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Methods in Enzymology

Volume XXVI

Enzyme Structure

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[17] The Strength of Hydrogen Bonding: Infrared Spectroscopy

By H. SUSI

General Background

Infrared spectroscopy has been widely used for detection of H-bonding, estimating H-bond characteristics from frequency shifts of specific infrared bands, and determining changes of thermodynamic parameters associated with H-bond formation.¹ Most investigations have involved infrared bands associated with covalent groupings capable of H-bond formation, such as $>N-H$, $-O-H$, and $>C=O$. Because of experimental difficulties, much less has been accomplished by investigating absorption bands associated with the hydrogen bond itself. (These bands occur at very low frequencies, $\sim 10-200\text{ cm}^{-1}$, beyond the range of most commercial instruments.)

Groupings capable of H-bonding generally give rise to infrared bands associated with bond stretching vibrations and bands associated with bond bending vibrations.² The stretching modes of a given grouping are observed at higher frequencies than the bending modes. $N-H$ and $O-H$ stretching modes absorb between ~ 3000 and $\sim 3500\text{ cm}^{-1}$, bending modes between ~ 1000 and $\sim 1600\text{ cm}^{-1}$.³ For $C=O$ groups the corresponding ranges are $\sim 1600-1800$ and $\sim 400-800\text{ cm}^{-1}$, respectively. There are several types of spectral changes associated with H-bonding: changes in band positions (frequencies), changes in band intensities, and changes in band shapes.

Changes in band positions have received most attention. It is a well substantiated observation that the frequencies of stretching vibrations are decreased and the frequencies of bending vibrations are increased by H-bond formation.^{1,3} The shifts are generally more pronounced for stretching modes. Shifts involving proton donors such as $O-H$ and $N-H$ are usually larger than shifts involving proton acceptors, such as $C=O$.¹ The majority of infrared investigations have therefore been

¹G. C. Pimentel and A. L. McClellan, "The Hydrogen Bond." Freeman, San Francisco, California, 1960.

²E. Herzberg, "Infrared and Raman Spectra of Polyatomic Molecules," p. 195. Van Nostrand, Princeton, New Jersey, 1945.

³L. J. Bellamy, "The Infrared Spectra of Complex Molecules," 2nd ed. Wiley, New York, 1958.

concerned with frequency shifts in the 3000–3500 cm^{-1} regions where O—H and N—H stretching modes are observed.

In biological macromolecules, including enzymes, H-bonding usually involves O—H or N—H groups as proton donors. In the past few years it has been suggested that C—H...O hydrogen bonding might also occur in some instances.^{4,5} Careful infrared studies indicate that the stretching⁶ as well as bending⁷ vibrations of H-bonded C—H linkages undergo changes which are quite analogous (although smaller) than corresponding changes in O—H and N—H bands.

General Correlations of Infrared Spectra with Hydrogen Bonding

The frequency shifts of N—H and O—H stretching bands (~ 3000 – 3500 cm^{-1}), commonly designated $\Delta\nu(\text{XH})$, have been correlated with various structural and physical parameters of H-bonds, such as the enthalpy change, ΔH , associated with H-bond formation⁸; the distance R between heavy atoms (e.g., O...N in O...N—H)^{9,10}; and the XH distance r .¹¹ As a general rule $\Delta\nu(\text{XH})$ increases with $(-\Delta H)$, decreases with R , and increases with r . Some early studies suggested that $\Delta\nu(\text{XH})$ might be a linear function of ΔH , but later investigations showed that this is not the case.¹ Many data are available on the dependence of $\Delta\nu(\text{XH})$ on R . A relationship of the form

$$\Delta\nu(\text{XH}) = a(b - R)$$

has been proposed, where a and b are constants which are different for each type of H-bond.¹⁰ The formula is in good agreement with experiment, provided that the value of R is not too high.¹ The relationship between $\Delta\nu(\text{XH})$ and r is more qualitative, partly because of the lack of appropriate data. None of the three correlations is precise enough to determine accurate values for R , r , or ΔH . They are useful in making qualitative judgments, especially for relative values of related systems.

Intensity variations have been studied in much less detail than frequency shifts, primarily because of experimental difficulties. (By intensity we mean an integrated value for a given band under unit con-

⁴ G. N. Ramachandran, V. Sasisekharan, and C. Ramakrishnan, *Biochim. Biophys. Acta* **112**, 168 (1966).

