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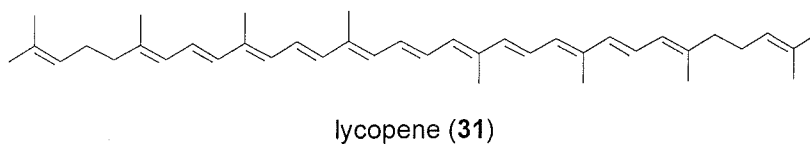
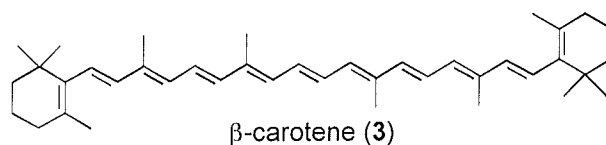
Chapter 9

Excited Electronic States and the Photochemistry and Photophysics of Carotenoids

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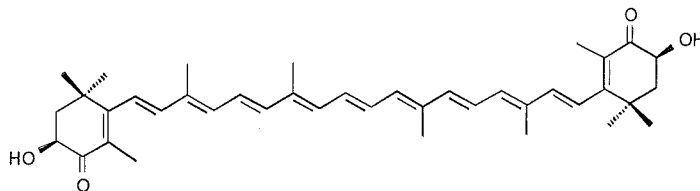
A. Introduction

The most striking characteristic of carotenoids is their palette of colours. Absorption of light in the visible region of the electromagnetic spectrum by molecules such as β -carotene (**3**) and lycopene (**31**) not only readily accounts for their colours but also signals the ability of these long-chain polyenes to serve as antenna pigments in diverse photosynthetic systems [1-4].



The absorption spectra of some carotenoids and related compounds undergo significant energy shifts upon binding to proteins and form the basis for biological coloration, *e.g.* astaxanthin

(406) in the lobster carapace (α -crustacyanin, *Chapter 6*) and retinal in vision (rhodopsin *etc.*, *Chapter 15*).



astaxanthin (406)

The spectra of β -carotene and lycopene given in Fig. 1 illustrate the absorption properties of carotenoids. The distinctive, strongly allowed absorption band that appears between 400 and 550 nm is responsible for the characteristic yellow, orange and red hues of carotenoids in solution and in biological matrices. These absorptions provide an important diagnostic for probing carotenoid photophysics and photochemistry.

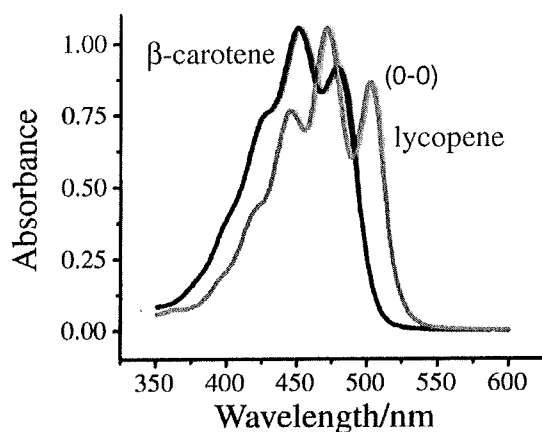


Fig 1. Room temperature absorption spectra of β -carotene (3) and lycopene (31). The electronic origin of lycopene is labelled (0-0).

Photosynthesis and other biological functions of carotenoids that are based on light absorption and energy transfer and transduction are covered in other chapters in *this Volume* and in some Chapters of *Volume 5*. This Chapter will focus on the absorption and fluorescence properties of carotenoids, including practical considerations central to the successful implementation of the methodology, and on the fundamental photochemistry and photophysics that underlie the natural functions.

B. Conceptual Background and Terminology

In order to understand the molecular details of the photochemistry and photophysics of carotenoids, it is important to establish the basic principles and terminology. A brief introduction is provided here. For a more comprehensive discussion of these terms, the reader is urged to consult any of a number of available texts on molecular photochemistry (*e.g.* [5]). The electronic structure of carotenoids and other polyenes is treated in detail in *Volume 1B, Chapter 1*, and the application of UV/Vis spectroscopy and spectrophotometry for the identification and quantitative analysis of carotenoids, with emphasis on the relationship between chromophore structure and absorption spectrum, in *Volume 1B, Chapter 2*.

1. Electronic structure and electronic states

(i). The **electronic structure** is a description of the distribution of electrons in a molecule [see *Volume 1B, Chapter 1*]. Electrons in low-energy (π) and high-energy (π^*) delocalized orbitals formed from a combination of $2p_z$ atomic orbitals are important in determining the photochemical properties of polyenes and carotenoids.

(ii). The **ground electronic state** is the electronic structure that gives the lowest possible electronic energy of a molecule. For carotenoids this is a singlet state, designated S_0 .

(iii). An **excited electronic state** is a higher energy electronic state of a molecule. For carotenoids this occurs by promotion of an electron from a low-energy molecular orbital to a higher one, by absorption of light. The excited states thus produced are normally also singlet states (S_1 , S_2 , *etc.*).

(iv). A **singlet state** is an electronic state of a molecule in which all the electronic spins are paired so that no net spin angular momentum exists.

(v). A **triplet state** is an electronic state that has two unpaired parallel electronic spins. A triplet state thereby has biradical character and may be detected and studied by EPR (Electron Paramagnetic Resonance) techniques. It is difficult to produce the lowest energy triplet state, T_1 , of a carotenoid by direct absorption of light into that state. Instead, it is usually formed by energy transfer from another triplet species, *e.g.* triplet chlorophyll, or by intersystem crossing, the radiationless interconversion of singlet states and triplet states.

(vi). The energy levels of a molecule may change in response to the application of an external electric field. This is known as an **electrochromic** or **Stark effect**.

2. Electronic transitions

(i). A change in the electronic state of a molecule is known as an **electronic transition**. It is typically brought about by the radiative processes of absorption or emission of light, but can also occur by non-radiative thermal processes. In carotenoids, a transition typically involves the promotion of an electron from a low-energy π orbital to a higher energy π^* orbital, or *vice versa*. The lowest energy excited configuration is generated from the ground state by promoting one electron from the highest-energy occupied molecular orbital (HOMO) to the lowest-energy unoccupied molecular orbital (LUMO), i.e. a **HOMO to LUMO transition**, subject to selection rules.

