Evaluation of Rate Constants for Enzyme-Catalysed Reactions by the Jackknife Technique

APPLICATION TO LIVER ALCOHOL DEHYDROGENASE

By ATHEL CORNISH-BOWDEN*

Department of Chemistry, University of Guelph, Guelph, Ont., Canada N1G 2W1

and J. TZE-FEI WONG

Department of Biochemistry, University of Toronto, Toronto, Ont., Canada M5S 1A8

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Steady-state measurements of enzyme-catalysed reactions are capable of providing more information about the rate constants of the individual steps than is commonly obtained. We have applied a combination of the jackknife and non-linear regression techniques to measurements of the rate of oxidation of ethanol by NAD^+ , catalysed by alcohol dehydrogenase from horse liver. This has permitted values and confidence intervals to be assigned to the eight rate constants that characterize the binding of ethanol and NAD^+ in random order to the enzyme, and to the net rate constant k_{cat} for the breakdown of the ternary complex.

In principle, transient-kinetic measurements of enzyme-catalysed reactions are more directly applicable than steady-state measurements to the estimation of the rate constants of the mechanism (see Hammes & Schimmel, 1970). It has often, and in our view mistakenly, been concluded from this that steady-state measurements can provide little information about the rate constants for the individual steps (e.g. Halford, 1974). On the contrary, there are many mechanisms for which all of the rate constants can be calculated from steady-state measurements, provided that no isomerization steps are formally included. For simple mechanisms, it is usually necessary to be able to follow the reaction in both forward and reverse directions, and considerable success has been had with such enzymes as liver alcohol dehydrogenase (Theorell *et al.*, 1955; alcohol- NAD^+ oxidoreductase, EC 1.1.1.1) and fumarase (Alberty & Pierce, 1957; L-malate hydro-lyase, EC 4.2.1.2). More recently it has been possible to assign values to many of the rate constants for the liver alcohol dehydrogenase reaction on the basis of measurements of the forward reaction alone (Hanes *et al.*, 1972; Wong *et al.*, 1972; Wong & Hanes, 1973), despite the fact that the mechanism is now known to be more complicated than that assumed by Theorell *et al.* (1955).

Steady-state methods are far more widely used than transient-state methods, not only because they

require simpler apparatus and much smaller quantities of enzyme, but also because a wide range of criteria have been developed for interpreting steady-state measurements and differentiating between mechanisms (see Wong, 1975; Cornish-Bowden, 1976). Accordingly, it is important to find general ways of evaluating rate constants from steady-state measurements, whenever the data are adequate to permit such evaluation. In the present study we have examined this problem in the context of the reaction catalysed by alcohol dehydrogenase from horse liver. We have found that, whereas conventional matrix-inversion methods for estimating the precision of fitted parameters give absurd and meaningless results when applied to complex rate equations, the jackknife technique (Quenouille, 1956; Tukey, 1958; Miller, 1974) gave such promising results with this example that it should be seriously considered as a general technique for analysis of enzyme kinetic results.

Theory

Mechanism of liver alcohol dehydrogenase

We shall be primarily concerned in this paper with the mechanism shown in Scheme 1, first because it applies to horse liver alcohol dehydrogenase at low concentrations of ethanol and in the absence of products (Hanes *et al.*, 1972), and is therefore of experimental interest, and second because it leads to a rate equation of sufficient complexity to provide a

* Usual address: Department of Biochemistry, University of Birmingham, P.O. Box 363, Birmingham B15 2TT, U.K.

Scheme 1. *Random-order ternary-complex mechanism*

The Scheme shows the mechanism proposed by Hanes *et al.* (1972) for the oxidation of ethanol (shown as Eth) by NAD^+ , catalysed by horse liver alcohol dehydrogenase. The mechanism is simplified by omitting product-binding steps, which are insignificant for measurements of the initial rate in absence of added product, and steps relating to substrate inhibition at high ethanol concentrations, which can be corrected for as described by Hanes *et al.* (1972). The ternary complex, shown as an enzyme- NAD^+ -ethanol complex, subsumes other ternary complexes, such as an enzyme-NADH-acetaldehyde complex, because steady-state measurements do not distinguish between such complexes.

