Indian Journal of Biochemistry & Biophysics
Vol. 63, October 2026, pp. 1135-1144
DOI: 10.56042/ijbb.v63i10.26408

Lactate dehydrogenase: Lactate oxidation and role of ROS

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Received 10 December 2025; *revised 11 September 2026*

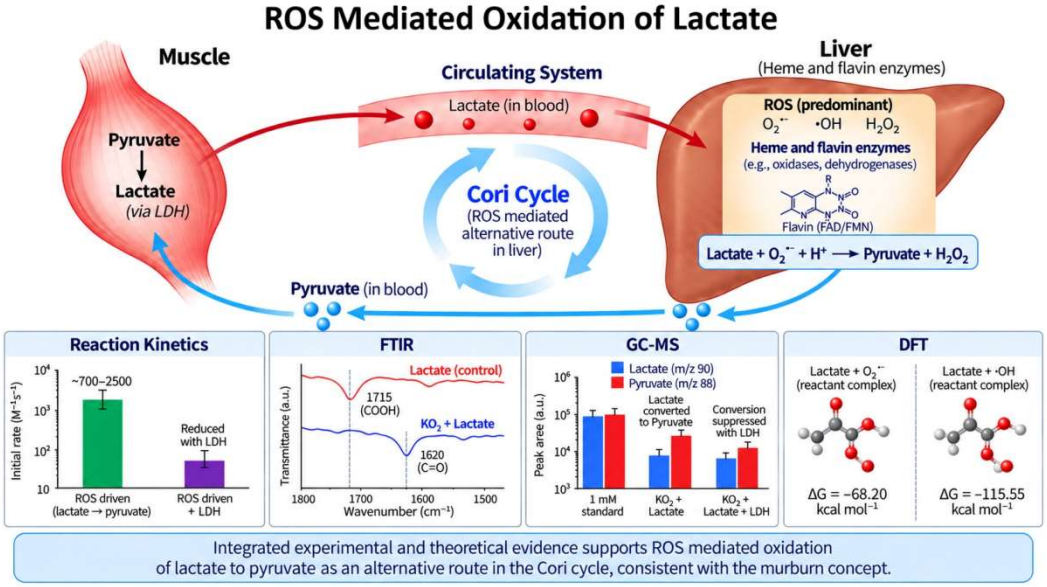
Lactate dehydrogenase is a well-studied nonspecific biomarker with profound implications in diseases like cancer. *In vitro* investigations into the mechanism of lactate dehydrogenase action described herein are based on our group's earlier reported thermodynamic calculations. While the reverse (pyruvate to lactate) reaction is thermodynamically favourable, the forward (lactate to pyruvate) reaction is unfavourable at physiological conditions. NADH consumption (lactate formation) rates were high, in the range of 2000-3000 M^{-1} s^{-1} contrary to the NADH formation (pyruvate formation) rates that were low (50 to < 300 M^{-1} s^{-1}) under classical reaction conditions. Interestingly, the lactate to pyruvate reactions driven with ROS (peroxide, hydroxyl radical or superoxide) alone demonstrated strikingly high reaction rates (~ 700-2500 M^{-1} s^{-1}). However, inclusion of lactate dehydrogenase into such reactions rapidly lowered the NADH formation rates. GC-MS and FT-IR results supported the superoxide-assisted oxidation of lactate to pyruvate in KO_2 containing reaction mixtures. DFT analysis further demonstrated the strong thermodynamic viability of ROS mediated oxidation of lactate. Comprehensively, the findings suggest an alternate route of lactate oxidation, *i.e.*, ROS mediated catalysis of lactate to pyruvate. The findings support the murburn concept because the observed ROS-dependent lactate oxidation is consistent with a central prediction of the murburn framework.

Keywords: Lactate, Murburn concept, Pyruvate, Reaction kinetics, Reactive oxygen species (ROS), Thermodynamics

Lactate dehydrogenase (LDH) is a widely studied oxidoreductase (1.1.1.27) that is present in almost all cell types in humans; it is also present in microbes, plants and animals[1]. Of the five isomeric forms in humans, the H_4/B_4/LDH1 tetramer is the major L-LDH type in heart (H) tissue, while M_4/A_4/LDH5 is the major isozyme in muscle (M) and liver tissues[2]. Type H is predominant in aerobic tissues, while type M is mostly associated with hypoxic tissues, such as skeletal muscle[3]. LDHD is a mitochondrial D-LDH that is expressed in almost all tissues; higher expression of LDHD is observed in certain cancers and during bacteremia.

LDH is a nonspecific biomarker for detection of 48 different human disease types[1]. It is a pharmacological target in many human ailments and is a nonspecific marker of tissue injury in diseases such as cancer, malaria, myocardial infarction, anemia as well as infectious diseases[4-6]. LDH is involved in anaerobic metabolism of pyruvate to lactate (P-L) in mammalian cells. It is known to catalyze pyruvate reduction to form lactate and the reversible oxidation of lactate back again to pyruvate with the help of the reduced and oxidized forms nicotinamide adenide dinucleotide coenzymes NADH & NAD^+, respectively. During Cori cycle, depletion of oxygen in skeletal muscle tissues leads to a build-up of L-lactate and depletion of pyruvate. The mitochondrial metabolic flux of pyruvate in the TCA cycle is reduced during hypoxic conditions[7] and L-lactate concentration increases in cells, shifting the lactate:pyruvate (L:P) molar ratio from ~10-20 (L):1 (P) to greater than 20:1[8]. In the cells of normal control subjects, the L:P ratio is around 10:1; the blood lactate concentration ranges between 0.31-2.00 mmol/L and pyruvate concentration is around 0.030-0.107 mmol/L[9]. The L:P molar ratio (L:P ratios of 25:1 or higher) is a diagnostic marker that can indicate poor oxygen circulation in diabetic patients, lactic acidosis, pediatric acute liver failure,

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Suppl. data available on respective page of NOPR



Graphical abstract

inherited respiratory disorders, defects in the TCA cycle and mitochondrial disorders[10-12].

