

## The Effect of Natural Selection on Enzymic Catalysis

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*(Received 30 June 1975)*

Analysis of the free energy changes in enzyme catalysis shows that maximum rates at low enzyme concentrations are attained for enzymes with Michaelis constants close to the concentrations of their substrates *in vivo*. For intracellular enzymes with no control function the values observed under natural conditions are generally in accordance with expectation. For enzymes where the maintenance of a steady flow of products is the prime objective it is preferable to evolve much lower Michaelis constants; extracellular digestive enzymes such as pepsin accordingly act under saturating conditions *in vivo*.

### 1. Introduction

In recent years there has been extensive study of the evolution of proteins, but this has been concerned much more with structure than with function. In consequence, although much is known about the evolution of primary sequences (see, e.g. Margoliash, 1972), very little is known about the evolution of the catalytic properties of enzymes.

It is most convenient to discuss the likely effects of selective pressure on the catalytic properties of enzymes in relation to the Michaelis-Menten equation

$$v = \frac{k_{\text{cat}}e_0s}{K_m + s} \quad (1)$$

which expresses the velocity  $v$  of many enzyme-catalysed reactions in terms of the total enzyme concentration  $e_0$ , the free substrate concentration  $s$ , and two constants,  $k_{\text{cat}}$  and  $K_m$ , known as the catalytic constant and the Michaelis constant, respectively. These are characteristic of the enzyme, and  $k_{\text{cat}}$  can be regarded as the first-order rate constant for the conversion of the enzyme-substrate complex into products, whereas  $K_m$  is the dissociation constant of the complex under steady-state conditions. In a rigorous discussion it is necessary to distinguish between  $K_m$  and  $K_s$ , the thermodynamic dissociation constant of the complex, but it is reasonable to equate the two for theoretical discussions of one-substrate enzymes in view of the observation that complex formation is generally diffusion-controlled (Hammes, 1968).

In two recent papers, Fersht (1974) and Crowley (1975) have argued that the likely effect of evolutionary pressure should be to increase both  $k_{\text{cat}}$  and  $K_m$ , so that  $k_{\text{cat}}$  becomes as large as chemically possible, and  $K_m$  becomes large in comparison with the physiological value of  $s$ . For example, Fersht states that "maximal rates are attained for  $K_s > 10s$ . Enzymes whose function is to turn over large amounts of

substrates rapidly, such as pepsin, lysozyme, chymotrypsin, etc., should have evolved to high values of  $k_{\text{cat}}$  and  $K_s$  to maximize the *rate* of reaction". This conclusion is surprising, first because it contradicts the widely held belief that strong binding (low  $K_m$ ) is an important component of enzymic catalysis, and second because it implies that evolution has been startlingly unsuccessful, at least in regard to the enzymes mentioned. Since all three have ill-defined natural substrates, it is impossible to make exact comparisons between  $K_m$  and  $s$ , but it is likely that all three are saturated under normal physiological conditions. For example, for pepsin  $K_m$  is much smaller than  $s$  for natural substrates (i.e. acid-denatured proteins) in the stomach. This is illustrated by the protein assay for pepsin (Anson & Mirsky, 1932), in which the rate is independent of haemoglobin concentration in the range 0.15% to 0.3%, i.e. the enzyme is saturated at low protein concentrations.

The extracellular hydrolases are in many ways atypical enzymes, and it may be more appropriate to examine some of the better-studied enzymes of intermediary metabolism, such as those involved in glycolysis. It is common to assume that metabolite concentrations *in vivo* are of the same order of magnitude as the Michaelis constants of the enzymes that catalyse their reactions, but it is difficult to be certain of this because of the difficulty of getting reliable evidence. Nonetheless, some efforts have been made to make the appropriate comparisons. For example, Lowry & Passonneau (1964) estimated the values of  $s$  and  $K_m$  for most of the carbohydrate metabolites of glycolysis in brain, with their appropriate enzymes. If hexokinase, phosphofructokinase and pyruvate kinase are excluded on the grounds that their functions are more complex than simply achieving maximum rates, the values of  $s/K_m$  ranged from 0.1 for phosphoglycerate kinase to 18 for aldolase, with several close to unity. These results suggest that whatever the direction of evolutionary pressure on the glycolytic enzymes its magnitude has not been sufficient to drive  $K_m$  values very far from  $s$  values.

