A Control Theoretic Analysis of Inhibitor Titration Assays of Metabolic Channelling

Key Words: *free energy transduction, localized chemiosmotic coupling, flux control, uncoupling, direct transfer, metabolic control theory, metabolic organization*

I. INTRODUCTION, CHANNELLING AND INHIBITOR TITRATIONS

A superficial inspection of biochemical charts of metabolic pathways might lead one to view the cell as a bag of enzymes. Indeed, the synthesis or uptake of every cell constituent would in principle be possible if all enzymes and metabolites were in one membrane-bounded compartment, therein free to communicate with one another by diffusion and at diffusion-limited rates. Yet, the eukaryotic cell consists of a number of compartments, the membranes of which contain carriers selective for certain compounds. Apparently the genetic burden of having these extra proteins is more than offset by the advantages of having metabolism compartmentalized. Advantages of such compartmentation include the simultaneous operation of otherwise incompatible metabolic pathways (e.g., because they require different pH values, or [ATP]/[ADP], or [NADH]/[NAD^+] ratios), a reduction of response and transit times, addition of extra control points, energization (e.g.,

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in the transport of adenine nucleotides across the inner mitochondrial membrane) and reduction in *amounts* of enzyme or substrate needed to achieve the required enzyme or substrate concentrations.

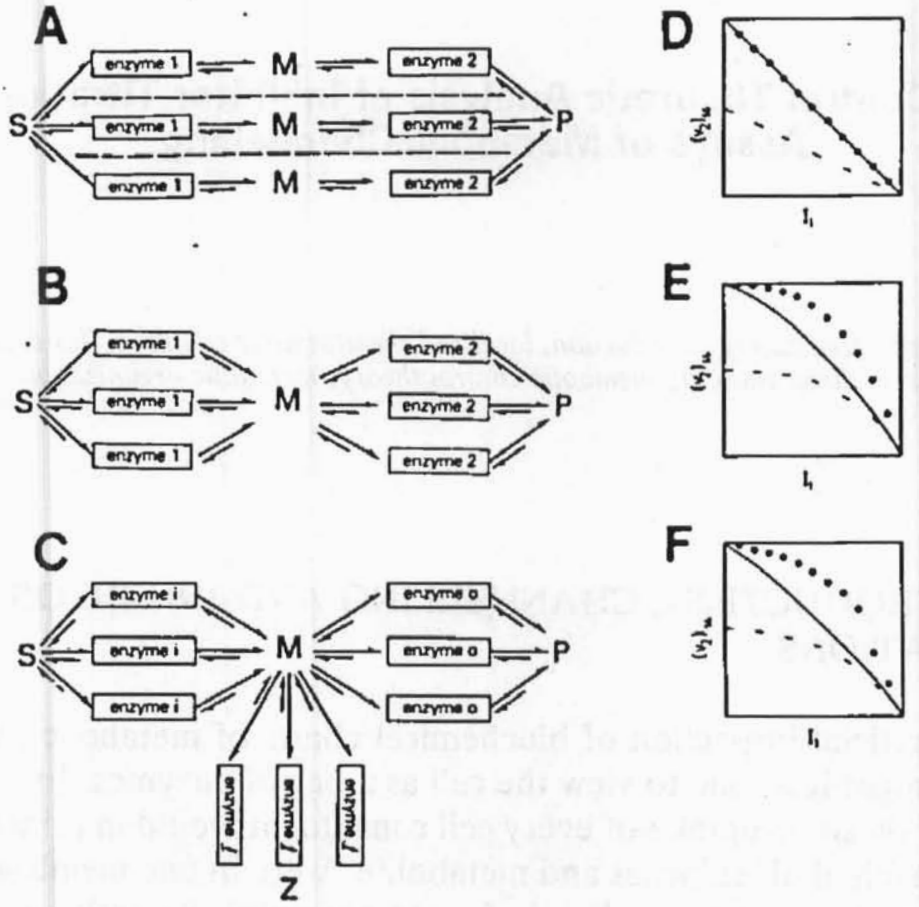

FIGURE 1 A generalized metabolic pathway, in which a substrate *S* is converted by enzyme 1 into an intermediate *M*, which is subsequently converted into product *P*([*S*] and [*P*] are considered to be fixed). A: Channelling behavior in which molecules of the intermediate *M* produced by a given molecule of enzyme 1 are not freely available to all molecules of enzyme 2 in the same membrane-limited compartment. In the (extreme) case shown, *individual* enzyme 1–enzyme 2 molecules act together, as a supercomplex, so that there is *no* inter-complex diffusion of intermediate *M* molecules. B: Pool behavior in which the intermediate *M* is freely diffusible, at a rate sufficiently high to ensure that it is available to all molecules of enzyme 2 in the membrane-bounded compartment of interest. In this case, it is useful to speak of the concentration of *M* as the number of moles of *M* divided by the volume of the compartment. C: A generalized scheme for a metabolic pathway or a free-energy coupling system containing a "leak" pathway. For the former case, substrate *S* is converted by the "input" enzyme (*i*) into an intermediate *M*, which may either be converted into the product of interest (*P*) by the "output" enzyme (*o*) or to another product (*Z*) by a "leak" (*ℓ*) pathway. The present figure is drawn to illustrate pool behavior. For the energy coupling case, we shall be especially

The matrix space of mitochondria, their intermembrane space, the aqueous cytoplasm, the lysosomes, the cisternae of the endoplasmic reticulum, the microsomes and the nucleus are the classical and prototypical compartments of eukaryotic cells, separated from each other by semipermeable membranes. However, reports are recurring (e.g., Refs. 1–4) of apparent subcompartmentation *within* these compartments. The extreme of such subcompartmentation would be that it occurs at the level of individual enzymes, a phenomenon that has been referred to as "direct cross talk," "microcompartmentation," "channelling," or "absence of a pool of intermediates." This channelling concept is illustrated in Fig. 1A. Here the boxes with enzyme 1 represent individual molecules of the enzyme converting molecules *S* to *M*. In Fig. 1B, representing the case where channelling is absent, any molecule *M* whose production is catalyzed by any molecule of enzyme 1 reacts equally rapidly with any molecule of enzyme 2. In the case of channelling (Fig. 1A), a molecule *M* produced by a molecule of enzyme 1 can only react with one (or a few) molecules of enzyme 2. In the case of channelling, it is as if the system consists of a large number of "subcompartments" each containing only one (or a few) enzymes 1 and one (or a few) enzymes 2.

A well-known case of channelling is found in the reaction catalyzed by the pyruvate dehydrogenase complex. Here the intermediary compound ("activated acetaldehyde") does not dissociate from the enzyme complex to be converted further by any of the

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concerned with the "delocalized" chemiosmotic coupling scheme for electron-transport-linked phosphorylation (see Ref. 51). Thus, enzymes *i* and *o* represent, respectively, redox-linked proton pumps and H^+-ATP synthase enzymes and ln[*M*] represents the proton electrochemical potential difference ($\Delta\tilde{\mu}_H$, proton-motive force) across the energy coupling membrane. The leak pathway then represents any back-flow of protons not coupled to ATP synthesis, and, at the level of the membrane vesicle, this scheme, by definition, exhibits delocalized or pool behavior. It is this scheme in particular which we shall be seeking to consider; if it fails, we must assume that quanta of free energy embodied in *M* are of a more microscopic character than those of a macroscopic thermodynamic intermediate such as the proton-motive force. Thermodynamic difficulties raised by such a situation are discussed elsewhere (Refs. 64–66). D: Predicted effect of an inhibitor (I_2) of enzyme 2 on the titration of the pathway flux with an inhibitor of enzyme 1, for the channelled case (cf. A). E: Same as D, but for the pool case (cf. B). F: Same as E, but for Fig. 1C. In D, E and F the full lines refer to the titration in the absence of inhibitor of enzyme 2, the dashed lines to that in the presence of the inhibitor of enzyme 2. The dotted lines result from normalization of the dashed lines.

other pyruvate dehydrogenase complexes, but remains on the same complex for its conversion. In other cases, the channelling may only be partial: there may be a preference of metabolite M produced by a given enzyme 1 to react with just one of the enzymes 2, but reaction with one of the other enzymes 2 may still occur to some extent. However, to clarify the issue, we shall here discuss channelling in terms of the extreme form illustrated in Fig. 1A.

There are a number of methods by which one might demonstrate channelling.[2,4] One is through rapid kinetic measurements. In the case of channelling (Fig. 1A), the first formation of product P after a sudden introduction of S into the system does not have to await the filling of a pool of *M* to a level (concentration) sufficient to allow for the synthesis of *P* at a rate commensurate with that determined by the enzyme kinetic properties as studied in the isolated enzymes; in other words, the transit time is reduced.[4] A second is to start the reaction with isotopically labelled *S* plus unlabelled M and to compare the specific activity of isolated *M* to that of isolated *P*; if the latter increases earlier than the former, there is channelling.

In this Comment we shall confine our attention almost exclusively to a third method. We refer to this method as the (dual) inhibitor titration method. In this approach one uses specific inhibitors to examine whether all enzymes 1 can indeed provide substrate (*M*) for any enzyme 2, as in the case of Fig. 1B. If, in such a case, there were to be an excess of the activity of enzymes 1, then elimination of a certain fraction of the enzymes 1 might be expected to have a less than proportionate effect on the steady-state rate of production of *P* (cf. Fig. 1E; full line), unless channelling was occurring (cf. Fig. 1D).

The argument becomes somewhat more sophisticated when the effect of inhibition of enzymes 2 on this apparent excess of enzymes 1 is considered. In a system exhibiting pool behavior, the proportional effect of an inhibitor of enzyme 1 on the overall pathway flux may be expected to be strongly decreased by the presence of a partially inhibitory titre of an inhibitor of enzyme 2 (and vice versa). This is the so-called rule-of-thumb of dual-inhibitor titrations, as indicated in Fig. 1E. For perfectly channelled metabolism, no such effect is to be expected; the inhibitory pattern will be essentially independent of the overall flux (Fig. 1D).

Recently, there has been a surge in the use of inhibitor titrations as assays for channelling in proton-mediated free-energy transduction, but the conclusion drawn by many of the authors of the ensuing papers, i.e., that this free-energy transduction is organized in "channels" [e.g., Refs. 5–9], has been challenged by others [e.g., Refs. 10–17] on the basis that the interpretation of the inhibitor titration results was insufficiently rigid. In view of the potential of inhibitor titrations for the detection of channelling in various metabolic pathways, it is important to develop a rigid framework for the interpretation of the results of such titrations. This is what we shall do in this Comment (Section II).

In a different field of biochemistry, the revival of the metabolic control theory developed by Kacser and Burns[18] and Heinrich and Rapoport[19] (see also Refs. 20–25, recently reviewed in Refs. 26 and 27) has led to important progress in our understanding of the control of steady-state metabolism [e.g., Ref. 28]. It therefore occurred to us[29–32] that this control theory might serve to provide a rigid framework for the analysis of the (dual) inhibitor titrations. However an important implicit assumption in most uses of control theory published to date is that the individual metabolites do exhibit pool behavior within the membrane-delimited compartments in question, i.e., that channelling is insignificant. The main purpose of this Comment are thus four-fold: (1) to give an outline of the metabolic control theory; (2) to discuss, within the framework of this metabolic control theory, the experimental behavior to be expected if pool behavior is indeed occurring; (3) to suggest that a lack of compliance of the experimental behavior observed, with the prediction of the metabolic control theory, may provide a *robust* criterion for channelling; and (4) to examine critically the protocols and findings of some recent experimental studies that have led their authors to suggest that channelling is indeed a widespread and significant feature of metabolism in general, and of membrane-linked energy coupling in particular.

To this purpose we shall start out by giving the theoretical basis of the control analysis of inhibitor studies in pooled metabolic systems. We then proceed by (Section III) indicating how the extent to which enzymes control fluxes can be determined from inhibitor titrations. This allows us to (Section IV) analyze experimental results obtained in free-energy transducing pathways in

terms of (variations in) flux control coefficients and conclude that at least some of the results are not consistent with that metabolism being "pooled."

The control analysis also allows us to quantitatively answer critiques of inhibitor titration experiments (Section V) and (Section VI) to deal with the possibility that some of the enzymes may "slip." Section VII focuses on titration endpoints.

Much further research is needed to understand how channelled metabolism is controlled. In Section VIII we indicate some of the special control properties. Section IX then summarizes our conclusions.

II. CONTROL THEORETIC ANALYSIS OF EFFECTS OF INHIBITORS ON METABOLISM

II.1. Elasticity Coefficients and Control Coefficients

In Sections III and IV we shall discuss three prototypes of (dual) inhibitor titration studies of channelling, first in terms of their original interpretation and then in terms of control theory. However, for the latter purpose we shall first (in the present section) have to introduce, in particular, two notions from control theory, i.e., Flux-Control Coefficient and Elasticity Coefficient.[18,19,26,27,32]

To make our discussion sufficiently general, we amend the system of Fig. 1B with an extra "leak" pathway for M. Thus, in Fig. 1C, the production of P and the consumption of S are no longer strictly coupled (i.e., with a constant, integral stoichiometry). As usual,[27] we shall only be considering transitions between steady states. We shall first discuss this system as if channelling is absent, and predict the results of inhibitor titrations. Failure of experimental results to comply with such predictions will then be taken as rejections of the scheme of Fig. 1C and, provided that all other aspects of Fig. 2 apply to the particular case, as indication of channelling.

An initial approach to interpreting the effect of an inhibitor of enzymes i (for "input," cf. Fig. 1C) on the rate of disappearance of S might be to calculate this effect from the effect of I in the rate equation for v_i. Let us consider the example where I is a

competitive inhibitor. The simplest rate equation for such an enzyme-catalyzed reaction is[32]:

$$v_i = \frac{V_S \cdot \frac{[S]}{K_S} - V_M \cdot \frac{[M]}{K_M}}{1 + \frac{[S]}{K_S} + \frac{[M]}{K_M} + \frac{[I]}{K_I}} \qquad (1)$$

where, as usual, v_i is the rate of production of M from S, V_S and V_M are, respectively, the maximal velocities (for enzyme i) of the forward and reverse reactions, K_S, K_M and K_I are Michaelis and inhibitor constants, and $[S]$, $[M]$ and $[I]$ are concentrations of S, M and I. If $[M]'$ is the concentration of M after the addition of inhibitor, the effect of the added δI on v_i is given by (it is assumed that δI increases the inhibitor concentration from $[I]$ to $[I]'$):

$$\delta v_i = \frac{V_S \cdot \frac{[S]}{K_S} - V_M \cdot \frac{[M]'}{K_M}}{1 + \frac{[S]}{K_S} + \frac{[M]'}{K_M} + \frac{[I]'}{K_I}} - \frac{V_S \cdot \frac{[S]}{K_S} - V_M \cdot \frac{[M]}{K_M}}{1 + \frac{[S]}{K_S} + \frac{[M]}{K_M} + \frac{[I]}{K_I}} \qquad (2)$$

For the time being, we take $[M]'$ and $[M]$ to be equal. Using this equation we would then predict that the effect of δI on v_i would depend on the concentration of S and M and on the kinetic constants of the reaction catalyzed by enzyme i. Following this line of reasoning, the kinetics and concentration of enzyme o (for "output") would seem to be irrelevant, which would, however, be surprising in view of the expectation that a significant reduction in the amount of enzyme o would reduce the effect of an inhibitor of the input enzyme on the consumption of S (cf., Fig. 1D).

