Sigmoidal Relation between Mitochondrial Respiration and log $([ATP]/[ADP])_{out}$ under Conditions of Extramitochondrial ATP Utilization. Implications for the Control and Thermodynamics of Oxidative Phosphorylation†

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ABSTRACT: Except for close to state 3, mitochondrial respiration has been observed to vary almost linearly with the extramitochondrial phosphorylation potential. For the understanding of the control, thermodynamics, and stoichiometries of oxidative phosphorylation, it is important if this linearity corresponds to an extension of a near-equilibrium flow–force relationship. Using three methods to determine the extramitochondrial ATP/ADP ratio, we observed that at high ATP/ADP ratios the relationship between respiratory rate and log (ATP/ADP) deviated in a sigmoidal fashion from linearity, if the amount of hexokinase present was modulated. In a titration with uncoupler, the sigmoidicity at high ATP/ADP ratios was absent. This difference between the flow–force relationships of these two experiments suggests that the sigmoidicity in the former case reflects a nonproportional flow–force relationship of the adenine nucleotide translocator. In the latter case, one measures the flow–force relationship of the redox-driven proton pumps alone, which turns out to be virtually linear. We determined the flow–force relation of the adenine nucleotide translocator for two ways of varying the force and confirmed the sigmoidicity in both cases. The implication is that the near-linearity of the flow–force relationships at intermediary respiratory rates does not correspond to an Onsager-type (near equilibrium) linearity. We discuss that this phenomenon requires the application of nonclassical forms of nonequilibrium thermodynamics and may be responsible for some of the control over oxidative phosphorylation that is exerted by the cytosolic ATP consuming processes.

During mitochondrial electron transport, oxidative free energy can be transduced into free energy of the synthesis of ATP from ADP and inorganic phosphate [see review by Westerhoff and Van Dam (1987)]. Because of the pivotal role of oxidative phosphorylation in cellular (energy) metabolism, its control, thermodynamics, and mechanisms have been studied intensely. The process has been described in terms of phenomenological nonequilibrium thermodynamics [Rottenberg et al., 1970; see review by Caplan and Essig (1983)]. In this formalism the, so-called, flow–force relations between the flows on the one hand (respiration and phosphorylation rates) and the forces on the other hand (free energies of respiration and phosphorylation) are assumed to be proportional and symmetrical. Proof for the validity of these assumptions is limited to processes less than 2 kJ/mol from equilibrium. However, the experimental flow–force relations were linear (except for the expected saturation effects at high rates) and, at times, even symmetrical (Rottenberg, 1973; Küster et al., 1976, 1981; Holian et al., 1977; Davis & Davis-Van Thienen, 1978; Stucki, 1980; Van der Meer et al., 1980).

The finding of linearity in the absence of a theoretical justification prompted various authors (Rottenberg, 1973; Rothshild et al., 1980; Van der Meer et al., 1980) to inspect what enzyme kinetics imply for flow–force relations. It turned out that one should expect sigmoidal flow–force relationships, with extensive linear regions around their inflection point. In general, these linear regions would not coincide with the "Onsager-domain" of proportional flow–force relationships near equilibrium. This generated the question whether the linear flow–force relations observed in mitochondrial oxidative phosphorylation do or do not coincide with a "near equilibrium" [or rather (Caplan & Essig, 1983) fully coupled static head] proportionality domain. The implications of the answer to this question extend to nonequilibrium thermodynamics, to the P/O and H^+/O stoichiometrics of oxidative phosphorylation, and also to the understanding of the efficiency (Stucki, 1980; Juretič & Westerhoff, 1987) and control of the process (Westerhoff et al., 1987a,b).

Since the near-equilibrium domain would lie around the (until now theoretical) reversal point of mitochondrial oxidative phosphorylation, the question would be answered if at high phosphate potentials (ΔG_p) a deviation from linear flow–force relations were observed. Earlier [Van der Meer et al., 1980; Wanders et al., 1981; cf. Nicholls and Bernson (1977)] we did observe indications for such deviations, but potential fallacies in the determination of ΔG_p kept us from drawing the conclusion. In this paper, we have measured the dependence of respiration and phosphorylation on the extramitochondrial phosphate potential in three independent ways and found them to exhibit sigmoidicity at high phosphate potentials. The adenine nucleotide translocator is the source of the nonlinearity.

There used to be considerable dispute concerning the role of the adenine nucleotide translocator in the control of oxi-

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dative phosphorylation [see review by Tager et al. (1983)]. The extent to which oxidative phosphorylation is controlled by the adenine nucleotide translocator has since been quantitatively assessed (Groen et al., 1982; Wanders et al., 1984a) using principles developed by Kacser and Burns (1973) and Heinrich and Rapoport (1975) [for reviews, see Westerhoff et al. (1984) and Westerhoff and Van Dam (1987)]. The flux control coefficient depends upon the rate at which respiration is poised, being zero in state 4 and increasing as the rate of respiration is increased to 75% of maximal respiration. Furthermore, the (flux) control exerted by the translocator on respiration depends strongly on the system used to regenerate ADP extramitochondrially [Wanders et al., 1984; see also Gellerich et al. (1983)].

The distribution of control in metabolic pathways is dictated by the thermodynamic and kinetic parameters (i.e., the so-called elasticity coefficients) of all enzymes constituting the pathway (Fell & Sauro, 1985; Westerhoff & Kell, 1987). For the adenine nucleotide translocator catalyzing the electrogenic exchange of ATP^{4-} for ADP^{3-} (Klingenberg, 1980), these properties are the dependencies of the enzymatic rates on the intramitochondrial ATP/ADP ratio, the extramitochondrial ATP/ADP ratio (Wanders et al., 1984), and the electrochemical potential difference for protons ($\Delta\tilde{\mu}_H$) (Westerhoff et al., 1987a). In this context, the implications of the sigmoidal flow–force relationship of the adenine nucleotide translocator for the control of oxidative phosphorylation will be discussed.

Materials and Methods

Rat liver mitochondria were isolated from male Wistar rats (200–250 g) by the method of Hoogeboom (1962) as described by Myers and Slater (1957), using 250 mM mannitol, 5 mM Tris-HCl, and 0.5 mM EGTA (final pH 7.4) as the isolation medium. The final pellet was taken up in 250 mM mannitol and kept on ice.

Mitochondria (0.5–0.8 mg of protein/mL) were incubated at 25 °C in a thermostatic oxygraph vessel (1.9 mL) equipped with a Clarke-type electrode, in a medium containing the following standard components: 100 mM KCl, 50 mM Tris-HCl, 1 mM EGTA, 10 mM potassium phosphate, 20 mM succinate, 2 mM $MgCl_2$, 1 μg of rotenone/mL, and 2 mM ATP. Further additions (see below) were made after a 30–60-s equilibration period. After a further 2.5-min incubation period, reactions were usually terminated with phenol/chloroform/isoamyl alcohol exactly as described before [method B of Wanders et al. (1984b)].

ATP and ADP were measured spectrophotometrically or fluorometrically according to standard procedures (Wanders et al., 1984b). Protein was determined as described by Cleland and Slater (1953) using egg albumin as a standard.

Carboxyatractyloside, nucleotides, and enzymes were purchased from Boehringer (Mannheim, FRG). All other reagents were of analytical grade.

Results

Sigmoidal Relationship between the Rate of Respiration and the Extramitochondrial ATP/ADP Ratio under Conditions of Extramitochondrial ATP Utilization. Some of the pitfalls inherent in the use of perchloric acid as quenching reagent in studies on the control of oxidative phosphorylation were described in previous publications [e.g., see Wanders et al. (1984b)]. Even if utmost care was taken to minimize the acid-catalyzed hydrolysis of ATP to ADP and of ADP to AMP, use of perchloric acid led to serious underestimations of true ATP/ADP ratios, especially when the latter were high. For determination of ATP/ADP ratios higher than 100, we therefore adopted a method which avoids extreme acidity, the phenol stop method as introduced by Slater et al. (1973).

