

Chapter 19 Spectrophotometry: Instruments and Applications

Problems: 1, 4, 6, 9, 13, 14

Bring in a Spec 20 to open up and show innards

19-1 The Spectrophotometer

Last week talked about a 'single beam' spectrophotometer. One of the simplest instruments. (light monochromator-sample-detector)

Remember how it had to be used?

Put nothing in the light - Take a 0% reading

Put in a blank take - reading for P_0 .

(actually we set a dial so P_0 was at 100 on the dial)

Put in a sample take a second reading. (P)

This has inherent error. The intensity of the light can shift significantly between one measurement and the next (sit and watch the dial move up and down).

A different design that is significantly more accurate is the double beam design. (Used in the Lambda machine) **Shown Schematically in figure 19-1 and 19-2**

In this design both your sample and your reference are in the machine at the same time, and mirrors are used to 'chop' the light hitting the detector between the sample and the reference very quickly (a fraction of a second) so lamp drift effects are minimized

Let's examine some of the components, keeping in mind that this basic design is used in many UV, vis and IR spectrometers

Light Source

For Vis an ordinary light bulb with a tungsten filament works great. The electricity running through the wire heats it to about 3,000K and it emits light from the near UV to the near IR 320 nm to 2500nm.

If you want to go into the UV, most instruments use a Deuterium lamp. Simply a tube filled with D_2 , and you run an spark (arc) through it. The energy of the spark dissociates the D_2 into two highly energized D molecules, and the light is emitted as the atoms lose their energy and reassociate. Discharge lamps filled with Xenon or Hg vapor are also used

If you want to generate IR light the most common source is a rod of silicon carbide (SiC) ceramic called a glowbar. When heated (again by running an electrical current through it) the glowbar generates light between 5000 and 200 cm^{-1}

While lasers are extremely bright sources they only generate a single wavelength of light, and so aren't useful for a spectrometer that needs to analyze a continuous spectrum.

Monochrometer

In a monochrometer the light is dispersed into its various wavelength, and then a small portion of this spectrum is passed through the sample at a time so the absorbance of the sample at that particular wavelength can be determined.

Older instruments used to use prisms to disperse the light into its wavelengths, But this is now done with gratings. A grating is a mirror with a series of closely spaced parallel lines. When the light bounces off this mirror, each line acts like a source of in phase radiation. Constructive interference will make the reflected light beam brighter at certain angles, while destructive interference will completely obliterate the light at other angles. Since each wavelength will have its optimum brightness at different angles, the net result is that the light is diffracted into a spectrum. The relationship between the light angles and the distance between the lines is given in the equation:

$$n\lambda = d(\sin \theta + \sin \phi) \quad \text{Figure 19-5}$$

where n is the order of the diffraction, d is the spacing between the lines and theta and phi are incident and emerging angles.

Since each order of the spectrum may overlap with another order, either, or even two monochrometers can be placed in series (a double monochrometer) to make sure the final light has only the correct wavelength.

Dispersive elements light prisms and gratings are usually combines with other light focusing elements like lenses and slits (figure 19-4) to insure that a single well focused light source hits the grating, and then the reflected beam is also well focused so only the narrowest range of wavelengths passed through the sample at a time.

Narrowing the slit width decreases the range of wavelengths passing through the sample so your analysis is cleaner, however is also decreases the total amount of light reaching the detector, so you noise increases. Sometimes you need to make a trade off between good signal and good spectral purity.

Detector

A detector is some electrical device that produces a signal when struck by light. Just like there were a range of materials that are used as light sources and produce a different range of wavelengths and intensities, there are also several different detectors, and each has its wavelength maximum and minimum. (Figure 19-8)

The simplest and cheapest detector is the phototube like the one used in the spec 20. In the phototube we have a vacuum tube that contains a plate of metal that is usually either tin or Cs. When light of a high enough energy (frequency) hit this metal, the photons have enough E to kick electrons out of the metal's outermost electron shell. Inside the tube is also a wire that is kept at a + potential with respect to the metal plate. The electrons are attracted to this + wire, so current flows out of the metal plate and into the wire in direct proportion to the amount of light hitting the metal.

