The following twelve questions deal with absorption spectrophotometers. Answer any eight.

a) The optics in most spectrophotometers has the path - source, monochromator, sample, detector. Why might this be advantageous compared to the path - source, sample, monochromator, detector?

With the second arrangement, all of the source output irradiates the sample during the entire time the spectrum is being recorded. This maximizes the chance that the sample will be damaged by photolysis. To further minimize the effect of photolysis, the spectrum should be scanned from the red towards the ultraviolet.

b) What effect will a fluorescent sample have on an absorption measurement? Propose an instrumental modification to eliminate the effect of fluorescence.

With a fluorescent sample some of the emitted photons are observed by the detector. This makes the measured absorption lower than the actual value. The least expensive way to minimize the problem is to move the detector far from the sample. This reduces the number of photons reaching the detector due to the 1/r^2 law. A better, but more expensive, fix is to place a second monochromator after the sample. Such a spectrophotometer used to be commercially available.

c) What effect will a scattering sample (proteins, polymers, cells) have on an absorption measurement? Propose an instrumental modification to eliminate the effect of scattering.

With a scattering sample some of the unabsorbed photons are removed from the beam. Thus the sample has a higher absorption than the actual value. This problem is the reverse of that in (b). The least expensive way to minimize the problem is to move the detector close to the sample. An "expensive" fix is to measure the difference in absorption between two closely spaced wavelengths. This gives a differential spectrum that is relatively free of scatter artifacts. A sample that both scatters and fluoresces, e.g. a tagged protein, has conflicting instrumental requirements.

d) Most absorption spectrophotometers can only reliably determine absorption values less than ~2.0. What parameter of the instrument determines the upper limit to measurable absorption.

When the source radiation reaches the monochromator grating, imperfections and roughness in the grooves of the grating scatter white light throughout the monochromator. The scattered white light that reaches the exit slit is called stray light. With ruled gratings, stray light is \(\sim 10^{-4}\) the total at the entrance slit. With a holographic grating the groove edges are smoother and stray light is \(\sim 10^{-8}\). As a sample absorbs more and more light, the stray light not absorbed by the sample reaches the detector and limits the maximum absorbance that can be measured.
e) A compound is known to have a strong absorption band at 300 nm and no absorption above 350 nm. When the spectrum is obtained a weak absorption band appears at 600 nm. What causes this instrumental artifact? Propose an instrumental modification to eliminate the artifact.

This artifact is observed with a grating monochromator. The Bragg diffraction equation is

\[ 2d \sin \theta = m\lambda \]

where \( d \) is the groove spacing, \( \theta \) is the angle of diffraction, \( m \) is the diffraction order, and \( \lambda \) is the wavelength of light. Thus, 300 nm can diffract at angles given by the following.

\[ \theta = \sin^{-1} \left( \frac{m\lambda}{2d} \right) \]

This means that the diffraction angle for the second order of 300 nm light is the same as the first order of 600 nm light. When the monochromator reaches 600 nm, 300 nm light reaches the detector. This gives a false absorption band. The remedy is simple - when the monochromator reaches 400 nm insert a 400 nm high pass glass filter after the monochromator and before the sample. This was the strategy used by the (no longer available) Bausch and Lomb 505. In contrast, the Cary line of spectrophotometers used a more expensive approach. These instruments send the light first through a prism monochromator and then a grating monochromator. Since prism monochromators do not have orders, the artifact is removed.

f) The absorption spectrum of a solution has two peaks, A and B. As the pH of the solution is increased, peak A decreases in absorbance while peak B increases. There is one wavelength between peak A and peak B where the absorption is independent of pH. What is chemically occurring, and why is the absorption fixed at one wavelength? The wavelength with fixed absorption has a name. What is it?

The solution contains A and B in a chemical equilibrium, i.e. \( \text{ArCOOH} \rightleftharpoons \text{ArCOO}^- \). As the pH changes the ratio of acid and base changes. The wavelength with a pH independent absorption is the one where the molar absorptivities of the two species have the same value.

\[ A_{\text{total}} = A_{\text{acid}} + A_{\text{base}} = \varepsilon_{\text{acid}} C_{\text{acid}} + \varepsilon_{\text{base}} C_{\text{base}} = \varepsilon C_{\text{total}} \]

Since the sum of the two concentrations is constant due to the conservation of mass, the absorbance does not change as a function of pH. This wavelength is called the isosbestic point, and is a terrific indicator of the presence of an equilibrium.
g) Before publishing an assay based on measuring absorption it is prudent to obtain the spectrum for a series of concentrations with a fixed pathlength x concentration product. For example, $10^{-6}$ M in a 10 cm cell; $10^{-5}$ M in a 1 cm cell; $10^{-4}$ M in a 0.1 cm cell; and $10^{-3}$ M in a 0.01 cm cell. What information is obtained from such a study?

