Nonparametric Estimation of Rate Equations for Nutrient Uptake

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Knowledge of the rate of a biological process is important for characterizing the system and is necessary for gaining a deeper understanding of the process. Consider measurements, Y, made over time on a system following the model $Y = f(t) + e$, where f is a smooth, unknown function and e is measurement error. Although most statistical methodology has focused on estimating $f(t)$ or $f'(t)$, in some applications what is of real biological interest is the relationship between f and f'. One example is the study of nitrogen absorption by plant roots through a solution depletion experiment. In this case $f(t)$ is the nitrate concentration of the solution surrounding the roots at time t and $-f'(t)$ is the absorption rate of nitrate by plant roots at time t. One is interested in the rate of nitrate absorption as a function of concentration; that is, one is interested in Φ, where $\Phi(f) = -f'$. Knowledge of Φ is important in quantifying the ability of a particular plant species to absorb nitrogen and in comparing the absorption ability of different crop varieties. A parametric model for Φ is usually not available, and thus a nonparametric estimate of Φ is particularly appropriate. This article proposes using spline-based curve estimates with the smoothing parameter chosen by cross-validation and suggests a method for obtaining confidence bands using a form of the parametric bootstrap. These methods are used to analyze a series of solution depletion experiments and are also examined by a simulation study designed to mimic the main features of such data. Although the true f is a monotonic function, simulation results indicate that for our specific application, constraining the estimate of f to be monotonic does not reduce the average squared error of the rate curve estimate, Φ. Although using a cross-validated estimate of the smoothing parameter tends to inflate the average squared error of the rate estimate, an analysis of a set of solution depletion experiments is still possible. Using the proposed methods, we are able to detect a difference in rate curves obtained under different experimental conditions. This is established by applying an analysis of variance (ANOVA)-like test to the estimated rate curves, where the critical value is determined by a parametric version of the bootstrap, and by examining confidence bands for the difference of two rate cures. This finding is important, because it suggests that the shape of Φ may not be constant under the experimental conditions examined.

KEY WORDS: Confidence bands; Cross-validation; Michaelis–Menten rate equation; Monotonicity constraints; Parametric bootstrap; Smoothing splines; Solution depletion experiments.

1. INTRODUCTION

Nitrogen is an essential nutrient for plant growth. Plants absorb nitrogen from the soil mainly in the form of nitrate or ammonium ions. At any given time, however, most soils contain only small amounts of these ions relative to the amount continuously required by plants (Epstein 1972). Nitrogen is added to the soil through the atmosphere (brought down by precipitation), through plant residues, through animal wastes, and through the application of nitrogenous fertilizers. But only a small portion of this nitrogen is in the form of nitrate or ammonium. In addition, ammonium is easily converted to nitrate, and nitrate is readily leached from the soil by rain or irrigation water, possibly becoming a water pollutant (Brady 1984). Therefore, knowledge of the nitrogen *uptake* (absorption) process by plant roots is necessary to avoid both nitrogen deficiency and leaching.

The rate at which roots absorb nitrate ions is a function of the nitrate concentration of the medium surrounding the roots (Epstein 1972). In this article it will be assumed that the dependence of the uptake rate, r, on the ambient concentration, c, follows a smooth function, $\Phi(c) = r$. Knowledge of the functional form of Φ is important in quantifying the ability of a particular type of plant to absorb a specific ion. The mechanisms in root cells that control the absorption of nitrate are not completely understood, so identifying Φ is a first step in understanding the cellular processes that mediate nitrate transport.

It is difficult to measure uptake rates directly, because the absorption process by the plant roots must invariably change the ambient concentration. One direct measure is to use a radioactively labeled ion and then assay the amount of labeled nutrient that is absorbed by the roots in a short period of time. Unfortunately, measuring nitrate absorption using this technique is difficult, because the radioactive isotopes of nitrogen have short decay times. An alternative method is to determine Φ indirectly by a solution depletion experiment (Claassen and Barber 1974). A single plant or set of plants is placed in a beaker containing a specific volume and prespecified initial concentration, c_0, of an aerated nutrient solution. Then at each of n predetermined time points, t_1, $t_2, \ldots t_n$, the solution in the beaker is sampled and the concentrations, $y_1, y_2, \ldots y_n$, are determined. Figure 1 illustrates typical results for solution depletion experiments that we have encountered for the uptake of NO_3^- (nitrate) by maize roots. Although the ability of the root material to deplete the solution of the nitrate ion over time is readily apparent from these time plots, the shape of the uptake rate curve, Φ, is not clear. Using nonparametric curve fitting, though, it is possible to recover reliable estimates of Φ from such depletion

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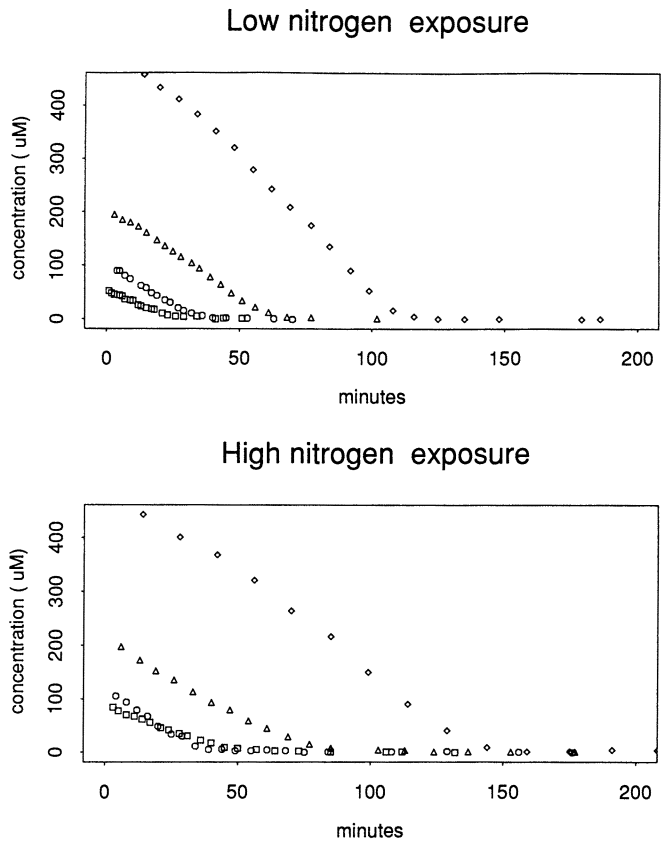

Figure 1. Nitrate Uptake by Maize Roots Over Time. The observed concentration of nitrate (μM) remaining in ambient solution is plotted as a function of time (1 μM = 1 μmol/1,000 ml). The time axes of the plots were truncated to 200 minutes so that the data were not compressed and difficult to distinguish. □, c_0 = 50 μM; ○, c_0 = 100 μM; △, c_0 = 200 μM; ◇, c_0 = 500 μM.

experiments. Although other types of experiments generate data from which Φ could be estimated, the solution depletion experiment is powerful because of its *relative* simplicity. (We stress the word "relative" here because this experiment is still not trivial to do.) Unlike more complicated designs, several solution depletion experiments can be done simultaneously, possibly under different experimental conditions.

It will be assumed that the depletion data follow the additive model

$$y_k = f(t_k) + e_k, \qquad 1 \le k \le n$$

and

$$0 = t_1 < t_2 < \cdots < t_n = T, \tag{1}$$

where the depletion curve, f, is a smooth function of time and $\{e_k\}$ are random, independent errors with constant variance σ^2. Typically, statistical methodology has focused on estimating f or its derivative from $\{(t_k, y_k)\}$. In this application and in many other biological applications, however, what is of real interest is not $f(t)$ or $f'(t)$ directly, but the *relationship* between f and f'. That is, one is interested in estimating the function Φ, where $\Phi(f) = -f'$. Because the biochemical basis of nitrate absorption has not been completely identified, it is difficult to specify parametric models for Φ. Also, because depletion experiments do not measure the uptake rate directly, it is not possible to readily identify empirical models for Φ based on a graphical examination of the data. For these reasons, it is appropriate to consider nonparametric regression estimates of Φ. Although this nonparametric approach may not give direct insight into the internal mechanisms of ion absorption, it does enable us to quantify how rates of absorption are affected by changes in various external factors or internal conditions. In this article we apply smoothing spline methods to estimate Φ, propose a method for obtaining confidence bands for the rate curve and for the difference between two rate curves, and suggest a simulation-based procedure to test for differences among rate curves for different treatment groups.

