

# Secrets of Life: Enzymes and Chemical Reactions

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## Summary

We will learn how enzymes accelerate the chemical reactions that make our bodies work. We will investigate the time course of an enzyme catalyzed reaction by ultraviolet spectroscopy. Our data analysis will reveal the kinetics of the reaction, and also explain enzyme specificity.

Concepts: Chemical reaction, enzymes, catalysis, macromolecule (protein), spectroscopy, chemical kinetics, rate of change, graphing of data

## Materials provided

3 workstations, including

UV/Vis spectrophotometer

Personal computer with molecular graphics software, data analysis software, spectrophotometer software

UV Cuvettes

“Green” solution: Green food coloring in water

Buffer solution: 50 mM Tris (hydroxymethyl) aminomethane (CAS# 77-86-1) in water, at pH 8 (also part of the sample solutions below)

## Sample solutions

OXA: 3.6 mM oxaloacetic acid (CAS# 328-42-7) + 1.2 mM NADH ( $\beta$ -Nicotinamide-adenine Dinucleotide, CAS# 606-68-8) in buffer, pH 8

SUC: 3.6 mM succinic acid (CAS# 110-15-6) + 1.2 mM NADH (CAS# 606-68-8) in buffer, pH 8

KGL: 3.6 mM  $\alpha$ -ketoglutaric acid (CAS# 328-50-7) + 1.2 mM NADH (CAS# 606-68-8) in buffer, pH 8

Enzyme: 0.6  $\mu$ M malate dehydrogenase from *Sus scrofa* (CAS# 9001-64-3) in buffer, pH 8

Note: These solutions must be kept frozen, and should not be thawed more than an hour before performing the experiments.

**Other materials required**

Laboratory gloves

Closed eyeglasses

Laboratory coat (optional)

Distilled water in squirt bottle

Paper towels

Pipettes

Glue, scissors

Calculator (optional)

Printer

## **Read this before you start**

### Hazards

The chemicals that are supplied with this experiment (for list, see above) may be harmful if swallowed, inhaled or absorbed through the skin. May cause skin, eye and respiratory tract irritation. In case of skin contact, wash off with soap and plenty of water. In case of eye contact, rinse thoroughly with plenty of water for at least 15 min. Contact a physician if irritation occurs. In case of ingestion, immediately contact a physician or poison control center.

### Laboratory practices

The laboratory environment should always be treated as potentially hazardous. Therefore, eating, drinking and smoking is strictly prohibited in the laboratory. Wear closed shoes (no sandals). During experiments, always wear gloves and eye protection (closed goggles). It is good practice to also wear a laboratory coat. After completing laboratory work, always wash hands with soap and water.

### Setup of equipment

Prepare required materials (see list above)

Warm supplied aliquots to room temperature, not more than one hour before the experiment

Connect SpectroVis Plus via the supplied USB cable to computer

### Disposal

The chemicals that are supplied with this experiment may be flushed down the drain with water.

## Activities

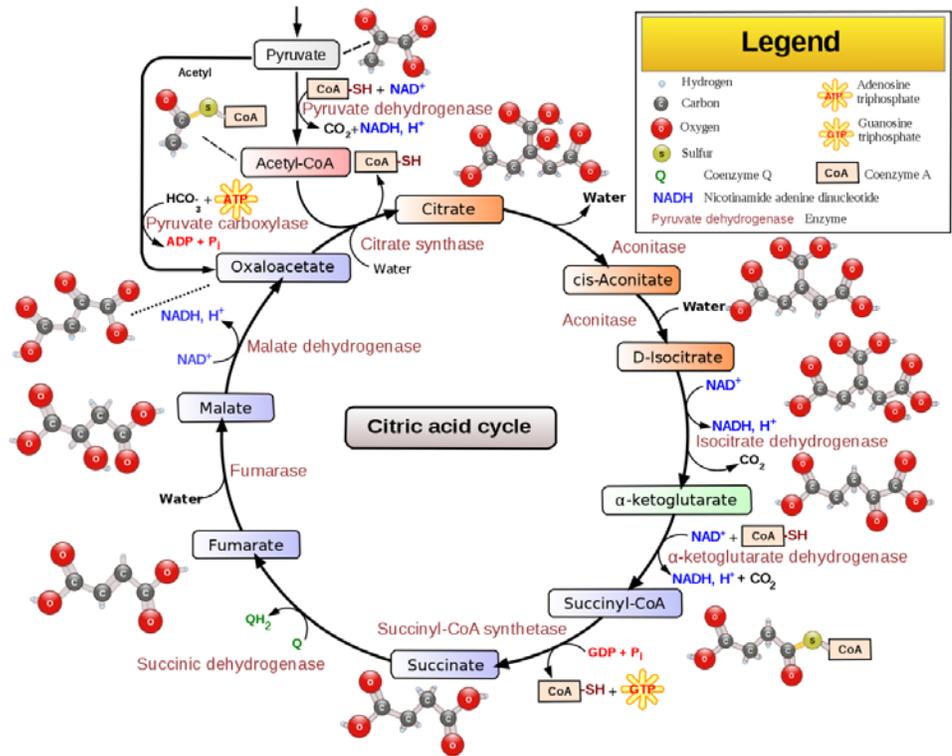
### Chemical reactions in our body

Have you ever wondered how our body can make us walk, feel, or even think? – For everything that we do, a practically uncountable number of chemical reactions occur in our cells, where molecules constantly change shape in a precisely orchestrated way. To get a perspective of how intricately complex this process is, look at the enclosed poster.<sup>1</sup> The poster shows some of the more important reactions that take place, pertaining to metabolism. Metabolism is the process by which our body turns food into energy, as well as into all of the molecules that our cells need.

Our understanding of the reactions that occur in our bodies, and how they play together to make a whole, living organism, is far from complete.

Scientists will still require many years to unlock more of these secrets of life.

The picture to the right shows an enlarged version of a part of the reactions that produce the energy in our body. You can see how molecules change into each other as you follow the arrows. If you look closely on the poster, you can find this reaction cycle near the lower left corner, inside of the yellow area.