Rise in cellular lactate concentrations can increase mitochondrial lactate uptake as well as lactate mobilization from extrahepatic tissues (specially, skeletal muscle cells) to the liver for reconversion to pyruvate (Cori cycle). Subsequently, gluconeogenesis occurs, leading to formation of glucose, which is released into the blood for utilization by extrahepatic tissues. The LDH reaction is considered to be reversible, with a ΔG of ~ +26 KJ/mol[13]. However, the ΔG of ~ +26 KJ/mol is still not favourable for a spontaneous reaction and hence, accounting the same should not facilitate Cori cycle with ease, as physiologically observed. Indeed, if LDH were able to catalyze a readily reversible reaction which can convert pyruvate into lactate (reverse reaction) and vice-versa (forward reaction), then there must be no need for Cori cycle.

Another discrepancy in literature (as yet unresolved) is: lactate to pyruvate reaction is more readily catalyzed by LDHB/H_4, and pyruvate to lactate reaction is catalyzed by LDHA/M_4[15]. There is no structural basis for this discrepancy (Suppl. Fig. 5), because the active site residues of the two protein monomers are identical[14]. Besides, some tissues contain mixtures of LDH isozymes 1-5 at varying proportions and this may not coincide with the idea that LDH_5/M_4 is present in relatively hypoxic environments and LDH1/LDHA/M_4 is present in aerobic tissues.

The traditional LDH reaction is considered a reversible process, whose direction depends on substrate/product concentrations and the redox state of NADH/NAD^+. The thermodynamic and kinetic features of the LDH-catalyzed lactate-pyruvate interconversion have been explored in great detail. Conversely, our earlier work suggested a different view where reactive oxygen species (ROS)/diffusible reactive species (DRS) may be involved in lactate oxidation and other cellular redox processes via the postulated murburn concept[14,17,18,34]. The present study is conceptually based on these earlier investigations. Although more experimental evidence is required to conclusively establish murburn as the operating mechanism, the present work investigates and strongly suggests the potential to employ ROS-associated chemistry for lactate-to-pyruvate conversion. The murburn mechanism is therefore treated as a testable hypothesis and the results of the present investigation are discussed in terms of the standard LDH pathway, rather than as an alternative to it.

Moreover, LDH assays for the lactate to pyruvate forward reaction involve non-physiological conditions, such as use of redox active agents and pH of close to 9.0 or even >9.0[16]. Manoj *et al*. (2021)[14] predicted that while NADH utilization could occur during the reduction of pyruvate to lactate, the forward reaction of lactate oxidation with/without the uphill reduction of NAD^+ "would necessitate alternative physiological approaches". The present *in vitro* study is a follow-up work which attempts to identify any alternative

physiological approaches which may be involved, such as ROS-mediated lactate oxidation. Liver microsomal CYP-mediated lactate metabolism may be a more feasible explanation for lactate mobilization to the liver in Cori cycle. This work also addresses the 'neglected' role of ROS in lactate oxidation. Since the liver possesses high concentrations of cytochrome P450s, it could provide an alternate redox route for lactate oxidation with a distinct energetics from the conventionally known NAD^+ dependent LDH reaction[14]. Also, the earlier observation that ROS produced during exercise enhances mitochondrial LDH-mediated lactate oxidation, and the fact that lactate enters the mitochondria, serving as an alternate fuel, is compatible with murburn-based interpretation of mitochondrial redox chemistry leading to lactate oxidation[17,18].

The LDH reaction is conventionally reversible, although standard biochemical thermodynamics favor reduction of pyruvate to lactate. The standard biochemical reduction potentials ($E^{\circ\prime}$) are −0.190 V for the pyruvate/lactate couple and −0.320 V for the NAD^+/NADH couple at pH 7. Using these values the $\Delta G^{\circ\prime}$ for the NAD^+-coupled oxidation of lactate to pyruvate is about +25 kJ mol^{-1}. Hence, lactate oxidation is thermodynamically not impossible but may be unfavorable under biochemical standard conditions. The actual reaction free energy depends on the concentrations and activities of the reactants and products. This difference is significant, because ΔG is the thermodynamic driving force and $\Delta G^{\ddagger}$ is the kinetic barrier. Therefore, the present study examines whether ROS-associated redox chemistry could provide an alternative mechanism for lactate oxidation that is thermodynamically distinct from the classic NAD^+-dependent LDH reaction.

