Some Examples of the Use of Computer-Produced Contour Plots in the Fitting of Enzyme Rate Equations to Reaction-Velocity Measurements

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(Received 12 July 1972)

The use of computer-based isotach plots, relating reaction velocity to simultaneous variation of two substrates or effectors of an enzyme, in producing estimates of the parameters of enzyme rate equations was investigated. The computer program ('SYMAP') incorporates an interpolation algorithm, and the superiority of this over visual estimation in producing interpolated velocity values for the estimation of parameter values by conventional double-reciprocal plots is described. The usefulness of the SYMAP program in monitoring the process of fitting data obtained by simultaneous changes in two experimental variables is also described. It is shown that if the residual errors are weighted by a procedure described elsewhere (Ottaway, 1971*b*, 1973), the percentage error of the computed velocity is distributed evenly over a plot which contains a 100-fold variation in the concentration of one substrate and a 500-fold variation in the concentration of Mg^{2+} , and in which the velocity of the reaction (that catalysed by NAD kinase) varies over a 60-fold range. The two-dimensional percentage error plot was used to assess the limits within which an incomplete inhibition equation is valid, and to detect a discrepancy in an expected good fit, caused by an impurity in one of the substrates.

The traditional method of determining the disposable constants of enzyme rate equations, the double-reciprocal plot, permits the determination of only two constants for each plot. For a two-substrate enzyme, therefore, unless the mechanism is Ping Pong, three plots are required in principle, and to obtain true estimates of the Michaelis constant for each substrate several more plots are required in practice to extrapolate to the limiting value. This is experimentally tedious, and at the same time there is a temptation to restrict the collection of data to regions which are favourable for the production of reciprocal plots, and not to explore the whole area of measurable catalytic activity.

The situation is even more complicated if inhibitors and activators must be taken into consideration, and here it is relevant that of the 970 enzymes listed by the International Enzyme Commission in 1964, no less than 350 have at least one substrate which is a derivative of phosphoric acid, and of these the vast majority are affected in some way by magnesium, so that terms involving Mg^{2+} , free substrate and complex ion must be inserted in the rate equation. There is therefore a need for computational methods that will permit the estimation of many constants from a single set of data, in the collection of which more than one substrate or effector had been simultaneously varied. It is now technically possible, by the use of optimization (function-minimizing) algorithms (Reich *et al.*, 1972; Ottaway, 1971*a*) to fit a complete

general rate equation to a suitable data set; it is, however, in our view desirable to use a somewhat simplified equation for which the fit to the data can be represented visually in some way, since the eye and the brain are still unrivalled in assessing the significance of discrepancies in the fit of theory to data. On a two-dimensional surface it is possible to represent three variables, for example two independent and one controlled, and we think that it is best to limit innovation to the manipulation of a matrix of results obtained by the simultaneous variation of only two of the possible substrates or effectors of the enzyme. In the present paper, this approach is used with some steady-state kinetic data obtained with NAD kinase (EC 2.7.1.23), at widely varying concentrations of ATP and Mg^{2+} ion. Previous kinetic studies of this enzyme (Apps, 1968, 1969, 1971*a*) have shown that under conditions of low [free ATP] and low [free Mg^{2+}] its activity can be described by the rate equation:

$$v_0 = \frac{V_{\max.}}{1 + \frac{K_A}{[\text{MA}]} + \frac{K_B}{[\text{NAD}^+]} + \frac{K_{AB}}{[\text{MA}][\text{NAD}^+]}} \quad (1)$$

where [MA] is the concentration of the complex MgATP^{2-} , and $K_{AB} = K_A \times K_B$, where K_A and K_B are the Michaelis constants for MgATP^{2-} and NAD^+ , respectively. More recently (Apps & Marsh, 1972) it has been shown that inhibition by free ATP is accounted for by the rate equation:

$$v_0 = \frac{V_{\max.}}{1 + \frac{K_A}{[MA]} \left(1 + \frac{[A]}{K_1}\right) + \frac{K_B}{[NAD^+]} \left(1 + \frac{[A]}{K_2}\right) + \frac{K_{AB}}{[MA][NAD^+]} \left(1 + \frac{[A]}{K_1}\right) \left(1 + \frac{[A]}{K_2}\right)} \quad (2)$$