We actually compared the phenol stop method and the perchloric acid stop method modified (Wanders et al., 1984b) to minimize ATP hydrolysis, as applied to state 4 mitochondria oxidizing succinate (Wanders, 1985). Under such conditions of high ATP/ADP ratios, intramitochondrial ADP contributes strongly to the amount of ADP found in total extracts. We therefore adopted the extrapolation method of Letko et al. (1980) for an exact determination of the intramitochondrial ADP concentration, so that the total amount of ADP could be corrected for the contribution of intramitochondrial ADP. To this purpose, various concentrations of mitochondria were incubated in the standard reaction medium containing 2 mM ATP. After 10 min at 25 °C, reactions were terminated either with perchloric acid (method A) or with phenol/chloroform/isoamyl alcohol (method B). Upon extrapolation to zero protein, different extramitochondrial ADP concentrations were obtained with the two denaturation procedures [cf. Wanders (1985)]: 2.4 and 1.0 μM for perchloric acid and phenol/chloroform/isoamyl alcohol, respectively. As a consequence, the calculated extramitochondrial ATP/ADP ratios differed considerably (752 versus 1880, respectively).

In Figure 1, the relationship between the rate of oxygen uptake and the extramitochondrial ATP/ADP ratio was studied. Mitochondria were incubated in the standard medium with 20 mM succinate plus 2 mM malate to keep the free energy driving respiration essentially constant. The glucose/hexokinase system was used to induce different steady rates of respiration. Reactions were terminated by using either the phenol stop method (○) or perchloric acid (●). The results depicted in Figure 1A show that, up to values of 100, identical ATP/ADP ratios were found with either denaturation procedure, whereas discrepancies arose at higher values. Most importantly, with both denaturation procedures, the relation between the rate of oxygen uptake and log $(ATP/ADP)_{out}$ was sigmoidal.

Because either of the methods used had the problem that 1% ATP hydrolysis would be sufficient to cause a 100% error in the [ATP]/[ADP] ratio, we used the creatine kinase equilibrium as an independent indicator of the extramitochondrial ATP/ADP ratio. Rat liver mitochondria were incubated in a medium supplemented with excess creatine kinase plus either 2 mM creatine or 2 mM creatine phosphate, thereby approaching equilibrium from either side. Hexokinase was again used to obtain different rates of respiration. The creatine phosphate/creatine ratio was allowed to adjust to the extramitochondrial ATP/ADP ratio. In order to reduce the time needed to reach equilibrium in the creatine kinase reaction, the experiment was carried out at 37 °C. Sigmoidal relations were found between the rate of oxygen uptake and both log (creatine phosphate/creatine) and log $(ATP/ADP)_{out}$ (cf. Figure 1B).

Sigmoidicity Resides in the Adenine Nucleotide Translocator Reaction. Figure 2 shows that the sigmoidicity in the relation J_o versus log $(ATP/ADP)_{out}$ largely disappeared if uncoupler (○) rather than hexokinase (●) was used to stimulate respiration. At identical rates of respiration, ATP/ADP ratios were found to be higher if uncoupler was used to stimulate respiration as compared to the glucose/hexokinase system. An important distinction between the two conditions of Figure 2 is the extent to which the adenine nucleotide translocator is involved in the overall process; flux through the translocator is much higher in the glucose/hexokinase system than with uncoupler. Hence, an obvious explanation for the

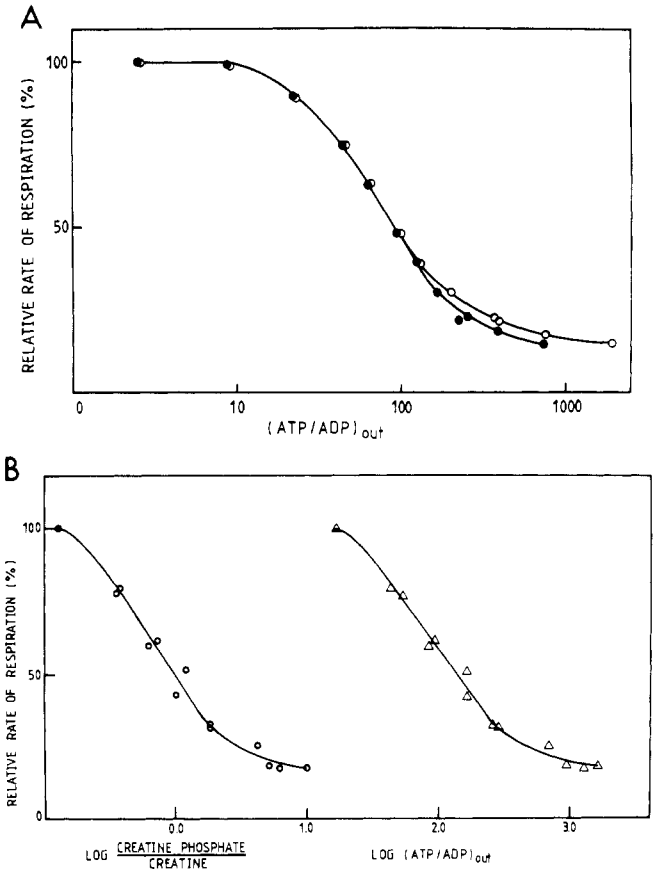

FIGURE 1: Relationship between the rate of oxygen uptake and the extramitochondrial ATP/ADP ratio under conditions of extramitochondrial ATP utilization. (A) Direct measurement of [ATP]/[ADP] by two methods. Rat liver mitochondria (0.93 mg of protein/mL) were incubated in the standard reaction medium, and respiration was adjusted to various rates by adding different amounts of hexokinase. Reactions were terminated via the phenol stop method (○) or with perchloric acid (●) (see Materials and Methods). Total ATP/ADP ratios were recalculated to true extramitochondrial ATP/ADP ratios by correcting for intramitochondrial ATP and ADP, as determined via silicone oil centrifugation. (B) Relationship between J_o and the logarithm of creatine phosphate/creatine as indicator. Rat liver mitochondria (0.5 mg of protein/mL) were incubated at 37 °C in a medium of the following composition: 200 mM mannitol, 50 mM Tris-HCl, 4 mM ATP, 10 mM succinate, 1 mM malate, 5 mM $MgCl_2$, 10 mM potassium phosphate, 1 mM EGTA, 20 mM glucose, 50 units/mL creatine kinase, 1 μg of rotenone/mL, and either 2 mM creatine or 2 mM creatine phosphate, final pH 7.00. Different amounts of hexokinase were used to obtain different rates of respiration. After 10 min, reactions were terminated with phenol/chloroform/isoamyl alcohol, and ATP, ADP (△) and creatine, creatine phosphate (○) were determined enzymically as described before (Wanders et al., 1984). From a single set of incubations, respiration is shown versus the (○) creatine phosphate/creatine ratio and (△) the ATP/ADP ratio.