Significance statement

This work questions the reversibility of the lactate dehydrogenase reaction. While pyruvate-to-lactate conversion is thermodynamically favoured, the contrary is not, i.e., formation of pyruvate from lactate involves NADH formation and H^+ consumption, and this reaction is not thermodynamically favoured. By tracking LDH driven rates of NADH consumption and formation, we demonstrate that NADH formation is not as favoured as NADH oxidation. Cori's cycle postulates the reconversion of lactate to pyruvate in liver tissue. We hypothesize this recycling to be an outcome of ROS dependent chemistry and hence the possible relevance of this chemistry to the Cori Cycle. We propose that the oxidative environment of liver tissue enables lactate oxidation and use GC-MS and IR spectroscopy to show that addition of ROS enables lactate to pyruvate conversion.

Materials and Methods

All chemicals used in this work were obtained from SRL, Merck, Himedia or Roche-Sigma India and were of analytical grade quality. NADH (54941), NAD^+ (26759), D-LDH (53110) from microorganism 400 U/mg and horseradish peroxidase (HRP) (28498) were obtained from SRL. Rabbit muscle L-LDH (10127230001) from rabbit muscle (10mg/2ml) which is 550 U/mg was obtained from Roche-Sigma. All solutions were prepared freshly and standardized just prior to the experiment.

Enzymatic assay

The activity of LDH was measured in two reactions: pyruvate reduction (NADH consumption) and lactic acid oxidation (NADH formation). LDH activity was measured in triplicate by monitoring the consumption or production of NADH at 340 nm (ε_{NADH} = 6.22 mM^{-1} cm^{-1}) in kinetics mode using a UV-Visible spectrophotometer (Shimadzu Model UV-1800 UV). Unless otherwise stated, final concentration used for lactate to pyruvate assay (forward reaction) in 3 mL reaction volume were: 1 mM L(+)-lactic acid, 1 mM NAD^+, 0.1 U D-LDH, 0.1 U L-LDH and 50 mM phosphate buffer (pH - 5.0, 7.0, 7.4 or 9.0). Assay reactions were carried out at room temperature (28±1°C), in accordance with the standard procedures[19]. The same concentrations of substrate (pyruvate), enzyme (D-LDH/L-LDH) and phosphate buffer were used for the reverse reaction (pyruvate to lactate). These standard assay conditions were used throughout this work and any variations in the assay mixture are indicated in the Figure legends.

Assay using liver tissue lysate

Fresh rat liver tissue was sliced and homogenized in phosphate buffered saline, pH 7.4 on an ice bath and the homogenate was filtered using Whatman No. 1 filter paper to remove the debris. The homogenate was stored on ice and used freshly for all further assays. Assay conditions employing liver tissue lysate for the forward reaction (lactic acid reduction) used 100 µL of liver tissue lysate and 0.1 U of D-LDH and 0.1 U L-LDH (wherever mentioned)[20].

Fenton mediated murburn reaction

Fenton reaction was performed to liberate hydroxyl radicals from hydrogen peroxide. The aim was to check if NADH formation could occur with the utilization of ROS (hydroxyl) radicals. Standard assay conditions for the reverse reaction or lactic acid reduction were as follows: 50 mM Phosphate buffer, 1 mM NAD^+, and 1 mM lactic acid, 0.1U L-LDH and 1:5 ratio of Fenton Reagent in a 3000 µL total volume. For analysis of the pH dependence of LDH activity, enzyme was assayed under optimal substrate concentrations in 50 mM phosphate buffer adjusted to different pH values (5.0, 7.0, 7.4 and 9.0) at room temperature (28±1°C) and activity was followed through kinetic analysis.

Peroxidase mediated murburn reaction

For performing peroxidase mediated murburn reactions we used 50 mM potassium phosphate buffer, 1 mM lactic acid, 1 mM NAD^+, 150 µM guaiacol, 1 mM KO_2, 1 mM H_2O_2, 10 nM HRP, 0.1 U D-LDH and 0.1 U L-LDH.

Peroxidase reactions are ROS generation. Hence, to check whether HRP-mediated ROS production could be involved in lactate oxidation, this experiment was performed. The assays were initiated by the addition of 50 mM phosphate buffer, lactic acid, NAD^+, HRP, H_2O_2, 150 µM Guaiacol, and D-LDH/L-LDH in 3 mL reaction volume. For pH dependent analysis, reaction was performed at different pH (5.0, 7.0, 7.4 and 9.0) at room temperature (28±1°C) and activity was measured through kinetic analysis at 340 nm.

Superoxide mediated reaction using ko_2

Since the results of the Fenton reaction showed that ROS generation from peroxide favoured lactate oxidation (studied via NADH formation), potassium superoxide was directly added to the reaction setup to check whether it could give a similar effect. Standard assay conditions for the reverse reaction or lactic acid reduction were as follows: The reaction was started by the addition of 50 mM phosphate buffer, NAD^+, lactic acid, KO_2 and D-LDH/L-LDH enzyme. For pH dependent analysis, reaction was performed at different pH (5.0, 7.0, 7.4 and 9.0) at room temperature (28±1°C) and activity was followed through kinetic analysis at 340 nm.

FTIR analysis

For FTIR analysis the test sample was prepared by adding 50 mM phosphate buffer, 1 mM lactic acid and 1 mM KO_2 and the transmittance was observed against standard 1 mM pyruvate and 1 mM lactic acid sample. The samples were analysed at ambient room temperature (28±1°C) (post-reaction), 20 µL of sample was taken for analysis. The beam diameter at the sample location was 6 mm. For each experiment, we used a resolution of 2.0 cm^{-1}, and acquisitions were performed in transmittance mode.