⁵ G. N. Ramachandran and R. Chandrasekharan, *Biopolymers* **6**, 1649 (1968).

⁶ S. Krimm and K. Kuroiwa, *Biopolymers* **6**, 401 (1968).

⁷ H. Susi, J. S. Ard, and R. J. Carroll, *Biopolymers* **10**, 1597 (1971).

⁸ R. M. Badger and S. H. Bauer, *J. Chem. Phys.* **5**, 839 (1937).

⁹ R. C. Lord and R. E. Merrifield, *J. Chem. Phys.* **21**, 166 (1953).

¹⁰ G. C. Pimentel and C. H. Sederholm, *J. Chem. Phys.* **24**, 639 (1956).

¹¹ K. Nakamoto, M. Margoshes, and R. E. Rundle, *J. Amer. Chem. Soc.* **77**, 6480 (1955).

ditions of path length and concentration.) The intensity of stretching vibrations generally increases considerably with H-bond formation. Diagnostically this increase is as important as frequency shifts, although by far not as well understood.

Changes in band shape with H-bond formation involve a substantial broadening of O—H and N—H stretching bands. H-bonding can frequently be instantly recognized by this band broadening. The phenomenon is not thoroughly understood, although several theoretical explanations have been offered.^{1,12}

In addition to changes in infrared bands which are associated with groups involved in H-bond formation (such as N—H, O—H, C=O), the H-bonds themselves give rise to new bands associated with stretching and bending modes of these bonds. They are observed at frequencies below 200 cm^{-1} and have not been extensively studied because of experimental difficulties. The stretching vibration of the C=O...H—N hydrogen bond absorbs close to 100 cm^{-1} and the bending modes are expected at frequencies ranging from a few cm^{-1} to $\sim 150 \text{ cm}^{-1}$.¹³ These vibrations are directly related to the H-bonds themselves, and a considerable amount of new information can be expected as low-frequency instruments become more widely available.

Finally, the usefulness of overtone and combination bands should be briefly mentioned. These arise from transitions involving more than one quantum of vibrational energy and are much weaker than the previously mentioned fundamental stretching and bending modes. In general, their frequencies and intensities cannot be correlated with hydrogen bonding in a way similar to the fundamental modes. The bands are nonetheless very useful for the determination of thermodynamic parameters (see below).

Hydrogen Bonding and Characteristic Amide Bands

The amide A band (essentially N—H stretching, $\sim 3000\text{--}3500 \text{ cm}^{-1}$ cf. this volume [22]) is most frequently used for hydrogen bonding studies. The "free" N—H stretching fundamental absorbs between 3400 and 3460 cm^{-1} . A corresponding mode for strongly hydrogen-bonded amide groups should, on the basis of theoretical calculations,¹⁴ absorb around 3180 cm^{-1} . No band is observed at this frequency in strongly hydrogen-bonded polypeptides and proteins. Instead, bands are found at $\sim 3100 \text{ cm}^{-1}$ (weak) and 3300 cm^{-1} (strong). This is caused by an

¹²H. Susi, in "Structure and Stability of Biological Macromolecules" (S. N. Timasheff and G. O. Fasman, eds.), Vol. 2. Dekker, New York, 1969.

¹³K. Itoh and T. Shimanouchi, *Biopolymers* 5, 921 (1967).

¹⁴T. Miyazawa, *J. Mol. Spectrosc.* 4, 168 (1960).

interaction (Fermi resonance) between the N—H stretching band and the overtone $2 \times$ N—H bending. The new bands are called amide A and amide B. Neither one can be used in a straightforward manner for estimating bond lengths and enthalpies as discussed in the previous section. Empirical correlations are nevertheless possible.¹⁵ The bands are also useful for thermodynamic calculations where the only requirement is that spectra of different species differ sufficiently to permit determinations of the concentration of "free" and bonded groups (see below). The amide A band has also been frequently used for estimating the direction of hydrogen bonds by methods of polarized infrared spectroscopy. Because of various factors, such estimations are necessarily of a qualitative nature.¹² (One must consider that the band is not a "pure" N—H stretching vibration.) Such measurements are, nevertheless, quite useful for qualitative purposes. Thus, in an α -helix the skeletal H-bonds are essentially parallel to the main axis and so is the polarization of the amide A band (see this volume [22]).

