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ACTIVATION BY BICARBONATE, ORTHOPHOSPHATE, AND SULFATE OF RAT LIVER MICROSOMAL GLUCOSE DEHYDROGENASE

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SUMMARY

1. The effects of the anions bicarbonate, orthophosphate, and sulfate on rat liver microsomal glucose dehydrogenase (β-D-glucose:NAD(P)$^+$ oxidoreductase, EC 1.1.1.47) have been studied in detail.

2. Significant activations of glucose dehydrogenase activity were produced by all these anions in the range from 2.5 to 50 mM. 30 mM sulfate, orthophosphate, and bicarbonate, respectively, effected increases in activity levels to approximately 550, 400, and 300% of control values.

3. Activations were noted at all of a variety of H^+ concentrations between pH 7 and 10. The pH optimum, 9.2 in the absence of effector, was shifted slightly towards neutrality in the presence of increasing concentrations of the various anions.

4. Kinetic studies revealed that K_m values for glucose and for nucleotide substrate were independent of second substrate concentrations. The various anions all effected increases in the v_{max} value and decreases in K_m values for both glucose and NAD.

5. Relatively small but significant inhibitions of glucose 6-*P* dehydrogenase activity also catalyzed by this multifunctional enzyme were produced by the various anions, while ATP was a potent inhibitor.

6. It is suggested on the basis of similarities of the effects noted with the two multifunctional enzymes that the mechanism of anion action previously proposed by HORNE *et al.*[3] (*Biochemistry*, 9 (1970) 610) for yeast glucose-6-*P* dehydrogenase also obtains, with a slight modification, for the mammalian microsomal glucose dehydrogenase.

7. Although the high K_m value for glucose (0.33–1.25 M) may contraindicate a physiological role for glucose dehydrogenase activity, the observation that in the presence of physiological levels of anionic activators glucose dehydrogenase activity with 0.5 M D-glucose may approach or exceed the level of total hepatic cellular glucose-6-*P* dehydrogenase activity (microsomal plus cytosolic) raises the possibility that, as previously has been suggested by METZGER *et al.*[38] a specialized, compartmentalized biological role for this hepatic activity is possible.

INTRODUCTION

Recently, ANDERSON AND NORDLIE[1], and ANDERSON *et al.*[2] described the marked activation of glucose dehydrogenase (β-D-glucose:NAD(P)$^+$ oxidoreductase, EC 1.1.1.47) activity of yeast glucose-6-*P* dehydrogenase (D-glucose 6-phosphate: NADP$^+$ oxidoreductase, EC 1.1.1.49) by the anions bicarbonate, sulfate, and orthophosphate. They[1,3–5] suggested that inhibition of glucose-6-*P* dehydrogenase by these same ions[1,2], especially bicarbonate (and by various nucleoside 5′-triphosphates and 5′-diphosphates as well)[3], may lead to the diversion of an increased proportion of cellular glucose-6-*P* to glycogen synthesis (see refs. 6 and 7), and that concomitant activation of glucose dehydrogenase activity of the enzyme by the smaller anions may provide a mechanism for continued generation of NADPH even in the presence of diminished glucose-6-*P* oxidation.

Recognizing the speculative nature of the suggested physiological significance of their observations with the yeast enzyme, ANDERSON AND NORDLIE[1] suggested that further investigation of the effects of these anions on glucose dehydrogenase and glucose-6-*P* dehydrogenase activities from additional sources should be conducted.

Such studies now have been carried out with an enzyme from rat liver microsomes[8,9] which has been demonstrated to be a single multifunctional catalyst capable of carrying out the oxidation of both unphosphorylated glucose and of glucose-6-*P* and other hexose phosphate esters[9]. Results of these studies, which indicate significant stimulation by sulfate, orthophosphate, and bicarbonate of glucose dehydrogenase activity, with either NADP$^+$ or NAD$^+$ as electron acceptor, are described in this paper.

MATERIALS AND METHODS

Livers used in these studies were from young adult, male albino rats (150 to 250 g) obtained from Sprague–Dawley, Inc., Madison, Wisc., which were maintained on Purina Laboratory Chow and tap water, *ad libitum*, until killed. Glucose and the sodium salts of glucose-6-*P*, NADP$^+$, and Tris buffer were obtained from Sigma Chemical Co., St. Louis, Mo. Sources of other chemicals ("Analytical Reagent" grade) were as follows: Na_2HPO_4 and $NaHCO_3$, Mallinckrodt Chemical Works, New York, N.Y.; Na_2SO_4, J. T. Baker Chemical Company, Phillipsburgh, N.J.; NaCl, Fisher Scientific Company, Fair Lawn, N.J. All reagents were prepared in distilled water which had been deionized by passage through an Amberlite IR-2 ion exchange resin.

Solutions of reagents were adjusted to the desired pH with dilute HCl or NaOH solution. Glucose solutions were allowed to stand and equilibrate between α and β forms before use as substrates. Glucose-6-*P*, NAD$^+$, and NADP$^+$ were assayed spectrophotometrically (see below) utilizing glucose-6-*P* dehydrogenase and an excess of the second substrate. Protein was determined by a modification of the biuret method, as described previously[10].

Rat liver microsomal glucose dehydrogenase ("hexose-6-*P* dehydrogenase") was prepared after the method of BEUTLER AND MORRISON[9]. After the rats were weighed and decapitated, their livers were removed and placed in a pre-weighed beaker containing an ice-cold solution of 0.1 M Na_2HPO_4, 1 mM EDTA, and 0.25 M sucrose.

