APPLICATION OF A NEW MODULATION METHOD FOR LINEAR DICHROISM STUDIES OF ORIENTED BIOPOLYMERS IN THE VACUUM ULTRAVIOLET

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To understand the relationship between spectral characteristics and conformational properties of biopolymers, detailed knowledge of ultraviolet spectra extended to the vacuum region is required. The classical absorption spectra, even with polarized light, are represented by broad structures. On the other hand, the small difference in absorption of parallel and perpendicular polarized light, known as linear dichroism (LD), reveals a much finer structure and also offers the advantage of having a sign associated with it. Specifically, such linear dichroism measurements are powerful for the resolution of overlapping absorption bands and thus should be particularly useful in theoretical and experimental investigation of biopolymer structure.

Several devices for LD measurements have been used for studying anisotropic crystals and polymer films.[1-7] Recently, Jaffe, Jaffe, and Rosenheck[7] proposed an ingenious device that consists of a polarizer and a multiwave plate. However, their device can give the full measurement of the LD only at the discrete, fixed wavelengths at which the plate has a half-wave retardation, and a correction has to be applied that depends on the resolution of the spectrophotometer associated with it. The actual instrument is limited to 1800 Å, but, even in principle, it will not work in the region of the line spectrum of a hydrogen lamp, that is, below 1670 Å.

The present method, which will be sketched below and described in detail elsewhere, allows LD measurement over a continuous range of wavelengths and, furthermore, the line spectrum of hydrogen may be exploited. It has a high resolving power, limited only by the resolution of the monochromator (about 2.5 Å), which is independent of wavelength. It is characterized by a high sensitivity of detection—LD 0.07 per cent (up to 1670 Å) and 0.2 per cent (1670–1490 Å). This is of special importance for the study of biopolymers, since their degree of orientation is often low. The method allows direct detection of the degree of polarization $p = (T_\perp - T_{||})/2T_m$, which is zero for a random conformation or orientation, and only ordered helical macromolecules with oriented chromophores can be distinguished.

In this paper we present the results of investigations that show four well-resolved polypeptide bands, and that demonstrate the presence in polynucleotides of new, far-ultraviolet transitions which can be related to the interactions of monomers in the helical array.

Methods.—A block diagram of the apparatus is presented in Figure 1. Light from a hydrogen discharge lamp S is dispersed by a normal incidence grating monochromator M. The light is made fairly parallel at the exit by the lens L_2 and is polarized by being passed through a synthetic quartz Rochon prism. This prism is mounted inside a pulley coupled

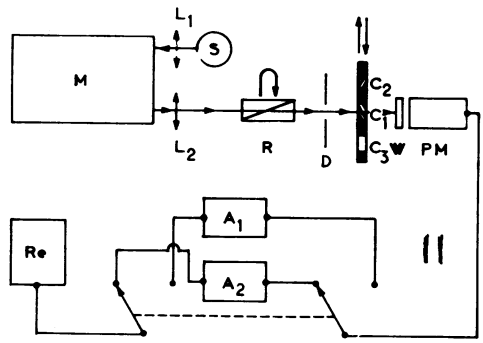

FIG. 1.—Block diagram of the apparatus: S, light source; L_1, L_2, lenses; M, monochromator; R, rotating Rochon prism; D, diaphragm; C_1, sample and fluorite window; C_2, fluorite window; C_3, hole; W, sodium salicylate-coated window; PM, photomultiplier; A_1, alternating-current amplifier and synchronous detector; A_2, direct-current amplifier; Re, recorder.

to a synchronous motor placed outside the vacuum chamber and rotates at 15 cps, which corresponds to a modulation frequency of polarization at 30 cps. Furthermore, a magnetic contact provides a signal for synchronous detection. A three-position sample holder permits measurements to be made consecutively on the window with the oriented film and on an identical reference window without film; the third position serves to control the intensity and modulation of the beam without any absorbing plate.

When the light beam emerging from the monochromator of intensity I_0 passes through the polarizer and a dichroic sample, its intensity is reduced and generates a signal determined by the equation:

$$I = \text{const.} \times \frac{I_0}{4} [\Sigma E + \Delta E \cos 2\,\Omega t],$$

where

$$\Sigma E = \exp(-(4\pi K_\perp l)/\lambda) + \exp(-(4\pi K_\parallel l)/\lambda),$$

$$\Delta E = \exp(-(4\pi K_\perp l)/\lambda) - \exp(-(4\pi K_\parallel l)/\lambda),$$

$$\Omega = 15 \times 2\pi,$$

$$\text{const.} = kS,$$

and S is the sensibility of the photomultiplier, $K_\perp$ and $K_\parallel$ are the two extinction indices of the anisotropic medium, and l is its thickness.

