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Drug analysis by Raman and micro-Raman spectroscopy*

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Abstract: The technique of Raman spectroscopy, resonance Raman spectroscopy and micro-Raman spectroscopy is described for application to drug analysis and investigation. Possibilities and limits are mentioned for qualitative and quantitative analyses as well as for studies of structure and interactions. Some principal interaction modes, such as hydrogen bonding, proton transfer, charge transfer and ion–molecule attraction, are shown to explain drug reactivity. Illustrations are given based on several drug families, in particular vitamins, anti-depressants, cardio-active and anticancer drugs.

Keywords: *Laser Raman spectroscopy; molecular interactions and reactivity; vitamins; cardio-active drugs; anticancer drugs; cancinogenesis mechanism.*

Introduction

Raman spectroscopy is a molecular and sub-molecular analytical and investigational technique. When a molecule is illuminated, the scattered light appears at frequencies which are characteristic of the vibrations involved in the displacement of its atoms. Each molecule therefore has its own Raman spectrum, and can be identified even when observed in a mixture.

Thanks to the availability of lasers used as sources of illumination, Raman spectroscopy has become a powerful analytical technique over the last twelve years [1].

Of course, one cannot do everything with this technique; but for drug analysis and research, Raman spectroscopy has been shown to be very helpful in many applications, especially: qualitative analysis; quantitative analysis; structural determination of intermediate and final products in drug development; control of potential drug–substrate interactions in galenical formulations; study of drug reactivity and mechanisms of action as a guide to drug research.

New developments in the technique, using the resonance Raman effect, allow the operating concentration of the material under investigation to be reduced one million times lower than usual [2, 3]. Moreover, by illuminating the sample through an optical microscope, one can now obtain Raman spectra of surfaces as small as 1 μm^2, so-called micro-Raman spectroscopy. Consequently, by combining the resonance Raman effect

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with the micro-Raman technique, the scope of this technique is substantially increased [4].

Theoretical Background

Ordinary Raman spectroscopy

When a molecule is illuminated by monochromatic incident radiation from a laser, strong scattered light at the same frequency as the incident radiation is observed (Rayleigh effect). In addition, scattered light of lower intensity is also observed at frequencies which are characteristic of the molecular vibrations. These frequencies are comparable to those observed in the infrared absorption spectrum of the same molecule. Although the band intensity in the two forms of spectroscopy varies differently with the symmetry of the vibrations in relation to the change in dipole moment and in polarizability of the molecule, the two spectroscopic methods are entirely complementary as they involve the same energy levels in the molecule (Fig. 1).

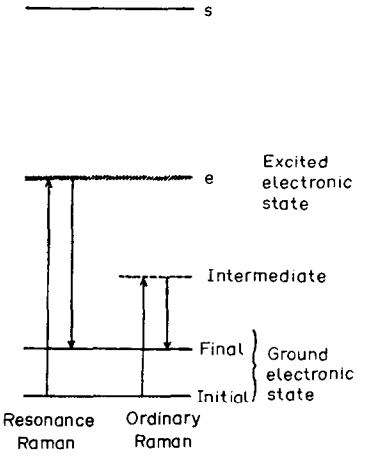

Figure 1
Energy levels involved in different molecular spectroscopies.

Applications

Raman spectroscopy can be used for samples in any physical state: gaseous, liquid, solid and living objects [5–8], as illustrated below. One of the advantages of this technique in structural studies, relative to direct structural methods such as X-ray diffraction, is the capability to investigate the same material in solid state, liquid state or in solution.

Ordinary Raman spectroscopy

Aqueous solutions. Figure 2 gives an example of a synthetic drug, paracetamol (4-amidophenol), both in the solid state and in aqueous solution. As is well known, it is not easy to obtain the infrared spectrum of aqueous solutions, because of the very strong, broad absorption of water itself, masking the spectrum of the dissolved molecule. Another advantage of this technique resides in its non-destructive nature, whereas other techniques give only elementary or partial analyses, accompanied by total or partial destruction of the molecule under investigation.