Although much of this article focuses on a specific application of nonparametric regression in soil science, there are two issues of more general interest: (1) the advantages and disadvantages of enforcing monotonicity constraints on an estimated depletion curve, and (2) the feasibility of using a data-based method for selecting the smoothing parameter (bandwidth). Basic scientific considerations imply that f should be a monotonically decreasing function in time. But with most nonparametric estimators, it is difficult to enforce monotonicity. We are interested in quantifying (in the context of our application to depletion experiments) the value of using monotonic estimators when the true function is indeed monotonic. The accuracy of the rate curve estimate depends primarily on the quality of the estimate of f'. Most data-based methods of smoothing parameter selection attempt to minimize the expected average squared error with respect to f. One important question is whether smoothing parameter estimates based on cross-validation for the observed data are also appropriate for estimates of Φ.

THe next section reviews some existing approaches to estimate rate curves and introduces an estimate of Φ based on smoothing splines. Section 3 gives the details of a particular depletion experiment designed to examine whether the uptake mechanism of nitrate ions in maize have "memory"—that is, does the form of Φ depend on the initial concentration of nitrate in the depletion experiment? Section 4 reports the results of a simulation study that investigated the distinction between constrained and unconstrained depletion curve estimates. Finally, Section 5 draws some conclusions from the analysis of the depletion experiment and considers more general statistical issues associated with the use of spline estimators.

2. RATE CURVE ESTIMATORS

2.1 Parametric Models

Epstein and Hagen (1952) first proposed the Michaelis–Menten model, $\Phi(c; \theta) = \theta_1 \cdot c/(\theta_2 + c)$, to describe ion uptake as a function of concentration, c. Michaelis and Menten (1913) originally derived this model from enzyme reaction equations, but Epstein and Hagen (1952) noted that the mechanism of ion absorption and enzyme activity are "quite analogous" and thus the Michaelis–Menten model was appropriate. Later, extensions of the Michaelis–Menten model and combinations of several Michaelis–Menten models were proposed (see Epstein 1972). Recently, it has been suggested that the uptake of ions, in particular nitrate,

by plant roots is much more complicated than what these earlier models suggest (Clarkson 1986; Jackson, Pan, Moll, and Kamprath 1986; Jackson, Volk, Morgan, Pan, and Teyker 1986). We have attempted to model these more complicated mechanisms of ion absorption using a biologically based compartmental model (Jacquez 1985). At this time, however, obtaining data for such a model is not yet feasible (Jackson, personal communication, January, 1988, Meier 1990). Therefore, this biologically based modeling approach was abandonded in favor of the more empirical approach proposed in this article.

Certain computational difficulties are associated with estimating parameters for the rate function, even if an adequate parametric model is available. To relate Φ to the observational data from a depletion experiment, it is necessary to solve the first order differential equation, $\Phi(f; \theta) = -f'$ for f, and then to compare the solution, f, to the observed data. In most cases, such as Michaelis–Menten kinetics, this equation must be solved numerically. Claassen and Barber (1974) first proposed fitting the Michaelis–Menten model to solution depletion data using this approach. They also suggested a nonparametric approach which is described in the next section.

2.2 Nonparametric Curve Estimates

If the depletion curve were known exactly, then the rate curve could be recovered by the relationship $\Phi(c) = -f' \circ g(c)$, where $g = f^{-1}$. We propose using a cubic smoothing spline to estimate f and then differentiating this estimate for the derivative estimate. The pairs of points $(\hat{f}(t), -\hat{f}'(t))$ can be interpreted as estimates of $(c, \Phi(c))$ with $c = f(t)$. If $\hat{f}$ is monotonic, then $\hat{\Phi}$ is actually a well-defined function with respect to c and can be written explicitly as

$$\hat{\Phi}(c) = -\hat{f}'(\hat{f}^{-1}(c)). \tag{2}$$

If $\hat{f}$ is not monotonic, then $\hat{\Phi}$ can not be expressed as a function across the entire range of observed concentrations. Several different methods have been proposed for estimating derivatives from noisy data (Anderssen and Bloomfield 1974a, b; Cullum 1971; Gasser and Müller 1984; Reinsch 1967; Rice 1986; Rice and Rosenblatt 1983; Silverman 1985; Wahba 1975). There is also literature on estimating growth (depletion) curves from data (for example, Gasser, Müller, Köhler, Molinari and Prader 1984; Wilson 1988; Zerbe 1979). But we are not aware of any published work that estimates a derivative of a function as it relates to the function itself (versus time) using the techniques proposed in this article. Claassen and Barber (1974) suggested obtaining point estimates of the rate curve at the observed concentrations by fitting a parabolic spline function (DuChateau, Nofziger, Ahuja, and Swartzendruber 1972) to solution depletion data or by fitting a cubic spline to the data with variable knot locations. They differentiated the splines to obtain point estimates of the rate at each observed concentration. These researchers did not address the problems of selecting the number of knots in the spline estimate nor how one might draw statistical inferences from the estimated rate curve.

Although our approach is similar to the nonparametric strategy suggested by Claassen and Barber (1974), we have added several improvements to the nonparametric estimator. Most notably, we consider monotonic estimates of the depletion curve and determine the amount of smoothing automatically by cross-validation. The details of our methods are given in the next subsection.

2.3 Smoothing Splines and Monotonicity Constraints

Although splines are popularly associated with piecewise polynomial functions, a more fundamental definition of a spline is as a solution to a variational problem. Let $\mathcal{H} = W_2^2[0, 1] = \{h: h, h' \text{ absolutely continuous}, h'' \in L^2[0, 1]\}$. For $\lambda > 0$, a cubic smoothing spline estimate (with natural boundary conditions) of the regression function f under Model (1) is the $h \in \mathcal{H}$ that minimizes $\mathcal{L}(h) = (1/n) \sum_{i=1}^{n} (y_i - h(t_i))^2 + \lambda \int_{[0,1]} (h''(u))^2 \, du$. The minimizer of $\mathcal{L}(h)$ will exist and is unique, provided that $n > 3$, and will be denoted as $\hat{f}_\lambda$ to emphasize its dependence on the smoothing parameter, λ. It is well known that $\hat{f}_\lambda$ can be written as a piecewise cubic polynomial with join points (knots) at each data point (Eubank 1988; Wahba 1990). Also, for a fixed λ, $\hat{f}_\lambda$ is a linear function of the data, $\mathbf{y}$, and one can define an $n \times n$ "hat" matrix $\mathbf{A}(\lambda)$ such that $\hat{f}_\lambda(\mathbf{t}) = \mathbf{A}(\lambda)\mathbf{y}$, where $\hat{f}_\lambda(\mathbf{t}) = [\hat{f}_\lambda(t_1), \hat{f}_\lambda(t_2), \ldots, \hat{f}_\lambda(t_n)]^T$. Smoothing splines are one of several types of nonparametric regression estimates that are linear functions of $\mathbf{y}$ and are essentially locally weighted averages (Nychka 1989; Silverman 1984). Here the smoothing parameter plays a similar role to the bandwidth for a kernel regression estimate, and an approximate expression for the average bandwidth of a cubic smoothing spline is $\lambda^{1/4}$.

The spline estimate just described may not be a monotonically decreasing function. This constraint can be imposed formally by simply restricting the minimization of $\mathcal{L}$ to $h \in \mathcal{H}$ such that $h' \leq 0$. Such estimators have been studied by Utreras (1985) and Villalobos and Wahba (1987) but are not readily computable. As an alternative, we choose to implement this constraint by exploiting the representation of the cubic smoothing spline as a linear combination of B-spline basis functions (Kelly and Rice 1990; Ramsay 1988). From the characterization of a cubic smoothing spline estimate as a piecewise polynomial, it is possible to represent the solution as a linear combination of cubic B-spline basis functions, $\{B_k\}$; see de Boor (1978) for their construction. Moreover, it is easy to construct monotonic functions with respect to this B-spline basis. Suppose that

$$h(t) = \sum_{k=1}^{n} c_k B_k(t)$$

and

$$c_1 \geq c_2 \geq \cdots \geq c_n, \tag{3}$$

then h will be monotonically decreasing. This property suggests a monotonic spline, $\hat{f}_{M\lambda}$, that is the minimizer of $\mathcal{L}(h)$ for all h satisfying the conditions at (3). The main advantage of this representation is that the inequality constraints are on the coefficients rather than on the function itself. This minimization problem is readily solved, as it is a quadratic

programming problem with inequality constraints (see Kelly and Rice 1990). Our work uses FORTRAN code, kindly provided by John Rice, and is based on de Boor's algorithm for evaluating B-splines (de Boor 1972) and on Lawson and Hanson's (1974, chap. 23) algorithm for computing constrained least squares estimates.

