Ultraviolet Germicidal Irradiation Handbook

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Chapter 2

UVGI Disinfection Theory

2.1 Introduction

Ultraviolet Germicidal Irradiation (UVGI) is electromagnetic radiation that can destroy the ability of microorganisms to reproduce by causing photochemical changes in nucleic acids. Wavelengths in the UVC range are especially damaging to cells because they are absorbed by nucleic acids. The germicidal effectiveness of UVC peaks at about 260–265 nm. This peak corresponds to the peak of UV absorption by bacterial DNA. The germicidal effectiveness of UVC radiation can vary between species and the broader range wavelengths that include UVB also make a small contribution to inactivation (Webb and Tuveson 1982). Although the methods and details of disinfection with ultraviolet light are fairly well understood, to the point that effective disinfection systems can be designed and installed with predictable effects, the exact nature of the effect of ultraviolet light on microorganisms at the molecular level is still a matter of intensive research. This chapter examines the fundamentals of the complex interaction between UV irradiation and cell DNA at the molecular level and provides detailed background information to aid in the understanding of the various biophysical processes that are involved in microbial inactivation.

2.2 UV Inactivation

The spectrum of ultraviolet light extends from wavelengths of about 100–400 nm. The subdivisions of most interest include UVC (200–280 nm), and UVB (280–320 nm). Although all UV wavelengths cause some photochemical effects, wavelengths in the UVC range are particularly damaging to cells because they are absorbed by proteins, RNA, and DNA (Bolton and Cotton 2008, Rauth 1965). The germicidal effectiveness of UVC is illustrated in Fig. 2.1, where it can be observed that germicidal efficiency reaches a peak at about 260–265 nm. This corresponds to the peak of UV absorption by bacterial DNA (Harm 1980). The germicidal effectiveness of UVC and UVB wavelengths can vary between species. Low pressure

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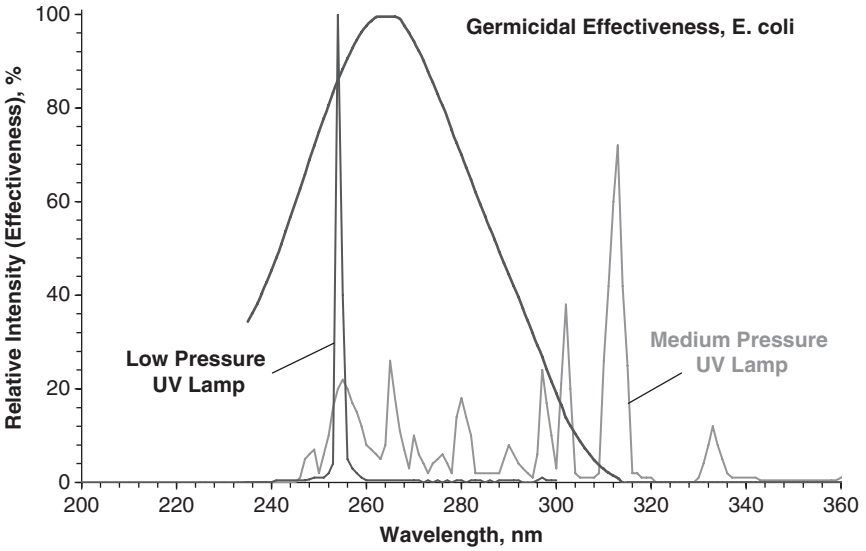


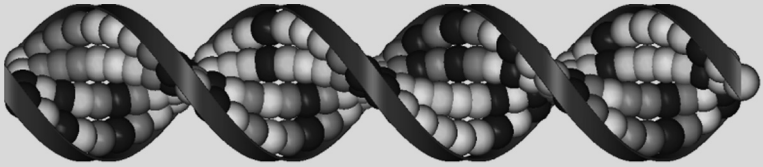
Fig. 2.1 Germicidal efficiency of UV wavelengths, comparing High (or medium) and Low pressure UV lamps with germicidal effectiveness for *E. coli*. Based on data from Luckiesh (1946) and IESNA (2000)

mercury vapor lamps radiate about 95% of their energy at a wavelength of 253.7 nm, which is coincidentally so close to the DNA absorption peak (260–265 nm) that it has a high germicidal effectiveness (IESNA 2000).

If we assume the LP and MP lamps in Fig. 2.1 produce the same total UV wattage, and multiply spectrum by the germicidal efficiency at each wavelength, we find the LP lamp has a net germicidal efficiency of 84% vs. 79% for the MP lamp. The optimum wavelength for inactivating *E. coli*, about 265 nm, is about 15% more effective than the UVC peak of 254 nm. The optimum wavelength for inactivating *Bacillus subtilis* is 270 nm, and this is about 40% more effective than 254 nm (Waites et al. 1988). The optimum wavelength for destroying *Cryptosporidium parvum* oocysts is 271 nm and this is about 15% more effective than 254 nm (Linden 2001). Although UVC is responsible for the bulk of the germicidal effects of broad-spectrum UV, the effects of UVB wavelengths cannot be discounted altogether. In a study by Elasri and Miller (1999) it was found that UVB had about 15% of the effect of UVC on *Pseudomonas aeruginosa*.

THE STRUCTURE OF DNA

Deoxyribonucleic acid (DNA) is a large, high molecular weight macromolecule composed of subunits called nucleotides. Each nucleotide subunit has three parts: deoxyribose, phosphate, and one of four nitrogenous bases (nucleic acid bases). The four bases are thymine (T), adenine (A), cytosine (C), and guanine (G). These four bases form base pairs of either thymine bonded to adenine or cytosine bonded to guanine. Since thymine always pairs with adenine, there will be equal amounts of thymine and adenine. Likewise, cytosine will always exist in amounts equal to guanine. The specific sequences formed by these base pairs make up the genetic code that forms the chemical basis for heredity (Atlas 1995). Nucleotides are the basic repeating unit of DNA and they are composed of nitrogenous bases called purines and pyrimidines. These bases are linked to pentoses to make nucleosides. The nucleosides are linked by phosphate groups to make the DNA chain.



DNA forms a double helix, as shown in the figure above, in which two complementary strands of nucleotides coil around each other. The two outside helices of DNA form a backbone that is held together by strong covalent bonds, locking in the stability of the hereditary macromolecule. Each helix terminates in a free hydroxyl group at one end, and a free phosphate group at the other, conferring directionality. The two halves of the DNA molecule run in opposite directions and coil around each other. Supercoiling may also occur as long chains of DNA fold and pack into the available space (i.e. in a cell or viral capsid). Several million nucleotides may be held together in sequence and they establish the genetic code for each species.

The two complementary chains of the DNA double helix are held together by hydrogen bonding between the chains. Two of the nitrogenous bases(C and T) are single-ring structures called pyrimidines and the other two (A and G) are double-ring structures called purines. The internal hydrogen bonds between the base pairs, which hold the entire structure together, have only about 5% of the strength of the covalent bonds in the outer helix. Thymine forms two hydrogen bonds with adenine, while cytosine forms three hydrogen bonds with guanine. The thymine/adenine bond, therefore, represents the weakest link in the structure.

Hydrogen bonds between complementary bases are not the primary stabilizing force of DNA since the energy of a hydrogen bond (2–4 kcal/bond) is insufficient to account for the observed stability of DNA. Ionic bonds between the negative phosphate groups and positive cations reduce the electrostatic repulsion between the negative charges of the sugar-phosphate backbone. The stability of DNA is also accounted for by the hydrophobic forces associated with stacking of the bases, which is due to mutual interactions of the bases and geometrical considerations (Guschlbauer 1976). In polynucleotide chains, this interaction results in a compact stack of bases that is restricted by the sugar-phosphate backbone and results in a narrow range of possible overlap angles between the bases (36° in DNA). The stacked bases form a hydrophobic core which favors hydrogen bonding between the complementary strands (see Fig. 2.2). The stacking and twisting of base pairs creates channels in which water may bond or be excluded depending of DNA conformation (Neidle 1999).

UVA was also found to have a lethal effect on *P. aeruginosa* although considerably more lamp power was needed (Fernandez and Pizarro 1996). The UVA inactivation effect, however, is relatively insignificant and may involve non-actinic effects (no photochemical changes). Throughout the following discussion only the actinic bands of UVC and UVB are considered to be operative.

Two general types of nucleic acids exist, ribonucleic acid (RNA) and deoxyribonucleic acid (DNA). Viruses contain DNA or RNA, but not both. During UV irradiation and inactivation, the most sensitive target of microorganisms is the DNA of bacteria, the DNA of DNA viruses, the RNA of RNA viruses, and the DNA of

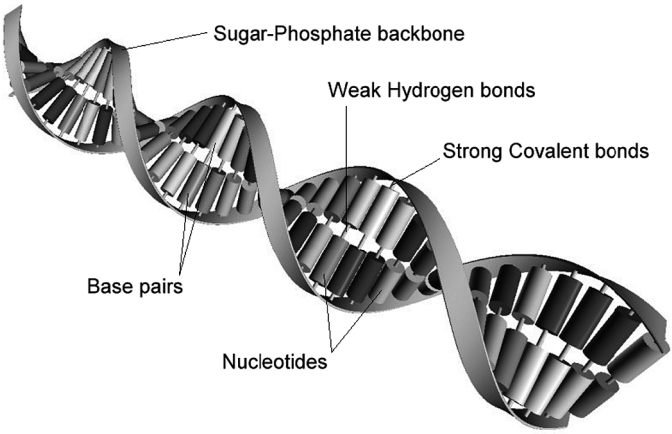


Fig. 2.2 The two helical backbones of DNA are connected by hydrogen bonds between the nucleotides

fungi. RNA has D-ribose as its main constituent and adenine, cytosine, guanine, and uracil as bases. DNA has 2-deoxy-D-ribose as its main constituent and adenine, cytosine, guanine, and thymine as bases. Hydrogen bonds link the bases. UV radiation can cause a crosslink between two thymine bases that is more stable than a hydrogen bond (Casarett 1968). Bacteria and fungi have DNA, while viruses may have either DNA or RNA. DNA and RNA are responsible for microbial replication and protein synthesis and damage to these nucleic acids results in inactivation or the failure to reproduce.

