Conclusions

From theoretical considerations, one would expect that the Fermi contact term would be dominant and that orbital and dipolar contributions would be small for light nuclei.[59] Thus, one might expect observations in proton–proton couplings would find analogy with carbon–proton and carbon–carbon couplings. The amount of carbon–proton and carbon–carbon data that is available does in fact show numerous parallels, and the similarity between J_{HH} values and J_{CH} and J_{CC} values is sufficiently clear to suggest that the use of J_{HH} values may be increasingly important to help establish correlations between J_{CH} and J_{CC} values and molecular structure.

Note Added in Proof. Some recent high-resolution work with furan[60] indicates some proton–carbon coupling assignments in five-membered heteroaromatic compounds should be reversed (see ref 33).

We wish to thank Dr. Michael Barfield for helpful discussions and for recording the carbon nmr spectra of some of our labeled compounds. We further acknowledge with pleasure the support of the Robert A. Welch Foundation (Grant No. B-325) and of North Texas State University Faculty Research.

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Resonance Raman Spectroscopy: a New Structure Probe for Biological Chromophores

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Received April 1, 1974

The central challenge of modern biochemistry is to elucidate biological function in terms of molecular structure. A considerable catalog of information on biomolecular architecture is already available from X-ray diffraction studies on crystalline or partially ordered materials. Numerous spectroscopic methods have been introduced to monitor structural features and detect changes which accompany biological function. Among them, vibrational spectroscopy offers high promise, since vibrational frequencies, available from Raman or infrared spectra, are sensitive to geometric and bonding arrangements of localized groups of atoms in a molecule.

The study of vibrational spectra has played a leading role in structural investigations of small molecules, and a substantial body of systematic knowlege has been formed.[1] Application to biological materials is beset with difficulties, however. Water, the ubiquitous biological medium, is an excellent absorber of infrared radiation, leaving only restricted "windows" for infrared spectroscopy. These can be somewhat extended by using D_2O as well as H_2O solutions.[2] Raman spectroscopy does not suffer as much from this limitation, since water is a poor Raman scatterer. Lasers now provide the high light power density required for Raman spectroscopy and allow examination of minute quantities (microliters) of material. The chief obstacle encountered with biological materials, however, is their complexity. A molecule containing N atoms has $3N - 6$ ($3N - 5$ for linear molecules) normal modes of vibration. The macromolecules of biology contain thousands of atoms and have far too many vibrational frequencies to be resolved, let alone assigned in a normal Raman or infrared spectrum. Fortunately these frequencies tend to group themselves into more-or-less discrete bands, which can be identified with certain classes of structure. These bands can then be used to monitor changes in gross conformation, and this technique has been fruitfully applied to proteins, nucleic acids, and lipids.[3,4]

If one is interested in structural features of a specific site of biological function within a macromolecule, then the myriad vibrations of the whole molecule are a serious interference. What is needed is a selective technique that samples only the vibrations of the atoms in the vicinity of the site. This can be provided by *resonance* Raman spectroscopy, if the atoms in the site give rise to an isolated electronic absorption band. A normal Raman spectrum is obtained by illumination of the sample in a transparent region of its spectrum. In resonance Raman spectroscopy, the illumination is within an absorption band. Most of the Raman bands are attenuated by the ab-

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sorption, but some bands may be greatly enhanced. This effect is due to a coupling of electronic and vibrational transitions, and the vibrational modes which are subject to enhancement are localized on the grouping of atoms which give rise to the electronic transition, *i.e.*, the chromophore. Resonance Raman spectroscopy therefore provides a means of monitoring vibrational frequencies of a chromophore, independent of its (nonabsorbing) matrix. Since biological chromophores (hemes, flavins, metal ions, etc.) are usually at sites of biological function, the technique offers high promise as a new probe of biological structure.

