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Infrared Internal Reflection Spectrometry of Aqueous Protein Films at the Germanium–Water Interface

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For twenty years, infrared spectrometrists have relied on the positions and intensities of the N–H stretching, amide I (primarily C=O stretch), and amide II (C–N stretch + N–H bend) bands as aids in interpreting the secondary structures of synthetic polypeptides and natural proteins. These three absorption bands provide essentially all of the secondary structural information that can be obtained from the infrared spectrum of a polypeptide (*1*). Two of the bands however, the N–H stretching band at approximately 3400 cm^{-1} and the amide I band at about 1650 cm^{-1}, are effectively obscured in aqueous solution spectra by the ~3380 cm^{-1} and 1639 cm^{-1} bands of liquid water. Various efforts to get around this problem have been employed, including the use of D_2O as a solvent, employing very thin cells, or using a compensating reference cell (*2–4*). The thin cell technique (*2–4*) is inadequate for protein solution spectrometry because of the concomitant requirement of high solute concentrations. D_2O solutions pose an additional problem, that of hydrogen–deuterium exchange. This results in a change in the intensity of the undeuterated amide II band, and formation of a deuterated amide II band at the same frequency as the HDO band (*1*).

Internal reflection spectrometry (*4*), using a high refractive index, infrared-transparent prism such as germanium, provides the thin sampling region necessary to avoid the total loss of energy at the water peak maxima. For an internal reflection system, the "depth of penetration," d_p, is defined (*4*) as the distance into the solution where the evanescent field amplitude decays to e^{-1} of its magnitude at the prism–solution interface. For germanium–water, at a 45° angle of incidence, this distance is 0.064 λ_0, where λ_0 is the *in-vacuo* wavelength (*5, 6*). Eighty-four percent of the peak intensity observed in an internal reflection spectrum is derived from absorbing molecules within one d_p of the prism–solution interface, and 95% of the observed peak intensity is due to molecules within two d_p. Thus, for the amide I peak at 1650 cm^{-1}, 95% of the band intensity comes from the 7750-Å thin region at the germanium–solution interface. This is true for the zero absorption case, but is also applicable for low values of k. Higher values (≥ 0.1) of k will depress d_p (*3*).

Using such a thin sampling region in combination with the signal-to-noise enhancing techniques of spectrum-averaging and mathematical smoothing, we have been able to obtain accurate spectra of aqueous proteins by subtraction of the solvent spectrum from that of the solution. Spectrum subtraction was demonstrated by Yang and Low (*7*), using a Fourier transform interferometer, for aqueous nitrate and nitrite solutions, but their use of difference spectra was restricted to regions where water bands would not interfere. In this paper, we show that, with the aid of a minicomputer interfaced to a standard dispersive infrared spectrophotometer, the obscured amide I band of a protein can be separated from the overlying water band at 1639 cm^{-1}.

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EXPERIMENTAL

The computer–spectrometer system consists of a Data General NOVA 1220, with three cassette tape drives, interfaced to a Perkin-Elmer Model 180 infrared spectrometer. The Model 180 standard interface (Perkin-Elmer) is connected to the NOVA via an interface of our own design. The interfacing and all of the software are described elsewhere (*8*). For the solution spectra, four scans at 5 cm^{-1}/sec were computer-averaged in both perpendicular and parallel polarizations. For the air-dried protein films, one scan provided an adequate S/N in parallel polarization, two scans were computer-averaged in perpendicular polarization. A precision chopper attenuator (Harrick Scientific), set at 30% transmittance, was in the reference beam, and a twice-normal spectrometer slit program was employed. The spectrometer was equipped with a wire grid polarizer (Perkin-Elmer).

The cell used to hold the germanium prism has been described in an earlier paper (*9*), except the aluminum contact to the germanium prism was replaced by platinum foil. Intrinsic germanium prisms (Harrick Scientific) employed were 20 × 52.5 × 1 mm oriented single crystals, with the 110 crystal face exposed to the solution. Twenty-two reflections were obtained at the germanium–solution interface.

