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The Effect of Ions on the Catalytic Activity of Enzymes: Yeast Glucose-6-phosphate Dehydrogenase

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1. The catalytic activity of yeast glucose-6-phosphate dehydrogenase preparations is markedly altered in the presence of various ions. At low ion concentrations there is a strong augmentation of catalytic activity, while at high concentrations there is an inhibition.

2. Anions are important in the activation process. The relative effectiveness of various anions for augmentation of catalytic rate is $F^- > PO_4^{\equiv} > Cl^- > Br^- > SO_4^{\equiv} > I^- > SCN^-$.

The concentration for maximum activation varies with each anion.

3. Cations also play a role in augmentation of finite catalytic rate. Calcium and magnesium ions are more effective than sodium or potassium ions.

4. The inhibition of *Zwischenferment* activity at high ion concentrations is apparently due to a competition with substrates glucose- 6-phosphate and TPN, for site on the enzyme surface.

5. Possible mechanisms for the augmentation of a finite catalytic rate by both anions and cations are discussed.

The marked influence of the ionic environment on physiological activity is usually explained in terms of ionic effects on enzymes in the biological system. Many purified enzyme preparations absolutely require a particular ion or closely related group of ions for catalytic activity; while the catalytic activity of many other enzyme preparations is influenced by ionic environment to a greater or lesser degree.

In cases where an absolute ionic requirement for enzyme activity has been demonstrated, specific monovalent and/or divalent metal ions have usually been implicated as the actual cofactor. The anionic species may usually be varied without observable effect. From this it has been inferred that the accompanying anionic species is relatively unimportant in the cases where a specific cationic requirement exists; and that the metal ions are intimately associated in the catalytic apparatus and are obligatorily involved in the catalytic process.

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There are in addition some observations which are more easily explained in terms of an augmentation of a finite catalytic rate. These effects may be the reflection of a general influence of ionic strength of the medium, or may be more specifically related to a particular cationic or anionic species. Of particular interest for the present discussion, for example, is the stimulation of the activity of the pancreatic and salivary α-amylases by chloride ions [1]; the augmentation of the catalytic activity of fumarase by polyvalent anions [2], the affects of both cationic and ionic species on the activity of β-galactosidase [3,4], and the influence of the ionic strength on the catalytic activity of urease [5].

The earlier observations from this laboratory concerning large effects of anions on certain flavoprotein systems [6,7] as well as with the reaction catalyzed by liver alcohol dehydrogenase [8] has prompted additional studies of ionic effects on other enzyme systems in order to ascertain, 1) the generality and magnitude of such effects, 2) the mechanism of stimulation, and, 3) the physiological significance of such phenomena.

The present paper presents the data which have been obtained in the *Zwischenferment* system *. It has been found that a variety of ions, including phosphate and the substrate glucose-6-phosphate stimulate enzymatic activity markedly. At high concentrations inhibition is observed. Possible mechanisms of ionic effects on the catalytic activity of this and other enzymes are discussed.

MATERIALS AND METHODS

Glucose-6-phosphate dehydrogenase (*Zwischenferment*) was prepared according to the procedures of Warburg and Christian [9], and of Kornberg [10]. The reaction was followed spectrophotometrically. The specific activity of these preparations is expressed as μmoles TPNH formed/min/mg protein under optimum assay conditions. The results obtained were independent of the preparation used. Crystalline barium glucose-6-phosphate (G-6-P) was converted to the sodium or potassium salt which in turn was used as the substrate.

The rates reported here are the initial rates when the reaction rate was zero order with respect to concentration of substrates. The experiments were performed at 23°C.

RESULTS

The initial experiments to be reported here concern the effects of certain ions on *Zwischenferment* activity. The addition of monovalent ions to the assay system is typified by the data presented in Fig. 1. In the presence of sodium chloride or sodium fluoride there was observed an increase in activity, especially on the acid side of the pH activity curve, and a change in the pH of optimum activity. It is noteworthy that fluoride and chloride did not elicit identical effects on catalytic activity. The strong role of the anion is indicated in the experiments summarized in Fig. 2 in which enzyme activity was measured as a function of anion concentration. A stimulation of activity was

* Abbreviations used in this manuscript are as follows: *Zwischenferment*, yeast glucose-6-phosphate dehydrogenase; G-6-P, glucose-6-phosphate; TPN, triphosphopyridine nucleotide; DPN, diphosphopyridine nucleotide; 6PGL, 6-phosphogluconolactone.

