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Pooling and Comparing Estimates from Several Experiments of a Michaelis Constant for an Enzyme

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Often the Michaelis constant of an enzyme will be determined several times. This may be done for various reasons such as ensuring reproducibility, comparing different enzyme preparations, or examining the effects of variations in experimental conditions. In these circumstances, two questions arise. First, how can the various estimates of the Michaelis constant be compared to determine whether they are the same within the limits of experimental variation? Secondly, if they are all the same, how can the values be combined to give an overall estimate? These questions are addressed here and a solution proposed in which the sets of data are pooled and analyzed with a separate maximum velocity for each set but a common Michaelis constant. The pooled data are partitioned in suitable ways and reanalyzed to examine, by means of a variance ratio test, whether a single Michaelis constant gives a satisfactory fit to the data. © 1990 Academic Press, Inc.

One of the fundamentals of science is that experiments be repeated and the results repeatable. In most cases, due to uncontrolled variations in technique and materials, identical results will not be obtained. A decision then must be made as to whether the observed variation is within acceptable limits.

A second and related problem concerning the results of several similar experiments is if, and how, the results can be combined. That is, when several experiments yield independent estimates of a particular quantity which may differ somewhat both in value and precision, how can we combine these estimates to give a single, overall value incorporating all the available information?

In some instances, such as when the quantity being determined is a mean, methods for comparing and combining estimates are extensively documented in standard statistical texts. However, there are other instances about which these texts offer no guidance.

A good example is where the Michaelis constant (K_m) of an enzyme has been determined several times, perhaps with different enzyme preparations and different numbers or combinations of substrate concentrations. We might expect that the maximum velocity (V_m) would vary from one experiment to another, while the K_m should remain constant. How do we decide whether the K_m is the same from all experiments, and how do we combine the several values of K_m to obtain a single value encompassing the entire set of data?

In a previous publication (1), several estimates of the K_m of human erythrocyte transketolase for thiamin diphosphate were combined, but no details of the method employed were given. Here I will provide these details and illustrate the method by example. Although the example is concerned specifically with Michaelis constants, the approach can be applied to a variety of other situations.

EXPERIMENTAL

The experimental observations consist of nine independent determinations of the V_m and K_m of *Escherichia coli* prephenate dehydratase. Each experiment consisted of between 5 and 14 rate measurements at concentrations of the substrate (prephenate) ranging between 0.1 and 5 mM although this full range was not used in any single experiment. These data were gathered during the course of another study which is reported elsewhere (2). Rates are expressed as ΔA_{340} per minute.

DATA ANALYSIS

Individual experiments were analyzed by nonlinear regression, fitting the Michaelis–Menten equation (Eq. [1]) to the data:

$$v = V_m/(1 + K_m/[A]). \quad [1]$$

Fitting was done using the DNRP53 computer program (3) and data were weighted on the basis that the

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error in rate determinations is proportional to the value. This analysis gave values and standard errors for both V_m and K_m together with a residual sum of squares (RSS). This latter value represents the weighted square of the difference between the fitted (ψ) and experimental rate, summed over all data points (Eq. [2]):

$$\mathrm{RSS} = \sum w_i(\psi_i - v_i)^2. \qquad [2]$$

To combine several experiments, it is necessary to fit an equation having different maximum velocities for each of them but a common K_m. This was achieved by using Eq. [3],

$$V_m = V_k, \qquad [3]$$

where k is an integer "indicator variable" which denotes the data set; i.e., $k = 1$ for the first set, $k = 2$ for the second set, and so on (see Appendix). A similar technique has been described by Bates and Watts (4) who have also recommended the variance ratio test (see below) for this type of analysis.

Different data analyses were compared using a variance ratio (F) test (Eq. [4]):

$$F = \{(\mathrm{RSS_r} - \mathrm{RSS_c})/(\nu_r - \nu_c)\}/\{\mathrm{RSS_c}/\nu_c\}. \qquad [4]$$

This test has been outlined elsewhere (5) but it will be explained briefly at this point. If data are fitted using a "complete" equation and a "reduced" equation, from which some parameters have been omitted, it is possible to make a statistical judgement as to whether the reduced equation adequately describes the data. The meanings of the complete and reduced equation in the present context will be detailed in Results.

