Enzyme Kinetic Data: Collection and Analysis

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Michaelis–Menten formalism undoubtedly is a very important first approximation for any new enzyme to be studied. Although simple and elegant, it has only a limited range of applicability. Not all enzymes adhere to Michaelis–Menten kinetics, and there are notable exceptions. Appropriate kinetic experimental design allows us to make this judgment. Collating good quality kinetic data is the first task in enzyme characterization.

17.1 Obtaining Primary Data: Practical Aspects

A reliable and robust assay method is a prerequisite for obtaining enzyme data. Good kinetic practices also ensure that best quality primary data is collected. Extensive coverage on both these aspects may be found in Chaps. 12 and 13. Additional considerations of importance, in generating primary kinetic data, are discussed below.

17.1.1 Reductionism in Experimental Design

The kinetic experimental design almost always takes a reductionist approach – varying one parameter at a time while keeping all others constant. The parameters that may be varied include $[S]$, $[P]$, pH, ionic strength, buffer species, activators, inhibitors, etc. Perhaps the most important and informative data set is the change in initial velocity versus substrate concentration (the $v \rightarrow [S]$ plot). Recall that even in a multi-substrate reaction, a series of bimolecular collisions take place to assemble the productive enzyme complex. Further, such reactions can be treated as pseudo-unimolecular with respect to one substrate by holding all others constant. In this sense, the primary data set is a $v \rightarrow [S]$ curve having six to ten data points for every substrate (see below). Complete kinetic analysis of a bi-substrate reaction ($n = 2$)

N. S. Punekar, *ENZYMES: Catalysis, Kinetics and Mechanisms*,
https://doi.org/10.1007/978-981-13-0785-0_17

therefore requires approximately 10^2 independent assays to be performed. As the number of reactants and modifiers increases, experimental data to be collected becomes enormous. Typically, the data volume increases as a power function of "*n*," where "*n*" is the number of reactants (substrate/product) and modifiers (activator/inhibitor) associated with that enzyme. For instance, *E. coli* glutamine synthetase is affected at least eight reactants/modifiers. Accordingly as many as 10^8 assays may be necessary to fully define its kinetics!

17.1.2 Choice of Substrate Concentrations

A major objective in the kinetic profiling of any enzyme is to determine its kinetic constants V_{max} (and hence k_{cat}) and K_M. Initial velocity measurements made at different [*S*] are the original data required to achieve it. Table 17.1 provides a sample of $v \rightarrow [S]$ data for arginase (initial velocities obtained under steady-state conditions). We shall use this data to demonstrate various aspects of subsequent kinetic analysis. The first and most straightforward way of analyzing the original data is to plot a graph of $v \rightarrow [S]$ as shown in Fig. 17.1. Reasonable estimates of V_{max} and K_M may be obtained from such plots – provided the data covers a broad range of [*S*].

Table 17.1 Variation of initial velocity with substrate concentration: original $v \rightarrow [S]$ data for *A. niger* arginase

[*S*] (Arginine, mM)	ΔA_{478}	[*P*] (Urea, mM)	*v* (μmol Urea × min^{-1} × mg^{-1})	[*S*] depleted (%)	$[\bar{S}]$ $(([S_t] + [S_f])/2)$ (mM)
6.3	**0.173**	0.86	**15.7**	13.7	5.8
12.5	**0.329**	1.65	**30.1**	13.2	11.7
14.2	**0.348**	1.74	**31.7**	12.2	13.3
16.2	**0.402**	2.01	**36.7**	12.4	15.2
20.0	**0.413**	2.06	**37.7**	10.3	19.0
35.0	**0.634**	3.17	**57.9**	9.1	33.4
50.0	**0.650**	3.25	**59.4**	6.5	48.4
100.0	**0.842**	4.21	**76.9**	4.2	97.9
150.0	**0.975**	4.88	**89.0**	3.3	147.6

Urea formed due to arginase action on L-arginine was estimated as ΔA_{478} by colorimetry (Archibald method). The original raw data is shown in black where [*S*] is independent variable (decided by the experimenter) and ΔA_{478} (reflecting urea formed) is the dependent variable. All other parameters are derived from this primary data set. For majority of kinetic analysis, [*S*] and v data (shown in bold face) are used

Concentration of urea in the 200 μl reaction (after a 10 min assay) is calculated from a urea standard curve (slope, 0.16). Initial velocity (*v*) is calculated (see Chap. 14 for details) from here using the amount of enzyme protein (0.35 μg per 200 μl assay). The percent of [*S*] depleted is obtained as follows: For example, 2.06 mM of urea is formed stoichiometrically from 20 mM of arginine. This amounts to 10.3% of substrate converted to product. Accordingly, $[\bar{S}]$ will be calculated as [20 mM + (20.0–2.06) mM]/2 = 19 mM

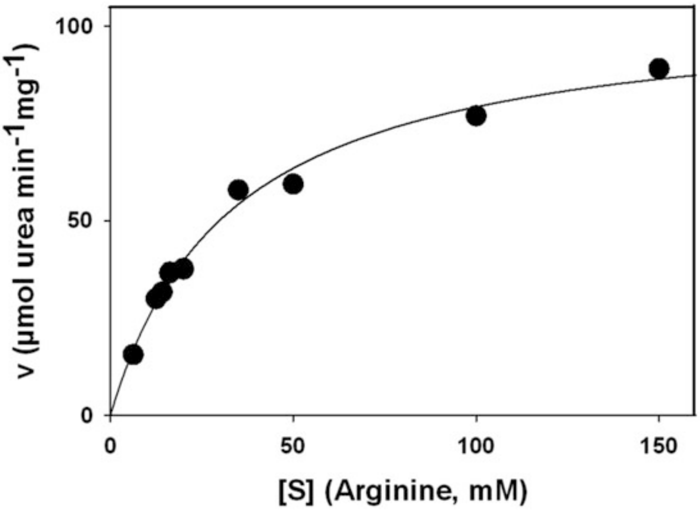

Fig. 17.1 **A v versus [S] plot of arginase data from Table 17.1**. The line drawn through the data points is the nonlinear least-squares best fit to Michaelis–Menten equation, representing a rectangular hyperbola

A broad range of [S] must be used to obtain reliable estimates of $V_{\max}$ and K_M. The two extreme cases [S] $<<K_M$ and [S] $>>K_M$, respectively, define $V_{\max}/K_M$ and $V_{\max}$ (Table 15.1) from this data set. One usually begins with the definition of $V_{\max}$ – the data representing high [S] (as will be discussed later). If only the lower [S] is covered, then the data will appear to be the first order with respect to [S]. This is defined by $v = (V_{\max}/K_M) \times [S]^1$, the limiting case of Michaelis–Menten equation (Fig. 15.2). On the other hand, measurements made only at higher [S] are biased toward $V_{\max}$. From such data, $V_{\max}$ may be estimated but there is no way to determine K_M. Clearly a range of substrate concentrations, between 0.1 K_M and 10 K_M (0.33 K_M to 3 K_M, at the least!), should be used to accurately determine the kinetic constants. The $v \rightarrow [S]$ graph around K_M is the region of maximum curvature for a rectangular hyperbola (Fig. 15.3). It is therefore wise to choose data points on both sides of K_M so that the curve is best defined!