whereas inhibition by free Mg^{2+} ion is satisfactorily described by the rate equation:

$$v_0 = \frac{V_{\max.}}{1 + \frac{[M]}{K_3} + \frac{K_A}{[MA]} \left(1 + \frac{[M]}{K_4}\right) + \frac{K_B}{[NAD^+]} \left(1 + \frac{[M]}{K_5}\right) + \frac{K_{AB}}{[MA][NAD^+]} \left(1 + \frac{[M]}{K_6}\right)} \quad (3)$$

In these equations [A] is the total concentration of free ATP, and [M] that of free Mg^{2+} and K_1 – K_6 are inhibition constants.

These rate equations were inferred from the apparent values of the parameters $V_{\max.}$, K_A , K_B and K_{AB} , which were derived from conventional double-reciprocal plots, in which the substrate concentrations were varied independently over a 10–20-fold range, with several discrete concentrations of each inhibitory species. It was decided to investigate whether a rate equation combining the terms in eqns. (2) and (3), and thus describing the dependence of the activity on $[ATPMg^{2-}]$, [ATP], $[Mg^{2+}]$ and $[NAD^+]$, would be valid over a wide range of concentrations of these species. Rate measurements were therefore performed with the total Mg^{2+} concentration varied over a 500-fold range, and the total ATP concentration over a 100-fold range, the concentration of NAD^+ being fixed. These data could be conveniently presented *in toto* on a contour or isotach plot (Fig. 1). Such plots have been used to present visually the effect of pH on the high-substrate inhibition of malate dehydrogenase activity (Thorne, 1962), the variation of $\Delta G'_0$ for the hydrolysis of ATP with pH and p (metal ion) (Shikama, 1971), the effect of $[Mg^{2+}]$ and [ATP] on pyruvate carboxylase activity (McClure *et al.*, 1971), and the effect of pH and ionic strength on the activity of bacteriophage T4 lysozyme (Jensen & Kleppe, 1972). Plots of this kind are experimentally easy to construct, as the total concentrations of ATP and Mg^{2+} (or any two independent variables) are varied on a simple logarithmic matrix; however, interpolation by hand of the rate values is difficult, and extraction of data for use in conventional (e.g. double-reciprocal) plots, which demand that only one component is varied at any time, is correspondingly laborious. The use of the computer for the generation and processing of interpolated values is described below.

Methods

The methods of purification of NAD kinase, and measurement of its activity, have been described elsewhere (Apps, 1968).

NAD^+ was from C. F. Boehringer und Soehne (Mannheim, Germany); ATP was from Sigma Chemical Co. (St. Louis, Mo., U.S.A.) (highest grade, from horse muscle). In all experiments the ionic strength was maintained at 0.4M, by the addition of KCl when necessary; rate measurements were all performed in 0.1M-triethanolamine-HCl buffer, pH 7.4.

The contour plots ('isotachs') shown in Figs. 1–5 were constructed by the SYMAP program (version 5) developed by and available from the Laboratory for Computer Graphics and Spatial Analysis, Harvard Centre for Environmental Design Studies, Graduate School of Design, Harvard University, Cambridge, Mass. 02138, U.S.A., and run on an IBM 360/50 computer. The program is widely used by geographers, and should be available in most university computer centres; the authors of this paper cannot supply copies. The computer lineprinter prints ten characters to the inch in each row, and either the usual six rows to the inch, or ten rows to the inch on lineprinters that have this facility. Thus each square of the map contains 60 or 100 symbols. The program computes an interpolated value for each of the points that is not already provided by the input data, by using a rather sophisticated two-dimensional interpolation algorithm; this interpolated value is then used to determine the correct symbol for each position on the printed map. There is no provision for using a different scale (in units per inch of print-out paper) for the two co-ordinates. Thus if the program is set for printing at six rows to the inch, the interpolation density per unit of concentration will only be six-tenths as great in the vertical dimension as in the horizontal. Unless the user wishes to scale the concentration units which are input into the program, this discrepancy may be avoided, when required, by specifying in the program that printing will be at ten lines to the inch; this guarantees that interpolation is equivalent in both dimensions. It should be pointed out that printing may actually be done at six lines to the inch when the program is set in this way. The resulting plot will be distorted in the vertical dimension but the accuracy of interpolation will have been preserved. The difference may be considerable.