(ii). The transitions are governed by selection rules, determined by symmetry considerations. The overall symmetry of all-*trans* carotenoids, characterized by a collection of **symmetry elements**, places them in the C_{2h} point group. The electronic state of a molecule can be classified as A or B, according to elements of the symmetry of its electronic distribution. A states show rotational symmetry, B states do not.

(iii). **Selection rules**: for most practical purposes, excitation by light absorption is possible if the transition involves a change in the symmetry designation $g \leftrightarrow u$ and a change in the pseudoparity sign $- \leftrightarrow +$. Transitions which do not involve such changes are forbidden. As discussed in Section C below, the transition from the ground state S_0 ($1^1A_g^-$) to the lowest energy excited state S_1 ($2^1A_g^-$) does not involve a change in symmetry nor in pseudoparity and is forbidden. The characteristic strong absorption in the visible region arises from a strongly allowed transition to the second excited state S_2 ($1^1B_u^+$).

(iv). The **electronic origin**, also referred to as the (0-0) band, indicates the transition between electronic states in their lowest energy vibrational states (zero-point vibrational levels). The electronic origin is the lowest energy band in absorption spectra and the highest energy band in emission spectra.

(v). The **oscillator strength** expresses the strength of light absorption by a molecule from the integration of the experimental absorption spectrum. It is proportional to the square of the magnitude of the **transition dipole moment**, *i.e.* the dipole moment of the molecule induced by its interaction with the electric field of the incident radiation. For the very strong $1^1A_g^-$ (S_0) \rightarrow

$1^1B_u^+$ (S_2) transition, the oscillator strength is *ca.* 1, and increases with extension of the π -electron conjugation.

(vi). The energy change associated with a transition between states is described by Planck's law, $\Delta E = h\nu$, where ν is the frequency of the light emitted or absorbed. Because $\nu = c/\lambda$, where c is the speed of light and λ is the wavelength, there is an inverse relationship between energy and wavelength.

(vii). The term **absorption cross-section** refers to a quantity that is proportional to the probability of light absorption by a molecule. It is used to indicate this probability.

(viii). The **potential energy surface** describes the energy of an electronic state as a function of nuclear coordinates. Potential energy surfaces are extremely useful for visualizing how energy is interconverted in carotenoids.

(ix). A **red shift** (bathochromic shift) is a shift of a spectral feature to longer wavelength. A shift to shorter wavelength is termed a **blue shift** (hypsochromic shift).

3. Interconversions and loss of excitation energy

(i). Excited electronic states may lose their excitation energy in a number of ways.

(ii). They may revert to the ground state by releasing the energy as radiation (emission). Emission from a singlet excited state is known as **fluorescence**, whereas **phosphorescence** is emission from an excited triplet state.

(iii). The radiationless deactivation of an excited state is known as **internal conversion**. This is the primary decay pathway of carotenoids from their excited states back to the ground state. The energy is released as heat.

4. Exciton interactions

When two or more chromophores are close together, their excited electronic states may interact. The excitation energy may be visualized as hopping from molecule to molecule, and this requires rather close spacing between donor and acceptor. When the inter-molecular distance becomes very close, *e.g.* as the two astaxanthins in crustacyanin, the energy levels of the individual molecules are perturbed and the spectral properties may be altered. Such systems involving pairs of molecules are termed '**exciton-coupled dimers**'.

5. Energy transfer

Especially significant in photosynthesis (*Chapter 14*), energy transfer processes are ones in which the excited state energy of one molecule is passed on to another. The lifetimes of the excited species are important for this. Typically for carotenoids, the lifetimes of the S_1 , S_2 and T_1 states are in the picosecond, femtosecond and microsecond range, respectively. Rapid radiationless decay (or rapid internal conversion) and the short singlet lifetimes of carotenoids present significant challenges for efficient energy transfer to chlorophyll. Donor and acceptor molecules must be close together, and the rate of energy transfer depends on the mechanism and interactions that control the process. In a dipolar (Förster) mechanism [6] the probability of transfer follows a $1/r^6$ dependence, where r is the distance between the donor and acceptor molecules. In the exchange (Dexter) mechanism [7] the probability follows an exponential dependence on r . The relative geometry between the donor and acceptor molecules is also a factor, and varies depending on the mechanism. Triplet energy from chlorophyll triplet excited states can be transferred in the other direction to form carotenoid triplet states.

6. Quantum yield

The term **quantum yield** is used as a measure of the efficiency of a photo-induced process. A process that is 100% efficient in terms of photons absorbed *versus* products formed has a quantum yield of unity.

C. Absorption Spectroscopy

The absorption of ultraviolet or visible light involves an electronic transition from a low-lying state, usually the ground state, to an excited state. A simple energy level diagram that describes the singlet-state energies and many of the photochemical properties of polyenes and carotenoids is shown in Fig. 2. The ground state, S_0 , and first excited singlet state, S_1 , have A_g symmetry elements in the idealized C_{2h} point group of all-*trans* configurations. The second excited state, S_2 , is a more energetic, excited electronic state of B_u symmetry. Higher excited singlet states, S_3 , S_4 , *etc.* are not indicated in Fig. 2, though it is important to point out that, in addition to the S_1 state, there may be other low-energy singlet states that are not easily detected by standard absorption measurements [8,9]. Symmetry labels for carotenoid electronic states are based on

the C_{2h} geometry of undistorted (all-*trans*)-polyenes. These designations and their implications for transition intensities, vibronic interactions, and radiative lifetimes also work remarkably well for a wide range of unsymmetrical *cis* and *trans* polyenes and carotenoids. Thus, the A_g and B_u labels are used throughout this Chapter.

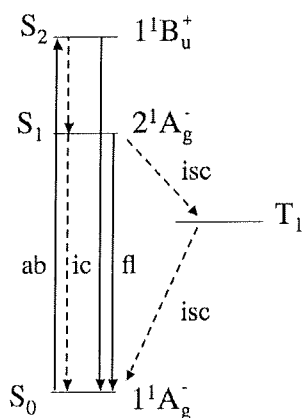


Fig. 2. Energy level diagram used to describe many of the photoprocesses that carotenoids and polyenes undergo, involving their singlet and triplet states. ab, absorption; ic, internal conversion; fl, fluorescence; isc, intersystem crossing.