The beaker was re-weighed and the weight of the liver calculated by difference. The livers and solution were transferred to a Potter–Elvehjem homogenizer and ground at high speed (600 rev./min) for 2 min with the homogenizer immersed in ice water during the procedure. The suspension was diluted with a 0.1 M Na_2HPO_4, 1 mM EDTA, and 0.25 M sucrose solution so that 1 g of tissue was contained in 5 ml of homogenate.

Mitochondria, nuclei, unruptured cells, and debris were separated from the homogenate by centrifugation at 3500 rev./min for 10 min (18 000 × *g*) in the International Model PR-2 centrifuge equipped with a high-speed attachment. The supernatant fraction was saved and combined with a wash from the precipitated fraction. A microsomal fraction was isolated from the above supernatant solution by centrifugation in the Number 30 head of the Spinco Model L preparative ultracentrifuge for 60 min at 27 000 rev./min (65 000 × *g*). The microsomes thus obtained were washed 3 times by resuspending them in an ice-cold solution containing 0.1 M Na_2HPO_4, 1 mM EDTA, and 0.25 M sucrose, and sedimenting them in the centrifuge as before. After the last wash the microsomes were suspended in a solution, pH 7.4, containing 0.1 M K_2HPO_4 and 0.5% (w/v) sodium cholate, and frozen at −20°.

After 12 h, such preparations were thawed at 0° and then centrifuged for 60 min at 27 000 rev./min (65 000 × *g*). The supernatant fraction resulting was then dialyzed against 0.25 M sucrose for 4 h and frozen at −20° in small quantities for later use. Immediately before use, such enzyme preparations were dialyzed at 4° for 6 h against 4 changes (500 ml each) of deionized water. The preparation was devoid of gluconate dehydrogenase activity and did not catalyze the oxidation of NADH or NADPH in the absence of other substrate.

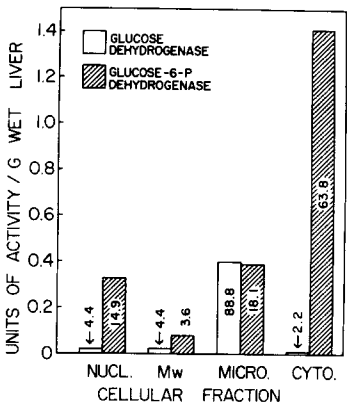
Dehydrogenase activities were assayed by modifying the methods of KORNBERG AND HORECKER[11] and STRECKER[12]. An increase in absorbance with a maximum at 340 nm accompanies the reduction of NAD^+ to NADH or of $NADP^+$ to NADPH[13]. The activities were measured spectrophotometrically by following the initial rate of change in absorbance at 340 nm with a Beckman Model DU spectrophotometer adapted for scale expansion (Gilford attachment) and continuous recording (Brown recorder). The temperature was maintained at 30 ± 0.1° by circulating water from a constant-temperature bath through thermospacers incorporated into the cuvette compartment of the spectrophotometer. Reaction mixtures were pre-incubated at 30° for 5 min prior to initiation of reaction by the addition of the enzyme. A reference molar absorbance index of $6.22 \cdot 10^3$ $M^{-1} \cdot cm^{-1}$ was employed in converting $A_{340\ nm}$ readings into molar concentrations[14].

Ionic strength was maintained constant at 0.20 in each series of experiments by the addition of appropriate supplemental amounts of NaCl to assay mixtures. Sodium salts of all substrates and effectors were used to avoid any possible selective effects due to cations. Additional details of individual experiments are given in the legends to the figures and tables.

RESULTS

Subcellular location of rat liver glucose dehydrogenase and glucose-6-P dehydrogenase activities

The subcellular location of glucose dehydrogenase activity in the rat liver cell



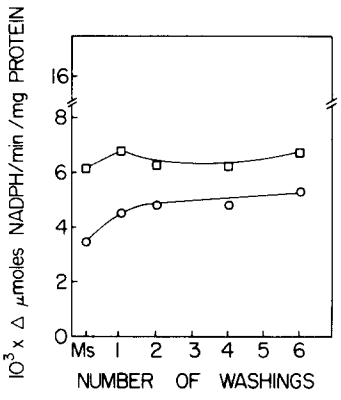


Fig. 1. Subcellular distribution of rat hepatic glucose dehydrogenase and glucose-6-*P* dehydrogenase activities. Livers were homogenized, and various subcellular fractions isolated by differential centrifugation as described for the preparation of microsomal glucose dehydrogenase in MATERIALS AND METHODS, except that nuclei *plus* cellular debris fraction was isolated by an initial centrifugation for 10 min at 640 rev./min (600 × *g*). Assay mixtures, pH 8.0, contained, in 3.0 ml, 100 mM Tris–HCl buffer, and either 0.5 M glucose and 0.1 mM NAD^+ (glucose dehydrogenase) or 0.017 mM glucose-6-*P* and 0.017 mM $NADP^+$ (glucose-6-*P* dehydrogenase). Nuclear (*plus* cellular debris), mitochondrial, microsomal, and cytosolic fractions are indicated by NUCL., Mw, MICRO., and CYTO., respectively. Numbers within the bars indicate the proportion of total liver activity present in the designated fraction.

