



Installation

See the Macro Installation.doc document.

Data Entry

The Exploratory EK macro is designed to work in conjunction with the Enzyme Kinetics Module or with data entered into a SigmaPlot worksheet.

Use with the Enzyme Kinetics Module

Open the worksheet that the EK Module created after you entered your data. The EK Module type of study must be Single Substrate – Single Inhibitor. Then run the Exploratory EK macro. It will recognize that this worksheet was created by the EK Module and will use the adjusted data in the worksheet. The adjusted data will be the same as the raw data that you entered if you have not entered an equation(s) to adjust it.

Entering Data into a SigmaPlot Worksheet

Data is entered in S, I, multiple-replicate-velocity format where S is a column of substrate values, I is a column of inhibitor values and multiple-replicate-velocity are groups of columns containing replicate velocity values. Each group contains the same number of columns for replicates. Replicates are entered rowwise. For example, the worksheet in Figure 1 shows substrate and inhibitor values in columns 1 and 2, respectively. There are 5 groups of replicate velocity values (only two are shown) corresponding to the 5 inhibitor values. Each group contains 3 columns of replicates. There are two replicate values and a missing value in the first row of the first replicate velocity group corresponding to S = 0.5 and I = 1.0.

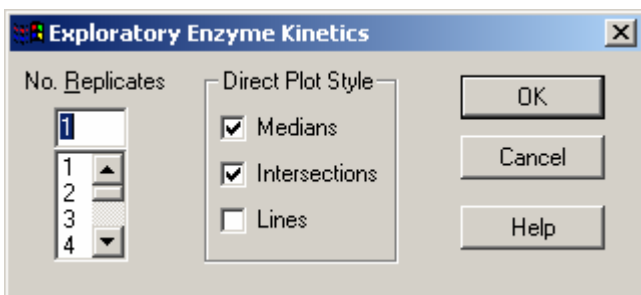
	1-S	2-I	3-v11	4-v12	5-v13	6-v21	7-v22	8-v23
1	0.5000	1.0000	3.8650		3.7040	3.3100	3.4260	3.1380
2	1.0000	2.0000	7.1920	6.9330	7.2180	6.1270	5.9880	5.8230
3	5.0000	5.0000	22.1020	29.4710	27.3540	21.1280	22.3070	19.7920
4	10.0000	10.0000	38.5220	38.5260	40.6130	30.7700	30.8910	31.5240
5	20.0000	20.0000	55.2780	49.4350	51.0530	40.4200	43.7810	38.7670
6	50.0000		58.9910	65.8090	63.6500	46.3720	47.4110	50.3450
7	100.0000		63.4080	72.0250	63.3970			61.2010

Figure 1. Data format in the SigmaPlot worksheet. Only two of the five replicate velocity groups are shown.

The data must be left-adjusted in the worksheet with substrate values in column 1. This is the same format used for data entry by the EK Module.

Using the Macro

Starting the macro produces the dialog



Enter the number of replicates if you have entered your data directly into a SigmaPlot worksheet. The number-of-replicates control will not be available if you are analyzing an Enzyme Kinetics Module worksheet. The macro obtains the number of replicates from this worksheet.

You can control the content of the direct plot. The most useful option is to show both the intersections and the medians – Figure 2A. Selecting the lines option is useful for tutorial purposes but for realistic data produces a cluttered graph which decreases your ability to visualize the intersection and median values – compare the direct linear plot in Figure 3A which contains the intersection lines with the graphs in Figure 2.

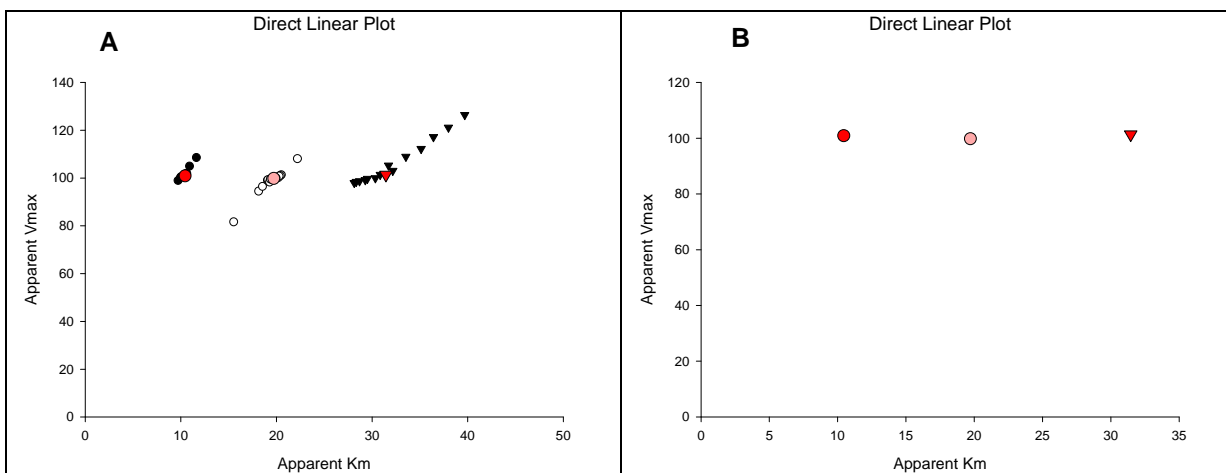


Figure 2. The direct linear plot with Medians and Intersections selected (A) and Medians only selected (B).

The median symbols are associated with increasing inhibitor concentration by symbol type and color. The SigmaPlot “doubles” scheme is used in conjunction with two colors. The symbol type cycle {circle, down-triangle, square, diamond, up-triangle, hexagon} is used with red and light red colors. Therefore, increasing inhibitor concentration is identified with the sequence {red circle, light-red circle, red triangle-down, light-red triangle-down, ...}.

Exploratory EK Results

The macro produces two graph pages and a numerical report. The first graph page contains two graphs – the direct linear plot and the Michaelis-Menten (v vs S for fixed I) plot. These two graphs allow examination of the raw data to understand the origin of second and third quadrant

data points in the direct linear plot. An example of the first graph page for simulated competitive inhibition data is shown in Figure 3A.

The second page contains the two secondary plots (apparent K_m/V_{max} vs I and apparent $1/V_{max}$ vs I) derived from apparent K_m and V_{max} median values (red and light-red symbols) computed for the direct linear plot. These are shown in Figure 3B. The secondary plots are useful for determining the type of linear inhibition and obtaining estimates for the inhibition dissociation constants.

