Glucose Dehydrogenase Activity of Yeast Glucose 6-Phosphate Dehydrogenase. II. Kinetic Studies of the Mode of Activation by Bicarbonate, Phosphate, and Sulfate*

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ABSTRACT: We (Anderson, W. B., and Nordlie, R. C. (1968), *Biochemistry 7*, 1479) recently reported that the anions bicarbonate, phosphate, and sulfate, which inhibit yeast glucose 6-phosphate dehydrogenase (D-glucose 6-phosphate:nicotinamide–adenine dinucleotide phosphate oxidoreductase, EC 1.1.1.49) activity, all stimulate quite markedly glucose dehydrogenase (β-D-glucose:nicotinamide–adenine dinucleotide phosphate oxidoreductase) activity also catalyzed by commercial (Boehringer) yeast glucose 6-phosphate dehydrogenase preparations. Further evidence for the common identity of these two dehydrogenase activities is presented in this paper, and the results of studies of the effects of the various anions on the reaction kinetics of both glucose 6-phosphate dehydrogenase and glucose dehydrogenase reactions are described. Inhibition of the former activity by anions was found to be competitive with respect to glucose 6-phosphate and noncompetitive with respect to nicotinamide–adenine dinucleotide phosphate. K_m values for both glucose and nicotinamide–adenine dinucleotide phosphate in the glucose dehydrogenase reaction were unaffected by activating concentrations of the various anions. Inhibition of glucose 6-phosphate dehydrogenase activity of all three anions, included in assay mixtures singly and in combinations of two and three, was of the "common-site" type. K_i values calculated for sulfate and phosphate, which functioned as competitive inhibitors of bicarbonate-effected activation of glucose dehydrogenase, agreed closely with corresponding K_i values for these anions acting as inhibitors of glucose 6-phosphate dehydrogenase activity. These results are interpreted in terms of a mechanism incorporating Koshland's induced-fit concept, and involving a single, common binding site for the anions which function both as activators of glucose dehydrogenase activity and as inhibitors of enzymic activity with glucose 6-phosphate as oxidizable substrate.

In a recent publication (Anderson and Nordlie, 1968), we described the selective, activity-discriminating effects of certain anions (phosphate, sulfate, and bicarbonate) on yeast glucose 6-phosphate dehydrogenase. A relatively slow oxidation of nonphosphorylated glucose is catalyzed by this enzyme (Colowick and Goldberg, 1963; Kuby and Noltmann, 1966; Salas *et al.*, 1965). This glucose dehydrogenase activity was observed by us to be quite significantly stimulated in the presence of the aforementioned anions,[1] while dehydrogenase activity with glucose 6-phosphate as substrate was inhibited by these ions. Since these selective anion effects present an interesting means of regulating the activities of this multifunctional enzyme, further investigations were dictated by these observations. Studies on the influence of these anions on the reaction kinetics for the two activities catalyzed by this enzyme are described in this paper. Experimental results, which indicate that all of these anions may affect both enzymic activities by binding at a common site on the enzyme, are presented along with a suggested explanation for their differential, activity-discriminating effects.

Materials and Methods

The commercial purified glucose-6-P[2] dehydrogenase preparation and reagents used, as well as the enzymic activity assays, were as described previously (Anderson and Nordlie, 1968). Studies were carried out with four different lots (lot control no. 6437591, 6018592, 6067475, and 6466473) of enzyme having a specific activity of approximately 140 units/mg of protein and obtained from Boehringer Mannheim Corp., New York. Experimental results described in this paper are typical of those obtained with all lots of the enzyme. Details of individual experiments are given in the Results section below and in legends to the individual figures and table. Activity was in all instances measured under conditions such that initial reaction velocities were determined. The results of kinetic studies are presented as

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[1] Stimulation by bicarbonate of glucose dehydrogenase activity of red blood cell glucose 6-phosphate dehydrogenase also has been reported recently (Kissin and Beutler, 1968).

[2] Abbreviation used that is not listed in *Biochemistry 5*, 1445 (1966), is: CoA, coenzyme A.

