

**PHOTOCHEMICAL
and
PHOTOBIOLOGICAL
REVIEWS**

Edited by Kendric C. Smith

7

Photochemical and Photobiological Reviews

Volume 7

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Volume 7

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Preface

The goals of the science of photobiology can be divided into four categories: to develop (1) ways to optimize the beneficial effects of light on man and his environment, (2) methods to protect organisms, including man, from the detrimental effects of light, (3) photochemical tools for use in studies of life processes, and (4) photochemical therapies in medicine.

To achieve these goals will require the knowledgeable collaboration of biologists, chemists, engineers, mathematicians, physicians, and physicists, because photobiology is a truly multidisciplinary science. While a multidisciplinary science is more intellectually demanding, it also has a greater potential for unexpected breakthroughs that can occur when data from several areas of science are integrated into new concepts for theoretical or practical use.

Photochemical and Photobiological Reviews continues to provide in-depth coverage of the many specialty areas of photobiology. It is hoped that these reviews will provide an important service to the younger scientists in the field and to senior scientists in related fields, because they provide a ready access to the recent literature in the field, and more importantly, they frequently offer a critical evaluation of the direction that the field is taking, or suggest a redirection when appropriate.

*Kendric C. Smith
Editor*

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Physiological Effects of Near-Ultraviolet Radiation on Bacteria

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1. INTRODUCTION

1.1. Scope of Review

Near-ultraviolet (near-UV) radiation (also called "UV-A") can be considered to lie in the wavelength range 320–400 nm. The long-wavelength limit represents the beginning of the visible spectrum, while the short-wavelength limit corresponds roughly to the point below which proteins and nucleic acids begin to absorb significantly. Below this region is the "mid-UV" region or UV-B (290–320 nm), where proteins and nucleic acids begin to absorb, and where sunburn and skin cancer are most effectively produced. "Solar-UV radiation" (UV radiation present in sunlight at the surface of the Earth at noon in clear weather) includes both the near-UV and the mid-UV regions.* Because nucleic acid and protein do not absorb significantly in the near-UV region, one can expect a wide variety of other chromophores to become important in this region, producing a wide variety of biological effects. It is also expected that the fluences required to produce these effects will be considerably higher than those required for killing by far-UV radiation ("UV-C"; 190–280 nm), because the target molecules are not as crucial to the cell as nucleic acid.

Near-UV radiation is now known to produce essentially all of the biological effects produced by far-UV radiation, as well as many additional actions. Killing and mutation are the most prominent effects of far-UV radiation, and these are also produced throughout the near-UV region. A full discussion of the lethality and mutagenicity of near-UV radiation, primarily in microorganisms, appears in the excellent review of Webb (1977); the present review deals with the remaining effects of near-UV radiation on bacteria. I call these "physiological effects." Some of them occur at sublethal and others at superlethal fluences. This review is a parallel for near-UV radiation of the review by Swenson (1976) of physiological responses of bacteria to far-UV radiation.

This review is restricted almost entirely to the bacteria, partly because this is an area with which I am familiar, but also because the physiological effects of near-UV radiation on eukaryotes has not been actively pursued by any one research group, and the reports are widely scattered in the scientific literature. One of the few attempts to pull together this widely distributed literature is represented by the excellent review of Klein (1978) on the effects of near-UV radiation on plants. The effects of

* "Near-UV" radiation was earlier defined as the wavelength region 300–380 nm and "far-UV" radiation as 120–300 nm (Jagger, 1973). It now appears desirable to modify these definitions (as indicated in the text) to provide for the mid-UV region, which is of so great importance for human responses to sunlight.

near-U.V. radiation on humans have received somewhat more concentrated attention, one of the best general reviews being the book by Parrish *et al.* (1978).

It may be noted that the effects discussed in this review are mostly deleterious (exceptions are photoreactivation and photoprotection). This reflects the fact that near-UV and mid-UV-radiation are the most energetic radiations in sunlight at the surface of the Earth. However, one might expect that evolution would have produced ways of harnessing this energy for positive results, and such effects are indeed found among plants, as noted by Drumm-Herrel and Mohr (1981). Near-UV radiation is quite effective in photosynthesis.

In the interest of economy, preference has been given to citation of recent papers, which often contain references to earlier literature; the original discoverers of an effect are therefore often not cited directly.

1.2. Chromophores for Near-UV Radiation

One of the fundamental laws of photochemistry states that a reaction cannot occur until a photon is absorbed. Although this is an obvious consequence of quantum mechanics, surprisingly little attention seems to have been paid to possible molecular absorbers (chromophores) for near-UV radiation-produced biological effects, even by those who determine action spectra. Thus, for example, the long-known fact that the thio group (—C=S) absorbs well in the near-UV region around 330 nm (Scott, 1955; Jagger, 1967) did not arouse suspicions of its importance in near-UV radiation photobiology until the close parallel between the absorption spectrum of 4-thiouridine and the action spectrum for growth inhibition was observed by Ramabhadran (1975). It would seem worthwhile, therefore, to devote some space in this review to a consideration of possible chromophores for near-UV radiation, even though some of these have not yet been shown to play a role in near-UV radiation photobiology.

A *chromophore* is the molecule or molecular group that absorbs the light that causes the biological effect under study. A *target* is the critical molecule or multimolecular structure that is damaged by the energy of the absorbed light. Chromophores are often, but not always, targets. One may say, for example, that pyrimidines, DNA, or even the cell nucleus, is the target for far-UV radiation lethality, and that the purines and pyrimidines, or DNA, are the chromophores for far-UV lethality.

What features make a molecule a good candidate as a chromophore for near-UV radiation? First, the *molar extinction coefficient* (ϵ) should be *high* (at least $1000 \text{ M}^{-1}\text{cm}^{-1}$ (see Table 1)). There are instances of

important chromophores having low extinction coefficients. One type of poorly absorbing but important chromophore is that which plays a critical function, such as the disulfide bonds of cystine in far-UV radiation inactivation of proteins, and the bases of DNA in killing by near-UV radiation. Also, some oxygen and nitrogen heterocyclic centers have $n \rightarrow \pi^*$ transitions of low absorption but very high intersystem crossing to the triplet state, which is very photoreactive. However, such chromophores are not common, and to consider them would mean evaluating a very large number of molecules. Second, the molecule should have a *crucial function* in the cell. Constituents of DNA of course satisfy this criterion. Some other molecules, such as DNA polymerases and some repair enzymes, are present in a sufficiently low number to make them vulnerable. Third, if the chromophore is not a crucial molecule, then there must be some mechanism for *amplification* of the action on the molecule. For example, pyrimidine adduct formation, following absorption of light by 4-thiouridine in bacterial transfer RNA, interferes with protein synthesis sufficiently to invoke the action of the *rel* gene, which then triggers an almost complete cutoff of protein and RNA synthesis.

1.2.1. Molecules of Electron-Transport Chain

The electron-transport chains of mitochondria and bacteria exhibit their remarkable abilities to pass electrons down a redox gradient because they consist of highly conjugated molecules whose valence electrons are quite mobile and loosely bound and thus are relatively easily excited or removed. This conjugation, of course, also makes them excellent absorbers of near-UV light.

Porphyrins. These molecules, present in chlorophyll and cytochromes, always have strong absorption around 420 nm, as well as considerable absorption at longer wavelengths. Biological systems containing them are therefore very often highly colored. The absorption at 420 nm is usually much higher than at any other wavelength in the 300–500 nm range, so that a rough action spectrum can quickly determine whether or not they are possible chromophores. Absorption of light by porphyrins usually does not destabilize them enough to inactivate them. Nevertheless, Epel, Butler, and co-workers (Epel, 1973) have shown that inhibition by violet light of growth and respiration, in the colorless alga *Prototheca zopfii* and in *Saccharomyces cerevisiae*, is due to light absorbed around 420 nm in cytochrome oxidase. The light fluences, however, are very high ($\sim 100 \text{ MJ/m}^2$).

Related to the porphyrins are the open-chain tetrapyrroles, such as bilirubin, phytochrome, and erythobilin. Neonatal jaundice is treated in

humans by irradiation with blue light, which is now known to photoisomerize bilirubin in the peripheral circulation to a more water-soluble, excretable product (McDonagh *et al.*, 1980), but similar reactions induced by near-UV light have not yet been shown to be important in microorganisms. Peters (1977) has demonstrated a role for ribonucleotide reductase (which converts all four common ribonucleoside diphosphates to the corresponding deoxyribonucleotides) in the near-UV radiation lethality of growing cultures of *Escherichia coli*. It is not known what part of the ribonucleotide reductase complex is sensitive, but it includes a non-heme-iron-containing protein (B2) whose absorption spectrum is similar to that of oxyhemerythrin (a protein with an erythobilinlike chromophore), showing a strong absorption throughout the near-UV region (Fig. 1 and Table 1). The near-UV radiation absorption of many tetrapyrrole compounds is largely due to metal-ion complexes; e.g., the 410 nm absorption of protein B2 is due to a tyrosine free radical, whose existence is closely linked to the presence of iron (Thelander and Reichard, 1979).

Flavins. The ribonucleotide reductase complex just mentioned utilizes as hydrogen donor the compound thioredoxin. This in turn must be reduced by thioredoxin reductase, a flavoprotein with typical flavin absorption in the near-UV region (Fig. 1), when in the oxidized state (Moore *et al.*, 1964). Thioredoxin reductase might also serve as a chromophore for near-UV radiation lethality. Brodie and Ballantine (1960) have shown that flavin mononucleotide (FMN) is moderately sensitive to near-UV light in *E. coli*, but is not as sensitive as vitamin K₁; flavin adenine dinucleotide (FAD) is more resistant than FMN.

Eisenstark and co-workers (Wang *et al.*, 1980) have shown that irradiation of a typical tissue-culture medium by black light results in a riboflavin-sensitized photooxidation of tryptophan and tyrosine, which results in the production of hydrogen peroxide. Such reactions might conceivably occur also within cells.

In a nondestructive role, flavins appear to be important in the phenomenon of photoreactivation, in which irradiation with near-UV light, after irradiation with far-UV radiation, reduces the biological effects of the far-UV radiation. Photoreactivation operates by means of a photo-reactivating enzyme. It has now been found by Eker *et al.* (1981) that the chromophore for this enzyme in *Streptomyces griseus* is a flavin derivative. Iwatsuki *et al.* (1980) also have evidence that the photoreactivating enzyme from baker's yeast is a flavoprotein, and they note the important fact that 4,5-reduced flavoproteins often lose their 450 nm absorption peak, retaining only the 375 nm peak.

Another example of nondestructive photobiology involving flavins is that of the photoactivation of the reduced nitrate reductase enzyme com-

plex in extracts of *Neurospora crassa* by light of 365–480 nm (Roldán and Butler, 1980). Light also appears to regulate nitrate reductase activity *in vivo*.

Quinones. Although hydroquinone shows little absorption above 320 nm, its oxidized form, benzoquinone, and the related compound, naphthoquinone, do absorb strongly in the near-UV region. Brodie and co-workers were the first to show the high sensitivity of quinones in bacterial systems (see Jagger, 1972, for review), and they showed that the naphthoquinone, vitamin K₁, was much more sensitive than the benzoquinone, ubiquinone (UQ). Jagger and co-workers have suggested for some time that quinones may be involved in effects at sublethal fluences in bacteria, but have been as yet unable to demonstrate this (see Section 2.1.2). Nevertheless, Madden *et al.* (1981) have shown that the naphthoquinone, menaquinone-8 (MQ-8; see Fig. 1), is extremely sensitive to near-UV light, showing an F₃₇ (fluence required to reduce activity to 37% of control values) at 334 nm of 1.3 kJ/m² *in vivo* and 3.6 kJ/m² *in vitro*. The biological consequences, if any, of this extreme sensitivity remain unknown. Taber *et al.* (1978) have shown that, at much higher fluences in the lethal range, menaquinone is a target molecule for near-UV radiation-induced growth delay in *Bacillus subtilis*. This is the only demonstrated case so far of quinone involvement in photobiological damage, although the photosensitivity of these compounds has long made them prime suspects.

Table 1. Molar Extinction Coefficient (ϵ) for Near-UV Absorption Peaks of Possible or Known Chromophores

Compound	λ (nm)	ϵ ($M^{-1}cm^{-1}$)
⁴ Srd	330	15,200 ^a
Riboflavin	375	10,600 ^b
Protein B2	360	6,000 ^c
Pyridoxal phosphate	390	5,500 ^d
MQ-8	334	4,500 ^e
<i>N</i> -formyl-kynurenone	318	3,200 ^f
<i>E. coli</i> DNA	310	23 ^g

^a Hall (1971).

^b Whitby (1953).

^c Brown *et al.* (1969).

^d Derived from data of Matsuo (1957).

^e Derived from data of Bishop *et al.* (1962).

^f Walrant and Santus (1974).

^g Per nucleotide; derived from data of Sutherland and Griffin (1981).

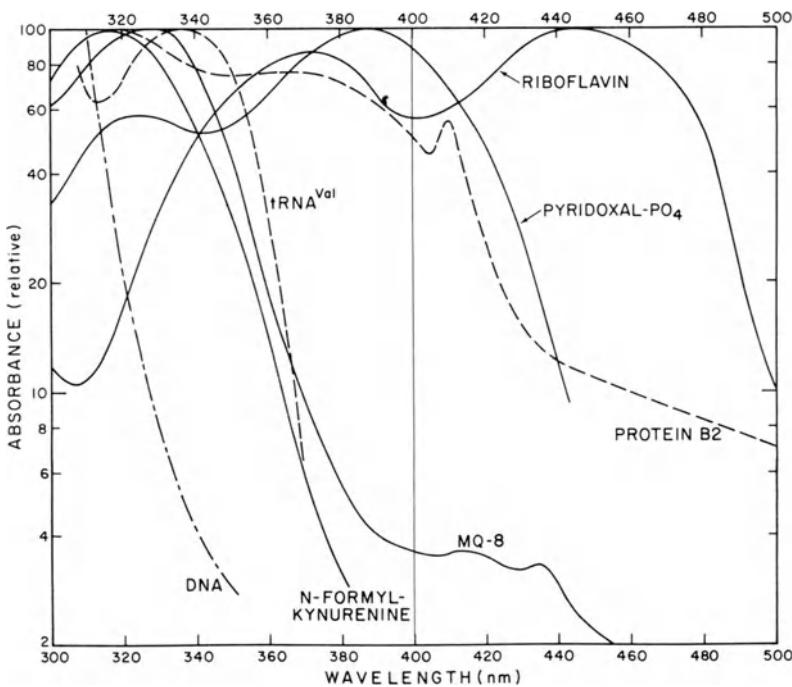


Fig. 1. Some near-ultraviolet absorption spectra. *E. coli* valyl transfer RNA ($tRNA^{Val}$) at pH 7 (dashed line; Favre *et al.*, 1969); *N*-formylkynurenine in phosphate buffer at pH 7.6 (Walrant and Santus, 1974); vitamin K₂ (MQ-8) in cyclohexane (Madden *et al.*, 1981); pyridoxal phosphate in phosphate buffer, pH 7.5 (Matsuo, 1957); riboflavin in phosphate buffer, pH 7, (Whitby, 1953); protein B2 of ribonucleotide reductase (dashed line; Brown *et al.*, 1969); *E. coli* DNA (dash-dot line; Sutherland and Griffin, 1981). These molecules (except DNA) all show high near-UV absorption (Table 1). Spectra are normalized to 100 at the highest peak (except DNA, normalized to 100 at 310 nm).

1.2.2. Other Molecules

Nucleic Acids. Nucleic acids containing the usual bases may absorb significantly but poorly above 300 nm (Fig. 1 and Table 1). This slight absorption might be due to direct triplet-state excitation (see K. C. Smith in Pollard, 1974). However, since solutions of pure nucleotides show little absorption above 320 nm, it seems more likely that the near-UV absorption of DNA is due to tenacious impurities (Sutherland and Griffin, 1981). Whatever its source, this absorption is sufficient to play a role in lethal effects up to 360 nm (Sutherland and Griffin, 1981). The unusual base, 4-thiouridine (⁴Srd), extends the high far-UV absorption of transfer RNA

into the near-UV region, with a peak at 340 nm (Fig. 1), producing a chromophore for a variety of sublethal biological effects (Section 2), but this is the only nucleic acid base that does this.* Nucleic acids might complex with proteins or other compounds to produce a distorted nucleic acid base with absorption at a longer wavelength (Jagger, 1957). There is evidence that such an event occurs in the interaction of *E. coli* with its photoreactivating enzyme, resulting in a (postulated) charge-transfer complex with a new longer-wavelength absorption band (Snapka and Fusilier, 1977; Wun *et al.*, 1977). A variety of dyes interact with DNA sufficiently to serve as effective photosensitizers, e.g., acridine orange (Prusik *et al.*, 1980), methylene blue (Ponce-De León and Cabrera-Juárez, 1970), and psoralen (Song and Tapley, 1979). Psoralen also interacts photochemically with RNA, with ${}^4\text{Srd}$ and adenine in tRNA, and even with proteins.

It is a remarkable fact that pure DNA is inactivated by radiation throughout the near-UV region. Transforming DNA from *B. subtilis* shows a 366-nm cross-section for genetic marker inactivation about 10^{-4} of that at 254 nm, and shows an oxygen effect in the near-UV region (Peak *et al.*, 1973). More highly purified transforming DNA from *Hemophilus influenzae* was reported not to show an oxygen effect, but this finding could not be repeated (M. J. Peak, personal communication); it still has a 366-nm cross-section about 10^{-5} of that at 254 nm (Cabrera-Juárez *et al.*, 1976). Phage T7 shows a similar sensitivity (Peak and Peak, 1978). These inactivations are probably not due to direct absorption of near-UV radiation in DNA, since substances that quench excited molecules or that scavenge free radicals, like histidine (Cabrera-Juárez *et al.*, 1976; Peak and Tuveson, 1979) and 2-aminoethylisothiouronium bromide hydrobromide (AET), provide protection against near-UV, but not far-UV inactivation (Peak and Peak, 1975). It would appear that some compound(s), so tightly bound to DNA that it resists extensive purification, can serve as a chromophore for the absorption of near-UV light and the consequent inactivation of the DNA. This substance(s) has not been identified, and it is not known whether it transfers energy into the DNA or simply forms a lethal complex with it. The observation of mutation shows that, although DNA itself may not be a chromophore in the near-UV region, it is clearly a target for wavelengths as far out as 460 nm.

Pyridoxal Phosphate. Pyridoxine compounds serve as prosthetic groups or coenzymes for about 50 different enzymes in which amino acids or amino groups are transformed or transferred. Pyridoxal phosphate shows strong absorption throughout the near-UV region, with peaks at

* A. Favre and co-workers (personal communication) have recently found that 5-methylaminomethyl-2-thiouracil ($\text{mam}'\text{s}^2\text{U}$) absorbs at shorter near-UV wavelengths than does ${}^4\text{Srd}$ and with much lower efficiency (see Section 2.1.3).

325 and 388 nm (Fig. 1), where the molar extinction coefficient is about $5500 M^{-1}cm^{-1}$ (Table 1). This compound therefore has long been suspected to be an important chromophore in the near-UV. Nevertheless, no important biological effects have yet been shown to be produced by absorption in pyridoxal phosphate or related compounds.

NADH. Nicotinamide adenine dinucleotide in its reduced form (NADH) absorbs light in the near-UV region (340 nm peak). The importance of NADH in oxidative metabolism, as well as its function as a cofactor for many enzymes, makes it a likely chromophore. However, the molecule is quite stable to irradiation in its pure state, and no evidence has yet been produced that it is an important near-UV radiation chromophore for biological effects. Stability of a molecule to photoinactivation does not of course preclude its serving as a chromophore that transfers excitation energy into a more sensitive molecule. For example, Shugar (1951) showed that D-glyceraldehyde-3-phosphate dehydrogenase may, during preparation, have its SH groups oxidized, with concomitant reduction of the closely associated NAD^+ to NADH. Illumination at 340 nm (absorbed by NADH) then reverses the SH oxidation, reactivating the enzyme activity. It is now known that many biologically active macromolecules, such as enzymes, may be converted from inactive to active forms by the absorption of light by photochromic effector molecules (Hug, 1981).

N-formylkynurenone. This compound (Fig. 1) is a metabolite of tryptophan and is one of its major photooxidation products. It is a remarkable photodynamic sensitizer of amino acids, proteins, or DNA *in vitro*, but a photobiological role has not yet been found. Its effectiveness is largely due to its long triplet lifetime (Walrant and Santus, 1974).

Formation of New Chromophores by Near-UV Radiation. Irradiation itself may sometimes produce a new chromophore. For example, near-UV irradiation of D-glyceraldehyde-3-phosphate dehydrogenase (previously treated with iodoacetate, which modifies an SH group at the active site) in the presence of excess NAD^+ produces a new substance with absorption at 325 nm (Ho and Tsou, 1979). The chromophore for the reaction is apparently tryptophan; the new chromophore is produced by a radiationless energy transfer from tryptophan to the new chromophore, which is unidentified, but presumably related to NADH.

It is well-known that near-UV irradiation may stimulate the biosynthesis of shielding chromophores, such as carotenoids (see Fraikin and Rubin, 1979). Irradiation of photosynthetic bacteria at 340 nm has been shown to induce synthesis of a tocopherol-like compound, which may function as an antioxidant (Fraikin and Rubin, 1979).

Metabolic Precursors. Simple cyclic compounds such as benzene

and pyrimidine typically absorb only in the far-UV region. However, only slight modifications of their structures may shift absorption into the near-UV region. A thio group at the 4-position will shift the absorption of either pyrone, uracil, or 1-methyl-pyridine to about 340 nm, with very high extinction coefficients (around $15,000 M^{-1}cm^{-1}$; Table 1). The addition of NH_2 and COOH groups to benzene to produce anthranilic acid, or OH plus CHO , to produce salicylaldehyde, also shifts absorption to the range of 340 nm, as will the addition of OH plus acetone, to produce 2-hydroxyacetophenone. Many such cyclic compounds may be important parts of metabolic pathways and may absorb well in the near-UV region and be readily inactivated by it. However, as we have noted above, absorption in the near-UV radiation region is not enough to make a compound a good candidate for a biologically important chromophore. There must be some additional factor, such as a critical function of the molecule in the cell, or some kind of amplification of the effect. Therefore, most such metabolic-pathway compounds will not be important near-UV chromophores: there are so many copies of them that inactivation of even a considerable fraction will usually not be deleterious to the cell.

2. EFFECTS AT SUBLETHAL FLUENCES

2.1. Growth Inhibition

2.1.1. General Characteristics

Inhibition of growth by near-UV radiation has been observed in bacteria, fungi, protozoa, algae, higher plants, and animal cells (Jagger, 1972). It is therefore a very widespread phenomenon. It often occurs, especially in bacteria, at fluences much below those required for lethality. In *E. coli*, these sublethal effects occur at $\sim 20 \text{ kJ/m}^2$ at 334 nm or $\sim 100 \text{ kJ/m}^2$ at 366 nm.

Figure 2a shows *growth inhibition* of *E. coli* B/r exposed to sunlight for times up to 30 min. Two phenomena occur: (1) a delay in the onset of growth (*growth delay*), and (2) a slight decrease in the rate of growth (*growth-rate depression*) after the population has recovered from its growth delay. The data show that growth inhibition is readily induced by sunlight. They also show that the population eventually recovers from the growth delay, but it may not recover completely from growth-rate depression.

These data may be analyzed in different ways. In our earlier work (Jagger *et al.*, 1964), we compared the times required to reach a given

optical density (after the cells had recovered from the growth delay) in the control and the irradiated cultures. However, such an analysis is dependent upon the level of growth at which one makes the comparison, and it does not reveal the separate contributions to growth inhibition made by growth delay and by depression of growth rate. We prefer now to analyze the data as shown in Fig. 2a, in which the straight-line portions of the (semilogarithmic) growth curves are extrapolated to the y-axis. A semilogarithmic plot of these extrapolation points will generate a kind of "survival curve" for growth (Fig. 2b), which will usually be exponential, and from which one can obtain an F_{37} , or fluence that results in a growth delay equivalent to what would be obtained if 37% of the population suffered no growth delay at all and the remainder was completely growth-inhibited. Such a curve then measures growth delay. Depression of growth rate is indicated by the slopes of the lines in Fig. 2a, which also can be plotted on a semilog plot vs. fluence, as shown in Fig. 2b. It is seen that depression of growth rate in this system is small compared to growth delay; growth delay is thus quantitatively almost equal to growth inhibition in some systems.

Action spectra for growth inhibition in the bacterium *E. coli* B and in the colorless alga *Prototheca zopfii* are shown in Fig. 3. Both spectra indicate a common chromophore absorbing around 340 nm. In addition, *Prototheca* shows evidence at longer wavelengths of some involvement of porphyrins, consistent with the finding of Epel and Butler (1969) of cytochrome oxidase alterations in both *Prototheca* and in baker's yeast (Epel, 1973). There is evidence that growth inhibition in some plant and animal systems shows an action spectrum similar to that found in bacteria. Thus, inhibition of growth occurs at 360 nm, but not at 400–500 nm, in *Parthenocissus* crown-gall tissue cultures and in HeLa cell cultures (Klein and Edsall, 1967; Klein, 1964). (Some growth inhibition occurs in these systems at wavelengths longer than 500 nm, but this is beyond the range of our discussion.)

Near-UV radiation-induced growth inhibition in bacteria was first observed by Hollaender (1943), and it is usually measured in a nutrient broth. Jagger *et al.* (1964) showed that it also occurs on nutrient-agar plates. Furthermore, for *E. coli* B, there is a considerable amount of filament formation on agar plates after near-UV (334 nm) irradiation, indicating that division on plates is even more delayed than growth, and suggesting induction of the SOS repair system, normally induced by far-UV. Later work of Phillips *et al.* (1967), using cell-counting techniques, showed that division delay and growth delay of *E. coli* B parallel each other in nutrient broth, suggesting that filament formation does not occur in broth. They also showed that the action spectrum for induction of

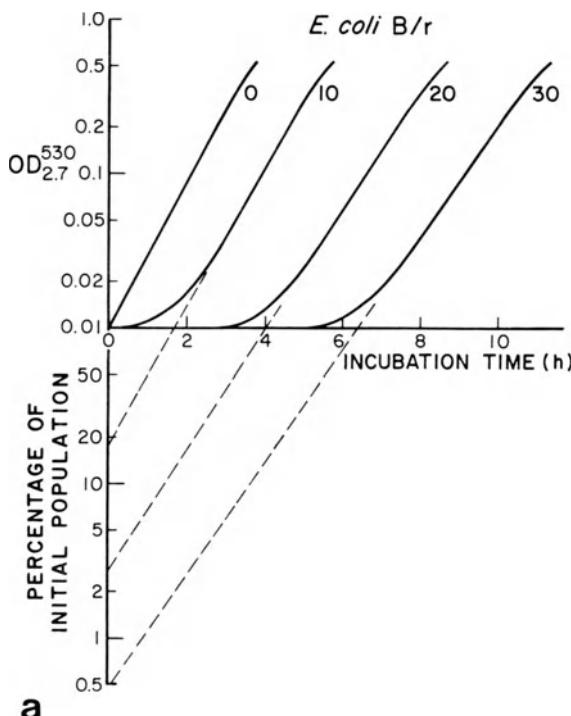


Fig. 2. (a) Growth of *E. coli* B/r (in a mineral salts medium supplemented with thiamine and casamino acids) after exposure (through window glass) to Texas noon sunlight for times (in min) indicated on curves. The broken lines are tangent to the mid-slopes of the curves (Jagger, 1975). (b) Semilogarithmic graphical analysis of growth delay (open circles), based on y intercepts in 2(a), and of growth rate depression (closed circles), based on slopes of curves in 2(a).

division delay is similar to that for growth delay. It would thus appear that the growth-delay phenomenon observed in *E. coli* at 334 nm is not particularly related to the ability of some *E. coli* strains to form filaments after far-UV irradiation. This is supported by the observation that a normal degree of growth delay is observed (Lakchaura, 1972) in *E. coli* K-12 AB2463 (*rec*⁻ *lon*⁺), which does not form filaments upon far-UV irradiation. Kubitschek and Peak (1980) have recently reported that the action spectrum for growth delay in *E. coli* B/r K (*lon*⁺) agrees closely with the action spectrum for growth delay in *E. coli* B (*lon*), which is a further indication that the *lon* gene product is not involved in growth delay.

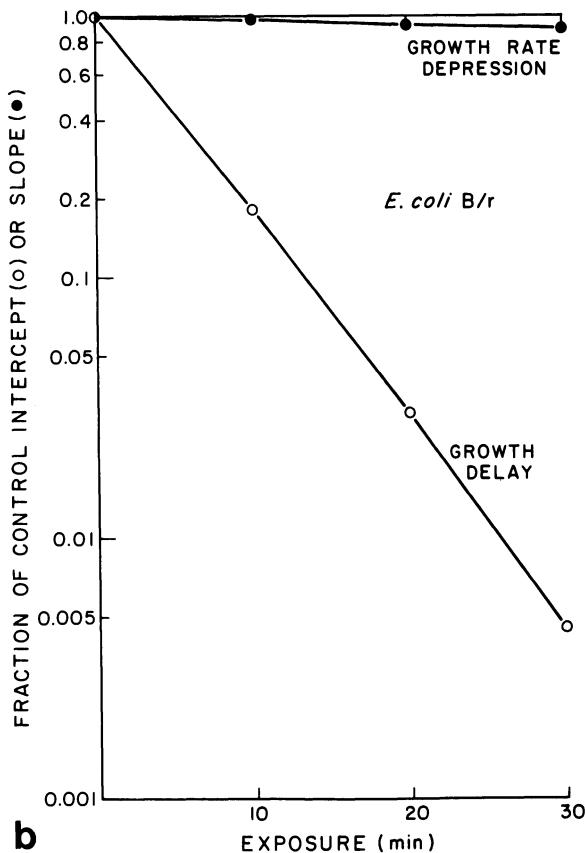


Fig. 2. (Continued)

2.1.2. The Chromophore and Target in *E. coli*

The early work of R. E. Beyer and of A. F. Brodie, on reactivation of near-UV radiation-damaged electron-transport systems by quinones, led Jagger (1972) to seek a correlation between growth delay and quinone content. He showed that both growth delay and photoprotection (Section 4.2.1b) correlate well with the ubiquinone (UQ; coenzyme Q) content, but not with the menaquinone (MQ; vitamin K) content of a variety of bacterial genera. In spite of this correlation, it now appears that UQ is not a chromophore for growth delay, because (1) neither its absorption spectrum nor its photomodification action spectrum agree with the action

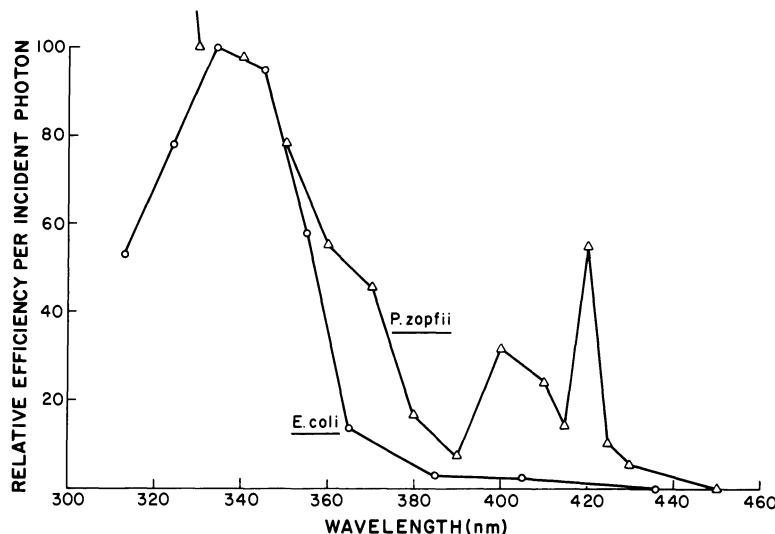


Fig. 3. Action spectra for growth inhibition in the bacterium *E. coli* B (circles; Jagger *et al.*, 1964) and in the alga *Prototheca zopfii* (triangles; Epel and Krauss, 1966). The curves are normalized to a value of 100 at 334 nm. The triangle at 320 nm would be off scale at a relative efficiency of 184.

spectrum for growth delay, and (2) the fluence required for its photo-modification (420 kJ/m² at 334 nm *in vivo*) is far too large (Werbin *et al.*, 1974). It is possible that the significant correlation in the data of Jagger (1972) is with gram-negativity rather than with quinone content, since all the genera showing high growth delay and UQ content are gram-negative.

In 1975, Ramabhadran and Jagger showed that near-UV radiation-induced growth inhibition in *E. coli* K-12 strains is not photoreactivable, and that it is not affected by dark-repair systems that operate on far-UV radiation-induced DNA damage. This work indicates that DNA damage is not involved. Further evidence that DNA is not a target for growth inhibition lies in the observation that growth delay induced in *E. coli* at 334 nm is not significantly affected by oxygen removal during irradiation (J. Jagger and T. Fossum, unpublished), while lethal and mutagenic actions of near-UV radiation, believed to involve DNA damage, are largely dependent upon oxygen during irradiation (Section 3.1).

4-Thiouridine (⁴Srd) is an unusual nucleoside that occurs at position-8 in about 65% of *E. coli* tRNAs (Carré *et al.*, 1974). Favre *et al.* (1971) showed that 334-nm irradiation of purified valyl tRNA containing ⁴Srd causes a cross-linking adduct to form between the ⁴Srd at position-8 and the cytidine residue at position-13, and it is now known that this adduct

can be produced by near-UV irradiation in all tRNAs of *E. coli* (about 50%) that have these two residues in these positions (Carré *et al.*, 1974). The absorption spectrum peak of ${}^4\text{Srd}$ in solution is at 328 nm, but it shifts slightly to 336 nm when it is present in tRNA^{Val} (Favre *et al.*, 1971). Ramabhadran (1975) showed that this tRNA absorption fits almost perfectly with the growth-delay action spectrum for *E. coli* B (Fig. 4), the

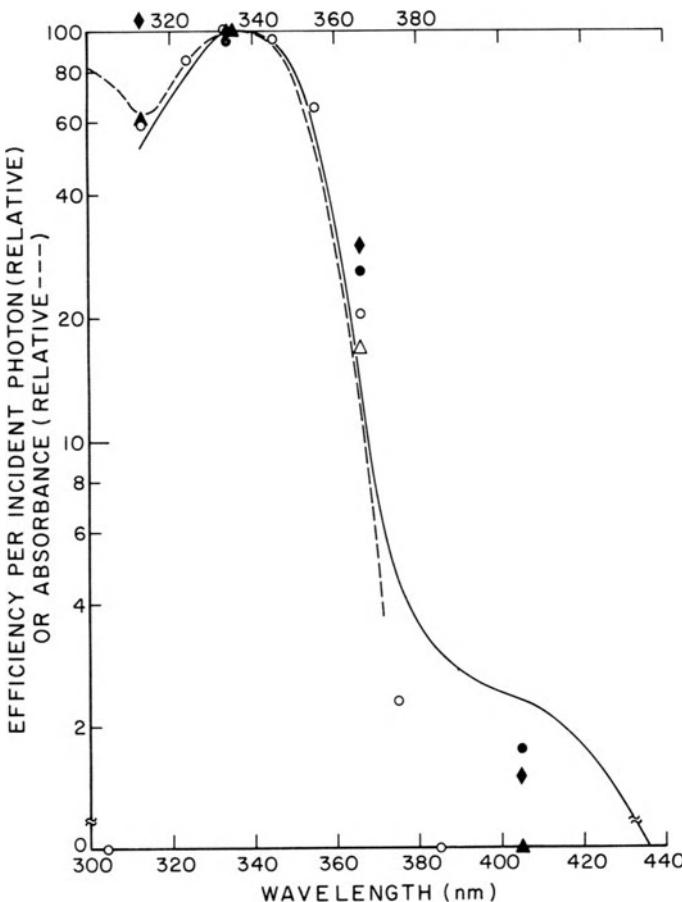


Fig. 4. Action spectra for growth inhibition in *E. coli* B (solid line; Jagger *et al.*, 1964); for photoprotection from 254 nm killing in *E. coli* B (open circles; Jagger *et al.*, 1964) and from 254 nm mutation in *E. coli* B/r (closed circles; from data of Tyrrell, 1980a); for inhibition of the induced formation of tryptophanase in *E. coli* B/r (triangles; Swenson and Setlow, 1970); and for reduction of *E. coli* B capacity for development of phage T7 (diamonds; Day and Muel, 1974). The absorption spectrum of *E. coli* tRNA^{Val} is also shown (broken line; Favre *et al.*, 1969). All spectra are normalized to 100 at 334 nm.

fit being much better than that for other possible chromophores, such as vitamin K₂ or NADH, and that the fluences required for growth delay are similar to those required for production of the ⁴Srd-Cyd adduct. Ramabhadran (1975) concluded that ⁴Srd was a major chromophore for growth delay in *E. coli*, and that the ⁴Srd-Cyd adduct was the probable photoproduct. This work was quickly repeated, for K-12 strains, by Thomas and Favre (1975; work published at the same time but submitted seven months after the Ramabhadran paper). Thomas and Favre (1975) also showed that the ⁴Srd-Cyd adduct is produced *in vivo* in *E. coli* K-12 at fluences that produce growth delay, and this result was confirmed by Ramabhadran *et al.* (1976a) for *E. coli* B/r. These findings lent support to the idea that the ⁴Srd-Cyd adduct was the major photoproduct responsible for near-UV radiation-induced growth delay in *E. coli*. Later work (see below) with ⁴Srd⁻ mutants, confirmed that ⁴Srd is indeed the chief chromophore for growth inhibition in *E. coli*, and that ⁴Srd, or tRNA containing it, is the chief target for growth inhibition in *E. coli*.

2.1.3. The Mechanism in *E. coli*

This strong evidence that the ⁴Srd-Cyd adduct was indeed the lesion responsible for growth delay was not comprehensible by itself. Even though it was known that the adduct could affect both the initial rate and the final level of tRNA amino-acylation *in vitro*, the effect is large only for a few tRNA species (Phe, Pro, Lys; Carré *et al.*, 1974). In fact, tRNA^{Phe} carrying ⁴Srd-Cyd adducts incorporates phenylalanine into polypeptides *in vitro* at about one-half the control rate (Chaffin *et al.*, 1971). Thus, if many of the tRNAs escape this damage, and if those damaged can still perform at a lower rate, it was not clear how this damage could result in a prolonged growth delay.

Ramabhadran (1975) had also observed that RNA synthesis (accumulation) was immediately and completely halted by broad-band near-UV irradiation of *E. coli* B/r, whereas DNA and protein synthesis showed less complete inhibition. He showed that the action spectrum for this inhibition of RNA synthesis was quite similar to the action spectrum for growth delay. It occurred to him that the "stringent" control system for RNA synthesis might be involved in growth delay. If *E. coli* cells are starved for a required amino acid, the uncharged tRNAs cause the ribosomes to produce guanosine tetraphosphate (ppGpp), and this blocks the transcription of genes for stable RNA (rRNA and tRNA), resulting in a sudden and complete shut-off of net stable RNA synthesis. This effect is not seen in "relaxed" (*rel*⁻) strains under similar conditions (Cashel, 1969). Ramabhadran and Jagger (1976) showed (Fig. 5) that the effects

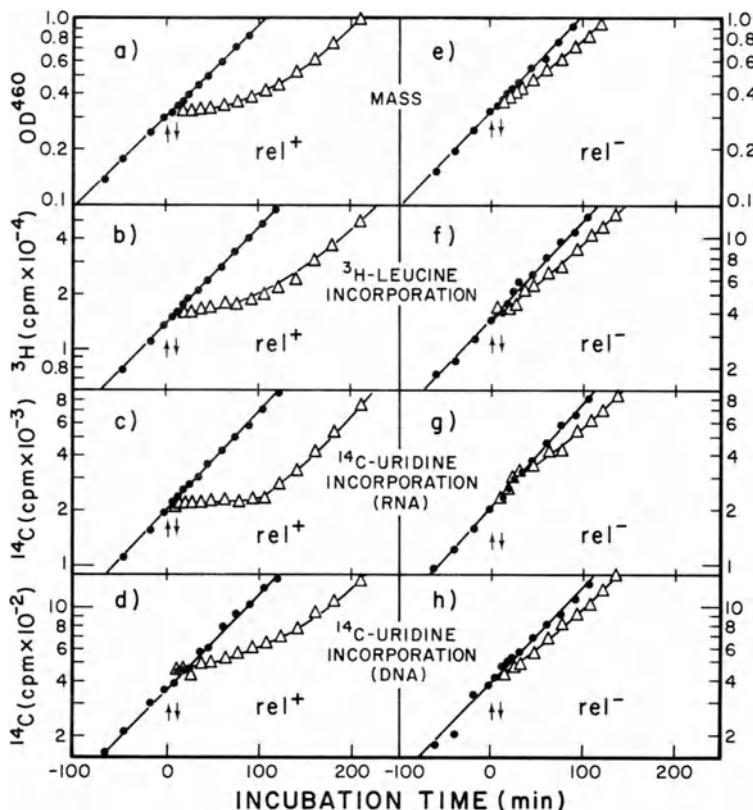


Fig. 5. Mass increase and protein, RNA, and DNA synthesis in *E. coli* B/r strains NC51 (*rel⁺*) and NC52 (*rel⁻*) irradiated with broad-band near-UV light (General Electric BLB lamps). Cells were grown for five generations in M9 medium supplemented with [^{14}C]uridine and [3H]leucine. At $OD^{460} \approx 0.3$, cells were irradiated for 10 min, starting at time = 0 (total fluence of 20 kJ/m²). Arrows indicate the beginning (\uparrow) and the end (\downarrow) of irradiation. (●) unirradiated, (\triangle) irradiated. (a-d) *rel⁺*; (e-h) *rel⁻* (Ramabhadran and Jagger, 1976).

produced on DNA, RNA, and protein synthesis in a *rel⁺* strain were not observed in an isogenic *rel⁻* strain, which exhibited no growth delay and only a small depression of the growth rate. Ramabhadran and Jagger (1976) also showed that ppGpp was produced upon near-UV irradiation of the *rel⁺* strain, but not the *rel⁻* strain. It now became clear how damage

to a relatively small fraction of the total tRNA of the cell could result in the sudden and virtually complete shut-off of RNA synthesis, followed by a great depression of protein synthesis, leading to a growth inhibition. The inability to synthesize new tRNA, owing to the stringent response, would result in an extended growth delay.

From these findings, Ramabhadran (1976) recognized that successive cycles of near-UV irradiation would produce a very strong selection for *rel*⁻ mutants, whose growth would not be delayed by the treatment. In the course of isolation of such growth-delay-minus mutants of B/r, Ramabhadran *et al.* (1976b) found a mutant (RJ) that showed no growth inhibition at all (Fig. 6) but was *rel*⁺, as indicated by cessation of RNA accumulation during amino acid starvation. They showed that this mutant lacked ⁴Srd, as indicated by less than 5% of the normal fluorescence at 436-nm produced by the reduced ⁴Srd-Cyd photoproduct. This finding was supported by the work of Lipsett (1978), who measured chemically the ⁴Srd in the RJ mutant and found it to be less than 10% of that in the wild type. Tsai and Jagger (1981), using the same technique, isolated a mutant of *E. coli* B that is ⁴Srd⁻; this mutant also shows no growth inhibition at all by broad-band near-UV radiation. The finding of these ⁴Srd⁻ mutants lacking growth inhibition supports the conclusions based on other evidence that ⁴Srd is both the chromophore and the target* for near-UV radiation-induced growth inhibition in *E. coli* B and B/r, and that the ⁴Srd-Cyd adduct is the primary photoproduct responsible.

The earlier finding of Ballini *et al.* (1976), that the binding of *E. coli* tRNA^{Phe} to its synthetase reduces the rate of photo-cross-linking between ⁴Srd and Cyd by a factor of 2.3, has been followed up by circular dichroism studies showing that the reduced cross-linking results from a decreased probability of the two residues to assume the highly reactive conformational state required (Favre *et al.*, 1979). Thus, one might expect that effects involving 4-thiouridine absorption will be larger in systems, such as starved cultures, in which less of the tRNA is charged with amino acid, and this has been generally observed to be true (J. Jagger and co-workers, unpublished).

In *E. coli* K-12, Thomas and Favre (1977a) found 13 mutants with lowered ⁴Srd content, one of which (Nop) had no ⁴Srd and showed "almost negligible" growth inhibition at 366 nm (see also Thomas and Favre, 1980). However, my analysis of their (1977) data shows ~20% of normal growth delay in the Nop strain. It thus appears that, unlike *E. coli* B and B/r, *E. coli* K-12 shows a component of growth delay that does not depend upon ⁴Srd. This component might represent a relatively inefficient near-

* tRNA may also be considered to be the target, since it is the only cellular site of ⁴Srd.

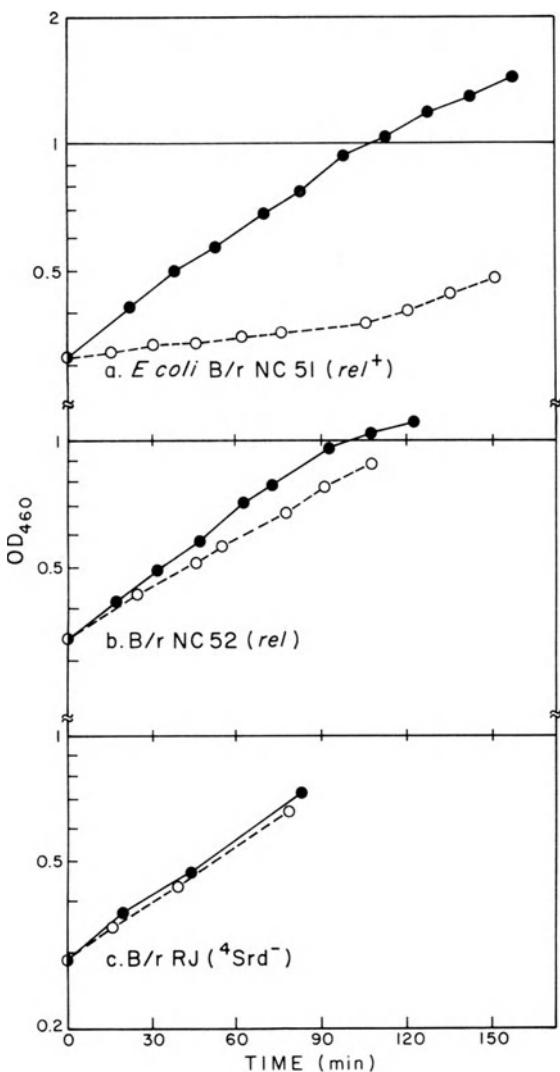


Fig. 6. Growth at 30°C of *E. coli* B/r strains in M9 minimal medium without (●) and with (○) exposure to 50 kJ/m² broad-band near-UV light (BLB lamps). Optical density at 460 nm is plotted on a logarithmic scale against time of postirradiation incubation. (a) NC51 (*valS*^{ts}), (b) NC52 (*valS*^{ts} *relA*), (c) NC32 RJ (*fuc valS*^{ts} *4Srd*⁻) (Tsai and Jagger, 1981).

UV radiation inactivation of tRNA by means other than ^4Srd absorption, as was suggested by Carré *et al.* (1974) and Thiam *et al.* (1980). Recent experiments by Favre and co-workers (Favre and Thomas, 1981, and A. Favre, personal communication) indicate that light at wavelengths <350 nm can be absorbed by 5-methylaminomethyl-2-thiouracil ($\text{mam}^5\text{s}^2\text{U}$), an unusual base found in the anticodon loops of the tRNA's of Glu, Lys, and Gln, leading to elevated ppGpp levels in a $^4\text{Srd}^-$ mutant (*nuv*) of K-12. The cross-section for absorption and the photoreactivity of this 2-thiouracil, however, is low compared to that of 4-thiouracil, except at the very short wavelength, 302 nm, where they are roughly equal. The 366-nm source used by Thomas and Favre (1977a) had a significant contaminant at 334 nm, sufficient to explain the residual growth delay as resulting from absorption in $\text{mam}^5\text{s}^2\text{U}$. Therefore, the K-12 strain appears to have a second minor chromophore for growth delay. *E. coli* B does not show evidence of such a minor chromophore, since its action spectrum fits the spectrum for ^4Srd -Cyd adduct formation (Kubitschek and Peak, 1980).

Recently, Thomas and Favre (1980) have shown that successive cycles of near-UV irradiation of mutagenized cultures of *E. coli* K-12 grown in glycerol provides a good yield of $^4\text{Srd}^-$ mutants, even in stationary-phase cells, which show no stringent response and therefore only the small growth delay due to the minor chromophore. The glycerol medium enhances the relative near-UV radiation-induced growth delay of the wild-type strain.

The mechanism for growth inhibition induced in *E. coli* B and B/r by near-UV radiation is diagrammed in Fig. 7.

It is important to recognize that, although there is no growth delay in *rel* $^-$ mutants of *E. coli* B/r, there is a small residual depression of growth rate (Figs. 5 and 6). This is to be expected, since damaged tRNA is produced even in *rel* $^-$ strains, and this alone slightly lowers the rate of protein synthesis at the ribosome (solid lines for *rel* $^-$ in Fig. 7). This growth-rate depression disappears in $^4\text{Srd}^-$ mutants.

Another, less certain, possibility for the *rel* $^-$ effect in B/r is a direct action of damaged tRNA on the genome. Modified tRNA might react directly with a regulatory system operating at the level of transcription, either through direct interaction with DNA or by allosteric combination with regulatory proteins (LaRossa and Söll, 1978). This route is shown by the broken lines in Fig. 7.

An *E. coli* K-12 gene (*nuv*) controlling the production of ^4Srd in tRNA has been located at 9.3 ± 0.2 min on the chromosome map (Thomas and Favre, 1977b). *E. coli* B/r NC32 RJ was shown by Lipsett (1978) to be deficient in factor A, one of the two proteins responsible for 4-thiolation of uridine (the levels of other thiouridines, including ^2Srd , were normal).

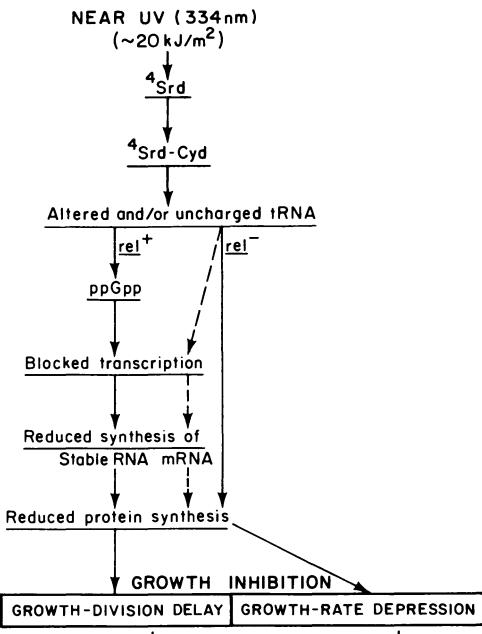


Fig. 7. Schematic diagram of the pathway for induction of growth inhibition (growth-division delay + growth-rate depression) in *E. coli* B and B/r by near-UV radiation. Broken arrows show a hypothetical pathway. This is part of the diagram shown in Fig. 10.

The site governing factor A lies roughly in the region of *nuv*. Lipsett proposed that *nuv* may be the structural gene for thiolation factor A, although Thomas and Favre (1977b) suggested that it may be a regulatory gene.

Kubitschek and Peak (1980) observed that the action spectrum for growth delay in *E. coli* B/r K fits very closely to the absorption spectrum for ⁴Srd in tRNA, but that this spectrum below 320 nm is lower than the action spectrum for the formation of the ⁴Srd-Cyd adduct (Thomas and Favre, 1975). They interpret this as an indication that, at wavelengths <320 nm, the adduct may not be the only photoproduct that contributes to growth delay. However, close examination of the fitting of a curve to their data for growth delay in strain B/r K indicates a significant departure from the action spectrum for the adduct only at the shortest wavelength (300 nm). The deviation is in the wrong direction to be explainable by the 2-thiouridine (²Srd) chromophore of Favre and co-workers (see above). Furthermore, at 300 nm, complications due to far-UV radiation effects enter the picture, making interpretations difficult.

Recent work by Madden *et al.* (1981) shows that menaquinone-8 (MQ-8; vitamin K₂) is extremely sensitive to near-UV light, showing an F₃₇ *in vivo* at 334 nm of 1.3 kJ/m² (Table 2, p. 37), a value 15 times lower than

that required for growth delay in *E. coli*. This high sensitivity might be due to the action of near-UV radiation on a trioxane photoproduct of MQ to produce a methyl ketone (Wilson *et al.*, 1980). The trioxane accumulates under blue light. However, Madden *et al.* (1981) grew and stored their cells under red light or in darkness. MQ-8 shows some small absorption in the region above 400 nm (Fig. 1) and the action spectrum for growth delay in *E. coli* also has a small component around 400 nm (Fig. 4). It would therefore appear that inactivation of MQ-8 might make some contribution to near-UV radiation-induced growth inhibition of *E. coli* at the longer wavelengths. However, even with broad-band near-UV light, virtually all growth inhibition is absent in ${}^4\text{Srd}^-$ strains of *E. coli* (Fig. 6).

Therefore, although MQ-8 alteration might account for some small fraction of near-UV radiation-induced growth inhibition in *E. coli* at longer wavelengths, it appears certain that it is not a chromophore for this effect in *E. coli* at 340 nm.

Kubitschek and Doyle (1981) have shown that rates of uptake of glucose and succinate in *E. coli* B/r are proportional to growth rate at all times during near-UV radiation-induced growth delay, and recovery from this growth delay. Rates of leucine uptake and oxygen utilization do not correlate with growth rate. They conclude that inhibition of carbon-source transport in *E. coli* (1) is a fundamental component, and (2) may be a primary mechanism (as opposed to the ${}^4\text{Srd-rel}$ mechanism) of near-UV radiation-induced growth delay. The first conclusion is acceptable, but may be a tautology, since cessation of uptake of carbon source would be one of the first consequences of a growth delay, however induced. The second conclusion appears to be incorrect, since (1) the evidence for the ${}^4\text{Srd-rel}$ mechanism is extensive and convincing, and (2) inhibition of transport systems requires about 5 times the fluence required for growth delay (Section 3.2). One need consider simply the fact that ${}^4\text{Srd}^-$ strains, irradiated with near-UV radiation fluences that produce growth delay in wild-type cells, grow at a normal rate in glucose medium.

2.1.4. Other Systems

Taber *et al.* (1978), using menaquinone-deficient (men^-) mutants, found that MQ is a target molecule for broad-band near-UV radiation-induced growth delay in *B. subtilis*. The F_{37} at ~ 350 nm required for growth delay in that system is ~ 300 kJ/m² (Table 2), about 5 times the fluence required in *E. coli*. It is curious that this growth delay therefore does not appear to involve ${}^4\text{Srd}$, which is known to be present in at least one *B. subtilis* tRNA (Tyr; Keith *et al.*, 1976); furthermore, ppGpp is present in *B. subtilis* (Silverman and Atherly, 1979), as well as adenine

analogs of ppGpp and pppGpp during sporulation (Rhaese and Groscurth, 1976).

One might wonder why the fluence required for growth delay is so high in *B. subtilis* if MQ-8 is so sensitive. We may suppose that damaged MQ-8 would affect electron transport and thereby the electrochemical proton gradient of the membrane. However, quite a massive reduction of the MQ-8 concentration would probably be necessary to produce a significant effect on the proton gradient. For example, Taber (1979) has found that the level of MQ in *B. subtilis* must be reduced to about 5% of normal before a significant effect is observed on the accumulation of gentamicin, and Brodie *et al.* (1979) showed that a near-UV radiation fluence that depresses oxidation in membrane vesicles of *Mycobacterium phlei* to 2% lowers proline transport only to 18%.

The findings of Taber *et al.* (1978) suggest that growth delay in *B. subtilis* reflects the time required for the removal of damaged quinone from the cell membrane, rather than the time required for resynthesis of MQ. This might serve as a model for near-UV-radiation induction of growth delay in the cells of higher organisms, which generally do not produce ppGpp (Silverman and Atherly, 1979).

2.2. Reduction of Bacterial Capacity for Phage Development

Near-UV radiation has long been known to reduce the capacity of bacteria to support phage growth. Day and Muel (1974) showed that the fluences required and the action spectrum for reduction of the capacity of *E. coli* B to support growth of phage T7 were similar to those for growth delay (Fig. 4). This suggested that the reduction of bacterial capacity by near-UV radiation might depend upon the *rel⁺* gene product and ⁴Srd. Wingo *et al.* (1980) demonstrated that this was so. Figure 8 shows that, at low fluences (up to about 40 kJ/m²), about 50% of capacity reduction for phage T2 (similar results were obtained with T7) requires the *rel⁺* gene, and at high fluences (40–100 kJ/m²), about 85% of capacity reduction requires the *rel⁺* gene (ratios based upon relative slopes). The data also show that about 95% of capacity reduction requires ⁴Srd. The low-fluence part of the capacity reduction curve for the wild-type strain (*rel⁺⁻*) shows an F_{37} of about 45 kJ/m², and the high-fluence part about 13 kJ/m², numbers which bracket the value of about 22 kJ/m² required for growth delay (Lakchaura *et al.*, 1976). The work of Wingo *et al.* (1980) suggested other parallels with bacterial growth delay, including confirmation of earlier observations of others that capacity reduction is reversible if the cells are allowed to incubate for about 2 h in nutrient broth at 37°C.

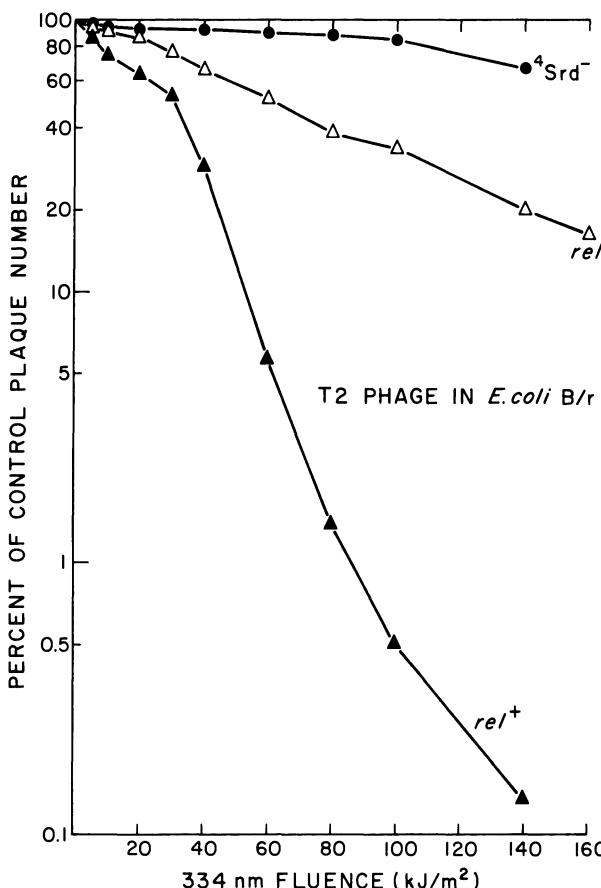


Fig. 8. *E. coli* B/r capacity for development of phage T2 as a function of monochromatic 334 nm fluence given to the host cells before infection. Solid triangles, NC51 *rel*⁺; open triangles, NC52 *rel*; circles, NC32 RJ (⁴Srd⁻) (Adapted from Wingo *et al.*, 1980).

For the *rel*⁺-gene-dependent part of capacity reduction, Wingo *et al.* (1980) suggested the following mechanism (Fig. 10): (1) near-UV radiation inhibits bacterial protein synthesis, (2) this causes a delay in the onset of phage growth, (3) this results in a greatly decreased burst size, and (4) the reduction in capacity reflects this decreased burst size. However, it is likely that depressed protein and RNA metabolism of the bacterium is not the only cause of decreased capacity: Donini (1970) has demonstrated a direct action of the *rel*⁺ gene product on phage DNA synthesis (see also Marchin, 1980). This route is shown by broken lines in Fig. 10. These effects of near-UV radiation on burst size and latent period are rather similar to those observed by Coetzee and Pollard (1974) on near-UV ra-

diation-induced reduction of the capacity of *E. coli* K-12 (λ) to support growth of phage λ , after induction by far-UV or gamma radiation. They interpreted their near-UV radiation effects as indicating a target in the cell envelope. This is quite different from the findings of a near-UV radiation-induced reduction in capacity for T2, which depends upon ^4Srd in tRNA.

For the fraction of capacity reduction not requiring the *rel⁺* gene (15–50%), it is clear that most of it requires ^4Srd , but the mechanism of action is unknown. It may result from lowered rates of transcription and translation due directly to damaged tRNA (same pathways as for growth-rate depression; Figs. 7 and 10).

2.3. Inhibition of Induced-Enzyme Synthesis

Webb and Bhorjee (1967) were the first to demonstrate that near-UV and visible radiation (300–450 nm) inhibited the induction of an enzyme (β -galactosidase in *E. coli* B). Although their treatment involved continuous irradiation, it is clear from their data that a fluence as low as 5 kJ/m² would totally inhibit β -galactosidase synthesis. They also found that irradiation was without effect if the cells were induced 15 min before irradiation, showing that the action was on the induction mechanism and not upon the enzyme itself or the machinery of protein synthesis.

In 1970, Swenson and Setlow did a thorough study of the inhibition of induction of tryptophanase in *E. coli* B/r by both monochromatic and broad-band (BLB; 300–400 nm) near-UV radiation. They obtained an action spectrum (Fig. 4) that was nearly identical to the action spectrum for growth delay of Jagger *et al.* (1964), thus strongly suggesting that the two phenomena share a common chromophore. They found that inhibition of tryptophanase induction showed an F_{37} at 334 nm of 5 kJ/m², a value only 20% of that required for growth delay, but that inhibition of β -galactosidase induction required higher fluences. They concluded that inhibition of tryptophanase induction was not a result of growth delay in the bacterium. They raised the possibility that pyridoxal phosphate might be the chromophore, since it is a cofactor for tryptophanase and has the appropriate near-UV absorption (Fig. 1 and Table 1). However, the results of several experiments ruled this out; these experiments included the finding that the irradiation of cells after induction did not affect the enzyme activity.

Sharma *et al.* (1981) have recently completed a study of 334 nm inhibition of tryptophanase induction in *E. coli* B/r, using *relA* and $^4\text{Srd}^-$ mutants. They found that about 50% of near-UV radiation-induced inhibition of tryptophanase induction depends upon the *relA⁺* gene, while

more than 90% of the effect requires ${}^4\text{Srd}$ (Fig. 9). For inhibition of induction, they obtained an F_{37} of 5.6 kJ/m^2 , nearly identical to that of Swenson and Setlow (1970), and supporting the idea that there is not a close connection between growth delay and inhibition of tryptophanase induction. One might suppose that a bacterial growth delay would cause an accumulation of catabolites that could cause catabolite repression of induced-enzyme synthesis, as appears to be true for the inhibition of β -galactosidase induction by far-UV radiation (Pardee and Prestidge, 1963). However, Swenson and Setlow (1970) have demonstrated that near-UV radiation inhibition of tryptophanase induction is not due to catabolite repression.

It therefore appears that, although ${}^4\text{Srd}$ is apparently the chromophore for inhibition of tryptophanase induction, this effect does not result from the induction of a growth delay. The 50% of inhibition of induced enzyme synthesis that requires the *rel⁺* gene product may reflect a direct action of ppGpp on the induction process (Fig. 10; solid lines to "blocked transcription," then broken lines to "reduced protein synthesis"). ppGpp is known to have direct actions on RNA polymerase, affecting its binding to promoters (van Ooyen *et al.*, 1976).

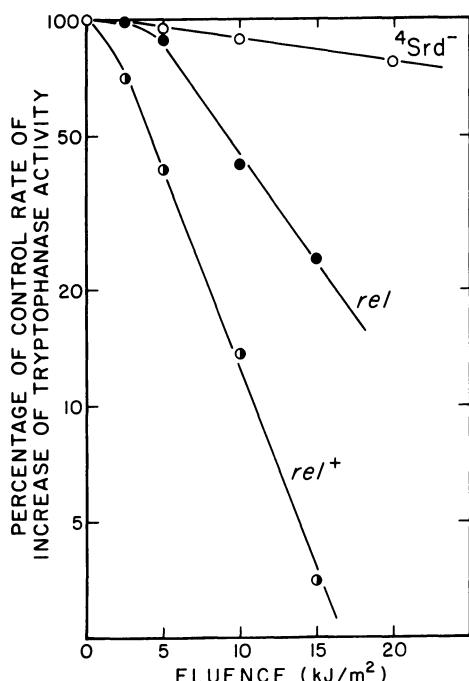


Fig. 9. Inhibition of induced tryptophanase activity as a function of monochromatic 334 nm near-UV fluence in *E. coli* B/r NC32 RJ ("Srd⁻") (○); *E. coli* B/r NC52 (*relA*) (●); *E. coli* B/r NC51 (*relA⁺*) (◐) (Sharma *et al.*, 1981).

Most of the remaining action requires ${}^4\text{Srd}$ but occurs in rel^- strains (Fig. 9). It is known that tRNA can react directly with DNA and that it can combine allosterically with regulatory proteins (LaRossa and Söll, 1978). It is possible that tRNA possessing ${}^4\text{Srd-Cyd}$ adducts may react directly with a regulatory mechanism of enzyme induction operating at the level of transcription, which could account for the inhibition of tryptophanase induction that is independent of the rel^+ gene (Fig. 10; broken-line pathway for rel^-).

2.4. Summary

It has now been demonstrated that ${}^4\text{Srd}$, which seems to exist only in the tRNA of prokaryotes, is required for all three of the sublethal effects in *E. coli* B and B/r so far discussed: growth inhibition, reduction of capacity to support phage growth, and inhibition of induced tryptophanase synthesis. This fact is clearly demonstrated by the virtual absence of all three effects in mutants that lack ${}^4\text{Srd}$.

It is of course theoretically possible that ${}^4\text{Srd}$ could be required for these sublethal effects but not be the actual chromophore. However, the very close agreement between the action spectra for these effects and the absorption spectrum of ${}^4\text{Srd}$ in tRNA (Fig. 4), as well as the agreement between the fluences required for these effects and the fluence required to produce the ${}^4\text{Srd-Cyd}$ adduct, provides very strong circumstantial evidence that ${}^4\text{Srd}$ is in fact the chromophore. Since ${}^4\text{Srd}$ occurs only in tRNA, it also follows that tRNA can be considered to be the target molecule for these effects.

There is excellent evidence, however, that menaquinone (MQ) is the chromophore and target for growth delay induced in *B. subtilis*. That this growth delay requires a fluence about 5 times greater than that involved in *E. coli* growth delay indicates that it represents a different system for induction of growth delay. Nevertheless, the high sensitivity to near-UV radiation of MQ (Section 2.1.3), as well as its favorable absorption spectrum (Fig. 1), leaves it still a prime candidate for a chromophore in other near-UV radiation-induced effects, and it may even play some small role in the sublethal effects discussed here. For example, both reduction of phage capacity (Fig. 8) and inhibition of induced tryptophanase synthesis in *E. coli* B/r (Fig. 9), but *not* growth inhibition (Fig. 6), show small residual effects in ${}^4\text{Srd}^-$ mutants. These might represent absorption by other chromophores in tRNA, such as ${}^2\text{Srd}$ (Section 2.1.3), but they might also result from actions on MQ.

Figure 10 is a schematic diagram of proposed pathways for induction

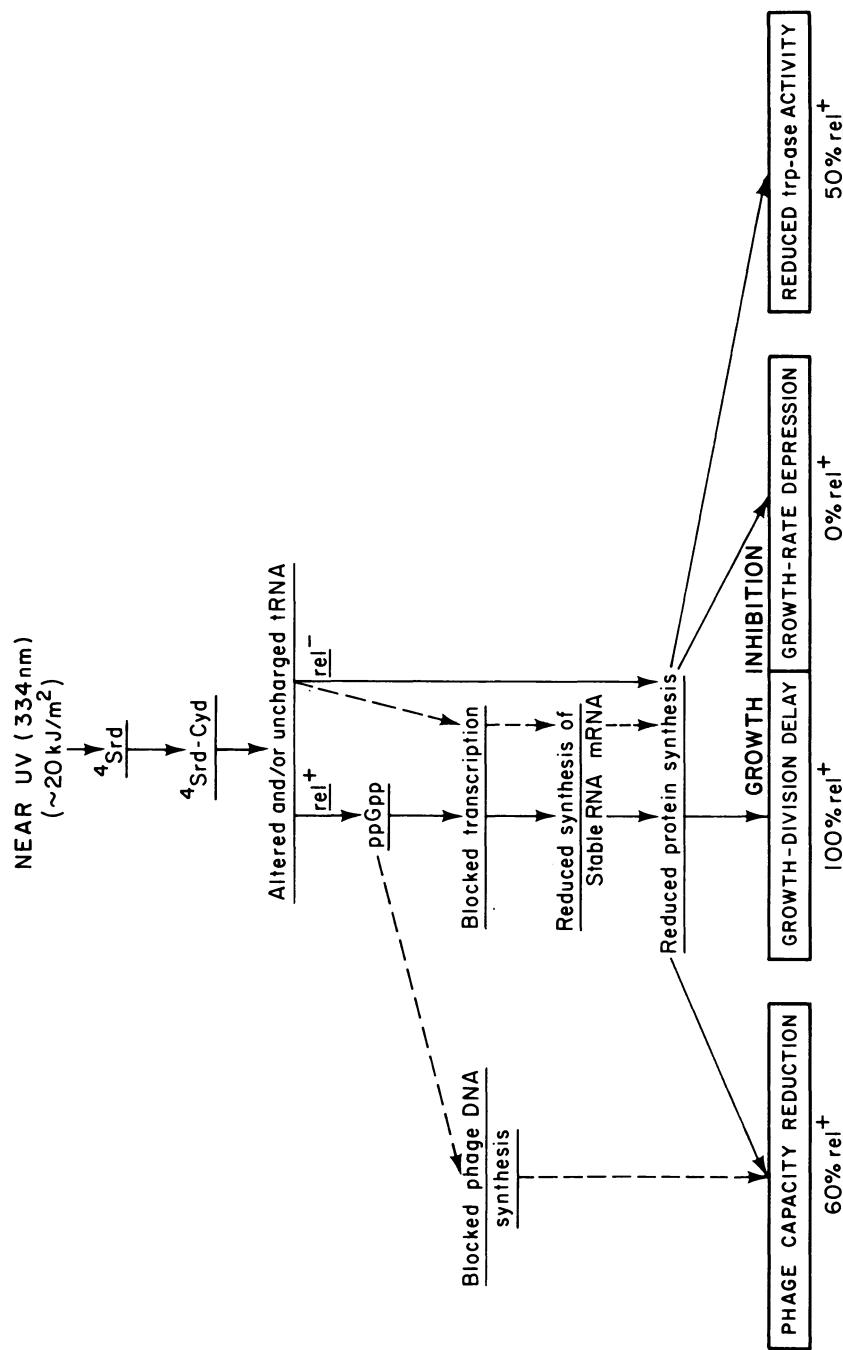


Fig. 10. Schematic diagram of proposed pathways for induction of several sublethal effects of near-UV radiation (reduced capacity for phage development, growth inhibition, reduction of tryptophanase activity) in *E. coli* B and B/r. Broken arrows show more hypothetical pathways.

of the sublethal effects of near-UV radiation in *E. coli* B and B/r. The central part of this diagram, showing pathways leading to growth inhibition, has already been discussed (Fig. 7). Growth inhibition consists of about 90% growth-division delay and about 10% growth-rate depression (Figs. 2b and 6). Growth delay shows 100% dependence upon the *rel*⁺ gene, and therefore probably results entirely from the induction of ppGpp. Growth-rate depression appears to be independent of the *rel*⁺ gene (cf., Figs. 2a and 6), but still requires ⁴Srd; it may represent direct actions of altered and/or uncharged tRNA on transcription and translation of messenger RNA.

Reduction of the capacity of bacteria to support phage growth shows approximately 60% dependence upon the *rel*⁺ gene, which therefore can be expected to result from inhibition of protein synthesis, with a possible contribution from direct action of ppGpp on phage DNA synthesis. Phage capacity reduction in *rel*⁻ strains might involve direct actions of altered tRNA on mRNA genes, which also would lead to reduced protein synthesis.

Inhibition of induced tryptophanase synthesis depends 50% upon the *rel*⁺ gene product. This may involve a direct action of ppGpp on the induction (transcription) process, affecting the binding of RNA polymerase. The remaining 50% of reduced tryptophanase activity that does not depend upon the *rel*⁺ gene may represent a rather specific effect on the induction process, produced by altered tRNA acting directly upon transcription (Fig. 10; broken-line pathway for *rel*⁻).

Finally, it must be emphasized that these pathways are proposed only for sublethal effects in *E. coli* B and B/r. The *lon* gene, which controls filament formation in *E. coli* B, and which appears to account for the difference in radiation sensitivity of *E. coli* B and B/r, does not seem to be involved. However, other strains of *E. coli* may respond somewhat differently; there is now good evidence for a secondary ²Srd short-wavelength chromophore for growth delay in the tRNA's of K-12 strains, and possibly in B/r at wavelengths <320 nm. The fact that higher organisms generally do not produce ppGpp, and seem to lack ⁴Srd, indicates that these pathways cannot be expected to apply to the higher eukaryotes.

3. EFFECTS AT LETHAL FLUENCES

3.1. Targets for Lethality

Although this review is not concerned with lethal and mutagenic effects (see Section 1), many physiological effects of near-UV radiation on bacteria occur at fluences in the lethal range (~100–1000 kJ/m²), and it

is therefore possible that the targets in some cases are identical with those for lethality. I therefore briefly review here current knowledge of lethal, and sometimes mutagenic, targets in the near-UV region (wavelengths >313 nm). The literature on this subject up to 1977 was thoroughly reviewed by Webb (1977), and a more recent analysis was presented by Tyrrell (1979a). Evidence for statements not referenced in this section will be found in those reviews.

One of the most important observations is that lethality in a variety of microorganisms shows a strong oxygen dependence above 313 nm, the sensitivity of *E. coli* in air being an order of magnitude greater than in nitrogen. A similar sensitivity is seen in transforming DNA (Section 1.2.2). This oxygen dependence alone means that the major mechanism of killing in the near-UV region is different from that in the far-UV region. Much other evidence supports this conclusion, such as the absence of photoreactivation in wild-type strains (Webb, 1977).

Nevertheless, in the near-UV region, DNA remains the primary target for lethality. Supporting evidence includes the observations that (1) many agents (such as glycerol or oxygen) that alter the sensitivity of transforming-principle DNA to near-UV radiation alter cell survival in a comparable way, (2) in stationary growth phase, a strain completely deficient for dark repair of DNA, *E. coli* K-12 AB2480 (*uvrA recA*), is ~ 70 times more sensitive to near-UV radiation than the wild-type strain (although it is 1000 times as sensitive to far-UV radiation), and (3) DNA repair inhibitors for damage produced by far-UV radiation, such as acriflavin, sensitize wild-type cells to near-UV radiation-induced killing. (For an excellent review of DNA repair systems for damage produced by far-UV radiation, see Witkin, 1976.)

Does DNA absorb the near-UV light directly? The recent determination of DNA absorption out to 360 nm (Section 1.2.2) indicates that the absorption cross-sections (per nucleotide, relative to 260 nm) at these wavelengths are an order of magnitude higher than the cross-sections (relative to 260 nm) for aerobic killing and two orders of magnitude higher than the relative cross-sections for anaerobic killing (Sutherland and Griffin, 1981) or for mutation. The roughly similar curve shapes of DNA absorption and of lethality action spectra (Sutherland and Griffin, 1981; Cabrera-Juárez *et al.*, 1976; Peak and Tuveson, 1979) suggest a DNA chromophore, but the differences in relative cross-section indicate that the quantum yields for direct DNA damage are lower in the near-UV region than in the far-UV region. However, it is likely that this absorption is not intrinsic to the DNA, but is caused by tenacious impurities (Section 1.2.2).

Aerobic killing probably involves both indirect damage to DNA (through intimately associated chromophores or sensitizers) and damage to other molecular systems, such as DNA repair systems, tRNA, or membranes. This hypothesis arises from a variety of observations of near-UV radiation effects in *E. coli*: (1) aerobic killing is oxygen-dependent and shows no photoreactivation in wild-type strains (Webb, 1977); (2) repair disruption by near-UV radiation is oxygen-dependent (Tyrrell, 1979a); (3) the action spectrum for aerobic killing shows detailed structure (Webb, 1977), suggesting absorption by dye-like molecules; (4) there is evidence that ⁴Srd in tRNA is a chromophore for lethality; and (5) there is evidence for lethality due to membrane damage (Section 3.2).

What are the lethal lesions produced in DNA by near-UV radiation? The oxygen dependence of near-UV radiation-induced killing, as well as the protection from killing by substances that quench excited molecules or scavenge free radicals (Section 1.2.2), is consistent with photooxidation being a major mechanism. Pyrimidine dimers and single-strand breaks are produced with roughly equal efficiency in DNA in air at 366 nm (Tyrrell, 1979a). Under *aerobic* irradiation conditions, however, pyrimidine dimers appear to be important lethal lesions only in some phages [T4 and λ (Tyrrell, 1979c)] and in completely repair-deficient cells or in cells that have photoreactivating enzyme but are irradiated at 0°C; under normal conditions, with photoreactivation-sufficient cells, the inactivating light itself photoreactivates most of the dimers (concomitant photoreactivation). Webb and Brown (1979a) have shown that some pyrimidine dimers induced by near-UV radiation in repair-deficient strains result from excited states that can be quenched by oxygen, and are therefore produced by a different mechanism than are those produced by far-UV radiation.

Some role in *anaerobic* killing may be played by pyrimidine-dimer formation, which is not enhanced by oxygen (Tyrrell, 1973). Pyrimidine dimers are induced only at wavelengths below 405 nm (Tyrrell, 1979a), and anaerobic killing is difficult to observe at wavelengths above 400 nm (Webb, 1977).

Under aerobic irradiation conditions, the principal lethal lesion may be single-strand breaks in DNA. Evidence supporting this hypothesis includes the observations that single-strand breakage by near-UV radiation (1) requires oxygen, (2) is found at 405 nm, where lethality occurs but dimers are not found, and (3) is highly dark-repairable in wild-type strains. Because of the oxygen dependence, these breaks are produced by a different mechanism (probably photosensitized oxidation) than those produced by far-UV radiation.

In some cases, DNA single-strand breaks seem not to be important

for lethality. For example, the synergism observed between near-UV radiation and H_2O_2 for lethality in phage T7 does not correlate with the induction of single-strand breaks in DNA (Ananthaswamy *et al.*, 1979), and Hartman *et al.* (1979) have shown that combined near-UV radiation and H_2O_2 inactivation of phage T7 produces DNA-protein crosslinks, which appear to be the lesion preventing injection of phage DNA into the host. However, other work in *E. coli* K-12 (Hartman and Eisenstark, 1978, 1980) indicates that *recA*-independent (high H_2O_2 concentration) synergistic killing by near-UV radiation and H_2O_2 does result from DNA single-strand breaks (later converted to double-strand breaks), possibly induced by photolysis of H_2O_2 in the presence of some unknown near-UV radiation chromophore (see Section 4.1).

Mutation appears to result from indirect damage to DNA. In repair-deficient strains, it involves pyrimidine dimers, but this is probably not true for wild-type strains. Evidence for this comes from the observations that (1) mutagenesis requires oxygen (Webb, 1977), (2) the action spectrum for mutation in *E. coli* shows detailed structure (Webb, 1977), suggesting absorption by dye-like molecules, and (3) mutation (but not killing) in a *uvrA* strain is highly photoreactivable.

Webb (1978) has shown that mutation (reversion to tryptophan independence), but not killing, in a *uvrA* mutant of *E. coli* WP2 is caused by pyrimidine dimers, since it is highly photoreactivable. It appears that mutation fixation in this system involves error-prone SOS repair. Since the mutagenesis at 365 nm is 4.5 times more efficient, for a given lethal effect, than at 254 nm, and shows 2-hit kinetics, it may involve interaction of nondimer oxygen-dependent lesions with pyrimidine dimers. Webb (1978) suggests that these nondimer lesions might be single-strand breaks or pyrimidine adducts. An adduct similar but not identical to the spore photoproduct (5-thyminyl-5,6-dihydrothymine) was found by Cabrera-Juárez and Setlow (1977) to be a possible lethal photoproduct in near-UV irradiated purified DNA. Earlier studies (see Jagger, 1976) failed to show any role, in the far-UV radiation killing of bacteria, of another pyrimidine adduct (6-4'-[pyrimidine-2'-one]thymine), which is photolysed by 313 nm radiation, but not reverted to the original pyrimidines.

There is much evidence that both light- and dark-repair systems are inactivated to some extent by lethal fluences of near-UV radiation, and this presumably causes the highly shouldered curves observed for near-UV radiation inactivation. At 366 nm, the break in this shouldered curve, for the *E. coli* K-12 wild-type strain, occurs at about 10^6 J/m^2 , at which fluence only about 30 single-strand breaks have been produced in the DNA of a completely dark-repair-deficient strain (Tyrrell, 1979a). Pre-

sumably most of these breaks are repaired in a wild-type strain, and the subsequent sharp drop in survival at higher fluences would, therefore, reflect the destruction of repair systems.

As we have already noted (Section 1.2.2), the major (oxygen-dependent) lethal effects in DNA are probably not produced by the direct absorption of light by the DNA. The action spectrum for killing of *E. coli* in air shows significant plateaus at 340, 410, and 500 nm (the 340 nm plateau also occurs in *Salmonella typhimurium*), suggesting absorption by several specific chromophores. Tsai and Jagger (1981) have recently reported an approximately 50% dependence of lethality in starved exponential-phase cells of *E. coli* B/r on the presence of ${}^4\text{Srd}$. This may be the 340-nm chromophore. Evidence apparently supporting the idea that ${}^4\text{Srd}$ is a chromophore for lethality is the finding of Cabrera-Juárez and Setlow (1979) that exponential-phase cultures of *Haemophilus influenzae* are killed with (1) and $F_{37} = 3 \text{ kJ/m}^2$ at 334 nm (1/100 of that required by *E. coli*), (2) an action spectrum like ${}^4\text{Srd}$ absorption, (3) no concomitant mutation, and (4) no influence of DNA repair abilities. However, there are difficulties with such an interpretation. It is difficult to see at this time how damage to ${}^4\text{Srd}$ would result in oxygen-dependent damage to DNA, especially since the near-UV-radiation induction of growth delay, produced by absorption in ${}^4\text{Srd}$ (at much lower fluences), is not dependent upon oxygen (Section 2.1.2). Clearly, one needs to measure DNA damage in isogenic strains that differ only at the ${}^4\text{Srd}$ locus.

Tuveson and Jonas (1979) have found a chromosomal gene (*nur*) of *E. coli* K-12 that confers near-UV radiation sensitivity to cells irradiated in the stationary growth phase. This gene is located at minute 58 on the *E. coli* linkage map, close to the *recA* allele (lacking recombination repair), which confers sensitivity to far-UV radiation. Isogenic strains representing all four combinations of these two genes have been isolated: RT-1 (*nur recA*), RT-2 (*nur*), RT-3 (*recA*) and RT-4 (wild type). The same high survival was observed for wild-type (RT-4) and *recA* (RT-3) strains, but both *nur* strains (RT-1, RT-2) showed the same much-lowered survival after near-UV irradiation (fluence-reduction factor of about 0.3). Thus, it would appear that the *nur* mutation, but not the *rec* mutation alone, produces sensitivity to near-UV radiation. These findings may explain some of the variable results found for near-UV radiation sensitivity dependence upon the *recA* gene, since *recA* and *nur* may be easily transduced at the same time. In later work (Tuveson, 1980) with strains containing all four possible combinations of *nur* and *uvrA* (lacking excision repair) genes, it was shown that, as was true for recombination deficiency, an excision-repair defect does not lead to near-UV radiation sensitivity

for cells irradiated in stationary phase. It therefore appears that lethal near-UV radiation damage produced in stationary-phase cells can be repaired as well by either recombination repair or excision repair as by both systems working together. Finally, Tuveson (1981) has found that the *polA1* mutation (lacking repair polymerase) sensitizes cells to near-UV radiation as much as does the *nur* mutation, suggesting that these two mutations may sensitize by an equivalent mechanism. This could explain the puzzling frequent failure of the *polA* gene to provide near-UV radiation sensitization. These results may not apply to rapidly growing (logarithmic-phase) cells.

Tuveson (1980) proposes that the sensitizing effect of the *nur* gene may result from an elevated concentration of a photosensitizer that absorbs near-UV radiation and, in the presence of oxygen, generates singlet oxygen; this proposal receives some support from the finding (Tuveson and March, 1980) that *nur* strains are particularly sensitive to inactivation by visible light in the presence of acridine orange. Further evidence is the observation by Peak and co-workers that both histidine and 2-aminoethylisothiouronium bromide hydrobromide (AET) (Peak and Peak, 1975), as well as 1,4-diazodicyclo(2.2.2) octane (DABCO) (Peak *et al.*, 1981), substances that are all effective scavengers of singlet oxygen, provide about a 50% protection against the inactivation of transforming DNA. However, recent work with conidia of *Neurospora crassa* indicates that killing by broad-band near-UV radiation, contrary to the case with visible light and photosensitizing dyes, is not due to singlet oxygen (Thomas *et al.*, 1981).

Peak and Peak (1980) have shown that glycerol protects transforming DNA against the actions of both X-rays and 366 nm UV radiation, but not against 254 nm UV radiation. This is a large effect (factor of 3 in the near-UV region), and the mechanism is not understood, but it is consistent with an indirect action of near-UV radiation on DNA.

Rapidly growing bacteria may have further dimensions of near-UV radiation sensitivity, as indicated by their much greater sensitivity to near-UV radiation-induced killing (Peak, 1970; Mackay *et al.*, 1976; Webb, 1977; Hartman and Eisenstark, 1978; Tuveson and Jonas, 1979). Peters (1977) has presented data implicating photoinactivation of the ribonucleoside diphosphate reductase (RDP-reductase) complex in the killing of exponentially growing *E. coli* by near-UV radiation. (Later unpublished determinations by J. Peters and J. Jagger indicate that the fluences reported in this paper are low by a factor of 30.) Peters and Jagger (1981) have presented evidence for a repair system induced by near-UV radiation in actively growing cultures of *E. coli* K-12, which repairs a major fraction of the lethal damage produced in these cultures by near-UV radiation.

This repair system is different from the error-prone "SOS" repair system for far-UV radiation-induced damage to DNA, being independent of the *recA* gene, and involving the induction of several new proteins different from the *recA*⁺ gene product. This induced repair system occurs also in a strain of *E. coli* B (PP-1) that lacks ⁴Srd and shows no photoprotection, and also in *E. coli* K-12 AB2480 (*recA uvrA*) (J. Peters, unpublished), indicating that (1) ⁴Srd is not the chromophore for the induction, so that the *rel*⁺ gene product is probably not involved, and (2) the recovery is not related to photoprotection, liquid-holding recovery, or excision repair.

A possibly similar effect, involving the apparent induction by white light of a repair protein for *far-UV* radiation damage, has been reported in the cyanobacterium *Anacystis nidulans* (Bhattacharjee, 1977).

Membrane damage may contribute to lethality under some circumstances (Section 3.2). Nevertheless, the evidence that DNA is the primary target for near-UV radiation lethality is extensive and includes the observation that stationary-phase *E. coli* AB2480 (*uvrA recA*), which is completely deficient in DNA dark repair, is ~70 times more sensitive to 365 nm near-UV radiation than is the wild-type strain (Webb, 1977). On the other hand, strains like AB2480 are often fastidious in their growth requirements and may have a defective membrane that is more easily damaged by near-UV radiation.

Many findings point to the advisability of doing near-UV radiation lethality studies with cells that are clearly in a particular growth phase. Sensitivity to the effects of near-UV radiation depends greatly upon growth phase, and actively growing (exponential-phase) cells appear to have additional mechanisms and/or degrees of inactivation and repair; these may be quantitatively much more important for such cells than the damage and repair systems operative in nongrowing cells.

