Cell Growth and the Structure and Mechanical Properties of the Wall in Internodal Cells of *Nitella opaca*

I. WALL STRUCTURE AND GROWTH

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WITH FOUR PLATES AND SIX FIGURES IN THE TEXT

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SUMMARY

This is the first of two papers dealing with the relationship between growth and the mechanical properties of the wall in internodal cells of *Nitella opaca* L.

The submicroscopic structure of the cell wall of this alga, as determined by chemical analysis, X-ray crystallography, polarizing microscopy, electron microscopy, swelling measurements, and infra-red spectrography, is described in detail and the changes during growth are recorded. It has been found that the wall contains cellulose in the form of cellulose I (type B). The constituent microfibrils are preferentially oriented, usually in slow helices with considerable angular dispersion about the common direction. They are arranged in discrete layers with pectic substances providing an amorphous matrix between microfibrillar-reinforced laminations. It is shown that, as the cell elongates, both the streaming direction in the cell and the mean microfibrillar orientation in the wall change in such a way as to allow the possibility of a causal connexion between streaming and microfibrillar orientation in a new wall lamella. The orientation in such a lamella is undoubtedly modified by subsequent passive extension much as implied in the multi-net growth hypothesis of Roelofsen.

INTRODUCTION

THE elegant work of Green and Chapman (1955) and Green (1954, 1958) has shown that the internodal cells of *Nitella* are most convenient objects for the study of wall structure and growth. These cells, moreover, are so large that the physical properties of the walls can also be measured with comparative ease. There seemed therefore reason to believe that these plants would form ideal material for a critical study of the interrelationships between growth and the physical properties of the wall, and the present series of papers presents the results of an investigation of this kind made during 1958 and 1959. Whereas Green worked with *Nitella axillaris*, the only species available for the present work was *Nitella opaca*, and it was therefore thought desirable first to check the wall structure in our species against that used by Green. As already briefly reported (Probine and Preston, 1958), certain significant

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differences have been found between the two species; in addition, it has been found possible to make observations with *Nitella opaca* which Green found impossible with *Nitella axillaris*. It is therefore the purpose in this first paper to describe the wall structure of *Nitella opaca*. The mechanical properties of the walls of growing cells will be dealt with in the second paper, and it will then be shown that the significance of correlations between these properties and cell growth can be examined in more detail than has so far proved possible with any other cell. Although, therefore, features of structure will be described below which are clearly concerned with growth, discussion of their significance will be postponed to the second paper.

The internodal cells of *Nitella* have long been objects of interest in the field of plant physiology. It seems therefore rather odd that up to now it has not been known with certainty that the walls contain cellulose. Although both Correns (1893) and Votava (1914) claimed that the *Nitella* cell wall contains a substance giving the staining reactions of cellulose, Debski (1898) was not able to identify the skeletal substances. Even Green (loc. cit.) does not offer any evidence of his own on the composition of the wall and, on the basis of the early work referred to above, merely assumes that the birefringence of the wall is due to a cellulose. Again, Nicolai and Preston (1952) included *Nitella* in a survey of the fine structure of the walls in about sixty species of filamentous green algae, using chiefly the methods of X-ray diffraction analysis, but found that the diagram was much too poor in diffraction arcs to allow a decision to be made on the nature of the skeletal substance. This is understandable in the light of the present investigation since it has been shown that some pretreatment of the wall is necessary before a satisfactory X-ray diagram is obtained.

The only information on the chemical nature of the wall, therefore, has rested on the evidence of staining tests. In view of the unreliability of staining and solubility tests for native cellulose (Nicolai and Preston, 1952) it was considered desirable to begin this investigation by an examination of the nature of the structural polysaccharide by the methods now available.

MATERIALS AND METHODS

The morphology of *Nitella* has already been reviewed by Green and Chapman (1955) but those features relevant to the present study will be described here for the sake of clarity.

The plant consists of a linear series of successive nodes and internodes. The nodes are multi-cellular with lateral cells of limited and of unlimited growth. The internodes, with which this paper is solely concerned, are single cylindrical cells which after being cut off from the apex proceed to elongate, without proportional increase in diameter, up to a length of several centimetres. Green (1954) has shown conclusively that growth in these cells is evenly distributed over the whole cell length. The walls of these elongated internodal cells are lined with cytoplasm surrounding a large central vacuole.

Within this parietal cytoplasm and close against the wall lies a single layer of densely packed discoid chloroplasts arranged in files slightly tilted from the cell axis in a steep helix. These chloroplasts are, however, lacking along two files on opposite sides of the cell which also pass along the cell in the same steep helix. In living cells the cytoplasm may be seen to be streaming along the direction of the files of chloroplasts, ascending on one side of the clear region and descending on the other, so that the direction of streaming may easily be measured as the direction of the files of chloroplasts. These clear regions, which are referred to as *striations*, thus represent the boundaries between the ascending and the descending streams.

