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Resonance Raman spectroscopy of carotenoids and carotenoid-containing systems

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Abstract - The application of Raman and resonance Raman spectroscopies to biochemical problems has developed mainly during the past decade. Among naturally occuring chromophores, carotenoids have proved to be very suitable for studies in resonance Raman spectroscopy. Vibrational spectra can be obtained at very low concentration (until 10^{-8}M) even if the chromophore is included in a complex biological medium ; thus a very active research area has been developed. After a brief presentation of resonant Raman spectroscopic properties of carotenoids, free and bound to proteins, a review, with some examples is given with special emphasis on two distinct advantages of Raman spectroscopy. Time resolution allows short lived species to be analysed on the nanosecond and picosecond time scales. Furthermore space resolution enables vibrational spectra of carotenoids to be obtained even from single living cells, circumventing the difficult biochemical purification of these pigments.

INTRODUCTION

Raman spectroscopy, like infrared spectroscopy, can provide detailed information on molecular vibrations, and has been successfully employed in many areas of investigation. The application of this method to the study of the conformations of biological molecules has developed only slowly because of numerous difficulties such as the great complexity of the spectra, poor quality spectra obtained from dilute solution and the large volumes needed. Thanks to the development of laser light sources (which now cover the 250-750 nm spectral range) and Raman instrumentation, much progress has been made, mainly in resonance Raman (RR) spectroscopy which allows some of the above problems to be overcome.

The strong RR enhancement (10^3 to 10^6 fold) observed when the radiation used to excite the Raman spectra lies in an electronic absorption band of a chromophore allows the analysis of specific vibrational modes of the chromophore, even if it is included in a complex biological medium at very low concentration. Since many natural chromophores are the key for important biological activities, RR spectroscopy is a valuable tool for probing such activity. Detection and structural analysis of very small concentrations of biological pigments is possible in the presence of large amounts of non absorbing species. Among naturally occuring chromophores, carotenes have proved to be the most accessible for studies in RR spectroscopy (Ref. 1, 4) , several spectra were recorded by Euler and Hellstron in 1932, just four years after the discovery of the Raman effect. After many years of inactivity, interest in carotenoid RR spectra was revived from a paper of Gill et al. (Ref.5) in which spectra from intact plant tissue were presented. In recent years, RR spectroscopy of carotenoids has become a very important research area for spectroscopists, theoreticians and biologists.

INTEREST OF RAMAN SPECTROSCOPY

When photons interact with matter, a small number of them undergo inelastic collisions with molecules and the frequencies are symmetrically shifted to higher (Raman-Stokes effect) and to lower frequencies (Raman-anti-Stokes effect). Each shift corresponds to a particular molecular vibrational frequency and is independent of the excitation radiation. Generally only the more intense Stokes part is considered ; the shift values are expressed as wavenumbers (cm^{-1}). The basic principles of the Raman experiment are very simple and can be described as follows. Monochromatic light from a laser is focussed into or onto a sample to produce a high photon density. Light scattered by molecules is collected by an optical objective and focussed onto a slit of a monochromator in order to analyze the vibrational frequencies and their intensities. Although the physical processes are different, the information obtained is essentially the same as that provided by infrared spectroscopy, and can be used to monitor the nature of chemical bonds, molecular structure and the interactions between the molecules and their environment.

Compared to infrared, Raman and RR spectroscopy present some special advantages in biochemical investigation.

- Since water exhibits very weak Raman lines, Raman spectra may be obtained for molecules in aqueous solutions.

- The size of the focussed laser beam (typically 1-2 μm^2) allows the analysis of very small amounts of material or sample included in heterogeneous media. A great flexibility in experimental arrangements leads to spatial resolution at the microscopic level (Ref. 6, 7, 8).

- The time scales of the Raman effect is essentially instantaneous. Raman spectra can be obtained within the nanosecond, or even picosecond range (Ref. 9,10) and allow the characterisation of transient species, photobiological processes and excited electronic states, with time resolution.

The two major disadvantages of Raman and RR spectroscopy are the unwanted photochemical processes produced by the high photon density and the fluorescence emission which can overcome the Raman signal (Réf. 8).