17.1.3 Pilot Experiments and Iteration

A new enzyme study often begins with a reliable assay method but no prior knowledge of its kinetic constants. How to choose the appropriate [S] range then? It is a common practice to conduct pilot experiments with data points spanning a broad range of [S]. From these preliminary data, a rough estimate of $V_{\max}$ and K_M is obtained. More reliable estimates can then be obtained by narrowing the [S] range from 0.33 K_M to 3 K_M (using a rough value of this K_M got from the pilot experiment). Finally, a large number of data points are generated within this range to calculate

$V_{\max}$ and K_M values. Obtaining reliable and useful $v \rightarrow [S]$ data set for an enzyme is thus an iterative process. Within couple of rounds, we should be able to reach the best range of substrate concentrations required.

17.1.4 Importance of Measuring Initial Velocities

Precise measurement of initial velocity is of prime importance in kinetic analysis. Underestimation of initial velocity is a common problem when nonlinear time courses are observed. Data obtained using a "continuous assay" is therefore more reliable. One practical remedy against nonlinear time course is to *use dilute enzyme solutions*. Working with suitably diluted enzyme also helps to (a) reduce rates to manageable levels, (b) conserve the precious enzyme, and (c) eliminate unwanted interactions, if any. Other difficulties in experimental measure of initial velocity at higher $[S]$ may be due to reasons of limited solubility, interference in measurements, sensitivity of detection method, etc. An example of one such limitation and how to overcome it is shown in the box below.

Monitoring NADP–Glutamate Dehydrogenase Reaction Progress
NADP-glutamate dehydrogenase reaction can in principle be continuously monitored by increase in absorption at 340 nm. This is due to the reduction of $NADP^+$ to NADPH during the reaction. The enzyme (from *A. niger*) exhibits a K_M of about 10 μM for its substrate $NADP^+$. A maximal absorbance difference at 340 nm ($\varepsilon = 6220\ M^{-1}\ cm^{-1}$) of 0.06 is obtained when all of 10 μM $NADP^+$ is converted to NADPH. If only 10% substrate conversion is permissible, this value can be 0.006. The ΔA_{340nm} values obtainable for $NADP^+$ concentrations below K_M (<10 μM) are even smaller! Attempts to achieve a larger ΔA_{340nm} will surely lead to higher substrate depletion, nonlinear time course, and erroneous initial velocity. The poor sensitivity of the spectrophotometric assay, particularly in this example, makes it unsuitable for use. *Switching to a more sensitive detection method is a better option*. For instance, fluorimetric estimation of the product (NADPH) allows precise initial velocity measurements, (a) even at $NADP^+$ concentrations below 10 μM and (b) with permissible (less than 10%) substrate depletion.

The elementary consideration in measuring the "$[S]$ versus v" data is that true initial rates be recorded, at every substrate concentration tested. This, in practice, is however easier said than done – particularly at low $[S]$ values. In the Michaelis–Menten formalism, we assume that $[S_t] \approx [S]$. In practice therefore, up to 5–10% depletion of $[S_t]$ may be tolerated over the assay period. This is because experimental errors (and variation) itself often contributes more than this substrate depletion effects. Depletion of substrate is a significant problem at lower $[S]$ ranges tested (Table 17.1). One should therefore ensure that less than 5–10% substrate is converted to product(s) while assaying the enzyme at the lowest [S] chosen. Any higher level of $S \rightarrow P$ conversion quickly results in deviation of rates from linearity – and underestimation of v. Since experimental errors are large at low [S] and it is desirable to limit substrate conversion to under 10%, many replicate measurements may be required at low [S] values.

Lee and Wilson suggested a modification to analyze kinetic data where significant substrate conversion (as high as 40% depletion) has occurred (Lee and Wilson 1971). Suppose the substrate concentration decreases from [S_t] to [S_f] by the end of the assay. Instead of using initial substrate concentration added ([S] total, i.e., [S_t]), the arithmetic mean of [S_t] and [S_f] is recommended. The enzyme does not see [S_t] throughout the assay period. Therefore the arithmetic mean ($[\bar{S}] = ([S_t] + [S_f])/2$) is a more appropriate measure of substrate concentration in $v \rightarrow [S]$ plots. For instance, suppose the initial substrate concentration of 5.0 mM reduces to 4.0 mM (at the end of the assay) because of a 20% conversion. The effective average substrate concentration ($[\bar{S}]$) felt by the enzyme during the assay is 4.5 mM (and not 5 mM!). This difference is even larger when substrate depletion becomes higher (for instance, see analysis of arginase data in Table 17.1). Finally, a word of caution is in order however. The *Lee–Wilson modification* works well only when substrate and product do not significantly inhibit the enzyme. This procedure is not suitable for enzymes where substrate inhibition is observed or significant product inhibition occurs at low [P] levels.

17.1.5 Utility of the Integrated Form of Michaelis–Menten Equation

A complete time course (the reaction progress curve) is actually more robust source of kinetic information. It allows us to characterize the rate behavior at different extents of substrate depletion and product accumulation. Kinetic analysis of a time course is possible with the integrated form of the corresponding rate equation. *Integrated rate equations are commonly used in chemical kinetics* but rarely in enzyme kinetics (of course with the exception of fast reaction kinetics – analysis of transients). Michaelis–Menten equation can be integrated, and this may be written as shown

$$\frac{[S_t] - [S]}{t} = -K_M \times \frac{1}{t} \times \ln \frac{[S_t]}{[S]} + V_{max}$$

where [S_t] and [S] are substrate concentrations at time zero and time t, respectively. Therefore, product formed after time t is ($[S_t]-[S]$). A plot of $([S_t]-[S])/t$ versus $1/t \times \ln([S_t]/[S])$ should give a straight line with V_{max} as its intercept and K_M its slope. In principle, with this integrated form of the equation, a single extended reaction time course analysis should suffice to obtain all the enzyme kinetic parameters. This also avoids mixing errors associated with initial rate methods. Then *why is it that initial rate studies are popular in enzyme kinetics*? One reason is historical. Second, the integrated form (as shown above) does not incorporate effects of product accumulation. When incorporated, however, the system quickly gets complicated – even for a single substrate–product example. Lastly, initial velocity measurement method is advantageous in that individual variables like [S], [P], [E_t], etc. can be manipulated individually and at will.

