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RELATIONSHIP BETWEEN CHEMIOSMOTIC FLOWS AND THERMODYNAMIC FORCES IN OXIDATIVE PHOSPHORYLATION

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Summary

A set of equations has been derived, describing quantitatively the relationships between flows and thermodynamic forces in the chemiosmotic model of oxidative phosphorylation.

Experimental tests of these equations give information on the stoichiometric coupling constants between the different flows.

Introduction

Mitochondria and many bacteria are able to catalyze the thermodynamically unfavourable synthesis of ATP from ADP and phosphate by coupling this reaction to exergonic oxidations. The mechanism of this coupling has been a matter of controversy for a long time [1].

The most widely accepted mechanism is that proposed by Mitchell [2]. According to this, so-called chemiosmotic, model the coupling between phosphorylation and oxidation reactions is effected by transmembrane movement of protons. According to the chemiosmotic model, these protons are derived from the substrates taking part in the phosphorylation and redox reactions. It has, however, also been proposed that the protons are primarily derived from the enzymes catalyzing the respective processes (see for instance Ref. 3).

Since a fundamental postulate of the chemiosmotic theory is the existence of a characteristic stoichiometry between the transmembrane proton fluxes and the rates of the chemical reactions [2], it is of obvious importance to verify this postulate.

Attempts to determine these stoichiometries have been numerous (for a

survey see for instance Ref. 4). Most methods involve a sudden disturbance of equilibrium through the addition of one of the substrates and measurement of the subsequent rate (or extent) of the proton flux (and sometimes of the chemical reaction). The conditions are chosen so that there is least interference of the measurement by back reactions, just as in initial-rate measurements of enzyme kinetics, but even so a number of technical and theoretical uncertainties precludes a final answer (Heinz et al., in preparation).

The fluxes under those conditions will approximately reflect the stoichiometries of coupling at the molecular level when the reactions proceed down a large gradient of free energy. When, however, oxidative phosphorylation occurs, the changes in free energy may be much smaller and it is important to verify that the stoichiometries also apply to that situation.

In previous papers [5,6], we have applied the principles of linear irreversible thermodynamics to derive a set of relations between flows and thermodynamic forces in oxidative phosphorylation. The important difference with earlier treatments along the same lines by Rottenberg and others [7,8] was that we were able to arrive at a description in which the different constants have a well-defined mechanistic meaning. Later, a similar but less complete description was given by Hill [9].

Recently, we have found that for enzyme-catalyzed reactions the proportional relation between flow and force can be more appropriately replaced by a linear relation (Van der Meer et al. [28]; see also Rottenberg [10]). Such a relation will hold over a large range of velocities for conservative systems, i.e. when the sum of substrate plus product is kept constant. Close to equilibrium the two descriptions become identical. Using these new findings, we describe in this article the derivation of some more refined equations, relating the velocities of the different fluxes with the thermodynamic forces in the chemiosmotic model of oxidative phosphorylation. Simultaneous measurement of the different fluxes and forces allows calculation of the stoichiometric coupling constants under any condition.

Materials and Methods

Rat-liver mitochondria were isolated according to the method of Hogeboom [11], as modified by Myers and Slater [12].

Protein was determined by the biuret method.

Rat-liver mitochondria (1—1.33 mg protein/ml) were incubated in a vessel with a movable stopper, from which samples could be taken without interference with the measurement by electrodes of $[O_2]$, $[H^+]$ or $[K^+]$. The incubation medium contained (Fig. 2) 235 mM sucrose, 10 mM Tris-succinate, 1 mM Tris-malate, 1 μg rotenone/ml, 10 mM KCl, 10 mM Tris-P_i, 10 mM glucose, 5 mM $MgCl_2$, 1 mM Na-EDTA, and 0.1 mM Na-ATP, final pH 7.0 or (Table I, Figs. 3,4 and 5) an oxygenated solution of 235 mM sucrose, 10 mM Tris-succinate, 1 μg rotenone/ml, 20 mM Tris-HCl, 10 mM Tris-P_i, 10 mM glucose, 4 mM $MgCl_2$, 2 mM Na-EDTA, 0.01 mM Na-ATP, and 0.5 μg valinomycin/ml, final pH = 7.2. In both cases variable amounts of dialysed hexokinase or 2,4-dinitrophenol were added.