RESONANCE RAMAN SPECTRA OF CAROTENOIDS

By exciting into the $\pi \rightarrow \pi^*$ transition of the polyene chain, very intense spectral features are observed in the 900-1600 cm^{-1} region as shown in Fig. 1. Changes in the end groups do not perturb strongly the RR spectra, provided that the end group vibrations do not mix strongly with the vibrations of the conjugated chain which are responsible for most of the observed bands. The main RR lines are resonant with the strong absorptions in the visible region, maximum intensity enhancement occurs with the 0-0 transition (Ref. 3).

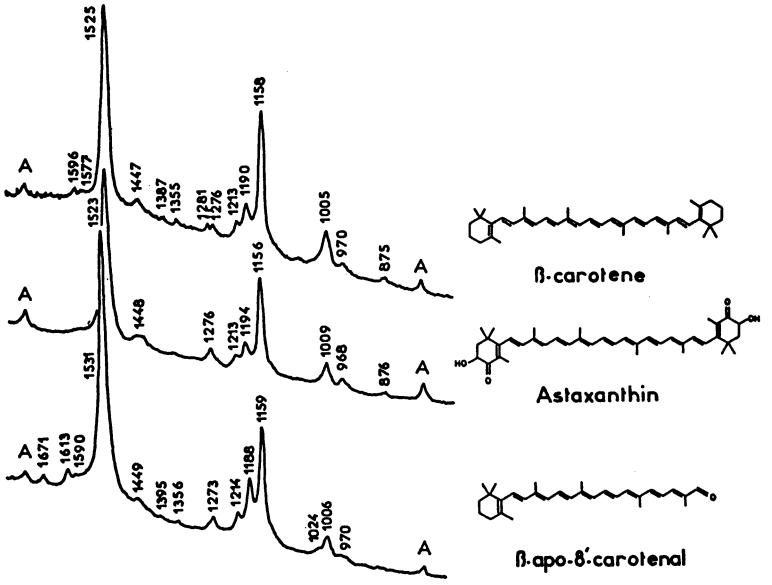

Fig. 1. Typical RR spectra of carotenoids in acetone with 488 nm excitation. Acetone bands are marked A.

The very strong RR band near 1520 cm^{-1} (ν_1) is assigned to the C=C stretching vibration. A recent normal coordinate analysis of β-carotene (Ref. 11) has shown that the C=C bonds in the chain move approximately in phase. Such displacements are expected to be larger in the central part and smaller toward the chain ends. The terminal C=C stretching, resonant with absorption in the ultra-violet region is observed near 1595 cm^{-1}. The ν_1 line can be used to monitor the degree of conjugation through the π electron system (Ref. 11). For carotenoids of different chain lengths in a variety of solvents a correlation between ν_1 and the absorption λ_{max} has been proposed by several authors (Ref. 12, 13). As for polyacetylenic molecules (Ref. 14), a relation is found between the ν_1 position and the number of the carbons in the chain (Fig. 2.). This shows the interrelationship of ν_1 and the delocalisation of the π electrons in the ground state.

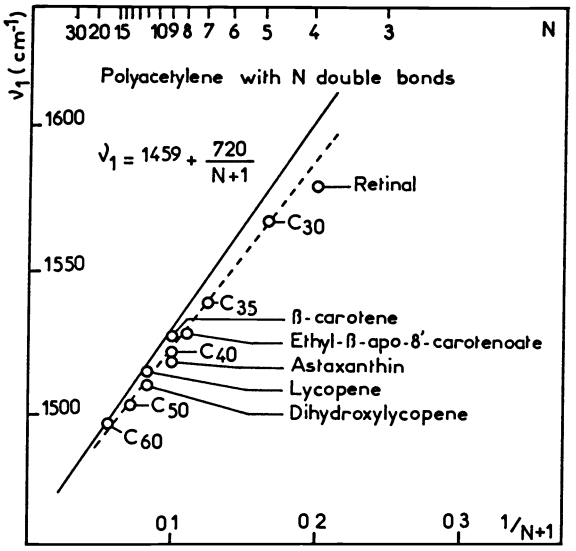

Fig. 2. Relation between C=C stretching frequency (ν_1) and the number of double bonds for polyacetylenes and carotenoids.

