Heme proteins and the development of resonance Raman spectroscopy – A personal account

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I recount here the events that led to the serendipitous discovery of inverse polarization and vibronic scattering in heme protein Raman spectra. These findings presaged the rapid development of the well-populated field of heme protein Raman studies. I emphasize the critical roles for discovery of persistence and of timely help from scientific friends.

THE discovery of the Raman effect was announced in 1928. Halfway through the intervening seven decades, I encountered my first Raman spectrometer, when I joined the Princeton faculty in 1963. It was a Cary 80, then state-of-the-art. It stood in the laboratory of Donald Hornig, the chemistry department chair, who had just hired me, but was himself on his way to Washington to become President Johnson's science advisor. I was fresh from a postdoc with Lars Gunnar Sillén, in Stockholm, where I became interested in the halide complexes of thallium [III]. I had taken Richard Lord's spectroscopy course as a graduate student at MIT, so I knew that Raman spectroscopy could be applied as a structure probe to molecules in aqueous solution. I persuaded Steve Kittleberger, Hornig's last graduate student at Princeton, to show me how to fire up the Toronto arc, fill the Wood's sample tube with thallium chloride solution, and run off spectra. To our amazement a huge peak appeared, arising from the Tl–Cl breathing mode, since, luckily for me, the highly polarizable thallium complexes are champion Raman scatterers. Thus was my career as a Raman spectroscopist launched[1].

By then lasers had been commercialized, and the Cary Corp. soon advertized a He–Ne laser attachment for its spectrometer, at a cost of only $20K! I was on the point of finding grant funds to buy one, when George Leroi, then on the Princeton faculty, persuaded me that the money would be better spent toward the construction of a new laser-based spectrometer, using the recently introduced design of the late Sergio Porto, which George proceeded to build. This instrument immediately eclipsed the Cary 80, and kept my career going, because the point-source scattering geometry permitted the study of coloured samples for the first time.

Soon, Thomas Strekas arrived as a graduate student and joined my small group. His project was to study a new class of synthetic oxygen-binding iridium complexes discovered by the inorganic chemist Laurie Vaska, but all these decomposed in the laser beam. In desperation, I asked Strekas to try nature's own oxygen carrier, hemoglobin, with the vague idea that the then-novel resonance Raman effect might produce a detectable signal. His first attempt failed, but fortunately a certified biochemist, Chien Ho, happened by the lab during a seminar visit, and asked what kind of hemoglobin we were using. When I pointed to the reagent bottle of Sigma bovine hemoglobin, Ho winced and told us it was no doubt mostly oxidized. He then explained how to prepare fresh hemoglobin from human blood, and Strekas donated a pint of his own blood to get his thesis going!

The outcome was spectacular, a beautiful spectrum with all kinds of peaks. We had no idea what they meant, especially when Strekas checked the polarizations and found they were the wrong way around; some of the strongest peaks showed up in perpendicular, but not in parallel polarization (Figure 1). Though I scoffed and urged him to change the polarizer, Strekas persisted in his claim. Thus was inverse polarization discovered[2].

It took a long time to figure out what was going on. A chance conversation at a conference with spectroscopist Herb Strauss pointed me to Martin McClain's paper on two-photon tensor symmetries[3], where I discovered that a tensor could be anti-symmetric. Strekas set to work averaging direction cosines, and showed that the polarization expected for such a tensor was indeed inverse. He then read Placzek's classic treatise[4], written forty years earlier, and discovered a footnote in which inverse polarization had been predicted!

What we had stumbled on was a dramatic example of vibronic scattering, offered by the heme chromophore of hemoglobin. Heme is a metalloporphyrin, a class of aromatic macrocycle (Figure 2) with high symmetry and unusual electronic spectra (Figure 3). The main features of these spectra were worked out by Martin Gouterman[5], and are illustrated in Figure 3. These are two closely-spaced HOMOs a_{1u} and a_{2u} and a degenerate pair of LUMO's, e_g. The electronic excitations are of the same symmetry (E_u) and are subject to configuration interaction, with the result that the transition moments add up in the higher energy B (or Soret) transition, and nearly cancel in the lower energy Q (or a) transition.

