

# Altered Kinetics Properties of Erythrocyte Lactate Dehydrogenase in Type II Diabetic Patients and Its Implications for Lactic Acidosis

Aniket V. Mali<sup>1</sup> · Sunita S. Bhise<sup>1</sup> · Surendra S. Katyare<sup>1</sup> · Mahabaleshwar V. Hegde<sup>1</sup>

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**Abstract** Recent studies have been noted that the erythrocytes from Type II diabetic patients show significantly altered structural and functional characteristics along with the changed intracellular concentrations of glycolytic intermediates. More recent studies from our laboratory have shown that the activities of enzymes of glycolytic pathway changed significantly in RBCs from Type II diabetic patients. In particular the levels of lactate dehydrogenase (LDH) increased significantly. Lactic acidosis is an established feature of diabetes and LDH plays a crucial role in conversion of pyruvate to lactate and reportedly, the levels of lactate are significantly high which is consistent with our observation on increased levels of LDH. Owing to this background, we examined the role of erythrocyte LDH in lactic acidosis by studying its kinetics properties in Type II diabetic patients.  $K_m$ ,  $V_{max}$  and apparent catalytic efficiency were determined using pyruvate and NADH as the substrates. With pyruvate as the substrate the  $K_m$  values were comparable but  $V_{max}$  increased significantly in the diabetic group. With NADH as the substrate the enzyme activity of the diabetic group resolved in two components as against a single component in the controls. The Apparent  $K_{cat}$  and  $K_{cat}/K_m$  values for pyruvate increased in the diabetic group. The  $K_i$  for pyruvate increased by two fold for the enzyme from diabetic group with a marginal decrease in  $K_i$  for NADH. The observed changes in catalytic attributes are conducive to enable the

enzyme to carry the reaction in forward direction towards conversion of pyruvate to lactate leading to lactic acidosis.

**Keywords** Diabetic erythrocytes LDH ·  $K_m$  and  $V_{max}$  · Catalytic efficiency

## Abbreviations

DM	Diabetes mellitus
HbA <sub>1c</sub>	Glycosylated haemoglobin
RBCs	Red blood cells
LDH	Lactate dehydrogenase
NADH	Nicotinamide adenine dinucleotide
G6P	Glucose-6-phosphate
F6P	Fructose-6-phosphate
FBP	Fructose-1,6-bisphosphate
DHAP	Dihydroxyacetone phosphate
GAP	Glyceraldehyde 3-phosphate
3PGA	3-Phosphoglyceric acid
EDTA	Ethylenediaminetetraacetic acid
NaCl	Sodium chloride

## Introduction

Diabetes mellitus (DM) is characterized by impaired glucose metabolism which is attributed either to inadequate synthesis of insulin as in Type I DM or by severe insulin resistance associated with defects in insulin receptor as in Type II DM [1, 2]. Increased glycosylated hemoglobin (HbA<sub>1c</sub>) is a characteristic feature of diabetes [3, 4] and pathies associated with diabetes have been well documented [5, 6]. However, more recently it has also been noted that the erythrocytes from Type II diabetic patients

✉ Mahabaleshwar V. Hegde  
mahabaleshwar@yahoo.com

<sup>1</sup> Center for Innovation in Nutrition, Health and Disease (CINHD), Interactive Research School for Health Affairs (IRSHA), Bharati Vidyapeeth Deemed University, Dhankawadi, Katraj, Pune, Maharashtra 411 043, India

**Table 1** Km and Vmax Values of Erythrocyte Lactate Dehydrogenase from Type II Diabetic Patients

Substrate	Subject	Component I		Component II	
		Km (mM)	Vmax	Km (mM)	Vmax
Na pyruvate	Control	0.136 ± 0.006	2.70 ± 0.08	–	–
	Diabetic	0.139 ± 0.013 NS	4.14 ± 0.31**	–	–
NADH	Control	–	–	0.069 ± 0.003	2.37 ± 0.18
	Diabetic	0.0093 ± 0.002	1.85 ± 0.081	0.047 ± 0.001***	3.04 ± 0.07*

The lactate dehydrogenase activity is expressed as nmol/min/10<sup>6</sup> RBCs. Results are given as mean ± SEM of 6 independent observations in each group

\*  $p < 0.006$ ; \*\*  $p < 0.001$ ; \*\*\*  $p < 0.0001$

**Table 2** Apparent Kcat and Kcat/Km values of erythrocyte lactate dehydrogenase from Type II diabetic patients

Substrate	Subject	Component I		Component II	
		Kcat × 10 <sup>-8</sup>	Kcat/Km × 10 <sup>-8</sup>	Kcat × 10 <sup>-8</sup>	Kcat/Km × 10 <sup>-8</sup>
Na pyruvate	Control	0.318 ± 0.009	2.34 ± 0.08	–	–
	Diabetic	0.486 ± 0.036**	3.53 ± 0.21***	–	–
NADH	Control	–	–	0.278 ± 0.020	4.12 ± 0.45
	Diabetic	0.216 ± 0.01	27.98 ± 5.36	0.357 ± 0.008*	7.69 ± 0.28****