In summary, killing of *E. coli* by near-UV radiation (>313 nm) involves DNA as a target, but is different from far-UV radiation-induced killing, showing a strong oxygen dependence. Nevertheless, DNA repair systems for far-UV radiation-induced lethal damage are also very effective for near-UV radiation-induced lethal damage. In addition, there is a *nur*⁺ gene involved in near-UV, but not far-UV, radiation-induced lethal damage in stationary-phase cells, and there is an induced repair system, different from the far-UV SOS system, for lethal damage in exponentially growing cells. DNA is damaged by near-UV radiation through photooxidation processes, possibly involving singlet oxygen. The oxygen-dependent lethal lesions in DNA are not pyrimidine dimers, but may be single-strand breaks. 4-Thiouridine in tRNA appears to be a subsidiary chromophore in log-phase cells, and membrane damage may contribute to lethality under certain conditions.

3.2. Membrane Targets

Although some effects of near-UV radiation may involve actions on cytoplasmic components (${}^4\text{Srd}$, RDP reductase), it is evident that, when lethal fluences are approached, effects on membranes do assume importance. Membranes contain a number of dye molecules that absorb near-UV radiation effectively (Section 1.2.1).

Shortly after the isolation of the isoprenoid naphthoquinone vitamin K₁, its sensitivity to sunlight was demonstrated by Almquist (1937); Ewing *et al.* (1943) later showed that 366-nm light was highly effective in the inactivation. Brodie and Ballantine (1960) explored this effect and showed that vitamin K₁ is drastically affected by irradiation *in vitro* with black light. Extensive further work, primarily by Brodie and co-workers in bacteria (see Brodie *et al.*, 1979), and by Beyer in mammalian mitochondria (see, e.g., Beyer, 1959), showed that near-UV radiation easily destroys naphthoquinones in the same fluence range that inactivates electron transport and oxidative phosphorylation, and that replacement by unirradiated naphthoquinones in particulate systems can restore both electron transport and oxidative phosphorylation. Madden *et al.* (1981) have recently shown that the naphthoquinone vitamin K₂ (menaquinone-8; MQ-8), can be exceedingly sensitive to near-UV light either *in vivo* or *in vitro*, showing an F₃₇ of only a few kJ/m² (Table 2).

There is thus considerable evidence that isoprenoid naphthoquinones, which function in the electron-transport systems of oxidative phosphorylation, may be important targets for near-UV radiation. However, there are complications. For one thing, the extreme sensitivity of MQ-8 found by Madden *et al.* (1981) is far greater than the sensitivity observed by Brodie *et al.* (1979). (Although the assays were different, loss of electrophoretic mobility being assayed by Madden *et al.* (1981) and loss of UV absorption by Brodie and co-workers, it is unlikely that this would result in the very large differences in their observations.) In addition, it is known that quinones added to particulate systems can bypass segments of the electron-transport system (Gel'man *et al.*, 1975), which may contain the true targets. Thus, although long suspected to be important chromophores and targets for near-UV radiation effects, naphthoquinones have been clearly demonstrated to be the chromophore and target in only one instance, that of near-UV radiation-induced growth delay in *B. subtilis* (Taber *et al.*, 1978; Section 2.1.4).

Isoprenoid benzoquinones (ubiquinones, UQ), which also operate in the electron-transport systems of mitochondria and of some bacteria, are much less sensitive to near-UV radiation. Werbin *et al.* (1974) have shown that the F₃₇ at 334 nm for loss of chromatographic mobility of ubiquinone-8 (UQ-8) in *E. coli* B is 100 kJ/m² *in vitro* and 420 kJ/m² *in vivo* (Table

Table 2. Fluences Required for Inhibition of Various Bacterial Membrane Systems

	334 nm	366 nm	405 nm
Systems			
Passive permeability ^a		~2000	
Electrochemical proton gradient ^b		~2000	
ATP synthesis ^c	140	≥ 750	~1800
Respiration		350–550 ^{a,d,e}	320 ^d
Growth delay (<i>B. subtilis</i>) ^f		400	
Sorbose transport (<i>S. cerevisiae</i>) ^g		750	
Ala/gly transport			
Glucose-grown	140 ^e	420, ^e 680 ^d	2500 ^e
Glycerol-grown		235 ^d	
Molecules			
Lactose permease	~160 ^h	~ 600 ^a	~ 900 ^h
Ubiquinone-8 ⁱ			
<i>In vivo</i>	420	850	4000
<i>In vitro</i>	100	260	510
Menaquinone-8 ^j			
<i>In vivo</i>	1.3	4.5	52
<i>In vitro</i>	4.2	9.7	55
Dehydrogenases			
NADH ^c	40	60	500
Lactic ^k			900
Succinic ^k			500
α-Glycerophosphate ^k			900

Note: Values shown are the F₃₇, the fluence in kJ/m² required to lower activity to 37% of control activity. Organism is *E. coli* except where noted.

^a Koch *et al.* (1976).

^b Sprott and Usher (1977); very large threshold (~1000 kJ/m²).

^c Lakchaura *et al.* (1976); thresholds observed for ATP synthesis ≈ 50 kJ/m² at 334 nm and 200 kJ/m² at 366 and 405 nm.

^d Sprott *et al.* (1976).

^e Derived from Sharma and Jagger (1981).

^f Taber *et al.* (1978). Source was General Electric BLB lamps with plate-glass filters, which cuts out much (but not all) of the radiation at 334 nm.

^g Doyle and Kubitschek (1976).

^h Robb and Peak (1979) show a value at 366 nm of 40 kJ/m², which is probably incorrect (F. T. Robb, personal communication). Their action spectrum is used to obtain relative values at 334 and 405 nm.

ⁱ Werbin *et al.* (1974).

^j Madden *et al.* (1981).

^k D'Aoust *et al.* (1980).

2). These data make ubiquinone a possible, but much less likely, target than menaquinone in the lethal fluence range.

There are reasons to believe that a large fraction of quinones would have to be destroyed before significant biological effects would be observed (see, e.g., Section 2.1.4). Therefore, although it is tempting to invoke quinone inactivation in near-UV radiation-induced biological effects, the evidence for such involvement must be better than circumstantial if it is to be convincing. No one has yet demonstrated clearly that ubiquinone is the target for any near-UV radiation-induced biological effect.

Flavins and cytochromes are generally more resistant to near-UV radiation than the quinones (see Jagger, 1972). Nevertheless, it has been demonstrated that the inactivation of cytochrome oxidase is responsible for the inhibition by violet light of growth and respiration in the colorless alga *Prototheca zopfii* and in the yeast *S. cerevisiae* (Epel, 1973). D'Aoust *et al.* (1974) reported that the flavoenzyme dehydrogenases for succinate, L- α -glycerophosphate, and D-lactate are inactivated by light of wavelength >400 nm ($F_{37} \approx 1$ MJ/m²), and they now report (D'Aoust *et al.*, 1980) that compounds known to react with flavin (cysteine, anthranilic acid) protect against such inactivation, with a fluence-modification factor ≈ 0.2 (Fig. 11). Sprott *et al.* (1976) reported that irradiation at 366 or 405 nm with similar fluences caused loss of flavoprotein and cytochrome absorption in *E. coli* membrane vesicles. These results show that flavoproteins may be targets at sufficiently high fluences. However, D'Aoust *et al.* (1980) do not rule out the possibility of flavin-photosensitized production of peroxides or free radicals that could inactivate parts of the electron-transport chain. Perhaps singlet oxygen is so produced.

There are thus few instances in which components of the electron-transport chain have been definitely shown to be involved in near-UV radiation-induced biological effects. Nevertheless, it seems likely that they are involved to some extent. For example, Sprott and Usher (1977) have shown that a fluence of 1 MJ/m² at 366 nm causes about an 80% inhibition of respiration, and about a 25% loss in the electrochemical proton gradient in *E. coli* (Table 2). Loss of electrochemical proton gradient shows a large threshold and is very rapid at fluences above 1 MJ/m². However, most of the physiological effects observed in the lethal-fluence range occur at fluences below 400 kJ/m², at which there are usually only small effects on respiration rate but no effect on electrochemical proton gradient (Table 2).

Some near-UV lethality may result from membrane alterations, to a considerably greater degree than is observed with far-UV radiation. For example, Moss and Smith (1981) have shown that stationary-phase *E. coli*

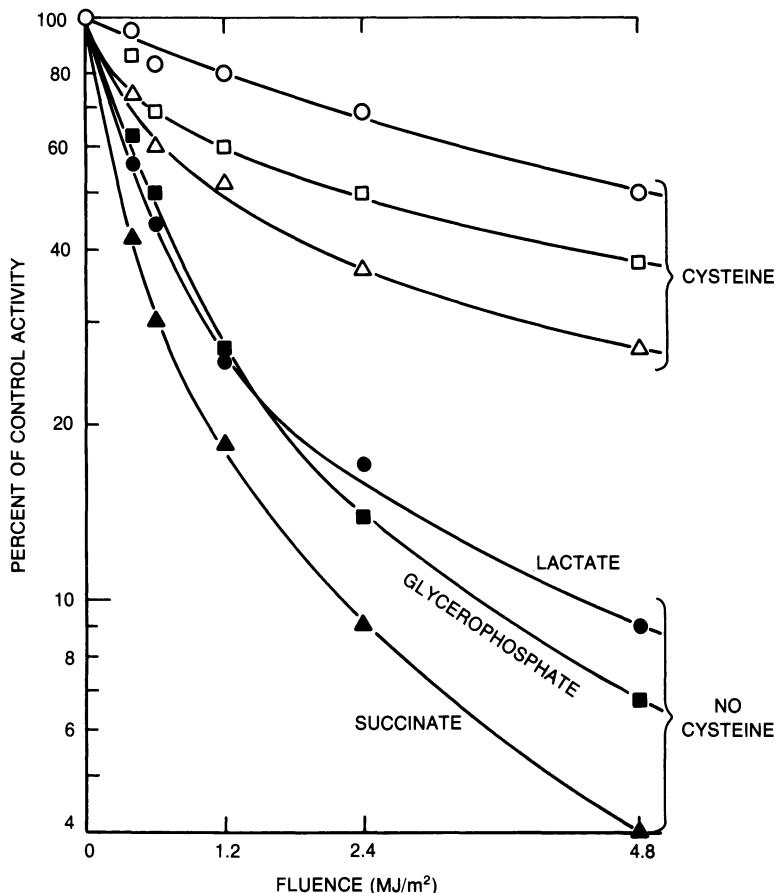


Fig. 11. Inactivation by light of wavelength >400 nm of the dehydrogenases of D-lactate, L- α -glycerophosphate, and succinate in intact cells of glycerol-grown *E. coli* ML308 in the presence and absence of cysteine (Fig. 1 of D'Aoust *et al.*, 1980, with abscissa changed to read absolute fluence).

K-12 (SR385), irradiated with broad-band near-UV radiation fluences of the order of 100 kJ/m^2 , show lower survival if plated on minimal medium, rather than on a complex (broth) medium (fluence-modification factor ≈ 1.4). The effect is largely oxygen-dependent and appears to reflect membrane damage, since it depends upon the salt concentration in the minimal medium and is enhanced by the addition of detergent. The fluences involved are too low to produce major effects on transport systems or on oxidative phosphorylation (Section 3.3).

Tuveson (1980) reported that *nur* strains of *E. coli* K-12 contain only 20% as many cyclopropane fatty acids (and therefore less saturation) in their membrane phospholipids as the *nur*⁺ strains. Assuming that near-UV radiation does indeed generate singlet oxygen (Section 3.1), Tuveson proposed that double bonds in fatty acids would be susceptible to peroxidation by singlet oxygen, which would make the *nur* strains more likely to be damaged, and might account for their increased sensitivity. If this hypothesis were correct, it would seem that the extra sensitivity of *nur* strains should be unaffected by DNA repair systems for far-UV radiation-induced damage, which is contrary to the suggestion that *nur* and *polA1* sensitize by equivalent mechanisms (Section 3.1). In more recent work, Klamen and Tuveson (1982) in fact retract their suggestion of the role of cyclopropane content in the sensitivity of *nur* strains, but they do find that the near-UV radiation sensitivity of *E. coli* increases with increasing degree of fatty acid unsaturation (number of double bonds per molecule), rather than amount of unsaturated fatty acid; the effect is observed only in cells irradiated in the logarithmic growth phase.

3.3. Specific Membrane Effects

3.3.1. Oxidative Phosphorylation

One would expect near-UV radiation to alter oxidative phosphorylation because of the large number of near-UV-absorbing compounds in the electron-transport system. Brodie and Ballantine (1960) found that oxidative phosphorylation with NADH in extracts of *Mycobacterium phlei* is considerably more sensitive at 360 nm than respiration alone, which showed a threshold type of inactivation curve. Later studies (Kashket and Brodie, 1962) showed that continuous illumination of *E. coli* W with black light produced a slightly lower growth rate when the cells were incubated in glucose medium, but an almost complete cessation of growth when the cells were incubated in succinate medium. This was evidence of damage to the respiratory system.

Later, more quantitative studies by Sprott *et al.* (1976) showed that oxygen uptake of glycerol-grown *E. coli* ML308 exhibits exponential inactivation (no threshold) in air with an F_{37} at 366 nm of 340 kJ/m², and that inactivation of oxygen uptake was identical to the inactivation of the uptake of glycine and alanine. Ascenzi and Jagger (1979) and Sharma and Jagger (1981) found the F_{37} at 366 nm for inhibition of uptake of these amino acids in glucose-grown *E. coli* B/r to be about 550 kJ/m² (Table 2). Lakchaura *et al.* (1976) showed that net ATP synthesis in *E. coli* K-12

AB2463 (*recA*) has an F_{37} at 366 nm of 750 kJ/m² for cells grown in and assayed for ATP synthesis in succinate medium. The relative sensitivity of respiration compared with oxidative phosphorylation found by these workers for *E. coli* is thus the opposite of that found by Brodie and Ballantine (1960) for *M. phlei*. Furthermore, Lakchaura *et al.* (1976) found that inactivation at 334 nm of ATP synthesis in succinate-grown cells is even less sensitive if the ATP synthesis is assayed during incubation in glucose medium instead of succinate medium [which parallels the finding of Kashket and Brodie (1962) for postirradiation growth in glucose and succinate media], whereas Sprott *et al.* (1976) found that inactivation of respiration (at 405 nm) is relatively little affected by carbon source. It thus appears that respiration is generally more sensitive to near-UV radiation at 366 nm than is oxidative phosphorylation. As a working hypothesis, we assume that respiration has an F_{37} at 366 nm in *E. coli* of about 450 kJ/m², regardless of substrate, and that oxidative phosphorylation (ATP synthesis) has an F_{37} at 366 nm of at least 750 kJ/m², dependent upon substrate (Table 2). ATP synthesis of cells grown and assayed in succinate medium is much more sensitive at 334 nm, showing an F_{37} of 140 kJ/m² in *E. coli* K-12 AB2463 (Lakchaura *et al.*, 1976). The sensitivity of respiration at 334 nm is unknown; it may be low, since it is lower at 366 than at 405 nm (Sprott *et al.*, 1976). Inhibition of ATP synthesis shows a threshold fluence of about 40 kJ/m² at 313 and 334 nm, and 200 kJ/m² or more at longer wavelengths (Lakchaura *et al.*, 1976; D'Aoust *et al.*, 1974). Therefore, inhibition of ATP synthesis is not expected to play a role in the sublethal effects discussed earlier (Section 2), although inhibition of respiration may have some role, especially at the longer wavelengths (see Table 2).

D'Aoust *et al.* (1974) showed that the flavoprotein dehydrogenases of L- α -glycerophosphate, lactate, and succinate in *E. coli* ML308 are comparatively sensitive to near-UV radiation, showing an $F_{37} = 500\text{--}900$ kJ/m² for light above 400 nm (Fig. 11 and Table 2); the dehydrogenases of malate and glucose-6-phosphate, and ATPase and *p*-nitrophenyl phosphatase, are much more resistant. Lakchaura *et al.* (1976) found that NADH dehydrogenase activity, measured in either cell extracts or whole cells of *E. coli* B, is exponentially inactivated with an F_{37} at 334 nm of only 40 kJ/m². From the action spectrum (Fig. 12), this would correspond to about 500 kJ/m² at 405 nm, the same as succinic dehydrogenase (Table 2).

Figure 12 shows action spectra for inhibition of ATP synthesis, NADH dehydrogenase activity, and respiration. The action spectrum for ATP synthesis is similar to the absorption spectrum of menaquinone-8, and the action spectrum for NADH dehydrogenase bears some resem-

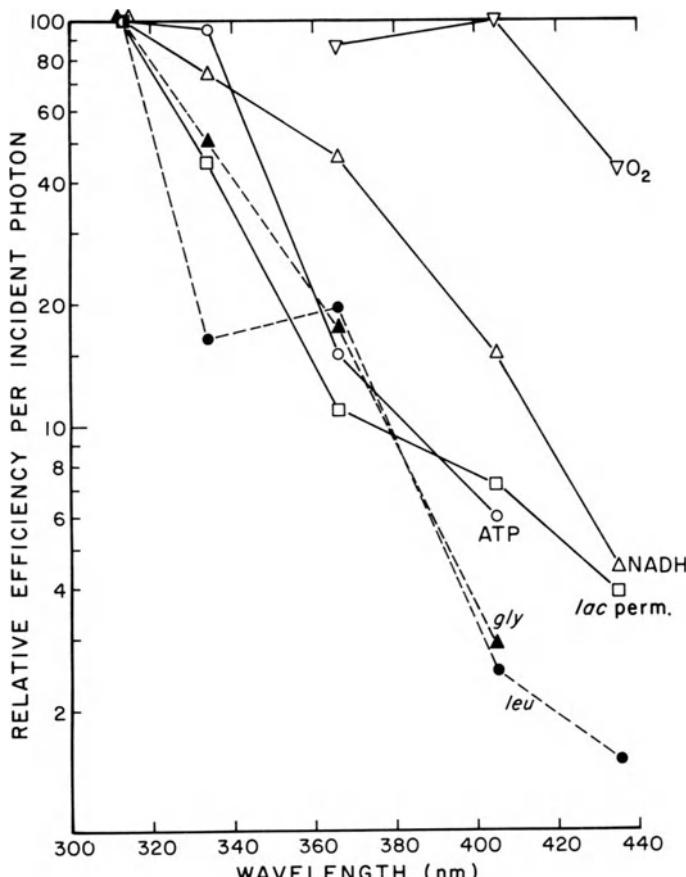


Fig. 12. Action spectra for inhibition of ATP synthesis (open circles) and NADH dehydrogenase activity (open triangles) in glucose-grown *E. coli* B (Lakchaura *et al.*, 1976), respiration (inverted triangles) in glycerol-grown *E. coli* ML 308 (derived from data of Sprott *et al.*, 1976), inactivation of lactose permease (squares) in *E. coli* (Robb and Peak, 1979), inhibition of leucine transport (closed circles; broken line) in *E. coli* K-12 (Robb *et al.*, 1978), and inhibition of glycine transport (closed triangles; broken line) in *E. coli* B/r (Sharma and Jagger, 1981). Curves are normalized to 100 at their maximum values.

blance to the absorption of ubiquinone-8, but no resemblance to the absorption spectrum of NADH; no other evidence has been adduced to indicate that these are the chromophores for these effects; in fact, the F_{37} for UQ-8 inactivation *in vivo* is 420 kJ/m² at 334 nm, some ten times that for inactivation of NADH dehydrogenase (Table 2).

Fujita *et al.* (1966) have shown that near-UV radiation inhibits oxi-

dation (respiration) and phosphorylation in *Micrococcus lysodeikticus* membrane extracts, and that these are restored by the addition of menaquinone (MQ-9). They showed in particular that MQ-9, extracted from this organism, was inactivated at the same rate as NADH oxidase activity. Eisenberg (1972) has extended these studies to show that both NADH oxidase and L-malate oxidase activities, measured by oxygen uptake in disrupted membranes of *M. lysodeikticus*, were inactivated by black light with exactly the same kinetics and were equally well restored by addition of MQ-4. MQ-9 was relatively ineffective in the restoration of activity, a problem also encountered in work on restoration of glutamic-acid-uptake activity (Section 3.3.2), which may reflect poor penetration of membranes by long-tailed quinones (Gel'man *et al.*, 1975). The similarity in behavior of NADH and L-malate oxidases, as well as the restoration by menaquinone, strongly suggests that the target molecule is the menaquinone, which is common to the oxidative pathways of both substrates, rather than the dehydrogenases of these two substrates, which have been shown to have greatly different radiation sensitivities. Nevertheless, we need to warn once again (see Section 3.2) that the interpretation of quinone reconstitution experiments is difficult, since quinones appear to be able to bypass other parts of the electron-transport pathway; one is also somewhat deterred by the fact that the native quinone (MQ-9) was relatively ineffective in reactivation. Finally, the demonstration by Taber *et al.* (1978; see Section 2.1.4) that MQ is a target molecule for near-UV radiation-induced growth delay in *B. subtilis*, at fluences comparable to those required for inhibition of respiration in *E. coli*, suggests that electron transport is the target system for this effect.

In summary, there is some evidence that flavins and menaquinones are involved in near-UV radiation effects on oxidative phosphorylation. Nevertheless, a clear demonstration of this involvement has not yet appeared.

3.3.2. Membrane Transport

Ions. In the first careful study of the action of near-UV radiation on bacteria, Hollaender (1943) showed that *E. coli* exposed to broad-band near-UV radiation (primarily 366 and 405 nm) fluences in the lethal range experienced further inactivation if held in a strong salt solution for several hours after irradiation, suggesting an effect on the ion permeability of the cell membrane. Further experiments under similar irradiation conditions showed that potassium retentivity in yeast, while very resistant to far-UV radiation in comparison with survival, was less resistant to near-UV radiation ($F_{37} = 4 \text{ MJ/m}^2$) than survival ($F_{37} = 7.5 \text{ MJ/m}^2$) (Bruce, 1958).

These early experiments clearly indicated that lethal fluences of near-UV radiation produced effects on the cell membrane that were not observed with lethal fluences of far-UV radiation. The *survival* curve obtained by Bruce (1958) is unusual, in that it is *exponential*, showing no threshold. The chromophore for these effects is unknown. It should be remembered that, in bacterial systems, Na^+ efflux is not directly coupled with K^+ uptake through a $\text{Na}^+ \text{-K}^+$ -transport ATPase, as it is in animal systems. Among the three well-established systems for K^+ transport in *E. coli*, the high-affinity one (which operates on very low external concentrations) probably does utilize a membrane ATPase (Silver, 1978). *E. coli* maintains an internal K^+ concentration of well over 0.1 M even when the external concentration is as low as 0.1 μM , so that any damage to the K^+ concentration systems would be expected to result in a rapid loss of potassium.

Sugars. Koch *et al.* (1976) provided evidence that changes in membrane passive permeability occur in *E. coli* with an $F_{37} \approx 2000 \text{ kJ/m}^2$ at 366 nm, while inactivation of the galactoside (lactose) permease has an $F_{37} \approx 600 \text{ kJ/m}^2$, and inactivation of energy-producing systems an $F_{37} \approx 400 \text{ kJ/m}^2$. Later work by Doyle and Kubitschek (1976) showed that facilitated transport (not requiring metabolic energy) of sorbose into *S. cerevisiae* was inhibited at 366 nm with an $F_{37} \approx 750 \text{ kJ/m}^2$, thus confirming their earlier conclusion that some protein carrier molecules are moderately sensitive to near-UV radiation (see Table 2).

Thus, it would appear that fluences in the range of 600 kJ/m^2 at 366 nm may destroy sugar permeases. The near-UV action spectrum for inactivation of the lactose permease is shown in Fig. 12. This spectrum shows involvement of a chromophore absorbing around 400 nm, with a probable contribution from another chromophore around 330 nm. The spectrum also involves the expected protein absorption at 280 nm (not shown).

Amino Acids. Among the first indications of lability of membrane transport systems other than ion transport to near-UV radiation was the report of D'Aoust *et al.* (1974) that visible light (polychromatic and above 400 nm) inhibited the uptake by *E. coli* of some amino acids and sugars, and also inactivated lactate and succinate dehydrogenases (Fig. 11). This work was soon extended to a demonstration by Sprott *et al.* (1976) that monochromatic wavelengths of near-UV radiation down to 366 nm were more effective than visible light, and that the effects were different for different groups of amino acids. They have shown (Sprott *et al.*, 1975a,b, 1976) a close parallel between the near-UV inhibition of glycine and alanine uptake by near-UV radiation and inhibition of respiration in *E. coli* grown in glycerol (but not in glucose or succinate), in agreement with

many observations of dependence of bacterial amino acid transport on respiration (Anraku, 1978). This parallel did not hold for inhibition of phenylalanine, tyrosine, and tryptophan uptake, which are more resistant to near-UV radiation. Sprott and Usher (1977) have shown a parallel between inhibition of phenylalanine uptake by near-UV radiation and loss of the electrochemical proton gradient.

The first action spectra for inhibition of membrane transport were presented by Robb *et al.* (1978). They showed complete UV action spectra for the inhibition of both the leucine system and the leucine-isoleucine-valine (LIV) system in *E. coli* K-12. These spectra were quite similar, showing a peak at 280 nm in the far-UV and another slight peak two orders of magnitude lower at 366 nm in the near-UV (Fig. 12). A somewhat similar spectrum was shown by Ascenzi and Jagger (1979) for inhibition of glycine uptake in *E. coli* B/r, with a slight peak at 334 nm, but this was not reproduced by Sharma and Jagger (1981) for either glycine or alanine uptake (Fig. 12). In summary, these spectra show evidence for a near-UV radiation chromophore(s) with an absorption peak at either 334 or 366 nm, but a much lower absorption efficiency at 400 nm than was evident in the spectrum of Robb and Peak (1979) for the lactose permease.

It is clear that the actions of near-UV radiation on the membrane transport of amino acids do not reflect the absorption of energy by DNA, since: (1) the action spectra do not resemble DNA absorption, (2) no photoreactivation of glycine uptake has been observed (Ascenzi and Jagger, 1979), and (3) the effects are observed within one minute of placing nonmetabolizing cells into a medium containing carbon source, before there has been time for expression of DNA genetic information. Furthermore, ⁴Srd in tRNA seems not to be involved, since identical kinetics for inhibition of glycine uptake are observed in mutants that contain or lack ⁴Srd (Ascenzi and Jagger, 1979).

The kinetics of inactivation of these transport systems is of considerable interest. Although small thresholds are sometimes observed (Robb *et al.*, 1978; Ascenzi and Jagger, 1979), the inactivation curves are generally exponential and show no threshold. Therefore, it is unlikely that inhibition of ATP synthesis (Section 3.3.1) or of the electrochemical proton gradient (Section 3.2) is responsible, since these show large thresholds. On the other hand, inhibition of respiration (Section 3.3.1) is exponential, and actually parallels the loss of glycine uptake (for cells grown in glycerol medium; Sprott *et al.*, 1976), thus implicating components of the electron-transport system. Now, the data of Robb and Peak (1979) strongly indicate a direct action on the lactose permease molecule, and the kinetic studies of Sharma and Jagger (1981) are also consistent with action on a permease. One may therefore adopt, as a working hypothesis,

the idea that the action of UV light on amino acid and sugar transport is primarily an action on the permease molecules, but that this action in the near-UV region may well result from the absorption of light by closely coupled components of the electron-transport chain, possibly menaquinone or the dehydrogenases of NADH or succinate.

To what extent can these conclusions be generalized to all of the large number of amino acid and sugar transport systems? It would appear that there is a considerable degree of uniformity: (1) the fluences and kinetics are fairly similar, and (2) the action spectra are rather similar (Sprott *et al.*, 1976, and Fig. 12). Nevertheless, Sprott *et al.* (1976) did observe sufficient differences in sensitivity and action spectrum as to indicate that the aromatic amino acids behave rather differently from the other amino acids (more resistant and showing no sensitivity at 405 nm) and that methyl-thio- β -galactoside (TMG) has an intermediate sensitivity and a flatter action spectrum. Some amino acids known to have common transport systems, such as alanine and glycine, show nearly identical kinetics and action spectra. These differences and similarities support the idea that the most important membrane-transport targets are the metabolite transport systems themselves, rather than components that alter respiratory electron transport.

3.4. Summary

In the lethal near-UV radiation fluence range below 1000 kJ/m², effects on membranes and membrane functions do not appear to result from actions on DNA or cytoplasmic components, but rather from direct effects on the membranes themselves.

Insufficient work has been done on near-UV radiation inhibition of ion transport to permit conclusions concerning mechanism; in any event, it appears to be affected only by higher fluences. Much more work has been done with amino acid and sugar transport systems, and here we can reach some tentative conclusions. Oxidative phosphorylation and the electrochemical proton gradient are relatively unaffected by fluences that inactivate metabolite transport. The respiratory electron-transport system, however, does appear to be sensitive in this range. Inhibition of transport of metabolites most likely involves direct actions on the transport systems themselves, specifically permease molecules, although electron-transport components may be involved [most bacterial amino acids are transported by H⁺-symport (Anraku, 1978)].

While the lethal actions of near-UV radiation appear to involve DNA

as the primary target, the membrane transport effects (at least those immediately observable) appear to be independent of DNA damage, even though they occur in the same fluence range. Under some circumstances, however, membrane damage may contribute to lethality, as for bacteria grown on high-salt minimal medium after near-UV irradiation.

4. INTERACTIONS OF DIFFERENT WAVELENGTHS

Synergisms and antagonisms have been observed between the effects of different wavelengths of near-UV radiation, as well as between the effects of wavelengths of near-UV and far-UV radiation. These interaction studies have concerned almost exclusively lethality and mutation; nevertheless, I treat them here because, although the end-point measured may be lethality or mutation, the interference with that end-point by near-UV radiation may be a physiological effect. Near-UV radiation also shows interactions with a variety of other damaging agents, such as heat, ionizing radiation, and alkylating agents, but I shall not consider these effects in this review.

The subject of radiation synergism and antagonism was thoroughly reviewed by Tyrrell (1978a), although several important papers have appeared since then.

4.1. Near UV–Near UV

Workers using near-UV radiation had often observed that they obtained different results if they used a polychromatic rather than a monochromatic source, but a published account of such effects did not appear until the report of Peak *et al.* (1975). They observed that the rate of inactivation of *trp* and *leu* markers of *B. subtilis* transforming DNA at 365 nm was tripled by simultaneous or pre-irradiation with completely non-inactivating fluences (up to 7 kJ/m²) of 334 nm radiation, or doubled by irradiation with a noninactivating fluence of 313 nm radiation. In later work, M. J. Peak and R. M. Tyrrell (unpublished work reported by Tyrrell, 1978a) found that such synergism did not occur if the fluence rate at the sensitizing wavelength was 100 times greater than that (0.6 W/m²) used by Peak *et al.* (1975). While this second result has not been further investigated, the first one is sufficiently dramatic to leave little doubt of its validity. The mechanism is unknown.

Rather similar results were obtained by Tyrrell and Peak (1978) for the inactivation of wild-type *E. coli* K-12 AB1157. They used wavelengths

334, 365, and 405 nm, and gave fluences of sensitizing radiation amounting to half of the shoulder value on the survival curve. The sensitizations observed were approximately a factor of two, in terms of a fluence-reduction factor, and there was a reduction in the shoulder of the survival curve, with little change in final slope. This suggested that these wavelengths were producing a common type of sublethal damage, which Tyrrell and Peak (1978) suggested is damage to repair systems. If this view is correct, then the bacterial mechanisms for near UV–near UV synergism are clearly different from those effective in transforming DNA.

This idea is also supported by the finding that any one of these wavelengths will sensitize to either of the other ones, with the exception that a 405-nm treatment *protects* the cells from the lethal effects of 365-nm radiation, the protection being in the form of an extension of the shoulder. It is conceivable that this represents an induced repair similar to that reported by Peters and Jagger (1981) for growing cells (Section 3.1): (1) the fluence is in the right range (1500 kJ/m^2 at 405 nm), (2) the Peters–Jagger repair effect has been observed at 405 nm (unpublished data), and (3) the cells were in the log phase of growth at the time of irradiation (required for the Peters–Jagger repair), although they were not in nutrient medium. Nevertheless, it is not obvious why such protection was not also observed when the order of the irradiations was reversed (365-nm pretreatment of cells later exposed to 405 nm).

Tyrrell and Peak (1978) ruled out photoprotection by 405-nm radiation against dimer damage by 365-nm radiation on the grounds that the near-UV radiation-induced dimer is not an important lethal lesion in wild-type strains (however, the only known photoprotection system enhances excision repair, which can operate on other lesions than dimers); furthermore, such a photoprotection ought to be much more effective for 334-nm than for 365-nm killing (since a higher fraction of dimers would be present), but 334-nm killing is merely increased by a 405-nm pretreatment. In addition, the sensitizing fluences used at 334 nm (150 kJ/m^2) and at 365 nm (1000 kJ/m^2), although nonlethal, are some ten times higher than those normally used for photoprotection against radiation-induced far-UV damage. Finally, photoprotection is not observed in *E. coli* B at 405 nm (but see Section 4.2.1b), although it is observed in *Pseudomonas aeruginosa* at 405 nm, with fluences of $\sim 1500 \text{ kJ/m}^2$ (Jagger and Stafford, 1962), the same fluence used in the work of Tyrrell and Peak (1978).

Eisenstark (1970) found that *recA* strains of *E. coli* and *S. typhimurium* are very sensitive to killing by broad-band near-UV radiation. Later work in his laboratory showed that exposure to black light in the presence of oxygen caused L-tryptophan to become toxic for *rec* mutants of *S. typhimurium* (Yoakum and Eisenstark, 1972), and that a biologically

important product of this photooxidation is H_2O_2 (McCormick *et al.*, 1976). A 50- to 100-fold synergistic effect was found between near-UV radiation and H_2O_2 in the killing of either *E. coli* bacteria (Hartman and Eisenstark, 1978) or T7 phage (Ananthaswamy *et al.*, 1979), with the action spectrum for synergism in phage showing a peak at 340 nm (Fig. 13). H_2O_2 absorbs poorly in the near-UV region, its absorption increasing monotonically with decreasing wavelength below 300 nm (Calvert and Pitts, 1966), and is presumably not the chromophore, but the H_2O_2 must be present during irradiation (Hartman and Eisenstark, 1978). A synergistic *mutagenesis* has also been reported between broad-band near-UV radiation and preirradiated tryptophan in *E. coli* (Webb *et al.*, 1978b).

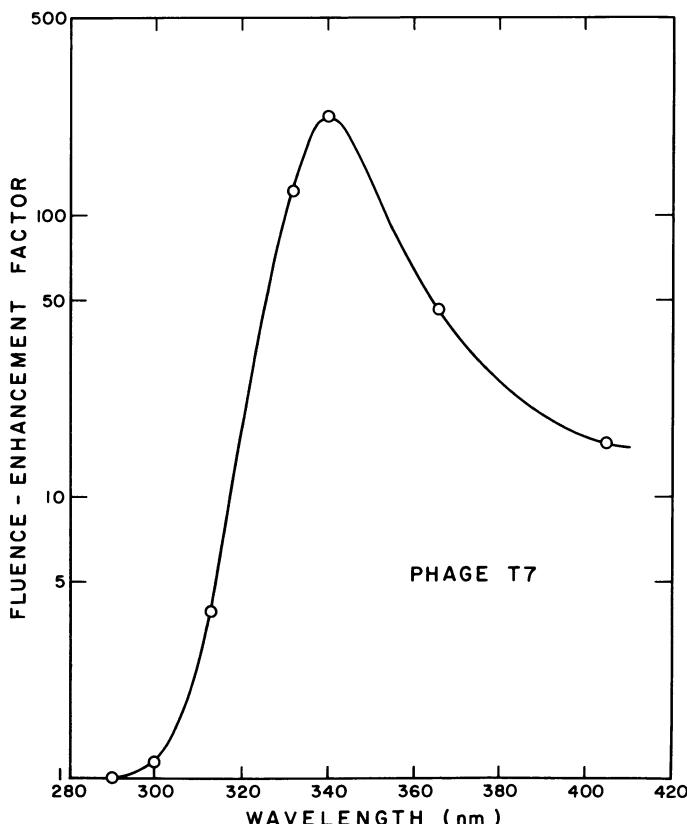


Fig. 13. Fluence-enhancement factor (inactivation constant in presence of H_2O_2 /inactivation constant in absence of H_2O_2) for inactivation of bacteriophage T7 by near UV plus H_2O_2 , as a function of wavelength (Ananthaswamy *et al.*, 1979).

These phenomena can be tentatively explained (see McCormick and Oczós, 1979; Walrant and Santus, 1974) in the following way: (1) with broad-band near-UV radiation, 310-nm light is absorbed by tryptophan; (2) in the presence of oxygen, this produces *N*-formylkynurenine, which absorbs at 317 nm, going into a triplet excited state and promoting photooxidation of DNA; (3) at the same time, *N*-formylkynurenine promotes the photoformation of singlet oxygen and/or superoxide anion, leading to formation of H₂O₂, which (4) enhances the damage to DNA. That the action spectrum for synergism peaks at 340 nm instead of at shorter wavelengths may be explained by the "masking model" recently proposed by Hartman (1981).

Wild-type *Sarcina lutea*, which are yellow, show a lower sensitivity to broad-spectrum radiation (1000-W tungsten-quartz iodine lamp) than colorless mutants (Mathews-Roth and Krinsky, 1970), but the effect is much smaller with monochromatic radiation and occurs only at wavelengths <420 nm (see Webb, 1977). Wild-type *S. lutea* has carotenoid pigments in its membrane, but studies with monochromatic light show protection only at wavelengths in the near-UV region, which are relatively poorly absorbed by the carotenoid. The much larger carotenoid protection observed with broad-spectrum light also has not been explained; it might result from the carotenoid removing wavelengths from the light beam that are highly synergistic with other wavelengths. Alternatively, it has been proposed that carotenoid pigments protect biological systems by quenching singlet oxygen (Webb, 1977).

At the sublethal level, little or no synergism among wavelengths of near-UV radiation has been found for producing photoprotection from far-UV killing in *E. coli*, which has been observed by Tsai and Jagger (1981) to be not significantly different when induced by fluorescent black light or by monochromatic radiation. Since photoprotection is mediated by the absorption of light in ⁴Srd (Section 4.2.1b), it seems likely that none of the sublethal effects utilizing this chromophore (Section 2) will show synergism.

A kind of near UV–near UV antagonism that may occur is *concomitant photoreactivation* (see Section 4.2.1), which involves photoreactivation of cells *during irradiation* by the same near-UV radiation that produced the damage. The damaging and reactivating wavelengths might be identical or different (shorter wavelengths are more effective in producing dimers, while longer ones are more effective in photoreactivation). Concomitant photoreactivation is observed in completely dark-repair-deficient strains. W. Harm (1978) suggested that concomitant enzymatic photoreactivation was operating partly on nondimer near-UV radiation-induced lesions, but this idea was questioned by Hodges *et al.* (1980).

Webb and Peak (1981) have provided strong arguments against the interpretation of W. Harm (1978), noting that (1) earlier studies have shown a parallel between dimer splitting and concomitant photoreactivation and that (2) it is essential in such studies to eliminate stringently any stray light of shorter wavelengths.

4.2. Near UV–Far UV

4.2.1. Antagonisms

In line with the definition proposed by Jagger (1967), I define a *protection* as a response reduction caused by a treatment applied before irradiation, and a *recovery* (or reactivation) as a response reduction caused by a treatment applied during or after irradiation. Since far-UV radiation effects are generally much more efficiently produced than are near-UV radiation effects, most of the synergisms and antagonisms observed with these radiations involve the effect upon a far-UV radiation response of a fluence of near-UV radiation that produces little response by itself. Near UV–far UV antagonisms are called *photoreactivation* if the near-UV radiation is given during or after the far-UV irradiation, and *photoprotection* if the near-UV radiation is given before the far-UV irradiation.

The words ‘‘protection’’ and ‘‘recovery’’ apply to a *treatment* and not to the thing protected from or recovered from. Thus, use of the term ‘‘photoprotection’’ for the action of chemical compounds that prevent sunburn (‘‘sunscreens’’) is inconsistent with the present convention; such action should be called ‘‘chemical protection’’ (from light damage). Nevertheless, the term ‘‘photoprotection’’ has been so widely used for sunscreens that it seems unlikely to be changed in the near future; this usage will continue to cause confusion.

4.2.1a. Photoreactivation

This subject has been recently reviewed (H. Harm, 1976), and will not be considered here in any detail. Killing and mutation are produced by far-UV radiation primarily by the induction of cyclobutane-type pyrimidine dimers. In the presence of near-UV and visible light (typically in the range 310–490 nm), these dimers may be split by a ‘‘photoreactivating enzyme’’ (‘‘photolyase’’). There are a variety of photolyases, with differing action spectra, and there is good evidence that at least two of them are flavoproteins (Section 1.2.1). Photoreactivation of killing or mutation typically shows a constant fluence-reduction factor (i.e., regardless

of the level of inactivation) of about 0.30, i.e., about 70% of the damage produced by far-UV radiation is normally repaired by this mechanism. Photoreactivation has been observed in a wide range of organisms, but exceptions are spotted within most of the taxonomic orders. Most bacteria show photoreactivation, but most human cell types do not.

4.2.1b. Photoprotection

Uequivocal photoprotection was first reported in bacteria by Weatherwax (1956), and the phenomenon was further characterized by Jagger and co-workers (Jagger, 1960; Jagger and Stafford, 1962; Jagger *et al.*, 1964). These studies showed that photoprotection in *E. coli* B occurs only when the far-UV radiation survival is above 0.1%, that the effect requires about 20 kJ/m² of 334-nm light, and that the action spectrum is nearly identical to that for growth inhibition (Fig. 4). It was also established that the reaction is photochemical, with no enzymatic component, showing little dependence on temperature and no dependence on fluence rate. *P. aeruginosa* shows an action spectrum very similar to that of *E. coli* B, except for moderate photoprotection at 405 nm, where *E. coli* B shows none. Studies with transforming principle indicated that the chromophore is not DNA. These early studies also showed that liquid-holding recovery (recovery after holding cells in non-nutrient medium after far-UV irradiation) shows a complete overlap with photoprotection, i.e., cells that have experienced full liquid-holding recovery show no further photoprotection, and *vice versa* (Fig. 14). Since liquid-holding recovery is known to require a functional excision repair system (Ganesan and Smith, 1969), it appeared likely that photoprotection also requires an excision repair system, and this was shown to be the case by Lakchaura (1972), who found excellent photoprotection in a *uvr*⁺ *rec*⁻ strain (*E. coli* K-12 AB2463), but no photoprotection in a *uvr*⁻ *rec*⁺ strain (AB2437). Indeed, it has generally been found that *rec* strains show much better photoprotection than wild-type strains (Lakchaura, 1972; Swenson *et al.*, 1975; Thomas and Favre, 1977c). This is consistent with the observations that (1) detection of liquid-holding recovery in K-12 strains requires a *recA* mutation (Tang and Smith, 1981), and (2) broad-band near-UV radiation mutagenesis in WP strains, as well as near-UV radiation-enhanced far-UV radiation mutagenesis, occurs only in *uvrA*⁻ *recA*⁺ strains (Turner and Webb, 1981; see also Sections 3.1 and 4.2.2), which would show no photoprotection.

Good photoprotection has been found among bacteria in *E. coli*, *P. aeruginosa*, and *Enterobacter aerogenes*. These three organisms also show good near-UV radiation-induced growth delay and at least some

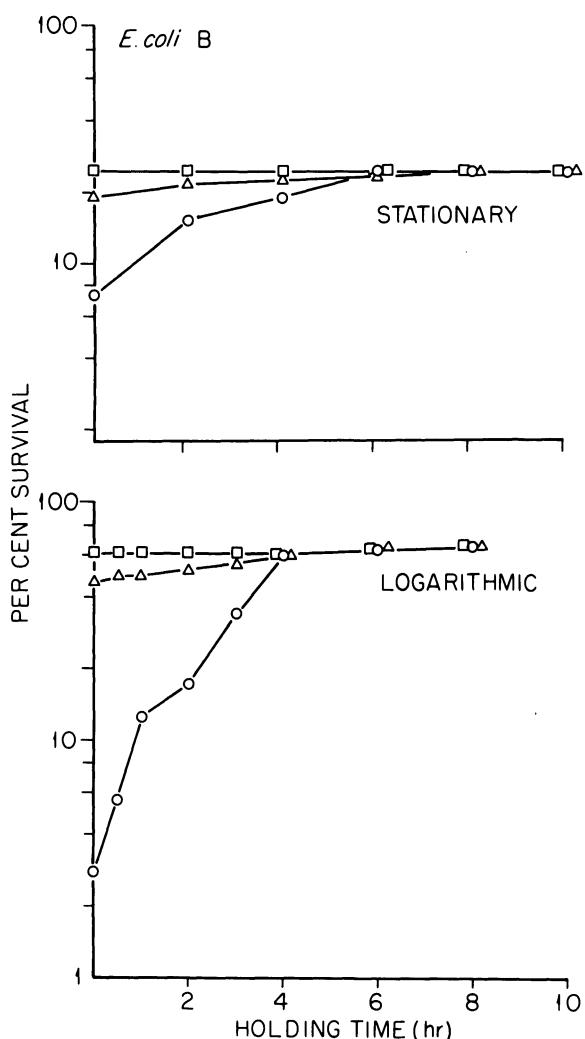


Fig. 14. Overlap of photoprotection and liquid-holding recovery in stationary-phase (upper curves) and starved exponential-phase (lower curves) cells of *E. coli* B. Percentage survival is plotted against hours held in phosphate buffer at 23°C after irradiation at 254 nm. Symbols correspond to the following fluences of 334 nm photoprotecting radiation: circles, 0; triangles, 38 kJ/m²; squares, 57 kJ/m² (From Jagger *et al.*, 1964).

liquid-holding recovery, whereas bacterial genera showing little or no photoprotection also show little or no near-UV radiation-induced growth delay or liquid-holding recovery (Jagger, 1972).

These correlations with growth inhibition and presence of an excision repair system led Jagger and co-workers (see Jagger, 1972) to propose that photoprotection in *E. coli* is a *result* of growth inhibition (a hypothesis that cannot be entirely correct—see below). A photoprotection treatment would inhibit growth, which in turn would provide more time for the action of the excision repair system. This growth delay (and division delay) would also result in much less repair by postreplication DNA repair systems, and therefore one would expect good photoprotection from mutation. This expectation was borne out by the observation by Kondo and Jagger (1966), of indirect photoreactivation of mutation to prototrophy for arginine, using a bacterial strain (*E. coli* H/r 30) that lacks photoreactivating enzyme. This was more directly and thoroughly explored by Tyrrell (1980a), who showed photoprotection from far-UV radiation-induced mutation (reversion to tryptophan independence), with efficiencies at 334 and 365 nm comparable to those effective in photoprotection from killing. However, he also found photoprotection from mutation at 405 and 434 nm, although the fluences required were very high. No photoprotection from mutation was observed in a *uvrA* strain of *E. coli* B/r, in accord with the earlier conclusion that photoprotection requires the *uvrA* gene product.

In early work, Jagger and Stafford (1965) observed some near-UV radiation “photoreactivation” in a strain of *E. coli* B isolated by Harm and Hillebrandt (1962) that shows no photoreactivation by white light and contains no active photolyase. Careful study of this phenomenon showed that the “photoreactivation” occurred at 334 nm but not at 405 nm, and that its induction was independent of fluence rate and nearly independent of temperature. Jagger and Stafford (1965) proposed that this phenomenon was really “photoprotection *after* far-UV irradiation,” and they named it “indirect photoreactivation.” Their studies provided indications that indirect photoreactivation also occurs in the parent strain, *E. coli* B; later studies (Jagger *et al.*, 1969) confirmed this interpretation, by showing that (1) indirect photoreactivation in strain B *phr*⁻ does not split thymine dimers, and (2) the amount of dimer splitting in the wild-type strain for a given amount of photoreactivation is only half as much at 334 as at 405 nm.

Neither photoprotection nor indirect photoreactivation occur to any great extent in truly wild-type strains, such as *E. coli* B/r and *E. coli* K-12, particularly if they are in the stationary phase of growth. Jagger *et al.* (1969) suggested that this could be explained by these strains having highly

efficient dark repair systems, so that they had no need of the additional excision repair that would be provided by the growth delay induced by a photoprotection treatment. Such a near-UV radiation-induced growth delay would be even less needed in stationary-phase cells, which already show a growth delay upon plating.

Mechanism. The similarities between photoprotection and growth inhibition are the following: (1) they have nearly identical action spectra; (2) they require the same fluences; (3) good photoprotection occurs only in strains that show good growth inhibition; and (4) photoprotection is always smaller in stationary-phase cells (which have a built-in growth delay upon plating) than in exponential-phase cells. There is also strong evidence that photoprotection requires the *uvrA* gene product, which explains the lack of photoprotection in the very radiation-sensitive strains *E. coli* K-12 AB2480 *uvrA recA* (Lakchaura, 1972) and *E. coli* B_{s-1} *uvrB exrA lon* (Jagger *et al.*, 1964). These findings all support the proposal by Jagger and co-workers that a photoprotection treatment induces a growth delay (and parallel division delay) that permits more time for excision repair before DNA replication.

However, Swenson *et al.* (1975) showed that, although a small photoprotection, and a near-UV radiation-induced growth delay, occurs in log-phase cells of *E. coli* B/r grown in and plated on a minimal salts medium containing glycerol, the kinetics of increase of viable cells (cell division), growing in liquid minimal medium with glycerol, is identical for photoprotected and unphotoprotected cultures. In other words, although near-UV radiation induces a growth delay, the time necessary for completion of repair appears to be the same in photoprotected and unphotoprotected cultures. This suggests that the growth delay is not in fact allowing more time for repair. Swenson *et al.* (1975) observed that, although respiration ceases after 60 min of growth after far-UV irradiation, photoprotected cells show only a decreased rate, and eventual recovery, of respiration. They proposed that this resumption of respiration permits the replacement of pyridine nucleotides that normally leak from the cell after far-UV irradiation (Swenson, 1976); this provides NAD, which is necessary for the action of DNA ligase and can therefore help in the repair of single-strand breaks in DNA. This work shows that photoprotection can occur under conditions in which there is no apparent benefit resulting from growth delay.

Tsai and Jagger (1981) have recently reported studies showing that *rel* mutants of *E. coli* B and B/r, grown in minimal medium with glucose, which undergo little growth inhibition after near-UV irradiation, exhibit 50% as much photoprotection as isogenic wild types, while ⁴Srd⁻ mutants show no photoprotection at all. Thus, photoprotection appears to utilize

⁴Srd as its sole chromophore in *E. coli* B and B/r.* But the results of Tsai and Jagger (1981) also show that half of the photoprotection is independent of the *rel*⁺ gene product and cannot be a result of growth delay.

These findings require modifications of the simple idea that photoprotection results from growth delay. Clearly, at least two mechanisms are involved, one related to the *rel* gene (*rel*⁺ photoprotection) and the other independent of the *rel* gene (*rel*⁻ photoprotection). Both mechanisms utilize ⁴Srd as a chromophore. In *rel*⁺ photoprotection, we may assume that photoprotection is indeed due to increased amounts of the *rel*⁺ gene product, caused by damage to tRNA resulting primarily from the induction of ⁴Srd-Cyd adducts (Fig. 10). The *rel*⁺ gene product produces a large increase in the concentration of ppGpp, which in turn exerts control over many genes involved in macromolecular synthesis (LaRossa and Söll, 1978). Many of the actions of ppGpp are inhibitory and thus lead to a growth delay. For cells grown in nutrient broth or other media containing glucose, *rel*⁺ photoprotection presumably results from this growth delay (by increasing the amount of excision repair and the ratio of excision repair to recombination repair). For cells grown in glycerol (or presumably any carbon source other than glucose; Swenson and Schenley, 1970), cyclic AMP levels are high, which induces a protein that inhibits respiration after exposure to far-UV radiation, and that increases the sensitivity of the cells to far-UV radiation [for *E. coli* B/r grown in glycerol, $F_{10} \approx 22 \text{ kJ/m}^2$ (Swenson *et al.*, 1975) compared to B/r grown in nutrient broth, where the $F_{10} \approx 40 \text{ kJ/m}^2$ (Tsai and Jagger, 1981)]. This extra sensitivity shows photoprotection (F_{10} goes to $\sim 40 \text{ kJ/m}^2$; Swenson *et al.*, 1975), but this photoprotection does not result from growth delay itself, but from events that normally precede growth delay.† For the glycerol-grown cells, the photoprotection treatment prevents induction of the respiration-inhibiting factor, presumably by action of ppGpp. This may be a result either of the general suppression of RNA and protein synthesis (Swenson and Schenley, 1970) or it may be a specific action of ppGpp.

* The key role of ⁴Srd in photoprotection was first shown by Thomas and Favre (1977c), using K-12 strains of *E. coli*. Although good photoprotection has been found only in bacteria with a high content of ubiquinone, the evidence that quinones are not the chromophores for either growth inhibition or photoprotection in *E. coli* is convincing; the correlation reported by Jagger (1972) (see above) may be with gram-negativity rather than quinone content.

† It may be noted that, for *E. coli* B grown in nutrient broth, the growth delay produced by near-UV + far-UV radiation is far greater than that produced by far-UV radiation alone (Jagger *et al.*, 1964), whereas, for *E. coli* B/r grown in glycerol medium, the growth delay produced by the two irradiations together is less than by far-UV irradiation alone (Swenson *et al.*, 1975).

It is known, e.g., that ppGpp can have a direct action on RNA polymerase, altering its binding to promoters (van Ooyen *et al.*, 1976). Finally, the cessation of respiration that occurs in cells grown on glycerol depends on the *recA*⁺ and *lexA*⁺ gene products (Swenson and Schenley, 1974). In general, *rec* strains (*rel*⁺) are quite sensitive to killing by far-UV radiation. The high photoprotection observed in these strains may be attributed to (1) the complete dependence upon the excision-repair system (the only dark-repair system favored by photoprotection), which must complete repair before DNA replication (a requirement aided by growth delay), since the gaps produced in daughter DNA strands opposite dimers during DNA replication cannot be repaired in *rec* strains, and (2) the total absence of respiration inhibition, which permits ample energy production (e.g., NAD, NADH, ATP) for the excision repair.

rel⁻ photoprotection also presumably results from photomodified tRNA, which, however, instead of producing ppGpp at the ribosome, as it does in *rel*⁺ strains, may then react directly with a regulatory mechanism of enzyme synthesis operating at the level of transcription, either through direct interaction with DNA or by combination with regulatory proteins (LaRossa and Söll, 1978). It is difficult at this time to suggest a mechanism of *rel*⁻ photoprotection, since the system has not been experimentally explored. It might, as an example, involve a direct stimulation of the excision-repair (*uvr*) system. It seems unlikely that the SOS repair system is directly involved, since a photoprotection treatment has very little effect, either positive or negative, on *rec*⁺ *lex*⁺ *uvr*⁻ strains (see, e.g., Lakchaura, 1972). Finally, although *rec* repair systems can be damaged by near-UV radiation, this requires fluences much higher than those used in photoprotection (Section 4.2.2).

Photoprotection in *E. coli* generally does not show oxygen dependence, but Webb and Brown (1979b) have now reported that exponential-phase cells of *E. coli* K-12 AB2463 (*uvr*⁺ *recA*) show less photoprotection under anaerobic conditions at the usual 365-nm fluences, although almost normal levels may be obtained at fluences $>10^6$ J/m².

A rather different kind of photoprotection has been reported by Masinovsky and Rubin (1976; see also Fraikin and Rubin, 1979) in the photosynthetic bacterium *Ectothiorhodospira shaposhnikovii*. Photoprotection and growth delay are produced with equally high efficiency at both 340 and 365 nm, and some photoprotection occurs at 405 nm. Thus, the action spectrum differs from that for *E. coli* (Fig. 4). The photoprotection shows separate "light" and "dark" steps. The "light" stage is apparently similar to that in *E. coli*, involving growth delay. The "dark" stage involves synthesis of a new tocopherol-like lipid, which may reduce damage by far-UV radiation because of its antioxidative properties.

4.2.2. Synergisms

Photoprotection by near-UV radiation against far-UV radiation-induced lethality and mutation is observed at sublethal 334-nm fluences below about 50 kJ/m². If one goes to very much higher fluences, however, the near-UV radiation treatment turns into a lethal synergism, first carefully studied by Tyrrell and Webb (1973). They showed that a fluence of 365-nm radiation that was high (1000 kJ/m²), but still in the shoulder region of the inactivation curve, increased the lethal action of 254-nm radiation on *E. coli* B/r by a factor of about five, the sensitized bacteria losing the entire shoulder of the far-UV radiation survival curve. These authors concluded that large (but still only slightly lethal) fluences of near-UV radiation will damage repair systems for far-UV radiation-induced DNA damage, and this conclusion is supported by their observation of a 1000-fold decrease in the efficiency of far-UV radiation-induced dimer excision after irradiation at these near-UV radiation fluences. Webb and Brown (1979b) have shown that this damage is largely oxygen-dependent. A strain deficient in recombination repair (*E. coli* K-12 AB2463 *recA*) shows photoprotection from killing to much higher fluences of 365-nm radiation, up to 1600 kJ/m², although fluences above 800 kJ/m² result in progressively less photoprotection, indicating the superposition of a lethal effect (Webb *et al.*, 1978a). A strain deficient in excision repair (*E. coli* K-12 AB1886 *uvrA*) shows no photoprotection from killing (as expected, Section 4.2.1b), but also no lethal synergism at 365-nm fluences up to about 1600 kJ/m² (Webb *et al.*, 1978a; Tyrrell, 1978b). These results suggest that excision repair is more sensitive to near-UV radiation damage than is recombination repair.

The effects on mutagenesis are somewhat different. In wild-type strains (*E. coli* K-12 and B/r), photoprotection from far-UV radiation-induced mutation to tryptophan independence is observed either with black light or with monochromatic 365-nm fluences up to 1600 kJ/m²; with *uvrA* mutants, however, mutation frequency is *increased* by a pretreatment with near-UV radiation (Tyrrell, 1978b; Turner and Webb, 1981).

4.2.3. Summary

Near UV–far UV interactions (other than photoreactivation) can be explained by the following hypotheses. In *wild-type cells*, photoprotection operates at relatively low fluences of near-UV radiation by enhancing excision repair of far-UV radiation-induced DNA damage. This produces increased survival and decreased mutation (since mutation is largely a result of recombination repair). The enhanced excision repair results from the absorption of light in ⁴Srd in tRNA, leading to elevated ppGpp levels,

suppression of RNA and protein synthesis, and consequent growth delay, conditions that permit (1) time for extra prereplication repair, and (2) continued respiration, to supply high-energy intermediates needed for repair.

Tyrrell (1978b) has proposed that the enhancement of excision repair caused by the normal photoprotection mechanism would, at higher fluences (around 1000 kJ/m²), become excessive, leading to large excisions, which begin to overlap, resulting in double-strand breakage of DNA. This would cause a decrease in survival, or a lethal synergism. Also, this excessive (and lethal) excision would remove from the population of survivors the most heavily damaged cells (in which the mutation frequency, due to induction of error-prone SOS repair, is high), the net effect of exposure to near-UV radiation thus being an overall decrease in mutation frequency. Thus, the photoprotection from mutation at high fluences may have a more complex mechanism than photoprotection from mutation at low fluences.

In *excision-repair-deficient mutants*, there is of course no excision repair, and no photoprotection occurs. There is no lethal synergism at high fluences (Tyrrell, 1978b), since there is no enhancement of excision repair. These near-UV radiation fluences, however, may damage constitutive recombination-repair systems, leaving most repair to be done by inducible error-prone systems. This would result in an *increase* in mutation frequency (Tyrrell, 1978b; see also de Moraes and Tyrrell, 1981).

It is apparent that near UV–far UV synergisms and antagonisms are complex, and further experimentation is needed to verify these hypotheses.

5. OVERVIEW

5.1. Roles of Nucleic Acids and Membranes

In the far-UV region, nucleic acid is the primary chromophore and target for most biological effects not simply because it absorbs light so well (so do proteins) but because of its critical genetic role. Most genes are represented only once or twice in a cell, and even minor damage to them can be crucial. Therefore, in the near-UV region, even though nucleic acids generally absorb very poorly (see Fig. 1), it is reasonable to suspect them of being targets, and possibly chromophores.

It is convenient to divide near-UV radiation effects into two classes: (1) those that are clearly sublethal, occurring typically in *E. coli* at fluences of about 20 kJ/m² at 334 nm, and (2) those that occur in the lethal range of fluences, around 200 kJ/m² at 334 nm or 1000 kJ/m² at 366 nm.

All the *sublethal effects* observed in *E. coli* appear to have action spectra with maxima around 340 nm and to be caused by absorption by ${}^4\text{Srd}$ in tRNA (Section 2). DNA seems not to be involved in these effects, both because of the low fluences involved and because of the shapes of the action spectra. ${}^4\text{Srd}$ seems to occur in the nucleic acids only in tRNA. Furthermore, ${}^4\text{Srd}$ has not been found in higher eukaryotes; it therefore seems likely that these sublethal effects will not be observed in those organisms. It is of interest that even the sublethal effects in *E. coli* are due to absorption in, and damage to, a nucleic acid. Although tRNA does not play a direct genetic role, it is nevertheless important in the control of gene action, both through the indirect mechanism of induction of ppGpp and through direct interactions of tRNA with transcription systems.

In the *lethal fluence range*, DNA is apparently the principal *target* for lethal effects themselves. DNA may also be a chromophore for these effects at the shorter wavelengths, but the major chromophores are probably other molecules intimately associated with DNA which transfer their excitation energy into the DNA target (Sections 1.2.2 and 3.1). The same is presumably true for near-UV radiation mutagenesis.

The membrane transport effects observed in the lethal fluence range appear not to involve damage to nucleic acid. For example, inhibition of glycine transport has been shown to be independent of the presence of ${}^4\text{Srd}$, and shows no photoreactivation (Ascenzi and Jagger, 1979). Critical molecules for these effects are probably membrane transport proteins, as well as components of the oxidative electron-transport chain, notably quinones, flavoproteins, and cytochromes. Iron-sulphur proteins are a possibility, although no evidence has yet appeared to implicate them.

The mid-UV region (290–320 nm) provides a separation between the far-UV and the near-UV regions that is significant, because of three effects that occur in the far-UV but not in the near-UV region: (1) high absorption by nucleic acids and proteins, (2) absence from sunlight at the surface of the Earth, and (3) absorption by many solids. No such criteria apply to the dividing point between the near-UV and visible regions at 380 nm, this division reflecting *only* the fact that humans do not see below 380 nm (many insects do). Therefore, one must expect a continuum of effects from the near-UV region into the violet, blue, and green regions of the visible spectrum. Thus, e.g., lethality is observed throughout the visible region, and mutation up to 500 nm (Webb, 1977). The absorption of many important near-UV radiation chromophores extends well into the visible region (Fig. 1). Some chromophores, however, such as 4-thiouridine, menaquinone, and *N*-formylkynurenone (Fig. 1) absorb chiefly in

the near-UV region and may be expected to be associated with effects that occur almost exclusively in the near-UV region.

Because lethal effects and membrane effects occur in the same fluence range, one may expect some interactions between them. Nevertheless, they seem frequently to be independent. We have noted above the evidence that the inhibition of glycine transport does not reflect damage to DNA. Lethality, on the other hand, appears to involve mostly damage to DNA, and sometimes to use ${}^4\text{Srd}$ as one chromophore (Section 3.1). Lethality seems generally not to involve damage to membrane chromophores in the near-UV region, but, as one moves into the visible, one may expect a greater contribution from chromophores residing in the membrane. Under some conditions, however, lethal consequences of membrane damage have been demonstrated even in the near-UV region (Moss and Smith, 1981).

5.2. Near-UV Radiation Effects as Tools in Biological Research

Identification of Target Molecules. The photoreactivating enzyme (photolyase) is specific for pyrimidine dimers. Such dimers are the principal lesion for many biological effects in the far-UV region, and they are also produced by mid-UV and near-UV radiation. If a biological effect can be photoreactivated, this is excellent evidence that pyrimidine dimers are the lesion responsible. These dimers are produced primarily in DNA, although in some systems they may also be produced in RNA. If photoreactivation is found not to occur, this does not mean that pyrimidine dimers have not been produced: they may have been produced, but subsequently repaired either by dark repair or, in the case of near-UV radiation effects, by concomitant photoreactivation by the damaging light itself. Photoreactivation has been widely and successfully used over the past decade or so as an indicator of pyrimidine dimer damage to DNA.

In prokaryotes, ${}^4\text{Srd}$ occurs only in the tRNA. Therefore, any biological effect that does not occur in an isogenic ${}^4\text{Srd}^-$ mutant is very likely caused by damage to tRNA. Such a conclusion is not certain, since it is always possible that the organism requires ${}^4\text{Srd}$ for some part of a dark reaction and is using a different molecule as the chromophore. However, this possibility can be virtually eliminated by determining that the action spectrum closely matches the absorption spectrum of ${}^4\text{Srd}$ in tRNA, and that ${}^4\text{Srd}$ -derived photoproducts are present in tRNA.

Mutant Selection by Growth Delay. By using multiple cycles of near-UV irradiation followed by several hours of incubation under growth

conditions, one may quickly select mutants that are deficient in near-UV radiation-induced growth delay, since such mutants will grow preferentially. If such a cycling technique is pushed far enough, one can select a variety of mutants for components involved in the induction of growth delay. For example, Ramabhadran (1976) developed this technique and first used it for the isolation of *rel*⁻ mutants of *E. coli* B/r. This technique was also used by Ramabhadran *et al.* (1976b), Thomas and Favre (1977a, 1980), and Tsai and Jagger (1981) for the isolation of mutants lacking ⁴Srd. The technique may also be used to isolate mutants deficient in other biological effects that stem from damage to ⁴Srd. For example, both Thomas and Favre (1980) and Tsai and Jagger (1981) have isolated mutants deficient in photoprotection by this technique. It is of interest that Tsai and Jagger (1981) found in this way mutants that were deficient in growth delay, but were *rel*⁺ and ⁴Srd⁺; these mutants must be deficient in some additional factor required for growth delay, and it would be very interesting to attempt to identify those components.

Solar Dosimetry. A biological dosimeter, based on a *B. subtilis* mutant spore lacking DNA repair systems, has been proposed for solar-UV radiation studies, since it essentially integrates the relative DNA-damaging capacity of sunlight (Tyrrell, 1978c; Munakata, 1981). This system is effective because of (1) the high sensitivity to sunlight, (2) the exponential nature of the inactivation curve, (3) the temperature independence of inactivation, (4) the possibility of assay long after irradiation, and (5) the simplicity of the system. Since the spore has only slight absorption in the solar spectral range, inactivation rates can be used directly to determine the equivalent incident dose at 254 nm, in terms of DNA-damaging capacity. This system is quite effective for measuring the *amount of sunlight* in the lethal range, which occurs mostly below 400 nm. However, it should be recognized that attempts to use such a dosimeter to predict the *lethal effect* in a particular vegetative biological system would be fraught with hazards. The strong synergisms observed in the near-UV range, for both lethality and mutation, as well as the existence of photoreactivation and photoprotection, which permit repair of the DNA damage produced by shorter wavelengths, are some of the complications that may be encountered (see Tyrrell, 1980b).

Other Applications. A variety of other applications of near-UV light as a biological probe, such as many uses of photodynamic action (see Amagasa, 1981), are available, but will not be discussed here because of limitations of space. Favre and Thomas (1981) provide an interesting review of probes of tRNA structure and function, using fluorescent and photoaffinity labels, both intrinsic and extrinsic. Song and Tapley (1979)

discuss applications of near-UV radiation interactions with psoralens, a process of interest because most of the biological effects involve DNA.

In summary, near-UV radiation can be successfully applied as a probe of biological function in a variety of ways. Because of the large number of chromophores in this region, and the probable resulting wide variety of biological effects, many of them yet to be discovered, we may expect that the usefulness of near-UV radiation as a biological probe will increase in the future.

5.3. Environmental Implications

The only radiations that have a significant ecological impact at the surface of the Earth are: (1) ionizing radiations, from radioactivity in the ground and the air, and from cosmic radiation; (2) mid-UV and near-UV radiation present in sunlight (solar-UV radiation); and (3) visible light from sunlight. The last category involves generally harmless, and usually beneficial, effects (Seliger, 1977). The first two categories involve primarily deleterious effects. The reasons why solar-UV radiation is damaging have been mentioned earlier, and relate to its being the most energetic radiation present in sunlight, and to the fact that it is effectively absorbed by, and can damage, many of the highly conjugated molecules that are so important in biological systems. These two things are not of course coincidental: complex organic compounds presumably arose on the surface of the Earth primarily through the action of UV radiation unfiltered by ozone. Molecules created by energetic radiation can also be readily broken down by energetic radiation (consider, e.g., the creation and splitting of thymine dimers). Although chemical evolution presumably no longer occurs, the ozone in the atmosphere still permits transmission of a significant amount of damaging radiation. There are, as has also been mentioned earlier, some beneficial aspects of this solar-UV radiation, but the deleterious effects seem to outweigh the beneficial ones.

Biological evolution would of course tend to eliminate organisms that are highly sensitive to solar-UV radiation, and to favor those that can tolerate it. Such tolerance may result from three different mechanisms: (1) physical screening of the radiation by a cell wall or membrane containing near-UV radiation-absorbing compounds, which reflect the light energy or degrade it into heat; (2) interference with the action of deleterious photoproducts, such as the proposed carotenoid pigment protection through the quenching of singlet oxygen (Webb, 1977); and (3) repair of DNA damage (Section 3.1). In spite of these measures, however, or-

ganisms retain various degrees of sensitivity to solar-UV radiation. Organisms normally sheltered from sunlight, such as coliform bacteria and many aquatic organisms, and perhaps even modern man, may show considerable sensitivity; some of these systems are discussed below.

Calkins and Thordardottir (1980) have conducted an interesting study of the tolerance of a variety of microorganisms to sunlight. The study includes marine diatoms of Iceland, and fresh-water bacteria, yeast, algae, protozoa, and arthropods of Kentucky. The survival of these organisms under simulated solar-UV radiation (280–320 nm) was compared to the typical sunlight exposures that they would experience in their natural aquatic environments. It was found that most of the organisms have only just barely enough resistance to survive in their natural environments, the Icelandic diatoms showing a much lower tolerance to sunlight than the Kentucky fresh-water organisms. This suggests that microorganisms expend no more energy than is absolutely necessary to resist the inactivating effects of solar radiation. In some cases, the measured sensitivity of the organisms is sufficiently great that it would not permit survival to the expected exposure in the natural environments. These paradoxes are explained in a variety of ways. For example, *E. coli* is very sensitive; work by Grigsby and Calkins (1980) shows that an entire day of full unfiltered sunlight will reduce coliform survival in lagoon water to less than 0.01%, in spite of the many pathways of DNA repair that exist in *E. coli*. The explanation given by Calkins and Thordardottir (1980) is that *E. coli* spends most of its life cycle in the sunlight-protected intestine of host animals, and therefore needs only short-term survival in natural waters, only long enough to enable it to transfer from host to host. *Paramecium aurelia* shows average resistance, but tends to remain at the surface of the water where the exposure is much above tolerable level; it copes with this by seeking shielded surface locations, such as are provided by rocks and plants. Another protozoan, *Coleps*, avoids light by moving deep into the water column. Both the marine diatoms and the unicellular fresh-water algae appear to obtain photosynthetic light with minimal solar-UV exposure by rising in the water in the early morning and late evening, and sinking at mid-day.

While it is thus apparent that many microorganisms live on the brink of destruction by solar-UV radiation in their natural environments, it is of interest that the level of exposure to ionizing radiation, for all organisms, is far below the lethal level. This presumably reflects the fact that a considerable fraction of ionizing radiation damage simply cannot be repaired and results in mutations that can have lasting effects in future generations. The implication, therefore, is that microorganisms must be able to protect themselves effectively from near-UV radiation-induced

mutation. That this is so is indicated by recent work of Tyrrell (1979b) and Turner and Webb (1981) showing that sunlight does not induce mutations in the DNA repair-competent B/r strain of *E. coli*, although it is strongly mutagenic for an excision-repair-deficient strain. This can be explained in terms of a photoprotection phenomenon (Section 4.2.1b), which permits the complete repair of mutagenic lesions in wild-type strains (see also Tyrrell, 1980a). It had earlier been shown by Jagger (1975) that 10 min. of exposure to bright sunlight would produce a large growth delay and photoprotection in *E. coli*, the fluence rate being equivalent to about 30 W/m^2 of growth-delaying near-UV radiation (action spectrum shown in Fig. 4). It should be recognized that these mutations are being induced primarily by the short wavelengths around 300 nm, which are producing far-UV radiation-type lesions in DNA, and it is this mutation, rather than that produced (at much lower efficiency) by longer wavelengths of near-UV radiation, that is being photoprotected against.

Finally, although it is not within the purview of this review, its importance nevertheless requires mention of the production of skin cancer in humans by near-UV radiation. This is presumably produced by direct damage to DNA, since the action spectra for a variety of related effects are closely correlated with the absorption spectrum of DNA (Setlow, 1974; Doniger *et al.*, 1981). This damage can presumably be repaired in normal adults by dark-repair systems, since patients with the disease xeroderma pigmentosum are defective in excision repair and are very susceptible to skin cancer (Setlow, 1978; see also Epstein, 1977). Photo-reactivation of UV radiation-induced cancers also has been reported in fish lung tissue (Hart *et al.*, 1977), and almost complete photo-reactivation (97%) of pyrimidine dimers induced by solar-UV radiation in humans (*in vivo*) has been reported by D'Ambrosio *et al.* (1981). The practical importance of this subject is underscored by the alarming rise in recent years of fatal malignant melanomas in the Northern Hemisphere (Parrish *et al.*, 1978), although the role played by UV radiation in the induction of malignant melanoma is still not clear (Baker-Blocker, 1980).

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The Electronic Spectroscopy of Photoreceptors (Other Than Rhodopsin)

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1. INTRODUCTION

A number of organisms ranging from prokaryotic bacteria to eukaryotic mammals directly respond to light of different wavelengths in terms of various photobiological reactions. A photobiological reaction entails the absorption of a specific wavelength of light by the functioning photoreceptor molecule within the photoreceptor organelle or apparatus. The general scheme of photoreceptor function is outlined diagrammatically in Fig. 1. As can be seen from this figure, the photoreceptor molecule absorbs a specific wavelength of light, generating its electronically excited

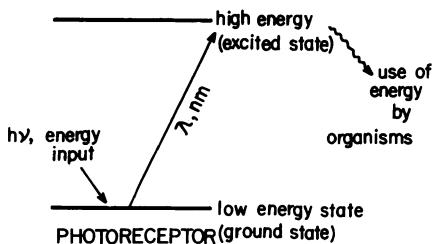


Fig. 1. The electronic excitation of photoreceptors and the primary photophysical and/or photochemical relaxation of the excited photoreceptor, leading to a series of energy transducing processes in photobiological responses of organisms.

state from which primary molecular processes leading to the measurable photoresponse reaction of an organism proceeds. It is readily recognized that the electronic spectroscopy of photoreceptors plays an essential part in describing the mechanisms of photobiological processes at the molecular level. This review is intended to provide the reader with the current knowledge of the electronic excited states of various photobiological receptors, except rhodopsin and bacteriorhodopsin, which are extensively reviewed elsewhere (Birge, 1981, and references therein).

The specific aim of this chapter is to provide the photobiology audience with basic information on the electronic spectroscopy of photoreceptors. In doing so, the quantitative treatments of concepts and the analysis of high resolution spectra have been omitted. In addition to the description of absorption spectra, the fluorescence properties of photoreceptors are briefly described. The latter are not discussed in detail, but are presented merely to reflect the spectroscopic nature of the longest wavelength absorption bands of photoreceptors.

As implied in Fig. 1, the wavelengths of light absorbed by the photoreceptor determines the spectral response of an organism to solar radiation (Song, 1977a). Through evolutionary adaptation of organisms to the solar radiation environment, organisms may have developed the spectral photosensitivity that is determined by the electronic spectral characteristics of photoreceptor molecules. The spectral photosensitivity of organisms can be represented in terms of what may be called "photobiological spectrum," which is illustrated in Fig. 2.

In the photobiological spectrum, various photoreceptors for light responses of organisms are superimposed over the solar spectrum, with their approximate absorption maxima and structures, where known, indicated by arrows. The identities of some of the photoreceptor chromophores indicated in Fig. 2 are by no means definitively established. For example, the photoreceptor chromophore for the photoreactivation by PRE (photoreactivating enzyme) is not known, although the thymine photodimer complexed to PRE may serve as a possible photoreceptor chromophore. Additionally, whether or not carotenoids can function as the pri-

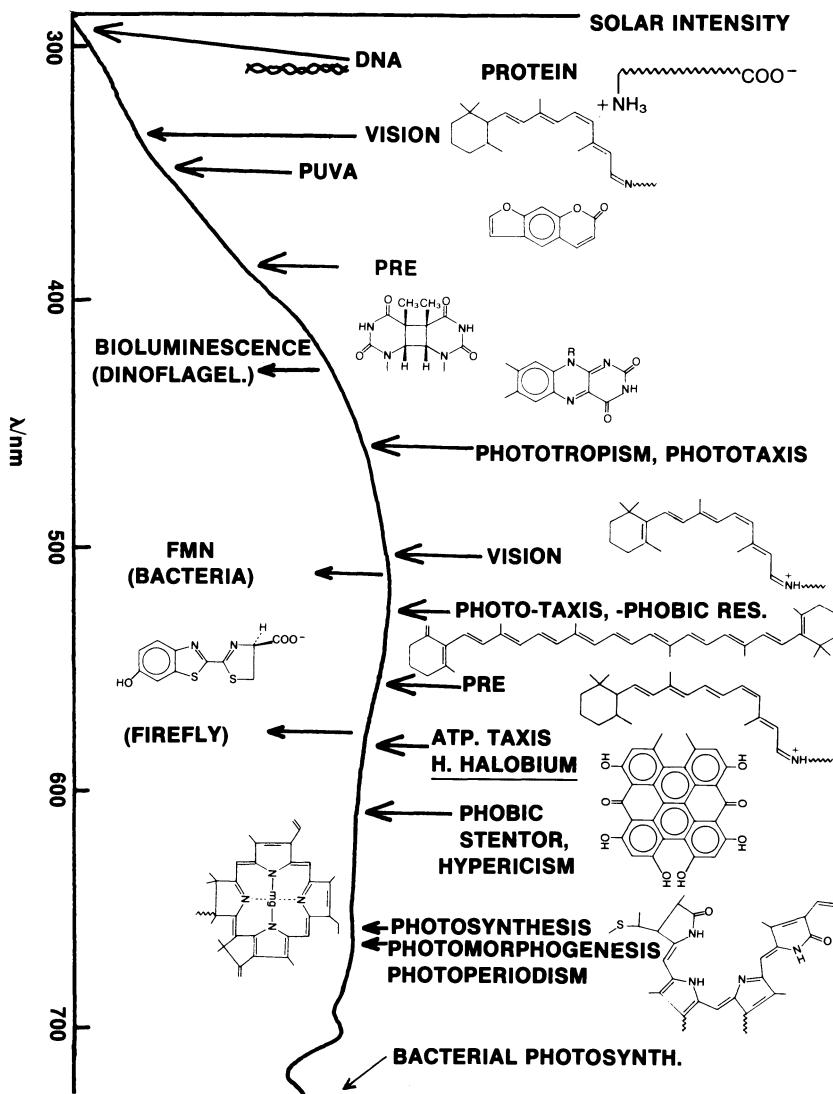


Fig. 2. The photobiological spectrum of photoresponses and their photoreceptor chromophores, including those involved in bioluminescent reactions. The arrows above and below the solar spectrum (solid line curve) represent the approximate absorption and emission maxima of the various photoreceptors, respectively. UV vision, in some insects; PUVA, psoralen-UVA photochemotherapy; PRE, photoreactivating enzyme.

mary photoreceptor for photophobic and phototactic responses in photosynthetic algae (e.g., *Gymnodinium*) is still an open question, although their participation as the secondary antenna pigment is less ambiguous.

There are a number of potential photoreceptor chromophores that are implicated in certain photoresponses and/or certain action spectra. For example, urocanic acids, desmosine, isodesmosine, quinones, and flavonoids are some of the possible chromophores of UV and near-UV radiation-induced responses. However, these will not be described in this review.

The photobiological spectrum shown in Fig. 2 illustrates the bioluminescence processes of certain bacteria, dinoflagellates, fireflies, and other organisms not listed in the figure. The present review will also exclude these luciferin molecules of bioluminescent organisms.

2. UV RADIATION PHOTORECEPTORS

Both prokaryotic and eukaryotic organisms exhibit diverse responses to UV and near-UV radiation, including thymine dimerization in DNA, mutagenesis, cell inactivation, and many others. In this section, we will review the electronic spectroscopy of nucleic acids (DNA and RNA) and proteins as endogenous photoreceptors, and psoralens as exogenous photoreceptors.

2.1. DNA

The UV absorption spectrum of DNA is determined by the composite spectra of DNA bases: adenine, thymine, guanine, and cytosine. The literature reports the absorption spectra of native (double helical) and denatured (single stranded) DNA. The most striking is the marked hypochromism of the absorption maximum at 260 nm. Although the absorption spectra of DNA do not show significant absorption at wavelengths longer than 300 nm, the native DNA solutions often absorb light of wavelength 300–310 nm.

The specific absorption coefficients of single- and double-stranded DNA are approximately 25 and 20 ml/mg/cm, respectively, at pH 7 and 260 nm. Comparison of the CD spectra of single- and double-stranded DNA shows no evidence of a unique transition for DNA at 300 nm or longer. Both the hypochromicity and the red edge absorption at wavelengths longer than 300 nm in native DNA can be explained in terms of

weakly coupled exciton interactions among the stacked base pairs (Rhodes, 1961; Tinoco, 1960). The long wavelength absorption in DNA is thus likely to be due to the weak exciton broadening of $\pi \rightarrow \pi^*$ bands of the composite bases of DNA, rather than due to the broadening of the $n \rightarrow \pi^*$ bands.

Except for the weak, hidden $n \rightarrow \pi^*$ bands (e.g., 294 nm in purine; Chen and Clark, 1969), the genealogy of UV bands of DNA bases can be described in terms of the benzene notation [i.e., $^1L_{a,b}$ and $^1B_{a,b}$ in free electron symbols or $^1B_{2u,1u}$ and $^1E_{1u}$ in symmetry notation (see review by Song, 1977b)]. Table 1 presents the absorption spectral characteristics of DNA bases.

The lowest $n \rightarrow \pi^*$ transition in purine bases is highly localized on N₃ (Hug and Tinoco, 1973, 1974). The 260 nm absorption band of adenine in solution (Table 1; 263 nm for adenosine, pH 7; $\epsilon \sim 1.49 \times 10^4$) is correlated to L_b or (B_{2u}); the second $\pi \rightarrow \pi^*$ band (L_a ; B_{1u}) is apparently hidden under the L_b band.

In Table 1, the $n \rightarrow \pi^*$ transition is designated as the lowest transition in adenine, thymine, and cytosine. However, it should be cautioned that the lowest electronic state, from which fluorescence emission and photochemistry originate may not necessarily be an n, π^* state,† since the 0–0 band of the $\pi \rightarrow \pi^*$ transition (L_b) may occur at wavelengths longer than 280 nm. In fact, the fluorescent state of these bases is likely to be a π, π^* state, as discussed later. It should also be noted that, except for the lowest $\pi \rightarrow \pi^*$ transition (L_b), the assignments of $\pi \rightarrow \pi^*$ transitions to higher electronic states (L_a , B_b , and B_a) are not unequivocally established at the present. For example, in some cases (see Table 1 for adenine) the second band is assigned to a $\pi \rightarrow \pi^*$ transition corresponding to the L_a state, which is polarized at some angle to the L_b transition moment direction. The difficulty with the L_a band assignment is that its oscillator strength is estimated to be lower than that of the L_b band, in contrast to what is expected from theory and spectral correlation with the benzene spectra (Song, 1977b). This difficulty is more severe with pyrimidine bases, as both first and second $\pi \rightarrow \pi^*$ transitions are strongly mixed (configuration interactions), resulting in both transitions polarized roughly along the N₁—C₄ axis.‡

The fluorescence characteristics of DNA bases are summarized in

† An electronic transition involving the promotion of a bonding orbital electron (e.g., n) to an antibonding orbital (e.g., π, π^*) is designated by an arrow (e.g., $n \rightarrow \pi^*$ or $\pi^* \leftarrow n$), whereas the excited state generated by a transition (e.g., $n \rightarrow \pi^*$) is designated by a comma between the orbitals involved (e.g., n, π^*).

‡ A landmark study of the polarized absorption spectra of purines and pyrimidines was reported by Stewart and Davidson (1963).

TABLE 1. The Electronic Transitions in DNA Bases

Base	Transition ($S_0 \rightarrow S_n$)	Notation ^a	λ/nm	$\log \epsilon (f)$	Polarization ($\Delta\theta$)	Remarks	Reference
Adenine	S_1	$n \rightarrow \pi^*$	280	(~0.004) ^b	o.p. i.p.	In poly dA	Bush and Scheraga (1969)
	S_2	$\pi \rightarrow \pi^*$	275	(0.28)	(-3 + 3°)	As 9-methyl-adenine crystal	Stewart and Jensen (1964)
	$L_b; B_{2u}$	(270) ^c	260	4.10		In trimethyl phosphate	Clark and Tinoco (1965)
S_3	$\pi \rightarrow \pi^*$	$L_a; B_{1u}$	255 (255)	(8 × 10 ⁻³) (0.30)	i.p.; ⊥ to L_b i.p.	9-methyladenine By MO calculation (277 nm, $f = 0.08$ for L_b)	Stewart and Jensen (1964) Song (1977b)
	$S_{4,5}$	$\pi \rightarrow \pi^*$	208 (206) ^d	4.27 (0.62) ^d	i.p. (⊥ to L_b) ^d	In TMP	Clark and Tinoco (1965)
Guanine	S_1	$B_{a,b}; E_{1u}$	185	4.20	i.p.	In TMP	Clark (1977)
	S_2	$n \rightarrow \pi^*$	300 (Weak)	o.p. (0.16)	i.p.	In 9-ethylguanine	Clark (1977)
	S_3	$\pi \rightarrow \pi^*$	278		(-4 ± 3°)	In 9-ethylguanine	Clark (1977)
	S_4	$L_b; B_{2u}$	254	(0.25)	i.p.	In 9-ethylguanine	Clark (1977)
S_1	$\pi \rightarrow \pi^*$	$L_a; B_{2u}$	275	3.97	i.p. (-75 ± 3°)	In 9-ethylguanine	Clark and Tinoco (1965)
	S_2	$\pi \rightarrow \pi^*$	$L_b; B_{2u}$	~264	o.p.	In DNA	Studdert and Davis (1974)
S_3	$n \rightarrow \pi^*$	$L_a; B_{1u}$	256	4.19	i.p.	In 9-ethylguanine	Clark and Tinoco (1965)
	$S_{4,5}$	$\pi \rightarrow \pi^*$	$B_{a,b}; E_{1u}$	203 190	4.30 4.44	i.p. i.p.	Clark and Tinoco (1965) In TMP

Thymine	S_1	$n \rightarrow \pi^*$	280	Weak (0.18)	o.p. i.p. (-20°)	In poly d(AT) In crystalline 1-me-thymine	Studdert <i>et al.</i> (1972) Clark (1972) ^e
	S_2	$\pi \rightarrow \pi^*$ $L_b; B_{2u}$	270				
		(264)	3.90	i.p. (-50°)		In water, pH 2 In crystalline 1-me-thymine	
S_3	$\pi \rightarrow \pi^*$	213	(0.29)	i.p. (-36°)		Clark (1972)	Clark (1972)
	$L_a; B_{1u}$			i.p. (-42°)		Clark (1972)	
	$\pi \rightarrow \pi^*$ $B_{a,b}; E_{1u}$	179	(0.31)			Clark (1972)	
$S_{4,5}$	$n \rightarrow \pi^*$	164	(0.17)				Rich and Kasha (1960) Callis and Simpson (1970) ^f
	$\pi \rightarrow \pi^*$ $B_{a,b}; E_{1u}$	280	Weak (0.18)	o.p. i.p.	In poly C In crystalline state		
	$n \rightarrow \pi^*$ $L_b; B_{2u}$	265					
Cytosine	S_1	$n \rightarrow \pi^*$	(277)	3.89	i.p. i.p. (-1°)	In TMP In crystal state	Clark and Tinoco (1965) Callis and Simpson (1970)
	S_2	$\pi \rightarrow \pi^*$ $L_a; B_{1u}$	230				
		(237)	3.54	i.p. i.p. i.p.	In TMP In TMP In TMP		
$S_{4,5}$	$\pi \rightarrow \pi^*$	204	4.08				Clark and Tinoco (1965) Clark and Tinoco (1965)
	$B_{a,b}; E_{1u}$	185	4.09	i.p.			

