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The Resonance Raman Light Scattering in Studies of the Structures and Functions of Flavins and Flavoproteins

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The application of the spontaneous and non-linear resonance Raman spectroscopic methods in studies on flavins and flavoproteins is examined. The biological functions of flavin systems are briefly surveyed and the parameters of the resonance Raman spectra and their relation with the electronic structure of the isoalloxazine ring in various oxidation states are described. The possibilities of identifying the flavin chromophore in a protein environment, in charge-transfer complexes, and in modified systems are discussed.
The bibliography includes 115 references.

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I. THE LINEAR AND NON-LINEAR RESONANCE RAMAN SPECTROSCOPIC METHODS

Resonance Raman light scattering (RRS) was observed in the 1950's before the appearance of universal Raman scattering (RS) excitation sources—lasers. "Resonance" implies in this sense the coincidence of the frequency of the exciting light with one of the frequencies of the electronic transition in the molecule. This term was first applied to the description of the large increase in the intensities of certain Raman lines of aromatic nitro-compounds on excitation in the long-wavelength absorption band region.[1] All the characteristic features of RRS were described in subsequent studies:[2-4] high intensity coefficients exceeding by a factor of 10^3–10^9 the intensity of the usual RS; selective intensification of only certain normal vibrations among those actually applicable to the given electronic absorption band; the appearance in certain cases in the Raman spectrum of progressions of intense overtones; relations between the RRS line intensities and the parameters of the electronically excited state. These properties made it possible to expand greatly our ideas about the interaction of light with matter and to obtain new information about molecular structure and have led to analytical possibilities unavailable in the traditional RS methods. The invention and theoretical application towards the end of the 1960's of lasers led to a vigorous growth of the applications of RS, in particular RRS, in the solution of a wide variety of problems in physics, chemistry, and biology.[5,6] At the same time numerous non-linear effects, arising in matter under the influence of coherent laser beams, were observed and foundations were laid for a new field—non-linear optics and one of its branches, namely non-linear scattering spectroscopy.

In contrast to the traditional spontaneous RS, arising from fluctuation (thermal) vibrations, in non-linear scattering spectroscopy one considers the interactions with forced intramolecular vibrations which are excited under certain conditions by the powerful light fields of lasers in a large volume of matter. It is important to emphasise that the coincidence of the phases (phase synchronisation) of the forced vibrations plays the main role in these processes, whereas their amplitudes in the strongest light field remain smaller by 2–3 orders of magnitude than the fluctuation (spontaneous) changes in the normal nuclear coordinates. The first of these phenomena observed was forced Raman scattering (FRS).[7] Although the spectroscopic possibilities of this method are extremely limited, it served as the physical foundation of a whole series of non-linear Raman spectroscopic methods developed in the subsequent decade[8] and based on measurements of the amplitude, polarisation and phase of coherent waves "scattered" by forced vibrations.[9,10] Although many of these processes are not scattering in the traditional sense of the word, their interpretation may be based on classical ideas about the modulation of polarisability by normal nuclear vibrations, which are widely used in spontaneous Raman spectroscopy and which only arose at the time of the publication of Placzek's book,[11] while the parameters of the non-linear scattering—the intensities, forms, half-widths, and positions of the signal—may be compared with spontaneous RS data.

The rapid progress in the technology of lasers with tunable frequencies resulted in what were initially unique measurements becoming routine measurements.[12] Two non-linear Raman spectroscopic methods, which have found most practical applications and whose variants analogous to spontaneous RRS have also been investigated, are briefly described below.

The Coherent Anti-Stokes (Stokes) Light Scattering (CALS and CSLS) Spectroscopy. Two coherent beams with the frequencies ω_1 and ω_2 such that their difference $\omega_1-\omega_2$ coincides with one of the intramolecular vibration frequencies ω_r can be excited and phase-synchronised against the background of the vibration with the frequency ω_r; a third (trial) wave then interacts with forced vibrations, is scattered, and gives rise to intense coherent components in the anti-Stokes and Stokes regions:[8]

$$\omega_{a,\,c}=\omega\pm(\omega_1-\omega_2)\ .$$

The most widely used version of this method is one where one of the pumping waves is used as the trial wave ($\omega=\omega_1$):

$$\omega_a=2\omega_1-\omega_2\ ,$$

Evidently, by varying the difference $\omega_1-\omega_2$, it is possible to obtain all the frequencies corresponding to the spontaneous Raman spectrum of the molecule. The abbreviations CALS and CSLS widely used nowadays in the foreign literature were introduced by Byer et al;[13] however, even earlier Akhmanov and co-workers[10] proposed for this process the term "active RS spectroscopy (ARSS) which we shall also use subsequently.

The analogue of spontaneous RRS, where the coherent pumping frequency ω_1 is close to the frequency of the electronic (one-photon) transition in the molecule ω_e, is nowadays

vigorously investigated in the CALS spectroscopy. On decrease of the difference $\omega_1-\omega_e$, a sharp increase in the intensity of the coherent signal was observed in the CALS spectra.[14,15] In the region of the one-photon resonance, the CALS and CSLS lines have extremely complex contours due to the interference of several contributions—electronic and vibrational (see, for example, Refs.9 and 10). This factor initially led to an erroneous interpretation of the observed phenomenon as inverse RS[15] (see below). Theoretical analysis within the framework of quantum-mechanical ideas has shown[16] that, when ω_1 is scanned in the region of a broad electronic absorption band, the contour of the anti-Stokes line can vary from positive Lorentzian through dispersion to negative Lorentzian, depending on the ratio of the parameters: the frequencies ω_1, ω_e, and ω_r and the decay constants Γ_e and Γ_r of the electronic and vibrational oscillators. For identical values of $\omega_1-\omega_e$, the CALS and CSLS signals have different forms (Fig.1). This has been observed experimentally for cytochrome *c*,[15] vitamin B_{12},[15] β-carotene,[17] and certain other biologically active substances.[9]

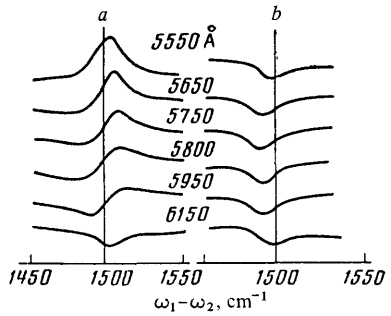


Figure 1. A model calculation of the CALS (*a*) and CSLS (*b*) band contours on resonance excitation;[16] the values of the parameters simulate the case of vitamin B_{12}; ω_e = 18,000 cm^{-1}, ω_r = 1500 cm^{-1}, Γ_e = 500, and Γ_r = 10.

The case of "double resonance", vibrational and electronic (one-photon), has also been investigated in inverse Raman spectroscopy. This method has a number of advantages, among which the absence of an intense permanent background, associated with the non-resonance electronic susceptibility and generating a serious interference in the CALS and CSLS spectra (under conditions remote from one-photon resonance), is important.[9,10] It has also been suggested that, in contrast to ARSS, in the intensification–attenuation scheme it is possible to obtain spectra fully corresponding to spontaneous RS, including the form of the signal.[19,20] In the first experiment on resonance inverse RS, direct measurements were made of the absorption.[21] Subsequently various modulation schemes were proposed[22,23] permitting a significant improvement of the detection limits.[24] The experiments on the excitation of inverse RS in the region of broad electronic absorption bands showed that[24] the form of the signal changes as a function of the ratios of ω_1, ω_e, and Γ_e. The results of a model parametric calculation of the resonance inverse RS line contour are presented in Fig.2.

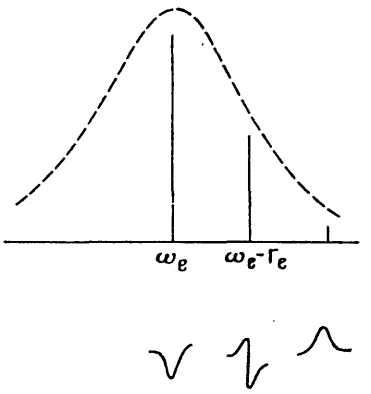


Figure 2. The contours of the inverse Raman signal (below) on excitation in the region of the absorption band (shown dashed); model calculation.[24]

Inverse Raman Spectroscopy. CALS and CSLS are four-photon processes. A lower-order non-linear process, namely a two-photon process, was also observed in the 1960's. When two coherent beams with the frequencies ω_1 (intense pumping) and ω_2 (weaker trial wave) were allowed to interact with the medium, Jones and Stoicheff[18] observed a weakening of the intensity of the trial wave, while the frequency difference $\omega_2-\omega_1$ coincided with the vibrational transition frequency ω_r:

$$\omega_2-\omega_1\simeq\omega_r;\quad \omega_1<\omega_2$$

This phenomenon was called the inverse Raman effect.[18] Instead of the weakening of the trial high-frequency wave in the field of powerful low-frequency pumping, one can employ another scheme and observe an intensification of the trial low-frequency ω_2 wave under the influence of the intense high-frequency ω_1 pumping.[19] The corresponding terms have been proposed in the Soviet literature: forced RS (FRS) in intensification and inverse RS (IRS) in attentuation.[10] These methods are sometimes referred to as modulation methods bearing in mind the change in the optical properties of the medium at the frequency of the trial wave.[10]

The advantages of coherent Raman spectroscopy in the study of rarefied gases and in the diagnostics of plasma, rapid combustion processes, etc. have been frequently noted.[8,10,12] In comparing the possibilities of spontaneous RRS and its coherent analogues in studies of the structures of complex molecules in condensed phases, one should note in the first place the difficulties associated with the extraction of information from coherent Raman spectra in the region of one-photon resonance; the treatment of spontaneous RRS data is in this case much simpler. The ARSS and inverse Raman spectra have been calculated on a computer by adjusting parameters. At the same time, the complexity of the form of the coherent signal and the changes in the latter in the resonance region due to the interference by several contributions make it possible to obtain, with the aid of parametric analysis, a series of additional data—in particular one can estimate the decay constants for the electronic and vibrational oscillators, determine the frequencies in the excited state of the molecule, and refine the position of the 0–0 transition. However, all these processes have so far been inadequately studied.

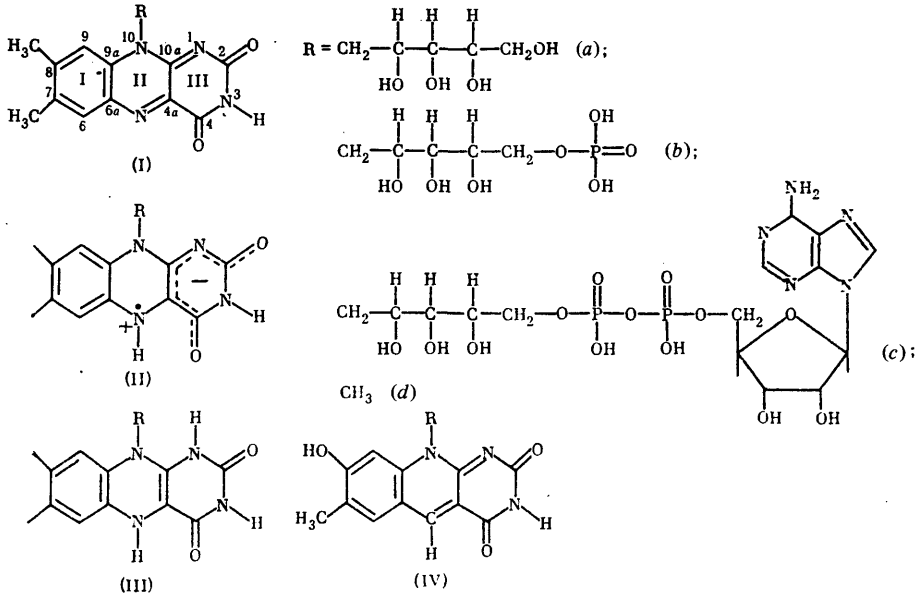


Figure 3. The prosthetic flavin groups and the numbering of the atoms in the isoalloxazine ring (I): *a*) riboflavin; *b*) flavin mononucleotide; *c*) flavin adenine dinucleotide; *d*) lumiflavin; II) semiquinone form; III) fully reduced form; IV) 5-deaza-8-hydroxyflavin.

The excitation in the region of resonance of complex and especially biological molecules is known to entail frequently the appearance of an intense fluorescence, which fully rules out the observation of spontaneous RRS. Since the CALS and inverse Raman signals are located in the anti-Stokes region and the fluorescence occurs in the Stokes region, they provide a unique possibility for recording Raman spectra in the presence of fluorescence. We may also note that the average level of the intensity of the irradiation of the specimen in the coherent spectroscopy is lower by 1–2 orders of magnitude than in the recording of spontaneous Raman spectra, which is important for photolabile substances.

