Laser Raman Spectroscopy of Biopolymers and Proteins

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The introduction of laser sources for Raman spectrometers has greatly increased the number of materials that may be readily studied using Raman spectroscopy. Included among these materials are the synthetic polymers and polypeptides. Although some of the information obtained from laser Raman spectroscopy is also available from infrared spectroscopy, both the method of sample presentation (including aqueous solutions) and the different selection rules suggest that Raman spectroscopy could be useful in studying macromolecules of biological interest. The applications of Raman spectroscopy to polypeptide conformation that have been made so far are discussed, using as examples spectra taken in our laboratory and from the literature.

Vibrational spectra of polyatomic molecules can be analyzed with varying degrees of sophistication, the level of discussion depending on the molecular complexity. Biological macromolecules have been treated at all levels, ranging from complete normal coordinate analyses for model compounds such as *N*-methylacetamide and polyglycine to mere suggestions of the presence of a certain conformation. Although vibrational spectroscopy has become a well-accepted physical method for studying biopolymer conformation, for the most part the vibrational information has been obtained from infrared spectroscopy, and a number of comprehensive reviews have appeared on the application of infrared spectroscopy to protein structure (Susi, 1969; Miyazawa, 1967; Krimm, 1962).

Prior to the 1940's infrared spectroscopy found only limited application to problems of chemical identification and molecular structure, but Raman's discovery of the inelastic scattering of light in 1928 appeared to have considerable potential and was widely applied. Raman spectroscopy depended on the scattering of light which could be confined to the visible region, and conventional spectroscopic techniques (*e.g.*, photographic detection) could be used. With the development of efficient infrared detectors and optical systems, the absorption technique competed directly with Raman spectroscopy and the experimental limitations of the latter led to a decline in its relative importance.

The possibility of using laser sources for Raman spectroscopy has resulted in a renaissance of the technique. These sources have increased the versatility of the technique and removed many of the experimental limitations; thus Raman spectroscopy has found many applications in organic, inorganic, polymer, and biopolymer chemistry during the last decade. The rapid increase in the application of Raman spectroscopy to biological areas does, in fact, reflect the possibility of obtaining additional vibrational information that is not available through infrared spectroscopy.

THE TECHNIQUE

Raman spectroscopy depends on an analysis of the radiation scattered from the surface of a material or by passing the incident beam through a transparent liquid or solid. If the incident radiation has a frequency denoted by ν_0 there will be some elastic scattering (Rayleigh scattering) of photons but, in addition, a few photons will be inelastically scattered. These inelastically scattered photons result in a spectrum of frequencies in the scattered beam due to the interaction of the incident photons with rotational, vibrational, or even electronic transitions. If ΔE represents the energy difference between two possible vibrational states of the molecules there will be two lines in the Raman spectrum corresponding to these two states. The first (Stokes line) corresponds to a loss of energy from the radiation due to the promotion of some molecules from the ground state to an excited state and has a frequency of $\nu_0 - \Delta E/h$; the second (anti-Stokes line) is due to the return of some excited molecules to the ground state with an increase in the energy of the radiation and has a frequency given by $\nu_0 + \Delta E/h$. Raman spectra are normally recorded from the Stokes lines or red shift since these lines are more intense due to the greater probability of finding molecules in the ground state. Even so, the scattered radiation has an intensity $\sim 10^{-6}$ that of the source intensity. Thus, successful experimentation requires a high source intensity, an efficient monochromator, and a sensitive photon detection device.

Since Raman spectroscopy depends on the scattering of photons it is possible to arrange for both the incident and the scattered radiation to be in the visible region of the electromagnetic spectrum. This makes it possible to use glass sample cells and simplifies the construction of lenses and gratings. This also avoids problems which arise in infrared spectroscopy due to the fogging and/or breakage of fragile crystals, particularly when aqueous solvents are used. In addition it means that one instrument can cover the whole range of vibrational spectroscopy, whereas the same range would require several infrared instruments for complete coverage. Sample presentation is also simplified since solid powders or composites may be examined directly without prior treatment by grinding and pressing into discs or making mulls. Thus compounds can often be examined in their natural environment. The various methods of sample presentation are illustrated in Figure 1.