The way out of this paradox is obtained by distinguishing two time scales; the effect of δI just after its addition might well differ from its effect some time later, especially because $[M]$ may change with time, i.e., from $[M]$ to $[M]'$. It is the former ("immediate") effect that is given by the above equation (2) with $[M]'$ equal to $[M]$. The latter effect will be a function of time for as long as $[M]'$ keeps changing.[32] In real systems, some time after the addition of I a new steady state is attained, in which v_i will usually lie in

between the v_i prior to and the v_i immediately after the addition of I. The change in v_i between the two steady-state situations is again given by Eq. (2), but now with $[M]'$ being equal to the new steady-state concentration of M. This magnitude of $[M]'$ will depend on the properties and relative organization of all enzymes in the system (see below). It is only the long-term ("steady-state") and not the immediate effect of I on v_i that is informative concerning the (channelling) properties of the system as a *system*.

As indicated in the above discussion, given the kinetic constants and the metabolite concentrations just before the addition of δI, the immediate effects on $[M]$ of adding $\delta I = I' - I$ will depend on the magnitude of δI. For the generality of the discussion, and also in order to eliminate the possible argument that the addition of δI may itself change the (non)channelling properties of the system, we shall consider the limiting case of (infinitesimally) small changes in inhibitor concentrations. To make our definitions independent of the units in which v_i and I are expressed, we shall take the ratio of the relative changes: $\delta v/v$ will be compared to $\delta I/I$ (the case where $I = 0$ will be discussed separately below).[18] Mathematically, this is the ln-ln or log-log differential of v_i with respect to I:

$$\lim_{\delta I \to 0} \frac{\delta v_i}{v_i} \Big/ \frac{\delta [I]}{[I]} = \frac{[I]}{v_i} \cdot \frac{dv_i}{d[I]} = \left(\frac{d \ln |v_i|}{d \ln [I]} \right)_{ss} \tag{3}$$

Here the subscript refers to the condition that before and after the change the system should be at steady state.

Using the chain rule this differential may be rewritten as:

$$\frac{[I]}{v_i} \cdot dv_i = \frac{[I]}{v_i} \cdot \left(\frac{\partial v_i}{\partial [I]} \right)_{[M]} \cdot d[I] + \frac{[I]}{v_i} \cdot \left(\frac{\partial v_i}{\partial [M]} \right)_{[I]} \cdot \left(\frac{d[M]}{d[I]} \right)_{ss} \cdot d[I] \tag{4}$$

This equation is the mathematical counterpart of the resolution of the paradox we stated in the paragraph before last: to understand

the change in consumption rate (v_i) of S (the first term in Eq. (4)), one must consider the immediate effect of I on v_i at constant $[M]$, i.e., the middle term in Eq. (4), and the effect arising from the change in M (the final term in Eq. (4)).

We shall first define the "Elasticity Coefficient" of enzyme i with respect to I as a measure of the immediate effect (the middle term in Eq. (4)):

$$\frac{[I]}{v_i} \cdot (dv_i)_{\text{imm}} \overset{\text{def}}{=\!=} \epsilon_I^{v_i} \cdot d[I] \overset{\text{def}}{=\!=} \frac{[I]}{v_i} \cdot \left(\frac{\partial v_i}{\partial [I]}\right)_{[M]} \cdot d[I] \qquad (5)$$

(Symbols and their meanings are listed in Table II). Equation (4) can now be rewritten as:

$$v_i \cdot C_I^{v_i} \cdot \frac{d[I]}{[I]} \overset{\text{def}}{=\!=} (dv_i)_{ss} = v_i \cdot \epsilon_I^{v_i} \cdot \frac{d[I]}{[I]} + v_i \cdot \epsilon_M^{v_i} \cdot C_I^M \cdot \frac{d[I]}{[I]} \qquad (6)$$

Here also the Elasticity Coefficient of the enzyme with respect to the metabolite M comes into play:

$$\epsilon_M^{v_i} \overset{\text{def}}{=\!=} \frac{[M]}{v_i} \cdot \left(\frac{\partial v_i}{\partial [M]}\right)_{[I]} \overset{\text{def}}{=\!=} \frac{[e_i]}{v_i} \cdot [t\epsilon]_M^i \overset{\text{def}}{=\!=} [a\epsilon]_M^{v_i}/v_i \qquad (7)$$

C_I^M is termed the Concentration-Control Coefficient of M with respect to I.[33] It indicates the steady-state effect of the inhibitor on the concentration of the metabolite M:

$$C_I^M \overset{\text{def}}{=\!=} \frac{[I]}{[M]} \cdot \left(\frac{d[M]}{d[I]}\right)_{ss} \qquad (8)$$

The Flux-Control Coefficient $C_I^{v_i}$ of v_i with respect to I is the effect of I on v_i after relaxation of the system to the subsequent steady state (referred to by the subscript *ss*). It is defined by the left-hand section of Eq. (6). The Absolute Elasticity Coefficient,[32] $[a\epsilon]_M^{v_i}$, and the Turnover Elasticity Coefficient, $[t\epsilon]_M^{ei}$ (first defined here), measure the change in v_i in absolute and turnover terms, respectively, rather than in relative terms. All Elasticity Coefficients can be determined by measuring, either *in situ* or with the isolated

enzyme, the reaction rate v_i as a function of the concentration of the metabolite M at constant concentrations of the inhibitor and of all metabolites and other modifiers. $[a\epsilon]_M^{v_i}$ is then the slope, at the point of interest, in the plot of v versus $\ln[M]$, while $[t\epsilon]_M^{e_i}$ is that slope divided by the enzyme concentration. In fact, $[t\epsilon]_M^{e_i}$ is the change in turnover number of enzyme i per e-fold change in concentration of M. $\epsilon_M^{v_i}$ is that slope divided by the reaction rate, or the slope of the double logarithmic plot (Eq. (7)). The word "elasticity" stems from the Greek ελαυνω, which means "to drive or to set in motion"; the Elasticity Coefficient of an enzyme with respect to a substance indicates how readily it is set in motion (in the sense of its catalytic action) by an increase in the concentration of that substance.

The purpose of defining both Control Coefficients and Elasticity Coefficients is to stress the distinction between systemic effects and effects at the level of the individual enzymes. The Control Coefficients express the behavior of the entire system as it relaxes between steady states, incorporating all the changes in the concentrations of metabolites and their concomitant effects. The Elasticity Coefficients describe the behavior of individual enzymes with respect to individual changes in system variables and parameters.

Equation (6) stresses that the immediate and the steady-state effects of I on v_i in the scheme of Fig. 1C differ unless either v_i is insensitive to changes in $[M]$ (i.e., $\epsilon_M^{v_i} = 0$) or there is no change in steady-state concentration of M upon changing $[I]$ (i.e., $C_I^M = 0$). Clearly, for the general interpretation of the steady-state effect of inhibitors, it is necessary to take account of the changes in $[M]$; one cannot discuss the situation solely in terms of the effect of the inhibitor on the activity of a single enzyme. Since the effect of an inhibitor I on $[M]$ runs entirely through its effect on the activity of the inhibited enzyme, we shall first examine how a change in that activity affects a steady-state metabolite concentration (i.e., we shall ask for the magnitude of C_I^M). Subsequently, we shall combine the latter effect with the effect of I on enzyme activity to find the effect of the inhibitor on steady-state fluxes (C_I^v).

The only terms in Eq. (1) that depend upon the enzyme concentration (i.e., V_S and V_M), are proportional to it. Hence the *immediate* effect of an $x\%$ increase in concentration of enzyme i will be an $x\%$ increase in v_i: leaving aside monomer–dimer equi-

libria, the Elasticity Coefficient of an enzyme with respect to its own concentration is 1. The Elasticity Coefficients with respect to the concentration of the other enzymes are zero, unless there are (anti-) cooperative interactions between the enzymes. The *steady-state* effect of this $x\%$ change in the concentration of enzyme i on v_i, however, may well differ from 1, because $[M]$ may change. Defining the Flux-Control Coefficient of v_i with respect to e_i as:

$$C_{e_i}^{v_i} \underset{\text{def}}{=\!=} \frac{[e_i]}{v_i} \cdot \left(\frac{dv_i}{d[e_i]}\right)_{ss} \tag{9}$$

we can write this consideration in mathematical terms. After relaxation to the new steady state

$$C_{e_i}^{v_i} = \epsilon_{e_i}^{v_i} + \epsilon_M^{v_i} \cdot C_{e_i}^{M} = 1 + \epsilon_M^{v_i} \cdot C_{e_i}^{M} \tag{10}$$

where the latter term multiplies the relative effect of a change in e_i on M ($C_{e_i}^{M}$, a steady-state effect) by the relative effect of such a change in M on v_i.

It should also be mentioned that an enzyme can exhibit a *negative* Flux-Control Coefficient, such that inhibiting it serves to *stimulate* the flux through the pathway of interest. A branched system in which a competing "leak" pathway is present is particularly germane to our considerations, since even if $C_i^o = C_o^o = 1$, then $\Sigma C_n^o = 1$ (i.e., adherence to the summation theorem) is still possible if $C_l^o = -1$ in the scheme of Fig. 1C.[32,34] This may occur because *inhibition* of the leak will *stimulate* the output flux. A Flux-Control Coefficient can be defined for any combination of the fluxes and the enzymes in the system.

II.2. Laws Governing Control Coefficients

If a Flux-Control Coefficient is equal to 1, then a certain percentage change in the enzyme gives rise to an identical percentage change in the flux; such an enzyme may then be viewed as "completely rate-limiting." Intuitively, it is often taken for granted that in a macroscopic system only one enzyme in a system can be "completely rate-limiting" with respect to a particular flux. If the input enzyme is completely rate-limiting, then the output in Fig. 1B could not exert significant flux control. This notion is paralleled mathematically by the summation theorem which (for systems exhibiting

pool behavior) states that the sum of the flux controls (quantified in terms of Flux-Control Coefficients) exerted by the enzymes in a system on any particular flux must be equal to 1:

$$C_i^v + C_o^v + C_\ell^v = 1 \qquad (11)$$

Here v can be any of the steady-state fluxes or reaction rates in the system. This is the so-called flux-control summation theorem.[18,19,27,32]

Since this theorem is the real basis for the notion "completely rate-limiting," it is clear that a given metabolic pathway may possess more than one "completely rate-limiting step" if a leak pathway with a Flux-Control Coefficient of -1 is also present.[34] That the *total* control on a flux must be unity leaves us with the question of how the control is distributed amongst the enzymes. This now is one of the primarily questions we must ask when studying the control of a metabolic pathway.[32]

A traditional view has it that the control is in the enzymes that catalyze the reactions whose steady-state mass-action ratios lie furthest from equilibrium. This view has been proven rather insecure.[18,27,32] Better correlation exists between the flux-control by an enzyme and its lack of responsiveness toward changes in metabolite concentrations. The mathematical formulation of this correlation is called the "flux-control connectivity theorem"[18,27]:

$$C_\ell^v \cdot \epsilon_M^{v_\ell} + C_o^v \cdot \epsilon_M^{v_o} + C_i^v \cdot \epsilon_M^{v_i} = 0 \qquad (12)$$

where v is any flux in the system. Below we shall leave out the v's from the ϵ superscripts.

II.3. Expressing Control Coefficients into Elasticity Coefficients

In Fig. 1C:

$$v_i \equiv v_o + v_\ell \qquad (13)$$

We stress that v_i, v_o and v_ℓ are defined in terms of numbers of molecules M produced, consumed or consumed per unit time, respectively. Since Eq. (13) remains true for the steady-state fluxes even if an enzyme concentration is changed, it may be differen-

tiated with respect to the concentrations of any enzyme e_k to yield (with Eq. (9)):

$$v_i \cdot C_k^i \cdot \frac{d[e_k]}{[e_k]} = dv_i = dv_o + dv_\ell$$

$$= v_o \cdot C_k^o \cdot \frac{d[e_k]}{[e_k]} + v_\ell \cdot C_k^\ell \cdot \frac{d[e_k]}{[e_k]} \quad (14)$$

where k can refer to any of the three enzymes in the system. The superscripts refer to the fluxes (v_i, v_o and v_ℓ, respectively). After dividing out v_i from this equation, we recognize how the controls that any enzyme k exerts on the three fluxes must be related to one another.

In Eq. (10) we have shown how the control of enzyme i on the rate of enzyme i may be expressed in terms of the Elasticity Coefficient of the rate with respect to M and the control exerted by the enzyme on M. Using such an expression for every Flux-Control Coefficient in Eq. (14), one obtains for $k = i$:

$$v_i + v_i \cdot \epsilon_M^i \cdot C_i^M - v_o \cdot \epsilon_M^o \cdot C_i^M - v_\ell \cdot \epsilon_M^\ell \cdot C_i^M = 0 \quad (15)$$

We define the "flux ratio" j of output to input flux, as:

$$j \overset{\text{def}}{=\!=} v_o/v_i \quad (16)$$

This "reduced *P*/2*e* ratio" j is an important property in that it indicates the yield of the free-energy-transducing process.[32] It corresponds to the relative number of molecules of M produced by e_i that are consumed by e_o. In the chemiosmotic model, M molecules are of course chemiosmotically active protons. Using Eqs. (13), (15) and (16), C_i^M can be expressed in terms of j and the Elasticity Coefficients.

Similarly, the Concentration-Control Coefficients of metabolite M with respect to the other two enzymes in the system can be expressed in terms of the Elasticity Coefficients and the flow ratio j (cf. Table I). The combination of these equations with equations such as (10) allows us to do the same for the Flux-Control Coefficients.[32] These are also given in Table I.