UV wavelengths inactivate microorganisms by causing cross-links between constituent nucleic acids. The absorption of UV can result in the formation of intrastrand cyclobutyl-pyrimidine dimers in DNA, which can lead to mutations or cell death (Harm 1980, Koller 1952, Kuluncsics et al. 1999). Pyrimidines are molecular components in the biosynthesis process and include thymine and cytosine (see Fig.2.2). Thymine and cytosine are two of the base pair components of DNA, the others being adenine and guanine. The primary dimers formed in DNA by UV exposure are known as thymine dimers (see the page on the Structure of DNA). The lethal effect of UV radiation is primarily due to the structural defects caused when thymine dimers form but secondary damage is also produced by cytosine dimers (David 1973, Masschelein 2002). Various other types of photoproducts are also formed that can contribute to cell death. Photohydration reactions can occur under UV irradiation in which the pyrimidines cytosine and uracil bond with elements of water molecules. The same reaction does not occur with thymine. The photohydration yield is independent of wavelength.

Double stranded RNA has a higher resistance to UV irradiation than single stranded RNA, and this may be due to various factors, including more structural stability (Becker and Wang 1989) and the redundancy of information in the complementary strands (Bishop et al. 1967). Ultraviolet light also causes photochemical reactions in proteins in the cell other than DNA and UV absorption in proteins peaks at about 280 nm. There is also some absorption in the peptide bonds within proteins at wavelengths below 240 nm.

Figure 2.3 illustrates how UV absorption can lead to cross-linking between adjacent thymine molecules and the formation of thymine dimers. When thymine bases happen to sit next to each other, the pair is called a doublet. The dimerization of thymine doublets by UV can lead to inactivation of the DNA, or RNA, with the result that cell may be unable to reproduce effectively.

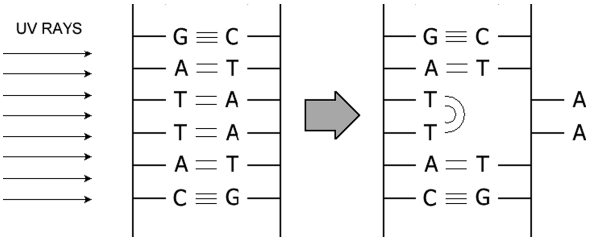


Fig. 2.3 Thymine dimers caused by UV absorption in adjacent nucleotides (thymine doublets)

The exact mechanism by which UV causes thymine dimers is not completely understood. Bhattacharjee and Sharan (2005) demonstrated that exposing *E. coli* DNA to UVC irradiation induced sparsely placed, dose-dependent, single strand breaks, and proposed that the conformational relaxation generates negative super-coiling strain on the DNA backbone.

It has been repeatedly demonstrated that thymine dimers produced by UV exposure result in the inactivation of bacteria and DNA viruses. A dose of 4.5 J/m^2 is reported to cause 50,000 pyrimidine dimers per cell (Rothman and Setlow 1979). It has been reported that 100 J/m^2 induces approximately seven pyrimidine dimers per viral genome in SV40, which is sufficient to strongly inhibit viral DNA synthesis (Sarasin and Hanawalt 1980). Thymine dimers form within 1 picosecond of UV excitation provided the bases are properly oriented at the instant of light absorption (Schreier et al. 2007). Only a few percent of the thymine doublets are likely to be favorably positioned for reaction and dimerization at the time of UV excitation. Figure 2.4 illustrates the dimerization process for a thymine doublet with the appropriate orientation.

The two most common conformations of DNA are called A-DNA and B-DNA. Molecular orientations can vary due to A and B conformation and vibrational or other movement in the DNA molecule. The average twist angle between successive base pairs differs between the A conformation and the B conformation is only a few degrees. The smaller amount of conformational variation in A-DNA vs. B-DNA explains the greater resistance of A-DNA to cyclobutane pyrimidine dimer

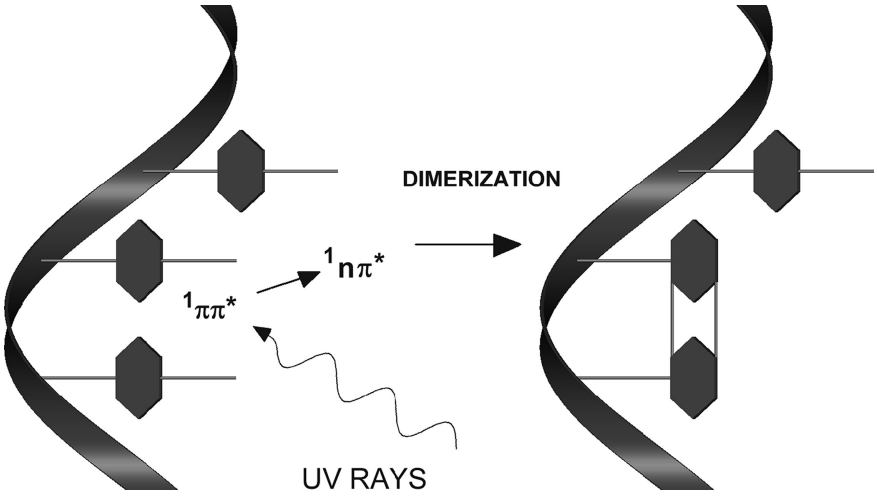


Fig. 2.4 The photodynamics of dimerization. A single strand of the DNA sugar-phosphate backbone is shown with thymine nucleotides. UV excitation populates the singlet state $\pi\pi^*$, which decays into the singlet $n\pi^*$ state (*left*). All energy is converted internally to form a thymine double hydrogen bond (*right*)

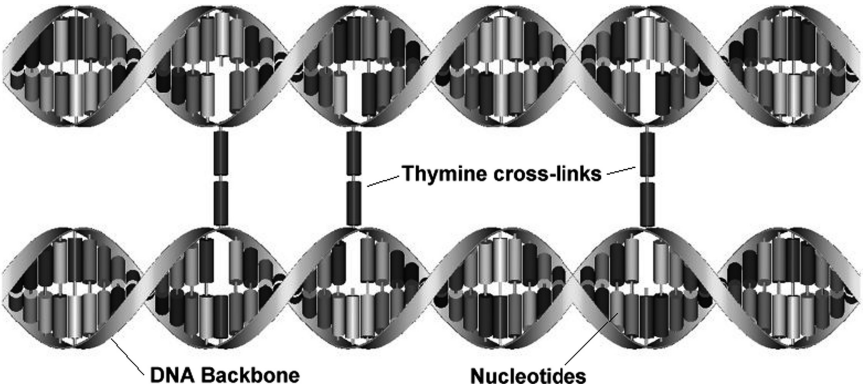


Fig. 2.5 Cross-linking between thymine nucleotides (or uracil nucleotides in the case of RNA viruses) can occur between adjacent strands of DNA (or RNA). It can also occur between the DNA (RNA) and the proteins of the capsid, for viruses

formation (Schreier et al. 2007). That is to say, the more dense packing of bases and lower flexibility in the B-DNA form ensures a higher probability that thymine doublets will be available for dimerization.

The exact sequences of thymines, cytosines, adenines, and guanines in DNA can directly impact the probability of dimerization. Adjacent pyrimidines (thymine and cytosine) are considerably more photoreactive than adjacent purines (adenine and guanine). Becker and Wang (1989) found that 80% of pyrimidines and 45% or purines form UV photoproducts in double-stranded DNA.

In addition to cross-links between adjacent thymines, UV may also induce cross-links between non-adjacent thymines, as illustrated in Fig.2.5. Cross-linking can also occur between the nucleotides and the proteins in the capsid of viruses, damaging the capsid of DNA viruses.

Cross-linking can also occur with cytosine and guanine, but the energy required is higher due to their having three hydrogen bonds instead of two for thymine/adenine bonds, and so thymine dimers predominate. Besides cross-links with adjacent thymine nucleotides and with thymine in adjacent strands of DNA, thymine may also form links with proteins, including proteins in the capsid (in the case of viruses) as shown in Fig. 2.6. Other biological molecules with unsaturated bonds like coenzymes, hormones, and electron carriers may be susceptible to UV damage.

In RNA, whether in prokaryotic cells, eukaryotic cells, or viruses, uracil takes the place of thymine. Inactivation of RNA viruses involves cross-linking between the uracil nucleotides and the creation of uracil dimers (Miller and Plageman 1974). Uracil dimers may also damage the capsid of RNA viruses. Some limited quantitative data is available on the specific nature of DNA damage produced by UV absorption. Miller and Plageman (1974) demonstrated that ultraviolet exposure of Mengovirus caused rapid formation of uracil dimers and that this appeared to be the

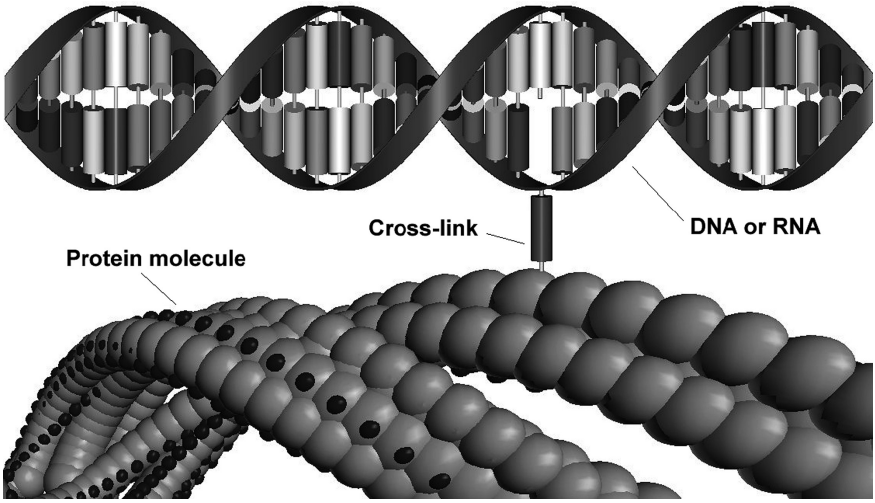


Fig. 2.6 Thymine dimerization can also occur between DNA/RNA and adjacent protein molecules, such as in cell cytoplasm or the capsid of a virus

primary cause of virus inactivation (i.e. loss of infectivity). A maximum of about 9% of the total uracil bases of the viral DNA formed dimers within 10 min of UV irradiation. Results also indicated that viral RNA became covalently linked to viral protein as a result of irradiation. A slower process of capsid destruction also occurred in which capsid proteins were modified and photoproducts were formed. UV irradiation of the virus also caused covalent linkage of viral RNA to viral polypeptides, apparently due to close proximity between the RNA and proteins in the capsid. The amount of protein covalently linked to the RNA represented not more than 1.5% of the total protein capsid. Smirnov et al. (1992) studied Venezuelan equine encephalitis (VEE) under UV irradiation and found evidence suggesting that the formation of uracil dimers led to extensive contacts of the RNA with protein in the nucleocapsid.

