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Methods in Enzymology

Volume XXVI

Enzyme Structure

Part C

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13. Flow Birefringence	MICHIKI KASAI AND FUMIO OOSAWA	289
14. Electric Birefringence	LEO D. KAHN	323
15. Dielectric Dispersion Measurement of Dielectric Constant and Conductivity	SHIRO TAKASHIMO	337

Section IV. Conformational Stability

16. The Determination of Activity Coefficients from Distribution Measurements	DWIGHT R. ROBINSON	365
17. The Strength of Hydrogen Bonding: Infrared Spectroscopy	H. SUSI	381

Section V. Conformation and Transitions

18. Dilatometry	SAM KATZ	395
19. Hydrogen-Tritium Exchange	S. W. ENGLANDER AND J. J. ENGLANDER	406
20. Potentiometric Titrations and Conformational Change	GERTRUDE E. PERLMANN	413
21. Measurements at High Pressure	KEIZO SUZUKI	424

Section VI. Conformation: Optical Spectroscopy

22. Infrared Spectroscopy-Conformation	H. SUSI	455
23. Raman Spectroscopy	MARVIN C. TOBIN	473
24. Nanosecond Fluorescence Spectroscopy of Macromolecules	JUAN YGUERABIDE	498
25. Environmentally Sensitive Groups Attached to Proteins	M. E. KIRTLEY AND D. E. KOSHLAND, JR.	578

Section VII. Resonance Techniques: Conformation and Interactions

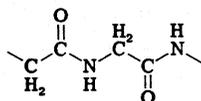
26. High-Resolution Proton Magnetic Resonance Spectroscopy of Selectively Deuterated Enzymes	JOHN L. MARKLEY	605
27. Preparation of Deuterated Proteins and Enzymes	HENRY L. CRESPI AND JOSEPH J. KATZ	627

[22] Infrared Spectroscopy—Conformation

By H. SUSI

General Background

The infrared spectra of high polymers are usually interpreted in terms of the vibrations of a structural repeat unit.¹ The approach is analogous to the treatment of vibrational spectra of molecular crystals.^{2,3} It has the physical implication that only those fundamental vibrations can be observed in infrared (or Raman) spectra in which corresponding atoms in all repeat units move in phase. Sometimes a further simplification is possible because interactions between chemically bonded atoms are in general much stronger than interchain interactions. It then becomes possible to study the vibrations of a single structural repeat unit of an isolated chain. It should be noted that even in the single-chain approximation, a structural repeat unit can contain more than one chemical repeat unit. Polyglycine I furnishes a simple example. The polymer has a simple pleated sheet structure ("β-structure").⁴ In the single-chain approximation the structural repeat unit ("the polymer molecule") is given by



in an all-trans zigzag conformation containing two molecular repeat units.⁵ Each molecular mode (e.g., the C=O stretching vibration) would, therefore, be split into two components. The two characteristic β-structure bands at approximately 1690 and 1630 cm⁻¹⁵ can indeed be interpreted on that basis. The two bands represent in-phase and out-of-phase stretching vibrations of the two carbonyl groups of the structural repeat unit (amide I mode). The true structural repeat unit of polyglycine I

¹ C. Y. Liang, S. Krimm, and G. B. B. M. Sutherland, *J. Chem. Phys.* **25**, 543, 549 (1956); C. Y. Liang and S. Krimm, *ibid.* **25**, 563 (1956).

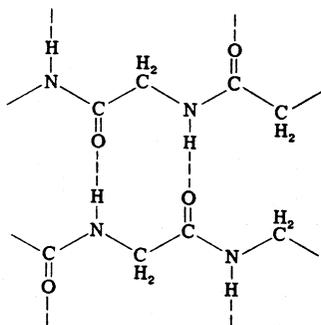
² D. F. Hornig, *J. Chem. Phys.* **16**, 1063 (1948).

³ H. Winston and R. S. Halford, *J. Chem. Phys.* **17**, 607 (1949).

⁴ C. H. Bamford, L. Brown, E. M. Cant, A. Elliott, W. E. Hanby, and B. R. Malcolm, *Nature (London)* **176**, 396 (1955).

⁵ K. Fukushima, Y. Ideguchi, and T. Miyazawa, *Bull. Chem. Soc. Jap.* **36**, 1301 (1963).

contains portions of two chains and a total of four chemical repeat units. The structure can be schematically presented as follows:



The true unit contains four peptide groups with each vibrational mode splitting into four branches. Three branches are infrared active (one of them very weak), and one is Raman active. Other polypeptide conformations, such as the α -helix and the parallel-chain pleated sheet can be treated in an analogous manner. Conformational studies of polypeptides are to a large degree based on the fine structure of absorption bands which results from the coupling between chemical repeat units within the structural repeat unit. A band will, in general, split into as many components as there are chemical repeat units in the structural unit. Some branches might not be infrared active and others might overlap or be too weak to be observed.⁶

Characteristic Amide Bands

The chemical polypeptide repeat unit gives rise to nine characteristic infrared absorption bands. The generally accepted nomenclature, the approximate frequencies, and the approximate descriptions of each mode are given in Table I.⁷ Some bands are more useful for conformation studies than others. The amide A, amide I, and amide II bands have been most frequently used for such investigations.^{6,8,9} More recently, the amide V band (which is beyond the reach of some older infrared spectrometers) has also received much attention.¹⁰ A typical spectrum of a

⁶ (a) H. Susi, S. N. Timasheff, and L. Stevens, *J. Biol. Chem.* **242**, 5460 (1967);

(b) S. N. Timasheff, H. Susi, and L. Stevens, *ibid.* **242**, 5467 (1967).