GC-MS metabolite characterization for determination of pyruvate and lactate

Derivatization Mixture Preparation: MTBSTFA: Triethylamine: ACN in the ratio of 5:4:1. Derivatization Process: 300 µL of properly mixed samples was taken in a glass vial and dried completely using N_2 gas, to the dried sample 100 µL of the derivatization mixture was added and incubated at 60°C for 30 min and the supernatant was collected and injected into GC-MS column.

For analysis the final concentration of reactants was 1 mM pyruvate, 1 mM lactic acid, 1 mM NAD^+, 1 mM NADH, 1 mM KO_2, 0.1U LDH used. To conclude murburn mediated peroxidase reaction involvement, three different reactions were analysed against the standard substrates (pyruvate and lactic acid). The first reaction with the combination of buffer, lactic acid, NAD^+, KO_2, and other reaction comprising buffer, lactic acid, NAD^+, KO_2, LDH. The final reaction consisted of the combination of buffer, pyruvate, NADH and LDH.

Density functional theory (DFT)

DFT analysis was performed using ORCA 6.1.1 and geometries were fully optimised at the (U) B3LYP level of the theory[21,22] with Grimme's D3 dispersion correction and Beck-Johnson damping[23,24] using 6-31+ G (d, p) basis set. Initial three dimensional structures for lactate, pyruvate, hydroxyl radical, water, hydrogen peroxide, superoxide and molecular oxygen were generated from SMILES strings using Open-Babel software[25].The lactate radical was constructed by removing the α-hydroxyl hydrogen atom from the lactate structure, which was optimised using Open Babel gen-3d, consistent with the proposed hydrogen atom transfer mechanism. The evaluation of elementary HAT processes was made possible by the inclusion of a solitary hydrogen atom (^2S, charge 0, multiplicity 2) as a reference species. NAD+ and NADH were represented using the literature-precedented truncated models 1- methylnicotinamide (MNA^+) and 1-methyl-1, 4-dihydronicotinamide, (MNAH) respectively. These models were retrieved as

three-dimensional structures from PubChem CID 457 and CID 192942. The calculated free energy of the ground-state triplet was combined with the experimentally determined vertical singlet-triplet excitation energy of O_2 (22.5 kcal/mol; Herzberg) to determine its free energy. Rather, the calculated free energy of the ground-state triplet species ($^3O_2$) was added to the experimentally determined vertical singlet–triplet excitation energy of O_2.

Statistical analysis

All the experiments were done in minimum sets of triplicate, unless mentioned otherwise, and the error bars represent the standard deviations. Two-way ANOVA was done with a confidence interval of 95% to check the significance of variance among the datasets followed by Tukey's multiple-comparison and is indicated with *, **, ***, **** for corresponding P-value of $P< 0.05$, $P< 0.01$, $P< 0.001$ and $P< 0.0001$ respectively and 'ns' corresponding to non-significant variance.

Results and Discussion

LDH is a thoroughly studied enzyme, and its action can significantly influence not just L:P ratio, but also NADH:NAD^+ ratio, a key indicator of cellular redox homeostasis. LDH can significantly affect the NADH:NAD^+ levels, particularly when LDH is overexpressed. As per the established mechanism, LDH can deplete NADH or catalyze NADH formation, depending on which way the reaction goes[26,27].

NADH depletion in pyruvate to lactate reaction

In Suppl. Fig. 1, the rate of NADH oxidation (also termed herein as NADH consumption) accompanying the reverse reaction (pyruvate to lactate) was monitored. The abbreviations used are: B = buffer, P = Pyruvate, L = Lactate, P-L = Pyruvate to Lactate (reverse reaction), L-P = Lactate to Pyruvate (forward reaction). The key observation from the data in (Suppl. Fig. 1) is that NADH consumption occurred in phosphate buffer systems and pH 7.4 was ideal for pyruvate to lactate reaction, as demonstrated earlier in literature[16]. Statistical significance was determined by two-way ANOVA followed by Tukey's multiple-comparison test ($F = 206.7$, $P< 0.0001$).

NADH formation in lactate to pyruvate reaction

NADH formation, indicating lactate to pyruvate conversion, is observed in the phosphate buffer (only in pH 7.4 and 9.0) set-ups. However, the key observation to take note of is that NADH formation rates (Fig. 1)

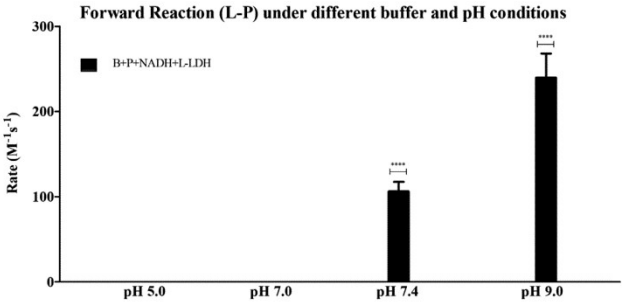


Fig. 1 — Formation of NADH in the lactate to pyruvate (L-P) reaction. No detectable NADH formation was observed at pH 5.0 and pH 7.0 under the experimental conditions. Feeble reactions were observed only at pH 7.4 and pH 9.0 during L-LDH-catalysed conversion of lactate to pyruvate. Data are presented as mean ± SD from n = 3 independent experiments. Statistical significance was determined using two-way ANOVA at a 95% confidence level ($P< 0.0001$)

