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A TRANSFORMATION/WEIGHTING MODEL FOR ESTIMATING MICHAELIS–MENTEN PARAMETERS

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ABSTRACT

There has been considerable disagreement about how best to estimate the parameters in Michaelis–Menten models. We point out that many fitting methods are based on different stochastic models, being weighted least squares estimates after appropriate transformation. We propose a flexible model which can be used to help determine the proper transformation and choice of weights. The method is illustrated by examples.

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SECTION 1: Introduction

The Michaelis–Menten relation between a response y and a predictor x is

$$y = Vx/(K+x) = \{\alpha_0 + \alpha_1/x\}^{-1}, \tag{1.1}$$

where $\alpha_0 = 1/V$ and $\alpha_1 = K/V$. The model applies at least approximately in a variety of biological and biochemical situations, see Cressie and Keightley (1981), Currie (1982), Johansen (1984) and Ruppert and Carroll (1985). In fisheries research, (1.1) is called the Beverton–Holt (1957) spawner–recruit model.

There is considerable debate as to how one can best estimate the parameters in model (1.1). There would certainly be less controversy if the error structure of the data were known, that is, if it were known to what degree the errors were skewed or heteroscedastic. However, before analysis of the data, the error structure is rarely, if ever, known precisely, and often is not known even approximately. In this paper we describe a method for identifying the error structure from the data. We analyze several examples and perform bootstrap simulations. Our method worked rather well in the simulations. It appears to provide more precise estimates of the Michaelis–Menten parameters than other available methods. First, we review some of these methods.

Since equation (1.1) is a hyperbola, a nonlinear approach could be used (Bliss and James (1966)). However, (1.1) can be linearized in a number of ways; the simplest method is the double reciprocal or Lineweaver–Burk transformation (Lineweaver and Burk (1934)):

$$1/y = \alpha_0 + \alpha_1/x; \tag{1.2}$$

regress 1/y on 1/x using least squares to estimate (α_0,α_1). Currie (1982) states that "virtually all of the papers examined in the *Journal of Biological Chemistry* used the Lineweaver–Burk double–reciprocal transformation". We have also found this transformation to be widely used. However, its frequent use appears due to tradition and simplicity rather than any claims of statistical efficiency.

A second common method is the Woolf linearizing transformation (Haldane (1959)), which multiplies through by x in (1.2), leading to the model

$$x/y = \alpha_1 + \alpha_0 x \,. \tag{1.3}$$

Another linearizing transformation due to Scatchard (1949) has been used heavily in the analysis of hormone–receptor assays. The Scatchard model is

$$y/x = (1/\alpha_1) - (\alpha_0/\alpha_1)y + \sigma\epsilon. \tag{1.4}$$

A fitting method not based on a linearization is the nonparametric direct plot of Cornish–Bowden and Eisenthal (1974). See also Eisenthal and Cornish–Bowden (1974), Dalgard and Johanson (1987), and Cressie and Keightley (1979).

There have been numerous studies in the literature to determine *the* best way to estimate parameters of the Michaelis–Menten relation (Dowd and Riggs (1965); Atkins and Nimmo (1975); Thakur, Jaffe, and Rodbard (1980); Currie (1982); Zivin and Waud (1982)). In many of these investigations the authors *artificially* generate data homoscedastic on the Scatchard or Nonlinear transformed scales. Little wonder that the Lineweaver–Burk and Woolf parameter estimators perform poorly in these studies, while the Scatchard and Nonlinear parameter estimators cannot help but be superior. For example, Currie (1982) uses homoscedastic errors and only one type of heteroscedastic errors in his study and concludes that "The Woolf transformation, the best among the linearizing transformations, ... provides unreliable parameter estimates" and therefore "Transformations should not be used", although he adds the qualifying statement "except in case where they stabilize the error". Such arguments are logically incomplete, since they include no evidence that the Monte Carlo sampling situations reflect typical error stuctures in practice. We do not criticize Currie for not presenting such evidence. There has been no well–understood methodology for studying error structure, though our aim here is to provide one.

It seems to us that the proper fitting method necessarily depends on the particular subject area, experiment, and underlying distribution. No general conclusions about a fitting method can or should be made without taking these considerations into account.

Fitting $(1.1) - (1.3)$ by unweighted least squares should be seen as a consequence of fitting different stochastic models for the data. If ϵ has mean zero and variance one, fitting $(1.1) - (1.3)$ is a consequence of assuming the models, respectively

(1.5) Nonlinear: $\quad y = \{\alpha_0 + \alpha_1/x\}^{-1} + \sigma\epsilon\,;$

(1.6) Lineweaver–Burk: $\quad y = \{\alpha_0 + \alpha_1/x + \sigma\epsilon\}^{-1}\,;$

(1.7) Woolf: $\quad y = \{\alpha_0 + \alpha_1/x + \sigma\epsilon/x\}^{-1}.$

Some empirical studies have attempted to find a "typical error structure". Storer, Darlison and Cornish–Bowden (1975) present real–life examples for which the standard deviation of y appears to be proportional to the mean or a power of the mean. This agrees with the experience of Zivin and Waud (1982) and an anonymous referee who report that a constant coefficient of variation (c.v.) is a common occurence. However, in the examples we examine there appears to be no "typical" error structure. Rather, the nature of the errors varies considerably between experiments.

What is needed is a flexible model, one that incorporates many possible errors structures, each structure determined by parameters that can be estimated from the data. The main purpose of this article is to describe the use of such a model. The three models $(1.4) - (1.6)$ are special cases of a simple generalization to the transform–both–sides (TBS) approach of Carroll and Ruppert (1984, 1987, 1988); see also Bates, Wolf, and Watts (1985) and Snee (1986) for nice applications of this technique. Define the usual Box–Cox (1964) power transformations:

$$v^{(\lambda)} = (v^{\lambda} - 1) \,/\, \lambda \qquad \text{for } \lambda \neq 0\,;$$

$$v^{(\lambda)} = \log(v) \qquad \text{for } \lambda = 0\,.$$

The transform–both–sides/power–of–x (TBS/PX) Michaelis–Menten Model is

(1.8) $$y^{(\lambda)} = \{Vx/(K+x)\}^{(\lambda)} + \sigma\, x^{\theta}\, \epsilon.$$

The name "transform–both–sides" is used because the same power transformation has been applied to both sides of the equation (1.1). "Power–of–x" refers to the errors in (1.8); the ϵ's are assumed to be (approximately) independent standard normal random variables, so

the errors (after the transformation) have a standard deviation proportional to a power of x. By choosing $\lambda = 1$ and $\theta = 0$, we obtain the Nonlinear model (1.4). The Lineweaver–Burk model (1.5) sets $\lambda = -1$, $\theta = 0$, while the Woolf model (1.6) sets $\lambda = \theta = -1$. The ordinary TBS model (which is TBS/PX with $\theta = 0$) is appropriate when the standard deviation of y is proportional to a power of the mean; this, of course, includes the constant coefficient of variation model where that power is 1. The TBS/PX model allows the standard deviation of $y^{(\lambda)}$ to depend on x. Table 1 gives the mean–standard deviation relationship for the TBS/PX model and selected submodels.

In section 2, it will be demonstrated that $\lambda = 0$, $\theta = 0$ is approximately the Scatchard model. The combination $\lambda = 0$, $\theta = 0$ is the logarithmic transformation which stabilizes the variance of data with a constant coefficient of variation (Bartlett (1947)).

The purpose of this paper is to illustrate the scope and flexibility of the TBS/PX model. In section 2, we discuss properties of the model and methods of fitting it. In section 3, we present examples. In section 4 a number of related issues, including robustness, are discussed. Section 5 is a summary.

SECTION 2: The Transform–Both–Sides/Weighting Model

We consider a slight generalization of model (1.8), one which permits any nonlinear regression model and a wider variety of models for the standard deviation. The transform–both–sides/weighting model is

$$y^{(\lambda)} = f(x,\beta)^{(\lambda)} + \sigma\, g(x,\theta)\, \epsilon. \tag{2.1}$$

Here $f(x,\beta)$ is the regression function, $g(x,\theta)$ is called the standard deviation function, and (λ,β,θ) are unknown parameters to be estimated. The error ϵ is assumed to be approximately normally distributed with mean zero and variance one. In (2.1), the standard deviations could also depend on the mean, but we do not discuss this refinement.

To make the identifications with (1.8), we have

$$\beta = (V,K)\ ;\ f(x,\beta) = Vx/(K+x)\ ;\ g(x,\theta) = x^{\theta}.$$

Assuming only symmetry of ϵ, under this model y has median $f(x,\beta)$ independent of the values of λ and θ. Snee (1986) has argued that for heavily skewed data in nonlinear regression, it is more natural to model the median rather than the mean, with the transformation taking care of skewness and some of the heteroscedasticity in the data.

The power transformation of y to $y^{(\lambda)}$ has two purposes, to remove skewness in y and to remove the dependence of the standard deviation of y upon the mean of y. As shown in Carroll and Ruppert (1984), the removal of skewness is only possible when the data are highly scattered, i.e., σ is large. For small σ, the transformation is nearly linear in ϵ (given x), so it has little effect on distributional shape. Removing the dependence of the standard deviation upon the mean is possible whether σ is large or small. In our experience, spawner–recruit data such as example (3.1) are in the "large σ" class while enzyme kinetic and hormone–receptor data are in the "small σ" category.

