Journal of Enzyme Inhibition and Medicinal Chemistry, December 2003 Vol. 18 (6), pp. 463–473

Taylor & Francis healthsciences

Equations of Substrate-inhibition Kinetics Applied to Pig Kidney Diamine Oxidase (DAO, E.C. 1.4.3.6)

G. IGNESTI*

Department of Pharmacology, University of Florence, V.le G. Pieraccini 6, 50139 Firenze, Italy

(Received 20 March 2003; In final form 5 July 2003)

Pig kidney diamine oxidase (DAO) and other semicarbazide-sensitive amine oxidases (SSAO) show clear substrate-inhibition kinetics and a reaction-scheme mechanism based on two substrate binding sites. We evaluated several reaction scheme mechanisms with a non-linear regression program (NCSS), estimating R^2, the constants of the equations and their standard errors and we determined the deviation of experimental data from theoretical equations. The best fit was obtained with a "dead end" mechanism with two binding sites. Based on this scheme, other schemes for a two-substrate reaction and for mechanisms of inhibition were constructed. These reaction schemes, even at low substrate concentration, fitted experimental data better than Michaelis-Menten kinetics, and provided information on the mechanisms of action of inhibitors. The presence of two substrate-binding sites on pig kidney DAO was confirmed by all experimental data.

Keywords: DAO; Kidney diamine oxidase; Substrate inhibition

INTRODUCTION

DAO is a key enzyme in the metabolism of histamine in several tissues such as thymus, placenta, kidney and intestinal mucosa;[1] DAO is often referred to as "histaminase", due to its activity on histamine and its regulating role on the effects of this mediator, although other enzymes of the class E.C. 1.4.3.6 may have histaminase activity.[2,3]

Moreover, DAO is involved in the deaminative oxidation of diamines such as putrescine and cadaverine and possibly in the regulation of the synthesis of polyamines, which are involved in cell proliferation control, ionic channel modulation and possibly in other cell functions. The deaminative oxidation of putrescine produces γ-amino-butyraldehyde and after oxidation, γ-aminobutyric acid (GABA), an inhibitory neurotransmitter; this reaction seems to be relevant for the regulation of GABA levels in pyloric gastric mucosa.[4] Furthermore, DAO acts on agmatine, interacting with inducible NO synthase activity.[5] A decrease in putrescine deaminative oxidation by DAO at high substrate concentrations was described by Kusche *et al.*[6] However, the hypothesis of substrate inhibition has been practically ignored in the calculation of kinetic parameters for DAO inhibitors, using the approximation of Michaelis-Menten kinetics,

$$\left(v = \frac{V\max \cdot S}{Km + S}\right) \tag{1}$$

for low substrate concentrations, in which substrate-inhibition is not evident. According to the general theories on enzyme activity,[7] substrate inhibition has at least two different substrate binding sites; therefore, at least two different enzyme-substrate complexes and two different equilibrium constants for the complexes have to be considered and not just one, as in equation (1). Therefore the use of equation (1) for the calculation of K_i of inhibitors or other enzyme kinetics parameters might not be correct.

In this study we considered many possible reaction schemes to explain the substrate inhibition of DAO, resolved the related equations and evaluated their fitting on experimental reaction-rate curves.

MATERIALS AND METHODS

Reagents

Reagents were from Merck (Darmstadt, Germany), or from Sigma-Aldrich (Milan, Italy).

*E-mail: ignesti@pharm.unifi.it

ISSN 1475-6366 print/ISSN 1475-6374 online © 2003 Taylor & Francis Ltd
DOI: 10.1080/1475636031000160543

DAO Activity

Pig kidney DAO was assayed according to Dimitrov *et al.*,[8] modified as follows: 150 µl of DAO (5 mg/ml) + 50 µl peroxidase (12 U/ml) + 150 µl of phenol (106 mM) + 150 µl of 4-aminoantipyrine (86 mM) and 50 µl of inhibitor (when used) were preincubated for 10 min at 37°C. The reaction was started with the addition of 50 µl of putrescine or other substrates and followed for 90 min at 37°C. The final reaction volume was 1.5 ml in Na-phosphate buffer M/15 at pH 7.4; all reagents were dissolved in this buffer. Blanks contained either no substrate or substrate and 50 µl of 30 mM semicarbazide. When two substrates were used, 50 µl of the second substrate were added together with putrescine. All samples were in duplicate and the experiments were replicated at least 3 times. The final putrescine, 1,6-diaminohexane, 1,7-diaminoheptane, cadaverine and spermidine concentrations for kinetic determination were 0.1, 0.25, 0.5, 1, 2.5, 5, 10, 15 and 20 mM; for histamine 0.033, 0.1, 0.25, 0.5, 1, 2.5 and 5 mM. The final concentrations of the second substrate or inhibitors are reported in the appropriate Tables. Calibration curves were obtained by adding 100 µl of H_2O_2 at concentrations ranging from 0.1 to 1.5 mM to the blanks; the absorption was read at λ 500 nm in a Lambda5 Perkin-Elmer spectrophotometer.

A similar assay was used for *Lathyrus cicera* DAO: 50 µl of a crude extract (obtained by centrifugation of a suspension of *Lathyrus cicera* seedling powder 1:10 (w/v) in Na-phosphate buffer 0.1 M pH 7.4), 1.25 ml of the same buffer, 50 µl of 6 mM 4-aminoantipyrine and 50 µl of 30 mM 3,5-dichloro-2-hydroxy-benzensulfonic acid (sodium salt) were preincubated for 10 min at 37°C; the reaction was started with 100 µl of histamine. The development of a colored complex was followed at λ 515 nm; the molar extinction coefficient of the complex is 26,000 $M^{-1}\,cm^{-1}$.

Rat Mesenteric Artery BzSSAO (SSAO with Benzylamine as Preferential Substrate) Assay

Hydrogen peroxide formation was measured as described by Ignesti *et al.*[2] The reaction mixture contained 100 µl of enzyme preparation, 10 µl of peroxidase (1000 U/ml), 10 µl of inhibitor solution, 340 µl of Na-phosphate buffer (ionic strength = 0.1) pH 7.4. After 10 min of preincubation at 37°C, 20 µl of homovanillic acid (5 mg/ml) and 20 µl of substrate solution were added. The reaction was carried out for 30 min, stopped by the addition of 0.1 M sodium hydroxide (2 ml) and the fluorescence developed was read at λ 426 nm with an excitation wavelength of λ 323 nm in a Shimadzu RF 5000 spectrophotofluorimeter. Blanks were incubated without substrates. Standard curves were obtained by adding 20 µl of H_2O_2 at different concentrations instead of substrates.[3]

Theoretical Equations

The kinetic equations were calculated by means of partial determinants of a square matrix in which the columns are the enzymatic forms (e.g. E, ES, ESS, EI ...) and rows were the variation of these compared to all the enzymatic forms (ΔE, ΔES, ΔESS, ΔEI ...). In the scheme the arrows that are going towards an enzymatic form are positive, while those going away from an enzymatic form are negative in respect to the variation of this enzymatic form. If there are no arrows going from an enzymatic form to another, the coefficient is zero (as detailed in the Appendix).