17.2 Analyzing Data: The Basics

Investigating the kinetic properties of an enzyme implies learning how it responds to changes in the environment. Most common variable is [S], and it is essential to work over a wide range of [S] where the rate changes appreciably. Provided an enzyme obeys Michaelis–Menten equation, good design of kinetic experiments requires that [S] values should extend on both sides of the K_M. Typically three to five data points below and an equal number above K_M are desirable. The data set for a well-represented $v \rightarrow [S]$ plot should thus have at least six to ten well spread points.

17.2.1 Variation, Errors, and Statistics

Whether Michaelis–Menten formalism operates or not, the $v \rightarrow [S]$ data for an enzyme represents a nonlinear relationship. Experimental data occupies only a segment of the rectangular hyperbola described by the equation (Fig. 15.3). Therefore one must start the kinetic analysis with high quality, original $v \rightarrow [S]$ data. Large errors are associated with measurements of v at lower [S]. This is because the initial velocity responds steeply in this [S] range. And many replicates may be required. This however requires care and attention especially when working with unstable enzyme preparations. For instance, suppose we wish to vary one parameter ([S]) at four different values of the other (say the second substrate). For ten points per $v \rightarrow [S]$ data set, a total of 40 assays need to be performed. The enzyme may lose significant activity toward the end of this lengthy experiment. This should be checked of course. It is possible to pool original $v \rightarrow [S]$ data from separate experiments while evaluating kinetic constants (like V_{max} and K_M). But it is best to use a full set of $v \rightarrow [S]$ data generated in a single experiment – this minimizes "between experiments" variation. Repeat measurements should be performed to obtain a reliable data set because *any degree of sophisticated analysis will not transform bad data into good data!*

Errors cannot be avoided while obtaining kinetic data. It is important to appreciate the nature of these errors and their scatter. Due to error scattering, it becomes difficult to decide whether the measured data fits the assumed rate equation (such as Michaelis–Menten equation and hyperbolic curve). Analysis of such data requires statistical tools and regression methods in particular. This treatment is very helpful for nonlinear curves where systematic deviations are more difficult to detect by the eye. The correlation coefficient indicates the consistency of the data with the assumed model (and rate equation). Residual plots are used to measure the deviation of each value from the assumed function (as per the rate equation). Rigorous

Table 17.2 Software available to analyze enzyme kinetic data

Name	Software details
Cleland's package	Suite of FORTRAN programs; *Methods in Enzymology* 63:103 (1979); Open source
SigrafW	Microsoft[R] Visual Basic Studio program; *Biochemistry and Molecular Biology Education* 33:399 (2005); Open source
Hyper and Median	Hyper.exe is a program for the analysis of enzyme kinetic data; http://homepage.ntlworld.com/john.easterby/abouthyp.html; Open source
Leonora	Steady-state enzyme kinetics by A. Cornish–Bowden; Supplement to *Analysis of Enzyme Kinetic Data*, Oxford University Press (1995)
DynaFit	BioKin, Ltd.; *Analytical Biochemistry* **237**, 260- (1996); http://www.biokin.com/; Open source/commercial
VisualEnzymics	Softzymics, Inc. 623 Brickhouse Road, Princeton, NJ 08540; http://www.softzymics.com/; Commercial
SigmaPlot	Enzyme Kinetics Module; Systat Software Inc.; http://www.sigmaplot.com/; Commercial
EnzFitter	BIOSOFT, PO Box 1013, Great Shelford, Cambridge, CB22 5WQ GB - United Kingdom; http://www.biosoft.com/w/enzfitter.htm; Commercial

statistical analysis reduces the dangers of subjectivity in interpretation. Nevertheless it is worth remembering that accuracy of calculation cannot compensate for the lack of accuracy in collecting or recording data.

Best curve fitting can be generated by nonlinear least-squares fit of the data to the rate equation, such as the Michaelis–Menten equation. Many graphics programs are available today to perform nonlinear curve fitting. A few common examples are listed in Table 17.2. One should be reasonably familiar with the limitations of such programs however. The two unknowns $V_{\max}$ and K_M are solved iteratively in such programs. Direct analysis of the untransformed data provides the most reliable estimates of $V_{\max}$ and K_M.

One objective of kinetic studies is to get an estimate of the intrinsic kinetic constants such as $V_{\max}$ (and hence k_{cat}) and K_M for the enzyme. Different means of analyzing enzyme kinetic data are discussed below.

17.3 Plotting *v* Versus [*S*] Data

17.3.1 The *v* Versus [*S*] Plot

Most valuable insight on enzyme kinetic behavior is found in the original $v \rightarrow [S]$ plot (e.g., Fig. 17.1). It is important to critically examine this plot before attempting to transform the original data into linear plot forms (e.g., Lineweaver–Burk plot, discussed later in this chapter). From the first look, the data may appear to follow a rectangular hyperbola. This can be easily checked as follows: obtain estimates of $V_{\max}$ and K_M from this data set as if the data fits a Michaelis–Menten equation; using these two constants, generate the rectangular hyperbola, and compare this computed curve with the original curve. Within the limits of error, any systematic deviations become obvious by this comparison. Software exists to do this nonlinear curve fitting exercise (curve in Fig. 17.1 fitted to data in Table 17.1).

Reasonable estimates of $V_{\max}$ and K_{M} may be obtained manually from the original $v \rightarrow [S]$ plot – provided the data covers a broad range of $[S]$. One plots the data on graph paper and draws a curve by simply connecting the data points by straight lines. A horizontal line is drawn at the apparent plateau value of v and its point of intersection with Y-axis then defines $V_{\max}$. The point on the Y-axis where $v = V_{\max}/2$ is then located. A horizontal line is drawn from this $V_{\max}/2$ point to the point of intersection with the data curve. A vertical line from this intersection to the X-axis then defines the value of K_{M} (for instance, see Fig. 15.2).

