
Technical Note

TWO SIMPLE FIBER DIFFRACTION EXPERIMENTS FOR STUDENTS TO OBTAIN BASIC STRUCTURAL PARAMETERS OF PROTEINS AND NUCLEIC ACIDS

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Historically basic structural parameters of proteins and nucleic acids have been established mainly through X-ray diffraction patterns of fibrous specimens. Fortunately, most of these parameters can be directly obtained as lattice parameters (including the "identity periods") or spacings of Bragg reflections. In this respect, for educational purposes, X-ray diffraction patterns from fibrous biopolymers have merits over those from single crystals of organic compounds or globular bio-polymers where lattice parameters and spacings usually have no direct significance with respect to molecular structure. In general, students majoring in the fields of organic chemistry, biological chemistry and molecular biology tend to be interested in only molecular structures. In contrast, students majoring in the fields of inorganic chemistry, petrology, metallurgy and solid state physics realize the value of studying and observing crystal structures. Of course, the molecular detail of crystals can be obtained by Fourier inversion of: a set of reflection intensity. However this process is hard to adapt to the level of student experimentation, since it requires tedious intensity data collection and the use of sophisticated computer programs to say nothing of the "phase problem". Furthermore, "dry lab" type exercises dealing with these procedures [such as the one described in (1)], although very much instructive, lack the excitement of directly observing nature.

During the past few years, we have offered the two fiber diffraction experiments described here to undergraduates at the Faculty of Pharmaceutical Sciences, University of Tokyo. As representatives of β -sheet type protein and B-form DNA, silk thread and salmon sperm DNA were used respectively. The

experiments require only two successive days (about 5 working hours per day). A reading assignment of "The Double Helix" by J. D. Watson (Athenum, New York, 1968) enhances the historical flavor.

The X-ray equipment

A (rotation) camera with a cylindrical (rather than flat) film cassette is preferred for two reasons: 1) calculation of layer line spacing becomes much simpler because the layer lines appear as straight lines and 2) the inner space of the cylindrical cassette can be made nearly airtight much more easily, a condition needed to maintain high relative humidity (R.H.) around the DNA fibers (see below). We use a Rigaku cylindrical camera with a radius of 34.7 mm (Cat. No. 1141) as schematically shown in Figure 1. The X-ray generator has a Phillips X-ray tube with a Cu target usually operated at 35 kV and 15 mA. A fine rather than normal, focus tube is desirable especially for the DNA experiment. Setting a Ni filter in front of the collimator is essential as the coexistence of $\text{CuK}\beta$ line can seriously disturb students. An observation telescope is set on the optical bench to facilitate centering of the sample fibers. The camera has a cap on top of the cylindrical cassette, allowing for the inner space to be made roughly airtight. For the DNA experiment, we hang a small petridish containing wet gauze on the cap and encircle the goniometer head with a "collar" consisting of wet gauze sandwiched between fine-meshed stainless steel netting (Fig. 1), in order to maintain the inner space at almost 100% R. H. Fiber specimens are mounted (with "compound" commonly used in crystallographic laboratories) across a hole of ~ 2 mm diameter placed on a sample plate of ca. 3 x 1 cm made of stainless steel.