TABLE I

Control Coefficients expressed in terms of Elasticity Coefficients and the flux ratio. Physically, $[a\epsilon]_{tot}$ represents the absolute Elasticity Coefficient with respect to M of the net formation rate of M and relates to the elasticity of the entire network

Control Coefficients for the Concentration of M

$$C_i^M = 1/\epsilon_{tot} = v_i/[a\epsilon]_{tot}$$

$$C_o^M = -j/\epsilon_{tot} = -v_o/[a\epsilon]_{tot}$$

$$C_\ell^M = -(1-j)/\epsilon_{tot} = -v_\ell/[a\epsilon]_{tot}$$

Control Coefficients for the Input Flux

$$C_i^i = 1 + \epsilon_M^i \cdot C_i^M = (j \cdot \epsilon_M^o + (1-j) \cdot \epsilon_M^\ell)/\epsilon_{tot}$$

$$= (e_o \cdot [t\epsilon]_M^o + e_\ell \cdot [t\epsilon]_M^\ell)/[a\epsilon]_{tot}$$

$$C_o^i = \epsilon_M^i \cdot C_o^M = j \cdot (-\epsilon_M^i)/\epsilon_{tot} = j \cdot e_i \cdot [-t\epsilon]_M^i/[a\epsilon]_{tot}$$

$$C_\ell^i = \epsilon_M^i \cdot C_\ell^M = (1-j) \cdot (-\epsilon_M^i)/\epsilon_{tot} = (1-j) \cdot e_i \cdot [-t\epsilon]_M^i/[a\epsilon]_{tot}$$

Control Coefficients for the Output Flux

$$C_i^o = \epsilon_M^o \cdot C_i^M = \epsilon_M^o/\epsilon_{tot} = e_o \cdot [t\epsilon]_M^o/(j \cdot [a\epsilon]_{tot})$$

$$C_o^o = 1 + \epsilon_M^o \cdot C_o^M = \{(-a_M^i) + (1-j) \cdot \epsilon_M^\ell\}/\epsilon_{tot}$$

$$= (e_i \cdot [-t\epsilon]_M^i + e_\ell \cdot [t\epsilon]_M^\ell)/[a\epsilon]_{tot}$$

$$-C_\ell^o = \epsilon_M^o \cdot (-C_\ell^M) = (1-j) \cdot \epsilon_M^o/\epsilon_{tot} = \left(\frac{1}{j} - 1\right) \cdot e_o \cdot [t\epsilon]_M^o/[a\epsilon]_{tot}$$

Control Coefficients for the Leak Flux

$$C_i^\ell = \epsilon_M^\ell \cdot C_i^M = \epsilon_M^\ell/\epsilon_{tot} = e_\ell \cdot [t\epsilon]_M^\ell/\{(1-j) \cdot [a\epsilon]_{tot}\}$$

$$-C_o^\ell = \epsilon_M^\ell \cdot (-C_o^M) = j \cdot \epsilon_M^\ell/\epsilon_{tot} = e_\ell \cdot [t\epsilon]_M^\ell \Big/ \left\{\left(\frac{1}{j} - 1\right) \cdot [a\epsilon]_{tot}\right\}$$

$$C_\ell^\ell = 1 + \epsilon_M^\ell \cdot C_\ell^M = \{(-\epsilon_M^i) + j \cdot \epsilon_M^o\}/\epsilon_{tot}$$

$$= \{e_i \cdot [-t\epsilon]_M^i + e_o \cdot [t\epsilon]_M^o\}/[a\epsilon]_{tot}$$

with:

$$\epsilon_{tot} \overset{\text{def}}{=\!=} \epsilon_M^i + j \cdot \epsilon_M^o + (1-j) \cdot \epsilon_M^\ell$$

$$[a\epsilon]_{tot} \overset{\text{def}}{=\!=} e_i \cdot [-t\epsilon]_M^i + e_o \cdot [t\epsilon]_M^o + e_\ell \cdot [t\epsilon]_M^\ell$$

Turnover Elasticity Coefficients, ([$t\epsilon$]'s), used in the equations of Table I and defined by Eq. (7) as $\partial(v_i/e_i)/\partial\ln[M]$, i.e., the change in enzyme turnover with a change in $\ln[M]$, have the property that at a constant concentration of M, they are independent of the enzyme concentration. Also, they are independent of $[M]$ in the special case that the reaction rates depend linearly on $\ln[M]$ (i.e., μ_M). It is important for the analyses of free-energy transduction that $\ln[M]$ may be replaced with an experimentally assessed or putative value for $\Delta\tilde{\mu}_H/RT$ (the proton electrochemical potential difference). Since[32] reaction rates tend to vary quasi-linearly with $\Delta\tilde{\mu}_H$ over rather large ranges of the latter, assumed constancy of the Turnover Elasticity Coefficients can be a first approach to understanding the results of inhibitor titrations in such systems (see Section IV).

The effect of an inhibitor on a steady-state flux can now be read from its effect on the activity of the enzyme it affects, and the Flux-Control Coefficient of that enzyme, e.g., for an inhibitor of the input enzyme[18,26]:

$$\left(\frac{dv}{v}\right)_{ss} = \frac{d[I]}{[I]} \cdot C_I^v = C_i^v \cdot \epsilon_I^i \cdot \frac{d[I]}{[I]} \tag{17}$$

where C_i^v would be looked up in Table I.

Because an inhibitor titration usually begins with zero inhibitor concentration, it is often useful to use a slightly different property instead of the Elasticity Coefficient, i.e., the inhibitor coefficient of I upon v_i, θ_I^i, defined by[35]:

$$\theta_I^i = \left(\frac{\partial v}{v} \Big/ \partial[I]\right)_{[M]} = [I] \cdot \epsilon_I^i \tag{18}$$

The above equation then reduces (from Eqs. (17) and (18)) to:

$$\left(\frac{dv}{v}\right)_{ss} = C_i^v \cdot \theta_I^i \cdot d[I] \tag{19}$$

Similarly, the Flux-Control Coefficient by inhibitor I ($C_I^{v_i}$ in Eq.

TABLE II

Glossary

Symbol	Definition	Equation	Name
$C_y^x = C_{e_y}^{v_x}$	$\left(\frac{d \ln\lvert v_x\rvert}{d \ln e_y}\right)_{ss}$	(6)	Flux-Control Coefficient of flux v_x with respect to enzyme e_y.
$C_y^M = C_{e_y}^{M}$	$\left(\frac{d \ln[M]}{d \ln e_y}\right)_{ss}$	(9)	Concentration-Control Coefficient of Metabolite M with respect to enzyme e_y.
$C_I^{v_x}$	$\left(\frac{d \ln\lvert v_x\rvert}{d \ln[I]}\right)_{ss}$	(20)	Flux-Control Coefficient of flux v_x with respect to inhibitor I.
C_I^M	$\left(\frac{d \ln[M]}{d \ln[I]}\right)_{ss}$	(8)	Concentration-Control Coficient of Metabolite M with respect to inhibitor I.
$\Gamma_I^{v_x}$	$\left(\frac{d \ln\lvert v_x\rvert}{d[I]}\right)_{ss}$	(21)	Steady-state effect of inhibitor I on the logarithm of flux v_x.
e_x		(7)	Activity of enzyme x.
j	v_o/v_i	(16)	Flow ratio, reduced $P/2e$ ratio.
$\tilde{n}_H^x$	(J_H/J_x)	(41)	Proton pumping stoichiometry of slipping pump.
v_x		(1)	Proton flux related to reaction x.
ϵ_M^x	$\frac{\partial \ln\lvert v_x\rvert}{\partial \ln[M]}$	(7)	Elasticity Coefficient of enzyme x with respect to metabolite M.
$[a\epsilon]_M^{v_x}$	$\frac{\partial v_x}{\partial \ln[M]}$	(7)	Absolute-Elasticity Coefficient of enzyme x with respect to metabolite M.
$[t\epsilon]_M^{v_x}$	$\frac{\partial(v_x/e_x)}{\partial \ln[M]}$	(7)	Turnover-Elasticity Coefficient of enzyme x with respect to metabolite M.
$[a\epsilon]_{tot}$	$a\epsilon_M^i + a\epsilon_M^o + a\epsilon_M^\ell$	Table I	Total Absolute Elasticity Coefficient.
θ_I^x	$\frac{\partial \ln\lvert v_x\rvert}{\partial[I]}$	(18)	Inhibitor Coefficient of enzyme x.

Sub- and Superscripts:

i	: input proton flux
o	: output proton flux
ℓ	: proton leak
M	: metabolite M
I	: inhibitor I
ochem	: output chemical reaction
imm	: immediate
ss	: steady-state

(6)) can be replaced with

$$\left(\frac{dv}{v}\right)_{ss} \Big/ d[I] \overset{\text{def}}{=\!=} \Gamma_I^v = \lim_{I \to 0} C_I^v[I] \tag{20}$$

such that

$$\Gamma_I^v = C_i^v \cdot \theta_I^i \tag{21}$$

Γ_I^v may be referred to as the Flux-Inhibition Coefficient.

The latter equation shows that[26,36] the Flux-Control Coefficient by an enzyme i can be obtained as the ratio of (i) the steady-state effect of the inhibitor on the flux Γ_I^v (i.e., Γ_I^v) to (ii) the immediate effect of the inhibitor on the reaction rate through enzyme i (θ_I^i). For convenience, we summarize the terms of the Metabolic Control Theory used here in Table II.

This section laid the theoretical groundwork for the quantitative interpretation of the effects of inhibitors on fluxes in nonchannelled metabolism. It showed how the immediate effect of added metabolite or added inhibitor on a reaction rate is given by the Elasticity Coefficient of the enzyme catalyzing that reaction. Steady-state effects of added inhibitors are given by Control Coefficients; they reflect the properties of all the enzymes in the system as well as their relative positions in the metabolic pathway. For the metabolic pathway of Fig. 1C we expressed the Control Coefficients into the Elasticity Coefficients.[32] Thus, by combining Eqs. (17)–(21) with Table I, effects of inhibitors on fluxes and concentrations in unchannelled metabolic pathways can be calculated from the enzyme properties (Elasticity Coefficients). The following section will describe how we can translate experimental data into the coefficients defined here.

III. HOW TO EXPERIMENTALLY DETERMINE CONTROL COEFFICIENTS

III.1. Reversible Noncompetitive Inhibitors

Figure 2A shows the generalized results of a titration of the steady-state output flux, v_o, of a system such as that in Fig. 1C, with an

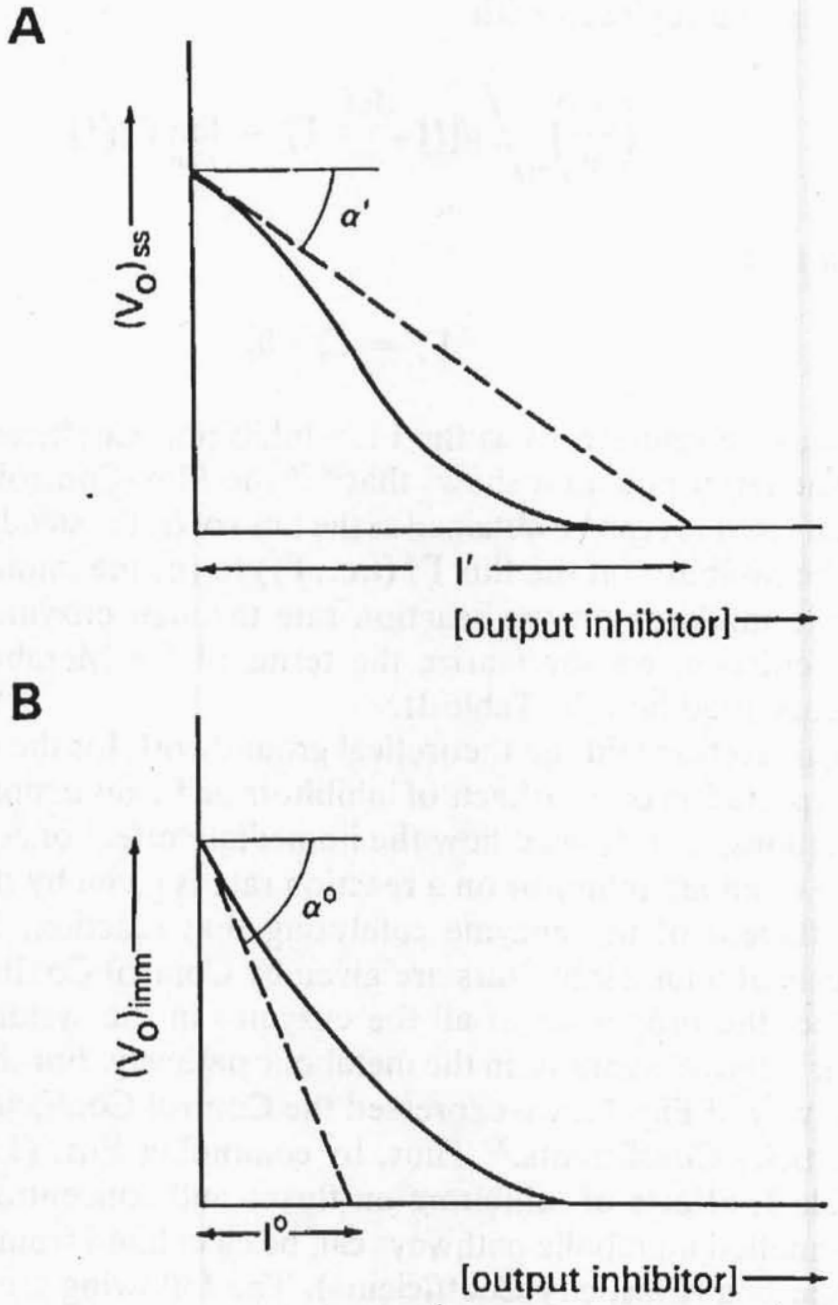

FIGURE 2 A generalized inhibitor titration of the flux through a metabolic pathway such as that in Fig. 2. The figure considers (A) the steady-state effect (when all transients have died down) and (B) the "immediate" or isolated-enzyme effect of the inhibitor (in this case of the output enzyme) on the flux rate through the enzyme. The (initial) slopes (tan α', tan α^0) and extrapolated intercepts (I', I^0) may be used to obtain the Flux-Control Coefficient (in this case, the enzyme o) as described in the text.

inhibitor of, say, the output enzyme. Figure 2B shows the result of titrating the same enzyme with the same inhibitor, but now considering the "immediate" effect, as it might be determined with the isolated enzyme (see below). This can be done either by meas-

uring the reaction rate immediately after the addition of the inhibitor (i.e., before $[M]$ has changed), or under conditions in which $[M]$ is kept constant by some other means. The starting point of both titrations (i.e., v_o, $[S]$, $[P]$, and $[M]$ at $I = 0$) should be identical. The tangent of α' in Fig. 2A is related to the Flux-Control Coefficient, C_o^o, of v_o with respect to the output enzyme (see below). However, $\tan(\alpha')$ is also dependent upon how effectively the inhibitor interacts with e_o (with effectiveness which is given by θ_I^o; cf. Eq. (18)):

$$\tan(\alpha^o) = \left(\frac{\partial v_o}{\partial [I]}\right)_{[M]} = v_o \cdot \theta_I^o = (v_o)_{\text{isolated}}/I^o \qquad (22)$$

where I_0 is determined as shown in Fig. 2B. Figure 2B serves to "calibrate" the inhibitor[26,36]:

$$\tan(\alpha') = \left(\frac{dv_o}{d[I]}\right)_{ss} = v_o \cdot \Gamma_I^v = v_o \cdot C_o^o \cdot \theta_I^o = (v_o)_{ss}/I' \qquad (23)$$

From Eqs. (22) and (23), one can obtain the Flux-Control Coefficient by comparing α^o to α' in the following way (I^o and I' are defined in Fig. 2):

$$C_o^o = \tan(\alpha')/\tan(\alpha^o) = I^o/I' \qquad (24)$$

i.e., the Flux-Control Coefficient simply equals the ratio of the extrapolated titers in the actual (I') and the calibration (I^o) titration.