In more expensive instruments where we want to be more sensitive to lower levels of light, we can enhance the sensitivity of phototube by building it into what is called a photomultiplier tube (show in figure 19-9) Here instead of simply collecting the electrons released from the metal in a wire, we make the wire into a larger plate, called a dynode, and coat the plate with a material that releases several electrons for every electron that strikes the plate. The electrons released from this dynode are then collected on a second dynode of even higher potential, and so on. Thus the number of electrons and the current produced is multiplied with each dynode, so we get a bigger and bigger signal. We can actually build several stages like this into a single tube, with each dynode me multiply or signal several fold. In the end for every single light photon that hits the tube, we can create a large enough current that can be measured by our instrument, so we can literally 'see' a single photon of light hitting the phototube.

Photodiode Array Spectrometer

In the above discussion we focused an machines where the light is dispersed into its component wavelengths, and then the machine must physically scan through each of these wavelengths to determine the absorbance at that wavelength. This scanning process takes time, sometime up to several minutes. Let's look at a different machine design, one that take the entire spectrum in a single reading!

A Photodiode array spectrometer does this by dispersing the light onto photodiode array. A photodiode array is a linear array of several hundred light sensing diodes light having a thousand phototubes, one at the sweet spot for every different wavelength.

The design of this kind of machine is somewhat different and simpler. (Figure 19-11) Light passes through the sample FIRST. Then it hits the monochrometer, and then it is dispersed onto the photodiode array.

Advantages - No moving parts, entire spectrum in <1 second.

Disadvantages - single beam, so absorbance not as accurate (people using in lab could see numbers change dramatically second by second) spectral resolution limited by size of diode

19-2 Analysis of a mixture

The absorbance of a solution at any wavelength is the sum of the absorbances of all the chemicals in the solution

$$A = \epsilon_1 l [X_1] + \epsilon_2 l [X_2] \dots + \epsilon_n l [X_n]$$

As you have seen in the lab, solutions that contain more than one component can be successfully analyzed for the amount of each component. All you have to do is to analyze the solution at several different wavelengths. It can be proven that mathematically that you need 1 different wavelength for every component in the solution.

You get the most accuracy if the selected wavelengths correspond to absorbance peaks for each component. Let's look at the bromophenol green you analyzed in the lab.

Figure X

Let's look at pH 5.26
at 5.26 $A_{616} = .60$ $A_{444} = .12$

In ⁻	$\epsilon_{616} = 51$	$\epsilon_{444} = 9700$
HIn	$\epsilon_{616} = 23300$	$\epsilon_{444} = 1240$

$$A_{444} = .12 = [\text{HIn}](9700) + [\text{In}^-]1240$$

$$A_{616} = .60 = [\text{HIn}](51) + [\text{In}^-]23300$$

Can solve as 2 equations with 2 unknowns and algebra, our use formula derived from algebra. Making a single spreadsheet that does this math is usually easier than solving the algebra every time you hit a problem like this.

Formula

$$\begin{aligned} A_1 &= [X]\epsilon_{x,\lambda 1} + [Y]\epsilon_{y,\lambda 1} & A_1 &= [X]A + [Y]B \\ A_2 &= [X]\epsilon_{x,\lambda 2} + [Y]\epsilon_{y,\lambda 2} & A_2 &= [X]C + [Y]D \end{aligned}$$

$$E = (\epsilon_{x,\lambda 1} \times \epsilon_{y,\lambda 2}) - (\epsilon_{y,\lambda 1} \times \epsilon_{x,\lambda 2}) \quad = (A \times D) - (B \times C)$$

$$[X] = 1/E (A_1 \times \epsilon_{y,\lambda 2} - A_2 \times \epsilon_{y,\lambda 1}) = 1/E (A_1 D - A_2 B)$$

$$[Y] = 1/E (A_2 \times \epsilon_{x,\lambda 1} - A_1 \times \epsilon_{x,\lambda 2}) = 1/E (A_2 A - A_1 C)$$

$$A_{444} = .12 = [\text{HIn}](9700) + [\text{In}^-]1240$$

$$A_{616} = .60 = [\text{HIn}](51) + [\text{In}^-]23300$$

$$E = (9700 \times 23300) - (1240 \times 51) = 2.26 \times 10^8 - 63240 = 2.26 \times 10^8$$

$$[\ln] = 1/2.26 \times 10^8 (.12 \times 23300 - .60 \times 1240)$$

$$[\ln] = 1/2.26 \times 10^8 (2796 - 744)$$

$$[\ln] = 1/2.26 \times 10^8 (2052)$$

$$[\ln] = 9.08 \times 10^{-6}$$

$$[H\ln] = 1/2.26 \times 10^8 (.60 \times 9700 - .12 \times 51)$$

$$[H\ln] = 1/2.26 \times 10^8 (5820 - 6.12)$$

$$[H\ln] = 1/2.26 \times 10^8 (5814)$$

$$[H\ln] = 2.57 \times 10^{-5}$$

19-3 Spectrophotometric titrations

No big deal. Don't get big changes like we see in pH or pX, so usually have lines and need to extrapolate where lines cross

19-4 What happens when a molecule absorbs light?

