Spectroscopic Techniques

Chapters 3 & 4 in "*Tietz* Fundamentals of Clinical Chemistry" or "*Tietz* Textbook of Clinical Chemistry".

Spectroscopic Techniques Employed in the Clinical Laboratory

- Ultraviolet/Visible (UV/Vis) Absorption
- Fluorescence and Phosphorescence
- Fluorescence Polarization
- Flame Emission and Atomic Absorption
- Turbidometry and Nephelometry

Nature of Light

Light can be described as a wave characterized by a wavelength (λ) and frequency (ν); where $\lambda\nu = c$, $c \approx 3 \times 10^8$ m/s (speed of light)

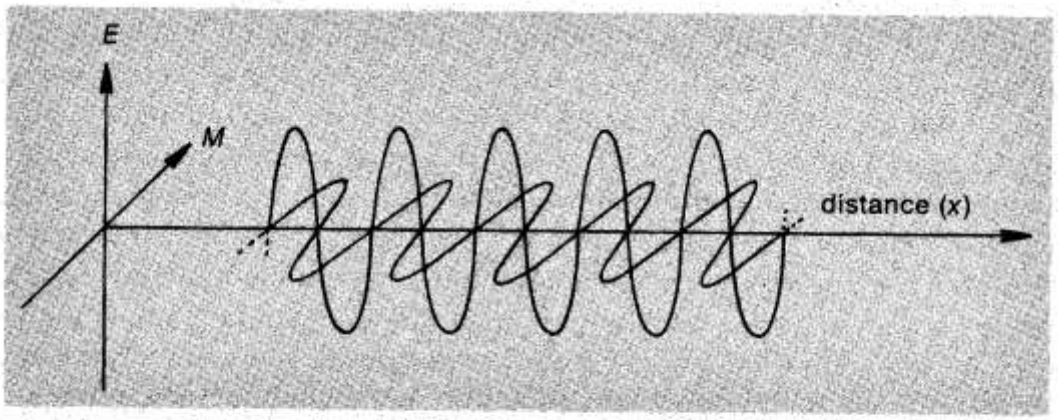


Figure 18–3. Portrayal of an electromagnetic wave, showing the electric (E) and magnetic (M) vectors; the wave is represented at some instant in time and is traveling through space in direction x.

Nature of Light

Photon energy is quantized and proportional to the frequency: $E = \hbar\nu$ (or $E = \hbar c/\lambda$), $\hbar$ is Planck's constant (divided by 2π).

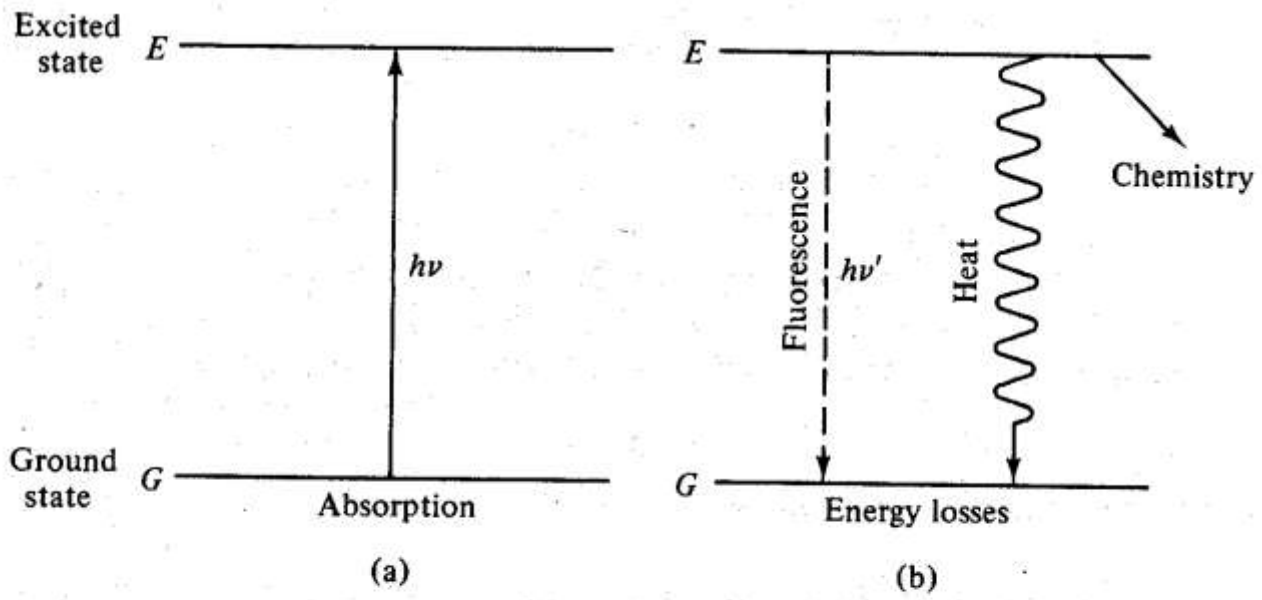


Fig. 10.3 Energy-level diagram: (a) the absorption of a photon of energy $h\nu$, and (b) three important processes by which the excitation energy is subsequently released or converted.

Electromagnetic Spectrum

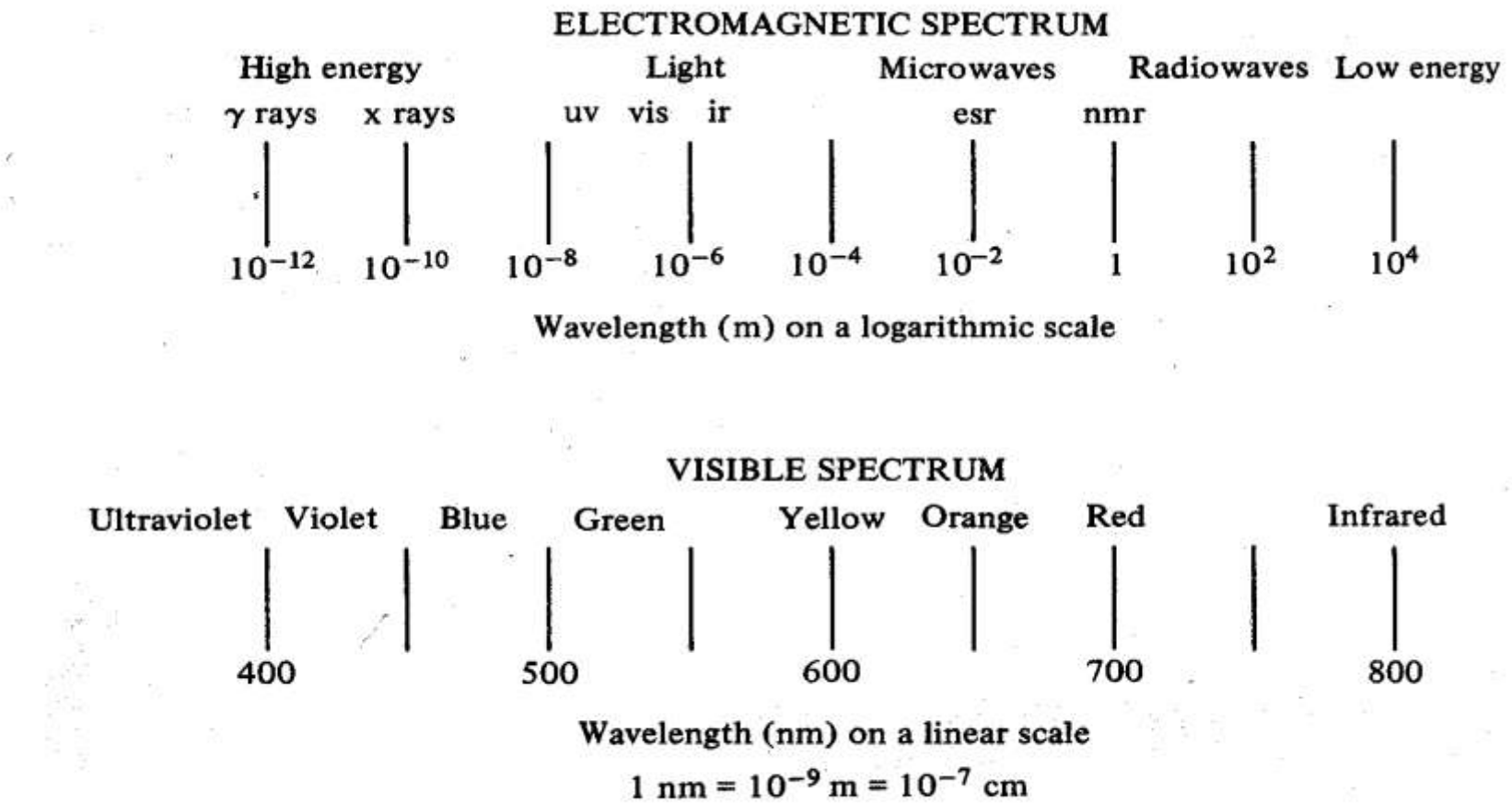


Fig. 10.1 Wavelength regions of the electromagnetic spectrum.

Molecular Energy Levels

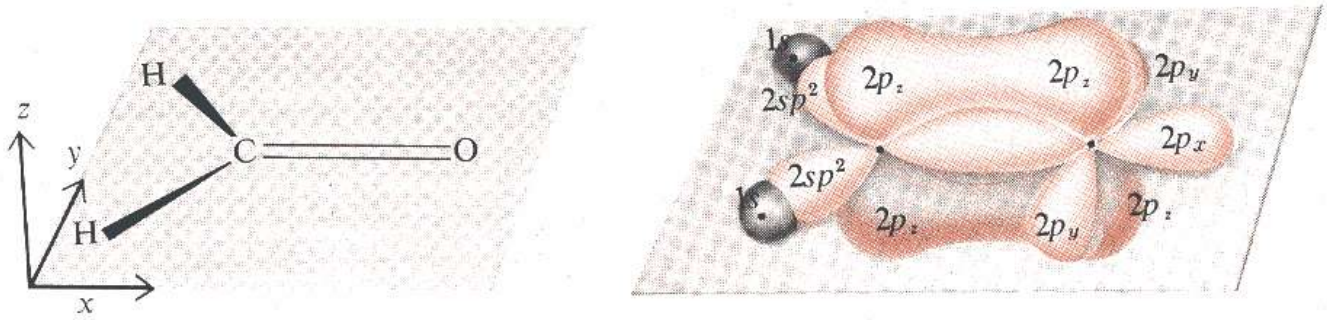
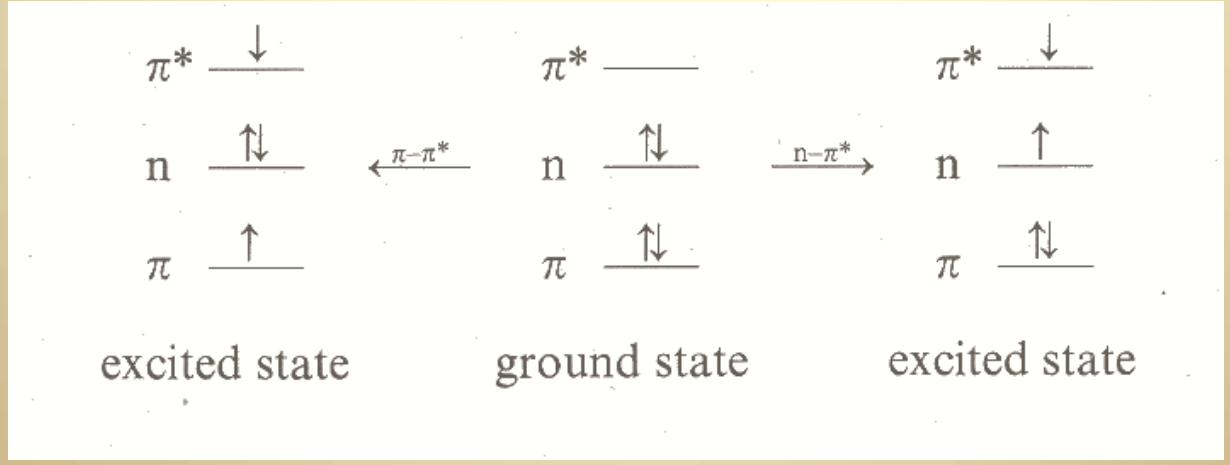


Figure 7-6

The formaldehyde molecule and a schematic diagram of its bonding.



Molecular Energy Levels

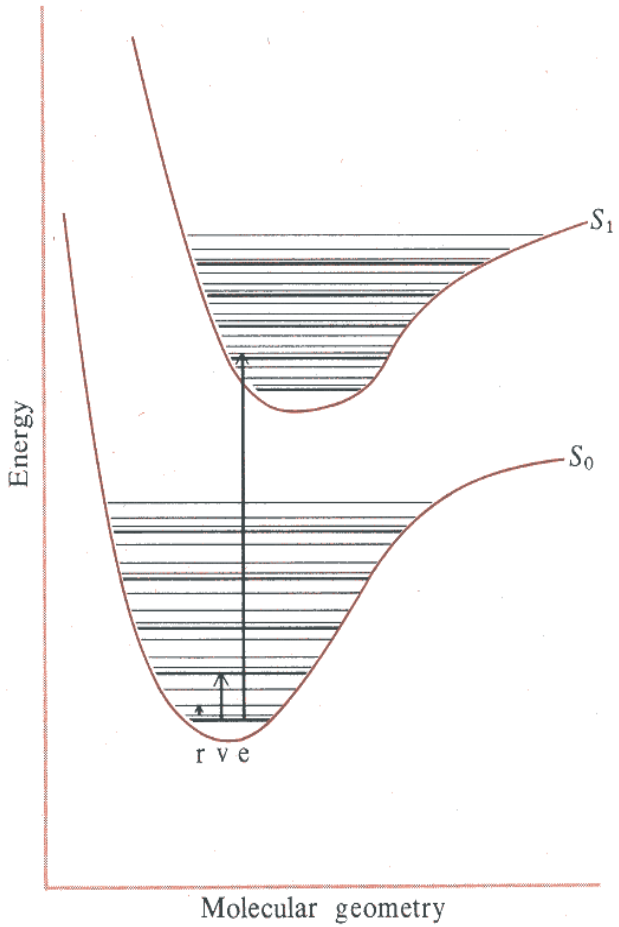


Figure 7-1

Energy levels of a small molecule. Selected rotational sublevels of the vibrational levels of each of two electronic states are shown. Transitions corresponding to electronic (e), vibrational (v), and rotational (r) spectra are indicated.

Electromagnetic Spectrum

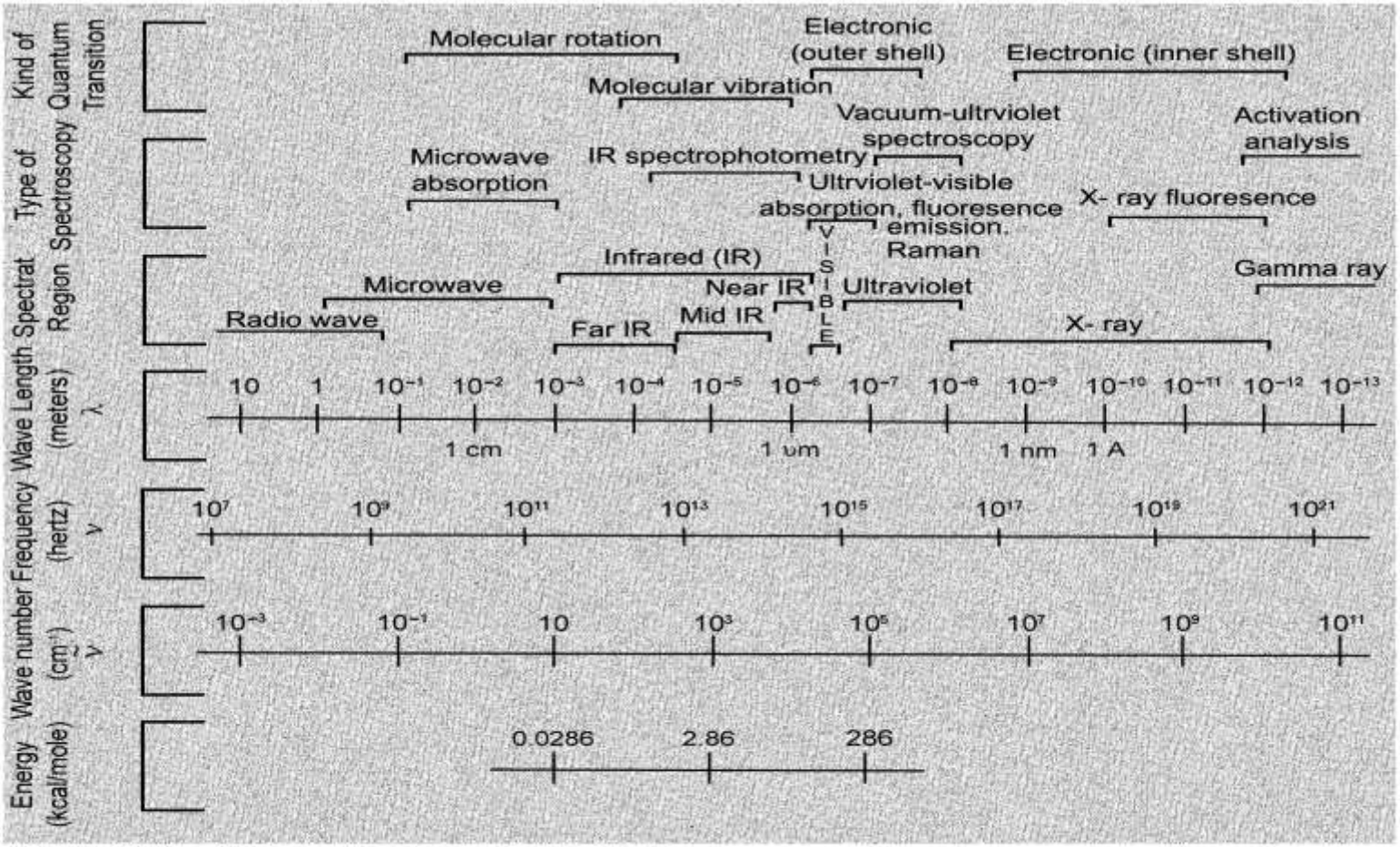
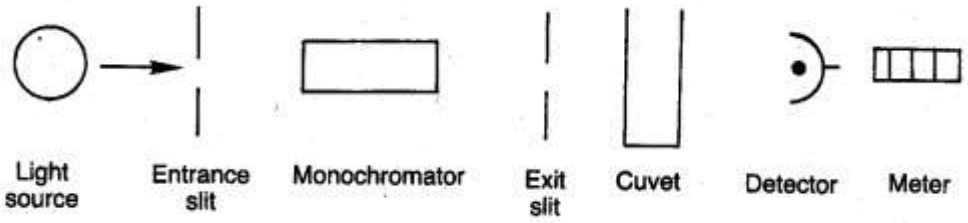


Figure 18-2. **The electromagnetic spectrum.**

* 1 Hz (hertz) = 1 cycle per second.

