Statistical Methods for Determination of Empirical Rate Equations for Enzyme Reactions

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The importance of considering an empirical rate equation as an estimate of the true steady-state rate equation is emphasized, and statistical methods of general applicability are described which may be used for estimation of the type of the rate equation as well as of kinetic coefficients in the equation. The methods are illustrated by determination of empirical rate equations for the ceruloplasmin catalyzed oxidation of derivatives of p-phenylenediamine. The diagnostic value of empirical rate equations for discrimination between reaction mechanisms of different degree is discussed.

Experimental determination of the steady-state rate equation, *i.e.* the functional relationship between the steady-state reaction velocity and concentrations of reactants in an enzyme reaction, is often a primary object of enzyme kinetic studies. Such rate equations may partly be used in a descriptive manner for characterization of the enzyme, and partly for elucidation of the reaction mechanism.[1] The common method for determination of empirical rate equations has been to postulate the type of equation to be fitted to the experimental data by subjective decision based on, for instance, theoretical considerations or graphical analysis of experimental results.[2] The main interest has then been centered to the problem of estimating kinetic coefficients for the chosen type of equation, and during the last ten years the classical methods of graphical analysis [3] have been considerably improved by the introduction of more precise and objective statistical methods for coefficient estimation.[2,4,5] The problem of making an adequate choice of the type of the postulated rate equation, which is extremely important for the interpretation of experimental results with respect to the reaction mechanism, does not appear to have been equally well investigated.

The present paper describes statistical methods and tests which may be used in order to make an objective choice of a proper type of rate equation. The question whether an adequately estimated rate equation always can be assumed to be identical with the theoretical rate equation for the actual mechanism will also be discussed.

THEORETICAL

Suppose that we want to determine an empirical steady-state rate equation with respect to a reactant R (substrate, product, or modifier) using n independent paired observations v_i and $[R]_i$, $i = 1, \ldots, n$. Reactant concentrations are considered as being accurately known, while steady-state reaction velocities v_i are assumed to be normally distributed with a constant variance.

As was pointed out in the preceding paper, almost all enzyme systems conform to a rate equation of the following general type

$$v = \frac{\sum\limits_{k=0}^{d} \alpha_k [R]^k}{1 + \sum\limits_{k=1}^{d} \beta_k [R]^k} \tag{1}$$

where d defines the degree of the rate equation, and where the kinetic coefficients α_k and β_k are independent of $[R]$. Unless contradictory information is available, a rate equation of this general type should be fitted to the experimental observations. This can be done by statistical standard methods for any value of d. The method of iterative regression analysis [6] may be used in the general case, and its application to eqn. (1) will be briefly described here.

Let α_k^* and β_k^* denote preliminary estimates of the kinetic coefficients. These estimates can be improved by addition of correction terms $\Delta\alpha_k^*$ and $\Delta\beta_k^*$ obtained as regression coefficients on determination of the multilinear regression through the origin of $v - v^*$ on $2d + 1$ variables defined by the regression equation

$$v - v^* = \sum_{k=0}^{d} \Delta\alpha_k^* \frac{\delta y^*}{\delta\alpha_k^*} + \sum_{k=1}^{d} \Delta\beta_k^* \frac{\delta y^*}{\delta\alpha_k^*} \tag{2}$$

v^* is obtained by substituting α_k^* and β_k^* for α_k and β_k in eqn. (1). The improved estimates may then be used for a second determination of correction terms, and this process is iterated until all correction terms become arbitrarily small. The final self-consistent set of coefficients represents an unbiased estimate of the different α_k and β_k in eqn. (1). The corresponding standard deviations $s(\alpha_k)$ and $s(\beta_k)$, as well as the residual variance s_k^2, can also be computed by the above method; the mathematical details of the method are described in statistical literature [6,7] and will not be treated here.