Protein solutions were prepared from ~72% clottable, ~90% bovine fibrinogen (Sigma), pH 7.2, 0.1*N* phosphate buffer, prepared with doubly-distilled, deionized water. The protein solutions were subjected to ultrasonic vibration to disperse the fibrinogen. After gathering the solution spectra, the prisms were removed from the cell, rinsed quickly with distilled water, and allowed to air-dry. Film thickness measurements on the dry films were made ellipsometrically.

RESULTS AND DISCUSSION

The separation of the 1639 cm^{-1} water band from the amide I band of fibrinogen is shown in Figure 1. The top spectra in Figure 1 are averages of four scans of the fibrinogen solution. In the protein solution, the single crystal germanium prisms exhibited an open-circuit potential of about −475 mV (to saturated calomel). At this potential and a pH of 7.2, intrinsic germanium should have a near-zero surface charge (*10, 11*). The amide II band is clearly visible at 1543 cm^{-1} in spectra $1_\parallel$ and $1_\perp$. The intensity of the amide II peak increased for the first 30 minutes after the protein solution was added to the cell, indicating a slow spontaneous adsorption of the protein at the germanium surface. This process can be accelerated and even enhanced by adjusting the surface charge on the germanium to more positive values (*8*), but this was not done for the experiment characterized by Figure 1. Spectra $2_\parallel$ and $2_\perp$ are the pH 7.2, 0.1*M* phosphate buffer base lines, with the $H_2PO_4^-$/HPO_4^{2-} band appearing at 1070 cm^{-1}. The top four spectra of Figure 1 are compressed to 0.375× for presentation purposes. Spectra $3_\parallel$ and $3_\perp$ are the simple absorbance differences of the protein solution spectra less the buffer base lines. The negative water peak at ~3400 cm^{-1} in spectra $3_\parallel$ and $3_\perp$ arises from the fact that protein molecules have replaced water in the region near the germanium surface. To correct for this, spectra $4_\parallel$ and $4_\perp$ are scaled absorbance subtractions, removing 90 and 88% of the buffer solution base lines. The final results are the protein spectra $4_\parallel$ and $4_\perp$ clearly showing the amide I band at 1640 cm^{-1} which is normally obscured by the 1639 cm^{-1} water band ($1_\parallel$ and $1_\perp$).