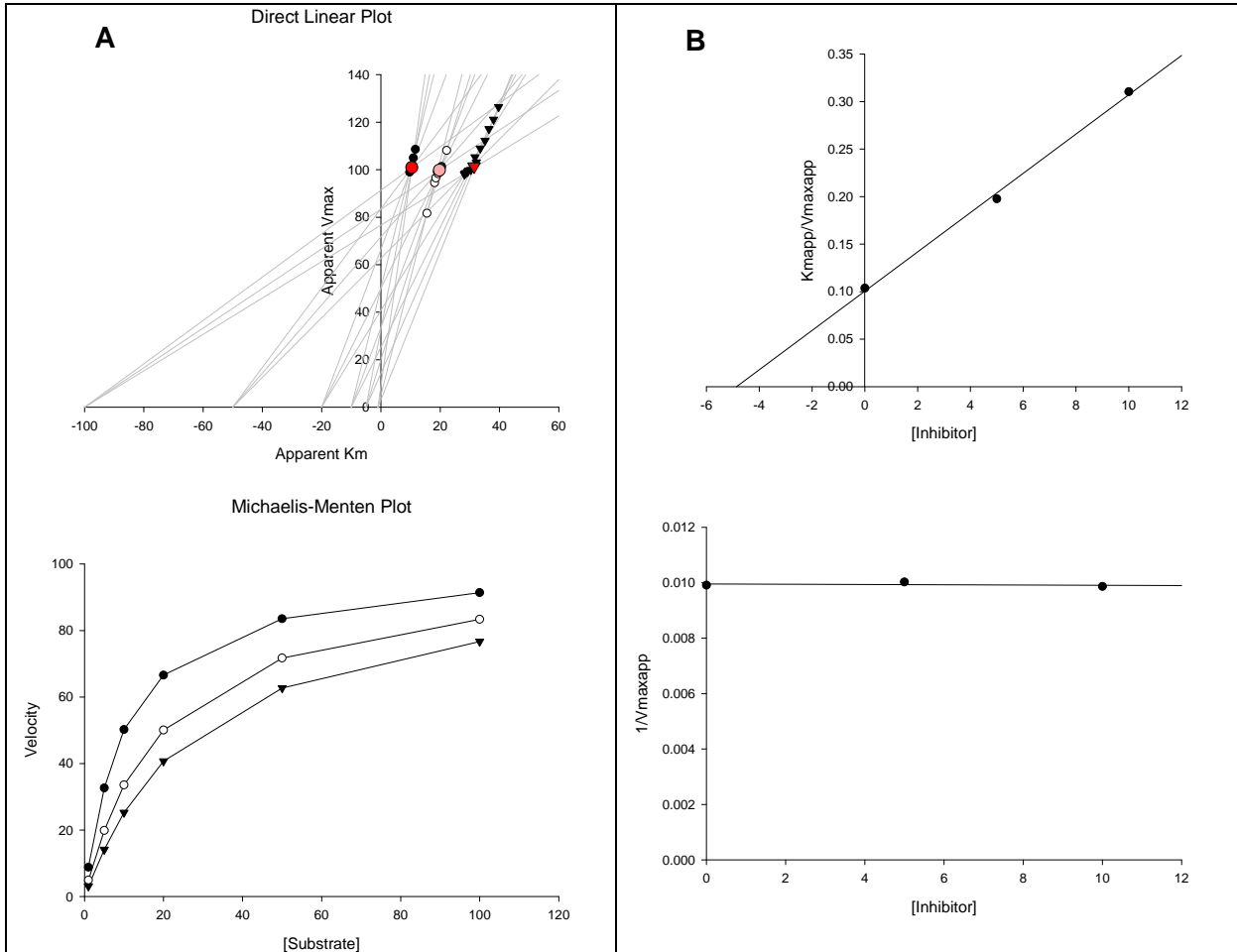


Figure 3. The two pages produced by the Exploratory EK macro. The first page with simulated competitive inhibition data is shown in A. The secondary plots on the second page are shown in B.

The numerical report is placed into a worksheet. Figure 4 shows the results for the data in Figure 3. The report contains median values for apparent K_m and apparent V_{max} and derived values used in the secondary plots. The total number of line intersections and the number in the third quadrant are also displayed. The intersections with the x-axis (inhibitor) by the secondary plot linear regression lines are used to obtain the inhibitor dissociation constants. The estimate for K_{iu} in Figure 4 is a large negative value since the regression line slope is slightly negative (but for practical purposes is zero for which K_{iu} is then infinite).

	1	2	3	4	5	6	7	8	9
1	[Inhibitor]	Kmapp	Vmaxapp	Kmapp/Vmaxapp	1/Vmaxapp	Intersections	3rd quad Inter	Kic	Kiu
2	0.0000	10.4600	100.9000	0.1036	9.9110e-3	15.0000	0.0000	4.8650	-2145.0000
3	5.0000	19.7200	99.7800	0.1977	0.0100	15.0000	0.0000		
4	10.0000	31.4500	101.4000	0.3103	9.8640e-3	15.0000	0.0000		
5									

Figure 4. The exploratory EK report results.

The Direct Linear Plot

The direct linear plot is the basis of the Exploratory EK macro. It is described in the text by Cornish-Bowden⁽¹⁾ and in his and Eisenthal's papers⁽²⁻⁴⁾. It is an excellent first-step for enzyme kinetics analysis since it examines the validity of the Michaelis-Menten assumption. It also gives useful information about the type of linear inhibition involved and, from secondary plots, generates inhibition dissociation constants.

The direct linear plot is different from the usual enzyme kinetics plots since it considers the parameters Vmax and Km to be variables and graphs Vmax vs Km. Enzyme kinetics graphs typically graph combinations of velocity v and substrate S against one another. The direct linear plot is derived by first assuming that Michaelis-Menten kinetics are applicable

$$v = \frac{V_{\max} S}{K_m + S} \quad (1)$$

and then rewriting this in terms of Vmax and Km as

$$V_{\max} = v + \frac{v}{S} K_m \quad (2)$$

For fixed values of v and S this is a straight line. The y-intercept (Km = 0) for this line is Vmax = v and the x-intercept (Vmax = 0) is Km = -S. Two pairs of (S,v) data values generate two straight lines which intersect as shown in the Vmax-Km graph in Figure 5A. The intersection defines the parameter values Vmax* and Km* that generated the two (S,v) data pairs. If you have more than two (S,v) pairs and these are error free, generated by Equation (1) say, then these lines will also intersect in a point as shown in Figure 5B.

