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A method for estimating the diffusion resistance of the unstirred layer of microorganisms

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A general enzyme-mediated flux equation which incorporates diffusion resistance was derived. By fitting the model to phosphate flux data from a blue-green alga, *Anacystis nidulans*, diffusion resistance, the half-saturation constant and maximal uptake velocity were estimated. Diffusion resistance for this organism was estimated to be 29 s/cm with a relative precision of about 10%. The parameters were also estimated by graphical analysis of a Woolf plot, but this method was less precise and tended to yield biased estimates.

Introduction

A variety of techniques have been used to estimate the diffusion resistance of the unstirred layer around cells and tissues, but most methods are unsuitable for work with microorganisms. The optical [1] electrophysiological [2,3] and ion-exchange [4] methods can be used only with tissues or large cells. Sha'afi et al. [5] used a method based on the delay in shrinkage of red blood cells after a sudden change in osmotic strength of the medium. This method, however, is not useful for studies with microorganisms with cell walls such as bacteria, yeast and algae. Moreover, it is a technically demanding method.

Winne [6], Pasciak and Gavis [7], and Lieb and Stein [8] have shown that diffusion resistance can affect the kinetics of enzyme-mediated transport. Pasciak and Gavis [7] quantitated their results with a dimensionless parameter, P, which lacked any physical interpretation, whereas Winne expressed his result in terms of the diffusion coefficient of the substrate and the thickness of the unstirred layer. This approach is appropriate only for diffusion to approximately flat surfaces. Lieb and Stein [8] used a permeability coefficient which made no assumptions about the geometry of the surface to account for the effects of diffusion resistance, and they described a graphical technique for estimating the permeability of the unstirred layer. Their method was based on net flux measurements, and their derivations were in terms of rate constants for certain mechanistic models that they were considering. The present paper derives an analogous method based on gross influx measurements made with radiotracers. The derivations are based on the conventional parameters of the Michaelis-Menten equation and a resistance parameter, R, which incorporates the effect of diffusion resistance on enzyme-mediated transport. The role of substrate efflux is considered, and it is shown that under certain circumstances substrate efflux will not influence the estimates of diffusion resistance of the unstirred layer. These results were applied to the analysis of phosphate transport by *Anacystis nidulans*, and estimates of diffusion resistance and the other parameters of the flux equation were obtained with a curve-fitting technique. These estimates were compared to the graphical technique of Lieb and Stein [8] with respect to precision and accuracy.

Methods

The procedures for raising *A. nidulans* and for studying phosphate transport are given elsewhere [9]. The Woolf equation is a linear transformation of the Michaelis-Menten equation [10]:

$$\frac{C_s}{J_{in}} = \frac{K_m}{J_m} + \frac{C_s}{J_m} \quad (1)$$

where C_s is the substrate concentration (nM) at the cell surface, K_m is the half-saturation constant (nM) and J_m is the maximal uptake velocity (pmol $\cdot$ cm^{-2} $\cdot$ s^{-1}) and J_{in} is the gross influx (pmol $\cdot$ cm^{-2} $\cdot$ s^{-1}). If diffusion resistance is negligible then C_s is equal to the concentration in the bulk phase, C_o. In this case, a plot of C_o/J_{in} vs. C_o yields a straight line, and the intercept and slope are used to estimate K_m and J_m. The quantity C_o/J_{in} can be interpreted as an apparent resistance, and will be hereafter designated R_a. In this study, the influx of [^{32}P]orthophosphate was measured, and provided that the specific activity at the surface of the cell is the same as in the bulk phase, R_a can be calculated from:

$$R_a = \frac{a_o(0)}{da_i(0)/dt} \quad (2)$$

where $a_o(0)$ is the initial external concentration of activity (dpm $\cdot$ cm^{-3}) and $da_i(0)/dt$ is the initial rate of uptake (dpm $\cdot$ cm^{-2} $\cdot$ s^{-1}).

Graphical analysis of Woolf plots was performed by equating the extrapolated and observed intercepts with the appropriate terms in Eqns. 10 and 13 and solving for the parameters. The slope of the linear portion of the Woolf plot and the extrapolated intercept were estimated by linear, least-squares regression.