⁷ T. Miyazawa, T. Shimanouchi, and S. Mizushima, *J. Chem. Phys.* **24**, 408 (1956).

⁸ S. Krimm, *J. Mol. Biol.* **4**, 528 (1962).

⁹ E. R. Blout, C. de Lozé, and A. Asadourian, *J. Amer. Chem. Soc.* **83**, 1895 (1961).

¹⁰ T. Miyazawa, K. Fukushima, S. Sugano, and Y. Masuda, in "Conformation of Biopolymers" (G. N. Ramachandran, ed.), Academic Press, New York, 1967.

TABLE I
CHARACTERISTIC INFRARED BANDS OF THE PEPTIDE LINKAGE

Designation	Approximate ^a frequency (cm ⁻¹)	Description
A ⁻ B	~3300 } ~3100 }	NH stretching in resonance with (2 × amide II) overtone
I	1600-1690	C=O stretching
II	1480-1575	CN stretching, NH bending
III	1229-1301	CN stretching, NH bending
IV	625-767	OCN bending, mixed with other modes
V	640-800	Out-of-plane NH bending
VI	537-606	Out-of-plane C=O bending
VII	~200	Skeletal torsion

^a Based on model compounds. Data from T. Miyazawa, T. Shimanouchi, and S. Mizushima, *J. Chem. Phys.* **24**, 408 (1956).

secondary amide (*N*-methyl acetamide) which exhibits all characteristic amide bands above 400 cm⁻¹ is shown in Fig. 1. A small model compound was chosen as an illustration because some bands are not as easily observed in polypeptides and proteins. It should be pointed out that the characteristic amide bands are by no means the only ones observed either in simple model compounds or in proteins. In the spectra of model compounds, there are bands associated with CH, CN, and CC linkages. In polypeptides and proteins bands associated with side chains are observed. The bands listed in Table I are the ones primarily associated with the vital CONH grouping.

In general, a spectrum can be properly analyzed only if all absorption bands are taken into consideration because, strictly speaking, there are no isolated vibrations involving only particular groups of atoms. For complex molecules it is, nevertheless, frequently possible to draw con-

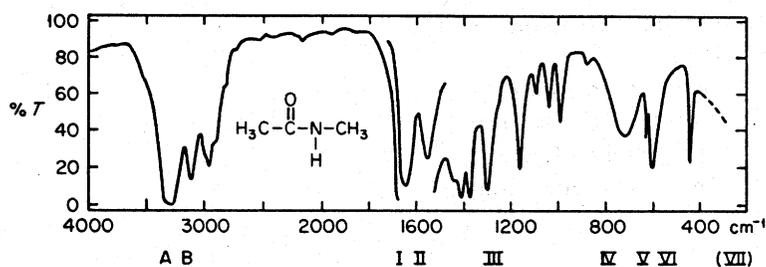


FIG. 1. Characteristic amide bands as exhibited by a capillary film of *N*-methyl acetamide. (Amide VII band not shown.)

clusions from the analyses of only a few selected bands if the corresponding vibrations can be regarded as "essentially localized," i.e., if they involve only a few atoms of a complicated overall structure. This is true for many of the listed characteristic amide bands.⁷

The amide A and B bands arise from the NH stretching vibration in resonance with the first overtone of the amide II (essentially NH bending) vibration.¹¹ These bands are very sensitive to hydrogen bonding and have been extensively utilized for investigations of hydrogen bonding in biological macromolecules and pertinent model compounds (see this volume [XVII]). In oriented samples the bands also show marked dichroism and can be utilized to estimate the direction of N—H bonds (see below).

The amide I and amide II bands split into components depending on the secondary structure of the molecules under investigation and have been most frequently used for conformational analysis. The fundamental theory was worked out by Miyazawa¹²; detailed applications have been described by Miyazawa and Blout,¹³ Krimm,⁸ Timasheff, Susi, and Stevens,⁶ and others. A more detailed review is given in reference cited in footnote 14. Although the fine structure of the amide I and amide II bands arising from various conformations results in principle in several branches for each conformation (two for the α -helix, three for the most common β -structure, i.e., the antiparallel-chain pleated sheet), many branches are too weak to be observed in practice. Table II lists the branches which are in fact observed under usual laboratory conditions for samples in the solid state. (Although solution work is of considerable importance, much less has been accomplished in solution than in the solid state because of experimental difficulties. A complete summary of solid state work is given by Krimm.⁸) In the solid state, the dichroism of the various branches of amide I and amide II bands is also of considerable importance, but care should be exercised in the interpretation of such measurements because no branch is polarized simply in the direction of any chemical bond of the CONH grouping.¹⁴ Oversimplified interpretation can lead to serious errors (see below).

Of the remaining amide bands, the amide V mode has received most attention.¹⁰ The mode is not "localized"; the nature of the vibration changes from one conformation to another. Partly because of this, the band is quite useful for conformational investigations. It occurs around

¹¹ T. Miyazawa, *J. Mol. Spectrosc.* **4**, 168 (1966).

¹² T. Miyazawa, *J. Chem. Phys.* **32**, 1647 (1960).

¹³ T. Miyazawa and E. R. Blout, *J. Amer. Chem. Soc.* **83**, 712 (1961).