Thus non-linear Raman spectroscopic methods significantly supplement the spontaneous spectroscopic method and sometimes constitute the only means of observing the Raman spectra. Yet another field, associated with studies on non-stationary processes and the determination of the dynamic characteristics of short-lived intermediate states, which is extremely important for biology, has developed rapidly during the last decade within the framework of non-linear spectroscopy (see, for example, Akhmanov and Koroteev,[10] Chapter 5). Progress in the technique of the generation of short and ultrashort [picosecond (10^{-12} s)] pulses and the creation of adequate recording methods[25–27] permitted the study of rapid biological processes, including the conformational rearrangements in the active centres of haemoproteins[28] and in visual pigments.[29]

The scale reached by studies with the aid of linear and non-linear resonance Raman spectroscopy in biology can be inferred from the appearance of the term "resonance Raman spectroscopy of haemoproteins",[30] "resonance Raman spectroscopy of visual pigments",[31] etc. The comparatively new rapidly developing field of flavoproteins has been least thoroughly investigated in this sense.[32–34] This review discusses resonance Raman spectroscopic data obtained for flavin and flavoproteins in connection with their structures and functions; the latest ideas concerning the role of flavoproteins in living nature and certain mechanisms of their action are also briefly surveyed.

II. THE STRUCTURES AND FUNCTIONS OF FLAVOPROTEINS

Flavins and Flavoproteins. This important class of natural products has been little investigated partly because of historical reasons† as well as a number of other factors: the great complexity of the molecules, the difficulty of isolation, the low content in natural objects, and, which is most important, the unusual wide variety of the functions of flavin-containing compounds in nature: in higher animal and plant organisms as well as micro-organisms. Flavoproteins are in most cases enzymes with a prosthetic group containing a substituted isoalloxazine ring (Fig.3), usually in the form of flavin mononucleotide (FMN), flavin adenine dinucleotide (FAD), or riboflavin. The highly conjugated tricyclic isoalloxazine system, in which there are comprehensive possibilities for electronic rearrangement by virtue of the presence of four nitrogen atoms and fragments analogous to the amide group, constitutes the centre of numerous biochemical reactions. The flavin system incorporated in a wide variety of protein environments—from comparatively short polypeptide chains (flavodoxins, ~130–170 aminoacid residues[35]) to enormous dimeric molecules (glutathione reductase, ~480 residues in each subunit[36,37])—affects the enzyme catalysis of the oxidation-reduction reactions of an enormous number of substrates, including amines, alcohols, amino- and hydroxy-acids, dithiols, etc.[34] The reactions of flavoproteins with molecular oxygen are no less varied. In certain oxidative processes, one oxygen atom of the O_2 molecule is transferred to the substrate, while the other is incorporated in a water molecule; in other reactions, molecular oxygen is reduced with formation of the superoxide O_2^-.

†The first natural flavin compound, namely riboflavin, was identified in 1935, while the most important prosthetic group, flavin adenine dinucleotide, was not identified until 1954.

Apart from its life supporting functions in organisms and plants, flavin enzymes play an enormous role in the turnover of the elements in nature effected by micro-organisms, in particular in the global turnover of carbon.[38] In the course of evolution, soil bacteria developed a wide variety of enzymes (monoxidases) catalysing the decomposition of residues from higher plants, mainly lignin, which is a stable natural polymer.[38] The function of flavin enzymes is just as important in the metabolism of nitrogen and especially sulphur, whose biological circulation effected by bacteria has been estimated[39] as amounting to 75% of the total content of sulphur on earth.

The applications of these compounds have extensive biomedical aspects. In the organisms of higher animals, the most important agent supporting the normal level of flavoproteins is riboflavin, which is ingested with food and is then transferred by special transfer proteins to the tissues, where it is bound in the form of FMN and FAD cofactors to other proteins.[40] One can apparently expect that in the immediate future it may be possible to approach an understanding of certain molecular aspects of diseases (including certain hereditary afflictions of the brain and the peripheral nervous system) associated with the deficiency of flavoproteins and the disturbance of their metabolism in the organism.[41] This is also indicated by the development of the medicinal antihypertensive agents and psychotropic preparations (derivatives of phenothiazine and tricyclic antidepressants), whose structure is close to that of riboflavin; their activity is apparently based on the inhibition of the conversion of riboflavin into FMN and FAD in the organism.[42] The functions of flavoproteins determining the occurrence of various detoxification processes, involving a wide variety of aromatic compounds, sulphur-containing derivatives, and mercury, have also been elucidated.

The multiplicity of functions of flavin enzymes has become evident only recently. On the basis of mainly biochemical data which have now accumulated, Massey and Hemmerich[43] suggested that five main classes of flavoprotein can be distinguished in accordance with the characteristic features of their catalytic cycles.

The field of flavins and flavoproteins is developing rapidly. Unusual flavin enzyme systems containing a modified isoalloxazine ring, including flavins with "double modification" of the ring—the 5-deaza-8-hydroxy-derivative (Fig. 3), the so-called F-420 cofactor of methane bacteria, have been discovered and investigated.[44,45] In contrast to haemoproteins, the flavin group does not form covalent bonds with the protein in most cases and is retained in the active centre mainly with the aid of strong hydrogen bonds, favourable electrostatic interactions, etc. However, whole series of flavoproteins have now been identified in which the isoalloxazine ring proved to be covalently bound to the apoprotein, usually in the C(8)–position via a CH_2 group to histidine, cysteine, or tyrosine residues.[46]

Nevertheless, despite the undoubted progress made, the field of flavoproteins has so far been developing mainly via the extensive accumulation of new data, leaving many problems unsolved. The true natural substrates are unknown for a whole series of flavoproteins distributed in animal and plant tissues; the biological functions of many flavoproteins, including one of the first compounds identified—"the old yellow enzyme", have not been elucidated either. The biological role of the covalent bond between the isoalloxazine chromophore and the protein in covalently bound flavoproteins is unknown. All the biological oxidation-reduction chains contain at least one flavoprotein, which transforms two-electron transport into one-electron transport to a "one-electron" acceptor (cytochrome or sulphur-containing protein). The unique mechanism on this $2e^-/1e^-$ switch still remains obscure.

Protein plays an enormous role in the functioning of flavin enzymes. The immediate protein environment of the isoalloxazine ring actually determines ultimately its specific function in the enzyme. However, our information about this field is still very limited and the structure (even the primary structure) of many vitally important flavoproteins remains unknown. X-Ray diffraction data have now been obtained for representatives of only three classes of flavoproteins: flavodoxins,[47] glutathione reductases,[36] and *p*-hydroxybenzoate hydroxylases.[48] Furthermore, it is evident that only knowledge of the details of the steric structure of the active centre (in certain cases even the static [resting-state] structure) would make it possible to reach well-founded conclusions about the mechanism of the catalysis and the character of the intermediate stages. The example of glutathione reductase is instructive from this point of view; according to X-ray diffraction data,[36,37] the immediate environment of FAD in this enzyme predetermines its functions as a two-electron transferring agent and blocks the structures necessary for the transfer of one electron.

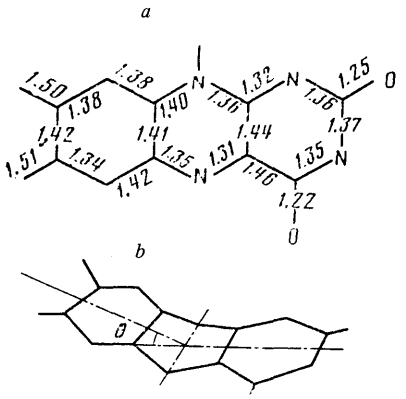


Figure 4. The bond lengths in the oxidised planar structure of the isoalloxazine ring (*a*) and the "butterfly" conformation of the fully reduced form (*b*); the energy minimum corresponds to the angle $\theta \cong 15°$.[50]

However, this example is exceptional and most of the proposed mechanisms of the biochemical reactions of flavoproteins remain extremely speculative. In order to account for the mechanism and to identify the intermediate stages, traditional biochemical methods are resorted to, in particular methods using the formation of artificial complexes of the enzyme with the inhibitors. Another procedure for the "probing" of the active centre is based on the ease of the separation of the flavin chromophore from the aproprotein,[49] which can then be joined to isoalloxazine chemically modified at points tentatively assumed to be of "key" importance for the kinetic and thermodynamic parameters of the catalysis. In order to deduce the environment of the isoalloxazine chromophore in the protein, indirect physical information is also used. For example, it is known that the intense fluorescence characteristic of flavins is quenched following addition to certain proteins. It has been suggested that this can occur only when the isoalloxazine ring interacts with the tyrosine and tryptophan in the active centres of the protein which are close to it in space.[35]

Electronic Absorption Spectra and Structural Parameters of the Isoalloxazine Ring. Three main biological functions of the isoalloxazine chromophore, namely (de)hydrogenation (only $2e^-$), activation of molecular oxygen ($2e^-$ or $1e^-$), and "pure" electron transport ($1e^-$), are possible by virtue of electronic rearrangements of three stable forms (Fig. 3): oxidised, half-reduced (semiquinone; radical), and fully reduced. In addition various ionisation and protonation states are possible for the last two forms.

The structural parameters of the isoalloxazine ring are indicated in Fig. 4. The conformational possibilities in the conjugated flavin system are extremely limited; according to X-ray diffraction data, the fully oxidised isoalloxazine ring in all the flavin systems investigated experimentally has a planar structure in conformity with the predictions of quantum-chemical calculations (see Dixon et al.[50] and the references quoted therein).

The "butterfly" conformation with a dihedral "folding" angle θ relative to the N(5)–N(10) axis of approximately 10° has been predicted for the fully reduced form by MO SCF calculations (in the INDO version—incomplete neglect of differential overlap)[50] (Fig. 4). The experimental values of θ for this form in model systems vary in the range 30–8°, whereas in proteins the angle θ can apparently be even smaller.[51] Calculation has shown[50] that the variation of the angle θ in the range 20–0° can be achieved for an energy expenditure of approximately 3.5 kcal mol^{-1}, which corresponds to slight conformational rearrangements of the aminoacid residues in the protein surrounding the flavin. For this reason, a planar structure is apparently preferable for the semiquinone form in the protein environment.[52]

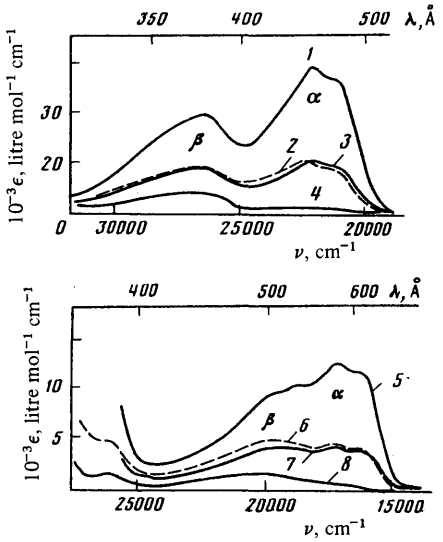


Figure 5. The electronic absorption spectra of an FMN-containing protein—flavodoxin from *Clostridium MP*; *a*) oxidised form; *b*) semiquinone form; the polarisation spectra of single crystals with $E \parallel$ crystal *c* axis (curves *1* and *5*) and $E_\perp$ crystal *c* axis (curves *4* and *8*) as well as the spectra of the solutions (curves *2* and *6*) are shown; curves *3* and *7* represent the calculated isotropic spectra of the crystals.[55]

The flavin chromophore has intense absorption bands in the long-wavelength part of the spectrum: one band at 20000–25000 cm^{-1} and another with centre at approximately 26400 cm^{-1},[53] which are due to the two lowest $\pi \rightarrow \pi^*$ transitions[54] and which will henceforth be designated as the α- and β-bands (Fig. 5).[55] Polarisation measurements of the absorption in crystals[55] and earlier studies on oriented films and the polarisation of the fluorescence of model flavins showed that the corresponding transitions are linearly polarised in the plane of the flavin ring in the direction close to the axis parallel to the plane of the ring and perpendicular to the N(5)–N(10) axis.[55]

III. THE RESONANCE RAMAN SPECTROSCOPY OF FLAVINS AND FLAVOPROTEINS

The Non-Linear and Spontaneous Resonance Raman Spectroscopy of Flavins. The resonance Raman spectroscopy of flavin systems, like that of certain proteins of other classes, constitutes in essence the only (with the exception of X-ray diffraction analysis) source of the most detailed information about the structure of the active centre. Indeed, until recently, the electronic rearrangements of the isoalloxazine ring in the functioning of the flavoprotein could be inferred from only one parameter—the change in the positions and intensities of the long-wavelength $\pi \rightarrow \pi^*$ transitions.[43] Apart from the usual limitations, the application of NMR is in this case complicated also by the fact that, in addition to the protons of the two methyl groups in the 7- and 8-positions and the CH bonds in the 6- and 9-positions, whose signals contain little information, the isoalloxazine ring contains a single proton at the N(3) atom; the low relative content of the flavin fragment in proteins hinders the application of ^{13}C NMR. The NMR data obtained hitherto refer mainly to the ribityl fragment of FMN and FAD.[56]