III.2. Irreversible Inhibitors

A particularly clear and convenient approach to the assessment of the flux-control exerted by an enzyme is possible when an "idealized," tight-binding, and specific inhibitor of that enzyme is available. In the extreme case of an "irreversible" inhibitor the binding constant of the inhibitor is much lower than the enzyme concentration and the dissociation rate constant is much smaller than any first-order catalytic rate constant, such that, for all practical pur-

poses, every added inhibitor molecule can be assumed to completely knock out one enzyme molecule. In contrast to the comparison in Figs. 2A and 2B, we then need consider only the (more easily measurable) steady-state. In Fig. 3 we plot, using linear scales, the output flux of the pathway (v_o) against the concentration of the inhibitor I. Curves like the one shown in Fig. 3A are observed in titrations of the state-3 mitochondrial respiration rate with carboxyatractyloside (e.g., Refs. 26, 36, and 37). As in Fig. 2A, we use α to denote the initial slope of the curve. Because of the stated tight-binding characteristics of the inhibitor, in the *initial* stages of the titration curve, every inhibitor molecule added is bound so that the initial slope is equivalent to $(dv_o)_{ss}/de$. In the terminal stages of the titration, as the number of inhibitor molecules added approaches the number of potentially inhibitable enzyme molecules, hyperbolic curvature of negative sign may be observed, as the concentration of free enzyme molecules approaches the (albeit small) K_D of the inhibitor. Thus, if we wish to obtain the number of enzyme molecules present, we must extrapolate the linear region of the plot of Fig. 3A to the abscissa, to obtain I_{max} ($\equiv[e]$). We may then obtain the slope of the straight line between the point $(0,v_o)$ and $(I_{max},0)$, which is given by β. The Flux-Control Coefficient, C_o^o, is given by $\tan(\alpha)/\tan(\beta)$ and (in Fig. 3A) is evidently less than 1.

The curve in Fig. 3A is sigmoidal. However, more linear behavior may also be imagined (Fig. 3B). In this case, the equivalent slopes to those is Fig. 3A are identical, so that the Flux-Control Coefficient, C_o^o, is identically 1.

It is perhaps worth stating here the properties required of "irreversible" inhibitors if the data obtained with them are easily interpreted by means of the graphical method. First, their K_D values should be much less than the concentration of target enzymes, so that an adequately large linear region may be observed without the necessity of varying the enzyme concentration and extrapolating to infinite enzyme concentration. Similarly, each molecule bound should indeed act fully to inhibit its target enzyme. (In electron transport-linked phosphorylation, one test would be to check that the amount of, say, energy transfer inhibitor necessary to inhibit the ATP synthase/hydrolase enzyme was the same for the two reactions.) Finally, trivial effects, such as the presence

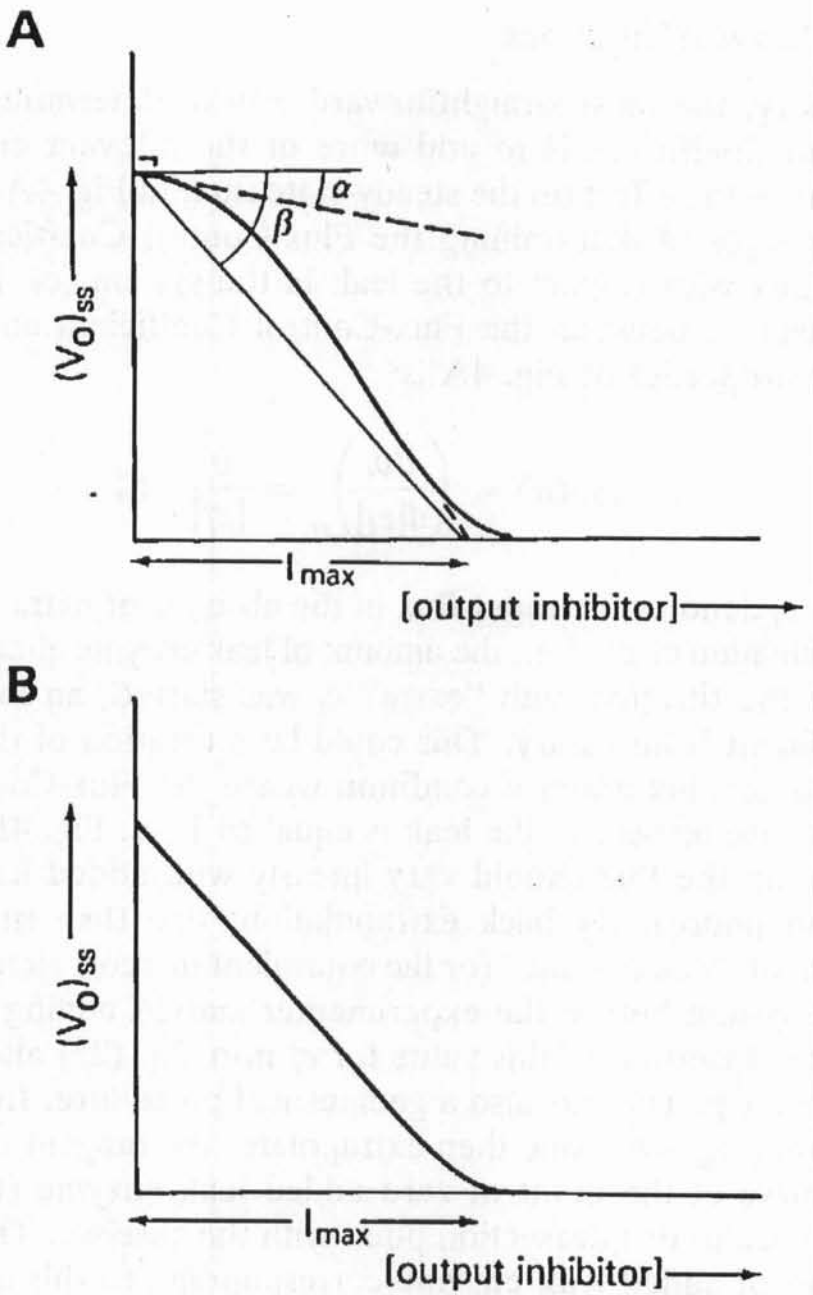

FIGURE 3 A generalized inhibitor titration of the flux through a metabolic pathway such as that in Fig. 2, using an "irreversible," tight-binding and specific inhibitor of one of the enzymes of the pathway, in this case, of enzyme *o*. Only steady-state effects are considered. The curves obtained may be of sigmoidal (A) or straight (B) shape, the former response indicating a Flux-Control Coefficient for enzyme *o*, $C_o^v < 1$ and the latter indicating $C_o^v = 1$. For further discussion, see text.

of catalytically inactive binding sites for the inhibitor, should be excluded. (It should be noted that failure to control each of these features will lead to an *underestimation* of the Flux-Control Coefficients, and thus possible appearance of pool behavior when channelling is in fact occurring.)

III.3. Enzyme Titrations

In theory, the most straightforward way of determining a Flux-Control Coefficient is to add more of the relevant enzyme and determine the effect on the steady-state flux. In Fig. 4A, we depict the example of determining the Flux-Control Coefficient of the input flux with respect to the leak in the system (cf. Fig. 1C).[36] The relation between the Flux-Control Coefficient and the geometric properties of Fig. 4A is:

$$\tan(\alpha) = \left(\frac{dv_i}{d[e_\ell]}\right)_{ss} = \frac{v_i}{[e_\ell^o]} \cdot C_\ell^i \qquad (25)$$

where v_i denotes the input flux in the absence of extra e_ℓ. For the determination of e_ℓ^o, i.e., the amount of leak enzyme already present before the titration with "extra" e_ℓ was started, an independent experiment is necessary. This could be a titration of the same or some other flux under a condition where the Flux-Control Coefficient with respect to the leak is equal to 1 (cf. Fig. 4B). In such a titration the flux should vary linearly with added leak enzyme (protonophore). By back extrapolation, one then finds e_ℓ^o, the amount of "leak enzyme" (or the equivalent thereof) already present in the system before the experimenter started adding extra leak enzyme. Insertion of this value for e_ℓ^o into Eq. (25) allows one to calculate $C_\ell^{v_i}$. There is also a geometrical procedure. In the actual titration (Fig. 4A), one then extrapolates the tangent to the titration curve at the point of zero added leak enzyme (the dashed line), back to its intersection point with the abscissa. The negative amount of added leak enzyme corresponding to this intersection point is then called the extrapolated virtual leak e_ℓ'. Since:

$$\tan(\alpha) = v_i/e_\ell' \qquad (26)$$

the Flux-Control Coefficient of the input flux with respect to the leak is then given by the ratio of extrapolated virtual leak in the calibration experiment (Fig. 4B) to the extrapolated virtual leak in the actual experiment (Fig. 4A) (combining Eqs. (25) and (26)):

$$C_\ell^{v_i} = e_\ell^o/e_\ell' \qquad (27)$$

provided that $(v_i)_{ss} = v_{cal}$ before the extra e_l was added.

One may also consider a titration of the output flow with leak

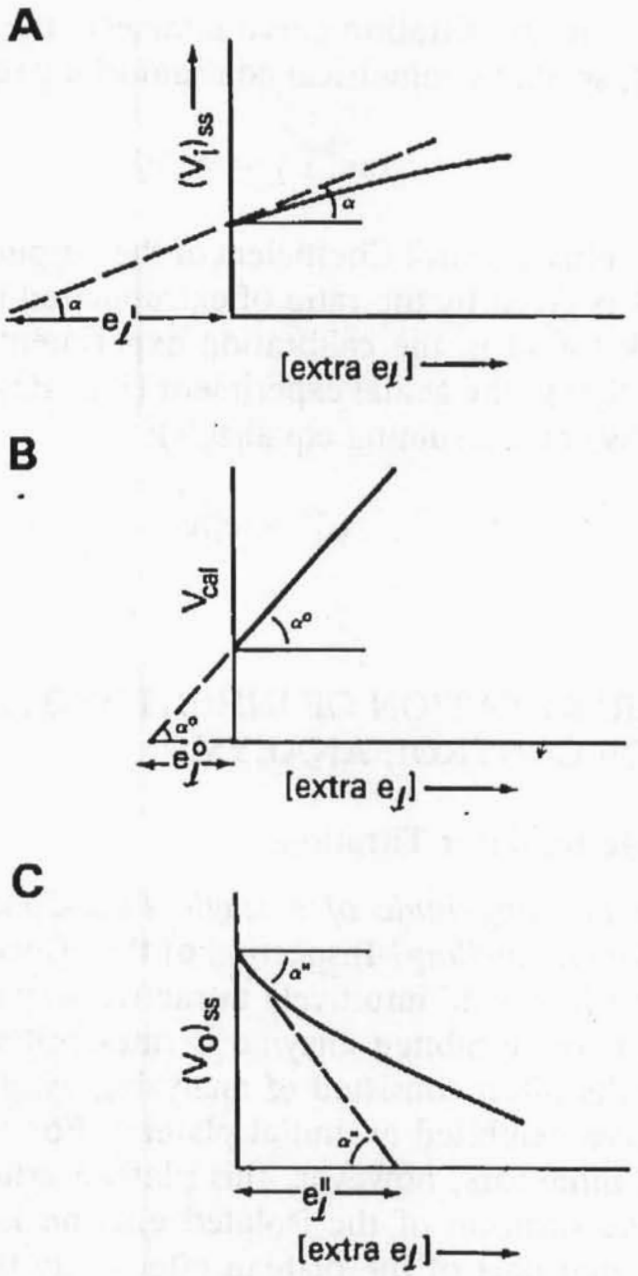

FIGURE 4 Assessment of Flux-Control Coefficients by means of adding extra enzyme molecules to the system. A: Steady-state effect of added leak enzyme on the input flux v_i. B: Calibration titration, titrating a flux completely dependent on leakage under conditions where the Flux-Control Coefficient of the leak equals 1. C: Steady-state effect of added leak enzyme on the output flux v_o. For further discussion, see text.

enzyme (cf. Fig. 4C). Here the Flux-Control Coefficient is given by:

$$\tan(\alpha'') = (v_o/e_l^o) \cdot C_l^{v_o} \qquad (28)$$

where v_o is the output flux in the absence of extra leak enzyme.

The tangent to the titration curve intersects the abscissa at an e_i value of e_i'', so that geometrical considerations tell us that:

$$\tan(\alpha'') = v_o/e_i'' \qquad (29)$$

Hence, the Flux-Control Coefficient of the output flux with respect to the leak is given by the ratio of extrapolated leaks, the extrapolated leak found in the calibration experiment (Fig. 4B) taken relative to that in the actual experiment (Fig. 4C) (combining Eqs. (28) and (29) and assuming equal v_o's):

$$C_i^{v_o} = e_i^o/e_i'' \qquad (30)$$

IV. INTERPRETATION OF INHIBITOR TITRATIONS IN TERMS OF CONTROL ANALYSIS

IV.1. Single Inhibitor Titrations

IV.1.a. *Is the magnitude of a single Flux-Control Coefficient a criterion for channelling*? Inspection of the effect of specific inhibitors on the flux is an intuitively attractive way of determining to what extent the inhibited enzyme is rate-limiting. Interpretation of the results often consisted of analyzing whether or not the titration curve exhibited an initial plateau. For the case of nonirreversible inhibitors, however, this plateau criterion is insecure, because the titration of the isolated enzyme is hyperbolic (Fig. 2B), such that part of the plateau effect may be concealed. The proper way of interpreting this type of experiment is by considering the ratio, $\tan(\alpha')/\tan(\alpha^o)$ in Fig. 2 as discussed above.