Extraction of the two constants from a straight forward $v \rightarrow [S]$ plot is error-prone because of the nonlinear relationship. $V_{\max}$ has to be obtained from the value of the asymptote to the X-axis. But this involves geometrical extrapolation, which is difficult. In practical terms, it may also not be feasible to test higher $[S]$ values – substrate solubility being one major consideration. K_{M} is nothing but $[S]$ at $V_{\max}/2$, and therefore errors of estimation in $V_{\max}$ are carried over into evaluation of K_{M}. Thus K_{M} directly determined from $v \rightarrow [S]$ plots is also subject to significant errors. Finally, a direct comparison of a group of hyperbolas obtained from different experiments is difficult. A number of data analysis procedures and manipulations of the Michaelis–Menten equation were therefore evolved and are in use over the years. Some of these important transforms are considered below.

17.3.2 Direct Linear Plot

This plot was suggested by Eisenthal and Cornish–Bowden (1974) where a series of "$v-[S]$" data pairs are directly plotted. For each "$v-[S]$" pair of data, we can generate a straight line by marking v on the Y-axis (the $V_{\max}$ axis) and $[S]$ on the negative side of the X-axis (the K_{M} axis). All these lines (n lines for as many "$v-[S]$" pair of data!) must intersect at a point in the first quadrant with K_{M} and $V_{\max}$ as its coordinates. This result follows from the following transformation of the original Michaelis–Menten equation. Taking reciprocals on both sides,

$$\frac{1}{v} = \frac{K_{\mathrm{M}} + [S]}{V_{\max}[S]}$$ and then on rearranging we obtain,

$$\frac{V_{\max}}{v} = \frac{K_{\mathrm{M}}}{[S]} + 1$$

According to this equation, when $K_{\mathrm{M}} = 0$, we get $V_{\max} = v$ and again for $V_{\max} = 0$, we obtain $K_{\mathrm{M}} = -[S]$. Therefore, (a) we plot $[S]$ on the negative side of the X-axis, and (b) the point of intersection of all the lines has K_{M} and $V_{\max}$ as its coordinates. A direct linear plot of the data from Table 17.1 is plotted, for example, in Fig. 17.2. The

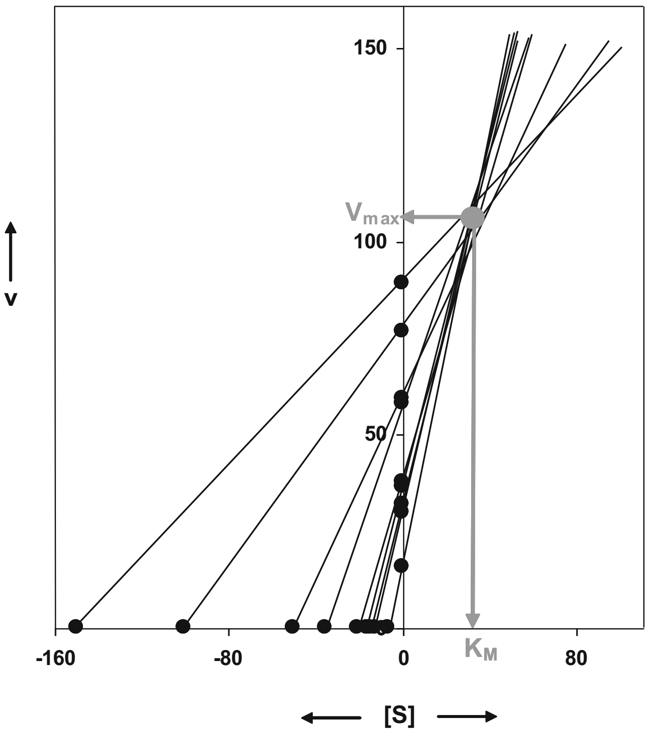

Fig. 17.2 Direct linear plot of Eisenthal and Cornish–Bowden. The graph of $v \to [S]$ data for arginase from Table 17.1 is shown

lines in practice do not neatly converge but intersect over a range of values – a clear reflection of experimental errors. This in itself is very useful in revealing poor data points. If it is certain that (a) the enzyme obeys Michaelis–Menten formalism and (b) nonlinear curve fitting is not feasible, then the direct linear plot provides the best estimates of K_M and V_{max} from $v \to [S]$ data.

17.3.3 *v* Versus log[*S*] Plot

Apart from the simple $v \to [S]$ plot in the original paper, Michaelis and Menten also plotted their data as v versus $\log[S]$. This plot is based on the rearrangement of the classical Michaelis–Menten equation as shown

$$v = \frac{V_{max}[S]}{K_M + [S]} \text{ may be rearranged to } \frac{V_{max}}{v} = \frac{K_M}{[S]} + 1$$

Taking logarithms,

$$\log\left(\frac{V_{\max}}{v}-1\right)=\log K_{\mathrm{M}}-\log[S]$$

The following variation of the above equation may appear more familiar:

$$p[S]=pK_{\mathrm{M}}+\log\left(\frac{V_{\max}-v}{v}\right)$$

This is comparable to dissociation of a weak electrolyte versus pH curve and takes a form very similar to the Henderson-Hasselbalch equation relating pH to pKa.

Advantage of a $v \longrightarrow \log[S]$ plot is that the points corresponding to lower $[S]$ are not crowded together. This semilog plot is particularly useful in (a) comparing velocities over a large range of substrate concentration and (b) plotting initial velocity data for enzymes with vastly different K_{M} values, on a single graph. For instance, substrate affinities of different liver hexokinase isozymes and glucokinase span from μM to mM (K_{M} for glucose). Plotting this on the same X-axis would require a very long graph paper! A $v \longrightarrow \log[S]$ plot, with fractional velocity ($v/V_{\max}$) on Y-axis, allows a convenient comparison on the same graph. The point of inflection (where $\log K_{\mathrm{M}} = \log[S]$ as seen by putting $v = V_{\max}/2$ in the above equation) provides a good estimate of K_{M}. Thus it is only required to identify the midpoint (inflection point) of the curve to determine K_{M} (Fig. 17.3). However, it is better to analyze $v \longrightarrow \log[S]$ plot by nonlinear curve fitting tools rather than manually.