demanding test of the versatility of steady-state methods for evaluating rate constants:

$$v/e_0 = \frac{(k_{+1}k_{+3}k_{+4}[\text{Eth}] + k_{+2}k_{+3}k_{+4}[\text{NAD}^+] + k_{+1}k_{-2}k_{+3} + k_{-1}k_{+2}k_{+4})k_{+5}k_{+6}[\text{Eth}][\text{NAD}^+]}{k_{+1}k_{+3}k_{+4}(k_{+5} + k_{+6})[\text{Eth}]^2[\text{NAD}^+] + k_{+1}k_{+4}k_{+6}(k_{-3} + k_{+5})[\text{Eth}]^2 + k_{+2}k_{+3}k_{+4}(k_{+5} + k_{+6})[\text{Eth}][\text{NAD}^+]^2 + [k_{+1}k_{+3}(k_{-2}k_{+5} + k_{-2}k_{+6} + k_{-4}k_{+6}) + k_{-1}k_{+2}k_{+4}(k_{+5} + k_{+6}) + k_{+4}k_{+6}(k_{+2}k_{-3} + k_{+3}k_{+5})][\text{Eth}][\text{NAD}^+] + [k_{+1}k_{-2}k_{+6}(k_{-3} + k_{-4} + k_{+5}) + k_{-1}k_{+4}k_{+6}(k_{-3} + k_{+5})][\text{Eth}] + k_{+2}k_{+3}k_{+6}(k_{-4} + k_{+5})[\text{NAD}^+]^2 + [k_{-1}k_{+2}k_{+6}(k_{-3} + k_{-4} + k_{+5}) + k_{-2}k_{+3}k_{+6}(k_{-4} + k_{+5})][\text{NAD}^+] + k_{-1}k_{-2}k_{+6}(k_{-3} + k_{-4} + k_{+5})} \quad (1)$$

In this equation the rate constants are as defined by Scheme 1, and $[\text{Eth}]$ represents the concentration of ethanol.

Data were obtained for horse liver alcohol dehydrogenase by Hanes *et al.* (1972) under a variety of conditions, and included studies of product inhibition and substrate inhibition at high ethanol concentrations. However, to keep the discussion within manageable limits we shall restrict it to the data shown in Table 1 for the uninhibited forward reaction. By a combination of trial-and-error and algebraic analysis, values were assigned to all of the ten rate constants (Wong *et al.*, 1972; Wong & Hanes, 1973). However, the procedures used were extremely laborious and required many months of analysis, and they gave no satisfactory indication of the precision of the result; they could not readily be generalized for routine use. In the search for more generally applicable procedures it seemed worth while instead to investigate the possibility that

computational methods that had proved efficient in simpler non-linear regression problems (Cornish-Bowden & Koshland, 1970; Wharton *et al.*, 1974) might provide a simpler way of fitting the data shown in Table 1 to eqn. (1).

The jackknife technique

The jackknife was first proposed by Quenouille (1956) as a technique for eliminating certain forms of bias from averages. It has subsequently proved to be very valuable for estimating the precision of parameters in non-linear models when conventional statistical methods based on linear approximations fail completely; it is for this latter purpose that we are concerned with the jackknife in the present paper. Although the technique, which has been reviewed by Miller (1974), is well known in applied statistics, it has been used very rarely in enzymological work (Dammkoehler, 1966; Herzfeld & Schlesinger, 1975; Gros *et al.*, 1976), despite being perhaps the most suitable of all methods for such work. Because it is almost wholly unknown in biochemistry, we now provide a brief introduction to the theory. To avoid making the description unduly abstract, we shall assume that there are 36 observations (as in Table 1) divided arbitrarily into six groups. Other numbers can be substituted in an obvious way.

Suppose we have a good (but possibly biased) method of getting an estimate $\hat{b}$ of an unknown parameter β . [For example, if β represented k_{+1} in eqn. (1), then $\hat{b}$ might be the estimate of k_{+1} obtained by minimizing the sum of squares of differences between observed and calculated values of v .] Then let $\hat{b}_0$ be the estimate of β obtained by using all 36 observations, and let $\hat{b}_{-i}$ be the estimate of β obtained by using the 30 observations left after omitting the i th set of six. Then a 'pseudo value' $\hat{b}_i$ may be calculated

Table 1. *Data for alcohol dehydrogenase from horse liver*

The Table shows values of v/e_0 obtained by Hanes *et al.* (1972) for the oxidation of ethanol by NAD^+ catalysed by alcohol dehydrogenase in 0.045 M-pyrophosphate buffer, pH 8.6, at 27°C. The values are expressed in s^{-1} and are corrected for substrate inhibition at high ethanol concentrations as described previously (Hanes *et al.*, 1972). The numbers in the body of the Table are prefixed with the letters A, B, C, D, E and F to show how the observations were grouped for application of the jackknife. For example, the first set of 30 observations consisted of groups B, C, D, E and F, the second of groups A, C, D, E and F, etc. The letters are arranged in the form of a latin square.