As mentioned above, the electronic states S_0 and S_1 are both A_g states, so transitions between them are forbidden by the powerful $g \leftrightarrow u$ selection rule for electronic transitions. Also, + and - signs frequently accompany the group theoretical labels of the electronic states of polyenes and carotenoids. These designate pseudoparity elements derived from orbital pairing relationships that emerge when high-level computations involving configuration interaction among singly excited configurations are done [10-12]. At this level of description, the ground state of a polyene belonging to the C_{2h} point group is represented as $1^1A_g^-$, and one-photon transitions between states having the same pseudoparity element are forbidden. So, for example, a transition from $1^1A_g^-$ (ground state) $\rightarrow 1^1B_u^-$ (excited state) is forbidden by pseudoparity selection rules that supplement the $g \leftrightarrow u$ selection rule derived from group theory. The strongly-allowed absorptions of polyenes and carotenoids (*e.g.* Fig. 1) correspond to $1^1A_g^-$ (S_0) $\rightarrow 1^1B_u^+$ (S_2) transitions that involve both $g \leftrightarrow u$ and $- \leftrightarrow +$ changes.

The emission of light by large organic molecules in condensed phases typically occurs from the lowest energy excited singlet or triplet electronic state following rapid radiationless decay from higher excited electronic states [5]. Longer polyenes and carotenoids, however, *e.g.* those

with more than five conjugated double bonds, often show $1^1B_u^+ (S_2) \rightarrow 1^1A_g^- (S_0)$ emission, with the ratio of $(S_2 \rightarrow S_0):(S_1 \rightarrow S_0)$ emission increasing with the length of conjugation. For carotenoids such as β -carotene and lycopene, the $S_1 \rightarrow S_0$ emission yields are almost negligible ($<10^{-5}$). Another important characteristic of the photophysics of carotenoids and polyenes is the apparent absence of reports of radiative decay (phosphorescence) from their lowest triplet states. The rate of the spin-forbidden phosphorescence apparently cannot compete with rapid, nonradiative $T_1 \rightarrow S_0$ relaxation from the low energy triplet states.

From an orbital standpoint, the absorption spectra shown in Fig. 1 are due to $1\pi\pi^*$ transitions of the conjugated π -system. Figure 3 shows the π and π^* molecular orbitals of butadiene as a simple illustration.

The energies of $\pi\pi^*$ transitions can be rationalized either by molecular orbital theory or by the free-electron model ('particle-in-a-box') [13]. The simplest versions of these models explain why the strongly-allowed, low-energy transition [$1^1A_g^- (S_0) \rightarrow 1^1B_u^+ (S_2)$ in Fig. 2] shifts to longer wavelength with increasing conjugation length. These models also predict that the energy of the $S_0 \rightarrow S_2$ transition can be approximated by $\Delta E = A + B/N$, where N is the effective number of conjugated double bonds and is proportional to the conjugation length. The experimental data suggest an asymptotic limit of ≈ 700 nm ($A \approx 14,000$ cm^{-1}) for the $S_0 \rightarrow S_2$ absorptions of infinite polyenes and carotenoids with alternating C-C and C=C bond lengths [13,14]. This stands in contrast to $A \approx 0$ for cyanine dyes and other linearly conjugated systems that lack alternation of the carbon-carbon bond lengths.

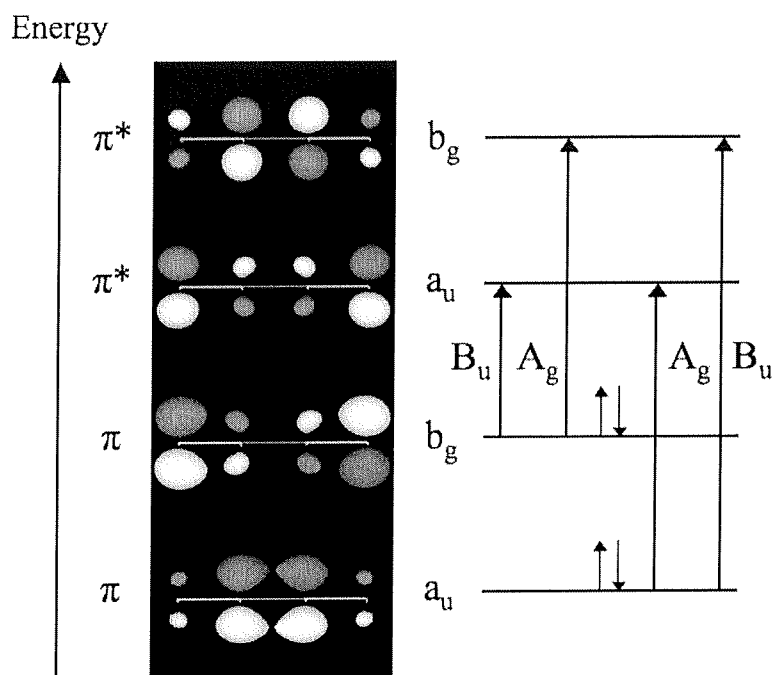


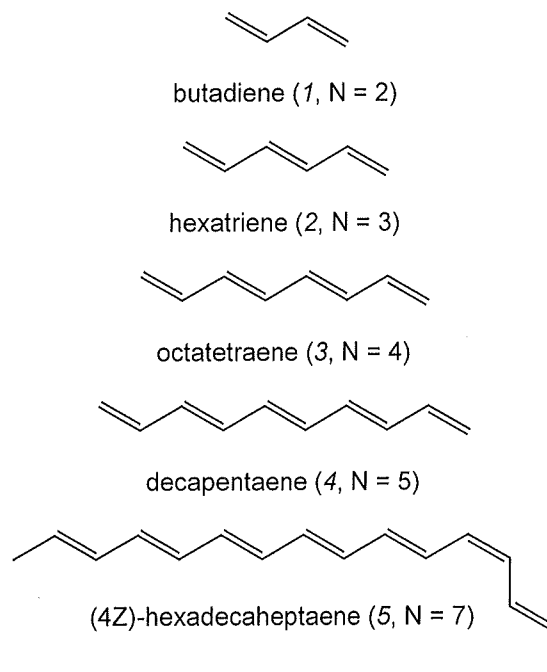
Fig. 3. The π molecular orbitals and $\pi\pi^*$ states in butadiene (1).