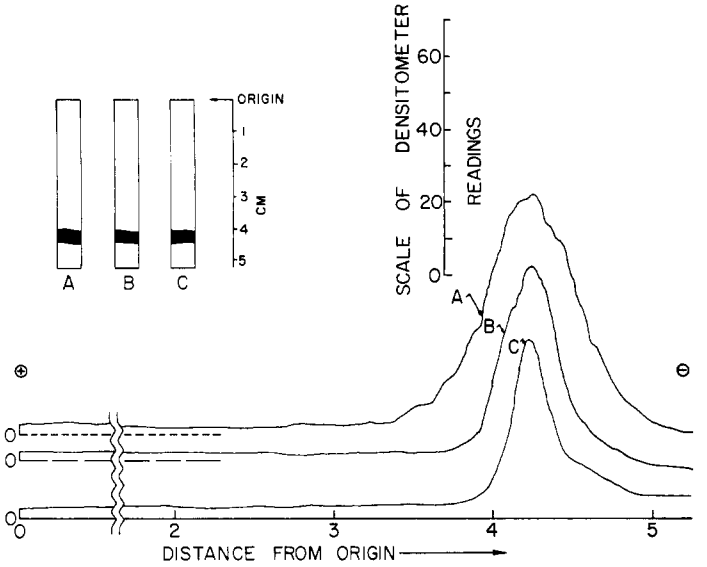

FIGURE 1: Disc electrophoresis studies of yeast glucose-6-P dehydrogenase preparation. Aliquots of enzyme preparation (25 μg of protein in 0.05 ml) were placed on columns of 6.5% polyacrylamide gel (pH 9.5) and electrophoresis was carried out with the Canalco Model 300A apparatus according to the "alternative procedure" described by Davis (1964). A current of 5 mA was applied for a period of 1.5 hr. Gel C was stained for protein with the aid of 1% Amido Schwarz solution and destained in 7.5% acetic acid solution. A second gel was divided longitudinally and one-half (A) was stained for glucose-6-P dehydrogenase activity while the other half (B) was stained for glucose dehydrogenase activity. These activities were visualized by incubating the gels in the dark in 5 ml of a solution of 0.05 M Tris-Cl buffer (pH 8.0), containing 1.0 mg of phenazine methosulfate, 0.5 mg of nitroblue tetrazolium, 1.5 μmoles of sodium NADP, and either 0.75 μmole of glucose-6-P or 60 mmoles sodium bicarbonate and 5 mmoles of D-glucose. In the inset in the upper left are line drawings of the patterns obtained. The remainder of the figure depicts tracings obtained by scanning the gels in a Densicord Model 542 recording electrophoresis densitometer. The vertical scale ("Scale of Densitometer Readings") applies to all tracings and indicates readings obtained with a D-2 response setting on the instrument. The tracings have been combined in a single figure for convenience of presentation; the "zero" readings for each scan are indicated in the lower left portion of the figure. Results of a typical study are presented. Multiple studies were carried out with each of the several lots of commercial enzyme described in the text. Occasionally second, minor, superimposable peaks of glucose-6-P and glucose dehydrogenase activities, constituting less then 5% of total activity in all instances, were noted immediately to the left of the peak depicted in the drawing.

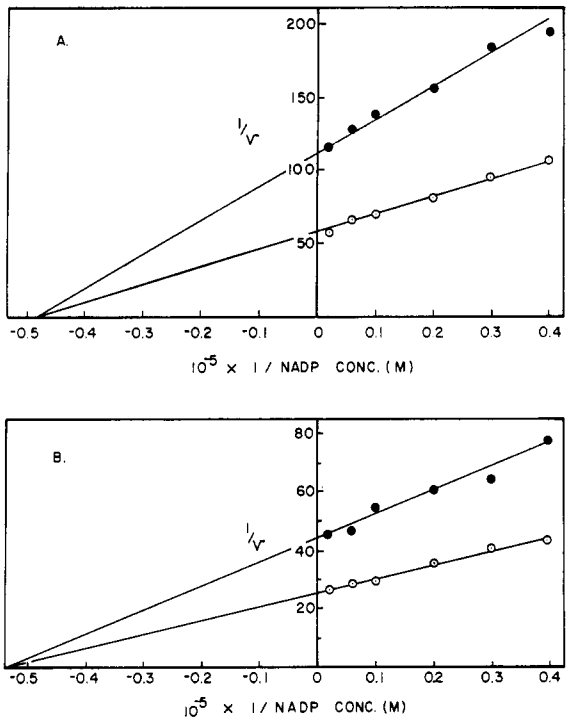

FIGURE 2: Inhibition of glucose dehydrogenase activity (A) and glucose-6-P dehydrogenase activity (B) by *p*-mercuribenzoate. Assay mixtures (pH 8.0) contained, in 3.0 ml, 8.3 mM Tris-Cl buffer, indicated concentrations of sodium NADP, and either 0.5 M equilibrated D-glucose and 12.5 μg of enzyme protein (glucose dehydrogenase), or 2×10^{-5} M sodium glucose-6-P and 0.25 μg of enzyme protein (glucose-6-P dehydrogenase). Activities, v, were measured both in the absence (○) and presence (●) of 0.5 mM *p*-mercuribenzoate. Ionic strength = 0.100 in all cases. K_i values, calculated as described by Dixon and Webb (1964b), were (A) 5.3×10^{-4} M and (B) 6.1×10^{-4} M. K_m values for NADP were 2.1×10^{-5} M for the glucose dehydrogenase reaction (A) and 1.9×10^{-5} M for the glucose-6-P dehydrogenase reaction (B). Velocity, v, is expressed as μmoles of NADPH formed/2.5 min.

conventional double-reciprocal plots (Lineweaver and Burk, 1934). Michaelis constants were calculated as negative reciprocals of x-axis intercepts of extrapolations of these plots, while V_{max} values were evaluated as reciprocals of y-axis intercepts of these same lines (see Dixon and Webb, 1964a). Inhibitor constant values also were calculated by the methods described by Dixon and Webb (1964b).

Kinetic studies of yeast glucose-6-P dehydrogenase have been described by several groups of workers (see Noltmann and Kuby, 1963). It was observed that the K_m value for NADP was not influenced significantly by the concentration of glucose-6-P present and that, correspondingly, the K_m value for glucose-6-P was independent of NADP concentration (Glaser and Brown, 1955). In supplementary experiments, we have noted similar results both with glucose-6-P dehydrogenase and glucose dehydrogenase activities of the enzyme. These observations simplified experimental design of the studies to be described, since for such a system where the affinity of each substrate for the enzyme is independent of the concentration of the second substrate, true Michaelis constant values may be determined directly by measuring activity as a function of varied concentration of one substrate while maintaining the second substrate at a single fixed concentration (Dixon and Webb, 1964c).

As in earlier studies (Anderson and Nordlie, 1968), supplementary sodium chloride was employed routinely to maintain constant ionic strength in assay mixtures, unless otherwise specifically noted.