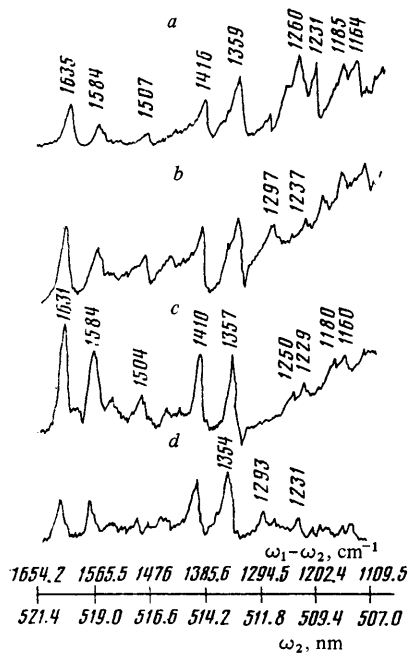


Figure 6. The resonance CALS spectra of flavin adenine dinucleotide [*a*) in H_2O; *b*) in D_2O] and riboflavin-binding protein [*c*) in H_2O; *d*) in D_2O]; pH 7; $\omega_1 = 480$ nm.[58]

In a free state the flavin chromophore (FMN, riboflavin) exhibits a very intense fluorescence, its intensity exceeding by several orders of magnitude (by a factor of ~10^4) that of the Raman lines. The first resonance Raman spectra of FAD and FAD-containing protein (glucose oxidases) were obtained by means of the "spatial" separation of the scattering and fluorescence in the anti-Stokes ARSS (CALS) method with excitation in the region of the α-band.[57] The frequency $\omega_1 \cong 480.0$ nm, tentatively coinciding with that of the 0–0 transition (ω_{00}), was used as the pumping frequency.[54] Further improvement of the experimental technique (the introduction of a second monochromator into the optical path of the signal) made it possible to reduce the background in the vicinity of the pumping frequency and to obtain CALS resonance spectra for FAD and two FAD-containing proteins, glucose oxidase and riboflavin-binding protein (Fig.6), starting from 300 cm^{-1} in certain cases.[58]

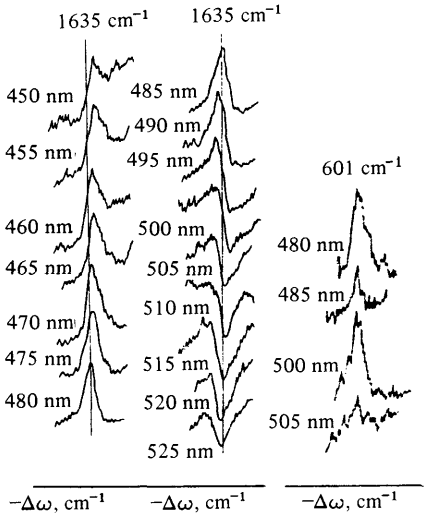


Figure 7. The $\omega_r \cong 1635$ and 601 cm^{-1} band contours in the resonance CALS spectra; the frequencies ω_1 are indicated in the Figure.[59]

As already mentioned, the major difficulties in the resonance excitation in the CALS are associated with the variation of the signal contour as a function of the ratios of ω_1, ω_e, ω_r, and a number of other parameters.[16] The FAD line contour ($\omega_r \cong 1635$ cm^{-1}) is illustrated in Fig.7 as a function of the varying ω_1 in the region of the α-band.[59] It is seen from these data that approximate estimates based on the semiclassical model[16,60] agree with experiment fairly satisfactorily (see also Fig.1). A positive Lorentzian contour is observed at $\omega_1 \cong \omega_0$; for higher and lower energies ($\omega_1 \cong \omega_{00} \pm \omega_{r/2}$), the signal becomes of the dispersion type and has a negative Lorentzian contour at $\omega_1 \cong \omega_{00} - \omega_r$.. We may note that, under the same excitation conditions, only a positive Lorentzian contour was observed for the $\omega_r \cong 601$ cm^{-1} lines, which the authors attributed to a large contribution of Franck–Condon factors.[59] In conformity with model calculations,[16] only dispersion line contours were observed for a coherent Stokes signal. Thus the exact determination of the frequency in the resonance CALS spectra may be difficult. The only practical procedure remaining so far is one involving the recording of a series of spectra at different pumping frequencies in order to discover the purely dispersion or purely Lorentzian contour.

Almost simultaneously with these studies, reports concerning the spontaneous resonance Raman spectra of flavins appeared. A group of Japanese workers[61]# used the ability, noted a long time ago, of the protein matrix to weaken greatly the fluorescence of the isoalloxazine chromophore and obtained the spectra of two riboflavin-binding proteins: from chicken egg yolk and white, and also of the 3-CH_3-, 2-CH_2COOH-, and 7,8-Cl-derivatives of riboflavin attached to the upper end of the apoprotein. Subsequently the resonance Raman spectra of several other flavoproteins and of the FMN and FAD prosthetic groups themselves were obtained; in order to eliminate the interfering fluorescent background, various procedures were employed; excitation by lines at longer wavelengths (600 nm; in the preresonance region)[63] or at shorter wavelengths (λ = 363.8, 351.1, 337.1, and 257.3 nm, in the region of high-energy electronic transitions of the chromophore), and also by adding quenching agents, mainly KI.[64,65]

In the first investigations,[57,58] it was already noted that only vibrations localised in the isoalloxazine ring are intensified by resonance and that, as might have been supposed, the vibrations associated with the substituents at N(10), namely ribose (in riboflavin), phosphoribose (in FMN), or adenosine diphosphate-ribose (in FAD), are not intensified. Typical FAD spectra are illustrated in Fig.6. We may note that some of the frequencies in the above study differ from those obtained subsequently in spontaneous Raman spectra (Table 1). Bearing in mind that positive CALS line contours are obtained at the pumping frequency $\omega_1 \cong 480$ nm[58] while negative contours are obtained at the frequency $\omega_1 \cong 495$ nm[66] the agreement between the frequencies in Table 1 can be regarded as satisfactory. It was shown subsequently that, under identical excitation conditions, the resonance Raman spectra of riboflavin, FMN, and FAD are almost identical in the region above 1100 cm^{-1} (for which reliable data are available) with the exception of small differences as regards the intensities of a number of bands.[64]

The Assignment of the Vibration Frequencies. In the very first studies, attempts were made to compare the resonance Raman frequencies with the structural elements of the isoalloxazine ring. The correlations were based initially on a single experiment involving isotope substitution [the replacement of the exchanging N(3)H proton by deuterium[58,61,63]] and also on data for 7,8-Cl-substituted derivatives[61] and intuitive guesses. However, a fundamental study by Kitagawa et al.,[67] who obtained the resonance Raman spectra for the N(3) and N(3)D forms of the isotope-substituted [2-^{13}C]-, [4a-^{13}C]-, [10a-^{13}C]-, [2,4,4a,10a-^{13}C]-, [5-^{15}N][1,3-^{15}N]- and [1,3,5-^{15}N]-riboflavins was soon published. In order to eliminate fluorescence, the authors used the ability of certain proteins, noted above, to exhibit a "quenching" effect on the fluorescence of isoalloxazine and the ease of the formation of its complexes with the apoprotein; the complexes of all the above derivatives with the riboflavin-binding protein from chicken egg protein were obtained in this way. The study of Kitagawa et al.[67] prepared a basis for a reliable calculation of the normal vibration frequencies of the flavin system, but these extremely rich data have remained virtually unused. The only calculation of the vibration spectrum of 10-methylisoalloxazine (lumiflavin) carried out hitherto[68] can be regarded as only preliminary.

The observation of a single isoalloxazine line on excitation with a UV laser had been reported in an earlier study.[62]

Table 1. The frequencies of the resonance Raman lines of the prosthetic flavin groups (cm^{-1}).

Band designation	FAD (Ref.58)[a]		FAD (Ref.63)[b]		FAD (Ref.64)[c]	FMN (Ref.63)[c]		FMN (Ref.66)[d]		FMN (Ref.64)[e]
	H_2O	D_2O	H_2O	D_2O	H_2O	H_2O	D_2O	H_2O	D_2O	H_2O
I	1635 s	1634 s	1633 w	1628 w	1629	1633	1628	1629	1628	1626
II	1584 m	1584 m	1585 m	1584 m	1582	1584	1584	1582	1580	1583
III	—	—	1551 m	1548 m	1548	1551	1548	—	—	1549
IV	1507 w	1507 m	1504 m	1502 m	1500	1503	1502	1503	1504	1500
V	—	—	1468 w	1469 m	1461	1469	1468	—	—	1459
VI	1416 s	1416 s	1412 s	1410 s	1408	1413	1411	1412	1414	1405
VII	1359 s	1359 s	1356 vs	1351 vs	1353	1355	1351	1355	1357	1350
VIII	—	—	—	—	1280	—	—	—	—	1278
X	1260 m	1297 m	1261 m	1300 m	1255	1261	1300	1261	1300	1256
XI	—	—	1234 s	1233 m	1228	1233	1230	1233, 1192	—	1228
XII	1185 w	1179 w	1190 w	1171 m	1182	1187	1172	1170	—	1183
XIII	1164 w	1144 w	1166 m	1147 m	1160	1166	1149	1151	—	1158

[a] CALS, ω_1 = 480 nm. [b] Spontaneous RS, λ = 600 nm. [c] pH 8.5 with added 5 M KI; spontaneous RS, λ = 488 nm. [d] In H_2O, CALS, ω_1 = 495 nm. [e] In H_2O with added 6 M KI; spontaneous RS, λ = 514.5 nm. s = strong (intense), vs = very strong, m = moderate, and w = weak.

The force constants of the valence bonds and angles and also the interaction constants were in the main transferred in the above study from benzene, pyrimidine, and uracil molecules, taking into account the bond length—force constant correlations (see the references in the paper of Bowman and Spiro[68]). At first sight, the force field generated in this way could in principle reflect the specific features of the flavin system; the large bond interaction constants (up to 15% of the diagonal force constant) are characteristic of molecules with a high degree of delocalisation of the electron density. Nevertheless, almost all the frequencies calculated in terms of this approximation differ appreciably from the experimental values and in certain cases the differences for the most intensive bands reach 80–100 cm^{-1}. However, it is more significant that the calculated data do not reproduce the line shifts on isotope substitution.[67] This is particularly clear for the ~1250 and 1160 cm^{-1} bands, which undergo the greatest shifts on deuteriation, and in certain isotope-substituted specimens. Among the reasons for the failure of the calculation, one of the main ones consists apparently in the incorrect initial assignment of certain frequencies. The selection of the force field, in which certain important interactions, for example, those of the N(5)–C(4*a*) and C(10*a*)–N(1) bonds, were altogether disregarded, was also relatively unsuccessful (Fig. 3). We may note that the initial approximation chosen by the authors proved to be relatively unsuitable also for uracil, where the calculation likewise reproduced unsatisfactorily the increase of the frequencies in the region of 1250 cm^{-1} on deuteriation.[69] Thus only preliminary conclusions may be made so far concerning the nature of the individual resonance Raman bands of flavins, mainly on the basis of isotope substitution data.[61,64,67]

Band I. The highest-frequency 1630 cm^{-1} band has been assigned in most investigations to vibrations with the predominant involvement of the benzene ring, mainly on the basis of the appreciable decrease of this frequency following substitution by halogen atoms in the 7- and 8-positions.[61,70] This Raman line proved to be almost insensitive to isotope substitution in the 2-, 4*a*-, 5, 1, and 3-positions;[67] it hardly shifts for the 1,3- and 5-deaza-derivatives[71] and is not intensified on resonance excitation at the frequency corresponding to the long-wavelength (α) absorption band. All the data obtained hitherto show that the symmetrical (ν_s) and anti-symmetrical (ν_{as}) vibrations of the C(2)=O and C(4)=O carbonyl groups are not manifested in the resonance Raman spectrum.

Band II (1548 cm^{-1}). This band changes appreciably on substitution of the N(5), C(4*a*), and C(10*a*) atoms in ring II[67] and disappears in 5-deaza-FMN.[71] However, the appreciable involvement of other coordinates is indicated by the shift of this band in the N(3)-substituted derivatives.[71] On resonance excitation at the frequency of the β-band (370 nm), it was possible to observe only this line.[62]

Table 2. The shifts of the resonance Raman lines of the N(3)H forms of the isotope-substituted riboflavin specimens.[67]

ν, cm^{-1}[a]	^{13}C			^{15}N		
	2-	4*a*-	2,4,4*a*,10*a*-	5-	1,3-	1,3,5-
1631 (1630)	2	1	2	1	1	2
1584 (1582)	3	8	13	4	1	5
1548 (1548)	6	5	21	3	2	4
1503 (1409)	3	2	7	1	2	4
1465 (1462)	5	6	8	1	3	6
1407 (1406)	3	5	12	1	4	6
1355 (1351)	4	3	15	0	1	1
1302 —	0	2	5	0	0	2
1282 —	0	2		0	0	0
1252 (1295)	14	0	17	0	10	10
1229 (1232)	0	3	4	0	2	2
1179 (1181)	3	4	8	2	4	6
1161 (1147) (1138)	1	2	5	1	1	2
1073 —	3	3	3	—1	4	2
994 (990)	2	0	4	0	1	3
834 (832)	2	0	2	5	5	10
789 (772)	2	0	4	2	2	6
740 (744)	1	0	2	1	0	2
633 (629)	1	—1	0	—1	1	2
605 (602)	4	1	4	1	1	4
576 —	3	4	5	9	2	9
521 (522)	1	1	2	4	1	7
496 (474)	1	2	—	1	2	4
429 (427)	2	2	2	3	0	6

[a] The frequencies for the N(3)D form are given in brackets.

