APPLICATIONS OF RAMAN SPECTROSCOPY

by

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Graduate Committee

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ABSTRACT I

The frequency of vibration of the C = 0 group in dimethylformamide, N-methylformamide, N-methylacetamide, acetone and methylacetate has been measured in a number of solvents. A marked contrast in the effect of the solvent on the solutes acetone, dimethylformamide and methylacetate on the one hand, and N-methylformamide and N-methylacetamide on the other hand, has been found. This suggests that the measurement of carbonyl stretching frequency as a function of different solvents can be an effective way to distinguish between solvent exposed and solvent protected carbonyl groups. In a plot of $\nu_{c=0}$ (carbonyl stretching vibration frequency) versus the dielectric constant ε would indicate free C = 0 groups, while an increase in $\nu_{c=0}$ with ε, followed by a decrease in $\nu_{c=0}$ would indicate partially protected or hydrogen bonded carbonyl groups. The use of Bellamy plots also distinguishes these two different cases.

ABSTRACT II

The preliminary Raman spectra of Fragment-1 prothrombin plus Ca^{2+} were obtained. Some differences may be present in the two spectra, possibly

in the α-helix and random coil contents, and in the carbonyl stretching vibration frequency. These dissimilarities may represent real conformational differences for Fragment-1 with or without Ca^{2+}.

Thesis Supervisor: Paul R. Schimmel
Professor of Biochemistry and Biophysics

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ABBREVIATIONS

CHAPTER I

ε : electric field associated with a wave

ε_o : maximum electric field

ν_{exc} : frequency of oscillation of the wave (exciting light)

t : time

μ : dipole moment

α : polarizability

Q_i : vibrational normal mode

ν_i : vibration frequency of the molecule

ν_{sc} : frequency of the scattered light

I : power radiated by a dipole

n : vibrational level

h : Planck's constant

k : Boltzmann constant

T : absolute temperature

CHAPTER II

$\nu_c=0$: carbonyl stretching vibration frequency

ε : dielectric constant

$\check{\nu}_c=0$: carbonyl stretch frequency of the vapor phase

CHAPTER III

Fragments of prothrombin:

I-1: Intermediate-1

F-1: Fragment-1

F-2: Fragment-2

F-1.2: Fragment-1.2

LIST OF FIGURES AND TABLES

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I also thank FAPESP-Brazil for supporting me during these years.

INTRODUCTION

INTRODUCTION

Raman scattering was discovered nearly 50 years ago, in 1928. During this period, Raman spectroscopy has advanced to take its place among other, older methods of investigating the structure and composition of matter, and its importance is still increasing.

The kind of information provided by laser Raman spectroscopy consists essentially of:

1. The frequencies of intra and inter-molecular vibrations in the range 10-4000 cm^{-1}.

2. The spectral line or band intensities associated with these frequencies. The intensity is to a good approximation a linear function of the molar concentration of the sample molecules.

The methods of drawing structural conclusions from this kind of information are mainly those of reasoning by analogy. Spectra of model compounds of known conformation and appropriate composition are obtained. Empirical correlations between frequencies (and sometimes intensities) and conformations can often be worked out and then used for structural studies of new systems. Clearly, this kind of structural study is vastly inferior to x-ray diffraction when the latter is applicable. Since most biopolymers are hard to obtain in crystalline form, and in any event are not crystalline <u>in vivo</u>, the structural information obtainable from their Raman spectra is potentially very useful.

In this work we present:

In chapter I we have a brief description of the Raman effect. A conceptual derivation of the scattering phenomenon is followed by a discussion of the advantages of the technique and then the experimental arrangement is described.

In chapter II we study the dependence of the carbonyl stretch vibration frequencies upon the environment of the carbonyl groups. The effect of solvent interactions is studied by dissolving a compound containing the carbonyl group in solvents with different polarity (e.g., CCl_4, $CHCl_3$, CH_3OH,...). Raman spectroscopy may be used to examine the conformational changes in a molecule as it binds ions or moves into a different chemical environment, and here it is used as a probe of hydrogen bonding. A further step would be to investigage the ability of compounds to complex selectivity with cations. By studying compounds which bind a wide variety of cations, information about the relative importance of steric and electrostatic forces in cation-ionophore interactions can be obtained.

In chapter III Raman spectroscopy is applied to a biological macromolecule: a fragment of prothrombin. This fragment is known to be the relevant part of the protein for the binding of Ca^{++} through carbonyl groups. The Raman spectrum of this fragment in the presence and absence of Ca^{++} is shown, and possible conformational differences are discussed.

CHAPTER I: THE PHYSICS OF THE PHENOMENON OF LASER RAMAN SPECTROSCOPY

LASER RAMAN SPECTROSCOPY

The Phenomenon

When light passes through a solution, a fraction of the light is scattered. Most of the scattered radiation has the same frequency as the incident radiation. This scattering, refered to as Rayleigh scattering, may be viewed as the elastic scattering of a photon by the molecule.

In 1928 Raman (1) discovered experimentally that there was sometimes also present in the scattered light weak radiation of frequencies different than the incident light. The frequency shifts from the incident frequency correspond to the vibrational frequencies of the scattering molecules and hence are a source of information on molecular structure. Raman scattering may be viewed as inelastic scattering of a photon by the molecule.

The Interpretation (2)

It is assumed that light is scattered from a free molecule as a result of the induced oscillations of the dipole of the molecule by the irradiating electromagnetic field.

Consider a free molecule subjected to an electromagnetic wave, where

$$\varepsilon = \varepsilon_o \cos(2\pi\nu_{exc}\, t)$$

that is, the wave is a monochromatic plane wave. Here, ε_o is the maximum electric field associated with the wave, ν_{exc} is the frequency of oscillation of the wave, and t is the time. This wave induces a

dipole moment μ in the molecule, where

$$\mu = \alpha\varepsilon$$

α is the polarizability of the molecule, i.e., a measure of the electronic deformation of the electron cloud by the field. The polarizability is a function of the interatomic distances of the molecule and is hence dependent upon the vibrational modes. Then, if the amplitude of the motion is small

$$\alpha(Q_i) = \alpha_o + \left(\frac{\partial\alpha}{\partial Q_i}\right)_o \cdot Q_i + \ldots$$

where Q_i is a particular vibrational normal mode of the molecule, i.e., Q_i is the displacement from equilibrium for some kind of possible harmonic motion. Q_i is assumed periodic in time

$$Q_i = Q_i^o \cos(2\pi\nu_i t)$$

with ν_i as the frequency of the vibration. Then

$$\mu = \left[\alpha_o + \left(\frac{\partial\alpha}{\partial Q_i}\right)_o Q_i^o \cos(2\pi\nu_i t) + \ldots\right] \varepsilon_o \cos(2\pi\nu_{exc}.t) =$$

$$= \alpha_o\varepsilon_o \cos(2\pi\nu_{exc}.t) + \tfrac{1}{2}\left(\frac{\partial\alpha}{\partial Q_i}\right)_o Q_i^o \varepsilon_o \left\{\cos 2\pi(\nu_{exc} + \nu_i)t + \right.$$

$$\left. + \cos 2\pi(\nu_{exc} - \nu_i)t \right\} + \ldots$$

Therefore if the polarizability of the molecule changes periodically in consequence of the vibration, then in the scattered radiation the sum and the difference of the incident frequency and the molecular vibration frequency will appear. These are respectively the anti-Stokes and the Stokes Raman scattering. In other words, if the net transition is from a lower to a higher energy level, so that $\nu_{sc} < \nu_{exc}$ (ν_{sc} is the frequency of the scattered light), the process is called Stokes

scattering; the opposite case, in which $\nu_{sc} > \nu_{exc}$ is termed anti-Stokes scattering (Figure 1).

In addition to the fundamental vibrations in the Raman effect, for which the frequency of a normal vibration has to be added or subtracted from the frequency of the incident radiation, there are also overtones and combination tones. The reasons for these are mechanical and electrical anharmonicity. This means, there are terms of a quadratic or higher order in the dependence of the polarizability on the displacements.

The total power I radiated by an oscillating dipole in classical electromagnetics (3) is

$$I \alpha |\mu|^2$$

Consideration of the energy level diagram (Figure 1) shows that the intensities of the Stokes and anti-Stokes Raman lines are not equal, as suggested by the preceding classical analysis. According to quantum theory (4) and in agreement with observation the anti-Stokes lines have a much smaller intensity (Figure 2), since the number of molecules in the initial state n = 1 of the anti-Stokes lines is only $\exp(-h\nu/kT)$ times the number of molecules in the initial state n = 0 of the Stokes lines (ground state). Here n numbers the vibrational levels, h is Planck's constant, k is the Boltzmann constant, ν is the vibration frequency, and T is the absolute temperature. One has to remember that the selection rule for the vibrational Raman effect is $\Delta n = \pm 1$, so that if one wants the ground state (n = 0) as one of the

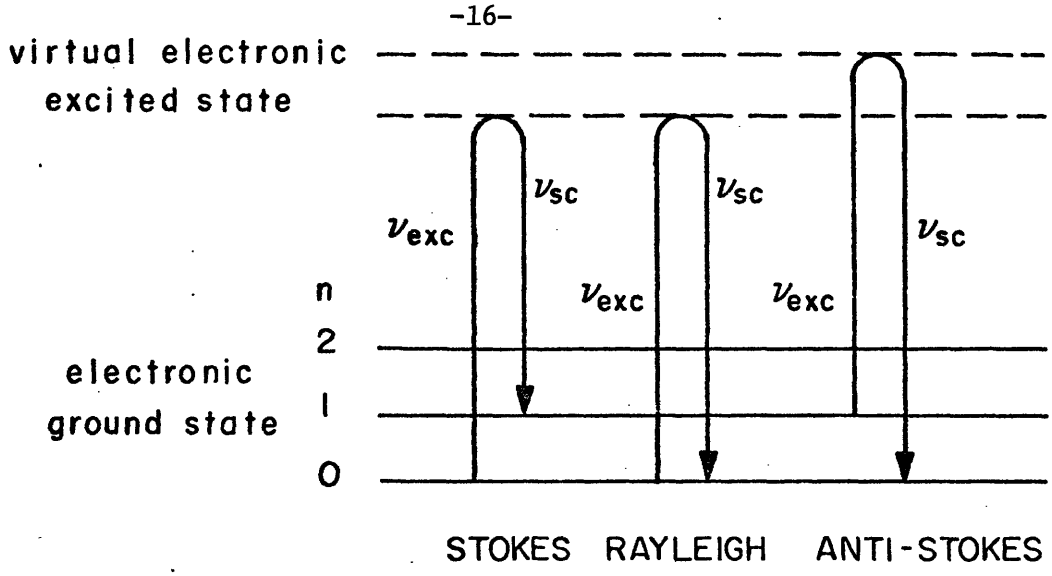


FIGURE 1: The transitions involved in the Rayleigh and Raman effect. The numbers at the left number the vibrational levels, ν_{exc} is the frequency of the incidente light and ν_{sc} is the frequency of the scattered light.