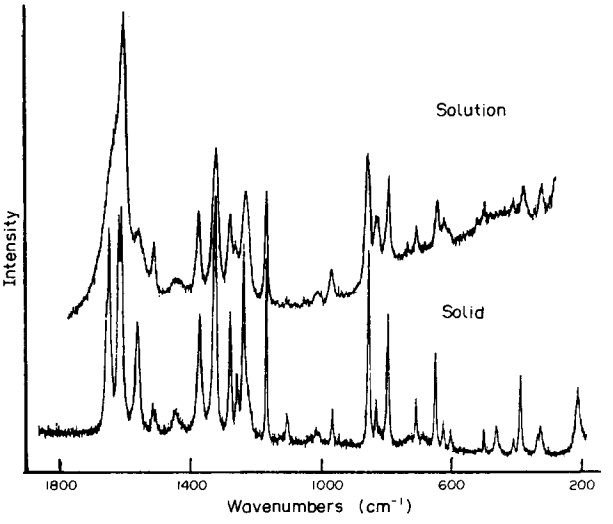

Figure 2
Raman spectrum of "Paracetamol" (4-amidophenol) in the solid state and in aqueous solution. The spectrum, as for all following spectra, was recorded on French spectrometers Coderg T 800 and Jobin-Yvon HG 2S and MOLE.

Direct analyses on suppositories. A suppository can be put in the sample holder without further preparation and the Raman spectrum of the active substance can be obtained, after removing the wax, as illustrated in Fig. 3.

Drugs in compressed tablets. Another advantage of Raman spectroscopy is its facility for obtaining a direct molecular spectrum by scattering through the bulk of the material, as well as by back-scattering from its surface. A tablet can be illuminated directly before collecting its Raman spectrum and compared to that of the pure drug (Fig. 4). Evidently, the protecting envelope, if any, should be removed before measurement.

Drugs of natural origin. The Raman spectrum can serve to establish the purity of an extracted substance (Fig. 5). In general, the purest product has the least number of vibrational bands.

Drugs accumulated in living systems. Raman spectroscopy also gives the possibility for direct observation of the presence of a drug in living systems. Moreover, by examining the shift of characteristic vibrational frequencies, one can deduce the binding properties of a drug in the biological medium.

Consider the simple example of iodine in hyperthyroidism. Many techniques allow the presence of iodine to be detected in this gland. But what is the valence state of iodine: I_2, I_3^- or $(I_2)_n$? The Raman spectrum of histological slice gives a weak band at 200 cm^{-1}. Knowing that free iodine vibrates at 213 cm^{-1} [9] and that this stretching frequency can be reduced by molecular interaction [10, 11], it can be deduced that in the thyroid gland iodine appears neither as I_3^-(112 cm^{-1}) nor as $(I_2)_n$ (180 cm^{-1}), but is present in the form of I_2 bonded with a relatively strong basic site, such as the carbonyl group.

Another example can be seen in drug-induced hepatitis, when the Raman spectrum of liver cell can reveal the accumulation of the drug in its walls (Fig. 6).

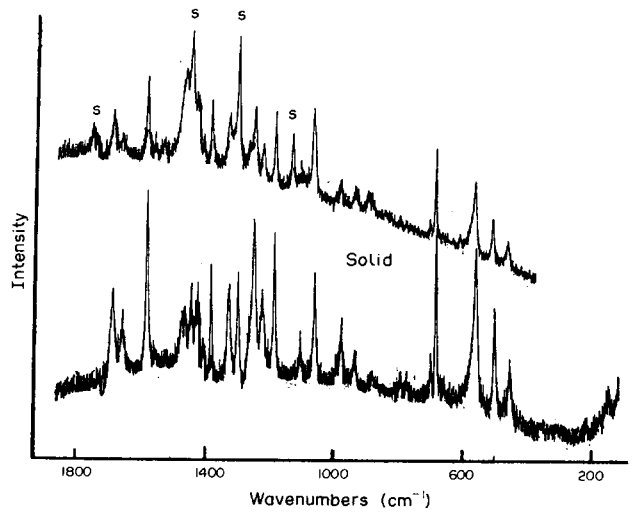

Figure 3
Raman spectrum of a suppository of "Aminophilline" in polyglycerides.

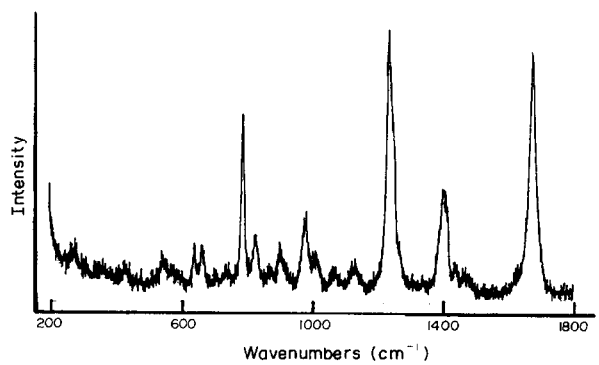

Figure 4
Raman spectrum of uracyl-monophosphate (UMP) in the solid state ($\lambda_0 = 514.5$ nm).