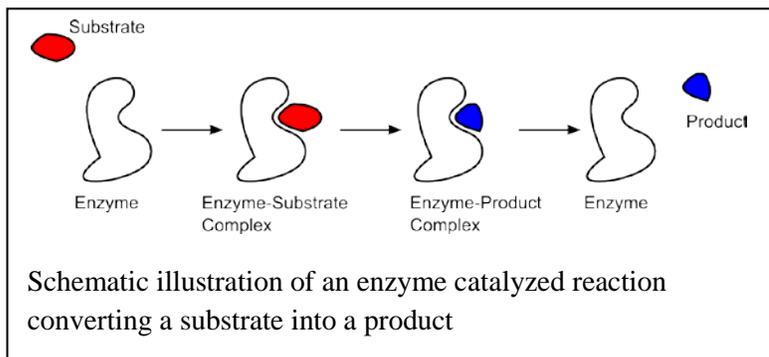


### Proteins as building blocks for our cells

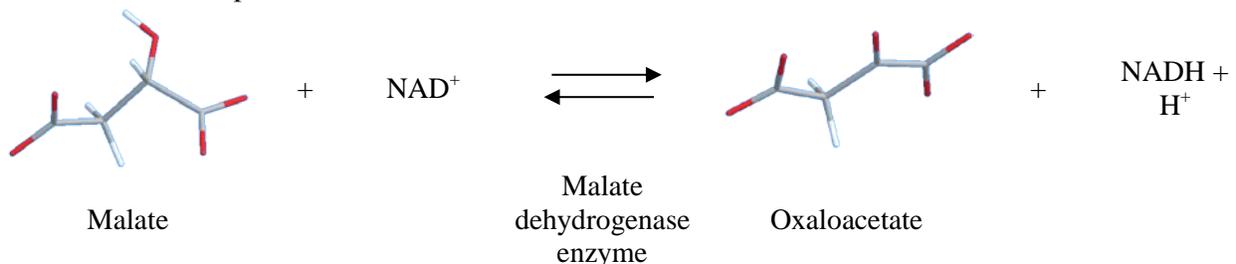
The poster is mainly concerned with the different “small molecules” that occur in our cells. The major building blocks of our cells, however, are proteins. Proteins are large molecules that our body creates by following the instructions contained in our DNA. Proteins are very special molecules. They are composed of only 20 different components, called amino acids, which are linked together like beads on a string. Proteins are more than just long strings. Depending on their sequence of amino acids, each folds up into a precisely defined shape. This shape is what gives each protein its function. Some act simply as blocks that build our cells. Others can interact with other molecules and make them undergo a chemical reaction. Those proteins are called enzymes.

### How enzymes make reactions happen

Enzymes are proteins that accelerate chemical reactions by binding to the reactants,<sup>1</sup> making the reaction occur, and releasing the products. This process is also called catalysis. It allows chemical reactions to occur that would otherwise be very unlikely to happen. Almost all of the reactions in our cells are catalyzed by an enzyme. On the poster and in the figure above, each arrow represents the action of an enzyme.



The enzyme that we will study today is called malate dehydrogenase, and it takes part in the reaction cycle that was discussed before. Malate dehydrogenase uses two reactants (also called substrates), and forms two reaction products:



In the stick drawings of the two molecules malate and oxaloacetate, oxygen atoms are red, carbon atoms gray and hydrogen atoms white. Below, the chemical structure drawings of the same molecules are shown. Look closely at the two molecules, and circle the position where the change in the molecule occurs. You can see that at the position you have circled, the malate molecule loses two hydrogen atoms. One of them is transferred to the NAD<sup>+</sup> molecule, which becomes NADH. The other one, indicated above as H<sup>+</sup>, is transferred to the enzyme.

We will now explore the enzyme in a molecular graphics program, and take pictures of it bound to the reactant molecules.

- Start the computer
- First, create a folder on the Desktop with your name (Right-click in the Desktop, select New -> Folder, and name the new folder with your name. Use this folder for saving all your files.
- Open the program “Chimera”<sup>2</sup> using the link on the Desktop, and maximize the window.
- Load the file 2dfd\_session1.py by selecting the menu option File -> Open. The file has been saved on the Desktop. Therefore, in the window that opens, click on Desktop. You will then find the file in the rightmost panel.

<sup>1</sup> Reactants are the molecules that undergo a reaction

2dfd\_session1.py has been saved from the entry 2DFD of the protein database.<sup>3,4</sup> It shows the malate dehydrogenase enzyme from humans.

Because the enzyme is a large molecule containing thousands of atoms, its picture can become confusing. Therefore, scientists often show only a cartoon of the “string” along which the different amino acids are arranged. This is the view that you obtain when you open the file. The “string” is also called the backbone of the protein. The cartoon shows how the protein is folded up into its three-dimensional shape.

- Use the left mouse button to rotate the molecule.
- Use the right mouse button to zoom in or out.
- Use the middle mouse button to move the molecule on the screen.
  
- Experiment with different viewing options. For example, in the menu Tools -> Depiction, you can select different coloring schemes. We (the authors) in particular like the rainbow colors.

Note: If you run into a problem, you can always go back to the original view by selecting File -> Close session, and then opening the file 2dfd\_session1.py again.

- Once you have found an overall view of the protein that you like, select File -> Save Image.
- In the window that appears click “Save As”. Then, in the right panel find and click on your folder that you have previously created on the Desktop. Type a filename of your choosing, such as “enzyme1”, and save your picture.
- To print your image, open your folder in Windows, and double click the image.
- Push the print button, then in the Wizard select “3.5x5 in prints”, and select “4” in “number of times to use each picture”. This way, you get one picture for each person in your group.

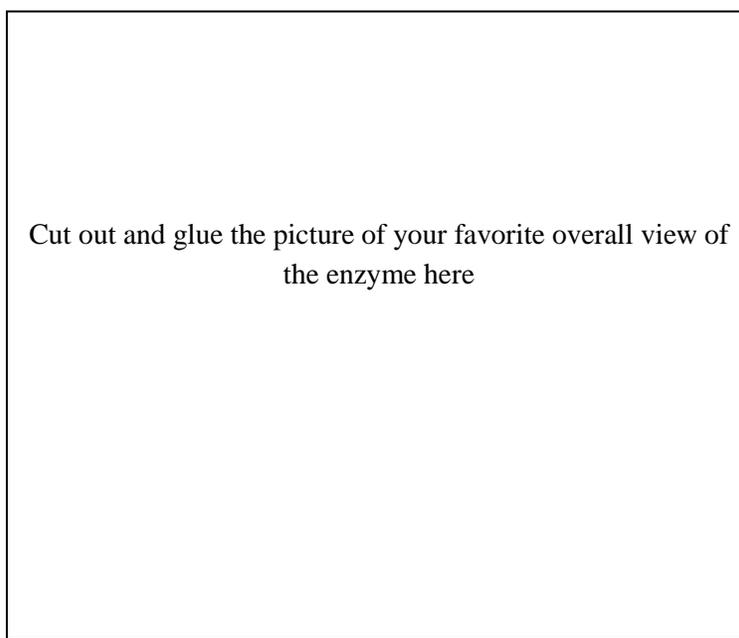


Image of malate dehydrogenase enzyme

In the picture, and on the screen, you will see that, in addition to the ribbon showing the protein, there are two smaller molecules shown in a stick representation. These are Malate and  $\text{NAD}^+$ . They are bound to the enzyme in the place, where the reaction occurs. This place is the most important part of the enzyme, where the action is, and is also called the active site.

