Biochimica et Biophysica Acta 859 (1986) 151–164
Elsevier

BBA 73148

The thermostatics and thermodynamics of cotransport revisited: a restatement of the Zeroth Law

Dieter Walz [a] and S. Roy Caplan [b]

[a] *Biozentrum, University of Basel, CH-4056 Basel (Switzerland), and* [b] *Department of Membrane Research, The Weizmann Institute of Science, Rehovot 76100 (Israel)*

(Received November 11th, 1985)
(Revised manuscript received April 8th, 1986)

Key words: Antiport; Cotransport stoichiometry; Static head; Symport; Thermodynamics; Zeroth law

The assertions by Naftalin ((1984) Biochim. Biophys. Acta 778, 155–175) that "(a) the static-head equilibrium state cannot exist; (b) the stoichiometry of cotransport ... does not affect the static-head distribution of cotransported ligands; ... (d) the only equilibrium state where there is zero net flow of both driving and driven transported ligand is at true equilibrium when the ligands are uniformly distributed across the membrane" are incorrect and hence misleading. These assertions are based on a faulty application of the lattice-gas models of Hill and Kedem ((1966) J. Theor. Biol. 10, 399–441), as well as a misunderstanding of the Zeroth Law of Thermodynamics. Furthermore the claim that "cotransport is not entirely an affinity-driven, but is partially an entropy-driven process" is unwarranted. A consistent treatment of the thermodynamics of cotransport is given which completely covers all the arguments put forward by Naftalin against the existence of static head.

Introduction

The importance of cotransport systems (both symport and antiport) in biology has recently been emphasized by Naftalin [1]. However, in discussing the thermodynamics of these systems Naftalin reached a number of conclusions which can readily be shown to be seriously in error. Since these conclusions bring into question an important aspect of transport systems, namely the existence of static head, they may lead to widespread misunderstanding and confusion. A clarification of the points at issue is therefore urgently required.

In the following sections we take the arguments put forward by Naftalin in succession, and show that they are based on incorrect premises or erroneous interpretations of thermodynamic relations.

Thermodynamics

The variation in Gibbs free energy (G) in a multicomponent, multicompartment system under constant temperature and pressure is given by

$$\delta G = \sum_i \sum_k \tilde{\mu}_{i,k} \delta n_{i,k} \quad (1)$$

where $\tilde{\mu}_{i,k}$ and $n_{i,k}$ denote, respectively, the electrochemical potential and the mole number of the ith species in the kth compartment. The summations are taken over all species and compartments. At equilibrium

$$\delta G = 0 \text{ (equilibrium)} \quad (2)$$

Eqn. 2 implies that $dG/dt = 0$ with the corollary,

in view of Eqn. 1, that

$$dn_{i,k}/dt = 0 \text{ (equilibrium)} \tag{3}$$

i.e., all spontaneous movement of species between compartments has ceased. Eqns. 1 and 2 can also be used to establish relationships between the electrochemical potentials of all species in all compartments. These relationships will permit us to draw conclusions about the presence or absence of restrictions on the transfer of species between the compartments.

Two compartments

Consider the reversible transfer by whatever means * of an infinitesimal number of moles of all species between the two compartments. Then

$$\delta n_{i,1} + \delta n_{i,2} = 0 \tag{4}$$

and combining Eqn. 4 with Eqns. 1 and 2 we obtain

$$\sum_i (\tilde{\mu}_{i,1} - \tilde{\mu}_{i,2}) \delta n_{i,1} = 0 \tag{5}$$

Since in practice $\delta n_{i,1}$ moles of a given species i can be chosen arbitrarily, independent of the choice $\delta n_{j,1}$ for all other species j, the only way in which Eqn. 5 can be satisfied is if

$$\tilde{\mu}_{i,1} - \tilde{\mu}_{i,2} = 0 \tag{6}$$

If this condition is found to hold for all species in a given system, it can be concluded that the system is at equilibrium without the necessity for checking whether spontaneous movement has ceased (cf. Eqn. 3). However, the conclusion that unrestricted diffusion of all species between the two compartments must have occurred in order for this state to be reached would be incorrect. For example, the compartments may be separated by a semipermeable membrane which permits only one species (the solvent) to permeate. Nevertheless if the composition is identical on both sides of the membrane and no differences in electrical potential or hydrostatic pressure exist between the compartments, Eqn. 6 is clearly fulfilled. On the other hand, if the condition expressed by Eqn. 6 is not fulfilled for all species, it does not necessarily mean that the system is not at equilibrium. A case in point is the Donnan equilibrium. Suppose one compartment contains a charged species of high molecular weight separated from the second compartment by a membrane permeable only to low molecular weight species. If no movement of any species between the two compartments is observed to occur, the system must be at equilibrium (cf. Eqn. 3). However, the condition expressed by Eqn. 6 is in this case not obeyed, from which we can conclude that there is a restriction to the movement of any species for which the difference in electrochemical potential between the two compartments is not zero. It should be stressed that for a spontaneous transfer of species in this system in response to a perturbation, Eqns. 4 and 5 continue to apply. Thus, if j represents the high molecular weight species,

$$\delta n_{j,1} = \delta n_{j,2} = 0 \text{ (for impermeant species)} \tag{7}$$

because of the impermeable barrier.

A similar situation arises in the case of coupled flows. Consider for example a cotransport system in which the transfer of ν_l moles of the lth species is obligatory when the transfer occurs of ν_j moles of the jth species, i.e. the cotransport is completely coupled, and no other pathway for these species is present. The quantities ν_j and ν_l are the stoichiometric coefficients of the cotransport process: they have equal signs in the case of symport and opposite signs in the case of antiport. Once again Eqn. 6 may be found to hold for all species, whereupon it can be concluded that the system is in equilibrium. On the other hand, as in the case of the Donnan equilibrium, Eqn. 6 may be found not to hold although no movement of any species between the two compartments can be observed. This situation comes about because of the restriction we have imposed, which gives rise to the additional condition:

$$\delta n_{j,k}/\nu_j = \delta n_{l,k}/\nu_l \text{ (for species undergoing coupled transport)} \tag{8}$$

* For example, this could be accomplished by withdrawing from compartment 1 an infinitesimal volume through a permselective membrane permeable only to the species i, and injecting it into compartment 2.

From Eqns. 1, 2, 4, and 8, we then obtain

$$\left[(\tilde{\mu}_{j,1}-\tilde{\mu}_{j,2})+(\tilde{\mu}_{l,1}-\tilde{\mu}_{l,2})\nu_l/\nu_j\right]\delta n_{j,1} + \sum_{i\neq j,l}(\tilde{\mu}_{i,1}-\tilde{\mu}_{i,2})\delta n_{i,1}=0 \tag{9}$$

Since $\delta n_{j,1}$ and all $\delta n_{i,1}$ may take on arbitrary values corresponding to any arbitrary perturbation of the system, Eqn. 9 can only be satisfied if the following conditions hold:

$$\tilde{\mu}_{i,1}-\tilde{\mu}_{i,2}=0 \text{ (for } i\neq j,l) \tag{10a}$$

$$(\tilde{\mu}_{j,1}-\tilde{\mu}_{j,2})+(\tilde{\mu}_{l,1}-\tilde{\mu}_{l,2})\nu_l/\nu_j=0 \tag{10b}$$

Hence an equilibrium state can be found for which

$$\nu_j(\tilde{\mu}_{j,1}-\tilde{\mu}_{j,2})=-\nu_l(\tilde{\mu}_{l,1}-\tilde{\mu}_{l,2}) \tag{11}$$

This is the state referred to as 'static head equilibrium' by Naftalin [1].

