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The Effect of Systematic Error on the Accuracy of Michaelis Constants and Maximum Velocities Estimated by Using the Integrated Michaelis–Menten Equation

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Systematic errors in initial substrate concentration (s_0), product concentration and reaction time give much larger errors in the Michaelis–Menten parameters unless s_0 is treated as an unknown parameter. These errors are difficult to detect because the fitted curve deviates little from the data. The effect of non-enzymic reaction is also examined.

The Michaelis–Menten parameters of an enzyme-catalysed reaction (K_m and V) can be found by fitting the integrated form of the Michaelis–Menten rate equation to progress curves of the reaction (Laidler, 1958; Atkins & Nimmo, 1973; Fernley, 1974). The main advantage of using progress curves rather than initial velocities is that, for the simplest type of reaction at least, K_m and V can be estimated in a single experiment.

One of us has been assaying the enzyme acetylcholinesterase (acetylcholine hydrolase, EC 3.1.1.7) by measuring the time-course of hydrolysis of acetylcholine using a continuous-titration technique and then fitting the integrated equation to the data (Newman, 1974). Since the variability of the estimates of K_m and V was greater than could be accounted for by random error [see Nimmo & Atkins (1974) for its quantitative effect], we have investigated and here describe the influence of different sorts of systematic error on the estimates. Our approach was to simulate 'perfect' (i.e. error-free) progress curves, and then to analyse them assuming that a systematic error had been made in either the initial concentration of substrate, the concentration of product or the time the reaction had been running. We have also assessed what effect a low rate of non-enzymic hydrolysis would have.

The results show that small systematic errors of these kinds lead to relatively much greater errors in K_m and V unless the initial substrate concentration is treated as an extra parameter to be estimated. They also show that the presence of the systematic error is extremely difficult to detect, because the best-fit curve still lies very close to the data.

Methods

The time-course of hydrolysis is determined by continuous titration in a pH-stat system as follows. At zero time known amounts of substrate and enzyme are mixed, giving an initial substrate concentration s_0, and titrant of known concentration is added to keep the pH constant. The concentration of product, p, at any time, t, is calculated from the volume of titrant added at that time. (It is assumed that the volume of titrant added is negligible; in the acetylcholinesterase assay it was between 0.5 and 1.0% of that of the reaction mixture.) The Michaelis–Menten parameters are estimated by fitting to the values of s_0, p and t the equation (Atkins & Nimmo, 1973):

$$V \cdot t = p - [K_m \cdot \ln(1 - p/s_0)] \qquad (1)$$

Systematic error could be present in s_0, p (if the concentration of the titrant or the calibration of the burette were wrong) and t (if the time at which the reaction was started were mistaken, or if the initial mixing were poor). A realistic magnitude for the errors in p and t is ±1%. The error in s_0 could be as great as 10%. [For example commercial preparations of acetylcholine iodide said to be 98–100% pure may in fact contain up to 5% by weight of impurities (Newman, 1974).]

To establish the effect of such errors, five 'perfect' progress curves were derived by setting $K_m = V = 1$ and s_0 to 0.5, 1.0, 2.0, 5.0 or 10.0, and then using eqn. (1) to calculate 15 roughly equispaced values of t from values of p in the range $0.08s_0 - 0.80s_0$. Eqn. (1) was then fitted to each curve by the iterative method of Fernley (1974) as adapted for curves of unknown origin by Nimmo & Atkins (1974). It was assumed (*a*) that s_0 was out by ±0.5%, ±2.0%, ±5.0% or ±10.0% of its true value, (*b*) that p was out by ±0.5% or ±2% of its true value, and (*c*) that t had an absolute error of ±0.01 (about 0.5% of the total running time) or ±0.04. [The iterative method was preferred to the linear one of Atkins & Nimmo (1973) because it gives 'better' answers when p is subject to random error (Nimmo & Atkins, 1974).]

