# The kinetics of slow-binding and slow, tight-binding inhibition: the effects of substrate depletion

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Inhibitors with dissociation constants in the micromolar to nanomolar range are important, but hard to characterize kinetically, especially when the substrate concentration in the assay is less than $K_m$. When inhibition increases during the course of the assay (slow-binding inhibition) the concentration of substrate may decrease appreciably. Methods that take substrate depletion into account are described for analysing experiments in which the initial substrate concentration is below $K_m$. Fitting progress curves gives the rate constants for the second (slow) step in a two-step mechanism. An approximate value for the overall dissociation constant may be determined from measurements of rates when the reaction is treated as a first-order process. When the concentrations of inhibitor and enzyme are comparable numerical methods are required. Procedures, suitable for implementation on a microcomputer, for the solution of the differential equations and the fitting of progress curves are described.

## INTRODUCTION

Powerful enzyme inhibitors are increasingly important, both for the information they provide about enzyme mechanisms and for their medical importance. However, the characterization of such inhibitors is often less than straightforward. The inhibition characteristically sets in during the course of the assay; the initial rate is not easy to measure: this is slow-binding inhibition. Moreover, powerful inhibitors are effective at low concentrations. Thus it may be necessary to use concentrations of the inhibitor that are comparable with the concentration of enzyme. Then it is no longer possible to ignore the depletion of the concentration of unbound inhibitor: the enzyme–inhibitor complex is present at concentrations comparable with that of the unbound enzyme. This is tight-binding inhibition. The two features mentioned often occur together: this is slow, tight-binding inhibition (Morrison, 1982; Morrison and Walsh, 1988). The present paper starts by discussing the problems posed by substrate depletion in slow-binding inhibition, in the absence of tight-binding inhibition.

The usual standby in characterizing enzymic reactions, measurement of initial rates, cannot be accurately implemented by the customary method of measuring the slope of a progress curve near the origin because the slope is changing too rapidly (see, e.g., Figure 1a). Instead, the main procedure for characterizing slow-binding inhibition consists of fitting progress curves to eqn. (1), here called the 'burst equation':

$$p = v_s t + (v_0 - v_s)(1 - e^{-kt})/k \quad (1)$$

Here $p$ is the concentration of product at time $t$, $v_0$ and $v_s$ are the initial and steady-state rates, and $k$ is the rate constant characterizing the transient. This distinction between the initial and steady-state rates is the hallmark of slow-binding inhibition. It is assumed that the slow-binding inhibitor brings about reversible, competitive inhibition (Morrison, 1982). An important assumption in the deduction of eqn. (1) is that the concentration of substrate does not change significantly. Now eqn. (1) is only applicable for as long as the reaction in the absence of inhibitor appears zero-order in time, i.e. when non-linearity of the progress curve is not detectable in the control reaction. Substrate depletion can only be neglected for a limited extent of reaction. The first part of the present work describes a method that takes into account substrate depletion in slow-binding inhibition.

When there is also tight-binding inhibition (i.e. slow, tight-binding inhibition) new problems arise, owing to depletion of the inhibitor by combination with comparable concentrations of the enzyme (Morrison, 1969; Sculley and Morrison, 1986). Indeed, this is commonly a more serious problem than depletion of the substrate, which may often be circumvented by raising the concentration of substrate; however, there may be practical limitations, such as the solubility of the substrate, or (in spectrophotometric assays) the absorbance of the substrate. Moreover, if the concentration of substrate is too high, the extent of inhibition will be too low. Thus it is worthwhile considering the situation in which substrate depletion, as well as inhibitor depletion, has to be taken into account. Here numerical methods are required, and these are described in the second part of the present paper and in the Appendix.

