*Theory of the Influence of the Dielectric Constant on the Rate of Reaction in Solution with Application to Enzyme Reactions. II. Application of the Theory to Enzyme Reactions**

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The enzyme molecule in solution has a number of charged groups, some of which play an essential role in the catalytic reactions. Rates of enzyme reactions, therefore, may be expected to be influenced by the dielectric constant of a medium as are generally observed in reactions involving simple ions or dipolar molecules. From this viewpoint, rates of several enzyme reactions have been measured in aqueous mixed solvents, and the results have been discussed in relation to the dielectric constant of the medium[1-8]. In treating catalase and peroxidase reactions, Ogura et al.[1] and Tonomura[3] considered the enzyme or the enzyme-substrate complex as a dipole, to which the expression for the reaction between dipolar molecules[9] was applied. On the other hand, for α-chymotrypsin[2] and myosin[4] catalyzed reactions, Laidler and his coworkers applied the coulombic law to the interaction between the charges of the substrate and the essential groups of the enzyme. However, since it is supposed that the reaction may occur in a local region near the surface of a large cavity of low dielectric constant formed by the enzyme molecule in solution, these treatments may be said to be over-simplified, and the validity of the conclusions deduced from them may be subject to criticism.

The Kirkwood's model for a spherical solute molecule having an arbitrary charge distribution, on which a general theory of the solvent effect has been developed in the preceding paper[10], seems to be appropriate as a model for a protein in solution. In this paper, the theory will be extended to include the solvent effect upon enzyme reactions.

Theoretical

Enzyme reactions in general consist of two stages, formation of an intermediate enzyme-substrate complex, and its breakdown into products and free enzyme.

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES \overset{k_2}{\rightarrow} E + P$$

The Michaelis constant K_m and the rate constant of breakdown k_2 can be obtained in the usual manner[11]. In certain cases in which $k_{-1} \gg k_2$, K_m may be identified with the reciprocal of the equilibrium constant K for the formation of ES complex[12]. In some instances, k_1 or k_{-1} can be measured directly[13], or may

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1) Y. Ogura, A. Hattori, Y. Tonomura and S. Hino, *Symposia on Enzyme Chem.* (*Kôso Kagaku Shimpojiumu*), 5, 28 (1950); Y. Ogura and H. Hasegawa, ibid., 7, 79 (1952).

2) M. L. Barnard and K. J. Laidler, *J. Am. Chem. Soc.*, 74, 6099 (1952).

3) Y. Tonomura, *J. Japan Biochem. Soc.*, (*Seikagaku*), 25, 175 (1953).

4) K. J. Laidler and M. C. Ethier, *Arch. Biochem. Biophys.*, 44, 338 (1953).

5) R. Lumry and E. L. Smith, *Discussions Faraday Soc.*, 20, 105 (1955).

6) S. Ono, K. Hiromi and Y. Sano, Presented as a lecture at the 8th Annual Meeting of Chem. Soc. Japan, Tokyo (1955); S. Ono, K. Hiromi and Y. Sano, to be published.

7) A. Stockell and E. L. Smith, *J. Biol. Chem.*, 227, 1 (1957).

8) T. Hosoya, *J. Biochem.* (Tokyo) in press.

9) S. Glasstone, K. J. Laidler and H. Eyring, "The Theory of Rate Processes", McGraw-Hill New York (1941), p. 419.

10) K. Hiromi, This Bulletin, 33, 1251 (1960).

11) For example, H. Lineweaver and D. Burk, *J. Am. Chem. Soc.*, 56, 658 (1934).

12) M. F. Morales, ibid., 77, 4169 (1955); K. J. Laidler, *Trans. Faraday Soc.*, 51, 540 (1955).

