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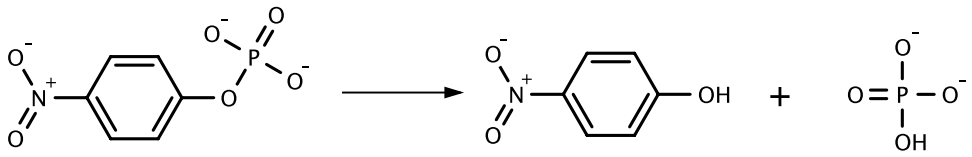
Biology 3515/Chemistry 3515
Biological Chemistry Laboratory
Spring Semester 2015
Quiz 3 - 23 April 2015

Please write your name on each page.
Be sure to show your work and include correct units in all of your answers!
50 points total.

Phosphatases are a large family of enzymes that hydrolyze phosphate esters formed with a variety of molecules, including proteins. Because phosphorylation and dephosphorylation of proteins is a major mechanism of cellular regulation, there is a great deal of interest in studying the enzymes that hydrolyze phosphate esters, as well as those that form them. Many phosphatases hydrolyze *p*-nitrophenylphosphate, a reaction that can be used in spectrophotometric assays:

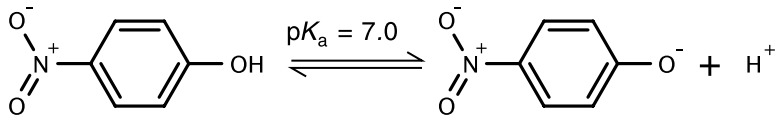


The product of this reaction, *p*-nitrophenol, absorbs visible light, with $\lambda_{max} \approx 405\,\text{nm}$, whereas the substrate does not absorb at this wavelength.

The questions begin on the next page.

Name:_____

1. (8 pts.) In order to analyze quantitatively the data from an assay using *p*-nitrophenyl-phosphate, it is necessary to know the extinction coefficient of the product. However, the situation in this case is a bit tricky, because *p*-nitrophenol undergoes a deprotonation reaction, and it is only the ionized form that absorbs light at 405 nm. The ionization equilibrium is:



The extinction coefficient for the deprotonated form is $18,300\,\mathrm{cm^{-1}M^{-1}}$, but the apparent extinction coefficient, ϵ_{app}, for a mixture of the conjugate acid and base depends on pH. (The apparent extinction coefficient represents the observed absorbance, in a 1 cm-cuvette, divided by the total concentration of the protonated and deprotonated forms.)

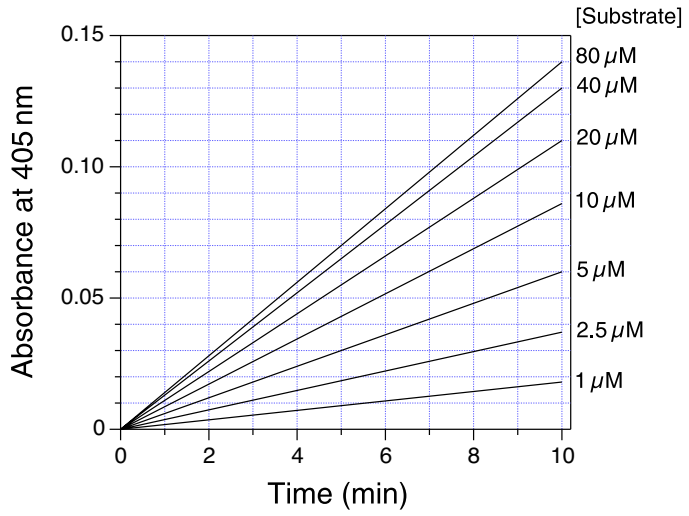
In the spaces below, calculate the fraction of *p*-nitrophenol that would be in the ionized state and the expected apparent extinction coefficient at pH 7 and 8.

(a) pH 7

(b) pH 8

Name:_____

2. (10 pts.) On the strength of your experience in Biology 3515/Chemistry 3515, you have been hired by the giant pharmaceutical company Proteins-R-Us and are working on a phosphatase that may prove to be a useful target for anti-tumor drugs. As a first step, you are characterizing the activity of this enzyme towards *p*-nitrophenol. You have done your first experiment using an enzyme concentration of 2.5 nM in a pH 7 buffer. The graph below represents the change of absorbance (in 1 cm-cuvettes) with time using different substrate concentrations, which are indicated by labels to the right of the graph.