0.5 ml samples were acidified with ice-cold perchloric acid, neutralized and assayed for ATP, ADP, AMP and glucose 6-phosphate by enzymatic methods

[13] and for P_i by a colorimetric method [14].

0.5 ml samples were centrifuged at $t = 1$, 3 and 5 min after starting the reaction through silicone into perchloric acid, and supernatant and acid layer analysed for P_i and K^+ (by atomic absorption spectrophotometer) to calculate $\Delta\tilde{\mu}_H$ [15]. In the calculation, it was assumed (and checked by [^{14}C]-sucrose distribution) that State-4 mitochondria have an internal volume of 1.8 µl/mg protein and an adhering water volume of 3.6 µl/mg under these conditions. Changes in internal volume were calculated from the changes in K^+ content relative to State 4, assuming osmotic equilibrium with a univalent K^+ salt [16].

The rate of phosphorylation was calculated from the rate of glucose-6-phosphate formation and found to be almost constant between $t = 1$ and 5 min. The attainment of a steady state during that period was also indicated by the constancy of the K^+ concentration.

Results and Discussion

According to the chemiosmotic model of oxidative phosphorylation, the energy-transducing membrane can be considered as having three independent functions: a redox-mediated H^+ pump, an ATPase-mediated H^+ pump and a passive H^+ leak (Fig. 1).

For each of the three functions a relation between the rate of the chemiosmotic process and the change in free energy of that process can be written [17,7,5]. This relation is most simple for the passive movement of H^+ across the membrane:

$$J_H^l = L_H^l \cdot \Delta\tilde{\mu}_{H^+} \qquad (1)$$

Close to equilibrium, a similar proportional relation between flux and total thermodynamic force will hold approximately for the other processes. However, the region of validity of this approximation is rather small. Furthermore, it is not a priori clear how the fact that these processes are catalyzed by enzymes would affect the relations.

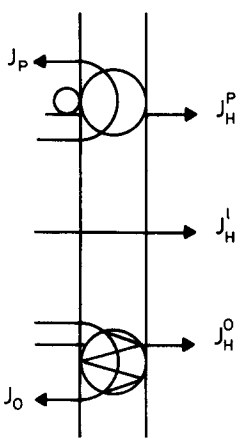

Fig. 1. Elements considered in the description. See text for explanation.

We have shown recently [28] that for an enzyme-catalyzed reaction over a large range of rates a linear relation between flux and change in free energy exists, provided that the total concentration of substrate plus product is kept constant. The proportionality constant of the relation between the flux and the force in this range may differ from that around equilibrium, depending on the values of the K_m and V of the enzyme in the two directions. The relation will have the general form

$$J = L\ \Delta G + \text{constant} \tag{2}$$

(ΔG in this equation has a positive sign if the reaction proceeds spontaneously). Close to equilibrium, the constant is zero and we have, for instance:

$$J_0 = L_0(\Delta G_0 + n_H^0\ \Delta\tilde{\mu}_H) \tag{3}$$

Further away from equilibrium, the constant (C_0) will appear and L_0 will assume a different value (L_0'). Since we may not expect a priori that the change in proportionality constant will be equal for the two right-hand terms in Eqn. 3, we allow for a difference in change by introducing an 'activity factor' γ^0. In the ideal case where the flow experiences a change in $\Delta\tilde{\mu}_H$ precisely n_H^0 times as strong as a change in ΔG_0, the value of γ^0 would be 1. The physical significance of the factor γ^0 may be visualized by remembering that, in the complex enzyme system under consideration, equal changes in the partial forces may have different effects. For instance, ΔG_0 may be varied by changing the oxygen concentration; if the concentration of oxygen remains far above the K_m for oxygen of cytochrome *c* oxidase, however, this will not result in a change in J_0.

