*Kinetics of Hydrolytic Reaction Catalyzed by Crystalline Bacterial α-Amylase. II. The Influence of Solvent**

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The electrostatic nature of a chemical reaction in solution can be inferred from the dependence of the reaction rate on the dielectric constant and on the ionic strength of the medium[1-3]. As for enzyme reactions, however, the study of the ionic strength effect is not so useful, because various ions often exert specific effects on enzyme reactions; moreover, the theoretical treatment of this effect is extremely difficult with such large complex ions as protein[4,5]. Thus the study of the dielectric constant effect becomes important in elucidating the electrostatic nature of enzyme reactions, for it would provide information concerning the reaction mechanisms.

There are, however, two difficulties in the study of the dielectric constant effect on enzyme reactions. The first, which is a theoretical one, is that the conventional theories that have been developed for simple ions or molecules[1-3] may no longer be applicable to enzyme reactions because of the complex charge structure of the molecule; the second is that the organic additives used to control the dielectric constant of the medium may possibly influence, directly or indirectly, the reaction rates from various causes other than the electrostatic one.

The effects of solvent on the rates of various enzyme reactions have been studied by several

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authors[6-16], some of whom have interpreted[6-10] the results in terms of simple electrostatic theories developed for simple ions or dipolar molecules. Recently one of us developed a theory of the influence of the dielectric constant of solvent on equilibria and reaction rates[17,18], using the Kirkwood model of a spherical solute molecule having an arbitrary charge distribution[4], which is most probably an appropriate model for a protein molecule in solution[5]. The theory is essentially applicable to the dielectric constant effect on reactions in solution, including enzyme reactions, in which any rearrangement of charges occurs within the spherical solute molecules involved. The applicability of the theory was tested for certain enzyme reactions[18], as well as for some simple organic reactions[17], and the results were considered satisfactory. Hosoya has also applied the theory to the interpretation of his data on the peroxidase reaction[15].

As for the second difficulty, we have tried in this paper to find some general kinetic procedures by which we can distinguish the true dielectric constant effect from the other non-electrostatic effects caused by the organic additives.

In Part I of this paper[19], the influence of pH upon the α-amylase-catalyzed hydrolysis of amylose was investigated, and the properties of the ionizable groups essential for the reaction were elucidated. In this paper, the influence of solvent upon the reaction rates will be studied kinetically, and the results will be interpreted on the basis of the theory developed by one of us[17,18]. The possible effects of the addition of the organic solvent will also be examined.

Experimental

Enzyme.—Six times-recrystallized bacterial amyloclastic α-amylase of *Bac. amyloliquefaciens* Fukumoto was kindly provided by Prof. J. Fukumoto and Dr. T. Yamamoto of Osaka City University. An aqueous solution of the crystalline enzyme was used as a stock solution. The enzyme activity of the stock solution was checked before each experiment.

Substrate.—Since amylose is precipitated from an aqueous solution by the addition of even a small volume of such an organic solvent as methanol, it can not be used as the substrate for the present purpose. Therefore, potato soluble starch, washed with cold water and with methanol and dried, was used as the substrate.

Solvent.—Water or a methanol-water mixture was used as the solvent. The methanol was purified with alkaline silver nitrate and twice redistilled.

Reaction Mixture.—The composition of the reaction mixture was as follows: Aqueous solution of soluble starch, 20 ml.; methanol, x ml,; water, $(8-x)$ ml.; diluted-enzyme stock solution, 2 ml. The final concentration of the substrate varied from 0.075% to 0.5%. The methanol concentration ranged from zero to 22.3 per cent by weight. In order to minimize the ionic strength of the medium[17,18] no buffer solution was used. The pH value of the reaction mixture, however, was maintained at 6.0 $\pm$ 0.1, sufficiently close to the optimum pH value of 5.85[19]. The reaction was carried out at 25.0°C throughout the experiments.

Procedure.—Two milliliters of the reaction mixture was pipetted into 1 ml. of 0.5 N sodium hydroxide to stop the reaction every minute during the first five minutes; 1 ml. of a 3,5-dinitrosalicylate reagent[20] was then added, and the mixture was heated at 65°C for one hour and diluted to 20 ml., and the red color which developed was measured photometrically, using maltose as the standard. Methanol slightly increases the color density, and this effect was calibrated.

Relative Rate-pH Curves.—In the investigation of the effect of methanol on the relative rate-pH curve, a Britton-Robinson buffer was used in its 1/30 concentration to adjust the pH values.

Even at the maximum methanol concentration used, no irreversible inactivation of the enzyme was detected during the reaction period at 25°C and pH 6.0.

Results

The Michaelis constant, K_m, and the first order rate constant for the breakdown of the ES complex, k_3, obtained from the Lineweaver-Burk plot[21] for nine different substrate concentrations at various methanol contents, are listed in Table I. Since the substrate soluble starch contains α-1,6 linkages, which are not hydrolyzed by this enzyme, the intrinsic substrate concentration cannot be

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TABLE I. THE VALUES OF K_m AND k_3 AT VARIOUS METHANOL CONCENTRATIONS AT 25°C AND pH 6.0

Methanol concn. % by wt.	Water concn. M	D[a]	K_m %	k_3 sec^{-1}
0	55.5	78.5	0.043	1750
5.34	51.9	76.3	0.043	1480
10.84	48.3	74.0	0.042	1210
16.50	44.8	71.6	0.040	970
22.33	41.3	69.1	0.042	750

a) Dielectric constant of the solvent. Interpolated from the values of Davis and Jones[22].

defined accurately. The Michaelis constants, therefore, is conveniently expressed in per cent units.

It is clearly indicated that K_m is constant over the range studied, while k_3 decreases with an increase in the methanol concentration, or with a decrease in the dielectric constant of the medium.