Optimization of parameter estimates for Eqn. 9b was based on least-squares, nonlinear-regression [11]. Quadruplicate measurements of R_a at a series of phosphorus concentrations from 0 to 300 nM P were used to estimate the variance of R_a with respect to the phosphorus concentration. These data (Table I) clearly indicated that the variance of estimates of R_a was not constant with respect to the substrate concentration. To correct for nonuniform variance, the sum of squares calculations were performed with weighting functions. These functions were simply the reciprocal of the variance in Table I, or for phosphate concentrations between the experimentally tested concentrations, the functions were estimated by cubic spline interpolation between the measured values. A Marquart's algorithm or computer search of the parameter space was used to locate the set of parameters which minimized the weighted sum of squared deviations, i.e., the optimum estimate of the parameters.

TABLE I

VARIABILITY OF ESTIMATES OF R_a WITH RESPECT TO PHOSPHATE CONCENTRATION

Bartlett's test for homogeneity of variance indicated highly significant differences between variances of R_a at the various phosphate concentrations ($\chi^2 = 24.33$, five degrees of freedom). At each concentration $n = 4$.

P conc. (nM)	Mean R_a (s/cm)	Variance (s/cm)^2	C.V. (%)
0	37.95	0.5426	1.94
20	40.41	1.066	2.55
75	67.15	6.012	3.65
150	122.4	171.5	10.7
225	160.7	28.10	3.30
300	213.1	17.16	1.94

Approx. 95% confidence contours for nonlinear model are the loci of points defined by [11]:

$$S = S_o + \frac{S_o P}{N - P} F_{P,N,0.95} \quad (3)$$

where N is the number of samples, P is the number of parameters, $F_{P,N,0.95}$ is the F-statistic with P and N degrees of freedom at a confidence level of 0.95, and S_o is sum of squared deviations of the data from the model [11]. These loci were located in various parameter planes with a computer search routine.

Results

Derivation of equations for interpreting the Woolf plot

The net flux of substrate, J_{net}, across the unstirred layer is given by:

$$J_{net} = \frac{C_o - C_s}{R} \quad (4)$$

where R is the diffusion resistance, and the net flux across the membrane is the difference between the gross influx, J_{in}, and the gross efflux, J_{out}. Assuming that J_{in} is specified by the Michaelis – Menten function,

$$J_{net} = \frac{J_m C_s}{K_m + C_s} - J_{out} \tag{5}$$

Since at steady state, diffusion through the unstirred layer and transport across the membrane are equal, Eqns. 4 and 5 can be equated and rearranged to yield a second-order equation in C_s. This equation is solved for C_s, and after eliminating the extraneous root one obtains:

$$C_s = 0.5\Big\{ C_o - R(J_{out} - J_m) - K_m + \sqrt{[K_m + R(J_m - J_{out}) - C_o]^2 + 4(C_o K_m + RJ_{out})} \Big\} \tag{6}$$

This equation can be simplified in certain situations. If J_{out} is constant or decreases as C_o increases, and $J_m \gg J_{out}$, then $J_m - J_{out} \approx J_m$. If J_m is substituted for $J_m - J_{out}$, and the terms under the radical expanded and then collected, one finds again that if $J_m \gg J_{out}$ that the remaining term, RJ_{out}, is negligible with respect to the other terms. Hence, under the specified conditions, Eqn. 6 reduces to:

$$C_s = 0.5\Big[C_o - K_m + RJ_m + \sqrt{(K_m + RJ_m - C_o)^2 + 4C_o K_m} \Big] \tag{7}$$

To determine the relationship between R_a and C_o, first note that Eqn. 5 can be transformed into:

$$\frac{C_o}{J_{net} + J_{out}} = \frac{C_o(K_m + C_s)}{J_m C_s} \tag{8}$$

Since $J_{net} + J_{out} = J_{in}$, Eqn. 8 is a formula for R_a. Substituting Eqn. 7 for C_s into Eqn. 8 and simplifying one obtains:

$$R_a = \frac{C_o + RJ_m + K_m + \sqrt{(K_m + RJ_m - C_o)^2 + 4C_o K_m}}{2J_m} \tag{9a}$$

or equivalently

$$R_a = \frac{2RC_o}{K_m + RJ_m + C_o - \sqrt{(K_m + RJ_m - C_o)^2 + 4C_o K_m}} \tag{9b}$$

When $C_o \ll K_m$, Eqn. 9 simplifies to:

$$R_a = \frac{K_m}{J_m} + R \tag{10}$$

This equation indicates that when $C_o \ll K_m$, R_a is independent of the substrate concentration. The intercept on the ordinate is equal to $K_m/J_m + R$. This may be compared to the intercept of the Woolf equation, K_m/J_m. The unstirred layer shifts the intercept by the quantity R.