[NAD ⁺] (mM)	[Ethanol] (mM)	...	v/e_0					
			0.171	0.356	0.603	1.065	4.630	100
0.0075			A 0.414	B 0.616	C 0.849	D 1.078	E 1.355	F 1.540
0.0160			B 0.572	F 0.915	D 1.276	C 1.536	A 2.002	E 2.244
0.0427			C 0.783	D 1.166	E 1.540	F 2.011	B 2.913	A 3.388
0.0962			D 1.012	A 1.443	F 1.848	E 2.367	C 3.441	B 3.916
0.481			E 1.165	C 1.725	A 2.310	B 2.842	F 3.832	D 4.277
10.000			F 1.210	E 1.826	B 2.446	A 2.992	D 3.982	C 4.453

by treating $\hat{b}_0$ as a weighted geometric mean of $\hat{b}_{-i}$ and $\hat{b}_i$:

$$\ln \hat{b}_0 = (30 \ln \hat{b}_{-i} + 6 \ln \hat{b}_i) / 36$$

from which we have

$$\ln \hat{b}_i = 6 \ln \hat{b}_0 - 5 \ln \hat{b}_{-i}$$

[The use of geometric rather than arithmetic means is not essential to the method; it acts as a stabilizing transformation (Miller, 1974) that prevents meaningless negative values from being assigned to the parameters, and is much more satisfactory in practice. The same transformation is used for the same reason in the non-linear regression program that we have used (Cornish-Bowden & Koshland, 1970).] One can then define a jackknifed estimate of $\ln \beta$ as the mean of all the $\ln \hat{b}_i$, i.e. $\ln \hat{b}_0 = \frac{1}{6} \sum \ln \hat{b}_i$.

According to a conjecture of Tukey (1958), the values of $\ln \hat{b}_i$ can be treated as if they were independent and identically distributed estimates of $\ln \beta$, and consequently it is a simple matter to estimate the standard error of $\ln \hat{b}_0$ by the ordinary methods of elementary statistics. Furthermore, if the original estimate $\ln b_0$ is biased, as is highly likely in a non-linear problem, the bias will be decreased in $\ln \hat{b}_0$: the extent of this decrease depends on the nature of the bias, but if it is inversely proportional to the number of observations, as is likely to be approximately true, it is completely eliminated by the jackknife.

Results

Fitting the data for alcohol dehydrogenase

The non-linear regression program that we used was essentially the same as that described previously (Wharton *et al.*, 1974), apart from the routine defining the equation to be fitted, which was replaced

by a FORTRAN equivalent of eqn. (1). Because of the complicated way in which every rate constant appears several times in eqn. (1), however, the problems of non-linearity were far more severe than those encountered previously. It was necessary to choose a parameter set very carefully to decrease correlation, and we were only partly successful in this. The equation contains ten rate constants, but only nine are independent because the tenth is specified by the thermodynamic constraint $k_{+1}k_{-2}k_{+3}k_{-4}/k_{-1}k_{+2}k_{-3}k_{+4} = 1$. Initially we took $(k_{+1}, k_{-1}, k_{+2}, k_{-2}, k_{+3}, k_{-3}, k_{+4}, k_{-4}, k_{+5}, k_{+6})$ as the parameter set, replacing k_{-4} in eqn. (1) with $k_{-1}k_{+2}k_{-3}k_{+4}/k_{+1}k_{-2}k_{+3}$, but this gave very poor results, not unexpectedly. Exploratory runs of the program with this parameter set showed that, whereas some functions of the rate constants were precisely defined by the data, others were barely defined at all. For example, good fits were never obtained unless $k_{+5}k_{+6}/(k_{+5} + k_{+6})$ was within a few per cent of 4.47s^{-1} , whereas good fits could be obtained for any values of k_{+5}/k_{+6} over a range at least as wide as 0.001–1000.