The relationship between pH and the relative rate, v/v_{max}, the ratio of the initial rate at a given pH value to that at the optimum pH value, is influenced by the addition of methanol, as is shown in Fig. 1. The curve is shifted towards the alkaline side by the addition of methanol (22.3 per cent by weight), which is probably due in part to the fact that the pK values of the essential ionizable groups of the enzyme have been influenced by the addition of methanol. It may be seen from Fig. 1 that the velocity is remarkably influenced by the addition of methanol at pH's remote from the optimum pH while the rate at the optimum pH is changed only insignificantly. In order to study the dielectric constant effect on the reaction rates, therefore, the reaction should be observed in the vicinity of the optimum pH. In the present case, in which the reactions were carried out at pH 6.0, the discrepancy between the curves is so small that the observed decreases in k_3 cannot be attributed to the shift of the pH optimum.

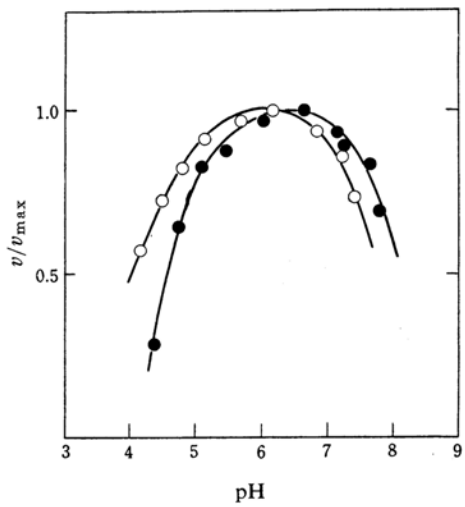

Fig. 1. Plots of v/v_{max} vs. pH.
○: without methanol
●: in 22.3 wt. % methanol
Substrate concn. 0.2%

Analysis of the Results

In the interpretation of the effect of a solvent upon the rate of enzyme reaction special caution is required, since the addition of an organic solvent may give rise to changes in reaction rates for various reasons. Six factors which may influence the rate on the addition of organic solvents and which are considered to be important in the present case are as follows: 1) a change in the dielectric constant of the solvent, which may directly influence the rate; 2) a decrease in the water concentration; 3) an inhibitory action of the organic additive; 4) an irreversible or reversible denaturation of the enzyme; 5) an aggregation of the substrate, and 6) a shift in the relative rate-pH curve. The first factor is the electrostatic one at which the present study is aimed. However, there may possibly exist influences from the other factors. In fact, for the enolase reaction[23] it was observed that the rate is the same function of the water concentration, irrespective of the nature of the organic additives used. In the myosine- and α-chymotrypsin-catalyzed reactions, it has been reported that methanol acts as a "water analogue" to yield methanolysis products, and the existence of the specific water site in these enzymes has been suggested [24,25]. The hydroxylaminolysis of methyl hyppurate catalyzed by α-chymotrypsin has also been observed[26]. On the other hand, by using a variety of organic substances as solvent additives, Castañeda-Agulló and Del Castillo [13,14] have shown that in α-chymotrypsin- and trypsin-catalyzed hydrolyses there is an obvious relationship between the rates and the dielectric constant. Thus, how to distinguish these possible factors is a general and important problem in the interpretation of solvent effects on enzyme reactions. The kinetic procedure for it will be worked out below.

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Kinetic Treatment Considering the Participation of the Water Molecule.—With respect to the role of water in enzyme-catalyzed hydrolytic reactions, two cases may be considered, based on the usual Michaelis-Menten-Briggs-Haldane mechanism, which includes a single intermediate enzyme-substrate complex[27].

Case 1. Water reacts with the ES complex directly from the bulk of the solvent.

The reaction scheme should be written as follows:

$$E+S \underset{k_2}{\overset{k_1}{\rightleftharpoons}} ES \tag{1}$$

$$ES+H_2O \xrightarrow{k_3'} E+P \tag{2}$$

instead of the ordinary scheme:

$$E+S \underset{k_2}{\overset{k_1}{\rightleftharpoons}} ES \xrightarrow{k_3} E+P \tag{3}$$

Obviously the apparent rate constant, k_3, is proportional to the water concentration $[H_2O]$:

$$k_3=k_3'[H_2O] \tag{4}$$

The k_3 values obtained are tentatively plotted against the water concentration in Fig. 2. It is apparent that the observed change in k_3 is too large to be accounted for by Eq. 4.

Case 2. The case in which there is competition between the water molecule and the organic solvent molecule for the water site on the enzyme.

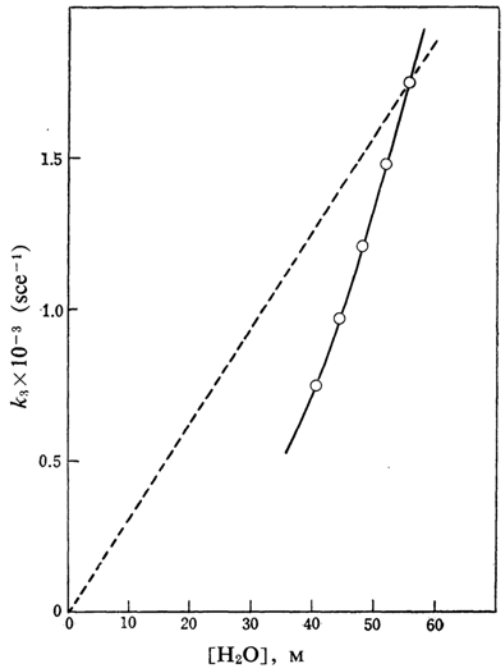

Fig. 2. Plot of k_3 vs. molar concentration of water. The dotted line represents the proportionality between them.