While its application to biology is new, the resonance Raman effect has long been familiar to physicists. Resonance enhancement was implicit in the treatment of Raman scattering theory given by Van Vleck[5] and by Placzek,[6] some 40 years ago, and has been the subject of several experimental studies in the interval.[7-9] Only recently, however, have multifrequency, and now tunable, lasers become available, with which one can deliberately tune in to the absorption bands afforded by biological chromophores. This Account provides a brief introduction to the principles of the technique and to some recent applications, particularly to heme proteins, which have proven to be fertile subjects for study.

Characteristics of Resonance Raman Scattering

The Scattering Process. Raman spectroscopy involves a light-scattering experiment in which the frequency of the scattered light is analyzed. Most of the scattered light emerges at the incident frequency (Rayleigh scattering), but occasionally a photon scatters inelastically from a molecule and is shifted from its original frequency by a quantum of energy corresponding to a molecular transition of the sample. The transition may be translational (*e.g.*, lattice modes of a crystal), rotational, vibrational, or electronic in nature. All of these processes have been detected in Raman scattering, but we are here concerned with the vibrational effect, a schematic representation of which is given in Figure 1. A photon can lose energy by raising a molecule to an excited vibrational state, or gain energy by inducing the reverse process, producing Stokes or anti-Stokes lines, respectively, in the Raman spectrum. Since the fraction of molecules occupying excited states decreases exponentially with increasing energy, the intensity of anti-Stokes lines falls off rapidly with increasing frequency shift.

Resonance enhancement of Raman bands comes into play when the energy of the incident light approaches that of an electronic transition. If the photon is actually absorbed in the electronic transition and then reemitted, the process is called fluorescence. The conceptual distinction between scattering and fluorescence under resonance conditions (having to do with the lifetime of the photon–molecule complex[10]) is subtle, but for practical purposes it is easy to distinguish the two processes by varying the exciting frequency. For Raman scattering the frequency shifts are independent of the exciting frequency, while for fluorescence, the absolute frequency remains constant. Moreover in liquids, fluorescence almost always occurs in a broad spectral band, because of molecular perturbations during the lifetime of the excited state while Raman bands are sharp. For this reason fluorescence can easily obscure resonance Raman spectra.

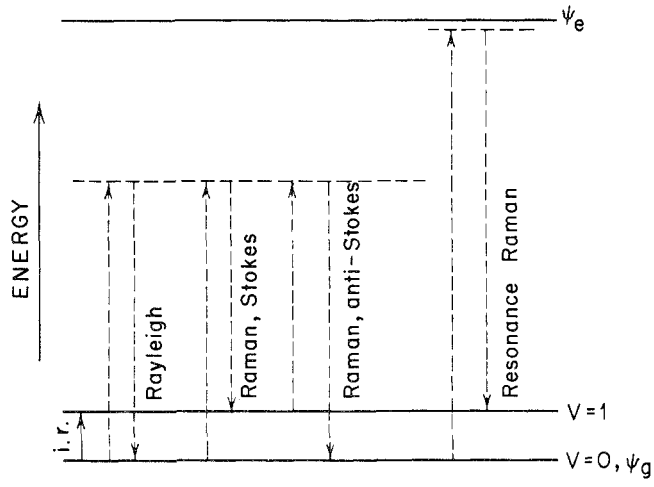

Figure 1. Energy level scheme illustrating the vibrational Raman scattering process.

A promising approach to reducing such interference is to time-resolve the emitted light, using pulsed laser sources. Because of the short lifetime of many fluorescence processes, however, there are practical difficulties in devising an effective "fluorescence filter."[11] Fortunately many biological chromophores have low fluorescence yields, thanks to effective quenching mechanisms.