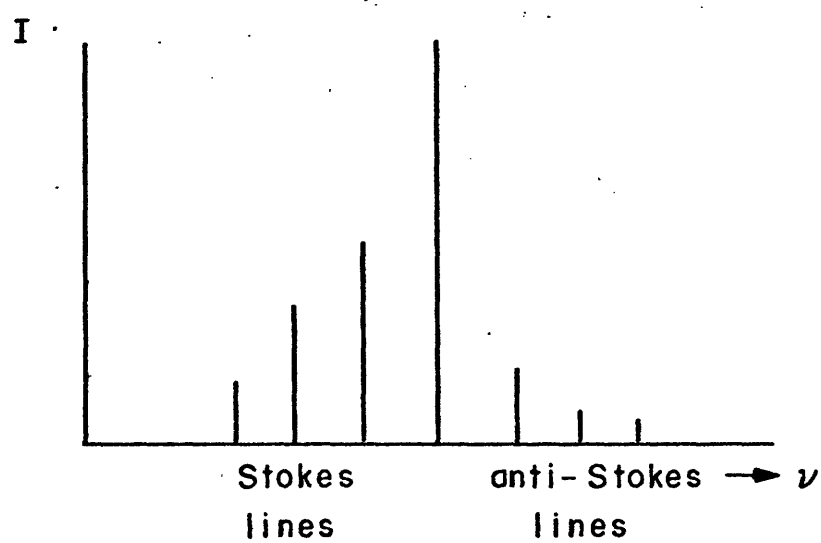


FIGURE 2: The anti-Stokes lines are usually less intense than the Stokes lines.

levels, the other has to be n = 1. The intensity ratio of the anti-Stokes lines to the corresponding Stokes lines is in agreement with the Boltzmann factor.

Comparison with Infrared Spectroscopy

The Raman effect of molecules provides the same kind of information as does infrared absorption, chiefly the characteristic frequencies of molecular vibrations. Only those vibrations that are connected with a change of dipole moment can give rise to an infrared transition, and only those vibrations that are connected with a linear change of polarizability can give rise to a Raman transition (4). Since selection rules for the Raman effect differ from those for the infrared absorption (5), the vibrational transitions observed by one method supplement those observed by the other, but sometimes one can be used when the other cannot. A great impediment to the use of infrared analysis of biological materials is the very strong absorption by water in the mid and far infrared. Raman spectra are then advantageous for study of biological materials (6-8) in aqueous solution (9).

In infrared spectroscopy one illuminates the sample with a variety of individual wavelengths of infrared light, and measures the transmitted intensity at different incident frequencies. In Raman spectroscopy one illuminates the sample with a fixed wavelength and measures the scattered intensity as a function of the frequency shift from the incident frequency - Raman spectrum (7, 10). One can repeat Raman experiments using a laser line of different frequency, and the resulting

spectrum is identical, so long as one plots ones data as a function of the shift in frequency (Figure 3). Raman spectrum does not change when one changes the exciting frequency, but fluorescence does. In fluorescence phenomena the laser excites an electronic level in a region where there are many closely spaced excited levels to which the molecules may decay (Figure 4). Then there is a final decay from some electronic level to the vibrational ground state. Thus the fluorescence signal competes with the Raman signal, and since fluorescence phenomena are generally intense (and since the noise is roughly proportional to the total light emitted) one may have difficulty seeing the Raman scattered light superposed on the fluorescence signal. One solution to this problem is to vary the frequency of the laser light until one is below the fluorescence band.

Technique

The laser is an ideal source for the excitation of Raman spectra and enables the study of samples as small as 1 mg or less. The Raman spectrum is recorded by directing a laser beam on the sample to be studied (often contained in a capillary) and collecting the scattered light into a monochromator-detector system that can measure the intensity of the light at different frequencies (Figure 5). The Raman effect is the result of inelastic scattering of the photons in the laser beam by molecules, in which process the energy of each photon may be decreased or increased by transfer of vibrational energy to or from the molecule. If the molecule receives energy (the more likely event) the scattered photons have lost it and thus have lower frequency.

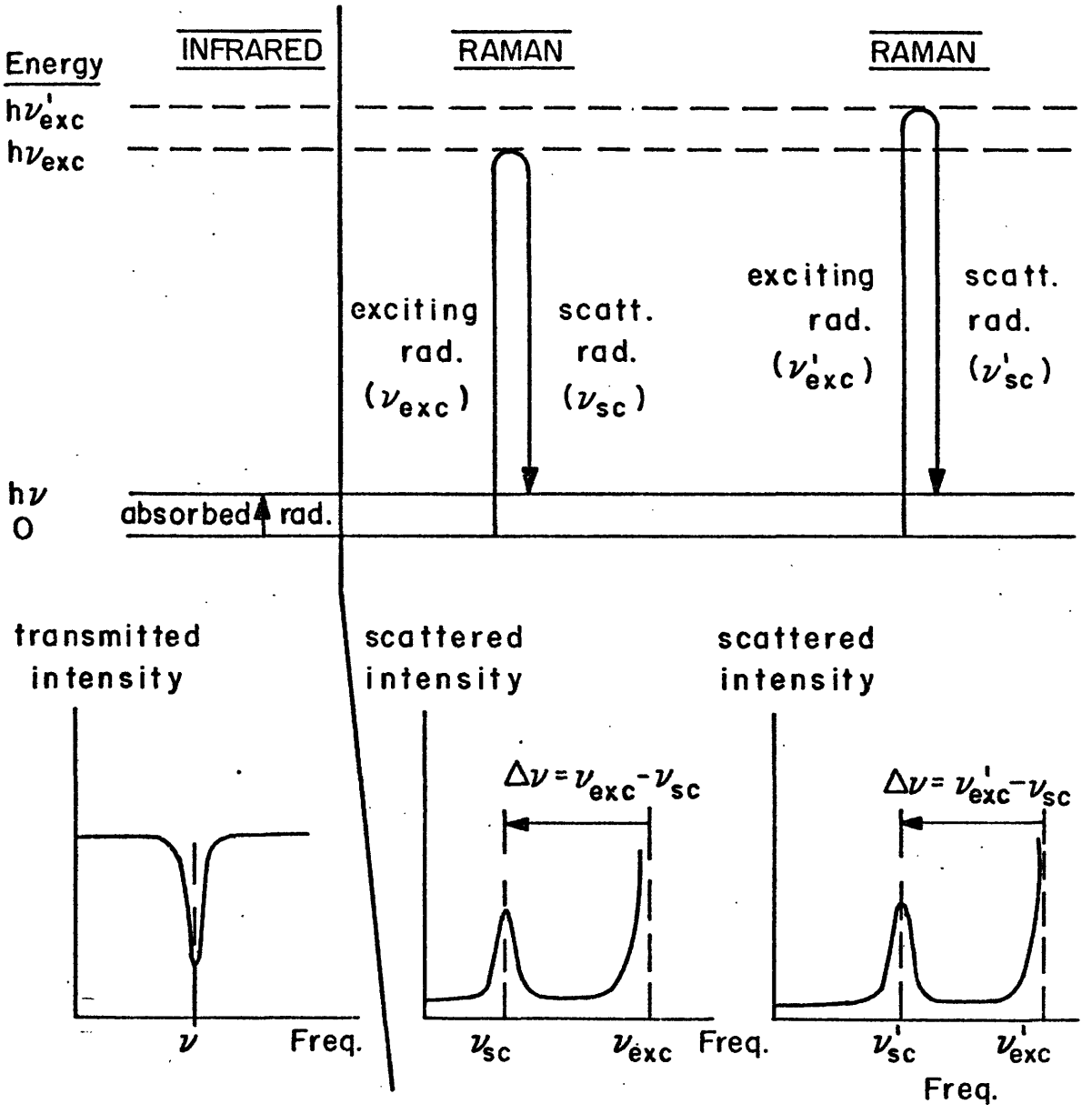


FIGURE 3: Infrared spectroscopy measures transmitted intensity as a function of incident frequency, while Raman spectroscopy measures scattered intensity as a function of frequency shift from a fixed incident light frequency.

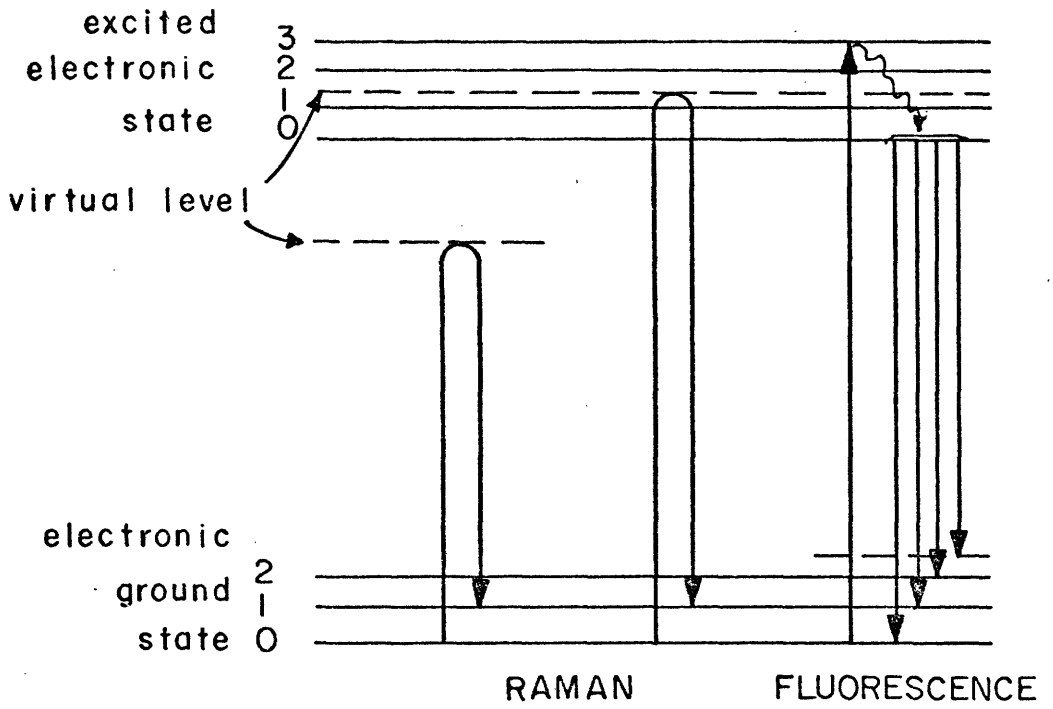


FIGURE 4: Fluorescenece phenomena are much stronger than Raman scattering. They can be removed in many cases by choosing a different laser frequency.

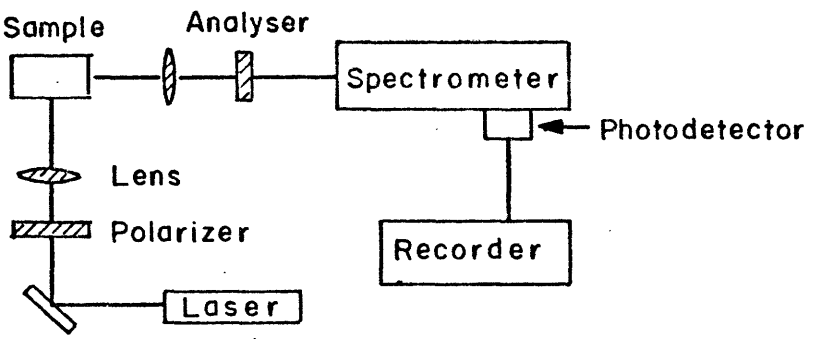


FIGURE 5: The light scattered by the sample is collected by a large lens and frequency analyzed by a double grating monochromator.