Fig. 2. Effects of repeated washings of rat liver microsomes on specific activities of glucose dehydrogenase (○) and glucose-6-*P* dehydrogenase (□). The microsomes were isolated as described in the text, and were washed the indicated number of times by resuspending them in a solution containing 0.1 M Na_2HPO_4, 1 mM EDTA, and 0.25 M sucrose and then sedimenting them by centrifugation for 60 min at 65 000 × *g*. After the last wash, the microsomes in each case were suspended in a solution containing 0.5% (w/v) sodium cholate and 0.1 M K_2HPO_4 at pH 7.4, and were then frozen at −20°, thawed at 0°, and assayed. Assay mixtures, pH 8.0, contained, in 3.0 ml, 100 mM Tris–HCl buffer, 0.17 mM sodium $NADP^+$, and either 0.5 M glucose (○) or 0.33 mM sodium glucose-6-*P* (□).

has been the subject of some controversy. ZAHEER *et al.*[16] reported the enzyme to be mitochondrial while METZGER *et al.*[8] and BEUTLER AND MORRISON[9] report the enzyme to be associated with the microsomal fraction from the cell. The results of studies of the distribution of NAD-linked glucose dehydrogenase activity in rat liver subcellular fractions separated by conventional methods of differential centrifugation, as described in the MATERIALS AND METHODS section, above, are presented in Fig. 1. It is evident that the activity is located principally in the microsomal fraction. Neither glucose dehydrogenase activity (Fig. 1) nor accompanying glucose-6-*P* dehydrogenase activity of the enzyme (see refs. 8 and 9) could be removed from microsomal preparations by repeated (1 to 6 times) washings with the original suspending medium (see Fig. 2).

Activation of glucose dehydrogenase activity by anions

The activating effects of sulfate, phosphate, and bicarbonate on liver microsomal glucose dehydrogenase activity, with either NAD or NADP as electron acceptor, was clearly demonstrated by experiments described in Table I. Activity was measured in the absence of anion and in the presence of 30 mM concentrations of each. Sulfate produced the greatest stimulation followed by phosphate and then bicarbonate.

TABLE I

ACTIVATION BY BICARBONATE, PHOSPHATE, AND SULFATE OF RAT LIVER MICROSOMAL GLUCOSE DEHYDROGENASE ACTIVITY

Assay mixtures, pH 8.0, contained, in 3.0 ml, 100 mM Tris–HCl buffer, 0.5 M glucose, either 0.1 mM NAD^+ and 1.12 mg enzyme protein or 0.1 mM $NADP^+$ and 0.44 mg enzyme protein as indicated and sodium salts of various anions as specified. $I = 0.20$ in all cases. Observed activity is expressed as μmoles of NADH or NADPH formed per 3 ml per 6 min. Relative activity = 100 × (activity observed in the presence of anion/activity observed without added anion).

Added anion	*Conc. (mM)*	*Activity with NAD⁺*		*Activity with NADP⁺*	
		Observed	*Relative (%)*	*Observed*	*Relative (%)*
None	—	0.040	100	0.013	100
HCO_3^-	30	0.091	228	0.039	300
HPO_4^{2-}	30	0.139	348	0.056	431
SO_4^{2-}	30	0.165	412	0.072	554

This order differs somewhat from that obtained in studies with the yeast enzyme with which bicarbonate activated most extensively followed by sulfate and then phosphate[1,2].

The possibility that increases in $A_{340\ nm}$ which routinely were observed with complete assay mixtures with added sulfate, phosphate, or bicarbonate could be due, in part, to the formation of complexes between the anion and some constituent of the assay mixture which might conceivably absorb light at this wavelength, was ruled out on the basis of experiments described in Figs. 3A and 3B. As indicated in Fig. 3A, reduction of NAD^+ or $NADP^+$ in the presence of 0.5 M glucose was a linear function of enzyme concentration, both in the absence (open symbols) and presence (closed symbols) of added anion. The data presented in Fig. 3B demonstrate that activation of glucose dehydrogenase activity, as measured by change in $A_{340\ nm}$, is

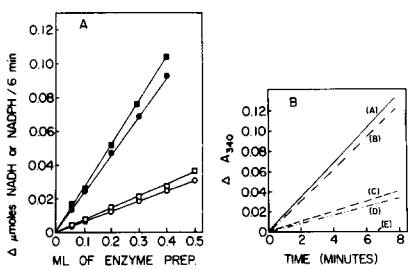


Fig. 3. A. Effect of enzyme concentration on glucose dehydrogenase activity. Assay mixtures, pH 8.0, contained, in 3.0 ml, 100 mM Tris–HCl buffer, 0.5 M glucose, the indicated aliquots of enzyme (0.22 mg protein per 0.1 ml), 0.2 mM sodium NAD^+ (squares) or 0.2 mM sodium $NADP^+$ (circles), and either no added anion (open symbols), 50 mM $NaHCO_3$ (●), or 50 mM sodium phosphate (■). B. Dependence of activation of glucose dehydrogenase by phosphate or bicarbonate on the presence of glucose, enzyme, and pyridine nucleotide. Complete assay mixtures, pH 8.0, contained, in 3.0 ml, 100 mM Tris–HCl buffer, 0.44 mg enzyme protein, 0.5 M glucose, and either (A) 50 mM sodium phosphate and 0.2 mM NAD^+, or (B) 50 mM $NaHCO_3$ and 0.2 mM $NADP^+$. When phosphate was omitted from assay mixture A or bicarbonate was omitted from assay mixture B, activity indicated by lines C and D, respectively, was obtained. When glucose, enzyme, or pyridine nucleotide was omitted from assay mixtures A or B, no activity was demonstrable (E).

dependent upon the simultaneous presence of enzyme, NAD^+ or $NADP^+$, and glucose. Activity noted with complete assay mixtures, devoid of activating anions, with NAD^+ or $NADP^+$ as electron acceptor, measured as a function of incubation time, is described by lines C and D, respectively. The maintenance of linearity with respect to time following the addition of 50 mM phosphate or 50 mM bicarbonate is apparent from an examination of lines A and B, respectively. No activity was observed when glucose, enzyme, NAD^+, or $NADP^+$ was omitted from otherwise complete assay mixtures containing activating anion (line E).