¹⁴ H. Susi, in "Structure and Stability of Biological Macromolecules" (S. N. Timasheff and G. O. Fasman, eds.), Vol. 2. Dekker, New York, 1969.

INFRARED SPECTROSCOPY

TABLE II
 PROMINENT AMIDE I AND II BRANCHES FOR DIFFERENT CONFORMATIONS^a
 IN THE SOLID STATE

Conformation	Strongest amide I component	Strongest amide II component	Weak amide I component, ca. 1690 cm ⁻¹
Unordered	1658	1520	—
ACPS ^b	1632 ⊥	1530	1685
PCPS ^c	1632 ⊥	1530	—
Parallel-chain polar sheet ^d	1648 (⊥)	1550 ()	—
α-Helix	1650	1546 ⊥	—
Triple helix (polyglycine II)	1648 ⊥	1558	—

^a Calculated values [S. Krimm, *J. Mol. Biol.* **4**, 528 (1962)].

^b Antiparallel-chain pleated sheet.

^c Parallel-chain pleated sheet.

^d Approximate polarization; not determined by symmetry.

600 cm⁻¹ for the α-helical conformation, ca. 650 cm⁻¹ for unordered samples, and ca. 700 cm⁻¹ for the β-structure. In the interesting example of lysozyme, all three bands are observed.

The amide III, IV, VI, and VII modes have not been studied in as much detail, and little information is available regarding their dependence on conformation. This is partly caused by the low intensity of some of the bands and by the low frequency of others. (Amide VI and VII modes absorb beyond the range of some conventional spectrometers.)

Instrumentation

The problem of instrumentation can be divided into two categories: (a) the nature of the spectrometer, and (b) sample handling and sample preparation. Part (b) will be discussed further below in conjunction with particular techniques.

Present-day commercial instruments can be roughly divided into "research-type" spectrometers with a resolution of ca. 0.5 cm⁻¹, and routine analytical instruments with a somewhat lower resolution and lower accuracy. Most research-type instruments use gratings as the dispersing element and have an accuracy of ca. ±1 to 2 cm⁻¹ and a wavelength range of 200–4000 cm⁻¹. Older instruments use prisms which have to be changed for different wavelength regions for optimum results (LiF ~ 4000 – 2500 cm⁻¹, NaCl ~ 2500 – 650 cm⁻¹, CsI ~ 650 – 200 cm⁻¹). The best instruments, if properly adjusted, are accurate to within one wavenumber. Nevertheless, the importance of proper calibration can

hardly be overemphasized in work with proteins and enzymes, where very small shifts can be quite significant.^{6,13,14} A rough calibration can be obtained by measuring the peaks of a polystyrene film (usually supplied with the instrument). More precise results are obtained with solutions of indene, and even better ones with various gases such as CH₄, H₂O, NH₃, and HCN.¹⁵ For the amide I and amide II bands, atmospheric water vapor bands are quite adequate for calibration. For the amide A and B regions, HCN is the best (if somewhat hazardous) reference material. HCN and DCN also give best results in the amide V region. For most purposes, indene is quite adequate over the conventional range of 600–4000 cm⁻¹.

If older prism-type instruments or the relatively inexpensive modern "routine analytical instruments" are used, calibration is an absolute necessity if any worthwhile results are to be obtained. The inexpensive latter-day routine instruments are usually adequate for most conformation studies as far as resolution is concerned. However, the wavelength readings must be very carefully checked.

The majority of modern instruments are of the double-beam type; i.e., absorption by the atmosphere is automatically canceled. Nevertheless, for best results the instrument should be purged with dry nitrogen or dry air because the sensitivity is greatly decreased at frequencies where the atmosphere absorbs. This includes specifically the amide I, II, and V regions.

Infrared Dichroism

In its simplest form, dichroic measurements consist ". . . simply in passing polarized radiation through the (oriented) sample in two directions at right angles and observing the intensity changes which occur in certain bands. In this way, the orientation of individual groups in relation to the whole can be studied."¹⁶ A simple formula

$$A \propto |\mathbf{PE}|^2 \cos^2 \alpha$$

is frequently given for estimating the direction of the transition moments (or even the direction of actual bonds) from dichroic measurements. *A* is absorbance, *E* the electric vector of the radiation beam, *P* the vibrational transition moment vector, and α the angle between the two vectors. Thus, one could simply determine the orientation of C=O or N—H bonds with

¹⁵ "Tables of Wavenumbers for the Calibration of Infrared Instruments," International Union of Pure and Applied Chemistry, Butterworth, London, 1961.

¹⁶ L. J. Bellamy, "The Infrared Spectra of Complex Molecules," 2nd ed. Wiley, New York, 1958.

respect to the main axes of a polypeptide by evaluating the transition moment directions of the amide I and amide A bands, respectively (cf. Table I).

