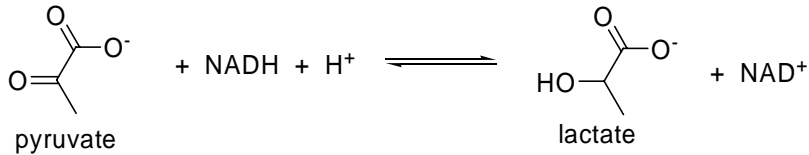


LACTATE DEHYDROGENASE

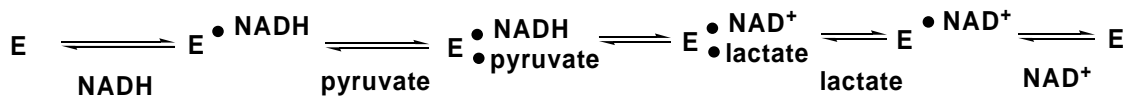
Enzyme Extraction, Purification, and Kinetic Behavior

Introduction: Lactate dehydrogenase (LDH) is a tetrameric enzyme which catalyzes the reduction of pyruvate by reduced nicotinamide adenine dinucleotide (NADH). Five isoenzymes are known. They consist of the various possible combinations of 4 subunits of the "H" and/or "M" type. The isoenzymes differ in molecular weight, isoelectric pH and K_m for substrates even though they catalyze the same reaction. The "M₄" type of LDH predominates in muscle; it is characterized by a low K_m for pyruvate, and thus promotes glycolysis by allowing removal of pyruvate as fast as it is formed. The H₄ LDH, found in heart, an actively respiring tissue, has a high K_m for pyruvate. This causes pyruvate to be funneled directly into the citric acid cycle for combustion, rather than sidetracking it into lactate. In emergencies, where the O₂ supply is not sufficient, the enzyme allows glycolysis to occur to produce needed ATP.

All isozymes of LDH catalyze the following reaction:



Kinetic studies indicate that there is a compulsory binding order:



The rate of the reaction can be followed by monitoring the absorbance of NADH at 340 nm (the oxidized form, NAD⁺, does not absorb at this wavelength).

Reduction of pyruvate can be inhibited by the presence of compounds structurally similar to pyruvate. Such compounds will form unreactive ternary complexes (*i.e.*, enzyme + inhibitor + NADH). This experiment will examine the inhibition by the anion of oxamic acid and by ethyl oxamate and will allow determination of K_m for pyruvate, the type of inhibition produced, and K_i values for the two inhibitors.

Enzyme purification: The enzyme will be extracted from fresh or frozen chicken or turkey muscle. Partial purification will be achieved by successive ammonium sulfate precipitations.

Extraction and partial purification of LDH

Note: All operations should be carried out in an ice bath. Work in pairs.

A. Extraction - keep all solutions and suspensions on ice at all times!

The initial part of the extraction will be done by the instructor assisted by a couple of volunteers. Cut about 60 g of chicken into small pieces. Add enough ice cold 0.03 M KOH to produce a runny slurry. Homogenize in a pre-chilled container using a Waring blender. Stir the suspension continuously for ten minutes. Filter through three layers of cheesecloth, squeezing gently. Portions of this crude extract will be passed out to individual work groups. Keep yours on ice!

B. First Ammonium Sulfate Precipitation - work on ice at all times!

Pour your crude extract into a 250 mL Erlenmeyer flask and estimate its volume. Swirl constantly while slowly adding powdered ammonium sulfate – use 258 mg of salt per mL of filtrate. Mixing must be vigorous enough to prevent local accumulations of undissolved salt, but not so vigorous as to produce extensive foaming. Rapid swirling is very effective; stirring rods are not unless the suspension is very thick.

When all the salt has dissolved, the solution is about 45% saturated in ammonium sulfate. The high ionic strength "ties up" a lot of water molecules and thus causes selective precipitation of some of the proteins. Let the mixture stand on ice for about 10 minutes to allow completion of precipitation.

Since ammonium sulfate is the salt of a strong acid and a weak base, its water solutions are weakly acidic (pH 5.5, in this case) due to hydrolysis. Normally one would worry about pH control, but LDH is a very tolerant enzyme so we need not adjust the pH.

After 10 minutes centrifuge the thick mixture for 5 minutes at medium speed (about 10000 rpm), using another group's tube or a weighed water-filled tube as a balance tube. Save the clear supernatant (filter through a glass wool plug if necessary to remove floating fat), discard the precipitate, and wash and shake dry the centrifuge tube.

C. Second Ammonium Sulfate Precipitation

Measure the volume of the supernatant, and bring it to 72% saturation (use the same mixing technique as above) by adding 190 mg of powdered solid ammonium sulfate per mL of supernatant.

After all the ammonium sulfate has dissolved, let the mixture sit for 10 minutes on ice, then collect the precipitate by centrifugation at 10000 rpm for 5 minutes. Discard the *supernatant* (keep the pellet).

