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A Linear Equation that Describes the Steady-State Kinetics of Enzymes and Subcellular Particles Interacting with Tightly Bound Inhibitors

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When an enzyme exhibits a high affinity for an inhibitor, the steady-state analysis of the mechanism is complicated by the non-linearity of normal dose–response plots or of reciprocal replots. It is shown here that dose–response measurements generate a linear plot of inhibitor concentration divided by degree of inhibition against velocity without inhibitor divided by velocity with inhibitor; the concentration of enzyme may be derived from the extrapolated intercept of such plots, and the mechanism of inhibition from replots of the variation of the slope with substrate concentration. The limiting cases where virtually all inhibitor molecules are bound or virtually all are free are described, together with the situation when a significant proportion of the substrate becomes bound. This type of analysis indicates that the inhibitors of oxidative phosphorylation, rutamycin and bongkrekic acid, are tightly bound to rat liver mitochondria.

When the association constant for the formation of an enzyme–inhibitor complex is high, a significant fraction of the inhibitor molecules in the system may become bound to the enzyme (Goldstein, 1944; Webb, 1963). This situation is particularly likely to occur when relatively high concentrations of enzyme are employed. Analyses of the kinetics of the inhibition based on the Michaelis–Menten equation are then invalid, because the assumption that the concentration of inhibitor that is free in solution is the same as the total inhibitor concentration is untrue (Webb, 1963). Several authors have derived steady-state equations that describe the reaction rates in the presence of tight-binding inhibitors that interact with the enzyme by 'competitive' or 'pure non-competitive' mechanisms (Fig. 1). In particular, the elegant treatise of Goldstein (1944) delineated the factors that control the appearance of such 'Mutual Depletion' kinetics. Goldstein (1944) showed that the ratio E_t/K_i† should be 0.01 or less for a Michaelis–Menten analysis to be valid, and that, with a sub-saturating concentration of inhibitor, virtually all of the added inhibitor molecules become bound to the enzyme if E_t/K_i exceeds 100; at intermediate E_t/K_i values, the total inhibitor is distributed between molecules free in solution and those complexed with the enzyme (reviewed by Webb, 1963). By a similar argument, the ratio E_t/K_m reveals the validity of the Michaelis–Menten treatment for rates obtained with substrate alone in the system (Goldstein, 1944; see also Webb, 1963; Cha, 1970; Rhoads & Garfinkel, 1971).

In all of the equations of Fig. 1, the derivations are facilitated by utilizing the ratio of v_i/v_0 or $v_i/V_{max.}$ instead of v_i alone, a principle central to the derivation given in this paper. The equations in Fig. 1 (*a*)–(*c*) apply only to the cases of competitive or non-competitive mechanisms of unireactant reactions. The equation in Fig. 1(*d*), a quadratic in v_i derived by Morrison (1969), is a more general form and is based on the general analysis of multi-substrate enzyme reaction mechanisms of Cleland (1963*a*,*b*, 1970). Morrison (1969) demonstrated that Lineweaver–Burk reciprocal plots become non-linear in the presence of tight-binding inhibitors (cf. Khoo & Russell, 1970), a conclusion implicit in the other equations of Fig. 1. Such non-linearity is not always apparent in experimental results, and the use of reciprocal plots could therefore lead to meaningless values of K_i or erroneous deductions as to the mechanism of inhibition. A linear form of the Morrison (1969) equation is derived below, in order to expedite the analysis of situations where a tight-binding inhibitor is (or is suspected to be) present.

Experimental Procedure

Rat liver mitochondria were prepared by the method of Johnson & Lardy (1967) with 0.25 M-sucrose–4 mM-tris–HCl–1 mM-tris–EGTA [ethanedioxybis(ethylamine)tetra-acetate], pH 7.4, as medium for both the washing and final suspension. Adenosine triphosphatase (ATPase) activity was assayed at 30°C by the procedure of Lardy & Wellman (1953) except that the reaction was initiated by addi-

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† Abbreviations: E_t = total concentration of enzyme; E_f = concentration of free enzyme; K_i = dissociation constant for inhibitor; I_t = total concentration of inhibitor; I_f = concentration of inhibitor free in solution; E_tI = enzyme–inhibitor complex; E_tA = enzyme–substrate complex; A_t = total concentration of substrate; v_i = velocity in the presence of inhibitor; v_0 = velocity without inhibitor.

$$(a)\ I_t = E_t i + K_i \frac{i}{1-i}$$

$$(b)\ I_t + \left(\frac{v_i}{v_0} - 1\right) E_t = K_i \left(\frac{v_0}{v_i} - 1\right)\left(1 + \frac{A_t}{K_a}\right)$$

$$(c)\ I_t = K_i \left[\left(\frac{A_t}{K_a} - \frac{aE_t}{K_a}\right)\left(\frac{1-a}{a}\right) - 1\right] + E_t\left[1 - a\left(1 + \frac{K_a}{A_t - aE_t}\right)\right]$$

$$(d)\ v_i^2 + N\left[\frac{1}{\sum \frac{N_i}{K_i}} + \frac{I_t - E_t}{D}\right] v_i - \frac{N^2 E_t}{D \sum \frac{N_i}{K_i}} = 0$$

Fig. 1. *Equations for enzyme reaction velocity in the presence of tight-binding inhibitors*

(*a*) Non-competitive, due to Easson & Stedman (1936) (see also Straus and Goldstein, 1943); $i=(1-v_i/v_0)$; (*b*) and (*c*) are for a competitive mechanism and are due to Krupka & Laidler (1959) and Goldstein (1944) (see also Huang & Niemann, 1951) respectively; $a=v_i/V_{max.}$; (*d*) is the general equation of Morrison (1969).

tion of carbonyl cyanide *m*-chlorophenylhydrazone after the mitochondria had been incubated for 3 min in portions of the reaction medium containing different amounts of inhibitor. For the bongkrekic acid experiment the medium consisted of 5 mM-ATP, 10 mM-phosphoenolpyruvate, 1.3 mM-$MgCl_2$, 67 μg of pyruvate kinase/ml, 30 mM-KCl, 13.3 mM-tris–HCl and 100 mM-sucrose, pH 6.6; the reaction was terminated by addition of trichloroacetic acid (final concn. 5%, w/v) after a measured time-interval during which not more than two-thirds of the phosphoenolpyruvate was hydrolysed. The amount of P_i released was corrected for that present at the time the uncoupling agent was added. For the rutamycin experiment the ATP concentration was 6.0 mM and the phosphoenolpyruvate and pyruvate kinase were replaced by 25 mM-sucrose. In this case small samples of the medium were inactivated with trichloroacetic acid at fixed time-intervals and the P_i in each sample was measured (Lardy & Wellman, 1953). The increase in P_i was linear with respect to time apart from a relatively small, but rapid, burst of P_i release immediately after addition of uncoupler. The rate was calculated as a least-squares fit of at least four measurements taken after the burst and before half of the ATP had been hydrolysed.

