

## **Oxalate: A potential ligand for differentiating *Plasmodium berghei* lactate dehydrogenase from host erythrocyte lactate dehydrogenase**

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### **Abstract**

*Plasmodium* lactate dehydrogenase is a valuable malaria diagnostic indicator. However, the difference between *Plasmodium* and host lactate dehydrogenase (LDH) activities in the presence of ligands has not been fully characterized. This study investigated the effects of  $\text{Co}^{2+}$  and oxalate on lactate dehydrogenase activities in *Plasmodium berghei*-infected erythrocyte (PBIE-LDH) and uninfected erythrocytes (UE-LDH) of mice. LDH activities in PBIE and UE were determined in the presence of varying concentrations of  $\text{Co}^{2+}$  and oxalate.  $V_{\max}$  of PBIE-LDH was three times higher (with reduced  $K_m$ ) than that of UE-LDH in the absence of the ligands.  $\text{Co}^{2+}$  activated both PBIE-LDH and UE-LDH activities with optimal concentrations at 10 mM and 5 mM respectively. Oxalate (0.1-10 mM) inhibited UE-LDH activity. Moreover, oxalate (from 0.1 to 2 mM) progressively inhibited PBIE-LDH activity but concentrations higher than 2 mM (up to 10 mM) progressively reversed this inhibition but not to the range of the enzyme activity in the absence of oxalate. Kinetic analyses revealed that  $V_{\max}$  of PBIE-LDH in the presence of 5 mM oxalate and 5 mM  $\text{Co}^{2+}$  was one and a half times and five times higher (with reduced  $K_m$ ) than those of UE-LDH respectively. These results suggest that the *P. berghei* LDH can be differentiated from host erythrocyte LDH in the presence of oxalate at concentrations higher than 2 mM. This could be of significance in antimalarial screening programmes and, by extension, effective diagnosis of malaria caused by other *Plasmodium* species and monitoring of recovery during treatment.

**Keywords:** Lactate dehydrogenase, *Plasmodium*, ligands, malaria diagnosis

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## Introduction

Malaria is a parasitic disease that claims approximately a million lives worldwide annually with 35 countries in central Africa bearing the highest burden of cases and deaths (National Institute of Allergy and Infectious Diseases, 2007; Agomo *et al.*, 2009 and WHO, 2011). Malaria is caused by a protozoan parasite called *Plasmodium*, belonging to the parasitic phylum *Apicomplexa*. Five *Plasmodium* species have been documented to cause human malaria, namely: *P. falciparum*, *P. vivax*, *P. ovale*, *P. malariae* and *P. knowlesi* (Kakkilaya, 2011). The global impact of malaria has spurred interest in developing effective diagnostic strategies not only for resource-limited areas where malaria is a substantial burden on society, but also in developed countries, where malaria diagnostic expertise is often lacking (Bell *et al.*, 2005). Various detection and diagnostic methods have been developed for malaria, but none of these methods is without limitations. Microscopy, generally known as the “gold standard” for malaria diagnosis, is the most widely used routine method (Kakkilaya, 2003; Murray, 2008 and Rosenthal, 2012). However, it is limited by its requirements of special skills and experience for effective diagnosis. It is also time and effort consuming. In addition, the many variations in staining and microscopic techniques between laboratories make comparison of results difficult (Cooke *et al.*, 1999). Thus, there is the need for a more rapid and standardized simple-to-use diagnostic method.

Several rapid diagnostic tests (RDTs) currently in the market can consistently detect over 95% of parasite infections at 200 parasites per microlitre, with 95% specificity. All RDTs are based on the same principle and detect malaria antigen in blood flowing along a membrane containing specific antimalarial antibodies; they do not require laboratory equipment (Tangpukdee *et al.*, 2009). *Plasmodium* LDH (pLDH) is one of the main groups of antigens detected by RDTs. pLDH is a soluble glycolytic enzyme produced by the asexual and sexual stages of the live parasites and it is present in, and released from, the parasite infected erythrocytes. It has been found in all human malaria species, and different isomers of pLDH exist for

each species. Previous studies have showed a correlation between levels of parasitemia and the activity of pLDH (Xu *et al.*, 2007).

