ANALYSIS OF THE KINETICS OF REVERSIBLE ENZYME INHIBITION BY A GENERAL ALGEBRAIC METHOD. APPLICATION TO MULTISITE INHIBITION OF THE PHOSPHOGLYCERATE KINASE FROM *TRYPANOSOMA BRUCEI*

JACQUES PUECH[a], MIA CALLENS[b] and MICHÈLE WILLSON[c],*

[a]*Laboratoire de Microscopie et Structure des Matériaux, Université Paul Sabatier, Toulouse, France;* [b]*Laboratory of Biochemistry, Catholic University of Louvain, Research Unit for Tropical Diseases-Institute of Cellular and Molecular Pathology, Brussels, Belgium;* [c]*Chimie Organique Biologique IMRCP UMR-CNRS 5623, Université Paul Sabatier, Toulouse, France*

The action of an inhibitor on a stationary enzyme reaction is described by a simple equation, which reflects how the progressive binding of inhibitor molecules influences the existence and the productivity of the enzyme forms. This allows deduction of the structure of the enzyme system from the experimental results, using new type of plots $(1/[I], 1/[I]^a v)$ where $a = 0, 1, 2, \ldots$ in complement to the usual graphs. A reaction scheme is thereby logically built. This method may be used without any theoretical calculation. It is valid whatever the inhibitor, when the association reactions of the substrates and the inhibitor to the enzyme are in rapid equilibrium, and with dead end inhibitors, more generally for steady state enzyme reactions. This method may be adapted to enzyme activation. An original inhibition mode is described with particular bifunctional molecules: cooperative binding of the inhibitor to the enzyme, outside the active site, by direct mutual interaction of two inhibitor molecules, and locking of the conformational changes that normally precede the release of the products.

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* Corresponding author. Fax: 05 61 25 17 33. E-mail: willson@iris.ups-tlse.fr.

INTRODUCTION

Whilst searching for new enzyme inhibitors we synthesized molecules whose kinetic behaviour appeared original and confusing. To explain the experimental results we set aside the usual criteria and denominations[1-4] and developed an algebraic procedure, which does not consider any arbitrary reaction scheme. We believe it may be useful in many circumstances.

METHOD

Let us consider, for example, the velocity of a sequential reaction with two substrates, A and B, in the presence of an inhibitor, I, and the absence of products:

$$\frac{v}{[E]_{total}} = \left\{ k_0[EAB] + k_1[EABI] + \cdots + k_p[EABI_p] \right\}$$
$$/ \left\{ [E] + [EA] + [EB] + [EAB] + [EI] + [EAI] + [EBI] \right.$$
$$\left. + [EABI] + \cdots + [EI_n] + [EAI_n] + [EBI_n] + [EABI_n] \right\} \quad (1)$$

(the complexes $EB, EBI, \ldots, EBI_n$ do not exist in the special case of an ordered mechanism). n is the largest number of inhibitor molecules bound to the enzyme. The complexes $EABI_{p+1}, \ldots, EABI_n$ are unproductive. Every macroscopic form may correspond to several microscopic states.

We assume here that the binding reactions of the substrates and the inhibitor to the enzyme are in rapid equilibrium. The macroscopic rate constants $k_0, k_1, \ldots, k_p$ are independent of concentrations. In the expression of $v/[E]_{total}$ each enzymic form introduces into the denominator and possibly into the numerator, a term with exponents for the concentrations of I, A and B equal to the number of inhibitor and substrate molecules bound to the enzyme. This result remains valid in general steady state kinetics, when the association reactions are not in rapid equilibrium, but only concerning the number of inhibitor molecules and only if it is a dead end inhibitor (Annexe A).

These mathematical properties may be very useful in the experimental study of an enzymic system. If the algebraic relationship of v with concentrations [A], [B], and [I] is deduced from the experimental results, the existence and the productivity of macroscopic states of the enzyme are immediately known. A reaction scheme is then logically built up.

Unfortunately, except in the simplest systems, the limited accuracy of the measurements makes it illusory to find all the terms of the expression of v, even using a least-squares method of calculation.

It is preferred to search at foremost for the extreme terms, as they give most information on the structure and properties of the enzymatic system. It is only necessary to study the limiting states of the enzyme at very high and very low concentrations of substrate and inhibitor.

The influence of the substrate concentration is represented by the standard graphs $(1/[S], 1/v)$ and $(v, v/[S])$ and $([S], [S]/v)$, which also show to what degree the inhibitor affects the enzyme's affinity for substrates and the catalytic activity of the enzyme–substrates complexes. To study the structure of the system, it is unwise to use secondary plots such as $([I], K'_m)$ or $([I], V'_m)$, because they are often dubious. It is better to use the Dixon plot $([I], 1/v)$. This graph is linear only if the enzyme has one inhibitor binding site (or several independent sites), and only if the inhibition is competitive, uncompetitive or non-competitive and total. In all cases the initial slope of the Dixon plot gives information on the enzyme complexes with only one inhibitor molecule (Annexe B). For the analysis of the highest enzyme–inhibitor complexes we propose new plots $(1/[I], 1/[I]^a v)$ where $a = 0, 1, 2, \ldots$ (Annexe C).

Near $1/[I] = 0$, the shape of these plots gives the number of enzyme–substrate complexes rendered unproductive by binding of inhibitor molecules, i.e. $n - p$. The maximum number of molecules of inhibitor, n, is generally more difficult to find: for $n = 1$ and 2 the graphs are simple and characteristic. If the experimental plots are clearly different, there are at least three inhibitor sites.

To illustrate this method we present a detailed kinetic study of the inhibition of the glycosomal phosphoglycerate kinase (PGK, EC 2.7.2.3) from *Trypanosoma brucei* by two compounds.

PGK catalyses the conversion of 3-phosphoglycerate into 1,3-diphosphoglycerate using ATP as cofactor. The glycosomal PGK is monomeric and reacts according to a sequential mechanism with rapid-equilibrium random binding of substrates 3-PG and ATP.[5]