Raman Intensities. The total intensity of a Raman line for randomly oriented molecules is given by

$$I = \frac{2^7\pi^5}{3^2c^4} I_0 \nu_s^4 \sum_{ij} |\alpha_{ij}|^2 \qquad (1)$$

where I_0 is the intensity of the incident light, ν_s is the frequency of the scattered light, and α_{ij} is an element of the scattering tensor.[12] This tensor is viewed classically as the molecular polarizability (Rayleigh scattering) or its derivative with respect to the nuclear displacements (Raman scattering). Its quantum mechanical expression, through second-order perturbation theory, is given by the Kramers–Heisenberg–Dirac dispersion equation[12]

$$(\alpha_{ij})_{mn} = \frac{1}{h}\sum_e \left[\frac{(M_j)_{me}(M_i)_{en}}{\nu_e - \nu_0} + \frac{(M_i)_{me}(M_j)_{en}}{\nu_e + \nu_s}\right] \qquad (2)$$

Here m and n are the initial and final states of the molecule, while e is an excited state, and the summation is over all excited states. The quantities $(M_j)_{me}$ and $(M_i)_{en}$ are electric dipole transition moments, along the directions j and i, from m to e and

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from e to n, while ν_e is the frequency of the transition from m to e, and ν_o and ν_s are the frequencies of the incident and scattered photons. In the nonresonance region $\nu_o \ll \nu_e$, and α_{ij} is independent of the exciting frequency. As ν_o approaches ν_e, however, α_{ij} is subject to preresonance enhancement through the first term on the right-hand side of eq 2. When $\nu_e - \nu_o$ becomes very small (it is prevented from reaching zero by inclusion of a damping constant to allow for a finite electronic line width), then one element in the summation, corresponding to the resonant electronic transition, dominates all others, assuming that the transition moments are sizable. When $\nu_e \gg \nu_o$ the resonance enhancement again decreases.

Equation 2 is unspecific about the initial and final states of the molecule and therefore gives no information as to which vibrations are subject to resonance enhancement. To examine this question it is customary to use the adiabatic approximation (separability of electronic and vibrational wave functions).[13] One can then expand the electronic wave function in a Taylor series of the nuclear displacements, using the Herzberg–Teller formalism.[12] When this is done, it becomes apparent that the vibrations which experience resonance enhancement are similar to those which (in the excited states) lend intensity to the electronic absorption spectrum.[12] Consequently resonance Raman spectroscopy can be considered as a form of high-resolution vibronic spectroscopy.

An alternative approach is to apply third-order time-dependent perturbation theory to the scattering process.[14] The results are similar to those stemming from the Herzberg–Teller approach, and the most convenient formulation[15] is

$$\alpha_{ij} = \frac{1}{h}\sum_{e,f}\left[\frac{(M_j)_{ge}h_{ef}{}^{\Delta\nu}(M_i)_{fg}}{(\nu_e - \nu_o)(\nu_f - \nu_s)} + \frac{(M_i)_{ge}h_{ef}{}^{\Delta\nu}(M_j)_{fg}}{(\nu_e + \nu_s)(\nu_f + \nu_o)}\right] \quad (3)$$

where the summation is over all excited electronic states e and f, taken in pairs, and g is the electronic ground state. Here $h_{ef}{}^{\Delta\nu}$ is a vibronic coupling matrix element, connecting states e and f by a particular vibration $\Delta\nu$. Some terms which are expected to be of lesser magnitude have been omitted from the full equation[14] for α_{ij}.

Two kinds of resonance process are expected to be important, corresponding to the conditions e = f and e ≠ f in eq 3, which Albrecht and Hutley[16] have called *A* and *B* terms, respectively. *A* terms involve vibrational interaction with a single excited electronic state, through the Frank–Condon overlaps. Only for totally symmetric vibrations are these overlaps nonzero for both the initial and final states of the molecule. *B* terms involve vibronic mixing of two excited states, e and f. The active vibrations may have any symmetry which is contained in the direct product of the two electronic transition representations. Moreover, *A* and *B* terms may be distinguished by the excitation frequency dependence of the preresonance enhancement.[16]