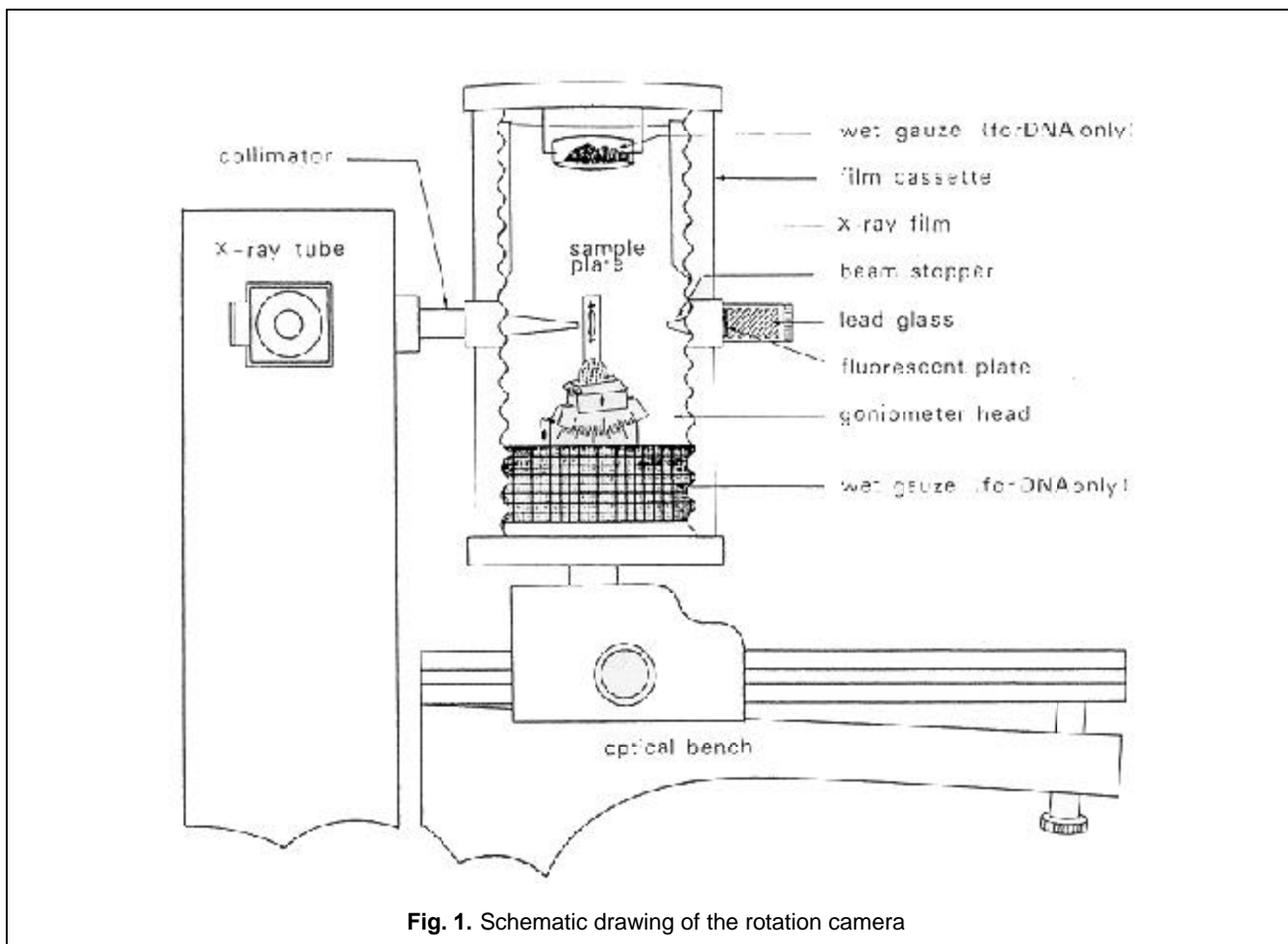


Fig. 1. Schematic drawing of the rotation camera

Use of crystallographic "compound" (rather than epoxy adhesives) make the operation of aligning and fixing a bundle of several fibers on the sample plate quite manageable.

The film coordinates that students must measure is the layer line height z and the radius r_E of a Bragg reflection appearing on the equator. Using the camera radius R , the layer line spacing τ can be obtained through the equations, $\tan\mu = z/R$ and $\tau \sin\mu = \lambda$, where μ is the layer line angle and λ is the wavelength (1.542 Å for $\text{CuK}\alpha$ radiation) of the incident beam. For example, for the n -th layer line, the identity period is given as n times τ . The scattering angle θ of an equatorial reflection is given by r_E/R and the corresponding spacing d is obtained through Bragg's equation $2d \sin\theta = \lambda$.