13) B. Chance, "Investigation of Rates and Mechanisms of Reactions", in "Technique of Organic Chemistry", edited by A. Weissberger, Interscience Publishers, New York (1953), Vol. VIII, p. 627.

be obtained from the analysis of overall reaction rates[14]. These kinetic parameters, k's and K, may be influenced by the dielectric constant D of the solvent, and equations similar to Eqs. 12 and 13 in the preceding paper[10] may be available. They involve the charge configuration functions L's of the substrate, enzyme, ES complex and/or ES$_*$, the activated complex of ES. The L's of the last three species involve the contribution of all the charges of the enzyme molecule, which we cannot evaluate explicitly because of our lack of knowledge about their spatial distribution over the enzyme molecule. However, as will be seen shortly, the following treatment may be allowable. Instead of L, the complete charge configuration function involving all the charges in the enzyme, we may use L^0, the effective charge configuration function concerned only with the small number of charges of special interest belonging to a limited reaction region, disregarding all the remaining charges in the enzyme which are considered to be kept fixed and not to come into play during the reaction process.

Consider the equilibrium, for example,

$$\mathrm{E} + \mathrm{S} \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} \mathrm{ES}$$

The variation of the equilibrium constant $K(=k_1/k_{-1})$ with D can be written as[10]

$$\frac{\mathrm{d}\log K}{\mathrm{d}(1/D)} = \frac{\varepsilon^2}{2.303(2kT)}\left(\frac{L_\mathrm{S}}{b_\mathrm{S}} + \frac{L_\mathrm{E}}{b_\mathrm{E}} - \frac{L_\mathrm{ES}}{b_\mathrm{ES}}\right) \tag{1}$$

where b's are the radii of the substrate, enzyme and ES complex, as indicated by the suffixes, L's are the corresponding complete charge configuration functions which contain the contribution of all the charges in E and ES, and the other symbols have the same meaning as those in the preceding paper[10]. For small substrates b_E and b_ES may be equated. Let E and ES have in the total M and M' charges, respectively, and let n of them be kept fixed in position and free from any change during the process. Thus

$$\left.\begin{aligned} M &= n+m \\ M' &= n+m' \end{aligned}\right\} \tag{2}$$

where m and m' are the number of charges of special interest which belong to the local reaction region in E and ES, respectively. L_E and L_ES are written as follows[10];

$$\begin{aligned} L_\mathrm{E} &= \sum_{k=1}^{M}\xi_k^2 f_{kk} + \sum_{k=1}^{M}\sum_{l\neq k}\xi_k\xi_l g_{kl} \\ &= \sum_{i=1}^{m}\xi_i^2 f_{ii} + \sum_{s=1}^{n}\xi_s^2 f_{ss} + \sum_{i=1}^{m}\sum_{s=1}^{n}\xi_i\xi_s g_{is} \\ &\quad + \sum_{i=1}^{m}\sum_{j\neq i}\xi_i\xi_j g_{ij} + \sum_{s=1}^{n}\sum_{t\neq s}\xi_s\xi_t g_{st} \end{aligned} \tag{3}$$

$$\begin{aligned} L_\mathrm{ES} &= \sum_{k=1}^{M'}\xi_k^2 f_{kk} + \sum_{k=1}^{M'}\sum_{l\neq k}\xi_k\xi_l g_{kl} \\ &= \sum_{i=1}^{m'}\xi_i^2 f_{ii} + \sum_{s=1}^{n}\xi_s^2 f_{ss} + \sum_{i=1}^{m'}\sum_{s=1}^{n}\xi_i\xi_s g_{is} \\ &\quad + \sum_{i=1}^{m'}\sum_{j\neq i}\xi_i\xi_j g_{ij} + \sum_{i=1}^{n}\sum_{t\neq s}\xi_s\xi_t g_{st} \end{aligned} \tag{4}$$

where the suffixes i and j refer to the charge number of the charges belonging to the m or m' group, s and t the n group, and f and g are the functions of the positions of charges as were defined by Eq. 4 in the preceding paper[10].