Results

Common Identity of Glucose-6-P Dehydrogenase and

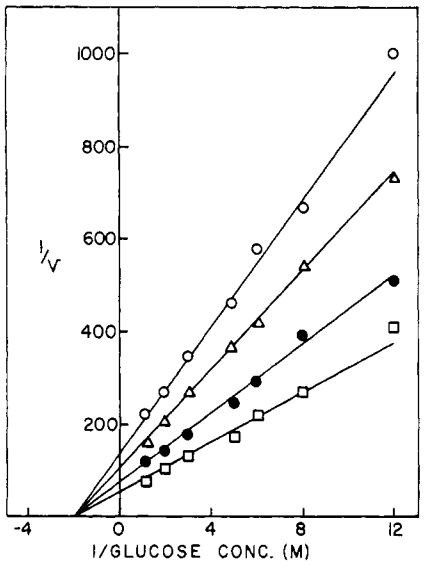

FIGURE 3: Effects of increasing ionic strength on the reaction kinetics of glucose dehydrogenase studied with respect to varied glucose concentrations. Assay mixtures (pH 8.0) contained, in 3.0 ml, 8.3 mM Tris-Cl buffer, 0.1 mM sodium NADP, 5 μg of enzyme protein, the indicated concentrations of equilibrated D-glucose, and either no (○), 10 mM (△), 50 mM (●), or 100 mM (□) sodium chloride. (Ionic strength of sodium chloride = its molar concentration.) v is expressed as in Figure 2. $K_{glucose}$ = 0.54 M in all cases.

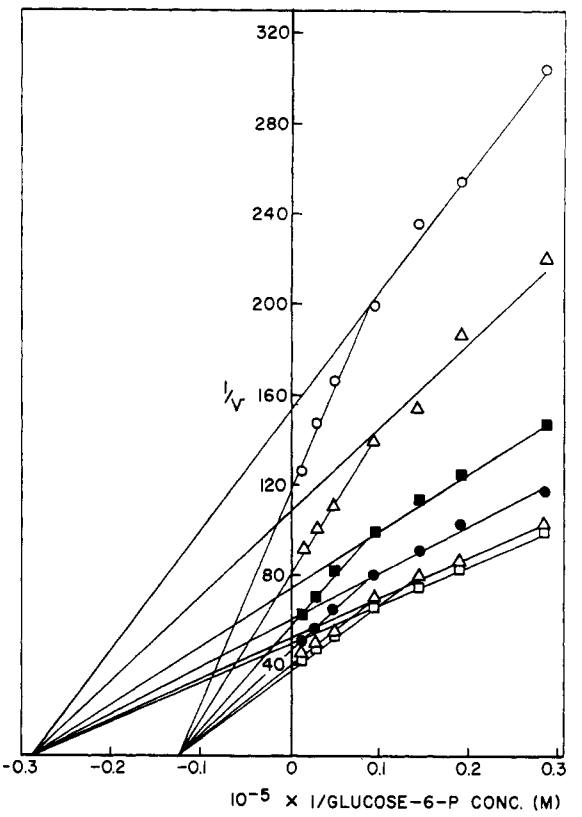

FIGURE 4: Effects of increasing ionic strength on the reaction kinetics of glucose-6-P dehydrogenase activity studied with respect to varied glucose-6-P concentrations. Assay mixtures (pH 8.0) contained, in 3.0 ml, 8.3 mM Tris-Cl buffer, 0.1 mM sodium NADP, 0.05 μg of enzyme protein, the indicated concentrations of sodium glucose-6-P, and either no (○), 10 mM (△), 30 mM (■), 50 mM (●), 75 mM (▲), or 100 mM (□) sodium chloride. (M of sodium chloride = μ.) v is expressed as in Figure 2. K_m^1 (for high glucose-6-P concentrations) = 8.0 × 10^{-5} M and K_m^2 (for low glucose-6-P concentration range) = 3.4 × 10^{-5} M.

Glucose Dehydrogenase. Glucose dehydrogenase activity previously has been observed with glucose-6-P dehydrogenase preparations from yeast (Colowick and Goldberg, 1963; Salas *et al.*, 1965; Kuby and Noltmann, 1966), from mouse and human liver microsomal fraction (Beutler and Morrison, 1967), and from human red blood cells (Kissin and Beutler, 1968). Included in these multifunctional preparations was a four-times-recrystallized enzyme from brewer's yeast (Kuby and Noltmann, 1966). We (Anderson and Nordlie, 1968) recently have demonstrated that glucose dehydrogenase and glucose-6-P dehydrogenase activities of commercial (Boehringer) preparations of the yeast enzyme are identically susceptible to partial thermal inactivation at a variety of temperatures, and also are extremely similar in their patterns of response to alterations in ionic strength of assay mixtures.

The presence of both dehydrogenase activities was noted by us with all of a number of commercial enzyme preparations tested (Boehringer lots 6437591, 6018592, 6067475, and 6466473). The differential effects of divalent cations on the two activities, which are described in this and an earlier paper (Anderson and Nordlie, 1968), also were observed with all of these preparations.

To further substantiate the involvement of a single enzyme in the catalysis of both reactions, a number of additional studies were carried out. The results of disc electrophoresis studies on acrylamide gel are described in Figure 1. Glucose dehydrogenase activity, glucose-6-P dehydrogenase activity, and protein were found to migrate as a single band.

