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EFFECT OF SALTS ON D-GLYCERATE DEHYDROGENASE KINETIC BEHAVIOR

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Summary

Bovine liver D-glycerate dehydrogenase (D-glycerate:NAD (NADP) oxidoreductase, EC 1.1.1.29) adapts its kinetic behaviour to a sequential mechanism. The presence of NaCl causes an appreciable variation in the K_m and V values. relative to the both substrates in the hydroxypyruvate/D-glycerate dehydrogenae/NADH system, which does not happen in the D-glycerate/D-glycerate dehydrogenase/NAD system. The former system is inhibited by high concentrations of NaCl and activated by low salt concentrations. The hydroxypyruvate concentration causing substrate inhibition increases as the concentration of NaCl increases; excess NADH inhibition is independent of the salt concentration.

The variation of the initial rates of both systems, in the presence of chlorides having monovalent and divalent cations, or sodium halides, Na_2SO_4 and $NaNO_3$ (at constant ionic strength) suggests that the anions have a specific action on the enzyme.

An increase in the NaCl concentration causes a displacement of the optimum D-glycerate dehydrogenase pH (with hydroxypyruvate and NADH as substrates) towards the acid area. The enzyme stability, at varying pH, varies with the salt concentration.

Introduction

D-Glycerate dehydrogenase (D-glycerate:NAD (NADP) oxidoreductase, EC 1.1.1.29) is involved in the non-phopshorylated pathway of serine metabolism. This enzyme occurs in the liver tissue, it is also found in the bovine spinal cord [1] and in the pig spinal cord [2]; it has been detected in rat brain [3] and is also found in spinach [4].

Inorganic salts affect the D-glycerate dehydrogenase activity; thus, halide, nitrate and sulphate ions activate the enzyme from bovine liver [5,6] and

Pseudomonas acidovorans [7]. This phenomenon could be explained by analogy with the changes undergone by certain proteins induced by electrostatic interactions of the ions with the amino acid rests of the protein [8,9]. When the substrates are negatively charged, the interaction of inorganic anions with the positively charged groups at the active site of the enzyme may affect the formation of the enzyme-substrate complex.

Optimal bovine liver D-glycerate dehydrogenase activation [5] is attained with defined anion concentration, but when these are exceeded, inhibition takes place. To interpret the salt effect, Kitagawa et al. [10] and Sugimoto et al. [11] suggested that the enzyme has two sites to which the hydroxypyruvate binds; the E · S complex would be formed at one of them and hydroxypyruvate would bind to the other, when excess substrate inhibition takes place. The competition between anions and hydroxypyruvate for the former site could explain the inhibition caused by the high salt concentrations; the competition for the latter site would explain the increase in hydroxypyruvate saturating concentration on the saline concentration increase in the medium.

On the other hand, contradictory aspects on the kinetic mechanism of D-glycerate dehydrogenase have been described since, while some authors postulate that the enzyme adapts itself to the Michaelis-Menten kinetics and is inhibited by excess hydroxypyruvate [12], others state that inhibition is only caused by excess NADP and that, in the presence of NAD(H), the double-reciprocal plot of v versus [S] is not linear [13].

The present paper describes the effects that various inorganic salts have on the kinetic parameters of bovine liver D-glycerate dehydrogenase on excess hydroxypyruvate inhibition and on the optimum enzyme pH. There is also a discussion as to whether the variations caused by the salt on the enzyme activity depend, specifically, on the nature of the ions or are due to the changes in the ionic strength induced in the medium.

Materials and Methods

The substrates are freshly prepared solutions, in the appropriate buffer, of the following: 98% lithium hydroxypyruvate (Sigma Chemical Co.); 98% sodium pyruvate, 78% NADH and 89% NAD (Boehringer); 98% dihydrated calcium D,L-glycerate (Koch Light); this last substrate was dissolved in presence of EDTA (Merck) to mask Ca^{2+}.