Effects of variations in anion concentrations and assay pH

The effects of increasing concentrations of sulfate, phosphate, or bicarbonate on glucose dehydrogenase activity, with either NADP or NAD as electron acceptor, are described in Figs. 4A and 4B, respectively. All three anions progressively activated glucose dehydrogenase activity in both instances. With NAD-linked glucose dehydrogenase activity (Fig. 4B), as with activity with NADP (Fig. 4A), progressive activation by all three anions—sulfate, phosphate, and bicarbonate—was observed. It may be seen by comparing Figs. 4A and 4B that the extent to which the anions activate rat liver glucose dehydrogenase activity is relatively independent of whether NAD^+ or $NADP^+$ is employed as coenzyme. The activation produced by 50 mM bicarbonate, phosphate, and sulfate was, respectively, more than 3-, 4-, and 6-fold with $NADP^+$ (Fig. 4A), compared with corresponding values of 2-, 3-, and 4-fold noted with NAD^+ (Fig. 4B).

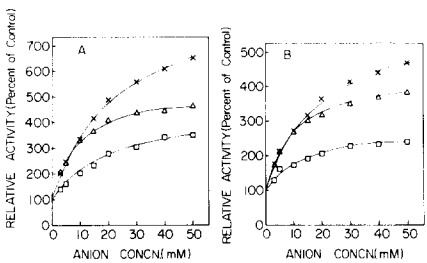


Fig. 4. Effects of varying concentrations of anions on glucose dehydrogenase activity. Assay mixtures, pH 8.0, contained, in 3.0 ml, 100 mM Tris–HCl buffer, 0.5 M glucose, the indicated concentrations of sodium salts of sulfate (×), phosphate (△), or bicarbonate (□), and (A) 0.1 mM $NADP^+$ and 0.44 mg protein or (B) 0.1 mM NAD^+ and 1.12 mg protein. $J = 0.20$ in all cases. Relative activity = 100 × (activity with anion present/activity without anion). Activity values, observed in the absence of anions, were for A and B, respectively, 0.013 and 0.040 μmole of nucleotide reduced per 6 min.

The effects of sulfate, phosphate, and bicarbonate on the pH–activity profiles of NADP-linked glucose dehydrogenase are evident from the data presented in Figs. 5A–5C. The optimum at pH 9.2 observed in the absence of added anion was progressively shifted towards a lower pH by the addition of 10 and 50 mM concentrations of each anion. For example, the optimum observed at pH 9.2 in the absence of anion was lowered to pH 9.0 with 10 mM $NaHCO_3$ and to pH 8.8 with 50 mM $NaHCO_3$, as indicated in Fig. 5A. Maximal activation by bicarbonate occurred at

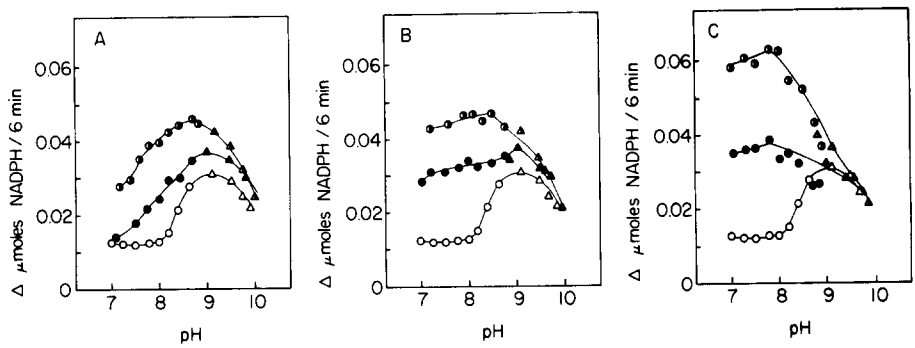


Fig. 5. Influence of varied concentrations of (A) bicarbonate, (B) phosphate, or (C) sulfate on activity–pH profiles of glucose dehydrogenase activity with $NADP^+$ as electron acceptor. Assay mixtures contained, in 3.0 ml, 50 mM Tris–HCl (circles) or 50 mM glycine (triangles) buffer, 0.44 mg enzyme protein, 0.2 mM $NADP^+$, 0.5 M glucose, and either no (open symbols), 10 mM (solid symbols), or 50 mM (half-shaded symbols) sodium salts of indicated anions. $I = 0.20$ in all cases. The pH values were obtained with reaction mixtures with the aid of a Beckman expanded scale meter immediately after incubations were completed.

pH 8.0; however, maximal activation by phosphate and sulfate extended from pH 7.0 to pH 8.0 (see Figs. 5B and 5C). It is evident from the effects of 10 and 50 mM phosphate on the pH–activity profile for glucose dehydrogenase activity with NAD^+ as nucleotide substrate (Fig. 6) that the activation here also is pH dependent.

Kinetics of the glucose dehydrogenase reaction

Studies to determine if second substrate concentration affects the affinity of the enzyme for NAD^+ or glucose are presented in Figs. 7A and 7B, respectively.