When $C_s \gg K_m$, it can be shown

$$C_s = C_o - J_m R \tag{11}$$

The formula for C_s given by Eqn. 11 is substituted into Eqn. 1 and after solving for C_o/J_{in} one obtains:

$$R_a = \frac{K_m}{J_m} + R\left(\frac{J_m}{J_{in}} - 1\right) + \frac{C_o}{J_m} \tag{12}$$

When $C_s \gg K_m$, $J_m/J \approx 1$ and provided that R is not much greater than K_m/J_m, Eqn. 12 simplifies to:

$$R_a \approx \frac{K_m}{J_m} + \frac{C_o}{J_m} \tag{13}$$

This is identical to the Woolf equation, and it indicates that R_a is approximately a linear function of the substrate concentration when $C_s \gg K_m$, and that the coefficients of the line have their conventional interpretation.

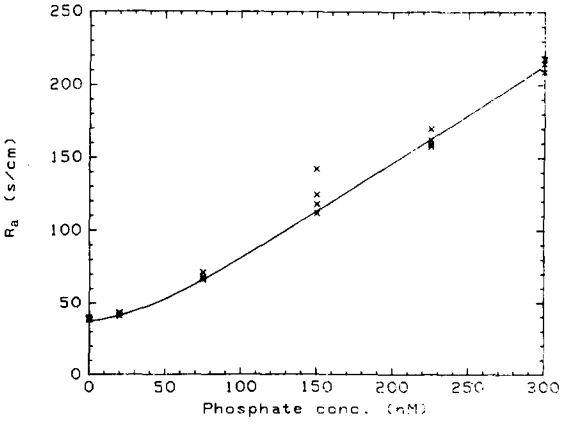

Fig. 1. Woolf plot of phosphate uptake by a blue-green algae.

TABLE II

ESTIMATES OF THE PARAMETERS OF EQN. 9b OBTAINED BY CURVE-FITTING PROCEDURES

The numbers in parentheses are approximate 95% confidence limits. Because of the small sample size in experiments C and D, confidence limits for the parameters were very large and could not be determined.

Expt.	R (s/cm)	K_m (nM)	J_m (pmol·cm^{-2}·s^{-1})	n
A	28.0 (±3.1)	13.0 (±3.6)	1.48 (±0.08)	24
B	31.4 (±7.6)	11.2 (±5.8)	1.07 (±0.04)	20
C	29.9	10.4	0.907	6
D	30.4	28.1	0.817	6
Mean	29.9	15.7	1.07	
S.E.	0.71	4.18	0.16	
C.V.(%)	4.7	53.2	28.0	

Optimal values of the transport parameters and the precision of the estimates

Excellent agreement was obtained between the observed values of R_a and the values of R_a calculated with Eqn. 9b using the optimal estimates of the parameters (Fig. 1). The parameter values for the illustrated data and for other experiments are presented in Table II. Reasonably good agreement was obtained from experiment to experiment even though only a small number of concentrations were tested in experiments C and D.

Approx. 95% confidence contours were determined in parameter planes passing through the optimum point in parameter space to estimate confidence limits for the parameters determined for experiments A and B. Two examples are shown in Figs. 2 and 3. Typically, the contours were nearly elliptic. The long axis in the J_m-R plane was nearly horizontal, but in the R-K_m plane it was clearly diagonally oriented. The long axis of the ellipse in the J_m-K_m plane (not shown) tended to have a diagonal orientation as well, but the slope was in the positive direction. The extreme values of the ellipses for each parameter in each plane were compared and the widest pair of limits were used for confidence estimates. Although the differences of the upper and lower bounds from the optimum were not identical, they were sufficiently similar to justify averaging the deviations to summarize the confidence limits with a single value

