Unified Theory of Enzyme Catalysis and Denaturation

II. *Some effects of anions on prostatic acid phosphatase behavior*

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ABSTRACT A system of kinetics was developed to show that a simple anion, chloride, bromide, or thiocyanate can inhibit an enzyme, prostatic acid phosphatase, in solution, both competitively with regard to substrate, and non-competitively. The non-competitive inhibition was related to alteration of charges on the protein molecule. The kinetics developed fit the experimental data. It was demonstrated that the anions studied were also effective accelerators of the thermal denaturation process. From the evidence, a theory was developed proposing that apart from the site of enzyme-substrate combination, secondary factors concerning charged sites and tensions within the enzyme molecular structure conferred specificity on the enzyme. These factors are designated as the "second order specificity," and are under the regulation of anions and protons.

In the first paper of this series (London *et al.*, 1958) it was demonstrated that a positively charged group and a hydrogen bonding type group were a likely pair which made up the active substrate combining site of the enzyme molecule. It was pointed out that anions capable of reacting with the positive site would behave as competitive inhibitors of the enzyme. Theoretically, there are many more positive sites than those at the substrate combining sites, and these too should be capable of reacting with the anions. It will be shown here that the presence of these anions regulates phosphatase function in a way other than by simple competitive inhibition, and that a theoretical treatment of the reactions between simple anions and the positive sites can explain these phenomena.

In that which follows, quasiequilibrium kinetics are utilized in order to simplify the mathematical treatment. It becomes very complicated if steady state kinetics are used without simplifying assumptions. In justification of the kinetics used, the value K_m, the conventional Michaelis constant for steady state kinetics obtained from a reaction velocity–substrate concentration plot, was almost equal to the average value of K_s, the equilibrium constant, obtained in the results which follow.

MATERIALS AND METHODS

The enzyme preparation used in this work was prostatic acid phosphatase purified 300 times from human glands. This was the same preparation used in earlier studies (London *et al.*, 1954). All assay procedures measuring enzymic activity using sodium β-glycerophosphate were similar to those used in earlier work (London *et al.*, 1958; London *et al.*, 1954), and the special experimental modifications employed for several tests will be described along with the experimental evidence. The denaturation procedures were similar to those used earlier (London *et al.*, 1954). It was shown then that the thermal denaturation process followed first order kinetics.

In the inhibition studies all the substances used were the best commercial grades available. Sodium-*p*-toluenesulfonate was purified by crystallizing it from an aqueous solution of purified *p*-toluene sulfonic acid after neutralizing with sodium hydroxide and concentrating the solution. Egg albumin and disodium ethylenediaminetetraacetate were used in trace quantities in the diluted enzyme solutions and stock substrate solutions as preservatives (London *et al.*, 1954). The stock solutions of diluted enzyme, inhibitor, substrate, and KH_2PO_4 standards were prepared in pH 5.0, 0.2 N acetate buffer.

A typical procedure for measuring inhibition is presented. For each test four tubes were used, a blank, uninhibited enzyme in duplicate, and enzyme with inhibitor. Into each tube 0.05 to 0.5 ml quantities of stock substrate were placed. Buffer was then added to bring the volume to 0.50 ml. Again 0.50 ml of buffer was added to each tube except the last, which received 0.50 ml of inhibitor solution. The mixtures were shaken and incubated at either 30° or 37°C after 0.50 ml of diluted enzyme was added. The variations in incubation temperature were adopted to bring assurance that thermal denaturation would not take place at 37° in the presence of certain ions which are also shown in this work to accelerate denaturation at higher temperatures. The length of incubation was varied in accordance with the concentrations of substrate and enzyme used so that the inorganic phosphate released could be readily measured. The reaction was usually stopped with 1.4 ml 5 N H_2SO_4 if the contents of the tubes were to be developed directly for their color production. If the concentrations of substances were sufficiently high as to interfere with color development the reaction was stopped with 1.0 ml 5 N H_2SO_4 and 0.5 ml or 1.0 ml was taken for color production. An additional 1.0 ml of 5 N H_2SO_4 was added and color was developed as usual.