Alternatively to (2.1), one might choose to model the mean–variance relationship directly, i.e., build a heteroscedastic nonlinear regression model without transformation. When σ is small, the two approaches are approximately the same. In this instance, the mean and median nearly coincide, skewness is little affected by transformation, and y has approximate standard deviation $\sigma g(x,\theta)f(x,\beta)^{1-\lambda}$. Hence, an approximation to the TBS/weighting model is the heteroscedastic model

$$E(y) = f(x,\beta);$$

(2.2)

$$SD(y) = \sigma g(x,\theta)f(x,\beta)^{1-\lambda}.$$

One can see from (2.2) that when $g(x,\theta) = x^{\theta}$, then SD(y) is proportional to the product of some power of f and another power of x. In the ordinary TBS model $\theta = 0$, and, as mentioned in section 1, SD(y) is proportional to a power of the mean; see Table 1.

The model (2.2) offers some insight when $g(x,\theta) = x^{\theta}$. Unless the range of x is wide, $f(x,\beta)$ given by (1.1) is approximately proportional to a power of x, so that θ and λ are difficult to distinguish. In most of the examples we have studied, we have found that the confidence regions for (λ,θ) are fairly long ellipses, but sufficiently narrow as not to include all three of the Nonlinear, Woolf and Lineweaver–Burk models; see Table 3.

The Scatchard model, (1.4), is strictly speaking not a member of the family of models (1.8), but an equivalent way to write (1.4) is

$$y = \{\alpha_0 + \alpha_1/x\}^{-1} (1 + \sigma\alpha_1\epsilon), \text{ for which} \tag{2.3}$$

$$SD(y) \propto E(y), \tag{2.4}$$

which is the constant coefficient of variation model. (Switching the two axes so that y is now plotted against y/x is another variant sometimes called the Eadie–Hofstee transformation.) Thus from (2.2), the TBS/PX model (1.8) with $\lambda = 0$, $\theta = 0$ approximates to the Scatchard and Eadie–Hofstee models.

Zivin and Waud (1982) advocate the Eadie–Hofstee estimate because they believe that (2.4) often holds in practice. Because the errors in (2.3) are multiplicative the Scatchard estimator is biased. The Eadie–Hofstee estimator is also biased, though not in the same way. See Zivin and Waud (1982) for further discussion of bias. Using the approximation $\log(1 + \sigma\alpha_1\epsilon) \cong \sigma\alpha_1\epsilon$, we see that the log transformation changes (2.3) to the TBS model with additive errors:

$$\log(y) = -\log(\alpha_0 + \alpha_1/x) + \sigma^*\epsilon, \text{ where } \sigma^* = \alpha_1\sigma.$$

Assuming the transformation achieves approximate normality, the log–likelihood for model (2.1) is given up to an additive constant by

$$\ell(\lambda,\theta,\beta,\sigma) = \sum_{i=1}^{N} \left[(\lambda-1)\log(y_i) - \log\{\sigma\, g(x_i,\theta)\} \right] - \sum_{i=1}^{N} \left[\{y_i^{(\lambda)} - f(x_i,\beta)^{(\lambda)}\} / g(x_i,\theta) \right]^2 / \{2\sigma^2\}.$$

When maximizing $\ell(\lambda,\theta,\beta,\sigma)$ by Fisher's scoring method or similar numerical techniques, occasionally σ becomes negative, especially with poor starting values of the parameters. There are several remedies, but the easiest is to maximize ℓ over σ analytically. For any fixed values of λ, θ, and β, ℓ is maximized in σ^2 by

$$\hat{\sigma}^2(\lambda,\theta,\beta) = N^{-1} \sum_{i=1}^{N} \left[\{y_i^{(\lambda)} - f(x_i,\beta)^{(\lambda)}\}/g(x_i,\theta) \right]^2,$$

and if $\hat{\sigma}(\lambda,\theta,\beta)$ is substituted for σ into $\ell(\lambda,\theta,\beta,\sigma)$ the result is

$$\ell_{max}(\lambda,\theta,\beta) = \sum_{i=1}^{N} \left[(\lambda - 1) \log (y_i) - \log \{\hat{\sigma}(\lambda,\theta,\beta) g(x_i,\theta)\} \right] - N/2. \tag{2.5}$$

One can maximize (2.5) by a general maximum likelihood program; we use a slight modification of MAXLIK in the GAUSS package. Initial values of the parameters (to start the iterations) can be obtained from a linearized model such as the Woolf–transformed Michaelis–Menten equation.

Those without access to a general optimization program are referred to Carroll and Ruppert (1988, chapter 5) and Giltinan and Ruppert (1987) where the fitting of transformation and weighting models by nonlinear regression software is discussed; the latter paper includes sample SAS programs. A grid search method was advocated formerly (Ruppert and Carroll (1985)), but now seems more cumbersome than direct optimization, but is useful because it produces a confidence region for (λ,θ); see section 3.1 for an example.

For hypothesis testing we used the following approach. Consider a model M_1 and a submodel M_2 with, respectively, k_1 and k_2 unconstrained regression, transformation, and variance parameters. For example, $R_1 = 4$ and $R_2 = 2$ if M_1 is the TBS/PX Michaelis–Menten model and M_2 is the Nonlinear Michaelis–Menten model. Let Λ be the likelihood ration, i.e., the ratio of density maximized over M_1 to the density maximized over M_2. The usual α–level large–sample test rejects if $2 \log \Lambda$ exceeds the $(1-\alpha)$ quantile of the χ^2 distribution with $(k_1 - k_2)$ degrees of freedom. In bootstrap

experiments we found this test too liberal. Therefore, we suggest an approximate F–statistic,

$$F_A = \frac{n - k_1}{k_1 - k_2} \{\Lambda^{2/n} - 1\},$$

where n is the sample size. M_2 is rejected at nominal level α if F_A exceeds the $(1 - \alpha)$ quantile of the F–distribution with $(k_1 - k_2)$ and $(n - k_1)$ degrees of freedom. It is well–known (e.g., Scheffé (1959), section 2.5) that this is an exact test for linear models with no transformation and regression parameters. Our bootstrap simulations indicate that the approximate F–test has an actual level close to α.

SECTION 3: Examples

In this section, we analyze two examples. The analyses are meant to be illustrative rather than complete investigations.

In the discussion of these examples we shall be concerned with testing which of the special submodels of TBS/PX, viz. Nonlinear, Lineweaver–Burk, Woolf, and Constant c.v. (approximate Scatchard), fit the data. In practice it is not necessary to use TBS/PX to select one of the models. It is much simpler to estimate the Michaelis–Menten parameters directly by TBS/PX, and that is our recommendation. Our intention here in testing these models is to see what types of error structures typically occur in practice. Our finding is that the submodels fitting the data vary substantially among and even within subject areas.

EXAMPLE 3.1: Skeena River Sockeye Salmon

The data in Table 2 and Figure 1, taken from Ricker and Smith (1975), concern the relationship between x = number of spawners and y = total return (or number of recruits) for Skeena River sockeye salmon. More specifically, when salmon return each year to their native river to spawn, x is the number of returning fish minus the catch in that

year, i.e., the fish that are available for spawning. The variable y is the number of fish spawned in that year that return in later years to spawn. Most Skeena sockeyes return in four or five years. The data, expressed in thousands of fish, are given for the years 1940 – 1967. We have deleted the observations from 1951 and 1955 that were affected by a rockslide in 1951 (Ricker and Smith 1975); the small return in 1955 is a consequence of the 1951 slide.

A possibility which we have considered but tentatively rejected is dependencies in the errors. Clearly the size of the spawner population and therefore the recruitment each year depends strongly on recruitment four and, to a lesser extent, five years earlier. However, the residual autocorrelation function (not reported here) shows no evidence that the errors in the model are dependent. Only independence of the errors is needed to justify the likelihood analysis of section 2.

The heteroscedasticity in the data is clearly evident in Figure 1. To identify a model for this heteroscedasticity, equation (1.8) was fit to the data and the maximum likelihood estimates for (λ,θ) were (0.34, 0.77); see Tables 3 and 4. Notice that for the Nonlinear, Woolf, and Lineweaver–Burk models, respectively, the approximate F–tests have observed significance levels of 0.108, .0286, and .0004, each F–test being based on 2 and 22 degrees of freedom. The approximate F–tests agree with informal residual analysis. The residuals are defined as

$$\left\{ y_i^{(\hat{\lambda})} - [(\hat{V}x_i/(\hat{K}+x_i)]^{(\hat{\lambda})} \right\} / x_i^{\hat{\theta}}$$

Here $\hat{\lambda}$ and $\hat{\theta}$ can be fixed values, say for the Lineweaver–Burk scale, or estimated values for the TBS/PX model. Deviations of the residuals from homoscedasticity and normality indicate that the assumed model for the error structure is inadequate. The Spearman rank correlation between the absolute studentized residuals and the predicted values is 0.60 for the Woolf model, and 0.50 for the Nonlinear model, and the residual plots show strong

heteroscedasticity. The Lineweaver–Burk residuals show no pattern of heteroscedasticity, but are noticeably left–skewed.