Eqn. 11 can, after some manipulation, readily be expressed in terms of concentrations and electrical potential differences. Recalling that

$$\tilde{\mu}_{i,k}=\mu^{\circ}_{i,k}+RT\ln(c_{i,k}f_{i,k})+z_iF\psi_k \tag{12}$$

where $\mu^{\circ}_{i,k}$, $c_{i,k}$, and $f_{i,k}$ denote, respectively, the standard chemical potential, concentration, and activity coefficient of the ith species in the kth compartment, while z_i and ψ_k denote, respectively, the charge number of the ith species and the electrical potential of the kth compartment (R, T, and F have their usual meanings) we obtain

$$\left(\frac{c_{l,1}}{c_{l,2}}\right)=\left(\frac{c_{j,1}}{c_{j,2}}\right)^{-\nu_j/\nu_l}\exp\left[-(z_l+z_j\nu_j/\nu_l)\frac{(\psi_1-\psi_2)}{RT/F}\right] \tag{13}$$

Here we have assumed that the standard chemical potentials and the activity coefficients are identical in the two compartments. There are three conditions under which the exponential term in Eqn. 13 reduces to unity: (a) $z_l=z_j=0$, (b) $z_l\nu_l=-z_j\nu_j$, and (c) $\psi_1-\psi_2=0$ which corresponds to Naftalin's electrical short-circuit condition [1]. In the case that at least one of these conditions hold, Eqn. 13 becomes

$$\left(\frac{c_{l,1}}{c_{l,2}}\right)=\left(\frac{c_{j,1}}{c_{j,2}}\right)^{-\nu_j/\nu_l} \tag{14}$$

(remember that symport occurs when $\nu_j/\nu_l>0$, antiport when $\nu_j/\nu_l<0$). Eqns. 13 and 14 include all the examples considered by Naftalin for a two-compartment system [1].

Three compartments in series

We now consider a three-compartment system in which the cotransport properties of the two membranes are not necessarily identical. Then equilibria of the type expressed by Eqns. 10 may be found between compartments 2 and 3 as well as between compartments 1 and 2. Rewriting Eqn. 13 for compartments 2 and 3, we obtain

$$\left(\frac{c_{l,2}}{c_{l,3}}\right)=\left(\frac{c_{j,2}}{c_{j,3}}\right)^{-\nu_j'/\nu_l'}\exp\left[-(z_l+z_j\nu_j'/\nu_l')\frac{(\psi_2-\psi_3)}{RT/F}\right] \tag{15}$$

where ν_j' and ν_l' denote the stoichiometric coefficients of the cotransport process between compartments 2 and 3. Multiplying Eqns. 13 and 15 yields

$$\left(\frac{c_{l,1}}{c_{l,3}}\right)=\left(\frac{c_{j,1}}{c_{j,3}}\right)^{-\nu_j/\nu_l}\left(\frac{c_{j,2}}{c_{j,3}}\right)^{(\nu_j/\nu_l)-(\nu_j'/\nu_l')} \times\exp\left[-(z_l+z_j\nu_j/\nu_l)\frac{(\psi_1-\psi_3)}{RT/F} + z_j\{(\nu_j/\nu_l)-(\nu_j'/\nu_l')\}\frac{(\psi_2-\psi_3)}{RT/F}\right] \tag{16}$$

If the ratios of stoichiometric coefficients for the two cotransport systems are identical, Eqn. 16 simplifies to

$$\left(\frac{c_{l,1}}{c_{l,3}}\right)=\left(\frac{c_{j,1}}{c_{j,3}}\right)^{-\nu_j/\nu_l}\exp\left[-(z_l+z_j\nu_j/\nu_l)\frac{(\psi_1-\psi_3)}{RT/F}\right] \tag{17}$$

In this case the three-compartment system at static head behaves in effect as if it were a two-compartment system. In all other cases it is seen

from Eqn. 16 that the concentration ratio of the lth species between compartments 1 and 3 at static head depends not only on the corresponding concentration ration of the jth species and the electrical potential difference $\psi_1 - \psi_3$, but also on the concentration of the jth species and the electrical potential in compartment 2.

In order to illustrate the above conclusion, consider a case in which only compartments 1 and 3 can be monitored, no access being available to compartment 2. An example of such a system might be an epithelium with one layer of cells mounted in an Ussing chamber. Suppose the system is found to be in equilibrium according to the condition expressed by Eqn. 3, and $c_{j,1}/c_{j,3} = 1$, $\psi_1 - \psi_3 = 0$. To conclude that the concentrations of all other permeant species must then necessarily be identical in compartments 1 and 3 would be unjustified. The epithelium may contain two cotransport systems on either side for species j and l that have different ratios of stoichiometric coefficients. If this is so, an equilibrium may be observed for which $c_{l,1}/c_{l,3} \neq 1$, and which is a true static head. This is readily verified on examination of Eqn. 16. In effect the concentration gradient of the lth species is balanced by the concentration gradient of the jth species between compartments 2 and 3, and for charged species additionally by $\psi_2 - \psi_3$.

Naftalin [1] gives two examples of such systems which are used in a reductio ad absurdum argument. Both examples involve uncharged species and the condition $c_{j,1}/c_{j,3} = 1$. The first, which considers two symporters with $\nu_j/\nu_l = 0.5$ and $\nu_j'/\nu_l' = 1$, yields $c_{l,1}/c_{l,3} = 0.1$. The second, which considers an antiporter and a symporter with $\nu_j/\nu_l = -1$ and $\nu_j'/\nu_l' = 1$, yields $c_{l,1}/c_{l,3} = 100$. These results are in perfect agreement with Eqn. 16, since in the first example $c_{j,2}/c_{j,3} = 100$ while in the second $c_{j,2}/c_{j,3} = 0.1$ (see Tables I and II, respectively, in Ref. 1).

The question arises as to whether a system left to itself can evolve to static head states such as those just discussed. That this is indeed the case will be demonstrated by simulations shown in a later section.

The Zeroth Law of Thermodynamics

Naftalin [1] argues that the static head states discussed in the above two examples are in conflict with the Zeroth Law of Thermodynamics *, and are therefore impossible. In support of this conclusion he argues that the Zeroth Law does not pertain only to thermal equilibrium states, and cites Tisza [3] as showing that (a) if a thermodynamic wall is non-restrictive of at least some chemical species i, it is also non-restrictive of entropy and energy, and (b) the criterion for equilibrium across such a wall consists of the simultaneous conditions $\mu_i' = \mu_i''$ and $T' = T''$. These conditions are exactly equivalent to the condition expressed by Eqn. 10a. Clearly all species to which the wall is restrictive are excluded from this condition, but are subject to different conditions determined by the nature of the restriction (as exemplified by Eqn. 10b).