Generic Spectrophotometer

FIGURE 3-4. Major components of a single-beam spectrophotometer.



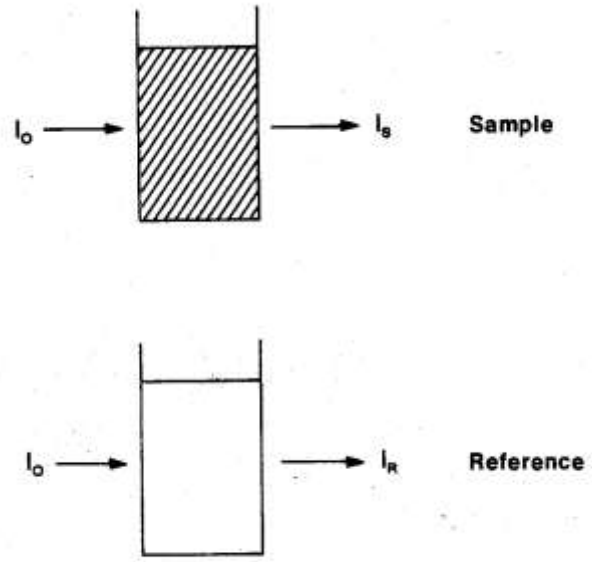


FIGURE 3-2. Transmittance of light through sample and reference cells. Transmittance of sample versus reference = I_S/I_R.

1) Light is generated with the desired intensity, frequency and polarity.
2) The light is directed onto an experimental sample, which affects it in some way.
3) Light emanating from the sample is measured (angle of detection may vary).

Ultraviolet-Visible Absorption Spectroscopy

- aka "UV-Vis spectroscopy"
- based on absorption of light with frequencies in the ultraviolet-to-visible range ($\lambda \sim 150 - 700$ nm)
- absorption generally corresponding to electronic transitions
- absorption spectrum dependent on chemical structure

Generic Absorption Spectrophotometer

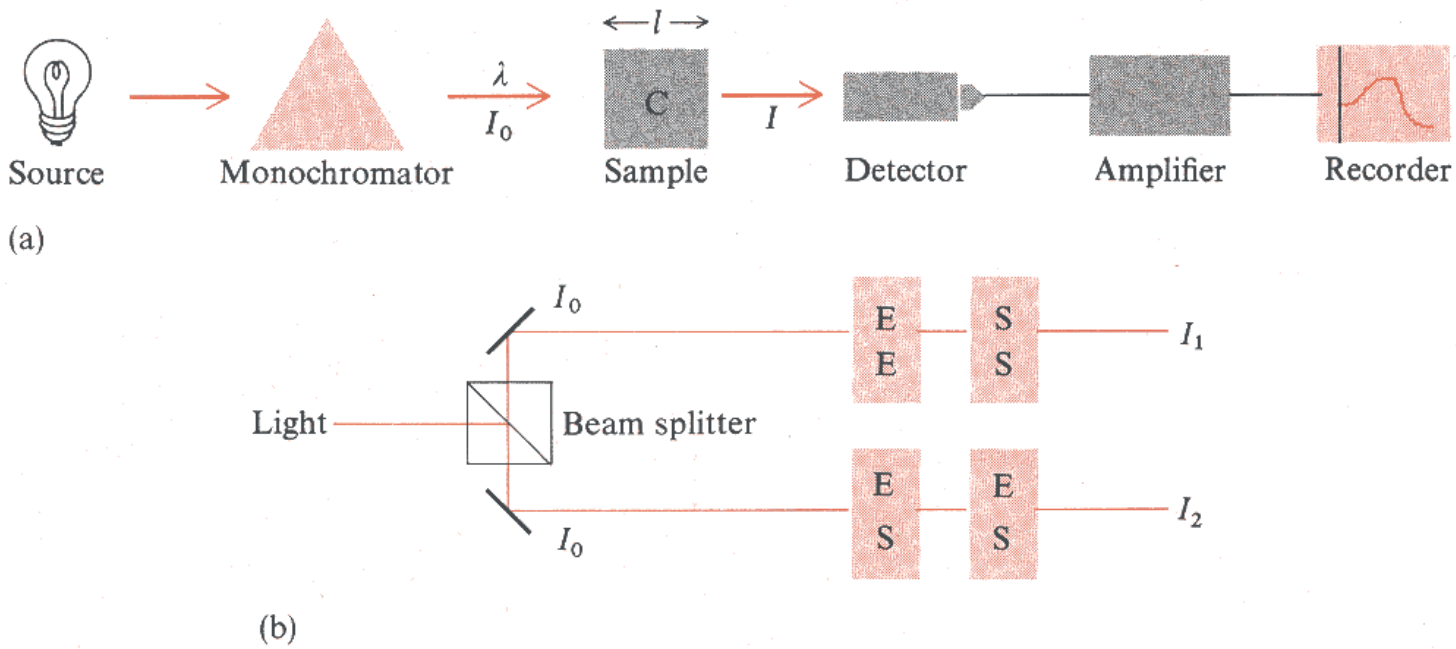
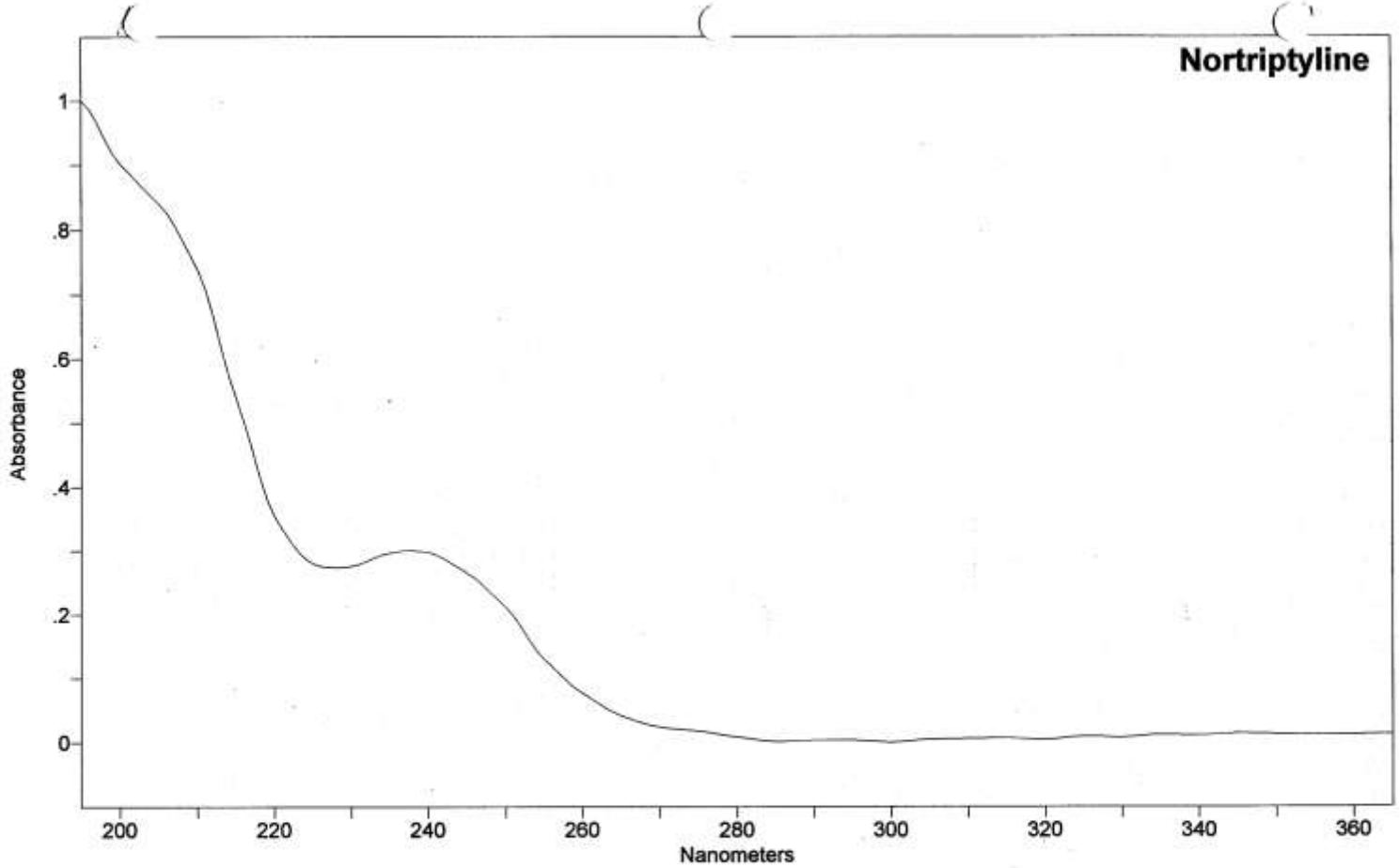


Figure 7-3

Schematic diagrams of spectroscopic experiments. **(a)** For measuring light absorption. **(b)** For difference spectrometry.



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YALE#9 **Nortriptyline** RRT = 0.54

UV/Vis Electronic Transitions

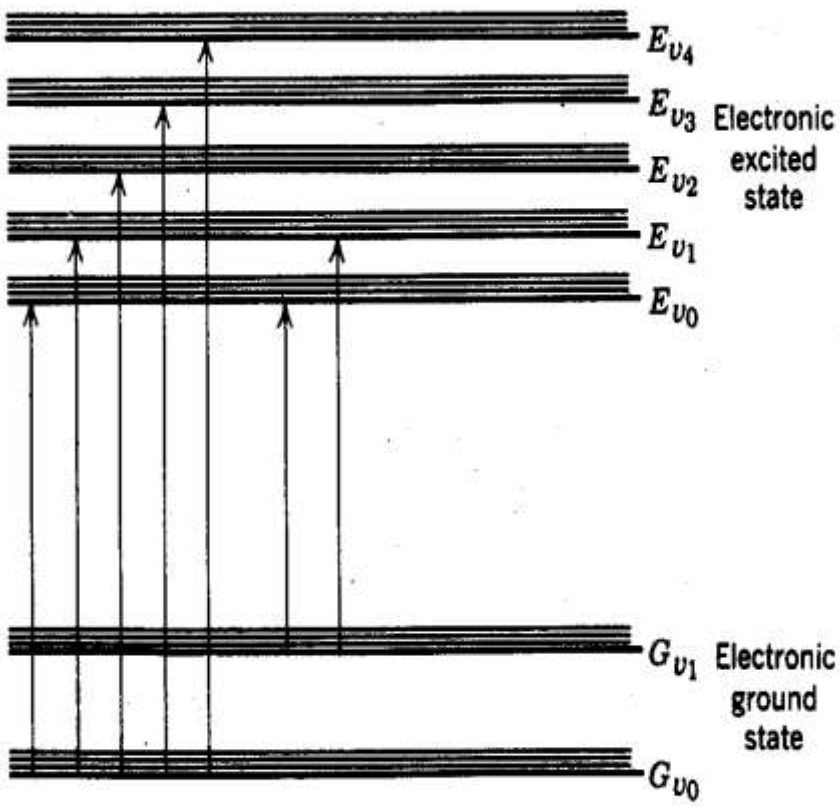


FIGURE 7.4. Energy level diagram of a diatomic molecule.

Each vertical arrow represents a single excitation to a higher energy site due to absorption of a photon with $E=h\nu$.

A distribution of transitions from different vibrational and rotational energy states results in a large number of transitions with slightly different energies.

The consequence of a large number of vibrational and rotational energy states is a "blurring" of the individual lines into a broader and more featureless absorption spectrum, as seen from the top to the bottom on the right.

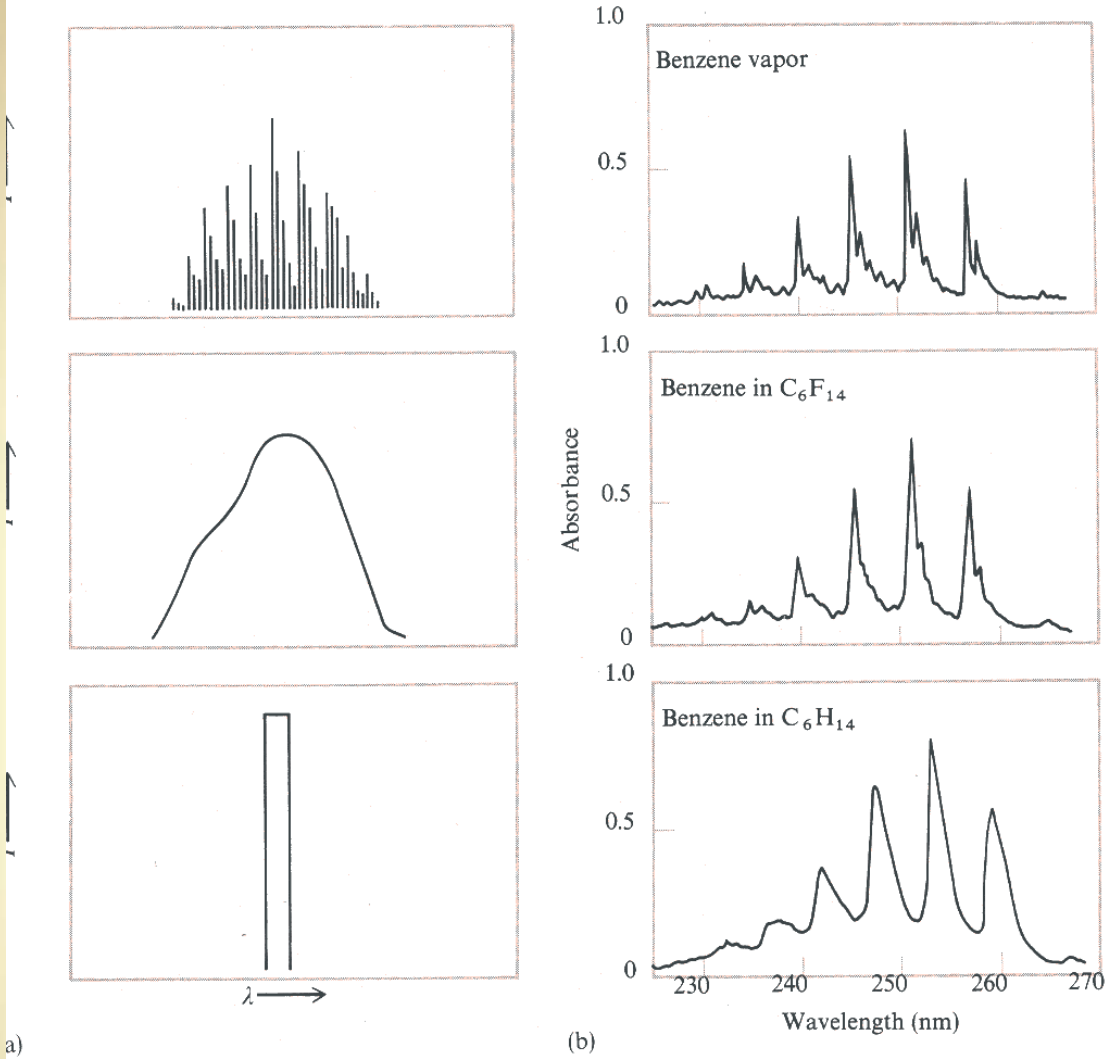


Figure 7-2

Electronic absorption spectra of small molecules. **(a)** Spectra of a typical small molecule. Shown, from top to bottom, are the gas-phase spectrum, the solution spectrum, and the theoretical spectrum usually dealt with in calculations. **(b)** Absorption spectra of benzene, showing solvent-induced broadening. [After J. B. Birks, *Photophysics of Aromatic Molecules* (New York: Wiley, 1970), p. 117.]

Different Chemicals Absorb at Specific, Reproducible Wavelengths

TABLE 7.1

Summary of Electronic Transitions

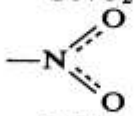
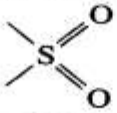
Example	Electronic Transition	λ_{max} (nm)	ϵ_{max}	Band[a]
Ethane	$\sigma \rightarrow \sigma^*$	135		
Water	$n \rightarrow \sigma^*$	167	7,000	
Methanol	$n \rightarrow \sigma^*$	183	500	
1-Hexanethiol	$n \rightarrow \sigma^*$	224	126	
n-Butyl iodide	$n \rightarrow \sigma^*$	257	486	
Ethylene	$\pi \rightarrow \pi^*$	165	10,000	
Acetylene	$\pi \rightarrow \pi^*$	173	6,000	
Acetone	$\pi \rightarrow \pi^*$	~150		
	$n \rightarrow \sigma^*$	188	1,860	
	$n \rightarrow \pi^*$	279	15	*R*
1,3-Butadiene	$\pi \rightarrow \pi^*$	217	21,000	*K*
1,3,5-Hexatriene	$\pi \rightarrow \pi^*$	258	35,000	*K*
Acrolein	$\pi \rightarrow \pi^*$	210	11,500	*K*
	$n \rightarrow \pi^*$	315	14	*R*
Benzene	Aromatic $\pi \rightarrow \pi^*$	~180	60,000	E_1
	Aromatic $\pi \rightarrow \pi^*$	~200	8,000	E_2
	Aromatic $\pi \rightarrow \pi^*$	255	215	*B*
Styrene	Aromatic $\pi \rightarrow \pi^*$	244	12,000	*K*
	Aromatic $\pi \rightarrow \pi^*$	282	450	*B*
Toluene	Aromatic $\pi \rightarrow \pi^*$	208	2,460	E_2
	Aromatic $\pi \rightarrow \pi^*$	262	174	*B*
Acetophenone	Aromatic $\pi \rightarrow \pi^*$	240	13,000	*K*
	Aromatic $\pi \rightarrow \pi^*$	278	1,110	*B*
	$n \rightarrow \pi^*$	319	50	*R*
Phenol	Aromatic $\pi \rightarrow \pi^*$	210	6,200	E_2
	Aromatic $\pi \rightarrow \pi^*$	270	1,450	*B*

[a]The *R* band, German *radikalartig; K* band, German, *konjugierte*; *B* band, benzenoid; *E* band, ethylenic; see: A. Burawoy, *Berichte*, **63**, 3155 (1930); *J. Chem. Soc.*, 1177 (1939); also see the chapter by E. A. Braude listed in the reference section.