The amide I band (C=O stretching) also moves downscale with hydrogen bonding. Free groups absorb at 1670–1700 cm^{-1} , hydrogen-bonded groups at 1630–1680 cm^{-1} . The fine structure and precise frequency of bands associated with hydrogen bonded groups depends on the secondary structure of the protein (see this volume [22]).

The amide II and III bands (N—H bending, C—N bending) move to slightly higher frequencies with hydrogen bonding as expected for bending modes. In the spectra of hydrogen-bonded species they are observed at ~ 1510 – 1570 cm^{-1} and ~ 1230 – 1300 cm^{-1} , respectively. The fine structure and precise frequencies again depend on the secondary structure (see this volume [22]).

Of the remaining bands only the amide V band (out-of-plane N—H bending, ~ 550 – 700 cm^{-1}) has been studied in any detail.¹⁶ The precise frequency is very sensitive to the secondary structure.

Qualitative Methods of Measurement

By qualitative infrared spectra we mean spectra which yield correct frequency values, but not intensities (absorbance values). The techniques for obtaining such spectra are quite similar to the ones described in this volume [22], last section. Mulls, pressed disks, or cast films are used if solid samples are investigated.

The pressed disk technique is in many ways the most convenient

¹⁵ J. A. Pullin and R. L. Werner, *Spectrochim. Acta* **21**, 1257 (1965).

¹⁶ T. Miyazawa, K. Fukushima, S. Sugano, and Y. Masuda, in "Conformation of Biopolymers" (G. N. Ramachandran, ed.). Academic Press, New York, 1967.

one. A very small amount of sample is required; there are no problems caused by the spectrum of the mulling agent; the sample is easily mounted in the sample chamber of the instrument. However, it should be pointed out that the use of pressed disks in an alkali halide matrix involves certain risks which are not present when other techniques are used. The sample is usually vigorously ground and then exposed to very high pressure in a liquid alkali halide environment. Conformational changes as well as changes in the environment of various outer groups can easily take place under these conditions, resulting in a spectrum which is not characteristic of the original material.

Spectra obtained with the help of a "mulling agent" (usually a high molecular weight hydrocarbon called Nujol) do not involve the above-mentioned difficulties, although the spectrum of the mulling agent is superimposed on the spectrum of the sample. Fortunately, the spectral regions where the most important amide bands absorb (~ 3000 – 3500 amide A and B; ~ 1500 – 1700 amide I and II; ~ 550 – 700 amide V; cf. this volume [22]) are free from Nujol absorption.

The thin film technique is limited to samples which are soluble in proper solvents and which do not undergo conformational changes while being dissolved and cast on an infrared transparent plate. It is the only technique suitable for the preparation of oriented samples which are necessary for polarization measurements.

To estimate the direction of hydrogen bonds by determining the approximate direction of the amide A dipole moment change, oriented samples and polarized radiation are used as described in this volume [22].

Solution spectra of proteins and enzymes are rarely obtained. If it becomes necessary to measure such spectra, the most important consideration is solvent absorption. It is necessary to work in a spectral region where the solvent has a "window." Usually model compounds are used to estimate the characteristics of hydrogen bonds in solution. Details are given in the section Quantitative Methods of Measurement.

Thermodynamics of Hydrogen Bonding

In thermodynamic investigations infrared spectroscopy serves essentially as an analytical tool. If A and B are two molecular species (or groups) which can form hydrogen bonds according to the schematic reaction



the thermodynamic parameters associated with hydrogen bonding can be determined if the concentrations of A, B and A-B can be measured

as a function of overall concentration and temperature. If the spectra of A, B and A-B differ sufficiently, the concentrations of these species can be determined by measuring the absorbance of characteristic absorption bands and by applying Beer's law to determine the concentration of individual species. By classical thermodynamics,

$$K_x = \frac{X_{A-B}}{X_A \cdot X_B} [\text{mole fraction}]^{-1}$$

where K_x is the equilibrium constant in terms of mole fractions.