Apparently Naftalin was aware of this exclusion, since he writes, in reference to the examples discussed in the previous section, "It could also be argued that in both examples, the thermodynamic walls between compartments 1 and 2 and 2 and 3 differ, so that the equilibrium between compartments 1 and 2 is dependent on a different process from that between 2 and 3 and hence, there is no necessity for an equilibrium state to exist between compartments 1 and 3" [1]. This argument, with which we concur, is then demolished on the grounds that it "is only correct at the microscopic level in considering the forms of mobile species which cross the membrane. However, at the macroscopic level, to which the thermodynamic laws all pertain, there is no distinction between any of the thermodynamic walls separating the compartments. They all permit the flow of components A and B. The relative rate at which A and B flow cannot affect the equilibrium state, which is time-independent" [1]. The latter quotation contains several fallacies:

(a) The restrictions we are concerned with apply both at the macroscopic and at the microscopic levels, and not just at the microscopic level as maintained by Naftalin. The walls are macroscopic devices, and the restrictions they impose can only be assessed on the macroscopic level.

* This law states that if two phases are in thermal equilibrium with a third phase, they are in thermal equilibrium with each other [2].

However, events occurring at the macroscopic level are simply time and space averages of events occurring at the microscopic level. Thus if transport on the microscopic level is governed by a fixed stoichiometric ratio, this stoichiometry must appear at the macroscopic level as well. If this were not so, independent pathways must be present, and these have been explicitly excluded.

(b) From the above considerations it is clear that the 'thermodynamic walls' are distinct and give rise to flows in a fixed ratio determined by the stoichiometries of their cotransport systems.

(c) The fixed flow ratios referred to above persist until the flows vanish at equilibrium, and hence influence the time-independent equilibrium state. (This point is clearly demonstrated in the simulations shown below.)

It should be noted that restrictions of the type considered above may also govern cotransport systems involving heat flow. For example, the flows of heat and electricity may be tightly coupled in a thermoelectric device such as a thermocouple [4]. Therefore, in order to avoid the pitfalls described above, we suggest reformulating the Zeroth Law as follows:

> If two phases are simultaneously in equilibrium with a third through walls which impose identical restrictions on the flows of mass and heat (but are not absolute barriers to any given flow), the two phases are in true equilibrium with each other.

Thus the Zeroth Law is not applicable to compartments 1 and 3 in the examples given by Naftalin.

Kinetics

Notwithstanding his argument that properties at the microscopic level are not expressed at the macroscopic level (see preceding section), Naftalin attempts to find additional support for his conclusions based on a consideration of microscopic kinetic schemes. In so doing he makes use of the 'lattice-gas' approach introduced by Hill and Kedem [5]. His 'analysis of microscopic forms', however, uses equilibrium conditions for the transmembrane transitions which apply to a carrier model although the lattice-gas schemes clearly represent a fixed binding site model. We therefore analyze, by way of example, a 1 : 1-symport of two uncharged species, A and B, in terms of a carrier model and two types of fixed binding site model.

Fig. 1A shows the lattice-gas representation used by Naftalin but with the states of the lattice numbered; p_i denotes the probability of the ith state. We commence with equilibrium conditions. Let α_{ij} be the first-order or pseudo-first-order rate constant for the transition from state i to state j. Then, from microscopic reversibility,

$$\alpha_{ij}p_i = \alpha_{ji}p_j \text{ (equilibrium)} \tag{18}$$

Transitions which involve binding of a species are characterized by a pseudo-first-order rate constant such that, for example,

$$\alpha_{12} = \alpha_{12}^{\circ}c_{A,1} \tag{19}$$

where α_{12}° is a second-order rate constant. The dissociation constant for release of species A into compartment 1 is

$$K_{A,1} = \alpha_{21}/\alpha_{12}^{\circ} \tag{20}$$

Using Eqns. 18–20 we obtain

$$p_1/p_2 = K_{A,1}/c_{A,1} \tag{21}$$

Similarly, we have

$$p_2/p_3 = K_{B,1}/c_{B,1} \tag{22}$$

The sum of the probabilities of the states which are accessible to compartment 1 is given by

$$P_1 = p_1 + p_2 + p_3 \tag{23}$$

From Eqns. 21–23 we find

$$p_1 = P_1/D_1 \tag{24}$$

and

$$p_3 = P_1\frac{c_{A,1}c_{B,1}}{K_{A,1}K_{B,1}}/D_1 \tag{25}$$

where

$$D_1 = 1 + \frac{c_{A,1}}{K_{A,1}} + \frac{c_{A,1}c_{B,1}}{K_{A,1}K_{B,1}} \tag{26}$$

Similar equations can be obtained for the side

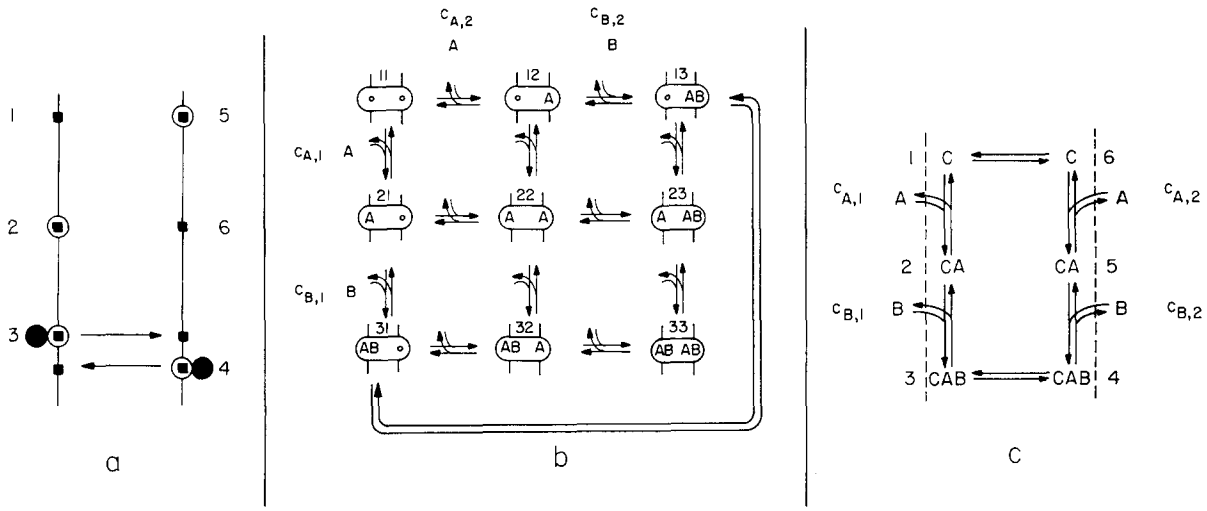

Fig. 1. Three models for a non-electrogenic symport of species A and B with 1:1 stoichiometry. Symport occurs across a membrane separating two compartments 1 and 2. The bulk concentrations in the two compartments are $c_{A,1}$, $c_{B,1}$ and $c_{A,2}$, $c_{B,2}$, respectively.
(a) Lattice-gas representation of a model with fixed but independent binding sites on each membrane surface. The scheme is identical to Fig. 3a in Ref. 1, except that the states are numbered from 1 to 6 according to the sequence of events characterizing the transport process. Open and closed circles represent species A and B, respectively, while the two lines indicate the membrane surfaces.
(b) Model with fixed binding sites that are located on a single entity incorporated into the membrane (e.g. an integral membrane protein). Transitions in each column (row) involve the consecutive binding of species A and B from compartment 1 (2). The transitions between states 31 and 13 represent the transfer of AB across the membrane.
(c) Cycle diagram for a six-state carrier model. The carrier molecule is embedded in the membrane (indicated schematically by the dashed lines). The states are numbered consecutively from 1 to 6.

facing compartment 2 (p_1 and p_3 are replaced by p_6 and p_4, respectively, and the subscript 1 on all other symbols is replaced by 2; obviously $P_2 = p_4 + p_5 + p_6$).