There is now considerable experimental evidence that the transition energies of other electronic transitions ($S_0 \rightarrow S_1$, $S_0 \rightarrow S_3$, $S_0 \rightarrow S_4$, *etc.*) also exhibit $1/N$ approaches to their asymptotic limits [13,15,16]. However, unlike the $S_0 \rightarrow S_2$ transition, there is little theoretical basis for this behaviour, particularly in the long polyene limit. Nevertheless, these empirical relationships prove extremely useful for describing how the excited state energies of intermediate length polyenes and carotenoids ($5 \leq N \leq 15$) vary with conjugation length.

The S_2 ($1^1B_u^+$) states are well described in simple molecular orbital or free-electron treatments as being of HOMO \rightarrow LUMO parentage ($a_u \rightarrow b_g$ or $b_g \rightarrow a_u$ where a_u and b_g refer to the irreducible representations of the π -electron molecular orbitals in Fig. 3), and the symmetry-allowed, S_0 ($1^1A_g^-$) \rightarrow S_2 ($1^1B_u^+$) transitions have long been appreciated to be responsible for the colours of long polyenes and carotenoids. However, satisfactory descriptions of the S_1 ($2^1A_g^-$) and other low-energy excited states demand a detailed consideration of the correlation of electron-electron interactions. Generally, this is approached by using configuration interaction (CI), which preserves the orbital description of the π electrons. Even a qualitative understanding of the experimentally observed ordering of the lowest lying excited states, $E(2^1A_g^-) < E(1^1B_u^+)$, requires the interaction of both singly excited, *e.g.* HOMO-1 \rightarrow LUMO and HOMO \rightarrow LUMO+1 ($b_g \rightarrow b_g$ or $a_u \rightarrow a_u$) and doubly excited (*e.g.* HOMO, HOMO \rightarrow LUMO, LUMO)

electronic configurations. A more quantitative agreement between theoretical and experimental $2^1A_g^-$ energies requires extensive configuration interaction. For example, the inclusion of all singly up to quadruply excited configurations explains the slight increase in the energy gap, $E(1^1B_u^+) - E(2^1A_g^-)$, as a function of increasing polyene length [15,17]. However, the computational effort for calculations at this level of CI increases exponentially with the number of π -electrons, and high level CI calculations for polyenes with more than five or six double bonds have proven prohibitive [15].

In addition to the semi-empirical, multi-reference CI calculations [15,17], *ab-initio* electronic structure calculations on polyenes of moderate size are now feasible [18-20]. However, calculating accurate energies for the $2^1A_g^-$ and $1^1B_u^+$ states remains a very challenging problem, especially for linearly conjugated systems that are comparable to the carotenoids. Time-dependent density functional theory (TDDFT) has been applied [20-22] to the simple, unsubstituted polyenes butadiene (1, $N = 2$), hexatriene (2, $N = 3$), octatetraene (3, $N = 4$), and decapentaene (4, $N = 5$). Whereas the $2^1A_g^-$ energies were described quite well, the results were considerably poorer for the $1^1B_u^+$ state, with theory underestimating the experimental excitation energies by ~ 4000 - 6000 cm^{-1} , which is comparable to the $1^1B_u^+ - 2^1A_g^-$ energy differences in these molecules. Although the TDDFT approaches and other *ab-initio* and empirical treatments account for the $1^1B_u^+$ and $2^1A_g^-$ state orderings and the general trends of decreasing excitation energies with increasing conjugation lengths, it is likely that our understanding of carotenoid excited states will rely on experimental data for the foreseeable future.



D. Fluorescence Spectroscopy

Absorption spectroscopy is not entirely adequate to resolve the complex character of the excited states of carotenoid molecules. Because the $S_0 \rightarrow S_1$ transition is forbidden, this has hindered the direct observation of this transition in an absorption spectroscopic experiment. In a small number of cases, *e.g.* for short, simple polyenes in solvent environments and at the low temperatures that lead to well-resolved spectra, the $S_0 (1^1A_g^-) \rightarrow S_1 (2^1A_g^-)$ transition can be observed in absorption [23-26]. However, even for *cis* polyenes and distorted *trans* isomers, this symmetry-forbidden, vibronically induced transition is very weak, with molar absorptivities $\sim 10^{-2}$ - 10^{-3} that of the allowed $S_0 \rightarrow S_2$ transition, which has ϵ_{\max} values of $\sim 10^5$ L/mol cm.

The inherent sensitivity of fluorescence spectroscopy provides a useful alternative for probing the energies and dynamics of the S_1 states in carotenoids, yet there are many technical impediments that prohibit the observation of fluorescence, including difficulties in obtaining samples free of fluorescing impurities, and the inherently low quantum yields of emission of longer polyenes and carotenoids. However, by combining HPLC to obtain ultra-pure samples, laser excitation for efficient and stable optical pumping, and photon counting to enhance the sensitivity of the detection of the weak emission, emission from carotenoids can be observed [13,27-29]. Although fluorescence can be detected readily, the carotenoid fluorescence bands often are broad and featureless, precluding an unambiguous assignment of the spectral origins.

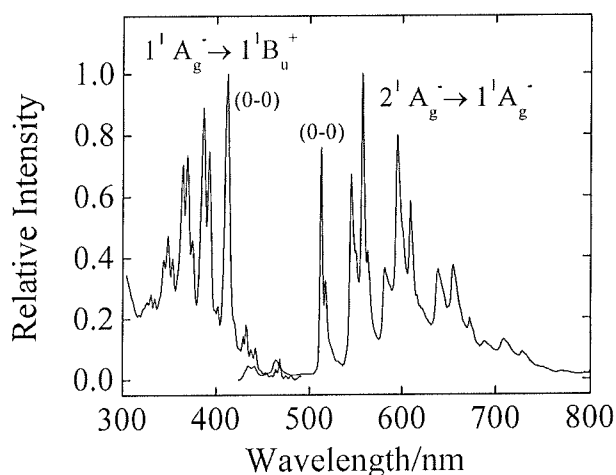


Fig. 4. Excitation and emission spectra of (4Z)-hexadecaheptaene (5) in *n*-pentadecane at 77 K. The spectra were normalized to their λ_{\max} values.