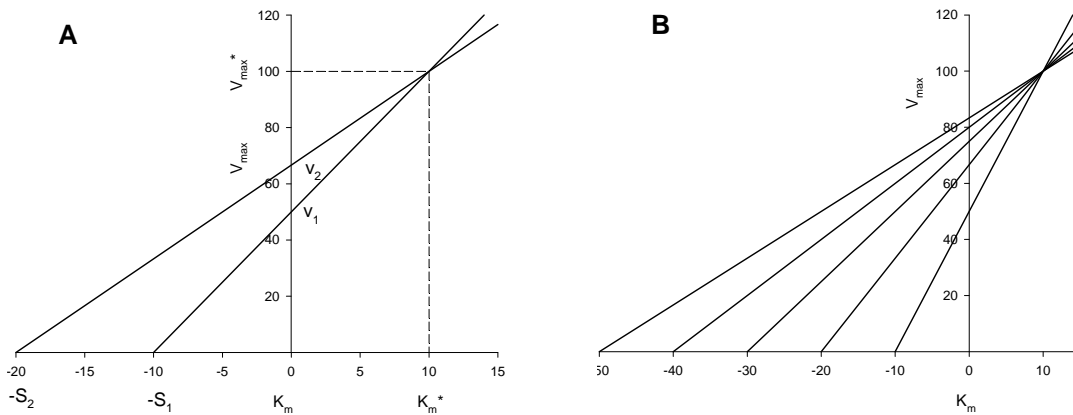


Figure 5. The direct linear plot is a graph of straight lines defined by (S,v) data pairs on a Vmax, Km coordinate system. The intersection of the lines defines the Vmax* and Km* parameter values which generated the (S,v) data.

Actual data will not be error free and so will not intersect at a point. A typical example is shown in Figure 6. The intersections are scattered around the true Vmax and Km values of 100 and 10, respectively.

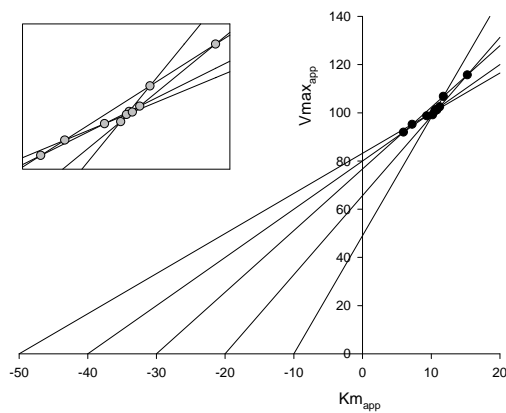


Figure 6. The direct linear plot for (S,v) data with error. The inset shows the ten line intersections that occur with five lines.

There will be $n(n-1)/2$ line intersections for n lines. Using the medians of the line intersection x-coordinates and y-coordinates as estimates for Km and Vmax, respectively, has been found to work well.

For positive S and v values the line intersections can occur in the first, second and third quadrants. Error-free Michaelis-Menten kinetics will result in intersections in the first quadrant. Intersections in the second and third quadrants can result from 1) random error in the data or 2) an enzymatic reaction which does not follow the Michaelis-Menten assumption. A second quadrant intersection results from two (S,v) data points at large S for which the velocity for the largest S is less than the other velocity. This can result from substrate inhibition where the velocity decreases for large S or from random error. A third quadrant intersection results from two (S,v) data points at small S for which the velocity for the smallest S is "too small" relative to the other velocity. "Too small" means with respect to the hyperbolic (S,v) shape for Michaelis-

Menten kinetics. This can occur for an allosteric enzyme with a sigmoidal (S,v) shape or from data error.

Computational Details

Replicates

Replicate velocity measurements are commonly made for each substrate concentration. Two different substrate concentrations are required for two direct-linear lines to intersect at a non-zero V_{max} value (Figure 5A). Therefore, replicate velocity values for a given substrate value provide no additional information for the corresponding direct-linear line. The Exploratory EK macro uses the mean of velocity replicates for each substrate value.

Third Quadrant Intersections

Cornish-Bowden and Eisenthal⁽⁴⁾ have shown that underestimates of $V_{max_{app}}$ and $K_{m_{app}}$ occur if third quadrant intersections are included in the computation of the medians. They also showed that the median computation method produces correct results if third quadrant intersections are considered to be first quadrant intersections at infinity. The Exploratory EK macro computes medians in this way with one modification. If the number of third quadrant intersections exceeds half the total (which would result in an infinite median value) then the maximum first quadrant $V_{max_{app}}$ (or $K_{m_{app}}$) value is used as the 'median'.

Linear Inhibition Examples

The Direct Linear Plot

A direct linear plot with one ($K_{m_{app}}$, $V_{max_{app}}$) median symbol, similar to the one shown in Figure 6, is obtained for each inhibitor concentration. Multiple inhibitor values result in multiple plots like that shown in Figure 6 superimposed in one graph. Examining the trajectory of the medians in the direct linear plot as the inhibitor concentration is increased gives information about the type of linear inhibition. In general, the relationship between inhibition type and the trajectory is

Inhibition Type	Trajectory
Competitive	Horizontal to the right
Mixed	Diagonal down and to the right
Noncompetitive	Vertically down
Uncompetitive	Toward the origin

Table 1. Direct linear plot median trajectory behavior.

Data was created by computer simulation for the four inhibition types to show realistic examples of direct linear plots – Figure 7. This figure gives you an idea of the amount of scatter that occurs for the intersections. It also shows the variability of the median values about the trajectory description in Table 1.