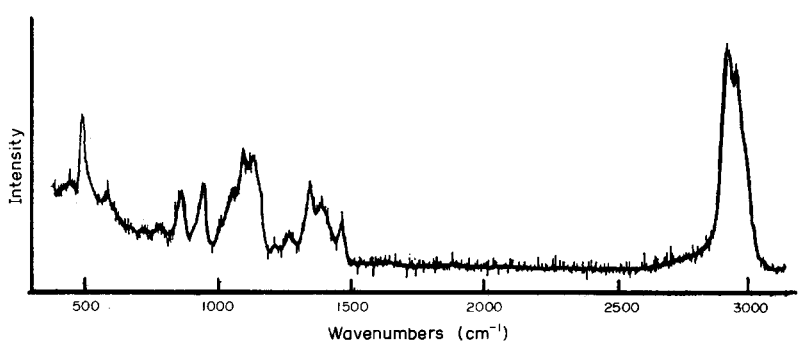

Figure 5
Raman spectrum of oyster glycogen.

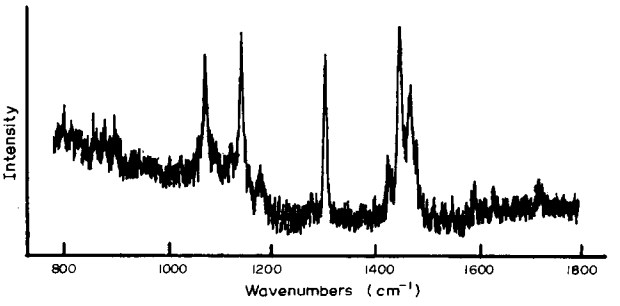

Figure 6
Raman spectrum of a liver-cell of a patient with hepatitis after administration of methotrexate (λ_0 = 514.5 nm).

Quantitative analysis. The Raman band intensity depends on the number of scattering molecules. It also depends on the configuration of the incident and scattered radiation relative to the symmetry of vibration in the sample. Therefore this technique can be a good method for quantitative analysis, both in the gaseous and in the liquid state. Usually, the Raman bands are much narrower than the corresponding infrared bands, so that quantitative analysis is much easier because of less overlap of the component bands.

In the solid state, quantitative analysis is delicate, but still possible when using an internal standard and digital averaging. In principle, for any given crystal (perfectly crystallized and oriented), up to six spectra with different relative intensities can be obtained.

The scope of all these analytical and investigational techniques can be readily increased as a result of the following developments.

Resonance Raman spectroscopy

The frequency of the laser can be chosen to correspond to a frequency close to a non-stationary electronic level of the analyte molecule. The resonance Raman effect is thereby induced for the chemical group associated with the electronic level. The observed vibrational frequencies are still the same as in ordinary Raman spectra, but their intensity is strongly enhanced [12]. Most laser sources emit in the visible region, therefore resonance Raman spectra of coloured compounds can be easily obtained. But enhanced resonance Raman effect can be induced in the ultraviolet, if the appropriate laser is used. An example is given with the antitumour drug elliptycine [2, 3].

In many cases, the intensity enhancement can be more than one million times, so that the concentration used for investigation can be reduced by the same factor. This opens new possibilities for sensitive detection.

Molecular vibrational spectra from one part per ten millions. Sometimes it is necessary to detect or identify a component at the one ppm level by a non-destructive technique. Resonance Raman spectroscopy can be helpful. Many examples can be found in the fields of enzymes and vitamins, as exemplified by Fig. 7. For the vitamin A series or carotenoids [13], lower concentrations can usually be sufficient. For β-carotene (Fig. 8), a concentration of 10^{-7} mole l^{-1} can be used.

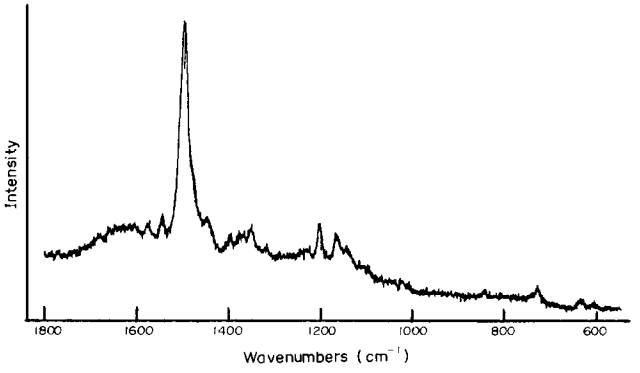

Figure 7
Resonance Raman spectrum of an aqueous solution of Vitamin B_{12} (Merck) (λ_0 = 514.5 nm).

