An Experimental Method Correcting for Absorption Flattening and Scattering in Suspensions of Absorbing Particles: Circular Dichroism and Absorption Spectra of Hemoglobin in Situ in Red Blood Cells†

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ABSTRACT: An experimental approach to interpretation of the anomalous absorption and circular dichroism (CD) spectra of hemoglobin in situ in red blood cells is reported. Absorption flattening effects have been overcome by use of high cell concentrations in very short light path cuvettes. Differential scattering contributions to circular dichroism have been resolved using a CD instrument capable of variable detection geometry. Scattering effects have also been resolved using media of high refractive index to match that of the red blood cell. The results are in agreement with a parallel calculational analysis of red blood cell CD spectra, which predicted the relative magnitudes of the flattening and differential scattering CD contributions. An experimental absorption spectrum has been obtained for hemoglobin in the red blood cell with scattering and flattening eliminated. This quantitatively simulates the spectrum of a hemoglobin solution. The methods described should be widely applicable to conformational studies of macromolecules in their native environment.

The optical activity and absorption spectra of intact biological structures contain valuable information on their composition, biopolymer conformation, and morphology (light scattering properties). Indeed spectroscopic methods offer one of the few nondestructive techniques for monitoring in situ changes in these properties which may accompany biological function. The spectra are difficult to interpret, however. The problem of interpretation has been clarified for a variety of complex systems including membranes (Schneider et al., 1970; Gitter-Amir et al., 1976a; Ji and Urry, 1969; Glaser and Singer, 1971; Litman, 1972; Gordon and Holzwarth, 1971), subcellular organelles (Rosenheck and Schneider, 1973), viruses (Dorman and Maestre, 1973; Holzwarth et al., 1974), and intact cells (Gitter-Amir et al., 1976b; Bryant et al., 1969). The main problem is the separation of light scattering and absorption flattening (Duysens, 1956; Amesz et al., 1961) effects from intrinsic structural contributions to the spectra. In some cases calculational methods (Schneider, 1971, 1973) have been able to resolve all contributions to the spectra and have even succeeded in quantitatively interpreting the visible absorption (Bryant et al., 1969) and ultraviolet optical activity spectra (Gitter-Amir et al., 1976b) of intact cells. The calculational methods, however, are lengthy and complex and not always convenient for rapid analysis of spectra. It is therefore desirable to develop experimental methods of accounting for and correcting the flattening and scattering effects on the spectra. This is the goal of the present work.

The main experimental approach to date has been to increase the spectrophotometer's solid angle of light detection, collect much of the scattered light, and thus prevent the scattering from being measured as an extinction or circular dichroism contribution. This approach has been used for some time to correct visible absorption spectra (Amesz et al., 1961; Shibata et al., 1954; Latimer and Eubanks, 1962; Keilin and Hartree, 1958). Several laboratories have recently extended large angle detection geometry to circular dichroism measurements (Dorman et al., 1973; Philipson and Sauer, 1973; Gregory and Raps, 1974; Nicolini et al., 1976). A less common method of reducing scattering losses has been the use of high refractive index suspending media (Barer, 1955; Rubinstein and Ravikovich, 1946). These methods can remove most of the scattering effects but the resulting spectra will still be subject to gross distortion because of the flattening artifact and may therefore not be ready for conformational analysis. Only very limited experimental attempts have been made to correct the flattening distortions (Jope, 1949; Lothian and Lewis, 1956; McCrae et al., 1961).

In the present work we present a simple experimental approach to absorption-flattening corrections. This approach together with two nondestructive methods for scattering corrections is applied to absorption and circular dichroism spectra of hemoglobin in situ in human red blood cells. For the flattening correction we use very short light path cuvettes coupled with a high concentration of particles (RBC[1]) in order to simulate a highly concentrated hemoglobin solution uniformly distributed across the area of the light beam. To correct for scattering in the visible absorption spectra, we have attempted to match the refractive index of the suspending medium to that of the cell using concentrated protein solutions. For the ultraviolet CD spectra we have made measurements with a Cary 6002 circular dichrometer modified by a refinement of the method of Dorman et al. (1973) to increase the solid angle of light detection.