Calcium phosphate gel used in the purification procedure was prepared from 150 ml $CaCl_2 \cdot 2H_2O$ (88.46 g/l) solution diluted with 1.5 l double-distilled H_2O, to which 150 ml $Na_3PO_4 \cdot 12H_2O$ (152 g/l) was carefully added. The gel was washed twice with double-distilled H_2O and adjusted to pH 7 by addition of 0.1 M acetic acid. Finally it was washed 4 times by suspension in 20 mM sodium phosphate buffer (pH 7)/0.1 mM dithiothreitol.

The D-glycerate dehydrogenase and lactate dehydrogenase activity is determined by reading the absorbance change at 340 nm and 30 ± 0.1°C in 1-cm light path cells in a PYE Unicam S-P 1700 recording spectrophotometer. The reaction was initiated by the addition of enzyme solution to 3 ml. The lactate dehydrogenase and D-glycerate dehydrogenase activity in the purification procedure was determined in a 20 mM sodium phosphate buffer (pH 7) with

0.1 mM NADH and 0.2 mM pyruvate or 1 mM hydroxypyruvate, respectively. In the kinetic studies with purified D-glycerate dehydrogenase, the activity of the hydroxypyruvate reduction system was determined in a 5 mM sodium phosphate buffer (pH 7), unless otherwise indicated.

In the reverse system, with D,L-glycerate and NAD, the buffer was 5 mM Tris-HCl (pH 9); the reaction was performed in the absence of hydrazine sulphate or semicarbazide hydrochloride, as proposed by some authors [1,13], since it has been observed that the absorbance change is linear during the first 5—7 min of the reaction.

The bovine liver D-glycerate dehydrogenase preparation was obtained by the following procedure: the tissue was cut into small pieces and homogenized in a Waring blendor (4°C) with 0.15 M KCl (1/2.5, w/v). The homogenate was centrifuged for 30 min at 12 000 $\times g$, at 4°C. To the supernatant fluid, 1 vol. 0.1 mM $MnCl_2$ solution was added. The suspension was dialysed for 2—4 h against 50 mM acetate buffer pH 6 (1/50, v/v) and was centrifuged as described above. $(NH_4)_2SO_4$ was added to the supernatant fluid and the precipitate having lactate dehydrogenase and D-glycerate dehydrogenase activity appearing between 30% and 60% saturation was collted by centrifugation. The precipitate was dissolved (1 g/30 ml) in 20 mM sodium phosphate buffer (pH 7)/0.1 mM dithiothreitol and was dialysed against the same buffer for 20 h at 4°C. The precipitate was separated by centrifugation. An equal volume of calcium phosphate gel suspension (20 mg/ml) was added to the active supernatant at 0°C; the suspension was centrifuged at 650 $\times g$ for 15 min at 0°C. $(NH_4)_2SO_4$ was added to the supernatant fluid to 60% saturation; the lactate dehydrogenase and D-glycerate dehydrogenase activity appeared in the precipitate. 10 ml dissolved precipitate (20 mg protein/ml) in 5 mM sodium phosphate buffer (pH 7)/0.1 mM dithiothreitol/0.1 M NaCl were dialysed for 24 h against the same buffer. This solution was added to a CM-Sephadex C50 chromatographic column (2 × 30 cm), previously equilibrated with the above buffer and eluted with the same buffer (1 ml/10 min), 10-ml fractions were collected. A linear NaCl gradient (from 0.1 to 0.3 M dissolved in the same buffer) was applied. The fractions eluted with NaCl from 0.125 to 0.150 M were only active with hydroxypyruvate as substrate; they were combined and dialysed at 0°C for 24 h against 5 mM sodium phosphate buffer (pH 7)/0.1 mM dithiothreitol. The fractions were concentrated by dialysis against 50% sucrose, 0.1 mM dithiothreitol. The solution was stored frozen (—15°C) without loss of activity for 6 months.

The preparation had a D-glycerate dehydrogenase activity of 446 U/mg prot. (1 U = $1 \cdot 10^{-2}$ ΔA/min), determined in the absence of NaCl in 20 mM sodium phosphate buffer (pH 7).