In the case of an irreversible, tight-binding inhibitor (cf. Fig. 3), Fig. 2B becomes a straight line (cf. Fig. 3B), such that the presence of a plateau in the steady-state titration (cf. Fig. 3A) *is* a criterion for non-rate (i.e., incomplete) limitation. In fact, one can readily evaluate the Flux-Control Coefficient in the way discussed in Section III.2.

Intuitively one might expect that in a nonchannelled system both the input and the output enzyme activity are present in excess, in the sense that elimination of a few input enzyme molecules would

hardly affect the steady-state flux because the other input molecules would take over. In this view, titration curves with irreversible inhibitors of either the input or the output enzyme, measuring the output flux, should exhibit a plateau. The control theory analysis tells us that in the absence of prior knowledge of Elasticity Coefficients, any Flux-Control Coefficient could take any magnitude (cf. Table I). The rigid criterion is the summation theorem (Eq. (11)), i.e., that the Flux-Control Coefficients of any flux with respect to the different enzymes should add up to 1. Therefore, if a Flux-Control Coefficient with respect to, say, the output enzyme amounts to 1, then the summation theorem could remain unviolated (and thereby the nonchannelling model unrefuted) if the sum of the Flux-Control Coefficients of the same flux with respect to the leak and the input enzyme would be zero.

It should be noted that this does not imply that the Flux-Control Coefficient with respect to the input enzyme would have to be equal to zero: the Flux-Control Coefficient with respect to the leak reaction may be negative. This latter possibility is rather feasible (and has been demonstrated experimentally[34,38]) when the flux considered is the output reaction rate, since an increased leak is likely to reduce that flux, such that the Flux-Control Coefficient of the output flux (v_o) with respect to the leaks would be negative. If the flux considered is the input reaction rate, increased leaks will usually stimulate the flux, i.e., the Flux-Control Coefficients with respect to the leak will usually be positive. For this flux, a Flux-Control Coefficient of 1 with respect to, say, the output enzyme would require the Flux-Control Coefficient with respect to the input enzyme to be nil, i.e., any inhibitor of the input enzyme would have to be without, or with negative, effect on the flux. Otherwise the nonchannelling model of Fig. 1C would have to be refuted.

IV.1.b. *Experimental results.* Only a few experiments have come close to determining the sum of the Flux-Control Coefficients for a certain flux. Groen and colleagues[36] made an almost, but not quite, complete determination of the Flux-Control Coefficients of mitochondrial respiration with respect to the enzymes participating in oxidative phosphorylation. The sum of these Flux-Control Coefficients did not significantly exceed 1. Titration of photophosphorylation in chromatophores[5–8] and reversed electron transfer

in submitochondrial particles[39,40,10,30,41] with electron-transfer inhibitors, H^+-ATPase inhibitors and protonophores have been carried out, but the absolute magnitudes of the Flux-Control Coefficients were not evaluated, partly because not all the inhibitors were of the irreversible type. Recently Pietrobon and colleagues have carried out such an evaluation and found the summation theorem to be violated, indicating channelling of the protons involved in energy coupling (D. Pietrobon, personal communication).[76]

IV. 2. Double Inhibitor Titrations

IV.2.a. *The rule of thumb.* The binding characteristics of even tight-binding inhibitors (such as rotenone[42] or antimycin[43]) can be nonlinear and it can be difficult to determine the binding characteristics under the same experimental conditions as those under which the steady-state flux is titrated. One way out of this is to compare the titration curve of, say, the output enzyme in the absence of any other inhibitor, with one in the presence of an inhibitor of, say, the input enzyme. Assuming that the binding characteristics of the titrated inhibitor do not depend on the activity of the system, they cancel in this comparison. The assumption can be validated by the symmetrical experiment.[29,31,44]

The intuitive basis for doing such dual inhibitor titrations in investigations of channelling was that, for the case where the metabolites were "pooled," inhibition of a significant fraction of the input enzymes would make the input enzymes more, and the output enzyme less, rate-limiting (and *vice versa*). Subsequent titration of the output enzyme would, at least initially, be less effective if pool behavior were exhibited (cf. Fig. 1E). These expectations have been used as rules of thumb in the interpretation of dual inhibitor titrations.[39,40,5,6]

IV.2.b. *How inhibition of the input enzymes affects the potency of an inhibitor of the output enzymes*

(b1) Expectations based on the control theoretic analysis. The initial part of the titration of the output flux with an irreversible inhibitor of the output enzyme is described by the Flux-Control Coefficient of the output flux with respect to the output enzyme, C_o^o. Table I gives its expression in terms of the Turnover Elasticity

Coefficients and the enzyme concentrations. If we use the first-order approximation in which the Turnover Elasticity Coefficients are independent of $[M]$, or, in the case of energy coupling, $\Delta\tilde{\mu}_H$, then the relevant expression in Table I predicts that upon exclusively inhibiting the input enzyme (i.e., lowering e_i), C_o^o should decrease. In other words, upon inhibition of the input enzyme, an inhibitor of the output enzyme should become less potent, which confirms the intuitive basis for this type of experiment which was given above.

At least two caveats emanate from the expression for C_o^o. The first is that the expected decrease in C_o^o might be so small as to be undetectable. This would occur if $e_o \cdot [t\epsilon]_M^o$ would be much smaller than $e_i \cdot [-t\epsilon]_M^i + e_\ell \cdot [t\epsilon]_M^\ell$, i.e., either if there is very little output enzyme (i.e., if e_o would be small), if the turnover Elasticity Coefficient of the output enzyme with respect to M would be small (output enzyme saturated with M; $[t\epsilon]_m^o$ small), if the system is rather leaky (i.e., if e_ℓ would be large), or if the leak has a high elasticity towards $M([t\epsilon]_M^\ell$ large). A consequence of this caveat is that, in the absence of further knowledge about the thermokinetic properties of the system, one should require that the input-enzyme inhibitor *increase* the control by the output enzyme inhibitor (as was observed in some cases,[39,10,30,6] like in Fig. 5B) before one may consider the notion of delocalized metabolism as potentially refuted.

The second caveat is that the prediction could be compromised if the Turnover Elasticity Coefficients were to be $[M]$-dependent in some peculiar way, e.g., such that $-[t\epsilon]_M^i$, $[t\epsilon]_M^\ell$ or $1/[t\epsilon]_M^o$ would greatly increase when the input enzyme is inhibited.

Here, we recall that the Turnover Elasticity Coefficients are the dependences of the enzymic turnover numbers on the logarithm of the activity (concentration) of M, or in other words, the dependencies on the electrochemical potential of M. In the case of protonic free-energy transduction, the $[t\epsilon]_{\Delta\tilde{\mu}H}^{v_x}$ are the slope of the plot of v_x/e_x versus $\Delta\tilde{\mu}_H/RT$. Large regions of linear $\Delta\tilde{\mu}_H$-dependence of reaction rates have been found.[32] Reported (e.g., Ref. 45) $\Delta\tilde{\mu}_H$ dependencies of ATP synthesis are most often nearly linear except (and there partly because the experimental set-up is biased towards ATP synthesis) at low rates of ATP synthesis [*pace* 46]. Inhibitor titrations of ATP synthesis are generally carried out at high rates of ATP synthesis.

An apparent exception to this rule of near linearity is the proton leakage, which tends to be "nonohmic."[46,47] It has become clear, however, that not much of this apparent "nonohmicity" is real. Much of it may really stem from slippage in proton pumps,[48–50] or from the very fact that free-energy transduction is organized in a channelled fashion.[3,32] If proton leakage were nonohmic in the sense suggested in Ref. 47, this would imply[49] that $[t\epsilon]^{i}_{\Delta\tilde{\mu}H}$ would decrease with decreasing $|\Delta\tilde{\mu}_H|$, hence with increasing inhibition of the input enzyme. This would not (cf. Table I) compromise the above prediction that inhibition of the input enzyme should lower the Flux-Control Coefficient, C^o_o, of the output enzyme.

(b2) Experimental results. In membrane-linked free-energy transduction, the effect of inhibitors of the primary proton pump on the control exerted by the secondary proton pump on the output flux has been studied by several authors. In ATP-energized reverse electron transport in submitochondrial particles, inhibition of the H^+-ATPase turned out to increase C^o_o, i.e., the flux control by the electron-transfer chain.[8,10,30,39] Within the assumption of constant Turnover Elasticity Coefficients, this would be in conflict with the expression for C^o_o, and thus be an indication of channelling.

An explanation of these experimental observations within the framework of nonchannelled energy coupling would have to invoke (cf. Table I, C^o_o) that upon inhibition of the input enzyme the Turnover Elasticity Coefficients of the input enzyme, $([-t\epsilon]^{i}_{\Delta\tilde{\mu}H})$, or of the leak $([t\epsilon]^{i}_{\Delta\tilde{\mu}H})$, would *increase* sufficiently to compensate for the immanent decrease in C^o_o through the reduction of e_i. Alternatively, the Turnover Elasticity Coefficient of the output enzyme for $\Delta\tilde{\mu}_H$ would have to *decrease* rather strongly upon inhibition of the input enzyme. Neither of these three changes is likely to occur in the required direction, however. Upon inhibition of input enzymes, one might expect $|\Delta\tilde{\mu}_H|$ to decrease. This would move the input reaction further away from equilibrium and thus,[32] if anything, probably *decrease* its Turnover Elasticity Coefficient. If proton leakage were nonohmic in the sense postulated by Nicholls,[51] and discussed by Jackson and colleagues,[46] then its Turnover Elasticity Coefficient would decrease with the decreasing $|\Delta\tilde{\mu}_H|$. Similarly, the decrease in $|\Delta\tilde{\mu}_H|$ would bring the output enzyme closer to equilibrium. Since in all the cases studied, the output

reactions seem to be of the kinetically reversible[32] type, one would expect this to lead to an increase in its Turnover Elasticity Coefficient. Consequently, we may stipulate that variation of the Turnover Elasticity Coefficients is not likely to account for the experimental observations in submitochondrial particles we just discussed.

In photophosphorylation in chromatophores antimycin A, an inhibitor of electron transfer (i.e., causing a decrease in e_i) increased, rather than (as expected for pooled metabolism) decreased C_o^o, i.e., the effect of oligomycin, an inhibitor of the H^+-ATPase (cf. Fig. 5B; the open dots give the titration with oligomycin in the presence of antimycin renormalized to 100% at zero oligomycin.[5,6] If the Turnover Elasticity Coefficient are $\Delta\tilde{\mu}_H$-independent, this decrease is incompatible with the unchannelled scheme of Fig. 1C. No effect of antimycin A was explicable in terms of the expression for C_o^o in Table I, if $e_o \cdot [t\epsilon]^o_{\Delta\tilde{\mu}H}$, i.e., the dependence of v_o on $\Delta\tilde{\mu}_H$, would be much smaller than $e_i[-t\epsilon]^i_{\Delta\tilde{\mu}H} + e_i[t\epsilon]^i_{\Delta\mu H}$. The Flux-Control Coefficient with respect to the H^+-ATPase (C_o^o) would have to equal 1 (cf. Table I). Other experiments (see below), however, suggested that C_o^o be close to zero.

IV.2.c. *How inhibition of the output enzymes affects the potency of an inhibitor of the input enzymes*

(c1) Expectations based on the control theoretic analysis. The symmetrical experiment (cf. Fig. 5A) considers how substantial inhibition of the output enzyme affects the titration of the output flux with an inhibitor of the input enzyme. Even within the approximation of $[M]$- (or $\Delta\tilde{\mu}_H$)-independent Turnover Elasticity Coefficients, the expression for C_i^o (Table I) by itself is not conclusive in predicting the outcome: the decrease in e_o due to the action of the output enzyme inhibitor (oligomycin in Fig. 5A; the open dots) would tend to decrease C_i^o, but a decrease in j might counteract this effect. Thus the validity of the intuitive picture of this version of dual inhibitor titration would seem to depend on near constancy of j (the reduced $P/2e$ ratio, cf., Eq. (16)).