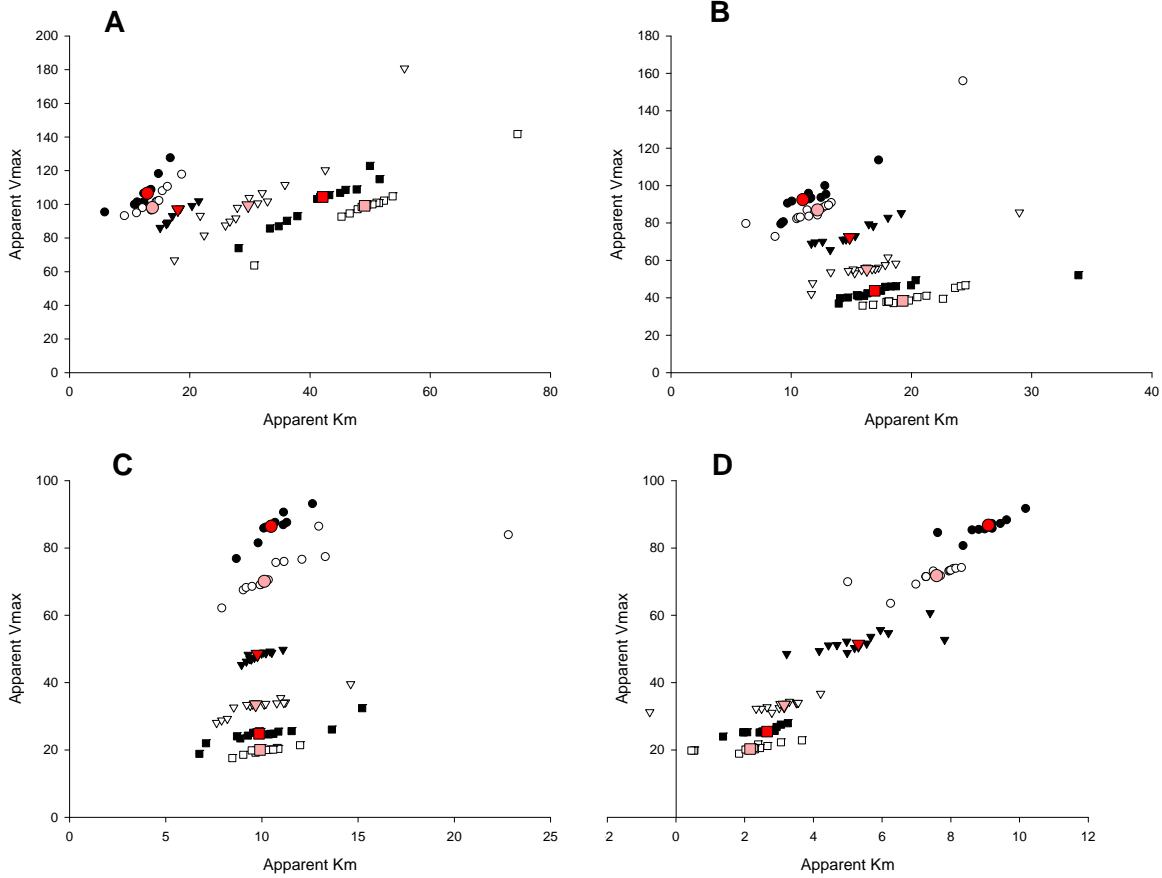


Figure 7. Median trajectories for different linear inhibition types. A - competitive inhibition, B - mixed inhibition, C - noncompetitive inhibition, D - uncompetitive inhibition. The second quadrant intersection in D results from data error. Increasing inhibitor concentrations are indicated by the median symbol type and color - red circle, light red circle, red triangle-down, light red triangle-down, red square and light red square. Simulated data: $V_{max} = 100$, $K_m = 10$, $K_i = 5$, 5% constant percentage data error.

The reason for the direction of the median trajectories can be understood by deriving the apparent V_{max} and K_m variables from the equation for mixed inhibition

$$v = \frac{V_{max}S}{K_m(1 + I/K_{ic}) + S(1 + I/K_{iu})} \quad (3)$$

K_{ic} and K_{iu} are the EI and ESI dissociation constants (Cornish-Bowden notation ⁽¹⁾). The Enzyme Kinetics Module uses the notation

$K_i = K_{ic}$
 $\alpha K_i = K_{iu}$.

Apparent K_m and V_{max} parameters can be derived from equation (3) for the various inhibition types by letting K_{iu} and K_{ic} approach infinity and then comparing (3) with the Michaelis-Menten equation (1). Consider two examples. For competitive inhibition, let K_{iu} be infinite (the inhibitor has zero affinity for the ES complex) to give $K_{m,app} = K_m(1 + I/K_{ic})$ and $V_{max,app} = V_{max}$. For noncompetitive inhibition, let $K_{ic} = K_{iu}$ which gives $K_{m,app} = K_m$ and $V_{max,app} = V_{max}/(1 + I/K_{ic})$. The results for all cases are shown in Table 2.

Linear Inhibition Type	$V_{max_{app}}$	$V_{max_{app}}/K_{m_{app}}$	$K_{m_{app}}$
Competitive	V_{max}	$\frac{V_{max} / K_m}{1 + I / K_{ic}}$	$K_m (1 + I / K_{ic})$
Mixed	$\frac{V_{max}}{1 + I / K_{iu}}$	$\frac{V_{max} / K_m}{1 + I / K_{ic}}$	$\frac{K_m (1 + I / K_{ic})}{1 + I / K_{iu}}$
Noncompetitive	$\frac{V_{max}}{1 + I / K_{iu}}$	$\frac{V_{max} / K_m}{1 + I / K_{ic}}$	K_m
Uncompetitive	$\frac{V_{max}}{1 + I / K_{iu}}$	V_{max} / K_m	$\frac{K_m}{1 + I / K_{iu}}$

Table 2. Apparent Vmax and Km relationship with inhibitor concentration I.

In Figure 7 the competitive inhibition median trajectory is horizontal since from Table 2 the apparent Vmax is constant and the apparent Km increases linearly with I. Similarly, for uncompetitive inhibition the median trajectory approaches the origin of the direct linear plot along a straight line since both apparent Vmax and Km approach zero proportionally as I increases to infinity.

Secondary Plots

The secondary plots can be used to determine the type of inhibition and to obtain estimates of the inhibition dissociation constants. This is demonstrated below using the same examples shown in Figure 7 and comparison with the “apparent” variables in Table 2.

Competitive Inhibition

The secondary plots for the median values shown in Figure 7A for competitive inhibition are shown in Figure 8. The macro performs a linear regression on the median data in both plots.

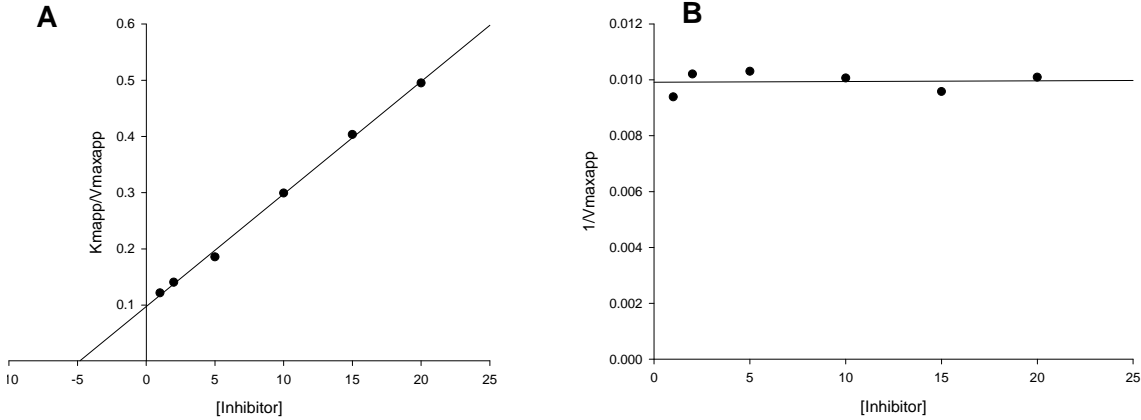


Figure 8. Secondary plots for competitive inhibition.