Results are given as mean ± SEM of 6 independent observations in each group

\*  $p < 0.004$ ; \*\*  $p < 0.001$ ; \*\*\*  $p < 0.0003$ ; \*\*\*\*  $p < 0.0001$

show significantly altered characteristics. These include: decreased/reduced life-span, change in deformability, increased red cell aggregation, and reduced membrane cholesterol, sphingomyelin and phosphatidylcholine, and decreased sialic acid content [7–9]. It has also been reported that the intracellular concentrations of intermediates of glycolysis change significantly in the RBCs of diabetic patients. In particular the concentration of glucose, G6P, F6P and lactate increased significantly, whereas those of FBP, DHAP + GAP, and 3PGA decreased [10]. More recent studies from our laboratory have shown that the activities of enzymes of glycolytic pathway changed significantly in RBCs from Type II diabetic patients. In particular the levels of lactate dehydrogenase (LDH) increased significantly [11].

Lactic acidosis is an established feature of diabetes and LDH plays a crucial role in conversion of pyruvate to lactate; as cited above the levels of lactate are significantly high which is consistent with our observation on increased levels of LDH [11, 12]. In normal healthy individuals erythrocytes contribute to about 20% of the plasma levels of lactate [13]. Obviously, as cited above [12] increased levels of lactate in the diabetic RBCs will lead to lactic acidosis.

The increased levels of LDH which we reported earlier could be either due to intrinsic changes and/or altered kinetics properties of the enzyme. We tried to illustrate these points by studying the substrate saturation, substrate binding and substrate inhibition characteristic of the enzyme from diabetic erythrocytes in comparison with

those from normal healthy individuals. The results of these investigations are described in the present communication which indicates that indeed significant changes were noted in the kinetics properties of LDH from diabetic RBCs.

## Materials and Methods

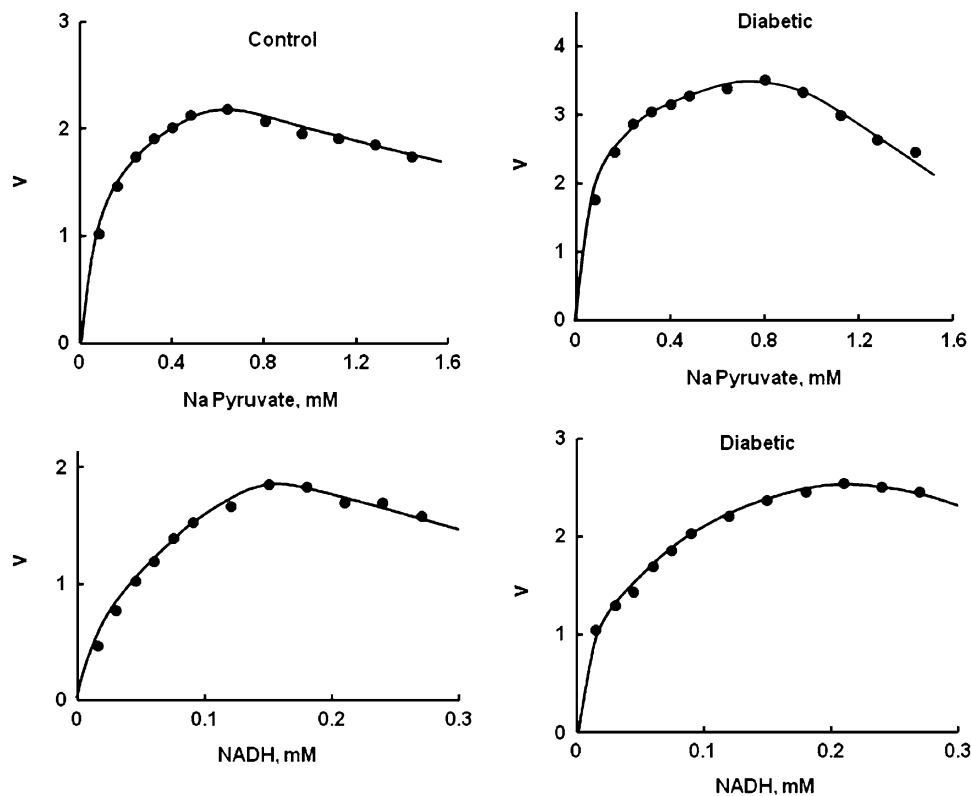
### Chemicals

Sodium salt of pyruvic acid and NADH (disodium salt) were purchased from SRL, Mumbai, India. All other chemicals were of analytical reagent grade and were purchased locally.