Electronic Transitions

If you remember from our introduction to spectroscopy absorbance occurs when a molecule absorbs a photon of light. We need to understand a bit more about what happens when the light is absorbed

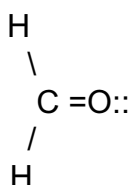
Let's start by remembering that absorption of visible and UV light involves photons with energies that change electronic transitions within molecules.. What are these electronic transitions? Well to understand that you need to think about the electrons in their orbitals in the molecule itself.

In Freshman chem you should have seen atomic orbitals like $1s^2$ and $3p^1$ and been introduced to molecular orbitals, orbitals formed by hybridizing atomic orbitals together. The σ bonds, with electrons localized between atoms, π bonds with the electrons to either side of a line joining the atoms, then the σ^* and π^* antibonding counterparts of these orbitals.

To help you hang a picture on what we are going to discuss next, let's focus on formaldehyde.

Formula H_2CO

Lewis structure?



Hybridization of central C? Sp^2 . 120° . triangular planar. Same for O

From Freshman chem remember the idea that molecular orbitals were orbitals that spanned the entire molecule, and were not just restricted to a single pair of atoms.

Let's look at the actual orbital shapes (figure 19-17)

Formaldehyde is a 4 atom molecule and is much more complicated than anything you looked at in Freshman chem, so without much justification let's look at the orbitals.

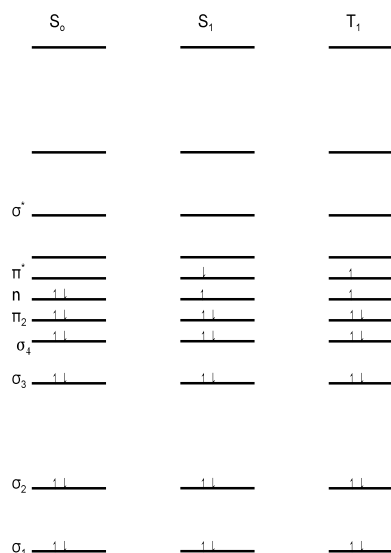
First you should expect 3 σ , 1 π , and 2 non bonding orbitals. Due to complications I won't go into you actually end up with one of the non-bonding orbitals mixing with the σ to get 4 σ orbitals, one of which is considered non bonding. Then you can see the π , the regular nonbonding and the first π^* .

Remember the idea that each orbital has an energy associated with it, with the σ at the lowest, then the π then the non-bonding then the π^* , then the σ^* ?

For this it is usually convenient to represent molecular orbitals in a ladder of energy.

Sigma bonds have the lowest energy then π then non-bonding then anti- π then anti sigma

In general you fill up the orbitals using electrons that are paired spin up with spin down. Our ground state usually has all bonding and non-bonding orbitals filled. Our absorption then kicks one of the electrons from the highest energy molecular orbital to the next



highest energy level. It can do this in two ways. It can either preserve our electron's spin so the ground and the excited states have no net electron spin (Called the **singlet** states, S) or it can change the spin of the electron so we now have a net spin of two electrons in the molecules (a **triplet state** (T))

In this transition it is literally moving the electron from an orbital of one shape into a different shaped orbital

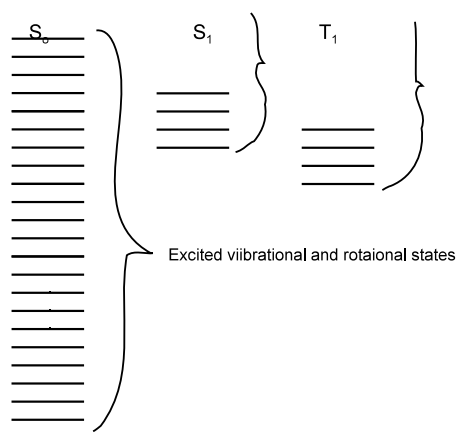
The triplet state is usually lower in total energy than the singlet state, so the energy that has to be absorbed is lower (the wavelength is larger For formaldehyde 397nm) however transition of singlets to triplets and vice versa do not occur very frequently so the overall absorbance of the transition may be extremely small. Singlet transitions usually a higher E (smaller wavelength (355 NM in formaldehyde) but occurs more frequently so give stronger absorbance band.