Clearly there is no point in using a regression equation on $2d + 1$ variables unless it gives a significantly better fit than a regression equation on a subset of these variables. A main problem in determinations of empirical rate equations will, therefore, be to *estimate* (not to *postulate*) the maximum value of d required for a proper fit of eqn. (1) to the experimental observations. Such an estimate of the appropriate degree can be made by fitting eqn. (1) for $d = 1$ and $d = 2$ by the above iterative method. The corresponding residual variance s_1^2 and s_2^2 are computed and used to calculate the quotient on the left hand side in eqn. (3):

$$\frac{(n-3)s_1^2 - (n-5)s_2^2}{2s_2^2} = F(2, n-5) \tag{3}$$

According to the likelihood ratio theory [8] this quotient becomes approximately F-distributed under the null hypothesis $\alpha_2 = \beta_2 = 0$ with the degrees of freedom indicated within brackets on the right hand side in eqn. (3). The null hypothesis (which is equivalent to the hypothesis $d = 1$) may consequently be tested by eqn. (3) at any chosen level of significance. If the hypothesis is rejected eqn. (1) must be fitted for $d = 3$, and the null hypothesis $\alpha_3 = \beta_3 = 0$ $(d = 2)$ be tested using

$$\frac{(n-5)s_2{}^2 - (n-7)s_3{}^2}{2s_3{}^2} = F(2, n-7) \tag{4}$$

If also this hypothesis is rejected the procedure can be analogously repeated until the appropriate value of d has been established. It may be observed, however, that only first and second degree empirical rate equations hitherto have been described in the literature, and it will probably rarely happen that both of the hypotheses $d = 1$ and $d = 2$ are rejected.

When the degree of the empirical rate equation has been properly estimated, one should proceed by testing the significance of the corresponding kinetic coefficients; in some cases the polynomial in the numerator in eqn. (1) may be of a lower degree than the polynomial in the denominator ($\alpha_d = 0$) and/or lack the constant term ($\alpha_0 = 0$). According to the theory of the iterative regression method [6,7] the quotient $\alpha_k / s(\alpha_k)$ becomes approximately t-distributed with $n - 2d - 1$ degrees of freedom under the null hypothesis $\alpha_k = 0$, and the significance of any α_k can be tested using

$$\frac{\alpha_k}{s(\alpha_k)} = t(n - 2d - 1) \tag{5}$$

If the hypothesis is accepted for a particular coefficient α_k, a new regression equation should be calculated under the restriction $\alpha_k = 0$ and with omission of the corresponding variable $\delta v / \delta \alpha_k$, before one proceeds by testing the significance of other coefficients. It must then also be observed that the degrees of freedom for test function (5) increase by one unit for each eliminated coefficient. The significance of the coefficients β_k can be analogously tested.

It frequently happens that one has theoretical and/or experimental evidence that, whatever the degree of the mechanism may be, $v = 0$ when $[R] = 0$; this is, for example, the case when R denotes an obligatory substrate.[1] In such a situation it may be advantageous to start by fitting eqn. (1) for $d = 1$ in order to obtain an estimate of α_0, and to test the significance of α_0 before estimating the degree of the rate equation. If the hypothesis $\alpha_0 = 0$ is accepted all further calculations should be carried out under the restriction $\alpha_0 = 0$ and with omission of the corresponding variable. This means that the test functions (3) and (4) must be changed to (6) and (7), respectively.

$$\frac{(n-2)s_1{}^2 - (n-4)s_2{}^2}{2s_2{}^2} = F(2, n-4) \tag{6}$$

$$\frac{(n-4)s_2{}^2 - (n-6)s_3{}^2}{2s_2{}^2} = F(2, n-6) \tag{7}$$

The objective of many kinetic investigations is to determine a rate equation with respect to two (or more) reactants R_1 and R_2, *e.g.* a substrate and an inhibitor. Such a combined rate equation can also be estimated by the above methods, using a series of experimental observations of the reaction velocity v as a function of R_1 at different constant levels of the second reactant R_2. Determination of the empirical rate eqn. (1) with respect to R_1 at each concentration of R_2, gives the different kinetic coefficients α_k and β_k as functions of $[R_2]$. According to the theory of Wong and Hanes [1] each α_k (β_k) is a quotient between two polynomials in $[R_2]$. Consequently, the functional dependence of α_k (β_k) on $[R_2]$ is exactly analogous to the dependence of v on $[R_1]$ expressed by eqn. (1), and can be estimated in the same manner.

