THE ORIENTATION OF DNA WITHIN 80-ANGSTROM CHROMATIN FIBERS

DAVID B. DUSENBERY and ROBERT B. URETZ

From the Department of Biophysics, The University of Chicago, Chicago, Illinois 60637.
Dr. Dusenbery's present address is the Division of Biology, California Institute of Technology, Pasadena, California 91109.

ABSTRACT

Squashing salivary glands of *Chironomus thummi* larvae, *Amblystoma tigrinum* erythrocytes, or *Spirostromum* frequently results in stretched chromatin having highly oriented DNA as determined by polarized fluorescence microscopy of acridine orange–stained preparations. The examination of such material from *C. thummi* in the electron microscope indicates that the individual chromatin fibers have an average thickness of 80 A as is usually found in embedded and sectioned material. It is thus concluded that the DNA lies nearly parallel to the axis of these chromatin fibers. Detailed calculations of the polarization expected from various models of DNA packing are contained in an appendix.

INTRODUCTION

It has been known for some time that chromatin of animal cells consists largely of submicroscopic nucleoprotein fibers. Extensive study has failed to resolve the question of their thickness in vivo. Embedding and sectioning the material results in fibers about 80 A (frequently rounded off to 100 A) in thickness (Kaye and McMaster-Kaye, 1966; Stevens and Swift, 1966; Wolfe, 1969). On the other hand, spreading on an air-water interface usually results in fibers averaging about 250 A thick (Wolfe, 1969). There is evidence suggesting that the thicker fibers are formed from naturally occurring thin fibers when the cell is disrupted during the spreading technique (Wolfe and Grim, 1967) and, on the other hand, evidence that the thinner fibers are formed from naturally occurring thick fibers during fixation in ordinary buffers (Ris, 1968). At the present time it is impossible to decide which type of fiber more accurately represents the in vivo situation, although it seems probable that both are closely related to the in vivo structure, and thus both are worthy of study.

Even less is known concerning the organization of DNA in nucleoprotein fibers. The various models which have been proposed include DNA lying parallel to the fiber axis (Wolfe, 1965), DNA coiled into a helix (DuPraw, 1965), and DNA lying along the axis with frequent side loops (Ris, 1967). Virtually the only evidence that has previously been obtained is that the length of the chromatin fibers appears to be much less (10- to 100-fold) than the length of DNA in them (DuPraw, 1966, 1968).

In addition, some indirect evidence comes from observations of DNA packing in related types of nucleoprotein fibers. In the first case, DNA and nucleoprotein fibers of the sperm of Orthoptera seem to lie predominantly parallel to the sperm axis (MacInnes and Uretz, 1968; Gibbons and Bradfield, 1957) and thus the DNA should lie predominantly parallel to the axis of the nucleoprotein fibers of these sperm. Secondly there is evidence that in purified nucleohistone the DNA is *not* parallel to the fiber axis (Ohba, 1966; Pardon et al., 1967). However, the model proposed by Pardon et al. predicts that the UV dichroism of

nucleohistone should be oriented perpendicular to that of purified DNA, and this was not observed by Ohba. In any case it cannot be assumed that the configuration of DNA in the nucleoprotein of either sperm or extracted nucleohistone is the same as that of chromatin fibers of somatic cells *in situ*.

In this work, we have studied the problem of DNA packing in 80 A chromatin fibers by attempting to determine the orientation of the DNA with respect to the fiber axis. A method of attacking this problem was suggested by the observation that squashing trypsin-treated *Drosophila* salivary glands frequently results in the production of regions of highly oriented DNA as observed by polarized fluorescence of acridine orange–stained preparations in the optical microscope (MacInnes and Uretz, 1966). It appeared that the trypsin was necessary to weaken the nuclear membrane, but whether or not it was also required to disrupt the fibers was not determined.

This observation indicated that, during the squashing process, forces are generated which often cause DNA, which was previously in an unoriented state, to become oriented. The appearance of these areas is frequently such as to indicate that the mass of chromatin has been stretched between two objects in the preparation. In material, such as this, which is composed of a mass of fibers, one would expect that stretching of the mass would tend to orient the individual fibers parallel to the direction of stretching. This could take place with or without elongation of the individual fibers, depending upon whether they are more likely to break, bend, slip or lengthen in response to stresses in the material. As an example, a fish net can be stretched a certain extent, resulting in orientation of individual pieces of twine without elongating them.