From Table 2 the y-axis variable for the secondary plot in Figure 8A is

$$K_{m,app} / V_{max,app} = \left(\frac{K_m}{V_{max}} \right) (1 + I / K_{ic}) \quad (4)$$

which increases linearly with inhibitor concentration and has an x-axis intercept equal to $-K_{ic}$. The K_{ic} estimate shown in Figure 8A is quite close to the actual value used in the simulation, $K_{ic} = 5.0$. The slope of the line in Figure 8B is nearly zero as it should be for competitive inhibition where from Table 2, $1/V_{max,app} = 1/V_{max}$.

Mixed Inhibition

The secondary plots for mixed inhibition shown in Figure 7B are shown in Figure 9.

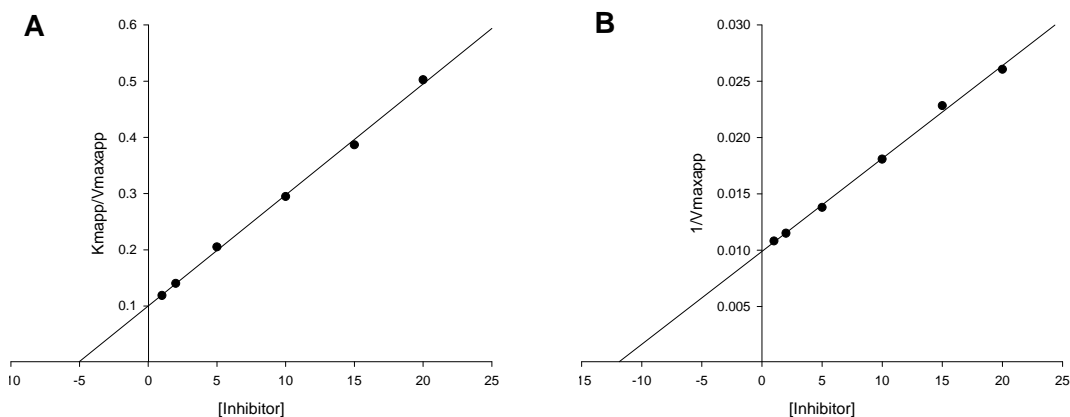


Figure 9. Secondary plots for mixed inhibition.

From Table 2 the mixed inhibition x-axis intercepts for the secondary plots are $-K_{ic}$ and $-K_{iu}$, respectively (the inverse of the relationships shown in columns 2 and 3 of that table are used for $1/V_{maxapp}$ and K_{mapp}/V_{maxapp} , respectively). The intercepts in Figure 9 are quite close to the values $K_{ic} = 5.0$ and $K_{iu} = 12.5$ used in the simulation.

Noncompetitive Inhibition

The secondary plots for the noncompetitive inhibition results shown in Figure 7C are shown in Figure 10.

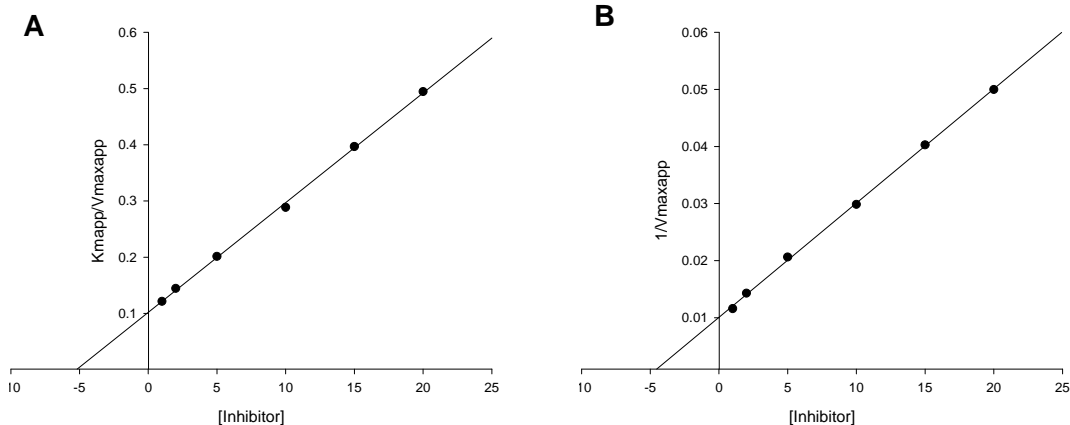


Figure 10. Secondary plots for noncompetitive inhibition.

Noncompetitive inhibition is the special case of mixed inhibition where $K_{ic} = K_{iu}$. Table 2 shows that the x-axis intercepts are $-K_{ic}$ ($= -K_{iu}$). The intercepts in Figure 10 are close to the $K_{ic} = 5.0$ value used in the simulation.

Uncompetitive Inhibition

The secondary plots for the uncompetitive inhibition results shown in Figure 7D are displayed in Figure 11.

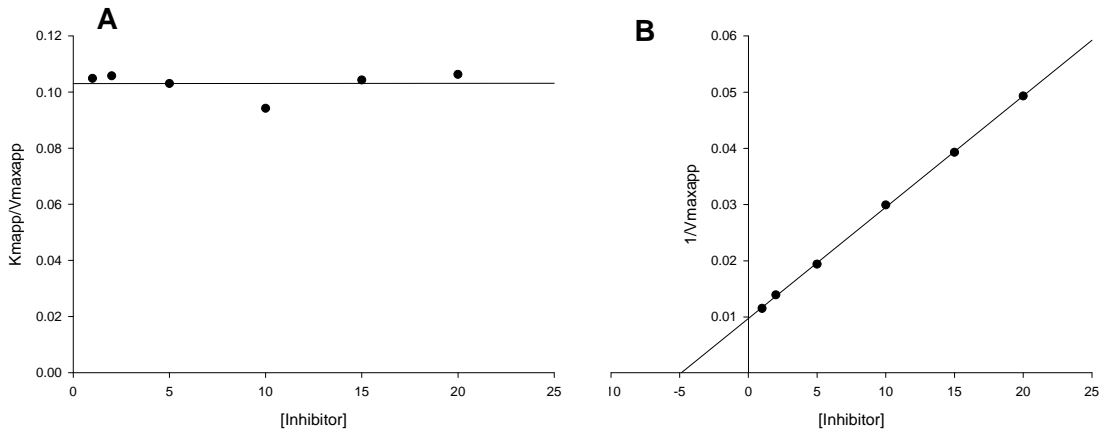


Figure 11. Secondary plots for uncompetitive inhibition.