The red blood cell represents a prototypical scattering particle, presenting problems of interpretation likely to be encountered in the spectra of other biological cells and of highly absorbing particles in general. In addition, the RBC is one of the best defined cells, having predominantly one protein (hemoglobin) whose structure and spectral properties are known. It thus has the built-in controls to test the extent to which the spectra can be corrected. Furthermore, the experimental resolution of the various contributions to the RBC spectra can be compared with the recent calculational analysis of the same spectra (Gitter-Amir et al., 1976b). Finally, the method could

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[1] Abbreviations used: uv, ultraviolet; CD, circular dichroism; ORD, optical rotatory dispersion; RBC, red blood cell; Hb, hemoglobin.

easily be extended to spectra of abnormal red blood cells (e.g., sickle cell anemia, hereditary spherocytosis) in order to determine whether changes in hemoglobin or membrane protein conformation contribute to the pathological transformation.

Materials and Methods

Red blood cells were obtained from fresh human blood and washed three times with isotonic Hanks Balanced Salt Solution (Grand Island Biochemical Co., N.Y.). The RBC were sphered in 200 milliosmolar Hanks Balanced Salt Solution and washed twice with this medium. The RBC suspensions were checked for hemolysis by examining the absorption spectrum in the Soret region (415 nm) of supernatant obtained by pelleting the washed cells. Washing with the 200 milliosmolar medium continued until no hemoglobin was detected in the supernatant. The total hemoglobin concentration in the final RBC suspension was determined by hemolyzing a given volume of RBC pellet into a known volume of distilled water. The resultant hemoglobin solution concentration was determined from the Soret absorption peak using a molar extinction coefficient of 860 l. (mol of peptide)$^{-1}$ cm^{-1} or 132 000 l. (mol of heme)$^{-1}$ cm^{-1}.

Visible and near-ultraviolet absorption spectra were measured on a Cary 11 absorption spectrophotometer in several path length cuvettes as indicated below and in the Results.

Circular dichroism measurements were made on a Cary 6002 spectropolarimeter modified for capability of variable detector acceptance angle in a manner similar to that of Dorman et al. (1973). After removing the ORD modulator and conventional phototube, an end-window photomultiplier tube (Hammatsu R375 S) was mounted on a movable carriage whose position could be continuously varied from 0 to 30 cm from the sample cuvette by means of a large threaded screw through the bottom of the carriage. The positioning dial for turning the screw was outside the instrument so that it was unnecessary to open the phototube housing when changing the detector acceptance angle. The lens and mask separating the sample and modulator compartments were removed. Variable aperture optical diaphragms were mounted both on a modified sample holder and in front of the phototube. The sample diaphragm aperture could be varied over a range of diameters from 0 to 14 mm and was generally held fixed at a relatively small aperture (4 mm) in order to minimize artifacts due to reflections between the phototube and sample cuvette windows. The small sample diaphragm aperture also permits closer simulation of a point scattering source. The phototube aperture could be varied from 3 to 42 mm depending on the desired acceptance angle. Generally we did not go below a 6-mm aperture in order to avoid excessive noise. By varying phototube position and apertures, we were able to obtain well-defined acceptance half-angles ranging from less than 1° to greater than the critical angle of reflection (~45° in the far uv). When the phototube is in the close position and the phototube diaphragm aperture at its maximum opening, an acceptance half-angle of 60° is subtended by the phototube. As noted above, however, light scattering at angles greater than the critical angle of reflection (~45°) will not leave the exit window of the cuvette. This is considered in reporting the data in the Results where we use the actual half-angle measured by the phototube while keeping in mind the above point. Also taken into account was the effect of refractive bending of light upon passing from the aqueous medium in the cuvette through the quartz exit window and air to the phototube. Light scattered at a particular angle inside the cuvette thus emerges from the exit window at a slightly larger angle; for example, light scattered at 7° emerges at ~9°. This was considered in comparing experimental and calculated spectra. The CD instrument modifications described above were made by Aviv Associates of Lakewood, N.J., in collaboration with Mr. Jack Aviv.

The modified CD instrument was calibrated with *d*-camphor-10-sulfonic acid, 1 mg/ml (Eastman-Organic Chemicals), according to standard procedures. In addition a series of checks was made for baseline artifacts due to optically inactive scatterers (latex particles, 1- and 5.7-μm diameters, and aluminum oxide particles, 1-μm diameter) and absorbers ($K_2Cr_2O_7$) as described by Dorman et al. (1973) and found to be generally less than 1 mdeg per unit OD for a variety of detector geometries. The effect of changing detection geometry on optical activity of nonscattering protein solutions (hemoglobin) was also checked and no artifact was found.