In addition to the features which are due to the scattering of radiation there are further features which arise from the use of lasers as Raman sources. The high power density, monochromaticity, coherence, and collimation of the laser beam make it an ideal source for Raman spectroscopy. Thus it is possible to use small samples or direct the focused beam onto a small area of a larger sample. Finally, the natural polarization of the laser beam means that line polarization, for unoriented transparent solids or liquids, may be readily determined, and oriented solids can be treated in a manner analogous to the more common infrared dichroism measurements, thus assisting in the assignment of the various vibrational modes to their symmetry species.

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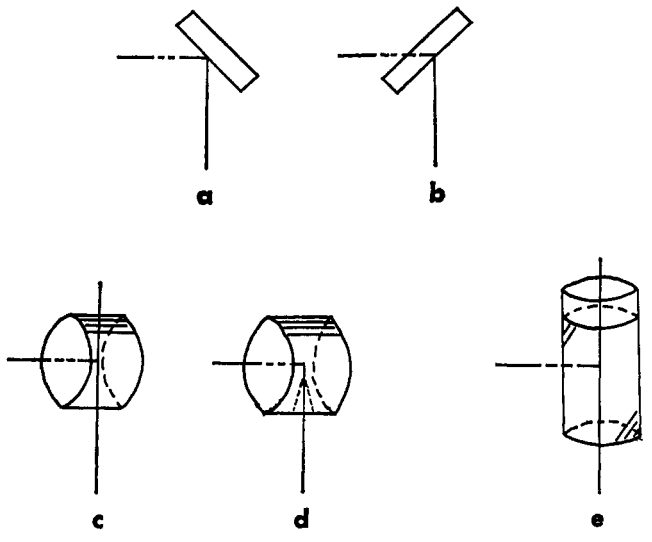

Figure 1. Sample techniques for Raman spectroscopy; (a) front reflection; (b) back transmission; (c) clear pellet; (d) drilled pellet transmission; (e) solution transmission

A vibrational mode will result in Raman scattering of the photons if there is a change in the polarizability or induced dipole moment of the molecule during the molecular motion. On the other hand infrared absorption occurs when the molecular motion interacts with the radiation by changing the dipole moment of the bond. Polar groups, which have large dipole moments, are strong absorbers of infrared radiation when undergoing vibrational motion, whereas nonpolar groups are readily polarized and thus give rise to strong Raman scatter. Thus certain groupings are recognized as giving rise to strong bands in infrared spectroscopy while other groupings may give rise to prominent Raman lines. Centrosymmetric molecules give rise to a Raman spectrum which has no lines in common with the infrared spectrum from the same molecule, and in general the more symmetrical a molecule, the greater the differences between the Raman and infrared spectra. In addition, symmetrical modes are generally strong Raman scatterers, whereas bands arising from antisymmetric modes tend to be more prominent in infrared spectra. Thus the two techniques will often complement each other and the comparison between the two spectra will often aid in the assignment of the frequencies of the various vibrational modes. Finally, theory often predicts that more vibrational modes will be Raman active than infrared active, and thus the Raman spectrum will be the richer of the two.

Although theoretical considerations may indicate that a particular mode will be active in both the infrared spectrum and the Raman spectrum of a given molecule, the limitations of instrumental sensitivity may prevent the observation of the vibrational mode by one of the two techniques, *e.g.*, the amide II mode seen in the infrared spectrum of a polypeptide is a weak Raman scatterer, whereas the C–H stretching mode is stronger in Raman spectroscopy. These intensity differences result from the way the photons interact with the molecular vibrations.

LASER RAMAN INSTRUMENTS

The experimental arrangement used to excite and detect Raman spectra is relatively simple. In our instrument, the source of excitation is an argon-ion laser having an output of 200 mW at 4880 Å. The beam passes through a narrow band pass filter to remove argon emission lines, and is then reflected upward and focused onto the sample. Light scattered by the sample is collected by a lens and focused onto the entrance slit of a Spex 1400 double monochromator. The output of the monochromator is detected by an ITT FW 130 startracker phototube which is cooled to −20° C by a thermoelectric cooler. The photoelectron pulses from the phototube are counted, passed through a discriminator barrier, and displayed on a strip chart recorder.

A Spectra Physics Model 700 is also being used in our laboratory coupled to a Coherent Radiation 1 W laser source. Other commercial instruments by Jarrell-Ash, Spex, Cary, Jelco, and Perkin-Elmer are available.