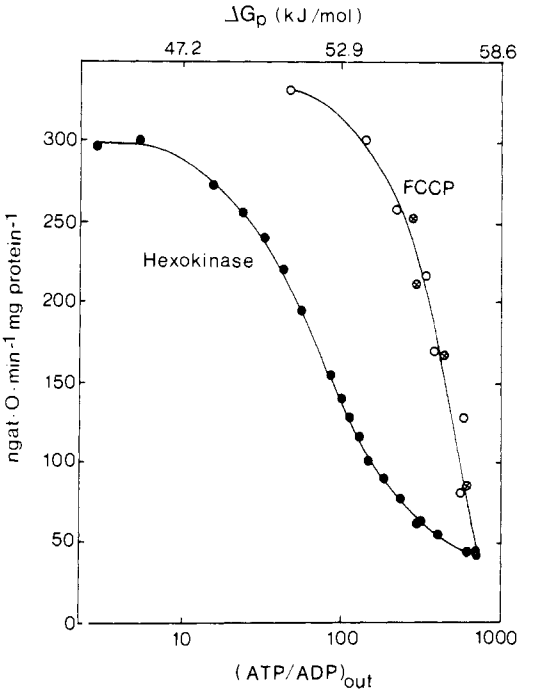

FIGURE 2: Relationship between the rate of mitochondrial respiration and $(ATP/ADP)_{out}$ under conditions of glucose/hexokinase- and uncoupler-induced rates of respiration. Rat liver mitochondria (0.59 mg of protein/mL) were incubated in the standard reaction medium except that [succinate] was 10 mM, [$MgCl_2$] was 10 mM, and [rotenone] was 2 μg/mL, at pH 7.4. After a 30–60-s equilibration, various steady-state rates of respiration were established through addition of limiting amounts of hexokinase (●) or FCCP (○, ⊗). Reactions were terminated by using the phenol stop method. Total ATP/ADP ratios were recalculated to extramitochondial ATP/ADP ratios by correcting for intramitochondrial ATP and ADP. In some experiments (⊗), a limiting amount of ADP (0.10 mM) was added to the mitochondria respiring in the presence of different amounts of FCCP, and reactions were terminated after return of the mitochondria to the original respiratory rate.

downward shift in the $(ATP/ADP)_{out}$ ratio for the hexokinase titration as compared to the FCCP titration observed in Figure 2 would be an increased free energy difference of the translocator under conditions of glucose/hexokinase-induced respiration.

In the FCCP experiment of Figure 2, the uncoupler was added to the mitochondria while the latter were respiring in state 4. Therefore, an alternative explanation for the high ATP/ADP ratios found with uncoupler would be that, due to a kinetic block at the level of the translocator [ATP^{4-} inhibiting competitively with respect to ADP^{3-} (Souverijn et al., 1973)], state 4 extramitochondrial ATP/ADP ratios did not drop after addition of uncoupler. This possibility was tested by adding a small amount of ADP to mitochondria already stimulated with the uncoupler. Addition of ADP led to a short increase in respiration, followed by a return to the same rate as observed before addition of ADP. Figure 2 shows that approximately identical ATP/ADP ratios were found irrespective whether the extra ADP was added (⊗) or not added (○), eliminating the possibility of artifactual, high ATP/ADP ratios due to a kinetic block of ATP hydrolysis.

The data of Figure 2 can be used to obtain information on the relationship between flux through the translocator and the free energy difference of adenine nucleotide translocation using the strategy described before (Wanders et al., 1981). The free energy difference of adenine nucleotide translocation (ΔG_T) is given by

$$\Delta G_T = \alpha F \Delta\psi + 2.3RT \log ([ATP^{4-}_{out}][ADP^{3-}_{in}]/[ATP^{4-}_{in}][ADP^{3-}_{out}]) \quad (1)$$

where α, the extent of electrogenicity, may be rounded off to 1 (Klingenberg, 1980). In principle, to calculate ΔG_T directly, $(ATP^{4-}/ADP^{3-})_{out}$, $(ATP^{4-}/ADP^{3-})_{in}$, and the transmembrane electric potential difference, $\Delta\psi$, have to be determined. However, in order to circumvent the disputed [see Ferguson and Sorgato (1982) for discussion] measurement of both $\Delta\psi$ and the free intramitochondrial ATP^{4-}/ADP^{3-} concentration ratios, the respiratory rate itself may be used as a probe. Two conditions are compared where the rate of O_2 uptake is the same. In condition 1, respiration is stimulated by addition of limiting amounts of hexokinase whereas in condition 2 small amounts of uncoupler were added to obtain the same rate of respiration.

In condition 1, eq 1 becomes

$$(\Delta G_T)_1 = F(\Delta\psi)_1 + 2.3RT \log ([ATP^{4-}_{out}][ADP^{3-}_{in}]/[ATP^{4-}_{in}][ADP^{3-}_{out}])_1 \quad (2)$$

In condition 2, eq 1 becomes

$$(\Delta G_T)_2 = F(\Delta\psi)_2 + 2.3RT \log ([ATP^{4-}_{out}][ADP^{3-}_{in}]/[ATP^{4-}_{in}][ADP^{3-}_{out}])_2 \quad (3)$$

According to the chemiosmotic coupling model, the variation of mitochondrial respiration with $\Delta\tilde{\mu}_H$ should be independent of whether hexokinase or uncoupler is used to cause the variation. Consequently, $(\Delta\psi)_1$ and $(\Delta\psi)_2$ should be equal. Various authors have attempted to check this deduction, with various results. The interpretation of many authors (the complications surrounding the chemiosmotic coupling model in this respect will be discussed more extensively under Discussion) is either that the methods used to measure the membrane potential may be of variable accuracy or that there is some other energetic intermediate that is more central to oxidative phosphorylation. There is no disagreement, however, that respiration would be uniquely related to that intermediate. Luckily, the uniqueness of the relationship, and not the identity of the intermediate, is what is important here. Thus, although $(\Delta\psi)_1$ and $(\Delta\psi)_2$ may not really correspond to the electric potential differences between the aqueous bulk phases on the two sides of the membrane, they can be assumed to be equal to one another.

The $\tilde{H}^+$-ATPase exerts little flux control on oxidative phosphorylation, probably because it is close to "constrained" equilibrium (Westerhoff & Van Dam, 1987). With equal membrane potential and equal respiratory rates under the two conditions, this would imply equal intramitochondrial ATP/ADP ratios. Indeed, Küster et al. (1981) have shown that the intramitochondrial ATP/ADP ratios were the same at one rate of respiration irrespective of whether respiration was stimulated with uncoupler (FCCP) or glucose plus hexokinase. Consequently, we shall assume that $([ADT^{4-}_{in}]/ADP^{3-}_{in}])_1$ equals $([ATP^{4-}_{in}]/[ADP^{3-}_{in}])_2$ at one respiration rate.

Substitution of $(\Delta\psi)_1 = (\Delta\psi)_2$ and $([ATP^{4-}_{in}]/[ADP^{3-}_{in}])_1 = ([ATP^{4-}_{in}/[ATP^{3-}_{in}])_2$ in eq 2 and 3 followed by subtraction yields the equation:

$$\Delta(\Delta G_T) \overset{\text{def}}{=} (\Delta G_T)_1 - (\Delta G_T)_2 = 2.3RT \log \{([ATP^{4-}_{out}]/[ADP^{3-}_{out}])_1/([ATP^{4-}_{out}]/[ADP^{3-}_{out}])_2\} \quad (4)$$

For every respiratory rate in Figure 2, we looked up $\log([ATP_{out}]/[ADP_{out}])_1$ (1 referring to the glucose hexokinase system) and the $\log([ATP_{out}/[ADP]_{out})_2$ (2 referring to the titration with uncoupler) and plotted the rate of glucose 6-phosphate production as a function of the difference of these two values, $\Delta(\Delta G_T)$ [(●) in Figure 3].

To the extent that the extramitochondrial ATP consumption in the absence of hexokinase can be neglected, the interpretation of Figure 3 is rather simple. For all uncoupler points in Figure 2, the adenine nucleotide translocator would be in equilibrium. Hence, $(\Delta G_T)_2$ *would* equal zero, and $\Delta(\Delta G_T)$ would equal the actual free energy difference across the translocator in the hexokinase titration $(\Delta G_T)_1$. Thus, (●) in Figure 3 should be an approximation of a flow–force relationship of the adenine nucleotide translocator.

Both the free energy difference across the adenine nucleotide translocator and the translocation rate are a function of five variables: $[ATP]_{in}$, $[ADP]_{in}$, $[ATP]_{out}$, $[ADP]_{out}$, and $\Delta\psi$. Under the present conditions of constant sum concentrations of $[ADP]_{in} + [ATP]_{in}$ and $[ADP]_{out} + [ATP]_{out}$, three variables remain: $([ATP]/[ADP])_{in}$, $([ATP]/[ADP])_{out}$, and $\Delta\psi$.