We reasoned that if DNA is held in some coiled configuration within the fibers, then by squashing without trypsin one should not be able to orient the DNA, unless the individual fibers were elongated during squashing so that the DNA lies straight within them. If substantial elongation of the individual fibers in fact took place, it would presumably result in a decreased average thickness which could be determined in the electron microscope. On the other hand, if the DNA is parallel to the fiber axis it should be possible to orient the DNA without the use of trypsin and without lengthening individual fibers.

The orientation of DNA was determined by measuring the direction and magnitude of polarization of fluorescence from acridine orange intercalated into the DNA. It was necessary to use this more sensitive technique rather than the more direct ones of UV-dichroism and birefringence because of the relatively small amount of DNA to be analyzed. This method is not in disagreement with the other methods where they have been compared. For instance, polarized fluorescence studies of *Drosophila* and *Chironomus* polytene chromosomes indicate no over-all alignment of DNA in the bands (MacInnes and Uretz, 1966, and unpublished observations), and this agrees with UV-dichroism studies on *Chironomus* (Wetzel et al., 1969). In addition, polarized fluorescence studies of orthopteran sperm heads indicate a high degree of DNA alignment parallel to the sperm axis (MacInnes and Uretz, 1968), and these results are consistent with the birefringence and polarized UV-irradiation measurements of Inoué and Sato (1966), although the "preliminary model" of DNA packing which Inoué and Sato presented to explain their data is not consistent with the magnitude of polarized fluorescence observed.

MATERIALS AND METHODS

Polarized Fluorescence

A *Spirostomum*, a salivary gland from larvae of *Chironomus thummi*, or a drop of blood from *Amblystoma tigrinum* was added to a drop of distilled water containing 100 μg/ml of purified acridine orange (Freifelder and Uretz, 1966) on a microscope slide. A cover slip was then pressed tightly over the drop and the edges were sealed with petroleum jelly (Vaseline). The slide was immediately examined in the fluorescence microscope, and measurements of the degree of polarization were made on all areas which looked as if the chromatin had been stretched. Individual measurements were well separated from one another, and four to eight different preparations of each material were examined.

The methods and instrumentation used to measure fluorescence polarization in the microscope have been previously described (MacInnes and Uretz, 1967). This instrument is basically a fluorescence microscope in which light from a selected area of the specimen can be passed through a rotating (6 rpm) dichroic filter to a photomultiplier tube. If the fluorescence is polarized the light intensity is modulated by the rotating filter, and the extent of modulation is a measure of the degree of polarization. We thus define the polarization, R, as the difference between maximum and minimum intensities divided by the maximum intensity, all multiplied by 100%.

Two modifications were made in the instrumentation for this study. One was the use of a 1.4 NA con-

denser without immersion oil. And the other was the replacement of the photometer with a lock-in amplifier (PAR model HR-8) and a light chopper, which interrupted the exciting light beam 1100 times per second and simultaneously provided a reference signal for the amplifier. This arrangement improved the signal-to-noise ratio of the system.

With the objective lens used (43 ×, 0.85 NA) and the specimen mounted in water the cone of light (from the specimen) which enters the instrument has a half-angle of 40°. Under these conditions the expected polarization of fluorescence from acridine orange completely intercalated in perfectly aligned DNA is calculated to be about 90% (equation 5 from MacInnes and Uretz, 1968).

The *Spirostomum* and *Amblystoma* were obtained from Turtox, Chicago, Ill. The *Chironomus* stock was obtained from Stevens and Swift (1966).

Electron Microscopy

The preparation of a squash so that a certain area could be viewed first by optical and subsequently by electron microscopy presented formidable obstacles. Fortunately, most of these had been previously overcome (Bloom and Leider, 1962).