Fig. 1. Plot of v_0 against $\log [\text{total ATP}]$ (abscissa) and $\log [\text{total Mg}^{2+}]$ (ordinate) for NAD kinase

$[\text{NAD}^+]$ was fixed at 1.06 mM. The contours were drawn at approximately 10, 20, 40, 60, 100, 135, 175 and 225 arbitrary velocity units. The region in which $v_0 < 10$ units is unshaded. The open circles represent experimental points.

The interpolation feature of the program was used to obtain estimates of the velocities corresponding to certain values of the two co-ordinates, i.e. to certain values of $[\text{total Mg}^{2+}]$ and $[\text{total ATP}]$. These estimates were then used in computing values of some of the constants in the rate equation. For example, if $[\text{free Mg}^{2+}]$ is fixed at 10 mM, it may be assumed that $[\text{free ATP}]$ is small enough to be neglected. A plot of $1/v_0$ against $1/[\text{MgATP}^{2-}]$ will then enable K_3 – K_6 to be calculated, if the procedure is carried out at a number of values of $[\text{NAD}^+]$. To obtain values of v_0 from the map, a series of values of $[\text{ATPMg}^{2-}]$ was chosen, and a small computer program written to carry out three operations.

(1) To calculate the corresponding values of $[\text{total Mg}^{2+}]$ and $[\text{total ATP}]$, by using the relationships:

$$\frac{[\text{ATPMg}^{2-}]}{[\text{free Mg}^{2+}][\text{free ATP}]} = K$$

$$[\text{total ATP}] = [\text{free ATP}] + [\text{ATPMg}^{2-}]$$

and

$$[\text{total Mg}^{2+}] = [\text{free Mg}^{2+}] + [\text{ATPMg}^{2-}]$$

the value of K being taken to be 20 mM^{-1} (Phillips, 1966).

(2) 100 $[\text{total ATP}]$ and 100 $[\text{total Mg}^{2+}]$ were converted into their respective logarithms, to correspond with the logarithmic co-ordinates which were

used in printing the map: for each chosen value of $[\text{ATPMg}^{2-}]$ the nearest pair of discrete co-ordinates, corresponding to a map symbol, were found which corresponded to the values of $[\text{total ATP}]$ and $[\text{total Mg}^{2+}]$, computed as in (1).

(3) The program we are now describing searched through this array and picked out the velocities corresponding to the co-ordinate pairs calculated as described in (2). These velocities were printed out and used for constructing reciprocal plots, initially by hand. In later studies this sequence was replaced by an optimization procedure (Nelder & Mead, 1964) which computed the best fit of all the experimental points to the velocities computed from a model rate equation (cf. Davis & Ottaway, 1972). The fit was obtained by minimizing the normalized sum of squares of the residual errors (Ottaway, 1971*b*, 1973). This procedure was also used to estimate constants of the rate equation, in experiments in which both $[\text{NAD}^+]$ and $[\text{ATPMg}^{2-}]$ were varied.

To visualize the fit of the various rate equations to the experimental data, the percentage error, $100 \times (v_{\text{exp.}} - v_{\text{calc.}})/v_{\text{exp.}}$, without regard to sign, was calculated for each experimental point, and was input as a 'height' into the SYMAP program, together with the co-ordinates corresponding to the substrate concentrations. A 'badness of fit' contour map was thus produced, with the darker symbols indicating large percentage deviations between calculated and experimental velocities (see Figs. 2–5). The advantages and limitations of this procedure are discussed below.

For reproduction in the *Biochemical Journal*, the plots were printed on 'reprographic' quality paper, and by using a new lineprinter ribbon.