In actual systems there are several factors which make the interpretation of dichroic data somewhat more complex. First, from the standpoint of physical optics the above equation is a poor approximation.¹⁷ Second, even "localized" vibrations are frequently complex and involve many atoms. Third, the polarization of absorption bands is determined by the orientation of the dielectric ellipsoid, not by the transition moment. The two do not necessarily coincide.^{18,19} Finally, characteristic vibrations are frequently split into different branches, depending on the geometry of the repeat unit. None of the branches necessarily coincides with the direction of a (hypothetical) "molecular" mode. A simple schematic example illustrating the coupling of vibrations is given in Fig. 2. The in-phase vibration results in a net dipole moment change along the "polymer axis" and the out-of-phase mode in a net dipole moment change perpendicular to the "polymer axes." It might appear that the whole picture is so complex that no useful information can be gained from infrared dichroism. This is not quite the case. By carrying out very careful measurements on highly oriented samples, it is possible to derive molecular transition moment directions which are in fair agreement with estimations based on geometry. Such measurements have been

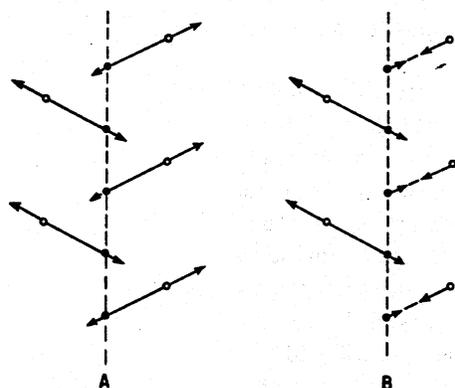


FIG. 2. Schematic presentation of a set of simple coupled oscillators. (A) In-phase vibration, polarized along the chain axis; (B) out-of-phase vibration, polarized perpendicularly to the chain axis.

¹⁷ M. Born and E. Wolf, "Principles of Optics." Pergamon Press, Oxford, 1959.

¹⁸ R. Newman and R. S. Halford, *J. Chem. Phys.* **18**, 1276 (1950).

¹⁹ H. Susi, *Spectrochim. Acta* **17**, 1257 (1961).

carried out on α -helical poly- γ -benzyl-L-glutamate,^{13,20} and a model has been proposed for the conformation of the entire polypeptide.²⁰

Very highly oriented samples (which permit studies leading to estimations of transition moment directions and relative orientation of groups) are difficult to obtain. The greatest value of polarization measurements is found in a somewhat different, although related, context. In previous sections patterns of unit-cell modes which are characteristic for various conformations were discussed. Table II summarizes the polarization of the stronger, more easily observed branches. The polarization of the individual branches is determined by symmetry. It thus becomes possible to identify a conformation without worrying too much about perfect orientation or local transition moment directions. Even qualitative studies are of great value in establishing the structure of unknown samples. Native proteins rarely have a uniform secondary structure. If more than one conformation is present, a complex pattern results which is not easily interpreted by frequency criteria alone. Krimm⁸ has untangled the amide I and II regions of eight proteins with a complex secondary structure, a task that would have been impossible without the help of polarization measurements.

An interesting, relatively recent example regarding conformational conclusions reached through polarized infrared studies concerns the "cross β " structure of some polypeptides.^{21,22} If such a structure is present, the observed frequencies conform with the antiparallel-chain pleated sheet structure (cf. Table II), but the polarization is opposite to the one expected.

From the experimental standpoint, obtaining sufficiently thin (~ 0.01 mm) oriented samples is more an art than a science. For fibrous proteins, stretching at room temperature or at slightly elevated temperatures frequently gives good results. For cast films, stroking with a spatula while the film is drying can induce sufficient orientation to produce worthwhile results. The film should, of course, be cast on an infrared transparent support such as AgCl, BaF₂, or one of the infrared transparent glassy materials which have recently become available. In the case of crystallizable model compounds, it is sometimes possible to obtain thin flakes by careful polishing. Or, alternatively, it may be possible to force the growth of single crystals from the melt between two rock salt plates.¹⁹

The method of polarizing the radiation beam is of little consequence

¹⁹ M. Tsuboi, *J. Polym. Sci.* **59**, 139 (1962).

²¹ R. D. B. Fraser, T. P. MacRae, F. M. Stewart, and E. Suzuki, *J. Mol. Biol.* **11**, 706 (1965).

²² S. Ikeda, H. Maeda, and T. Isemura, *J. Mol. Biol.* **10**, 223 (1964).

unless very exact data are desired. (In this case, the sample should also be perfectly oriented.) The most common and entirely adequate polarizers consist of a pile of tilted silver chloride plates, but any method of polarization will suffice for qualitative and semiquantitative measurements (the only ones possible on films of globular proteins and enzymes).

If grating instruments are used, the polarization caused by the grating itself should be taken into consideration. For maximum efficiency and accurate results, it is best to align the polarizer parallel to the polarizing axes of the grating and to rotate the sample to obtain the desired relationship between sample and polarizer.

Conformation Measurements in the Solid State

Although infrared investigations of solid proteins (such as the keratins and silk fibers) are not very closely related to structural investigations on enzymes, these investigations form the background on which conformational studies of globular proteins are based.^{6,8} Many aspects have already been discussed. Very thin solid films, or films cast on infrared transparent windows must be prepared. It is desirable to induce as much orientation as possible by one of the methods listed in the preceding section. Polarization measurements are of great value even for the study of partially oriented samples. This is especially true if the protein under investigation has segments with different secondary structures. If this is the case, various branches of absorption bands, characteristic for different conformations, can overlap, and the resultant apparently complex band is not easily unscrambled into different components without dichroic measurements. An excellent review and summary has been given by Krimm.⁸

It frequently happens that the area of the sample film is not enough to fill the whole cross section of the infrared beam in the sample compartment. (The beam cross section in some commercial instruments is as large as $\sim 3 \times 25$ mm, in others only $\sim 2 \times 10$ mm.) If the sample area is too small, a compensating grid or some similar device should be placed into the reference beam of double beam spectrometers. As a rule of thumb, with modern instruments meaningful measurements are possible with ca. 10% transmittance in the sample beam (at a frequency where the sample does not absorb) and with proper compensation in the reference beam. If the sample beam transmittance is even lower, a "beam condensing device" or an "infrared microscope" should be used. Such devices are available for most good quality commercial instruments. The beam condensers come in various designs; some are equipped with mirrors, others with rock salt or KBr lenses. It is important to study the optics of the selected device in some detail in order to decide what in-

fluence it will have on the polarization characteristics of the radiation beam.