## SUBSTRATE DEPLETION IN SLOW-BINDING INHIBITION

The kinetic mechanism for slow-binding inhibition that is most widely discussed (Morrison and Walsh, 1988) describes the binding of inhibitor (I) as a two-step process (Scheme 1): there is a fast formation of a non-covalent enzyme–inhibitor complex (EI), followed by a slower step in which EI is transformed into a more stable (sometimes covalently bound) complex, EI*. The equilibrium constant for the first step is $K_i$, the overall dissociation constant is $K_i^*$, and the rate constants for the interconversion of EI and EI* are $k_{+2}$ and $k_{-2}$. The reaction of the single substrate (S) is considered effectively irreversible, either innately or because a coupled reaction removes the product; as usual, there may be a second substrate present at a high enough concentration for its concentration not to change; the inhibitor is assumed to bind only to free enzyme. The rate of disappearance of substrate is:

$$dy_1/dt = -C \cdot e \cdot y_1$$

where $y_1$ is the concentration of substrate (initially $s_0$), $C$ stands for $k_{cat.}/K_m$, and $e$ is the concentration of free enzyme (total concentration $e_0$). It is more convenient here to express rates in terms of the free, not the total, enzyme concentration. The stoicheiometry for the enzyme is:

$$e + ei + ei^* + es = e_0$$

where lower-case symbols denote the concentrations of the

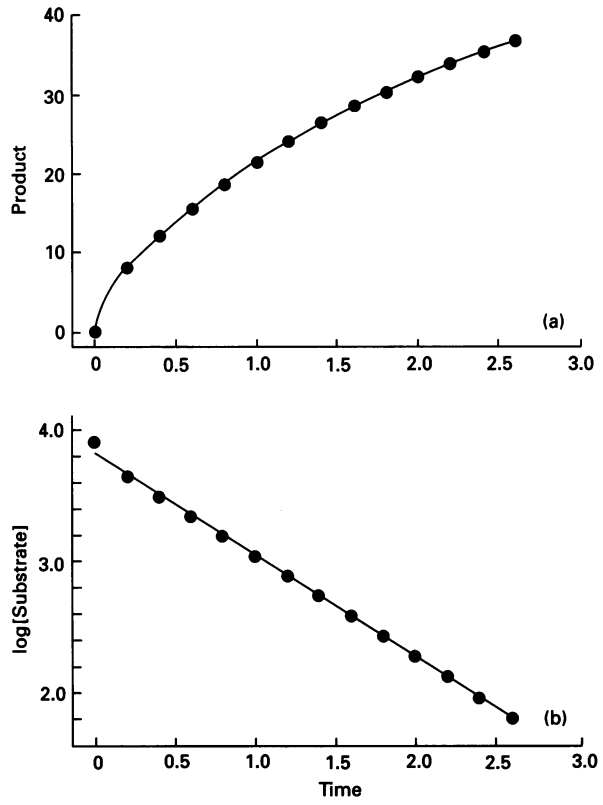


**Figure 1 Progress curve for slow-binding inhibition fitted by the 'low-*s*' method**

The initial concentrations of substrate, enzyme and inhibitor were 50 $\mu$M, 5 $\mu$M and 500 $\mu$M respectively, and $k_{cat.} = 50\ min^{-1}$, $K_m = 50\ \mu M$, $k_{+2} = 60\ min^{-1}$, $k_{-2} = 3.57\ min^{-1}$ and $K_i = 1700\ \mu M$. (The unit of time is taken as minutes rather than seconds here, so that the simulated experiment matches usual times.) (**a**) The points show the simulated experimental data and the curve shows the fit obtained by the low-*s* method described in the text. (**b**) Logarithm of the concentration of substrate, fitted by linear regression.

$$E + I \underset{}{\overset{K_i}{\rightleftharpoons}} EI \underset{k_{-2}}{\overset{k_{+2}}{\rightleftharpoons}} EI^*$$