The spline estimate is sensitive to the choice of the smoothing parameter. Although λ is often chosen subjectively in the process of data analysis, we also recommend considering a data-based estimate of the smoothing parameter based on cross-validation. For the unconstrained spline, one estimate of λ is obtained by minimizing the generalized cross-validation function (Craven and Wahba 1979; Wahba 1990)

$$\mathrm{GCV}(\lambda) = \frac{1}{n}\left[\sum_{k=1}^{n} (y_k - \hat{f}_\lambda(t_k))^2\right] \div [(1 - \mathrm{trace}(\mathbf{A}(\lambda))/n]^2. \quad (4)$$

This criterion depends on the estimate being a linear (or approximately linear) function of $\mathbf{y}$. For the constrained case where linearity does not hold, Villalobos and Wahba (1987) suggested an approximation for trace $(\mathbf{A}(\lambda))$.

Rice (1986) has suggested an alternative method for choosing lambda when estimating a derivative based on minimizing a nearly unbiased estimate of the integrated mean squared error. But it is not clear how to extend this method to constrained spline estimates. To compare the performance of constrained and unconstrained smoothing splines, therefore, we estimated λ based on minimizing the adjusted cross-validation function (AdCV), because for the monotone splines at least it was clear what the criterion was estimating. Let $\hat{f}_\lambda^{[k]}$ be the spline estimate of f, having omitted the kth data point, and define the adjusted cross-validation function as

$$\mathrm{AdCV}(\lambda) = \frac{1}{n-2}\sum_{k=2}^{n-1} (y_k - \hat{f}_\lambda^{[k]}(t_k))^2. \quad (5)$$

AdCV(λ) is an estimate of the expected average prediction error of the spline for a particular choice of λ. The first and last values were omitted from the criterion because these were not available from our constrained spline software. For unconstrained splines, $y_k - \hat{f}_\lambda^{[k]}(t_k)$ can be shown be to equal to $(y_k - \hat{f}_\lambda(t_k))/(1 - \mathbf{A}_{kk}(\lambda))$; therefore, if $\hat{f}_\lambda$ and the diagonal elements of $\mathbf{A}(\lambda)$ are known, evaluating AdCV(λ) is only an order n calculation. Although these computational shortcuts do not carry over to constrained splines, minimization of AdCV(λ) is still feasible, taking roughly 10 minutes ($n = 21$) on a SUN SPARCstation 1.

2.4 Testing for Equality of Rate Curves

In our application we are specifically interested in testing whether a set of rate curves generated from different depletion curves are the same. The comparison is made on the rate curve rather than on the depletion curves, because the soil scientists are interested in the rate curves and because comparisons of just the depletion curves can give ambiguous results. Two depletion curves that differ by only a horizontal shift will yield the same rate curve on the range of common concentrations. We know a priori that the depletion curves will differ at least by a horizontal shift due to the different initial conditions of the experiment; therefore, we are not interested in simply testing whether the depletion curves are different. As a result, the techniques proposed in the literature for comparing nonparametric curve estimates (Hall and Hart 1990; Härdle and Marron 1990; King 1989; Raz 1990) do not directly apply. One might develop a test for detecting a horizontal difference among the depletion curves, although such a test would be complicated because it would involve estimating shift parameters that align the curves. Instead, our attention focuses on developing a test for the difference among rate curves. In this section we first propose a simulation-based method for comparing rate curve estimates and for constructing confidence intervals. We begin by defining some notation.

Suppose that one has M depletion experiments yielding rate curve estimates $\{\hat{\Phi}_1, \ldots, \hat{\Phi}_M\}$, corresponding residual sums of squares $\{\mathrm{RSS}_1, \ldots, \mathrm{RSS}_M\}$ where $\mathrm{RSS} = \sum_{i=1}^{n} (y_i - \hat{f}_\lambda(t_i))^2$, and effective number of parameters $\{\nu_1, \ldots, \nu_M\}$ (ν is the trace of $\mathbf{A}(\hat{\lambda})$, where $\hat{f}_\lambda(\mathbf{t}) = \mathbf{A}(\hat{\lambda})\mathbf{y}$). Let $\bar{\Phi}$ denote an "average" rate curve across these experiments. Although there are several ways to construct $\bar{\Phi}$, we chose $\bar{\Phi}$ based on the following criteria: $\bar{\Phi}$ is smooth, $\bar{\Phi}$ is defined over the largest range of concentrations (this condition is necessary so that $\Phi(f) = -f', f(0) = c_0$ can be solved numerically for $c_0 = (50, 100, 200, 500)$), and the averaging process is somehow weighted according to the number of rate estimates at observed concentrations in a particular range. Based on these criteria, one reasonable way to obtain $\bar{\Phi}$ is to fit a cubic smoothing spline to the data set obtained by combining all pairs of estimated concentrations and rates across the different experiments. Under the assumption that trace$(\mathbf{A}(\hat{\lambda}))$ is a reasonable measure of the degrees of freedom associated with the model for a depletion curve, consider the pooled estimate of σ^2, $s_p^2 = (\sum_{k=1}^{M} \mathrm{RSS}_k)/(N - \nu_T)$, where $\nu_T = \sum_{k=1}^{M} \nu_k$ and $N = \sum_{k=1}^{M} n_k$. With these definitions, let

$$F^* = \frac{\sum_{k=1}^{M} \int \nu_k(\hat{\Phi}_k - \bar{\Phi})^2 dc}{s_p^2}. \quad (6)$$

The definition of F^* is analogous to the usual one-way analysis of variance (ANOVA) F statistic, except that an integral replaces the sum of squared differences between treatment and grand means. This idea of using an L_2 measure to compare curves was used by Zerbe (1979).

We wish to test the null hypothesis that the rate curves are equal against the alternative that they are not all equal. Clearly, the distribution of F^* will not have a simple form, even under the null hypothesis, and is more easily approximated by simulation methods. These simulations are based on the assumptions that $\bar{\Phi}$ is the true rate curve across all experiments and that the measurement error is normally distributed with variance $\hat{\sigma}^2 = s_p^2$. Given these specifications for the model, pseudodata is generated by adding normal errors to the individual depletion curves obtained by numerically solving $\bar{\Phi}(f) = -f', f(0) = c_0$ for $c_0 = (50, 100,$

200, 500)). The rate curve estimates are then computed exactly as they were with the original data (including using GCV for selecting the smoothing parameter), and the test statistic at (6) is computed. Percentage points from the distribution of F^* can be estimated and are used to set approximate critical values for the test statistic evaluated at the observed data.

It should be noted that the reference distribution for the test statistic is only an approximation to the true distribution, because it is computed with respect to a pooled estimate of Φ rather than the true rate curve and because normal errors have been assumed. Simulation methods using the estimated spline with normal errors have proven useful in approximating the distribution of the estimated smoothing parameter (Nychka 1991). This experience suggests that the simulated distribution of F^* may be a valid approximation to the distribution of the test statistic.

Using a form of the parametric bootstrap, "confidence" bands can be constructed for each rate curve, Φ_k, $k = 1, \ldots, M$, and for the difference between two rate curves, $\Phi_i - \Phi_k$, $i = 1, \ldots, M$, $k = 1, \ldots, M$, $i \neq k$. The idea is to treat the estimated rate curves as "truth," simulate data from these curves, estimate new rate curves from the pseudodata, and then construct confidence bands based on the distribution of the new rate estimates. Specifically, let $\hat{f}_k$ denote the depletion curve estimate corresponding to the rate curve estimate, $\hat{\Phi}_k$, from the kth experiment, $k = 1, \ldots, M$. Pseudodata is generated for $\hat{f}_k$ under the assumption that the measurement error is normally distributed with variance $\hat{\sigma}_k^2 = \mathrm{RSS}_k/(n_k - \nu_k)$. A new rate curve estimate is then computed from each generated set of data in exactly the same way as it was with the original data. This new rate curve estimate is a realization of the random curve, which we will denote as $\hat{\tilde{\Phi}}_k$. (Hereafter, we will omit the treatment subscript k unless it is necessary for clarity).