Hitchens and Kell[5-8,29] pointed out that in the case of photophosphorylation by chromatophores, the $P/2e$ ratio (which in the absence of uncoupling equals j) is not affected by inhibition of the output enzyme. Thus, their result that C_i^o was not affected by

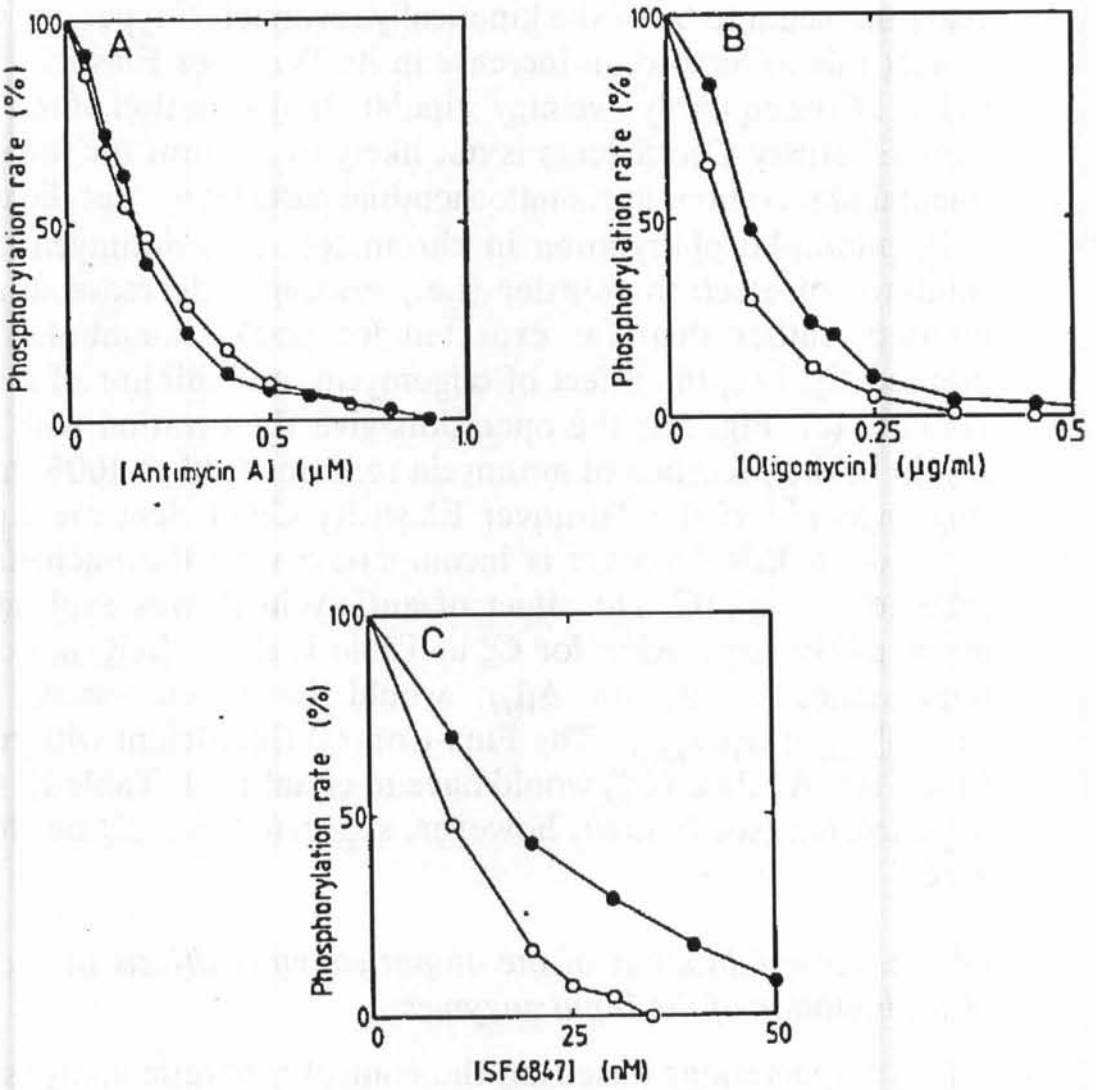

FIGURE 5 Double inhibitor titrations of photophosphorylation by bacterial chromatophores. In each case,the rate of photophosphorylation (in a system corresponding to that of Fig. 2) is titrated with an inhibitor of e_i (antimycin-A) or of e_o (oligomycin or dicyclohexylcarbodiimide, DCCD). Rates are given as percentages of the values in the absence of titrated inhibitor. A: Titration of v_o with antimycin in the absence (•) or presence (○) of a 50% inhibitory titre of DCCD. B: Titration of v_o with oligomycin, in the absence (•) or presence (○) of a 50% inhibitory titer of antimycin A. It may be seen that (i) initial plateaus are absent, and (ii) the "normalized" titration curves map onto each other (A), or (B) the one inhibitor even slightly fortifies the other. C: Uncoupler inhibitor titration of photophosphorylation by bacterial chromatophores, using the uncoupler SF6847 (3,5-di(*t*-butyl)-4-hydroxybenzylidene malononitrile) and the energy transfer (e_o) inhibitor venturicidin. Venturicidin was either absent (•) or present (○) at a 63% inhibitory concentration. It may be observed that in this case the uncoupler's potency is actually increased by the e_o-inhibitor. Data From Refs. 5, 6 and 8.

elimination of some 50% of the output enzymes (cf. Fig. 5A) can only be reconciliated with the abscence of channelling if (i) $e_o \cdot [t\epsilon]^o_{\Delta\tilde{\mu}H}$ is by far the largest term in $[a\epsilon]_{tot}$, or (ii) if, upon inhibition of the output enzymes, the $\Delta\tilde{\mu}_H$ sensitivity of the leak or the input process suddenly drops from a dominating magnitude (see the expression for C^o_i in Table I).

We shall first examine the former possibility. Since there was evidence that the P/2e ratio was insensitive to inhibition of either the input or the output system, the leak was presumably negligible and j was equal to 1 (i.e., $P/2e$ equal to the theoretical $P/2e$ ratio). The strong inhibitory effect of antimycin on photophosphorylation observed by Hitchens and Kell (Fig. 5A) may be consistent with $e_o \cdot [t\epsilon]^o_{\Delta\tilde{\mu}H}$ being the largest term in $[a\epsilon]_{tot}$: Table I then predicts a Flux-Control Coefficient, C^o_i, near 1. However, a consequence of the dominance of $e_o \cdot [t\epsilon]_{\Delta\tilde{\mu}H}$ and the sheer absence of leakage would be that (Table I) C^o_o would have to be close to zero (see also Eq. (11)). Hitchens and Kell (Fig. 5B), however, observed that both oligomycin and DCCD, inhibitors of e_o (Fig. 1E), did have a strong effect on photophosphorylation, an effect that was not reduced by inhibiting the input system (see above, and closed circles in Fig. 5B). This indicated (see also Section b(1) above) that C^o_o would be close to 1. We conclude that possibility (i) is not realistic.

For the possibility (ii), $e_o \cdot [te]^o_{\Delta\tilde{\mu}H}$ would have to be insignificant with respect to either of the other terms in $[a\epsilon]_{tot}$. It is unlikely that $[t\epsilon]^\ell_{\Delta\tilde{\mu}H}$ would decrease with $\Delta\tilde{\mu}_H$ since all reported nonohmic conductance of the leak is such that $[t\epsilon]^\ell_{\Delta\tilde{\mu}H}$ increases rather than decreases with $\Delta\tilde{\mu}_H$. The remaining possibility is that $[-t\epsilon]^i_{\Delta\tilde{\mu}H}$ is dominant in $[a\epsilon]_{tot}$ and strongly decreases upon inhibition of the output reaction. This would amount to the input reaction becoming insensitive to changes in $\Delta\tilde{\mu}_H$ at higher magnitudes of the latter, a phenomenon not yet observed experimentally, but expected from theoretical calculations for "kinetically irreversible proton pumps."[32] However, if indeed $e_i \cdot [-t\epsilon]^i_{\Delta\tilde{\mu}H}$ would be much greater than $e_o \cdot [t\epsilon]^o_{\Delta\tilde{\mu}H}$, then the flux-control by the input reaction would be very small. This is in apparent contrast with the finding of Hitchens and Kell[5,6] (reproduced in Fig. 5A) that the effect of antimycin on photophosphorylation is quite strong. Indeed, from Fig. 5A, one can estimate (cf. Section III) that the Flux-Control Coefficient

of photophosphorylation with respect to the input enzyme is very close to 1. Consequently, also, possibility (ii) disappears. We conclude that if indeed the P to $2e$ ratio in these experiments remained equal to its magnitude at zero (negligible) leak, the observation (Fig. 5A) that DCCD did not affect the flux-control by antimycin may be taken as a confirmation of channelling.

(c2) Further experimental results. Baum[39,40] had preceded Hitchens and Kell[5,6] by carrying out the same type of titration in reversed electron transfer in submitochondrial particles (cf. Refs. 10, 30 and 41) and had observed that the flux-control by the input enzyme was slightly increased or unaffected by partial inhibition of the output enzyme. In reversed electron transport in submitochondrial particles, the rate of ATP hydrolysis is usually only marginally affected by inhibition of the electron transfer chain (Ref. 52), perhaps because under the prevailing conditions ATP hydrolysis hardly responds to changes in $\Delta\tilde{\mu}_H$ (contrast Ref. 53; according to Table I a small $[-t\epsilon]^i_{\Delta\mu H}$ implies a small C^i_o). On the other hand, inhibition of the electron-transfer chain has a strong effect on reversed electron flow (C^o_o is high, arguably, cf. Table I, because the $\Delta\tilde{\mu}_H$-dependence of the proton leakage reaction is stronger than that of the reverse electron transport). As a consequence, inhibition of the output enzyme in the case of reversed electron transport by submitochondrial particles strongly decreases the flow ratio j, even such that j is nearly proportional to e_o. Thus the effect of the inhibition of the output enzyme on C^o_i through the reduction of the factor e_o in the numerator of the expression for C^o_i in Table I might be cancelled (but see below) by the decrease in j, leaving the effect on C^o_i of the inhibition through decreasing e_o in $[a\epsilon]_{tot}$ (Table I). An agonistic effect of inhibition of the output enzyme on the effectiveness (with respect to the output flow) of inhibition of the input enzyme might thus be consistent with the absence of channelling. Hence, according to our present analysis (but see below), the results obtained by Baum *et al.* and Westerhoff *et al.* (*op. cit.*) for the effect of rotenone on the inhibitory effectiveness of oligomycin with respect to ATP-energized reversed electron transport in submitochondrial particles would not of themselves falsify the absence of channelling; they are consistent with a delocalized scheme (Fig. 1B), as well as with a channelled scheme (Fig. 1A).

IV.2.d. *If small amounts of both inhibitors are used.* Although in submitochondrial particles the conclusiveness of the second type of dual inhibitor titrations is thus jeopardized by the uncertainty about how j changes when a significant fraction of the output enzymes is eliminated, this may not be so if the discussion were to be limited to cases where only *small amounts* of inhibitor of the output enzyme are added. One may analyze this case quantitatively by taking the mixed second derivative of the steady-state output flux with respect to both inhibitors.[16] Such a mixed derivative describes the effect of adding one inhibitor to the effectiveness of the other inhibitor. We here amend this approach by taking the derivative of $-C_i^o$ with respect to the logarithm of the activity (e_o) of the output enzyme. (Because C_i^o describes the control of the output flux by the input enzyme, $-C_i^o$ relates to the effect of an inhibitor of the input enzyme on the output flux). A negative value for this derivative implies that (a little) inhibition of the output enzyme would decrease the output-flux control coefficient with respect to the input enzyme, C_i^o, which corresponds to the intuitively (see above) expected result. Using the expression for C_i^o (Table I), and assuming the Turnover Elasticity Coefficients to be constant, we find for this derivative:

$$\frac{d\,-\,C_i^o}{d\,\ln\,e_o} = -e_o \cdot [t\epsilon]_M^o \cdot \left\{ e_i \cdot [-t\epsilon]_M^i + e_t \cdot [t\epsilon]_M^t - [a\epsilon]_{\text{tot}} \left(\frac{\mathrm{d}\,\ln\,j}{d\,\ln\,e_o} \right) \right\} \Big/ \{ j([a\epsilon]_{\text{tot}})^2 \} \quad (31)$$

If the reduced *P/2e* ratio, i.e., j, is not significantly affected by the inhibition of the output enzyme, this implies that $d\,\ln j/d\,\ln e_o$ can be neglected. Unless there is substrate-inhibition (i.e., $[t\epsilon]_M^o$, or $[t\epsilon]_M^t < 0$), or product enhancement (i.e., $[t\epsilon]_M^i < 0$) by M, this would leave the right-hand side and thus the left-hand side of Eq. (31) negative definite. Thus we see that for small amounts of added inhibitors and if the *P/2e* ratio and Turnover Elasticity Coefficients are essentially constant, inhibition of the output enzymes should lead to the intuitively reasonable result of a decreased potency of an input-enzyme inhibitor.

If we could predict generally how the *P/2e* ratio (j) would vary

with the concentration of added inhibitor of the output enzyme, then Eq. (31) could also be used to predict (still within the non-channelled framework) the result of this type of dual inhibitor titration experiment in the more general case in which j does vary. Using control theory, this is indeed possible.[32] We may define the Control Coefficient of the flow ratio, j, with respect to the output enzyme as:

$$C_o^j \overset{\text{def}}{=\!=} \left(\frac{d \ln j}{d \ln e_o}\right)_{ss} \tag{32}$$

With Eqs. (16) and (9), this can be written as (using the fact that $\ln (j) = \ln (v_o) - \ln (v_i)$):

$$C_o^j = C_o^o - C_o^i \tag{33}$$

Using the expressions for C_o^o and C_o^i given in Table I, the control exerted by the output enzyme on the $P/2e$ ratio (j) can be expressed in terms of the Turnover Elasticity Coefficients, j, and the enzyme concentrations[32]:

$$C_o^j = \{(1 - j) \cdot e_i[-t\epsilon]_M^i + e_t \cdot [t\epsilon]_M^t\}/[a\epsilon]_{\text{tot}} \tag{34}$$

It is of some interest to ask under which circumstances the reduced $P/2e$ ratio, j, is, according to Eq. (34), not affected by inhibitors of the output enzyme. One circumstance is if there were no leak, i.e., $j = 1$ and $e_t = 0$. A second is a vast excess of output enzyme, or if the output enzyme were highly elastic with respect to M (e.g., because it is close to equilibrium).[27] Equation (34) also shows that for small e_t and high $P/2e$ ratios, C_o^j is close to zero. Hitchens and Kell[5] have stressed that this applies to the chromatophores with which they carried out their dual inhibitor titration experiments.

Combination of Eqs. (31) and (34) allows the expression (in terms of Turnover Elasticity Coefficients, enzyme concentrations and $P/2e$ ratio) of the effect of output inhibitor on the output-flux control by the input enzyme:

$$-\frac{dC_i^o}{d \ln e_o} = -e_o \cdot [t\epsilon]_M^o \cdot e_i \cdot [-t\epsilon]_M^i/([a\epsilon]_{\text{tot}})^2 \tag{35}$$

Except in the exceptional cases of product stimulation (i.e., positive $[t\epsilon]_i^i$), or substrate inhibition (i.e., negative $[t\epsilon]_i^o$), the right-hand side of this equation is negative, implying that in the unchannelled situation, inhibition of the output enzyme would be expected to decrease the effect of an inhibitor of the input enzyme on the output flux. This corresponds to the rule of thumb for this type of dual inhibitor titration. It should be kept in mind that in the derivation of Eqs. (34) and (35) the Turnover Elasticity Coefficients were assumed to be constant.

One may wonder if an expression similar to Eq. (35) might exist that would indicate the effect of a small amount of input inhibitor on the potency of the output inhibitor. The answer is yes. Moreover, it is the identical expression, since:

$$\frac{dC_o^o}{d\ln e_i} = \left(\frac{d^2 \ln v_o}{d\ln e_i \cdot d\ln e_o}\right)_{ss} = \left(\frac{d^2 \ln v_o}{d\ln e_o \cdot d\ln e_i}\right)_{ss} = \frac{dC_i^o}{d\ln e_o} \tag{36}$$

In view of Eq. (35) and the fact that also small amounts of output inhibitors already seemed to increase, rather than, as predicted by Eq. (35), decrease the potency of the output inhibitor in reversed electron transport in submitochondrial particles,[10,30] we may now reinterpret the results of these experiments and conclude that, at constant Turnover Elasticity Coefficients, the variation of j (the reduced $P/2e$ ratio) could not have been such as to produce the experimentally observed behavior in a nonchannelled coupling system. Consequently, either the Turnover Elasticity Coefficients in the submitochondrial particles depend significantly on $\Delta\tilde{\mu}_H$ in one of a set of particular ways[13,16] (but see Section IV.2.c), or free-energy transduction in this system must be channelled.

