INVESTIGATION OF NEGATIVELY STAINED PLANT
FLAGELLAR MICROTUBULES BY OPTICAL DIFFRACTION

R. BARTON. From the Department of Botany, University College of Wales, Aberystwyth, Cards.,
United Kingdom

During an investigation of the fine structure of dis-
integrated flagella from Pteridium sperm cells by
using negative staining (1), the author had at-
ttempted to use Markam’s linear integration tech-
nique to analyze repeating subunits in the walls of
doublets. This method, although providing useful
information about the size and shape of the basic
35-A protein subunits, was less successful when
used to determine higher orders along each
doublet compared with those deduced from optical
diffraction of negatively stained cilia of Tricho-
nympha by Grimstone and Klug (3). Following the
demonstration by Gall (2) that a light microscope
may be used as an optical diffractometer, it has
now been possible to investigate the negatively
stained flagella of Pteridium sperm cells by his
technique.

The chief findings are presented in this paper. In
general, the diffraction patterns that were ob-
tained from doublets and whole flagellar regions
from this plant material are closely comparable
with those previously published by Grimstone and
Klug (3) for the protozoan Trichonympha.

MATERIALS AND METHODS

A Vickers Patholux microscope fitted with a mercury
arc light, which had been built in the department,
was used for optical diffraction. It was set up, accord-
ing to Gall (2), with a pinhole light source made from
a 100 µ EM aperture and a green interference filter
transmitting a peak wave-length of 546 mµ and a
band width of 13 mµ. A normal condenser, a X 10
objective, and a X 6 eyepiece were used. The dif-
fraction objects were contrasty prints from parts of
the original electron micrographs reduced to a con-
venient magnification of 10,000 onto 35 mm film. A
wide range of exposures was made initially, and the
35 mm film was scanned with a dissecting microscope
to select the most contrasty exposure. The area deter-
nined for diffraction in the chosen micrograph was
then outlined by a rectangular-shaped aperture,
which was made with photographic opaque by using
a fine brush and the dissecting microscope. After
drying, the diffraction masks were cut out to a
roughly spherical shape so that they could be readily
fitted across the ledge at the back of the microscope
objective ready for diffraction. With some practice,
the masks could be centralized, by using forceps,
with the upper part of the microscope removed.
Diffraction patterns were recorded on the 35 mm
camera attachment with exposures of 1–5 min.

OBSERVATIONS

A considerable number of regions from micro-
graphs of negatively stained flagellar components
were examined, in the above manner, in order to
obtain a representative sample of diffraction pat-
terns. On the whole, relatively few examples gave
well-defined patterns which could be analyzed
with any certainty. This is presumably due to the
poor preservation of detail over long stretches of
doublets.

Diffraction patterns from doublets released from
disintegrated flagella were easiest to interpret, and
representative examples are shown in Figs. 1, 3,
and 5. The equatorial spots were always well de-
veloped and of high intensity. The chief of these
were two elongated, 50-A spots found on each side
of the center spot. Clearly, there is a direct rela-
tionship between these two spots and the parallel
protofibrils previously reported to be in this ma-
terial (1), which were shown to average about 50 A
center-to-center spacing. In some diffraction pat-
FIGURE 1 Optical diffraction pattern from doublet prepared as described in text. In this pattern the equatorial spots are well preserved, with two elongated spots, which have been derived from the longitudinally running protofibrils in the material, on each side at 50 A. The circular spots nearer the center spot are thought to be non-Bragg reflections (see text). Spots are present on the 44-, 88-, and 176-A layer lines. Furthermore, there are spots lying much nearer the center spot which might well indicate even higher repeats in this series. Only the 44 A spot is marked in the figure.

FIGURE 2 Approximate region of the negatively stained tubule from which the diffraction pattern shown in Fig. 1 was taken. X 240,000.

FIGURE 3 Optical diffraction pattern taken from a group of doublets running parallel to each other. The equatorial axis is not so clearly developed as in Fig. 1, but two 50-A spots may also be seen here. Spots are present on the 44-, 88-, and 176-A layer lines, but in this case there is also a weakly developed spot at about 58.5 A. The 44 A spot is marked.