The equation for the redox-mediated H^+ pump will then be

$$J_0 = L_0'(\Delta G_0 + \gamma^0 \cdot n_H^0\ \Delta\tilde{\mu}_H) + C_0 \tag{4}$$

Similarly, we can derive for the ATPase-mediated H^+ pump

$$J_p = L_p'(\Delta G_p + \gamma^p \cdot n_H^p\ \Delta\tilde{\mu}_H) + C_p \tag{5}$$

The concomitant fluxes of H^+ can be written down directly, assuming that the enzymes are fully coupled at the molecular level (one of the fundamental postulates of the chemiosmotic theory):

$$J_H^0 = n_H^0 J_0 = n_H^0 L_0'(\Delta G_0 + \gamma^0 n_H^0\ \Delta\tilde{\mu}_H) + n_H^0 \cdot C_0 \tag{6}$$

$$J_H^p = n_H^p J_p = n_H^p L_p'(\Delta G_p + \gamma^p n_H^p\ \Delta\tilde{\mu}_H) + n_H^p \cdot C_p \tag{7}$$

The net flux of H^+ ions across the membrane is the sum of the separate fluxes:

$$J_H = J_H^1 + J_H^0 + J_H^p \tag{8}$$

We now have a complete set of equations, relating the fluxes in the system to the thermodynamic forces. In mitochondria, an extra complication arises since the substrates react at the inside of the organelle. Unfortunately, it is rather difficult to measure the substrate concentrations in this compartment (the so-called matrix). It can be shown that the thermodynamic forces ΔG_0 and ΔG_p may also be measured outside the mitochondria and used in the equations [7,5]. However, the proportionality constants will then again have a different

value (L *), as they will now include a contribution of the resistance for substrate movement posed by the different translocators [5]. Furthermore, the validity of the equations has been shown only for the steady-state condition in which the matrix concentration of substrates is constant.

Keeping these restrictions in mind, we arrive at the following set of equations, describing mitochondrial oxidative phosphorylation

$$J_0 = L_0^* \Delta G_0^{ex} + \gamma^0 n_H^0 L_0^* \Delta\tilde{\mu}_H + C_0^* \tag{9}$$

$$J_H = n_H^0 L_0^* \Delta G_0^{ex} + (\gamma^0 (n_H^0)^2 L_0^* + \gamma^p (n_H^{p*})^2 L_p^* + L_H^1) \Delta\tilde{\mu}_H + n_H^{p*} L_p^* \Delta G_p^{ex} + n_H^0 \cdot C_0^* + n_H^{p*} \cdot C_p^* \tag{10}$$

$$J_p = \gamma^p n_H^{p*} L_p^* \Delta\tilde{\mu}_H + L_p^* \Delta G_p^{ex} + C_p^* \tag{11}$$

in which the asterisks denote the fact that the proportionality constants include transport components; furthermore, n_H^{p*}, the number of protons translocated per ATP hydrolysed, now includes any net proton involved in the virtual exchange of ATP for ADP and phosphate. A fundamental difference between these equations and those published before is the inclusion of the constants C and the activity factors γ [7,5,9].

Measurements of the different fluxes and forces can be subjected directly to Eqns. 9—11 to obtain the values of the proportionality constants and, more importantly, of the stoichiometric coupling constants.

For some purposes, however, it is more convenient to look at special cases which will simplify the relations.

