TRUE AND APPARENT ACTIVATION ENERGIES OF ENZYMIC REACTIONS

by

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It is now generally accepted that enzyme catalysis obeys the equation of MICHAELIS AND MENTEN[31], or its modification by BRIGGS AND HALDANE[4]. The two essential postulates on which these theories are based are that the reaction occurs in two steps, the formation of a complex between enzyme and substrate followed by its breakdown to the products of the reaction,

$$E + S \underset{k_2}{\overset{k_1}{\rightleftharpoons}} ES \xrightarrow{k_3} \quad ;$$

and that the rate of the second step determines the rate of the reaction. The equation derived from this argument,

$$v = k_3 p = \frac{k_3 es}{s + K_M} \qquad (1),$$

where e and s are the total concentrations of enzyme and substrate and p is the concentration of enzyme-substrate complex, has been found to describe the kinetics of all enzymes to which it has been applied. An exactly similar equation can be derived from the adsorption isotherm of LANGMUIR[25], and WEBER[46] has shown that it can be derived on the assumption that a finite number of molecules is activated at one time, whether after complex formation or at a distance. In all cases the form of the equation implies that the reaction occurs in two steps, of which the second determines the rate.

It is also widely acknowledged that the variation with temperature of a chemical reaction follows the equation of ARRHENIUS[1],

$$\frac{\partial \log k}{\partial\ (1/T)} = -E/R$$

$$\text{or} \quad k = Ae^{-E/RT} \qquad (2),$$

where E is a constant, the energy of activation. In (2), k is the specific reaction rate, and is independent of the concentration of the reactants. In a reaction involving more than one step, the rate of one of the steps will often determine the rate of the overall reaction, and it is to this step that the ARRHENIUS equation applies.

The rate of an enzymic reaction is determined by the rate of breakdown of the MICHAELIS complex (equation 1). A plot of log k_3 against $1/T$ will therefore have a slope of $-E/R$, where E is the energy of activation of this step. If p, the concentration of enzyme-substrate complex, is kept constant while the temperature is varied, v will be

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proportional to k_3, and $\frac{\partial \log v}{\partial (1/T)}$ will equal $\frac{\partial \log k_3}{\partial (1/T)}$. The simplest condition for p to be constant and finite is that the concentration of substrate shall be very large; p will then tend to e, the total concentration of enzyme. Thus a plot of log v_{max} against $1/T$ will have a slope of $-E/R$; *i.e.*

$$\frac{\partial \log v_{max}}{\partial (1/T)} = \frac{\partial \log k_3}{\partial (1/T)} = -E/R \tag{3}.$$

However at lower concentrations of substrate,

$$p = \frac{es}{s + K_M} \tag{4}.$$

If s, the concentration of substrate, were varied in such a way as to keep p constant at all temperatures, $\frac{\partial \log v}{\partial (1/T)}$ would still be equal to $\frac{\partial \log k_3}{\partial (1/T)}$. But if s and e are kept constant themselves while the temperature is varied,

$$\begin{aligned}\frac{\partial \log v}{\partial (1/T)} &= \frac{\partial}{\partial (1/T)}\left[\log\left(\frac{k_3 es}{s + K_M}\right)\right] \\ &= \frac{\partial \log k_3}{\partial (1/T)} - \frac{\partial K_M/\partial (1/T)}{s + K_M} \\ &= \frac{\partial \log k_3}{\partial (1/T)} - \left(\frac{K_M}{s + K_M}\right)\frac{\partial \log K_M}{\partial (1/T)}\end{aligned} \tag{5}.$$

Writing μ for the apparent activation energy derived from a plot of log v against $1/T$ under these conditions, we have

$$\frac{\partial \log v}{\partial (1/T)} = -\mu/R = -E/R - \left(\frac{K_M}{s + K_M}\right)\frac{\partial \log K_M}{\partial (1/T)},$$

$$\text{and} \qquad \mu = E + \left(\frac{K_M}{s + K_M}\right) R \frac{\partial \log K_M}{\partial (1/T)} \tag{6}.$$

It is seen that μ will differ from E by an amount which will vary with the concentration of substrate, being greatest at low concentrations and falling to zero as the concentration is made very large. Further, since K_M is not in general independent of temperature, $(\mu - E)$ will also vary with temperature, although this effect may be expected to be rather small.