### Sample Collection

3.0 mL blood samples were collected in vacutainer tubes containing EDTA from six normal healthy individuals and six Type II diabetic patients after written informed consent, as per guidelines approved by the Institutional Ethics Committee. For the normal male volunteers the mean age was 40.3 ± 2.01 years (ranging from 33 to 45 years) and the mean blood sugar level was 95.30 ± 3.75 mg/dL (ranging from 91 to 107 mg/dL). The mean age of the diabetic patients was 45.8 ± 3.89 years (ranging from 33 to 53 years) and the mean blood sugar level was 326.9 ± 22.32 mg/dL (ranging from 223 to 422 mg/dL). The samples were centrifuged immediately at 0–4 °C at 4000 rpm to sediment RBCs. The RBCs were washed 3

**Fig. 1** Substrate saturation curves for Na Pyruvate and NADH for lactate dehydrogenase from Control and Diabetic erythrocytes. The enzyme activity  $v$  (nmoles substrate transformed/min/ $10^6$  RBCs) is plotted on abscissa versus substrate concentration (mM) on the ordinate. The results are typical of 6 independent observations



times with physiological saline (0.9% NaCl) and then were reconstituted to original volume with physiological saline. Known aliquots were diluted 1:10 in 10 mM sodium phosphate buffer, pH 7.4 as described previously and the lysate was used within 2–3 h for kinetics studies [11, 14]. The RBC count was determined in hemocytometer using separate aliquots.

### Enzyme Kinetics

The basic assay mixture contained: 50 mM sodium phosphate buffer, pH 6.5, 0.8 mM sodium pyruvate and 0.18 mM NADH in a total volume of 1.0 mL. 10  $\mu$ L RBC lysate was used as the source of the enzyme [11, 14].

With pyruvate as the substrate, concentration of NADH was kept constant at 0.18 mM and the concentration of pyruvate was varied from 0.08 to 1.44 mM in 12 steps. To study the kinetics parameters for NADH as substrate, concentration of sodium pyruvate was kept constant at 0.8 mM and the concentration of NADH was varied from 0.015 to 0.27 mM in 12 steps. The reaction was monitored at 25 °C by recording decrease in optical density at 340 nm at 5 s intervals for up to 1 min in a LABINDIA analytical UV/VIS spectrophotometer model UV 3000<sup>+</sup> and the linear rates of reaction were taken into consideration for calculations of the activity. The activity is expressed as nmoles of substrate transformed/min/ $10^6$  RBCs.

### Data Analysis

Computer analysis of substrate kinetics data was carried out using Sigma Plot version 6.1 to obtain Lineweaver–Burk, Eadie–Hofstee and Eisenthal and Cornish-Bowden plots for calculation of  $K_m$  and  $V_{max}$  values [15]. The  $K_m$  and  $V_{max}$  values obtained by the three methods were in close agreement and were averaged for final presentation of the data. Component with low  $K_m$  is designated as component I and the component with high  $K_m$  designated as component II (e.g. see Tables 1, 2; Figs. 2, 3).

Hill plot analysis was carried out to find out the substrate binding properties while Murray plot analysis was performed to determine the values of  $K_i$ ; the concentration of substrate inhibition constant [15].

The apparent  $K_{cat}$  values were calculated from the corresponding  $V_{max}$  values essentially according to methods described previously [16–18]. Thus the apparent  $K_{cat}$  represents

#### Apparent $K_{cat}$

$$= \frac{V_{max} \text{ in } \mu\text{mole of substrate transformed per second}}{1.42 \times 10^4}$$

where  $1.42 \times 10^4$  is the molecular weight of lactate dehydrogenase [19], and the values are expressed as  $K_{cat} \times 10^{-8}$ . Similarly, values of  $K_{cat}/K_m$  are given as  $K_{cat}/K_m \times 10^{-8}$ .  $K_{cat}$  represents the number of substrate

molecules turned over per enzyme molecule per second and hence  $K_{cat}$  may also be considered as the turnover number.  $K_{cat}/K_m$  is thought to be a measure of enzyme efficiency. Either a high value of  $K_{cat}$  (rapid turnover) or a low value of  $K_m$  (high affinity for substrate) will increase the catalytic efficiency [16].