At concentration c_j, an approximate $(1 - \alpha)100\%$ pointwise confidence interval for $\Phi(c_j)$ is $\hat{\Phi}(c_j) \pm u_j$, where u_j is chosen so that $P(|\hat{\tilde{\Phi}}(c_j) - \hat{\Phi}(c_j)| \leq u_j) = 1 - \alpha$. To obtain m simultaneous confidence intervals, $\hat{\Phi}(c_1) \pm b_1$, $\hat{\Phi}(c_2) \pm b_2$, $\ldots$, $\hat{\Phi}(c_m) \pm b_m$, it is necessary to find $b_1, b_2, \ldots, b_m$ such that $P(\cap_{j=1}^m \{|\hat{\tilde{\Phi}}(c_j) - \hat{\Phi}(c_j)| \leq b_j\}) = 1 - \alpha$. One way to achieve $1 - \alpha$ as this minimum simultaneous coverage is to make use of Bonferroni's theorem. That is, choose b_j such that $P(|\hat{\tilde{\Phi}}(c_j) - \hat{\Phi}(c_j)| \leq b_j) = 1 - \alpha/m$. One disadvantage of this conservative approach is that a very large number of realizations of $\hat{\tilde{\Phi}}$ are needed to reasonably estimate extreme quantiles.

An alternative approach is to determine the widths for simultaneous coverage directly. Take $b_j = u_j D$, where D is the factor needed to inflate pointwise confidence intervals to achieve simultaneous coverage. D can be estimated directly from the simulated rate estimates. First, from N_1 realizations of $\hat{\tilde{\Phi}}$, we estimate u_j such that $P(|\hat{\tilde{\Phi}}(c_j) - \hat{\Phi}(c_j)| \leq u_j) = 1 - \alpha$ for $j = 1, \ldots, m$. Then, from N_2 independent realizations of $\hat{\tilde{\Phi}}$, we estimate D such that $P(\sup_{1 \leq j \leq m}\{|\hat{\tilde{\Phi}}(c_j) - \hat{\Phi}(c_j)|/u_j\} \leq D) = 1 - \alpha$. Simultaneous confidence intervals are then formed as $\hat{\Phi}(c_j) \pm u_j D, j = 1, \ldots, m$. Strictly speaking, simultaneous confidence intervals pertain to coverage only at a discrete set of points. But it is reasonable to connect the intervals to form a band, if the number of intervals is large and the underlying function is sufficiently smooth. Confidence bands for the difference between two curves, say $\Phi_i - \Phi_k$, are obtained in the same manner but with $\hat{\tilde{\Phi}}$ and $\hat{\Phi}$ being replaced by the differences, $\hat{\tilde{\Phi}}_i - \hat{\tilde{\Phi}}_k$ and $\hat{\Phi}_i - \hat{\Phi}_k$.

3. A SOLUTION DEPLETION EXPERIMENT

3.1 Background

An uptake experiment begins with an initial concentration, c_0, of the solution that surrounds the plant roots. The depletion period is kept as short as possible so that the capacity of the uptake system is not altered. Despite these controls, one question is whether the resulting uptake rate curve depends on the initial concentration. Suppose that Φ and Φ^* are rate curves resulting from depletion experiments based on initial concentrations c_0 and c_0^*, where $c_0 > c_0^*$. Under what circumstances will Φ and Φ^* coincide? Equality of these curves suggests that under these conditions the absorption mechanism does not depend on previous absorption of the nutrient. That is, when the root material has depleted the solution from c_0 to c_0^*, the subsequent nutrient uptake is the same as a solution depletion experiment with an initial concentration of c_0^*. Alternatively, a dependence of Φ on c_0 has both practical and biological implications. If the underlying rate curves are different, then one should not directly compare depletion results obtained at different initial concentrations. A biological implication is that the Michaelis–Menten model, which does not allow for a dependence of Φ on c_0, should not be regarded as a biologically based mechanistic model for ion absorption. In the next two subsections we describe and analyze solution depletion data for nitrate absorption by the roots of corn seedlings (8-day-old maize, P-3320), to study the effect of different initial concentrations on the shape of the uptake curve.

3.2 Experiment Design

Eight solution depletion experiments were carried out following a 4×2 factorial design. There were four initial concentrations, c_0 (50, 100, 200, and 500 μM), and two different nitrate exposure levels of the growing medium (low and high). The latter factor was included to study the dependence of the uptake rate on the nitrate level in the growing medium. The four initial concentrations were chosen because they correspond to what is typically observed in a plant's normal environment (Barber 1984). Each depletion was based on four plants in a container with 200 ml of nutrient solution. The depletions were carried out simultaneously under constant light conditions over an 8-hour period, and the solutions were sampled at 21 different times. The details of the actual experiment were planned and performed by Dr. William Jackson and coworkers in the Department of Soil Sciences, North Carolina State University.

Traditionally, the sampling times in a solution depletion experiment are chosen to be equally spaced. We designed the eight experiments to have unequally spaced sampling points, with more samples taken at the onset of the experiment. Typically, a depletion curve decreases rapidly at high

concentrations and changes relatively slowly at low concentrations. The disadvantage of equally spaced sampling points is that fewer observations are taken when the concentration curve is high and changing rapidly. Based on data from previous depletion experiments, we designed sequences of sampling points yielding measurements that were approximately equally spaced with respect to concentration rather than time. (The details of this procedure are in Meier 1990.)

3.3 Analysis of the Data

For each of the eight data sets displayed in Figure 1, we examined four different estimates of Φ obtained from the following depletion curve estimates:

1. unconstrained cubic smoothing spline with λ determined by generalized cross-validation, GCV (4)
2. unconstrained cubic smoothing spline with λ determined by adjusted generalized cross-validation, AdGCV
3. monotonic spline estimate defined at (3) with λ determined by adjusted cross-validation, AdCV (5)
4. parametric estimate based on the Michaelis–Menten model.

We define the AdGCV to be that in (4) where the sum is taken from 2 to $n - 1$ instead of from 1 to n.

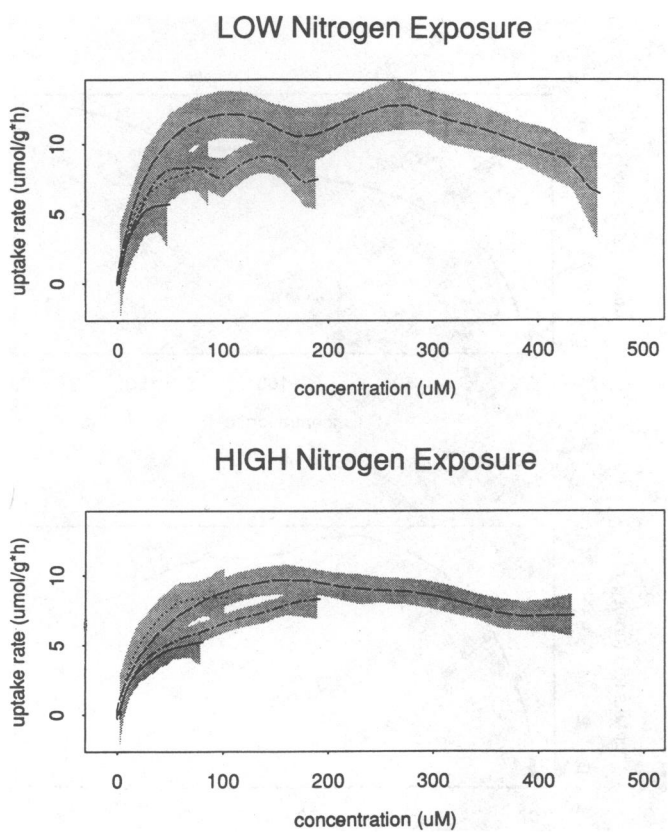

Figure 2. Estimated Rate Curves With 95% Confidence Bands. Each nonparametric rate curve estimate is based on an unconstrained estimate of the depletion curve. Lambda is the minimizer of GCV(λ). C_0 is the initial nitrate concentration of the ambient solution in μM. The uptake rate is given in units of μmol nitrate per gram root mass per hour. ———, C_0 = 50 μM; -----, C_0 = 100 μM; — · — · —, C_0 = 200 μM; — — —, C_0 = 500 μM.