Results

The data available were: (1) matrices of 126 or 156 rate measurements, with $[\text{total Mg}^{2+}]$ and $[\text{total$

$\text{ATP}]$ varied independently over the range 0.2–100.0 and 0.2–20.0 mM, respectively; $[\text{NAD}^+]$ was fixed at 2.0 or 1.0 mM; and (2) a series of 25- or 36-point matrices, in which both $[\text{NAD}^+]$ and $[\text{total ATP}]$ were varied independently. $[\text{Total Mg}^{2+}]$ was varied to produce a fixed concentration of free Mg^{2+} or free ATP throughout each matrix.

As a first step, the SYMAP interpolation algorithm was used, as explained in the Methods section, to provide interpolated values of v_0 on a 'profile', i.e. a projection, on the velocity surface of the isotach, of a line drawn in the $([\text{total Mg}^{2+}], [\text{total ATP}])$ plane. This line was drawn by fixing a value of either $[\text{free Mg}^{2+}]$ or $[\text{free ATP}]$, which fixes a unique relationship between the total concentrations of Mg^{2+} and of ATP. From the interpolated rates for selected values of $[\text{ATPMg}^{2-}]$ reciprocal plots were then produced manually. The results were comparable with those obtained previously (Apps & Marsh, 1972), although not all the inhibition constants could be estimated by this method, as one of the substrate concentrations, $[\text{NAD}^+]$, was not varied.

Next, the optimization routine was employed; as explained in the Methods section, this used directly all the information provided by rate measurements, yielding optimum values of the Michaelis and inhibition constants in the various rate equations. This was used in three ways.

(1) It was tested with four small data sets, to obtain estimates of $V_{\text{max.}}$, K_A , K_B and K_{AB} (when the concentrations of free Mg^{2+} and free ATP were negligible), which were then used in the calculation of the six postulated inhibition constants (when either $[\text{free Mg}^{2+}]$ or $[\text{free ATP}]$ were significant). These estimates are in good agreement with those obtained from conventional double-reciprocal plots (Apps & Marsh, 1972); the values are compared in Table 1.

(2) The optimization routine was then used with the large data set. The six inhibition terms of equations

Table 1. *Values of Michaelis and inhibition constants in the NAD kinase rate equation, as defined in eqns. (1), (2) and (3)*

The values compared were derived from: (a) small data sets with both substrate concentrations varied in the presence of fixed inhibitor concentrations, by using reciprocal plots (Apps & Marsh, 1972); (b) the same data, by using the optimization program (Nelder & Mead, 1964); (c) a large data set, where total $[\text{ATP}]$ and total $[\text{Mg}^{2+}]$ were varied, $[\text{NAD}^+]$ being fixed at 2.0 mM, by again using the optimization program.

Method of derivation	Parameters								
	K_A (mM)	K_B (mM)	K_{AB} (mM)	K_1 (mM)	K_2 (mM)	K_3 (mM)	K_4 (mM)	K_5 (mM)	K_6 (mM)
(a) Reciprocal plots	1.69	0.47	0.79	13.9	11.9	150	19	27	27
(b) Optimization (small data sets)	1.42	0.37	0.63	6.3	18.9	151	14	25	26
(c) Optimization (large data set)	3.33	0.35	1.17	11.3	8.7	1811	1173	221	7

Fig. 2. Percentage error $[100 (v_{\text{exp.}} - v_{\text{calc.}})/v_{\text{exp.}}]$ for the fit of data to an equation predicting inhibition by both free Mg^{2+} and free ATP (combination of eqns. 2 and 3) to calculate v_0

The error averages 10% over the whole plot, except for the bottom left-hand corner. No penalty was imposed on the residual errors. The contours were drawn at 7.5, 15, 25 (and 50)% deviation from the experimental results. The area in which percentage error is less than $\pm 7.5\%$ is unshaded.