The protein concentration were determined by the Warburg and Christian method [14]. The variation of initial reaction rates against substrate concentrations were determined graphically by Lineweaver-Burk [15], Eadie-Hoffstee [16] and Vestling-Florini method [17]. The standard errors of the data were evaluated by least-squares adjustments, carried out on the UNIVAC 1100 Computer for which appropriate programs were written.

Results

Kinetic constants of the hydroxypyruvate/D-glycerate dehydrogenase/NADH system

When working with 15 mU/ml of D-glycerate dehydrogenase in 5 mM sodium phosphate buffer pH 7, the double-reciprocal plots of the initial rate values against the [NADH] (15.6—157 μM) or hydroxypyruvate (20—80 μM) concentrations, give non-parallel straight lines with an intercept at the left of the ordinate axis. The enzyme behaviour, under these conditions, is Michaelian and suggests a sequential type kinetic mechanism. Slopes and intercepts replots are also linear; the values of the kinetic parameters (see Materials and Methods) are the following:

$V = (11.464 \pm 0.215) \cdot 10^{-2}\ \Delta A/\text{min per ml preparation}$

$K_{m(NADH)} = 6.156 \pm 0.019\ \mu M$

$K_{m(hydroxypyruvate)} = 3.846 \pm 0.013\ \mu M$

Effect of NaCl at various concentrations of substrate

The influence of different concentrations of NaCl (0, 10, 25, 50, 100, 250 and 500 mM) on the D-glycerate dehydrogenase activity (18 mU/ml), was determined against variable concentrations of hydroxpyruvate (12 μM to 8 mM) and a constant and kinetically saturating concentration of NADH (156 μM); Lineweaver-Burk plot of the values obtained gives straight lines with no common point of intersection, the slope of the straight lines varies with the NaCl concentration. The V of the reaction increases with an increase of NaCl up to 0.2 M, but diminishes if the salt concentration is greater (Fig. 1a). The $K_{m(hydroxypyruvate)}$ values grow when the NaCl concentration is higher than 10 mM (Table I). The enzyme is inhibited by excess hydroxypyruvate and the phenomenon depends on the salt concentration; as this increases, the hydroxypyruvate concentration required to produce inhibition also increases; thus, without NaCl, hydroxypyruvate saturating concentration is 80 μM, with 25 mM of NaCl is 0.1 mM and with 250 mM of NaCl is 1 mM.

When working with the same system, if the NADH concentration (30 μM) is held constant and below the kinetically saturating level, the double-reciprocal plot of v versus [hydroxypyruvate] is very similar to that of the one described above. The secondary plots of the apparent V values against the NaCl concentration (Fig. 1a) show that the NADH protects the enzyme from the inhibition produced by the NaCl in this system; the inhibition portions of the curves corresponding to Fig. 1a are much more notable when NADH concentration is 30 μM. It is seen, moreover, that the NaCl concentrations producing inhibition are lower. The K_m values obtained working with NADH 30 μM are shown in Table I.

When working with hydroxypyruvate having a constant and kinetically saturating concentration (1 mM) or a concentration below the saturating level (40 μM) and with varying concentrations of NADH (7.8—156 μM), in absence or presence of varying concentrations of NaCl (10—500 mM) the double-reciprocal plots of v versus [NADH] give straight lines that have no common