Note: f , oscillator strength; $\Delta\theta$, polarization direction in accordance with the DeVoe and Tinoco sign convention (1962), e.g. +12° and -3° for the $S_0 \rightarrow S_2$ transitions of cytosine and adenine, respectively, refer to counter-clockwise and clockwise angles with respect to the N-C₄ and C₄-C₅ axes, respectively. o.p. and i.p. represent out-of-plane and in-plane polarization, respectively.

^a Along with symmetry designations, we use the Platt Notations (L_a and L_b , etc.) for the excited states. Strictly speaking, these notations are inappropriate for use in non-alternant heterocyclic molecules such as the DNA bases and tryptophan.

^b In solution.

^c In stretched film (Fucaloro and Forster, 1971).

^d 9-methyladenine crystal (Stewart and Jensen, 1964).

^e Quoted from Daniels (1976).

^f See Lewis and Eaton (1971) for the polarization data.

TABLE 2. The Fluorescence Characteristics of DNA Bases

Base	λ_F/nm (0-0)	ϕ_F	τ_F/ns	Solvent	Temperature (K)	Reference
Adenine	281	2.6×10^{-4}	0.001	pH 7.3	300	Daniels and Hauswirth (1971)
	318	0.06	2.9 ^a	pH 7	77	Longworth <i>et al.</i> (1966)
Guanine	299	3.0×10^{-4}	0.0014	pH 6.3	300	Eisinger and Shulman (1968)
	313	0.06	5 ^a	pH 7	77	Daniels and Hauswirth (1971)
Thymine	290	1.02×10^{-4}	0.0009	pH 6.7	300	Longworth <i>et al.</i> (1966)
	316	0.21 ^c	3.2 ^d	pH 7	77	Daniels and Hauswirth (1971)
Cytosine	316	0.82×10^{-4}	0.0002	pH 6.5	300	Daniels and Hauswirth (1971)
	312	0.06	5.5	pH 7	77	Longworth <i>et al.</i> (1966)

^a GMP (Daniels, 1976).^b Hönnås and Steen (1970).^c AMP (Blumberg *et al.*, 1968).^d TMP (Daniels, 1976).

Table 2. It can be seen that the fluorescence quantum yields and lifetimes are extremely low at room temperature. The radiative fluorescence lifetimes of DNA bases range from 2.9 (adenine; Table 2) to 12 ns (GMP; Daniels, 1976). Such short lifetimes are indicative of an emission from a π,π^* state, rather than an n,π^* state. Thus, it is reasonable to assume that the lowest electronic state, from which DNA bases undergo photo-reactions, is a π,π^* (L_b) state. §

2.2. RNA

The absorption spectra of RNA are determined by the composite spectra of RNA bases, adenine, guanine, cytosine, and uracil, and they resemble single-stranded DNA (Section 2.1). The absorption maximum is at 260 nm, with virtually no absorbance above 300 nm. On the other hand, tRNA absorbs slightly above 300 nm due to minor bases such as 4-thiouracil in *E. coli* tRNA and Y-base in yeast tRNA. Table 3 summarizes the absorption spectral characteristics of uracil, thiouracil, and Y-base. The fluorescence properties of these bases are presented in Table 4.

The assignments and polarization of the UV absorption bands of uracil (Table 3) may be regarded as reasonably well established; however, the location of the n,π^* state in solution remains ambiguous. However, the room temperature fluorescence quantum yield, radiative lifetime in the ns range, and the mean lifetime in the ps range at room temperature are consistent with the lowest singlet electronic state being of the π,π^* type.

4-Thiouracil exhibits a rather unusual absorption spectrum for a nitrogen heterocyclic system, with its anomalously intense and weak absorption for the first and second absorption bands, respectively (Table 3). The $n \rightarrow \pi^*$ band is probably hidden under the intense absorption band. It is likely that the first band is composed of two $\pi \rightarrow \pi^*$ transitions, as tentatively assigned in Table 3. 4-Thiouracil is unusually photoreactive; 4-thiouridine adds to cytidine within *E. coli* tRNA (Favre *et al.*, 1971) and produces a fluorescent species with a 200-ns lifetime upon excitation (Favre, 1974). The latter may be attributed to a rare example of room temperature phosphorescence rather than fluorescence (Shalitin and Feitelson, 1973). However, at 77°K, 4-thiouridine phosphoresces maximally at 470–480 nm (Favre, 1974). Thus, it is not certain that phosphorescence

§ The reader is referred to a very recent review of the spectroscopy of DNA and its bases by Callis (1983).

TABLE 3. The Electronic Transitions in RNA Bases

Base	Transition ($S_0 \rightarrow S_n$)	Notation	λ/nm	$\log \epsilon (f)$	Polarization ($\Delta\theta$)	Remarks	Reference
Uracil	S_1	$\pi \rightarrow \pi^*$ $L_b; B_{2u}$	276	0.19	i.p. ($0^\circ \sim 7^\circ$)	In 1-methyl-uracil crystal	Eaton and Lewis (1970)
	S_2	$n \rightarrow \pi^*$	264	2.39	o.p.	In 1-methyl-uracil crystal	Eaton and Lewis (1970)
	S_3	$\pi \rightarrow \pi^*$ $L_a; B^{1u}$	270	0.19	i.p. (-9°)	In 1-methyl-uracil crystal	Clark (1972)
	S_4	$n \rightarrow \pi^*$	222	0.06	o.p.	In 1-methyl-uracil crystal	Clark (1972)
	S_5	$\pi \rightarrow \pi^*$ $(B_b; E_{1u})?$	213	0.27	i.p. (-53°)	In 1-methyl-uracil crystal	Clark (1972)
	S_6	$n \rightarrow \pi^*$	184	0.11	o.p.	In 1-methyl-uracil crystal	Clark (1972)
	S_7	$\pi \rightarrow \pi^*$ $(B_a; E_{1u})?$	179	0.30	i.p. (-33°)	In 1-methyl-uracil crystal	Clark (1972)
	S_2	$\pi \rightarrow \pi^*$ $L_b; B_{2u}$	258	3.91	i.p.	In TMP	Clark and Tinoco (1965)

S_3	$\pi \rightarrow \pi^*$ $L_a; B_{1u}$?	?	In TMP
S_4	$\pi \rightarrow \pi^*$ $B_b; E_{1u}$	203	3.91	i.p. (?)
S_5	$\pi \rightarrow \pi^*$ $B_a; E_{1u}$	181	4.07	i.p.
G-Thiouracil	$\pi \rightarrow \pi^*$ $L_{b,a}; B_{2u,1u}$	330	4.31	(i.p.)?
S_1	$\pi \rightarrow \pi^*$ $(B_b; E_{1u})?$	245	3.66	(i.p.)?
S_2	$\pi \rightarrow \pi^*$ $(L_a; B_{1u})?$	305	3.86	i.p., short axis
S_3	$\pi \rightarrow \pi^*$ $(L_b; B_{2u})?$	271	~3.9	i.p.
Y-base	$\pi \rightarrow \pi^*$ $(n \rightarrow \pi^*)?$	260	~3.9	(o.p.)?
S_1^a	$\pi \rightarrow \pi^*$ $(L_b; B_{2u})?$	238	~3.8	i.p.
S_2	$\pi \rightarrow \pi^*$ $(B_b; E_{1u})$	230	4.25	i.p.
S_3	$\pi \rightarrow \pi^*$ $(B_a; E_{1u})$			

^a The MO (INDO/S) calculation predicts S_1 to be due to a $n \rightarrow \pi^*$ transition at 322 nm (Gryczynski *et al.*, 1979b).

TABLE 4. The Fluorescence Characteristics of Selected RNA Bases

Base	λ_F/nm (0–0)	ϕ_F	τ_F/ns	Solvent	Temperature (K)	Reference
Uracil	280	4.5×10^{-5}	0.0007	pH 6.8 Ethylene glycol-	300	Daniels and Hauswirth (1971)
	287	8×10^{-4}	4.5 ^a	H ₂ O (1:1)	77	Longworth <i>et al.</i> (1966)
4-Thiouracil	287	1×10^{-2b}		Ethylene glycol-	77	Eisinger and Shulman (1968)
	550 (max) ^c	3×10^{-4}	200	H ₂ O	298	Favre (1974)
Y-Base	530 (max)	$< 10^{-4}$	300000	H ₂ O	77	Shalitin and Feitelson (1973)
		1.5×10^{-3}		In tRNA	300	Pochon <i>et al.</i> (1968)
4-UMP	~365	0.07		H ₂ O	293	Eisinger <i>et al.</i> (1970)
	432 (max)			In PVA	250	Eisinger <i>et al.</i> (1970)
4-Thiouridine (see text)	447	0.035				Gryczynski <i>et al.</i> (1981a,b)
	(460) ^d	0.521				

^a Radiative lifetime.^b UMP.^c 4-Thiouridine (see text).^d Beardsley and Cantor (1970).

accounts for the 550 nm emission maximum of 4-thiouridine at room temperature. Apparently, 4-thiouridine also acts as the photoreceptor for the near-UV radiation-induced growth delay in *E. coli* (Ramabhadran, 1975; Ramabhadran *et al.*, 1976).

The lowest singlet transition is tentatively assigned to the L_a transition in Table 3. This assignment is based on the theoretical result of Gryczynski *et al.* (1979b) that the transition moment lies along the short axis of the Y-base, in analogy to the polarization of the L_a band along the short axis of naphthalene and anthracene. The molecular orbital theory (INDO/S) predicts that $n \rightarrow \pi^*$ transitions occur at wavelengths longer than the first $\pi \rightarrow \pi^*$ band. Experimental verification of this is still lacking, although the relatively small differences in dipole moment between the ground and excited states (Gryczynski *et al.*, 1979b) is at least qualitatively consistent with the lowest state being the n, π^* type. However, the fact that the Y-base is strongly fluorescent (Table 4) suggests that the lowest singlet excited state is of the π, π^* type.

2.3. Proteins

The polypeptide backbone of a protein absorbs UV radiation of wavelengths less than 230 nm. The UV absorption spectrum of a polypeptide is due to the weak $n \rightarrow \pi^*$ ($S_o \rightarrow S_1$; *ca.* 220 nm, out-of-plane polarized with respect to the peptide plane) and $\pi \rightarrow \pi^*$ ($S_o \rightarrow S_2$; *ca.* 190 nm, in-plane polarized) bands of individual peptide groups. The latter splits into two components in an α -helical conformation due to the exciton coupling of the peptide transition dipoles (Moffitt, 1956; Moffitt and Yang, 1956). The lower energy $\pi \rightarrow \pi^*$ exciton transition occurs at 205–207 nm, whereas the higher energy exciton transition is located at 190–192 nm. These transitions are polarized parallel and perpendicular to the helix axis, respectively (Moffitt, 1956; Brahms *et al.*, 1968).

It is unlikely that the peptide group of proteins serves as a primary photoreceptor chromophore for UV radiation effects on proteins and organisms (cf., Fig. 1). Histidine, phenylalanine, tyrosine, and tryptophan exhibit absorption at $\lambda \geq 230$ nm. Of these amino acid residues in protein, cystine ($\lambda_{\max} \sim 246$ nm; $\epsilon \sim 3200$), tyrosine ($\lambda_{\max} \sim 275$ nm; $\epsilon \sim 1400$), and tryptophan ($\lambda_{\max} \sim 280$ nm; $\epsilon \sim 5600$) absorb UV radiation of wavelengths longer than 280 nm, with tryptophan absorbing to 312 nm ($\epsilon \sim 10$; Fasman, 1976). Table 5 summarizes the spectral properties of tyrosine, tryptophan, and cystine. Table 6 presents the fluorescence data for the two aromatic amino acids.

The L_b (0–0) and L_a assignments for the 289 and 268 nm bands in tryptophan (Table 5) were deduced from the overlapping absorption band

TABLE 5. The Electronic Transitions in Tyrosine, Tryptophan, and Cystine

Amino acid	Transition ($S_0 \rightarrow S_n$)	Notation	λ/nm	$\log \epsilon$	Polarization	Remarks	Reference
Tyrosine	S_1	$\pi \rightarrow \pi^*$ $L_b; B_{2u}$	275	3.15	i.p., short axis	pH 7.1	Fasman (1976)
	S_2	$\pi \rightarrow \pi^*$ $L_a; B_{1u}$	223	3.94	i.p., long axis	pH 7.1	Fasman (1976)
	$S_{3,4}$	$\pi \rightarrow \pi^*$ $B_b; B_a; E_{1u}$	212 193	3.85 4.71	i.p.	pH 7.1	Fasman (1976)
Tryptophan	S_1^a	$\pi \rightarrow \pi^*$ $L_b; B_{2u}$	289	3.68	i.p. ^a	pH 7.1	Fasman (1976)
	S_2	$\pi \rightarrow \pi^*$ $L_a; B_{1u}$	267 ^a	3.70	i.p. ^a	pH 7.1	Fasman (1976)
Cystine	$S_{3,4}$	$\pi \rightarrow \pi^*$ $B_{b,a}; E_{1u}$	214 200	3.51 3.51	i.p. ^a i.p. ^a	Crystal	Yamamoto and Tanaka (1972)
	S_1	$\ell \rightarrow \sigma(?)^b$	246	3.30	S-S axis ^b	pH 7	Johnson (1971)
	S_2	$\sigma \rightarrow \sigma(?)^b$	207	3.30	S-S axis ^b	pH 7	Johnson (1971)

^a See text.^b ℓ , lone pair orbital; σ , sigma orbital (-S-S-).

TABLE 6. The Fluorescence Characteristics of Tyrosine and Tryptophan

Amino acid	λ_F/nm	ϕ_F	τ_F/ns	Solvent	Temperature (K)	Reference
Tyrosine	303	0.21	2.6 ^a	pH 7	298	Teale and Weber (1957)
		0.14		pH 7	296	Chen (1967)
Tryptophan	348 (357) ^c	0.20	2.6 ^b (2.9) ^c	pH 7	298	Teale and Weber (1957)
	356	0.14	2.8	pH 7	300	Eisinger and Navon (1969)
	319	0.6		Ethylene glycol	80	Eisinger and Navon (1969)

^a Chen *et al.* (1967).^b Chen (1967).^c Weinryb and Steiner (1968), at pH 7.5, 298°K.

with its maximum at 280 nm. The L_b and L_a bands are not well resolved due to the considerable overlap and vibronic mixing between these two transitions (Song and Kurtin, 1969; Valeur and Weber, 1977; Suzuki *et al.*, 1977; Umetskaya and Turoverov, 1978). UV absorption at 260–275 nm is usually assigned to the L_a band, although the L_b intensity underlying the former contributes to the overall spectral shape in this region.

The photoreactive state, i.e., the lowest excited S_1 state, in tryptophan is not necessarily identified with the L_b state (cf., Table 5). From the fluorescence polarization of the long wavelength absorbance edge at 305–310 nm, the L_a (0–0) band has been assigned for indole and tryptophan in propylene glycol at 215°K (Valeur and Weber, 1977). The L_b (0–0) band of tryptophan is resolved at 291 nm, whereas the L_a band maximum is located at 267 nm. The magnetic circular dichroism (MCD) spectrum of tryptophan also resolves the L_b transition at 290 nm (Barth *et al.*, 1972). The (0–0) bands of L_a and L_b origins for *N*-acetyltryptophan have been assigned to 297.5 and 290.5 nm, respectively, at 77°K (Strickland *et al.*, 1969). Thus, the photoreactive S_1 state is likely to be L_a , particularly in polar solvents (Andrews and Forster, 1972; Sun and Song, 1977). In nonpolar solvents, the L_a (0–0) band occurs at 284.5 nm, whereas L_b (0–0) is positioned at 287.9, according to Gaussian component analysis of the tryptophan spectrum (Strickland *et al.*, 1970). However, these assignments have been recently questioned (Umetskaya and Turoverov, 1978).

The L_b and L_a transition moments of indole are oriented 49° to the long molecular axis and nearly parallel to the N₁–C₄ axis (42° from the long axis), respectively, according to a polarized single-crystal absorption study (Yamamoto and Tanaka, 1972). Glycyl-tryptophan shows slightly different polarization directions, with L_b oriented 58° to the long molecular axis. Molecular orbital calculations predict the angle between the two transition moments to be 54°, as the two transitions are polarized nearly along the short axis and the N₁–C₄ axis, respectively (Sun and Song, 1977). The linear dichroic spectra of indole revealed an alternative polarization direction ($\pm 25 \sim 29$ ° with respect to the long molecular axis) for the L_b band assigned as $S_o \rightarrow S_1$ transition in polyethylene film (Umetskaya and Turoverov, 1978). The polarized single-crystal absorption spectroscopy of indole led to the resolution of B_b and B_a bands at 213.7 and 200 nm, respectively, and the transition moments are oriented 18° and –61° with respect to the long axis (Yamamoto and Tanaka, 1972).

There seems to be no doubt that the emitting state in tyrosine is L_b . However, the nature of the fluorescent state in tryptophan remains uncertain because the L_a and L_b states are closely disposed in energy, as discussed above. The situation is further clouded by the fact that the

fluorescence decay of tryptophan is non-exponential (Fleming *et al.*, 1978; Szabo and Rayner, 1980). The fluorescence emission maximum of tryptophan in nonpolar solvents is 328–330 nm, which red shifts to $\lambda \geq 350$ nm in polar solvents. It is reasonable to assign the fluorescent state of tryptophan to L_b in nonpolar solvents. However, different points of view have been advocated regarding the nature of fluorescence from tryptophan in polar solvents. These are (a) fluorescence emission from the exciplex state of the indole ring (see review by Lumry and Herschberger, 1978), (b) emission from the solvent-stabilized L_a state as the result of increase in permanent dipole moment upon excitation (e.g., Sun and Song, 1977), and (c) emission from different rotamers or conformers of the alanyl side chain of tryptophan (Szabo and Rayner, 1980).

In contrast to the case of indoles, the fluorescence decays of tryptophan, tryptophan ethyl ester, and 5- and 6-methyltryptophan in aqueous solution are non-exponential with two lifetime components. For tryptophan, these two components exhibit lifetimes of 3.1 ns (emission maximum resolved at 350 nm) and 0.51 ns (emission maximum at 335 nm; Szabo and Rayner, 1980). These two components have been attributed to the emission from different rotamers or conformers of the side chain [viewpoint (c) above].

The double exponential decay of tryptophan and the ground state association[†] of the indole ring have important implications in describing the nature and heterogeneity of the tryptophan fluorescence in proteins. For example, the fluorescence decay of tryptophan-25 (fluorescence emission maximum at 350 nm) in glucagon at pH 8.2 shows double exponential decays with lifetimes of 1.11 and 3.26 ns, even though the protein does not contain any other tryptophan residues (Cockle and Szabo, 1981). Double exponential decays have been attributed to different conformers of the indole ring or of the tryptophanyl peptide chain. For a tabulation of fluorescence data of various proteins, the reader may refer to the *Handbook* edited by Fasman (1976). A brief review of the literature on protein emission up to 1979 has been published (Galley and Milton, 1979). Table 6 summarizes the fluorescence data for tyrosine and tryptophan.

2.4. Psoralens (Furocoumarins)

Exogenously applied psoralens serve as photoreceptors for a number of near-UV radiation photoresponses in both prokaryotic and eukaryotic

[†] See Skalski *et al.* (1980) for the origin of the Stokes' shift due to ground state complex formation between 1-methylindole and polar solvents.

TABLE 7. The Electronic Transitions in Coumarin and Psoralen

Compound	$S_0 \rightarrow S_n$	Notation	λ/nm	$\log \epsilon (f)$	Polarization	Remarks	Reference
Coumarin	S_1	$\pi \rightarrow \pi^*$ L_b	310	3.78	i.p., ca. long axis	Ethanol, 77°K	Song and Gordon (1970)
	S_2	$n \rightarrow \pi^*$			o.p.?	Ethanol, 77°K	Mantulin and Song (1973)
Psoralen	S_3	$\pi \rightarrow \pi^*$ L_a	274	4.02	i.p., ca. long axis	Ethanol, 77°K	Song and Gordon (1970)
	$S_{4,5}$	$\pi \rightarrow \pi^*$ $B_{a,b}?$	238 210	Shoulder	i.p., short axis	Ethanol, 77°K	Moore <i>et al.</i> (1971)
Psoralen	S_1	$\pi \rightarrow \pi^*$ L_b			i.p. (along $C_3-C'_5$ axis)	PVA, 298°K	Minegishi <i>et al.</i> (1978)
	S_2	$n \rightarrow \pi^*$			o.p.?	Ethanol, 77°K	Moore <i>et al.</i> (1971)

S_3	$\pi \rightarrow \pi^*$ L_a	290	i.p. (nearly parallel to S_1)	Ethanol, 77°K	Song <i>et al.</i> (1981a)
S_4	$\pi \rightarrow \pi^*$ $B_a?$	250			Song <i>et al.</i> (1981a)
S_1	$\pi \rightarrow \pi^*$ L_b	(340) ^a 345	3.5 ^a	i.p. (nearly along short axis)	Song <i>et al.</i> (1981a)
S_2	$n \rightarrow \pi^*$		o.p.?	Ethanol, 77°K	Song <i>et al.</i> (1981a)
S_3	$\pi \rightarrow \pi^*$ L_a	300	4.1 ^a	i.p. (~20° to S_1)	Ethanol, 77°K
$S_{4,5}$	$\pi \rightarrow \pi^*$	263	Shoulder	i.p.	Ethanol, 77°K
	$B_{a,b}?$	252	i.p.	Ethanol, 77°K	Song <i>et al.</i> (1981a)

^a At room temperature (Sasaki *et al.*, 1977).

TABLE 8. The Fluorescence Characteristics of Coumarin and Psoralens

Compound	λ_F/nm	ϕ_F	τ_F/ns	Solvent	Temperature (K)	Reference
Coumarin	380	0.009	$\sim 0.01^a$	Ether	77	Mantulin and Song (1973)
	390			Ethanol	77	Mantulin and Song (1973)
Psoralen	409	0.019	1.8	Ethanol	77	Mantulin and Song (1973)
	440	0.013		Ethanol	77	Mantulin and Song (1973)
8-Methoxypsoralen	470			Ethanol	298	Ou <i>et al.</i> (1977); Poppe and Grossweiner (1975)

^a Calculated mean lifetime.

organisms. For example, psoralen plus near-UV radiation is responsible for skin erythema and phototherapy (vitiligo and psoriasis) in humans, mutagenesis and killing of yeast and bacterial cells (see recent review by Song and Tapley, 1979). The photobiological activation of psoralens can be correlated with their photoreactivity toward pyrimidine bases of DNA via cycloaddition, which results in interstrand crosslinking of the DNA duplex (see reviews by Song and Tapley, 1979; Hearst, 1981).

Due to its structural and spectral similarity with psoralen, coumarin serves as a useful model for elucidating spectroscopic characteristics of psoralen and its derivatives (Moore *et al.*, 1971; Mantulin and Song, 1973). The lowest singlet excited state of coumarin and psoralens is assigned to $^1\pi,\pi^*$ with the $^1n,\pi^*$ state lying slightly higher. Table 7 summarizes the absorption spectra of psoralens.

Low fluorescence polarization and short fluorescence lifetime (Table 8) at 77°K suggest that the $^1n,\pi^*$ state localized on the carbonyl group must be in the vicinity of the lowest $^1\pi,\pi^*$ state in coumarin and psoralens. The location of the $^1n,\pi^*$ state determines not only the relative populations (and lifetimes) of the singlet and triplet π,π^* states, but it also affects the photoreactivity of furocoumaryl compounds toward nucleic acid bases.

Since psoralen is isoelectronic with anthracene, it is reasonable to assign the first and second bands as L_b and L_a in origin (Table 7). The third band in psoralens, which likely contains contributions from a fourth transition, can then be assigned as B_a and/or B_b . It is clear that the first band arises from the coumarin moiety, since the hydrogenation at the pyrone 3,4-C=C bond results in a benzofuran-like spectrum (Song *et al.*, 1981a). The spectral genealogy of psoralens with coumarins is predictable in view of the fact that the first absorption band in both psoralen and coumarin is long-axis polarized (Moore *et al.*, 1971).‡

Psoralens fluoresce moderately (Table 8; Mantulin and Song, 1973). The fluorescence properties of psoralens are solvent dependent. For example, the fluorescence emission maximum and quantum yield of 8-methoxypsoralen depend strongly on the solvent dielectric constant (Sasaki *et al.*, 1977).

3. BLUE LIGHT PHOTORECEPTORS

Among the photobiological receptors reviewed in this chapter and elsewhere, the identities of the blue light photoreceptors are the least

‡ In agreement with the earlier work, the recent linear dichroic study of coumarin showed that the first absorption band is polarized along the long molecular axis (Minegishi *et al.*, 1978). The second and third $\pi \rightarrow \pi^*$ bands are polarized along the long and short molecular axes, respectively.

substantiated. For a review of the current status of blue light receptors, the reader is referred to the two most recent symposium volumes on the subject (Senger, 1980; Cossens and Prue, 1982).

Flavins and carotenoids are generally regarded as the most likely chromophores of the photoreceptors for a variety of blue light responses in both prokaryotic and eukaryotic organisms. This reviewer has questioned the likelihood of carotenoids as the primary photoreceptor chromophore on grounds of their photophysical and photochemical inadequacies (Song and Moore, 1974; Song, 1980). Nonetheless, we include these molecules for review in Section 3.2, as they likely function as secondary (antenna) photoreceptors in a number of blue light responsive systems.

3.1. Flavins and Flavoproteins

Flavins show three major absorption bands at about 450, 370 and 270 nm. These bands are attributable to $\pi \rightarrow \pi^*$ type transitions, although other types of transitions such as $n \rightarrow \pi^*$ may contribute to some of these band intensities either directly by overlapping band or via vibronic mixing between $n \rightarrow \pi^*$ and $\pi \rightarrow \pi^*$ transitions. Table 9 summarizes the absorption spectral properties of flavins.

Recently, the action spectra of blue light responses have been analyzed in detail in terms of quantum-chemically predicted $\pi \rightarrow \pi^*$ transitions, yielding an apparent agreement between the observed and calculated spectra of lumiflavin in the near-UV and blue regions (Nakano *et al.*, 1979, 1980). However, this analysis cannot be valid, since the theory erroneously assigns the blue absorption band at 450 nm to the second $\pi \rightarrow \pi^*$ ($S_0 \rightarrow S_2$) transition, their predicted $S_0 \rightarrow S_1$ transition being in the near-IR region. Other molecular orbital calculations have reasonably reproduced the observed $\pi \rightarrow \pi^*$ transition energies of flavins (Sun *et al.*, 1972; Grabe, 1972, 1974).

The polarized single-crystal absorption of flavin mononucleotide (FMN) in flavodoxin indicates that both S_1 and S_2 bands are polarized along the long molecular axis (Eaton *et al.*, 1975) making an angle of 20° between the two transition moments. Johansson *et al.* (1979) argue that this angle is in fact 29° (cf., Table 9). The Kronig-Kramers transformation of specular reflection spectra of crystalline bis(10-methyliso-alloxazine) copper (II) perchlorate tetrahydrate yields corresponding absorption spectra (Yu *et al.*, 1976). Again, S_1 ($f \sim 0.16$) and S_2 ($f \sim 0.15$) transitions are found to be polarized along the long molecular axis, with an angle of 25° between them. Considering this, agreement between the two sets of polarization data is satisfactory. Likewise, earlier theoretical transition

moment directions (Sun *et al.*, 1972; Grabe, 1972) calculated from Pariser-Parr-Pople (PPP) (S_1 approximately along C₈-N₃ and S_2 along C₇-C₂) are generally in good agreement with the above experiments. Polarization directions of S_1 and S_2 bands are not significantly altered by substituting N₅ with carbon, as in deazaflavin, yielding an angle of 25° (from fluorescence polarization) and 27° between the two transitions (from molecular orbital calculations; Sun and Song, 1973). For the tabulation of polarization angles between S_1 and S_n ($n = 2, 3$) transitions deduced from different techniques and workers, the reader is referred to Schmidt (1981).

Hydrogen bonding affects the absorption spectrum of a flavin (Nishimoto *et al.*, 1978). The first absorption band of riboflavin displays a blue shift upon initial H-bonding of trichloroacetic acid to the flavin nucleus, followed by a red shift with further H-bonding. The second absorption band shifts to red upon H-bonding. These effects have been used to probe the flavin microenvironment and the photoreactivity of flavoproteins (Yagi *et al.*, 1980).

Acidic media exert more drastic effects on the absorption spectra of flavins than does hydrogen bonding (Sun *et al.*, 1972; cf., Table 9). Thus, the two characteristic absorption bands of riboflavin at 445 and 375 nm collapse into one band at 399 nm in concentrated HCl solutions (Table 9) and 402 nm in phosphoric acid at room temperature (Sun *et al.*, 1972). The degrees of fluorescence excitation polarization of riboflavin in phosphoric acid are considerably lower than those of the neutral flavin in ethanol, but the long wavelength edge at $\lambda \geq 450$ nm approaches a theoretical maximum degree of 0.5. These results suggest two $\pi \rightarrow \pi^*$ transitions corresponding to the $S_0 \rightarrow S_1$ and $S_0 \rightarrow S_2$ bands, respectively, which make up the single, overlapped absorption band of maximum absorbance at 402 nm for the cationic flavin. The molecular orbital calculation of protonated riboflavin predicts $\pi \rightarrow \pi^*$ transitions at 457 nm ($f = 0.40$) roughly polarized along the long molecular axis (20° clockwise from the axis) and 410 nm ($f = 0.42$) polarized along the long axis (3° counterclockwise). Note that the 23° angle between the two transitions is only qualitatively consistent with the experimental angle of *ca.* 40° from the polarized fluorescence excitation spectrum (Sun *et al.*, 1972). The MCD and CD spectra of protonated flavins also suggest the presence of two transitions in the long-wavelength band (Tollin, 1968).

Substituents around the flavin nucleus have profound effects on the spectral shape of the characteristic absorption spectra of flavins (see reviews by Schmidt, 1980; Massey, 1980). Among the substituents, the 8-amino-substitution as roseoflavin (Kasai *et al.*, 1975) exhibits the most drastic effects including convergence of the blue and near-UV bands into a single absorption peak at 488 nm (in ethanol) or 500 nm (in water, pH

TABLE 9. The Electronic Transitions in Flavins

Flavin	Transition ($S_0 \rightarrow S_n$)	Notation	λ/nm	$\log \epsilon (f)$	Polarization ($\Delta\theta$)	Remarks	Reference
Riboflavin	S_1	$\pi \rightarrow \pi^*$ A'_1	445	4.07 (0.14) ^a	i.p. (58°) ^b	pH 5.1	Fasman (1976)
	S_2	$\pi \rightarrow \pi^*$ A'_2	375	4.0 (0.11) ^a	i.p. (97°) ^b	pH 5.1	Fasman (1976)
	$S_3(?)^a$	$\pi \rightarrow \pi^*$ A'_3	267	4.51 (0.43) ^a	i.p. (119°) ^b	pH 5.1	Fasman (1976)
	$S_4(?)^a$	$\pi \rightarrow \pi^*$ A'_4	223	4.48	i.p.	pH 5.1	Fasman (1976)
	S_1	$\pi \rightarrow \pi^*$ A'_1	470	(0.068)	i.p.	pH 7.0, Gaussian resolved	Tollin and Edmondson (1971)
			449 (~max)	(0.053)	i.p.	pH 7.0, Gaussian resolved	Tollin and Edmondson (1971)
			420	(0.080)	i.p.	pH 7.0, Gaussian resolved	Tollin and Edmondson (1971)
	S_2	$\pi \rightarrow \pi^*$ A'_2	381 (~max)	(0.073)	i.p.	pH 7.0, Gaussian resolved	Tollin and Edmondson (1971)
			359	(0.071)	i.p.	pH 7.0, Gaussian resolved	Tollin and Edmondson (1971)
			334	(0.003)	i.p.	pH 7.0, Gaussian resolved	Tollin and Edmondson (1971)
Riboflavin tetrabutyrate	S_1	$\pi \rightarrow \pi^*$ A'_1	443	404	i.p.	Cyclohexane-dioxane (20%)	Koziol (1966)
	S_2	$\pi \rightarrow \pi^*$ A'_2	336	3.91	i.p.	Cyclohexane-dioxane (20%)	Koziol (1966)
	$S_3(?)^a$	$\pi \rightarrow \pi^*$ A'_3	271	4.52	i.p.	Cyclohexane-dioxane (20%)	Koziol (1966)
	$S_4(?)^a$	$\pi \rightarrow \pi^*$	224	4.49	i.p.	Cyclohexane-dioxane (20%)	Koziol (1966)

Roseoflavin	S_1	$\pi \rightarrow \pi^*$ A'_1	505	4.49	i.p.	pH 6.99	Kasai <i>et al.</i> 1975
	S_2	$\pi \rightarrow \pi^*$ A'_2	407 (sh)		i.p.	pH 6.2	Song <i>et al.</i> (1980a)
	S_3	$\pi \rightarrow \pi^*$ $A'_3 (?)$	314	3.90	i.p.	pH 6.99	Kasai <i>et al.</i> (1975)
	S_4	$\pi \rightarrow \pi^*$ $A'_4 (?)$	275 (sh)	4.31	i.p.	pH 6.99	Kasai <i>et al.</i> (1975)
Riboflavin cation	$S_{1,2}^c$	$\pi \rightarrow \pi^*$ $A'_{1,2}$	399	4.32	i.p. ^c	12 M HCl	Knowles and Roe (1968); Fasman (1976)
	$S_3 (?)$	$\pi \rightarrow \pi^*$ A'_3	268	4.49	i.p. ^c	12 M HCl	Knowles and Roe (1968); Fasman (1976)
	$S_4 (?)$	$\pi \rightarrow \pi^*$ A'_4	222	4.33	i.p.	12 M HCl	Knowles and Roe (1968); Fasman (1976)
Riboflavin anion	S_1	$\pi \rightarrow \pi^*$ A'_1	451	4.06	i.p.	1 N NaOH	Knowles and Roe (1968); Fasman (1976)
	S_2	$\pi \rightarrow \pi^*$ A'_2	357	4.06	i.p.	1 N NaOH	Knowles and Roe (1968); Fasman (1976)
	$S_3 (?)$	$\pi \rightarrow \pi^*$ A'_3	271	4.56	i.p.	1 N NaOH	Knowles and Roe (1968); Fasman (1976)
FMN	S_1	$\pi \rightarrow \pi^*$ A'_1	450	4.07	i.p. (57° or 75°) ^d	pH 7.0	Yagi (1956)
	S_2	$\pi \rightarrow \pi^*$ A'_2	375	4.02	i.p. (37° or 95°) ^d	pH 7.0	Yagi (1956)
	$S_3 (?)$	$\pi \rightarrow \pi^*$ A'_3	265	4.43	i.p. (119°) ^e	pH 7.0	Yagi (1956)

(continued)

TABLE 9. (Continued)

Flavin	Transition ($S_0 \rightarrow S_n$)	Notation	λ/nm	$\log \epsilon (f)$	Polarization ($\Delta\theta$)	Remarks	Reference
FMN (L-amino acid oxidase)	S_1	$\pi \rightarrow \pi^*$ A'_1	497	(0.044)	i.p.	pH 7.0, Gaussian resolved	Tollin and Edmondson (1971)
			465 (~max)	(0.065)	i.p.	pH 7.0, Gaussian resolved	Tollin and Edmondson (1971)
			438	(0.074)	i.p.	pH 7.0, Gaussian resolved	Tollin and Edmondson (1971)
			405	(0.008)	i.p.	pH 7.0, Gaussian resolved	Tollin and Edmondson (1971)
	S_2	$\pi \rightarrow \pi^*$ A'_2	391	(0.092)	i.p.	pH 7.0, Gaussian resolved	Tollin and Edmondson (1971)
FMN (glucose oxidase)	S_1	$\pi \rightarrow \pi^*$ A'_1	362 (~max)	(0.108)	i.p.	pH 7.0, Gaussian resolved	Tollin and Edmondson (1971)
			481	(0.064)	i.p.	pH 7.0, Gaussian resolved	Tollin and Edmondson (1971)
			451 (~max)	(0.079)	i.p.	pH 7.0, Gaussian resolved	Tollin and Edmondson (1971)
			424	(0.090)	i.p.	pH 7.0, Gaussian resolved	Tollin and Edmondson (1971)
			387	(0.027)	i.p.	pH 7.0, Gaussian resolved	Tollin and Edmondson (1971)
			361 (~max)	(0.076)	i.p.	pH 7.0, Gaussian resolved	Tollin and Edmondson (1971)
			340	(0.149)	i.p.	pH 7.0, Gaussian resolved	Tollin and Edmondson (1971)

FAD	S_1	$\pi \rightarrow \pi^*$ A'_1	450	4.05	i.p.	pH 7.0	Yagi (1956)
	S_2	$\pi \rightarrow \pi^*$ A'_2	375	3.97	i.p.	pH 7.0	Yagi (1956)
	$S_3(?)$	$\pi \rightarrow \pi^*$ A'_3	263	4.43 ^f	i.p.	pH 7.0	Yagi (1956)
FAD (D-amino acid oxidase)	S_1	$\pi \rightarrow \pi^*$ A'_1	269 ^g	4.37 (0.40) ^g	i.p.	pH 7.0	Miles and Urry (1968)
			485.0 (sh)	3.89 (0.045)	i.p.	pH 8.3, Gaussian resolved	Shiga and Shiga (1973)
			460.5 (~max)	3.95 (0.047)	i.p.	pH 8.3, Gaussian resolved	Shiga and Shiga (1973)
			439.5 (sh)	3.92 (0.051)	i.p.	pH 8.3, Gaussian resolved	Shiga and Shiga (1973)
			416.5 (sh)	3.77 (0.056)	i.p.	pH 8.3, Gaussian resolved	Shiga and Shiga (1973)
	S_2	$\pi \rightarrow \pi^*$ A'_2	386.0 (~max)	3.96 (0.064)	i.p.	pH 8.3, Gaussian resolved	Shiga and Shiga (1973)
			363.0 (sh)	3.92 (0.061)	i.p.	pH 8.3, Gaussian resolved	Shiga and Shiga (1973)
			343.5 (sh)	3.79 (0.045)	i.p.	pH 8.3, Gaussian resolved	Shiga and Shiga (1973)

^a For 3,7,8-trimethyl-10-(*n*-octadecyl)-isoalloxazine; Eweg *et al.* (1982). It is possible that the S_3 and S_1 bands may actually represent higher transitions, as there are hidden transitions corresponding to $n \rightarrow \pi^*$ (360–370 nm) and $\pi \rightarrow \pi^*$ (\sim 300 nm) (Sun *et al.*, 1972; Song *et al.*, 1972a).

^b For lumiflavin; measured clockwise relative to the N_5-N_{10} axis (Johansson *et al.*, 1979).

^c See Sun *et al.* (1972) for the overlap of the two bands (S_1 and S_2) in acidic media.

^d In flavodoxin. For the choice of either of the two values given, see Eaton *et al.* (1975) and Johansson *et al.* (1979). The latter choose 57° and 95° for the S_1 and S_2 transitions, respectively.

^e Johansson *et al.* (1979).

^f After correction for absorption by the adenine moiety.

^g Gaussian-resolved from CD (Miles and Urry, 1968).

6.2). 8-Hydroxyriboflavin also shows a single peak absorption in the blue region (Massey, 1980). These substituent effects are similar to the protonation effect, except the blue absorption band has been red-shifted.

The molecular orbital calculation of roseoflavin predicts that the first $\pi \rightarrow \pi^*$ transition is polarized approximately along the C₄–C₉ axis, due to a strong charge transfer perturbation by the 8-amino group (Song *et al.*, 1980a). A weak, second transition hidden under the short wavelength side or the blue absorption maximum is polarized roughly along the C₂–C₇ axis, making an angle of 33° between the two transitions, which is in reasonable agreement with the photoselection-deduced angle of *ca.* 25° between the S₁ (492 nm) and S₂ (407 nm) transition moments.

Environmental effects on the flavin absorption spectra are also well known. For example, both low temperature and nonpolar solvents enhance the vibrational resolution of the blue absorption band and alter the extinction ratio of the blue to near-UV absorption bands (e.g. Koziol, 1966; Sun *et al.*, 1972). Riboflavin tetrabutyrate in CCl₄ shows absorption at 472 nm (shoulder: $\epsilon = 9.1 \times 10^3$), 447 nm (maximum: $\epsilon = 1.23 \times 10^4$) *ca.* 420 nm (shoulder: $\epsilon = 9 \times 10^3$) (Kotaki *et al.*, 1967).

The riboflavin adsorbed on dehydrated SiO₂ and in amorphous solid state show drastically different absorption spectra from those of flavins in solution (Lyalin *et al.*, 1973; Schmidt, 1982). In both cases, the near-UV band is obscured and the blue band is red-shifted. These examples illustrate how sensitive the absorption spectral shape of flavin is with respect to substitution and environment around the flavin nucleus. Thus, caution should be exercised in identifying the photoreceptors for blue light effects as either flavin or carotenoid (Song and Moore, 1974). In this regard, the binding effects of flavins to protein on the absorption spectra of flavins have been reviewed (Song *et al.*, 1972b; Massey, 1980; Schmidt, 1981). Table 7 presents a detailed description of the effect of the binding of flavin adenine dinucleotide (FAD) to D-amino acid oxidase in terms of spectral resolution of the absorption and CD spectra (Shiga and Shiga, 1973). Earlier, Tollin and Edmondson (1971) reported the spectral solution of several free and protein-bound flavins (Table 7).

The location and intensity of n → π* transitions in flavins remain undetermined mainly due to the strong, overlapping π → π* bands and possible vibronic mixing with π, π* states. Theoretically, n → π* transitions are localized on the C₂=O and C₄=O carbonyl group and N₅ nitrogen of the flavin nucleus.† It has been suggested that n → π* transitions are located under the near-UV band of flavins (Miles and Urry, 1968; Song, 1969), but CD spectroscopy of free and protein-bound flavins has not resolved the n → π* transition (Tollin and Edmondson, 1971).

† The n → π* transition localized on N₁ is predicted to be of higher energy transition.

On the basis of the red shift in nonpolar solvents, the shoulder on the long wavelength side of the blue absorption band of flavin has sometimes been attributed to an $n \rightarrow \pi^*$ transition. This cannot be correct, however, since flavins are strongly fluorescent. The near-UV band shifts to the blue in nonpolar solvents, e.g., riboflavin absorption at 375 nm in water and 335 nm in carbon tetrachloride. This shift has been attributed to a change in the transition monopole at N₁₀ as the result of interactions involving N₁₀-side chain and the isoalloxazine nucleus, and not due to the presence of an $n \rightarrow \pi^*$ transition (Scolar-Nagelschneider and Hemmerich, 1972), in agreement with the conclusion reached earlier (Tollin and Edmondson, 1971).

The specular reflection spectrum of a flavin does not resolve an $n \rightarrow \pi^*$ transition, indicating that such a transition, if present, is too weak to be detectable (Yu *et al.*, 1976). It has been argued that the lowest $n \rightarrow \pi^*$ transition occurs in the near-UV region because the flavin tautomers, allooxazines, with their absorption maximum ($S_0 \rightarrow S_1$ band) at 380–390 nm are markedly less fluorescent than their parent, isoalloxazines. This argument is only qualitatively suggestive of an n, π^* state lying slightly above the fluorescent π, π^* state (Koziol, 1971; Sun *et al.*, 1972). In this argument, it is assumed that tautomers do not affect $n \rightarrow \pi^*$ energy, compared to $\pi \rightarrow \pi^*$ energy.

In 8-substituted 6,7-dimethylumazines, a forbidden transition is resolved at 320 nm (Harders *et al.*, 1974), but it is not certain whether this transition represents an $n \rightarrow \pi^*$ or a third $\pi \rightarrow \pi^*$ ($S_0 \rightarrow S_3$) transition (see footnote *a* in Table 7). In any case, it appears difficult to resolve an $n \rightarrow \pi^*$ band in flavins, as a "pure" $n \rightarrow \pi^*$ transition is not likely (Eweg *et al.*, 1982).

Flavins fluoresce strongly. Both the emission maximum and quantum yield are sensitive to solvent polarity (Koziol, 1966; Kotaki *et al.*, 1967; Yagi *et al.*, 1969; Kotaki and Yagi, 1970; Yagi *et al.*, 1972, 1980), temperature (Sun *et al.*, 1972; Schmidt, 1981), and pressure (Li *et al.*, 1976). Table 10 presents fluorescence data for selected flavins. The reader is referred to McCormick in Fasman (1976) for a more complete compilation of the fluorescence data on flavins.

Most flavoproteins are nonfluorescent. D-Amino acid oxidase and lipoamide dehydrogenase are two of the exceptions (Table 10). The lack of fluorescence in most flavoproteins is likely due to the static quenching of flavin at its binding site. However, in D-amino acid oxidase, both static and dynamic quenchings play important roles in the reduction of the fluorescence quantum yield of bound flavin relative to free FAD (Nakashima *et al.*, 1980). Free FAD in solution exists in both folded and unfolded forms between the flavin nucleus and adenine moiety (Spencer and Weber, 1972). In aqueous solutions, 18% of FAD takes the unfolded, open

TABLE 10. The Fluorescence Characteristics of Flavins

Flavin	λ_F/nm	Φ_F	τ_F/ns	Solvent	Temperature (K)	Reference
Riboflavin	531	0.234	5.2 ^a	pH 7	298	Koziol (1966)
	536	0.24	pH 7		298	Kotaki and Yagi (1970)
	523	0.32	5.7	EtOH	298	Fugate and Song (1976)
	540	0.172 ^b	3.7	HAc	298	Fugate and Song (1976)
	490	0.60 ^c		EtOH	77	Sun <i>et al.</i> (1972); Sun and Song (1973)
				CCl ₄	298	Kotaki and Yagi (1970)
Riboflavin tetrabutylrate	525	0.46		Dioxane	298	Kotaki and Yagi (1970)
	526	0.58		HAc	298	Kotaki and Yagi (1970)
	535	0.27		EtOH	298	Kotaki and Yagi (1970)
	534.5	0.38		pH 7	298	Song <i>et al.</i> (1980 ^a)
Roseoflavin	550	0.00032	4.70 ^d	pH 7	298	Weber and Teale (1957)
	530	0.25		pH 7	298	Weber and Teale (1957)
	530	0.02	2.82 ^d	pH 7	298	
FAD (D-amino acid oxidase)			2.83 ^e			
	520	<i>e</i>	0.04 (monomer)	pH 8.3	293	Nakashima <i>et al.</i> (1980)
			0.13 (dimer)			

FAD (lipoamide dehydrogenase)	515	2.6 ^f 3.5 2.9	pH 7 pH 7.2 pH 7.6	298 298 295	Fukuzawa and Aki (1979); Song (1980 ^c); Veeger <i>et al.</i> (1976)
FMN (lactate oxidase)	507	0.04	pH 7	298	Ghislia <i>et al.</i> (1975)
FMN, reduced (lactate oxidase)	476	0.21	pH 7	298	Ghislia <i>et al.</i> (1975)
FMN (flavodoxin)	530	0.004	pH 7	298	Ghislia <i>et al.</i> (1975)
FAD, reduced ^h (D-amino acid oxidase)	520	0.019	pH 7	298	Ghislia <i>et al.</i> (1975)
FAD, reduced (L-amino acid oxidase)	520	0.02	pH 7	295	Veeger <i>et al.</i> (1980)
		2.05 ^e 1.3 ^{g,i} 5.4	pH 7	298	Ghislia <i>et al.</i> (1975)
		4.3	pH 7	295	Ghislia <i>et al.</i> (1975)
		1.33 ^e	pH 7	295	Veeger <i>et al.</i> (1980)

^a Vierstra *et al.* (1981).^b Koziol (1966).^c Uncorrected for refractive index of ethanol at 77°K.^d At 293°K, pH 6.9 (Wahl *et al.*, 1974).^e Nakashima *et al.* (1980). The fluorescence quantum yield depends on enzyme monomer \leftrightarrow dimer and free \leftrightarrow bound FAD equilibria.^f For resolution of the fluorescence lifetime into two components (0.49 and 2.03 ns), see Visser and van Hoek (1979).^g Non-exponential; Visser *et al.* (1979). Also, see Veeger *et al.* (1980).^h C₄₀-adduct.ⁱ Borohydride-reduced (Visser *et al.*, 1975).

form, which is more fluorescent than the folded form. The fluorescence of plasma membrane-bound flavin isolated from phototropically active corn coleoptile tips exhibits relatively short lifetimes due to dynamic quenching (Song *et al.*, 1980*b*; Sarkar *et al.*, 1982).

Reduced, non-planar flavins lose characteristic flavin absorption maximum in the blue region ($\epsilon \sim 2000$ at 400 nm), although it has been predicted that the planar form of reduced flavin will absorb at wavelengths longer than 450 nm (Song, 1969). This long wavelength (~ 450 nm) absorption depends on the planarity of the reduced flavin nucleus (Dudley *et al.*, 1964; Hemmerich and Haas, 1975).

The absorption spectrum of the reduced flavin of D-amino acid oxidase extends to above 500 nm (Visser *et al.*, 1979). The fluorescence polarization degree in the 350–430 nm region is constant and approximates the theoretical maximum, i.e., 0.46. Visser *et al.* (1979) assigned this region of absorption to $S_0 \rightarrow S_1$, whereas the 320 nm region with declining degrees of polarization (average ~ 0.32) was assigned to $S_0 \rightarrow S_2$. If these assignments are correct, both transitions are polarized roughly parallel to each other ($\sim 30^\circ$). On the other hand, the molecular orbital calculation of a fully planar, reduced flavin predicts that the $S_0 \rightarrow S_1$ and $S_0 \rightarrow S_2$ transitions are polarized perpendicular to each other, with the former polarized along the short axis (Song, 1969). Clearly, spectroscopic assignment of reduced flavins requires more information on their conformation and the resolution of the broad absorption spectra of reduced flavins.

Protein binding is likely to induce planarity, thus enhancing the blue absorption of reduced flavins (Hemmerich and Haas, 1975). In fact, some of the protein-bound reduced flavins are also weakly fluorescent (Ghisla *et al.*, 1975; Visser *et al.*, 1979). Table 10 presents fluorescence data for several reduced flavoproteins. Finally, the high resolution fluorescence of flavins in vapor phase (Eweg *et al.*, 1980) and in Shpol'skii matrix (Platenkamp *et al.*, 1980) has been briefly reviewed by Müller (1981).

3.2. Carotenoids and Carotenoproteins

In contrast to cyclic polyenes, linear polyenes show strong absorption at the first absorption band, with considerably weaker absorption at shorter wavelengths. First, we shall review the spectroscopic notation using a "four-orbital" (or butadiene) model according to the traditional description of polyene absorption spectra.

For a polyene within a "four-orbital" framework (e.g., butadiene), the electronic transitions arise from electron configurations corresponding

to the change in orbital quantum number, $\Delta n = 1$ for $N(\text{HOMO}) \rightarrow (N + 1)$ (LEMO) and $\Delta n = 2$ for $(N - 1) \rightarrow (N + 1)$ and $N \rightarrow (N + 2)$ configurations.[‡] The degeneracy of the latter excitations (c.f., Coulson-Rushbrooke theorem) is lifted via the configuration interactions between them. It is well-known that the first transition, $A_g \rightarrow {}^1B_u$ (or $A \rightarrow {}^1B$ in Platt notation), is strongly electric dipole allowed with its polarization direction along the long molecular axis. Both oscillator strength ($f \sim 0.74$ for all-*trans* butadiene; ~ 3.4 for all-*trans* β -carotene) and λ_{\max} increase with conjugation. The latter converges to an asymptotic value of 610 nm for “infinitely” long polyenes as a result of C—C and C=C bond-order alternations which restrict the electron motion in a 1-dimensional periodic potential and/or π -electron correlation effects.

The $A_g \rightarrow {}^1A_g^+$ and $A_g \rightarrow {}^1A_g^-$ transitions are symmetry-forbidden, but the former can be readily observed even in *trans*-polyenes, and it becomes strongly allowed in *cis*-polyenes (“*cis* peak”) due to the loss of the center of symmetry. The doubly forbidden ($g \rightarrow g$, $- \rightarrow -$) $A_g \rightarrow {}^1A_g^-$ transition is mainly the result of a doubly excited configuration. In contrast to the 1B_u state, which is ionic in character, the ${}^1A_g^-$ excited state is covalent with a high degree of double bond character for the C—C single bond relative to the 1B_u or A_g ground state.

The long-held traditional assignment of polyene spectra described above has been questioned on both experimental and theoretical grounds in recent years (Hudson and Kohler, 1974). The new assignment puts the ${}^1A_g^-$ excited state below the strongly allowed 1B_u state. The lowering of the ${}^1A_g^-$ state arises from configuration interactions among singly and multiply excited configurations (Schulten and Karplus, 1972).

The low-lying ${}^1A_g^-$ state is consistent with the valence bond picture of polyenes, in contrast to the free electron and simple LCAO MO methods. The valence bond picture indicates that the ${}^1A_g^-$ state is lowered in energy, as the conjugation of polyenes is extended, and that the $A_g \rightarrow {}^1A_g^-$ transition may occur below the $A_g \rightarrow {}^1B_u$ transition (Hudson and Kohler, 1974).

The assignment of ${}^1A_g^-$ to the lowest excited ${}^1\pi$, π^* state has a profound implication in reinterpreting the spectroscopic and photochemical properties of linear polyenes. Recent results further confirm that the ${}^1A_g^-$ state is the lowest excited singlet state for polyenes of up to four conjugated double bonds (Granville *et al.*, 1979; Birks *et al.*, 1978; Fang *et al.*, 1978; Andrews and Hudson, 1979; Birge and Pierce, 1979; D’Amico *et al.*, 1980; Birge, 1981). However, the location of the ${}^1A_g^-$ state in longer

[‡] N is the number of conjugated double bonds. HOMO, highest occupied molecular orbital ($=N$); LEMO, lowest empty molecular orbital ($=N + 1$).

polyenes, including carotenoids, has not been resolved unambiguously, and consequently, its assignment remains to be established (Song, 1977b; Chae *et al.*, 1977; Hotchandani *et al.*, 1978).

The blue absorption band, 1B_u , in β -carotene and other carotenoids is predicted to be polarized along the long axis of the carotenoid conjugation. It has been shown that the 1B_u band of all-*trans* β -carotene is polarized at an angle of between 0 and 40° to the long molecular axis (Chapman *et al.*, 1967; Parkhurst and Anex, 1966). Linear dichroic results of carotenoids in stretched polyethylene films and in liquid crystals are interpretable in terms of the long molecular axis polarization of the main band (Moore and Song, 1974a; Chae *et al.*, 1977). The main absorption polarization in *cis*-carotenoids is also expected to be along the long molecular axis.

The nature of the weak absorption bands at λ shorter than λ_{\max} in carotenoids are not well established, except for the "c*is* peak" (e.g., 337 nm, 15,15'-*cis*- β -carotene in hexane at room temperature; 350 nm at 77°K), which becomes strongly allowed in *cis*-carotenoids. The *cis* peak can be safely assigned to the ${}^1A_g^+$ state (${}^1A \rightarrow {}^1C$ in Platt notation). The polarization of the *cis*-band has been deduced from linear dichroic spectroscopy of β -carotene and several other carotenals (Moore and Song, 1974a), and its polarization is perpendicular to the long molecular axis.

A new band has been observed for β -carotene film, 536 and 537 nm, for all-*trans* and 15,15'-*cis* isomers, respectively. On the basis of the resonance Raman excitation profile spectrum, Thrash *et al.* (1977) proposed that the $S_0 \rightarrow S_1$ transition in β -carotene is due to a transition to the ${}^1A_g^-$ state as the lowest state. However, the ${}^1A_g^-$ assignment for β -carotene cannot be viewed as definitively established until the possibility of impurities present in and produced during spectral measurements has been rigorously dealt with.

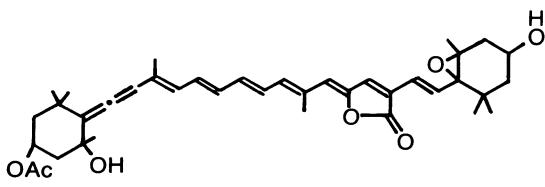
Carotenoids are generally nonfluorescent ($\phi_F \leq 10^{-6}$) even at low temperatures. Protein-bound carotenoids have also been investigated for their fluorescence emission, but none have been shown to fluoresce. However, energy transfer from carotenoids to chlorophyll has been demonstrated in a few cases of carotenoproteins, peridinin in the photoreceptor proteins (four peridinins and one chlorophyll *a* per protein) of dinoflagellates being the most clear-cut example (Song *et al.*, 1976, Koka and Song, 1977).

Peridinin shows the 0-0 band (1B_u) at about 506 nm, with three broad bands at 320, 295 and 240 nm. The bands at 320 and 295 nm develop vibrational resolution at 77°K, which is also found in other carbonyl-containing carotenals (Chae *et al.*, 1977). The "c*is*-band" transition moment ($S_0 \rightarrow S_2$; $A \rightarrow C$ in Platt notation) is predicted to be oriented 32°

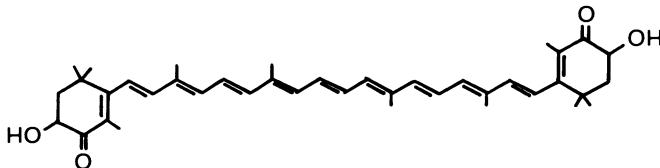
from the main transition moment [$S_0 \rightarrow S_1; A \rightarrow B (B_u)$] (Song and Lee, 1979).

The blue absorption moments of the four peridinin molecules in the peridinin-chlorophyll *a*-protein complexes of dinoflagellates are oriented about 45° with respect to the Q_y -transition moment direction of chlorophyll *a*.

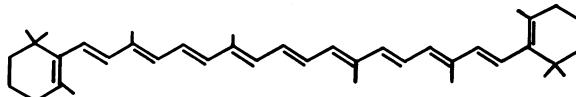
The spectral properties of astaxanthin in solution and in carotenoproteins (ovoverdin, ovorubin, and crustacyanins) have been studied by means of absorption and optical rotatory dispersion (ORD) (Buchwald and Jencks, 1968*a,b*), resonance Raman excitation profiles (Salares *et al.*, 1976, 1977, 1979; Clark *et al.*, 1980), and photoacoustic spectroscopy (Mackenthun *et al.*, 1979). Unlike β -carotene, which exhibits some vibrational fine structure over the blue absorption band even at room temperature, astaxanthin shows a broad $\lambda_{\text{max}} (^1B_u)$ at 488 nm in CHCl_3 at 296°K, apparently due to the effect of two conjugated carbonyl groups (Salares *et al.*, 1976).



Peridinin



Astaxanthin



All-trans β -carotene

Other carotenals and their Schiff's bases show similarly broad, structureless absorption bands at room temperature (e.g., Chae *et al.*, 1977;

Moore and Song, 1974b). However, astaxanthin develops its vibrational fine structure at low temperatures, exhibiting vibronic peaks at 463, 490, and 524 nm, and with the 0–0 band at 541 nm (Salares *et al.*, 1976), although the low temperature enhanced resolution of the absorption maximum is not as dramatic as in β -carotene. In the latter case, vibrational resolution is also accompanied by an overall shift of λ_{\max} , which is partly due to an increased effect of polarizability in the rigid matrix at 77°K, along with a conformational modification of the Franck–Condon envelope for the blue absorption band of β -carotene.

As demonstrated by the temperature effect on the absorption spectra of β -carotene, environmental factors exert strong spectral perturbations on carotenoids in general and astaxanthin in particular (Buchwald and Jencks, 1968a). Table 11 illustrates just one such example in terms of solvent effects on the values of λ_{\max} and ϵ_{\max} for astaxanthin. Thus, the strong dependence of the absorption spectra of carotenoids on environmental factors (e.g., binding site on proteins) presents an intrinsic difficulty in identifying the photoreceptor from the photobiological action spectrum in terms of carotenoid.

One of the strongest spectral perturbations in carotenoids is the effect of aggregation, as shown by the bleaching of the blue absorption band and the appearance of an intense near-UV band, such as astaxanthin in aqueous solution in the presence of sodium perchlorate and related salts (Buchwald and Jencks, 1968a) and β -carotene and lutein in ethanol-water mixtures (Song and Moore, 1974). These spectral perturbations can be explained in terms of a strong exciton interaction between the two stacked carotenoid molecules.

A similar exciton spectral shift from λ_{\max} 480 nm to 410 nm occurs upon the binding of astaxanthin to a lobster shell protein, yielding a yellow protein (Salares *et al.*, 1977). Salares *et al.* (1979) discussed three types of mechanisms for the spectral shift in carotenoproteins such as ovooverdin

TABLE 11. The λ_{\max} and Molar Extinction Coefficient of Astaxanthin in Different Solvents at Room Temperature^a

Solvent	λ_{\max} (nm)	$\epsilon_{\max}(M^{-1}cm^{-1}) \times 10^{-5}$	Refractive index
Methanol	473	1.23	1.329
Hexane	472	1.24	1.365
Benzene	488	1.13	1.501
Pyridine	492	1.12	1.509
Carbon disulfide	506	—	1.624

^a Buchwald and Jencks, 1968a.

and crustacyanins from the lobster *Homarus americanus*. The first type entails a large blue shift in the astaxanthin absorption band, as mentioned above. The exciton coupling between the two astaxanthin molecules on the yellow protein adequately accounts for the shift. The second type is given by the 460 nm absorption ($A \rightarrow B$ transition) maximum in ovooverdin. This absorption is due to the essentially unperturbed π -electron system of astaxanthin "frozen" in a hydrophobic binding site of the protein. The third type mechanism involves the large red shift of the astaxanthin absorption band found at 640 nm in ovooverdin and crustacyanins. The large red shift may be ascribed to a substantial twisting about a C=C bond in astaxanthin (Buchwald and Jencks, 1968b; Zagalsky, 1976). Lee *et al.* (1980) proposed an exciton model for α -crustacyanin on the assumption that each subunit protein contained two astaxanthin molecules (Buchwald and Jencks, 1968b; Zagalsky and Cheesman, 1963; Cheesman *et al.*, 1966). However, both the twisting model and exciton mechanism have been rejected on the grounds that the resonance Raman excitation profiles of crustacyanins yield a good correlation between the vibrational frequency ($\nu_{C=C}$) and $1/\lambda_{max}$, suggesting that the large red shift is possibly caused by greater electron delocalization in the ground state of astaxanthin ("polarization effect"). The latter is envisaged as being induced by the presence of proximal charge groups or hydrogen bonding. However, it does not seem likely that hydrogen bonding to the carbonyl oxygen of astaxanthin leads to such a large red shift in crustacyanins and ovooverdin. As to the presence of proximal charges, no structural evidence is currently available. There is indirect evidence that the astaxanthin binding site includes tryptophan residues. It has been proposed that the charge transfer interaction between the tryptophan residue(s) and astaxanthin contributes to the red shift of the absorption ($A \rightarrow B$) band (Lee *et al.*, 1980). Also, the effect of a C=C bond twisting cannot be ruled out as a contributory factor, in spite of the $\nu_{C=C}$ vs. $1/\lambda_{max}$ correlation (Clark *et al.*, 1980). Finally, no fluorescence from the carotenoid group of carotenoproteins has ever been observed and characterized.

3.3. Bilirubin

Bilirubin serves as a blue light receptor in the phototherapy of infant jaundice, in which the concentration of toxic bilirubin isomer in circulation is decreased (McDonagh *et al.*, 1980; Lamola *et al.*, 1981). The first transition in bilirubin is clearly of the $\pi \rightarrow \pi^*$ type, since its molar extinction coefficient is high (6.65×10^4 in $CHCl_3$). It is probably polarized along the long axis of the dipyrryl methene group (Blauer and Wagniere,

1975). The $n \rightarrow \pi^*$ transition is predicted at 320 nm by the CNDO calculation of the same authors, but its location has not been resolved experimentally.

Bilirubin is unusual in that only one major absorption band in the blue region constitutes its UV-visible absorption spectrum. The blue absorption maximum and its intensity (ϵ) are sensitive to solvent polarity (Holzwarth *et al.*, 1980). The natural isomer (4Z,15Z) shows a λ_{max} at 438 nm ($\epsilon = 47,200$) in phosphate buffer, pH 7.4 (Lamola *et al.*, 1979a), whereas the absorption maximum shifts to 450 nm in chloroform with an increase in the molar extinction coefficient. Upon binding to human serum albumin, bilirubin absorbs maximally at 460 nm ($48,500 M^{-1} \text{ cm}^{-1}$; pH 7.4; Lamola *et al.*, 1979a). The photoisomerization of the Z,Z isomer to "photobilirubin" isomers (4Z,15E; 4E,15Z) is accompanied by some changes in λ_{max} and ϵ_{max} , but the most significant change occurs in the CD spectra of these isomers (Lamola *et al.*, 1981).

The CD spectrum of bilirubin bound to human serum albumin exhibits a conservative split spectrum with the positive band centered at 462 nm and the negative band at 410 nm (Lamola *et al.*, 1981). The origin of the split CD band is not known. It may be speculated that the split CD bands arise from an exciton coupling between the two dipyrryl methenes, resulting in an exciton split of *ca.* 2750 cm^{-1} ; the fact that the exciton split calculated from the CD split is smaller than the absorption half-bandwidth (4500 cm^{-1}) is qualitatively consistent with the exciton model. The exciton model can also explain spectral changes, particularly with respect to the observed difference spectra in the 410- and 495-nm regions, upon photoisomerization of bilirubin to its photoisomers.

Although bilirubin (dimethyl ester) is a ready fluorescer at 77°K ($\phi_F = 0.31$ in ethanol), its fluorescence quantum yield at room temperature is weak (7.6×10^{-4} ; Holzwarth *et al.*, 1980), partly due to efficient photoisomerization from the singlet excited state. However, bilirubin becomes a stronger fluorescer at room temperature upon binding to human serum albumin ($\phi_F 1.6 \times 10^{-3}$; Lamola *et al.*, 1979b). Photoisomers are even less fluorescent than bilirubin. The fluorescence lifetime of bilirubin dimethyl ester is 1.9 ns in ethanol at 77°K (Holzwarth *et al.*, 1980). The fluorescence lifetimes of this compound in other solvents are non-exponential, due to the existence of two isomeric forms of the solute.

4. RED LIGHT PHOTORECEPTORS

Red light responses exhibited in the form of photosynthesis and photomorphogenesis are universal in the plant kingdom. In this section, we

review the spectroscopy of stentorin, chlorophylls, and phytochrome. Only a passing remark will be made concerning the spectroscopy of the photosynthetic accessory pigment proteins, phycobilins (see review by Scheer, 1981). Both phytochrome and phycobilin chromophores of bili-proteins contain conjugated, open tetrapyrrole chromophores, in contrast to bilirubin (Section 3.3) which is a nonconjugated tetrapyrrole. The absorption spectral characteristics of tetrapyrroles are correlated to the porphyrin origin, which is briefly described in this review. It has been suggested that porphyrins and/or hemes may serve as photosensory receptors (Hong *et al.*, 1981). In addition, the spectroscopy of stentorin is reviewed here, as this photoreceptor elicits the photoresponsive behaviors of *Stentor coeruleus* when stimulated with red light.

4.1. Stentorin

The absorption spectra of hypericin and stentorin are virtually identical, except for the red shift of a few nanometers of the red absorption maximum of the latter (586 *vs.* 593 nm). The blue absorption maximum occurs at 465 nm. The molecular orbital calculations of hypericin (assumed to be coplanar) yield the spectral shape, in terms of relative oscillator strength distribution over the 230–420 nm region, in agreement with the absorption spectrum. However, the calculated transition energies are higher than the absorption band maxima by more than 100 nm. For example, the lowest $\pi \rightarrow \pi^*$ transition is calculated at 410 nm, whereas the observed maximum is at 586 nm in hypericin. This large discrepancy can be attributed to the possibility that the hypericin chromophore is severely distorted, as can be expected from repulsive steric crowding between the pairs of neighboring hydroxyl and methyl groups (see structure in Fig. 3; Walker *et al.*, 1979; Song, 1981b).

The red absorption band of hypericin and stentorin at 586 and 593 nm is attributable to the first $\pi \rightarrow \pi^*$ transition, which is polarized along the short axis (*x*-polarized; Fig. 3). The polarized excitation spectra of hypericin and stentorin are virtually identical (Walker *et al.*, 1979). The second $\pi \rightarrow \pi^*$ transition ($S_0 \rightarrow S_2$; *y*-polarized) occurs at *ca.* 465 nm and is polarized perpendicular to the first $\pi \rightarrow \pi^*$ transition ($S_0 \rightarrow S_1$), since the fluorescence excitation polarization of the blue absorption band approaches a theoretical minimum value of $-1/3$. Molecular orbital calculations are consistent with the mutually perpendicular polarization directions of the first and second $\pi \rightarrow \pi^*$ transitions (Fig. 3).

The fluorescence quantum yields of hypericin and stentorin at room temperature are shown in Table 12. The fluorescence quantum yield of

TABLE 12. The Fluorescence Characteristics of Hypericin and Stentorin

Compound	λ_F/nm	Φ_F	τ_F/ns	Solvent	Temperature (K)	Reference
Hypericin	612	0.27 ± 0.02	5.24	EtOH	295	Walker (1980)
	596		6.05	EtOH	77	Walker (1980)
Stentorin	615	0.23 ± 0.02	5.35	EtOH	291	Walker (1980)
	~610	~0.004	1.35	Pi buffer, pH 7.4	291	Song <i>et al.</i> (1981b)
Hypericin anion	664		1.15	Pi buffer, pH 10	291	Walker (1980)
	660		4.96	Pi buffer, pH 7.4	291	Song <i>et al.</i> (1981b)

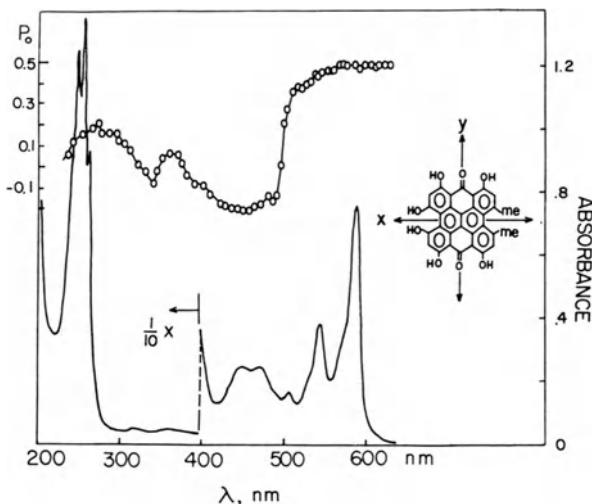


Fig. 3. The room temperature absorption spectrum (solid line) and fluorescence polarization spectrum at 77°K (open circles) of hypericin in ethanol. Emission wavelength was set at the fluorescence emission maximum. The inset shows the hypericin molecule with the polarization axis for the first (*x*-polarized) and second (*y*-polarized) $\pi \rightarrow \pi^*$ transitions. The absorption and fluorescence polarization spectra of stentorin are similar to those of hypericin (Song, 1981a).

hypericin is considerably lower in water than in organic solvents, probably due to aggregation (Giese, 1980). At 77°K, a sharp fluorescence emission peak occurs at 596 nm, which shifts to 612 nm with a shoulder at 664 nm at room temperature. The fluorescence emission spectrum is approximately the mirror image of the red absorption band. The fluorescence quantum yield of stentorin in ethanol is similar to that of hypericin (Table 12), and the fluorescence lifetimes of both pigments are also similar, suggesting that the chromophore in stentorin is not significantly altered. Walker (1980) discussed evidence for the binding of the hypericin chromophore to protein in stentorin.

4.2. Porphyrins and Chlorophylls

The π -electron network of porphyrin and its hydrogenated derivatives (e.g., chlorins and chlorophylls) can be treated as cyclic polyenes. The absorption spectra of these compounds are, therefore, characterized by red and near-UV (Soret) absorption bands in the regions of 550–800 and 350–450 nm, respectively. The latter are considerably more intense

($\epsilon \sim 1-3 \times 10^5$, $f \sim 1-2$) than the former ($\epsilon \sim 1-8 \times 10^4$, $f \sim 0.01-0.3$), in contrast to the well-known spectral features of linear polyenes such as carotenoids, i.e., an intense visible band with much weaker UV- or near-UV-bands.

The cyclic polyenes can be qualitatively described by their combined, free electron and four-orbital configuration interactions (see Song, 1981*a*, for review). According to this model, the red absorption bands are weaker than the Soret bands because the former arise from "forbidden" or partially forbidden transitions involving greater changes in orbital angular momentum (e.g., $\Delta q = \pm 9$ for porphyrins and chlorophylls), while the Soret bands arise from transitions involving $\Delta q = \pm 1$. The validity of the four-orbital model has recently been supported by *ab initio* configuration-interaction calculations (Petke *et al.*, 1979).

Figure 4 shows four possible excited state configurations in por-

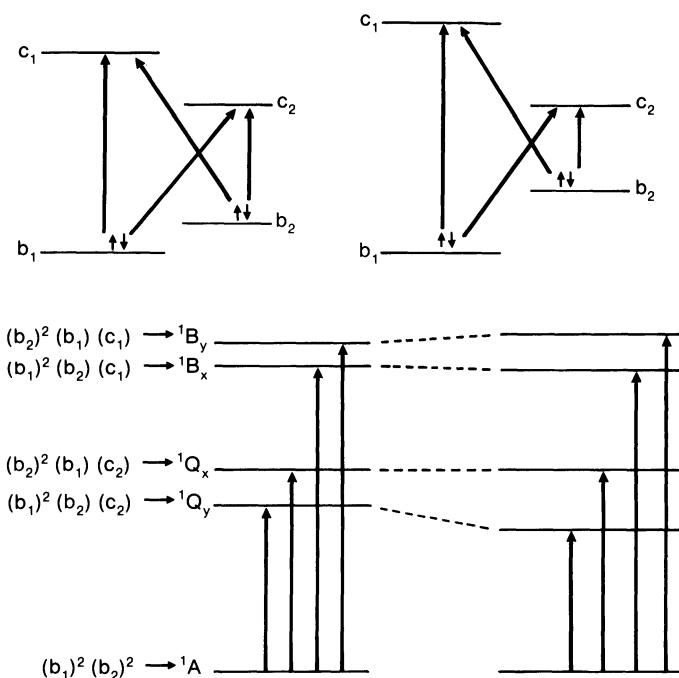


Fig. 4. A qualitative description of orbital energy levels (upper) and spectroscopic states (lower) for chl *a* (left panel) and Bchl *a* (right panel). Energy levels are in arbitrary scale. See Weiss (1972) for quantitative energy level diagrams. Major contributing configuration to each state is indicated at the far left.

phyrin. The configurations arising from cross electron promotions, i.e., $b_1 \rightarrow c_2$ and $b_2 \rightarrow c_1$, involve $\Delta q = \pm 9$. The resulting transitions, $A \rightarrow ^1Q_x$ and $A \rightarrow ^1Q_y$, usually occur in the visible region.

The degenerate Q and B bands split into Q_x , Q_y , and B_x, B_y , respectively, (Platt notation adopted here), as the symmetry of the metalloporphyrin-like system descends from D_{4h} to D_{2h} (e.g., free base porphyrin and bacteriochlorin) and C_{2v} (e.g., chlorin and pheophytin). In addition to the four state ($Q_{x,y}$ and $B_{x,y}$) absorption spectrum, one prominent vibronic satellite appears for each Q_x 0–0 and Q_y 0–0 origin, thus producing the well-known visible bands I (Q_x 0–0), II (Q_x 1–0), III (Q_y 0–0), and IV (Q_y 1–0) for free base porphyrins. Similarly, four bands (I-IV) appear in chlorins and bacteriochlorins, except that bands I and III are designated Q_y 0–0 and Q_x 0–0, respectively; x and y axes of polarization contain N-N and NH-NH directions, respectively.

The absorption spectra of chlorophylls can be approximately treated by the four-orbital model for porphyrins. For example, protochlorophyll *a* exhibits absorption spectrum similar to porphyrins.

The spectroscopy of chlorophyll *a* (chl *a*) and bacteriochlorophyll *a* (Bchl *a*) is representative of photosynthetic pigments molecules. The absorption properties of other derivatives are listed in Table 13. The red absorption band maximum at 660 nm (band I: Q_y 0–0, $f \sim 0.151$ in solution; Shipman, 1977) is polarized along the y -axis (along the ring I-III axis). Various methods have been employed in the determination of the polarization axis of the Q_y band (Sauer, 1975; Thurnauer and Norris, 1977; Vermeglio *et al.*, 1978; Rafferty and Clayton, 1979; Shuvalov and Asadov, 1979; Boxer and Roelofs, 1979; Frank *et al.*, 1979). However, an unambiguous determination of the polarization direction is likely to come from the polarized absorption spectroscopy of pyrochlorophyllide-apomyoglobin crystals (Boxer *et al.*, 1982).

Band II of chl *a* is made up of two components, one at *ca.* 632 nm with a fluorescence polarization degree of *ca.* 0 and another at 613 nm with a positive fluorescence polarization degree of 0.28 in ethanol at 77°K (Koka and Song, 1977). These bands may be assigned to Q_y (1–0) and Q_y (2–0), respectively. If this assignment is correct, the 575 nm band with a polarization degree of 0.03 is likely to be Q_x (0–0) (band III). Magnetic circular dichroism results are consistent with this assignment (Houssier and Sauer, 1970).

The four-orbital model (Gouterman, 1978), *ab initio* (Petke *et al.*, 1979), and semiempirical molecular orbital (Weiss, 1972; Koka and Song, 1977) theories predict that the Q_x transition dipole is polarized along the x -axis (connecting rings II and IV) and is thus perpendicular to the Q_y polarization axis. However, minimum fluorescence polarization in the

TABLE 13. The Electronic Transitions in Chlorophylls

Chlorophyll	Transition ($S_0 \rightarrow S_n$)	Notation ^a	λ/nm	$\log \epsilon(f)$	Polarization	Remarks	Reference
Protochl <i>a</i>	S_1	Q_y	622	3.34	i.p., y-axis (?)	Mineral oil, RT	Houssier and Sauer (1969, 1970)
	S_2	Q_x	602	3	i.p., x-axis (?)	Mineral oil, RT	Houssier and Sauer (1969, 1970)
	S_3	B_x	~440 (sh)	~4 (sh)	i.p., x-axis (?)	Mineral oil, RT	Houssier and Sauer (1969, 1970)
	S_4	B_y	431	4.26	i.p., y-axis (?)	Mineral oil, RT	Houssier and Sauer (1969, 1970)
	Protopheophytin <i>a</i>	$Q_y(?)$	638		i.p., y-axis (?)	Mineral oil, RT	Houssier and Sauer (1969, 1970)
	chl <i>a</i>	Q_y	661	4.94 (0.124) ^b	~y-axis	Ether, RT	Clayton (1965) ^c
chl <i>a</i>	S_2	Q_x	575	3.84	~x-axis	Ether, RT	Clayton (1965) ^c
	S_3 , S_4	$B_{x,y}$	428	5.04	i.p.	Ether, RT	Clayton (1965) ^c
	S_1	Q_y	664	(0.124) ^b	i.p., ~y-axis	EtOH, 298	Koka and Song (1977)
	S_2	Q_x	579		i.p., ~x-axis	EtOH, 298	Koka and Song (1977)
Pheophytin <i>a</i>	S_3	B_x	431		i.p., ~x-axis (?)	EtOH, 298	Koka and Song (1977)
	S_4	B_y	414		i.p., ~y-axis (?)	EtOH, 298	Koka and Song (1977)
	S_1	Q_y	667	4.74	i.p., ~y-axis	Ether, RT	Morton (1975)
	S_2	Q_x	534 (?)	4.03	i.p., ~x-axis (?)	Ether, RT	Morton (1975)
chl <i>b</i>	$S_{3,4}$	$B_{x,y}$	409	5.05	i.p.	Ether, RT	Morton (1975)
	S_1	Q_y	667		i.p., ~y-axis	Castor oil, RT	Goedheer (1966)
	S_1	Q_y	642	4.72	i.p., ~y-axis	Ether, RT	Clayton (1965)
	S_2	Q_x	590(sh)	4	i.p., ~x-axis	Ether, RT	Clayton (1965)
$S_{3,4}$	$B_{x,y}$	453		5.17	i.p.	Ether, RT	Clayton (1965)

Pheophytin <i>b</i>	S_1	Q_y	655	4.56	i.p., \sim y-axis	Ether, RT	Morton (1975)
	S_2	Q_x	599 or 526	3.91 or 4.08	i.p., \sim x-axis (?)	Ether, RT	Morton (1975)
	$S_{3,4}$	$B_{x,y}$	434	5.27	i.p.	Ether, RT	Morton (1975)
chl <i>c</i> ^d	S_1	Q_y	628	5.27	i.p., \sim y-axis	Ether, RT	Morton (1975) ^c
	S_2	Q_x	580	4.25	i.p., \sim x-axis	Ether, RT	Morton (1975) ^c
	$S_{3,4}$	$B_{x,y}$	447	5.28	i.p.	Ether, RT	Morton (1975) ^c
chl <i>d</i>	S_1	Q_y	688	4.97	i.p., \sim y-axis	Ether, RT	Morton (1975) ^c
	S_2	Q_x	595	3.91	i.p., \sim x-axis	Ether, RT	Morton (1975) ^c
	S_3	B_x	459 (sh)	4.82	i.p., \sim x-axis	Ether, RT	Morton (1975) ^c
Bchl <i>a</i>	S_4	B_y	447	4.92	i.p., \sim y-axis	Ether, RT	Morton (1975) ^c
	S_1	Q_y	769	4.93	i.p., \sim y-axis	Ether, RT	Clayton (1965); ^c Morton (1975) ^c
	S_2	Q_x	573	4.29	i.p., \sim x-axis	Ether, RT	Clayton (1965); ^c Morton (1975) ^c
Bacteriopheophytin <i>a</i>	S_3	$B_x^e(?)$	392(sh)	4.65	i.p., \sim x-axis	Ether, RT	Clayton (1965); ^c Morton (1975) ^c
	S_4	B_y^e	357	4.84	i.p., \sim y-axis	Ether, RT	Clayton (1965); ^c Morton (1975) ^c
	S_1	Q_y	749	4.81	i.p., \sim y-axis	Ether, RT	Morton (1975) ^c
Bacteriopheophytin <i>a</i>	S_2	Q_x	526	4.44	i.p., \sim x-axis	Ether, RT	Morton (1975) ^c
	S_3	$B_x^e(?)$	384	4.78	i.p., \sim x-axis (?)	Ether, RT	Morton (1975) ^c
	S_4	B_y^e	358	5.04	i.p., \sim y-axis (?)	Ether, RT	Morton (1975) ^c

^a These assignments remain to be established.^b *f* value for Q_y (0-0) (Shipman, 1977).^c See therein for original references.^d See Jeffrey (1972) for chl *c*₁ and *c*₂.^e Alternatively, the S_3 band may be N_x (see text). If this is the case, B_x will be S_4 .

visible absorption band region of chl *a* is only about zero, suggesting that the Q_y and Q_x transition dipoles are not nearly perpendicular to each other. The fluorescence polarization spectra of chl *a* in solution and in protein (e.g., Song *et al.*, 1976; Koka and Song, 1977) are similar, although some of the polarization spectral resolution is lost in the latter. This observation suggests that the protein binding does not greatly affect the polarization directions of Q_y and Q_x bands in chl *a*.

The Soret band of chl *a* and its derivative is even more complex. The *ab initio* configuration-interaction calculation of ethyl chlorophyllide *a* predicts that the Soret band is composed of no less than ten $\pi \rightarrow \pi^*$ transitions, in contradiction to the four-orbital model; the latter yields two transitions, B_x and B_y (Petke *et al.*, 1979). In the case of ethyl pheophorbide *a*, an $n \rightarrow \pi^*$ transition is also predicted in the Soret region (Petke *et al.*, 1979). In chl *a*, $n \rightarrow \pi^*$ localized in ring IV is the lowest among possible $n \rightarrow \pi^*$ transitions (Boxer *et al.*, 1974).

Bacteriochlorophyll *a* absorbs at *ca.* 770 nm in organic solvents. The large red shift of the Q_y band in Bchl *a* relative to that in chl *a* can be qualitatively understood in terms of the four-orbital model as shown in Fig. 4. The Pariser-Parr-Pople molecular orbital calculations of Bchl *a* place Q_y at 718 nm and Q_x at 562 nm, with the former about nine times more intense than the latter (Chin, 1975), in reasonable agreement with the absorption spectrum of Bchl *a*. The polarization directions of Q_y and Q_x bands are predicted to be along the *y*- and *x*-axis, respectively. The Soret band in Bchl *a* is blue-shifted relative to that in chl *a*, as predicted by the molecular orbital calculation (Weiss, 1972; Chin, 1975). The blue shift is such that Weiss (1972) suggested that the N_x band is a major contributor to the Soret band. The weak UV bands of the chlorophylls are not accounted for in the four-orbital model, and they are poorly understood. Petke *et al.* (1979) have discussed various electronic transitions in the UV region within the framework of an *ab initio* molecular orbital theory.

The effect of Mg^{2+} ion in chlorin and bacteriochlorin macrocycles is slightly hypsochromic on the Q_y band. Weiss (1978) discussed the effects of various substituents, including the cyclopentanone ring, on the band shifts and intensities of the visible and Soret bands. A positive point charge placed near the chlorophyll macrocycle caused a small blue shift; it is possible that a negative charge shifts the Q_y band to the red (Davis *et al.*, 1981).

Porphyrin dimers and aggregates usually show a bathochromic shift of the visible band. The spectral shift of the dimers and aggregates of chlorophylls is also well-known. The most prominent type of interactions in these aggregates appears to be dimeric. Therefore, the red shift of the

Q_y band in chlorophylls has been described in terms of a dimeric exciton model. Dimeric and oligomeric interactions play an important role in determining the spectral properties of chlorophyll-protein complexes. For example, in Bchl *a* both the Q_y band maximum and its molar extinction coefficient dramatically increase upon the binding of Bchl *a* to its protein (Table 13). Recently, it has been proposed that the red shift of the Q_y band of chl *a* in protein is attributable to both dimeric exciton interactions and a p_σ -orbital overlap between the $2p_z$ orbitals of the vinyl group (ring I substituent) of one monomer and the ring III-cyclopentanone ring of the other monomer (Warshel, 1979). The red shift of the Q_y band in Bchl *a* has also been described in terms of exciton interactions (Pearlstein and Hemenger, 1978). However, the red shift of the Q_y band in chlorophyllide-apomyoglobin complexes is probably not due to exciton interactions (Pearlstein *et al.*, 1982).

Recently, Wasielewski *et al.* (1981) obtained evidence for the possible involvement of a monomeric chl *a* enol as the primary electron donor in photosystem I. "Trapped" enol and synthetic enol compounds exhibit a blue shift of the Q_y band. However, it is possible that the enol form may exist as an enolate anion or its ion pair on the protein, thus causing a red shift of the Q_y band (Chin, 1975).

In analyzing the 800-nm absorption band and circular dichroic spectra of the Bchl *a*-protein complex from *Prosthecochloris aestuarii*, it is necessary to assume that the lowest transition is *x*-polarized (along the axis connecting rings I and III; Pearlstein and Hemenger, 1978). However, this possible reordering of the Q_y and Q_x states of Bchl *a* in the protein is not unambiguously confirmed, as the magnetic circular dichroism of Bchl *a* in solution and in the protein are qualitatively similar (Sutherland and Olson, 1981). It is, however, interesting to note that the enolization of the cyclopentanone ring exerts a strong perturbation to the band position and polarization direction of the Q_y and Q_x bands in Bchl *a* (unpublished calculations). In free Bchl *a* in solution, these transitions are mutually perpendicularly polarized, consistent with predictions of the four-orbital and fluorescence polarization models (Goedheer, 1966; Ebrey and Clayton, 1969).