(2) and (3) were combined in a general rate equation, describing inhibition by both free Mg^{2+} and free ATP; this equation was applied to the entire matrix of results, all the constants being allowed to vary. The fit is satisfactory overall (Fig. 2) except when the free Mg^{2+} concentration is 90mM or over, and the concentration of ATPMg^{2-} less than 1 mM (cf. also Fig. 3). Eqn. (3) does not predict the inhibition of the enzyme in this region, but its failure to do so is not regarded as serious. Except in this region of the plot, the percentage error was less than 20%, in large

areas much less than this. Moreover, as would be expected if the distribution of the experimental error was unbiased, the error was just as frequently negative as positive, and errors of either sign were rather randomly distributed over the graph. Therefore, in arranging the symbolism for Fig. 2, the sign of the error was neglected, and the symbol '+' for example, indicates all errors lying between +7.5 and +15.0%, and between -7.5 and -15.0%. The Michaelis and inhibition constants computed by this procedure also appear in Table 1. The values of K_A , K_B , K_1 and K_2

are in good agreement with the other estimates in Table 1; however, inhibition by free Mg^{2+} is adequately described, in this experiment, by a single finite term (i.e. that containing K_6), since K_3 , K_4 and K_5 , as defined in eqn. (3), are very large in the optimized solution. This simply confirms that inhibition by free Mg^{2+} is competitive with respect to $ATPMg^{2-}$, as shown previously (Apps & Marsh, 1972). Since $[NAD^+]$ was not varied in this plot, it cannot be deduced that inhibition by free Mg^{2+} is virtually uncompetitive with respect to this sub-

strate; however, the data of Table 1, lines 1 and 2, obtained from matrices in which $[NAD^+]$ was variable, confirm this type of inhibition, which is required by the mechanism deduced by Apps & Marsh (1972). It is noteworthy that K_5 and K_6 (Table 1, lines 1 and 2) are equal to the independently measured dissociation constant of the complex formed between NAD^+ and Mg^{2+} , also as predicted (Apps & Marsh, 1972). A further factor in the discrepancy between K_3 – K_6 in Table 1, lines 2 and 3, may be that the values in Table 1, line 3, apply to an

Fig. 3. Percentage error plot for the fit of data to rate eqn. (3), which predicts inhibition by free Mg^{2+} only, plotted against log [total ATP] and log [total Mg^{2+}]

The heavy lines indicate boundaries between a positive error (apparent activation) in the central region and a negative error (inhibition). Above and to the right of the upper line inhibition by free ATP was important; elsewhere the restricted equation fitted the data satisfactorily. Positive and negative errors are shown separately; for symbolism see the accompanying key.

equation describing inhibition by free Mg^{2+} over the concentration range 0.2–100mM, as discussed above, whereas Apps & Marsh (1972) presented some evidence for anomalous behaviour in the region 0–10mM-free Mg^{2+} , a region not explored in the other data used to produce the constants in Table 1.

The optimization routine was used to investigate this further by using eqns. (2) and (3), which take account of inhibition only by free ATP, and only by free Mg^{2+} , separately. These equations were first

used on restricted regions of the large data matrix where respectively [free Mg^{2+}] and [free ATP] could be assumed to be negligible. The fit of each equation was visualized by the SYMAP program, and found to be satisfactory, i.e. percentage error fairly randomly distributed, and generally less than 15%. The SYMAP program was then used to try to determine the limits within which rate eqn. (3) is valid; Apps & Marsh (1972) had inferred that the equation did not apply when [free Mg^{2+}] was less than 10mM. Fig. 3

Fig. 4. Percentage error plot for the same data and equations as in Fig. 3

In this optimization the square of the residual error was multiplied by 3 if $v_{\text{calc.}}$ lay between 92.5 and 85% of $v_{\text{exp.}}$, by 9 if it lay between 85 and 75%, and by 27 if it was less than 75% of $v_{\text{exp.}}$. No penalty was imposed if $v_{\text{calc.}}$ was greater than 92.5% of $v_{\text{exp.}}$. Note the improvement in the fitting to the data in the central region. Contour boundaries and shading are as in Fig. 3.

shows the error plot when eqn. (3) was applied to the complete data set. At first sight the equation does not appear to fit the data at all, since the percentage error is greater than 20% everywhere except in narrow regions on either side of the heavy lines drawn on the graph. However, the error is not now randomly distributed; within the area bounded by the lines it is uniformly positive, indicating an apparent activation, most intense in the centre of the area. The lower left and the upper right areas of the plot, on the other hand, show a uniform inhibition of activity with respect to that predicted by eqn. (3). The former is due to the failure of the complete equation at very high $[Mg^{2+}]$, already mentioned; the latter delineates the region in which free ATP is present in significant concentrations, or by corollary, the concentration of free Mg^{2+} is negligible.