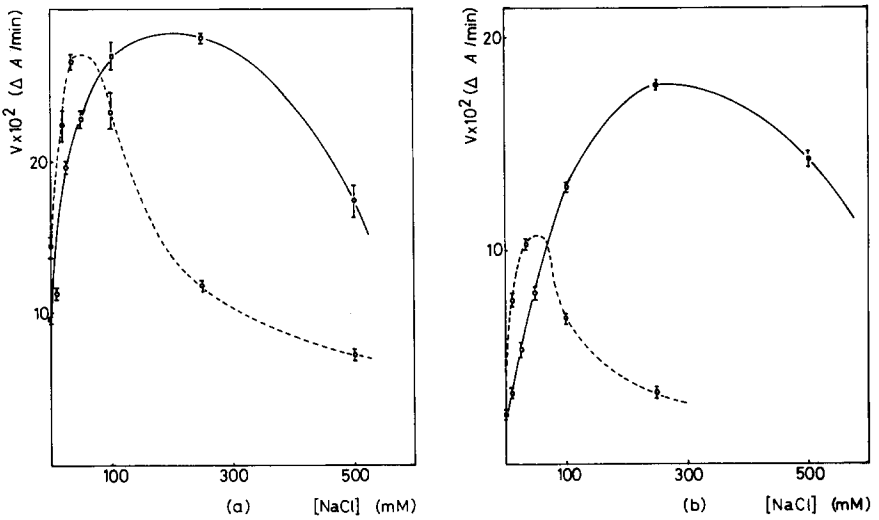

Fig. 1. Effect of sodium chloride concentration on V and K_m kinetic parameters. Hydroxypyruvate/D-glycerate dehydrogenase/NADH systems; 5 mM sodium phosphate buffer pH 7; (a) hydroxypyruvate: 12 μM to 8 mM; ———, [NADH] = 156 μM; 18 mU/ml of D-glycerate dehydrogenase — — —, [NADH] = 30 μM; 12.2 mU/ml, (b) [NADH]; 7.8–156 μM ———, [hydroxypyruvate] = 1 mM; 6 mU/ml of D-glycerate dehydrogenase; — — —, [hydroxypyruvate] = 40 μM; 5 mU/ml D-glycerate dehydrogenase.

point of intersection, the slope of each of them varies with the NaCl concentration. The plot of the apparent V values against the NaCl concentration, calculated for saturating or unsaturating hydroxypyruvate concentrations are shown in Fig. 1b. The apparent K_m values obtained in this case are shown in Table I. The kinetically saturating concentration of NADH does not vary with the NaCl concentration.

The effect of NaCl on the kinetic parameters of the hydroxypyruvate/D-glycerate dehydrogenase/NADH system was also calculated by simultaneously varying the NADH concentration (15.6–156 μM) and the hydroxy-

TABLE I

K_m VALUES VARIATION WITH NaCl CONCENTRATIONS IN HYDROXYPYRUVATE REDUCTION SYSTEM

NaCl (mM)	$K_{m(hydroxypyruvate)}$(μM)		$K_{m(NADH)}$(μM)	
	[NADH](μM)		[Hydroxypyruvate](mM)	
	156	30	1	0.04
0	23.0 ± 0.99	23.8 ± 4.3	34.7 ± 0.1	15.2 ± 0.3
10	16.0 ± 1.4	—	10.9 ± 0.4	11.7 ± 0.7
20	—	36.6 ± 8.2	—	19.7 ± 0.3
25	36.2 ± 1.6	—	9.7 ± 0.4	—
35	—	66.8 ± 4.1	—	35.4 ± 0.8
50	44.6 ± 4.1	—	19.0 ± 1.2	—
100	74.0 ± 9.3	109.2 ± 25.6	41.9 ± 0.7	64.1 ± 0.9
250	282.7 ± 5.8	223.1 ± 5.9	148.0 ± 1.1	198.4 ± 3.7
500	—	743.9 ± 0.9	376.4 ± 12.2	—

pyruvate concentration (20 μM to 1 mM) for different concentrations of NaCl (15—300 mM) and a constant enzyme concentration of 7.3 mU/ml in a 5 mM sodium phosphate buffer pH 7. The plot of V, $K_{m(NADH)}$ and $K_{m(hydroxypyruvate)}$ against the NaCl concentration shows that the curve profile is completely analogous to those observed for the apparent V and K_m values.

The oxidation of the D-glycerate (5 mM) with NAD (87 μM to 1.4 mM), catalysed by D-glycerate dehydrogenase (133 μl purified preparation/ml incubated) at pH 9 (Tris-HCl buffer, 5 mM) was studied; the double-reciprocal plot of v versus NAD at different NaCl concentration (0, 20, 40, 60, 100 and 200 mM) show that the V values and the K_m values vary very little as the salt concentration increases (Table II). The results have the opposite sing to those observed in the hydroxypyruvate reduction. With variable D-glycerate concentration (1—5 mM), a constant NAD concentration (2.6 mM), with 66 μl pure enzyme preparation/ml at pH 9 (5 mM Tris-HCl buffer), the K_m values vary with the increase in the NaCl concentration and the V remains practically constant (Table II).