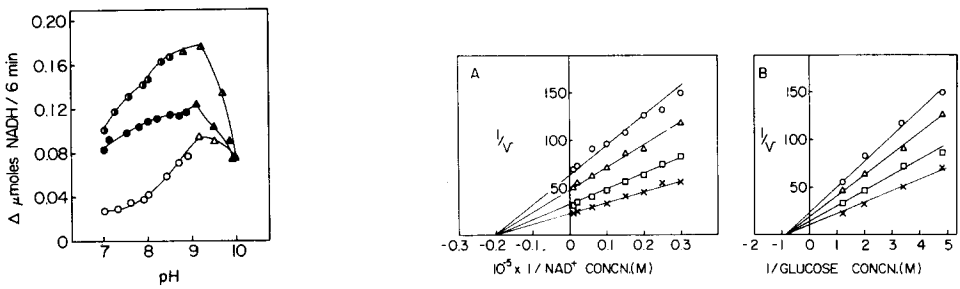


Fig. 6. Influence of varied concentrations of phosphate on activity–pH profiles of glucose dehydrogenase activity with NAD^+ as electron acceptor. Assay mixture compositions and other experimental details are identical with those in Fig. 5B, except that NAD^+ replaced $NADP^+$ and protein concentration was 1.12 mg per assay mixture.

Fig. 7. Effects of varied concentrations of NAD^+ (A) and glucose (B) on glucose dehydrogenase activity. All assay mixtures, pH 7.5, contained, in 3.0 ml, 50 mM Tris–HCl buffer and 0.56 mg enzyme protein. In A, glucose concentrations were held constant at 0.21 M (○), 0.29 M (△), 0.50 M (□), or 0.83 M (×), and initial reaction velocity, v, was determined as a function of NAD^+ which was varied from 0.033 to 1.0 mM. K_m for NAD^+, calculated as the negative reciprocal of x-axis intercepts of extrapolations of experimental plots, is $5 \cdot 10^{-5}$ M in all cases. In B, NAD^+ concentrations were held constant at 0.033 mM (○), 0.05 mM (△), 0.10 mM (□), or 1.0 mM (×), and initial reaction velocity was measured as a function of concentration of glucose which was varied from 0.21 to 0.83 M. K_m for glucose = 1.25 M in all cases. Velocity in all cases is expressed as μmoles of NADH formed per 6 min; $I = 0.200$.

Studies were carried out in which the concentration of glucose was held at various constant levels and v determined as a function of varied NAD^+ concentration (Fig. 7A), and in which NAD^+ was held constant at various levels and v determined as a function of glucose concentration (Fig. 7B). Data obtained were plotted in conventional double-reciprocal fashion[17].

When the glucose concentration was varied from 0.21 to 0.83 M for the glucose dehydrogenase reaction, Fig. 7A, the K_m values for NAD^+, calculated as the negative reciprocals of x-axis intercepts of extrapolations of experimental plots in Fig. 7A[18], remained unchanged. Also, the K_m value for glucose in the glucose dehydrogenase reaction, similarly calculated, was not affected by variations in the concentration of NAD^+, as indicated in Fig. 7B. Similar patterns of results previously have been obtained with yeast glucose dehydrogenase activity[2,19]. Rate Eqn. 1 has been shown to be applicable to two-substrate enzyme systems, the kinetics of which are as in Figs. 7A and 7B[20].

$$v = \frac{v_{max}}{\frac{K_{NAD^+}}{[NAD^+]} + \frac{K_{Glc}}{[Glc]} + \frac{K_{NAD^+} \cdot [Glc]}{[NAD^+] \cdot [Glc]}} \quad (1)$$

An equation of this form, (and experimental kinetic plots such as those in Figs. 7A and 7B), have been shown to be applicable, if it is assumed that compound A does not affect the affinity of enzyme from substrate B, and *vice versa*, either (a) to a reaction mechanism involving a compulsory order of binding of substrates to enzyme or (b) to a mechanism involving random order of addition of substrates to enzyme (if the additional assumption is made of the existence of two kinetically significant ternary enzyme–substrate complexes whose interconversions are rate-limiting)[20–22]. Although the existence of a binary enzyme–$NADP^+$ complex has been demonstrated with yeast glucose-6-*P* dehydrogenase[23] and precedence[24] for the involvement of a compulsory ordered mechanism of CLELAND's[25] "ordered Bi–Bi" type, in which $NADP^+$ binds first, exists based on studies of glucose-6-*P* dehydrogenase from human erythrocytes, it cannot be concluded, *a priori*, that such a mechanism (although reasonable) obtains with the multifunctional rat liver glucose dehydrogenase system.

Effects of anions on the kinetics of glucose dehydrogenase

That the K_m values were not affected by the concentration of the second substrate simplifies the kinetic 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[26].