## 2. Theory

### (a) Free energy levels in enzymic catalysis

In his discussion of the evolution of enzymic catalysis, Crowley (1975) considered the effects of slow changes in each of the rate constants that compose the empirical quantities  $k_{\text{cat}}$  and  $K_m$ . This approach has the advantage that there is a simple correspondence between the observable parameters and those that are assumed to evolve, but it does not give much insight into the structural changes that must accompany any changes in catalytic properties. It is preferable therefore to use the approach of Fersht (1974), who discussed the Michaelis-Menten equation in terms of the thermodynamic properties of the presumed intermediate(s) and transition state(s) of the reaction.

The simplest model is represented in Figure 1, in which the reaction is considered to involve a mixture of free enzyme and substrate,  $E + S$ , with free energy  $G_0$ , a Michaelis complex  $ES$ , with free energy  $G_1$ , a transition state  $X^\ddagger$ , with free energy  $G_2$ , and a mixture of free enzyme and product,  $E + P$ , with free energy  $G_3$ . No transition state is shown between  $E + S$  and  $ES$ , in recognition of the fact, noted above, that the formation of  $ES$  is normally diffusion-controlled. Although the free energy of activation of a diffusion-controlled process is not zero, because of a finite entropy term, it is usually small, and can be ignored in this discussion.

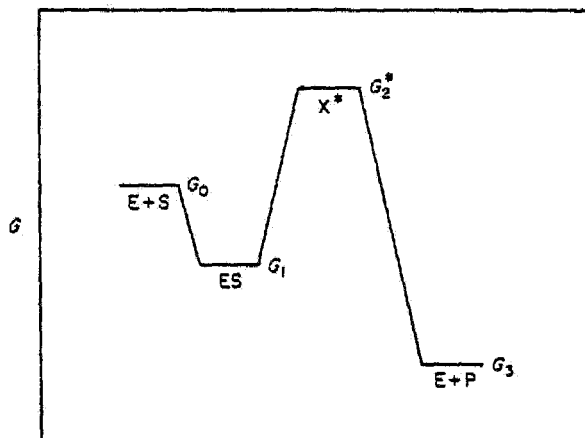


FIG. 1. Free energy relationships in the simplest Michaelis-Menten formulation of enzymic catalysis. The initial binding of substrate to enzyme to give the enzyme-substrate complex ES is assumed to be maintained at equilibrium and accordingly no energy barrier is shown for this step. The standard state of the substrate should be taken as the concentration that exists in the cell at the temperature and pH of the cell. This convention avoids any confusion that might result if the system were related to a very unnatural standard state.

The difference between  $G_0$  and  $G_3$  is fixed by the nature of the reaction catalysed, and cannot be altered by any effect of the enzyme, unless it is present at a high enough concentration to bind a significant fraction of the reactants (cf. Theorell & Bonnicksen, 1951), in which case the observed change in equilibrium constant is apparent rather than real. It is convenient to assume that  $G_0 - G_3$  is large enough for the reaction to be essentially irreversible. Although the absolute values of  $G_0$  and  $G_3$  may change, any changes can equally well be regarded as opposite changes in  $G_1$  and  $G_2^*$ , so that  $G_0$  can be treated as a constant and  $G_1$  and  $G_2^*$  are the only energy levels whose variation under selective pressure need be considered.