A *Chironomus* salivary gland was dissected out in Ringer's solution and placed on a cover slip which had been treated with a silicone parting agent and then carbon coated (Bloom and Özarslan, 1965). This cover slip was pressed on top of a second, similarly treated cover slip which held a drop of 10% formalin (or 4% formaldehyde in 0.1 M sodium phosphate buffer). The two cover slips which remained stuck together were placed in a container of the same formaldehyde solution. After about 0.5 hr the cover slips were frozen in an ethanol–dry ice mixture and separated with a razor blade. Each cover slip was rinsed in distilled water and mounted in an acridine orange solution (10 μg/ml distilled water). Under the fluorescence microscope an area showing polarized fluorescence was located and the position was marked by scratches on the cover slip. The specimen side of the cover slip was treated with 1% osmium tetroxide, dehydrated, embedded in Epon and, when that had hardened, a Lucite rod was attached with Epon directly under the selected area act as a handle. When this had hardened, the area was centered (by means of the scratches in the cover slip) under cross hairs in a dissecting microscope. The cover slip was then pried off and the block around the selected area was trimmed for sectioning. The sections were stained in uranyl acetate and examined in a modified RCA EMU-3G electron microscope.

Altogether, 10 preparations were carried through this procedure. Of these, five were found to have insignificant amounts of biological material in the sections, two were found to have material but no clearly defined chromatin, and three were found to have chromatin fibers. The reasons for the low rate of success were not evident apart from the complexity of the procedure. The other materials were not examined by electron microscopy since their lower DNA content was expected to make them even more difficult than the *Chironomus* squashes.

RESULTS

Three types of materials were examined for oriented DNA after squashing. These were the macronucleus of the protozoan *Spirostomum*, salivary glands from larvae of the fly *Chironomus thummi*, and erythrocytes of the salamander *Amblystoma tigrinum*. Many regions of highly oriented DNA were found. Fig. 1 shows the appearance of one of these regions.

Fig. 2 shows the various degrees of polarization that were measured in the three materials. The measurements were only made on areas that appeared to be highly oriented. Thus the histograms do not represent all the material but do show that that the maximum polarizations frequently observed are around 50% for *Spirostomum* and 75%

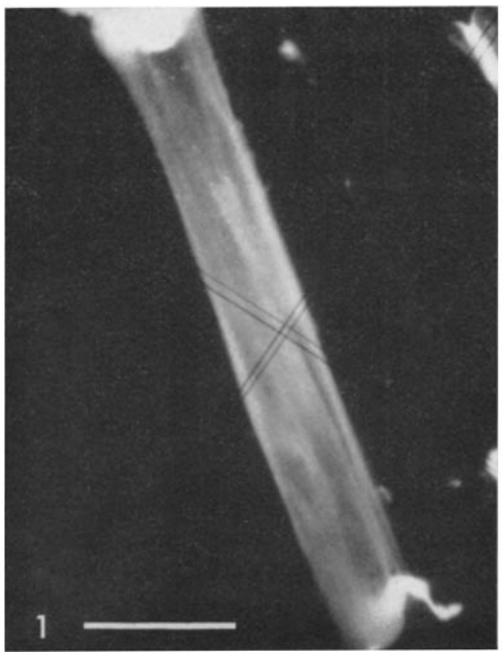

FIGURE 1 Stretched, acridine orange–stained chromatin from an *Amblystoma* erythrocyte as viewed in the fluorescence microscope. The measured degree of fluorescence polarization from the region indicated by the cross hairs was 67%. Scale, 50 μ. × 400.

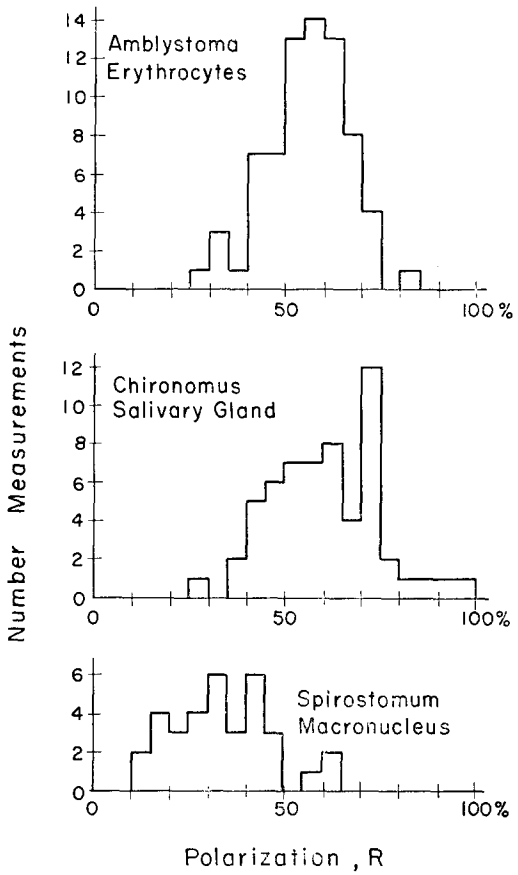

FIGURE 2 The magnitude of polarized fluorescence observed in acridine orange-stained squashes of three types of chromatin.