were in the range of 100-300 $M^{-1}s^{-1}$. NADH consumption rates were much higher in pyruvate to lactate reaction (1000-2500 $M^{-1}s^{-1}$ as shown in Suppl. Fig. S1). This points to a possibility that NADH formation rates are not equal to its consumption rates within the LDH system. Another point to note is that the NADH formation rate was very low at physiological pH of 7.4, indicating negligible conversion of lactate to pyruvate. The pH that was preferred for NADH formation, in contrast to NADH oxidation was 9.0, which was once again in line with the conditions that Gay *et al*. (1968) had reported for the lactate to pyruvate reaction[16]. Statistical significance was determined by two-way ANOVA followed by Tukey's multiple-comparison test ($F = 27.96$, $P< 0.0001$). Such low rates of lactate conversion to pyruvate are expected since the ΔG is ~+26 kcal/mol, which isn't a favorable value for driving a reaction.

Lactate to pyruvate reaction in liver tissue lysate

Liver tissue lysate was used to check for NADH formation in the lactate to pyruvate reaction (Fig. 2). Compared to the control set-up comprising of only liver tissue in phosphate buffer (set-up A), set-up B did not show any change w.r.t. control. In set-up C, there was a minor increase in NADH formation rate. But, in set-ups D and E, it is very clear that NADH formation rates declined significantly upon addition of both L- and D-LDH enzymes. Since the same liver tissue lysate was used for this entire set of experiments, distribution of its constituents is homogeneous and hence the recycling of NADH (rate of NADH production) observed is similar even when lactate is added to the system. This non-significant variation observed in the first three setups (2A-2C) also indicates the equilibrium of NADH

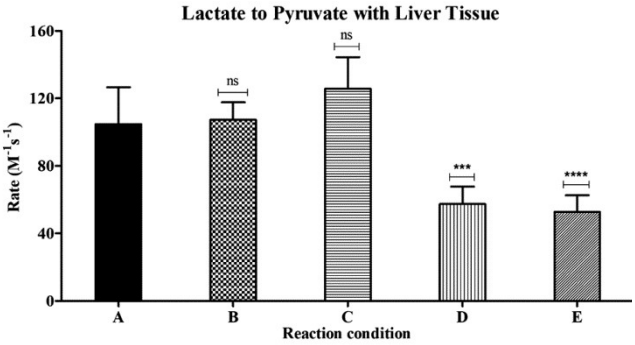


Fig. 2 — Lactate to Pyruvate conversion at pH 9 by liver tissue lysate. The various conditions depicted are 'A' = buffer (B) + liver tissue (LT); 'B' = B + LT + lactic acid (LA); 'C' = B + LT + LA + NAD^+; 'D' = B + LT + LA + NAD^+ + L-LDH; 'E' = B + LT + LA + NAD^+ + D-LDH. Rates are still subdued upon addition of LDH. Data are presented as mean ± SD (n = 3). Statistical significance was determined using two-way ANOVA at a 95% confidence level; $P< 0.001$ and $P< 0.0001$ are denoted by ***, and ****, respectively; ns for not significant

recycling in the liver lysate sample. The reduction in net NADH accumulation following LDH addition suggests that NADH-consuming processes prevail under these conditions; this finding is consistent with, but does not independently establish, the proposed thermodynamic concept as predicted by us earlier. The much greater NADH consumption in P-L reaction with respect to bare NADH formation in L-P reactions demonstrate a pronounced directional kinetic asymmetry under the assayed conditions towards P-L reactions. Therefore, addition of LDH is prone to cause NADH depletion rather than NADH formation. Suppl. Fig. 2 represents the results for a reaction that was performed on three random days to check the rate of NADH formation (lactate to pyruvate reaction) in different preparations of fresh liver tissue lysate. Once again, a similar trend of LDH-associated NADH depletion can be seen in the different days of experimentation.

Decline of NADH formation rates in presence of LDH

Some literature reports indicate that LDH is involved in the production of H_2O_2 as well as other ROS species[28,29]. Superoxide has been shown to be involved in LDH-catalyzed depletion of NADH to form hydrogen peroxide in aqueous milieu[28]. In the pyruvate to lactate reaction, an active site process (as given by the conventional mechanism) would be involved in depletion of NADH. However, in the lactate to pyruvate reaction NADH that is formed could be depleted back by LDH, as observed in (Figs. 2-4). As a result, NADH oxidation observed therein could be via the classical active site process of NADH depletion and superoxide-mediated hydrogen peroxide formation driven by LDH.