**Scheme 1 Two-step binding mechanism**

species concerned. Hence the usual equilibrium assumptions (Cha, 1968) lead to:

$$e = (e_0 - y_2)/(1 + y_1/K_m + i/K_i) \qquad (2)$$

Here $y_2$ replaces $ei^*$. Then the second differential equation is:

$$dy_2/dt = (k_{+2} \cdot i \cdot e/K_i) - k_{-2} \cdot y_2$$

Thus there are three parameters to be found: $k_{+2}$, $k_{-2}$ and $K_i$; values of $k_{cat.}/K_m$ are presumed to be known from experiments in the absence of inhibitor. It can be a help that it is $k_{cat.}/K_m$ that is required, because it is sometimes easier to determine $k_{cat.}/K_m$ than $k_{cat.}$ and $K_m$ individually. The concentration of inhibitor ($i$) is here assumed to be constant; this assumption is relaxed when tight-binding inhibition is considered. The overall dissociation constant is then given by:

$$K_i^* = \frac{K_i}{1 + \frac{k_{+2}}{k_{-2}}}$$

## Analytical solutions

It is customary to estimate the initial rate from the slope near the origin of a progress curve. In slow-binding inhibition this may be hard. Thus in Figure 1(a) there is no initial linear phase. Hence an equation is required to fit the progress curve. There are two procedures for simplifying the equations, so that analytical solutions for progress curves can be obtained. The concentration of substrate can either be regarded as constant throughout the experiment, or it can be regarded as small, as explained below.

## Constant substrate concentration

The usual treatment (Morrison and Walsh, 1988) treats the concentration of substrate as constant, an approximation that is valid near the start of the reaction and when the concentration of enzyme is much less than that of the substrate. In practice, an upper limit of 10% for the extent of reaction is often used; the control reaction will be linear if the substrate concentration is several times the $K_m$. The use of extrapolation in the context of this approximation is discussed below.

## Low substrate concentration

When a larger extent of reaction is measured in an experiment in which the concentration of substrate is below $K_m$, then a useful approximation is to drop the term $y_1/K_m$ in eqn. (2) so that the concentration of free enzyme is given by:

$$e = \frac{e_0 - y_2}{1 + \frac{i}{K_i}}$$

Now we can substitute this value for $e$ in the equation for $dy_2/dt$, and solve the equation for $y_2$, and then substitute in the equation for $dy_1/dt$ to obtain an analytical solution for *Fprod*, the fraction of product formed. This leads to a simple method for estimating the overall dissociation constant, described below [eqn. (3)].

$$y_2 = \frac{\alpha}{\beta}(1 - e^{-\beta t})$$

$$\alpha = \frac{k_{+2} i e_0}{K_i + i}$$

$$\beta = \frac{k_{+2} i}{K_i + i} + k_{-2}$$

Thus *Fprod* is given by:

$$Fprod = 1 - e^{-[\gamma t + \delta(1 - e^{-\beta t})]}$$

$$\gamma = \frac{k_{cat.}(e_0 - \alpha/\beta)}{K_m(1 + i/K_i)}$$

$$\epsilon = \frac{k_{cat.}\,\alpha/\beta}{K_m(1 + i/K_i)}$$

$$\delta = \epsilon/\beta$$

**Table 1 Kinetic parameters in simulated slow-binding and slow tight-binding inhibition**

The first data set had initial concentrations of substrate, enzyme and inhibitor of 50 $\mu$M, 5 $\mu$M and 500 $\mu$M respectively, and $k_{cat.} = 50\ s^{-1}$, $K_m = 80\ \mu M$; the theoretical values were $k_{+2} = 60\ s^{-1}$, $k_{-2} = 3.57\ s^{-1}$ and $K_i = 1700\ \mu M$. The second data set had initial concentrations of substrate, enzyme and inhibitor of 2 $\mu$M, 30 nM and 30 nM respectively, and $k_{cat.} = 80\ s^{-1}$, $K_m = 15\ \mu M$; the theoretical values were $k_{+2} = 2.6\ s^{-1}$, $k_{-2} = 0.08\ s^{-1}$ and $K_i = 11$ nM. Random errors from a normal distribution with mean zero and S.D. 0.1 were added to the second data set. S.D. values are given below the corresponding values. The terms SBI and STBI stand for slow-binding inhibition and slow tight-binding inhibition. The third row of values gives the results obtained when three data sets were fitted together: one was the second data set defined above, and the two others had initial concentrations of enzyme and inhibitor of 120 nM and 30 nM respectively, and of 120 nM and 120 nM respectively. The 'low-$s$' approximation method and the 'burst equation' methods are described in the text. The column 'Dist' is 100 times the Euclidean distance of the found parameters from the theoretical parameters, both as their logarithms. The overall inhibition constant is $K_i^*$ (theoretical values: 95.5 $\mu$M and 0.328 nM for the first and second data sets; the units in the Table are $\mu$M or nM for the first or second set).