Analysis of *Plasmodium* enzymes showed that host- and parasite- specific enzymes could be distinguished. *Plasmodium falciparum* LDH can be differentiated from that of human LDH by using 3-acetylpyridine adenine dinucleotide (APAD) as the coenzyme instead of  $\beta$ -nicotinamide adenine dinucleotide (NADH or NAD<sup>+</sup>) (Makler *et al.*, 1993; Makler and Hinrichs, 1993). pLDH can utilize APAD at 200 fold more rapidly and effectively than host LDH isoforms. This property permits measurement of pLDH and malaria diagnosis in man (Riandey *et al.*, 1996). However, the cost of APAD is very expensive relative to NADH or NAD<sup>+</sup>. Besides, the difference between pLDH and host LDH activities in the presence of ligands has not been fully characterized. In this study, the effects of two ligands (Co<sup>2+</sup> and oxalate) on *Plasmodium berghei*-infected erythrocyte LDH (PBIE-LDH) and uninfected erythrocyte LDH (UE-LDH) activities of mice were investigated using NAD<sup>+</sup> as cofactor with a view to differentiating pLDH from host LDH for effective diagnosis of malaria.

## **Materials and Methods**

### **Animals and Reagents**

Four Swiss male albino mice were obtained from the Animal Holding Unit, Department of Biochemistry, University of Ilorin, Ilorin, Nigeria.  $\beta$ -nicotinamide adenine dinucleotide (NAD<sup>+</sup>) salt and sodium salts of L-lactate, pyruvate and oxalate were products of Sigma-Aldrich, UK. All other reagents used were of analytical grade.

### **Parasite**

The malaria parasite, *Plasmodium berghei* strain, was obtained from the Institute of Medical Research and Training (IMRAT), University of Ibadan, Ibadan, Nigeria.

### **Inoculation of Mice**

Two albino mice were inoculated intraperitoneally with *Plasmodium berghei* using standard inoculum size (0.2ml of  $1 \times 10^7$  infected erythrocytes). The parasitemia was allowed to build up in the mice up to 10%. The parasitemia was determined by making blood smears from the tail of the mice, which was fixed in methanol, stained with Giemsa and examined under microscope ( $\times 1000$  magnification).

### **Isolation of Lactate Dehydrogenase**

Blood was collected from the infected and uninfected mice. This was anaesthetised with diethyl ether, by cardiac puncture. The collected blood was dispensed into ethylene diamine tetraacetic acid (EDTA)-containing sample bottles. The blood samples were centrifuged at  $3500 \times g$  for 10 minutes and the blood plasma (supernatant) was separated from the packed blood cells (pellet) into separate bottles. The separated red blood cells were subsequently subjected to a series of thawing and freezing to enhance lysis of the cells for the release of erythrocyte lactate dehydrogenase from both parasitised and non-parasitised cells.

### **Determination of Lactate Dehydrogenase Activities**

PBIE-LDH and UE-LDH activities were determined by measuring the amount of pyruvate produced from the dehydrogenation of lactate using  $\text{NAD}^+$  as coenzyme according to the method described by Kubowitz and Otto (1943). To a reaction mixture containing 0.3 ml of 0.1M glycine buffer (pH 10.0) and 1.0ml of 150mM buffered lactate salt (substrate) solution, 0.1ml of the crude enzyme was added except for the blank and the mixture was left to equilibrate in a water bath at  $37^\circ\text{C}$  for 5 minutes. Reaction was initiated by the addition of 0.1ml of 7.5 mM  $\text{NAD}^+$  solution to the mixture and incubated at  $37^\circ\text{C}$  for 15 minutes. Thereafter, 0.5 ml of 1 mM DNPH solution was added and the mixture was again incubated at  $37^\circ\text{C}$  for another 15 minutes. 0.1 ml of the enzyme solution was then added to the blank before the reaction was terminated by the addition of 1.0ml of 1M NaOH. The absorbance was read at 440nm against the reagent blank using a Spectrumlab 752S UV-Visible spectrophotometer. Absorbance was

compared with the standard graph to determine the concentration of pyruvate produced [the standard graph was obtained by reacting known concentrations of pyruvate solution (0.0167 $\mu$ M-0.167 $\mu$ M) with 1 mM DNPH and plotting a graph between concentration of pyruvate and absorbance]. One unit of lactate dehydrogenase activity was defined as the amount of enzyme that could dehydrogenate lactate and release 1 micromole of pyruvate per minute at 37°C.

#### **Determination of Protein Concentration**

Protein concentration was determined using Biuret method with bovine serum albumin (BSA) as standard (Gornall *et al.*, 1949).