In terms of a model with fixed but independent binding sites on both sides of the membrane, $P_1 = P_2 = 1$. With N_1 and N_2 denoting, respectively, the total number of binding sites on the two sides of the membrane, the equilibrium condition for the transmembrane transitions (rate constants α_{lr} and α_{rl}) indicated by the arrows in Fig. 1a reads

$$\alpha_{lr} N_1 p_3 N_2 p_6 = \alpha_{rl} N_2 p_4 N_1 p_1 \tag{27}$$

Instead of Eqn. 27 Naftalin used the conditions $p_3 = p_4$ and $p_1 = p_6$ (cf. Eqns. 9.8 and 9.9 in Ref. 1), which are clearly wrong since neither states 3 and 4 nor states 1 and 6 are connected through a transmembrane transition (in contrast to the carrier model represented in Fig. 1c). By means of Eqns. 24,25, and the analogous equations for the membrane side facing compartment 2, we obtain from Eqn. 27 after rearranging

$$\frac{c_{A,1} c_{B,1}}{c_{A,2} c_{B,2}} = \frac{\alpha_{rl} K_{A,1} K_{B,1}}{\alpha_{lr} K_{A,2} K_{B,2}} \tag{28a}$$

Eqn. 28a holds for all possible equilibrium states, including the one in which both species A and B are separately equilibrated (i.e. $c_{A,1} = c_{A,2}$ and $c_{B,1} = c_{B,2}$). The latter state requires that the right-hand side of Eqn. 28a be equal to 1 (detailed balance). Hence, Eqn. 28a can be rewritten as

$$\frac{c_{B,1}}{c_{B,2}} = \frac{c_{A,2}}{c_{A,1}} \tag{28b}$$

which is identical to Eqn. 14 for the case $A = j$, $B = l$, $\nu_j = \nu_l = 1$, and $z_j = z_l = 0$.

Fig. 1b presents the scheme for a model where the binding sites facing the two compartments are located on one entity (e.g. an integral membrane protein). We apply the equilibrium condition expressed in Eqn. 18 to the 12 transitions involving binding of species A or B, and introduce the dissociation constants according to Eqns. 19 and

20. Together with the obvious relation

$$\sum_{i,j} p_{ij} = 1 \tag{29}$$

this yields equations for the p_{ij}, in particular

$$p_{31} = \frac{c_{A,1} c_{B,1}}{K_{A,1} K_{B,1}} / D \tag{30a}$$

and

$$p_{13} = \frac{c_{A,2} c_{B,2}}{K_{A,2} K_{B,2}} / D \tag{30b}$$

$K_{A,1}$, $K_{B,1}$ and $K_{A,2}$, $K_{B,2}$ are the dissociation constants for the transitions in the left column and the top row, respectively. Note that the transitions in the other rows and columns can have different dissociation constants. D is of the form 1 + sum of eight terms consisting of ratios of concentrations of species A or B in the compartments and pertinent dissociation constants (cf. Ref. 5). The equilibrium condition for the transfer of AB between the two binding sites is

$$\alpha_{lr} p_{31} = \alpha_{rl} p_{13} \tag{31}$$

which, by virtue of Eqns. 30, immediately yields Eqns. 28.

Carrier-mediated symport is most conveniently analyzed by means of the diagram method developed by Hill [6] as a massive extension of the classical King-Altman technique. The carrier is a membrane-bound macromolecule which can exist in six states, and Fig. 1c shows the cycle diagram relating the states. The steps involving binding of species are described by Eqns. 23–26 together with similar equations for the membrane side facing compartment 2. Obviously

$$P_1 + P_2 = 1 \tag{32}$$

From Eqn. 18 we have, at equilibrium,

$$\alpha_{34} p_3 = \alpha_{43} p_4 \tag{33a}$$

and

$$\alpha_{61} p_6 = \alpha_{16} p_1 \tag{33b}$$

Note that for $\alpha_{34} = \alpha_{43}$ and $\alpha_{16} = \alpha_{61}$, Eqns. 33 yield the conditions erroneously used by Naftalin for the fixed binding site model. Upon substitution of the p_i's in Eqns. 33 by means of the equations pertinent to binding, we obtain

$$\frac{c_{A,1} c_{B,1}}{c_{A,2} c_{B,2}} = \frac{\alpha_{43} \alpha_{16}}{\alpha_{34} \alpha_{61}} \frac{K_{A,1} K_{B,1}}{K_{A,2} K_{B,2}} \tag{34}$$

Eqn. 34 is fully analogous to Eqn. 28a, and detailed balance immediately leads to Eqn. 28b. Thus, irrespective of which model we choose, we

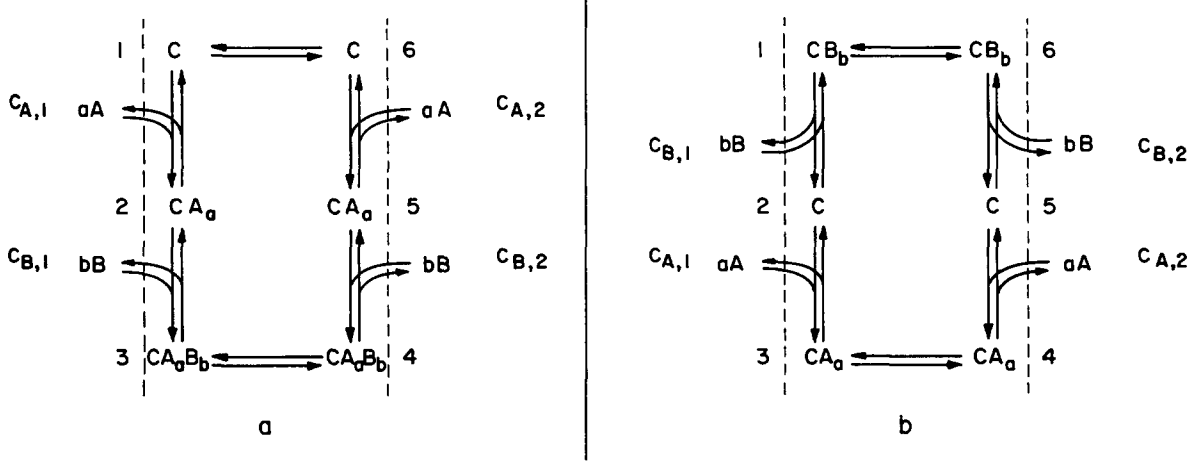

Fig. 2. Cycle diagrams for a non-electrogenic carrier model with generalized stoichiometry: (a) symport, (b) antiport. The transitions involving binding or release represent, in each case, a sequence of a or b steps each involving the binding of a single molecule of A or B, respectively.

always arrive at the same condition for equilibrium. This is identical to that obtained from thermodynamics, as it should be.