(Can I get a UV Spectrum to put in here?)

After the electrons move, the atom positions readjust to relieve unfavorable electronic interactions. You can see in **figure 19-16** that the S_1 state is not the same as the original molecule.

Vibrational and Rotational States

The molecule also vibrates and rotates so focusing only on electronic transition is only part of the story. The $S_0, S_1,$ and T_1 electronic state have a plethora of smaller vibration and rotation state within them so our if we include these we get a slightly different picture of energy:

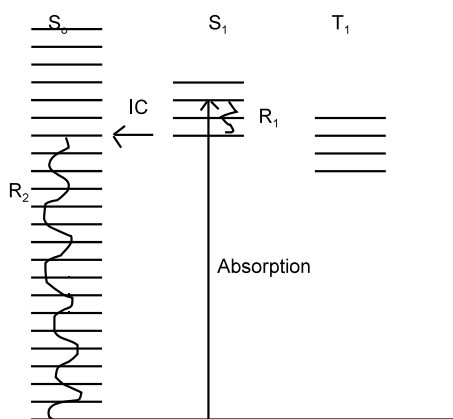


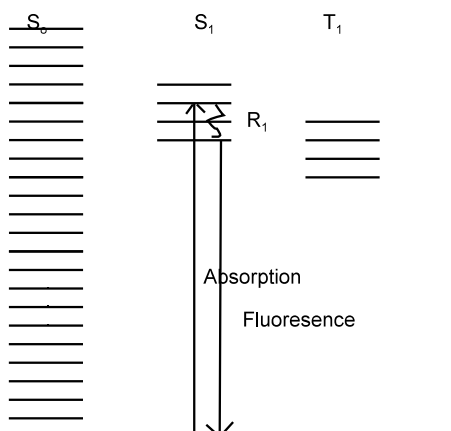
(Note that energies are not equally spaced and continue upward)

Does anyone remember energies are associated with these transitions (IR vibration Microwave rotations)

Now the photon we absorb can take us from the ground state to any number of different vibration and rotationally excited S_1 state, so our absorbance transition is broad.

Once we get to the S_1 state, what happens? The molecules vibrates and rotates, bumping in to other molecules and giving the excess vib and rot energy as heat. That takes us to the ground S_1 state. At this point we have two options to get rid of the remaining energy. Either we can perform an **internal conversion** where the molecule changes to an excited form of the S_0 state with the same energy, and we continue to dump energy as heat,

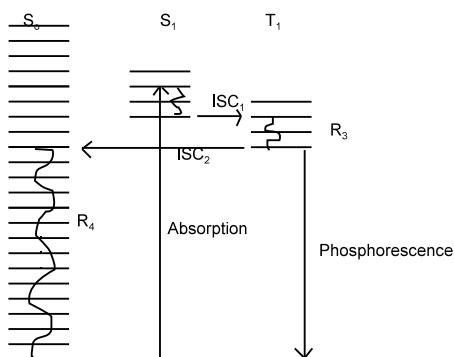




or we can **fluoresce** and re-radiate the energy as light. Note that the energy here is always less than the absorbance was.

There is also a third option. I said earlier that singlet to triplet conversions are rare, but they do occur. Once you get to the ground S_1 state you can undergo a **Intersystem crossing** and get converted to an excited T_1 state. From here you can undergo a second intersystem crossing to the S_0 state or you can emit light of even lower energy.

This kind of emission is called **phosphorescence**.



Both fluorescence and phosphorescence are relatively rare. The lifetime of fluorescence is short (10^{-8} - 10^{-4} sec) while the lifetime of phosphorescence is longer (10^{-4} - 10^2 sec)

Both these mechanisms together are referred to as **Luminescence**. In compounds like transition metal where states other than singlets and triplets occur we can't actually differentiate between fluorescence and phosphorescence.