Detection of the low-lying S_1 state, especially in shorter polyenes and carotenoids, is illustrated in the low-temperature absorption and fluorescence spectra of (4Z)-hexadecaheptaene (5) (Fig. 4). The vibronic structure exhibited in these spectra is broadened for carotenoids of photobiological interest, particularly for molecules such as β -carotene (3) where non-planarity between the central polyene chain and terminal cyclohexenylidene rings results in a distribution of absorbing and emitting species [30,31]. The well-resolved spectra of unsubstituted, model polyenes at low temperature facilitates the unambiguous identification of electronic origins [(0-0) bands] and the precise measurement of $1^1B_u^+$ and $2^1A_g^-$ electronic energies. Figure 4 shows the characteristic gap between the onsets [(0-0)s] of the strongly allowed $1^1A_g^- \rightarrow 1^1B_u^+$ absorption and the $2^1A_g^- \rightarrow 1^1A_g^-$ emission. The S_1 ($2^1A_g^-$) \rightarrow S_0 ($1^1A_g^-$) fluorescence yields decrease steadily with increasing conjugation length, ranging from ~ 1 in low temperature octatetraene [32] to $< 1 \times 10^{-5}$ in molecules such as β -carotene [33,34] and most other carotenoids of biological interest [35-40].

The vibronic features of absorption and emission spectra of polyenes are worth noting. Higher-resolution versions of the spectra of (4Z)-hexadecaheptaene (5), obtained in low-temperature, mixed crystals, have been discussed [23]. The electronic spectra are dominated by combinations of totally symmetric (a_g) C-C and C=C stretching modes of ~ 1200 cm^{-1} and ~ 1600 cm^{-1} , with frequencies decreasing (as $\sim 1/N$) with increasing conjugation. These details are easily identified in the S_1 ($2^1A_g^-$) \rightarrow S_0 ($1^1A_g^-$) fluorescence spectrum, which shows Franck-Condon maxima that characteristically involve at least one quantum of the double-bond stretch. In the broader, less well resolved absorption and emission spectra of carotenoids, the vibronic features corresponding to single-bond and double-bond stretches often coalesce into progressions of what appears to be a single, intermediate frequency of 1300-1400 cm^{-1} . Note that the absorption spectrum of the heptaene 5 in Fig. 4 has its maximum intensity in the (0-0) band; it is likely that this reflects the relatively small geometry change experienced in the S_0 ($1^1A_g^-$) \rightarrow S_2 ($1^1B_u^+$) transition. This is consistent with theory, which predicts a more significant transposition of the ground state π -bond orders in the $2^1A_g^-$ state [41]. The vibronic features in Fig. 4, those seen in low-temperature spectra of mixed crystals [23,25,26,42,43], and the highly detailed vibronic development observed in high-resolution spectra of isolated tetraenes in supersonic expansions [44] all are consistent with planar $2^1A_g^-$ and $1^1B_u^+$ excited states in longer polyenes. With long polyenes and carotenoids, there is no evidence for the substantial deviations from planarity experienced by the excited states of dienes and trienes [45-47].

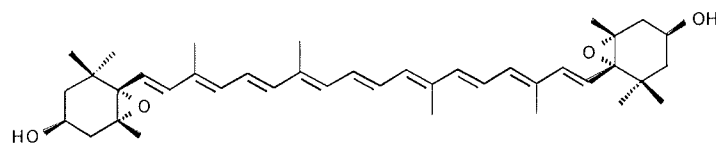
Carotenoids with eight or fewer carbon-carbon double bonds exhibit fluorescence bands associated with the $S_1 \rightarrow S_0$ transition. In longer-chromophore carotenoids, the fluorescence is weak but dominated by the $S_2 \rightarrow S_0$ transition. The crossover from S_1 to S_2 fluorescence can be explained by increases in the rates of $S_1 \rightarrow S_0$ non-radiative decay due to a combination of

smaller S_1 - S_0 energy gaps and the increased density of S_0 -accepting modes in the longer molecules [48]. This leads to the disappearance of $S_1 \rightarrow S_0$ fluorescence, allowing the weaker, residual $S_2 \rightarrow S_0$ fluorescence to dominate the emissions of longer conjugated systems. This idea is supported by significant decreases in $S_1 \rightarrow S_0$ lifetimes and quantum yields observed as the extent of conjugation increases. The $S_2 \rightarrow S_1$ internal conversion rates and fluorescence yields are relatively constant for carotenoids [9,13]. The abrupt decrease in $S_1 \rightarrow S_0$ fluorescence with increasing conjugated chain length accounts for the crossover from S_1 to S_2 fluorescence. Fluorescence is clearly the most direct method by which the forbidden $S_1 \rightarrow S_0$ transition may be observed and the S_1 state energy assigned.

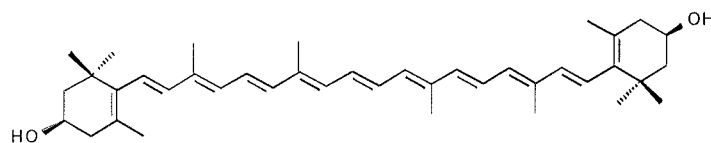
E. Other Optical Techniques and Aspects

1. Pump-probe spectroscopy

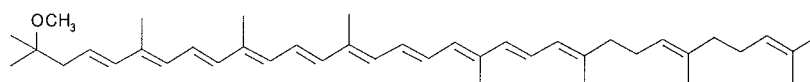
The $S_0 \leftrightarrow S_1$ absorption and emission transitions are forbidden by symmetry and are difficult to detect, especially in longer polyenes and carotenoids. However, the $S_1 \rightarrow S_2$ transition is symmetry allowed and exhibits a substantial oscillator strength. Femtosecond lasers have been used [49] to excite carotenoids from S_0 to S_2 , which then relaxes to the zero-point vibrational level of S_1 . The $S_1 \rightarrow S_2$ absorption spectrum then can be detected by means of an infrared probe laser. Subtraction of the energy of the spectral origin of the $S_1 \rightarrow S_2$ transition from the energy of the spectral origin of the $S_0 \rightarrow S_2$ transition gives the S_1 energy of the carotenoid. This approach was applied to violaxanthin (259), zeaxanthin (119) [49], and spheroidene (97) [50,51].