#### **Substrate Kinetics of PBIE-LDH and UE-LDH activities**

Reaction mixture containing 0.1ml of 1M glycine buffer (pH 10.0), buffered lactate solution ranging from 0.5mM to 62.5mM and 0.1ml of the crude enzyme were pre-incubated at 37°C for 5 minutes. Reaction was initiated by the addition of 0.1ml of 7.5 mM NAD<sup>+</sup> solution and then incubated at 37°C for 15 minutes. Thereafter, 0.5 ml of 1mM DNPH solution was added and the mixture was again incubated at 37°C for another 15 minutes. The reaction was terminated by the addition of 1.0ml of 1M NaOH. The absorbance was read at 440nm against a reagent blank. The data obtained were fitted to the Lineweaver-Burk plot using Microsoft Excel and kinetic constants, maximum reaction rate ( $V_{max}$ ) and Michaelis constant ( $K_m$ ), were calculated.

#### **Effects of Cobalt Ions and Oxalate on PBIE-LDH and UE-LDH activities**

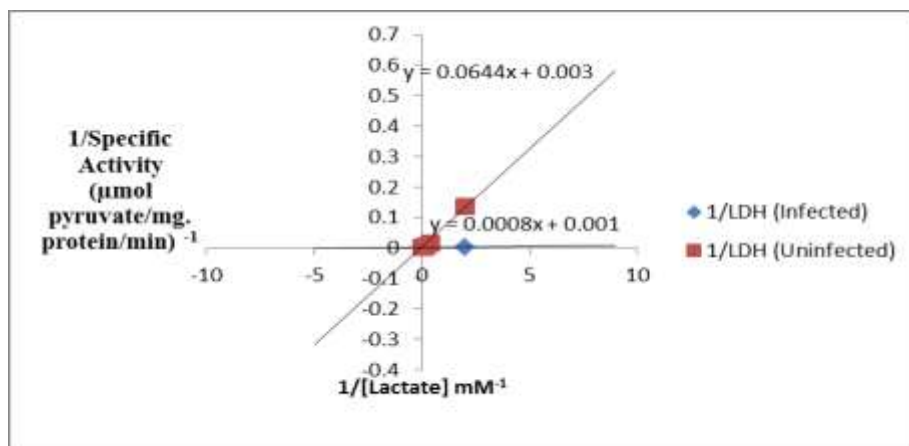
Reaction mixture containing 0.1ml of 1M glycine buffer (pH 10.0), 50 mM buffered lactate solution and 0.1ml of the crude enzyme were incubated at 37°C for 5 minutes. Reaction was initiated by the addition of 0.1ml of 7.5 mM NAD<sup>+</sup> solution and 0.2 mM-10 mM Co<sup>2+</sup> or oxalate and incubated at 37°C for 15 minutes. Thereafter, 0.5 ml of 1mM DNPH solution was added and the mixture was again incubated at 37°C for another 15 minutes. The reaction was terminated by the addition of 1.0ml of 1M NaOH. The absorbance was read at 440nm against a reagent blank. For the kinetics of

LDH activity in the presence of  $\text{Co}^{2+}$  or oxalate, reaction mixture containing 0.1ml of 1M glycine buffer (pH 10.0), buffered lactate solution ranging from 0.5 mM to 62.5mM and 0.1ml of the crude enzyme were pre-incubated at 37°C for 5 minutes. Reaction was initiated by the addition of 0.1ml of 7.5 mM  $\text{NAD}^+$  solution and 5 mM  $\text{Co}^{2+}$  or oxalate and then incubated at 37°C for 15 minutes. Thereafter, 0.5ml of 1mM DNPH solution was added and the mixture was again incubated at 37°C for another 15 minutes. The reaction was terminated by the addition of 1.0ml of 1M NaOH. The absorbance was read at 440nm against a reagent blank. The data obtained were fitted to the Lineweaver-Burk plots using Microsoft Excel and kinetic constants ( $V_{\max}$  and  $K_m$ ) were calculated.

