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Teaching Enzyme Kinetics and Mechanism in the 21st Century

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Abstract

The teaching of enzyme kinetics has been neglected in recent years, with the growth in influence of molecular biology, but its importance has not diminished. Elementary aspects of enzyme inhibition have always been central to the understanding and design of pharmacological agents and pesticides, and both kinetics and metabolism have acquired a new role for making sense of the flood of genome data that has appeared in the past decade. Although at one time it was hoped that sequence analysis alone would be sufficient for deducing phenotypic information from genomic data, it has become clear that it has to be combined with stoicheiometric analysis, knowledge of metabolic networks and analysis of enzyme regulation. Presentation of kinetics in general textbooks has always been very poor, and the decline of specialized teaching has made the inadequacy of these textbooks more serious than it already was in the past.

Introduction

The basis of enzyme kinetics as it is commonly taught today derives from the work of Henri [1] and Michaelis and Menten [2], and one may wonder why it should continue to be regarded as an essential component of biochemistry courses nearly a century later. However, despite a decline in interest in kinetics over recent decades the subject remains fundamental to several currently active fields of research. The development of genetic techniques for producing artificially modified enzymes has increased the importance of precise methods for

estimating kinetic parameters, because modern research requires the ability to quantify small differences in activity between mutant forms. Enzyme inhibition remains central to the development of new drugs, pesticides and other products of biotechnology, and needs to be well understood for such development to proceed efficiently. For these and other reasons to be developed in this chapter, satisfactory teaching of enzyme kinetics is no less important for the training of biochemists than it has ever been. Unfortunately, however, the majority of current general textbooks of biochemistry fall far short of providing an adequate treatment of the subject. At the same time although specialized textbooks exist [3 – 5] they are less numerous than they once were.

Drugs typically act by inhibiting enzymes, and this implies two things that must be important for designing them, first an understanding of the differences between the different kinds of inhibition (competitive, uncompetitive, etc.), and second, less obvious but at least as important, an understanding of why measurements of inhibition made in controlled conditions in a spectrophotometer may provide very little guide as to how much inhibition can be expected to occur (even at the same concentration of inhibitor) *in vivo*. Both points arise out of the same central fact: experiments in the spectrophotometer are typically done at concentrations of inhibitor, substrate, product etc., that are chosen and fixed by the experimenter, but concentrations of all metabolites (both substrates and products of enzyme-catalysed reactions) *in vivo* can vary by very large factors when conditions change; differences between inhibition types that are small enough to pass unnoticed when substrate and product concentrations are fixed can be very large when these concentrations are allowed to vary. Both points are considered in more detail in this chapter. For the moment it is sufficient to note that an inhibitor concentration that produces a large decrease in the rate of an enzyme-catalysed reaction considered in isolation will often produce no detectable effect on the rate *in vivo*. This problem does *not* arise primarily from an inability to achieve the same inhibitor concentration in a cell as one can readily achieve in the spectrophotometric (though that may well be a serious additional problem). Anyone setting out to design a pharmacological agent needs both to recognize the basic fact that kinetic behaviour *in vivo* is usually different from that *in vitro* (observed as well as theoretically predicted) and to understand why it happens.

Elementary Enzyme Kinetics in Current Textbooks

A major difficulty for understanding the (slightly) less elementary aspects of kinetics discussed later in this paper is that even the most elementary points are often presented very badly in general textbooks of biochemistry. It is a matter of simple arithmetic to calculate that if a reaction follows the Michaelis–Menten equation,

$$v = \frac{V_a}{K_m + a} \tag{1}$$

in which v is the rate at substrate concentration a, V is the limiting rate and K_m is the Michaelis constant, then $v = 10V/11$ when $a = 10K_m$, in other words v is nearly 10% below V at this concentration. As the calculation is so simple one can only be amazed that so many textbook authors are apparently unable to do it. Of general biochemistry textbooks published in the past decade, three [6 – 8] illustrate the dependence of v on a given by eqn. 1 with grossly inaccurate curves, three [9 – 11] show curves that are reasonably accurate on one page and inaccurate on another, and fewer than half [12 – 15] manage to present the relationship accurately. Notice that one of the textbooks that has it incorrect is now in its 6th edition, having appeared originally in 1975 [17]: evidently the correction of elementary errors is not a high priority when successful textbooks are revised. However, in the past even some authors of specialized books on enzyme kinetics were unable to draw the curve correctly [16], something that would be unthinkable today, so at least there has been some progress.