- By comparing to the image of malate in the previous section, locate this molecule in the structure (rotate and zoom appropriately).

You can also see the other substrate molecule,  $\text{NAD}^+$ , which is the larger molecule located right next to the malate. By bringing the two molecules together in just the right way, the enzyme makes the reaction happen. A hydrogen atom is transferred from malate to  $\text{NAD}^+$ . The image also shows two of the amino acids in stick representation (if you have previously colored the protein, they may be shown in uniform color). Those two amino acids are also important for the reaction to occur. One of the hydrogen atoms from malate transfers to the amino acid with the ring structure (histidine) during the reaction.

The reactant molecules bind to the enzyme by tightly fitting into the binding pocket, like a key into a lock. The shape of the binding pocket is more easily visualized by looking at the actual, solid shape of the protein instead of the ribbon representation. When thinking of the binding pocket, it is also important to realize that proteins are quite flexible. Often, enzymes slightly change their shape when binding the substrate, reacting and releasing the product.

- In the menu, select Actions -> Surface -> Show

You now see that the malate is in fact bound deep inside of the protein. By rotating the molecule just right, you may still be able to look into the binding pocket and catch a glimpse of it. In order to better see the binding pocket, you can slice through the protein.

- Select the menu option Tools -> Viewing controls -> Side view
- In the window that opened, use the mouse to move the yellow lines. Anything that is outside of the two yellow lines will be cut away
- Try to arrange the protein, by rotating, zooming and adjusting the slice positions (yellow lines) so that you get a good view of the malate molecule in the binding pocket of the protein
- As before, save your picture (File -> Save Image) and print using Windows

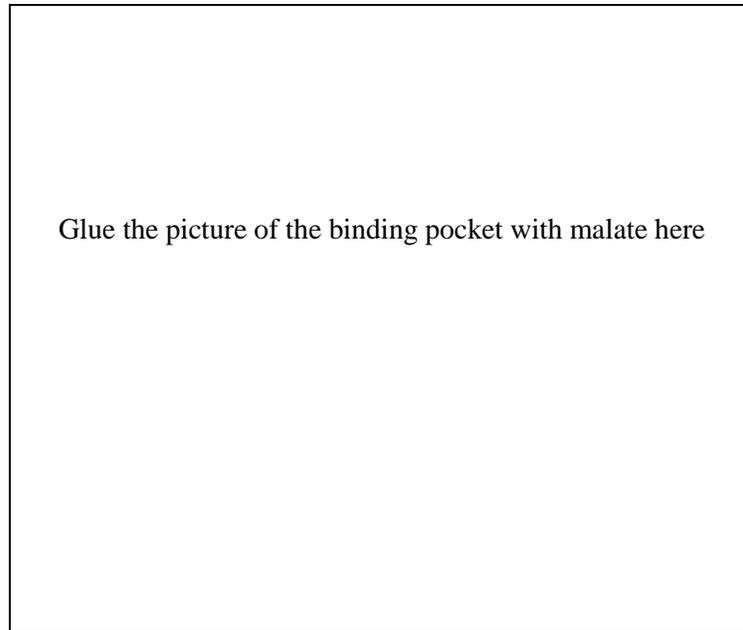


Image of binding pocket of malate dehydrogenase with malate

There are many additional things that you can explore if you have extra time now or at the end of the lesson. Here are a few suggestions:

You can try to view the protein in 3D stereo mode. Since you do not have stereo glasses, you will have to rely on “twisting” your eyes, which takes some getting used to. So don’t be frustrated if you do not immediately succeed in seeing a three-dimensional image.

- In Chimera, select the menu option Tools -> Viewing controls -> Camera
- In the window that opens, select “cross-eye stereo” for camera mode

You now have two images on the screen. The trick is to look at the left image with your right eye, while at the same time looking at the right image with your left eye.

- Sit squarely in front of the screen, about 3 feet away.
- With only your left eye open, use your left hand to cover half of the view, so that you see only the right image. Do not move the left hand after that.
- Similarly, with only your right eye open, use your right hand to cover half of the view so that you see only the left image. Do not move the right hand after that.
- Now, open both eyes. Since you left your hands in place, each eye will be able to see only one picture.
- Twist your eyes until the pictures from both eyes merge into one. You now see the molecule in stereo.
- At this point, you may remove the two hands while continuing to view the molecule.
- Use the mouse to move, rotate or zoom. If you succeed, the effect is quite stunning

Note: Once you have practiced a few times, you may no longer require the use of your two hands to initially block the images.

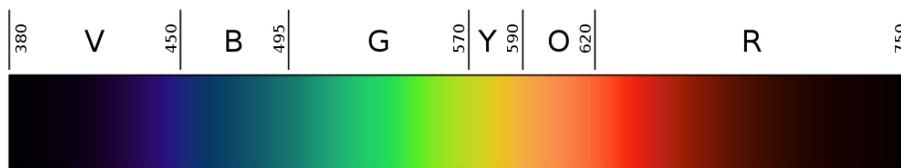
If you decide that stereo viewing is not for you today, you can also explore other views of the molecule.

- Hide the surface and ribbon by choosing Actions -> Surface -> hide and Actions -> Ribbon -> hide
- Show all of the atoms of the protein by selecting Actions -> Atoms/Bonds -> Show and Actions -> Atoms/Bonds -> Sphere
- You may further experiment with other options in the Actions menu

### Using spectroscopy to detect molecules

Since proteins and the other molecules that we have encountered are far too small to be seen by eye, or even by microscopy, scientists need other techniques to observe them. The picture of the protein that we have explored before was made by X-ray crystallography, which sometimes requires the use of synchrotrons as large as a football field.

Today, we will use a simpler technique, optical spectroscopy, for observing the molecules. We will not be able to take pictures of molecules,

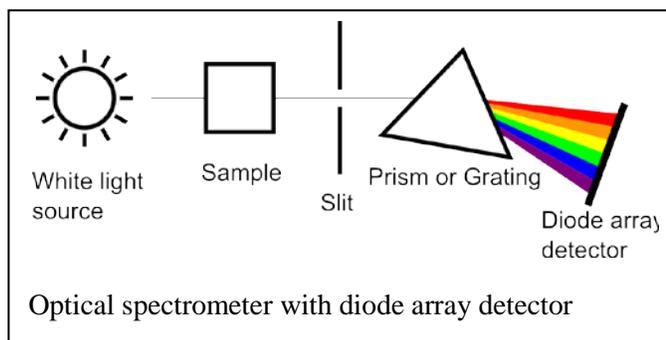


Spectrum of visible light. The wavelengths in nanometers (nm) are indicated at the top. The letters represent the colors.

but we will be able to determine their concentration, and how it changes over time as a reaction occurs.