When substrate is hydrolysed in a first-order non-enzymic reaction as well as enzymically (as may be the case for acetylcholine), the rate equation is:

$$\frac{dp}{dt} = \frac{V \cdot (s_0 - p)}{K_m + (s_0 - p)} + k \cdot (s_0 - p) \qquad (2)$$

where k is the first-order rate constant. On integration eqn. (2) gives:

$$k\cdot(V+k\cdot K_m)\cdot t = -k\cdot K_m\cdot\ln(1-p/s_0)-V\cdot\ln\left(1-\frac{k\cdot p}{V+k\cdot(K_m+s_0)}\right) \quad (3)$$

Progress curves were again simulated with $K_m = V = 1$ and $s_0 = 0.5$, 1.0, 2.0, 5.0 or 10.0, but with the use of eqn. (3) to calculate t from p. The rate constant k was set to either 0.005 or 0.02 (at $s_0 =$ 1.0 the rate of non-enzymic hydrolysis is then either 1% or 4% of the initial rate of enzymic hydrolysis). Eqn. (1) (which assumes no non-enzymic hydrolysis) was fitted to these curves.

Since the errors in K_m and V caused by errors in s_0 were relatively large, s_0 was next considered to be an additional unknown parameter to be estimated and eqn. (1) was refitted to each progress curve, again assuming that systematic errors had been made. This was done by including on the right-hand side of the adjustment regression used by Nimmo & Atkins (1974) the term

$$+\frac{\Delta s_0\cdot K_m}{D}\left[\frac{1}{s_0-(\hat{p}-p_0)}-\frac{1}{s_0}\right]$$

Results and discussion

Table 1 gives the results obtained when the different sorts of systematic error are present and the initial substrate concentration (s_0) is assumed to be a known constant. If the error is in s_0 or the product concentration (p), V is always the better estimated parameter, and the error in K_m is greatest at the lowest s_0 but does not decrease much as s_0 becomes increasingly greater than K_m. The size of the error in K_m is striking: even at $s_0 = 10.0$ a 2% error in s_0 or p causes an error of about 10% in K_m.

The error in time (t) is independent of s_0, and thus affects the low s_0 estimates more than the high ones. Since the error of ±0.04 corresponds to one of between 2 and 3s in a reaction lasting about 2min ($s_0 = 0.5$) or 10min ($s_0 = 10.0$), it is clear that poor initial mixing of the reactants may substantially alter K_m and V.

Another striking feature of these results is that even in the presence of any of the systematic errors the best-fit curve lies very close to the data. Thus if the lowest s_0 is overestimated by 5%, K_m is overestimated by 103% and V by 68%; but the observed and predicted concentrations of product always differ by less than 1%, and usually by less than 0.1%. This means it would be very difficult to establish if systematic error were in fact present.

When s_0 is assumed to be an unknown parameter instead of a known constant the results are much better. Since the data are otherwise perfect the correct values of K_m and V are obtained even when the initial estimate of s_0 is wrong. When p contains error, the percentage errors in K_m and V are just equal to that in p. For comparison, had initial velocities been used, an error in substrate concentration would have given the same percentage error in K_m and the correct

Table 1. *Effect of systematic error on the estimates of K_m and V*

The Table gaves the percentage errors in K_m and V resulting from the different sorts of systematic error, when s_0 is assumed to be a known constant.