One such special case is that in which $J_H = 0$, i.e. the steady state in which no further net transmembrane movement of H^+ occurs. This condition allows elimination of one of the thermodynamic forces. We choose to eliminate $\Delta\tilde{\mu}_H$, since it is technically the most difficult to determine. This is most easily done by taking $J_H = n_H^0 J_0 + n_H^{p*} J_p + J_H^1 = 0$ and using Eqns. 1 and 9. Without further assumptions elimination leads to the equation:

$$J_p = -\left\{\frac{n_H^0}{n_H^{p*}} + \frac{L_H^1}{\gamma^0 n_H^0 n_H^{p*} L_0^*}\right\} J_0 + \frac{L_H^1}{\gamma^0 n_H^0 n_H^{p*}} \Delta G_0^{ex} + \frac{L_H^1 C_0^*}{\gamma^0 n_H^0 n_H^{p*} L_0^*} \tag{12}$$

A relation of similar form could be derived, starting from 'simple' proportional equations used before [7,18]. The important point is that the more complicated linear equations, including the constants, still lead to a relatively simple conclusion. The model thus predicts that, at constant ΔG_0^{ex}, there must be a linear relation between the rate of ATP synthesis and the rate of oxygen uptake by mitochondria. The slope of the line relating these two fluxes should increase with increasing leakiness of the membrane towards protons (L_H^1 increases). In the earlier treatment by Rottenberg et al. [7], there was no explicit dependence of any of the 'phenomenological' constants on the proton permeability of the membrane. Thus, it could not be predicted a priori whether an uncoupler would increase or decrease the slope of this line. A fortiori, the Rottenberg treatment did not allow the systematic analysis of a series of such lines, obtained at different uncoupler concentrations. Eqn. 12 predicts that a set of such lines, each at a different value of L_H^1, should intersect at a point where $-J_p/J_0 = n_H^0/n_H^{p*}$ [18]. Since n_H^0 and n_H^{p*} are the molecular stoichiom-

etric coupling constants (including substrate transport), this ratio represents a theoretical maximal P/O ratio. The experiment in Fig. 2 was designed to test these predictions, using succinate as the substrate. The slope of the lines increases indeed with the concentration of the uncoupler.

Furthermore, the lines intersect at a common point, where the ratio $-J_p/J_0 = 1.4 \pm 0.1$ (S.E.). In a series of 8 independent experiments this value was 1.46 ± 0.04 (S.E.M.). According to these experiments, the theoretical maximal P/O ratio with succinate under our experimental conditions is 1.46 (cf. Ref. 19).

Another special case is that in which there is no more net ATP synthesis (State 4). This is the condition where the ATP driven proton pump is at equilibrium, so that the simplified form of Eqn. 11 (without γ^p and C_p^*) holds. We find

$$-\left(\frac{\Delta G_p^{ex}}{\Delta\tilde{\mu}_H}\right)_{J_p=0} = n_H^{p*} \tag{13}$$

This simple relation was derived earlier also by others [7] and has been tested by several investigators [20—23]. The values for n_H^{p*}, calculated from the measurement of ΔG_p^{ex} and $\Delta\tilde{\mu}_H$, are always greater than 2. We have found values around 2.5, increasing upon addition of protonophorous uncouplers [20].

Since the ATPase driven H^+ pump functions relatively close to equilibrium in either direction, it may be assumed to function as an enzyme in which the value of the additional constant in Eqn. 11 (C_p^*) vanishes and γ^p becomes equal to 1 (cf. Ref. 28). Thus, we could rewrite Eqn. 11 as

$$J_p = n_H^{p*} L_p^* \Delta\tilde{\mu}_H + L_p^* \Delta G_p^{ex} \tag{14}$$

or

$$\frac{\Delta G_p^{ex}}{J_p} = -n_H^{p*} \frac{\Delta\tilde{\mu}_H}{J_p} + \frac{1}{L_p^*} \tag{15}$$

This equation allows us to use data at different rates of phosphorylation for the calculation of n_H^{p*}. The experiments of Table I and Fig. 3 show that a linear

