A COMPUTER PROGRAMME FOR THE DETERMINATION OF THE KINETIC CONSTANTS OF TWO ENZYMES ACTING SIMULTANEOUSLY ON THE SAME SUBSTRATE

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SUMMARY: A computer programme permitting rapid and accurate estimation of the kinetic parameters (apparent K_m and V_m) of two enzymes acting simultaneously on the same substrate is described. No starting estimates of the parameters are required. The only additional parameter needed is an inflexional substrate concentration. This is obtained from the last half of the segment of major curvature on the Lineweaver-Burk plot, which is not linear under these conditions. The value of this substrate concentration need not be precisely determined. This procedure is shown to give significantly better results than general non-linear regression procedures. The programme is written in the Fortran IV language.

An upwards-convex Lineweaver-Burk plot [1] is obtained when two enzymes, with different kinetic properties, act simultaneously on the same substrate [2]. Having established that two enzymes are present it is often desirable to estimate apparent K_m and V_m values directly from the non-linear Lineweaver-Burk plot. To do so by extrapolation of the apparent linear regions of the plot can lead to very unreliable estimates [3].

Cleland [4] was able to fit this type of data to equation (1).

$$v = \frac{A(s^2+Ds)}{s^2+Bs+C} \qquad (1)$$

A, B, C and D are constants and v and s represent observed velocity and substrate concentration, respectively. This programme requires graphical estimates (from the Lineweaver-Burk plot) of slope and intercept values as $s \longrightarrow 0$ and as $s \longrightarrow \infty$. This procedure is therefore very sensitive to errors in the measured velocities at low substrate concentrations. Further arithmetic manipulations are also required to obtain the apparent K_m and V_m values.

Spears et al [3] described an alternative approach employing linear regression on the two apparently linear regions of the s/v against s plot. This procedure suffers from the disadvantage of using linear regression on approximately linear regions of a non-linear replot of the experimental data.

Here is described a novel regression procedure which fits the experimental data to equation (2) (which is the expanded form of equation (1)) using the non-linear regression procedure of Wilkinson [5].

$$v = \frac{VMH\ s}{KMH+s} + \frac{VML\ s}{KML+s} \qquad (2)$$

VMH, KMH, VML and KML represent the apparent V_m and K_m of the enzyme with higher and lower values of these parameters, respectively. The only additional parameter required for the regression procedure is an inflexional substrate concentration which is chosen from the last half of the segment of the Lineweaver-Burk plot showing the most severe curvature (see fig. 1). This value tentatively divides the experimental data into two groups corresponding to the substrate concentration ranges where one enzyme may be the major contributor to the measured velocity.

The programme has been developed using the IBM 360/67 computer of the University of Newcastle upon Tyne. Copies of the programme are available, on request, from the author.

Description of iterative technique

The measured velocities and substrate concentrations are initially divided into two arrays , above and below the inflexional substrate concentration (IS, fig. 1). The regression analysis is then initiated by fitting the data belonging to the high substrate concentration range, in which the measured velocities are predominantly due to the high K_m enzyme, to the Michaelis-Menten equation using the procedure of Cleland [6]. The resulting K_m(KMH) and V_m(VMH) estimates are then used to compute estimates of the net velocities due to the high K_m enzyme over the low substrate concentration range ($s < IS$). By subtraction of these velocities from the