Theory

Derivation of a linear form of the Morrison equation

The initial steady-state velocity of an enzyme reaction may be represented by the general eqn. (1) (Morrison, 1969):

$$v_0 = \frac{NE_t}{D} \qquad (1)$$

The numerator term, N, contains the velocity constants and substrate concentrations that determine the maximum velocity of the reaction, and the denominator, D, is the sum of several terms, each of which represents the distribution of the enzyme in a particular form (Cleland, 1963*a*,*b*; Morrison, 1969); the nature of each term contributing to N and D depends on the mechanism of the reaction (Cleland, 1963*a*). Morrison (1969) also gives a general equation for the velocity when an inhibitor combines with several of the enzyme forms to produce 'dead-end' complexes with different dissociation constants:

$$v_i = \frac{NE_t}{D + I_f \sum \frac{N_i}{K_i}} \qquad (2)$$

N_i is the term in the denominator representing the distribution of the enzyme in the form that combines with the inhibitor (Cleland, 1963*b*). Additional terms must be introduced into eqn. (2) when the enzyme–inhibitor complex is not 'dead-end', but undergoes conversion by the enzyme into product(s) (Morrison, 1969). Also, terms containing I_t^2 or higher powers of I_t may be necessary if, for example, more than one molecule of inhibitor combines with a single form of the enzyme. If only one enzyme–inhibitor complex is formed, the fraction of the enzyme in the complex is given by eqn. (3) (Morrison, 1969):

$$\frac{E_i I}{E_t} = \frac{N_i \frac{I_f}{K_i}}{D + N_i \frac{I_f}{K_i}} \qquad (3)$$

When the inhibitor combines with more than one enzyme form it is easily shown that:

$$\frac{\Sigma E_i I}{E_t} = \frac{I_f \sum \frac{N_i}{K_i}}{D + I_f \sum \frac{N_i}{K_i}} \quad (4)$$

where $\Sigma (E_i I)/E_t$ is the total fraction of the enzyme combined with the inhibitor.

Eqn. (1) can be used to eliminate NE_t from eqn. (2), so that:

$$v_i = \frac{v_0 D}{D + I_f \sum \frac{N_i}{K_i}} \quad (5)$$

Rearrangement of eqn. (5) gives:

$$D + I_f \sum \frac{N_i}{K_i} = \frac{v_0}{v_i} D \quad (6)$$

$$I_f \sum \frac{N_i}{K_i} = \left(\frac{v_0}{v_i} - 1\right) D \quad (7)$$

$$I_f = \frac{\left(\frac{v_0}{v_i} - 1\right) D}{\sum \frac{N_i}{K_i}} \quad (8)$$

By substitution from eqns. (6) and (7), eqn. (4) becomes:

$$\frac{\Sigma E_i I}{E_t} = \frac{\left(\frac{v_0}{v_i} - 1\right) D}{\frac{v_0}{v_i} D} \quad (9)$$

Hence:

$$\Sigma E_i I = E_t \left(1 - \frac{v_i}{v_0}\right) \quad (10)$$

The conservation equation for inhibitor is:

$$\underset{\text{TOTAL}}{I_t} = \underset{\text{BOUND}}{\Sigma E_i I} + \underset{\text{FREE}}{I_f} \quad (11)$$

Therefore, from eqns. (10) and (8):

$$I_t = E_t \left(1 - \frac{v_i}{v_0}\right) + \frac{D\left(\frac{v_0}{v_i} - 1\right)}{\sum \frac{N_i}{K_i}} \quad (12)$$

Linear forms of eqn. (12) may be derived by dividing through by $(1 - v_i/v_0)$ or by $(v_0 - v_i)$:

$$\frac{I_t}{\left(1 - \frac{v_i}{v_0}\right)} = E_t + \frac{D}{\sum \frac{N_i}{K_i}} \cdot \frac{v_0}{v_i} \quad (13)$$

and

$$\frac{I_t}{(v_0 - v_i)} = \frac{E_t}{v_0} + \frac{D}{\sum \frac{N_i}{K_i}} \cdot \frac{1}{v_i} \quad (14)$$

As $(1 - v_i/v_0)$ is the degree of inhibition, i (Easson & Stedman, 1936; Straus & Goldstein, 1943; Webb, 1963) eqn. (13) may be written:

$$\frac{I_t}{i} = E_t + \frac{D}{\sum \frac{N_i}{K_i}} \cdot \frac{1}{1 - i} \quad (15)$$

Eqn. (15) can be rearranged to a quadratic in i:

$$(i^2 E_t) - i\left(I_t + E_t + \frac{D}{\sum \frac{N_i}{K_i}}\right) + I_t = 0 \quad (16)$$

This form has been utilized in computer-assisted simulation studies to determine the effect of different mechanisms on the parameters of eqns. (12)–(15) (see below).

Eqn. (12) can also be derived by substitution of $v_0 D$ for NE_t in Morrison's (1969) equation (Fig. 1*d*) (Henderson, 1971). It is more readily apparent from eqn. (12) than from the original Morrison (1969) equation that the equations of Fig. (1*a*) (Easson & Stedman, 1936) and Fig. (1*b*) (Krupka & Laidler, 1959) are particular cases of the general form (Fig. 1*d*) (cf. Webb, 1963).

Estimation of E_t by 'dose–response' measurements

If the concentration of enzyme and substrate(s) are kept constant in an experimental system, then eqns. (13) and (14) predict that measurements of v_0 and v_i at increasing concentrations of inhibitor, 'dose–response' measurements, should give linear plots

$$\frac{I_t}{1 - \frac{v_i}{v_0}} \text{ against } \frac{v_0}{v_i} \text{ or } \frac{I_t}{v_0 - v_i} \text{ against } \frac{1}{v_i}.$$

of Eqn. (13) yields the value of E_t from the intercept directly, in contrast with eqn. (14), and so eqn. (13) is referred to exclusively in the following discussion. It should be noted that v_0/v_i has a minimum value of 1, and estimation of the intercept on the

$I_t(1 - v_i/v_0)$ axis therefore requires extrapolation from experimentally determined points.

It is instructive to consider two limiting cases of this treatment. As Goldstein (1944) pointed out, when $E_t/K_i > 100$, virtually all of the inhibitor molecules are bound to the enzyme, so that eqn. (12) becomes:

$$I_t = E_t\left(1 - \frac{v_i}{v_0}\right) \qquad (17)$$

Similarly, when $E_t/K_i < 0.01$, virtually all of the inhibitor molecules are free, and:

$$I_t = \frac{D}{\sum \frac{N_i}{K_i}}\left(\frac{v_0}{v_i} - 1\right) \qquad (18)$$

In the case of eqn. (17) a plot of $I_t/(1 - v_i/v_0)$ against v_0/v_i is horizontal, but the intercept still yields the