The effects of the three anions, phosphate, sulfate, and bicarbonate, on the kinetics of the glucose dehydrogenase reaction with NAD^+ at pH 8.0 are described as classical double-reciprocal plots[17] in Figs. 8A and 8B. The addition of 10 mM $NaHCO_3$, 10 mM sodium phosphate, or 5 or 50 mM Na_2SO_4 in all instances both increased v_{max} values (reciprocals of y-axis intercepts) and lowered K_m values (negative reciprocals of x-axis intercepts) in experiments in which either the concentration of NAD^+ (Fig. 8A) or glucose (Fig. 8B) was varied. The K_m for glucose was lowered from 1.25 M in the absence of added anion to 1.0 M (10 mM bicarbonate),

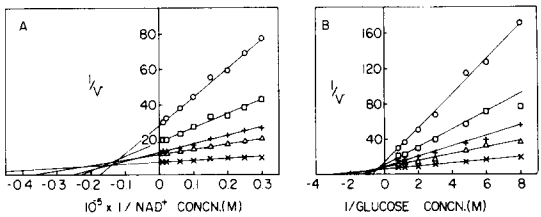


Fig. 8. Kinetics of activation by phosphate, sulfate, and bicarbonate of glucose dehydrogenase activity, studied with respect to (A) NAD^+, or (B) glucose. Assay mixtures, pH 7.5, contained, in 3.0 ml, 50 mM Tris–HCl buffer; 0.56 mg enzyme protein; either no supplemental anion (○), 10 mM $NaHCO_3$ (□), 5 mM Na_2SO_4 (+), 10 mM sodium phosphate (△), or 50 mM Na_2SO_4 (×); and (A) 0.5 M glucose and varied (0.033 mM to 1.0 mM) concentrations of NAD^+, or (B) 0.1 mM NAD^+ and varied (0.125 to 1.25 M) concentrations of glucose. I and expression of velocity, v, are as in Fig. 7. Apparent K_m values for NAD^+ (A), determined in the absence of added anion and in the presence of 10 mM bicarbonate, 5 mM sulfate, 10 mM phosphate, or 50 mM sulfate, were $5.7 \cdot 10^{-5}$ M, $4.4 \cdot 10^{-5}$ M, $2.7 \cdot 10^{-5}$ M, and $1.2 \cdot 10^{-5}$ M, respectively. Corresponding K_m values for glucose, determined from data in B, were 1.25 M, 1.00 M, 0.73 M, 0.50 M, and 0.32 M.

TABLE II

INHIBITION OF GLUCOSE-6-*P* DEHYDROGENASE ACTIVITY OF RAT LIVER MICROSOMAL GLUCOSE DEHYDROGENASE BY BICARBONATE, PHOSPHATE, SULFATE, AND ATP

Assay mixtures, pH 8.0, contained, in 3 ml, 100 mM Tris–HCl buffer, 33 μM $NADP^+$, 17 μM glucose-6-*P*, the indicated concentrations of inhibitors, and 0.39 mg microsomal protein. $I = 0.20$ in all instances. Activity is expressed as μmoles of NADPH formed per 3 ml per 6 min. Inhibition = 100 × [(activity noted without inhibitor − activity noted in the presence of indicated concentration of inhibitor)/activity noted in the absence of inhibitor].

Inhibitor added	*Conc. (mM)*	*Observed activity ($\times 10^4$)*	*Inhibition (%)*
None	—	415	—
HCO_3^-	2.5	403	3
	10	398	4
	20	382	8
	30	369	11
	50	336	19
HPO_4^{2-}	2.5	390	6
	10	382	8
	20	378	9
	30	369	11
SO_4^{2-}	2.5	403	3
	10	388	6.5
	20	369	11
	30	365	12
	50	353	15
ATP	1	340	18
	2.5	303	27
	5	261	37
	7.5	224	46
	10	191	56

0.73 M (5 mM sulfate), 0.50 M (10 mM phosphate), or 0.33 M (50 mM sulfate) (Fig. 8B). The K_m for NAD^+, Fig. 8A, was lowered from $5.7 \cdot 10^{-5}$ to $4.4 \cdot 10^{-5}$ M (10 mM bicarbonate), $4.2 \cdot 10^{-5}$ M (5 mM sulfate), $2.7 \cdot 10^{-5}$ M (10 mM phosphate), or $1.2 \cdot 10^{-5}$ M (50 mM sulfate).

Inhibition by anions and by ATP of glucose-6-P dehydrogenase activity of microsomal glucose dehydrogenase

The inhibition of glucose-6-*P* dehydrogenase activity of the rat liver microsomal enzyme by several concentrations of the anions bicarbonate, phosphate, and sulfate, as well as by ATP, is described in Table II. Consistent with our earlier observations with the multifunctional yeast enzyme[1-3], inhibitions progressive with increasing concentrations of these compounds were noted in all cases. The extent of inhibition by the smaller anions was relatively small, even with 30 or 50 mM concentrations, however (compare data in Table II with those in refs. 1 and 2) although ATP was a relatively potent inhibitor, as it was also with the yeast enzyme[3].

DISCUSSION

The stimulation by the anions sulfate, phosphate, and bicarbonate of glucose dehydrogenase activity of glucose-6-*P* dehydrogenase, which was first described by ANDERSON AND NORDLIE[1] and ANDERSON *et al.*[2], for the yeast enzyme, and by KISSIN AND BEUTLER[27], for the human erythrocyte enzyme (bicarbonate activation, only), has now been extended by the studies described above to obtain with the rat liver microsomal enzyme as well. Experimental results presented in Figs. 1–8 and Tables I and II, above, provide a characterization of the phenomenon as observed with the latter preparation.

A comparison of experimental observations made with rat liver microsomal enzyme with those previously made with yeast glucose-6-*P* dehydrogenase[1-3] indicates many similarities in the two enzymes, particularly with respect to the nature of responses to anions, as well as a few basic differences.