The latter technique can best be illustrated by means of a simple example. The combined rate eqn. (8) can be applied to all inhibition mechanisms which are of the first degree with respect to both the substrate ($R_1 = S$) and the inhibitor ($R_2 = I$):

$$v = \frac{\alpha_{00} + \alpha_{10}[S] + \alpha_{01}[I] + \alpha_{11}[S][I]}{1 + \beta_{10}[S] + \beta_{01}[I] + \beta_{11}[S][I]} \tag{8}$$

Re-writing this equation in a form corresponding to eqn. (1) we get

$$v = \frac{\alpha_0 + \alpha_1[S]}{1 + \beta_1[S]} \tag{9}$$

where

$$\alpha_0 = \frac{\alpha_{00} + \alpha_{01}[I]}{1 + \beta_{10}[I]} \tag{10}$$

$$\alpha_1 = \frac{\alpha_{10} + \alpha_{11}[I]}{1 + \beta_{01}[I]} \tag{11}$$

$$\beta_1 = \frac{\beta_{10} + \beta_{11}[I]}{1 + \beta_{01}[I]} \tag{12}$$

showing that the functional dependence of α_0, α_1 or β_1 on $[I]$ is of the general type indicated in eqn. (1).

After having established (by the above F-tests) that eqn. (9) represents the empirical rate equation with respect to S, and that no second degree terms are significant in the relationships between α_0, α_1 or β_1 and $[I]$, one may proceed by testing the significance of each α_{jk} and β_{jk} in eqns. (10) – (12). Non-significant α_{jk} and β_{jk} are eliminated in eqn. (8), which then represents the estimated combined rate equation and hence gives information on the inhibition mechanism. The above technique can readily be generalized and is applicable to combined rate equations with respect to any number of reactants.

The statistical methods described above are based upon the assumption that the variance of the experimental reaction velocities is constant. If this is not the case all fits have to be properly weighted in order to obtain unbiased estimates and to make the tests valid. Methods of weighting the observations in statistical analysis of enzyme kinetic data have been described and discussed by Cleland.[2]

RESULTS

The practical application of the above statistical methods will be illustrated by two representative examples from our current studies on the ceruloplasmin catalyzed oxidation of mixtures of dimethyl-p-phenylenediamine (DPD) and Wurster's red (DPD^+); a complete description of the latter investigations will be given elsewhere. Table 1A shows experimental determinations of the enzymatic reaction rate v as a function of the concentration of DPD^+ in the absence of DPD. Reciprocal plots of the data (see Fig. 1) indicated that the empirical rate equation was of the first degree with respect to $R = [DPD^+]$, and eqn. (13) was fitted to the observations using the corresponding regression eqn. (14):

Table 1. Enzymatic steady-state reaction rate v as a function of: (A); $[R]=[DPD^+]$ at $[DPD]=0$; (B), $[R]=[DPD]$ at $[DPD^+]=0.30$. One concentration unit equals 9.5×10^{-5} M. One rate unit equals 1.69 moles substrate oxidized per minute per mole enzyme. Columns marked "final fit" give reaction rates as being calculated from the estimated rate equations.

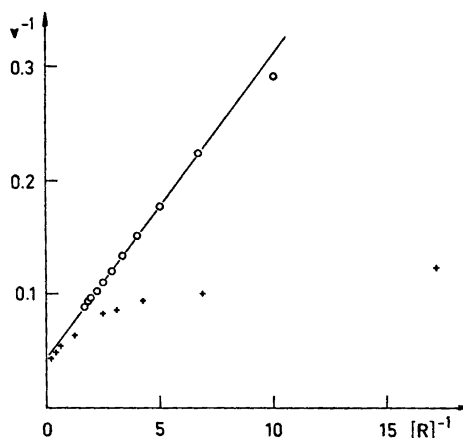| Experiment A			Experiment B		
[R]	v	final fit	[R]	v	final fit
0.10	3.45	3.26	0.58	8.20	8.41
0.15	4.47	4.56	1.46	9.95	9.60
0.20	5.67	5.69	2.34	10.60	10.64
0.25	6.60	6.68	3.22	11.70	11.55
0.30	7.54	7.57	4.10	12.00	12.37
0.35	8.36	8.36	8.50	15.65	15.42
0.40	9.06	9.06	17.3	18.60	18.77
0.45	9.75	9.70	26.1	20.50	20.58
0.50	10.41	10.28	34.7	21.90	21.69
0.55	10.70	10.81	43.7	22.40	22.48
0.60	11.31	11.30			