When visible radiation is used for excitation, the scattered radiation still lies in or near the visible spectrum because the vibrational energy transferred is a small fraction of the energy of visible photons. Hence the optical components and instrumentation for Raman spectra are those of visible spectroscopy.

The spectrum is usually recorded with the intensity of the Raman scattering in relative units on the ordinate scale and wavenumber displacement in cm^{-1} from the laser exciting frequency, which is taken as the zero of the abcissa scale.

REFERENCES

1. Raman, C.V. (1928) Indian J. Phys. 2, 387

2. Schutte, C.J.H. (1976) The Theory of Molecular Spectroscopy (North Holland, Elsevier)

3. Jackson, J.D. (1962) Classical Electrodynamics (New York, John Wiley)

4. Hibben, J.B. (1939) The Raman Effect and its Applications (New York, Reinhold)

5. Herzberg, G. (1945) Infrared and Raman Spectra (New Jersey, D. Van Nostrand)

6. Koenig, J.L. (1972) J. Polymer; Sci. part D 60, 59

7. Lord, R.C. (1971) XXII Int. Congr. Pure Appl. Chem. 7, 179

8. Whittman, R. (1974) Transport and Diffusion in Red Blood Cells (London, Edward Arnold)

9. Yu, N.T.; Lui, C.S. and O'Shea, D.C. (1972) J. Mol. Biol. 70, 117

10. Tobin, M.C. (1971) Laser Raman Spectroscopy (New York, John Wiley)

CHAPTER II: SOLVENT EFFECTS ON THE CARBONYL STRETCH VIBRATION FREQUENCY

INTRODUCTION

It has been shown that the stretching frequency of a carbonyl group, $\nu c=0$ is sensitive to many factors including inductive effects, hydrogen bonding, and ionic interactions (1-5). Hence it is often difficult to draw any conclusions about the local interactions of a carbonyl group from the absolute value of $\nu c=0$. We are interested in investigating whether the changes of $\nu c=0$ as a function of different dielectric and polar solvents can be used to probe the local environment of a carbonyl group. For example, it has been found that the $\nu c = 0$ of intramolecular hydrogen bonded amide carbonyl groups of valinomycin increases in frequency with a increase in the dielectric of the solvent, whereas non-hydrogen bonding ester carbonyl groups decrease in frequency. It is not known, however, to what extent these changes are due to direct solvent effects on the carbonyl groups versus induced conformational changes of the molecule (6).

One of the first attempts to relate $\nu c = 0$ to changes in properties of the solvent medium was made by Kirkwood, Bauer and Magat (7,8). Their theory relates the frequency shift $\Delta\nu$, where $\Delta\nu=(\overset{v}{\nu}c=0)-(\nu c=0)$ and $\overset{v}{\nu}c=0$ is the carbonyl stretch frequency of the vapor phase, to the dielectric constant ε of the solvent by

$$\frac{\Delta\nu}{\nu c=0} = \frac{C(\varepsilon-1)}{2\varepsilon + 1} \quad \ldots . \quad (1)$$

where the factor C is complex and depends on details of the model used for the vibrating solute dipole. In a test of this theory, Thompson

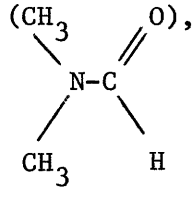
has shown (9) that if $\Delta\nu/\nu c=0$ is plotted against $(\varepsilon-1)/(2\varepsilon+1)$, a straight line can be drawn from the origin which passes through points corresponding to nonpolar solvents he measured, but not for most other solvents. He suggested that the deviation does not seem to arise from hydrogen bonding, since this should lower $\nu c=0$ and increase $\Delta\nu(1)$, whereas the converse is found.

Detailed studies by Josien et al. (10-16) and by Bayliss et al. (17,18) have also shown there is a relationship between frequency shifts and bulk solvent properties, in those cases where such specific interactions such as hydrogen bonding are minimal. Bellamy (19,20) has studied the carbonyl frequencies of a wide range of compounds in many solvents and concluded that all the frequency shifts follow a common pattern and seem to be produced mainly by local association effects, and not by dielectric constant factors. The order of solvent effectiveness in lowering $\nu c=0$ is therefore always the same, and a quantitative relationship exists between the effects of one solvent and another. Thus, the relative frequency shifts ($\Delta\nu/\nu c=0$) of any one carbonyl group in a series of solvents can be plotted directly against the values for some other carbonyl group in the same solvents, to give a straight line. The failure of the slopes of these lines to correlate directly with the proton-accepting powers of the carbonyl group, shows that the use of relative carbonyl shifts cannot safely be used as a measure of the hydrogen bonding powers of different solvents.

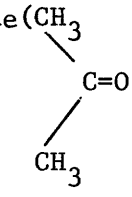
The effect of the solvent on $\nu c=0$ for various carbonyl compounds has been examined in terms of bulk dielectric constant also by

Kagarise (21). He suggested that dispersion forces, bulk dielectric effects and specific interaction effects all make a contribution to $\Delta\nu$. He suggested that the stronger the proton donating tendency of the solvent, the greater the proportion of $\Delta\nu$ arising from the formation of stable compounds possessing large interaction energies and well defined structures.

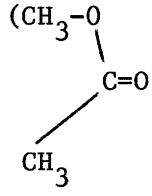
Although these studies indicate that $\nu c{=}0$ cannot be directly interpreted easily in terms of microscopic interactions, it is possible that $\nu c{=}0$ for a group of solvents can be used to distinguish between two different classes of carbonyl groups, i.e., those strongly hydrogen bonded and those non-hydrogen bonded. For this purpose, we have studied $\nu c{=}0$ for three compounds, dimethylformamide



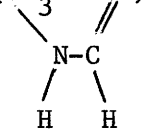
, acetone



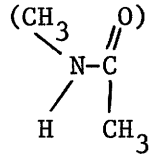
, and methylacetate



which do not form strong intermolecular bonds with themselves, and two compounds, N-methylformamide



and N-methylacetamide



which can form hydrogen bonds with themselves.

MATERIALS AND METHODS

The solvents used were: carbon tetrachloride, chloroform, dichloromethane, 1-hexanol, 2-butanol, 1-butanol, 1-propanol, ethanol, methanol, and water. The solvents and solutes were obtained from Baker and Allied Chemical and had purity for spectroscopic studies.

The solutions were prepared immediately prior to usage and sealed in glass capillaries. They were centrifuged to eliminate bubbles. The molar ratio of solute to solvent was 1/8 for all samples.

Raman spectra were measured using a Spex Ramalog 4 system and a Spectra-Physics Model 164-03 Ar laser (488 nm excitation). The incident power was 300-400 mw, resolution 3-4 cm^{-1} and scanning speed 1.2-30cm^{-1}/min.

Only the Raman scattered light with polarization parallel to the polarization of the incident light was measured (the laser light used to illuminate the sample is typically plane polarized perpendicular to the plane determined by the incident light beam and the entrance slit of the spectrometer).

For more details of the Raman spectroscopy system see ref. 6.

RESULTS AND DISCUSSION

Carbonyl stretch vibrations occur in the 1600-1800 cm^{-1} region. Table I lists $\nu c=0$ for each solute as a function of solvents. If we plot $\nu c=0$ of each solute in a particular solvent as a function of $\nu c=0$ for acetone in the same solvent (Bellamy plot) (see Fig. 1a and 1b), we find dimethylformamide and methylacetate fall on straight lines whereas N-methylformamide and N-methylacetamide clearly are not on straight lines.

If we also plot the stretching frequency versus the dielectric constant of the solvent (Fig. 2 to 6), dimethylformamide, acetone, and methylacetate showed a similar behavior: a decrease in the stretching frequency with an increase in the dielectric constant. The points are almost in a straight line with the consistent exception of carbon tetrachloride and chloroform. However, for N-methylformamide and N-methylacetamide there is a jump in $\nu c=0$ for carbon tetrachloride, chloroform, and dichloromethane to higher dielectric solvents such as hexanol, and then a decrease in $\nu c=0$ with increasing ε. Here, at least for N-methylformamide, the points fit a straight line. For N-methylacetamide, the point using water as solvent may be wrong due to interference of the carbonyl stretching vibration peak with the water peak. If this point is 10 cm^{-1} higher, the points for N-methylacetamide will also fit roughly a straight line.

From inspection of this data it is evident that two kinds of patterns are found for hydrogen bonding versus non-hydrogen bonding (with itself) solutes. The decrease in the stretching frequency with

TABLE I - $\nu c{=}0$ for each solute in different solvents (the dielectric constant of the solvents is also given)

	ε	Dimethyl-formamide	Acetone	N-methyl-acetamide	N-methyl-formamide	Methyl-acetate
Carbon tetrachloride	2.24	1679.5	1714.5	1652.0	1666.0	1748.0
Chloroform	4.81	1672.0	1710.0	1655.0	1662.0	1735.0
Dichloromethane	9.08	1674.5	1712.0	1654.5	1666.5	1741.0
1-hexanol	13.30	1671.5	1709.5	1661.0	1677.0	1733.5
2-butanol	15.80	1673.5	1710.5	1663.0	1679.5	1734.0
1-butanol	17.80	1670.5	1709.5	1662.0	1678.0	1733.0
1-propanol	20.08	1670.0	1709.0	1660.5	1677.5	1733.5
Ethanol	24.30	1671.5	1709.0	1661.5	1676.5	1733.0
Methanol	33.62	1667.5	1707.0	1660.5	1672.5	1732.0
Water	80.37	1654.0	1699.5	1634.5	1658.0	1713.0

($\nu c{=}0$ is given in cm^{-1})