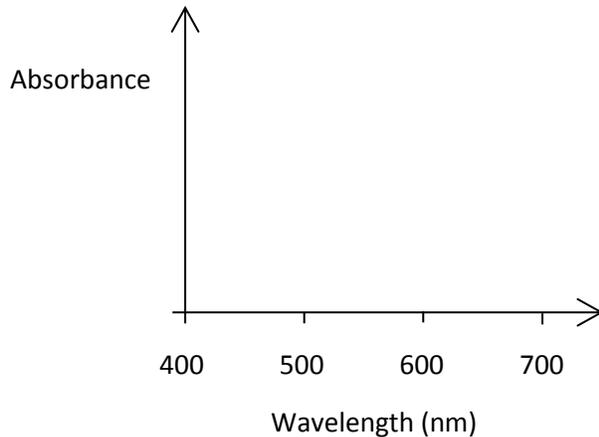
Optical spectroscopy detects the presence of molecules when light interacts with their electrons. Through this interaction, light of a certain wavelength range is being absorbed. Since white light is a combination of light of all colors, if a certain part of the spectrum is removed (absorbed), the object will appear colored.

The spectrophotometer that we will be using is capable of measuring the absorbance of a liquid in function of the wavelength. It does this by passing white light through the liquid, and detecting how much of each wavelength arrives at the other end. A high absorbance value for a given wavelength means that a large part of the light of that wavelength is absorbed by the substance.



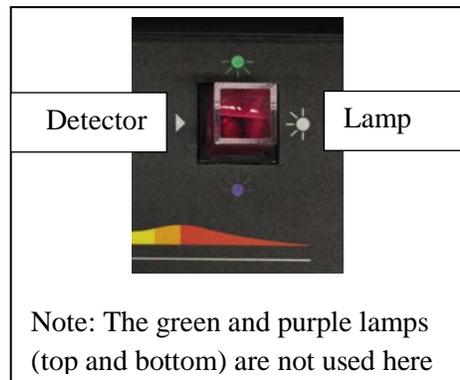
We will now record a spectrum of a green solution, and identify the wavelengths corresponding to different colors...

...but first, stop for a moment. Discuss with your group members how you would expect that spectrum to look like. Here is some space to draw it:

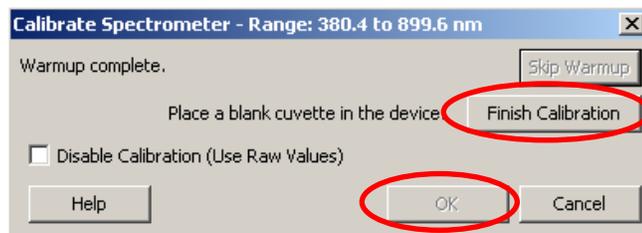
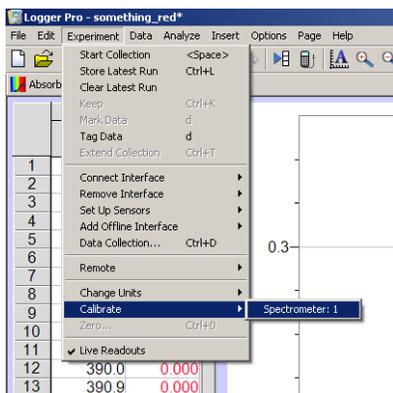


If you can't figure it out, it's OK. Just proceed to measure the spectrum:

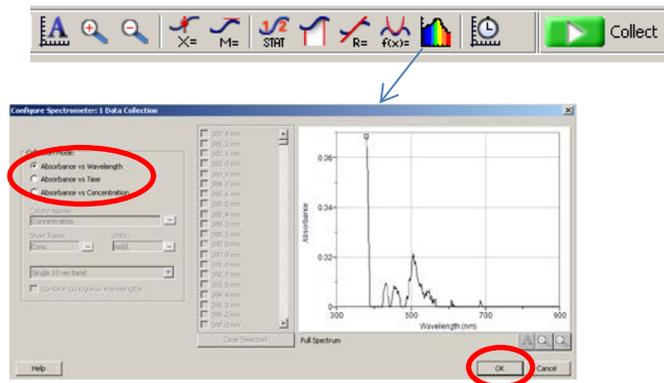
- If you have not already done so, put on your goggles and gloves.
- Take the cuvette, and identify the smooth face. This face is where the light of the spectrometer will pass through. To find which way the cuvette should be inserted into the spectrometer, find the direction of the light path.
  - Before filling the cuvette, take it out of the spectrometer



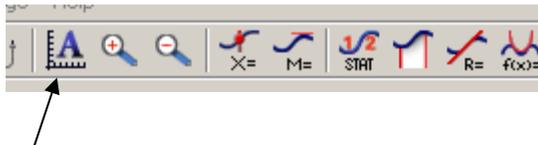
- Fill the cuvette to about 2/3 with water. Take a paper towel and carefully dry the outside of the cuvette.
  - Make sure that there is no liquid on the outside of the cuvette, as this could damage the spectrometer.
- Insert the cuvette into the spectrometer
- Start the program "LoggerPro".<sup>5</sup> The icon is located on the Desktop.
- Choose the option to calibrate (Experiment -> Calibrate; see also picture below). This procedure determines the reference point of how much light is detected from a transparent sample such as water. This sample is also called a "blank".



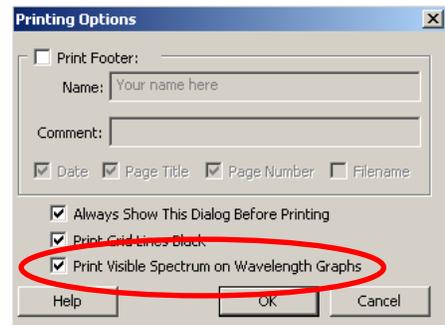
- Now, set up the spectrometer to measure absorbance vs. wavelength (see picture below)

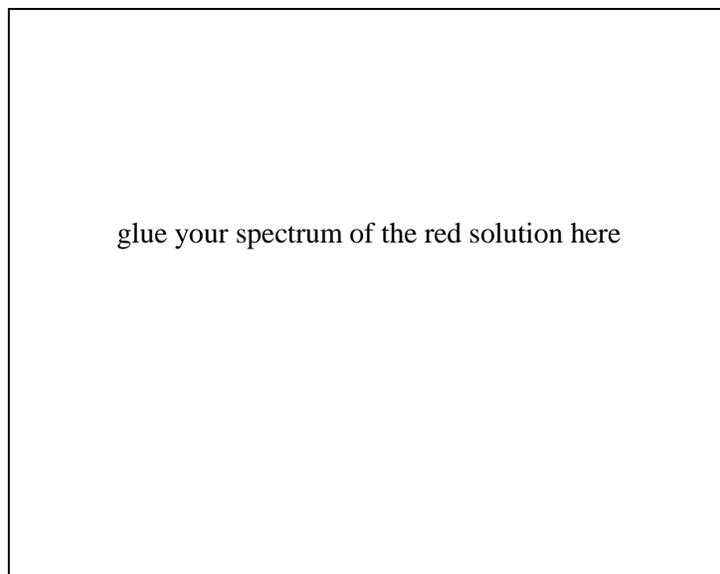


- Remove the cuvette, empty it and fill it with the green solution
- Insert into the spectrometer and collect the spectrum (push the green “Collect” button)
  - As always, make sure that there is no liquid on the outside of the cuvette.
- Once the spectrum has appeared, push the red stop button.
- Click on the topmost number on the vertical scale, and enter a number so that the spectrum fills the entire screen. You can also try to use the “A” button in the toolbar (see picture) to scale automatically.