If the theory of MICHAELIS AND MENTEN[31] is assumed to apply, K_M is the reciprocal of an equilibrium constant, and the VAN 'T HOFF isochore gives

$$\frac{\partial \log K_M}{\partial (1/T)} = \frac{\Delta H_M}{R} \tag{7},$$

where ΔH_M is the change in heat content accompanying the formation of the MICHAELIS complex. Equation (6) then becomes

$$\mu = E + \left(\frac{K_M}{s + K_M}\right)\Delta H_M \tag{8},$$

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a relation which applies also to the LANGMUIR adsorption isotherm, and to any kinetic theory which assumes an equilibrium for the first step of the reaction. However, in the kinetic theory of BRIGGS AND HALDANE[4], K_M no longer represents an equilibrium constant, and so this relation is no longer true, unless $k_3 \ll k_2$.

The physical meaning of equation (8) is represented in Fig. 1. The modern theoretical basis of the ARRHENIUS equation[11] is that the reacting molecules form an unstable complex of higher energy than themselves, which can break down to give the reaction products. In Fig. 1, A represents the initial reactants E and S. These combine reversibly (presumably through an intermediate activated complex B) to form C, the MICHAELIS complex, the process being accompanied by a change in heat content ΔH_M. The complex then acquires an amount of energy E and becomes activated (D); the activated complex then breaks down. Thus ΔH_M is the change in heat content accompanying formation of the MICHAELIS complex, and E is the energy required to activate this complex. It

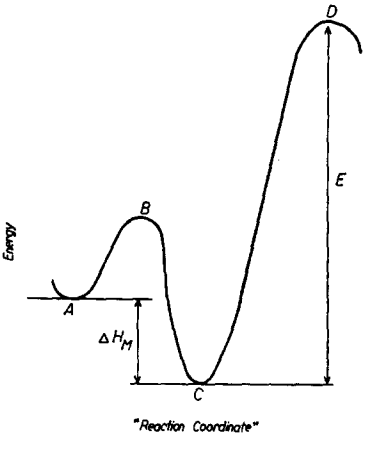

Fig. 1.

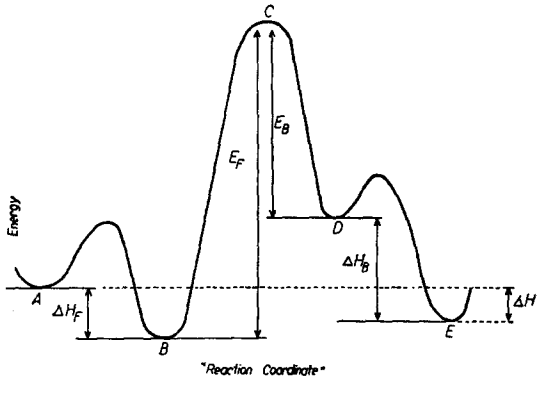

Fig. 2.

should be pointed out that this process is completely analogous to surface catalysis of a reaction in the gas phase, where there is an exactly similar process of adsorption followed by activation[11]. Equation (8) is equally applicable to this phenomenon.

In a reversible reaction, the MICHAELIS theory holds for the reaction in either direction, and therefore there are two steps involved in the activation of either substrate. This implies the formation of two distinct MICHAELIS complexes, one with each substrate; and the rate in either direction must be governed by the rate of interconversion of the two complexes. This is shown diagrammatically in Fig. 2. Here A and E are the initial and final states, B and D the two MICHAELIS complexes, and C the activated complex. Many more steps may of course be involved in such a reaction; however this is the minimum number required to describe a reversible enzymic reaction, which obeys the MICHAELIS equation in both directions. Fig. 2 diagrammatically represents HALDANE's[18] "completer theory" of a reversible reaction,

$$E + S \underset{k_2}{\overset{k_1}{\rightleftharpoons}} ES \underset{k_4}{\overset{k_3}{\rightleftharpoons}} EP \underset{k_6}{\overset{k_5}{\rightleftharpoons}} E + P.$$

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It can be seen from Fig. 2 that $(E_F + \Delta H_F) - (E_B + \Delta H_B)$ should equal ΔH, the difference in heat content between initial and final reactants. This relation can also be derived from the equation of HALDANE[18] connecting maximum initial velocities and K_Ms with the equilibrium constant by differentiating with respect to temperature. It has been shown to hold for fumarase[30].