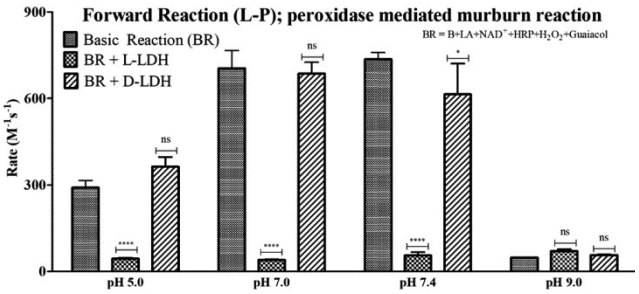


Fig. 3 — Peroxidase mediated murburn conversion of Lactate to Pyruvate. Evident generation of pyruvate in presence of ROS generated during peroxidase reaction over a wide range of pH. It is suppressed significantly in presence of L-LDH and is either suppressed slightly or unaffected in presence of D-LDH (note: there is no enhancement in the production of pyruvate as expected in presence of LDH). Data are presented as mean ± SD (n = 3). Statistical significance was determined using two-way ANOVA at a 95% confidence level; $P< 0.05$ and $P< 0.0001$ are denoted by * and ****, respectively; ns for not significant

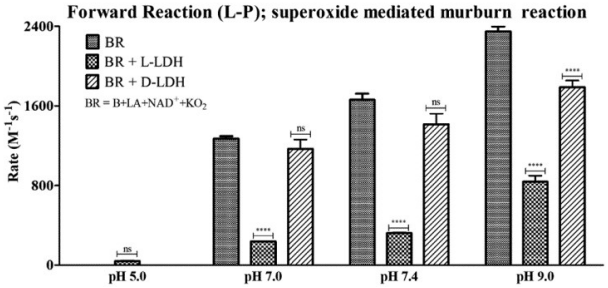


Fig. 4 — Superoxide mediated conversion of Lactate to Pyruvate. Again an extremely triggered production of pyruvate in presence of ROS (here, superoxide) is observed at pH 7, 7.4 and 9.0. It is suppressed significantly in presence of L-LDH and is either suppressed or unaffected in presence of D-LDH (note: there is no enhancement in the production of pyruvate as expected in presence of LDH). Data are presented as mean ± SD (n = 3). Statistical significance was determined using two-way ANOVA at a 95% confidence level. **** indicates $P< 0.0001$; ns, not significant

In Figures 3 & 4, the data shows a rapid decline in NADH formation rates in the presence of LDH in lactate to pyruvate reactions.

Role of ROS in LDH reaction

We anticipated that ROS present in liver tissue, owing to the high CYP450 and CYP450 reductase concentrations, would be able to facilitate the reconversion of lactate to pyruvate. Lactate metabolism, in hepatocytes, when coupled to xenobiotic metabolism, was calculated to be a thermodynamically feasible and facile route for reconversion of lactate to pyruvate[14,34]. LDH can catalyze pyruvate to lactate reaction via the classical mechanism; but, the ROS mediated route is chemically distinct from the standard NAD^+ coupled LDH reaction and may provide an alternative pathway

for lactate oxidation to pyruvate. Moreover, based on the results shown in (Suppl. Fig. 3), Fenton reaction was able to yield a very high rate of NADH formation and once again, incorporation of LDH into the Fenton reaction set-up led to a decline in the NADH formation rate. To further understand the two observations highlighted above - a) the decline of NADH formation rates in the presence of LDH and b) neglected role of ROS in LDH reaction, the following set of experiments were explored.

In our own investigations in the peroxidase field, we have observed the role of antioxidant substrates and the relevance of Fenton reaction in heme peroxidase-mediated one-electron oxidation reactions[30-32]. It is therefore plausible for diffusible reactive/reduced species (DRS/ROS) produced from heme peroxidase to facilitate an energetically favorable lactate oxidation in tandem with peroxidase-mediated one-electron oxidation reactions. To investigate whether this is possible, reaction set-ups containing HRP and peroxidase substrate (guaiacol, in an untraceable amount) were performed to check the rate of NADH formation, as represented in (Fig. 3). Addition of L-LDH caused a dramatic decrease in NADH formation rates which were observed in the basic reaction containing LDH. There was some indication that D-LDH also was able to utilize HRP-driven DRS for NADH formation. The differences may be owing to the radical dissimilarity in the structures of D-LDH (a flavoenzyme) and L-LDH, a nicotinamide-dependent enzyme. Statistical significance was determined by two-way ANOVA followed by Tukey's multiple-comparison test ($F = 166.1$, $P < 0.0001$).

Figure 4 indicates that there is no reaction observed at pH 5. Superoxide, when added to a reaction mixture containing buffer, lactate, and NAD^+ exhibited significant NADH formation rates, but once again, addition of LDH caused a highly significant drop in the rates. This reaction was more favourable at higher pH (particularly, pH 9.0). Another important point to acknowledge is the higher stability of superoxide as well as hydroxyl radicals at higher pH conditions. Since hydroperoxyl radical (a weak acid) has a pKa of 4.88, superoxide anion (conjugate base of hydroperoxyl radical) predominantly exists in neutral pH. Superoxide was estimated to have a half-life of ~5 seconds at physiological pH; however, in proton-lacking media (at higher pH), superoxide anions can have greater longevity. This supports the fact that lactate to pyruvate reaction is highly favoured at pH 9.0 or greater. Taken together, the data indicate a strong kinetic bias against net NADH accumulation in the lactate-to-pyruvate direction under the conditions tested and necessitates the consideration of additional oxidation routes. Many inhibitors of LDH (*e.g*. epigallocatechin gallate) are antioxidants which can also scavenge free radicals. Lactate was termed to be a moderate hydroxyl radical scavenger at pH 9.0[33]. Lactate, on its own, can serve as an antioxidant by reacting within the liver tissue (with the involvement of CYP-CPR system), or in the mitochondrial respiratory system with oxygen to form hydrogen peroxide[14,34]. Statistical significance was determined by two-way ANOVA followed by Tukey's multiple-comparison test ($F = 217.4$, $P < 0.0001$).