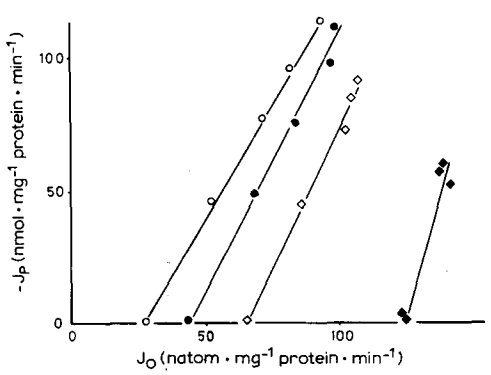

Fig. 2. Relation between rate of phosphorylation and rate of oxygen uptake at different concentrations of a protonophore. Rat-liver mitochondria were incubated and analysed as described under Materials and Methods, in the presence (from left to right) of 0, 2, 5, 10 μM 2,4-dinitrophenol.

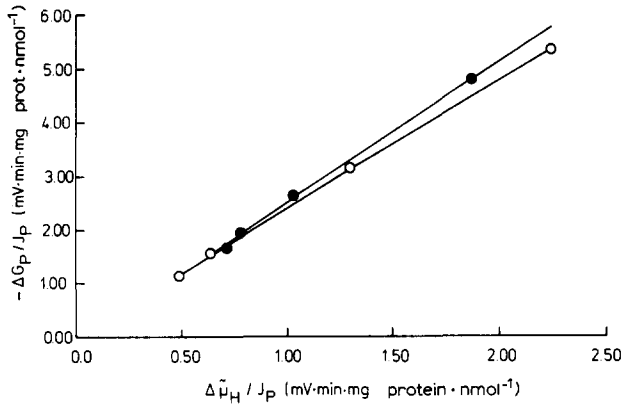

Fig. 3. Relation between $\Delta G_p/J_p$ and $\Delta\tilde{\mu}_H/J_p$. Rat-liver mitochondria were incubated and analysed as described under Materials and Methods. The rate of phosphorylation was varied by the addition of hexokinase. ○———○, without or ●———●, with 5 μM 2,4-dinitrophenol.

relation exists between $\Delta G_p^{ex}/J_p$ and $-\Delta\tilde{\mu}_H/J_p$. The slope of the line relating these parameters is $n_H^p{}^* = 2.4$. Since this value is equal to that at $J_p = 0$, the stoichiometric coupling constant is the same at any rate of phosphorylation ($\gamma^p \cdot n_H^p{}^*$ is constant).

The oxidation-driven H^+ pump is not readily reversible and, therefore, the simplifying assumptions made for the ATPase driven H^+ pump can not be used here. We can, however, test Eqn. 9 directly without knowledge of the magnitude of the constants C_0^* and γ^0. Fig. 4 shows an experiment demonstrating that J_0 indeed depends linearly on $\Delta\tilde{\mu}_H$ at constant ΔG_0^{ex} (ΔpH was essentially constant). In contrast to Padan and Rottenberg [24] and Azzone et al. [23] we find that it makes no difference whether $\Delta\tilde{\mu}_H$ is varied by activation of the flux through the ATPase or by addition of protonophore. The explanation for the different findings must lie in the different conditions; for instance, the other authors have varied the total adenine nucleotide concentration in their experi-

TABLE I

FLUXES AND FORCES DURING SUCCINATE OXIDATION BY RAT-LIVER MITOCHONDRIA

Experimental conditions as described in Materials and Methods.