The calculation of the kinetic parameters of the D-glycerate oxidation system, was made in the presence of 50 mM NaCl, NAD (87 μM to 1.6 mM), D-glycerate (1—5 mM) at pH 9 (5 mM Tris-HCl buffer). Linear plots were obtained in all cases and the following values were calculated:

$V = (9.666 \pm 0.095) \cdot 10^{-3}\ \Delta A$/min per ml preparation

$K_{m(NAD)} = 220.30 \pm 0.02\ \mu M$

$K_{m(D\text{-glycerate})} = 522.79 \pm 0.05\ \mu M$

Effect of different salts on the D-glycerate dehydrogenase activity

The effect produced by different salts on the D-glycerate dehydrogenase activity in the reduction of hydroxypyruvate have been determined at one constant ionic strength (80 mM) provided by the corresponding salts. The incubated samples contain variable hydroxypyruvate concentration (20—100 μM) at a constant NADH concentration (156 μM), 5 mU/ml of D-glycerate dehydrogenase, the corresponding salt concentration to guarantee a ionic strength of 80 mM and the 5 mM sodium phosphate buffer pH 7. In all cases, the double-reciprocal plots of V values against the hydroxypyruvate concentra-

TABLE II

APPARENT K_m AND V VALUES VARIATION WITH NaCl CONCENTRATION IN D-GLYCERATE OXIDATION SYSTEM

NaCl (mM)	Respect to NAD		Respect to D-glycerate	
	K_m(M) × 10^5	$V(\Delta A$/min) × 10^3	K_m(M) × 10^4	$V(\Delta A$/min) × 10^3
0	7.13 ± 0.46	6.45 ± 0.08	1.49 ± 0.14	5.15 ± 0.04
20	9.99 ± 0.77	5.88 ± 0.09	1.19 ± 0.13	5.65 ± 0.04
40	7.98 ± 0.21	4.94 ± 0.02	1.17 ± 0.24	5.43 ± 0.07
60	12.45 ± 0.34	5.95 ± 0.03	7.34 ± 0.27	6.74 ± 0.06
100	11.76 ± 0.19	6.48 ± 0.02	9.62 ± 0.32	7.03 ± 0.06
200	9.22 ± 0.76	4.66 ± 0.08	36.21 ± 0.98	9.49 ± 0.12

tions are linear. The results obtained are expressed in Table III. In the reduction of hydroxypyruvate, the sodium, potassium, and cesium chlorides produce an analogous effect on the apparent V and K_m/V parameters, while sodium sulphate causes more noticeable reduction of V value and sodium nitrate causes great inhibition.

In the same way, concentration of 30 mM of sodium halides (chloride, bromide and iodide) in 5 mM sodium phosphate buffer pH 7, modify the hydroxypyruvate (12 μM to 8 mM)/D-glycerate dehydrogenase (1.5 mU/ml)/NADH (156 μM) system activity (Table IV). I^- is the one producing a more noticeable effect. The presence of the halides causes different displacements of the point where the hydroxypyruvate inhibition appears; I^- is the one inducing the maximum displacement. The fact suggest that the salt's effect on the enzyme activity depends on the nature of the salt's anion. This fact is confirmed by the results obtained when experimenting with chlorides having divalent cations (Mg^{2+}, Ca^{2+} and Ba^{2+}, 30 mM) in the hydroxypyruvate (12 μM to 8 mM)/D-glycerate dehydrogenase (1.5 mU/ml)/NADH (156 μM) system, with 12 mM Tris-HCl buffer pH 7.2; the apparent V and K_m/V values obtained without salt are $(7.77 \pm 0.81) \cdot 10^{-2}$ ΔA/min and $(2.14 \pm 0.35) \cdot 10^{-4}$ M/ΔA per min, respectively, while with salts these values are comprised between $(29.94 \pm 0.98) \cdot 10^{-2}$ and $(34.32 \pm 2.42) \cdot 10^{-2}$ ΔA/min and $(2.15 \pm 0.37) \cdot 10^{-4}$ and $(2.54 \pm 0.19) \cdot 10^{-4}$ M/ΔA per min respectively, it show that the divalent cations affect the D-glycerate dehydrogenase activity in the same way.