19-5 Luminescence

The reason we are studying fluorescence is that it can be thousand of time more sensitive than absorbance. Why is this? We remember how we measure absorbance? We place a sample in a beam of light and measure how much light is absorbed. (Sketch physical set up) If you remember I said that absorbance measurements aren't very good if the $OD < 1$ This is because you are trying to measure a small change in intensity. But look at luminescence. Here we shine a light at the sample and look at the light emitted at right angles. Looking for a firefly [How about this analogy looking at a firefly on your car's headlight while staring straight in to it. Then look at it from the side](#) This makes is an important analytical tool

You can see from the treatment of luminescence I just gave you that the energy fluorescence and phosphorescence should be at lower energy of the exciting light, this translates to **longer wavelengths**. Further the **emission spectrum** usually looks like the mirror image of the absorption spectrum. The book explains why

Excitation and Emission Spectra

Sketch general Luminescent set up like [figure 19-23](#)

In analyzing luminescence we want to know both what the excitation profile looks like and the emission profile. Thus our spectrophotometer contains 2 monochrometers, one for the excitation light and a second to analyze the emitted light

If we hold the excitation wavelength constant λ_{ex} and scan the emitted radiation we get an **emission spectrum**. Graph of emission intensity vs wavelength. [Figure 19-21](#)

If we look at a single emission wavelength λ_{em} and scan the excitation wavelength we get an **excitation spectrum**. Graph of emission intensity vs excitation λ . The excitation spectrum looks very much like an absorbance spectrum because the more light that is absorbed, the more molecules are excited and can emit light.

In either of the above measurements we are directly measuring the intensity of the emitted light directly, and the response of our detector and the intensity of the light may vary with wavelength, so the overall measurement may not accurately reflect the actual response of the molecule. For a single analytical measurement this may be no big deal, but for exact measurements of these profiles you must carefully calibrate machine to remove these artifacts. Thought question - Why don't we worry about this in normal absorption measurements?

Emission Intensity

[Skip section in blue](#)

[Let's look in more detail at the measurement of luminescence. What is the relationship between \$P_0\$ the intensity of the light we are shining at the sample and \$I\$, the intensity of the emitted light?](#)

[Let's start by taking a more detailed look at the actual set up of the instrument. Notice how our sample monochrometers are set up. We have slits that adjust how wide the light beam entering the cell is and how wide with light beam that we are analyzing is. Why would you want to vary one or the other?](#)

[\(Narrower beam better resolution wider beam more light for both excitation and emission\)](#)

[You can see from this geometry that some of the light will pass through the sample before it hits the sweet spot of our cell where we will analyze it. Thus we will start our math by calculating how much light actually remains after passing through this 'dead' zone](#)

[Remember our basic Beer's Law](#)

$$A = \epsilon bc = \log P_0/P_1$$

Where P_0 is the power of the incident light and P_1 is the light left after absorbance

Taking the antilog of both sides we have

$$10^{\epsilon l c} = P_0/P_1$$

$$P_1 = P_0/10^{\epsilon l c}; \quad P_1 = P_0 10^{-\epsilon l c}$$

if b_1 represents the distance from the front of the cell to the start of our observing region, and ϵ_{ex} represents the molar absorptivity of the sample at the excitation wavelength then our incident intensity, P_1 is

$$P_1 = P_0 10^{-\epsilon(ex)b(1)c}$$

The intensity of our luminescence (I') will be directly proportion to the amount of light that is absorbed by our sample in the observation area times some constant that will depend on the electronic properties of the molecule, its concentration, and other solution properties.

The amount of light absorbed by the sample is equal to the amount of light that is present at b_1 minus the amount of light that remains at b_2 . I will call this P_2

$$P_2 = P_1 10^{-\epsilon(ex)b(2)c}$$

-and-

$$I' = k(P_1 - P_2)$$

Not all of the emitted light will make it to the detector either! Some of it will be absorbed by the solution just as the excitation light was. Thus

$$I = I' 10^{-(em)b(3)c \epsilon}$$

where ϵ_{em} is the molar absorptivity of the chromophore at the emission wavelength and b_3 is the pathlength the emitted light travels in the cell.