The signal may be applied separately to two amplifiers (Fig. 1), either a d-c amplifier that allows the measurement of $kS\ (I_0/4)\ \Sigma E$, or to an a-c synchronous amplifier that gives $kS\ (I_0/4)\ \Delta E$. The possibility of averaging the transmittance of the sample allows the measurement of ΣE.

This yields:

(1) the average transmittance,

$$T_m = \frac{\Sigma E}{2} = \frac{\exp(-(4\pi K_\perp l)/\lambda) + \exp(-(4\pi K_\parallel l)/\lambda)}{2},$$

and (2) the degree of polarization of the light emerging from the sample, polarizance

$$p = \frac{T_\perp - T_\parallel}{2T_m} = \frac{\exp(-(4\pi K_\perp l)/\lambda) - \exp(-(4\pi K_\parallel l)/\lambda)}{\exp(-(4\pi K_\perp l)/\lambda) + \exp(-(4\pi K_\parallel l)/\lambda)},$$

where $T_\perp$ and $T_\parallel$ are the transmittances of the two orthogonal states of polarization perpendicular and parallel, respectively, to the axis of orientation of the polymer. The degree of polarization p is a measure of the LD, which is defined classically as $K_\perp - K_\parallel$.

The sensitivity of the method is determined by the value of the parameter p obtained in the absence of any sample; this so-called residual p was smaller than 0.07% in the con-

tinuous region of the hydrogen spectrum. This is further confirmed by the low value of p; less than 0.1% was obtained with a DNA film in the disordered random coil conformation prepared from a solution containing 80% trifluoroethanol. The amount of stray light was less than 0.02%.

Sample preparation: Oriented polymer films were prepared by unidirectional stroking with relatively concentrated solutions (1–1.5%) deposited on a fluorite disk under a current of air to hasten evaporation. Poly-γ-ethyl-L-glutamate was dissolved in chloroform. The solvent used for poly (A + U) and DNA (thymus) was an aqueous solution containing 0.002 M KF at pH 7.0. Under these conditions and at room temperature, polyadenylate and polyuridylate form double-stranded helical complexes.[8]

The orientation of poly (A + U) was relatively good (p about 0.4–0.5) and was not changed at a low degree of relative humidity (r.h.). In contrast, DNA-oriented samples decrease in $\Delta T/2T_m$ values (p about 0.2) when exposed to low r.h. conditions under vacuum, in agreement with Falk's[9] observation. Because of high values of the coefficient of molar absorption ϵ in the vacuum UV region, thin films were prepared and only well-oriented uniform films with a minimum amount of scattering were selected for study. The orientation of the sample was controlled with a polarizing microscope.

Results.—Polypeptides: The degree of polarization p and the absorption spectrum of an oriented film of helical poly-γ-ethyl-L-glutamate are compared in Figure 2. It is immediately seen that in the spectral region from 1500 to 2500 Å the spectrum of p reveals four well-resolved sharp bands of different sign contribution, whereas the structure of the absorption spectrum appears only as a monotonic change of intensity. One can consider separately each absorption band of the polypeptide spectrum. The curve of the degree of polarization clearly indicates the presence of the weak first band of negative p sign centered in the region of 2200–2300 Å. The negative sign allows the transition to be assigned as perpendicular to the helical axis. It is a weak band with an ϵ previously estimated to be about 100 by Hamm and Platt[10] and an oscillator strength determined by Gratzer *et al.*[4] to be about 0.004 in helical polypeptides. This is in agreement with its assignment as an $n \rightarrow \pi^*$ transition,[1, 10] whose transition moment must be out-of-plane. This was demonstrated experimentally by Gratzer *et al.*;[4] from theoretical considerations, Schellman and Oriel[11] pointed out its importance for rotatory power of polypeptides and proteins. However, since this first band is rather broad, it may be considered as composed of two perpendicular overlapping bands, e.g., of a mystery band of Rhodes and Barnes[12] and of an $n \rightarrow \pi^*$ band.