Fig. 1. Reciprocal plot for the observations given in Table 1 (A, $\bigcirc$; B,$+$).

$$v = \frac{\alpha_0 + \alpha_1[R]}{1 + \beta_1[R]} \tag{13}$$

$$v - \frac{\alpha_0^* + \alpha_1^*[R]}{1 + \beta_1^*[R]} = \Delta\alpha_0^* \frac{1}{1 + \beta_1^*[R]} + \Delta\alpha_1^* \frac{[R]}{1 + \beta_1^*[R]} - \Delta\beta_1^* \frac{\alpha_0^*[R] + \alpha_1^*[R]^2}{(1 + \beta_1^*[R])^2} \tag{14}$$

The preliminary estimates $(\alpha_0^*, \alpha_1^*, \beta_1^*)$ required for the iterative regression method were obtained by putting $\alpha_0^* = 0$ and calculating α_1^* and β_1^* from the slope and the intercept of the straight line indicated in Fig. 1; this line was assumed to be described by (cf. eqn. (13))

$$\frac{1}{v} = \frac{\beta_1^*}{\alpha_1^*} + \frac{1}{\alpha_1^*} \frac{1}{[R]} \tag{15}$$

The calculated regression coefficients $(\alpha_0^*, \alpha_1^*, \beta_1^*)$ were then used for correction of the preliminary estimates, and the regression analysis was iterated until a self-consistent set of coefficient had been obtained at the desired level of precision (see Table 2).

Table 2. Iterative fit of eqn. (13) to the observations given in Table 1A.

Coefficient	α_0	α_1	β_1
Preliminary	0	32	1.35
First correction term	+0.3320	+3.19	+0.141
First corrected estimate	0.3320	35.19	1.491
Second correction term	+0.0031	−0.03	−0.008
Second corrected estimate	0.3351	35.16	1.483
Third correction term	−0.0004	0.00	+0.001
Third corrected estimate	0.3347	35.16	1.484
Fourth correction term	0.0000	0.00	0.000
Self-consistent estimate	0.3347	35.16	1.484
Standard deviation	0.2238	2.164	0.169

Calculation of test function (5) for the values of α_0 and $s(\alpha_0)$ given in Table 2 showed that $t(8) = 0.3347/0.2238 = 1.50$. Consequently, the hypothesis $\alpha_0 = 0$ cannot be rejected even at the 5 % level of significance (for rejection $t(8)$ must exceed 1.86), and the iterative regression analysis was repeated using the rate equation

$$v = \frac{\alpha_1[R]}{1 + \beta_1[R]} \tag{16}$$

which corresponds to the regression equation

$$v - \frac{\alpha_1^*[R]}{1 + \beta_1^*[R]} = \Delta\alpha_1^* \frac{[R]}{1 + \beta_1^*[R]} - \Delta\beta_1^* \frac{\alpha_1^*[R]}{1 + \beta_1^*[R]} \tag{17}$$

The self-consistent values of α_1 and β_1 obtained on fitting eqn. (13) (see Table 2) were taken as preliminary estimates for the fit of eqn. (16), and the results of this fit are given in Table 3. It can be seen from the t-tests that both α_1

Table 3. Iterative fit of eqn. (16) to the observations listed in Table 1A.

Coefficient	α_1	β_1
Preliminary estimate	35.16	1.484
Self-consistent estimate	38.16	1.709
Standard deviation	0.663	0.070
t(9) in test function (5)	57.5	24.4
Level of singificance	<0.0005	<0.0005
Residual variance	0.009197	

and β_1 in eqn. (16) are most significant, and the rate equation cannot be further simplified.