The mirror image relationship between the Q_y absorption and emission bands in porphyrins is not rigidly obeyed when examined closely. This is also true for chlorophylls. For example, the intensity of the fluorescence band II (shoulder appearing at wavelengths longer than the fluorescence maximum) which corresponds to the Q_y (0-1) emission is weaker than that of Q_y (1-0) absorption in protochlorophyll *a* and other chlorophylls, as expected from the vibronic theory (Perrin *et al.*, 1969). However, the single-site absorption spectroscopy of chl *a* and pheophytin

a in *n*-octane at 4.2°K dramatically illustrates the breakdown of mirror image symmetry (Platenkamp *et al.*, 1980). Nonetheless, at least an approximate mirror image relationship between the absorption and emission bands has been confirmed in several cases, e.g., chl *b*.

Bacteriochlorophyll *a* shows its fluorescence maximum at 802 nm in methanol. Table 14 presents data on the fluorescence properties of selected chlorophyll derivatives. It should be noted that the fluorescence quantum yield of chlorophylls varies with substitution including Mg²⁺ ion, solvent, temperature, and other variables. Chlorophyll dimers and oligomers are generally nonfluorescent or only weakly fluorescent.

4.3. Phytochrome and Phycocyanobilins

The absorption spectra of phytochrome and phycocyanobilins are qualitatively similar to those of porphyrins and chlorophylls with their characteristic visible and Soret bands, except that the Soret band intensity is significantly weaker than the corresponding Soret band in porphyrins and chlorophylls. Although the relative intensity of the Soret band in these tetrapyrrolic chromoproteins appears to be considerably lower than the Q_y band intensity, its integrated intensity, i.e., oscillator strength, is comparable to that of the Q_y band. In spite of the oversimplification of the π -electron network and the implied neglect of the noncoplanarity of the tetrapyrrole rings, the spectral analogy between porphyrins and bilinoids is a useful one (Song, 1977b; 1981b).

In this section, we focus our discussion on the spectroscopy of the two forms of phytochrome, Pr and Pfr, with only a passing remark on the spectroscopy of phycocyanobilins.

In analogy to the cyclic polyene systems, phytochrome (Pr form) absorbs strongly in the red region (667 nm). Recently, the molar extinction coefficient of the absorbance maximum has been revised by two independent methods, yielding 1.02×10^5 (per mol wt 120000, oat phytochrome; Roux *et al.*, 1982) and $1.09\text{--}1.18 \times 10^5 M^{-1}cm^{-1}$ (Brandlmeier *et al.*, 1981). Consequently, the molar extinction coefficient of the Pfr form of phytochrome has been revised; 4.35×10^4 at 724 nm (Roux *et al.*, 1982).

As mentioned earlier, the intensity ratios of visible to near-UV (Soret) bands in porphyrins and of the blue to UV (*cis*) bands are strongly dependent on the shape of the π -electron network. Thus, the intensity ratio is significantly less and significantly greater than unity for cyclic and linear polyenes, respectively. In the case of both forms of phytochrome, the oscillator strength ratio, $f_{Q_{x,y}}/f_{B_{x,y}}$, is approximately unity, which sug-

TABLE 14. The Fluorescence Characteristics of Chlorophylls

Chlorophyll	λ_F/nm	Φ_F	τ_F/ns	Solvent	Temperature (K)	Reference
Protochl <i>a</i>	626	6.2 ^a		Acetone	RT	Morton (1975) ^b
Protopheophytin <i>a</i>	626			Acetone	RT	Morton (1975) ^b
chl <i>a</i>	668	0.33	5.1	Ether	RT	Brody and Rabinowitch (1957); French <i>et al.</i> (1956)
	668	0.32	4.4 ^c	Ether	RT	Weber and Teale (1957)
	669	0.35	5.3 ^d	Acetone	RT	Wasielewski <i>et al.</i> (1981)
Pheophytin <i>a</i>	673	0.14	5.3 ^e	Ether	RT	Livingston (1960)
chl <i>b</i>	648	0.16	3.0 ^d	Ether	RT	Latimer <i>et al.</i> (1956)
		0.11	3.9	Ether	RT	Brody and Rabinowitch (1957)
		0.12	5.1 ^f	Ether	RT	Weber and Teale (1957)
		0.09	4.3 ^d	Acetone	RT	Weber and Teale (1957)
chl <i>c</i> ₁	633	0.16		Acetone	RT	Jeffrey (1972)
chl <i>c</i> ₂	635	0.15		Acetone	RT	Jeffrey (1972)
chl <i>d</i>	699			Ether	RT	French <i>et al.</i> (1956)
Bchl <i>a</i>	802		4.7 ^g	MeOH	RT	French <i>et al.</i> (1956)
Bacteriochlorophytin <i>a</i>	762			Ether	RT	Gaedheer (1966)
chl <i>a</i> (Chlorella)	~685	0.027	0.5~0.6		RT	Latimer <i>et al.</i> (1956); Brody and Rabinowitch (1957); Müller and Lumry (1965)
chl <i>a</i> (spinach)					RT	Murata <i>et al.</i> (1966)

^a Rubin *et al.* (1962).^b See therein for original reference.^c At 77°K., Butler and Norris (1963).^d Dmitrievsky *et al.* (1957).^e Fujimori and Livingston (1957).^f Brody and Rabinowitch (1957).^g Rubin and Osntsikaya (1963).^h Measured under conditions where no photochemistry occurs.

gests unique chromophore configurations, neither fully cyclic nor fully extended linear molecular structures (Song *et al.*, 1979; Song and Chae, 1979).

Fluorescence polarization yields an angle of *ca.* 50° between the polarization axes of the Q_y and $B_{x,y}$ bands, which agrees with the predicted angle of 56° (or 63°) between the Q_y and $S_0 \rightarrow S_5$ (or $S_0 \rightarrow S_4$) polarization axes for a semicircular chromophore conformation for Pr (Song and Chae, 1979; Kwak, 1981). In contrast, a cyclic conformation (as in porphyrin) adequately accounts for the spectrum of a synthetic Pr model in terms of the oscillator strength ratio (calc. 3.23 *vs.* exp. 4) and the polarization angle between the Q_y and B transitions (calc. 88°; obs. ~ 75°; Song and Chae, 1979). In the phototransformation of Pr to Pfr, a reorientation of the chromophore is proposed on the basis of a variety of probes (see Song, 1982, for review). Björn (1982, to be published) deduced a dichroic angle between the Pr and Pfr chromophore *in vivo*, which agrees with the above model.

The oscillator strength ratio for Pfr is also approximately unity, indicating that the chromophore conformation has not been altered substantially in the $\text{Pr} \rightarrow \text{Pfr}$ phototransformation. Ring A in Pr is "activated" in its 1Q_y state, as the excited state functions of Pr contain significant contributions from the local excitation and charge transfer character (Song *et al.*, 1977). Precise structural changes accompanying the $\text{Pr} \rightarrow \text{Pfr}$ phototransformation *in vivo* are not fully known at the present. However, any one of the proposed ionic and enolic structures of the chromophore (Lagarias and Rapoport, 1980; Song *et al.*, 1980b; Sarkar and Song, 1981) could account for the red shift of the Q_y band in the native Pfr form. In the case of denatured Pfr and its chromopeptide, other structures including conformational isomers (e.g., $Z \rightleftharpoons E$) are possible (Rüdiger, 1980; Thümmler *et al.*, 1981; Scheer, 1981). Kwak (1981) calculated the electronic spectra of several possible Pfr chromophores in the semicircular configuration. For example, enolic (ring A with proton shifted to ring C nitrogen) and tautomeric (biliverdin-like) structures yield a red shift of the Q_y band by 3–4 kcal, in agreement with the observed red shift of 3.9 kcal (from 660 nm for Pr to 725 nm for Pfr). However, these results should be regarded as entirely tentative until the chromophore structure of the native Pfr is experimentally determined.

The fluorescence spectra and temperature dependent quantum yield of phytochrome have been reported (Song *et al.*, 1973; Song *et al.*, 1975). The fluorescence lifetimes have also been measured (Song *et al.*, 1979). The fluorescence of Pr is substantially enhanced in D_2O over that in H_2O , provided the Pr form has been photocycled through the Pfr form in D_2O (Sarkar and Song, 1981).

The electronic spectra of phycocyanobilins can be treated as spectroscopic derivatives of the porphyrin spectra, in analogy to the description of the electronic spectra of phytochrome. However, the visible to near-UV band intensity ratio tends to be somewhat greater than that of phytochrome. Furthermore, these chromophores produce complex absorption spectra in their native proteins, due to subunit interactions. Scheer (1981) has recently reviewed the electronic spectroscopy of biliproteins and their chromophores. Of particular interest is phycocromes, the photosensory pigments in blue-green algae (Björn, 1979; Ohki and Fujita, 1979; Ohad *et al.*, 1979). Phycocromes exhibit photoreversible transformations similar to the phototransformation of phytochrome. The spectral properties of these photochromic biliproteins have been reviewed by Björn and Björn (1980).

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Photodynamic Agents as Tools for Cell Biology

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1. INTRODUCTION

A light quantum, upon absorption, can elevate the absorbing substance to an excited state and it, in turn, may affect a critical component of the cell, under certain conditions, by using this excitation energy. Such a substance can either be exogeneous, or a normal component of the cell, and is called a photosensitizer. The extent of the cellular effects produced by a photosensitizer is dependent upon a number of factors that include: (i) how easy the sensitizer is excited to a particular state by light of a given wavelength, (ii) how accessible the sensitizer is to the particular site for the relevant photobiological interaction in the cell, and (iii) the importance of the biological role played by the affected substrate in the cell. Contributions of all these factors must be properly worked out before any systematic view on biological photosensitization can be constructed.

Enough information about the first factor for many substances of biological importance can be obtained without much difficulty. However, we still have limited knowledge about the second and the third factors. There has been a rapid expansion of cellular research in the past several years, and this review summarizes the present status of biological photosensitization. It is based mainly on information obtained at the cellular level, particularly of bacteria, yeasts, and cultured mammalian cells. Hence, it remains largely a study of basic photobiology. Logically, however, it provides a major basis for phototherapeutic applications, for which empirical knowledge still predominates. The readers who wish to obtain a better background in this subject should consult the reports by Spikes and Livingston (1969) for basic chemical and biological aspects of photosensitization, Castellani (1977), Singh and Petkau (1978), and Rodgers and Powers (1981) for a broad coverage of recent photosensitization studies, Kittler and Löber (1977) for the photochemistry of nucleic acids, and Jung (1976) and Regan and Parrish (1982) for phototherapeutic aspects that are not dealt with in this review. Also, the reader may find useful articles in *Trends in Photobiology* (Hélène *et al.*, 1982) which is the proceedings of a recent international photobiology meeting.

2. FUNDAMENTALS OF CELL PHOTOSENSITIZATION

2.1. Basic Experimental Procedures

2.1.1. Two Principal Techniques

The general procedures for sensitizing cells in suspension are of two types. In the first type, the cells are incubated in the dark, with sensitizers

in an appropriate suspension medium, for a sufficient time for equilibrium to occur. The period of incubation may be variable, but it usually takes 30 min or longer at the physiological temperature. Then the system is irradiated with visible light or in some cases with near-UV radiation, depending on the absorption range of the sensitizers. After irradiation, the treated cells are plated for survival or tested for specific functions. The second type differs from the first in that excess sensitizers in the medium are removed by centrifugation or some other means after incubation, and before irradiation. This procedure is somewhat difficult to perform with cells that are sensitive to such operations.

2.1.2. General Location of Sensitizers

The period of incubation prior to irradiation is critical in evaluating the biological effects, because sufficient information on the cell-sensitizer interaction occurring in this period is often lacking (Ito, 1980). Certain types of sensitizers may remain outside of the cell even after long incubation times (e.g., 1 h). Another group of sensitizers may only be bound to the surface of the cell. Lipid soluble sensitizers are often incorporated into the hydrophobic region of the cell membrane (Wagner *et al.*, 1980, 1982; Pooler and Valenzeno, 1978, 1979a, 1979b; Valenzeno and Pooler, 1982). Certain types of sensitizers may get into the interior of the cell and be bound to various parts of the cell (Lochmann and Micheler, 1973; Ito, 1978).

Whether or not the free sensitizers in the medium are removed from the cell suspension before irradiation is also an important consideration for the sensitizer location. The presence of the light-absorbing sensitizer in the medium may shield the light coming to a particular cellular site. In addition, the initial distribution of the sensitizer (supposed to be in equilibrium) could change during prolonged irradiation due to the progression of the cellular damage, resulting in an uncalculable influence on the observed effects (Ito, 1977). On the other hand, removing the sensitizers is not necessarily a perfect condition, since it may affect the equilibrium distribution once attained in the presence of sensitizers.

2.1.3. Variations of Cell Sensitization

There are other variations of sensitizations of cells. The cells can be sensitized on nutrient agar plates or in liquid nutrient medium that contains sensitizers. The cells in suspension can also be sensitized by immobilized sensitizers, immobilized on plastic beads that have dimensions comparable to or larger than the cells (Bezman *et al.*, 1978; Hoober and

Franzi, 1980). Rose Bengal immobilized on porous polystyrene beads is commercially available. Rapid mixing of sensitizer solution and cell suspension has been used, in combination with a short period of illumination, to investigate transient aspects of the cell-sensitizer interaction (Ito, 1980). Of course, cells in tissue or in animals may be sensitized topically, orally, or by injection.

2.1.4. Irradiation of Sensitized Cells

Irradiation by visible light or by near-UV radiation may be performed using any lamp whose emission covers the desired range of wavelengths with a sufficient fluence rate. Often monochromatic radiation is advantageous over broad band radiation in permitting one to draw a simpler conclusion. Flashes and a short pulse of light have been used for particular types of experiments. A laser and, in particular, a tunable dye laser may find suitable applications (Cameron *et al.*, 1972; Anders *et al.*, 1976). A xenon arc lamp (e.g., 500 W) with a stabilized power supply is recommended as a standard light source for quantitative work in the visible region (e.g., Kobayashi and Ito, 1976). For a near-UV radiation source, the black light lamp is a standard type. More elaborate equipment is required for monochromatic near-UV radiation.

It is both convenient and accurate to use a standard spectrophotometry cuvette for irradiation, although the requirements by specific experiments may force one to choose other variations. The estimation of fluence is easier this way than when using a test tube or a container with a round surface. The fluence needed to kill the sensitized cells may vary widely depending on many factors, but the requirement of the order of 10^4 J/m^2 for the inactivation of a typical yeast cell sensitized by acridine orange (10^{-5} M) would give some idea. The deliberate bubbling with oxygen or air may be required for special situations, but ordinary aerobic experiments require only stirring during illumination under open air. The decrease of oxygen pressure in the suspension would be negligible under such conditions.

2.2. Photochemical Processes in Sensitized Molecules

The sensitizers may be bound to substrate molecules or may remain free in the bulk medium when they sensitize. The literature indicates that the binding or nonbinding could cause a substantial divergence in the photochemical processes to follow (Bellin, 1964, 1968; Youtsey and Grossweiner, 1967; Amagasa, 1981).

Our knowledge of photosensitization in the presence of molecular oxygen converges into two pathways called the Radical Mechanism and the Singlet Oxygen Mechanism (Bourdon and Schnuriger, 1967; Foote, 1976; Spikes and Straight, 1981), both being mediated by the triplet of sensitizers. This state has a number of advantageous properties over the excited singlet state for chemical reactions (Turro, 1969).

Photosensitization processes occurring in a deoxygenated reaction system have less relevance to the present purpose. Therefore, it is only touched on occasionally, although it provides useful and often indispensable information in the mechanistic analysis.

2.2.1. Radical Mechanism

Elevated to the first excited singlet state by the absorption of photons, the sensitizer molecules may undergo intersystem crossing with a given probability, reaching the triplet state, which has a relatively long lifetime. In the radical mechanism, the sensitizer may be reduced via a hydrogen or an electron transfer reaching a semireduced state, leaving the reacted substrate as radicals or radical ions. The resulting radicals undergo a wide variety of reactions such as a reaction with oxygen and other accessible substrates, or the initiation of chain reactions. The products include hydroperoxides, alcohols, ketones, epoxides, etc. Appropriate radical scavengers and electron acceptors are useful for diagnosis. Considerations of the redox potentials of sensitizer and substrate molecules can be a useful guide to understand the photodynamic oxidation reaction through the radical mechanism (Grossweiner and Kepka, 1972; Kittler and Löber, 1977; Kittler *et al.*, 1980).

2.2.2. Singlet Oxygen Mechanism

In this mechanism, the triplet state of the sensitizer is formed as above, and then reacts with molecular oxygen to generate excited singlet oxygen (${}^1\Delta_g \text{O}_2$) (Kearns and Khan, 1969; Kearns, 1971; Wilson and Hastings, 1970). This concept originated in the work of Kautsky (1937, 1939), and was later substantiated. The basic condition for this process to occur is that the oxygen must be accessible in competition with the substrate in the reaction mixture (Foote, 1968, 1976). Of course there are a number of factors that influence the rate of this process. It apparently depends on the photophysical properties of sensitizers and other environmental conditions such as pH (Bonneau *et al.*, 1975; Pottier *et al.*, 1975).

Because of the low energy level of singlet molecular oxygen ($\Delta E \cong 1 \text{ eV}$), many sensitizers in their triplet state have sufficient energy to

convert ground-state oxygen to the excited state. Once generated, singlet oxygen may undergo oxygenation reactions with the substrate molecules which, in particular cases, gives a specific product distinguishable from radical-induced products (Kulig and Smith, 1973; Smith and Stroud, 1978, and therein). It is important to note that since singlet oxygen is now the active intermediate, the sensitizer-substrate specificity is no longer critical as in the radical mechanism. Rather, the reactivity of the substrate with the excited state of oxygen is to be considered (Ito, 1978). The importance of the singlet oxygen mechanism has gained increasing credits in biological photosensitizations since Foote (1968) brought it to the wide attention of photobiologists. A recent dramatic surge of the singlet oxygen mechanism is due to two important discoveries. Merkel *et al.* (1972) found a large solvent effect on singlet oxygen, e.g., the lifetime of singlet oxygen is ten times longer in D₂O than in H₂O, and Hasty *et al.* (1972) found that azide ion (N₃⁻) quenches singlet oxygen with a high specificity. The enhancement effect of D₂O and the protective effect of N₃⁻ not only find valuable applications in photochemistry, but they are also important diagnostic aids in cellular systems (Krinsky, 1977; Ito, 1978). The precautions for applying these and other diagnostic aids are also discussed in a number of papers (e.g., Krinsky, 1979). A monograph by Wasserman and Murray (1979) may be very useful for the chemical and biochemical aspects of the reactions of singlet oxygen.

2.3. Comments on Cell Photosensitization

2.3.1. Extrapolation from a Homogeneous Solution System

Whatever the primary short-lived product of photosensitization may be, it may undergo further changes. The degradation of guanine residues in nucleic acid, or the inactivation of enzyme function can be easily measured in a homogeneous solution system. One can even estimate which of the above-mentioned two mechanisms operates by a variety of means. Many studies have appeared since 1972, and the references may be found in a number of reviews already mentioned. Despite all of this knowledge of homogeneous systems, recent research at the cellular level reveals more and more uncertainty about what is going on in the cell system, since greater degrees of heterogeneity prevent effective analyses.

The use of micellar systems was introduced in order to not only mimic the biological membranous structures, but also to provide a model of heterogeneous systems. Such structures are not new in radiation chemical studies (e.g., Wallace and Thomas, 1973). Recent photochemical studies with aqueous micellar dispersions have been providing, as would be an-

ticipated, necessary information for linking solution photochemistry and events in complex biological systems (Bagno *et al.*, 1979; Kraljic *et al.*, 1979; Gorman *et al.*, 1976; Gorman and Rodgers, 1978; Lindig and Rodgers, 1979; Lindig and Rodgers, 1981; Miyoshi and Tomita, 1979; Sconfienza *et al.*, 1980; Rossi *et al.*, 1981; Usui *et al.*, 1978, for singlet oxygen-related studies; Sconfienza *et al.*, 1980; Patterson and Grätzel, 1975; Grätzel *et al.*, 1975; Almgren and Thomas, 1980; Rossi *et al.*, 1981, for photoinduced electron transfer with charged micellar systems). The sensitizers and substrates incorporated inside or within the membrane structure of micelles separately or combined in various combinations, provide valuable experimental situations. With these systems one can study such problems as electron migration, the diffusion of singlet oxygen from the bulk phase to the micelles and *vice versa*, the reactivity of these species within the membrane, and general reaction kinetics in the heterogeneous system. The use of micellar systems is a step forward to the biological system. It is, however, still not comparable to biological complexity and orderedness. Nonetheless, it is extremely useful for the investigation of the basic parameters involved in cellular photosensitizations. A lucid review emphasizing micellar systems and their link to the cellular phenomena was recently published (Jori and Spikes, 1981). The reader is also referred to Lindig and Rodgers (1980) for the recent literature in this field. There are a number of other investigations with the end points being membrane destruction or the chemical change of biomolecules embedded in the membrane of liposomes. These systems of higher structural complexity may be more suitable models that simulate biological structures. The effect of fluidity of lipid matrix or region-specific reactions among different phases could be conveniently studied (Copeland *et al.*, 1976; Kondo and Kasai, 1974; Muller-Runkel *et al.*, 1981; Suwa *et al.*, 1977, 1978).

2.3.2. Considerations of Uniqueness of Cell Level Photosensitization

As pointed out above, the extrapolation of chemical knowledge obtainable in a homogeneous system, or even from a liposome system, does not seem to secure a proper view of cellular photosensitization. To enumerate some obvious difficulties: the pH inside the cell is extremely difficult to control by outside conditions; the distribution of a given substance, hence, the local concentration is most difficult to assess; the steady concentration of oxygen is rarely known; the behavior of exogenously added substances (quenchers and scavengers) as diagnostic aids are only a guess in the cellular environment.

The uniqueness of cellular systems is best explained by the daily experience that a simple prediction from test-tube knowledge often fails in cell-system experiments. The failure of the quantitative observation of D₂O enhancement in nearly all cellular systems has prevailed in the recent literature (Ito, 1978, and the literature therein). The poor accessibility of D₂O to the target site is often mentioned as the reason. It is also not at all clear why the protective effect of N₃⁻ differs greatly depending on the growth stages of the cell (Kobayashi and Ito, 1976). A higher concentration of N₃⁻ is usually required for *in vivo* protection than in a homogeneous system. This may be partly ascribed to the presence of large quantities of competing singlet oxygen quenchers and acceptors in the interior of the cell (Kobayashi and Ito, 1976; Arnason *et al.*, 1981). The use of electron acceptors and radical scavengers as diagnostic aids would also be subject to the same biological complications unique to the cellular system, and the interpretation of the results would require special considerations. The stability of sensitizers in the cellular environment, the great variation of cell physiology over the cell cycle, and the restoring ability of cells, all add to the uniqueness in dealing with photosensitization at the cellular level.

3. SELECTIVE ACTION OF PHOTODYNAMIC AGENTS AT THE CELLULAR LEVEL

3.1. Introductory Remarks

In the presence of light, acridines, thiazines, xanthenes, and many other dyes, light absorbing drugs (e.g., Utsumi and Elkind, 1979), and natural pigments or products such as furocoumarins, polyacetylenes, and porphyrins (Giese, 1971, 1981; Towers *et al.*, 1977; Wat *et al.*, 1977, 1980; Arnason *et al.*, 1981; Gommers *et al.*, 1982) act as sensitizers on many kinds of biological systems. When the sensitization involves oxygen molecules in the reaction pathways it has been called photodynamic action (Blum, 1941). Furocoumarins act both by an oxygen-requiring pathway (Poppe and Grossweiner, 1975; Singh and Vadasz, 1978; de Mol and Beijersbergen van Henegouwen, 1979, 1981; de Mol *et al.*, 1981) and a pathway that does not require oxygen (Musajo and Rodighiero, 1970), the latter being studied more extensively (see Musajo and Rodighiero, 1972; Löber and Kittler, 1977 for reviews). As outlined earlier (Section 2.2), in the oxygen-requiring pathways, the sensitizer activates either oxygen molecules by energy transfer (Type II) or produces, by electron or hydrogen transfer, substrate radicals that later react with ground-state ox-

ygen (Type I). In the pathways not requiring oxygen, the sensitizer reacts in most cases with a given substrate forming a complex (absence of photooxidative reaction), the so-called photoaddition products (e.g., monoadduct to DNA), or in some cases the sensitizer becomes a bridge to connect two different entities (e.g., cross-links of two DNA strands). A deoxygenated environment is favorable for the latter type of reaction because oxygen, if present, would quench the excited state of sensitizers, and thus prevent it from reacting with the substrate. The present discussion is concerned with the photosensitization via the oxygen-requiring pathways.

Since many types of compounds could act as photosensitizers, it is expected that those that have different chemical properties behave differently towards the cell. However, it is only recently that the importance of this predetermined condition has been recognized. In the virus inactivation experiments, workers have long noticed the importance of the preillumination dark reaction with virus particles (e.g., Mayor, 1962; Melnick and Wallis, 1975). In cell studies, Mathews (1963) and Mathews-Roth (1967) first examined the difference in photosensitizing action between acridine orange and toluidine blue in terms of their interaction with bacterial cells. They found that toluidine blue acted on the surface of the cells, while acridine orange damaged intracellular entities. Apparently, the difference is related to the preillumination interaction of these dyes with the cells.

A classical work by Peacocke and Skerrett (1956) showed that proflavine binds to nucleic acids specifically. Later work by Lerman (1961, 1963) and others (e.g., Prichard *et al.*, 1966) established the formation of the intercalated state. Since then, it has become clear that there are two states in the binding of acridines to nucleic acids: intercalation and stacking (outside of a polynucleotide chain). Lochmann and Micheler (1973) were probably the first to recognize the importance of these binding modes in acridine-sensitized photodynamic action at the cellular level. In my laboratory, the specific binding of acridine orange to cellular DNA was deliberately used to observe the strandedness in the photosensitized induction of mutations in yeast cells (Ito *et al.*, 1963).

The continuation of comparative photodynamic studies of various sensitizers, in conjunction with their permeability and specific interaction with cell components, contributed to the current view of cellular photodynamic action (Ito and Kobayashi, 1977b; Ito, 1978). Needless to say, the developments in research on the photochemical processes in dye-sensitization in recent years (see Bourdon and Schnuriger, 1967 for review) have provided the chemical basis. Above all, provocative accounts on the involvement of singlet oxygen in many biological sensitization

phenomena (Foote, 1968), the findings of the specific means for the modification of singlet oxygen reactivity (Merkel *et al.*, 1972; Hasty *et al.*, 1972), and the demonstration of its applicability to cellular systems as a diagnostic means for the primary photochemical events (Ito and Kobayashi, 1974; Kobayashi and Ito, 1976), stimulated further studies.

It is also important to note that if the photodynamic action in question was once inferred to occur via the singlet oxygen mechanism, then the primary photochemical process would have occurred in the vicinity of the location of sensitizers in the cell, within the diffusion distance related to its short lifetime (3 μ s in aqueous medium according to Gorman and Rodgers, 1978). Conversely, for example, if the location of damage is predetermined in the cell as nuclear DNA, the sensitizers must be located in close proximity to DNA. Thus, localization of sensitizers and appropriate systems for the detection of induced damage will provide, in combination, a useful tool in cell biology. One could get a variety of specific damage, nuclear, cytoplasmic, and membranous damage, by choosing an appropriate sensitizer and sensitizing conditions. As knowledge accumulates, the damaged site could be defined better, i.e., we may discriminate the damage in the protein component from that of the fatty acid component in the membrane, for example. Thus, the cell may be dissected *in situ* to an extent that other techniques do not accomplish. The present review is devoted to a discussion on the selective action of sensitizers, based on their locations and their interaction with cell components.

The reader should also consult recent review articles: Ito (1978) for cellular and subcellular photodynamic action with emphasis on the singlet oxygen hypothesis; Lochmann and Micheler (1979) for current research on molecular and biochemical aspects of photodynamic action; a rather comprehensive review by Pooler and Valenzeno (1981) focusing on the photodynamic inactivation of yeast cells, excitable cells, erythrocytes, and cultured mammalian cells; Spikes (1982) for a thorough survey on the photodynamic reactions at various levels of organisms including viruses and biomolecules.

3.2. Nuclear Damage

3.2.1. Acridines

Nuclear damage may be induced by a sensitizer that localizes in the nucleus. Acridine derivatives usually penetrate across the cell wall and cell membrane, and reach the nuclear area (Ito, 1974). Kinetic data on this penetration have been obtained by Ito (1973a) (see also Section 3.5.2).

It is not certain whether or not the finding that acridines intercalate or stack to DNA in solution is applicable to cellular systems. These binding modes probably hold as such for prokaryotic cells, but need some modification of the details in eukaryotic cells. The presence of nuclear proteins complexed with DNA reduces the number of binding sites, as would be expected (Traganos *et al.*, 1976). Acridine dyes also bind to cytoplasmic DNA, which has a simpler structure than the chromosome.

Cells of a wide range of origins are inactivated by acridine sensitization. Because of the selective localization of acridines with nucleic acids, the major photodamage responsible for inactivation is likely in DNA. The acridine-sensitized induction of genetic changes, including gene mutation, gene conversion, recombination, and chromosome aberration, are well known. However, RNA is also damaged by the photodynamic treatment with acridine dyes in solution (Amagasa and Ito, 1970), and could be an important site of photodynamic cell damage under certain physiological conditions (Section 3.5.2).

The action spectrum for acridine orange sensitization of yeast cell inactivation is somewhat skewed in shape from the absorption spectrum of acridine orange itself (Ito *et al.*, 1967). It reflects the binding of acridine orange to the nucleic acids. Bagchi and Basu (1979) showed, in *E. coli* cells, that acriflavine remaining outside the cell does not contribute to photodynamic inactivation, suggesting the importance of bound acridines. They also reported recently that the presence of acriflavine in the photodynamically treated cell (loosely bound or free) inhibited DNA repair (Basu and Bagchi, 1982), being in accord with the recognition of the importance of DNA damage.

Genetic changes were induced more effectively by 510 nm illumination than by 470 nm illumination, as judged by survival studies (Ito, 1973b). It is known that 510 nm light is close to the absorption peak of the intercalation type binding, and 470 nm corresponds to the absorption of the aggregate of acridine orange along the outside of the nucleic acid chains (stacking type). In consonance with the above observation, 460 nm light was much less mutagenic than 500 nm light in acridine-sensitized *E. coli* cells (Hass and Webb, 1979).

By illumination with proflavine, DNA and RNA syntheses were inhibited immediately in HeLa cells (Roberts, 1981a). This photooxidative effect was reduced by free radical scavengers. Extending the analysis, Roberts (1981b) concluded that thymine and cytosine residues of DNA, in contrast to the commonly believed guanine residue, are the sites of photodamage.

Isolated DNA (ϕ X174) of the double-stranded form was 10 times more sensitive compared with the single-stranded form by proflavine sensit-

zation, in contrast to the other DNA attacking agents (e.g., UV radiation, X-rays; Piette *et al.*, 1978a), where the single-stranded form is usually significantly more sensitive. This supports the notion that the strong binding (intercalation in double-stranded DNA) is more effective in producing DNA damage (Piette *et al.*, 1978b). Incidentally, these reactions are claimed to occur via ionization accompanying the absorption of two photons. The involvement of various photochemical pathways has been proposed for the inactivation and the formation of free radicals in phage DNA sensitized by proflavine (Piette *et al.*, 1977, 1978b,c). But the exact pathways leading to the biological effects or even to the strand breaks need more work to be settled (see also Berg, 1978). The selective degradation of guanine residue might be the primary damage responsible for the formation of the strand breaks or the alkali-labile bonds (Kittler and Löber, 1977). Not only with bacterial cells, but with human fibroblast cells from xeroderma pigmentosum patients, the formation of DNA strand breaks was reported for proflavine plus light (Regan and Setlow, 1977).

The DNA strand breaks are usually seen in dye-sensitized DNA only after intense irradiation; kinetics for the strand breaks and for the infectivity loss in proflavine-sensitized phages was not directly related (Piette *et al.*, 1979). Recently, Piette *et al.* (1981) reported an alteration of guanine residues in proflavine-sensitized DNA under mild treatment. This guanine degradation process was markedly decreased in the presence of the singlet oxygen quencher, N_3^- , indicating that this particular process involves singlet oxygen. Although the implications of this finding await further experiments, the process may well be related to a loss of biological activity.

Both the inactivation and the induction of genetic changes in acridine orange-sensitized yeast cells were reduced by the addition of N_3^- (Ito and Kobayashi, 1974; Kobayashi and Ito, 1976). Evidence indicates that there is a significant difference in the relative contribution of the singlet oxygen mechanism between 470 and 510 nm photoeffects for the induction of genetic changes (Kobayashi and Ito, 1977). A differential role of the singlet oxygen mechanism in the production of different types of genetic changes (Kobayashi, 1978) suggest that the acridine-sensitized damage of DNA is not unique.

3.2.2. Thiazines, Xanthenes, and Other Sensitizers

Thiazine dyes like methylene blue or toluidine blue are reported to induce mutations photodynamically in bacterial cells (Nakai and Saeki, 1964; Imray and MacPhee, 1975; Gutter *et al.*, 1977a; Webb *et al.*, 1979; Mathews, 1963). Neutral red is also reported to be mutagenic in *Sal-*

monella cells (Gutter *et al.*, 1977b). In the cases tested, the induced mutation was the base-substitution type.

This is not the case with yeast cells: toluidine blue (Ito, 1977), methylene blue (Ito and Kobayashi, 1977b), thiopyronine (Marquardt and von Laer, 1966; Ito and Nishiyama-Watanabe, unpublished data), Rose Bengal, eosin Y (Ito and Kobayashi, 1977b), hematoporphyrin (Ito, 1981b) and acridine (lipid soluble) (Ito, unpublished data) are not usually competent for the photosensitized induction of mutational damage in yeast cells. Since these dyes all appear to stay out of the nucleus of yeast cells, the observed action mode is expected. In prokaryotic cells (*E. coli*), however, the hydrophobic acridine (believed to be taken up only within the cell membrane) induced DNA strand breaks (Wagner *et al.*, 1980) which, nonetheless, seemed to be minor for the lethality (Section 3.3.2). Also, halogenated fluoresceins (Yoshikawa *et al.*, 1978) and methylene blue (Jacob, 1975) were reported to induce DNA damage in bacteria.

Moan and Boye (1981) observed the photoinduction of DNA single-strand breaks in cultured human cells by hematoporphyrin sensitization, but the quantity induced was not as much as by X-rays on the basis of survival. Gomer (1980) made the same observation with the so-called hematoporphyrin derivative. The induction was not related to the loss of colony-forming ability based on the nonparallelism in the modification of the two types of effects by the substitution of H₂O with D₂O in the medium. More DNA damage was induced at higher pH, and this was attributed to the increased uptake of hematoporphyrin by the cell (Moan *et al.*, 1980). Riboflavin (Speck *et al.*, 1975; Hoffmann and Meneghini, 1979) and neutral red (Speck *et al.*, 1979) are also photodynamically active in producing DNA damage in mammalian cells.

These instances of variable results on the ability of inducing genetic and DNA changes by thiazine dyes and hematoporphyrin, indicate the several factors that may affect the sensitivity of a cell to DNA damage. These may include the difference among organisms, in particular, prokaryotic *E. coli* cells and eukaryotic cells (the presence of a membrane-DNA complex in *E. coli* cells, for example). Other differences in membrane structure may well be important factors. The sensitizers discussed above are usually more effective than acridines in producing cell membrane damage (Section 3.3). Therefore, it is conceivable that as the damage in the cell membrane proceeds with the treatment, the cell tends to permit the sensitizers to penetrate into the cell. This possibility was proved to be true for toluidine blue in yeast cells (Ito, 1977), and it was also indicated in other cases including *Neurospora* conidia for toluidine blue (Shimizu-Takahama *et al.*, 1981), and human cells for hematoporphyrin (Moan *et al.*, 1979; Moan and Christensen, 1981). In combination with

other conditions like the concentration gradient of the sensitizers across the cell membrane, a sufficient amount of sensitizers may reach the interior of the cell. Since thiazine dyes bind to DNA in solution and damages guanine in the presence of light (Simon and van Vunakis, 1962), it would not be surprising if methylene blue or toluidine blue were to sensitize DNA *in vivo* under certain experimental conditions. *In vitro* experiments show that DNA is also modified by hematoporphyrin photodynamically (Gutter *et al.*, 1977c; Boye and Moan, 1980). Another intriguing possibility is that the diffusion of photoinduced active intermediates occurs over a much longer-than-expected distance from the site of generation (sensitizer location) through a hydrophobic path in the cell, so that it reaches the region where DNA is located. A very small increase of gene conversion in toluidine blue-sensitized yeast cells, which was recently noticed (Ito, 1980), and other seemingly contradicting observations mentioned above may have the explanation in one of these directions.

With 8-methoxypsoralen, which undergoes photoaddition reactions to DNA in the absence of oxygen, induction of mutational changes (nuclear) was recently reported in *E. coli* cells via the singlet oxygen mechanism (de Mol, 1980). It is known that this compound penetrates into the cell.

3.3. Cell Membrane Damage

Certain types of dye sensitizers virtually do not penetrate across cell membranes into the cytoplasmic region. This includes toluidine blue, methylene blue, xanthenes, porphyrins, and acridine (lipid soluble). (Note that acridine orange and other acridine derivatives are water soluble and can easily penetrate into the cell.) Since these compounds are photodynamically active in cell inactivation, the basis for this activity must be sought in the changes inflicted on the surface of the cells. Much recent research has attempted to establish that the cell surface is the major or only site for this cellular inactivation. Some of these compounds induce photohemolysis of erythrocytes. The photohemolysis has long been regarded as a consequence of membrane-associated damage.

There exists a large variety of hydrophobicity among these active compounds and their derivatives. This and other factors, such as molecular weight, affect the degree of the penetration (solubility) into the cell membrane. Of course, the photophysical properties such as the yield and the lifetime of the triplet state, and the oxygen quenching efficiency, are other important factors. In view of the reactivity of membrane components comprising proteins, sterols, and unsaturated fatty acids, both the singlet oxygen and the radical mechanisms are anticipated to play a crucial role in the photosensitizations.

3.3.1. Toluidine Blue

By the comparative studies using acridine orange and toluidine blue, the former being established as predominantly a nucleus attacking agent, Mathews-Roth (Mathews, 1963, 1967) observed in *Sarcina* (*Micrococcus*) cells the inactivation of membrane bound enzymes but not enzymes in the cytoplasm with toluidine blue. Later Ito (1977) studied toluidine blue-sensitization with a yeast strain that is useful for the detection of a genetic change (conversion from tryptophan requirement to nonrequirement). In this system it was shown that toluidine blue had essentially no photodynamic effect on the induction of gene conversion; the frequency remains nearly at the spontaneous level at the survival of about 10% (see Section 3.2). Furthermore, the photoreactivation test for far-UV (254 nm) radiation-induced gene conversion in the photodynamically treated cell (survival, 80%) showed that the prephotodynamic treatment caused no appreciable damage in the cell's ability for enzymatic photoreactivation, presumably located in the nuclear area. Moreover, the attainment of a normal rate of induction of acridine orange-sensitized gene conversion in the simultaneous presence of toluidine blue suggested that no competitive interaction of acridine and toluidine blue existed at the level of the chromosome, a logical consequence if toluidine blue does not interfere with DNA in any effective way as to the binding of acridine orange. The disruption of the permeability barrier was also shown in toluidine blue-sensitized cells (Ito and Kobayashi, 1977a). Direct evidence for the change of nonspecific membrane permeability was recently demonstrated with toluidine blue sensitization by measuring the hydrolysis of a normally impermeable analog substrate using induced intracellular maltase (Ito and Ito, unpublished observation). This type of damage develops surprisingly fast; it can be detected within 30 min after irradiation. Physiological studies by Tijssen et al. (1981) demonstrated that toluidine blue was indeed bound to the yeast cell surface without penetrating into the cell, the binding site being polyphosphates outside the plasma membrane. Both the enhancing effect of D_2O and the protecting effect of N_3^- were quite remarkable in the toluidine blue-sensitized cell inactivation (Ito, 1977), indicating the involvement of singlet oxygen. Thus, it may be concluded that toluidine blue acts on cells through singlet oxygen-mediated damage occurring in membranes. Supporting evidence is also accumulating with other organisms; *Sarcina* (Mathews-Roth, 1977), *E. coli* (Wakayama *et al.*, 1980), and *Neurospora* conidia (Shimizu-Takahama *et al.*, 1981).

Interesting observations have been made by Prebble and Huda (1973) that toluidine blue (or methylene blue) sensitized the components of the respiratory chain associated with cell membranes. This is particularly

interesting in view of the work that showed the feasibility of electron transfer from mitochondrial components to singlet oxygen based on its high reduction potential (Peter and Rodgers, 1980). This may suggest that singlet oxygen does not necessarily act on cells through oxygenation, but rather by diverting electron flow. Another example indicates that toluidine blue sensitized-yeast inactivation occurred via the semireduced state of dyes in the presence of a good electron donor (ascorbate; Ito and Ito, 1982). In this case, singlet oxygen may be excluded as the intermediate even in toluidine blue sensitization.

Recently a report appeared that membrane-attacking photodynamic agents (such as toluidine blue) could cause damage in cellular DNA in *E. coli* (Nishida *et al.*, 1980). Damage in a membrane-DNA complex was suggested. (See also, Section 3.2.2 for DNA damage by poorly penetrable sensitizers.) In this connection it is interesting to note that malondialdehyde, an oxidation product of polyunsaturated fatty acids, was able to induce mutations in *Salmonella* (Mukai and Goldstein, 1976) and *E. coli* (Yonei and Furui, 1981).

3.3.2. Porphyrins, Xanthenes, and Other Sensitizers

In the hematoporphyrin sensitization of cultured mammalian cells, a severe deformation of cell membrane was observed (Fritsch *et al.*, 1976; Moan *et al.*, 1979). In protoporphyrin sensitization of leukemic cells a drastic damage (large holes or many blebs) was also observed in the cell membrane (Malik and Djaldetti, 1980). Other papers also suggested that the cell membrane would be the major damaged site responsible for the porphyrin-sensitized cell inactivation regardless of the type of organism (Dougherty *et al.*, 1981; Stenström *et al.*, 1980; Ito, 1981b).

There are a number of instances in which DNA damage has been detected in porphyrin-sensitized cells (Section 3.2). The significance of this DNA damage in cell inactivation is, however, suspected. One exception would be the observation that the intracellular presence of singlet oxygen quencher significantly suppressed the photoinactivation (Weisshaupt *et al.*, 1976). Mammalian cells, in contrast to yeast cells, appear to take up appreciable hematoporphyrin or its derivative inside the cell (Gomer and Smith, 1980; Moan and Christensen, 1981). Tumor cells and normal cells of human origin (Chang and Dougherty, 1978) or of mouse origin (Moan *et al.*, 1981) showed no major difference in their uptake. Although these experiments have demonstrated that there is cellular uptake of hematoporphyrin and its derivative, the exact location is not necessarily clear. Many lines of evidence indicate that the major part locates in the cell membrane. As discussed later (Section 3.3.4), the uptake of

porphyrin species consists of very complicated processes occurring in the cell membrane region.

The following observations further support the cell membrane damage as the primary cause of mammalian cell inactivation by sensitization with porphyrin species. (1) Replacement of H_2O with D_2O in the cell suspension medium only enhances cell inactivation and not DNA damage (Moan and Boye, 1981). (2) Release of lysosomal enzymes occurs after most of the cells are killed (Christensen *et al.*, 1981a). (3) Early events of photosensitization are the leakage of K^+ and membrane (plasma) bound enzymes (Sandberg *et al.*, 1981) and not lysosome damage as reported earlier (Allison *et al.*, 1966). (4) Significant effects on various cell-surface phenomena occurs, at treatment levels that markedly reduce cell viability, without affecting an intracellular event (Kessel, 1977).

It has been known that the so-called hematoporphyrin derivative includes several components separable by chromatography. Recent studies have shown that various porphyrin derivatives are not significantly different from each other in the photophysical parameters such as triplet quantum yield, oxygen quenching, etc. (Bonnett *et al.*, 1980; Truscott, personal communication). Nonetheless, for human carcinoma cells the hematoporphyrin derivative was twice as effective as hematoporphyrin. This was attributable to the least polar components contained in larger proportion in hematoporphyrin derivative than in hematoporphyrin, which is also a mixture (Moan, personal communication). Porphyrins are generators of singlet oxygen (Bodaness and Chan, 1977; Cannistaro *et al.*, 1978), O_2^- (Buettner and Oberley, 1979) OH^- *in vitro* (Buettner, 1979), and OH^- *in vivo* (Hariharan *et al.*, 1980). It was shown that the efficiency of singlet oxygen production among hematoporphyrin, mesoporphyrin, protoporphyrin, and deuteroporphyrin are different by a factor of 5, the highest being hematoporphyrin (Jori *et al.*, 1980). Photooxidation in micellar systems proceeds via either singlet oxygen or radicals (Sconfienza *et al.*, 1980; Rossi *et al.*, 1981; Spikes, 1981), depending on the conditions, in particular, the geometrical relation of substrate and sensitizer in the system. Diagnostic tests for singlet oxygen (D_2O , N_3^- , singlet oxygen scavengers) in different cell systems commonly indicate that singlet oxygen is the major reacting species (Weishaupt *et al.*, 1976; Moan *et al.*, 1979; Stenström *et al.*, 1980; Ito, 1981b; Moan and Stenström, 1981; Moan and Boye, 1981; Sandberg and Romslo, 1981).

Bezman *et al.* (1978) found that immobilized Rose Bengal in porous plastic beads could still kill bacterial cells in the presence of oxygen and light. This proved that cells can be inactivated by attack on the surface by a diffusive intermediate generated in bulk medium alone. This was confirmed with yeast cells (Ito and Ito, unpublished observations). Eosin

also acts on the membrane in the inactivation of yeast cells (Cohn and Tseng, 1977). Allison *et al.* (1966) also observed cell membrane damage by Rose Bengal. Lipid soluble acridine was shown to inactivate bacterial cells through membrane damage (Wagner *et al.*, 1980; Wagner *et al.*, 1982). Valenzeno and Pooler (1982) extended their previous work on nerve membranes to erythrocytes, and indicated that a proper location of fluoresceins in the membrane is a critical condition for sensitized photohemolysis (Section 3.3.4).

Naturally occurring polyacetylenes and α -terthienyl sensitize human erythrocytes for photohemolysis by oxygen requiring pathways (Wat *et al.*, 1980). Many related compounds had photodynamic activity on yeast and bacterial cells (Towers *et al.*, 1977; Wat *et al.*, 1977, 1980; Arnason *et al.*, 1981; Gommers *et al.*, 1982). There must also be unidentified endogenous sensitizers located either in the cell membrane or its vicinity to cause membrane damage in the presence of visible light or near-UV radiation. A portion of the so-called near-UV radiation effects appears to be such photosensitization (Jagger, this volume; Webb, 1977; Pileni *et al.*, 1978).

Photodynamic action has usually been viewed as a destructive photodamage, but an entirely different aspect has emerged with the finding that a new membrane protein (21 kilodalton) was rapidly synthesized in *Arthrobacter* cells when sensitized by several dyes (e.g., Rose Bengal and methylene blue) (Hoover, 1977, 1978; Hoover and Franzi, 1980). This phenomenon occurred during illumination via oxygen-requiring pathways. In view of the nature of the sensitizers used and the presence of an effective inhibition by histidine, the photooxidative damage appears to locate in the vicinity of the membrane. The significance of this response in cells remains to be studied.

3.3.3. Molecular Damage

The molecular damage in the membranes produced by membrane-attacking photodynamic agents has attracted many researchers. The physiological changes in the membrane functions in *E. coli* cells (Wagner *et al.*, 1980), *Sarcina* cells (Mathews, 1963) and yeast cells (Ito and Kobayashi, 1977a), were mentioned earlier. Recently, with erythrocyte ghosts Deziel and Girotti (1981) concluded that there are two types of damage other than catastrophic breakdown; the leakage of small ions and much larger molecules (M.W. 10,000), each corresponding to damage in proteins and lipids, respectively. Photohemolysis seems to occur abruptly as if it is related to the accumulation of damage. Yeast cells also lose permeability control abruptly in toluidine blue sensitization. All the inactivation

curves of cells, where membrane damage is presumed to occur, proceed rapidly after a large shoulder.

Erythrocytes have long been a favorite material for investigating molecular damage by photodynamic actions. Photohemolysis occurs in erythrocytes from erythropoietic protoporphyrin patients in association with the presence of high concentration of protoporphyrin (Allison *et al.*, 1966; Hsu *et al.*, 1971). The work by Lamola *et al.* (1973) indicated that the singlet oxygen-mediated oxidation product of cholesterol could lead to the hemolysis. With protoporphyrin, de Goeij *et al.* (1975) have demonstrated a photoinduced rearrangement of intramembrane particles and severe mutilation of the membrane by the freeze-fracture method. The progressive cross-linking of proteins involving spectrin was shown to be independent of lipid peroxidation (de Goeij *et al.*, 1976). Cross-linkings of protein components in the membrane were also shown with protoporphyrin (Girotti, 1976a) and bilirubin (Girotti, 1975) sensitization. Peroxidation of unsaturated membrane lipids occurs undoubtedly, preceding the association of proteins. To determine which lesions trigger the lysis has been the focus of current research (see Lamola, 1977). The nature of the cross-link has also been investigated. While —S—S— bonds seem to be excluded (Girotti, 1976a; 1978), evidence indicates that a reaction between free amino groups of proteins and a photooxidation product of histidine residues is involved in the cross-linking reaction (Dubbelman *et al.*, 1978). It was shown that a subunit of ATPase which is buried in the mitochondrial membrane (and only in this membrane-bound state) was susceptible to cross-links to membrane components by bilirubin photosensitization (Hackney, 1980).

Different primary photochemical damage can result in membrane destruction at later stages. Cholesterol hydroperoxide-induced hemolysis does not involve protein cross-links, indicating that lysis is not necessarily coupled with it. Moreover, Lamola and Doleiden (1980) pointed out that the effects of oxygen reduction on photohemolysis and on the cross-linking of proteins do not proceed in parallel. With resealed erythrocyte membranes sensitized by bilirubin, Deziel and Girotti (1980) showed that antioxidant protected against lysis but not against the cross-linking reaction of proteins. Later the cross-linking was conclusively shown to be a secondary chemical reaction following primary photooxidation, which resulted in a stable product (Dubbelman *et al.*, 1980b). Moreover, lipid peroxidation by H_2O_2 treatment did not parallel the modification of transport processes (Dubbelman *et al.*, 1980a). However, the first-order kinetics for the inactivation of a membrane-bound enzyme (ATPase; Girotti, 1976b) or the modification of the transport processes do not match the general sigmoidal fluence-response of photohemolysis. Thus, a satisfac-

tory model of photohemolysis accommodating these data is still to come. Perhaps because of technical difficulties, comparable work with nucleated cells are few (e.g., Schothorst *et al.*, 1980). Preliminary data show that such membrane-attacking sensitizers as toluidine blue, eosin Y or hematoporphyrin all produce lipid peroxidation in yeast cells in contrast to the absence of such damage by the nucleus-attacking acridine orange (Watanabe and Ito, unpublished data).

The photodynamic reactivities of various porphyrin species were differentiated according to hydrophobicity using murine leukemic cells (Kohn and Kessel, 1979; Kessel, 1981) (see Section 3.3.4) and rat liver mitochondria (Sandberg and Romslo, 1980; 1981). In the latter work, for succinate dehydrogenase (inner membrane), the effectiveness decreased with decreasing hydrophobicity, namely, in the order: protoporphyrin > uroporphyrin-octamethylester \gg uroporphyrin, regardless of being intact or sonicated, while for glutamate dehydrogenase (water soluble), the order was reversed if the mitochondria were sonicated. Tryptophan photooxidation in aqueous solution was most effective with uroporphyrin. For the photooxidation of mitochondria as a whole, the order was again protoporphyrin > corprotoporphyrin > uroporphyrin. Thus, in summary, there is a parallelism between the effectiveness of photodamage of mitochondria by porphyrin species and the solubility of the porphyrins in the hydrophobic membrane region that gives the porphyrins access to the target molecules. Photohemolysis of erythrocytes was also induced more severely by protoporphyrin, and least by uroporphyrin, indicating the importance of damage to the inner membranes.

3.3.4. Kinetic Aspects

One of the current focuses of research is: exactly where is the sensitizer when it is acting (or producing the active intermediate like singlet oxygen) on cells. The problem of sensitizer-cell relationship in space and time is extremely important, not only for membrane damage but also for nuclear damage. In the conventional technique of cell photosensitization (Section 2), the cell and the sensitizers are mixed and incubated for a long time (e.g., 30 min) and in the usual experimental setup the irradiation lasts 5 to 30 min or more. Thus, an initial phase of cell-sensitizer interaction, say, within 1 min cannot be reflected in the results. Moreover, even for the slowly occurring interaction in 10 min or so, the details may well be masked. Ito (1980) has attempted to solve the problem by shortening both incubation and irradiation periods. The incubation time was selected in a range of from 20 ms to 1 min, and the irradiation time was shortened by increasing the fluence rate of the light so that the times

ranged from fractions of a second to 1 min to achieve enough inactivation. This setup allowed the choice of any combination of the lengths of times for the incubation–irradiation sequence. By this method, it can be studied how the sensitizer develops the photodynamic activity in less than 1 min after mixing with the cells. Use of such a device provided the following information with yeast cells (Ito, 1980; Ito, 1981b): (1) toluidine blue acts in two modes on cell inactivation, one is very fast in the order of 20 s and the other is slow of the order of 20 min at 30 °C, (2) on the contrary, acridine orange acts both on inactivation and mutagenesis almost immediately, and steadily increases in effectiveness at least up to 20 min, (3) hematoporphyrin and lipid soluble acridine (unpublished observation) behave as if they have only one mode of action (comparable to the fast mode in the toluidine blue case mentioned above), and (4) thiopyronine penetrates very fast with a corresponding increase in effectiveness, but this activity is limited to the inactivation alone (no mutagenesis; unpublished observation).

It seems that the photodynamic agents that are thought to be membrane specific are qualitatively classified into two groups. Those that exhibit only the fast interaction mode may remain free in the cell suspension or be adsorbed on the cell surface when acting. Those that undergo the slow interaction probably penetrate into membranes within a matter of several minutes to develop their optimum effectiveness. But adsorption, penetration, or any interaction with the cell is, in principle, time dependent. Thus, a certain type of sensitizer may behave in a complex way. Some porphyrins and xanthene dyes exhibit such properties. According to the analysis of the time-dependent location of the acting site of hematoporphyrin in bovine fibroblasts (Fritsch *et al.*, 1976), two stages were distinguished in the time course. A 5-min exposure to hematoporphyrin (with a short illumination) induced membrane damage, and a 2-h exposure combined with a relatively long illumination induced nuclear damage. Apparently, the first type of damage was caused by the hematoporphyrin localized on the cell surface.

Sery (1979) has made a number of interesting observations using a unique system, retinoblastoma cells in suspension. Hematoporphyrin was effective immediately after mixing with cells (the sensitizer only in the medium). Cells incubated with hematoporphyrin long enough (e.g., 3.5 h) were inactivated even after washing (the sensitizers probably being incorporated in the cell)—the former condition being far more effective though. A remarkable suppressive effect of serum was observable on the hematoporphyrin-sensitized inactivation of the cell. The kinetics of uptake of hematoporphyrin derivative by Chinese hamster cells was dependent on the presence of serum, and the equilibrium was attained in its

presence, but not in saline (Chang and Dougherty, 1978). The two-step uptake with a very fast and much slower rate was noted in the absence of serum in the media. Recent kinetic experiments with yeast cells on the time course of photoinactivation by hematoporphyrin demonstrated that there would be no increase of photoinactivating effectiveness for the incubation period from practically zero to 35 min at 30°C (Ito, 1981b). Protoporphyrin, however, in the same system exhibited a slow "build up" in the effectiveness during the incubation period (unpublished data), indicating the progression of solubilization into the cell membrane.

According to the recent work by Kessel (1981), where several chromatographically separated components from hematoporphyrin derivative were examined in murine L1210 cells, the most hydrophobic components were the most potent photosensitizers in a 10-min porphyrin-loading incubation. These were readily removed by washing with serum-containing medium, whereas hydrophilic components were gradually bound during a longer period of incubation time (24 h) to low molecular weight membrane components. Kessel (1981) suggested that the latter components have preferential affinity to neoplastic cells. Other experiments showed that during longer incubation times, mesoporphyrin (relatively hydrophobic) gradually partitions to more hydrophobic loci with a diminished photosensitizing effectiveness. Thus, the hydrophobicity of porphyrins plays a crucial role in the effects on cells, too, through a differential localization in the cell. When incubated with cells, mesoporphyrin exhibits the absorption spectrum of monomeric type such as observed in dilute alcohol solution (Kessel and Kohn, 1980). With rat hepatoma cells, the highest affinity site of hematoporphyrin locates in the external membrane, although the secondary binding site appears to be inside (nuclear membrane) the cell (Cozzani *et al.*, 1981). Hepatoma cells retain hematoporphyrin longer than normal cells do. The observation by fluorescence microscopy of human carcinoma and monkey cells sensitized with a relatively hydrophilic porphyrin, *meso*-tetra(*p*-sulfonyl)porphyrin, showed that the porphyrin localized after a long incubation in the cytoplasm and in the nucleolus, but not in the cell membrane; the intracellular pattern differed among cell types, however. The fluorescence intensity decreased with time. The major cytological changes after illumination, as observed by electron microscopy, occurred in mitochondria and endoplasmic reticulum (Austen *et al.*, 1978).

The sensitizers in category (2) and (4) may be excluded from the cell membrane attacking agents in view of the mode of action. These agents would be more important to cause nuclear (already discussed) or cytoplasmic damage (Section 3.4).

Finally, the work on nerve membranes with xanthene and thiazine

dyes (Pooler and Valenzano, 1978; 1979*a,b*) deserves comment. According to their experiments, the kinetics for the photodynamic blocking of sodium channels differs widely among sensitizers. With eosin Y, which is virtually impermeable, a saturation of the effectiveness was reached within a few minutes of preillumination incubation. Some of the other fluorescein dyes showed a slow continued rise in effectiveness for longer times related to their partition coefficients in an octanol/sea-water mixture. Considering other factors such as relative absorption, Pooler and Valenzano postulated an interesting supposition that there would be a hydrophobic sink in the membrane, from which the accumulated sensitizers diffuse out to the sites of actual sensitization. Combined tests of D₂O and N₃⁻ effects indicated that intramembraneously generated singlet oxygen was the major active intermediate. It was later claimed that this concept may be applied to the photodynamic lysis of erythrocytes (Valenzano and Pooler, 1982).

3.4. Cytoplasmic Damage

The occurrence of damage in the cytoplasmic region may also be understood according to the compartment concept of the location of sensitizers. Once a given sensitizer crosses the membrane region and, if it is sufficiently hydrophilic, it may dissolve in the aqueous phase in the cytoplasm. Then certain types of sensitizers may bind to the protein components exposed to the aqueous phase. If the sensitizer has more hydrophobic properties, it may dissolve in the membrane structures of the cytoplasmic organelles. It is not at all clear how the complex network of the endoplasmic reticulum behaves in this situation. Furthermore, since most of the photodynamically active compounds would also be subject to the redox processes in the cell, access to the intracellular redox system, such as the mitochondrial electron transport chain, would be an important element to be considered. In this situation, only the sensitizers that survived such a dark interaction could cause photodynamic action in the light. For certain sensitizers (thiazines) the excited state is more reducing than the ground state (Kramer and Maute, 1972; Bonneau *et al.*, 1974). This should also be taken into account in a specified microenvironment of the cells. The behavior of sensitizers in the cytoplasm appears much more complex than in the cell membrane or the nucleus.

3.4.1. Thiopyronine

The most prominent sensitizer known to date as the cytoplasmic agent is thiopyronine. Thiopyronine has long been the subject of studies

by German workers. Most characteristically, it penetrates across cell membranes, but does not or only poorly exhibits the capability to induce mutational (nuclear) changes in the light (Lochmann *et al.*, 1964; Marquardt and von Laer, 1966). A report exists, however, that there was an increase of nuclear mutation by the thiopyronine sensitization in one particular mutant (photodynamic sensitive and respiratory deficient, isolated by Roth *et al.*, 1978) of *Saccharomyces cerevisiae* (Kenter and Laskowski, 1978).

Thiopyronine was photodynamically active for the induction of DNA strand breaks and alkali labile bonds if the DNA was sensitized in solution (Triebel *et al.*, 1978). *In vitro* studies of RNA and protein synthesis indicate that template DNA, RNA polymerase, polyribosomes and enzymes related to protein synthesis were all affected by the thiopyronine sensitization (Micheler and Nishiyama-Watanabe, 1977; Nishiyama-Watanabe and Schulz-Harder, 1977).

Micheler and Lochmann (1971) have concluded that the uptake of thiopyronine by yeast cells is a simple diffusion process, and depends on the extracellular concentration, the temperature, etc. By fractionating the stained cells, the major location of thiopyronine was found to be the cytoplasm (Lochmann *et al.*, 1976). Extensive work has been performed on RNA and protein synthesis in thiopyronine-sensitized yeast cells (Lochmann *et al.*, 1964; Lochmann, 1967; Lochmann and Pietsch, 1967). The two synthesis processes were inhibited immediately after photodynamic treatment. From the general location of thiopyronine mentioned above, it is expected that thiopyronine affects various metabolic functions associated with the cytoplasm. Nishiyama-Watanabe (1976) observed that respiration and fermentation were inhibited; the former paralleled the decrease of survival. It is conceivable that thiopyronine causes mitochondrial damage by photodynamic activity in view of its location in the cells. More recent *in vivo* studies indicate that the synthesis of mRNA is hardly affected while both the rate of synthesis and the quantity of free ribosomes are greatly decreased. Although it is not yet clear whether the synthesis of rRNA is inhibited (a nuclear feature), or a failure in ribosome assembly occurs (a cytoplasmic feature), they suggest, based on their *in vitro* and *in vivo* studies, that the action site of thiopyronine is the cytoplasm but not the nucleus (Lochmann *et al.*, 1981a,b). Few investigations are available about the mechanistic aspects of the thiopyronine sensitization; however, it is likely that the singlet oxygen mechanism is operating.

It is interesting to note that thiopyronine is bleached (reduced) intracellularly both in the dark and in the light. Jacob (1974) has noted a large mass of bacterial cells caused the conspicuous bleaching of thio-

pyronine in the dark. This bleaching, probably the reduction to the leuco form, started in 10 min or so after mixing with the concentrated cells. He noted at the same time that the decrease of thiopyronine concentration in the bulk medium was much faster than the onset of bleaching. Experiments with yeast cells in this laboratory indicated that thiopyronine accumulates at the major sensitization site in about 2 min at 24°C (unpublished observation). These are important observations when one considers the intracellular behavior of thiopyronine in connection to the photodynamic activity. It may be speculated that thiopyronine, when bound to an electron transport system, might extract an electron, bypassing it from the normal flow in the dark. In the presence of light this tendency would become more so since the triplet state could be more electrophilic as in thiazine dyes (Kramer and Maute, 1972; Bonneau *et al.*, 1974). Thus, the situation is of a complex nature.

A potentially important development related to thiopyronine sensitization is the isolation by Roth *et al.* (1978) of yeast mutants that are sensitive or resistant to thiopyronine photosensitization. The mutants were isolated as such on thiopyronine-containing agar plates. The character was shown to be controlled by a single Mendelian gene. How they are sensitive or resistant is not known. It is to be noted that the major action site of thiopyronine is not DNA.

3.4.2. Xanthenes and Other Sensitizers

Eosin Y is not (Allison *et al.*, 1966; Cohn and Tseng, 1977), or poorly (Ito and Kobayashi, 1977b), penetrable into the cells, but can sensitize the cells for inactivation. Eosin Y and Rose Bengal, however, do not cause genetic changes in the presence of light (Ito and Kobayashi, 1977b), indicating that other than the nuclear region may be the site of the photodynamic action. The cell membrane has been mentioned as the major damaging site.

Toluidine blue and methylene blue are also not usually penetrable into the cell, as mentioned earlier (Section 3.3). These dyes, therefore, would not have a capability to affect the cytoplasmic (and/or nuclear) region under ordinary conditions. Some cases that are not well accommodated in this statement have been discussed in Section 3.2.2.

Mathews-Roth (1967) reported that an intracellular enzyme was inactivated in *Sarcina* cells sensitized by acridine orange. The membrane-associated enzyme was also inactivated by the acridine-orange sensitization, but both membrane and cytoplasmic damage by acridines seemed to be minor compared to nuclear DNA damage in the cell inactivation, because carotinoid pigments were able to prevent the destruction of mem-

brane enzymes but not the lethal action (see also Sections 3.2.1. and 3.5.1.).

Some porphyrin species have photodynamic effects on the cytoplasmic organelles of mammalian cells as discussed earlier (e.g., Austen *et al.*, 1978; Sandberg *et al.*, 1981). Lysosomes have long been claimed as the first cytoplasmic organelle to be destroyed by porphyrins and light (Allison *et al.*, 1966), although recent work indicates that the damage caused in the cell membrane is more critical in cell inactivation (see also Section 3.3.2.).

It is to be added that some sensitizers exhibit a large dependence on the cell stage in their effectiveness; thus part of the increased sensitivity in the acridine sensitization at the exponential growth phase is apparently attributed to the damage associated with RNA components located in the cytoplasmic region. The loss of the amino acid-accepting ability of tRNA (Tsugita *et al.*, 1965; Kuwano *et al.*, 1968; Amagasa and Ito, 1970) and of other RNA activities by sensitization with acridines (see Lochmann and Micheler, 1973) are well documented. This aspect is discussed in more detail in Section 3.5.2.

3.5. Complex Modes of Photodynamic Action

3.5.1. Stability of Sensitizers in the Cellular Environment

There is an old observation that the photosensitized virus inactivation by methylene blue was significantly protected in the presence of live bacterial (*Staphylococcus*) cells (Perdrau and Todd, 1933). The cells were not acting by adsorbing the sensitizers or taking up the viruses. Dead cells were not effective. In view of the consideration mentioned in Section 3.3, it may be conceivable that methylene blue might be reduced by the contact with live cells. In the presence of a suitable electron donor, toluidine blue is readily reduced in the light under anaerobic conditions (Ito and Ito, 1982), but is reoxidized in the presence of sufficient oxygen (Oster and Watherspoon, 1954). The reduction of thiopyronine occurs upon contact with a high concentration of cells (Jacob, 1974) as mentioned in Section 3.4.1. It is not reoxidized by oxygen. Since thiopyronine is taken up in the cytoplasm of cells, it seems as if the dye is bleached as it enters the interior of the living cells. Thus, the selectivity of a given sensitizer with respect to its photodynamic effectiveness also appears to be governed delicately by the microenvironment of the cell, in addition to the nature of the sensitizers themselves. The change of chemical structure should also be mentioned since, as seen in a number of works (Pottier *et al.*,

1975; Bonneau *et al.*, 1975), the quantum yield of singlet oxygen of thiazines is related to the chemical form they take under a specified pH. In contrast, acridine dyes show no large systematic dependence on pH (Kobayashi and Ito, unpublished observations).

3.5.2. Cell Stage Dependence

Since acridines have a much weaker binding capacity to proteins than nucleic acids, this group is not expected to act as dominant sensitizers on the cytoplasm. In a growing cell, RNA components increase tremendously in yeast cells. The RNA-bound acridine orange is clearly seen under the fluorescence microscope as a yellowish-orange fluorescence all over the cell (Ito, 1974). The relative contribution of cytoplasmic damage and nuclear damage in cell inactivation in this situation may have been changed from that of the nondividing cells. The variation in sensitivity over the cell cycle is much bigger for acridine orange than for far-UV radiation. For toluidine-blue sensitization such a variation is much smaller (Ito, 1978). During differentiation or high transcriptional activity, binding sites for acridine orange in cellular DNA increase (Rigler and Killander, 1969; Smets, 1973; Traganos *et al.*, 1976). Conformational changes in nuclear chromatin is thought to provide the increased intercalating binding sites (Burns, 1980).

After mixing yeast cells with acridine orange, the frequency of gene conversion was taken using aliquots sampled at various times. The illumination time was set sufficiently short and kept constant (Ito, 1973a). In this way, provided that induction frequency by a fixed fluence is proportional to the accumulated acridine orange in DNA, one can estimate the rate of penetration of acridine molecules and the saturation level of the binding site in DNA. The time constant corresponding to 63% of the saturation level was 18 min under a given set of conditions. It shortened to several minutes when the dividing cell was tested. Not only that, the saturation level increased by several times when DNA was in the replicating phase (Ito and Kobayashi, 1975).

E. coli cells in exponential phase, where the cell is usually more sensitive than at stationary phase, exhibit an increased resistance toward acridine orange-sensitized inactivation when preincubated in leucine-deprived medium (Matsumoto, 1974). The interaction of cells with a given sensitizer may be affected by a change in the physiological conditions. Recently Ito (1981a) reported that the temperature has an increasing effect on the photodynamic effectiveness of toluidine-blue sensitization in stationary phase yeast cells, possibly being brought about by a change in membrane fluidity. A number of workers have also shown a similar tem-

perature dependence of sensitized cell inactivation (Cohn and Tseng, 1977; Stenström *et al.*, 1980; Moan and Christensen, 1981). Suwa *et al.*, (1978) have reported a similar fact with a hematoporphyrin-bound liposome system. Since membrane fluidity changes in the cell cycle, the stage dependence of photodynamic effect could be explained based on the change of physical properties of the membranes. Christensen *et al.* (1981b) obtained a stage-dependent inactivation pattern with hematoporphyrin derivative and found that *S* phase was most sensitive and *G₁* was least sensitive for several cell lines, as previously obtained with hematoporphyrin (Christensen and Moan, 1979), although other workers reported little dependence for Chinese hamster cells (Gomer and Smith, 1980). The higher uptake of hematoporphyrin is generally thought to be the cause of the increase in sensitivity. The higher uptake could be associated with a membrane fluidity change. However, the possibility that the overall reactivity of the active intermediate of photodynamic action is affected by the fluidity change may not be excluded. Thus, the cell stage could have multiple effects on photodynamic action. In *Rhodotorura* cells, the age dependent accumulation of protective pigments was related to the photodynamic sensitivity (Macmillan *et al.*, 1966; Maxwell *et al.*, 1966).

3.5.3. Repair

Another aspect that deserves comment is the cell's repair ability for photodynamic damage. Repair intervenes between the initial photosensitized incidence and its final fixation as the damage. If the repair capability towards any insult by the environment has evolved during the evolution of organisms, there may be no reason why the repair would not operate on the photodynamic damage since all organisms cannot be free from numerous exogeneous and endogeneous sensitizers and the light. Particularly, damage in DNA would have been the object of such repair as amply proved in the cases of far-UV and ionizing radiations, and many DNA-attacking chemicals. Discussions on the repair of photodynamic inactivation based on the literature up to 1976 are available in previous review (Ito, 1978).

It has become increasingly clear, that certain type(s) of photodynamic damage in DNA is repairable, notably being associated with *rec* gene functions (Hass and Webb, 1979; Basu and Bagchi, 1982). The photo-sensitized inactivation of ϕ X174 DNA by proflavine is also enhanced in a *recA* strain (Calberg-Bacq *et al.*, 1977). Hass and Webb (1979) proposed a *recA*⁺ dependent error-free recombination process for the repair of photodynamic damage by acridine orange in chemostat culture of *E. coli*. More than a ten-fold increase of mutation rate was observed in a recom-

bination-deficient strain. It is important to note that they used 500 nm light in these experiments, since the intercalated dye in DNA may be assumed as the site of the initiation of the action. Basu and Bagchi (1978) has shown that acriflavine sensitized-reversion mutation ($his^- \rightarrow his^+$) is probably refractory to the error-free excision repair pathway. The inhibition of repair operation for lethal DNA damage occurred in the presence of intracellular acriflavine during holding in liquid, and this was not observable in *recA* mutant cells, which also indicates the involvement of *rec* gene function in the repair (Basu and Bagchi, 1982). There are conflicting results about the presence of multiplicity reactivation of photodynamically damaged phages. Phage reactivation through the induction of an SOS repair mechanism was accompanied by a significant increase in reversions (amber mutant to wild type; Piette *et al.*, 1978b).

With methylene blue, whose primary site of attack on the cell is the cell membrane, Jacob and Hamann (1975) have reported a rapid repair of *Proteus* cell membrane (within 5 min after treatment) from osmotic fragility; the repair occurring faster than DNA repair in this organism (Jacob, 1975). The nature of this restoration of membranes is left for further investigation. Recently, with the hydrophobic sensitizer acridine, which was active for photodynamic inactivation but was not taken up by the cell, Wagner *et al.* (1980) showed, with a split dose experiment, the existence of recovery from inactivation in *E. coli* strains regardless of the mutants (DNA repair proficient and deficient) and, interestingly, they also showed that this recovery needed 30–45 min of postirradiation incubation. Moreover, the cells previously sensitized to lysozyme by acridine plus near-UV radiation lost that sensitivity to lysozyme upon subsequent incubation of 45 min under growth conditions. Likewise (Wagner *et al.*, 1982), the susceptibility of treated cells to a number of membrane perturbation agents (detergent, osmotic shock) was lost upon incubation. Furthermore, among other evidence, the fact that the inhibition of phospholipid synthesis or cell wall synthesis eliminated the split dose recovery response suggests an involvement of membrane repair in response to damage by acridine photosensitization. All of these observations seem to exclude any association with DNA in this type of recovery.

Only a few examples may be cited for the presence of repair in porphyrin-sensitized cellular damage. In Chinese hamster cells, the induced single-stranded DNA was reduced within 30 min of postincubation in growth medium nearly to the level of untreated samples (Gomer, 1980). This was very similar to the results of X-ray experiments performed in parallel. A difference, however, was noted by a sudden inability of repair at a low survival (7×10^{-3}) in the case of porphyrin sensitization. It is not certain that the DNA repair and the increase of survival are directly

related. In skin fibroblasts sensitized by protoporphyrin, Schothorst *et al.* (1980) reported a recovery of SH groups of both membrane and cytosol, and of the tryptophan content by postirradiation incubation in growth medium. The same condition regenerated the surviving cells.

3.6. Summary

Selectivity of various photodynamic sensitizers in causing damage to the nucleus, cell membrane, and cytoplasm was discussed based on the specific localization of the sensitizers. Even though the selectivity may depend on a number of factors relating to the type and physiology of the cell, the importance of the elucidation of cell component-sensitizer interaction may be emphasized. Despite of all the problems that remain to be investigated, the field has now reached a state where one can formulate with reasonable confidence a general framework by which the selectivity of cellular photodynamic action for many sensitizers may be predicted, if not completely, at least to a degree that is useful for further experimentation. Studies of detailed action mechanisms and the evaluation of the knowledge derived not only afford one with fine tools in cell biology, but also may help to place much of the empirical knowledge currently employed in clinical trials on a sound scientific basis.

4. PROBLEMS RELATED TO PHOTOTHERAPY

Recent concerns on the effectiveness and the safety of the photodynamic techniques for the treatment of herpes simplex virus infection has brought about the needs of further basic research (Bockstahler *et al.*, 1977). The photodynamic treatment consists of application of a sensitizer dye to the herpes lesion and subsequent exposure to visible light. This inevitably involves viruses, sensitizers, and cells as a system. Photodynamic sensitizers often used for the therapeutic purposes are proflavine, methylene blue, and neutral red (Bockstahler *et al.*, 1977; Melnick and Wallis, 1977; Caldas *et al.*, 1982). As discussed at length in the previous sections, proflavine belongs to the class of acridines whose major site of action is cellular DNA. Thus, as already noted, proflavine plus light is mutagenic and may well be carcinogenic, as shown by Santamaria *et al.* (1980). This latter notion would also gain a general support from the current thinking that carcinogenesis would involve DNA damage and repair (Smith, 1981). It is also conceivable on the same basis that the induction of virus occurs from its prophage state in acridine orange-sensitized *E.*

coli cells (Freifelder, 1966). Infectious simian virus (SV40) was induced by proflavine-mediated photodynamic action from SV40-transformed cells (Bockstahler and Cantwell, 1979). SV40 is known to produce tumors in hamsters and can transform mammalian cells in culture. The possibility of the unmasking of oncogenic potentials of tumor virus by photodynamic treatment is a major warning against its therapeutic applications (Rapp *et al.*, 1973; Li and Rapp, 1976; Rapp and Kemeny, 1977; Oxman, 1977). Also, the possibility of the enhancement of incorporation of viral DNA sequences into photodynamically treated cells is suggested based on the increased biochemical transformation of virus (UV inactivated) infected cells sensitized with proflavine at a low treatment level where no major inhibition of either viral production or cellular DNA synthesis occurs (Verwoerd and Rapp, 1978). The interaction of viral and cellular nucleic acids is amply shown under proflavine-mediated photodynamic treatment by the presence of Weigle-reactivation and a Luria-Latarjet type experiment (Lytle and Hester, 1976).

Some of the possible dangers of tumor induction by the treatment of virus infection might be avoided if DNA-attacking photodynamic sensitizers are avoided. Acridine (lipid soluble) could be one such agent. As mentioned earlier this acridine damages virus coat (lipid bilayers) in the presence of light, and prevents the infection (Snipes *et al.*, 1979). Cells are also killed by the same agent via membrane damage. There are many sensitizers known to act on membranes (Section 3.3) or cytoplasmic organelles (Section 3.4). Methylene blue is clearly different from proflavine or acridine orange in the mode of photodynamic action; it acts on cellular DNA to a much lesser degree but might act on intracellular virus DNA under appropriate conditions. According to the experiments of methylene blue with *E. coli* cells by Caldas *et al.* (1982), the phage-infected cells were much more sensitive than the cells or phages treated separately. A similar finding was reported with cultured mammalian cells infected by herpes simplex virus. The sensitizer in this case was proflavine (Lytle and Hester, 1976). Thiopyronine, whose major action site is the cytoplasm, has been thought of as a candidate deserving clinical trials (Lochmann and Micheler, 1979). Neutral red is similar to thiopyronine in its action mode, but much less basic information is available. Every effective sensitizer is not ideal for therapy, but as knowledge on the mode of sensitizing action advances we may even be able to tailor a photodynamic agent with optimum potential for a specified purpose.

Hematoporphyrin derivative has recently been used successfully for the phototherapy of certain cancer metastatic to skin (Kelly *et al.*, 1975; Dougherty *et al.*, 1978). We can reason from cellular level studies that this compound has superior characteristics in its action mode. The major

damage that inactivates the cell seems to be membrane damage, and DNA damage should play a minor role (Section 3.3.2).

To have a proper insight on the action of porphyrin species on malignant tissues it seems important to recognize the difference between isolated cells and tissues. The basic knowledge about the uptake and the localization of porphyrin species in the cell has only begun to accumulate (Section 3.3.2-3.3.4). Since porphyrins can sensitize not only living cells but also many kinds of biological molecules (Spikes, 1975), they would induce multiple photodynamic damage in the cell depending on the localization. There are other complications for tissues that contain several types of cells differentiated for specific functions. In malignant tissues, the administered hematoporphyrin derivative is not retained in tumor cells themselves but mostly in the vascular systems, and vascular collapse is the first to be observed upon irradiation of malignant tissues (Dougherty *et al.*, 1981). It is interesting to note that Konrad *et al.* (1975) observed probably the same vascular photolysis in protoporphyrin mice upon irradiation; the selective destruction of endothelial cells and basal lamina of the blood vessels occurred. It was reasoned that protoporphyrin circulating in the serum sensitized endothelia as the primary target of photochemical reaction. Perhaps, more rigorous experimentations are desirable on the system which consists of multicomponents (tumor cells, surrounding tissues and other environmental components such as serum albumin and oxygen). Eighty percent of intraperitoneally injected hematoporphyrin in rats, upon reaching the liver, was recovered in the extracellular compartment, and this was readily eliminated after several hours (Cozzani *et al.*, 1981). Serum albumin, due to its strong binding affinity to porphyrins and also due to its potential to affect the photochemical properties of porphyrin species when bound, may well play an important role in the whole process of porphyrin photosensitization of tissues (Moan and Sommer, 1981; Reddi *et al.*, 1981).

Finally it may be pointed out that in most of the *in vivo* situations the concentration and the solvent conditions are favorable for the aggregation of the porphyrins even for a relatively hydrophilic species. The state of aggregation could affect the adsorption and accessibility to the effective sensitizing sites in the cell or tissues besides anticipated changes in photochemical reactions. Such basic questions as the effectiveness and the reaction mechanisms in aggregated or nonaggregated state of porphyrins must also be studied in relation to localization both in *in vivo* and a model system.

Phototherapy of a newborn child with abnormal bilirubin metabolism has also been questioned for the risk of possible DNA damage by near-UV radiation. Near-UV radiation by itself could cause unwanted muta-

tions or even tumor induction (Speck *et al.*, 1980). It is reasonable to think that possible endogenous substances may act in multiple ways as a photosensitizer when near-UV radiation is applied. Although the recent development in near-UV radiation studies is remarkable (Jagger, this volume; Webb, 1977), the present knowledge is still not sufficient to predict all of its action pathways at the cellular level.

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Photoacoustic Spectroscopy and Related Techniques Applied to Biological Materials

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1. INTRODUCTION

The absorption of light by living organisms is important both as a probe of biochemical processes at the molecular level, and as the stimulus for myriad photobiological processes. Typically, light absorption may be characterized by measuring either the transmission or the reflectance spectrum; however, most biological systems *in situ* are not amenable to these measurements due to opacity, scattering, poorly defined or heterogeneous surface properties, etc. Thus, it is of interest to have a technique for measuring the absorption of light that is less constrained by the nature of the material under study. Photoacoustic spectroscopy (PAS) clearly meets this requirement while offering new information that arises uniquely from the combination of spectroscopic and calorimetric phenomena. In certain respects PAS is a qualitative spectroscopic technique, the spectra (except in special cases) are only similar to conventional absorption spectra; also for complex biological samples there is no general method of extracting extinction coefficients or concentrations from the observed signal. On the other hand, photophysical parameters such as quantum yields, lifetimes, and energies, characterizing the various excited states and relaxation pathways of photobiological systems *in situ*, can sometimes be measured by PAS. In considering PAS, it is perhaps useful to keep in mind that just as the fluorescence excitation spectrum is the action spectrum for fluorescence, the photoacoustic (PA) spectrum is the action spectrum for the production of heat.

Several general reviews have recently appeared (Rosencwaig, 1978; Cahen *et al.*, 1980; Somoano, 1978; Balasubramanian and Rao, 1981). The present review focuses on applications, examples, and techniques germane to biological systems.

2. DESCRIPTION OF THE PHOTOACOUSTIC EFFECT; IMPORTANT ASPECTS FOR BIOLOGICAL EXPERIMENTS

2.1. Photoacoustic Spectroscopy

A number of quantitative treatments of PAS have appeared, and an essential consensus has emerged regarding the origin of the phenomenon

(Rosencwaig and Gersho, 1976; Murphy and Aamodt, 1977; Aamodt *et al.*, 1977; Bennett and Forman, 1977; McDonald and Wetsel, 1978; McDonald, 1980). Consider Fig. 1 and assume a solid or liquid sample in an enclosed volume allowing a small air-space containing a coupling gas (air) above the sample. Light from a monochromator, which can be scanned, is chopped and is incident on the sample surface exposed to the air-space. A fraction of the light absorbed by a molecule at a point in the sample is converted to heat, which then diffuses, *via* thermal conduction, to the sample surface. At the surface, a thin boundary layer of gas is slightly heated, expands, and creates a pressure wave (sound wave) that fills the enclosed volume and is sensed by a transducer such as a microphone. This sequence of events, a thermal wave in the sample driving thermal expansion in the thin layer of gas that in turn drives a pressure wave in the cell chamber, accounts for the PA response in most cases.

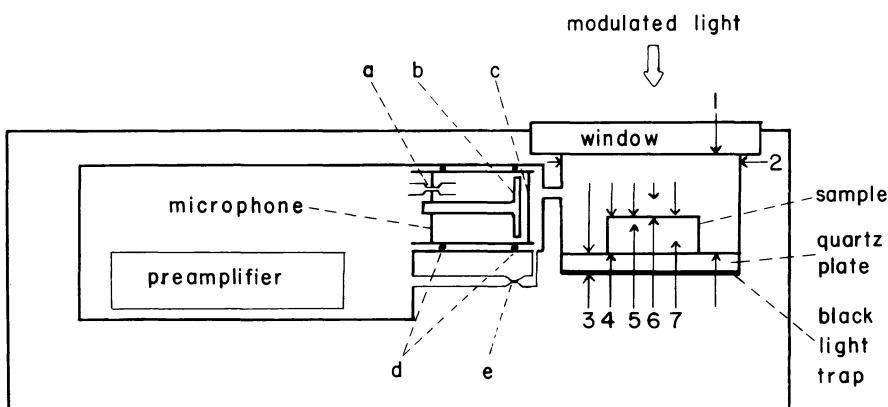


Fig. 1. Schematic diagram of a typical PAS cell including sample chamber, microphone, and preamplifier. The sample chamber is larger than scale. The numbers refer to the various lengths that determine the photoacoustic response of the sample and are defined as follows: (1), (2) sample chamber dimensions, (3) thickness of sample backing or transparent quartz plate covering a black light trap, (4) sample thickness, (5) one thermal diffusion length in the sample, μ_s , (6) one thermal diffusion length in the coupling gas, μ_g , (7) optical absorption length, $\beta_{\text{optical}}^{-1}$, where $\beta_{\text{optical}} = (2.303)$ (optical density per cm). The letters refer to the principal parts of a condenser microphone installed in a PA cell: (a) controlled equalization vent for the microphone; this vent determines the low frequency limit of the microphone alone, (b) electrode or plate behind the diaphragm; the change in capacitance as the diaphragm is deflected between the diaphragm and this plate is the basis for signal generation by a microphone, (c) microphone diaphragm, (d) sealing rings preventing pressure leakage around the microphone, (e) controlled equalization vent connecting the sample chamber with the large chamber containing the microphone and preamplifier; this vent determines the low frequency limit of the PA cell. The cell must also have a means of changing the sample such as a removable window and electrical connectors for the signal and preamplifier power supply.

However, under certain conditions one may detect the much smaller acoustic wave produced in the sample along with the thermal disturbance upon relaxation of the excited state (McDonald and Wetsel, 1978). This acoustic wave propagates in the sample as sound (not according to thermal conduction), and is conveniently sensed by a piezo-electric detector attached to the sample itself. In the photoacoustic experiments discussed herein (involving solid or semisolid samples, a coupling gas, and microphone detection), this acoustic wave *in the sample* makes only a small contribution to the signal.

The periodic fluctuations in the coupling gas, driven at the chopping frequency of the incident light, produce a periodic alternating current (AC) signal at the microphone output, which is amplified and converted to a direct current (DC) signal by a lock-in amplifier. With microphone detection, the photoacoustic response is intrinsically AC; the microphone has an equalization vent across the diaphragm (Fig. 1a) that determines its limiting low frequency response. In other words, the microphone does not produce a signal at constant (DC) pressure. As can be seen from Fig. 1, the limiting low frequency response of the PA cell is determined by the equalization vent (e).

2.2. Photoacoustically Detected Absorption Spectrum

Provided PA saturation [in Fig. 1 the length (7) \gg (5), Section 2.3] is avoided, scanning the monochromator produces a spectrum with the signal (S) at each wavelength given by

$$S \propto \Phi_h \beta_{\text{optical}}(\lambda) P(\lambda) R(\omega) \quad (1)$$

where $R(\omega)$ describes the photoacoustic response of the sample and cell. $R(\omega)$ is a complex function of modulation frequency (ω) and, as indicated in Fig. 1, the sample thickness and thermal diffusion lengths in the sample and surrounding gas (Section 2.3). $P(\lambda)$ is the optical power as a function of wavelength of the incident, modulated light beam; $\beta_{\text{optical}}(\lambda)$ is 2.303 times the optical density per cm as a function of wavelength, and Φ_h is the fraction of the absorbed light converted to heat (Murphy and Aamodt, 1977; Rosencwaig and Gersho, 1976). The function $S[P(\lambda)]^{-1}$ is proportional to β_{optical} , and thus to the absorption spectrum. $P(\lambda)$ is obtained by measuring the spectrum of a black body absorber, usually soot or carbon powder. The general intent of detailed theories is the evaluation of R so that S can be quantitatively related to optical and calorimetric parameters of the sample. In certain cases, especially liquid samples,

β_{optical} can be determined quantitatively (Poulet *et al.*, 1980; Malkin and Cahen, 1981). Work in this area is clearly important to future photobiological applications.