The questions below should not require any graphing, curve fitting or other extensive calculations. You will, however, need a value for the extinction coefficient of *p*-nitrophenol at pH 7, from Question 1a. Any error in this value will not be counted against you again for this question.

(a) From the graph above, estimate $V_{\max}$, in units of μM/min.

(b) Estimate k_{cat}, in units of min^{-1}, from the data.

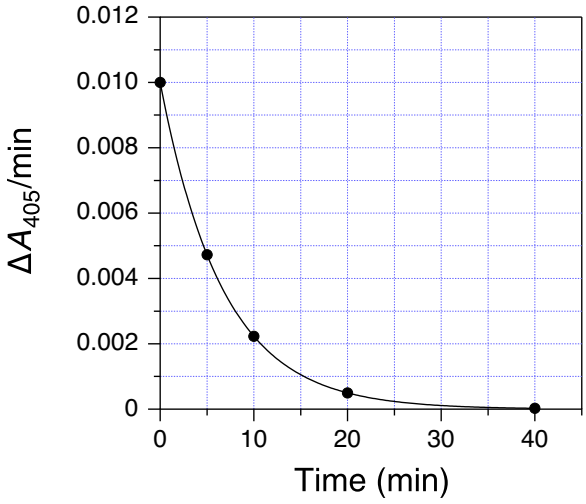
(c) Estimate K_{m}, in units of μM.

3. (12 pts.) The chemical synthesis group at Proteins-R-Us has synthesized a new compound designed to be an irreversible inhibitor of the phosphatase you are studying. To test this compound (tentatively called PIX, for phosphatase inhibitor X), you have set up a reaction in which the 1 nM enzyme is incubated with 2 μM inhibitor. At time intervals, you withdraw samples of reaction mixture and measure the remaining phosphatase activity using the spectrophotometric assay, with *p*-nitrophenol as the substrate.

   (a) What is the *single most critical* control that should be included in this experiment? What information will you obtain from this control?

Name:_____

(b) The graph below shows the results of your irreversible inhibition experiment. The data points represent the measured absorbance change per minute for samples withdrawn from the reaction at the indicated times. The curve is a fit of the data to an exponential decay function.



The excellent fit of the exponential function to the experimental data demonstrates not only your exemplary technique, but also shows that the reaction follows pseudo first-order kinetics. From the data, estimate the apparent first-order rate constant, k_{app}, with appropriate units.

(c) Calculate the second-order rate constant, k_2, with appropriate units.

Name:_____

4. (12 pts.) The amino acid sequence of the phosphatase has been determined by other scientists at Proteins-R-Us, who have given you the following list of the ionizable residue types found in the protein, with the number of each residue type.

Residue	Number of residues
Aspartate	14
Arginine	7
Glutamate	11
Histidine	8
Lysine	10

(a) You would like to run a non-denaturing gel on a sample of the phosphatase and have found three buffer solutions in the lab that might be suitable, with pH values of 3, 5 and 7. For each of these pH values, calculate the net charge of the protein, ignoring the effects of the amino- and carboxyl-terminal groups. Then, indicate whether that pH would be your first, second or third choice for your experiment, and why you have ranked it this way. For your first and second choices, indicate how you would connect the bottom electrode to the power supply (i.e., to the positive or negative outlet).

pH 3:

pH 5:

pH 7:

Name:_____

5. (8 pts) After running and staining your non-denaturing gel, you are pleased to find just a single clear band, suggesting that the sample is quite pure. However, when you run an SDS gel, there are three distinct bands, with relative mobilities indicative of molecular weights of 25,000, 50,000 and 75,000 Da.

   (a) Suggest *two* different possible explanations for the apparent discrepancy between the two results.

   (b) Suggest an experiment, using techniques that you used in this class, that could help decide between your two different explanations. Clearly describe how the results of your experiment would distinguish between the possibilities. Also, if you think that the result might not be conclusive, explain why.