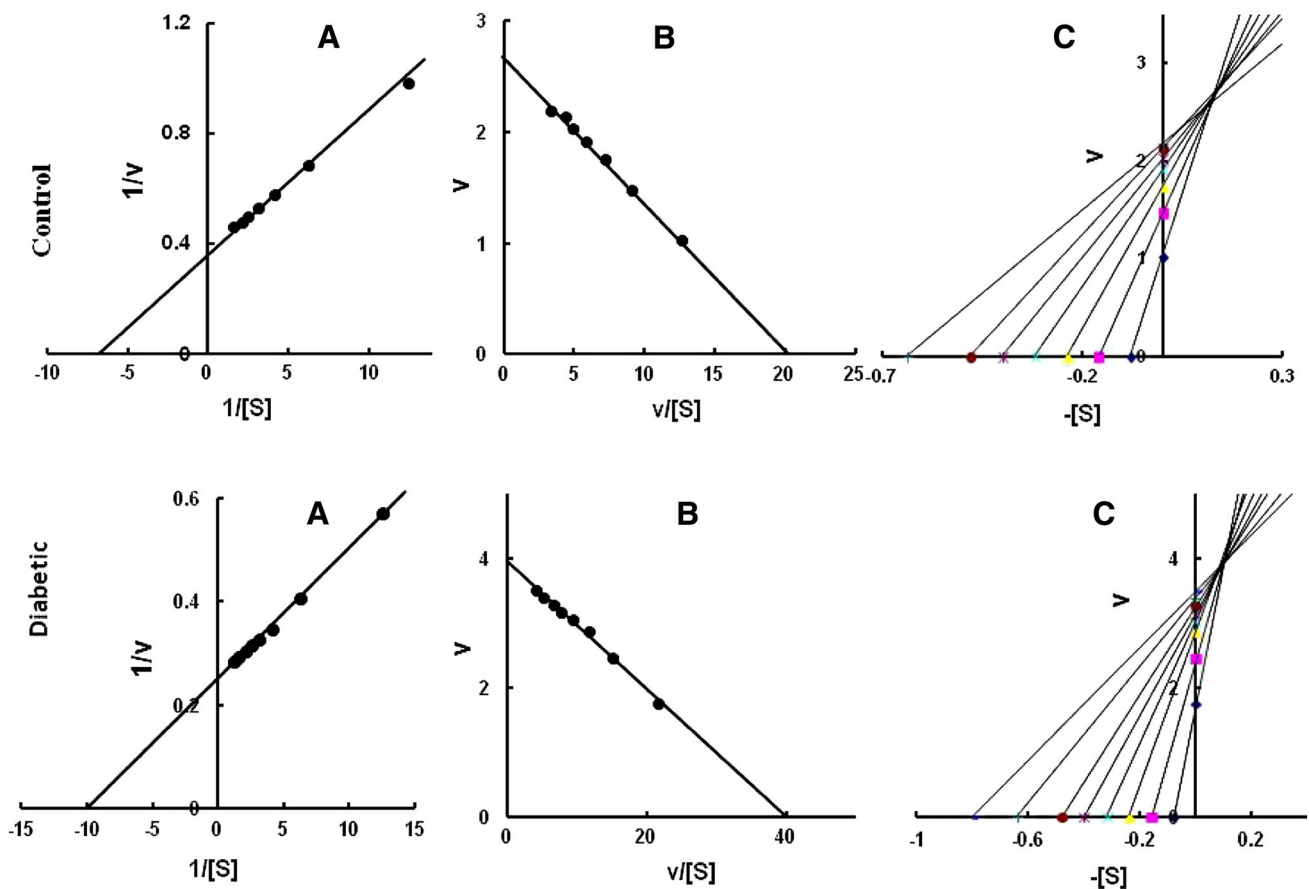
Statistical analysis of the data was carried out using GraphPad Prism version 5 and  $p$  values less than 0.05 were considered statistically significant.

### Results

The substrate saturation curves for pyruvate and NADH are shown in Fig. 1. The corresponding Lineweaver–Burk, Eadie–Hofstee and Eisenthal–Cornish-Bowden plots were obtained from substrate saturation kinetics data. These plots for pyruvate as the substrate for control and diabetic

