A Simple Generalized Equation for the Analysis of Multiple Inhibitions of Michaelis-Menten Kinetic Systems*

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TING-CHAO CHOU AND PAUL TALALAY†

From the Laboratory of Pharmacology, Memorial Sloan-Kettering Cancer Center, New York, New York 10021, and the Department of Pharmacology and Experimental Therapeutics, The Johns Hopkins University School of Medicine, Baltimore, Maryland 21205

The summation of the effects of two or more reversible inhibitors of various types on the initial velocity of enzyme systems obeying Michaelis-Menten kinetics is described by the general relation:

$$\frac{1}{v_{1,2,...,n}} = \sum_{i=1}^{n} \frac{1}{v_i} - \frac{n-1}{v_0}$$

wherein $v_{1,2,...,n}$ is the velocity of reaction in the simultaneous presence of $n$ inhibitors, $v_i$ is the velocity observed in the presence of each individual inhibitor, and $v_0$ is the velocity in the absence of inhibition. The derivation is based on the assumption that each enzyme species may combine with no more than one of the inhibitors (i.e. the inhibitors are mutually exclusive). The above relationship holds irrespectively of the number of inhibitors, the type of inhibition (competitive, noncompetitive, or uncompetitive), or the kinetic mechanism (sequential or ping-pong) of the enzyme reaction under consideration. Deviations from this equality define synergism or antagonism of inhibitors depending on whether the value of the left side of the above equation is greater or smaller than the right, respectively. Knowledge of the kinetic constants for substrates and inhibitors is not required. If two or more inhibitors act independently (i.e. are not mutually exclusive), their combined effects are necessarily synergistic. Under certain circumstances, described in the text, mutually nonexclusive inhibitors obey the fractional velocity product relationship:

$$\frac{v_{1,2,...,n}}{v_0} = \left(\frac{v_1}{v_0}\right) \times \left(\frac{v_2}{v_0}\right) \times \left(\frac{v_3}{v_0}\right) \times \cdots \left(\frac{v_n}{v_0}\right)$$

The present paper offers a novel, generalized, and exceptionally simple analysis of the effects of more than one inhibitor on the initial velocities of enzymatic reactions obeying Michaelis-Menten kinetics. We derive a relationship applicable to multiple, reversible, and mutually exclusive inhibitors, irrespective of their kinetic behavior (competitive, noncompetitive, or uncompetitive), and independent of the number of substrates involved, or whether the mechanisms are of the ordered (sequential) or of the ping-pong type. This rigorous definition of the summation of inhibitory effects makes possible the quantitative descriptions of synergism or antagonism among inhibitors.

Enzymatic reactions obeying Michaelis-Menten kinetics in the presence of varying concentrations of single inhibitors have been described in terms of three boundary conditions, in accordance with the effects of inhibitors on double reciprocal plots of initial reaction velocity with respect to substrate concentration (1, 2). Thus, the inhibitor may change the slope (competitive), the intercept on the ordinate (uncompetitive), or both (noncompetitive) of such graphs. In the case of single-substrate reactions, these conditions are the consequences of the binding of the inhibitor to free enzyme, $E$, only (competitive), to $E$ and enzyme-substrate complex, $EA$, (noncompetitive), or to $EA$ complex only (uncompetitive).† This report considers only pure boundary conditions and their permutations. Equations for mixed types of inhibitions can be derived similarly by introducing interaction factors (3, 4).

It is well known that for a given enzymatic reaction and inhibition mechanism, rate equations specific for each circumstance can be derived with steady state or rapid equilibrium analyses (3-6). Such rate equations always contain the maximum velocity term as well as the kinetic constants and concentration factors for each of the substrates and inhibitors. Algebraic rearrangement of these equations leads to useful alternative equations or graphical representations (7-13). We show herein that the algebraic rearrangement of these individual equations, and substitutions in each of them for multiple inhibitors, result in the cancellation of all kinetic constants, concentration parameters, and the maximum velocity term. An exceptionally simple general equation is thus obtained, which correlates the reaction rates in the presence of each inhibitor alone, with that observed in the simultaneous presence of all of these inhibitors. A preliminary account of this work has appeared (14).

There are several excellent experimental and theoretical studies of multiple inhibitions of individual enzymes (3, 4, 15-20). Many workers have made the simple assumption that the

† To whom inquiries should be addressed at The Johns Hopkins University School of Medicine.