Figure 2 plots the estimated rate curves (estimate 1) separated into the low and high nitrogen exposure group. All eight of the unconstrained depletion curve estimates were monotone at concentrations above 2.5 μM. (*Note:* 2 μM is the lowest concentration that can be reliably measured.) Figure 3 displays estimates 2 and 3. The sharp upward spike at the right boundary of some of the monotone estimates is most likely due to boundary effects. These boundary effects also appear in some of the simulation results discussed in Section 4. The loops in the unconstrained estimate at low concentrations are due to lack of monotonicity of the depletion curve. Besides these differences, the two estimates in Figure 3 have a similar appearance. But both estimates yield rougher estimates of the rate curve than those displayed in Figure 2. In particular, the rate estimates based on GCV are much smaller than those estimates based on AdGCV. This general phenomenon was not observed in the simulation study described in Section 4. For these particular data sets, we found that this difference was due to the fact that the minimum of both the GCV function and the AdGCV function occurred in relatively flat portions of the functions. Although the difference between the two functions was almost 0 in these flat portions, the actual minimizers (values of λ) differed by several orders of magnitude, resulting in different amounts of smoothing.

All three nonparametric rate curve estimates yielded some curves with bumps. One possible explanation for these bumps is spurious structure due to undersmoothing. That is, for this experimental data the lambda that minimizes the GCV function (or the AdGCV or AdCV function) underestimates the appropriate amount of smoothness necessary to yield a smooth rate curve. An alternative choice for lambda is the value such that the variance estimated from the smoothing spline estimate, $\hat{\sigma}_s^2$ (see Craven and Wahba 1979), is equal to or at least as large as an independent estimate of σ^2 (Reinsch 1967). For this experiment, an independent estimate of σ^2 is .04. All of the unconstrained rate estimates based on GCV—except for the case c_0 = 200 and LOW nitrate exposure—yielded estimates where $\hat{\sigma}_s^2$ was slightly larger than .04. For the one case where $\hat{\sigma}_s^2$ was less than .04, a new rate curve estimate was computed where lambda was chosen so that $\hat{\sigma}_s^2 \approx .04$. The resulting new estimate could not be distinguished from the original estimate. Thus the estimated rate curves appear to be in agreement with the expected amount of measurement error.

Given the proposed rate curve estimates, the goal is to determine whether there is evidence that the true underlying rate curves are different. Recall that a difference would suggest some dependence on the initial concentration. It is already known that the level of nitrogen exposure will have some influence on the uptake rates. The main interest is to determine whether there is a significant difference among the rate curves within each pretreatment group. We used the rate curve estimates corresponding to the unconstrained estimates with λ determined by generalized cross-validation as the basis for our comparison for two reasons. First, from the simulation study reported in Section 4, it was found that a monotonic estimate was less accurate (larger average squared error) than an unconstrained estimate. Second, the

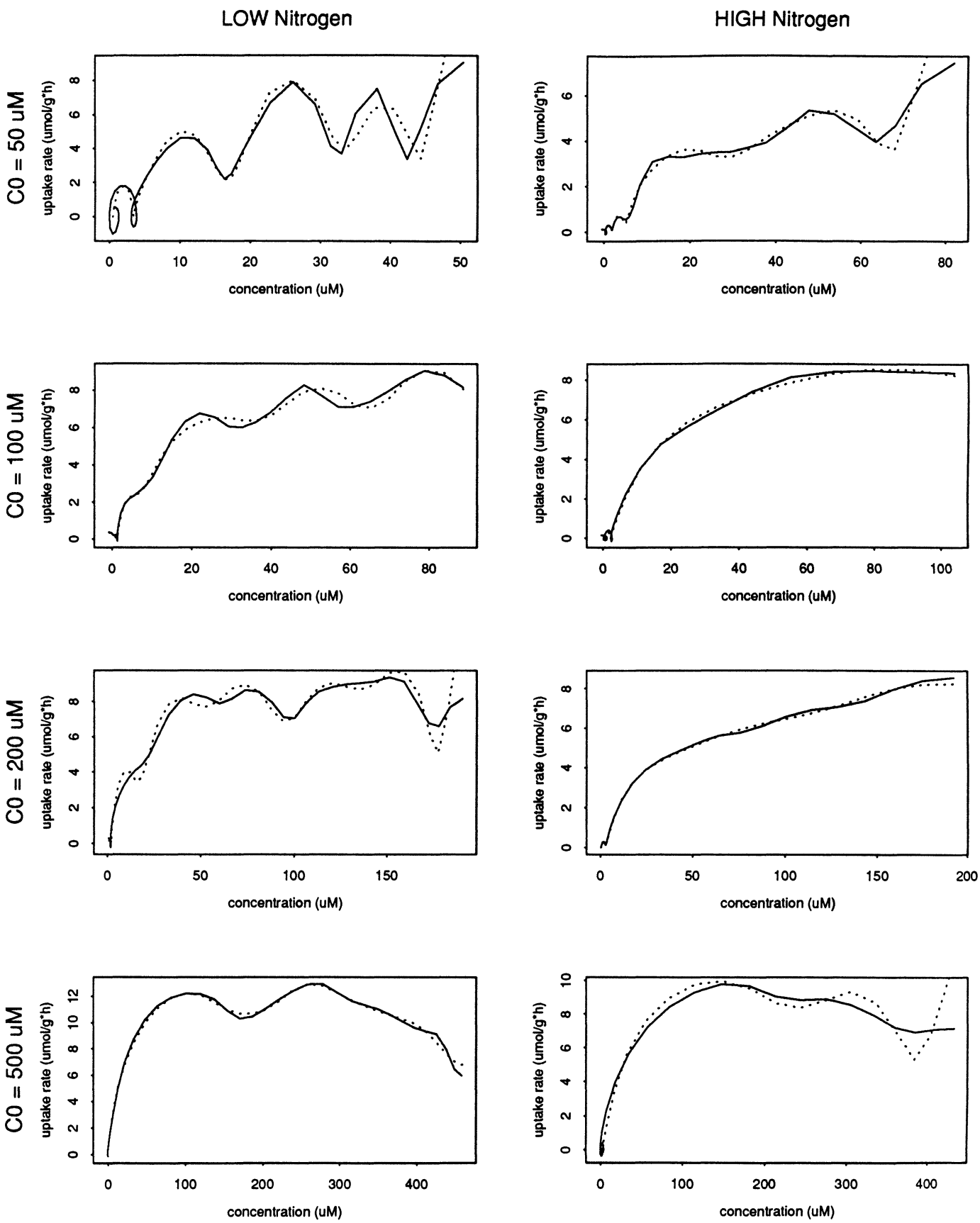

Figure 3. Comparison of Nonparametric Rate Curve Estimates Based on Unconstrained (Solid Line) and Monotone (Dashed Line) Estimates of the Depletion Curve. For the unconstrained estimates, lambda is the minimizer of AdGCV(λ); for the monotone estimates, lambda is the minimizer of AdCV(λ).

unconstrained estimates were actually monotonic over almost the entire range of concentrations. As the simplest hypothesis, then, the rate curves were compared over a restricted range of concentrations, where the lower bound was set to ensure the estimates were functions (2.5 μM) and the upper endpoint was the highest concentration common to all the experiments (50 μM) (see Fig. 4). We used the method described next to test for differences among these curves; the results are reported in Table 1.

For each null hypothesis, the distribution of the test statistic under the null hypothesis was calculated as described in Section 2.4, using 10,000 simulated data sets. In these simulated data sets, some of the depletion curves were not monotone over the concentration range of interest (2.5–50 μM) and, therefore, the corresponding rate curve estimates were not true functions. Because all eight depletion curves estimated from the observed data were monotone, we conditioned our test on those cases where all of the depletion curves needed to compute the test statistic were monotonic. That is, we generated the distribution of the random variable F^* conditional on the rate curves being monotonic. As a result, the actual number of samples used in calculating percentage points for the distribution of the test statistic varied between 5,462 and 8,770.

Perhaps the most important test result is evidence for significant differences (p value = .008) among the rate curves for the high exposure group of depletions. For both pretreatment groups, the rate curves tend to be ordered, with the higher initial concentrations yielding slightly higher rates (at the same solution concentration). The only departure in this pattern is the 100-ml depletion curve associated with the high nitrate pretreatment group. This group of four curves had a significantly smaller p value when testing for equality, and we were concerned about the dependence of this result on the unusually high curve estimate for the 100-ml depletion. A test was done with this rate curve excluded, and the results are also reported. We found that the p value for this reduced set of curves increases roughly by a factor of four, to .033.