The standard free energy change, ΔF° is given by

$$\Delta F^\circ = -RT \ln K_x$$

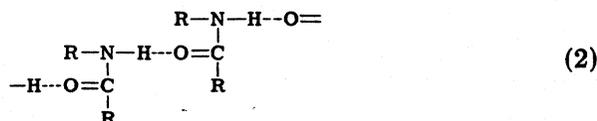
the enthalpy change by

$$\frac{d \ln K}{dT} = \frac{\Delta H^\circ}{RT^2}$$

and the standard entropy change by

$$\Delta S^\circ = (1/T)(\Delta H^\circ - \Delta F^\circ)$$

It should be noted that equilibrium constants in terms of molarities can be used only in very dilute solution, where the mole fraction of the solvent can be regarded as constant, regardless of the association or dissociation of the solute. Because reactions of the type (1) are not easily observed with macromolecular species, thermodynamic calculations are usually based on equilibria between small model compounds. Two types of model compounds have been used to investigate the kind of hydrogen bonding which occurs in proteins, i.e., bonding between N-H and C=O groups of the polypeptide repeat unit: small trans secondary amides such as *N*-methyl acetamide, and cyclic cis amides such as δ -valerolactam. The former have the advantage that the trans configuration is more closely related to the polypeptide groups of proteins. Unfortunately, actual calculations are complicated because of the formation of chain polymers of the type:



Multiple equilibria have to be considered which involve a number of equilibrium constants $K_2, K_3, K_4, \dots, K_n$.^{17,18} The dimerization constant

¹⁷ M. Tsuboi, *Nippon Kagaku Zosshi* **756**, 376 (1955).

¹⁸ I. M. Klotz and J. S. Franzen, *J. Amer. Chem. Soc.* **84**, 3461 (1962).

K_2 and the characteristic absorbance values of the monomer have to be determined by extrapolation to zero concentration. Cis amides are not as closely related to polypeptides, but their investigation is greatly simplified because only monomeric species and dimers of the type



have to be considered. Furthermore, it is possible to determine the absorbance of monomeric species without extrapolation, provided that an absorption band exists which does not overlap with the spectrum of the dimer. For a dimerization reaction¹⁹

$$K_x = \frac{C - M}{2M^2} \left(\frac{C + M}{2} + C_s \right)$$

$$M = A/\epsilon d$$

$$K_x = \frac{C - A/\epsilon d}{2(A/\epsilon d)^2} \left(\frac{C + A/\epsilon d}{2} + C_s \right) \quad (4)$$

where K_x is the dimerization constant in mole fraction units, C the total solute concentration in moles per liter, C_s the solvent concentration in moles per liter, M the monomer concentration in moles per liter, A the absorbance of monomers, ϵ the absorptivity of monomers, and d the path length.

Rearrangement leads to

$$C_s/A = (\epsilon d/A^2)(C^2/2 + C_s C) - (4K_x + 1)/(2\epsilon d)$$

The unknowns K_x and ϵ are determined by setting

$$Y = C_s/A \quad (5a)$$

$$X = (1/A^2)(C^2/a + C_s C) \quad (5b)$$

and plotting Y vs. X . A straight line is formed. ϵd is given by the slope, K_x is calculated from the intercept.

If model compounds of the type (2) are employed, Eqs. (4) and (5) are valid only in very dilute solution where only monomers and dimers are observed. In practice, the unknowns ϵ and K_x are then evaluated by extrapolation to infinite dilution. The equations are simplified because C_s can be taken as a constant. Alternatively, in very dilute solutions a standard state in terms of molarities can be used. In this case

¹⁹ H. Susi, S. N. Timasheff, and J. S. Ard, *J. Biol. Chem.* **239**, 3071 (1964).

$$K_c = \frac{[C_D]}{[C_M]^2} \text{ [liter} \cdot \text{moles]} \quad (6)$$

$$C_D = A_D/\epsilon_D d; C_M = C - 2C_D \quad (7)$$

$$C_M = A_M/\epsilon_M d; C_D = (1/2)(C - C_M) \quad (8)$$

where C_M is the molarity of monomers, C_D the molarity of dimers, and C the overall molarity in terms of monomers. Equations (6) and (7) are used if the measured quantity is dimer absorbance; Eqs. (6) and (8) if the monomer absorbance is monitored.