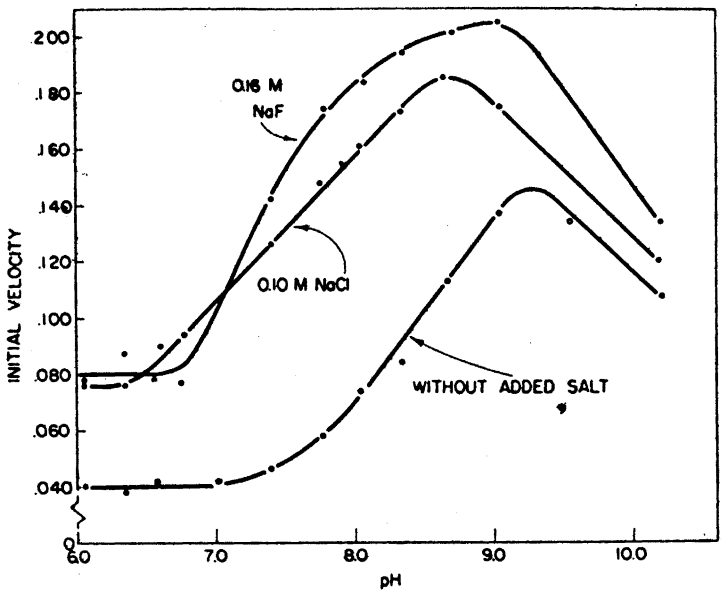

Fig. 1. Influence of ions on the *Zwischenferment* reaction as a function of pH. The reaction mixtures contained 6 μM G-6-P, 0.3 μM TPN, 100 μg *Zwischenferment* preparation, 100 μM buffer. (Histidine pH 0.6—7.0; glycyl-glycine pH 7.0—9.0, glycine pH 9.0—10.2) salt added when indicated, final volume 3.0 ml, temperature 23°C. Initial velocity is expressed in terms of change in optical density change at 340 mμ/min. The pH of each reaction was tested imediately after assay of enzymatic activity.

observed at low concentration and an inhibition at higher concentrations; the degree of stimulation and concentration required for stimulation and inhibition varied with the anionic species. The "effectiveness" of various anions in augmentation of *Zwischenferment* activity may be stated qualitatively $F^- > PO_4^{\equiv} > Cl^- > Br^- > SO_4^{\equiv} > I^- > SCN^-$; and for inhibition of catalytic rate $SCN^- > I^- > SO_4^{\equiv} > PO_4^{\equiv} > Br^- > Cl^- > F^-$. The position of $PO_4^{\equiv}$ in these series is noteworthy. The wide variation in effects elicited by anions precludes ionic strength as the sole cause of activation and inhibition phenomena. It is true, however, that certain of the data, for example the stimulation by low concentrations of phosphate, chloride, and fluoride may be so related. The finite activity observed without the further addition of ions does not represent activity of a salt free system, but that in the presence of 3×10^{-2} M glycylglycine buffer and 2×10^{-3} M glucose-6-phosphate. The ionic strength of the solution, therefore, was high enough to allow the possibility of an absolute requirement for added ions for enzyme activity. Suitable experiments with dialyzed preparations, however, showed that glycylglycine as well as versene and citrate give practically no activation or inhibition of this system. These observations make it unlikely that the activation by ions is due to the solvation of a contaminating inhibitory metal ion, and also emphasize the role of the anionic species in the activation process.