Optical cuvettes used included circular, 1-cm path length, far-uv quartz, and also a removable window, circular, short-path cuvette with path length of 10 ± 3 μm, supplied by Hellma Cells, Inc., Jamaica, N.Y. Calibration of the cuvette with hemoglobin and bovine serum albumin solutions of known concentration indicated a path length of 13 ± 1.5 μm. Great care was required in filling and reopening the short-path cuvette in order to avoid hemolysis of the RBC. After placing a small drop of cell suspension (~5 μl) on the cuvette window having the raised ground-glass ring, the second window was placed over it very gently to avoid any pressure on the window which could hemolyze the cells. To check for hemolysis the cells were reopened after the spectral measurements by dropping the closed cuvette into 5 ml of buffered salt solution and letting the two windows spontaneously come apart. No significant hemolysis was found to occur when this procedure was used, whereas attempts to open the short path cuvette by sliding one window over the other gave appreciable hemolysis.

Suspensions of RBC in concentrated bovine serum albumin solutions (the high-refractive-index media) were prepared by mixing 25 μl of RBC pellet with the same volume of a 40% boving serum albumin solution in 200 milliosmolar Hanks Balanced Salt Solution. Approximately 5 μl or less of this suspension was then used to fill the short-path cuvette as described above.

Results

In the experimental results below we first present the ultraviolet CD spectra measured with a variety of detector geometries in order to determine the relative size of the scattering vs. flattening contributions for the red blood cell. These are shown in Figure 1 for detector acceptance half-angles of 1, 8, and 45° together with a hemoglobin solution spectrum for comparison. The differential scattering contribution to the spectrum, $[\theta_s]_\phi$, for a given acceptance half-angle, ϕ, is estimated by subtracting the absorptive contribution, $[\theta_A]$, from the total measured signal, $[\theta_T]$. Since for red blood cells most of the scattered light is concentrated in a narrow cone around the forward direction (Gitter-Amir et al., 1976b), an acceptance half-angle of 45° should yield a CD spectrum equivalent to the absorptive contribution, or $[\theta_T]_{45°} \simeq [\theta_A]$. This concentration of the scattering around the forward direction is also apparent in Figure 1, where the effect of collecting scattered light between 1 and 8° is considerably greater than between 8 and 45°. The differential scatter is shown for $\phi = 8°$ as $[\theta_s]_{8°}$ and is seen to be relatively small (1000 deg cm^2 dmol^{-1}). By contrast, the reduction in CD intensity due to flattening is very large (~20 000 deg cm^2 dmol^{-1}) as measured by the difference between the hemoglobin solution spectrum and the absorption

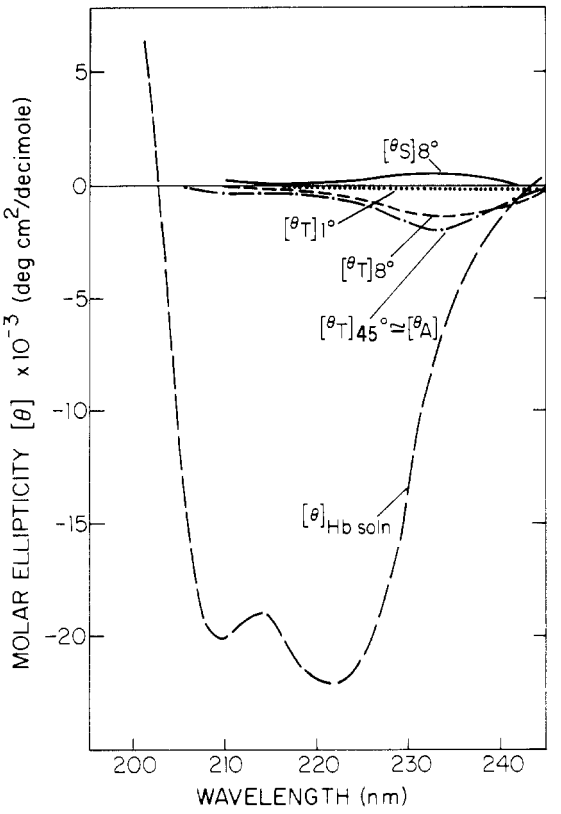

FIGURE 1: Experimental CD spectra of red blood cells measured at various detector acceptance half-angles, as indicated. $[\theta_T]$ is total measured CD, $[\theta_s]$ is scattering contribution, and $[\theta_A]$ is absorptive contribution to CD. $[\theta]_{Hb}$ curve represents CD of hemoglobin solution.