The results of kinetic studies of inhibition of both enzymic activities by *p*-mercuribenzoate are described in Figure 2A,B. Inhibitions, observed with both activities, were noncompetitive with respect to NADP as indicated by the convergence of extrapolations of the experimental plots on the x axis in both Figure 2A,B. K_i values calculated with both activities were in excellent agreement (see legend to Figure 2). (Inhibitions were found in supplementary studies also to be noncompetitive with respect to glucose and glucose-6-P.) In addition, it is apparent from the studies described in Figure 2A,B, that K_m values for NADP serving as substrate in both dehydrogenase reactions (2.1 × 10^{-5} M in the glucose dehydrogenase reaction and 1.9 × 10^{-5} M in the glucose-6-P dehydrogenase reaction) were in excellent agreement.

In other, additional studies not otherwise described, support for the common identity of the two activities also was obtained. ATP (and other nucleotides), which previously has been reported to inhibit glucose-6-P dehydrogenase activity (Passonneau *et al.*, 1966; Avigad, 1966), was found to inhibit both dehydrogenase activities competitively with respect to glucose and glucose-6-P. At pH 7.5 and μ = 0.100, K_i values with

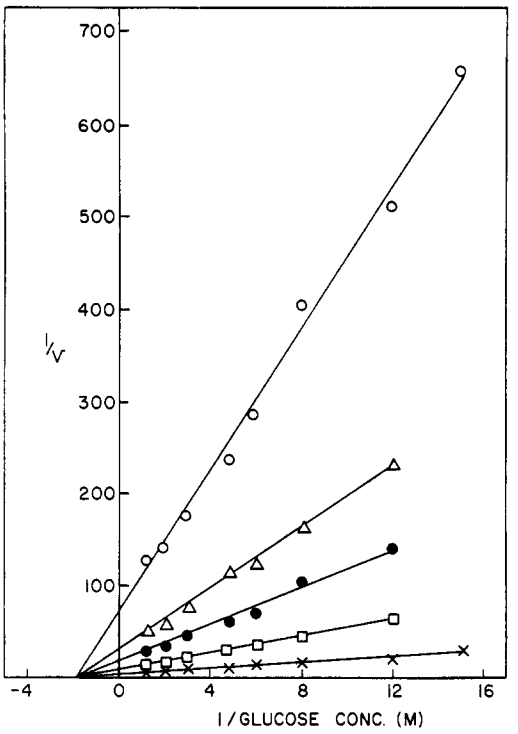

FIGURE 5: Effects of phosphate, sulfate, and bicarbonate on the kinetics of glucose dehydrogenase activity studied with respect to various glucose concentrations. Assay mixtures (pH 8.3) contained, in 3.0 ml, 8.3 mM Tris-Cl buffer, 0.1 mM sodium NADP, 5.0 μg of enzyme protein, the indicated concentrations of equilibrated D-glucose, and either 50 mM sodium chloride (○), 10 mM sodium phosphate (△), 10 mM sodium sulfate (●), 10 mM sodium bicarbonate (■), or 50 mM sodium bicarbonate (X). All mixtures were adjusted to an ionic strength of 0.05. v is expressed as in Figure 2. $K_{glucose}$ = 0.54 M in all cases.

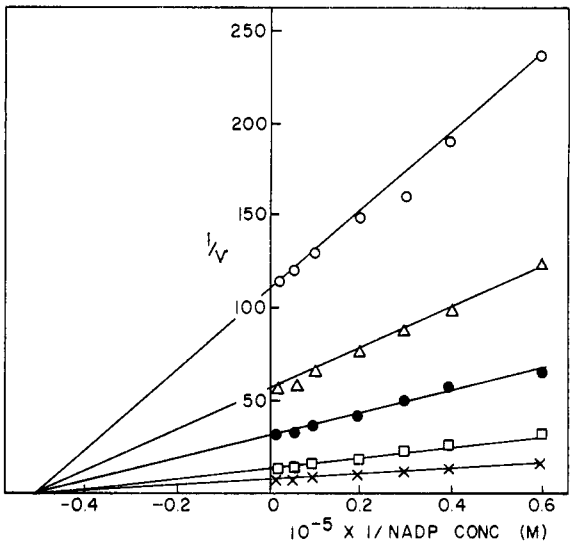

FIGURE 6: Effects of phosphate, sulfate, and bicarbonate on the kinetics of glucose dehydrogenase activity studied with respect to varied NADP concentrations. Assay mixtures (pH 8.3) contained, in 3.0 ml, 8.3 mM Tris-Cl buffer, 0.5 M equilibrated D-glucose, 5.0 μg of enzyme protein, the indicated concentrations of sodium NADP, and either 50 mM sodium chloride (○), 10 mM sodium phosphate (△), 10 mM sodium sulfate (●), 10 mM sodium bicarbonate (■), or 50 mM sodium bicarbonate (X). All mixtures were adjusted to an ionic strength of 0.05. v is expressed as in Figure 2. $K_{NADP} = 2.0 \times 10^{-5}$ M in all cases.

glucose and glucose-6-P as substrates were, respectively, 6 and 8 mM.

At pH 8.0, in the presence of 0.1 mM sodium NADP and either 0.1 mM sodium glucose-6-P or 0.5 M equilibrated D-glucose, inhibitions of glucose-6-P and glucose dehydrogenase activities, respectively, by long-chain fatty acyl-CoA compounds (see Taketa and Pogell, 1966) were observed as follows: 4.1 μM palmitoyl-CoA, 63 and 74%; 41 μM palmityl-CoA, 95 and 94%; and 4.1 μM stearoyl-Co A, 92 and 87%. Under these same basic assay conditions, both dehydrogenase activities were stimulated by Mg^{2+}, Ca^{2+}, and Ba^{2+}, and were inhibited by Co^{2+}.