The next higher energy band is observed from 2160 to 2020 Å, centered at 2090 Å. This intense narrow band has a positive p sign and hence is polarized parallel to the helical axis. At 2160, 2020, and 1720 Å the contribution of the two components is identical and the value of p drops to zero. The region from 2020 to 1720 Å has a negative p sign centered at 1900 Å and thus can be assigned as a perpendicular band. The presence of these two bands confirms the assumptions of the exciton splitting of the $\Pi_0 \rightarrow \Pi^*$ transition (NV_1) of the monomer, which lies in the plane of the amide group, into two dipole-allowed transitions in the α-helix, one polarized perpendicular to and the other parallel to the helical axis.[13] The data agree well with the splitting and polarization predictions of Moffitt[13] and with previous experimental results obtained with different methods by Gratzer *et al.*,[4] Holzwarth and Doty,[14] and Jaffe, Jaffe, and Rosenheck.[7] How-

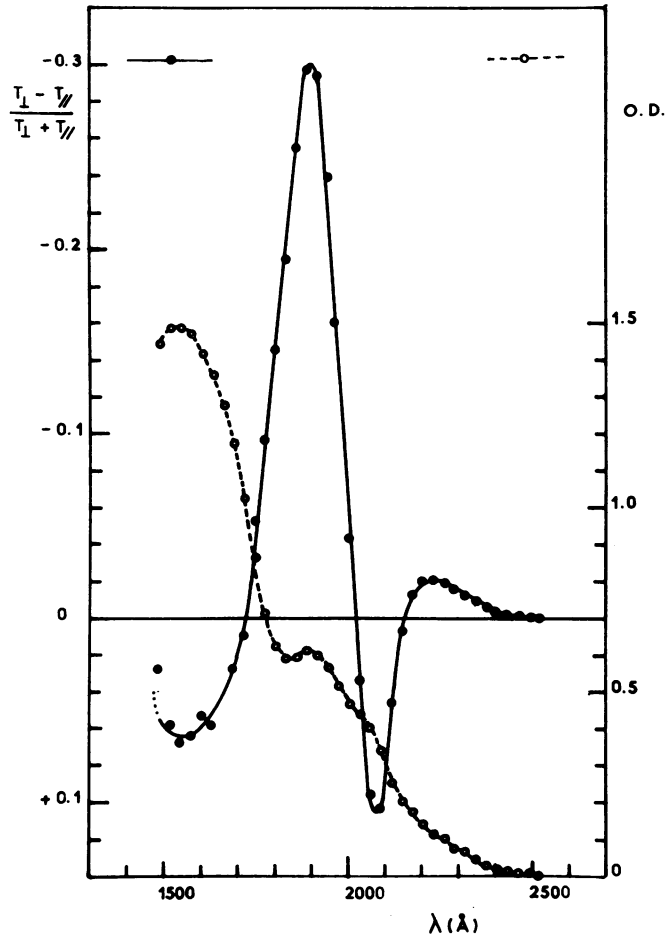

FIG. 2.—The spectra of oriented poly-γ-ethyl-L-glutamate: (—●—●—), linear dichroism transmittance expressed as $p = (T_\perp - T_{||})/2T_m$; —(O— —O—), conventional absorbance (optical density).

ever, the high resolution of the present method allows the separation of overlapping bands and the determination of the direction of transition dipole moments relative to the polymer axis.

In the region of 1600 Å we observe the presence of a new band. The positive p sign indicates that the transition dipole moment is parallel to the helical axis and is certainly in the plane of the amide chromophore.

For the assignment of this new band one has to consider the following possibilities:

(1) an $n' \rightarrow \Pi^*$ transition that occurs at 1650 Å according to the studies by Peterson and Simpson[1] on simple myristamide,

(2) an $n \rightarrow \sigma^*$ transition occurring at 1500 Å, which, according to Tinoco, Halpern, and Simpson,[16] is polarized in the amide plane and perpendicular to the carbonyl bond,

(3) or, finally, a theoretically predicted band not directly observed, but assumed to occur at 1520 Å; it is assigned as a $\Pi+ \rightarrow \Pi^*$ transition (NV_2) by Peterson and Simpson[1] and Caldwell and Eyring.[17] Since this new 1600-Å band is polarized parallel to the helical axis, the first assignment ($n' \rightarrow \Pi^*$) is excluded, because such an assignment would involve the orientation of the dipole moment perpendicular to the amide plane. We are left with a choice between the two assignments $n \rightarrow \sigma^*$ and NV_2 transition. Both these assignments will be in agreement with the transition dipole moment orientation parallel to the helical axis. This new band may be of importance for the studies of polypeptides and protein conformation.