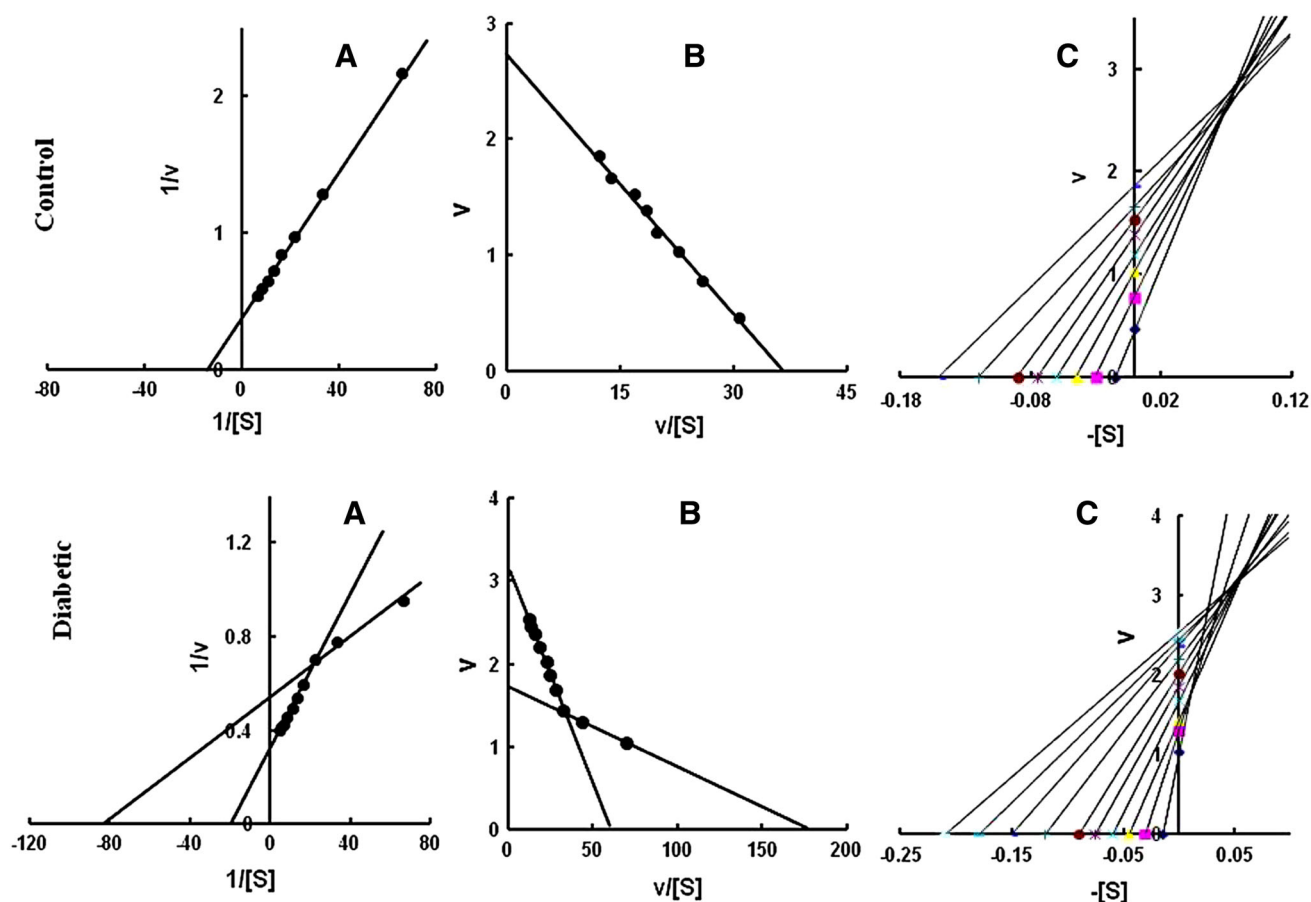
RBCs are shown in Fig. 2a–c respectively. Similar plots for NADH as the substrate are shown in Fig. 3a–c respectively. As is evident, (Fig. 2) for pyruvate as the substrate the activity resolved in a single component in both the groups. By contrast, the picture was very different for NADH as the substrate. Thus in the control group the activity resolved in the single component whereas for the diabetic group two components, one with low  $K_m$  low  $V_{max}$  and second one with high  $K_m$  and high  $V_{max}$  was apparent (Fig. 3). The  $K_m$  and  $V_{max}$  values were calculated by the above three methods and averaged for final presentation of data.

As can be noted from data in Table 1, in the diabetic group, the  $K_m$  for pyruvate was unchanged. However, consistent with our earlier report [11] the value of  $V_{max}$  was significantly high (53% increase). With NADH as the substrate, in the control group the  $K_m$  was 69  $\mu\text{M}$  and the  $V_{max}$  was comparable to that noted with pyruvate as the substrate. In the diabetic group the component with low



**Fig. 2** Corresponding Lineweaver–Burk (a), Eadie–Hofstee (b) and Eisenthal–Cornish–Bowden (c) plots for lactate dehydrogenase from Control and Diabetic erythrocytes with Na pyruvate as the substrate. For Lineweaver–Burk plots (a) the reciprocal of enzyme activity ( $1/v$ ) is plotted on abscissa versus reciprocal of substrate concentration ( $1/[S]$ ) where substrate concentration is in mM. For Eadie–Hofstee plot

(b) the reaction velocity,  $v$  is plotted on abscissa versus  $v/[S]$  on the ordinate. In the Eisenthal–Cornish–Bowden plots (c) the reaction velocity,  $v$  is plotted on abscissa versus substrate concentration is in mM on negative scale. The results are typical of 6 independent observations



