, B. (1976) J. Biol. Chem. 251, 3112–3116
125 Hong, J.-S., Hunt, A.G., Masters, P.S. and Lieberman, M.A. (1979) Proc. Natl. Acad. Sci. U.S.A. 76, 1213–1217
126 Lieberman, M.A. and Hong, J.-S. (1974) Proc. Natl. Acad. Sci. U.S.A. 71, 4394–4399
127 Hong, J.-S. (1977) J. Biol. Chem. 252, 8582–8588
128 Lieberman, M.A. and Hong, J.-S. (1976) Arch. Biochem. Biophys. 172, 312–315
129 Michels, P.A.M., Michels, J.P.J., Boonstra, J. and Konings, W.N. (1979) FEMS Microbiol. Lett. 5, 357–364
130 Mandel, K.G. and Krulwich, T.A. (1979) Biochim. Biophys. Acta 552, 479–491
131 Harold, F.M. and Spitz, E. (1975) J. Bacteriol. 122, 266–277
132 Brodie, A.F., Lee, S.-H. and Kalra, V.K. (1979) Microbiology pp. 46–53, American Society for Microbiology, Washington
133 Jacobs, A.J., Kalra, V.K., Prasad, R., Lee, S.-H., Yankofsky, S. and Brodie, A.F. (1978) J. Biol. Chem. 253, 2216–2222
134 Lee, S.-H. and Brodie, A.F. (1978) Biochem. Biophys. Res. Commun. 85, 788–794
135 Lee, S.-H., Cohen, N.S., Jacobs, A.J. and Brodie, A.F. (1979) Biochemistry 18, 2232–2239
136 Heinz, E., Geck, P., Pietrzyk, C. (1975) Ann. N.Y. Acad. Sci. 264, 428–441
137 Schafer, J.A., Rickey, B.E. and Williams, A.E. (1977) J. Gen. Physiol. 69, 681–704
138 Löw, H., Crane, F.L., Grebing, C., Tally, M. and Hall, K. (1978) FEBS Lett. 91, 166–168
139 Löw, H. and Crane, F.L. (1978) Biochim. Biophys. Acta 515, 141–161
140 Kilberg, M.S. and Christensen, H.N. (1979) Biochemistry 18, 1525–1530
141 Christensen, H.N. (1977) J. Supramol. Struct. 6, 205–213
142 Garcia-Sancho, J., Sanchez, A., Handlogten, M.E. and Christensen, H.N. (1977) Proc. Natl. Acad. Sci. U.S.A. 74, 1488–1491
143 Lagarde, A. (1977) Biochem. J. 168, 211–221
144 Mitchell, W.J., Booth, I.R. and Hamilton, S.W. (1979) Biochem. J. 184, 441–449
145 Niven, D.F. and Hamilton, W.A. (1974) Eur. J. Biochem. 44, 517–522
146 Cockburn, M., Earnshaw, P. and Eddy, A.A. (1975) Biochem. J. 146, 705–712
147 Ramos, S. and Kaback, H.R. (1977) Biochemistry 16, 854–859
148 Seaston, A., Inkson, C. and Eddy, A.A. (1973) Biochem. J. 134, 1031–1043
149 Ramos, S. and Kaback, H.R. (1977) Biochemistry 16, 4271–4275

150 Schuldiner, S. and Fishkes, H. (1978) Biochemistry 17, 706 - 711
151 Rottenberg, H. (1976) FEBS Lett. 66, 159 163
152 Booth, I.R., Mitchell, W.J. and Hamilton, W.A. (1979) Biochem. J. 182, 687 696
153 Stevenson, R. and Silver, S. (1977) Biochem. Biophys. Res. Commun. 75, 1133 1139
154 Zilberstein, D., Schuldiner, S. and Padan, E. (1979) Biochemistry 18, 669 673
155 Collins, S.H., Jarvis, A.W., Lindsay, R.J. and Hamilton, W.A. (1976) J. Bacteriol. 126, 1232 - 1244
156 Horiuchi, T. and Novick, A. (1971) Cold Spring Harbor Symp. Quant. Biol. 26, 239 245
157 Horiuchi, T., Tomizawa, J.-I. and Novic, A. (1962) Biochim. Biophys. Acta 55, 152 163
158 Morville, M., Reid, M. and Eddy, A.A. (1973) Biochem. J. 134, 11 26