The equations of all depicted schemes for two-substrate binding sites kinetics (see: Appendix, Table VI) were simplified through the reunion of all the coefficients of S, 1/S, S^2 or $1/S^2$ in a simple factor; the simplified formulas (Table I) were satisfactorily fitted by a non linear regression curve using the program NCSS 6.0 by Jerry Hintze, 1996, which utilizes the Levenberg-Marquardt evaluation method.[9] To evaluate the best fit of the general simplified equations of Table I we considered:

1) $\mathbf{R}^2$, calculated by NCSS; 2) the fraction: **e/Val** = asymptotic standard error (NCSS)/estimated parameter (NCSS); 3) the sum (**Σ**) of the absolute value of the differences between experimental values and theoretical values divided by

TABLE I Simplified formulas of general reaction schemes with two binding sites

$\frac{V\max}{1+\frac{a}{S}+\frac{S}{b}}$	$\frac{V\max}{1+a+\frac{b}{S}+\frac{S}{c}}$	$\frac{V\max}{1+\frac{a}{S}}$	$\frac{V\max}{1+a+\frac{b}{S}}$	$\frac{V\max}{1+\frac{a}{S}+\frac{b}{S^2}}$	$\frac{V\max}{1+a+\frac{b}{S}+\frac{c}{S^2}+\frac{S}{d}}$
F1	F2	F3	F4	F5	F6
1	14b; 14c; 14; 8 × 1; 8 × 2; 99; 99c; 8 × 5	MM	01; 20; 0	0bis; 8 × 4 01bis; solvable as: $\frac{1}{c+\frac{d}{S}+\frac{a}{S^2}}$	8 × 3

the experimental values. The absolute value of differences allowed the evaluation of the sum of all positive or negative differences and the ratio with experimental values permitted all variations to have the same specific weight in Σ. The theoretical values were obtained by substituting in the general equation the values of the constants obtained by NCSS analysis and calculating the values of the reaction rate at the substrate concentrations used in the experiment. Fitted functions having estimated parameters $<10^{-10}$, or negative, or $>10^4$ and/or standard error $\gg$ estimated parameter or 0 values were rejected; for the remaining the related Σ was calculated. The curve with minimal Σ, minimal **e/Val** and maximal R^2 was taken as the best fit.

RESULTS

One substrate kinetics: As reported in Figure 1, some SSAOs showed substrate inhibition. The fact that the oxidation of spermidine (Figure 1a), which apparently does not show clear substrate inhibition, might be carried out by other enzymes such as spermine oxidase or polyamine oxidase was excluded; in fact, no oxidation of spermine was observed and spermidine behaved as an inhibitor when added to

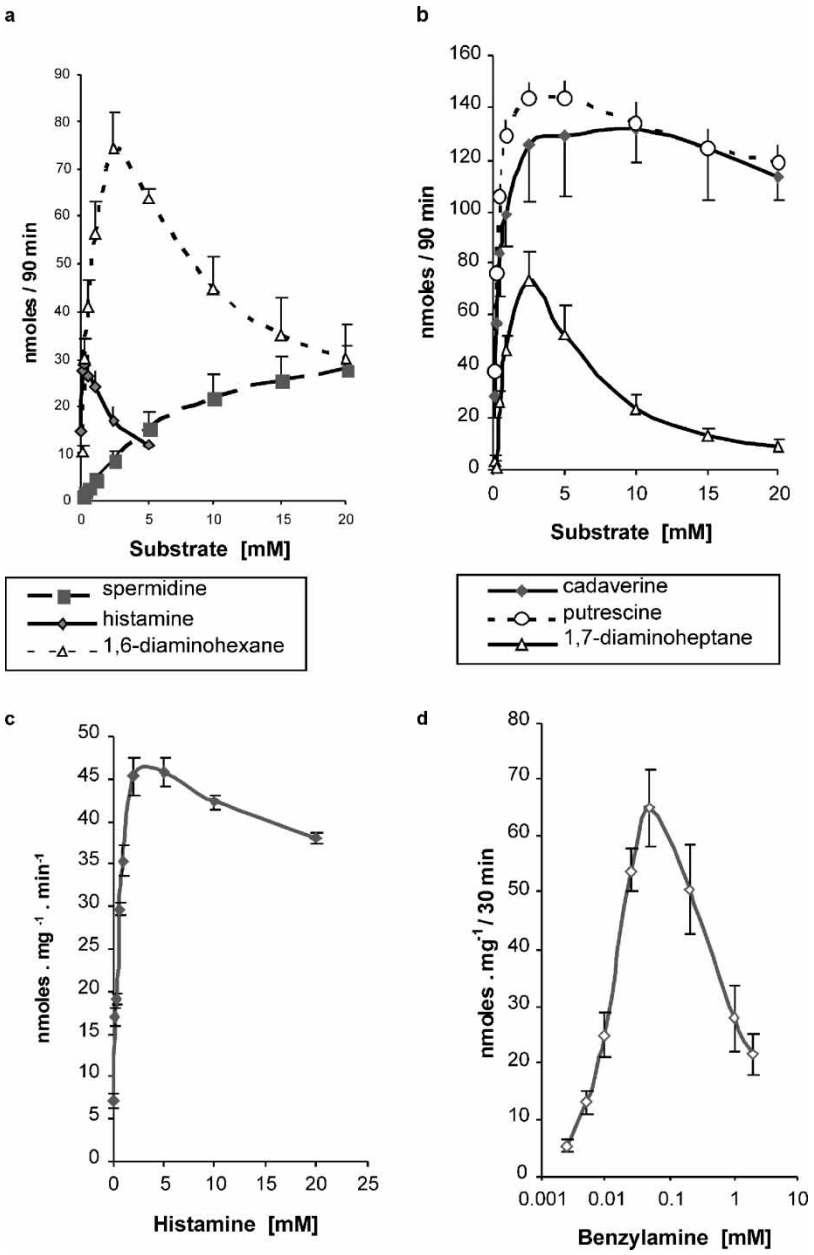


FIGURE 1 Kinetics of different substrates of pig kidney DAO (a,b), *Lathyrus cicera* DAO (c) and rat mesenteric artery BzSSAO (d).