Band III. Large isotope shifts (21 cm^{-1} in the 2,4,4*a*,10*a*-^{13}C-substituted derivatives (Table 2) have been observed for the low-intensity 1548 cm^{-1} line.

Bands IV, V, and VI. The weak bands at 1500 cm^{-1} (IV) and 1462 cm^{-1} (V) and the intense 1407 cm^{-1} band (VI) are approximately equally sensitive to isotope substitution. The 1407 cm^{-1} vibration is appreciably delocalised; the shifts of this band are large on ^{13}C and ^{15}N isotope substitution in ring (III) and amount to approximately 10 cm^{-1} in the 7,8-dichloro-derivatives.[61]

Band VII. The 1355 cm^{-1} band is one of the most intense in the resonance Raman spectrum. It has been noted that it is intensified on resonance with the electronic absorption α-band (450 nm) and is the most intense on excitation in the pre-resonance region.[63] Table 2 shows that the corresponding vibration is very sensitive to ^{13}C substitution and is relatively insensitive to ^{15}N substitution. However, this finding is not in any "conflict" with the decrease of the frequency of this line in the deaza-derivatives,[71] since a different system of conjugated bonds is formed on replacement of the N(1) and N(5) atoms by carbon atoms.

Bands VIII and IX. The very weak lines at 1302 cm^{-1} (VIII) and 1282 cm^{-1} (IX) are almost insensitive to isotope substitution.

Bands X, XI, XII, and XIII (1255, 1228, 1182, and 1160 cm^{-1}). The greatest changes occur in this group of bands on replacement of the N(3)H proton by deuterium and were noted in the very early studies;[58] not only the line frequencies but also the relative line intensities change significantly under these conditions (Fig.6). Evidently the $\nu = 1295$ cm^{-1} band is the analogue of the intense 1255 cm^{-1} band in the spectrum of the deuteriated specimen. The increase of the frequency by more than 40 cm^{-1} is usually explained, after Kitagawa,[61] by the breakdown of the "resonance" interaction of the C–N stretching vibration (tentatively at 1290 cm^{-1}) with the in-plane NH deformation vibration, which reduces this frequency in the non-deuteriated specimen.§ However, we may note that the idea of "resonance" in interactions of the vibrations is altogether unnecessary for the explanation of the observed changes. Evidently, the frequency considered can increase even as a result of a slight involvement of the fairly rigid N–D bond when the amplitude of the stretching vibration of the deuterium atom is large. If this hypothesis is correct, then the replacement of the N(3)H hydrogen by the methyl-group should entail an increase in frequency, which has been observed experimentally for *N*(3)-methyl-lumiflavin.[70] It is evident from these ideas that the change in the 1255 cm^{-1} frequency can serve as a criterion of the strength of the hydrogen bonding of the N(3)H group of flavin with the solvent or the aminoacid residues in the active centre of the protein; the frequency should increase as a result of the increase of the effective rigidity of the C–N(3)–H angle as a consequence of the additional binding of the hydrogen atom (see below).

Among the remaining bands of this group, the 1179 cm^{-1} band partly corresponds to the most "delocalised" vibration. The 1179/1160 cm^{-1} band pair undergoes an appreciable change in the relative intensities on isotope substitution[67] also in *N*(3)-derivatives[61] and also on substitution by heavy atoms in the 7- and 8-positions.[61] Evidently any kind of reliable assignments in this region are impossible without a reliable calculation of the normal vibration frequencies; there appears to be little justification for the scheme of frequency shifts on deuteriation proposed by Schmidt et al.[64] (with the exception of the 1255 → 1290 cm^{-1} shift discussed above). Data for the region below 1100 cm^{-1} have also been obtained;[67] the greatest isotope shifts are apparently localised in ring II and have been observed for the 834 cm^{-1} band, while the greatest shifts on deuteriation are those of the 789 and 496 cm^{-1} frequencies.

The Hydrogen Bonds Involving the Isoalloxazine Ring in the Active Centres. Since the prosthetic flavin group does not in most cases form a covalent bond with apoprotein, hydrogen bonding is important for the stabilisation of the structure. The change in the distribution of electron density in the isoalloxazine ring on formation of hydrogen bonds is evident from the changes in the electronic absorption spectra in model systems (see Yagi and co-workers[73,74] and the references in the latter communication). As already mentioned, the frequencies and intensities of the bands in the range 1160–1300 cm^{-1} are sensitive to hydrogen bonds in the resonance Raman spectra. The hydrogen bonding of flavins has been studied experimentally by Schmidt et al.,[64] who compared the spectra of model flavins in H_2O (a powerful hydrogen bond donor and acceptor), dimethyl sulphoxide (DMSO) (a polar solvent which is a weaker acceptor than H_2O), and acetonitrile (a polar solvent). Potassium iodide was added to suppress fluorescence. On passing from aqueous solutions to DMSO, a change in the relative intensities of bands II and X and a decrease of the frequency of band X were noted. In the subsequent discussion the weak 1281 cm^{-1} band was used as an "indicator" of hydrogen bonding; however, it has been shown[67] that the corresponding vibration is insensitive to any kind of isotope substitution in rings III and II, i.e. at the sites where the hydrogen bonds are localised.

On the basis of these model studies, the authors attempted to examine the hydrogen bonds in the active centres of three FAD-containing flavoproteins of different classes and reached the following conclusions: (1) the acyl-CoA-dependent general aliphatic acid dehydrogenase is characterised by a "somewhat reduced" hydrogen bonding compared with FAD in H_2O; (2) acyl-CoA-oxidase does not form at all or forms only weak hydrogen bonds in all the potential N(1), N(5), C(2)=O, C(4)=O, and N(3) hydrogen bonding centres; (3) glutathione reductase is bound to the same extent as the free FAD group in water.

However, examination of the data of Schmidt et al.[64] (Fig.8) shows that the real situation is much more complex than the above simplified picture. In the case of acyl-CoA-dehydrogenase, the 1253 cm^{-1} band has an unusually high intensity, which may be a result of strong hydrogen bonding. X-Ray diffraction data have not as yet been obtained for acyl-CoA-oxidase, but the paradoxical conclusion that there is no hydrogen bonding in flavins of this class conflicts both with the entire experience gained in the steric studies on flavoproteins[34] and with the UV spectroscopic data.[74] An attempt has been made[74] to estimate the influence of hydrogen bonding on the electronic transition energies and absorption band intensities by modelling the hydrogen bond involving the heteroatom in the isoalloxazine ring (O or N) in the MO SCF calculation by variations in the ionisation potential of the valence state. The authors carried out calculations in this way for all the possible variants of hydrogen bonding; in the light of these results, the experimental electronic spectroscopic data for oxidases should correspond to a strong hydrogen bond at N(5).[74] The resonance Raman spectrum of glutathione reductase in the region 1300–1200 cm^{-1} has a complex contour made up of four incompletely resolved bands,[64] which differs completely from the picture for free FAD in aqueous solution. The resonance Raman

§ As already mentioned, this shift has an analogy with that observed in the Raman spectrum of uracil;[72] however, the increase of the frequency in the latter on deuteriation is only by 16 cm^{-1}.

spectroscopic data refer to glutathione reductase from yeast; the three-dimensional structure of the glutathione reductase from erythrocytes has been determined recently with a high resolution;[36,37] in this complex protein the isoalloxazine ring brings the subunits into contact by means of hydrogen bonding between the N(3)H proton of the FAD in one subunit and the histidine residue in the main chain of another subunit; the N(1) and N(5) atoms form strong hydrogen bonds with the aminoacid residues of the active centre. Since glutathione reductase contains two FAD domains, it is quite possible that the complex contour of the resonance Raman spectrum can be accounted for by certain differences in the orientation and strength of the hydrogen bonds of the two isoalloxazine rings.

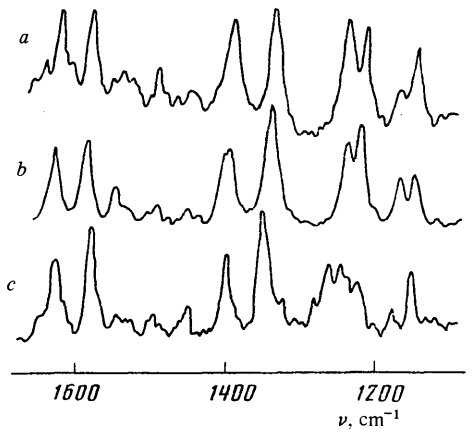


Figure 8. The resonance Raman spectra of aqueous solutions of certain flavoproteins:[64] *a*) acyl-CoA-dependent general aliphatic acid dehydrogenase (pH 8.4; λ = 488 nm); *b*) aliphatic acid acyl-CoA-oxidase (pH 8.0; λ = 488.0 nm); *c*) yeast glutathione reductase (pH 7.6; λ = 514.5 nm).

Flavodoxins. Flavodoxins are the smallest of the FMN-containing proteins; both long (~170 aminoacid residues)[75] and "short" (138 [35] and 148 [76] residues) flavodoxins fulfil identical biological functions; they transport one electron to other flavin systems, proteins with Fe/S centres, or haemoproteins. The two one-electron oxidation-reduction potentials of these molecules differ significantly, which makes it possible to characterise three forms; the oxidised form, the semiquinone (radical), and a reduced form, which makes the flavodoxins convenient model compounds. The resonance CALS spectra with excitation frequency of the long-wavelength absorption band have been obtained[66,78] for representatives of two classes of flavodoxins isolated from the bacteria *Clostridium* and *Desulfovibrio*, which differ in the aminoacid sequences, electronic spectra, and ability to bind riboflavin.[77]

High-resolution X-ray diffraction data are available for the same proteins,[35,76,79] which permits a comparison with the possible interactions with the resonance Raman spectroscopic parameters. It has been shown for flavodoxins of both types that the ribityl component "immersed" in the protein environment forms strong hydrogen bonds with the latter. The planar isoalloxazine ring is disposed nearer to the surface of the globule, but its contacts with the solvent or with an electron donor are strictly limited by the protein environment, which "closes" the pyrimidine and central pyrazine windows, so that only the dimethylbenzene component is accessible to external contacts. Fig. 9 illustrates the system of hydrogen bonds postulated for FMN in the active centre of the *Desulfovibrio vulgaris* flavodoxin.[76] Evidently, with the exception of N(1), all the donor and acceptor atoms are hydrogen-bonded, i.e. the situation in this instance is analogous to FMN in an aqueous environment. Nevertheless, on comparing the CALS spectrum (Table 3) of this flavodoxin with the spectrum of FMN in H_2O, the authors[66] noted a change in frequencies (cm^{-1}): 1629 (free)–1624 (bound); 1582–1573; 1412–1407; 1261–1251; 1233–1227; 1192–1185. The intensity of certain bands changes even more appreciably than the frequency, which is particularly notable in the region 1100–1300 cm^{-1}. As already mentioned, the low frequency (1251 cm^{-1}) and the low intensity of this line

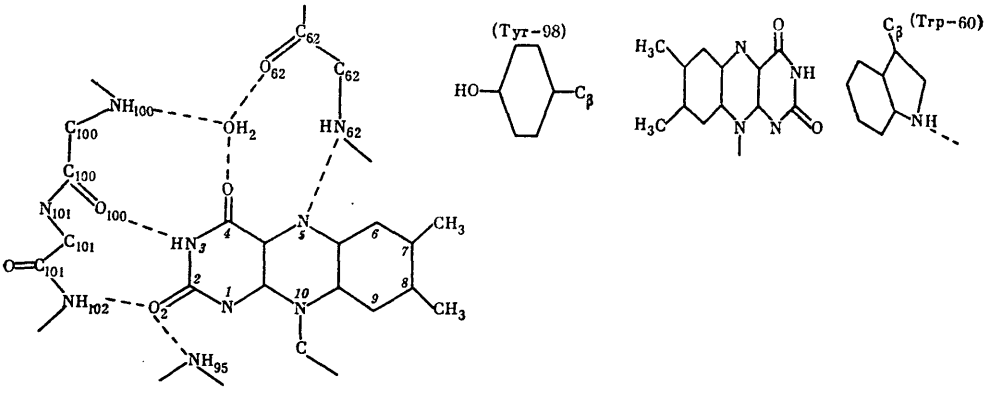


Figure 9. A tentative system of hydrogen bonds of the isoalloxazine ring in the active centre of the flavodoxin of *Desulfovibrio vulgaris*;[76] the numerals represent the numbering of the aminoacid residues in the protein chains; slight rotation of the residue 62 permits the formation of a hydrogen bond with the N(5) atom; the orientation of the flavin relative to the tryptophan and tyrosine residues is shown above.

most probably indicate a decrease of hydrogen bonding along the section C(2)O–N(3)–C(4)O. Among the remaining differences, the decrease of the frequency and intensity of the 1571 cm^{-1} band are the most significant; bearing in mind the sensitivity of the corresponding vibration to isotope substitution,[67] one can postulate a change in electron density in ring II as a result of the binding of N(5). However, there is also another possibility; the isoalloxazine ring in the active centre of flavodoxin is sterically close to the indole ring of the tryptophan-60 residue and the aromatic ring of tyrosine-98 (Fig. 9). It is known that the intense fluorescence of the FMN in flavodoxins is entirely quenched; a stacking interaction of the isoalloxazine ring with tryptophan has been suggested as the quenching mechanism.[79] One cannot rule out the possibility that the change in the intensity of the 1573 cm^{-1} band is associated with an interaction of this type. However, we may note that the flavin and indole rings are in this case non-coplanar and make an angle of 45°, which renders the charge-transfer interaction problematical.

