Some equations and formulae that might or might not be useful:

$\Delta G = \Delta H - T\Delta S$	$K_w = [H^+][OH^-] = 10^{-14}\ M^2$
$\Delta G = \Delta G^0 + RT\ lnQ$	$pH = -\log[H^+]$
$\Delta G = \Delta G^0 + RT\ ln(\{[products]\}/\{[reactants]\})$	$K_a = [H^+][A^-]/[HA]$
$K_{eq} = \{[products]\}_{eq}/\{[reactants]\}_{eq}$	$pH = pK_a + \log([A^-]/[HA])$
$\Delta G^0 = -RT\ ln(K_{eq})$	$[A^-]/[HA] = 10^{pH-pKa}$
$K_{eq} = exp(-\Delta G^0/RT)$	$pH = pK_a + \log[x/(c_0 - x)]$
$R = 8.31\ J/K/mol = 0.00831\ kJ/K/mol$	(c_0 is the initial molar concentration of a weak acid, and x denotes molar equivalents of the conjugate base)
$T(K) = t(^oC) + 273.16^o$	

Table 1: Peptide bond cleavage reagents

Treatment	Cleavage points	Cleavage location at
Trypsin	Lys, Arg	carboxyl side of these residues
Chymotrypsin	Phe, Tyr, Trp	carboxyl side of these residues
Asp-N protease	Asp, Glu	amino side of these residues
Pepsin	Phe, Tyr, Trp	amino side of these residues
Cyanogen bromide	Met	carboxyl side of this residue

Problem 1. (15 points)

Consider a hypothetical reaction A + B $\rightleftarrows$ C which takes place at 25ºC. The initial molar concentrations of A and B were 30 mM and 50 mM, respectively, and there was no C. When the molar concentration of B dropped to 30 mM, the ΔG for the forward reaction was -2.0 kJ/mol. Determine the value of ΔG^0 and the equilibrium constant for this reaction.

(10 points)

$\Delta G = \Delta G^0 + RT \ln(Q)$ ➔ $\Delta G'^o = \Delta G - RT \ln(Q)$, where $Q=[C]/([A][B])$.

Since [B] = 0.03 M at the time point of interest, using the matter conservation law we get [A] = 0.01 M and [C] = 0.02 M. Substituting these values in the above equation (remember: you need to use the concentrations in M units!) gives:

$\Delta G^0 = \Delta G - RT*\ln(0.02/(0.03*0.01)) = -2000$ J/mol $- RT*\ln(66.6667) = -2000$ J/mol -10403 J/mol = -12.403 kJ/mol

Note after grading: unfortunately, a common mistake was to plug into the above equation the concentrations in mM units without converting them into M. That resulted in the ΔG^0 value that is wrong not only numerically but also of the positive sign.

(5 points)

Once you know the ΔG^0, calculating the equilibrium constant is straightforward, but don't forget that K_{eq} has units – it is not dimensionless for this reaction:

$K_{eq} = \exp(-\Delta G^0/RT) = 149.48\ M^{-1}$

Note after grading: unfortunately, a common mistake was to ignore the fact that K_{eq} for this reaction has units.

Problem 2. (25 points) This problem deals with arginine.

A. For what pH values can arginine amino acid be used as an effective buffer?

Amino acid Arg has 3 titratable groups: the amino group (NH_2, pK_2=9.7), the carboxyl group (COOH, pK_1=2.3) (assuming the "common" pKa values for the terminal groups) and the guanidino group (pKa=12.48). Following the convention that the buffering range is ±1 pH unit around the pKa value, these ranges are: 1.3 – 3.3; 8.7 – 10.7, and 11.48 –13.48.

Note after grading: Unfortunately, when answering this question some students considered only the R (guanidino) group of arginine and forgot that the amino- and carboxyl groups of arginine amino acid are ionizable and have buffering capabilities within ±1 pH unit of their respective pKa values (pK_2 and pK_1).