To obtain a confidence region for (λ,θ), the TBS/PX model was fit with λ and θ fixed at each point on the grid $\lambda,\theta = -1\ (.2)\ 1$. Values of (λ,θ) accepted by a .05–level likelihood ratio test are in the region; see Table 3. Of course, many choices of (λ,θ) adequately explain the skewness and heteroscedasticty in these data, and no choice is perfect. However, $\lambda = \theta = .5$, $(F_A = .5)$ and $\lambda = \theta = 1$ $(F_A = 1.0)$ both seem adequate.

The choice $\lambda = \theta = 1$ has the feature of modeling heteroscedasticity without data transformation, which some will find an advantage. The residual plot for this model is given in Figure 2 and shows no indication of model inadequacy. In this model, the standard deviation is proportional to the number of spawners, which changes by a factor of about 4 over the observed data. Alternatively, one might model the variance as proportional to a power of the mean, see Carroll and Ruppert (1988, Chapter 3 and example 6.4.1).

A common assumption in stock–recruitment analysis is multiplicative lognormal errors which imply that the log transformation $(\lambda = 0,\ \theta = 0)$ is correct. This assumption seems acceptable $(F_A = 1.5)$.

Estimates of the regression coefficients are given in Table 6 for two fitting methods, TBS/PX and Nonlinear. The two parametrizations, (V,K) and (α_0,α_1), are compared. Judging from the standard errors, $\hat{V}$ and $\hat{K}$ seem unstable, particularly the former. Since $\hat{V}$ is a highly nonlinear function of $\hat{\alpha}_0$, curvature appears the culprit. In Figure 1 one sees that most of the data are in the x region where the mean response is well below its asymptote, V. This may explain why V is particularly poorly determined in this example. In fisheries analysis, the (α_0,α_1) parametrization is as useful as (V,K), and inference is best made about (α_0,α_1). In enzyme kinetics and hormone–receptor studies, V and K are the parameters of interest, and then inference based on standard errors and pivotal

confidence intervals is risky. Profile likelihoods (Bates and Watts 1988) or bootstrap confidence intervals (Efron 1987) are preferred.

A small bootstrap of 200 simulations was performed, using the TBS/PX maximum likelihood estimates of V, K, λ, and θ as the "true parameters". The errors were generated by sampling with replacement from the set consisting of the residuals from TBS/PX plus these residuals multiplied by -1. (The latter refinement guarantees that the bootstrap error distribution is perfectly, rather than merely approximately, symmetric.) The bootstrap standard errors for $\hat{V}$ dramatically illustrate the instability mentioned above, but they are somewhat misleading. The bootstrap sampling distribution of $\hat{V}$ is extremely heavy–tailed, and the standard errors reflect the outliers, not the typical deviations of $\hat{V}$ from the true parameter. Bootstrap confidence intervals will not be heavily influenced by these outliers, unless the confidence coefficient is nearly equal to 1. The sampling distributions of $\hat{\alpha}_0$ and $\hat{\alpha}_1$ are not heavy–tailed, but $\hat{\alpha}_0$ can get near to zero which induces outliers in $\hat{V}$.

The bootstrap biases and standard errors show that TBS/PX provides less variable estimates of α_0 and α_1 than nonlinear least–squares. Neither method has appreciable bias.

This is evidence that the TBS/PX can not only identify error structure, but also produces more precise estimates of the regression parameters than nonlinear least squares.

In Table 7 the bootstrap performance of approximate F–tests is shown. The probabilities of rejection are not estimated precisely with only 200 simulations, yet some conclusions can be drawn. The tests seem somewhat too liberal. The approximate F–tests perform better for the kinetic data below, even though that data has fewer observations and more parameters. Perhaps the problem here is that the Skeena data are not optimally "designed" for estimating the Michaelis–Menten parameters. The Nonlinear model is usually rejected in the simulations, even using level .01.

EXAMPLE 3.2: An Enzyme Kinetics Study

Data set #1

Table 8 shows the data from three enzyme kinetics experiments performed at the Becton Dickinson Research Center at Research Triangle Park, NC. The independent variables are substrate concentration, S, and inhibitor concentration, I. The response, v_o, is the initial velocity. The postulated model is the Michaelis–Menten equation

$$v_o = (V_{max}\, S) \,/\, (K_m(I) + S), \tag{3.1}$$

where V_{max} is the maximum velocity and $K_m(I)$ is the Michaelis parameter which depends on I. It is believed that the binding is competitive so that V_{max} is independent of I. Although there are parametric models for the dependence of $K_m(I)$ on I, we will use the model with a separate value of K_m for each level of I. Throughout the discussion of the three Becton Dickinson data sets, "Michaelis–Menten Model" will mean this model with a common V_{max} and separate $K_m(I)$.

Here we discuss the first data set in detail. Because the other two data sets exhibit some deviation from the Michaelis–Menten model, they are more difficult to analyze and will be discussed separately.

The data are plotted in Figure 3. The data at $S = 400$ show some deviations from the hyperbolic model, but these deviations show no systematic trend across the four levels of inhibitor and appear due to heteroscedasticity.

The TBS/PX model and various submodels were fit to the data set, and the results are summarized in Tables 4 and 5.

Residual analysis indicates that the TBS/PX Michaelis–Menten model fits BDRC #1 very well. The residuals appear symmetric and homoscedastic; in particular there is no evidence that the residual variance depends on S, I, or the mean of v_o. The TBS/PX model is not designed to remove a dependence of the residual variance on I. It is merely fortuitous that no dependence is present. However, if the variance had appeared to depend on I, we could have modeled this within the TBS/PX framework by allowing σ in

(1.8) to depend on I. Residual analysis also supports the assumption that V_{max} is independent of I, and which we shall test formally below.

From Table 4 one sees that $\hat{\lambda}$ is almost equal to 1. The PX model ($\lambda = 1$) fits extremely with $\hat{\theta}$ nearly .5. In fact, the simple model, $\lambda = 1$, $\theta = .5$ provides an excellent fit, indistinguishable from the fit to TBS/PX.

An advantage of TBS/PX over nonparametric estimation is the ease with which more complex models can be fit and tested. As an illustation we test whether the assumption of a common V_{max} value is reasonable for BDRC #1. This is done by fitting the model with separate values of V_{max} for I = 0, 3, 10, and 30. The extra three parameters only increase the log–likelihood by 1.43, and the approximate F–statistic is .48 with 3 and 9 degrees of freedom (p = .70), which strongly supports the hypothesis that V_{max} is independent of I.

The Lineweaver–Burk plot, Figure 4, shows I = 30 as the inhibitor concentration with intercept differing most from the others. Thus, a more powerful test might be obtained by comparing the common intercept model with a model having a common intercept for I = 0, 3, and 10 and a separate intercept for I = 30. However, since the log–likelihood ratio will be at most 1.43, F_A can be at most 1.78 (p = .21).

A graphical check of the common–intercept assumption uses a Lineweaver–Burk plot and inspects whether the intercepts $(1/V_{max})$ of v_o^{-1} versus x^{-1} are independent of I. However, it is important that this informal check be supplemented by a rigorous test procedure such as the likelihood ratio test. Graphical tests suffer from a common difficulty; in cases that are not clear–cut, there is no p–value or other formal test statistic to help decide among the hypotheses. In this example, the Lineweaver–Burk plot alone is insufficient for deciding whether the lines have a common intercept. In particular, the heteroscedasticity of the Lineweaver–Burk plot for this data set makes interpretation difficult.

One can, of course, test for common intercepts by F–tests on the Lineweaver–Burk scale. However, such tests are based on the assumption of homoscedasticity on this scale and are invalid, in fact drastically so in this case. The test of common intercepts versus separate intercepts has $F = 2.65$, $p = .1006$. The test of common intercepts versus a separate intercept for $I = 30$ gives $F = 7.05$, $p = .0065$. These results are very different than those for the approximate F–test using the TBS/PX model, and dramatically illustrate the danger of using tests based on untested assumptions.

In Figure 4, one sees that the apparently anomalous intercept for $I = 30$, is due to a single outlying high–leverage point, which is indicated by an arrow. The outlier was induced by the reciprocal transformation; this case is outlying neither on the original nor the TBS/PX scale.

In Table 9 the estimates and their standard errors are given for V_{max} and $K_m(I)$ for BDRC #1. The estimates are given for the TBS/PX model and selected submodels. The standard errors are calculated from Huber's (1967) asymptotic theory of the maximum likelihood estimator under model misspecification, so they are consistent even under submodels not fitting the data. The standard errors show that using a slightly nonfitting model such as Nonlinear, results in a noticeable loss of efficiency, and using a poorly fitting model such as Lineweaver–Burk can be disastrous.

We performed a small bootstrap of 200 simulations, and the results are given in Table 9. In the simulation, the true values of λ and θ were 1 and .5, respectively, and V_{max} and K_m were set equal to the estimates on this scale. The bootstrap standard errors for TBS/PX and Nonlinear are similar to the standard errors from asymptotic theory, except for $\hat{K}_m(0)$ and $\hat{K}_m(30)$ where the asymptotic standard errors seem too small.