Since evidence has been obtained for the existence of a specific water site in some hydrolytic enzymes[24,26], it is possible that there is a specific water site on this enzyme for which the water molecule competes with the organic solvent molecule. The reaction scheme may then be written as follows:

$$\begin{array}{ccccc} EM & \overset{K_M}{\rightleftharpoons} & E & \overset{K_W}{\rightleftharpoons} & EW \\ {\scriptstyle K_S''}\downarrow\uparrow & & {\scriptstyle K_S}\downarrow\uparrow & & {\scriptstyle K_S'}\downarrow\uparrow \\ EMS & \underset{K_M'}{\rightleftharpoons} & ES & \underset{K_W'}{\rightleftharpoons} & EWS \end{array} \xrightarrow{k_o} E+P \tag{5}$$

where W and M represent water molecule and organic solvent molecule, respectively, and where only the ternary complex EWS is assumed to break down into the hydrolytic product P with the rate constant k_o. The K_S' are the association constants for the binding of S, W and M to E, as is indicated in the scheme; five of these are independent. A quasi-equilibrium treatment leads to the equation:

$$v=\frac{k_oK_s'K_wswe}{(1+K_ww+K_Mm)+(K_s+K_s'K_ww+K_s''K_Mm)s} \tag{6}$$

where w, m, s and e are the molar concentration of water, the organic solvent, the substrate and the total enzyme, respectively. The K_m and k_3 obtained from the Lineweaver-Burk plot are now represented by:

$$K_m=\frac{1+K_ww+K_Mm}{K_s+K_s'K_ww+K_s''K_Mm} \tag{7}$$

$$k_3=\frac{k_oK_s'K_ww}{K_s+K_s'K_ww+K_s''K_Mm} \tag{8}$$

If the substrate binding is not affected by the binding of water and that of the organic solvent molecule (it may be true with water and an organic solvent molecule of a small size, such as methanol), i.e., if $K_S=K_S'=K_S''$, K_m reduces to $1/K_S$, which is independent of the solvent composition. The density of a mixed aqueous solvent relative to pure water, ρ, is approximately expressed by $\rho=1+bm$, where b is a constant characteristic of the organic solvent for not so large an organic solvent content (e.g., up to ca. 30% by volume for methanol and ethanol). Using this relation we have:

$$w+pm=55.5 \tag{9}$$

26) S. A. Bernhard, W. C. Coles and J. F. Nowell, ibid., 82, 3043 (1960).

27) Similar treatments have been worked out by Inagami and Sturtevant[16] in the case of two intermediate complexes; according to them, the ratio of the apparent k_3 value to the apparent K_m value should be independent of the water concentration, although each of them is dependent on it. These features are obviously inconsistent with our results.

p is a constant expressed by $p=(M_M-1000b)/M_W$, where M_M and M_W are the molecular weights of the organic solvent and water respectively. (For a methanol-water mixture, $b=-0.0059$ and $p=2.10$.)

From Eqs. 8 and 9 we derive the following equation:

$$\frac{1}{k_3}=\left(\frac{K_S+55.5K_S''K_M/p}{k_OK_S'K_W}\right)\frac{1}{w}+\left(\frac{K_S'K_W-K_S''K_M/p}{k_OK_S'K_W}\right) \quad (10)$$

which predicts a linear relationship between $1/k_3$ and $1/w$. As may clearly be seen from Fig. 3, however, the linearity does not hold for the present case. Thus, this mechanism is not consistent with the observed results.

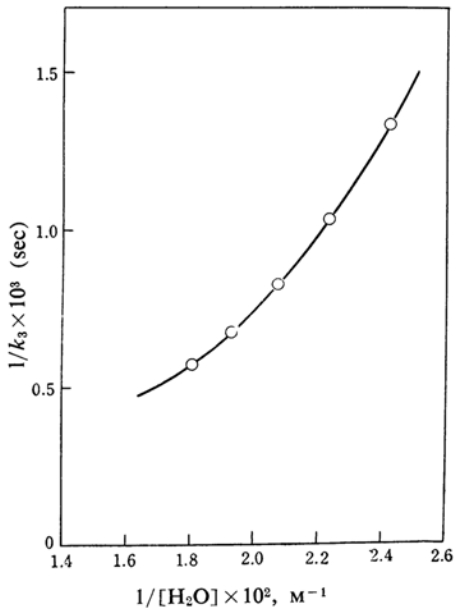

Fig. 3. Plot of $1/k_3$ vs. $1/[H_2O]$.

The Inhibitory Action of the Organic Additive.—Apart from the inhibitory action of the organic additive as a water analogue discussed above, there is a possibility that the organic additive may act as a simple inhibitor, either competitive, or non-competitive. At least qualitatively, the results obtained in this study are not inconsistent with a possible interpretation that methanol acts as a non-competitive inhibitor. If this is the case, k_3 may be written as:

$$k_3=\left(\frac{K_i}{K_i+m^n}\right)\cdot k_3^{\circ} \quad (11)$$

where m and K_i denote the methanol concentration and the dissociation constant of the inactive complex EM_n (or ESM_n) according to the scheme:

$$E\ (\text{or } ES)+nM \overset{K_i}{\rightleftharpoons} EM_n\ (\text{or } ESM_n) \quad (12)$$

where n is the number of methanol molecules combined with the enzyme molecule to form the inactive enzyme-methanol complex, and k_3° is k_3 at $m=0$. If we define α as the ratio k_3/k_3°, we have:

$$\frac{1-\alpha}{\alpha}=\frac{m^n}{K_i} \quad (13)$$

Thus, a plot of $\log\left(\frac{1-\alpha}{\alpha}\right)$ versus $\log m$ should give a straight line of slope n. This plot is shown in Fig. 4, which does not give a straight line of an integral number of slope, but does give a concave curve with an average slope of ca. 1.4. Thus, the results are not consistent with a typical non-competitive inhibitory mechanism involving the stoichiometric binding of the organic solvent molecule to the enzyme molecule.