Different Functional Groups Absorb at Specific, Reproducible Wavelengths

TABLE 7.3

Absorption Data for Isolated Chromophores

Chromophoric Group	System	Example	λ_{max} (nm)	ϵ_{max}	Transition	Solvent
Ethylenic	RCH=CHR	Ethylene	165	15,000	$\pi \rightarrow \pi^*$	Vapor
			193	10,000	$\pi \rightarrow \pi^*$	
Acetylenic	R—C≡C—R	Acetylene	173	6,000	$\pi \rightarrow \pi^*$	Vapor
Carbonyl	$RR_1C{=}O$	Acetone	188	900	$\pi \rightarrow \pi^*$	*n*-Hexane
			279	15	$n \rightarrow \pi^*$	
Carbonyl	RHC=O	Acetaldehyde	290	16	$n \rightarrow \pi^*$	Heptane
Carboxyl	RCOOH	Acetic acid	204	60	$n \rightarrow \pi^*$	Water
Amido	$RCONH_2$	Acetamide	<208		$n \rightarrow \pi^*$	
Azomethine	>C=N—	Acetoxime	190	5,000	$\pi \rightarrow \pi^*$	Water
Nitrile	—C≡N	Acetonitrile	<160		$\pi \rightarrow \pi^*$	
Azo	—N=N—	Azomethane	347	4.5	$n \rightarrow \pi^*$	Dioxane
Nitroso	—N=O	Nitrosobutane	300	100		Ether
			665	20		
Nitrate	$—ONO_2$	Ethyl nitrate	270	12	$n \rightarrow \pi^*$	Dioxane
Nitro		Nitromethane	271	18.6	$n \rightarrow \pi^*$	Alcohol
Nitrite	—ONO	Amyl nitrite	218.5	1,120	$\pi \rightarrow \pi^*$	Petroleum ether
			346.5[a]		$n \rightarrow \pi^*$	
Sulfoxide	S=O	Cyclohexyl methyl sulfoxide	210	1,500		Alcohol
Sulfone		Dimethyl sulfone	<180			

[a]Most intense peak of fine structure group.

Source: A. E. Gillam and E. S. Stern, *An Introduction to Electronic Absorption Spectroscopy in Organic Chemistry*, 2nd ed., London: Edward Arnold, 1957.

Examples of Absorption Spectra For a Group of Related Molecules

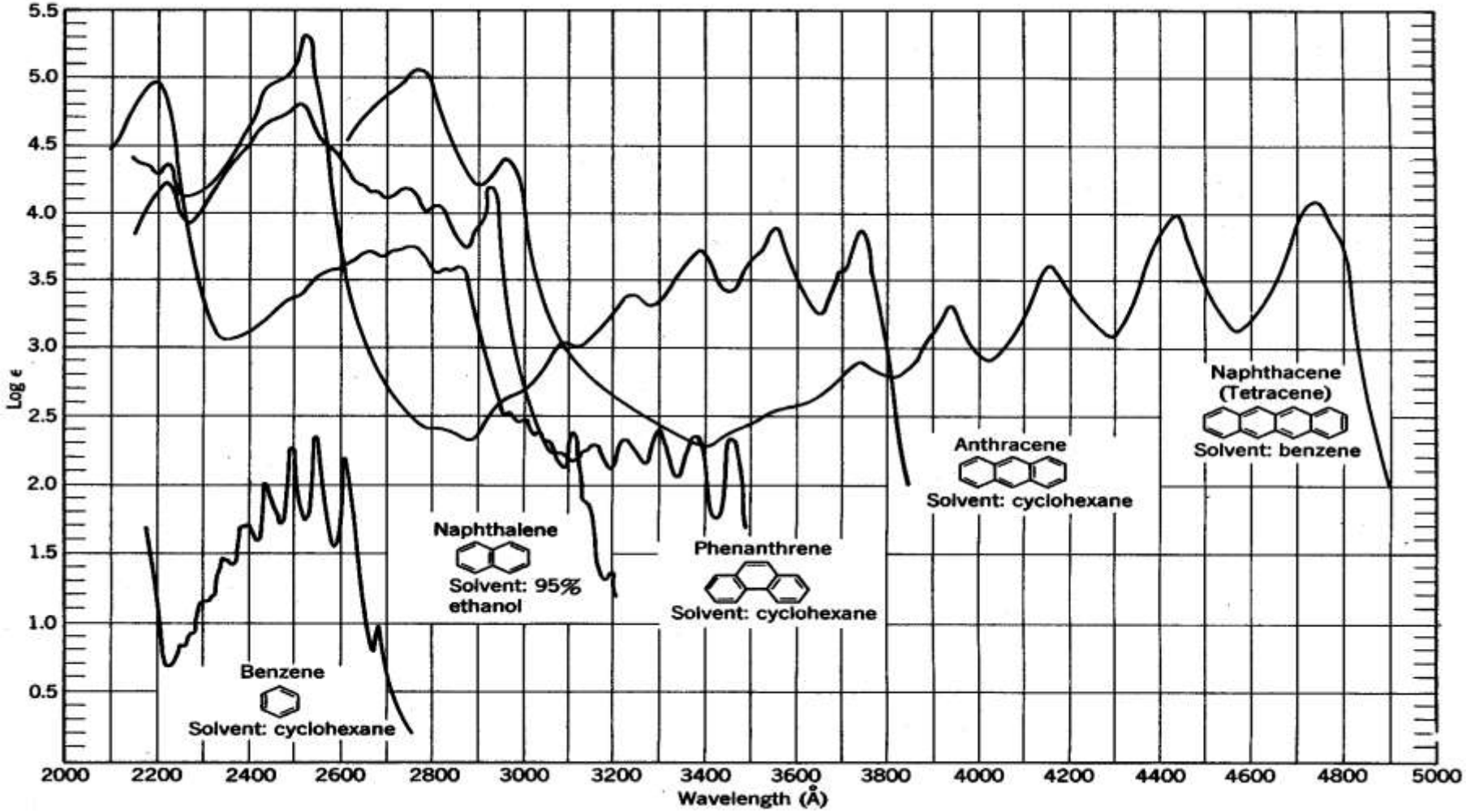


FIGURE 7.7. Electronic absorption spectra of benzene, naphthalene, phenanthrene, anthracene, and naphthacene.

Beer's Law ($A = \varepsilon lc$)

- Absorption of light is directly proportional to concentration of the absorbing molecule.

- Absorbance (A) = $-\log(I/I_o)$, where I and I_o are the intensity of the transmitted and incident light, respectively.

- **$A = \varepsilon lc$**, where **ε** is *absorptivity* (extinction coefficient), **l** is the path-length and **c** is the concentration of the absorbing molecule.

- Absorbance is linear with concentration from approximately 0.1 – 1.1 (safest) or 0.01 – 2.0 (under ideal conditions).

Linearity of Beer's Law

Instrumental/Technical (common)

1. At a very low absorbance, the intensity of the transmitted light is very similar to the incident light (i.e. $I \sim I_0$). For example, if $A = 0.01$ then $I/I_0 \sim 0.98$.
   - At these low absorbances, contributions due to stray light, scattering due to dust, contamination of other absorbing compounds, etc. are significant and the value is less accurate.
2. At a very high absorbance, the absolute intensity of the transmitted light is very small. For example, if $A = 2$, then $I/I_0 = 0.01$.
   - At these very high concentrations, accuracy is dependent on the dynamic range (or the noise threshold) of the instrument, i.e. when it is calibrated to properly measure "I_0", then can it still distinguish a very weak "I" from instrumental noise or stray light?

Chemical (rare)

- Photochemistry: light absorption may change the molecule in some way.
- Deviations in absorptivity coefficients at high concentrations (>0.01M) due to electrostatic interactions between molecules in close proximity.
- Fluorescence of phosphorescence of the molecule.
- Change in refractive index of the solution due to increased concentrations of the molecule.

Advantages/Disadvantages of UV/Vis Spectroscopy

Advantages

- simple and inexpensive instrumentation (portable)
- most organic molecules absorb UV/Vis light
- quantitative (Beer's law)

Disadvantages

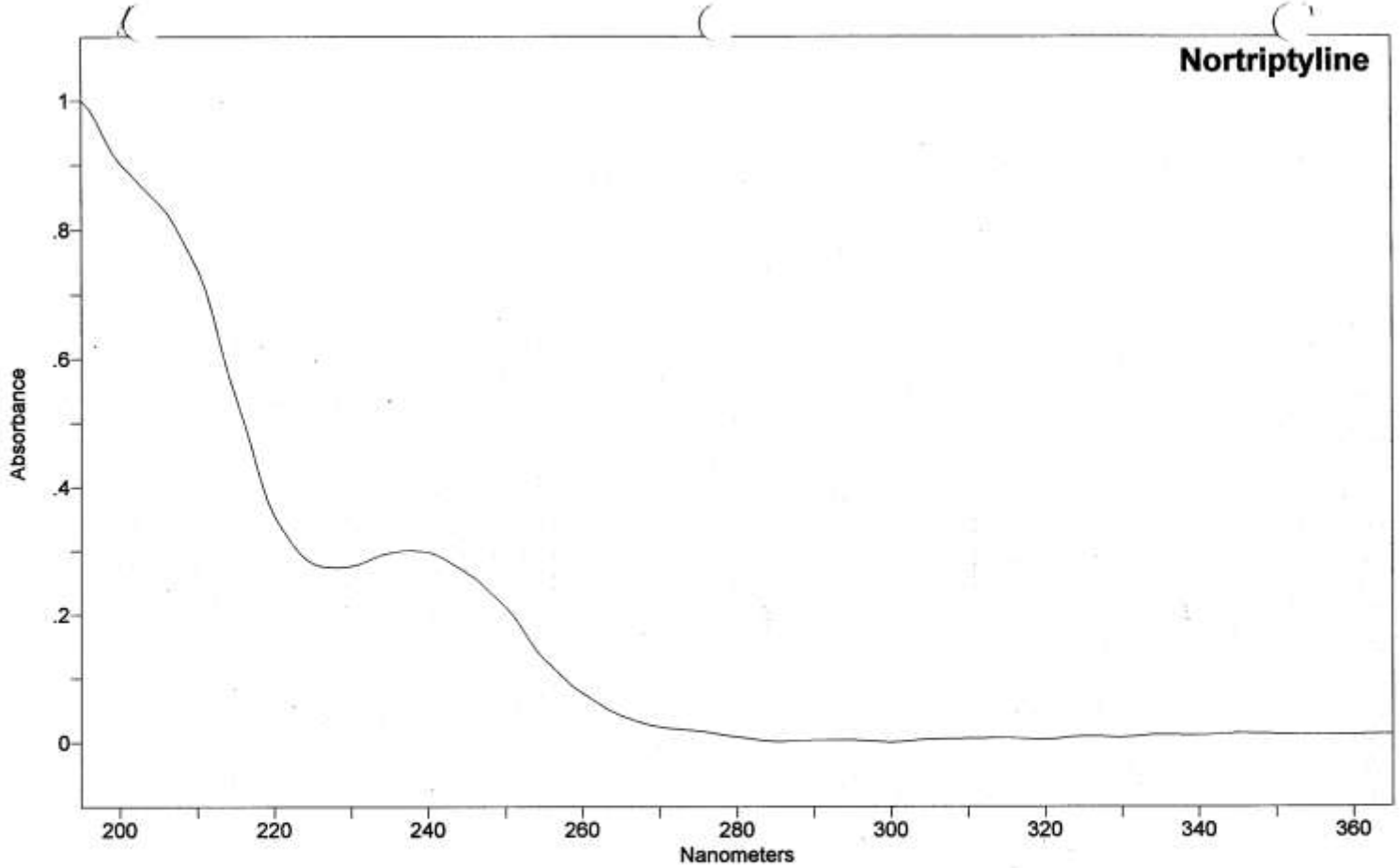
- mixtures of molecules can be a problem due to overlap (hence, routinely requires significant sample preparation)
- spectra are not highly specific for particular molecules
- absorption can be dependent on solution conditions; hence, often optimal to combine with a HPLC in order to standardize solution conditions

Uses of UV/Vis Spectroscopy in the Clinical Laboratory

1) A Majority of Routine Automated Analyzers.
2) Detector for HPLC instruments (e.g. TCA by HPLC).
3) Hemoglobin oximetry (OxyHgb, DeoxyHgb, MetHgb, COHgb, CNHgb, etc.).
4) Many manual methods such as the total barbiturate assay, cholinesterase, delta OD_{450}, etc.

TCA by HPLC with UV/Vis Detection (diode array).

- After an organic extraction of serum (and back-extraction into aqueous phase), drugs are separated using HPLC and detected using a UV/Vis "diode array" detector.
- A diode array can simultaneous detect a wide range of wavelengths, allowing essentially real-time acquisition of UV/Vis absorption spectra of individual HPLC "peaks" (as opposed to continuous monitoring of absorption at a single wavelength).
- Based on characteristic "relative retention times" and UV/Vis absorption spectra, a wide range of drugs can be identified.
- Only routine and official use at YNHH is for TCA quantitation, but the method can also be borrowed as consult-based screen for other drugs. We do this frequently for the CAP toxicology surveys.



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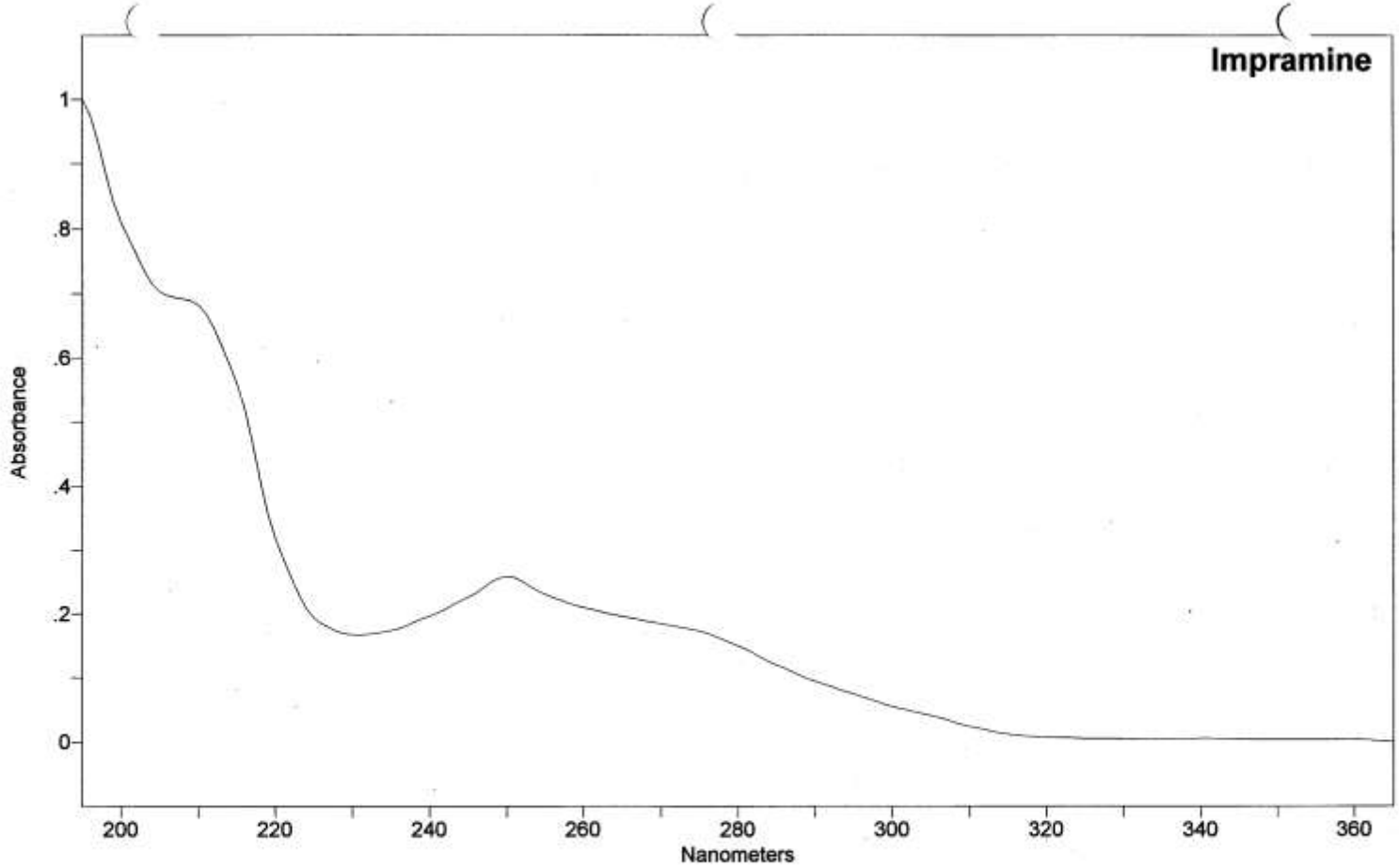