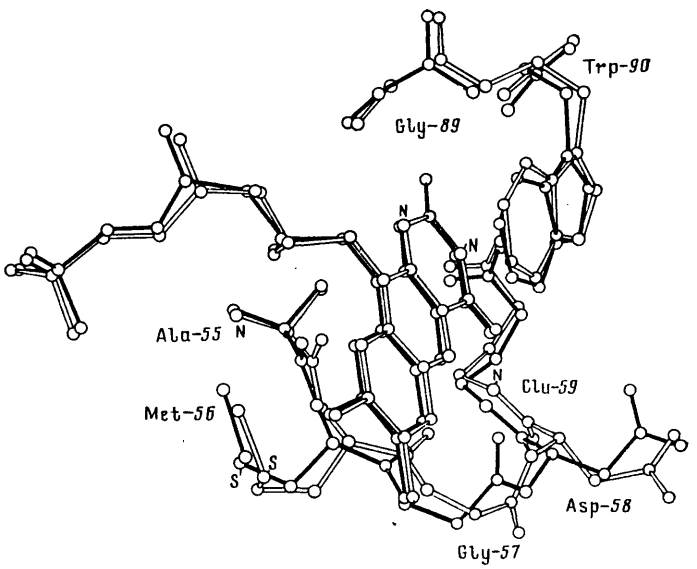


Figure 10. The orientation of the aminoacid residues 55–59 and 89–90 relative to the isoalloxazine ring in the active centre of the oxidised (light lines) and semiquinone (dark lines) form of the flavodoxin of *Clostridium MP*.[35]

Another flavodoxin from a *Desulfovibrio* of the same class (*Desulfovibrio gigas*), whose three-dimensional structure is unknown, has also been investigated by resonance Raman spectroscopy.[66] While the aminoacid compositions of the *gigas* and *vulgaris* flavodoxins are similar, their abilities to bind FMN were found to be different.[75] The CALS spectra demonstrate unambiguously that the immediate environment of the FMN in the active centres of the two proteins considered should be completely identical.[66]

A representative of another class of flavodoxins (from *Clostridium*), fulfilling analogous biological functions, differs significantly in many details of the environment of the isoalloxazine ring, whilst there is an overall homology of the three-dimensional structure with those of the proteins of the *Desulfovibrio* type examined above and the binding of the ribityl phosphate component is very similar.[35,79] However, despite the differences in the chemical sequences, in the active centres of proteins of these classes there is a considerable analogy of the steric structures, which apparently in fact plays a decisive role in their functioning. In the *Clostridium* flavodoxin the pyrimidine and pyrazine rings are likewise "close", the typtophan-90 residue is close to the pyrimidine component (at an angle of 27°), and the orientation of the Gly-57 residue, the interaction with which is tentatively believed to stabilise the semiquinone form (see below), is equivalent to the orientation of Gly-61 in the *Desulfovibrio* flavodoxin.[76] The CALS spectrum of the *Clostridium* flavodoxin[78] is in general similar to the spectra of the *Desulfovibrio* proteins obtained under the same excitation conditions (ω_1 = 480 nm[66]); a decrease of the frequency and intensity of the 1577 cm^{-1} line (tentatively assigned to the flavin–tryptophan interaction) is observed in this case also, but the changes in the relative intensities of the bands in the range 1100–1300 cm^{-1} indicate a different character of the hydrogen bonding in the active centre of the *Clostridium* flavodoxin. The high quality of the CALS spectrum,[78] for which the authors were able to obtain reliable data in the region below 1000 cm^{-1}, is noteworthy. The character of the low-frequency section of the spectrum proved to be significantly different than for free FMN.[63] There is no doubt that analysis of the low frequencies made it possible to obtain subsequently additional information about the electronic rearrangements and interactions of the prosthetic flavin group in the active centres of the proteins.

Table 3. The frequencies (cm^{-1}) in the resonance Raman spectra of certain flavoproteins.

Band designation	RBP (Ref.67)[a]	Flavodoxins			GO (Ref.58)[e]	CoA (Ref.164)[f]	CoA (Ref.64)[g]	GR (Ref.64)[h]
		gigas (Ref.66)[b]	*vulgaris* (Ref.66)[c]	Cl.MP (Ref.78)[d]				
I	1631	1624	1626	1628	1626	1624	1698	1627
II	1584	1573	1571	1577	1578	1583	1586	1580
III	1548	1545	—	—	—	1544	1551	—
IV	1503	1501	1501	1505	1501	1496	1498	—
V	1465	—	—	—	—	1454	1460	—
VI	1407	1407	1407	1409	1404	1407	1412	1402
							1403	
VII	1355	1352	1354	1356	1364,1345	1352	1351	1353
VIII	1302	—	—	—		—	—	1281
IX	1282	—	—	1279	—	—	—	1261
X	1252	1251	1254	1257	—	1253	1251	1246
XI	1229	1227	1223	1231	1230	1231	1233	1224
XII	1179	1173	1185	1179	1182	1182	1182	1180
XIII	1161	1163	1164	1165	1158	1162	1162	1155

[a] RBP = riboflavin-binding protein from chicken egg protein, pH 7, λ = 488.0 nm. [b,c] CALS, ω_1 = 480 nm. [d] Clostridium MP, pH 7.3, CALS, ω_1 = 480 nm. [e] Glucose oxidase, CALS, ω_1 = 480 nm. [f] Acyl-CoA-dependent aliphatic acid dehydrogenase; here and henceforth there was spontaneous RS with λ = 488.0 nm at pH 8.4. [g] Acyl-CoA-oxidase (from yeast), pH 8, λ = 488.0 nm. [h] Glutathione reductase (from yeast), pH 7.6, λ = 514.5 nm.

The Semiquinone Form. A key property of flavoproteins, as one-electron transferring agents, is the thermodynamic stabilisation¶ of the intermediate radical state—the so-called

¶ See, for example, the references in the paper of Smith et al.[35] concerning the association of the semiquinone form with the particular protein compared with the oxidised form on energy grounds.

semiquinone form (Fig.3). The electronic absorption spectra of model systems and flavoproteins with a stabilised semiquinone form contain intense bands due to the $\pi \rightarrow \pi^*$ transitions in the range 500–600 nm (Fig.5),[55] which indicates the retention by the isoalloxazine ring of the planar structure characteristic of the oxidised form; this is also demonstrated by the electronic nuclear double resonance (ENDOR) spectra.[80] These conclusions have been fully confirmed by X-ray diffraction data.[35]† It follows from Fig.10, where the disposition of the aminoacid residues closest to the flavin in the active centre is illustrated for the oxidised and semiquinone states of the flavodoxin, that the formation of the semiquinone form is unaccompanied by any radical changes in the orientation of the isoalloxazine ring in the active centre. At the same time the conformational rearrangements of certain aminoacid residues, particularly along the Gly-57–Asp-58 section, are apparently decisive for the stabilisation of the semiquinone. A special role has been attributed[35] to the change in the orientation of the Gly-57 residue by approximately 180°; as a result, conditions are created in the semiquinone for the formation of the N(5)...O (Gly-57) hydrogen bond and the dissociation of the O(4)...NH (Asp-58) hydrogen bond, which may exist in the oxidised form. According to Smith et al.,[35] the N(5)...O (Gly-57) binding is a decisive stabilising factor;# the shift of the atoms of the other residues in the active centre (with the exception of Asp-58) is on average 0.5 Å; the orientation of the tryptophan residue relative to the plane of the isoalloxazine ring also changes somewhat. We may note that these conformational rearrangements fully correspond to 3.4 kcal mol^{-1}–the preferential energy of the association of the semiquinone form relative to the oxidised form. A study[55] of the polarisation absorption spectra of flavodoxin crystals showed that the electronic α- and β-transitions in the oxidised and semiquinone forms (in terms of the author's notation[55]) are linearly polarised in the plane of the flavin ring with the direction of the transition moment closer to the N(5)–N(10) axis (Fig.6) for both the α- and β-bands. The most interesting conclusion reached by the above investigators[55] is that, in the electronic spectra of the semiquinone structure, both low-energy $\pi \rightarrow \pi^*$ transitions take place between orbitals of the same type as in oxidised FMN. This hypothesis is apparently confirmed by the following experimental observations:[55] (1) similar band polarisation ratios; (2) similar β-band contours and similar frequencies observed in the diffuse vibrational structure of the α-band (the 0–0 and 0–1 transitions respectively at 21000 and 22000 cm^{-1}) for the oxidised form and at 1620 and approximately 17300 cm^{-1} for the semiquinone form; (3) similar correspondingly normalised intensities. The identical signs for the α- and β-bands observed in the circular dichroism spectra of both forms and the similar anisotropies of the transitions may also serve to support the above hypothesis.[81] Thus if the hypothesis is valid, the formation of the semiquinone structure should be represented by the addition of one electron to the unoccupied π orbital of the oxidised form without a significant redistribution of electron density.

Apart from the information about the change in electron density in the semiquinone, the identification of this structure on the basis of Raman spectroscopic data may prove to be especially useful also because the manifestations of the semiquinone form in the electronic spectra of flavoproteins are frequently similar to those observed on formation of charge-transfer complexes (see, for example, Massey and Hemmerich[43] and the references quoted therein). The resonance Raman spectra have now been obtained for the semiquinone forms of two proteins, namely flavodoxin[78] and the riboflavin-binding protein,[82] and also for the semiquinone forms of FMN and FAD stabilised by the methylation of the N(5) atom.[83] As might have been supposed, the change in bond orders in ring II led to changes in almost all the vibration frequencies of the flavin system (Table 4). On excitation in the region of the β-transition of the semiquinone (λ = 514.5 nm; Fig.11), the 1611 cm^{-1} band predominates in the spectrum;[83] as the α-transition is approached, its intensity diminishes[82] and, after excitation at the frequency corresponding to the long-wavelength slope of the absorption α-band, the band at approximately 1340–1370 cm^{-1} becomes most intense.[83] It has been suggested[78,83] that the intense 1611 cm^{-1} line of the semiquinone is an analogue of the 1580 cm^{-1} line of the oxidised form and that the corresponding vibration is localised mainly in ring II. However, bearing in mind the reduced bond orders in ring II and also the displacement of the unshared electron pair of the N(5) atom from the conjugation system on formation of the semiquinone structure, a decrease (and a fairly large one) of the corresponding frequency might

Table 4. The frequencies of the resonance Raman lines of the semiquinone structures of flavins[a].

Flavin	ν, cm^{-1}												Refs.
RBP[b] (H_2O)	1617 s	1582 w	1553 w	—	1407 w	1393 с,п	1350 m	—	1273 w	1230 m	1160 v.w	1082 w	
" (D_2O)	1618	1585	1535		1407	1387	1353					1089, 1060	[82]
Flavodoxin[c] (H_2O)	1611 s	—	—	—	—	1391 s	1378 m	1333 w	1269 m	1232 s	—	—	[78]
" (D_2O)	1611					1386							
5-Me-FAD[d]	1611 v.s	1590 m	—	1476 w	—	1372 w	1351 m	1330 m	1278 w	1224 w	—	—	
" [e]	1611 m	1590 m	1516 m	1432 m		1372 v.s	—	1330 v.s	1278 w	1224 m	1174 m		[83]
5-Me-FMN[f] (H_2O)	1613 m	1590 m	1516 w	1443 v.w	—	1376 v.s	—	1334 v.s	1282 v.w	1224 m	1179 m	—	
" (D_2O)	1611 m	1588 m	1514 w	1446 w		1372 v.s		1334 v.s	1256 v.w	1204 w	1178 m		[83]
8-Cl-5-Me-FMN[f]	1613 w	1590 m	1516 w	1443 w	—	1378 v.s	—	1334 s	—	1226 w	1170 m	—	[83]

[a] For the region above 1000 cm^{-1}. [b] λ = 514.5 nm. [c] CALS, ω_1 = 620 nm. [d] λ = 514.5 nm. [e] λ = 647.1 nm. [f] λ = 641.7 nm.