In our search for inhibitors of glycolytic enzymes[6,7] we have exploited a unique feature of some trypanosome glycosomal enzymes: the positively charged clusters on their surface called hot spots[8] which do not exist on the homologous mammalian glycolytic enzymes. We have synthesized a series of symmetrical long chain compounds with negative charges or strong dipoles at each end. Several of these compounds inhibited the glycosomal enzymes selectively when compared with the homologous enzymes from
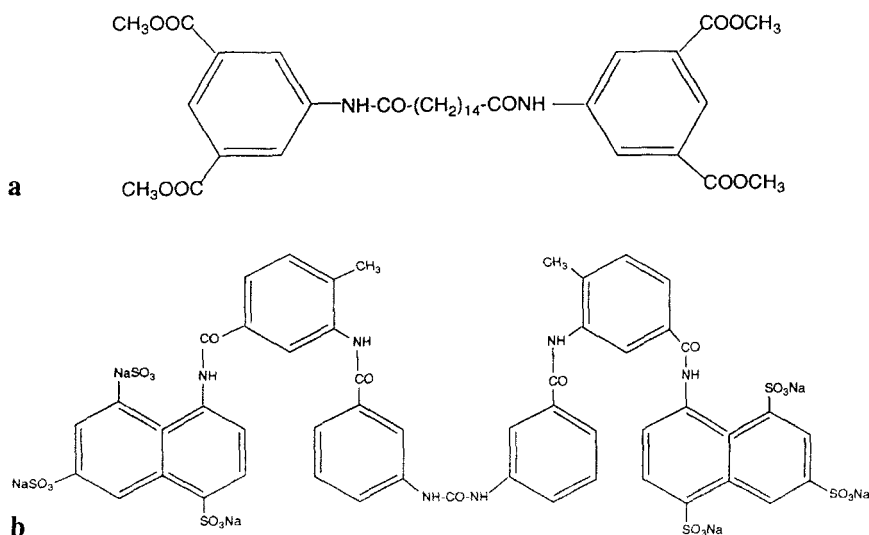
FIGURE 1 Two strong inhibitors of the glycosomal PGK from *Trypanosoma brucei* (a) N,N'-(3,5-dicarboxymethylphenyl)hexadecanediamide-1,16, (b) suramin.

other organism.[6] This is the case for N,N'-(3,5-dicarboxymethylphenyl)-hexadecanediamide-1,16 (Figure 1). Moreover suramin, a drug for the treatment of human trypanosomiasis (African sleeping sickness), has a net negative charge at each end of the molecule on the trisulphonated naphthyl-amine group at physiological pH (Figure 1). It is suggested that this drug may act through its interaction with the positively charged clusters on the surface of the glycosomal enzymes.[5]

Both compounds, in most cases, exhibit complex inhibition kinetics revealing both competitive and non-competitive inhibition through multiple sites of interaction on the enzyme.

METHODS AND RESULTS

Materials and Methods

Enzyme, Substrates and Inhibitors

Glycosomal PGK was isolated from the bloodstream form of *T. brucei* as described.[9,10] 3-Phosphoglycerate, ATP, rabbit muscle glyceraldehyde phosphate dehydrogenase and NADH were purchased from Boehringer GmbH,

Germany, or from Sigma Chemical Co. Suramin was purchased from Aldrich Chemical Co. The synthesis of the inhibitor used in this study has been described elsewhere.[6]

Inhibition Studies

PGK activity was evaluated by spectrophotometric measurement of the rate of oxidation of NADH in the presence of an excess of glyceraldehyde phosphate dehydrogenase.[5,9] The inhibition was determined by variation of the concentration of one of the two substrates (in the range 0.35–10 mM for 3-PG and 0.1–2 mM for ATP) with the other substrate present in excess (10 mM ATP or 20 mM 3-PG). The inhibitor concentrations were varied in the range from 1.25 to 50 μM for suramin and from 1.5 to 20 μM for the aminophthalate derivative. For the aminophthalate derivative, the remaining activity was calculated using a reference without inhibitor, containing the same volume of solvent (DMSO) used for the inhibitor. At a solvent concentration below 10%, no significant effect on enzyme activity was observed.

Suramin

The Dixon plots for suramin inhibition with respect to ATP and 3-PG (Figure 2(a) and (b)) give upward concave curves indicating that more than one molecule of suramin can bind to the same molecule of enzyme (Annexe B).

Figure 2(c) and (d) expressing $1/[I]v$ and $1/[I]^2v$ versus $1/[I]$, also gives non-linear upward concave curves. When the inhibitor concentration tends towards an infinite value, the product $[I] \cdot v$ tends to a finite value differing from zero and the product $[I]^2 \cdot v$ towards infinity. We can deduce that in the algebraic expression of v the degree of the numerator is lower than that of the denominator by one.

Therefore the enzyme–substrate complexes that are bound to a maximum number, n, of inhibitor molecules are not productive and at least one complex bound to $n - 1$ inhibitor molecules is productive.

The plots $1/[I]v$ versus $1/[I]$ (Figure 2(c) and (d)) indicate moreover that there are at least three inhibitor binding sites per enzyme molecule. Indeed the ordinate of the minimum is greater than twice the ordinate of the intercept of the asymptote. If n were equal to 2, the binding of the inhibitor would be cooperative (Annexe C, Figure 8); however that is very unlikely as the enzyme is monomeric and suramin is highly charged at physiological pH.
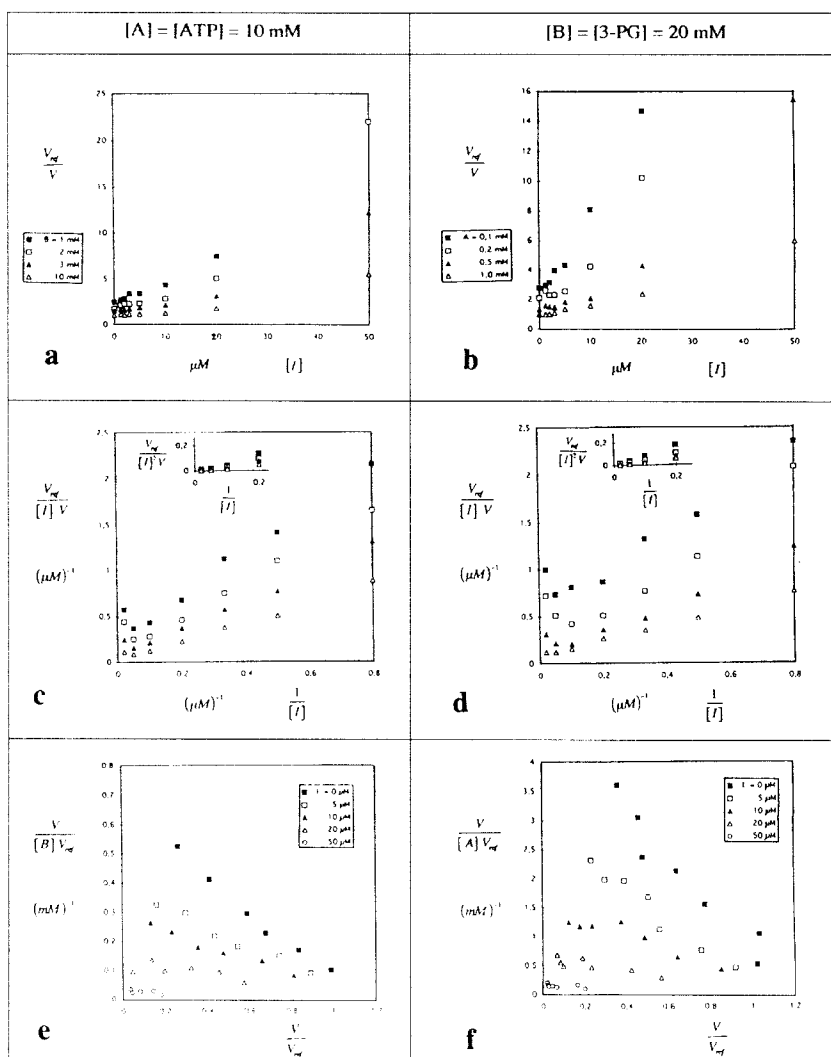
FIGURE 2   Inhibition of the *T. brucei* glycosomal PGK by suramin: experimental results. v/v_{ref} is the ratio of the velocity in the presence of the inhibitor to that measured, for the same concentration of enzyme, in a reference solution containing standard concentrations of the substrate. For the measurements of v one substrate is saturating ([ATP] = 10 mM or [3-PG] = 20 mM): (a) and (b) Dixon plots; (c) and (d) "Z_1 and Z_2 plots" (Annexe C); (e) and (f) Eadie–Scatchard plots.