Antiport systems can be dealt with in exactly the same way, and Naftalin's treatment again involves the same erroneous relations between probabilities of states at equilibrium. Moreover, the treatment can easily be extended to stoichiometric ratios other than ± 1, e.g. in the case of the carrier model the general result can be written down immediately by inspection of the cycle diagram (Fig. 2) using Hill's technique [6]. In the steady state, the cycle flux J, i.e. the net flux around the cycle using the convention that the counter-clockwise direction is positive, is given by

$$J = N(\Pi_+ - \Pi_-)/\Sigma \tag{35}$$

where N is the number of moles of carrier, Π_+ and Π_- are the products of first-order and pseudo-first-order rate constants in the positive and negative directions, respectively, and Σ is a sum of products of first-order and pseudo-first-order rate constants [6]. In the most general case the cycle involves a moles of A and b moles of B, and the flows of A and B are, respectively, $J_A = aJ$ and $J_B = bJ$ (symport) or $-bJ$ (antiport). In any equilibrium condition $J_A = J_B = J = 0$, i.e.

$$\Pi_+ = \Pi_- \tag{36}$$

Accordingly,

$$\alpha^{o}_{12} c^{a}_{A,1} \alpha^{o}_{23} c^{b}_{B,1} \alpha_{34}\alpha_{45}\alpha_{56}\alpha_{61} = \alpha^{o}_{65} c^{a}_{A,2} \alpha^{o}_{54} c^{b}_{B,2} \alpha_{43}\alpha_{32}\alpha_{21}\alpha_{16} \quad \text{(symport)} \tag{37a}$$

and

$$\alpha_{12} \alpha^{o}_{23} c^{a}_{A,1} \alpha_{34}\alpha_{45}\alpha^{o}_{56} c^{b}_{B,2} \alpha_{61} = \alpha_{65} \alpha^{o}_{54} c^{a}_{A,2} \alpha_{43}\alpha_{32}\alpha^{o}_{21} c^{b}_{B,1} \alpha_{16} \quad \text{(antiport)} \tag{37b}$$

From the consideration of microscopic reversibility, i.e. remembering that at true equilibrium the concentrations of both species are equal on either side, it follows that the products of the rate constants are also equal on either side of Eqns. 37a and 37b. Hence, reverting to our earlier use of signed stoichiometric coefficients, both equations yield

$$\left(\frac{c_{B,1}}{c_{B,2}}\right) = \left(\frac{c_{A,1}}{c_{A,2}}\right)^{-\nu_A/\nu_B} \tag{38}$$

Eqn. 38 is again identical to Eqn. 14 (uncharged species). It should be noted that the more general case (charged species), which includes the effects of electrical potentials on rate constants, can be dealt with similarly [6] and yields Eqn. 13.

Nonequilibrium thermodynamics

In an effort to cope with what he considers is "the failure of equilibrium thermodynamics to account adequately for static-head accumulation or depletion of driven solute" [1], Naftalin concludes that models of cotransport where the driving force is solely determined by the thermodynamic affinities of the transported species are inappropriate. Accordingly he introduces an alternative mode of cotransport, claimed to be entropy driven. This comprises an ATP-energized pump, which maintains a steady-state gradient of species A, in parallel with the cotransport system. In this model the cotransport of species A and B is assumed to be incompletely coupled, hence static head does not correspond to equilibrium and the entropy production always exceeds zero. Indeed Naftalin takes the view that the degree of coupling of a cotransport system must always be less than unity, and on this basis he argues that such systems are driven by entropy.

Since the pump is not directly coupled to the cotransporter, the phenomenological equations for the latter in a linear range sufficiently close to equilibrium are straightforward. Assuming uncharged solutes,

$$J_A = L_{AA}(\mu_{A,1} - \mu_{A,2}) + L_{AB}(\mu_{B,1} - \mu_{B,2}) \tag{39a}$$

$$J_B = L_{BA}(\mu_{A,1} - \mu_{A,2}) + L_{BB}(\mu_{B,1} - \mu_{B,2}) \tag{39b}$$

where the L_{ij}'s are phenomenological coefficients. Using the terminology of Kedem and Caplan [4], and taking into account that $L_{AB} = L_{BA}$ and species A drives the cotransport of species B, we can define the degree of coupling $q = L_{AB}/\sqrt{(L_{AA} \cdot L_{BB})}$ and the phenomenological stoichiometry $Z = \sqrt{(L_{BB}/L_{AA})}$. We then have

the static head condition [4]

$$(\mu_{B,1} - \mu_{B,2})/(\mu_{A,1} - \mu_{A,2}) = -q/Z \tag{40}$$

and consequently

$$\left(\frac{c_{B,1}}{c_{B,2}}\right) = \left(\frac{c_{A,1}}{c_{A,2}}\right)^{-q/Z} \tag{41}$$

Eqn. 41 was also obtained by Naftalin, but he failed to appreciate its relationship to Eqn. 38 – the latter is simply the limiting case of the former as q tends to unity. This follows from an important inequality, readily proved by explicit consideration of either external or internal leakage pathways [7], which for symport takes the form:

$$q \leqslant \frac{Z}{\nu_B/\nu_A} \leqslant \frac{1}{q} \tag{42}$$

(a similar result may be obtained for antiport). Hence, when $q = 1$, $Z = \nu_B/\nu_A$. From this it is seen that the ratio ν_B/ν_A is not independent of the membrane parameters which control flow, as claimed by Naftalin, and as a consequence q and Z cannot be treated as completely independent parameters, as Naftalin has done. Eqn. 42 always imposes a condition on Eqn. 41, so the ratio ν_B/ν_A certainly affects the static head distribution of cotransported ligands whether or not coupling is complete. No new insight regarding static head equilibrium is obtained from this approach, since there is no a priori reason why q should not be unity, or so close to unity as to make the difference experimentally indistinguishable. The usual thermodynamic forces (affinities) acting in the system determine the distribution of matter either in a steady state or at equilibrium, and no necessity arises to introduce the concept of entropy production as an additional driving process. At static head equilibrium, of course, the entropy production disappears anyway.

Simulations

The approach to and the attainment of a true static head can be demonstrated by computer simulations for multicompartment systems, starting from a variety of initial conditions. The transport of species through the membranes separating the compartments is described either on a phenomenological (or macroscopic) level by means of flow-force relations, or on a mechanistic (or microscopic) level using kinetic schemes for the carriers involved.

Let us first assume that the linear flow-force relations of Eqns. 39 hold over the entire concentration ranges of species encountered in a given setup and not just close to equilibrium. This assumption is generally not justified but does not invalidate the point to be made in the context of Naftalin's statements. For completely coupled flows we have

$$L_{AA} = L,\ L_{AB} = L_{BA} = (\nu_B/\nu_A)L, \text{ and } L_{BB} = (\nu_B/\nu_A)^2 L \tag{43}$$

where L is a generalized permeability of the cotransport system. It is proportional to the mole number of carriers and comprises the rate constants of the cotransport. Denoting the flow of species X (A or B) through the membrane between compartments j and l by $J_{X,jl}$, and remembering that $J_{X,jl} = -\mathrm{d}n_{X,j}/\mathrm{d}t = \mathrm{d}n_{X,l}/\mathrm{d}t$ yields the following relations for the change in concentrations of species in a system with three compartments in series

$$V_1 \mathrm{d}c_{X,1}/\mathrm{d}t = -J_{X,12}, \quad V_2 \mathrm{d}c_{X,2}/\mathrm{d}t = J_{X,12} - J_{X,23},$$

$$V_3 \mathrm{d}c_{X,3}/\mathrm{d}t = J_{X,23} \tag{44}$$

V_j denotes the volume of the solution in compartment j which is assumed to be constant, i.e. the membranes are impermeable to the solvent, and the minor changes in volume due to the transport of species A and B are neglected.