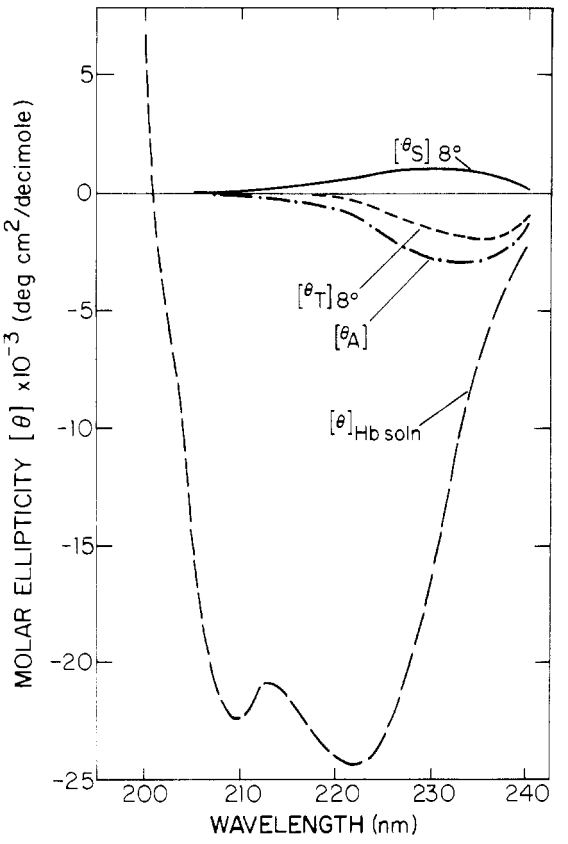

FIGURE 2: Calculated CD spectra of red blood cells from Gitter-Amir et al. (1976b). Legend same as in Figure 1.

contribution to the RBC spectrum.

In Figure 2 we show a similar set of calculated CD spectra from the work of Gitter-Amir et al. (1976b). Upon comparing the experimental and calculated spectra it is seen that the calculations have correctly predicted the relative size of the scattering and flattening effects, the sign of the scattering effects, the order of magnitude reduction in total CD intensity, and the large red shift in CD trough of the RBC spectrum relative to that of the solution.

We now attempt to correct the huge flattening distortion in the RBC spectra using highly concentrated cell suspensions in short light path cuvettes. We consider the visible absorption spectra of RBC since it shows large flattening effects and is easier to work with than the far uv. Results achieved here should demonstrate the underlying principles and be applicable to other spectral bands. Figure 3 compares the Soret region absorption spectra for RBC as measured in a conventional 1-cm cuvette with parallel measurements in an ultrashort path cuvette (~13 μm). The product of cell concentration and path length is comparable for the two cases, and the spectra are reported in terms of molar residue extinction coefficients. The ordinary hemoglobin solution spectrum is also shown as the correct reference spectrum we ultimately hope to simulate. We first note that the conventional 1-cm path RBC spectrum resembles an elevated featureless straight line due to the flattening of the absorption peak regions and elevation of the shoulder regions by scattering. We next note in the upper curve of Figure 3 the reappearance of the spectral bands of hemoglobin in situ in the RBC when a highly concentrated cell suspension is measured in an ultrashort light path (~13 μm). We now have a recognizable spectrum of hemoglobin in situ in the cell. However, the scattering is still present, causing the