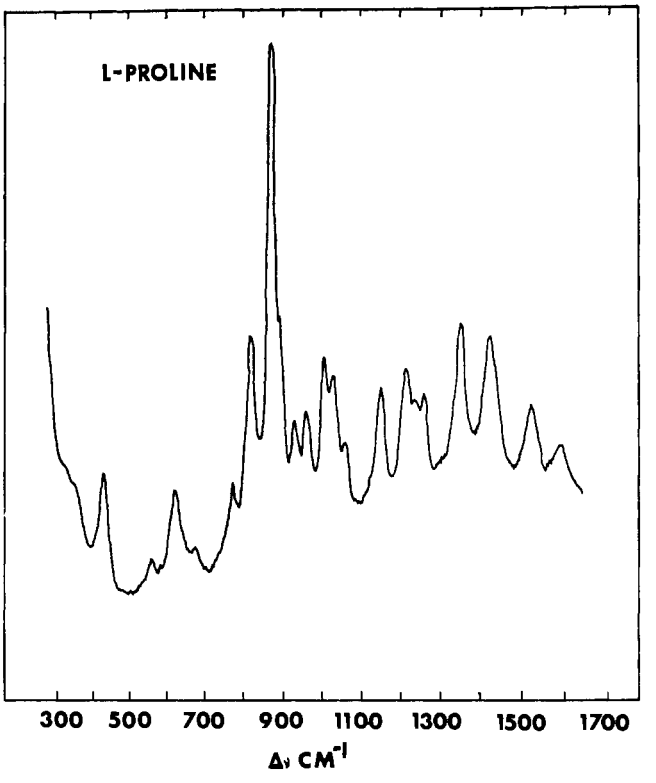

Figure 2. Raman spectrum of L-proline

APPLICATIONS TO BIOLOGICAL MATERIALS

Although Raman spectroscopy deals with molecular vibrations and is thus similar to infrared spectroscopy, the differences mentioned above may mean that the Raman spectrum of a compound can be more useful to biological studies than merely supplying more complete vibrational information or aiding mode identification due to the different selection rules. Figure 2 illustrates the quality of the spectra obtained with our instrument for low molecular weight materials. Historically, spectra reported for proteins have generally been of poor quality with poorer signal to noise ratio, as seen in Figure 3. It has been surprising, therefore, to find that reasonably good spectra could be obtained from bone chips and teeth, as shown in Figures 4 and 5. Our approach, to date, has been to concentrate on spectra from synthetic polypeptides which give reasonable spectra after some purification. Figure 6 shows typical spectra obtained from poly-L-proline form I before and after cleanup.

One of the important features of Raman spectroscopy arises from the weak Raman scattering of the most important biological solvent, water. Only restricted regions of the vibrational spectrum are available to infrared spectral studies in aqueous solutions because of the strong absorption of water.

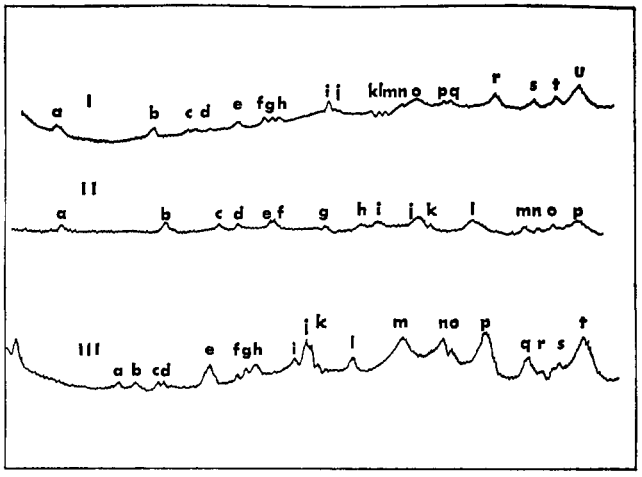

Figure 3. Raman spectra of crystalline. I-pepsin, line positions (cm^{-1}): a, 239; b, 521; c, 623; d, 645; e, 760; f, 831; g, 856; h, 879; i, 1006; j, 1032; k, 1126; l, 1156; m, 1177; n, 1207; o, 1250; p, 1313; q, 1341; r, 1455; s, 1554; t, 1614; u, 1674. II-lysozyme, line positions: a, 505; b, 768; c, 876; d, 900; e, 1003; f, 1011; g, 1139; h, 1202; i, 1251; j, 1336; l, 1430; m, 1512; n, 1591; o, 1625; p, 1667. III-α-chymotrypsin, line positions: a, 512; b, 568; c, 627; d, 644; e, 758; f, 830; g, 855; h, 878; i, 976; j, 1007; k, 1034; l, 1127; m, 1245; n, 1341; o, 1358; p, 1453; q, 1551; r, 1578; s, 1619; t, 1667. Redrawn with permission of M. C. Tobin, *Science* 161, 68 (1968)