The Eadie–Scatchard plots ($v/[S]$ versus v) (Figure 2(e) and (f)) show that the rate of the reaction does not depend on the inhibitor concentration when both substrates are saturating. This means that (1) the inhibitor may bind at the active site since there is mutual exclusion of the two substrates,

and (2) the presence of inhibitor on other sites has no influence on the catalysis as $k_0 = k_1 = \cdots = k_{n-1}$.

Derivative Aminophthalate

This compound is not very soluble in water. We did not vary our inhibitory measurements above a concentration of $20 \, \mu M$ because aggregation starts to occur.[6] As will be seen, the tetramethylester aminophthalate interacts with the enzyme by successive binding of several monomeric molecules, or also by direct binding of a polymeric molecule initially formed by aggregation of monomers. Since both processes are thermodynamically equivalent, the second does not need to be explicitly accounted for, if the association reactions are in rapid equilibrium. Then the inhibition concentration for theoretical interpretation must be that of the free monomer. But the graphs were drawn using the concentration of inhibitor added to the solution. The exact plots would use free inhibitor concentrations in the monomer state. The Dixon plots would be more concave and the curves $(1/[I], 1/[I]v)$ would be further away from the origin of the coordinates. This strengthens the conclusions below.

As in the case of suramin the Dixon plots (Figure 3(a) and (b)) show that several molecules of inhibitor can bind to the enzyme. For very high concentrations of both substrates, the Dixon plot shows two interesting features: (1) The initial slope is very small, nil at limit, as for suramin. There is, therefore, at least one inhibitor site distinct from the active site. The presence of a single inhibitor molecule has no influence on catalysis (unless it occupies the active site, which does not happen here, as will be seen). (2) The plot is strongly concave. Assuming that only two inhibitor binding sites exist per molecule of enzyme, we deduced approximate values of the equilibrium constants (Annexe A(ii)). We plotted $([I]^2 v/v_0 - v$ versus $[I])$ at high concentration of both substrates, $[ATP] = 2 \, mM$ and $[3\text{-}PG] = 20 \, mM$. Except for low inhibitor concentrations the curve is close to its asymptote, which intersects the axis on $-k_0/k_1 K_{1\infty}$ and $1/K_{1\infty} K_{2\infty}$, with $k_1 = k_0$. We used the same plot for $[ATP] = 10 \, mM$ and $[3\text{-}PG] = 10 \, mM$. The values obtained were $K_{1\infty} = 0.06 \, \mu M^{-1}$ and $K_{2\infty} = 0.14 \, \mu M^{-1}$.

The binding is cooperative, because the coefficient $c = 4K_{2\infty}/K_{1\infty}$ is clearly greater than 1. This cooperativity is very surprising. Normally such phenomena are observed only in oligomeric protein molecules.

The plots $(1/[I], 1/[I]v)$ and $(1/[I], 1/[I]^2 v)$ (Figure 3(c) and (d)) show also in this case, that the binding of the last molecule of inhibitor causes
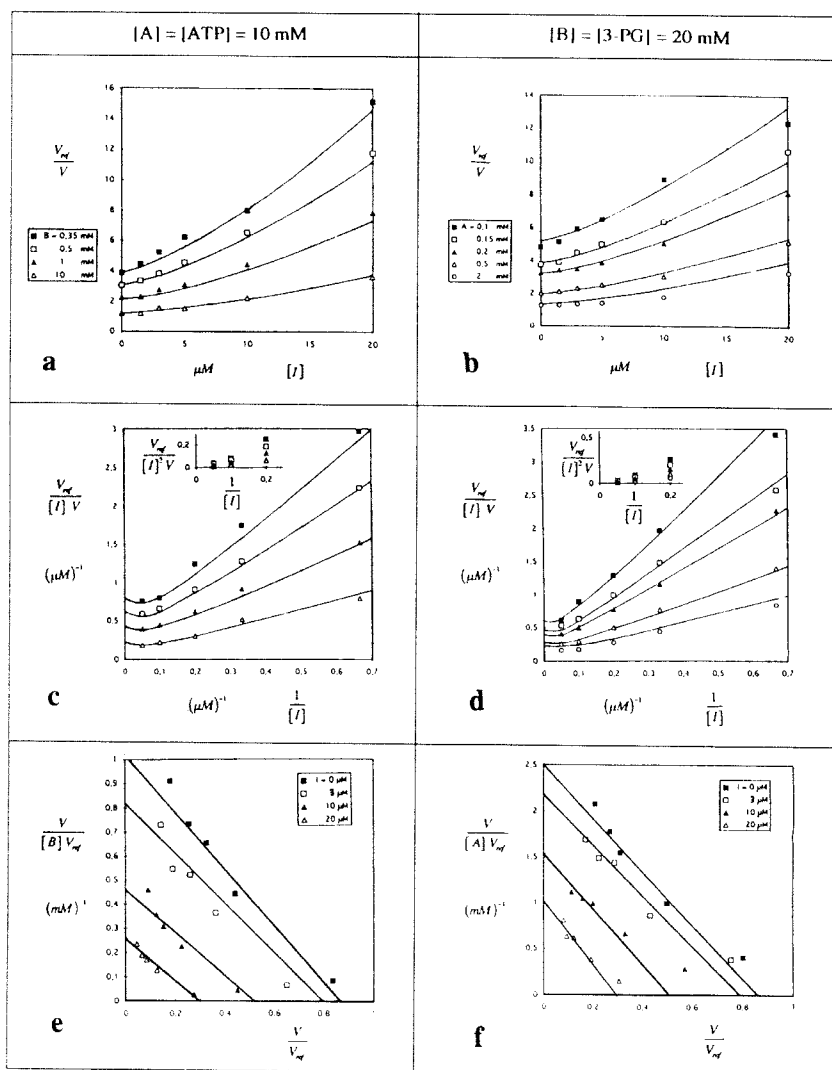
FIGURE 3 Inhibition of the *T. brucei* glycosomal PGK by the aminophthalate derivative: experimental results at high concentration of ATP or 3-PG. Same conditions and plots as for Figure 2. The theoretical curves are drawn for $K_i = 30\,\mu M$ (see the reaction scheme (Figure 5), see the text for the values of other parameters).

complete inhibition. In contrast with suramin, nothing suggests the existence of more than two inhibitor sites per enzyme molecule.

In spite of the scatterring of the measurements, the Eadie–Scatchard plots (Figure 3(e) and (f)) look like straight and roughly parallel lines.

The inhibitor sites are obviously distinct from the active site. The inhibitor strongly influences the catalytic stages of the reaction.