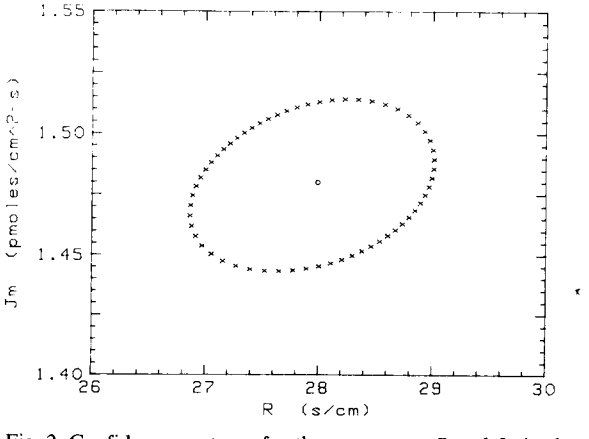

Fig. 2. Confidence contours for the parameters R and J_m in the plane passing through the optimum. The circle marks the optimum, and the x's are loci on the confidence contour. The loci were located at 6° increments around the optimum.

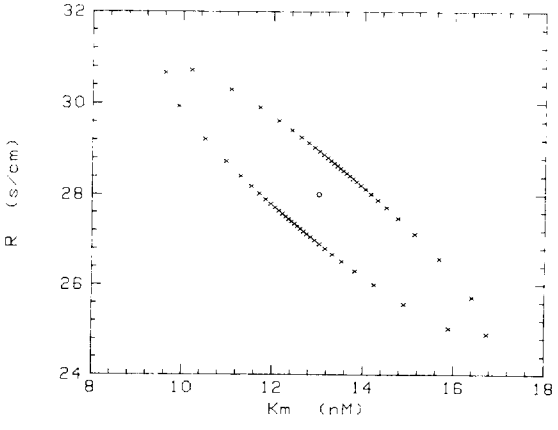

Fig. 3. Confidence contours for the parameters K_m and R. See Fig. 2 for explanation of symbols.

TABLE III

ESTIMATES OF PARAMETERS OBTAINED BY APPLICATION OF EQNS. 10 AND 13

The numbers in parentheses are 95% confidence limits that were calculated under the assumption that the data were unbiased.

Expt.	R (s/cm)	K_m (nM)	J_m (pmol·cm^{-2}·s^{-1})	n
A	7.56 (±10.3)	46.4 (±17.4)	1.58 (±0.12)	24
B	7.61 (±5.97)	38.4 (±8.33)	1.15 (±0.056)	20
C	15.4 (±27.1)	26.7 (±27.0)	0.908 (±0.12)	6
D	31.7 (±62.2)	27.9 (±48.6)	0.728 (±0.50)	6
Mean	15.6	34.9	1.09	
S.E.	5.68	4.66	0.184	
C.V.(%)	72.8	26.7	33.8	

(Table II). R was determined with a relative precision of 11 and 24% in experiments A and B, respectively. The relative precision of the estimates of K_m were twice as large, but for J_m the relative precision was better than 10%.

The confidence contours of experiments C and D were very large and extended into the negative values of parameter space. The function R_a, of Eqn. 13 is not defined for most negative values of the parameters, hence the complete contours could not be determined. Since the only difference between the first and last pairs of experiments was the number of data points, the large confidence limits in the last two experiments can be attributed to the small sample size.

Estimation of parameters by graphical analysis

The estimates of J_m made by graphical analysis agreed reasonably well with the numerically estimated values (compare Tables II and III). The values of K_m and R, however, differed noticeably. In experiments C and D, the numerical estimates fell within the wide confidence limits of the graphical estimates; the difference in these estimates cannot be considered significant. In experiments A and B, however, the estimates of K_m and R differed significantly between the two methods. In both experiments, graphical analysis overestimated K_m and underestimated R with respect to the numerically determined values.

Discussion

The theoretical analysis by Winne [6] showed that the resistance of the unstirred layer could alter the linearity of the Lineweaver-Burke plot. Pasciak and Gavis [7] showed theoretically and experimentally that a similar deviation occurred in the Woolf plot, and the theoretical work of Lieb and Stein [8] demonstrated that the deviation could be used to estimate the permeability of the unstirred layer. The present paper derived an equation for apparent transport resistance in terms of the coefficients of the Michaelis-Menten equation and a diffusion resistance parameter, and demonstrated that the parameters could be estimated with a nonlinear curve-fitting technique. The method is particularly well-suited to studies with cell suspensions. Since previous techniques were not amenable to studies of diffusion resistance of small cells, the method is an useful addition to the study of diffusion resistance of the unstirred layer.

