December 20, 2004 name________________

Final Examination – 115:413 Experimental Biochemistry – Annotated Answers

The two versions of the exam differed in placement of the correct answers in the multiple choice section, and in the numbers in the problems. This account should explain both versions.

Part A – multiple choice; answer by circling the letter of the correct answer. 2 pts each.

1. One of the following is *not* good practice in micropipetting:
   Sticking the end of the tip into the receiving solution to deliver its contents.
   It is best to deliver onto the wall above the solution, particularly to avoid contamination of the tip if you are going to use it to deliver into more tubes.

2. If one average or slope (for instance the slope of a plot of absorbance vs. ml unknown) is divided by another (for instance the slope of a plot of absorbance vs. mg standard protein), the standard error (SE) of the quotient a/b is found from the standard error of a (SE_A) and the standard error of b (SE_B) as $\frac{a}{b}\sqrt{\left(\frac{SE_a}{A}\right)^2+\left(\frac{SE_b}{B}\right)^2}$ The standard error must have the same units as the quotient of which it is the error, and it must be a fraction of the quotient.

3. It requires 10 ml 0.5 M KOH to change the color of 50 ml of an indicator-containing solution from yellow to blue. Which of the following is true?
   a. The solution contains at least 5 millimoles of a buffering compound.
   b. A pK_a of the indicator lies in the pH range being titrated.
   c. The pK_a of the indicator is similar to that of the buffering compound.
   d. **All of the above**.

4. Which of the following is *not* used directly in calculating pH by the Henderson-Hasselbalch equation? **Total concentration of buffer, $[B]_{total}$.** I stress that the concentrations of the basic and acidic forms, not the total concentration, appear in the H-H equation. Their sum, of course, is equal to the total concentration; but that is a separate equation.

5. A stock solution of ovalbumin, 10 mg/ml, is diluted 1:25. Twenty-five microliters of the diluted solution contains this amount of ovalbumin:
   0.01 mg. The 1:25 dilution is 0.4 mg/ml, x .025 ml = .01 mg.

6. Which protein method is *least* sensitive to the amino acid composition of the protein being measured? **Biuret**, because it measures the complex of Cu^{++} with the peptide bonds.

7. Which protein method is *most* sensitive to protein concentration?
   Coomassie Blue, as you calculated in answering a question on the protein lab.

8. Absorbance equals **ε·c·l**. The basic statement of the Beer-Lambert Law.

9. Absorbance of a protein at 280 nm measures its content of **tyrosine & tryptophan**, the aromatic amino acids with absorbance in this range.

10. An individual sugar can have how many interconvertible forms in solution? **Five** - α and β pyranose forms, α and β furanose forms, the open-chain form.

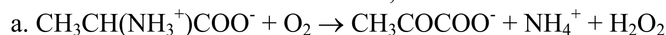
11. The most sensitive (highest absorbance/μmole) method for sugars is **Nelson-Somogyi**. You just had to remember this, 0.4 ml 0.1 mg/ml glucose gave A_{66} close to 2.

12. The change of optical rotation after a crystalline sugar is dissolved in water is called **mutarotation**. Again, just remembering the correct term.

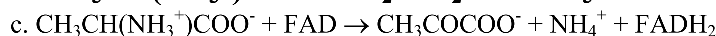
13. Why is the indole method fairly specific for ketoses? **Ketoses form the five-membered ring of furfural faster.** I stressed this enough that almost everyone got it right.

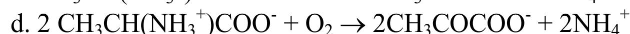
14. One of the following is *not* a reducing sugar: **sucrose**, which has the two hexoses sharing what would otherwise be the glycosidic oxygen.

15. D-Amino acid oxidase is yellow because of its bound **flavin adenine dinucleotide.**

16. The D-Amino acid oxidase reaction, in the absence of catalase, is
    
    a. $CH_3CH(NH_3^+)COO^- + O_2 \rightarrow CH_3COCOO^- + NH_4^+ + H_2O_2$

b. $CH_3CH(NH_3^+)COO^- + O_2 + H_2O \rightarrow CH_3COCOO^- + NH_4^+ + H_2O_2$


c. $CH_3CH(NH_3^+)COO^- + FAD \rightarrow CH_3COCOO^- + NH_4^+ + FADH_2$


d. $2\ CH_3CH(NH_3^+)COO^- + O_2 \rightarrow 2CH_3COCOO^- + 2NH_4^+$

It has to include the water molecule to come out balanced. The last equation is in **presence** of catalase.