DISCUSSION

The study of the algebraic significance of the experimental results of stationary enzyme kinetics often tells us how many molecules of substrate and inhibitor are bound to one enzyme molecule in given circumstances. As the inhibitor is polyfunctional, we cannot always simply deduce the number of binding sites, because the inhibitor can bind to the enzyme in several ways, and it is not definite that all the sites are distinct. Even more so than in the case of a unifunctional inhibitor, the development of a reactional model must also take into account chemical information on the enzyme, the substrates and the inhibitor.

Suramin

Its interaction with the active site was already explained by the analogy between the sulfonic groups of this molecule and the phosphate groups of both substrates. Moreover the present kinetic studies confirm the existence of at least two other sites of interaction which are probably the "hot spots".

The reaction scheme (Figure 4) represents some of the reactions connecting all of the enzymic forms (because for a rapid equilibrium, it is not necessary to explicitly write other reaction paths with the same start and end). For example, EI represents the form in which the inhibitor is bound to the active site, for $\overset{I}{E}$ it is bound to an external site and for $\overset{I}{EAB}$ to the two external sites with substrates occupying the active site. To simplify, it is supposed that the external sites are identical and mutually independent and that their interaction energy with the active site is not influenced by the state of other parts of the enzyme molecule.

The rate equation is then reduced to:

$$\frac{1}{v} = \frac{1}{k[\mathrm{E}]_{\mathrm{tot}}} \left\{ 1 + \frac{K'_{\mathrm{A}}}{[\mathrm{A}]} \left[\frac{1 + [\mathrm{I}]/(\mu K'_{\mathrm{i}})}{1 + [\mathrm{I}]/(\lambda \mu K'_{\mathrm{i}})} \right]^2 + \frac{K'_{\mathrm{B}}}{[\mathrm{B}]} \left[\frac{1 + [\mathrm{I}]/(\lambda K'_{\mathrm{i}})}{1 + [\mathrm{I}]/(\lambda \mu K'_{\mathrm{i}})} \right]^2 \right.$$

$$\left. + \frac{L^2}{[\mathrm{A}][\mathrm{B}]} \frac{\left[1 + [\mathrm{I}]/K'_{\mathrm{i}} \right]^2 + [\mathrm{I}]/K_{\mathrm{i}} \left[1 + [\mathrm{I}]/(\varepsilon K'_{\mathrm{i}}) \right]^2}{\left[1 + [\mathrm{I}]/(\lambda \mu K'_{\mathrm{i}}) \right]^2} \right\} \qquad (2)$$

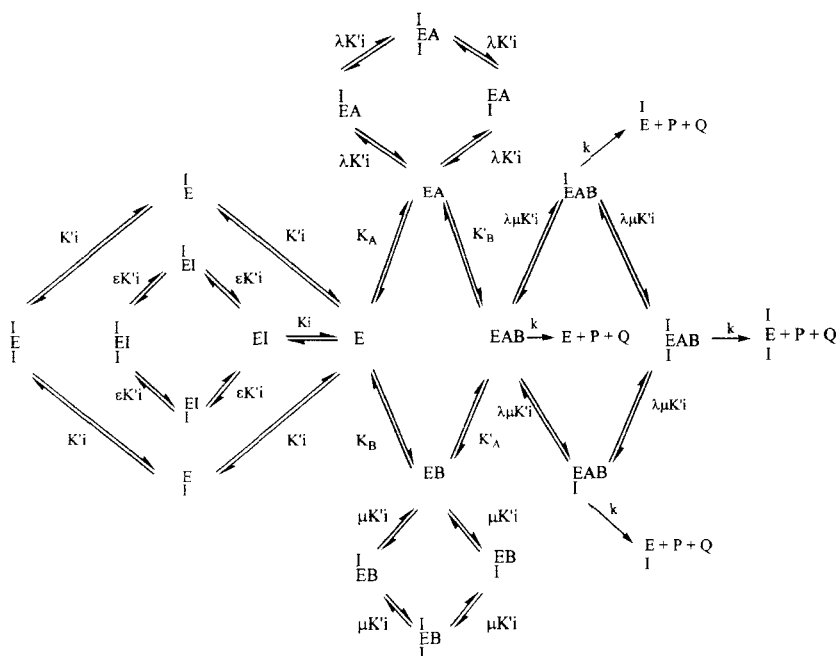with $L^2 = K_{\mathrm{A}} K'_{\mathrm{B}} = K_{\mathrm{B}} K'_{\mathrm{A}}$.

FIGURE 4 Inhibition of the *T. brucei* glycosomal PGK by suramin: reaction scheme EI represents the enzyme form in which the inhibitor is bound to the active site, for $\overset{I}{E}$ it is bound to an external site and for $\overset{I}{EAB}$ to the two external sites with substrates occupying the active site. K_A, K_i, $\lambda\mu K_i'$ are the values of the equilibrium dissociation constants of the reaction of the enzyme with substrates and the inhibitor. K is the velocity constant for release of products.

Suramin does not equally influence the association reaction of ATP and 3-PG with the enzyme, as can be seen by comparing Figure 2(e) and (f); the slopes of the lines are differently changed by the inhibitor concentration. The coefficients λ and μ are therefore not equal. Apart from k, K_A', K_B' and L^2, there are five independent parameters.

Aminophthalate Derivative

This bifunctional compound (Figure 2) strongly inhibits glycosomal PGK from *Trypanosoma brucei*. We have checked that the monofunctional derivative is inactive.[6] The strength of inhibition by the difunctional compound depends on the distance between the two functions, and is maximum for the molecule with 14 methylene groups. The kinetic studies were made with this molecule.

Its length is 4 nm, which is also the distance between the enzyme's "hot spots". Unexpectedly the corresponding carboxylic acid molecule, with negatively charged extremities at physiological pH, is a much weaker inhibitor than the ester. It is therefore unlikely that the inhibitor interacts with the "hot spots". We observed no inhibition with analogues of the amino-phthalate derivative missing the aromatic rings.

As already mentioned, the results of the kinetic studies show that the inhibitor does not bind to the enzyme's active site. The presence of the first inhibitor molecule does not affect catalysis but binding of the last molecule blocks catalysis completely.