The C-C stretching mode is usually attributed to the intense band near 1157 cm^{-1} (ν_2) but a strong mixing with C-H in plane bending modes perturbs this vibration. A decrease in the π order of the C=C bond exemplified by the downshift of the ν_1 mode should result in an increase of the ν_2 position, but for carotenoid and polyene chains ν_2 exhibits the opposite behavior (Ref. 15) (Fig. 3.). In contrast to the C=C stretching mode the relationship among the C-C stretching modes is disturbed by the presence of CH_3 groups (Ref. 11) so in polyacetylenic molecules, where CH_3 groups are lacking, the ν_2 band is generally 20 cm^{-1} lower than for carotenoids with the same chain length.

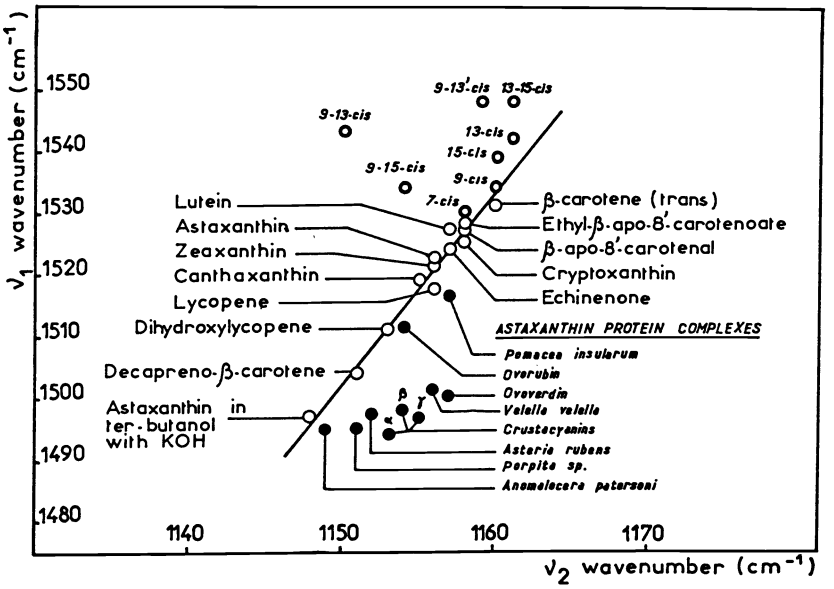

Fig. 3. Plot of C=C stretching frequency (ν_1) vs. C-C stretching frequency (ν_2). Values for ß-carotene isomers provide from Ref. 20, values for astaxanthin-protein complexes are obtained from Ref. 25-29.

The ν_3 line, which is the third line enhanced by resonance effects, is assigned to the CH_3 in-plane rocking mode. The 1100-1400 cm^{-1} spectral range is called the "finger print region" of the carotenoid and contains weak lines sensitive to both the nature of the end groups and the chain conformation. The RR lines observed in this region can be sometimes used as key bands for structural identification and conformational studies. Analysis of a wider spectral range (1600-5000 cm^{-1}) shows many combination and overtone bands (Ref. 16).

CIS-TRANS ISOMERISATION

As expected from the above discussion, RR spectra of carotenoids vary appreciably when a cis-trans isomerisation occurs. RR spectra of different configurational isomers of β-carotene have been recently described and analysed by several authors (Ref. 11,17-20). The order of increasing frequency of the ν_1 band when compared to the all-trans isomer correlates well with the blue shift of the absorption band when the chain is perturbed. Generally centrally bent-isomers show larger Raman shifts than peripherally-bent isomers.

The ν_2 region (1100-1300 cm^{-1}) is very sensitive to the configuration of the chain. The spectral pattern is unique for each of the isomers studied (Fig. 4) (Ref. 20) and can be ascribed to changes in the mixing of the C-C stretching vibrations. Because of the twisting about the double bonds, the ν_1 vs. ν_2 correlation is not obtained (Fig. 3).

Cis carotenoids have been established in the reaction centers of several photosyntetic bacteria by using RR spectroscopy. Lutz and coworkers (Ref. 17,18) suggested a di-cis configuration while Koyama and coworkers (Ref. 19,20) have pointed out that the reaction center carotenoids are more like 15-cis-β-carotene than any other cis isomers.