However, there is only limited interference by the Raman active vibrational modes of liquid water in the 3200 cm^{-1}–2000 cm^{-1} range (vibrational transitions in Raman spectroscopy are strictly frequency shifts away from the exciting line, but these are referred to as frequencies) and the 2000 cm^{-1}–200 cm^{-1} region is completely accessible, if the solute is concentrated enough and a sufficiently good Raman scatterer, in either H_2O or D_2O solution (see Figure 7).

Thus, it is possible to obtain spectra in aqueous solutions and compare them with spectra obtained from solids and determine the effect of solvent molecules on molecular conformation. In addition, comparisons can be made between deuterated and nondeuterated solute molecules in solution and information regarding the effect of isotopic exchange obtained using Raman spectroscopy.

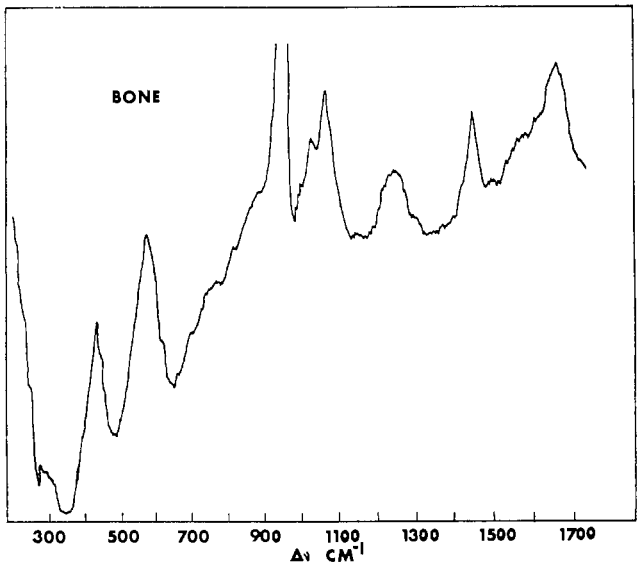

Figure 4. Raman spectrum of defatted ox tibia

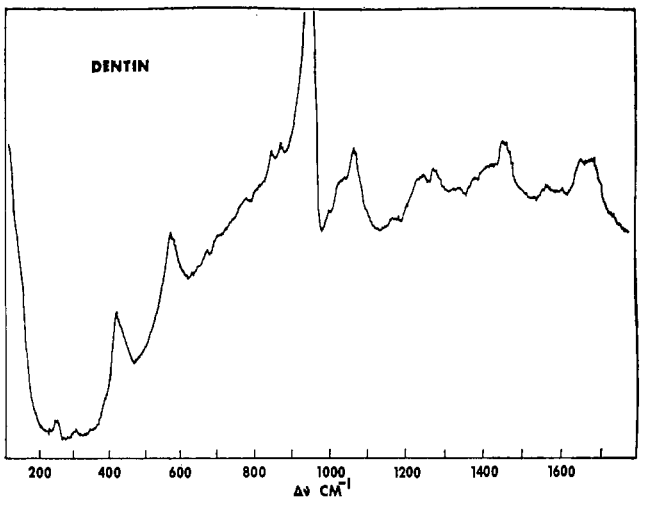

Figure 5. Raman spectrum of human dentin

Sample presentation is also ideally suited to biologica studies where the amount of sample available may be limited or local small areas of larger samples, which cannot be broken down, may need to be studied. Also, the material can be observed in its natural state without the influence of foreign materials such as salts or oils, with their unknown effects on the conformation of biological macromolecules.