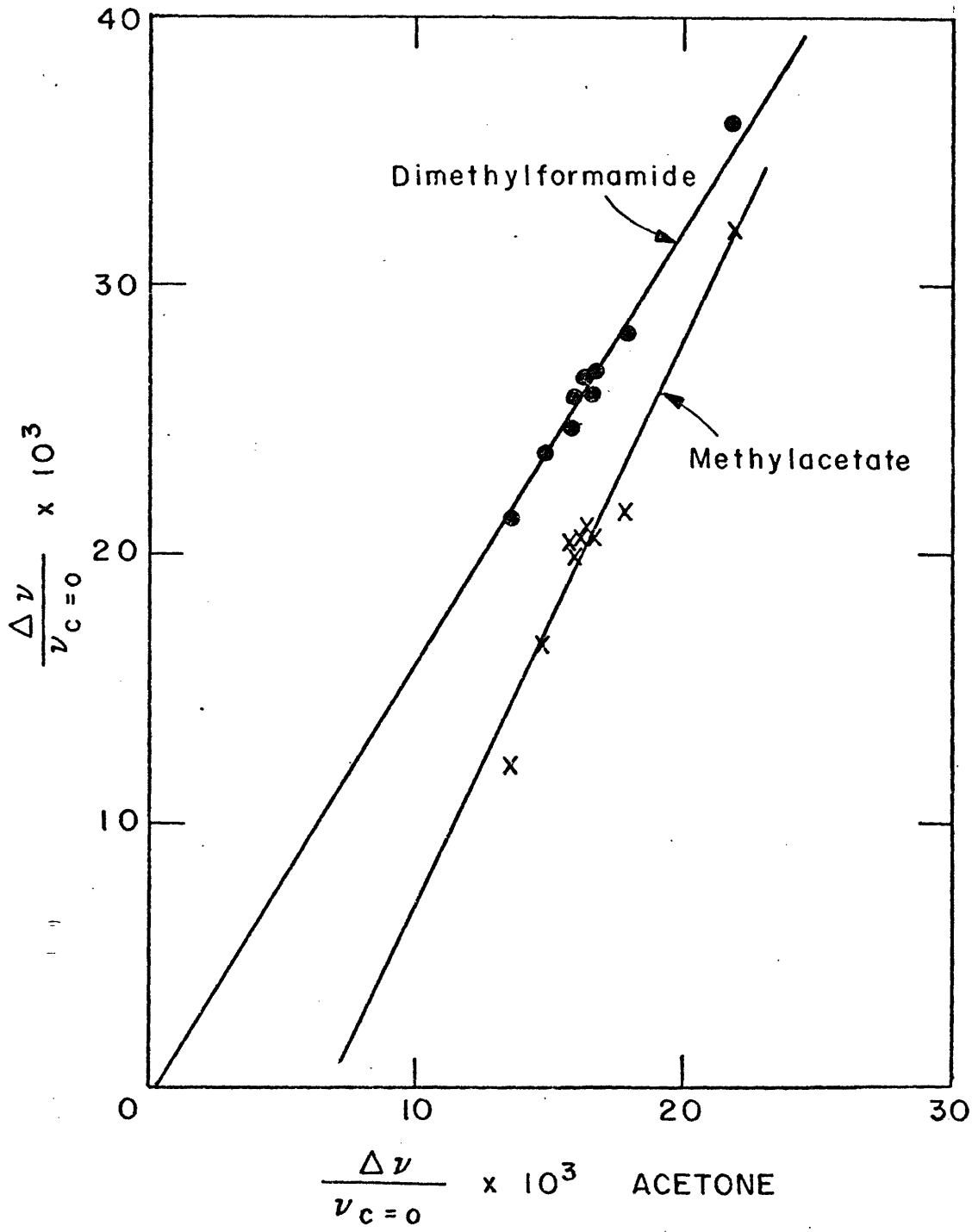


FIGURE Ia Bellamy Plot

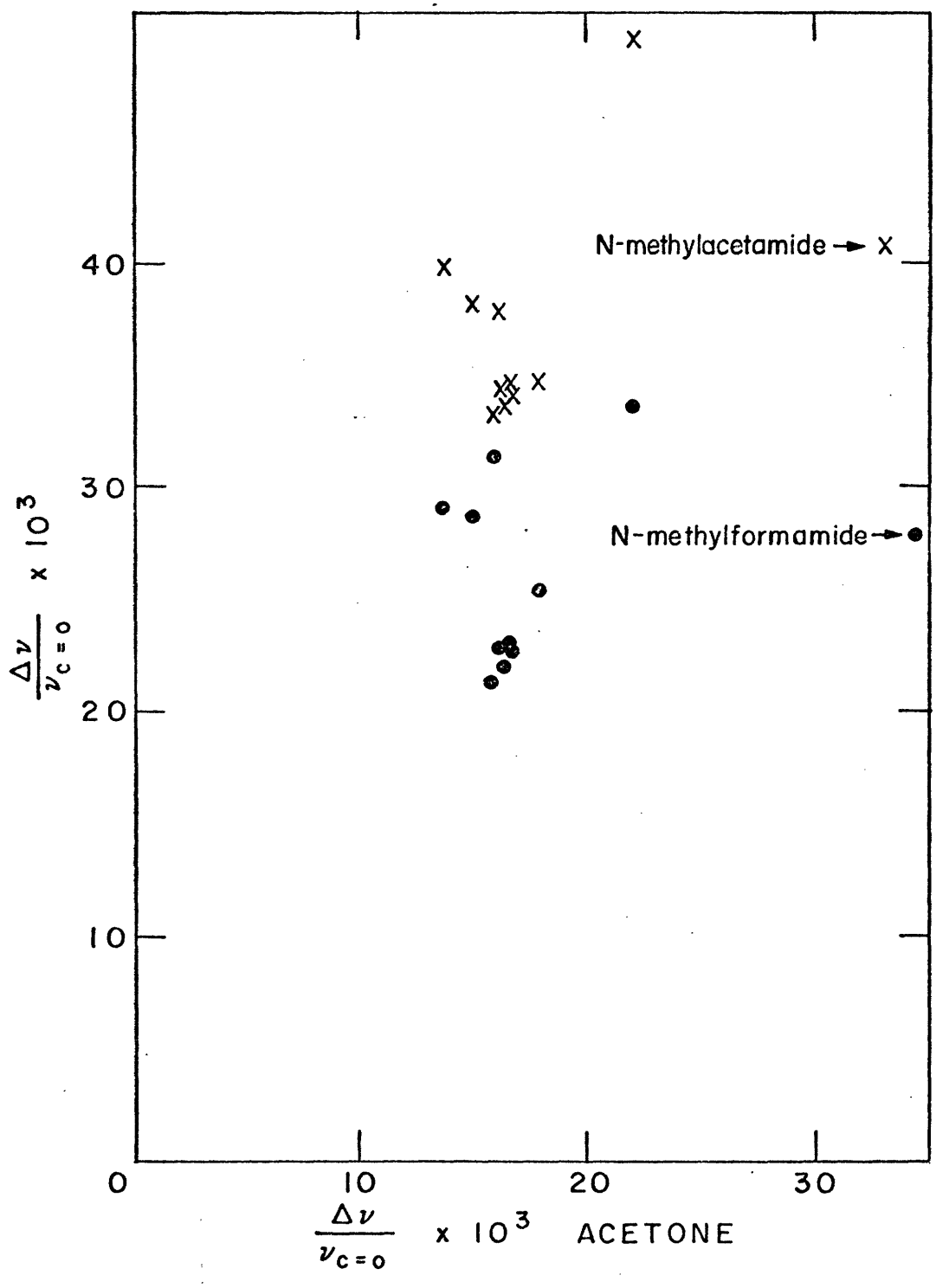


FIGURE Ib – Bellamy Plot

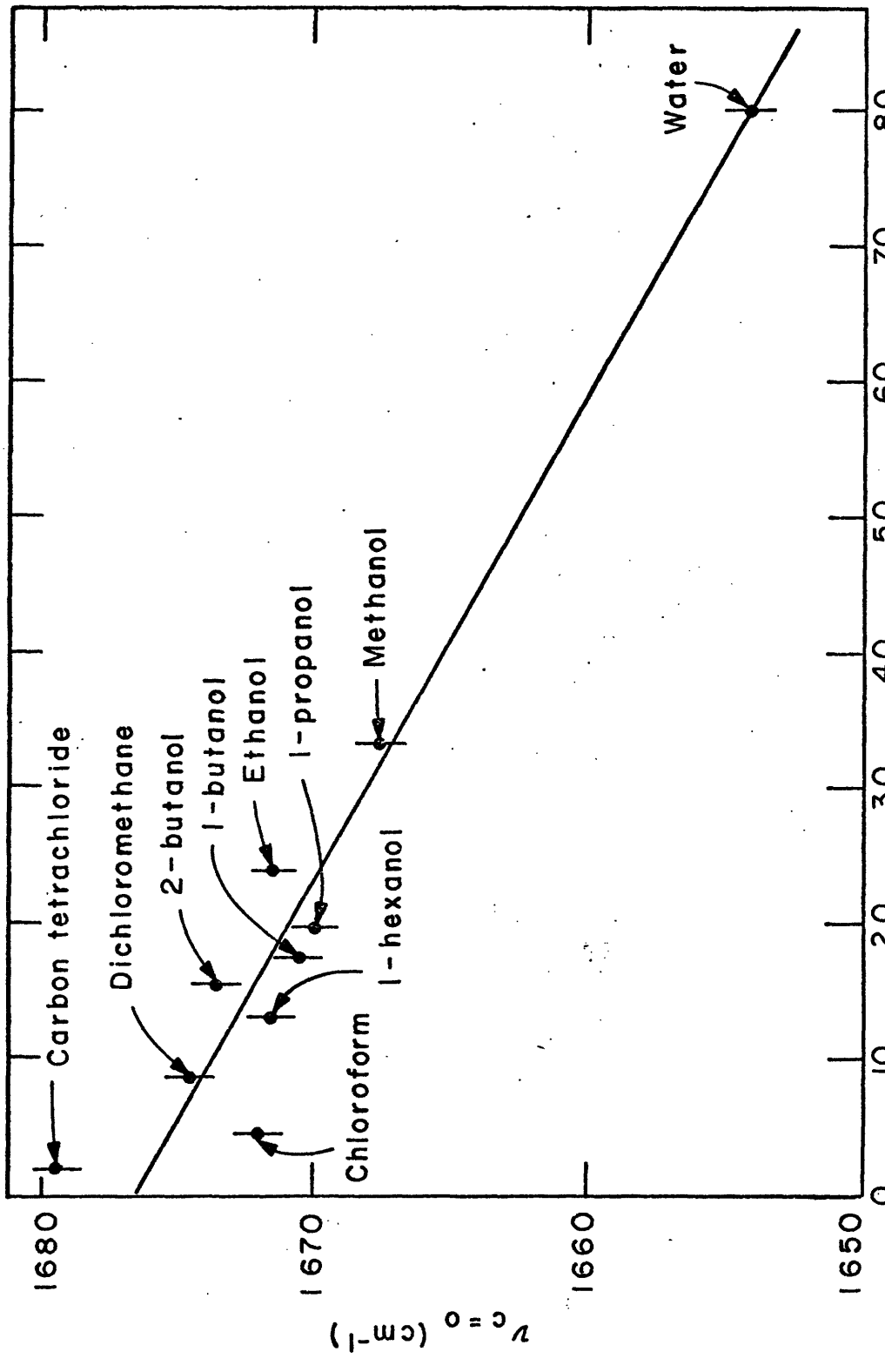


FIGURE 2 - $\nu_{c=o}$ versus $\epsilon_{solvent}$ Solute - dimethylformamide

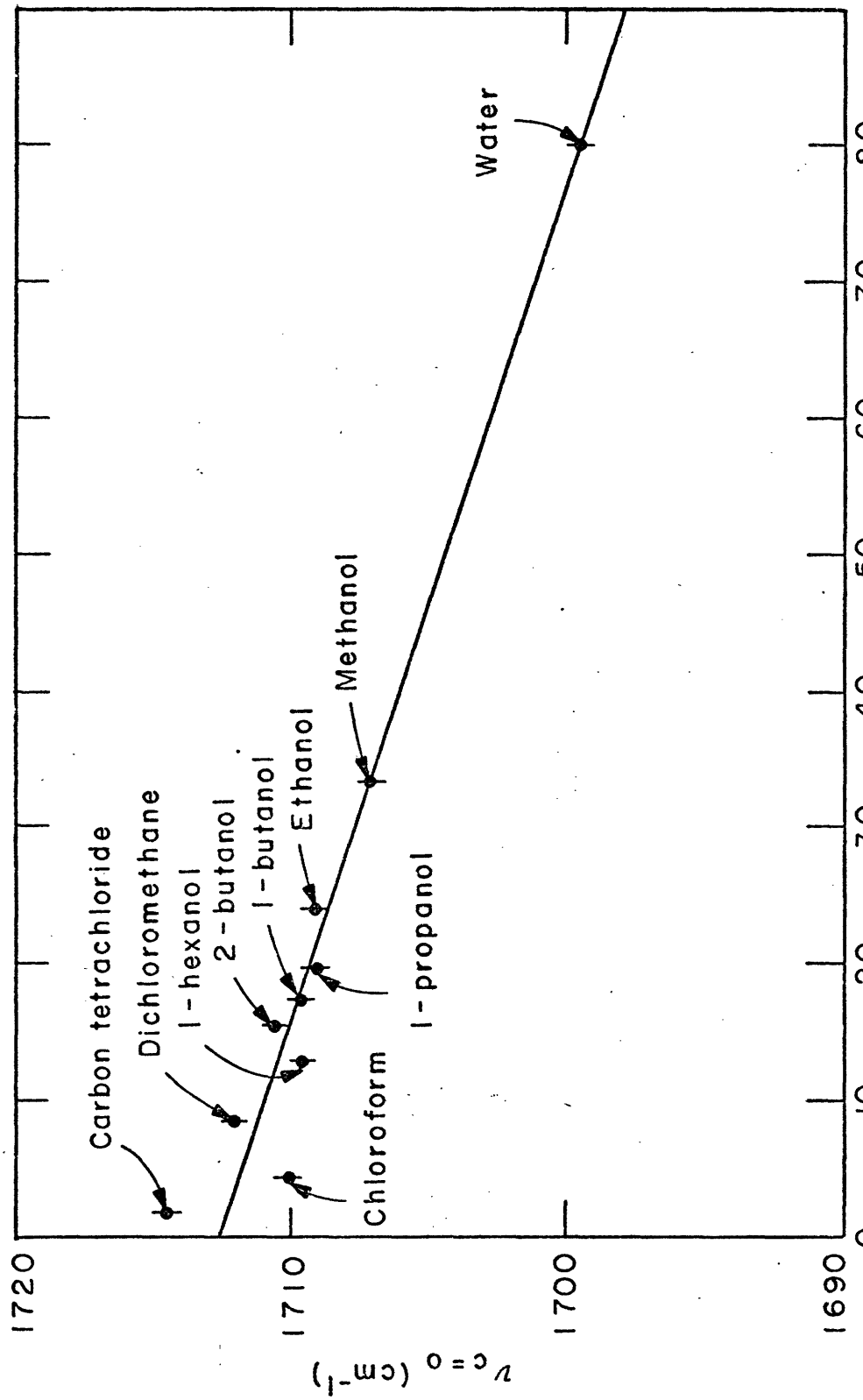


FIGURE 3 - $\nu_{C=O}$ versus $\epsilon_{solvent}$ solute - acetone

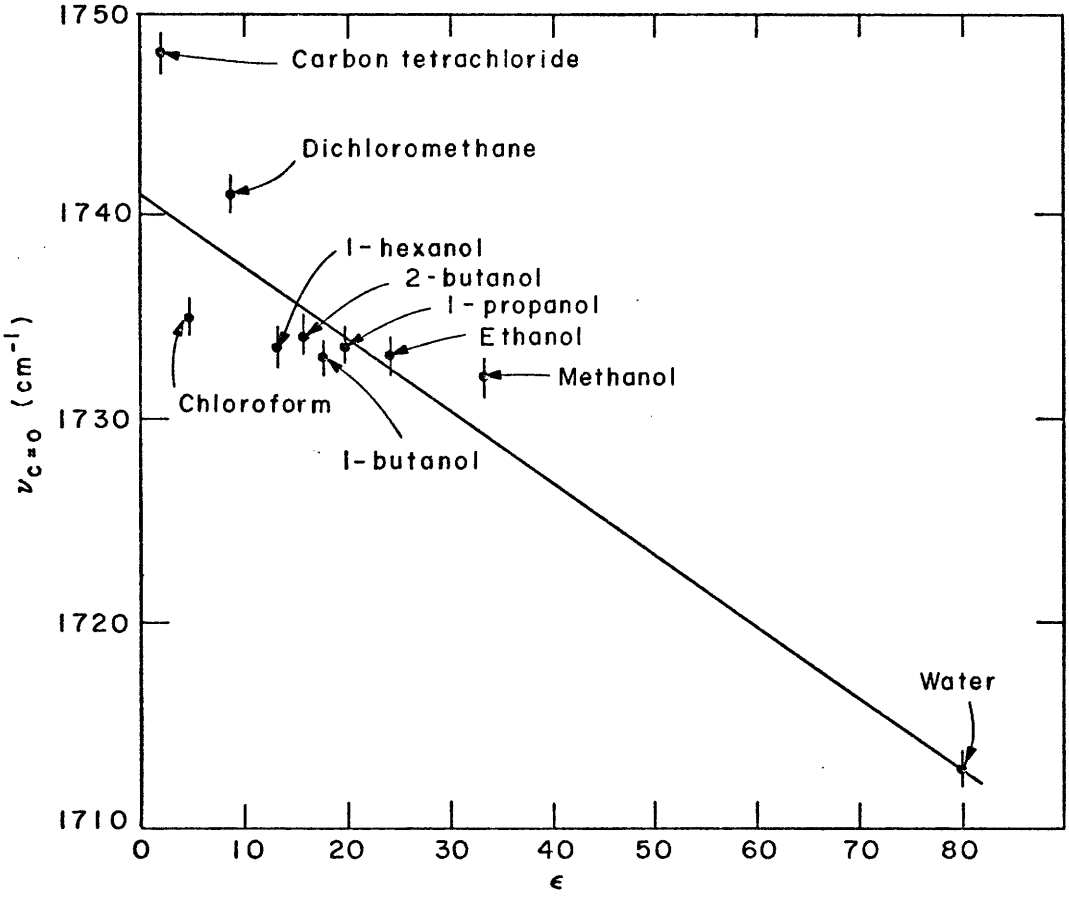


FIGURE 4 $\nu_{C=O}$ versus $\epsilon_{solvent}$ Solute – methylacetate

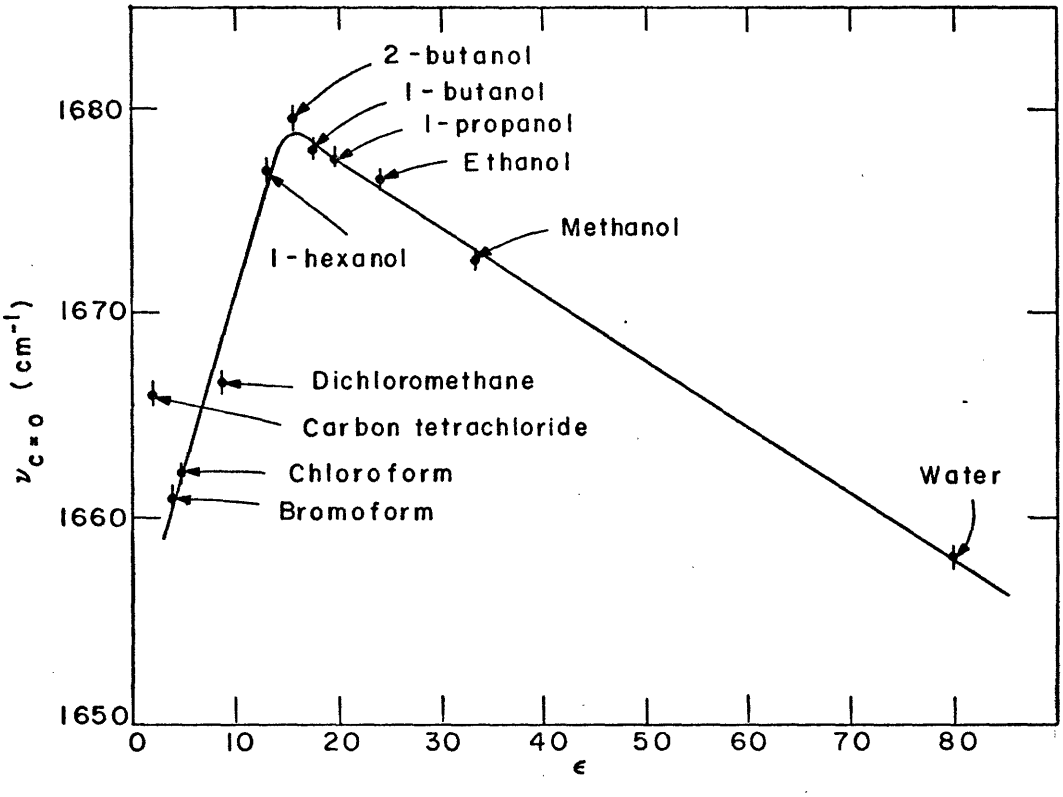