| Method | Data set | $k_{+2}$ ($s^{-1}$) | $k_{-2}$ ($s^{-1}$) | $K_i$ ($\mu$M) | Dist (%) | $K_i^*$ ($\mu$M or nM) |
|---|---|---|---|---|---|---|
| SBI | 1st | 60.55 | 3.57 | 1714 | 1.2 | 95.4 |
| STBI | 2nd | 2.62 | 0.0833 | 0.0109 | 4.2 | 0.336 |
| | | (0.02) | (0.012) | (0.001) | | |
| STBI | 2nd | 2.65 | 0.0803 | 0.0112 | 2.65 | 0.329 |
| | | (0.2) | (0.0056) | (0.0012) | | |
| Low-$s$ | 2nd | 1.40 | 0.249 | 0.0206 | 144 | 0.313 |
| Burst | 1st | 44.0 | 2.80 | 1170 | 54.3 | 70.1 |
| Low-$s$ | 1st | 40.9 | 4.33 | 948 | 72.9 | 90.7 |

The kinetic parameters can then be found from the following equations by non-linear regression (see the Appendix):

$$K_i = \frac{i}{\left(\frac{k_{cat.}\, e_0}{K_m}\right)/(\gamma+\epsilon)-1}$$

$$k_{+2} = \frac{\beta}{\frac{i}{i+K_i}+k_{-2}}$$

$$\frac{k_{-2}}{k_{+2}} = \left(\frac{\gamma}{\epsilon}\right)\left(\frac{i}{i+K_i}\right)$$

The use of the 'low-substrate' approximation has been tested on several simulated data sets. Although accurate values for $k_{+2}$, $k_{-2}$ and $K_i$ were obtained only when the initial concentration of substrate, $s_0$, was considerably below $K_m$ (e.g. one-tenth), values of $K_i^*$ accurate to better than 10% were returned when $s_0 < K_m$ (Table 1). Incidentally, the $s_0/K_m$ term is compared with $1 + i/K_i$, so that the larger $i/K_i$ is, the better the 'low-$s$' approximation. Although the procedure of fitting progress curves has the potential for determining all three kinetic parameters, only $K_i^*$ may be required. The approximate 'low s' treatment provides a simple and satisfactory method for estimating $K_i^*$. When the logarithm of the concentration of substrate remaining (or, in a spectrophotometric assay, the logarithm of $|A - A_\infty|$) is plotted against the time, the curve soon approximates closely to a straight line (Figure 1b). Then $K_i^*$ can be found from the numerical value of the slope (*slope*) from:

$$C = \frac{k_{cat.}\, e_0}{K_m}$$

$$K_i^* = \frac{i}{\frac{C}{slope}-1}$$

This arises because, at times much greater than $1/\beta$, the term $e^{-\beta \cdot t}$ is small, and the slope of the plot gives $\gamma$, which may be written, in terms of $K_i^*$, as:

$$\gamma = \frac{C}{1+\frac{i}{K_i^*}} \qquad (3)$$

The line drawn in Figure 1(b) gave a value for $K_i^*$ of 93.2 $\mu$M, where the theoretical value was 95.5 $\mu$M. Here the initial concentration of substrate was equal to (rather than much less than) $K_m$. For many practical purposes a convenient way to obtain $K_i^*$ accurate to about 20% is all that is required. When a lower concentration of substrate (5/8 of $K_m$; data set 1 in Table 1) was used, a value for $K_i^*$ of 93.7 $\mu$M was obtained. The extent of reaction was about 70% in these simulations, and the lines were least-square fits. Values of $K_i^*$ accurate to about 20% were obtained under a variety of conditions. The procedures given do not allow for depletion of the concentration of inhibitor, and so are strictly not applicable to tight-binding inhibition. Indeed, the values found by the non-linear regression method for most of the parameters in the last, and antepenultimate, rows of Table 1 were in error. Nevertheless, satisfactory values of $K_i^*$ were obtained (perhaps owing to a cancellation of errors) even when the concentration of inhibitor was equal to that of the enzyme (Table 1). The linear–log-plot procedure for $K_i^*$ was not satisfactory for the second data set of Table 1 when the concentrations of enzyme and inhibitor were equal.

The above analysis refers to experiments in which the concentration of substrate is less than $K_m$. As mentioned in the Introduction, this situation is probably less usual than the alternative one in which the substrate concentration is greater than $K_m$.

## Numerical solution of the differential equations for slow-binding inhibition

The differential equations for $y_1$ and $y_2$ have been given; the values at zero time are $s_0$ (initial substrate concentration) and 0 respectively. Zero time refers to the time before product or $ei^*$ have been formed. It is assumed, as usual, that the concentration of substrate is much greater than the concentration of enzyme, and so $es$ will be small compared with $s_0$ at zero time. Before considering finding the parameters, it is essential to test how sensitive the solution is to the values of the parameters. The example in Figure 2 shows that variation of the parameters had a marked effect on the goodness-of-fit, as measured by the sum-of-squares criterion; e.g. a 20% increase in $k_{+2}$, $k_{-2}$ or $K_i$ gave a sum of squares of residuals of 61, 39 or 66 respectively and, as the Figure shows, there are even larger changes for a 20% decrease.

The harder part is coupling the solution of the equations with a least-squares procedure to determine the parameters; the lack of symmetry referred to above implies that convergence may be

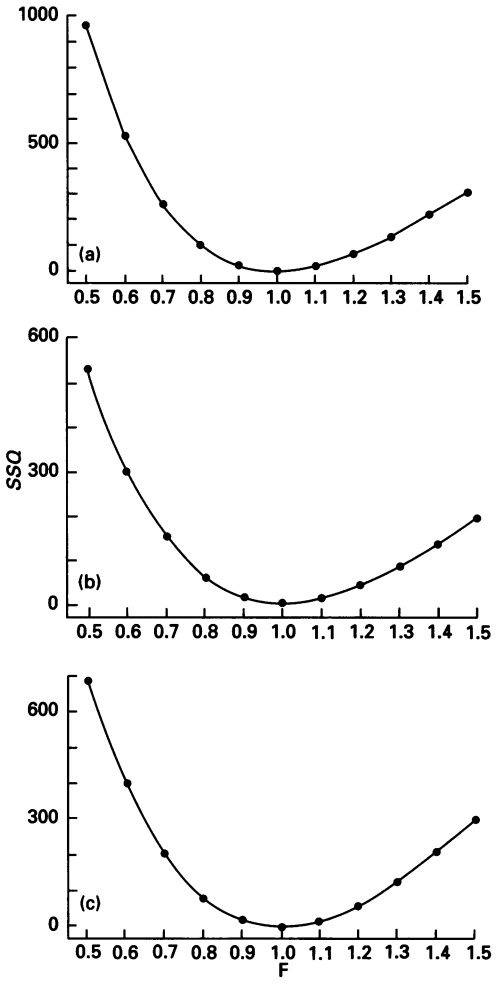


**Figure 2 Dependence of lack-of-fit on values of parameters in slow-binding inhibition**

The value of each parameter in turn was varied by a factor (F) and the sum of the squares of the residuals was determined. The parameters were: (**a**) $K_i$, (**b**) $k_{-2}$ and (**c**) $k_{+2}$, and the data were the first set given in Table 1.

difficult unless good starting values are used; this is a common feature in fitting differential equations. The approximate procedures given above are useful for providing good starting values for the kinetic parameters. The numerical procedures are outlined in the Appendix. Although this procedure is satisfactory (see Table 1), it is limited in that the concentration of inhibitor is assumed constant, so further testing was carried out with the more general method in which depletion of inhibitor is allowed for.

