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Deviation from Michaelis–Menten Kinetics for Fumarase

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A study of the steady-state kinetics of fumarase over an extended concentration range, using novel methods of analysis, reveals an initial-rate equation of at least fourth degree for malate as substrate at pH 7.0, with no kinetically significant dead-end complex formation even up to concentrations of 100 mM. In the absence of demonstrable enzyme-aggregation phenomena, this is interpreted as indicating co-operative effects overlooked previously, although a mixture of isoenzymes, each individually of high degree and giving a complex curve, may be a contributing factor.

Fumarase is usually represented as an enzyme obeying Michaelis–Menten kinetics up to $5 \times K_m$ followed by a region of substrate activation (Hill & Teipel, 1971; Alberty & Bock, 1953) and finally a region of dead-end substrate inhibition (Rajender & McColloch, 1967) up to 1 M. Second-degree rate equations have been suggested (Hill & Teipel, 1971; Alberty & Bock, 1953; Rajender & McColloch, 1967), and much interest has been shown in the subunit structure, some concluding that the enzyme is a homogeneous species consisting of four identical subunits (Teipel & Hill, 1971), whereas others maintain that there are a variety of distinct enzyme forms, with six species of subunits resulting in deviations from Michaelis–Menten kinetics (Penner & Cohen, 1971). The aim of the present paper is to show how a detailed inspection of the initial-velocity data leads to the conclusions that a rate equation of at least fourth-degree is required, but that, up to 100 mM-malate concentration, there are no kinetically significant dead-end complexes formed.

Materials and Methods

Pig heart fumarase (L-malate hydrolyase, EC 4.2.1.2) was obtained as a crystalline suspension from Boehringer Corp., London, W.5, U.K. All other chemicals were of the highest grade obtainable. Enzyme activity was measured by the method of Racker (1950), by using a Cary 118C recording spectrophotometer fitted with multi-zero suppression accessory, automatic cell changer and a cell-rotating turret, thermostatically controlled at 20°C by a Grant circulator. All reactions were carried out in 3 ml quartz cells, at a constant ionic strength ($I = 0.2$ M) in sodium/potassium phosphate buffer, pH 7.0 (checked by a Pye Research pH-meter). Spectrophotometric accuracy was checked by running ten cuvettes consecutively, giving highly reproducible linear rates over 10 min at 10 mM-L-malate concentration (S.D. < 5% with no obvious drift) and solutions were pipetted by using calibrated Gilson automatic pipettes (accuracy checked to be better than ±5%). A plot of rate of reaction against enzyme concentration at 10 mM-malate was found to be approximately linear over a 100-fold range of enzyme (0.005–0.5 unit/3 ml of assay medium). All kinetic data were obtained by using 0.07 unit of fumarase per assay. All rates are shown corrected for non-enzymic fumarate production (with L-malate as substrate) from blank cells not containing fumarase. At L-malate concentrations below 20 μM, experimental error gives rise to unreliable data; similarly, with fumarate as substrate, high absorbance renders accurate and consistent results less easily obtainable.

Results

Fig. 1 shows ten separate determinations of the v-versus-[S] profile over the range 0.5–100 mM. In all cases there is a shoulder on the ascending limb of the curve, followed by a pronounced maximum, a trough, a second shoulder and finally a horizontal asymptote. Fig. 2 shows the means of these points and additional low-concentration points plotted in various ways to extract information as to the minimum degree of the rate equation.

Discussion

The minimum aim of enzyme kinetics is to find the simplest reaction scheme consistent with the experimental data, and any pronounced inflexions or turning points in the v versus [S] or $(1/v)$ versus $(1/[S])$ profiles must indicate a higher-degree rate equation than 1:1 as long as such features are not due to artifacts. It is clear from Fig. 1 that interpretation of fumarase kinetics in terms of the Michaelis–Menten