The rat liver enzyme closely resembles the yeast enzyme[1,2] with respect to the following properties: (a) Enzymes from both sources are multifunctional, exhibiting glucose dehydrogenase as well as glucose-6-*P* dehydrogenase activity. (b) Glucose dehydrogenase activities from both sources have relatively very high K_m values for glucose. (c) The anions bicarbonate, phosphate, and sulfate all activate glucose dehydrogenase activity and inhibit glucose-6-*P* dehydrogenase activity of the enzymes from both sources. (d) The various anions effect shifts towards acidic values in the pH-activity profiles of glucose dehydrogenase activity from both sources. (e) Increases in the degree of activation of glucose dehydrogenase activity progressive with elevations in anion concentrations are observed with bicarbonate and with lower (< 5 mM) concentrations of phosphate and sulfate, with preparations from both sources. (f) Glucose dehydrogenase and glucose-6-*P* dehydrogenase activities of the yeast enzyme are inhibited by ATP[3], as are bovine liver glucose dehydrogenase[28], glucose-6-*P* dehydrogenase activity of rat liver glucose dehydrogenase (Table II), and rat liver cytosolic glucose-6-*P* dehydrogenase[29].

Differences in the modes of action of small anions with respect to their effects on yeast and rat liver glucose dehydrogenase activities include the following: (a) Sul-

fate > phosphate > bicarbonate in effectiveness as activators of rat liver glucose dehydrogenase (Table I and Figs. 4A and 4B), whereas a somewhat altered order of effectiveness was noted with the yeast enzyme, where bicarbonate > sulfate > phosphate[1]. (b) While activations progressive with increasing anion concentrations were observed with the mammalian enzyme with all concentrations of anions studied (1–50 mM; Figs. 4A and 4B), maximal activations of the yeast enzymic activity were obtained with 5 mM phosphate or sulfate, and higher levels of these ions resulted in a decrease rather than an increase in extent of activation[1]. (c) Activating anions were without effect on K_m values for either glucose or pyridine nucleotide with the yeast enzyme[2], but effected quite marked reductions in K_m values for glucose and NAD^+, as well as an increase in v_{max} values, with rat liver microsomal glucose dehydrogenase (see Figs. 8A and 8B). (d) Glucose dehydrogenase activity of the yeast enzyme, as assayed in the absence of added anion, maximally constitutes less than 1% of activity observed with glucose-6-*P* as oxidizable substrate, a value which can be elevated to as high as 20% by optimal amounts of bicarbonate[1]; whereas with the rat liver enzyme, glucose dehydrogenase activity observed with 0.5 M glucose was approximately equal to that obtained with 0.17 mM glucose-6-*P* in the absence of anion (see Fig. 1), and increased to a ratio value of 3 or more in the presence of appropriate concentrations of anions (see Table I and Figs. 3A, 4A and 4B, for example).

On the basis of the similarities in the responses noted with the enzyme from rat liver microsomes (described above) and from yeast[1–3], it appears reasonable that the mechanism of anion action previously proposed[2,3] for this multifunctional enzyme from the latter source also is generally applicable, with one modification, to the rat liver enzyme. This mechanism as previously proposed by ANDERSON *et al.*[2,3], involves the binding of glucose and anion to the active enzymic site in a manner imitating that of glucose-6-*P*. The presence of the anion on the enzymic site involved in the binding of the phosphoryl portion of the glucose-6-*P* molecule would explain the observed inhibition with glucose-6-*P* as oxidizable substrate, while a "flexation"[30] in the enzyme molecule resulting from the binding of anion would facilitate the binding of glucose and would hence result in activation with respect to glucose dehydrogenase. Such a mechanism appears generally applicable on the basis of experimental observations described for the rat liver enzyme in this paper, although the added qualification that the binding of anion also may produce conformational alterations resulting in an increase in the affinity of the enzyme for NAD^+ as well as glucose appears predicated on the basis of experimental observations on the effects of anions on K_m values described in Figs. 8A and 8B.

Physiological implications

It is apparent from data in Fig. 1 that, while more than half of liver glucose-6-*P* dehydrogenase activity is present in cytosol, quite significant amounts also are present in the nuclear and microsomal fractions, as also observed earlier by BEUTLER AND MORRISON[9]. Glucose dehydrogenase activity, in contrast, is confined almost exclusively to the microsomal fraction. It is of particular interest that the level of this latter activity, determined in the absence of activating anion and with 0.5 M glucose present, approximately equals that of glucose-6-*P* dehydrogenase activity of the microsomal enzyme measured with saturating levels of substrates (see Fig. 1). In

this respect, the mammalian microsomal dual-function dehydrogenase contrasts rather sharply with its yeast counterpart for which the ratio of activity with glucose/ activity with glucose-6-*P* is less than 0.01 in the absence of anion effectors.

It is also apparent from data in Table I and in Figs. 1, 4A and 4B, for example, that, in the presence of physiological levels (see refs. 31–37) of certain anions, the level of glucose dehydrogenase activity may not only significantly exceed that of microsomal glucose-6-*P* dehydrogenase activity but may approach that of total glucose-6-*P* dehydrogenase activity present in the hepatic cell.

Based on their observations on the metabolic disposition of isotopically labelled metabolites under a variety of conditions and the fact that the necessary complement of enzymes for the further metabolism of gluconolactone, the immediate product of glucose oxidation, are present in liver, METZGER *et al.*[38] have suggested that glucose dehydrogenase may play a physiologically significant role in rat hepatic glucose metabolism. Our observations on the magnitude of hepatic glucose dehydrogenase activity which may be manifest with high concentrations of the hexose in the presence of physiological levels of activating anions, along with the rather significant decreases in K_m values for glucose brought about by such anions (see Fig. 8B), may be interpreted to support this hypothesis. However, it must be pointed out that the lowest K_m value observed for glucose—0.33 M in the presence of 50 mM sulfate (Fig. 8B)—is more than 60 times the normal blood sugar level. It thus appears that if the enzyme conceivably may be involved in a physiologically relevant metabolic process, such a process likely must be initiated in a specialized cellular compartment under marked local hyperglycemic conditions.