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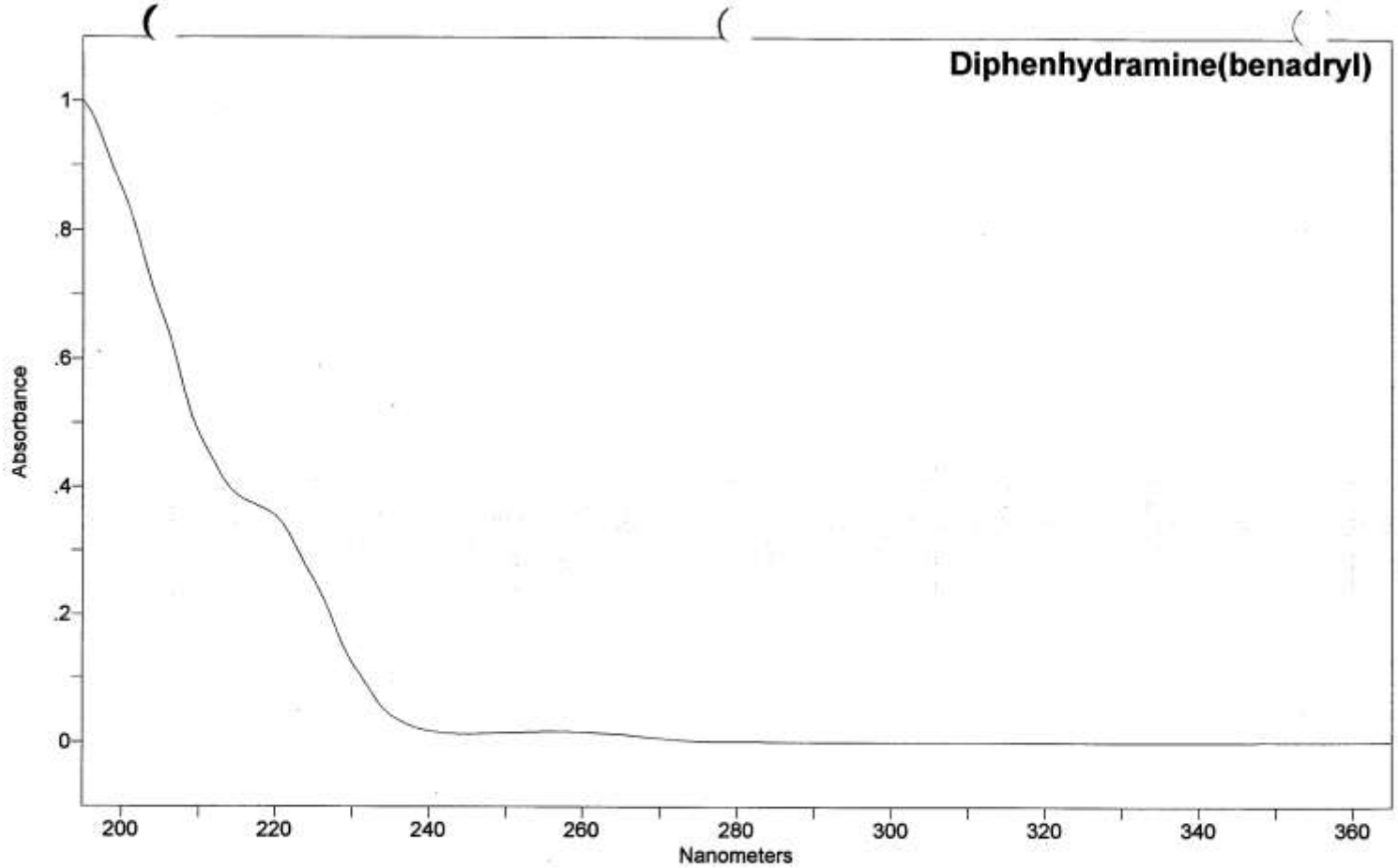
YALE#10 **Impramine** RRT = 0.67



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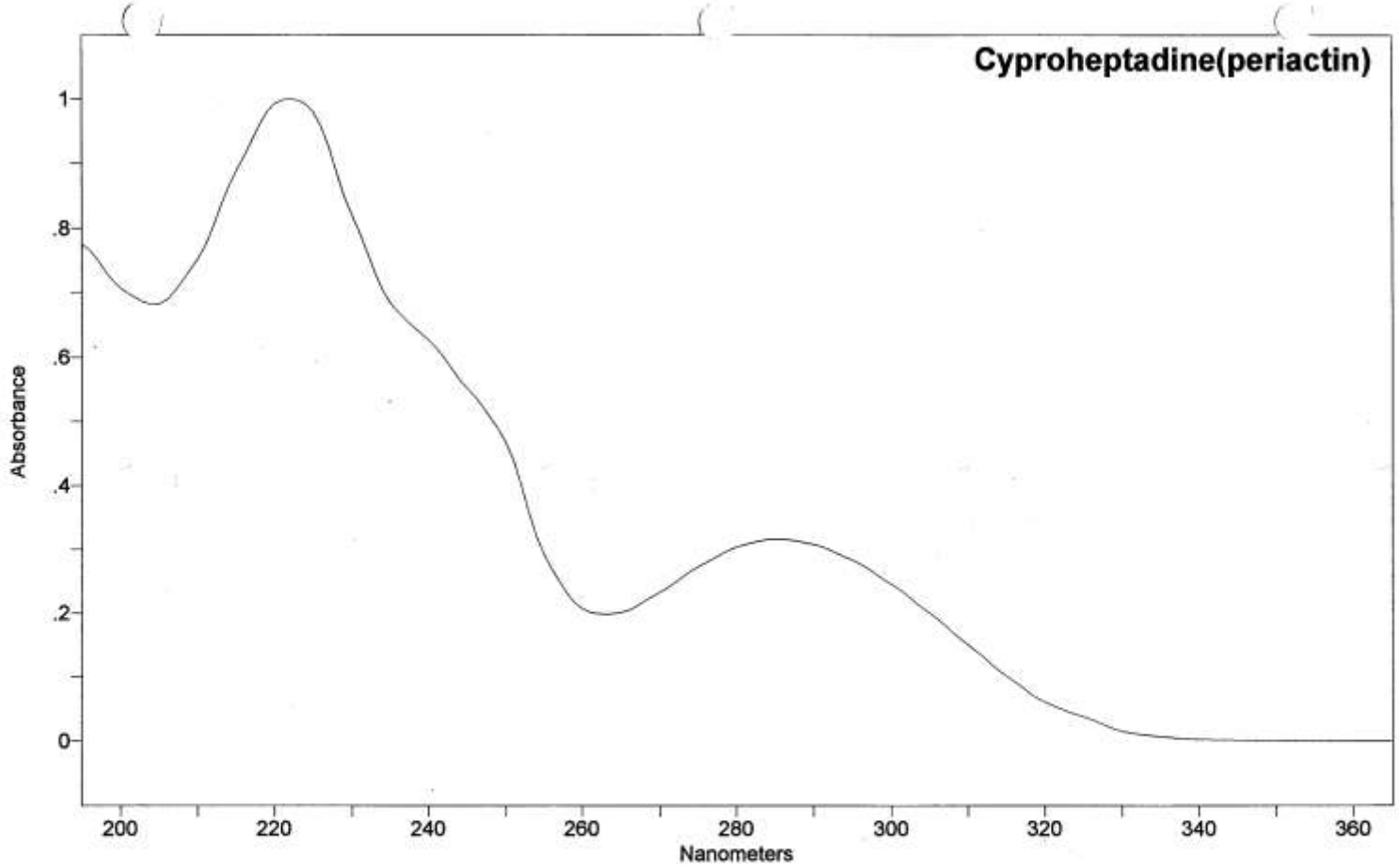
YALE#31 Diphenhydramine(benadryl)

RRT-0.35



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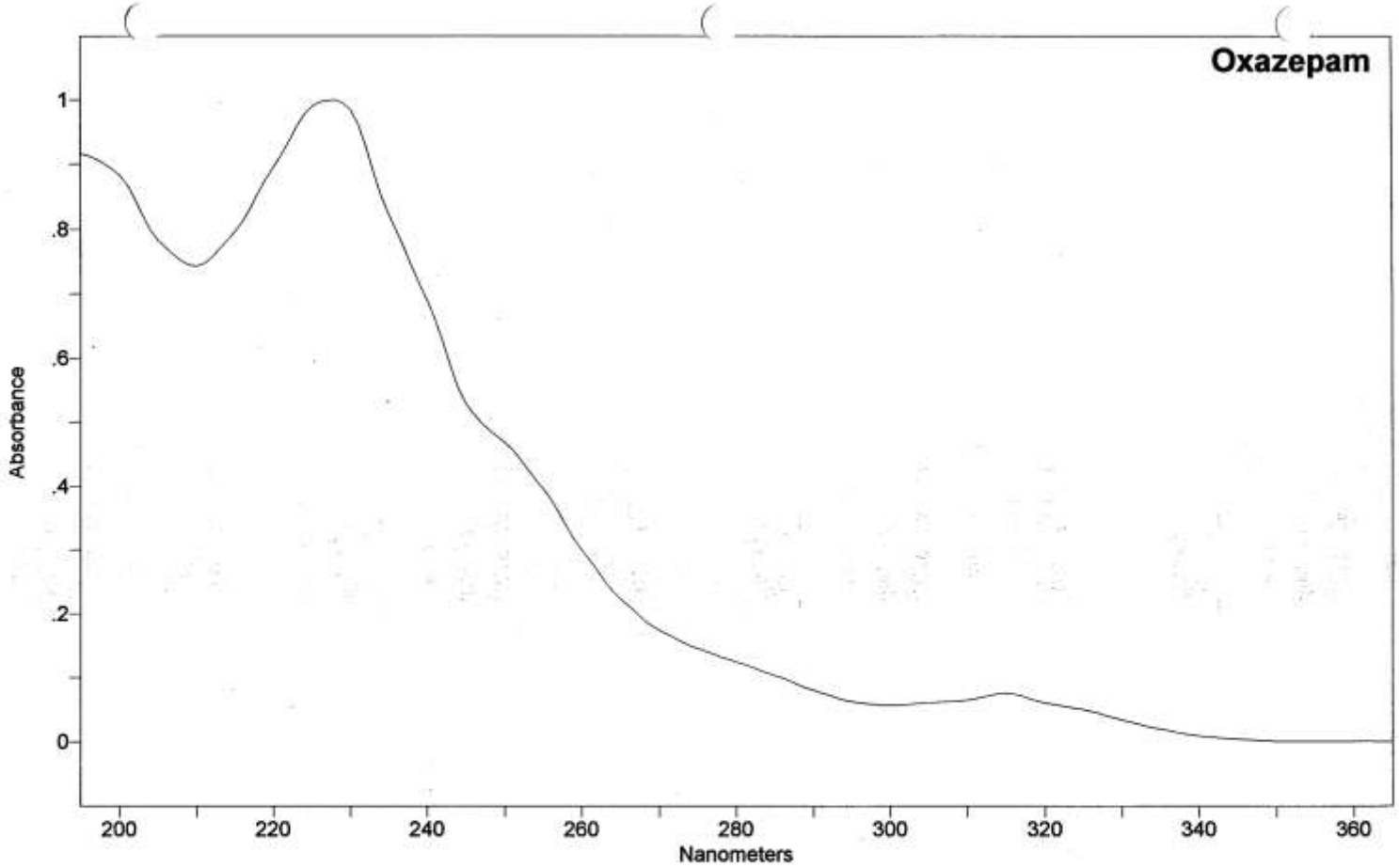
YALE#29 Cyproheptadine(periactin)

Rht=0.70



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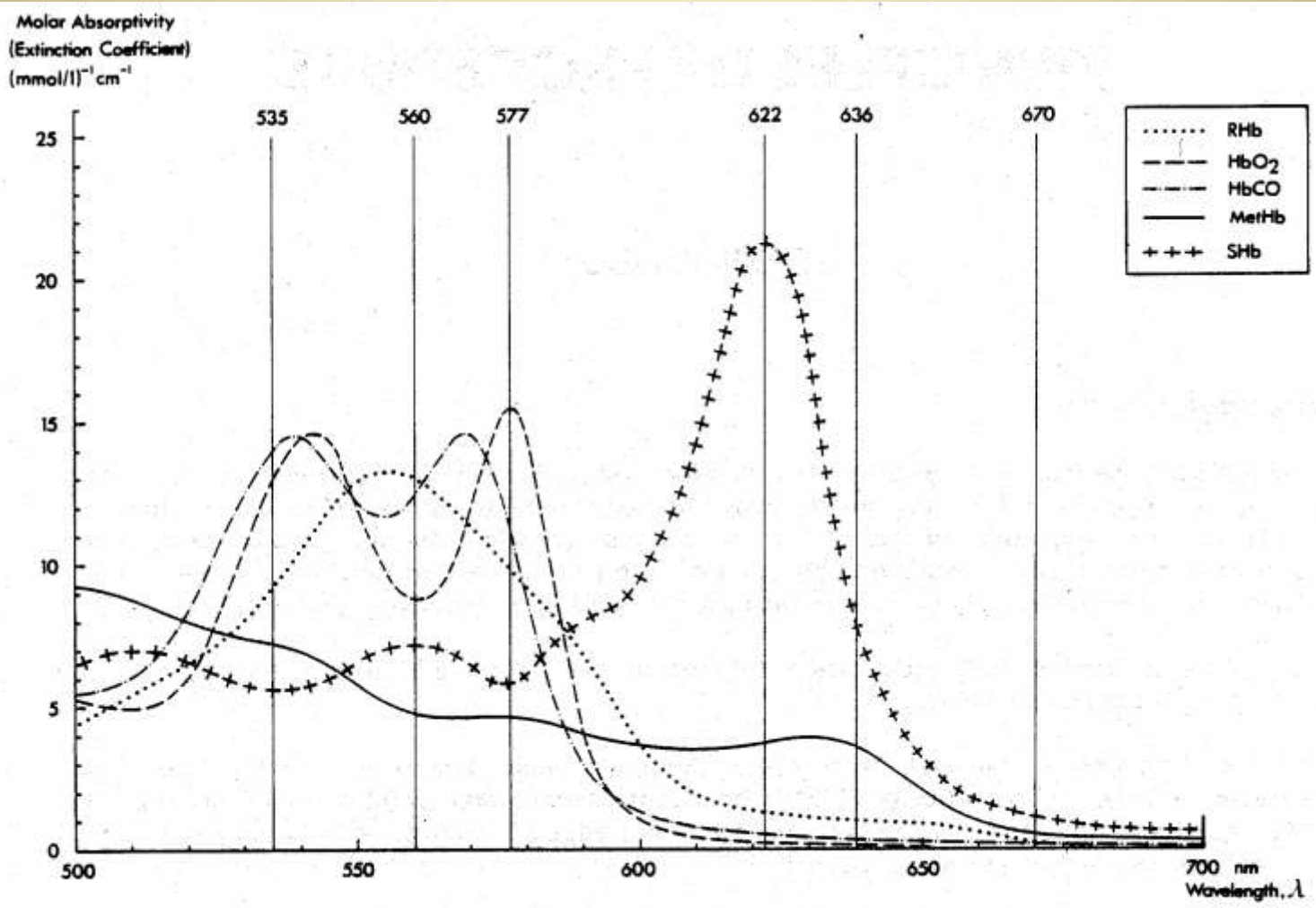
YALE#35 **Oxazepam**

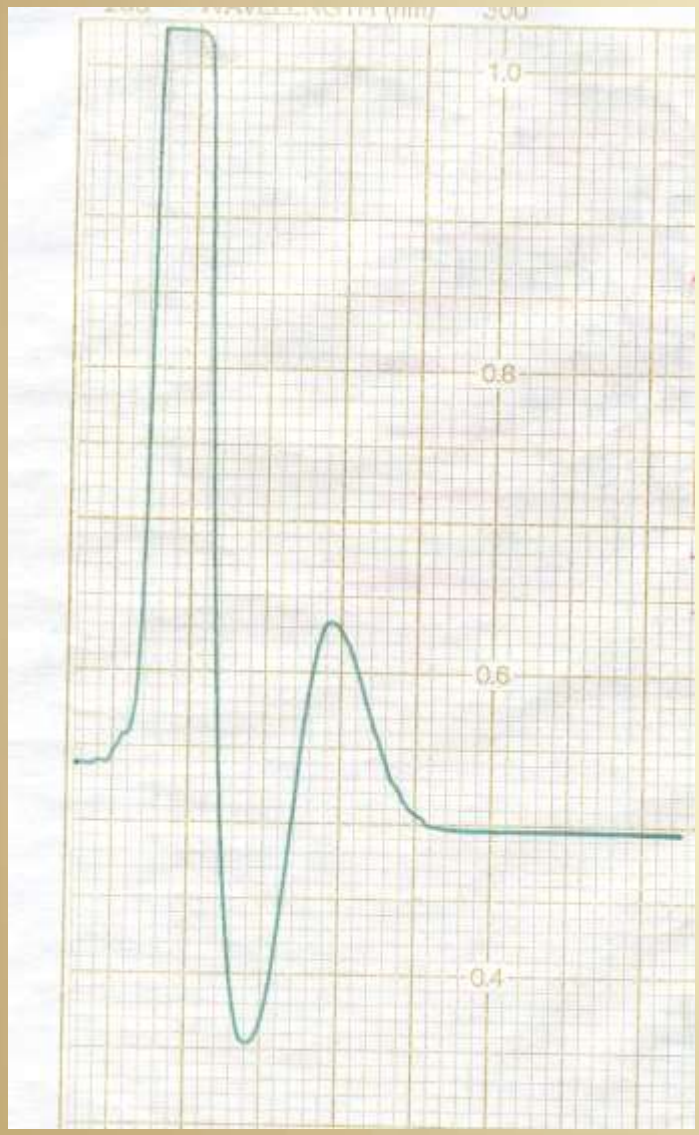
RRT= 0.40

Hemoglobin Oximetry



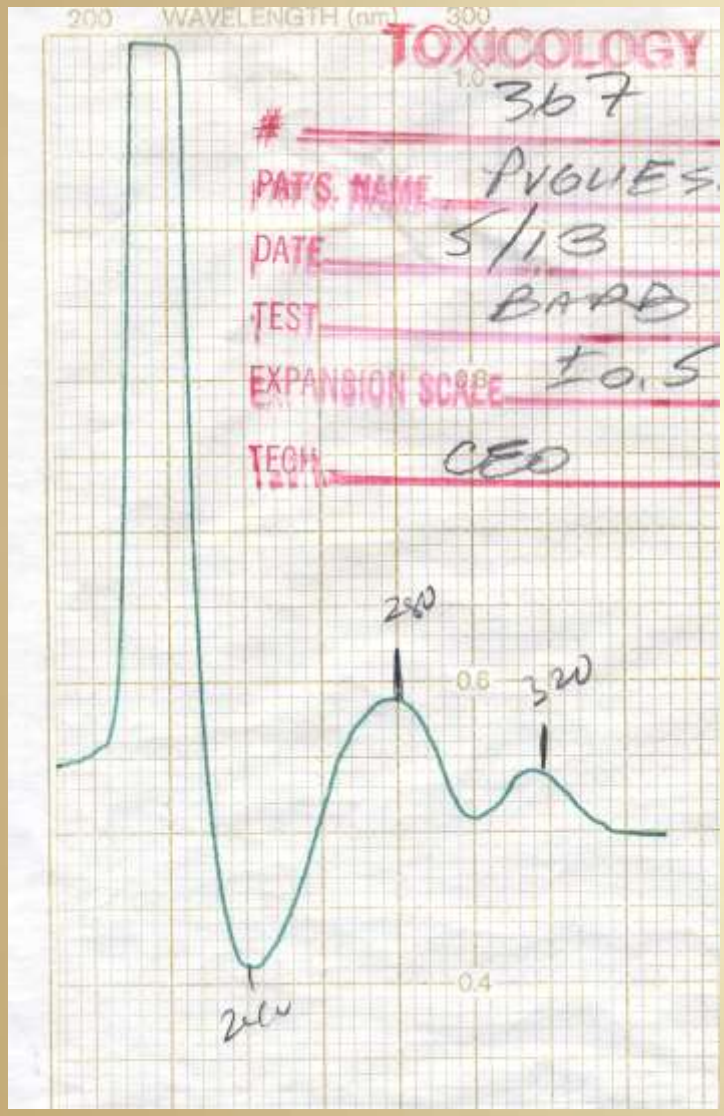
Characteristic spectra of hemoglobin derivatives (Ref. Zijlstra, W.G., private communication).