FIGURE 5 $\nu_{C=O}$ versus $\epsilon_{solvent}$ Solute – N-methylformamide

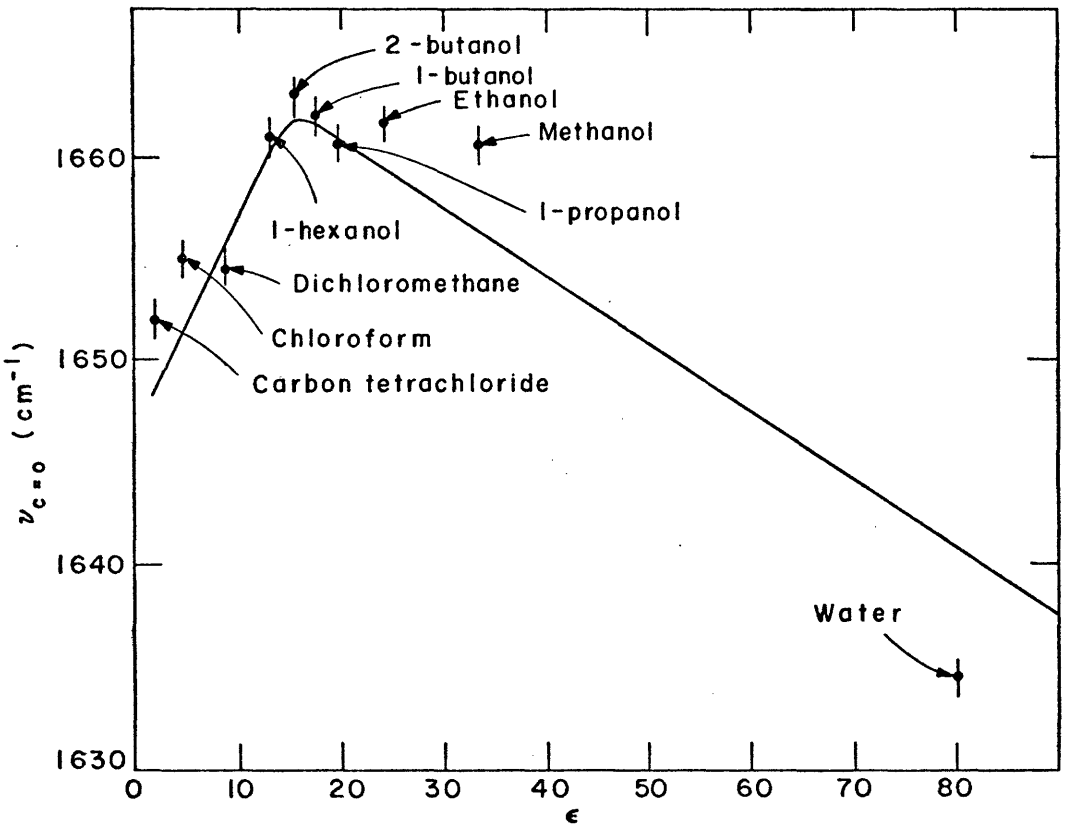


FIGURE 6 $\nu_{c=o}$ versus $\epsilon_{solvent}$ Solute - N-methylacetamide

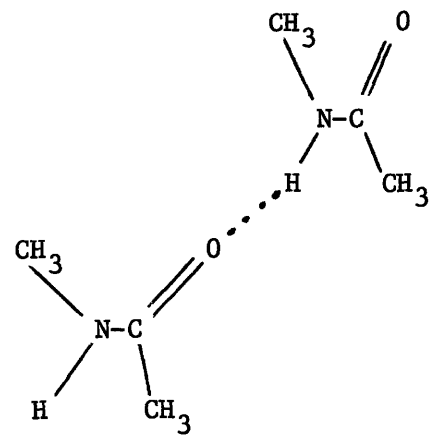
an increase in the dielectric constant is similar to that observed by Rothschild et al. (6) for the non-hydrogen bonded ester carbonyl groups of valinomycin. They found also an initial jump in $\nu c{=}0$ and then a decrease with increasing ε for amide carbonyl groups which are believed to be able to form hydrogen bonds.