B. What ***fraction*** of arginine has its guanidino group in the deprotonated form at pH=12?

Designating Arg with the protonated guanidino group as R^+ and deprotonated as R, we get

$[R]/[R^+] = 10^{pH-pKa}$, where pKa = 12.48 for the guanidino group. At pH=12, this ratio equals $r=10^{-0.48} = 0.3311$.

The deprotonated fraction of R is $[R]/([R]+[R^+])= r/(1+ r) = 10^{-0.48}/(1+ 10^{-0.48}) = 0.2488 \approx 0.25$.

C. You have a 200 mL sample of 50 mM solution of arginine at the pH value that equals the pKa of the arginine's guanidino group. You need to bring the pH of the sample to 12 (see previous question). You have at your disposal 1M HCl and 1M NaOH. Which of the two reagents and in what volume you need to add to your sample in order to achieve your goal? *Explain your reasoning and assumptions.*

200 mL of 50 mM solution of arginine contains 10 mmol of Arg. At the pH= pKa = 12.48, we have 5 mmol of base (deprotonated guanidine, R) and 5 mmol of acid (protonated guanidine, R^+). To decrease the pH to 12 we need to add an acid, hence HCl.
Based on the answer to the previous question, at pH 12 we will have 2.488 mmol of deprotonated guanidino groups (base, R) and 7.512 mmol of protonated guanidino group (acid, R^+). Thus we need to add 5-2.488 = 2.512 mmol of HCl. This corresponds to 2.512 mL of 1M HCl.

Problem 3. (23 points) Consider a penta-peptide with the following amino acid sequence: **NIHRC**

A. Estimate the isoelectric point of this peptide. *Show your calculation and explain your reasoning.*

B. Draw the **complete structure** (including hydrogens) of the penta-peptide. Show the main forms of all ionizable groups at the isoelectric point.

C. You want to use ion-exchange chromatography to purify this peptide. The peptide is in Tris buffer at pH 7.2. What type of column do you need if you want the peptide to be retained on the column and not washed out in the flow-through? *Explain your reasoning.*

The same questions are repeated below on this and next page to give you more space for your answers

A. Estimate the isoelectric point of this peptide. *Show your calculation and explain your reasoning.* (10 points)

First let's identify all ionizable groups in this peptide: N-terminal amine (pK_2= 9.7), side chains of His (pKa = 6.0), Arg (pKa = 12.48), and Cys (pKa = 8.18), and the C-terminal carboxyl (pK_1 = 2.3).

Let's now consider a pH grid and check the predominant charge states of the individual groups and the total charge of the peptide.