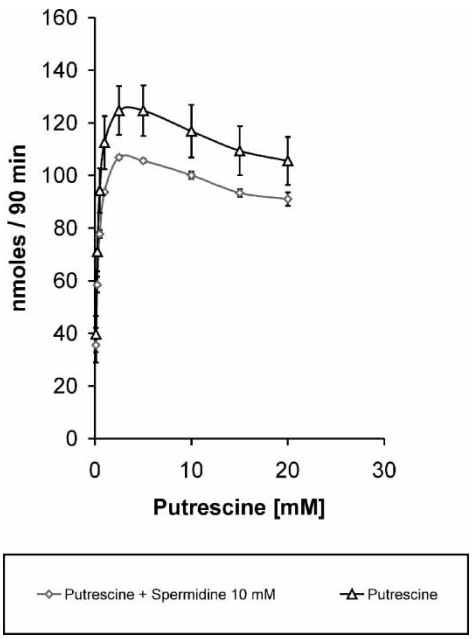


FIGURE 2 Kinetics of pig kidney DAO with putrescine or putrescine plus spermidine.

putrescine (Figure 2). With putrescine, cadaverine, 1,6-diaminohexane, 1,7-diaminoheptane, histamine and spermidine as substrate the best fit was obtained with the general formula F1, corresponding to Scheme 1 (see Appendix, Table VI), the equation of which is

$$v1 = \frac{V\max}{1 + \frac{Km}{S} + \frac{S}{Ks}}$$

The Σ for spermidine was similar for F1, F2 and F4; but F1 had the lowest **e**/**Val** (Table II). The values of *V*max, *Km* and *Ks* are reported in Table III.

Two substrates kinetics: We designed several additional schemes based on Scheme 1 for a two-substrate reaction or for an inhibited reaction (see: Appendix).

Putrescine at variable concentrations and the second substrate at fixed concentration were used in the experiments. The fit of the various schemes (see: Appendix, Table VII) was analyzed as previously described, introducing in the formula the values of *Km* and *Ks* of putrescine (Table III); the results are reported in Table IV. With spermidine 10 mM the only logical and acceptable result was obtained with Scheme 2sub24s. The best results with cadaverine 5 mM or 1,6-diaminohexane 1 mM were obtained with Scheme 2sub12s; with histamine, with the Scheme 2sub25 (for the equations of the schemes, see Appendix, Table VII).

Inhibition kinetics: The inhibitory mechanism of cimetidine, triethylamine and diethylamine on pig kidney DAO was analyzed using putrescine as substrate. The analysis included an evaluation according to a Lineweaver-Burk plot for a Michaelis-Menten equation, using low substrate concentrations, and for all substrate concentrations with the schemes derived from Scheme 1 (see: Appendix, Table VIII).

The values of *V*max, *Km* and *Ks* of putrescine were substituted in the formulas for NCSS calculation of non-linear equations. This analysis (see Table V) indicated a best fit with mixed inhibition for all inhibitors using Michaelis-Menten schemes; for schemes derived from Scheme 1 different results were obtained: cimetidine had the best fit with Scheme 4, the other inhibitors with Scheme 3a (Appendix: Table VIII). It has to be noted that Σ for a Michaelis-Menten scheme was always higher than Σ for schemes derived from Scheme 1.

DISCUSSION

The possibility that two different substrate binding sites are present in DAO can be inferred from literature data considering that two different research groups found an active site differing not only in a difficult to isolate cofactor (Pyrrole-Quinoline Quinone[10] or TOPA-Quinone[11]), but also in the amino acid composition, which is readily determined. Our data show a clear substrate inhibition with some SSAO; the analysis of kinetic data for pig kidney DAO with various substrates, with two substrates at the same time and inhibitors indicated that the best fit of the experimental data was always obtained through Scheme 1, a classical "dead end" mechanism,[7] with two binding sites and its derived schemes, also at low substrate concentrations. As a consequence, the use of Michaelis-Menten approximation at low substrate concentrations is incorrect; moreover, the definitions of inhibitor (competitive, non competitive, etc.) are inadequate and it is misleading to describe the inhibition mechanism, not only with DAO, but with other enzymes showing substrate-inhibition. In fact, diethylamine and triethylamine, which are mixed inhibitors according to Michaelis-Menten kinetics, bind to both binding sites of DAO; cimetidine, which has a mixed inhibition mechanism with Michaelis-Menten kinetics, binds only to the first binding site (catalytic) (see related schemes in Appendix, Table VIII).

We propose that the use of the mechanistic analysis suggested in this paper might be useful for the synthesis of new reversible inhibitors of DAO or other enzymes showing substrate inhibition. Moreover, the study of specific molecules binding

TABLE II Results of the kinetic analysis with several substrates [note: $(E + X) = 10^x$]

Simplified formula	F1	F2	F3	F4	F5	F6
Histamine						
R^2	0.73	0	0.009	0.008	0.081	0.413
Vmax (e/Val)	0.168	E+04	0.162	E+04		3840
A (e/Val)	0.747	<0	5.395	E+12	0.156	<0
B (e/Val)	0.543	<0		E+04	0	<0
C (e/Val)		E+04			2.843	E+14
D (e/Val)						3847
Σ	1.377		2.555			
Putrescine						
R^2	0.998	0.912	0.901	0.790	0.927	0.937
Vmax (e/Val)	0.009	478	0.037	898		3664
A (e/Val)	0.031	E+14	0.234	E+15	0.038	E+15
B (e/Val)	0.052	480		899	0.832	3660
C (e/Val)		483			0.808	3668
D (e/Val)						3663
Σ	0.071		0.704		0.576	
1,7-diaminoheptane						
R^2	0.999	0.999	0.628	0.587	0.754	0.999
Vmax (e/Val)	0.015	18.323	0.111	1580		47.8
A (e/Val)	0.025	18.36	0.533	<0	0.178	72
B (e/Val)	0.0029	18.34		1585	<0	47.84
C (e/Val)		21.86			0.45	51.29
D (e/Val)						47.84
Σ	0.060	0.072	1.926			0.064
1,6-diaminohexane						
R^2	0.990	0.990	0.384	0.359	0.489	0.964
Vmax (e/Val)	0.073	96.95	0.141	2237		0.175
A (e/Val)	0.139	163.3	1.017	E+14	1.044	<0
B (e/Val)	0.135	97.13		2240	0	E+17
C (e/Val)		97.04			0.124	0.507
D (e/Val)						0.35
Σ	0.333	1.551	2.864			
Cadaverine						
R^2	0.995	0.981	0.981	0.978	0	0.982
Vmax (e/Val)	0.027	261.94	0.023	198.4		959.4
A (e/Val)	0.082	261.91	0.117	198.8	<0	962.2
B (e/Val)	0.281	267.11			E+13	969.2
C (e/Val)		E+18		E+19	0.045	<0
D (e/Val)						969.1
Σ	0.171		0.265			
Spermidine						
R^2	0.997	0.997	0.997	0.997	0.997	0
Vmax (e/Val)	0.161	263.3	0.035	107.12		E+06
A (e/Val)	0.245	268.6	0.086	250.4	0.042	E+07
B (e/Val)	8.644	264.1		107.1	0.051	E+19
C (e/Val)		1456			<0	<0
D (e/Val)						E+06
Σ	1.320	1.328	1.594	1.307		

to the second site might offer new perspectives for studying enzyme activators. In fact, at least in theory, a molecule that displaces the substrate from the second site, but does not interfere with the catalytic function of the first site, might function as an activator (see: Appendix, Table VIII); in several physio-pathological functions, such as in controlling the levels of histamine or putrescine, a DAO activator could be very useful.