for the other two materials. Since the objective lens limits the polarization to less than about 90% and since other factors which have not been taken into account also lower the polarization, it is clear that, on the assumption that the dye samples all orientations of the DNA equally, the DNA in these preparations is frequently highly oriented. The difference between *Spirostomum* and the other materials may reflect a difference in the extent of orientation of the chromatin fibers rather than a difference in the organization of DNA within the chromatin.

From the appearance of a stretched mass of chromatin one usually is able to determine the direction in which stretching took place. In all cases the polarization was such as to indicate that the DNA was aligned parallel to the direction of stretching.

Fig. 3 is an electron micrograph of a portion of a region exhibiting polarized fluorescence from a *Chironomus* preparation. It may be seen that the chromatin fibers have indeed been oriented. Measurements of the widths of the fibers in this preparation are shown in Fig. 4. The widths in the other two preparations were similar although the fibers were not as distinct as in this preparation. These widths fall in the range 60–100 A which agrees well with the range 50–100 A previously reported for unsquashed preparations from this tissue (Stevens and Swift, 1966), and for other unsquashed materials prepared in the same way (Kaye and McMaster-Kaye, 1966; Wolfe, 1969), suggesting that little or no elongation of the individual fibers occurred during squashing. Unfortunately, we were unable to determine the thickness of unoriented fibers in the squash preparations because of uncertainty in distinguishing unoriented chromatin from other materials in the squashes.

DISCUSSION

In order to relate the observed polarizations to the models of DNA configuration, calculations (presented in the Appendix) were performed of the expected polarizations for several models. The models chosen were based on those that had previously been proposed. The suggestion that DNA lies parallel to the fiber axis (Wolfe, 1965) is represented by the parallel loop model or any of the others at the point of full extension. The proposal that DNA lies in a tight helix (DuPraw, 1965; DuPraw and Bahr, 1968) is represented by a helix of large pitch since a helix of small pitch leads to a polarization that indicates that the DNA is at right angles to the fiber axis (MacInnes and Uretz, 1968), and this has never been observed. Also helices of helices (DuPraw and Bahr, 1968) are excluded by the fact that the observed magnitude of the polarization exceeds 30% (MacInnes and Uretz, 1968). Finally, the suggestion that the DNA lies along the fiber axis with frequent loops to the side (Ris, 1967) is represented by two models. In one the loops are randomly oriented in a plane perpendicular to the axis of the fiber, and in the other the loops are oriented completely at random.

All of these models can have varying degrees of extension from nearly straight DNA to a relatively compact structure. In order to facilitate comparison of the different models, we define a parameter, E, the extension, as the ratio of end-to-end lengths to contour length.

The results of the calculations are presented in Fig. 5 where the polarization, R, is plotted against