The bootstrap standard errors for constant c.v. were huge, largely because of a few extremely poor bootstrap estimates. In Table 9 we report results for constant c.v. after trimming the bootstrap samples with the 10 largest and 10 smallest values of $\hat{K}_m(30)$.

These trimmed standard errors are two orders of magnitude smaller than those without trimming. Nonetheless, the bootstrap standard errors for constant c.v. are higher than for TBS/PX where we did not trim.

In the bottom of Table 9, we compare the bootstrap performance of TBS/PX estimates with weighted least–squares ($\lambda = 1$ and $\theta = .5$). The latter estimator represents the ideal situation, where the correct transformation (here no transformation) and weights are known a priori. Although there is some increase in standard errors due to estimation of λ and θ, it is not appreciable. The biases of both TBS/PX and WLS are small compared to the standard errors, and squared bias is a negligible proportion of mean–squared error.

Data sets 2 and 3

We obtained several similar data sets from Becton Dickinson. This seemed a good opportunity to investigate whether the error structure stays constant over repetitions of the laboratory procedure. However, when analyzing some of these data sets, we found deviations from Michaelis–Menten behavior. Upon inquiry, the biochemist who provided these data warned that such deviations might be expected. The reason is as follows. In the reaction of interest, the enzyme and inhibitor rapidly form a loosely bound complex. This enzyme–inhibitor complex is converted slowly into another complex, one that is more tightly bound. The data are collected as rapidly as possible to mitigate biasing caused by the second reaction. Apparently, the experimenters are sometimes successful at this, since data set #1 shows no deviation from Michaelis–Menten kinetics, although some bias may occur. Nonetheless, at Becton Dickinson these data are routinely analyzed using the Michaelis–Menten model, which is believed to yield adequate estimates of the rate constants for the fast reaction.

Here we introduce a more complicated model for two purposes: (1) to test for systematic deviations from the Michaelis–Menten model, and (2) to identify the structure of the random deviations after the systematic deviations have been eliminated. In data sets #2 and #3, the residuals from the Michaelis–Menten model exhibit a quadratic trend, so we use the empirically derived model

$$v_o = \{[V_{max}\, S]/[K_m(I) + S]\}\ \{1 + \alpha(S - \bar{S})^2\}. \tag{3.2}$$

Here $\bar{S}$ is the average of substrate concentration, about which S is centered. In model (3.2) the quadratic term α is assumed to be the same for all inhibitor levels. This assumption seems consistent with the residual plot from the Michaelis–Menten model.

For BDRC #2, approximate F–statistic for testing the Michaelis–Menten against the quadratic model is $F_A = 2.44$ with 1 and 12 degrees of freedom ($p = .144$) (both models use TBS/PX for error structure). This is slight evidence against the Michaelis–Menten model. For BDRC #3, $F_A = 13.0$ with 1 and 12 degrees of freedom ($p = .0036$); the approximate F–test and the residual plot (not shown here) both provide strong evidence that the Michaelis–Menten model does not fit BDRC #3. The BDRC #1 $F_A = .06$.

In Figures 5 and 6 are, respectively, a plot of the raw data and a Lineweaver–Burk plot for BDRC #3. One sees little evidence against the hyperbolic model in Figure 5 or against the linearity in Figure 6. This suggests that likelihood ratio tests are more sensitive in detecting departures from Michaelis–Menten behavior than graphs, especially graphs using scales with substantial heteroscedasticity. The near linear appearance of the Lineweaver–Burk plot suggests that the deviation from the Michaelis–Menten model may not be of practical significance in this data set. Nonetheless, the ability to detect lack–of–fit is important, if not here then certainly in other situations.

In Table 4 one finds $\hat{\lambda}$ and $\hat{\theta}$ under the Michaelis–Menten and quadratic models. It appears that minor systematic deviations from the Michaelis–Menten model, e.g., data set #2, have only minor biasing effect on $\hat{\lambda}$ and $\hat{\theta}$. More serious systematic departures (data set #3), cause more bias but the bias is still not severe.

Thus it appears that the TBS/PX Michaelis–Menten model can approximately identify the structure of the random errors despite the systematic errors caused by the slow reaction. In Table 5, we see that the submodels TBS and PX each fit all three Becton Dickinson data sets at .05, but none of the "fixed error structure" models (NL, LB, WF, and CCV) fit the three data sets at .05. It seems unlikely that one can find a "typical" error structure that nearly always fits data from such experiments; the flexibility of TBS/PX, or at least TBS or PX, is needed.

SECTION 4: Further Remarks

Is TBS/PX better than what is used in practice?: The Lineweaver–Burk scale appears to be the most widely used estimation method for enzyme data (Currie 1982). We have seen that the reciprocal transformation can induce severe heteroscedasticity and unstable estimates. In our examples, TBS/PX provides much better estimates than the Lineweaver–Burk transformation.

As the dangers in routine use of linearizing transformations become better appreciated, a trend may develop toward nonlinear least–squares. However, we have never encountered homoscedastic Michaelis–Menten data, and we expect nonlinear least–squares to be, in general, less efficient that TBS/PX. The bootstrap simulations support this conclusion for the examples analyzed here.

Is the Lineweaver–Burk transformation always bad?: There are examples where the Lineweaver–Burk transformation provides an excellent fit. In the Atlantic menhaden fishery, recruitment is highly right–skewed and the reciprocal transformation seems needed

to induce symmetry (Carroll and Ruppert 1984). In an earlier draft of this paper, Carroll, Cressie, and Ruppert (1987), we analyzed a hormone–receptor data set and found the Lineweaver–Burk model to fit almost as well as the TBS/PX model.

Robustness: A common view is that error structure cannot be estimated without very large sample sizes but that robust methods are insensitive to the nature of the errors, and therefore robust estimators should be used. We disagree with the premises of this statement, but not necessarily with the conclusion.

First, though error structures cannot be identified precisely, they can be identified sufficiently well to prevent improper weighting of the observations. Second, most robust estimators implicitly assume homoscedastic data since they accord equal weight to all observations. In the presence of gross heteroscedasticity, robust estimation can fail. We applied a robust bounded–influence estimator to BDRC #1 on the Lineweaver–Burk scale. The results are in Table 9. The standard errors of the bounded–influence estimator are much smaller than those of the least–squares estimator, but neither estimator on the Lineweaver–Burk scale is nearly as efficient as the TBS/PX estimator. Of course, looking at standard errors ignores the question of bias. Both estimates on the Lineweaver–Burk scale differ substantially from other estimators, but the true value of the parameter is unknown so that the nature of the biases is uncertain. On the basis of standard errors, though, we can conclude that the robust estimator cannot correct for the heteroscedasticity induced by the reciprocal transformation.

Another point to remember is that tests and confidence intervals that assume a constant variance will not hold their level under heteroscedasticity, even if they are "robust" to distributional shape.

There are two reasons why robust estimation and influence diagnostics should be used in conjunction with weighting and transformation. First, a number of authors (Cornish–Bowden and Eisenthal (1974); Atkins and Nimmo (1975); Cornish–Bowden

(1981)) have noted that enzyme kinetics data are prone outliers and have suggested robust estimation. Some outliers noticed in the past are undoubtedly due to inappropriate transformation and weighting. Besides these, however, there may be genuine outliers. These are observations that are outlying on a scale where the rest of the data appear homoscedastic. Such cases can greatly influence the estimates of the transformation and weighting parameters; see Carroll and Ruppert (1987 and 1988) for examples, including the year 1951 of the Skeena River data (deleted here). For simplicity, we did not discuss robust estimation in the previous sections. However, we did apply the robust bounded–influence estimators of Carroll and Ruppert (1988, chapter 6) to Skeena River and Becton Dickinson data sets. Except for year 1951 in the Skeena River data, we found no unusual or influential observations.

A second reason for using robust estimators is that error structures cannot be identified precisely, and robust estimators may compensate for the minor heteroscedasticity due to the use of estimated weights. In fact, in Carroll and Ruppert's (1983) Monte Carlo study of robust estimation in another class of heteroscedastic models (random coefficient models), it was found the robust methods could be more slightly efficient than maximum likelihood even for normally distributed data.

Lineweaver–Burk and other plots: In this paper we are concerned with the efficient estimation of Michaelis–Menten parameters. Efficient estimation and graphical presentation of data are two distinct issues. Although we recommend, in general, against the routine use of either the Lineweaver–Burk, Woolf, or Scatchard plots for parameter estimation, we believe that such plots are useful ways to graph the data.

Curvature: Identification of error structure is only one step, albeit an important one, towards efficient estimation. As in all nonlinear problems, curvature may make such pivotal–method confidence intervals inaccurate, and inference based on the profile likelihood

is preferable. See Bates and Watts (1980, 1988) for an introduction to curvature in nonlinear regression.

One parameter or two for error structure?: In the TBS/PX model, λ and θ are not well determined jointly; there is a whole ridge of values, roughly of the form $\lambda - \theta = c$ for some constant c, such that all pairs of (λ,θ) on this ridge provide an acceptable fit. The one parameter TBS and PX models may be adequate for most purposes. We have used the TBS/PX model since many standard estimators are implied by submodels of TBS/PX.