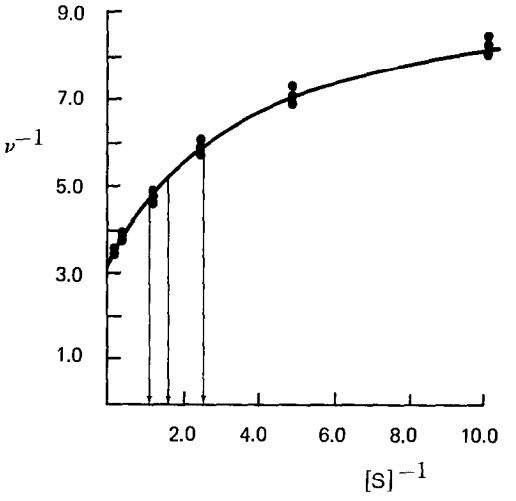

Fig. 1. A Lineweaver-Burk plot [1] obtained from equation (2). The curve drawn represents the computed best fit to the synthetic data points shown. These data were generated using the following values of the kinetic parameters; KMH=0.60, VMH=0.20, KML=0.02 and VML=0.10. The inflexional substrate concentration used was 0.40. The vertical lines represent the position of the reciprocal values of the inflexional substrate concentrations used in table 2. The units of velocity and substrate concentration have been omitted for clarity.

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corresponding total (i.e. measured) velocities estimates of the net low K_m enzyme velocities in this substrate concentration range are obtained. These velocities are used to obtain estimates of K_m(KML) and V_m(VML) of the low K_m enzyme with the regression procedure employed earlier. These values of KML and VML are then used to estimate the net high K_m enzyme velocities in the high substrate concentration range ($s > IS$), by a procedure analogous to that used for the low K_m enzyme. These velocities and the corresponding substrate concentrations are again fitted to the Michaelis-Menten equation to give improved estimates of KMH and VMH. Improved estimates of the net low K_m enzyme velocities (in the low substrate concentration range) can also now be computed, thereby starting another iterative cycle.

The iterative process is terminated once the changes in the values of the parameters, between successive iterations, is less than

0.1%. This is a criterion of termination similar to that used by Atkins [7]. If the data fails to give convergence the iterative process is terminated once the iterative limit, which is defined in the data input, has been exceeded.

The standard errors of the parameters are computed at each iteration stage using the procedure of Cleland [6]. The printout control allows optional suppression of all intermediate values of parameters and standard errors. The programme will also print out tables of the computed net velocities for each separate enzyme together with the fraction contributed by each enzyme to the experimental observed velocity over the experimental substrate concentration range.

Evaluation and testing of the computer programme

The programme has been tested using random, normally distributed, synthetic data with defined standard deviations, generated using sub-routine Gauss of the IBM Subroutine Package [8]. Two levels of standard deviation of the dependent variable (i.e. velocity) were used to give best resemblance to experimental data. The standard deviation of velocities for substrate concentrations higher than KMH was half that for velocities of substrate concentrations lower than KMH. The standard deviations used varied from 2.5-20%.

The synthetic data covered substrate concentration ranges varying between 1-5xKMH and 0.1-1xKML, using duplicate estimates of velocities. The substrate concentrations were allowed to decrease in a geometrical fashion, each value being half of the preceeding value. In this way most sets of data contained 16-28 data points. The quality of the parameter-estimates was not markedly affected by this variation in the substrate-concentration range, the important factor being that substrate concentrations at each side of the major curvature of the Lineweaver-Burk plot are included in the data set.

Results and discussion

The programme gave very reliable estimates of the kinetic parameters (KMH, VMH, KML and VML) provided that at least one parameter from each enzyme differed by more than a factor of five. Results obtained using synthetic data fulfilling this criterion are shown in table 1. The computed estimates are not critically dependant on the exact value of the inflexional substrate concentration (IS) (table 2).

Parameter	Parameter Starting guesses	Computed Estimates G	S	Theoretical values	
KMH	25.0	25.9(*)	24.7	24.0	
VMH	38.0	22.5	33.1	33.0	SL = 0.8
KML	12.0	21.1	1.6	2.0	
VML	29.0	10.7	2.6	3.5	
KMH	5.2	5.3	4.4	4.6	
VMH	8.4	5.4	8.2	8.3	SL = 1.0
KML	2.6	2.9	0.05	0.08	
VML	5.6	2.9	0.09	0.12	
KMH	0.5	0.6(*)	1.5	1.5	
VMH	3.3	3.0	2.3	2.3	SL = 0.5
KML	0.2	0.0	0.2	0.2	
VML	1.9	0.28	1.3	1.3	
KMH	1.0	0.8	o.8	0.8	
VMH	1.3	1.2	1.3	1.3	SL = 0.2
KML	0.04	0.05	0.008	0.02	
VML	0.05	0.1	0.05	0.07	
KMH	13.0	20.1	15.9	15.0	
VMH	20.0	27.2	23.8	23.0	SL = 1.0
KML	3.6	3.3	0.4	0.4	
VML	5.9	3.2	0.6	0.6	