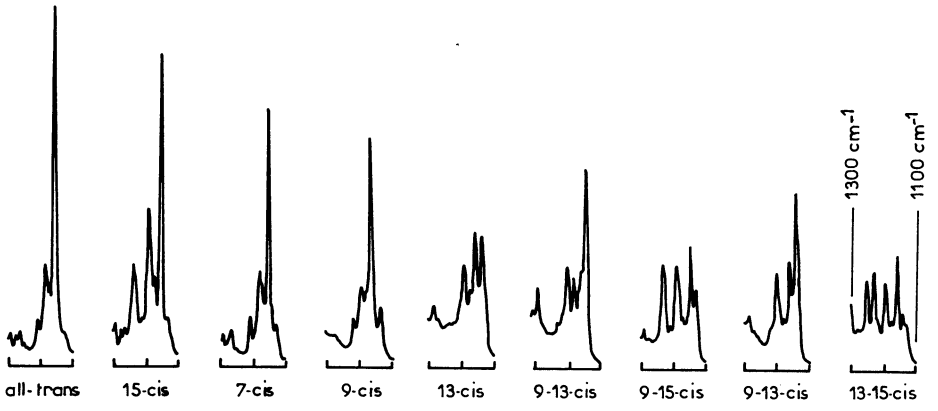

Fig. 4. Raman lines of β-carotene isomers in the 1100-1300 cm^{-1} region. From Y. Koyama, T. Kakii, K. Saiki and K. Tsukida (Ref. 20)

CAROTENOID-PROTEIN COMPLEXES

RR spectra of carotenoproteins, generally exhibit many more vibrational bands than the free carotenoids, but no bands assignable to the protein are observed. The ν_1 shift is in concordance with the shift of the electronic absorption band.

Different kinds of information can be obtained from the RR spectra. Band position is a property of the electronic ground state ; changes in intensities yield information about differences between ground and excited electronic states. With a judicious choice of excitation, small changes in absorption maxima can give rise to large changes in the intensity ratio of the ν_1 and ν_2 modes ; a sensitive measure of membrane potential has been obtained by this method (Ref. 21). The RR spectral change can reflect conformational changes when included in a membrane ; mixtures with phospholipid dispersions (Ref. 22), human blood platelet (Ref. 23) and frog sciatic nerves during conduction (Ref. 24) are some examples of applications.

Two types of proteins, both containing astaxanthin, are responsible for the pigmentation of the lobster shell studied by Carey's group (Ref. 25-27). The yellow protein has absorption and RR properties identical to those of aggregates of astaxanthin in aqueous solution. The blue shift of the absorption band (410 nm) is not accompanied by changes in the ν_1 and ν_2 band positions. This indicates that a large perturbation of the electronic excited state takes place while the ground state conformation is minimally perturbed (Ref. 25). On the basis of the correlation between RR and absorption shifts evident for some carotenoproteins the large red shift for the three crustacyanins which absorb in the 600 nm region has been accounted for by a charge polarisation mechanism (Ref. 26). By using red exciting lines, the RR spectra of the intact shell supports the hypothesis that a new astaxanthin-bearing pigment, not yet isolated and characterised, is present (Ref. 27).

Other astaxanthin proteins have been investigated by Clark and coworkers (Ref. 28,29). If a high degree of similarity between the astaxanthin conformations has been found, twisting about double bonds is postulated in order to explain both the ν_1 shift and the new bands observed in the carotenoprotein RR spectra.

The causes of the spectral shifts in carotenoproteins have not yet been exactly determined

but among numerous effects due to astaxanthin environments, two factors seem to be dominant : charge polarisation and twisting about the double bonds. If the chief factor affecting the spectral properties were the extent of π-electron delocalisation along the chain, the linear correlation of the ν_1 and ν_2 frequencies, as well as the dependance of the ν_1 frequency on the absorption λ_{max}, should be preserved. By dissolving astaxanthin in tert-butanol saturated with KOH, an anion is formed and a blue color develops as a result of a charge polarisation mechanism. The frequencies of the ν_1 and ν_2 lines recorded from this blue solution (Ref. 26) are in agreement with the two above mentioned relations.