violaxanthin (259)

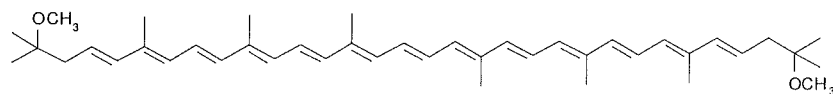


zeaxanthin (119)

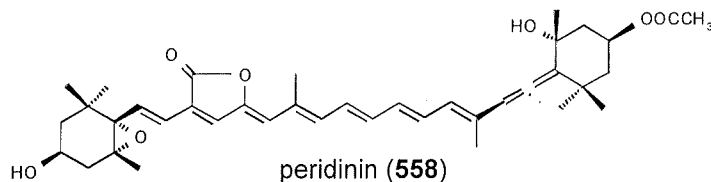


spheroidene (97)

Semi-empirical and *ab-initio* quantum calculations [15,17,21,52,53] have suggested that additional excited singlet states may lie in the vicinity of S_1 or between S_1 and S_2 . These are states ($^1A_g^-$ and $^1B_u^-$) into which absorption from the $^1A_g^-$ ground state is symmetry forbidden. Different variations on ultra-fast laser technology have been used to seek evidence for these states. For example, ultra-fast pump-probe spectroscopy was used [54] to obtain results on several carotenoids including lycopene (31) and β -carotene (3) and, from this, the existence of an intermediate singlet state (S_x), which facilitates internal conversion between the S_2 ($^1B_u^+$) and S_1 ($2^1A_g^-$) states, was postulated. A wavelength dependence of the dynamics of spirilloxanthin (166) was observed [55] and interpreted in terms of a singlet electronic state, denoted S^* . Recent experiments on several cyclic xanthophylls and open-chain carotenoids have suggested that S^* is associated with twisted conformations of carotenoids (see Section G). Application of pump-probe optical techniques to β -carotene [56] suggested yet another carotenoid excited state, referred to as S^\ddagger , formed directly from S_2 ($^1B_u^+$). Spectroscopic features have been assigned to a low-lying $^1B_u^-$ state [16], and the presence of an intramolecular charge transfer state, S_{ICT} , has been invoked [57,58] to explain spectroscopic observations on peridinin (558). Details of these results and other experiments are summarized in a recent review [9].



spirilloxanthin (166)



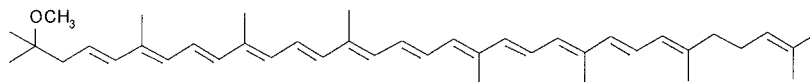
peridinin (558)

2. Two-photon spectroscopy

Two-photon transitions in π -electron conjugated molecules are symmetry-allowed between states that have the same parity [10]. Therefore, in principle, the $S_0 \rightarrow S_1$ ($1^1A_g^- \rightarrow 2^1A_g^-$) transition should be observable by, for example, fluorescence-detected, two-photon excitation. This technique has been exploited in high-resolution optical studies of short polyenes in mixed crystals [59] and as cold, isolated molecules in supersonic jets [44]. However, the very low quantum yields of S_1 emission in longer polyenes have precluded broad application of the technique to purified carotenoids in solution. The $S_0 \rightarrow S_1$ transition ($1^1A_g^- \rightarrow 2^1A_g^-$) has been observed for carotenoids bound in pigment-protein complexes from photosynthetic organisms [60-67] where the two-photon excitation profile can be monitored by using the emission from the highly fluorescent bound chlorophylls to which carotenoids typically transfer energy. These studies are important because they provide information on the energies and dynamics of the S_1 states of carotenoids bound in these pigment-protein complexes.

3. Resonance Raman spectroscopy

Resonance Raman spectroscopy is a powerful technique for analysing the electronic structures and dynamics of excited carotenoids in solution as well as those bound to proteins [68]. Both steady-state and time-resolved methods have been used extensively to probe carotenoids in their ground and excited singlet and triplet states. A variation of the technique, resonance Raman excitation spectroscopy, monitors resonance Raman line intensities as a function of the excitation energy of the photons used to induce scattering. The technique has been applied to several carotenoids including β -carotene (**3**), lycopene (**31**), spheroidene (**97**), anhydrorhodovibrin (**91**), and spirilloxanthin (**166**) [16,69-72].



anhydrorhodovibrin (**91**)

The experiments employ crystalline carotenoids to induce strong self-absorption of the resonance Raman lines associated with the strongly allowed $S_0 \rightarrow S_2$ absorption. In this manner, signals associated with optically forbidden transitions may be revealed. The excitation profiles are strongly dependent on the concentration of the sample and the geometry of the optical set-up, and assigning the vibrational progressions to particular electronic states is difficult. Nevertheless, signals have been assigned to the $1^1B_u^+$, $1^1B_u^-$ and $2^1A_g^-$ states of several

carotenoids, and the energies of the $2^1A_g^-$ states, based on resonance Raman excitation profiles, are in reasonable agreement with the values obtained from steady-state fluorescence methods.

The experiments and computations described above have suggested a much more complex energy level diagram (Fig. 5). At this stage, it is not clear how many, if any, distinct electronic states lie between $1^1B_u^+$ (S_2) and $2^1A_g^-$ (S_1), or what their significance may be for biological functions of carotenoids, but this clearly will remain an active area of research.

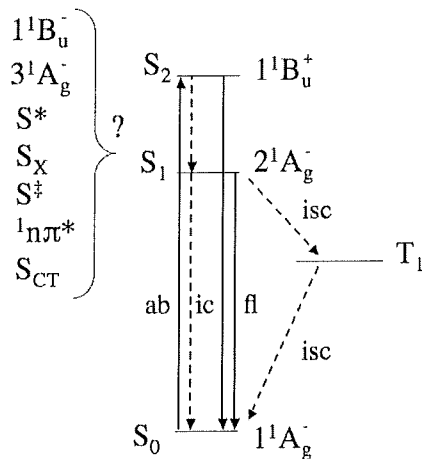


Fig. 5. Energy level diagram depicting the notation of various additional excited singlet states under investigation.