Viruses containing many thymine dimers may still be capable of plaque formation (Rainbow and Mak 1973). An *E. coli* chromosome exposed to UVB produced pyrimidine photoproducts in the following proportions: 59% thymine dimers, 34% thymine-cytosine dimers, and 7% cytosine-cytosine dimers (Palmeira et al. 2006).

Figure 2.7 shows the rate at which uracil dimers form under irradiation, shown in terms of the uracil bonds remaining intact in RNA. This plot is shown alongside the decay rate for Mengovirus. It can be observed that the virus is rapidly inactivated while the formation of uracil dimers proceeds relatively slowly. The scale of the chart is limited, but the virus goes through six logs of reduction before 9% of the uracil is cross-linked. Clearly, it takes but little cross-linking to inactivate a virus. The ratio of the microbial inactivation rate to the dimer production rate should be a constant for any given species. Theoretically, each species should have a characteristic inactivation rate that is a function of the dimerization probability.

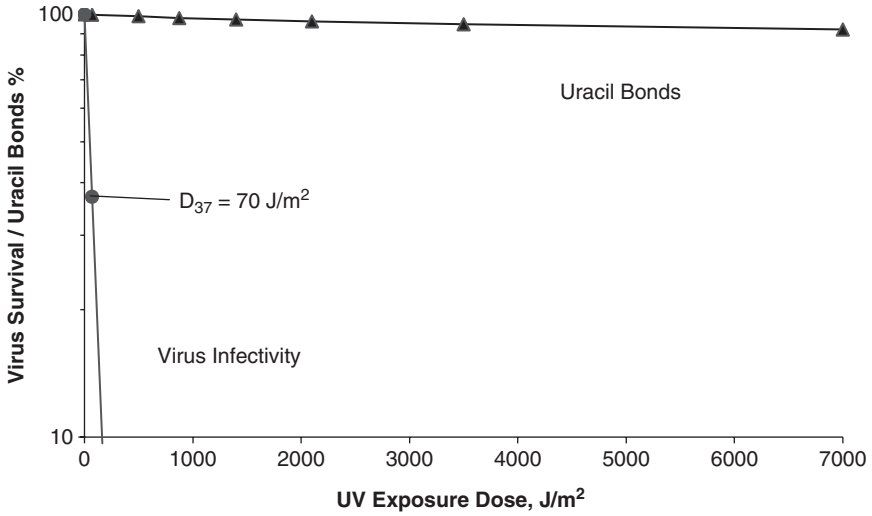


Fig. 2.7 Survival of Mengovirus under UV irradiation, plotted along with the percentage of intact uracil bonds in viral RNA. Based on data from Miller and Plageman (1974)

2.3 UV Absorption Spectra

An absorption spectrum is a quantitative description of the absorptive capacity of a molecule over some specified range of electromagnetic frequencies. The absorption of ultraviolet light by a molecule will result in altered electronic configuration, conversion into radiant energy, rotation, and vibration. When these energy levels are at a minimum the molecule is in a ground state. The energy imparted to a molecule by UV absorption produces an excited state. The capacity of a molecule to absorb UV energy over a band of wavelengths is described in terms of an absorption spectrum. The intensity of absorption is generally expressed in absorbance or optical density. The intensity of an absorption band is directly related to the probability that the particular transition will take place when a photon of the right energy comes along. Figure 2.8 represents the absorption spectra for the four DNA bases, which have peaks in the UVC band, and also below 220 nm, which is in the VUV range. Thymine and cytosine both have strong peaks near 265 nm.

Pyrimidines (thymine, cytosine, and uracil) absorb about ten times more UV than purines. The quantum yield at 254 nm is $\phi \sim 10^{-3}$ for pyrimidines and for purines $\phi \sim 10^{-4}$. The capacity of a molecule to absorb light of a particular wavelength depends on both the electronic configuration of the molecule and on its available higher energy states (Smith and Hanawalt 1969). An absorption spectrum may be regarded as a summation of a series of individual absorption bands, each corresponding to a transition between two particular electronic configurations (Hollaender 1955). This transition typically occurs when an orbital electron is raised from the normal ground state to an excited state. These transitions occur only in

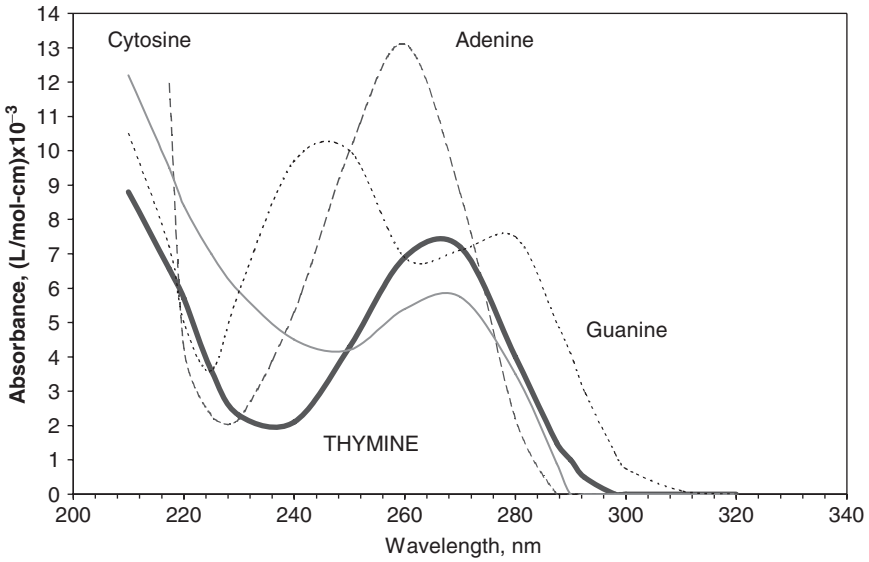


Fig. 2.8 Comparison of response spectra for the four main nucleotides

discrete jumps, and therefore only a specific quantum of energy can be absorbed. The electronic configuration of an excited molecules can be a very transitory event, and only those excitations of sufficient duration will have a high probability of absorption.

An absorption band may be described by the width of the band (or the range of wavelengths) and the degree of absorption (absorptivity). The width of the band is defined as the spectral separation between the points of half-maximum (50%) absorption. The width of a band is inversely dependent upon the duration of the excited electronic state. Integration of the absorption band over the width determines the probability that the particular transition will occur when a photon of the right energy comes along.

The absorption spectrum is typically measured by beaming light through a transparent solution containing microbes or molecules and comparing it against the pure solution. The transmittance (or transmissivity), T, of a solution is defined as:

$$T = \frac{I}{I_0} \tag{2.1}$$

where

I = irradiance of light exiting the solution, W/m^2
I_0 = irradiance of light entering the solution, W/m^2

Beer's Law establishes a relationship between the transmittance in Eq. (2.1) and the absorbance, A, as follows:

$$T = \frac{I}{I_0} = 10^{-A} \tag{2.2}$$

The absorbance (or absorptivity), also called the optical density, OD, is defined as:

$$A = \varepsilon lc \tag{2.3}$$

where

ε = molar absorptivity, liters/mole-cm
l = thickness of the solution, cm
c = concentration of solute, moles/liter

An alternate form of Beer's Law is:

$$\frac{I}{I_0} = e^{-nsl} \tag{2.4}$$

where

n = number of molecules per unit volume
s = absorption cross-section, m^2 or μm^2

The absorption cross-section represents the product of the average cross-sectional area of the molecule and the probability that a photon will be absorbed. The absorption cross-section is related to the molar absorptivity by the following relation:

$$s = 3.8x10^{-21}\varepsilon \tag{2.5}$$

Strong absorption bands in the ultraviolet region correlate with molecular structures containing conjugated double bonds. Ring structures, such as the pyrimidines and purines, exhibit particularly strong absorption and can define the overall absorption spectrum of DNA. Von Sonntag (1986) reports that DNA has a peak of UV absorption not only at 265 nm but also at 200 nm. Most of the absorption at 200 nm occurs in the DNA backbone molecules of ribose and phosphate. At 265 nm, most of the absorption occurs at the nucleotide bases, thymine and adenine, and cytosine and guanine, but dimers of thymine are by far the most common UV photoproducts. In RNA-based microbes, uracil is also involved in UV absorption in place of thymine but not necessarily to the same degree. Figure 2.9 compares the absorption spectrum of uracil with that of thymine. It can be observed that not only are the absorption spectra very similar for these nucleotides, but that the mercury emission line at 254 nm is more nearly aligned with the peak absorption of uracil.

Carbohydrates make up about 41% by weight of nucleic acids, but they show essentially no UV absorption above about 230 nm and would not be expected to

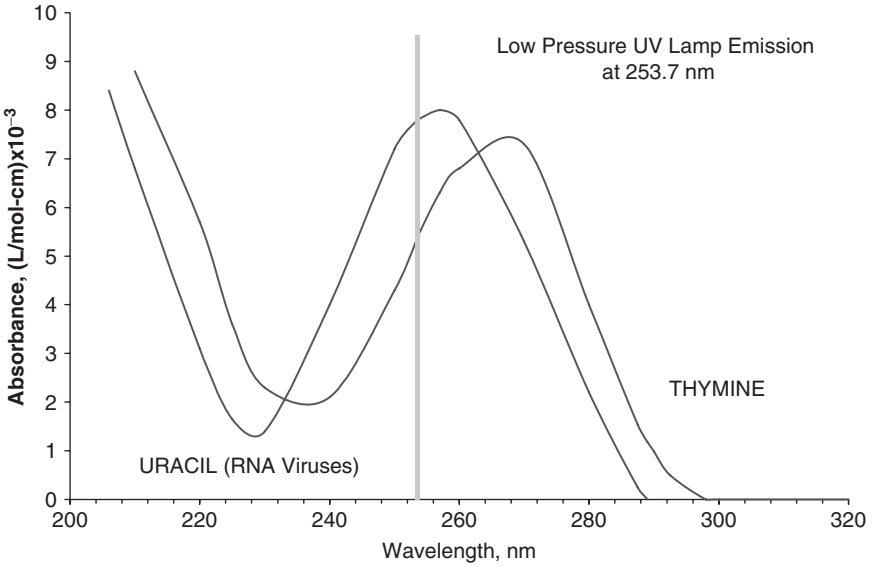


Fig. 2.9 Comparison of thymine UV absorption spectra with uracil, the nucleotide that takes the place of thymine in RNA viruses

participate in photochemical reactions at around 254 nm. However, certain photochemical processes that produce uracil radicals can result in chemical alterations to the carbohydrates of nucleic acid (Smith and Hanawalt 1969).