2.3. Thermal Diffusion Length and Photoacoustic Saturation

The modulation frequency of the incident light defines a key parameter, the thermal diffusion length (μ). μ is one of the lengths in R (Eq. 1) and is

$$\mu = \left(\frac{2\alpha}{\omega} \right)^{1/2} \quad (2)$$

where α is the thermal diffusivity ($\text{cm}^2 \text{ s}^{-1}$), and ω ($\omega = 2\pi f$) is the modulation frequency. There is a thermal diffusion length in both the gas (μ_g) and in the sample (μ_s). μ_g defines the boundary layer of gas that is slightly heated upon diffusion of heat to the sample surface and for air has a value of 0.6 mm at a chopping frequency of 20 Hz. μ_s may be thought of as the distance that heat travels in the sample during the period of the modulation. Each successive cycle damps the fluctuation induced by its predecessor, thus limiting the depth from which a PA signal can be obtained. μ_s defines the optical path length in PAS experiments, and ranges from $\sim 4 \mu\text{m}$ (2 kHz modulation) to $\sim 170 \mu\text{m}$ (1 Hz modulation) for a thermal diffusivity of $0.001 \text{ cm}^2 \text{ s}^{-1}$, a value characteristic of biological materials. Quantitatively, μ_s is the distance over which the thermal wave in the sample is damped to e^{-1} of its original magnitude.

Photoacoustic saturation (McClelland and Kniseley, 1976) occurs when $(\beta_{\text{optical}})^{-1} \leq \mu_s$, that is, a significant fraction of the incident light is absorbed within one μ_s . In this case, the signal amplitude no longer depends upon β_{optical} , and the spectrum becomes the power spectrum $P(\lambda)$ mentioned above. The onset of saturation is shown by peak flattening, since division by $P(\lambda)$ (a carbon soot sample is completely saturated at all wavelengths) yields a constant. It should be noted, however, that in certain cases spectra may be obtained from phase measurements even from very concentrated [$(\beta_{\text{optical}})^{-1} \ll \mu_s$] samples (Poulet *et al.*, 1980). Also, several techniques have been presented for “diluting” concentrated (pure solid) samples so that saturation is avoided (Fuchsman and Silversmith, 1979; Lin and Dudek, 1979). In order to avoid complicated three dimensional effects (Quimby and Yen, 1979; McDonald, 1980, 1981) it is important that the edge of the sample not be illuminated, i.e., the exciting light should not be closer than several times μ_s to the edge of the sample.

There should also be a space of several times μ_g between the illuminated portion of the sample and any cell wall or the cell window.

2.4. Amplitude and Phase of the Photoacoustic Signal

As mentioned above, the signal from the microphone is AC, and hence carries in addition to frequency two kinds of information, amplitude and phase (Mandelis *et al.*, 1979; Poulet *et al.*, 1980; Adams and Kirkbright, 1977). In general, PA spectra are plots of signal amplitude vs. wavelength. The amplitude of the PA signal is strongly dependent on a number of factors, including characteristics of the sample such as surface area, the presence and thickness of a waxy coat, and the reflectivity and opaqueness of the sample. Since PAS is a single beam technique, the major factors affecting signal amplitude are instrumental factors. Signal amplitude may be related to the concentration of the absorber by a calibration curve, provided sample preparation and instrumental factors are carefully controlled (Castleden *et al.*, 1979). Also, fluorescence quantum yields may be measured by a careful comparison of signal amplitudes between a nonfluorescent reference sample and the fluorescent unknown sample (Section 2.5.2). However, considerable information, especially in the case of *in situ* biological systems, is contained in the phase of the PA signal. Furthermore, except in the case of a two phase lock-in analyzer, one must, in fact, select a phase in order to record a spectrum.

The measured phase angle of the signal (θ_m) is defined with respect to the modulated incident light. Factors affecting θ_m include: (1) processes intrinsic to the PA phenomena such as thermal diffusion and the production of the pressure wave, and herein designated θ , (2) processes such as the relaxation of excited states with lifetimes of the order of the modulation period (ϕ), and (3) strictly experimental processes. Fortunately, it is often possible, in at least a semiquantitative way, to separate these contributions so that photobiologically significant information can be extracted from the phase of the signal.

Experimental contributions include such things as the phase shift due to the microphone, which is especially large and frequency dependent at low frequencies, and phase shifts in the signal processing electronics. It must be remembered that the major contribution to the absolute phase of the signal is the angular relationship (around the light chopping wheel) between the incident light beam and the electro-optic pick-up for the lock-in reference. Except in cells designed to operate at resonance, the dimensions of the enclosed volume [(1), (2) in Fig. 1] are usually much less than the wavelength of sound at usual frequencies so that acoustic resonance and concomitant phase shifts are not observed.

For a homogeneous, thermally thick [in Fig. 1, (4) \gg (5)] sample the phase θ of the PA signal is given by (Poulet *et al.*, 1980).

$$\tan \theta = \beta_{\text{optical}} \mu_s + 1 \quad (3)$$

From this expression it is clear that for cases where $\beta_{\text{optical}} \mu_s \ll 1$ there is no appreciable phase shift as a function of β_{optical} , i.e., over the absorption band. In the case where $\beta_{\text{optical}} \mu_s$ is ≥ 0.1 , this equation may be used to determine β_{optical} , and thus the absorption spectrum quantitatively (Poulet *et al.*, 1980).

If one considers a biological sample in which different chromophores are present at different depths from the sample surface, the phase and modulation frequency dependence of the signal are quantitatively complicated (Fernelius, 1980). However, certain information about this chromophore distribution at depths ranging from $\sim 1 \mu\text{m}$ to $\sim 200 \mu\text{m}$ from the sample surface can be obtained from the frequency and phase of the PA signal. The phase of the signal is completely specified by the in-phase and 90° out-of-phase (quadrature) components. It is reasonable to think of one of these components as biased towards response from the upper half of μ_s , and the other component as biased towards response from the lower half of μ_s . Thus, the signal from the sample "interior" is phase shifted by the transit time for heat diffusing to the surface. In this way the overall depth of analysis is controlled by frequency (ω) through μ_s while the upper or lower parts of μ_s can be selected by the appropriate choice of phase. This approach is sometimes referred to as depth profiling, and has been described in detail by Adams and Kirkbright (1977).

2.4.1. Stratified Chromophores, Model Studies

An example of the use of phase information for a layered sample is presented in Fig. 2. A plastic film approximately $25 \mu\text{m}$ thick was marked on the upper surface with a blue dye and on the lower surface with a red dye. The lower surface was sealed with a light film of grease to the aluminum surface of the sample holder. The spectrum was scanned using a chopping frequency such that $\mu_s > 20 \mu\text{m}$. Referring to the inset, it can be seen that two pieces of information completely characterize the response of the sample to the modulated light: either the resultant $A(\lambda)$ and the angle $\theta_m(\lambda)$ or $A \cos \theta_m$ and $A \sin \theta_m$ may be recorded (the use of a two-phase lock-in analyzer and a two channel analog to digital converter allows simultaneous recording of both spectra during a single scan). Once these two spectra are obtained, there is no additional PA information available at the selected chopping frequency. From the phasor diagram it is clear, e.g., that measuring along the 0° axis will yield a signal that is

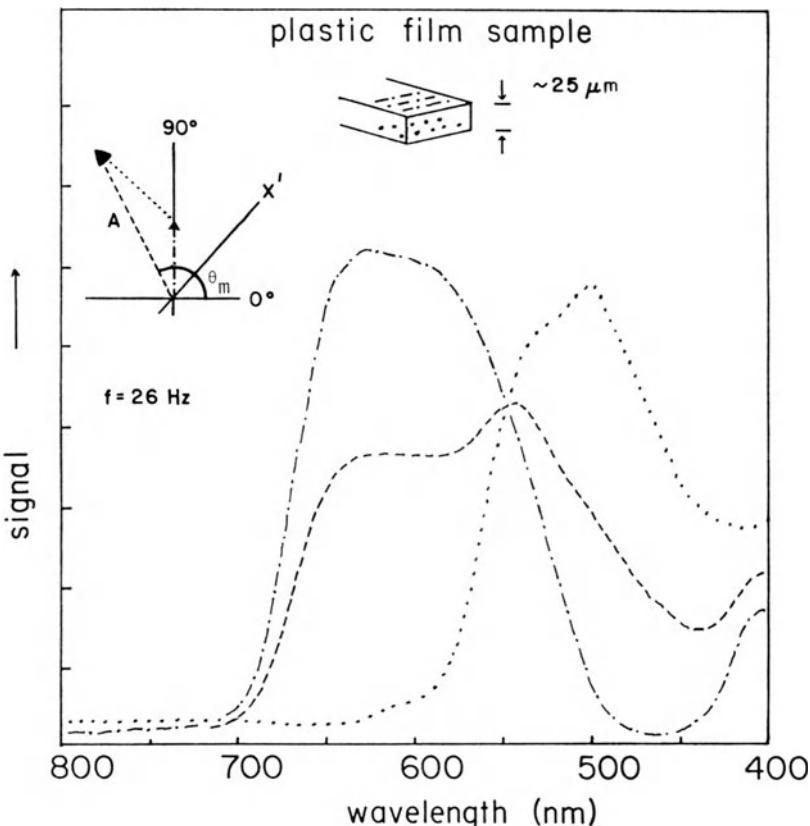


Fig. 2. Two-layer chromophore model. Plastic film, approximately $25 \mu\text{m}$ thick, with a blue dye (—·—·—) on the upper surface and a red dye (·····) on the lower surface, optical density at $\lambda_{\max} \sim 0.5$ for each. The dyes did not diffuse significantly into the plastic, and the lower surface was sealed to the aluminum sample holder with a light film of silicon grease. The composite spectrum (-----) was recorded at a phase setting so that both dyes contributed to the signal. A mixed spectrum was also recorded with a two phase lock-in amplifier set in the phase independent, vector magnitude, or resultant mode. In the inset phasor diagram the phase of the signal from each dye is indicated by a vector of the appropriate symbol. θ_m is the measured phase angle and is with respect to the signal from the electro-optic pickup for the reference channel of the lock-in amplifier. Application of the phasor diagram is fully described in the text. The amplitudes of the spectra cannot be compared to each other.

the projection of the lower surface signal on the 0° axis, but contains no component from the upper surface. Likewise, measurement along the X' axis will be pure upper surface signal uncontaminated by signal from the lower surface. It is important to note that the 0° axis is not in general orthogonal to X' . It is very convenient to have a computer program rotate

the axis and calculate the two spectra at any desired phase setting (Bettidge *et al.*, 1979). As can be seen from comparing Fig. 2 with Fig. 3, the spectra of each dye alone, an essentially uncontaminated spectrum of each dye can be extracted from the data (O'Hara *et al.*, 1981). Similar model studies have shown excellent agreement between the experimental phase shifts and those calculated from theory (Morita, 1981; Helander *et al.*, 1981).

This resolution of depth is unique to PAS; both transmission and reflectance techniques can only provide the sum of these absorbances.

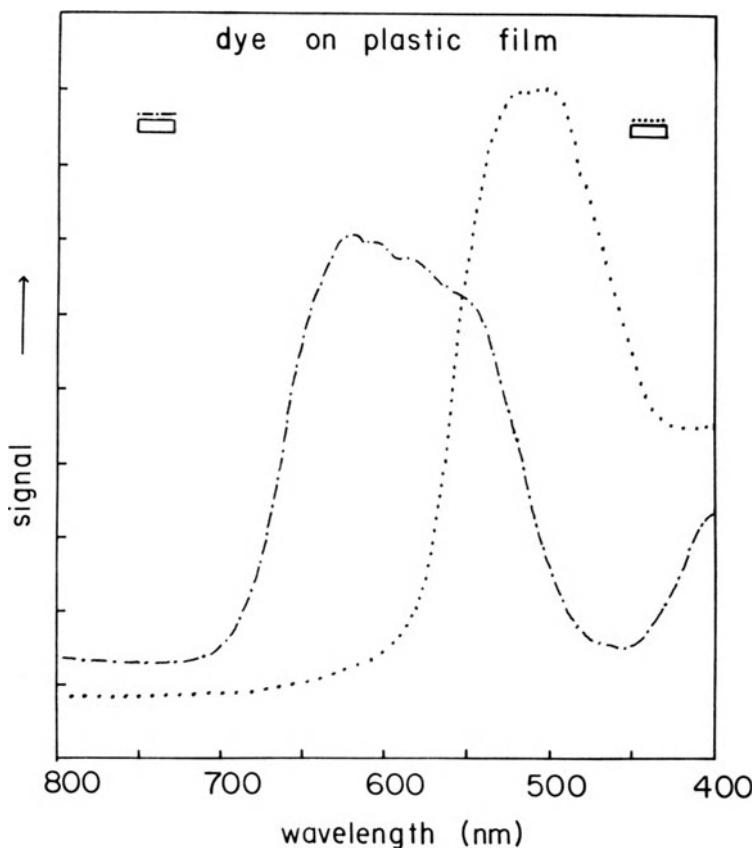


Fig. 3. PA spectra of red ($\cdots \cdots \cdots$) and blue ($- - - - -$) dyes on individual films recorded separately. Experimental conditions are similar to those in Fig. 2. The two phase lock-in amplifier was in the resultant mode. Notice that the spectrum of the blue dye (surface) in Fig. 2 matches exactly the spectrum shown here, whereas the spectrum of the red dye in Fig. 2 is slightly distorted by the filter effect (shading by absorption) of the long wavelength side of the blue dye.

One must, however, proceed with great caution when making measurements on unknowns. The key problem is to determine which phase angle yields the true spectrum. By examining a series of models similar to the one just described, and from a quantitative treatment of the phasor diagram, it is possible to develop certain guidelines for the investigation of unknowns.

(i) At high modulation frequency (1 kHz), and thus short μ_s , one can restrict the response to within a few μm of the surface. Then, at a phase setting shown from models to be surface sensitive at this frequency, one can be confident of recording a spectrum from approximately the first μm of sample. If the spectrum quadrature to the surface is the same as the surface spectrum, then it is likely that there is no layering of chromophores over this μ_s .

(ii) If, at lower modulation frequency and with the phase selected for interior sensitivity, spectra are recorded which differ from the spectra in (i), then it is likely that a different chromophore has been located. From the in-phase and quadrature spectra at this modulation frequency the spectra are calculated as a function of the rotation of the axis. An axis of rotation is searched for that nulls a known spectral band from the outermost chromophore. The spectrum at this rotation will be that of the interior relatively uncontaminated by the more surface chromophore.

(iii) Similarly, a rotation is searched for that nulls a specific band of the interior chromophore. The spectrum at this rotation will be relatively uncontaminated by the interior chromophore. This procedure is facile when one of the chromophores absorbs in a region where the other does not. The correct rotation yields a flat baseline in the spectral region where only the interior chromophore absorbs.

(iv) It is, in principle, possible to make use of specific stains to mark known regions of cells or organelles. For example, a cell wall stain could be used to mark the cell wall of an algae, thus allowing one to determine the correct phase for observing chromophores located in the cell wall.

It is important to realize that the spectral shapes of underlying chromophores may be distorted by the filter effect of the chromophores nearest the surface. In special cases it may be possible to correct for the filter effect by calculating the light attenuation from known absorption bands. Furthermore, referring to Eq. 3, in cases where $\beta_{\text{optical}} \mu_s \geq 0.1$ at the absorption maximum, then there will be a phase shift between the signal from the band maxima and the signal from other regions of the band or other bands of the same chromophore with lower β_{optical} . In these cases spurious spectra arise upon rotation of the axis. Our model studies have clearly shown that, subject to $\beta_{\text{optical}} \mu_s \leq 0.1$, rotation of the axis results

in uniform reduction of the spectrum to the baseline for samples having a nonlayered distribution of chromophores.

2.4.2. Examples of Naturally Occurring Stratified Chromophores

An early example of the resolution of a naturally occurring stratified system is the work by Adams *et al.* (1976), in which light absorption by the waxy cuticular layer of a green leaf was clearly resolved from the light absorbed by the chloroplasts. The chlorophyll-like spectrum was recorded at a phase setting optimizing the signal at 650 nm, and the spectrum rich in cuticular absorption recorded quadrature to this phase. Figure 4 presents the spectrum of an oat seedling taken in our spectrometer; spectra of a variety of leaf types have clearly reproduced the results of

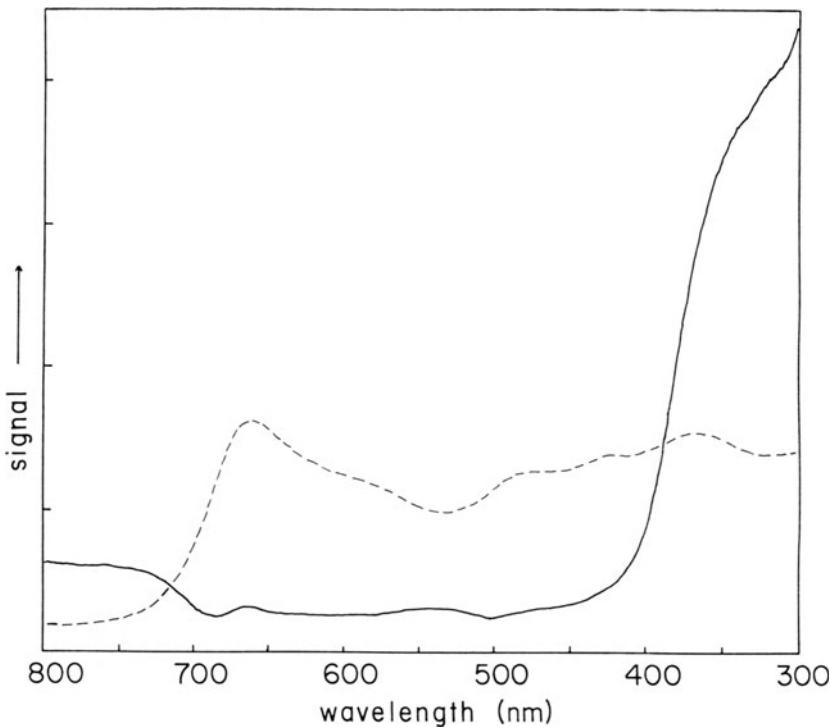


Fig. 4. PA spectrum of an oat seedling after axis rotation to null the 500–650 nm signal from the chloroplasts (—), and to null the cuticular signal in the near-UV region (— —). The optical bandpass was 10 nm, the modulation frequency was 12 Hz, and the lock-in amplifier output bandwidth was 0.1 Hz.

Adams *et al.* (1976), and illustrate the use of (i)–(iii) above in selecting modulation frequencies and finding the best phase for a particular spectrum.

We have measured the PA spectrum of lobster shell at different modulation frequencies and phases, and have found an unexpected arrangement of protein–pigment complexes (carotenoproteins) in the pigmented layer of the shell (Mackenthun *et al.*, 1979). The carotenoproteins having the longest wavelength absorption maxima were found in the interior-most region of the pigmented layer. This anisotropic distribution of carotenoproteins as a function of depth is destroyed by denaturing the protein, clearly indicating that tertiary and quaternary structural features are involved in the pigment-protein interaction.

In Section 3.2, a study of pigment stratification in lichens is presented.

2.5. Photoacoustically Detected Relaxation of Excited States; The Measurement of Photophysical Parameters

2.5.1. Quantum Yields, Energies, and Lifetimes of Metastable States

A unique feature of the PA effect is that absolute values of photophysical parameters such as quantum yields, energies, and lifetimes of long-lived (from submillisecond to subsecond) metastable excited states can be determined from relative phase shift measurements (Moore, *et al.*, 1982). Consider the Jablonski diagram (Fig. 5) in which the metastable state in this case is the lowest excited triplet state. It can be seen that the relaxation rate from $T_1 \rightarrow S_0$ will be characteristic of the triplet lifetime, whereas the relaxation processes $S_1 \rightarrow S_0$ and $S_1 \rightarrow T_1$ will be much faster. Thus, at an appropriate modulation frequency the heat produced from $T_1 \rightarrow S_0$ relaxation ($h_{\text{slow}}(\omega)$) will be phase shifted from that produced by $S_1 \rightarrow S_0$ and $S_1 \rightarrow T_1$ processes (h_{fast}). Quantitatively, for sinusoidal modulation

$$h_{\text{slow}}(\omega) = \frac{k_{\text{isc}}}{k_s} \cdot \frac{\epsilon_T}{1 - i\omega\tau} \quad (4)$$

and

$$h_{\text{fast}} = \Delta\epsilon_1 + \frac{k_r}{k_s} \Delta\epsilon_0 + \frac{k_{\text{nr}}}{k_s} \epsilon_s + \frac{k_{\text{isc}}}{k_s} (\epsilon_s - \epsilon_T) \quad (5)$$

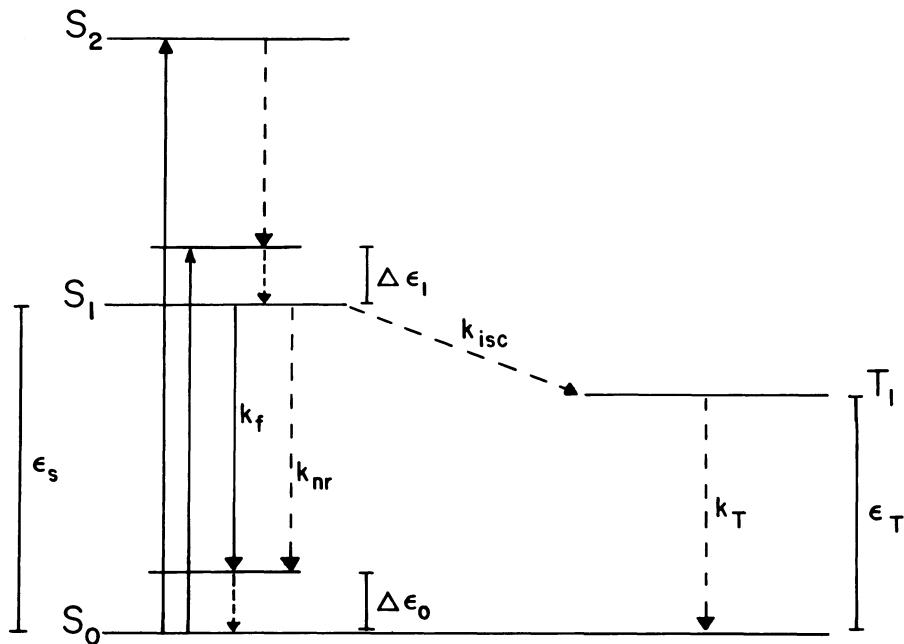


Fig. 5. Jablonski diagram. The vibrational levels in the ground (S_0) and first excited singlet (S_1) state are replaced with a single level corresponding in the case of absorption to the excitation wavelength, and in the case of emission to the fluorescence maximum, ϵ_s , $\Delta\epsilon_1$, $\Delta\epsilon_0$ and ϵ_T are the energy differences between the states indicated by vertical bars. Radiative (—) and non-radiative (----) processes are indicated; phosphorescence is ignored under the conditions of this experiment. k_f , k_{nr} , k_{isc} and k_T are the radiative, nonradiative, intersystem crossing and triplet decay rate constants, respectively.

where τ is the triplet lifetime and k_s is the total decay rate of S_1 . The complex amplitude $P(\omega)$ of the resulting PA signal has the form

$$\begin{aligned} P(\omega) &= R(\omega)[h_{\text{fast}}(\omega) + h_{\text{slow}}(\omega)] \\ &= P_{\text{fast}}(\omega) + P_{\text{slow}}(\omega) \end{aligned} \quad (6)$$

where $R(\omega)$ is the response function describing the diffusion of heat in the sample and its coupling to the gas. Although $R(\omega)$ is not known explicitly, it is the same function for both fast and slow processes. This fact allows one to design experiments to measure the relative phase shift. The phase lag ϕ between the slow and fast components is determined by h_{slow} and h_{fast} , and is given by $\tan \phi = \omega\tau$, and the phase θ , between $P(\omega)$ and

P_{fast} is given by

$$\tan \theta_r = \frac{a\omega\tau}{a + b(1 + \omega^2\tau^2)} \quad (7)$$

where

$$a = \frac{k_{\text{isc}}}{k_s} \epsilon_T \quad (8)$$

and

$$b = \Delta\epsilon_1 + \frac{k_r}{k_s} \Delta\epsilon_0 + \frac{k_{\text{nr}}}{k_s} \epsilon_s + \frac{k_{\text{isc}}}{k_s} (\epsilon_s - \epsilon_T) \quad (9)$$

This function depends only on $\omega\tau$ and the parameters of the energy level scheme; from a plot of $\tan \theta_r$ vs ω values of a and b can be determined. From the value of the parameter a , one can determine either the energy of the metastable state or the quantum yield of its formation. In order to measure θ , it is necessary to prepare a reference sample in which there is no slow decay component. This can be accomplished by preparing a reference sample using an absorber such as β -carotene in which the triplet state is not populated, and therefore all relaxation is fast. It is extremely important that the optical, thermal, and geometric properties of the reference and sample be exactly matched in order that $R(\omega)$ is the same for each. Alternatively, the reference could be the sample in which the lifetime of the metastable state is markedly shortened by quenching processes. The necessity of a matched reference sample probably limits this method to solutions of biomolecules or subcellular preparations in either liquid solvents or plastics. It would be difficult to prepare the proper reference for chromophores *in situ* (however, see Section 2.5.3 for a reference system based on the PA signal in the presence of a strong continuous light).

Fortunately, it is possible to carry out this experiment in a way in which the sample itself acts as the reference. From the Jablonski diagram (Fig. 5) it can be seen that excitation to S_2 results in fast decay to S_1 followed by the photophysical processes, whereas excitation to S_1 results directly in these photophysical processes. In this way the fast relaxation from $S_2 \rightarrow S_1$ provides the reference fast heat. Quimby and Yen (1980) have used essentially this method to measure the quantum yield of fluorescence in ruby and report an accuracy of 0.90 ± 0.05 . It is expected that these experiments can be carried out *in situ* in some photobiological

systems, thus making possible the measurement of photophysical parameters of intact chromophore systems in their native environment.

2.5.2. Fluorescence Quantum Yields

By measuring the ratio of the signal amplitude of a fluorescent sample to that of a nonfluorescent reference material, the energy yield of radiationless relaxation γ can be determined. Φ_f is related to γ by

$$\Phi_f = \frac{\lambda_a}{\lambda_f} (1 - \gamma) \quad (10)$$

where λ_a is the excitation wavelength and λ_f is the mean fluorescence wavelength (Callis, 1976). These measurements rely on the fact that $R(\omega)$ is the same for sample and reference materials. This can be assured in solution studies since β_{optical} can be matched and the thermal, surface, and geometric properties are those of the solvent. Adams *et al.* (1977) have measured Φ_f for a series of dyes in solution by using the fully quenched dye as the reference.

Adams *et al.* (1980, 1981) have also devised a way to measure Φ_f of solid samples by using carbon soot as a reference, even though $R(\omega)$ is not the same for sample and reference. In this case Φ_f can be extracted from a plot of the ratio of the sample signal amplitude to the reference signal amplitude vs wavelength of exciting light over a range of wavelengths where the sample is photoacoustically saturated. This technique may well be the most accurate method for obtaining Φ_f of solids.

2.5.3. Photochemical Energy Conversion

Callis (1976) has pointed out that the heat produced (Q) in a photochemical process excited by N photons of energy E_{in} is

$$dQ = N(E_{\text{in}} - Q_p E_p) \quad (11)$$

where Q_p is the quantum yield of stable product with energy E_p above the ground state. Malkin and Cahen (1979) have developed a general theory for the photoacoustically detected enthalpy changes in a photoactive sample. The relative PA signal of a photoactive sample is given by

$$S(\omega, \lambda) = F_{\beta(\lambda)} G(\omega) \left[1 - \frac{\sum_i \Phi_{pi} \Delta E_{pi(\lambda, \omega)}}{N\hbar\nu} \right] \quad (12)$$

where $S(\omega, \lambda)$ is the PA signal compared to a photoacoustically saturated reference with no photochemical activity; $F_{B(\lambda)}$ is the fraction of the incident radiation ($N\hbar\nu$) absorbed in the distance μ_s ; $G(\omega)$ is an instrumental factor and Φ_{p_i} and ΔE_{p_i} are the quantum yield and stored enthalpy of product p of photochemical reaction i . ΔE_p is a function of modulation frequency since in order to be observed, the product (in many cases intermediate) must have a lifetime of the order of ω^{-1} . By analyzing S as a function of ω , different intermediates are observed; the fastest ones at high frequency and the slowest ones at very low frequency. In favorable cases qualitative rate constants may be assigned. In addition to measuring energies of the intermediates above (or below) the reactant ground state this equation can be used to extract information about the wavelength dependency of photochemical processes. As described below (Section 4) the photocycle of *Halobacterium halobium* has been extensively studied using this approach.

2.5.4. Flash Calorimetry

Callis *et al.* (1972) have devised a strategy for directly determining the enthalpy changes of photophysical and photochemical processes by measuring the volume change of a solution with a capacitor microphone following flash excitation. This technique differs from the photoacoustic measurements discussed herein in two important ways. First, the measurement is in the time domain rather than the frequency domain, and second, the volume change of the liquid solution is measured by the deflection of the microphone diaphragm in contact with the solution (there is no air-space in the cell). An advantage of the time domain measurement for experiments on complicated photocyclic systems such as photosynthesis and *Halobacterium* photoconversion is that the system is allowed to return to a well-defined initial state between excitation flashes. Although volume changes for a given amount of heat production are much smaller in liquid than in gas, the time domain measurements are readily signal averaged and volume changes of $3 \times 10^{-10} \text{ cm}^3$ and enthalpy changes of 1 μcal in the interval of 100 μs to 1 s after the flash were reported.

Volume changes after the flash can arise from two sources (Arata and Parson, 1981, 1982). Expansion or contraction of the solution due to heating or cooling and, in the case of photochemical processes, a volume difference between reactants and products. The total volume change after the flash is given by

$$V = (nE_i - NE_p) \left(\frac{\zeta}{\rho C_p} \right) + N\Delta V_r \quad (13)$$

where n is the number of systems excited by the flash; E_i is the average energy of the photons; N is the number of systems reaching product; E_p is the energy of the product relative to the ground state (defined as zero); ζ is the thermal expansion coefficient; ρ is the density; C_p is the heat capacity per g of solution, and ΔV is the difference in volume between reactants and products. By using the appropriate reference solutions and by making measurements at two temperatures, it is possible to separate the contributions to ΔV and to find the enthalpy changes of the photochemical process. Flash calorimetry has been used effectively in determining triplet state quantum yields in molecular systems (Callis, 1976), in studies of enthalpy changes and proton translocation in photosynthetic systems (Arata and Parson, 1981, 1982; Callis *et al.*, 1972), and in studies of the *H. halobium* photocycle (Ort and Parson, 1978, 1979*a,b*).

2.6. Experimental Techniques Important in Photoacoustic Studies of Biological Systems

2.6.1. Two-Beam Experiments

In spectroscopic studies of photobiological systems undergoing photocyclic or photochromic reactions, it is often desirable to apply two beams of light, one to hold the system in the desired state and one to use for interrogation. PAS is ideally suited for these kinds of measurements since only the modulated light beam will contribute to the signal. Furthermore, it is not possible to saturate the detector (microphone) with a very strong continuous beam even at the measuring wavelength, since DC levels are not detected.

Two beam experiments have been used in the study of the bacteriorhodopsin photocycle (Garty *et al.*, 1978; Cahen *et al.*, 1978*a*), in photosynthetic studies (Callis *et al.*, 1972; Lasser-Ross *et al.*, 1980; Bults *et al.*, 1981) and in our laboratory in the search for signals from phytochrome photoreceptors.

2.6.2. Masking Unwanted Signals

The nature of PAS offers a simple method for restricting the signal to desired regions of the sample. First, of course, the best signal-to-noise ratio will be obtained by tightly focusing the light on the portion of the sample of interest. In this connection, note that the window shown in Fig. 1 can be replaced by a lens. Further restriction of the photoacoustically active region of the sample is obtained by coating it with a film of transparent wax, plastic, or silicone grease. The coating should be several

thermal diffusion lengths thick, and transparent to the wavelength range used in the experiment. Under these conditions the signal from the masked (coated) regions of the sample will be essentially completely attenuated (Fernelius, 1980; Helander *et al.*, 1981). In this way it is not necessary to dissect out for spectroscopic study a single region or organelle from an intact biological specimen. This procedure has been employed in our laboratory in the study of insect eye photopigments *in situ*, and to attenuate the signal from very near the cut edge of leaf samples.

2.6.3. Low Temperature Experiments

PAS experiments have been reported at temperatures as low as 5°K (Pichon, *et al.*, 1979; also Section 2.6.6). One technique is to cool the microphone with the sample, thus avoiding the large thermal gradient (noise) and volume necessary if the microphone is maintained at room temperature (Murphy and Aamodt, 1977). Inexpensive electret microphones such as the Radio Shack 33-1058 or Sony ECM 16 work well at low temperature after modification in which the original J-type FET impedance translator is replaced with a MOS-FET such as the RCA 3N 187. However, Bechthold *et al.* (1982) have developed a cell that can be used with a commercial PA spectrometer and is continuously variable from 90–320°K, and the microphone remains at room temperature. A similar variable temperature cell has been reported by Schneider *et al.* (1982). Boucher and LeBlanc (1981) have used low temperature to block the photobleaching sequence in cattle rhodopsin (rod outer segments), and have measured the spectrum of the photostationary mixture of rhodopsin, bathorhodopsin, and isorhodopsin. Although not yet reported, the use of two beams and low temperature should allow the observation of relatively pure rhodopsin and bathorhodopsin spectra in intact retina.

2.6.4. Photoacoustically Detected Circular and Linear Dichroism

There is an obvious interest in the study of conformationally sensitive chiroptical characteristics of biomolecules *in situ*. Generally, this is not possible using conventional transmission detected circular dichroism due to the opaque nature of biological material. Experiments on solid samples using PA detection of both linear and circular dichroism have been reported (Fournier *et al.*, 1978; Palmer *et al.*, 1979), and a theoretical analysis has been done defining conditions under which the PA amplitude and phase are directly related to $\Delta\epsilon$ (Saxe *et al.*, 1979). While this method may hold considerable promise, a potential problem is the loss of integrity of the circularly polarized light upon multiple reflections (scattering) prior to absorption (scattered light not absorbed within μ_s is of no concern).

In this connection, and subject to the above *caveat*, it should be feasible to measure photoacoustically the linear dichroism of chromophores *in situ*.

2.6.5. Photothermal Radiometry (PTR)

Nordal and Kanstad (1979) have shown that the periodic temperature fluctuation in the sample induced by the absorption of the modulated light may be detected directly as a change in the black body emission from the sample. From the Stefan–Boltzmann's Law

$$W = \epsilon\sigma T^4 \quad (14)$$

and

$$\delta W = 4 \epsilon\sigma T^3 \delta T \quad (15)$$

where W is the total radiant emittance, ϵ is the emissivity, σ is the Stefan–Boltzmann constant, and T is the temperature. It can be seen that modulation of T will modulate W . A detailed theoretical description of PTR has recently appeared in which the conditions under which the signal is linearly proportional to β_{optical} have been discovered (Tom *et al.*, 1982). Although δW may be considerably less than 10^{-7} W at the detector, the experiment is feasible since infrared detectors with noise-equivalent-powers of $10^{-12} \text{ W (Hz)}^{1/2}$ are available.

This technique requires essentially no sample preparation and no sample cell or container. The emission from the sample is imaged onto the detector with appropriate lenses in much the same way fluorescence is gathered and focused onto a detector. The potential of this technique is illustrated by the spectrum of a green leaf with signal to noise ratio ≥ 50 obtained using an Xe arc lamp monochromotor combination of modest output ($\geq 1 \text{ mW}$) and an electrical time constant of 3 s (Nordal and Kanstad, 1981). It is also worth noting that infrared detectors with response times of 10^{-7} s are available, making it possible to monitor relatively fast kinetic processes *in vivo* by PTR.

2.6.6. Photothermal Deflection Techniques (Mirage Effect)

Recall from Section 2.1 that upon absorption of light by the sample a thin boundary layer of gas (μ_g) at the sample surface is heated. Boccara *et al.* (1980*a,b*) have shown that the resulting change in refractive index of the boundary layer of gas can be detected from the deflection of a laser beam passing through the gas boundary layer. For an opaque sample, the

laser beam should be parallel to the sample surface and within one thermal diffusion length in the gas ($\mu_g \sim 0.4$ mm in air at 50 Hz modulation frequency) of the surface. A knife edge blocking half the beam diameter followed by a photodetector makes an excellent position sensitive detector. Boccara *et al.* (1980a) reported visible spectra at room temperature of both an inorganic powdered sample ($\text{Cs}_3\text{Cr}_3\text{Cl}_9$) and of a single crystal ($\text{Nd}_2(\text{MoO}_4)_3$) and, as an illustration of the versatility of this method of detection, they also measured absorption and magnetic circular dichroism of $\text{Nd}_3(\text{MoO}_4)_3$ at 2°K and 0.7°K, respectively. In the low temperature work the refractive index change in the superfluid helium was observed as a standing wave in the tail of the helium Dewar.

These authors reported sensitivity comparable to that of conventional microphone detection, and noted that there is essentially no sample preparation or sample cell (for room temperature work) needed for these experiments.

2.6.7. Transform Techniques in Photoacoustic Spectroscopy; Infrared Photoacoustic Spectroscopy

Due to the detector limited signal to noise ratio usually observed in PA experiments, transform techniques offer considerable time advantage. The need for this advantage was first felt in infrared PAS, where source intensity is especially weak. It is now relatively easy to convert a Fourier transform infrared (FTIR) spectrometer to PA detection (FTIR-PAS, Maugh, 1980) and, indeed, the major manufacturers of FTIR instruments offer PAS cells. Examples of FTIR instruments converted to PA detection include Digilab (Krishnan, 1981) and Nicolet (Chalmers *et al.*, 1981). This makes it possible to obtain infrared spectra of materials that do not transmit infrared light, e.g., most biological materials, without solubilization or other sample preparation techniques that destroy *in vivo* ultrastructures and interactions. Spectra of mg-amounts of protoporphyrin, bovine hemin, hemoglobin and horseradish peroxidase have been measured by Rockley *et al.* (1980). Although certain features in the spectral range where the protein absorbed strongly were unresolved, the structural information characteristically available from infrared spectroscopy is readily apparent.

Fourier transform techniques have been extended into the visible range, and a detailed comparison to the conventional technique presented (Lloyd *et al.*, 1980). In special cases the Fourier transform time advantage could be very useful, but it appears that applications in the visible range to biological systems will be limited by technological (interferometer alignment) and fundamental (it is difficult to extract phase and modulation

frequency, i.e., depth, information) problems. Gray *et al.* (1977) have suggested the use of Hadamard transform techniques, perhaps a very good suggestion especially for improving the signal to noise ratio in the ultraviolet region where source intensity is weak.

3. PHOTOACOUSTIC STUDIES OF CHLOROPHYLL-BASED PHOTOSYNTHESIS

3.1. Chloroplasts, Reaction Centers, and Leaves

Callis *et al.* (1972), using the flash calorimeter, measured volume, and enthalpy changes upon flash excitation of *Chromatium* chromatophores in order to resolve into enthalpy and entropy components the free energy storage upon light-driven electron transfer from cytochrome C₅₅₅ to a quinone acceptor (Q_B). Several novel experiments were pioneered with this work, and have been used in subsequent efforts. A photochemically saturating continuous light incident on the sample was used to provide a reference. This reference has exactly the thermal and optical characteristics of the sample; it differs only in that the flash does not elicit photochemical changes. Also, ionophores, uncouplers, and various buffers were used in order to perturb the chromatophores in a predictable way. These authors found that the increase in free energy upon flash excitation of these chromatophore preparations is almost totally in the form of a large negative entropy change.

Later work by Arata and Parson (1981, 1982) with light-driven electron transfer from P₈₇₀ to quinone Q_A in reaction center preparations of *Rhodopseudomonas sphaeroides* indicates that in the state $P^+Q_A^-$ free energy is stored as negative entropy. However, in preparations containing both Q_A and Q_B, the formation of the state $P^+Q_AQ_B^-$ results in a substantial enthalpy increase and little change in entropy. These results and the contrast between the results from *Chromatium* chromatophores and *R. sphaeroides* reaction centers is fully discussed in the references above.

Cahen *et al.* (1978b) reported a study of photosynthesis in a chloroplast preparation using the more conventional coupling gas-microphone technique. They found that the PA spectrum very nearly matched the absorption spectrum obtained with an integrating sphere. In this preliminary report they also noted that treatment with 3-(3,4-dichlorophenyl)-1,1-dimethyl urea (DCMU) changed the ratio of blue to red region signal strength, and that the extent of this change depended on the chopping

frequency. In later work (Lasser-Ross, *et al.*, 1980) on this system, a strong continuous (DC) light was used to saturate the photochemical processes of photosynthesis and the expected increase in the PA signal was observed [e.g., less photochemical loss (Malkin and Cahen, 1979)]. Furthermore, measurements of this photochemical loss as a function of wavelength and electron acceptors such as methylviologen could be interpreted in terms of cyclic photodriven electron flow around Photosystem I. These authors made the observation that fluorescence and PA techniques seem to report on different aspects of the photosynthetic process.

When Bults *et al.* (1981) extended their work to include whole leaves, an important observation was made. At low modulation frequency the PA signal *decreased* upon application of a strong DC light. The authors interpreted this result in the following way. At low modulation frequency the PA signal consists in large part of modulated O₂ evolution. The strong DC light drives the O₂ evolution to saturation, and hence the modulated O₂ evolution and concomitant PA signal decreases. At high modulation frequency, above 200 Hz, the slower O₂ evolution process does not contribute to the PA signal and the expected increase in signal upon application of the DC light was observed. DCMU vacuum infiltrated into the leaf completely abolished the change in PA signal upon application of DC light at any modulation frequency. Further evidence was provided by the work of Bults *et al.* (1982) in which PTR was used in a similar experiment (see also Canaani *et al.*, 1982; Kanstad *et al.*, 1983). Since PTR is sensitive only to heat production, the evolution of O₂ will not contribute to the signal. Indeed, it was found that over the frequency range 15–120 Hz (poor signal to noise ratio prevented experiments at higher frequency) the application of a saturating DC light always resulted in an increase in the photothermal signal. This observation supports the interpretation that modulated O₂ evolution does contribute to the PA signal at low frequency. These results lay the groundwork for facile methods of measuring the action spectrum for the production of O₂ and the production of heat and should open up new avenues of research in photosynthetic energy conversion.

Inoue *et al.* (1979) reported the transient variation of the PA signal from intact leaf sections upon irradiation with modulated light. The transient signals are large, undergo light and dark adaptation reminiscent of the Kautsky effect, are inhibited by DCMU or heating, and are kinetically distinct from the transient fluorescence changes. In preliminary work in our laboratory we have observed large changes in the PA signal from oat seedlings over a period of minutes. These changes in signal amplitude depend on the light/dark history of the leaf and the wavelength and intensity of a continuous light. It seems clear that perturbations of

the photosynthetic process give rise to large changes in the PA signal. These signals contain information about the partitioning of excitation energy between the two pigment systems, the energies and kinetics of intermediate states, the extent of saturation of each photosystem, and the effects of drugs.

At this point we mention again (Section 2.4.2) the work by Adams *et al.* (1976) in which the spectrum of the waxy cuticula of a leaf was resolved from the spectrum of the interior chloroplasts. As Adams *et al.* (1976) suggested, it is clear from these results that the cuticular layer attenuates the near-UV light, and thus may play a role in protecting the photosynthetic membranes. However, it must also be pointed out that the intensities of the spectra in Fig. 4 are qualitative. One can say only that the absorption of near-UV radiation by the cuticular layer is similar in strength to the absorption of red light by the chloroplasts. We have found that, as expected, the very thick cuticular layer found on certain desert plants (*agave*) essentially masks the PA signal from the chloroplasts.

It is reasonable to expect that PA techniques will play a significant role in research on energy conversion in photosynthetic membranes. An understanding of the initial forms of free energy storage in the native system is not only of intrinsic value, but may also serve to guide efforts in biomimetic solar energy conversion schemes.

3.2. Algae and Lichens

The PA spectra of a series of freeze dried phytoplankton cultures has been reported by Ortner and Rosencwaig (1977). Excellent signal to noise ratios were obtained from as few as 200 cells mm^{-2} , and in several cases the spectra were better resolved than the corresponding absorption spectra. Recently Yoon *et al.* (1981) measured the PA spectra of several examples of *Porphyra sp.* in which the contributions of the light harvesting carotenoids, biliproteins and chlorophylls to the absorption over the entire visible range can clearly be distinguished. The various peaks correspond very well to those present in the absorption spectra; however, the relative intensities of the bands in the two types of spectra are markedly different. Probably the major factor affecting the relative band intensities in these systems is the disposition of excitation energy between Photosystem I and II. The spectra of these cells was found to be independent of modulation frequency indicating that there are no wavelength dependent photochemical rate processes in the *ms* to *s* range, and no layering of pigment systems along the direction normal to the cell surface (μm range).

The PA spectrum of a monolayer of living cells of *Porphyridium*

cruentum is shown in Fig. 6. Chromatic adaptation is easily shown by comparing the spectra of cells grown under different light conditions. The relative peak heights in these spectra depend strongly on the intensity and wavelength of a continuous light irradiating the cells. Furthermore, the amplitude of the signal from the accessory pigments phycocyanin, phycoerythrin, and carotene increases relative to the chlorophyll band at ~ 670 nm as the concentration of DCMU is increased from 1×10^{-8} M to 1×10^{-7} M (cells grown for several days in DCMU-treated agar). Even though a detailed analysis is not complete, it seems clear that these observations are related to energy distribution between the photosystems.

Figure 7 presents the spectrum at several modulation frequencies of a living sample (~ 4 mm 2) of the lichen *Acarospora schleicheri* (O'Hara *et al.*, 1981). Note that at high frequency the response is limited to the fungal region of the lichen and yields the *in vivo* spectrum of the dominant

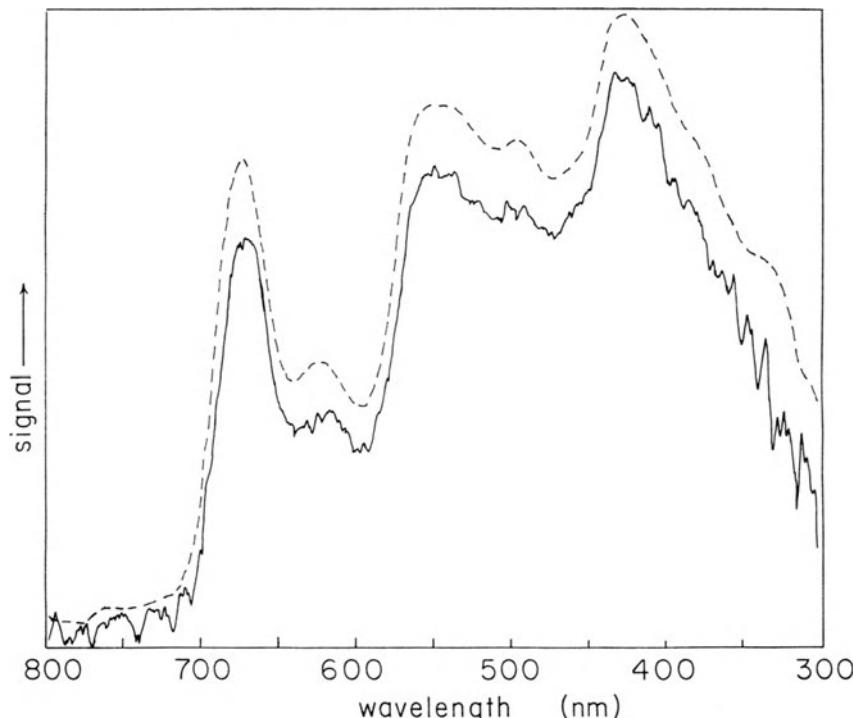


Fig. 6. PA spectra of a monolayer of living *Porphyridium cruentum* cells on agar. (----) Spectrum at 26 Hz modulation frequency and (—) spectrum taken at 1015 Hz. Note the change in signal to noise ratio as the modulation frequency is increased. The optical bandpass was 10 nm, and the lock-in amplifier output bandwidth was 0.1 Hz.

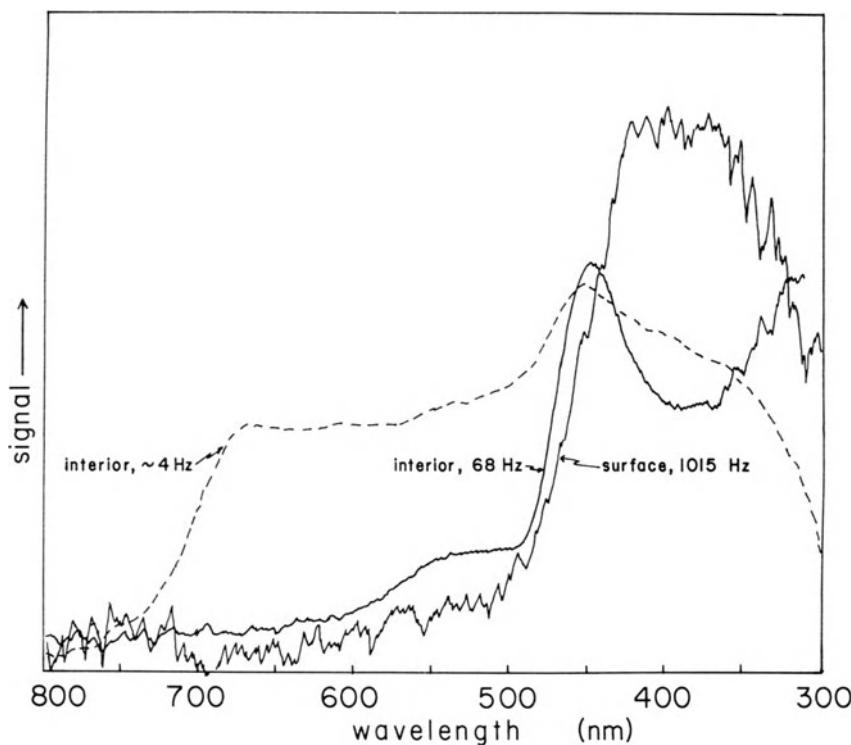


Fig. 7. PA spectra of the lichen *Acarospora schleicheri*. A small section $\sim 4 \text{ mm}^2$ of lichen growing on a rock was placed in the sample chamber. The spectrum at 1015 Hz was taken with the phase optimized for surface response. The spectra of 68 Hz and 4 Hz were recorded with the phase optimized for response from the interior. The optical and electrical bandwidths were as in Fig. 6.

fungal pigment, rhizocarpic acid. It is worth noting that the spectrum of rhizocarpic acid in the native environment differs sharply from that in solution (peak 275 nm, shoulder 375 nm). At this frequency there was no evidence of absorption by the chloroplasts; in order to record a signal from the chloroplasts it was necessary to lower the frequency to 4 Hz (see Section 7.1 for information on microphones that operate at very low frequency). This spectrum clearly shows the chloroplast absorption, making it possible to monitor the algae photosynthesis *in vivo*. We have observed that simultaneous irradiation with a DC source decreases the PA signal, consistent with the results of Bults *et al.* (1981). This response only occurs with hydrated lichen samples. Dry samples, known to be dormant, do not respond to the application of a DC light.

4. PHOTOACOUSTIC STUDIES OF *H. halobium* PHOTOCYCLE

Ort and Parson (1978) reported studies of the purple membrane using the flash calorimeter to measure the solution volume change after flash excitation. A fast, buffer-dependent component of the volume increase following the flash was interpreted as electrostriction of the solvent due to proton release by the membrane. A slower, buffer-independent component of the volume increase could be due to proton translocation on the membrane. The kinetics of the volume change could not be correlated with the kinetics derived from the studies of the photocycle by optical spectroscopy. By making use of the large volume change occurring when protons enter the solution, it was possible to measure the quantum yield of proton release (Φ_{H+}), an important parameter in the mechanism of the proton pump (Ort and Parson, 1979b). At low ionic strength a value of $\Phi_{H+} = 0.25$ was found, in good agreement with the quantum yield for the formation of intermediate, M , and suggesting one proton per photocycle. However, at high ionic strength $\Phi_{H+} = 0.43$ was found while the quantum yield for the formation of M was unchanged, implying that two protons are pumped per photocycle. Additionally, the work of Renard and Delmelle (1981) on the PAS of films of purple membranes demonstrates that the quantum yield of photocycle is independent of pH over the range 5–9. Taken with earlier work by these authors (Renard and Delmelle, 1980), the suggestion was made that $2H^+$ are pumped per photocycle at pH 5–7 and $1H^+$ is pumped per photocycle at alkaline pH. This conclusion is in contrast to the observation by Ort and Parson (1978) that Φ_{H+} does not depend on pH over the range 6.0–8.75. However, a direct comparison is not possible since the work by Ort and Parson (1978) is on membrane fragments in solution while Renard and Delmelle (1981) used films of membrane on filter paper. It is also noted here that for solution samples the time domain liquid-coupled microphone technique used by Ort and Parson (1978) is perhaps better suited for measuring proton flux than the gas-coupled microphone in which the solution volume change makes only a very small contribution to the signal.

Enthalpy changes during the photocycle have also been reported by Ort and Parson (1979a). They find that fast relaxation, proton release and the slow component of expansion are exothermic, and that proton uptake to return to the original state is endothermic by approximately 35 kcal/mol. Since return to the starting state is spontaneous, entropy changes must be rather large, and thus play a significant role in the bioenergetics of the proton pump. It is noteworthy that Garty *et al.* (1980), using the gas coupled microphone and modulation techniques (Section 2.5.3), reported a qualitatively similar picture of the enthalpy changes during the photo-

cycle and, using a continuous light in a two beam experiment, they have recorded a very nice spectrum of the intermediate M_{412} in a film of purple membrane. Again, it appears that although some of the intermediate steps may correlate with those observed by optical techniques, there is no general agreement. However, complete agreement between photoacoustic and optical techniques is not expected since thermochemical changes do not necessarily result in observable optical changes, further illustrating the fact that these techniques are complementary. Garty *et al.* (1981) have discussed in detail the use of modulation techniques in *H. halobium* to extract enthalpy changes and the energy stored at each step of the photocycle.

Bechtold *et al.* (1982) have reported the spectrum of purple membranes in water and in a glycerol-water mixture at temperatures as low as 90°K. Marked spectral changes occur with irradiation time and as a function of temperature, but these results do not parallel those from transmission measurements.

In the case of visual rhodopsin, the only report of PAS is mentioned in Section 2.6.3.

5. PHOTOACOUSTIC STUDIES OF SKIN TISSUE

Rosencwaig and Pines (1977a,b) have reported a rather extensive study of newborn rat stratum corneum. Spectra with a good signal-to-noise ratio in the UV region (220–400 nm) were presented; the peak at 280 nm, due to the aromatic amino acids in the proteins, was clearly resolved. As the tissue matured from birth to age 60 h, a marked change in the 280 nm band was observed. The peak position shifted ~5 nm to the red, and the line width approximately doubled. After considering a variety of structural and chemical changes during keratinization, the authors tentatively suggested that the enzymatic modification of tyrosine, such as hydroxylation, could account for the observed changes. Also, since thermal properties of the tissue are affected by the extent of hydration, it was possible to correlate the signal amplitude with the tissue water content.

Campbell *et al.* (1977, 1979) have carried out extensive studies of the hydration profile or water concentration gradient of human skin. A multilayer theoretical model was developed for the dependence of signal on modulation frequency and thermal properties, and was used to interpret the experimental data. It was found that the transition from hydrated to dry tissue occurred approximately halfway through the stratum corneum, consistent with the view that the stratum corneum is the diffusional barrier

of the epidermis. Further illustrating the application of PAS to dermatological research, the authors reported the detection of tetracycline (down to ~0.01% solution topically applied), and its diffusion through both plantar and abdominal skin. The time constant for diffusion was found to be ~10 times greater in the case of abdominal skin.

Several other studies related to skin tissue have appeared. Sunscreen effectiveness is directly related to the attenuation of light as a function of penetration, wavelength, time, environment of the skin, etc. PA techniques can be used to measure *in situ* the absorption of light by the chemical sunscreen as a function of these parameters (Pines, 1978). Lerman *et al.* (1978) used PAS to measure the absorption of normal, aging, and cataractous human eye lenses. An analysis of skin lipids by infrared PAS at sensitivities of better than one μg can be accomplished simply by rubbing a cotton swab lightly against the skin and transferring a drying stain to a small aluminum plate (Kanstad *et al.*, 1981). Rather than a gas-microphone arrangement, these authors used a piezoelectric detector bonded directly to the aluminum plate.

These reports have clearly established the underpinning work for the use of PA techniques in studies of both photobiological and biochemical/biophysical properties and functions of skin tissue. It is expected that applications will expand in this area and include studies of photoproducts of skin sensitizing chemicals and their distribution in the epidermis, the diffusion of chemicals in tissue, and the disease conditions affecting surface and near surface tissue. In this connection, developments such as open-ended photoacoustic cells (Fishman and Bard, 1981) and radiometric detection (Section 2.4.5) could lead to true *in vivo* spectroscopy of a variety of living things.

6. CONCLUSION

Both qualitative and quantitative information can be extracted from measurements involving PA techniques. The observation from early experiments that it is in general extremely difficult to determine quantitatively β_{optical} , and hence the concentration of a chromophore, remains correct. On the other hand, calorimetric aspects of photoacoustics can be exploited to yield absolute values of energies or quantum yields of photoprocesses in an extremely wide range of samples including living things. Furthermore, with proper attention to experimental technique (saturation, phase, etc.) unambiguous absorption maxima can be measured and therefore the transition energy of chromophores *in vivo* determined.

Certainly the usefulness of PAS in certain types of strongly scattering materials is questionable (Tilgner, 1981). However, it is clear that the unique sensitivity of PA techniques to optical, thermal, and thermochemical properties of the sample will assure its place in studies of biochemical and especially energy-transducing photobiological systems. Capabilities are expanding rapidly, partly as a consequence of novel techniques such as PTR, mirage effect, and FTIR-PAS; it seems safe to continue the prediction (Smith, 1977) that PA techniques will find increasing use in the study of biological material.

7. EXPERIMENTAL NOTES

7.1. Microphone

We have found that the selection of a high quality microphone is rather important. This is especially true in the case of photobiological experiments, since achieving an acceptable signal to noise ratio by using a high light level may sometimes damage or alter the sample. In other words, choosing the most sensitive transducer will result in a given signal to noise ratio at a minimum light level. In order to derive maximum benefit from a sensitive microphone, it is necessary to have a cell with sufficient acoustic and seismic isolation so that the noise floor is electrical from the preamplifier stage. We have found that the Brüel and Kjaer model 4165 (condenser) or 4175 (electret) microphones to have the highest sensitivity and widest frequency response of any commercially available microphones; with special sealing kits these microphones have low frequency response to <0.1 Hz. Furthermore, they have quartz-coated diaphragms so that corrosive chemicals are less of a problem.

7.2. Light Scrambler

Since the PA spectrum results from the point by point division of the sample signal by the signal from a black body absorber, it is essential for the beam to have the same power spectrum when irradiating sample and reference. The power spectrum of a compact arc lamp (Xe) varies markedly over the distance between the electrodes, and if the source optics are of high quality, the image of the arc at the sample will not be homogeneous with respect to power spectrum. Since the sample is often not the same size or it is not in the same position in the cell as the black

body absorber, serious errors (up to 50%) can occur in the observed spectrum upon division. We partly solved this problem by installing a spherical mirror behind the arc lamp so that an inverted image of the arc falls on the arc, and completely solved the problem by having Maxlight (Phoenix, Arizona, USA) build a quartz light guide with the fibers randomized. The exit slit is imaged onto this light guide with an ellipsoidal mirror, and the light guide scrambles and transmits light to the cell.

Arc lamps with ellipsoid reflectors arranged such that the arc is coaxial with the ellipsoid axis may not suffer from this problem, since the heterogeneous power spectrum is along the optical axis, a direction not resolved when the image is formed on the sample.

7.3. Bifurcated Light Guide

An ideal way to carry out two beam experiments is a beam combiner in the form of a bifurcated light guide. Again, Maxlight custom builds these with randomized, quartz fibers.

7.4. Stirred Arc

A well-known technique for suppressing arc wander is to place a magnetic stirrer or solenoid near the lamp so that the arc is stirred or averaged over all available sites on the electrodes at relatively high frequency. In this way the random wander (noise), which can cause sudden intensity changes of as much as 50% every few minute (very low frequency noise), is translated up in frequency so that it is averaged by the time constants in the system.

7.5. Photoacoustic Cell Signal

Signals arising from absorption by the walls of the PA cell can distort the sample spectrum, and are especially troublesome in the case of phase measurements. We have experienced considerable difficulty with these spurious signals and have not completely solved the problem. The walls of the PA cell are illuminated by scattered light and sample fluorescence; it is difficult to remove these signals since the empty PA cell signal is not the correct blank for the PA cell contribution when the sample is present. A good strategy is to construct the PA cell from Plexiglass or aluminum with all interior surfaces polished. Additionally, a light trap, as shown in Fig. 1, may be used. The quartz cover plate must be carefully sealed at the edges with silicone grease. It is also important that the cell be designed

so that light does not fall on the microphone diaphragm. The design of PA cells is discussed in the general reviews cited earlier; good examples of useful reports of the sensitivities and signal to noise performance of cells are given by Cahen (1981), Ducharme *et al.* (1979) and Gray *et al.* (1977).

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Photobiology and Radiobiology of *Micrococcus (Deinococcus) radiodurans*

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1. INTRODUCTION

Micrococcus radiodurans, a gram-positive, nonsporing, red-pigmented bacterium, is the type species of a small group of bacteria, the members of which are characterized by extreme resistance to both the lethal and mutagenic effects of ionizing and ultraviolet (UV) radiation. They show no loss of viability up to doses of 500 krad or 500 Jm^{-2} of ionizing or UV radiation, respectively, and this has made them particularly useful for studying aspects of DNA damage and repair in populations in which every member is a survivor. Those scientists working with *Escherichia coli* will realize what a luxury this is. Nevertheless, these bacteria have been regarded largely as a scientific curiosity (a recent proposal has been made to change their generic name to *Deinococcus*, meaning "strange berry")! and the research effort put into them has been paltry compared with that devoted to *E. coli*. Thus, although the first paper on them was published twenty-seven years ago, this is the first review of the literature on their remarkable property of radiation resistance.

2. THE RED-PIGMENTED MICROCOCCI

2.1. Isolation

Rumor has it that the radiation-resistant bacterium *M. radiodurans* was isolated from the cooling water of an atomic reactor at Oak Ridge National Laboratory in Tennessee (personal communication from several

ill-informed sources). In fact, its origin was much more prosaic, having been isolated in Oregon from cans of meat that had supposedly received a sterilizing dose of γ radiation (Anderson *et al.*, 1956). On storage, there was evidence of spoilage and when the cans were opened proteolysis of the meat occurred fairly rapidly. A nonsporing bacterium that formed salmon-pink colonies on agar was found to be present in pure culture. Investigation showed that this bacterium comprised only 0.05 to 0.1% of the contaminating microbial population of the unirradiated meat, but after doses of γ -radiation of 2×10^6 rep or greater was the sole surviving species, even bacterial spores having been inactivated. Doses considerably greater than 3×10^6 rep were required to completely sterilize the meat. When pure cultures of the radiation-resistant bacterium, isolated from both unirradiated and irradiated meat were γ -irradiated on agar slants or in liquid media, some cells survived a dose of 6×10^6 rep and led to *M. radiodurans*, as this bacterium was subsequently named (Anderson *et al.*, 1961), being described in the Guinness Book of Records (1974) as the "toughest bacterium in the world". This strain is referred to as *M. radiodurans R₁*.

An investigation into the ecology of the organism showed it to be present in creek water upstream from the meat plant, on the hair and hide of live beef cattle that were watered from the creek, and in samples of ground meat from the packing plant (Krabbenhoft *et al.*, 1965). *M. radiodurans* is reported to have been isolated only twice since then, and from different geographical locations. It was isolated in Ontario as a contaminant in a hospital laboratory (Murray and Robinow, 1958), although its identification was delayed for some years since its main feature of interest was its unusual mode of cell division. This strain is referred to as *M. radiodurans Sark*. More recently, *M. radiodurans* has been isolated in Japan from irradiated sawdust used for mushroom culture (Ito, 1977).

M. radiodurans is not unique in its radiation-resistance, since several other red-pigmented micrococci have been isolated from various γ -irradiated materials, and these have similar properties. *M. roseus* (ATCC 19172) was isolated from irradiated haddock tissue (Davis *et al.*, 1963), *M. radiophilus* from irradiated Bombay duck (a dried fish; Lewis, 1971, 1973), and *M. radioproteolyticus* from the irradiated faeces of a llama (Kobatake *et al.*, 1973).

The taxonomy of these bacteria has been a subject for discussion for some years. It became clear that *M. radiodurans*, on the basis of its unusual and complex cell wall (Work, 1964; Thornley *et al.*, 1965; Work and Griffiths, 1968; Schleifer and Kandler, 1972; Sleytr *et al.*, 1973; Lancy and Murray, 1978) and lipid constituents (Knivett *et al.*, 1965; Girard, 1971) as well as its radiation resistance, was not related to the genus

Micrococcus (Baird-Parker, 1965, 1970). A recent study of the red-pigmented micrococci (Brooks *et al.*, 1980) has shown them to be closely related to each other and on the basis of a comparative study of ribonuclease digests of 16 S ribosomal RNA they form, on their own, one of the eight recognized groups of the true bacteria, the Eubacteria (Fox *et al.*, 1980). They have also been shown to be related by transformation. DNA extracted from rifampicin-resistant mutants of each of the four species transforms wild-type *M. radiodurans* to rifampicin-resistance, while DNA from *M. luteus* and *M. sodonensis* does not (Tempest, 1978). As already mentioned, there is at present a proposal that the nomenclature of the radiation-resistant cocci should be changed, to avoid the confusion caused by the generic name *Micrococcus*, to *Deinococcus* (Gr. deinos meaning strange or unusual; Brooks and Murray, 1981). Thus, *Micrococcus radiodurans* would become *Deinococcus radiodurans*, and would be the type species of the genus. *M. roseus* (ATCC 19172) would become *D. radiopugnans*, while *M. radiophilus* and *M. radioproteolyticus* would become *D. radiophilus* and *D. proteolyticus*, respectively. However, although it is likely that this proposal will be accepted, the generic name *Micrococcus* will be used in this review.

Since most of the work on the radiation-resistance mechanisms of these bacteria has concerned itself with the type species *M. radiodurans*, this review will concentrate on this species with only an occasional reference to its relatives. It is likely, however, that the mechanisms of resistance described for *M. radiodurans* will be similar in all the species of this group of organisms.

Before discussing the effects of ionizing and UV radiation on *M. radiodurans*, it is necessary to describe its growth and morphology, and to review what is known about the nature and arrangement of its DNA.

2.2. Culture of *M. radiodurans*

M. radiodurans is normally grown in TGY broth (Bactotryptone 0.5%; glucose 0.1%; yeast extract 0.3%; in distilled water) at 30°C with shaking or aeration. Under such conditions, it has a doubling time of about 80 min. On TGY agar (1.5% agar), colonies can be counted after incubation at 30°C for two days. The maximum temperature for growth on agar is 40–41°C, and it is possible to grow *M. radiodurans* in broth at 37°C, but at this temperature some cultures “clump” quite badly and this makes them unsuitable for the quantitative analysis required for radiation studies. The TGY medium has been supplemented in some studies with either methionine (Raj *et al.*, 1960) or aspartic acid (Moseley and Laser,

1965a), although an increase in growth rate or yield of the bacterium was not demonstrated. Chemically defined media have been described (Raj *et al.*, 1960; Little and Hanawalt, 1973; Shapiro *et al.*, 1977) that are sometimes useful for the radioactive labeling of DNA and necessary for the isolation of auxotrophic mutants, but they have not been used extensively because they give slow and often erratic growth. The fastest doubling time for *M. radiodurans* in a chemically defined liquid medium at 30°C is 3.5 to 4 h, while colonies can be counted on agar only after 4 to 5 days (Moseley, unpublished observation).

M. radiodurans is rarely observed as a single cell. The smallest unit is a diplococcus, which after dividing at right angles to the previous division-plane forms a tetracoccus (Anderson *et al.*, 1956). In liquid culture, this soon forms two diplococci, which then proceed to the tetracoccus stage and, in an exponentially growing population having a doubling time of 80 min, the majority of cells are in the diplococcus form with less than 10% in the tetracoccus form (Hansen, 1978). On agar, since the bacterium divides in only two planes, a sheet of cells is formed from a diplococcus and loses this regular arrangement only when friction between the agar surface and the sheet of cells causes the latter to fracture (Murray and Robinow, 1958; Driedger, 1970a; Moseley and Copland, 1975a). Mutants have been isolated that maintain the sheet formation for many more division cycles than does the wild-type strain (B. E. B. Moseley, unpublished result).

2.3. The DNA of *M. radiodurans*

The DNA of *M. radiodurans* forms between 1.5 and 2.0% of its dry weight, and has a G + C content of 66–68% (Moseley and Schein, 1964; Schein, 1966). Values lower than these have been reported (Alexander *et al.*, 1965; Setlow and Duggan, 1964), but it is now generally accepted to be 67% (Brooks and Murray, 1981). No unusual bases, e.g., 5-methylcytosine or 6-methyladenine, have been observed using chromatographic procedures that would have detected one mole of an unusual base per 100 moles of base (Schein, 1966). Radioactive tracer methodology and immunochemical techniques have failed to reveal any methylated bases in the DNA or any DNA methyltransferase activity (Schein *et al.*, 1972; Störl *et al.*, 1979).

Values for the amount of DNA per cell vary. The methodology is to measure the amount of DNA in a volume of culture (Burton, 1956) and then to calculate the amount of DNA per viable unit or per cell, having previously observed by microscopy the numbers of diplo- and tetracocci.

The amount of DNA per (monococcal) cell in exponential-phase growth has been calculated as 3.5×10^{-14} g (= 2.1×10^{10} daltons; Okazawa and Matsuyama, 1967), 2.2×10^{10} daltons (Hansen, 1978) and $2.07 \pm 0.65 \times 10^{10}$ daltons (Moseley and Evans, unpublished results). The time required to replicate the genome (C) has been calculated by Hansen (1978) to be between 60 and 80 min at 30°C. Using temperature shift experiments with the mutant ts1, which is temperature-sensitive for DNA synthesis but has the same generation time of 80 min at 30°C as the wild-type strain (Moseley *et al.*, 1972a), the genome replication time has been estimated to be between 55 and 60 min, and the time from the end of genome replication to cell division (D) to be 35 min (Moseley, unpublished observations). Substituting values of 60 and 35 min for C and D respectively into the formula of Pritchard and Zaritsky (1970) for calculating the number of chromosome equivalents in an exponential cell, gives a value of 1.79. Thus, the size of the genome, assuming each cell to be haploid (Driedger, 1970a), is $2.1 \times 10^{10}/1.79 = 1.17 \times 10^{10}$ daltons, equivalent to 1.9×10^7 base pairs. A lower value of 8.0×10^9 daltons, equivalent to 1.2×10^7 base pairs, was obtained by Driedger (1970b), who treated cultures with lysozyme to release single cells from their cell walls in an attempt to make more accurate cell counts. However, since the numbers of single, liberated cells were determined by using a counting chamber under a microscope, which is not a particularly accurate method of counting (Mallette, 1969), there is no reason to prefer this value. Other values for the amount of DNA per viable unit of 30×10^{-14} g (= 1.8×10^{11} daltons) appear to be too high unless the culture was clumping somewhat (Freedman and Bruce, 1971, 1972).

The question of whether *M. radiodurans* is haploid or not is relevant because polyploid *Saccharomyces*, e.g., is more resistant to radiation than the haploid form (Mortimer, 1958). Setlow and Duggan (1964) excluded ploidy as an explanation for the extreme UV-resistance of *M. radiodurans* on the basis that the exponential slope of the survival curve, which should be independent of ploidy, expresses too high a level of resistance. Driedger (1970a), taking advantage of the formation of sheets of cells on agar, showed that in cultures of *M. radiodurans* treated with the mutagen N-methyl-N'-nitro-N-nitrosoguanidine (MNNG) lethal mutations were expressed with a high frequency in the first generation. This would be consistent with one copy of the genetic information per cell, i.e., haploidy. The appearance of aberrant colonies following treatment of *M. radiodurans* with MNNG had been observed previously (Moseley, 1967b) and can be simulated using sublethal, nonmutagenic treatments (Moseley and Copland, 1975a). The apparently lethal sectors grow very slowly, even-

tually forming colonies in which morphologically normal cells appear and may, therefore, be a physiological phenomenon rather than a genetic one.

Hansen (1978) measured the complexity of the genome by DNA renaturation kinetics, and calculated it to be $2.0 \pm 0.3 \times 10^9$ daltons. The number of genome equivalents per cell was then calculated from the complexity and the amount of DNA per cell. He concluded that there were four genome equivalents per resting cell and up to ten in exponentially-growing cells. This work has been extended by measuring the amount of DNA per stainable nucleoid, and by dividing by Hansen's value for the genome complexity it has been calculated that there are 4.7 ± 0.9 genome complexes per nonreplicating, stainable nucleoid (Moseley and Evans, 1981). A model to accommodate these results will be discussed in Section 13.1.

2.4. Plasmids in the Red-Pigmented Micrococci

Strains of all four species of the radiation-resistant Micrococci have been carefully screened for the presence of plasmid DNA (Mackay and Moseley, unpublished observations). Two size classes of plasmid have been identified in each of the strains *M. radiodurans* Sark (38.5 and 45.3 kb), *M. roseus* ATCC 19172 (2.5 and 28.6 kb) and *M. radioproteolyticus* (99.3 and 139.1 kb) while three size classes have been found in *M. radiophilus* (11.2, 28.2 and 92 kb). However, in spite of intensive efforts no plasmids have yet been identified in *M. radiodurans* R₁ so that no role can be ascribed to the plasmids especially in relation to radiation resistance.

3. RADIATION SURVIVAL CURVES

The ionizing and UV radiation survival curves for *M. radiodurans* each have a large shoulder, indicating that the bacteria can initially absorb radiation energy with no loss of viability, i.e., they can accumulate sub-lethal damage, followed by an exponential loss of viability (Fig. 1). The shape of the curve can be characterized by two parameters, the $1/_{e}$ dose (or D_O), the dose required to reduce viability on any region of the exponential part of the survival curve by 63%, and the intercept dose (or D_Q), defined by extrapolating the exponential part of the survival curve to unit survival, which is thus a measure of the size of the shoulder. (An interesting refinement used by Dewey (1969) was to extrapolate the exponential part of the survival curve to the value four times unit survival,

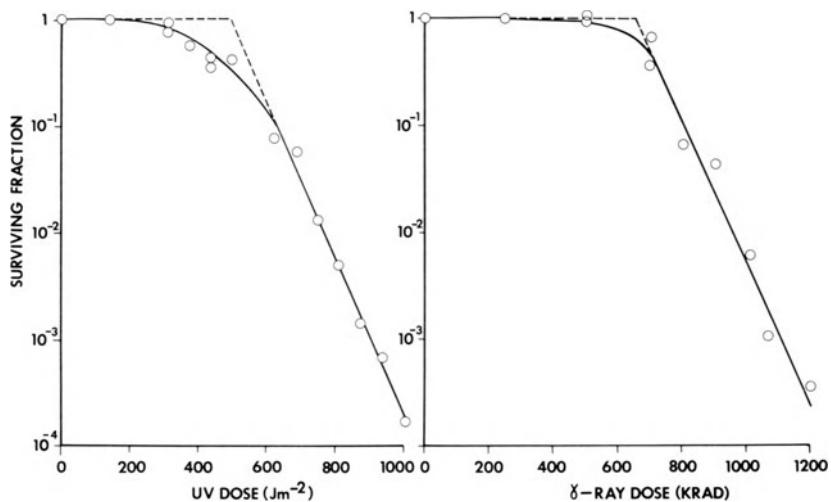


Fig. 1. Typical ultraviolet and γ -radiation survival curves of wild-type *Micrococcus radiodurans*. The exponential parts of the survival curves have been extrapolated to unit survival to indicate the intercept doses of 515 J m^{-2} and 640 krad, while the $1/e$ doses are 60 J m^{-2} and 60 krad, respectively (data from Moseley and Mattingly, 1971).

and to use this dose as a measure of the shoulder. It was reasoned that since all the bacteria irradiated were in the tetrad form and if each cell of the tetrad were inactivated by a single hit, the survival curve would extrapolate back to 4.) Together, the intercept and $1/e$ doses completely define the exponential part of the survival curve. No simple parameters can be given to define in absolute terms the shapes of the initial rounded shoulder. It makes for tedious reading if the intercept and $1/e$ doses are given in a text for each survival curve, and it is more convenient to use the D_{37} , i.e., the dose required to leave 37% of the original population viable or the dose required to kill one viable unit. The D_{37} does not *per se* define any stretch of the survival curve, but empirically it turns out that the D_{37} is usually close to the sum of the intercept and $1/e$ values.

3.1. Ionizing Radiation

The first ionizing-radiation survival curve for *M. radiodurans* showed the D_{37} to be about 10^6 rep (Anderson *et al.*, 1956). The conditions for the experiment were somewhat unusual. The cultures were grown in Corvallis, Oregon for 24 h at 35°C in TGY broth, tubes of the culture were hermetically sealed into a can, placed at -20°C for 24 h and then shipped

either to the Materials Testing Reactor at Arco, Idaho or to the Stanford Research Institute, California to be γ -irradiated. The cans were refrigerated with dry ice and returned to Corvallis for the measurement of the numbers of viable bacteria.

In order to make valid comparisons between results from different laboratories, it is necessary to use a standard protocol. The normal procedure is to incubate a freshly diluted culture in TGY broth until the turbidity of the culture has increased by a factor of about 6 to 7, i.e., about 2.5 generation times. The culture is then centrifuged, the bacteria resuspended in a neutral buffer and irradiated with X-rays or ^{60}Co - γ rays either with oxygen being bubbled through the culture during irradiation to maintain aerobic conditions, or with oxygen-free nitrogen for some minutes before and during irradiation to ensure anaerobic (anoxic) conditions. However, even with a standard procedure, survival curves vary and almost every published survival curve is different in detail from all the others even when from the same laboratory.

To give an indication of this variability, values for *M. radiodurans* irradiated aerobically in buffer include a D_{50} of 700 krad (Lee *et al.*, 1963, quoted in Krabbenhoft *et al.*, 1965), 640 and 64 krad for intercept and $1/e$ doses (Moseley and Mattingly, 1971), and a D_{37} of 500 krad (Sweet and Moseley, 1976). It would seem reasonable to give 600 krad as a representative value for the D_{37} , and this has been used as the basis for the calculations in Table 1. For anoxic radiation, Dean *et al.* (1966), using

TABLE 1. Amount of Radiation-Induced Damage to DNA Required to Inactivate *Micrococcus radiodurans*^a

Lesion	Numbers of lesions per:			Average distance between lesions (bps)
	Genome	Nuclear body	Cell	
Ionizing radiation				
DNA single-strand breaks	3100	15000	33000	1000
DNA double-strand breaks	240	1100	2500	13000
5,6-dihydroxydihydrothymine	1400	6800	15000	2200
UV radiation				
Thymine-containing pyrimidine dimers	12000	57000	128000	260
5,6-dihydroxydihydrothymine	600	2800	6400	5400

^a Expressed as lesions per genome (2.0×10^9 daltons, Hansen, 1978), lesions per stainable nucleoid (9.4×10^9 daltons, Moseley and Evans, 1981) and lesions per cell (2.1×10^{10} daltons). The D_{37} values used were 600 krad for ionizing radiation and 570 J m^{-2} for UV radiation.

220 kVp X-rays, obtained a D_{37} of 740 krad while Fox and Hopkins (1970), using 10 MeV electrons given at a very high dose rate (5 krad s⁻¹), obtained values of 800 and 85 krad for the intercept and $\frac{1}{e}$ doses respectively. The fact that in a culture of *M. radiodurans* most of the cells are in either a diplococcus or tetracoccus form does not give a spurious view of the resistance of single cells. In a study in which irradiated diplococci and any first and second generation offspring were micromanipulated onto agar and their growth observed, the resulting survival curve did not show them to be more sensitive than a survival curve obtained using standard procedures (Driedger *et al.*, 1970).

3.1.1. Effect of Growth Conditions

The resistance of *M. radiodurans* to ionizing radiation is dependent on the age of the culture, the medium in which it is grown prior to irradiation, and the agar medium on which it is plated to assay survival. When cultures at different ages in TGY broth were given single doses of 1.85 Mrad, bacteria became gradually more resistant as the culture aged from 2 h (survival 10⁻⁶) to 50 h (10⁻²; Duggan *et al.*, 1959). This result was confirmed by Serianni and Bruce (1968), who found that the D_{37} of stationary-phase cells was about three times that of exponentially-growing cells. The resistance of exponentially growing cells did not increase when chloramphenicol was added to inhibit protein synthesis (Serianni and Bruce, 1968), nor was the increased resistance of stationary-phase cells dependent on the DNA-protein ratio (Freedman and Bruce, 1972). The resistance of exponentially growing cells varies with the growth rate and, therefore, with the growth medium. Freedman and Bruce (1971) showed that, in common with other bacteria, as the growth rate increased (by growth in different media) and the cell size increased, resistance to ionizing radiation decreased. The increased sensitivity was tentatively attributed to the larger size of the macromolecular targets in fast-growing cells. They also showed that survival increased when the irradiated bacteria were plated on minimal medium. There was no correlation between the changes in resistance of *M. radiodurans* and the amounts of possibly-radioprotective sulphydryl compounds (Serianni and Bruce, 1968; Freedman and Bruce, 1971).

Krabbenhoft *et al.* (1967) found that growing *M. radiodurans* in TGY broth to which a tryptic digest of casein had been added caused a significant increase in sensitivity to γ -radiation compared to growing it in TGY, the D_{37} being reduced from 700 to 350 krad. The growth rate was not different, and a satisfactory explanation is still lacking.

3.1.2. Linear Energy Transfer (LET) Effects

Dewey (1969) examined the shapes of the survival curves of *M. radiodurans* following irradiation at high LET and plated in the presence and absence of acridine orange. The bacteria were mounted on gelatin discs and bombarded with beams of fully stripped nuclei of helium, lithium, boron, carbon, oxygen, neon, and partially-stripped argon nuclei, all accelerated to about 10 MeV per nucleon. The length of the shoulder of the survival curve increased as LET increased whereas the final slope had a definite LET optimum (equivalent to boron) for maximum sensitivity, very near that for bacterial spores, haploid and diploid yeast, and mammalian cells in culture. The effect of the presence of acridine orange was to reduce the shoulder without changing the slope.

Kitayama *et al.* (1970) irradiated *M. radiodurans* suspended in phosphate buffer with 26 MeV α -particles and found that cell killing was exponential. Double-strand breaks caused by the α -particles were repaired during postirradiation incubation at about the same rate as those caused by γ -rays, and the authors concluded that DNA must not be the only primary target for cell killing by α -particles. The apparently contradictory results of Kitayama *et al.* (1970) and Dewey (1969) in the absence and presence, respectively, of a shoulder to the survival curves is probably the result of the different irradiation methods used (Kitayama, personal communication). Thus, Dewey (1969) used a track segment method while the method of Kitayama *et al.* (1970) caused energy deposition at the "Bragg peak." At the "Bragg peak" more energy is deposited in a small region so that cells receive more lethal damage than cells at the track. When Kitayama *et al.* (1977) used the track segment method the survival curve for α -particles had the same shape as that for γ -rays, i.e., a large shoulder.

3.2. UV Radiation

The first UV radiation survival curve was published by Duggan *et al.* (1959), who showed *M. radiodurans* to be much more resistant than *E. coli*, *Sarcina lutea*, or spores of *Bacillus globigii*. The D_{37} value depended upon the stage of growth of the culture, but when the cells were maximally resistant it was 626 J m^{-2} , although it fell to about half that at other stages of the growth cycle. This variation of D_{37} with the stage of growth of a culture was not confirmed either by Setlow and Boling (1965), or by Serianni and Bruce (1968) who showed there to be no differences

in the sensitivity of cultures to UV radiation over a period of 40 h. One possible explanation, given by Boling and Setlow (1966) for the results of Duggan *et al.* (1959), is that the irradiated bacteria were assayed for survival in molten agar and the heat of the agar may have influenced their sensitivity. The results of Setlow and Duggan (1964), obtained using monochromatic light at 265 nm, gave a D_{37} value of 600 Jm^{-2} . The results from my own laboratory have always given much higher values than this because the output of the UV lamp was overrated by the manufacturer, and has recently been derated from 2.25 Jm^{-2} to 1.05 Jm^{-2} . This means that the published value of 1220 Jm^{-2} for the D_{37} is in fact 570 Jm^{-2} (Moseley and Mattingly, 1971).

One report suggests that cells that have been in resting phase for periods from two to seven days have exponential survival curves (D_{37} , 350 Jm^{-2}) while one day-old cultures have a substantial shoulder to their survival curves, and approximately the same exponential rate of inactivation (D_{37} , 600 Jm^{-2} ; Vilenchik, 1970). However, the conditions of the experiment were unusual in that the bacteria were harvested from agar and irradiated at a cell density of 10^4 ml^{-1} , which seems surprisingly low. Although the same phenomenon was observed when *E. coli* cultures of varying age were exposed to ionizing radiation, no results were given for *M. radiodurans* and ionizing radiation.

The resistance of the other radiation-resistant red-pigmented cocci to UV radiation has been measured under conditions similar to those used to investigate *M. radiodurans* (Tempest, 1978), and the survival curves are shown in Fig. 2. The D_{37} values for each of the species are *M. radiodurans*, 504 Jm^{-2} ; *M. roseus* (ATCC 19172), 513 Jm^{-2} ; *M. radiophilus*, 670 Jm^{-2} ; and *M. proteolyticus*, 896 Jm^{-2} . Thus, *M. radiodurans* is the most sensitive of these micrococci to UV radiation.

An usual feature in the shape of the UV survival curve of *M. radiophilus* has been reported in that after survival has fallen to 10^{-3} , at a dose approaching 2000 Jm^{-2} , the remaining bacteria appear to be completely refractory to killing (Lewis and Kumta, 1972; Lavin *et al.*, 1976).