The extremely high correlation between k_{+5} and k_{+6} is illustrated by the sum-of-squares contours in Fig. 1. Not only are the contours highly elongated, but the correlation is non-linear, as the contours are not even approximately elliptical, either in the logarithmic co-ordinates used in Fig. 1 or in linear co-ordinates. In consequence, almost any linear combination of $\ln k_{+5}$ and $\ln k_{+6}$ (or of k_{+5} and k_{+6}) will give the impression of being well-defined if one measures the sum of squares as a function of it. For example, the contours make it quite clear that the point marked ● in Fig. 1 does not correspond to the minimum sum of squares, and is not even particularly close to the true minimum (marked ○). Nonetheless, linear exploration in any of the directions indicated would suggest that the false minimum lay at or very close to the minimum of a steep-walled valley. So not only would

Fig. 1. Non-linear correlation between k_{+5} and k_{+6}

For values of k_{+5} and k_{+6} in the ranges shown the sum of squares was calculated for the complete data set listed in Table 1. The sum of squares was defined as given by Wong & Hanes (1973), i.e. as $\sum(1 - \hat{v}/v)^2$, where $\hat{v}$ and v represent calculated and observed initial rates respectively. The results are shown as a series of contours. The other kinetic constants were held at the following values: $k_{+1} = 6.08 \text{ mM}^{-1} \cdot \text{s}^{-1}$, $k_{+2} = 31.2 \text{ mM}^{-1} \cdot \text{s}^{-1}$, $k_{-3} = 54.1 \text{ s}^{-1}$, $k_{-4} = 74.6 \text{ s}^{-1}$, $k_{\text{cat.}}/K_s^{\text{Ethanol}} = 436 \text{ mM}^{-1} \cdot \text{s}^{-1}$, $k_{\text{cat.}}/K_m^{\text{Ethanol}} = 9.49 \text{ mM}^{-1} \cdot \text{s}^{-1}$, $k_{\text{cat.}}/K_m^{\text{NAD}^+} = 310 \text{ mM}^{-1} \cdot \text{s}^{-1}$. These values are close to but not identical with the best values found; consequently the true minimum in this projection, shown as ○, is close to but not identical with the true global minimum in nine dimensions. The point shown as ● is neither at nor close to the true minimum, but could be thought close to the true minimum if tested in over-simple ways, such as by exploring the behaviour of the sum of squares along the directions indicated by the arrows.

individual tests of k_{+5} and k_{+6} , as described by Wong & Hanes (1973), suggest that this point was either at or very close to the minimum, but so also would most tests of simple combinations of k_{+5} and k_{+6} . One should expect, therefore, that any attempt to estimate the precision of k_{+5} and k_{+6} by methods based on linear approximations would grossly underestimate the actual uncertainty.

We were able to overcome the problem of correlation between k_{+5} and k_{+6} almost entirely by replacing them as parameters with $k_{+5}k_{+6}/(k_{+5} + k_{+6})$, which we shall refer to hereafter as $k_{\text{cat.}}$, and k_{+5}/k_{+6} , for which the correlation is negligible. Further improvements in the definition of the sum-of-squares surface were obtained by replacing k_{-1} , k_{-2} , k_{+3} and k_{+4} in the parameter set with $k_{\text{cat.}}/K_m^{\text{Ethanol}}$, $k_{\text{cat.}}/K_m^{\text{NAD}^+}$, $k_{\text{cat.}}/K_s^{\text{NAD}^+}$ (these parameters are defined in Table 2, below) and k_{-4} . Nonetheless, severe correlation and non-linearity remained. Consequently the progress of the regression program was extremely slow once the vicinity of the minimum was reached and we were never able to satisfy the fundamental

criterion for a minimum sum of squares, i.e. we were never able to find values of the parameters such that the first partial derivative of the sum of squares with respect to each parameter was close to zero. Nonetheless, the consistency of results obtained with different starting guesses and the excellent fit to the data leave little doubt that the final fit was essentially the same as that at the true minimum.

Estimation of the value of k_{+5}/k_{+6}

As shown in Fig. 2, it proved possible to obtain an excellent fit to the data with any value of k_{+5}/k_{+6} in the range 0.001–1000, and doubtless also for values outside this range. Thus it was clear that no definitive values could be assigned to the rate constants without using additional information. Fortunately, such additional information existed in the form of studies of inhibition by acetaldehyde, from which Wong *et al.* (1972) had been able to place the following limits on k_{+6} , which differ from those given by Wong

