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Chemistry

Coherent anti-Stokes Raman scattering (CARS) spectra, with resonance enhancement, of cytochrome *c* and vitamin B_{12} in dilute aqueous solution

(resonance Raman spectroscopy/non-linear optics)

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ABSTRACT **Coherent anti-Stokes Raman scattering (CARS) spectra have been obtained for ferrocytochrome *c* and cyanocobalamin in aqueous solution at millimolar concentrations, using a pair of tunable dye lasers pumped by a pulsed nitrogen laser. Resonance enhancement was obtained by tuning the ω_1 laser to the visible absorption bands of the samples. The spectral features correspond to those observed in the conventional resonance Raman spectra. It appears that CARS spectroscopy, with its advantageous fluorescence rejection, can be usefully applied to biological samples by exploiting resonance enhancement. While the background scattering from water is 10 times higher than that of benzene and other aromatic solvents, it is actually at the low end of the scale for most liquids. The anomalously low background of aromatic liquids is thought to result from competition by the unusually efficient stimulated Raman scattering which they display. Off-resonance spectra for both cobalamin and cytochrome *c* contain negative peaks, i.e., absorption bands in the background. These are interpreted as inverse Raman processes induced by the ω_1 photons in the presence of the continuum provided by the background scattering. While both CARS and the inverse Raman effect are subject to resonance enhancement, the wavelength dependence of CARS is evidently steeper.**

The generation of Raman spectra by the nonlinear four-photon mixing process of coherent anti-Stokes Raman scattering (CARS) offers some significant advantages over conventional methods employing continuous laser excitation (1). An important one is its ability to spatially eliminate the underlying fluorescence background which often degrades or completely obscures the ordinary Raman spectrum of many materials, especially biological samples (2). This consideration is of particular importance in the case of resonance Raman spectroscopy, where the excitation frequency is chosen to correspond to an electronic transition of the scattering molecule. Resonant excitation produces a substantial enhancement of the scattered intensity of the vibrational modes associated with the chromophore. At the same time, however, the probability of exciting fluorescent states is also increased. Resonance Raman spectra have been obtained for several biological chromophores (3), but many applications have been frustrated by high fluorescence levels.

From its initiation, CARS was believed to be susceptible to resonance enhancement (4) similar to that of resonance Raman spectroscopy. The recently reported CARS spectrum of diphenyloctatetraene in benzene (5) shows appreciable preresonance enhancement. We report here resonance CARS spectra of ferrocytochrome *c* and cyanocobalamin (vitamin B_{12}) obtained in dilute aqueous solution. The CARS and resonance Raman spectra show the same resonance enhancement of porphyrin bands, but the CARS spectra are devoid of the fluorescent background of the resonance Raman spectra.

Coherent anti-Stokes emission results from the interaction, through the third-order molecular polarizability, of two laser photons of frequency ω_1 and one laser photon of frequency ω_2, producing a fourth photon, ω_3, of frequency $2\omega_1 - \omega_2$. A Raman emission occurs whenever

$$\omega_1 - \omega_2 = \Delta\omega$$

where $\Delta\omega$ is a Raman-active vibrational frequency. The energy level scheme describing this process in detail has been reproduced elsewhere (1). Additionally, conservation of momentum requires the wave vector equality

$$2k_1 - k_2 = k_3$$

where $k_i = \omega_i n/c$, n is the index of refraction of the interaction medium, and c is the velocity of light. This requirement is fulfilled if the two incident beams intersect at a small angle within the solution. The generated anti-Stokes beam, ω_3, is spatially distinct and easily separable from the other two.

The efficiency of the CARS process is a function of the third power of the incident laser intensity; it is therefore advantageous to use pulsed sources with high peak power. The conversion efficiency is also proportional to the square of the third order polarizability which contains the Raman cross section (1, 6). Enhancement of the CARS efficiency in dilute absorbing solutions is believed to occur when the ω_1 and/or ω_3 frequencies are in resonance with electronic transitions of the molecule.