Thus

$$I = k(P_1 - P_2) 10^{-(em)b(3)c \epsilon}$$

$$= k(P_0 10^{-\epsilon(ex)b(1)c} - P_1 10^{-\epsilon(ex)b(2)c}) 10^{-(em)b(3)c \epsilon}$$

$$= k(P_0 10^{-\epsilon(ex)b(1)c} - P_0 10^{-\epsilon(ex)b(1)c} 10^{-\epsilon(ex)b(2)c}) 10^{-(em)b(3)c \epsilon}$$

$$= k P_0 10^{-\epsilon(ex)b(1)c} (1 - 10^{-\epsilon(ex)b(2)c}) 10^{-(em)b(3)c \epsilon}$$

The important parts of this equation

I proportional to P_0

What happens to I as a function of c?

$$\text{At } c=0, \quad 10^{-\epsilon(ex)b(1)c} \text{ and } 10^{-(em)b(3)c \epsilon} = 1 \text{ and } (1 - 10^{-\epsilon(ex)b(2)c}) = 0$$

As C increases the 10^c term decrease from 0 to 1

And the $1-10^c$ term increases from 0 to 1

Net effect is that I increases due to $1-10^c$ term and I proportional to c

If C gets too large the 10^c terms dominate and I decreases Figure 19.16

In the low concentration range it can be shown that

$$I = kP_0c \text{ (See section 8.2 in Willard and Merit)}$$

So intensity of emission is proportional to a constant times the power of the light times the concentration. Bottom line for analytical intensity directly proportional to concentration. So as long as concentration not too high can be used to evaluate concentration (Rule of thumb, absorbance of solution must be $<.05$! So this begins where standard absorption ends)

A couple of thing to watch. 1.) since we know that the response of the emission changes at high concentration you always need to check you assay for linearity through the range of concentrations you are working with. 2.) Notice also proportional to amount of light. If have low sensitivity can simply make lamp brighter! (Why doesn't this work for absorption? (Because absorption is ratio of P/P_0))

Not all compounds have luminescence. Typically you need aromatic systems or multiple conjugated double bonds in a compound before you will see fluorescence. It also help to be a rigid ring system since this makes the system less prone to losing energy through collision deactivation. Fluorescent properties also can vary strongly with solvent Thus cannot use for everything, but works nicely for many biologicals and drugs. Also can chemically attach fluorescent tags

Also have to watch out for quenching. That is other compounds in your solution that can quench the fluorescence interaction. **How??**

Many more details in Willard Merrit & Dean if you want them.

Immuno Assays (Do I want this here? It is a valid analytical technique but has little to do with optical spectroscopy)

Since fluorescence is used in many immuno assays, the book next briefly discusses immuno assays. Since immuno assays are in determining blood levels of many compounds, it is of interest to pharmacist to understand a little better how these assays work. Indeed as a pharmacist you sell pregnancy test that work via immuno assays, so I think that I will spend a little extra time here.

One assay method that is being used extensively in the clinical lab, and is being used more and more in OTC test like home pregnancy test is the immuno assay. Immuno assays aren't in your textbook, but because they are very important to the health field, I though I would take some time to talk about it now. For some background material you can check your Biochemistry book, Lehninger, Nelson & Cox Page 144-145 "The Antibody-Antigen Interaction is used to quantify and localize proteins "

I. BACKGROUND

All of these methods rely on the use of **Antibodies**. What is an antibody? Antibodies are one part your body's immune response mechanism. They are a protein your body makes to bind foreign materials so they can be disposed of. The foreign substance is termed and **Antigen** and the material made by the body to bind it is the **Antibody**. An antibody is a large protein Y shaped with sites for binding the antigen at the end of each Y-tip. Thus it is designed to bind two antigens. This allows the antibody to either bind to the antigen in two places, or to bind two different antigen molecules together. If you have an equal amount of antigen and antibody, and you have multiple binding sites on the antigen, you can get a mass of antigen-antibodies bound together that make a precipitate (This is a simple agglutination test). In fact you can think of this as the simplest immuno assay possible, if you have antibody and you add it to a solution ant you get a precipitate, then you must have the antigen in the solution, and the concentrations of the antigen and the antibody must be about the same.

The antigen-antibody binding is useful for two reasons, first is can be very selective. Antibodies are designed to bind one substance selectively, and to have very little **cross-reactivity** it other substances. For instance you may have an antibody to a 100 residue protein, sometimes a change as little as replacing a single amino acid, will completely block the binding of the protein to the antibody.

Second, the binding of the antibody to the antigen can be very strong $K_{\text{bind}} > 10^{10}$. This allows you to use very low concentration of both antibody and antigen in your assay. A good example of this is that immune assays can be used to detect hormone levels in the blood that are as low as 10^{-12} M! Thus immune assays are both very selective and very sensitive.