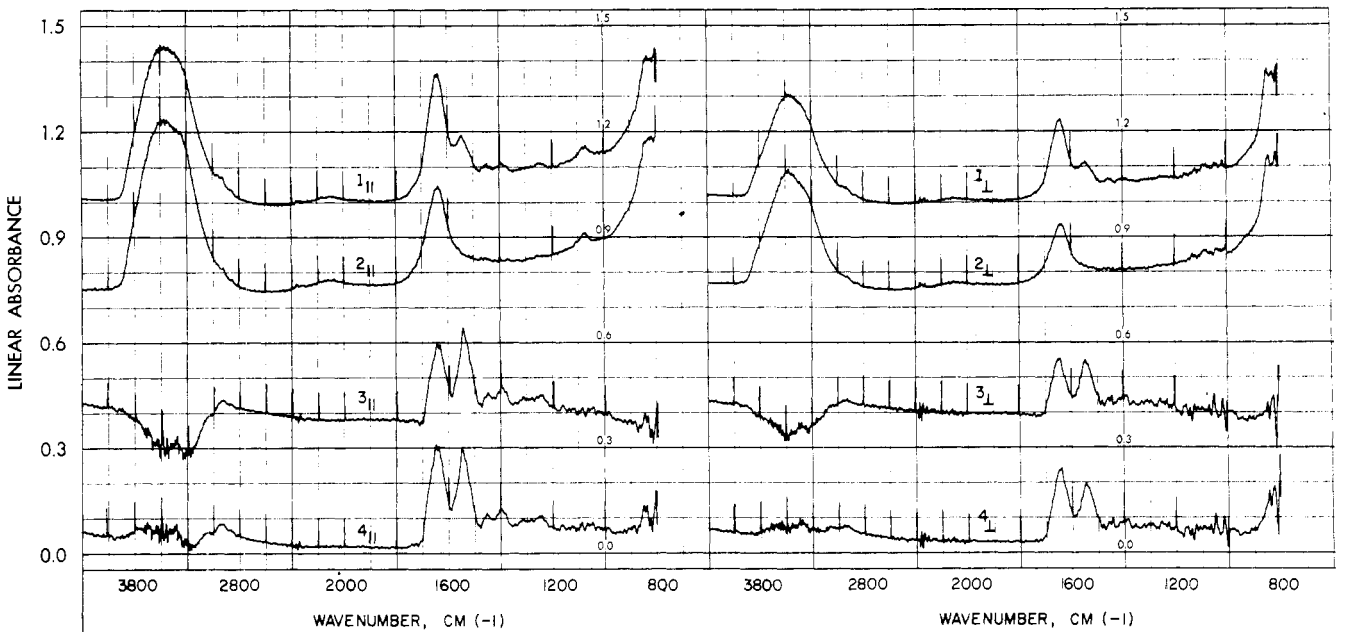

Figure 1. Spectra $1_{\parallel}$ and $1_{\perp}$: 0.375X ordinate-expanded, once-smoothed average of four scans of 0.36% solution of 72% clottable bovine fibrinogen in pH 7.2, 0.10*M* phosphate buffer. Spectra $2_{\parallel}$ and $2_{\perp}$: Background spectra of buffer solution only, as in $1_{\parallel}$ and $1_{\perp}$. Spectra $3_{\parallel}$ and $3_{\perp}$: Direct subtraction of spectra $2_{\parallel}$ and $2_{\perp}$ from $1_{\parallel}$ and $1_{\perp}$, 1.0X ordinate expansion, twice-smoothed. Spectra $4_{\parallel}$ and $4_{\perp}$: Scaled subtraction of $0.90 \cdot 2_{\parallel}$ and $0.88 \cdot 2_{\perp}$ from $1_{\parallel}$ and $1_{\perp}$, respectively, 1.0X ordinate expansion, twice-smoothed

