Practical 3: Enzymes
Assay of lactate dehydrogenase activity

Introduction

Lactate dehydrogenase is an important cytosolic enzyme that occurs in different isoenzyme forms in different tissues (named $LD_1 - LD_5$). These experiments take advantage of the fact that LD_1 is the dominant enzyme in heart muscle in most higher vertebrates, while LD_5 predominates in skeletal muscle. The isoenzymes differ in their kinetic parameters and you will be able to determine quantitatively these factors during this practical. For these experiments, samples of lactate dehydrogenase (the enzyme is extracted commercially from bovine sources of heart and skeletal muscle) are supplied diluted in phosphate buffer, pH 7.5.

Aims of the practical

1. to measure the initial velocity of an enzyme-catalysed reaction, using a recording spectrophotometer.

2. to demonstrate the effect of increasing substrate concentration on the rate of an enzyme-catalysed reaction.

3. to make a graphical analysis of the kinetic data by two methods: the Michaelis-Menten plot and the Lineweaver-Burk plot.

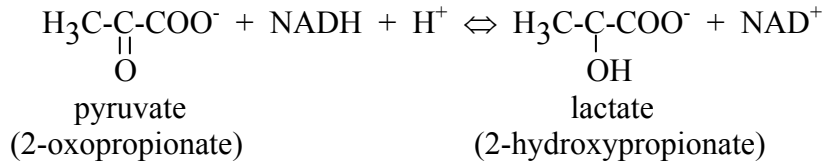
4. to estimate the values of K_m and V_{max} for the heart and skeletal muscle isoforms of the enzyme

5. to demonstrate that the isoenzyme forms of lactate dehydrogenase in different tissues have different kinetic parameters.

Experimental

Principle of the assay

Lactate dehydrogenase (LDH) is an NAD^+- dependent enzyme that catalyses the interconversion of pyruvate and lactate:-



The enzyme exists in five different forms, which are examples of isoenzymes (see Addendum). The enzyme is assayed conveniently by using the physical properties of the coenzyme NAD^+/NADH. The oxidised form (NAD^+) does not absorb light at 340nm, but at this wavelength the reduced form (NADH) shows maximum absorbance. The eye is not sensitive to ultraviolet light of this wavelength, but fortunately instruments that are sensitive at this wavelength are available. The spectrophotometer in front of you is an example. Since lactate dehydrogenase catalyses a reversible reaction the enzyme may be assayed either using pyruvate and NADH or by using lactate and NAD^+. You will be using lactate and NAD^+ (which is more chemically stable – and cheaper - than NADH!) Thus as the reaction proceeds the absorbance

of the reaction mixture at 340nm *increases* with time and the rate of increase in absorbance can be related to the rate of the enzyme catalysed reaction.

In all enzyme assays it is important that parameters that might affect the rate of the reaction are kept constant. In these experiments only the substrate concentration will be varied. The pH and ionic strength of the reaction will be constant (the same buffer at pH 7.5 will be used); the temperature will be constant (the centrally-heated laboratory temperature is about 22^{o}C); the enzyme and co-enzyme (NAD^{+}) concentrations will be constant (the same volumes will be used throughout).

Procedure

The initial rate of lactate dehydrogenase activity will be measured over a range of substrate concentrations. This information permits calculations of the K_m value of the enzyme for lactate. The two isoenzyme forms of lactate dehydrogenase that you will investigate differ in their K_m values. One-half of the group will use the heart enzyme (LDH_1) and the other group will use the skeletal muscle source of the enzyme (LDH_5). The results of the whole class will be compared at the end of the practical session.

The equipment used for the enzyme assay is a spectrophotometer (set at 340nm) the output from which is recorded continuously by a chart recorder. The NAD^{+} (coenzyme) solution and the lactate (substrate) solutions will be dispensed from glass dispensers set to the appropriate volumes. The enzyme solution will be measured using a Gilson auto-pipette set to deliver 100 µl. You will need to be very accurate in the use of pipettes and other apparatus in order to obtain reproducible data.

Setting up

The instructions indicated in steps 1 - 12 show you how to assay a series of seven cuvettes containing identical concentrations of lactate dehydrogenase and NAD^{+} (0.1mM) in buffer, but with a range of different concentrations of lactate as indicated in the list below: You will use a range of seven sodium lactate concentrations that, when diluted with the other components in the cuvette, will give final concentrations as shown in the table.