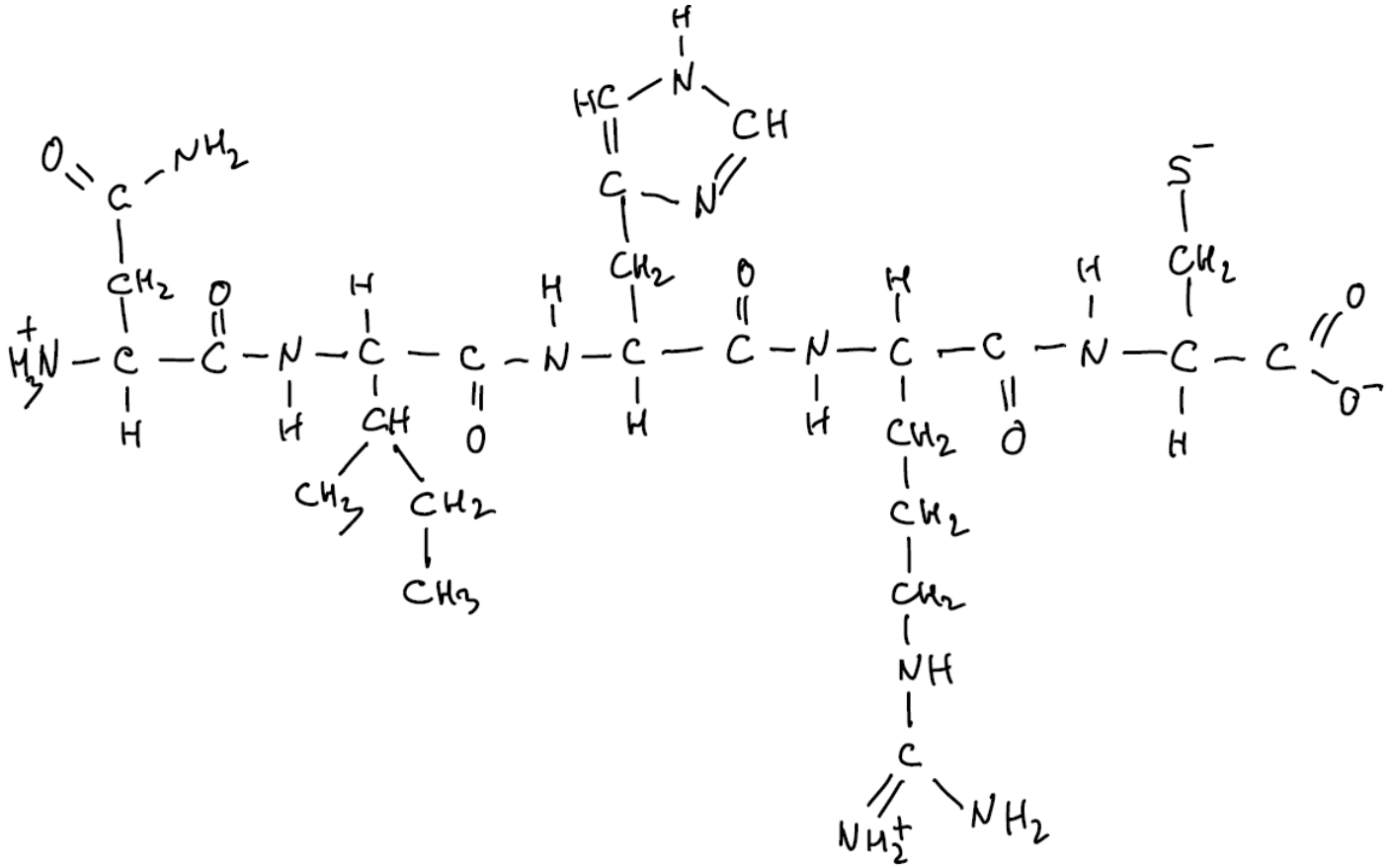
pH	NH_2	His	Arg	Cys	COOH	Total charge
1	+	+	+	0	0	+3
3	+	+	+	0	-	+2
5	+	+	+	0	-	+2
7	+	0	+	0	-	+1
8	+	0	+	0	-	+1
9	+	0	+	-	-	0 (zw)
12	0	0	+	-	-	-1
14	0	0	0	-	-	-2

Thus, the isoelectric point can be estimated as pI = (pK_{Cys}+pK_2)/2= 8.94.

B. Draw the **complete structure** (including hydrogens) of the penta-peptide. Show the main forms of all ionizable groups at the isoelectric point.
(10 points)

At the isoelectric point, the N-terminal amine and the guanidine group of Arg are predominantly positively charged, the thiol and the carboxyl of Cys are negatively charged, while the imidazole of His is neutral/uncharged.

Note that the placement of the hydrogen as attached to ε2-nitrogen in the histidine ring in my drawing here is different from the one in the Lehninger textbook where the H is attached to δ1-nitrogen. Both drawings are fine: they represent two tautomeric forms of the neutral imidazole ring. (From my experience, at least in ubiquitin, at pH above the pKa of histidine the ε-tautomer is more populated than the δ-tautomer.)