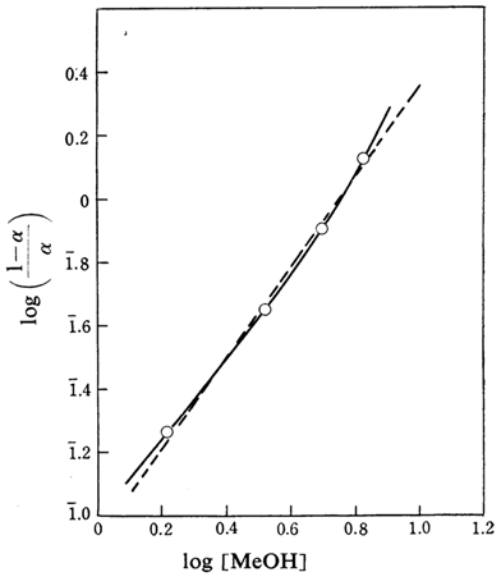

Fig. 4. Plot of $\log\left(\frac{1-\alpha}{\alpha}\right)$ vs. log [MeOH].
The dotted line represents a straight line whose slope is equal to 1.42.

Denaturation of the Enzyme.—Although an irreversible denaturation of the enzyme caused by the addition of the organic solvent could easily be found out experimentally, a rapid, reversible denaturation would be difficult to detect. If the enzyme undergoes a reversible denaturation in the presence of the organic solvent, the scheme may be written as follows:

$$\left.\begin{aligned} &E+S \underset{k_2}{\overset{k_1}{\rightleftharpoons}} ES \overset{k_3}{\rightarrow} E+P \\ &E \overset{K}{\rightleftharpoons} E' \\ &E'+S \overset{K_{m'}}{\rightleftharpoons} E'S \end{aligned}\right\} \quad (14)$$

where E' denotes the reversibly denatured form of the enzyme, which may (or may not) combine with the substrate to form an inactive complex E'S, where K_m' is the dissociation constant of this complex, and where K represents the equilibrium constant for the denaturation of the enzyme, which, in general, may increase with an increase in the concentration of the organic solvent. Then the initial rate v becomes

$$v=\frac{k_3es}{K_m(K+1)+(K_mK/K_m'+1)s} \quad (15)$$

where $K_m=(k_2+k_3)/k_1$. The apparent Michaelis constant, K_{ma}, and the apparent breakdown rate constant, k_{3a} which are obtained from the Lineweaver-Burk plot, are expressed as follows:

$$K_{ma}=\frac{K_mK_m'(K+1)}{K_mK+K_m'} \quad (16)$$

$$k_{3a}=\frac{k_3K_m'}{K_mK+K_m'} \quad (17)$$

K_{ma} will be independent of the solvent composition if $K_m=K_m'$. Assuming that k_{3a} and K_{ma} in water (or in the absence of the organic additive) are equal to k_3 and K_m, respectively, at a given concentration of organic solvent can be calculated from k_{3a}/K_{ma} (or directly from k_{3a} if $K_m=K_m'$). The K' values were tentatively calculated from the data on the assumption of this mechanism. There is, however, no linear relationship between K and the methanol concentration nor between $\log K$ and $1/D$, as may be seen from Figs. 5 and 6. Thus, the experimental results are inconsistent with this assumption.

Aggregation of the Substrate.—If the addition of the organic solvent gives rise to aggregation of the substrate, the effective concentration of the substrate will be reduced from s to γs, where γ is a positive number less than unity. The initial rate, v, becomes

$$v=\frac{k_3es}{K_m/\gamma+s} \quad (18)$$

Thus, in the presence of the organic solvent, the apparent Michaelis constant will be increased by a factor of $1/\gamma$, while k_3 will remain constant. Obviously this is not consistent with our results.

As was mentioned earlier, it was experimentally confirmed that neither the irreversible inactivation of the enzyme (one of factor 4)

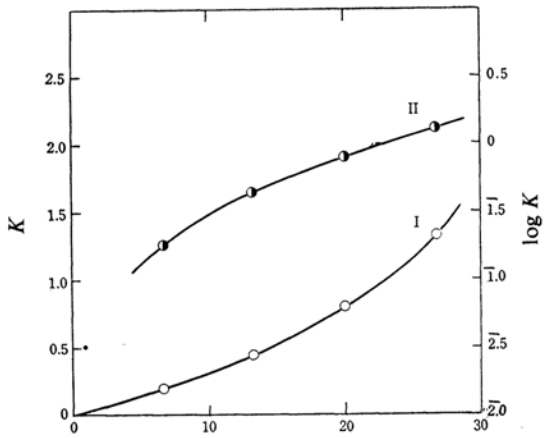

Fig. 5. Plots of K (Curve I) and $\log K$ (Curve II) vs. methanol concentration.

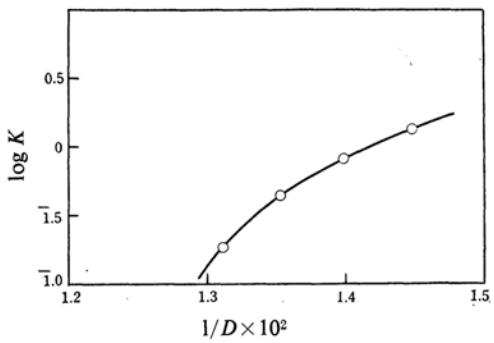

Fig. 6. Plot of $\log K$ vs. $1/D$.

nor the shift of pH curve (factor 6) can be the cause of the present results.