Cuvette number	Final concentration of sodium lactate
1	0.25mM
2	0.50mM
3	0.75mM
4	1.00mM
5	1.50mM
6	2.00mM
7	3.00mM

Performing the assay

1. Your demonstrator will show you how to use the spectrophotometer and recorder. The spectrophotometer wavelength control will be set to 340nm and the reading on the recorder will be set just above zero.

2. Add 1.9ml of the NAD^+ solution (from the dispenser) into each of seven spectrophotometer cuvettes (1cm light path-length).

3. Add 1ml of each of the different lactate solutions (provided in the pre-set labelled dispensers; final concentration range from 0.25mM – 3.00mM) to the series of cuvettes, remembering the order from lowest to highest concentration.

4. Mix the contents of the first cuvette by covering with a square of plastic film (Nescofilm or Parafilm) and inverting the cuvette twice. Wipe any liquid from the outer surfaces of the cuvette, place it in the spectrophotometer with the *clear* faces in the light path, press CAL and check that the recorder reads just above zero (the zero position on these instruments can be varied arbitrarily.)

5. Start the recorder chart paper and place the pen on the paper. There should be a steady horizontal trace, with no change in absorbance as there is no enzyme present yet to catalyse the conversion of NAD^+ to NADH.

6. When you are confident you know how to use the recorder, you can get ready to add the enzyme to start the reaction. The initial velocity is the most important part of the reaction, when little product has formed. Mixing of enzyme and substrate as indicated in step 7 & 8, must be done quickly and the cuvette replaced in the spectrophotometer as rapidly as possible (3 - 4 seconds maximum). Delayed measurements of the reaction rate can give very misleading results.

7. To start the reaction remove the cuvette from the spectrophotometer and add 100µl of the enzyme solution using a Gilson auto-pipette. *Lightly wipe the pipette tip before you make that addition.*

8. Rapidly mix the cuvette contents by inversion, as before. Quickly wipe the outside of the cuvette and replace it in the spectrophotometer. Record the increase in absorbance at 340nm for about 5 minutes. You will have produced a 'progress curve', i.e. change of the concentration of NADH in the cuvette with time. Label your chart paper clearly so that you can identify the individual traces.

9. Whilst the recording is proceeding, get ready to set up the next assay with a different lactate concentration. For each lactate concentration repeat the procedure from step 4 above. A recording from 2 – 5 minutes should be long enough to produce a curve to which you can draw a tangent to the initial part

10. After completing the experiment with all seven different lactate concentrations, measure the tangents to each of the progress curves to determine the initial velocities and fill in the data in **Table 1** on the Results sheet. *Repeat any obviously erroneous measurements. Note that full scale deflection across the chart paper (100 divisions) represents 0.5 absorbance units; the chart speed is set to 1cm/min.*

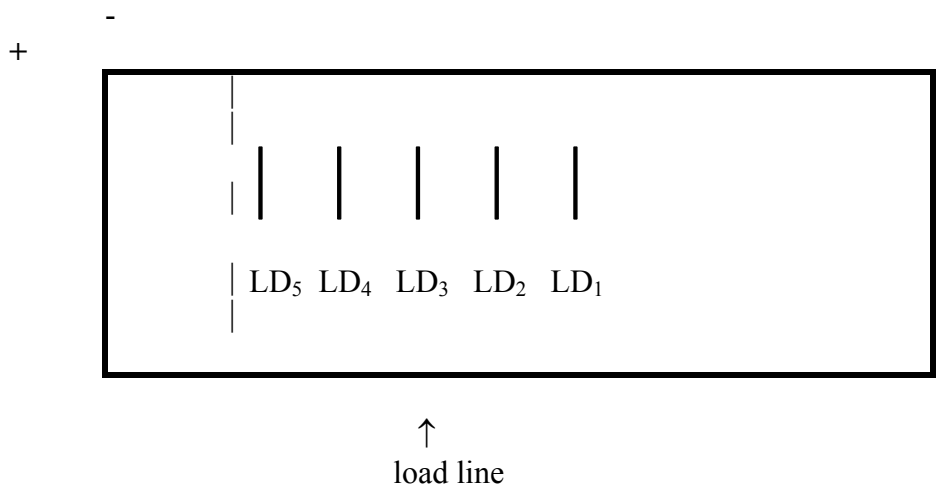
11. Plot the data by hand on graph paper by both the Michaelis-Menten plot and the Lineweaver-Burk plot. From the latter derive values of K_m and $V_{max.}$

12. Then compare the values you have obtained with those from a group using the other tissue source of the enzyme. Fill in the values of K_m and V_{max} in **Table 3**.