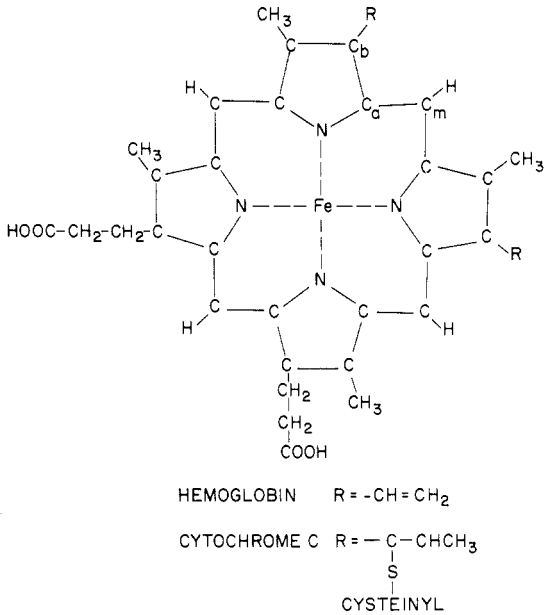

Figure 2. Structure of heme, indicating pyrrole substituents which occur in hemoglobin and cytochrome *c* (from ref 17).

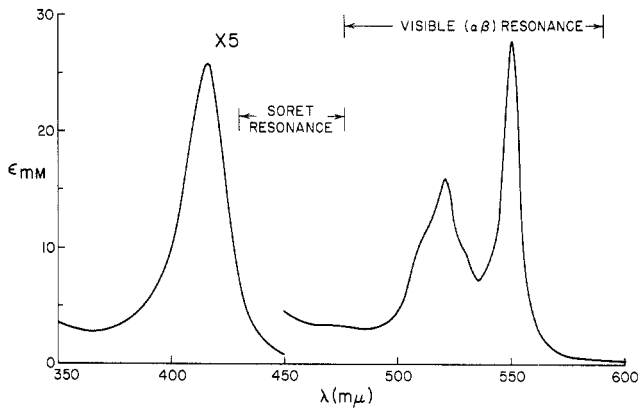

Figure 3. The near-uv (Soret) and visible (α–β) absorption spectrum of ferrocytochrome *c*. Arrows span the approximate regions in which resonance with each of the two kinds of optical transitions dominates the Raman spectrum (from ref 17).

Heme Scattering. These characteristics are nicely illustrated by the resonance Raman spectra of heme proteins,[17] whose chromophore is an iron porphyrin complex (Figure 2). The Raman spectra are dominated by the porphyrin vibrational modes which are enhanced by resonance with the allowed electronic transitions in the visible and near-ultraviolet region. These are π–π^* transitions,[18] polarized in the porphyrin plane, and the strong Raman bands are at frequency shifts (1000–1700 cm^{-1}) appropriate for in-plane stretching vibrations of the ring. Within this set different bands are enhanced depending on whether the excitation wavelength lies in the vicinity of the visible (α and β) or near-ultraviolet (Soret) bands. Figure 3 shows a typical heme absorption spectrum, that of reduced cytochrome *c*, while Figure 4 illustrates the alteration in the Raman spectra of oxy- and deoxyhemoglobin on changing the excitation wavelength.