The dielectric plot can be explained considering that the solute will form hydrogen bonds with the solvents and will decrease $\nu c{=}0$. The strong specific interaction apparent in polar solvents is due to more or less stable complexation with the solute molecules. Such complexes are formed by way of a hydrogen bond, e.g., in the case of methanol:

$$\rangle C = O \; . \; . \; . \; . \; H - O - CH_3$$

The $\nu c{=}0$ frequency decrease with an increase in the strength of the hydrogen bond is a reflection of a lowered C=O bond force constant (22). This explanation may be accepted with confidence, as it is entirely consistent with the independent experimental observation that the C=O distance increases with decreasing O....O distance (in the case of methanol) (23). It is well established that for a given type of bond a decrease in force constant goes together with a bond lengthening (24). However, no theoretical explanation was found for the linearity of the frequency of vibration with the dielectric constant.

For those compounds in which a solute-solute hydrogen bond is possible (N-methylformamide and N-methylacetamide (16)), there is an increase in $\nu c{=}0$ with increasing ε (for low values of ε). The self-association in N-methylacetamide is as follows:



Either weakening or breaking this hydrogen bond by solvent could cause an increase in $\nu_{C=O}$. But if the solute-solvent hydrogen bond formed is stronger than the solute-solute one, a decrease in the stretching frequency will occur. This happens for high dielectric constant solvents.

In Fig. 2 to 4 the two points that are consistently out of a straight line are carbon tetrachloride and chloroform. It is known that chloroform solutions give exceptionally large frequency shift compared with its dipole moment and correlation time (25). This might explain its position below the line compared to dichloromethane. In contrast, carbon tetrachloride is somewhat above the line which may reflect the relative weakness of the

interaction.

These results seem to indicate that the lack of a straight line for the Bellamy plots and the lack of monotonically decreasing dielectric versus $\nu_{C=O}$ plot, can be used as an indication that the C = O group

in question is not able to freely interact with the solvent. In the case of amide C=O groups of valinomycin, this is caused by intramolecular hydrogen bonds, whereas N-methylformamide and N-methylacetamide it is caused by intermolecular solute-solute hydrogen bonding. Here specific information might be derived from deviations in the normal position of carbon tetrachloride and chloroform as defined by our standard solutes. In this regard, the fact that $\nu c=0$ of the ester carbonyl groups in valinomycin for carbon tetrachloride does not appear raised could indicate some type of local interaction or protection of the ester carbonyl groups.

Our next step would be to try this method with other materials. Enniatin-B, for example, is an antibiotic related to valinomycin that cannot form intramolecular hydrogen bonds since it contains $N-CH_3$ instead of the N-H present in valinomycin . Interestingly its Raman spectrum shows peaks that could correspond to hydrogen bonded carbonyl groups (26). It may be possible to distinguish between valinomycin hydrogen bonded carbonyl groups and enniatin-B carbonyl groups on the basis of a Bellamy plot or $\nu c=0$ versus ε plot, as discussed in this paper. Further application of this method might be generally applicable where one wishes to determine if an intramolecular C=O group is solvent exposed or solvent protected.

REFERENCES

1. Bellamy, L.J. (1975) Advances in Infrared Group Frequencies (London, Chapman and Hall Ltd.)

2. Whetsel, K.B. and Kagarise, R.E. (1962) Spectrochim. Acta 18, 315

3. Kagarise, R.E. (1955) J. Amer. Chem. Soc. 77, 1377

4. Kosower, E.M. (1958) J. Amer. Chem. Soc. 80, 3253

5. Schuber, W.M. (1960) J. Amer. Chem. Soc. 82, 1353.

6. Asher, I.M.; Rothschild, K.J. and Stanley, H.E. (1974) J. Mol. Biol. 89, 205

7. West, W. and Edwards, R.T. (1937) J. Chem. Phys. 5, 14

8. Bauer, E. and Magat, M. (1939) J. Phys. Radium 9, 319

9. Heald, C. and Thompson, H.W. (1962) Proc. Roy. Soc. A268, 89

10. Josien, M.L. and Fuson, N. (1954) J. Chem. Phys. 22, 1169

11. Josien, M.L. and Fuson, N. (1954) J. Chem. Phys. 22, 1264

12. Josien, M.L. and Lascombe, J. (1954) Compt. Rend. 239, 51

13. Josien, M.L. and Lascombe, J. (1955) J. Chem. Phys. 52, 162

14. Josien, M.L; Leicknam, J.P. and Fuson, N. (1958) Bull. Soc. Chim. France 188

15. Josien, M.L.; Lascombe, J. and Leicknam, J.P. (1958) Compt. Rend. 246, 1418

16. Rey-Lafon, M.;Lascombe, J. and Josien, M.L. (1963) Ann. Chim. 8, 493

17. Bayliss, N.S.; Cole, A.R.H. and Little, L.H. (1955) Australian J. Chem. 8, 26

18. Bayliss, N.S.; Cole, A.R.H. and Little, L.H. (1959) Spectrochim Acta 15, 12

19. Bellamy, L.J. and Williams, R.L. (1960) Proc. Roy. Soc. (London) 255A, 22

20. Bellamy, L.J. and Williams, R.L. (1959) Trans. Faraday Soc. 55, 14

21. Kagarise, R.E. and Whetsel, K.B. (1962) Spectrochim. Acta 18, 341

22. Davies, M.M. and Sutherland, G.B.B.M. (1938) J. Chem. Phys. 6, 755

23. Nakomoto, K.; Margoshes, M. and Rundle, R.E. (1955) J. Amer. Chem. Soc. 77, 6480

24. Badger, R.M. (1935) J. Chem. Phys. 3, 710

25. Koga, K. (1973) J. Mol. Spect. 47, 107

26. Rothschild, K.J. , personal communication.

CHAPTER III: APPLICATION OF RAMAN SPECTROSCOPY TO FRAGMENT-1 OF PROTHROMBIN

INTRODUCTION

Bovine prothrombin is a glycoprotein (1-3) of molecular weight 68,000 to 74,000 (4-7) and consists of a single polypeptide chain (6,8, 9). Activation of prothrombin yields a serine protease thrombin (10,11) with a molecular weight of 37,000 (12,13). The amino acid sequence of bovine thrombin has been reported (9). It has been known for a long time that thrombin accounts for only one-half of the mass of the prothrombin molecule (14). What is the function of the nonthrombin- forming half of prothrombin? Two other products are formed (designed Fragment-1 (F-1) of molecular weight 22,000 to 24,000 and Fragment-2 (F-2) of molecular weight 12,800) derived from the amino terminal half of prothrombin (14). The structural information is summarized by the schematic diagram in Figure 1:

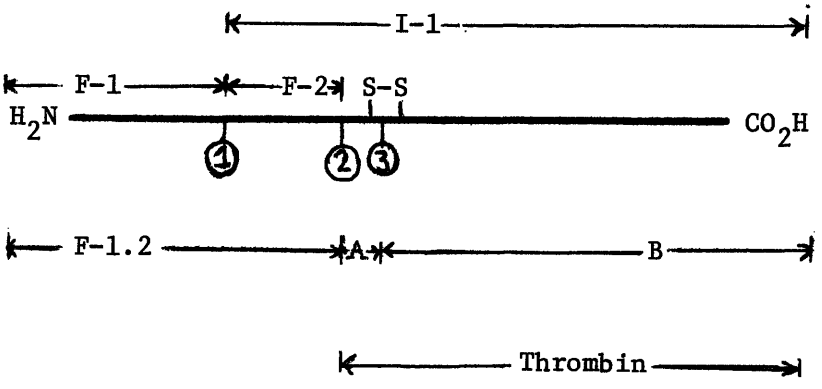


FIGURE 1: A schematic model for the prothrombin molecule. The symbol ❶ represents the peptide bonds in prothrombin which are cleaved during activation: Factor X_a (a blood clothing factor) cleaves bonds ② and ③ (14,15); and thrombin cleaves bond ① (15).

Fragment-1, the NH_2-terminal region of the propiece (Fragment 1.2) has been shown to be responsible for the binding of prothrombin to phospholipids in the presence of Ca^{2+}(16). Other studies have shown that the Fragment-1 region contains the sites for Ca^{2+} binding, and that all Ca^{2+} binding to prothrombin occurs via the Fragment-1 region, consistent with the Ca^{2+} requirement for phospholipid binding (17, 18).

Fragment-2, the COOH-terminal region of the propiece, is required for acceleration of prothrombin activation by Factor V_a (19).

The amino acid composition of prothrombin, Intermediate-1 (I-1), and Fragment-1 are given in Table 1 (20).