- Then use the menu File -> “Save As...” to save the spectrum in your folder (located in Desktop), so you can later load it again if you need to. Select a file name of your choosing.
- Print the spectrum using File -> Print. If you like, you can first set the option to print the background colors (see picture).





Spectrum of green solution

Explain how you can see from the spectrum that this is a green object \_\_\_\_\_

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One thing that a spectrophotometer can do better than our eyes, is measure exactly how much light is absorbed at a given wavelength. This is shown by the vertical axis on the graph of your spectrum.

Since the amount of light absorbed is also related to the amount of absorbing molecules present, we can use this measurement to determine the amount of molecules present.

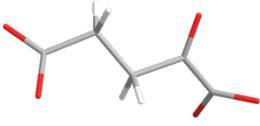
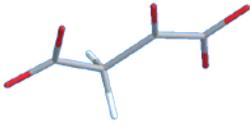
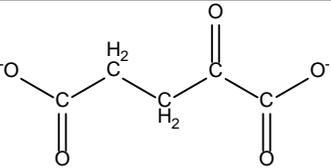
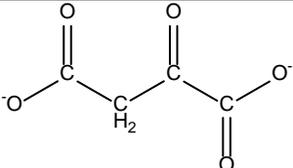
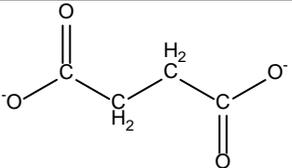
Beer's law relates the concentration of absorbing molecules to the absorbance of a solution.

$$A = \epsilon lc$$

$A$  is the absorbance,  $l$  the length of the light path, and  $\epsilon$  is called the extinction coefficient. The extinction coefficient is different for each molecule, and for a given molecule is also different for each wavelength.

### Measuring reaction progress by optical spectroscopy

We will examine a set of possible substrates (reactants) for the enzyme malate dehydrogenase, and determine for which ones the enzyme works. Our reaction mixtures consist of NADH, and the potential reactant. We will use optical spectroscopy to find whether a reaction occurs. These are our reactants, shown both in stick representation and as chemical structure drawings:

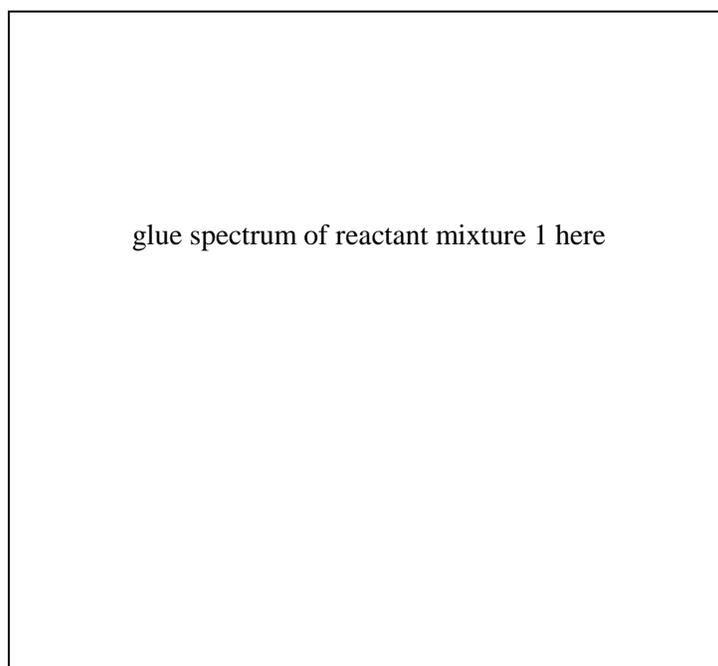
$\alpha$ -ketoglutarate	Oxaloacetate	Succinate
		
		

You can see that all of them are related in the sense that they contain similar arrangements of atoms (also called functional groups).

A note, which is not as important for the experiment, but mentioned here for completeness: In our experiment, we run the reaction in the opposite direction compared to the natural citric acid cycle. In principle, all reactions can occur in both directions, but one direction is more likely than the other. In the citric acid cycle, the “forward” reaction can only occur because the reaction product is continuously removed by the next step of the cycle.

In the first step, we will measure a spectrum of a substrate (reactant) solution.

- Empty the cuvette and wash it out with distilled water two to three times, to make sure that none of the previous solution remains
- Using a fresh pipette, fill the cuvette with the buffer solution.
- As always, make sure that there is no liquid on the outside of the cuvette.
  
- Insert the cuvette and calibrate the spectrometer (Experiment -> Calibrate; same way as above). Because all the solutions we will measure are in this buffer, the calibration will remain the same from now on and does not need to be repeated.
  
- Wash out the cuvette again and fill with reaction mixture “AKG” ( $\alpha$ -ketoglutarate + NADH) using a fresh pipette. Use the entire content of the vial provided.
- Set up the spectrometer to measure absorbance vs. wavelength
- Measure the spectrum (click Collect, then Stop), set up axes, save (File -> “Save As...”) and print (File -> Print)