Hence we have

$$\begin{aligned} L_\mathrm{E} - L_\mathrm{ES} &= \Big(\sum_{i=1}^{m}\xi_i^2 f_{ii} + \sum_{i=1}^{m}\sum_{j\neq i}\xi_i\xi_j g_{ij}\Big)_\mathrm{E} \\ &\quad - \Big(\sum_{i=1}^{m'}\xi_i^2 f_{ii} + \sum_{i=1}^{m'}\sum_{j\neq i}\xi_i\xi_j g_{ij}\Big)_\mathrm{ES} \\ &\quad + \Big(\sum_{i=1}^{m}\sum_{s=1}^{n}\xi_i\xi_s g_{is}\Big)_\mathrm{E} - \Big(\sum_{i=1}^{m'}\sum_{s=1}^{n}\xi_i\xi_s g_{is}\Big)_\mathrm{ES} \end{aligned} \tag{5}$$

The last two terms of the right hand side in Eq. 5 are those from the pair-wise contribution between the m or m' charges of special interest and the n remaining fixed charges in E and ES. If the n charges consist of approximately equal numbers of positive and negative charges, and they are distributed at random over the enzyme molecule, these terms may be appreciably small and may be dropped without serious error. Hence

$$\left.\begin{aligned} L_\mathrm{E} - L_\mathrm{ES} &= L^0_\mathrm{E} - L^0_\mathrm{ES} \\ L^0_\mathrm{E} &= \Big(\sum_{i=1}^{m}\xi_i^2 f_{ii} + \sum_{i=1}^{m}\sum_{j\neq i}\xi_i\xi_j g_{ij}\Big)_\mathrm{E} \\ L^0_\mathrm{ES} &= \Big(\sum_{i=1}^{m'}\xi_i^2 f_{ii} + \sum_{i=1}^{m'}\sum_{j\neq i}\xi_i\xi_j g_{ij}\Big)_\mathrm{ES} \end{aligned}\right\} \tag{6}$$

where L^0_E and L^0_ES are the effective charge configuration functions of E and ES, concerned only with the charges of special interest. Eq. 1 becomes, therefore,

$$\frac{\mathrm{d}\log K}{\mathrm{d}(1/D)} = \frac{\varepsilon^2}{2.303(2kT)} \times\left[\frac{L_\mathrm{S}}{b_\mathrm{S}} + \frac{1}{b_\mathrm{E}}(L^0_\mathrm{E} - L^0_\mathrm{ES})\right] \tag{7}$$

Similarly for the breakdown process of ES, we have

$$\frac{\mathrm{d}\log k_2}{\mathrm{d}(1/D)} = \frac{\varepsilon^2}{2.303(2kT)}\left(\frac{L^0_\mathrm{ES}}{b_\mathrm{ES}} - \frac{L^0_{\mathrm{ES}*}}{b_{\mathrm{ES}*}}\right) \tag{8}$$

14) H. Gutfreund, *Discussions Faraday Soc.*, **20**, 167 (1955); E. C. Slater, ibid., **20**, 231 (1955).

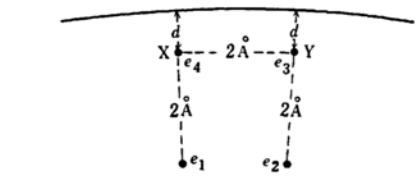
where L^0_{ES*} is the effective charge configuration function of ES_*. Similar equations for k_1 or k_{-1} if necessary, may readily be obtained.

Application to Some Typical Models

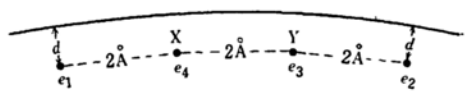
In Table I are summarized the data of the solvent effect upon the kinetic parameter $\mathfrak{K}$ which have been obtained for various enzyme reactions[1-8]. $\mathfrak{K}$ represents the rate constant or equilibrium constant specified in each item. The slopes $d \log \mathfrak{K}/d(1/D)$ have been calculated under the assumption that the observed changes in $\mathfrak{K}$ are due entirely to the variation in dielectric constant of the mixed solvent. The radii of the enzymes are computed from the molecular weight and the specific volume[15] assuming the spherical shape of the enzymes. In many cases, the slopes have considerably large negative values of the order of -200.

It is worthwhile to see whether the present theory can reasonably account for the slopes of the observed order of magnitude, considering only the electrostatic rearrangement in the local reaction region.

