

## Annotated Answers to Final Examination – 115:413 Experimental Biochemistry

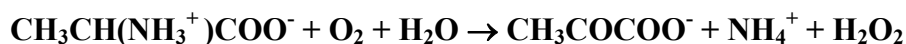
The two versions of the exam were the same except for letter of the correct answer in A and numbers in the problems.

- One of the following is *not* good practice in micropipetting:  
**sucking up solution into the tip as quickly as possible**, as everyone remembered.
- A data point statistically has less than one chance in 20 of being valid if its value is more than **two** standard deviations above or below the mean.
- Phthalic acid has  $pK_1 = 3.0$ ,  $pK_2 = 5.0$ . The pH of a solution of potassium biphthalate is **4.0**, between the two  $pK_a$ s, since in potassium biphthalate one of the two carboxyls is ionized.
- Solving pH problems generally requires using  **$pH = pK_a + \log ([A^-]/[HA])$  and total  $[A] = [A^-] + [HA]$** . I state this whenever lecturing on pH problems, though the second equation is usually implicit rather than explicit.
- You have a 5 mg/ml solution of ovalbumin. You wish to use a dilution of it for a protein standard curve. How much would you dilute this solution so that 0.25 ml contained 0.1 mg protein? **1:12.5**. If your final solution contains 0.1 mg/0.25 ml, its concentration is 0.4 mg/ml,  $5/0.4 = 12.5$ , a 1:12.5 dilution.
- Which method for protein determination is *most* sensitive to the amino acid composition of the protein? **UV** depends essentially on the tryptophan and tyrosine content of the protein.
- Values of the molar extinction coefficient of D-amino acid oxidase at 460 nm calculated by students, using the Lowry reaction to determine the protein concentration, frequently are higher than expected. This could be due to **low** content of tyrosine and cysteine in D-amino acid oxidase, which would result in less reaction in the Lowry method than expected for the amount of protein present, which would mean that the amount of protein present was underestimated, so that in the equation  $\epsilon = A/c \cdot l$  the concentration  $c$  is too small (smaller than it really is) and the value of  $\epsilon$  is therefore too large. This was my conclusion after seeing many high values of  $\epsilon$  in enzyme reports
- In which protein determination should you *not* read absorbance against a reagent blank (as we performed the method)? **Coomassie Blue**, where you should read against water in order to get values for both  $A_{595}$  and  $A_{466}$  and their ratio.
- Most methods for determination of solution concentration of carbohydrates depend on reactions of which type of group? **carbonyl**, whether oxidized in reducing sugar methods or used in forming the furfural ring in acid methods.
- The *primary* function of the sulfuric acid in the phenol method is to **dehydrate the sugars to furfurals**. The colors come from reaction of other compounds with furfurals.
- The symbol (+) before the name of a compound indicates that **its solutions rotates the plane of polarization of light to the right**. (-) would indicate rotation to the left.
- Only one of the following compounds will show mutarotation when dissolved in  $H_2O$ : **lactose**, the only one with a free glycosidic hydroxyl. Sorbitol has no ring, and the other two have the glycosidic hydroxyl(s) tied up in glycosidic linkage.
- The concentration of 0.25 mM Na pyruvate can also be expressed correctly as **0.25  $\mu$ mole/mL**. Just checking that you know that 1 ml contained 0.25  $\mu$ mole.
- In a table recording purification of a protein, yield is defined as **total units at that step/total units in crude homogenate**. A matter of definition.
- In high speed centrifugation, the relative sedimenting force depends on **the square of the speed of rotation and the radius of the centrifuge head**. Not something I stressed, but you should at least have noted that sedimenting force increases more than linearly with the rpm.