**Fig. 3** Corresponding typical Lineweaver–Burk (a), Eadie–Hoffstee (b) and Eisenthal–Cornish–Bowden (c) plots for lactate dehydrogenase from Control and Diabetic erythrocytes with NADH as the substrate. For Lineweaver–Burk plots (a) the reciprocal of enzyme activity ( $1/v$ ) is plotted on abscissa versus reciprocal of substrate concentration ( $1/[S]$ ) where substrate concentration is in mM. For Eadie–Hoffstee plot

(b) the reaction velocity,  $v$  is plotted on abscissa versus  $v/[S]$  on the ordinate. In the Eisenthal–Cornish–Bowden plots (c) the reaction velocity,  $v$  is plotted on abscissa versus substrate concentration is in mM on negative scale. The results are typical of 6 independent observations

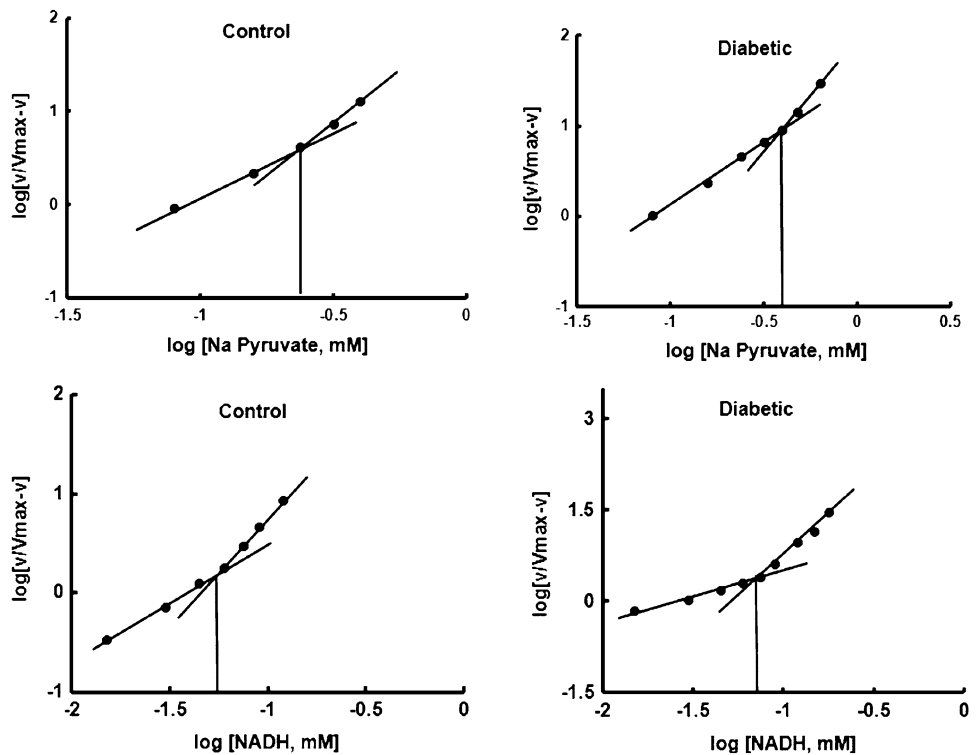
$K_m$  (Component I) showed a very low  $K_m$  i.e.  $9 \mu\text{M}$ . However, the  $V_{\text{max}}$  was almost comparable to that noted for pyruvate in the control group as well as that noted for control with NADH as the substrate. Even for component II, the  $K_m$  was significantly lower than that noted for the control group ( $47 \mu\text{M}$  against  $69 \mu\text{M}$ ), however the  $V_{\text{max}}$  was significantly high (Table 1).

In view of the observed changes in the  $K_m$  and  $V_{\text{max}}$ , it was of interest to find out how these factors influenced the catalytic efficiency of the enzyme. This was determined in terms of apparent  $K_{\text{cat}}$  and apparent  $K_{\text{cat}}/K_m$  values. The results are given in Table 2. As can be noted, with pyruvate as the substrate the apparent  $K_{\text{cat}}$  as well as apparent  $K_{\text{cat}}/K_m$  values increased significantly in the diabetic patients (53 and 51% increase respectively). With NADH as the substrate the apparent  $K_{\text{cat}}$  value for component I was close to that noted for pyruvate in the control group but the value of apparent  $K_{\text{cat}}/K_m$  was about seven folds higher. For component II the value for apparent  $K_{\text{cat}}$  was

comparable to that of pyruvate in the control group but the apparent  $K_{\text{cat}}/K_m$  values almost doubled (Table 2).