It is concluded on the basis of this body of supportive evidence that glucose dehydrogenase and glucose-6-P dehydrogenase activities observed are due to a common enzyme.

Effects of Varied Ionic Strength on Reaction Kinetics. Both glucose dehydrogenase and glucose-6-P dehydrogenase activities of this enzyme were shown in previous studies (Anderson and Nordlie, 1968) to be quite sensitive to variations in ionic strength. These variations in levels of activities with altered ionic strengths also are quite apparent in the experiments described in Figures 3 and 4. Maximal activity (note y-axis intercept values) increased progressively as the concentration of supplementary sodium chloride (which for this salt is equal to ionic strength) was increased from 0 to 100 mM; however, no change was noted in the values for either $K_{glucose}$ (Figure 3) or $K_{glucose\text{-}6\text{-}P}$ (Figure 4). Similarly, no effects on K_{NADP} values were noted in supplementary experiments in which NADP concentrations were varied.

Of particular interest is the bimodal nature of the double-reciprocal plots noted at pH 8.0 in the experiments in which glucose-6-P concentration was varied (Figure 4). As indicated by the figure, variations in ionic strength did not influence either the biphasic nature of the plots or the two K_m values for glucose-6-P (designated as K_{m1} for the high-substrate activity and K_{m2} for the low-substrate activity). Bimodal plots of this sort, which previously have been observed by other workers (Rutter, 1957; Pinto *et al.*, 1966), were not obtained at 7.3, but became pronounced as pH was increased above this value. The effect was not observed at any pH when glucose, rather than its phosphate ester, served as substrate (see, for example, Figure 3).

Some discrepancies exist in the literature concerning the type of inhibition exerted by phosphate toward glucose-6-P dehydrogenase. Rutter (1957) and Passonneau *et al.* (1966) have reported inhibition to be competitive with respect to glucose-6-P, while Glaser and Brown (1955) have described this anion as inhibiting competitively relative to NADP. Differences between the results presented in this paper and those reported by the latter workers may be attributable to differences in assay conditions employed. The studies of Glaser and Brown (1955) were completed with a partially purified

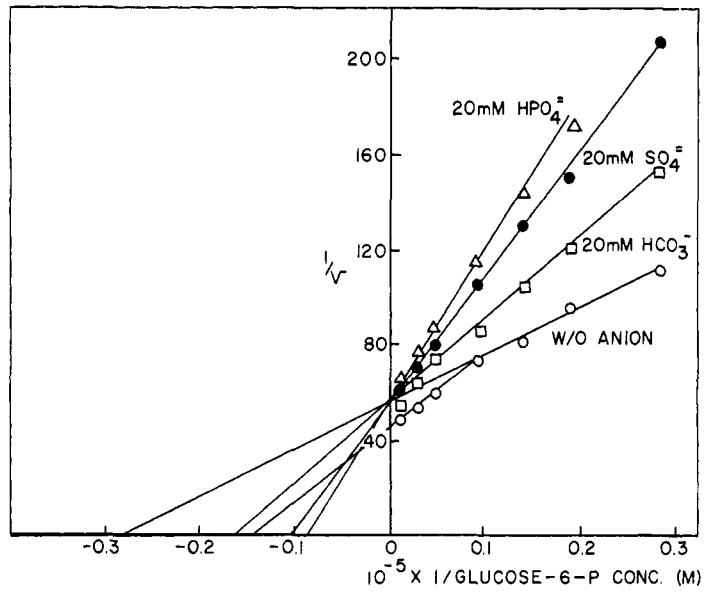

FIGURE 7: Kinetics of inhibition of glucose-6-P dehydrogenase activity by sulfate, phosphate, and bicarbonate, studied at pH 8.1 with respect to varied concentrations of glucose-6-P. Assay mixtures contained, in 3.0 ml, 8.3 mM Tris-Cl buffer, 0.1 mM sodium NADP, 0.05 μg of enzyme protein, the indicated concentrations of sodium glucose-6-P, and either 20 mM sodium phosphate (△), 20 mM sodium sulfate (●), 20 mM sodium bicarbonate (□), or 60 mM sodium chloride (○). Mixtures were adjusted to an ionic strength of 0.06. v is expressed as in Figure 2. K_i values for bicarbonate, sulfate, and phosphate were, respectively, 26.6, 12.0, and 9.0 mM.

enzyme preparation, and very high phosphate concentrations (150 and 217 mM) were employed with no allowance made for drastic variations in ionic strength inherent in their studies.

Effects of Various Anions on Reaction Kinetics. The studies to be described were carried out both at pH 7.3 (at which maximal inhibition of glucose-6-P dehydrogenase activity by phosphate or sulfate previously was noted (Anderson and Nordlie, 1968)) and at approximately pH 8.2 (where maximal stimulation of glucose dehydrogenase by bicarbonate was observed (Anderson and Nordlie, 1968)). With one exception data presented were obtained at the latter pH. However, similar patterns of results also were noted in all cases at the lower pH.

Data obtained with the glucose dehydrogenase system are described in Figures 5 and 6. Although the addition of 10 mM sodium phosphate, 10 mM sodium sulfate, or 10 or 50 mM sodium bicarbonate in all instances increased V_{max} values in experiments in which either the concentration of glucose (Figure 5) or NADP was varied (Figure 6), no alteration in the K_m values for either glucose or NADP was noted in any instance (note that the x-axis intercepts, which are equal to $-1/K_m$ values, remained unchanged in both Figures 5 and 6).