In the case of cast films, caution should be exercised in drawing conclusions regarding the original solution from infrared spectra of the films. During the evaporation process the pH can undergo drastic changes, resulting in a final film which is in effect cast from a concentrated solution with a pH quite different from the original solution. If the conformation of the sample is likely to change with pH, this effect should be taken into consideration.

Conformation in Aqueous Solution and in D₂O Solution

Solid-state investigations are in many ways easier to carry out than solution studies. No solvent absorption need be considered; dichroic measurements are possible. Despite the difficulties encountered in solution work, studies in aqueous as well as other solvents are of considerable interest. Conclusions carried over from solid state studies necessarily involve the tacit assumption that the conformation is not changed by the phase change (cf. preceding section). Any verification of this assumption is worthwhile. Furthermore, conformation changes (e.g., denaturation) caused by changes of pH or solvent composition can be directly observed only by studying the solutions themselves.

Several considerations must be taken into account in any attempt to apply infrared spectroscopy to aqueous solutions. Among the most important ones are specific solute-solvent interactions, refractive index effects, and spectroscopic interference by solvent bands. The last complication is particularly disturbing in aqueous solution where the strong HOH bending mode practically coincides with the amide I mode. One way to circumvent this difficulty is to investigate D₂O solutions. However, if hydrogen-deuterium exchange occurs (which is to some extent true for all polypeptides), the characteristic frequencies change markedly. Furthermore, in D₂O solution it is extremely difficult to avoid small amounts of HOD. The bending frequency of HOD is close to the amide II mode of COND (the so-called amide II' mode).

Very thin sample cells (ca. 0.01 mm) and very precise balancing of H₂O absorption is necessary for studies carried out in H₂O solution. This can be achieved with precision cells of variable path length and CaF₂ windows,⁶ such as the model VT-01 cells of the Research and Industrial Instruments Co., London, England. The spectra of some proteins obtained by this technique are shown in Fig. 3. These data suggest that the difference in amide I and II frequencies is very small between the solid state and aqueous solution (except for random conformations).

Some spectra obtained in D₂O solution are shown in Fig. 4. Only the

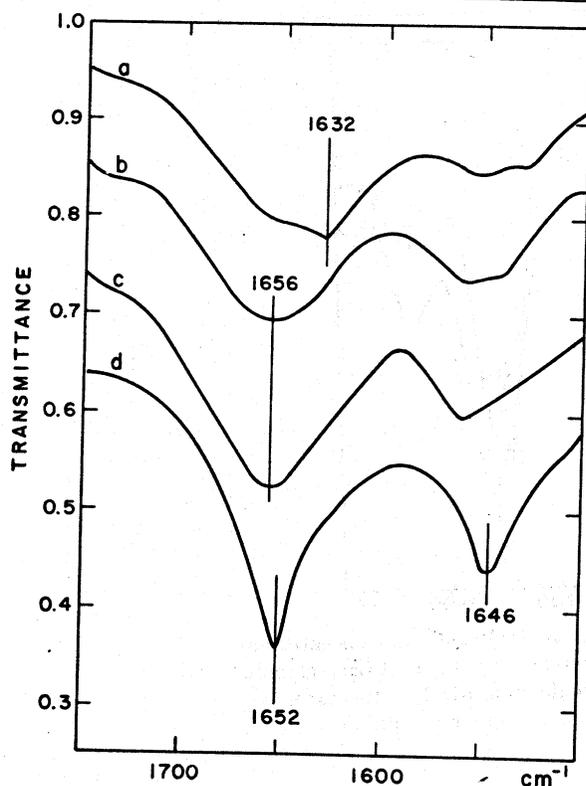


FIG. 3. Spectra of proteins in aqueous solution. Concentration, 3% by weight. Path length, about 0.01 mm. Pure H₂O as reference. X2 ordinate scale expansion. Curve a, native β -lactoglobulin, pH 6.5; b, denatured β -lactoglobulin, pH 12.3; c, α_s -casein, pH 10.6; d, myoglobin, pH 7.6. Consecutive spectra displaced by 0.1 scale unit.

amide I bands are shown because in deuterated samples the amide II mode coincides with absorption caused by traces of HOD.⁶ Nevertheless, it is clearly possible to distinguish between the α -helix, the pleated sheet conformation, and apparently unordered conformations on the basis of the amide I frequencies. Characteristic frequencies for the amide I mode under various conditions are summarized in Table III.