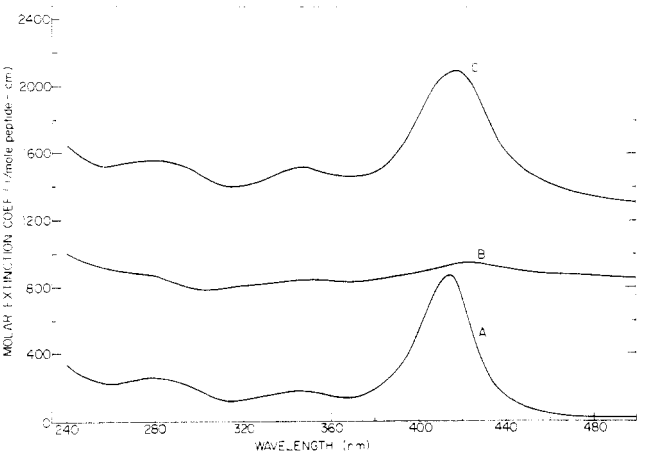

FIGURE 3: Effect of path length of flattened absorption spectrum of red blood cells. (A) Hemoglobin solution; (B) red blood cell suspension in 1-cm path length; (C) red blood cell suspension in short path length (13 μm) cuvette.

whole spectrum to be shifted upward, resting on the elevated scattering background. We can remove the scattering by suspending the cells in a nonabsorbing medium whose refractive index approximates that of the RBC, namely, a concentrated bovine serum albumin solution. The results are shown in Figure 4 in which an RBC spectrum in short path and with matched-medium refractive index is compared with a conventional spectrum in 1-cm path in buffered salt solution. The short-path RBC spectrum with matched-refractive-index medium is seen to have most of its scattering removed and now simulates the spectrum of a hemoglobin solution. We have thus effected an essentially complete correction of the hemoglobin

spectrum in situ within the red blood cell. The small remaining difference between the RBC short-path spectrum and the hemoglobin solution spectrum is roughly within experimental error of our combined concentration and short path length determinations.

In order to be sure that the effects reported above are bona fide flattening and scattering corrections of the spectra and not artifacts due to partial hemolysis of the cells in short path cuvettes, we have performed several controls. The Soret absorption of supernatants prepared from the same cells previously in short path cuvettes, then pelleted, were measured and compared with the total hemoglobin from deliberately lysed cells. Less than 2% hemolysis had occurred. Further controls were done by diluting the cells which had been in short path cuvettes to an appropriate concentration ($\sim 1/1000$ pellet) and remeasuring their visible absorption spectrum in a 1-cm path cuvette. If any significant hemolysis had occurred, it would show up as the appearance of the Soret band. The resultant spectrum was quite similar to the totally flattened original spectrum of the dilute RBC in 1-cm path cuvettes with no evidence of the Soret band. Thus hemolysis had not occurred and free hemoglobin could not account for the spectral corrections we obtain.

Discussion

We have now obtained an experimental absorption spectrum of hemoglobin in situ in the intact red blood cell which is essentially free of the gross distortions caused by absorption flattening and light scattering and which closely resembles the familiar spectrum of a dilute hemoglobin solution. In addition we have obtained ultraviolet circular dichroism spectra with differential scattering removed, thus allowing a resolution of the scattering and flattening effects in the CD spectrum of an intact cell. We have thus far had some limited success in our efforts to extend experimental flattening corrections to the ultraviolet CD. Preliminary measurements down to 230 nm in the 13-μm cuvette gave a CD spectrum with recovery of most of the intensity lost by flattening. Below 230 nm the sample became opaque due to the high uv absorption. Future refinement of the technique with yet shorter path lengths should make possible the complete correction of uv CD spectra from which conformational information can be directly obtained.

The data obtained in Figure 1 with variable detection geometry experimentally demonstrate that the effect of differential scatter on the RBC CD spectra is relatively small and the order of magnitude reduction of CD intensity relative to a hemoglobin solution is caused by the flattening effect. When the differential scatter is removed with large angle detection geometry, the resultant spectrum $[\theta_A]$ is still an order of magnitude lower than the molar ellipticities of hemoglobin in solution. We thus caution against attempting to give a structural interpretation to spectra of absorbing particles which have had only their differential scatter removed, since such spectra may still be significantly distorted by the flattening effects. The experimental results in Figure 1 are seen to confirm the previous calculational analysis of the RBC CD spectrum, Figure 2 (Gitter-Amir et. al., 1976b), in almost all respects including the relative importance of flattening and scattering, the shifts in RBC relative to hemoglobin solution spectra, the sign and the shape of the differential scatter, $CD_{scat\ 8°}$, and the changes with variation of detection geometry.