The derivation of Eqn. 7 and subsequent formulae was based on the premise that $J_m \gg J_{out}$. For certain substances, e.g., growth-limiting nutrients that are actively transported into the cell, one would expect a priori that J_{out} would be much smaller than J_m. Literature data bear out this supposition for phosphate. Perry [12] simultaneously measured J_{in} and J_{net} with a marine alga over a wide range of phosphate concentrations from substrate-limiting to carrier-limiting conditions and could not detect a significant difference. That is, J_{out} was very small, and clearly J_{out} was much smaller than J_m. Working with natural phytoplankton from an oligotrophic lake at very low phosphate concentration, Lean and White [13] were able to detect a difference between J_{in} and J_{net}, but the difference was independent of substrate con-

centration. These observations indicate that the assumption $J_m \gg J_{out}$ is valid for phosphate transport by phosphate-starved algae.

For the method to be applicable, the resistance of the unstirred layer would have to be large enough to make a significant contribution to the total transport resistance of an enzyme-mediated system. As Winne [6] indicated, the quantity K_m/J_m can be interpreted as the minimum resistance of the transporter. The sum, $K_m/J_m + R$ is the total minimum resistance. In the present study, the estimate of R was about twice as large as K_m/J_m, and with such a large contribution to the total resistance, R could be determined with reasonable accuracy. If the contribution of R were smaller, the precision of the estimate would decrease. From a practical standpoint, R would probably have to be at least half as large as K_m/J_m to be estimated with reasonable precision with the methods described in this paper.

To obtain minimum variance, unbiased estimates of parameters by curve-fitting procedures, the error in the dependent variable must be uniform with respect to the independent variable. The condition of constant variance is seldom met in practice and the use of weighting functions is required. If the model is correct and the distribution of errors is symmetric, the estimated parameters are also unbiased. The residuals from the fitted curve were examined to assess possible bias. There was no apparent trend in the distribution or central tendency of the weighted residuals as a function of substrate concentration. The mean and variance of the residuals were very close to 0 and 1, respectively. Also, the Geary kurtosis test statistic and coefficient of skewness indicated no significant kurtosis and skewness, respectively. These observations suggest that there was no lack of fit or nonsymmetry in the distribution of errors, and tentatively we can conclude that the values of the parameters in Table II are minimum variance, unbiased estimators.

The variance in R_a in the present study varied in a rather unusual manner. This result is probably peculiar to the specific experimental conditions and to the method of measuring the initial uptake rate. At low substrate concentration, the variance was quite low. It increased markedly at the mid value of the concentration range studied, and decreased at high levels of substrate. At low and high concentration of substrate, the uptake curves were highly curvilinear. In the former instance, the rapid depletion of radioisotope from the medium was responsible. At high concentration, the rapid uptake of substrate probably saturated the cell quickly and caused a decrease in the net flux of radioisotope into the cell. At 150 nM, however, the uptake curves were only slightly curvilinear. At this concentration, there was evidently sufficient phosphate to prevent depletion of the medium, and yet it was not so high that the cells were saturated in the course of the experiment. Since the lines were only slightly curvilinear, second-order regression led to considerable uncertainty in the first-order coefficient. The degree of curvature, and hence error, would have been different with other cell densities, other sampling schemes and with other algae. Consequently, the variances associated with R_a observed in this study represent a special case and should not be generalized to other situations.

Lieb and Stein [8] described a graphical technique for estimating the permeability of the unstirred layer. Diffusion resistance is simply the reciprocal of the unstirred layer permeability, and their method was adapted in the present paper and applied to the data on phosphate transport (see below for further comments on their method). The estimates of K_m and R obtained by graphical analysis were larger and smaller, respectively, than the estimates obtained by numerical analysis. Lieb and Stein [8] noted that the graphical technique would tend to produce biased results due to the nature of the approximations inherent in the derivation of equations used for interpreting the graph. Above a substrate concentration of 50 nM, the error in Eqn. 13 is small, but it systematically declines over the interval 50–300 nM. Consequently, the slope of this portion of the Woolf plot is less than the true value of $1/J_m$ when diffusion resistance makes a significant contribution to the total resistance. Although the resultant error in the estimate of J_m is small, extrapolation of this line to the ordinate leads to a significant overestimate of K_m/J_m. When K_m is calculated from the ratio, intercept/slope, the bias of the slope adds to the bias of the intercept such that the total bias in K_m is large. When this overestimated value of K_m is

used to calculate R from Eqn. 8, R is seriously underestimated. The bias from graphical analysis could be minimized by using a very high range of substrate concentration, but since the distance over which the line would be extrapolated would increase, the precision of the estimate would worsen. Despite these limitations, the graphical technique is useful because it allows determination of preliminary estimates of the parameter. The numerical determination of the parameters relies on iterative correction procedures, and the graphical estimates are excellent starting points for numerical analysis.