Since the concentration-pathlength product is constant, the same spectrum should be obtained for each measurement. This approach is used to identify the presence of concentration-dependent chemical changes such as solvation and dimerization. As an example, aqueous solutions of rhodamine B will show a change in the spectrum at millimolar concentrations. The same study in ethylene glycol shows no spectral change. This is one of the reasons why dye lasers use ethylene glycol as the solvent. A second example would be metal chelates where the spectrum changes at low concentrations due to the solvent (at a very high concentration) displacing the lower concentration ligand.

h) Samples are usually held within a 1 cm quartz cell. What optical losses are introduced by the quartz cell? How are these losses taken into account when measuring a spectrum?

The quartz/air and quartz/solvent boundaries cause reflections due to differences in refractive index. Each quartz/air boundary reflects $\sim$4% of the light, while a quartz/water boundary reflects $\sim$0.4% of the light. This means that the quartz cell reflects $\sim$8.8% of the light. If uncompensated, this introduces an absorption artifact of 0.04 absorption units. When taking a spectrum this artifact is removed by using a reflection-matched cell in the reference. Cells can be purchased in matched pairs. Note that the matched pairs should not be mixed with other spectrophotometer cells!

i) How might you verify the accuracy of the wavelength scale of an absorption spectrophotometer?

The simplest method involves replacing the spectrophotometer source with a mercury pen lamp, blocking one beam and measuring the resultant spectrum. This operation is sufficiently common that many spectrophotometers have the option built into them. A less intrusive method involves using an NIST standard solution of holmium oxide.

j) How might you verify the photometric accuracy of an absorption spectrophotometer?

A crude calibration might involve measuring the spectrum of an organic compound with a known molar absorbivity. A better method involves using NIST spectra published for copper sulfate, ammonium sulfate, or potassium chromate. NIST also sells calibrated glass standards.

k) Give an order of magnitude estimate of the smallest absorption you would expect to measure at a $SNR = 3$ for a commercial spectrophotometer.

$10^{-3}$ to $10^{-4}$ depending upon the instrument. As an example, the Cary 400 has an accuracy of $1.1\times10^{-4}$ at an absorption of 0.3 (50% of the light is absorbed).
1) *A solution has a distinct yellow color.* At about what wavelength will you expect to find the absorption band?

Use the rough color/wavelength guide: 400 nm is violet, 450 is blue, 500 is green, 550 is yellow, 600 is orange, and 650 is red. Then use the color complement to convert observed color into absorbed color, i.e. red and green, orange and blue, yellow and violet. Thus a yellow solution will absorb in the violet near 400 nm.
BIOCHEMISTRY CUMULATIVE EXAM

Signal Transduction
August 25, 2001

1. 15 A. Describe the general mechanism by which steroid hormones regulate gene expression.

15 B. Describe a feasible mechanism by which the drug tamoxifen (shown below) works in the treatment of breast cancer.

5 C. How could this approach be exploited for the treatment of other types of cancers?

152. Explain why some children with retinoblastoma develop multiple tumors of the retina in both eyes, while others have a single tumor only in one eye.

3. Signal transduction cascades can be regulated by changes in protein-protein interactions and through the intracellular targeting of key proteins. The Ras protein is an important component of receptor tyrosine kinase-mediated signal transduction cascades.

A. What is the enzymatic activity of Ras in this pathway?

B. How could mutation of the Ras protein lead to cancer in the context of this pathway?

C. How is Ras regulated?

D. Where is Ras localized in the cell and how is it targeted there?

D. Farnesyltransferase inhibitors are a new class of compounds being developed to treat Ras based cancers. What is the biochemical basis for this work?
1. A. Describe the general mechanism by which steroid hormones regulate gene expression.

   The steroid/thyroid hormone receptor superfamily (e.g. glucocorticoid, vitamin D, retinoic acid and thyroid hormone receptors) is a class of proteins that reside in the cytoplasm and bind lipophilic steroid/thyroid hormones. These hormones are capable of freely penetrating the hydrophobic plasma membrane after being carried to the target tissue on serum binding proteins. Upon binding ligand, the hormone-receptor complex undergoes a conformational change and translocates to the nucleus where it binds to specific DNA sequences termed hormone response elements (HREs). The binding of the complex to an HRE results in altered transcription rates of the associated gene either increasing or decreasing the rate of mRNA formation.