TABLE III Kinetic parameters obtained through Scheme 1

Substrates	Vmax*	Km (mM)	Ks (mM)
Cadaverine	110.9	0.335	93.1
1,6-Diaminohexane	123.3	1.406	6.94
1,7-Diaminoheptane	114.9	0.836	6.30
Histamine	46.5	0.028	3.48
Putrescine	126.3	0.25	56.1
Spermidine	34.97	7.89	1163.4

*nmoles $mg^{-1} h^{-1}$. Vmax = 106.8 nmoles $mg^{-1} h^{-1}$ and $K_m = 0.1579$ mM were obtained through the analysis of putrescine kinetics by the Michaelis-Menten equation. e/**Val** for each parameter is reported in Table II.

The occupation of the first site might allow occupation of the second site also, and the bound molecule might regulate the binding to the second site. In our experiments, when cadaverine or 1, 6-diaminohexane were incubated together with putrescine, only putrescine was bound to the second site. In the case of histamine incubated with putrescine, only histamine was bound to the second site; when spermidine was used together with putrescine, the molecules bound to the first and second site were the same (see Schemes 2sub12s,

TABLE IV Analysis of two substrate kinetics

Schemes	2sub12s	2sub24s	2sub25s	2subMM
Equation	$\frac{Vmax}{1+\frac{S}{Ks}+\frac{1}{\frac{S}{Km}+\frac{I}{Kmi}}}$	$\frac{Vmax}{1+\frac{S}{Ks}+\frac{Km}{S}+\frac{I^2\cdot Km}{Kmi\cdot Kii\cdot S}}{1+\frac{I\cdot Km}{Kmi\cdot S}}$	$\frac{Vmax}{1+\frac{Km}{S}+\frac{I}{Kii}+\frac{I^2\cdot Km}{Kmi\cdot Kii\cdot S}}{1+\frac{I\cdot Km}{Kmi\cdot S}}$	$\frac{Vmax}{1+\frac{1}{\frac{S}{Km}+\frac{I}{Kmi}}}$
2nd substrate				
Spermidine 10 mM				
Vmax (e/val)		0.07		0.036
Kmi (e/val)		0.559		1.045
Kii (e/val)		0.32		
R^2		0.997		0.879
Σ		0.081		0.707
Cadaverine 5 mM				
Vmax (e/val)	0.014			
Kmi (e/val)	0.26			
Kii (e/val)				
R^2	0.914			
Σ	0.208			
1,6-diaminohexane 1 mM				
Vmax (e/val)	0.061			
Kmi (e/val)	0.128			
Kii (e/val)				
R^2	0.545			
Σ	1.231			
Histamine 0.25 mM				
Vmax (e/val)	0.031		0.339	
Kmi (e/val)	0.285		0.538	
Kii (e/val)			0.573	
R^2	0.505		0.949	
Σ	0.533		0.180	

Note: In the equations, S represents putrescine and I the second substrate. Other schemes (see: Appendix Table VII) had worse or void results.

TABLE V Analysis of inhibitors

	Schemes			
Inhibitor	$\frac{Vmax}{1+\frac{Km\cdot\left(1+\frac{I\cdot\left(1+\frac{I}{Kii}+Ks\right)}{Ki}\right)}{S}+\frac{S}{Ks}+\frac{I}{Kii}}$	$\frac{Vmax}{1+\frac{I\cdot Km}{Ks\cdot Ki}+\frac{Km\cdot\left(1+\frac{I}{Ki}\right)}{S}+\frac{S}{Ks}}$	$\frac{Vmax}{1+\frac{I}{Ki}+\frac{Km\cdot\left(1+\frac{I}{Kii}\right)}{S}}$	
	3a	4	MM mixed	
Cimetidine				
R^2		0.992	0.991	
Ki (e/Val)		0.077	0.508	
Kii (e/Val)			0.152	
Σ**		0.283		
Σ*		0.239	0.242	
Diethylamine				
R^2	0.996		0.995	
Ki (e/Val)	0.081		0.118	
Kii (e/Val)	0.047		0.110	
Σ**	0.307			
Σ*	0.247		0.338	
Triethylamine				
R^2	0.990		0.994	
Ki (e/Val)	0.204		0.109	
Kii (e/Val)	0.107		0.278	
Σ**	0.156			
Σ*	0.069		0.288	

**Sum of the errors of all 9 substrate concentrations. *Sum of the errors of the initial 5 substrate concentrations. In the formula's, S is the substrate and, I is the inhibitor. Other reaction schemes (see: Appendix, Table VIII) give worse or inconsistent results.

2sub25 and 2sub24 in Appendix, Table VII). Regarding the inhibitors used, diethylamine and triethylamine had free access to the second site while cimetidine did not (see Schemes 3a and 4 in Appendix, Table VIII). Some SSAO were recently described as vascular-adhesion-protein (VAP-1),[12–17] able to attract lymphocytes. Hypothetically, the presence of a second substrate-binding site in VAP-1 could be a signalling mechanism for the interaction with VAP-lymphocytes.

Our method of analysis is based on logical considerations alone and is partially manual at moment. However, an automatic and rapid mathematical method for the evaluation of the fit of this type of non-linear equation can be easily developed in the future.

Acknowledgements

This work was supported by a MURST (ex 60%) grant. I thank Dr Marilina Longo, Dr Claudia Betti and Dr Marco Malavolti for helping in carrying out the kinetic measurements and in developing the calculation methods.