The ν_1 and ν_2 values obtained for different astaxanthin-protein complexes (Ref.26-28, 29) agree neither with the linear correlation (Fig. 3) nor with the expected absorption spectral shift (Ref. 29). Thus we can postulate that polarisation of the carotenoid is not the major contributing factor to the observed spectral changes and that two competitive effects must be considered :
(i) Neighbouring charged groups of proteins or hydrogen bonds can produce a polarisation of the π-electron system resulting in a simultaneous decrease in both ν_1 and λ_{max} values.
(ii) Twisting about the double bond, which leads to a distorsion of the chain, can in some cases minimise the first effect and produce an increase in the ν_1 value. Further RR studies of other carotenoproteins may reveal finer details of carotenoid-protein interaction.

Canthaxantin-lipovitellin has been recently investigated by Zagalsky et al. (Ref. 31, 32) ; twisting of the polyene chain and asymmetric binding were proposed.

TIME-RESOLVED RESONANCE RAMAN SPECTROSCOPY

Excited states of biologically important polyenes such as retinals and carotenoids have been studied by various methods in order to clarify the role of these transient states in vision and photosynthesis.

Pulsed multichannel Raman spectrometry developed by Bridoux and coworkers (Ref. 9, 10) has the unique ability of recording the entire Raman spectrum excited by a single laser pulse on the nanosecond or picosecond time scale. Pulsed radiolysis techniques have been used to create long-lived excited states (Ref. 15, 33, 34) but it appears that tunable pulsed lasers are more effective for pumping at selected wavelengths and allowing the study of the population of states from transmission of the sample as a function of the laser intensity (Ref. 35). RR probing of these states with time resolution can be performed by using another pulsed laser during or after the pump laser pulse.

Excited triplet states of carotenoids have been studied by several authors (Ref. 10, 33-36). Triplet Raman spectra obtained from all-trans and 15-cis β-carotene are similar (Ref. 34).For similar decreasing wavenumber shifts ot the ν_1 and ν_2 RR lines the interpretations are quite different. Woodruff and coworkers suggest a change of the interaction between C=C and C-C vibrational modes when the triplet state is created (Ref. 34). Wilbrandt et al. consider a decrease of the double bond order and a twisting around the inner double bonds to be important (Ref. 33). In photosynthetic bacteria the triplet carotenoids bound to reaction centers retain a cis-conformation and a marked difference from the all-trans carotenoid triplet state was found (Ref. 36). To explain these results a cis-configuration around one or more of the single bonds was postulated (Ref. 34).

The ν_1 and ν_2 part of the vibrational spectrum of the 265 fs lifetime singlet state of β-carotene has been recorded (Ref. 37). Within experimental error the ν_1 and ν_2 frequencies are the same as the ground state but 10 cm^{-1} broadening was found.

The short-lived conformational state of retinal bound to proteins (rhodopsin and bacteriorhodopsin) during photoisomerisation processes have also been extensively studied by RR spectroscopy in order to establish the conformation of the intermediate (Ref. 13).

IN SITU AND IN VIVO RESONANCE RAMAN STUDIES

One of the distinct advantages of RR spectroscopy is that spectra can frequently be obtained from chromophores in situ ; the strong resonance enhancement allows one to observe selectively vibrational modes of a chromophore without interference of the non-resonant scattering of a complex medium such as a biological material. The first RR spectra from live carrot root and live tomato fruit was obtained by Gill and coworkers (Ref. 5), and RR studies of the pigmentation of lobster shell have been performed in the shell of the live lobster without extraction or purification (Ref. 25-27).

Carotenoids in hard skeletal parts of shells and corals have been chemically investigated (Ref. 38) : carotenoproteins or inorganic complexes were proposed in order to explain both the shift of the visible absorption band and the great chemical stability of the colours. For quite a time the problem was unresolved because no pigments could be extracted from calcareous skeletons (Ref. 39).

RR spectra obtained from intact specimens are shown on Fig. 5. The two strong lines observed

in the 800-1600 cm^{-1} region are typical of a trans conjugated double bond system. Though a carotenoid can be characterized in Pinna nobilis pearl and Favonia vaughanii branches by the strong bands near 1160 and 1520 cm^{-1}, some differences indicate that the polyene chain is different in structure in the other specimens (Ref. 40), the wavenumber of ν_2 line is lower, and the weaker spectral features are different ; two small lines are present near 1300 and 1010 cm^{-1} for all the specimens studied.