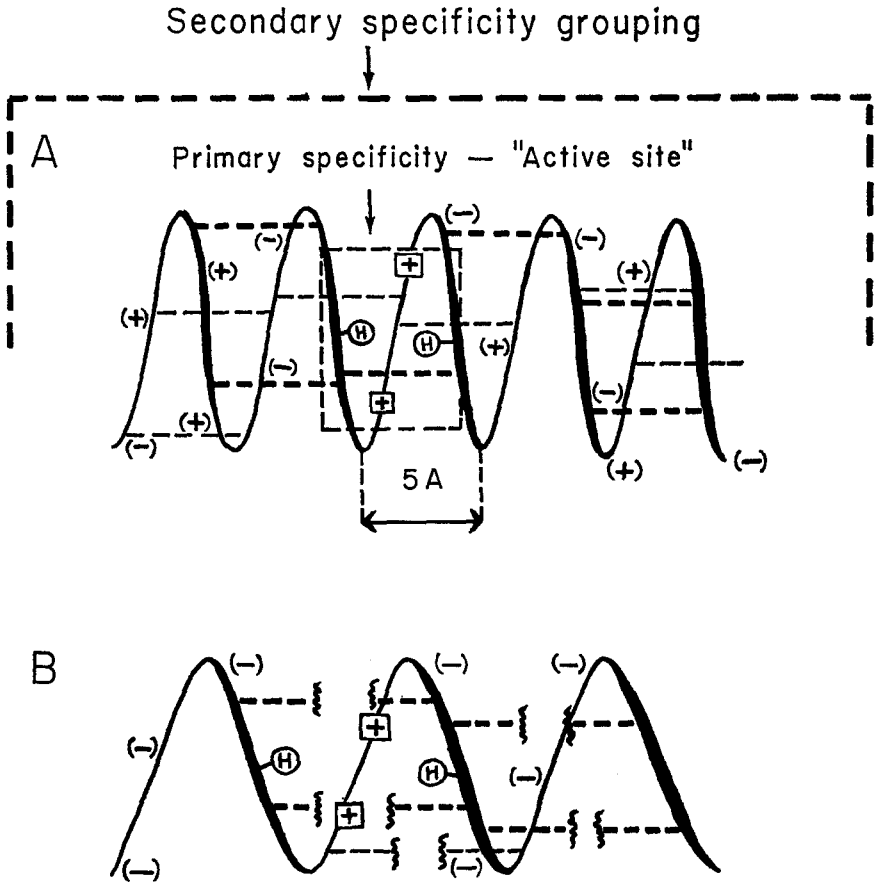


FIGURE 1 A. An enzyme species that may be capable of activity. The designation of "Secondary specificity" will be differentiated from that of the active site later in this work. B. An exaggerated view of an enzyme species in the process of denaturing. The horizontal bonds holding the molecule in its coiled form are rupturing.

KINETIC ANALYSIS

Consider a portion of the enzyme molecule shown in Fig. 1 (as described earlier in London *et al.*, 1958). It consists of a helix with the critical positive sites (the sites of direct substrate attachment) designated as [+] and many other positive sites, too. Part A of the figure represents the molecular species which are predominant near the isoelectric point. These species are thermally stable and catalytically active. The pH values of the isoelectric point (Derow and Davison, 1953), optimal thermal stability (London *et al.*, 1954), and optimal activity (5.0 measured in this laboratory), all exist within a narrow range, 4.6–5.0.

A conception of the distribution of the charged forms which exist at pH 5.0 is shown in Fig. 2 B. The distribution is based on probability, and is of the type discussed by Edsall (Edsall, 1943). The curves of Fig. 2 only approximate the types of distribution discussed. Assuming that the partly shaded area represents the active enzyme form, a redistribution of forms can be accomplished in either of two ways. The pH can be increased or anions with a strong affinity for positive sites can be added. In this way a distribution

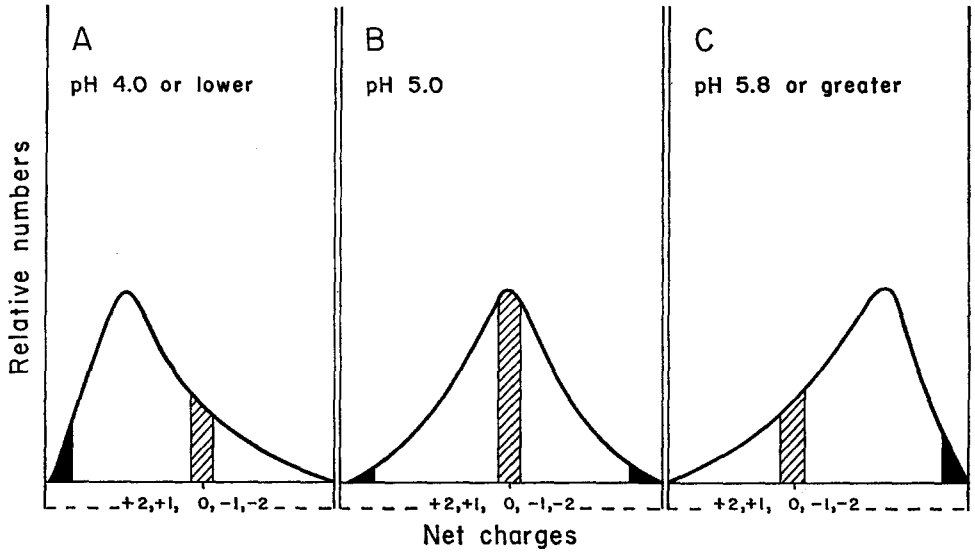


FIGURE 2. A qualitative description of the distribution of the charged forms of the enzyme at three pH values in solutions of low anionic concentration. The solid black areas represent the forms which denature most readily because of the stretching of the molecule due to coulombic forces. The shaded areas represent the neutral or slightly charged forms which have catalytic activity. All the forms shown are in equilibrium and may change from one to another by acquiring or losing protons. According to an hypothesis presented earlier (London *et al.*, 1954) these shifts in form may also come about as the result of the association or dissociation of anions.

resembling C of Fig. 2 can be attained. As this occurs the enzymatic activity decreases and thermal denaturation if measured at high enough temperature increases. It was demonstrated (Scatchard and Black, 1949; Scatchard *et al.*, 1950) that the alkali metal ions do not show nearly the range of binding affinities for a protein, serum albumin, as do the anions that were tested. Sodium ion binding was very much weaker than that of chloride, the anion of least affinity. Therefore, in this work sodium salts were used and the effects of cation in binding to negative sites of the enzyme molecule will be neglected.

If one considers that $\bar{n}$ is the average number of positive sites that must be occupied by anions to cause the shift from B to C and beyond (Fig. 2), a

treatment using Michaelis-Menten kinetics of inhibition is possible. The exact significance of an average number may seem unclear, since the quantitative distributions of Fig. 2 are unknown. The values of $\bar{n}$ will be treated theoretically as single whole numbers as in most conventional kinetic treatments, but the experimental values of $\bar{n}$ do not have to be whole numbers. Preliminary evidence obtained from monovalent anions indicated that $\bar{n}$ must be greater than unity and prompted the following derivations.