†The "folding" angle relative to the N(5)–N(10) axis (Fig.5) is 2.5° in the semiquinone.[35]

#In the flavodoxins of the *Desulfovibrio* class,[76] this role is fulfilled by the Gly-61 residue; the H(62)–O(4) bond is possible in the oxidised form of the protein.

have been expected. Furthermore, the 1611 cm^{-1} line is almost insensitive to either methylation of the N(5) atom[83] or to the deuteriation of the semiquinone structures in which an exchangeable proton is present at the N(5) atom.[76,82] Thus the resonance Raman spectra can be used as a reliable criterion in the identification of the semiquinone structure, which has in fact been done[84] in studies on the NADP complex with adrenodoxin reductase. However, the Raman resonance spectroscopic data obtained hitherto cannot be used to either confirm[78,83] or reject Eaton's hypothesis.[55] In order to solve this problem, detailed studies of the dependence of the resonance Raman lines on the exciting frequency (the excitation spectra) in the region of the α- and β-electronic transitions of the semiquinone are necessary. On the other hand, the proposed correlations of the resonance Raman frequencies of the semiquinone and oxidised structures[83] can be reliably established only on the basis of data for isotope-substituted specimens.

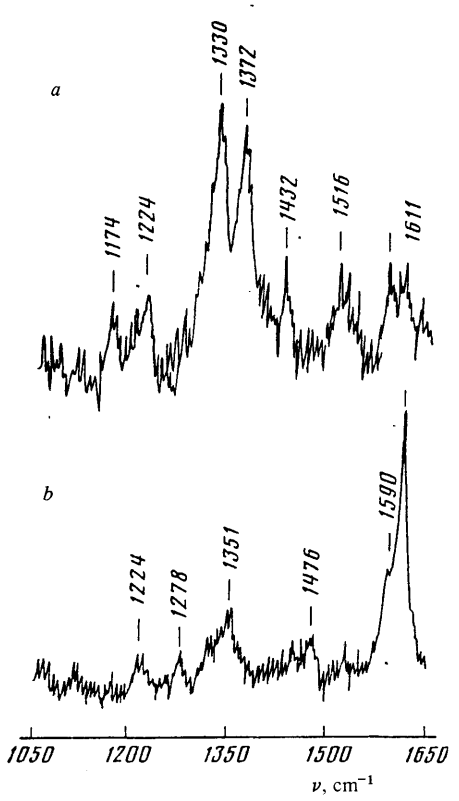


Figure 11. The spectra of *N*(5)-methyl-FAD in the semiquinone form; spontaneous RRS; excitation wavelengths (nm): *a*) 647.1; *b*) 514.5.[83]

Reduced Flavins. The fully reduced isoalloxazine ring undergoes electronic transitions at shorter wavelengths than the oxidised and semiquinone forms (Fig.12), a factor making difficulties in the measurement of the resonance Raman spectra. The latter have been obtained comparatively recently only for structures where the reduced form gives rise to a charge-transfer complex. The so-called "purple complexes", formed at the intermediate stages of the reaction of D-aminoacid oxidase (DAO) with D-alanine and D-proline, have been investigated.[85-88] These complexes have a characteristic long-wavelength absorption extending to approximately 750 nm. On excitation with light at wavelengths in the region of the long-wavelength band, a resonance intensification of the lines of both the substrate (alanine and proline) and of the active centre of the enzyme (FAD) was observed. Using numerous verions of isotope substitution in the isoalloxazine ring and in the substrate molecules, the authors[85-88] assigned the frequencies with exceptional reliability and demonstrated that, (1) the isoalloxazine ring in the purple complex exists in the fully reduced form, (2) the ~1605 cm^{-1} vibration of the flavin, which intensifies more than others on excitation at wavelengths corresponding to the charge-transfer band, is localised in the C(10*a*)–C(4*a*)–C(4) = 0 fragment, and (3) the 1692 cm^{-1} band in the complex with D-alanine and the 1658 cm^{-1} band in the complex with D-proline refer to the C=N§ stretching vibrations of aminoacids, which are thus involved in the imino-form in the complex formation reaction. An interesting and hitherto unexplained feature of the complexes with D-alanine is the sensitivity of the ~1540 cm^{-1} line to isotopic substitution in both the substrate molecule and in FAD.[88]

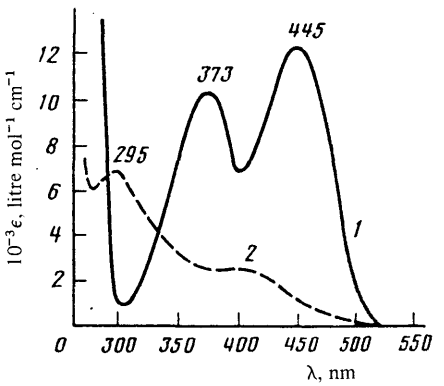


Figure 12. The electronic absorption spectra of the fully oxidised (curve *1*) and fully reduced (curve *2*) forms of FMN.[43]

The resonance Raman data obtained provide direct evidence that the C(10*a*)–C(4*a*)–C(4)=O fragment of reduced FAD in the purple complex is in close contact with the HN = C–COO^- plane of the substrate, ensuring an interaction of the charge-transfer type.

Charge-Transfer Complexes. One of the most important features which is apparently essential for the functioning of many flavin systems is their ability to form charge-transfer complexes (CTC); such complexes are formed by almost all flavoenzymes containing pyridine nucleotide.[89] Data obtained recently on the three-dimensional structure of glutathione reductase[36,37] confirmed that the NADP ring in the active centre is indeed located above the isoalloxazine ring in a conformation favourable for the formation of a

§ The changes in these frequencies in the deuteriated specimens indicate an appreciable involvement of δ(NH) in the vibration.

charge-transfer complex. Certain flavo-proteins are known to be capable of undergoing charge-transfer interactions also with other substrates, in particular with benzene derivatives.[89] The mechanisms of the formation of such complexes, their structure, and their catalytic value are still in many respects obscure. The formation of CTC of flavins is usually inferred from the appearance of a long-wavelength absorption in the range 500–700 nm, which is accompanied as a rule by a decrease of the intensity of the α- and β-bands, but these changes, as already stated, are extremely similar to those observed for semiquinone flavin structures. Resonance Raman spectroscopic data have been obtained recently for a series of model CTC of flavoproteins: it is now already evident that the resonance Raman spectra make it possible to identify reliably the CTC of flavins and can yield, in principle, unique information about the structure and about the site of the charge transfer.

The first model for the study of the CTC of flavins was the so-called old yellow enzyme (OYE) containing the FMN prosthetic group. In nature, this protein exists in the "green" form—a complex with *p*-hydroxybenzaldehyde;[90] it is also capable of forming artificial complexes with a wide variety of aromatic and heteroaromatic derivatives containing hydroxy-groups.[43,90] Japanese workers[91,92] investigated the resonance Raman spectra of the products of the interaction of OYE with a set of substituted phenols on excitation at the wavelength corresponding to the long-wavelength absorption band. Analysis of the data obtained shows in the first place that, in the complexes investigated, the flavin is present in the oxidised state; the 1628, 1585, 1548, 1415, 1356, 1260, and 1160 cm^{-1} bands present in the spectra of all the specimens have virtually the same frequencies as in the spectra of FMN and riboflavin. At the same time a resonance intensification of the bands (especially in the low-frequency section), specific to the substituted substrate, is observed in the resonance Raman spectra at 477, 390, 475, 386, and 547 cm^{-1} respectively for *p*-CH_3, *p*-Cl, *p*-F, *p*-NO_2, and other substituted phenols respectively. In the high-frequency region, it is also possible to identify bands referring to the phenolic component, which is especially apparent for complexes with pentachloro- and pentafluorophenols.[92] Studies on the frequency dependence of certain resonance Raman lines in the charge-transfer band[92] have shown that the 1588 cm^{-1} frequency of isoalloxazine and the 1475 and 454 cm^{-1} frequencies of phenol make the greatest contribution to the vibrational structure of the long-wavelength electronic absorption band (Fig.13).

In this case the spectra do not exhibit frequencies referring directly to the C–OH fragment of phenol, which is characteristic of, for example, the complexes of the phenoxide anion with metals, but the interaction takes place locally via the phenolic oxygen atom. The character of the dependence of the intensity on the excitation frequency $I(\nu)$ for the 1588 cm^{-1} line[92] yields direct evidence for charge transfer to the flavin ring. Since the corresponding vibration is associated predominantly with the N(5)–C(4*a*)–C(10*a*) fragment, these data make it possible to infer the location of the charge transfer. The occurrence of such interaction requires a sandwich conformation with parallel isoalloxazine and phenol rings. The constancy of the frequency of the 1588 cm^{-1} band (as for the remaining frequencies of the isoalloxazine ring) shows that all the changes in electron density refer exclusively to the excited state of the molecules. The greatest charge transfer occurs in the complex with *p*-nitrophenol, which can be inferred from the change in the distribution of intensities in the resonance Raman spectrum.

The same characteristic features have been noted in the resonance Raman spectra of the charge-transfer complexes of other flavoenzymes obtained at present. In the study of the complexes of the CoA-dependent aliphatic acid dehydrogenase (containing FAD) with acetoacetyl-coenzyme A, the authors observed a resonance intensification of the 1586 and 1550 cm^{-1} lines,[94] confirming thereby the fact that the electron transfer in the interaction with the most varied donors is directed to the N(5) and C(4*a*) centres of the isoalloxazine ring, in agreement with quantum-chemical calculations.[95] Artificial complexes of D-aminoacid oxidase with amino- and hydroxy-benzoates have also been studied:[96] the latter are known to be effective inhibitors of the enzyme. On excitation at the frequency corresponding to the long-wavelength absorption band (λ = 632.8 nm) of the complex of DAO with *o*-aminobenzoate, the 1583 cm^{-1} (isoalloxazine ring) and 568 cm^{-1} (*o*-aminobenzoate) lines were observed. It is of interest that resonance intensification has not been observed for the *meta*- and *para*-aminobenzoates, which indicates severe conformational limitations in the formation of the CTC.

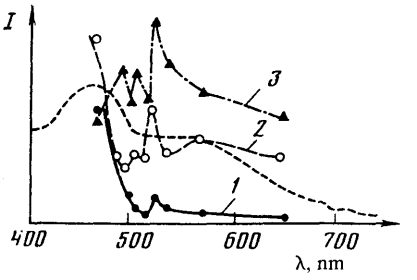


Figure 13. Dependence of the intensity of certain spontaneous resonance Raman lines of the complex of old yellow enzyme with pentachlorophenol on the frequency of the exciting light; the intensification of the lines of the flavin component [*1*) 1628 cm^{-1}; *2*) 1588 cm^{-1}] and the ligand [*3*) 1475 cm^{-1}] can be seen; the contour of the absorption band is shown by a dashed line.[92]

In the reaction of pig liver DAO with β-cyano-D-alanine, the intermediate stages also consist of CTC of the oxidised flavin according to resonance Raman spectroscopic data.[97] We may note that, in the spectra of all the DAO complexes investigated, the "hydrogen bond indicator" band is at 1240 cm^{-1},¶ [96,97] i.e. is appreciably displaced towards lower frequencies compared with other flavins, which indicates extremely specific interactions of FAD with the DAO apoprotein. Charge-transfer complexes have been identified also in the intermediate stages of the anaerobic reduction of adrenodoxin reductase by the NADP coenzyme.[98]

Metal Complexes. The problem of the interaction of flavins with metals is important for the understanding of the mechanisms of the functioning of flavoproteins containing Fe/S centres and also molybdenum.[99] The model complexes of FMN with silver and ruthenium[100] and the complexes of the riboflavin-binding protein (RBP) and 10-methylisoalloxazine with ruthenium(II) have been investigated.[100,101] The electronic absorption spectra of the silver(I) complex of FMN contain a weak shoulder at approximately 530 nm on the

¶ The assignment of this band in the spectra of the DAO complexes has been confirmed by the 1242 → 1284 cm^{-1} shift on deuteriation.

long-wavelength slope of the α-band, while an intense band at ~630 nm appears in the spectra of the complexes $(NH_3)_4Ru^{II}$ (Fig.14).