Tracking lactate to pyruvate conversion using FT-IR and GC-MS

To further consolidate the findings, FTIR analysis and GC-MS (Fig. 5) analysis was carried out with the superoxide-based set-up. GC-MS results provided clear chemical evidence for the loss of lactate concomitant with pyruvate formation in mixtures containing KO_2. These data, in conjunction with

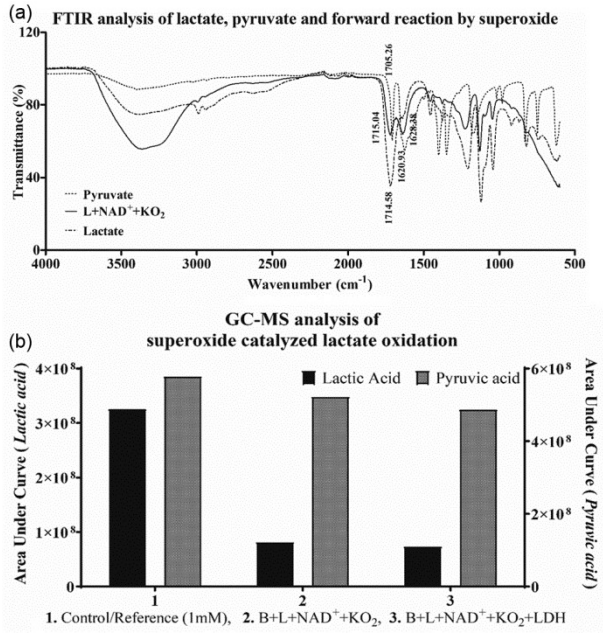


Fig. 5 — FT-IR (A) and GC-MS analysis (B) for lactate to pyruvate conversion in presence of KO_2. Emergence of a ketone peak at ~1600 cm^{-1} in the FT-IR spectra in the reaction catalysed by superoxide supports the conversion of lactate to pyruvate in presence of ROS, which is further backed up by the mass spectral data for same set of reactions. Disappearance of lactate and appearance of pyruvate as observed in 'B' is evident. Corroborating further is condition 3 in 'B' which indicates suppression of pyruvate formation in presence of LDH as observed in the former *in vitro* set of experiments

FT-IR results, support ROS-associated lactate oxidation and are consistent with a murburn-based interpretation. The red and green spectral traces indicate standard pyruvate and lactate, respectively, while the blue trace indicates KO_2 catalyzed lactate to pyruvate reaction. Emergence of the peak at ~1620 (ketone) and diminishing of the peak at ~1715 (carboxylic acid) in the spectrum of KO_2 catalyzed lactate oxidation (blue) conclusively indicates the conversion of lactate to pyruvate.

GC-MS was performed to confirm the oxidation of lactate to pyruvate by superoxide generated from KO_2. As seen in Figure 5B, area under the curve for lactate and pyruvate are plotted on left and right Y-axis respectively. The first set of bars represents the area under the curve for 1 mM lactate/pyruvate and serves as the reference. The second and third set of bars represent the area under the curve corresponding to lactate and pyruvate after lactate conversion to pyruvate via reaction with superoxide generated by KO_2. A significant amount of lactate was converted to pyruvate with the aid of superoxide. But again, this was mildly suppressed upon incorporation of LDH in the reaction mixture (in line with our previous observations). These observations strongly support murburn driven conversion of lactate to pyruvate.

DFT analysis of lactate oxidation

The NADH-dependent conversion of pyruvate to lactate was found to be thermodynamically favourable while the NAD^+ dependent conversion of lactate to pyruvate was unfavourable, as presented in (Table 1). Further, the ROS mediated reactions demonstrate quite favorable ΔG values. DFT calculated values as high as -68.20 and -115.55 kcal/mol for superoxide

