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Lactate dehydrogenase: Lactate oxidation and role of ROS

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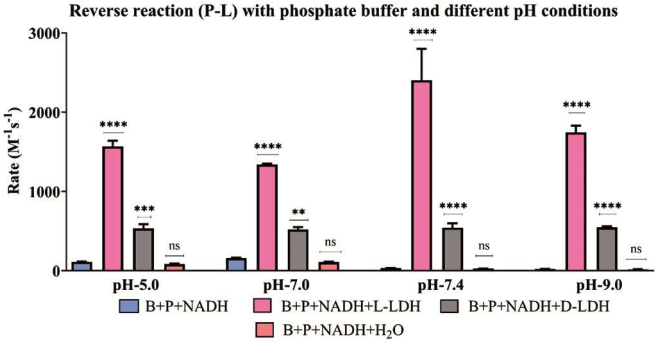
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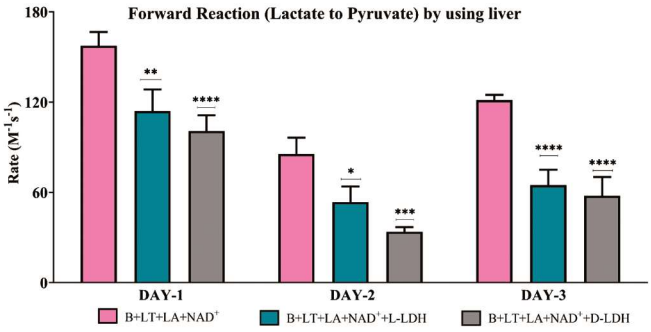
Supplementary data



Suppl. Fig. 1 — Pyruvate to Lactate conversion with phosphate buffer different pH conditions

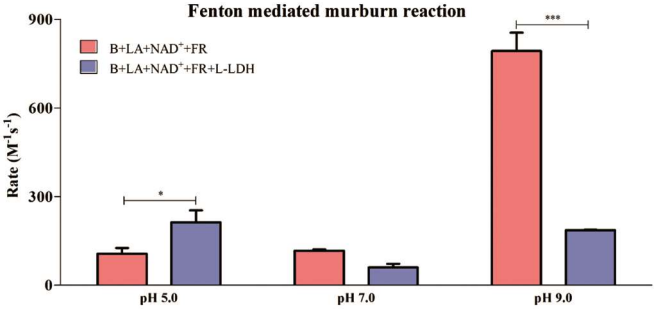
Control (B+P+NADH) was employed to check for NADH autocatalysis in different buffer set-ups of phosphate with various pH conditions. The B+P+NADH+H_2O reaction served as another control, as the same volume of water was added instead of the D-LDH and L-LDH in these test reactions. The first major observation in the experiment comparing the differences in pH-dependent NADH consumption is that no drastic differences in the controls can be seen in Figure S1. While D-LDH mediated NADH consumption was almost the same across a range of pH, the NADH consumption rates were higher in all pH conditions in test reactions containing L-LDH and pyruvate. Both D- and L-LDH consumed NADH but in the case of D-LDH (B+P+NADH+D-LDH), the rate of NADH consumption was almost similar with varying pH. On the other hand, with L-LDH (B+P+NADH+L-LDH) in the reaction, the NADH consumption rates were higher with the general trend that rates were in the order of pH 7.4 > 9.0 > 5.0 > 7.0.

From the data herein, it is evident that pH 7.4 and 9.0 had the most favoured conditions for NADH consumption by LDH. As per the classical mechanism of LDH action, H195 (in many papers it is H193) in the active site pocket is involved in protonation of pyruvate C=O group and the positively charged R109 (R107) and R171 (R169) attract the negatively charged carboxylate group of α-hydroxy acids like pyruvate and lactate. The mechanism of LDH action involves direct transfer of a hydride ion from the NADH nicotinamide ring to the carbonyl carbon atom of pyruvate, generating lactate in the process. In the forward reaction of L-P, His195 serves as a H^+-acceptor, and NAD^+ is reduced to NADH + H^+ (Holbrook and Gutfreund, 1973). Hence, the pH of the medium should have a remarkable effect on NADH oxidation rates. However, the NADH oxidation rates were better at pH 7.4 and 9.0, bettering the rates obtained at pH 5.0. The pH optimum of normal serum was found to be 7.2 for the P-L reaction and >9.0 for L-P reaction (Buhl and Jackson, 1978). The L-P reaction *in vivo*, however, cannot occur at the non-physiological condition of >9.0 pH.