Spectrum of  $\alpha$ -ketoglutarate + NADH solution

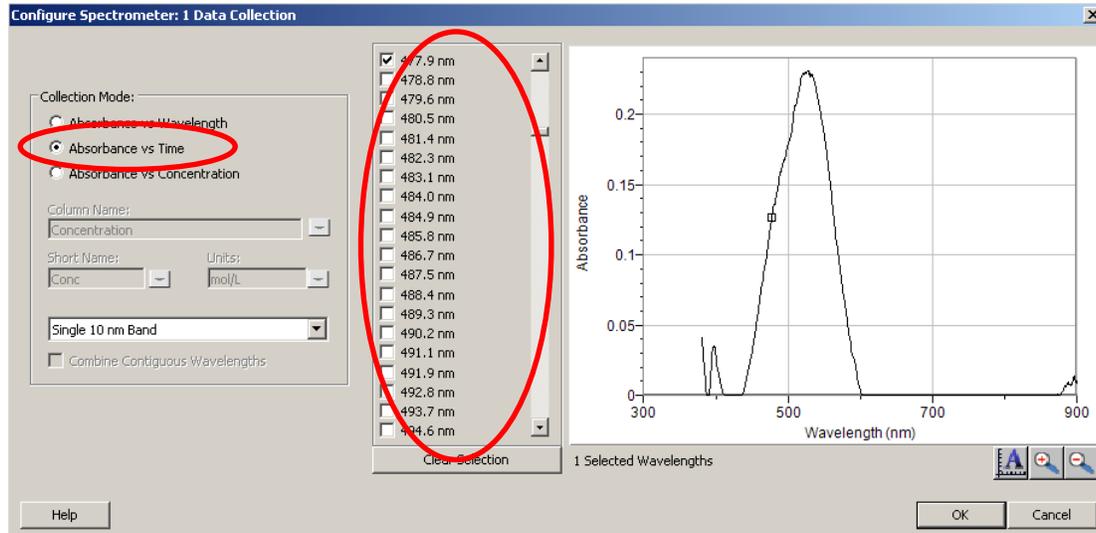
In fact, the only compound of our reaction that has absorbance in the wavelength range covered by our spectrometer is NADH. Which is the best wavelength to use for our subsequent observations, where we will monitor the absorbance at just one wavelength? (you can use the mouse to move the crosshair cursor and read the corresponding wavelength in the lower left corner of the spectrum window)

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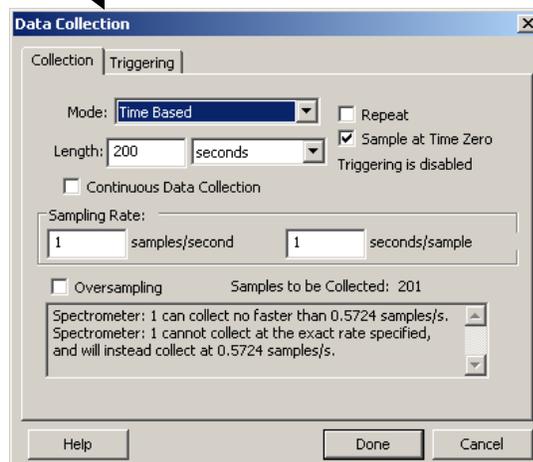
We will now test the activity of the malate dehydrogenase enzyme. The enzyme that we will use in our experiments is from pig instead of human, because it is easier to obtain samples. The enzyme is however similar in structure to the human enzyme.

At the wavelength that you chose, we will measure the absorbance in function of time. This type of measurement is also called a kinetics measurement. During the reaction, the amount of substance that has reacted changes with time, just as during a drive the distance traveled by a car changes. For a car, the velocity determines the amount of distance traveled in a given time. For chemical reaction catalyzed by an enzyme, the rate of catalysis determines the amount of reactant consumed in a given time.

- Set up the spectrometer in time dependent mode. Click the button indicated below.



- In the window that opens, select “Absorbance vs. Time”
- In the panel near the center of the window, select the wavelength that is closest to the one you have determined above, which you would like to monitor.
- Click OK. Since you have already saved your data previously, you can select “No” when asked whether you want to save the existing data.
- Use the button with the image of a stopwatch to choose the time settings. Set a measurement time of 200 seconds.

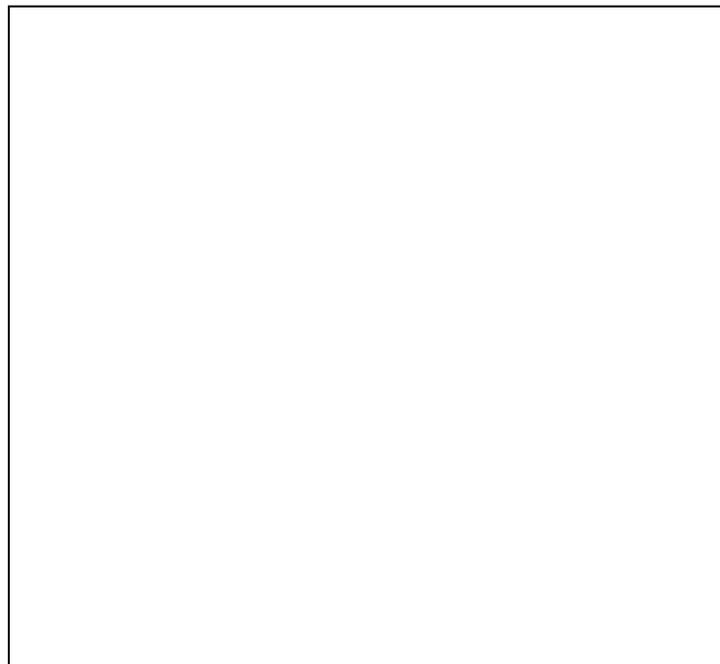


- Start the measurement with the green button, and watch the progress
- After the measurement has ended, as before, use File -> Save as to save, and File -> Print to print the resulting curve. There is space on the next page to glue your result.

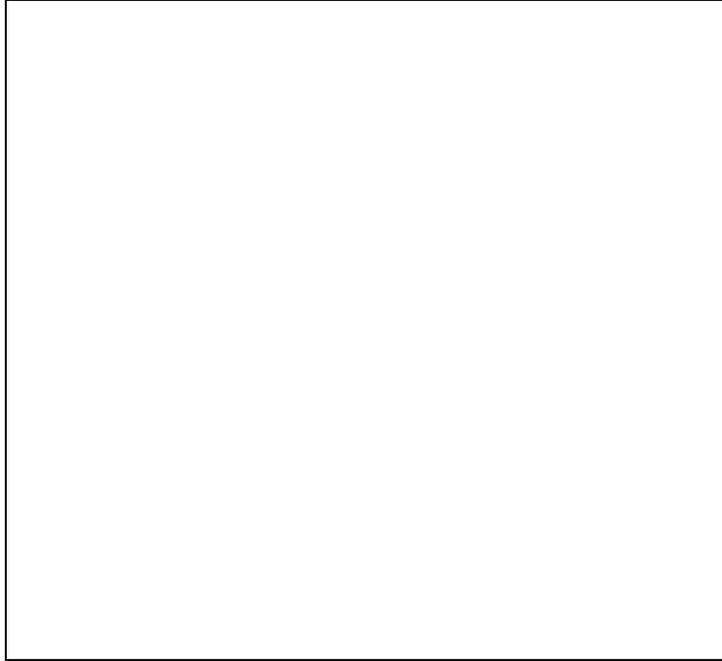
Did a reaction occur or not? How can you tell from looking at the curve that you have measured? \_\_\_\_\_