Consider that at pH 5.0 the fraction of E_o (the total amount of enzyme that is capable of being active) is fixed before the anions are added. The activity then is proportional to E_o, and it represents the total activity in the development which follows. The anions can combine with any + sites, including [+] sites which are part of the active enzymic sites. For the mathematical development which follows, equations are presented which represent the ways in which the enzyme and anionic inhibitor may combine, and enzyme and anionic substrate may combine. The corresponding equilibrium expressions are given too.

The Possible Reactions	*Corresponding Dissociation Equations*
(*a*) $E + S \rightleftharpoons ES \rightarrow P + E$	(*a*) $\frac{[E]\,[S]}{[ES]} = K_s$;
(*b*) $E + I \rightleftharpoons EI$(competitive inhibition)	(*b*) $\frac{[E]\,[I]}{[EI]} = K_i$;
(*c*) $E + \bar{n}I \rightleftharpoons EI_{\bar{n}u}$(non-competitive inhibition)	(*c*) $\frac{[E]\,[I]^{\bar{n}}}{[EI_{\bar{n}u}]} = K_u$;
(*d*) $EI_{\bar{n}u} + S \rightleftharpoons EI_{\bar{n}u}S$(inactive)	(*d*) $\frac{[EI_{\bar{n}u}]\,[S]}{[EI_{\bar{n}u}S]} = K_{us}$;
(*e*) $ES + \bar{n}I \rightleftharpoons EI_{\bar{n}u}S$(inactive)	(*e*) $\frac{[ES]\,[I]^{\bar{n}}}{[EI_{\bar{n}u}S]} = K_{su}$;

$$ES + I \nrightarrow EIS$$

$$EI + S \nrightarrow EIS$$

(Products on right hand side of reaction equations (*d*) and (*e*) are identical.)

Definitions E, S, and I represent uncombined forms of enzyme, substrate, and inhibitor, respectively. ES is the symbol for the complex formed between enzyme and substrate. EI and $EI_{\bar{n}u}$ are two types of complexes formed between enzyme and inhibitor, which are inactive; the first being the complex of competitive inhibition, and the other non-competitive inhibition.

$EI_{\bar{n}u}S$ is the inactive complex formed between enzyme, substrate, and inhibitor. The conservation equations are:

$$[E_o] = [ES] + [E] \text{ (uninhibited system)} \tag{A}$$

$$[E_o] = [ES] + [EI] + [EI_{\bar{n}u}] + [EI_{\bar{n}u}S] + [E] \text{ (inhibited system)} \tag{B}$$

$[I_o] = [I]$ since $[I_o] \gg [E_o]$ and $[S_o] = [S]$ since $[S_o] \gg [E_o]$.

Products of E and I which are formed as a result of combination only at the non-critical sites, and where less than $\bar{n}$ molecules of I are involved, are not inhibited, and are not considered. It is assumed that these forms are equal to those molecules of E which have not reacted with I at the non-critical sites. They are not eliminated from the equations since they are distributed as parts of ES, EI, and E. After making the proper substitutions in (B) and rearranging,

$$[ES] = \frac{[E_o]}{\frac{K_s[I]}{K_i[S]} + \frac{K_s[I]^{\bar{n}}}{K_u[S]} + \frac{[I]^{\bar{n}}}{K_{su}} + \frac{K_s}{[S]} + 1}. \tag{C}$$

Correspondingly, substituting in (A) and rearranging,

$$[ES] = \frac{[E_o]}{1 + \frac{K_s}{[S]}}. \tag{D}$$

$$\alpha \text{ is the ratio } \frac{[ES] \text{ (inhibited system)}}{[ES] \text{ (uninhibited system)}}.$$

The reaction rates are taken as proportional to $[ES]$, and an equation can be expressed using α by dividing (C) by (D).

The $[E_o]$ terms can be cancelled since the same initial concentrations of enzyme are added in any experiment which measures α, and multiplying numerator and denominator by $[S]$

$$\alpha = \frac{[S] + K_s}{\frac{K_s[I]}{K_i} + \frac{K_s[I]^{\bar{n}}}{K_u} + \frac{[S]\,[I]^{\bar{n}}}{K_{su}} + K_s + [S]}. \tag{E}$$

Let r be defined by the equation $[S] = rK_s$. Substituting for $[S]$, making the appropriate cancellations, multiplying both sides of the equation by $[I]/(1 - \alpha)$, and again simplifying we have

$$[I]\frac{\alpha}{1-\alpha} = \frac{(r+1)}{\frac{1}{K_i} + \frac{[I]^{\bar{n}-1}}{K_u} + \frac{r[I]^{\bar{n}-1}}{K_{su}}}. \tag{F}$$

It is likely that $K_{su} \simeq K_u$ because the non-competitive inhibitor is acting at sites removed from where substrate may be attached, and

$$[I]\frac{\alpha}{1-\alpha} \simeq \frac{(r+1)K_i K_u}{(r+1)[I]^{\bar{n}-1}K_i + K_u} \quad \text{is obtained.} \qquad (G)$$

In the region where r is small (*ca.* unity) ($[S]$ is low) and $[I]$ is low equation (G) is reduced to

$$[I]\frac{\alpha}{1-\alpha} \simeq \frac{K_i[S]}{K_s} + K_i\,. \qquad (H)$$

This is the equation derived for competitive inhibition as described by Massart (1950).