Implications for experimental design: Currie (1982) discusses the design of Michaelis–Menten experiments. It should be emphasized that designs that are optimal for one assumed error structure may be poor for other error structures. Although there appears to be no typical error structure, certain error structures, e.g., homoscedasticity, can perhaps be ruled out as uncommon. This information should be useful when designing experiments.

Theoretical or empirical models for error structure?: TBS/PX is an empirical model; experience has taught us that power transformations and weighting by a power of x generally induces nearly normal errors with a constant variance. Rudemo, Ruppert, and Streibig (1987) have introduced theoretical models where the error structure is modeled as having two components. The first is due to measurement error in y and "equation error". The second comes from variability in the model parameters (here V and K) or from an error in x. These theoretical models are applicable to the Michaelis–Menten model, and their use in that context is worth exploring. However, these models are somewhat more complicated than TBS/PX, involving three or more parameters for describing the error structure. Also, they do not include standard models, e.g., Lineweaver–Burk and Woolf, as submodels.

SECTION 5: Summary

In fitting a Michaelis–Menten model to data, we have demonstrated that many standard techniques are consequences of various distributions for the responses. It is clear that no single fixed distribution will fit all situations, so it should also be clear that no single fitting technique will be universally appropriate. The proper model and its associated fitting techniques should be allowed to vary among subject areas and even within a subject area.

In the examples we have shown that the TBS/PX method can be used to estimate the error structure of Michaelis–Menten data. Bootstrap simulations indicate that error structure can be identified sufficiently well to provide good estimates of Michaelis–Menten parameters. Moreover, approximate F–tests within the TBS/PX model allows one to check hypotheses such as a common value of V_{max}. An F–test on a linearized scale is often invalid because the variance is far from constant on that scale.

We view TBS/PX as a reasonable, flexible and effective method for helping choose a model for data. The method is easily computed and enables standard model checks to be performed. Naturally, as indicated in section 2, the model is not restricted to Michaelis–Menten relationships.

We do not advertise this method as a panacea. It clearly cannot solve all problems. In the enzyme kinetics data, for example, TBS/PX by itself it cannot correct the bias caused by a second reaction. In nonlinear models, curvature must be addressed, but this is an issue separate from the identification of error structure. Nor do we advocate that TBS/PX be used as a black box. All formal fitting methods should be used in conjunction with graphical displays and diagnostics that check for problems with the data or the model. While the technique is much more widely applicable than techniques assuming a fixed error structure, the model will not fit all data sets purported to satisfy the Michaelis–Menten equation.

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TABLE 1

The relationship between the mean and standard deviation of the response implied by the TBS/PX Michaelis–Menten model and several of its submodels.

Model	Relationship between SD(y), f, and x
TBS/PX	$SD(y) = \sigma\,(f^{1-\lambda}\,x^{\theta})$
TBS ($\theta = 0$)	$SD(y) = \sigma\,(f^{1-\lambda})$
PX ($\lambda = 1$)	$SD(y) = \sigma\,x^{\theta}$
Nonlinear ($\lambda = 1,\ \theta = 0$)	$SD(y) = \sigma$
Lineweaver–Burk ($\lambda = -1,\ \theta = 0$)	$SD(y) = \sigma\,(f^{2})$
Woolf ($\lambda = -1,\ \theta = -1$)	$SD(y) = \sigma\,(f^{2}/x)$
Constant c.v. ($\lambda = 0,\ \theta = 0$) (Approximate Scatchard)	$SD(y) = \sigma\,(f)$

TABLE 2

Skeena River Sockeye Salmon data. Years 1951 and 1955 have been deleted in the illustrative example of section 3. Data are in millions of fish.

YEAR	SPAWNERS	RECRUITS
1940	0.963	2.215
1941	0.572	1.334
1942	0.305	0.800
1943	0.272	0.438
1944	0.824	3.071
1945	0.940	0.957
1946	0.486	0.934
1947	0.307	0.971
1948	1.066	2.257
1949	0.480	1.451
1950	0.393	0.686
1951	0.176	0.127
1952	0.237	0.700
1953	0.700	1.381
1954	0.511	1.393
1955	0.087	0.363
1956	0.370	0.668
1957	0.448	2.067
1958	0.819	0.644
1959	0.799	1.747
1960	0.273	0.744
1961	0.936	1.087
1962	0.558	1.335
1963	0.597	1.981
1964	0.848	0.627
1965	0.619	1.099
1966	0.397	1.532
1967	0.616	2.086

TABLE 3

Confidence region for λ and θ for the Skeena River sockeye salmon data; "1" denotes presence in the 95% confidence region. λ and θ are restricted to lie between -1 and 1 on a grid of width .2.

						θ					
	−1	−.8	−.6	−.4	−.2	0	.2	.4	.6	.8	1
λ											
−1	·	·	·	·	·	·	·	·	·	·	·
−.8	·	·	·	·	·	1	1	1	1	·	·
−.6	·	·	·	1	1	1	1	1	1	1	1
−.4	·	·	·	1	1	1	1	1	1	1	1
−.2	·	·	·	1	1	1	1	1	1	1	1
0	·	·	·	1	1	1	1	1	1	1	1
.2	·	·	·	1	1	1	1	1	1	1	1
.4	·	·	·	·	1	1	1	1	1	1	1
.6	·	·	·	·	·	·	1	1	1	1	1
.8	·	·	·	·	·	·	·	1	1	1	1
1	·	·	·	·	·	·	·	·	·	1	1

TABLE 4

Estimates of transformation and variance parameters. Asymptotic standard errors are in parentheses and bootstrap standard errors are in square brackets. MM = Michaelis–Menten model. BDRC #2 and #3 were not bootstrapped. TBS and PX were not fit to the quadratic models.

Data Set	Model			
	TBS/PX		TBS	PX
	$\hat{\lambda}$	$\hat{\theta}$	$\hat{\lambda}$	$\hat{\theta}$
Skeena	.34 (.31) [.65]	.77 (.33) [.66]	−.103 (.29) [.40]	1.16 (.36) [.35]
BDRC #1	1.04	.567	.50	.54
MM	(.35) [.33]	(.23) [.43]	(.14) [.27]	(.11) [.31]
quadratic	1.00 (.35)	.50 (.26)	——	——
BDRC #2	.017	.76	−.69	1.45
MM	(.35)	(.23)	(.39)	(.19)
quadratic	.18 (.49)	.68 (.40)	——	——
BDRC #3	−.21	−.10	−.13	.87
MM	(.35)	(.38)	(.21)	(.17)
quadratic	−.13 (.54)	−.30 (.46)	——	——

TABLE 5

P–values from the approximate F–tests for testing submodels of the TBS/PX model. The submodels are transform–both–sides (TBS), power–of–x (PX), Nonlinear (NL), Lineweaver–Burk (LB), Woolf (WF), and constant coefficient of variation (CCV).

Data Set	Model					
	TBS	PX	NL	LB	WF	CCV
Skeena	.099	.187	.011	.029	.0004	.248
BDRC #1	.095	.806	.058	.0000	.0000	.035
BDRC #2	.220	.151	.0065	.354	.052	.167
BDRC #3	.802	.055	.0109	.011	.273	.907

TABLE 6

Estimates of the regression parameters and simulation results for the Skeena River data. "Asym. s.e." is asymptotic standard error. "Boot. s.e." and "Boot. bias" are bootstrap standard error and bias, respectively. The bootstrap used 200 simulations.

Model	Estimated Regression Parameters			
	$\hat{V}$	$\hat{K}$	$\hat{\alpha}$	$\hat{\alpha}$
TBS/PX				
estimate	3.81	1.12	.26	.29
asym. s.e.	2.14	.84	.14	.059
boot. s.e.	27.3	.0249	.139	.071
boot. bias	2.72	−.0041	.020	−.003
NL				
estimate	2.78	.60	.36	.22
asym. s.e.	1.13	.43	.14	.070
boot s.e.	20.1	.069	.183	.111
boot. bias	−2.67	−.030	−.048	.012

TABLE 7

Performance of approximate F–tests in a bootstrap experiment of 200 simulations. The "truth" of the null hypothesis refers to the bootstrap distribution.

Data Set	Null hypothesis	Nominal level	Proportion of rejections in 200 simulations (Standard error)
Skeena	$\lambda = .33$ (true)	5%	8.5% (2.0%)
		1%	5% (1.5%)
	$\lambda = .33$, $\theta = .77$ (true)	5%	7.5% (1.9%)
		1%	4% (1.4%)
	$\lambda = 1$, $\theta = 0$ (false)	5%	87.5% (2.4%)
		1%	76% (3.0%)
BDRC #1	$\lambda = 1$ (true)	5%	4.5% (1.5%)
		1%	0.5% (0.5%)
	$\lambda = 1$, $\theta = .5$ (true)	5%	5.5% (1.6%)
		1%	1% (0.7%)
	$\lambda = 1$, $\theta = 0$ (false)	5%	34% (1.0%)
		1%	11.5% (2.3%)
	$\lambda = 0$, $\theta = 0$ (false)	5%	86.5% (2.5%)
		1%	70.5% (3.2%)

TABLE 8

Enzyme kinetics data from Becton Dickinson.