## TIGHT-BINDING INHIBITION

The kinetics of tight-binding inhibition has been much studied and procedures for estimating $K_i^*$ have been described (Morrison, 1969; Cha, 1975, 1980; Greco and Hakala, 1979; Morrison and Walsh, 1988). Although the usual procedure is to fit experimentally determined rates, it may be preferable instead to fit the progress curve to the solution of the differential equation, essentially as is done below. In either case, the rate equation (with $s$ replacing $y_1$) is:

$$\frac{ds}{dt} = -\frac{k_{cat.}\,s}{2(K_m+s)}\left\{\left[\left(K_i^*\left(1+\frac{s}{K_m}\right)+i_0-e_0\right)^2 + 4K_i^*\left(1+\frac{s}{K_m}\right)e_0\right]^{0.5} - \left[K_i^*\left(1+\frac{s}{K_m}\right)+i_0-e_0\right]\right\}$$

Whether it is better to use rates than progress curves seems unclear; a detailed comparison of methods (Greco and Hakala, 1979) assumed that the rates were determined without bias; if there is a (perhaps undetected) slow-binding component, this assumption will not hold. When there is also slow-binding inhibition the situation is more complex, and this is now discussed.

### Substrate and inhibitor depletion in slow tight-binding inhibition

The depletion of inhibitor is important when the concentrations of enzyme are comparable (Morrison, 1969; Morrison and Walsh, 1988). When the depletion of inhibitor by the formation of EI is taken into account, the concentration of free enzyme ($e$) is given by:

$$H = 1+y_1/K_m$$
$$D = e_0-y_2$$
$$G = i_0-e_0$$
$$B = H\cdot K_i+G$$
$$Q = -[B-\sqrt{(B^2+4H\cdot D\cdot K_i}]/2$$
$$e = Q/H$$

and the concentration of free inhibitor is given by:

$$i = \frac{i_0-y_2}{1+\frac{e}{K_i}}$$

The procedure is robust: one set of data with added random, normally distributed errors (mean zero, S.D. 0.01) was fitted 50 times. Here the concentrations of enzyme and inhibitor were equal and the final concentration of product was 0.77 $\mu$M. The parameters were obtained with adequate accuracy (Table 1). Fitting several sets of data simultaneously did not lead to an appreciable improvement in the overall fit (Table 1). A similar procedure was used by Williams et al. (1979). The present procedure, suitable for microcomputers, complements methods described for main-frame computers (Barshop et al., 1983; Zimmerle and Frieden, 1989).

## USE OF EXTRAPOLATION

There is a quite different way to overcome the difficulty of substrate depletion in slow-binding inhibition. This is to carry out a series of experiments and to extrapolate. Thus a series of simulated experiments with the conditions specified in Table 1 (first data set), except that the enzyme concentration was varied, have been reported (Crompton and Waley, 1989). When the values of the parameters found by use of the 'burst equation' were plotted against the concentration of enzyme, accurate values were obtained by extrapolation. This procedure has the advantage that familiar equations can be used. The obvious disadvantage is that more experiments have to be undertaken,

and also it is not always convenient to use a low enough concentration of enzyme. The use of extrapolation in the context of slow, tight-binding inhibition does not appear to have been reported.