Table 1. Comparison of computed estimates of the kinetic parameters obtained using the general non-linear regression procedure (G) of Atkins [7] with those obtained using the programme described here (S). The starting estimates for the general non-linear procedure was obtained by extrapolation of the approximately linear regions of the Lineweaver-Burk plot. "IS" represent the inflexional substrate concentrations used. The data range covered here was 2xKMH - 0.5xKML using duplicate velocity estimates, and 2.5% and 5% standard deviations. The parameter sets labelled with (*) did not converge within 24 iterations. Using the present specific programme convergence was always achieved within 10 iterations. The units of the kinetic parameters have been omitted for clarity.

IS	KMH	VMH	KML	VML	Iterations
0.40	0.64	0.20	0.02	0.10	17
0.60	0.58	0.21	0.01	0.09	21
0.90	0.36	0.26	negative values		no convergence

Table 2. The effect of the value of the inflexional substrate concentration (IS) on the computed estimates of parameters. The theoretical value of the parameters are those of the functions shown in fig. 1; KMH = 0.60, VMH = 0.20, KML = 0.02, VML = 0.10. The synthetic data were generated using standard deviation of 2.5 and 5%. The iteration limit was set to 30 iterations, and the number of iterations required for convergence are also tabulated. The synthetic data covered a substrate concentration range identical to that shown in fig. 1. The inflexional substrate concentration is defined as the concentration above which velocity changes are largely due to the high K_m enzyme. The units of the kinetic parameters have been omitted for clarity.

Unacceptable values will lead to negative estimates of the parameters, illogical values (e.g. KML > KMH), and/or failure to converge (table 2).

The reliability of the estimates of the standard errors of the parameters was investigated using data generated from the same parameter set, but at three different levels of standard deviation of the velocities. These results (table 3) show that a decreased precision of the synthetic data leads to a proportional increase in the standard errors of the kinetic parameters (table 3). These standard errors therefore are true reflections of the precision of the data used in the regression analysis.

This approach gives better results than a general non-linear regression program [7] (table 1). Similar results were also obtained using the non-linear regression program of Cornish-Bowden and Koshland [9] (results not shown). A strong dependence between starting estimates of the kinetic parameters and the computed values may explain why general

Parameter	Theoretical value	Standard deviation of synthetic data/computed parameter estimates 2.5/5.0%	5.0/10.0%	10.0/20.0%
KMH	3.66	3.39(0.31)	3.11(0.56)	2.47(0.84)
VMH	4.00	3.91(0.10)	3.83(0.20)	3.69(0.36)
KML	0.10	0.10(0.01)	0.10(0.01)	0.10(0.02)
VML	2.50	2.48(0.06)	2.46(0.012)	2.37(0.24)
Iterations		9	9	10
Residual variance		0.00635	0.0127	0.0254

Table 3. The effect of the precision of the synthetic data on estimates of the parameters and their standard errors (in parenthesis). The lower value of the synthetic data standard deviation refers to that of the velocities at substrate concentration higher than KMH. The residual variance and the number of iterations required for convergence are also tabulated. The units of the kinetic parameters have been omitted for clarity.

non-linear regression analysis, on equation (2), appears less reliable.

Conclusion.

A non-linear regression procedure resolving the kinetic parameters of a mixture of two enzymes has been developed, and has been shown to offer significant improvements compared to previously published procedures [3,4]. The programme is simple to use and should be of benefit in practical enzymological work.

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