C. You want to use ion-exchange chromatography to purify this peptide. The peptide is in Tris buffer at pH 7.2. What type of column do you need if you want the peptide to be retained on the column and not washed out in the flow-through? *Explain your reasoning.*
(3 points)

As pH 7.2 is lower than the pI, the peptide will be positively charged on average. Therefore you will need to use a cation exchange column for this purpose.

Problem 4. (23 points) Suppose you have a mixture of five proteins listed in the table below.

Protein	pI	Number of amino acids
A	10.6	250
B	9.6	700
C	7.5	100
D	4.5	620
E	3.5	80

1. (3 points) Predict the order in which these proteins will migrate on the PAGE SDS gel, starting with the fastest.

Smaller proteins generally migrate faster in SDS-PAGE. So the proteins will migrate in the following order **E, C, A, D, B**

2. (3 points) Predict the order in which these proteins will elute from a gel-filtration (size-exclusion) column, starting with the fastest.

Bigger proteins generally migrate faster through the size-exclusion column. So the proteins will elute in the following order: **B, D, A, C, E**

3. (5 points) You loaded an aliquot (small portion) of this mixture on a cation column (that bears negatively charged groups). The proteins are in sodium acetate buffer at pH 5.0. List the proteins that will bind to the column.

At this pH proteins A, B, and C will be positively charged, and D and E will be negatively charged. The former three proteins bind to the column: **A, B, C**

4. (5 points) You also loaded another aliquot of the mixture, this time on an anion column (that bears positively charged groups), and the buffer is HEPES (pH 7.5). List the proteins that will bind to the column.

At this pH proteins A and B will be positively charged, C will be neutral, and the rest will be negatively charged, hence bind to the column: **D, E**

5. (7 points) Now it's time to do the separation. Devise a scheme that will allow you to unambiguously separate and purify all these proteins using a combination of the preparative chromatographic methods mentioned in the previous questions. Explain your reasoning. Remember, you goal is to separate the proteins unambiguously.

There are several scenarios. Take advantage of the differences in the size and the pI values. What you want to avoid is trying to separate proteins that are close in size (e.g. 80 from 100 a.a. or 620 from 700 a.a.) using size exclusion or those that are close in pI (e.g. 3.5 from 4.5 or 9.6 from 10.6) by ion exchange.
For example, using your answers to questions 3-4, you can
(1) first separate D and E from the rest of the proteins using cation exchange column and a buffer with pH 5.5 or slightly higher – this will result in A,B,C sticking to the column and D and E flow through.

(2) then take the flow through of the cation column (which contains D and E) and separate them from each other using size-exclusion chromatography: you should be able to separate 80 a.a. and 620 a.a. proteins;

(3) then elute proteins A,B,C from the cation column by adding high-salt buffer and separate them using size-exclusion chromatography you should be able to separate 100 a.a. protein from 250 a.a. protein from 700 a.a.

protein. You might also be able to separate these proteins by when they elute from the cation column when using a shallow salt gradient.

Or in step (1) you can separate D and E from A, B, C using an anion column with pH 7.5 or slightly lower: D and E will stick to the column while A, B, C will flow through. Then follow steps similar to (2) and (3) to separate D from E and A from B from C.

Or you can first use size-exclusion chromatography to separate D+B from A and from C + E, and then use the cation (as in question 3) or anion (as in question 4) exchange column to separate B from D and C from E.
Or some other scenario….