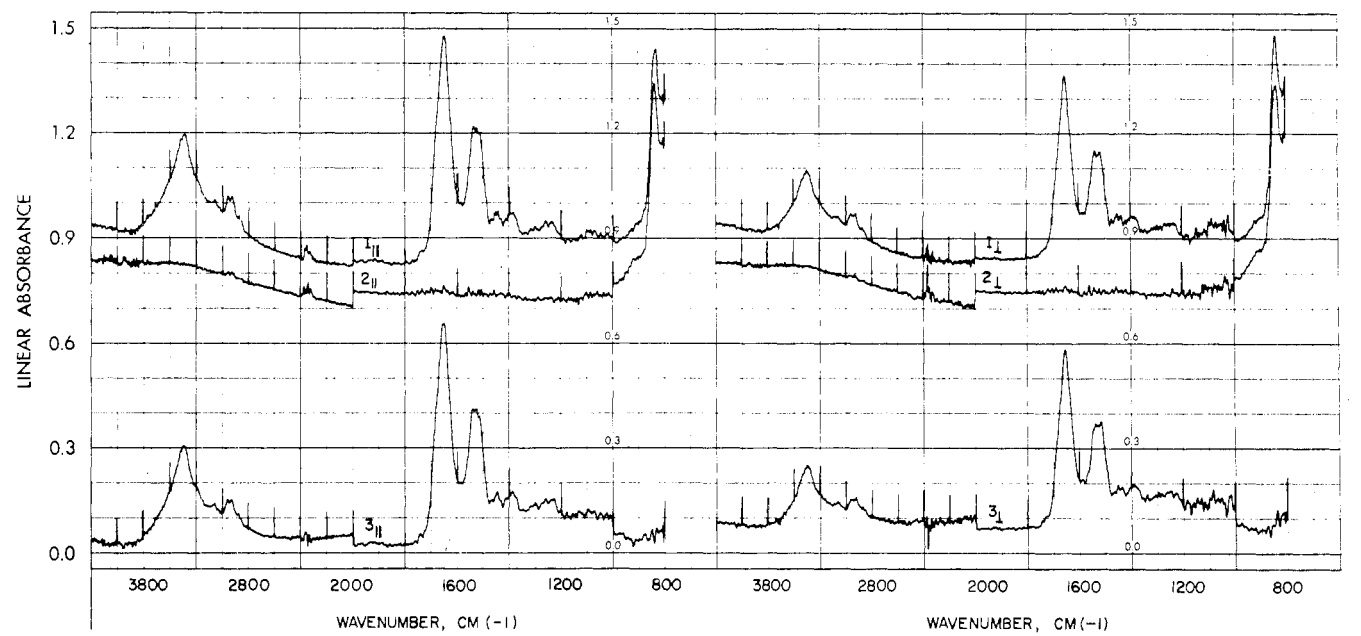

Figure 2. Air-dried protein film. Spectra $1_{\parallel}$ and $1_{\perp}$: spectra of the dried film, 1 represents a single scan, $1_{\perp}$ is an average of two scans, both are once-smoothed, 1.0X ordinate expansion. Spectra $2_{\parallel}$ and $2_{\perp}$: Single-crystal, oriented (110) germanium prism base lines. Spectra $3_{\parallel}$ and $3_{\perp}$: Difference spectra of raw data, twice smoothed, 1.0X ordinate expansion

Spectra $4_{\parallel}$ and $4_{\perp}$ of Figure 1 also show the C–H stretching bands of the protein in the 2800–3000 cm^{-1} region, and the weaker amide bands in the 1200–1500 cm^{-1} region. For symmetry reasons, we would not expect to see the N–H absorption band at ~3400 cm^{-1} in an aqueous solution.

After rinsing and air-drying the protein film which had been spontaneously adsorbed on the germanium surface during the above experiment, the spectra shown in Figure 2 were obtained. The top spectra are those of the air-dried prism. Spectra $2_{\parallel}$ and $2_{\perp}$ are base lines obtained with a different single-crystal prism, and the difference spectra $3_{\parallel}$ and $3_{\perp}$ are those of the dried protein film. Comparing spectra $3_{\parallel}$ and $3_{\perp}$ of Figure 2 to $4_{\parallel}$ and $4_{\perp}$ of Figure 1, we note that the shift in the amide I band position in going from the wet to the dried film is only from 1640 to 1650 cm^{-1}, not a very large change (*12*).

The thickness of the air-dried film shown in Figure 2 was measured ellipsometrically to be 970 ± 100 Å. (The uncertainty in the film thickness is due to the uncertainty in the refractive index of the dried protein. For $n = 1.5$, the thickness was actually computed to be 970 ± 10 Å.) Since the dried film obviously occupied more space in the wet state, spectra $4_{\parallel}$ and $4_{\perp}$ of Figure 1 are due to combinations of adsorbed and solution protein. A careful examination of the amide I peaks in spectra $4_{\parallel}$ and $4_{\perp}$ of Figure 1 reveals a splitting of the peak maximum, with maxima at both 1640 and 1650 cm^{-1}, possibly due to the combinations of strongly adsorbed and more "native" protein in the interphase.