The free energy levels define the values of  $K_m$  and  $k_{cat}$  in accordance with thermodynamics and the transition-state theory as follows,

$$K_m = K_s = \exp[(G_1 - G_0)/RT] \quad (2)$$

$$k_{cat} = \frac{RT}{Nh} \exp[(G_1 - G_2^*)/RT] \quad (3)$$

$$k_{cat}/K_m = \frac{RT}{Nh} \exp[(G_0 - G_2^*)/RT], \quad (4)$$

where  $R$  is the universal gas constant,  $T$  is the absolute temperature,  $N$  is Avogadro's number, and  $h$  is Planck's constant. So the Michaelis-Menten equation may be written as follows,

$$v = \frac{RT \exp[(G_1 - G_2^*)/RT] e_0 s}{Nh (\exp[(G_1 - G_0)/RT] + s)} \quad (5)$$

By considering the velocity only at very low substrate concentrations, one can regard evolution simply as an effect on  $k_{cat}/K_m$ , ignoring any individual effects on  $k_{cat}$  and  $K_m$ . Because  $k_{cat}e_0/K_m$  is a first-order rate constant it can be directly compared with the first-order rate constant of the uncatalysed reaction, so it provides

a convenient index for the chemist to assess the efficiency of enzymic catalysis. But it seems unlikely that the main aim of evolution has been to impress theoretical chemists and it is more realistic to discuss how the reaction velocity can be maximized under the conditions that actually exist, i.e. finite substrate concentrations. In this case one cannot discuss evolution solely in terms of  $k_{\text{cat}}/K_m$ .

The conclusions that can be drawn about selective pressure are determined by the assumptions made about the degrees of freedom in the design of an enzyme. It is important therefore that these assumptions be stated clearly and justified, to permit assessment of the validity of any conclusions. The reasonable range of assumptions is likely to be bounded by two extreme cases: (i)  $G_1$  and  $G_2^\ddagger$  are treated as independent variables, which is effectively the approach of Fersht (1974); and (ii)  $G_1$  and  $(G_2^\ddagger - G_1)$  are treated as independent variables. These will first be discussed separately and then be merged into a plausible compromise.

(b)  $G_1$  and  $G_2^\ddagger$  treated as independent variables

Partial differentiation of equation (5) gives

$$\frac{\partial \ln v}{\partial G_1} = \frac{s}{RT(K_m + s)} \quad (6)$$

$$\frac{\partial \ln v}{\partial G_2^\ddagger} = -1/RT. \quad (7)$$

The first of these is positive and the second is negative under all conditions, so if  $G_1$  and  $G_2^\ddagger$  can be manipulated independently, the effect of evolution should be to increase  $G_1$  and decrease  $G_2^\ddagger$ , i.e. to increase both  $K_m$  and  $k_{\text{cat}}/K_m$  without limit. (More rigorously, a limit to the increase in  $K_m$  would be reached if  $G_2^\ddagger - G_1$  were to become very small or negative; in this event it would no longer be possible to ignore the transition state in the formation of ES and it would not be valid to equate  $K_m$  with  $K_s$ .) This agrees with the conclusion of Fersht (1974).

(c)  $G_1$  and  $(G_2^\ddagger - G_1)$  treated as independent variables

Let  $\Delta G^\ddagger = G_2^\ddagger - G_1$ . Then partial differentiation of equation (5) gives

$$\frac{\partial \ln v}{\partial G_1} = \frac{-K_m}{RT(K_m + s)} \quad (8)$$

$$\frac{\partial \ln v}{\partial \Delta G^\ddagger} = -1/RT. \quad (9)$$

Thus if  $G_1$  and  $\Delta G^\ddagger$  can be manipulated independently, it is advantageous to decrease both without limit, i.e. to make  $K_m$  as small and  $k_{\text{cat}}$  as large as possible. For  $K_m$  this conclusion is the opposite of that for the previous case.

(d)  $G_1$  and  $G_2^\ddagger$  treated as interdependent

It is clear from the contrary conclusions about  $K_m$  required by the two extreme cases that their defining assumptions must be examined to decide whether either can be regarded as reasonable, or whether some intermediate case might be better. On the one hand it is implausible to propose that  $G_2^\ddagger$  can be altered by structural modifications to the enzyme without any concomitant change in  $G_1$ . Plainly there