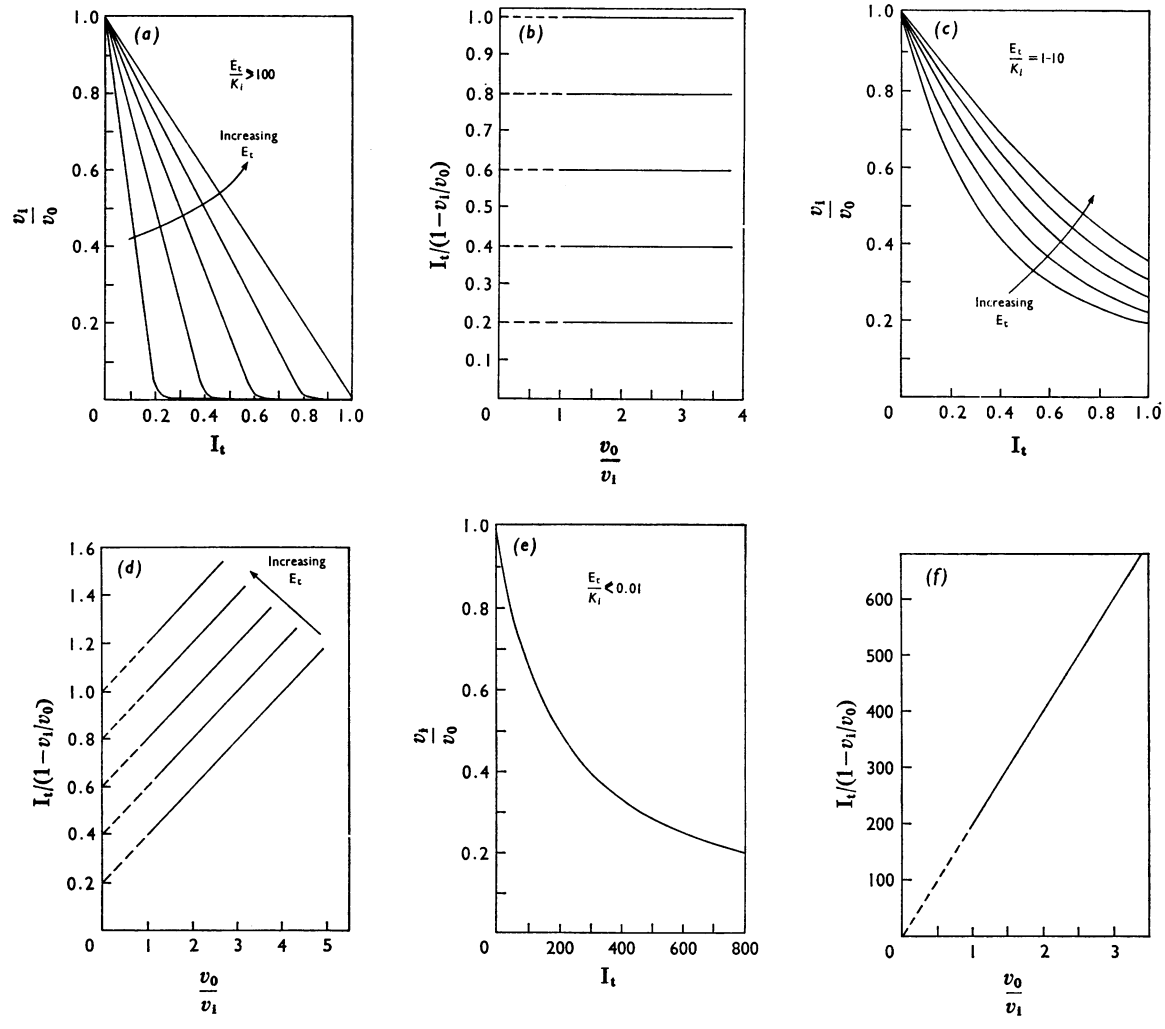

Fig. 2. *Relationship of dose–response curves and replots to changes in enzyme concentration at different affinities of enzyme for inhibitor*

Eqn. (16) was solved for i and $1/(1-i)$, i.e. $(1-v_i/v_0)$ and v_0/v_i, for the case of competitive inhibition (Fig. 4). The values of the parameters are $K_a=100.0$; $A_t=100.0$; $E_t=0.2$, 0.4, 0.6, 0.8 and 1.0; $K_i=0.0001$ (*a* and *b*), 0.10 (*c* and *d*) and 100.0 (*e* and *f*); I_t was varied as indicated in the diagrams; a unit of concentration for these parameters is omitted because the shapes of the curves depend only on the ratio of A_t/K_a, I_t/K_t, E_t/K_i and E_t/K_a. In (*e*) and (*f*) the same line was obtained at all values of E_t.

concentration of enzyme in the system (Fig. 2*b*). A simpler plot would be a normal dose–response type of v_i/v_0 against I_t; this is linear, and E_t can be obtained from the slope, $-1/E_t$, and the intercept on the abscissa, E_t (Fig. 2*a*). In the case where nearly all the inhibitor is free (eqn. 18), then a plot of $I_t/(1-v_i/v_0)$ against v_0/v_i is not horizontal, but has a slope of $D/\sum \frac{N_i}{K_i}$. Also, the extrapolated intercept crosses the axes at the origin (Fig. 2*f*) and so E_t cannot be evaluated. A graph of v_i/v_0 against I_t is not linear and its position is independent of E_t (Fig. 2*e*). However, a graph of v_0/v_i against I_t is linear, the intercept on the abscissa has the value $-D/\sum \frac{N_i}{K_i}$, and the slope is the reciprocal of this, i.e. both are related to the mechanism of inhibition. The latter is formally equivalent to a 'Dixon' plot (Dixon, 1953; Laidler, 1954) and illustrates the fact that reciprocal plots based on the Michaelis–Menten equation are valid in the region where $E_t/K_i < 0.01$. Eqn. (18) is therefore a generalized form of this type of equation.

At intermediate values of E_t/K_i, the full eqn. (12) applies. Normal dose–response curves are non-linear, and their position depends on E_t (Fig. 2*c*). Linear graphs are obtained only by plots of eqns. (13) (Fig. 2*d*) and (14) and E_t may be estimated from the extrapolated intercepts (Fig. 2*d*). The plots of $I_t/(1-v_i/v_0)$ against v_0/v_i should be parallel when E_t is varied and the dose–response study is repeated (Fig. 2*d*). Provided that E_t/K_i remains >0.01, the several intercepts should then yield a reliable estimate of E_t.

Provided that E_t/K_i is between 0.01 and 100.0, both $D/\sum \frac{N_i}{K_i}$ and E_t can be evaluated and eqn. (12) then enables the amounts of inhibitor bound to the enzyme and free in solution to be calculated from the measured velocities. When the binding of the inhibitor can be measured, the predicted binding can be conveniently compared with the measured binding by generating a linear Scatchard plot (bound inhibitor against bound/free inhibitor ratio; Scatchard, 1949), as depicted in Fig. 3. A discrepancy could indicate the presence of binding sites that are not related to the measured reaction, for example. Also, if more than one molecule of inhibitor combines per receptor site, the derivation of a completely general equation is not possible, and plots based on eqn. (13) are non-linear.

Relationship of slope to mechanism of inhibition

In the region where $E_t/K_i < 100$ the slope of a linear plot of eqn. (13) or (14) is $D/\sum \frac{N_i}{K_i}$. This expression contains the terms that reflect the mechanism of the reaction of the enzyme and its interaction with the inhibitor (Morrison, 1969). Thus, changes in the slope under different conditions should be diagnostic of the mechanism of inhibition and reveal the form(s) of the enzyme that combines with the inhibitor.