Having established that Raman spectroscopy should be a very useful tool for studying biological systems, it is perhaps surprising that there has not been a more general application of the technique now that laser sources are readily available. Probably the major reason for the delay is that, in addition to the Raman scatter discussed above, many biopolymers give rise to a high background of scattered radiation, which masks the very weak Raman effect. This is known as fluorescence or luminescence, and is probably the greatest single problem facing the Raman spectroscopist. However, there are signs that this problem is being overcome by various purification procedures and even the simple expedient of leaving the material in the laser beam for some time prior to taking the spectra frequently improves the quality. Thus there have been a number of reports of Raman spectra from biological compounds in the last 2 years and we can look forward to increased activity in this developing field.

Some 7 years after the discovery of the Raman effect in 1928, Raman spectra were reported for a number of amino acids and fatty acids (Edsall, 1936). This study included the effect of water on the frequency of the Raman lines for the fatty acids and the change in the carbonyl stretching frequency accompanying the ionization of the amino acids. Subsequently, further reports of spectra from amino acids and related compounds appeared (Edsall, 1943, 1950; Edsall and Scheinberg, 1940; Garfinkel and Edsall, 1958a) leading up to spectra from polylysine and lysozyme (Garfinkel and Edsall, 1958b). However, all these spectra were taken prior to the advent of the laser and were recorded photographically from aqueous solutions.

With the introduction of the laser and the improvements in instrumentation that have taken place over the last decade, it has now become possible to obtain Raman spectra from a large range of synthetic polymers and a few proteins. Studies to date may be divided into several categories. First, there have been reports which illustrate the sensitivity of the technique to the conformation of biopolymers by studying those polymers which exist in two conformational forms. Secondly,

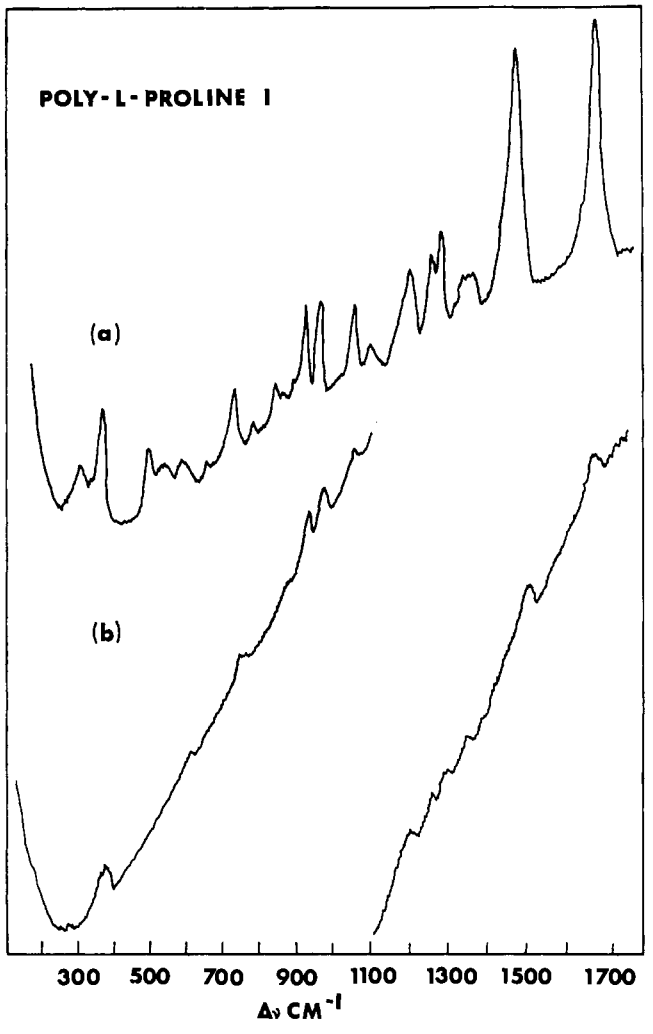

Figure 6. Raman spectrum of poly-L-proline I. (a) After treatment; (b) Before treatment

several papers have studied oligomers with the view of establishing the minimal length of a polypeptide required for the formation of a conformation which has been established for the polymer. Thirdly, the possibility of using aqueous solutions and hydrated solids for spectral studies has been utilized to determine the effect of solvent molecules on conformation. Finally, there have been several reports on the Raman spectra of natural materials such as hard tissue, purified globular proteins, and nucleic acids.