Table 1. Results of Testing for Equality of Rate Curves Obtained From Different Initial Concentrations for a Particular Exposure Level

	Null Hypothesis			
	Low	*High*	*All*	*High, excluding c_0 = 100 μM*
p value[a]	.108	.008	.017	.033
% monotone[b]	87.7	54.6	62.2	61.3

[a] Approximate p value based on the simulated distribution of F^* conditional on monotone depletion curve estimates.

[b] The percent of simulated samples out of 10,000 that yielded monotone estimates for each treatment.

Besides testing the equality of the rate curves, we computed confidence bands for each rate curve and confidence bands for the difference of each pair of rate curves, using the method described in Section 2.4. Similar to the hypothesis test, these bands were constructed conditional on the simulated depletion curve estimates being monotone. That is, realizations of $\hat{\Phi}$ were discarded if the corresponding depletion curve was

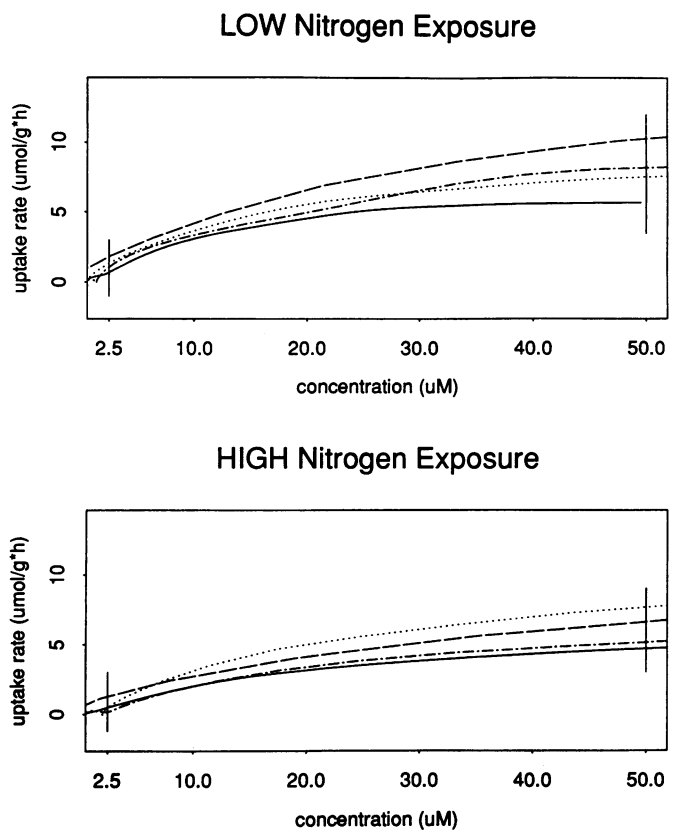

Figure 4. A Magnification of Figure 2. The vertical lines indicate the range where the test statistic at (6) was computed. ———, C_0 = 50 μM; -----, C_0 = 100 μM; — · — · —, C_0 = 200 μM; — — —, C_0 = 500 μM.

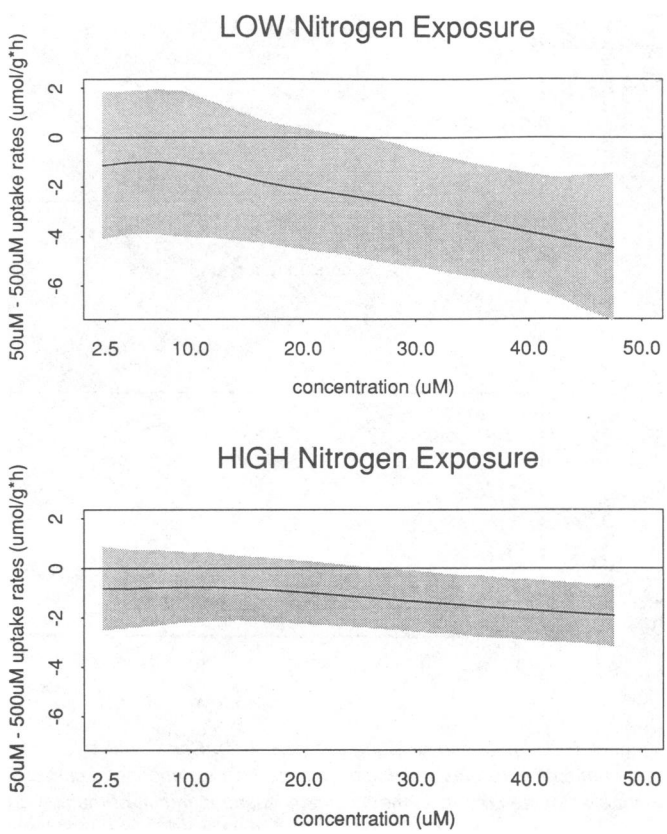

Figure 5. 95% Confidence Bands for the Difference Between the Two Rate Curves Corresponding to C_0 = 50 μM and C_0 = 500 μM.

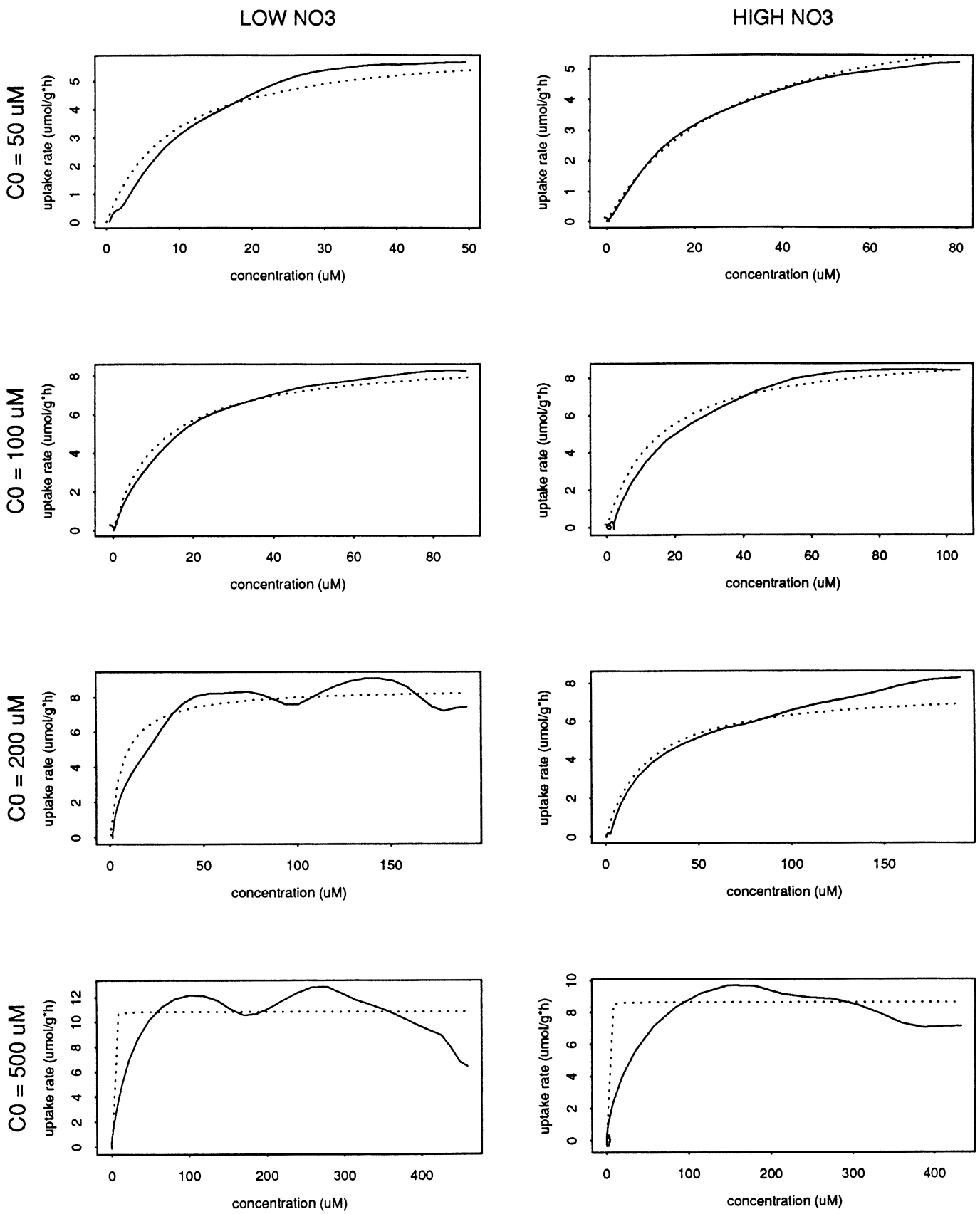

Figure 6. Comparison of Nonparametric Rate Curve Estimates (Solid Line) and Best Fit of the Michaelis–Menten Model (Dashed Line). Each nonparametric rate curve estimate is based on an unconstrained estimate of the depletion curve; lambda is the minimizer of GCV(λ). The best Michaelis–Menten model is the minimum least squares fit of the numerically integrated Michaelis–Menten model.