†† In single-substrate reactions, uncompetitive inhibition is only a hypothetical situation. However, in many multisubstrate reactions, particularly those with ping-pong mechanisms, inhibition with respect to the secondary substrate is obligatorily uncompetitive.
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The effects of the simultaneous presence of two inhibitors can be predicted from the product of the fractional velocity obtained in the presence of each inhibitor individually (3, 21). We show that this relationship is theoretically sound only under very restricted circumstances. Most of the earlier analyses have invoked the use of kinetic constants for substrates and inhibitors or have required the accumulation of extensive experimental measurements in order to obtain valid graphical representations. The derivations presented in this paper lead to quantitative descriptions of the summation of effects of multiple inhibitors of various types, require few measurements, and do not invoke the kinetic constants of substrates or inhibitors. Furthermore, our generalized relationships are readily applicable to the simultaneous action of more than two inhibitors.

To our knowledge, Lienhard et al. (22) are the only workers who have recognized the possibility of such simple relationships. In the course of work on transition state inhibitors of ribonuclease, these authors (22) mention the relation 1/νo = 1/ν1 + 1/ν2 - 1/ν0 for a single-substrate reaction and two noninteracting inhibitors of competitive or noncompetitive type. However, the theoretical basis for this derivation and the range of its applicability were not developed.

NOMENCLATURE

The symbols and notations follow those proposed by Cleland (5):

νo: initial velocity of uninhibited reaction;

ν1, ν2, ν3, ..., νn: initial velocity in the presence of inhibitors I1, I2, I3, ..., I4, respectively;

νi: initial velocity in the simultaneous presence of inhibitors I1 and I4;

ν1,2,3, ..., νn: initial velocity in the simultaneous presence of inhibitors I1, I2, I3, ..., I4;

A, B: concentrations of substrates A and B, respectively;

KmA, KfA: Michaelis constants for substrates A and B, respectively;

KmI1, KfI1: inhibitor constants for inhibitors I1 and I4, respectively;

K dissociation constant for substrate A;

I1, I2, I3, ..., In: concentrations of inhibitors I1, I2, I3, ..., In, respectively;

V: maximum velocity of reaction;

fi: fractional velocity = νi/νo;

(fj1,2,3 ..., fj1,2,3, ..., fn): fractional velocity in the simultaneous presence of n inhibitors;

fi: fractional inhibition = (1 - fi),.

ANALYSIS

Our initial analysis assumes that classical Michaelis-Menten kinetics is obeyed, that the inhibitors combine reversibly with the enzyme, and that each enzyme-inhibitor complex species contains only a single species of inhibitor, i.e., the inhibitors are mutually exclusive (4). We consider in turn, the relationships between the uninhibited initial velocities and those observed in the presence of one or more inhibitors, for reactions involving one or more than one substrate.

Case 1 - One substrate reaction with two inhibitors: I1 is competitive, I2 is competitive.

νo = VA/(KmA + A) (1)

ν1 = VA/(KmA + I1/A) + A (2)

ν2 = VA/(KmA + I1/A) + I2/A (3)

Combining Equations 1, 2, and 4, hence:

1/ν1,2 = 1/ν1 + 1/ν2 - 1/νo (5)

Case 2 - One substrate reaction with three inhibitors selected at random: I1 is competitive, I2 is noncompetitive, and I3 is uncompetitive.

νo = VA/(KmA + I1/A) + A (6)

ν2 = VA/(KmA + I2/A) + A (7)

ν1,2,3 = VA/(KmA + I1/A) + I2/A + I1/A (8)

Combining Equations 1, 2, 6, 7, and 8, hence:

1/ν1,2,3 = 1/ν1 + 1/ν2 + 1/ν3 - 2/νo (9)

Extending the above arguments to four inhibitors of any classes, it may be seen that:

1/ν1,2,3,4 = 1/ν1 + 1/ν2 + 1/ν3 + 1/ν4 - 3/νo = 1/ν1,2,3 + 1/ν4 - 1/νo

= 1/ν1,2,3 + 1/ν4 - 2/νo

Thus, the numerator of the reciprocal of the νo term is equal to the number of partitions (the preceding terms) minus one. More generally, the velocities of single-substrate reactions in the presence of n inhibitors belonging to any combination of competitive, noncompetitive, and uncompetitive classes are expressed by the relation: 3

1/νo = Σ 1/n - 1 - n - 1/νo (10)

Case 3 - Two substrate reactions with two inhibitors: Ping-Pong Bi Bi Mechanism.