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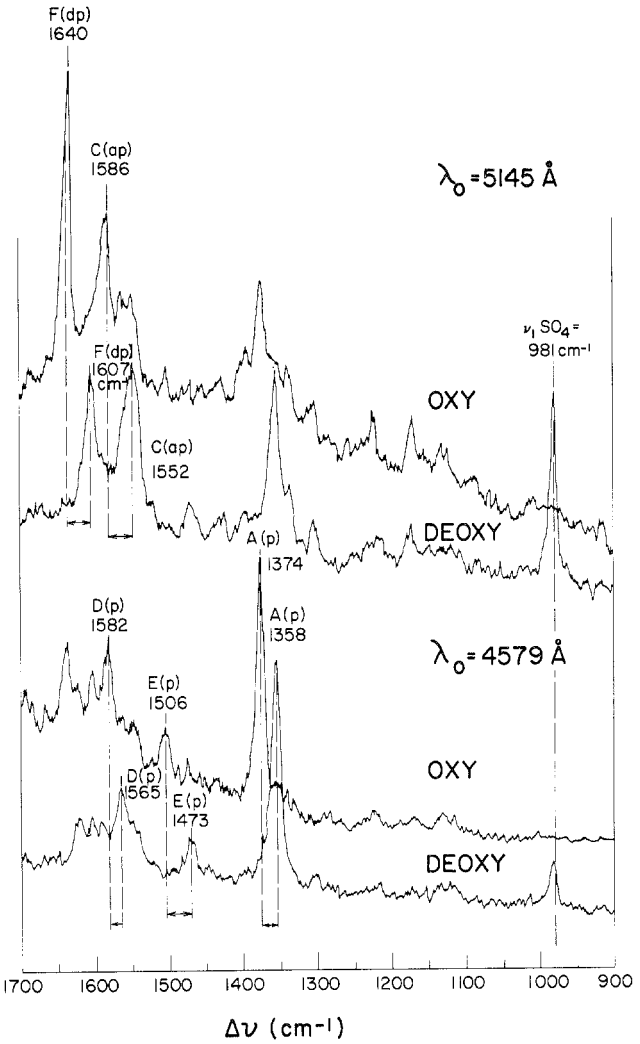

Figure 4. Resonance Raman spectra of oxy- and deoxyhemoglobin in the α-β (λ_0 5145 Å) and Soret (λ_0 4519 Å) scattering regions. The solutions were 0.68 and 0.34 mM in heme for oxy- and deoxyhemoglobin, respectively, and the latter contained 0.4 M $(NH_4)_2SO_4$, the $\nu_1(SO_4^{2-})$ band (981 cm^{-1}) of which is indicated. Frequency shifts for corresponding bands are marked by the arrows (from ref 17).

The modes which are in resonance with the intense Soret band are all of type A, and are totally symmetric.[19,20] No A terms are seen in the α-β region, presumably because the absorption (transition moment) is much lower in the α than in the Soret band. A rich variety of vibronic, type B, modes are observed,[21] however. The β band is known to be a vibronic side band on the α band, the result of vibronic mixing between the α and Soret transitions.[18] Both are of E_u symmetry, under the effective D_{4h} point group of the chromophore, and the allowed symmetry of the vibronically active modes is $E_u \times E_u = A_{1g} + B_{1g} + B_{2g} + A_{2g}$. The A_{1g} modes, however, have been shown to be ineffective in vibronic mixing.[22] Indeed all of the bands which are in-resonance with the α-β bands are either depolarized (B_{1g} or B_{2g}) or are anomalously polarized, the latter having greater intensity in the perpendicular than in the parallel scattering component. Anomalously polarized bands have contributions from A_{2g} modes, which have antisymmetric scattering tensors, *i.e.*, $\alpha_{xy} = -\alpha_{yx}$. Observation of these bands[21] in hemoglobin and cytochrome c was the first experimental confirmation of antisymmetric vibrational scattering, which is forbidden in the nonresonance region, although the phenomenon was predicted 40 years ago by Placzek.[6] Recent experiments[23,24] have also confirmed Placzek's suggestion,[6] recently reemphasized by McClain,[25] that antisymmetric scattering can be measured directly using circularly polarized as well as linearly polarized illumination.

Equation 3 predicts resonance maxima at both the incident and the scattered frequency. This prediction has been confirmed for heme proteins for the visible electronic transition. The plot of intensity *vs.* excitation wavelength, called the excitation profile, peaks at the center of the α band,[26] *i.e.*, the position of resonance of the incident frequency, $\nu_e = \nu_0$. As the excitation frequency is increased into the region of the β band, the Raman intensity increases again and reaches a second maximum, which, however, lies at increasingly higher frequency with increasing vibrational frequency of the individual Raman bands.[21] The intensity maxima are at positions corresponding to the sum of the electronic and vibrational frequencies, $\nu_e + \Delta\nu$. This is just the position of resonance of the scattered frequency, $\nu_e = \nu_s = \nu_0 - \Delta\nu$.