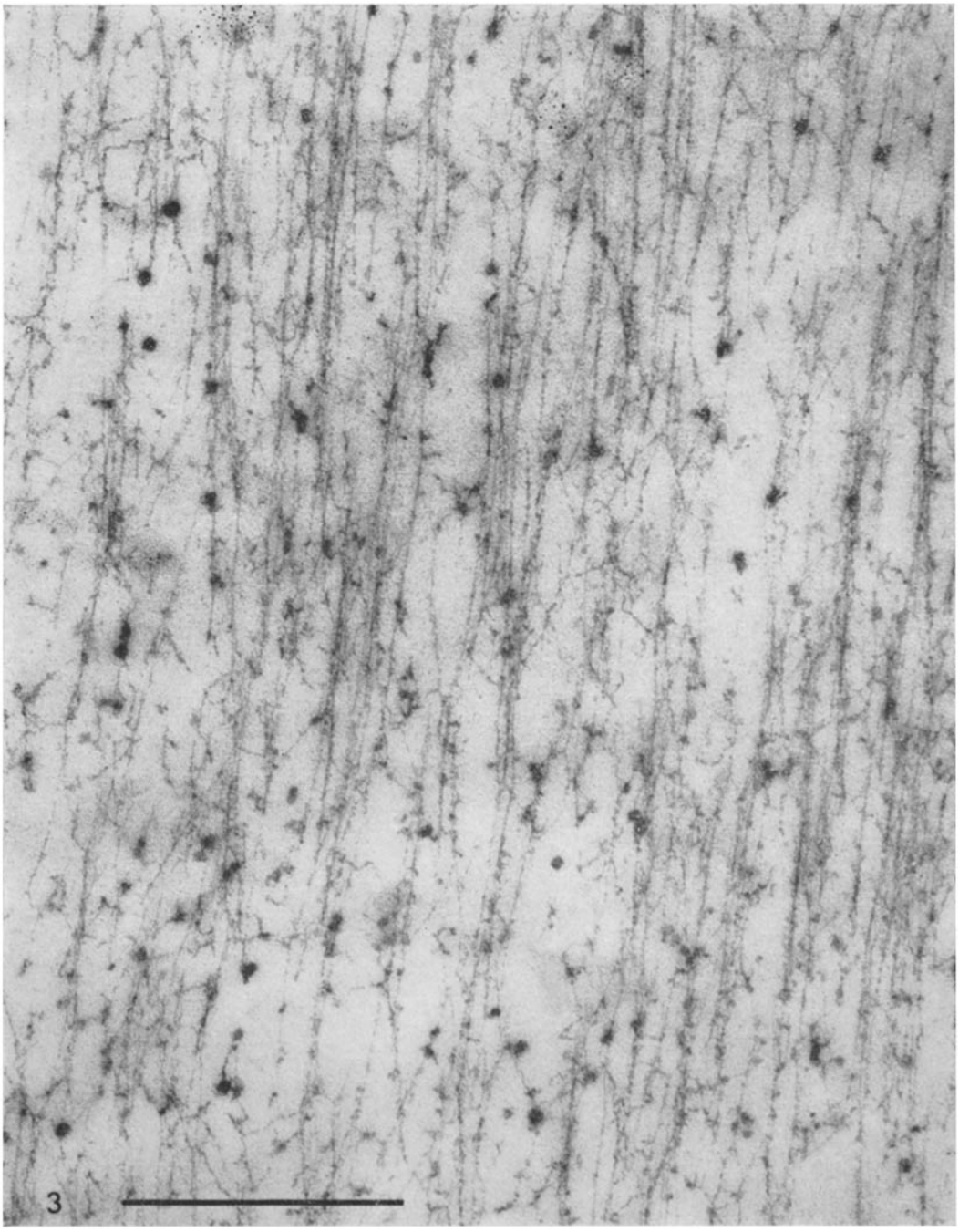

FIGURE 3 Electron micrograph of a region of chromatin which exhibited polarized fluorescence. Scale, 1.0 μ. $\times$ 41,000.

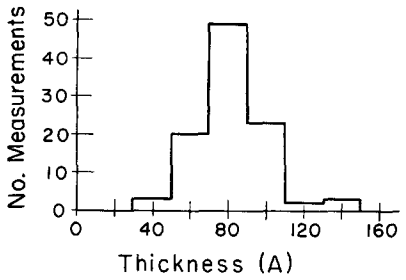

FIGURE 4 The widths of chromatin fibers from the same preparation as Fig. 3.

the extension of the DNA for each of the models. It may be seen that the high polarizations observed (about 80% of expected maximum) indicate that the DNA must be highly extended (end-to-end length greater than 70% of contour length) unless the DNA is looped back on itself such that nearly all the DNA lies parallel to the fiber axis.

As a result it seems clear that DNA in the oriented chromatin fibers lies predominantly parallel to the fiber axis (making the reasonable assumptions that acridine orange does not bind preferentially to parallel DNA or increase the orientation of DNA in the fibers). Since the oriented fibers from *Chironomus* chromatin are not measurably thinner than those in intact nuclei prepared by the same methods but without squashing, it appears that the fibers were oriented without significant elongation and concomitant decrease in fiber diameter. Thus, assuming that the oriented fibers are equivalent to the fibers of fixed intact nuclei, we conclude that DNA *in situ* probably lies preferentially parallel to the axis of the chromatin fibers.