νo = VA/(KmA + A) (11)

ν1 = VA/(KmA + I1/A) + A (12)

ν2 = VA/(KmA + I2/A) + A (13)

ν1,2 = VA/(KmA + I1/A) + I2/A + I1/A (14)

Combining Equations 11, 12, 13, and 14, again gives Equation 5, i.e.,

1/ν1,2 = 1/ν1 + 1/ν2 - 1/νo (5)

It is shown in the supplement that the general relation

3 Alternative forms of this relationship, not involving the uninhibited velocity νo term, are as follows:

(fj1,2,3 ..., fj1,2,3, ..., fn) = 1 - [Σ (fj1,2,3 ..., fj1,2,3, ..., fn)] (10a)

= 1 - [Σ (fj1,2,3 ..., fj1,2,3, ..., fn)] (10b)

The limit descriptor J is used here in place of i in order to avoid ambiguity. These alternative formulations are useful in analyzing inhibitory effects in which the uninhibited velocity is unknown (see Appendix V).
Mutually Nonexclusive Inhibitors and the Fractional Inhibition Concept

A useful method for expressing the degree of inhibition of a reaction is in terms of the fractional velocity \((f_i)\) which is the ratio of the velocity in the presence \((v_a)\) to that in the absence \((v_b)\) of the inhibitor. Consequently, the fractional inhibition \((f_i)\) is \((1 - f_i)\). Numerous authors have intuitively assumed that the fractional reaction velocity in the presence of two or more inhibitors may be expressed as the product of the fractional velocities observed in the presence of each of the inhibitors individually. Thus, Webb (see Ref. 3, pp. 507–508) states that this relation describes a summation of inhibitory effects, since Webb (3) further proposes that synergism and antagonism among inhibitors should be defined in terms of deviations from Equation 15.

Our own analysis does not support this supposition, as may be seen from the following. Equation 15 may be transformed as follows:

\[
\frac{v_{i_1,i_2}}{v_a} = \frac{v_{i_1}}{v_{i_1}} \times \frac{v_{i_2}}{v_{i_2}}
\]

whence

\[
v_{i_1,i_2} = v_{i_1} v_{i_2} v_a
\]

The generalized relationship developed in this paper for reciprocal velocities for two inhibitors (Equation 10) may be transformed as follows:

\[
v_{i_1,i_2} = \frac{v_{i_1} v_{i_2} v_a}{v_{i_1} + v_{i_2} v_a}
\]

or

\[
(f_{i_1,i_2} - [f_{i_1} \times f_{i_2}]/[f_{i_1} + f_{i_2}]) - [f_{i_1} \times f_{i_2}]
\]

Clearly Equations 15 and 18 are not identical.

The inhibited velocities calculated from the product of fractional velocities (Equation 15 or 16) will always be smaller than those predicted by Equation 10. In the case of more than two inhibitors, the disagreement between values given by Equation 10, and those calculated from the product of fractional velocities (Equations 15 or 16) becomes even larger. We conclude that if the assumptions of mutual exclusivity by reversible inhibitors obeying Michaelis-Menten kinetics apply, the analysis of multiple inhibitions by the product of fractional velocities (Equations 15 or 16) will always indicate synergism of inhibition (in comparison to the results predicted by Equation 10 for summation of inhibitory effects). The magnitudes of these discrepancies are illustrated in the supplement (Appendix II). However, it is shown in the supplement (Appendix III) that the product of fractional velocities accurately describes the behavior of two nonexclusive inhibitors provided at least one of these inhibitors is noncompetitive.

GENERALIZATIONS

Equation 10 describes the initial velocities of enzymatic reactions in the presence of multiple exclusive inhibitors. This relationship is independent of the number of substrates, the reaction mechanism, and the types or mechanisms of inhibitors. Consequently, we propose the following definitions of the effects of two inhibitors acting on a single target enzyme under steady state conditions:

**Summation:**

\[
1/v_{i_1,i_2} = 1/v_i + 1/v_a - 1/v_0
\]

**Synergism:**

\[
1/v_{i_1,i_2} > 1/v_i + 1/v_a - 1/v_0
\]

**Antagonism:**

\[
1/v_{i_1,i_2} < 1/v_i + 1/v_a - 1/v_0
\]

By analogy, these relationships may be extended to larger numbers of inhibitors.

For mutually nonexclusive inhibitors, synergisms will be invariably observed. Moreover, for noncompetitive, nonexclusive inhibitors, the relationship between inhibited and uninhibited velocities is given by the product of the respective fractional velocities.

\[
\frac{v_{i_1,i_2,...,i_n}}{v_0} = \frac{v_{i_1}}{v_{i_1}} \times \frac{v_{i_2}}{v_{i_2}} \times \ldots \times \frac{v_{i_n}}{v_{i_n}}
\]

\[
\prod_{i=1}^{n} \frac{v_i}{v_0}
\]

\[
(f_{i_1,i_2,...,i_n} = f_{i_1} f_{i_2} f_{i_3} \ldots f_{i_n} = \prod_{i=1}^{n} f_i)
\]

REFERENCES

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5. Ordered II Mechanism (Sequential Mechanism)

\[ r = \frac{v}{K + [S]} \]