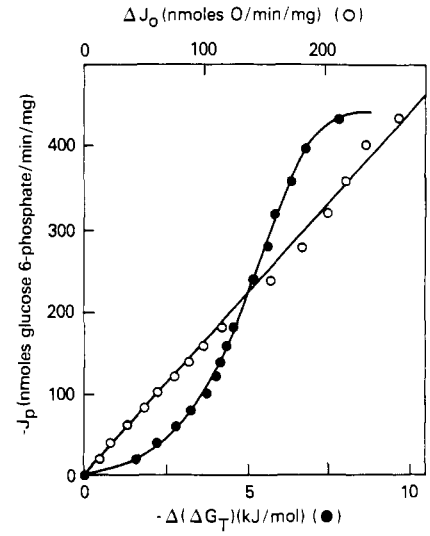

FIGURE 3: Relationship between the rate of glucose 6-phosphate production and the free energy difference across the adenine nucleotide translocator reaction (●). The extramitochondrial ATP/ADP ratios of Figure 2, as found under conditions of identical glucose/hexokinase- and uncoupler-induced rates of respiration, were used to calculate the difference in ΔG_T across the translocator as described in the text. The rates of glucose 6-phosphate formation were calculated from the difference in the concentration of glucose 6-phosphate in samples taken from the mitochondrial incubation at time intervals of 150 s. Also shown (○) is how the respiratory rate varied with the phosphorylation rate; ΔJ_o is the difference between the actual respiratory rate and the rate in the absence of both hexokinase and uncoupler.

Consequently, there are different ways in which ΔG_T can be changed (e.g., by increasing $([ATP]/[ADP])_{in}$ at constancy of $([ATP]/[ADP])_{out}$ and $\Delta\psi$ or by increasing $\Delta\psi$ at constant ratios). In principle, an identical change in ΔG_T, but brought about by a different change in $(ATP/ADP)_{in}$, $(ATP/ADP)_{out}$, or $\Delta\psi$, could effect the translocation rate differently [see Westerhoff and Van Dam (1987)]: away from equilibrium, the flow–force relationship may depend on the way in which the force is varied. In Figure 3, ΔG_T varied with *concomitant* variation of $(ATP/ADP)_{in}$, $(ATP/ADP)_{out}$, and $\Delta\psi$.

The most direct way to obtain a flow–force relationship of an enzyme is to vary the concentrations of its substrates and products and measure the effect on its reaction rate. However, in cases where it is difficult to dictate the concentrations of substrates or products from the outside, there are other possibilities. In fact, any perturbation induced should provide information about the flow–force relationship. A method we employ here is to perturb the system by actually eliminating a fraction of the enzyme under study. This will cause a change in the concentrations of its substrates and products, as well as a change in the reaction rate. Knowledge of what fraction of the enzyme molecules has been eliminated allows us to calculate the flux per active enzyme at the altered concentrations of its substrates and products. In fact, our approach here is a little bit more complicated, because we wish to determine the flow–force relationship if ΔG_T is varied at constant $\Delta\psi$ and $(ATP/ADP)_{in}$. To keep the latter two forces constant, we also add hexokinase such that we return to the same respiratory rate [and hence the same $\Delta\psi$ and $(ATP/ADP)_{in}$] as before, eliminating the fraction of adenine nucleotide translocators.

More specifically, addition of a limiting amount of carboxyatractyloside (a tight-binding inhibitor of the adenine nucleotide translocator; Vignais et al., 1973) to mitochondria respiring at an intermediary rate (j) may lead to a partial inhibition of respiration. By addition of an appropriate amount of extra hexokinase, the original respiratory rate (j) can be

readjusted, ensuring that $\Delta\psi$ and $(ATP/ADP)_{in}$ will be identical with their original values. $(ATP/ADP)_{out}$ will be lowered by this procedure; there must be an increase in the free energy difference across the adenine nucleotide translocator, since an identical rate is now maintained by fewer translocator molecules. This increase in the free energy difference across the translocator, $\Delta'(\Delta G_T)$, induced by addition of carboxyatractyloside can be calculated directly from the $(ATP/ADP)_{out}$ ratios found in the absence (one asterisk) and presence (two asterisks) of carboxyatractyloside, respectively (cf. the derivation of eq 4):

$$\Delta'(\Delta G_T) \overset{\text{def}}{=} \Delta G_T(I = i, \text{hexokinase} = h, J_o = j) - \Delta G_T(I = 0, \text{hexokinase} = 0, J_o = j) = 2.3RT \log \{([ATP^{4-}_{out}]/[ADP^{3-}_{out}])^{**}/([ATP^{4-}_{out}]/[ADP^{3-}_{out}])^{*}\} \quad (5)$$

Assuming a constant P/O (J_p/J_o) ratio [where J_p now includes the flux through the adenine nucleotide translocator in state 4, cf. (O) in Figure 3], the total flux of adenine nucleotide translocation is the same under both conditions. However, since under the two-asterisk condition a number of translocators have been inactivated by carboxyatractyloside, the flux per active translocator will be higher than under the one-asterisk condition. The dependence of the flux per active translocator on ΔG_T is what we are interested in. We therefore divide the respiratory rates observed in the presence of carboxyatractyloside by the fraction of adenine nucleotide translocator calculated (from the amount of inhibitor added relative to the amount of inhibitor needed to completely knock out all the translocators; carboxyatractyloside is a tight-binding inhibitor) to be active and thus find the flux that would have been obtained at the same magnitudes of membrane potential and ATP/ADP ratios, in the absence of the inhibitor. The plot of this flux versus the $\Delta'(\Delta G_T)$ of eq 5 should reflect the flow–force relationship of the adenine nucleotide translocator.

In line with this strategy, mitochondria were incubated under state 4 conditions, and carboxyatractyloside was added in increasing amounts. Removal of up to 70% of translocator activity did not lead to a change in respiration so that readjustment with hexokinase was not necessary. For each carboxyatractyloside point, we calculated both the fractional rate through the translocator and also the increase in free energy difference across the translocator. The fractional translocation rate was plotted versus the increase in free energy difference across the translocator, giving a flow–force relationship of the adenine nucleotide translocator at the state 4 magnitudes of $(ATP/ADP)_{in}$ and $\Delta\psi$ for varying $(ATP/ADP)_{out}$. The results (Figure 4) indicate that also under these conditions a sigmoidal flow–force relationship is obtained.

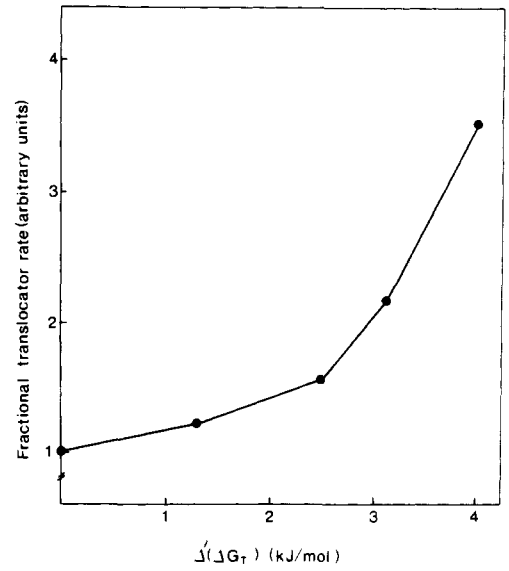

FIGURE 4: Relationship between the fractional rate of the translocator and the increase in free energy difference across the translocator at constant $(ATP/ADP)_{in}$ and $\Delta\psi$. Rat liver mitochondria (0.7 mg of protein/mL) were incubated in the standard reaction medium with succinate (plus malate) as respiratory substrate, either in the absence or in the presence of different amounts of carboxyatractyloside. The fractional rate of the translocator under each condition was calculated from the amount of carboxyatractyloside (i) added as $J_p I_{max}/(I_{max} - i)$. This uses that the binding of carboxyatractyloside is so tight as to always have more than 90% of added inhibitor bound to adenine nucleotide translocators (Vignais et al., 1973). I_{max} is defined as the amount of inhibitor needed to completely inactivate all the adenine nucleotide translocators, if its binding were infinitely tight. It is equal to the concentration of adenine nucleotide translocators and was determined by extrapolation of the linear portion of the titration of respiration to the point where it intersects respiration in the absence of adenine nucleotides [cf. Groen et al. (1982)]. Thus, $I_{max} = 0.32$ nmol/mg of protein. Reactions were terminated via the phenol stop method. The total ATP/ADP ratios obtained were recalculated to true $(ATP/ADP)_{out}$ ratios by correcting for intramitochondrial ATP and ADP. The increase in free energy difference across the translocator, $\Delta'(\Delta G_T)$, was calculated from the $(ATP/ADP)_{out}$ ratios as described in the text.