17. Which of the following makes the pyruvate assay for D-Amino acid oxidase more sensitive than the peroxidase and polarograph assays? **Longer time of incubation**. The extinction coefficient is lower than for the peroxidase assay, and you use more dilute enzyme (which is a measure of being a more sensitive assay!)

18. The D-amino acid oxidase stuck to the column of DEAE-Sepharose by
charge interactions between the negatively charged protein and positively charged DEAE
You had to remember that it was ionic interaction, and that DEAE (diethylaminoethyl) groups are positively charged.

The next three questions refer to the following table:

step	vol (ml)	units/ml	total units	mg prot/ml	mg/total	units/mg	yield, %
crude extract	800	2.086	1668.8	19.47	15576	0.107	100
acid supernatant	620	1.949	1208.4	11.47	7111	0.170	72.4
AS precip. before dialysis	51	15.2	775.2	4.33	221	3.51	46.5
AS precip. after dialysis	51	18.7	953.7	2.95	150	6.34	57.1
DEAE-Sepharose eluate	50	11.17	558.5	0.87	43.5	12.84	33.5
final enzyme	3.5	27.66	96.8	1.07	3.7	25.85	5.8

19. Which step gave the greatest purification (increased specific activity the most)?
AS precip. before dialysis. This is generally the best step, and this could be seen by comparing specific activities. of each step with that at the preceding step.

20. Which step had the largest loss? **Final enzyme**, where the yield was only about one-fifth that at the previous step. I can't understand why there should be poor yield at this step, but these were actual results (with the results for AS precipitate before dialysis filled in, since this group forgot to determine them).

21. How might the apparent regain of activity after dialysis be explained?
The stored sample lost more activity than the main preparation being dialyzed. This was perhaps unfair, since I had not stressed this answer (though I believe it to be true); but you dilute the enzyme so much for assay that the other two answers should not be correct, the $(NH_4)_2SO_4$ present should be diluted out.

22. The curvature of the chart trace in the polarograph assay is due to
Decrease of concentration of substrate oxygen. O_2 is present at a much lower concentration than D-alanine (0.26 mM vs 50 mM), and this concentration is far below the K_m for O_2 (if there is one), so the rate will decrease as the $[O_2]$ decreases.

23. Which of the following is a likely reason why your calculated ε_{463} for D-amino acid oxidase was lower than Yagi's value, 11,000 L/mole·cm?
a. Presence of other proteins contributing to c in $\varepsilon = A/c \cdot l$.
b. Greater response of your protein determination method per mg D-amino acid oxidase than per mg ovalbumin.
c. Use of the correct mol. wt. 39,000 mg/mmole rather than Yagi's value 50,000.
d. **all of the above** could explain it, though presence of other proteins is probably the major reason.

24. To calculate the activity in the peroxidase assay in μmoles/min, you should
a. divide ΔA_{500}/min by 14.25.
b. **divide ΔA_{500}/min by 14.25, multiply by 3.**
c. divide ΔA_{500}/min by .01425, multiply by 3.
d. multiply ΔA_{500}/min by 14.25, divide by 3.

25. In electrophoresis in presence of sodium dodecyl sulfate, mobility of proteins depends on
a. charge density of the protein molecule
b. shape of the protein molecule
c. **size of the protein molecule**. Shape is lost due to denaturation, and association of dodecylsulfate molecules with the peptide bonds makes the charge density approx. constant.

26. Gelling of the polyacrylamide gel is initiated by addition of **ammonium persulfate**
Allen argued that tetramethylethylenediamine should also be an answer, but that is an accelerator, not the primary source of the necessary free radical ions.

27. Relative movement (R_m) in gel electrophoresis is measured as **cm from top of running gel to band/cm from top of running gel to dye front (ink)**. You have to remember to measure from the *top*. Next year I want to place more emphasis on determining R_m on all representations of the gel (actual gel before staining; picture of the stained gel; blot), since the actual size of the representation may be different (storage of gel in acetic acid may shrink it before transfer to the blot), but R_m, a ratio, shoul;d be the same in all cases.