## Results

### Substrate Kinetics of PBIE-LDH and UE-LDH activities

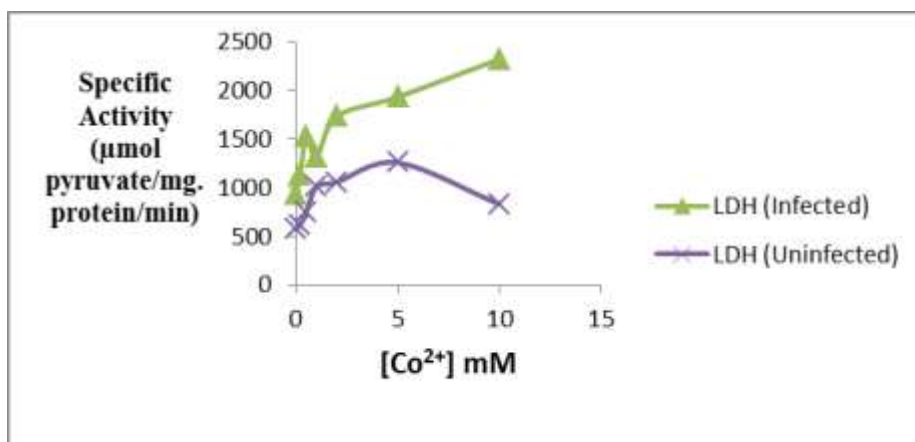
The Lineweaver-Burk plot for the substrate kinetics of PBIE-LDH and UE-LDH activities is shown in Figure 1. The maximum reaction rate ( $V_{\max}$ ) of PBIE-LDH was three times higher with decreased  $K_m$  than that of UE-LDH (Table 1).



**Figure 1: Lineweaver-Burk plot for the Substrate Kinetics of Lactate Dehydrogenase Activities in *Plasmodium berghei*- infected and Uninfected Erythrocytes of Mice**

### Effects of Cobalt Ions and Oxalate on PBIE-LDH and UE-LDH activities

$\text{Co}^{2+}$  activated both PBIE-LDH and UE-LDH activities with optimal concentrations at 10 mM and 5 mM respectively (Figure 2). On the other hand, oxalate (0.1-10 mM) inhibited UE-LDH activity (Figure 3). In addition, oxalate inhibited PBIE-LDH activity progressively from 0.1 to 2 mM concentrations but concentrations higher than 2 to 10 mM of oxalate progressively reversed the inhibition observed at lower concentrations but not to the range of the enzyme activity in the absence of oxalate (Figure 3). The Lineweaver-Burk plots for the effects of cobalt ions and oxalate on PBIE-LDH and UE-LDH activities are shown in Figures 4 and 5 respectively. Kinetic analyses showed that the  $V_{\max}$  of PBIE-LDH in the presence of 5 mM  $\text{Co}^{2+}$  was five times higher with decreased  $K_m$  than that of UE-LDH (Table 1). Also, the  $V_{\max}$  of PBIE-LDH in the presence of 5 mM oxalate was one-half times higher with decreased  $K_m$  than that of UE-LDH (Table 1).



**Figure 2: Effects of Cobalt Ion on Lactate Dehydrogenase Activities in *Plasmodium berghei*-infected and Uninfected Erythrocytes of Mice**

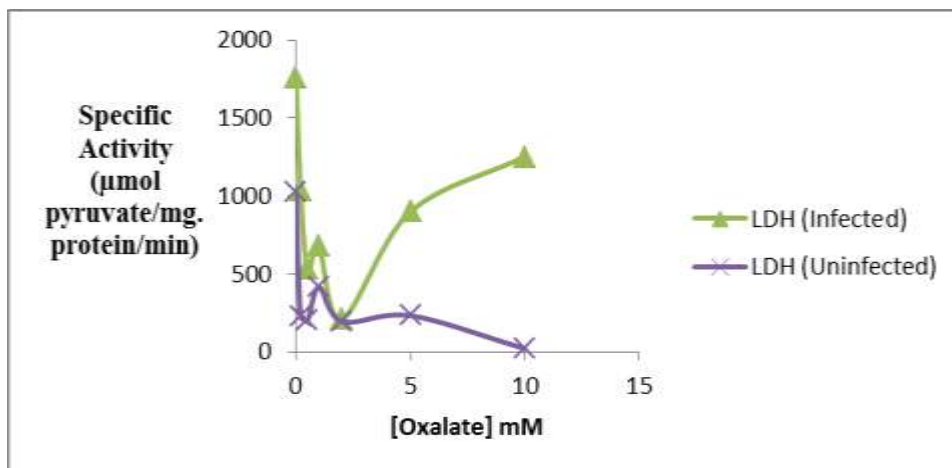


Figure 3: Effects of Oxalate on Lactate Dehydrogenase Activities in *Plasmodium berghei*- infected and Uninfected Erythrocytes of Mice