Analysis of the Results by the Electrostatic Theory.—The above arguments have shown that no reasonable interpretation of the results can be obtained on the basis of factors 2 to 6 mentioned above. We will next, examine the possibility of the factor 1, i. e., the electrostatic influence of the dielectric constant of the medium. According to the theory of the dielectric constant effect on enzyme reactions which has been developed by one of the present authors [17,18], the variation of the rate parameters, K_m and k_3, with the dielectric constant, D, of the solvent can be expressed in the following equations:

$$-\frac{\mathrm{d}\log K_m}{\mathrm{d}(1/D)}$$

$$=\frac{\varepsilon^2}{2.303(2kT)}\left[\frac{L_S}{b_S}+\frac{1}{b_E}(L_E-L_{ES})\right] \quad (19)$$

$$=\frac{\varepsilon^2}{2.303(2kT)}\left[\frac{L_S}{b_S}+\frac{1}{b_E}(L^o{}_E-L^o{}_{ES})\right] \quad (20)$$

$$\frac{d \log k_3}{d(1/D)} = \frac{\varepsilon^2}{2.303(2kT)} \cdot \frac{1}{b_{ES}} (L_{ES} - L_{ES^{\neq}}) \quad (21)$$

$$= \frac{\varepsilon^2}{2.303(2kT)} \cdot \frac{1}{b_{ES}} (L^{O}_{ES} - L^{O}_{ES^{\neq}}) \quad (22)$$

where ε is the protonic charge, k the Boltzmann constant, T, the absolute temperature, b's are the radii of the species denoted by the suffixes, and L's are their "charge configuration functions" as defined elsewhere[17]. L is a dimensionless quantity which is determined by the radius and the charge state of the molecule; it is calculated by the aid of Tables I and II of Ref. 17, provided the charge configuration within the molecule is given. Although the spatial distribution of every charge in E, ES or $ES^{\neq}$ is not yet known, and the exact evaluation of the L values of these species is impossible, it can be shown that under certain conditions we may use the "effective charge configuration function"[18] denoted by L^{O} instead of the complete charge configuration function, L. L^{O} is the charge configuration function constructed with respect only to the small number of charges within the local region of the enzyme or the enzyme-substrate complex in which the catalytic reaction is considered to occur, neglecting all the other charges, which are considered to be unimportant for the reaction and to be fixed during the course of the reaction. The radii of E, ES and $ES^{\neq}$ may be equated for small substrates. Thus, Eqs. 20 and 22 are the approximate equations to be used in treating enzyme reactions. These equations predict that the plot of $\log K_m$ or $\log k_3$ against $1/D$ will give a straight line, the slope of which will be determined by the radii and the effective charge configuration functions of the species involved. In Fig. 7 are shown the plots of $\log K_m$ and $\log k_3$ versus $1/D$. The linearity of the plots is in accordance with the theory.

Since L is usually small for a neutral molecule unless it is largely polarized, the contribution of the L_S/b_S term in Eq. 20 to the slope may be no more than the experimental error of the slope. (For example, this contribution has been found to be negligibly small for several uncharged organic acids[17]). Thus, the fact that the slope $d \log K_m/d(1/D)$ is zero implies that L^{O}_{ES} is equal to L^{O}_{E} (or $L_{ES}=L_E$); i. e., there is no appreciable change in charge state during the formation of the ES complex because a change in the charge state would be reflected in a change in L^{O}. The force operating in the ES complex formation, therefore, is supposed to be non-electrostatic in origin, i. e., van der Waals force or hydrogen bonding, or both.

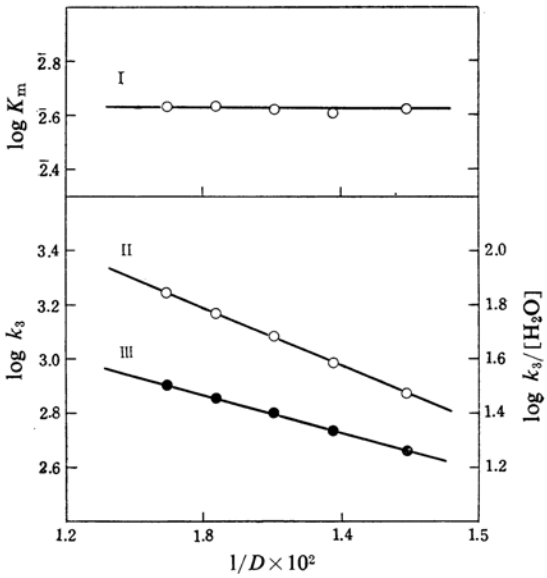

Fig. 7. Plots of log K_m (Curve I), log k_3 (Curve II) and log $k_3/[H_2O]$ (Curve III) vs. $1/D$.

On the other hand, the plot of log k_3 versus $1/D$ gives a straight line of negative slope, indicating that the effective charge configuration function of the activated state of the ES complex ($L^{O}_{ES^{\neq}}$) is larger than that of the normal ES complex (L^{O}_{ES}). If we calculate L^{O}_{ES} and $L^{O}_{ES^{\neq}}$, assuming certain charge configurations of ES and $ES^{\neq}$, and compare the slope $d \log k_3/d(1/D)$ thus calculated with that observed experimentally, we can pick up some of the charge configurations as plausible; this may give information about the reaction mechanism.