Background scattering

While CARS efficiency is much greater than that of normal Raman scattering, its sensitivity is limited by structureless background emission arising from the nonresonant component of the third-order electronic susceptibility. In dilute solutions, this background level is due to the solvent molecules. The CARS spectrum of water has been reported (7). It shows vibrational structure at high frequency and a nonresonant baseline tailing into the low frequency region. We have estimated backgrounds for a number of solvents using $\lambda_1 = 1/\omega_1 = 4800$ Å, setting $\omega_1 - \omega_2$ to a value where none of the liquids have vibrational bands, and adjusting the crossing angle for proper phase-matching. The following levels, relative to water = 1.0, were obtained: methylene chloride, 2.6; chloroform, 2.6; carbon tetrachloride, 2.6; tetrahydrofuran, 2.3; ethanol, 1.8; methanol, 1.0; D_2O, 1.0; benzyl chloride, 0.12; *m*-xylene, 0.09; toluene, 0.09; benzene, 0.09.

We suggest that the anomalously low background levels of the aromatic solvents may be due to competition from stimulated Raman emission (8, 9), which we have observed in ben-

Abbreviation: CARS, coherent anti-Stokes Raman scattering.
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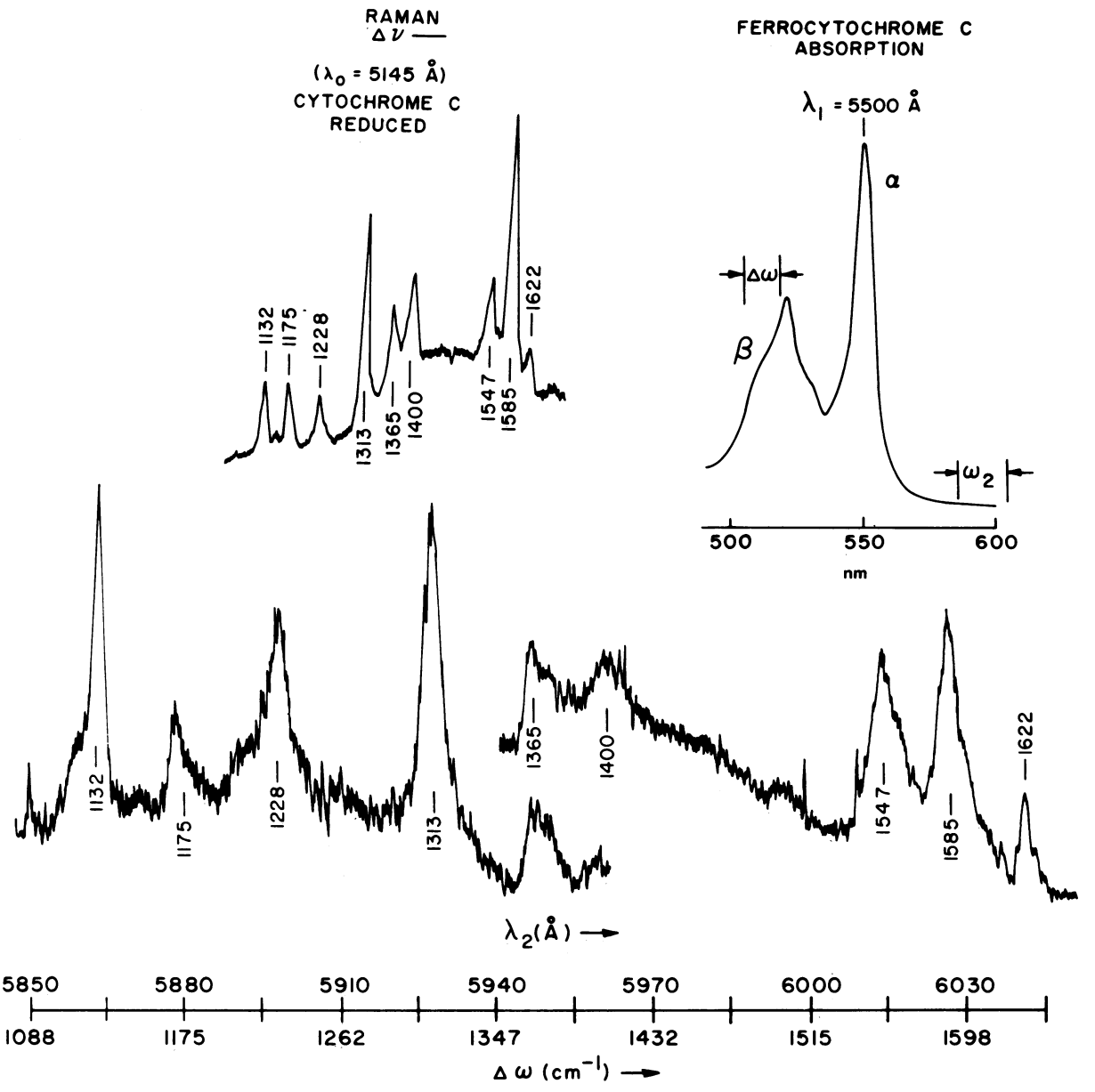