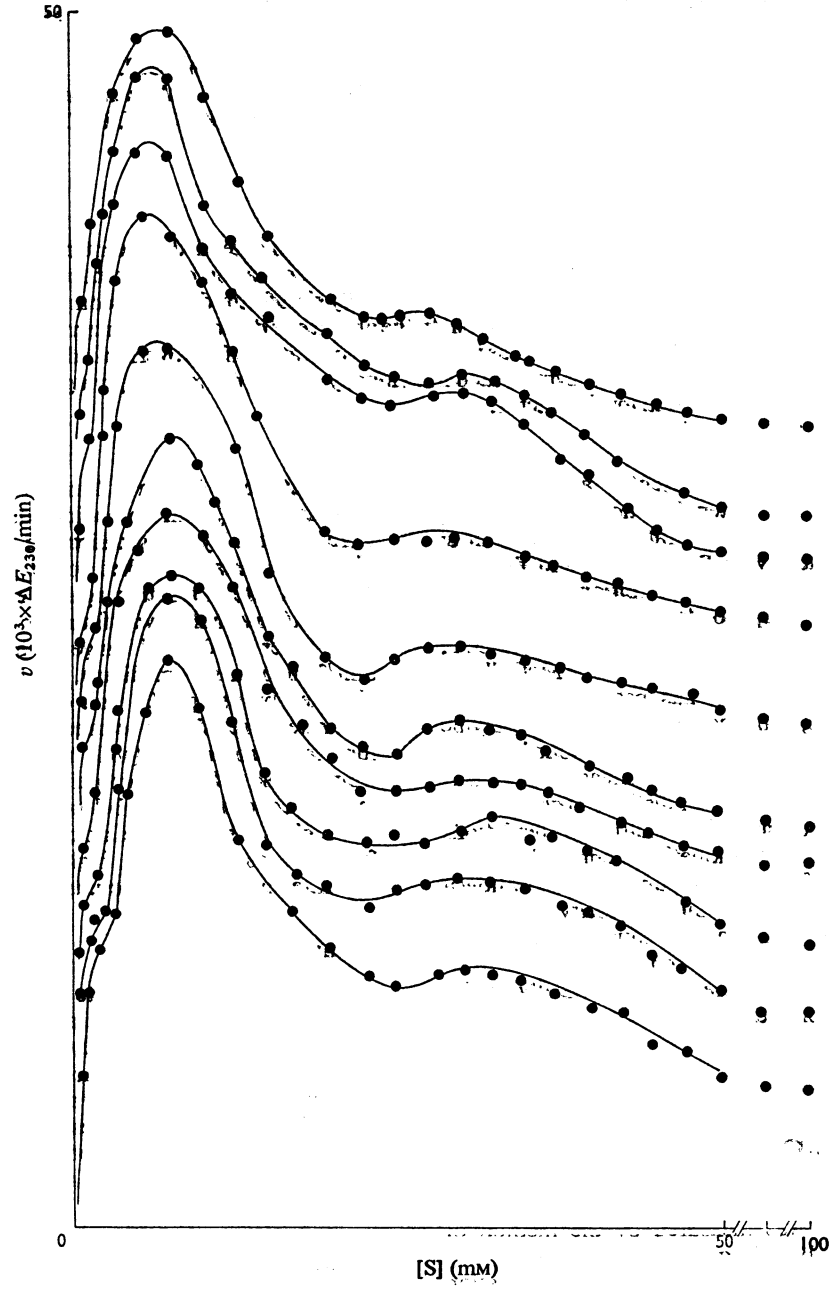

Fig. 1. *Initial velocity* (v) ($10^3 \times \Delta E_{230}/min$) *as a function of malate concentration* ([S]), *for fumarase*

The curves are stacked by successive displacement of the origin [by 2 ($10^3 \times \Delta E_{230}$/min) units] and show that a shoulder on the ascending limb and second maximum followed by a final horizontal section up to 100mM can be reproduced in ten separate experiments. This shape requires a rate equation of minimum degree 4:4 although the actual degree could be higher.