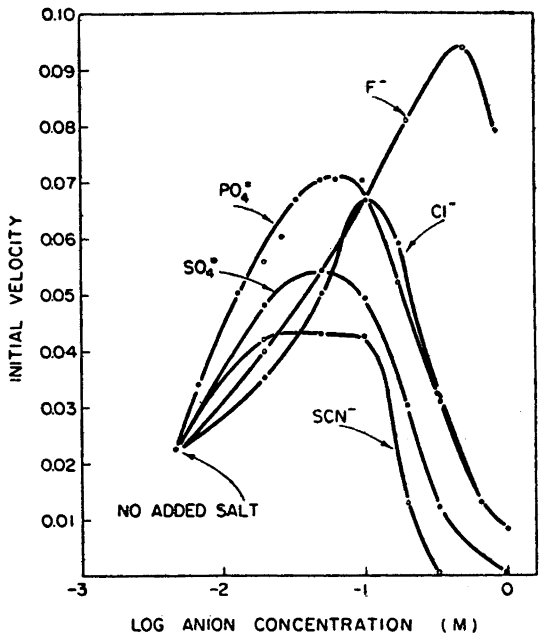

Fig. 2. Effect of anions on the rate of the *Zwischenferment* reaction.
The reaction mixtures contained 6 μM G-6-P, 0.3 μM TPN, 100 μg *Zwischenferment*, 100 μM glycyl-glycine pH 7.4, anions as indicated, water to 3.0 ml, temperature 23°C. All of the anions were added as the sodium salts.
The rate expressed as optical density change at 350 mμ/min.

Fig. 3 shows the effect of substrate concentration in the presence and absence of added ions. Glucose-6-phosphate does, itself, stimulate enzyme activity at concentrations higher than those needed to saturate the enzyme, but it is less effective than the simple inorganic anions. The presence of other anions markedly influences the apparent affinity of the enzyme for its substrate (*i. e.*, the K_m of the substrate). At a concentration of 0.5 M NaCl, for example, at least ten times more glucose-6-phosphate is required to obtain maximum activity than at 0.1 M NaCl. The early reports [11,12] concerning the phosphate inhibition of *Zwischenferment* activity, could, on the basis of the present investigation, be related to the concentrations of glucose-6-phosphate and/or TPN present in the assay system used.

Fluoride ions are more effective than chloride or other ions in augmenting the catalytic rate, especially at low substrate concentrations. The small size of the fluoride ion could conceivably allow more effective collisions of enzyme and substrate.

In Fig. 4 are presented Lineweaver-Burk plots of representative data on the interrelationship of ions and G—6—P in the *Zwischenferment* reaction at a constant TPN concentration. There is an apparent competitive relationship between ions and the substrate. The strong activation by high concentrations

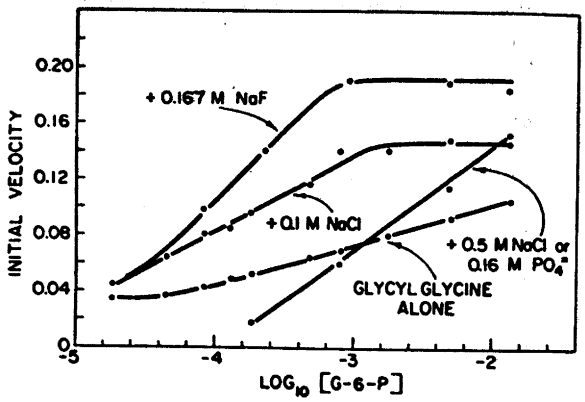

Fig. 3. Ion-glucose-6-phosphate interrelationships in the *Zwischenferment* reaction. The general reaction conditions are as indicated in Fig. 2. The molar G-6-P- concentration was varied as indicated. The sodium salts of anions were employed.

of the substrate is evidenced by the rather marked inflection in the curve. It is not possible to say definitely whether G—6—P itself is the activating ion, or whether contaminating ions (traces of sodium sulfate, *e. g.*, any sodium) are responsible for this effect.

Similar data concerned with the influence of ions on the reaction of *Zwischenferment* with TPN at constant glucose-6-phosphate concentration are