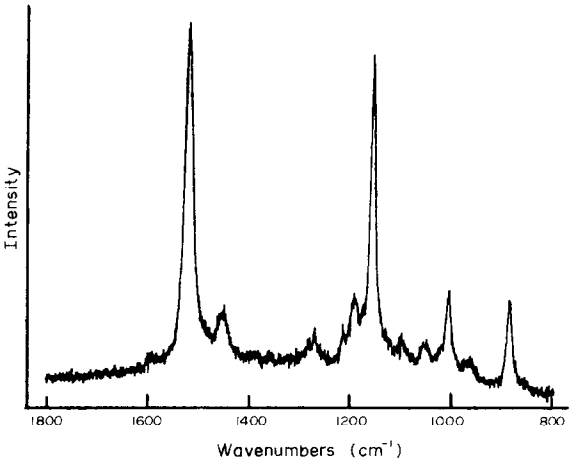

Figure 8
Resonance Raman spectrum of β-carotene (Fluka) in ethanol (λ_0 = 514.5 nm).

In all these cases, the spectra were recorded with only one scan. Spectrometers are now, however, coupled with computers, thus enabling repetitive scanning, thus enabling lower concentrations to be used.

Even at these low concentrations, micro-chemical identification is still easy. For instance, in the case of vitamin A aldehyde, the Schiff's base and its protonated form can be clearly distinguised by the stretching vibrations C=O, C=N and $C=N^+-H$ [14].

Selective sub-molecular resonance Raman spectra. For molecules having many distinct electronic transitions, one can create the resonance Raman effect by choosing a laser frequency which excites a given level; then only Raman bands of this chromophore appear, the rest of the spectrum being lost in the noise. By changing the frequency of the incident radiation, one can hit another level and enhance the Raman lines of the corresponding group. Selective enhancement is obtained in this way. This new possibility is helpful when only the properties of a specific site in a large molecule are of interest.

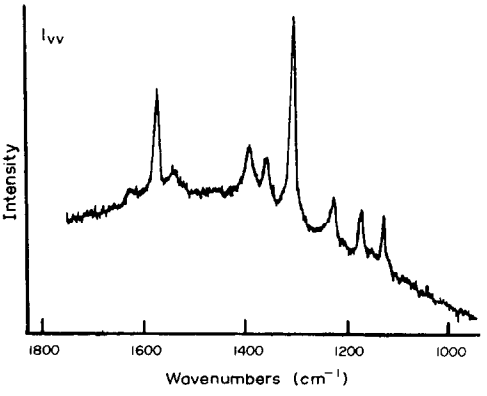

Figure 9
Resonance Raman spectrum of an aqueous solution of cytochrome-C (Sigma) (λ = 514.5 nm).

This is illustrated in the case of the resonance Raman spectrum of cytochrome C (Fig. 9), where only the vibrational bands of the metalloporphyrin core are apparent.

Micro-Raman spectroscopy

If the incident light is transmitted through an optical microscope (magnification × 1000) before reaching the sample, the back-scattering from an area of a few micro-meters in size can be collected before transmission to the monochromator of a Raman instrument to obtain its Raman spectrum.

Obtaining the Raman spectrum of a pure compound from an impure sample. Some substances used as drugs are of limited purity. In the ordinary Raman technique, the illuminated surface is relatively large, so that the spectrum of the whole is collected, including the contributions of undesirable impurities which can sometimes strongly fluoresce. In the micro-Raman technique, the substance under investigation appears on the micrometer scale as small crystals or particles generally separated from the particles of the others. By illuminating a chosen crystal selectively its Raman spectrum can be obtained while the impurities are excluded from the investigation zone.

A very old drug, glutathione, known for more than sixty years [14], has been only very recently found to have anticancer activity [16]. It often contains some 4% of fluorescing impurities. It is not possible to record its vibrational spectrum by the ordinary Raman technique using lasers emitting in the visible region. But by the micro-Raman technique, its vibrational spectrum can be easily obtained (Fig. 10a). In this spectrum, one can clearly identify, among others, the characteristics S–H vibration at 2526 cm^{-1}, the carboxylic C=O at 1712 cm^{-1}, the amide C=O at a slightly lower frequency, and the NH and NH_2 vibrations around 3300 cm^{-1}. This product is also known as reducing agent in many enzyme reactions. In the oxidized form, the SH band disappears completely, while a new band at 516 cm^{-1} appears (Fig. 10b) which is characteristic of the formation of the S–S bond.