Fig. 2. Lack of information about the value of k_{+5}/k_{+6} . The data shown in Table 1 were fitted to eqn. (1) for each value shown for k_{+5}/k_{+6} , the other eight parameters being allowed to vary independently. Note that a highly expanded scale is used for the ordinate and that the sum of squares varies little over the range of k_{+5}/k_{+6} values shown. The arrow indicates the value of 18.5 assumed for k_{+5}/k_{+6} on the basis of other information. For $k_{+5}/k_{+6} = 18.5$ the value of the sum of squares is 0.03171, which is not significantly greater than the minimum of 0.03055; both give the same mean square of 0.00113 when divided by the numbers of degrees of freedom (28 and 27 respectively).

et al. (1972) by a factor of 4.4s^{-1} because of a difference in units:

$$4.44\text{s}^{-1} < k_{+6} < 5.06\text{s}^{-1} \quad (2)$$

Taking a value of 4.75s^{-1} as representative of this range for k_{+6} , and assuming that $k_{\text{cat.}} = k_{+5}k_{+6}/(k_{+5} + k_{+6}) = 4.47\text{s}^{-1}$, we obtained a value of about 18.5 for k_{+5}/k_{+6} . We assumed this value in the remainder of the work described in this paper. Although it does not give the smallest possible sum of squares (see Fig. 2), the difference is not significant. (The variance ratio F is 1.0, which is not significant at any level of confidence.)

Estimation of the precision of the fitted parameters

The program that we have used attempts to estimate variances for the best-fit parameters from the inverse of the matrix of second partial derivatives of the sum of squares with respect to the parameters. This is a widely used method, but one that is of questionable validity when applied to severely non-linear models (see Draper & Smith, 1966). It gave meaningless results with the data shown in Table 1, presumably because of the gross non-linearity of the sum-of-squares surface (cf. Fig. 1, which can be regarded as a two-dimensional projection of a surface in nine dimensions). The program sometimes obtained negative variances and sometimes variances that were ridiculously small, e.g. a

coefficient of variation of 0.5% for a parameter that was certainly not precisely defined.

In view of the clear failure of this method of estimating the precision of the parameters, we decided to investigate the possibility of using a jackknife instead. For this purpose we divided the 6×6 grid of observations according to a latin square (as indicated in Table 1), to give six exclusive sets of six observations, such that within each set each NAD^+ concentration was represented once and each ethanol concentration was represented once. We then obtained six sets of 30 observations, with each set of six observations omitted in turn, and fitted eqn. (1) to each of these sets of 30 observations, as well as to the complete set of 36. The jackknife was applied to the results of this fitting exercise, as detailed in Table 2, and the parameter values and precision estimates shown in Table 3 were obtained.

Discussion

In this work we have shown that it is possible for some mechanisms of enzyme-catalysed reactions to obtain values of the elementary rate constants from steady-state measurements alone. For some of the simpler mechanisms this can be done by rearranging the definitions of the 'observable parameters' in terms of the rate constants (Cleland, 1963). However, this method does not provide information about the precision of the values determined for the rate constants and is thus of limited utility. Alternatively one can fit the data directly to a rate equation expressed in terms of rate constants, and we have found that this method can be applied to a mechanism as complicated as that shown in Scheme 1. In contrast, transient-state methods have met with limited success with such mechanisms.

The conventional matrix-inversion method produced absurd results, as described in the Results section, when used to assess the precision of the rate constants obtained with the data in Table 1. This was not unexpected, for the reasons outlined in the Theory section. The jackknife technique, on the other hand, gave results (Table 3) that are entirely reasonable. Parameters such as $k_{\text{cat.}}$, which is known to be well-defined by the data at high substrate concentrations, were assigned small coefficients of variation; parameters such as k_{-1} , which has comparatively little effect on the calculated rate, were assigned large coefficients of variation.

The jackknife has a further advantage over other methods of assessing precision, in that it can be applied as easily to derived parameters as to the primary parameters used in the fitting procedure. For example, K_s^{Ethanol} is not a member of the parameter set used for fitting the data in Table 1; instead it is a derived parameter with the value $(k_{\text{cat.}})(k_{\text{cat.}}/K_m^{\text{NAD}^+})/[(k_{\text{cat.}}/K_s^{\text{NAD}^+}) - (k_{\text{cat.}}/K_m^{\text{Ethanol}})]$. Now al-

Table 2. *Application of the jackknife to the data in Table 1*

The parameter set used for fitting the data comprised all of the parameters listed in this Table apart from K_s^{Ethanol} , which is included to show that the jackknife can be applied just as readily to derived parameters as to primary parameters. The letters A, B, C, D, E and F in the top line show which groups of observations from Table 1 were used to obtain the $\ln \hat{b}$ values shown beneath. The corresponding $\ln \bar{b}_i$ values were calculated from the formula $\ln \bar{b}_i = (36 \ln \hat{b}_0 - 30 \ln \hat{b}_{-i})/6$.