Applications

Heme Structure. The dominant features of the resonance Raman spectra of heme proteins are porphyrin ring modes in the 1000–1700-cm^{-1} region.[17] Vibrations involving the iron atom are unfortunately not strongly enhanced, presumably because the resonant π-π^* transitions are largely localized on the porphyrin ring.[18] Assignment of the numerous ring modes is difficult, since they contain contributions from many internal coordinates. They can be readily catalogued, however, *via* their three different states of polarization, and overlapping bands can often be resolved by varying the excitation wavelength to take advantage of their differential enhancement. Consequently corresponding modes can reliably be correlated from one heme derivative to another.[17]

Figure 5 shows a frequency correlation diagram for a series of heme protein derivatives, chosen for their well-characterized spin (high spin or low spin) and oxidation (Fe(II) or Fe(III)) states. The hemoglobin spectra display more bands than do those of cytochrome c, probably because of the two peripheral vinyl substituents on protoporphyrin IX, known to be conjugated with the ring, which are replaced in cytochrome c by thioether links to the protein (Figure 2). The vinyl groups may themselves be responsible for one or two high-frequency vibrations, and can also induce Raman activity into infrared-active modes (E_u) since their asymmetric disposition destroys the center of symmetry of the chromophore. Most of the frequencies remain nearly constant for all the derivatives, but a few display substantial

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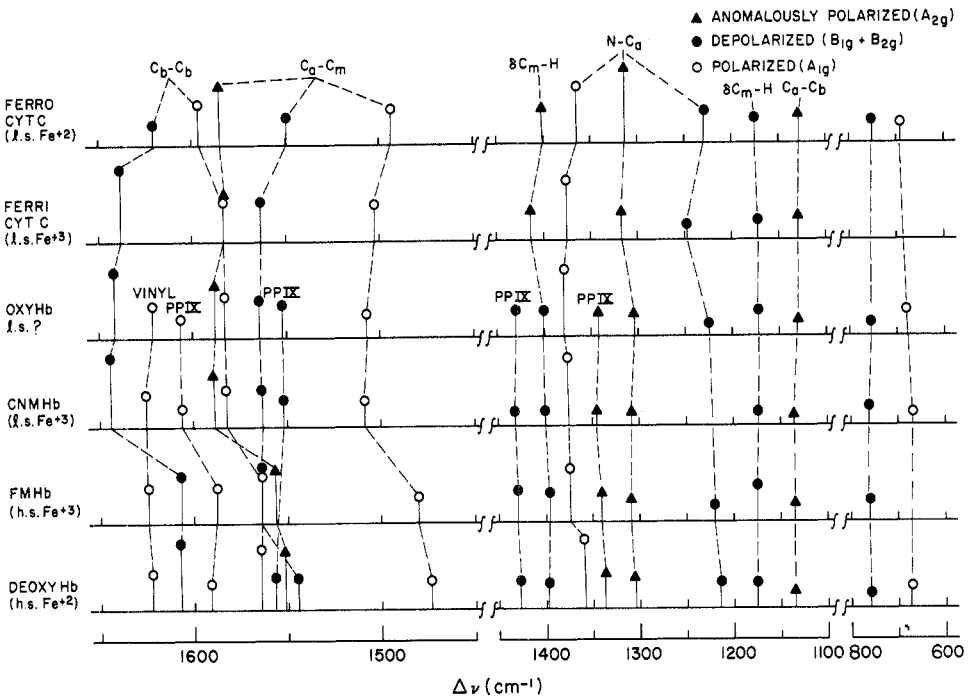

Figure 5. Correlation diagram for resonance Raman bands of hemoglobin and cytochrome *c*. Spin and oxidation states of the various species are indicated. See text for discussion of the oxyhemoglobin oxidation state. The lengths of the solid lines are roughly proportional to the observed relative intensities at 5145 Å excitation (4965 Å for FMHb) for anomalously polarized (▲) and depolarized (○) bands, and at 4579 Å for polarized (○) bands. Suggested assignments (approximate) to various heme internal coordinates are indicated at the top. The bands marked PPIX (protoporphyrin) and VINYL are observed for hemoglobin derivatives only (from ref 17).