It is interesting to examine the extent of adsorption in the wet state, considering the fact that a scale factor of 0.88–0.90 was required in the subtraction of the water base line before the negative water peaks could be eliminated. Using a value of about 0.735 for the partial specific volume of the protein (*13*), we compute that a base-line subtraction scale factor of 0.9975 would be expected for a 0.360 weight percent protein solution. To produce the observed scale factor of 0.90, an average concentration of 12.8% protein would be necessary in the region sampled by the evanescent field. The solubility of fibrinogen is less than 1% by weight in aqueous solution, supporting the conclusion that the solution protein has spontaneously precipitated out on the germanium surface. Since 95% of the observed amide I peak comes from the first 7750 Å of solution, a scale factor of 0.90 could be explained by a nearly solid protein film in the first 1000 to 1500 Å, and a 0.36% solution from there on out. To better describe the wet film structure, a model will have to be proposed for the film–solution interphase, and we do not feel that such an exercise is appropriate in this report.

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Sensitive Method for Spectrophotometric Determination of Amines

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Amines are alkyl or aryl derivatives of ammonia and exhibit alkaline reactions in many solvents. Therefore, in the past years, amines have mainly been determined by acid base titrations in aqueous and nonaqueous solutions. Some colorimetric methods are available in the literature for the determination of amino compounds (*1–4*). Copper sulfate has been used for the determination of ethylenediamine (*5*). Colorimetric determination of small amounts of aromatic amines by diazotization was reported by Kuritsyn *et al.* (*6*). A photometric method for the determination of small amounts of aniline has also been reported (*7*). De-Atley reported a method for the determination of diphenylamine and its mono nitro derivatives by oxidation with iron(III) in sulfuric acid solution. All these spectrophotometric methods suffer from two limitations: they are less sensitive and cannot be applied for all types of amines. We have, therefore, decided to find a sensitive method for the determination of aliphatic as well as aromatic primary, secondary, and tertiary amines. Small amounts of the amino compounds react with acetyl chloride and ferric ion to produce a greenish violet colored complex. The use of this reaction for the determination of amines is described in this report.

EXPERIMENTAL

Apparatus. A Bausch & Lomb Spectronic-20 (U.S.A.) was used for the spectrophotometric work. Elico pH meter Model L1-10 (India) was employed for pH measurements.

Reagents. All the reagents were of analytical grade.

Solution of Amino Compounds. A 1% solution of the organic amino compound was prepared in conductivity water and those compounds which were insoluble in water were dissolved in 1-propanol.

Solution of Ferric Nitrate. A 5% solution of ferric nitrate was prepared in conductivity water.

Solution of Acetyl Chloride. A 1% solution of acetyl chloride was prepared by taking 1 ml of acetyl chloride and diluting with conductivity water to a total volume of 100 ml.

Buffer solutions of pH 0–5.5 were prepared as reported (*8*).

Recommended Procedure. A small amount of amino compound (50 μg–10 mg) was taken in a test tube; to this was added gelatin and 1 ml of 1% acetyl chloride solution and 2 ml of 5% ferric nitrate solution; after shaking 10 ml of pH 1.8 buffer solution was added; and after heating on water bath at 65 °C for 20 minutes, a greenish violet color was observed. Then the volume was made up to 25 ml with conductivity water and the absorbance was taken at the optimum wavelength (550 nm).

RESULTS

The absorbance of the violet colored complex formed by the action of acetyl chloride and ferric nitrate with amines was noted at varying wavelengths. The maximum absorbance (in the form of a plateau) was obtained at 550 nm. In order to get the optimum conditions for the determination of amines, the effect of possible variables was studied. The results are summarized as follows:

Effect of Temperature. One ml of 1% aniline (v/v) was taken in a boiling tube. To this was added 1 ml of 1% acetyl chloride and 1 ml of 5% ferric nitrate solution. The reaction mixture was kept at the specified temperature for 20 minutes in a temperature-controlled water bath. This was then transferred to a standard flask of 25-ml capacity and the volume was made up to the mark with water. The absorb-