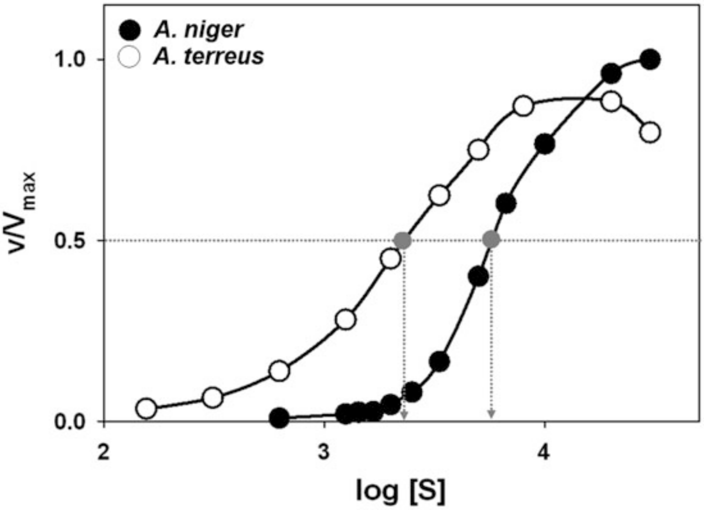

Fig. 17.3 The plot of $v \longrightarrow \log[S]$. 2-Oxoglutarate (substrate) saturation of NADP-glutamate dehydrogenases from *Aspergillus niger* [●] and *Aspergillus terreus* [○] (redrawn from the original data in Fig. 15.4). Fractional velocity ($v/V_{\max}$ which is dimensionless) is plotted on the Y-axis and arrows point to the inflection point on the two curves. The curve for *A. niger* enzyme is steeper (higher *h* value)

The $v \rightarrow \log[S]$ plot is a useful method to diagnose the presence of cooperativity in enzyme kinetics. While such graphs are always sigmoid, the steepness of the curve at the inflection point provides a measure of cooperativity. The steepness (and hence cooperativity) is much more obvious in this semilogarithmic plot than the original $v \rightarrow [S]$ curve. Its steepness is also directly related to h – the Hill coefficient (Cooperative kinetics, Chap. 15). Such a graph for two NADP-glutamate dehydrogenases (with distinct h values) in Fig. 17.3 succinctly illustrates the point.

In fact, dose–response curves for enzyme inhibition also take a similar semilogarithmic form. More on dose–response curve and the graphical determination of IC_{50} value (*i*nhibitor *c*oncentration giving 50% inhibition) is discussed in a later section (Fig. 22.8, Chap. 22). Effective range for binding in general extends over two logarithms of the ligand concentration. We may recall that acetic acid (pKa of 4.8) is mostly in the ionized state (CH_3COO^-) at pH 5.8 and is mostly in the unionized state at pH 3.8.

17.3.4 Hill Plot

We noted earlier (Cooperative Kinetics, Chap. 15) that Hill equation (and not Michaelis–Menten equation) better describes the effect of cooperative interactions on the measured enzyme rate. The Hill coefficient h is a convenient and commonly used index of cooperativity. For Michaelian enzymes h is one. It however takes other values (including non-integers) for cooperative enzymes. Hill coefficient is considered to represent the *minimum number* of interacting binding sites on an oligomeric enzyme (Hill 1910). Constant $K_{0.5}$ is similar (not same!) to K_M but also contains terms related to the effect of substrate binding at one site to the binding at other sites. The Hill equation represents a nonlinear relationship between v and [S] of an enzyme. The $v \rightarrow [S]$ data can be directly fit to this equation (through nonlinear curve fitting protocols) to extract the three parameters – V_{max}, $K_{0.5}$, and h. Otherwise, the velocity data can be analyzed by using the linear form of the Hill equation shown below. Taking logarithms after rearranging the Hill equation we obtain,

$$\log\left(\frac{v}{V_{\max} - v}\right) = h \times \log[\mathrm{S}] - \log K_{0.5}$$

A plot of $\log[v/(V_{max} - v)]$ as a function of $\log[S]$ should therefore yield a straight line graph with a slope of h and a Y-axis intercept of $-\log K_{0.5}$. Hill plots for three different enzymes are illustrated in Fig. 17.4.

A precise V_{max} value should be known beforehand to plot $\log[v/(V_{max} - v)] \rightarrow \log[S]$ graph. This is not easily obtained because of the nonlinear $v \rightarrow [S]$ relation in the first place. If present, errors in V_{max} get carried further. Lastly, the linear region of this plot is the most meaningful portion of the curve (Fig. 17.4); and linearity prevails only over a limited region of substrate concentration (around $[S] = K_{0.5}$). For these reasons, it is desirable to determine V_{max}, $K_{0.5}$, and **h** from direct nonlinear curve fits to the Hill equation itself.

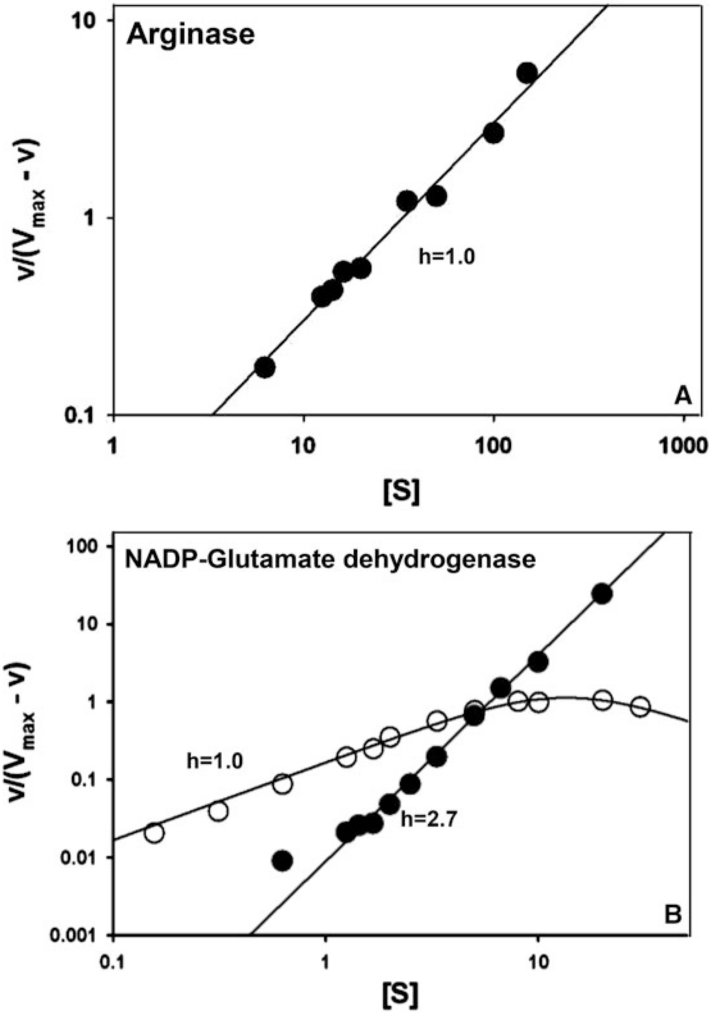

Fig. 17.4 **The Hill plot**. The log[$v/(V_{\max}-v)$] → log[S] plots for (**A**) arginase ($v \rightarrow [S]$ data from Table 17.1) and (**B**) two NADP-glutamate dehydrogenases (data from Fig. 15.4) are shown. Note that in both cases axes are marked in the logarithmic scale. Only the data points in the linear portion of the curves are used to obtain the Hill coefficient (h) and $K_{0.5}$