D. Third Ammonium Sulfate Precipitation

Add a volume of water equal to the volume of the pellet and stir gently to dissolve the pellet. Estimate the volume of the dissolved protein solution, and add to it an equal volume of saturated ammonium sulfate solution. Mix well, remove from the ice bath and allow to stand at room temperature for the next 10 minutes. Collect the precipitate by centrifugation for 5 minutes at 10000 rpm. Decant most of the supernatant. Resuspend the precipitated enzyme in the remaining supernatant and transfer the suspension to a capped, labeled vial. The enzyme can be stored in the refrigerator in this state for many months.

Study of LDH Kinetics: Determination of K_m and K_i

A. Working solution of LDH and assay

You will need a simple assay to detect the presence of LDH. All assay mixtures in this part and the next must be at room temperature. As a start, prepare 4 tubes containing:

0.1 M phosphate buffer pH 7 (2.0 mL)
2 mM NADH (0.2 mL)
1 mM pyruvate (0.7 mL)

To make a stock solution of LDH, add a drop or two of your ammonium sulfate suspension to 5 mL of 0.1 M phosphate, pH 7.0. A working solution of LDH is prepared by diluting the stock solution to an appropriate concentration – one which, under the assay conditions, gives a rate that is neither too fast nor too slow. The working solution of LDH should give a decrease of 0.25-0.30 A_{340}/min . when 0.1 mL of it is added to one of the assay tubes. A_{340} should start high and decline over time as NADH is oxidized. Take data every 5 sec. From the computed rate, adjust your LDH concentration until the rate is in the correct range. You should have about 10 mL of working solution (no less than 5!) – you cannot afford to run out.

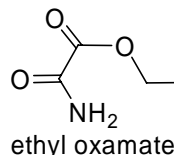
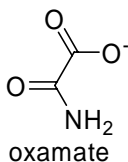
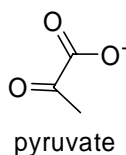
B. Determination of K_m for pyruvate (NOTE: This part and the next must be done with the same working solution of LDH on the same day.)

Set up the following assays and find the reaction rate for each (remember to start the reaction by addition of enzyme, mix well by inversion, and begin taking readings as soon as possible after mixing - you want to get the initial slope). Remember that slow samples may heat up in the sample compartment, affecting the rate. NOTE: Total volume in each assay is 3.0 mL.

Tube #	1	2	3	4	5	6
contents (mL)						
~2mM NADH*	0.2	0.2	0.2	0.2	0.2	0.2
~1mM pyruvate*	0.1	0.2	0.3	0.5	0.8	1.1
0.1 M P_i , pH 7.0	2.6	2.5	2.4	2.2	1.9	1.6
Enzyme (when ready to start)	0.1	0.1	0.1	0.1	0.1	0.1

*masses in *stock solutions*: NADH = about 1.48 mg/mL, pyruvate = about 0.11 mg/mL - **BUT CHECK THE LABEL and record actual molarity**. Note that these actual molarities are **NOT the final concentrations** in the tubes assayed above. When you work up your data you must use the final concentrations in the assayed tubes.

C. Inhibitor effects



Set up 12 tubes according to the following protocol and determine the reaction rates as above.

tube #	1	2	3	4	5	6	7	8	9	10	11	12
contents (mL)												
~2mM NADH	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2
~1mM pyruvate	0.1	0.2	0.3	0.5	0.8	1.1	0.1	0.2	0.3	0.5	0.8	1.1
0.1 M P_i , pH 7.0	2.5	2.4	2.3	2.1	1.8	1.5	2.5	2.4	2.3	2.1	1.8	1.5
~10mM Na oxamate*	0.1	0.1	0.1	0.1	0.1	0.1	0	0	0	0	0	0
~10 mM ethyl oxamate*	0	0	0	0	0	0	0.1	0.1	0.1	0.1	0.1	0.1
enzyme (when ready to start)	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1

*masses in *stock solutions*: sodium oxamate = about 1.11 mg/mL, ethyl oxamate = about 1.17 mg/mL; **CHECK THE LABELS and record actual molarities**. Again, these are **NOT the final concentrations** in the assayed tubes.

D. Data presentation

As you write your experimental section, remember that final concentrations of important chemicals are needed (*i.e.*, those in the assayed tubes), not the stock bottle concentrations.

Present the data collected in parts B and C of this protocol as follows:

- (a) format data from all three experiments according to either the Lineweaver-Burk or the Hanes-Wolff method. Plot all on a single chart to determine the type of inhibition caused by oxamate and by ethyl oxamate. *Note: Discard obvious outliers before imposing a best-fit line. Straight lines that you impose on data points must be extended so that they cross the x-axis (L-B) or the y-axis (H-W). Make the plotting program do this from the Options tab on the "add Trendline" screen.* In a table, report (**with proper units**) K_m for pyruvate, K_I for oxamate and K_I for ethyl oxamate.
- (b) attach an appendix in which you show how you computed the values in your report. *Be careful: the type of plot you choose dictates how you must compute K_m and K_I .*
- (c) also in the Appendix, include a plot of V vs. $[S]$, on which you show all 3 experiments suitably identified.

In your discussion, comment on the type of inhibition found for each inhibitor, including a discussion of the relationship between molecular structure and behavior/effectiveness as inhibitor.

By what factor would K_m estimated from the V vs. $[S]$ plot in the Appendix differ from the more accurate value obtained from your reciprocal plot? (Compare as a ratio whose denominator is the value from the reciprocal plot.)