28. Visualization of D-amino acid oxidase after native gel electrophoresis depends on **reduction of phenazine methosulfate by $FADH_2$** The phenazine is the essential electron carrier between $FADH_2$ (reduced by the D-amino acid) and the Nitro Blue tetrazolium whose reduced product you actually see.

29. Which component of the western blot procedure is most specific for a particular protein?
The first antibody is what is specific for the particular protein; the second antibody is specific for the first antibody, wherever it may have bound.

30. In isoelectric focusing, a drop of water (or kerosene) is placed between the gel and the bed. This is to ensure **good heat transfer from the gel to the cooling bed.** Gavin talked about this.

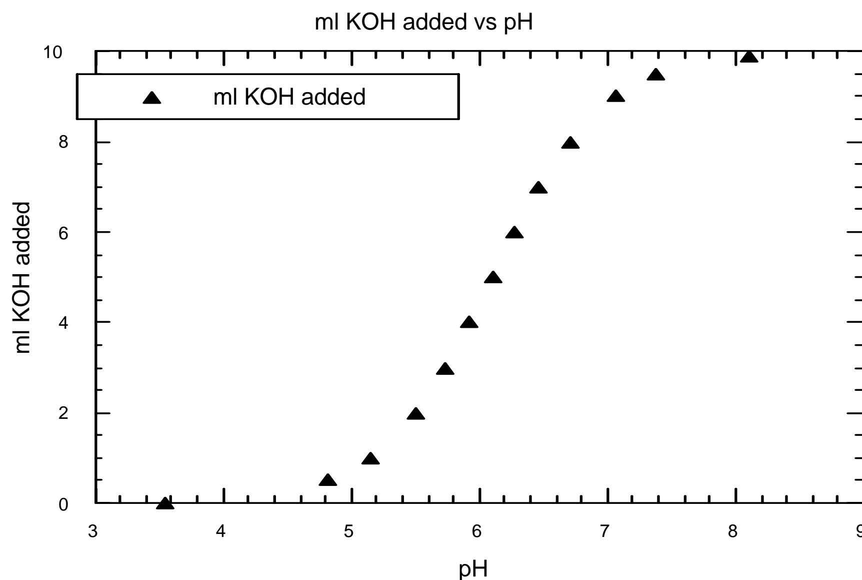
Part B. In the peroxidase assay, the second minute's chart trace may have a higher slope than the first. Explain in your own words why. (5 pts) Hint: this is a coupled reaction.

The rate of the peroxidase reaction depends on the concentration of peroxide when that concentration is low. It takes a minute for the peroxide concentration to build up to the level at which peroxidase is acting just as fast to remove peroxide as D-amino acid oxidase is generating it. (An analogy would be pouring water into a leaky bucket at a constant rate. The rate of water flowing out the leak depends on the pressure above it. Thus if water is poured in at a constant rate, the water level in the bucket will rise until the pressure is sufficient to make the outflow occur at the same rate as the rate at which water is poured in.) I covered this in lecture – for those who attended.

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

1. The graph shows a (rather perfect) titration of a buffering compound. Determine the pK_a. (5 points)

The titration is complete at the top of the graph (10 ml or 8 ml, depending on the version). Therefore it is half complete half



way up the graph (5 or 4 ml). Draw a line across the graph at that level, read down to the pH axis, and find that pH (at which [acidic form] = [basic form]) = pK_a - **6.1** in the version shown above, **7.8** in the other.

2. Results of determining A_{595} and A_{466} for a standard ovalbumin solution, 1.0 mg/ml, are:

ml	0	.005	.01	.02	.04	.10
A_{595}	.260	.312	.360	.445	.615	1.075
A_{466}	.400	.374	.362	.338	.315	.304

Results for a 1:10 dilution of an unknown protein solution are:

ml	0	.005	.01	.02	.04
A_{595}	.260	.299	.323	.371	.466
A_{466}	.400	.391	.378	.358	.334

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.