On determining the influence exercised by the concentration of the sodium phosphate buffer pH 7 on the enzyme activity (Table V) it is seen to cause an increase in the V values with an increase of buffer concentration up to 90 mM but diminishes if the buffer concentration is greater, the analogous effect is observed with the K_m values and that the inhibition caused by hydroxypyruvate does not vary so noticeably on varying the buffer concentration as was the case in the presence of NaCl.

In the oxidation of D-glycerate at a constant concentration (5 mM), by NAD (variable concentrations between 0.087 mM and 1.6 mM), catalysed by D-glycerate dehydrogenase at pH 9 (Tris-HCl buffer, 50 mM), the chlorides having monovalent cations (Li^+, Na^+, K^+, Cs^+ and NH_4^+, 100 mM) do not sensibly affect the enzyme activity, since the K_m values are comprised between $(7.13 \pm 0.46) \cdot 10^{-5}$ and $(9.87 \pm 0.51) \cdot 10^{-5}$ M and the V values between 0.95 ±

TABLE III

EFFECT OF DIFFERENT SALTS ON THE D-GLYCERATE DEHYDROGENASE ACTIVITY

Apparent kinetic parameters respect to hydroxypyruvate. See in the text other experimental conditions. Ionic strength: 80 mM.

Salt	$V(\Delta A/\text{min}) \times 10^2$	$K_m/V(\text{M}/\Delta A/\text{min}) \times 10^5$
Without	6.31 ± 0.03	8.21 ± 0.27
NaCl	50.74 ± 0.23	51.71 ± 0.08
KCl	66.75 ± 1.67	57.49 ± 0.34
CsCl	18.47 ± 0.20	41.92 ± 0.45
Na_2SO_4	7.26 ± 0.08	43.63 ± 1.00
$NaNO_3$	3.01 ± 0.15	1255.90 ± 15.80

TABLE IV

EFFECT OF SODIUM HALIDES IN THE HYDROXYPYRUVATE REDUCTION SYSTEM

Apparent kinetic parameters respect to hydroxypyruvate. Other experimental conditions in the text.

Salt (30 mM)	$V(\Delta A/\text{min}) \times 10^2$	$K_m/V(M/\Delta A/\text{min}) \times 10^4$	Hydroxypyruvate saturating concentration (mM)
Without	12.24 ± 1.17	2.55 ± 0.23	0.08
NaCl	22.20 ± 1.61	4.22 ± 0.15	0.1
NaBr	17.53 ± 1.35	4.44 ± 0.26	0.3
NaI	15.49 ± 0.97	7.94 ± 0.38	1.0

$0.05) \cdot 10^{-2}$ and $(1.12 \pm 0.06) \cdot 10^{-2}$ ΔA/min. The action caused by different salts on the same system is given in Table VI, showing that the NaCl does not affect sensitively the kinetic parameters of the system in accordance to exposed above and suggest that the inhibition caused (decrease of V values, increase of K_m/V values) depends on the nature of the anion, in accordance to the effect of anions on the hydroxypyruvate reduction system.

Influence of NaCl concentrations on the optimum operating pH of the hydroxypyruvate/D-glycerate dehydrogenase/NADH system

The variation of the D-glycerate dehydrogenase activity with the pH in absence of NaCl and in presence of varying concentrations (25, 73 and 150 mM) has been determined. The results obtained (Fig. 2c) show that the optimum pH value moves towards the acid region as the salt concentration increases.