F. Experimental Considerations

1. Purity of carotenoids

For the spectroscopic experiments described above, it is imperative that carotenoid samples be of high purity. Fortunately, for most optical experiments, analytical-scale chromatography yields sufficient material. Purification of carotenoids generally uses combinations of column chromatography, TLC and/or recrystallization, followed by HPLC, as treated in *Volume 1A*.

In a typical procedure used by the authors [40], carotenoids were extracted from anaerobically grown cells of *Rhodobacter sphaeroides* wild-type strain 2.4.1 with methanol/acetone and fractionated by column chromatography on alumina. The fraction containing spheroidene (**97**) was then subjected to reversed-phase HPLC, either on a NovaPak C_{18} column with gradient elution or a YMC C_{30} column with isocratic elution (methyl *t*-butyl ether/methanol, 11:89). (*all-E*)-Spheroidene and several *Z*-isomers were resolved, and the

isomerically pure (all-*E*)-spheroidene, which had the longest retention time on the C₃₀ column, was collected for use in spectroscopy experiments.

2. Fluorescence spectral corrections

Fluorescence spectra should be corrected for the wavelength dependences of the optical components (including lenses, gratings, and photomultipliers) by using correction factors traceable to NIST or comparable illumination standards and, normally, a quartz-halogen tungsten coiled filament lamp.

3. Band-pass corrections

When emission spectra are displayed on a wavenumber ($\tilde{\nu}$) scale, it is important to recognize that, although wavelengths may be converted into wavenumbers by taking the reciprocal, $\tilde{\nu} = 1/\lambda$, the bandpass on a wavelength scale ($\Delta\lambda$) is not simply the inverse of the bandpass on a wavenumber scale ($\Delta\tilde{\nu}$) [73]. For an emission spectrum obtained with a fixed wavelength bandpass, the usual experimental method, the bandpass on a wavenumber scale decreases with wavelength. This stems from the relationships: $d\tilde{\nu} = -d\lambda/\lambda^2$ or $|\tilde{\nu}| = |\Delta\lambda|/\lambda^2$. For an emission spectrum obtained as photons/sec nm, $I(\lambda)/\Delta\lambda$, conversion into emission intensity in photons/sec cm⁻¹, $I(\tilde{\nu})/\Delta\tilde{\nu}$, requires multiplying each value of the intensity by the square of the detection wavelength [73]. Thus, $I(\tilde{\nu}) = \lambda^2 I(\lambda)$ gives the corrected spectral response for the conversion of an emission spectrum from wavelength to wavenumber scales. This correction does not apply to absorption or fluorescence excitation spectra. In these cases the signals are related to **ratios** of intensities, and $I(\tilde{\nu})/I_0(\tilde{\nu}) = I(\lambda)/I_0(\lambda)$ where $I(\tilde{\nu})$, $I(\lambda)$, *etc.* represent the average of signals (*e.g.* photons/s) integrated over the band pass.

4. Gaussian deconvolution

Absorption and fluorescence spectra may be deconvoluted into vibronic components by fitting the lineshape to a sum of Gaussian functions. Given the fundamental importance of energy in quantum mechanics and spectroscopy, spectra are typically converted to wavenumber or other energy scales before spectral fitting and analysis of vibronic spacings and intensities. ‘Origin’ software [74], which employs a Levenberg-Marquardt curve-fitting algorithm, is a typical package for accomplishing this. The frequencies (in cm⁻¹) of the vibronic bands are initially estimated from the locations of the peaks and shoulders in the experimental spectra, and the frequencies, widths, and amplitudes can then be allowed to vary through several iterations of the

non-linear least squares program. In this manner, best-fit parameters for each of the Gaussian bands comprising the spectra are obtained. These parameters should not depend on the initial estimates of band positions and their intensities.

5. Excitation spectra

Fluorescence excitation spectra should be corrected for the intensity of light incident on the sample. Most spectrometers accomplish this by splitting the excitation beam and detecting the incident light by means of a quantum counter such as Rhodamine 610 in ethylene glycol (0.3 g/100 mL) [75] or a calibrated photodiode. The sample emission is then divided by the incident light response, to yield a corrected excitation spectrum. The quantum counter technique is limited by the absorption cut-off (~600 nm for Rhodamine) of the fluorescent dye. The use of a calibrated photodiode can extend the correction range into the infrared.

6. Correlation of absorption and excitation spectra

Fluorescence excitation spectra can be used to identify the emitting molecular species and, by comparing the excitation and absorption spectra, to determine the efficiencies of energy transfer between molecules, but caution should be exercised in making these comparisons.

Fluorescence intensity (F) as a function of the excitation wavelength (λ) is given by

$$F(\lambda) = I_0(1 - 10^{-\epsilon b C})Q_f = I_0(1 - 10^{-A})Q_f = I_0(1 - T)Q_f \quad (1)$$

where I_0 is the intensity of incident light, $\epsilon b C$ is the sample absorbance (A) at the wavelength of excitation, T ($= I/I_0$) is the transmission of the sample, and Q_f is the quantum yield of the fluorescence. If $A = \epsilon b C$ is small, then the series

$$10^{-A} = 1 - A(2.302) + A^2[(2.302^2)/2!] - A^3[(2.302^3)/3!] + \dots \quad (2)$$

rapidly converges. Keeping only the first order term in A gives

$$F(\lambda) = I_0[A(2.302)]Q_f \quad (3)$$

For samples with low absorbance, fluorescence intensities thus will be linearly proportional to A , and the fluorescence excitation spectrum should be superimposable with the absorption spectrum [$A(\lambda)$]. At higher concentrations, the series convergence is not rapid and the

fluorescence excitation spectra then should be compared with (1-T) not Absorbance (A). For example, comparing $F = I_0[A(2.302)]Q_f$ with $F = I_0(1 - 10^{-\epsilon b C})Q_f$ at $A = 0.03$ yields an approximate 4% error which increases to 12% for $A = 0.1$. This will result in an apparent distortion of relative intensities in the fluorescence excitation spectrum, if compared with $A(\lambda)$, rather than $[1-T(\lambda)]$.