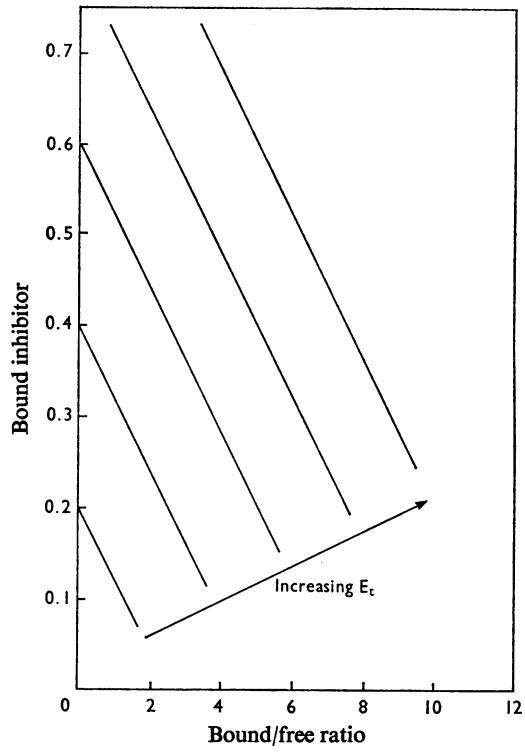

Fig. 3. *Scatchard plots for inhibitor at different concentrations of* E_t

Eqn. (16) was solved for $(1-v_i/v_0)$ by using the parameters of Fig. 2 except that $K_i=0.2$ and I_t is varied between 0.05 and 2.0; the value of $(1-v_i/v_0)$ was then used to generate values of bound and free I from eqn. (12).

For example, consider the common case where an enzyme reaction velocity (in the absence of products) is described by:

$$v_0 = \frac{V_{max.} A_t}{K_a + A_t} \qquad (19)$$

K_a may be the true Michaelis constant for a single substrate enzyme, or the apparent Michaelis constant of a multi-substrate enzyme reaction for which one substrate concentration is varied as the other substrates remain constant (Cleland, 1963*b*,*c*, 1970). Then:

$$\frac{E_f}{E_t} = \frac{K_a}{K_a + A_t}$$

E_f becomes the form with which the varied substrate, A_t, combines for the multi-substrate reaction. For the case where inhibitor combines only with the same form of enzyme as the substrate, i.e. competitive inhibition (Cleland, 1963*b*,*c*):

$$\sum \frac{N_i}{K_i} = \frac{K_a}{K_i}$$

When inhibitor combines with equal affinity to all enzyme forms (simple non-competitive):

$$\sum \frac{N_i}{K_i} = \frac{A_t + K_a}{K_i}$$

and with different affinities (mixed non-competitive):

$$\sum \frac{N_i}{K_i} = \frac{A_t}{K_{it}} + \frac{K_a}{K_{is}}$$

$$\frac{I_t}{1 - \frac{v_i}{v_0}} = E_t + K_i\left(\frac{A_t + K_a}{K_a}\right)\frac{v_0}{v_i} \quad \text{Competitive}$$

$$\frac{I_t}{1 - \frac{v_i}{v_0}} = E_t + K_i\left(\frac{A_t + K_a}{A_t}\right)\frac{v_0}{v_i} \quad \text{Uncompetitive}$$

$$\frac{I_t}{1 - \frac{v_i}{v_0}} = E_t + K_i\left(\frac{v_0}{v_i}\right) \quad \text{Non-competitive}$$

$$\frac{I_t}{1 - \frac{v_i}{v_0}} = E_t + \left(\frac{A_t + K_a}{\frac{K_a}{K_{is}} + \frac{A_t}{K_{it}}}\right)\frac{v_0}{v_i} \quad \text{Mixed non-competitive}$$

Fig. 4. *Forms of eq.* (13) *for different mechanisms of inhibition*

For details see the text.

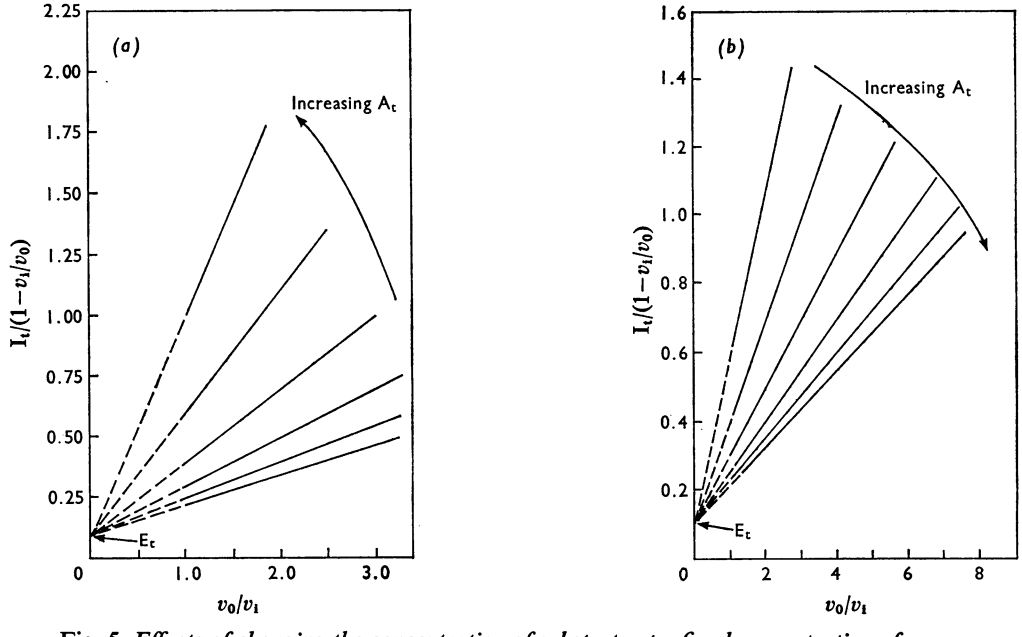

Fig. 5. *Effects of changing the concentration of substrate at a fixed concentration of enzyme*

The appropriate eqn. of Fig. 4 was used to generate dose–response curves with the parameters $E_t = 0.1$, $K_i = 0.1$, $K_a = 100.0$, and a concentration of I_t between 0.025 and 1.0. The fixed values of A_t were 25.0, 50.0, 100.0, 200.0, 400.0 and 800.0. (*a*) Competitive; (*b*) uncompetitive.

The forms of eqn. (13) for the different mechanisms are presented in Fig. 4, including uncompetitive, which may be of academic interest only in the case of single-substrate reactions (W. W. Cleland, personal communication).