For the present, let us confine our attention to the breakdown process of ES complex in which the bond X–Y of a substrate molecule is to be split. During the activation process a change in charge state may occur in the local reaction region which involves the bound substrate molecule and the catalytic center of the enzyme. We may assume that ES and ES_* are spheres of equal radii b_{ES}, and that the substrate molecule is situated near the surface of the sphere. It is reasonable to suppose that in the activated complex the bond to be split is polarized so that a positive charge and a negative charge will be produced on the atoms X and Y forming the bond, as a preliminary stage of bond splitting[2]. It may lead to a change in L^0 by which the slope $d \log k_2/d(1/D)$ will be determined. The increment in L^0, and hence the slope, may depend on the magnitude of the charges produced, their position in the sphere (or the depth of the charges from the surface), and the situation of the neighboring charges. Two typical models for ES_* are shown in Fig. 1. e_3 and e_4 are the charges produced on the atoms X and Y in ES_* at a depth d from the surface of the sphere. e_1 and e_2 are the neighboring charges which represent, for example, the charges of the active site of the enzyme, or the other charges the substrate may bear. In both models, the distance between e_3 and e_4 is taken as 2 Å, which is nearly equal to 50% stretched bond length of C–O or C–N



Model I



Model II

Fig. 1. Models of ES_*.
e_1 and e_2 represent the neighboring charges, and e_3 and e_4 the charges produced on the atoms X and Y in the activated state.

bond over their normal ones. In Model I, e_1 and e_2 are assumed to be situated by 2 Å inside on the lines drawn from the center to the charges e_4 and e_3, respectively. In Model II, e_1 and e_2 are situated on both sides of e_4 and e_3, 2 Å apart from them, respectively, at a depth d from the surface. ξ's denote the valences of the charges. The increment in the effective charge configuration function, ΔL^0_* $(=L^0_{ES*} - L^0_{ES})$, accompanied with the activation of ES complex, can be calculated from Eq. 6, using Tables I and II in the preceding paper[10], and the slope $d \log k_2/d(1/D)$ from Eq. 8. In the present case, calculations were made under the following conditions; i) the charges e_3 and e_4 are both zero in ES, ii) $-\xi_3=\xi_4=1$ or 1/2 in ES_*, iii) e_1 and e_2 are either positive or negative unit charges and are fixed during the activation process, iv) d is taken as 0.5 Å or 1.0 Å, and v) $b_{ES}=b_{ES*}=21$ Å and the temperature is 25°C. The results are summarized in Table II. It is seen that the slope is determined primarily by the magnitude of the charges produced and by the depth of the charges below the surface, d, and is also dependent upon the situation of the neighboring charges especially in Model II. The slope of the observed order of magnitude is reasonably accounted for, if a pair of charges of opposite sign whose valence is close to unity are produced in the activated complex at the depth of 0.5~1.0 Å below the surface. These values for depth of charges are in reasonable agreement with those determined for the simple systems in the preceding paper[10]. On the other hand, Laidler-Landskroener's equation[16] applied to the above models, yields a slope, at the greatest, of about

15) E. J. Cohn and J. T. Edsall, "Proteins, Amino Acids and Peptides", Reinhold Publishing Co., New York (1943), p. 370.

16) K. J. Laidler and P. A. Landskroener, *Trans. Faraday Soc.*, 52, 200 (1956). (cf. Eq. 14 in the preceding paper).