Sensitivity of Raman Spectroscopy to Biopolymer Conformation. A spectrum has been obtained from poly-L-proline in a right-handed 10_3 helix with cis peptide bonds and a residue translation of 1.9 Å (form I) and compared with the spectrum for a solid with a left-handed 3_1 helix with trans peptide bonds and 3.12 Å translation per residue (form II) (Rippon *et al.*, 1970). In addition to the line at 957 cm^{-1}, which corresponded to the infrared band at 960 cm^{-1}, form I had unique Raman lines at 781 cm^{-1}, 662 cm^{-1}, and 363 cm^{-1}. In addition, the carbonyl stretching mode of form I had a reduced intensity of scatter and there was reversal of the intensities of the lines at 1264 cm^{-1} and 1239 cm^{-1} when the form I spectrum was compared with the form II spectrum. The difference between the carbonyl stretching modes was not observed in the infrared spectra and could reflect the sensitivity of the Raman effect to symmetric A modes, rather than the E modes which dominate in infrared spectroscopy.

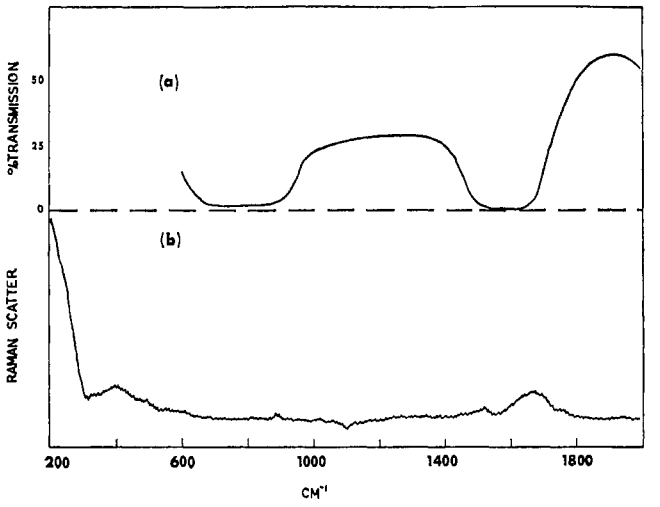

Figure 7. Raman and infrared spectra of water. (a) Infrared spectrum with a 0.025-cm cell path length; (b) Raman spectrum with instrument settings used for polypeptide solution spectra

Raman spectra have also been reported for the two forms of polyglycine (Small *et al.*, 1970; Smith *et al.*, 1969a). Form I is an antiparallel β sheet and form II is in a 3_1 helix. An interesting feature of these spectra was that for polyglycine I (β form) the infrared spectra showed an amide I band split into a weak shoulder at 1685 cm^{-1} and a strong band at 1636 cm^{-1}, whereas the Raman spectrum had one strong line at 1674 cm^{-1}. However, the shape of the Raman line was consistent with the presence of unresolved components. In addition, a very strong line has been reported at 1672 cm^{-1} for the β form of poly-L-lysine, which also has antiparallel chains (Wallach and Graham, 1970). Finally hexa-L-alanine, which also appears to form an antiparallel β sheet structure, was reported to have infrared bands at 1690 cm^{-1}, 1650 cm^{-1}, and 1623 cm^{-1} and a single strong Raman line at 1663 cm^{-1} (Sutton and Koenig, 1970). These authors did a perturbation calculation (Miyazawa, 1960) based on an assignment of the infrared bands to the $\nu(0,\pi)$, $\nu(\pi,\pi)$, and $\nu(\pi,0)$ vibrational modes of the amide I, respectively, and calculated that the $\nu(0,0)$ mode would be seen at 1662 in the Raman. This suggests that the strong Raman lines seen in the other examples are also due to the $\nu(0,0)$ mode. This mode is totally symmetric and would be inactive in the infrared spectrum, but it is expected to be the dominating line in the Raman spectrum. There seems to be evidence that the shift of the $\nu(0,0)$ mode from ~1673 cm^{-1} for polyglycine and poly-L-lysine to 1663 cm^{-1} for hexa-L-alanine is due to the latter being an oligomer rather than the change in amino acids. This conjecture is further substantiated by the fact that pentaglycine appears to exist in an antiparallel β sheet and also has a single strong Raman line at 1665 cm^{-1} (Smith *et al.*, 1969a). Thus, the shift in frequency of the amide I $\nu(0,0)$ mode probably reflects the limited number of chemical repeats. An earlier perturbation calculation for polyglycine (Smith *et al.*, 1969a) was reported but was based on the amide I $\nu(0,0)$ mode seen at 1665 cm^{-1} for pentaglycine. Miyazawa (1962) has calculated the $\nu(\pi,\pi)$ mode for polyglycine to be at 1668 cm^{-1}. However, he used interaction constants derived from Nylon 66 and these same constants give a $\nu(0,0)$ frequency of 1648 cm^{-1}. The Raman line at 1674 cm^{-1} could be the 1668 $cm^{-1}\nu$ (π,π) within the limits of experimental measurement and calculation, but it is most unlikely that the $\nu(\pi,\pi)$ mode would be strong in the Raman spectrum, since