The effects of these same anions on kinetics of the glucose-6-P dehydrogenase reaction at pH 8.3 and 7.3 are described in Figures 7 and 8, respectively. Inhibition, which is perhaps more clearly defined at pH 7.3 in the absence of bimodal effects (Figure 8), was of the classical competitive type, as indicated by the common point of intersection of all plots on the y axis. As indicated by the data in Figure 7, the bimodal phenomenon which was observed at pH 8.3 in the absence of added anions, was absent when bicarbonate, phosphate, or sulfate was present. Inhibition by these ions was competitive with respect to glucose-6-P in the lower range (0.035–0.106 mM) and was mixed with respect to higher concentrations of the sugar phosphate substrate. K_i values, calculated in terms of competitive inhibition, are indicated in the legends to Figures 7 and 8. In supplementary experiments, inhibitions of glucose-6-P dehydrogenase activity considered with respect to NADP substrate were found to be of the classical noncompetitive type, and consequently did not involve any alterations in K_{NADP} value.

Evidence for a Common Site of Anion Action. The following studies were carried out to gain insight regarding the possible modes of interaction of bicarbonate, phosphate, and sulfate in (a) activating glucose dehydrogenase activity and (b) inhibiting glucose-6-P dehydrogenase activity. Sulfate and phosphate were found in earlier studies (Anderson and Nordlie, 1968) to be much less effective activators of glucose dehydrogenase activity than was bicarbonate. Studies in which activation

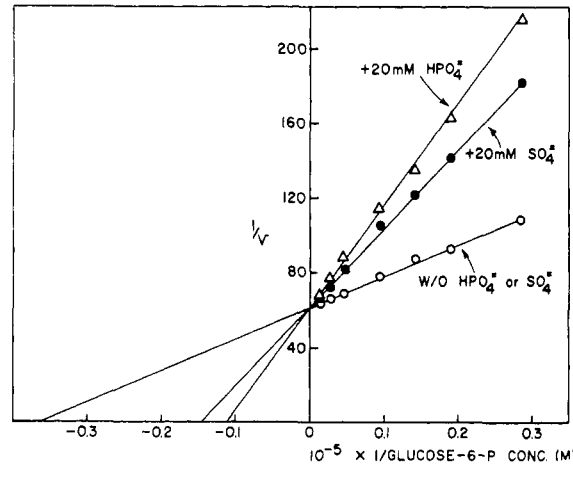

FIGURE 8: Kinetics of inhibition of glucose-6-P dehydrogenase activity by sulfate and phosphate, studied at pH 7.3 with respect to varied concentrations of glucose-6-P. With the exception of the different pH, assay mixture compositions and other details are as in Figure 7, with either 20 mM sodium phosphate (△), 20 mM sodium sulfate (●), or 60 mM sodium chloride (○) included. K_i values for sulfate and phosphate, respectively, were 10.6 and 7.2 mM.

TABLE I: Effects of Simultaneous Addition of Anionic Inhibitors on Glucose-6-P Dehydrogenase Activity.[a]

Inhibitor (30 mM)			Experimental		Theor Inhibn	
Phosphate	Sulfate	Bicarbonate	Act.[b]	Inhibn (%)	(%)	K_i (mM)
I	II	III	IV	V	VI	VII
			2.14	0.0	0.0	
+			1.22	42.9		10.0
	+		1.40	34.3		14.8
		+	1.66	22.5		25.2
+	+		0.93	56.4	56.2	
+		+	0.99	53.5	51.6	
	+	+	1.12	44.0	45.0	
+	+	+	0.81	62.1	61.3	

[a] Assay mixtures (pH 8.0) contained, in 3.0 ml, 33.2 mM Tris-Cl buffer, 0.1 mM sodium NADP, 0.1 mM sodium glucose-6-P, 0.05 μg of enzyme protein, and the indicated concentrations of anionic inhibitors ("+" indicates that designated anion was present). Ionic strengths of all assay mixtures were adjusted to 0.200 by the addition of supplementary sodium chloride. [b] Activity is expressed in terms of 100 × μmoles of NADP produced per 2.5 min.

of glucose dehydrogenase by various concentrations of bicarbonate was studied in the absence and presence of 10 mM phosphate or sulfate are described in Figure 9. Activation by bicarbonate in the absence of other anionic effectors followed Michaelis–Menten kinetics as indicated by the linearity of the double-reciprocal plot obtained. Both phosphate and sulfate competitively inhibited this activation by bicarbonate concentrations >10 mM (data to left of vertical arrow in Figure 9). However, at lower concentrations of bicarbonate, the plots of data observed in the presence of the supplemental phosphate or sulfate deviated from linearity in a manner suggesting that, in the presence of relatively low concentrations of bicarbonate, these other anions also manifested their potential to activate (although not as effectively as bicarbonate). These observations are consistent with the idea that all three of the tested anions activate glucose dehydrogenase through interactions with a common site on the enzyme. Further, the K_i values calculated for phosphate and sulfate functioning as competitive inhibitors of bicarbonate-effected activation of *glucose dehydrogenase*, 9.8 and 14 mM, respectively, agree well with K_i values for these same anions acting as competitive inhibitors of *glucose-6-P dehydrogenase* activity of the enzyme (see Figures 7 and 9).