TABLE I. SOLVENT EFFECTS UPON VARIOUS ENZYME REACTIONS

Substrate	Enzyme	Radius b_E, Å	Temp. °C	$\mathfrak{K}$	Solvent[a]	$\frac{d \log \mathfrak{K}}{d(1/D)}$	Reference No.
Carbobenzoxy-glycyl-L-tryptophan	Carboxy-peptidase	21	25	k_2	W-M	−200	
					W-E	−180	5
					W-D	− 30	
Methyl hydrocinnamate	α-chymo-trypsin	20	25	k_2	W-M	−260	2
				K	W-M	−240	
α-Benzoyl-L-argininamide	Papain	18	38	k_1	W-M	−160	
			38	k_1	W-*i*Pr	−160	7
			5	k_1	W-M	−180	
Soluble starch	Bacterial α-amylase	23	25	k_2	W-M	−210	6
				K	W-M	0	
ATP	Myosin	—	25	k_2	W-M	80	4
				K	W-M	330	
H_2O_2	Catalase	42	0	k_3[b]	W-E	−160	1
H_2O_2+guaiacol	Peroxidase (Japanese radish)	22	25	k[c]	(W-E, W-A W-D)	−200	3
H_2O_2+guaiacol	Peroxidase (Turnip) A_1	23	20	k_4[d]	(W-M, W-A)	−210	
	A_2	23	20	k_4	W-M	−220	8
	D	23	20	k_4	W-M	−250	

a) W: water, M: methanol, E: ethanol, D: dioxane, *i*Pr: *iso*-propanol, A: Acetone.

b) $ES \xrightarrow{s} E + P$ (s: H_2O_2) $\quad \xrightarrow{k_3}$

c) $ES \xrightarrow{a} E + P$ (a: guaiacol) $\quad \xrightarrow{k}$

d) The apparent rate constant of the reaction between complex II and guaiacol.

TABLE II. PREDICTION OF THE THEORY FOR TYPICAL MODEL REACTIONS

Charges produced	Model	Neighboring charges ξ_1	Neighboring charges ξ_2	Charge configuration in $ES_\ddagger$[a]	$\Delta L^0_\ddagger$ $d=$ 0.5 Å	$\Delta L^0_\ddagger$ $d=$ 1.0 Å	$-d \log k_2/d(1/D)$ $d=$ 0.5 Å	$-d \log k_2/d(1/D)$ $d=$ 1.0 Å
	I, II	0	0	(○ ●)	46.3	12.8	269	74.2
	I	+1	+1	(○ ● / ○ ○)	46.3	12.8	269	74.2
$\xi_3=-1$	I	+1	−1	(○ ● / ○ ●)	55.1	16.8	320	97.4
$\xi_4=+1$	I	−1	+1	(○ ● / ◕ ○)	37.5	8.8	218	51.0
	II	+1	+1	(○ ○ ● ○)	46.3	12.8	269	74.2
	II	+1	−1	(○ ○ ● ●)	80.4	34.1	466	198
	II	−1	+1	(◕ ○ ● ○)	13.0	−8.4	75.4	−48.7
	I, II	0	0	(∘ •)	11.6	3.2	67.3	18.6
	I	+1	+1	(∘ • / ○ ○)	11.6	3.2	67.3	18.6
$\xi_3=-1/2$	I	+1	−1	(∘ • / ○ ●)	16.0	5.2	92.8	30.2
$\xi_4=+1/2$	I	−1	+1	(∘ • / ● ○)	7.2	1.2	41.8	7.0
	II	+1	+1	(○ ∘ • ○)	11.6	3.2	67.3	18.6
	II	+1	−1	(○ ∘ • ●)	28.6	13.8	169	80.0
	II	−1	+1	(● ∘ • ○)	−5.4	−7.4	−31.3	−42.9

a) The charge configurations in $ES_\ddagger$ (Models I and II) are schematically illustrated with the white and the black circles. The large white or black circle represents a positive or a negative unit charge, and the small white or black circle a positive or a negative half unit charge, respectively.

-0.8, by a factor of 10^2 less than the observed values.

Discussion

The present theory which is based on the Kirkwood's model may be more suited for treating the solvent effect upon enzyme reactions than the previous ones[1-4], although some simplifications inevitable in dealing with the complex enzyme molecule have still been made. The application of the theory to the typical models has shown that it successfully accounts for the experimentally obtained slopes, considering only the local rearrangement of charges in the enzyme-substrate complex. An advantage of the present theory over the existing theories is that it can deal with any microscopic change in charge state in any position in the spherical solute. It eliminates the disadvantage of the treatment which regards the enzyme or the enzyme-substrate complex as a dipole[1,3], according to which an extraordinarily large dipole moment had to be assigned to the activated complex in order to account for the slope of the observed order of magnitude[17].