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APPENDIX

Calculations have previously been made for the expected polarization from acridine orange–stained DNA coiled into a helix or a helix of a helix (MacInnes and Uretz, 1968). In this appendix we will make the calculations for several other possible models of DNA packing. With these models a somewhat different analysis is convenient.

For the sake of comparison we wish to determine the polarizations of the different models in terms of a common parameter. To this purpose we have chosen the extension (E) which is defined as the ratio of end-to-end length to contour length of the DNA. In the models with side loops, E is equal to the proportion of DNA not in the loops, since we assume all loops are closed. In the case of the helix, E equals the sine of the pitch.

Consider the diagram in Fig. 6. Here we let the vector $\mathbf{r}$ represent the local direction of the DNA double helix and the vector $\mathbf{A}$ represent the polarization of the light emitted by a particular dye molecule. Since the transition moment of an acridine dye lies in the plane of the dye and since the dye is intercalated between adjacent base-pairs of the DNA, the polarization is at right angles to the DNA axis ($\mathbf{A} \perp \mathbf{r}$). Furthermore, since the dye molecule can lie at any angle within this plane, in

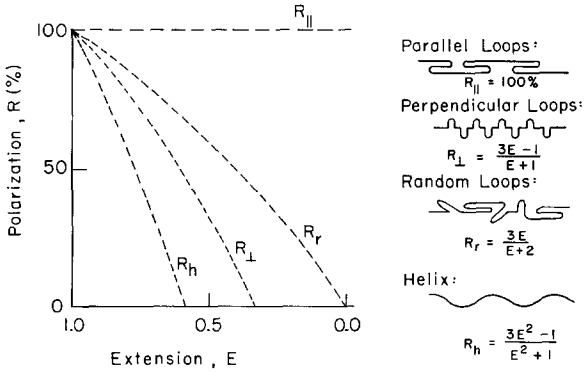

FIGURE 5 The degree of polarized fluorescence expected for various models of DNA packing in chromatin fibers.

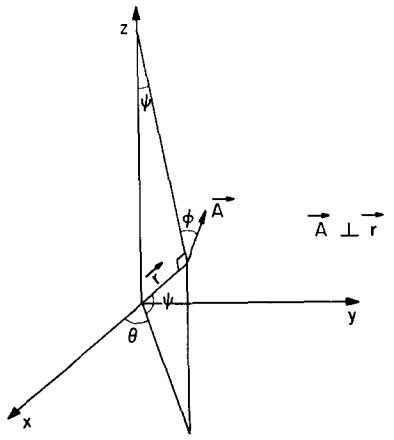

FIGURE 6 Geometry used in calculation of the expected degrees of polarized fluorescence. **r** represents the local direction of the DNA axis; **A** represents the direction of polarization of fluorescence from acridine orange bound to the DNA. Axes x, y, and z represent potential fiber orientations and viewing directions.

the integrations that follow one must average all these positions by integrating from ϕ equals 0 to 2π. The calculations require that we determine the projections (A_x, A_y, A_z) of **A** on the axes and take their square to obtain the intensity of light polarized along a particular axis ($I_x = A_x^2$, etc.). The intensity for the configuration as a whole is then determined by integrating the intensity over the angles appropriate for that configuration. The projections in general are:

$$A_x = -A \sin\phi \sin\theta - A \cos\phi \sin\psi \cos\theta$$
$$A_y = A \sin\phi \cos\theta - A \cos\phi \sin\psi \sin\theta$$
$$A_z = A \cos\phi \cos\psi.$$

Parallel Loops

For DNA parallel to the x-axis $\theta = \psi = 0$, and the projections reduce to:

$$A_x = 0$$
$$A_y = A \sin\phi$$
$$A_z = A \cos\phi.$$

The intensities are then:

$$I_x = (2\pi)^{-1} \int_0^{2\pi} 0 d\phi = 0$$

$$I_y = (2\pi)^{-1} A^2 \int_0^{2\pi} \sin^2\phi d\phi = (\tfrac{1}{2})A^2$$

$$I_z = (2\pi)^{-1} A^2 \int_0^{2\pi} \cos^2\phi d\phi = (\tfrac{1}{2})A^2.$$

Since $I_y = I_z \geq I_x$ the polarization viewed along the y- or z-axes is:

$$R = \frac{I_y - I_x}{I_y} \times 100\% = 100\%.$$

Perpendicular Loops

For DNA restricted to the y-z plane, $\theta = \pi/2$, and the projections reduce to:

$$A_x = -A \sin\phi$$
$$A_y = -A \cos\phi \sin\psi$$
$$A_z = A \cos\phi \cos\psi.$$