159 Seaston, A., Carr, G. and Eddy, A.A. (1976) Biochem. J. 154, 669 - 676
160 Jacquez, J.A. (1972) Math. Biosci. 13, 71 93
161 Vidaver, G.A. and Shepherd, S.L. (1968) J. Biol. Chem. 243, 6140 6150
162 Lieb, W.R. and Stein, W.D. (1974) Biochim. Biophys. Acta 373, 178 196
163 Stein, W.D. (1977) Biochim. Biophys. Acta 467, 376 385
164 Geck, P. and Heinz, E. (1976) Biochim. Biophys. Acta 443, 49 53
165 Heinz, E. and Geck, P. (1978) in Membrane Transport Processes (Hoffman, J.F., ed.), Vol. 1, pp. 13 30, Raven Press, New York
166 Heinz, E. (1978) Mechanics and Energetics of Biological Transport, Springer-Verlag, Berlin
167 Hodgkin, A.L. and Katz, B. (1949) J. Physiol. (London) 180, 37 77
168 Heinz, E., Geck, P. and Wilbrandt, W. (1972) Biochim. Biophys. Acta 255, 442 461
169 Thomas, E.L. and Christensen, H.N. (1971) J. Biol. Chem. 246, 1682 1688
170 Kaczorowski, G.J. and Kaback, H.R. (1979) Biochemistry 18, 3691 3697
171 King, E.L. and Altman, C. (1956) J. Phys. Chem. 60, 1375 1378
172 Britton, H.G. (1966) Arch. Biochem. Biophys. 117, 167 183
173 Hopfer, U. (1977) J. Supramol. Struct. 7, 1 13
174 Geck, P., Pietrzyk, C. and Heinz, E. (1976) in Amino Acid Transport and Uric Acid Transport (Silbernagl, S., Lang, F. and Greger, R., eds.), pp. 33 40, Georg Thieme, Stuttgart
175 Pietrzyk, C. (1978) Inaugural Dissertation, J.W. Goethe-Universität, Frankfurt am Main
178 Aronson, P.S. and Sacktor, B. (1975) J. Biol. Chem. 250, 6032 6039
177 Hamilton, T.R. and Nilsen-Hamilton, M. (1975) Proc. Natl. Acad. Sci. U.S.A. 73, 1907 1911
178 Fass, S.J., Hammerman, M.R. and Sacktor, B. (1977) J. Biol. Chem. 252, 583 590
179 Hammerman, M.R. and Sacktor, B. (1977) J. Biol. Chem. 252, 591 595
180 Lever, J. (1977) J. Biol. Chem. 252, 1990 -1997
181 Von Baeyer, H., von Conta, C., Haeberle, D. and Deetjen, P. (1973) Pflüger's Arch. 343, 273 286
182 Schultz, S.G. and Curran, P.F. (1970) Physiol. Rev. 50, 637 718
183 Tannenbaum, C., Toggenburger, G., Kessler, M., Rothstein, A. and Semenza, A. (1977) J. Supramol. Struct. 6, 519 -533
184 Komor, E. and Tanner, W. (1971) Biochim. Biophys. Acta 241, 170 179
185 Komor, E., Haass, D. and Tanner, W. (1972) Biochim. Biophys. Acta 266, 649 660
186 Komor, E. and Tanner, W. (1974) J. Gen. Physiol. 64, 568 581
187 Komor, E. and Tanner, W. (1976) Eur. J. Biochem. 70, 197 204
188 Lenzel, F., Deck, M., Haass, D. and Tanner, W. (1977) Eur. J. Biochem. 72, 509 514
189 Simoni, R.D. and Postma, P.W. (1975) Annu. Rev. Biochem. 43, 523 554
190 Schwab, W.G.W. and Komor, E. (1978) FEBS Lett. 87, 157 160
191 Mitchell, P. (1968) Chemiosmotic Coupling and Energy Transduction, Glynn Research, Bodmin
192 Mitchell, P. and Moyle, J. (1974) Biochem. Soc. Spec. Publ. 4, 91 -111
193 Mitchell, P. (1977) Symp. Soc. Gen. Microbiol. 27, 383 423