The theoretical estimation of the slope, $d \log k_3/d(1/D)$, will be made on the basis of the following assumptions, all of which are considered plausible: 1) In the activated state of ES, designated by $ES^{\neq}$, the C_1 atom and the glucosidic oxygen atom of the substrate molecule are located near the surface of the sphere of $ES^{\neq}$, with their centers situated d Å below the surface (d was taken to be 0.3 Å to 1.0 Å). The bond length between these atoms is assumed to be ca. 50% stretched over the normal value. (For convenience of calculation, the angle between the lines adjoining the center of the sphere to the C_1 atom and the oxygen atom was kept constant at 5°26'40''. This angle corresponds to the bond length of 2.15 Å when d is 0.5 Å. The bond length ranges form 2.10 Å to 2.17 Å depending on the value of d[28].) As a consequence of the

28) This minor change in the bond length scarely affects the value of the slope.

charge separation in ES‡, two charges of equal magnitude but of opposite signs are produced on the C_1 atom and the glucosidic oxygen atom between which the linkage is to be split. In the ground state of ES, however, no such charge separation is assumed to occur. 2) The essential dissociable groups of the enzyme, the carboxylate and the imidazolium (or ammonium) group, which are considered essential in the breakdown process of ES[19], are assumed to be located below the glucosidic bond and to attack the bond simultaneously as a base and as an acid, respectively[19]. The water molecule is considered to be situated between the carboxylate group and the C_1 atom. The positions of these essential groups are assumed to be kept fixed in ES and ES‡. The geometrical disposition of the atoms assumed in the calculation is shown in Fig. 8. The C_1

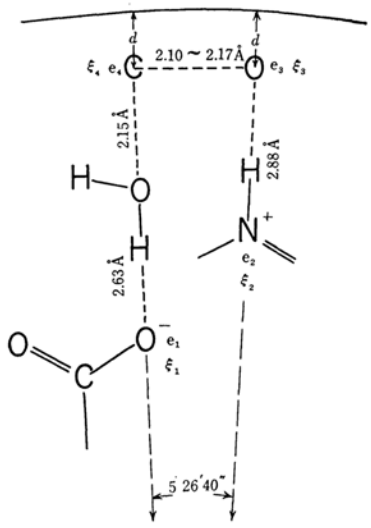

Fig. 8. Model of ES‡.
e_1 and e_2 represent the charges of the essential ionizable groups of the enzyme, and e_3 and e_4 represent the charges produced in the glucosidic linkage in the activated state.

atom, the O–H bond of the water molecule and the carboxylate oxygen atom are assumed to lie on one radius, and the glucosidic oxygen and the N–H bond of the imidazolium group on another radius. The hydrogen-bonded distances of O–H···O and N–H···O are taken as 2.63 Å and 2.88 Å respectively[29]. The charges to be considered (or to be involved in the effective charge configuration functions) are those on carboxylate oxygen, imidazolium nitrogen, glucosidic oxygen, and the C_1 atom; they are designated by $e_1, e_2, ..., e_4$, respectively, as is shown in Fig. 8. ξ's are the valences of the charges. In ES, it was assumed that $\xi_1=-1$ and $\xi_2=+1$ and $\xi_3=\xi_4=0$. In ES‡, calculations were carried out for several cases, i. e., $\xi_3=-\xi_4=\pm1$ and $\pm1/2$; $-\xi_1=+\xi_2=1$, 1/2 or 0, and $d=0.3$, 0.5, 0.7 and 1.0 Å.

The expressions for L^0_{ES} and $L^0_{ES‡}$ are as follows[18,19]:

$$L^0_{ES}=\xi_1^2 f_{11}+\xi_2^2 f_{22}+2\xi_1\xi_2 g_{12} \tag{23}$$

$$L^0_{ES‡}=\sum_{k=1}^{4}\xi_k^2 f_{kk}+\sum_{k=1,l\neq k}^{4}\sum\xi_k\xi_l g_{kl}=\xi_1^2 f_{11}+\xi_2^2 f_{22}+\xi_3^2 f_{33}+\xi_4^3 f_{44}+2\xi_1\xi_2 g_{12}+2\xi_1\xi_3 g_{13}+2\xi_1\xi_4 g_{14}+2\xi_2\xi_3 g_{23}+2\xi_2\xi_4 g_{24}+2\xi_3\xi_4 g_{34} \tag{24}$$

f_{kk} is a function of $(r_k/b)^2$, where r_k is the distance of the charge, e_k, from the center of the molecule. g_{kl} is a function of $(r_k r_l/b^2)$ and $\cos\theta_{kl}$, where θ_{kl} is the angle between r_k and r_l. The values of f_{kk} and g_{kl} may be readily obtained from the tables[17] if r_k's and θ_{kl}'s are calculated from Fig. 8.

The results of the calculation are summarized in Table II, in which the slopes theoretically predicted for various cases are presented. The theoretical slopes are negative in all the cases considered, the magnitude depending primarily upon the valences of the charges produced on the glucosidic linkage (ξ_3 and ξ_4) and on the depth of the charges, d, and being relatively insensitive to the situation of the neighboring charges, e_1 and e_2. The experimentally observed magnitude of the slope, -210, may reasonably be accounted for if $d=0.6$ Å and $-\xi_3=+\xi_4=\pm1$, i. e., if a considerably large separation of charges occurs in the glucosidic linkage in the activated state, ES‡. The value of d is not of an unreasonable order of magnitude, although it appears to be somewhat smaller than that of the carboxylic oxygen atom (ca. 1.0 Å)[17], calculated from the dependence of the pK values of carboxylic acids on the dielectric constant of the medium.