The silk experiment

Commercially available silk thread, mainly consisting of Tussah silk fibroin, is used. First the thread is unwound into individual silk strands, and several of these are aligned on the sample plate and

fixed so that the resultant fiber is of $\sim 0.5\text{mm}$ diameter. After exposure to X-rays for ca. half an hour, a diffraction pattern (shown in Fig. 1 of reference (2) and schematically shown in Fig. 2) is obtained reflecting the antiparallel β -sheet structures shown in Figure 3. Usually only the reflection a is clearly seen on the equator. According to reference (2), this is the (200) reflection from the orthorhombic unit cell with $a=9.44 \text{ \AA}$, b (fiber axis) $=6.95 \text{ \AA}$ and $c=10.60 \text{ \AA}$, space group $P2_12_12_1$. As shown in Figure 3, the spacing of the (200) reflection (d_{200}) represents the average distance between the β -strands in the β -sheet ("interchain distance") known to have a common value around 4.75 Å in antiparallel β -sheet structures (3). Fortunately (100) reflection is almost non-existent because the β -strands A and B (see Fig. 3) look fairly similar in the projection along the fiber (b -) axis. For a similar reason, the (001) reflection is also almost non-existent. The spacing d_{002} is dependent on the kind of sidechains attached to the α -carbons. Given the amino acid composition of Tussah silk (glycine = 27%, alanine=44%, serine=12% and

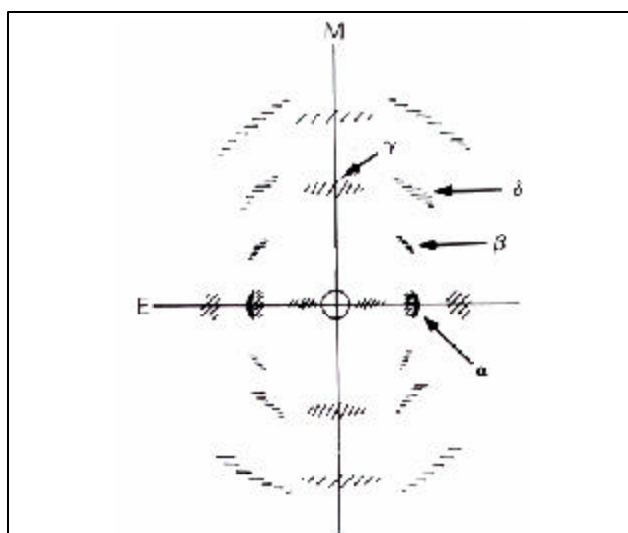


Fig. 2 Schematic drawing of a diffraction pattern from silk fibroin. E: equator, M:meridian.

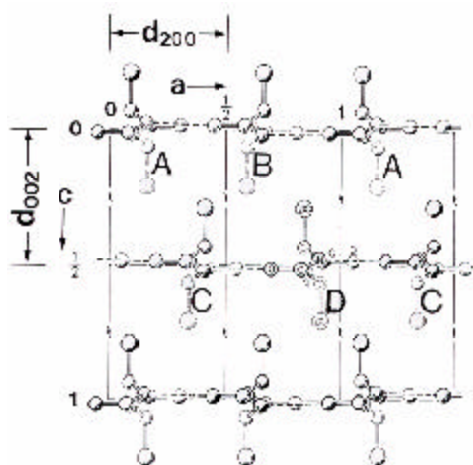


Fig. 3 A representation of the unit of structure of Tussah silk viewed along the fiber (*b*-) axis. An alanyl residue is shown for each β -strand polypeptide chain running perpendicular to the plane of the paper. β -Strands A, B, C and D constitute one unit cell (see text). (adapted from Figure 2 of reference (2)).

tyrosine = 5%), however, the d_{002} spacing is scattered around an average value 5.3 Å, resulting in a weak blurred streak attached to the inside of the reflection α (see Fig. 2). Because of this, if the exposure time is too long, students may well find an unreasonably large d_{200} .

The identity period of polypeptide chain in β -strand conformation can be derived either as the layer line spacing of the 1st layer line (reflection β in Fig. 2) or as twice that of the 2nd layer line (reflections γ and δ). The identity period corresponds to two amino acid residues (see e.g. Fig. 6 of reference (4)) and is

commonly about 7.0 Å in antiparallel β -structures. We have found that students can obtain, the "inter-chain distance" and the identity period with at most a 2% error. During the course of the experiment, the following drawing exercise is assigned for homework: Given unit cell parameters, space group, equivalent positions, and fractional coordinates of the atoms belonging to two independent residues in the asymmetric unit [all taken from Table 3 of reference (2)], 1) draw the polypeptide chains contained in one whole unit cell, 2) decide whether the β -sheet is parallel or anti-parallel, 3) indicate the inter-strand NH \cdots O=C hydrogen bonds, and 4) calculate the corresponding distances.