IV.3. Uncoupler-Inhibitor Titrations

Another type of dual inhibitor titration applied to the question of channelling in proton-mediated free-energy transduction is that in which the effect is measured of inhibition of either the input or the output reaction on the titration of the output flux by an activator of the leakage (cf. Fig. 5C). In the chemiosmotic model, so-called uncouplers act as such activators, due to their membrane-protonophoric properties such that they decrease the magnitude of $\Delta\tilde{\mu}_H$. The expression for the control coefficient C_i^o of the output

flux by the leaks is given in Table I. The control is negative, but we shall discuss it in terms of its absolute magnitude.

The usual experiment examines the effect of eliminating some 50% of the output enzyme molecules on the effectiveness with which an uncoupler reduces the output flow. The qualitative picture (rule of thumb) is that inhibition of the output enzymes would, if anything, cause an increase in $M(\Delta\tilde{\mu}_H)$, such that more, or at least the same amount, of uncoupler would be needed to reduce the remaining output flow by a given percentage. With the first-order approximation that the Turnover Elasticity Coefficients are independent of $\Delta\tilde{\mu}_H$, the expression for C_i^o would make the same prediction, if one neglected effects on the reduced *P/2e* ratio (i.e., on *j*) *and* if $j < 1$ (*but see below*). In the presence of output inhibitor, e_o would be reduced and hence $-C_i^o$ would become smaller such that more uncoupler would be needed to effect the same fractional reduction in output flux. However, if the inhibition of the output reaction were to cause a reduction in $j(v_o/v_i)$, this effect would tend to lead to an increased control of the output flux by the uncoupler.

The experimental observations both in reversed electron transport in submitochondrial particles[40,10,54] and in photophosphorylation in chromatophores[7,8] were such that inhibition of the output reaction increased the effectiveness of uncouplers. As discussed above, in submitochondrial particles the flow ratio *j* does decrease upon inhibition of the output process, possibly even proportionately with the decrease in e_o; because the decrease in $[a\epsilon]_{tot}$ caused by the decrease in e_o is bound to be less than proportional, this would predict the observed increase in control of output flow by the leakage reaction.

In photophosphorylation in chromatophores, *j* (assessed as the *P/2e* ratio) is not significantly affected by partial inhibition of the H^+-ATPases.[55] Consequently, it seemed[7,8] that within the non-channelled paradigm the *increased* effectiveness of uncouplers upon inhibition of the output pump in the latter system can only be explained through $e_o \cdot [t\epsilon]_H^o$ being very small compared to the other terms in $[a\epsilon]_{tot}$ (Table I) *and a more than proportionate* decrease in those other terms upon inhibition of the output pump. As discussed above (Section IV.2), $[t\epsilon]_{\Delta\tilde{\mu}H}^i$ would rather be expected to increase upon inhibition of the output enzyme. $e_i \cdot [t\epsilon]_{\Delta\tilde{\mu}H}^i$ might indeed decrease, but it would then have to be much

greater than $e_o \cdot [t\epsilon]^o_{\Delta\tilde{\mu}H}$; however, this would require the control of the output flux by the input reactions (C^o_i) to be very small, whereas the observations plead for it to be close to 1 (see above). Thus it would seem that in the chromatophores, with their constant *P/2e* ratio, the observation that output-enzyme inhibitors potentiate uncouplers constitutes evidence against a nonchannelled ("delocalized") organization of free-energy transduction in that system.

In our argumentation, however, there has been a jump in logic: we assumed simultaneously that $j < 1$ (i.e., incomplete coupling) *and* that j would not be affected by partial inhibition of output enzymes. As we discussed in Section IV.2.d, the simplest (but not the only) scenario for having j independent of the activity of the output enzyme would be to have no leak. However, this would make j equal to 1 which is not compatible with our present condition. Further inspection of Eq. (34) demonstrates that the condition that j is independent of the activity of the output enzyme requires that $j = 1$ *and* that there be no leakage. Thus, for constant *P/2e* ratio, the argumentation in the preceding paragraph for the chromatophores is unrealistic and hence its conclusion is unjustified.

It is therefore also instructive to go through the analysis where the effect of addition of *very small amounts* of output-enzyme inhibitor on the uncouplers' potency to inhibit the output flux is considered. As in Section IV.2.d, we assume all Turnover Elasticity Coefficients to be constant (i.e., independent of $\Delta\tilde{\mu}_H$) and take the negative derivative of C^o_i with respect to the logarithm of the output enzyme concentration (we use the expression for C^o_i given in Table I)

$$\frac{-d - C^o_i}{d \ln e_o} = -e_o \cdot [t\epsilon]^o_M \cdot \left\{ e_i \cdot [-t\epsilon]^i_M + e_i \cdot [t\epsilon]^i_M \cdot (1 - j) - [a\epsilon]_{tot} \cdot \left(\frac{d \ln j}{d \ln e_o} \right) \right\} \Big/ \{ j \cdot ([a\epsilon]_{tot})^2 \} \quad (37)$$

Application of Eq. (34) to Eq. (37) allows us to determine whether or not, at constant Turnover Elasticity Coefficients, one should expect the output enzyme to decrease the potency of the uncoupler

(which we here define as $-C_i^o$):

$$-\frac{d - C_i^o}{d \ln e_o} = e_o \cdot [t\epsilon]_M^o \cdot e_i \cdot [t\epsilon]_M^i/([a\epsilon]_{tot})^2 \qquad (38)$$

Since this expression is positive definite, one would expect the output enzyme inhibitor to *potentiate* the uncoupler. This result then is in contrast to the rule of thumb for this experiment (see above and in Refs. 7 and 8) but in line with conclusions drawn from thermokinetic simulations of free-energy transducers,[12,15,49] which did employ constant Turnover Elasticity Coefficients and which suggest that an inhibitor of the output enzyme should in fact be expected (to a greater or lesser extent) to potentiate the uncoupler in inhibiting the output flow. This is what was found experimentally. It urges a reconsideration of the conclusions drawn from uncoupler-inhibitor experiments *re*-channelling: the observations may, in fact, be in keeping with free-energy transduction in those systems being delocalized (unchannelled).

The symmetrical experiment considers the effect of inhibition of the input reaction on the effectiveness of uncoupler to inhibit the output flux. Also here the observations point toward a synergism. Also in this case this is in line with the predictions made for the unchannelled case: the inhibitor causes a decrease in e_i which tends to increase $-C_i^o$ (cf. Table I). Effects through j would work in the same direction. Consequently, this type of uncoupler-inhibitor titration is particularly unsuited to distinguish channelled from delocalized metabolism.

V. CRITIQUES OF DUAL INHIBITOR TITRATION STUDIES

The interpretations of dual inhibitor titration results as evidence for channelling have been criticized by earlier authors. Here we shall discuss these critiques in terms of the present analysis.

Parsonage and Ferguson[11] have pointed out that upon elimination of 50% of the output enzymes $\Delta\tilde{\mu}_H$ (M) might *not* increase, due to nonohmic conductance of the proton leaks (although much

of the leak pathway is sometimes[46] taken to be via the F_0F_1 ATP synthases). Consequently, the effectiveness of the inhibitor of the input enzymes would have remained the same. A similar reasoning leads to the expectation that the effectiveness of an *uncoupler* would also be unchanged upon elimination of 50% of the *output* enzymes.

Although this would indeed cancel the intuitive basis for the interpretation of this type of experiment, it is not immediately obvious if it would really work out this way: the fact that $\Delta\tilde{\mu}_H$ would be constant does not absolutely show that the effectiveness of the uncoupler would be unchanged. This is illustrated by the left-hand part of the expression for C_i^o (Table I): if $\Delta\tilde{\mu}_H$ (M) is unchanged then $\epsilon^o_{\Delta\tilde{\mu}H}$ is unchanged, but this does not tell us that C_ℓ^M is unchanged and hence whether C_ℓ^o is unchanged. (Note that C_ℓ^M addresses what happens if the leak is increased; then $\Delta\tilde{\mu}_H$ is still expected to change.) From this expression it also follows that the supposed increased in $v_\ell = v_o \cdot (1 - 1/j)$ (due to the supposed nonohmic conductance of the leak), which would indeed predict an increased control by uncoupler of $\Delta\tilde{\mu}_H$ and hence of the output flow, might be compensated by the increase in $[t\epsilon]^\ell_{\Delta\tilde{\mu}H}$ in $[a\epsilon]_{tot}$, the supposed nonohmicity of the leak, plus the decrease in e_o. A proper question to ask here is suggested by the expression for $|C_\ell^o|$ itself (Table I). The postulated increase in $[t\epsilon]^\ell_{\Delta\tilde{\mu}H}$ would still be expected to decrease the flux-control by the protonophore, provided that the flux ratio not be affected (decreased) by the addition of the output inhibitor. As discussed above (but see the pertinent discussion of Eq. (38) and its conclusion) this is the case in photophosphorylation in chromatophores, though not in reversed electron transfer in submitochondrial particles. Thus, at least in the former system, the argument of Parsonage and Ferguson[11] was not conclusive.

O'Shea and Thelen[67] have used another argument to criticize the existing interpretations of the increased effectiveness of uncoupler in the presence of an inhibitor of the output reaction. These authors pointed out that in the stationary state the input flux must equal the output flux plus the leak flux. They then assumed both the leak and the output flux to be proportional to $\Delta\tilde{\mu}_H$, such that:

$$v_i = (L_\ell + L_o) \cdot \Delta\tilde{\mu}_H \qquad (39)$$

where L_ℓ and L_o would be proportional to the activity of the uncoupler and the output proton pump, respectively. Essentially, the argument then continues:

$$v_o = L_o \cdot \Delta\tilde{\mu}_H = L_o \cdot v_i/(L_o + L_\ell) \qquad (40)$$

It is then pointed out that the magnitude of L_ℓ needed to decrease v_o to half its magnitude in the absence of uncoupler is equal to L_o. Consequently, this amount of uncoupler would decrease upon reduction of the activity (L_o) of the output enzymes, due to addition of an inhibitor of the latter.

This argumentation is inadequate because it oversimplifies the problem (see also Ref. 56). Disregarding the demonstrable insufficiency of a proportionality assumption between the phosphorylation or leak rate and $\Delta\tilde{\mu}_H$ (note that such an assumption is much stronger than a linearity assumption, which is simply equivalent to assuming $\Delta\tilde{\mu}_H$-independent Turnover Elasticity Coefficients (see above), and demonstrably erroneous (e.g., Refs. 46 and 57)), we next point out two assumptions implicit in the above argumentation that were not mentioned by O'Shea and Thelen.[67] First, they assumed the input flux not to be affected by inhibition of the output enzymes. In our terminology, the authors assumed $j = v_o/v_i$ to vary proportionately with the activity of the output enzymes. As we argued in the previous section, one then indeed may expect an increased effectiveness of uncouplers. However, in the experimental system of Hitchens and Kell, criticized by O'Shea and Thelen, j is constant (see above) and certainly not proportional to v_o. The second assumption was that addition of the uncoupler would not increase v_i. In realistic well-coupled systems the uncoupler will always tend to increase v_i, and therefore the amount of uncoupler needed to inhibit v_o by 50% is not just a function of the activity of the output enzymes (L_o), but also of the extent to which the input enzyme decelerates upon an increase in $\Delta\tilde{\mu}_H(\epsilon^i_{\Delta\tilde{\mu}H})$. We conclude (see also Ref. 56) that the "analysis" of dual inhibitor titrations by O'Shea and Thelen is too simplistic to deal with the problem.

A third critique of dual inhibitor titration studies[13,16] (but also J. B. Jackson, personal communication; M. Wikström, personal communication) has been that systems like that in Fig. 1C with

specific rate equations for the different reactions, when solved for the steady state, would exhibit, despite their organization with a "pool," some of the results of dual inhibitor titrations that have been presented as evidence for channelling. Our above analysis does not contradict these results: if the ($\Delta\tilde{\mu}_H$-dependence of the) Turnover Elasticity Coefficients are allowed to take arbitrary magnitudes, then the results of individual dual inhibitor titrations may be anything. What we hope to have pointed out by the above analysis is that: (i) inhibition of the input pump increasing or leaving unaffected the output-flux-control by the output pump is an unlikely result in a nonchannelled model, and (ii) inhibition of the output pump increasing the flux-control by the input pump is similarly unlikely, especially if the reduced *P*/2*e* ratio (j) is unaffected by that inhibition.

A fourth critique[10] of dual inhibitor titrations pointed out that the inhibitors used (e.g., DCCD) might not be specific and that inhibitor binding might depend on $M(\Delta\tilde{\mu}_H)$. Obviously, this criticism is important and has to be considered for every actual dual inhibitor titration experiment individually. However, there is evidence that these criticisms do not apply to the experiments considered here[29,31,44,58] and we do not here consider this further.

A fifth critique[14] was exceptional in that it was completely experimental in nature. Its strategy was to use an experimental model system of which one would be certain that its metabolism would be organized in the nonchannelled fashion. Van der Bend and colleagues chose the system of partly purified yeast mitochondrial H^+-ATPase and pure bacteriorhodopsin coreconstituted into liposomes. By virtue of the unrelatedness of these two proton pumps, it was considered likely that any components possibly organizing *in vivo* free-energy transduction in the channelled fashion would be either absent or nonfuctional. The results obtained were that up to 69% inhibition of the H^+-ATPase did not affect the control exerted by bacteriorhodopsin on photophosphorylation. The symmetrical experiment gave the analogous result. It was[14] argued that these results contradicted the expectations of the original rules of thumb used by Hitchens and Kell,[5,6] interpreting the results obtained with this type of experiment in bacterial chromatophores which were, in a delocalized situation, that 50% inhibition of one type of proton pump (i.e., input or output) should substantially

reduce the flux-control exerted by the other type. In line with what we concluded in Section IV.2.c, the proper rule of thumb for this type of experiment is indeed that partial inhibition of the input pumps should *not increase* the flux-control by the output pumps and *vice versa*. With respect to the uncoupler-output-inhibitor experiment, Van der Bend *et al.*[14] found results that, also according to the criteria of Hitchens and Kell,[7,8] would be inconsistent with the presumed nonchannelled organization of the free-energy transduction in the system. Similarly, therefore, the uncoupler-inhibitor titration results obtained by Hitchens and Kell[7,8] would not prove channelling.