$\mathrm{RSS_r}$ and $\mathrm{RSS_c}$ are the residual sums of squares for the reduced and a complete equation, respectively, while ν_r and ν_c are the corresponding numbers of degrees of freedom. The complete equation is taken to be a perfect fit so that $\mathrm{RSS_c}/\nu_c$ represents the variance due to experimental error only. However, $\mathrm{RSS_r}$ will contain a "lack of fit" component which can be estimated by subtracting $\mathrm{RSS_c}$, and the variance due to lack of fit then calculated. The ratio of these variances (lack of fit, relative to experimental error) is then tested for statistical significance.

From the results of such an analysis it is possible to decide whether the K_m values found in different experiments can be regarded as the same.

The DNRP53 computer program and its use in fitting Eqs. [1] and [3] to experimental data is described briefly in the Appendix. More detailed information and the program itself can be obtained from the author.

RESULTS AND DISCUSSION

Initially the method will be illustrated using three experiments (data sets 3, 4, and 6) selected from the nine which were available. Equation [1] was fitted to each set separately, to yield V_m and K_m values shown in the upper section of Table 1. The V_m differs considerably from one experiment to another, reflecting the different amounts of enzyme which were used. However, the K_m appears to be fairly constant and the theoretical lines drawn according to these fitted parameters (Fig. 1A) confirm this view, in that all intersect the abscissa at similar positions.

TABLE 1

Analysis of Prephenate Dehydratase Substrate Saturation Data

Data set	$10^4 \times V_m$	K_m (μM)	N	$10^2 \times$ RSS	ν
Separate analysis					
3	238 ± 13	557 ± 60	10	3.8710	8
4	134 ± 10	617 ± 88	5	1.3410	3
6	622 ± 25	479 ± 41	14	3.8392	12
				$\mathrm{RSS_c}$ = 9.0512	ν_c = 23
				$10^2 \times \mathrm{RSS_r}$	ν_r
Combined analysis					
3	232 ± 8	531 ± 33	29	10.1278	25
4	124 ± 5				
6	652 ± 21				

Variance ratio

Calculated: $F = \{(10.1278 - 9.0512)/(25 - 23)\}/\{9.0512/23\}$

$= 1.37$

From tables: $F_{2,23} = 1.73$ (80%)

Note. For the section labeled separate analysis, the V_m and K_m were determined by fitting Eq. [1] to each of three sets of data, while for the section labeled combined analysis, the data were pooled and analyzed by fitting Eqs. [1] and [3] to yield a V_m for each of set of data, but a common K_m. The value of $\mathrm{RSS_r}$ was obtained directly from this combined analysis while $\mathrm{RSS_c}$ was calculated by summing the individual sums of squares from the separate analyses. The variance ratio (F) was calculated using Eq. [4].

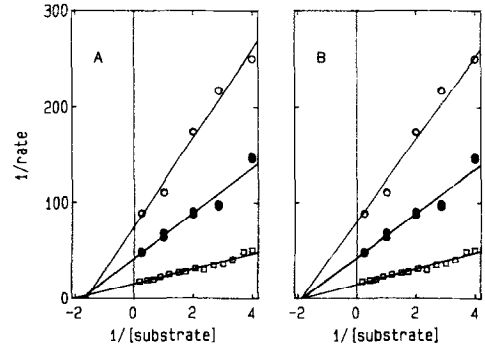

FIG. 1. Double reciprocal plot of substrate saturation data for prephenate dehydratase. Three experiments were selected; there are data sets 3 (filled circles), (open circles) and 6 (open squares). The lines are calculated from the fitted parameters given in Table 1 from the separate analysis (A) and the combined analysis (B).