Total Barbiturate Assay



- All barbiturates (mild acids with pKa's ~ 7.5 – 8.0) are extracted from serum (barbs are neutral in charge at pH < 7) into an organic phase and then back-extracted into a weak base (they are positively charged at pH > 8.5).
- UV/Vis absorption spectra are collected at two different pH values and the difference is plotted.
- The absorption peak for barbiturates shifts from 240 nm to 260 nm with the change in pH, but the co-extracting contaminants do not, and will be "blanked" by the difference.
- Absorbance at 240 nm and 260 nm (the 240 nm band appears negative) is added and then multiplied by a factor of 65 to get the final total concentration of barbiturates in ng/ml.

Total Barbiturate Assay



- Because the extraction is not 100% specific for barbiturates (but pretty good because of the unusual pKa's of barbiturates), sometimes other drugs contaminate the final solution.
- If these contaminating drugs also absorb near 240 or 260 nm ***in a pH-dependent fashion***, then they will interfere with the assay.
- Thiopental (shown on the left) is a common problem. Thiopental is rapidly metabolized to pentobarbital; it is usually sufficient to request a subsequent sample, in which all the thiopental will be gone and pentobarbital can be measured.
- Acetaminophen in overdose levels can also interfere in this same way.

Fluorescence/Phosphorescence

- When some molecules absorb UV light, they convert some of the stored energy into heat and then emit light (in all directions) at a lower frequency (corresponding to the lower energy).
- The emitted light is best detected at a right angle to the incident light. Must be careful about stray light.
- Fluorescence and phosphorescence differ in their quantum mechanical mechanism for the dissipation of the stored energy.
  - Generally, fluorescence emits UV light and phosphorescence emits visible light.
  - However, most importantly, in both cases the emitted light is generally linearly proportional to the analyte concentration.
- Luminescence is generally more sensitive than absorption because it is easier to accurately measure a small amount of *absolute* light than to accurately detect a small decrease in relative light.

Mechanism of Fluorescence and Phosporescence

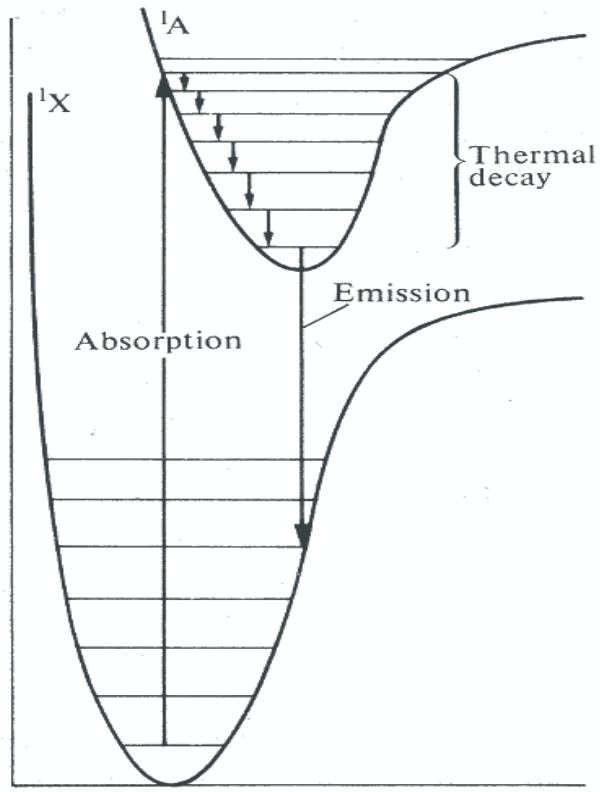


Fig. 12.13. The mechanism of fluorescence.

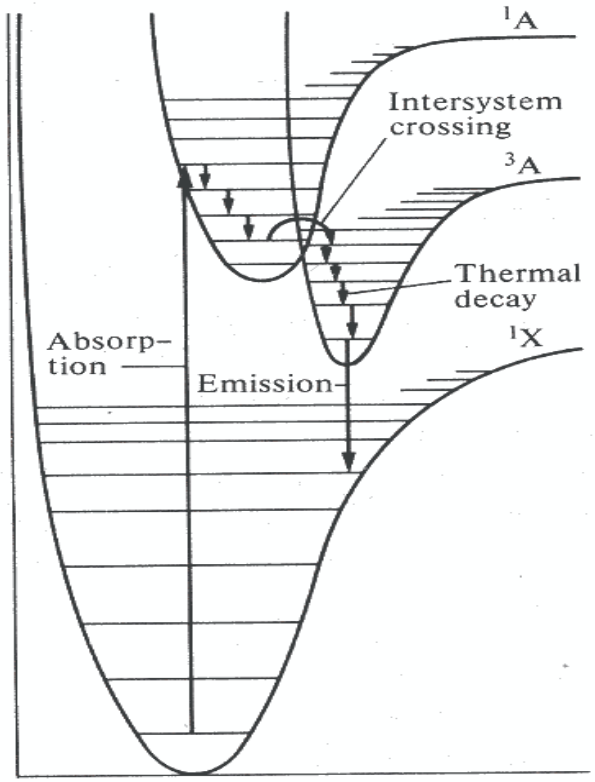


Fig. 12.14. The mechanism of phosphorescence.

Mechanism of Fluorescence and Phosphorescence

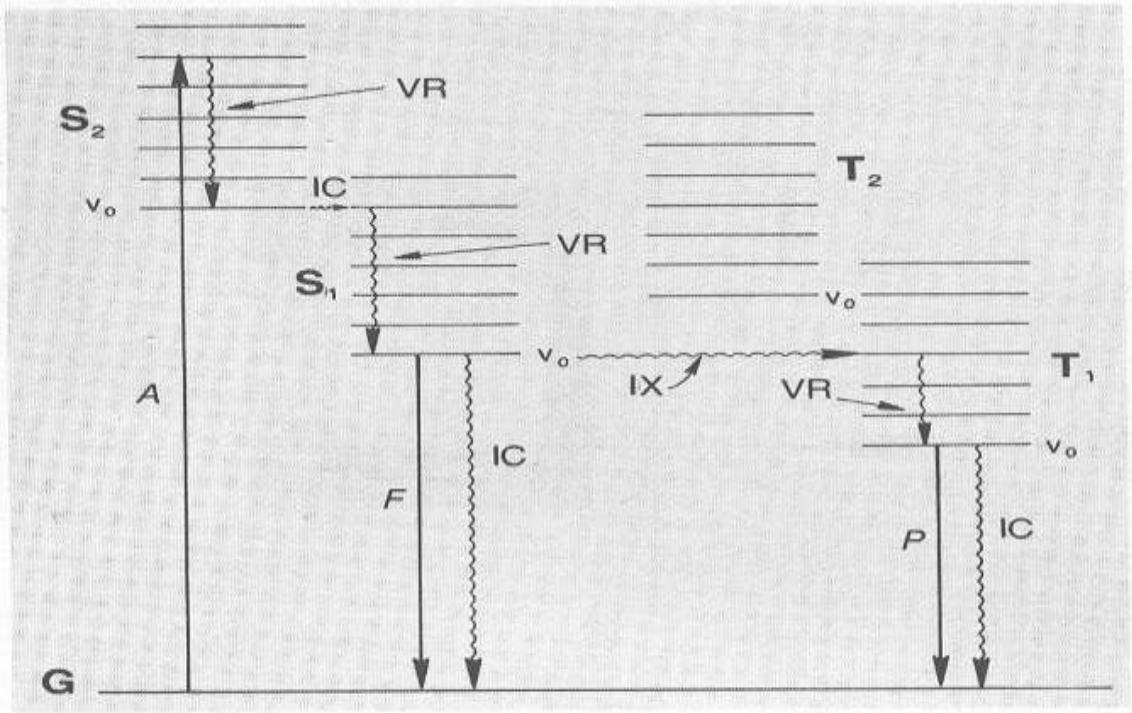


Figure 19–19. Electronic energy-level diagram for a molecule with ground state (**G**) and excited singlet (**S**) and triplet (**T**) states. Radiationless transitions between states are represented by wavy arrows; *A* is absorption, *F* is fluorescence, *P* is phosphorescence, VR is vibrational relaxation, IX is intersystem crossing, and IC is internal conversion. (See text for discussion.)

Generic Luminescence Detector

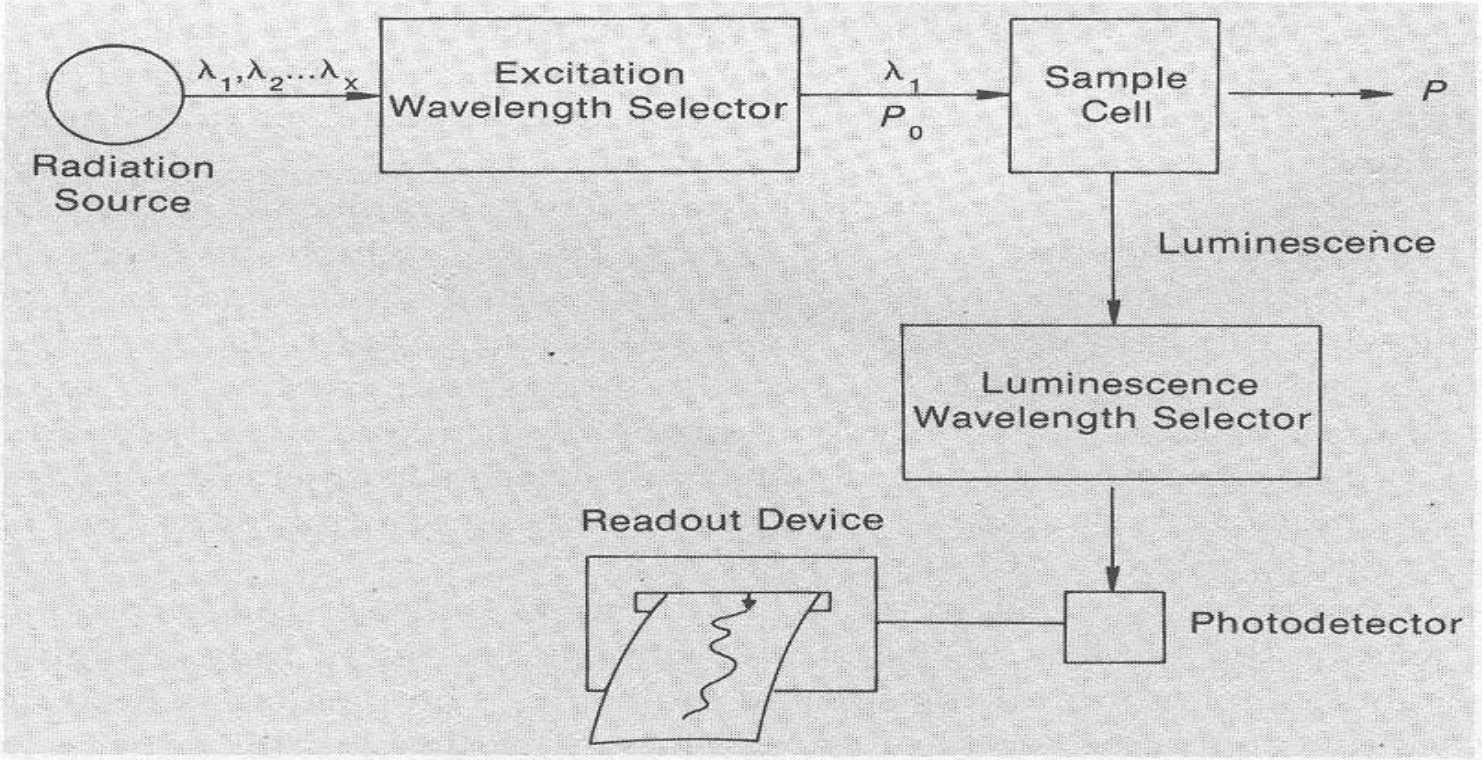


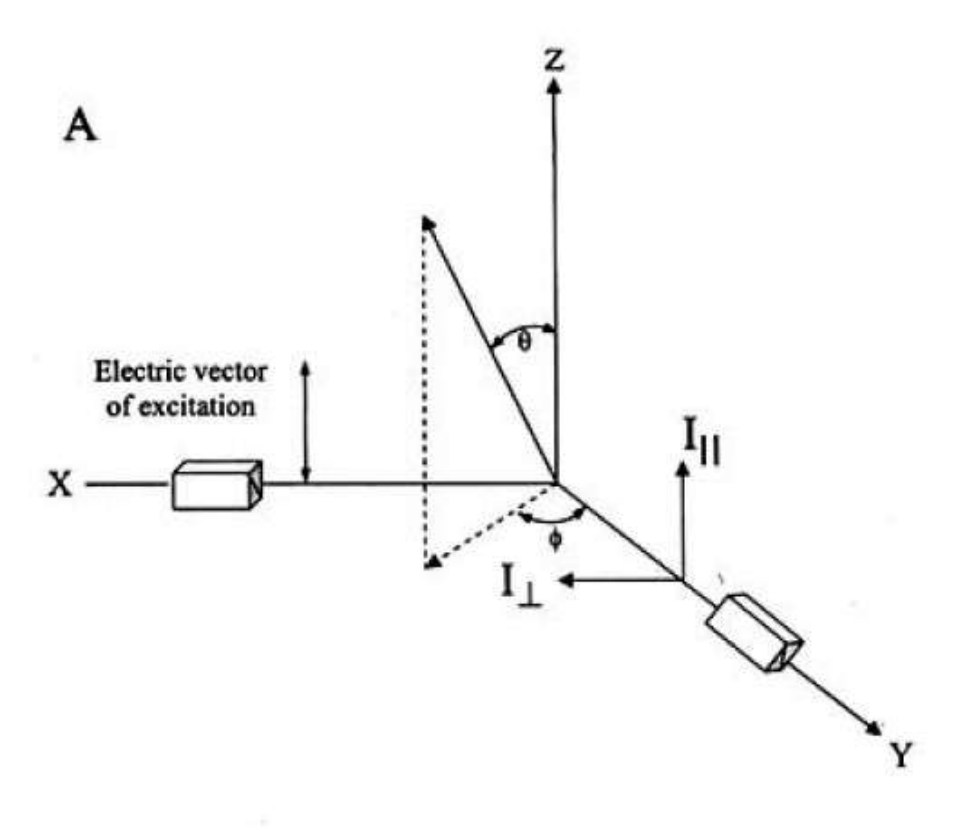
Figure 19–20. Generalized block diagram of a luminescence spectrometer. (See text for discussion.)

(Luminescence is a generic word for fluorescence and phosphorescence)

Fluorescence Polarization

- Generic method for detecting binding of a *small* molecule (e.g. a drug or hormone) to a *large* protein or particle.
- Concept is that if polarized light is used to excite a fluorescent molecule, then the emitted light is also polarized.
- However, if the molecule rotates during the "fluorescent lifetime" then the plane of polarization will change and this can be quantified.

General Design of a Fluorescence Polarization Spetrophotometer



Fluorescence Polarization: Equations

$$\text{Polarization Value (P)} = \frac{(\text{Intensity}_{\parallel}) - (\text{Intensity}_{\perp})}{(\text{Intensity}_{\parallel}) + (\text{Intensity}_{\perp})}$$

$$\text{Anisotropy Value (A)} = \frac{(\text{Intensity}_{\parallel}) - (\text{Intensity}_{\perp})}{(\text{Intensity}_{\parallel}) + 2(\text{Intensity}_{\perp})}$$

Both polarization and anisotropy vary between 0 and 1.