At present we shall have to be content with the successful results of the application of the theory to the typical examples stated above. However, when our knowledge has accumulated about the value of d to be assigned in the individual cases, we may reasonably hope that we can have an insight into the microscopic charge configuration in the activated state from the slope obtained by the study of the solvent effect on the reaction rate, which may serve as a tool for elucidating the mechanism of the reaction.

However, in interpreting the experimental data of solvent effect, attention must be paid to the possibility that the observed effects may involve contributions of various factors other than the purely electrostatic effect of dielectric constant on the rate. The factors that may possibly influence the rate by changing the composition of mixed solvents are enumerated as follows; 1) decrease in water concentration which might result in reduction of rates, if the rate determining step involves participation of the water molecules especially for hydrolytic enzymes, 2) shift of the activity-pH curve which may decrease or increase the rate when the experiments are carried out at constant pH[6], 3) possible inhibition by some component of the mixed solvent, 4) irreversible or reversible denatuation of the enzyme caused by the addition of organic solvents, 5) change in viscosity which may influence the rate if the reaction is diffusion controlled[18-20]. In most cases, the addition of organic solvents may lead to a decrease in rates due to these factors, in addition to a decrease in the dielectric constant of the medium. If this is the case, the observed slopes may possibly have been over-estimated in the negative direction, which would lead to larger values of d in the above calculation.

For non-zero values of ionic strength, the slope may be affected by the inclusion of the term dependent on ionic strength as already discussed in the preceding paper[10]. The contribution of this term may be small except for bimolecular processes in which both of the reactants have non-zero net charges. Of course, the contribution is greater at higher ionic strength. A rough estimate reveals that for ionic strength of 0.02 and the radius of the enzyme taken as 20 Å[21], the contribution to the slope may be less than about ± 40 for a bimolecular process between reactants having univalent net charge. It is desirable that the reaction should be carried out at an ionic strength as low as possible, in order to elucidate the effect of dielectric constant on the rate.

The theory may also be applied to the influence of solvent on binding of substrates or inhibitors to the enzyme, although there are restrictions inherent to the Kirkwood's model.

Summary

The theory of solvent effect developed in the preceding paper has been extended to include the enzyme reactions. The application to some typical model reactions which resemble the breakdown of enzyme-substrate complex has shown that the order of magnitude of experimentally observed effects can reasonably be accounted for by the theory.

Factors which may possibly influence rates of enzyme reactions by changing the composition of mixed solvents have also been discussed.

The author expresses his hearty thanks to Professor Sôzaburo Ono for his kind guidance

17) For example, if the slope d log k_2/d(1/D) of the order of -200 is interpreted by the equation for dipolar molecules[1,3,9]

$$\frac{\mathrm{d}\log k_2}{\mathrm{d}[(D-1)/(2D+1)]} = \frac{1}{2.303\,kTb_{ES}^3}(\mu_{ES^\ddagger}^2 - \mu_{ES}^2)$$

where μ's are the dipole moments, it is necessary that $\mu_{ES^\ddagger}^2$ be larger than μ_{ES}^2 by ca. 2.4×10^5 Debye^2, for b_{ES} = 21 Å at 25°C. Accepting this large increase in μ, one might be forcibly led to the conclusion that a profound change in shape or charge distribution in the whole body of the enzyme molecule had been brought about during the activation process.

18) E. Ackerman, G. K. Strother and R. L. Berger, "The Influence of Temperature on Biological Systems", Papers Symposium, Storrs, Conn. (1956), p. 25.

19) R. A. Alberty and G. G. Hammes, *J. Phys. Chem.*, 62, 154 (1958).

20) H. Mitsuda et al., *Symposia on Enzyme Chem.*, (*Kôso Kagaku Shimpojiumu*), in press.

21) Accordingly κa (cf. Eq. 21 in the preceding paper) at room temperature is about unity.

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Note: The present theory was recently applied by Hosoya to the interpretation of his data of solvent effect upon the peroxidase reactions. (See Ref. 8.)