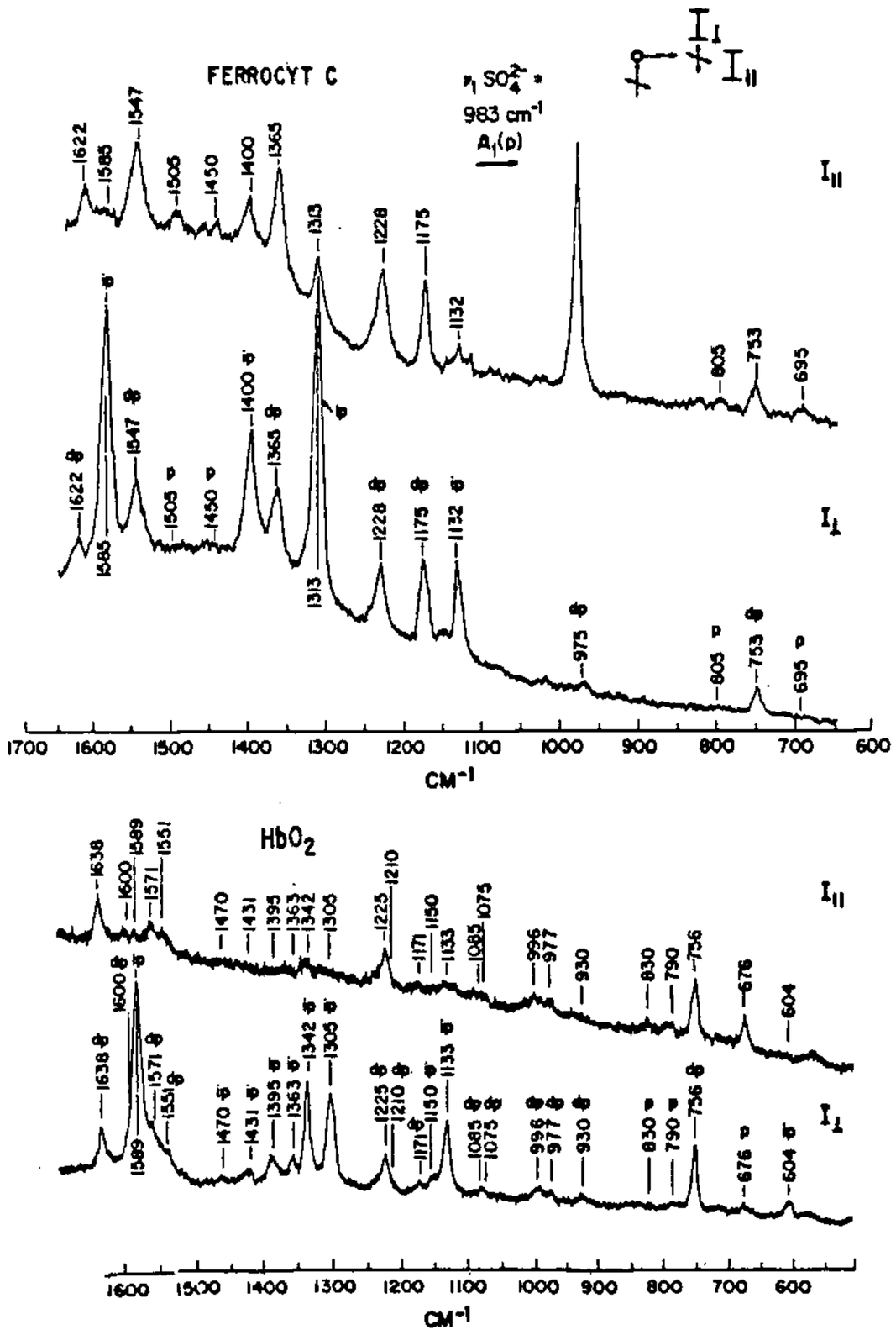

Figure 1. RR spectra of ferrocytochrome *c* (514.5 nm excitation) and of oxy-hemoglobin (568.2 non excitation) showing the parallel and perpendicular scattering components, and illustrating inverse polarization for the strongest bands (from ref. 2).

But the Q transition steals back some (~10%) of the intensity through vibronic mixing, giving rise to a vibronic side-band, Q_v (or β), some 1300 cm^{-1} above Q_0. The mixing vibrations are of B_{1g}, B_{2g} and A_{2g} symmetry. The A_{2g} vibrations have anti-symmetric scattering tensors. Thus Strekas had discovered the A_{2g} vibrations of the heme group.

These vibrations can only be observed on resonance. Scattering contributions from the 0–0 and 1–0 vibrational levels of the excited state interfere destructively for anti-symmetric modes, and they cancel off-resonance[6]. Strekas' discovery depended on our first laser being an Ar^+/Kr^+ mixed gas model (discontinued long since), which had a 568.2 nm line, exactly between the 0–0 and 1–0 wavelength of the Q transition in oxy-hemoglobin.