Table 2 indicates that the secondary plot in Figure 11A should be a constant equal to $1/V_{max} = 0.01$ (the simulation used $V_{max} = 100$). The y-axis intercept is very nearly this value. The x-axis intercept in Figure 11B is $-K_{iu}$ which is very nearly equal to the value of 5.0 used in the simulation.

Other Examples

Substrate Inhibition

Simulated data for substrate inhibition is shown in Figure 12A. The initial velocities decrease for large substrate values. The second quadrant intersections that result from this are shown in Figure 12B.

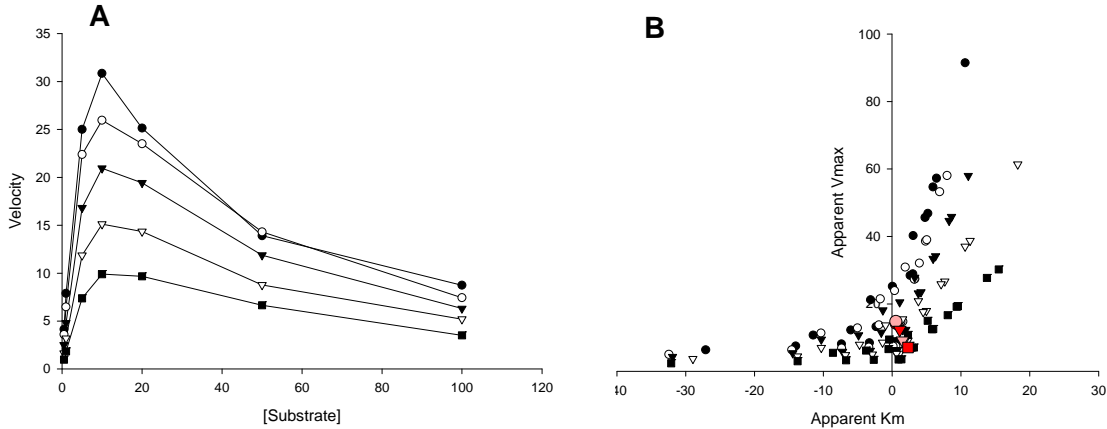


Figure 12. Second quadrant intersections that occur with substrate inhibition.

Multisite System Inhibition

Simulation of 'pure competitive inhibition, exclusive at both substrate sites' ⁽⁵⁾ results in the sigmoidal velocity shapes shown near the origin of Figure 13A. This results in the third quadrant intersections shown in B of this figure.

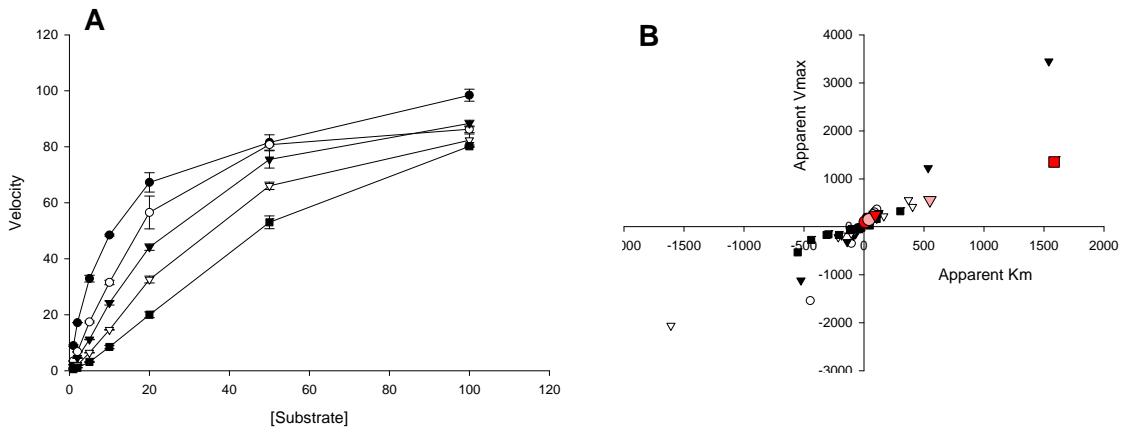


Figure 13. Third quadrant intersections that occur with multisite or allosteric inhibition.

Nonlinear Inhibition

The Exploratory EK macro was used on the data shown in the Michaelis-Menten plot in Figure 14A. The direct linear plot obtained is shown in Figure 14B. There were third quadrant intersections caused by random error and also intersections in the first quadrant with large Vmax

values. The axis scaling was changed in Figure 14B to exclude these intersections in order to better show the median values.

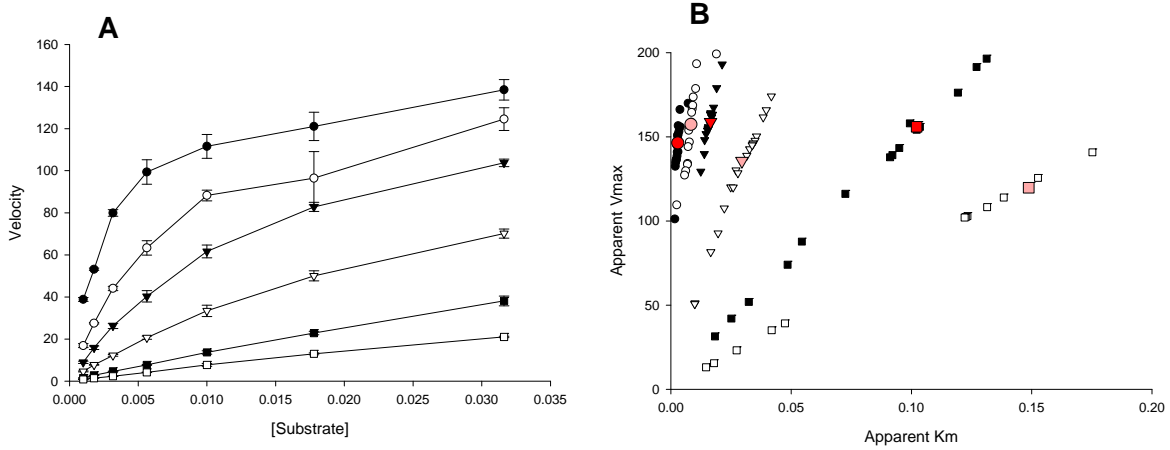


Figure 14. A. Michaelis-Menten plot of raw data. B. Direct linear plot for this data. Third quadrant and some first quadrant intersections have been excluded.

The median trajectory appears to be down and to the right suggesting mixed inhibition (Tables 1 and 2). The secondary plots are shown in Figure 15.