Analysis of the combined data using Eqs. [1] and [3] gave the results shown in the middle section of Table 1. Each set is described by its own V_m but a common K_m describes all three sets. As illustrated in Fig. 1B, this overall fit is a satisfactory description of the combined data. As expected, the common K_m is within the range of values found in the separate analysis. Moreover, it has a smaller standard error since it is obtained from more information than any of the separate determinations.

Table 1 also illustrates the use of the variance ratio test as a means for examining whether a common K_m gives a satisfactory fit to the data. The separate analyses each give a residual sum of squares, and these may be added together, giving the RSS_c value of 9.0512. In effect, the entire set of 29 points is being fitted using Eq. [1] except that there is a separate V_m and K_m for each set; thus there are six parameters extracted from the data, which leaves 23 degrees of freedom (ν_c). From the combined analysis, RSS_r is calculated and this has 25 degrees of freedom (ν_r) because there are only four fitted parameters. From these RSS and ν values, the variance ratio can be calculated from Eq. [4], as shown in the lowest section of Table 1.

These calculations yielded an F value of 1.37, which is lower than the corresponding value at the 80% level from tables of 1.73. In other words, there is a better than 20% probability that the increase in sum of squares between the complete and the reduced equation has arisen by chance and it is reasonable to conclude that a common K_m describes all of the data.

In the data analysis shown in Table 1, comparison was made between two alternative ways of grouping the three data sets. These groupings were with the three sets combined, and with each of them separate. Other groupings involving pairs of data sets could have been used and there are circumstances in which this would be sensible. For example, if we had an experiment in the presence of a potential inhibitor and two controls in its absence, then the most informative comparison might be between the combined data and the grouping in which the data from the two control experiments are pooled.

With multiple sets of data, the combinations are almost limitless, but the comparisons undertaken would be dictated by circumstances. Consider the present situation (Table 2) in which there are nine sets of data. Since all nine experiments were performed under similar experimental conditions there is no reason to expect them to give different results but, as can be seen when each set was analyzed alone, the K_m varied between 479 and 790 μM. To test whether all sets were consistent with a single K_m, a comparison was made between an analysis with all nine sets combined (i.e., a single K_m and nine separate V_m values) and one in which one data set was segregated from the rest (i.e., a single K_m for eight sets plus a separate K_m for the segregated set, and again nine separate V_m values). This comparison was repeated with each of the nine sets being segregated in turn. In most cases the calculated F value was below the 80% level from tables (with 1 and 64 degrees of freedom) of 1.68. Three of the values (sets 1, 2, and 6) fall in the 95–99% range (F values between 3.99 and 7.05), meaning that there is a 1–5% probability that the decrease in the sum of squares resulting from the extra freedom of including an additional parameter has arisen by chance. As discussed elsewhere (6), it is this author's experience that probabilities of less than 1% are required to reject the null hypothesis; i.e., to be sure that the K_m is truly different. Nevertheless, to find three sets falling in the 1–5% range must raise some suspicions.

The enzyme used in these experiment, *E. coli* prephenate dehydratase, is involved in phenylalanine biosynthesis and is subject to inhibition by phenylalanine (7). This inhibition changes the substrate saturation curve from an apparently hyperbolic shape to one which is clearly sigmoidal. While the present experiments were done in the absence of phenylalanine, it is possible that a trace of sigmoidicity is present. In these circumstances, fitting the Michaelis–Menten equation to the data would be an approximation; moreover, the value obtained for the K_m might be expected to depend on the range of substrate concentrations used in the experiment. It would be anticipated that experiments performed at lower substrate concentrations would tend to

TABLE 2

Detection of Aberrant Michaelis Constants for Prephenate Dehydratase

Data set	Set omitted		Set alone			
	K_m (μM)	RSS	K_m (μM)	RSS	RSS_c	F
1	557 ± 19	0.17755	790 ± 40	0.00104	0.17859	6.325
2	556 ± 20	0.18010	694 ± 48	0.00383	0.18393	4.283
3	572 ± 21	0.15729	557 ± 60	0.03871	0.19600	0.078
4	565 ± 20	0.18133	617 ± 88	0.01341	0.19474	0.493
5	573 ± 21	0.18628	523 ± 46	0.00847	0.19475	0.490
6	592 ± 21	0.13991	479 ± 41	0.03839	0.17830	6.439
7	572 ± 21	0.19443	513 ± 22	0.00049	0.19492	0.433
8	563 ± 20	0.18527	654 ± 52	0.00659	0.19186	1.461
9	576 ± 22	0.16094	534 ± 42	0.03315	0.19409	0.709