Our method for collecting scattered light with variable CD detection geometry is essentially the same as that described by Dorman et al. (1973), but with one important refinement. In our system we have included variable aperature diaphragms after the sample cuvette and in front of the end window phototube, thus permitting well-defined acceptance angles to be obtained down to less than 1°. This is important for large particle scattering which is known to be concentrated around the forward direction. For red blood cells, for example, Gitter-Amir et al. (1976b) have calculated (and we have demonstrated in Figure 1) that most of the scattered light falls within a cone of less then 8° from the forward direction. The CD variable detection geometry described by Dorman et al. (1973) operates with a minimum acceptance angle of 11° and would thus not detect most of the differential scatter of particles such as red blood cells.

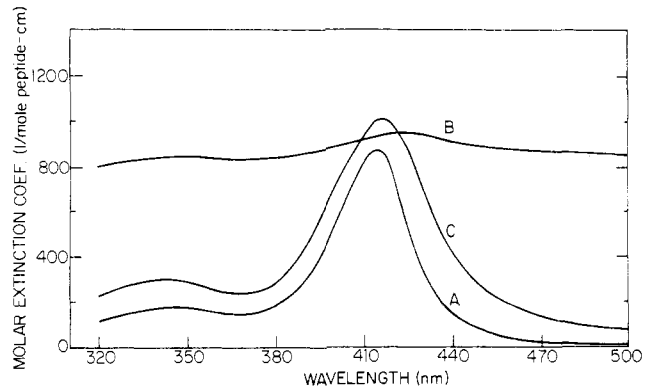

FIGURE 4: Effect of high suspending medium refractive index and short-path cuvette on scattering and flattening in absorption spectrum of red blood cells. (A) Hemoglobin solution; (B) red blood cell suspension in buffered salt solution in 1-cm path length; (C) red blood cell suspension in high refractive index medium, in short path length (13 μm) cuvette. High refractive index medium is concentrated bovine serum albumin in buffered salt solution.

The technique of matching medium and particle refractive index in order to reduce scattering has been used previously by Rubinstein and Ravikovich (1946) and Barer (1955) on red blood cells with resultant partial recovery of the Soret absorption band. We have simply used this approach in conjunction with the short-path cuvettes to simultaneously reduce both scattering and flattening effects. The variation of medium refractive index also offers some interesting possibilities of studying the subtleties of the scattering process; for example, by adjusting the relative particle-to-medium refractive index to specific intermediate values and observing the resultant scattering effects on the spectra, it should be possible to move along various regions of the oscillating Mie scattering curve (scattering cross section vs. relative refractive index). This in turn should result in sign changes in differential scatter and oscillation of scattering extinction loss.

The method we use for reduction of absorption flattening effects has not been previously described and the present technique represents the first quantitative experimental approach to recovery of spectral flattening losses. Our method was partly inspired by Duysens' (1956) calculation of a hypothetical solution spectra of pigmented cubes. Duysens considered a model in which a suspension of oriented cubes was pressed to one face of the cuvette thus forming a solid layer in which the pigment distribution is homogeneous. He then used this model to calculate the equivalent solution spectrum. We have tried to do the same thing experimentally by using highly concentrated cell suspensions in short-path cuvettes in order to approximate a homogeneous distribution of pigment. In addition to the Duysen's calculational model, there has been one experimental approach that foreshadowed our use of short-path cuvettes, namely, that first reported by Jope (1949)

using vertical illumination through settled layers of red blood cells. Jope (1949) achieved the partial recovery of the Soret band in settled layers of cells. However, he did not quantitate how much of the flattening he had removed. Similar measurements on settled cells using vertical illumination were later made by Lothian and Lewis (1956) and McCrae et al. (1961).

The problem of interpreting the spectra of particulate biological suspensions is becoming increasing relevant as interest grows in understanding the state and response of macromolecules in situ in functioning biological complexes, such as membranes, viruses, and intact cells. For the red blood cell the state of aggregation and conformation of hemoglobin in the normal and pathological (e.g., sickle) cell are certainly questions of great physiological and biochemical interest. The last 5 years have seen a substantial improvement in our ability to obtain useful information from the spectra of such systems, principally using calculational approaches. The present results point the way to experimental resolution of these spectra. These techniques should find wide use among bioscientists interested in probing the conformational state of proteins, nucleic acids, and other bioactive molecules in their native environment.

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