Hexokinase (μg/ml)	2,4-Dinitrophenol (μM)	J_0 (natoms O/mg/min)	$-J_p$ (nmol P/mg/min)	ΔG_p (mV)	ΔG_0 (mV)	$-\Delta\tilde{\mu}_H$ (mV)
0	0	63	0	505	1603	218
1	0	127	95	505	1603	214
2	0	173	162	508	1603	210
4	0	277	311	489	1603	199
10	0	334	394	441	1603	192
0	5	240	0	509	1603	199
1	5	301	105	500	1603	196
2	5	342	189	498	1603	195
4	5	369	234	451	1603	182
10	5	388	254	422	1603	182

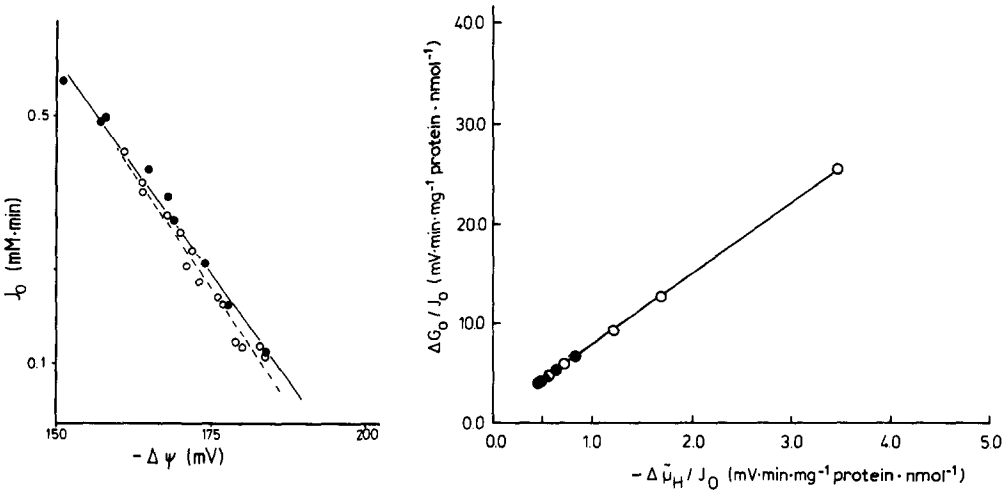

Fig. 4. Relation between J_0 and $\Delta\psi$. Rat-liver mitochondria were incubated and analysed as described under Materials and Methods. The rate of oxygen uptake was varied by the addition of hexokinase (○- - - - -○) or 2,4-dinitrophenol (●————●).

Fig. 5. Relation between $\Delta G_0/J_0$ and $\Delta\tilde{\mu}_H/J_0$. Rat-liver mitochondria were incubated and analysed as described under Materials and Methods. The rate of oxygen uptake was varied by the addition of hexokinase. ○ without, or ● with 5 μM 2,4-dinitrophenol. The data of this figure and of Fig. 3 were obtained in the same incubations.

ments, which may not influence not only $\Delta\tilde{\mu}_H$ but also some constants (at least L_p^*; Van der Meer et al. [28]).

A convenient way to further test Eqn. 9 is to use the derived equation

$$\frac{\Delta G_0^{ex}}{J_0} = -\gamma^0 n_H^0 \frac{\Delta\tilde{\mu}_H}{J_0} + \frac{1}{\Lambda_0} \tag{16}$$

with

$$\Lambda_0 = (L_0^* \Delta G_0^{ex} + C_0^*)/\Delta G_0^{ex}$$

and

$$\gamma^0 = \gamma_0/(1 + C_0^*/(L_0 \Delta G_0^{ex}))$$

Fig. 5 is a plot of $\Delta G_0^{ex}/J_0$ versus $-\Delta\tilde{\mu}_H/J_0$ for an experiment (Table I) in which ΔG_0^{ex} was kept constant. The observed straight line is in agreement with Eqn. 16. In this case the slope of the line does not directly represent the stoichiometry n_H^0. Its value (7.1) may be compared with two independent estimates that we can obtain of n_H^0. The first follows from the foregoing measurements of $n_H^{p\,*}$ and of $n_H^0/n_H^{p\,*}$:

$$n_H^{p\,*} \times n_H^0/n_H^{p\,*} = 2.4 \times 1.46 = 3.5 = n_H^0$$

The other was obtained by performing an oxygen pulse experiment in the same medium as used for the measurement of oxidative phosphorylation and with the same batch of mitochondria. Since the medium was too strongly buffered to follow pH changes, we measured the change in K^+ concentration and

assumed a 1 : 1 K^+/H^+ exchange. The number of K^+ moving per O turned out to be 3.8.