Note. Nine sets of data were pooled and analyzed by fitting Eqs. [1] and [3], which gave values for the nine maximum velocities (not shown), a K_m of 569 ± 19 μM and RSS_r of 0.19624 (ν_r = 65). The data were then reanalyzed by omitting set 1 to give the K_m and RSS values of 557 ± 19 μM and 0.17755, respectively, as shown in the second and third columns of the first row. Set 1 was analyzed alone to give the K_m and RSS values of 790 ± 40 μM and 0.00104, respectively, as shown in the fourth and fifth columns of the first row. Summing the values of RSS gave RSS_c = 0.17859 as shown in the sixth column, with ν_c = 64. Finally, F was calculated from Eq. [4], which gave a value of 6.325 (seventh column). This process was repeated with each data set omitted in turn.

give high K_m values and vice versa. Thus it is of interest that the two experiments (sets 1 and 2) which gave the highest K_m values were the only two in which substrate concentrations as low as 0.1 mM were used, while the highest substrate concentration used was 5 mM in experiment 6, which gave the lowest K_m. Thus, while no curvature is visually discernible in a double-reciprocal plot (Fig. 1), it appears that the substrate saturation curve for this enzyme has some sigmoidicity. This was confirmed by fitting the Hill equation (8) to data set 6 and to the combined nine data sets, which gave Hill coefficients of 1.303 ± 0.146 and 1.153 ± 0.036, respectively.

Setting aside the sigmoidicity, there may appear to be an inconsistency between Tables 1 and 2 in that data set 6 is judged to have the same K_m as sets 3 and 4 from Table 1, while the results in Table 2 raise some doubts about this equality. The reason for these apparently conflicting conclusions is the different amounts of information on which they are based. Table 2 includes six more data sets than Table 1, and it turns out that all the extra sets give a K_m higher than data set 6. Thus, it is quite reasonable that the aberration in data set 6 can be seen more clearly in the light of this extra information.

The example discussed here concerns a common problem in enzyme kinetics but the solution proposed can be applied much more widely. Any time a mathematical model is fitted to experimental data a series of parameters will be obtained, some of which will be expected to vary from one experiment to another while the remainder stay constant. The results can be compared and analyzed by the pooling method and F test outlined in this report. For example, a first-order decay process with different amounts of starting material should yield an unchanging rate constant. A suitable analysis for this situation is readily adapted from that described here.

APPENDIX

The DNRP53 computer program is a general nonlinear regression routine which can be used to fit a wide variety of equations to experimental data. The program, which is written in BASIC, requires that the equation to be fitted be inserted at a particular place and uses a prescribed syntax in which the dependent variable is referred to by the symbol G while the independent variables are represented by X(1), X(2) and so on. The fitted parameters are referred to as B(1), B(2), B(3) and so on.

To fit Eq. [1] to a set of substrate saturation data for an enzyme-catalyzed reaction, V_m is represented by B(1), K_m by B(2), and the substrate concentration by X(1). Thus, the following lines is inserted into the program:

```
5100 G = B(1)*X(1)/(B(2) + X(1)).
```

In the case where several subsets of data are pooled and fitted with a common K_m but a different V_m for each subset, a second independent variable (X(2)) is used as an indicator variable to select the particular V_m to be applied to that subset of data. The lines to be inserted into the program are shown below; it should be noted that M is a predefined variable in the program which is equal to the number of parameters:

```
5100 VM = B(X(2))
5110 G = VM*X(1)/(B(M) + X(1)).
```

The DNRP53 program, together with a user's manual, can be obtained on a disk suitable for an IBM-compatible computer from the author for a small fee.

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