In the region where r is large and $[I]$ is not low equation (G) becomes

$$[I]\frac{\alpha}{1-\alpha} = \frac{K_u}{[I]^{\bar{n}-1}}\,. \qquad (I)$$

It implies that for any concentration of I an $[I]\ \alpha/(1-\alpha)$ against $[S]$ plot produces straight lines independent of $[S]$, and is similar to the equations derived by Massart for univalent non-competitive inhibitions.

Transforming (I) into a logarithmic equation, rearranging, and cancelling terms,

$$\log\frac{1-\alpha}{\alpha} = \bar{n}\log[I] - \log K_u \quad \text{is obtained.} \qquad (J)$$

Plotting $\log\ (1-\alpha)/\alpha$ against $\log\ [I]$ allows for a graphical solution of $\bar{n}$, the slope.

Assuming that the nature of anionic inhibition is governed by the kinetics just described, the values for all the constants can be obtained by applying equations (J) and (H). The value of K_s is already known from a Lineweaver-Burke plot (Lineweaver and Burke, 1934), or from values obtained earlier (London *et al.*, 1958) and can be used to correlate with the values found here.

RESULTS

In Figs. 3–6 are shown typical α against $[S]$ plots for sodium chloride and ammonium sulfate inhibition, and the corresponding $[I]\ \alpha/(1-\alpha)$ against $[S]$ plots. The errors in measuring α are small, but in computing the function, $[I]\ \alpha/(1-\alpha)$, these errors are increased several fold, especially where $\alpha \to 1$. Therefore, in order to obtain more precise values of $[I]\ \alpha/(1-\alpha)$, the values of α where they become constant at high values of $[S]$ were taken from the

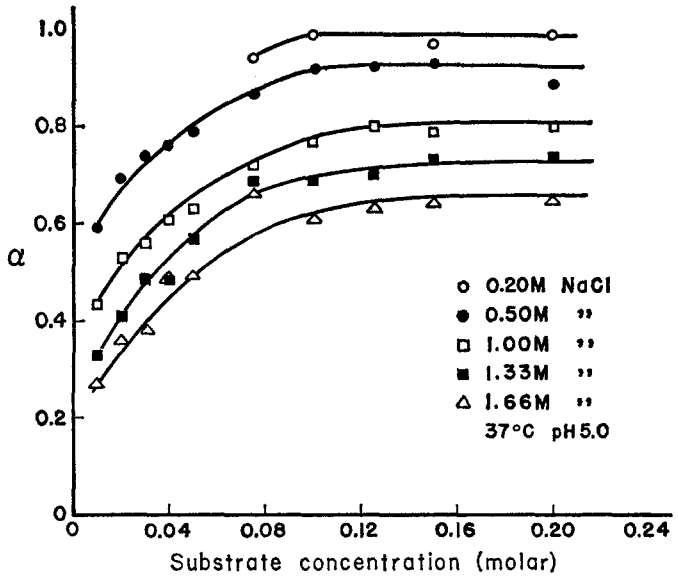


FIGURE 3. Plot of α against $[S]$ for the inhibitor, sodium chloride.

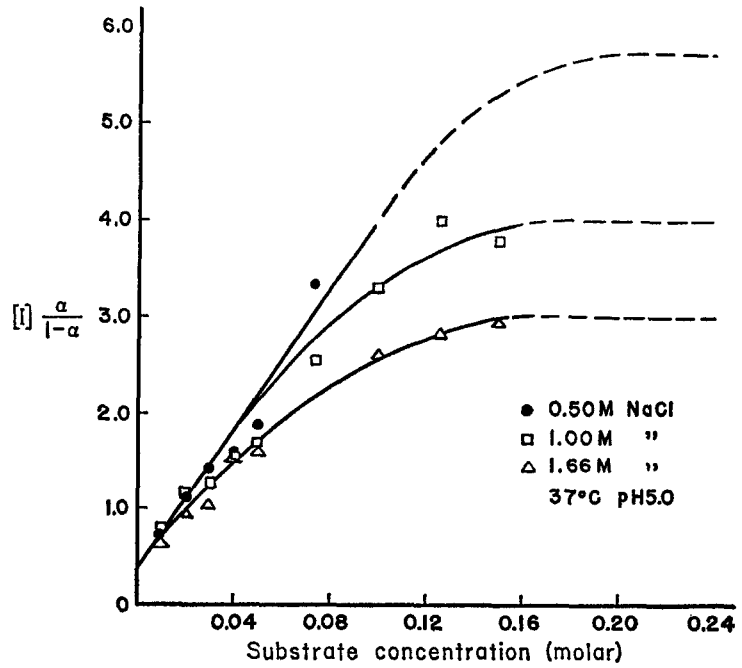


FIGURE 4. Plot of $[I]\ \alpha/(1-\alpha)$ against $[S]$ for the inhibitor, sodium chloride. This was the simplest ion system studied.

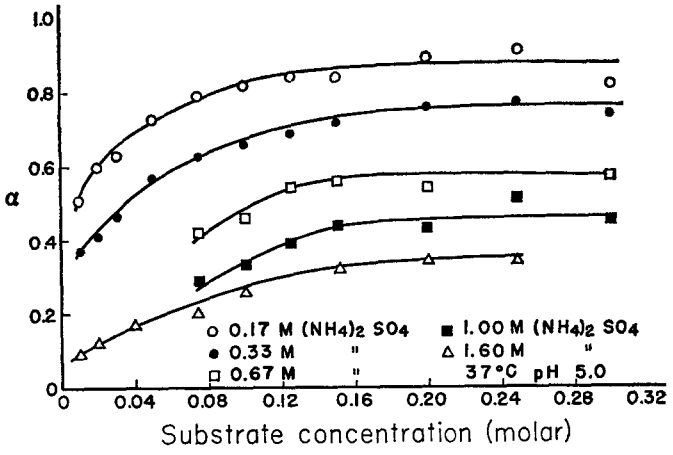


FIGURE 5. Plot of α against $[S]$ for the inhibitor, ammonium sulfate.