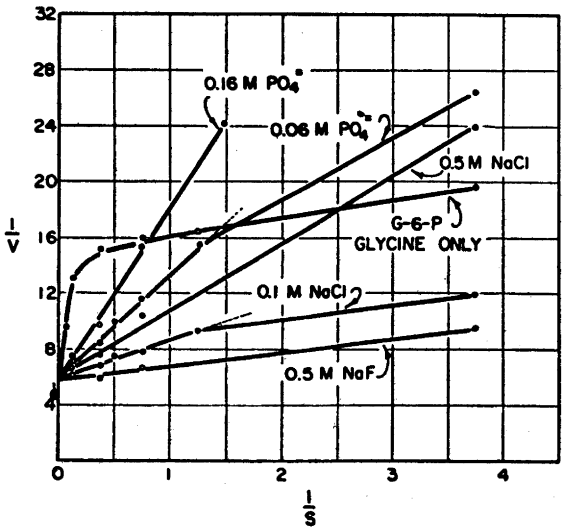

Fig. 4. Ion-glucose-6-phosphate relationships in the *Zwischenferment* reaction. The general reaction conditions are as indicated in Fig. 3. In these Lineweaver-Burk plots the units of reaction velocity are optical ensity change at 340 mμ/min and the units of substrate concentration are μM/ml reaction medium.

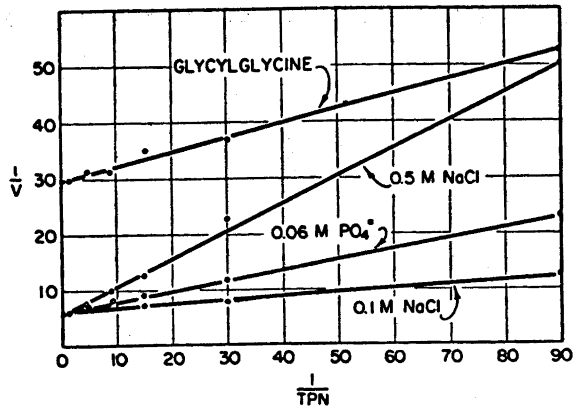

Fig. 5. Ion-TPN relationship in the *Zwischenferment* reactions.
General reaction conditions are as described in the legend of Fig. 2, 6 μM G-6-P present, and TPN concentration varied as indicated. In these Lineweaver-Burk plots, the units of reaction velocity are — O.D. 340 mμ/min, and units of TPN concentration μM/ml reaction medium.

presented in Fig. 5. Again there is a competitive relationship between TPN and the ions tested, presumably for a site on the enzyme surface. TPN in the concentrations used is not effective as an ion activator, and may be contrasted to glucose-6-phosphate in this respect.

The difference between the maximum velocities in the presence and absence of added ions is considered a point of some significance. At concentrations of G—6—P below those at which activation occurs (10^{-3} M) and in the absence of added activating ions, either a large fraction of total enzyme molecules are catalytically inactive, or, perhaps more likely, catalysis proceeds at all active sites of the enzyme population, but at a much slower rate. Some of the inflec-

Table 1. Effect of substrate and TPN in overcoming anion inhibition of glucose-6-phosphate dehydrogenase

In 0.5 molar NaCl Additions	Initial rate
1. 0.4 μmoles G-6-P 0.18 μmoles TPN	0.020
2. 10 μmoles G-P-6 0.18 μmoles TPN	0.030
3. 0.4 μmoles G-6-P 1.5 μmoles TPN	0.042
4. 10 μmoles G-6-P 1.5 μmoles TPN	0.042

Each reaction mixture contained 1.5 μmoles NaCl, 100 μg *Zwischenferment*, 100 μM glycyl glycine buffer, pH 7.4, and the concentration of G-6-P and TPN indicated above. The final volume was 3.0 ml., temperature 23°C.
The initial rate is expressed in terms of change in optical density.

Table 2. Effect of cations on *Zwischenferment* activity.

Ion	μ	% Stimulation
NaCl	0.02	87
	0.04	175
	0.10	312
	0.15	250
KCl	0.04	175
$CaCl_2$	0.02	300
	0.05	387
	0.07	425
	0.10	437
	0.15	437
	0.20	437
$MgCl_2$	0.02	287
	0.05	350
	0.10	400
	0.15	387
$MnCl_2$	0.02	312
	0.05	350
	0.10	300

Each reaction mixture contained 1 μmole G-6-P, 0.3 μmoles TPN, 100 μmole glycyl glycine, pH 7.4. 100 μg *Zwischenferment* (lyophilized preparation, original specific activity approximately 1.0). Ions added when indicated, final volume 3.0 ml, temperature 23°C. The figures represent stimulations of the rate observed in the absence of added ions (Δ O.D./min = 0.032).

tions in the Lineweaver-Burk curves in Fig. 4 (which have been consistently observed) may be the result of transitions between the two types of catalysis. The possibility that anions influence the dissociation of the enzyme-product complex will be considered in the discussion section.