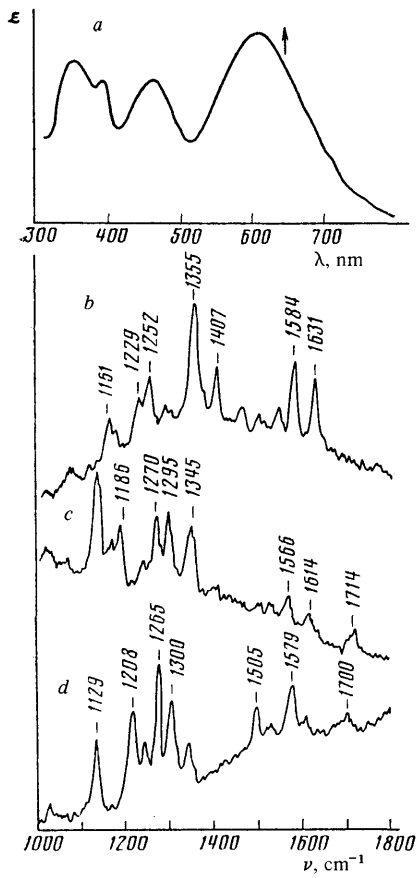
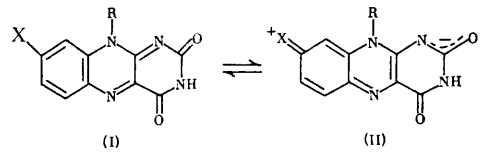


Figure 14. The electronic absorption spectrum of the complex of the riboflavin-binding protein with $Ru^{II}(NH_3)_4$ (*a*) and the spontaneous resonance Raman spectra: *c*) the RBP–$Ru^{II}(NH_3)_4$ complex; *d*) the complex of $Ru^{II}(NH_3)_4$ with 10-methylisoalloxazine; excitation at λ = 647 nm (indicated by an arrow on the absorption spectrum) was used in both cases; *b*) the spectrum of RBP (λ = 488.0 nm) is presented for comparison.[101]

The resonance Raman spectroscopic data may permit the conclusion that the interaction in the Ag^+ FMN complex is of the charge transfer type. Apart from the N(5) atom (whose involvement can be inferred from the increase of the intensity of the 1580 and 1540 cm^{-1} lines), the oxygen atom closest to N(5) apparently also participates in the reduction; this is indicated by the changes in the symmetric and antisymmetric vibrations of the carbonyl groups in the infrared spectrum of the complex Ag^+ FMN.[100] On formation of the complex of $Ru^{II}(NH_3)_4$ with RBP,[101] the frequencies and relative intensities of almost all the lines in the resonance Raman spectrum are altered. The frequencies and particularly the distribution of the intensities on excitation at 647.1 nm (Fig.14) resemble most closely those observed for the semiquinone form (Section 5). However, this hypothesis requires further tests. It is of interest that bands at ~1700–1710 cm^{-1}, referring to the carbonyl groups, are manifested in the resonance Raman spectrum of Ru^{II}RBP as well as in the spectrum of the model ruthenium(II) complex of 10-methylisoalloxazine.[101]

The data obtained for the model metal complexes established[102] that there is no direct electron transfer between the Fe/S cluster and the flavin in the ETF hydrogenase,† an FAD-containing flavoprotein, which incorporates Fe/S centres and functions in the electron transport chain, although such transfer might have been postulated on the basis of the EPR spectra (see the references in the paper of Schmidt et al.[102]).

Modified Flavins. As already mentioned, in many cases the prosthetic group of the flavin can be readily separated from the protein without its denaturation, modified by the introduction of substituents, and then rejoined to the apoprotein. Such modifications have been widely used to obtain information about the interaction of the flavin with the protein, mainly from kinetic data–the binding constants, the changes in catalytic activity, and the changes in the electronic and circular dichroism spectra. The 1- and 5-deaza-derivatives or substitution of the methyl groups in the 7- and 8-positions are most often used for such purposes.# Evidently the results obtained cannot as a rule be interpreted unambiguously, since several factors can change simultaneously in the modified compound: the conformations of several aminoacid residues in the active centres (and hence the character of the interactions with the prosthetic group), the steric conditions for the approach of the substrate, and finally, the electronic structure of the isoalloxazine ring itself. Extensive data have been obtained recently on the resonance Raman spectra of modified flavins, both free and bound to different proteins,[65,70,71,103,104] which makes it possible, in principle, to differentiate these factors. It follows from resonance Raman spectroscopic data that the presence of halogen atoms in the 7- and 8-positions changes little the distribution of electron density in the isoalloxazine ring; the changes in the resonance Raman spectra[70] can be accounted for in this instance by the mass effect. However, the introduction of groups containing unshared electron pairs (the hydroxy-, amino-, or mercapto-groups) in these positions leads to radical changes in the resonance Raman spectroscopic parameters (Fig.15). It had been suggested earlier on the basis of UV spectroscopic data (see Massey and Hemmerich[43] and the references quoted therein) that tautomeric (sic) transformations of the type



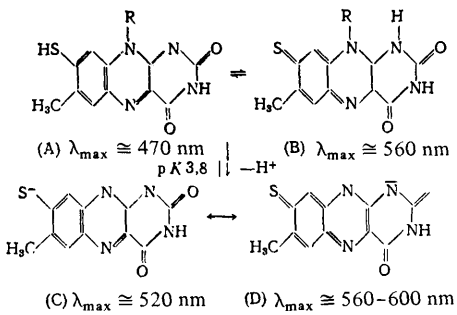
(I) (II)

i.e. equilibrium between the benzenoid (I) and quinonoid (II) forms, are possible for compounds of this kind. In all cases the bathochromic shift and an increase of the intensity of the long-wavelength absorption band were noted for the suggested quinonoid structure.

†ETF = electron-transporting flavoprotein.

#In the flavoproteins with known structures,[35,36] the isoalloxazine ring is oriented in such a way that the benzene component and the 7- and 8-methylgroups are on the "surface" and are least in contact with the aminoacid residues of the active centre. This apparently actually accounts for the ease of the binding of the 7,8-modified analogues to the protein.

A large decrease of the frequencies of bands II and III (by 30–40 cm^{-1}), changes in the range 1400–1200 cm^{-1}, and the appearance of a whole series of new bands in the range 1100–1000 cm^{-1} are characteristic of the resonance Raman spectra of these derivatives (Table 5). The changes are especially large in the resonance Raman spectra of 8-mercaptoflavins exhibiting a long-wavelength absorption (λ_{max} = 500–600 nm), greatly exceeding in intensity the absorption by other derivatives (Fig.16). In conformity with UV spectroscopic data, the following tautomeric structures have been proposed for 8-mercaptoflavins as a function of the environment and the ionisation potential:[43]



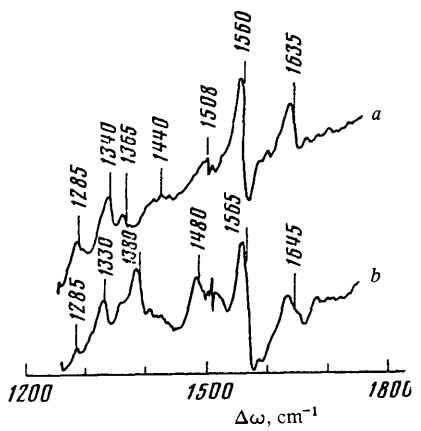


Figure 15. The inverse spectra (resonance excitation) of 8-aminoriboflavin (*a*) and 8-hydroxyriboflavin (*b*) at pH 7.3.[65]

The resonance Raman spectra of mercaptoriboflavin in various states have been obtained.[103] It follows from these data that the frequencies of lines I and II for the protonated form (pH 2) are reduced (by 9 and 14 cm^{-1} respectively relative to the riboflavin), but the constancy of the positions of the remaining lines and, what is more significant, of the distribution of the band intensities indicate the retention of the "benzenoid" character of the electron density, as in the halogeno-derivatives.[70,104] According to resonance Raman data, 8-mercaptoriboflavin[103] and 8-methylmercaptoriboflavin[104] add precisely in this form to the riboflavin-binding protein. The transition to conditions for the formation of the anionic (thiolate) form (C) (accompanied by a bathochromic shift of the absorption band) leads to radical changes in the resonance Raman spectrum, in which the 1616 and 1545 cm^{-1} bands are dominant; the latter band is usually regarded as an analogue of the 1584 cm^{-1} band of the oxidised form, the corresponding vibration being localised in the N(5)–C(4*a*)–C(10*a*) section[67] and it is therefore regarded as an indicator of the double bond character of this fragment.[65,103] The large decrease of the frequency of line II has been interpreted in all investigations as evidence for the decrease of the order of the π-bonds in this section of the isoalloxazine ring and as decisive evidence in support of the quinonoid structures (B) and (D). The study of the variation of the frequency of the 1545 cm^{-1} band[103] has shown that the corresponding normal vibration does indeed contribute to the long-wavelength electronic transition. However, we may note that no convincing evidence has been obtained so far in support of the fact that this vibration is localised in the same fragment as the 1584 cm^{-1} vibration of the oxidised form.

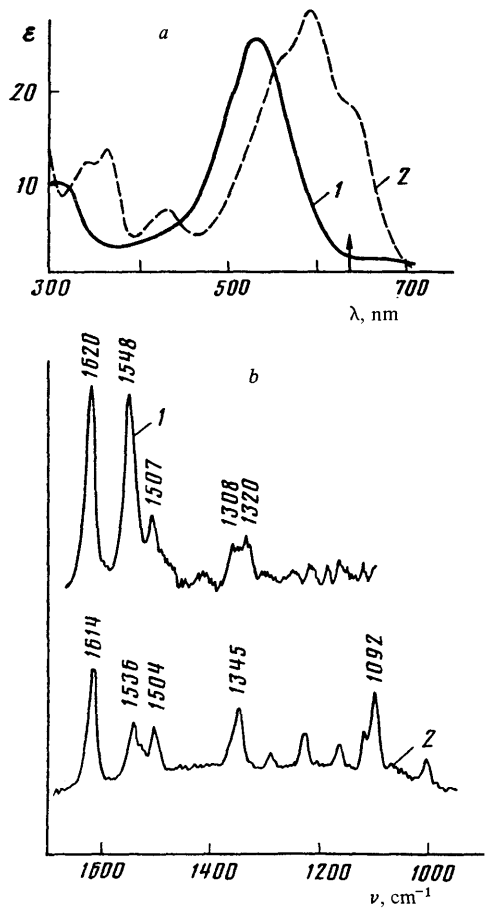


Figure 16. *a*) The electronic absorption spectra of modified flavoproteins: *1*) 8-mercaptoflavodoxin; *2*) D-aminoacid 8-mercapto-oxidase;[43] *b*) the spontaneous resonance Raman spectra: *1*) 8-mercaptoriboflavin (pH 8.4; λ = 514.5 nm); *2*) lactate 8-mercapto-FMN-oxidase (pH 7; λ = 632.8 nm).[65]

It had been noted earlier that the binding of 8-mercapto-FMN by certain proteins does not entail significant changes in the colour of the "red thiolate" form (flavodoxin),[105] while

the addition of the modified prosthetic group to lactate oxidase, glucose oxidase, *D*-aminoacid oxidase, and old yellow enzyme results in a change of the red colour to a deep blue colour (Fig.16).[105] Since all the flavoproteins enumerated belong to the class of oxidases, it has been suggested that oxidases stabilise the "blue benzoquinone" structure (D) and that the presence of the aminoacid residue, stabilising the negative charge on the N(1)–O(2) section of isoalloxazine, in the active enzyme centre of the oxidase is essential:

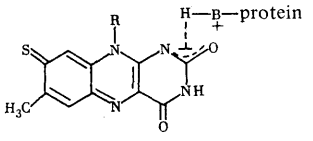


Table 5. The frequencies of the resonance Raman lines (cm^{-1}) of modified flavin systems.

Band designation	RF (Ref.65)[a]	8-OH-RF (Ref.65)[b]	8-OCH_3-RBP (Ref 82)	8-SCH_3-RBP (Ref.104)	8-NH_2RF (Ref.6)	8-$NH(CH_3)$-RBP (Ref.71)	8-$N(CH_3)_2$-RF (Ref.65)
I	1631	1635 (1638)	1638	1624	1645	1641	1629
II	1582	1560 (1555)	1578	1564	1565	1567	1558
III	1547	—	1542	1533	—	1537	1519
IV	1501	1508	1506	1505	—	1531	—
V	1462	1440	—	1476	1480	—	—
VI	1407	1365	1413	1405	—	1407	1405
			1387	—	1380	—	1375
VII	1354	1340 (1337)	1358	1348	1330	1360	1335
VIII	1304	—	—	—	—	—	—
IX	1281	1285 (1286)	1276	—	1285	—	1281
X	1250	—	1237	1246	—	—	1251
XI	1228	(1215)	—	—	—	—	1212
XII	1178	—	—	1194	—	—	—
XIII	1161	—	1152	1150	—	1162	1133
XIV	1072	—	1071	1084	—	—	—
				1014	—	—	—

Note. RF = riboflavin; RBP = riboflavin-binding protein.
[a] The discrepancies with the data of Nishina et al.[70] for RBP do not exceed -2 cm^{-1}. [b] The data of Dutta et al.[71] are given in brackets.