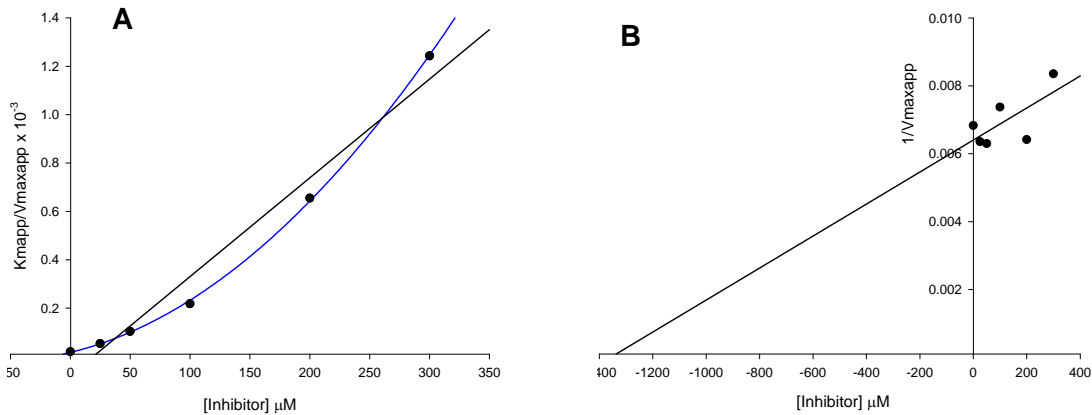


Figure 15. Secondary plots for the data shown in Figure 14.

A straight line is a poor fit to the apparent K_m/V_{max} median data. It intersects at a positive inhibitor value that corresponds to a negative inhibition constant, which doesn't make biochemical sense. A quadratic function fits this data very well, $R^2 = 0.9996$, suggesting a nonlinear inhibition. The intersection of the straight line fit on the negative inhibitor axis of the apparent $1/V_{max}$ secondary plot suggests a mixed inhibition just as the direct linear plot did. But the median data in Figure 15B is much more variable than in Figure 15A and the intersection on the inhibitor axis gives a very large $K_{iu} = 1385 \mu\text{M}$ (notice the difference in the inhibitor axis scales in Figure 15). Thus there is some evidence for mixed inhibition but the small slope in Figure 15B may be due to random error.

Two nonlinear inhibition equations, competitive and mixed, were created to use the Akaike criterion in the Enzyme Kinetics Module to determine which equation fit the data best and if the

nonlinear equations fit the data significantly better than the linear inhibition equations. These equations were adapted from Equations 5 and 6 in the paper by Willemoes⁽⁶⁾.

Nonlinear Competitive Inhibition

$$v = \frac{V_{\max} S}{K_m (1 + I/K_{i1} + I^2 / K_{i1} K_{i2}) + S} \quad (5)$$

Nonlinear Mixed Inhibition

$$v = \frac{V_{\max} S}{K_m (1 + I/K_{i1} + I^2 / K_{i1} K_{i2}) + S(1 + I/K_{ii})} \quad (6)$$

The two equations were added to the Single Substrate – Single Inhibitor section of the Enzyme Kinetics Module. All equations in that section were then fit to the data and ranked by the Akaike criterion AICc with the results shown in Table 3.

Rank by AICc	Equation	R ²	AICc	Sy.x	Runs	
					Test	Converg.
1	Competitive (Full) + Quadratic Inhib	0.98533	409.176	4.94401	pass	Yes
2	Mixed (Full) + Quadratic Inhib	0.98533	411.382	4.96440	pass	Yes
3	Mixed (Full)	0.96561	516.517	7.56955	fail	Yes
4	Mixed (Partial)	0.96561	518.722	7.60076	fail	Yes
5	Competitive (Full)	0.96219	526.290	7.90458	fail	Yes
6	Competitive (Partial)	0.96219	528.459	7.93691	fail	Yes
7	Noncompetitive (Full)	0.93181	600.599	10.61557	pass	Yes
8	Uncompetitive (Full)	0.86878	683.064	14.72522	fail	Yes
9	Uncompetitive (Partial)	0.86879	685.232	14.78538	fail	Yes
10	Noncompetitive (Partial)	0.34625	887.574	33.00246	fail	Yes

Table 3. Comparison of linear and nonlinear equations fit to the data shown in Figure 14A.

The Akaike criterion separates the equations into several groups. Lower AICc values correspond to better fits to the data. The absolute value of AICc is not important. It can be positive or negative. It is the difference between AICc values for different equations that determines whether one equation provides a better fit than the other. A very rough rule-of-thumb is that one equation provides a better fit to the data over another if its AICc value is 2 units less. The first group containing the two nonlinear equations is separated from the next nearest group by approximately 100 Akaike units. Thus the linear equations may be safely removed from further consideration. The nonlinear competitive inhibition equation is the primary candidate since its Akaike value is greater than two units from the nonlinear mixed inhibition equation. Thus with the present data set, the Enzyme Kinetics Module analysis does not support the qualitative Exploratory EK results suggesting a mixed inhibition mechanism. Two Akaike units is a borderline difference and additional data should be collected if it is important to differentiate between these two mechanisms.

Partial Inhibition

Partial inhibition is also called hyperbolic inhibition due to the hyperbolic shape of the secondary plots. Apparent Km/Vmax for partial competitive inhibition is described by

$$\frac{K_m^{\text{app}}}{V_{\text{max}}^{\text{app}}} = \frac{K_m}{V_{\text{max}}} \frac{\left(1 + \frac{I}{K_i}\right)}{\left(1 + \frac{I}{\alpha K_i}\right)}$$

This function intersects the apparent K_m/V_{max} y-axis at K_m/V_{max} and is asymptotic to $\alpha K_m/V_{\text{max}}$ for large inhibitor concentrations (see Figure 17A). The competitive (partial) equation from the Enzyme Kinetics Module was used to simulate partial competitive inhibition. The parameters used were $V_{\text{max}} = 100$, $K_m = 10$, $K_i = 2$, $\alpha = 10$ and 7% constant percentage error. The direct linear plot had one third-quadrant intersection that was caused by random error. To show the median values clearly, the graph was rescaled to ignore this intersection and two others in the first quadrant with large V_{max} values. The Michaelis-Menten and direct linear plots are shown in Figures 16A and B, respectively. The median trajectory moves horizontally to the right reflecting the competitive inhibition.