Since the extent of inhibition by bicarbonate (and other anions) of dehydrogenase activity of the microsomal enzyme with glucose-6-*P* as substrate is relatively small (see Table II), it appears doubtful that the glycogenic effects of this anion[6,7] are manifest at this site. A thorough study of the effects of the anion on cytosolic glucose-6-*P* dehydrogenase would thus appear to be suggested.

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REFERENCES

1 W. B. ANDERSON AND R. C. NORDLIE, *Biochemistry*, 7 (1968) 1479.
2 W. B. ANDERSON, R. N. HORNE AND R. C. NORDLIE, *Biochemistry*, 7 (1968) 3997.
3 R. N. HORNE, W. B. ANDERSON AND R. C. NORDLIE, *Biochemistry*, 9 (1970) 610.
4 R. C. NORDLIE, *Ann. N.Y. Acad. Sci.*, 166 (1969) 699.
5 J. E. D. DYSON, W. B. ANDERSON AND R. C. NORDLIE, *J. Biol. Chem.*, 244 (1969) 560.
6 A. B. HASTINGS AND W. J. LONGMORE, *Advan. Enzyme Regulation*, 3 (1965) 147.
7 W. J. LONGMORE, C. M. NIETHE AND M. J. MCDANIEL, *J. Biol. Chem.*, 244 (1969) 6451.
8 R. P. METZGER, S. S. WILCOX AND A. N. WICK, *J. Biol. Chem.*, 240 (1965) 2767.
9 E. BEUTLER AND M. MORRISON, *J. Biol. Chem.*, 242 (1967) 5289.
10 R. C. NORDLIE AND H. A. LARDY, *J. Biol. Chem.*, 238 (1963) 2259.

11 A. KORNBERG AND B. L. HORECKER, *Methods Enzymol.*, 1 (1955) 323.
12 H. J. STRECKER, *Methods Enzymol.*, 1 (1955) 335.
13 O. WARBURG AND W. CHRISTIAN, *Biochem. Z.*, 287 (1936) 291.
14 B. L. HORECKER AND A. KORNBERG, *J. Biol. Chem.*, 175 (1948) 385.
15 *Circular OR-7*, Pabst Brewing Co., Milwaukee, Wisc., 1961, pp. 5–30.
16 N. ZAHEER, K. K. TEWARI AND P. S. BRISHMAN, *Arch. Biochem. Biophys.*, 120 (1967) 22.
17 H. LINEWEAVER AND D. L. BURK, *J. Am. Chem. Soc.*, 56 (1934) 658.
18 M. DIXON AND E. C. WEBB, *Enzymes*, Academic Press, New York, 1964, p. 68.
19 W. B. ANDERSON, Ph. D. Dissertation, Univ. North Dakota, 1968, p. 40.
20 R. A. ALBERTY, *Advan. Enzymol.*, 17 (1956) 1.
21 R. A. ALBERTY, *J. Am. Chem. Soc.*, 75 (1953) 1928.
22 J. R. FLORINI AND C. S. VESTLING, *Biochim. Biophys. Acta*, 25 (1957) 575.
23 R. H. YUE, E. A. NOLTMANN AND S. A. KUBY, *J. Biol. Chem.*, 244 (1969) 1353.
24 S. J. SOLDIN AND D. BALINSKY, *Biochemistry*, 7 (1968) 1077.
25 W. W. CLELAND, *Biochim. Biophys. Acta*, 67 (1963) 104.
26 M. DIXON AND E. C. WEBB, *Enzymes*, Academic Press, New York, 1964, pp. 37 and 72.
27 C. KISSIN AND E. BEUTLER, *Proc. Soc. Exptl. Biol. Med.*, 128 (1968) 595.
28 N. G. BRINK, *Acta Chem. Scand.*, 7 (1953) 1090.
29 G. AVIGAD, *Proc. Natl. Acad. Sci. U.S.*, 56 (1966) 1543.
30 D. E. KOSHLAND, JR., *Symp. Quant. Biol.*, 28 (1963) 473.
31 S. RAPOPORT, *J. Biol. Chem.*, 161 (1945) 429.
32 C. LONG, *Biochemists Handbook*, Van Nostrand, Princeton, 1961, p. 681.
33 J. SACKS, *Am. J. Physiol.*, 172 (1953) 93.
34 J. M. LOONEY AND E .M. JELLINEK, *Am. J. Physiol.*, 118 (1937) 225.
35 J. H. BLEND, *Clinical Recognition and Management of Disturbances of Body Fluids* Saunders, Philadelphia, 2nd ed., 1956, p. 103.
36 F. W. SUNDERMAN AND F. BOERNER, *Normal Values in Clinical Medicine*, Saunders, Philadelphia, 1950, p. 161.
37 P. L. ALTMAN, in D. S. DITTMER, *Blood and Other Body Fluids*, Federation of Am. Soc. for Exptl. Biol., Washinton, D.C., 1961, p. 188.
38 R. P. METZGER, D. PISZKIEWICS, L. A. MAYER, P. W. MOBLEY AND A. N. WICK, *Biochim. Biophys. Acta*, 141 (1967) 231.