In order to test the degree of the empirical rate equation, the self-consistent estimates in Table 3 were taken as preliminary estimates α_1^* and β_1^* for the iterative fit of eqn. (18) to the observations; α_2^* and β_2^* were both put equal to zero.

$$v = \frac{\alpha_1[R] + \alpha_2[R]^2}{1 + \beta_1[R] + \beta_2[R]^2} \tag{18}$$

The residual variance was now found to be 0.0071, which has to be compared with the value of 0.0092 obtained on fitting eqn. (16). Test function (6) thus becomes $(n=11)$ $F(2,7) = (9 \cdot 0.0092 - 7 \cdot 0.0071)/2 \cdot 0.0071 = 2.34$, showing that the fit cannot be significantly improved by using the second degree rate eqn. (18); at the 5 % level $F(2,7)$ must exceed 4.74 for significance.

The empirical rate equation, as estimated by the above methods, is thus given by eqn. (16) with the coefficient estimates listed in Table 3. The value (0.0092) of the residual variance may be taken as a measure of the precision of the fit, values of the reaction rate calculated from the empirical rate equation are given in Table 1A.

Table 1B shows experimental determinations of the enzymatic reaction velocity as a function of $R = [DPD]$ at a constant concentration of the second substrate DPD^+. The results obtained on fitting eqn. (13) to the observations are listed in Table 4, which also gives t-tests of the kinetic coefficients. In this case the hypothesis $\alpha_0 = 0$ was rejected on the 0.05 % level of significance and eqn. (19) was fitted to the data.

$$v = \frac{\alpha_0 + \alpha_1[R] + \alpha_2[R]^2}{1 + \beta_1[R] + \beta_2[R]^2} \tag{19}$$

Comparison of the residual variances obtained on fitting eqns. (13) and (19), respectively, using test function (3) gave $F(2,6) = 2.39$. Consequently, the null hypothesis $d = 1$ was accepted (rejection at the 5 % level of significance

requires that $F(2,6)$ exceeds 5.14), and in the final t-tests of α_1 and β_1 (Table 4) both coefficients were found to be significant on the 0.05 % level. The empirical rate equation is thus given by eqn. (13) with the coefficient estimates

Table 4. Iterative fit of eqn. (13) to the observations listed in Table 1B.

Coefficient	α_0	α_1	β_1
Preliminary	7	2	0
Self-consistent estimate	7.539	2.204	0.08285
Standard deviation	0.2503	0.1751	0.008054
t(8) in test function (5)	30.1	12.6	10.3
Level of significance	<0.0005	<0.0005	<0.0005
Residual variance		0.06764	

indicated in Table 4. Fitted values of the reaction rate calculated from the empirical equation are given in Table 1B.

The above experimental material may be also used as an illustration of the technique used for determination of combined rate equations. Experimental estimation of the rate equation with respect to $R = [DPD]$ was carried out as described for the observations listed in Table 1B at several constant levels of $[DPD^+]$, and thus yielded a set of α_0-values at corresponding concentrations of DPD^+. The observations given in Table 1A represent this set of paired values of $v = \alpha_0$ and $R = [DPD^+]$, and were used to determine the functional dependence of α_0 on $[DPD^+]$. The dependence of the remaining coefficients on $[DPD^+]$ may be analogously determined, in order to obtain the complete combined rate equation.

DISCUSSION

The great advantage of statistical methods for coefficient evaluation over the commonly used graphical methods has been emphasized in several reports.[2,4,5] Statistical analysis eliminates subjective influence on the results and yields better estimates of kinetic coefficients as well as a measure of the precision of these estimates in the form of their standard deviation. Furthermore, a large number of kinetic investigations must be expected to give results which cannot be evaluated by any graphical methods. Such results are frequently encountered in the study of two-substrate mechanisms, where rate equations generally are of a higher degree than unity.[1] The present investigation also gives an illustrative example of observations (Table 1B, Fig. 1) which cannot be reliably evaluated graphically even though they conform to a rate equation of the first degree.