shifts. Conversion from Fe(III) to Fe(II) without change in spin state produces a slight but systematic lowering of the ring frequencies (compare ferri- with ferrocytochrome *c* (low spin), and fluoromethemoglobin with deoxyhemoglobin (high spin)), suggesting a concomitant weakening of the bonds in the ring. Such weakening may be attributable to population of π^* antibonding ring orbitals *via* back-donation of electrons from the iron d_π orbitals, which should be greater for Fe(II) than for Fe(III).[17]

Conversion from low- to high-spin states, without change in oxidation state, produces pronounced frequency lowering for some of the Raman bands (compare cyano (low spin) with fluoromethemoglobin (high spin) (Fe(III)) and ferrocytochrome *c* (low spin) with deoxyhemoglobin (high spin) (Fe(II)). These shifts, which are much larger than those accompanying oxidation-state change, can be related to the alterations in heme structure which are known to accompany spin-state changes.[27] In low-spin hemes, the iron atom, whether Fe(II) or Fe(III), lies in the plane of the four pyrrole nitrogen atoms, but high-spin Fe(II) or Fe(III), in which the antibonding d orbitals are half-occupied, are too large to fit into the central porphyrin cavity. In high-spin hemes the iron atom moves out of the porphyrin plane, and the pyrrole groups tilt, with the nitrogen atoms pointed at the iron atoms, producing a doming of the entire heme group.[27] It is no doubt this doming which produces the observed frequency lowering of the ring modes, through changes both in kinematic coupling and in the force field.

Electron Distribution in Oxy- and Carbonmonoxyhemoglobin. While deoxyhemoglobin contains high-spin Fe(II), oxyhemoglobin is diamagnetic. Over the years a considerable controversy has developed over whether the iron–oxygen complex should be thought of as $Fe^{2+}O_2$ (low spin)[28,29] or as $Fe^{3+}O_2^-$ (low spin, with exchange coupling between unpaired electrons on Fe^{3+} and O_2^-),[30] or even as $Fe^{4+}O_2^{2-}$.[31] It is therefore of some interest that, on the basis of its resonance Raman frequencies, oxyhemoglobin classifies unambiguously as low-spin Fe(III).[17,32] Figure 5 shows that all of its frequencies are nearly the same as those of cyanomethemoglobin. Consistent with this, the O–O stretching frequency, recently located in the infrared spectrum of packed red cells,[33] is at a frequency, 1104 cm^{-1}, characteristic of superoxide.

On the other hand the resonance Raman frequencies of carbonmonoxyhemoglobin are found to be the same as those of O_2Hb, including the band at 1377 cm^{-1}, which is a reliable oxidation-state marker (see Figure 6).[17] Yet the formulation $Fe^{3+}CO^-$ is uncongenial, as the CO^- radical would be highly unstable. Relief of charge on a metal center *via* back-donation to π-acceptor ligands such as CO and O_2 is a familiar concept in inorganic chemistry, however. Indeed, the lowering of the stretching frequency[34] of carbon monoxide on binding to hemoglobin is comparable to that found in transition-metal carbonyls. One need not insist that oxyhemoglobin contains superoxide radical to allow that substantial back-donation to the bound O_2 (or CO) molecule occurs, leaving the iron atom with about the same charge as in low-spin Fe(III) hemes.