Suppl. Fig. 2 — NADH formation rate profile in L-P reaction in liver tissue lysate

The experiments show a similar trend (Figure S2), where addition of both D- and L-LDH into the reaction with phosphate buffer caused NADH formation rates to decline, instead of increase- despite the addition of lactate with the aim to stimulate the L-P reaction.



Suppl. Fig. 3 — NADH formation in L-P reaction in Fenton reaction setup

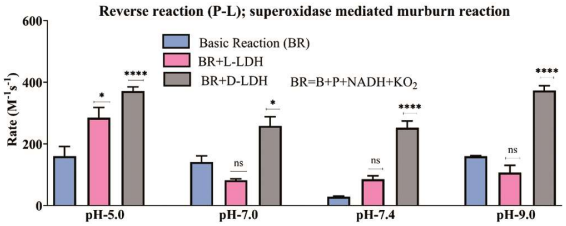
The Fenton reaction, named following Henry J. H. Fenton, explains how Fe^{2+} reacts with H_2O_2 (Fenton, 1894). The Fenton reaction generally works optimally under mildly acidic conditions, between pH 3 and 5. Within this range of pH, ferrous iron (Fe^{2+}) will stay in solution and hydrogen peroxide be stable. Under these acid conditions, the reduction of Fe^{3+} back to Fe^{2+} is enhanced, a process necessary for the continuation of the reaction cycle. At pH 5, hydroxyl radicals (•OH) are formed with high efficiency. These are very active free radicals that may provide effective oxidation. At pH 9, ferrous iron Fe^{2+} tends to precipitate into iron hydroxides, generally reducing its availability for the Fenton reaction. However, if some iron is still in a soluble form or if there is a continuous supply of iron in a soluble form, then the reaction can proceed, albeit less efficiently. Although the reaction is less efficient at pH 9 because of iron precipitation, hydrogen peroxide decomposes more slowly at higher pH. This may mean that the production of radicals would be extended over a longer time, perhaps accounting for the higher rates observed at pH 9.0. The Fenton reaction has tremendous relevance in physiological settings (Meyerstein, 2021; Prousek, 2007) and this is why we assayed NADH formation in Fenton reaction-mediated lactate oxidation (Figure S3).

Although superoxide is not known to reduce NAD^+ to yield NADH, we observed a high rate of NADH formation with just superoxide addition (Figure 4). At higher pH conditions, the Fenton reaction might lead to the formation of other reactive oxygen species, such as superoxide anion, that would be able to participate in oxidation reactions and thus potentially lead to higher oxidation rates (Andrés *et al*., 2023; Xiao *et al*., 2024). Superoxide is very transient and can be readily converted to hydrogen peroxide via SOD catalysis or LDH activity *in vivo* (Brawn and Fridovich, 1980). In phosphate buffer, the rate of NADH formation was highly favoured at pH 9.0 with just Fenton reagent. However, addition of L-LDH enzyme into the reaction caused the rate of NADH formation to decrease several (~ 3.5) folds, particularly at pH 9.0. From the results in Figure S3, it is clear that ROS alone, in the absence of L-LDH enzyme, could be capable of catalyzing the conversion of

lactate to pyruvate. Lactate is known to serve as an antioxidant, with free radical scavenging roles in cellular physiology; it was reported to scavenge both superoxide and hydroxyl radical using EPR spectroscopy (Groussard *et al*., 2000). Lactate concentrations of 30 mM or higher are detected in muscle tissue during strenous exercise (Bartoloni *et al*., 2024) and hence, the concentration of 1 mM used in this work is well within physiological limits. Besides the involvement of superoxide in LDH-mediated peroxide formation, there could be several other reaction equilibriums and multiple competing reactions involving diffusible reactive species (DRS) within the reaction milieu (Manoj *et al*., 2016b). Our earlier works in heme peroxidase kinetics reveal the futile generation of alternative products, which could be generated by depletion of precious reduced equivalents such as NADPH and NADH (Manoj *et al*., 2010). While ROS are generated in other parts of cells (to an extent), mitochondria are the major site of cellular ROS production. Millions of ROS are generated per mitochondrion per day. Mitochondrial DNA are susceptible to ~100,000 ROS-attacks per day (Ames *et al*., 1993), indicating the prevalence of a highly oxidizing environment therein. We have previously investigated the role of flavin in cytochrome P450 reductase, the electron transfer flavoprotein present in liver microsomes (Manoj *et al*., 2016a). As opposed to the heme activates oxygen hypothesis, the flavin activates oxygen hypothesis (Massey, 1994) supports the validity of murburn concept in drug metabolism.