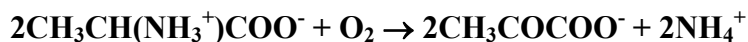
16. Why did you dialyze D-amino acid oxidase? **To remove  $(\text{NH}_4)_2\text{SO}_4$**  so that the enzyme would bind to the DEAE-Sepharose column.
17. Which amino acids in a protein are critical for its chromatography on DEAE-Sepharose? **Glutamic & aspartic acids**, since the column is positively charged, it must be the negatively charged amino acids which interact with it. But you may not have remembered what DEAE stands for and therefore that it is positively charged.
18. D-amino acid is eluted from DEAE-Sepharose by increasing the concentration of **chloride**, the anion competing with the anionic groups of the protein for the positive charges of the column. Crotonate is also an anion, but its concentration does not change during the elution, while [chloride] increases thanks to the gradient-making apparatus.
19. The absorbance at 460 nm of D-amino acid oxidase is due to **bound FAD**. Benzoate, tryosine and tryptophan absorb only in the UV range, hemoglobin is not D-amino acid oxidase.
20. To determine units of D-amino acid oxidase/mL stock enzyme from one pyruvate assay tube, **divide  $A_{560}$  by the slope of the std curve, by 10 min and by the volume of enzyme used, multiply by the dilution factor**. All those elements are needed. Some people *divided* by their dilution factor in figuring activity in their enzyme reports.
21. One hypothesis to explain the ‘leveling off’ of pyruvate production by D-amino acid oxidase after 10 minutes is that the enzyme is not stable, losing activity after that time at pH 8.3. To test this, you followed activity over time at 2 levels of enzyme. If this hypothesis is correct, **pyruvate production should level off at the same rate at both levels of enzyme**. This called for a bit of thought. Simple loss of enzyme activity should happen at the same rate whether the enzyme is dilute or concentrated; whereas if the ‘leveling off’ is due to exhaustion of  $\text{O}_2$  this should happen faster at the higher level of enzyme (some saw this, some didn’t).
22. Since benzoate is known to be a competitive inhibitor vs. D-alanine,  $V_{\text{max}}$  values derived from  $v$  vs. [D-alanine] at increasing benzoate concentrations are expected to **be the same**. The hallmark of competitive inhibition is that Lineweaver-Burk lines at different [I] cross *on* the y axis, which is equivalent to stating that  $V_{\text{max}}$  is the same.
23. Why can’t we assay D-amino acid oxidase in the gel after SDS gel electrophoresis? **The enzyme is unfolded by SDS**. We can get substrate to the enzyme – see the native gel – and we could correct the pH, but the enzyme would have to refold and rebind FAD to be active.
24. Mobility of a protein in SDS gel electrophoresis depends on **size of the protein**. In presence of SDS the charge density is dominated by the SDS and is  $\approx$  constant.
25. The critical feature of the stacking gel in gel electrophoresis is **its pH,  $\approx 2$  pH units closer to neutrality than the running gel buffer**. At this pH the proteins have greater mobility than the reservoir buffer (because so little of the latter is present as the anion), and concentrate between it and the stacking gel buffer.
26. Which way must the gel ‘sandwich’ face during transfer? **membrane side facing the positive electrode**. The proteins must move as anions, toward the positive electrode, so the membrane must be between the gel and the positive electrode in order to intercept them.
27. In the print-out from laser densitometry of a gel, the  $R_m$  may be calculated as 
$$\frac{\text{STOP value} - \text{band value}}{\text{STOP value} - \text{dye front value}}$$
 One had to remember that the top of the gel = STOP value, measurements were made from this point.
28. In the visualization system for our western blot, the second antibody is an antibody to **rabbit antibodies**, so that the reagent is general, whatever the specific protein is that you wish to visualize.

**Part B - Short Answers.**

- a. (3 pts) Write the balanced chemical reaction for the reaction catalyzed by D-amino acid oxidase.



1a (3 pts **EXTRA CREDIT**) Write the balanced chemical reaction for the net reaction catalyzed by D-amino acid oxidase + catalase:



**Part C - Problems.** Show all work and indicate your answer **clearly**.

(5 pts) A 2 mg/ml solution of ovalbumin (mol. wt. 45,000) has  $A_{278} = 1.68$  in a 1 cm path length cell. Calculate the molar extinction coefficient. Specify units.

$$\frac{2 \text{ mg/ml}}{45,000 \text{ mg/mmole}} = 4.45 \times 10^{-5} \text{ M} \quad \frac{1.68}{4.45 \times 10^{-5} \text{ M}} = \mathbf{37,800 \text{ L/mole}\cdot\text{cm}}$$

2. An ovalbumin standard solution, 0.5 mg/ml, gives the following results in the Coomassie Blue method for protein determination:

ml ovalbumin	0	0.01	0.02	0.05	0.1	0.2	0.3
mg ovalbumin		<b>0.005</b>	<b>0.01</b>	<b>0.025</b>	<b>0.05</b>	<b>0.1</b>	<b>0.15</b>
$A_{595}$	.400	.450	.500	.575	.690	.930	1.16
$A_{466}$	.533	.500	.495	.433	.373	.338	.318
$A_{595}/A_{466}$	<b>.750</b>	<b>.900</b>	<b>1.01</b>	<b>1.328</b>	<b>1.85</b>	<b>2.75</b>	<b>3.65</b>

A solution of another protein gives the following results:

ml unknown	0	0.01	0.02	0.05	0.1	0.2
$A_{595}$		.460	.508	.595	.720	1.005
$A_{466}$		.493	.485	.427	.366	.323
$A_{595}/A_{466}$	<b>.750</b>	<b>.925</b>	<b>1.04</b>	<b>1.38</b>	<b>1.95</b>	<b>3.10</b>

a. (3 pts) Calculate, or draw on the graph below, a standard curve for protein determination, based on the results with ovalbumin. State slope and intercept.