must be many respects in which changes in the interactions between enzyme and substrate in the ES complex must be matched by similar changes in the interactions in the transition state. This must surely be true of most interactions between groups that undergo no chemical changes during the reaction, for example, between the hydrophobic parts of the enzyme and substrate in pepsin catalysis. On the other hand, the structures of the substrate and the transition state are not identical, and so it is unreasonable to assume that all changes in  $G_1$  are exactly paralleled in  $G_2^\ddagger$ , as implied in the second extreme case. A reasonable compromise is to permit changes in  $G_1$  and  $G_2^\ddagger$  in the ratio of  $1:r$ , where  $r$  is a constant fraction in the range 0 to 1. If  $G_1$  and  $G_2^\ddagger$  are referred to arbitrary datum points  $G_{1_0}$  and  $G_{2_0}^\ddagger$ , they may be defined as

$$G_1 = G_{1_0} + \lambda \quad (10)$$

and

$$G_2^\ddagger = G_{2_0}^\ddagger + r\lambda, \quad (11)$$

where  $\lambda$  is a parameter that may be adjusted under evolutionary pressure at constant  $r$ . Then equation (5) may be written

$$v = \frac{RT \exp[(G_{2_0}^\ddagger - G_{1_0} + (1-r)\lambda)/RT] e_0 s}{N h (\exp[(G_{1_0} - G_0 + \lambda)/RT] + s)} \quad (12)$$

and differentiation gives

$$\frac{d \ln v}{d\lambda} = \frac{s(1-r) - rK_m}{RT(K_m + s)}. \quad (13)$$

Thus  $v$  is a maximum when  $K_m = s(1/r - 1)$ . So if the degree of dependence of  $G_2^\ddagger$  on  $G_1$  is assumed to be between 10% and 90%, i.e.  $0.1 < r < 0.9$ , then the optimum value of  $K_m$  should be within one order of magnitude of  $s$ .

(e) *Magnitude of selective pressure at non-optimum values of  $K_m$*

Although the value of  $\lambda$  for which  $v$  is a maximum clearly provides a target for evolution, it may not necessarily be reached if the selective pressure becomes negligible first. The value of  $RT(d \ln v/d\lambda)$  provides a convenient measure of selective pressure and is plotted against  $K_m/s$  for various values of  $r$  in Figure 2. In no circumstances is there strong pressure to take  $K_m/s$  far outside the range 0.1 to 10; in other words,  $K_m$  is likely to be within an order of magnitude of  $s$ , even if its theoretical target is zero ( $r = 1$ ) or infinity ( $r = 0$ ).

### 3. Discussion

(a) *Physiological examples and apparent exceptions*

Evolution appears to have been successful in achieving appropriate  $K_m$  values for the enzymes of glycolysis, and indeed of the whole of intermediary metabolism if the commonly held views about likely physiological substrate concentrations are correct. But the extracellular hydrolytic enzymes, such as pepsin and chymotrypsin, appear to be exceptions in that they have physiological  $K_m$  values much smaller than one might expect, and it is appropriate to enquire why this should be. The most likely explanation is found in the need of many higher animals to maintain constant energy and metabolite supplies without continuous feeding. For energy this is achieved to a