To obtain the experimental quantities A_D and/or A_M , absorbance measurements are usually carried out in the fundamental N—H stretching region ($\sim 3000\text{--}3500\text{ cm}^{-1}$) or in the first overtone region of N—H fundamentals ($\sim 6000\text{--}7500\text{ cm}^{-1}$).^{18,19}

In the fundamental region both monomer and dimer (or polymer) absorption is observed for cis and trans secondary amides.¹ In the first overtone region only monomeric cis amides absorb, whereas trans amides show both monomer and dimer absorption. Either the fundamental or the overtone region can be used for measurements in solvents with no O—H groups, such as CCl_4 , HCCl_3 , or dioxane. If the solvent contains O—H groups (water or alcohols), the overtone region must be used because the solvent interferes too much in the fundamental region to make meaningful measurements possible.^{18,19}

No matter which spectral region is chosen, the following considerations must be taken into account to obtain meaningful results.¹²: (a) For systems of type (2), multiple equilibria involving H-bonded chain polymers can occur. (b) End groups such as the "free" N—H grouping of the schematic dimer $\text{O}=\text{CR}-\text{RN}-\text{H} \cdots \text{O}=\text{CR}-\text{RN}-\text{H}$ absorb at frequencies very close to monomer absorption. (c) Absorption bands of monomeric and polymeric species frequently overlap. (d) The absorptivity ϵ of a given species might change with temperature.

SOLUTION AND CELL CHARACTERISTICS OF THE MODEL δ -VALEROLACTAM AS
STUDIED IN THE $6000\text{--}7500\text{ cm}^{-1}$ REGION^{a,b}

Solvent	Path length (mm)	Concentration range (moles/liter)	Association constant, K_x
Water	2	4-9	0.77
Dioxane	25	0.1-0.8	4.1
Chloroform	50	0.02-0.4	28
Carbon tetrachloride	100	0.005-0.03	1180

^a H. Susi, S. N. Timasheff, and J. S. Ard, *J. Biol. Chem.* **239**, 3071 (1964); and H. Susi and J. S. Ard, *Arch. Biochem. Biophys.* **117**, 147 (1966).

^b Cell windows: quartz. Temperature range: $25\text{--}65^\circ$.

(e) Solvent absorption can seriously interfere with the absorption of dissolved species.

Quantitative Methods of Measurement

To obtain spectra which yield reliable absorbance and absorptivity values, solutions of known concentration must be investigated in cells of known path length and controlled temperature. Temperature control is usually achieved by circulating water from a constant temperature bath through a jacket which is an integral part of the absorption

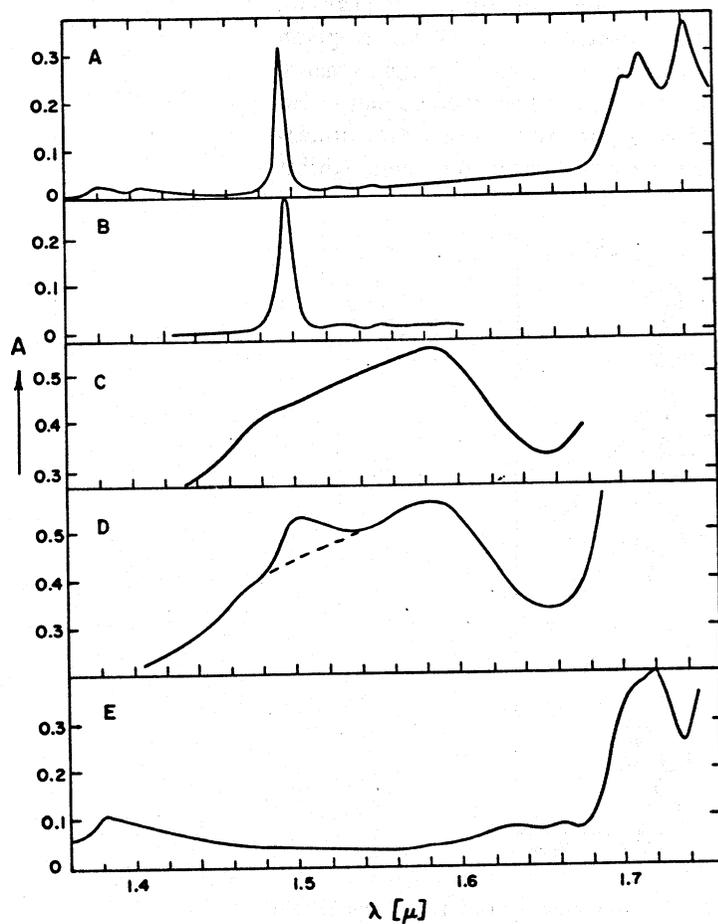


FIG. 1. The first overtone of "free" N—H stretching vibrations of the model compound δ -valerolactan in (A) CCl_4 , (B) HCCl_3 , (D) MeOH. (C) Pure MeOH for comparison. (E) Pure lactam (all dimerized).