This is not a trivial matter, because if one thinks that v reaches V at a substrate concentration a few times greater than K_m it is impossible to understand why V cannot be determined by direct measurement, so that K_m could be obtained from a simple measurement of the a value at which $v = V/2$. Without this understanding it is impossible to understand why linear transformations of eqn.1 [18 – 21] played such a great role in the development of biochemistry, and why they remain important today for illustrating results and in teaching.

The presentation of the more elementary aspects of kinetics in the general biochemistry textbooks of today may be poor[1], but the treatment of more advanced aspects is nonexistent. Not since 1972 has any widely used general textbook [23] included any attempt to go beyond the Michaelis–Menten equation and competitive inhibition, and so anyone who wants to do this has no choice but to go to more specialized sources.

Deducing Phenotypes from Genotypes

When genome sequencing on a large scale first became practicable, and in particular when it first became realistic to expect the entire human genome to become known, there were hopes, not always clearly stated, that deducing phenotypes from gene sequences would be relatively straightforward. In the event, however, although the human genome project has certainly had and is having major effects on the diagnosis and treatment of many diseases, it has proved much more difficult than was widely expected to pass directly from genotype to phenotype, which is far from being a one-step transition.

[1] It is depressing to note that IUPAC's recommendations on quantities, units and symbols in physical chemistry [22], published as recently as 2007, assert that k_{-2}, defined as the rate constant constant for binding of the product to the free enzyme, is negligibly small. Astonishingly, the compilers of the recommendations did not wish to acknowledge that this was an error when it was brought to their attention. If experts on physical chemistry cannot understand that an algebraic expression is zero if any *one* of its factors (in this case the product concentration) is zero, without implying anything about the magnitudes of its other factors, what hope is there for undergraduate students?

In the first place the genes present in a genome need to be identified, and this is by no means an error-free process. When we discussed the steps involved in deducing phenotypes some years ago [24] we reported an estimate [25] that as many as half of the proteins in *Caenorhabditis elegans*, with a much more compact and non-repetitive genome than the human, might be incorrectly identified; even in as thoroughly studied an organism as *Escherichia coli* as many as 40% of its 4405 genes are still without experimentally determined functions [26].

So the first difficulty in trying to deduce a phenotype from genome information is the need to correct the errors of identification, and to identify the genes with unknown functions. As an example of how this can be done, consider the work of Schuster *et al.* [27], who examined the problem of identifying the genes of *Treponema pallidum*, the organism responsible for syphilis, for which the genome was known but was accompanied by extremely little biochemical information. Comparison with the genome of *E. coli* allowed many genes to be tentatively identified, and stoicheiometric analysis allowed some of the others to be identified. Assuming that the metabolism of *T. pallidum* is not totally different from that of *E. coli* and indeed from those of other organisms for which the information is available, one must explain the apparent absence of a gene for the enzyme transaldolase by supposing that it is indeed present but has not been recognized. The logic here is that in *E. coli* and all other known cases transaldolase occurs only in organisms that use the transketolase reaction, because stoicheiometric analysis shows that transketolase would have no function to fulfil in the absence of transaldolase. As two different genes for transketolase were found in *T. pallidum* there must be at least one transaldolase gene as well even if none had been found. It would of course be extremely laborious to analyse an entire genome in this way, but it can be very helpful nonetheless. However, there is also a more fundamental difficulty, that it offers away of detecting *similarities* between organism, whereas the main reason for studying an organism such as *T. pallidum* is to understand its *differences* from a more thoroughly studied organism like *E. coli*. Why does *T. pallidum* cause syphilis, whereas *E. coli* does not? We cannot learn this by noticing respects in which *T. pallidum* resembles *E. coli*.