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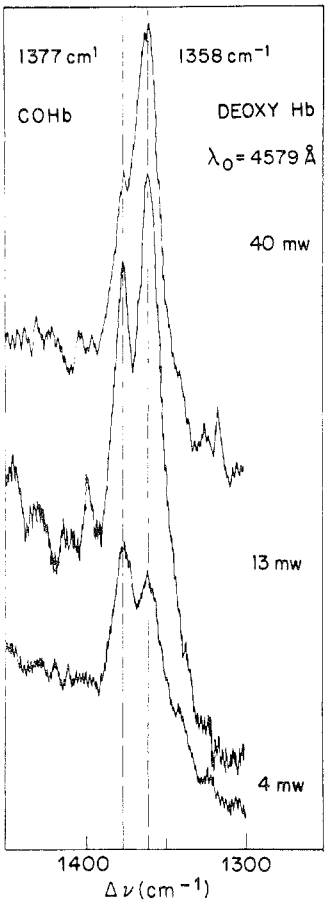

Figure 6. Portion of the Raman spectrum of carbonmonoxyhemoglobin (1377-cm^{-1} peak), showing reversible photodissociation to deoxyhemoglobin (1358-cm^{-1} peak). The sample, 0.66 m*M* COHb with sodium dithionite added, was placed under argon and run in a (sealed) rotating cell, at ~2000 rpm. The changing peak heights correspond to changing ratios of COHb and deoxyHb at the indicated power levels (measured at the sample) of 4579-Å Ar^+ laser radiation. The spectra were recorded in order of *decreasing* power levels, and the absorption spectrum of the solution at the end of the experiment corresponded to that of pure COHb. Photodissociation is less pronounced with longer wavelength excitation (from ref 17).

Other Applications. The number of resonance Raman applications to biological systems is increasing rapidly, and space precludes more than a listing of some of the chromophores for which spectra have recently been reported. Cobalt-substituted hemoglobin gives Raman spectra[35] nearly identical with those of protein-free cobalt porphyrins, suggesting very little doming of the heme group, in contrast to native hemoglobin. Raman spectra of oxidized cytochrome *c'* are indicative of a heme structure intermediate between those of low- and high-spin hemes,[36] as are those of horseradish peroxidase.[37,38] Cytochrome oxidase spectra[20,39] give some indication of inequivalence of heme a and a_3.[39] Chlorophyll[40,41] and vitamin B_{12}[42,43] give spectra similar to those of hemes. Carotenes give intense resonance scattering,[44,45] readily observable *in situ* in plant tissues.[46] Rhodopsin may involve a charge-transfer complex between retinal and aromatic protein side chains, as evidenced by resonance enhancement of both retinal and aromatic amino acid vibrations upon excitation in the rhodopsin absorption band.[47] Transferrin spectra show enhancement of tyrosine modes,[48-50] confirming tyrosine coordination of the bound Fe^{3+}. Resonance Raman spectra of the iron-sulfur proteins rubredoxin[51,52] and adrenodoxin[53] show Fe–S stretching modes, while those of "blue" copper proteins show both Cu–S and Cu–N (or Cu–O) stretching modes.[54] Oxygen bound to hemerythrin[55] and hemocyanin[56] has been shown by the resonance Raman technique to be peroxide-like; an O_2^{2-} group apparently bridges two Fe^{3+} or Cu^{2+} ions, respectively, in the oxyproteins. It is possible to introduce an artificial chromophore, *i.e.*, a "resonance Raman label," into biological sites. Thus the structural consequences of binding methyl orange to bovine serum albumin,[57] and of binding chromophoric haptens to their antibodies[58] have been explored with resonance Raman spectroscopy.

Most of the studies cited in this account have appeared within the last 2 years. It is clear that applications of resonance Raman spectroscopy to biological systems, still in their infancy, are growing rapidly. It should be stressed, however, that the technique is limited to systems with intense absorption at wavelengths corresponding to available laser sources, which currently operate in the red or blue regions of the spectrum. This limitation is being rapidly overcome by the development of lasers with increasingly wide tuning ranges. It will soon be possible to tune Raman spectrometers through the ultraviolet region, where nucleotides and aromatic amino acids absorb strongly. The scope of resonance Raman studies will then become wide indeed.

The author is indebted to his coworkers, whose names appear in the literature cited. This research has been supported by grants from the National Science Foundation and from the National Institutes of Health.

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