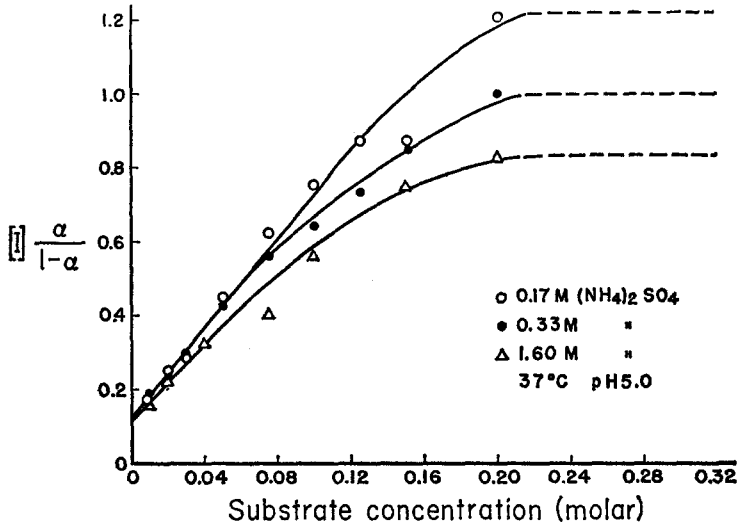


FIGURE 6. Plot of $[I]\ \alpha/(1-\alpha)$ against $[S]$ for the inhibitor, ammonium sulfate. It shows that despite the use of divalent anion and a different cation the general kinetic system is the same as that of sodium chloride.

α against $[S]$ plot and used to obtain $[I]\ \alpha/(1-\alpha)$ in corresponding curves. The dashed portions of the lines in Figs. 4 and 6 are taken directly from the corresponding smooth curves of Figs. 3 and 5, rather than being drawn through individual experimental points. Similar families of curves were obtained for the sodium salts of bromide, thiocyanate, sulfate, and toluenesulfonate.

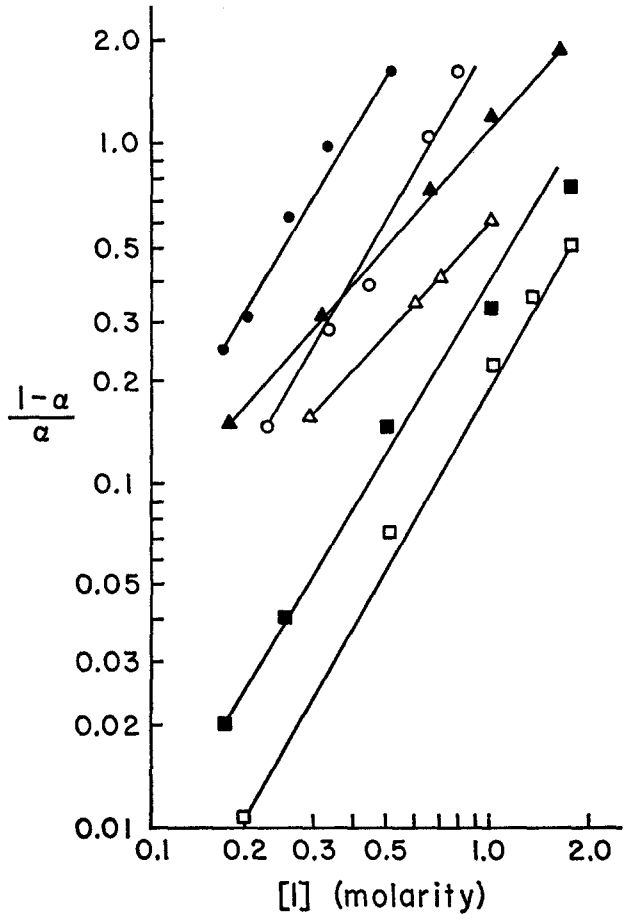


FIGURE 7. Plots of log $(1 - \alpha)/\alpha$ against log $[I]$. The slopes of the plots enable one to calculate $\bar{n}$ values. The closeness of fit of each set of points to a straight line demonstrates the truly constant character of the $\bar{n}$ value for each ion. Symbols are □, sodium chloride, ■, sodium bromide, △, sodium sulfate, ▲, ammonium sulfate, ○, sodium thiocyanate, and ●, sodium *p*-toluenesulfonate. To obtain the points shown the inhibitions were all measured at substrate concentrations of 0.2 M or greater, where α no longer changed with substrate concentration.

In Fig. 7 the series of plots of log $[(1 - \alpha)/\alpha]$ against log $[I]$ is shown. According to equation (*J*), $\bar{n}$ and K_u may be obtained from this plot. It is apparent from the slopes of the chloride, bromide, thiocyanate, and *p*-toluenesulfonate lines that the $\bar{n}$ values are constant.

The kinetic constants evaluated from these families of curves are listed in Table I. Equation (*G*) fits all the data within 22 per cent when use is made of the constants listed in Table I.