Problem 5. (14 points) In the course of amino-acid sequence determination of an unknown peptide enzymatic cleavage was combined with subsequent sequencing. You obtained the following results.
1. Chymotrypsin (see Table 1 on the equations sheet) cleaved the peptide into the following three fragments:

NLW
IAQF
KGEILRVY

2. Treatment with trypsin produced three penta-peptides.

A. Based on these data determine the correct amino acid sequence of the peptide. *Briefly explain how you arrived at your answer.*
(10 points)

The sequence of the peptide is **IAQFKGEILRVYNLW**

Since all three fragment peptides end with an aromatic residue, it is not immediately obvious from the chymotrypsin cleavage which of them is the C-terminal fragment. However, you can realize that because trypsin cleaves after R and K, the peptides **NLW** and **IAQF** cannot be adjacent to each other, and, furthermore, the long fragment **KGEILRVY** must be in the center of the peptide. In order for trypsin cleavage at K to result in a penta-peptide, the **IAQF** fragment must precede K. Likewise, in order to get a penta-peptide after cleavage at R, we need 3 more amino acids after VY – this positions the **NLW** fragment at the C terminal end.

B. Now that you *know* the sequence of the peptide, design/propose a reaction using only one of the cleavage reagents from Table 1 that will allow unambiguous determination of the peptide's sequence in a single step.
(4 points)

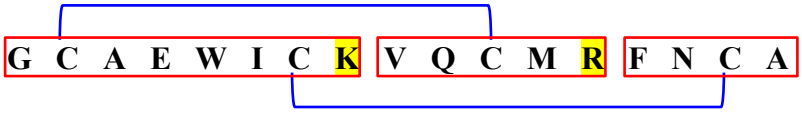
Of the cleavage reagents in Table 1, the only one that will cut the peptide in two pieces (thus no other steps will be required) is Asn-N. It will result in **IAQFKG** and **EILRVYNLW**, which can be unambiguously "assembled" into the correct peptide sequence. Pepsin will give 3 fragments, while cyanogen bromide will not cleave at all.

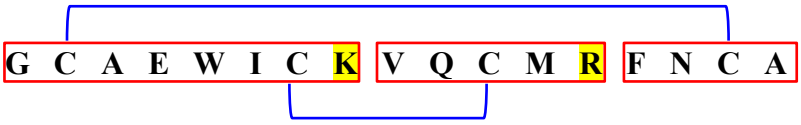
Bonus Problem. (10 points) If you are done with the above problems, consider this one for an extra credit.

A peptide has the following sequence: **GCAEWICKVQCMRFNCA**. It is known that the structure of this peptide is stabilized by two disulfide bonds, but their exact location is unknown. In order to determine their location, you decided to break the peptide into fragments using a cleavage reagent. You do not have equipment for sequencing or mass determination, all you are capable of doing is to determine the number of fragments/peptides resulting from the cleavage. So, you treated the peptide with trypsin and found that trypsin cleavage resulted in two peptides.
Based on this observation, determine the location of the –S–S– bonds. Show them schematically on the sequence (below) by connecting the corresponding residues. *Explain your reasoning.*

(4 points for each correct disulfide bond + 2 points for meaningful reasoning/explanation)

Since trypsin cleaves at the carboxyl end of K and R, the peptide will be cleaved into three fragments, unless disulfide bonds hold some of them together. The three possible ways to form two disulfide bonds between four cysteines are shown below. Only the first scenario will result in two disconnected peptides after cleavage by trypsin. General considerations: two of the three cleaved fragments must be held together. If any of the two cysteines of the long fragment is bonded to a Cys of a short fragment, the second Cys would have to be connected to a Cys of the other short fragment (scenarios 2 and 3) – but such bonding will still hold all three trypsin-cleaved fragments together. Thus, one disulfide bond must be between the two cysteines of the long fragment, while the 2nd disulfide bond must be between the cysteines of the two short fragments.





Notes after grading:
(1) cysteine is the only amino acid residue that forms disulfide bonds, and, obviously, with another cysteine not with any other residue;
(2) this question is about disulfide bonds holding cleaved fragments together *after* trypsin cleavage. This question has nothing to do with whether a disulfide bond might somehow hinder/obstruct trypsin cleavage of the initial peptide at K or R or whether two cysteines form a "weaker" or "stronger" disulfide bond depending on where they are located in the peptide's sequence.