The body uses antibodies primarily to bind large objects like bacteria or viruses so they don't normally bind to small molecules like hormones or drugs with out some special coaxing. (That we won't get into). Also the body's natural immune response will elicit not just one antibody for each antigen, but a large variety of antibodies, each of which may bind to a different site on the antigen. Each of these different antibodies, however, is being expressed by a different cell in the body. Thus if you can isolate that single cell, you will get that single antibody.

One of the tricks of the Biotechnology industry is to do just that. Raise antibodies to a particular substance, and find the cells that are making those antibodies. You then select one of these cells and clone it. You then produce this cell on the industrial scale and purify the antibody it makes and you have a **monoclonal antibody**. This allows you to obtain gram quantities of a substance that will uniquely and strongly bind your molecule of interest.

While many of the assay methods I will talk about were developed using everyday garden variety antibodies, Most diagnostic systems or assays now use monoclonal

antibodies to enhance their day-to-day reproducibility.

II. ASSAY METHODS

There are a large variety of different Immuno assays. I will talk about and illustrate a few of the simpler ones, but be aware that there are lots of other methods out there.

Radioimmunoassay or RIA.

I mentioned earlier that in the simplest immuno assay you simply look for the appearance of a precipitate. To get visible precipitates takes large amounts of both antigen and antibody, as a result the assay is not terribly sensitive. In the RIA you modify either the antibody or the antigen itself to contain radioactive molecules like ^{125}I . With the radioactive label use can use Geiger counters or scintillation detectors to detect much minute quantities of your labeled material and this makes the assay lots more sensitive.

The simplest RIA method involves a competition assay. It is called a competition assay because you have the compound you want to measure in one solution, and you have a second solution with a known amount of that same compound radioactivity labeled. You do the assay by mixing these solutions together to let them compete for the binding site on the antibodies. This is illustrated in this overhead.

OVERHEAD1

Notice several important points. First you have a limited amount of antibody and an excess amount of drug so the labeled and unlabeled drug can come to equilibrium with the antibody. Next, you have to physically separate the free drug from the bound drug. Assays where you have to do separations are called **heterogeneous assay systems**. This separation is an extra step that can introduce errors and would be nice to avoid if possible. Finally notice the sense of the results. If we look at the radioactivity of the antibody bound drug, more radioactivity means there was a low concentration of the compound we were looking at and vice-versa. Finally note that as outlined this assay looks simple, but to do it right takes careful set-up. You have to have the relative concentrations just right you have to have control experiments to measure non-specific binding, and you need to do some kind of calibration curve.

What is the tie to this and fluorescence? Well radioactive compounds are expensive, and very nasty to get rid of. If you replace the radioactive iodine with a fluorescence compound you get an assay that is almost as sensitive, but is much cheaper and safer to use.

In the above assay one difficulty was that at some point you had to separate the

antibody from the solution so you could see what was bound to it. There are two ways this can be done. Does the term Size exclusion or gel filtration Chromatography ring a bell (you should have had in Biochem) A second way this is achieved on a massive scale is to chemically link the antibody to the plastic walls of the dish you do the assay in. That way you can simply rinse the dish out to remove the unbound material! If you are going to be doing lots of samples they make plastic rack with 96 little holes in them in so you can do all the experiments and controls you want.

(Can you bring in a titer plate example?)

Even when you use fluorescence, you don't get enough sensitivity, so there are other techniques to amplify a weak signal for instance:

Enzyme-linked immunosorbent assays ELISA

In this assay we will have our antibody linked to the plastic of the dish, and we will again put in a solution containing our unknown that will bind to the antibody. Now, let's add a second antibody that binds to a different site on the antigen. Also on this antibody we will covalently attach a useful enzyme.

So what happens when you wash this plate off? the more antigen(Drug) you have, the more of the enzyme is bound to the walls of the plate. If the enzyme is our old friend glucose oxidase, it can reduce glucose to gluconic acid, producing hydrogen peroxide, you add a little peroxidase and a dye, and you can see a color change. The more color change, the more enzyme was bound to the well, the more antigen(Drug) there was to begin with. Thus you can quantitate the amount of the antigen without needing to use radioactive compounds!

This particular kind of ELISA is called a sandwich ELISA because our compound gets sandwiched between two antibodies

This is pushed even farther in an assay method called an EMIT. EMIT stands for enzyme-multiplied immunosorbent assay. You again have a solution with a known concentration of a labeled drug, but in this case the label is an enzyme like glucose oxidase. A second part of this label is that it is placed on the drug in such a way that when the drug is bound to its antibody the enzyme is inactivated.