4. CONDITIONS THAT SENSITIZE *M. radiodurans* TO RADIATION

4.1. The Oxygen Effect

Very little has been published on the survival of *M. radiodurans* following ionizing radiation under oxic versus anoxic conditions. The oxygen-enhancement ratio (OER) for stationary-phase cells at the 1% level

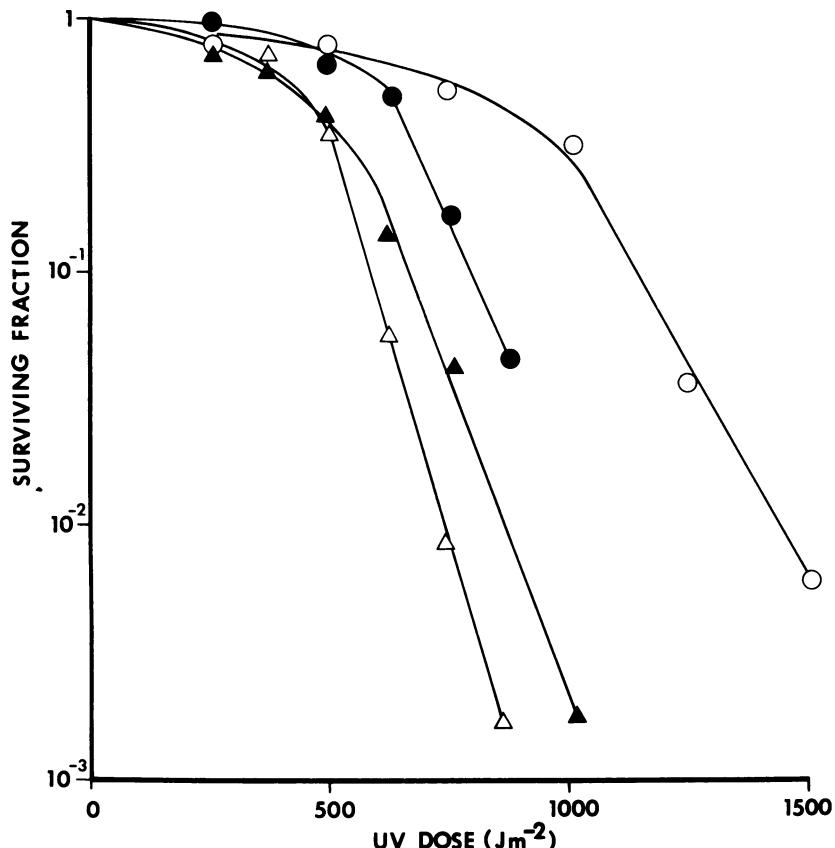


Fig. 2. Ultraviolet radiation survival curves of four radiation-resistant red-pigmented micrococci. (Δ) *M. radiodurans*, (▲) *M. roseus* ATCC 19172, (●) *M. radiophilus* and (○) *M. proteolyticus* (data from Tempest, 1978).

of survival is 1.3 to 1.4 (Okazawa and Matsuyama, 1967), while a recent study of exponentially growing cells shows an OER of 1.7 (Myers and Johnson, 1977).

4.2. Heat

Although *M. radiodurans* is very resistant to radiation, it is very sensitive to heat, a temperature of 60°C reducing its viability by a factor of 10⁸ in 6 min in a beef puree (Duggan *et al.*, 1963). Nevertheless, Bridges *et al.* (1969) concluded that there is a general correlation between re-

sistance to ionizing radiation and the ability of strains of *E. coli* to survive incubation at 52°C, and showed that *M. radiodurans* was considerably more resistant to incubation at 52°C than the most resistant *E. coli* strain, AB1157. Heated bacteria were more sensitive to ionizing radiation than unheated ones. The reverse order of treatment, i.e., radiation followed by heat, was not tested.

However, Cho *et al.* (1974) incubated irradiated *M. radiodurans* at the nonpermissive growth temperature of 42°C in TGY broth and found that irradiated cells lose their colony-forming ability as a result. The loss of viability was a function of both the irradiation dose and the time of incubation at 42°C. About 10^{-3} of the population was refractory to the heat treatment. The incubation at 42°C did not inhibit the repair of double-strand breaks; the molecular weight of the DNA, reduced by a dose of 100 krad, being restored to control size in 3 h. This was taken to mean that the mending of strand breaks is insufficient in itself to explain cell survival. Kitayama *et al.* (1977) extended this study to show that incubation at 42°C for 6 h posttreatment caused extensive loss of colony formation after irradiation with γ -rays, α -particles, or treatment with mitomycin C.

Some preliminary experiments on the effect of heating a culture of *M. radiodurans* on its resistance to UV radiation, and *vice versa*, have been carried out (Moseley, unpublished observations). Comparing cultures heated at 48°C for 0, 20 and 40 min the D_{37} values for UV radiation were 630, 345, and 140 Jm^{-2} , respectively. When cultures were irradiated with 0, 190, 380, and 560 Jm^{-2} the D_{37} values at 48°C were 200, 90, 8.25, and 4.0 min respectively.

4.3. Iodoacetamide and Iodoacetic Acid

M. radiodurans irradiated in the presence of iodoacetamide shows a very great increase in sensitivity to ionizing radiation. Under aerobic conditions, 3×10^{-4} M iodoacetamide reduced the D_{37} from 263 to 37 krad, while a concentration of 10^{-3} M reduced it to 3 krad. The shoulder of the survival curve was reduced and the slope of the exponential part increased, i.e., both components became more sensitive (Dean and Alexander, 1962). Although the results were by no means as dramatic, the presence of 10^{-4} M iodoacetate during aerobic or anoxic radiation also sensitized *M. radiodurans* to ionizing radiation (Lee *et al.*, 1963). Both iodoacetamide and iodoacetate have to be present during the irradiation since incubation of bacteria with the compounds before or after irradiation, or incubation of irradiated bacteria in irradiated solutions of the compounds do not sensitize the bacteria. The effect of iodoacetamide can

be accounted for by the production of extremely short-lived toxic products formed by radiation from the iodoacetamide (Dewey and Michael, 1965). Incidentally, *N*-ethylmaleimide, which sensitizes *E. coli* to ionizing radiation under anoxic conditions does not sensitize *M. radiodurans* (Lee *et al.*, 1963).

4.4. *p*-Hydroxymercuribenzoate

When *M. radiodurans* is incubated for 30 min prior to irradiation with *p*-hydroxymercuribenzoate (HMB), a sulfhydryl-binding agent, it is sensitized to ionizing radiation. Bruce and Malchman (1965) obtained an eight-fold reduction in the D_{37} at a concentration of 0.1 mM HMB, both the shoulder and the exponential slope showing an increase in sensitivity. The sensitization was not lost by repeatedly washing the cells, because after two washes the amount of HMB bound to the cells remained constant at $1.03 \pm 0.20 \times 10^{-16}$ moles per cell, which was equivalent to the 0.8 to 2.0×10^{-16} moles per cell of a radioprotective component described by Bruce (1964).

4.5. 5-Bromodeoxyuridine

Progressive replacement of thymine by 5-bromouracil in the DNA of *M. radiodurans* results in the progressive removal of the shoulder of the X-ray survival curve, although there is little change in the final slope (Lett *et al.*, 1970). There is no effect on the number of DNA strand-breaks formed during radiation or on the properties of the system rejoicing the breaks. However, the loss of the shoulder was paralleled by a reduction in the rate and amount of postirradiation degradation of DNA. The rate of release of degradation products, although independent of the irradiation dose, was slower than in unsubstituted DNA, and less DNA was degraded per unit dose. However, the time taken to complete degradation was longer, whereas there was only a small increase in the time taken to resume DNA synthesis. At the level of thymine substitution by 5-bromouracil where the shoulder of the survival curve completely disappeared, there was no degradation of DNA.

4.6. Thymineless Death

Thymine-requiring mutants of *M. radiodurans* that have parental resistance to UV radiation are not resistant to thymineless death, i.e., when

suspended in a medium lacking thymine there is, following an initial lag of 2 h at 37°C, an exponential loss of viability (Little and Hanawalt, 1973). Cells removed from the medium during the exponential phase are considerably more sensitive to UV radiation, e.g., after 60 min of thymine starvation the D_{37} was reduced from 350 to 120 J m⁻², the effect being entirely on the shoulder of the survival curve. This change in sensitivity was not reflected by a change in the rate or extent of pyrimidine dimer excision, but by an increased breakdown of DNA in thymine-starved bacteria (Budayová and Sedliaková, 1977).

5. QUANTIFICATION OF DNA DAMAGE

The extent of radiation-induced damage to the DNA of *E. coli* is normally calculated as the number of lesions per genome (Howard-Flanders and Boyce, 1966; Setlow and Setlow, 1972; Moseley and Williams, 1977). This is because the size of the *E. coli* genome is known and the general concept of DNA repair is that radiation-induced lesions in DNA are blocks to replication, and once the replication fork has proceeded the length of the (irradiated) genome the major part of repair is complete, lesions having been repaired or by-passed, to be repaired subsequently. Thus, the number of lesions per nonreplicating genome is more pertinent than the number of lesions per replicating genome. In *M. radiodurans*, however, the structure of the DNA within the cell is the subject of speculation, the most recent calculation for the size of the genome being 2×10^9 daltons, although this represents only one-eight to one-tenth of the total DNA of a replicating cell (Hansen, 1978), and it is likely that four or five genome copies are present in each nuclear structure (Moseley and Evans, 1981). For this reason, the numbers of lesions have been calculated for each genome, for each stainable nucleoid, and for the total DNA in an exponential-phase cell (Table 1). The average distance between each type of lesion in base pairs is also given. The data on which the calculations are based are as follows.

5.1. Ionizing Radiation

Although estimates vary for the energy deposition required per double-strand break in the DNA, the value derived by Burrell *et al.* (1971) has been used. They showed that above a dose of 50 krad of X-rays in oxygen, a plot of reciprocal molecular weight against dose was linear up to 700 krad. That is, the number of DNA double-strand breaks

was directly proportional to dose, with an efficiency of one double break being made per 520 ± 50 eV deposited (1 break per krad in 5.10^9 daltons); and they occur at a frequency some thirteen-fold lower than DNA single-strand breaks (efficiency 40 eV/single break; Dean *et al.*, 1969).

Hariharan and Cerutti (1971, 1972a) developed a reduction assay for the classification and determination of the major radiolysis products of thymine and its derivatives. Radiolysis products of the type 5,6-dihydroxy-5,6-dihydrothymine and 5-hydroperoxy-6-hydroxy-5,6-dihydrothymine were reductively cleaved with sodium borohydride, and a four-carbon alcohol was released containing the thymine-methyl group (Fig. 3). If thymine derivatives carried a radioactive label in the methyl group, the amount of four-carbon fragments produced by sodium borohydride (mainly 2-CH₃-glycerol after irradiation in air) could be determined by radioactivity measurements, and is a measure of thymine radiolysis. The efficiency of formation of damaged thymine residues was calculated to be one per 90 eV in air or one per 110 eV in nitrogen and, therefore, the amount formed in the DNA of irradiated *M. radiodurans* is 1.2×10^{-6} per rad per 10^6 daltons in air, and 10^{-6} per rad per 10^6 daltons in nitrogen (Hariharan and Cerutti, 1972a). Thus, thymine destruction occurs with an efficiency comparable to the production of single-strand breaks, and since ionizing radiation undoubtedly causes damage to cytosine, guanine, and adenine it appears that total nucleic acid base damage is by far the predominant type of lesion caused by ionizing radiation in *M. radiodurans*. The assay procedure has been improved and now involves an alkali-acid degradation (Hariharan and Cerutti, 1974), or an alkali degradation procedure (Hariharan, 1980).

5.2. UV Radiation

The number of pyrimidine dimers produced by UV radiation is based on two estimates. Firstly, Setlow and Duggan (1964) showed a linear

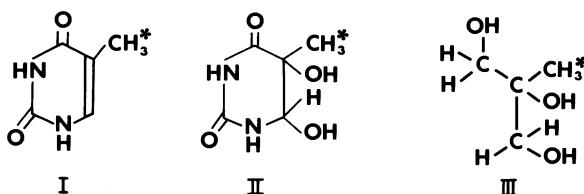


Fig. 3. Structural formulae of: I, thymine; II, 5,6-dihydroxy-5,6-dihydrothymine, a product of thymine formed in the DNA of *M. radiodurans* by ionizing or UV radiation; III, 2-methyl glycerol formed by reductive cleavage of II. A tritiated methyl group (*) enables a quantitative estimation of 5,6-dihydroxydihydrothymine-type radiation products to be made.

increase in labeled thymine appearing in pyrimidine-dimer form with increasing dose of UV radiation; 1% of the label being associated with dimer for each 500 Jm^{-2} . Moseley *et al.* (1972a) found that 0.52% of labeled thymine was present as dimer following a dose of 250 Jm^{-2} , equivalent to 1.04% for 500 Jm^{-2} , and since the D_{37} is 570 Jm^{-2} (Setlow and Duggan, 1964; Moseley and Copland, 1975b) this has been used to calculate the number of thymine-containing pyrimidine dimers in Table 1. The extra burden of cytosine-cytosine dimers has not been included. The value for thymine products of the 5,6-dihydroxydihydrothymine type is based on the above value, since approximately one nondimeric product is formed for every 20 pyrimidine dimers (Hariharan and Cerutti, 1977).

6. POSTIRRADIATION DEGRADATION AND SYNTHESIS OF DNA

6.1. Ionizing Radiation

One immediate consequence of the sublethal irradiation of *M. radiodurans* is the limited degradation of DNA. This was first observed by Dean *et al.* (1966) who followed the amount of DNA in irradiated cultures using the Burton (1956) chemical assay, and later quantified by studying the loss of radioactive label from the DNA of irradiated cells prelabeled with tritiated thymidine (Lett *et al.*, 1967a). The label first appeared in the perchloric acid-soluble fraction of the cell, and then appeared in the growth medium mainly associated with thymine or thymidine. It was subsequently shown that the primary degradation products were oligonucleotides of about 7×10^5 daltons (about 2000 bases), which were rapidly degraded in the growth medium by exonucleolytic enzymes released by both irradiated and unirradiated populations (Vukovic-Nagy *et al.*, 1974). The rate of release of degradation products was independent of the radiation dose, 0.12% of the DNA being degraded per minute at 30°C , although the amount of label released was dose-dependent and appeared to be linearly related to dose, about 5% of the DNA being degraded for each 100 krad anoxic radiation, at least up to 500 krad (Lett *et al.*, 1967a). Individual reports give varying values per 100 krad from 4.3% (Lett *et al.*, 1967a), through 6.2% (Driedger *et al.*, 1970) to 7.0% (Fox and Hopkins, 1970). Thus, the time taken for degradation to cease following a dose of 200 krad would be $10/0.12 = 83$ min. Driedger *et al.* (1970) calculated that a dose of 200 krad would create 4800 single-strand gaps per genome (as then determined) and, on the basis of the total label released, concluded that 850 bases were being excised per break, although as Dean *et al.* (1970) pointed out, some breaks might be made during incubation as

a result of other damage such as defective bases, i.e., by excision. The amount of DNA degraded, although not its initial rate, which remains at 0.12% per min, can be increased by incubation in chloramphenicol (Dean *et al.*, 1970; Driedger and Grayston, 1971a), nalidixic acid (Driedger and Grayston, 1971b), or phenethyl alcohol (Driedger and Grayston, 1971c). Thus, following a dose of 200 krad (anoxic), 10% of the DNA is degraded, but if the irradiated bacteria are incubated in the presence of chloramphenicol more than 40% of the DNA is degraded after incubation for 7 h, and what should have been a nonlethal dose now kills more than 90% of the cells (Dean *et al.*, 1970). Chloramphenicol also inhibits the slow phase of single-strand rejoining that occurs in growth medium (see Section 7.1). The effects of nalidixic acid and phenethyl alcohol, both of which inhibit DNA synthesis, the former by inhibiting DNA gyrase, are less clear because although both cause a substantial increase in the amount of DNA degraded. Neither inhibits the mending of DNA single-strand breaks (except at very high concentrations of nalidixic acid that inhibit protein synthesis and, therefore, mimics chloramphenicol) and, surprisingly, no effort was made to show that increased DNA degradation caused a decrease in viability. Since DNA breakdown continued in the presence of these compounds even after strand-mending was complete, Driedger and Grayston (1971b,c) concluded that the X-ray-induced DNA breakdown is due partly to damage to a non-DNA target, such as the cell membrane. An interesting feature of nalidixic acid-inhibition of DNA gyrase, which has not been explained, is that quite a low concentration of nalidixic acid ($5 \mu\text{g ml}^{-1}$) causes 70% inhibition, while the remaining 30% is about 125 times as resistant to inhibition (Driedger and Grayston, 1971b). Kitayama and Matsuyama (1970) studied the inhibition of DNA, RNA, and protein synthesis by ionizing radiation and suggested that, since all three were significant at high doses, inhibition of RNA and protein synthesis might have an important role in the primary events leading to cell death.

There is some variation in the estimates for the time taken for DNA synthesis to reach control levels in irradiated cells. Dean *et al.* (1966) showed a dose-dependent delay, approximately linear with dose, 495 krad of X-rays given anoxically delayed normal rates of DNA synthesis for 140 min (equivalent to a 56 min delay per 200 krad). Lett *et al.* (1967a) obtained values for DNA synthesis delay that were not quite linear with dose up to 400 krad, i.e., a dose of 200 krad caused a delay of 100 min, and 400 krad caused a delay of 156 min. Moseley and Copland, (1976) using γ -rays aerobically, obtained a value of 85 min delay for 100 krad (equivalent to 170 min per 200 krad). However, in all these cases it is clear that the time taken for DNA synthesis to resume at normal rates

exceeds the time required for DNA degradation to end. The cause and effect of this coincidence has not been determined, although Moseley and Copland (1976) showed that the normal rate of DNA synthesis is reached immediately after the completion of recombination repair of γ radiation-induced damage.

6.2. UV Radiation

DNA degradation occurs as one of the consequences of UV irradiation (Moseley, 1967a; Varghese and Day, 1970). Recently it has been shown that in the wild-type strain the rate of UV radiation-induced DNA degradation is independent of the dose, 0.1% of the total DNA being degraded (and appearing in the growth medium as short oligonucleotides) per min at 30°C (Evans and Moseley, unpublished observations). The amount of DNA degraded is linearly dependent on the dose of UV radiation, and degradation ceases abruptly at a time which coincides with the completion of the excision of pyrimidine dimers. DNA degradation appears to be under the control of products synthesized in response to UV radiation damage, since irradiated cells incubated in the presence of chloramphenicol are unable to stop DNA degradation. In mutants unable to incise their DNA after UV irradiation, there is no degradation.

The delay in DNA synthesis returning to control rates is linearly related to the dose (Setlow and Boling, 1965); doses of 100 and 200 Jm⁻² giving delays of 50 and 110 min, respectively, i.e., a delay of 1 min per 2 Jm⁻². Moseley and Copland (1976) obtained similar results, 100 Jm⁻² giving a delay of 40 min, i.e., 1 min delay per 2.5 Jm⁻². In the case of UV radiation, however, *M. radiodurans* appears to resume a normal rate of DNA synthesis before the completion of recombination repair of UV radiation-induced damage, and from the kinetics of recombination repair and resumption of DNA synthesis it was postulated that cell death at the lethal dose of radiation is caused by a second round of replication of DNA on a DNA template that is still undergoing recombination repair (Moseley and Copland, 1976).

7. REPAIR OF IONIZING RADIATION-INDUCED DNA DAMAGE

Prior to the demonstration that *M. radiodurans* is resistant to radiation because it possesses DNA-repair mechanisms, a number of other explanations were proposed. These included the possession of energy-transfer mechanisms in which the carotenoid pigment may have played

a role (Kilburn *et al.*, 1958), and radical-trapping mechanisms involving a sulphydryl compound (Bruce, 1964; Bruce and Malchman, 1965). However, nonpigmented mutants were isolated that had the same resistance to radiation as the wild-type strain (Moseley and Laser, 1965a; Suhadi *et al.*, 1971, 1972), and radiation-sensitive mutants were isolated that had both the same amount of carotenoid pigment and sulphydryl content as the wild-type strain (Moseley, 1967b). This is not to imply that sulphydryl compounds have no role in the resistance of *M. radiodurans* to ionizing radiation but it is not the major role.

The first experimental evidence for the repair of ionizing radiation-produced damage in *M. radiodurans* came from split-dose experiments (Moseley and Laser, 1965a). Following a dose of 350 krad of X-rays, cell division was inhibited for about 300 min and then resumed at a rate comparable with that of an unirradiated culture. When a second dose of 350 krad was given towards the end of the period of division-inhibition, the bacteria were as resistant as unirradiated cells, i.e., cells that had not undergone division had repaired the damage inflicted by 350 krad of X-radiation. That the damage was to DNA was inferred from experiments in which *M. radiodurans* was irradiated with ionizing radiation followed immediately by UV radiation or *vice versa* (Moseley and Laser, 1965b). The ability of a cell to repair UV radiation-induced damage decreased proportionally with the increase in dose of the prior γ -irradiation and *vice versa*. When the initial dose of γ -radiation was sufficiently large that inactivation of cells was exponential, the subsequent UV radiation also killed the cells exponentially, and *vice versa*. By superimposing the survival curves and comparing the dose scales it is possible to equate 1 J m^{-2} UV radiation with 1 krad of γ radiation in terms of repair ability of the bacterium. Since at that time it was known that UV-resistance in *E. coli* was dependent upon the removal of pyrimidine dimers from DNA (Setlow and Carrier, 1964; Boyce and Howard-Flanders, 1964), this was taken as evidence for the repair of ionizing radiation damage being that of damage in DNA. Incidentally, almost all radiation-sensitive mutants isolated since then have been sensitive to both types of radiation (Section 14).

7.1. Repair of DNA Single-Strand Breaks

The demonstration of the repair of DNA single-stranded breaks awaited the development of a method (McGrath and Williams, 1966) in which bacteria radioactively labeled in their DNA were lysed on the top of alkaline sucrose gradients, and the sedimentation velocity of the single-stranded DNA through the gradient was measured. The number of DNA

single-strand breaks was originally thought to depend upon whether the bacteria were irradiated aerobically or anaerobically (Lett *et al.*, 1967b); a single-strand break being formed for every 70 eV deposited in the presence of oxygen and for every 150 eV in the absence of oxygen. However, using 0.02 M EDTA to inhibit the rejoicing of broken DNA strands, Dean *et al.* (1969) showed that there was no significant difference in the numbers of single-strand breaks introduced into the DNA when the cells were irradiated either in the presence or absence of oxygen; 50 eV being required to produce one single-strand break. When cells were irradiated in the absence of oxygen and in the absence of EDTA, fewer DNA single-strand breaks were found; one single-strand break being formed for every 150 eV deposited. Thus, more than 60% of the single-strand breaks introduced into the DNA under anoxic conditions were sealed during the irradiation procedure at 0°C. This type of break mending was later observed in *E. coli* and was interpreted as indicating the existence of a very rapid repair system—Type I repair (Town *et al.*, 1972; reviewed by Town *et al.*, 1973). Subsequent experiments, however, showed that in both bacterial and mammalian DNA the oxygen enhancement ratio for strand breaks is caused essentially by the influence of fast chemical processes (Roots and Smith, 1974; Palcic and Skarsgard, 1975; Johansen, 1975). In *E. coli* B/r these chemical processes occur up to 0.2 s after irradiation while enzymatic excision and strand rejoicing processes are observed only after 2 s (Sapora *et al.*, 1975). Of the breaks remaining at the completion of irradiation, half are rapidly mended within 5 min of incubation postirradiation, even by cells incubated in buffer (Dean *et al.*, 1970). This is equivalent to the Type II repair in *E. coli* (Town *et al.*, 1971a), which was defined to include all the repair processes that rejoin DNA single-strand breaks in buffer. In *E. coli*, DNA polymerase I (the *polA* gene product) plays a major role in Type II repair. This mode of repair was found to be absent in the radiation-sensitive mutant of *M. radiodurans*, UV17 isolated by Moseley (1967b; Bonura and Bruce, 1974).

The single-strand breaks remaining in *M. radiodurans* after this rapid repair are mended more slowly. Complete restitution takes 2 h in TGY medium in cells exposed to 200 krad of X-rays under anoxia (Dean *et al.*, 1970), and 5 h in cells irradiated with 200 krad in oxygen (Burrell *et al.*, 1971). In *E. coli* K-12 incubated in minimal medium, it takes 40 to 60 min to complete the repair of damage caused by 15 to 20 krad (Ganesan and Smith, 1972). This type of repair was called Type III in *E. coli* (Town *et al.*, 1973), and is defined as the slow rejoicing of single-strand breaks that occurs only in bacteria incubated under conditions which allow growth. This repair is inhibited in *M. radiodurans* if chloramphenicol is added to

the growth medium (Dean *et al.*, 1970; Hariharan and Cerutti, 1972b). In *E. coli*, the *recA*, *recB*, and *recC* gene products are all required for Type III repair; *recA* mutants are completely deficient in Type III repair while *recB* and *recC* mutants have considerably less Type III rejoicing ability (Kapp and Smith, 1970; Youngs and Smith, 1973). The only mutant of *M. radiodurans* that is known to be deficient in recombination is the *rec30* mutant, and this strain is more than 100 times as sensitive to γ -radiation as the wild-type strain (Moseley and Copland, 1975b). Other genes involved in this type of repair in *E. coli* are *lexA* (*exr*) (Youngs and Smith, 1973), *dnaB*, *ror* (Glickman *et al.*, 1971), and presumably *lig* (Dean and Pauling, 1970). Type III repair requires DNA replication of the semiconservative type, but is completed in less than is necessary for one round of replication (Ganesan and Smith, 1972). This observation excludes a mechanism of recombination between homologous daughter chromosomes in a manner analogous to the recombination repair of UV radiation-induced damage, while recombination between homologous parent chromosomes is excluded by the result of Bridges (1971) showing that acetate-grown cells of *E. coli*, which are mononucleate, are only slightly more sensitive than glucose-grown cells, which are binucleate. Using a quite different technique to measure the rate and time of completion of recombination repair of ionizing radiation damage in *M. radiodurans*, Moseley and Copland (1976) found that, for doses up to 120 krad, recombination repair was complete before DNA synthesis resumed at normal rates.

Since Type III repair occurs only under conditions that allow growth, and it has been known for many years that different growth conditions can alter the radiation sensitivity of a culture. Town *et al.* (1971b) studied the effect of different growth conditions on both the survival of *E. coli* B/r and on the Type III single-strand mending process. They found that *E. coli* B/r grown to stationary phase in a peptone medium containing glucose was 3.4 times more resistant to radiation than when grown without glucose, and that late exponential-phase cells growing in brain-heart medium plus glucose were 1.6 times more sensitive than stationary phase cells. In each case the more sensitive bacteria degraded more of their DNA during postirradiation incubation, while in the latter case this was associated with a decreased ability to repair single-strand breaks. This may well explain the increased resistance of stationary-phase cells of *M. radiodurans* compared to exponential-phase cells, and the greater sensitivity of exponential-phase cells from media in which the growth rate was faster compared with similar cells from a medium that could only support lower growth rates (Serrianni and Bruce, 1968; Freedman and Bruce, 1971).

7.2. Repair of DNA Double-Strand Breaks

Using an enzyme derived from *Streptomyces albus* to lyse *M. radiodurans* to make the isolation of DNA feasible, Dean *et al.* (1966) provided the first evidence for the repair of ionizing radiation-induced DNA double-strand breaks in *M. radiodurans*. Their method measured the intrinsic viscosity of DNA extracted from unirradiated and from irradiated bacteria either immediately after irradiation or after incubation in TGY medium under growth (low cell density) or nongrowth (high cell density) conditions. The viscosity of the DNA was reduced as a result of irradiation, but during incubation under growth conditions the viscosity increased to control values after times that were dose-dependent. Under nongrowth conditions there was no increase in the viscosity of DNA from irradiated cells. It was suggested, on the basis of the large radiation doses and the reduced viscosity of irradiated DNA, that *M. radiodurans* is capable of repairing double-strand breaks in DNA. In the same year it was concluded that a double-strand break in the DNA of *E. coli* is a lethal event (Kaplan, 1966), although more recently it has been demonstrated that the repair of a small number of double-strand breaks in *E. coli* is possible provided a recombination function and an homologous chromosome are present (Krasin and Hutchinson, 1977).

The technique used by Kaplan (1966) was to measure the sedimentation velocity of DNA from unirradiated and irradiated bacteria through neutral sucrose (5–20%) gradients. This method has been used to investigate the repair of DNA double-strand breaks in *M. radiodurans* (Kitayama and Matsuyama, 1968, 1971a; Burrell *et al.*, 1971). Kitayama and Matsuyama (1971a) calculated double-strand scissions to occur at the rate of one per 800 eV and, following a dose of 220 krad of ^{60}Co γ -rays; their repair was complete after 3 h. The mean rejoining time, i.e., the time to reduce the remaining fraction of DNA double-strand breaks to 0.37 was 52 min. The repair of double-strand breaks did not occur when chloramphenicol, actinomycin D, or tetracycline were added to the postirradiation medium (Kitayama and Matsuyama, 1968, 1971b). Burrell *et al.* (1971), using a butanol-pretreatment to make the cells susceptible to lysis with lysozyme and sodium lauryl sulfate (Driedger and Grayston, 1970), estimated that the efficiency of double-strand break formation was one per 520 ± 50 eV (1 break per krad per 5×10^9 daltons) and that repair was efficient following doses up to 200 krad in oxygen.

The question that arose then was how can *M. radiodurans* hold together the two free ends of a DNA molecule at a double-strand break so that its repair can take place in such a way that the continuity of the genetic message encoded in the DNA is not impaired? One suggestion

was that the chromosome of *M. radiodurans* has multiple attachment points to cell membrane (Driedger, 1970c; Burrell *et al.*, 1971).

7.3. The DNA-Membrane Complex

During studies on the repair of DNA double-strand breaks, it was observed that although following doses in excess of 50 krad of X-rays, the DNA appeared as freely sedimenting molecules; below 50 krad, much of the DNA was present in a complex (density about 1.4), and more than 70% of the DNA from unirradiated cells was present in such a complex (Burrell *et al.*, 1971). It was suggested that the chromosome in *M. radiodurans* is attached to a lipoprotein membrane at only one point by a bond that is resistant to X-irradiation, the lysing procedure, and high salt concentrations, but by many more bonds that are unstable to such conditions. Irradiation of the cell causes random shearing of the DNA; the production of double-strand breaks being linear with dose up to 700 krad in oxygen at 0°C, to produce freely sedimenting molecules, which during repair become reassociated with the membrane complex. Following a dose of 200 krad in oxygen, all the DNA became associated with the membrane complex by 3 h. The reformation of the membrane complex was preceded by the appearance of a second rapidly sedimenting DNA-membrane complex of density 1.5 to 1.6, caused ostensibly by the attachment of broken DNA molecules to membrane fragments. This component was not observed in the experiments of Dardalhon-Samsonoff and Rebeyrotte (1975).

Burrell *et al.* (1971) calculated that the minimum distance between points of attachment of DNA to membrane by stable bonds would be equivalent to that of 2×10^9 daltons, i.e., one per genome of the size calculated by Hansen (1978). Driedger (1970c) had attempted to demonstrate multiple points of attachment between the bacterial DNA and cell membrane. He treated cells with spermidine before fixing them with osmium tetroxide for electron microscopy. The appearance was consistent with multiple attachment sites between DNA and the membrane but, as the author pointed out, such results can always be dismissed as artifacts of the procedure.

Studies on the DNA-membrane complex have been made by Dardalhon-Samsonoff and Rebeyrotte (1975) and Dardalhon-Samsonoff and Averbeck (1980), who followed restitution of the membrane complex after X-irradiation. They found that the time taken for the DNA-membrane complex to reassemble to its unirradiated form in cells is a function of the irradiation dose. It depends more upon ligation than polymerase ac-

TABLE 2. Radiation Survival Characteristics of Sensitive Strains of *Micrococcus radiodurans*

Strain	Ultraviolet radiation				Ionizing radiation			
	Intercept (Jm ⁻²)	1/e (Jm ⁻²)	D ₃₇ (Jm ⁻²)	Ratio D ₃₇ wild type (570 Jm ⁻²) D ₃₇ mutant	Intercept (krad)	1/e (krad)	D ₃₇ (krad)	Ratio D ₃₇ wild type (600 krad)/D ₃₇ mutant
GW1	ND	ND	ND	145	215	360	1.7	Suhadi <i>et al.</i> (1971, 1972)
GR1	ND	ND	ND	90	85	175	3.5	Suhadi <i>et al.</i> (1971, 1972)
KH840	ND	ND	ND	27	8	35	17	Kitayama and Matsuyama (1975)
UV17	76	10.5	86.5	6.5	127	15	142	Moseley (1967b)
UV38	31	13.5	44.5	1.3	100	45	145	Moseley (1967b)
UV22	29.3	11.2	40.5	14	115	20	135	Moseley (unpublished)
UV47	5.2	2.3	7.5	.76	10	7.5	17.5	Moseley (unpublished)
UV50	15.7	5.5	21	27	24	13	37	Moseley (unpublished)
Rec30	22.5	15.5	38	15	0	5.9, 9.1	5.9	102
261 ^a	22	8	30	19	59	10	69	Moseley and Copland (1978)
262	440	73	513	1.1	480	70	550	Moseley and Copland (1978)
263	20.5	5.0	25.5	22	43	7	50	12
301 ^a	10	8	17	33.5	90	8	98	Moseley and Copland (1978)
302	440	115	555	1	614	56	670	6
303	14	8	22	26	85	10	95	Moseley and Copland (1978)
UVS78 ^a	33	8	40	14	695	25	585	6.5
UVS10 ^a	33	8	41	14	ND			Moseley and Copland (1978)
UVS9 ^a	0	54	54	10.5	375	25	400	1.5
UVS25 ^a	0	70	70	8	815	25	840	Moseley (unpublished)
								0.7

^a These strains contain two mutations. Strains 261 and 301 possess the mutations present singly in 262 and 263 and 302 and 303 respectively. Strains UVS9, 25, and 78 all carry the mutation which is present in strain 302 plus another mutation that makes them UV radiation sensitive.

tivity since the complex can be almost completely restored before there is any significant DNA synthesis, and that DNA breakdown can be correlated with the loss of complex formation. Complex assembly is inhibited if irradiation is carried out in the presence of iodoacetamide, a radiosensitizing agent, while phenethylalcohol enhances the extent of DNA degradation and delays complex reassembly.

The picture that emerges is one in which the genome copies are attached to the plasma membrane, each by one stable bond, and that although radiation causes DNA double-strand breaks that fracture the individual double helices, the resulting component pieces are held together by lysis-labile bonds that allow repair to occur.

7.4. Repair of 5,6-Dihydroxydihydrothymine-Type Damage

Radiolysis products of thymine of the 5,6-dihydroxydihydrothymine-type formed in the DNA of *M. radiodurans*, given a dose of 150 krad of γ -radiation, were released from the DNA into the cytoplasm, and then into the growth medium during postirradiation incubation (Hariharan and Cerutti, 1971, 1972a). There were two phases; a rapid phase lasting 30 min during which 75% of the damaged bases were released, followed by a temporary halt, and a second phase starting at about 60 min and lasting until 200 min, during which the remaining 25% were released. Only the release of thymine radiolysis products in the second phase was inhibited by chloramphenicol. It was hypothesized by Hariharan and Cerutti (1971, 1972a) that the initial rapid release of the thymine product was the result of exonucleolytic degradation beginning at radiation-induced DNA single-strand breaks, and that strand breaks and thymine radiolysis products are clustered in the DNA, so that protein synthesis is not necessary for this phase. The second phase for which protein synthesis is necessary, was assumed to be an endonucleolytic incision and release. On average about 300 undamaged thymine residues were removed from the DNA for every damaged residue during the 240 min of postirradiation repair (Hariharan and Cerutti, 1972a). An attempt has been made to correlate the slightly increased radiation sensitivity of strain 262 (see Table 2) with reduced excision repair of 5,6-dihydroxydihydrothymine-type damage, but the results are not convincing (Targovnik and Hariharan, 1980).

8. REPAIR OF UV RADIATION-INDUCED DNA DAMAGE

The repair of UV radiation-induced damage in *M. radiodurans* was demonstrated in a series of investigations in J. K. Setlow's laboratory, (Setlow and Duggan, 1964; Setlow and Boling, 1965; Boling and Setlow,

1966). It was first shown that, although the bacterium was very resistant to UV radiation, its DNA was not exceptionally resistant as indicated by thymine dimerization, just over 1% of the thymine being present as dimer after 600 J m^{-2} , approximately the D_{37} . More recently the association of the chromosome with manganese ions has been suggested to reduce the yield of UV radiation photoproducts (Leibowitz *et al.*, 1976); but this can only play a minor role in the overall resistance of *M. radiodurans* to UV radiation. The thymine-containing pyrimidine dimers were excised from the DNA at an exponential rate that was dose dependent, but in contrast to *E. coli* where TCA-soluble fragments of DNA that contain dimers remain in the cell, the dimers appeared in the growth medium of *M. radiodurans* (Boling and Setlow, 1966). The fact that the rate of excision was not a linear function of the dose was taken to mean that the substrates in the reaction (dimers in DNA) were in excess over the enzymes presumed responsible for their repair (Boling and Setlow, 1966). Similar results have been reported for *M. radiophilus* (Lavin *et al.*, 1976). Excision was not inhibited by chloramphenicol.

A cytosine-thymine adduct accounts for about 17% of the total thymine-derived photoproducts in UV-irradiated *M. radiodurans*, and this proportion remains constant during the excision repair process (Varghese and Day, 1970). This indicates that the cytosine-thymine adduct is excised at the same rate as the thymine-thymine dimer, confirming an earlier report (Moseley, 1969). The time delay for DNA synthesis to resume at the normal rate was about 20-fold less than in *E. coli* B/r for the same dose (Setlow and Duggan, 1964), but was greater than the time required to excise almost all the dimers (Boling and Setlow, 1966).

By measuring the efficiency of killing and the delay in DNA synthesis in *M. radiodurans* irradiated at different wavelengths of UV radiation, Setlow and Boling (1965) concluded that killing and the length of the DNA synthesis-delay must be, at least in part, the result of lesions other than thymine dimers, and they suggested that deoxycytidine and protein damage are involved in these two effects, since the exponential slope of the survival curve is the same at both 260 and 280 nm. Two UV radiation-sensitive mutants of *M. radiodurans*, UV17 and UV38 (Moseley, 1967b), were found to have reverted to the normal bacterial action spectra, i.e., killing was more efficient at 260 nm than at 280 nm (Moseley, 1969).

9. RADIATION-INDUCED MUTATION

9.1. Ionizing Radiation

Okazawa and Matsuyama (1967) reported that γ -radiation could induce in a population of wild-type cells, a mutation that resulted in a less-

pigmented phenotype. The frequency of such induced mutants was extremely high, reaching 20% of an irradiated stationary-phase population showing almost no loss of viability, and 40% of the population surviving 800 krad. It is very difficult to take this result as serious evidence for mutation induction. It is possible to obtain results in which all (Moseley, 1967b), or an increasing proportion (Moseley, 1963), of the colonies from cells surviving various treatments contain such "mutants." However, this appears to be a consequence of the breakdown of the normally disciplined cell division and can be simulated using treatments that are not mutagenic (Moseley and Copland, 1975a).

To investigate mutation induction properly, it is necessary to have a selectable phenotype with a sufficiently low spontaneous mutation frequency in the population so that small increases in the numbers of induced mutants are not hidden by the background level of spontaneous mutants. Using the induction of resistance to trimethoprim in a trimethoprim-sensitive population, i.e., wild-type *M. radiodurans* (spontaneous mutation frequency about 10^{-5}), Sweet and Moseley (1976) were unable to detect any γ -radiation induced mutation. However, using a different marker, i.e., induction of mutation in the wild-type strain to rifampicin-resistance (spontaneous mutation frequency about 3×10^{-8}), the induced mutation frequency at doses of 300 krad and above was 4×10^{-7} , i.e., about 13 times the spontaneous level (Tempest, 1978). This is an exceedingly low induced mutation frequency, being about 1000 times less than that obtained using MNNG. The weak mutability by γ -rays may be ascribed to misreplication caused by the presence of miscoding nucleotides, or by damage to a DNA polymerase rather than to error-prone repair (Tempest, 1978).

9.2. UV Radiation

The forward mutation to trimethoprim resistance and the reverse mutation of a temperature-sensitive strain to temperature resistance could not be induced by UV radiation, although both were sensitive to mutation by MNNG (Sweet and Moseley, 1974). Nor could induced mutation be detected when the irradiated bacteria were incubated in caffeine. It was concluded that the repair of UV radiation-induced damage in *M. radiodurans* is accurate. A similar conclusion was reached by Kerszman (1975), who studied mutation induction of resistance to low levels of streptomycin in both the wild-type and the UV-sensitive mutant, UV17. UV radiation-induced rifampicin-resistant mutants were not detected by Tempest (1978). It must be concluded that error-prone repair of UV radiation-induced damage is absent from *M. radiodurans* (Tempest and Moseley,

1982). This is in marked contrast to the role played by the inducible error-prone repair system in *E. coli* (Witkin, 1976). Similar results have been obtained for the other red-pigmented radiation-resistant Micrococci. *M. roseus* (ATCC 19172), *M. radiophilus*, and *M. radioproteolyticus* were all given UV radiation doses that resulted in a survival of between 1% and 10%. The resolution was such that a minimum of a four-fold increase over the spontaneous level of mutation to rifampicin-resistance, which was between 1 to 4×10^{-8} , would have been detected. No UV radiation-induced mutants were found in any of these species. In contrast, two true members of the genus *Micrococcus*, namely, *M. luteus* and *M. sodonensis*, which had D_{37} values of 109 and 145 J m^{-2} , respectively, were mutated by UV radiation, showing at least a 30–50-fold increase over the spontaneous levels of rifampicin-resistant mutants (Tempest, 1978). At present it appears that all very radiation-resistant bacteria lack the error-prone repair of UV radiation-induced damage (Tempest and Moseley, 1982).

10. REPAIR MECHANISMS

10.1. Photoreactivation

Photoreactivation repair of pyridimine dimers does not occur in *M. radiodurans*. Its survival following UV irradiation cannot be influenced by its being exposed to photoreactivating wavelengths of light (Moseley and Laser, 1965a). Since the repair of UV radiation-induced damage by dark repair mechanisms is very efficient, it might be that a photoreactivating repair mechanism is present, but cannot be demonstrated. However, an increase in survival of either of two UV-irradiated UV-sensitive mutants *M. radiodurans*, *rec30* (Moseley and Copland, 1975b) and strain UV47 (see Table 2 for radiation-survival characteristics), did not occur following photoreactivation treatment (Moseley, unpublished observation). Photoreactivating enzyme was also not detected by an *in vitro* assay in which a cell free extract of *M. radiodurans* was used in an attempt to photoreactivate UV-irradiated *Haemophilus influenzae* DNA (J. K. Setlow, personal communication).

10.2. Excision Repair

Evidence has already been given for the existence of an excision mechanism for the release of pyrimidine dimers from DNA. It is inter-

esting to note that in spite of the considerable ease with which radiation-sensitive mutants of *M. radiodurans* can be isolated, no excision-deficient mutants have been reported. In *E. coli*, e.g., the excision-deficient mutants were among the first radiation-sensitive mutants to be isolated (Hill, 1958; Howard-Flanders and Theriot, 1962). It was thought that perhaps the contribution of excision repair to the total repair capacity of wild-type *M. radiodurans* was a minor one compared with that of recombination repair, and that the protocol used to isolate radiation-sensitive mutants would not detect strains that had only a slightly increased sensitivity to UV radiation (Moseley *et al.*, 1972b). However, it now appears that *M. radiodurans* has at least two UV endonucleases that incise DNA after UV irradiation prior to the release of pyrimidine dimers from its DNA. Therefore, two mutations have to be present that block the formation of both endonucleases before the UV-sensitive phenotype can be seen (Evans and Moseley, unpublished results).

Strain 302 of *M. radiodurans* is mutant in gene *mtcA*, which causes it to be about 40 times as sensitive as the wild-type strain to the lethal action of mitomycin C while retaining wild-type resistance to UV radiation (Moseley and Copland, 1978). It is also much more mutable than the wild-type strain by the alkylating agents MNNG, methyl methanesulphonate and ethyl methanesulphonate (Tempest and Moseley, 1978, 1980). It is, therefore, a much more useful strain than the wild-type strain with which to start when trying to isolate mutants by MNNG mutagenesis, and the *mtcA* mutation can easily be removed subsequently by selecting for resistance to mitomycin C after transformation with wild-type DNA. Several strains, mutant in different genes, have now been isolated and found to be sensitive to UV radiation, but resistant to methyl methanesulphonate, and are incision deficient. However, when the *mtcA* gene, which is not linked to the new mutant genes, is restored to wild-type by transformation, the new mutants all become UV-resistant, i.e., two genes have to be mutant for the UV-sensitive phenotype. The restoration of either gene to the wild-type gives a UV-resistant phenotype. This is consistent with there being two UV endonucleases capable of acting independently prior to the excision repair of UV radiation-induced damage. These mutations are now being investigated in detail.

Preliminary results indicate that the two endonucleases either have associated exonuclease activities or make different substrates for subsequent exonuclease action. Thus, when one endonuclease is absent in a mutant strain, the associated exonuclease activity is also absent. The two exonuclease activities can be distinguished by the fact that one of them requires a UV radiation-inducible product to limit its activity, while the other does not (Evans and Moseley, unpublished observations).

10.3. Recombination Repair

The physical evidence for postreplication recombination repair, of the kind produced for *E. coli* (Rupp and Howard-Flanders, 1968; Rupp *et al.*, 1971), has not been obtained for *M. radiodurans*. The evidence has been largely circumstantial. For example, it was found that a mutant of *M. radiodurans*, ts1, temperature sensitive for DNA synthesis (Moseley *et al.*, 1972a), gradually became sensitive to both ionizing and UV radiation when held at its restrictive temperature (39°C), the shoulder of the survival curve eventually disappearing, although the slope of the exponential part of the survival curve only increased by a factor of two. The rate of loss of shoulder was correlated with the rate of loss of recombinational ability, as measured by transformability (Moseley *et al.*, 1972b). When this mutant was given sublethal doses of ionizing or UV radiation and then incubated at the restrictive temperature (thus depleting recombinational ability) before plating at a permissive temperature, a very radiation-sensitive feature was the loss of disciplined cell-division. Although most of the irradiated bacteria survived and produced colonies, the latter only appeared after four days of incubation instead of two, and were highly sectored and irregular (Moseley and Copland, 1975a). The radiation survival curves for normal colony formation under the conditions described were virtually identical to the survival curves of a recombination-deficient mutant *rec30*, which is 15 times as sensitive to UV radiation, 100 times as sensitive to γ -radiation, and 300 times as sensitive to mitomycin C, as the wild-type strain (Moseley and Copland, 1975b).

Because the immediate exposure of the sublethally irradiated mutant ts1 to its restrictive temperature before plating caused the loss of normal colony formation, it followed that postirradiation incubation at a permissive temperature that allowed recombinational repair to occur should eventually produce a culture as resistant to the loss of normal colony formation as an unirradiated population. This approach was used to measure the rates of recombination repair in *M. radiodurans* ts1, and showed that during postirradiation incubation in broth at 30°C the damage caused by 1.2 krad of γ -radiation or 1.8 J m^{-2} of UV radiation was repaired per minute (Moseley and Copland, 1976).

10.4. Error-Prone (SOS) Repair

There is substantial evidence (discussed in Section 9.2.) that *M. radiodurans* does not possess an inducible error-prone repair pathway, and that its repair of UV radiation-induced damage is error free. Evidence for

E. coli suggests that an error-prone function is needed to repair lesions that cannot be repaired by constitutive mechanisms. Sedgwick (1976) proposed that overlapping daughter-strand gaps in two homologous chromosomes, produced as a consequence of the replication of pyrimidine dimer-containing DNA, may be such lesions. He suggested that any non-pairing base, such as a pyrimidine dimer, an alkylated base or a γ -ray damaged base, opposite a gap or noncoding base cannot be repaired in an error-free manner, and requires random nucleotide insertion for effective, though mutagenic, repair. It follows that in the radiation-resistant Micrococci, either such lesions are produced infrequently or they are repaired by a mechanism not present in *E. coli*. One possibility is that the size of the gap produced when the DNA polymerase encounters a dimer and reinitiates synthesis at a site past the dimer could be much smaller than the 1000 or so base gap produced in *E. coli* (Iyer and Rupp, 1971), so that the probability of two such gaps overlapping is very small. However, there is no information on the gap size in *M. radiodurans*.

An alternative, but most attractive, hypothesis is that the nuclear body of *M. radiodurans* is multigenomic (Moseley and Evans, 1981), and that overlapping single-strand gaps opposite dimers would have to be present in all genome copies for the genetic information in that region not to be recoverable by recombinational exchange. Such a circumstance would only arise at very high radiation doses. In fact, before such a situation could exist, the high UV dose may already have created a lethal event in a non-DNA target (Setlow and Boling, 1965).

11. THE ENZYMOLOGY OF REPAIR

Virtually nothing is known of the enzymology of repair in *M. radiodurans*. An exonuclease released from *M. radiodurans* by ionizing radiation has been shown not to be related to the repair of ionizing-radiation damage (Gentner and Mitchel, 1975). Gentner (1973, 1974) gave two brief reports of a magnesium-dependent DNA polymerase associated with exonuclease activity, which had been partially purified from *M. radiodurans*. Postirradiation incubation of cells after sublethal doses of X-rays gave an increase of several-fold in specific activity of the polymerase in the absence, but not the presence, of chloramphenicol. The radiation-sensitive mutants, UV17 and UV38 (Moseley, 1967b), had only 2 and 25% of the polymerase activity of the wild-type strain, respectively, but normal levels of exonuclease activity. In a later investigation of DNA polymerases (Kitayama and Matsuyama, 1977; Kitayama *et al.*, 1978), two polymerase fractions of MW 140,000 were originally isolated that catalyzed

the condensation of deoxyribonucleoside triphosphates on to a 3'-hydroxyl primer terminus, but they differed in their pH optimum and residual activity in the absence of a full deoxyribonucleoside triphosphosphate complement. Deoxyribonuclease activity was associated with the polymerase fraction that had the higher activity in the absence of such a complement. Both fractions showed a lower activity on an irradiated DNA template than on an unirradiated one. This last result was disappointing because in a permeable-cell system of *M. radiodurans* (Kitayama and Matsuyama, 1976) DNA synthesis was stimulated by irradiation of the permeable cells with γ -rays, and this effect was abolished with NAD, which also partially inhibited one of the polymerases *in vitro*.

Using an improved technique, Kitayama *et al.* (1978) demonstrated three polymerase activities (DNA polymerases I, II, and III), the exonuclease activity being associated with DNA polymerase I. The two activities could not be separated, showing the same optimum pH and heat inactivation kinetics. The exonuclease hydrolyzes preferentially double-stranded DNA from both ends of the duplex, releasing mostly deoxyribonucleoside 5'-monophosphates. It is tempting to ascribe a proof-reading function to this 3' \rightarrow 5' exonuclease activity, associated with a polymerase catalysing DNA synthesis in a 5' \rightarrow 3' direction, but evidence for this is not forthcoming at present. However, a mutant possibly defective in the exonuclease activity is UV47, which is very radiation sensitive and has an enhanced spontaneous-mutation frequency (Tempest, 1978).

A recent approach to the study of enzymes associated with DNA repair in *M. radiodurans* is to identify proteins induced in response to irradiation (Hansen, 1980). After a nonlethal dose of UV radiation, *M. radiodurans* was incubated for 30 min with [35 S]methionine and the newly-synthesized proteins located by two-dimensional electrofocussing and SDS polyacrylamide gel electrophoresis. Four proteins were preferentially synthesized in UV-irradiated cells, and had molecular weights between 26,000 and 36,000. For three of the proteins, there was a 30-fold increase over the constitutive level and a 90-fold increase for another. Damage caused by ionizing radiation or mitomycin C also caused induction of these proteins, but in different proportions, while treatment with nalidixic acid or deprivation of thymine in a Thy⁻ strain did not. Progress in this direction will be made when the induced proteins (or lack of them) can be related to mutant phenotypes.

12. DEATH OF *M. radiodurans* FOLLOWING IRRADIATION

After discussing the type of DNA-repair mechanisms present in *M. radiodurans*, it is pertinent to consider the reasons why these eventually

fail to retain the viability of the cell at high doses of radiation. One theory that emerges from the published literature is that the semiconservative synthesis of DNA, temporarily inhibited by radiation, eventually resumes, but at high doses, however, it occurs on a DNA template that has not yet been repaired, and this has lethal consequences. Lett *et al.* (1970) showed that, in cells that survived X-irradiation, the resumption of DNA synthesis followed the end of the period of release of DNA degradation products. However, at the dose corresponding to the end of the shoulder of the survival curve there was a "crossover point" where DNA synthesis resumed before the release of degradation products was complete. Boling and Setlow (1966) demonstrated that following a nonlethal UV radiation-dose of 300 J m^{-2} , almost all the dimers were removed during a 70 min postirradiation incubation; but the delay in the rate of DNA synthesis returning to normal took almost twice as long. Moseley and Copland (1976) showed that postreplication recombination repair of UV radiation-induced damage took longer per unit dose of radiation than the resumption of DNA synthesis, so that at the lethal dose the DNA replication fork would have reached DNA still undergoing recombination repair. Finally, Little and Hanawalt (1973) considered the theory that thymineless death is caused by the normal growing point region of DNA, upon restoration of thymine, encountering damaged regions of the DNA before they had a chance to be repaired, with lethal results. All of these models have the replication of DNA on an unrepaired template having lethal consequences in common.

13. GENETICS OF *M. radiodurans*

13.1. Transformation

M. radiodurans is a genetically transformable species (Moseley and Setlow, 1968; Tirgari and Moseley, 1980). However, transformation is not a useful method for mapping the chromosome, e.g., to locate the mutations in repair genes in order to see whether they are clustered or not, because it involves the uptake and integration of only very small pieces of DNA. However, it is useful in other respects. For example, it is possible to treat the isolated DNA in various ways and to correlate such treatment with its subsequent biological activity. Thus, when transforming DNA is UV irradiated, the ability of recipients to repair the induced pyrimidine dimers and express the carried marker can be tested and compared to the efficiency with which the dimers are repaired by photoreactivating enzyme *in vitro*. After treatment of UV-irradiated *M. radiodurans* DNA

with purified yeast photoreactivating enzyme such that almost all the pyrimidine dimers were monomerised there was no increase in the survival of biological activity, indicating that *M. radiodurans* can repair irradiated transforming DNA as efficiently as it does its own DNA (Moseley and Setlow, 1968). Both the wild-type strain and the radiation-sensitive mutant UV17 repair UV- or γ -irradiated DNA to the same extent, and this has been explained in terms of the two strains being equally efficient at repairing lesions that are present in only one strand of a DNA helix, the complementary strand being undamaged host DNA (Moseley and Mattingly, 1971). The radiation sensitivity of the mutant is shown up, however, if the wild-type and UV17 strains are irradiated and then transformed, since the loss of transformability is a more radiation-sensitive parameter in UV17 than the wild-type strain. In the mutant, the D_{37} for loss of transformability has the same value as the $1/\epsilon$ dose for loss of cell viability, i.e., the shoulder of the curve for loss of viability is missing (Moseley and Mattingly, 1971). In these experiments DNA uptake into the cell is not affected.

An unusual feature of transformation of *M. radiodurans* is that there is no transient competent stage during which the cells take up transforming DNA. So long as the cells remain in the exponential phase of growth they are competent to take up DNA (Tirgari and Moseley, 1980). This is convenient because, since the integration of transforming DNA involves recombination, recombinational ability can be assayed by measuring the level of transformation, checking, of course, that the uptake of DNA into the cell is not reduced. This method was used to show that a temperature-sensitive mutant (ts1) held at its restrictive temperature gradually lost recombinational ability at the same rate as the reduction in length of the shoulder of the survival curve (Moseley *et al.*, 1972b). This led to the suggestion that the shoulder of the survival curve was largely determined by a recombinational repair mechanism. The lack of transformability, although not of DNA uptake, was used to assign the radiation- and mitomycin C-sensitive mutant *M. radiodurans rec30* its Rec⁻ phenotype (Moseley and Copland, 1975b).

Transformation is also valuable in enabling mutations that give similar mutant phenotypes to be assigned to the same or different genes. Thus, if DNA from one of the mutants transforms a similar mutant and restores the wild-type phenotype at level consistent with the transformation frequency for a single marker, it can be assumed that both strains are mutant in different genes (Moseley *et al.*, 1972a; Moseley and Copland, 1978).

Recently, after improving the methodology for transformation to give relatively high transformation frequencies (in excess of 1%), attempts to measure the competent fraction of the population using the unlinked

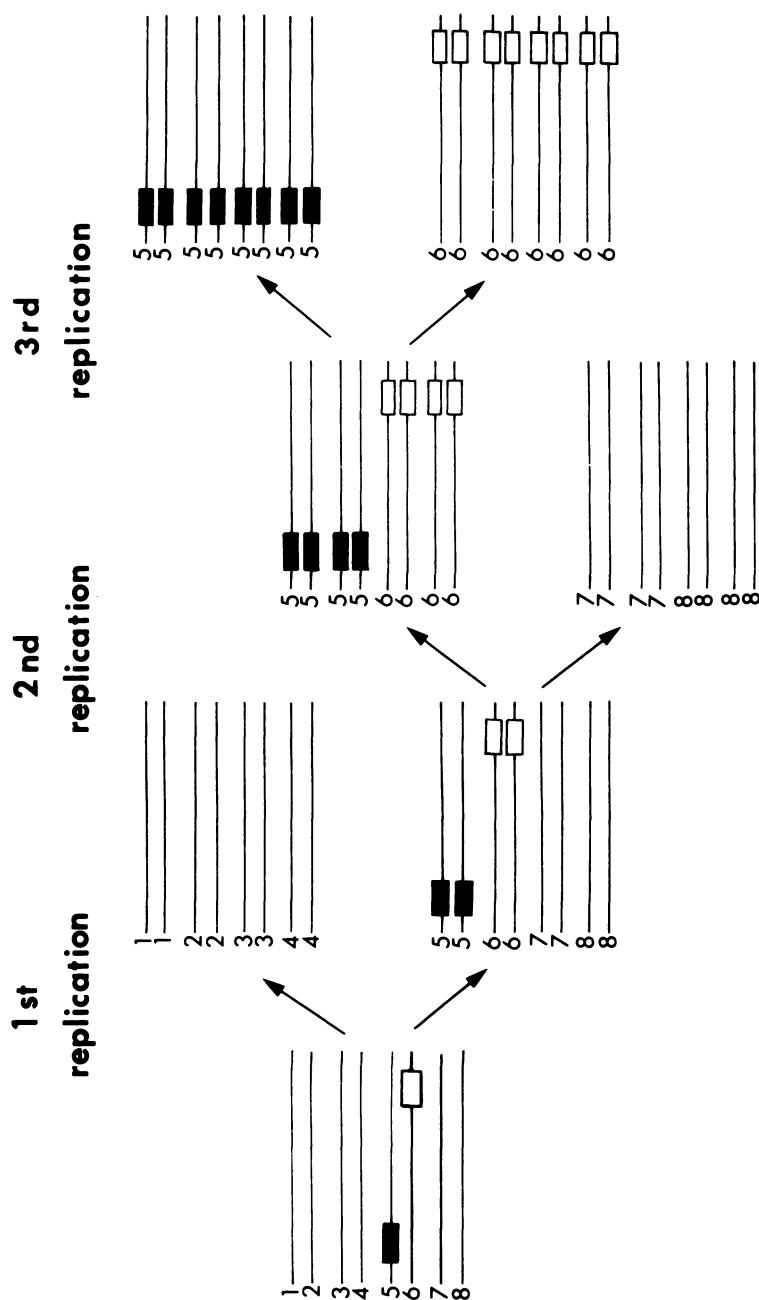


Fig. 4. Proposed scheme for the segregation of transformable units (single strands of DNA). The nucleoid is shown having four homologous chromosomes in a cell doubly transformed to rifampicin-resistance (which behaves as a dominant marker) in strand 5 and streptomycin-resistance (which acts as a recessive marker) in strand 6. Eventually, progeny become homozygous for each of the DNA strands in the original transformed cell and thus express recessive markers.

marker technique led to values of 400% or greater (Tirgari and Moseley, 1980). This has been interpreted to mean that *M. radiodurans* has a multiple number of transformable units (single strands of DNA) that are independent and segregate independently. When the amount of DNA per nuclear body was assayed it became clear that four to five genome copies were present in each stainable nucleoid (Moseley and Evans, 1981), and a scheme for their segregation is shown in Fig. 4. The nuclear arrangement is a novel one in the sense that the genome copies must be identical, otherwise recessive mutations could not be expressed as they obviously are. This means that recombinational exchange should be possible between homologous regions of the several identical genome copies. An alternative mechanism to allow the expression of recessive genes would involve a very complicated scheme of gene conversion.

The presence of multiple genome copies per nuclear body appears not to be unique to the radiation-resistant Micrococci, since there is evidence that the nuclear bodies of the relatively radiation-sensitive species *M. luteus* and *M. sodonensis* also contain multiple genome equivalents (Purvis and Moseley, unpublished observations). It follows that the mere possession of such a genome arrangement is not sufficient in itself to cause radiation resistance. Incidentally, *Azotobacter vinelandii* which contains at least 40 chromosomes per cell (Sadoff *et al.*, 1979) is very sensitive to UV radiation (personal observation). The number of genome equivalents of DNA per cell of *M. radiodurans* has been varied experimentally from 5.4 to 9.4 by varying the growth medium. These cultures showed differences in their resistance to UV and γ radiation, but the differences could not be correlated with the differences in genome equivalents per cell (Harsjø *et al.*, 1981). The authors concluded that if reconstruction of an intact genome from intact segments of different chromosomes is responsible for the radiation resistance of *M. radiodurans*, then five genome equivalents per cell are sufficient.

13.2. Transduction and Conjugation

In spite of a long and exhaustive search, no phages have been isolated that plaque on *M. radiodurans* (Moseley, unpublished), and hence no transduction system is available. All attempts to develop a conjugation system have likewise been unsuccessful.

14. THE ISOLATION OF RADIATION-SENSITIVE MUTANTS

The successful isolation of radiation-sensitive mutants is dependent on the use of nitroso compounds such as MNNG (Sweet and Moseley,

1974, 1976) or *N*-methyl-*N*-nitrosourethane (Tempest, 1978), since they are the only compounds useful for mutagenesis of wild-type *M. radiodurans*. The first isolation of radiation-sensitive mutants employed UV radiation followed by MNNG treatment in order to reduce the viability of the treated population to 10^{-2} – 10^{-3} , but in retrospect the UV radiation was unnecessary (Moseley, 1967b).

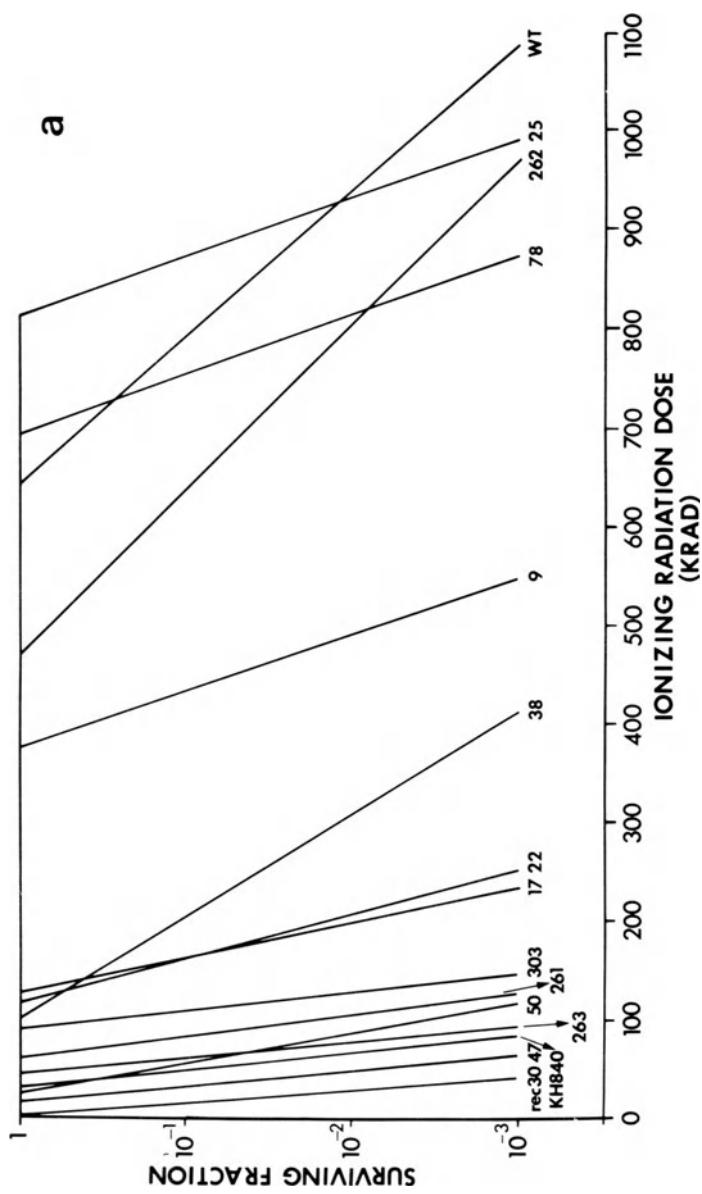
Selective methods are not necessary, although they have been successful. Suhadi *et al.* (1971, 1972) isolated two temperature-sensitive ionizing radiation-sensitive mutants following MNNG mutagenesis of the wild-type strain by irradiating the population with a sublethal dose, incubating the cells with 5-bromodeoxyuridine (5-BU) at 37°C for 4 h, and then irradiating again. Radiation-sensitive mutants would be likely to have a long delay in DNA synthesis caused by the first dose of radiation, would not take up 5-BU (as a thymine analogue) at the higher temperature, and would, therefore, be relatively more resistant to a second dose of radiation than wild-type bacteria (Lett *et al.*, 1970). In fact, the mutants GR1 and GW1 were not very sensitive to radiation.

The normal method following MNNG mutagenesis would be to allow sufficient (usually overnight) incubation for a mutation in one chromosome of a tetrad of cells to segregate into a homozygous viable unit, plate for single colonies, replicate onto agar, and identify radiation-sensitive clones by irradiating one copy of each plate. A mutant has been isolated (Moseley and Copland, 1978) that is about 50 times more sensitive to MNNG mutagenesis than the wild-type strain (Tempest and Moseley, 1978, 1980) and it would be prudent for most purposes to start with this strain, *M. radiodurans* 302.

A list of all the radiation-sensitive mutants and their radiation characteristics is given in Table 2, and the majority of their survival curves (somewhat idealized) are shown in Fig. 5.

Apart from the first three mutants listed in Table 2, all were isolated on the basis of their sensitivity to UV radiation, and it transpired that most were also sensitive, to a greater or lesser degree, to ionizing radiation. The exceptions are those double mutants (UVS78, UVS25 and UVS9) that are incapable of excising pyrimidine dimers. While not having exactly wild-type resistance to γ -radiation, these mutants are nonetheless very resistant to γ -rays. All the UV radiation-sensitive mutants are sensitive also, again to a greater or lesser degree, to the lethal action of mitomycin C, an anti-tumor drug that can covalently cross-link DNA. However, not all mitomycin C-sensitive mutants are UV radiation-sensitive, since strains 302 and 262, mutant in *mtcA* and *mtcB* respectively, have wild-type resistance to UV radiation (Moseley and Copland, 1978).

Very few of the radiation-sensitive mutants have been characterized with regard to their repair defect. The UV17 and UV38 mutants appear



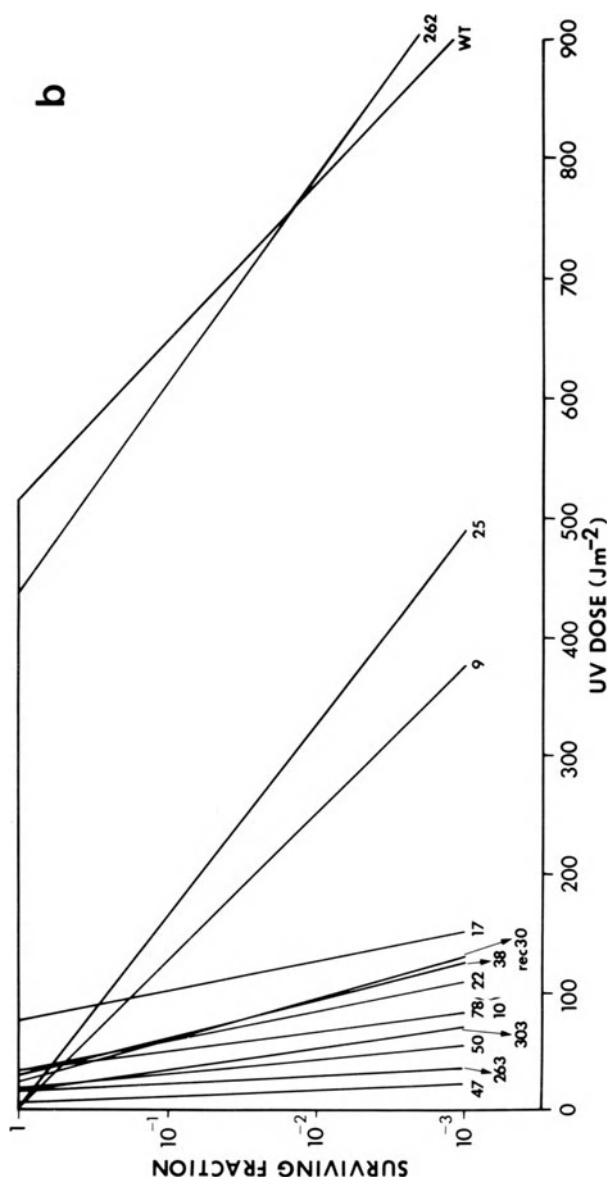


Fig. 5. Idealized ionizing (a) and UV (b) radiation survival curves for the radiation-sensitive mutants of *M. radiodurans*, compared with those of the wild-type (WT) strain. The survival curves have been constructed by extrapolating the exponential inactivation slopes to unit survival (data from Table 2).

to be defective in DNA polymerase activity (Gentner, 1974; Bonura and Bruce, 1974), while the *rec30* strain is deficient in recombinational activity (Moseley and Copland, 1975b). The remainder have still to be investigated.

15. THE REPAIR OF ALKYLATION DAMAGE

Although not strictly relevant to this review, a brief section on the repair of alkylation damage is of interest. The wild-type strain is able to repair potentially lethal damage caused by the bifunctional alkylating agent mitomycin C (MTC), the monofunctional alkylating agent decarbamoylmitomycin C (DCMTC), methyl methanesulfonate (MMS), ethyl methanesulfonate (EMS), and MNNG. The mutant 302 is 40 times as sensitive as the wild-type strain to the lethal action of MTC and DCMTC, and is much more sensitive to the mutagenic, but not lethal, action of MMS, EMS, and MNNG, the defect in MMS repair being entirely in the removal of mutagenic lesions (Moseley and Copland, 1978; Tempest and Moseley, 1978, 1980). The wild-type strain can excise the modified bases *O*⁶-methylguanine, 7-methylguanine and 3-methyladenine from its DNA, although whether by glycosylase or endonuclease activity has not been determined (Tempest, 1978).

The mutability of strain 302 to the methylating agents MMS and MNNG is likely to be caused by that strain's inability to excise the mispairing base *O*⁶-methylguanine while the mutant UV22 appears to be unable to excise 3-methyladenine (Tempest, 1978).

16. IONIZING RADIATION-INDUCED RELEASE OF CELL COMPONENTS INCLUDING AN EXONUCLEASE

M. radiodurans, irradiated in aqueous suspension, loses up to 30% of its wet weight and 6% of its dry weight, the maximum loss being reached at the sublethal dose of 400 krad (Mitchel, 1976). This appears to be due to the loss of polysaccharide, including a polymer of glucose and N-acylated glucosamine, into the growth medium from a hydrophobic site in the middle, lipid-rich, cell-wall layer. The polysaccharide is present in the cell wall as a high molecular weight polymer, but the radiation, generating free radicals from the surrounding water, especially hydroxyl radicals, causes some degradation prior to release. Muramic acid from the peptidoglycan layer is not released.

The radiation-induced loss of polysaccharide material into the me-

dium is similar to the release from this organism of an exonuclease and other proteins from the same cell-wall layer (Mitchel, 1975a,b). The exonuclease (orthophosphoric diester phosphohydrolase EC 3.1.4.1), which is not normally excreted, is released into the medium when cells are exposed to ionizing radiation, less than 10% remaining bound at 400 krad. The amount released is dependent only on the radiation dose, being independent of dose-rate, temperature, and the presence or absence of oxygen during irradiation (Gentner and Mitchel, 1975; Mitchel, 1975b). Since more than 80% of the exonuclease is released, but only a small amount of protein (less than 0.5% at 400 krad), its specific activity is increased 150-fold in crude extracts. The releasing agent appears to be hydroxyl radicals generated by the radiolysis of water, since the release of the nuclease is inhibited by ethanol, a good hydroxyl-radical scavenger. Although the enzyme is not released by UV irradiation alone, it is released when hydrogen peroxide is added to the cell suspension in order to generate hydroxyl radicals (Mitchel, 1975a). The exonuclease exists in the cell as a noncovalently linked dimer whose molecular weight is 260,000, and is released in this form into the surrounding medium where it is rapidly split by further aqueous radical attack into two subunits whose molecular weights are 130,000 (Mitchel, 1980). There is no evidence that this exonuclease has a role in the repair of DNA damaged by ionizing radiation (Gentner and Mitchel, 1975). A 5'-nucleotidase is also present in the cell envelope of *M. radiodurans* (Mitchel, 1973), but is not released by ionizing radiation.

17. SUMMARY AND SUGGESTIONS FOR FUTURE RESEARCH

M. radiodurans and its relatives are very resistant to the lethal and mutagenic effects of ionizing and UV radiation. The D₃₇ values for *M. radiodurans* are approximately 600 krad for ionizing radiation and 570 Jm⁻² for UV radiation. Following ionizing irradiation, the DNA is degraded at 0.12% per min independent of dose, although the amount degraded is dose dependent. Both single- and double-strand DNA breaks are repaired at an exponential rate, 63% of both being mended in about 1 h at 30°C. The repair begins during irradiation and proceeds for up to five hours in growth medium, depending on the radiation dose. Thymine radiolysis products of the 5,6-dihydroxydihydrothymine type, numerically equal to the strand breaks, are released into the growth medium, initially probably, as a consequence of DNA degradation from strand breaks.

After UV irradiation, the DNA is incised by either of two independent

endonucleases and the pyrimidine dimers are excised at exponential rates. Those dimers that have not been excised when the replication complex reaches them are by-passed by recombination-dependent repair processes, and may be excised later. Neither photoreactivation nor error-prone repair mechanisms are present.

M. radiodurans probably has a nuclear structure in which a number of identical genomes (four or five) are arranged in such a way that recombinational exchange involving homologous regions of all copies may occur. This would obviate the need for an error-prone repair mechanism, and allow error-free repair even at very high radiation doses.

Three areas of research are in need of investigation. First, the detailed structure of the nucleus should be unravelled and physical evidence obtained for the involvement of multiple genome copies in recombinational exchange. Second, knowledge of the enzymology of repair is rudimentary and needs developing. However, since a number of repair-deficient mutants are available, and others could be isolated quite easily, these would form the basis of such research. Third, the development of a mapping system, either by chromosome mobilization or spheroplast fusion would be welcome, since a large number of mutations affecting both repair and other functions are begging to be mapped.

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Cherenkov Radiation: Its Properties, Occurrence, and Uses

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1. INTRODUCTION AND HISTORICAL BACKGROUND

When a fast charged particle, for example an electron, plunges through a transparent medium such as water or glass, it produces a faint emission of radiation, which occurs predominantly in the visible and ultraviolet (UV) regions of the electromagnetic spectrum. This is known as Cherenkov radiation, named after the Russian experimental physicist who studied the phenomenon in some detail between the years 1934 and 1938.

It was, however, many years prior to this detailed study and interpretation, that Cherenkov radiation had been observed by individual researchers working in the field of radioactivity. As far back as 1910 Mme. Curie (Curie, 1941), in her exhaustive studies of the radioactivity of radium, noticed an uncanny blue light emanating from her bottles of concentrated solutions. Preoccupation with the radioactivity itself probably stifled any investigation into the causes of this faint luminescence. As these early observations were made during the epoch when many physicists were engaged in studies of fluorescence and phosphorescence caused by the irradiation of liquids and solids by the then newly discovered ultraviolet and X-radiations, it was generally supposed that this faint emission of bluish-white light from solutions of radioactive substances was likewise some form of fluorescence.

It was somewhat later that the Frenchman Mallet (1926, 1928, 1929) carried out a series of valuable experiments in an attempt to understand the phenomenon. Mallet's contribution to the subject, this writer feels, has often been forgotten or ignored. He found that the light emitted from a wide variety of transparent bodies placed close to a radioactive source always had the same bluish-white quality, and that the spectrum was continuous, not possessing the line or band structure characteristic of fluorescence. He was the first to appreciate the generality of the effect and to notice that in a number of other respects, it was very different from fluorescence and other known forms of luminescence. Unfortunately

Mallet did not pursue the work, nor did he attempt to offer an explanation for the origin of the light. The subject then lay dormant until Cherenkov (1934) commenced an exhaustive series of experiments, which he continued until 1938. These experiments were remarkable for their simplicity and for the excellent agreement between their results and the theory, which had in the meantime been proposed by Frank and Tamm (1937). Cherenkov appears to have been unaware of the earlier work of Mallet, though he too met the problem accidentally through studies of fluorescence; his experiments covered a wider range than did those of Mallet. The details of these early experiments have been summarized by Jelley (1958).

The essential features of the radiation which were revealed by these experiments were that the spectra were continuous, as Mallet had shown, (i.e., they did not show the line and band structure associated with fluorescence), that the radiation was produced in all of 16 liquids used for the tests, that it occurred in the purest of liquids, that it was polarized (unlike fluorescence), and that the light was emitted in only a certain direction relative to the direction of the exciting radiation. The direction depended on the refractive index of the medium and the velocity of the particle.

In the USSR it was customary for many years to call the phenomenon the Vavilov-Cherenkov effect, due to Vavilov who made some contribution to the work. It was he (Vavilov, 1934) who at first suggested that the observed light might be associated with the slowing down of the electrons in the medium, but this idea presented difficulties and was found to be untenable. We will allude later (Section 2) to the phenomenon of radiation from the deceleration (or acceleration) of charges, which is known as Bremsstrahlung ("braking radiation").

It must be remembered that all the work both by Mallet and by Cherenkov was carried out by visual and photographic techniques, long before the development of the highly sensitive photomultiplier. Thus, in these early experiments, relatively strong radioactive sources had to be used, with the radiation as weak as it was, combined with long photographic exposures.

The war years caused a lapse in research in the field, though at the same time they heralded the development of the photomultiplier. The advent of this remarkable instrument, the most sensitive light detector ever developed, gave a great impetus to the subject which has since developed at an ever increasing rate. With a photomultiplier it became quite easy for the author to detect the light from even single charged particles in simple liquids (Jelley, 1951). Owing to the unique features of Cherenkov radiation, there evolved a whole new range of particle detectors that have

proved to be of great use to physicists, especially in the fields of high-energy particle and cosmic-ray physics; these are described in Section 4.

It was not long after Cherenkov's experimental work began, that a satisfactory theoretical interpretation of the phenomenon was produced by Frank and Tamm (1937), who were theorists in the Lebedev Institute, the same Institute in which Cherenkov carried out his experimental work. The theoretical treatment of Frank and Tamm (1937), based on classical electrodynamics, is accepted by everyone as correct. The next contribution was due to Ginsburg (1940), who produced a quantum theory of the effect. The difference between the results obtained by these two very divergent approaches are however quite trivial, and need not concern us in this review.

Of what interest is Cherenkov radiation to the biologist, and what is its possible role? It is only in recent years that its potential significance in the biochemical and biological spheres has been appreciated. The basic consideration is that Cherenkov radiation, appearing in the visible, and especially in the UV region of the spectrum, is produced *within* living tissue, partly from the natural radioactivity of certain isotopes of some elements that occur in biological material, and also from the ever present and considerable flux of cosmic rays, whether at the Earth's surface, deep in mines, or at high altitudes, such as the Altiplana in Bolivia. There is considerable evidence that UV radiation causes damage to DNA, especially at certain rather specific wavelengths, and it is primarily for this reason that the subject of Cherenkov radiation has become of interest to biologists. The role of Cherenkov UV radiation as a contributing factor to damage in biological material is discussed in Section 7.

Cherenkov radiation has also been discussed in a totally different area. Astronauts on some of the Apollo flights reported seeing flashes of light in their eyes. This phenomenon, not anticipated before the flights, has received many interpretations, one of which is that the flashes arise from Cherenkov radiation produced by multiple charged primary cosmic rays in space, above the upper layers of the atmosphere. This subject is discussed in Section 6.

Since Cherenkov radiation differs in character in many respects from other forms of electromagnetic radiation, and also because this review is intended primarily for biochemists and biologists, the author feels it would be appropriate to introduce the subject with a discussion of the basic features of electromagnetic radiation in general. Moreover, although the writer is a physicist, he will endeavour to describe and interpret the phenomenon of Cherenkov radiation in as descriptive a way as possible, with the absolute minimum of mathematics.

2. THE GENERAL FEATURES OF ELECTROMAGNETIC RADIATION

2.1. Maxwell's Theory

It was the English theoretical physicist James Clerk Maxwell, who, in 1873, showed that the main properties of light could be interpreted in terms of the already accepted theories of electricity and magnetism. From his theory developed the prediction that much longer-wave radiation could be produced and detected, and indeed, this prediction was fulfilled by the experiments conducted by Hertz in Germany in 1886. This of course led to the exploitation of Hertz's experiments, by Marconi and others, leading in due course to the development of radio, and subsequently TV.

Other, much shorter waves were also found. In particular, Roentgen discovered X-rays in 1895, and somewhat later, the even shorter wavelength γ -rays were discovered in the course of the fundamental studies of radioactivity. It is now known that all electromagnetic waves travel at the same velocity in free space, in accord with the predictions of Maxwell's theory. This velocity, which is always denoted by the symbol c , has the numerical value $3 \times 10^{10} \text{ cm}\cdot\text{s}^{-1}$.

What is an electromagnetic wave? We show, schematically in Fig. 1 a monochromatic wave travelling in free space from left to right. By monochromatic we mean that the wave has a single frequency (or single wavelength). The wave shown in this diagram is sinusoidal in character, and it will be seen that it consists of a varying or oscillating component of

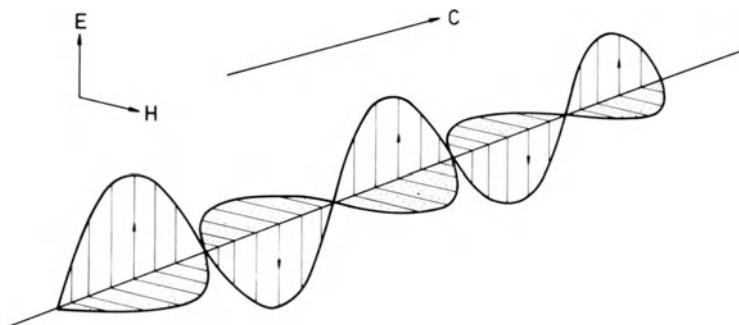


Fig. 1. The electric, E , and magnetic, H , vectors associated with a sinusoidal monochromatic wave travelling at a velocity, c , from the left to the right of the diagram. The hatched areas represent the horizontal plane of the H vector, while the vertical lines indicate the plane of the E vector.

electric stress called an electric vector, E , and a corresponding component of magnetic stress termed a magnetic vector, H . E and H are always in phase, and orthogonal to each other and to the direction of propagation of the wave.

Monochromatic waves, or waves of one single wavelength, are emitted by lasers and by radio transmitters, except that the latter carry a small spread in wavelength, known as sidebands. It is these sidebands which contain the audio and TV signals that we utilize every evening in our homes.

2.2. The Electromagnetic Spectrum

We have already mentioned that many superficially very different types of radiation are all basically the same in character. In Fig. 2 we have displayed the predominantly important part of the electromagnetic spectrum, noting that it covers a band of wavelengths from 10^{-11} to 10^4 m, i.e. a span of 15 decades. The interesting region, as far as the present context is concerned, has been expanded in Fig. 2, and this covers the range of wavelengths between 100 nm (hard UV) to 800 nm (far-red), i.e., a bandwidth of ~ 1 decade, out of the 15 mentioned above. Longer wavelength bands in the infrared and microwave regions are utilized in the medical world for therapy. X-rays and γ -rays are of course also used in the medical field, both for radiography and for therapy.

2.3. Frequency and Wavelength

The frequency, ν , and the wavelength, λ , of a wave are related to its velocity in free space, by the simple relation:

$$\nu\lambda = c \quad (1)$$

In the visible band, e.g., in the violet region, where $\lambda \sim 400$ nm, ν is extremely high, in fact $\sim 7.5 \times 10^{14}$ Hz, which could be compared with the 3×10^9 Hz for a TV UHF transmission.

2.4. Polarization

If, in a beam of radiation, all the waves have their E vectors in the same orientation, and so likewise their H vectors, the radiation is said to

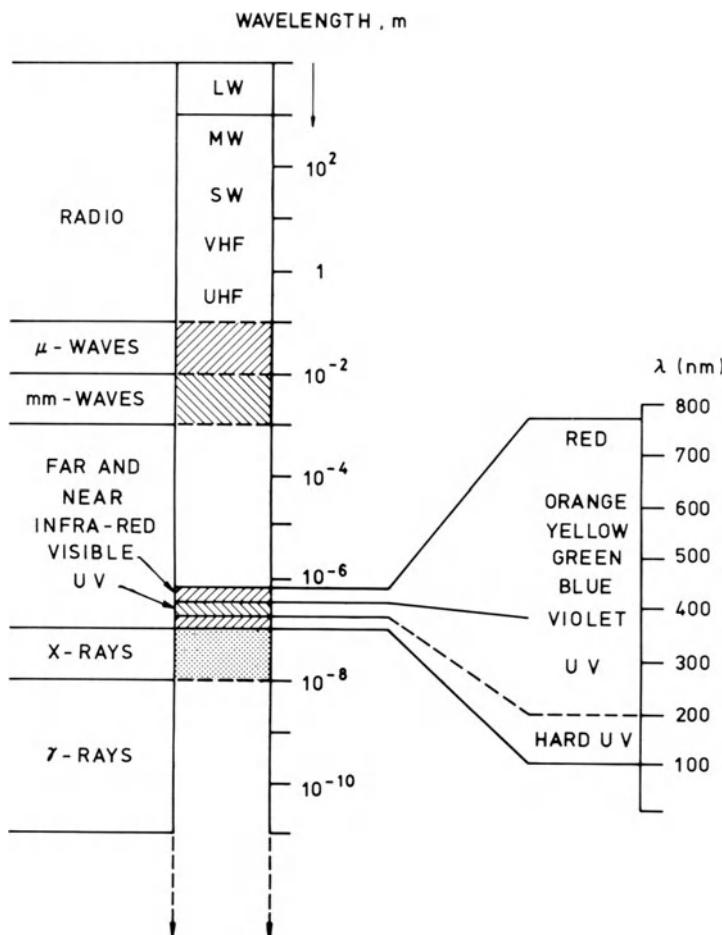


Fig. 2. The full electromagnetic spectrum, with the visual and ultraviolet regions shown on an expanded scale to the right of the diagram. The symbols in the radio regions are designated as follows: LW, long wave; MW, medium wave; SW, short wave; VHF, very high frequency; and UHF, ultra-high frequency.

be *plane polarized*. In general, as in sunlight or the light emitted by domestic lighting, the radiation is *unpolarized*, which means that the E (and H) vectors of the individual waves are totally random in their relative orientations. Cherenkov radiation, as we shall see in the next section, has a very definite character of polarization.

2.5. Coherence

Monochromatic radiation is said to be coherent if all the waves that go to make up a beam are in phase with one another. This is the case for light emitted by a laser, and it is also true in the case of the radio waves generated by a transmitter antenna. Cherenkov radiation is also coherent. On the other hand, light from the sun and domestic lighting is incoherent.

2.6. Refractive Index and Dispersion

Light travelling through a liquid or transparent solid moves at a velocity that is less than c , due to the presence of the medium. The speed is (c/n) where n is the refractive index of the medium. Thus, in water for example, for which $n = 1.33$, the velocity of light passing through it will be $2.3 \times 10^{10} \text{ cm}\cdot\text{s}^{-1}$. The refractive indices of all media vary with the wavelength of the radiation, and rise with decreasing wavelength; this effect is called dispersion. n increases rapidly in the UV region until an absorption band in the medium is reached, whereupon it collapses to unity and even goes below unity, leading to anomalous dispersion. There will be, in general, a few bands further into the UV region where n again becomes >1 .

2.7. The Dual Nature of Light

We have so far described light and UV radiation in terms of waves. It was however discovered by Planck that light can also be described in terms of small packets or quanta, and that for any individual frequency ν (see Eq. 1) the amount of energy in an individual quantum is $h\nu$ where h is Planck's constant ($6.6 \times 10^{-27} \text{ erg}\cdot\text{s}$). Quanta of visible light, UV radiation, X-rays or γ -rays are frequently referred to as photons, the two terms being synonymous. These two alternative views of the nature of radiation present difficulties even among many physicists, but it is not necessary to discuss them further in these notes.