The enzyme stability with variable pH was determined by incubation of D-glycerate dehydrogenase samples in 50 mM sodium phosphate buffer. The activity of each of the samples was determined by addition of 0.2 ml of the solution in the spectrophotometric cells containing appropriate amounts of the substrates, without NaCl or with NaCl at the optimum pH for each of the salt concentrations. The results obtained (Fig. 2a) show that the enzyme is not sensitive to the pH changes. On incubating the enzyme at the different pH

TABLE V

EFFECT OF BUFFER CONCENTRATION IN THE HYDROXYPYRUVATE REDUCTION SYSTEM

Buffer: sodium phosphate pH 7; [NADH] = 156 μM; [hydroxypyruvate]: 12 μM to 1 mM; D-glycerate dehydrogenase: 7.3 mU/ml.

Buffer (mM)	$V(\Delta A/\text{min}) \times 10^2$	$K_m(M) \times 10^5$	Hydroxypyruvate saturating concentration (mM)
18.4	9.57 ± 0.04	0.914 ± 0.018	0.06
45.4	12.84 ± 0.05	1.62 ± 0.02	0.08
90.4	26.73 ± 0.19	9.39 ± 0.11	0.08
129.5	13.75 ± 0.14	4.50 ± 0.12	0.10
180.4	12.73 ± 0.16	4.42 ± 0.15	0.10

TABLE VI

EFFECTS OF VARIOUS SODIUM SALTS ON D-GLYCERATE DEHYDROGENASE ACTIVITY

D-Glycerate oxidation system: 5 mM Tris-HCl buffer pH 9, 133 μl purified preparation/ml; [D-glycerate] = 5 mM; [NAD]: 87 μM to 1.6 mM; sodium sulphate concentration: 33 mM; other salts concentration: 100 mM.

Salt	$V(\Delta A/\text{min}) \times 10^2$	$K_m/V(M/\Delta A/\text{min}) \times 10^2$
Without	1.23 ± 0.02	0.516 ± 0.057
NaCl	0.972 ± 0.002	0.534 ± 0.005
NaBr	0.625 ± 0.010	1.374 ± 0.091
NaI	0.467 ± 0.003	1.361 ± 0.040
$NaNO_3$	0.864 ± 0.051	1.961 ± 0.440
Na_2SO_4	0.375 ± 0.014	3.698 ± 0.540

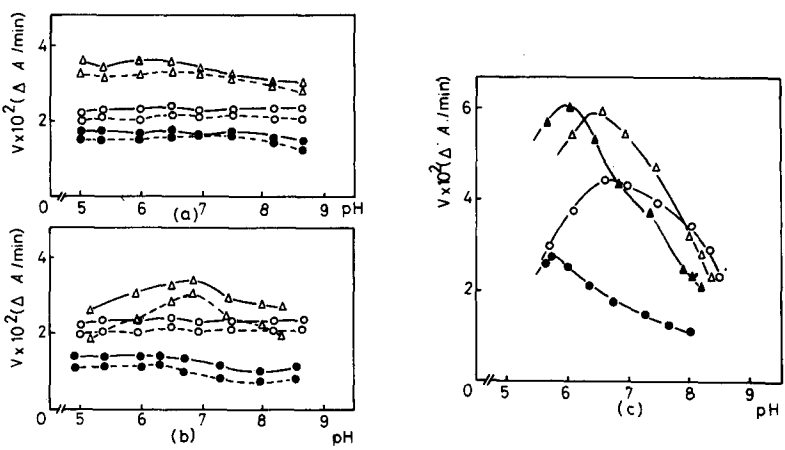

Fig. 2. Effect of sodium chloride on D-glycerate dehydrogenase optimum pH. [hydroxypyruvate] = 40 μM; [NADH] = 78 μM; [NaCl] mM: (○) 0· (△) 25; (▲) 73; (●) 150; (a) and (b) enzyme stability from pH. Enzyme concentration: 2.9 mU/ml. Incubation time: (———) 10 min; (— — —) 75 min; (a) incubation without salt; (b) incubation with salt (c) optimum pH of enzyme determined with or without salt. Enzyme concentration: 3.6 mU/ml. Other experimental conditions in the text.

values in presence of NaCl (Fig. 2b), it is shown that only in presence of the lowest NaCl concentration tested does the stability curve acquire a slightly bell profile.