Groups	ABCDEF	BCDEF	ACDEF	ABDEF	ABCEF	ABCDF	ABCDE	
Parameter	$\ln \hat{b}_0$	$\ln \hat{b}_{-1}$	$\ln \hat{b}_{-2}$	$\ln \hat{b}_{-3}$	$\ln \hat{b}_{-4}$	$\ln \hat{b}_{-5}$	$\ln \hat{b}_{-6}$	$\ln \bar{b}_0^*$
$k_{+1}/\text{mm}^{-1} \cdot \text{s}^{-1}$	1.805	1.815	1.794	1.744	1.801	1.832	1.817	1.826 ± 0.063
$k_{+2}/\text{mm}^{-1} \cdot \text{s}^{-1}$	3.443	3.413	3.380	3.592	3.448	3.407	3.475	
k_{+3}/s^{-1}	3.996	4.003	3.987	3.911	3.997	4.031	3.992	3.398 ± 0.155
k_{+4}/s^{-1}	4.313	4.324	4.292	4.382	4.325	4.330	4.296	
$k_{\text{cat.}}/\text{s}^{-1}$	1.499	1.499	1.495	1.493	1.508	1.498	1.498	4.044 ± 0.082
$k_{\text{cat.}}/K_m^{\text{Ethanol}}/\text{mm}^{-1} \cdot \text{s}^{-1}$	2.250	2.254	2.260	2.257	2.233	2.257	2.238	
$k_{\text{cat.}}/K_m^{\text{NAD}^+}/\text{mm}^{-1} \cdot \text{s}^{-1}$	5.735	5.741	5.730	5.738	5.714	5.765	5.727	4.260 ± 0.066
$k_{\text{cat.}}/K_s^{\text{NAD}^+}/\text{mm}^{-1} \cdot \text{s}^{-1}$	6.079	6.066	6.183	6.065	6.085	6.025	6.078	
$K_s^{\text{Ethanol}}/\text{mm}$	-1.095	-1.081	-1.218	-1.091	-1.096	-1.018	-1.091	4.497 ± 0.011
		-1.172	-0.483	-1.119	-1.093	-1.483	-1.119	2.250 ± 0.023
								5.732 ± 0.035
								6.057 ± 0.108
								-1.078 ± 0.133

* Mean and S.E.M. of six pseudo values.

Table 3. *Kinetic parameters for horse liver alcohol dehydrogenase*

The Table shows the values and precision estimates of the rate constants and other kinetic parameters obtained after applying the jackknife as detailed in Table 2. Abbreviation: c.v., coefficient of variation.

Parameter	Definition	Units	$\ln(\text{Value}) \pm \text{S.E.M.}$	Value $\pm$ c.v.
k_{+1}		$\text{mm}^{-1} \cdot \text{s}^{-1}$	1.826 ± 0.063	$6.21 \pm 6.5\%$
k_{+2}		$\text{mm}^{-1} \cdot \text{s}^{-1}$	3.398 ± 0.155	$29.9 \pm 17\%$
k_{+3}		$\text{mm}^{-1} \cdot \text{s}^{-1}$	6.236 ± 0.053	$510 \pm 5.5\%$
k_{+4}		$\text{mm}^{-1} \cdot \text{s}^{-1}$	2.845 ± 0.045	$17.2 \pm 4.6\%$
k_{-1}		s^{-1}	0.747 ± 0.165	$2.11 \pm 18\%$
k_{-2}		s^{-1}	-1.164 ± 0.215	$0.31 \pm 24\%$
k_{-3}		s^{-1}	4.044 ± 0.082	$57.1 \pm 8.5\%$
k_{-4}		s^{-1}	4.260 ± 0.066	$70.8 \pm 6.8\%$
$k_{\text{cat.}}$	$k_{+5}k_{+6}/(k_{+5} + k_{+6})$	s^{-1}	1.497 ± 0.011	$4.47 \pm 1.1\%$
K_m^{Ethanol}	$\frac{k_{+4}k_{+6} + k_{+5}k_{+6}}{k_{+3}(k_{+5} + k_{+6})}$	mm	-0.750 ± 0.029	$0.472 \pm 3.0\%$
$K_m^{\text{NAD}^+}$	$\frac{k_{-3}k_{+6} + k_{+5}k_{+6}}{k_{+3}(k_{+5} + k_{+6})}$	mm	-4.237 ± 0.041	$0.0145 \pm 4.2\%$
K_s^{Ethanol}	k_{-1}/k_{+1}	mm	-1.078 ± 0.133	$0.340 \pm 14\%$
$K_s^{\text{NAD}^+}$	k_{-2}/k_{+2}	mm	-4.564 ± 0.110	$0.0104 \pm 12\%$
$k_{\text{cat.}}/K_m^{\text{Ethanol}}$		$\text{mm}^{-1} \cdot \text{s}^{-1}$	2.250 ± 0.023	$9.85 \pm 2.3\%$
$k_{\text{cat.}}/K_m^{\text{NAD}^+}$		$\text{mm}^{-1} \cdot \text{s}^{-1}$	5.733 ± 0.035	$309 \pm 3.6\%$
$k_{\text{cat.}}/K_s^{\text{Ethanol}}$		$\text{mm}^{-1} \cdot \text{s}^{-1}$	2.574 ± 0.131	$13.1 \pm 14\%$
$k_{\text{cat.}}/K_s^{\text{NAD}^+}$		$\text{mm}^{-1} \cdot \text{s}^{-1}$	6.058 ± 0.108	$427 \pm 11\%$