We can account for all these results by assuming the existence of only two inhibitor sites per enzyme molecule. The reaction scheme (Figure 5) represents the microscopic enzyme forms and sufficient reactions to bind them.
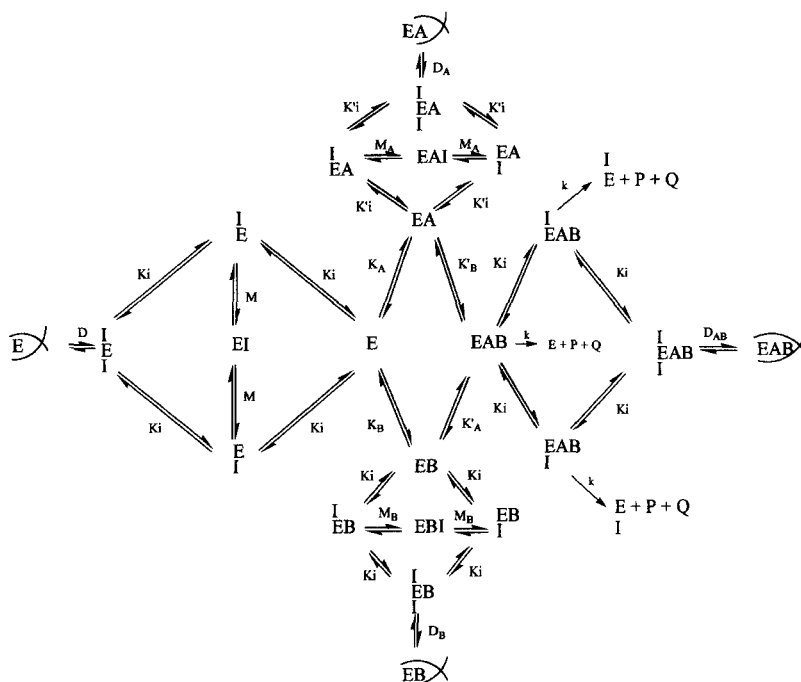

FIGURE 5 Inhibition of the *T. brucei* glycosomal PGK by the aminophthalate derivative: reaction scheme. In $\overset{\text{I}}{\text{E}}$ for example, an inhibitor molecule is bound to the enzyme by one of its extremities, in EI by both. In $\overset{}{\text{E}}$ two inhibitor molecules are bound to the enzyme and also each other by their other extremities. K_A, K_i are equilibrium dissociation constants. M, M_A, D, D_{AB} are equilibrium dissociation constants. M, M_A, D, D_{AB} are equilibrium isomerisation constants. k is the velocity constant for release of products.

In $\overset{\mathsf{I}}{\mathrm{E}}$, for example, an inhibitor molecule is bound to the enzyme by one of its extremities. It is supposed that the equilibrium constant of dissociation is independent of the state of the other sites. If the other inhibitor site is free the other extremity of the inhibitor molecule can occupy it. EI represents the result of such bridging. If the other site is already occupied by another molecule of inhibitor, which is represented by $\overset{\mathsf{I}}{\mathrm{E}}$, the free extremities can bind each other, probably by hydrophobic interaction of the aromatic rings; $\overset{\mathsf{I}}{\mathrm{E}}\!\!\times$ symbolizes the result of this tangling.

This event is in fact highly probable because the distance between the two inhibitor molecules bound to the enzyme cannot be greater than 5 nm and because, as mentioned above, the inhibitor begins to aggregate as its concentration approaches 20 μM, i.e. when the average distance between molecules is around 40 nm.

It may also happen that two inhibitor molecules, already bound to each other within the solution, bind together to the two sites of the enzyme molecule. With the concentrations used in our experiments, this event is less frequent than the former. As already mentioned, supposing that the association reactions are in rapid equilibrium, this second phenomenon need not appear explicitly in the reaction scheme, because it is thermodynamically equivalent to the previous one.

Whatever happens, these direct interactions between inhibitor molecules are responsible for the cooperativity of association revealed by kinetic studies, with this monomeric enzyme molecule, and not the allosteric transitions observed in oligomeric proteins.

On the other hand, the possible association of two enzyme molecules through the difunctional inhibitor is of very little importance here, because the concentration of the enzyme is low and the inhibitor has a moderate affinity.

According to the experimental results, complexes of enzyme and substrates with two inhibitor molecules are unproductive. The yeast PGK enzyme is known to undergo ample conformational changes during its catalytic cycle.[11] In the enzyme from *Trypanosoma brucei*, these changes are presumably hindered by two aminophthalate derivative molecules.

The rate equation is derived from the reaction scheme (Figure 5)

$$\frac{k[\mathrm{E}]_{\text{total}}}{r} = [(1 + (1 + D_{\mathrm{AB}})J_2] + \frac{K'_{\mathrm{A}}}{[\mathrm{A}]}[1 + M_{\mathrm{B}}J_1 + (1 + D_{\mathrm{B}})J_2]$$

$$+ \frac{K'_{\mathrm{B}}}{[\mathrm{B}]}[1 + M_{\mathrm{A}}J_1 + (1 + D_{\mathrm{A}})J_2] + \frac{L^2}{[\mathrm{A}][\mathrm{B}]}[1 + MJ_1 + (1 + D)J_2]$$

$$(3)$$

with

$$J_1 = \frac{[I]/K_i}{1 + 2([I]/K_i)} \quad \text{and} \quad J_2 = J_1 \frac{[I]}{K_i}.$$

Attempts to calculate the numerous parameters by a least-squares method. We were unsuccessful, probably because of the scattering of experimental results. However the experimental plots allowed estimation of the parameters.

Notably, it can be seen that M_A and M_B are not equal to zero, as the initial slope of the Dixon plot generally differs from zero (Figure 3(a) and (b)). This proves that, according to our hypothesis, one inhibitor molecule can bind to the enzyme by both of its extremities.

The horizontal intercepts of the straight lines in the Eadie–Scatchard plots (Figure 3(e) and (f)) give an approximate value of $V_{reference}/k[E]_{total}$, K_i and D_{AB}. The vertical intercepts in the same plots give respectively K'_B,
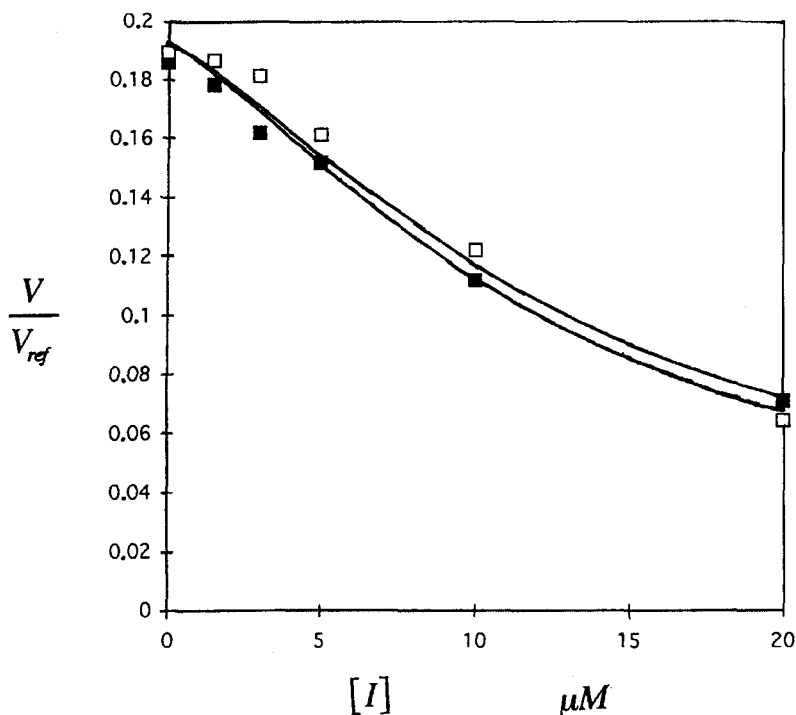

FIGURE 6   Inhibition by the aminophthalate derivative: results at moderate concentrations of both substrates, ■ [ATP] = 0.2 mM and [3-PG] = 3 mM; □ [ATP] = 0.5 mM and [3-PG] = 1 mM. The theoretical lines are drawn for the same parameters as in Figure 3.