The resonance Raman spectra of the "blue benzoquinone" structures have been obtained by Schopfer et al.[65] (Table 6). The spectrum of 8-mercapto-FMN lactate oxidase excited at $\lambda = 632$ nm (denoted by an arrow) is illustrated in Fig.16; the data for glucose oxidase and old yellow enzyme are presented in Table 6. These results demonstrate that the same electronic structure of the 8-mercaptoflavin prosthetic group, differing appreciably from the traditional hydroxy-form of riboflavin, FMN, and FAD, is characteristic of all the proteins enumerated. Certain changes in the frequencies of the principal bands within the limits of each spectrum evidently reflect the specific features of definite protein–flavin interactions in the active centre of the enzyme. In the literature these spectra have been interpreted as referring to the "fully quinonoid" structure (D),[65] while the spectra of derivatives of 8-hydroxy, 8-amino-, 8-mercapto-,[65] 8-methylamino-,[71] 8-methoxy-,[70] and 8-methylthio-riboflavins[104] are thought to be "intermediate" between those corresponding to the typical quinonoid and benzenoid structures with "greater" or "lesser" quinonoid character.[65] Apart from a number of discrepancies (the differences between the resonance Raman spectra of derivatives for which identical "contributions" by the quinonoid structure have been predicted on the basis of electronic spectra,[43,65] a significantly "benzenoid" distribution of intensities in the case of the 8-methoxy- and 8-methylthio-derivatives accompanied by major shifts of band II in the latter case, etc.), the very concept of describing the electronic states as mixtures of resonance structures and of the resonance Raman line shift as a result of a particular "percentage content" of the quinonoid form is unproductive and cannot yield any specific information either about the distribution of electron density in the isoalloxazine ring or about the interactions of the latter with the protein. The correlations of the positions of certain resonance Raman bands with the Hammett constants,[65,104] disregarding the influence of the masses and kinematic interactions on the vibration frequencies (see, for example, the correlations for band I in the study of Schopfer and Morris[104]), are also of little use. Despite the very unique nature of the resonance Raman spectra obtained for modified flavins, they have been scarcely used. The interpretation of these results requires additional experiments with isotope-substituted specimens, which would permit reliable assignments of the vibration frequencies, and also quantum–mechanical calculations of the electron density in the modified flavins.

Table 6. The frequencies (cm^{-1}) in the resonance Raman spectra of 8-mercaptoflavins.[65]

RBP[a]	8-SH-RF[b]	8-SH-FMN-LO[c]	8-SH-FAD-GO[d]	8-SH-FMN-OYE[e]
1631	1620	1614	1606	1611
1582	1548	1536	1530	1528
1547	1507	1504	1501	1500
1501	—	—	—	—
1462	—	—	—	—
1407	—	—	—	—
1354	1360, 1320	1345	1337	1343
1304	—	—	—	—
1281	—	1292	1263	—
1250	—	1223	1209	1220
—	—	—	—	—
1228, 1178, 1161	—	1161	1159	1168
—	—	1110	—	1111
—	—	1092	1099	1087
1072	—	1063	1079	1039

[a] Riboflavin-binding protein, quoted for comparison. [b] 8-Mercaptoriboflavin, pH 8.4 with added 3 M KI. [c] L-lactate 8-mercapto-FMN-oxidase, pH 7. [d] Glucose 8-mercapto-FAD-oxidase, pH 7. [e] 8-Mercapto-FMN derivative of old yellow enzyme, pH 7.

Stacking Interactions of Flavins. It has been frequently suggested in the literature that the free FMN and FAD groups in solutions exist under certain conditions in the form of stacking complexes. In particular, the changes in the fluorescence properties of flavins have been attributed to interactions of this type. It is known that the fluorescence of FAD in aqueous solution is appreciably quenched compared with an aqueous solution of FMN at the same concentration; it has been suggested[106] that one of the probable causes of the quenching may be the stacking interaction of the isoalloxazine and adenine rings. The possibility of the formation of intramolecular stacked structures from the isoalloxazine and adenine rings in the FAD molecule has been discussed.[107] When 30% of DMSO is added to the solvent, such interaction is significantly weakened and the FAD fluorescence is intensified by a factor of 40, approaching that of FMN. Attempts have been made to detect stacking interactions with

the aid of resonance Raman spectra. Comparison of the frequencies in the spectra of aqueous FMN solutions at a concentration of approximately 60 mM,[63] where the FMN molecules should be fully dimerised, with the CALS spectra of dilute aqueous solutions and solutions in the H_2O–DMSO mixture[66] failed to reveal any significant differences. One should note however that these results are difficult to compare, because the data of Nishimura and Tsuboi[63] refer to spontaneous RRS under conditions of virtually non-resonance excitation (λ = 600 nm), while the data of Irvin et al.[66] refer to the excitation of the anti-Stokes ARSS (ω_1 = 495 nm), which gives rise to a negative Lorentzian contour. On comparing the CALS spectra of FAD in water and aqueous DMSO, the authors noted small changes in certain frequencies.[66] However, it was subsequently confirmed that the FMN spectra in solutions at high concentrations, ensuring stacking interactions (> 50 mM), do not differ from the spectra of dilute solutions (< 2 mM).[64]

Flavocytochromes. These compounds, which function in electron transport chains, contain two centres for oxidation-reduction reactions: flavin and cytochrome (haem group). The mechanism of the functioning of flavocytochromes is in many respects still obscure;[108] little is known also about an interesting feature of these compounds—the postulated intramolecular tunnelling from flavin (electron donor) to cytochrome (electron acceptor). Furthermore, the study of this interaction is of general importance for many-centre enzymes.

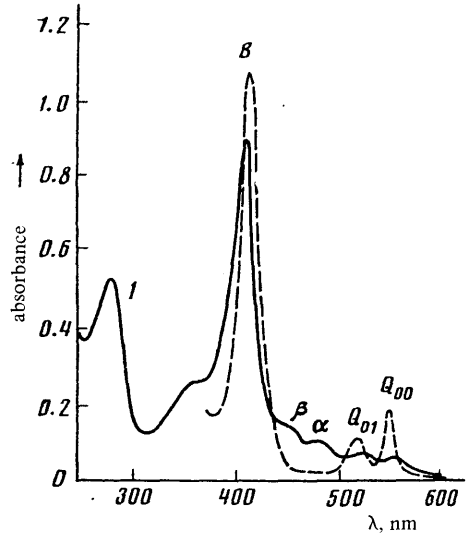


Figure 17. The electronic absorption spectra of flavocytochrome c_{552} in the oxidised (curve *1*) and reduced (curve *2*) forms; the symbols *B*, Q_{01}, and Q_{00} denote the absorption bands of haem *c* and α and β denote the absorption bands of the flavin component.[113]

Flavocytochrome *c* present in purple and green phototropic bacteria, which play an important role in the biological reduction of sulphates and in the general sulphur turnover on Earth, has been most thoroughly investigated in the class of cytochromes.[39] Flavocytochrome c_{552}, isolated from *Chromatium vinosium*, consists of two non-equivalent subunits, one of which (M_r = 21 000) contains two haem *c* groups, while the other (M_r = 46 000) contains one FAD group.[109] The isoalloxazine ring is in this case covalently bound to the protein by a sulphide linkage in the 8-position.[110] According to EPR data,[111] the two haem *c* groups are non-equivalent; the immediate environment of one of these groups (apparently consisting of the ligands) varies with the pH.

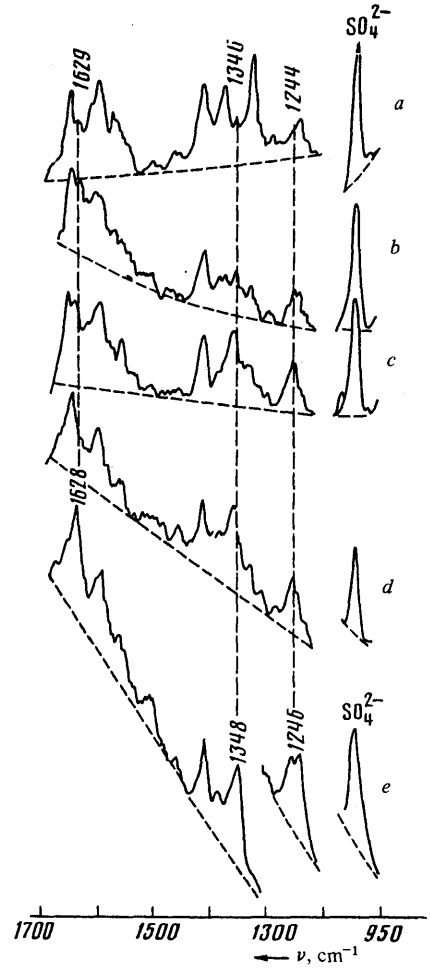


Figure 18. The resonance Raman spectra of flavocytochrome c_{552} (pH 6.5) on excitation with light corresponding to the 514.5 (*a*), 501.7 (*b*), 496.5 (*c*), 488.0 (*d*), and 476.5 nm (*e*) lines; internal standard—SO_4^{2-} ion (ν = 983 cm^{-1}); the vertical dashed lines indicate the positions of the resonance Raman lines of flavin.[113]

Flavocytochrome c_{552} has been investigated by the resonance Raman method simultaneously by two groups of investigators.[112,113] Owing to the unsuccessful choice of the exciting frequencies in the study of Ondrias et al.,[112] it was not possible to identify the resonance Raman lines which could be assigned to the isoalloxazine rings. The electronic absorption spectrum of the enzyme (Fig.17) consists of a superposition of the intense absorption by haem [the *B* bands (Soret bands) Q_{00} and Q_{01}] and less intense bands at approximately 450 and 480 nm referring to the flavin fragment. By altering the frequency of the exciting line, the authors[113] isolated the 1629, 1346, and 1244 cm^{-1} lines of the

isoalloxazine ring together with the intense resonance Raman lines of the cytochrome at 1645, 1566, and 1366 cm^{-1}, which are close to the frequencies observed for horse ferricytochrome *c* (1637, 1563, and 1316 cm^{-1} respectively[114]). The intensity of the isoalloxazine lines is highest on excitation at $\lambda = 476.5$ nm, in the region of the bands due to the electronic transitions of flavin (Fig.18).

The resonance Raman spectrum of the reduced (ferro) form of flavocytochrome c_{552} contains lines referring exclusively to the haem groups;[113] their number, polarisation, and frequencies are virtually the same as for other ferrocytochromes *c*.[115] Judging from the symmetrical contours of the sharp 1588 and 1316 cm^{-1} lines, both haem groups in the ferrocytochrome c_{552} molecule should be identical. The absence of bands due to the isoalloxazine ring shows that the flavin exists in this case in the fully reduced form and not in the semiquinone form.[113]

The photoreduction of the entire enzyme and its constituent cytochrome- and flavin-containing submits separately as well as the interaction with strong inhibitors (sulphite and cyanide) have been investigated with the aid of RRS.[113] The changes in the intensities of the resonance Raman bands in the spectra of the complexes with cyanide and sulphite suggest that both adducts are formed as a result of the addition to the N(5) atom of the isoalloxazine ring, which is the cause of the inhibition of the enzyme. It has been shown[113] that in the photoreduction, which in this case proceeds exclusively in the presence of ethylenediaminetetra-acetic acid, the first prosthetic group to be reduced is the flavin system, while the haem groups cannot be reduced in the absence of flavin. These experiments constitute the first direct evidence for the occurrence of intramolecular transfer from the flavin group to the cytochrome in this class of enzymes.

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Thus extensive data have now been obtained on the resonance Raman spectra of flavins. Evidently the method can be used successfully for the identification of the isoalloxazine ring and the derivation of information about its structure in all possible oxidation and ionisation states. Under conditions where the scope of NMR is extremely limited, resonance Raman spectroscopy remains in essence the only method potentially capable of yielding extensive information about the ground and excited states of flavin systems. Nevertheless, these data have so far been used to a small extent. Compared with the novel methods of isolation and synthesis of, for example, the modified specimens and with the exceptionally sophisticated experimental spontaneous and nonlinear resonance Raman spectroscopic technique, the results of the interpretation appear to be modest. This situation is caused mainly by the inadequate development of the theoretical basis of the analysis of the normal vibrations and quantum-mechanical calculations of the electron density in model flavin systems. Evidently the description of the changes in the electronic states of the isoalloxazine ring in terms of the percentage contents of the resonance structures cannot promote the discovery of the real situation. Despite the abundance of data available on isotope-substituted specimens and modified systems, there has been so far no adequate calculation for the normal vibrations of flavin. In view of the quantum-chemical and vibrational spectroscopic methods which have now been developed and the present level of computational techniques, such theoretical data could be readily obtained after the correct choice of model. All the information about the frequencies, intensities, and polarisations of the resonance Raman lines could be fully employed on this basis in order to deduce the state of the isoalloxazine ring and its interactions with the protein environment.

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