though formulae exist for calculating variances of derived quantities, these are complicated if several correlated variables are involved and they are predicated on assumptions that may well be incorrect. The jack-

knife avoids such complexities altogether because $\hat{b}_0$ and $\hat{b}_{-i}$ values for the derived parameters can be calculated before the jackknife is applied rather than afterwards. Thus for each of the 17 parameters listed

in Table 3 the precision was estimated independently, even though the model contains only eight independent parameters (one of the original nine being lost when the value of 18.5 was assumed for k_{+5}/k_{+6}). It is for this reason that the values in Table 3 are not precisely consistent; for example, division of $k_{\text{cat.}} = 4.47 \text{ s}^{-1}$ by $K_m^{\text{Ethanol}} = 0.472 \text{ mM}$ gives $9.47 \text{ mM}^{-1} \cdot \text{s}^{-1}$, slightly different from the value of $9.85 \text{ mM}^{-1} \cdot \text{s}^{-1}$ obtained by applying the jackknife independently to $k_{\text{cat.}}/K_m^{\text{Ethanol}}$.

The main disadvantage of the jackknife is that it requires much more computation than the matrix-inversion approach. In the work described in this paper it was necessary to fit the data seven times by a non-linear least-squares technique rather than once. If the 36 observations had been divided into more than six groups the amount of computing required would have been correspondingly higher. In the limit 36 groups (each containing one observation) could have been used, and 37 runs of the regression program would have been required. Although this difficulty presents a serious obstacle to carrying out simulation studies, we do not feel that it outweighs the advantages of the jackknife as a technique for analysing real data, because even with seven or more runs of the regression program the computing cost is likely to be small in comparison with the cost of collecting the data.

Although our main concern in this work has been to study the methodology required for the analysis of complex kinetic data, it is pertinent to compare the results obtained with other measurements of the kinetic parameters of alcohol dehydrogenase from horse liver; the remainder of the paper will be devoted to such a comparison.

Hanes *et al.* (1972) observed activation of the enzyme by an impurity in acetaldehyde, which is rapidly formed during storage at -20°C and present even in freshly distilled acetaldehyde. As a result they could estimate only an upper limit of 4.4 s^{-1} for the saturation rate constant for acetaldehyde reduction, i.e. the same as that for the forward reaction, ethanol oxidation. According to Table 3, k_{-1} is small compared with k_{-3} , and k_{-2} is small compared with k_{-4} , so the saturation rate constant for the reverse direction should be close to $(k_{-1} + k_{-2})$, or 2.42 s^{-1} . This is consistent with the observations of Hanes *et al.* (1972) for the reverse reaction, though it should be noted that k_{-1} and k_{-2} are the least precisely defined values in Table 3.

Brooks *et al.* (1972) determined directly the rate constant for the chemical step in the reaction, i.e. the conversion of the enzyme-NAD⁺-ethanol ternary complex into the enzyme-NADH-acetaldehyde complex, and obtained a value of 155 s^{-1} . Steady-state measurements cannot separate this step from the subsequent acetaldehyde-release step, and consequently k_{+5} in Scheme 1 refers to the sequence of

these two steps and cannot exceed either of the two individual rate constants. If $k_{+5}/k_{+6} = 18.6$, as assumed above, the value of $k_{\text{cat.}} = 4.47 \text{ s}^{-1}$ implies that k_{+5} is about 90 s^{-1} , which is less than 155 s^{-1} and therefore consistent with the observations of Brooks *et al.* (1972).