If the real value of n_H^0 is 3.5—3.8 then the term

$$\frac{\gamma^0}{1 + C_0^*/(L_0^*\Delta G_0^{ex})}$$

must have a value of approximately 2. This can reflect any case between $\gamma^0 \approx 2$ and $-C_0^* << L_0^*\Delta G_0^{ex}$ or $\gamma^0 = 1$ with $C_0^* \approx -\frac{1}{2}L_0^*\Delta G_0^{ex}$. At present we cannot make a choice between these possibilities.

One make now ask the question: what is the significance of non-integral stoichiometric coupling constants? According to the simplest form of the chemiosmotic concept, coupling at the molecular level will always involve whole atoms or molecules and, thus, the coupling constants must be whole numbers. Evidently, there are a number of possible complicating factors that will affect the outcome of experiments designed to measure the constants. To mention just a few: the presence of inverted vesicles (submitochondrial particles [25]) will cause oxygen uptake with concomitant movement of H^+ in the opposite direction, heterogeneity of the mitochondria will cause erroneous averaging in the calculation of $\Delta\tilde{\mu}_H$ from ion distribution, some of the steady-state conditions may not have been fulfilled completely. Taking these complications into account we must reckon with some systematic errors in the determination of the stoichiometric constants. In some of the calculations these errors cancel each other to a large extent, for instance in the determination of $n_H^0/n_H^{p\,*}$, described in Eqn. 12 and Fig. 2.

It is uncertain, however, whether the corrections that possibly have to be applied will lead to an integral number for $n_H^{p\,*}$. A possible explanation for the anomalous finding of a non-integral number for $n_H^{p\,*}$ may be that the transport of the substrates of the ATPase requires a fractional number of protons. If the adenine nucleotide translocator could operate in two modes: electroneutral or electrogenic, the relative contribution of the two modes might be variable and thus a fractional number of protons might apparently be involved in the transport (see, however, Ref. 26). If an uncoupler would affect the relative contribution of the two modes this might incidentally also explain why the apparent value of $n_H^{p\,*}$ changes with addition of an uncoupler [20].

Concluding remarks

The major advantage of our thermodynamic description, starting from the molecular reactions, over that given by others lies in the insight it gives in the meaning of the proportionality constants. Furthermore, our treatment explicitly allows the quantitative evaluation of the effect of, for instance, changes in proton permeability of the membrane or changes in stoichiometric numbers on the fluxes and forces. In this respect, we have given a physical meaning to the coupling coefficient q, introduced by Kedem and Caplan [27]. In a more exhaustive treatment it will be possible to explicitly account for incomplete coupling at the level of oxidative and phosphorylative proton pumps. Since the description should be helpful in interpreting and in designing experiments, this is very important. It is, for instance, inherent to our descrip-

tion that it is not allowed to apply the equations directly to a series of conditions in which the concentration of substrates plus product is varied.

Although as a first approximation linearity between flows and forces may be satisfactory, it may turn out later that refinements have to be made. In practice, this can be done by allowing for a (second-order) variability of the L's or the other constants. In that case the derivative at any point of the curve will still give an interpretable phenomenological stoichiometry at that particular point. As illustrated in this paper, elimination of some of the forces can be achieved using appropriate steady-state assumptions, yielding more easily testable relationships between flows and forces.

Ultimately, the stoichiometric numbers derived from our equations must be consistent with numbers derived from other types of approach. In cases where the agreement is still incomplete, the discrepancies may lead to a critical reconsideration of the assumptions inherent in each of the approaches.

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