The ultraviolet absorption spectrum of a polymer is not necessarily the linear sum of its constituents. This nonadditivity is referred to as *hyperchromicity*. If the absorbance of a given oligonucleotide is higher than its constituents molecules, it is hyperchromic. Hyperchromicity is largely explained by the coulombic interaction of the ordered bases in the polymer. Hyperchromicity is a kind of resonant electronic effect in which the partial alignment of transition moments by base stacking results in coupled oscillation. A relatively small number of bases in a DNA strand are required for such coupling and about 8–10 base pairs can exhibit roughly 80% of the hyperchromism of an infinite helix. Becker and Wang (1989) present data that indicates that hyperchromicity may add about twice the number of photoproducts when strings of eight or more thymines occur sequentially.

2.4 UV Photoprotection

Microbes have various mechanisms by which they can protect themselves from UV exposure, including nucleocapsids and cytoplasm which may contain UV absorbing proteins (i.e. dark proteins). The absorption of UV in any surrounding complex of proteins will reduce the density of photons reaching the nucleic acid and thereby provide photoprotection. Comparisons of virus inactivation with

inactivation of purified DNA show the absorption spectrums are not identical, the implication being that UV is absorbed in the envelope, the nucleocapsid, or other protein-laden constituents of the viroid, although in some cases the nucleic acid is more resistant in isolation (Zavadova et al. 1968, Furuse and Watanabe 1971, Bishop et al. 1967). In bacteria, the cytoplasm may offer photoprotection due to its UV absorptivity. Unrau et al. (1973) have suggested that there is a *in vivo* shielding effect in *Bacillus subtilis* since dimer formation is doubled when its DNA is irradiated separately, although they do not attribute this effect to the cytoplasm. Fungal spores are among the most resistant microbes and they often have melanin-containing dark pigmented conidia. The photoprotective component melanin increases the survival and longevity of fungal spores (Bell and Wheeler 1986). *Aspergillus niger* conidia are more resistant to UV due to the high UV absorbance of their melanin pigments (Anderson et al. 2000). Various studies on fungi have suggested that lighter-pigmented thin-walled conidia are more susceptible to UV than thicker-walled dark-pigmented conidia (Boyd-Wilson et al. 1998, Durrell and Shields 1960, Valero et al. 2007). UV scattering (addressed later) can also contribute to photoprotection.

Figure 2.10 illustrates photoprotection mechanisms in viruses – UV scattering by the envelope, UV absorption by the envelope, and UV absorption by the nucleocapsid the latter being mostly negligible. UV scattering, occurs when the particle is in the Mie scattering size range, and the effective scattering cross-section my be much larger than the actual physical cross-section of the particle (see Sect. 2.13).

Chromophores are chemical groups in molecules that are capable of absorbing photons. Polyatomic molecules have fairly broad absorption bands. In proteins, the molecular groupings which give rise to absorption are principally amino acids,

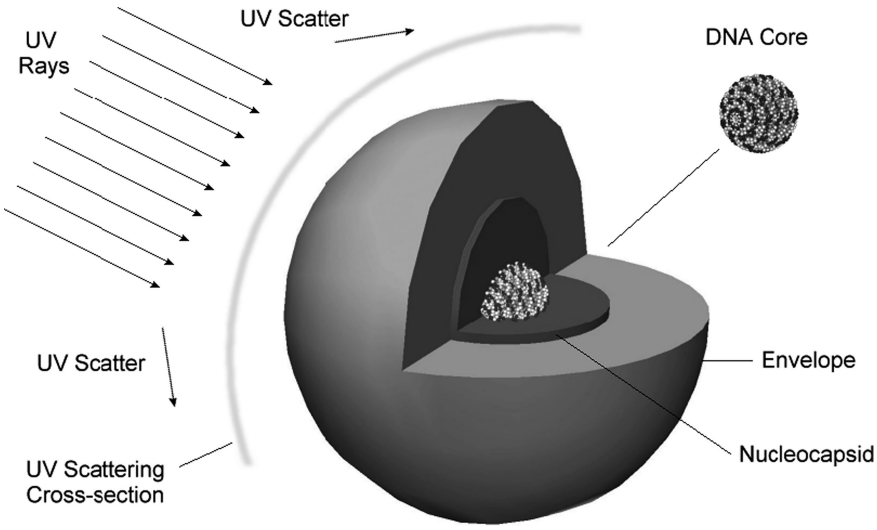


Fig. 2.10 Schematic illustration of the levels of photoprotection of an enveloped virus, including the UV scattering cross-section, the envelope, and the nucleocapsid

which have absorption peaks at about 280 nm (Webb 1965). For the nucleic acids, the chromophores responsible for the absorption peak around 265 nm are the bases, the purines and pyrimidines, the dimers of which are considered primary products of the biocidal action of UV. The chromophores that are likely to confer protection to these bases are those in the cytoplasm or cell wall of bacteria, in the capsid or protein coat, if any, of the virus, and the spore coat and cortex of a spore.

Some amino acids are optically transparent above UV wavelengths of about 240 nm, while others, the chromophores, have high molar absorptivities at or near 253.7 nm. The molar absorptivity of a compound is the probability that the wavelength will be absorbed. Figure 2.11 shows the molar absorptivity of amino acids at 253.7. These might be called 'dark proteins' because of their relatively high absorbance for UV wavelengths. Microbes that contain higher proportions of chromophores will likely absorb UV photons that would otherwise be absorbed by DNA and cause dimerization. However, degradation of the proteins in a cell may also contribute to cell death.

Enzymes are proteins that function as efficient biological catalysts that increase the rate of a reaction. Biological systems depend on enzymes to lower the activation energy of a chemical reaction and thereby facilitate processes of growth, and repair (Atlas 1995). Enzymes consist of various proportions of the amino acids including those in Fig. 2.11 and their quantum yield will vary accordingly. The quantum yield indicates the probability that absorbed UV light will induce a chemical change. Table 2.1 lists several enzymes, their chromophore constituents, and their measured quantum yields, based on data from Webb (1965).

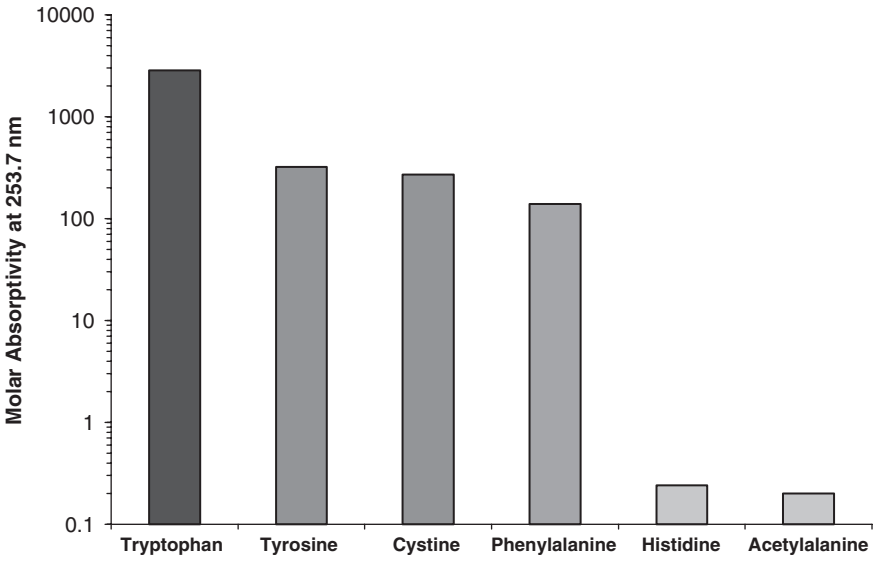


Fig. 2.11 Molar absorptivity of 'dark proteins' or amino acids with a relatively high probability of absorbing light at 253.7 nm. Based on data from Webb (1965)

Table 2.1 Quantum yields for enzyme inactivation by UV at 253.7 nm

Protein	Chromophores	Quantum Yield
Ribonuclease	$Cys_4 \cdot His_4 \cdot Phe_3 \cdot Tyr_{16}$	0.027
Lyzsozyme	$Cys_5 \cdot His_1 \cdot Phe_3 \cdot Tyr_8 \cdot Tyr_2$	0.024
Trypsin	$Cys_6 \cdot His_1 \cdot Phe_3 \cdot Tyr_4 \cdot Tyr_4$	0.015
Insulin	$Cys_{18} \cdot His_{12} \cdot Phe_{18} \cdot Tyr_{24}$	0.015
Subtilisin A	$His_5 \cdot Phe_4 \cdot Try_1 \cdot Tyr_{13}$	0.007
Japaneses Nagarse	$His_6 \cdot Phe_3 \cdot Try_4 \cdot Tyr_{10}$	0.007
Subtilisin B	$His_6 \cdot Phe_3 \cdot Try_4 \cdot Tyr_{10}$	0.006
Chymotrypsin	$Cys_5 \cdot His_2 \cdot Phe_6 \cdot Tyr_7 \cdot Tyr_4$	0.005
Pepsin	$Cys_3 \cdot His_2 \cdot Phe_9 \cdot Tyr_4 \cdot Tyr_{16}$	0.002
Carboxypeptidase	$Cys_2 \cdot His_8 \cdot Phe_{15} \cdot Tyr_6 \cdot Tyr_{20}$	0.001

Since enzymes are catalysts, they are not consumed during normal biological processes and are relatively few in number, and may therefore contribute little protective effect. However, their destruction will inhibit repair processes during or after UV exposure and may limit the effective UV rate constant. Inactivation of enzymes can be higher at wavelengths other than 253.7 and broadband UV irradiation is reported to be more effective at eliminating repair enzymes than narrow band UVC (Zimmer and Slawson 2002, Hu et al. 2005). Enzymes are associated with bacterial cells and not with viruses, although some viruses (i.e. bacteriophages) may employ enzymes for self-repair from the cells they parasitize.

Powell (1959) used optical density measurements to estimate the reduction of UV absorption at 265 nm in Herpes Simplex virus due to shielding by the host cell. The cells had a radius of 6 μm and this thickness was estimated via the Beer-Lambert law to result in a transmission, including corrections for scattering, of 40%, which is an attenuation of 60%. Such levels of photoprotection may be possible for other bacterial cells in this size range. Viruses, however, have such relatively thin coats that it seems unlikely that any chromophores present would provide any significant photoprotection.