The DNA experiment

Materials

Salmon sperm DNA [Calbiochem., catalogue No. #262011 (sodium salt, highly polymerized); older product #2620 seems less suitable to this experiment]

Spermine tetrahydrochloride (sigma, #S 2876)

Lithium chloride (Sigma, #L 0505)

Background

DNA is known to undergo a transconformation dependent upon the relative humidity (R.H.) of the atmosphere surrounding the fiber specimen (5). At 92% or higher R.H., it is in the "B-form" (5), the double-helical structure discovered by Watson and Crick, while at 75% or lower R.H. it is found in the "A-form" (6). In the intermediate R.H. range, the A-form and B-form coexists. The B-form exists *in vivo* and thus appears in regular biochemistry text books. As shown in Figure 4, the diffraction pattern (B-pattern) of B-form DNA exhibits a pair of very strong "meridional" reflections having a spacing of ca. 3.4Å which is the separation between the planes of base pairs stacked perpendicular to the helix axis. The diffraction pattern of A-form DNA, of which the biological significance is not yet clear, is much more complicated (6). From our experience, making oriented fibers from concentrated DNA solution is both time-consuming and beyond the skills of students. Moreover such fibers easily give rise to A-pattern or (A+B) pattern if the humidity control is imperfect.

It has been known that addition of polyamine to DNA makes the fiber making procedure much easier and the A-B transition much slower (at least several days are needed for complete transition) due to the extensive intra- and inter-double-helical cross-

bridging by polyamine (7). In addition, such fibers remain solid even at 100% R.H. unlike pure DNA fibers which would extensively swell and finally break in two. Using spermine $[\text{NH}_3^+ (\text{CH}_2)_3 \text{NH}_2^+ (\text{CH}_2)_4 \text{NH}_2^+ (\text{CH}_2)_3 \text{NH}_3^+]$ as a polyamine, we found that X-ray diffraction experiment of DNA fibers can be made sufficiently easy for undergraduates.

The experiment

1) Prepare 25 ml of Solution A, the aqueous solution containing 2mM DNA (the concentration is with respect to nucleotide) and 2mM LiCl [replacement of sodium ion attached to DNA by lithium ion may improve the quality of the diffraction pattern (5)]. This solution should be made at least 12 hrs prior to the start of the experiment in order to let the DNA dissolve completely.

2) Prepare 25 ml of Solution B, the aqueous solution containing 40 mM spermine.

3) Place 2 ml of Solution A in a 10 ml beaker and gradually add Solution B by micropipet while stirring by a magnetic stirrer until no new precipitate is formed. Note that one molecule of spermine, having four plus charges at neutral pH can neutralize four nucleotide phosphate groups, each having one minus charge. Nevertheless, excessive addition of Solution B seems to cause no unfavourable effect. The stirring appears essential for orienting the DNA molecules.

4) Pick out the resultant fibrous precipitate using a tweezer and place it on a microslide. Remove any excessive water with a strip of filter paper. After this, gently stretch out the fiber using two tweezers, continually applying tension as the fiber dries. The resultant fibers usually have diameter of 0.1 to 0.3 mm.

5) To obtain a strong enough diffraction intensity, several of such fibers should be aligned on the sample plate making the net diameter about 0.5 mm. To align the fibers, first fix one end of the bundle of fibers to the sample plate using standard crystallographic "compound". Next, completely rewet the fibers by placing a droplet of water on them, wait for a few minutes, remove the excess water and then fix the other end of the bundle. Without rewetting, the bundle of fibers will bend due to swelling, after it has been set in the humidity-controlled camera, and may well escape from the X-ray beam.

6) Set the sample plate on the goniometer head, adjust the R.H. of the space around the specimen to as close to 100% as possible (procedure described in *X-ray equipment* section), and start exposure to X-rays.