194 Mitchell, P. (1976) Biochem. Soc. Trans. 4, 399 430
195 Komor, E., Schwab, W.G.W. and Tanner, W. (1979) Biochim. Biophys. Acta 555, 524 - 530
196 Lancaster, J.R. and Hinkle, P.C. (1977) J. Biol. Chem. 252, 7657 - 7661
197 Lancaster, J.R. and Hinkle, P.C. (1977) J. Biol. Chem. 252, 7662 7666
198 Teather, R.M., Hamelin, O., Schwarz, H. and Overath, P. (1977) Biochim. Biophys. Acta 467, 386 395
199 Winkler, H.H. and Wilson, T.H. (1966) J. Biol. Chem. 241, 2200 2211
200 Mitchell, P. (1963) Biochem. Soc. Symp. 22, 142 -168

201 Pavlasova, E. and Harold, F.M. (1969) J. Bacteriol. 98, 198–204
202 Lancaster, J.R., Hill, R.J. and Struve, W.G. (1975) Biochim. Biophys. Acta 401, 285–298
203 Barnes, E.M. and Kaback, H.R. (1970) Proc. Natl. Acad. Sci. U.S.A. 66, 1190–1198
204 Robbie, J.P. and Wilson, T.H. (1969) Biochim. Biophys. Acta 173, 234–244
205 Kepes, A. and Cohen, G.N. (1962) in The Bacteria: The Physiology of Growth (Gunsalus, I.C. and Stanier, R.V., eds.), Vol. 4, pp. 179–221, Academic Press, New York
206 Kepes, A. (1970) in Current Topics in Membranes and Transport (Bronner, F. and Kleinzeller, A., eds.) Vol. 1, pp. 101–134, Academic Press, London
207 Wong, P.T.S., Kashket, E.R. and Wilson, T.H. (1970) Proc. Natl. Acad. Sci. U.S.A. 65, 63–69
208 Wilson, T.H. and Kusch, M. (1972) Biochim. Biophys. Acta 255, 787–797
209 West, I.C. and Wilson, T.H. (1973) Biochem. Biophys. Res. Commun. 50, 551–558
210 Reeves, J.P., Shechter, E., Weil, R. and Kaback, H.R. (1973) Proc. Natl. Acad. Sci. U.S.A. 70, 2722–2726
211 Schuldiner, S., Kerwar, G., Kaback, H.R. and Weil, R. (1975) J. Biol. Chem. 250, 1361–1370
212 Schuldiner, S., Kung, H., Kaback, H.R. and Weil, R. (1975) J. Biol. Chem. 250, 3679–3682
213 Schuldiner, S., Spencer, R.D., Weber, G., Weil, R. and Kaback, H.R. (1975) J. Biol. Chem. 250, 8893–8896
214 Schuldiner, S., Weil, R. and Kaback, H.R. (1976) Proc. Natl. Acad. Sci. U.S.A. 73, 109–112
215 Schuldiner, S., Weil, R., Robertson, D. and Kaback, H.R. (1977) Proc. Natl. Acad. Sci. U.S.A. 74, 1851–1854
216 Kaback, H.R., Ramos, S., Robertson, D.E., Stroobant, P., Tokuda, H. (1977) J. Supramol. Struct. 7, 447–461
217 Overath, P., Teather, R.M., Simoni, R.D., Aichele, G. and Wilhelm, U. (1979) Biochemistry 18, 1–11
218 Cecchini, G., Payne, G.S. and Oxender, D.L. (1977) J. Supramol. Struct. 7, 481–487
219 Bardin, C. and Johnstone, R.M. (1978) J. Biol. Chem. 253, 1725–1732
220 Fairclough, P., Malathi, P., Preiser, H. and Crane, R.J. (1979) Biochim. Biophys. Acta 553, 295–306
221 Kusaka, I. and Kanai, K. (1978) Eur. J. Biochem. 83, 307–311
222 Hirata, H., Sone, N., Yoshida, M. and Kagawa, Y. (1977) J. Supramol. Struct. 6, 77–84
223 Amanuma, H., Mutojima, K., Yamagudu, A. and Anraku, Y. (1977) Biochem. Biophys. Res. Commun. 74, 366–373
224 Schultz, S.G. and Soloman, A.K. (1961) J. Gen. Physiol. 45, 355–369