Recently, we[9,15] discussed in detail some of the possible shortcomings of the approach of Van der Bend *et al.*[14] One is the possibility that the mere 2% of *in vivo* ATP synthesis functional in this system would still be organized in a channelled fashion, owing to reconstitution of the necessary factors, which might be present as impurities in the H^+-ATPase preparation. The fact that low concentrations of (*in vivo* potent) uncouplers did not eliminate the energy coupling in this system was considered not in favor of this possibility,[15] but of course the presence of background uncoupler binding sites could also have been responsible for this observation.

VI. THE IMPACT OF SLIP

Above we have analyzed the system in terms of three reactions only: input, output and leakage. Pietrobon and colleagues[48,50] (see also Ref. 32) have pointed out that one should consider the possibility that the coupling of the input proton pumping to the chemical reaction that drives it is not complete. Similarly, the output proton flux may not be completely coupled to the ATP synthesis reaction. However, measurements of redox-linked proton translocation contra-indicate a $\Delta\tilde{\mu}_H$-*dependent* slip in the experiments of Hitchens and Kell.[44,58] We[3] have noted that the experimental indications for molecular slip reported by Pietrobon and colleagues[48] could also be explained in terms of completely coupled proton pumps functioning in a channelled system. In the present context, however, we should examine the possibility of whether a model with slipping input and output enzymes organized in a nonchan-

neled fashion could account for the experimental observations that have been taken to be indicative of channelling.

We shall assume that inhibitors of input and output enzymes act by eliminating all activities of either enzyme molecule (i.e., both the coupled reaction, the proton slip and the chemical slip). In this case the formulae we derived above remain valid, with the provision that the fluxes mentioned are the proton fluxes, not the fluxes through the chemical reactions. The observed fluxes are usually those through the chemical reactions coupled to the proton fluxes. For instance, with respect to the output chemical reaction one finds (using Eqs. (7) and (17)) (cf. Table I):

$$C_i^{\text{ochem}} = \epsilon_M^{\text{ochem}} \cdot C_i^M = n_H^o \cdot e_o \cdot [a\epsilon]_M^{\text{ochem}}/(j \cdot [a\epsilon]_{\text{tot}}) \quad (41)$$

$$\begin{aligned} C_o^{\text{ochem}} &= 1 + \epsilon_M^{\text{ochem}} \cdot C_o^M \\ &= \{e_i \cdot [-t\epsilon]_M^i + e_\ell \cdot [t\epsilon]_M^\ell + e_o \cdot ([t\epsilon]_M^o - n_H^o \\ &\quad \cdot [t\epsilon]_M^{\text{ochem}})\}/[a\epsilon]_{\text{tot}} \end{aligned} \quad (42)$$

$$\begin{aligned} -C_\ell^{\text{ochem}} &= \epsilon_M^{\text{ochem}} \cdot (-\epsilon_\ell^M) \\ &= \left(\frac{1}{j} - 1\right) \cdot n_H^o \cdot e_o \cdot [t\epsilon]_M^{\text{ochem}}/[a\epsilon]_{\text{tot}} \end{aligned} \quad (43)$$

Here $[t\epsilon]_M^{\text{ochem}}$ represents the Turnover Elasticity Coefficient of the chemical reaction partially coupled to the output proton flux:

$$[t\epsilon]_M^{\text{ochem}} \underline{\underline{\text{def}}} \left(\frac{\partial \ln |v_o^{\text{chem}}|}{\partial \ln [M]}\right) \cdot \frac{v_o^{\text{chem}}}{e_o} = \frac{1}{e_o} \cdot \left(\frac{\partial v_o^{\text{chem}}}{\partial \ln [M]}\right) \quad (44)$$

and $\tilde{n}_H^o$ is the (now variable) stoichiometry of the number of protons per chemical turnover in the output reaction. It is useful to elaborate on the relationship between $[t\epsilon]_M^{\text{ochem}}$ and $[t\tilde{\epsilon}]_o^o$ by using the fact that $v_o = \tilde{n}_H^o \cdot v_{\text{ochem}}$:

$$[t\epsilon]_M^o = \tilde{n}_H^o \cdot [t\epsilon]_M^{\text{ochem}} + \frac{v_o}{e_o} \cdot \left(\frac{\partial \ln \tilde{n}_H^o}{\partial \ln [M]}\right) \quad (45)$$

With this, the above expression Eq. (42) for C_o^{ochem} can be rewritten as:

$$C_o^{\text{ochem}} = \left\{ e_i \cdot [-t\epsilon]_M^{\text{ochem}} + e_t \cdot [t\epsilon]_M^t + v_o \cdot \left(\frac{\partial \ln \bar{n}_H^o}{\partial \ln [M]} \right) \right\} \Big/ [a\epsilon]_{\text{tot}} \quad (46)$$

In the term $v_o \cdot \partial \ln (\bar{n}_H^o)/\partial \ln [M]$, we find an effect of M-dependent slippage on the Flux-Control Coefficient. This term will usually be positive, as the absolute amount of slip in the output reaction may be taken to tend to increase with $[M](\Delta\bar{\mu}_H)$.[32] It is, however, not *a priori* clear how the presumed decrease in $[M]$ associated with inhibition of the input reaction will affect this factor. In order to explain the experimental result of an increase in C_o^{ochem} the factor $\partial\bar{n}_H^o/\partial \ln [M]$ would have to increase dramatically with $[M]$, since it must more than compensate for the decrease in v_{ochem}. Thus the $\ln [M]$- or $\Delta\bar{\mu}_H$-dependence of the slip would have to increase dramatically with decreasing $\Delta\bar{\mu}_H$. Though this may seem unlikely (in fact, one would rather expect a decrease) the possibility cannot yet be ruled out completely.

With respect to the observation that inhibition of the output reaction does not reduce the flux control by the input reaction, whereas $v_o^{\text{ochem}}/v_i^{\text{ochem}}$ (i.e., the $P/2e$ ratio) is constant, we rewrite the above expression for C_i^{ochem} (Eq. (41)):

$$C_i^{\text{ochem}} = e_o \cdot [t\epsilon]_M^{\text{ochem}} \cdot \bar{n}_H^i \cdot v_i^{\text{chem}}/(v_o^{\text{ochem}} \cdot [a\epsilon]_{\text{tot}}) \quad (47)$$

Inhibition of the output enzyme would tend to increase $\Delta\bar{\mu}_H$ and thus decrease $\bar{n}_H^i$. Thus the effect of slip would not be in the direction that would explain a nondecreasing flux control by the input enzymes. Phenomena of slip in the input proton pumps therefore can not alone be used to explain the results of dual inhibitor titrations in terms of pool behavior.

Finally we discuss the observation that uncouplers become more effective upon elimination of some of the output enzymes, at constant initial $v_o^{\text{chem}}/v_i^{\text{chem}}$. We rewrite the above expression for $-C_t^{\text{ochem}}$ as:

$$-C_\ell^{\text{ochem}} = \left(\frac{\tilde{n}_H^i \cdot v_i^{\text{chem}}}{v_o^{\text{chem}}} - \tilde{n}_H^o\right) \cdot e_o \cdot [\iota\epsilon]_M^{\text{ochem}}/[a\epsilon]_{\text{tot}} \quad (48)$$

The effect of slip here would be that upon inhibition of the output enzyme, the increase in $\Delta\tilde{\mu}_H$ would cause an increase in $\tilde{n}_H^o$ and a decrease in $\tilde{n}_H^i$. This then would account for a decrease in $-C_\ell^{\text{ochem}}$, which is contrary to what was observed.

We conclude that, within the context of a delocalized chemiosmotic coupling scheme, $\Delta\tilde{\mu}_H$ dependent slip in the proton pumps cannot readily account for the experimental observations that have been taken as support for channelling.

VII. TITRATION ENDPOINTS

Up to this point we have mainly considered the initial parts of inhibitor or uncoupler titrations of the output flux, simply because at this point the titrating substance has had the least chance to mess up the system. There is however a different point in the titration curves that is extremely informative on channelling. This is the point of intersection of the output flux with the abscissa (the inhibitor concentration axis). It gives the amount of inhibitor of the input enzymes or uncoupler needed to lower $M(\Delta\tilde{\mu}_H)$ such that the output reaction has come to a halt. In a model without channelling this intersection point should be independent of the number of output enzymes (provided that there are no other enzymes present that carry out an uncoupled reverse version of the output reaction). Especially with the uncoupler titrations, the results tend to be such that less uncoupler is needed to *completely* abolish the output flux when some of the output enzymes have been eliminated; a model without channelling is again insufficient (see also Refs. 12 and 17 and Fig. 5C).

VIII. RESULTS OF DUAL INHIBITOR TITRATIONS EXPECTED IF METABOLISM WERE CHANNELLED

Up to this point, dual inhibitor titration results have been compared to predictions on the basis of the unchannelled model of

Fig. 1C. It was inferred that failure of the results to match predictions would reject the model given by Fig. 1C. In so far as the description of the actual experimental system consisting of input, output and leak enzymes was correct, this would then imply that the assumption of the absence of channelling had been incorrect. As such, indications for (at least partial) channelling have been found. In this section we briefly discuss the experimental results from another point of view: (to what extent) are they consistent with "complete" channelling?

In this discussion we take the free-energy transducing system as homogeneous: it simply consists of a large number of channels such as shown in Fig. 1A, each with one input enzyme molecule, one output enzyme molecule and one leak. A first characteristic of channelled systems is that the definition of Flux-Control Coefficients as a simple (log/log) derivative of flux with respect to enzyme activity is not possible, because the addition of a small amount of enzyme to such a system will not have an effect opposite to the effect of the elimination of a quantitatively equal fraction of those enzymes. Whereas the latter will have a dramatic effect (for enzymes 1 and 2 in Fig. 1A), the former will have little effect.[59] Here we shall define the Flux-Control Coefficients in the sense of eliminating enzyme molecules.

With regard to the output flow, both the Flux-Control Coefficient of the input and that of the output enzyme would be expected to equal 1, as elimination of $x\%$ of the input (or output) enzymes would simply reduce the output flux by $x\%$. Also, these Flux-Control Coefficients would be independent of inhibition of any of the enzymes. It is somewhat more difficult to predict what the Flux-Control Coefficient by the leaks would be, as doubling of the number of leakage "enzymes" per channel might have any effect between almost nil and complete inhibition of the output flux. In general, however, the Flux-Control Coefficient of the leak will be somewhere between zero and -1. A consequence is that the flux-control summation theorem (Eq. (11)) will generally not apply to the output flux in channelled systems.[29–32] Also, for a completely channelled system the control by the leak on the output flux (when measured in terms of the apparent Flux-Control Coefficient C_ℓ^o) is predicted to be independent of inhibition of output enzymes.

Whereas the former predictions are all reasonably well reflected by the (dual) inhibitor titrations of proton-mediated biological free-energy transducers (see above), the latter prediction seems at odds with the result that, after addition of input or output inhibitor, *less* uncoupler is needed to obtain the same relative inhibition of the output flux, especially if the concentrations of uncoupler $< e_i$ or e_o ("substoichiometric uncoupling"). Hitchens and Kell have attributed this phenomenon to the ability of the low molecular weight uncoupler molecules to shuttle between the channels in which output flux can be uncoupled from input flux; after elimination of some of the input or some of the output enzymes, the number of *such* channels would be decreased and less uncoupler would be needed to do the uncoupling. It is also required, for this analysis, that it is the uncoupling step *itself* (and not the rate at which uncoupler molecules diffuse between uncoupling sites) which is the (most) rate-determining step in uncoupler action, at least for non-pore-forming uncouplers.[7,8,44,54] However, one might also expect the uncoupler to visit the channels with inhibited output pump, and, since these might (in a pool analysis) have a higher average energization state than the uninhibited channels, the uncoupler might need more time there.[15] Thus the argument of Hitchens and Kell[7,8] might alone seem to be insufficient. A possible way out of this dilemma would be that uncouplers interact specifically with an energized state that could only arise from the direct (e.g., collisional[60]) or functional interactions of active input and active output enzyme molecules. Here, however, it would be unclear how uncouplers stimulate electron transfer in the presence of inhibitors of the H^+-ATPases, except that the effectiveness of an uncoupler is likely to be a very sensitive function of the "local" permittivity, and there are abundant indications for important uncoupler-ATP synthase interactions.[31]

Parenthetically, we note that this "substoichiometric" behavior is possible only for "irreversible" reactions, and that the application of metabolic control theory to inhibitor titrations generally might well be extended to the *fundamental* problems of agonist action in chemical pharmacology, a problem for which an understanding is still lacking.[61]

It is of some interest how the control of the *input* flux would be

distributed in the channelled case. We expect a flux control of +1 by the input enzymes, something between 0 and +1 for the leaks and positive but perhaps not quite +1 (in the case of absence of respiratory control) for the output enzymes. Also for the control of the input flux the summation theorem should be violated in the case of channelling.

IX. CONCLUSION

In this Comment we have used control theory to discuss the validity of the dual inhibitor titration approach as an assay for channelling in metabolism. We conclude *inter alia*: (i) *that increased effectiveness of an inhibitor of input enzymes upon elimination of some of the input enzymes is a reasonable criterion* for channelling; and (ii) that increased effectiveness of an output inhibitor upon elimination of some of the input enzymes is such a criterion, especially if the ratio between output and input flux is not affected by the said elimination. Strictly speaking, these conclusions depend, to different degrees, on assumed near independence of Turnover Elasticity Coefficients from $\Delta\tilde{\mu}_H$ (i.e., nearly linear relations between reaction rates and $\Delta\tilde{\mu}_H$). However, we have shown that the known or postulated nonlinear relationship does not work out so as to invalidate these criteria for some of the experimental systems examined to date. With respect to uncoupler-inhibitor titrations, we conclude that they are not as yet wholly robust criteria for channelling, except as regards the endpoints of such titrations.

Because of the multitude of factors to be considered (including the question of specificity of the inhibitors used), we appreciate that dual inhibitor titrations should never be the *sole* criterion for channelling. While the present analysis would indicate that the dual-inhibitor approach is indeed a potent means of assessing the presence of channelling in a metabolic or free-energy transducing system, proof of channelling should always include indications obtained through other approaches.[3,4,62–66]

For recent reviews of such approaches as applied to free-energy transduction, see Refs. 68–70. Some interesting results for free-energy transducing systems are reported in Refs. 71–76.

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