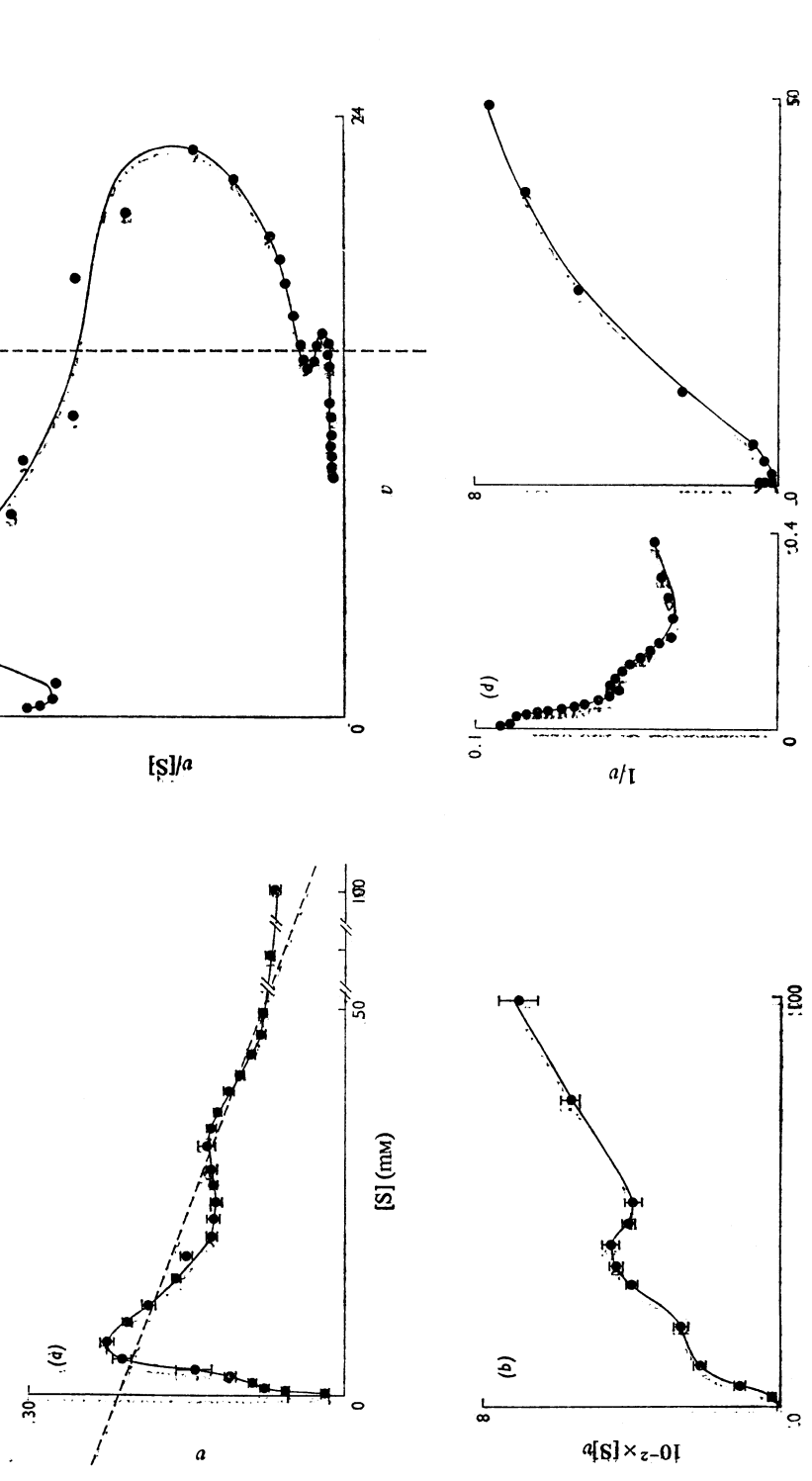

Fig. 2. *Initial-velocity data* ($10^3 \times \Delta E_{230}/min$) *as a mean of the ten points from Fig. 1, plotted in several spaces to elucidate the minimum degree*

(*a*) The mean of ten points is plotted ± 1 S.E.M., and the arbitrary sloping line intersects the experimental curve five times, requiring a minimum degree of 4 + 4 in the rate equation. (*b*) The plot of [S]/*v* versus [S] has a positive gradient in the range 50–100 mM, indicating that no kinetically significant dead-end complexes are formed at these saturating concentrations of substrate. (*c*) The arbitrary vertical line cuts the Scatchard plot four times, requiring minimum degree 4 : 4, and the overall deviation from Michaelis–Menten kinetics is most easily seen in this space. Each point is the mean of ten determinations. (*d*) Even though the double-reciprocal plot suppresses deviation from Michaelis–Menten kinetics, it is not possible to regard any extended section as truly linear, and a variety of values of K_m and V could be obtained, depending on the region extrapolated. Each point is the mean of ten experimental velocity determinations as in Fig. 1 and error limits are omitted in this and Fig. 2(*c*) for clarity.

equation is of limited validity, since the deviations were reproducible on ten separate occasions. Accordingly, we shall attempt analysis of these data by using techniques specifically designed to extract the minimum degree of the rate equation (Bardsley & Childs, 1975; Bardsley, 1976; Childs & Bardsley, 1976) and hence the simplest possible mechanistic complexity. The minimum degree and form of the rate equation should be regarded as a further piece of experimental evidence to be considered before a plausible reaction scheme is decided upon, a rate equation calculated and best fit obtained to the experimental points.

Fig. 2(*a*)

We assume a rate equation of the form

$$v = \sum_{1}^{n} \alpha_i [S]^i \Big/ \sum_{0}^{n+r} \beta_i [S]^i \qquad (1)$$

which for $n = 3$ and $r = 0$ can have up to five inflexions, and one maximum and one minimum in the plot of v versus [S] (Childs & Bardsley, 1976; W. G. Bardsley, unpublished analysis of the 3:3 function). Fig. 2(*a*) has some five inflexions, two maxima and one minimum, and thus, from turning-point analysis, the degree is at least 4:4, the horizontal asymptote suggesting $r = 0$. Intersection of the arbitrary line $v = \lambda[S] + \mu$ with the locus of eqn. (1) indicates intersections at roots of

$$\left\{\lambda \sum_{0}^{n+r} \beta_i [S]^{i+1} + \mu \sum_{0}^{n+r} \beta_i [S]^i - \sum_{1}^{n} \alpha_i [S]^i\right\} = 0$$

Applying Descartes' rule of signs, it is seen that a line from the origin can have up to n intersections, but an arbitrary line can have up to $n + 1$ intersections for λ, $\mu > 0$ or up to $n + r + 1$ intersections for λ and μ of opposite sign. In the present case, since $r = 0$, a horizontal line is easily seen to cut the curve up to four times (requiring degree 4:4), and the arbitrary sloping line shown has up to five intersections with the curve, establishing the minimum degree as 4:4.