not monotonic. Confidence bands were formed by connecting simultaneous confidence intervals at 20 equally spaced concentrations and are based on two sets of 1,000 realizations ($N_1 = N_2 = 1{,}000$) from the conditional distribution of $\hat{\tilde{\Phi}}$. Some of the results are displayed in Figures 2 and 5. Note that the confidence bands for the difference between the two curves (Fig. 5) corresponding to $c_0 = 50$ and $c_0 = 500$ for both the low and high exposure groups do not always include 0, indicating that the curves are different. Our overall "F" test was only able to detect differences for the high-exposure group.

Historically, the Michaelis–Menten model has been used to describe the rate curve for ion absorption and to compare different rate curves. We have fit the Michaelis–Menten model to our data; the results are displayed in Figure 6. These plots indicate that there are some differences between the Michaelis–Menten fit and our nonparametric estimate. In particular, the rate curves for the highest levels of initial concentration appear to have much more structure than the sharp step suggested by the Michaelis–Menten estimate. It is also worth noting that for each of the eight treatments, the Michaelis–Menten estimate is consistently higher than the nonparametric estimate at lower concentrations and consistently lower than the nonparametric estimate at the higher concentrations. We have not specifically tested the adequacy of the Michaelis–Menten model.

4. SIMULATION EXPERIMENT

4.1 Objectives and Design

We conducted a simulation study to examine two aspects of estimating rate equations, using the smoothing spline estimator, $\hat{\Phi}_\lambda$. We were interested in the value of using cross-validation for determining λ and in the possible effects on the average squared error and smoothness of the rate curve when monotonicity constraints are imposed on the estimated depletion curve.

This study consists of 12 cases following a 4×3 factorial design with four test functions (listed in the Appendix) and three levels of σ (.02, .2, .5). The particular test functions (rate curves) used were chosen to be realistic for nitrate uptake by maize and the middle level of σ, $\sigma = .20$, is one that is typically observed in the laboratory for solution depletion experiments. For each of these 12 test cases, 240 samples of size 20 were simulated according to Model (1) with normal errors. For each of these samples, estimates 2 and 3 listed in Section 3.3 were calculated along with two other "optimal" estimates described later.

A global criterion to measure the closeness of the rate estimates to Φ is the integrated mean squared error, $\mathrm{IMSE}(\hat{\Phi}) = \int (\hat{\Phi}(u) - \Phi(u))^2\, du$. As an approximation to this integral, we computed the average squared error (ASE) to assess correctness of $\hat{\Phi}$, where

$$\mathrm{ASE}(\hat{\Phi}) = \frac{1}{m} \sum_{k=1}^{m} (\hat{\Phi}(u_k) - \Phi(u_k))^2, \qquad (7)$$

$u_k = \hat{f}(t_k)$, and $m = 500$. This quantity is an approximation to the IMSE divided by $(u_m - u_0)$. The grid points $\{t_k\}$, $1 \le k \le 500$ were chosen so that the *true* depletion curve would generate an equally spaced sequence of concentration values at these time points. In this manner $\hat{\Phi}(\hat{f}(t_k))$ is uniquely defined for all t_k and will be evaluated on a roughly equally spaced grid with respect to estimated concentration. The ASE was computed for both constrained and unconstrained rate estimates and is the basis for comparison among the estimators.

Finally, to calibrate the data-based methods of determining the smoothing parameter, the optimal choice for λ (with respect to ASE) was also calculated. These minimizations were carried out by a coarse grid search that was refined using a golden section minimization.

4.2 Results of Simulation Study

Figure 7 and Table 2 provide a summary of the simulation results pertinent to the analysis of the uptake experiment. The figure gives an overview of the accuracy of the rate curve estimates under the 12 cases and across four different estimates.

For data-based methods of estimating λ (labeled as gcv u and cv m), Figure 7 reveals that the distribution of the ASE's have roughly the same variability. One systematic difference, however, is that the median ASE associated with the data-based unconstrained estimate (gcv u) is smaller than the ASE for the constrained estimate (cv m). This pattern is also true for the rate curve estimates using the optimal value for λ. Here we see similar scatter, but the distribution of the ASE

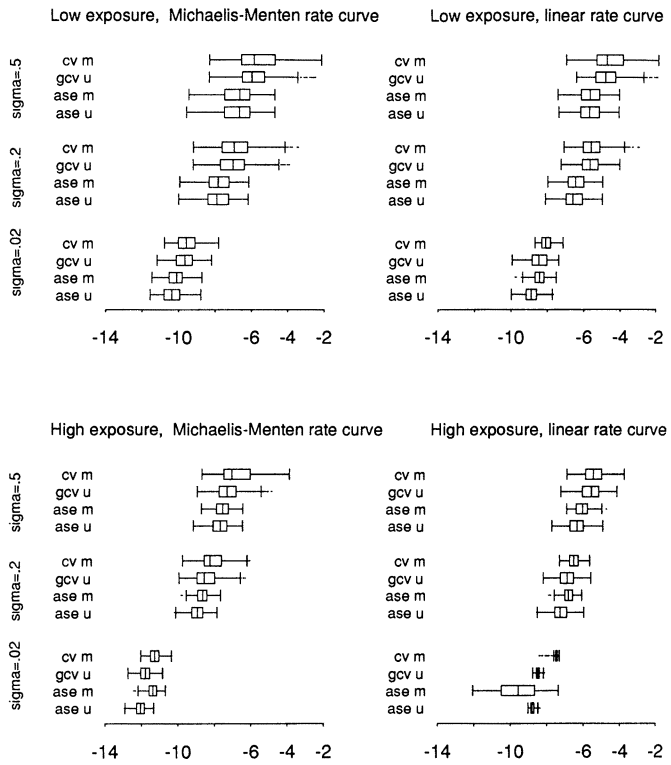

Figure 7. Simulation Results Comparing the Log Average Squared Error (ASE) of Different Nonparametric Estimates of Rate Curves. Boxplots of the log (ASE) of 240 rate curve estimates are displayed for each of four different types of estimates: unconstrained estimate, lambda is the minimizer of AdGCV (gcv u); unconstrained estimate, lambda is the minimizer of ASE (ase u); monotone estimate, lambda is the minimizer of AdCV (cv u); and monotone estimate, lambda is the minimizer of ASE (ase m).