FIG. 1. Resonance CARS spectrum of ferrocytochrome *c*, 1 mM in H_2O. λ_1 = 5500 Å, λ_2 scan speed = 2 Å/min, laser repetition rate = 10 pulses per sec, 30 pulses averaged. The discontinuity in the spectrum is due to the limited tuning range of the apparatus (5). Insets: resonance Raman and visible absorption spectra of the same solution (10).

zene. The stimulated scattering would deplete the pump laser intensity to some extent, causing a decrease in the energy available for coherent anti-Stokes generation. The aromatic solvents are unusually prone to stimulated scattering due to the exceptional intensity and narrow bandwidth of the symmetric breathing mode of the benzene ring at about 1000 cm^{-1} (4). The background level for water is at the low end of the normal range. Nevertheless, the background does seriously limit the sensitivity of CARS for aqueous solutions. For example, the detection limit of sodium benzoate in water, using the intense 1000 cm^{-1} benzene band, is about 0.1 M. It is evident that resonance enhancement is required if CARS is to be applied to dilute aqueous samples (or other dilute nonaromatic solutions).

Experimental

The CARS spectra reported here were recorded using equipment identical to that described in ref. 5. Successful acquisition of resonance Raman spectra requires careful attention to sample concentration and/or optical path length in order to avoid excessive absorption of the incident and scattered light. Absorption tolerances for resonance CARS are even more stringent since reduction in sample width is limited by the interaction length of the incident beams, which is about 1 mm for liquids. Within this constraint, the sample concentration has an optimum value which we have found, by experiment, to be about 1 mM for cytochrome *c* in a cell with a 1 mm path length. This corresponds to an absorbance of 2.8 at the λ_1 wavelength (5500 Å).

Another potential problem, photo-induced damage to the absorbing sample, did not develop with the present experimental arrangement. Although peak power levels are high, about 100 kW, the pulses are short, <10 nsec, and average power levels are low, <10 mW. We observed no chemical

degradation in the samples, even after long periods of exposure to laser pulses.

Cytochrome *c* spectrum

Among lasers currently available, the pulsed nitrogen-tunable dye system has the advantage of the widest tuning range, allowing for the systematic exploration of resonance effects. This system, which is described in detail in ref. 5, was used to record the CARS spectra of ferrocytochrome *c* and cyanocobalamin. The cytochrome *c* spectrum is shown in Fig. 1, where it is compared with the conventional resonance Raman spectrum, obtained with 5145 Å excitation. The inset absorption spectrum shows the resonance conditions for the CARS experiment: the λ_1 beam was set at 5500 Å, the center of the α absorption band, and scanning of the λ_2 beam produced a range of anti-Stokes frequencies within the β band. The resonance Raman bands are known to correspond to vibrations of the porphyrin ring. All of these bands are observed in the resonance CARS spectrum, including the inverse polarized bands (*ip*) which are known to arise from A_{2g} porphyrin vibrations and which become allowed only in resonance scattering (10). The CARS spectrum was obtained with unpolarized laser beams; the polarization characteristics of the bands remain to be explored. The somewhat different relative intensities seen in the two spectra are attributable to the different resonance conditions employed. Although ferrocytochrome *c* is generally considered to be nonfluorescent, a broad fluorescent band can be seen to underlie the Raman spectrum. This is absent in the CARS spectrum, whose slightly sloping baseline is due to changing laser power levels with wavelength.

The peak ratio of signal-to-background intensity in the CARS spectrum has a maximum value of about 4. The overall photon flux is substantially higher in the CARS spectrum than in the comparable resonance Raman spectrum; however, the signal-to-noise ratio is lower due to pulse-to-pulse variations in the pump laser intensity. Ratio detection should raise the CARS signal-to-noise ratio above that of the resonance Raman spectrum.