17.4 Linear Transforms of Michaelis–Menten Equation

Accurate extraction of kinetic constants directly from $v \rightarrow [S]$ plots is error-prone because these graphs are nonlinear. There are several ways to transform the hyperbolic relation into linear form. This is often attempted because linear relations are

Table 17.3 A few standard transforms of original $v \rightarrow [S]$ data for arginase

$[S]$ (Arginine, mM)	v (μmol $\times$ min^{-1} $\times$ mg^{-1})	$1/[S]$	$1/v$	$v/[S]$	$[S]/v$
6.3	**15.7**	0.159	0.0637	2.492	0.401
12.5	**30.1**	0.080	0.0332	2.408	0.415
14.2	**31.7**	0.070	0.0315	2.232	0.448
16.2	**36.7**	0.062	0.0272	2.265	0.441
20.0	**37.7**	0.050	0.0265	1.885	0.531
35.0	**57.9**	0.029	0.0173	1.654	0.604
50.0	**59.4**	0.020	0.0168	1.188	0.842
100.0	**76.9**	0.010	0.0130	0.769	1.300
150.0	**89.0**	0.007	0.0112	0.593	1.685

The original $v \rightarrow [S]$ data from Table 17.1 is shown in bold

better amenable to extrapolations – and hence extraction of kinetic constants. The standard transforms of original $v \rightarrow [S]$ data for arginase (from Table 17.1) for linearization are listed in Table 17.3. These will be used to demonstrate various plots described subsequently.

17.4.1 Lineweaver–Burk Plot

The Lineweaver–Burk plot (also known as *double-reciprocal plot*) is a historically important, often-used linear transform of the Michaelis–Menten equation (Lineweaver and Burk 1934). This is done by taking reciprocals on both sides to obtain,

$$\frac{1}{v} = \frac{K_M}{V_{\max}} \frac{1}{[S]} + \frac{1}{V_{\max}}$$

This equation describes a straight line and is of the form $y = mx + C$. A graph of $1/v \rightarrow 1/[S]$ should be linear with slope $m = K_M/V_{\max}$ and intercept $C = 1/V_{\max}$. When $[S] = \infty$ (and hence $1/[S]$ on X-axis is zero), the Y-axis intercept should relate to maximal velocity and zero-order rate constant ($1/V_{\max}$, as expected). While achieving $[S] = \infty$ may be nearly impossible in practice, extrapolation ($[S] = \infty$) is possible due to this linear transform, and $V_{\max}$ is conveniently evaluated. The value of K_M can be obtained from the slope and intercept (dividing m by C) of such a plot. A representative double-reciprocal plot (of the data from Table 17.3) is shown in Fig. 17.5.

The double-reciprocal plot overcomes the analysis difficulties due to nonlinear (hyperbolic) $v \rightarrow [S]$ relation. The plot should however be used with much discretion. Casual evaluation of $V_{\max}$ and K_M from this plot can be flawed. The practical considerations associated with Lineweaver–Burk plot can be serious and need attention. These aspects are described in the box below.

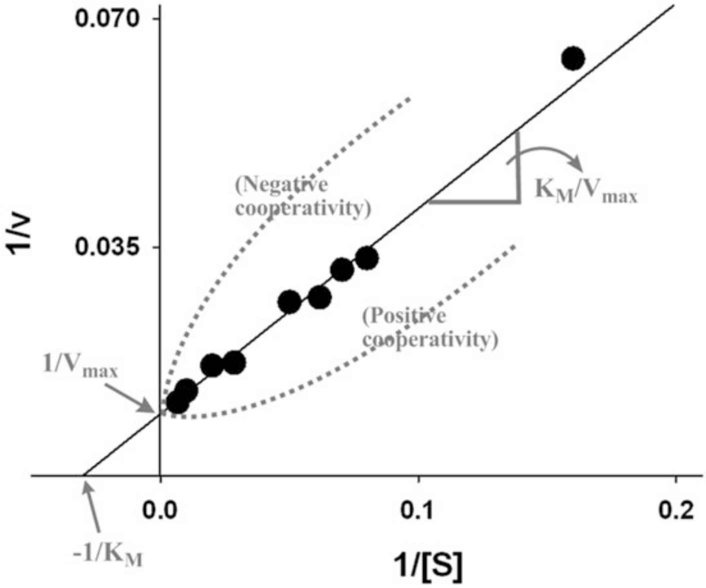

Fig. 17.5 **The double-reciprocal plot**. A graph of 1/*v* → 1/[*S*] data for arginase from Table 17.3 is shown. The curved dotted lines (schematic and in gray) represent the nonlinear plots that may be obtained when cooperativity of substrate binding is manifest

Practical Aspects of Double-Reciprocal Analysis

- Common practical problem encountered with Lineweaver–Burk plot is the data spread. One should carefully choose [*S*] values such that they lead to evenly spaced points on the 1/[*S*] axis.

With evenly spaced [*S*] values used for saturation, the data points as their reciprocals tend to cluster toward the Y-axis (see Fig. 17.5). A good experimental design and careful choice of initial [*S*] values can take care of this problem. One approach could be as follows: first, decide on the highest [*S*] to be used. Accordingly prepare a 10× stock solution – on adding 0.1 ml of this to a reaction mixture (1.0 ml final volume), desired highest [*S*] is obtained. Second, dilute the original 10× substrate solution into 1:2, 1:3, 1:4, 1:5, 1:6, 1:7, 1:8, etc., to generate working stock solutions of 1/2, 1/3, 1/4, 1/5, 1/6, 1/7, 1/8, etc. strengths. When these are used (by adding 0.1 ml to 1.0 ml reaction), a decreasing concentration series of 0.500, 0.333, 0.250, 0.200, 0.167, 0.143, 0.125, etc. is obtained. On plotting their reciprocals (1/[*S*]), the data points will now be equally spaced at intervals of 1, 2, 3, 4, 5, 6, 7, 8, etc. For arginase example – starting with the highest concentration of 150 mM (Table 17.1) – data spread for an ideal Lineweaver–Burk plot would be to use 150 mM,

(continued)

75 mM, 50 mM, 37.5 mM, 30 mM, 25 mM, 21.4 mM, 18.8 mM, 16.7 mM, 15 mM, etc. This range should also satisfy the additional important condition of data points on both sides of the K_M.