Fig. 2(*b*)

The plot of [S]v versus [S] would become horizontal as [S] $\rightarrow \infty$ for the case $r = 1$, but would tend to a positive slope if $r = 0$ in the rate equation (Bardsley & Childs, 1975). Since Fig. 2(*b*) has a positive gradient throughout the range 50–100 mM, we conclude that $r = 0$, i.e. there is no formation of kinetically significant dead-end complexes up to this concentration. Our data support partial substrate inhibition rather than the dead-end type that has been claimed at the extreme concentration of 1 M (Rajender & McColloch, 1967). It becomes difficult to ensure reasonable values of constant ionic strength in work above substrate concentrations of 100 mM, and results obtained in this range would have to be analysed with restraint.

Fig. 2(*c*)

A vertical line is seen to intersect the experimental curve up to four times in this Scatchard plot, requiring a minimum degree of 4:4 for the initial-rate equation (Childs & Bardsley, 1976). Also, it should be noted how deviation from Michaelis–Menten kinetics over the whole substrate concentration range is easily seen in Scatchard space.

Fig. 2(*d*)

The inconvenience of the double-reciprocal plot for presenting data over extended substrate-concentration ranges is easily seen by comparing Fig. 2(*d*) with Fig. 2(*c*). Despite the split scale, however, it is easy to see that Fig. 2(*d*) has more than the two inflexions allowed in the plot of $1/v$ versus $1/[S]$ for the 3:3 function. Analysis shows that the Scatchard, double-reciprocal and ([S]/v) versus [S] plots have exactly the same points of inflexion (Bardsley, 1976), and in the present case this requires minimum degree 4:4. This double-reciprocal plot could be extrapolated to give a variety of apparent K_m and V values, all equally unreliable.

Conclusion

We have demonstrated that the rate equation for fumarase must be at least 4:4, and interpretation of this result is briefly attempted. Obviously discussion in terms of kinetic constants or simple substrate activation or inhibition mechanisms with second-degree rate equations are not in accord with the more detailed investigation of initial-rate data presented in the present paper, and we now seek the constraints necessary on a mixture of isoenzymes to give such complex curves. Suppose the velocity (v, say) were the result of a mixture of different enzyme forms (v_1, v_2, ... v_n, say) all acting on the same substrate. Taking derivatives with respect to [S] (denoted by primes) we find:

$$\begin{aligned} v &= v_1 + v_2 + \ldots + v_n \\ v' &= v_1' + v_2' + \ldots + v_n' \\ v'' &= v_1'' + v_2'' + \ldots + v_n'' \end{aligned}$$

Hence such a complex curve as we are considering could not result from isoenzymes obeying Michaelis–Menten kinetics individually, for here $v_i > 0$, $v_i' > 0$ and $v_i'' < 0$ for all i requiring $v > 0$, $v' > 0$ and $v'' < 0$ necessarily, and a similar analysis can be carried out on the other algebraic graphs leading to the same conclusion, i.e. featureless smooth curves for mixtures of 1:1 isoenzymes. Neither can the curve result from addition of 1:2 rate equations, but it is conceivable that mixtures of individually high-degree isoenzymes could be responsible, since this condition can give rise to complex curves, but only provided

that the isoenzymes individually are complex. In other words, a complex v versus [S] curve cannot result from a mixture of high-degree isoenzymes that individually give smooth curves with v, $v' > 0$ and $v'' < 0$ as discussed above. The fact that the plot of v versus enzyme concentration was tolerably linear does seem to eliminate a possible explanation based on enzyme aggregation for fumarase, but it is also possible that we have here an example of co-operative interaction previously overlooked. Final resolution of this problem must await the availability of fumarase in an unambiguously homogeneous form, but, since even isoenzymes, if they did exist, would have to give individually complex kinetics, the most likely explanation is co-operative subunit interactions. The degree of the rate equation for fumarase must be at least 4:4 by the techniques used in the present study, indicating that at least four distinct enzyme species interact with substrate in any mechanistic scheme that is to be seriously considered to account for the experimental findings. However, the degree could actually be higher than 4:4 and so, at this stage, until the problem of possible heterogeneity is resolved, it is not profitable to calculate an actual rate equation and attempt a best fit to the experimental data.

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