I should have provided rows for mg protein (same as ml in this example, half the ml in the other version) and for the ratio A_{595}/A_{466}; but you could do it, less accurately, using A_{595} alone. This version was constructed to have A_{595}/A_{466} = **32(mg) + .675**, the other to have A_{595}/A_{466} = **34(mg) + .660**.

b. (3 pts) Using the standard curve, determine the concentration of the unknown protein solution.

This version had the slope of the unknown A_{595}/A_{466} = 18 per ml; thus 18/ml ÷ 32/mg = 0.5625 mg/ml for the 1:10 dilution, therefore **5.625 mg/ml** for the stock solution. For the other version the slope of the unknown was 15.3/mg, thus 15.3/ml ÷ 34/mg = 0.45 mg/ml for the dilution, **4.5 mg/ml** for the stock.

3. The $[\alpha]_D$ of maltose is +129°. Its molecular weight is 342 g/mole.

a. A solution of maltose, 15 ml in a 2 dm polarimeter cell, has an optical rotation of +8.82°. Calculate 1) its concentration in g/100 ml, 2) its molar concentration. (3 pts).

Since optical rotation = $[\alpha_D]\cdot c\cdot l/100$, $c = 100(\text{opt. rotation})/[\alpha_D]\cdot l$, in this case 882/129·2 = **3.42 g/100 ml** = 34.2 g/L. Since the molecular weight is 342 g/mole, this is **0.1 mole/L.**

b. To this solution is added 0.1 ml of a concentrated solution of an α-glucosidase (which will hydrolyze maltose to two glucose molecules, $[\alpha]_D$ = +52.8°). If the activity of the glucosidase solution is 125 µmoles/min·ml, how much maltose will it hydrolyze in 30 min? (1 pt) What will the optical rotation then be? (5 pts) (Neglect dilution by the added enzyme.)
I wanted to give you the optical rotation after 30 min an ask you to calculate the activity of the enzyme, but I felt that would be too difficult. **EXTRA CREDIT**: derive an equation which will tell you the molar concentration of glucose formed (and maltose hydrolyzed) from the optical rotation (5 pts).

Since the enzyme's activity is 125 µmole/min·ml (200 in the other version), and 0.1 ml is used, the enzyme will hydrolyze 12.5 µmoles/min for 30 min, or 375 µmoles (600 in the other version) (**0.375 or 0.6 mmole**). The original solution contained 15 ml x 0.1 mmol/ml = 1.5 mmoles maltose. The hydrolysis decreased this by 0.375 or 0.6 mmoles, leaving a residual 1.125 or 0.9 mmoles maltose, while forming **two** mmoles glucose for each maltose hydrolyzed, 0.75 or 1.2 mmoles.

Now convert these amounts to molar concentrations and then into g/100 ml:
1.125 mmoles maltose ÷ 15 ml = 0.075 M = 25.65 g/L = 2.565 g/100 ml
0.9 mmoles maltose ÷ 15 ml = 0.06 M = 20.52 g/L = 2.052 g/100 ml.
0.75 mmoles glucose ÷ 15 ml = 0.05 M, x 180 g/mole = 9 g/L = 0.9 g/100 ml
1.2 mmoles glucose ÷ 15 ml = 0.08 M, x 180 g/mole = 14.4 g/L = 1.44 g/100 ml.

Now calculate the resulting optical rotations :

$$OR_{maltose} = \frac{+129°\cdot 2.565\cdot 2}{100} = +6.62°$$ (+5.29° in the other version)

$$OR_{glucose} = \frac{+52.8°\cdot 0.9\cdot 2}{100} = +0.95°$$ (+1.52° in the other version)

Add them together: +6.62° + +0.95° = **7.57**°, +5.29° + +1.52° = **6.82**°.

EXTRA CREDIT: express $[\alpha]_D$ in molar terms (assume a 2 dm cell):
for maltose, 1 M = 342 g/L = 34.2 g/100 ml, optical rotation = +129°·34.2·2/100 = +88.24°
for glucose, 1 M = 180 g/L = 18 g/100 ml, optical rotation = +52.8°·18·2/100 = +19.01°, but this must be multiplied by 2 since two glucose are produced per maltose hydrolyzed. Then the optical rotation = initial rotation – 88.24(M hydrolyzed) + 38.02 (M hydrolyzed), where M = mmoles/ml of solution; combining terms, OR = $OR_{initial}$ – 50.22 (M hydrolyzed).
Rearranging, 50.22 (M hydrolyzed) = $OR_{initial}$ – OR, then M hydrolyzed = ($OR_{initial}$ – OR)/50.22. Multiplying this by the volume of solution (here 15 ml) gives the mmoles of maltose hydrolyzed; the mmoles of glucose formed is twice this, since 2 glucose are formed per maltose hydrolyzed. But this was far beyond everyone.