Raman spectra of the components of a heterogenous sample. Medicines often comprise mixtures of two or more components. Under the objective of a micro-Raman spectrometer, mixed powders appear as separated crystals, the vibrational spectra of

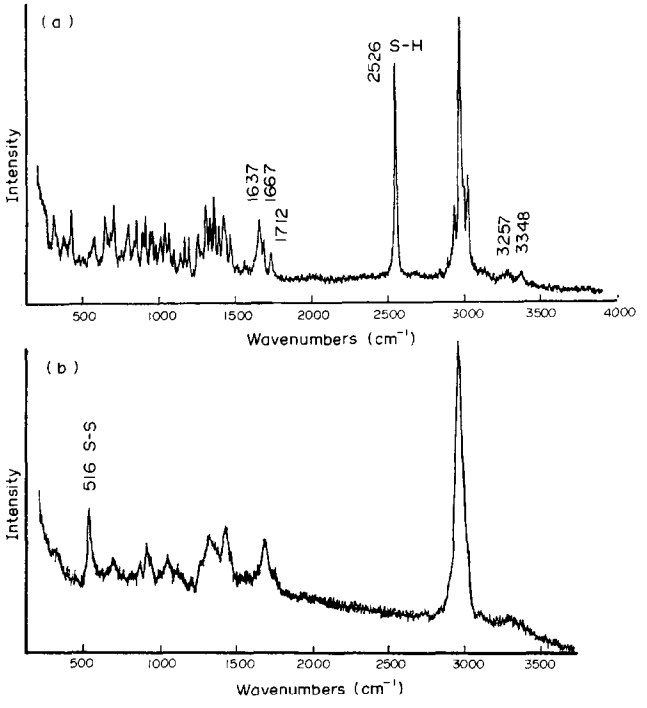

Figure 10
Micro-Raman spectrum of anticancer drug glutathione (Aldrich). (a) reduced; (b) oxidised (λ_0 = 514.5 nm).

which can be selectively recorded, as discussed above. For example, this is illustrated in the case of 'Tensophoryl' (a powder formulation of dopamine, boric acid, amobarbital and ascorbic acid), where different spectra can be recorded from different particles of the powder (Fig. 11). However, one must always be careful, because of the possibility that a single crystal may give different polarized spectra. In similar fashion, two different spectra can be recorded from the powder of Nikethamide (Fig. 12). In all these examples, no previous chemical separation of the components was needed.

Combination of resonance Raman effect and micro-Raman technique. When combining the micro-Raman technique with the resonance Raman effect, the analytical possibilities are tremendously increased. Detection becomes much easier for micro-quantities of substances alone or in other environments.

Detection of drug-interactions and mechanisms of action

An isolated molecule is represented by characteristic Raman vibrational frequencies. When this molecule interacts with other agents, its Raman spectrum changes and the band shifts depend on the nature, force and localization of such interaction. When the interaction is localized in a special part of the molecule, only vibrations concerning this part are perturbed. Conversely, by examining selective changes in the Raman spectrum of a drug after administration, one can localize the site of interaction.

The mixing of two chemical components can give a reaction yielding new chemical entities. Such reactions can be desirable for some drugs. Sometimes, however, a

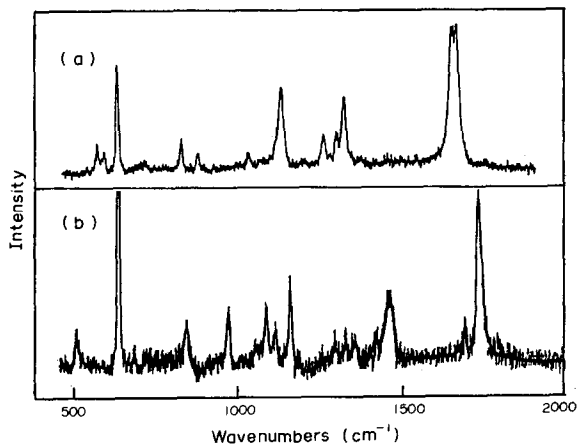

Figure 11
Raman spectra of some particles of "Tensophoryl". (—) dopamine.