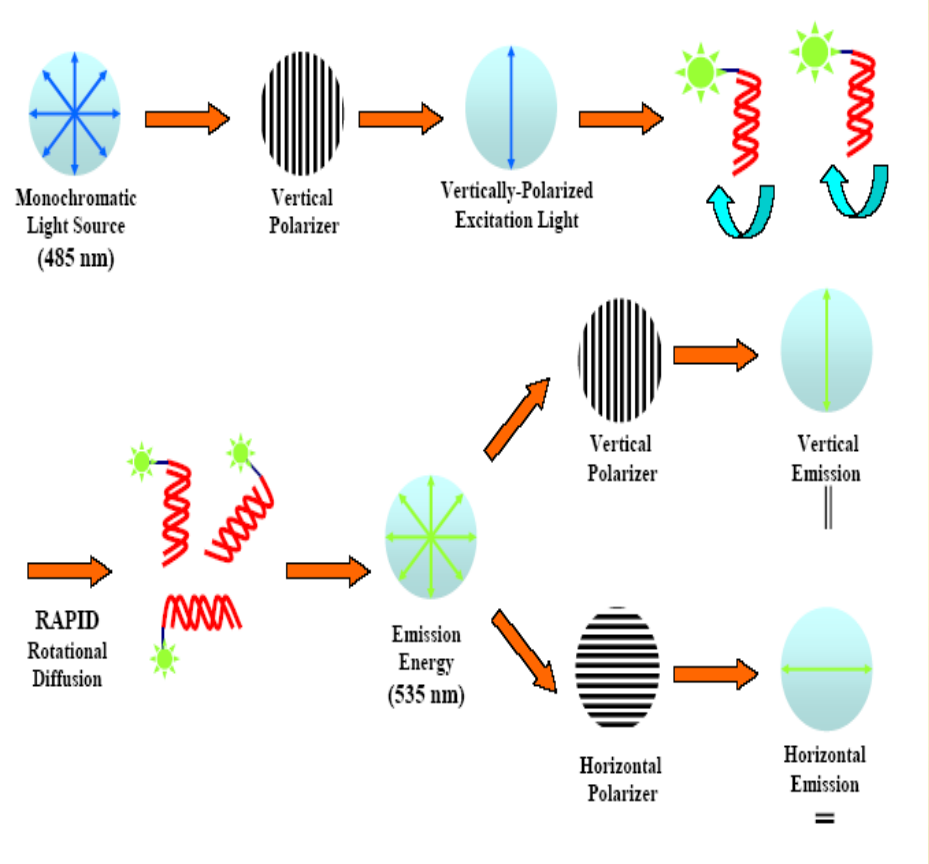
Small, rapidly rotating molecule:

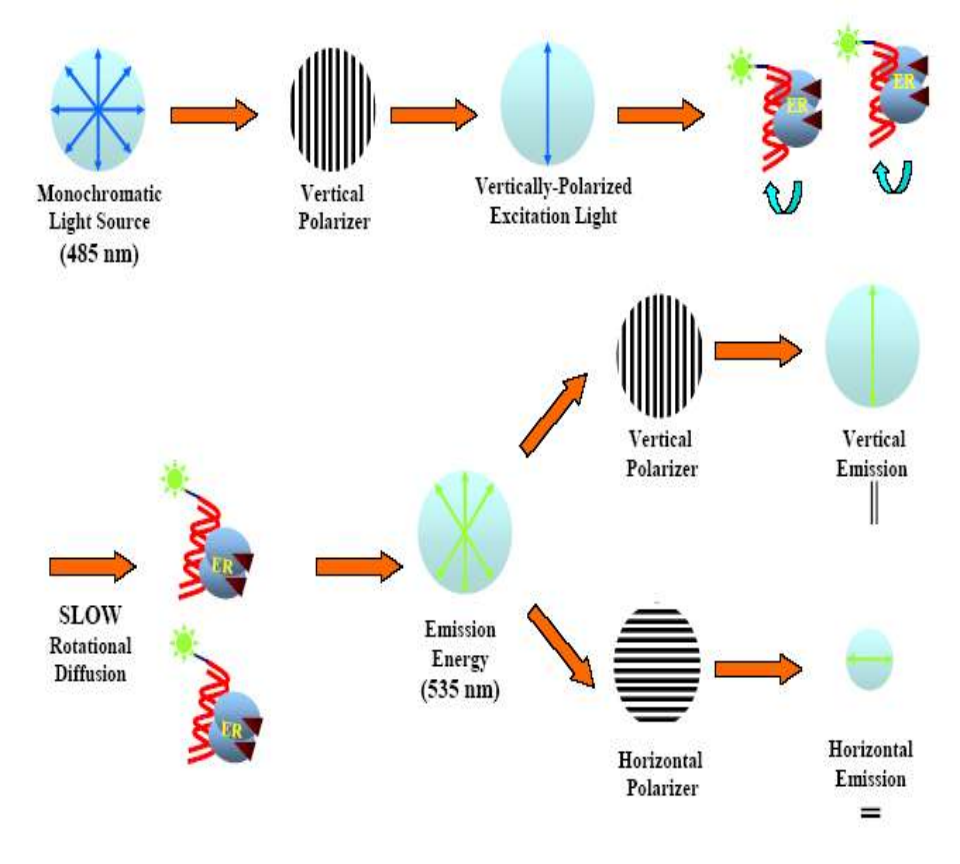
$P = A = 0$

Large, slowly rotating molecule:

$P = A = 1$

A Pretty Example of Fluorescence Polarization





$I_{||} \sim I_{=}$ and $P \sim 0$

$I_{||} >> I_{=}$
and P close to 1

FPIA: Fluorescence Polarization Immunoassay

Among the first commercial instruments designed to use FPIA for clinical diagnostic purposes was the Abbott “TDx” introduced in 1981. Currently, many of our assays on the Abbott “AxSym” use FPIA.

The basic principle of FPIA is to:

(1) Add a fluorescent analog of a target molecule, e.g. a drug, to a solution containing an antibody to the target molecule.

(2) Measure the fluorescence polarization, which is high when the fluorophore is bound to the antibody and low when it is free in solution.

(3) Add the appropriate biological fluid (blood, plasma, urine, etc.) and measure the decrease in polarization as the target molecules in the sample fluid bind to the antibodies and displace the fluoroescent analogs.

Advantages and Disadvantages of Fluorescence (Luminescence)

Advantages

- More sensitive than UV/Vis absorption (can detect low levels of a compound).
- More specific than UV/Vis absorption (only fluorescent molecules detected).

Disadvantages

- Very few molecules are naturally luminescent.
- More complicated equipment required compared to UV/Vis absorption.
- Similar to absorption, luminescence can be dependent on solution conditions; hence, often optimal to combine with HPLC in order to standardize solution conditions.

Uses of Luminescence in the Clinical Laboratory

- Fluorescence and Phosphorescence
  - Fluorescence Polarization Immunoassay (FPIA) on the AxSym.
  - Flow cytometry and immunofluorescence (using standard "tag" molecules, i.e. FITC, PE, ...)
  - A few rarely found manual assays (e.g. platelet serotonin).

- Chemiluminescence has great potential.
  - based on the conversion of chemical energy into light (like a firefly); takes away the need for incident light
  - hence, ***very*** sensitive and specific
  - Immulite analyzer in Chemistry uses chemiluminescence.

Atomic Spectroscopy

- UV/Vis absorption spectra of organic molecules in solution are ***not*** highly specific due to broad, overlapping absorption bands.

- This is generally a feature of chemical bonds, which have more complex electronic transitions (complicated by bond vibrations and rotations).

- Isolated elements (Pb, Cu, etc.) in a gaseous state are NOT complicated by chemical bonding or solvent interactions.

- Therefore, their "atomic spectra" are highly specific.

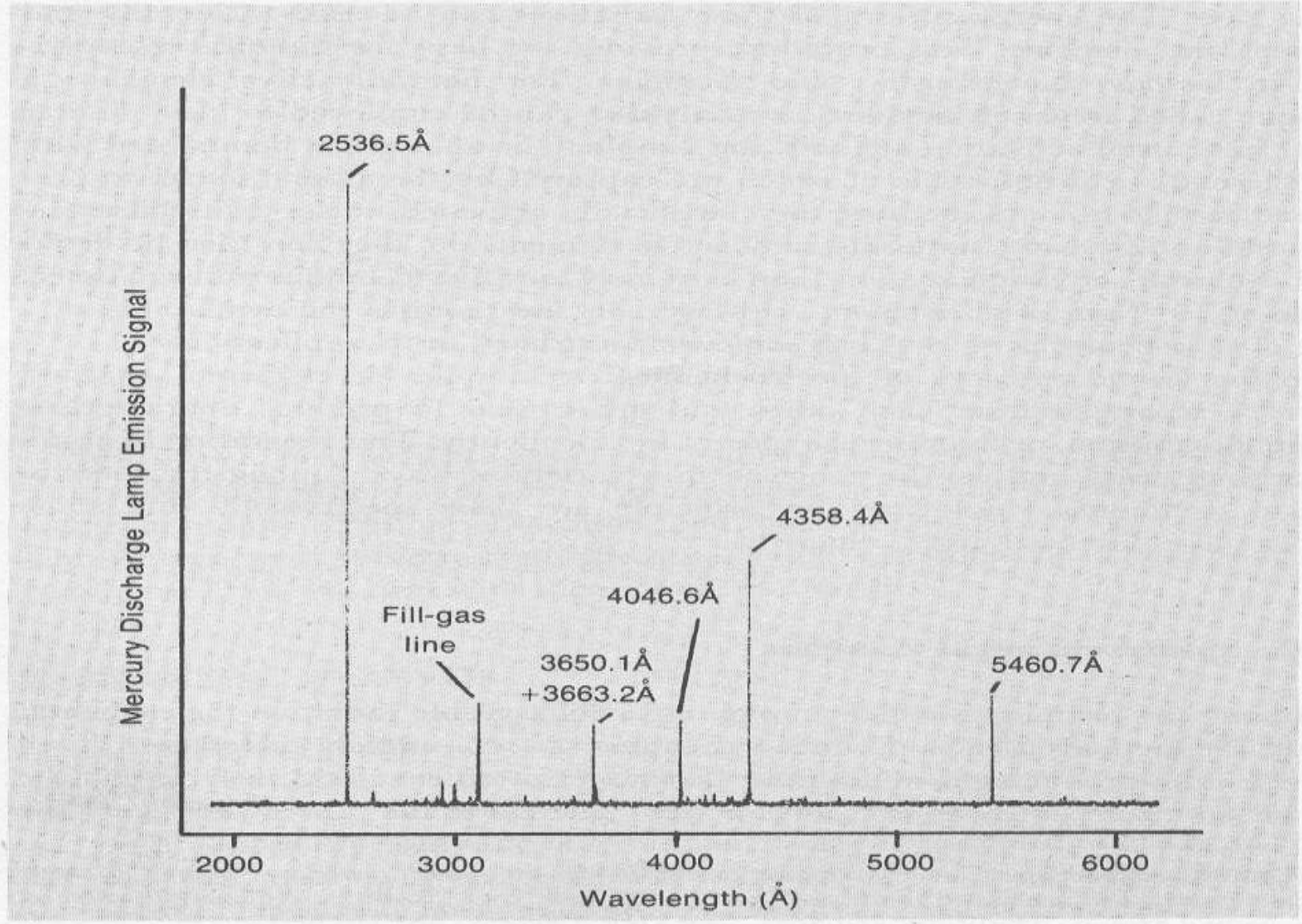


Figure 20–1. Emission spectrum of mercury atoms excited in a low-power electrical discharge. Prominent, characteristic spectral lines are indicated. A fill-gas emission line is produced by excitation of an inert gas present in the mercury discharge lamp.

Flame Emission Spectroscopy

- Thermal energy from the flame excites some elements (alkali metals) into a higher energy electronic state.

- When the excited electrons relax to their ground state, they emit light with very specific frequencies, which is quantitated by a detector.

- Originally used for Sodium, Potassium, Lithium and other alkali metals but has been replaced by ion selective electrodes (ISE).

- Homework: try dumping table salt onto a gas burner and see the pretty yellow color; whereas salt substitutes containing KCl are violet...

Generic Flame Emission Spectrophotometer

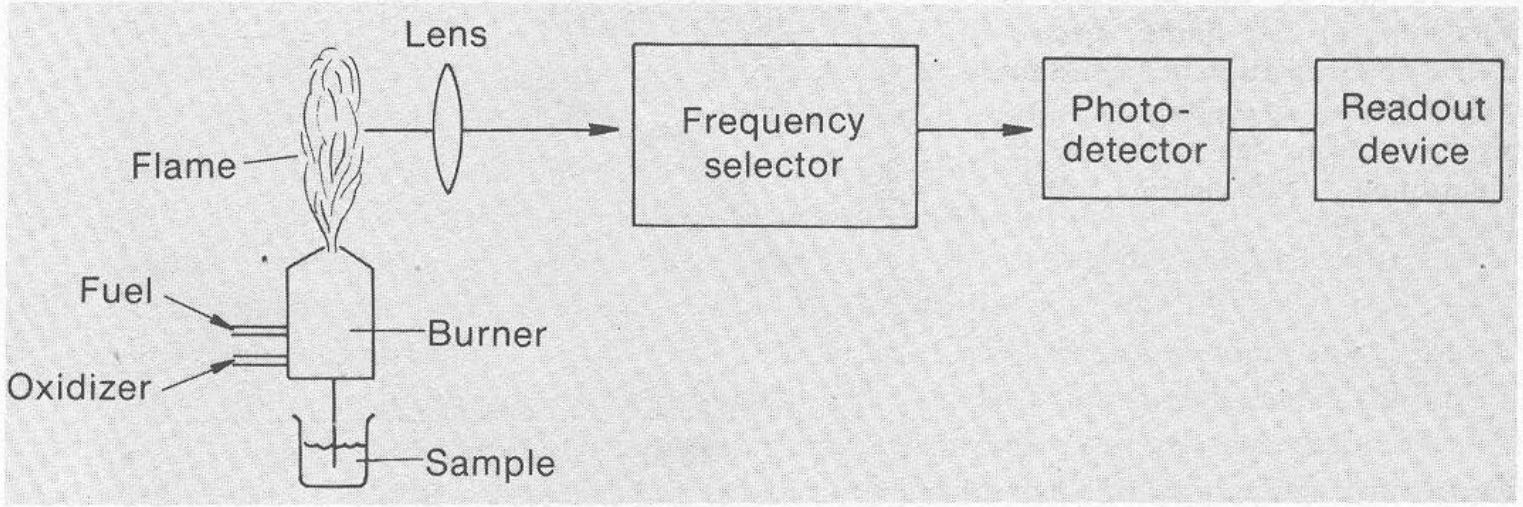


Figure 20–6. Generalized, schematic diagram of a simple flame emission spectrometer.

Atomic Absorption Spectroscopy

- Most elements are not easily excited to higher electronic states by thermal energy.

- However, their narrow-energy and highly specific electronic transitions can still be detected by standard absorption of light (just like UV/Vis spectroscopy).

- A (reducing) flame is still part of the apparatus in order to separate the element from chemical bonds and solute interactions.

- A "hollow cathode" lamp specific for individual elements is used as the light source. These lamps contain the element (typically a metal) to be analyzed and emit only the specific frequencies which can be absorbed by the element in the flame (ingenious, no?).

Generic Atomic Absorption Spectrophotometer

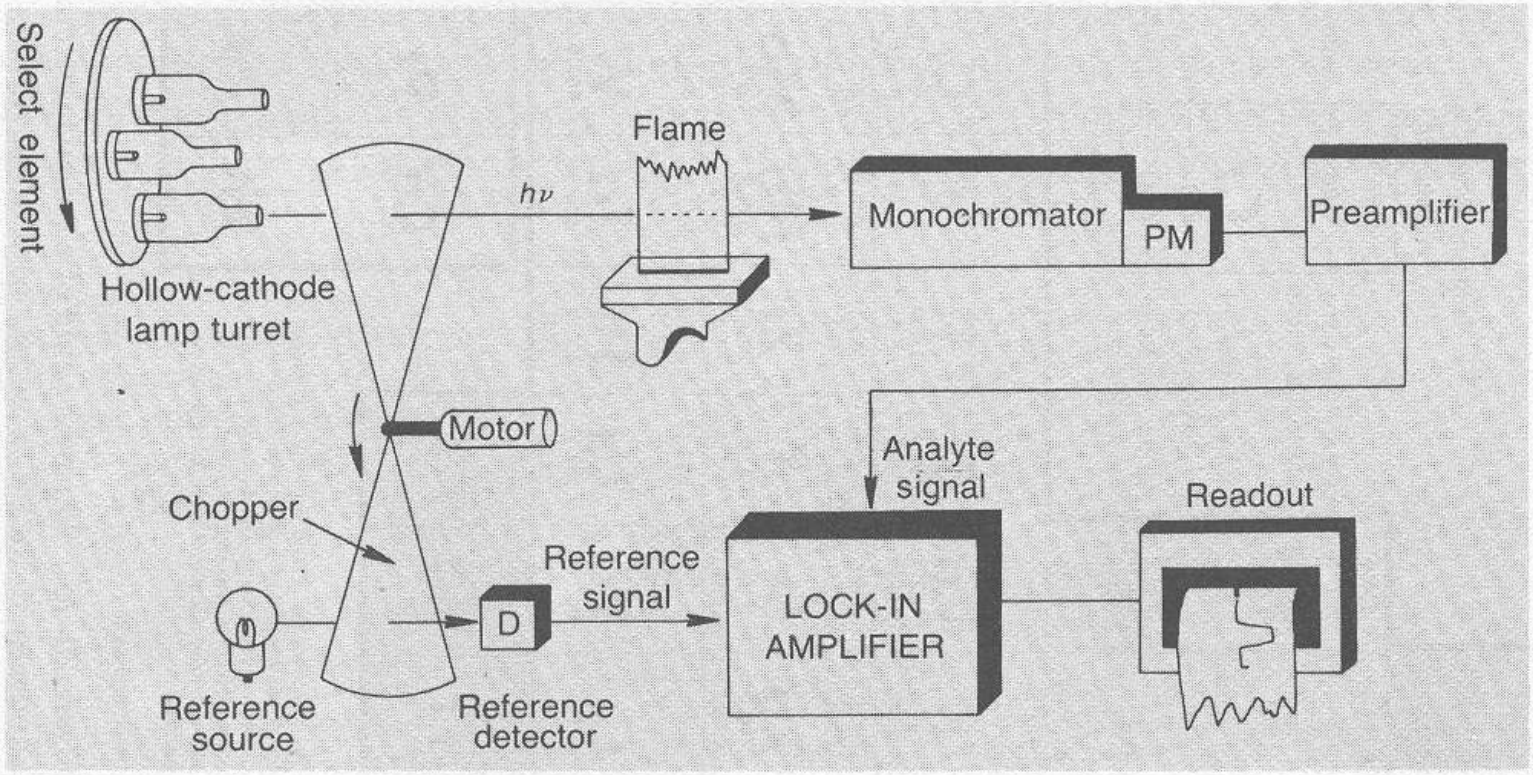


Figure 20–14. Modern atomic absorption spectrometer, with a multiple hollow-cathode lamp turret which can be rotated to select the desired lamp and a sophisticated signal-processing system; PM is a photomultiplier tube.

Hollow Cathode Lamp Emission Spectrum

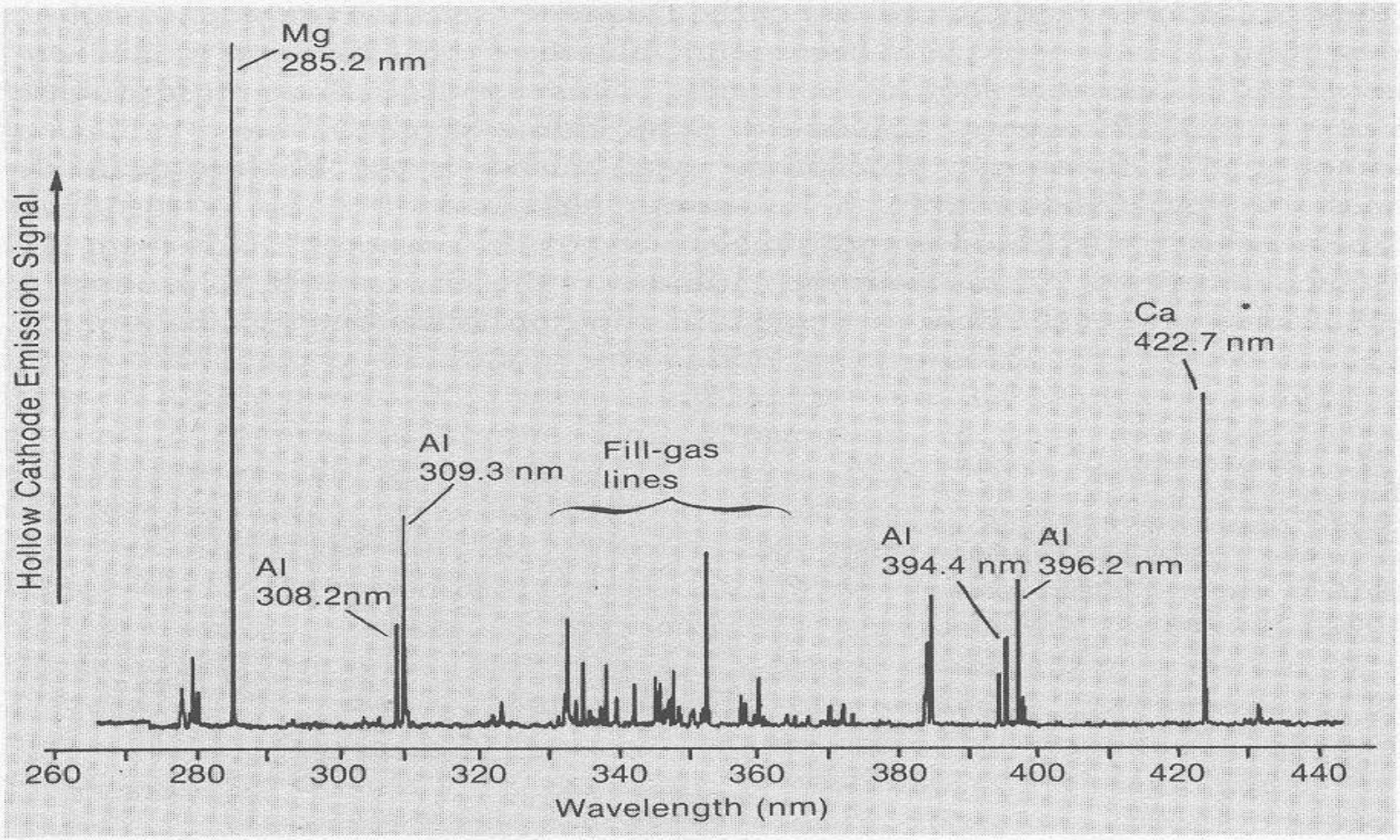


Figure 20–12. Spectrum of the emitted radiation from a three-element (Ca, Mg, Al) hollow-cathode lamp. Note the presence of spectral lines from the fill-gas. Wavelengths of the prominent lines are given in nanometers (nm).