it evolves out of phase motions of the peptide groups. It is now possible to recalculate the fourth amide I band for polyglycine, based on an assignment of the two infrared bands at 1636 cm^{-1} and 1684 cm^{-1}, and the Raman line at 1674 cm^{-1} to the $\nu(\pi,0)$, $\nu(0,\pi)$, and $\nu(0,0)$ modes, respectively. Using these frequencies and the method introduced by Miyazawa (1960), the unperturbed frequency ν_0, intrachain interaction constant D_1, and interchain interaction constant D_1 are calculated to be 1660.5 cm^{-1}, 19 cm^{-1}, and -5.5 cm^{-1}, respectively, and the $\nu(\pi,\pi)$ mode would be expected at 1647 cm^{-1}.

Further illustrations of the sensitivity of Raman spectroscopy to biopolymer conformation are found in the aqueous solution spectra reported for poly-L-glutamic acid and poly-L-lysine (Wallach and Graham, 1970). These authors report the effect of pH on the spectra for these polymers which undergo conformational transitions associated with the ionization of the residue carboxylic acid or amine groups. It is interesting to note that this work provides evidence for the presence of antiparallel β sheets in aqueous solution, since the amide I line is seen at 1672 cm^{-1}, which is in the same range as the amide I $\nu(0,0)$ mode of solid polyglycine and away from the region expected for disordered or other structure. This argument is strengthened since the splitting of the amide I band into the identifiable components depends on inter- as well as intrachain interactions. Such antiparallel chains need not arise from molecular aggregation but could be the result of chain folding, such as has been postulated for other polypeptides (*e.g.*, Andries and Walton, 1969, 1970; Rippon *et al.*, 1971). Finally there has been a recent report on the polarized Raman spectra obtained from oriented, helical, poly-L-alanine fibers. From this study it was possible to assign a number of the observed lines to A, E_1, and E_2 symmetry species (Fanconi *et al.*, 1969).

Raman Spectroscopy in the Study of Oligomer Conformation. Raman spectra have been reported for glycine, diglycine, triglycine, tetraglycine, and pentaglycine (Smith *et al.*, 1969a) in both aqueous solutions and the solid state. Precipitation of the oligomers from aqueous solutions with acetone resulted in a β sheet conformation. However, the aqueous solutions of diglycine and triglycine yielded spectra exhibiting a number of differences, when compared with the solid state spectra for these same oligomers. Most prominent of these were the methylene bending modes at 1446 cm^{-1} and 1420 cm^{-1} for the solid and solution spectra, respectively, and the methylene wagging modes at 1406 cm^{-1} for the solid and 1391 cm^{-1} for the solution. Since these modes were shown to be sensitive to the polyglycine conformation, the solution spectra were attributed to the formation of different rotational isomers.

The Raman studies of alanine oligomers reported by Sutton and Koenig (1970) indicated that di-L-alanine was randomlike, tri-L-alanine and hexa-L-alanine formed β sheets, and poly-L-alanine favored a helical conformation. It was also noted that dissolution of the di-L-alanine in water gave little change in the spectrum, whereas dissolution of solid tri-L-alanine in water resulted in a destruction of the interchain hydrogen bonding which permitted the array to take up a nonplanar conformation.