Table 2. Simulation Results Comparing Several Nonparametric Estimates of the Rate Curve

Test function[a]	Value for λ	Median average squared error[d] Unconstrained	Monotone	Unconstrained/ monotone
M-M (LOW)				
σ = .02	CV[b]	.632e−4	.683e−4	.960
	Opt.[c]	.311e−4	.406e−4	.822
σ = .20	CV	.904e−3	.955e−3	.992
	Opt.	.368e−3	.396e−3	.958
σ = .50	CV	.257e−2	.292e−2	.993
	Opt.	.129e−2	.131e−2	.982
Linear (LOW)				
σ = .02	CV	.760e−5	.127e−4	.575
	Opt.	.579e−5	.114e−4	.516
σ = .20	CV	.193e−3	.263e−3	.757
	Opt.	.132e−3	.177e−3	.790
σ = .50	CV	.673e−3	.868e−3	.868
	Opt.	.460e−3	.527e−3	.885
M-M (HIGH)				
σ = .02	CV	.209e−3	.302e−3	.717
	Opt.	.135e−3	.214e−3	.686
σ = .20	CV	.346e−2	.369e−2	.993
	Opt.	.136e−2	.155e−2	.909
σ = .50	CV	.836e−2	.911e−2	.100
	Opt.	.344e−2	.351e−2	.978
Linear (HIGH)				
σ = .02	CV	.203e−3	.573e−3	.365
	Opt.	.151e−3	.698e−4	2.160
α = .20	CV	.102e−2	.147e−2	.714
	Opt.	.689e−3	.110e−2	.666
α = .50	CV	.383e−2	.426e−2	.918
	Opt.	.175e−2	.240e−2	.769

[a] Test functions are listed in the Appendix.
[b] Smoothing parameter is found by either AdGCV (unconstrained spline) or AdCV (monotone spline).
[c] Optimal choice of smoothing parameter are found by minimizing the average squared error (ASE).
[d] Median values of ASE's and ratios of ASE's are based on 240 simulation estimates per test function.

for the unconstrained estimate tends to be shifted to the left (or to lower values) compared to that for the constrained estimate. One case that stands out in these boxplots is the ASE for the high exposure linear rate curve with the optimal choice of λ under monotonicity constraints and σ = .02. When we examined plots of several of these rate estimates, we found that they did a poor job reproducing the true curve only at the boundaries. The corresponding unconstrained estimates did a poor job at only one boundary: the high concentrations. The estimates from the Michaelis–Menten curve did not have trouble at the boundaries.

Another important feature of these results is the increasing variability and skewness of the ASE for the data-based estimates when σ increases. Usually these poor estimates are associated with values for λ that substantially undersmooth the data. Table 2 lists the median values for these results and quantifies the general patterns apparent in Figure 7. For example, when σ = .2, estimating λ by AdGCV for the unconstrained estimate has the effect of inflating the square root of the median ASE in a range of 20 to 60%.

5. DISCUSSION AND CONCLUSIONS

Our analysis of a depletion experiment differs from that of most other researchers in that we do not assume that the rate curve must satisfy a particular parametric form. Using a nonparametric regression estimate of the rate curve, we suggest an inference procedure to detect differences between curves based on Monte Carlo simulation. We are not comfortable basing an inference on the historically used Michaelis–Menten model, because we feel that it does not adequately describe the data. It should be noted that if the nitrate uptake did follow Michaelis–Menten kinetics, then a *single* set of parameters should describe the uptake rate for all treatment groups. This is not the case, as the rate curves are shown to differ. If Michaelis–Menten models are used, then they really function as a convenient parametric family of curves rather than as a biologically based model for the underlying absorption process.

Several qualifications need to be made regarding these conclusions. One explanation of the observed differences is that over longer periods of depletion time, the capacity of the uptake system may actually be changing. Thus in these experiments the effect due to initial concentration is confounded with the length of the depletion experiment (see Fig. 1). Another issue is the lack of replication for this experiment. Systematic effects among containers cannot be estimated from the single depletion experiments done at each of the four initial concentrations. Another problem with these conclusions is the influence of the depletion with c_0 = 100 ml on the significance of the hypothesis test. Although there are possible scientific explanations for the unexpected ordering of the 100-ml rate curve relative to the others, it is also possible that this outcome was due to uncontrolled variables in the depletion experiment. For this reason, the p value associated with the high exposure group omitting this depletion is a more conservative measure of the significance. Note that in the low exposure group there is still some evidence for differences among the rates, based on the confidence bands for the difference between the c_0 = 50 and c_0 = 500 case.

One interesting result from the simulation study was the decreased accuracy associated with constrained estimates. Although one might argue that enforcing monotonicity adds more information to the estimate, this was not the case for the sample sizes and rate curves appropriate for depletion experiments. For our application, we found that the unconstrained estimates were monotonic over the most relevant range of concentrations; thus the need to enforce constraints was not necessary.

The simulation results also point to substantial variability in the individual estimates of λ. Evidence of this problem is the skewed distributions of ASE for the large values of σ in Figure 7. Some alternatives to cross-validation have been proposed that may have much less variability (e.g. Jones, Marron, and Park 1991). These procedures require detailed calculations of the asymptotic bias and variance of the curve estimate, however. Such formulas are difficult to derive for splines and may not be accurate due to the differences between cubic smoothing splines and second-order kernel estimators.

One basic objection to the formulation of the spline estimate is that the roughness penalty is associated with the depletion curve rather than the function of interest, Φ. It is

possible to formulate a variational problem that depends directly on the rate function. Let K be an operator such that $f = K(\Phi)$ will be the solution to the rate equation with $f(0) = c_0$. Under the assumption that c_0 is known, one might consider minimizing

$$\mathcal{L}(\Phi) = \frac{1}{n}\sum_{i=1}^{n}(y_i - K(\Phi)(t_i))^2 + \lambda \int_{[0,c_0]} (\Phi''(u))^2\, du \quad (8)$$

over all rate curves such that $\Phi \geq 0$, $\Phi(0) = 0$ and $\int_{[0,c_0]} (\Phi''(u))^2\, du < \infty$. In this way the roughness constraint is placed directly on the rate curve. Some of the problems with monotonicity arise for low concentrations, where the depletion curve is essentially following an exponential decay rate. One advantage of this formulation is that in the case of $\lambda = \infty$, Φ will be a linear function. If Φ is linear, then the corresponding depletion curve will be an exponential function and may provide a better representation of the data for low concentrations.

From a theoretical standpoint, this direct estimate of Φ may seem better suited for this problem. We did not pursue this method, however, because of numerical considerations. Besides the basic issue of whether a minimizer of (8) even exists, computation of the estimate would be formidable. Evaluating K at a particular Φ would require the numerical solution of the rate equation for f. In addition, it would be difficult to estimate the smoothing parameter, because this is a nonlinear problem. Given the rapid growth in computing power, however, we hope that such problems may eventually become feasible. Differential equations describing the rate of a process are a useful tool for understanding biological systems, and we believe that this is rich field for the application of nonparametric (and computer-intensive) methods.

APPENDIX: RATE CURVES USED IN THE SIMULATION STUDY

Observations were generated from the depletion curves corresponding to the following four rate functions:

$$\Phi(u) = \frac{12 \cdot u}{40 + u}, \qquad c_0 = 50 \quad \text{(Michaelis–Menten [LOW])}$$

$$\Phi(u) = \frac{12 \cdot u}{40 + u}, \qquad c_0 = 500 \quad \text{(Michaelis–Menten [HIGH])}$$

$$\Phi(u) = -.076 \cdot u, \qquad c_0 = 50 \quad \text{(Linear [LOW])}$$

$$\Phi(u) = .017 \cdot u, \qquad c_0 = 500 \quad \text{(Linear [HIGH])}.$$

To obtain the corresponding depletion curve, for each of these rate curves the first-order system $\Phi(f) = -f', f(0) = c_0$ was solved for f. The Michaelis–Menten equations do not have an explicit, closed-form solution; thus they were solved numerically, using fourth-order Runge–Kutta methods (Boyce and DiPrima 1969). The numerical solution consisted of the pairs $(x_h, f(x_h))$, where $c_0 = x_0 \leq x_1 \leq \cdots \leq x_n$, and the step size, $x_{h+1} - x_h$, was chosen to be less than or equal to .01. The solution was evaluated up to x_n where $f(x_n)$ was less than or equal to 2 for the first time. This corresponds to a solution depletion experiment where the solution is sampled until its concentration falls below 2 uM. To obtain 20 time points, $\{t_k, 1 \leq k \leq 20\}$, that yield equally spaced $\{f(t_k\}, 1 \leq k \leq 20\}$, the following method was used. An interpolating spline was fit to the "inverse" data $\{(f(x_h), x_h)\}$, and then this interpolating spline was evaluated at 20 equally spaced points, $\{f(t_k), 1 \leq k \leq 20\}$. The result was a grid of unequally spaced time points, $\{t_k, 1 \leq k \leq 20\}$.

Unlike the Michaelis–Menten equations, the linear equations can be solved explicitly as $f(t) = 50\, e^{-.076t}$ (LOW) and $f(t) = 100\, e^{-.017t}$ (HIGH). These particular exponential functions were chosen because they minimized the residual sum of squares between the function and the numerical solution to the corresponding Michaelis–Menten rate equation. The 20 time points that yield equally spaced $\{f(t_k), 1 \leq k \leq 20\}$ were found directly from the exponential form.

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