The "Chopper" Eliminates Background Signals

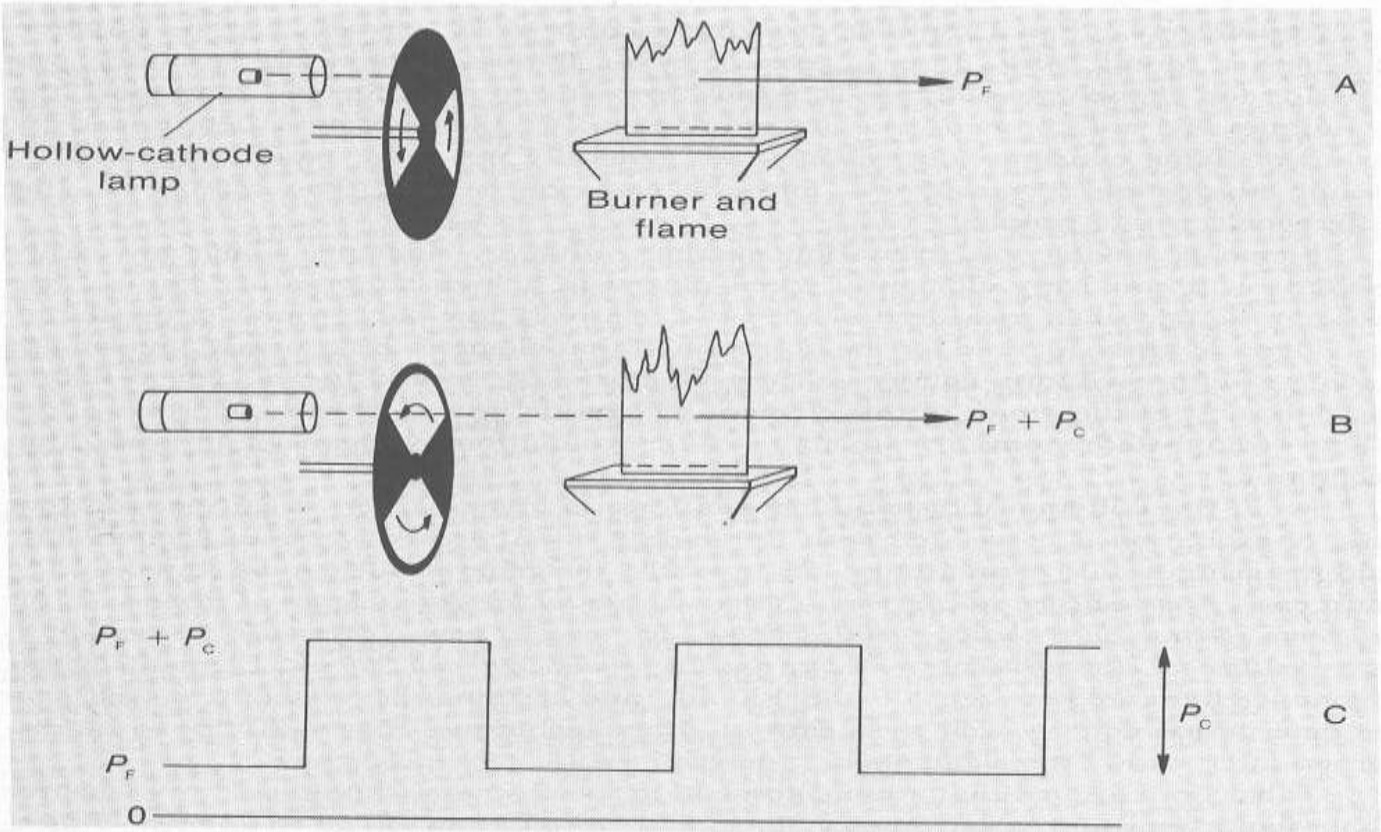


Figure 20–13. Elimination of emission background in atomic absorption spectrometry through the use of a rotating chopper; P_F is the radiant power emitted by the flame and P_C is the desired radiant power from the hollow-cathode lamp transmitted by the flame. (See text for discussion.)

Flaming Conditions are Critical

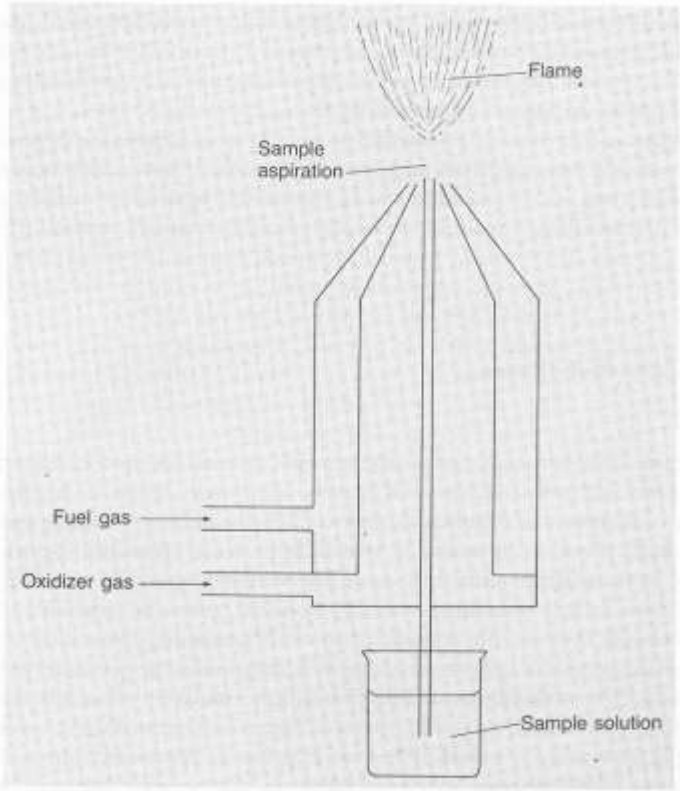


Figure 20–3. Cutaway diagram of a total-consumption nebulizer-burner.

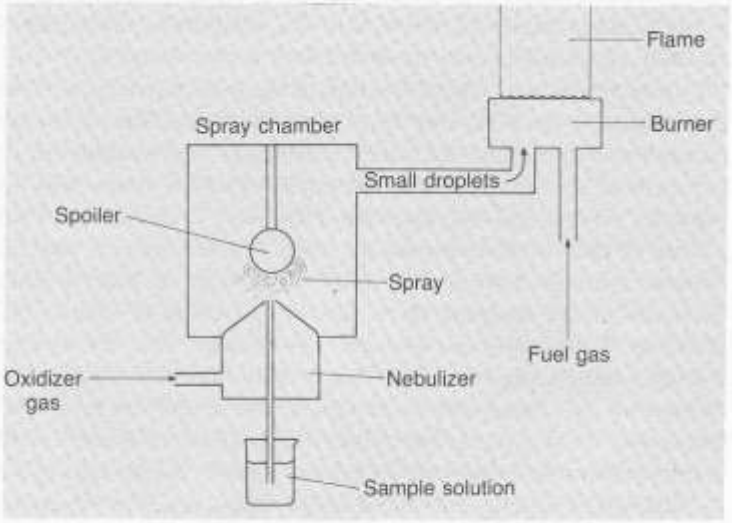


Figure 20–2. Premix burner-nebulizer system used in flame spectrometry.

- *Nebulization* (spraying), *desolvation* and *vaporization* (including chemical separation and reduction) of solutes during flaming is desired.
- Ionization of solutes is avoided.

Interferences with "Flame" Atomic Spectroscopy

- Despite the high specificity of atomic absorption spectroscopy, interferences from other molecules in the nebulized sample are possible.
- They can be classified as *vaporization* interferences, *spectral* interferences and *ionization* interferences.
- *Vaporization* interference results when some component of the sample interferes with the vaporization process, often by chemically interacting with the analyte. An example is Ca-phosphate precipitation during desolvation. One solution is to add an excess of phosphate to standardize the effect. Another solution is to add a "releasing agent" such as EDTA which tightly binds to the Ca in solution but is also destroyed upon vaporization within the flame.

Interferences with "Flame" Atomic Spectroscopy

- *Spectral* interferences result when two elements absorb or emit light at similar frequencies. This problem is more common in flame emission spectroscopy where the emitted light cannot be controlled. However, in either case this problem is easily handled by selecting different frequencies for quantitation.

- *Ionization* interferences result when a component of the sample affects the degree of ionization of the measured analyte. A common example is due to the easy ionization of potassium in a flame, which "dumps" free electrons (increased reductive potential) into the flame and reduces the ionization of the measured analyte. Therefore, variations in potassium levels in samples will vary the signal from the analyte causing an interference. The solution is to add an excess of an element which also ionizes easily (potassium or lithium), thereby maximally reducing the ionization of the measured analyte.

Atomic Absorption vs. Flame Emission Spectroscopy

- Flame Emission
  - simpler instrumentation as hollow cathode lamps and "choppers" are not required
  - few elements are easily excited thermally
  - historical use for Na, K, Li but has been replaced by ISE
- Atomic Absorption
  - very high sensitivity (especially graphite furnace/Zeeman)
  - very high specificity (partially due to hollow cathode lamps)
  - complex instrumentation for only a few biomedically relevant analytes (only Pb & Ca at YNHH, but also Zn & Cu elsewhere)
  - usually reserved as a "gold standard" reference method
  - also has use for complex samples such as tissue biopsies (Fe, Cu and Zn in liver biopsies)

“Flameless” (Graphite Furnace) Atomic Absorption Spectroscopy

- Some samples are too complex to adequately account for all possible vaporization or ionization interferences. An example is tissue biopsy specimens submitted for heavy metal analysis (liver is common).
- These samples are homogenized and dissolved into harsh solutions (strong base and heavy oxidizer) and analyzed with “flameless” atomic absorption spectroscopy.
- The sample is placed in a well on a carbon rod within an enclosed chamber (a graphite furnace) and heated in successive steps to dry, char and atomize the sample into the chamber, where the atomic absorption is measured in typical fashion.
- Matrix effects are effectively eliminated by a proper heating sequence which destroys organic components and separates other inorganic elements from the measured analyte.
- The reduction in background or interference signal greatly increases the overall sensitivity of the method.

Background Correction Based upon the "Zeeman" Effect

- The atomized sample from a graphite furnace is contained between two electromagnets, which are cycled on and off at regular intervals.
- The desired atomic absorption signal is modulated in a predictable way by this effect, but the undesired background signal is not.
- By proper comparison of the signal in the presence and absence of the magnet field, background interferences can be reduced even further giving rise to extremely high sensitivities.
- We use this method for Pb allowing a very low limit of detection and quantification. Good for monitoring low level lead exposure compared to standard electrochemical method.

Zeeman Background Correction

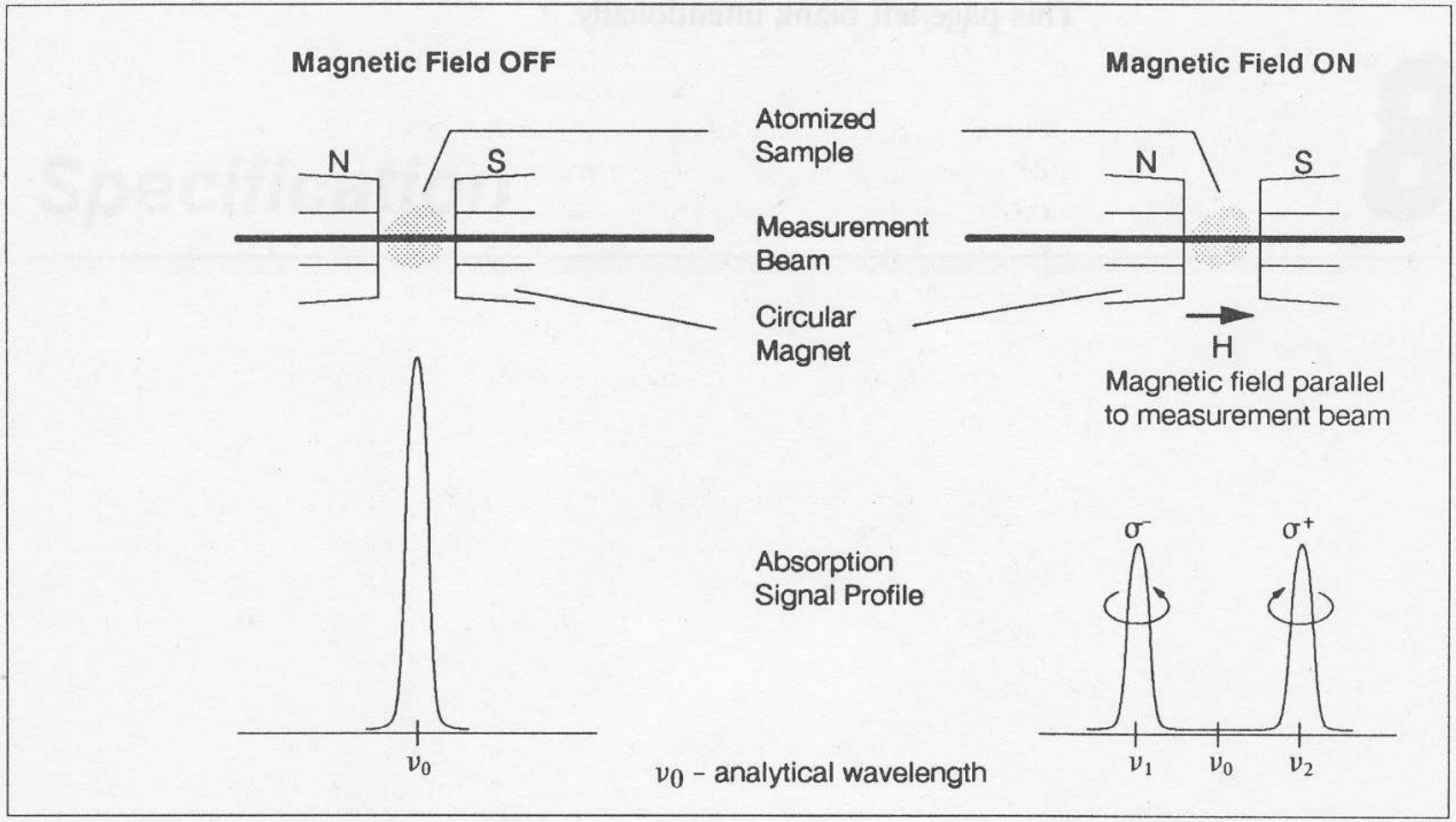


Figure 7-5 Analyte Signals Obtained with the Longitudinal Zeeman Effect

Zeeman Background Correction

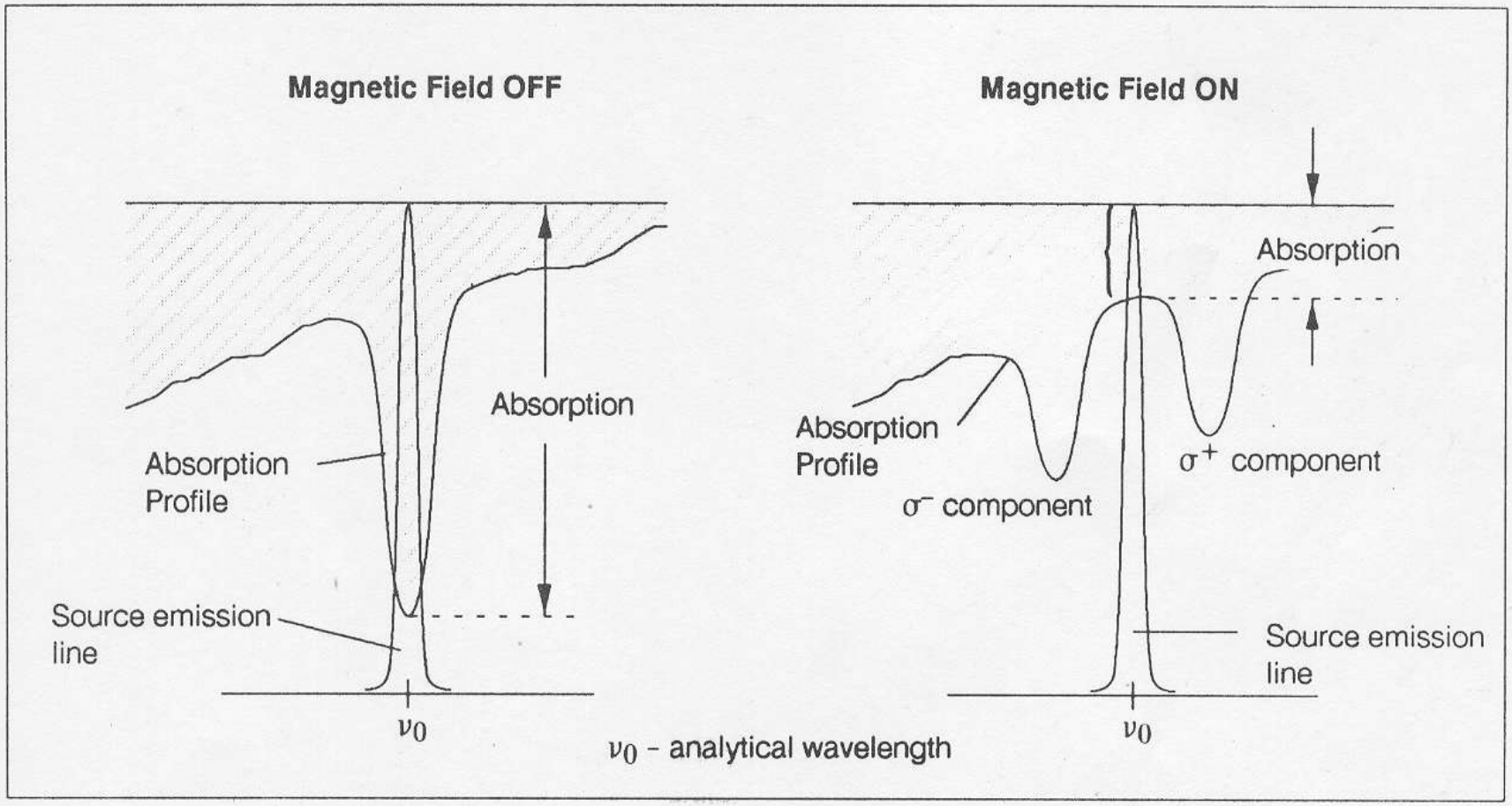


Figure 7-6 Background Correction with the Longitudinal Zeeman Effect

Light Scattering

- Particles scatter light.
  - Scattering is essentially the induction of an oscillating "dipole" in a molecule due to the varying electromagnetic field of the light.
  - This oscillating dipole acts like an antenna and disperses (i.e. scatters) some of the energy in other directions.
- However, there are many important factors.
  - Scattering varies with the size of the particle
  - Scattering varies with the wavelength of light
  - Scattering varies with the angle of observation