In the present paper the wall is examined chemically by hydrolysis followed by paper partition chromatography and physically by the methods of polarization microscopy, X-ray diffraction analysis, infra-red absorption spectrophotometry, and electron microscopy. For chemical analysis of the wall, whole plants were treated according to the methods of Cronshaw, Myers, and Preston (1958), modified from Jermyn and Isherwood (1956). Otherwise cell walls were isolated by cutting away each end of the internodal cell and brushing out the cytoplasm from the hollow cylinder thus produced. For some observations it was found necessary to subject the walls to chemical treatments and these will be described in the appropriate place. Determinations of major extinction positions were made by the standard method using a Red I plate. For X-ray analysis, CuK_α radiation was used, collimated to a beam 0·5 mm. diameter with a specimen/film distance of about 3 cm., calibrated accurately against a silver diagram. For electron microscopic observation the wall was either ground in a blender or dissected into lamellae as described in the body of the paper. A few observations were made on ultra-thin sections imbedded in the usual way in a mixture of methyl and butyl methacrylate.

RESULTS

(*a*) *The Identification of the Skeletal Material as Cellulose*

The first step in the determination of structure was the chemical fractionation of the wall and the identification of the sugars produced by hydrolysis of the various fractions. Identification was achieved by comparison with the positions and colours of standard sugars run on the same chromatogram, and the results are given in Tables I and II.

TABLE I

Proportion of various constituents in the wall

(*per cent. dry weight*)

Water-soluble fraction	29·9
Alkali-soluble fraction	34·8
Chlorite-soluble fraction	18·6
α-cellulose	16·7

TABLE II

Sugars in the hydrolysis products of wall fractions

	Water-soluble fraction	Alkali extract	α-cellulose
Galacturonic acid . .	M	—	trace
Galactose . . .	M	V.W.	—
Glucose	S	S	S
Mannose . . .	V.W.	V.W.	W
Xylose	trace	M	W
Rhamnose . . .	trace	trace	—

S (strong); M (moderate); W (weak); V.W. (very weak).

The water-soluble fraction of the wall amounts to about a third of the initial dry weight. Although this is a fairly large fraction of the total wall material, it is, nevertheless, small compared with that of most of the algae examined by Cronshaw, Myers, and Preston (1958). Comparable figures for the water-soluble fraction in that investigation were of the order of 50 to 70 per cent. Only *Cladophora*, *Enteromorpha*, and *Ptilota* had water-soluble fraction below 40 per cent. Galacturonic acid in the water extract indicates the presence of pectic substances in the wall. There is X-ray evidence that the strong glucose spot is due partly to hydrolysis of starch (p. 265). The α-cellulose fraction (17 per cent.) is not as high as that of *Cladophora* (28·2 per cent.), *Chaetomorpha* (41 per cent.), and, particularly, *Valonia* (75 per cent.). It is, however, comparable with *Enteromorpha*, *Ulva*, *Laminaria*, *Ptilota*, and *Griffithsia*. The fact that sugars other than glucose occur in the hydrolysate of this fraction (which was observed in the electron microscope to contain microfibrils only), puts *Nitella* outside the unique group which yield only glucose (Myers and Preston, 1959), though the trace of galacturonic acid has possibly been carried over from earlier stages in the extraction.

At each stage of the chemical treatment a small sample of the residue was examined by X-ray diffraction analysis. The principal spacings determined from the X-ray diagrams of powdered wall material are listed in Table III in order of increasing intensity. The table also includes corresponding accepted spacings of cellulose I and II and of starch. The diagrams will be discussed in reverse order. The X-ray spacings of the final residue ('post chlorite', col. 7) are similar to those of cellulose II, both in value and in the relative intensity of the corresponding arcs. This close agreement, taken with the fact that glucose is the principal sugar in this material, undoubtedly means that this residual component must at least be something like cellulose II. Although the spacing at 9·6 Å. is longer than any accepted for cellulose, spacings of this order have been recorded in the cellulose both of higher plants (Sen and Woods, 1948; Preston and Singh, 1950) and of algae (Myers, Preston, and Ripley, 1956; Cronshaw, Myers, and Preston, 1958).

The diffraction diagram of the material after alkali treatment (Table I, col. 6) shows much more background scatter than was present on the 'post chlorite' diagram, indicating the removal of much amorphous material by

chlorination. There is, however, little doubt that at this stage the crystalline material is already cellulose in the form of cellulose II.