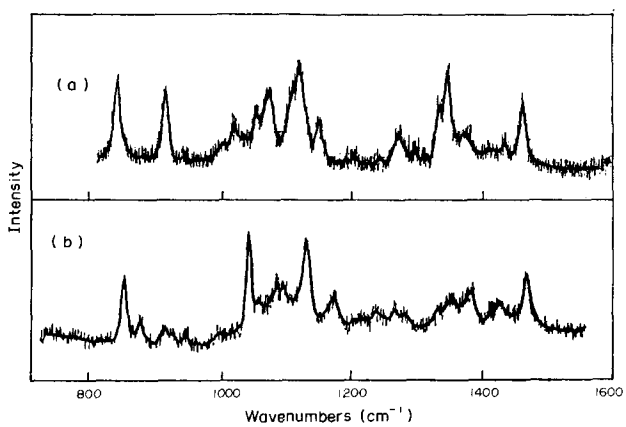

Figure 12
Raman spectra of different particles of nikethamide (Ciba–Geigy).

chemical reaction is not at all as anticipated. Raman spectroscopy allows the detection of the final product in such cases. For example, sodium salicylate dissolved in water gives salicylic acid (Fig. 13).

The vibrational technique is also a sensitive means of detecting physical interactions between components, even when no chemical reaction occurs. Such cases include hydrogen bonding, charge transfer and ion–molecule attraction.

In the case of suppositories, such interactions can explain the retention of drug in the suppository-wax and determines the release rate of the active substance into the body cavity [16a].

In the liquid phase, hydrogen bonding between some drugs and alcohol media can explain any observed secondary effects. In other cases, the hydrogen bonds are strong enough to allow the proton to transfer from one partner to another. Such proton transfers occur in many mechanisms of action *in vivo*.

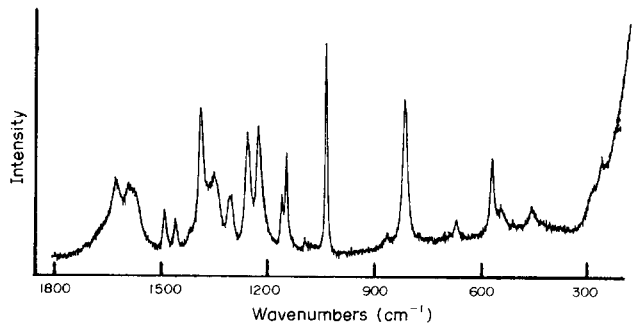

Figure 13
Raman spectrum of an aqueous solution of sodium salicylate.

Proton transfer and enolization in thio-barbiturates. An exciting development is the study on how the sulphur atom in a barbiturate sharply increases its anti-depressant effect in the central nervous system. Thanks to vibrational spectroscopy, which readily gives characteristic spectra of barbiturates (Fig. 14), one can follow the spectral change corresponding to substitution of a given atom (S to O) or to changes in molecular environment. The author's group has clearly established that for oxygenated barbiturates, interactions primarily involve hydrogen bonding. But for thio-barbituric acid, proton transfer can occur, leading to the enol form [17, 18].

Double proton transfer in amino-acids. An amino-acid, such as γ-amino-butyric-acid (GABA), which plays an important role as an inhibitory transmitter in the central nervous system, can be isolated in an inert medium [19], when it appears in the form of a neutral molecule, $H_2N(CH_2)_3CO_2H$. But in the condensed state, its vibrational characteristics (Fig. 15) indicate that this compound exists as a zwitterion $^+H_3N{-}(CH_2)_3CO_2^-$.

By analysing concentration effects in the spectra of the GABA molecules in an inert medium by vibrational spectroscopy, one obtains evidence of every stage of interaction of GABA in the condensed state. These are: hydrogen bonding, then proton transfer from the carboxylic group to the amine nitrogen atom. The author's group have also shown that proton transfer involves a tri-molecular mechanism:

$$-CO_2^- \ldots H_3{}^+N{-}(CH_2)_3{-}CO_2^- \ldots H_3N^+{-}.$$

This double proton-transfer mechanism can explain the role of GABA as a transmitter in the central nervous system.