Scattering of Light by a Particle

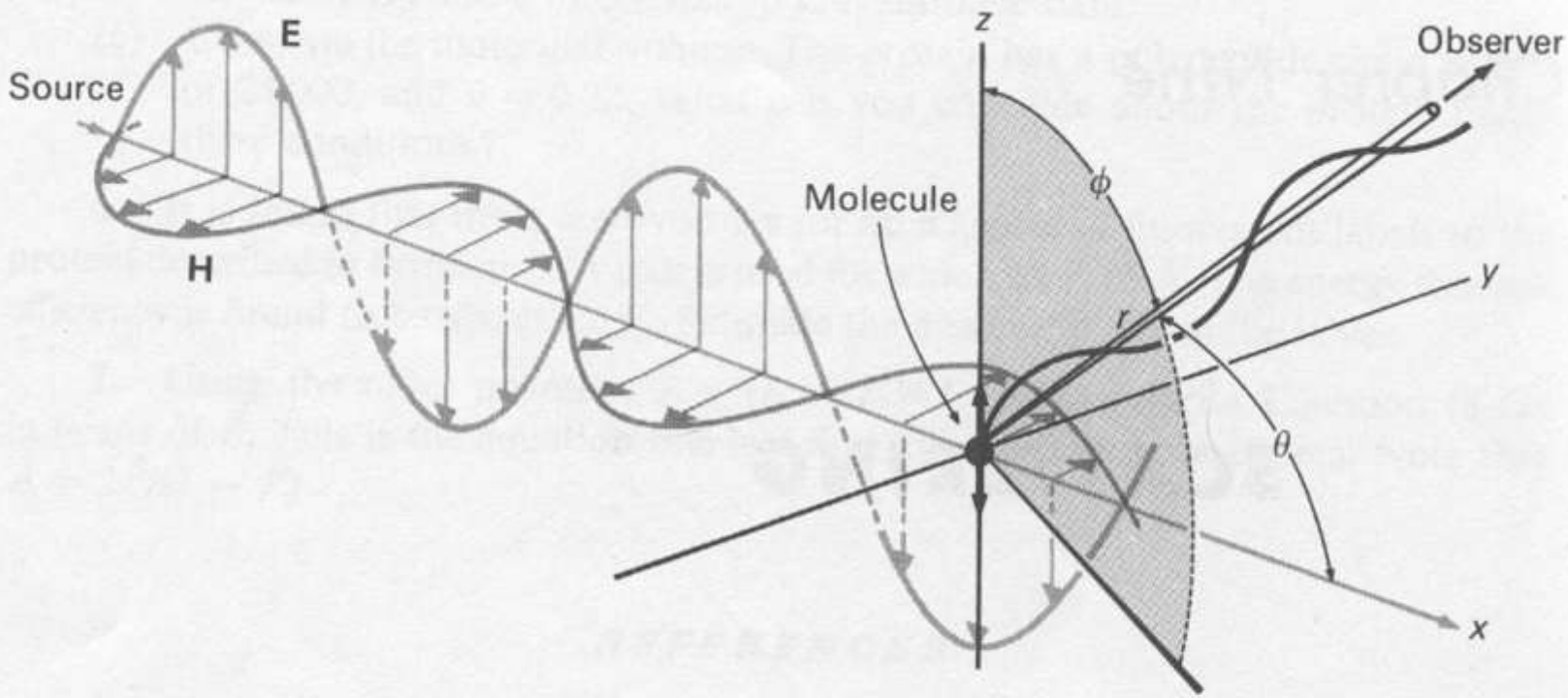


Figure 9.1 Scattering of radiation by a particle. Only the electric vector of the radiation scattered in the particular direction given by θ and ϕ is shown. Radiation scattered in this direction is polarized in the plane defined by the z axis and r.

Note that the angle θ is located in the XY plane and the angle ϕ is located in the YZ plane.

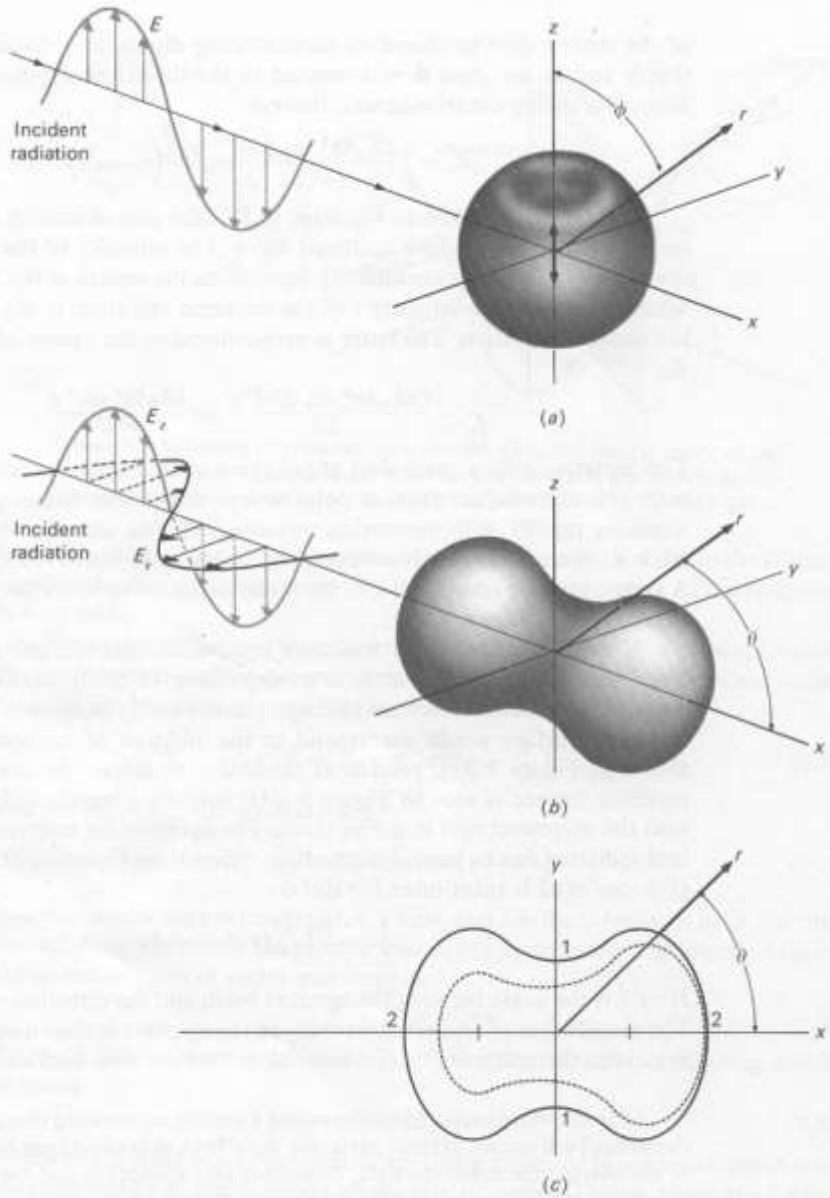


Figure 9.2 (*a*) Distribution of intensity of scattering of incident light polarized in the *z* direction. In this figure, and in (*b*), the distance from the origin to the surface along a direction *r* shows the intensity in that direction. (*b*) Distribution of scattering intensity for unpolarized incident light. (*c*) A section in the *xy* plane of the surface of (*b*). The solid line shows the intensity of scattering as a function of θ for Rayleigh scattering (small particle). The dotted line is for a larger particle.

Rayleigh Scattering (small particle size relative to wavelength) for polarized incident light:

[redacted]

Rayleigh Scattering (small particle size relative to wavelength) for non-polarized incident light:

[redacted]

Debye-Rayleigh Scattering (large particle size relative to wavelength) for non-polarized incident light.

- No simple equation. Has to do with summing up scattering from separate "bits" of the particle.

Rayleigh Scattering Explains why the Sky is Blue and Sunsets are Red

As sunlight passes through the atmosphere at indirect angles (i.e. not aiming directly at the earth), it is scattered by the molecules present in the air. Because of the powerful 4th power dependence of scattering on wavelength (λ), blue light, which has a higher frequency/energy and lower wavelength, is scattered much more than red and we see a blue sky.

On the other hand, when the sun "sets" in the west light aimed directly at your vision has passed through a LOT of atmosphere (and a lot of smog). The blue light has all been scattered, primarily leaving behind red light. As well, because smog contains large particles and because the Debye effect is more effective for longer wavelengths, the red light is scattered directly ahead, contributing to the effect.

Neat, eh?

Dependence of Light Scattering on Molecular Size and Angle of Observation

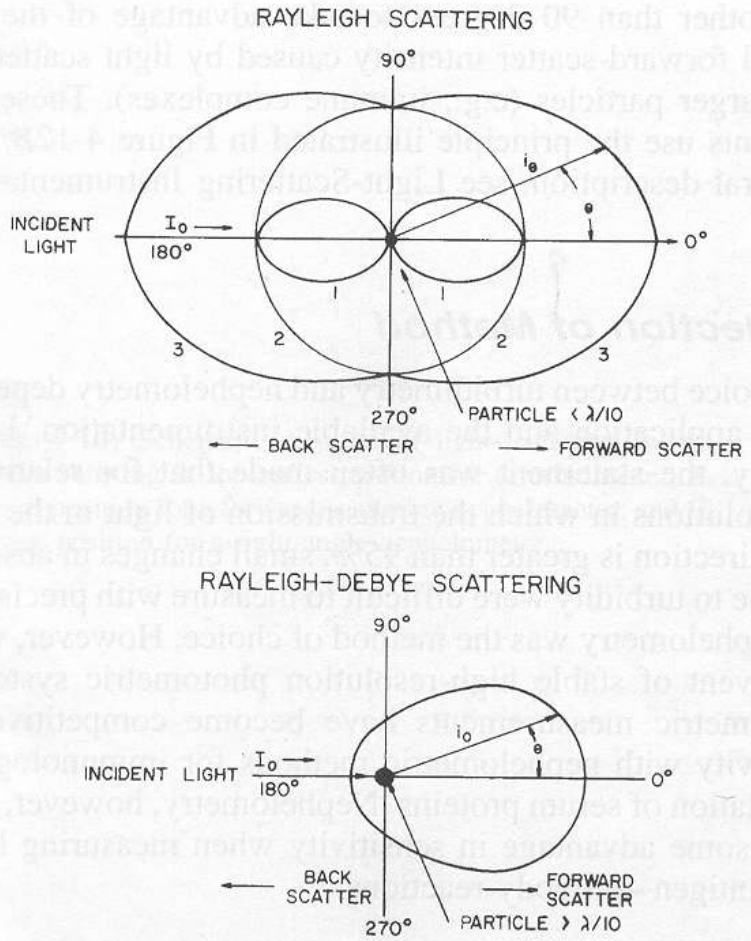


FIGURE **4-12.** The angular dependence of light-scattering intensity with nonpolarized and polarized incident light for small particles (*A*) and the angular dependence of light scattering with nonpolarized light for larger particles (*B*).

(A) Small particles scatter light approximately equally to the side (90 degrees) as forward (0 degrees).

(B) Larger particles scatter proportionally more light forward (and at longer wavelengths) than small particles.

Nephelometry and Turbidometry

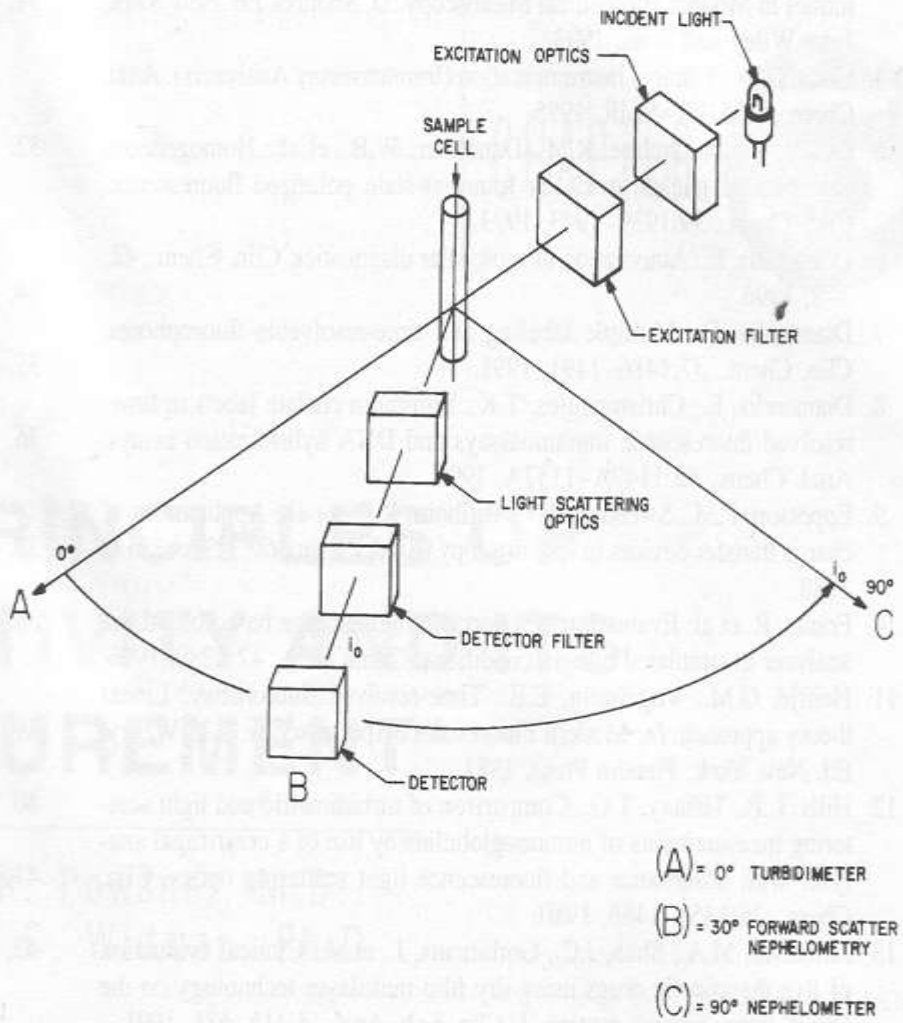


FIGURE **4-13.** Schematic diagram of light-scattering instrumentation showing *A*, the optics position for a turbidimeter; *B*, the optics position for a forward-scattering nephelometer; and *C*, the optics position for a right-angle nephelometer.

Uses of Nephelometry and Turbidometry in the Clinical Laboratory

- Outside of flow cytometry, primary use of both is to quantitate the presence of large immune complexes in serum or urine.
- Basically, when an antibody to a multivalent antigen is added to plasma, large oligomeric/polymeric immune complexes form in proportion to the degree of antigen.
- The large immune complexes scatter light, which can be quantified by either turbidometry or nephelometry.
- Calibration curves can be generated from standard solutions of the antigen similar to any other antibody-based method.
- Note that these techniques are subject to the same classical "hook-effect" as any other antibody-based assay.
- Generally, nephelometry is advantageous when measuring low-level antibody-antigen reactions. However, this advantage has diminished with improvement in turbidometry optics.
- The advantage of turbidometry is simpler instrumentation.

Turbidometry

- Basically, turbidometry is entirely analogous to absorption spectroscopy, where an attenuation of transmitted light through a sample is measured.
- However, with turbidometry the attenuation is due to light scattering instead of electronic absorption.
- Equations are straightforward:
  - $I/I_o = \exp(-bt)$, where I/I_o is the ratio of transmitted light in a sample relative to a blank or control, "b" is the pathlength (typically 1 cm) and "t" is the *turbidity*.
  - Rearranging, $tb = \ln(I/I_o)$. Note that Beer's Law can be rearranged to $abc = \ln(I/I_o)$, where a is the absorptivity and c is the analyte concentration.
  - When turbidity is related to analyte concentration, the analogy of turbidometry with absorption spectroscopy is complete.

Nephelometry

- Nephelometry attempts to directly quantify the scattered light. To do this it must measure light at an angle to the incident light.
  - Both “side” scatter (at 90) and “forward” scatter (at 30) instruments are available.
- In theory, nephelometry is more sensitive than turbidometry, because it is easier to detect a small amount of scattered light than to accurately detect a small (< 5%) attenuation of transmitted light.
  - However, for sensitive detection of low-level antigen-antibody complexes, it is critical to avoid “stray” light directed at the detector (e.g. reflected light of the cuvette).
  - For this reason, “forward” scatter instruments are often preferred to take advantage of the increased forward-scatter intensity from larger particles (i.e. immune complexes).