Discussion

The above calculations have revealed that the observed results can reasonably be explained in terms of the electrostatic effect of the dielectric constant of the solvent on the reaction rates only, although slightly smaller values had to be assigned for d. However, it may also be possible that, in addition to the dielectric constant effect, some non-electrostatic effects, e. g., a decrease in the water concentration or an inhibitory effect of the organic solvent, might have been involved in the observed results. Although it is not a simple matter to determine the magnitude of the effect which is really due to the dielectric

29) G. C. Pimentel and A. L. McClellan, "The Hydrogen Bond", Freeman & Co., San Francisco (1960), pp. 284, 289. These are the average values for carboxylic acids and for ammonium, respectively. A minor change in these values does not affect the resultant slopes seriously.

TABLE II. THE THEORETICALLY PREDICTED SLOPES $d \log k_3/d(1/D)$

No.	Charge state	Schematic representation of the charge state[a]	$-d \log k_3/d(1/D)$ for $d=$ 0.3 Å	0.5 Å	0.7 Å	1.0 Å
1	$\begin{cases}\xi_3=-\xi_4=+1\\ \xi_1=-\xi_2=-1\end{cases}$		656.5	290.2	160.6	85.6
2	$\begin{cases}\xi_3=-\xi_4=-1\\ \xi_1=-\xi_2=+1\end{cases}$		504.9	256.8	136.9	66.9
3	$\begin{cases}\xi_3=-\xi_4=+1\\ \xi_1=-\xi_2=-1/2\end{cases}$		615.5	279.5	152.6	79.3
4	$\begin{cases}\xi_3=-\xi_4=-1\\ \xi_1=-\xi_2=-1/2\end{cases}$		539.7	262.7	140.7	70.0
5	$\begin{cases}\xi_3=-\xi_4=\pm1\\ \xi_1=-\xi_2=0\end{cases}$		576.5	270.3	145.9	74.1
6	$\begin{cases}\xi_3=-\xi_4=+1/2\\ \xi_1=-\xi_2=-1\end{cases}$		183.1	76.7	43.1	23.7
7	$\begin{cases}\xi_3=-\xi_4=-1/2\\ \xi_1=-\xi_2=-1\end{cases}$		107.2	60.0	31.2	14.3
8	$\begin{cases}\xi_3=-\xi_4=+1/2\\ \xi_1=-\xi_2=-1/2\end{cases}$		161.0	70.1	38.0	19.8
9	$\begin{cases}\xi_3=-\xi_4=+1/2\\ \xi_1=-\xi_2=-1/2\end{cases}$		123.0	61.8	32.1	15.0
10	$\begin{cases}\xi_3=-\xi_4=\pm1/2\\ \xi_1=-\xi_2=0\end{cases}$		141.0	65.1	34.3	16.9

a) 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.

constant of the solvent itself, we may be able to take the non-electrostatic effect to some extent into consideration by plotting the logarithms of $k_3/[H_2O]$ instead of k_3 versus $1/D$. This case corresponds to case 1 considered above, where water reacts with the ES complex directly from the bulk of solvent (see Eq. 4), or to a special case of case 2 (the case in which $K_W=K_M/p$ in Eq. 8), where methanol competes for the water site with the water molecule. This plot is shown in curve III of Fig. 7, where a straight line with a slope of -124 is obtained. Table II shows that this order of magnitude of the slope is reasonably accounted for if $-\xi_3=+\xi_4=\pm1$ and $d=0.75\sim0.8$ Å.

Although there is a little ambiguity in the value of d to be assigned, it may be reasonable to conclude that the main effect operating in the present case is the electrostatic one, i. e., the effect of the dielectric constant on the rate, and the results of calculation are consistent with the hypothesis that the charge separation occurs at the glucosidic linkage of the substrate molecule to be hydrolyzed in the activated state of the ES complex.

Electrostatic Entropy.—Electrostatic entropy is often discussed in relation to the dielectric constant effect on reaction rates[1-3,9,10]. As will be shown in the Appendix, we can estimate the electrostatic part of the entropy change involved in the formation of the ES complex, $\Delta S_{e.s.}$, and of the entropy of activation involved in the breakdown of the ES complex, $\Delta S_{e.s.\ddagger}$, if we assume that the temperature dependence of the internal dielectric constant of the cavity is much smaller than that of the solvent. From Eqs. A-13 and A-14 in the Appendix, with water as the solvent and at 25°C, we obtain:

$$\Delta S_{e.s.} \fallingdotseq -0.159 \times \frac{d \log K_m}{d(1/D)} \tag{25}$$

$$\Delta S_{e.s.\ddagger} \fallingdotseq 0.159 \times \frac{d \log k_3}{d(1/D)} \tag{26}$$

Since $d \log K_m/d(1/D)=0$, and $d \log k_3/d(1/D)=-210$ or -124 according as the non-electrostatic effect of the solvent is disregarded or not, we can estimate the electrostatic entropies to be:

$\Delta S_{e.s.} \fallingdotseq 0$

$\Delta S_{e.s.\ddagger} \fallingdotseq -33$ or -20 cal. deg^{-1} mol^{-1}

The negative entropy of activation suggests that the electrostriction of the solvent is caused by the charge separation in ES$\ddagger$.

Summary

The influence of methanol on the rate of the hydrolytic reaction of soluble starch catalyzed by crystalline bacterial amyloclastic α-amylase has been studied at 25°C and pH 6.0, over the methanol concentration of 0~22.6% by weight.

The apparent Michaelis constant, K_m, is independent of the methanol concentration,

while the breakdown rate constant of the ES complex, k_3, decreases with an increase in the methanol concentration.

The plot of log k_3 versus the reciprocal dielectric constant of the solvent gives a straight line with a negative slope.

Various possible effects on the reaction rates caused by the addition of methanol have been examind, and it has been concluded that the dielectric constant effect is predominantly operative.

The results have been interpreted quantitatively on the basis of the electrostatic theory previously developed by one of the present authors. The results of calculation have been found to be consistent with the hypothesis that charge separation occurs in the glucosidic linkage to be hydrolyzed in the activated state of the ES complex.