The substrate binding characteristics as derived from Hill Plots (Fig. 4) are given in Table 3. Thus for sodium pyruvate as the substrate, the enzyme from both the groups bound one molecule of substrate in low concentration range whereas in the high substrate concentration range three molecules of the substrate were bound. The transition substrate concentration for the two groups was comparable i.e.  $250$  and  $240 \mu\text{M}$ . With NADH as the substrate the enzyme from both the groups bound one molecule of the substrate in the low concentration range. In the high concentration range the enzyme bound two molecules of substrate in the control group; by contrast in the diabetic group three molecules of substrate were bound. The transition concentrations also differed significantly. For the control group the transition concentration was  $54 \mu\text{M}$  whereas in the diabetic group this value shifted to higher concentration of  $91 \mu\text{M}$  (e.g. see Fig. 4).

**Fig. 4** Corresponding Hill plots for lactate dehydrogenase from Control and Diabetic erythrocytes with Na pyruvate and NADH as the substrate. The values of  $\log[v/V_{max} - v]$  on abscissa are plotted against  $\log$  substrate concentration (mM). *Perpendicular line* indicates point of Transition Concentration. The results are typical of 6 independent observations



**Table 3** Hill plot analysis of erythrocyte lactate dehydrogenase activity from Type II diabetic patients

Substrate	Subject	Hill coefficient		Transition concentration ( $\mu\text{M}$ )
		$n_1$	$n_2$	
Na pyruvate	Control	$1.05 \pm 0.06$	$2.74 \pm 0.05$	$250.0 \pm 12.8$
	Diabetic	$0.64 \pm 0.13$	$2.83 \pm 0.12$	$237.7 \pm 30.0$ NS
NADH	Control	$0.75 \pm 0.05$	$2.24 \pm 0.08$	$54.2 \pm 3.5$
	Diabetic	$0.75 \pm 0.01$	$3.11 \pm 0.22^*$	$90.5 \pm 6.0^{**}$

Results are given as mean  $\pm$  SEM of 6 independent observations in each group

\*  $p < 0.004$ ; \*\*  $p < 0.0004$

In the next set of experiments we tried to evaluate substrate inhibition characteristics based on Murray plots. Typical Murray plots are shown in Fig. 5 and the values of  $K_i$  are given in Table 4. As can be noted, there was marginal but reproducible decrease in the  $K_i$  value for Na Pyruvate (23% decrease). The picture was opposite for NADH as the substrate. Thus the value of  $K_i$  in the control group was 1.15 mM; this value became almost three fold higher in the diabetic group.

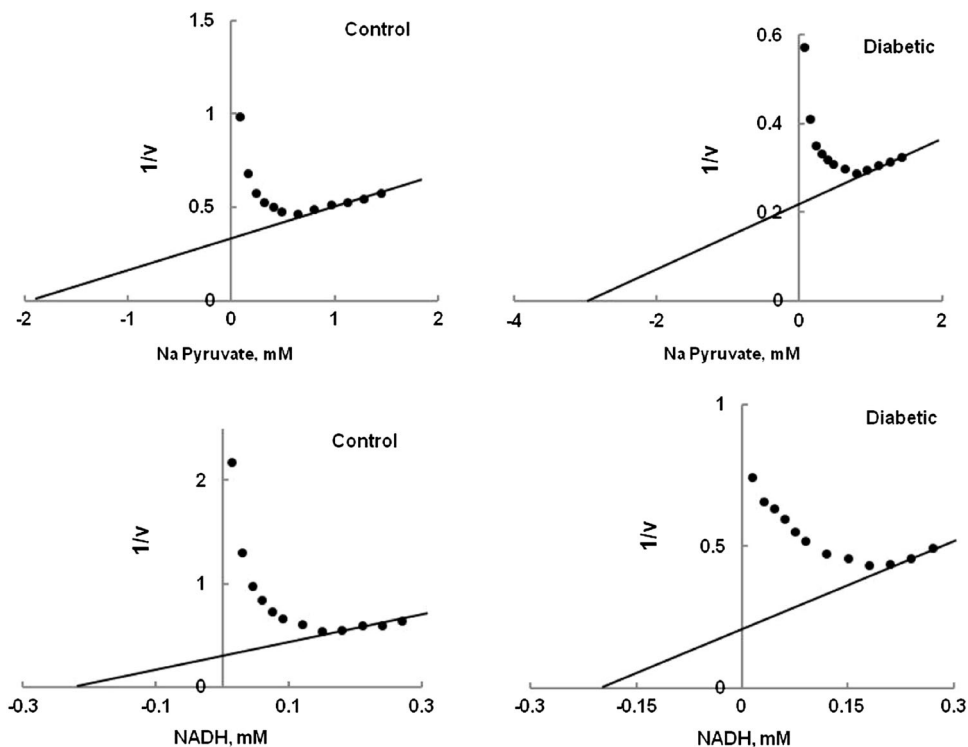
**Discussion**

From the data presented it is clear that with pyruvate as the substrate the  $K_m$  of LDH from diabetic erythrocytes was unchanged. Our observations on the  $K_m$  for pyruvate agree with the values reported in the literature [20]. Interestingly,

however, the  $V_{max}$  value increased by 52% in the diabetic group which agrees well with our previously reported observation [11]. Kinetics studies with NADH as the substrate displayed an entirely different picture. Thus the control group showed presence of a single component whereas in the diabetic group the system resolved in two components: one with low  $K_m$  and the second one with high  $K_m$ .