Shore & Gilleland (1970) measured the binding of NAD⁺ to enzyme in the presence of excess pyrazole, and obtained a combination rate constant of $840 \text{ mM}^{-1} \cdot \text{s}^{-1}$. This value would be comparable with k_{+2} in Scheme 1 if no enzyme-pyrazole binary complex is formed, but with k_{+3} if such a complex is formed. As pyrazole acts as an inhibitor competitive with respect to ethanol, one may assume that an enzyme-pyrazole complex analogous to the enzyme-ethanol complex is formed in the presence of excess pyrazole. The value of $k_{+3} = 510 \text{ mM}^{-1} \cdot \text{s}^{-1}$ from Table 3 for the binding of NAD⁺ to the enzyme-ethanol complex is similar in magnitude to the value of $840 \text{ mM}^{-1} \cdot \text{s}^{-1}$ obtained by Shore & Gilleland (1970) for binding of NAD⁺ to the enzyme-pyrazole complex, and indicates that both binary complexes bind to NAD⁺ much more rapidly than the free enzyme, for which k_{+2} from Table 3 is $29.9 \text{ mM}^{-1} \cdot \text{s}^{-1}$.

On the basis of a compulsory-order mechanism in which the coenzyme binds to the enzyme before the substrate in both forward and reverse reactions, Dalziel (1963) found k_{+2} to be $830 \text{ mM}^{-1} \cdot \text{s}^{-1}$ and k_{+4} to be greater than $10 \text{ mM}^{-1} \cdot \text{s}^{-1}$. The value of $k_{+4} = 17.2 \text{ mM}^{-1} \cdot \text{s}^{-1}$ from Table 3 is consistent with these results, but the value of $k_{+2} = 29.9 \text{ mM}^{-1} \cdot \text{s}^{-1}$ is not. There are likely to be several reasons for this disagreement. Dalziel (1963) used phosphate/glycine buffer, pH 9.0, at 23.5°C , and an enzyme preparation now known to contain multiple isoenzymes with non-uniform kinetic properties (Theorell *et al.*, 1970; Lutstorf *et al.*, 1970; Gurr *et al.*, 1972). The measurements of Hanes *et al.* (1972) that yielded the rate constants of Table 2 were done in pyrophosphate buffer, pH 8.6, at 27°C , with a single purified isoenzyme. Perhaps more important, the mechanism used by Dalziel (1963) for calculating the rate constants given above did not allow for the occurrence of an enzyme-ethanol complex. The existence of such a complex was suggested by Theorell & McKinley-McKee (1961) and Sund & Theorell (1963), and has been amply confirmed by the analysis of Hanes *et al.* (1972). The compulsory-order mechanism is, moreover, inconsistent with the observation by Silverstein & Boyer (1964) that exchange between NAD⁺ and NADH could not be eliminated by saturating the enzyme with acetaldehyde and ethanol. By contrast, this finite NAD⁺-NADH exchange is required by the mechanism of Hanes *et al.* (1972), which proposes random additions of NAD⁺ and ethanol to the enzyme in the forward direction, but a compulsory order with addition of NADH before acetaldehyde

in the reverse direction. Excess ethanol and acetaldehyde do not therefore block the exchange between free and enzyme-bound NAD^+ . A similar exchange for NADH is blocked along the main reaction pathway, but permitted along a side reaction branch generated by the reaction of excess ethanol with the enzyme-NADH complex (Wong & Hanes, 1964). Accordingly, the finite NAD^+ -NADH exchange in the presence of excess ethanol and acetaldehyde is expected to be much slower than the ethanol-acetaldehyde exchange in the presence of excess NAD^+ and NADH, in complete agreement with the observations of Silverstein & Boyer (1964).

Taniguchi *et al.* (1967) determined the dissociation constant of the enzyme-NAD⁺ binary complex directly as a function of pH; the value for pH 8.6, interpolated from their data, is $12.3 \mu\text{M}$. This agrees to within 20% with the value of $k_{-2}/k_{+2} = 10.4 \mu\text{M}$ given in Table 3.

In conclusion, the good agreement between the results in Table 3 with comparable values obtained in other ways provides some support for the validity of the approach suggested in this paper.

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