DISCUSSION

Sigmoidal Flow–Force Relation for the Adenine Nucleotide Translocator. In this paper, we reinvestigated the dependence of respiration by rat liver mitochondria on the extramitochondrial ATP/ADP ratio, paying special attention to the determination of the ratio at high magnitudes. Because of the possibility of generation of ADP from ATP under acid conditions, we (i) accelerated the usual perchloric acid extraction method, (ii) employed the phenol extraction method, and (iii) used the creatine/creatine phosphate ratio as an indicator in the presence of creatine kinase. With all three methods, we found the relationship between respiratory rate and the logarithm of [ATP]/[ADP] to be sigmoidal, such that at the highest ratios respiration varied less than at intermediary ratios. This is in contrast to some [Rottenberg, 1973; Küster et al., 1976; Holian et al., 1977; Davis & Davis-Van Thienen, 1978; Stucki, 1980; cf. Lemasters and Billica (1984); Berry et al., 1987] but not all (Nicholls & Bernson, 1977; Van der Meer et al., 1980; Wanders et al., 1981) measurements of this relationship. We attribute the discrepancy to the probability (Wanders et al., 1984) that under acid extraction conditions part (e.g., 1%) of the extracted ATP is hydrolyzed to ADP. At ATP/ADP ratios exceeding 100, this would cause the determined ATP/ADP ratio to be much lower than the actual one. As a consequence, the sigmoidicity of the flow–force relation would disappear.

This interpretation is supported by the observations that (i) the sigmoidal relationship between J_o and log $(ATP/ADP)_{out}$ as observed in Figures 1 and 2 was lost if in the experiment of Figure 1 samples were left in acid for 20 min before neutralization and no corrections for intramitochondrial ADP and ATP were made (not shown), (ii) when we used both extraction methods in a single incubation, the acid extraction method revealed less of the sigmoidicity than the phenol extraction method (Figure 1A), (iii) the creatine kinase method, which lacked the possibility of a similar artifact because the creatine phosphate/creatine ratio was much closer to 1, revealed the same sigmoidicity as did the phenol extraction method (Figure 1B), and (iv) (suggesting that peculiarities of the phenol extraction and creatine kinase method do not generate the "tail" of the sigmoidicity where there is none) we observed a nonsigmoidal flow–force relationship with the

phenol method when respiration was titrated with uncoupler rather than hexokinase.

In the experiments discussed in the preceding paragraphs, respiration was varied by titration with hexokinase in the presence of adenine nucleotides and excess glucose. Therefore, our conclusion that the variation of respiration with log ([ATP]/ADP) or ΔG_p is sigmoidal is limited to our condition of varying workload. In fact (Van der Meer et al., 1980), the flow–force relation should be expected to depend on other factors, such as the sum concentration of adenine nucleotides, the degree of coupling of the mitochondria [cf. Westerhoff and Van Dam (1987)], and the concentrations of phosphate and Mg^{2+}.

The dependence of mitochondrial respiration on the extramitochondrial phosphate potential is indirect (see, however, below): it involves the dependence of respiration on the electrochemical potential difference for protons ($\Delta\tilde{\mu}_H$), the stoichiometry at which protons are pumped, the dependence of the rate of intramitochondrial ATP synthesis on $\Delta\tilde{\mu}_H$ and ΔG_p, the dependence of the rate of adenine nucleotide translocation on its free energy difference, ΔG_T, and also the dependence of the proton leak on $\Delta\tilde{\mu}_H$. If all elemental flow–force relations were linear and slip (Pietrobon et al., 1981) were negligible, then respiration would depend linearly on ΔG_p^{out} (Westerhoff & Van Dam, 1987). Since it does not, one may wonder if the sigmoidicity of the relationship between respiration and phosphate potential at varying workload resulted from nonohmicity of the proton leak (Nicholls, 1974) or from the slip in the mitochondrial proton pumps [Pietrobon et al., 1982; Zoratti et al., 1986; cf. Murphey and Brand (1987)], rather than from an inherently nonlinear flow–force relationship. However, in the hexokinase titration of Figure 2, the phosphorylation rate increased strictly linearly with the respiration rate (cf. the open circles in Figure 3) which [cf. Westerhoff and Van Dam (1987)] suggests (though it does not prove) that the proton leak is rather ohmic and that the pump stoichiometries are fairly constant over the range of this experiment.

In state 3, the flux through the mitochondrial ATP synthesizing system (i.e., $\tilde{H}^+$-ATPase plus adenine nucleotide translocator) is high. In state 4, i.e., in the absence of added hexokinase, the flux is comparatively small, being commensurate to the rate at which extramitochondrial ATP is hydrolyzed by contaminating ATPases and damaged mitochondria (Duszyński & Wojtczak, 1985). Addition of uncoupler instead of hexokinase should leave the flux through the $\tilde{H}^+$-ATPase and nucleotide translocator small. Consequently, the relationship between respiration and extramitochondrial phosphate potential at varying concentrations of uncoupler, but in the absence of added hexokinase, should reflect the dependence of respiration on $\Delta\tilde{\mu}_H$ rather than the flow–force relationship of the adenine nucleotide translocator plus $\tilde{H}^+$-ATPase.

The experiment [(○) in Figure 2] revealed a nonsigmoidal relationship between respiration and ΔG_p^{out}, suggesting that the sigmoidicity under conditions of varying workload [(●) in Figure 2] was due to nonlinearity in the flow–force relation of the adenine nucleotide translocator plus $\tilde{H}^+$-ATPase. The inference that the dependence of respiration on $\Delta\tilde{\mu}_H$ is linear over the range studied is in line with all experimental observations known to us [e.g., see Padan and Rottenberg (1973), Wilson and Forman (1982), Azzone et al. (1978), Van Dam et al. (1980), and Küster et al. (1981)].

Earlier [Wanders et al., 1981; see also Küster et al. (1981)] it was shown that the sigmoidicity in the relationship between respiration and the logarithm of the extramitochondrial ATP/ADP ratio under conditions of varying amounts of hexokinase was accompanied by a linear variation of respiration with the logarithm of the *intra*mitochondrial ATP/ADP ratio. This suggests that the origin of the sigmoidicity may lie in the adenine nucleotide translocator. Indeed [cf. Westerhoff and Van Dam 1987)], the $\tilde{H}^+$-ATPase is likely to be closer to equilibrium than the adenine nucleotide translocator, and its flow–force relationship could well be proportional.

Using empirical rate equations and kinetic constants, Bohnensack (1981) predicted the flow–force relationships of the adenine nucleotide translocator to be sigmoidal. As discussed by Krämer and Klingenberg (1982), the sigmoidal flow–force relation is most probably caused by the fact that the apparent V_{max} at which intramitochondrial ATP exchanges for extramitochondrial ADP greatly exceeds the V_{max} of the reverse exchange combination in which extramitochondrial ATP is exchanged for intramitochondrial ADP.

Having concluded that the nonlinearity is in the flow–force relationship of the adenine nucleotide translocator, we tried to derive its flow–force relationship from our experimental observations and confirm this conclusion. If the free energy difference across the adenine nucleotide translocator in the titration with uncoupler were negligible, (●) in Figure 3 would correspond to the flow–force relationship of the adenine nucleotide translocator under conditions of varying workload (causing $\Delta\psi$, ΔG_{in}^p, and ΔG_p^{out} to vary) (cf. eq 4). However, in state 4, there *is* extramitochondrial ATP consumption: in the experiment of Figure 2 for instance, saturating amounts of carboxyatractyloside led to a 25% reduction of oxygen consumption under state 4 conditions. Consequently, in the titration with uncoupler, there is flux through the translocator, so that $(\Delta G_T)_2$ in eq 4 does not equal zero. If $(\Delta G_T)_2$ varied between the different points in Figure 3, then Figure 3 would not represent the relationship between flux through the translocator and the force $(\Delta G_T)_1$ across the translocator.