The mixed gas laser had several lines in the Q band region, permitting Strekas to make a second discovery[2]. The excitation profiles of another heme protein, cytochrome *c*, peaked in the Q_v, region, but at wavelengths

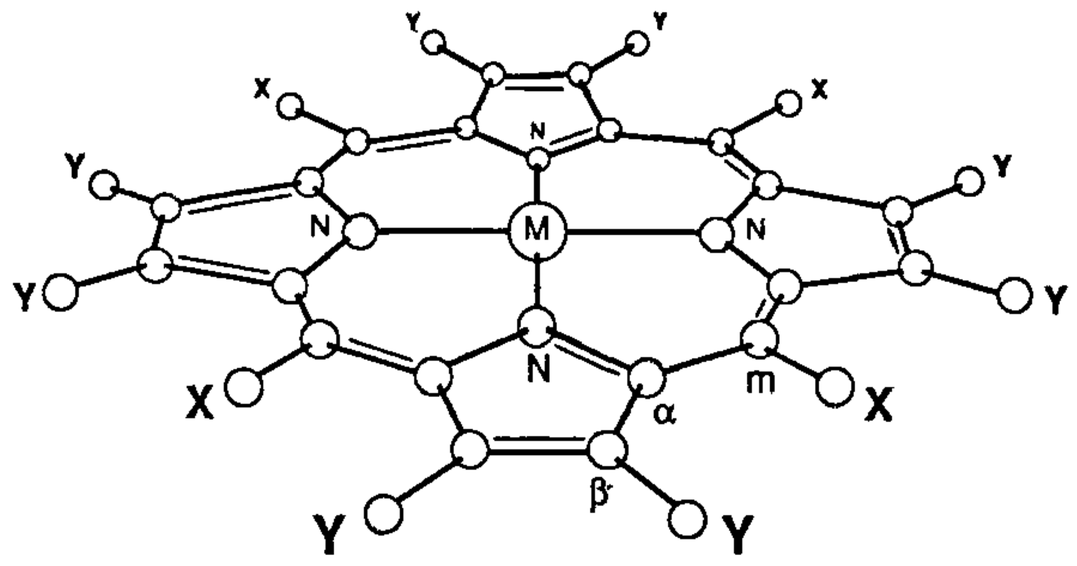

Figure 2. Molecular structure of a metalloporphyrin illustrating the high (D4h) symmetry. In heme proteins, the *X* substituents are *H*, and the *Y* substituents are methyl, vinyl and propionic acid groups.