Introducing Eqn. 12 (with $z_i = 0$, uncharged species) into Eqns. 39 yields, together with Eqns. 44, a set of differential equations which, upon integration starting from initial values, determine the time course of the concentration for both species in all compartments. This integration, however, is not straightforward but rather difficult. It is therefore more convenient to represent the transport system in terms of an electric network, i.e. to use the tool offered by network thermodynamics, and let the integration be done by one of the available electric network simulation programs such as SPICE. A full account of this technique can be found elsewhere (see e.g. Refs. 8

and 9). It is sufficient here to note that chemical species are represented by electrons, hence mole number, flow, and concentration are equivalent to charge, electric current, and voltage, respectively, while volumes are represented by capacitors. Obviously, a separate circuit has to be devised for each species, and the dependence of flows on concentrations is then introduced through so-called controlled sources whose current is determined by voltages taken from anywhere in the whole circuitry. The logarithmic term involved in the chemical potential can be assessed by means of a diode whose current-voltage characteristic is exponential.

A more realistic assessment of the dependence of flows on concentrations is obtained by means of the Hill cycle diagrams for the carriers as shown in Figs. 1c and 2. In fact, such simulations are equivalent to performing actual experiments, provided that the membranes used and the carriers embedded therein display the same properties as assumed in the simulations. The differential equations resulting from such a description are even more complex than in the case of linear flow-force relations, and the use of network thermodynamics is almost unavoidable. A Hill cycle is then represented by a set of capacitors whose voltages are equivalent to the probabilities of the states in the cycle. The capacitors are connected through controlled current sources which define the transitions between the states (see Kinetics section). The current through sources pertinent to transitions which involve binding or release of a species represents the flow of this species into or out of the carrier.

Simulations performed with networks pertinent to three-compartment systems in series whose membranes contain either two symporters with stoichiometric ratios 2 : 1 and 1 : 1 or an antiporter and a symporter both with stoichiometric ratios 1 : 1 have shown that any set of initial values for the concentrations of species A and B, and given values for the volumes in the three compartments, leads to a unique and stable static head. The evolution of the static head equilibria discussed by Naftalin [1] are illustrated in Fig. 3 for linear flow-force relations, and in Fig. 4 for kinetic schemes, respectively. Some points are worth discussing.

Varying the generalized permeabilities, L, of the two cotransporters but holding the initial concentrations and the volumes constant affects the time courses which, however, always converge to the same static head (see Fig. 3b). Similarly, in the case of kinetic schemes, varying the dissociation constants gives rise to different time courses without changing the static head (Fig. 4). This should not be mistaken for support of Naftalin's statement that static head is independent of the molecular transport mechanisms. Obviously, a change in L can arise from a variation in the number of carrier molecules or an appropriate rescaling of the rate constants, which also causes an alteration of the dissociation constants. This occurs, however, without affecting the stoichiometric ratios ν_B/ν_A which clearly determine the static head to be attained.

When the volume of one of the compartments is altered, the initial concentrations in this compartment have to be adjusted in order that the same static head will be reached (see Fig. 3a). Although flows are determined by concentrations, the changes of the latter created by the former are dependent on the volume (cf. Eqn. 44) which constitutes a capacitance for chemical species like a capacitor for charges. Accordingly, when the volume of a compartment is changed, the concentrations of species in it follow different time courses, though starting from the same initial values, which eventually leads to a different static head. Moreover, decreasing the volume (i.e. decreasing the capacitance of species) without changing the permeabilities (or resistances) causes the system to relax faster to the static head equilibrium, in full analogy to the RC-law in electric networks.

The fact that initial concentrations and volumes definitely determine which static head will be reached by a system has an intriguing consequence for the case that the middle compartment is an epithelium which cannot be directly accessed. Intuition would tell us that the same static head should develop whenever we mount a particular epithelium between two solutions with given volumes and initial concentrations. Even if we assume that the volume of the epithelium is constant this expectation is wrong. Clearly, the static head depends also on the initial concentrations of

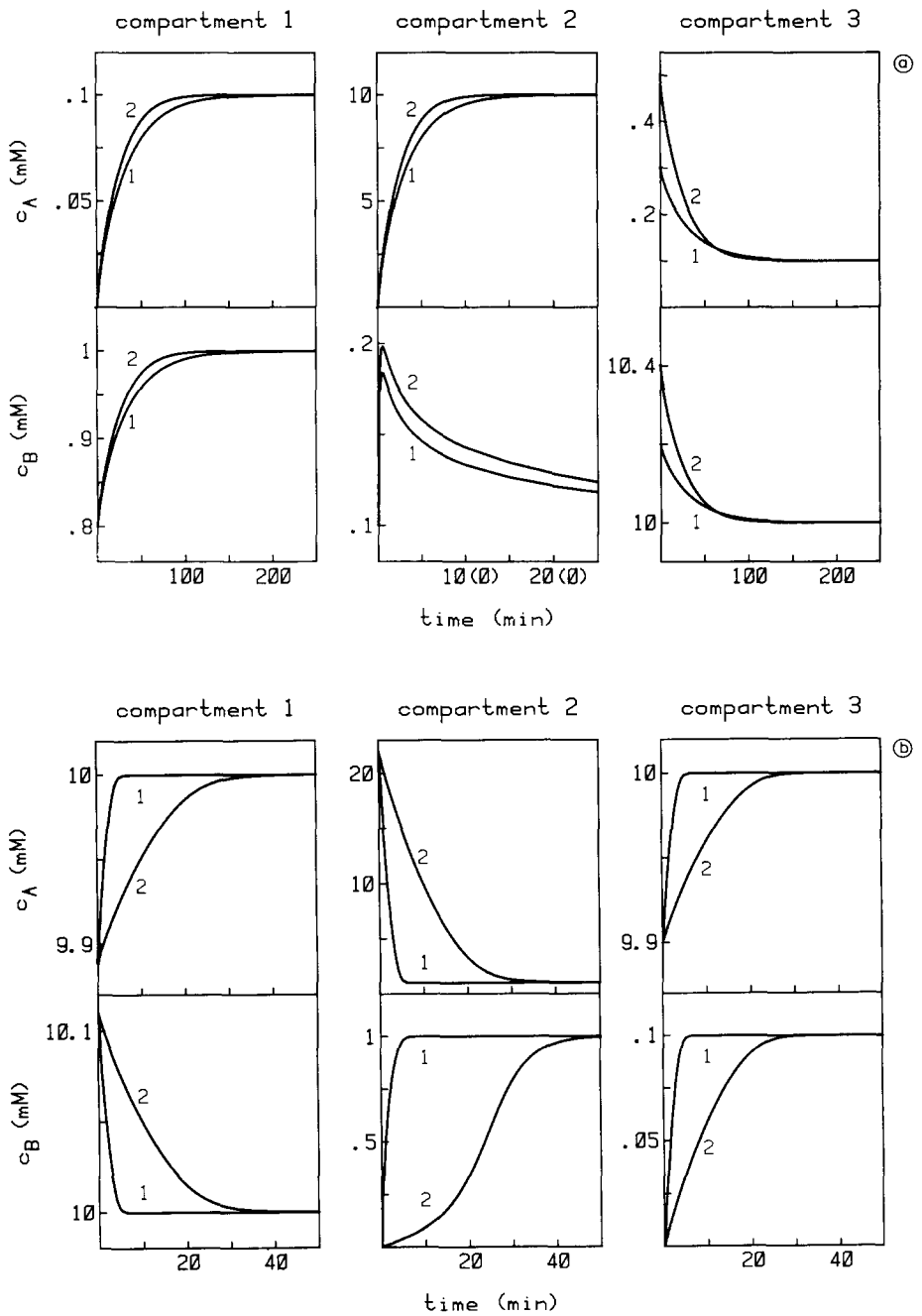

Fig. 3. Time course of concentrations of species in a three-compartment system in series, calculated with linear flow-force relations. The membranes between the compartments are permeable only to species A and B whose flows are completely coupled. The volumes are (if not otherwise stated) 10 ml for compartments 1 and 3, and 0.1 ml for compartment 2. Unprimed and primed parameters are pertinent to the transport from compartments 1 to 2 and 2 to 3, respectively. Generalized permeabilities (cf. Eqn. 43) are given in $\mu mol^2 \cdot J^{-1} \cdot min^{-1}$. $T = 25°C$.