From survey of literature we could not get information regarding the  $K_m$  of LDH from RBCs; apparently the major emphasis has been to delineate the kinetics properties with pyruvate as the substrate. Thus it would appear that we are reporting for the first time the substrate saturation kinetics properties with NADH as the substrate. As is evident (Table 1; Fig. 3) in the control group the  $K_m$  for NADH was about half that for pyruvate. This would imply that NADH may not be rate limiting and reaction can

**Fig. 5** Corresponding Murray plots for lactate dehydrogenase from Control and Diabetic erythrocytes with Na pyruvate and NADH as the substrate. The values of reciprocal of reaction velocity ( $1/v$ ) is plotted on abscissa versus substrate concentration (mM) on the ordinate. The results are typical of 6 independent observations



**Table 4** Murray plot analysis of erythrocyte lactate dehydrogenase activity from Type II diabetic patients

Substrate	Ki (mM)	
	Control	Diabetic
Na pyruvate	$1.15 \pm 0.032$	$3.32 \pm 0.077^{**}$
NADH	$0.26 \pm 0.014$	$0.20 \pm 0.018^*$

Results are given as mean  $\pm$  SEM of 6 independent observations in each group

\*  $p < 0.025$ ; \*\*  $p < 0.0001$

proceed in the forward direction towards formation of lactate. In the case of diabetic RBCs component I showed about 7 times lower  $K_m$  compared to that in the control groups. Even for component II the  $K_m$  was lower by 32%, however, the  $V_{max}$  was high. The results thus suggest that the catalytic efficiency of the enzyme from diabetic RBCs is significantly high. This is exemplified by data in Table 2. Thus for pyruvate the  $K_{cat}$  and  $K_{cat}/K_m$  values were significantly high. However, most importantly with NADH even component I showed  $K_{cat}$  values comparable to that of control and the  $K_{cat}/K_m$  value increased seven folds. For component II also  $K_{cat}$  value was about 30% higher and that of  $K_{cat}/K_m$  increased by 86%. Even substrate binding characteristics for NADH changed significantly. Thus the transition concentration increased from 54 to 91  $\mu\text{M}$  but in higher concentration range the enzyme from diabetic erythrocytes bound 3 molecules of NADH as

against 2 molecules bound by enzyme from control group; in lower concentration range enzyme from both the group bound only one molecule of NADH. These changes are once again consistent with enhanced catalytic activity of the enzyme from diabetic RBCs (Table 3). One additional feature was increased resistance to inhibition by pyruvate in the diabetic group where the  $K_i$  almost doubled; there was only marginal decrease in  $K_i$  for NADH (Table 4).

Taken together the results suggest that in diabetic erythrocytes there are significant alterations in the kinetic properties of lactate dehydrogenase which are favorable for efficient conversion of pyruvate to lactate. As is well recognized, the tissues which have predominately anaerobic glycolysis convert pyruvate to lactate which is released in the plasma. In normal individual RBCs contribute about 20% of the plasma lactate level [13]. Obviously these levels would be significantly higher in diabetes. As can be inferred from the data presented increased catalytic efficiency together with increased resistance to inhibition by higher concentrations of pyruvate are the major influencing factors.

It has been reported that LDH 1 and LDH 2 are the major isoforms in the erythrocytes from normal individuals and make up about 80% of the total activity. It has been reported that the  $K_m$  for heart type of LDH (LDH 1 and LDH 2) is in the range of 0.1 mM whereas that of muscle type of LDH (LDH 4 and LDH 5) is in much higher ranging from 0.35 to 3 mM. It may therefore be inferred that the enzyme in the diabetic erythrocyte shows entirely

different and unique characteristics especially with NADH as the substrate. Non-enzymatic glycosylation is a distinguishing feature of diabetes. It is possible but not clear if such structural changes could influence the catalytic activity of LDH. Alternately, it is possible that the LDH isozyme pattern might have changed in the diabetic RBCs. These possibilities need to be ascertained by more direct experiment. Nonetheless data of our present studies emphasize the significant role of altered kinetic properties of LDH in diabetic lactic acidosis.

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**Compliance with ethical standards**

**Conflict of interest** None.

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