Since the reaction of both G—6—P and TPN with the enzyme is inhibited in a competitive manner by a variety of ions, the question arises whether the binding of one substrate to the enzyme influences the binding of the other; that is whether the competitive inhibition by anions of the reaction of G—6—P with *Zwischenferment* is independent of TPN and vice versa. The experiment reported in Table 1 was performed to test this possibility. At high salt concentrations, increasing the concentration of either substrate (G—6—P or TPN) increases the reaction rate. This observation is most easily explained in terms of an increased affinity for the second substrate, when the enzyme is combined with the first substrate.

The fact that anions elicit more or less specific effects on the catalytic activity of *Zwischenferment* does not, of course, rule out a role of the cationic species. An experiment, therefore, was designed to test the effects of various chloride salts on enzyme activity. As shown in Table 2, the divalent metals ions, Ca^{++}, Mg^{++}, and Mn^{++} are more effective than sodium and potassium, especially at low ionic strengths *. Moreover, inhibition of the reaction by

* Dr. Hans Klenow, Universitetets Institut for Cytofysiologi, Copenhagen, Denmark, has independently observed a stimulation of *Zwischenferment* by certain ions (private communication).

chloride salts occurs according to the concentration of chloride ions, and is not, apparently, simply related to the overall ionic strength; thus at 0.1 M NaCl there is already a significant decrease in activity, while at this concentration of $MgCl_2$ and $CaCl_2$ maximum activity is still observed. The report of Kornberg [10] that magnesium ions stimulate *Zwischenferment* activity is thus confirmed, but it is emphasized that there is no apparent specific requirement for magnesium or other divalent metal ions in the system [13]. This is not to deny the possible existence of a non-dissociable metal ion in the enzyme itself. The possible role of divalent metal ions as non-specific activators will be considered in the discussion.

DISCUSSION

The ionic effects observed here are more easily interpreted in terms of an augmentation of catalytic rate, rather than an absolute requirement for added ion. It would not be wise, however, to suggest that there is not an absolute requirement for ions, since the H_3O^+ and OH^- ions of the solvent are doubtless contributory in the system. As a matter of fact, one explanation for the comparative lack of ionic effect in the basic portion of the pH activity curve, may be the increasing number OH^- ions in solution. The marked influence of ions on the catalytic rate cannot be a simple function of the ionic strength, since widely varying effects were observed with different ionic species at the same ionic strength. Any interpretation of the data must consider the role of both cationic and anionic species in the catalytic process.

Michaelis and Pechstein[14] have advanced a theory to explain ionic stimulation of enzymatic activity. According to their argument, enzymatic activity is related to both acidic and basic dissociable groups; the optimum catalytic activity being at that pH where maximum dissociation of both acidic and basic groups occurs. The presence of salts then may affect catalytic activity by altering the dissociation of these groups involved in the catalytic process. This theory extended by Alberty [15] has satisfactorily explained much of the data concerned with the effect of ions on enzymatic activity. The extensive studies on the influence of ions on fumarase activity by Massey [2], and Massey and Alberty [16], for example, suggest effects of anions on the dissociation of groups at, or near, the active center.

This type of explanation may at least in part have validity here. It is true that the apparent affinity (K_m) of the enzyme for G—6—P and TPN is markedly affected by the ionic environment. The strong stimulation of catalytic activity on the acidic side of the pH-activity curve would presumably be related to the influence of anions present, and the smaller but significant increase on the basic side of the curve, to the influence of cations.