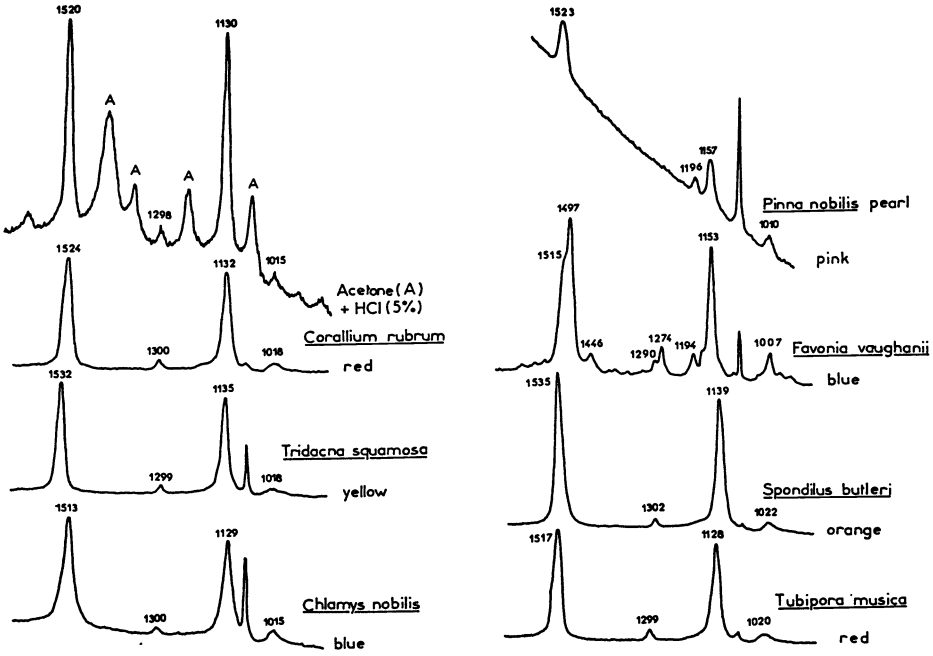

Fig. 5. in situ RR spectra of some calcareous skeletons with 488 nm excitation

By dilution of Corallium rubrum in acetone slightly acidified with HCl (5 %), a yellow solution which exhibits the same lines as the in situ study can be obtained (Fig. 5). From litterature data (Ref. 14,41,42), a polyenic molecule lacking CH_3 groups can be proposed ; similar spectra are observed for synthetic trans-polyacetylene (Ref. 14,43). If we consider that the polyene chain in acetone is free of any perturbation, a length of 11 double bonds in trans-configuration without important interactive terminal groups can be proposed.

The changes in shape, relative intensity and wavenumber for the two more intense bands in passing from one specimen to another can be related to the observed colour ; they shift to lower wavenumber if the colour goes from yellow, to orange to violet. Different chain lengths or a polarisation of the π-electron system can explain both the colour and the observed spectral shift. By varying the position of a charged group about the chain, a gradual change in spectral properties and colour can be achieved.

For the blue coral Favonia vaughanii, an astaxanthin-protein complex has been determined after an EDTA digestion of the calcareous skeleton (Ref. 40).

Live bacteria and algae can be studied by using RR techniques (Ref. 44). Sixteen types of carotenoid containing microorganisms have been studied in aqueous suspension using rapid flow techniques through a capillary (Ref. 45) ; but use of the techniques of laser microanalysis developed by Delhaye and Dhamelincourt (Ref. 6,7) enable one to obtain good vibrational spectra of pigments from a single living cell.

The laser beam which excites the Raman scattering can be focussed onto a very small spot (2 μm^2) on the component of the sample to be analysed through a microscope objective ; the same objective collects the scattered light which is analysed by a spectrometer. In order to minimise thermal effects which can destroy the cell components, the illumination of the sample must be performed with care either by defocussing the laser beam or by using a global illumination system (Ref. 8). The first result obtained by this technique is for Pyrocystis lunula which is known to contain a peridinin-chlorophyll-protein complex (Ref. 46). By illuminating the cytoplasm of a single cell, the RR spectrum of peridinin was recorded. The characteristic bands are superposed onto a very strong fluorescence emission assigned to the chlorophyll (674 nm) and luciferin (518-542 nm) (Fig. 6).