Inspection of Table I reveals several interesting relationships. The values

of $\bar{n}$ are quite constant for the monovalent anions tested, and also for the two sulfate salts. The values of K_u are greater than the corresponding K_i values by factors varying from about 2 to 10. This can be expected if one considers a fundamental similarity among all the (+) sites. There is no known metal requirement for the enzyme, and it is likely that the [+] and (+) are all either charged amine, guanidine, or imidazole groups. Therefore, it would seem that in each case the anion would be expected to approach a charged nitrogen group.

Examination of Fig. 8 demonstrates a portion of the theme of this work. The order of variation of k, the first order thermal denaturation rate constant at pH 5.0 and 50.3°, with anion concentration parallels the order found for

TABLE I
CONSTANTS OF REACTIONS BETWEEN THE ENZYME AND ANIONS OR SUBSTRATE

Salts	Constants*			
	$\bar{n}$	K_u (or K_{su})	K_i	K_s
Sodium chloride	1.68	4.5	0.40	0.012
Sodium bromide	1.65	2.7	0.30	0.008
Sodium sulfate	1.11‡	1.6	0.17	0.013
Ammonium sulfate	1.14‡	0.87	0.13	0.020
Sodium thiocyanate	1.76	0.44	0.21	0.012
Sodium *p*-toluenesulfonate	1.72	0.19	0.045	0.014

* The values for $\bar{n}$ were obtained from the slopes of the curves in Fig. 7, and the values for K_u from the intersections of these curves with the line log $I = 0$. The curves in Fig. 7, of course, pertain to the high substrate concentration portions of the curves of Figs. 4 and 6 and of similar graphs plotted according to equations (*H*) and (*I*). Conversely, values for K_i and K_s were obtained from the very low substrate portions of the latter curves.

‡ The two sulfate values are almost equal, indicating that the effects observed are solely due to that ion rather than to the influence of sodium and ammonium as observed in the data for K_u.

the ability of anions to inhibit non-specifically: toluenesulfonate > thiocyanate ≫ sulfate > bromide > chloride. The log-log plot indicates that k is determined by a fractional power of the anion concentration at low concentrations, the power increasing until $k \propto C^4$ at higher concentrations. C is expressed as molarity. Reexamination of Fig. 2 enables one to see how the theory developed thus far allows for this observed function of k. At low values of C only the extremely negatively charged forms are shifted to the right sufficiently to be capable of being denatured. As the C values increase, more enzyme species from the less negatively charged groups are capable of acquiring negative charges sufficient to be denatured.

From theoretical considerations it should be possible to obtain concentrations of several salts used at more acid pH values (*viz.* 3.6–4.0 using 0.18 M

acetate buffers), which are capable of protecting the enzyme. This situation requires a low salt concentration containing a weakly binding cation, and a strongly binding anion. It is the only way to alter the distribution in Fig. 2 A and decrease the solid black area (representing the relative number of denaturing species) on the left without having a compensating rise of denaturing

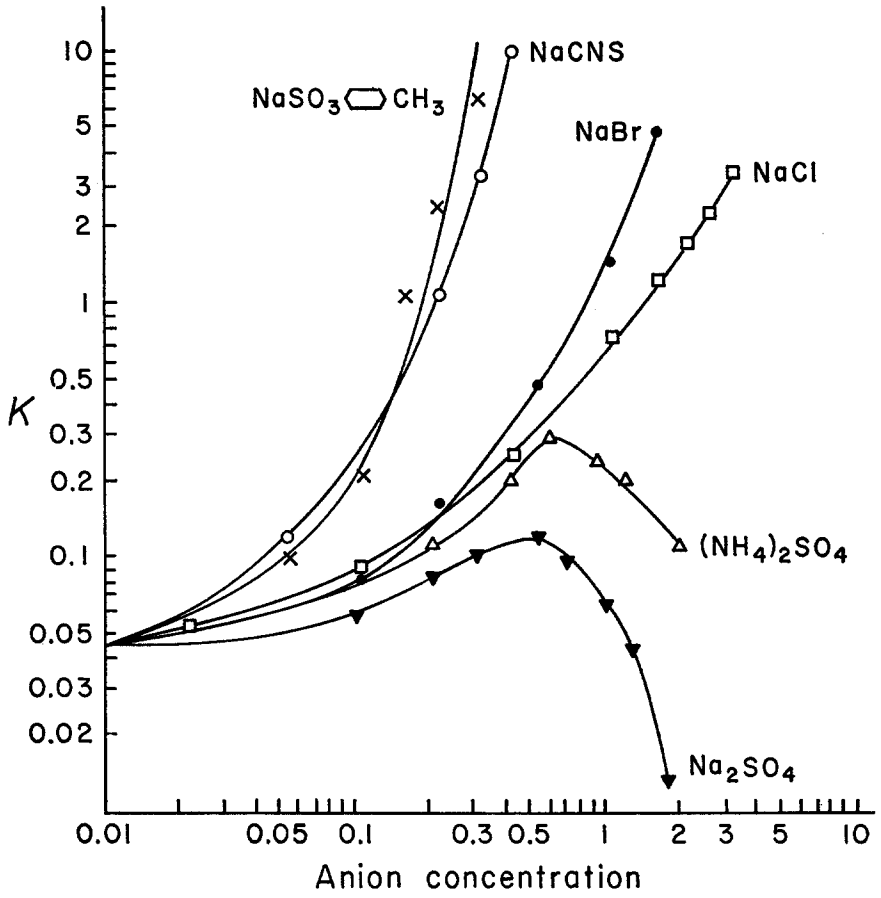


FIGURE 8. Demonstration of the ability of anions to govern the rate of thermal denaturation of prostatic acid phosphatase. The curves are plots of first order denaturation rate constants against the concentrations of simple anions.

species at the right. Strong binding by cations would cause a relative increase in denaturing forms at the left.