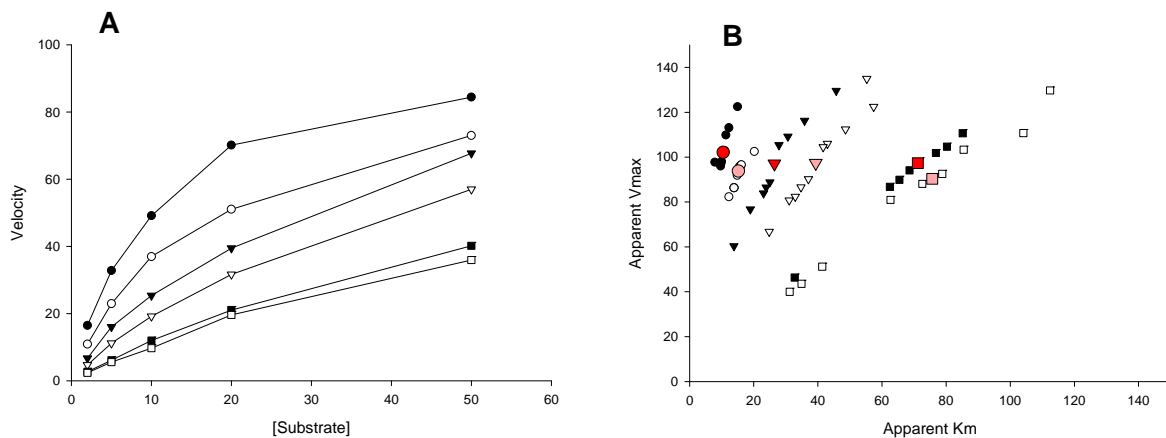


Figure 16. A. The Michaelis-Menten plot for partial competitive inhibition. B. The direct linear plot.

The secondary plots are shown in Figure 17. A straight line does not fit the apparent K_m/V_{max} data well – Figure 17A. The SigmaPlot hyperbolic function “Rational, 3 Parameter I” fit this data very well ($R^2 = 0.999$). The partial competitive inhibition parameters can be computed from the hyperbolic fit as $K_i = 1.85$ and $\alpha = 10.5$ (error-free values are $K_i = 2.0$ and $\alpha = 10.0$). The linear fit to the apparent $1/V_{\text{max}}$ data in Figure 17B is approximately constant (the K_i estimated from the regression line parameters is 1345.) as is expected from this competitive inhibition simulation.

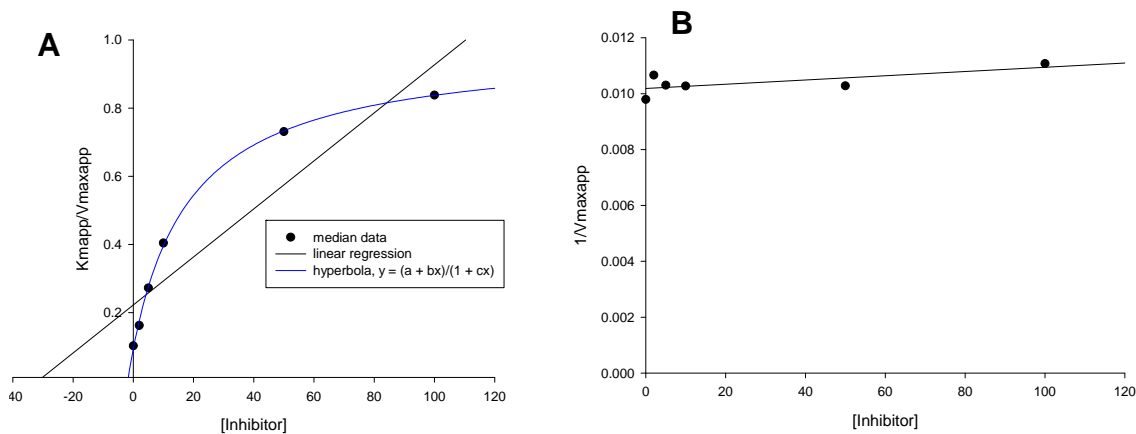


Figure 17. A. The K_m/V_{max} secondary plot shows the excellent hyperbolic function fit. B. The $1/V_{max}$ secondary plot is nearly constant reflecting the competitive inhibition.

Analysis of the initial velocity data with all equations in the Single Substrate – Single Inhibition section of the Enzyme Kinetics Module produced the equation comparison shown in Table 4. The table is sorted by the Akaike criterion AICc. It separates candidate equations into groups ⁽⁷⁾. The competitive (partial) equation has an AICc value 2 units less than the mixed (partial) equation and, given this data set, is the best candidate. Though the 2 unit AICc difference is considered to define a difference between equations it is not a large difference, so if determining the mechanism type is important then collecting additional data is warranted.

Rank by AICc	Equation	R ²	AICc	Sy.x	Runs Test	Converg.
1	Competitive (Partial)	0.98375	204.778	3.00676	pass	Yes
2	Mixed (Partial)	0.98379	206.845	3.02051	pass	Yes
3	Noncompetitive (Partial)	0.95465	297.117	5.02213	pass	Yes
4	Competitive (Full)	0.93093	332.741	6.16233	fail	Yes
5	Mixed (Full)	0.93093	334.985	6.19806	fail	Yes
6	Noncompetitive (Full)	0.90242	363.845	7.32470	fail	Yes
7	Uncompetitive (Full)	0.86781	391.170	8.52549	fail	Yes
8	Uncompetitive (Partial)	0.86952	392.241	8.51920	fail	Yes

Table 4. Comparison of Enzyme Kinetics Module single substrate – single inhibition equation fits to competitive (partial) simulated data.

The excellent fit of the competitive (partial) equation to this data is shown by the Lineweaver-Burk plot from the Enzyme Kinetics Module in Figure 18.

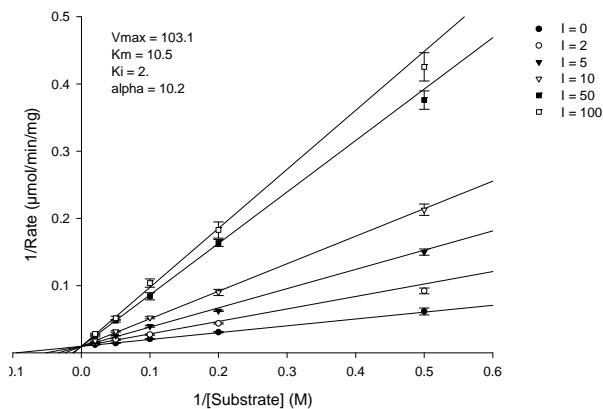


Figure 18. Lineweaver-Burk plot of the competitive (partial) equation fit to simulated data. Good inhibition parameter estimates were obtained for the experimentally realistic 7% constant percentage error.

References

- 1) Cornish-Bowden, A., Fundamentals of Enzyme Kinetics, Princeton University Press, 2nd edition, 1999, ISBN 1 85578 072 0
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