2.5 Covalent Bonding and Photon Interaction

Chemical bonding between atoms occurs when a single electron is shared between more than one atomic nucleus. The wave function between the two atomic orbitals is called a molecular orbital. Two kinds of bonding molecular orbitals may be involved in complex molecules, σ (sigma) orbitals, and π (Pi) orbitals. The σ orbitals are localized around two nuclei and the π orbitals are nonlocalized and may involve two or more nuclei. The larger the nonlocalized orbital the more spread out is the electron probability distribution, and the longer the wavelength for electrons in that orbital. The longer wavelength means lower energy and more stability. Conjugated ring structures like the pyrimidines and the purines have large nonlocalized π orbitals and stable structures (Smith and Hanawalt 1969). A single bond is usually

a σ bond while double bonds may involve both σ and π orbitals. There can be no free rotation about a double bond.

An incoming UV photon will promote an electron to an orbital of higher energy, which may be a new set of antibonding orbitals called σ^* or π^* orbitals, which may result in weakening of the bonds. A $\pi\pi^*$ transition involves the excitation of a π electron into a π^* state. The $\pi\pi^*$ transitions are responsible for the most intense absorption bands in molecular spectra.

An ultraviolet photon at 253.7 nm has an energy of 4.9 eV and if this were totally converted to vibrational energy it would be sufficient to break chemical bonds, but the energy becomes distributed over many possible vibrational modes. Upon absorption of a UV photon, which may take 10^{-15} s, molecules may briefly exist in an excited state before the energy is dissipated, either by re-emission or by vibration and rotational modes. Differential quantized modes of vibration can be represented as levels of potential energy, the first of which is the singlet state. The triplet state may also be stimulated and it is one in which the system has two electrons with unpaired spins. The triplet state may persist for 10^{-3} s. The triplet state does not, as a rule, degrade directly back to the ground state, but it allows more time for photochemistry to occur and the probability of a chemical reaction is briefly increased.

2.6 UV Photoproducts

Thymine dimers are formed when two thymine molecules are cross-linked between their respective 5 and 6 carbon atoms, forming a cyclobutane ring. There are six possible isomers of the thymine dimer. Dimers can both be formed by UV exposure and separated or monomerized by UV. At longer UV wavelengths (about 280 nm) the formation of the dimer is favored while at shorter wavelengths (about 240 nm) monomerization occurs due to differences in the absorption spectra of thymine and its dimer and in the quantum yields for the formation and splitting of the dimer. The maximum yield of cyclobutane dimers is dependent on equilibrium between the formation and splitting of dimers. The reversal of dimerization by wavelengths of UV or visible light is known as photoreactivation, as is the repair of dimers by enzymes.

Thymine dimers can be formed by wavelengths of light that are not directly absorbed if the thymine molecule is in close proximity to other molecules that absorb these wavelengths. This process is called molecular photosensitization and it requires that the triplet state of the absorbing species (the photosensitizer) be slightly higher in energy than the triplet state of the thymine. Upon absorption, the triplet energy of the photosensitizer is transferred to the thymine molecule where it may induce dimerization. There are at least five other dimers of the natural pyrimidines, including cytosine dimers, cytosine-thymine dimers, uracil dimers, uracil-thymine dimers, and uracil-cytosine dimers. Cytosine dimers are formed at lower rates than thymine dimers and are readily converted to uracil dimers.

Cytosine hydrate, a water addition photoproduct, can be formed in RNA and single-stranded DNA but is not commonly found in irradiated double-stranded DNA (Smith and Hanawalt 1969). Uracil hydrates can be formed from the excited singlet

state. Uracil can be derived from the monomerization of cytosine dimers. The formation of hydrates is greatly favored in single-stranded DNA.

Many other pyrimidine photoproducts, besides hydrates and cyclobutane dimers, can be produced and may be at least partly responsible for damage to nucleic acids or to a cell. Chief among these is the spore photoproduct, also called the azetane thymine dimer. The spore photoproduct, so named because it was first noted in spores, can be formed from as much as 30% of the thymine. The spore photoproduct is a type of thymine dimer that cannot be photoreversed (although it can be repaired) and the yield of this product can approach the maximum determined from the number of thymines that are nearest neighbors in DNA. In the normal B conformation of DNA the planes of the bases are parallel to each other and perpendicular to the helical backbone, and the cyclobutane dimers are favored. In the dehydrated A conformation, which is the form in which DNA is held by spores, the planes of the bases are parallel but they are inclined at an angle of 70° to the axis of the helix, a conformation which favors the spore photoproduct.

DNA cross-linking can occur under UV irradiation and this apparently involves cyclobutane dimers. Cross-linking can be highly fatal to DNA but such lesions do not appear to play a major role in UV inactivation since the cyclobutane dimers and other photoproducts are largely responsible for the inactivation effect. Per Edenberg (1983), the hypothesis that DNA replication forks are halted upon encountering thymine dimers in the template strand is consistent with data on inhibition of Simian virus replication by ultraviolet light. Per Stacks et al. (1983), the percentage of repaired and completed molecules containing dimers increases with time after irradiation ceases, and they postulate that the cellular replication machinery can accommodate limited amounts of UV-induced damage and that the progressive decrease in simian virus 40 DNA synthesis after UV irradiation is due to the accumulation in the replication pool of blocked molecules containing levels of damage greater than that which can be tolerated.

DNA may also cross-link to proteins in the cell wall, nucleocapsid, or cytoplasm, forming potentially fatal lesions. Amino acids that may contribute to photoreactivity in DNA and that may impact cross-linking include cysteine, cystine, tyrosine, serine, methionine, lysine, arginine, histidine, tryptophan, and phenylalanine. Under dry conditions (A DNA) the yield of thymine dimers is greatly decreased but there is an increase in the amount of DNA cross-linked to protein (Smith and Hanawalt 1969). Per Becker and Wang (1989), the ability of UV to damage a given base in DNA by inducing dimers or photoproducts is determined by two factors, the DNA sequence and the flexibility of DNA. Upon absorption of a UV photon, only those bases that are in a geometry capable of easily forming a photodimer can photoreact.

2.7 DNA Conformation

DNA molecules can exist in two conformations, A or B (Eyster and Prohofsky 1977). The UV susceptibility differs between the conformations. In the A conformation the bases are tilted with respect to the helix axis. In the B conformation the

bases are roughly perpendicular to the double helix axis. The interaction of electrostatic and van der Waals forces at the molecular level are influenced by the presence of water. The B conformation is fully hydrated (i.e. in solution or even in air at 100% relative humidity) and the A conformation could therefore be considered to be the dehydrated state. The dry A conformation shrinks in length in comparison with the wet DNA, and transitions through a phase when the population is mixed with cells in both A and B conformations. In general, microbes in high relative humidity or in water (B-DNA), have a higher resistance to UV (Peccia et al. 2001). Microbes transition from A to B when humidity or moisture increases and it is possible that the more compact A conformation (see Fig. 2.12) lends itself to more cross-links, but it is also possible that the presence of moisture or bound water provides extra protection or improved self-repair mechanisms, or that a combination of these factors is responsible for the difference in UV susceptibility between the A and B conformation.

DNA undergoes conformational transition from the B form to a disordered form as the relative humidity is lowered from about 75% to 55%. At the lower relative humidity, the dry condition, the bases are no longer stacked one above the other but are slightly angled with reference to the helix and DNA films equilibriated between 75 and 100% RH show no conformational changes and are assumed to be entirely in the B-form (Rahn and Hosszu 1969). The yield of thymine dimers remains constant

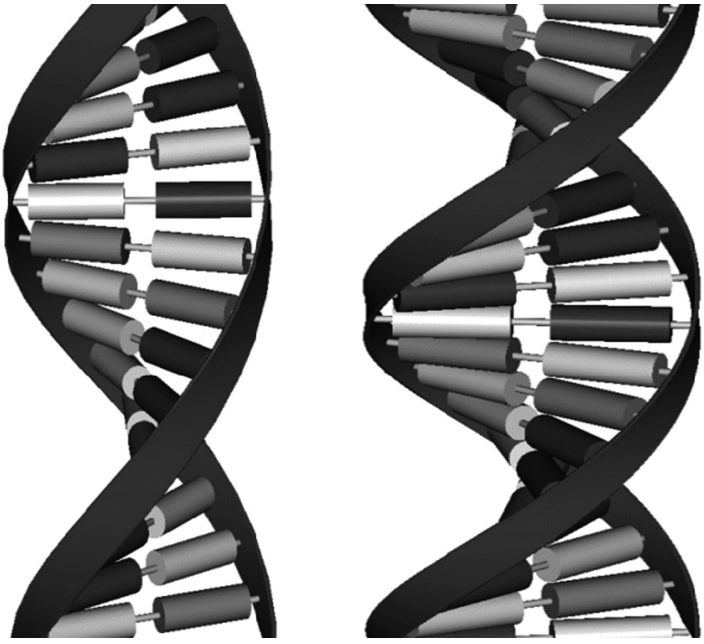


Fig. 2.12 DNA can exist in two states, the hydrated B conformation (*left*), and the dehydrated A conformation (*right*) with tighter packing of the nucleotides

in this range and is the same as that found in solution. Although most air-based UV rate constants in the range of 75–100% RH tend to converge towards water-based UV rate constants, they do not appear to become equivalent. One possible reason for this nonequality is that the refractive index of UV in air is different from that in water, causing differences in the photoprotective effect due to UV scattering.

At lower relative humidities, DNA transitions to the A form with more order and less probability of contact between thymine bases during irradiation, and there is a reduction in the rate of thymine dimer formation. The bases have different affinities for water and can trap available water molecules. The purines have two principal hydration sites in each of the major and minor DNA grooves, while the pyrimidines have only one hydration site in each groove (Neidle 1999). The individual hydration sites for bases in the A and B conformations are much the same, the major difference being that in B-DNA water is found in both grooves equally while in A-DNA more water is found in the major groove than in the minor groove.

The B form of DNA contains more bound water molecules, including those that attach to the internal grooves of DNA. The A form of DNA leads to the exposure of more hydrophobic portions of the sugar units of the backbone compared to the B form (Neidle 1999). In A-DNA, water molecules are displaced from the shallow groove, creating a local environment of low water content that favors and stabilizes the A form. The mere presence of water is insufficient to induce a conversion from A-DNA to B-DNA, instead, the water molecules must be able to contact the DNA directly over its entire length. In spores, the DNA is typically maintained in a tightly packed hydrophobic environment which prevents the DNA from going into the B-form even under high humidity or in solution which partly explains their higher UV resistance. The interaction of water molecules with DNA indicates that water forms an integral part of DNA structure and stability, and can impact UV inactivation rates.