When E_t is kept constant and dose–response measurements are repeated at increasing concentrations of A_t, the slopes of plots of $I_t/(1 - v_i/v_0)$ against v_0/v_i increase with A_t for the competitive case and decrease for the uncompetitive (Fig. 5). In the simple non-competitive case the slope is unrelated to A_t and gives the true K_i value directly (Fig. 6). Further replots of slopes against A_t (Fig. 6) or slopes against $1/A_t$ are linear for competitive and uncompetitive inhibitions respectively, and the extrapolated intercepts on the ordinate yield the true K_i values (Fig. 6). For mixed inhibition the behaviour of the slopes depends upon the relative values of K_{ii} and K_{is} [see Cleland (1963*b*) for definition]; replots of the slopes against A_t or $1/A_t$ are non-linear, but K_{ii} or K_{is} can still be evaluated from the extrapolated intercepts (Fig. 7). The non-linearity is not very apparent when $K_{ii}/K_{is} > 10$ or < 0.1 (see, e.g., Fig. 8). Hence the appearance of linearity in slope replots indicates that the inhibition is predominantly competitive or uncompetitive; experimental results would have to be examined very carefully for evidence of non-linearity before the case of mixed inhibition could be excluded.

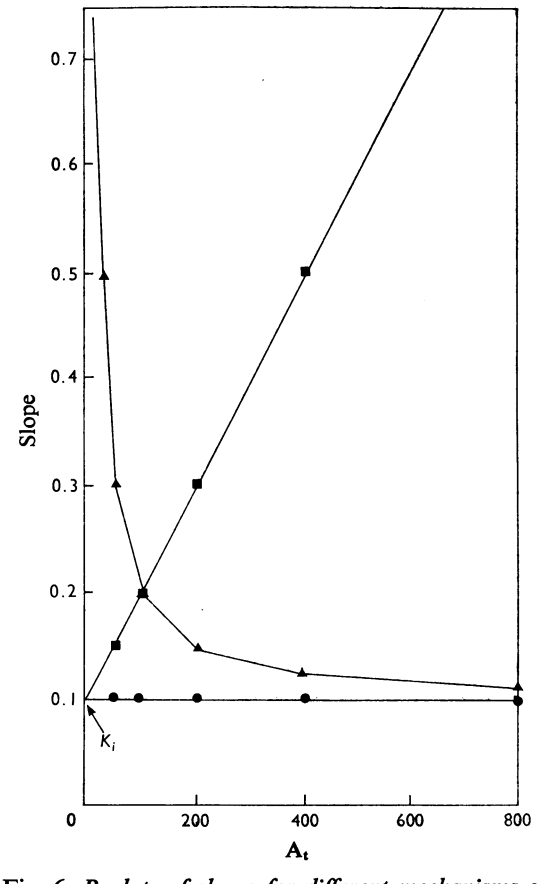

Fig. 6. *Replots of slopes for different mechanisms of inhibition*

Values of the parameters were as in Fig. 5. ■, Competitive; ▲, uncompetitive; ●, non-competitive.

When all inhibitor is bound to the enzyme and eqn. (17) applies rather than eqn. (13), it is obviously not possible to obtain changes in slope for mechanistic studies (cf. Goldstein, 1944).

For a multi-substrate reaction of known mechanism, the patterns of slope behaviour may be predicted from eqn. (16). Thus for the ordered Bi Bi mechanism used as an example by Morrison (1969) [see Cleland (1963*a*) for nomenclature]

$$i^2 E_t - i\left[I_t + E_t + \frac{K_{ia}K_b + K_a B + K_b A + AB}{\sum \frac{N_i}{K_i}}\right] + I_t = 0$$

where N_i may be the expression representing E_f, EA, EAB + EPQ, or EQ (Cleland, 1963*a*), or combinations of these terms. However, there are 14 possible types of interaction of inhibitor with this system, and the results would have to be accurate in order that some of the possibilities could be distinguished by replots of eqn. (13) alone. Nevertheless, if it is feasible also to measure equilibrium binding of the inhibitor to the enzyme in the presence of substrates, it should be possible to elucidate completely the enzyme form(s) that combines with inhibitor. For a multi-substrate reaction of unknown mechanism the mode of investigation can be the same as for a normal inhibitor, i.e. to vary the concentration of one substrate while keeping that of the others fixed (Cleland, 1970), but also to perform dose–response plots at each concentration of the varied substrate. This procedure allows linear replots from eqns. (13) or (14) to be used to deduce the 'competitive', 'uncompetitive' etc. nature, instead of non-linear reciprocal replots (Morrison, 1969; Khoo & Russell, 1970). The technique of deducing whether the tight-binding inhibitor combines 'upstream' or 'downstream' of the varied substrate is then the same as for normal inhibitors.

Effects of tight-binding substrates on linear plots

When the affinity of an enzyme for its substrate is sufficiently high for E_t/K_a to become greater than 0.01, the concentration of bound substrate is a significant fraction of A_t; rate equations may be adjusted for this by substitution of $(A_t - E_t A)$ for A_t

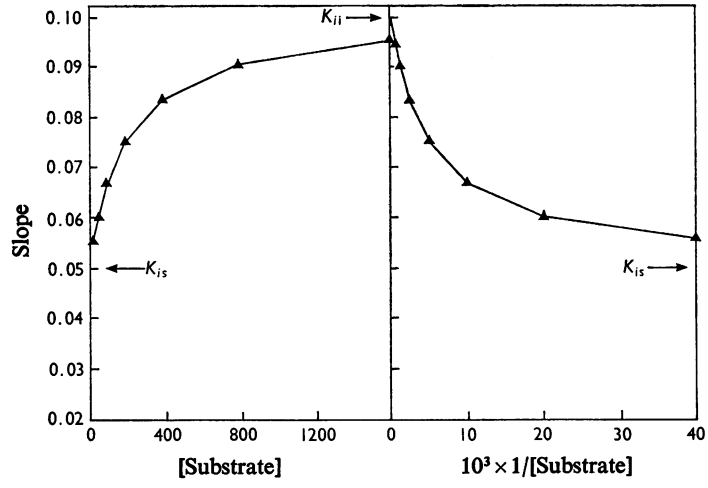

Fig. 7. *Replots of slopes for mixed non-competitive inhibition*

Values of parameters are as in Fig. 5, except that $K_{is}=0.05$ and $K_{ii}=0.10$.

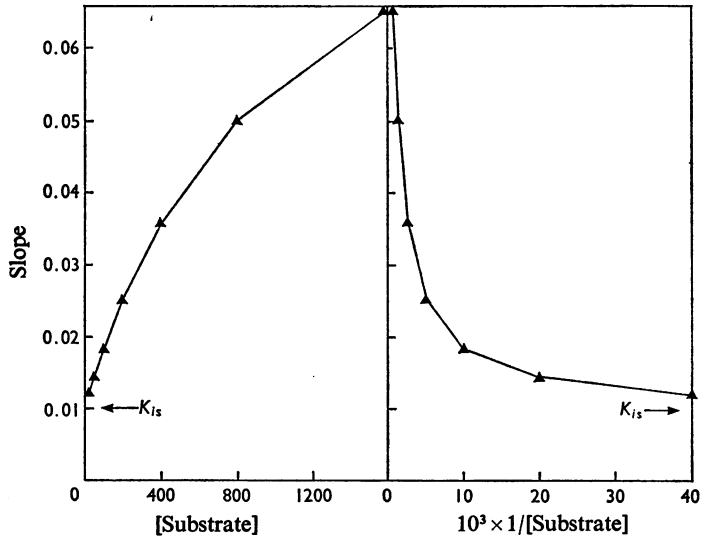

Fig. 8. *Replots of slopes for predominantly competitive mixed inhibition*

Parameters etc. are as in Fig. 7, except that $K_{is}=0.01$ and $K_{ii}=0.10$.