**Over the most linear points, 0 – 0.05 mg,  $A_{595}/A_{466} = 21.6(\text{mg}) + .779$ ; over all points,  $A_{595}/A_{466} = 19.15(\text{mg}) + 0.818$ . One may also use just  $A_{595}$ : over 0.01 to 0.15 mg  $A_{595} = 4.713(\text{mg}) + .455$ . But the  $A_{595}$  line curves near the y axis.**

b. (5 pts) Using the standard curve, determine the concentration of the other protein solution.

**For these data, the slope of  $A_{595}/A_{466}$  vs ml = 11.57 A/ml;  $\frac{11.57 \text{ A/ml}}{19.15 \text{ A/mg}} = 0.604 \text{ mg/ml}$ . For the**

**other set of data,  $A_{595}/A_{466} = 12.41(\text{mg}) + 0.797$ , i.e. slope = 12.41 A/ml.  $\frac{12.41 \text{ A/ml}}{19.15 \text{ A/mg}} = 0.648$**

**mg/ml.**

3. (8 pts) Below are shown  $[\alpha]_D$  values for the carbohydrates in our carbohydrate experiment.

Sugar	$[\alpha]_D$	Sugar	$[\alpha]_D$	Sugar	$[\alpha]_D$
D-glucose	+52.8°	L-fucose <sup>@</sup>	-75.6°	D-trehalose <sup>o</sup>	+178°
D-fructose <sup>#</sup>	-92.0°	L-rhamnose <sup>@</sup>	+8.9°	D-sucrose <sup>o#</sup>	+66.5°

D-galactose	+81.7°	D-sorbitol (glucitol) <sup>◇</sup>	-2.0°	D-maltose <sup>∘</sup>	+129.0°
L-sorbose <sup>#</sup>	-42.7°	L-arabinose <sup>*</sup>	+104.0°	D-lactose <sup>∘</sup>	+52.3°
D-mannose	+14.1°	D-xylose <sup>*</sup>	+18.6°	D-cellobiose <sup>∘</sup>	+34.5°

\*Pentose #Ketose <sup>∘</sup>Disaccharide @6-deoxyhexose <sup>◇</sup>Sugar alcohol The others are aldohexoses.

A 10% (10 g/100 mL) solution of a carbohydrate has optical rotation (20 cm cell) +10.5°. A 1:1000 dilution is used in various reactions. It reacts similarly to glucose in the phenol-H<sub>2</sub>SO<sub>4</sub>, orcinol and indole reactions. Nelson-Somogyi and glucose oxidase reactions are as follows:

Sugar	Nelson-Somogyi, A <sub>660</sub>	glucose oxidase, A <sub>500</sub>
Glucose	0.4 ml → 1.62	0.5 ml → 0.503
Unknown	0.4 ml → 0.930	0.5 ml → 0.010

What is the sugar?

$[\alpha]_D = 100(\text{optical rotation})/\text{concentration} \cdot \text{path length}$  (where c is in g/100 ml and path length in decimeters).  $[\alpha]_D = 1050/10 \cdot 20 = +52.5^\circ$ . This is consistent with either glucose or lactose; but the unknown does not react in the glucose oxidase reaction, therefore it must be lactose. Consistent with this identification, the reaction in the Nelson-Somogyi reaction is a little over half as much as that of glucose, as expected since the mol. wt. of lactose is almost twice that of glucose and therefore an 0.1 mg/ml solution contains a little over half as many mmoles/ml.

4. (5 points) Below is a copy of the chart of a polarograph assay of D-amino acid oxidase. If the assay volume was 3 ml, the chart speed 2 cm/min, and the amount of enzyme 0.030 ml of a 1:10 dilution, calculate the activity (μmoles/min-ml) of the stock enzyme. Solubility of O<sub>2</sub> in water at 37° = 0.26 mM. Show how you determined v (chart width/min, cm/min).

Typically, a tangent to the initial part of the chart trace went from 1.0 at 0 min to 0.380 at 3 min. 0.62 chart width/3 min = 0.2067 chart width/min. This times  $\frac{0.78 \mu\text{moles } O_2}{\text{chart width}} \times \frac{10}{0.03 \text{ ml}} = 53.7 \text{ u/ml}$ .