Chromatosomes of Palaemon serratus exhibit yellow, red and blue parts which have been investigated in vivo by RR spectroscopy. From ν_1 and ν_2 lines a trans-carotenoid molecule has been

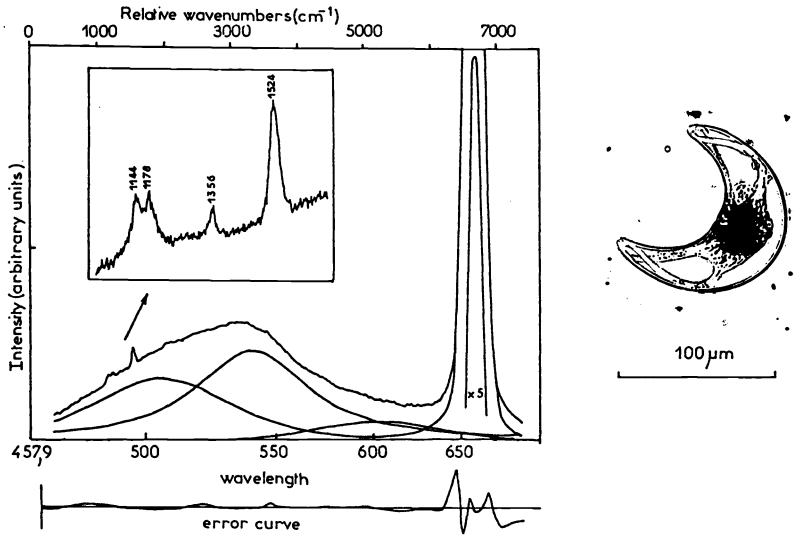

Fig. 6. In vivo fluorescence and RR spectrum (frame insert) of single Pyrocystis lunula cell (right) with 457.9 nm excitation.

unambigously identified but no lines assignable to other cell components are observed (Fig. 7) The RR spectrum of the yellow pigment is very similar to that obtained from a solution of astaxanthin in acetone. The wavenumber shifts for the ν_1 line and the new weak spectral features observed in the RR spectra of the red and blue parts characterise carotenoproteins which are present in the same chromatosome (Ref. 47). This example illustrates well the potential of the method ; the fresh collected sample was placed directly in sea water under the microscope objective without other preparation.

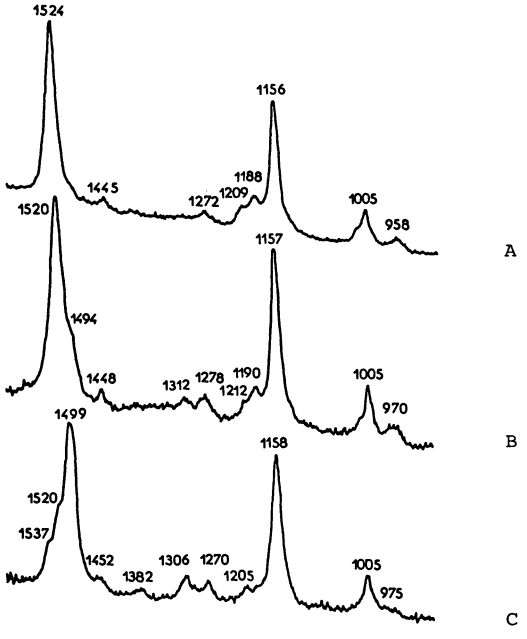

Fig. 7. In situ RR spectra of Palaemon serratus chromatosomes with 514.5 nm excitation (8 mW) ; A-yellow part ; B-red part ; C-blue part.

Other applications by several groups show that this technique is very useful in characterizing and in studying cellular chromophores in live conditions. RR spectra of visual pigments recorded directly from a single photoreceptor cells (Ref. 48) allow the in situ analysis of photostationary steady-state mixtures. Spectra of a plant-cell wall (pimento) exhibit characteristic lines of carotenoid (Ref. 49). A promising extention of this area is the use of a new multichannel Raman spectrometer (Ref. 7) which leads to a significant gain in time recording.

PROSPECT

Only a limited number of applications have been reviewed, but thanks to the characteristic RR properties of carotenoid molecules which can be detected as traces in complex materials even under live conditions, many domains of study can be considered. This research area is still being developed and needs some improvement both in the technology of Raman spectrometry and in the knowledge of the spectroscopic properties of carotenoids. Collaboration between spectroscopists, biochemists and biologists could lead shortly to a increasingly more powerful analytical method.

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