Polyribonucleotides: The spectra of the oriented polyriboadenylate and polyribouridylate double-stranded helical complex and the curve of its degree of polarization, $p = \Delta T/2T_m$, are shown in Figure 3.

In the long-wavelength region, the main 2600-Å band shows great similarity

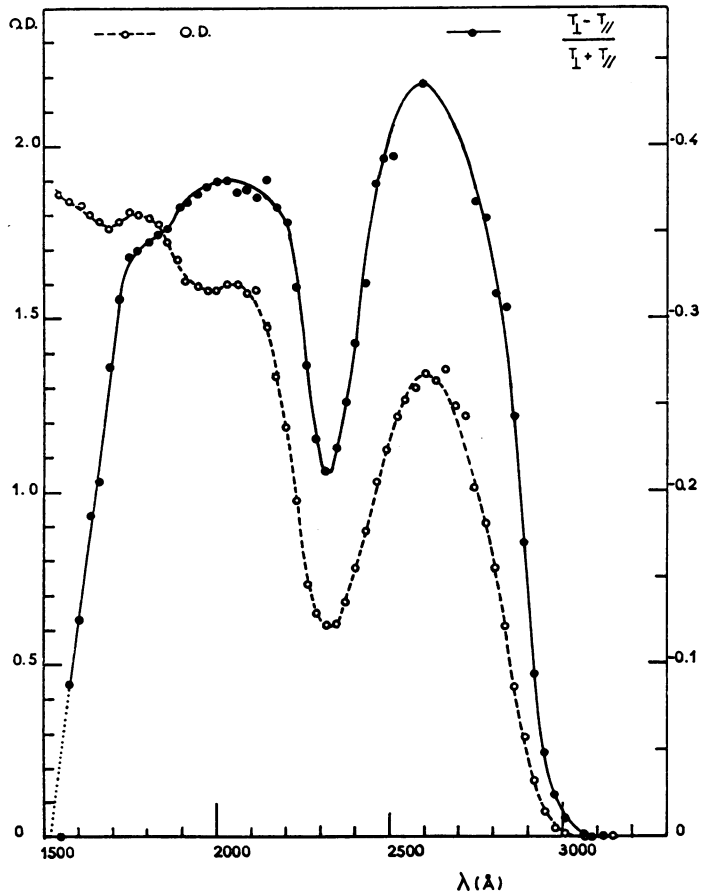

FIG. 3.—The spectra of oriented poly (A + U) double-stranded helical complex: *solid line,* degree of polarization expressed as a ratio $(T_\perp - T_{||})/2T_m$; *dashed line,* absorbance (optical density).

between the absorption spectrum and the spectrum of the degree of polarization (p), which is of negative sign. Thus the predominant contribution to the absorption in this region arises from electronic transitions perpendicularly oriented to the helical axis. This is also true for the transitions located at 2060 and 1770 Å. However, below 1800 Å a significant decrease of the perpendicular component of the polarization appears, whereas the unpolarized absorption is still rising to a higher value. Very likely one should expect positive p values on the high-energy side. Our results in the near UV region confirm previous evidence, indicating possible correlation of the spectra of purine and pyrimidine bases to the absorption bands of benzene by Mason,[18] Clark and Tinoco,[19] and Miles, Robins, and Eyring.[20] According to these authors, the near UV 2600-Å band will be derived from the benzene B_{2u} and B_{1u} transitions and, of course, as a $\Pi \rightarrow \Pi^*$ transition polarized in the plane of the base.

Furthermore, there is now convincing evidence from X-ray data on films of double-stranded helical RNA and poly (A + U)[21] that the bases are almost perpendicular to the helical axis. We have not detected the presence of an n–π^* transition due to the nonbonding electron pairs of the N and O atoms, which are expected to occur at longer wavelengths and which must be perpendicular to the base plane.[5,18] Further detailed studies are necessary.