However, $(\Delta G_T)_2$ probably did remain approximately constant in the greater part of Figure 3. In the part of the uncoupler experiment that is relevant to Figure 3, $(ATP/ADP)_{out}$ varied between 150 and 650. This implies that variation in the ATP concentration of 2 mM is by less than 0.5%. The ADP concentration varied between 13 and 3 μM. These ADP concentrations are below the K_I's for ADP of most uncoupled ATPases (Tonomura, 1986) as well as of hexokinase [cf. Wanders et al. (1981)]. As a consequence, the rate of extramitochondrial ATP consumption and hence the rate of adenine nucleotide translocation will have varied little in the uncoupler titration of Figure 2. (Here it is also assumed that the uncoupler does not greatly stimulate extramitochondrial ATP hydrolysis through an effect on contaminating submitochondrial particles.) In experiments with rat liver mitochondria incubated under state 4 conditions, it was found that up to 75% of translocator activity can be taken away by addition of carboxyatractyloside without any effect on respiration. Under these conditions, however, the extramitochondrial ATP/ADP ratio drops to less than 100. Assuming a constant J_p/J_o ratio [cf. (○) in Figure 3], this confirms that the rate of extramitochondrial ATP utilization in the absence of added hexokinase is independent of the extramitochondrial ATP/ADP ratio over the whole range of ATP/ADP ratios measured in Figure 2. Because in Figure 3 the amount of active adenine nucleotide translocator remained constant, the implication is that $(\Delta G_T)_2$ remained constant too. We conclude that Figure 3 indeed represents an approximative flow–force relationship

of the adenine nucleotide translocator except that the force across the translocator in the absence of hexokinase has been subtracted. This confirms that the relationship between translocator flux and the free energy difference of adenine nucleotide translocation can be sigmoidal.

Because only in the near-equilibrium domain the flow–force relation of a process has to be independent of the manner in which the force is varied (Westerhoff & Van Dam, 1987), the possibility remained that the flow–force relation of the adenine nucleotide translocator is sigmoidal only if its force is varied as in the hexokinase titration of Figure 2. To investigate this, we devised a method to measure the flow–force relationship of the adenine nucleotide translocator at constant magnitudes of $\Delta\psi$ and ΔG_p^{in}. Also this flow–force relationship (Figure 4) turned out to be sigmoidal. It is shifted with respect to the true flow–force relationship by the ΔG_T across the translocator at state 4 in the absence of carboxyatractyloside.

In the derivation of eq 4 and 5, it was assumed that under the conditions of our experiments respiration be uniquely related to $\Delta\psi$. This corollary of the chemiosmotic coupling hypothesis [see, however, Rigoulet et al. (1987)] has been confirmed by some (Nicholls & Bernson, 1977; Van Dam et al., 1980), but not all (Padan & Rottenberg, 1973; Azzone et al., 1978; Küster et al., 1981; Wilson & Forman, 1982), laboratories. The discrepancies found in the latter laboratories were significant only close to state 3. The sigmoidicity we are studying here occurs at respiratory rates lower than half the state 3 rates.

To account for other conflicts between experimental results and chemiosmotic predictions as well, a number of authors [see reviews by Westerhoff et al. (1984b), Rottenberg (1985), and Slater (1987)] have suggested that the energy coupling protons may not delocalize into the bulk aqueous phase adjacent to the membrane but may remain in, or close to, the membrane: $\Delta\tilde{\mu}_H$ as determined experimentally would not monitor the true "high-energy intermediate", i.e., the local proton gradient. Assuming that also the adenine nucleotide translocator senses the local rather than the delocalized potentials, the $\Delta\psi$ terms in eq 2 and 3 would have to be interpreted as the local $\Delta\psi$'s. Also in this interpretation of energy coupling in oxidative phosphorylation, the relationship between the local (but not the delocalized) proton gradient and the rate of respiration would be independent of whether the extramitochondrial ATP/ADP ratio or the proton permeability of the membrane would be varied [see, however, Westerhoff et al. (1988)]: $(\Delta\psi)_1$ and $(\Delta\psi)_2$ in eq 2 and 3 would still be identical, leaving eq 4 and 5 and hence the conclusions of this paper valid.

We therefore think that our experiments do reveal flow–force relations of the adenine nucleotide translocator. The debate about how protons are actually involved in the coupling of respiration to phosphorylation is important and cannot be solved here. Until that moment, we shall have to be satisified with a flow–force relation of the translocator in the absence of certainty of the exact identity of the electric component of the total force. The conclusions we draw in this paper do not depend on this identity.

Implications for the Thermodynamics of Oxidative Phosphorylation. The sigmoidicity of the relationship between respiration, as well as phosphorylation (cf. Figures 2 and 3), and ΔG_p^{out} indicates that whenever respiration is out of state 4, neither oxidation nor phosphorylation can be close to equilibrium. Indeed, from Figure 3 we can conclude that the adenine nucleotide translocator alone must then be further than RT (=2.7 kJ/mol) displaced from equilibrium: Linear back-extrapolation of Figure 3 to a $-J_p$ of −16 nmol min^{-1} mg^{-1} [i.e., assuming a $-J_p/J_o$ from (○) in Figure 3 and 25% of state 4 respiration as if being caused by extramitochondrial ATP hydrolysis] suggests that $(\Delta G_T)_2$ exceeds 1.2 kJ/mol, so that, when respiration amounts to 30% of the state 3 rate, the free energy difference of adenine nucleotide translocation would be approximately equal to 6 kJ/mol. This estimate is comparable to the earlier estimate of 9 kJ/mol (Wanders et al., 1981) and confirms that, throughout oxidative phosphorylation, adenine nucleotide translocation and hence phosphorylation of extramitochondrial ADP are not close to equilibrium. LaNoue and colleagues (LaNoue et al., 1986) found that the ratio of unidirectional forward to unidirectional reverse rate of ATP synthesis is not close to the equilibrium value of 1. Our results (Wanders et al., 1981; present paper) extend this conclusion to state 4 (in the presence of Mg^{2+}) and pinpoint the adenine nucleotide translocator as the (or at least a) nonequilibrium step.

Observing that under their conditions the flux control coefficient of the adenine nucleotide translocator with respect to respiration was small, Forman and Wilson (1983) concluded that the translocator has to be close to equilibrium. However [cf. Kacser and Burns (1973) and Westerhoff et al. (1984a)], this is not a closed argument: the flux control coefficient of an enzyme does not depend uniquely on its displacement from equilibrium. An enzyme with a high elasticity coefficient will have a low flux control coefficient even if that enzyme is far from equilibrium. Below we shall argue that this is indeed the case for the adenine nucleotide translocator.

The observations then that the phosphorylation reaction of extramitochondrial ADP is not close to equilibrium invalidate equilibrium thermodynamic approaches to oxidative phosphorylation. How about nonequilibrium thermodynamic approaches? Caplan, Essig, and Rottenberg were the first to apply nonequilibrium thermodynamics to the process of mitochondrial oxidative phosphorylation. As a first approximation, they used the near-equilibrium type of nonequilibrium thermodynamics, which assumes proportional and Onsager-symmetrical relationships between reaction rates ("flows") and free energy differences ("forces") [reviewed in Caplan and Essig (1983)]. Thus, the rate of oxidation (J_o) and the rate of phosphorylation ($-J_p$) would depend on ΔG_o (the redox potential difference across the respiratory chain) and ΔG_p (the phosphate potential) as follows:

$$J_o = L_{oo}\Delta G_o + L_{op}\Delta G_p \quad (6)$$

$$J_p = L_{op}\Delta G_o + L_{pp}\Delta G_p \quad (7)$$

Several other authors have also applied the near-equilibrium type of nonequilibrium thermodynamics to interpret experimental data obtained in oxidative phosphorylation, which led to interesting conclusions such as those concerning the efficiency of oxidative phosphorylation [e.g., see Stucki (1980), Lemasters et al. (1984), and Jensen et al. (1986); for review, see Westerhoff and Van Dam (1987) and Westerhoff et al. (1988b)].