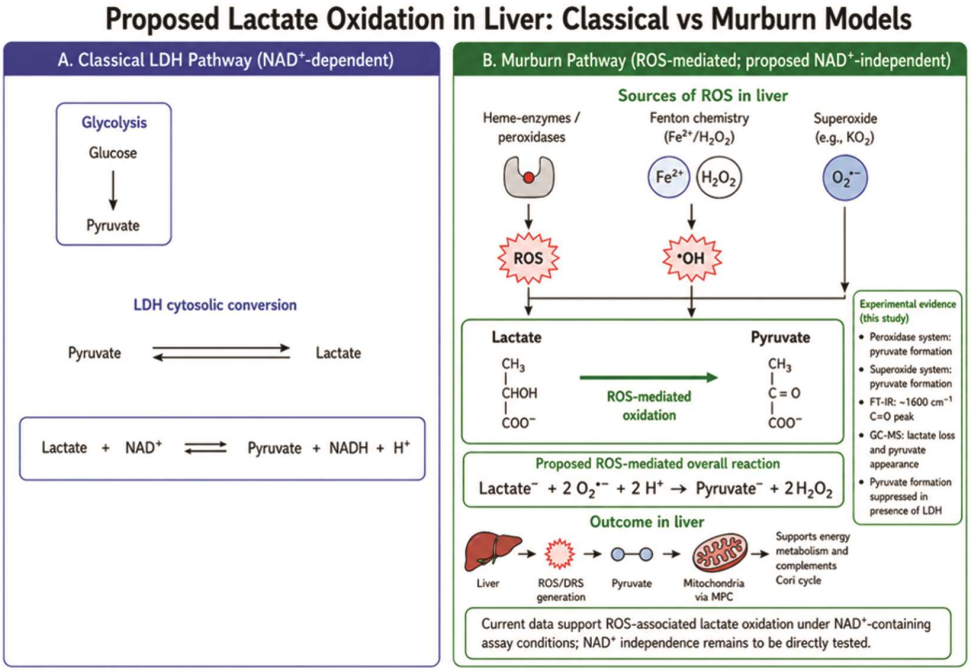


Fig. 6 — Proposed murburn-mediated lactate oxidation in liver. Schematic comparison of classical LDH-mediated lactate to pyruvate conversion with the proposed ROS-mediated murburn pathway. ROS/DRS generated by heme-containing systems may promote lactate oxidation independently of NAD^+ and LDH. Experimental support is provided by Figs. 3–5, while the physiological role of the proposed pathway remains to be validated

Table 1 — DFT calculated thermodynamic free energy variations (ΔG) for the interconversion of lactate and pyruvate mediated by NADH, NAD^+, superoxide, and hydroxyl radicals. Negative ΔG values signify thermodynamically favorable reactions, while positive ΔG values denote unfavorable reactions under specific conditions

S. No.	Reaction	ΔG (kcal/mol)	Thermodynamic stability
1	Pyruvate → Lactate (NADH-dependent)	-15.79	Favourable
2	Lactate → Pyruvate (NAD^+-dependent)	+15.79	Unfavourable
3	Lactate → Pyruvate (superoxide-mediated)	-68.20	Strongly Favourable
4	Lactate → Pyruvate (·OH-mediated)	−115.55	Most favourable of all four

and hydroxyl radical driven lactate oxidation respectively, indicates an alternate ROS favored reaction mechanism as per our hypothesis.

Conclusion

Liver tissue is saturated with oxygen, and it is loaded with heme-enzymes which can generate lots of DRS for the murburn mediated catalysis of lactate oxidation. The results of this work clearly demonstrate that LDH catalyzes NADH depletion at a faster rate than NADH formation. Addition of LDH favours NADH depletion. Lactate oxidation by ROS is also evident from the results of this work, both in the horseradish peroxidase-assisted murburn reaction and the Fenton-based setups involving a) Fenton reagent (results in supplementary information) and b) added superoxide (KO_2). Liver is also a major site of glycolysis owing to the large volumes of glucose influx through the hepatic portal vein. The switch between glycolysis and Krebs cycle demands the transport of pyruvate to the mitochondrial matrix through a transport protein known as mitochondrial pyruvate carrier. This surely is a rate limiting step with a much slower rate of transfer than the production of pyruvate. Hence, pyruvate conversion to lactate is catalyzed by LDH, perhaps through the conventional active site mechanism. However, reconversion of lactate to pyruvate is hampered; incidentally, even though the liver has lower LDH-5/M_4 concentrations than the muscle, these high concentrations of LDH are unable to afford rapid reconversion of lactate into pyruvate, thus setting the Cori cycle into motion. Presence of LDH in the liver can perhaps serve as a means to avoid accumulation of pyruvate, which can inhibit the glycolytic pathway and could also be toxic at higher concentrations. LDH, therefore, works as a shuttle and a regulatory enzyme to prevent such outcomes. The *in vitro* results suggest that ROS-dependent lactate oxidation could occur in redox-active physiological environments such as the liver or mitochondria; however, this remains a testable hypothesis. Heart tissue contains more mitochondria than other body tissues. D-LDH, which is housed in mitochondria, contains flavin groups that are capable of generating DRS. Similarly, as pointed out earlier by Manoj *et al*. (2021), the cytochrome P450 & P450 reductase system present in liver cells could be involved in the generation of copious amounts of ROS which can support lactate reconversion to pyruvate as per our observations demonstrated in this manuscript.

ROS/DRS mediated reactions can be explained within the murburn framework as we have postulated in our earlier papers[14,17,18,34]. In this work, the framework is employed as a mechanistic interpretation of the observed ROS-associated lactate oxidation, and not as an established replacement of the standard LDH process. Thus, the experimental evidence supports ROS mediated lactate oxidation but the precise attribution of this process to the murburn mechanism is a hypothesis that needs further mechanistic and physiological validations. Taken together, these findings suggest that ROS-mediated lactate oxidation may contribute to lactate reconversion to pyruvate and provide a potential murburn-based perspective for understanding aspects of the Cori cycle and is shown as a hypothetical scheme in (Fig. 6).The overall reaction according to the proposed alternative route for oxidation of lactate to pyruvate can be written as:

$$Lactate^{-}+2O_2^{\bullet-}+2H^{+}\rightarrow Pyruvate^{-}+2H_2O_2$$

Acknowledgement

NS and AP thank the advance research centre in the Department of Biotechnology and Centre of Excellence at Vignan's Foundation for Science, Technology and Research (VFSTR) for providing the space and facilities for conducting the necessary experiments. DAG thanks Fr. Roshan Castelino at St. Joseph's University for providing an opportunity to serve as a dedicated Research Faculty. We also acknowledge Ms. Sakshi G. Deheri for helping us with DFT calculations.

Conflict of interest

All authors declare no conflict of interest.

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