Even if we brush aside all the problems inherent in establishing a list of putative proteins present in an organism, there remain several steps to be taken before we arrive at a real phenotype. For the first of these stoicheiometric analysis is again very helpful, as it can provide a metabolic map, from which one may deduce a possible phenotype. However, a possible phenotype is not a real phenotype, and there is no way in which purely stoicheiometric considerations allow one to proceed any further. It is not at all sufficient to know what reactions are present and hence what is stoicheiometrically possible. One also needs some information about kinetics and regulation. Perhaps in the future it may be possible to deduce this sort of information from genomic data, but it is certainly not possible now, and there is no alternative to undertaking some real biochemical experiments.

Characterizing Inhibition *in vitro*

As enzyme inhibition is central to the design of pharmacological agents[2], it is essential to consider how it should be measured and reported. Biochemists have long used *inhibition constants*, i.e. the parameters K_{ic} and K_{iu} that appear in the following equation:

$$v = \frac{V_a}{K_m\left(1+\frac{i}{K_{ic}}\right)+a\left(1+\frac{i}{K_{iu}}\right)} \qquad (2)$$

in which v is the rate at substrate concentration a and inhibitor concentration i, and V and K_m are the parameters that define the kinetics of the uninhibited reaction (as in eqn. 1). In the past it was common to consider only the competitive component of the inhibition, defined by K_{ic}, but it is now usual to recognize that the uncompetitive component, defined by K_{iu}, must also be considered. For reasons that will be discussed, this is actually essential for characterizing inhibition *in vivo*. Many methods are available for determining the inhibition constants: among the more widely known are the plots of $1/v$ against i for determining K_{ic} as the abscissa coordinate of the common intersection point of lines plotted at different values of a [28], and of a/v against i for determining K_{iu}, again as the abscissa coordinate of the common intersection point of lines plotted at different values of a [29].

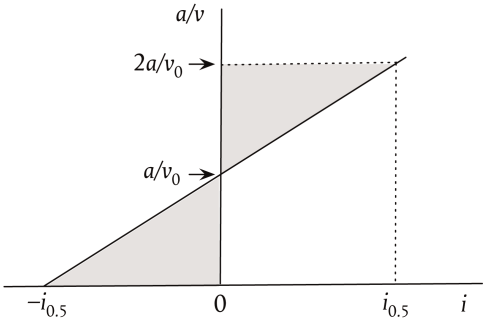

Figure 1. Determination of $i_{0.5}$ from standard inhibition plots. The value of a/v when i is extrapolated to zero is a/v_0 by definition, and likewise the value when $i = i_{0.5}$ is $2a/v_0$. Rotation of the shaded triangle 180° about the point $(0,a/v_0)$ gives a congruent triangle, from which it follows that the intercept of the line on the abscissa must occur at $i=-i_{0.5}$. Although the construction is illustrated for a/v as ordinate axis [29] the logic applies equally well to $1/v$ as ordinate axis [28].

In contrast, pharmacologists continue to characterize inhibition in terms of a parameter that has become largely obsolete in biochemistry, the *inhibitor concentration for half-inhibition*, the concentration needed to arrive at $v = 0.5V$, which can be symbolized as $i_{0.5}$. This would

[2] Enzyme activation is important only in some special circumstances, exemplified by liver hexokinase, as discussed later.

be a trivial difference if there were a simple relationship relating $i_{0.5}$ to K_{ic} and K_{iu}, but in reality no such relationship exists, as one may deduce from the fact that K_{ic} and K_{iu} are independent of the substrate concentration a whereas $i_{0.5}$ is not. For the limiting cases, competitive and uncompetitive inhibition, it is *never* true (even at a carefully selected substrate concentration) either that $i_{0.5}=K_{ic}$ or that $i_{0.5}=K_{iu}$. For mixed inhibition, when both competitive and uncompetitive components make significant contributions, it is possible to choose values of a for which $i_{0.5}=K_{ic}$ or $i_{0.5}=K_{iu}$, but these are special cases of no particular interest or importance. Only when $K_{ic}=K_{iu}$ do we find $i_{0.5}=K_{ic}=K_{iu}$, but this is also a special case, known as pure non-competitive inhibition. It is often given undeserved attention in elementary accounts of inhibition because, many years ago, Michaelis contrasted it with competitive inhibition in his studies of the inhibition of invertase [31] and maltase [32]. Except in the case of inhibition by protons it has very little importance in the real world.