Now you simply mix the labeled and unlabeled drugs together and let them compete for the antibody. If there is lots of the free drug, most of it binds to the antibody, so little of the drug-enzyme complex binds. If little of the drug-enzyme complex binds, it can be active and you can get it to make a colored solution. On the other hand, if you only have a little of the free drug, mostly drug-enzyme binds to the antibody, thus inactivating the enzyme and lowering the amount of color produced.

Also note that this can be made fluorescent, if the enzyme performs a reaction that converts a non-fluorescent reactant into a fluorescent product.

Notice that in this system you didn't have to separate the complex from the free drug. This is called a **homogeneous** assay method and is simpler to perform because you don't have any rinsing steps.

(NO picture hard to get right)

FLUORESCENT POLARIZATION IMMUNO ASSAY (FPIA)

This is another homogeneous technique (1-step mixing with no washing). IF is also another competition assay between labeled and unlabeled compound, only in this case our label is a small fluorescence molecule. To understand how this works we need to brush up on what fluorescence is.

One thing we didn't talk about when we discussed absorption and fluorescence was light polarization. Light can be oriented. Remember Physics 101 where you picture light as a beam of electronic vectors that oscillate? Well in ordinary light these oscillations occur in all directions, but in plane polarized light we restrict them to a single plane.

If you use plane polarized light in a fluorescence experiment, and the molecule doesn't move before it re-emits the light, then the emitted light will also be polarized. If the molecule moves around between absorbing the light and emitting it, then the emitted light will not be polarized.

BACK TO ASSAY

Let's assume the molecule we are quantitating is small, and the fluorescent probe we add to it is also small. Thus it tumbles around in solution quickly and reorients. If you shine polarized light at it, it reorients quickly and the fluorescent light is not polarized.

What happens if this molecule is bound to an antibody. Antibodies are large, and move relatively slowly. Thus if you shine polarized light at the molecule when it is bound to the antibody, it doesn't move much before it re-emits the light, and the emitted light is polarized. This allows you to look at the emitted light and tell if the compound is bound to the antibody by monitoring fluorescence polarization. Low polarization low binding, high polarization-high binding.

Now let's put this compound in competition with an unknown amount of unmodified compound we wish to assay. If we have lots of material in our sample, most of that will bind to the antibody, and little of our labeled material will bind, so you get low fluorescence. On the other hand if our sample contains little free compound, most of our modified compound will bind to the antibody and polarization will be high. Thus we have yet another way to quantitate drug compounds!

(Can I get a picture?)

Having gone over these standard assay techniques, let's now go over an everyday pharmacy example. IN the pharmacy you sell, over the counter, many different immuno assays. they aren't radioactive or fluorescence, but they are very useful. They are home pregnancy tests.

Let's examine one of these test to see how the immunochemistry is used.

The Fact + test is one of the easier ones to understand, it is kind of neat because the manufactures have gone through lots of thinking and design to get a very simple appearing test. You put some urine on a pad, and in a few minutes you get a + if you're pregnant and a - if you're not. With no electronic or fancy devices to be plugged in.

How does this work. First you need to know some physiology. Human chorionic gonadotropin (HCG) is a hormone that is present in the bloodstream and therefore in the urine only during pregnancy. While we call it a hormone it is a relatively large glycoprotein it has two subunits, α and β . α has 92 residues and β has 147 residues, polysaccharides make up an additional 30% more molecular mass. For many year doctors determined if a woman was pregnant by using rabbit antibodies to HCG to see if this hormone was present in the blood. The over-the-counter methods do the same thing, only they are packaged and simple to use.

The fact plus test uses different antibodies to HCG that bond to the molecule at two different places. It uses a soluble antibody that is bound to a dye, and a second antibody that is chemically linked to part of the +/- sign of the test strip. This later is not soluble, does not have a visible dye attached to it, and bind to a different site on HCG than the first antibody.

The bound antibody is on the | part of the + sign. Along the - part of the sign the test strip contain HCG bound chemically bound to the test strip. (See Fig 1)

So what happens when you add urine?

If there is no HCG in the urine, then the water from the urine simply solubilized the antibody-dye, and as it flows across the +/- sign, it binds to the hormone immobilized along the - part of the +/- sign, and leave color along the -.

(See Fig 2)

If you are pregnant, then you have HCG in your urine, and this will bind to some of soluble HCG antibody that is colored. These molecules will bind to the | part of the +/- sign and give you the positive results.

(See Fig 3)