Newer research investigations have focused on the shuttling and oxidation of lactate by mitochondrial LDH (mLDH) (Passarella and Schurr, 2018). Skeletal muscle, despite their net lactate release, significantly uptake lactate and reconvert it with the help of mLDH present in mitochondrial intermembrane space (Van Hall, 2000). The entry of lactate into mitochondria is controlled by mitochondrial monocarboxylate transporters (mMCTs) (Hashimoto and Brooks, 2008). Analyses of LDHA, LDHB, LDHC and mLDH (LDHD) protein sequences and active site residues therein show that there are no major differences in LDH A, B and C and hence, there need not be any mechanistic differences in either substrate binding or catalysis in these isozymes. However, LDHD (mLDH) is an FAD-dependent enzyme which catalyzes D-lactate and other D-2-hydroxy acids containing hydrophobic moieties, but does not act on L-lactate (Jin *et al*., 2023). This can explain how D-LDH and lactate oxidase, by virtue of their flavin moiety, could catalyze lactate oxidation in the mitochondria.

An interesting component in the overall mechanism of LDH reaction is the presence and availability of ROS, which has been detected by numerous researchers. Lactate clearance in muscle tissues is enhanced during exercise, which is associated with increased ROS production (Hashimoto and Brooks, 2008; Van Hall, 2000). Hence, the pertinent question arises: if mLDH does not catalyze L-lactate oxidation, how does L-lactate get metabolized in the mitochondria? L-lactate oxidase can catalyze L-lactate oxidation, but then this again questions the role of LDH in L-P reaction, hence rendering it to be a 'pyruvate reductase' rather than a lactate dehydrogenase. Our prediction of lactate-dependent peroxide formation is justified by observations of hydrogen peroxide production in LDH reactions. Lactate can also form a stable complex with Fe^{3+} ion (1:2 complex of Fe^{3+}: lactate) which can react with hydrogen peroxide to form hydroxyl radicals (Ali *et al*., 2000). Therefore, there is precedent from literature to suggest that lactate can sponsor hydrogen peroxide generation as well as hydrogen peroxide consumption to produce hydroxyl radicals- and these independent contexts can be dictated by the prevailing redox equilibriums. Superoxide, on its own, can react with lactate to form pyruvate. One group investigated LDH-mediated hydrogen peroxide formation involving superoxide utilization and found that both LDHA and LDHB were capable of producing hydrogen peroxide; when LDHA and LDHB genes were knocked out in HeLa or 4T1 cells, there was a dramatic decline in hydrogen peroxide levels and restoration of LDHA gene partially restored the high peroxide levels observed in cancer cells (Wu *et al*., 2021). We also had earlier predicted that lactate can produce hydrogen peroxide.



Suppl. Fig. 4 — Effect of KO_2 on Pyruvate to Lactate conversion in presence of LDH

When tracking NADH consumption in the P-L reaction in the presence of superoxide and pyruvate (Figure S4), there is clear indication once again that NADH consumption rates are higher with addition of either D- or L-LDH. The presence of LDH in the reaction is a driving force for NADH consumption.

Location of LDH in cells: LDHA was even found to be located in the nucleus (Liu *et al*., 2018). LDHB was found to localize to mitochondrial membrane of fermenting mammalian cells (Young *et al*., 2020). A readily reversible reaction occurring primarily in the cytosol does not necessarily explain why these membrane associations of LDH would need to occur. The shuttling of lactate into mitochondria is another point to consider, as it favours a ROS-based L-P reaction. Hence, lactate oxidation could be supported by the mitochondrial milieu. While LDH is predominantly found in cytosolic milieu, the entry of lactate into mitochondria as well as liver cells (which are rich in cytochrome P450 and P450 reductase and are known to generate copious amounts of ROS) supports the necessity of an oxidative environment, viz., the agency of ROS in lactate oxidation (Manoj *et al*., 2010). A flavin-dependent enzyme present in the mitochondrial intermembrane space, L-lactate oxidase, is involved in L-lactate oxidation to yield pyruvate and H_2O_2 as a by-product (Ma *et al*., 2004). This enzyme is distinct from LDH. Flavin containing enzymes can utilize oxygen and form DRS (diffusible radicals) / DROS (diffusible reactive oxygen species) (Manoj *et al*., 2010), which can be involved in lactate oxidation. The flavoenzyme D-LDH may utilize flavin to generate ROS, using which, it could convert lactate to pyruvate.