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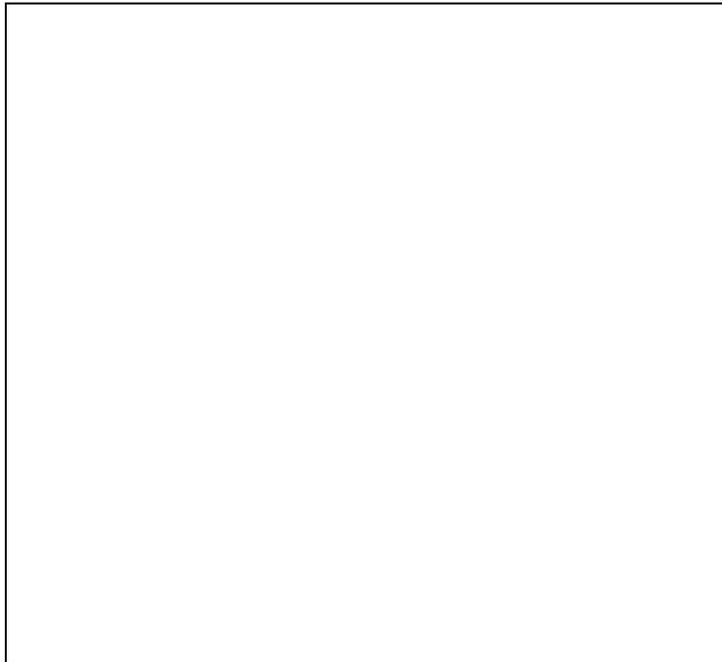
- Now, remove the cuvette from the spectrometer, and add the enzyme. Immediately mix with the pipette by taking up solution from the cuvette and squirting it back two or three times.  
 Make sure that you do not take more than a few seconds for mixing
- Wipe any liquid from the outside of the cuvette, and insert into spectrometer
- Start measurement
- Use File -> "Save As..." to save, and File -> Print to print the resulting curve
  
- Repeat the measurement with and without enzyme for reaction mixture "OXA" (oxaloacetate + NADH)
- If you have time, also repeat the measurement with and without enzyme for reaction mixture "SUC" (succinate + NADH)



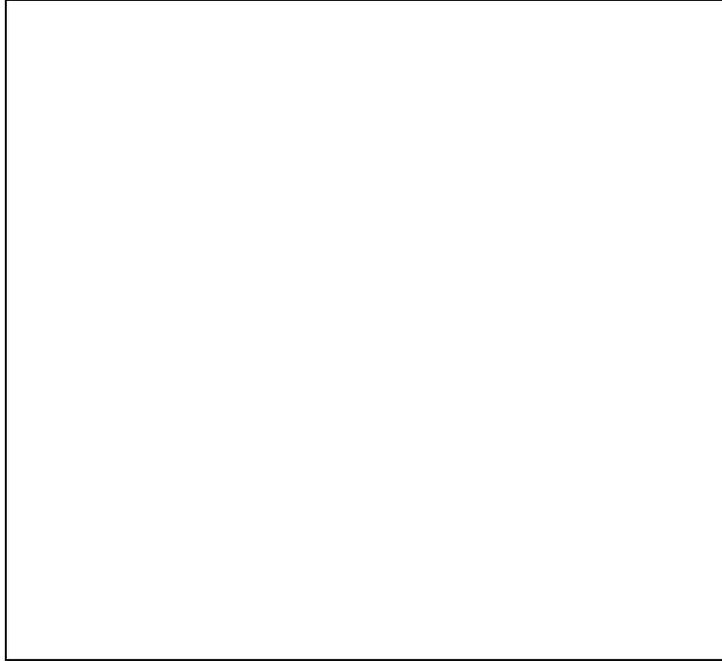
$\alpha$ -ketoglutarate + NADH without enzyme



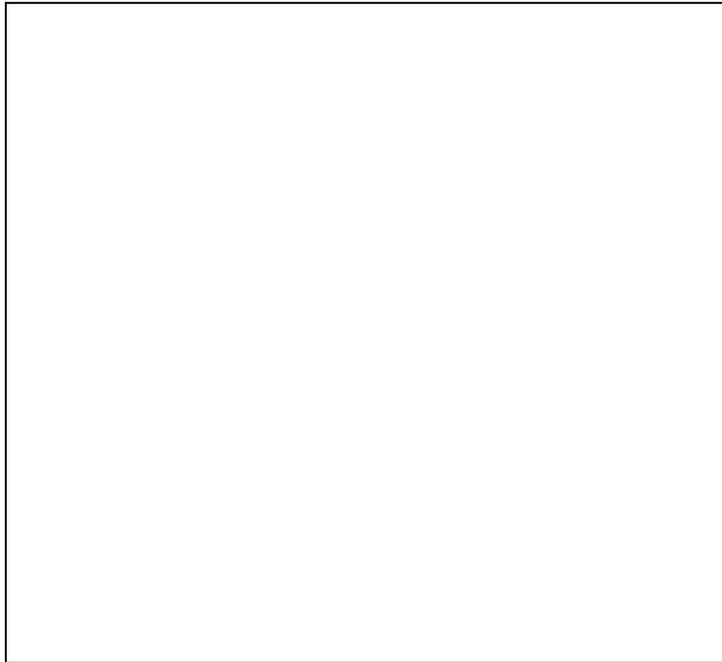
$\alpha$ -ketoglutarate + NADH with enzyme



Oxaloacetate + NADH without enzyme



Oxaloacetate + NADH with enzyme



Succinate + NADH without enzyme



Succinate + NADH with enzyme

Summarize your results by completing the table:

Substrate 1	Substrate 2	Enzyme (yes/no)	Reaction occurred (yes/no)
$\alpha$ -ketoglutarate	NADH	No	
$\alpha$ -ketoglutarate	NADH	Yes	
Oxaloacetate	NADH	No	
Oxaloacetate	NADH	Yes	
Succinate	NADH	No	
Succinate	NADH	Yes	

Even though the molecules you tested have a similar chemical structure, the reaction did not occur with all molecules. The fact that an enzyme works only with one kind of molecule (or a small number of related molecules) is called “specificity”. Why do you think it is important that the enzymes in our body have specificity? \_\_\_\_\_

\_\_\_\_\_

For each of the substrates with no reaction, what do you think is the foremost reason that no reaction occurred (recall what you found when looking at the structures of the different molecules, and of the enzyme)? \_\_\_\_\_

\_\_\_\_\_

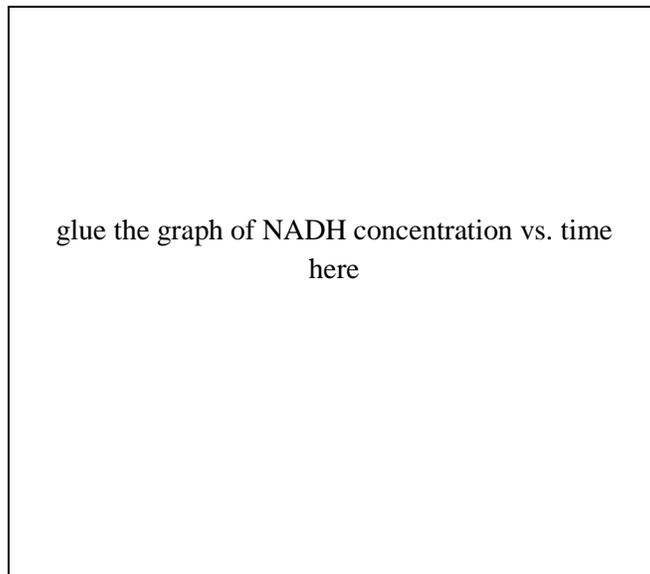
\_\_\_\_\_

### Calculations

This section contains more advanced questions. Solve them if your instructor asks you to do so.