There is, however, another interpretation of effects observed in this and in certain other cases. As is the case with any enzymatically catalyzed reaction which is presumed to proceed through the formation of an enzyme substrate complex, the catalytic process associated with *Zwischenferment* may be considered to be composed of several partial reactions: (a) the association of the enzyme with its substrates, (b) the catalytic process *per se*, and (c) the dissociation of the enzyme from the products of the reaction. For the *Zwischenfer-*

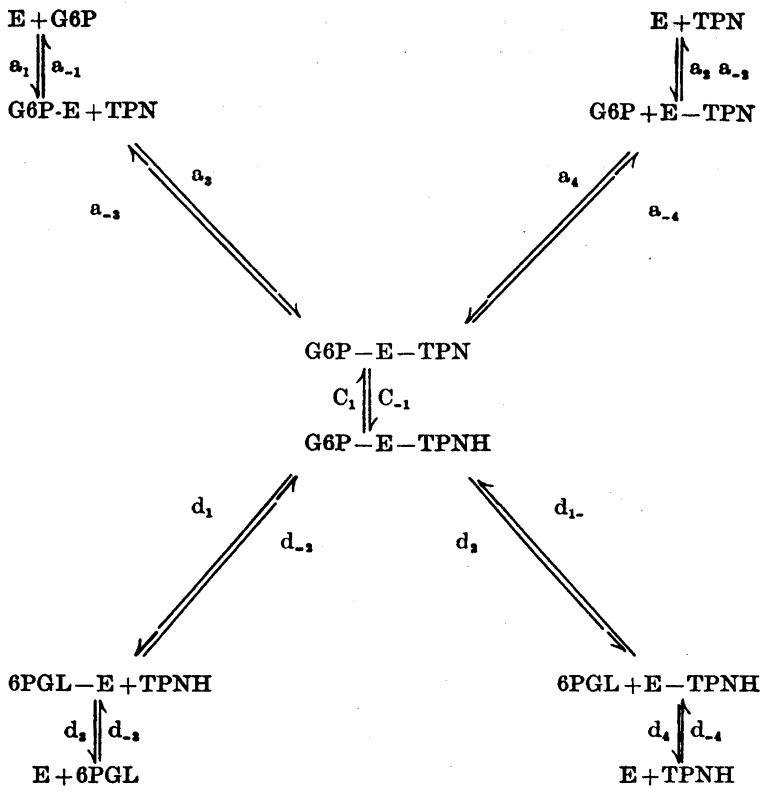

Fig. 6. Hypothetical kinetic mechanism for the *Zwischenferment* reaction.

ment system, the partial reactions formulated in Fig. 6 have been arbitrarily selected as an expression of the overall process. The actual catalytic reaction is represented by C, which is a composite constant of the partial reactions involved in this process. Clearly the observed overall rate of the reaction will be a function of the rate constants for the partial reactions, and in particular instances could, therefore, be a reflection of association of enzyme and substrates, the catalytic reaction, or of dissociation to enzyme and products, or combinations of these. If the formation of the enzyme-substrate complex can be considered according to the ion association theory, then at low dielectric constant (in the absence of salts), there would exist a greater tendency for the enzyme and substrate, to associate, and at higher dielectric constant (*i. e.*, at higher ionic strength), a greater tendency to dissociate since both charged species would then be solvated. The stimulatory effects of ions on *Zwischenferment* activity are explicable on this basis if it be assumed that the dissocia-

tion of products is rate limiting *. An increase in the anion concentration would simultaneously decrease the association reaction, and increase the dissociation reaction. The net result would be a stimulation of activity together with an increased K_m for the substrate. In the observations presented in this paper, note that at a given substrate concentration a stimulation of activity followed by an inhibition was observed as the ionic concentration increased. The degree of effectiveness of a particular anion, either as an activator or inhibitor, would then be related to its effectiveness in displacing the product from the complex, and the ease with which it in turn was displaced by the substrate. Thus, phosphate is especially effective as an activator at low concentrations, while sulfate inhibits at comparatively low concentrations presumably because it would be less readily displaced by the substrate. The increased effectiveness of the divalent metal ion can be easily visualized, since these cations would readily form an ion pair with the divalent phosphate groups on the product molecules, enhancing the rate of dissociation. The pH optimum of the enzyme in the absence of added ions then might be expected to be in the basic region where the hydroxyl ions could increase dissociation rate of enzyme product complex. The change in pH optimum with salt concentration could conceivably be a reflection of the relatively smaller effect of hydroxyl ions in the presence of salt. It would be expected that effects of added anions would be proportionally greater at lower pH values, as has been observed in these studies.

The argument presented provides a means of explaining the effect of ions on catalytic activity of glucose-6-phosphate dehydrogenase, and could be applied to other cases of ion stimulation and inhibition, especially when concomitant effects on the apparent affinity of the enzymes for the substrates are also observed.