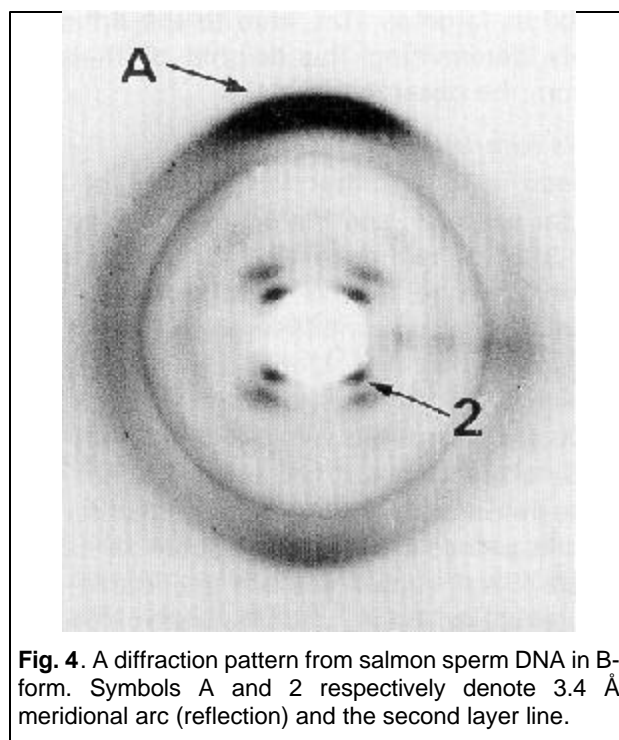


Fig. 4. A diffraction pattern from salmon sperm DNA in B-form. Symbols A and 2 respectively denote 3.4 Å meridional arc (reflection) and the second layer line.

7) The exposure time should be several hours. If the DNA experiment is done after the silk experiment, this exposure is most conveniently done overnight, with an appropriately diminished tube current. Note that the required exposure time is roughly inversely proportional to the tube current when the tube voltage is kept constant.

Interpretation of the diffraction pattern

The strong arc A indicated in Figure 4 is the meridional reflection mentioned above. As is well known, one turn of the DNA double helix contains ten base pairs (paired through Watson-Crick type hydrogen-bonding) each of which is separated from the neighboring pair by a distance (measured along the helix axis) corresponding to the layer line spacing of this meridional reflection, the 10-th layer line. A value close to 3.4 Å can be easily obtained usually with less than 1% error. Very often the second and third layer lines, especially the former (indicated by "2" in Fig. 4), are clearly visible even on the student's photograph (the first layer line is not seen in Fig. 4 as it is hindered by the beam trap). Unfortunately, however, calculation of the identity period (or the helix repeat), which should be approximately 34Å, is not recommendable as a student exercise since the error here can easily be as large as 10%, due to the difficulty in precisely determining the heights of these layer lines from the observed arcs.

Possible extension

As seen in ideal diffraction patterns of B-form DNA [for example, see Plate I of reference (5) or Figure 3(a) of reference (7)], the second most prominent feature of the B-pattern is a pair of strong equatorial arcs appearing close to the center of the pattern. In Fig. 4, this arc is obscured by the beam trap set in front of the X-ray film to trap direct X-ray beams. However, if finer X-ray beams and a smaller beam trap are used, this arc can be easily seen. Since this is the (100) reflection from a hexagonal unit cell having one double-helical DNA molecule (5), the outer radius of the double-helix is simply given by $d_{100}/\sqrt{3}$. To observe this equatorial arc (the spacing is ca. 21 Å at 92% R.H.), the diameter of the beam trap should be less than 5.2 mm.

Caution

It seems essential to use salmon sperm DNA in order to make fiber preparing procedure reasonably easy. When *E. coli* DNA (Sigma #D 4889, Na salt), calf thymus DNA (Sigma #D 1501, Na salt) or herring sperm DNA (Sigma #D 1632) was used, the precipitate formed upon addition of spermine was much less elastic and step 4) of *The experiment* section became considerably more difficult. This is most likely due to the fact that salmon sperm DNA is of considerable higher molecular weight than the other two DNA's. In any case, it seems that DNA used in this experiment should be of as high as possible molecular weight or, that is, as intact as possible.

Sometimes a diffraction pattern resembling Fig. 3(b) of reference (7) is observed, in which the B-pattern and A-pattern are superimposed. This is an indication that the R.H. inside the camera is not high enough (say, less than 85%). In this case, gauze shown in Fig. 1 should be rewetted. Fortunately, however, even from such (A+B) patterns, the inter base-pair separation can be obtained fairly accurately since the 3.4 Å meridional reflection of the B-pattern is much stronger than the reflections of the A-pattern that may appear around it.

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