2.8 Photon Density and Single-Hit Concepts

It can be informative to consider UV energy incident upon a microbe in terms of the number of photons, or the photon density per unit surface area. Each photon carries an amount of energy called a quantum, ϵ, determined from quantum mechanics as (Modest 1993):

$$\varepsilon = hv \tag{2.6}$$

where

h = Planck's constant, 6.626×10^{-34} Js
v = frequency, cycles per sec or Hz

The energy of a mole of photons is called an Einstein. It is defined as:

$$Einstein = Nhv \tag{2.7}$$

where N = Avogadro's number, 6.022×10^{23}

The frequency of UVC light at a wavelength of 253.7 nm is 1.18×10^{15} Hz, and the energy of UVC is computed to be 7.819×10^{-19} J/photon. Inverting this value gives us 1.279×10^{18} *photons/Joule*. A UV dose of 10 J/m^2 produces 1.279×10^{19} photons per m^2. A virus of 0.1 micron diameter has a cross-sectional area of 3.14×10^{-14} m and will be subject to the passage of about 401,000 photons when exposed to 10 J/m^2, which is sufficient to highly inactivate most viruses.

Despite of the vast number of photons passing through a virus, only an extremely small number are absorbed. Klein et al. (1994) report that Vaccinia virus experienced some 15 dimers per genome after a dose of 8 J/m^2. Miller and Plageman (1974) found that 1.7 uracil dimers were formed per PFU of inactivated Mengovirus. Based on data from Rainbow and Mak (1973), 100 J/m^2 produced about 102 dimers in Adenovirus Type 1, and a lethal hit (D_{37}) involved 30 thymine dimers and one single strand break. Per Ryan and Rainbow (1977), 0.3 dimers and 3.5 uridine hydrates were formed per three lethal hits in herpes simplex virus. Cornelis et al. (1981) reports that UV dosing of Parvovirus H-1 produced 10 dimers per genome, and that 80 dimers were formed in Simian virus SV40. Peak and Peak (1978) report that a frequency of 0.3 single-strand breaks occurs per lethal hit in phage T7. Sarasin and Hanawalt (1978) report that a 100 J/m^2 dose results in 7 pyrimidine dimers per the SV40 genome. Studies with phage T7 DNA suggest a rate of damage of 0.21 sites per 10,000 base pairs per 10 J/m^2 (Hanawalt et al. 1978). Clearly, very few photons out of the total interact photochemically with the nucleotides, implying that virions are virtually transparent to UV.

The First Law of Photochemistry (Grotthus-Draper Law) states that light must be absorbed by a molecule before any photochemical reactions can occur. The Second Law of Photochemistry (Stark-Einstein Law) states that absorbed light may not necessarily result in a photochemical reaction but if it does, then only one photon is required for each molecule affected (Smith and Hanawalt 1969). Since not every quantum of incident energy is absorbed by a molecule, there is an absorption efficiency that describes photochemical absorptivity. This efficiency is called the quantum yield, ϕ, and it is defined as:

$$\Phi = \frac{N_c}{N_p} \tag{2.8}$$

where

N_c = Number of molecules reacting chemically
N_p = Number of photons absorbed

The number of photons absorbed is sometimes specified in Einsteins, or moles of photons as defined in Eq. (2.7). Quantum yields may be extremely low for macromolecules of low absorptivity. Since most of these photochemical excitations of molecules do not lead to chemical reactions, energy is dissipated by various means. Light may be re-emitted at a different wavelength, and energy absorption may result in molecular vibrations that translate into heat.

The inactivation of nucleic acids involves quantum yields on the order of 10^{-3} to 10^{-4} and at the UV doses typically applied, it is clear that a relatively small number

of photons photoreact, and that they are absorbed at discrete genomic sites – normally those bases that produce dimers. Based on UV inactivation studies of *E. coli*, only about 0.025% of the DNA molecule is photochemically altered at the point that 99% of *E. coli* are killed (Smith and Hanawalt 1969). A UV dose of 180 J/m^2 dimerizes only 0.1% of the total thymine. This dose represents (180 J/m^2)(1.279×10^{18} photons/J) = 2.3×10^{20} photons/m^2. With a diameter of about 0.5 microns, and a DNA size of 5490 kb, this would imply that 1.8×10^8 photons impinged upon the bacterial cell to produce about 1372 photochemical reactions. Even if we ignore the cell space that is not occupied by the bacterial DNA, it is clear that it takes a relatively large number of photons to induce a relatively limited number of photoreactions sufficient to inactivate a cell. Figure 2.13 shows a comparison of the typical number of photons necessary for certain processes to provide some further perspective.

Rauth (1965) measured the inactivation and absorption cross-sections of several viruses and computed the quantum yields to be in the range of 5–65×10^{-4}. The quantum yield is computed from the inactivation cross-section divided by the absorption cross-section as follows:

$$\Phi = \frac{\sigma}{S} \tag{2.9}$$

where

σ = inactivation cross-section, m^2/photon
S = absorption cross-section, m^2/photon

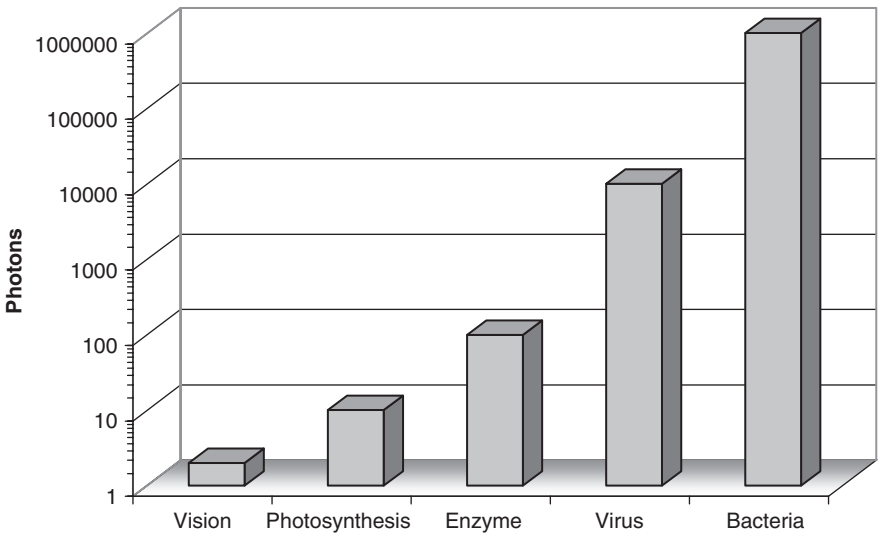


Fig. 2.13 Typical numbers of absorbed photons necessary for certain processes or to inactivate enzymes, bacteria, and viruses. Based on Setlow and Pollard, 1962

The absorption cross-section can be determined by various means but the measurement typically involves putting specific densities of cells (or virions) in solution and measuring the difference in irradiance that passes through the solution containing cells versus a solution containing no cells. The inactivation cross-section is equivalent to the UV rate constant, which is normally given in units of m^2/J.

2.9 Photochemistry of RNA Viruses

RNA viruses are characteristically in the A conformation and this partly dictates the type of photoproducts produced under UV irradiation. The main photoproducts produced are pyrimidine hydrates and cyclobutadipyrimidines, while other photoproducts, like altered purines and pyrimidine dimers, occur at much lower rates, if at all (Fraenkel-Conrat and Wagner 1981). Remsen et al. (1970) found that inactivation of R17 phage at 280 nm was a log-linear function of the number of uridine hydrates formed and that no cyclobutapyrimidines were formed. RNA animal viruses and phages demonstrate little or no photoreactivation, and the photoreactive effects that have been observed are attributed to host cells (Fraenkel-Conrat and Wagner 1981). RNA viruses have no repair enzymes and any photoreactive effects may be due strictly to thermal, visible light, or near-UV light effects.

Much information on the photochemistry of RNA viruses has come from studies of Tobacco Mosaic Virus (TMV), and although plant viruses are not the subject of this text some useful information can be garnered from UV studies regarding the protective effect of protein coats. The quantum yield for inactivation of the whole TMV virus is barely 1% that of RNA at longer wavelengths of the absorption spectrum (Fraenkel-Conrat and Wagner 1981). The relative insensitivity to UV of the whole virus is a property of the coat protein, which modifies the UV photoproducts formed in RNA. Protein inhibits the formation of pyrimidine photoproducts (cyclobutadipyrimidines) and inhibits the formation of other photoproducts by reducing the quantum yield for photoreactions. This could occur through shielding of the RNA, through quenching of the excited states of RNA, and by surrounding the bases with a hydrophobic environment and limiting the mobility of the individual bases. Although the protein coat reduces the overall rate of photoreactions, it allows the formation of noncyclobutane-type dipyrimidines and of uridine hydrates. In irradiated TMV, the number of uridine hydrates formed was about two per lethal hit (D_{37} or 37% survival), while only about one dimeric photoproduct was formed.

RNA bacteriophages, which are viruses that infect bacteria, typically consist of a capsid composed principally of one protein, with small numbers of one or two other proteins. The action spectra of several RNA phages has been studied by Rauth (1965), who showed that the quantum yield for virus inactivation was approximately the same as the quantum yield for RNA inactivation, which suggests that not only is the RNA the primary target but that the protein coats of the phages studied contribute little protective effect. Under UV irradiation, a lethal hit for mengovirus (at 70 J/m^2) produced 1.7 uracil dimers but no apparent structural damage to the RNA (Miller and Plageman 1974).

Most RNA virus coats consist of one major protein and sometimes one or two other proteins. There are five known viral proteins, M, G, L, N, and NS. The protein of the common strain of TMV consists of three tryptophan and four tyrosine residues, both of which have high molar absorptivities (see Fig. 2.11). The coat protein itself may suffer UV photodamage and may become cross-linked to RNA, but the extent to which this contributes to overall inactivation may be of limited significance (Fraenkel-Conrat and Wagner 1981).

Double stranded RNA viruses tend to be much more resistant to UV exposure than single stranded RNA viruses, by almost an order of magnitude. Zavadova (1971) showed that the D_{90} for double stranded encephalomyocarditis virus RNA was about six times that for the single stranded version.