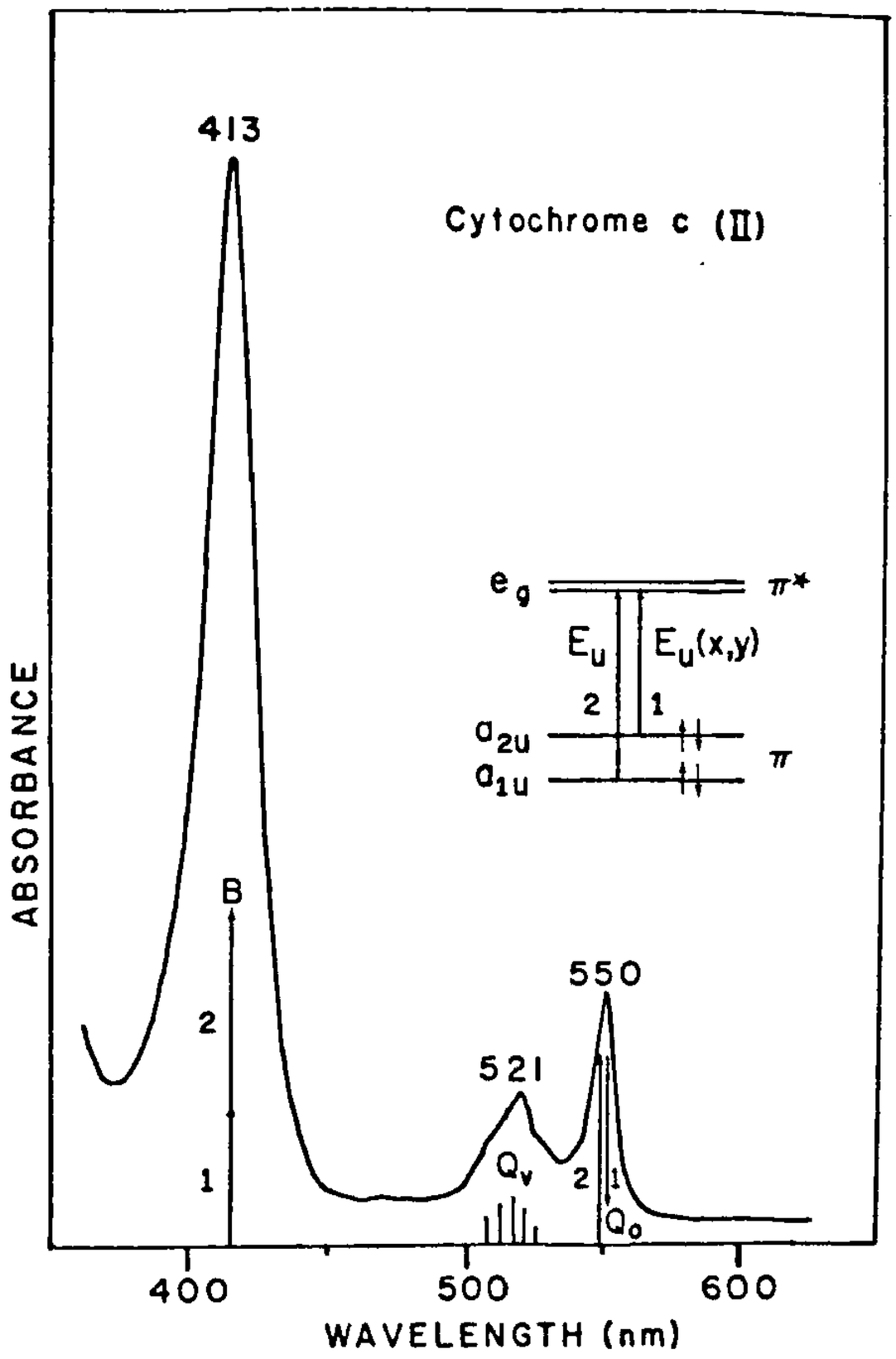

Figure 3. Absorption spectrum of ferrocytochrome *c*, illustrating the orbital excitations that combine to form the porphyrin *B* and *Q* electronic transition. The Q_v band is a collection of vibronic transition, built on Q_0.

which decreased systematically with increasing vibrational frequency of the band being measured (Figure 4). Simply adding the ground state frequency to the Q_0