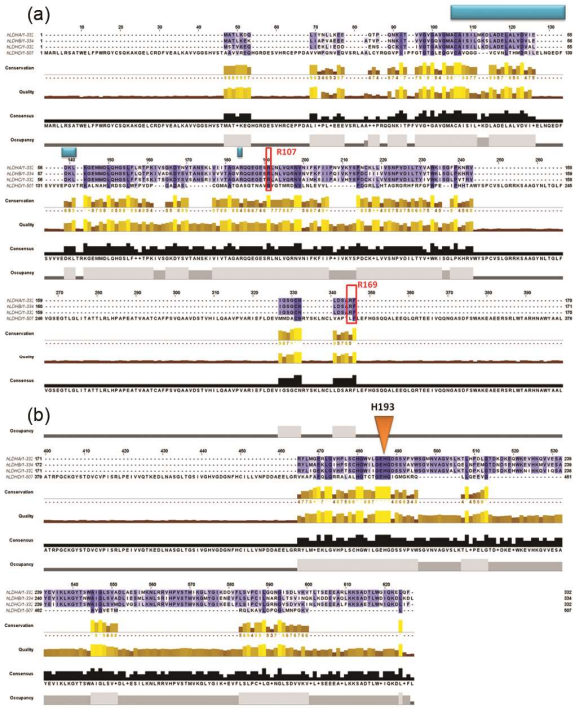


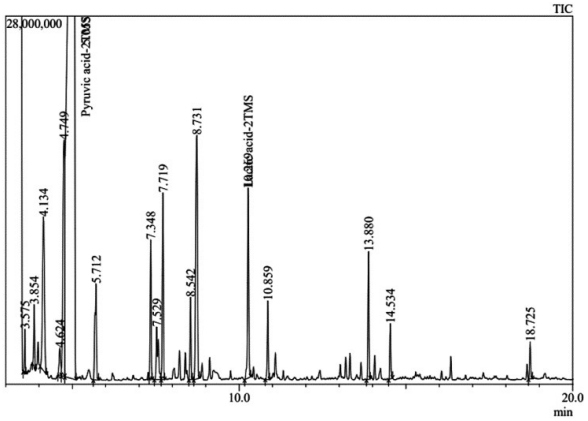
Figure S5 — Comparison of human LDH protein sequences. LDH proteins have an NADH/NAD+ binding site (indicated by a blue bar) and a substrate binding site (characterized by R107 and R169). The het catalytic residue of H193 is conserved in all the LDHs. The other active site pocket residues like N138 and T248 are conserved in LDHA, B and C, but are not conserved in LDHD.

From Figure S5, it is clear that the key catalytic H193 and substrate binding R107 amino acids are conserved in all human LDHs compared herein (LDHA, LDHB, LDHC and LDHD, aka mLDH). R169 is not conserved only in LDHD/mLDH. The sequence comparison demonstrates that LDHA, B and C are almost identical and therefore, the idea that LDHB catalyzes lactate oxidation (L-P reaction) and LDHA catalyzes pyruvate reduction (P-L reaction) is questionable.

GC-MS analysis

Pyruvate

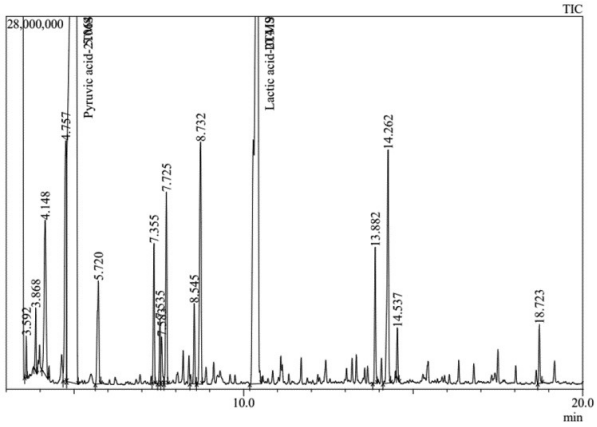
GC-MS analysis revealed different chromatographic profiles of the lactate and pyruvate samples. In the lactate sample, lactic acid-2TMS was found at 10.419 min, while pyruvic acid-2TMS was found at 5.055 min in the pyruvate sample as the main identified component. In the pyruvate sample pyruvic acid-2TMS accounted for 40.92% of the total chromatographic area, while lactic acid-2TMS represented 2.63%. These results provide chromatographic evidence for the presence of the respective lactate and pyruvate derivatives in the analyzed samples and support their characterization in the lactate-pyruvate reaction system.