Data Set	Substrate	Inhibitor concentration			
		0	3	10	30
BDRC #1	25	.0328	.0153	.0087	.0039
	50	.0510	.0327	.0146	
	100	.0697	.0536	.0231	.0094
	200	.0934	.0716	.0305	.0175
	400	.0924	.0904	.0658	.0398
		0	.25	1	4
BDRC #2	25	.0195	.0134	.0092	.0055
	50	.0327	.0249	.0159	.0103
	100	.0540	.0428	.0298	.0205
	200	.0667	.0612	.0464	.0295
	400	.0729	.0706	.0582	.0452
		0	1	3	10
BDRC #3	25	.0171	.0118	.0068	.0028
	50	.0289	.0195	.0137	.0056
	100	.0426	.0335	.0235	.0103
	200	.0542	.0471	.0355	.0180
	400	.0582	.0543	.0474	.0276

TABLE 9

Estimates of V_{max} and $K_m(I)$ for BDRC #1. Asymptotic standard errors in parentheses. Bootstrap standard errors in square brackets. The estimators are based on the following models: Transform–both–sides/power–of–x (TBS/PX), Nonlinear (NL), log transform = Constant c.v. = approximate Scatchard (CCV), Lineweaver–Burk (LB), Lineweaver–Burk with a robust bounded–influence estimator (LB–BI), and Woolf. The asymptotic standard errors use Huber's (1967) asymptotics for the MLE under a misspecified model. The bootstrap standard errors for CCV involve trimming; see text. The boostrap used 200 simulations.

Estimators and standard errors

	Model					
Parameter	TBS/PX	NL	CCV	LB	LB–BI	Woolf
V_{max}	.1189	.1169	.1122	.0668	.0744	.1171
	(.0053) [.0057]	(.0067) [.0059]	(.0069) [.0094]	(.1736)	(.0262)	(.0159)
$K_m(0)$	66.4	65.1	60.3	36.4	25.5	68.1
	(4.79) [7.03]	(7.60) [8.19]	(6.64) [11.80]	(290.6)	(18.8)	(29.9)
$K_m(3)$	138	126	131	75	84	128
	(14.6) [13.7]	(16.6) [16.2]	(18.3) [19.4]	(294.4)	(62.5)	(38.7)
$K_m(10)$	402	402	361	174	207	402
	(51.0) [45.5]	(68.3) [49.7]	(41.7) [56.6]	(577)	(115)	(83.3)
$K_m(30)$	969	901	857	458	504	904
	(111) [180.1]	(121) [192.2]	(118) [241.0]	(1520)	(673)	(167)

Bootstrap comparison of TBS/PX and WLS

		V_{max}	$K_m(0)$	$K_m(3)$	$K_m(10)$	$K_m(30)$
Bias	TBS/PX	−.0002	−.33	.30	−.13	9.8
	WLS	−.0000	−.31	.35	1.24	15.8
Std. err	TBS/PX	.0057	7.03	13.7	45.5	180
	WLS	.0055	6.18	12.1	39.3	164

FIGURE 1

Plot of spawners and recruits for Skeena River. Years 1951 and 1955 not shown.

FIGURE 2

Residuals from $\lambda = 1,\ \theta = 1$ plotted against the logarithms of the predicted values. Skeena River data. Data are in millions of fish.

FIGURE 3

BDRC #1 data. Data points at each level of the inhibitor concentration are connected.

FIGURE 4

Lineweaver–Burk plot for BDRC #1. At each level of the inhibitor concentration the data points are connected by dotted lines and the least squares fit is shown by a solid line.

FIGURE 5

BDRC #3 data.

FIGURE 6

Lineweaver–Burk plot of BDRC #3. The data within each level of inhibitor concentration are connect by lines.

FIGURE 1

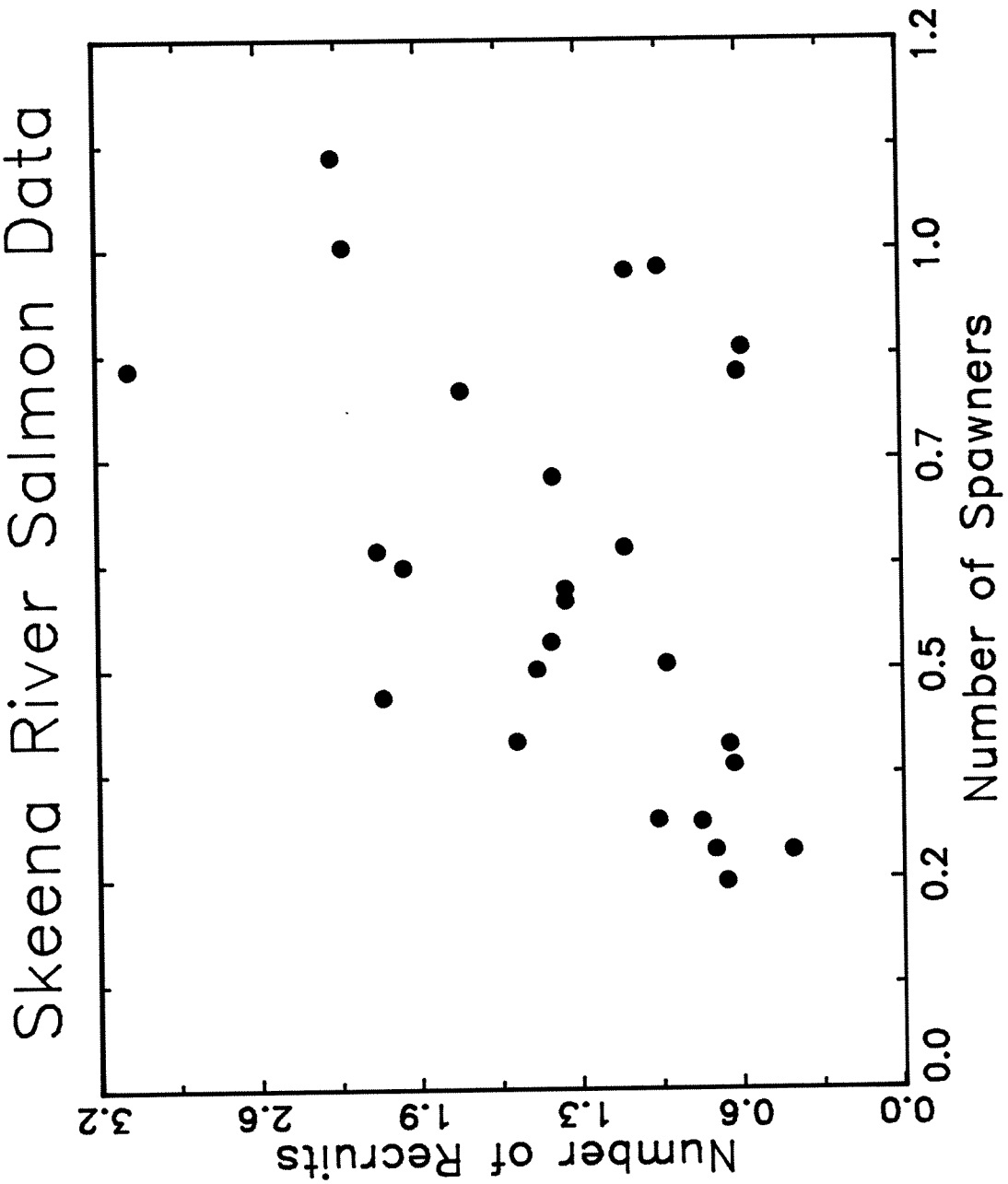

Skeena River Salmon Data
Number of Recruits
0.0
0.6
1.3
1.9
2.6
3.2
Number of Spawners
0.0
0.2
0.5
0.7
1.0
1.2