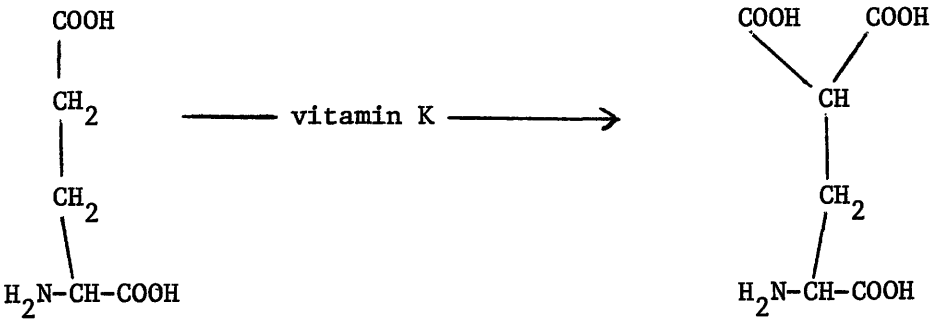
The Fragment-1 region

The Fragment-1 region is responsible for the Ca^{2+} mediated binding of prothrombin to phospholipid vesicle surfaces (16,18) and contains the modified glutamyl residues (21) which are necessary for normal Ca^{2+} binding by prothrombin (21-24). Carboxylation of 10 glutamyl to γ-carboxyglutamyl residues (23, 25) in the Fragment-1 region has been shown to occur as a consequence of the action of the vitamin K (26) and to be carried out postribosomally (26). Sequence analysis have indicated that these residues are found primarily in pairs (25, 27-29) and it has been proposed that the cluster of 4 carboxyl groups provided by this structure is responsible for Ca^{2+} binding by these proteins. Abnormal prothrombin (induced by the vitamin K antagonist, dicoumarol) lacks these modified glutamic acid residues (the only protein primary structure difference from normal prothrombin) and that is the reason why

Residue	Prothrombin		Intermediate 1		Fragment 1		I-1 + F-1[a]
	Micro-moles	Residues per mole	Micro-moles	Residues per mole	Micro-moles	Residues per mole	(Residues per mole)
Asp	0.222	62.0	0.130	46.1	0.180	16.2	62.3
Thr	0.102	28.6	0.0180	17.0	0.114	10.3	27.3
Ser	0.145	40.5	0.0770	27.3	0.158	14.2	41.5
Glu	0.263	73.6	0.138	49.0	0.259	23.4	72.4
Pro	0.115	32.2	0.0618	22.0	0.112	10.1	32.1
Gly	0.173	48.4	0.108	38.3	0.133	12.0	50.3
Ala	0.120	33.6	0.066	23.7	0.113	10.2	33.9
½Cys	0.070	19.6	0.033	12.0	0.101	9.1	21.1
Val	0.114	31.9	0.0637	22.6	0.101	9.1	31.7
Met	0.0187	5.2	0.0132	4.7	0.0109	0.98	5.7
Ile	0.0619	17.3	0.0386	13.7	0.0414	3.7	17.4
Leu	0.162	45.4	0.101	35.9	0.112	10.1	46.0
Tyr	0.0639	17.9	0.0394	14.0	0.0428	3.9	17.9
Phe	0.0683	19.1	0.0439	15.6	0.0437	3.9	19.5
Lys	0.110	30.8	0.0713	25.3	0.0558	5.0	30.3
His	0.0292	8.2	0.0170	6.0	0.0215	1.9	7.9
Arg	0.154	43.2	0.0810	28.8	0.155	14.0	42.8
Trp		18.6		14.6		4.8 (3)[b]	19.4
Gal + Man[c]		18.0		5.2		11.5	16.7
GlcN[d]	0.0338	9.4	0.0101	3.6	0.0706	6.4	10.0
AcNeu[e]		7.2 (8)[f]		3.2 (3)[f]		4.9 (5)[f]	9.1

TABLE I: Amino acid composition of bovine prothrombin and products of thrombin-catalyzed proteolysis of prothrombin (from reference 20)

abnormal prothrombin does not bind Ca^{2+} and is non-functioning in blood coagulation (18, 21, 30).



Some investigators have observed positive cooperativity in the binding of Ca^{2+} to prothrombin (23, 31), whereas others claim that exists two distinct classes of binding sites (17,32). Some authors (24,33) found that more than glutamate residue carboxylation is required for the normal Ca^{2+} and phospholipid binding by the vitamin K related proteins. The results of their experiments have led to the discovery that positive cooperativity in the Ca^{2+} binding to Fragment-1 depends upon having intact disulfide bridges (there are four disulfide bridges in Fragment-1) and, by inference, specific protein tertiary structure. These results do not eliminate the possibility that groups in the Fragment-1 other than the carboxyl groupd of γ-carboxyglutamic acid are involved in tight calcium binding and the detailed calcium-binding structure is still unknown.

Stenflo (34) did a structural comparison of normal and discoumarol-induced bovine prothrombin. He found that the two prothrombins have the same main antigenic determinants and that the sedimentation co-

efficients, the Stokes molecular radius, the tyrosine titration curves, and the fluorescence emission spectra were identical indicating that there is no gross difference involving the entire molecule. However, the quantitative immunological precipitin curves suggest that there is a conformational difference between normal prothrombin with and without calcium ions.

Investigation of the conformation of the normal and abnormal (dicoumarol-induced) prothrombins in the presence and absence of Ca^{2+} was reported using optical rotatory dispersion (ORD) and circular dichroism (CD) (35). Both techniques showed no conformational differences between the two proteins, in the absence of Ca^{2+}. A small α -helix content was found. But, normal prothrombin exhibits a marked change in the aromatic region of the spectrum on binding of Ca^{2+} ions, whereas no such conformational change was found in the case of the dicoumarol-induced protein. This suggests that the conformational change is local and occurs in the neighborhood of an aromatic amino acid (28).

An interesting comparison between a vitamin K dependent peptide from human prothrombin to the known corresponding bovine segment has shown that a number of residues which could be considered as the most important are the same (36). The 8 glutamic acid residues which have been proved to be modified in the bovine peptide are present in the human material as well as the two cysteine residues which are involved in a disulfide bridge in the bovine molecule. The main differences are the absence of proline in the human polypeptide and conversely the presence of valine and tyrosine which are absent in the bovine material (the amino

acid composition from reference 20 indicates the presence of 32 VAL and 18 TYR residues per mole of bovine prothrombin - see Table 1).

The ORD and CD results (37) indicate the existence of marked alterations in the secondary structure of Profragment-1 obtained from human prothrombin as a result of changes in hydrogen ion and calcium ion concentration. These results are consistent with the hypothesis that ionization of the γ-carboxyglutamyl side chain carboxyl groups, localized in a quite small region of Profragment-1 leads to considerable electrostatic repulsion resulting in a conformational change and a nonfunctional configuration near neutral pH. Addition of Ca^{2+} diminishes the electrostatic repulsive forces by charge neutralization and induces shifts to a conformation resembling the low pH form of Profragment-1.

Binding of protein to phospholipid through calcium ions, a function ascribed to γ- carboxyglutamic acid, can be fulfilled by other structures as evidenced by S-100 protein from brain which binds to phospholipid through calcium ion (38) but contains no γ-carboxyglutamic acid (39).

Preliminary investigations of Ca^{2+} binding to Fragment-1 by difference ultraviolet absorption spectroscopy indicates a significant environment change for tryptophanyl and possibly tyrosyl chromophores during Ca^{2+} binding (Peng and Jackson, unpublished observation).

We decided to study the binding of Ca^{2+} to Fragment-1, using Raman spectroscopy. We hoped to see the effect of binding in the carbonyl stretching frequence (1600-1800 cm^{-1} region of the spectrum) and possible conformational changes. Also the pH and ionic strength dependence could be investigated.

MATERIAL AND METHODS

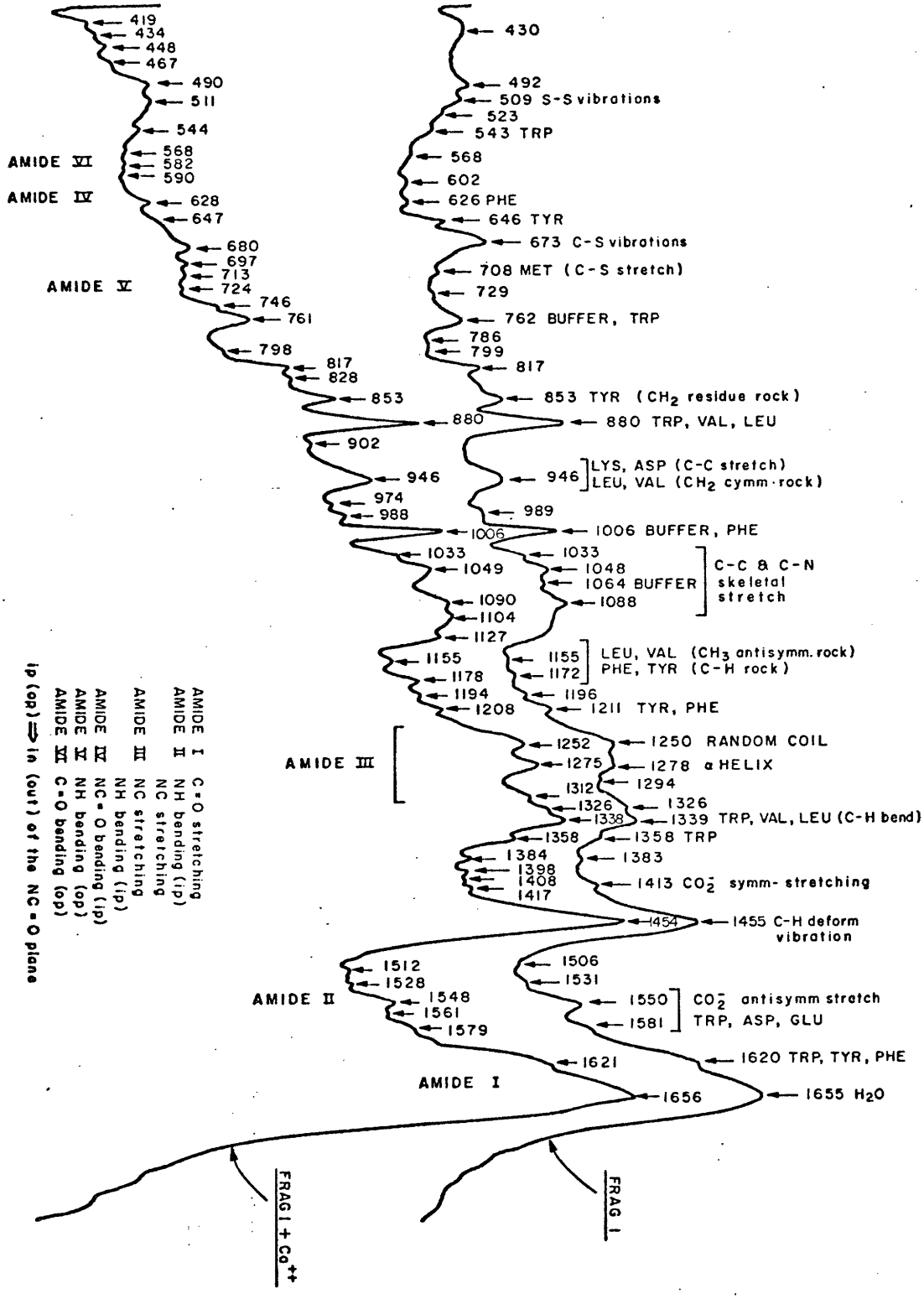
Fragment-1 samples were provided by Dr. Craig Jackson from Washington University. Purification of prothrombin and isolation of Fragment-1 is described in reference 20. Bovine Fragment-1 was used at a concentration of 60 mg/ml in Tris-buffer, with (10 mM) or without Ca^{2+}. The Raman system used was improved with a computer and accumulation and manipulation of the data made it possible to increase the signal: noise ratio.

RESULTS AND DISCUSSION

The spectra of buffer, Fragment-1 without Ca^{2+}, and Fragment-1 plus Ca^{2+} is shown. These samples were made in water and the carbonyl stretching frequency (1600-1800 cm^{-1}) is not accessible due to the water peak. Samples in D_2O are necessary to study this region.