Discussion

The behaviour of bovine liver D-glycerate dehydrogenase, when catalysing the D-glycerate oxidation or hydroxypyruvate reduction is adapted to the Michaelis-Menten kinetics under the experimental conditions described. These results agree with those postulated by Kitagawa et al. [10] and Sugimoto et al. [11] for the same source enzyme and disagree with those described by Rosemblum et al. [13], who observed deviations from linearity in the double-reciprocal plots of v versus the coenzyme (NAD, NADH) or the substrate concentrations. The determination of the initial rates, in presence or absence of NaCl, for varying substrate concentrations allows it to be said that the

D-glycerate dehydrogenase follows a sequential mechanism.

The presence of the salts had been considered only to modify the association of the enzyme with the hydroxpyruvate [11]; the results described in this paper show that since the NaCl acts on the D-glycerate dehydrogenase, the apparent kinetic parameter (V and K_m) relative to NADH and hydroxypyruvate are affected in a similar way. The plot of $1/v$ versus 1/hydroxypyruvate or 1/NADH, in the presence of NaCl give straight lines, without a common intercept, showing that the salt exercises a double activating and inhibiting action on the enzyme; therefore, the nature of the interaction (competitive, non-competitive or uncompetitive) may not be defined, as was defined by other authors [11]. The double activating and inhibiting effect of NaCl on the D-glycerate dehydrogenase activity is better shown in the secondary plots of apparent V (Fig. 1). The bell shaped form of the V plot suggests that the presence of NaCl affect the limiting state of the catalytic rate. None of the plots is of a sigmoidal nature, so that the NaCl may not be considered to be a positive effector of D-glycerate dehydrogenase. The variation of the apparent $K_{m(hydroxypyruvate)}$ and $K_{m(NADH)}$ values, with the NaCl concentration, suggests that the affinity of both substrates for the enzyme diminishes in the presence of the salt.

It may, therefore, be postulated that the action of NaCl on the D-glycerate dehydrogenase affects the binding of the hydroxpyruvate and of the NADH to the enzyme in a similar way. This hypothesis is correlated to the protective effect exercised by both substrates in the inhibition of the enzyme by NaCl. Thus, when the hydroxypyruvate or NADH concentrations are held constant and below the kinetically saturating value, the reduction in the V value (Fig. 1) is more noticeable than if the substrate concentration is saturating. This might suggest, therefore, that some competition takes place between the salt ions and the hydroxpyruvate or NADH.

The presence of NaCl determines the increase in the kinetically saturating concentration of hydroxpyruvate, but the effect is not observed with NADH. The fact suggest that the presence of NaCl reduces the affinity of the excess hydroxypyruvate for the D-glycerate dehydrogenase whilst that of NADH remains unaltered; consequently, the excess of both substrates may be assumed to bind to different sites of the enzyme molecule.

In the D-glycerate oxidation system, the D-glycerate dehydrogenase activity is scarcely altered by the presence of NaCl, since the values of V and K_m depend very little on the salt concentration.

The effect of the NaCl on the D-glycerate dehydrogenase activity could be interpreted through the variations in the medium ionic strength due to the salt concentration. Whatever these effects are, it is seen if the ionic strength is held constant, but the nature of the salt is varied, the activity variations depend only on the anion, as shown by the V and K_m values. Experiments with several salts show that the anions having a greater ion volume are the ones inducing more noticeable effects and also cause the hydropyruvate concentration required for producing substrate inhibition to increase. The iodide and nitrate ions are the ones causing the most noticeable variations.

The above described results reveal the existence of a specific anion-protein interaction which modifies the binding of the enzyme to its substrate and

perhaps the sensitivity of the enzyme to the pH. Effectively, it has been observed that the optimum pH of the hydroxypyruvate reduction system moves towards the acid zone as the NaCl concentration in the medium increases. This phenomenon may be attributed to the variation of the pK values of the enzyme ionisable groups, induced by the variation of the ionic strength caused by the presence of the salt, or to the appearance of new groups. The enzyme stability, at different pH levels, varies with the salt concentration. Both facts suggest that the D-glycerate dehydrogenase may undergo structural changes depending on the NaCl concentration.

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