FIGURE 2

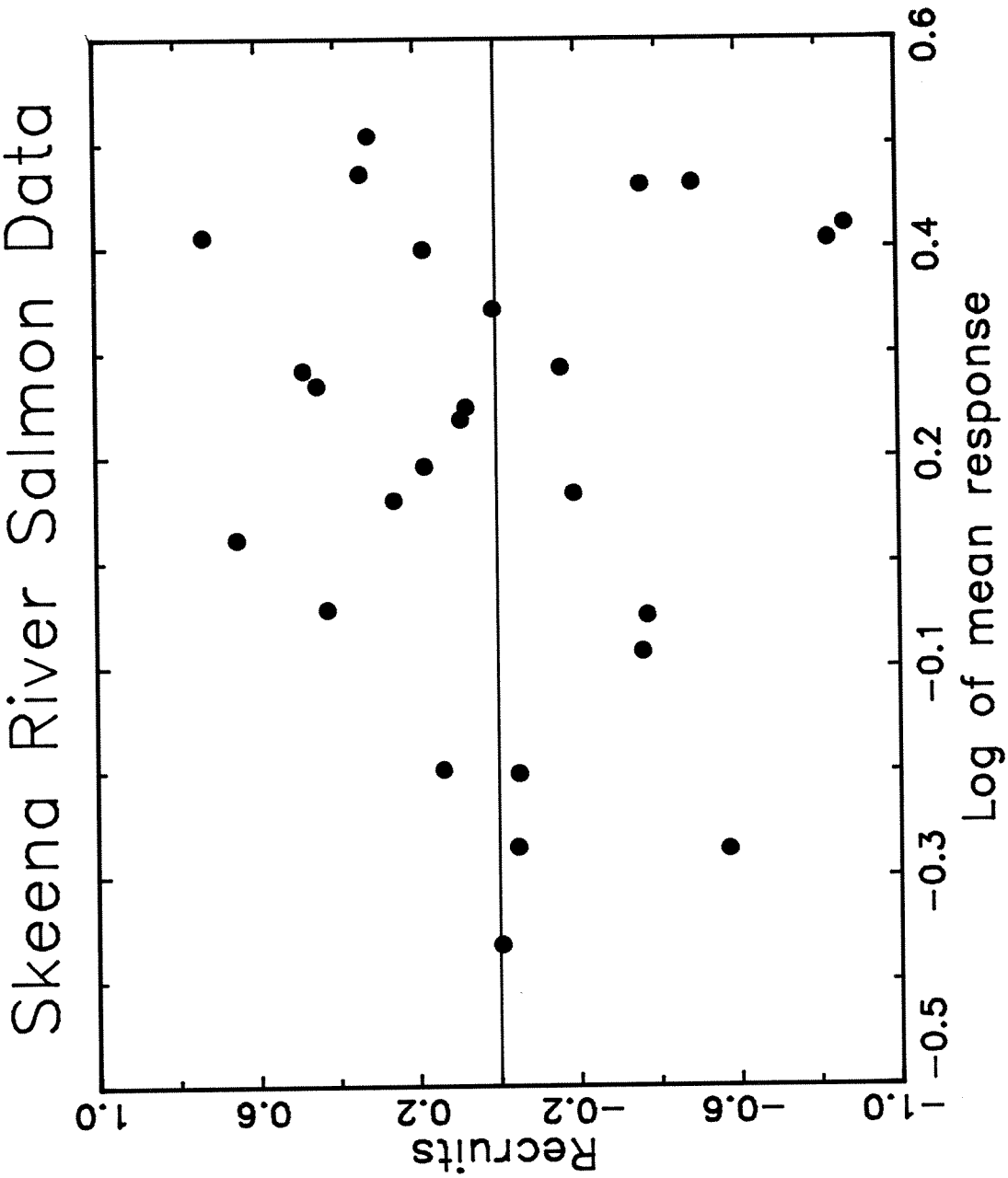

Skeena River Salmon Data
Recruits
1.0
0.6
0.2
−0.2
−0.6
−1.0
Log of mean response
−0.5
−0.3
−0.1
0.2
0.4
0.6