The model for R_a (Eqn. 9) was developed around the concept of diffusion resistance, and the ability of the model to explain the data suggests that this interpretation of the data is correct. However, alternate tests of this interpretation are desirable, and alternate explanations should be considered. Activation energy analysis was performed with *Anacystis* [9] at substrate-limiting and substrate-saturating conditions. In the former instance, the activation energy was 5.6 kcal/mol which is close to the activation energy for diffusion of ions and small molecules through water [11]. However, under substrate-saturating conditions (i.e., when uptake was limited by the carrier), the activation energy was 11.9 kcal/mol, which is suggestive of an enzyme-mediated process. Since the relative contribution of diffusion resistance to the total resistance would be greatest at low substrate concentrations and negligible at high substrate concentration, these results are consistent with the interpretation used in this paper. Another experiment which might be useful is to determine R_a in an unmixed and in a highly agitated medium. If diffusion resistance is a significant component of R_a, R_a should be less in the agitated medium. This may not work for small cells, however, because their unstirred layer is evidently very small [5]. Artifacts should also be considered; in particular, loss of substrate by adsorption to container walls. If this were to happen, an error in the intended substrate concentration would arise. This error could systematically decline as the substrate concentration increased, and it could produce a curvilinear relationship between R_a and the intended C_o. In the experiments reported in this paper, this possibility was checked by comparing the total radioactivity in the medium during or at the end of an experiment to the amount added. Finally, if other models are available that might fit the data the ability of those models to explain the data should be compared to results obtained with Eqn. 9. In particular, the residuals should be carefully examined for lack of fit. Any indication of lack of fit of an optimumized model to the data should raise doubts as to the validity of that model.

Lieb and Stein [8] concluded from their theoretical analysis that the permeability of the unstirred layer (i.e., the reciprocal of diffusion resistance) could be estimated from a plot of C_o/J_{net} against C_o. In the notation of the present paper, an analogous conclusion can be derived by substituting Eqn. 7 for C_s into Eqn. 4 and solving for C_o/J_{net}. The result is identical to the right side of Eqn 9b and leads to the apparent contradiction that $J_{in} = J_{net}$. This anomaly arises because $C_s \approx C_o$ at low substrate concentration, and even a small error in C_s leads to a large error in their difference. Since C_s does indeed have a small error due to neglect of the efflux component, the substitution of Eqn. 7 into Eqn. 4 produces an equation for J_{net} that is unreliable at low substrate concentration, unless J_{out} is neglibible with respect to J_{net}. This is not likely to be the case at low substrate concentration. Since flux measurements at low substrate concentrations are critical for accurately estimating diffusion resistance, the use of net flux data as suggested by Lieb and Stein [8] should be considered with caution.

From the experience obtained in this study, the following recommendations are made with regard to determination of the resistance of the unstirred layer of cell suspensions. A suitable enzyme-mediated transport system must be selected. Literature data may be useful in this regard, or a preliminary experiment could be performed to make rough estimates of K_m and J_m using graphical analysis and Eqn. 12. After an estimate of K_m is obtained, R_a should be estimated at a large number (e.g., 15) of substrate concentrations in the interval 0–K_m and a few values (e.g., 5) estimated in the interval K_m–$20\ K_m$. If diffusion resistance is significant with respect to the total transport resistance, a deviation from linearity of R_a will be observed at low substrate concentration. A final experiment

should be performed with replication (4 or more) at each substrate concentration to determine the variance in R_a with respect to substrate concentrations. A large number of concentrations need not be tested (6–8 should be suitable), but they should be selected so that each linear region is adequately estimated, e.g., at multiples of K_m of 0, 0.1, 0.25, 1, 4, 12 and 16 if R is comparable to K_m/J_m. The total number of measurements of R_a should exceed 20. From these data optimum estimates of R, K_m and J_m can be calculated. Confidence contours should also be determined, and adequacy of the estimates evaluated. If further improvement is desired, the design should be rigorously optimized using procedures outlined by Currie [15].

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