HALDANE[19], reviewing the work of NELSON AND BLOOMFIELD[33] on invertase, states " . . . we should expect to obtain true activation energies only at high substrate concentrations. Submaximal concentrations should give spuriously low or high temperature coefficients, the increase in the velocity of transformation of the enzyme-substrate compound being partly counteracted by decreased formation of the compound or augmented by its increased formation". This statement qualitatively describes equation (6). Further[20], he says "Calling the enzyme and substrate E and S, the molecule ES requires a certain energy of activation before it can undergo chemical change"; he thus distinguishes the two steps shown in Fig. 1.

DANN[7] considered the MICHAELIS equation, and pointed out that there are two quantities to be distinguished: 1. the heat of formation of the MICHAELIS complex (ΔH_M in equation 8); 2. the energy of activation of this complex (E in equation 8). He calculated the latter quantity for the citric dehydrogenase system of peas from the values of the maximum initial velocity at two different temperatures.

KIESE[23] studied the association of CO_2 and water catalysed by carbonic anhydrase. He calculated ΔH_M by means of the VAN 'T HOFF isochore (equation 7); he also determined μ, from the values of the rate constants at concentrations of CO_2 which were adjusted so that exactly half of the enzyme would be combined in the form of the MICHAELIS complex at any temperature. By this means p is kept constant, so that $\mu = E$.

KAUFMAN, NEURATH AND SCHWERT[22] calculated μ for the chymotryptic hydrolysis of two substrates from the corresponding specific rate constants (k_3 in equation 1) at different temperatures. The latter were determined from an equation derived from the MICHAELIS equation by integration with respect to time, by substituting the values of K_M into this equation. This procedure leads to the same result for μ as does that of DANN; so that in this case also the observed activation energy is equal to E. These authors also calculated $\frac{\partial \log K_M}{\partial (1/T)}$ for the reactions studied. A somewhat similar procedure was employed by LUMRY, SMITH AND GLANTZ[28], who studied the action of carboxypeptidase on two synthetic substrates.

Apart from these examples, the significance of the MICHAELIS equation seems to have been ignored when the effects of temperature on enzymic reactions have been studied. It has been the practice generally to determine activation energies for such reactions from a plot of log v against $1/T$, or from the Q_{10} of the reaction velocity, at constant enzyme and substrate concentrations, the latter being usually quite arbitrary. The values so obtained cannot be considered to have any physical significance by themselves, since (equation 8) they represent a combination of E with a function of K_M, s and ΔH_M; they will also vary with substrate concentration. Such a practice would only lead to values of μ independent of substrate concentration if v were found to be directly proportional to k, the specific rate constant to which the ARRHENIUS

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equation applies (equation 2); it has already been pointed out above that in the case of enzymic reactions this constant is k_3, and that the above relation is only true at maximum initial velocity (equation 3). It seems to have been assumed in much of the literature that v is proportional to k under all conditions; thus GREENBERG AND MOHAMED[16] state that "ln k is proportional to the logarithm of the reaction velocity". Similarly, BODANSKY[3] identifies "ln k" with "the natural logarithm of the observed reaction velocity". Again, HADIDIAN AND HOAGLAND[17] use the equation $v = ze^{-E/RT}$, where z is constant; comparison with equation (2) shows that here also the identity $v \propto k$ is implied. v would in fact be proportional to k at all values of s if the enzyme were a reactant and were changed by the reaction; then, the reaction would be a bimolecular one, and v would be connected with k by the relation $v = kes$. v would also be directly proportional to k at all substrate concentrations if the enzyme took no part whatever in the reaction, so that the reaction was unimolecular (in this case v would equal ks). Either alternative contradicts the undoubted fact that enzymes are catalysts.

In Table I some of the data from the literature have been tabulated, and the values of $K_M/(s + K_M)$ worked out. In a few cases ΔH_M has also been recorded, so that it has been possible to work out $(\mu - E)$. However not all the data need recalculating on the basis of equation (6). The work of DANN[7], of KIESE[23], of KAUFMAN *et al.*[22], and of LUMRY *et al.*[28] has already been mentioned. In other cases the concentration of substrate at which μ was measured was sufficiently high for $[K_M/(s + K_M)]\ \Delta H_M$ to be neglected in comparison with the experimental error. An example is the work of GLICK[14] on tropine esterase. This author measured μ at a concentration of atropine of 0.25%, which he found to be the lowest concentration at which the rate of hydrolysis was maximal, so that μ here would not be far different from E. Further examples in which it is considered that the difference between μ and E is probably of the same order as the experimental error are given in Table II.