Experimentally it is much simpler to obtain protein spectra in D₂O solution than in H₂O solution. Any good sealed cells with insoluble windows and a path length of ca. 0.1 mm can be used for solutions of ca. 3% by weight. (In H₂O solution the maximum path length is around 0.01 mm.) Under favorable conditions it is possible to observe conformational changes as they occur in deuterium oxide solution. Figure 5 shows

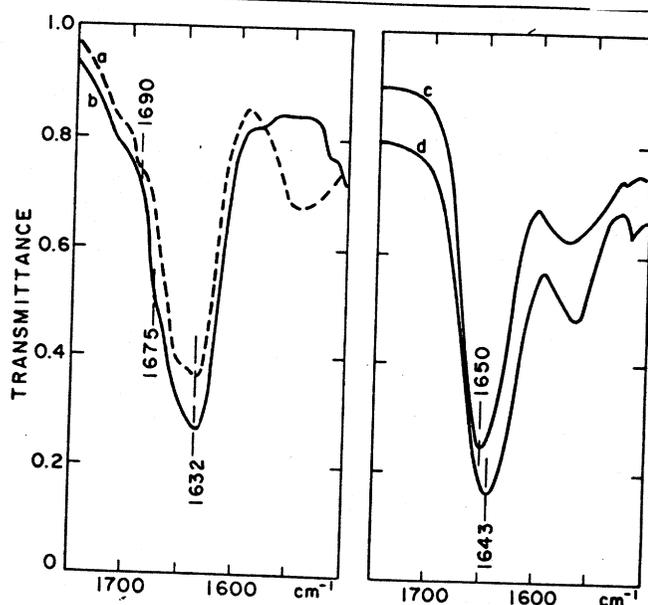


FIG. 4. Amide I' bands of N-deuterated proteins in D₂O solution. Concentration, 2 wt. %; path length, 0.1 mm; pure D₂O as reference. Curve a, β -Lactoglobulin (largely ACPS), pD 2.1, before exchange; b, β -lactoglobulin, pD 2.4, after exchange; c, myoglobin (largely α -helix), pD 7.0; d, α_s -casein (unordered), pD 10.9.

TABLE III
OBSERVED AMIDE I AND AMIDE I' FREQUENCIES OF PROTEINS
UNDER VARIOUS CONDITIONS

Conformation	Mode	D ₂ O solution ^a	H ₂ O solution	Solid	Sample
Antiparallel-chain pleated sheet	$\nu(\pi, O)_A$	1632 ~1630	1632	1632	β -Lactoglobulin Lysozyme
	$\nu(O, \pi)_A$	1675	1690	1630-1634 1690	Fibrous proteins ^b β -Lactoglobulin
α -Helix	$\nu(O)_\alpha$	1649 ^c 1650	1652	1695-1697 1652	Fibrous proteins ^b β -Lactoglobulin Myoglobin
		1650 1643 1643	1656 1656	1650-1653	Fibrous proteins ^b Lysozyme β -Lactoglobulin α_s -Casein
Unordered	$\nu(\mu)$	1650 1643 1643	1656 1656	1660-1664	Fibrous proteins ^b

^a After complete exchange.

^b S. Krimm, *J. Mol. Biol.* 4, 528 (1962).

^c In D₂O-CH₃OD mixed solvent.

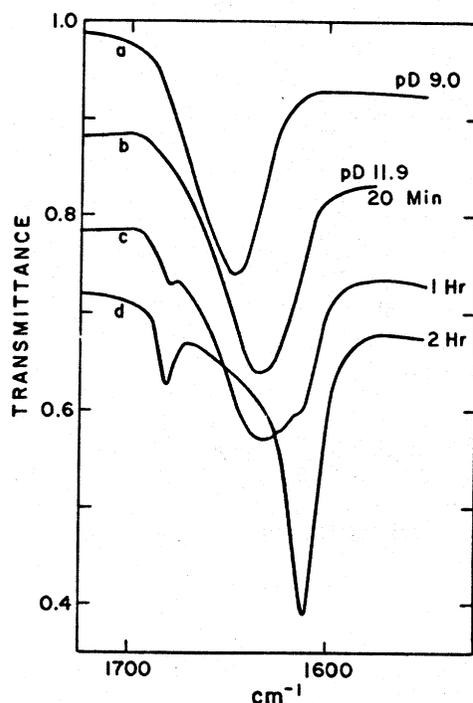


Fig. 5. The amide I band of poly-L-lysine in D_2O solution. Path length, 0.1 mm; concentration, 0.4% by weight. Pure D_2O as reference. Consecutive spectra displaced by 0.1 scale unit (upper curve is on scale). Curve a, random conformation; b, α -helix; c, transition between α -helix and antiparallel-chain pleated sheet; d, antiparallel-chain pleated sheet.

the rearrangement of poly-L-lysine from the random state to the α -helix and to the β -structure as a function of pD (note that the characteristic frequencies for this synthetic polypeptide are somewhat different from protein frequencies). Similar studies can be performed with actual proteins,^{6b} although the results are not as clean-cut. A correlation of infrared data with results of optical rotatory dispersion measurements is shown in Fig. 6.

In complex proteins which contain segments with different conformations, the results are not always as clean and obvious as the simpler examples in Fig. 4 might suggest. Figure 7 shows the amide I band of several enzymes in D_2O solution.^{6b} The strongest β -structure band shifts from 1632 to 1637–1640 cm^{-1} . Despite the complexity, it is still possible to obtain a fair qualitative idea about the predominant conformation of each sample. Based on amide I absorption, most globular proteins can be

CONFORMATION: OPTICAL SPECTROSCOPY

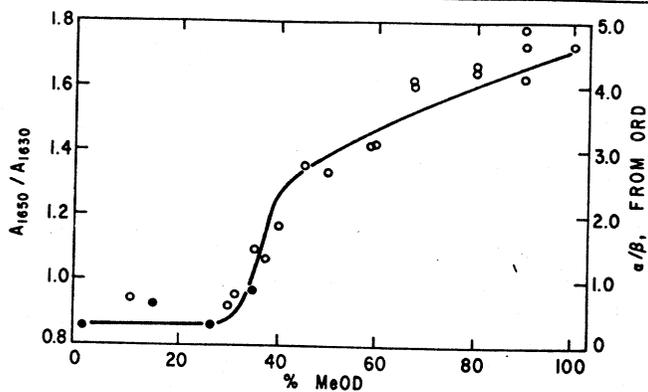


FIG. 6. Relative change of absorbance of β -lactoglobulin A solutions at 1650 and 1630 cm^{-1} , as a function of CH_3OD concentration. The points at high protein concentration were obtained immediately after mixing to avoid the onset of aggregation. Solid line, α/β from ORD measurements. \circ . 0.4 g/l; \bullet . 4.0 g/l.