Finally, Raman spectra have been reported for tri-L-proline, tetra-L-proline, and penta-L-proline (Rippon *et al.*, 1970). The results indicated that tetra-L-proline and penta-L-proline recovered from the polymerizing solvent had a conformation similar to that of poly-L-proline II, an observation previously reported from infrared measurements (Isemura *et al.*, 1968) and consistent with conformational calculations (Hopfinger and Walton, 1969). However, spectra taken from the aqueous solutions of the oligomers indicated that the trimer could also approach the poly-L-proline II conformation. In addition, freeze-drying this aqueous solution led to the recovery of a solid which gave a spectrum similar to that of the polymer (see Table I). There was, however, broadening of the line due to the carbonyl stretching mode which could be accounted for if water of hydration was present hydrogen-bonded to the carbonyl group. The infrared spectrum from the freeze-dried solution was consistent with there being water of hydration, and subsequent drying in a vacuum oven removed this water and destroyed the organization of the trimer. Thus, the binding of solvent molecules to the trimer apparently stabilized the helical form, even though only one turn was possible. This helical form does not appear to be present in the solid recovered from organic solvents and thus depends on specific solvent/solute interactions for its stability.

Table I. Raman Spectra of Proline Containing Oligomers and Poly-L-proline

Solid state spectra			Aqueous solution spectra	
AOC-(proline)$_3$		Poly-L-	AOC-	Poly-L-
Dry	Hydrated	proline II	(proline)$_3$	proline II[a]
1658	1650	1650	1631	1631
1272[b]	1263[b]	1261[b]	1267[b]	1265[b]
1238[b]	1241[b]	1241[b]	1246[b]	1246[b]
1201[b]	1196[b]	1198[b]	1194[b]	1192[b]
1083	1093	1093	1093	1096
972	1000	1000	1002	1003
842	869	869	872	871
823	830	838	828	820
736[b]	729[b]	733[b]	731[b]	731[b]
523	530	530	534	528
474	509	496	505	500
383[b]	399[b]	401[b]	403[b]	399[b]

[a] Smith *et al.*, (1969b). [b] Indicates lines which are sensitive to poly-L-proline I/II conformation.

Aqueous Solution Spectra. Reference has been made to aqueous solution spectra in each of the above sections, and the influence of solvent on the conformation of the polymers and oligomers has been emphasized. The possibility of such comparisons between solid state conformation and aqueous solution conformation is one of the more important aspects of Raman spectroscopy as far as biological research is concerned. This is further illustrated by the spectra obtained from poly-L-proline in aqueous solution and in the solid state (Smith *et al.*, 1969b). The results suggested that the solid state conformation, stabilized by steric factors, since hydrogen bonding is not possible with this polyimino acid, was maintained in the presence of aqueous solvent. Similar results have also been reported for poly-L-hydroxyproline (Deveney *et al.*, 1971).

Raman Spectra of Proteins. It was several years after the initial report of the Raman spectrum of lysozyme (Garfinkel and Edsall, 1958b) before further reports giving the Raman spectra of proteins appeared. This delay was, in part, due to the difficulty of obtaining samples of high purity and the problem of fluorescence mentioned earlier. Since laser Raman instruments have become available, there have been reports of several protein spectra. Tobin (1968) has reported laser Raman spectra for crystalline lysozyme, pepsin, and α-chymotrypsin, and there has been a report of spectra from aqueous solutions of bovine serum albumin (Careri *et al.*, 1970).

Another interesting application of laser Raman spectros-

copy to biological tissue has been the report of a Raman spectrum from a chip of ox tibia (Figure 4) (Walton *et al.*, 1970). This spectrum has prominent sharp lines assigned to the vibrational modes of the inorganic phosphate superimposed on the broader less intense lines due to the organic matrix. The latter contains a high proportion of collagen and in this indirect way the spectrum of collagen may be seen. In addition, a Raman spectrum has been obtained from tooth dentin (Figure 5) which is similar to the bone spectrum in general form. These appear to be the first examples of spectra obtained from fibrous proteins and natural tissue. These reports, coupled with the ability to focus the laser source to a small selected area, suggest the possibility of probing the molecular basis for abnormalities in these hard tissues.

CONCLUSION

This review has attempted to show the impact the use of laser sources has had on the applications of Raman spectroscopy. The availability of such sources, coupled with other improvements in instrumentation, has enabled Raman spectroscopy to become as versatile a technique as its absorption counterpart. However, there are additional advantages in using Raman spectroscopy when biological materials are to be investigated. It is thus anticipated that the next few years will see continued increase in the applications of Raman spectroscopy to problems of macromolecular structure.

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