4. a) The following rates of enzymatic oxidation of samples of 0.04 M DL-norvaline **(20 mM D-norvaline)** are observed in the pyruvate assay (assay volume 1.0 ml, slope of standard curve 3.1 A/μmole, assay time 10 min):

ml DL-nvl/PP_i	0.015	0.03	0.06	0.1	0.2	0.3	0.5
[D-nvl], mM:	**0.3**	**0.6**	**1.2**	**2**	**4**	**6**	**10**
A_{560}	0.310	0.465	0.620	0.715	0.809	0.845	0.877

a. (4 pts) Calculate D- norvaline concentration (above), and either [S]/v (Woolf plot) or 1/[S] and 1/v (Lineweaver-Burk plot) and plot on the graph below. It is simplest to plot v as A_{560} and calculate μmoles/min etc. only for V_{max}. Label the axes clearly

1/[S]	**3.33**	**1.67**	**0.833**	**0.5**	**0.25**	**0.167**	**0.1**
1/v	**3.276**	**2.15**	**1.61**	**1.4**	**1.236**	**1.183**	**1.14**
[S]/v	**0.967**	**1.29**	**1.935**	**2.797**	**4.94**	**7.10**	**11.4**

An actual plot of [S]/v vs [S], where v is expressed as A_{560}, gave an intercept of 0.7, a slope = (7.23 – 0.7)/6 = 1.088 = $1/V_{max}$, V_{max} = 0.92 A_{560}/10 min, K_m = 0.64 mM. I actually constructed the above values from V_{max} = 0.93 A_{560}, K_m = 0.6 mM. The other version was constructed from V_{max} = 0.4 μmoles/10 min = 1.23 A_{560}, K_m = 0.8 mM. Thus the intercept of a Woolf plot would be 0.65, the slope 0.813.

b. (4 pts) Determine V_{max} (in A_{560}) and K_m (mM) from the data above. **See above.**

c. (1 pt) If the enzyme used was 0.1 ml of a 1:100 dilution, what is the V_{max} in μmoles/min· **stock** enzyme? If A_{560} = 0.93,

÷ 3.1A/μmole → 0.3 μmoles, x $\frac{1}{10\,\text{min}}$ x $\frac{1}{0.1\,ml}$ x 100 = **30 μmoles/min·ml.** 40 for the other version.

d. (1 pt) If the stock enzyme had a protein concentration of 0.6 mg/ml, and the molecular weight (per subunit) is 39,000, what is the turnover number?

$\frac{0.6\,mg/ml}{39{,}000\,mg/mmole}$ = 1.54 x 10^{-5} M = ,0154 μmoles/ml. $\frac{30\,\mu moles}{\text{min}\cdot ml}$ x $\frac{ml}{.0154\,\mu moles}$ = **1950min^{-1}** In the other version the concentration is 0.8 mg/ml, and the turnover number comes out the same.

5. (5 points) Observed R_ms 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.
aprotinin	0.95	6,500	ovalbumin	0.437	45,000
α-lactalbumin	0.74	14,200	albumin, bovine serum	0.336	66,000
trypsin inhibitor	0.65	20,000	β-galactosidase	0.187	116,000
carbonic anhydrase	0.55	29,000	myosin	0.037	205,000

An unknown protein has an R_m of 0.45 on the same gel. Calculate its molecular weight (use a graph below, or fit the standard molecular weights to an appropriate equation) .

The problem was constructed for the standards to fit the equation $R_m = 3.26 - 0.6067 \log \text{mol wt.}$, which can be rearranged to $\log \text{mol. wt.} = 5.37 - 1.65 R_m$. The R_ms in the two versions (0.45, 0.59) gave mol. wts. of **42,816** and **25,168** respectively. The plot below on semilog graph paper (I think I have to instruct students in its use) indicated a mol. wt. = 43,000 – pretty good.