Structural Raman spectroscopy in cancer drug research

Blue complexes in cancerous blood and tissues. Recently, blue complexes have been observed by Huong and Plouvier [20, 21] in blood and tissues of cancerous and leukaemic patients, before and after cultivation of the tissues on pre-warmed blood gelose. Micro-Raman studies reveal that these particles are copper–organo-complexes and characteristic metal–ligand vibrations, such as Cu–O, Cu–N and Cu–S, have been localized. The spectral analysis has been facilitated by the knowledge of the Raman

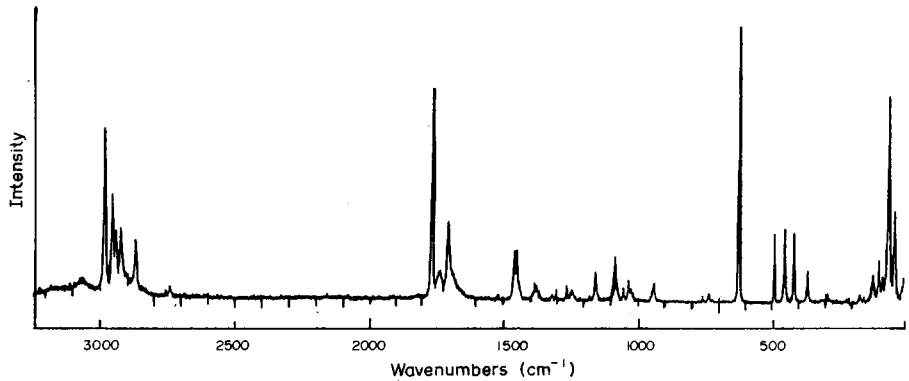

Figure 14
Raman spectrum of the barbiturate, barbital.

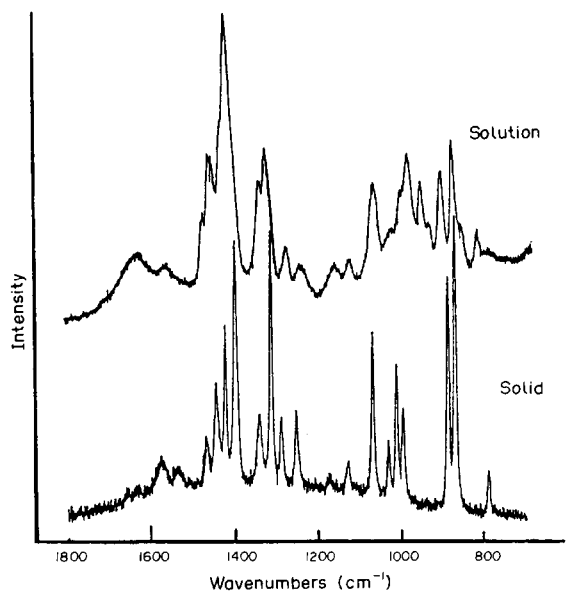

Figure 15
Raman spectrum of solid γ-amino-butyric acid (GABA) (Sigma).

spectra of model copper–protein complexes [22] and of copper–nucleotide complexes [23].

The observation of blue complexes is now general for a large number of cancers and the nature of the metal, copper, has been reconfirmed by micro-fluorescence-X coupled with electron microscopy [24]. Furthermore, in many cancerous samples, highly concentrated depots of metal have also been found in the neighbourhood of the blue complexes.

A mechanism of carcinogenesis. These findings favour mechanism of carcinogenesis based on the local disorder induced by metallo–organo-complex formation [25].

The author considers that the presence of localised metal concentrations, caused by bacteria or other agents, facilitates the formation of organo–metallic complexes. The Raman spectra of these species indicate that the organic moiety is not only composed of nucleotide bases, but also of proteins. Copper is well known to coordinate with many and different ligands [22]. These organic bases, taken out of the regular living structure, may cause the disorder which induces the loss of coding and messages in the biosynthesis. This mechanism can be examined in parallel to that of intercalation.

Directions for research in anticancer drugs. In the intercalation mechanism of carcinogenesis, the perturbing agent intercalates between two rings of a helical structure and induces the loss of coding and messages. In this theory, it is not easy to imagine how to remove the intercalating agent, because of the great number of strong hydrogen bonds established.

By contrast, in the theory of disorder created by metallo–organic formation recently proposed [25], it is suggested that competition occurs by complexing the metal with suitable drugs. Future anticancer complexing drugs could have similar structure to that of nucleotides or basic proteins, but the basicity must be modified by appropriate substitution, to complex with the metal in the equilibrium. Of course, the basicity of the drugs must not be too high, because an organism also needs a certain amount of metals. New chemicals similar to 5-fluorouracil (Fig. 16) could correspond to this proposed profile.