References

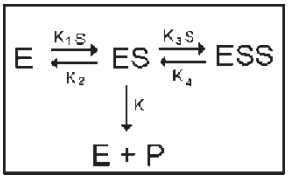
[1] Buffoni, F. (1966) *Pharmacol. Rev.* **18**, 1163–1199.
[2] Ignesti, G., Banchelli, G., Raimondi, L., Pirisino, R. and Buffoni, F. (1992) *Agents Actions* **35**, 192–199.
[3] Banchelli, G., Ignesti, G., Pirisino, R., Raimondi, L. and Buffoni, F. (1994) *J. Neural Transm.* **41**(Suppl.), 445–448.
[4] Hardt, J., Larsson, L.I. and Hougaard, D.M. (2000) *J. Histochem. Cytochem.* **48**(6), 839–846.
[5] Satriano, J., Schwartz, D., Ishizuka, S., Lortie, M.J., Thompson, S.C., Gabbai, F., Kelly, C.J. and Blantz, R.C. (2001) *J. Cell Physiol.* **188**, 313–320.
[6] Kusche, J., Richter, R., Hersterberg, J., Schmidt, J. and Lorenz, W. (1973) *Agents Actions* **3**, 148–156.
[7] Webb, J.L. (1963) Chapter 4, *Enzyme and Metabolic Inhibitors* (Academic Press, New York and London) **vol. 1**, pp 112–113.
[8] Dimitrov, O., Pavlov, V. and Jotova, L. (1996) *Experientia* **52**(8), 795–798.
[9] Press, W.H., Flannery, B.P., Teukolsky, F. and Vetterling, W. (1988) *Numerical Recipes in C: the Art of Scientific Computing* (Cambridge Univ. Press, New York).
[10] Van der Meer, R.A., van Wassenar, P.D., van Brouweshaven, J.H. and Duine, J.A. (1989) *Biochem. Biophys. Res. Commun.* **159**, 726–733.
[11] Janes, S.M., Palcic, M.M., Scaman, C.H., Smith, A.J., Brown, D.E., Dooley, D.M., Mure, M. and Klinman, J.P. (1992) *Biochemistry* **31**, 12147–12154.
[12] Salmi, M. and Jalkanen, S. (1992) *Science* **257**, 1407–1409.
[13] Salmi, M., Tohka, S., Berg, E.L., Butcher, E.C. and Jalkanen, S. (1997) *J. Exp. Med.* **186**(4), 589–600.
[14] Yoong, K.F., McNab, G., Hubscher, S.G. and Adams, D.H. (1998) *J Immunol.* **160**(8), 3978–3988.
[15] Jaakkola, K., Kaunismaki, K., Tohka, S., Yegutkin, G., Vanttinen, E., Havia, T., Pellinierni, L.A., Virolainen, M., Jalkanen, S. and Salmi, M. (1999) *Amer. J. Pathol.* **155**(6), 1953–1965.
[16] Jaakkola, K., Jalkanen, S., Kaunismaki, K., Vanttinen, E., Saukko, P., Alanen, K., Kallajoki, M., Voipio-Puikki, L.M. and Salmi, M. (2000) *J. Am. Coll. Cardiol.* **36**(1), 122–129.
[17] Alexander, J.S. and Granger, D.N. (2000) *Circulation Res.* **86**(12), 1190–1192.

APPENDIX

Mathematical Solution of Reaction Schemes

Example of scheme solutions: the kinetic equations were calculated by means of partial determinants of a square matrix in which the columns are the enzymatic forms (e.g.: E, ES, ESS, EI . . .) and rows are the variation of these in respect to all the enzymatic forms (ΔE, ΔES, ΔESS, ΔEI . . .). In the scheme arrows that are going towards an enzymatic form are positive, while those leaving an enzymatic form are negative compared to the variation in this enzymatic form. If there are no arrows from an enzymatic form to another, the coefficient is zero.

Scheme 1 (dead-end mechanism)



In this scheme the resulting matrix is:

$$\begin{array}{c|ccc} & \mathrm{E} & \mathrm{ES} & \mathrm{ESS} \\ \Delta\mathrm{E} & -k_1{\cdot}S & k_2+k & 0 \\ \Delta\mathrm{ES} & k_1{\cdot}S & -(k_2+k+k_3{\cdot}S) & k_4 \\ \Delta\mathrm{ESS} & 0 & k_3{\cdot}S & -k_4 \end{array}$$

To obtain an enzymatic form we resolved the determinant of a reduced matrix obtained by deleting a row (e.g. ΔES) and the column related to the enzymatic form to be resolved. So that

$$\mathrm{E} = \begin{vmatrix} k_2+k & 0 \\ k_3{\cdot}S & -k_4 \end{vmatrix} = -k_4{\cdot}(k_2+k)$$

$$\mathrm{ES} = \begin{vmatrix} k_1{\cdot}S & 0 \\ 0 & -k_4 \end{vmatrix} = k_4{\cdot}k_1{\cdot}S$$

$$\mathrm{ESS} = \begin{vmatrix} -k_1{\cdot}S & k_2+k \\ 0 & k_3{\cdot}S \end{vmatrix} = -k_1{\cdot}k_3{\cdot}S$$

The rate of reaction products formation being $v = k{\cdot}\mathrm{ES}$ and the theoretical maximal reaction rate (Vmax) if all the enzyme forms are able to give reaction products $V\mathrm{max} = k\cdot(\mathrm{E}+\mathrm{ES}+\mathrm{ESS})$, then we have:

$$\text{(B)}\quad \frac{v}{V\mathrm{max}} = \frac{\mathrm{ES}}{\mathrm{ES}+\mathrm{E}+\mathrm{ESS}} = \frac{1}{1+\left(\frac{\mathrm{E}+\mathrm{ESS}}{\mathrm{ES}}\right)}.$$

By substituting the absolute values of E, ES, ESS previously obtained, the final equation is:

$$v = \frac{V\max}{1 + \frac{k_2 + k}{k_1 \cdot S} + \frac{k_3 \cdot S}{k_4}}$$

Denoting $Km = (k_2 + k)/k_1$ and $Ks = k_4/k_3$ we have:

$$v = \frac{V\max}{1 + \frac{Km}{S} + \frac{S}{Ks}}$$

An Additional Calculation Method

Considering that, at the steady state, there is an equal amount of any enzymatic form appearing or disappearing, we can write for E: $-k_1 \cdot E \cdot S + (k_2 + k) \cdot ES = 0$, for ESS: $k_3 \cdot \mathrm{ES} \cdot \mathrm{S} - k_4 \cdot \mathrm{ESS} = 0$; by solving in respect to ES we obtain:

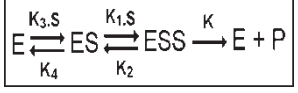
$$E = \frac{k_2 + k}{k_1 \cdot S} \cdot \mathrm{ES} \quad \text{and} \quad \mathrm{ESS} = \frac{k_3 \cdot S}{k_4} \cdot \mathrm{ES}$$

and for substitution in (B) the same results are obtained. A similar procedure might be applied to resolve all proposed schemes for enzymes with 2 or more binding sites.