wherever A_t occurs in the full equation (Straus & Goldstein, 1943; Webb, 1963; Reiner, 1969; Cha, 1970; Rhoads & Garfinkel, 1971). The treatments by these authors show that in the absence of inhibitor:

$$E_i A = 0.5[(E_t + A_t + K_a) - \sqrt{(E_t + A_t + K_a)^2 - 4A_t E_t}]$$

In the presence of inhibitor, this value of $E_i A$ may be utilized to substitute $(A_t - E_i A)$ for A_t in a selected mechanistic equation from Fig. 4. The slope of a plot of $I_t/(1-v_i/v_0)$ against v_0/v_i then becomes a function of E_t. Thus the plot remains linear and the intercept on the ordinate is still E_t, but lines obtained at different E_t values are no longer parallel. When

E_t is kept constant and A_t is varied, the plots remain linear but replots of the slopes are non-linear for simple competitive or uncompetitive inhibition; this contrasts with the linearity obtained when $A_f=A_t$ (Fig. 6). The conversion of substrate into product(s) will be rapid under these conditions and it is possible that the steady state is short-lived or not achieved at all, so invalidating the use of equations based on the Morrison (1969) treatment. The problem may be avoided by utilizing a second reaction system to maintain a constant substrate concentration and/or remove products (Cha & Cha, 1965; McClure, 1969).

Scatchard plots in the presence of a high-affinity substrate

The binding of high-affinity substrate and inhibitor to the mitochondrial adenine nucleotide translocase enzyme has been measured (Weidemann *et al.*, 1970*a*; Klingenberg *et al.*, 1970; Vignais *et al.*, 1970), and it is of interest to predict effects that may occur under these conditions. The following procedure has been used. One of the equations of Fig. 4 is solved for $(1-v_i/v_0)$, by using (A_t-E_iA) instead of A_t; here E_iA is the substrate bound in the absence of inhibitor. The concentrations of I_f and E_iI are then obtained by substituting $(1-v_i/v_0)$ in the appropriate term of the original equation, and the process is repeated at different values of I_t. Thus a Scatchard plot of E_iI against E_iI/I_f is readily obtained, and further plots over the same range of I_t values but at different fixed values of A_t may be generated. To find E_iA in the presence of inhibitor, the value of I_f is used in the corresponding mechanistic equation for E_iA derived in the Appendix; A_f becomes (A_t-E_iA), and so Scatchard plots at different concentrations of A_t and fixed values of I_t may be generated.

Even in the presence of a tight-binding substrate, the Scatchard plots for inhibitor binding are linear for the four mechanisms studied, and they can be extrapolated to intercept the ordinate at the value of E_t. However, binding plots for substrate in the presence of inhibitor are non-linear for the competitive (Fig. 9) and uncompetitive cases, and cannot be extrapolated to E_t in the uncompetitive and non-competitive cases. The parameters in Fig. 9 were chosen because they clearly demonstrate the non-linearity; the plots tend to linearity if the affinity of the enzyme for substrate is greater than that for inhibitor or as the fraction of substrate bound decreases. Clearly, the estimation of E_t from substrate-binding studies should utilize enzyme preparations proved to be free of any endogenous inhibitor. Also, the appearance of non-linearity in Scatchard plots of substrate binding may indicate the presence of an inhibitor rather than multiple binding sites

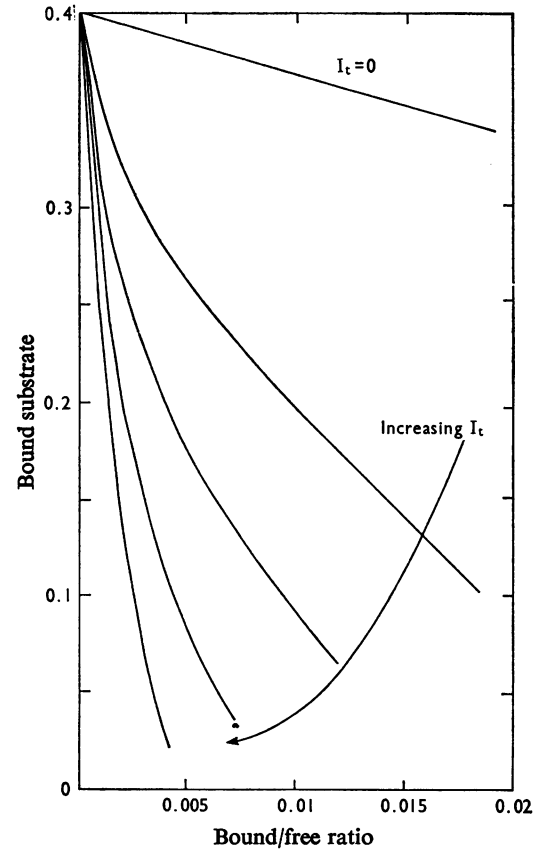

Fig. 9. *Scatchard plot for a tightly bound substrate at different fixed concentrations of a tight-binding competitive inhibitor*

The curves were generated as described in the text, by utilizing the values $E_t=0.4$, $K_a=3.15$, $K_i=0.02$, and values of A_t from 3.15 to 315.0. The fixed concentrations of I_t are 0.125, 0.25, 0.375, 0.50 and 0.625.

(Klotz & Hunston, 1971). The relevance of these predictions to binding experiments with isolated mitochondria will be discussed further below.

Linearity of normal reciprocal plots when all inhibitor molecules are bound to the enzyme

When $E_t/K_i>100$, eqn. (12) simplifies to eqn. (17) because virtually all inhibitor molecules are in combination with the enzyme. The observed extent of inhibition is then independent of the mechanism of interaction between inhibitor and enzyme (see the Appendix) and, unlike the case where the full eqn. (12) applies, a plot of $1/v_i$ or E_t/v_i against $1/A_t$

is linear. When repeated at different concentrations of inhibitor the intercept on the ordinate varies but that on the abscissa is a constant, $1/K_a$. This means that, whatever the true mechanism of combination between inhibitor and enzyme, a non-competitive pattern is apparent in Lineweaver–Burk reciprocal plots; Fig. 10 illustrates that a competitive mechanism is virtually indistinguishable from a non-competitive mechanism under these circumstances. The presence of a completely bound inhibitor may be diagnosed from a replot of the slopes of the reciprocal plot against I_t; the replot is concave and asymptotic to a vertical line drawn where $I_t=E_t$. This contrasts with the linear or parabolic replots obtained with normal inhibitors (Cleland, 1963*b*).