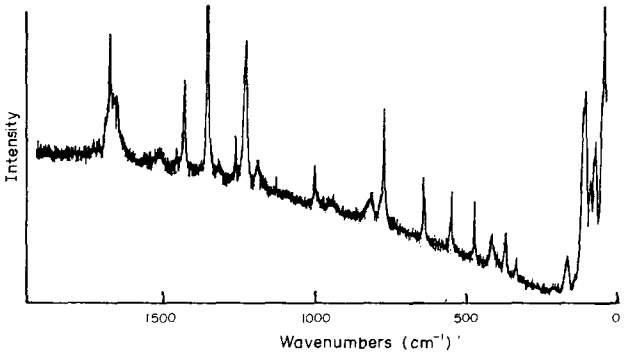

Figure 16
Raman spectrum of anticancer drug 5-fluorouracil (Ega).

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References

[1] P. V. Huong, Chemical applications of resonance Raman spectroscopy, in *Vibrational Spectra and Structure* (J. R. Durig, Ed.). Elsevier, New York (1980).
[2] P. V. Huong, in *Raman Spectroscopy, Linear and non-Linear* (J. Lascombe and P. V. Huong, Eds). Wiley, New York (1982).
[3] P. V. Huong, New analytical possibilities of Raman and resonance Raman spectroscopy, in *Review on Analytical Chemistry* (L. Niinisto, Ed.). Akademia Kiado, Budapest (1982).

[4] R. Cavagnat, F. Cruege and P. V. Huong, *Biochimie* **63**, 927 (1981).
[5] P. V. Huong and R. Giege, *Biochimie* **63**, 921 (1981).
[6] P. V. Huong, R. Giege, D. Moras, M. B. Comarmond and J. C. Thierry, in *Raman Spectroscopy* (J. Lascombe and P. V. Huong, Eds), p. 705. Wiley, New York (1982).
[7] P. V. Huong, E. Audry, R. Giege, D. Moras, J. C. Thierry and M. B. Comarmond, *Biopolymers* **23**, 71 (1984).
[8] R. Giege, P. V. Huong and D. Moras, *Spectrochim. Acta* **42A**, 387 (1986).
[9] P. V. Huong, N. Platzer and M. L. Josien, *J. Am. Chem. Soc.* **91**, 3669 (1969).
[10] P. V. Huong and F. Cruege, *J. Chim. Phys.* **78**, 5 (1981).
[11] W. Kiefer and H. J. Bernstein, *J. Raman Spectrosc.* **1**, 417 (1973).
[12] P. V. Huong and J. C. Pommier, *C. R. Acad. Sci.* **285C**, 519 (1977).
[13] P. V. Huong, *C. R. Acad. Sci.* **286C**, 25 (1978).
[14] P. V. Huong, R. Cavagnat and F. Cruege, in *Lasers in Physical Chemistry and Biophysics* (J. Joussot-Dubien,. Ed.), p. 425. Elsevier, Amsterdam (1975).
[15] C. Voegtlin, J. M. Johnson and H. A. Dyer, *J. Pharmacol. Exp. Ther.* **27**, 466 (1926).
[16] A. M. Novi, *Science* **212**, 541 (1981).
[16a] L. Casahoursat, P. V. Huong and D. Larrouture, *Sci. Pharm.* **2**, 700 (1986).
[17] J. P. Bideau, P. V. Huong and S. Toure, *Acta Crystallogr.* **B52**, 481 (1976).
[18] P. Berrouet, Doctoral Thesis, University of Bordeaux (1978).
[19] P. V. Huong and J. C. Cornut, *J. Chem. Phys.* **65**, 4748 (1976).
[20] P. V. Huong and S. R. Plouvier, *J. Mol. Sruct.* **115**, 489 (1984).
[21] S. R. Plouvier and P. V. Huong, *Biorheology*, Suppl. 1, 345 (1984).
[22] J. Lorosch, W. Haase and P. V. Huong, *J. Inorg. Biochem.* **27**, 53 (1986).
[23] P. V. Huong, P. Carmona and J. Lorosch, in *Proceed. ICORS X* (W. Peticolas, Ed.). Eugene, USA (1986).
[24] P. V. Huong, S. R. Plouvier and P. Lambert, in *Spectroscopy of Biological Molecules*, (Alix, Bernard, Manfait, Eds), p. 231. Wiley, New York (1985).
[25] P. V. Huong, *J. Mol. Struct.* **141**, 203 (1986).

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