M_A, D_A and K_B', M_B, D_B. L^2, D and M are found by fitting the theoretical equation with experimental results for moderate concentrations of ATP and 3-PG (Figure 6). Finally the following values were obtained: $K_A = 1.5\,\text{mM}$, $K_B = 3.8\,\text{mM}$. $K_A' = 0.30\,\text{mM}$, $K_B' = 0.76\,\text{mM}$, $M = 0.5$, $M_A = 1.9$, $M_B = 1.3$, $D = 5.6$, $D_A = 13$, $D_B = 4.8$, $D_{AB} = 9$, $V_{\text{reference}}/k[\text{E}]_{\text{total}} = 1.12$, $K_i = 30\,\mu\text{M}$.

The theoretical lines drawn for these values of the parameters agree with the experiemental results (Figures 3 and 6). The above value $K_i = 30\,\mu\text{M}$ is somewhat overestimated, because it was obtained using the concentration of inhibitor added to the solution, instead of that of the free monomer. Nevertheless the exact value should be relatively high.

So the design of a bifunctional molecule may give a strong inhibition with a functional group of moderate affinity for the enzyme.

CONCLUSIONS

The method used leads to the logical construction of a reaction scheme. It only uses some simple algebraic criteria. It avoids theoretical calculations, except if a final numerical check is desired. The method can be applied to activation phenomena as well as to inhibition.

Here, the two particularly interesting inhibitors act in very different ways on the *T. brucei* phosphoglycerate kinase. Several molecules of suramin bind to the active site and to other site. The aminophthalate derivative binds to the enzyme only outside the active site. The linking up of two inhibitor molecules both bound to the enzyme hinders conformational changes and stops the catalytic cycle. The binding sites of the aminophthalate derivative are unknown. The specificity and the efficiency of this new type of inhibitor undoubtedly depend on two factors: the distance between the functional groups and the tendency for self-association.

References

[1] Cleland, W.W. (1963) *Biochim. Biophys. Acta.* **67**, 173–187.
[2] Webb, J.L. (1963) *Enzyme and Metabolic Inhibitors*, pp. 149–191. Academic Press; New York.

[3] Todhunter, J.A. (1979) *Meth. Enzymol.* (Purich, D.L., ed.), Vol. 63, pp. 383–391. Academic Press; New York.
[4] Segel, I.H. (1993) *Enzyme Kinetics.* Wiley; New York.
[5] Misset, O. and Opperdoes, F.R. (1987) *Eur. J. Biochem.*, **162**, 493–500.
[6] Willson, M., Callens, M., Kuntz, D.A., Périé, J. and Opperdoes, F.R. (1993) *Biochem. Parasitology*, **59**, 201–210.
[7] Trinquier, M., Périé, J., Callens, M., Opperdoes, F.R. and Willson, M. (1995) *Biorgan. Med. Chem.*, **11**, 1423–1427.
[8] Wierenga, A.K., Swinkels, B., Michels, P.A., Osinga, K., Misset, O., Van Beeumen, J., Gibson, W.G., Potsma, J.P., Borst, P., Opperdoes, F.R. and Hol, W.G. (1987) *EMBO J.*, **6**, 215–221.
[9] Misset, O., Bos, O.J.M. and Opperdoes, F.R. (1986) *Eur. J. Biochem.*, **157**, 441–453.
[10] Misset, O. and Oppperdoes, F.R. (1984) *Eur. J. Biochem.*, **144**, 475–483.
[11] Watson, H.C., Walker, N.P.C., Shaw, P.J., Bryant, T.N., Wendell, P.L., Forthergill, L.A., Perkins, R.E., Copray, S.C., Dobson, M.J., Tuite, M.F., Kingsman, A.J. and Kingsman, S.M. (1982) *EMBO J.* **12**, 1635–1640.
[12] Volkenstein, M.V. and Goldstein, B.N. (1966) *Biochem. Biophys. Acta*, **115**, 471–477.

ANNEXE A

Velocity in Terms of Concentration

In the case of a sequential mechanism, the velocity is expressed as:

$$\frac{v}{[\text{E}]_{\text{total}}} = \left\{ k_0[\text{EAB}] + k_1[\text{EABI}] + \cdots + k_p[\text{EABI}_p] \right\}$$

$$/ \left\{ [\text{E}] + [\text{EA}] + [\text{EB}] + [\text{EAB}] + [\text{EI}] + [\text{EAI}] + [\text{EBI}] \right.$$

$$\left. + [\text{EABI}] + \cdots + [\text{EI}_n] + [\text{EAI}_n] + [\text{EBI}_n] + [\text{EABI}_n] \right\}. \quad (4)$$

If the association reactions of the enzyme with its substrates and the inhibitor are in rapid equilibrium, the concentration of each enzymic form is easily expressed in terms of concentrations of free enzyme, substrates and inhibitor. For example with two substrates:

$$[\text{EABI}_n] = [\text{E}][\text{A}][\text{B}][\text{I}]^n [\underset{\rightarrow}{\amalg} K], \quad (5)$$

where $[\underset{\rightarrow}{\amalg} K]$ is the product of the equilibrium constants calculated along any reversible path from E to EABI_n.

Transferring the concentration of each enzyme form into the above expression of $v/[\text{E}]_{\text{total}}$ the common factor [E] disappears. So, the first numerator term becomes proportional to [A][B], the second to [A][B][I], ..., the last to $[\text{A}][\text{B}][\text{I}]^p$. The first denominator term is now equal to 1, the second proportional to [A], ..., the last to $[\text{A}][\text{B}][\text{I}]^n$.

In the general steady state the algebraic expressions of the concentration of the enzyme forms are often much more complicated. But a special

case of wide interest is the dead end inhibition. The concentration of each enzyme form is still proportional to the inhibitor concentration with an exponent equal to the number of bound inhibitor molecules. In the absence of the inhibitor the reaction may be ordered or random, eventually with substrate inhibition. The inhibitor may not be a substrate or a product. It must act as a dead end inhibitor as soon as a first molecule binds to the enzyme. Several dead ends of equal or different degrees may exist in the reaction scheme. The reason for this general property of the dead end inhibition is that, referring to the theory of graphs,[12] it is seen that whatever base tree of whatever base contains a number of inhibitor associating branches equal to the number of bound inhibitor molecules to the enzyme in this base. Concerning the substrate concentration, the proportionality remains for all enzyme forms in the case of a single substrate reaction and a dead end inhibitor, even in presence of substrate total inhibition, but generally does not remain in the other circumstances.