FIGURE 4 Negatively stained tubules used to obtain the diffraction pattern shown in Fig. 3. X 240,000.
Figure 5  Optical diffraction pattern obtained from a single tubule in which the higher repeats on the longitudinal axis are not developed. The equator shows the two typical spots at 50 A and also the two small spots between the center spot and the 50-A streaks on each side. An elongated spot is present on the 44-A layer line (this is marked on the figure).

Figure 6  Negatively stained tubule from which the diffraction pattern at Fig. 5 was obtained. × 240,000.

Figure 7  Optical diffraction pattern taken from an area of intact flagellum. This case is taken from the type of preparation in which the outer membrane had been retained. The equatorial axis is difficult to interpret because of the number of the surrounding spots. The 50-A spots are poorly developed, occurring as unusually small spots. This result would fit in with the fact that it is difficult to pick out the protofibrils visually in this preparation (see Fig. 8). There are spots on the 44-A layer line and also on the 88- and 176-A lines. The 44 A spot is marked.

Figure 8  Negatively stained region of intact flagellum which was used to obtain diffraction pattern in Fig. 7. The area used is indicated by brackets. Note the presence of the surface membrane in these preparations. × 160,000.
equator on the 176-A layer line (Figs. 1 and 3). Thus, the chief periodicity would therefore be the 176-A repeat, and the 88- and 44-A units the second and fourth orders of it. Furthermore, in a few cases a third order, namely a diffraction spot at about 58.5 A, was also found (Fig. 3). As in the diffraction patterns published by Grimstone and Klug (3), there was also a series of less distinct spots much nearer the equator which might well be even higher repeats in this series. Unfortunately, because of the distortion produced here by the Airy Disc pattern around the center spot, it was too difficult to investigate these spots more accurately. As all these spots were invariably found to one side of the meridian, it is inferred that the longitudinal repeating units are arranged in a helical pattern around each tubule.

In some cases the diffraction patterns obtained were much simpler than those described above, in that the 88- and 176-A spots were absent (Fig. 5). From this, it would appear that, at least in parts, some microtubules are composed of only simple parallel protofibrils. Grimstone and Klug (3), encountering similar patterns in their material, account for the findings by presuming that when microtubules spread out they lose the special longitudinal repeats and take up a simple linear form.

In the original negatively stained preparations, several examples of regions of intact flagella were observed in which some details of the substructure could be seen. In general these regions were of two different types. First, the region in which the flagellar membrane had remained intact, and the phosphotungstate had penetrated into the axonemes of the under surface and revealed some structures in them. Second, the membrane which had apparently disintegrated, although the axonemes had been retained as a compact bundle. Optical diffraction patterns were taken from selected areas, from the two different types. Because of the overlapping of different doublets and the difficulty of obtaining clear photographs, these diffraction patterns were usually complex and difficult to interpret. In the main, however, similar diffraction patterns were obtained from intact

**Figure 9** Diffraction pattern obtained from region of intact flagellum from which the membrane had been dispersed. On the equator the 50-A spots are not observed. Spots are present, however, on the 44-A layer line and on the 88- and 176-A layer lines. The 44 A spot is marked.

**Figure 10** Negatively stained bundle of units from a complete flagellum. The area used for diffraction is indicated by brackets. \( \times 120,000 \).
flagella as from isolated doublets which are described above. Figs. 7-10 illustrate the two different types of intact flagella found here and also the diffraction patterns from the indicated areas.

**DISCUSSION**

The use of an optical diffraction method has therefore suggested a much more complex structure in negatively stained images of *Pteridium* flagella than had hitherto been detected by direct observation and by the Markham linear integration technique (1). The higher repeating pattern found here is presumably caused by perturbations of the spherical subunits previously reported in this material, the higher orders thus representing larger orders of helices around the tubule. Grimstone and Klug (3) have put forward convincing evidence relating images observed in negatively stained material to some of the higher repeats detected by optical diffraction. For instance, the second order periodicity was attributed to a slight displacement of alternate protein subunits giving a zigzag appearance, and the first order periodicity, was caused by a pushing in of every fourth subunit. In the *Pteridium* material it was possible to find evidence for this zigzag arrangement of subunits in parts, but there was no convincing evidence for a structure relating clearly to the 176 Å repeat.

In order to interpret these diffraction patterns in relation to the globular subunits known to be present in the material, it is clear that further information is needed about the nature of the subunits themselves.

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**REFERENCES**