2.8. The Basic Radiation Process

We now ask how electromagnetic radiation is generated. The underlying process in all cases is that of the *acceleration* or *deceleration* of electric charges, and in no case will a uniformly moving charge produce radiation. Cherenkov radiation, as we shall see in the next section, ap-

pears to arise from a particle traveling at a constant velocity and therefore seems to contradict the statement above. This will be explained in detail in due course.

In Fig. 3 we show schematically three apparently totally different radiation processes, but all three resulting from the acceleration of charges. Fig. 3(a) is a representation of thermal or “black body” radiation, which is the source of light and heat from the sun, and from the incandescent filament lamps in our homes and cars. In this case, the free electrons in a hot material, represented in the diagram by black dots, suffer changes in velocity as they move close to other electrons or positive ions of the atoms of the material.

In Fig. 3(b), a single *very fast* electron, e , is plunging through the electron cloud, C , of a single atom, and is deflected (i.e., accelerated) by the positively charged atomic nucleus, N . The resulting radiation in this case is termed Bremsstrahlung, and is important in the generation of high-energy photons, mainly in the X and γ regions of the spectrum.

In Fig. 3(c), a fast electron is entering the region of a strong magnetic field, represented by dots indicating magnetic field lines orthogonal to the plane of the paper. Again the particle is being deflected. The radiation from this process is called either synchrotron radiation or magneto-bremsstrahlung.

2.9. Continuous and Discrete Spectra

Radiative processes are broadly divided into two classes: those that emit radiation over a wide band of the spectrum, and those that emit radiation in well-defined and narrow regions of the spectrum. In the former class fall the three radiation mechanisms outlined in Fig. 3. Cherenkov radiation, as we shall see, also falls into this category. The other class of processes is that which arises from transitions between excited states of nuclei, atoms, or molecules. These occur in different parts of the spectrum depending on the energy levels involved. γ -Rays arise from transitions between levels within atomic nuclei, X-rays and UV radiation from the tightly bound electrons in the central regions of atoms, light from the outer atomic shells, and infrared and millimeter waves from molecules.

2.10. Interactions between Radiation and Matter

The processes by which radiation interacts with matter are too numerous and intricate to discuss systematically here, but since we will be

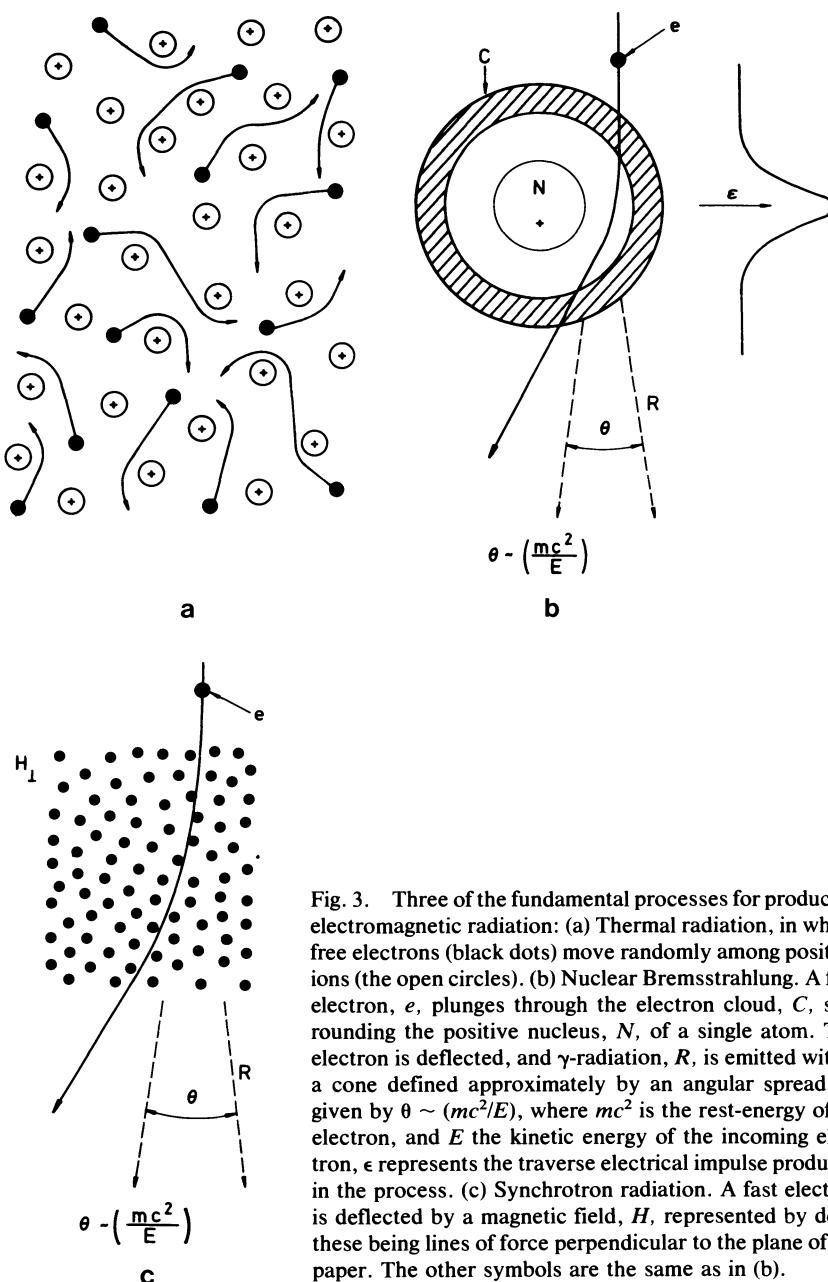


Fig. 3. Three of the fundamental processes for producing electromagnetic radiation: (a) Thermal radiation, in which free electrons (black dots) move randomly among positive ions (the open circles). (b) Nuclear Bremsstrahlung. A fast electron, e , plunges through the electron cloud, C , surrounding the positive nucleus, N , of a single atom. The electron is deflected, and γ -radiation, R , is emitted within a cone defined approximately by an angular spread, θ , given by $\theta \sim (mc^2/E)$, where mc^2 is the rest-energy of an electron, and E the kinetic energy of the incoming electron, ϵ represents the traverse electrical impulse produced in the process. (c) Synchrotron radiation. A fast electron is deflected by a magnetic field, H , represented by dots, these being lines of force perpendicular to the plane of the paper. The other symbols are the same as in (b).

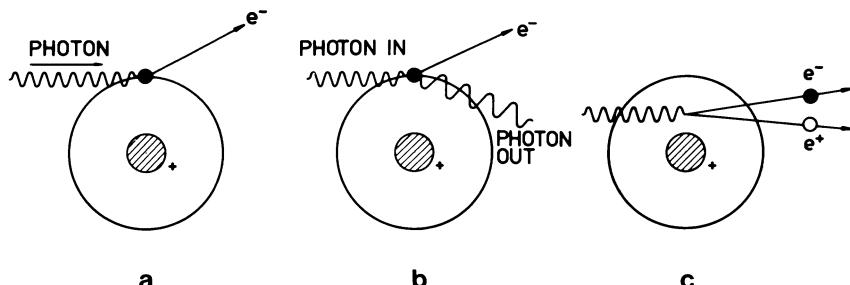


Fig. 4. The three main processes for absorption of X and γ radiation. (a) Photoelectric absorption: a photon ejects an electron, e^- , from its orbit around the nucleus of an atom (shown shaded), the photon being totally absorbed. (b) Compton scattering: a similar process except that the emitted electron only carries away some of the energy, the photon being reemitted, but at reduced energy. (c) Pair production: an incoming photon interacts directly with the electric field close to the nucleus of an atom. The photon is totally absorbed and a pair of electrons, one positive and one negative, are produced in the process.

involved with the UV, X-ray, and γ -ray spectral regions, it is important to at least touch on the subject, especially in view of the fact that the electrons that cause Cherenkov radiation have been derived in their turn from γ -ray interactions.

For γ -rays there are basically three processes. In the first, the photoelectric effect, Fig. 4(a), the photon is totally absorbed by an atom and an electron ejected. This process is important at low photon energies and for atoms of high atomic number. In the next process, Fig. 4(b), the Compton effect, the photon collides with an electron in the atom, is scattered with reduced energy, and an electron is ejected from the atom as shown. This process is the dominant one for light atoms and photon energies in the region of a few MeV. At very high energies, and especially for atoms of high atomic number, the third process, Fig. 4(c) becomes important; this is known as pair production. The photon interacts directly with the strong electric field close to the nucleus of an atom and generates an electron-positron pair, both particles being ejected from the atom.

With this brief review of some of the properties of electromagnetic radiation we proceed to discuss Cherenkov radiation.

3. THE BASIC PHYSICS OF CHERENKOV RADIATION

3.1. A Descriptive Account of the Effect

In Fig. 5 we illustrate a small section of an amorphous solid or liquid transparent medium in which the atoms or molecules are represented by

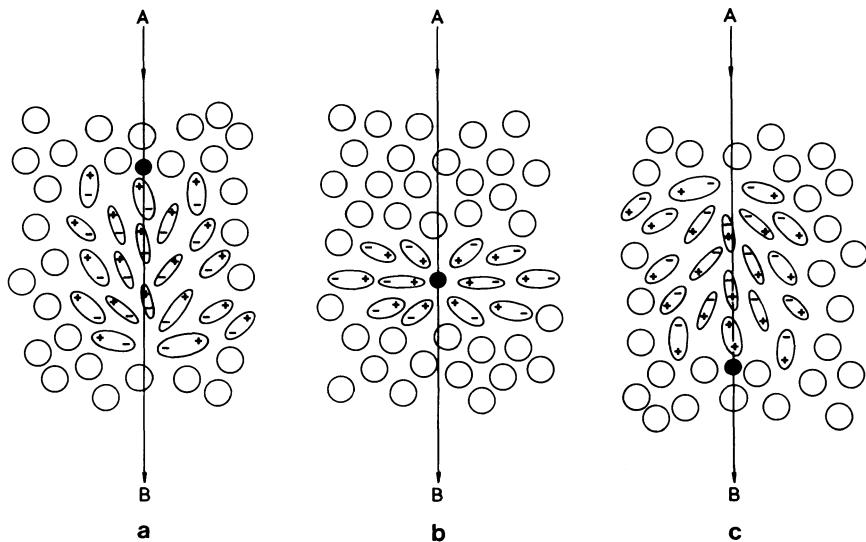


Fig. 5. The local polarization produced in an amorphous medium during the passage of a fast charged particle; (a) the distortion of the atoms (elliptical shapes) in a particular region of the medium just ahead of the instantaneous position of the fast particle (black dot) as it travels along its path from A to B; (b) the same region, at the moment the particle is near the center; and (c) the situation when the particle is leaving the region.

circles. To give some idea of scale, the diameter of an atom is of the order of 1 nm. In the normal quiescent mode, all the atoms will be uncharged and in their normal state (i.e., not excited); such atoms are represented in this figure by clear open circles. Suppose now that a fast electron, such as a β -particle from a radioactive nucleus, an electron from a Compton-scattered γ -ray, or a cosmic-ray particle, plunges through the medium. When this fast electron reaches the point represented by the black blob on its track AB [Fig. 5(a)], the atoms ahead of it will be slightly polarized, i.e., the atomic electron orbits will be slightly disturbed so that the atoms, though still electrically neutral, will develop a slight positive charge at one end and a negative one at the other, as shown. When the electron has reached the center of this small section of material, Fig. 5(b), the polarized atoms will be symmetrically disposed as shown. Later, when the electron has passed further down the track, Fig. 5(c), the polarization will be as for Fig. 5(a), but with the charges reversed. We should note at this stage, because we will be discussing it later, that the electron is moving, but the *atoms remain stationary*.

Before we go further, looking at these diagrams closely, we see that

the fast-moving electron actually goes *through* some of the atoms. This is significant because those particular atoms will be either excited, or ionized, and this is one of the main causes of damage in the biological sense. While this ionization only occurs over the diameter of a single atom or molecule, the range of influence of the fast-moving electron, as far as polarizing the medium is concerned, is over a much greater range. In fact, the range is comparable to the wavelength of the radiation emitted, i.e., say 400 nm for blue light, or about 100 times the diameter of a single atom.

When we mention that the clear circles in Fig. 5 represent atoms or molecules, it should be pointed out that these may be simple atoms or molecules like H_2O , or they may equally be complex organic molecules; it makes no difference to the basic physical process of local polarization of the medium by the passage of a fast particle. Also note that we said the medium was amorphous. This phrase was inserted because, in a crystalline material like quartz or diamond, the effects are considerably modified because the atoms are precisely aligned.

Figure 5 is of course the view of the local polarization of the medium in a single plane, and it will be appreciated that this polarization is completely symmetrical about the axis of the fast-moving electron.

It is appropriate to mention at this stage that strictly speaking Fig. 5(a) and 5(c) are not precisely similar because of what the physicist calls "retarded potentials." What we mean by this is that in spite of the very high speed of travel of light ($3 \times 10^{10} \text{ cm}\cdot\text{s}^{-1}$), it takes a finite time for the atoms to become aware of the presence of the passage of a fast charged particle. In other words, the instantaneous situation with regard to the local polarization of the medium, lags very slightly behind the instantaneous position of the particle.

Let us now look at Fig. 6(a). Consider a point S in the medium at some place slightly off to the side of the track of the electron, represented by $ABCD$. The local polarization of the medium is depicted by the vector P . Owing to the retarded potentials mentioned above, when the electron is at B , the vector P is directed to a point A slightly behind B , in time. As the electron flashes by, the polarization vector turns over from P_1 to P_2 , to end up directed to the point C , again slightly behind the real position of the electron which will now be at point D . The polarization vector P may be resolved into two components, which, as a function of time, are represented in Fig. 6(b). The radial component P_r is a single pulse as shown, while the axial component P_z consists of two δ -functions of opposite sign. Owing to the axial symmetry, an observer well away to the side of the track will see no effects from the radial component, but will readily sense the existence of the axial component.

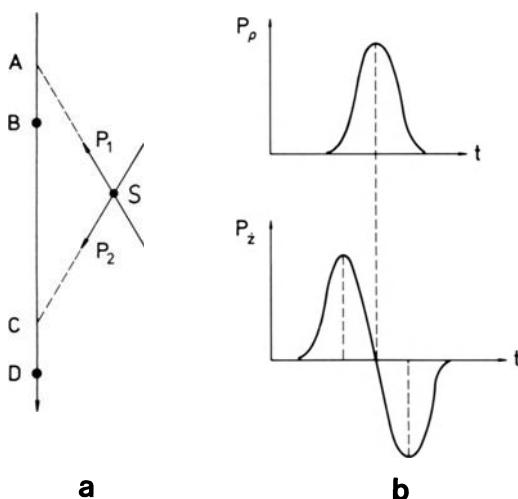


Fig. 6. (a) A fast electron is moving along the track from A to D passing a particular point S somewhat to the side of the track. When the electron is at the point B the polarization vector, P , at S is directed somewhat behind the true position of the electron, to the point A , and similarly when the electron is really at D it appears only to have reached C . (b) The variation of the radial P_p , and the axial P_z , components of the polarization vector, P , at S as a function of time, t .

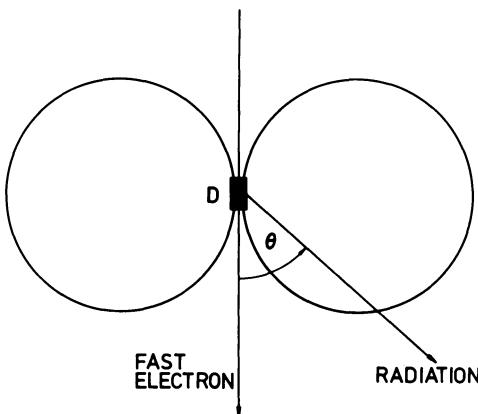
This transient axial component of polarization will generate a minute pulse of radiation; it will appear as a minute dipole, D (Fig. 7), similar to a single element of a TV antenna. The distribution of the intensity of this radiation will be of the form $\sin^2\theta$, where θ is the angle between the track of the particle and the direction along which the radiation is emitted.

We have, so far, discussed the local polarization of the medium along a single element of track, perhaps only ~ 100 nm in length. This picture, so far, represents the situation when a charged particle of any velocity traverses just a small section of amorphous material.

We are now in a position to ask what happens in the real situation when we consider the passage of the electron through an appreciable pathlength in the medium; by appreciable we mean a large pathlength compared with the wavelength of light. Reverting temporarily to the picture we have had of the local transient polarization effects in a small element of material, the rapidly changing vertical component of this polarization will in itself generate a minute amount of radiation on the basis of the simple points made in Section 2.8, that electromagnetic radiation is always produced from the acceleration (or deceleration) of charge.

In Fig. 8(a) we depict the passage of a fast electron through the medium from point A to another point B . AB might be 10 μm , 1 mm, or

Fig. 7. An elementary dipole, D , is formed in the medium coincident with the instantaneous position of the fast moving charged particle, the dipole itself remaining stationary in the medium. The circles represent the distribution of intensity of the emitted radiation as a function of the emission angle, θ . The intensity is proportional to the length of the vector from D to its intersection with the circle.



perhaps 1 cm or more. It is here that we meet the concept of coherence. The velocity of the particle, v , may be expressed as a fraction $\beta = v/c$ of the velocity of light, c . If we now consider three arbitrarily chosen points X_1 , X_2 , and X_3 on the particle track, AB , each will radiate a minute burst of radiation from the elements of track, as shown earlier, in Fig. 7.

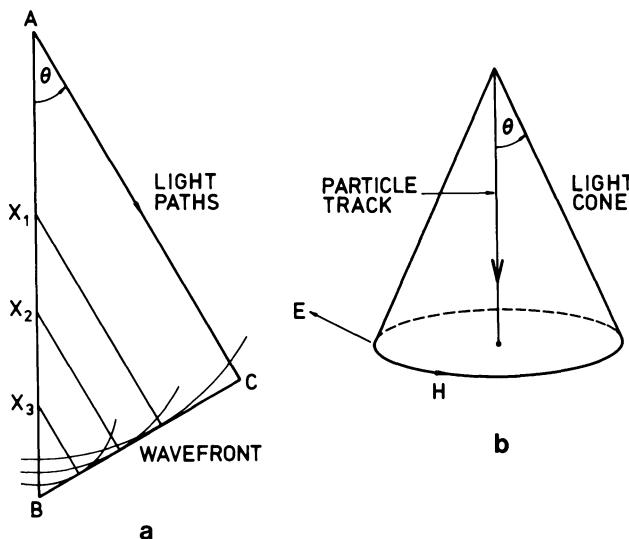


Fig. 8. Cherenkov radiation: (a) the coherence condition and (b) the formation of the Cherenkov cone. In (a) a fast electron is travelling from A to B and the emitted light travels out at every point along AB , at an angle θ , to form a wavefront BC . Three waves are shown, arising from three arbitrary points X_1 , X_2 and X_3 along the path. This sketch applies only to one plane, while in (b) we see the total Cherenkov cone, in which the electric vectors, E , are everywhere perpendicular to the surface of the cone, and the magnetic vectors are everywhere tangential to the surface.

Owing to the refractive index of the medium, designated by the symbol n , any electromagnetic radiation produced travels much more slowly than the velocity of light in a vacuum; to be precise it travels at a speed of (c/n) . Returning to Fig. 8(a), we find that the various waves of radiation from each of the three points X_1 , X_2 , and X_3 , will present a *coherent wavefront* along BC at one specific angle with respect to the line of the track.

In a finite time δt , the particle will travel the distance AB , when $\delta t = (AB/\beta c)$, while any light or other electromagnetic radiation will travel from A to C in this same time, when $\delta t = AC/(c/n)$. These two conditions can only be simultaneously satisfied for one specific value of the angle θ , i.e., the angle between the axis of the fast moving electron and the direction of the emitted light. For this direction the light is said to be coherent, i.e., all the waves from the different elements of track are in phase.

From these two simple equations we derive the relationship

$$\cos \theta = (AC/AB) = (1/\beta n) \quad (2)$$

which is known as the Cherenkov relation. Two conditions immediately follow from Eq. 2. First, for a given n , there is a threshold velocity:

$$\beta_{\min} = 1/n \quad (3)$$

below which no radiation takes place, and $\theta = 0$, and secondly, for an ultra-relativistic particle, i.e. when $\beta \rightarrow 1$, there is a maximum angle of emission, given by:

$$\theta_{\max} = \cos^{-1}(1/n) \quad (4)$$

There is also a third condition, namely that Cherenkov radiation can only occur at those wavelengths for which n is greater than one. So while the radiation can appear in the microwave, infrared, visible, and UV parts of the spectrum, it cannot be observed in the X-ray and γ -ray regions of the spectrum, because in these bands the refractive index is less than unity, i.e., $n < 1$.

Figure 8(a) has been drawn in one plane only. There is of course complete symmetry about the axis of the particle. The light originating from each element of track is propagated along the surface of a cone whose apex is at this element, whose axis coincides with the track, and whose semivertical angle is the angle θ . The distribution in θ of the light intensity approximates to a δ -function, and the polarization is such that the electric

vector, E , is everywhere perpendicular to the surface of the cone, and the magnetic vector, H , is tangential to this surface [Fig. 8(b)].

From what has already been said, it will be seen that the phenomenon is in some ways analogous to the V-shaped shock wave observed in acoustics when a projectile travels through the air at a velocity in excess of the speed of sound. The quantity βn takes the place of the Mach number in aerodynamics. A more familiar case is that of the formation of a bow wave from a ship moving through water when its speed is greater than that of surface waves in the water.

Let us look at Fig. 9 which is designed to illustrate the threshold condition, the coherence phenomenon, and the shock-wave nature of the radiation. We suppose someone to be at the bow of the ship with a heavy weight on the end of a line, the weight being caused by oscillate up and

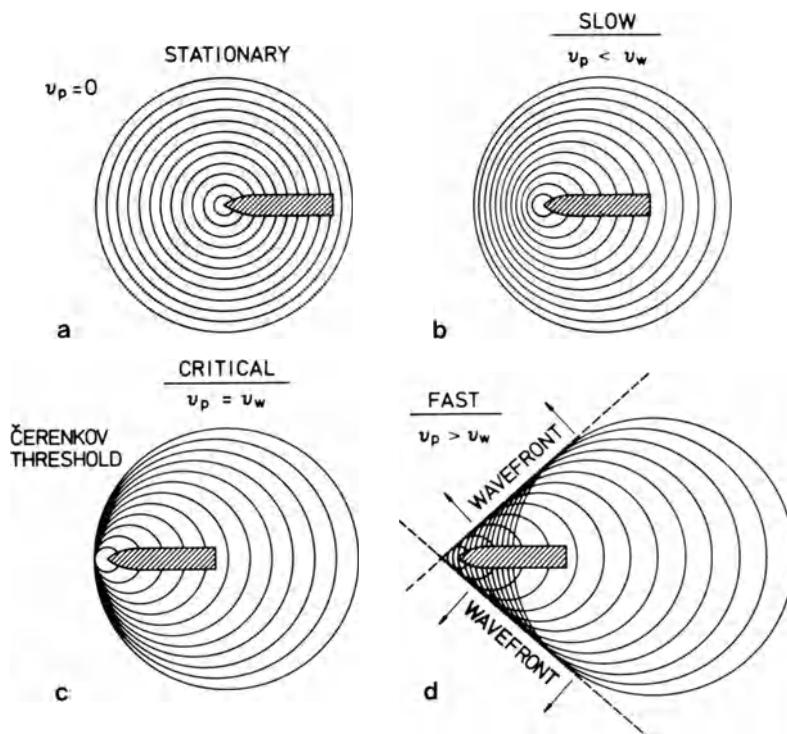


Fig. 9. An illustration to show the analogy of Cherenkov radiation with the passage of a ship through water. In (a) the ship is stationary and its speed, v_p , is therefore zero. In (b) it is moving slowly, but its speed is below that of the speed of surface waves on the water, v_w . At (c) a critical situation when $v_p = v_w$, and in (d) when $v_p > v_w$, a wavefront is produced; the familiar bow wave.

down at the surface of the water. When the boat is stationary [Fig. 9(a)], or even moving slowly [Fig. 9(b)], the waves do not form a coherent front. However, there comes a condition [Fig. 9(c)] when the speed of the boat is precisely the same as the speed of waves on the surface of the sea. This corresponds to the threshold condition. When the boat is going faster than the waves, a coherent wavefront builds up [Fig. 9(d)]. As one sees from Eq. 2, the higher the velocity of the electron, the larger the angle θ . This then is just the reverse of the situation which we discussed in Section 2.8 for the processes of Bremsstrahlung and synchrotron radiations. The reason for this is very fundamental. In these two processes it is the particle itself that is radiating, while in the *Cherenkov effect*, it is the medium that is radiating, not the particle, and the medium stays at rest. This point can never be overstressed.

In Fig. 10, we have plotted the variation of the cone angle, θ , as functions of the kinetic energy E , and β values for the specific case of fast electrons in pure water for which $n = 1.33$. The choice of pure water is a reasonable approximation to dilute biological material as far as refractive index is concerned, although of course the question of absorption of visible light and UV radiation will play an important role when one comes to discuss the significance of Cherenkov radiation in biochemical and biological situations.

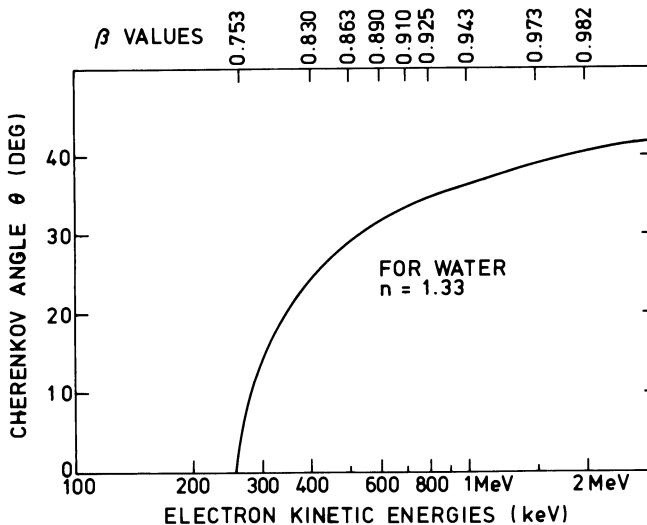


Fig. 10. The Cherenkov angle θ as a function of β and the kinetic energies, for fast electrons in water.

3.2. The Radiation Yield

We are now in a position to discuss the intensity or the yield of visible light or UV radiation in the Cherenkov process. We will not derive the mathematical formulation, as this can be found in the original paper by Frank and Tamm (1937) or in the work by Jelley (1958).

The fundamental expression for the energy production, per unit path-length, derived originally by Frank and Tamm (1937), is

$$dW/dl = (e^2/c^2) \int_{\beta n > 1} [1 - (\beta^2 n^2)^{-1}] \omega d\omega \quad (5)$$

where e and c are the charge on the particle and the velocity of light respectively, and ω and $d\omega$ are the frequencies and bandwidths, expressed in radians s^{-1} over the total band covered in the integral by the condition that $\beta n > 1$. ω and $d\omega$ are $2\pi\nu$ and $2\pi d\nu$, respectively, where ν and $d\nu$ are frequencies in Hz. As we saw in Section 2.3, the frequency of a wave is related to the wavelength λ , and the free-space velocity of the waves, c by the relation $\nu = (c/\lambda)$.

It is frequently more convenient to rewrite Eq. 5 in terms of the number of photons per centimeter of path, between wavelength limits λ_2 and λ_1 (provided that over this band $\beta n > 1$). When this transformation is carried out, and taking into account Eq. 1, the radiation yield is then expressed as follows:

$$(dN/dl) = (2\pi/137) [\lambda_2^{-1} - \lambda_1^{-1}] \sin^2\theta \quad (6)$$

We see at once, from both Eq. 5 and Eq. 6 that the radiation per unit bandwidth or per unit wavelength interval increases steeply towards the UV end of the spectrum. For a relativistic electron ($\beta \rightarrow 1$) in water ($n = 1.33$), that is, for kinetic energies above 1 MeV, the yield amounts to about $200 \text{ photons}\cdot\text{cm}^{-1}$ between wavelengths of 350 to 550 nm (the visible band), or about $59 \text{ photons}\cdot\text{cm}^{-1}$ over the biologically interesting UV band of 250–270 nm.

There are variants of the spectral distributions expressed by Eq. 5 and Eq. 6 depending on whether one is describing the energy production or the photon-number yield, and whether one wishes to express these in terms of unit wavelength interval or unit angular frequency interval. There are essentially four alternative forms:

$$(d^2W/dl\cdot d\omega) \propto \omega \quad \begin{matrix} \text{energy per unit path, per unit frequency} \\ \text{interval} \end{matrix} \quad (7)$$

$$(d^2W/dl \cdot d\lambda) \propto \lambda^{-3} \quad \text{energy per unit path, per unit wavelength interval} \quad (8)$$

$$(d^2N/dl \cdot d\omega) = \text{const} \quad \text{number of photons per unit path per unit frequency interval} \quad (9)$$

$$(d^2N/dl \cdot d\lambda) \propto \lambda^{-2} \quad \text{number of photons per unit path per unit wavelength interval} \quad (10)$$

3.3. The Comparison between the Ionization Energy Loss and the Yield of Cherenkov Radiation

It is appropriate at this stage to emphasize the extreme weakness of Cherenkov radiation, at least as far as energy production is concerned. In Fig. 11 we show the specific ionization of an electron passing through a medium composed mainly of elements of low atomic number. We at once see that for a relativistic particle, i.e., an electron of kinetic energy of ~ 1 MeV, the specific ionization is around $2 \text{ MeV} \cdot \text{g}^{-1} \cdot \text{cm}^{-2}$, or, for materials of density around unity, $2 \text{ MeV} \cdot \text{cm}^{-1}$. Against this, we find that the yield of Cherenkov radiation, even at higher energies and over the whole visible band, is only $200 \text{ photons} \cdot \text{cm}^{-1}$. Taking an average photon

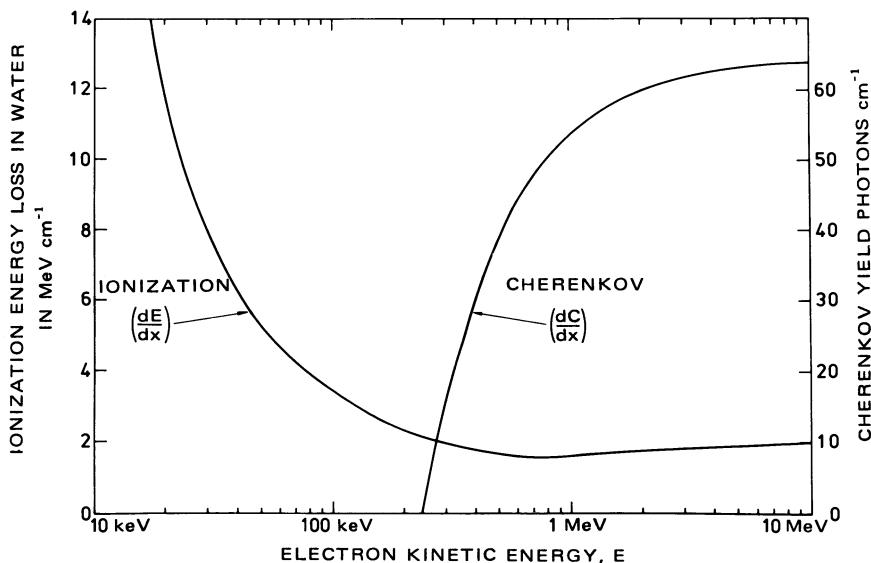


Fig. 11. Curves showing the ionization energy loss (dE/dx) and the Cherenkov radiation production (dC/dx) for fast electrons in water.

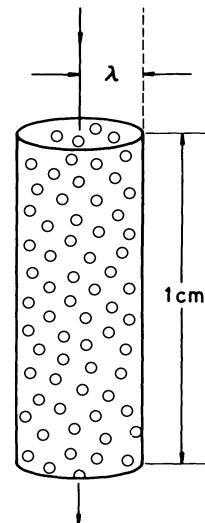


Fig. 12. An illustration to show the number of atoms involved in the Cherenkov process over a path length of 1 cm, in water. The individual atoms are represented by small circles within a cylinder of length 1 cm and radius λ , the wavelength of the Cherenkov light emitted by a fast charged particle travelling down the axis of the cylinder.

in the blue region to possess an energy of ~ 3 eV, this represents a yield $\sim 600 \text{ eV} \cdot \text{cm}^{-1}$. With these two figures we then find that the rate of energy loss of this relativistic electron by ionization is $[(2 \times 10^6)/600]$, i.e., about 3000, times that of the yield of Cherenkov light, expressed as energy. This will be discussed further in Section 7.

3.4. Energy Exchange with Individual Atoms

We will now interpret the weakness of the Cherenkov radiation in terms of the energy exchange between the fast electron and an *individual* atom of the medium, to demonstrate first, that Cherenkov radiation is a macroscopic phenomenon involving the medium as a whole, and second, that the energy exchanges with individual atoms are quite negligible compared with the energy levels of excitation (or ionization) of these same atoms.

We show, in Fig. 12, a section of the medium through which the particle has passed. The radius-of-action, as far as polarization of the medium is concerned, will be $\sim \lambda$, the wavelength of the radiation emitted, so the volume of this small sample of the medium over a path length of 1 cm, will be $V = \pi\lambda^2$. The number of atoms affected will be $N = \pi\lambda^2\rho$ where ρ is the density of the medium, expressed in numbers of atoms per cm^3 . With $\rho \sim 10^{23} \text{ atoms} \cdot \text{cm}^{-3}$ and $\lambda \sim 300 \text{ nm}$, $N = 3 \times 10^{14}$ atoms. We saw that the total yield of Cherenkov radiation through the visible

range of the spectrum was $\sim 200 \text{ photons} \cdot \text{cm}^{-1}$ or $\sim 600 \text{ eV} \cdot \text{cm}^{-1}$ of path, so therefore the energy exchange with an *individual* atom is a mere $(600/3 \times 10^{14})$, or $2 \times 10^{-12} \text{ eV/atom}$, which is negligible compared with the energy levels in any of the atoms of the medium. From this we realize at once that this radiation process is a macroscopic phenomenon, in no way related to the interaction of a charged particle with an individual atom.

3.5. Kinetic Energy and the Velocity Ratio

So far, the Cherenkov relation (Eq. 2), the threshold condition (Eq. 3), and the radiation yield (Eqs. 5 and 6) have been expressed as a function of β , the ratio of the velocity of the particle to that of visible light (or UV radiation) *in vacuo*. It is usually of more interest to know what kinetic energies, E , of the electrons are involved, and it is therefore essential that we can derive E from β . Because the electrons are in general relativistic, i.e., their velocity is comparable to that of light *in vacuo*, we must use the relativistic formulae. The rest mass energy of a particle is $m_o c^2$, so the kinetic energy, E , is related by:

$$E = m_o c^2 [(1 - \beta^2)^{-1/2} - 1] \quad (11)$$

from which we derive the expression for β :

$$\beta = [(m_o c^2 + E)^2 - (m_o c^2)^2]^{1/2} (m_o c^2 + E)^{-1} \quad (12)$$

The nuclear radiations within living matter arise from naturally occurring radioactivity on the one hand, and from the ever present cosmic rays on the other. These contributions are comparable at sea level, though at high altitudes the cosmic-ray component increases by a factor of about 30. While the naturally occurring radioactivity in nature consists mainly of electrons and positrons, the cosmic radiation at sea level has a high proportion of particles of high mass, such as μ -mesons and protons.

It follows from Eq. 11 that particles of different mass will have different kinetic energies, E , for the same threshold value of β . In Table 1 we list the masses of the various particles, in terms of the rest mass, m_e , of the electron, and the corresponding kinetic energies, E_o , at the Cherenkov threshold in water.

The naturally occurring radioactivity in biological material consists of β -radiation (electrons or positrons), γ -radiation which, as we have seen in Section 2.2, is an electromagnetic radiation, and α -radiation. α -particles are fast moving helium nuclei, but with their high mass (four times that of the proton), the Cherenkov threshold is $\sim 2 \text{ GeV}$, far above the energy levels of any of the α -active elements.

TABLE 1. Threshold Kinetic Energies, E_o , for Producing Cherenkov Radiation in Water, for Particles of Different Mass, Expressed in Terms of the Rest Mass, m_e , of an Electron

Particle	Rest mass (m_e)	Threshold (E_o)
Electron/positron	1	260 keV
μ -Meson	207	52 MeV
π -Meson	273	70 MeV
Proton	1836	500 MeV

While γ -radiation cannot itself produce Cherenkov radiation directly, its secondary products will do so. As we saw in Section 2.10, γ -rays produce Compton electrons, and electron-positron pairs by the process of pair production.

The other component of potentially damaging radiation, the cosmic rays, consists of particles of extremely high energy, with a remarkably constant flux all over the Earth's surface and over long periods of time. Their flux is approximately one particle per square centimeter per minute, over the 2π solid angle of the sky, and their rate shows a negligible diurnal variation. Moreover, due to the high particle energies, this cosmic-ray background is not easily screened, so that it is basically the same indoors or out, and only down in deep mines is its intensity appreciably reduced. At sea level, approximately 70% of all cosmic-ray particles are μ -mesons, and the remaining 30% consist of electrons, positrons, protons, γ -rays, and neutrons.

4. CHERENKOV DETECTORS AND COUNTERS

4.1. General Comments

Weak though it is, Cherenkov radiation in the visible and UV regions of the electromagnetic spectrum can readily be detected, mainly as a result of the development of the photomultiplier [e.g., see Rodda (1953) and Sommer (1951)].

In view of this, Cherenkov radiation has been used over the years, especially by high-energy particle physicists and cosmic-ray researchers, as a very valuable tool in these two fields of research. To be sure, by far the most important applications of the effect, are spread over these two areas. Since, however, this aspect is only of secondary importance to the biochemist and biologist, we will keep this section short. For anyone wishing to pursue the subject, refer to Chapter 6 of the book by Jelley

(1958), and the review articles by Hutchinson (1960), and by Fabjan and Fischer (1980).

While there are a wide variety of applications of Cherenkov radiation in high-energy particle and cosmic-ray physics, and these particle detectors may take on many forms, they fall broadly into three classes.

4.2. Threshold Detectors

Since the Cherenkov threshold depends on the velocity rather than the energy of the particle, the existence or nonexistence of a flash of light from a Cherenkov detector may be used to determine the mass or nature of a fast nuclear particle, when the detector is used in conjunction with other types of detector. For example, it played a crucial role in the discovery of the anti-proton by Chamberlain *et al.* (1955).

4.3. Direct Velocity Measurements

Since the angle of emission of the light is a function of the velocity of the particle, it is possible to measure the energy of a particle to some considerable accuracy, provided one knows its mass. This application is, however, limited to a rather narrow energy band above the threshold. An example of an instrument developed to do just this is illustrated in Fig. 13 [see Sutton *et al.* (1955)]. In this device, used in proton–proton scattering experiments, the high-energy protons travelling down the particle beam along the track *AB* produced Cherenkov light in a small but thick slab of glass, *G*. A proportion of the Cherenkov cone was focused by the lens, *L*, to be collected and detected by a photomultiplier, *P*, situated behind a defining slit, *S*. The signal strength varied with the angle θ in such a way that the full width at half-height of the distribution was ~ 30 MeV, at the proton energy of 437 MeV, which they were using, i.e., the energy resolution was $\sim 8\%$.

4.4. “Deep” Counters, or Total Absorption Spectrometers

We saw in Sections 2.8 and 2.10, how, at very high energies, γ -rays can produce electron–positron pairs and also how high-energy electrons and positrons can produce γ -rays by the process of nuclear Bremsstrahlung. Thus, a very high energy γ -ray can generate a cascade of electrons, positrons, and further γ -rays. If the depth of the material is sufficient for the entire cascade to develop and ultimately dissipate, then, by measuring the total Cherenkov light from the charged particles, we have a measure

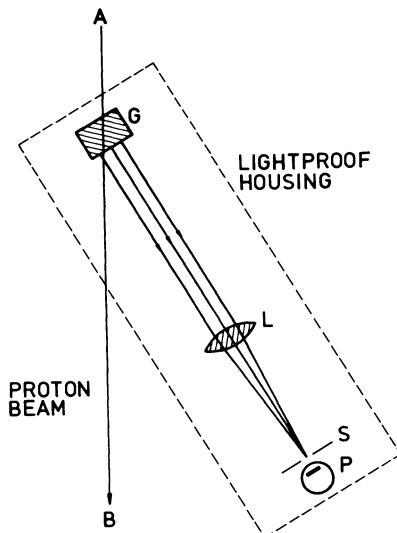


Fig. 13. A simple Cherenkov detector for measuring directly the velocity, and hence the energy, of a beam of fast protons (see text). The proton beam, travelling along a path from A to B, traverses a thick slab of glass, G. A small fraction of the Cherenkov light cone emitted in G is focused by a lens, L, to impinge on the photocathode of a photomultiplier, P, situated behind a defining slit, S.

of the energy of the primary γ -ray. This is the basis of the total absorption spectrometer, sometimes termed a calorimeter by those in the field of cosmic-ray physics.

Since both the electron-positron pair production process, and the Bremsstrahlung mechanism increase with Z^2 , where Z is the atomic number of the material of the medium in which the cascade develops, it is clear that we need transparent materials heavily loaded with elements of high atomic number. For this reason lead-loaded glasses have been developed, and an example of such a "total absorption spectrometer" has been described by Brabant *et al.* (1957). The problem with lead-loaded glass is that it tends to be yellowish, and therefore very absorbant in the UV region, the very region where the Cherenkov radiation is most intense. There are many other variants of Cherenkov detectors, but they are naturally of little interest to biochemists and biologists.

5. CHERENKOV RADIATION IN GASES, IN THE ATMOSPHERE, AND IN THE DEEP OCEANS

5.1. Cherenkov Radiation in Gases

The refractive indices of gases at normal temperatures and pressures are so close to unity that the Cherenkov threshold kinetic energies, E_o , are extremely high and the radiation yields correspondingly low. In air

for example, the refractive index $n = 1.000293$, and E_o are 21 MeV, 4.3 GeV, 5.6 GeV and 38 GeV, for electrons, μ -mesons, π -mesons, and protons, respectively. At ultra-high energies, i.e., $\beta \rightarrow 1$, the maximum cone angle $\theta = 1.3^\circ$, and the radiation yield is as low as 0.3 photon cm $^{-1}$. These figures are in sharp contrast to those with which we are familiar in the case of solids and liquids, recalling, e.g., that in water, $n = 1.33$, $\theta(\text{max}) = 41^\circ$, $(dN/dl) = 250$ photons·cm $^{-1}$, and E_o (electrons) = 260 keV.

In spite of the extreme weakness of Cherenkov light in gases, it is nevertheless possible to detect it, even from single particles. The first experiments in which this detection was achieved were conducted contemporaneously by Ascoli Balzanelli and Ascoli (1953), and by Barclay and Jelley (1955). It would seem that the effect is of virtually no significance in the biological field.

5.2. Cherenkov Radiation in the Atmosphere

Realizing that Cherenkov radiation should be observable in gases as well as in liquids and solids, Blackett (1948) showed that a very small fraction of the light of the night sky, $\sim 10^{-4}$, must be contributed by Cherenkov radiation produced by the incoming charged-particle component of the cosmic radiation. As the fraction was so small, and also because the cosmic radiation cannot be turned off, it is quite unfeasible to detect this effect.

However, it was later realized (Galbraith and Jelley, 1953) that when very high energy primary cosmic rays strike the top of the atmosphere, a very marked *temporary* increase in the Cherenkov radiation must exist, and that this would probably be detectable. Early and very simple experiments proved this to be the case. When these very highest energy primaries strike the top of the atmosphere, they generate cascade showers of electrons, positrons, and γ -rays, much as in the total absorption spectrometers already discussed in Section 4.4. Because these showers are developed in the atmosphere, and their particles spread out to impinge on a large area of the ground, they are classified as “extensive air showers” (EAS).

The Cherenkov light flashes that are generated by the EAS are of extremely short duration, ~ 10 ns, and cover a vast area of ground, a pool of light of diameter ~ 200 m, or an area of $\sim 3 \times 10^4$ m 2 . That such a large area of ground is flooded with light in this way arises from the fact that much of the light is generated at great altitudes, and the angular spread of the light is determined not so much by the 1.3° cone angle of the Cherenkov light, but rather by the divergence of the shower particles as they pass through the atmosphere.

enkov radiation, but rather by the angular spread of the particles in the cosmic-ray showers, due to multiple Coulomb scattering between 5° and 10° or so.

These light flashes are readily detected with large searchlight mirrors and sensitive photomultipliers. They cannot be detected directly with the human eye, partly because the pupil of the eye is of far too small an area to collect enough light, and partly because the response time of the eye (about 0.06 s) is far too slow in comparison with the statistical fluctuations in the background light of the night sky in that time.

A detailed discussion of this phenomenon, of the light flashes from the night sky associated with ultra-high energy primary cosmic rays, may be found elsewhere (Jelley, 1967).

Over the last 20 years or so, this phenomenon has been developed into a technique of great value in γ -ray astronomy (see, e.g., Turver and Weekes, 1978).

In Fig. 14 we depict the sequence of development of an electron-positron cascade in the atmosphere, generated by an ultra-high-energy γ -ray. The primary γ -ray photon generates an initial electron-positron pair by the process discussed in Section 2.10, and each of these in turn generate further γ -rays by Bremsstrahlung, and so on, until the total energy of the primary γ -ray has been shared among this huge swarm of secondary particles. Whether the final products of this cascade reach the ground or not is of little importance because, on a clear night, the Cherenkov light from the electrons and positrons at *all* altitudes will reach the ground.

The potential of this, the atmospheric Cherenkov technique, as a means of detecting ultra-high γ -rays of celestial origin, arises from the fact that the effective collecting area, of even a simple installation, is determined by the size of the pool of light, rather than by the size of the searchlight mirror. It is, in fact, the *only* technique available for studying celestial γ -ray sources in the extreme energy region of $10^{11} - 10^{13}$ eV.

We mentioned above that there is an analogy between this technique and that of the lead-loaded glass total absorption spectrometers mentioned in Section 4.4. It is mainly a question of scale. Our detector is now a vast volume in the atmosphere, approximating to a cylinder of effective radius $r \sim 100$ m, and of height $h \sim 7$ km, since this is the lapse rate, i.e., the altitude at which the pressure in the atmosphere falls to approximately $(1/e)$ of its value at sea level. The volume is thus $\sim \pi r^2 h$, or ~ 0.2 km 3 . If we take the density of air over this altitude to be one half of its value at sea level, i.e., 0.5 mg·cm $^{-3}$, then the mass of this giant detector is 10^8 kgm. It differs in at least one respect from the solid detectors described in Section 4.4; it is not enclosed in a housing and is therefore exposed to the quite considerable constant stray light from the night sky, and this

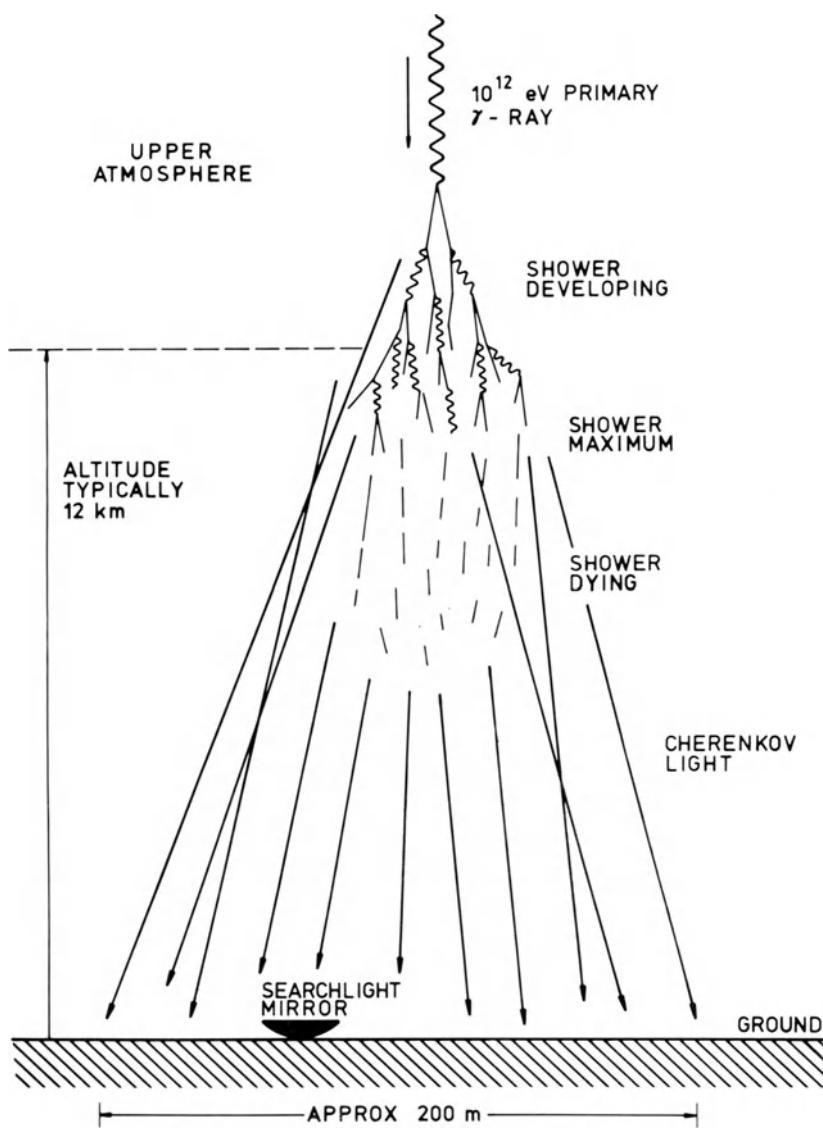


Fig. 14. The development of an extensive cosmic-ray shower in the atmosphere, with its associated Cherenkov light.

sets severe restrictions on its ultimate sensitivity. It nevertheless combines a huge collecting area with good directionality, essential for point source γ -ray astronomy.

5.3. Cherenkov Radiation in the Deep Oceans

Ambitious plans have been made in recent years to devise an experiment to detect high-energy neutrinos from celestial sources. It is not appropriate to discuss this in any detail or to introduce the subject of neutrino astronomy. Suffice it to say, the neutrino is an elusive particle of zero charge and zero mass, which always travels at the speed of light, and which interacts with matter so weakly that it is barely attenuated even by passage through the Earth. This project is called DUMAND (Deep Underwater Muon and Neutrino Detector).

The objective is to set up a vast array of large area photomultipliers at a great depth in the ocean, in one of two trenches off the Hawaiian Islands, and to look for electron-photon cascade showers produced in the deep water from celestial neutrino sources, through the Cherenkov radiation that they would produce. The reason for going to such depths, of perhaps 1 to 2 km, is to substantially reduce the cosmic ray background.

Even at these depths, however, a few cosmic rays are known to penetrate, mainly μ -mesons. The purpose of mentioning this in this review is simply to point out that even in the deepest oceans there will be occasional flashes of Cherenkov light. At an earlier stage in the history of this project, it was suggested that bioluminescence might be a serious problem, or at least a contributor to the ambient light. This, however, is no longer believed to be the case, because it is so dark at these depths that life forms could not exist if life depended exclusively on photosynthesis. In fact, life in the deep oceans is known to exist in many forms, and this is attributed to chemosynthesis (Macdonald and Luyendyk, 1981).

As an indication of how dark it is at these depths, the attenuation length τ for light in sea water has been measured, yielding numerical values which lie between approximately 16 and 40 m, depending on the site.

The light flux from the zenith sun at the surface of the sea is approximately 2.5×10^{17} photons $\text{cm}^{-2} \text{s}^{-1}$, so that with $\tau = 16 \text{ m}$, and a depth of 1 km, the light flux in daytime will be $2.5 \times 10^{17} \times \exp[-1000/16] = 1.8 \times 10^{-10}$ photons $\text{cm}^{-2} \text{s}^{-1}$. At these depths, therefore, it will be essentially as dark in daytime as at night. One should add that although Cherenkov radiation will exist at these depths, both it and

the ionization from these residual cosmic rays will be quite negligible, though of course there remains the ionization associated with the naturally occurring radioactive species of various elements dissolved in the water, e.g., radium and ^{40}K , etc.

6. CHERENKOV RADIATION WITHIN THE HUMAN EYE

The detection of light from *single* charged particles has always been carried out using photomultipliers, as these have a high quantum efficiency and are capable of detecting single photons. Since, however, the dark-adapted human eye has a sensitivity comparable to that of the photocathode of a photomultiplier, there have been many attempts, if only out of curiosity, to see isolated flashes of light from suitable radiators placed in front of the eye. Various people have lain down and held Lucite or glass cylinders, or cones, vertically above the eye, in attempts to observe flashes of Cherenkov light as cosmic-ray μ -mesons passed through these radiators. None, however, were seen, and this has been attributed to three factors: the small solid angle subtended by these devices on the celestial sphere, certain unfavourable geometrical and optical situations, and, above all, to the very small aperture of even the dilated pupil of the eye.

Over the years, however, a very considerable field of research has developed around the general topic of various visual phenomenon associated with the passage of ionizing radiations through the eye itself. One of the many processes that can cause visual effects is of course Cherenkov radiation. The experiments and observations fall broadly into three categories, as follows:

6.1. The Passage of Single Cosmic-Ray Particles through the Eye, at Sea Level

In a pioneering experiment, D'Arcy and Porter (1962) attempted to detect single cosmic ray μ -mesons passing through the eyes of various subjects lying in the dark in a supine position. They calculated that such a particle travelling diametrically through the eye would emit a total of 600 photons, that the photon density would be $10 \text{ photons} \cdot \text{mm}^{-2}$ at the point on the retina where the particle would emerge, and that the corresponding flux at the periphery would be $\sim 0.5 \text{ photons} \cdot \text{mm}^{-2}$. Such fluxes would not be vastly different from the then known limits for threshold vision.

The particles were selected by conventional cosmic-ray techniques.

There appeared to be a significant positive effect though the subjects reported "points" of light, rather than the "diffuse patches" which might have been expected if Cherenkov radiation were generated in the vitreous humour.

A rather more refined version of this same experiment was carried out nine years later by Charman and Rowlands (1971), who also found a slight positive effect. They found, however, that the flash rate was the same whether the subjects were lying in the supine or prone position. This result seemed inconsistent with the Cherenkov hypothesis, and this, combined with reports of the nature and shapes of the images observed, suggested instead some mechanism of "direct excitation" at the retinal level. One interesting result, however, emerged: they were able to show that the observed flashes did not occur when the particles traversed the visual cortex, i.e., that the phenomenon was directly associated with the eye itself.

6.2. Observations of Light Flashes by Astronauts in Translunar Flight

During the extensive space-flight program, crew members of Apollos 11, 12, and 13 reported seeing flashes of light in their eyes, during the long periods of translunar flight. It was at once realized that these flashes of light were probably caused by high energy primary cosmic rays, which penetrated the cabin walls of the spacecraft and the astronaut's eyes. Two basic mechanisms were proposed, either that the flashes arose from Cherenkov radiation generated in the ocular media or the retina of the eye, or alternatively, that the receptors or their accompanying cells in the retina were being excited by direct ionization.

As we shall see, it is likely that both mechanisms were occurring. The Cherenkov interpretation has, however, the advantage that it is possible to predict the flash rates with reasonable accuracy. The yield of light, the spectrum, and the angular characteristics of the Cherenkov radiation are all accurately known, as are the fluxes of the various components of the primary cosmic radiation in space. Furthermore, because the stimulus is optical and hence operates only through the light receptors, whose sensitivity is well known, there is relatively little uncertainty in the calculation of the flash rate.

Let us now calculate what we would expect on the Cherenkov model. First consider a relativistic proton (i.e., $\beta \rightarrow 1$) passing through the eye and impinging on the retina normal to its surface. Since the Cherenkov light is so intrinsically weak, we will only be concerned with scotopic, or

rod, vision, and also, since the cosmic rays in space well away from the Earth are isotropic in direction, we will be mainly concerned with the peripheral regions of the retina, where in any case, the rod population is highest. Near the visual threshold, the retina exhibits areal integration, but this is very restrictive. So, the effective path length of the particle is also restricted, and hence the amount of available light. Taking 20 arc min for the diameter of the basic unit of detection area, the area believed to be served by a single ganglion cell (Graham and Bartlett, 1939), we deduce that with a posterior nodal distance of 17 mm, the absolute radius of this region on the retina is only 290 μm . With $n = 1.34$, leading to $\theta(\text{max}) = 42^\circ$, the effective track length is only 320 μm which, we note, is comparable with the thickness of the retinal layer itself, typically 360 μm (Graham 1965; see Fig. 15). The light intensity falls off as $(1/r)$, where r is the radius out from the point where the particle emerges from the back of the retina.

From the light-yield (Eq. 6), we find that with $n = 1.34$ and a visual bandwidth for scotopic vision of $\Delta\lambda = 380 - 620 \text{ nm}$, $(dn/dl) = 210 \text{ photons}\cdot\text{cm}^{-1}$ or 6.7 photons over the pathlength of 320 μm mentioned above. Allowing for the scotopic luminosity factor (Crawford, 1949), and correcting for the transmission of the ocular media, the *effective* average number of photons is reduced from 6.7 to 2.9.

For the absolute sensitivity of the dark adapted eye, Hecht *et al.* (1941) showed that between 5 and 14 quanta are required in the retina (on a 10 arc min diameter area) to produce a detectable signal at the 60% probability level, i.e., a round figure of 10 quanta. This threshold, we see, falls considerably higher than the calculated yield of 2.9 photons per fast proton, quoted previously. It is for this reason that there is some difficulty in interpreting the observations of D'Arcy and Porter (1962), and of Charman and Rowlands (1971), for sea level μ -mesons, at least in terms of the Cherenkov effect as the dominant mechanism.

In this analysis we have discussed only the light yield from singly-charged primary cosmic-ray protons. In outer space, the primary cosmic rays consist not only of protons, but of stripped nuclei of heavier atoms, in more or less the abundance ratios corresponding to the "solar abundance." That is to say, there are α -particles, i.e., helium nuclei, and nuclei of lithium, beryllium, and boron, and heavier nuclei, like carbon, nitrogen, oxygen, and fluorine, and others besides. The light yield of Cherenkov radiation, as expressed in Eqs. 5 and 6 refers only to particles of single charge, i.e., electrons, positrons, μ -mesons, and protons. The Cherenkov light yield in fact is a function of the square of the atomic number, Z , of the particle, so that nuclei of helium, lithium, beryllium, boron, carbon, nitrogen, oxygen, and fluorine (with Z -values of 2, 3, 4, 5, 6, 7, 8, and 9,

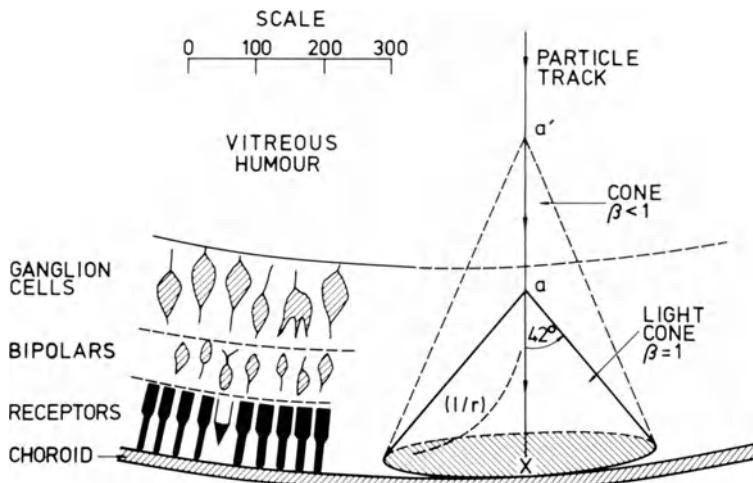


Fig. 15. The essential features of a portion of the retina of a human eye, in relation to cones of Cherenkov light produced within the retinal layer by highly charged primary cosmic-rays in outer space; the resulting flashes of light are observed by astronauts under favorable circumstances. Scale in microns.

respectively) will produce Cherenkov radiation, if these nuclei are relativistic (i.e., $\beta \rightarrow 1$), which is more intense than that from protons ($Z = 1$), by factors of Z^2 , i.e., 4, 9, 16, 25, 36, 49, 64, and 81, respectively.

It was on this basis that Fazio *et al.* (1970) proposed that the observations by the crew members of the three Apollo flights could be readily interpreted on the basis of the Cherenkov mechanism. Taking into account the relative abundances of the various light nuclei in the primary cosmic radiation, Fazio *et al.* (1970) were able to show that the flash rates reported by the astronauts were consistent with the interpretation of the observations in terms of the Cherenkov effect.

The astronauts described a variety of forms of the flashes of light. There were reports of "pinpoint-like" flashes, "streaks" and even "double points." This in itself is no surprise. Referring to Fig. 15, one would probably expect to observe a pin-point flash, in view of the $(1/r)$ variation of the light flux from the axis of the track. If, however, a cosmic-ray primary crossed the retinal layer tangentially, then a "streak" could well be produced, and "double points" could occur if a particle crossed a single eye at two places in turn, or even passed through two eyes in succession.

Having discussed the Cherenkov interpretation of the visual effects observed, one has to admit that alternative explanations may also have

to be invoked. For example, Fremlin (1970) and Tobias *et al.* (1970) showed that neutrons could also cause visual effects. Since their neutron energies were relatively low, it was presumed that these observed effects were due to direct excitation of the retina by either knock-on protons in the hydrogenous media in the retina, or by recoil nuclei of carbon, nitrogen, or oxygen.

It may be of some interest to note that the observations by the crews of the Apollo spacecraft referred entirely to the periods of translunar flight. Light flashes, it seems, have never been reported by astronauts in earth or lunar orbit. The lack of positive effects in earth orbit might in principle be attributed to the effects of the earth's magnetic field, although, when the field strengths are inserted into the relevant equations, such effects are predicted to be quite small. It may well be that the lack of data from astronauts in lunar orbit arises from the fact that they were then preoccupied with other far more pressing activities.

6.3. Observations with Artificially Accelerated Particles

Following these reports from the astronauts, it was only to be expected that there would arise a host of experiments using artificially accelerated particles from nuclear machines. With such accelerators one could control the energy and flux of the particles, select their atomic number, precisely determine their direction relative to the ocular axis, and above all, have observers who were fully dark adapted, rested in an atmosphere of normal pressure and oxygen content, and subject to the normal gravitational field on earth.

There is now a considerable area of literature based on experiments of this type, so we will only quote from a few. For example, Budinger *et al.* (1971) detected light flashes in the eye resulting from neutrons at a flux-level of 10^3 to $10^4 \text{ cm}^{-2} \cdot \text{s}^{-1}$, having a maximum energy of 25 MeV, and an average energy of ~ 8 MeV. They suggested that the observed effects were due to either recoil protons, or to α -particles from neutron reactions on ^{14}N and ^{16}O nuclei in the ocular media. In either case the energies were well below the Cherenkov threshold, so that the observed effects must have been due to some mechanism of direct excitation. In some experiments by our own group, also with neutrons (Charman *et al.*, 1971), visual effects were observed, again with energies well below the Cherenkov threshold.

Such effects, initiated by neutrons, would not have been seen by the astronauts since the neutron content of the primary cosmic-rays in space is negligible. However, the neutron experiments, by providing secondary

particles of low energy *within* the ocular medium, have shown that visual effects due to some direct excitation mechanism by charged particles can be produced in some circumstances.

There have been a variety of experiments carried out with accelerated particles of high charge. For example, using the Princeton Particle Accelerator, visual sensations were induced by relativistic nitrogen nuclei ($Z = 7$) at energies of 531 MeV per nucleon, just above the Cherenkov threshold. The group carrying out this work (McNulty *et al.*, 1972) offered three alternative interpretations for these observations, namely Cherenkov radiation, isomerization of the photochemical rhodopsin molecules by collisions, or optical scintillations produced in the lens of the eye. In connection with the last of these suggestions, McCaulay (1971) obtained direct observations of scintillations induced by 5.3 MeV α -particles in the eye-lenses of freshly killed bullocks.

Convincing evidence that Cherenkov radiation plays a significant role in the interpretation of at least some of these visual effects stems from the work of McNulty *et al.* (1975), using bursts of μ^+ and π^- mesons at energies well above the Cherenkov thresholds, by further experiments of McNulty and Pease (1976), using relativistic μ -mesons, and especially by McNulty *et al.* (1978), who carried out experiments with relativistic carbon ions at energies above and below the Cherenkov threshold.

For a more general account of this subject the reader is referred to the review by Wick (1972).

7. CHERENKOV RADIATION AND CELL DAMAGE

7.1. The Contribution of Cherenkov Ultraviolet Radiation to the Inactivation of Cells Exposed to Ionizing Radiations

Extensive investigations have been carried out over the last few years to understand and interpret the mechanism or mechanisms responsible for the formation of photoreactivable damage caused by ionizing radiation, to a particular strain of the bacterial cells *Esherichia coli*, see, e.g., the works of Myasnik and Morozov (1977), Redpath and Tortorello (1977), Wang and Smith (1978), and Vinicombe *et al.* (1978).

In earlier experiments, damage that could be repaired by photoreactivation (PR) had been considered to occur only in cells irradiated with UV radiation, the sensitive UV range being a relatively narrow band around 250–270 nm. In the more recent work cited previously, evidence was found in certain UV-sensitive strains of *E. coli*, for damage that could partially be photoreactivated after exposure to X-rays or fast electrons.

Since ionizing radiations, e.g., Compton electrons from γ -rays, photoelectrons from X-rays, or primary electrons direct from accelerating machines, could apparently cause damage similar to that by UV radiation, it became important to consider any sources of UV radiation that might be associated directly with the ionizing particles.

The extent of the photoreactivable damage was found to be dependent on the energy of the ionizing radiation. This observation led to the realization that Cherenkov radiation might contribute a significant or even dominant role in providing sufficient UV radiation in the relevant wavelength, to account for the photoreactivable damage observed, following the irradiation of *E. coli* (*uvrA recA*) by ionizing radiation.

This area of work may indeed represent the first time that Cherenkov radiation has appeared as a possible physical process of interest to biologists, other than that of the interpretation of the visual phenomena already discussed in Section 6.

Morozov and Myasink (1980) were the first to publish their demonstration of the role of the Cherenkov process in producing photoreactivable damage in *E. coli*, though three other groups have been working independently in the same field, namely Moss and Smith (1981), Michael *et al.* (1981), and Redpath *et al.* (1981).

Moss and Smith (1981) using ^{137}Cs γ -rays, the Compton electrons from which are mostly above the Cherenkov threshold, produced evidence that the major portion of the photoreactivable damage was caused by Cherenkov UV radiation. They irradiated a suspension of *E. coli* (*uvrA recA*) in a quartz or glass tube located at the center of a beaker that could be filled with water. Using the quartz tube, and with water in the surrounding beaker, there was a marked increase in the inactivation produced and in the photoreactivable sector (PRS) of the damage, compared with the experiment with a glass tube, which does not transmit significantly below 300 nm, or with the surrounding water removed. From these observations they inferred that the additional photoreactivable damage was caused by Cherenkov radiation produced in the surrounding water and transmitted through the quartz tube.

In a second series of experiments, UV radiation-absorbing DNA was introduced in increasing concentrations, in solution in the bacterial suspension. It was shown that approximately 70% of the PRS could be removed due to the absorption of the UV radiation, but a 30% component remained, which could thus not be attributed to the Cherenkov-generated UV radiation. A small PRS component of similar magnitude was observed when 50 keV X-rays, which could not produce Cherenkov radiation, were used instead of ^{137}Cs γ -rays.

Thus, it was concluded by Moss and Smith (1981) that, for ionizing

radiation above the Cherenkov threshold, the major part of the photoreactivable damage produced when the Cherenkov UV radiation could reach the *E. coli* suspension was due to this cause, a smaller component being due to some other process, direct excitation being suggested as one possibility.

In connection with this interpretation, we should stress the point made in Section 3.3, in which we showed that the output of Cherenkov light over the entire visible spectrum, or even more so, over the relatively narrow band of 250–270 nm, is but an extremely small fraction of the energy loss by ionization itself. It is therefore not surprising that there may be a measurable component of photoreactivable damage caused by direct excitation.

It is important at this stage to note that at least as far as pure water is concerned, there is no evidence that there is *any* UV or visible radiation produced by fast electrons *other* than Cherenkov radiation. Two such experiments have been performed. Greenfield *et al.* (1953) studied the spectral distribution of the light emitted in triple distilled water, in the spectral region 313–473 nm, using γ -rays from radium and the β -rays (i.e., electrons) from ^{32}P . Rich *et al.* (1953) carried out a very similar type of experiment over a much wider spectral band. This group used a 3400 Ci ^{60}Co source and, with a spectrograph having a dispersion of $3 \text{ nm} \cdot \text{mm}^{-1}$, found that when they used deionized water, there was no evidence for any light other than that of the Cherenkov radiation, and moreover that the light yield observed agreed closely with that derived by Frank and Tamm (1937). The spectral range covered was 320–600 nm, and there was no evidence for fluorescent or any other optical radiation from the water.

Returning to the biological experiments relating to Cherenkov effects; in the experiments by Michael *et al.* (1981), three types of radiation were used: 250 keV X-rays, ^{60}Co γ -rays, and a pulsed beam of electrons at 1.8 MeV. We will consider here only that section of the work relevant to the interpretation of their results in favor of the role of Cherenkov radiation. The bacterial suspension was placed in a spectrophotometer cuvette having 1 mm-thick walls of high-purity fused-silica (Spectrosil), and a solution depth of 5 mm in the direction of the beam. This cuvette could thus be irradiated directly by the 1.8 MeV electrons, which would readily penetrate both the walls of the container and the solution. By interposing a 5-mm-thick optically polished disk, also of Spectrosil, the electrons could be stopped but their associated Cherenkov UV radiation would be transmitted.

The results obtained were as follows: direct exposure to the electrons and their associated Cherenkov UV radiation showed responses with and without PR similar to those found using ^{60}Co γ -rays. With the Spectrosil

disk in place, considerable cell killing still resulted, indicating clearly that Cherenkov UV radiation was responsible. Moreover, on further interposing a 0.05 mm thick sheet of black paper it was found that photoreactivable damage was completely eliminated, confirming the interpretation that Cherenkov UV radiation was playing a dominant role in the photoreactivable damage to their particular strain of *E. coli*.

In the somewhat similar experiments by Redpath *et al.* (1981), who used an accelerator generating 6 MeV γ -rays, it was concluded that Cherenkov UV radiation was the source of PR damage to their strain of *E. coli*. One point of interest raised in this paper is that since the Cherenkov spectrum extends over the whole optical band, it was suggested that perhaps some of the PR may be occurring *during the period of the radiation itself*. However, one has to bear in mind that this is rather unlikely, because the wattage of the lamps used in PR, taking into account the distance of the lamps from the culture samples, together with allowance for exposure times, suggests that the total optical illumination required during the PR process is many orders of magnitude above the level of the optical Cherenkov light accompanying the UV radiation during the irradiation.

It is of course difficult in this area of work, which interfaces between physics and biology, to use even the same nomenclature, and especially, to interpret observations on a really quantitative basis. Since, therefore, many experiments have already been carried out with γ -ray sources, and others in the future may well be based on such sources, it seems appropriate that we should discuss some of the features of the Compton electron spectrum.

7.2. The Compton Electron Energy Distribution

While some of the experiments just described were carried out with electrons from accelerators, these electrons having energies well above the Cherenkov threshold, and others with X-rays, with energies well below the Cherenkov threshold, the majority of observations have been carried out with γ -rays from artificially produced radioactive isotopes.

Of the three processes by which γ -rays can produce electrons, (see Section 2.10) the Compton scattering process is the only significant one for light elements, which make up the bulk of biological suspensions, in the γ -ray energy range encompassed by most of the isotopes used in this work, i.e., for ^{137}Cs and ^{60}Co . Thus, in view of the importance of the Compton process we will now amplify the brief description presented in Section 2.10.

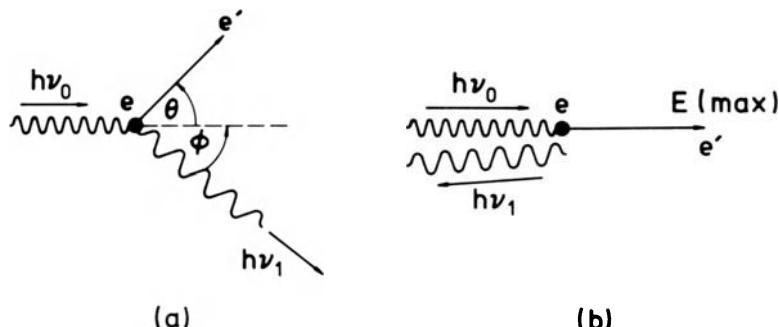


Fig. 16. Compton scattering: (a) the general case and (b) the case for maximum energy transfer. In both cases the incoming photon is represented by an oscillating wave of energy $h\nu_0$ (where h is Planck's constant, and ν_0 the wave-frequency) and the outgoing photon, always of the lower energy, is similarly represented as $h\nu_1$. e is the struck electron, and e' is the scattered electron. The maximum energy given to the electron is $E(\max)$; for details see text.

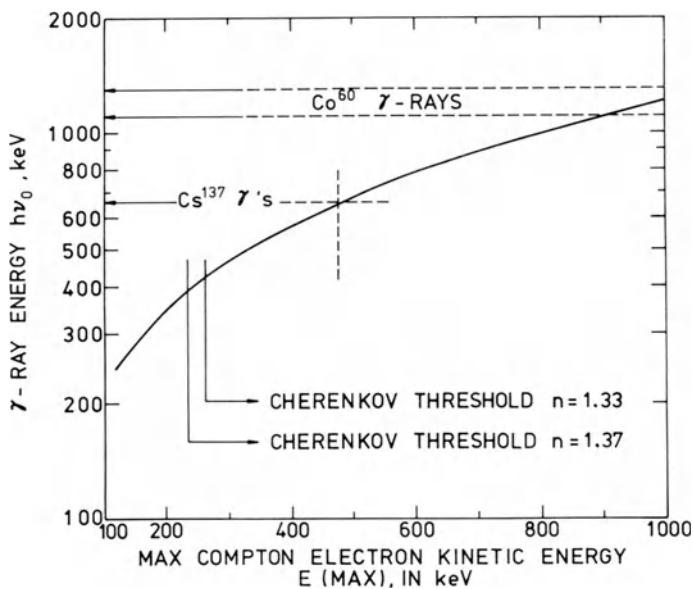


Fig. 17. The maximum Compton-electron energy $E(\max)$ as a function of the γ -ray energy, $h\nu_0$.

The collision of a photon of energy, $h\nu_o$, with an electron of rest energy, $m_o c^2$, can be likened to a collision of billiard balls where the incident ball (the photon) is lighter than the ball being struck (the electron). In the general case, the photon and electron go off at angles of ϕ and θ , respectively, relative to the initial direction of the incident photon, see Fig. 16(a). If we write $\alpha = (h\nu_o/m_o c^2)$, then, in the general case, the kinetic energy, E , of the struck electron, is:

$$E = h\nu_o \cdot \alpha (1 - \cos\theta) / [1 + \alpha(1 - \cos\theta)] \quad (13)$$

There is a maximum value of E that the electron can take up, this occurring when the electron goes straight ahead and the photon is back-scattered along its original path, see Fig. 16(b). This occurs when $\theta = 180^\circ$, so that, from Eq. 13 we obtain

$$E_{\max} = h\nu_o / [1 + (1/2\alpha)] \quad (14)$$

A plot of E_{\max} as a function of $h\nu_o$ is shown in Fig. 17 over the energy range of interest. It is most important to realize that in the Compton

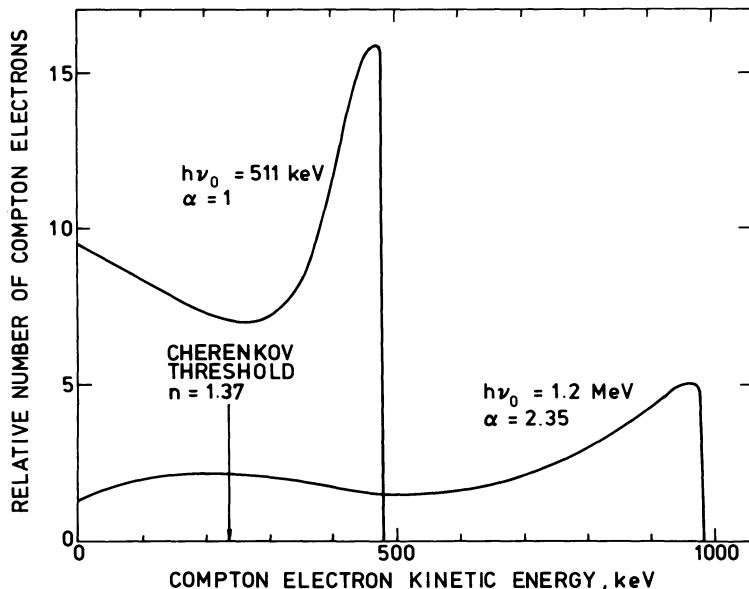


Fig. 18. Typical Compton-electron energy spectra. In both curves, $h\nu$ represents the photon-energy of the incoming γ -ray, and α is the ratio $(h\nu_o/mc^2)$, where mc^2 is the rest-energy of the electron, i.e., 0.51 MeV. n is the refractive index of typical biological material.

process the maximum electron energy always falls short of the γ -ray energy, the difference between the two depending on the value of $h\nu_o$. When $\alpha \gg 1$, i.e., $h\nu_o \gg m_oc^2$, the back-scattered γ -ray has an energy of $(m_oc^2/2)$ or 250 keV, and the energy interval $(h\nu_o - E)$ is likewise 250 keV.

For a γ -ray beam, the photon scattering will occur with random impact parameters and with a cross section for the process that varies for different values of ϕ and corresponding θ . Thus, by no means all the electrons will have the maximum energy E_{\max} .

After integrating the appropriate energy equations over all possible angles of photon scatter, one obtains an energy distribution for the scattered electrons. This distribution, two examples of which are shown in Fig. 18, reveals that while there is indeed a fairly narrow peak close to the "Compton Edge," defined as the energy corresponding to E_{\max} , there is nevertheless a considerable fraction of the distribution showing up at lower energies. This will be important if the Cherenkov threshold is only a little below E_{\max} , and we shall return to this later.

7.3. Toward a Quantitative Estimate of Cherenkov UV Radiation Damage from γ -Ray Sources

In assessing the photoreactivable damage potential of Cherenkov UV radiation, for the particular strain of *E. coli*, there has been little attempt to make the calculations quantitative. So far, the most that has been done, at least for those experiments based on γ -ray sources, is to note the energy of the Compton edge of the electron spectrum relative to the Cherenkov threshold, and then to calculate the UV radiation yield over the wavelength range of interest, namely 250–270 nm, assuming E_{\max} for all the Compton electrons.

To perform the calculation rigorously is indeed difficult, as it includes functions that probably have not been measured in the media concerned, in this wavelength band. We will, however, suggest a procedure that might be adopted if sufficient data were available.

1. Starting with the known γ -ray energy, compute the Compton electron spectrum via standard published curves, or directly, from the Klein-Nishina formula (Evans, 1955).
2. Convolute this with the Cherenkov yield spectrum, over the energy band of interest, bearing in mind that the refractive index is itself a function of the wavelength, especially in the UV region, if the spectral band is near or includes any absorption bands.
3. Correct the resultant or "effective" Cherenkov yield, for absorption of the medium over this wavelength band; this will entail

measuring the spectral absorption curve for the medium, and this in turn may contain geometrical factors associated with the shape of the container, etc.

4. Most difficult of all, determine some Lethality Factor as a function of the wavelength. This could in principle be carried out using a calibrated weak UV continuum source in conjunction with a monochromator whose through-put has likewise been calibrated.

Summarizing the above, we could express the effective photoreactivable damage, D , by an equation of the following form:

$$D \propto \int_{\beta n > 1} \int_{250 \text{ nm}}^{270 \text{ nm}} C_\gamma(E_e) \cdot \text{Ch}(E_e, \lambda) \cdot A(\lambda) \cdot L(\lambda) dE d\lambda \quad (15)$$

where $C_\gamma(E_e)$ represents the Compton electron energy spectrum for an input γ -ray of energy, E_γ , Ch is the Cherenkov spectrum, A the absorption spectrum of the medium, and L some lethality factor, by which we mean the relative potency of radiation at different wavelengths.

Even the above expression is not complete, because it does not allow for geometrical factors or for the slowing down of the Compton electrons in any medium of appreciable thickness interposed between the γ -ray source and the bacterial sample.

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Neurospora crassa: A Unique System for Studying Circadian Rhythms

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1. INTRODUCTION

Progress in a particular field of biology has often been the result of the development of an organism or system especially well suited to research on that problem. The value of *E. coli* to molecular biology, the mammalian red blood cell to membrane biochemistry, and the oat coleoptile to plant physiology has been well-documented.

For the past 30 years or so, research on circadian (daily) rhythms has focused on identification of circadian rhythms in a wide variety of organisms and on defining the formal properties of such rhythms and their similarities among different organisms. Such research has been crucial in documenting the ubiquity and adaptive significance of circadian rhythms and the basic nature of circadian organization in eucaryotic cells (Pittendrigh, 1960, 1974).

As a result of such convincing and overwhelming evidence of the general importance of circadian rhythms, increasing efforts have been directed toward understanding the physiological and biochemical mechanisms underlying such rhythmicity, i.e., at elucidating the mechanisms of the circadian clock, the endogenous timer which underlies and controls the observed daily rhythmicities. Organismal studies have shown that all circadian rhythms share a number of properties. They persist in constant environmental conditions and their period lengths under such conditions are close to, but not exactly equal to, the 24-h period of the earth's rotation. In addition, this "free-running" period length does not change over a wide range of physiological temperatures (temperature compensation), and rhythmicity can be synchronized to the external environment by light or temperature cycles.

Despite such similarities in the general features of circadian organization, it is already clear that there are likely to be both major and minor differences in the underlying mechanisms in different organisms. For example, there are strikingly different roles for the pineal gland and hypothalamus between birds and mammals (Takahashi and Menaker, 1979; Rusak, 1979), and among arthropods, different parts of the brain are important for circadian rhythmicity in cockroaches (optic lobes) and *Dro-*

sophila (pars intercerebrallis; Konopka, 1981; T. Page, personal communication). Even a cursory knowledge of comparative physiology reveals a vast diversity of neural and endocrine systems, and it would seem only reasonable that circadian clocks in different organisms would utilize their own uniquely adapted physiology to control their rhythmicity.

At the cellular and biochemical levels, however, there has been a tendency to try to accommodate data from organisms as diverse as *Acetabularia*, a giant single-celled alga, *Neurospora*, a filamentous fungus, and *Aplysia*, a marine mollusc, into a unified or consensus molecular mechanism for the circadian clock (Hastings and Schweiger, 1976). While certain features of clock organization may well be similar among different organisms, many specific features will surely differ. Since much of the biochemical data on circadian clocks comes from the ability of various drugs to alter the timing mechanism, it is not surprising that much apparently conflicting data has arisen. One needs only to consider the diversity of mechanisms of gene regulation in a single bacterium, *E. coli*, not to mention the variety of types of genetic and cellular organization in eucaryotes, to imagine that the details of circadian regulation are likely to vary at the molecular level.

There can be no doubt that the comparative approach has been valuable in helping to define the kinds of experimental questions one needs to ask. For example, it has served to differentiate clearly between the overt rhythmicity (the "hands" of the clock) and the underlying timer (the clock mechanism itself) and to define experimental protocols which distinguish between the two (Hastings, 1960). It has also uncovered several generalities about the biochemical mechanisms, such as the need for 80S (cytosolic) protein synthesis. However, it now seems as if significant progress will depend on an intensive and detailed analysis of a limited number of systems especially well suited for research on circadian rhythms.

In attempting to identify such systems, various criteria could be applied. One would like an organism with several known rhythms, at least one of which can be assayed easily, and an organism which is amenable to as wide a variety of biochemical, molecular, and genetic techniques as possible. Clearly, no "perfect" organism satisfying all criteria exists, and efforts will continue on several, including *Gonyaulax*, *Acetabularia*, *Euglena*, *Drosophila*, and *Neurospora*.

Neurospora crassa is a filamentous fungus with a long history of research in genetics and biochemistry. As a result, a vast backlog of information and techniques has been developed, and a large community of researchers is working on many aspects of *Neurospora* biology including gene organization and expression; transport and other aspects of

membrane structure and function; energy metabolism; mitochondrial structure, function, and biogenesis; mechanism of mutation, repair, and recombination; photobiological responses such as induction of carotenoid biosynthesis; and developmental responses such as sexual differentiation, spore formation, and germination. These studies provide not only a significant understanding of many facets of the life cycle and behavior of the organism, but also offer many new tools and experimental approaches useful for studies on circadian rhythmicity.

In this review it is our purpose to describe the features of the *Neurospora* clock system, since there has been a considerable increase in the use of this organism for clock studies in recent years. We will also describe the various approaches that can be taken with this organism, present some preliminary results obtained in several areas, and indicate some of the directions likely to be taken in the near future. Several aspects of the biochemistry of circadian rhythms have been reviewed in this series previously, including the nature of the photoreceptors (Ninnemann, 1979) and the involvement of membranes (Engelmann and Schrempf, 1980).

2. ASSAY OF CIRCADIAN RHYTHMICITY

2.1. The Conidiation Rhythm

One reason that *Neurospora* has become such a favored organism for studying circadian clocks is that it has one of the most easily assayed rhythms presently under study—the circadian rhythm of conidiation (asexual spore formation). In practice, this rhythm is expressed in nearly all strains of *Neurospora* (Sargent and Woodward, 1969), but several mutants, *patch* and *band*, exhibit the rhythm much more clearly and under a much wider variety of conditions than do the wild-type strains. The rhythm is expressed by cultures growing across an agar surface, usually on petri dishes or in cylindrical tubes known as “race” tubes. The race tubes are usually 12–16 mm diameter glass tubing and 20–60 cm long. At each end about 6 cm of the tube are bent upwards at a 45° angle so that the tube will hold agar medium. A *Neurospora* culture is inoculated at one end of the tube, placed in the appropriate light and temperature conditions, and allowed to grow until it reaches the other end of the tube or until the experiment is terminated. As the culture grows along the surface of the agar, the growth alternates between surface mycelium alone and surface mycelium plus aerial hyphae which pinch off to form the conidia. Once the mycelium is laid down on the agar, it does not “fill in.” Hence, at the end of an experiment the region of aerial hyphae and conidia appears

as a patch or band of dense growth in contrast to the relatively sparse growth of the surface mycelium, referred to as the interband. Petri dish cultures can be inoculated at the edge of the dish, and the culture forms bands in the shape of arcs of a circle as it grows across the agar. Rectangular baking dishes of somewhat larger dimensions have also been used in biochemical experiments requiring greater amounts of material (S. Brody, personal communication; J. Perlman, unpublished experiments). This rhythm of alternating growth forms exhibits all of the defining characteristics of a circadian rhythm (Sargent *et al.*, 1966). In a 24-h light-dark cycle, there is one band of conidia per cycle, with the center of the band occurring in the late night or early morning. In constant dark and constant temperature, the rhythm persists, usually with a period length of 21–22 h and is temperature-compensated, i.e., the period length is approximately the same at temperatures from 18–30°C. A single flash of light can shift the phase of the rhythm, and the magnitude and sign of the phase shift is dependent on the phase of the cycle at which the light pulse is given. These three properties—synchronization to external light-dark cycles, persistence of the rhythm with an approximately one-day period length in constant conditions, and temperature compensation of the period length—are the defining characteristics of a circadian rhythm (Pittendrigh, 1960).

In actual practice, cultures are placed in the light for about a day, then transferred to constant dark, and the position of the growth front of each culture is marked in red safelight at 24-h intervals throughout the experiment. After the experiment is terminated, the position of the conidial bands is marked, usually as the middle of the band, and the time at which each band occurred is calculated by interpolating between the growth fronts. This interpolation assumes that linear growth along the surface of the mycelium is constant throughout the day, which is approximately correct (Sargent *et al.*, 1966). The period length is then defined as the time from the center of one conidial band to the next, and the period length of the culture can be calculated as either the average period length of the cycles in the race tube or as the slope of a least squares regression line through a plot of the time of each band vs. age of the culture. Usually, phase can be determined to about ± 1.0 h and period length to about ± 0.3 h, but these values depend greatly on the strain, the medium, the temperature, the method of assay (petri dish vs. race tube), and the duration of the experiment.