Addendum - Lactate dehydrogenase isoenzymes

Each isoenzyme of LDH is a tetramer consisting of four polypeptide chains formed by a random combination of two distinct types of polypeptide (different gene products) referred to as M (muscle) and H (heart). (Note that lactate dehydrogenase is an example of a protein with a quaternary structure; you are already familiar with haemoglobin). Thus there are five LDH isoenzymes which contain 4H chains (LD_1), 3H + 1M (LD_2), 2H + 2M (LD_3), 1H + 3M (LD_4), and 4M (LD_5). These isoenzymes have approximately the same molecular weight of 135,000da. The M and H chains have different primary amino acid compositions and carry different electric charges and can thus be separated by electrophoresis; LD1 is (by definition) the most electropositive at pH 8.0.

Migration of lactate dehydrogenase isoenzymes upon electrophoresis at pH 8.0.



Lactate dehydrogenase assay – results sheet

Table 1. Initial rates of reaction (v_i) at seven different concentrations of sodium lactate. (You may take the data from colleagues who have used a second isoenzyme form.)

Lactate concentration in cuvette [S](mM)	Initial rate of reaction (v_i) ΔA_{340}/min. heart muscle enzyme	Initial rate of reaction (v_i) ΔA_{340}/min. skeletal muscle enzyme
0.25		
0.50		
0.75		
1.00		
1.50		
2.00		
3.00		

Plot the curves for the initial rates of reaction, (v_i), against the substrate concentration, [S], for both the heart and skeletal muscle LDH preparations, using the same sheet of graph paper. This graph is usually called a Michaelis-Menten curve. Use sensible parameters; you do <u>not</u> have to fill the page with your graph – common sense will achieve a satisfactory effect! Calculate the data to three significant figures. You will use this data to calculate the reciprocal values in Table 2.

Table 2. The reciprocal values of (v_i) and [S] derived from Table 1 for determining kinetic parameters by graphical means (Lineweaver-Burk plot)

[S](lactate concentration in cuvette {mM})	[1/S](reciprocal lactate concentration in cuvette {mM})*	$1/v_i$ (reciprocal initial rate of reaction {ΔA_{340}/min.}* heart muscle enzyme)	$1/v_i$ (reciprocal initial rate of reaction {ΔA_{340}/min.}* skeletal muscle enzyme)
0.25			
0.50			
0.75			
1.00			
1.50			
2.00			
3.00			

Plot the data for $1/v_i$ against 1/[S] for both the heart and skeletal muscle lactate dehydrogenase preparations, using the same sheet of graph paper. Calculate the data to three significant figures. This graph is usually called the Lineweaver-Burk plot. Use sensible parameters; you do <u>not</u> have to fill the page with your graph – common sense will achieve a satisfactory effect! From the graph determine the kinetic parameter for each lactate dehydrogenase preparation and insert the values in Table 3.

*To be strictly accurate the units of the reciprocal concentration and of reciprocal ΔA_{340}/min should be used here. However, since the final results are expressed in terms of mM concentration and ΔA_{340}/min the more simplistic approach has been adopted in this table.

Table 3 Summary of kinetic parameters for lactate dehydrogenase preparations from skeletal muscle and heart muscle.

Lactate dehydrogenase preparation	$1/K_m$ (mM)*	K_m (mM)	$1/V_{max}$ (ΔA_{340}/min.)*	V_{max} (ΔA_{340}/min.)
Skeletal muscle				
Heart muscle				

*To be strictly accurate the units of the reciprocal concentration and of reciprocal ΔA_{340}/min should be used here. However, since the final results are expressed in terms of mM concentration and ΔA_{340}/min the more simplistic approach has been adopted in this table.

Tutorial 3 also covers enzyme kinetics, but please make sure that you have understood your results before you leave the laboratory, as no additional material relating to this practical will be made available to you later.

Have you signed the register?

This practical will be assessed by a short multiple choice test, which will take place after all students have completed the practical. See pages 75 and 78 for information on the format of the questions and computer marked forms. Questions will cover properties of enzymes, enzyme kinetics, and background to and conduct of the experiment. It is unnecessary (and pointless) to learn the specific values you obtained for Km and Vmax.

You will need a calculator for the test.