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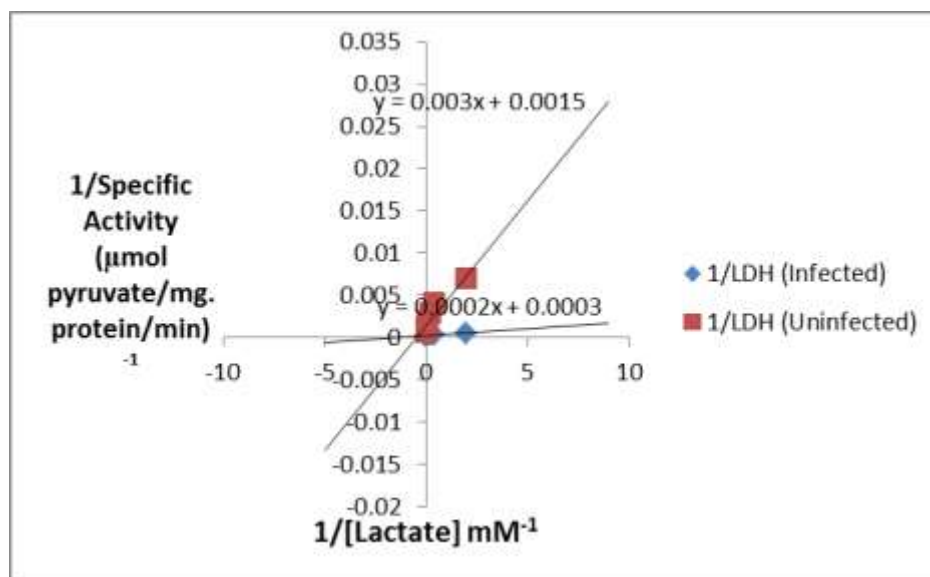
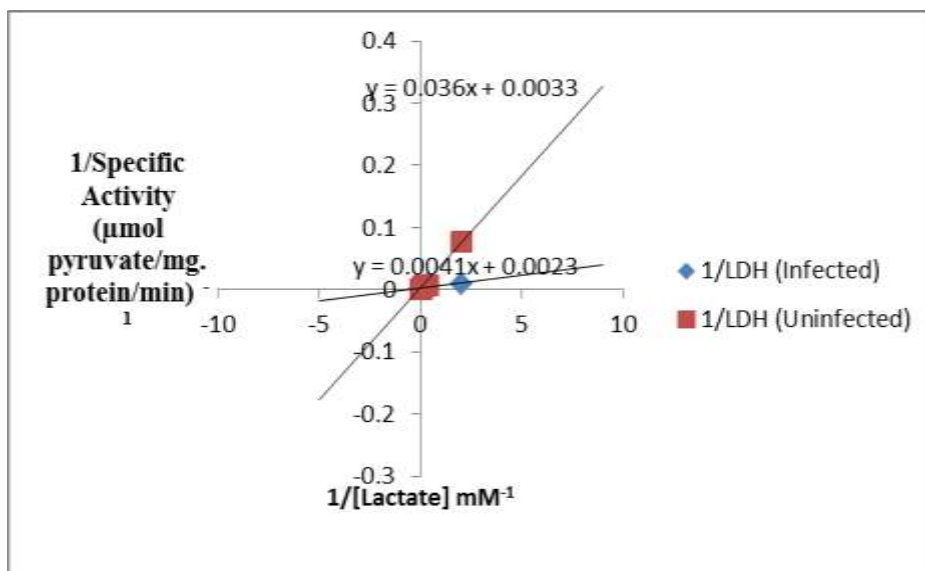


Figure 4: Lineweaver-Burk Plot of the Effect of Cobalt Ion on Lactate Dehydrogenase Activities in *Plasmodium berghei*- infected and Uninfected Erythrocytes of Mice



**Figure 5: Lineweaver-Burk Plot for the Effect of Oxalate on Lactate Dehydrogenase Activities in *Plasmodium berghei*-infected and Uninfected Erythrocytes of Mice**

**Table 1: Effects of Cobalt Ion and Oxalate on the Kinetic Parameters of Lactate Dehydrogenase in *Plasmodium berghei*-infected and Uninfected Erythrocytes of Mice**

| Parameters                              | $V_{\max}$ ( $\mu\text{mol}$ pyruvate/mg protein/min) | $K_m$ (mM) |
|-----------------------------------------|-------------------------------------------------------|------------|
| LDH (Uninfected)                        | 333.33                                                | 21.47      |
| LDH (Infected)                          | 1000.00                                               | 0.80       |
| LDH (Uninfected) + 5mM $\text{Co}^{2+}$ | 666.67                                                | 2.00       |
| LDH (Infected) + 5mM $\text{Co}^{2+}$   | 3333.33                                               | 0.67       |
| LDH (Uninfected) + 5mM Oxalate          | 303.03                                                | 10.91      |
| LDH (Infected) + 5mM Oxalate            | 434.78                                                | 1.78       |