cell.¹⁷⁻¹⁹ The temperature is measured by a thermocouple inserted in the solution outside the path of the radiation beam. The concentration should be chosen so as to cover a range where the association coefficient shows a marked change. Thus, in CCl_4 solution (high association) very high dilution must be employed, whereas in H_2O solution no marked association is observed before very high concentrations are reached. The table illustrates the choice of concentration range and path length for the model compound δ -valerolactam as studied in the $6000\text{--}7500\text{ cm}^{-1}$ range (the first overtone region). Other typical values are found in references cited in footnotes 17-20.

To obtain quantitative values of monomer or dimer absorption, it is desirable to choose characteristic absorption bands for a given species which do not overlap with absorption bands of other species. Figure 1 shows the first overtone of the monomeric model compound δ -valerolactam in various solvents and as a pure substance (total dimerization). The bottom curve shows that dimeric lactam does not exhibit

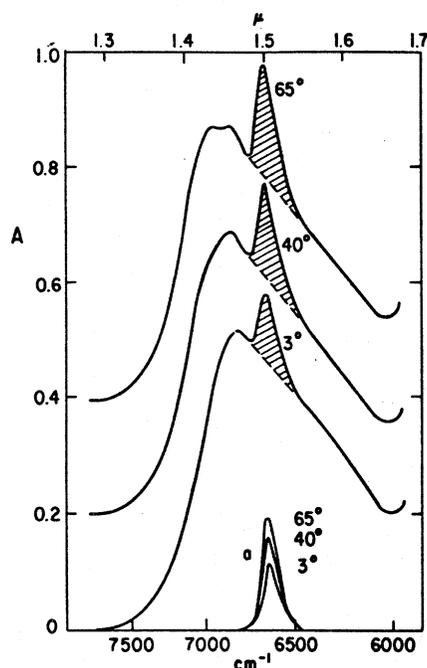


FIG. 2. The first overtone of "free" N—H stretching vibrations of the model compound δ -valerolactam in H_2O solution as a function of temperature. Absorbance measured by the baseline method. (a) Estimated amide absorption.

²⁰ H. Susi and J. S. Ard, *Arch. Biochem. Biophys.* **117**, 147 (1966).

an absorption band in the vicinity of 1.5μ ($\sim 6670 \text{ cm}^{-1}$) and that the band in curves A, B, and D must be assigned to the monomer. Absorbance values are measured by the baseline method (cf. this volume [22]) as illustrated in Fig. 1D and in Fig. 2. Figure 2 also shows the change of amide absorbance with temperature, as measured by the baseline method. If baseline absorbance values as obtained from Fig. 2 are inserted in Eq. (4) and a plot of Y vs. X is formed according to Eq. (5) at various temperatures, a graph is obtained¹⁹ which leads to $\epsilon = 0.120 M^{-1} \text{ cm}^{-1}$, $K_{x,298} = 0.75 \text{ mf}^{-1}$ and $\Delta H^\circ = -5500 \text{ cal/mole}$ for the dimerization of δ -valerolactam in aqueous solution. Since two hydrogen bonds are formed, the enthalpy change per bond is ca. 2750 cal/mole. It should be emphasized that this is the enthalpy change for the overall reaction in aqueous solution, involving solute-solute, solute-solvent, and solvent-solvent interactions.¹⁹

In general, the following practical rules have been proposed for infrared measurements for obtaining information on hydrogen bonding¹:

- (a) The solvent should be selected with attention to its H-bonding properties.
- (b) The concentration should be varied to determine the sensitivity of the spectrum to this variable. If possible, sufficiently low concentrations should be used to permit control of intermolecular association.
- (c) The temperature should be rigorously controlled.
- (d) Solvent, temperature, and concentration should be specified in publications.
- (e) The cell temperature should be varied to detect and identify H-bonded species.
- (f) Assignments should be verified by deuterium substitution.
- (g) The solvent should be rigorously dried.
- (h) Alcohol (added as an antioxidant) should be removed from chloroform solutions.
- (i) Solid samples should be annealed.
- (j) Care should be exercised in using KBr pellet spectra.