   B. Describe a feasible mechanism by which the drug tamoxifen (shown below) works in the treatment of breast cancer.

   The use of tamoxifen in the treatment of breast cancer exploits the specific nature of the steroid hormone and receptor interaction. By inspection, the structure of tamoxifen is similar to that of estrogen. Tamoxifen effectively competes with estrogen for binding to the estrogen receptor but the tamoxifen-receptor complex has no effect on gene expression. Since the growth of some types of breast cancer depends on the continued presence of estrogen, competition with tamoxifen can effectively slow or stop the growth of the cancerous cells.

   C. How could this approach be exploited for the treatment of other types of cancers?

   Other cancerous cells that are hormone-dependent for their growth could be targeted similarly with these types of antagonists.

2. Explain why some children with retinoblastoma develop multiple tumors of the retina in both eyes, while others have a single tumor only in one eye.

   The retinoblastoma protein (Rb) participates in a mechanism that arrests cell division in G1 of the cell cycle if DNA damage is detected. Therefore, Rb functions to regulate cell division. Children who develop multiple tumors in both eyes were born with every cell in the retina already having one defective copy of the RB gene. Early in their lives, as retinal cells divided, several cells each independently suffered a second mutation that damaged the one remaining good copy of the RB gene, producing a tumor. The single tumor form of the disease occurs in children who at birth had two good copies of the Rb gene in every cell. In a single cell, two mutations make both copies of the Rb gene defective. It is very unlikely that this will happen twice in the same person, and these children therefore develop only one tumor and in only one eye.
3. Signal transduction cascades can be regulated by changes in protein-protein interactions and through the intracellular targeting of key proteins. The Ras protein is an important component of receptor tyrosine kinase-mediated signal transduction cascades.

A. What is the enzymatic activity of Ras in this pathway?

Ras is a GTP binding protein and a GTPase, capable of hydrolyzing GTP to GDP. When GTP is bound to ras, the protein is activated and is capable of transmitting regulatory growth signals from cell surface receptors to the nucleus. GTP binds to ras by replacing a GDP nucleotide already bound to the ras protein. The ras protein returns to the inactive state by removal of the terminal phosphoryl group (or hydrolysis) of the bound GTP leaving GDP bound to the protein. This hydrolysis function, or "GTPase activity", is an integral function of the ras protein.

B. How could mutation of the Ras protein lead to cancer in the context of this pathway?

Mutant ras proteins have impaired or altered GTPase activity, forcing the protein to remain in the active state. Thus the cell is unable to inactivate the cell proliferation function of the ras protein, that is, GTP remains bound to the ras protein. This results in alterations in cellular growth and metabolism common to tumor cells. Mutant ras proteins have been identified in 25-30% of human cancers. This impairment of GTPase activity could result from a change in the intrinsic ability of Ras to hydrolyze GTP or from an improper interaction with GAP.

C. How is Ras regulated?

Ras activity is regulated by two proteins. These are: 1) a GTPase activating protein (GAP) and 2) a guanine nucleotide exchange factor (GEF). The GTPase activity is very slow in the ras protein. It hydrolyses only one GTP every 35 minutes. Consequently ras would normally remain active upon binding of GTP. However the interaction of the ras protein with GAP protein increases Ras GTPase activity. GTP hydrolysis increases to 100-1000 GTP molecules per minute. In this state, Ras is bound to GDP and is inactive. Ras is cycled back to its active form by a nucleotide exchange factor (GEF). Binding of GEF to the Ras-GDP complex causes release of GDP and binding of GTP. An example of GEF is the Sos protein.

D. Where is Ras localized in the cell and how is it targeted there?

Ras is localized to the plasma membrane. It is initially synthesized with the C-terminal sequence –CaaX where C is cysteine, a is an aliphatic amino acid and X is any amino acid. It is targeted from the cytoplasm to the plasma membrane by a series of posttranslational modifications including farnesylation of the cysteine, proteolysis of the three terminal amino acids and α-carboxyl-methylesterification of the newly exposed farnesylcysteine residue. Ras is also subsequently palmitoylated at an adjacent cysteine residue. These additions of hydrophobic groups target Ras to the plasma membrane.
E. Farnesyltransferase inhibitors are a new class of compounds being developed to treat Ras based cancers. What is the biochemical basis for this work?