Inverse Raman

CARS spectra of cyanocobalamin reveal an unexpected phenomenon, illustrated in Fig. 2. The observed band at 1500 cm^{-1} is identifiable with the strongest band found in the resonance Raman spectrum. Its shape is normal when λ_1 is close to resonance with the first absorption maximum at 5500 Å. As λ_1 is tuned to longer wavelengths, however, a dispersion line shape develops and eventually the band becomes completely negative; i.e., it is seen as an absorption relative to the background. A dispersion line shape is not unexpected in CARS spectra, due to interference between Raman and background scattering (11). We believe, however, the absorption band in the baseline is due to the onset of the inverse Raman effect (12, 13), which dominates when the solution is transparent at the ω_1 frequency. When ω_1 is intermediate between the absorption and the transmission maxima, the CARS and inverse Raman effects are competitive, accounting for the gradual transition from a positive to a negative band shape. Inverse Raman is an independent nonlinear optical effect which can be observed when a material is simultaneously illuminated by an intense monochromatic field at frequency ω_1 and a relatively broad continuum (12, 13). Absorption bands appear in the continuum at frequencies $\omega_1 + \Delta\omega$, while re-emission occurs at the pump frequency ω_1. This is formally equivalent to the inverse of spontaneous Raman scattering.

In the present case observation of inverse Raman is made possible by the presence of the non-resonant coherent anti-Stokes background level, which functions as the continuum. We have also detected the presence of additional inverse Raman absorption bands at 1172 cm^{-1} and 1208 cm^{-1} in the cobalamin spectrum when ω_1 is away from resonance. All of these bands are polarized in the resonance Raman spectrum (14). Inverse Raman bands also occur in the spectrum of ferrocytochrome *c* when ω_1 is on the low energy side of the α absorption band. In this case, all the bands of the resonance Raman spectrum appear, with the exception of the inverse polarized modes at 1132, 1313, and 1585 cm^{-1}. From a recent report (15) of inverse Raman spectra observed for dilute solutions of highly fluorescent dyes, it is evident that the inverse Raman effect is also subject to resonance enhancement. Our results imply, however, that the dependence on laser wavelength is different for inverse and CARS amplitudes. CARS dominates at resonance while the inverse Raman effect dominates off-resonance.

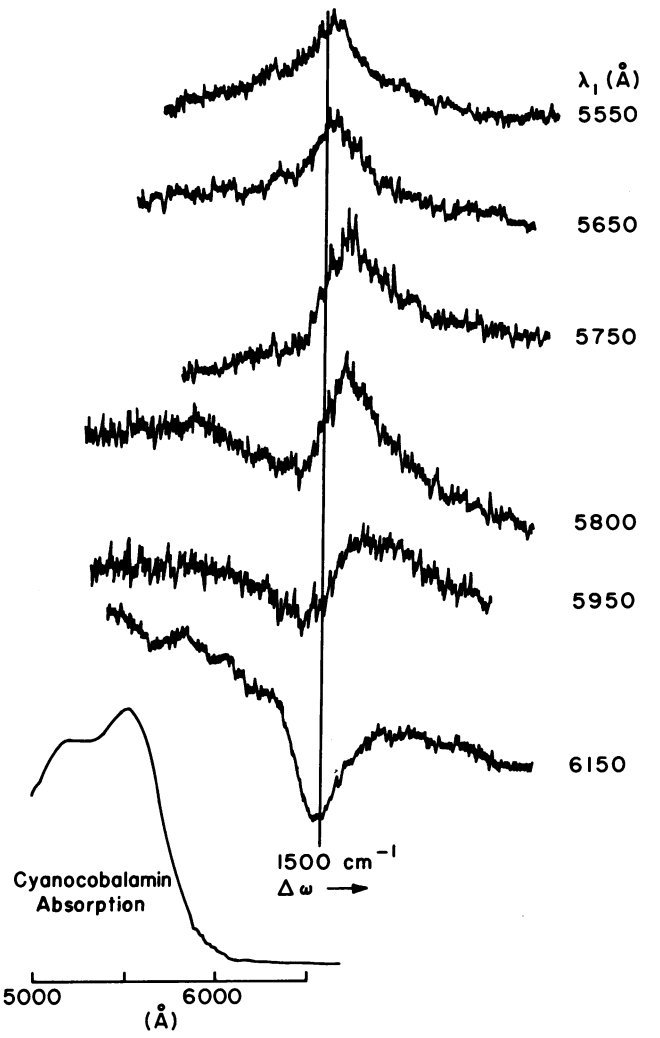

FIG. 2. Resonance CARS scans of the 1500 cm^{-1} band of cyanocobalamin, 1 mM in H_2O, as a function of λ_1 wavelength. λ_2 scan speed = 7.5 Å/min, laser repetition rate = 10 pulses per sec, 10 pulses averaged.

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