Only the sodium salts of thiocyanate and *p*-toluenesulfonate produced significant stabilization at low concentrations at pH 4.0. The bromide salt produced a barely detectable stabilization and chloride produced no such effect. It could not be observed at pH 3.6 or 4.5 either. Fig. 9 demonstrates the stabilization produced by sodium thiocyanate.

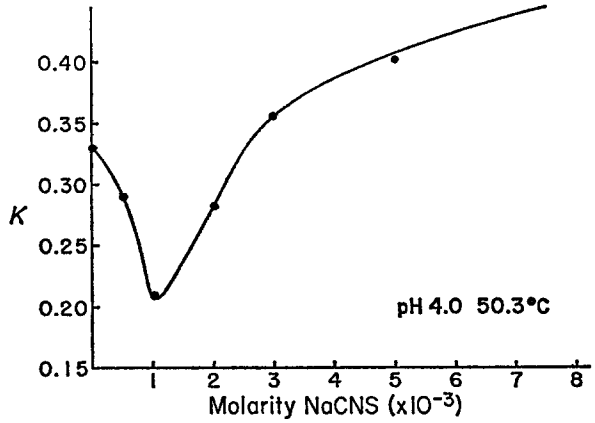


FIGURE 9. Demonstration of the stabilizing effects of thiocyanate ion under a unique set of conditions, predictable from theory. This ion under most other conditions enhances denaturation.

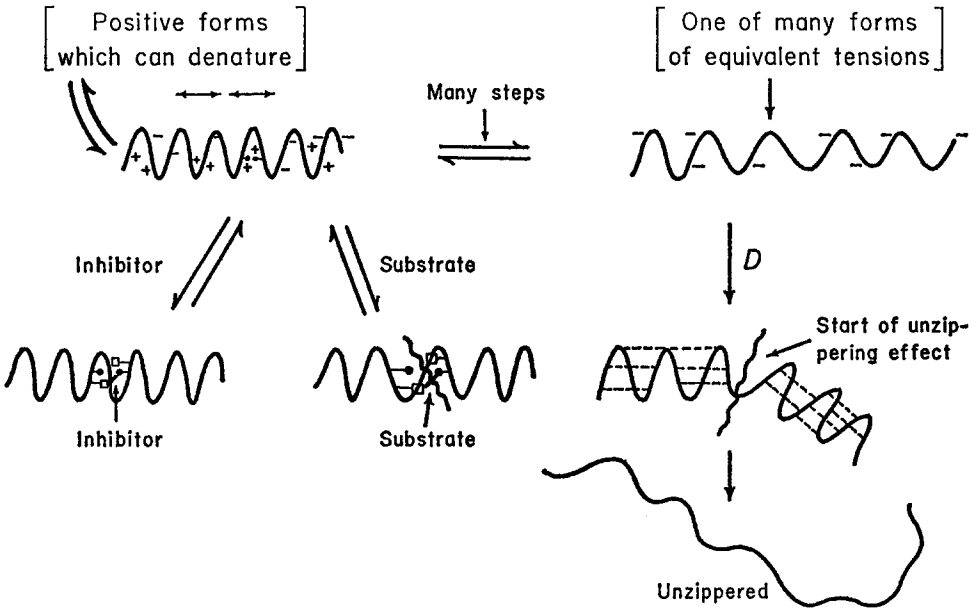


FIGURE 10. The interrelationships of the effects of charges in regulating enzyme functions. *D* stands for denaturation.

Experiments were performed to determine whether under similar conditions the activity of the enzyme could be enhanced by a shift of charged forms. It seems that the inhibition of the enzyme by reaction with critical [+] sites was the predominant reaction, and the phenomenon of enhanced activity could not be demonstrated. Anagnostopoulos (1953) demonstrated

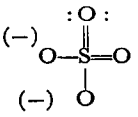
that low concentrations of citrate (which has a strong protein-binding capacity in this system, London *et al.*, 1954) enhanced enzymatic activity greatly at pH 4.0, but not at pH 5.0.

Steinhardt (1941) in his excellent work on combining ability of anions (with protein) used in titration, demonstrated extreme variations in affinity of anions for the protein combining sites. There are certain striking parallels between these affinities and the anion-enzyme reactions already described. Steinhardt measured the relative affinities of chloride, bromide, nitrate, toluenesulfonate, trichloroacetate, and picrate (as part of a much greater series). They were related by the factors 1.0, 1.9, 3.2, 3.9, 6.2, and 758. The same order for denaturation was found here, but we have no basis for calculating affinity constants. Our values for trichloroacetate and picrate are not reported here, and the much greater affinities of these compounds were noted too. The denaturation phenomena in these cases could only be studied at lower temperatures.

DISCUSSION

According to the kinetic analysis, the constant values of $\bar{n}$ observed in Fig. 7 and Table I imply that on the average almost two anions associate with one enzymic moiety to inhibit the enzyme non-competitively. Both sulfate curves indicate that $\bar{n}$ is little more than unity. This is in agreement with the theory since each sulfate ion carries twice the charge of the other anions. It also indicates that groups acquiring these charges are not specific since the sulfate ion must attach itself with its two negative charges to the same (+) sites and the other ions must select two (+) sites to accomplish the same end. It is unlikely that sulfate ion reacts with two (+) sites located at the end of two nearby side chains, as with two lysine end amino groups since such a complex is unstable. The (+) sites cannot be those at the critical region since substrate does not compete with the anions for these (+) sites.