Farnesyltransferase inhibitors have been shown to effectively inhibit protein farnesylation and are remarkably non-toxic to normal cells. For Ras based cancers, it was originally thought that FTase inhibitors would function by preventing association of activated Ras with the membrane thereby preventing cell growth. However, it appears that inhibition of farnesylation of other proteins including RhoB may suppress the transformed phenotype of Ras based cancer cells.
Answer Key
Inorganic Chemistry Cumulative Exam
Purdue University
August 25, 2001

Question 1:
A cone angle is the three dimensional space, or “cone” occupied by a ligand from the donor atom out, away from the metal. Fewer of the larger cone angle ligands can fit around a given metal ion. A PMe₃ ligand has a cone angle of 118° and a the larger PPh₃ has an angle of 145°.

Question 2:
A delta bond is a quadruple bond between metal ions. A σ bond, then two π bonds form, preceding a δ bond. The δ bond results from overlap of dₓᵧ orbitals between two metals.

Question 3:
HgS is called “cinnabar,” the naturally occurring form of mercury. Many different iron ores are found. Some examples include Fe₂O₃ (hematite), Fe₃O₄ (magnetite), [FeO(OH)] (limonite), and FeCO₃ (siderite). Hard-soft acid base theory states that soft metals such as mercury bind best to soft ligands such as sulfide. Conversely, the hard iron ions prefer hard oxygen ligands.

Question 4:
When iron is in the higher oxidation state, the charge density on the ion is higher. And the metal ion is more electron deficient. Thus, the metal-ligand bonds tend to become stronger with an increase in oxidation state. So the water will bind more tightly to Fe(III) relative to Fe(II) and be less able to exchange with solvent water.

Question 5:
A) fac and B) mer

Question 6:
Cobalt has two predominant oxidation states: +2 and +3. These two states have very different coordination environments, unlike Fe²⁺/³⁺. Co(II) tends to have lower coordination numbers than Co(III). These drastic changes can be detrimental to the precise organization needed in an enzyme active site. The redox processes capable of converting between Co(II) and Co(III) may also be damaging to the enzyme (e.g., oxidative damage). Also, Co(III) is kinetically very inert. Reaction chemistry at this metal center is often so slow, there is no benefit to having this metal ion present to accelerate chemical reactions.
A. In one simple model, the classical oscillating electromagnetic field of the light interacts perturbatively with the electronic dipole of a molecule to cause its wavefunction/energy to change with time. (This change is described by the time-dependent Schrödinger equation with a Hamiltonian that includes the field-molecule perturbation.) Energy flows from the field to the molecules. As the molecules absorb light, they undergo transitions from initial to final energy states separated by the frequency of the light field. In the process the molecules can acquire (or loose) vibrational energy, and/or change rotational state (this by transfer of angular momentum from the electromagnetic field- or, if you prefer, photon angular momentum). The result is an electronic transition, in which a distribution of molecules in an initial state reach a distribution of final states, from the energy separation that is evident in this spectrum the structure present must be rotational.

B. In simplest terms, we assume

\[ H = H_e + H_v + H_R \]

So that

\[ \psi' = \psi_e \psi_v \psi_R \]

Thus, we can use quantum numbers associated with each separable sub-system.

For example,

\[ |\psi\rangle = |n\rangle |v\rangle |N\rangle \]

Where \( n \) stands for all electronic quantum numbers, \( v \) for those of the vibrational state, and \( N \) for rotation. In the present case:

\[ |N\rangle \]

\[ |N''\rangle \]

\[ |n\rangle |v\rangle \]

\[ |n''\rangle |v''\rangle \]
Intensifies differences are caused mainly by the thermal population differences in the initial state

C. The spectrum clearly shows P and R rotational branches that arise from the selection rule $\Delta N = \pm 1$

D. A finite perturbation leading to absorption in the dipole approximation requires a non-zero integral of the form

$$<\psi' | \mu | \psi''>$$

(transition dipole) Where $\psi'$ and $\psi''$ are final and initial total wave functions and $\mu$ is the dipole operator. To be non-zero the integral $<\psi' | \mu | \psi''>$ must be totally symmetric. The dipole operator ($\rightarrow$) has odd parity, therefore the initial and final states must be of opposite parity. In a diatomic molecule, when ground and excited states are non-degenerate (of $\Sigma$ symmetry), this parity reversal only occurs (in transitions allowed by angular momentum conservation) for initial and final state pairs which differ in rotational quantum number by one. Thus, N must change by one on going from $N''$ to $N'$.