Studies indicating that anion inhibition of glucose-6-P dehydrogenase also involves the interaction of bicarbonate, sulfate, and phosphate with a common enzymic site are presented in Table I. In these experiments, inhibitions due to these three anions, included singly and in various combinations in reaction mixtures, were measured under conditions of constant ionic strength (μ = 0.200). K_i values for each anion under these experimental conditions were calculated from activity values (column IV), obtained in the absence and presence of each individual anion, with the use of eq 1 and 2. V_{max} was first evaluated with the aid of eq 1 and the activity value, v, observed in the absence of inhibitor. Inhibitor constant values for the various anions then

$$v = \frac{V_{max}(S)}{K_m + (S)} \quad (1)$$

$$v_i = \frac{V_{max}(S)}{K_m\left(1 + \frac{(I)}{K_i}\right) + (S)} \quad (2)$$

were calculated by substituting the V_{max} value above and activity values, v_i, observed in the presence of the individual inhibitors (column IV, Table I) into eq 2 and solving for K_i (eq 2 conveniently was rearranged in the form indicated in eq 2a). K_i values thus obtained (see column VII in Table I) were in good agreement with corresponding values obtained in more elaborate kinetic studies which were carried out at μ = 0.060 (see Figure 7).

$$K_i = \frac{K_m(I)}{(S)\left(\frac{V_{max}}{V_i} - 1\right) - K_m} \quad (2a)$$

These K_i values and eq 3 (Webb, 1963) were employed to calculate theoretical inhibitions for combinations of two and three of the anions included together in assay mixtures. Equation 3 (Webb, 1963) was derived on the basis of the assumption that the several inhibitors compete for a common binding site on the enzyme. The excellent agreement obtained between experimental and theoretical values (compare values in column V with those in VI, Table I) strongly supports a mechanism in which phosphate, sulfate, and bicarbonate ions all inhibit glucose-6-P dehydrogenase activity by binding competitively to a common inhibitory site on the enzyme, and is inconsistent with "cumulative" and "additive" types of inhibition (see Woolfolk and Stadtman, 1967).

$$i = \frac{\frac{(I_1)}{K_i^1} + \frac{(I_2)}{K_i^2} + \ldots}{1 + \frac{(S)}{K_m} + \frac{(I_1)}{K_i^1} + \frac{(I_2)}{K_i^2} + \ldots} \quad (3)$$

where i = fractional inhibition in the presence of two (or more) competitive inhibitors acting at a common enzyme site; (I_1), (I_2), etc., indicate molar concentrations of individual inhibitors; K_i^1, K_i^2, etc., are inhibitor constants for I_1, I_2, etc.; (S) is substrate concentration; and K_m is Michaelis constant for the substrate S toward which competitive inhibition by compounds I_1, I_2, etc., is manifest.

Discussion

The bimodal response noted for yeast glucose-6-P dehydrogenase at pH 8.0 previously has been observed with the yeast (Rutter, 1957) and erythrocyte (Pinto *et al.*, 1966) enzymes, and a number of alternative explanations for this phenomenon have been advanced. In the following discussion, inhibition of glucose-6-P dehydrogenase by various anions at pH 8.1 (Figure 7) is considered with respect to glucose-6-P in the lower concentration range (0.035–0.106 mM) since (a) the bimodal effect was not noted in studies at a lower pH (pH 7.3; see Figure 8 and text), and (b) mechanistic interpretation of the phenomenon under consideration in this paper, the differential effects of various anions on activities of the yeast enzyme, is most clearly defined on such a basis.

Any mechanistic consideration of the described differential effects of anions on yeast glucose dehydrogenase and glucose-6-P dehydrogenase activities must take into account the following observations. (1) A single enzyme is involved (see text). (2) The affinity of enzyme for NADP in both glucose-6-P dehydrogenase and glucose dehydrogenase reactions is the same (see Figure 2A,B). (3) The studied anions inhibit glucose-6-P dehydrogenase competitively with respect to sugar–phosphate substrate (Figures 7 and 8) while having no effect on the K_m value for NADP. (4) Activation of glucose dehydrogenase by these same anions does not involve alterations in K_m values for either glucose or NADP substrates (see Figures 5 and 6). (5) Inhibition of glucose-6-P dehydrogenase activity appears to involve the binding of any of the three anions at a common inhibitor site (see Table I). (6) Phosphate, sulfate, and bicarbonate all appear to compete for a common site for the activation of glucose dehydrogenase (Figure 9). (7) A common site for anions functioning both as activators of glucose dehydrogenase and as inhibitors of glucose-6-P dehydrogenase is indicated by the excellent agreement of K_i values for phosphate and sulfate acting both as direct competitive inhibitors of the latter activity (see Figure 6) and as competitive inhibitors of bicarbonate-effected activation of the former activity (Figure 9).

A hypothetical mechanism incorporating the above experimental observations is presented in highly schematic form in Figure 10. This scheme incorporates the