So, if $i_{0.5}$ cannot be understood as an inhibition constant, how should it be understood in standard biochemical terms? In fact it is the abscissa intercept of a line plotted in either of the ways mentioned, i. e. either in a plot of $1/v$ against i or in one of a/v against i [30]. The logic of these relationships is illustrated in Fig. 1. In addition, plots of $1/i_{0.5}$ against v_0/V allow the type of inhibition to be determined from measurements of $i_{0.5}$ at different values of a: with all linear types of inhibition this plot gives a straight line, with a negative slope of $-1/K_{ic}$ and an abscissa intercept at $v_0/V = 1$ if the inhibition is competitive, a positive slope of $1/K_{iu}$ and a line passing through the origin if the inhibition is uncompetitive, and intermediate behaviour for mixed inhibition [30]. This approach gives results in accordance with theoretical expectation for inhibition of lactate dehydrogenase by different kinds of inhibitor [30].

Inhibition *in vivo*

The equation for competitive inhibition is a limiting case of eqn. 2 with the term in i/K_{iu} omitted:

$$v = \frac{V_a}{K_m(1+i/K_{ic})+a} \tag{3}$$

and the equation for uncompetitive inhibition is at the opposite extreme with the term in i/K_{ic} omitted:

$$v = \frac{V_a}{K_m+(1+i/K_{iu})} \tag{4}$$

These two equations are not only similar in appearance; they are also similar in the quantitative behaviour they predict when i is varied at fixed a. That is why experiments in the spectrophotometer often lead them to be distinguished poorly or not at all (Fig. 2), even in published work, and the uncompetitive component sometimes passes unnoticed. For this

reason one must always treat reports of competitive inhibition with suspicion: did the authors really exclude the possibility of an uncompetitive component, or did they just ignore it?

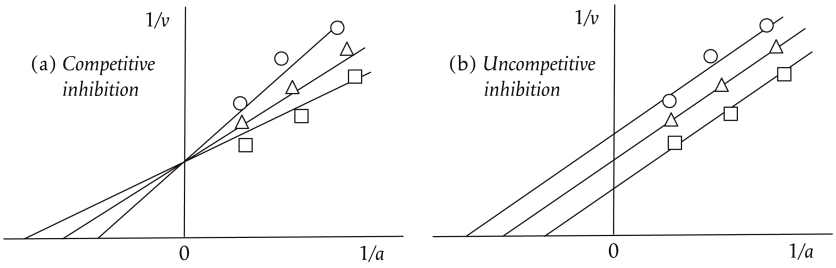

Figure 2. On a casual inspection the double-reciprocal plots in (**a**) made at three different inhibitor concentrations illustrates competitive inhibition, with the lines intersecting on the ordinate axis, where the plots in (**b**) show parallel lines, and hence uncompetitive inhibition. However, inspection of the data points reveals that they are identical in the two cases, so what the plots illustrate is not different kinds of inhibition but different *interpretations* of the same data.

One maybe tempted to dismiss the (invented) example in Fig. 2 as an exaggeration of what happens with real data, but in reality one can find published examples (e. g. [33]) that are much worse than what is illustrated here. The current tendency of journal editors to discourage the presentation of primary data is making the problem progressively less obvious, but that does not mean the problem is disappearing, only that it is becoming more difficult to recognize.

Matters are drastically different if i is varied at fixed v, with a allowed to vary freely. To see this the two equations need to be rearranged to show a/K_{ic} as a function of i in each case. For competitive inhibition, rearrangement of eqn. 3 yields

$$a \Big/ K_m = \frac{1+i/K_{ic}}{V/v-1} \tag{5}$$

from which it is immediately evident that a is a linear function of i under these conditions. Rearrangement of eqn. 4 shows that the corresponding behaviour with uncompetitive inhibition is very different, however:

$$a \Big/ K_m = \frac{1}{V/v-1-i/K_{iu}} \tag{6}$$

Not only is this a non-linear function, it is also one that allows a to become infinite when $i/K_{iu}=V/v-1$, a condition that is easily satisfied in practice: it means, for example, that at a substrate concentration of K_m in the absence of inhibitor it is sufficient for i to reach K_{iu} for the steady state to be lost [34], as illustrated in Fig. 3.