For a substrate with reaction, make a plot of the concentration of NADH in function of time, based on the measured spectra. To do this, export the data from LoggerPro, and then use a spreadsheet program (Excel). You will also need the Beer-Lambert equation.

Here is some space for calculations:



glue the graph of NADH concentration vs. time  
here

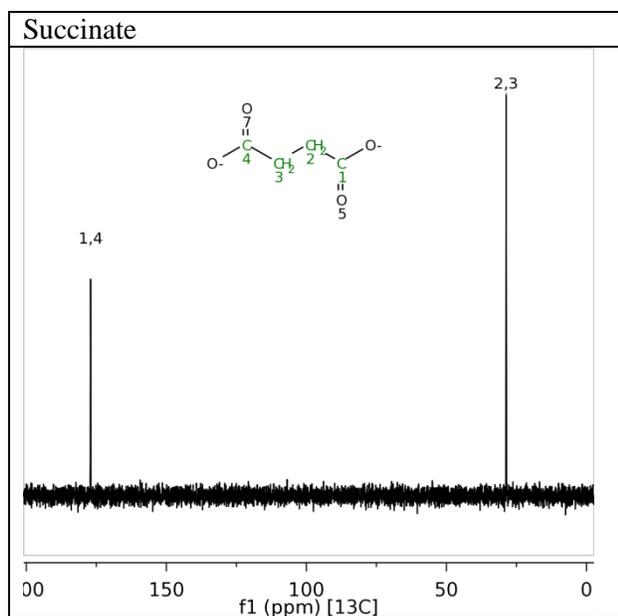
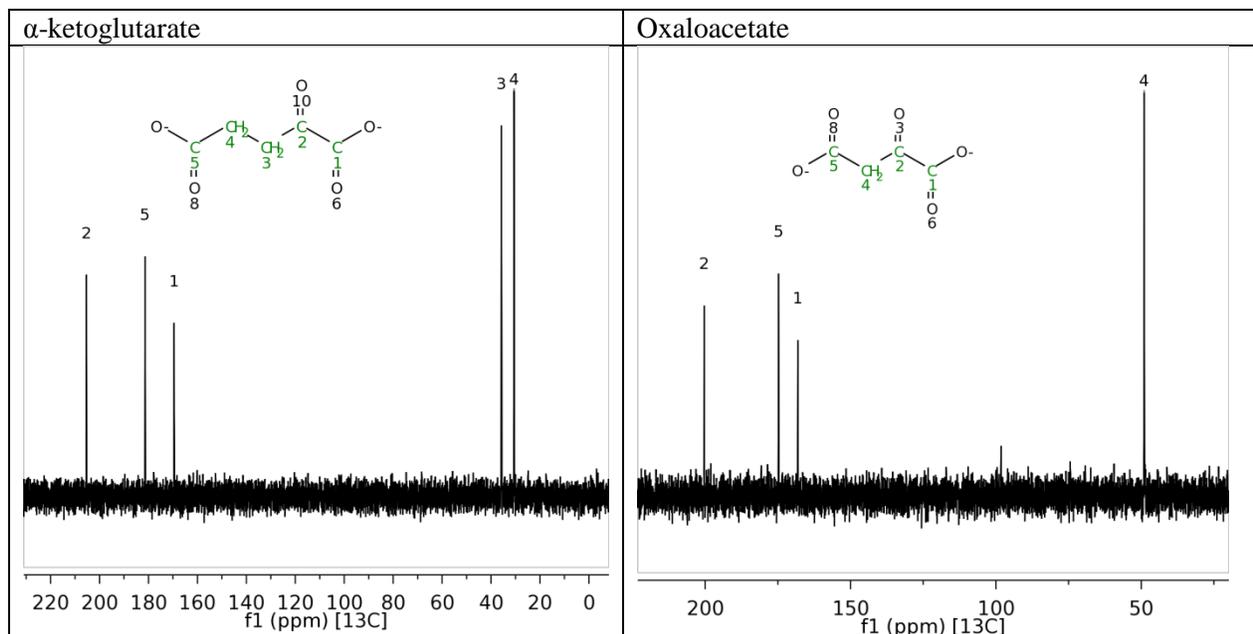
NADH concentration vs. time

If an ample quantity of reactant is present, the rate of catalysis is determined solely by how efficient the enzyme is. In that case, the graph of absorbance vs. time is a straight line. As soon as the reaction runs out of a reactant, or reaches an equilibrium, where enough product is present for the forward and reverse reaction to be in a balance, the rate of reaction will slow and the graph will curve towards becoming horizontal. In the graph above, between which times is the rate of catalysis constant? \_\_\_\_\_

What is the rate of catalysis in that time window? (include units in your answer) \_\_\_\_\_

### Other analytical techniques

One final question you might ask is, since all of the solutions are clear, how could you find out whether they actually contained the compounds marked on their labels. To answer this question, scientists could use nuclear magnetic resonance (NMR), which is another type of spectroscopy that works with radio waves. It requires a larger instrument than the optical spectrometer we have used here, but it can measure distinct signals from the different atoms in the molecules. Therefore, it can be used to find the structure of unknown molecules. Printed below are the NMR spectra of the substrates that you have used (spectra were measured in pure water, with pH adjusted to 6.5). The numbers above the peaks indicate the carbon atoms in the structure of the molecules that give rise to the peaks.<sup>6,7</sup>



(chemical shifts not referenced)

A possibility for further reading: Find other analytical techniques that you could also use to analyze the composition of the samples.

### **Disclaimer and requirements**

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### **Acknowledgments**

Image of citric acid cycle: Wikipedia users Narayanese, WikiUserPedia, YassineMrabet, TotoBaggins

Image of spectrum of light: Wikipedia user Gringer

### **References**

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- (2) <http://www.cgl.ucsf.edu/chimera/>
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- (4) [http://www.sgc.ox.ac.uk/structures/MDH2A\\_2dfd.html](http://www.sgc.ox.ac.uk/structures/MDH2A_2dfd.html)
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- (6) <http://bmrbl.wisc.edu>
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