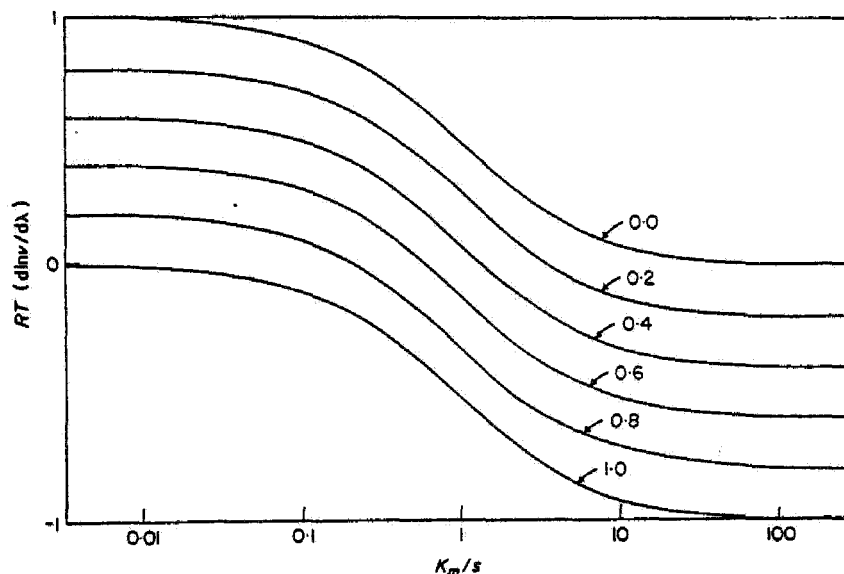


FIG. 2. Selective pressure to modify  $K_m$ , plotted as a function of  $K_m$ . Curves are shown for various values of  $r$ , as indicated, where  $r$  is the degree of dependence of changes in  $G_2^2$  on changes in  $G_1$ .

large extent by storing carbohydrate in the liver as glycogen. But no comparable storage polymer seems to exist for amino acids, and there would therefore be a positive disadvantage in permitting protein digestion to proceed very rapidly, with a half-time of minutes or seconds. It is far more valuable to have a half-time of several hours, to permit a fairly constant flow of amino acids into the body at all times. Since the digestive system is much more tolerant than any cell of fluctuations in its composition, it is advantageous to retain surplus amino acids in the gut rather than to permit a wildly fluctuating rate of absorption. This requirement is satisfied by ensuring that the digestive system is saturated under normal conditions.

Support for this interpretation of the requirements for efficient digestion is provided by the occurrence of chymosin (rennin) instead of pepsin in the stomachs of young mammals. Since pepsin has chymosin activity, but chymosin has very restricted pepsin activity, particularly in the hydrolysis of casein (Foltmann, 1967), it is clear that chymosin fulfils a need for an enzyme with limited activity, and that it is too simple to regard very rapid protein digestion as a universal evolutionary aim.

#### (b) *Effect of inhibition*

Physiological reactions often proceed in the presence of products and other inhibitors at sufficient concentrations to cause significant inhibition. Since such inhibition is usually either wholly or largely competitive, it follows that  $K_m$  values estimated under idealized conditions may significantly underestimate the functional values *in vivo*. For example, Kahana *et al.* (1960) have estimated for glucose phosphate isomerase that inhibitors are present in the cell in sufficient concentrations to raise the  $K_m$  threefold for glucose 6-phosphate. But this may well be an extreme case and a more typical factor might be in the range 1.0-fold to 1.5-fold, which would not appreciably alter the conclusions of this paper.

(c) *Catalysis at high enzyme concentrations*

Another important difference between standard practice in steady-state experiments and the situation in the cell is in the concentration of enzyme, which may well be several orders of magnitude greater in the cell (Srere, 1968). In some special cases, such as glutamate dehydrogenase (Frieden & Colman, 1967), the kinetic properties of the enzyme may be drastically altered by association at high concentrations; but more generally all enzymes show deviations from the Michaelis-Menten equation at enzyme concentrations greater than about  $0.1 K_m$ . These result from the fact that at high enzyme concentrations it is invalid to assume that the free and total substrate concentrations are equivalent. The steady-state rate is given by an equation of the following form (Laidler, 1955),

$$v = \frac{1}{2}k_{\text{cat}}(K_m + s_0 + e_0) \{1 - [1 - 4e_0s_0/(K_m + s_0 + e_0)^2]^{\frac{1}{2}}\}, \quad (14)$$

in which  $s_0$  is the total substrate concentration. In spite of its very different appearance, equation (14) defines a dependence of  $v$  on  $s_0$  that is qualitatively similar to the Michaelis-Menten equation, as illustrated in Figure 3. The main difference is that the