(a) $\nu_B/\nu_A = 2$ and $\nu'_B/\nu'_A = 1$ (both symport). $L = L' = 10$ for curves 1 and 2, but the volume in compartment 3 is 5 ml for curves 2. Note that the time scale for $c_{B,2}$ is restricted to 25 min in order to show the fast initial changes (both curves eventually converge to 0.1 mM).

(b) $\nu_B/\nu_A = -1$ (antiport) and $\nu'_B/\nu'_A = 1$ (symport). $L = L' = 40$ (curves 1) and $L = 4$, $L' = 40$ (curves 2).

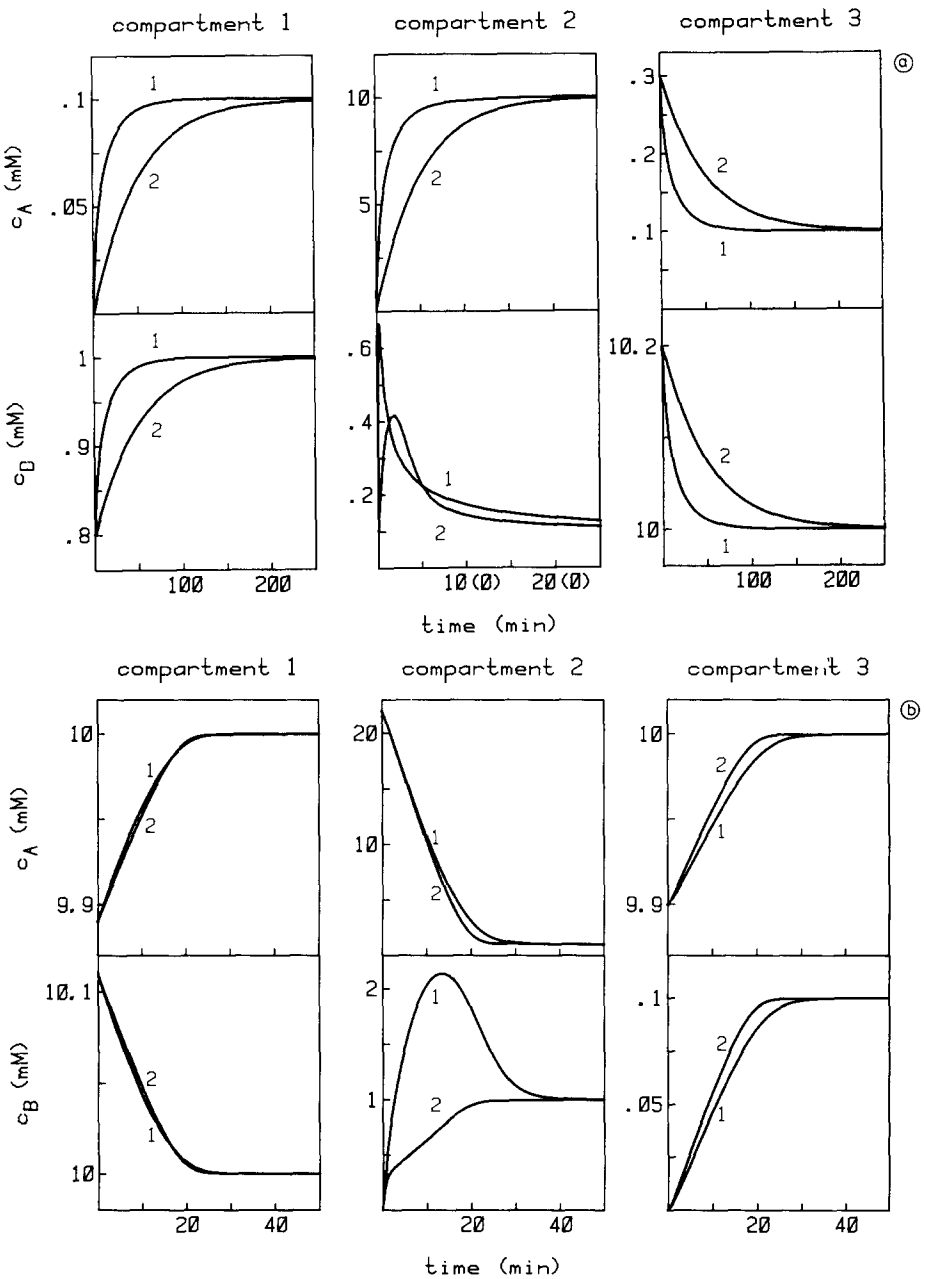

Fig. 4. Time course of concentrations of species in a three-compartment system in series, calculated by means of kinetic schemes for the carriers. Same conditions as given in the legend to Fig. 3, except for the generalized permeabilities which are replaced by first and second order rate constants, α_{ij} and α_{ij}° (see Kinetics section). If not stated otherwise, $\alpha_{ij} = 1000\ \text{min}^{-1}$ for all transitions. Values for α_{ij}° are chosen such that the values for the dissociation constants K (cf. Eqn. 20) occur as listed below.

(a) $\nu_B/\nu_A = 2$ and $\nu'_B/\nu'_A = 1$ (both symporters); mole number of carriers, $N = N' = 10$ nmol. $K_{A,i} = K'_{A,i} = K_{B,i} = K'_{B,i} = 1$ mM for all i (curves 1). $K_{A,1} = K_{B,2} = 0.1$ mM, $K_{B,1} = K'_{B,2} = K'_{A,3} = 1$ mM, $K_{A,2} = K'_{A,2} = K'_{B,3} = 100$ mM. Detailed balance requires adjustment of at least one α_{ij} pertinent to the transmembrane transfer of the 1 : 2-symporter; in the case of curves 2, $\alpha_{45} = 100\ \text{min}^{-1}$ (transition of loaded carrier from compartment 1 to 2). Note that the time scale for $c_{B,2}$ is restricted to 25 min in order to show the fast initial changes (both curves eventually converge to 0.1 mM).