However, the assumed proportionality and Onsager symmetry of the relations between flows and forces have only been proven to hold near-equilibrium (i.e., if all $\Delta G \ll RT$; Caplan & Essig, 1983; Westerhoff & Van Dam, 1987).. Since many processes occur far from equilibrium, the approach has been criticized [see, e.g., Wilson (1982)]. Yet, experimental determinations of the dependence of mitochondrial respiration and/or phosphorylation on the phosphate potential (ΔG_p) showed linear (but not necessarily proportional) relationships between the flows and forces. (At the highest respiratory rates,

deviations were observed. However, this can be regarded as an expeded limitation of the linear approach due to the saturation property of enzymes.) Calculations of the dependence of reaction rates on the free energy difference across a reaction, using simple enzyme kinetics (Rottenberg, 1973; Rothschild et al., 1980; Van der Meer et al., 1980; Wilson, 1980; Bohnensack, 1981; Pietrobon & Caplan, 1985; Westerhoff & Van Dam, 1987), suggested a solution to the paradox of linear flow–force relations away from equilibrium: these dependencies are usually sigmoidal, so that around their inflection point, a rather large region of the velocity range (v/V) can be well approximated by a straight line. Importantly, however, this linear approximation often differed from the near-equilibrium linear approximation, giving rise to linear but not proportional domains away from equilibrium.

The finding that the relationship between J_o and log $(ATP/ADP)_{out}$ and hence $(\Delta G_p)_{out}$ (the concentration of phosphate was essentially constant under the conditions used) is sigmoidal indicates that this explanation of the observed quasi-linear flow–force relationships may be correct. As a consequence, the near-equilibrium thermodynamics of eq 6 and 7 cannot always be used to describe oxidative phosphorylation under conditions of active extramitochondrial ATP utilization. To describe the dependence of respiration on ΔG_p^{out} over its entire range, a curvilinear rather than a proportional relation would have to be used. However, for a large number of applications, it may be sufficiently accurate to describe the part of the relationship that centers around its inflection point by a straight line [Westerhoff & Van Dam, 1987; cf. Rottenberg (1973)]:

$$J_o = L_{oo}\Delta G_o + L'_{op}(\Delta G_p - \Delta G_p^{\#}) \quad (8)$$

$\Delta G_p^{\#}$ is independent of ΔG_p. The essential difference between eq 6 and 8 is that in the quasi-linear region of the flow–force relation, $\Delta G_p^{\#}$ is *not* assumed to equal zero and that L'_{op} may differ from the slope of the line near equilibrium (the tail part near state 4 in Figures 1–4) but be equal to the higher slope in the inflection point of the flow–force relationship.

Implications for the Control of Oxidative Phosphorylation. The slope in Figure 4 is equal to the partial derivative of the rate of adenine nucleotide translocation with respect to the logarithm of the extramitochondrial [ATP]/[ADP] ratio (or ΔG_p^{ex}) at constant $([ATP]/[ADP])_{in}$ and constant $\Delta\psi$. Thus, these slopes are equal to the so-called (Westerhoff et al., 1987a) absolute elasticity coefficient of the adenine nucleotide translocator with respect to the extramitochondrial [ATP]/[ADP] ratio:

$$[a\epsilon]^T_{([ATP]/[ADP])_{out}} = \left(\frac{\partial|J_T|}{\partial \ln ([ATP]/[ADP])_{out}}\right)_{\Delta\psi,(ATP/ADP)_{in}} \quad (9)$$

Here, the T refers to the adenine nucleotide translocator, and ln is the natural logarithm. The following theorem holds (Wanders et al., 1984; Westerhoff et al., 1987ab):

$$C^J_T/C^J_{load} = [a\epsilon]_{\Delta G_p{}^{out}{}^{load}}/-[a\epsilon]_{\Delta G_p{}^{out}{}^{T}} \quad (10)$$

for the case where an ATP consuming reaction (the load) is present in addition to mitochondria catalyzing oxidative phosphorylation. C^J_T and C^J_{load} represent the coefficients of flux control by the adenine nucleotide translocator and the load, respectively, with respect to any flux (J) in the system. This equation illustrates that it is the (absolute) elasticity coefficients of the enzymes in a system that determine the distribution of the flux control among the enzymes. As we have noted elsewhere (Westerhoff et al., 1987b), the fact that flow–force relationships tend to be sigmoidal rather than proportional bestows them with the property that the absolute elasticity coefficient around their inflection point can become quite high [cf. eq 4.50 of Westerhoff and Van Dam (1987)]. As illustrated by eq 10, such high magnitudes of the absolute elasticity coefficient of an enzyme tend to reduce the control the enzyme exerts on the flux. For the case of the adenine nucleotide translocator, this implies that its control of oxidative phosphorylation (away from state 3) is smaller than if the relationship were simply proportional ("ohmic"). In cell biological terms, this property would be useful as it transfers control over oxidative phosphorylation away from the mitochondria to the ATP consuming process. Also, this property, rather than (Forman & Wilson, 1983) proximity to equilibrium, is responsible for the fact that the flux control coefficient of the adenine nucleotide translocator is less than 0.5 (Groen et al., 1982; Forman & Wilson, 1983; Halangk et al., 1987).

Finding that when the sum concentration of ADP plus ATP is not kept constant respiration does not depend uniquely on the phosphate potential, Jacobus et al. (1982) concluded that oxidative phosphorylation is not regulated by the extramitochondrial phosphate potential but rather by the concentration of ADP (even though the dependence on ADP concentration was not unique either). LaNoue and colleagues (LaNoue et al., 1986) came to a similar conclusion on the basis of their finding that the phosphorylation system is not close to equilibrium halfway between state 3 and state 4, a finding confirmed by (this paper) and confirming (Wanders et al., 1981) us.

In fact, there is no discrepancy between the observations of these two groups and our observations: At constant concentrations of redox substrates and products and of phosphate, oxidative phosphorylation will, in general, depend on the concentrations of both ADP and ATP in the medium. For those interested in energetics, it is useful instead to consider the process as a function of [ATP] + [ADP] and [ATP]/[ADP]. The former tends to be conserved, whereas the latter is logarithmically related to an energy parameter of paramount interest, e.g., the phosphate potential, ΔG_p^{out}. At different magnitudes of [ADP] + [ATP], the relationship between the rate of oxidative phosphorylation and ΔG_p would be shifted (Van der Meer et al., 1980; this paper). All observations [including the presence of some flux control in the adenine nucleotide translocator (Groen et al., 1982; Halangk et al., 1987)] indicate that there is no control of oxidative phosphorylation by the phosphorylation potential in the sense of *equilibrium* thermodynamics [as initially proposed by Wilson et al. (1979)]. There is control, however, in the sense of *non*equilibrium thermodynamics [as outlined above and in Westerhoff and Van Dam (1987) and Westerhoff et al. (1987a)]. The distinction between this "nonequilibrium thermodynamic control" and "kinetic control" is one of concept rather than substance.

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REFERENCES

Azzone, G. F., Pozzan, T., Massari, S., Bragadin, M., & Pregnolato, L. (1978) *Biochim. Biophys. Acta 501*, 296.

Berry, M. N., Gregory, R. B., Grivell, A. R., Henly, D. C., Phillips, J. W., Wallace, P. G., & Welch, G. R. (1987) *FEBS Lett. 224*, 201.