7. Quantum yields

The fluorescence quantum yields of carotenoids can be measured by using standards such as Rhodamine 590 in methanol ($\phi_r = 0.95$) [76]. The evaluation of the quantum yields is based on equation 4 [77]:

$$\phi_c = \phi_r \left(\frac{1 - 10^{-A_r \lambda}}{1 - 10^{-A_c \lambda}} \right) \left(\frac{I_{r\lambda}}{I_{c\lambda}} \right) \left(\frac{n_c^2}{n_r^2} \right) \left(\frac{D_c}{D_r} \right) \quad (4)$$

where ϕ_c and ϕ_r are the quantum yields of the carotenoid and reference (standard) solutions, respectively. I_c and I_r are the relative intensities of the excitation light at wavelength λ for the carotenoid and standard solutions. A_c and A_r are the absorbances of the carotenoid and standard solutions at λ . n_c is the refractive index of the solvent used for the carotenoid, and n_r is the refractive index of the solvent used for the Rhodamine 590 standard. In quantum yield experiments, the solvent for the carotenoid and standard solutions should preferably be the same, so the ratio of the indices of refraction can be set to unity. The ratio of the excitation light intensities can be obtained by use of a reference photomultiplier. D_c and D_r are the integrated areas of the corrected emission spectra of the carotenoid and standard solutions and should be obtained under identical instrumental conditions of slit width, gain, *etc.*

8. Solvents

The absorption spectrum associated with the $S_0 \rightarrow S_2$ ($1^1A_g^- \rightarrow 1^1B_u^+$) transition of carotenoids is influenced by solvent. Dispersion interactions between the solvent environment and the large transition dipole moment shift the spectral profiles [78-81]. For nonpolar carotenoids in nonpolar solvents, the magnitude of this effect depends linearly on solvent polarizability given by $R(n) = (n^2-1)/(n^2+2)$ where n is the refractive index of the solvent [79]. The energies of the $S_1 \leftrightarrow S_0$ ($2^1A_g^- \leftrightarrow 1^1A_g^-$) transitions of carotenoids are not as strongly affected by changes in the solvent due to the much smaller dipole moment associated with this transition [82]. Solvent

shifts of the $S_1 \leftrightarrow S_0$ ($2^1A_g^- \leftrightarrow 1^1A_g^-$) transition are $<ca.10\%$ of those associated with the $S_0 \leftrightarrow S_2$ ($1^1A_g^- \leftrightarrow 1^1B_u$) transition. An exception is peridinin (**558**), which exhibits a pronounced solvent effect on the wavelength of the $S_1 \rightarrow S_0$ ($2^1A_g^- \rightarrow 1^1A_g^-$) fluorescence and on the lifetime of its lowest excited singlet state [57]. An examination of the spectroscopic behaviour and dynamics of peridinin and other carbonyl-containing carotenoids revealed that the lifetime of the lowest excited singlet state of these molecules is strongly dependent on solvent polarity, $P(\epsilon) = (\epsilon-1)/(\epsilon+2)$ where ϵ is the dielectric constant of the solvent, In general, carotenoids containing carbonyl groups also have complex transient absorption spectra and show a pronounced dependence of the excited singlet state lifetime on solvent environment. These effects have been related to the presence of an intramolecular charge transfer state strongly coupled to the S_1 ($2^1A_g^-$) excited singlet state.

G. Recent Developments

1. Geometrical isomerization

A recent report of spectroscopic studies on *cis* and all-*trans* isomers of the simple polyene, hexadecaheptaene, indicates a low barrier for conversion between *cis* and all-*trans* isomers in the $2^1A_g^-$ state [24]. In solutions at room temperature, the essentially non-fluorescent *trans* isomer is in equilibrium, on the $2^1A_g^-$ potential surface, with non-symmetric, fluorescent *cis* isomers. These experiments suggest: (i) that the S_1 states of longer polyenes and carotenoids have local energy minima corresponding to a range of conformations and isomers; and (ii) that these minima are connected by relatively low energy barriers. Steady-state and time-resolved optical measurements on the S_1 states thus sample a distribution of conformers and geometrical isomers, even for molecules represented by a single, dominant ground-state structure. Complex S_1 potential energy surfaces may help explain the complicated $S_2 \rightarrow S_1$ relaxation kinetics of carotenoids and reduce the need to invoke many of the intermediate electronic states indicated in Fig. 5. These recent experiments highlight the limitations in using fluorescence techniques to study the S_1 ($2^1A_g^-$) states of longer all-*trans* polyenes and carotenoids. Previous reports of fluorescence from these symmetrical systems can probably be attributed to distorted *trans* isomers and/or *cis* impurities. Excited state absorption experiments [49,51] thus have the important advantage of exploiting an allowed electronic transition to probe the energy levels of all-*trans* samples with signals that reflect accurately the distribution of symmetrical and asymmetrical species.

Other recent work [83,84] supports the essential features of the three-level energy level diagram given in Fig. 2. At least some of the spectroscopic transients observed in pump-probe experiments on carotenoids and assigned to the states included in Fig. 5 can be attributed to two-photon processes induced by the high light intensities of these pulsed experiments. Also, recent ultrafast, time-resolved experiments on xanthophylls [85] and open-chain carotenoids [86], with increasing number of π -electron carbon-carbon double bonds, have focused on elucidating the nature of the state denoted S* implicated as an intermediate in the depopulation of S₂ ($1^1B_u^+$) and as a pathway for the formation of carotenoid triplet states in light-harvesting complexes [55]. The experimental data are supported by quantum computations which suggest that S* is simply an S₁ state with a twisted conformational structure, the yield of which increases as the π -electron conjugated chain length of the molecule increases. Thus, upon photo-excitation of the carotenoids into the S₂ ($1^1B_u^+$) state, relaxation into the S₁ ($2^1A_g^-$) state is accompanied by conformational twisting leading to branched decay pathways of the molecules that populate various conformers with different spectroscopic features. These then decay independently at different rates back to the ground state. Further work will resolve the precise structures of these intermediates in the excited-state decay pathways, thereby increasing our understanding of their role in controlling the important photochemical processes that carotenoids undergo in biological systems.

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