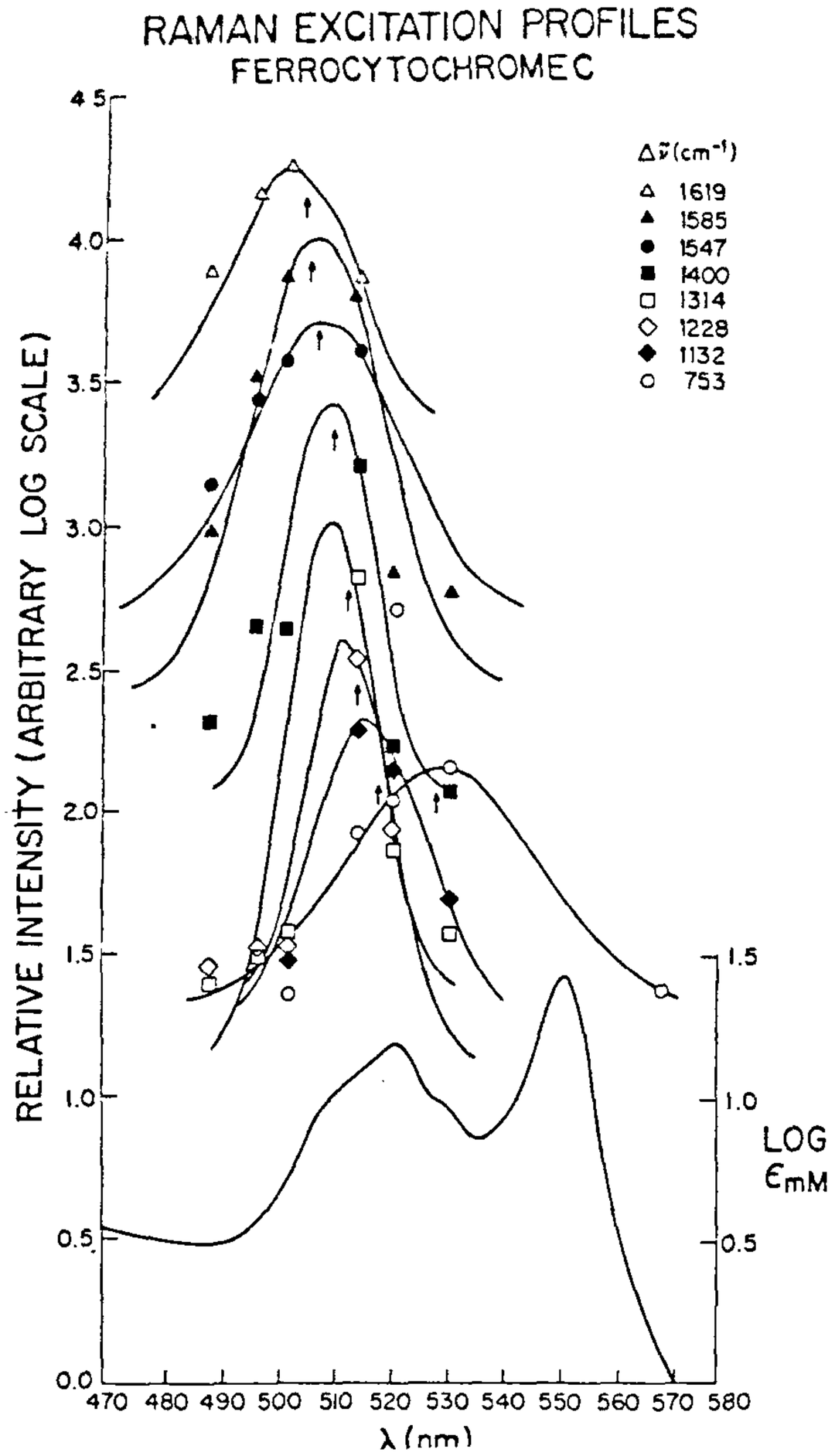

Figure 4. Ferrocytochrome *c* excitation profiles for the indicated RR bands. The arrows mark the wavelengths obtained by adding the (ground state) vibrational frequency to the Q_0 frequency (from ref. 2).

frequency reproduced the excitation profile peak wavelengths quite well. This pattern provided striking confirmation of Albrecht's then-novel theory of vibronic scattering in RR spectra[7]. The excitation profiles are all expected to peak at the Q_0 energy (this was later confirmed[8]), but the Q_v peak is mode-dependent, as each mode comes into resonance with its own 1–0 energy. The actual situation is complicated by multimode effects and multi-state interferences[9], but Strekas' rather sparse excitation profiles managed to get the basic picture right.

These findings generated much interest in the Raman community, and stimulated experiments in many laboratories. Useful spectra–structure correlations were discovered, involving the iron oxidation state, the porphyrin core size, as modulated by the ligation and spin state, and the extent of Fe π back-bonding[10]. Enhancement was discovered for the stretching mode of the iron–histidine bond[11], which is the sole covalent linkage between the heme and the hemoglobin polypeptide chain. Its frequency was found to be sensitive to the functional state of the protein, and its depressed value in deoxy-hemoglobin provided the first direct evidence for molecular tension in the T quaternary state[11], as had been postulated by Perutz[12]. This band has provided a useful probe of protein dynamics, via time-resolved RR techniques[13]. Vibrations of exogenous ligands were discovered, including the physiologically-important ligands CO, NO and O_2 (ref. 14). These vibrational monitors of the bound ligand have weighed importantly in arguments about the binding geometry[15], and are

providing useful information about interactions with the surrounding protein[16].

The porphyrin RR modes have been assigned in detail[17], thanks to their variable sensitivity to excitation wavelengh. They have been used to examine the electronic structure of radical cations and anions, and of excited states[18]. Reliable force-fields have been obtained, most recently using *ab initio* computations[19]. The eigenvectors are accurate enough to calculate RR intensities, with the aid of INDO-level calculations of the excited states[20]. These computational advances make quantitative modelling of heme protein RR spectra a feasible goal.

Princeton's late Hubert Alyea, an education enthusiast who lectured tirelessly around the globe on the wonders of chemistry, always invoked a homily from Einstein, that scientific discoveries require 'lucky accidents and the prepared mind'. At the beginnings of heme protein RR spectroscopy, our minds were hardly prepared, but with the aid of scientific friends, we eventually pieced together the explanation of the strange phenomena on which we happened. It is worth recalling how much science is a genuinely communal enterprise. This is a great source of strength and progress.

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