TABLE VI Reaction schemes with 2 substrate-binding sites

$$v0 = \frac{V\max}{1 + \frac{Km \cdot \left(1 + \frac{Ks}{S}\right)}{S} + \frac{k}{k_3 \cdot S}} \qquad v0bis = \frac{V\max}{1 + \frac{(k_4 + k) \cdot \left(1 + \frac{k_2}{k_1 \cdot S}\right)}{k_3 \cdot S} + \frac{k}{k_1 \cdot S}}$$

Scheme 0

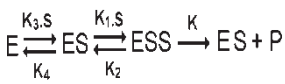


Scheme 0: The enzyme is active when 2 substrate molecules are consecutively bound (ternary complex) and the formation of reaction products regenerates a free enzyme E. Scheme 0 bis: The same as scheme 0, but the constants are changed ($k1 \leftrightarrow k3, k2 \leftrightarrow k4$).

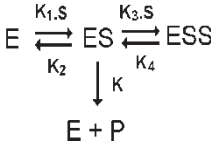
Scheme 01: The enzyme is active when 2 substrate molecules are bound (ternary complex) and the formation of reaction products regenerates the binary complex ES. Scheme 01bis: The same as scheme 0, but the constants are changed ($k1 \leftrightarrow k3, k2 \leftrightarrow k4$).

$$v01 = \frac{V\max}{1 + \frac{Km \cdot \left(1 + \frac{Ks}{S}\right)}{S}} \qquad v01bis = \frac{V\max}{1 + \frac{Km \cdot \left(1 + \frac{Ks}{S}\right)}{S}}$$

Scheme 01



Scheme 1



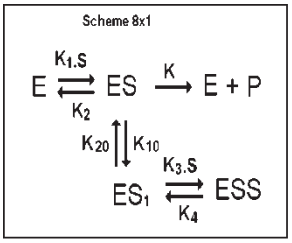
Scheme1: the first site which binds the substrate is the catalytic site, this first binding allows the binding of a second substrate molecule at the inhibitory site and forms a ternary inactive complex.

Scheme 8X5: Like Scheme 1, but the ternary complex should evolve into a further inactive binary complex ($\mathrm{ESS} \overset{K_1 \cdot S, K_2}{\longleftrightarrow} \mathrm{ES_2}$).

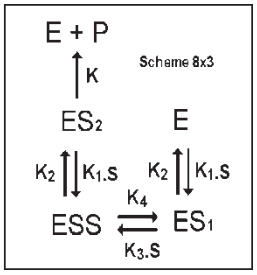
$$v1 = \frac{V\max}{1 + \frac{Km}{S} + \frac{S}{Ks}} \qquad v8x5 = \frac{V\max}{1 + \frac{Km}{S} + \frac{S \cdot \left(1 + \frac{k_2}{k_1 \cdot S}\right)}{Ks}}$$

Scheme 8X1: the first site binding for the substrate is the catalytic site and produces a reaction product or is modified to an intermediate form allowing the binding of a second substrate molecule with the formation of an inactive ternary complex.

Scheme 8x1



Scheme 8x3



$$v8x1 = \frac{V\max}{1 + \frac{Km}{S} + \frac{k_{10} \cdot \left(1 + \frac{S}{Ks}\right)}{k_{20}}}$$

Scheme 8X3: After the formation of an inactive binary complex, a ternary inactive complex is formed, from which a binary active complex is further formed.

Scheme 8X4: like 8X3, but the formation of ES_2 is irreversible ($\mathrm{ESS} \overset{K_2}{\rightarrow} \mathrm{ES_2}$).

$$v8x3 = \frac{V\max}{1 + \frac{k \cdot (k_2 + k_4)}{k_3 \cdot S^2 \cdot k_1} + \frac{k_4 + k}{k_3 \cdot S} + \frac{k_4 \cdot (k_1 \cdot S + k)}{k_3 \cdot S \cdot k_2} + \frac{k_1 \cdot S + k}{k_2} + \frac{k}{k_1 \cdot S}}$$

$$v8x4 = \frac{V\max}{1 + \frac{k \cdot \left(1 + \frac{k_2 + k_4}{k_3 \cdot S}\right)}{k_2} + \frac{k \cdot \left(1 + \frac{k_2 + k_4}{k_1 \cdot S}\right)}{k_1 \cdot S}}$$

Scheme 14: there are two binary complexes, ES_1 active, ES_2 inactive; ES_2 evolves into a ternary

complex which is still inactive. In Schemes 14b and 14c the constants varies in respect to Scheme 14;

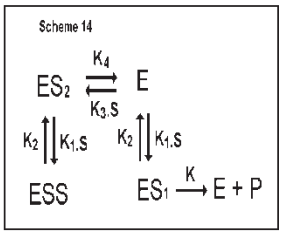
$$14b: \left(E \overset{k_{20},k_{10}\cdot S}{\longleftrightarrow} ES_2 \overset{k_4,k_3\cdot S}{\longleftrightarrow} ESS\right),$$

$$14c: (ES_2 \overset{k_4,k_3\cdot S}{\longleftrightarrow} ESS).$$

$$v14 = \frac{V\max}{1+\frac{Km}{Ks}+\frac{Km}{S}+\frac{(k_2+k)\cdot S}{Ks\cdot k_2}}$$

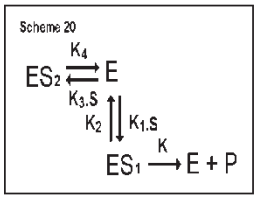
$$v14b = \frac{V\max}{1+\frac{Km}{S}+\frac{Km\cdot k_{10}\cdot\left(1+\frac{S}{Ks}\right)}{k_{20}}}$$

$$v14c = \frac{V\max}{1+\frac{Km}{Ks}+\frac{Km}{S}+\frac{S\cdot Km\cdot k_{10}}{Ks\cdot k_{20}}}$$



Scheme 20: The inactive form is a binary complex different from the active one.

Scheme 99: Like Scheme 20, but the active binary complex forms an inactive ternary complex ($ES_1 \overset{k_4,k_3\cdot S}{\longleftrightarrow} ESS$), and ($E \overset{k_{20},k_{10}\cdot S}{\longleftrightarrow} ES_2$). In Scheme 99c the constants vary in respect to Scheme 99 ($k_4 \leftrightarrow k_{20}, k_3\cdot S \leftrightarrow k_{10}\cdot S$).