Collection of data from the race tubes, including the positions of the daily growth fronts and conidial bands, can be done with a digitizer interfaced to a microcomputer programmed to carry out these calculations. As a result, complete processing and storing of data from a single race

tube takes about 15 sec. In addition, because the culture leaves a permanent record of the position (= time) of the conidial band, no automated equipment is needed to monitor the cultures continuously. Consequently, one can carry out literally hundreds of experiments in a single refrigerator-sized incubator.

2.2. Factors Affecting Expression of the Conidiation Rhythm

The expression of the circadian conidiation rhythm is very sensitive to a number of factors, although the temporal properties of the clock controlling the rhythm are not. This distinction between the overt rhythm (conidiation) and the underlying clock which determines its timing is an important one which affects the design and interpretation of many clock experiments. Therefore, although an analysis of factors affecting expression of the conidiation rhythms is not likely to generate direct insights into clock mechanisms themselves, they are an important practical aspect of using the *Neurospora* system.

2.2.1. Strains

If one inoculates a wild-type strain of *Neurospora* on a race tube or petri dish, in most cases one will not observe a circadian conidiation rhythm in constant conditions. Instead, there will be one or two bands at the beginning of the tube and one or two bands at the end, but only mycelial growth in between. However, Sargent and Woodward (1969) showed that most wild-type strains could be induced to express a conidiation rhythm if a slow, constant stream of air was blown through the race tube in the opposite direction from the growth of the culture. Since the airflow itself was not rhythmic, this experiment demonstrated that these wild type cultures possess a circadian clock, but express a conidiation rhythm only under certain conditions. It was later shown (Sargent and Kaltenborn, 1972) that conidiation is highly sensitive to, and inhibited by, CO₂, and that removal of the accumulated CO₂ by the air stream allowed conidiation to occur rhythmically.

The need for continuous air flow through a race tube is a cumbersome process that significantly reduces the usefulness of the system. Fortunately, several mutants have been isolated which allow expression of the rhythm under a much wider variety of environmental conditions, including the absence of air flow.

The first demonstration of circadian rhythmicity in *Neurospora* was by Brandt (1953), who showed that a particular proline auxotroph when

grown along the surface of agar medium showed alternating regions of dense and sparse mycelium with strong conidiation in the dense regions. When such cultures were grown in "race" tubes, the dense regions appeared as patches of conidia, and as a result, Brandt named this strain *patch*. Physiological experiments on *patch* (Pittendrigh *et al.*, 1959) demonstrated that this rhythmicity had the characteristics of circadian rhythms in other organisms—synchronization to light-dark cycles, persistence in constant dark and constant temperature, and temperature compensation of period length. Genetic experiments (Stadler, 1959) showed that the proline auxotroph contained a second mutation which was responsible for the expression of rhythmicity, and it was this mutant gene that was then called *patch*. Although the biochemical basis of the *patch* mutation is not known, it is probably related to the synthesis of polysaccharides or their sugar precursors, since the *patch* strain is partially resistant to the toxic and morphogenetic effects of sorbose, an inhibitor of phosphoglucomutase. The relationship between sorbose resistance and expression of circadian rhythmicity is unknown.

It was recognized in these early studies (Pittendrigh *et al.*, 1959) that the genetic manipulation of *Neurospora* offered unique opportunities to study circadian clock mechanisms, but such efforts lay dormant for many years. This occurred in part because the rhythm of the *patch* strain was somewhat "sloppy," i.e., it was difficult to measure exactly the beginning, middle, or end of each conidial band. As a result, precise measurements of phase and period length of the rhythm were difficult.

Several years later a new mutant of *Neurospora* was discovered (Sussman *et al.*, 1964) which exhibited a somewhat different type of rhythmicity referred to as a hyphal branching rhythm. Although this mutant was termed *clock*, the rhythmicity did not show the properties normally associated with a circadian clock. Although under some conditions, the period length of the rhythm was close to 24 h, it was highly dependent on both the temperature and the composition of the medium, and the rhythm was insensitive to light. It could neither be synchronized to external light-dark cycles nor be phase shifted by single pulses of light.

It was also observed that certain wild-type strains could be induced to exhibit a hyphal branching rhythm if grown on medium containing the appropriate concentrations of sorbose, i.e., one could produce a phenocopy of the *clock* mutation in wild-type strains (Sussman *et al.*, 1964). Later experiments (Feldman and Hoyle, 1974) showed that the properties of the rhythm in the phenocopy were similar to those in the *clock* mutant, i.e., they were not typical of circadian clocks.

Major progress in the use of *Neurospora* for circadian clock studies began with the discovery by Sargent *et al.* (1966) of a new strain which

exhibited circadian conidiation. This strain, originally called *timex*, was shown to contain two mutations—*inv* (invertaseless) and *bd* (*band*, for conidial banding patterns on race tubes). The *inv* mutation was later shown (Sargent and Woodward, 1969) to be dispensable for expression of the circadian conidiation. The conidiation rhythm of *bd* was similar to that of *patch* in that it exhibited all of the defining properties of a circadian clock. In addition, the *bd* strain had two major advantages over the *patch* strain: (1) rhythmicity was expressed on a defined medium containing glucose, salts, and arginine, whereas *patch* required a complex medium containing yeast extract for its expression, and (2) the clarity of the conidial bands was much sharper in *bd* than in *patch*, thus allowing much more precise measurements of phase and period. Since the discovery of the *bd* strain, nearly all circadian clock experiments in *Neurospora* have been done on strains carrying this mutation. Although the biochemical nature of the *bd* mutation has not been elucidated, Sargent and Kaltenborn (1972) demonstrated that conidiation, which is very sensitive to CO₂ in wild-type strains, is resistant to CO₂ in the *band* strain. It appears, therefore, that rhythmicity in the *bd* strain is due to the insensitivity of this strain to the accumulation of CO₂ in the race tube. The mutation obviates the need for air flow to remove the CO₂.

Brody and co-workers have introduced the use of the *csp*⁻ mutation into *bd* strains for use in petri dish cultures (see Mattern *et al.*, 1982). This mutation prevents the separation of conidia from mycelium (Selitrennikoff *et al.*, 1974) and allows much easier manipulation of the cultures for both physiological and biochemical experiments. However, since one cannot make clean conidial suspensions with this strain, it is more difficult to obtain a uniform inoculum for liquid culture experiments (see Section 2.2.3).

2.2.2. Medium Composition

The composition of the medium has a significant effect on the expression of the conidiation rhythm. For the *patch* strain, experiments were carried out using Gray's medium, an undefined medium containing yeast extract, glucose, salts, and phosphate buffer. There were very few attempts to find other media that would support expression of circadian conidiation in this strain.

For the *band* strain, however, this question has been investigated to a considerable extent. Sargent *et al.* (1966) were able to obtain circadian conidiation on a medium containing glucose, salts, and casamino acids, and further showed that L-arginine could replace the requirement for casamino acids. Brody and Harris (1973) developed a medium containing

maltose and inositol for use on petri dish cultures. Maltose supports somewhat denser bands of conidia than glucose. Sargent and Kaltenborn (1972) carried out an extensive investigation on the effects of amino acids and sugars on this rhythm, measuring such parameters as band density, persistence of banding along the length of the race tube, and period length. They found that among the amino acids, arginine, tryptophan, histidine, and alanine strongly favored circadian conidiation, while lysine, cysteine, glycine, methionine, and tyrosine were strongly inhibitory. Among the sugars, maltose, xylose, and trehalose gave dense bands and persistence for at least seven cycles, while with ribose and galactose, banding was less dense but persisted longer. Sucrose, glucose, and fructose yielded 3–4 dense bands, while glycerol supported 3–4 weak bands. Despite the dramatic effects of these substances on the expression of the rhythm, they had little effect on the period length of the rhythm, although small differences of 1–2 h were detected with a few compounds. No obvious metabolic patterns emerged from these studies.

Although the glucose–arginine and maltose–inositol media give clear and persistent expression of the rhythm, the width of the conidial bands often spans $\frac{1}{3}$ to $\frac{1}{2}$ of the circadian cycle, and the accuracy of phase determination is reduced somewhat as a result. In order to find conditions under which phase was more accurately determined, Feldman and Hoyle (1973) utilized acetate as carbon source, Vogel's salts, and casamino acids in race tubes. On acetate, growth is about 25 mm/day at 25°C, in contrast to about 40 mm/day on glucose, but the conidial bands usually occupy only $\frac{1}{4}$ to $\frac{1}{5}$ of the cycle, i.e., the ratio of conidial/mycelial growth is much less on acetate than on glucose. As a result, determination of the center of the conidial band, the phase reference point, is more accurate. Despite the significant difference in growth rates between acetate and glucose, the properties of the rhythm itself, including light-entrainability, period length, and temperature compensation, are nearly identical on the three media.

West (1975) found that both the pH of the medium and the concentration of biotin affected the clarity and persistence of conidial banding. Using only glucose and salts, he found that clear and persistent banding could be obtained with the *bd* strain if the pH of the medium was adjusted to the range of 6.5–7.0. More acidic media resulted in a rhythm which damped out after several cycles in the race tube unless the culture was aerated. Since all strains of *Neurospora* are biotin auxotrophs, this vitamin must be added to all culture media. West (1975) found that he could obtain clear and persistent conidial banding with the wild-type (i.e., *bd*⁺) strain on minimal medium containing only glucose and salts, adjusted to pH 7.0, if the biotin concentration was reduced to limiting values (0.025

$\mu\text{g}/\text{l}$). Similar results were obtained with the *bd* strain at pH 5.5; on limiting concentrations of biotin, banding was clearer and more persistent. West suggested that there was a relationship between the inhibiting effects of high biotin concentration and high CO_2 concentrations, since biotin is a cofactor for two enzymes involved in dark CO_2 fixation.

2.2.3. Other Factors

The expression of the conidiation rhythm is greatly affected by the size and geometry of the culture vessel in which the rhythm is assayed. For example, strains which show a clear and persistent rhythm on race tubes may exhibit poor banding on petri dishes, or vice versa, even if the same medium is used in both cases. Banding may occur in a race tube of one diameter but not another, and the manner in which the lid is placed on a petri dish culture (sealed or unsealed) may affect banding. Presumably, this is due to the inhibitory effect of CO_2 on banding, but this problem has not been analyzed extensively and most culture systems are the result of trial and error.

There have been a number of reports of the effects of magnetic or electrical fields on circadian rhythms in a number of organisms (see Bunning, 1973; chapter 9). However, Bitz and Sargent (1974) were unable to find any effects of magnetic fields on either the growth rate or circadian conidiation of race tube cultures of *Neurospora*.

2.3. Indirect Assay of the Clock

2.3.1. Cultures on Solid Medium

The organism's decision to make aerial hyphae, and hence form a conidial band, is made within a few mm of the hyphal tip, i.e., very near the growing front of the culture (Dharmananda and Feldman, 1979). This can be seen from the fact that once mycelium is laid down on the agar surface, it does not "fill in" at a later time. It is, in fact, this failure to fill in that allows one to assay the clock at the end of the experiment. Since the information for the clock, as observed by conidial banding on race tubes or petri dishes, is therefore contained within the first few millimeters of the growing front, one can ask whether there is a functioning clock in the "old" mycelia, mycelia which was formed one, two, three, or more days prior to the growing front.

This problem was studied by the development of an indirect assay of the circadian clock. Dharmananda and Feldman (1979) inoculated cul-

tures of the *bd,csp* strain onto petri dishes. At various times during the growth of the culture, a piece of mycelium was cut out and transferred to a race tube. This piece of mycelium then grew onto the new agar surface where the phase and period of the rhythm were determined. Using this cut and transfer method, it was first shown that the procedure itself did not shift the phase of the clock in the growing region of the culture; mycelia cut from the growth front of a given culture at different phases of the cycle all showed the same phase in the new race tubes and these cultures were all in phase with the original culture. However, it was found that the *bd,csp* strain had a free-running period length about 1 h shorter than that of the *bd* strain, and that cultures of *bd,csp* growing in petri dishes had period lengths about 0.9 h shorter than cultures growing in race tubes.

This technique was then extended to regions of mycelium behind the growth front, old mycelium whose age was dependent on the distance behind the growth front. These experiments showed that the clock was running in this old mycelium as many as 9 or 10 days after it was initially formed. Surprisingly, however, the older mycelium was not entirely in phase with that at the growth front. In fact, a phase gradient existed across the plate, with the older mycelium losing about 1.5 h/day relative to younger mycelium. The gradient was shown not to be an artifact of the cut and transfer method by exposing an entire petri dish culture to a 24-h pulse of white light, which normally resets the *Neurospora* clock to circadian time 12 (CT 12), i.e., the phase it would assume at "lights off" in a 12:12 light-dark cycle. When the phase of both old and new mycelium was assayed after this light pulse, mycelia from all regions of the culture showed the same phase, and that phase was the expected phase based on a resetting by the light pulse to CT 12. The interpretation of the phase gradient was that the clock of young mycelium at the growth front was running about 1.5 h faster than the clock in the older mycelium. In this way, the old mycelium would lose 1.5 h each day relative to the growth front.

These experiments demonstrated, then, that the clock is running in mycelium which is not actively expressing a conidiation rhythm, or in fact, which is not even growing. Since biochemical experiments dealing with rhythmicity were complicated by the biochemical events involved in conidiation itself, it seemed useful to determine whether other types of cultures not expressing the conidiation rhythm or not exhibiting morphological differentiation could be shown to have a functional circadian clock. As a next step in this procedure, petri dish cultures were formed by spreading a lawn of conidia over the agar surface. Such cultures grew uniformly with no evidence of conidial bands, and if examined early

enough, without evidence of any conidiation. Through use of the cut and transfer technique, it was shown that a functional clock was running in these lawn cultures, and that mycelium taken from different locations in the petri dish culture were in phase with each other (Perlman, 1981).

2.3.2. Rhythmicity in Liquid Cultures

With the information from the lawn cultures in hand, it was only a small step to substitute liquid medium for the agar. Standing liquid cultures inoculated with conidia form a mycelial mat on the surface of the culture. Such a mat can be cut into small mycelial disks, and the disks can be transferred to race tubes and assayed for phase and period. Such cultures have shown considerably more sensitivity than the solid cultures to phase shifting by the cut and transfer procedure. However, it has been possible to find conditions in which the procedure does not shift the phase of the transferred mycelium (Perlman *et al.*, 1981; Nakashima, 1981), and under these conditions the liquid cultures have a normally functioning circadian clock.

These experiments have involved two different types of media. In one system (Perlman *et al.*, 1981) a pantothenate-requiring strain (*pan-2*) is first grown in the light in liquid medium containing excess pantothenate to produce a mycelial pad. The pad is then put into constant dark and transferred to medium containing limiting amounts of the vitamin. After the transfer, growth slows down and eventually stops, but the clock runs normally for 48–60 h in the dark, and the mycelium can be transferred to race tubes containing full growth-supporting nutrients to assay the rhythm. This system can be used with either acetate or glucose as carbon source. In the other system (Nakashima, 1981), which uses glucose as the carbon source, the need for the pantothenate auxotroph is obviated by transferring the mycelial pad to a medium containing limiting levels of glucose. Again, growth of the culture slows and eventually stops but the clock runs normally in the liquid medium in the dark for up to 72 h and can be assayed by transferring the mycelium to race tubes.

These procedures have become the basis for a wide variety of biochemical experiments on the clock of *Neurospora* not previously possible, including the determination of effects of drug or inhibitor pulses, assay of biochemical parameters during the cycle, and measurement of biosynthetic phenomena such as RNA and protein synthesis through incorporation of appropriate pulses of radioactive isotopes.

It should be pointed out that since many disks can be cut from one mycelial mat and since the rhythm in the cultures is assayed indirectly

in race tubes, no automated equipment is needed for liquid cultures either, and a large number of cultures can be manipulated quickly and in a relatively small space.

A few experiments were also carried out which demonstrated that a functional clock exists in shaking liquid cultures (Perlman, 1981), but little has been done with this mode of growth.

2.4. Biochemical Rhythms

In addition to the morphological rhythms described above, a variety of physiological and biochemical oscillations have been catalogued in *Neurospora*. It is worth noting at the outset, however, that there is much reason to believe that all of the rhythms thus far described can be tied fairly directly to the morphological and biochemical changes associated with the conidiation process. These changes include an increased rate of accumulation of cellular mass, production of aerial hyphae, hyphal branching and budding of proconidia, and the final septation and separation of mature conidia. Associated intracellular events involve active nuclear division, a general decrease in glycolysis and increase of the glyoxylate and Krebs cycles, enhanced biosynthesis of reducing power via the pentose phosphate shunt, and deposition of different classes of lipids, carbohydrates, and proteins (Turian and Bianchi, 1972; Schmit and Brody, 1976; Hochberg and Sargent, 1974). While clearly not all of these changes can occur during the 6- to 8-h when a "band" is laid down, they are characteristic of the extensive physiological changes that the commitment to conidiation must entail.

2.4.1. Rhythm of CO₂ Production

The first physiological rhythm described in *Neurospora* was one in CO₂ production (Woodward and Sargent, 1973). Although also noted in standing liquid cultures, it is best expressed in race tube cultures where the amplitude can reach 20 ppm. Since the rhythmic production of CO₂ peaks midway through the formation of a conidial band, it probably reflects the increased rate of accumulation of mycelial mass and necessarily enhanced Krebs cycle activity associated with the conidiation process. The finding that the short period circadian clock mutants *frq-1* and *frq-2* both exhibit short period CO₂ rhythms (Sargent, personal communication) is taken as evidence that the CO₂ rhythm is controlled by the circadian clock.

2.4.2. Rhythms in Enzyme Activities

In an effort to confirm that the clock-controlled morphological changes were correlated with presumptive clock-controlled enzymatic changes, Hochberg and Sargent (1974) examined representative enzymes of glycolysis, the Krebs and glyoxylate cycles, and the hexose-monophosphate shunt from agar-grown cultures under conditions both permitting and not permitting rhythmicity. As expected, (glycolysis associated) glyceraldehyde phosphate dehydrogenase activity when normalized to mycelial mass, oscillated 180 degrees out of phase with conidiation. Glyoxylate and Krebs cycle enzymes (isocitrate lyase, citrate synthase) were completely in phase with conidiogenesis, as was nicotine adenine dinucleotide glycohydrogenase (NADase), an enzyme previously shown to be developmentally associated with, but not required for, spore formation (Nelson *et al.*, 1975). Enzymes associated with the hexose monophosphate shunt (glucose-6-phosphate dehydrogenase and 6 phosphogluconate dehydrogenase) displayed oscillations which rose in anticipation of conidiogenesis, peaking during the 6 h bracketing its initiation. Also peaking during conidiation was a rhythm in total soluble protein content (normalized to mycelial mass).

The question of whether the rhythmic enzyme activities were directly associated with the biological clock or associated only through the rhythmic conidiation process was examined in three ways. The concentration of CO₂ was varied from nearly zero to enough to damp the oscillations in both the *bd*⁺ and *bd* strains (Sargent and Kaltenborn, 1972); a loss of enzyme rhythmicity was noted only when the morphological rhythms also disappeared. In addition, in the nonconidiating mutant *fluffy*, the absence of overt morphological rhythmicity was associated with an absence of rhythmicity in the associated enzymes. Finally, cultures of *bd* were examined over time at a particular region behind the growing front and at one time in sections all along the surface previously traversed by rhythmic growth. Rhythmicity was found only at the growing front, meaning that once laid down, an area of high activity remained high relative to surrounding regions. Taken together, these data are all consistent with the finding that the activities of enzymes described were rhythmic only through their associations with the clock controlled rhythmic conidiation process. This finding was further enforced by the work of Halaban and Feldman (1973a) described in later sections.

At least in passing, it should be noted that rhythmicity in enzymes, even in these pathways, is not universal. Malate dehydrogenase which is associated with both the glyoxylate and Krebs cycles failed to show any rhythmicity, thus suggesting that enzymes not associated with junction

points of pathways are perhaps not as tightly regulated. Other activities constant over time were glutamate dehydrogenase, constitutive and repressible alkaline phosphatase, and acid phosphatase, all of which are associated more with nutrient acquisition than intermediary metabolism. Also consistent with the general lack of rhythmicity is the finding of Schmit and Brody (1976), that, despite rhythmic morphological changes, mycelial content of glucosamine and galactosamine is constant over time. The possibility that the relative rates of synthesis of various cellular proteins were different at different times in the cycle has also been examined (Perlman, 1981) by two-dimensional gel electrophoresis (O'Farrell, 1975) followed by autoradiography. In these experiments, cultures were pulse labeled near midday and midnight with [³⁵S]methionine, and the proteins extracted and fractionated into phosphate-buffer-soluble and insoluble fractions. Despite resolution of more than 100 distinct polypeptides on gels from each fraction, no differences were found between samples from different times of day.

2.4.3. Rhythms in Nucleic Acid Metabolism

Rhythms in nucleic acid metabolism paralleling the protein and enzyme studies noted above have also been examined (Martens and Sargent, 1973). Long term trends in DNA and RNA content along with ribonuclease activity were described in standing and shake cultures of *bd* and wild type; however, as with the CO₂ rhythm in liquid culture, the amplitude of any circadian component in the temporal variation was too small to be seen. For this reason, subsequent experiments were performed by sampling, over time, the surface and aerial hyphae from the growing front of baking dish cultures. This revealed approximately 24-h oscillations in both DNA and RNA content (normalized to mycelial mass). The oscillation was in phase with the conidiation rhythm (as predicted from the finding that conidia contain more DNA and RNA per mg than mycelia), and trends in the amplitudes of the two oscillations followed each other. Daily cycles in the synthesis of nucleic acids were followed by measuring the incorporation of [³H]juridine into DNA and RNA, with the expected result that the synthesis rhythms (normalized either to mycelial mass or DNA/RNA content) phase led the nucleic acid content rhythms by about 6 h.

In an effort to determine whether the rhythm was tied directly to the clock or was forced by the morphological changes, DNA and RNA content were analyzed over time in the mutant *fluffy*. This aconidial strain, while producing abundant aerial hyphae, nevertheless fails to complete the septation process required for the formation of free conidia (Turian

and Bianchi, 1972). Rhythms in both DNA and RNA content were found, albeit with an amplitude about $\frac{1}{3}$ to $\frac{1}{2}$ that of *bd*. This result supports the notion that the oscillator was tied directly to the circadian clock. However, the possibility exists that the aerial hyphae continued to be produced rhythmically but with a smaller amplitude of oscillation than seen in the *bd* strain. More importantly, the phase relationship to the previously entraining light-dark cycle was not that expected of a true circadian rhythm. After only two days in constant conditions, the peaks were about 12 h (180 degrees) out of phase with those noted for *bd* and wild type. Finally, while the nucleic acid rhythm in *bd* tightly followed the conidiation rhythm, that of wild type seemed to phase lead the conidiation rhythm by several hours, and furthermore displayed a period length of more nearly 24 than 21–22 h. It is possible that these inconsistencies can be explained through small but uncontrolled effects of medium composition on period and phase (Sargent and Kaltenborn, 1972), differences in period length on plates or baking dishes vs. race tubes (Dharmananda, 1980), possible strain differences, and the difficulty of following a rhythm in a substance which must be normalized to weight. An important finding in this context is the observation (Sargent, personal communication) that the short period clock mutant *bd, frq-1* also displays a rhythm in nucleic acid content with an appropriately shortened period. In light of this, there can be little doubt that the oscillation in *bd* (and therefore probably wild type) are driven by the circadian clock. However, other questions, particularly regarding the 12 h phase difference with *fluffy*, must remain unresolved for the present. Thus, as with the enzyme rhythms, the only known circadian oscillations are associated with a visible conidiation rhythm. This may also serve as a cautionary note that, until the relationships between growth, the circadian conidiation rhythm, and the noncircadian hyphal branching rhythm are fully resolved, it will remain important in biochemical experiments to establish that a rhythm is truly circadian if it is entrainable by a light-dark cycle (or phase shiftable by a light pulse in otherwise constant darkness), or has a period length which is altered in a strain carrying a mutation (such as *frq-1* above) known to modify the period of the circadian clock.

2.4.4. Rhythms in Adenylate and Pyridine Nucleotides

Based on the realization that Sargent's CO₂ rhythm probably reflected changes in respiratory metabolism and therefore perhaps mitochondrial function, Delmer and Brody (1973, 1975) examined the growing front of banding agar grown cultures for variations in the levels

of adenine nucleotides. While the concentrations of ATP and ADP showed no systematic trend over a circadian cycle, AMP was reported to oscillate from 0.5–6 $\mu\text{M/g}$ dry weight, with a peak in the growing front near the midpoint of a conidiating phase and a trough during the time when only surface hyphae were being produced. In the absence of specific intracellular compartmentalization of these nucleotides, this would represent a daily change in energy charge of from 0.65 to 0.93. It should be noted however, that a change in energy charge normally denotes a change in the extent of phosphorylation of a relatively constant amount of adenine nucleotide (Atkinson, 1968, 1970). In the present case, however, the organism is apparently undergoing a rather massive daily cycle in synthesis and/or processing of adenine nucleotides. The apparent change in energy charge as calculated from the standard formula could thus be alternatively viewed as a necessary by-product of this cycle in nucleotide metabolism, exclusive of energetic considerations. In any case, the strict circadian nature of the daily cycle was verified by demonstrating that constant bright light damped the rhythm and, more importantly, that the phase of the rhythm was set by the time of the culture's light to dark transition (rather than the time of its inoculation, as would have been expected from the hyphal branching rhythm). Since neither regions behind the growing front nor cultures not displaying morphological rhythms were examined, it cannot be determined whether the oscillation was only a reflection of the morphological rhythm or was controlled independent of it. The oscillation in the level of AMP could be due to changes in the synthesis and/or degradation of the adenosine moiety or to changes in its degree of phosphorylation. The authors concentrated on the phosphorylation interpretation and favored an explanation involving rhythmic partial uncoupling of mitochondrial oxidative phosphorylation.

Spatial and temporal changes in the levels and ratios of oxidized and reduced pyridine nucleotides (NAD, NADP, NADH, and NADPH) from petri dish cultures have also been examined (Brody, 1973; Brody and Harris, 1973; Dieckmann, 1980). The most recent investigations utilized improved extraction procedures and a cycling assay (Lowry *et al.*, 1961) that allowed analysis of samples about 1000 times smaller than was previously possible (Dieckmann, 1980). Present data indicate that (1) there is a significant decrease in NAD as cultures age on an agar surface (without regard to circadian time); (2) any changes in nucleotide levels at the growing front over time are quite small; and (3) the redox ratio is strictly controlled and is constant throughout the circadian cycle. The spatial differences found in pyridine nucleotide levels between different morphological areas of a colony (Brody and Harris, 1973) are thus not due to a rhythm in the level of these nucleotides (Dieckmann, 1980).

2.4.5. Rhythms in Fatty Acids

Since unsaturated fatty acids were known to act *in vitro* as at least partial uncoupling agents (Borst *et al.*, 1962), the hypothesis of rhythmic partial mitochondrial uncoupling led logically to the analysis of mycelial fatty acid content over time (Roeder *et al.*, 1982). In cultures of both *bd* and wild type, the mole percentage of linolenic (18:3) and linoleic (18:2) acids were found to oscillate in a reciprocal manner. Minima in 18:3 (corresponding to maxima in 18:2) occurred about 40 and 59 h after the light to dark transition, times corresponding to the ends of conidiating phases. Maxima in 18:3 were near 51 and 73 h slightly after the start of a band. The mole percentages of palmitic (16:0), stearic (18:0), and oleic (18:1) were constant. A previous preliminary report (Brody and Martins, 1976) of a circadian oscillation in the ratio of saturated to unsaturated fatty acids was later shown by workers in the same laboratory to have been in error (Brody, personal communication). These data are interesting not only because of the implications for mitochondrial uncoupling and membrane fluidity, but also because the occurrence of a rhythm out of phase with conidiation and of possibly undiminished amplitude in wild type (in the absence of a highly visible morphological rhythm) suggests that this oscillation may be coupled directly to the clock (however, see Section 5.5.3). Such a conclusion, however, will require more information regarding the sensitivity of the rhythm to light or mutation.

3. LIGHT AND TEMPERATURE RESPONSES

3.1. Light-Induced Phase Shifting

Neurospora can be entrained to light-dark cycles of 12 h light and 12 h dark. Under these conditions, the center of the conidial band occurs just before lights on (circadian time 22; CT 22). Single 5-min pulses of light once per cycle will also entrain the organism to a 24-h cycle (Gardner, unpublished data). *Neurospora* can also be entrained to cycles that differ from 24 h, but the upper and lower limits of entrainment have not been systematically determined. It would be interesting to examine these limits, not only in the *bd* strain but in clock mutants with altered period lengths.

Single pulses of visible light can induce a steady-state phase shift of all circadian clocks, and the magnitude (number of hours) and direction (advance or delay) of this phase shift depend on the phase of the cycle at which the pulse is given. A plot of phase shift vs. phase of the cycle at which the pulse was administered is called a phase response curve

(PRC), and this PRC is a direct measure of the behavior of the underlying oscillator (Pittendrigh, 1967). In *Neurospora*, a light PRC has been determined by a number of workers whose results all basically agree. Sargent *et al.* (1966) first showed that the *Neurospora* light PRC was similar to that in many other organisms, with phase delays occurring during the late subjective day and early subjective night, and phase advances occurring during the late subjective night and early subjective day. Maximum phase shifts were large (12–14 h). More recently, Nakashima and Feldman (1980) obtained similar results for cultures at 25°C, but also showed that light-induced phase shifting was temperature sensitive. Although phase shifts were large (12–14 h) at 25°C, the maximum phase shifts obtained at 30°C were about half those at 25°C, and at 34°C, there was little or no phase shifting by light. The basis for this temperature dependence of light-induced phase shifting is not known but resembles observations of temperature sensitivity of light-induced carotenoid synthesis in *Neurospora* (Harding, 1974).

Pulses of ultraviolet light also induce phase shifts of the circadian conidiation rhythm. West (1976) found that 5-min pulses of short wavelength UV light from a germicidal lamp (maximum wavelength of 254 nm) could induce large (up to 11–12 h) phase advances when given during the late subjective night and possibly some small advances during the early subjective night. Qualitatively, the phase response curve for phase shifting by UV light was similar to that obtained for visible light, but some quantitative differences were also seen. Although it was suggested that this implied a role for nucleic acids in the phase-shifting mechanism, no further work has been done along these lines.

3.2. Nature of the Photoreceptor

Sargent and Briggs (1967) first showed that constant light caused damping (or suppression) of the conidiation rhythm at wavelengths of 350–500 nm, with a maximum effect at 465 nm and other peaks at 485, 415, and 375 nm. This action spectrum is typical of so-called “blue light photoreceptors” found in a wide variety of prokaryotes and eucaryotes (Briggs, 1976) and resembles the absorption spectrum of both carotenoids and flavins. However, since the photoresponse of a double mutant *al-1*, *al-2* (*al* = *albino*) blocked at two steps in the pathway of carotenoid biosynthesis was unaltered (Sargent and Briggs, 1967), it appeared that the photoreceptor was more likely a flavin than a carotenoid.

However, in some organisms, damping of a circadian rhythm by constant light may not be a measure of the response of the clock mechanism

itself (Sweeney, 1979). This appears to be the case in *Neurospora*. Paietta and Sargent (1983b) have isolated three mutants whose sensitivity to light, as measured by suppression of the conidiation rhythm, is reduced 50–80 fold. However, the sensitivity of the clock to light-induced phase shifting in these mutants appeared normal. It is important, therefore, that Dharmannanda (1980) has obtained an action spectrum for light-induced phase shifting which is quite similar to that for suppression of conidial banding and which is also characteristic of a blue light photoreceptor.

Additional evidence for the flavin nature of the photoreceptor has come from experiments demonstrating that riboflavin auxotrophs, grown under conditions which resulted in decreased levels of total cellular flavins, showed a significant decrease in their sensitivity to light for both the damping and phase shifting responses (Paietta and Sargent, 1981). Furthermore, addition of exogenous riboflavin analogues restored light sensitivity to these strains (Paietta and Sargent, 1983a).

Briggs and his colleagues (Brain *et al.*, 1977*a,b*) have obtained evidence that the clock photoreceptor is actually a flavin coupled to a b-type cytochrome in the plasma membrane. They have isolated a plasma membrane fraction that shows a flavin-mediated blue light photoreduction of a b-type cytochrome with the same action spectrum as that obtained for phase shifting the clock. They have also shown (1) that the respiratory mutant *poky*, which has a significant reduction in the amount of this cytochrome, also shows a significantly reduced sensitivity to light for both phase shifting and photosuppression, and (2) that the addition of the photodynamically active dye methylene blue sensitizes both the *in vitro* blue light-induced photoreduction and the *in vivo* light induced phase shifting and photosuppression to red light (W. R. Briggs, personal communication). Such results would suggest the possibility that light-induced phase shifting involves an interaction with electron transport phenomena and possibly ion (proton?) gradients across the plasma membrane.

It has also been suggested (Klemm and Ninnemann, 1979; Ninnemann and Klemm-Wolfgramm, 1980) that nitrate reductase, a molybdo-flavoprotein containing a b-type cytochrome, is involved in blue light stimulated conidiation. However, this enzyme does not seem to be important for photosuppression or phase shifting of circadian conidiation, since Paietta and Sargent (1982) showed that three mutants defective in nitrate reductase activity (*nit-1*, *nit-2*, and *nit-3*; see Marzluf, 1981) exhibited normal photoresponses for these phenomena and that cultures grown under conditions where nitrate reductase activity was repressed also showed normal responses.

Recently Nakashima (1982*a*) has identified several inhibitors of light-induced phase shifting in cultures grown in liquid medium and has found

that the pH of the culture medium significantly influenced both the effects of these inhibitors and the overall light sensitivity of the *Neurospora* mycelium. For example, the light-sensitivity of cultures grown at pH 5.7 was about 8 times greater than the sensitivity of cultures grown at pH 6.7; a saturating response for the pH 5.7 cultures was obtained with a light pulse of 60 ft candle-sec, while the pH 6.7 cultures required a pulse of 480 ft candle-sec for saturation. In addition, two inhibitors of the H⁺-translocating plasma membrane ATPase, diethylstilbestrol (DES) and N,N-di-cyclohexylcarbodiimide (DCCD), inhibited light-induced phase shifting of cultures grown at pH 6.7 but not pH 5.7. These effects of pH were the result of some relatively stable change induced in the mycelium during its growth, since transfer of the culture to medium of a different pH 1 h prior to treatment did not alter the response. Thus, it was the pH of the medium in which the culture grew, not the pH of the treatment medium, that was important. In contrast, inhibitors of mitochondrial ATPase, including venturicidin and oligomycin, did not significantly inhibit light-induced phase shifting at either pH. Azide, which inhibits both plasma membrane and mitochondrial ATPase, also inhibited light-induced phase shifting, but this effect was due to inhibition of the plasma membrane enzyme, since inhibition of respiration, and hence of mitochondrial ATPase, was dependent on the pH of the treatment medium.

Nakashima and Fujimura (1982) have extended these studies by showing that transfer of mycelium from culture medium to buffer inhibited light-induced phase shifting for cultures grown at pH 6.7 but not at pH 5.7. Again, the pH of the growth medium, but not the buffer, was important. They also found that addition of NH₄⁺ to the buffer restored light sensitivity to the cultures, but K⁺, Na⁺, Cl⁻, or NO₃⁻ did not. These results, coupled with those of Briggs and his colleagues and Paietta and Sargent on the flavin/b-cytochrome nature of the photoreceptor, focus additional attention on the role of ionic and electrochemical gradients across the plasma membrane in the mechanism of light-induction phase shifting of the *Neurospora* clock.

3.3. Temperature Responses

One of the primary characteristics of circadian rhythms is that of temperature compensation of period length. Over a wide range of physiological temperatures the period length in constant conditions remains nearly constant. In *Neurospora* this is true only at temperatures below 30°C, where the Q₁₀ is approximately 1 (Sargent *et al.*, 1966; Nakashima and Feldman, 1980). Above 30°C, however, the Q₁₀ increases to about

1.3. The period length of the *Neurospora* clock also shows a lack of response to changes in nutritional conditions, but this "nutritional compensation" also occurs only at temperatures below 30°C. Above this temperature period length becomes strikingly dependent on the composition of the medium (Nakashima and Feldman, 1980). This result is consistent with the suggestion (Pittendrigh and Caldarola, 1973) that temperature compensation in circadian rhythmicity is but one example of a general homeostatic mechanism which the clock has evolved for maintaining accurate timing. In *Neurospora*, conditions under which the clock is no longer temperature compensated also appear to result in a loss of "nutritional compensation" as well.

The phase shifting effects of temperature steps and pulses have also been studied in *Neurospora* (Francis and Sargent, 1979). In general, these responses are similar to those found in other organisms. Upward temperature steps induce phase advances, while downward temperature steps cause phase delays. Temperature pulses are roughly equal to the sum of the effects of a step up or step down, although a number of interesting exceptions have been found. A formal model relating temperature compensation to the phase shifting effects of temperature steps and pulses has been proposed (Pavlidis *et al.*, 1968), but no concrete biochemical models which account for this behavior have been proposed.

4. GENETIC ANALYSIS

4.1. Genetic Approaches

As mentioned previously, one of the major advantages of using *Neurospora* is the ability to isolate mutants and carry out a wide range of genetic, cytogenetic, and molecular analyses (Davis and deSerres, 1970; Perkins and Barry, 1977). The application of genetic approaches to the study of circadian clocks in general, and in *Neurospora* in particular, has recently been reviewed (Feldman, 1982). Therefore, we will present only a brief summary of these experiments here.

The use of mutants to analyze the *Neurospora* clock has fallen into one of three categories (1) the isolation of clock mutants with altered clock properties, such as period length, light sensitivity, or temperature compensation, and their subsequent genetic, physiological, and biochemical analysis, (2) the isolation of biochemical mutants with known lesions in specific metabolic pathways and the determination of the effects of such alterations on clock function, and (3) the use of drug-resistant mutants to identify the specific cellular target of an inhibitor known to alter clock

function. The first of these topics will be considered here, while the other two will be discussed in Section 5.

4.2. Clock Mutants

Approximately 20 mutants with altered free-running period lengths have been isolated (Feldman *et al.*, 1979), of which 12 have been studied in detail. These 12 mutants map to six sites, with one mutant at each of five loci, called *chr*, *prd-1*, *prd-2*, *prd-3*, and *prd-4*, and the other seven mutants all at a single locus called *frq*. The six loci are unlinked to each other and are located on five of the seven chromosomes of *Neurospora*.

Most efforts have focused on the *frq* mutants, which have period lengths ranging from 16.5 hours (*frq-1*) to 29 hours (*frq-7* and *frq-8*). Fine structure analysis suggests that there are at least two functional regions of the *frq* locus, the long period mutants (*frq-3*, *frq-7* and *frq-8*) being in one region, and the short period mutants (*frq-1*, *frq-2*, *frq-4*, and *frq-6*) being in the other.

Mutants at the *frq* locus exhibit a number of properties which suggest that this locus or the gene product(s) derived from it play a key role in the circadian clock.

1. An equal number of "fast" and "slow" mutants have been obtained. This is unusual in that most mutations result in loss of function and would be expected to be similar or to have phenotypes altered in the same direction. The existence of more than one gene at this locus might explain this result.
2. All *frq* mutants show incomplete dominance in heterocaryons, while most other kinds of mutants are recessive.
3. All *frq* mutants show gene dosage effects, i.e., the amount of alteration of period length is proportional to the fraction of mutant nuclei in heterocaryons. From these results, it appears as if the *frq* gene(s) or gene product(s) is part of, or closely related to, the clock mechanism itself, since it seems unlikely that the strict proportionality between gene dosage and clock phenotype would be maintained through a sequence of pleiotropic or secondary events subsequent to the primary gene function.
4. All *frq* mutants show normal growth and development. The only phenotypic alteration detected so far is the period length of their circadian clock.
5. All *frq* mutants are altered in the same part of their circadian cycle (Dharmananda, 1980). A 7-h portion of the late day-early

- night part of the cycle of the wild-type strain is shortened to 2 h in the shortest mutant (*frq-1*) and lengthened to 14.5 h in the longest (*frq-7, frq-8*).
6. The period lengths of the *frq* mutants are clearly not random. At 25°C they differ from the wild-type (21.5 h) period length by an integral multiple of 2.5 h.

Thus, there are mutants that are 2.5 and 5.0 h shorter than wild type, and 2.5 and 7.5 h longer than wild type. Furthermore, of the seven mutants, three independent isolates have the same 2.5-h short period, while two independent isolates have the 7.5-h-long phenotype. This result suggests that there may be a 2.5-h quantum element controlled by the *frq* locus. In this regard, it is interesting that all of the *frq* mutants were isolated after mutagenesis with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine, and that no *frq* mutants were isolated after mutagenesis with UV light, although several mutants at other loci were recovered in the latter case. One usually associates MNNG mutagenesis with events at the replication fork during DNA synthesis. However, in this case the mutagenesis treatment was carried out on conidia during the first hour of germination, well before the start of DNA replication (Serna and Stadler, 1978). It may be that the *frq* locus replicates much earlier than the bulk of chromosomal DNA, but no evidence for this exists. An alternative explanation comes from observations that MNNG can cause chromosomal aberrations in nonreplicating cells (Botstein and Jones, 1969), and it may be that the *frq* mutations involve some event other than a single point mutation, such as a deletion, and inversion, or two point mutations. Genetic analysis has not yet revealed any evidence in support of such a hypothesis either. It should also be mentioned that these mutants were screened on the acetate-casamino acids medium and that other types of mutants which would not grow or conidiate well on this medium would not have been detected.

The temperature compensation properties of all of the mutants have been characterized (Gardner and Feldman, 1981). Most intriguing have been the results with the *frq* mutants, for which it has been shown that those with long periods have a significantly altered temperature compensation, i.e., period length is significantly more dependent on temperature than in the wild-type strain; those with short periods exhibit normal temperature compensation. This is consistent with the genetic data described above that indicate that the long and short period *frq* mutants are in different functional regions of the locus. Mutants at other loci exhibit a variety of alterations of their temperature compensation but have not been studied in detail.

In addition to genetic analysis, studies on the detailed nature and structure of the *frq* region can now be carried out at the molecular level.

Recombinant DNA technology can be used to clone *frq* DNA, and the ability to transform *Neurospora* allows a functional test of the cloned material. DNA sequence analysis of the cloned *frq* DNA will determine directly the organization of this locus and the nature and position of the various *frq* mutants. In addition, the cloned DNA could potentially be used to identify *frq* messenger RNA to determine whether the *frq* locus is transcribed periodically and to isolate *frq* mRNA for use in *in vitro* translation studies to isolate and identify polypeptide(s) encoded by *frq* DNA. Such experiments are currently underway in our laboratory.

Recently, Nakashima (personal communication) has isolated two additional clock mutants. One, with a period length of about 17 h, maps close to or at the *frq* locus, while a second, whose period length is about 23 h, is unlinked to *frq*.

4.3. Mixing Experiments

Efforts to identify a "phasing substance" for the circadian clock have been carried out in a number of organisms, including *Euglena* and *Gonyaulax*, by mixing together cultures previously entrained to different phases. All such experiments have failed to detect such a substance, as the phase or phases of the mixed culture always appeared to be equal to the sum of the phases of the individual pre-mixed cultures. A variation of this type of study has been undertaken in *Neurospora* by Woodward (personal communication). By constructing race tubes in the shape of a "Y", he was able to make heterocaryons in the dark between two strains which had previously been entrained to different phases. In such experiments, the heterocaryons ultimately express the phase of only one of the two strains rather than the sum or some average of the two, although during the first few days after heterocaryon formation, evidence of both phases often can be seen. These surprising results strongly suggest that a significant part of the *Neurospora* clock mechanism is compartmentalized. Furthermore, it was found that certain phases of the cycle are dominant over other phases, the dominant phases occurring primarily during the late subjective day and early subjective night. Coupled with the first observation, this result may indicate that a substance is produced at certain phases of the circadian cycle which can entrain other clock systems in the same cytoplasm. The possible existence of a phasing substance in *Neurospora* does not conflict with the negative results in other organisms, since in those experiments the substance might have been diluted by the culture medium or been unable to cross the plasma membrane of the cells.

Woodward's (unpublished) results are consistent with the observa-

tion that race tube cultures occasionally show two conidial bands during each circadian cycle, especially during the first several days after being placed in constant dark. Ultimately, the band at the "wrong" phase is lost and the culture resumes its normal banding pattern. It is possible that *Neurospora* mycelium contains a population of oscillators, that occasionally some of these oscillators assume a different phase relationship to the previous light-to-dark transition and induce a second conidial band, and that ultimately the dominant phase entrains these wayward clocks to the "correct" phase.

5. BIOCHEMICAL ANALYSIS

In addition to the analysis of clock mutants, two other approaches have been employed in efforts to elucidate the molecular mechanism of circadian rhythms. These concern the examination of circadian rhythms in organisms possessing known biochemical defects, and the use of inhibitors thought to be specific for known metabolic functions. In each case, the rationale is the same. If a metabolic function or biochemical pathway can be blocked either by genetic lesion or antibiotic inhibition without any resulting permanent change in the clock (as measured by period length or phase), then flow of metabolites through that pathway or the use of products of that function are not important to the operation of the clock within the timespan of the inhibition. There are few problems with this reasoning as stated and, as such, this method can and has been used to test distinct and specific models for the clock. Its converse, however, that positive genetic or inhibitor effects necessarily imply intimate involvement of a pathway or function in the oscillator, is certainly not true. The logical fallacies lie in the facts that few inhibitors are absolutely specific in their site of action and even when the primary site of inhibitor action or genetic lesion is specific and known, the results can often be at least partially pleiotropic. While methods exist for dealing with the first of these problems (Sargent *et al.*, 1976), the second must be considered inherent to the method of analysis. In this section, a brief summary of data describing elements not involved in the clock will be followed by data showing positive effects dealt with on an individual basis.

5.1. Nutritional Mutants—Negative Effects

Nutritional mutations which, when fully supplemented, have been shown to be without effects on the biological clock are listed in Tables 1 and 2. There are few surprises here and the data are included mostly for the sake of completeness. Due to the nature and results of the experi-

TABLE 1. Nutritional Mutations in *Neurospora crassa* Not Affecting Clock Function

Class of mutants	Metabolite	Strain	Reference
Amino acid biosynthesis	Tyrosine	<i>try-1</i>	Brody and Martins, 1973
	Histidine	<i>his-3</i>	
	Tryptophan	<i>trp-3</i>	
		<i>nt</i>	
	Methionine	<i>met-7</i>	Feldman and Widelitz, 1977
	Arginine	<i>arg-10</i>	
	Serine	<i>ser-3</i>	
	Serine	<i>ser-5</i>	
	Ammonia	<i>am²</i>	Dunlap, unpublished
		<i>nit-1²</i>	J. Paietta and M. L. Sargent (1982a)
Nitrogen assimilation	Nitrate	<i>nit-3</i>	
	Pantothenic acid	<i>pan-1</i>	Feldman, unpublished ^{a,b}
	Nicotinic acid	<i>nit-2</i>	S. Brody, personal communication
Enzyme cofactor biosynthesis	Pyridoxine	<i>nic-3</i>	
	Choline	<i>pdx-1</i>	
	Inositol	<i>chol-2</i>	S. Brody, personal communication
		<i>inos (inl)</i>	H. Nakashima, unpublished
Fatty acid precursors or membrane biosynthesis	Ergosterol	<i>erg-1</i>	
	Sucrose	<i>erg-3</i>	
		<i>inv</i>	Sargent and Woodward, 1969
		<i>acu-1</i>	Halaban and Feldman, 1973a
		<i>acu-3</i>	
Carbohydrate metabolism	Acetate	<i>acu-5</i>	
		<i>acu-6</i>	
		<i>acu-7</i>	

^a Also no effect on clock function when concentrations of supplement are lowered to the point where growth is appreciably slowed.

^b Also not involved with light induced phase resetting of the clock.

ments, it is probably also not a complete list; negative data are often not published. In nearly all of these cases (see however footnote *a*, Table 1), the mutant was supplemented with the deficient metabolite so that growth, and presumably intracellular levels, returned to normal. These studies thus establish only that the normal uninterrupted flow of metabolites through these pathways can be eliminated as being involved in the clock. It is perhaps of interest that these include the glyoxylate, Krebs and urea cycles. The conidiation rhythm, and indeed the rate of growth itself, is also clearly not tied to the clock in any sort of feedback mechanism. With the exception of methionine, arginine, serine, and pantothenate, however, it has not been established that a significantly lowered level of a metabolite

TABLE 2. Morphological Mutations Not Affecting Clock Function in *Neurospora crassa*

Mutant designation	Phenotypic defect
<i>Band</i> (<i>bd</i>)	Sensitivity to CO ₂ inhibition of conidiation
Patch	Sensitivity to CO ₂ inhibition of conidiation
Albino (<i>al-2</i>)	Carotenoid synthesis
Fluffy	Septation and disarticulation of aerial hyphae
<i>Crisp-3</i>	Genesis of aerial hyphae
Conidiation (<i>csp</i>)	Cell wall autolyzing enzyme
Spreading colonial (<i>spco-6</i>) ^a	Unknown
Spreading colonial (<i>spco-9</i>) ^a	Unknown
Spreading colonial (<i>col-4</i>)	Unknown
Spreading colonial (<i>pile</i>) ^a	Unknown

^a Growth rate slowed sufficiently that small changes in period length of phase may not have been detected.

might not affect the clock. In this context, an interesting case in point deals with mutants in sulfur assimilation and cysteine biosynthesis.

5.2. Cysteine Auxotrophs

When some auxotrophs requiring sulfur compounds for growth were partially starved for this nutritional supplement, growth was slowed to 60% of wild type and more importantly, the period of the clock decreased by about 2 h, i.e., the clock speeded up (Feldman and Widelitz, 1977). These properties were originally detected in a new cysteine isolate, *cys-X*, and were later extended to previously isolated mutants *cys-4* (with which *cys-X* may be allelic) and *cys-12*. In each case, the growth rate (reductions up to 70%) and the period length (reductions up to 3 h) can be titrated by the amount of exogenously added organic sulfur (as cysteine, cystathionine, homocysteine, or methionine). In each case, however, the degree of growth inhibition and the extent of period shortening produced by the limiting sulfur are not tightly correlated. This suggests that the defect is not simply due to an unavailability of amino acids, or even the unavailability of sulfur-containing amino acids. This conclusion is borne out by the finding (Taylor and Feldman, 1982) that a qualitatively similar period shortening is seen when *arg-13* is grown on limiting arginine. However, when the auxotrophs *met-7* and *arg-10* are starved respectively for methionine or arginine, growth is slowed but the period length of the clock remains unchanged. A further complication lies in the observation that the period-shortening effects are seen only when cultures are using acetate rather than glucose as a carbon source (Taylor and Feldman, 1982).

These observations have recently been extended by analyzing the period effects resulting from genetic lesions at different points in the cysteine biosynthetic pathway (W. Taylor, personal communication). Serine auxotrophs *ser-3* and *ser-5* when grown on limiting serine still retain a normal period length despite decreased growth. Since cysteine is a condensation product (via cysteine synthase) of O-acetylserine and sulfide, serine limitation probably entails cysteine limitation. Therefore, the lack of period effects in this case suggests that the results seen with the cysteine mutants above cannot be a straightforward result of intracellular cysteine limitation, but rather must be due to abnormal cycling or levels of intermediates involved in sulfate reduction and assimilation. In this context, the possibility that it is the abnormal levels of intermediates (and their perception by the cell's regulatory machinery) that is causing the effect suggests that an examination of sulfur regulatory mutants (e.g., *scon*) could prove insightful.

5.3. Other Amino Acid Auxotrophs

Dieckmann (1980) noted in her controls for heterokaryon analyses that several different amino acid auxotrophs had altered period lengths even when fully supplemented. Of particular note were *leu-3* (period length of 23.1 h) and *arg-5* (25.4 h). A confirmation of these results and further analysis is certainly warranted.

5.4. Cyclic AMP

Adenosine 3'-5' cyclic monophosphate is phylogenetically ubiquitous and has been implicated in a variety of regulatory schemes in different organisms (Pastan, 1972). Furthermore, the enzymes responsible for cAMP metabolism, phosphodiesterase (PDE) and particularly adenylate cyclase (AC), are often membrane associated (Ross and Gilman, 1980) and therefore are in a good position to be central in importance to a cell's response to external stimuli (e.g., as with *Dictyostelium* [Bonner *et al.*, 1972] and hormone responses [Means and Dedham, 1980]). In *Neurospora*, intracellular levels of cAMP and the activities of PDE and AC have been shown to be of critical importance to the maintenance of normal cellular morphology (Scott, 1976a; Mishra, 1977 for review). It is thus inevitable that a possible involvement of cAMP metabolism in the biological clock be proposed (Cummings, 1975) and analyzed.

In the first reported experiments (Feldman, 1975), several different methylxanthine inhibitors of the *Neurospora* cAMP PDE were shown to

cause a dose-dependent period lengthening of the clock in *bd* and the period mutants *frq-1*, *frq-2*, and *frq-3*. The concentrations of inhibitors required to produce the effect (1–5 mM) did not result in any inhibition of growth and had previously been shown under nearly identical growth conditions (Scott and Solomon, 1973, 1975) to inhibit the PDE and thereby raise intracellular cAMP levels. In all cases, most effective was aminophylline followed by caffeine and theophylline; period lengthening of up to 5 h was obtained. While these data imply that the intracellular level of cAMP may be important to clock functions, the inevitable ambiguities surrounding inhibitor studies render the data only suggestive in this regard.

An analysis of the importance of steady state levels of cAMP to the normal functioning of the clock (Feldman *et al.*, 1979) was undertaken using the morphological mutant *crisp-1* which has greatly reduced levels of AC and cAMP (Terenzi *et al.*, 1974). Partial revertants of the tight colonial morphology were isolated so as to allow measurement of the rhythm in conidiation. Despite greatly reduced levels of AC (<1% of wild type) and intracellular cAMP (9.1% and 15.4% of wild type in two different revertant strains), the biological clock ran with a normal period length and could still be entrained to 12:12 light-dark cycle. Thus, the maintenance of wild-type levels of intracellular cAMP or AC are clearly not necessary for a functional clock.

The effects of pulses of drugs which interfere with cAMP metabolism were studied by Perlman (1981), who repeated and expanded Feldman's (1975) original work. In addition to the caffeine and aminophylline effects, isobutylmethylxanthine (IBMX), a synthetic PDE inhibitor (Beavo *et al.*, 1970), was also effective, albeit less so, increasing the period only about 2 h at equivalent concentrations. In each case, although growth was slowed at the high concentrations used, there existed no correlation between the drugs' growth-inhibiting and period-lengthening efficacy. Quinidine, an inhibitor of AC (Scott and Solomon, 1975), resulted in virtually no effect or slight shortening of the period length at drug concentrations which allowed sufficient growth to monitor the rhythm (up to 1 mM). Analysis of phase shifting by these drugs was accomplished utilizing the newly developed liquid culture system (Perlman *et al.*, 1981). Unexpectedly, 3 h pulses of aminophylline, caffeine, IBMX, and quinidine all yielded essentially the same curve of phase shift, vs. phase of application (equivalent PRC's). Delays in the late subjective night were followed by advances in the early subjective morning with the drugs' relative effectiveness being 5 mM IBMX (2- to 3-h phase shifts) < 10 mM aminophylline < 10 mM caffeine (4- to 5-h shifts) < 10 mM quinidine (8-h delays and 10-h advances). The effectiveness of quinidine is especially noteworthy con-

sidering its lack of effect on period length, although it should be noted that because the drug was added and then washed out a short time later, considerably higher concentrations could be used in these experiments. The validity of the advance and delay phase shift assignments was confirmed by dose response curves done in the midsubjective night and early morning. Delays of up to about 10 h (at 10 mM quinidine, 20 mM aminophylline, 40 mM caffeine) occurred in a dose dependent manner when cultures were pulsed 30 h after the light to dark transition, while advances of up to 5 h (10 mM quinidine) or 4 h (30 mM aminophylline or 40 mM caffeine) were seen at 14 h after the light to dark transition.

Taken *in toto*, it is probably reasonable to assume that the drugs are exerting their effects by their activity on the AC, PDE, cAMP system. Since the PRC effects are not well-correlated in time with response of the clock to light or temperature, it is unlikely that they are mimicking these physical phenomena. Caffeine has been shown to inhibit protein synthesis in *Neurospora* (Costantini *et al.*, 1978), and the time of sensitivity of the clock to inhibitors of protein synthesis is the same as that for caffeine. However, whereas cycloheximide (Nakishima *et al.*, 1981, J. Dunlap, unpublished) and anisomycin (J. Dunlap, unpublished) produced far larger advances than delays, caffeine resulted in equal and relatively small advances and delays. Nonetheless, this common mode of action must remain a possibility. Alternatively, if their action is via effects on cAMP, then the period lengthening data is most easily interpreted as meaning that increases in the intracellular levels of cAMP (resulting from the methylxanthine PDE inhibitors) cause the clock to run slower (period lengthening), while tonic decreases in the level below that of wild type (as with the *crisp-1* and quinidine), are essentially without effect. It should be noted here that the effects of these drugs and this mutation on *Neurospora* cAMP levels have previously been determined under conditions essentially identical to those used for the measurement of period length (Scott and Solomon, 1973, 1975; Scott, 1976b). Thus, there is little ambiguity as to whether they are having the desired effect; the only question is whether the clock effect is derived directly from the primary effect or from some secondary consequence of the initial inhibition.

The phase shifting data are somewhat more difficult to rationalize since in the steady state the drugs differ in their effects on cAMP levels. After 3–5 days growth in liquid culture, theophylline and quinidine act to lower cAMP levels, whereas caffeine raised its level (Scott and Solomon, 1975). The discrepancy with the two methylxanthines is due to the fact that in liquid culture they inhibit both AC and PDE (to different extents with each drug), so that the final result in each case is the differential of the two primary effects. Also, part of the explanation may lie

in the kinetics of the drugs' action. For example, another inhibitor (chloroquine; Scott and Solomon, 1975) acts *in vitro* to inhibit both AC and PDE but *in vivo* fails to fully penetrate the mycelia and thus acts like a drug affecting only AC and thereby decreasing intracellular cAMP. If caffeine were similarly slow in penetrating the cell, during a 3-h pulse it might be having its primary effect on the membrane-bound AC (which it would encounter first) rather than on the soluble PDE. Thus, short term exposure could lower intracellular cAMP whereas on the long term, intracellular drug concentrations would stabilize resulting in an enhanced level of cAMP. Verification of this possibility must await chemical analysis of cAMP during and following short drug pulses. Nevertheless, the simplest, albeit necessarily tentative, summary might be that phase shift advances and delays of approximately equal magnitude are brought about by transient decreases in the intracellular level of cAMP. The unification of this hypothesis with the somewhat paradoxical one involving period changes (that whereas tonic increases in intercellular cAMP result in period lengthening, tonic decreases are without effect) must await further study.

5.5. Fatty Acid Metabolism and Mitochondrial Function

5.5.1. The *cel⁻* Mutation

There has existed for quite some time in the study of biological clocks an interest in the possible involvement of cellular membranes (Njus *et al.*, 1974; Sweeney, 1974; Engelmann and Schrempf, 1980). This background, coupled with the hypothesis that the rhythm in intracellular AMP was derived from a rhythmic uncoupling of oxidative phosphorylation (Delmer and Brody, 1975), has prompted Brody and co-workers to undertake a more in depth examination of the involvement of fatty acid metabolism and mitochondrial function in the clock. Central to part of this work has been an examination of the effects of fatty acid supplementation on the clock of the partial fatty acid auxotroph *cel⁻* (Perkins *et al.*, 1962; Henry and Keith, 1971). Elovson (1975) showed this strain to possess a fatty acid synthetase complex deficient in the covalently bound 4'-phosphopantotheine prosthetic group (2% of wild type). It could not be determined whether the effect was due to a modification in the fatty acid synthetase complex itself (rendering it unable to accept the prosthetic group) or due to deficiency in the enzyme normally attaching the prosthetic group to the synthetase. Thus, although the phenotype of *cel⁻* is partial fatty acid auxotrophy (almost fully supplementable by pal-

mitic acid), the possibility remains that the fatty acid synthetase is not the only defective enzyme in this strain.

Measured at 21°C, the clock of strains carrying the *cel*⁻ mutation was normal although growth was slowed by 45% (Brody and Martins, 1979). Complete supplementation of this deficiency with palmitate (16:0) or stearate (18:0) did not alter clock function, but attempts to supplement growth with oleate (18:1), linolenate (18:3), or linoleate (18:2) resulted in extraordinary increases in period length (up to 26 h, 33 h, and 40.5 h respectively). The effect was proportional to the amount of supplement added and could be reversed by the addition of nonstoichiometric amounts of 16:0 in excess of the unsaturated fatty acids. Controls showed no effect of supplementation on wild-type strains and, importantly, full entrainment of even 40-h rhythms by 12:12 light-dark cycles (Brody and Martins, 1979). This was of particular importance since the long period hyphal branching rhythm is known to be susceptible to nutritional manipulation, but is not entrainable to light-dark cycles (Feldman and Hoyle, 1974).

Mattern and Brody (1979), extended these studies to saturated fatty acids. When *cel*⁻ was supplemented, the clock functioned normally during the first four days of growth, but on subsequent days a significant period lengthening was noted. Its magnitude was dependent upon the length of the fatty acid carbon chain in the supplement; chain lengths of 8:0 to 13:0 gave the effect, with a maximum of 33 h seen with 9:0, while no change was seen with 14:0 or longer. As with the unsaturated fatty acids, the period lengthening was fully reversible by adding excess 16:0, lengthened clocks were fully entrainable to light-dark cycles, and there was no effect of supplementation on *cel*⁺ (wild-type) strains. In all cases with *cel*⁻, fatty acids affecting the period length actually inhibited the already slowed growth of the auxotroph, rather than acting as supplements. This lack of phenotypic correction suggested that the supplements were not being incorporated, a conclusion borne out by careful analysis of the medium and culture itself. Significantly, there was no statistical difference in uptake of fatty acids between *cel*⁺ and *cel*⁻, and incorporation studies using radiolabeled 12:0, showed that only 2–5% of the label actually appeared as membrane lipid, the remainder being metabolically transformed (3–11%) or oxidized to CO₂ (>85%). In other words, the *cel*⁻ mutation must somehow only render the clock susceptible to modification by fatty acids since *cel*⁻ and *cel*⁺ both utilize the supplements to a similar extent, but the clock is modified only in *cel*⁻. It was not determined, however, whether there was a change in the evolution of CO₂ by *cel*⁻ concurrent with the appearance of the period lengthening effect at 4–5 days. This remains an interesting possibility.

Recently much of the physiology data has been unified by obser-

vations on the effect of temperature on the supplementation induced period effects (Mattern *et al.*, 1982). The period length of *cel⁺* is approximately the same from 18–30°C, despite the large changes in the rate of growth, i.e., it is temperature compensated. *Cel⁻* has lost this compensation below 22°C, although supplementation with 14:0 or 16:0 returns the compensation to normal. Supplementation with either short chain fatty acids (as exemplified by 12:0) or long chain unsaturated fatty acids (18:1, 18:2, or 18:3) merely exacerbates the deficiency, i.e., they render altered compensation even worse, such that 12:0, 18:2, and 18:3 cultures are not compensated below 26°C and 18:1 cultures not at all, regardless of the temperature. Another way of stating this is to say that the supplementation effects are temperature dependent such that at some temperature for any supplement the clock will run with a normal period length.

Taken *in toto*, these data are important for a number of reasons. First of all, it should be noted that single gene mutants have previously been isolated, affecting at the same time both period length and temperature compensation (Gardner and Feldman, 1981). Thus, compensation may be a direct result of the oscillation mechanism itself rather than an external characteristic coupled to the oscillator. These *cel⁻* studies then demonstrate the alteration of a fundamental clock property by a known (at least knowable) biochemical lesion which is furthermore fully supplementable. Second, the supplementation effects are seen as a result of the addition of normal cellular constituents, not antimetabolites, activators, or inhibitors. Finally, the supplemented *cel⁻* cultures display an apparent full loss of temperature compensation without a loss of the rhythm itself. Nevertheless, several questions remain to be answered. Incorporation studies have apparently eliminated the straightforward hypothesis that the fatty acids were acting directly via incorporation into membranes with resulting changes in membrane fluidity. Remaining possibilities include the effect being due to a localized membrane change such as might affect the activity of the ATP synthetase complex or separately to the metabolism of the supplement. In either case questions remain regarding what protein or proteins are involved.

5.5.2. Oligomycin-Resistant Mutants

From among the several possible answers to these questions, Brody and co-workers have proposed that the effect is due to modification of the activity of an integral membrane protein by a fatty acid-induced localized membrane effect. Coincidentally, one such protein complex, the mitochondrial ATP synthetase (at least in yeast), contains a 4'-phospho-

pantothenate prosthetic group (Criddle *et al.*, 1977), the same moiety found to be deficient in the fatty acid synthetase complex from *cel⁻* mutants. For this reason, and because of the hypothesized rhythm in mitochondrial oxidative phosphorylation uncoupling (Delmer and Brody, 1975), they have chosen to concentrate on subunit 9 of this ATPase complex, an extremely hydrophobic protein of M_r about 8000 which, when mutated, can render *Neurospora* resistant to the antibiotic oligomycin. Several *oli^r* mutations have been independently isolated in two different laboratories (Sebald *et al.*, 1977; Edwards and Unger, 1978); Dieckmann has analyzed the genetics and clock properties of strains carrying this mutation.

When the oligomycin-resistant mutants of Edwards and Unger (1978) were examined for rhythmicity, period lengths were found to be reduced by 2–3 h at 22°C (Dieckmann and Brody, 1979, 1980) but essentially not at all at 26°C or 30°C (Dieckmann, 1980). More recently, Brody (1981) has found the same effect at 22°C using Sebald's (Sebald *et al.*, 1977) mutants where, furthermore, the degree of period shortening was correlated with the extent of oligomycin resistance. No data were given regarding the dependence of this correlation with period shortening upon temperature. Additional experiments showing that (1) period shortening and oligomycin resistance cosegregate in crosses and (2) revertants of *oli^r* to oligomycin sensitivity have normal period lengths (Dieckmann and Brody, 1980) provide strong evidence that the change is not due to specific strain, background, or nutritional effects. These results clearly implicate mitochondrial function, and in particular the F_o portion of the mitochondrial ATP synthetase, in clock function.

Preliminary genetic mapping studies reported that a particular *oli^r* mutant, *oli* 16-3, mapped 0.43 map units distal to *frq-1* on chromosome VII, a finding that placed the two genes within 16,000 base pairs of each other (making appropriate assumptions regarding recombination frequencies and the size of the *Neurospora* genome; Perkins and Barry, 1977). This led to the suggestion (Dieckmann, 1980; Dieckmann and Brody, 1980) that *frq-1* and *oli^r* might be allelic and might code for the same, or possibly separate, subunits of the mitochondrial ATP synthetase.

In order to test this prospect, Dieckmann (1980) undertook to examine more carefully the physiology of mutants at the *frq* locus and also the behavior of such mutations in heterokaryons or double-mutants in association with *oli^r*. If *frq* and *oli^r* were coding for the same biochemical function, the effects of the two mutations on period length would probably not be additive (Feldman *et al.*, 1979; Gardner and Feldman, 1981). However, in three out of four cases the period lengths of the double mutants *oli^r,frq-1* were shorter than either parent and showed additivity. (In the

fourth case, where the *frq-1,oli^r* double mutant had a period length similar to *frq-1* alone, the particular *oli^r* lesion used did not shorten the period length as a single mutant.) Therefore, these results tended to support the idea that *frq-1* and *oli^r* are separate, albeit closely linked, genes. Complementation studies with heterocaryons yielded equivocal results and have not yet helped to resolve this question. However, a possible relationship between *frq* and *oli* came from the observation that *frq-1* (but not *frq-2* or *frq-3*) appeared to be slightly more resistant to the effects of oligomycin on linear growth rate than wild type (Dieckmann, 1980).

These questions have recently been reexamined in more detail (A. Richman and J. Feldman, unpublished experiments). With the use of closely linked outside markers, the equivalent of more than 2500 progeny from each of four crosses between each of two *oli* alleles (*oli* 16-1 and *oli* 16-16) and each of two *frq* alleles (*frq-1* and *frq-7*), have indicated that (1) *oli* is proximal to *frq*, not distal, and (2) that the two genes are about two map units apart. Furthermore, double mutants between each of the *frq* and *oli* alleles have all shown additive effects on circadian periodicity. Furthermore, the effects of oligomycin on the growth rate of all seven *frq* mutants in liquid culture has shown no detectable resistance of these strains to oligomycin. It seems, therefore, likely that *frq* and *oli^r* are separate genes. However, the final resolution of this issue must await either conclusive data from heterokaryons or actual physical analysis of the *frq* and/or *oli^r* genes.

It will be recalled that the fatty acid synthetase complex and the F_o portion of the mitochondrial ATP synthetase share in common the 4'-phosphopantothenate prosthetic group found to be lacking in the fatty acid synthetase of *cel⁻* mutants. It was thus of interest to examine the interaction of *oli^r* and *cel⁻* mutations on period length. Brody and Forman (1980) noted that supplementation of *cel⁺*, *oli^s*, i.e., wild-type, strains with linoleic acid (18:2, the one causing the greatest period lengthening effect in *cel⁻* strains) conferred partial resistance to oligomycin. Furthermore, when the *oli^r* mutation was crossed into *cel⁻* strains, the latter were rendered insensitive to the period lengthening effects of 18:2. More recently, Lakin-Thomas and Brody (1981) have reported that clocks of *cel⁻* strains lengthened by supplementation with 18:2 can still be further lengthened or shortened by the simultaneous presence in the genome of different *frq* mutations, i.e., the period length of *frq-1, cel⁻* supplemented with 18:2 is shortened compared to supplemented *frq⁺, cel⁻* by the same amount that *frq-1* is shortened as compared to *frq⁺*. This strict additivity (S. Brody, personal communication) is seen with two of the short period mutants (*frq-1* and *frq-2*) but is lost to varying degrees in the long period

mutants *frq-3* and *frq-7*. It is interesting to note that temperature compensation is also lost to varying degrees in these two long periods *frq* mutants (Gardner and Feldman, 1981). This serves as a further suggestion of the link between fatty acids and temperature compensation already noted (Mattern *et al.*, 1982). A clock mutation at a different unlinked locus (*prd-1*) conferred resistance in *cel⁻* strains to the period lengthening effects of 18:2, just as had *oli^r* above, i.e., *cel⁻,prd-1* supplemented with 18:2 has the same period length as *prd-1*, irrespective of supplementation with 18:2. Thus, the *oli^r* and *prd-1* mutations are either interacting with *cel⁻* or overriding it in such a way as to correct the clock phenotype deficiency.

In summary, while there is a great deal of genetic and physiological data dealing with fatty acids and mitochondrial function, it is somewhat difficult to unite all of it in a simple model. One focus is that supplementation of the fatty acid auxotroph *cel⁻* (but not *cel⁺*) with either short or unsaturated long chain fatty acids results in a significant slowing down of the clock. Incorporation studies show that in neither strain is there sufficient incorporation of the supplement to significantly change the composition of the organism's mycelial lipids (Henry and Keith, 1971; Mattern and Brody, 1979). Thus, the effect must be due either to differences in metabolism of the supplement or to very localized membrane effects of long chain unsaturated fatty acids on a particular enzyme or complex. Brody and colleagues favor the latter possibility and furthermore theorize that the primary clock effect is due to a localized effect of the F_o (membrane associated) part of the mitochondrial ATP synthetase. Oligomycin resistant strains are theorized to have mutated so that the activity of this F_o portion, in addition to having become oligomycin resistant, is also (1) rendered insensitive to the localized effects of long chain unsaturated fatty acids which caused period lengthening in *cel⁻* strains and (2) confers upon the organism a fast running clock at temperatures below 25°C. Correlated with this model are data showing that (1) 18:2 confers resistance to oligomycin in *cel⁻* or wild type (Brody and Forman, 1980); (2) the degree of period lengthening is correlated with the extent of oligomycin resistance (Brody, 1981); and (3) supplementation of *cel⁻* with excess 16:0 relieves the period lengthening effects of 18:2 supplementation (Brody and Martins, 1979). A second focus of the data centers on temperature compensation and temperature effects in that (1) *cel⁻* strains have lost temperature compensation below 22°C (Mattern *et al.*, 1982); (2) supplementation of *cel⁻* with short chain saturated fatty acids extends to high temperatures the range over which temperature compensation is lost (Mattern *et al.*, 1982); and (3) *oli^r* results in period shortening at 22°C

or lower but has no effect on period length (although still conferring resistance and slow growth) at temperatures of 25°C or above (Dieckmann, 1980).

While not necessarily contradictory, the following data await further explanation or reconciliation with this scheme: (1) the lack of period effects in unsupplemented *cel*⁻ strains above 20°C despite grossly altered membrane and lipid composition and, in particular, a large change in the ratio of saturated to unsaturated fatty acids (Henry and Keith, 1971); (2) the lack of period effects in supplemented *cel*⁺ (wild-type) strains despite uptake and incorporation of the supplement equal to that seen in *cel*⁻ strains (Brody and Martins, 1979; Mattern and Brody, 1979); (3) if the degree of oligomycin resistance is correlated with period shortening (Brody, 1981) then *frq* and *oli*^r cannot be in the same gene, since none of the *frq* mutants shows a significant level of oligomycin resistance; (4) why the *oli*^r mutation results in period shortening while supplementation of the *cel*⁻ strain results in period lengthening if in each case the lesion is causing partially altered function of the mitochondrial ATP synthetase; and (5) why the clock in unsupplemented *cel*⁻ strains is lengthened below 22°C where the clock of *oli*^r is shortened, if in both cases the molecular change is in the same or functionally related proteins. The continued evolution of this area of research is certain to prove interesting, particularly with regard to resolving whether the clock effects are due to the ATPase itself or rather, in some secondary and pleiotropic way, to the changing of intracellular ATP levels and energy charge. In this context, it is important to note that even large and sudden bursts in respiratory activity (CO₂ evolution and O₂ consumption), such as occur when 48-h starved *Neurospora* are presented with respirable carbon sources (glucose or ethanol), fail to result in any shifting of the clock phase (Nakashima, 1981). Thus, while the data of Brody and colleagues seem to indicate an importance of the level of mitochondrial function, these data suggest that neither constant levels of electron transport, nor presumably oxidative phosphorylation (via the mitochondrial ATP synthetase) are required for normal operation of the clock.

5.5.3. Fatty Acid Content

A different but somewhat related approach has been used by Nakashima (personal communication) in the study of the involvement of membranes in the clock. In analyzing the phospholipids from strains carrying different clock mutations at the *frq* locus, he found a small but reproducible difference in the ratio of linolenic to linoleic acids (18:3 to

18:2) between the long period mutants (*frq-7* or *frq-8*) and any of the others (or *bd*). Furthermore, with increasing temperature, the ratio of 18:3 to 18:2 fell, decreasing especially fast above 30°C. Initially, these experiments seemed quite exciting since both cases (*frq-7* or *8* and temperatures above 30°C) showing reduced ratios were ones where a partial loss in temperature compensation had been noted (Gardner and Feldman, 1981). Significantly, however, these changes were proven to be correlative rather than causal by the observation (H. Nakashima, personal communication) that low concentrations of phenethyl alcohols can cause gross changes in membrane phospholipid composition with no significant concomitant changes in clock function. This interesting study, then, may serve as a cautionary note concerning the extent to which causal relationships can be inferred from biochemical correlations.

5.5.4. Inhibitors of ATPases

The relative importance of the plasma membrane ATPase (as opposed to the mitochondrial ATP synthetase) to the phase shifting of the clock was also examined by Nakashima (1982b). Diethylstilbestrol (DES) and related compounds (dienestrol, DIE; hexestrol, HEX; diethylstilbestrol-dipropionate, DESP; dienestroldiacetate; DIEA) display different dose response curves with respect to the inhibition of the plasma membrane ATPase versus whole cell O₂ consumption (as a measure of the ATP synthetase). It was thus hoped that an examination of their relative efficacies in phase shifting the clock would reveal a pattern implicating one but not the other enzyme. DES, DIE, and HEX cause large phase shifts while DESP and DIEA were largely without clock effect. Unfortunately, DIE resulted in near maximal phase shifting at concentrations (50 μM) where there was essentially no detectable inhibition of the plasma membrane ATPase *in vitro*. And, while DES, DIE, and HEX were all indistinguishable in their inhibition of O₂ consumption *in vivo* (from 30–60% between 10 and 30 μM), DES was about five times more effective in phase shifting the clock. Thus, on the assumption that measurement of intracellular ATP levels in the presence of the drugs corroborates the O₂ consumption data, neither enzyme can be thought to be wholly responsible for the phase shifting effect.

A similar effort to distinguish between the action of the plasma membrane and mitochondrial ATPase relied on the use of selective inhibitors. Azide is a specific inhibitor for the mitochondrial ATPase, vanadate is specific for the plasma membrane enzyme, and DCCD (*N,N'*-dicyclohexylcarbodiimide) acts on both proteins (Bowman *et al.*, 1978). Results of recent experiments, however, show that all three inhibitors are capable

of causing phase shifts (N. Koyama, unpublished; R. Schultz, unpublished), and support the belief that phase shifting may be a result of inhibition of the action of either ATPase.

5.6. Inhibitors of Membrane Function and Energy Metabolism

Nystatin is a polyene antibiotic whose mode of activity requires interaction with membrane steroids (Gottlieb and Shaw, 1970). In *Neurospora*, after treatment with nystatin, most of the antibiotic is found in the plasma membrane where the primary steroid is ergosterol. The steroid requirement for the action of nystatin has been exploited for the isolation of ergosterol deficient mutants in *Neurospora* (Grindle, 1973), and has prompted a preliminary analysis of the importance of this aspect of membrane structure and function to the clock.

Two ergosterol deficient mutants, *erg-1* and *erg-3*, are respectively ten times and two times more resistant to nystatin than wild type as judged by their linear growth rate on agar in the presence of the drug. However, both mutants have normal biological clocks at 25°C (H. Nakashima, personal communication; N. Koyama, unpublished). Pulses of nystatin result in dose, pulse duration, and phase dependent shifting of the wild-type (*bd*) clock, with 3-h pulses yielding phase delays (16 h) in the late night and advances (3 h) in the early morning. These phase shifts are significantly attenuated in the ergosterol deficient mutants, with *erg-1* being even more resistant than *erg-3* (N. Koyama, unpublished). These results strongly suggest that the nystatin phase shifting effect is mediated through the binding and interaction of nystatin with plasma membrane ergosterol. Since treatment with nystatin results in the loss of K⁺ from *Neurospora* (Slayman and Tatum, 1964), it was suggested that the K⁺ ionophore valinomycin might also cause phase shifts. This was confirmed by finding that 3-h pulses of valinomycin, like nystatin, cause phase shifts in a dose- and phase-dependent manner and result in a PRC of very similar shape to that seen with nystatin (N. Koyama, unpublished). One possible interpretation of these results may be that both nystatin and valinomycin are acting to promote the loss of potassium from *Neurospora*, thereby causing a depolarization of the plasma membrane which results in a phase shift. Since the phasing of these PRC's are not dissimilar to those seen with pulses of visible light, it may be that phase shifting by light could be given a similar mechanistic interpretation. However, the validation of such speculations must await further work on the biochemical effects of these inhibitors.

Since valinomycin is known to affect mitochondrial function, another

possible interpretation is that valinomycin is exerting its effect there. Consistent with this possibility is the observation that cyanide and dinitrophenol, two known inhibitors of electron transport and oxidative phosphorylation, are capable of phase shifting the *Neurospora* clock (N. Koyama, unpublished). Another effect of nystatin treatment is the inhibition of cellular protein synthesis (Gottlieb and Shaw, 1970). However, it seems unlikely that its clock effect is being exerted in this way, since the phasing of the PRC's induced by inhibition of protein synthesis (see Section 5.7) differ from those induced by nystatin treatment (N. Koyama, unpublished).

5.7. Macromolecular Synthesis

The first experiments demonstrating phase shifting (Karakashian and Hastings, 1963; Feldman, 1967a) and period lengthening (Feldman, 1967b) of biological clock cycles with inhibitors of eucaryotic cytosolic protein synthesis are now 15–20 years old. While some early experiments (Sargent, 1969) in which race tube cultures of *Neurospora* were grown in the presence of protein synthesis inhibitors seemed to indicate that the *Neurospora* clock was insensitive to these drugs, this apparent lack of effect can be attributed to interference of the drugs with the growth required for the expression of the rhythm. Thus, the fact that the generalized phenomenon of protein synthesis inhibitor action on biological clocks (Jacklet, 1981) has been extended to *Neurospora* (Nakashima *et al.*, 1981a) comes as little surprise. However, aspects of the genetics and biochemistry of *Neurospora* allow additional types of analysis of this effect and serve to emphasize the unique aspects of this organism as a clock system.

Short (2–4 h) pulses of the 80 S ribosomal protein synthesis inhibitor cycloheximide can result in large phase shifts of the *Neurospora* clock. Maximum sensitivity to phase shifting is in the early to mid-subjective day; small phase delays (up to 6 h) are followed in time by large advances (up to 15 h), both of which act to shift the clock to phases in the early to middle subjective night (the time at which pulses have little or no effect on the clock). This general pattern of phase resetting is the same at 20°C and 25°C and continues to cycle for at least three days under conditions of constant light and temperature and carbohydrate starvation (Nakashima, 1981).

Unambiguous interpretation of inhibitor studies has often proved difficult in previous work on the clock (Sargent *et al.*, 1976). For example, Frelinger *et al.* (1976) found that chloramphenicol, an inhibitor of mitochondrial protein synthesis, shortened the period length of the conidiation

rhythm when added to the medium of race tube cultures. However, they found that both the L(+) and D(−) optical isomers of the drug had the same effect on periodicity but only the D(−) isomer significantly inhibited mitochondrial protein synthesis. Furthermore, tetracycline, another inhibitor of mitochondrial protein synthesis, did not alter the period length of the rhythm. The authors concluded that the effect of chloramphenicol on clock periodicity was not related to its inhibition of protein synthesis but rather to some other effect such as interference with electron transport by a destabilization of the mitochondrial membrane.

However, in the case of cycloheximide, several independent lines of evidence support the notion that the effect of this drug is due directly to an inhibition of 80s protein synthesis rather than to some other effect. Phase shifting is seen both with cycloheximide and with the structurally dissimilar inhibitor anisomycin (J. Dunlap, unpublished). In each case, dose response curve threshold and saturation levels were approximately the same for both inhibition of protein synthesis and phase shifting (Nakashima *et al.*, 1981a; J. Dunlap, unpublished). The strongest evidence that the effect is actually due to protein synthesis inhibition comes from the finding (Nakashima *et al.*, 1981b) that, in single gene ribosomal mutants whose protein synthetic machinery is partially resistant to cycloheximide, the resistance to phase shifting closely paralleled the resistance to protein synthesis inhibition. Since the genetic backgrounds of the strains were otherwise the same, the phase shifting cannot be due to secondary effects of cycloheximide mediated via cellular processes other than protein synthesis. Thus, either the action of making proteins or the appearance of certain proteins themselves is important to the normal operation of the clock at certain specific stages in its cycle.

Some further insight into this question has been gained by the comparison of cycloheximide induced phase shifting in clock mutants (at the *frq* locus) and nonclock mutant (*bd*) strains (Dunlap and Feldman, 1982). In the short period and intermediate long period mutants (*frq-1*, *frq-4*, *frq-3*), phase shifting by cycloheximide appears to be normal (when compared to *bd*) with concentration-dependent resetting at approximately the same subjective clock times and no major differences in the response between 25°C and 30°C. The long period mutant *frq-7*, however, appears to be relatively insensitive to phase shifting by cycloheximide at both 25°C and 30°C. Drug concentrations even ten-fold higher than those resulting in 95% inhibition of synthesis and causing 10- to 15-h advances in *bd*, produce at most 2–3 h shifts during the sensitive phase in *frq-7*. The typically smaller delay shifts are also attenuated, although somewhat less so. Cycloheximide pulses given at different times in the cycle show that the sensitive phase has not simply moved from early morning to some other time in the cycle, and dose-response curves done at three different

times show that the inhibition of protein synthesis itself by cycloheximide was not altered in *frq-7*. This novel and completely unexpected result suggests that we may need to reassess our ideas concerning the manner in which protein synthesis-mediated phase shifting occurs. The clock in *frq-7* strains has a long period and is partially deficient in its temperature compensation (Gardner and Feldman, 1981), but nonetheless is functional in controlling conidiation and is fully entrainable by light-dark cycles. The possibility of a connection between protein synthesis and temperature compensation is thus intriguing. These results further suggest that in *frq-7*, the normal clock requirement for the synthesis of certain protein(s) at specific times in the cycle has apparently been eliminated, either by bypassing entirely the need for such proteins or by increasing their level (for instance by changes in synthesis or degradation rates) such that they need not be synthesized at a particular phase of the cycle.

The proposal that protein synthesis may somehow be important to the operation of the wild-type clock immediately suggests that RNA synthesis may also be important. Previous experiments in the clock field have been somewhat ambiguous in this regard (Sargent *et al.*, 1976), with many of the more straightforward results coming from one rather anomalous organism (the giant algal cell *Acetabularia*) which is known to have extremely long-lived messenger RNA and which can live for weeks at a time without a nucleus. We have begun a re-examination of this question in *Neurospora* using the antimetabolite 6-methylpurine (J. Dunlap, in preparation). Three-hour pulses of this compound given through the circadian cycle result in small (up to 6 h) phase shifts; the time of maximum sensitivity occurs about 3 h before the sensitive phase for cycloheximide. Since 6-methylpurine inhibits total RNA synthesis by lowering precursor pool sizes (Pendyala *et al.*, 1979), its effects may be somewhat pleiotropic so that caution must be exercised in interpretation of these results. Still, that 6-methylpurine does inhibit RNA synthesis and that the daily sensitive period occurs a few hours before the sensitive time for protein synthesis both suggest a possible requirement for RNA synthesis by the clock at certain times during the day. More work is required to evaluate this possibility, and in this regard the availability of mutant strains resistant to the primary effect of 6-methylpurine (Pendyala *et al.*, 1979) should prove helpful, as was the case with protein synthesis.

6. SUMMARY

Neurospora crassa now offers a wide range of genetic, physiological, biochemical, and molecular biological approaches that can be applied to the study of circadian rhythmicity. Clock mutants have been isolated at

six different loci and show a variety of alterations in their responses to light and temperature. One locus, called *frq*, appears to play a key role in the organization of the circadian clock, and experiments to isolate and clone this locus using recombinant DNA techniques are well underway, while mutants at several other loci offer similar opportunities. Such experiments could ultimately lead to the identification of the primary gene product encoded in a "clock gene" and to its role in the mechanism of circadian rhythmicity.

Significant progress has also been made toward identification of the photoreceptor for the *Neurospora* clock, which appears to be a b-type cytochrome coupled to a flavin moiety in the plasma membrane. If correct, this would direct attention to electron transport, membrane potentials, and ion (proton?) gradients as key elements in the mechanism of light entrainment and phase shifting of the clock. Altered clock behavior in mutants defective in ATP synthesis and fatty acid metabolism have also led to the suggestion that mitochondrial energy metabolism plays a significant role in the operation of the clock, while inhibitor studies have offered strong evidence that macromolecular synthesis is also an important element in clock function.

It appears, then, that *Neurospora* offers a unique opportunity to combine the strengths of a number of different approaches into a truly interdisciplinary analysis of the cellular and biochemical basis of circadian rhythmicity. While it seems likely that the molecular interactions that regulate the *Neurospora* clock will not be identical to those in other organisms, especially higher eucaryotes, it seems important at this stage of circadian clock research to focus on a limited number of systems in order to learn as much as possible about a single organism. In this regard *Neurospora* appears to offer many advantages as a model system. The progress made during the past ten years offers significant hope for continued advances during the coming decade.

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