The iterative regression method outlined in the theoretical section can be used for estimation of kinetic coefficients in any postulated type of rate equation. A similar method for coefficient evaluation in non-linear rate equations has recently been described by Cleland,[2] and the main contribution

of the present paper is to introduce the concept of and give methods for estimation of not only kinetic coefficients but also the degree of the rate equation. The great importance of such methods is evident in view of the results obtained by Wong and Hanes, who showed that steady-state rate equations for enzyme mechanisms adhere to the general form shown in eqn. (1), but differ primarily in their degree with respect to the reactants.[1] The diagnostic value of direct correlations between rate behaviour and mechanism will, consequently, be dependent upon the possibility to distinguish between rate equations of different degree, and Wong and Hanes anticipated that unequivocal delineation of such diagnostic features of rate behaviour might prove to be difficult and demand refinement of procedures for processing the experimental data.

Estimates of the degree of a rate equation of the type indicated in eqn. (1) may be obtained in several different ways, but the method described in the theoretical section (which is based on analysis of variance combined to t-tests of individual coefficients) is of general applicability and hence easily programmed for a digital computer. We have, further, found this method to be more rapid and easy to handle than other methods tested. For example, an estimation of the degree can be based exclusively on test function (5), which may be considered as a special case of the F-tests required for analysis of variance. Eqn. (1) should in that case be fitted to the experimental observations for a value of d which deliberately has been chosen at least one unit too large. The significance of the coefficients may then be tested using eqn. (5) for one coefficient at the time (in the order α_d, β_d, α_{d-1}, β_{d-1}, $\cdots$) until the appropriate degree has been established by elimination of insignificant coefficients. The latter method requires one complete recalculation of the regression equation for each eliminated coefficient, and will usually be less rapid than the method based on F-tests where two coefficients may be eliminated for each recalculation. Furthermore, it appears that the preliminary estimates α_k^* and β_k^* must be fairly precise when the number of variables in the regression eqn. (2) is large. For this reason, it is advantageous to start by fitting an equation of the lowest reasonable degree, and to use the estimates thus obtained for the fit of higher degree equations.

The statistical methods described in the present paper require a large number of calculations and should preferably be programmed for a digital computer. Such programs are routinely used in our laboratories, and Fig. 2 shows the operation scheme for a standard program which carries out all calculations required for a complete determination of the empirical rate equation with respect to one reactant. This program was used for the experimental data given in Tables 1A and 1B; the operation route followed was $A1 - A3 - R6$ and $R1 - A2 - R6$, respectively. The program should not be used when the experimental observations exhibit features (e.g. maximum or minimum values in the dependence of v on [R]) which clearly can be ascribed to the presence of higher degree terms in the rate equation. In such cases it is advantageous to start by fitting an equation of the second degree.

Application of the above statistical methods and test functions successfully establishes the significance of the empirical rate equation. The power of the tests can, evidently, be increased by increasing the number and the
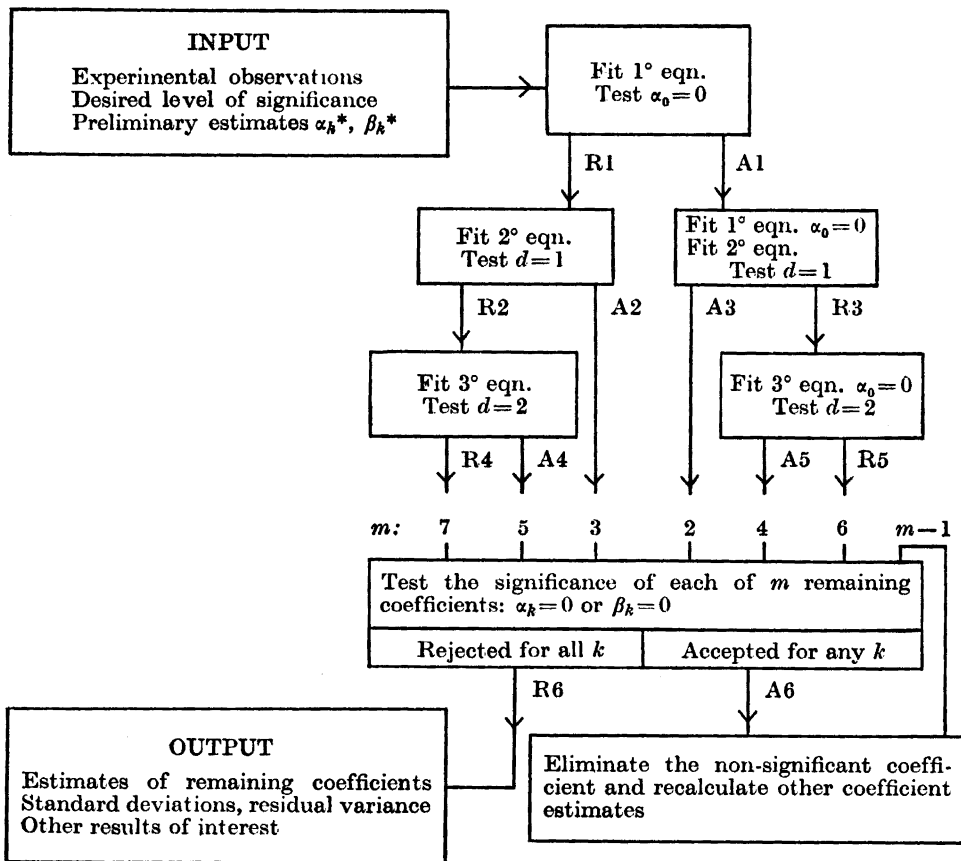
Fig. 2. Operation scheme for a computer program for determination of empirical rate equations with respect to one reactant. A (R) stands for the route followed when the null hypothesis tested is accepted (rejected).