## Discussion

The effects of the two ligands ( $\text{Co}^{2+}$  and oxalate) on LDH activities investigated in this study were aimed at establishing a difference between *Plasmodium* and host erythrocyte LDH activities as a diagnostic indicator for malaria. The kinetic properties of LDH activities in plasmodium-infected and uninfected erythrocytes of mice in the absence of ligands were compared. The higher turnover rate of lactate to pyruvate catalysed by PBIE-LDH when compared with UE-LDH as observed in this study is in conformity with the report by Cameron *et al.* (2004). A comparison of the crystal structures of both *Plasmodium falciparum* LDH and human LDH showed two key differences namely: the positioning of the NADH factor, reflecting sequence changes in the cofactor binding pocket, and a change in the sequence (including a 5-residue insertion) and secondary structure of a loop region that closes down on the active site during catalysis. These changes combine to produce an increase in the volume of the active site cleft in *Plasmodium* LDH relative to its human counterpart (Cameron *et al.*, 2004), thus enhancing higher catalytic activity. The fact that the genome of *P. berghei* has high similarity both in structure and content, with that of *P. falciparum* (Janse *et al.*, 2006), may also be responsible for the observed higher  $V_{\text{max}}$  of PBIE-LDH. In addition, the stronger binding affinity of PBIE-LDH than that of UE-LDH could have been responsible for the higher activity.

Furthermore, the effects of  $\text{Co}^{2+}$  and oxalate on PBIE-LDH and UE-LDH activities were compared.  $\text{Co}^{2+}$  activated both LDH activities. This observation can be corroborated with the report of Sailaja *et al.* (2013) who showed that  $\text{Co}^{2+}$  caused a significant increase in LDH activity accompanied by a decrease in the lactic acid content and an increase in the pyruvate content of gonads of silkworm, *Bombyx mori* L. Kinetic analyses showed that the turnover rate of lactate to pyruvate catalysed by PBIE-LDH in the presence of 5 mM  $\text{Co}^{2+}$  was five times higher with stronger binding affinity of the enzyme for lactate than that of UE-LDH. The inhibitory effect of oxalate as observed in this study corroborates earlier reports (Mohrenweiser

and Novotny, 1982; Schurr and Payne, 2007; Zeczycki *et al.*, 2010). Most studies on the inhibitory effect of oxalate had been on human lactate dehydrogenase isozymes (Novoa *et al.*, 1958; Zeczycki *et al.*, 2010), and LDH of some microorganisms (Bacchi *et al.*, 1970; Assa *et al.*, 2005). Oxalate has been described as a non-competitive inhibitor of LDH that competes with lactate in its binding to LDH-NAD<sup>+</sup> complex to form the ternary complex LDH-NAD<sup>+</sup>-lactate (Mohrenweiser and Novotny, 1982; Cameron *et al.*, 2004). Bacchi *et al.* (1970) worked on *Trypanosoma conorhini* LDH and reported that the human serum LDH was ten times more sensitive to oxalate than the parasite enzyme although pyruvate was used as substrate (i.e. forward reaction). Oxalate inhibition varies with the source of enzyme, irrespective of substrate; it inhibited LDH<sub>1</sub> and LDH<sub>2</sub> of heart more than LDH<sub>4</sub> and LDH<sub>5</sub> from muscle and inhibited *T. conorhini* LDH at approximately the same concentration regardless of substrate (Bacchi *et al.*, 1970). Thus, the gradual reversal of inhibition in *Plasmodium berghei*-infected erythrocyte LDH activity as opposed to further decline of the uninfected LDH activity in the presence of 2 to 10 mM oxalate could be due to the presence of the *Plasmodium berghei* LDH isozyme. However, further studies on purified *Plasmodium-berghei* LDH enzyme are needed to establish the observed effect.

Findings from this study revealed that the *Plasmodium berghei* LDH can be differentiated from host erythrocyte LDH in the presence of oxalate at concentrations higher than 2 mM. This could be of significance in antimalarial screening programmes. Due to the fact that the genome of *P. berghei* has high similarity with that of *P. falciparum* (the most virulent species), by extension, oxalate may also be useful in distinguishing *P. falciparum* LDH from host erythrocyte LDH, which will be of importance in effective diagnosis and monitoring of recovery during treatment of malaria.

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