Bohnensack, R. (1981) *Biochim. Biophys. Acta 634*, 203.
Caplan, S. R., & Essig, A. (1983) *Bioenergetics and Linear Non Equilibrium Thermodynamics*, Harvard University Press, Cambridge, MA.
Cleland, K. W., & Slater, E. C. (1953) *Biochem. J. 53*, 547.
Davis, E. J., & Davis-van Thienen, W. I. A. (1978) *Biochem. Biophys. Res. Commun. 83*, 1260.
Duszyński, J., & Wojtczak, L. (1985) *FEBS Lett. 182*, 243.
Fell, D. A., & Sauro, M. M. (1985) *Eur. J. Biochem. 148*, 555.
Ferguson, S. J., & Sorgato, M. C. (1982) *Annu. Rev. Biochem. 51*, 185.
Forman, N., & Wilson, D. F. (1983) *J. Biol. Chem. 258*, 8649.
Gellerich, F. N., Bohnensack, R., & Kunz, W. (1983) *Biochim. Biophys. Acta 722*, 381.
Groen, A. K., Wanders, R. J. A., Westerhoff, H. V., Van der Meer, R., & Tager, J. M. (1982) *J. Biol. Chem. 257*, 2754.
Halangk, W., Dietz, H., Bohnensack, R., & Kunz, W. (1987) *Biochim. Biophys. Acta 893*, 100.
Heinrich, R., & Rapoport, T. A. (1975) *BioSystems 7*, 130.
Holian, A., Owen, C. S., & Wilson, D. F. (1977) *Arch. Biochem. Biophys. 181*, 164.
Hoogeboom, G. H. (1962) *Methods Enzymol. 1*, 16.
Jacobus, W. E., Moreadith, R. W., & Vandegaer, K. M. (1982) *J. Biol. Chem. 257*, 2397.
Jensen, B. D., Gunter, K. K., & Gunter, T. E. (1986) *Arch. Biochem. Biophys. 248*, 305.
Juretič, D., & Westerhoff, H. V. (1987) *Biophys. Chem. 28*, 31.
Kacser, H., & Burns, J. A. (1973) in *Rate Control of Biological Processes* (Davies, D. D., Ed.) pp 65–104, Cambridge University Press, London.
Klingenberg, M. (1980) *J. Membr. Biol. 56*, 97.
Krämer, R., & Klingenberg, M. (1982) *Biochemistry 21*, 1082.
Küster, U., Bohnensack, R., & Kunz, W. (1976) *Biochim. Biophys. Acta 440*, 391.
Küster, U., Letko, G., Kunz, W., Duszyński, J., Bogucka, K., & Wojtczak, L. (1981) *Biochim. Biophys. Acta 636*, 32.
LaNoue, K. F., Jeffries, F. M., & Radda, G. K. (1986) *Biochemistry 25*, 7667.
Lemasters, J. J., Grunwald, R., & Emaus, R. K. (1984) *J. Biol. Chem. 259*, 3058.
Letko, G., Küster, U., Duszyński, J., & Kunz, W. (1980) *Biochim. Biophys. Acta 593*, 196–200.
Murphey, M. P., & Brand, M. D. (1987) *Nature (London) 329*, 170.
Myers, D. K., & Slater, E. C. (1957) *Biochem. J. 67*, 558.
Nicholls, D. G., & Bernson, V. S. M. (1977) *Eur. J. Biochem. 75*, 601.
Padan, E., & Rottenberg, H. (1973) *Eur. J. Biochem. 40*, 431.
Pietrobon, D., & Caplan, S. R. (1985) *Biochemistry 24*, 5764.
Pietrobon, D., Zoratti, M., Azzone, G. F., Stucki, J. W., & Walz, D. (1982) *Eur. J. Biochem. 127*, 483.
Rigoulet, M., Guerin, B., & Denis, M. (1987) *Eur. J. Biochem. 168*, 275.
Rothschild, K. J., Elias, S. A., Essig, A., & Stanley, M. E. (1980) *Biophys. J. 30*, 209.
Rottenberg, H. (1973) *Biophys. J. 13*, 503.
Rottenberg, H. (1985) *Mod. Cell Biol. 4*, 47.
Rottenberg, H., Caplan, S. R., & Essig, A. (1970) in *Membranes and Ion Transport* (Bittar, E. E., Ed.) pp 165–191, Interscience, New York.
Slater, E. C. (1987) *Eur. J. Biochem. 166*, 489.
Slater, E. C., Rosing, J., & Mol, A. (1973) *Biochim. Biophys. Acta 292*, 534.
Souverijn, J. H. M., Huisman, L. A., Rosing, J., & Kemp, A., Jr. (1973) *Biochim. Biophys. Acta 305*, 185.
Stucki, J. W. (1980) *Eur. J. Biochem. 109*, 269.
Tager, J. M., Wanders, R. J. A., Groen, A. K., Kunz, W., Bohnensack, R., Küster, U., Letko, G., Böhme, G., Duszyński, J., & Wojtczak, L. (1983) *FEBS Lett. 151*, 1.
Tonomura, Y. (1986) *Energy-Transducing ATPases—Structure and Kinetics*, Cambridge University Press, Cambridge, U.K.
Van Dam, K., Westerhoff, H. V., Krab, K., Van der Meer, R., & Arents, J. C. (1980) *Biochim. Biophys. Acta 591*, 240.
Van der Meer, R., Westerhoff, H. V., & Van Dam, K. (1980) *Biochim. Biophys. Acta 591*, 488.
Vignais, P. V., Vignais, P. M., & Defaye, G. (1973) *Biochemistry 12*, 1508.
Wanders, R. J. A. (1985) *Quantitative Studies on Control of Flux*, Academisch Proefschrift, University of Amsterdam.
Wanders, R. J. A., Groen, A. K., Meijer, A. J., & Tager, J. M. (1981) *FEBS Lett. 132*, 201.
Wanders, R. J. A., Groen, A. K., Van Roermund, C., & Tager, J. M. (1984a) *Eur. J. Biochem. 142*, 147.
Wanders, R. J. A., Van den Berg, G. B., & Tager, J. M. (1984b) *Biochim. Biophys. Acta 767*, 113.
Westerhoff, H. V., & Kell, D. B. (1987) *Biotechnol. Bioeng. 30*, 101.
Westerhoff, H. V., & Van Dam, K. (1987) *Thermodynamics and Control of Biological Free Energy Transduction*, Elsevier, Amsterdam.
Westerhoff, H. V., Groen, A. K., & Wanders, R. J. A. (1984a) *Biosci. Rep. 4*, 1.
Westerhoff, H. V., Melandri, B. A., Venturoli, G., Azzone, G. F., & Kell, D. B. (1984b) *Biochim. Biophys. Acta 768*, 257.
Westerhoff, H. V., Plomp, P. J. A. M., Groen, A. K., Wanders, R. J. A., Bode, J. A., & Van Dam, K. (1987a) *Arch. Biochem. Biophys. 257*, 156.
Westerhoff, H. V., Plomp, P. J. A. M., Groen, A. K., & Wanders, R. J. A. (1987b) *Cell Biophys. 11*, 239.
Westerhoff, H. V., Kell, D. B., Kamp, F., & Van Dam, K. (1988a) in *Microcompartmentation* (Jones, D. P., Ed.) CRC, Boca Raton, FL.
Westerhoff, H. V., Ferrer, F., & Astumian, R. D. (1988b) in *Integration of Mitochondrial Function* (Lemasters, J. J., Hackenbrock, C. R., Thurman, R. G., & Westerhoff, H. V., Eds.) Plenum, New York.
Wilson, D. F. (1980) *Biochim. Biophys. Acta 616*, 371.
Wilson, D. F. (1982) *Trends Biochem. Sci. (Pers. Ed.) 7*, 275.
Wilson, D. F., & Forman, N. G. (1982) *Biochemistry 21*, 1438.
Wilson, D. F., Owen, C. S., & Erecinska, M. (1979) *Arch. Biochem. Biophys. 195*, 495.
Zoratti, M., Favaron, M., Pietrobon, D., & Azzone, G. F. (1986) *Biochemistry 25*, 760.