E. The initial and final states must both be of $\Sigma$ symmetry. Otherwise, both symmetric and antisymmetric forms of the total wave function exist at all values of N and the selection rule becomes $\Delta N = 0, \pm 1$ (a Q branch appears).

F. Let's say the ground state is $^1\Pi$. Then the relevant every diagram is

This spectrum would include a Q-branch formed by $\Delta N = 0$ transitions.
G. The R(0) and P(2) transitions originate from \(N'' = 0\) and 2 to both terminate on \(N' = 1\). The frequencies of these transitions, estimated from the figure are 44,620 and 44,640 cm\(^{-1}\). The 20 cm\(^{-1}\) difference is the separation between levels with rotational energies: \(B_0(0+1)\) and \(B_2(2+1)\) or \(6B\). So \(6B\) is 20 cm\(^{-1}\), or \(B = 3.33\) cm\(^{-1}\). Applying the formulas given, \(I = 5.063\) amu Å\(^2\) and with \(r = 1.6456\) Å, \(\mu = 1.8697\) amu. Using the mass given for \(A\) (which must be deuterium), we can calculate \(m_B = 26.1\) amu, which is closest to the 27 amu mass of \(Al\). Thus, the carrier of the spectrum is \(AlD\).
Olefin metathesis (Eq. 1) has emerged as one of the most versatile C-C bond-forming reactions.

\[
\begin{align*}
R^1\text{CH}=\text{CHR}^1 & \quad \xrightarrow{\text{cat. } \Phi \text{CH}=\text{RuL}_n} \quad 2 \quad R^1\text{CH}=\text{CHR}^2 \\
R^2\text{CH}=\text{CHR}^2 & \quad L_n = \text{Cl}_2 [\text{P(\text{C}_{\text{Pr}}\text{e})_3}]_2
\end{align*}
\]  
(1)

It has been shown to proceed through metallacyclobutane intermediates (Chauvin mechanism).

1. Write a mechanistic scheme for the transformation shown in Eq. 1. You may use \( L_n \) to show any ligands.

\[
\begin{align*}
\text{R'}\text{CH} = \text{CHR}^1 & \quad + \quad \Phi \text{CH} = \text{RuL}_n \quad \rightarrow \quad \Phi \text{CH} = \text{Ru} \text{CHR}^1 + L_u \\
\Phi \text{HC} = \text{CHR}^1 & \quad \rightarrow \quad \text{RuL}_u + C_{\text{HR}^1} \\
\Phi \text{HC} & \quad \rightarrow \quad \text{RuL}_u \quad \text{CHR}^1 \quad \rightarrow \quad \text{CHR}^1 \quad + \quad \text{L}_u \quad \text{CHR}^2
\end{align*}
\]

\[
\begin{align*}
\text{R}^2\text{CH} = \text{CHR}^2 & \quad + \quad \Phi \text{HC} = \text{CHR}^1 \quad \rightarrow \quad \text{CHR}^1 \quad + \quad \text{CHR}^2 \\
\Phi \text{HC} & \quad \rightarrow \quad \text{RuL}_u \quad \text{CHR}^1 \quad \rightarrow \quad \text{CHR}^1 \quad + \quad \text{L}_u \quad \text{CHR}^2
\end{align*}
\]
Olefin metathesis is very useful for preparing cyclic compounds. Recently, a fragrant compound, muscone, was synthesized as shown in Eq. 2.

\[
\text{cat. PhCH=RuL}_n \rightarrow \quad \text{1} + \quad \text{2}
\]

9-Decenol (3) is an inexpensive chemical. The other moiety containing an asymmetric carbon center can be readily synthesized from geraniol or nerol by another Ru-catalyzed reaction known as Noyori asymmetric catalytic hydrogenation (Eq. 3).

\[
\text{H}_2 \quad \text{cat. RuL}_n \rightarrow \quad \text{4(98 - 99% ee)}
\]

Write a clear synthetic scheme for the synthesis of 2 from 3 and 4 showing all pertinent reagents. Your synthesis should not require more than 5 – 6 steps.
3. The following synthesis of anolignan A is another example showing the synthetic utility of olefin metathesis.

\[
\begin{align*}
\text{cat. PhCH=RuL} & \quad \rightarrow \quad \text{anolignan A} \\
\end{align*}
\]

Assuming that the reaction first generates an active intermediate \(\text{CH}_2=\text{RuL}_n\), write a plausible mechanism for Eq. 4. Use \(\text{Ar}^1\text{CH}_2\text{C} = \text{CCH}_2\text{Ar}^2\) and so on to save your time.