## CONCLUSIONS

An important feature of experiments with slow-binding, and slow, tight-binding inhibitors is that the concentration of enzyme can alter the kinetics appreciably. Thus, in slow-binding inhibition, if there is a problem due to substrate depletion, this will be lessened at low concentrations of substrate (Crompton and Waley, 1989). Similarly, tight-binding inhibition will be less marked the lower the enzyme concentration. Moreover, for a fixed enzyme concentration, an increase in the concentrations of both substrate and inhibitor may eliminate the tight-binding component of what would otherwise be slow, tight-binding inhibition. The slow-binding component may be eliminated if the enzyme and inhibitor are preincubated and the reaction started by addition of substrate (see, e.g., Frieden et al., 1980). Thus the investigator may be able to simplify the kinetics by altering the conditions.

When a discontinuous assay has to be used, slow-binding inhibition may not be detected. Then the rate, estimated from the concentration of product formed after (say) 10% reaction, may not be the steady-state rate. Nevertheless, Lineweaver–Burk or Hanes plots may still appear linear, but will yield misleading values for the kinetic parameters (Frère et al., 1983). In practice, the concentration of product must be measured at enough different times to tell whether there is slow-binding inhibition.

The analysis of slow-binding inhibition depends on whether there is substrate depletion. If the uninhibited (control) reaction is linear, then the 'burst' equation (eqn. 1) can be used; if not, then the 'low-substrate-concentration' procedure is applicable. At first sight, it seems that the first equation could be corrected for substrate depletion by writing $V_{max.}/(K_m+s)$ for the steady-state rate, $v_s$. This manoeuvre was tried in the present work, and was used previously (De Meester et al., 1987), but the correct parameters were not returned accurately in simulated experiments. Then I realized that the deduction of the 'burst equation' depended on the assumption that the substrate concentration was not changing, and so the 'burst equation' cannot be simply corrected.

Slow-binding inhibitors may demand a time-consuming conformation change of the enzyme, but it is far from obvious why this change should take longer than any step in normal turnover (Crompton et al., 1988). Causes of slow binding have been discussed; the slow release of water molecules bound tightly in the active site is one possibility (Rich and Northrop, 1989).

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## APPENDIX

### Non-linear regression

For non-linear regression, the DNRPEASY program has been found very satisfactory (obtainable from R. G. Duggleby, Department of Biochemistry, University of Queensland, Brisbane, Queensland 4072, Australia). This program was derived from the DNRP53 program (Duggleby, 1984), which utilizes the well-established Marquardt (1963) procedure (see, e.g. Bevington, 1969). However, one of the more commonly used similar programs (see, e.g., Leatherbarrow, 1990) would be perfectly adequate.

### Numerical solution of differential equations

For the numerical solution of the differential equations the NAG (Phillips, 1986, and see the main paper) programs D02EBF or D02BBF, or the ODEINT program (Press et al., 1986) were used on a Viglen II (PC, AT type) micromputer. The difference between the NAG programs is that D02EBF should be used if the rate constants differ very greatly in magnitude. For the least-square minimizations, the NAG program E04FDF was less successful with the data sets used than the simplex method (Nelder and Mead, 1965) in the AMOEBA program (Press et al., 1986). The difference between these approaches is that the E04FDF program uses a Gauss–Newton method for minimization [see, e.g., Bevington, 1969), whereas the simplex method is a direct-search algorithm for finding the minimum of a function, which is known to cope well even with difficult problems. There are other FORTRAN implementations of the simplex procedure (O'Neill, 1985), as well as implementations in BASIC (Sprott, 1991; Nash and Walker-Smith, 1987) and Pascal (Caceci and Cacheris, 1984). The use of the simplex procedure is now outlined. The function required by the main program returns the sum of the squares of the residuals (*SSQ*), where a residual is the difference between the observed and calculated concentration of product at a given time (*ndp* is the number of data points):

$$SSQ = \sum_{i=1}^{ndp} (prod_{obs.} - prod_{calc.})^2$$

The concentration of product formed in each time interval is calculated with a trial set of parameters with one of the programs mentioned above. The parameters are then adjusted by the simplex routine to minimize *SSQ*.

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