There are substancial frequency differences between the Raman spectrum of Fragment-1 and Fragment-1 plus Ca^{2+} which may represent real conformational differences. The disulfide bonds vibration is seen, in both spectra, around 509 cm^{-1}, and the presence of these bonds is confirmed for both samples. The α-helix and random coil contents shown by the peaks around 1275 and 1250 cm^{-1} respectively seem to be different for the two spectra. The region around 1400 cm^{-1} shows also dissimilarities. In this region is the CO_2^- stretching vibration frequency and the binding of Ca^{2+} to the γ-carboxyglutamyl residues is supposed to cause variation in this frequency. It was not possible to decide if there is or if there is not an environmental change for tryptophanyl and tyrosyl residues upon Ca^{2+} binding. The vibrations of the aromatic amino acids appear in various regions of the spectrum. One of these frequencies is not the same in the two spectra. The C-H rocking vibration appears at 1172 cm^{-1} for Fragment-1, and at 1178 cm^{-1} for Fragment-1 plus Ca^{2+}, and this may indicate a different environment for tyrosyl or tryptophanyl residues.

We have to remember that the buffer spectrum is superimposed to the protein spectrum, and it would have to be subtracted from the overall


419
434
448
467
490
511
544
AMIDE VI
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582
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AMIDE IV
628
647
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AMIDE V
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746
761
798
817
828
853
880
902
946
974
988
1006
1033
1049
1090
1104
1127
1155
1178
1194
1208
AMIDE III
1252
1275
1312
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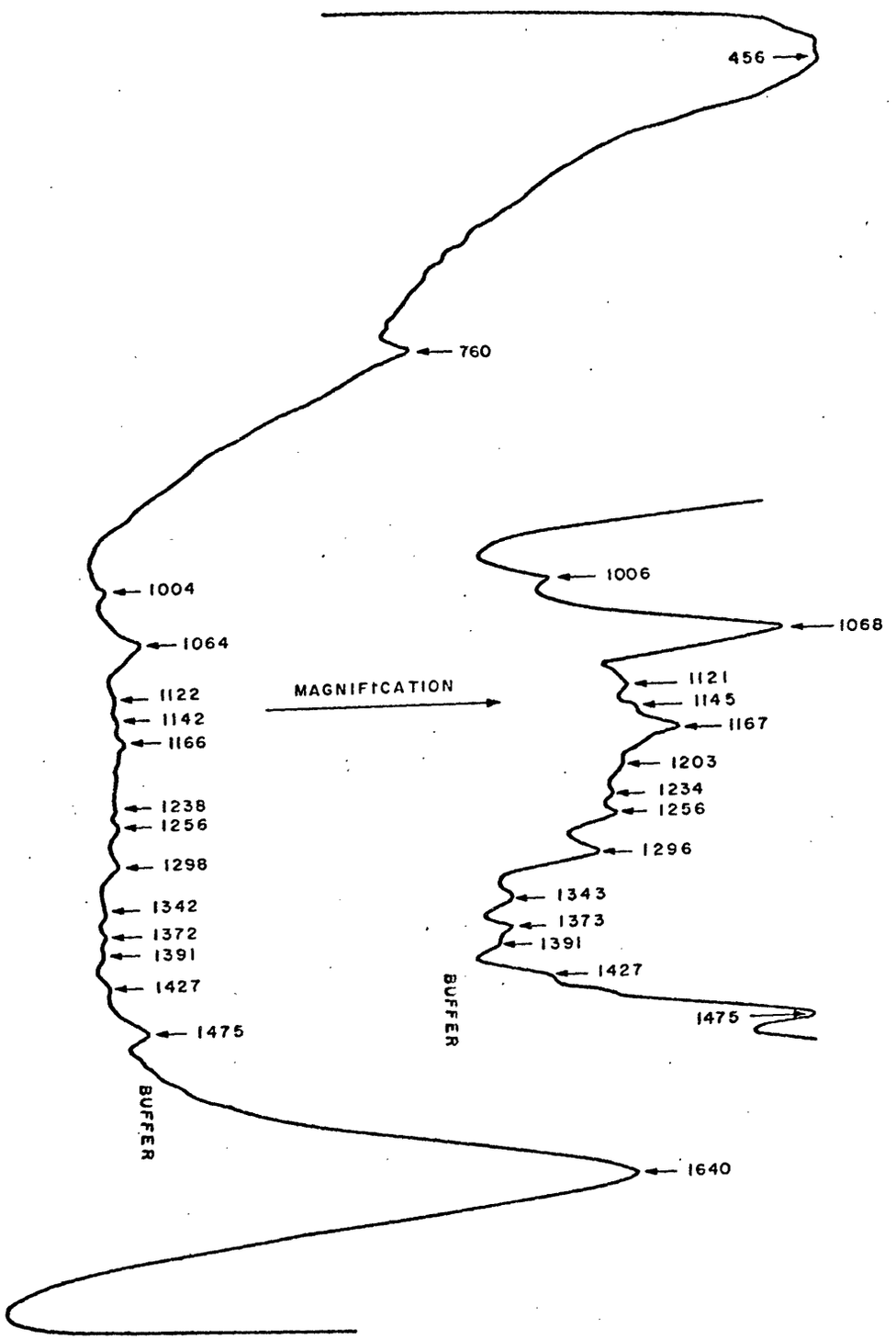
1338
1358
1384
1398
1408
1417
1454
1512
1528
AMIDE II
1548
1561
1579
1621
AMIDE I
1656
430
492
509 S-S vibrations
523
543 TRP
568
602
626 PHE
646 TYR
673 C-S vibrations
708 MET (C-S stretch)
729
762 BUFFER, TRP
786
799
817
853 TYR (CH2 residue rock)
880 TRP, VAL, LEU
946
LYS, ASP (C-C stretch)
LEU, VAL (CH2 cymm·rock)
989
1006 BUFFER, PHE
1033
1048
1064 BUFFER
1088
C-C & C-N skeletal stretch
1155
1172
LEU, VAL (CH3 antisymm. rock)
PHE, TYR (C-H rock)
1196
1211 TYR, PHE
1250 RANDOM COIL
1278 α HELIX
1294
1339 TRP, VAL, LEU (C-H bend)
1358 TRP
1383
1413 CO2- symm- stretching
1455 C-H deform vibration
1506
1531
1550
1581
CO2- antisymm stretch
TRP, ASP, GLU
1620 TRP, TYR, PHE
1655 H2O
AMIDE I C=O stretching
AMIDE II NH bending (ip)
NC stretching
AMIDE III NC stretching
NH bending (ip)
AMIDE IV NC=O bending (ip)
AMIDE V NH bending (op)
AMIDE VI C=O bending (op)
ip (or) ⇒ in (out) of the NC=O plane
FRAG I + Ca++
FRAG I


456
760
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1122
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BUFFER
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MAGNIFICATION
1006
1068
1121
1145
1167
1203
1234
1256
1296
1343
1373
1391
1427
1475
BUFFER

spectrum for a quantitative analysis of the data.

The next step would be to try a set of samples in D_2O, so that the water peak at 1655 cm^{-1} would be removed and this region analysed.

REFERENCES

1. Miller, K.D. and Seegers, W.H. (1956) Arch. Biochem. Biophys. 60, 398

2. Magnusson, S. (1965) Ark. Kemi 23, 285

3. Nelsestuen, G.L. and Suttie, J.W. (1972) J. Biol. Chem. 247, 6096

4. Harmison, C.R.; Landaburu, R.H. and Seegers, W.H. (1961) J. Biol. Chem. 236, 1693

5. Tishkoff, G.H.; Williams, L.C. and Brown, D.M. (1968) J. Biol. Chem. 243, 4151

6. Ingwall, J.S. and Scheraga, H.A. (1969) Biochemistry 8, 1860

7. Cox, A.C. and Hanahan, D.J. (1970) Biochim. Biophys. Acta 207, 49

8. Thomas, W.R. and Seegers, W.H. (1960) Biochim. Biophys. Acta 42, 556

9. Magnusson, S. (1970) Thromb. Diath. Haemorrh. 38 (suppl), 97

10. Bailey, K. and Bettelheim, F.R. (1955) Biochim. Biophys. Acta 18, 495

11. Laki, K; Gladner, J.A.; Folk, J.E. and Kominz, D.R. (1958) Thromb. Diath. Haemorrh. 2, 205

12. Baughman, D.J. and Waugh, D.F. (1967) J. Biol. Chem. 242, 5252

13. Winzor, D.J. and Scheraga, H.A. (1964) Arch. Biochem. Biophys. 104, 202

14. Esmon, C.T.; Owen, W.G. and Jackson, C.M. (1974) J. Biol. Chem. 249, 606

15. Stenn, K.S. and Blout, E.R. (1972) Biochemistry 11, 4502

16. Gitel, S.N.; Owen, W.G.; Esmon, C.T. and Jackson, C.M. (1973) Proc. Nat. Acad. Sci. USA 70, 1344

17. Benson, B.; Kisiel, W. and Hanahan, D.J. (1973) Biochim. Biophys. Acta 329, 81

18. Stenflo, J. (1973) J. Biol. Chem. 248, 6325

19. Esmon, C.T. and Jackson, C.M. (1974) J. Biol. Chem. 249, 7782

20. Owen, W.G.; Esmon, C.T. and Jackson, C.M. (1974) J. Biol. Chem. 249, 594

21. Stenflo, J; Fernlund, P; Egan, W. and Roepstorff, P. (1974) Proc. Nat. Acad. Sci. USA 71, 2730

22. Nelsestuen, G.L.; Zytkovicz, T.H. and Howard, J.B. (1974) J. Biol. Chem. 249, 6347

23. Stenflo, J. and Ganrot, P.O. (1973) Biochem. Biophys. Res. Commun. 50, 98

24. Nelsestuen, G.L. and Suttie, J.W. (1972) Biochemistry 11, 4961

25. Magnusson, S; Sottrup-Hensen, L. and Peterson, T.W. (1974) FEBS Lett. 44, 189

26. Shah, D.V. and Suttie, J. (1974) Biochem. Biophys. Res. Commun. 60, 1397

27. Howard, J.B. and Nelsestuen, G.L. (1975) Proc. Nat. Acad. Sci. USA 72, 1281

28. Stenflo, J. (1974) J. Biol. Chem. 249, 5527

29. Enfield, D.L.; Ericsson, L.H.; Walsh, K.A.; Neurath, H. and Titani, K. (1975) Proc. Nat. Acad. Sci. USA 72, 16

30. Nelsestuen, G. and Suttie, J. (1972) J. Biol. Chem. 247, 8;76

31. Mann, K.G.; Bajaj, S.P.; Heldebrant, C.M.; Butkowski, R.J. and Fass, D.N. (1973) Ser. Haemat. 6, 479

32. Yue, R.H.; Starr, T. and Gertler, M.M. (1972) Fed. Proc. 31, 241

33. Henriksen, R.A. and Jackson, C.M. (1975) Arch. Biochem. Biophys. 170, 149

34. Stenflo, J. (1972) J. Biol. Chem. 247, 8167

35. Bjork, J. and Stenflo, J. (1973) FEBS Lett. 32, 343

36. Ellion, J.; Bernarous, R. and Labie, D. (1976) Thrombos. Haemostas. 35, 82

37. Gabriel, D.A. (1975) Thromb. Res. 7, 839

38. Calissano, P.; Alema, S. and Fasella, P. (1974) Biochem. 13, 4553

39. Nelsestuen, G.L.; Broderius, M.; Zytkovicz, T.H. and Howard, J.B. (1975) Biochem. Biophys. Res. Commun. 65, 233