FIGURE 3

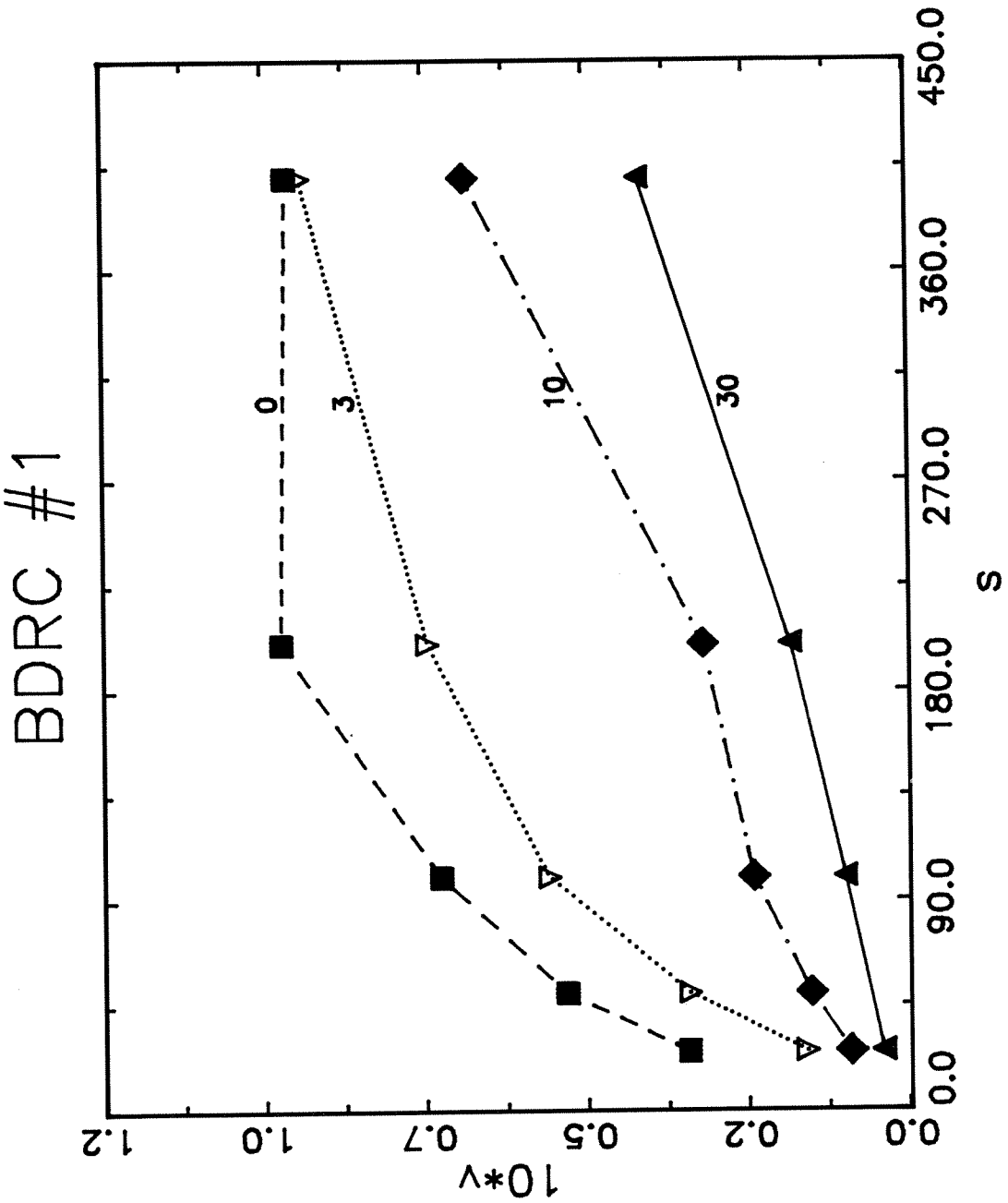


FIGURE 4

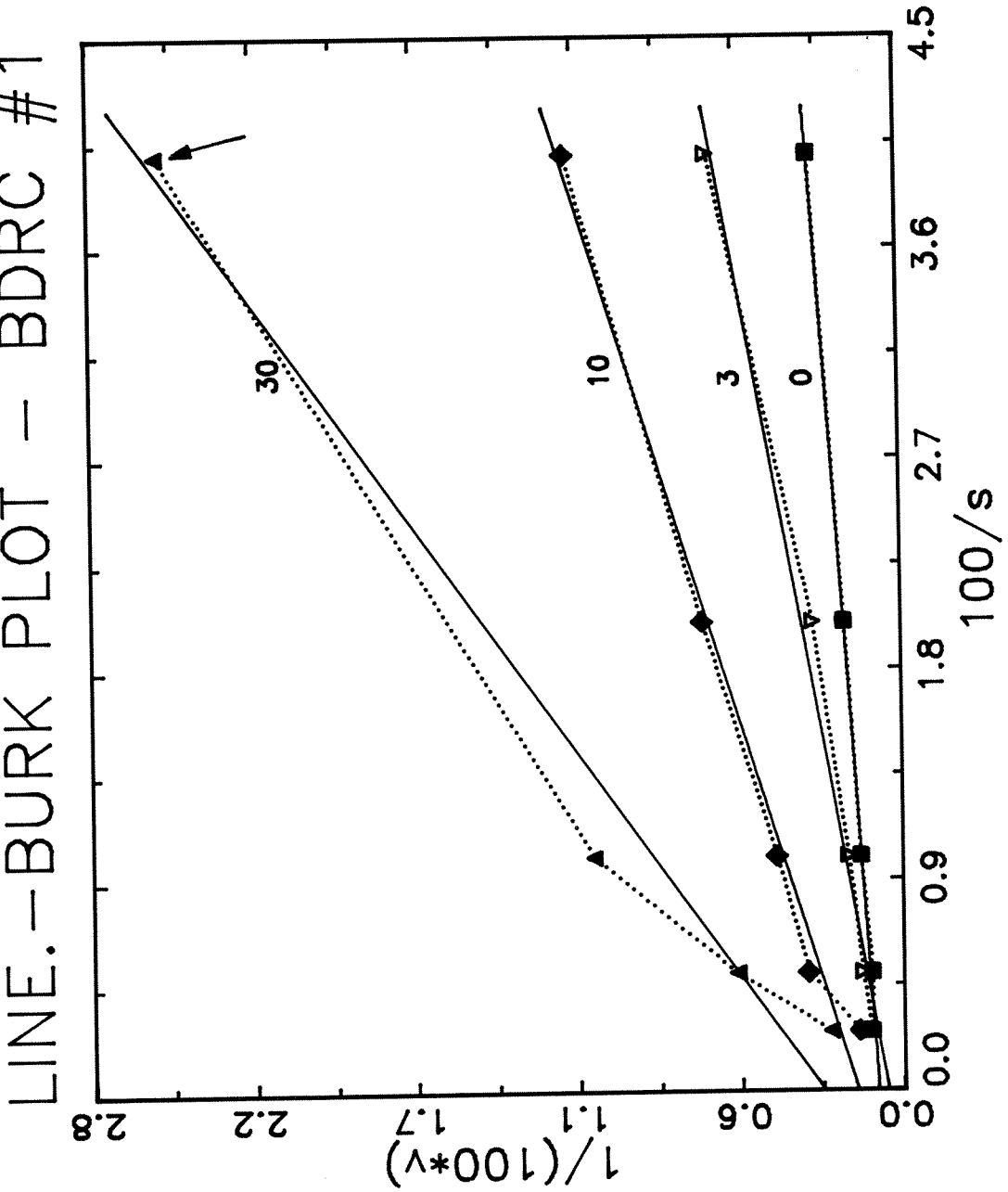

LINE.-BURK PLOT - BDRC #1
1/(100*v)
0.0
0.6
1.1
1.7
2.2
2.8
100/s
0.0
0.9
1.8
2.7
3.6
4.5
30
10
3
0

FIGURE 5

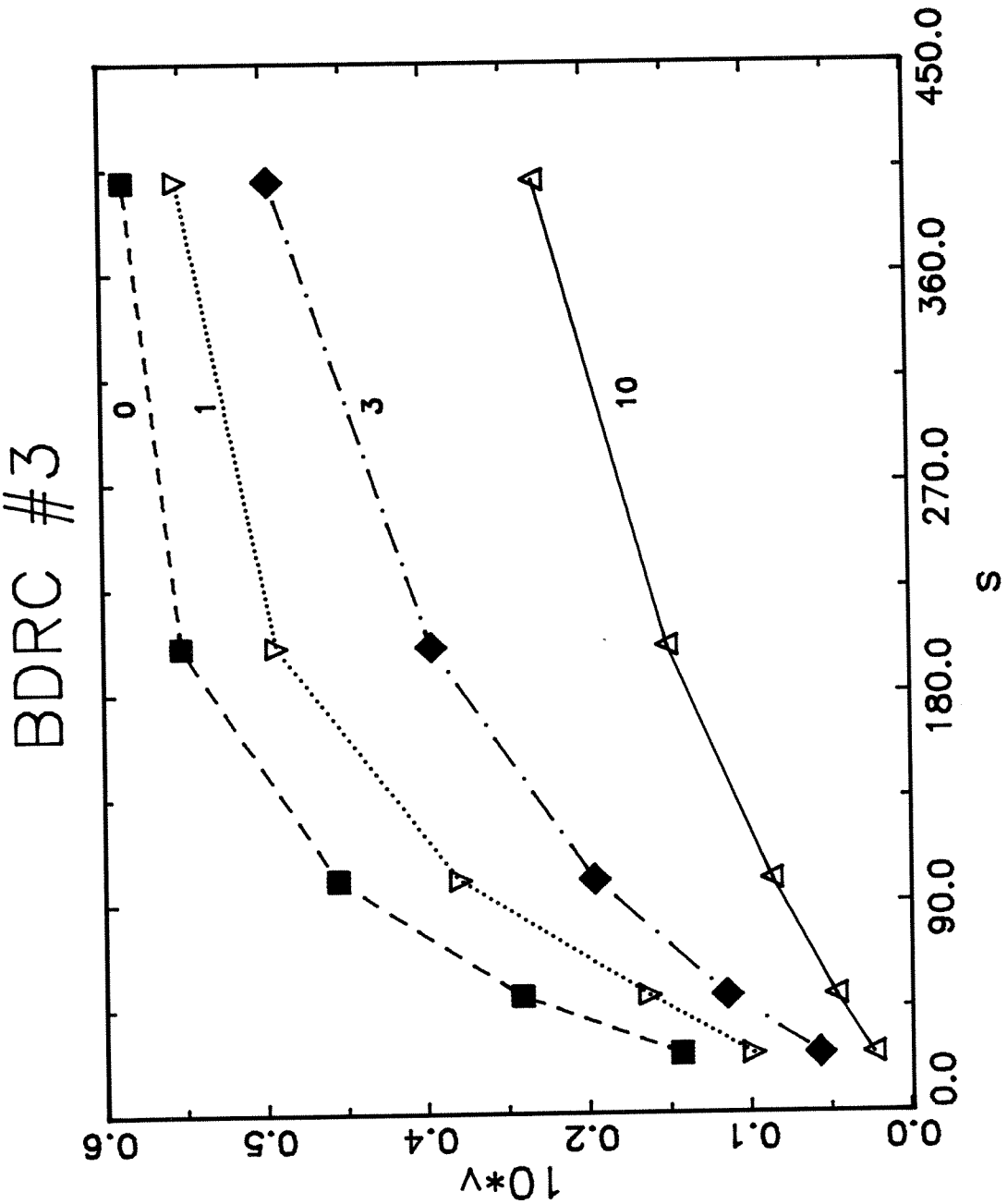
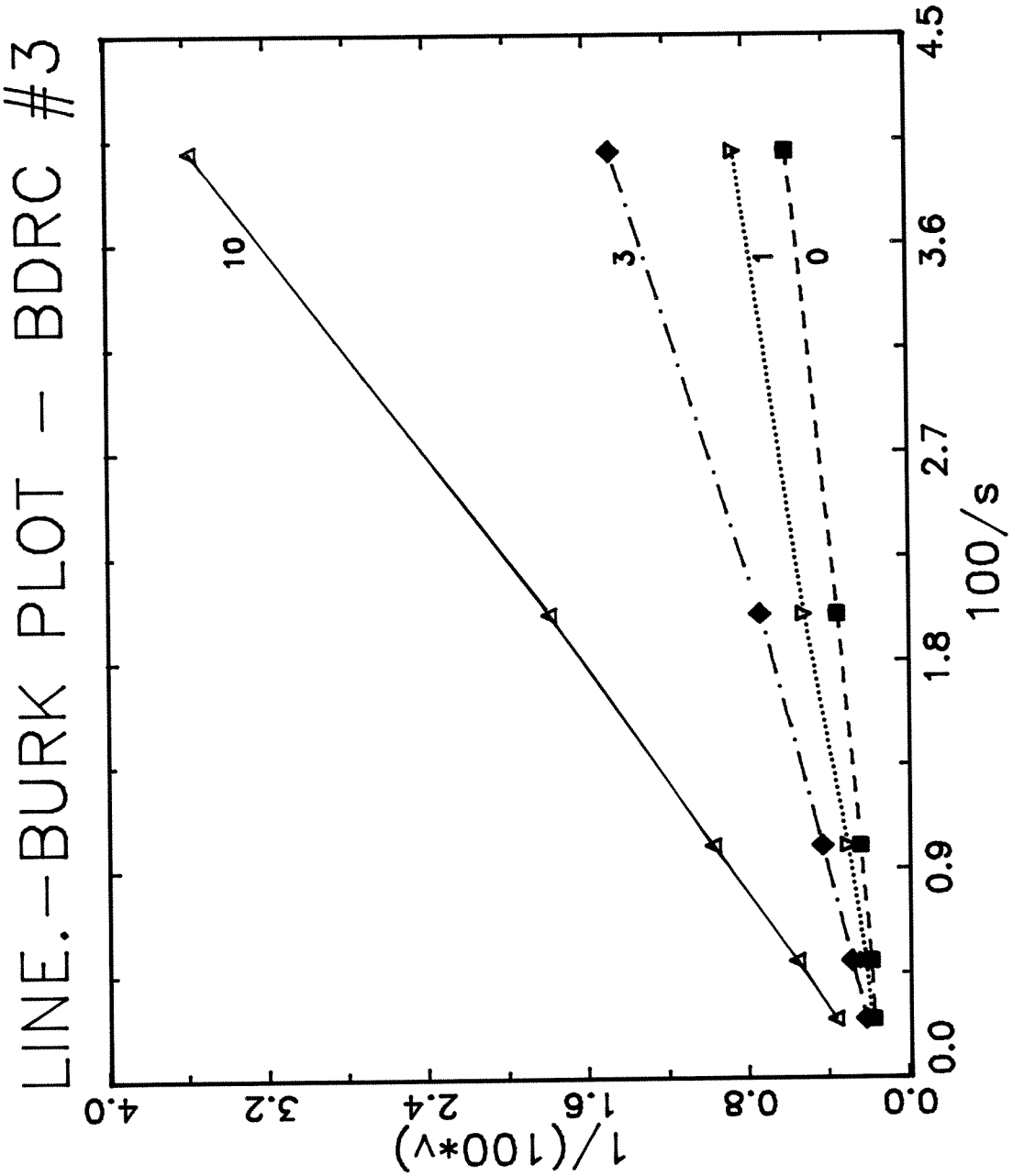


FIGURE 6


LINE.-BURK PLOT - BDRC #3
1/(100*v)
0.0
0.8
1.6
2.4
3.2
4.0
100/s
0.0
0.9
1.8
2.7
3.6
4.5
10
3
1
0