The clear separation of positive and negative error has been emphasized by using different symbols for the shading in Fig. 3 (and Fig. 4). The reason for the apparent activation in the centre of the plot is that the optimization program minimizes the sum of squares over the entire matrix of results, and 'compensates' for the inhibition in the top right-hand and bottom left-hand corners of Fig. 2 by an apparent activation in the centre of the plot. The latter can in principle be removed by imposing a penalty function on the optimization. If each normalized squared deviation is multiplied by a factor greater than 1 when $v_{calc.} < v_{exp.}$, the optimum solution can be forced to fit more exactly in the region in which eqn. (3) holds, with a correspondingly more severe inhibition in regions of poor fit. Fig. 4 demonstrates the principle; the apparent activation in the centre of the plot could be removed completely by the imposition of a greater penalty. The validity of this explanation is shown by the fact that if the data points in the upper right-hand section were omitted from the data set a perfectly satisfactory fit of eqn. (3) to all the remaining data could be obtained without the imposition of penalties. Similarly, a satisfactory fit of eqn. (2) could be obtained to a restricted matrix of results from which points corresponding to a significant concentration of free Mg^{2+} had been removed.

Although penalty functions may be useful in allowing one to discriminate between genuine and apparent activations and inhibitions, it is nevertheless dangerous to apply them, because one may be obscuring genuine deviations from the expected behaviour of the enzyme. The importance of the 'compensation' phenomenon in limiting the general approach to fitting equations to enzyme-kinetic data is referred to again in the Discussion section.

The results of this investigation confirmed the view of Apps & Marsh (1972) that there may be anomalies in the behaviour of the enzyme if $[free\ Mg^{2+}]$ is less than 10 mM. Below this concentration, departure from

obedience to eqn. (3) appeared to be independent of $[free\ ATP]$, but to depend on the concentrations of both free Mg^{2+} and of $ATPMg^{2-}$.

Discussion

The work reported in the present paper has shown that it is possible to use computer-produced isotach plots for several purposes. For those who wish to continue using double-reciprocal plots, the interpolation algorithm of the SYMAP program is a valuable aid in producing reliable estimates of the velocity appropriate to a particular concentration of the substrate, when the latter is not pre-determined by the experimental protocol. Its usefulness is well demonstrated in the present instance, when the experimental variables $[total\ Mg^{2+}]$ and $[total\ ATP]$, which were also the co-ordinates of the plot, were not the species which appear in the rate equations. Although we did not attempt to do so, it would be perfectly possible to store the interpolated velocities on a temporary file in the computer and to use them as input data for a regression program which would calculate the desired constants directly. The interpolation algorithm does not produce a smoothed contour, as can be seen very clearly at the boundary of the densest shading in Fig. 1.

A second use of these plots is to supervise the progress of optimization programs that fit the data directly to rate equations. Various authors (Hanson *et al.*, 1967; Kowalik & Morrison, 1968; Wieker *et al.*, 1970) have devised iterative procedures to solve particular problems, or have suggested a general strategy (Reich *et al.*, 1972) for determining the parameters of rate equations. A problem common to all such programs is that of weighting the data so that all observations are of equal importance in determining the best values of the parameters. This has been solved fairly satisfactorily for rate equations written in terms of a single variable substrate (see, e.g., Wilkinson, 1961; Cleland, 1967), but the distribution of the error for a non-linear equation containing two or more variables has not been studied. Reich *et al.* (1972), for example, give three methods of weighting which could be applied to non-replicate data, but leave the choice very much to the intuition of the user. The optimizations shown in Figs. 2–5 weighted the residual errors by a method described elsewhere (Ottaway, 1971b, 1973). It is a modification of the fractional error. A valuable feature of the present study is that it has shown that this device leads to the even distribution of the residual (fractional, or percentage) error over the whole matrix of measurements when a suitable rate equation is provided (Fig. 4), and that the estimates of the parameters produced are comparable with those produced by least-squares fitting of the conventional double-reciprocal plot (Table 1). Since the observations

covered a 60-fold range of velocity units, this is a fairly severe test.