Although eqns. 5 – 6 are useful for understanding the great differences between kinetics at fixed rate and kinetics at fixed substrate concentration, they oversimplify the problem of understanding the differences between *in vivo* and *in vitro* kinetics, because one cannot equate *in vivo* conditions with fixed-rate conditions. For a minority of enzymes, those that act of substrates like glucose that are maintained at essentially constant concentrations by regulatory mechanisms, the conditions may, in fact, resemble those in the spectrophotometer; such enzymes can be expected to respond just as well to inhibition *in vivo* as they do *in vitro*. They are the exceptions, however. More often an enzyme finds itself in the middle of a metabolic pathway where it has little influence on the flux through the reaction that it catalyses, but considerable influence on the concentrations of its substrate and product. In the language of metabolic control analysis [4] it typically has a small flux control coefficient for the flux through its own reaction, but large concentration control coefficients (negative and positive respectively) for the concentrations of its product and substrate. Effectively it must process its substrate at the rate at which it arrives, but can adjust the concentrations to satisfy its kinetic equation. As expected, therefore, computer modelling of a ten-step pathway in which the fifth enzyme is inhibited by an added inhibitor gave results for both competitive and uncompetitive inhibitors that closely resembled the calculated behaviour for fixed-rate conditions and were very different from those for fixed-concentration conditions [34].

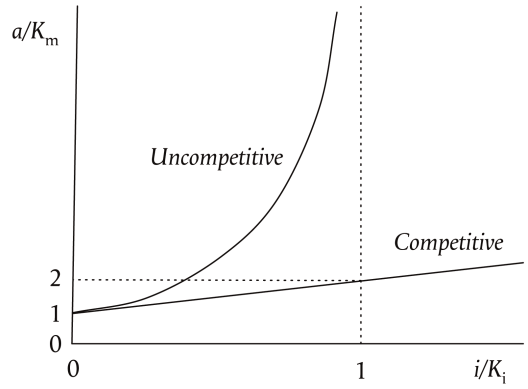

Figure 3. Inhibition at constant rate. When the concentration of an inhibitor is varied and the rate is held constant the substrate concentration needs to change in order for the rate equation to be satisfied. If the inhibition is competitive the substrate concentration varies linearly with the inhibitor concentration, and, starting from an initial state of $a/K_m = 1$ at $i = 0$ the substrate concentration is simply doubled at $i = Ki$. For uncompetitive, however, the behaviour is very different, and the same condition $i = K_i$ is sufficient to incur loss of the steady state.

From the point of view of designing inhibitors as drugs or pesticides, the conclusion to be drawn from this discussion is that in most circumstances (i. e. when the enzyme to be inhibited acts under conditions of constant or nearly constant flux through its reaction) there needs to be an uncompetitive component in the inhibition if it is to be useful *in vivo*.

Practical examples of this principle include Li^+, an uncompetitive inhibitor of *myo*-inositol monophosphatase [35] used to treat manic depression, and Glyphosate (“Roundup”), an uncompetitive inhibitor of 3-phosphoshikimate 1-carboxyvinyltransferase [36] and the herbicide with the greatest commercial success in history.

Designing an Uncompetitive Inhibitor

A difficulty that will occur to anyone who wishes to apply the principle developed in the preceding section is that whereas it is easy to design a competitive inhibitor, as the structural characteristics of a typical competitive inhibitor are obvious, it is almost impossible to design an uncompetitive inhibitor, as there are no general structural properties that make a particular molecule likely to be an uncompetitive inhibitor of a particular enzyme. The reality, however, is not nearly as discouraging as this suggests. First of all it is not necessary for the inhibition to be purely uncompetitive, because as long as a mixed inhibitor displays a sufficient uncompetitive component it can have useful effects *in vivo*. In fact the uncompetitive component may dominate the behaviour *in vivo* even if the inhibition is predominantly competitive [37, 38]. So, although true uncompetitive inhibition is rare [34] mixed inhibition is not, and inhibitors with structural characteristics that suggest them to be competitive inhibitors often act as mixed inhibitors.