Equation (8) also explains the statement of SIZER[36] that μ for the inversion of sucrose by yeast invertase is independent of the concentration of sucrose. This statement is based on measurements of μ with 16% and 40% sucrose. Since K_M for invertase is about 0.7%[33], $K_M/(s + K_M)$ will in one case be about 0.04, and in the other about 0.015. Taking the maximum value for ΔH_M as being 5,000 cals. (for a discussion of this quantity see STEARN[44]), $(\mu - E)$ will in one case be less than 200 cals. and in the other less than 75 cals.; the difference is within the experimental error.

In view of equations (6) and (8), it is impossible to attach much significance to values of μ which have been recorded for complex systems, unless the mechanism of the reaction and the rate-determining step are known. The former is unknown in most reactions involving the hydrolysis of a large molecule in the presence of a hydrolytic enzyme, *e.g.* casein and trypsin[43], starch or amylose and amylases[9, 10, 27, 34]; and in such reactions as haemolysis[2]. In these cases the enzyme combines with many degradation products of the original substrate, having a different affinity for each, so that no value can be assigned to K_M. If it is quite certain that the enzyme is saturated with substrate, and that the velocity is not increased by increasing the substrate concentration, μ can be taken to be equal to E. E may be considered to have some significance in the case of, say, β-amylase acting onam ylose; here only α-1:4-glucose links are attacked, and the reaction is fairly clear-cut. In other cases, such as protein hydrolysis, it is doubtful if E can have much meaning, and ΔH_M must remain meaningless, at least until the reaction mechanism is known. The rate-determining step is very often unknown in

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TABLE I

Enzyme	*Substrate*	*pH*	μ *cals.*	s *M*	*Ref.*	K_M *M*	*Temp.* °C	*Ref.*	$\frac{K_M}{s + K_M}$	ΔH_M *cals.*	*Ref.*	$\mu - E$ *cals.*	E *cals.*
Cholinesterase (horse serum)	Acetyl choline	7.4	5,100[a]	.0083	13	.0011[a]	25	12	.12				
Urease	Urea	7.0	8,700[a] or 11,700	.25	38, 39, 40	about .025 [a, b]	20	45	about .091				
		7.0	8,830[a]	.25	24				about .091				
Chymotrypsin	Sturin	7.5	11,800	about .002	5	about .0025[c]	37½	5	about .55				
Arginase (liver)	Arginine	7.5	13,850	.005	16, 32	.0063[d]	40	16	.56				
		9.2	16,000	.005	16, 32	.013 [d]	40	16	.72				
Hydantoinase (liver)	Hydantoin	7.8	5,300	.02	8	.032	37½	8	.62	zero	8	zero	5,300
Hydantoinase (jack bean)	Hydantoin	7.8	11,600	.02	8	.016	37½	8	.45	zero	8	zero	11,600
Malic dehydrogenase (thermophilic bacteria)	L-malate	8.0	3,700	.054	29	.012	37	29	.18				
Fumarase	Fumarate (above 18°)	5.6	7,300	.0167	30	.0018	23	30	.097	+ 7,100	30	+ 700	6,600
		6.35	6,700	.0167	30	.0018	23	30	.097	+ 4,200	30	+ 400	6,300

[a] μ and K_M determined under different experimental conditions.
[b] K_M estimated from data of VAN SLYKE AND CULLEN[45].
[c] K_M taken from a graph of v against s.
[d] K_M taken from a graph of K_M against pH.

TABLE II

Enzyme	*Substrate*	*pH*	μ *cals.*	s *M*	*Ref.*	K_M *M*	*Temp.* °C	*Ref.*	$\frac{K_M}{s+K_M}$	ΔH_M *cals.*	*Ref.*	$\mu - E$ *cals.*
Invertase	Sucrose	var.	7,700 to 16,000	.29	33	about .020	var.	33	about .07			
		6.0	11,000	.47	37				about .04			
Phosphatase (bone)	β-glycerophosphate	9.2	9,900[a]	.0127	3	.0012[a]	37	35	.086			
Zymohexase	Hexose diphosphate	7.3	16,500[b]	.0125	21, 41	.001	38	21	.08			
Fumarase	Fumarate (below 18°)	5.6	13,000	.0167	30	.0018	23	30	.097	+800	30	+80
		6.35	10,600	.0167	30	.0018	23	30	.097	+400	30	+40
	Fumarate	7.9	4,900	.0167	30	.0010	23	30	.056	+4,200	30	+230
	L-malate	6.35	13,100	.033	30	.0040	23	30	.11	−1,200	30	−120
		7.9	15,600	.033	30	.0062	23	30	.16	−1,200	30	−190