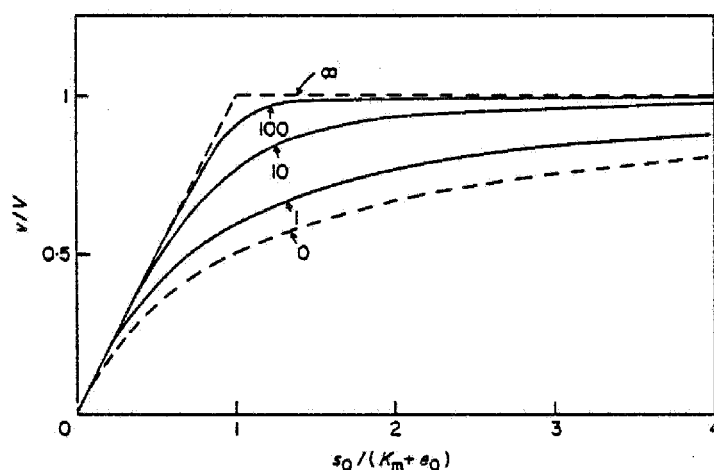


FIG. 3. Dependence of velocity on total substrate concentration at high enzyme concentrations. Each curve is labelled with the value of  $e_0/K_m$ , and the abscissa scales are normalized to show the equivalent behaviour at low substrate concentrations and to permit comparison of the complete curves.

transition from first-order to zero-order dependence on  $s_0$  is more abrupt, so that as  $e_0$  increases the curve tends towards the type of curve presented in most textbooks of biochemistry as a rectangular hyperbola.

The practical effect of working at high enzyme concentrations may be judged from the fact that the half-saturation point is raised by  $\frac{1}{2}e_0$  (J. R. Griffiths, personal communication; cf. also Myers, 1952), so that one can consider the effective Michaelis constant to be about  $K_m + \frac{1}{2}e_0$ . Since  $e_0$  is unlikely to exceed  $K_m$  by a significant amount under physiological conditions, this effect will not be large enough to invalidate the conclusions of this paper.

(d) *Response to changes in flux*

It may be questioned whether the achievement of high rates of reaction at low enzyme concentrations has been the only major objective of evolution. Any metabolic pathway should possess the capacity for enduring abnormal circumstances, such as an increase in flux above normal levels. For an enzyme obeying the Michaelis-Menten equation the capacity to respond to changes in flux depends on the normal value of  $s/K_m$ , as discussed by Atkinson (1969). Thus, an enzyme needs a safety factor to protect the cell against catastrophic increases in metabolite concentrations, which must be weighed against the demands of efficiency under normal conditions.

It is difficult to predict how large the safety factor ought to be; Atkinson (1969) has argued that a pathway should be able to tolerate a flux of more than double the normal maximum, but this may underestimate the capacity of a healthy organism for maintaining homeostasis and a much smaller safety factor may be adequate. An excessive safety factor *per se* may well be wasteful of catalytic capacity (Cleland, 1967), although the analysis in this paper provides some support for Fersht's (1974) view that a small value of  $s/K_m$  may evolve for reasons quite independent of the need for a safety factor.

(e) *Conclusions*

If catalytic efficiency is considered solely from the point of view of maximizing flux, then it follows that enzymes should have evolved to possess  $K_m$  values within one order of magnitude of the physiological concentrations of their substrates. But this conclusion must be seen in the light of the various complicating aspects that exist. Both the presence of inhibitors and high enzyme concentrations indicate that the effective physiological  $K_m$  value is likely to be somewhat greater than the value measured under idealized conditions. In addition, the need for a safety factor suggests that  $K_m$  should exceed the normal value of  $s$ .

The glycolytic enzymes appear to support this analysis, except that aldolase, with  $s/K_m = 18$  (Lowry & Passonneau, 1964), is nearly saturated *in vivo*. But this ratio may need to be adjusted downwards, to allow for the inhibition of the enzyme by various metabolites (Spolter *et al.*, 1965), and the difficulties of measuring concentrations and Michaelis constants *in vivo* are such that one stray value is not surprising.

Several classes of enzymes may have suffered evolutionary pressures rather different from a simple demand for a maximum rate. For control enzymes the most crucial need in evolution was probably to establish a high sensitivity to environmental changes. For enzymes involved in digestion the achievement of a constant rate of product release may well have been more important than attainment of a high rate. For enzymes primarily used for detoxication the safety factor may assume paramount importance. But for most enzymes catalysing reactions of intermediary metabolism under conditions of homeostasis, the attainment of high rates is likely to have been the prime evolutionary objective; this leads one to expect  $K_m$  values close to physiological substrate concentrations.

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