Results

Some observations on inhibitors of mitochondrial reactions

To investigate the behaviour of plots of $I_t/(1-v_i/v_0)$ against v_0/v_i the ATPase activity of intact mitochondria has been measured at increasing concentrations of the inhibitors rutamycin (Lardy *et al.*, 1965; Slater & Ter Welle, 1969) and bongkrekic acid (Henderson *et al.*, 1970; Weidemann *et al.*, 1970*b*). The dose–response measurements were repeated at different concentrations of protein so that effects due to tight binding of the inhibitor would be apparent. As a first approximation an unweighted least-squares analysis (see the next section) has been applied to find the slope and intercept of an assumed linear relationship, and the results are depicted in Fig. 11. With bongkrekic acid the intercepts on the ordinate increased proportionately to the protein concentration; also, the slopes of the plots were very small, even at the lowest protein concentration. In the light of the previous discussion, it may be deduced

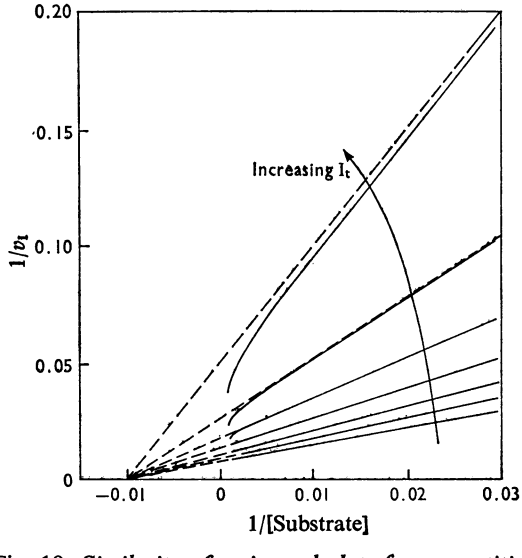

Fig. 10. *Similarity of reciprocal plots for competitive and non-competitive inhibition when I_t is very tightly bound to the enzyme*

Values of the parameters are $E_t=0.1$, $K_i=0.0001$, $K_a=100.0$, $V_{max.}=150.0$, and A_t was varied between 25.0 and 800.0. The fixed concentrations of I_t are at intervals of 0.0125 between 0.0125 and 0.0875. ----, Non-competitive; ——, competitive.

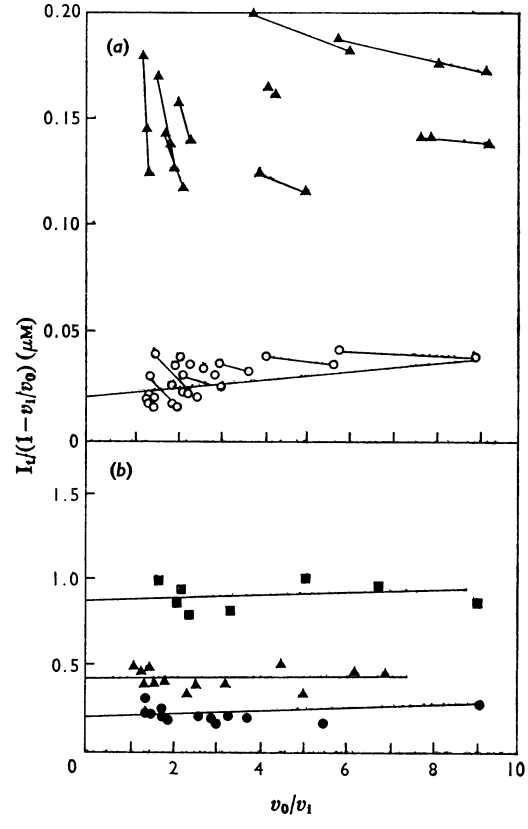

Fig. 11. *Analysis of the inhibition of mitochondrial ATPase by rutamycin and bongkrekic acid*

Dose–response measurements were performed as described in the text and replotted according to eqn. (13). Protein concentrations are: ■, 2mg/ml; ▲, 1mg/ml; ●, 0.4mg/ml; o, 0.2mg/ml. (*a*) Rutamycin; (*b*) bongkrekic acid. The connected points in the rutamycin experiment are replicate measurements of velocity at a single inhibitor concentration (performed in collaboration with R. W. Ebel).

that this inhibitor is tightly bound to the mitochondria, that the apparent K_t values

$$\text{slope} = D \Big/ \sum \frac{N_i}{K_t}$$

are 12×10^{-9}M, 1.8×10^{-9}M and 8.8×10^{-9}M, and that the concentration of receptor sites at pH6.6 is 0.44, 0.50 and 0.41 nmol/mg of protein. For rutamycin the concentration of receptor sites (intercept) at a protein concentration of 0.2mg/ml was 0.11nmol/mg of protein and the apparent K_t was 2.6×10^{-9}M. At the higher protein concentration the scatter of the transformed data points was too great to deduce accurately the slope of the line for rutamycin but the receptor-site concentration was between 0.13 and 0.17nmol/mg of protein. Again, as the positions of the lines are different at the different protein concentrations and the intercepts do not pass through the origin, rutamycin must be a tight-binding inhibitor. If the mechanism of inhibition is simple non-competitive, the slopes are the true K_t values; however, as these inhibitors appear to be very tightly bound it is not possible to elucidate the mechanisms, for the reasons already discussed.

A prior incubation of each inhibitor with the mitochondria was done before initiation of the reaction, as both these inhibitors take some minutes to reach maximum potency (Slater & Ter Welle, 1969; Henderson *et al.*, 1970; cf. Myers, 1952). The rates were linear during the assay period, so that the requirement for a steady state was fulfilled.

Statistical treatment of linear replots

The calculation of the best slope and intercept of linear plots of eqns. (13) or (14) is complex because of the stochastic dependence of the variables (Johansen & Lumry, 1961; Acton, 1959). This makes the calculation of the correct weighting factors to be applied at different values of $(1-v_i/v_0)$ extremely difficult. When the rules of Wilkinson (1961) are used to estimate the variances of $I_t/(1-v_i/v_0)$ and v_0/v_i (the variance of v_i was homogeneous throughout the range of measured v_i values for the experiments in Fig. 11), it can be shown that the variance of $I_t/(1-v_i/v_0)$ should be minimum at high inhibitor concentrations and the variance of v_0/v_i should be minimum at low inhibitor concentrations; such behaviour is apparent in the replicate measurements of the upper part of Fig. 11. Thus calculations should be weighted in favour of points in the range $(v_i/v_0)=$ 0.4–0.6. In the absence of a rigorous treatment, it should be emphasized that measurements of velocity at each inhibitor concentration should be repeated as often as necessary for the observed mean value to be a good approximation of the true mean; this condition is met when the standard deviation does not change greatly on the addition of further velocity measurements. The accuracy of the transformed variables will then be correspondingly enhanced.

Discussion

This study was initiated by the observation that the potency of many inhibitors of mitochondrial reactions depends systematically on the concentration of protein in the assay system. The principles described by Goldstein (1944) and Morrison (1969) clearly indicated that such effects are a result of the high affinity for the inhibitor, but did not give a reasonably simple and general method for analysing rate measurements. The linear plots of eqns. (13) and (14), based directly on Morrison's (1969) treatment, have provided the basis for a relatively rapid quantitative method of showing the high affinity of mitochondrial enzymes for two such inhibitors. These principles should be applicable to other enzyme systems interacting with tightly bound inhibitors (see, e.g., Khoo & Russell, 1970), but this discussion will be limited to some implications for the study of mitochondria.