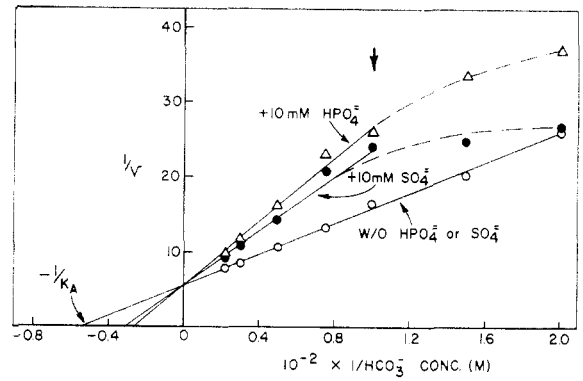

FIGURE 9: Kinetics of the phosphate and sulfate inhibition of bicarbonate-effected activation of glucose dehydrogenase. Assay mixtures (pH 8.1), contained, in 3.0 ml, 8.3 mM Tris-Cl buffer, 0.1 mM sodium NADP, 0.5 M equilibrated D-glucose, 5.0 μg of enzyme protein, the indicated concentrations of sodium bicarbonate, and either 10 mM sodium phosphate (△), 10 mM sodium sulfate (●), or 30 mM sodium chloride (○) (μ = 0.03). Control reaction mixtures contained sodium chloride of ionic strengths comparable with those of the varied sodium bicarbonate concentrations. Velocities, v, of the bicarbonate-stimulated activity are expressed as (μmoles of NADPH formed/2.5 min in the presence of bicarbonate plus anionic effector) − (μmoles of NADPH formed/2.5 min in the presence of sodium chloride of corresponding ionic strength), in a manner analogous with that employed by Vardanis (1967) for the expression of data for the glycogen synthetase system. K_A for bicarbonate (see figure) was calculated to be 19 mM. K_i values for phosphate (9.8 mM) and sulfate (14 mM) were evaluated by the general method described by Dixon and Webb (1964b) for classical competitive inhibitors.

view of Koshland (1963) that an enzyme's active site is flexible, and that the substrate is able to induce a change in the shape of this active site resulting in a catalytically active fit of enzyme and substrates.

The free enzyme, with binding sites A, B, and C for glucose-6-P, is depicted in I. NADP is postulated to bind initially[3] to this enzyme to produce the binary complex II. As shown in III, glucose-6-P may then bind to this enzyme–NADP complex in the absence of anion, and induce a rearrangement of the active site such that NADP and the hemiacetal of the sugar–phosphate substrate would be spatially oriented properly so that reaction may ensue. Glucose by itself would be unable to produce such a "flexation" in the active site, as indicated in IV, However, the addition of anion, binding to hypothetical sites B and C, could transpose the enzyme–NADP complex into the "flexed" state indicated by V. According to this mechanism, the anion would compete with glucose-6-P for binding sites B and C as indicated by VI in the diagram. Glucose dehydrogenase, on the

[3] An ordered sequence of enzyme–substrate binding appears to apply with glucose-6-P dehydrogenase (Soldin and Balinsky, 1968) as well as with a variety of other dehydrogenase systems (see Mahler and Cordes, 1966). For these reasons, and for the sake of simplicity of presentation, NADP is assumed in Figure 10 to bind to the free enzyme prior to the binding of sugar (phosphate) substrates. The mechanism of anion action depicted would also hold if NADP were to bind either randomly or after the attachment of glucose or glucose-6-P.

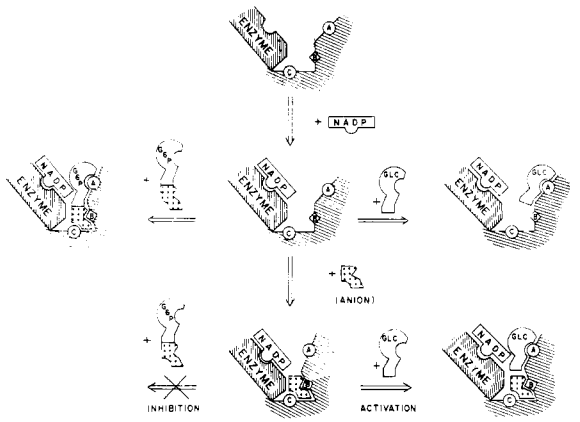

FIGURE 10: Possible mechanism of action of anions in selectively controlling glucose dehydrogenase and glucose-6-P dehydrogenase activities of the yeast enzyme.[3] This highly schematic diagram incorporates Koshland's (1963) induced-fit concept. Details are presented in the Disscussion.

other hand, would be catalytically enhanced by bound anion, as indicated in VII. While the affinity, K_m, of glucose for the enzyme would not be altered by bound anions, the glucose molecule, when bound to this "flexed" enzyme, would now be in a favored position with respect to NADP (VII), and the dehydrogenase reaction would procede (alternatively, the production of VII could also involve the binding of anion to the glucose–enzyme–NADP complex (IV)).

The relative effectiveness of the various anions to activate, or inhibit, may also be explained in terms of the scheme depicted in Figure 10. The size of each anion would be of importance in determining its ability to activate glucose dehydrogenase. Not only would the extent of "flexing" of the native enzyme molecule be influenced by the size of the activating anion, but also the presence of the additional oxygen atom on phosphate and sulfate (compared with bicarbonate) might interfere to some degree with the hydroxyl group on the number six carbon atom of the glucose molecule. Thus, even though phosphate and sulfate have a greater affinity for the enzyme than does bicarbonate (compare K_i values for the former two anions with K_A value for bicarbonate, recorded in Figure 9), the latter, smaller ion is the most effective activator of glucose dehydrogenase.

The degree of inhibition exerted by a particular anion on the glucose-6-P dehydrogenase activity, however, would be related directly to the affinity of the enzyme for the effector ion. This affinity in turn would be a consequence of anion size, shape, and charge suitable for binding to hypothetical sites B and C. Since glucose-6-P is definitely the preferred substrate for this enzyme, it would be predicted that phosphate would be the most potent of the tested inhibitory ions, followed by sulfate and then bicarbonate. This predicted order of effectiveness of inhibitors is in agreement with the experimental results obtained.

Although kinetic studies by themselves cannot unequivocally establish the mechanism of an enzymic reaction, the scheme for anion action presented in Figure 10 does appear to explain nicely the rather larger number of individual experimental observations described in this paper, and it presently is being used as the basis for the formulation of further studies on the differential control of activities of this multifunctional enzyme by a variety of other factors.

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