Another approach is to exploit the rough estimate of K_M from a pilot experiment. Using this as a point in the middle, prepare a series of *relative* substrate concentrations of 1.0, 1.11, 1.25, 1.43, 1.67, 2.0, 2.5, 3.33, 5.0, and 10.0. Supposing the K_M is around 1.0 mM, then we can have the range as 0.5 mM, . . .0.1.0 mM, . . .0.5.0 mM. In this arrangement, the relative substrate concentration of "2" corresponds to 1.0 mM.

- Second important issue with Lineweaver–Burk analysis is the way experimental errors are reflected in this plot. Errors are unevenly weighted in the form of reciprocals. Appropriate weighting to data points is best achieved by reliable curve fitting programs (Table 17.2) that account for the nonlinear error distribution. Small errors in lower v values lead to substantial errors in $1/v$, whereas similar errors in large v values lead to barely noticeable errors in $1/v$. A linear regression method cannot recognize this distortion because the errors themselves are nonlinear. This problem can be sorted by using suitable weighting factors – less importance to data points with large errors.

Lineweaver–Burk analysis is possible even in the absence (nonavailability rather) of programs that incorporate nonlinear error distribution. The procedure involves the following steps: original $v \rightarrow [S]$ data is first directly fit to Michaelis–Menten equation. The K_M and V_{max} values so obtained are then plugged into the double-reciprocal equation (given above) to obtain a straight line. This straight line fit is without the systematic errors arising from improper weighting of data points. The trick therefore is to avoid the temptation of directly fitting a straight line to $1/v \rightarrow 1/[S]$ values.

Finally, it is best to obtain data of such good quality that the result is also obvious without statistical analysis. Quoting Henry Clay, "Statistics are no substitute for judgment."

Despite its limitations (mentioned above), Lineweaver–Burk plots are of considerable value. Of the various approaches to linearize the Michaelis–Menten equation, only the *Lineweaver–Burk plot permits the individual display of v and [S] on the two axes*. In all others (see below), at least one coordinate is a composite of both v and $[S]$. The second big advantage of this plot is our ability to follow changes in the first-order and zero-order rate constants of an enzyme-catalyzed reaction simply by inspection. Recall that the reciprocal of its slope is V_{max}/K_M (first-order rate constant) while the reciprocal of its intercept is V_{max} (zero-order rate constant). *An increase in slope or intercept indicates a decreased first-order or zero-order rate constant, respectively.* We live in real time, and reciprocal analysis (of this type) makes it difficult to grasp the reality. Beginners in enzymology may find it awkward to appreciate the reciprocal relationship. Nevertheless, this information is particularly useful (a) in diagnosing the mechanistic details of multi-substrate enzymes and (b) to probe the mode of interaction between an enzyme and its inhibitor (see Chap. 18).

If and when Lineweaver–Burk plots of enzyme kinetic data are nonlinear, then it is obvious that the original assumptions inherent in the Michaelis–Menten equation do not hold! Failure to measure true initial velocity, a common problem, should be quickly checked. Further, the kinetic data should not be forced to fit a straight line. Instead, other kinetic models should be explored to address deviations from hyperbolic kinetics. Nonlinear Lineweaver–Burk plots result if multiple enzyme forms acting on the same substrate (isozymes with distinct kinetic characteristics) exist in the assay. With pure enzyme samples, however, curved double-reciprocal plots could mean any of the following: (a) substrate activation, (b) substrate inhibition, (c) multiple binding of substrate molecules, or (d) cooperativity of substrate binding. Some of these will be elaborated at appropriate places later. The curvature in the double-reciprocal graph is concave upward for positive cooperativity and concave downward for negatively cooperative enzymes (gray dotted lines schematically shown in Fig. 17.5). Departures from linearity are less obvious in a Lineweaver–Burk plot but are better viewed in others like the Eadie–Hofstee plot and the Hanes–Woolf plot described below.

17.4.2 Eadie–Hofstee Plot

This plot (Eadie 1942; Hofstee 1952) is one other way to transform the hyperbolic relation into a linear form for further analysis. The classical Michaelis–Menten equation can be rearranged by cross multiplying as shown

$$v(K_{\mathrm{M}} + [S]) = V_{\max}[S]$$

Dividing both sides by [S] and rearranging, we obtain,

$$v = -K_{\mathrm{M}}\frac{v}{[S]} + V_{\max}$$

This is again of the form $y = mx + C$ when v is plotted against $v/[S]$. A graph of $v \rightarrow v/[S]$ should be linear with negative slope $m = K_{\mathrm{M}}$ and the Y-axis intercept $C = V_{\max}$. The arginase data from Table 17.3 is plotted in this form in Fig. 17.6, for example. This method of plotting original $v \rightarrow [S]$ data is a linearization through a single reciprocal; only $[S]$ is in the reciprocal form. Since the X-axis represents a composite value ($v/[S]$), it is conceptually more difficult to appreciate the velocity changes as a function of $[S]$ in this plot. Because of different spread of data points and error distribution, the Eadie–Hofstee plot is better suited to track departures from the typical Michaelis–Menten kinetics. The nonlinearity arising due to cooperativity is better viewed in this plot. However, actual quantitative analysis of cooperativity should be done through a Hill plot.