A second important point is that nearly all metabolic reactions have two or more substrates and two or more products, and an inhibitor that is competitive with respect to one substrate is often mixed or uncompetitive with respect to another. This is, in fact the case for Glyphosate: it is a structural analogue of one substrate of 3-phosphoshikimate 1-carboxyvinyltransferase, phospho*enol*pyruvate and is competitive with respect to it, but it is predominantly uncompetitive with respect to the other substrate, 3-phosphoshikimate.

In practice, therefore, designing an uncompetitive inhibitor may not be as difficult as it appears at first sight. In any case, the degree of difficulty is ultimately not the point: it is better to search for a difficult solution to a problem that has a chance of succeeding than to search for an easy solution that cannot work.

Does Enzyme Activation Ever Have a Useful Pharmacological Role?

The tendency of most enzymes to have small or negligible control coefficients for the flux through their own reactions not only means that inhibiting them will typically have little or no effect or this flux *in vivo* (though it may, if the inhibition has an uncompetitive component, have a major effect on some metabolite concentrations); it also means that activating them will also have little or no effect on the flux. In fact even activating them by large factors will usually have a negligible effect, because flux control coefficients typically decrease when the enzyme activity increases. There is, however, an important exception to

this generalization. As discussed elsewhere [39], the resistance of most metabolic fluxes to changes in enzyme activity, mainly due to the summation relationship [40], is in part due to the regulatory design of pathways in terms of supply and demand. As most pathways respond to changes in demand they resist changes in supply. An important exception, however, concerns uptake of glucose by the mammalian liver followed by phosphorylation to glucose 6-phosphate: this is not primarily regulated by the liver's need for glucose, but by the need to maintain homeostasis, and particular to maintain a constant blood-glucose concentration [41]. In other words it must be regulated according to supply: increases in glucose availability need to be followed by increased uptake in the liver. It follows that hexokinase D, the enzyme responsible for phosphorylation of glucose in the liver, has a high flux control coefficient for its own reaction [42], and as a result is capable of responding *in vivo* to activators. Hence the current commercial interest in finding good activators of this enzyme is much better founded than it would be for most other enzymes. The example is discussed in more detail elsewhere [43].

Computer Analysis of Kinetic Experiments

For many years an unsatisfactory aspect of practice in enzyme kinetics was the almost universal tendency to estimate kinetic parameters by visual inspection of double-reciprocal plots, even after it was pointed out that the deviation of $1/v$ from its theoretical value provided an extremely misleading indication of the corresponding deviation in v and satisfactory methods of calculation became available [44]. Practice has, however, changed drastically since desktop computers became generally available, and it is now almost universal to use commercial software for estimating rate constants. Unfortunately, this is not necessarily an improvement: when double-reciprocal plots appeared in every paper it was at least possible for a critical reader to understand what had been done, but this has become almost impossible when crucial details are often hidden by a cryptic sentence to the effect that a particular commercial program was used. Even when authors are aware of the statistical assumptions implicit in the software they rarely reveal this to their readers, who have no way of knowing whether the observations were appropriately weighted or not. More detail may be found in kinetics textbooks [3 – 5]: the important point is that computer fitting is an advance over fitting by eye only if it is correctly done, with the added disadvantage that it is difficult for the reader to judge.

Concluding Remarks

There are many aspects of enzyme kinetics that form a less visible part of biochemistry courses than they did 40 years ago, though they remain essential to the proper understanding of the subject, and in particular to its application to biotechnology. As noted, development of pharmacological agents, metabolic engineering, etc., typically involve knowledge of enzyme inhibition and the kinetics of multienzyme systems, but the first of these tends to be taught in a superficial way and the second often not at all.

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