Suppl. Fig. 6: Analysis of Pyruvate

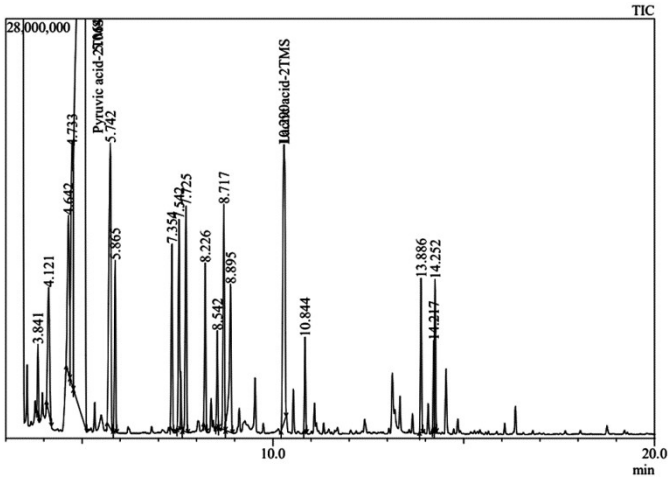
Lactate

The GC–MS analysis of lactate samples revealed a significant chromatographic peak at retention time 10.419 min with relative peak area 19.01% corresponding to lactic acid-2TMS. Pyruvic acid-2TMS was also found at 4.757 min which accounts for 4.35 % of total chromatographic area. The simultaneous determination of lactic and pyruvic acids confirms the presence of the lactate-pyruvate metabolite pair in the material under analysis.



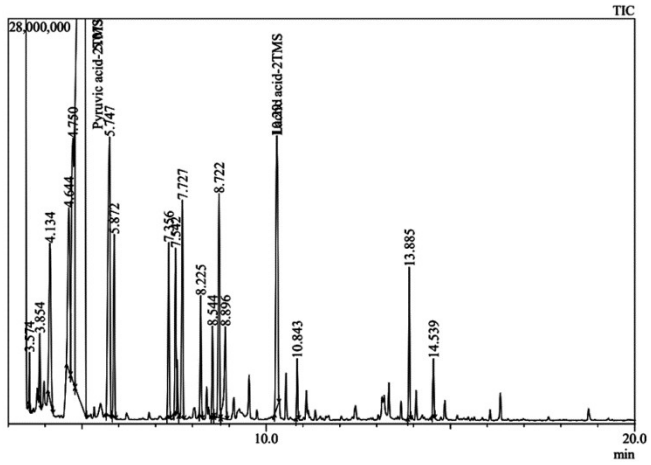
Suppl. Fig. 7: Analysis of Lactate

Superoxide mediated lactate to pyruvate reaction using KO_2 (without L-LDH) GC–MS analysis of the buffer + lactate + NAD + + KO 2 reaction mixture showed a large peak assigned to pyruvic acid-2TMS at 5.064 min (39.49% relative peak area) along with a peak attributed to lactic acid-2TMS at 10.290 min (5.39% relative peak area). The presence of pyruvic acid in the reaction mixture containing lactate is compatible with the production of pyruvate under the experimental circumstances. These observations offer a qualitative GC–MS proof of the transition of lactate to pyruvate in the reaction system.



Suppl. Fig. 8: Analysis of superoxide (KO_2) mediate reaction without enzyme (L-LDH)

Superoxide mediated lactate to pyruvate reaction using KO_2 (with L-LDH) GC–MS analysis of the reaction mixture comprising buffer, lactate, NAD+, KO2 and LDH showed a major peak corresponding to pyruvic acid-2TMS with a retention time of 5.073 min with a relative peak area of 38.91%. Lactic acid-2TMS was identified at 10.291 min with a relative peak area of 5.07 %. Detection of pyruvic acid-2TMS gives qualitative proof of the existence of pyruvate in the reaction mixture under the experimental conditions.



Suppl. Fig. 9: Analysis of superoxide (KO_2) mediate reaction with enzyme (L-LDH)

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