(i) To compare the velocity v with the velocity v_0 measured in the absence of inhibitor, for the same total enzyme and free substrate concentrations, the above expression is transformed:

$$\frac{v}{[E]_{tot}} = \frac{k_0[EAB]}{[E] + [EA] + [EB] + [EAB]}$$
$$\times \frac{1 + \dfrac{k_1}{k_0}\dfrac{[EABI]}{[EAB][I]}[I] + \cdots + \dfrac{k_p}{k_0}\dfrac{[EABI_p]}{[EAB][I]^p}[I]^p}{1 + \dfrac{[EI] + [EAI] + [EBI] + [EABI]}{\{[E] + [EA] + [EB] + [EAB]\}[I]}[I] + \cdots + \dfrac{[EI_n] + [EAI_n] + [EBI_n] + [EABI_n]}{\{[E] + [EA] + [EB] + [EAB]\}[I]^n}[I]^n}.$$

$$(6)$$

The first term concerns enzyme–substrate reactions. It is the rate of the reaction divided by the total concentration of the enzyme in the absence of inhibitor. Due to the independence of this quotient from the total enzyme concentration, it is also equal to $v_0/[E]_{total}$, thus

$$v/v_0 = \frac{1 + N_1[I] + \cdots + N_p[I]^p}{1 + D_1[I] + \cdots + D_n[I]^n}. \qquad (7)$$

The coefficients

$$N_1 = k_1[EABI]/k_0[EAB][I] \cdots$$
$$D_1 = \{[EI] + [EAI] + [EBI] + [EABI]\}/\{[E] + [EA] + [EB] + [EAB]\}[I] \cdots$$

depend on [A] and [B] but not on [I] either in rapid equilibrium, or in general steady state with a dead end inhibitor.

N_1 is nil if binding a single inhibitor molecule on EAB suffices to render this complex unproductive, or all the more if the complex EABI cannot exist, in other words, if there is only one inhibitor site and if the inhibitor and at least one substrate are mutually exclusive. The coefficient D_1 is never nil.

(ii) Only in the case of rapid equilibrium to examine any cooperativity of the successive enzyme–inhibitor association reactions are apparent equilibrium constants introduced:

$$K_1 = \{[EI] + [EAI] + [EBI] + [EABI]\}/\{[E] + [EA] + [EB] + [EAB]\}[I],$$
$$K_2 = \{[EI_2] + [EAI_2] + [EBI_2] + [EABI_2]\}$$
$$/\{[EI] + [EAI] + [EBI] + [EABI]\}[I].$$

These macroscopic equilibrium constants depend on substrate concentrations. If $K_{1\infty}, K_{2\infty}, \ldots$ are their values at saturating substrate concentrations, it may be deduced that

$$\frac{v}{v_0} = \frac{1 + k_1/k_0 K_{1\infty}[I] + k_2/k_0 K_{1\infty} K_{2\infty}[I]^2 + \cdots + k_p/k_0 K_{1\infty} K_{2\infty} \cdots K_{p\infty}[I]^p}{1 + K_1[I] + K_1 K_2[I]^2 + \cdots + K_1 K_2 \cdots K_n[I]^n}.$$

$$(8)$$

In the special case $n = 2$, the inhibitor association reactions are cooperative if the index $c = 4K_2/K_1$, exceeds 1.

ANNEXE B

Some Properties of the Dixon Plot ([I], $1/v$)

$$\frac{1}{v} = \frac{1}{v_0} \times \frac{1 + D_1[I] + D_2[I]^2 + \cdots + D_n[I]^n}{1 + N_1[I] + N_2[I]^2 + \cdots + N_p[I]^p} \quad \text{(cf. Annexe A)}. \quad (9)$$

(i) The plot is a straight line only if $n = 1$ where the numerator is of the first degree (only one inhibitor binding site) or reduces to a binomial of first degree (independent sites) and $p = 0$ where the denominator contains no term [I], in other words if the enzyme substrate–inhibitor complexes do not exist (competitive inhibition: mutual exclusion of the inhibitor and at least one substrate by competition for the same site, or very strong negative

interaction between different sites), or are unproductive (uncompetitive inhibition, more generally non-competitive total inhibition with $K_i' = K_i$ or $K_i' \neq K_i$)

(ii) If there is only one inhibitor binding site per enzyme molecule and if the plot is curved, its concavity is necessarily downwards. Because of inhibition

$$v = v_0 \frac{1 + N[\mathrm{I}]}{1 + D[\mathrm{I}]}$$

must decrease with [I], hence N is smaller than D.

The second derivative

$$\frac{\mathrm{d}^2(1/v)}{\mathrm{d}[\mathrm{I}]^2} = \frac{2N(N-D)}{v_0(1 + N[\mathrm{I}])^3}$$

is therefore negative.

(iii) No matter what the number of inhibitor sites, the initial slope of the Dixon plot is equal to $D_1 - N_1/v_0$. As all substrates are saturating, it often happens that the initial slope is nil. Then, at least one microscopic form EABI exists and k equals k_0. In other words, at least one inhibition site differs from the active site and the presence of a molecule of inhibitor has no effect on the catalytic stages.

ANNEXE C

Plots $(1/[\mathrm{I}], 1/[\mathrm{I}]^a v)$

These plots represent the quantities $Z_a = 1/[\mathrm{I}]^a v$ for $a = 0, 1, 2, \ldots$ as functions of the inverse of the inhibitor concentration $x = 1/[\mathrm{I}]$:

$$\frac{v}{v_0} = \frac{1 + N_1[\mathrm{I}] + N_2[\mathrm{I}]^2 + \cdots + N_{p-1}[\mathrm{I}]^{p-1} + N_p[\mathrm{I}]^p}{1 + D_1[\mathrm{I}] + D_2[\mathrm{I}]^2 + \cdots + D_{n-1}[\mathrm{I}]^{n-1} + D_n[\mathrm{I}]^n}, \tag{10}$$

v_0 being the velocity in the absence of inhibitor for the same concentrations of substrates (Annexe A), the function Z_a is so expressed:

$$Z_a = \frac{1}{v_0} x^{a+p-n} \left\{ \frac{x^n + D_1 + D_2 x^{n-2} + \cdots + D_{n-1} x + D_n}{x^p + N_1 x^{p-1} + N_2 x^{p-2} + \cdots + N_{p-1} x + N_p} \right\}.$$
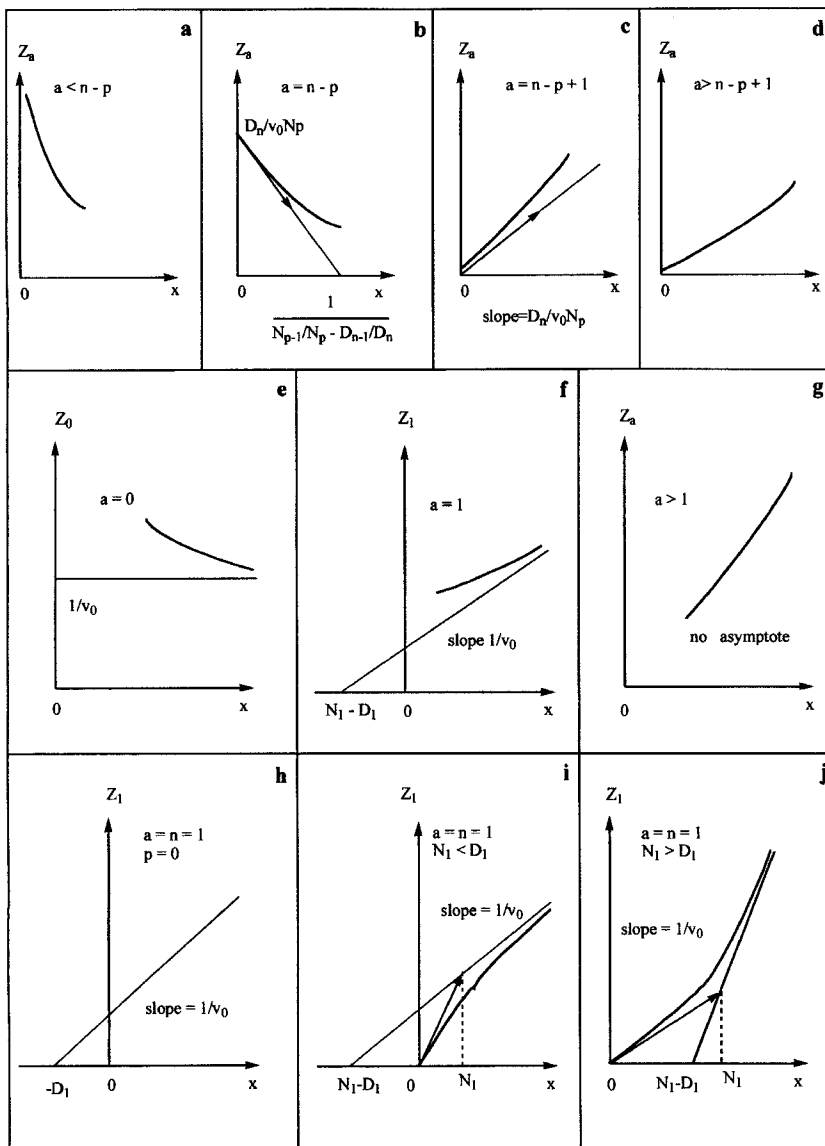
FIGURE 7 Plot ($x = 1/[I]$, $Z_a = 1/[I]^a v$) (Annexe C). Properties for small values of x ((a)–(d)) and large values ((e)–(g)). Special case $n = 1$: competitive, uncompetitive or non-competitive and total inhibition (h), inhibition (i), activation (j).

$$Z_1 = 1/[I]v = 1/v_0(x^2 + D_1x + D_2/x + N_1)$$
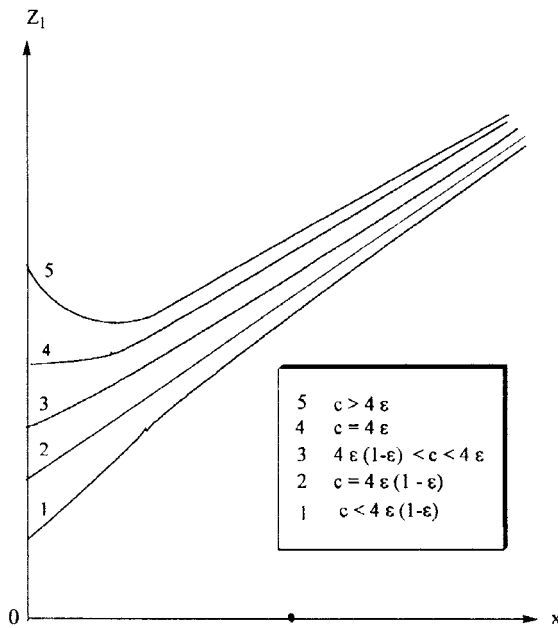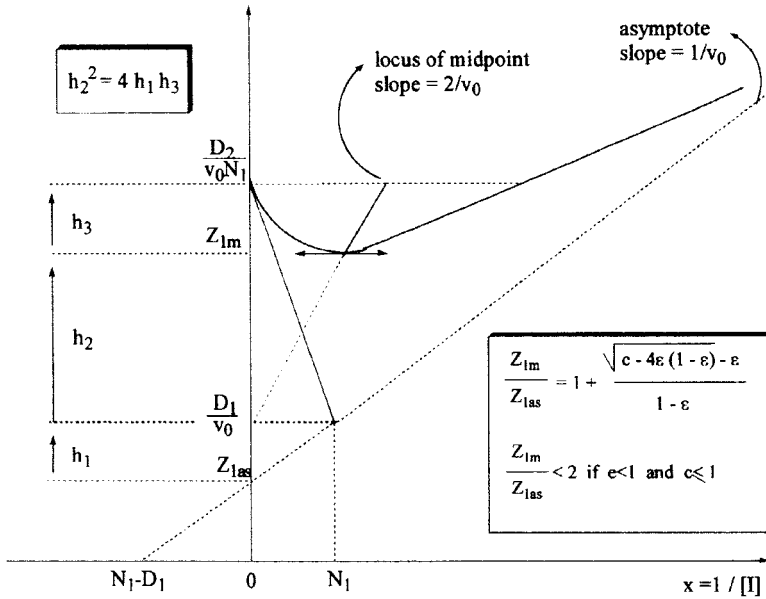

FIGURE 8   Plot Z_1 for the case where $n = 2$ and $p = 1$. Characteristic properties and shape for different values of the cooperative index c and the inhibition index ε (Annexe C).

Limit behaviour:

$$x \to 0 \quad Z_a \approx \frac{1 D_n}{v_0 N_p} x^{a-(n-p)} \left\{ 1 + x \left[\frac{D_{n-1}}{D_n} - \frac{N_{p-1}}{N_p} \right] \right\} \qquad \text{(Figure 7(a)–(d))}.$$

$$(11)$$

As plot z_{n-p} has a minimum, the slope of plot z_{n-p+1} may vary abruptly near the origin. Then the intercept, on the ordinate, of z_{n-p} cannot be obtained from the slope of z_{n-p+1}:

$$x \to \infty \quad Z_a \approx \frac{1}{v_0} x^a \left(1 + \frac{D_1 - N_1}{x} \right) \qquad \text{(Figure 7(e)–(g))}, \qquad (12)$$

$1/v_0$ and $D_1 - N_1/v_0$ are also, respectively, the ordinate and the slope at the origin of the Dixon plot.

Special Cases

$a = 1$

The quantity $v_0 z_1 = v_0/[I]v$ represents the quality of the inhibitor, since it is as large as the inhibitor is efficient at dilute concentrations. It varies with inhibitor and substrate concentrations.

$n = 1, a = 1$ *(Figure 7(h)–(j))*

If the plot is a straight line, there is competitive, uncompetitive or non-competitive and total inhibition.

$n = 2, p = 1$

It is useful to introduce the cooperativity index $c = 4D_2/D_1^2$, which exceeds 1 if the binding of two inhibitor molecules is cooperative, and the inhibition index $\varepsilon = N_1/D_1$ which is smaller than 1 if weak concentrations of I are effective in inhibition. The characteristic properties of the plot Z_1 are given in Figure 8.