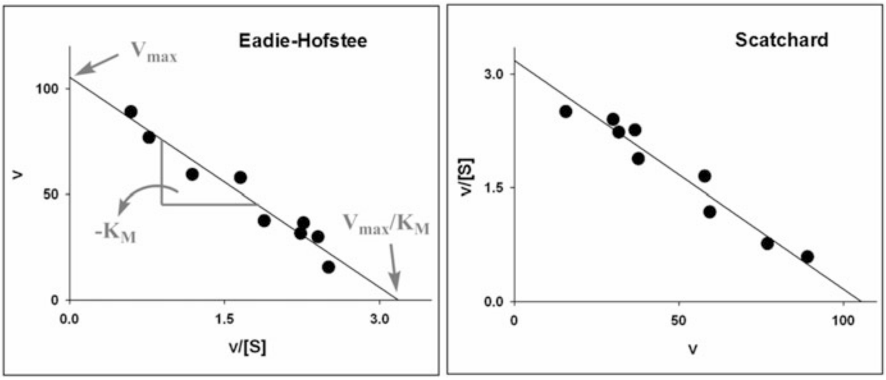

Fig. 17.6 The single-reciprocal plot according to Eadie–Hofstee. Arginase data from Table 17.3 was graphed. Both the Eadie–Hofstee ($v \rightarrow v/[S]$; left panel) and Scatchard ($v/[S] \rightarrow v$; right panel) versions of the plot are shown

Another version of the Eadie–Hofstee plot is obtained when the two axes are interchanged (Fig. 17.6). Thus a plot of $v/[S]$ against v is equivalent to a Scatchard plot (normally used in binding analysis) (Scatchard 1949). The linear form of the Michaelis–Menten equation corresponding to this plot is

$$\frac{v}{[S]} = -\frac{1}{K_{\mathrm{M}}}v + \frac{V_{\max}}{K_{\mathrm{M}}}$$

In this representation, the ratio of $[S]_{\mathrm{bound}}/[S]_{\mathrm{free}}$ (corresponding to $v/[S]$) is plotted against $[S]_{\mathrm{bound}}$ (corresponding to v). Also, the K_{M} becomes the equivalent of K_{D} in Scatchard analysis for ligand binding.

17.4.3 Woolf–Hanes Plot

Woolf–Hanes plot is a single-reciprocal analysis of $v \rightarrow [S]$ data where $[S]/v$ is plotted against $[S]$ (Haldane 1957). It is derived simply by multiplying the Lineweaver–Burk transformation throughout by $[S]$. The following linear transform of the Michaelis–Menten equation is thus obtained:

$$\frac{[S]}{v} = \frac{1}{V_{\max}}[S] + \frac{K_{\mathrm{M}}}{V_{\max}}$$

This is again of the form $y = mx + C$ when $[S]/v$ is plotted against $[S]$. The kinetic parameters are extracted from the slope ($1/V_{\max}$) and intercept ($K_{\mathrm{M}}/V_{\max}$) of such a plot (Fig. 17.7). Since the Y-axis represents a composite value ($[S]/v$), the plot is conceptually more difficult to appreciate. This plot is not so commonly used in enzyme literature.

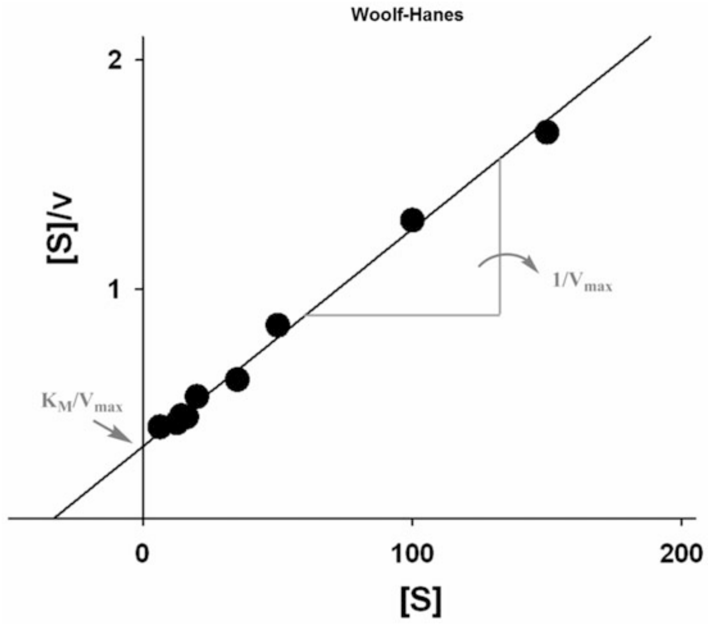

Fig. 17.7 **The Woolf–Hanes single-reciprocal plot**. Arginase data from Table 17.3 was graphed as $[S]/v \rightarrow [S]$ plot

Table 17.4 **Linear transforms of Henri–Michaelis–Menten equation: a summary**

Plot	Lineweaver–Burk (1934)	Eadie–Hofstee (1942)	Scatchard (1949)	Woolf–Hanes (before 1932)
Plot of (Y → X)	$1/v \rightarrow 1/[S]$	$v \rightarrow v/[S]$	$v/[S] \rightarrow v$	$[S]/v \rightarrow [S]$
Y-axis intercept	$1/V_{\max}$	$V_{\max}$	$V_{\max}/K_M$	$K_M/V_{\max}$
X-axis intercept	$-1/K_M$	$V_{\max}/K_M$	$V_{\max}$	$-K_M$
Slope	$K_M/V_{\max}$	$-K_M$	$-1/K_M$	$1/V_{\max}$
Features	Individual display of v and [*S*] on two axes; rate constants directly visualized from intercept and slope	View nonlinearity due to cooperativity and departure from hyperbolic kinetics	Same as Eadie–Hofstee but axes interchanged; ligand binding studies	Weighting of errors from original data is least distorted

Note $V_{\max}$ and $V_{\max}/K_M$, respectively, correspond to zero-order and first-order rate constants in the Michaelis–Menten formalism

Salient features of the common linear transforms of classical Henri–Michaelis–Menten equation are collated and compared in the tabular form below (Table 17.4).

17.5 Summing Up

A very fundamental piece of enzyme kinetic insight is the primary *initial velocity* versus *substrate concentration* data set. The $v \rightarrow [S]$ data is a gold mine of kinetic information. Therefore, it is prudent to obtain reliable data in the first place – because any degree of sophisticated analysis will not transform bad data into good data! While collecting and analyzing enzyme kinetic data, the following key issues should be critically considered:

- Ensure that true initial velocities are measured at all the concentrations of substrate tested, particularly at lower $[S]$ values.
- Use the untransformed data to figure out whether it actually fits the Michaelis–Menten kinetics or this model is being imposed/forced on the data.
- A sufficiently broad range of substrate concentration should be tested to obtain the original $v \rightarrow [S]$ data. And ensure that the data is not biased to either low $[S]$ or high $[S]$.
- Use suitable and rigorous statistical analysis to account for experimental errors and data scatter.

Analysis of enzyme kinetic data must involve the relevant statistical analysis. Fancy statistical packages should not be used without clearly understanding what they can and cannot do! *Statistics should be used as a lamp post – to illuminate but not to lean on to poor data.*

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