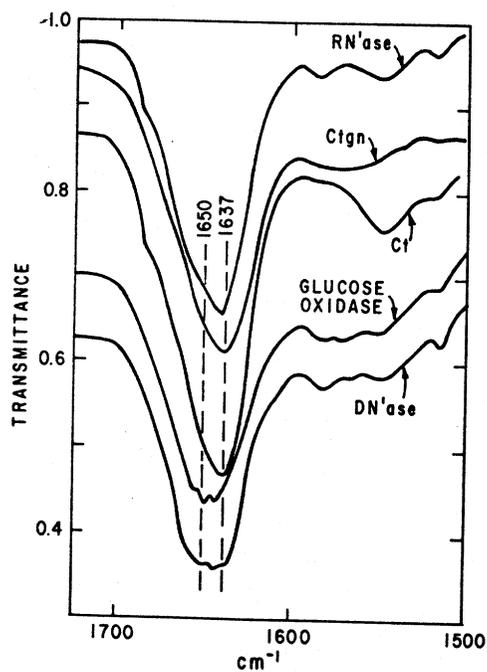


FIG. 7. Infrared spectra of various proteins in D_2O solution. Ctgn, chymotrypsinogen; Ct, α -chymotrypsin; RNase, ribonuclease; DNase, deoxyribonuclease.

divided into two categories^{6b}: (1) the ones with amide I absorption maxima close to 1650 cm^{-1} (essentially helical), and (2) proteins with complex amide I bands, indicating a considerable amount of " β -structure" and/or apparently random segments.

Kinetics of Hydrogen-Deuterium Exchange as Related to Conformation

A considerable amount of infrared spectroscopy has been carried out in connection with hydrogen-deuterium exchange in polypeptides.^{9,23,24} In these studies infrared spectra are used merely as an analytical device to determine the amount of exchange as a function of time. Band assignments are immaterial, as long as absorbance at one or more frequencies is sufficiently different for hydrogenated and deuterated species. In earlier days absorbance at the amide A (NH stretching) and amide A' (ND stretching) frequencies was used to monitor hydrogen-deuterium exchange. This method has the disadvantage that H_2O , HOD, and D_2O absorb strongly in the same region as NH and ND groups. It is much more convenient to base such investigations on apparent intensity changes of the amide II band.⁹ This band does not interfere too badly with absorption bands of H_2O , HOD, or D_2O . Upon deuteration it shifts from ca. 1550 cm^{-1} to ca. 1450 cm^{-1} . (The precise position and splitting into components is determined by the backbone conformation, as previously discussed.)

Figure 4 (a and b) shows the disappearance of the 1550 cm^{-1} region amide II band of β -lactoglobulin as the peptide groups become deuterated. The amide II band behaves in the same manner in all polypeptides and proteins. For greatest convenience and accuracy, the spectra should be measured in absorbance units. (Not transmittance, as is the usual custom.) Some modern instruments permit direct absorbance measurements. For simpler instruments, transmittance values should be converted to absorbance by the relationship: $A = \log T^{-1}$. Relative concentrations can be evaluated either by measuring the area of the band or the peak intensity. For most purposes, peak intensities are sufficiently accurate to provide useful semiquantitative data. Whichever method is chosen, reference points close to the measured absorption band are necessary because infrared spectra seldom yield an unequivocal I_0 line. The most frequently employed method is the so-called "base-line technique."²⁵ It can be shown that "base-line absorbance" values are proportional to the

²³ K. Linderstrøm-Lang, in "Symposium on Protein Structure" (A. Neuberger, ed.), Methuen, London, 1958.

²⁴ A. Hvidt and S. O. Nielsen, *Advan. Protein Chem.* **21**, 288 (1966).

²⁵ W. J. Potts, Jr., "Chemical Infrared Spectroscopy, Vol. I. Wiley, New York, 1963.

concentration of the material under investigation, even if the bracket points do not correspond to frequencies of zero absorbance. The method can be used either with cells of known thickness (absorbance directly proportional to concentration) or by using a ratio method, i.e., measuring the base-line absorbance of a band which does not change by deuteration as reference.