Initial substrate concentration (s_0)		0.5		1.0		2.0		5.0		10.0	
Source of error	Size of error	K_m	V	K_m	V	K_m	V	K_m	V	K_m	V
Initial substrate concn. (s_0)	−10.0%	*	*	−52.7	−26.6	−51.2	−18.6	−47.1	−9.0	−45.8	−4.9
	−5.0%	−43.2	−29.3	−30.9	−15.2	−29.8	−10.0	−25.4	−4.5	−24.2	−2.4
	−2.0%	−21.1	−14.1	−13.7	−6.6	−12.4	−4.2	−10.6	−1.8	−10.0	−1.0
	−0.5%	−5.9	−3.9	−3.6	−1.7	−3.2	−1.1	−2.7	−0.5	−2.5	−0.2
	0.5%	6.4	4.2	3.8	1.8	3.3	1.1	2.8	0.5	2.6	0.2
	2.0%	29.4	19.4	16.0	7.6	13.8	4.5	11.3	1.9	10.5	1.0
	5.0%	103.5	67.5	45.5	21.2	37.5	12.0	29.5	4.8	27.1	2.4
	10.0%	617.0	395.5	116.8	53.0	86.8	26.9	63.8	9.9	57.3	4.8
Product concn. (p)	−2.0%	27.5	17.5	14.0	5.6	11.8	2.5	9.3	−0.1	8.5	−1.0
	−0.5%	5.9	3.7	3.3	1.3	2.8	0.6	2.3	0.0	2.1	−0.3
	0.5%	−5.4	−3.4	−3.1	−1.2	−2.7	−0.6	−2.2	0.0	−2.0	0.3
	2.0%	−19.1	−12.1	−11.7	−4.6	−10.4	−2.2	−8.6	0.2	−8.0	1.0
Time (t)	−0.04	42.2	27.7	16.0	7.6	9.1	3.0	3.7	0.6	1.9	0.2
	−0.01	8.7	5.7	3.8	1.8	2.2	0.7	0.9	0.2	0.5	0.0
	0.01	−7.7	−5.2	−3.6	−1.7	−2.2	−0.7	−0.9	−0.2	−0.5	0.0
	0.04	−26.6	−17.9	−13.7	−6.6	−8.4	−2.8	−3.6	−0.6	−1.9	−0.2

* These values of K_m and V were negative.

Table 2. *Effect of non-enzymic hydrolysis on the estimates of K_m and V*

The Table gives the percentage errors in K_m and V caused by the presence of non-enzymic hydrolysis, calculated assuming s_0 is either a known constant or an additional parameter to be estimated.

Initial substrate concentration (s_0)		0.5		1.0		2.0		5.0		10.0	
Treatment of s_0	Rate constant (k)	K_m	V	K_m	V	K_m	V	K_m	V	K_m	V
Constant	0.005	0.8	1.3	1.3	1.6	2.2	2.1	6.4	3.7	18.4	6.4
Parameter	0.005	1.0	1.4	1.8	1.8	3.4	2.5	13.0	4.8	46.6	9.0
Constant	0.02	3.3	5.2	5.0	6.4	8.7	8.6	26.8	15.2	74.4	26.7
Parameter	0.02	3.9	5.6	7.1	7.5	13.9	10.4	55.8	20.6	236.5	42.2

V, whereas an error in p would have given the same percentage error in V and the correct K_m. When the error is in t rather than s_0 or p the values of K_m and V are again virtually correct. There is, however, a disadvantage of treating s_0 as a parameter rather than a constant. It is that, when p contains random error, the standard errors of K_m and V increase by about an order of magnitude.

The effect of non-enzymic hydrolysis is shown in Table 2. It differs in two ways from that of the systematic errors already considered. First, the errors in K_m and V are greatest when s_0 is high and the initial rate of reaction close to zero-order. Secondly, they are greater when s_0 is assumed to be an unknown parameter rather than a known constant. For comparison, the errors in K_m and V, calculated from the five initial velocities by the iterative method of Wilkinson (1961), are: $K_m = +9.8\%$, $V = +5.6\%$ ($k = 0.005$); and $K_m = 41.7\%$, $V = +23.6\%$ ($k = 0.02$).

We have therefore concluded that, provided there is no possibility of non-enzymic hydrolysis, progress curves can be used with confidence to estimate K_m and V so long as s_0 is also treated as an unknown parameter. On the other hand, if s_0 is not treated in this way, the estimates are very sensitive to small systematic errors. If non-enzymic hydrolysis is a possibility, as, for instance, when acetylcholine is incubated at a pH above about 8.0 (Newman, 1974), the best approach might be to work at an s_0 which is not much greater than K_m and then to consider it as a known constant.

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