The assignment of the 2000-Å band in purine and pyrimidine bases and nucleosides was made by correlation to the doubly degenerate E_{1u} band of benzene,[20] which in uridine can be detected at 2100 and 1900 Å, and in adenosine at 2060 and 1900 Å.[22] In the poly (A + U) double-stranded helical structure we have also observed two maxima in the absorption at about 2060 and 1770 Å. It is of interest that in the high-energy region, below 1770 Å, the sharp decrease of the $\Delta T/2T_m$ curve will lead to a change of sign, i.e., to a contribution of a parallel polarized transition. In addition to the negative 1770-Å band, the appearance of a positive contribution to the p curve may be interpreted as indicative of the exciton splitting of the monomer transition into two parallel and perpendicular components. Simple consideration of the splitting energy V_{12} by the dipole-dipole approximation indicates that the splitting will be proportional to the intensity of the transition, i.e.,

$$V_{12} = \frac{I}{R^3}\left[\boldsymbol{\mu}_1 \cdot \boldsymbol{\mu}_2 - \frac{3(\mathbf{R}_{12} \cdot \boldsymbol{\mu}_1)(\mathbf{R}_{12} \cdot \boldsymbol{\mu}_2)}{R^2}\right].$$

Thus one can expect that the splitting must be very important in the case of electronic transitions of strong intensities such as those occurring below 2000 Å when compared to the 2600-Å weaker band. The presence of optically active bands in the far ultraviolet region and its importance for the polynucleotide helical conformation investigation was predicted on the basis of the application of reciprocal relations between circular dichroism and optical rotatory dispersion (ORD).[23]

DNA: The DNA spectra were measured at higher and lower ratios of r.h. The samples maintained at a high ratio of r.h. were much better oriented (see *Methods*). However, the use of the present method allows the study of macromolecules having a relatively small per cent of orientation. Figure 4 shows the

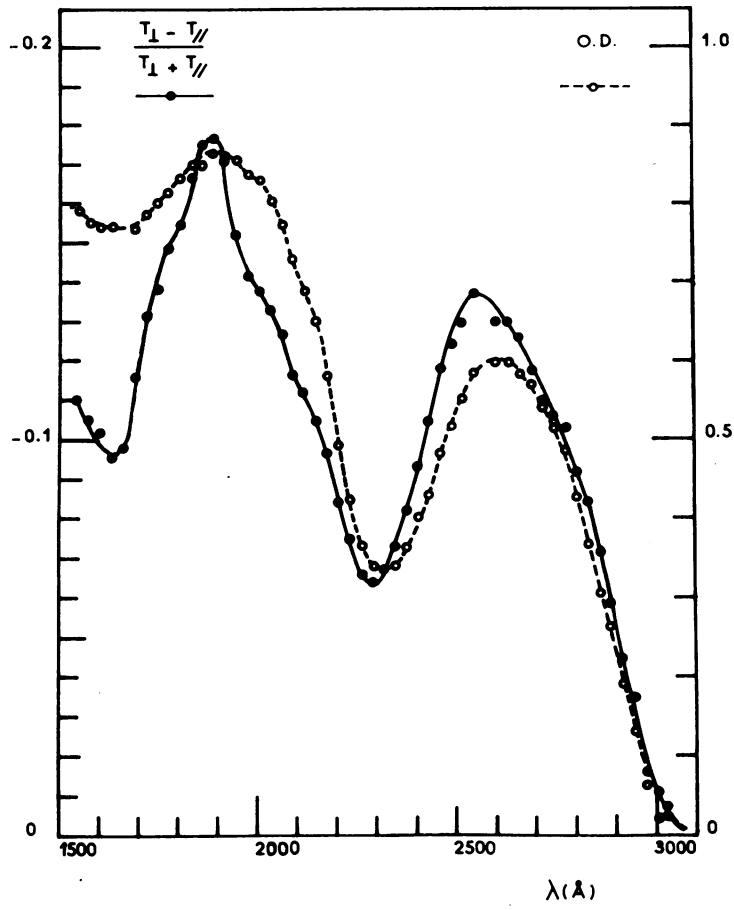

FIG. 4.—The spectra of oriented thin DNA (thymus) film: *solid line*, degree of polarization; *dashed line*, absorbance (optical density).