First, it has been observed that the specific activity of the ATPase of intact rat liver mitochondria varies moderately from preparation to preparation, and this variation is quite marked when different protein concentrations of the same preparation are used (R. W. Ebel, unpublished work; cf. Harris, 1971). These effects may be due to variations in the ratio of endogenous adenine nucleotides (Mitchell & Moyle, 1971; Harris, 1971); to variable amounts of fatty acyl compounds, as these have been found to impair the adenine nucleotide translocase activity of mitochondria (Shug *et al.*, 1971); or to a significant proportion of substrate being bound to the enzyme, since apparent K_a values are in the range 0.1–100μM (see, e.g., Weidemann *et al.*, 1970*a*,*b*; Klingenberg *et al.*, 1970; Mitchell & Moyle, 1971). Since the results in Fig. 11 indicate that the concentration of oxidative phosphorylation enzymes is in the region of 0.1–2.5μM for experiments conducted at mitochondrial protein concentrations of 1–5mg/ml, it is evident that mutual-depletion kinetics apply to binding of substrate as well as of inhibitor. Also, if an endogenous, tightly bound, inhibitor is present, then the non-linearity of Scatchard plots for substrate or inhibitor binding to the adenine nucleotide translocase (Klingenberg *et al.*, 1970; Weidemann *et al.*, 1970*a*; Vignais *et al.*, 1970) is not necessarily caused by the presence of multiple binding sites, as indicated by these authors.

Hammes & Hilborn (1971) utilized Lineweaver–Burk plots to conclude that the inhibition of ox heart mitochondrial ATPase by oligomycin (very similar to rutamycin; Lardy *et al.*, 1965) is non-competitive. They reported that their oligomycin concentrations were at least ten times that of the enzyme present, but

it may be that their initiation of the reaction by addition of enzyme allowed time for only a fraction of the oligomycin to equilibrate (Slater & Ter Welle, 1969). If this were the case it may not be valid to deduce the mechanism from Lineweaver–Burk analysis of the results, for the reasons indicated in the Theory section.

Clearly the theoretical approach described in the present paper is applicable to the analysis of mitochondrial reactions, and further studies on this basis should help to elucidate the nature of inhibitor effects on the enzymes of oxidative phosphorylation.

I am deeply indebted to Professor Henry A. Lardy, in whose laboratory this study was conducted, for his encouragement and helpful advice both in this work and throughout my stay at the Enzyme Institute. Professor W. W. Cleland kindly suggested several improvements that have been incorporated into the manuscript. The excellent technical assistance of David Goldberg and Brian Warnecke greatly facilitated the computational analysis and the preparation of the manuscript for publication. This investigation was supported by grants from the National Institutes of Health (AM 10334), and the National Science Foundation (GB-6676X). Computer programs were processed on a Burroughs 1108 machine operated by the Madison Academic Computing Center; the Center is supported by a grant from the National Science Foundation.

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APPENDIX

Calculation of the amount of substrate bound to the enzyme

From the work of Cleland (1963*a*,*b*) and Morrison (1969) the following general equation for the proportion of enzyme complexed with the substrate in the presence of inhibitor may be derived:

$$\frac{\Sigma E_i A}{E_t} = \frac{\Sigma N_a}{D + I_f \sum_i \frac{N_i}{K_i}} \qquad (A1)$$

$E_i A$ is an enzyme–substrate complex and N_a is a term of the denominator representing the proportion of the enzyme in that complex. For a unireactant reaction that follows the Michaelis–Menten rate equation and interacts with inhibitor in a mixed non-competitive manner, eqn. (A1) becomes:

$$\frac{E_i A}{E_t} = \frac{A_t - E_i A}{(A_t - E_i A) + K_a + \frac{I_f K_a}{K_{is}} + \frac{I_f}{K_{ii}}(A_t - E_i A)} \qquad (A2)$$

As discussed in the text, A_t is replaced by $(A_t - E_i A)$ because a significant proportion of A_t is bound to the enzyme. Hence:

$$E_i A = \frac{A_t E_t - (E_i A) E_t}{A_t\left(1 + \frac{I_f}{K_{ii}}\right) + K_a\left(1 + \frac{I_f}{K_{is}}\right) - E_i A\left(1 + \frac{I_f}{K_{ii}}\right)} \quad \text{(A3)}$$

After cross-multiplication and arrangement in a quadratic form, we obtain:

$$E_i A^2\left(1 + \frac{I_f}{K_{ii}}\right) - E_i A\left[A_t\left(1 + \frac{I_f}{K_{ii}}\right) + K_a\left(1 + \frac{I_f}{K_{is}}\right) + E_t\right] + E_t A_t = 0 \quad \text{(A4)}$$

For the competitive case, K_{ii} is ∞ and:

$$E_i A^2 - E_i A\left[A_t + K_a\left(1 + \frac{I_f}{K_{is}}\right) + E_t\right] + E_t A_t = 0 \quad \text{(A5)}$$

For the uncompetitive case, K_{is} is ∞ and:

$$E_i A^2\left(1 + \frac{I_f}{K_{ii}}\right) - E_i A\left[A_t\left(1 + \frac{I_f}{K_{ii}}\right) + K_a + E_t\right] + E_t A_t = 0 \quad \text{(A6)}$$

For simple non-competitive inhibition, $K_{ii} = K_{is}$.

To generate values of $E_i A$ for theoretical Scatchard plots, K_{ii}, K_{is}, K_a, E_t and A_t are assigned the same values used to calculate I_f from one of the eqns. of Fig. 4 (main paper); the coefficients of the quadratic eqns. (A4–A6) corresponding to the mechanism are then calculated and $E_i A$ is obtained from the general solution:

$$\frac{1}{2p}(-q - \sqrt{q^2 - 4pr}) \quad \text{(A7)}$$

Form of the Lineweaver–Burk equation when all inhibitor molecules bind to the enzyme

Under this condition, eqn. (17) of the main paper applies, i.e.

$$I_t = E_t\left(1 - \frac{v_i}{v_0}\right) \quad \text{(A8)}$$

For a reaction obeying eqn. (19) of the main paper one can substitute for v_0, so that

$$I_t = E_t - v_i E_t \frac{A_t + K_a}{V_{max.} A_t} \quad \text{(A9)}$$

This assumes that virtually all substrate molecules are free. Hence:

$$v_i\left(\frac{E_t}{V_{max.}} + \frac{K_a E_t}{V_{max.} A_t}\right) = E_t - I_t \quad \text{(A10)}$$

and:

$$\frac{1}{v_i} = \frac{1}{A_t}\frac{K_a E_t}{V_{max.}(E_t - I_t)} + \frac{E_t}{V_{max.}(E_t - I_t)} \quad \text{(A11)}$$

or:

$$\frac{1}{v_i} = \frac{1}{A_t}\frac{K_a}{V_{max.}\left(1 - \frac{I_t}{E_t}\right)} + \frac{1}{V_{max.}\left(1 - \frac{I_t}{E_t}\right)} \quad \text{(A12)}$$

Thus a plot of $1/v_i$ against $1/A_t$ is linear.

When the plots are repeated at different concentrations of I_t, the lines have a common intercept, $1/K_a$, on the $1/A_t$ axis but different intercepts on the $1/v_i$ axis, which is the pattern for simple non-competitive inhibition.

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