Decrease of the amide II absorbance around 1550 cm^{-1} thus provides a measure for the decrease of the number of N—H bonds and furnishes a way to investigate the kinetics of hydrogen-deuterium exchange. The latter, in turn, is related to the conformation of the protein or polypeptide. For instance, for the sodium salt of poly- α -L-glutamic acid (random), the exchange takes approximately 10 minutes, whereas more than 24 hours are required for polyglutamic acid (helical). Frequently many days are necessary for complete exchange, and it then becomes possible to evaluate a percentage of "hard-to-exchange amide hydrogens" (HEAH).⁹ The numerical values of HEAH provide a measure for the number of peptide groups which for some reason or another are not easily deuterated. There is in general no direct correlation between HEAH and the "amount" of a particular conformation, such as the α -helix. It has been emphasized⁹ that such oversimplified interpretations could easily lead to serious errors. There might be areas in the polypeptide chain so surrounded by hydrophobic regions that the exchange is slow regardless of the local conformation of the backbone chain. Even if exposed to the solvent, some extended structures appear to exchange very slowly, and it is by no means justified to associate slow exchange specifically with the α -helix.^{6,14,26} Measurements of HEAH do provide a measure for the "tightness" of a secondary structure but, taken in isolation, give little information about the nature of the tight structure.

Methods of Measurement

The types of spectrometers used for obtaining infrared spectra have been discussed in the section on instrumentation. In the following paragraphs methods of sample preparation, cell design, polarization measurements, and absorbance measurements are briefly summarized.

Three basic sample preparation techniques are available for obtaining qualitative spectra of solid samples: the oil-mull technique, the pressed disk technique, and the thin film technique. All three yield information regarding frequencies and relative intensities, but not absolute intensities or absorptivities.²⁵

A mull is made by grinding solid sample to a fine particle size

²⁵ S. N. Timasheff and H. Susi, *J. Biol. Chem.* **241**, 249 (1966).

while the particles are suspended in highly purified mineral oil (Nujol) or some other convenient suspension medium. The resulting paste is spread between two infrared-transparent plates (usually NaCl or KBr) and the sandwich inserted in the sample space of the spectrometer. Dismountable cells consisting of proper plates and sample holders are available from instrument manufacturers.

The pressed disk technique consists of grinding a few milligrams of the sample with ca. 0.5 g of alkali halide (usually KBr) and compressing the mixture to a disk of about 1 mm thickness under a pressure of 500–1000 atm. The disk is placed into the sample beam of the spectrometer with the help of an appropriate sample holder, and the spectrum is obtained.

The solid film technique involves casting a thin film (~ 0.01 mm) of sample from a solution onto an infrared transparent plate. AgCl plates are frequently used for samples cast from aqueous solution.

Each technique has its advantages and disadvantages. It is, therefore, frequently desirable to use more than one approach. The oil-mull technique produces spectra with bands of the mulling agent. To obtain a complete spectrum of the sample it is desirable to use two mulling agents which absorb at different wavelengths (e.g., Nujol and a fluorocarbon). The pressed disk technique leads to spectra with no interfering bands, but the conformation (and the spectrum) can undergo changes during the vigorous grinding and pressing necessary for sample preparations. The main advantage is the small amount of sample required. The solid film technique is limited to samples soluble in a convenient solvent. No disturbing bands are observed, but relative intensities might be distorted by accidental orientation of the sample molecules. Each method can be adapted to micro samples by using commercially available beam condensers.

Quantitative absorbance measurements are very difficult to perform on solid samples. Fortunately relative intensities are sufficient for many applications. For dichroic measurements (cf. Infrared Dichroism) spectra of the same oriented sample as obtained at various polarizer and sample positions are compared to obtain dichroic ratio values. The latter are independent of true absorbance values.¹⁹ For hydrogen-deuterium exchange measurements the absorbance of the amide II band (~ 1550 cm^{-1} , largely NH bending) can be compared with the amide I band (~ 1650 cm^{-1}) which, in first approximation, is independent of deuteration.

The absorbance of infrared bands is commonly determined by the "base-line method."²⁵ The procedure consists of selecting points of maximum transmittance on either side of the band of interest, drawing a straight line between the points and measuring the transmittance (or

absorbance) of the band center relative to the straight "base line." The necessity to use "base-line absorbances" stems from the difficulties in determining a true I_0 value in the infrared region; this, in turn, is related to reflection and scattering phenomena, and the high number of closely spaced and overlapping bands in most infrared spectra.²⁵ [See this volume [17], Fig. 2.]

For polarization measurements oriented samples are required. The most common procedure involves "stroking" of a cast film with a spatula while the solvent is being evaporated. At best only qualitative results can be expected. Frequently even these qualitative results are very useful in drawing structural conclusions.⁸ For quantitative polarization data, very thin single crystals must be used. These are in most cases very difficult to obtain. The great majority of dichroism studies have been carried out with incompletely oriented films, and the results are at best semiquantitative.

For work in aqueous solution very thin precision cells must be used because water itself is a strong absorber in the spectral region of greatest interest. The thickness of the water film in the reference beam of double beam instruments must precisely compensate for water absorption in the sample beam. This can be achieved only with highly complex cells of variable path length.⁶ Work in H_2O solutions is not recommended unless necessitated by special problems.

Solution studies in D_2O are experimentally much easier to carry out than studies in H_2O because the absorption spectrum of D_2O has a convenient "window" in the region where amide I and II bands are observed. Sealed cells of 0.1–0.2 mm path length and CaF_2 or BaF_2 windows, easily available from several manufacturers, can be used.^{6,14} For some time water-insoluble infrared-transparent cell windows made from special materials such as "Irtran 2" (polycrystalline zinc sulfide, Eastman Kodak) have been commercially available. They frequently have a very high refractive index which can cause disturbing interference patterns superimposed on the observed spectrum. The interference can be reduced by roughening the inside of the windows with sandpaper. The phenomenon is usually absent or much less pronounced if CaF_2 or BaF_2 windows are used.