spectra of a DNA film. The first, near UV band has a negative sign for the ratio $\Delta T/2T_m$, confirming previous evidence that the main contribution to the absorption spectra in mixed bases arises from $\Pi \rightarrow \Pi^*$ transitions polarized in the plane of the bases and perpendicular to the helical axis.[18–20] However, we observe that the maximum of the degree of polarization $\Delta T/2T_m$ is at 2555 Å and does not correspond to the maximum of an ordinary absorption curve. This shift may be interpreted as indicating a contribution of other transitions polarized parallel to the helical axis and of weaker intensity, in agreement with Kasha.[24]

The second main band situated in the far ultraviolet is complex and shows the presence of the following four singularities: At 2130, 2000, and 1770 Å shoulders can be clearly situated, and at 1890 Å a distinct sharp maximum appears. These details are difficult to observe in conventional absorption. The assignment of these four maxima can be made on the basis of the correlation of the mixed-base spectra with the degenerate E_{1u} band of benzene.[20] The most interesting fact is that below 1800 to 1650 Å the degree of polarization dras-

tically decreases. Again we interpret this sharp change as due to the contribution of another parallel transition, not fully observable, which may reflect the splitting of the monomer high-energy band into two perpendicular and parallel components. The shape of the curve, in this spectral region, is similar to that of poly (A + U) (Fig. 3), but the decrease is less marked in DNA owing to the smaller degree of orientation and perhaps to the mixed nature of the four different bases. The presence of optically active far ultraviolet bands dependent on a DNA conformation was predicted on the basis of the application of the reciprocal relation between absorption (circular dichroism) and dispersion (ORD).[23]

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[1] Peterson, D. L., and W. T. Simpson, *J. Am. Chem. Soc.*, **79**, 2375 (1957).
[2] Seeds, W. E., *Progr. Biophys.*, **3**, 27 (1951).
[3] Stewart, R. F., and N. Davidson, *J. Chem. Phys.*, **39**, 255 (1963).
[4] Gratzer, W. B., G. M. Holzwarth, and P. Doty, these PROCEEDINGS, **47**, 1785 (1961).
[5] Rich, A., and M. Kasha, *J. Am. Chem. Soc.*, **82**, 6197 (1960).
[6] Yamada, R., and R. S. Stein, *J. Polymer Sci.*, **B2**, 1131 (1964).
[7] Jaffe, J. H.. H. Jaffe, and K. Rosenheck, *Rev. Sci. Instr.*, **38**, 935 (1967).
[8] Massoulié, J., Thesis, University of Paris (1966).
[9] Falk, M., K. A. Hartman, Jr., and R. C. Lord, *J. Am. Chem. Soc.*, **85**, 391 (1963).
[10] Hamm, J. S., and J. R. Platt, *J. Chem. Phys.*, **20**, 336 (1952).
[11] Schellman, J. A., and P. Oriel, *J. Chem. Phys.*, **37**, 2114 (1962).
[12] Rhodes, W., and D. G. Barnes, *J. Chim. Phys.*, in press; Barnes, D. G., and W. Rhodes, manuscript in preparation.
[13] Moffitt, W., these PROCEEDINGS, **42**, 736 (1956).
[14] Holzwarth, G., and P. Doty, *J. Am. Chem. Soc.*, **87**, 218 (1965).
[15] Moffitt, W., these PROCEEDINGS, **42**, 736 (1956).
[16] Tinoco, I., Jr., A. Halpern, and W. T. Simpson, in *Polyamino Acids, Polypeptides and Proteins*, ed. M. A. Stahmann (Madison: Univ. Wisconsin Press, 1962), p. 147.
[17] Caldwell, D. J., and H. Eyring, *Ann. Rev. Phys. Chem.*, **15**, 281 (1964).
[18] Mason, S. E., *J. Chem. Soc.*, 2071 (1954).
[19] Clark, L. B., and I. Tinoco, Jr., *J. Am. Chem. Soc.*, **87**, 11 (1965).
[20] Miles, D. W., R. K. Robins, and H. Eyring, these PROCEEDINGS, **57**, 1138 (1967).
[21] Abott, S., M. H. F. Wilkins, W. Fuller, and R. Langridge, *J. Mol. Biol.*, **27**, 535 (1967); Fuller, W., private communication.
[22] Voet, D., W. B. Gratzer, R. A. Cox, and P. Doty, *Biopolymers*, **1**, 193 (1963).
[23] Brahms, J., *J. Mol. Biol.*, **11**, 785 (1965).
[24] Kasha, M., *J. Chim. Phys.*, **51**, 916 (1961).