(b) $\nu_B/\nu_A = -1$ (antiporter) and $\nu'_B/\nu'_A = 1$ (symporter); mole number of carriers, $N = N' = 1$ nmol. $K_{A,i} = K'_{A,i} = K_{B,i} = K'_{B,i} = 1$ mM for all i (curves 1). $K_{A,2} = K_{B,2} = K'_{A,2} = K'_{B,2} = 1$ mM, $K_{A,1} = K_{B,1} = K'_{A,3} = 10$ mM, $K'_{B,3} = 0.1$ mM.

species within the epithelium and hence on the conditions the epithelium has been subjected to before we use it in our experiment. It should be added, however, that this behaviour results from the membranes being impermeable to species A and B except for the completely coupled flows through the carriers. This condition, which was chosen by Naftalin, is rarely met by real membranes which usually have leak pathways for the species and/or incompletely coupled carriers. The system then goes through a pseudo static head and eventually reaches overall equilibrium. Thus, the concentrations of species in a real epithelium can usually be adjusted to any values by long enough incubation in large volumes of appropriate solutions.

Discussion and Conclusions

The analysis given above has of necessity been somewhat detailed, in order to provide a complete refutation of the very detailed arguments put forward by Naftalin [1] in favor of his thesis that static head equilibrium does not exist. Even so, we have not dealt with all the pitfalls that await the unwary reader of Naftalin's paper. For instance, two examples are given of the stoichiometric equations of cotransport, in which transport is presented as a sequence of chemical reactions. Within the membrane transport is considered to occur between two 'phases' numbered 2 and 3, and the over-all reaction for symport is found to be (cf. Eqn. 8.2e in Ref. 1)

$$nA^2 + B^2 = nA^3 + B^3$$

where n is a stoichiometric coefficient and the superscripts refer to phase numbers. In the subsequent mathematical manipulation (addition of Eqns. 8.1, 8.2e, and 8.3a,b in Ref. 1) Naftalin implicitly separates the above equation into two equations,

$$nA^2 = nA^3$$

$$B^2 = B^3$$

This impermissible operation leads him to the incorrect conclusion that the stoichiometric coefficient for the phase transfer reaction across a membrane for all transported species is always 1, and hence the equilibrium distribution of solutes is unaffected by the stoichiometry of ligand interaction with microscopic components within the membrane. To examine these arguments further would be invidious.

The essential error in Naftalin's paper is his failure to recognize the correct conditions for equilibrium. Correlations between the probabilities of different states in a kinetic scheme exist only for those states which are directly connected through transitions. The correlations are determined by the pertinent rate constants (see Eqn. 18) and express the fact that, at equilibrium, the transitions in both directions are equally frequent, i.e. there is no net transition between the states. Thus, the probabilities of the states in a carrier cycle (Figs. 1c and 2) adopt such values at static head equilibrium that net transitions between all states vanish, as is clearly demonstrated by the simulations (not shown here). This, however, does not mean that the probabilities of corresponding states on both sides of the membrane are always equal at equilibrium as assumed by Naftalin. Such a situation can arise for some of the states under certain conditions; in the cycle of Fig. 1c, for example, $p_1 = p_6$ and $p_3 = p_4$ provided that $\alpha_{16} = \alpha_{61}$ and $\alpha_{34} = \alpha_{43}$, respectively. However, one cannot then conclude that p_2 is also equal to p_5.

For similar reasons, the argument put forward by Naftalin in terms of the chemical potential of a bound species X, μ_{Xb}, fails to prove his point. Besides the fact that the equations used (Eqns. 9.29–9.31 in Ref. 1) are inadequate (Eqn. 9.29 has been criticized by Hill [10], and Eqn. 9.31 applies only to an all or none binding of n molecules of one species), the driving force for cotransport defined by Naftalin as the difference in μ_{Xb} on both sides of the membrane (Eqn. 9.35 in Ref. 1) is not correct. The connection between states of bound species as expressed by a chosen kinetic scheme has to be considered. Thus, in the lattice-gas representation of a 1 : 1-antiport where a bound species A on one side of the membrane exchanges binding sites with a bound species B on the other side of the membrane (see Fig. 3c in Ref. 1), the driving force would be $\mu'_{Ab} + \mu''_{Bb} - \mu''_{Ab} - \mu'_{Bb}$ (prime and double prime indicating the two sides of the membrane). This quantity vanishes at equi-

librium, but it does not follow that $\mu'_{Ab} = \mu''_{Ab}$ and $\mu'_{Bb} = \mu''_{Bb}$. Adopting the latter conclusion would constitute a mistake similar to that discussed at the beginning of this section.

We are well aware of the fact that completely coupled cotransport through membranes is rarely found in real systems, and that the subject of this paper may therefore be considered as purely academic. However, we have been compelled to adhere to this condition since it is inherent in all of Naftalin's arguments, except for those dealing with 'entropy driven cotransport systems'. As shown in the section entitled 'Nonequilibrium thermodynamics', incompletely coupled systems are not fundamentally different but merely arise when we include leak pathways in the membrane and/or so-called 'futile' transitions in the kinetic schemes *. It should be added that a static head equilibrium can no longer be reached by such systems. The static head attained is a stationary state when the concentrations of one species in the compartments are clamped, or a pseudostationary state in a closed system with a sufficiently large capacity for at least one of the species. Again, the concentration ratios at static head clearly depend on the molecular stoichiometry of the cotransport mechanism (see Eqns. 41 and 42).

To sum up, it is seen that all approaches are essentially equivalent and lead to identical conclusions. For completely coupled cotransport these are:

(a) the static head equilibrium state exists and is stable;

(b) the stoichiometry of cotransport influences the static-head distribution of cotransported ligands.

Furthermore, the above conclusions are entirely consistent with the Zeroth Law of Thermodynamics as reformulated in the Thermodynamics section above.

Acknowledgements

One of us (D.W.) is grateful to EMBO for a short term fellowship which enabled him to visit the Weizmann Institute where the studies on the use of network thermodynamics for simulating transport systems were initiated. He also acknowledges financial support by the Swiss National Science Foundation.

References

1 Naftalin, R.J. (1984) Biochim. Biophys. Acta 778, 155–175
2 Denbigh, K.D. (1964) The Principles of Chemical Equilibrium, Cambridge University Press, London
3 Tisza, L. (1977) Generalized Thermodynamics, The M.I.T. Press, Cambridge, MA
4 Kedem, O. and Caplan, S.R. (1965) Trans. Faraday Soc. 61, 1897–1911
5 Hill, T.L. and Kedem, O. (1966) J. Theor. Biol. 10, 339–441
6 Hill, T.L. (1977) Free Energy Transduction in Biology, Academic Press, New York
7 Caplan, S.R. (1981) Proc. Natl. Acad. Sci. USA 78, 4314–4318
8 Mikulecky, D.C. (1979) Biophys. J. 25, 323–340
9 Mikulecky, D.C. (1983) in Membrane Biophysics II: Physical Methods in the Study of Epithelia (Dinno, M., Rozell, T., and Calahan, A., eds.), Alan R. Liss, Inc., New York
10 Hill, T.L. (1983) Proc. Natl. Acad. Sci. USA 80, 2922–2925

* 'Futile' transitions (see e.g. Ref. 6) are so called because they are futile in terms of coupling of the transport of the two species. They cause what is known as 'slip' in the coupling device. An alternative notion is 'internal leakage pathways', since futile transitions give rise to leakage of species through the molecular machinery reminiscent of the independent movement of species through leaks in the membrane ('external leakage pathways'). In the schemes of Fig. 1, futile transitions could occur between states 2 and 6 or 1 and 5 (Fig. 1a), between states 21 and 12 (Fig. 1b), and between states 2 and 5 (Fig. 1c).