precision of the experimental observations, but is also dependent upon factors which cannot be subjected to experimental variation. This means that the empirical rate equation, although being adequately estimated, must not necessarily be identical with (it may be simpler than) the theoretical steady-state equation for the investigated mechanism. An important consequence of this fact is that an empirical rate equation of a certain degree d cannot, in general, be used for discrimination between possible reaction mechanisms for which the degree with respect to the actual reactant equals or is higher than d.

The latter situation may, for example, be encountered in the classical enzyme kinetic studies where initial reaction velocities are measured at various initial substrate concentration in absence of products. Such experiments have frequently been observed to yield apparently linear reciprocal rate plots

and experimental data have, consequently, been fitted to a Michaelis-Menten type of equation which is an adequate treatment for descriptive purposes. For the purpose of elucidating the reaction mechanism, however, it may be completely misleading to assume that the empirical rate equation is identical with the theoretical one, and hence that the reaction mechanism is of the first degree with respect to the actual substrate. The number and/or the precision of the experimental observations may have been insufficiently large for establishing the presence of higher degree terms in the rate equation, and the mere exhibition of linear reciprocal rate plots can never be taken as evidence for elimination of possible higher degree mechanisms. The latter conclusion is further substantiated by the results obtained in the preceding paper, which showed that reciprocal rate equations become asymptotically linear for any higher degree mechanism at low reactant concentrations, and hence easily can be mistaken for first degree equations.

On the other hand, when the significance of higher degree terms in an empirical rate equation has been established, all mechanisms of a lower degree may be definitely excluded at the corresponding level of significance. Taking this fact into consideration, and observing that enzymatic reaction mechanisms in general must be expected to be of a higher degree than unity,[1] it becomes evident that experiments always should be planned with proper regard to the possibility of detecting higher degree terms (*e.g.* by application of the above F-tests), also in cases where experimental data can be fairly well fitted to a rate equation of the Michaelis-Menten type.

As was mentioned in the introduction, the common approach in steady-state kinetic investigations has been to postulate a certain rate equation (or to postulate the mechanism leading to this equation), the point of the investigation being to estimate the corresponding kinetic coefficients. For the purpose of establishing a mechanism, however, the question of coefficient evaluation is of minor interest, the major problem being to establish the degree of the theoretical rate equation. This problem is not easily solved, and generally requires a considerable amount of information (a large number of experimental observations of high precision). The method described in the present investigation allows a more cautious approach to be made. The method is not assumed to give a correct determination of the theoretical rate equation, but the best estimate of the true equation will be obtained for any given amount of information. This approach is satisfactory both for descriptive purposes and for the purpose of elucidating the reaction mechanism. The significance of the estimated rate equation is directly given by the tests applicated, and the levels of significance at which other types of rate equations can be excluded can be obtained by calculation of the power of the tests.

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