Case 5. The inhibitors: \( I_2 \) in competition with substrate \( S \) and noncompetition with \( I_1 \); \( I_2 \) in competition with substrate \( S \) and noncompetition with \( I_1 \)

\[ r = \frac{v}{K + [S]} \]

Combining Equations (5), (6), and (7), we have

\[ r = \frac{v}{K + [S]} \]

Case 6. The inhibitors: \( I_2 \) in noncompetition; \( I_1 \) in noncompetition

\[ r = \frac{v}{K + [S]} \]

Combining Equations (5), (6), and (7), we have

\[ r = \frac{v}{K + [S]} \]

Case 7. The inhibitors: \( I_2 \) in noncompetition; \( I_1 \) in noncompetition

\[ r = \frac{v}{K + [S]} \]

Combining Equations (5), (6), and (7), we have

\[ r = \frac{v}{K + [S]} \]

Case 8. The inhibitors: \( I_2 \) in noncompetition; \( I_1 \) in noncompetition

\[ r = \frac{v}{K + [S]} \]

Combining Equations (5), (6), and (7), we have

\[ r = \frac{v}{K + [S]} \]

Therefore, in which substrate saturation and kinetics are the subject of the main text, and the main statements are in the main text. Section 5.

The Directed Synthesis

A. Firing II Mechanism

\[ \frac{d}{dt} \frac{x}{y} = \frac{v}{K + [S]} \]

Combining Equations (10), (11), (12), and (13), we have

\[ \frac{d}{dt} \frac{x}{y} = \frac{v}{K + [S]} \]

Equation (II) may also be written:

\[ \frac{d}{dt} \frac{x}{y} = \frac{v}{K + [S]} \]

The values in which substrate saturation and kinetics are the subject of the main text, and the main statements are in the main text. Section 5.

APPENDIX II

Comparison of Kinetic Methods for Calculating Initial Reaction Velocities

Table II

<table>
<thead>
<tr>
<th>Initial Reaction Velocity</th>
<th>Equation from Equation (II) for Calculating Initial Reaction Velocities</th>
</tr>
</thead>
<tbody>
<tr>
<td>( v_{0.1} )</td>
<td>( v_{0.1} = \frac{v}{K + [S]} )</td>
</tr>
<tr>
<td>( v_{0.2} )</td>
<td>( v_{0.2} = \frac{v}{K + [S]} )</td>
</tr>
<tr>
<td>( v_{0.3} )</td>
<td>( v_{0.3} = \frac{v}{K + [S]} )</td>
</tr>
<tr>
<td>( v_{0.4} )</td>
<td>( v_{0.4} = \frac{v}{K + [S]} )</td>
</tr>
</tbody>
</table>

Equations (14), (15), (16), and (17) are used for the calculation of initial reaction velocities.
Multiple Inhibitions of Michaelis-Menten Systems

UPPER III

Multiple Inhibitions of Michaelis-Menten Systems

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Multiple Inhibitions of Michaelis-Menten Systems

A. General Equation for Multiple Inhibitions

\[ I = I_1 + I_2 + I_3 + \cdots \]

B. Specific Equation for Multiple Inhibitions

\[ I = \frac{K_{i1}}{K_{i2}} + \frac{K_{i2}}{K_{i3}} + \cdots \]

C. General Equation for Multiple Inhibitions

\[ I = \frac{K_{i1}}{K_{i2}} + \frac{K_{i2}}{K_{i3}} + \cdots \]

D. Specific Equation for Multiple Inhibitions

\[ I = I_1 + I_2 + I_3 + \cdots \]

E. General Equation for Multiple Inhibitions

\[ I = \frac{K_{i1}}{K_{i2}} + \frac{K_{i2}}{K_{i3}} + \cdots \]

F. Specific Equation for Multiple Inhibitions

\[ I = I_1 + I_2 + I_3 + \cdots \]

G. General Equation for Multiple Inhibitions

\[ I = \frac{K_{i1}}{K_{i2}} + \frac{K_{i2}}{K_{i3}} + \cdots \]

H. Specific Equation for Multiple Inhibitions

\[ I = I_1 + I_2 + I_3 + \cdots \]

TABLE III

Inhibition of Liver Alcohol Dehydrogenase by Ethanol, Phenobarbital, and Indomethacin

<table>
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<th>Compound</th>
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UPPER IV

Inhibition of Liver Alcohol Dehydrogenase by Ethanol, Phenobarbital, and Indomethacin

A comparison in vivo between the inactivation velocities \( V_i \) of the three liver alcohol dehydrogenase preparations, of \( \Delta \), a small liver plate (L), and of \( \Delta_2 \), a small liver plate (L), shows that the inactivation velocities \( V_i \) are virtually identical. The results are shown in the Table IV.

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A simple generalized equation for the analysis of multiple inhibitions of Michaelis-Menten kinetic systems.

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