2.10 Photochemistry of DNA Viruses

UV irradiation of DNA produces photoproducts called pyrimidine dimers as well as non-dimer photoproducts. Dimers produced in DNA can consist of thymine:thymine, thymine:cytosine, and cytosine:cytosine. Thymine dimers, the most common photoproducts, were the focus of much early investigation in UV irradiation experiments. The number of thymine dimers produced per lethal hit in the DNA of phage ϕX174 is about 0.3 (David 1964). For coliphage lambda, about two dimers were produced per lethal hit (Radman et al. 1970). For phage T4, 10.2 dimers were formed per lethal hit (Meistrich 1972). For a given dose, more dimers are produced when AT-rich DNA is irradiated than GC-rich DNA, but this difference is not more than twofold. The relative efficiency of dimer formation in DNA is in the order TT > CT > CC (Setlow and Carrier 1966). However, this can vary with the thymine content since, for viruses with high GC content, the number of CC dimers produced under UV exposure can exceed the number of TT dimers (Matallana-Surget et al. 2008). Photoproducts other than dimers are also produced, including pyrimidine adducts, which occur at about a tenth of the frequency of dimers, and others which occur at even lower frequencies (Wang 1976). Cytidine-derived photoproducts include cytidine hydrate (or the deamination product, uridine hydrate) and cytosine dimer (deamination product, uracil dimer).

2.11 Photochemistry of Bacteria

The kinetics of bacterial inactivation by ultraviolet light are much the same as in DNA viruses since they contain DNA, except that many bacteria have more photoprotection and often have the ability to photorecover or photoreactivate. About 65 dimers are produced per every 10^7 nucleotides in the DNA of *E. coli*, for every J/m^2 of UV irradiation (Fraenkel-Conrat and Wagner 1981). The number of dimers formed varies from one species to another but the ultraviolet sensitivities of bacteria with varying GC content are not directly proportional to the TT frequency,

indicating that thymine dimers are not the sole cause of lethality. David (1973) inferred that for a constant G+C content (or T+A content), the sensitivity to UV radiation is a reciprocal function of the molecular weight of the genome, suggesting that the smaller the DNA molecule, the higher the probability that a hit would be lethal.

Bacteria invariably contain enzymes and other repair mechanisms that may allow for photoreactivation and photorecovery from UV exposure (Atlas 1995). The quantum yield for inactivation of an enzyme is approximately proportional to its cystine content and is roughly inversely proportional to its molecular weight (Smith and Hanawalt 1969). The latter is explained by the fact that the cystine content of proteins is inversely proportional to their molecular weight. The action spectrum for an enzyme can be resolved into the contributions from its constituent chromophores.

2.12 Photoreactivation

Photoreactivation is a natural process in which bacterial cells can partially recover from ultraviolet damage when visible and UV wavelengths of light reverse DNA damage by monomerizing cyclobutane pyrimidine dimers. It was first identified in *E. coli* by Prat (1936) and later demonstrated by Kelner (1949), and has since been noted to occur in many other bacteria. Photoreactivation is an effect that primarily operates on bacteria and spores. Viruses and certain bacteria seem to have very limited capability to self-repair or photoreactivate, including *Haemophilus influenzae, Diplococcus pneumoniae, Bacillus subtilis*, and *Deinococcus radiodurans* (Masschelein 2002). David et al. (1971) reports photoreactivation rates of 40–56% in mycobacteria. Little evidence exists for the photoreactivation of animal viruses since they lack enzymes although they may be photoreactivated by host-cell repair mechanisms (Samad et al. 1987). Photoreactivation has never been observed in animal virus RNA (Bishop et al. 1967). The photoreactivation effect may be dependent on RH, with the effect possibly absent when RH is less than approximately 65%. Evidence suggests that the conformation change in DNA that occurs at higher RH may allow microbes to experience photoreactivation (Rahn and Hosszu 1969, Munakata and Rupert 1974).

Many bacterial cells possess repair enzymes that can repair gaps and defects in the DNA. Thymine dimers formed by UV irradiation of DNA are hydrolyzed by specific DNases and are replaced with correct sequences by repair enzymes (Guschlbauer 1976). The maximum yield of thymine dimers in irradiated bacterial cells depends on the wavelength as well as the conditions (i.e. RH%). After a sufficient UV dose has been imparted to the bacteria a steady state is reached in which the relative numbers of dimers do not change (Smith and Hanawalt 1969). Dimer formation is a reversible process and thymine dimers may revert to free thymines via the absorption of UV and visible light. Photoreactivation cannot completely reverse damage to DNA since UV may cause other types of photoproducts but it can effectively limit UV damage.

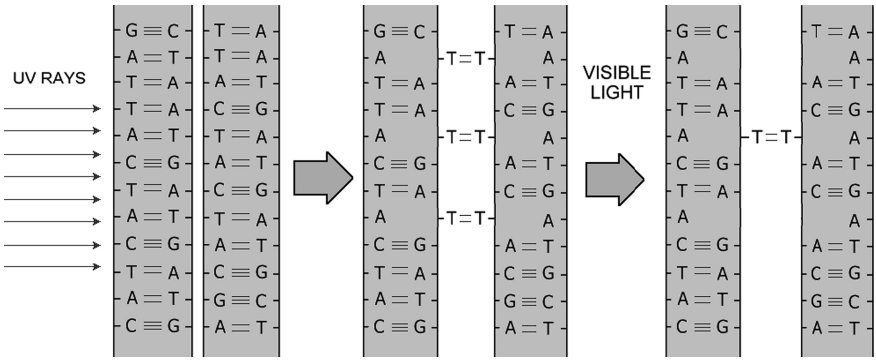


Fig. 2.14 After inactivation by UV irradiance, exposure to visible light for 2–3 h may produce photoreactivation, in which many broken thymine links (thymine dimers) are repaired by enzymes

Thymine dimers absorb light in the visible range (blue light) and this leads to self-repair of the nucleotide bonds, as illustrated in Fig. 2.14 . Reactivation can also occur under conditions of no visible light, or what is called dark repair. The ability to self-repair can depend on the biological organization of the microorganism, as well as the amount of UV damage inflicted on the cell.

Photoreactivation can be catalyzed by enzymes, which are commonly present in bacterial cells. The process occurs in two stages; the first involves the production of an enzyme-substrate complex at the DNA lesion site in the absence of light, and the second is a photolytic reaction in which light energy is absorbed and the lesion is repaired (Fletcher et al. 2003). Enzymatic photoreactivation is facilitated by visible light and results in the splitting of pyrimidine dimers, called monomerization. Thymine dimers are more efficiently eliminated than other types. Only polynucleotide strands containing adjacent pyrimidines are photoreactivable and a minimum length of about nine bases appears to be necessary for the enzyme to attach and excise dimers.

In photoreactivation, repair is due to an enzyme called photolyase. Photolyase reverses UV-induced damage in DNA. In dark repair the damage is reversed by the action of a number of different enzymes. All of these enzymes must initially be activated by an energy source, which may be visible light (300–500 nm) or nutrients that exist within the cell. Masschelein (2002) suggests that the enzymatic repair mechanism requires at least two enzyme systems: an exonuclease systems (i.e. to disrupt the thymine-thymine linkage), and a polymerase system to reinsert the thymine bases on the adenosine sites of the complementary strain of DNA.

In DNA repair mechanisms, the damaged strand is excised by the enzyme and then the complementary strand of DNA is used as a template for inserting the correct nucleotides. Failure to repair UV damaged DNA can result in errors during the replication process during which base substitutions can occur and result in the development of mutants. The most favored sites for base substitutions leading to mutants involve transitions from GC to AT at sites with adjacent pyrimidines (Miller 1985).

Enzymes can be damaged by broadband UV wavelengths other than 253.7 nm, and it has been reported that the use of medium pressure UV lamps inhibits photoreactivation due to the fact that broadband wavelengths inflict damage on photorepair enzymes (Kalisvaart 2004, Quek and Hu 2008).

No types of DNA damage other than that produced by UV can be photoreactivated. In cell systems with efficient dark repair mechanisms, like *D. radiodurans*, little or no photoreactivation occurs. UV damage produced at 253.7 nm can be attenuated by exposure to wavelengths between 330 and 480 nm (Hollaender 1955). The enzymatic monomerization of pyrimidine dimers operates when pyrimidine dimers are the primary type of photodamage, and when photodamage is due to other photoproducts, like the spore photoproduct, photoreactivation appears to be absent.

2.13 UV Scattering

Another kind of photoprotection, other than shielding or photoreactivation, occurs when light is scattered from microbes. Scattering of UV light from microbes is a phenomenon that is routinely observed during the measurement of optical density of microbes in solution to obtain absorption spectra, and during which corrections for scattering must often be made (Holler et al. 1998). Luria et al. (1951) used corrections of 10–20% for scattering at 260 nm while Zelle and Hollaender (1954) found the absorbance corrections for phages T2 and T7 were somewhat greater than 20%. In studies on phage T2 (0.065–0.095 μm), Dulbecco (1950) found that for wavelengths longer than 320 nm the absorption closely followed Rayleigh's law of scattering, and that the photosensitive pigments were part of the phage (but not the DNA) and tended to darken after exposure. Rauth (1965) found that for small viruses like MS2 and ϕX174, the corrections for scattering are almost negligible and only become appreciable above about 280 nm, where they can approach 20–25% depending on virus size. Powell (1959) found that UV scattering effects accounted for no more than 25% attenuation in water. According to Jagger (1967) the UV transmission through an *E. coli* cell is only 70% at 254 nm, leaving a maximum of 30% to be scattered or absorbed.

Scattering may cause appreciable loss of light when the exposed microbes have dimensions comparable with UV wavelengths (Hollaender 1955). The scattering effect is reduced as the index of refraction of the microbe approaches the index of refraction of the medium (i.e. air or water). The scattering effect increases, however, when the size parameter (a function of the diameter) approaches the wavelength of the ultraviolet light (van de Hulst 1957).

Mie scattering is the dominant form of light scattering in the micron-size range of viruses and small airborne bacteria (Bohren and Huffman 1983). Scattering can have significant impact on the amount of UV that actually reaches the nucleocapsid or DNA of a microbe in air and the effect appears to become significant at diameters of about 0.03 microns and greater. Scattering is a protective effect and not dependent on the protein content of nucleocapsids or cell walls, since most microbes appear to have similar indices of refraction (i.e. about 1.05–1.08).