For the other version only the amount of enzyme differed, yielding 64.45 μmole/ml enzyme.

5. a) The following rates of enzymatic oxidation of samples of 0.02 M DL-valine, diluted to an assay volume of 3.0 ml, are observed in the peroxidase assay:

ml DL-val/PP <sub>i</sub>	0.03	0.06	0.12	0.25	0.50	1.0	2.0
[D-val], mM:	0.1	0.2	0.4	0.833	1.667	3.333	6.67
ΔA <sub>500</sub> /min	0.044	0.080	0.133	0.204	0.270	0.323	0.357

(Nearly everyone forgot to divide by 2, to make it the concentration of D-valine.)

a. (3 pts) Calculate D-valine concentration, and either [S]/v (Wolf plot) or 1/[S] and 1/v (Lineweaver-Burk plot) and plot on the graph below. The rate (v) is most easily plotted as ΔA<sub>500</sub>/min. Label the axes clearly. ε<sub>500</sub> of peroxidase product = 14,250 L/mole·cm.

1/[S]	10	5	2.5	1.2	0.6	0.3	0.15
1/v	32.26	17.54	10	6.1	4.29	3.40	2.95
[S]/v	3.225	3.51	4	5.08	7.15	11.34	19.67

b. (5 pts) Determine  $V_{\max}$  and  $K_m$  (mM) from the data above.

**However plotted, the graph should yield  $V_{\max} = 0.4 A_{500}/\text{min}$ ,  $K_m = 1.2 \text{ mM}$  (slope/intercept). I did not take off again for failure to convert [S] to mM D-valine. In the other version  $V_{\max}$  is the same but  $K_m$  is 0.8 mM. In the Woolf plot above the intercept is 2, the slope =  $(18.67 - 2)/6.67 = 2.5$ , thus  $V_{\max} = 1/\text{slope} = 0.4$ ,  $K_m = \text{int} \cdot V_{\max} = 0.8 \text{ mM}$ .**

c. (1 pt) If the enzyme used was 0.1 ml of a 1:50 dilution, what is the  $V_{\max}$  in  $\mu\text{moles}/\text{min} \cdot \text{stock enzyme}$ ?

$$0.4 A_{500}/\text{min} \times \frac{3 \text{ ml}}{14.25 \frac{A \cdot \text{ml}}{\mu\text{mole}}} \times \frac{50}{0.1 \text{ ml}} = 42.1 \mu\text{mole}/\text{min} \cdot \text{ml}$$

d. (1 pt) If the stock enzyme had a protein concentration of 0.75 mg/ml, and the molecular weight (per subunit) is 39,000, what is the turnover number? (Units:  $\mu\text{mole product}/\mu\text{mole enzyme} \cdot \text{min}$ )

$$\frac{42.1 \mu\text{mole}}{\text{min} \cdot \text{ml}} * \frac{\text{ml}}{0.75 \text{ mg}} * \frac{39 \text{ mg}}{\mu\text{mole}} = 2189.5 \text{ min}^{-1}$$

6. (5 points) Observed  $R_m$ s and molecular weights of the standard proteins for molecular weight determination by SDS gel electrophoresis are as follows:

Protein	$R_m$	mol. wt.	Protein	$R_m$	mol. wt.
lysozyme	0.85	14,300	ovalbumin	0.375	45,000
trypsin inhibitor	0.71	20,000	albumin, bovine serum	0.22	66,000
carbonic anhydrase	0.56	29,000	phosphorylase b	0.075	93,000

An unknown protein has an  $R_m$  of 0.35 on the same gel. Calculate its molecular weight (use one of the graphs below, or fit the standard molecular weights to an appropriate equation).

**The equation used to set up the values was  $R_m = 4.81 - .953 \log \text{ mol. wt.}$ , or  $.953 \log \text{ mol. wt} = 4.81 - R_m$ . Then  $\log \text{ mol. wt} = \frac{4.81}{.953} - \frac{R_m}{.953} = 5.0472 - \frac{0.35}{.953} = 5.0472 - 0.367 = 4.68$ ; antilog 4.68 =**

**47,858**

**For the other version  $\log \text{ mol. wt.} = 5.047 - (0.65/0.953) = 5.047 - 0.682 = 4.365$ , antilog = 23,182. Values within  $\pm 5\%$  of these were accepted, further off resulted in a point off. No one seemed to know how to use the semilog graph (just plot the mol. wt. values directly); I should instruct in how to do this!**