The affinities of sulfate ion for the critical [+] sites are about one magnitude greater than those for the non-critical (+) sites. One of the mesomeric structures of the sulfate ion,



indicates that it is possible for a more intense bonding to be made because of a two point contact. As shown earlier (London *et al.*, 1958) the minimum requirement of a negative charge separated from a free electron pair by 2.5 to 3.0 A is available and the bond can be made at the critical site. The structure shown may contribute enough to the mesomeric system to

cause the one magnitude difference observed. K_i and K_u for the sulfate reactions are slightly dependent on the cation involved, and the values of K_s are fairly constant, agreeing with the values found earlier. Apparently the ammonium ion cannot be completely neglected in evaluating the K_s.

The difference in ability of the two sulfate salts to enhance thermal denaturation can be explained by presuming that a small but significant number of ammonium ions react with negatively charged sites of molecules to the left of center of Fig. 2 B, while the anion reacts with the molecules to the right of center. Actually, the ions react with all enzyme species regardless of net charge, but only molecules at the extremes, left and right, denature. Sodium ion being less reactive produces a slightly lesser denaturation.

The doubly charged anion, sulfate, in the case of both the ammonium and sodium salts exhibits maximum denaturation enhancement at almost the same concentrations, as in Fig. 8. It was already shown (London *et al.*, 1954) in the same system that citrate too has a maximum, but at even lower molar concentration. From the limited evidence on hand it seems that multiple charges on an anion may introduce a stabilizing factor. At the highest concentrations of sodium sulfate they stabilize the enzyme. Extrapolation of the ammonium sulfate curve indicates that at higher concentrations that salt would be a stabilizer too. At higher concentrations than were used in this study the sulfate salts precipitate the enzyme. Dehydration or salt bridges may cause this stabilization.

Mechanism Hypothesis The kinetic picture of the inhibitions caused by these anions can be explained in many different ways. However, it was possible for us to outline the following hypothesis consistent with the facts of this paper and other evidence available about this phosphatase, which describes an enzyme mechanism.

If we assume that charges on the enzyme molecule (other than those charged sites specifically associated with the active center) determine enzymatic activity, these charges of themselves confer a degree of specificity to the enzyme activity. A high degree of specificity is implied because despite the means of altering the charge, number, and perhaps the order of distribution, the proper charges must be present for an enzyme to be active. The region of activity of an enzyme molecule consists of a site of attachment and an array of charged groups. The distribution of charged groups on a matrix will be referred to as the second degree of specificity, the first being the combining site. A mechanism of secondary specificity was suggested earlier (London *et al.*, 1958) and is now elaborated.

Fixed charges (they do not move about and remain associated or undissociated with protons for short periods of time) determine the tension within the protein helix in the region of the charges. It is postulated that there is a

thermal vibration causing the helix to vibrate like a spring. When the combination of tension and vibration is great enough, the enzyme denatures as explained earlier (London *et al.*, 1954). Within the region of charges considered there are also the four critical points, or substrate binding sites, as shown in Fig. 1. Substrate when bound straddles the critical seam (London *et al.*, 1958). In this position it can oppose the tension and vibration, thus stabilizing the enzyme. Similarly, the tension and vibrations exert their actions on the substrate.

An enzyme species should have at least one natural vibration frequency, depending on its size and distribution of tensions (charged groups and various bonds) like any other spring with fixed tension.

Thus, if a submolecular group straddles the seam, the vibrations of the very much larger helix are imposed on this group and the substrate is actually part of the entire vibrating system. Should the natural frequencies of the attached group and the active center coincide, or one is a harmonic relative to the other, a large portion of the kinetic energy of the vibrating enzyme molecule can be transmitted to the substrate. This is mechanical resonance providing the energy of activation.

The activation energy, 10 to 20 kilocalories per mole, required to break a covalent bond is much greater than the net energy of any hydrogen bond. This requires the rejection of simple hypotheses of strain as a means of enzyme action. Bending or stretching a P—O bond must strain the hydrogen bond and break it before any appreciable strain is placed on the P—O bond. Other hypotheses which require mesomeric or inductive effects are also objectionable because of the loose coupling that the substrate makes with the enzyme.

The hypothesis of the secondary specificity factor is supported by the following observations. Denatured phosphatases and other proteins may possess the [+]—(H) unit spaced at 2.5 to 3.0 A and the same mesomeric or inductive conditions as the enzyme, but are inactive. There are no substantiated experiments to demonstrate that an active enzyme remains after being broken down to fragments so small as to destroy the integrity of the matrix and group of charges. Model systems of active centers fail to be fully enzymatic by only accelerating the splitting of unstable bonds. Competitive inhibitors of the phosphatase, sulfamate and diphenylphosphate (London *et al.*, 1958) straddling the active center should split at the S—N and P—O bonds not unlike that of the P—O bond of any substrate by a hypothesis of strain, mesomerism, or induction.

Vibrational stretching of the helix that permits 15 per cent elongation from the mean position of the spring exerts an almost negligible effect on the individual covalent bonds of the polypeptide backbone, a 2 or 3° average deviation of the bond angles. The cross-linking hydrogen bonds are not

stretched as much as the straddling bond (P—O) on a percentage basis. This mechanism may account for a number of hydrolyses.

Recapitulation Fig. 10 is a pictorial summary of all the phenomena and their interrelationships discussed in this and the previous work (London *et al.*, 1958) that are regulated by ionic combination.

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