For DNA randomly oriented in the y-z plane, integrate ψ from 0 to 2π and the intensities become:

$$I_x = (2\pi)^{-2} A^2 \int_0^{2\pi} \int_0^{2\pi} \sin^2\phi d\phi d\psi = (\tfrac{1}{2})A^2$$

$$I_y = (2\pi)^{-2} A^2 \int_0^{2\pi} \int_0^{2\pi} \cos^2\phi \sin^2\psi \, d\phi d\psi$$
$$= (\tfrac{1}{4})A^2$$

$$I_z = (2\pi)^{-2} A^2 \int_0^{2\pi} \int_0^{2\pi} \cos^2\phi \cos^2\psi \, d\phi d\psi$$
$$= (\tfrac{1}{4})A^2.$$

Combining the intensities for DNA in perpendicular loops with those previously calculated for parallel DNA we obtain:

$$I_x = (1 - E)(\tfrac{1}{2})A^2$$
$$I_y = I_z = (1 - E)(\tfrac{1}{4})A^2 + E(\tfrac{1}{2})A^2.$$

For $E \geq \tfrac{1}{3}$, $I_y = I_z \geq I_x$ and thus the polarization viewed along the y- or z- axis is:

$$R = \frac{I_y - I_x}{I_y} \times 100\% = \frac{3E - 1}{E + 1} \times 100\%.$$

Random Loops

For DNA randomly oriented it is necessary to integrate over both θ and ψ, which requires that the integration be weighted according to the solid angle swept by **r**. A simpler method is to take advantage of the fact that in this case

$$A_x = A_y = A_z,$$

and since

$$A^2 = A_x^2 + A_y^2 + A_z^2,$$

then

$$A_x^2 = A_y^2 = A_z^2 = (1/3)A^2,$$

and

$$I_x = I_y = I_z = (1/3)A^2.$$

Combing the intensities for DNA in random loops with those previously calculated for parallel DNA we obtain:

$$I_x = (1 - E)\ (1/3)A^2$$

$$I_y = I_z = (1 - E)\ (1/3)A^2 + E(1/2)A^2.$$

Since $I_y = I_z \geq I_x$ the polarization viewed along the y- or z-axes is:

$$R = \frac{I_y - I_x}{I_y} \times 100\% = \frac{3E}{E + 2} \times 100\%.$$

Helix

For a helix along the z-axis, ψ equals the pitch, a constant, and θ varies from 0 to 2 π. Thus the intensities become:

$$I_x = (2\pi)^{-2} A^2 \int_0^{2\pi} \int_0^{2\pi} (\sin\phi \sin\theta - \cos\phi \sin\psi \cos\theta)^2\, d\phi d\theta$$

$$= (1/4)A^2 (1 + \sin^2\psi)$$

$$I_y = (2\pi)^{-2} A^2 \int_0^{2\pi} \int_0^{2\pi} (\sin\phi \cos\theta - \cos\phi \sin\psi \sin\theta)^2\, d\phi d\theta$$

$$= (1/4)A^2 (1 + \sin^2\psi)$$

$$I_z = (2\pi)^{-2} A^2 \int_0^{2\pi} \int_0^{2\pi} \cos^2\phi \cos^2\psi\, d\phi d\theta$$

$$= (1/2)A^2 \cos^2\psi.$$

For $\psi > 35°$, $I_x = I_y \geq I_z$ and the polarization observed along the x- or y- axis is:

$$R = \frac{I_y - I_z}{I_y} \times 100\% = \left[1 - \frac{2\cos^2\psi}{1 + \sin^2\psi}\right] \times 100\%.$$

Since the extension of the DNA, E, equals $\sin\psi$,

$$R = \frac{3E^2 - 1}{E^2 + 1} \times 100\%,$$

for $E \geq 1/\sqrt{3}$.

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