The electrostatic entropy of activation in the breakdown of the ES complex has also been estimated.

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Appendix

The Evaluation of the Electrostatic Entropy.— The electrostatic part of the entropy of reaction and that of the entropy of activation may be estimated from the variation of the equilibrium constant and of the rate constant with the dielectric constant of the medium respectively, in the following manner. For example, consider an equilibrium:

$$A+B=C+D \tag{A-1}$$

with an equilibrium constant of K. We have:

$$\Delta G=-\boldsymbol{R}T\ln K \tag{A-2}$$

where ΔG is the standard free energy change, which is divided into the non-electrostatic part, $\Delta G_{n.e.s.}$, and the electrostatic part, $\Delta G_{e.s.}$. Thus

$$\Delta G=\Delta G_{n.e.s.}+\Delta G_{e.s.} \tag{A-3}$$

The electrostatic free energy, $\Delta G_{e.s.}$, is expressed in terms of the work of the charging, W, of each species, in the following equation:

$$\Delta G_{e.s.}=\boldsymbol{N}\Delta W=\boldsymbol{N}(W_C+W_D-W_A-W_B) \tag{A-4}$$

where $\boldsymbol{N}$ is the Avogadro number. ΔW of a species for zero ionic strength, designated as W_0, is expressed in the form:

$$W_0=\frac{\varepsilon^2}{2b}\left(\frac{L'}{D_i}+\frac{L}{D}\right) \tag{A-5}$$

where ε is the protonic charge, b, the radius of the species, L, the charge configuration function, and L', a parameter also dependent on the charge configuration of the species. (The full expressions of L and L' will be found in Eq. 3 of Ref. 17.) D_i, the internal dielectric constant of the spherical cavity formed by the solute species, was taken by Kirkwood to be 2 for all the molecules or ions. D is the dielectric constant of the solvent.

The electrostatic part of the entropy change, $\Delta S_{e.s.}$, is obtained by differentiating $\Delta G_{e.s.}$ with respect to the absolute temperature, T. Thus we have:

$$\Delta S_{e.s.}=-\frac{\partial(\Delta G_{e.s.})}{\partial T}=-N\cdot\frac{\partial(\Delta W)}{\partial T}$$
$$=-\frac{N\varepsilon^2}{2}\left[\frac{\partial(1/D_i)}{\partial T}\cdot\Delta\left(\frac{L'}{b}\right)+\frac{\partial(1/D)}{\partial T}\cdot\Delta\left(\frac{L}{b}\right)\right] \tag{A-6}$$

where $\Delta(L'/b)$ and $\Delta(L/b)$ stand for:

$$\Delta\left(\frac{L'}{b}\right)=\frac{L'_C}{b_C}+\frac{L'_D}{b_D}-\frac{L'_A}{b_A}-\frac{L'_B}{b_B} \tag{A-7}$$

$$\Delta\left(\frac{L}{b}\right)=\frac{L_C}{b_C}+\frac{L_D}{b_D}-\frac{L_A}{b_A}-\frac{L_B}{b_B} \tag{A-8}$$

The dielectric constant of a liquid can be expressed as a function of temperature by the empirical formula[30]:

$$D=D^0e^{-T/\theta} \tag{A-9}$$

where D^0 and θ are the constants characteristic of the liquid. Since L and L' are considered to be independent of temperature, if we assume that $\partial(1/D_i)/\partial T$ is much smaller than $\partial(1/D)/\partial T$, i.e., the temperature coefficient of the reciprocal dielectric constant of the cavity is negligibly smaller than that of the solvent, Eq. A-6 becomes:

$$\Delta S_{e.s.}=-\frac{\varepsilon^2\boldsymbol{N}}{D\theta}\cdot\Delta\left(\frac{L}{b}\right)$$
$$=\frac{\varepsilon^2\boldsymbol{N}}{D\theta}\left(\frac{L_A}{b_A}+\frac{L_B}{b_B}-\frac{L_C}{b_C}-\frac{L_D}{b_D}\right)$$
$$=\frac{4.606\,\boldsymbol{R}T}{D\theta}\cdot\frac{\mathrm{d}\log K}{\mathrm{d}(1/D)} \tag{A-10}$$

since

$$\frac{\mathrm{d}\log K}{\mathrm{d}(1/D)}=\frac{\varepsilon}{4.606\,kT}\left(\frac{L_A}{b_A}+\frac{L_B}{b_B}-\frac{L_C}{b_C}-\frac{L_D}{b_D}\right) \tag{A-11}$$

Thus, the electrostatic entropy, $\Delta S_{e.s.}$, is expressed in terms of the slope, $\mathrm{d}\log K/\mathrm{d}(1/D)$, which can be obtained experimentally. Quite similarly, for a rate process, we have:

$$\Delta S_{e.s.\neq}=\frac{4.606\,\boldsymbol{R}T}{D\theta}\cdot\frac{\mathrm{d}\log k}{\mathrm{d}(1/D)} \tag{A-12}$$

where $\Delta S_{e.s.\neq}$ and k are the electrostatic entropy of activation and the rate constant, respectively.

With water as solvent and at 25°C, $\theta=219$[30], $D=78.5$ and $T=298$; therefore, we have

$$\Delta S_{e.s.}\fallingdotseq 0.159\times\frac{\mathrm{d}\log K}{\mathrm{d}(1/D)} \tag{A-13}$$

$$\Delta S_{e.s.\neq}\fallingdotseq 0.159\times\frac{\mathrm{d}\log k}{\mathrm{d}(/1D)} \tag{A-14}$$

30) R. W. Gurney, "Ionic Processes in Solution", McGraw-Hill, New York (1953), p. 16.