[a] μ and K_M determined under somewhat different conditions.
[b] μ calculated by SIZER[41] from data of HERBERT *et al.*[21]

biological oxidations and reductions. Even where it is known, it will be necessary to determine the values of both E and ΔH_M for the limiting step before the activation process can be adequately described. A simple value of μ, taken at arbitrary concentrations of hydrogen-donor and acceptor[6,15,42], will not have much significance.

It is evident from the above, and from Tables I and II, that much more work must be done if enzymic activation is to be understood. In place of the somewhat haphazard method of measuring μ at arbitrary substrate concentrations, it will be necessary to determine μ at maximum initial velocity, where it is equal to E, and also to determine ΔH_M. ΔH_M may be determined from a plot of log K_M against $1/T$, which has a slope of $\Delta H_M/R$ (equation 7). It may also be determined from a plot of log v against $1/T$ at very low substrate concentrations; under these conditions $K_M/(s + K_M) \rightarrow 1$, and $\mu \rightarrow E + \Delta H_M$ (equation 8). Both methods will have about the same degree of error, as they both require the accurate measurement of reaction velocities at low substrate concentrations. Perhaps the simplest method of determining both E and ΔH_M would be to observe reaction velocities at various substrate concentrations and temperatures, but constant enzyme concentration; a graph of $1/v$ against $1/s$ would then give the values of v_{max} and K_M at a particular temperature[26], and from a series of these E and ΔH_M could be calculated by means of equations (3) and (7). However until measurements of both E and ΔH_M become general practice it will not be possible to draw any justifiable conclusions about enzymic activation from the recorded data.

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SUMMARY

1. From a consideration of the MICHAELIS equation and the ARRHENIUS equation, it is shown that it is necessary to distinguish two quantities in enzymic activation: a. the heat of formation of the enzyme-substrate complex, and b. the energy of activation of this complex; and an equation is derived connecting these two quantities with the activation energy observed at any concentration of substrate.

2. Some activation energies recorded in the literature have been briefly examined in the light of this equation. It has been found that, with a few notable exceptions, the values recorded for activation energies of enzymic reactions are open to criticism on the grounds that they represent a combination of the two quantities above, and hence by themselves have no physical significance.

RÉSUMÉ

1. En se basant sur les équations de MICHAELIS et d'ARRHÉNIUS les auteurs montrent qu'il est nécessaire de distinguer deux quantités lors de l'étude de l'activation enzymatique: a. la chaleur de formation du complexe enzyme-substrat et b. l'énergie d'activation de ce complexe. Ils établissent une équation reliant ces deux quantités à l'énergie d'activation observée à une concentration de substrat quelconque.

2. Quelques énergies d'activation de la littérature ont été examinées brièvement à la lumière de cette équation. Les auteurs ont constaté que, à part un petit nombre d'exceptions, les valeurs indiquées pour les énergies d'activation de réactions enzymatiques sont critiquables parce qu'elles représentent des combinaisons des deux quantités susnommées et n'ont donc pas de signification physique en elles-mêmes.

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ZUSAMMENFASSUNG

1. Auf Grund der Gleichungen von MICHAELIS und ARRHENIUS wird gezeigt, dass bei der Enzym-Aktivation zwei Grössen unterschieden werden müssen: a. die Bildungswärme des Enzym-Substrat-Komplexes und b. die Aktivierungsenergie dieses Komplexes. Eine Gleichung wird abgeleitet, welche diese beiden Grössen mit der bei irgend einer Substratkonzentration beobachteten Aktivationsenergie verbindet.

2. Einige Aktivationsenergien aus der Literatur werden im Lichte dieser Gleichung kurz geprüft. Es wurde gefunden, dass mit einigen wenigen Ausnahmen, die für Aktivationsenergien von Enzymreaktionen angegebenen Werte beanständet werden müssen, da sie Kombinationen der beiden obengenannten Grössen darstellen und deshalb keine eigene physikalische Bedeutung haben.

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