Absorption of photons takes place as ultraviolet radiation penetrates a particle. Light that is not absorbed may be scattered from a particle in the virus and bacteria size range (0.02–20 μm) by three different mechanisms: (1) reflection of photons from the particle, (2) refraction of photons that pass unabsorbed through the particle, and (3) diffraction of photons that pass through or near a particle. Diffraction may alter the path of photons even though they are not in the direct path of the particle. This latter phenomena can result in a particle scattering more light than it would actually intercept due to its physical size alone (Modest 1993). The interaction between ultraviolet wavelengths and the particle is a function of the relative size of the particle compared with the wavelength, as defined by the size parameter:

$$x = \frac{2\pi a}{\lambda} \tag{2.10}$$

where

a = the effective radius of the particle
λ = wavelength

If the size parameter, x << 1, then Rayleigh scattering dominates and for simple spherical particles of diameters less than λ/10 the scattering will approximately vary with the inverse of the wavelength raised to the fourth power ($1/\lambda^4$). If x>>1, the principles of normal geometric optics may be applied. If $x \approx 1$, Mie scattering dominates, and this is the case for small viruses and bacteria. For Mie scattering in air, the size parameter can be written as follows (Chen et al. 2003):

$$x = \frac{\pi d n_m}{\lambda} \tag{2.11}$$

where

n_m = refractive index of the medium (air)
d = particle diameter (typically nanometers)

For nonspherical microbes where the length is significantly greater than the diameter (i.e. aspect ratio > 5), the size parameter for rods may be used. In such cases the length is merely substituted for the diameter (from Stacey 1956). The scattering of light is due to differences in the refractive indices between the medium and the particle (Modest 1993, Garcia-Lopez et al. 2006). The scattering properties of a spherical particle in any medium are defined by the complex index of refraction:

$$m = n - i\kappa \tag{2.12}$$

where

n = real refractive index
κ = imaginary refractive index (absorptive index or absorption coefficient)

Since the refractive index of ultraviolet light approaches 1 in air (or about 1.00029 for visible light), Eqs. (2.10) and (2.11) are virtually identical. If scattering is not affected by the presence of other surrounding particles, and this is generally the case for airborne microbes since concentrations will never be so high as to even be visible, the process is known as *independent scattering*. The process of independent Mie scattering is also governed by the *relative refractive index*, defined with the same symbol (m) as follows:

$$m = \frac{n_s}{n_m} \tag{2.13}$$

where n_s = refractive index of the particle (microbe)

Water has a refractive index of approximately $n_m = 1.4$ in the ultraviolet range and about 1.33 in the visible range. The refractive index of microbes in visible light has been studied by several researchers. Balch et al. (2000) found the median refractive index of four viruses to be 1.06, with a range of 1.03–1.26. Stramski and Keifer (1991) assumed viruses to have a refractive index of 1.05. Biological cells were assumed by Mullaney and Dean (1970) to have *relative refractive indices* of about 1.05 in visible light. Klenin (1965) found *S. aureus* to have a refractive index in the range 1.05–1.12. Petukhov (1964) gives the refractive index of certain bacteria in the limits of 1.37–1.4. There are no studies that address the real refractive index of bacteria or viruses at UV wavelengths except Hoyle and Wickramasinghe (1983) who suggest $n_s = 1.43$ as a reasonable choice for coliform bacteria. Garcia-Lopez et al. (2006) state that for soft-bodied biological particles n is between 1.04 and 1.45. For the imaginary refractive index (the absorptive index or absorption coefficient) in the UV range no information is currently available. Per Garcia-Lopez et al. (2006), hemoglobin has a κ of 0.01–0.15, while polystyrene has a κ of 0.01–0.82.

The mathematical solution of Mie scattering is so complicated as to generally require the use of advanced computational methods. For details of these solutions see van de Hulst (1957), Bohren and Huffman (1983), and Modest (1993). A variety of software packages are freely available for solving the scattering problem for small particles in air, such as DDSCAT, and tables have also been published for use (Draine and Flatau 2004).

Per Eq. (2.13) the refractive index for microbes in air would be about (1.05)(1.33) = 1.4. Figure 2.15 shows two examples of scattering effects in microbes of 0.2 μm (small virus) and 1 μm (large virus or small bacteria) when light is incident from the left passing to the right. The scattering was evaluated for a 253 nm wavelength, a real refractive index of 1.4, an imaginary refractive index of –1.4, a medium refractive index of 1.0003 (air), and wide dispersion of particles (negligible concentration). Computations were performed using the Mie Scattering Calculator (Prahl 2009).

The amount of scattering and absorption by a particle is defined by the scattering cross-section, C_{sca}, and the absorption cross-section, C_{abs}. The scattering cross section is defined as the area which when multiplied by the incident irradiance gives the total power scattered by the particle. The absorption cross section is the area which when multiplied by the incident irradiance gives the total power absorbed.

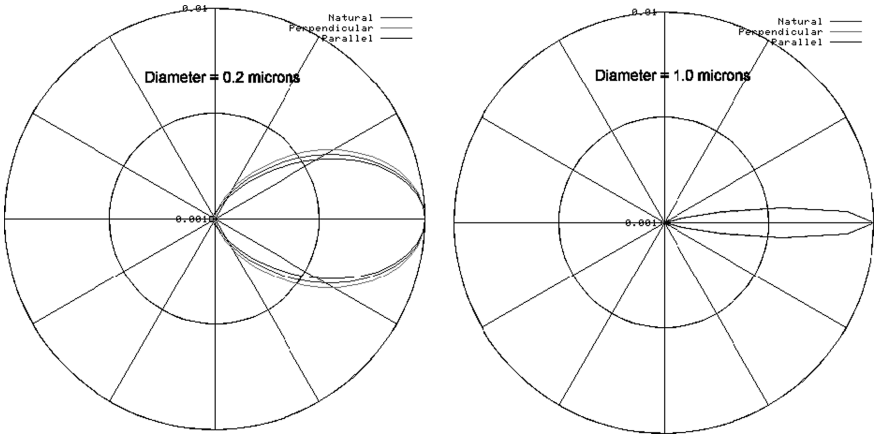


Fig. 2.15 Angular UV light scattering functions for spherical microbes in air, with diameters as indicated. Plots reprinted courtesy of Scott Prahl, Oregon Medical Laser Center

The total amount of absorption and scattering is the extinction cross-section, C_{ext}, defined as the area which when multiplied by the incident irradiance gives the total power removed from the incident wave by scattering and absorption.

$$C_{ext} = C_{abs} + C_{sca} \tag{2.14}$$

The fraction of UV that is scattered from the total incident irradiation, S_{uv}, can be computed as follows (Kowalski et al. 2009):

$$S_{uv} = \frac{C_{sca}}{C_{abs} + C_{sca}} = \frac{C_{sca}}{C_{ext}} \tag{2.15}$$

Equation (2.15) effectively defines the correction factor (as a complement) for UV incident on a particle that scatters UV. Efficiency factors used in Mie scattering are the cross-sections divided by the area, and include the absorption efficiency factor, Q_{abs}, the scattering efficiency factor, Q_{sca}, and the extinction efficiency factor, Q_{ext}, defined as follows:

$$Q_{abs} = \frac{C_{abs}}{\pi a^2} \tag{2.16}$$

$$Q_{sca} = \frac{C_{sca}}{\pi a^2} \tag{2.17}$$

$$Q_{ext} = \frac{C_{ext}}{\pi a^2} \tag{2.18}$$

The extinction efficiency factor is equal to the sum of the other two factors:

$$Q_{ext} = Q_{abs} + Q_{sca} \tag{2.19}$$

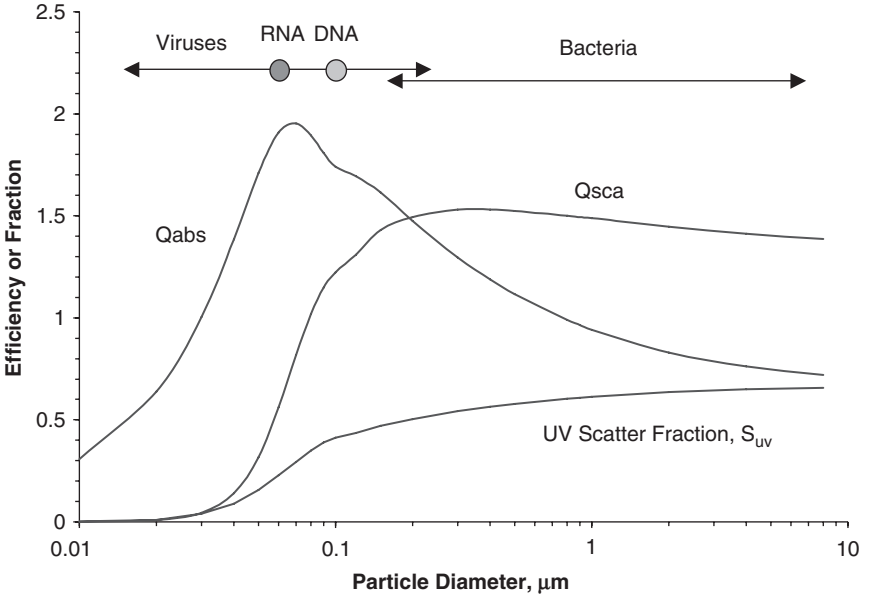


Fig. 2.16 UV Scattering Efficiency, Absorption Efficiency, and Scatter Fraction for spherical particles in air. Real refractive index = 1.4, imaginary absorptive index = –1.4, n_m = 1.0003. Dots show approximate logmean diameters of RNA and DNA viruses

The degree of scattering increases from small viruses up to small bacteria. Figure 2.16 shows an example of the scattering efficiency, absorption efficiency, and scatter fraction of spherical viruses and bacteria in the 0.02 – 8 μm size range in air, based on computations performed using Mie scattering software (Prahl 2009). The refractive index (real component) used for this example is n=1.4, while the absorptive index (the imaginary component) is assumed the same as water, κ=1.4. Note that the scattering efficiency increases dramatically above about 0.06 μm. The range of sizes for viruses and bacteria are also shown, and it can be seen that DNA viruses and bacteria would be most impacted by scattering effects. This graph is revisited for water in Chap. 4, where it will be seen that although the medium changes the efficiency factors, it has little effect on the scattering fraction.

The refractive index and the absorptive index of individual species of microbes may be somewhat different from the values assumed above, but testing of alternate values indicates that the general pattern of behavior observed in Fig. 2.16 remains essentially unchanged for all possible microbial values previously cited. The UV scatter fraction confers some significant UV photoprotection, especially to larger viruses and to bacteria. Such photoprotection translates into an effective UV dose lower than that to which the microbe is exposed. The subject of UV scattering as a mechanism of photoprotection, and how it relates to predicting UV susceptibility will be revisited in Chap. 4.

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