By using the investigations reported here as a paradigm, we are not convinced that a completely general approach is valid, for several reasons.

(i) The complete rate equation, which predicts the behaviour of NAD kinase for most combinations of substrates and the two inhibitors Mg^{2+} and ATP^{4-} (or $ATPH^{3-}$) at constant ionic strength, contains ten disposable constants. This is a very general equation which can describe many mechanisms, and it is doubtful whether a meaningful decision between mechanisms can be made on the basis of treating a

single set of data. If, on the other hand, the data are restricted, the programs will tend to fit the data to the equation by manipulating the fewest possible disposable constants. For example, the large data set used for the preparation of Figs. 1–5 was produced at fixed $[NAD^+]$. When the optimization routine fitted either eqn. (3) alone, or a combination of eqns. (2) and (3), it made the constants K_3 , K_4 and K_5 very large, since the effect of inhibition by free Mg^{2+} can be allowed for by multiplying the term $K_A/[MA]$ alone by an inhibition factor. Although this correctly predicts competitive inhibition with respect to $ATPMg^{2-}$, it does not give correct values for any of

Fig. 5. Percentage error plot for a different set of data (see the text) from that used in Figs. 2–4, fitted by the complete equation used for Fig. 2

The unexpected inhibition on the right of the plot indicates an inhibitory impurity in the ATP. Contour limits are as in Fig. 2.

the four constants, which were only correctly obtained from sets in which both $[ATPMg^{2-}]$ and $[NAD^+]$ had been varied.

(ii) It has been pointed out above that optimization programs produce the best fit to the data *in toto* without taking account of regions in which a good fit is not to be expected. A deliberate example of this is shown in Fig. 3; the fact that eqn. (3) fits the large central portion of the data very well could not be appreciated without in some way visualizing the results. The general strategy therefore neglects the advantages of procuring a good fit to a restricted range of data, in other words, the piecemeal acquisition of understanding. An example of this is shown in Figs. 2, 3 and 4, where it is clear that the equation describing inhibition by Mg^{2+} fails to hold at very high Mg^{2+} concentrations. The aberration is trivial, especially since it occurs at such unphysiologically high concentrations of the ion, but a more accurate estimate of the constants would have been obtained if this region had been omitted from the optimization. A more serious example occurred in the work of Apps (1971b) and D. K. Apps & A. Marsh (unpublished work); in their studies there was an anomalous inhibition at high concentrations of free ATP, leading to an apparent effect on V_{max} , so that eqn. (2) failed to hold over the whole range of [total ATP]. The fitting of these data to the complete equation is shown in Fig. 5. (In the present studies this inhibition was not found, as evidenced by a good fit of the complete equation at the right-hand side of the plot in Fig. 4.) As mentioned elsewhere (Apps & Marsh, 1972) this inhibition is assumed to have been due to the presence of an impurity in the ATP used in the previous (unpublished) studies; ATP obtained from a different manufacturer was used in the work reported here. The spurious inhibition is severe enough to distort the isotach plots considerably, with a compensatory 'activation' at the top centre of Fig. 5. Artifacts of this kind must be not uncommon in enzyme studies, and in our opinion it is desirable to verify their absence by a visual check of the results. For a long time it has been customary to do this by means of reciprocal plots involving one controlled variable. The isotach plot enables a con-

siderable advance to be made in the information to be obtained in a single computation, while at the same time retaining the safeguard of visual examination of the results.

The optimization procedure of Nelder & Mead (1964) was used in these investigations. Although it is rather slow it is relatively independent of scaling factors (Davis & Ottaway, 1972), and it is possible to adapt it easily to work with a boundary constraint (J. H. Ottaway, unpublished work). However, other faster methods, such as that of Powell (1964), would work equally well.

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