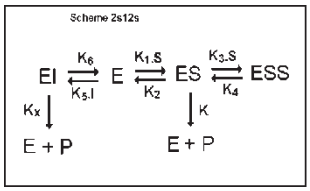
$$v20 = \frac{V\max}{1+\frac{Km}{Ks}+\frac{Km}{S}}$$

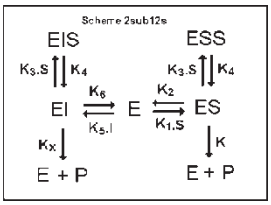
$$v99 = \frac{V\max}{1+\frac{Km\cdot\left(1+\frac{S\cdot k_{10}}{k_{20}}\right)}{S}+\frac{S}{Ks}}$$

$$v99c = \frac{V\max}{1+\frac{Km\cdot\left(1+\frac{S}{Ks}\right)}{S}+\frac{S\cdot k_{10}}{k_{20}}}$$

Notes: $Km = k_2 + k/k_1$, $Ks = k_4/k_3$. Schemes involving a ring of intermediate steps have more complex equations and their fit is worse than the previous schemes.

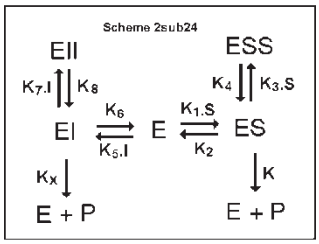
TABLE VII Two-substrate schemes derived from Scheme 1 or MM or two distinct enzymes

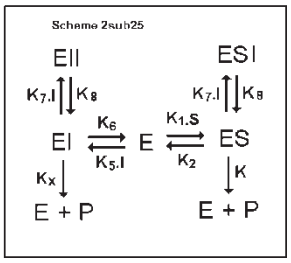




Scheme 2s12s: The second substrate binds to the catalytic site and does not allow any to bind to the second site. Scheme 2sub12 s: The second substrate binds to the catalytic site and allows the binding of the first substrate to the second site.

$$vs12s = \frac{V\max}{1+\frac{\frac{S}{Ks}+\frac{Km}{S}}{1+\frac{I\cdot Km}{Kmi\cdot S}}} \qquad v2sub12s = \frac{V\max}{1+\frac{S}{Ks}+\frac{1}{\frac{S}{Km}+\frac{I}{Kmi}}}$$

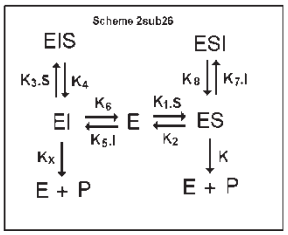


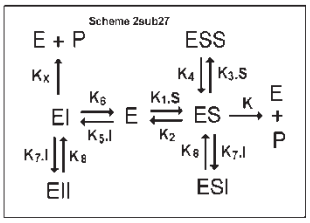


Scheme 2sub24: The second substrate binds to the catalytic site and allows itself to bind to the second site. Scheme 2sub25: The second substrate binds to the catalytic site and to the second site of the enzyme, giving simple or mixed ternary complexes.

$$v2sub24s = \frac{V\max}{1+\frac{S}{Ks}+\frac{Km}{S}+\frac{I^2\cdot Km}{Kmi\cdot Kii\cdot S}}{1+\frac{I\cdot Km}{Kmi\cdot S}}$$

$$v2sub25s = \frac{V\max}{1+\frac{Km}{S}+\frac{I}{Kii}+\frac{I^2\cdot Km}{Kmi\cdot Kii\cdot S}}{1+\frac{I\cdot Km}{Kmi\cdot S}}$$

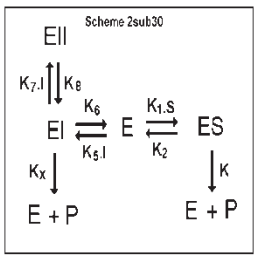


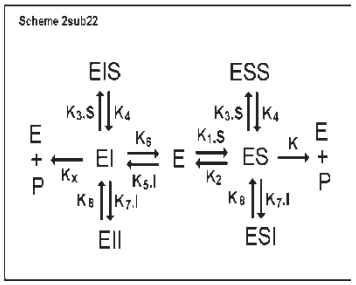


Scheme 2sub26: There is formation of mixed ternary complexes (EIS or ESI) but not simple ternary complexes (as EII or ESS). Scheme 2sub27: There is formation of a simple ternary complex with both substrates, but only a ternary mixed complex with the first substrate bound to the catalytic site and the second substrate bound to the inhibitory site.

$$v2sub26s = \frac{V\max}{1+\frac{Km}{S}+\frac{I\cdot Km}{Kmi\cdot Ks}+\frac{I}{Kii}}{1+\frac{I\cdot Km}{Kmi\cdot S}}$$

$$v2sub27s = \frac{V\max}{1+\frac{I}{Kii}+\frac{\frac{Km}{S}+\frac{S}{Ks}}{1+\frac{I\cdot Km}{Kmi\cdot S}}}$$





Scheme 2sub30: The second substrate forms a simple ternary complex, the first substrate does not; no mixed ternary complexes are allowed.

Scheme 2sub22: All types of binary or ternary complexes are formed.

$$v2sub30s = \frac{V\max}{1 + \dfrac{1 + \dfrac{I^2}{Kmi \cdot Kii}}{\dfrac{S}{Km} + \dfrac{I}{Kmi}}}$$

$$v2sub22s = \frac{V\max}{1 + \dfrac{1}{\dfrac{S}{Km} + \dfrac{I}{Kmi}} + \dfrac{S}{Ks} + \dfrac{I}{Kii}}$$

Scheme 2subMM: two substrates are at the same time utilized in a Michalis-Menten kinetic.

$$v2subMM = \frac{V\max}{1 + \dfrac{1}{\dfrac{S}{Km} + \dfrac{I}{Kmi}}}$$

Scheme 2e and 2eMM: two different enzymes act on the first or on the second substrate according to Scheme 1 or Michaelis-Menten respectively.

$$v2e = \frac{V\max 1}{1 + \dfrac{Km1}{I} + \dfrac{I}{Ks1}} + \frac{V\max}{1 + \dfrac{Km}{S} + \dfrac{S}{Ks}}$$

$$v2eMM = \frac{V\max 1}{1 + \dfrac{Km1}{I}} + \frac{V\max 2}{1 + \dfrac{Km}{S}}$$

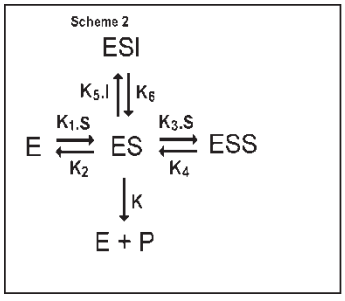
Using a constant concentration of one substrate, one of the two terms of the equation is a constant.

Notes: I = second substrate, at constant concentration S = first substrate, at variable concentration (e.g. putrescine here). The second substrate binds to the catalytic site also. Binary complex: ES or EI; simple ternary complex: EII or ESS; mixed ternary complexes: EIS or ESI.

$$Kmi = \frac{k_6 + kx}{k_5}, \quad Kii = \frac{k_8}{k_7}, \quad Ks = \frac{k_4}{k_3}, \quad Km = \frac{k_2 + k}{k_1}.$$

Some other possible mechanisms (MM10, MM11, MM12) involving the formation of an enzyme-inhibitor complex and one or two enzyme-inhibitor-substrate complex were investigated; the resulting equation was the same general equation as *vMM9*, but with different values of *Ki* or *Kii*.

b) Substrate inhibition kinetics derived from Scheme 1.