It is not a novel idea that the rate limiting reaction of an enzymatic catalysis be the association or dissociation of enzyme-substrate complex. Theorell and Chance [19] have, for example, shown that in the reaction of ethanol with DPN catalyzed by alcohol dehydrogenase, the dissociation of Enzyme-DPNH is in all probability the limiting step.

The above treatment allows the qualitative prediction of this type of ionic effects on the rate constants in the catalytic process. For example, an increase in appropriate ion concentration should increase A_{-1}, A_{-2}, A_{-3}, A_{-4}, d_1, d_2, d_3, d_4, and decrease A_1, A_2, A_3, A_4, d_{-1}, d_{-2}, d_{-3}, d_{-4}, in the *Zwischenferment* system as written. It is conceivable that ionic affects of the type suggested, could be observed on the catalytic step C_1 and C_{-1}. In this case, however, the manner in which C_1 and C_{-1} would be affected would be considerably more difficult to predict.

* It is true in the present instance, that ionic effects observed might also be related to a conversion of the product, 6-phosphogluconolactone (6PGL) to a compound not involved in the reaction, namely, 6-phosphogluconic acid [14]. Since the observations were initial reaction rates, it is believed the large effects observed cannot be solely related to this additional reaction. The results also cannot be effects on the lactonizing enyme, since Ca++ ions stimulate the present reaction while magnesium, manganese, or cobalt ions are required for gluconolactonase; and in addition, fluoride ions are efficient activators of *Zwischenferment*, yet they inhibit gluconolactonase [15].

An effect of ions on the dissociation of groups involved in the combination of the substrate or products with the enzyme, or in the catalytic reaction itself could on the other hand alter the individual rate constants in either direction, depending on the chemical circumstances.

According to the nature of the reacting groups the present hypothesis provides an explanation for ionic effects on reactions involving groups which are completely ionized under the conditions imposed. It is an adjunct to the more often expostulated theory which concerns the effects of added ions on reactions involving groups which dissociate under the conditions imposed; together, they provide a general mechanism for the interpretation of cases in which there is a general ionic influence in catalytic activity, but no specific ion erquirement.

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REFERENCES

1. Bernfeld, P., Staub, A. and Fischer, C. H. *Helv. Chim. Acta* **31** (1948) 2165.
2. Massey, V. *Biochem. J.* **53** (1953) 67.
3. Cohn, M. and Monod, J. *Biochim. et Biophys. Acta* **7** (1951) 153.
4. Kuby, S. A. and Lardy, H. A. *J. Am. Chem. Soc.* **75** (1953) 890.
5. Kistiakowsky, G. B. and Shaw, W. H. A. *J. Am. Chem. Soc.* **75** (1953) 2751.
6. Theorell, H. and Nygaard, A. P. *Acta Chem. Scand.* **8** (1954) 877, 1649.
7. Walaas, E. and Walaas, O. *Acta Chem. Scand.* **10** (1956) 122.
8. Theorell, H., Nygaard, A. P. and Bonnichsen, R. *Acta Chem. Scand.* **9** (1955) 1148.
9. Warburg, O. and Christian, W. *Biochem. Z.* **254** (1932) 438.
10. Kornberg, A. *J. Bio.. Chem.* **182** (1950) 805.
11. Theorell, H. *Biochem. Z.* **275** (1953) 416.
12. Negelein, E. and Haas, E. *Biochem. Z.* **282** (1935) 206.
13. McNair-Scott, D. B. and Cohen, S. S. *Biochem. J.* **55** (1953) 23.
14. Michaelis, L. and Pechstein, H. *Biochem. Z.* **59** (1914) 77.
15. Alberty, R. A. *J. Am. Chem. Soc.* **76** (1954) 2494.
16. Massey, V. and Alberty, R. A. *Biochim. et Biophys. Acta* **12** (1954) 354.
17. Cori, O. and Lipmann, F. *J. Biol. Chem.* **194** (1952) 417.
18. Brodie, A. F. and Lipmann, F. *J. Biol. Chem.* **212** (1955) 677.
19. Theorell, H. and Chance, B. *Acta Chem. Scand.* **5** (1951) 1127.

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