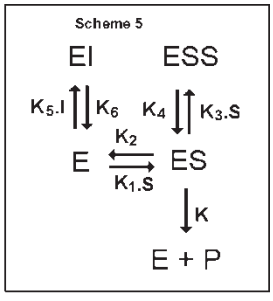


TABLE VIII Reaction schemes of inhibitors derived from Scheme 1 or Michaelis-Menten

Note: When the inhibitor is bound the enzymatic activity is absent. I=inhibitor

a) Michaelis-Menten (MM) kinetics

Scheme MM1: The inhibitor binds to the complex ES

$vMM1 = \frac{V\max}{1 + \frac{Km}{S} + \frac{I}{Ki}}$ (non competitive).

Scheme MM2: The inhibitor binds to the free enzyme and blocks the binding of the substrate.

$$vMM2 = \frac{V\max}{1 + \dfrac{Km \cdot \left(1 + \dfrac{I}{Ki}\right)}{S}} \text{ (competitive).}$$

Scheme MM8: The inhibitor binds both to the enzyme and to the enzyme-substrate complex with the same affinity constant

$$vMM8 = \frac{V\max}{1 + \dfrac{Km \cdot \left(1 + \dfrac{I}{Ki}\right)}{S} + \dfrac{I}{Ki}} \text{ (uncompetitive).}$$

Scheme MM9: The inhibitor binds to the enzyme and to the enzyme-substrate complex with different affinity constants (mixed).

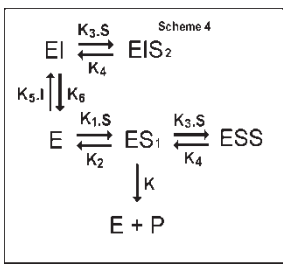
$$vMM9 = \frac{V\max}{1 + \dfrac{I}{Ki} + \dfrac{Km \cdot \left(1 + \dfrac{I}{Kii}\right)}{S}}$$

Scheme 2: The inhibitor binds to the enzyme-substrate complex (ES)

$$v2 = \frac{V\max}{1 + \dfrac{Km}{S} + \dfrac{I}{Ki} + \dfrac{S}{Ks}}$$

Scheme 5: The inhibitor only binds to the free enzyme (E) giving (EI)

$$v5 = \frac{V\max}{1 + \dfrac{Km \cdot \left(1 + \dfrac{I}{Ki}\right)}{S} + \dfrac{S}{Ks}}$$



Scheme 4: The inhibitor only binds to E and the substrate might bind to EI giving EIS.

$$v4 = \frac{V\max}{1 + \frac{I \cdot Km}{Ks \cdot Ki} + \frac{Km \cdot \left(1 + \frac{I}{Ki}\right)}{S} + \frac{S}{Ks}}$$

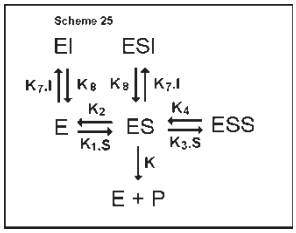
Scheme 60: As Scheme 4 with the formation of a ternary complex EII.

$$(\mathrm{EI} \overset{K_8, K_7 \cdot I}{\longleftrightarrow} \mathrm{EII})$$

$$v60 = \frac{V\max}{1 + \frac{S}{Ks} + \frac{Km \cdot \left(1 + \frac{I \cdot \left(1 + \frac{I}{Kii}\right)}{Ki}\right)}{S}}.$$

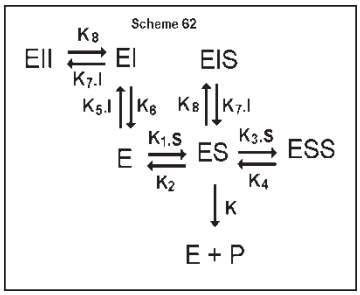
Scheme 25: The inhibitor binds to E and to ES (giving ESI) with the same affinity constant.

$$v25 = \frac{V\max}{1 + \frac{Km \cdot \left(1 + \frac{I}{Ki}\right)}{S} + \frac{S}{Ks} + \frac{I}{Ki}}$$



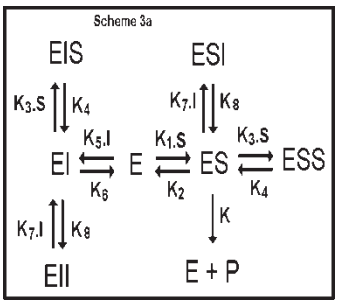
Scheme 62:
The inhibitor binds to E, EI and ES.

$$v62 = \frac{V\max}{1 + \frac{I}{Kii} + \left(\frac{S}{Ks} + \frac{Km}{S}\right) \cdot \left(1 + \frac{I}{Ki}\right)}$$



Scheme 3a:
All ternary and binary complexes are formed

$$v3a = \frac{V\max}{1 + \frac{Km \cdot \left(1 + \frac{I \cdot \left(1 + \frac{I}{Kii} + Ks\right)}{Ki}\right)}{S} + \frac{S}{Ks} + \frac{I}{Kii}}$$

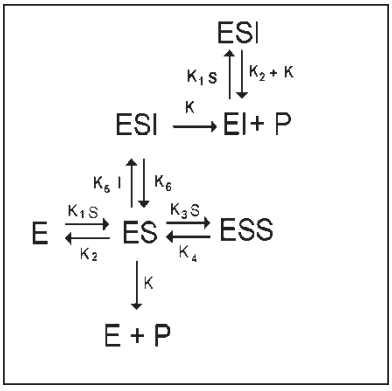


Scheme 7: Like scheme 3a, but the formation of EI from E and ESI from ES have the same constant

$$v7 = \frac{V\max}{1 + \frac{Km \cdot \left(1 + \frac{I \cdot \left(1 + \frac{I}{Kii} + \frac{S}{Ks}\right)}{Ki}\right)}{S} + \frac{S}{Ks} + \frac{I}{Ki}}$$

c) A particular case of a partial inhibitor (that binds to the complex ES, but does not block the enzymatic activity) is reported in the scheme: The related equation is

$$v = \frac{V\max}{1 + \frac{Km}{S} + \frac{S}{Ks \cdot \left(1 + \frac{I}{Ki}\right)}}$$



It can be noted that there is an increase in the reaction rate in respect to $v1$. The higher the ratio I/K_i, the lower the substrate inhibition.