After extraction of the water-soluble material (Table III, col. 3), the spacings compare very favourably with the principal spacings of cellulose I (with the exception of the long spacing at 8·9 Å., to which the remarks on long spacings above also apply). There is no evidence of the presence of cellulose II; for instance, the ring at 7·35 Å. is absent and it will be shown below that the spacing listed at 5·25 Å. refers to 10$\bar{1}$ planes, consistent with the 5·39 (10$\bar{1}$) of cellulose I but not the 5·19 (020) of cellulose II. The indications are, clearly, that the crystalline substance present after removal of the water-soluble material and before the alkali treatment, is something like cellulose I. Treatment with 4N. KOH converts this to something very like cellulose II.

TABLE III

Principal spacings in order of intensity calculated from powder diagrams

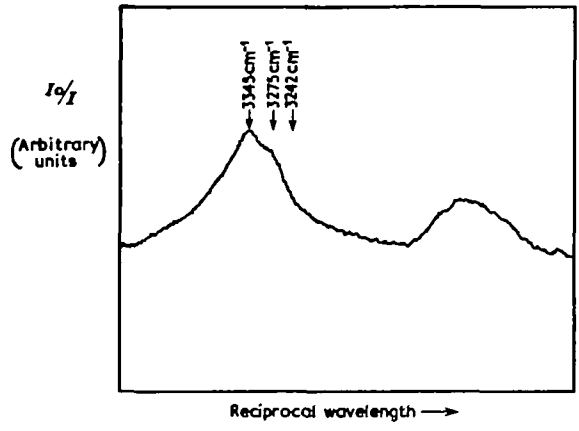
Post-alcohol	Starch-B	Post-water	Cellulose I	Miller index cellulose I	Post-alkali	Post-chlorite	Cellulose II	Miller index cellulose II
5 18	5 17 *s*	3·90	3·93	002; 301	4 41*–4·46*	4 39	4·42; 4·38	002; 021
16·00	15·6 *s*	4 25	4·34	021	4·03	4 01	4·03	002
4·00	4·00 *m*	8 94	—	—	7·35	7 23	7·35	101
8·33	—	5·25	5 39	10$\bar{1}$	—	3 10	3·14	031; 131; 130
6 11	6·29 *m*; 5 89 *m*	2·58	2·58; 2·59	040; 212; 311	2·58	2·58	2·58	040
4·43	4 51 *m*	2·14	5·97	101	2·13	2·13	2·21	202; 103
2 58	—	6·10	1·95; 2·16	004; 240; 042	—	9·64	—	—
3·69	3·68 *m*				—	5·15	5·19	020

Notes: (1) The spacings marked * are very diffuse.
(2) The 4·42 and 4·38 spacings of cellulose II are not resolved and have been bracketed.
(3) *s*, *strong*; *m*, *moderate*.
(4) The order of intensity is approximate only and is intended to serve as a guide to identification, and not as a criterion.

The X-ray powder diagram of the material after the alcohol extraction (col. 1) resembles closely that of starch type B (col. 2) (Bear and French, 1941), the type of starch known to be present in some algae (Frei and Preston, 1960). It seems likely, therefore, that the cellulose I diagram has here been partially masked by a starch diagram type B. The absence of starch diagram after boiling in water and the presence of glucose in the hydrolysate of the water extract can be regarded as confirmatory.

The general conclusion reached from inspection of Table III is, therefore, that the crystalline component of the wall is cellulose I. The comparison between the *Nitella* skeletal material and *Cladophora* eucellulose illustrated in the sector diagram in Pl. I, Fig. 1, fully confirms this view. It is clear that there is complete correspondence of spacings within the limits of resolution of the photograph. There appear, however, to be some slight differences between the two diagrams, in that the relative intensities of reflection from the various crystal planes are different. In particular, the difference between the intensity of the reflection from the 101 planes (inner ring), and the intensity of that from the 002 planes (fourth ring from centre) appears to be greater in *Cladophora* than it does in *Nitella*. Similarly, reflection from the 230 plane is stronger, relative to the intensity of that from the 040 plane, in *Cladophora*

than in *Nitella*; but the combined reflection from the 130, 031, and 131 planes is stronger relative to that from the combined 221 and 122 planes in *Nitella*. The reason for the difference in relative intensity of the rings is not clear. It may be due to a difference in molecular packing in the crystal or to a difference in degree of crystal perfection which might be associated with the presence in *Nitella* cellulose of mannose and xylose. Whatever the cause it is probably not due to a major difference in crystal structure. One other point should be mentioned. The rings are much more diffuse in the *Nitella* diagram than in the *Cladophora* diagram. This, again, might be due to a less perfect crystal structure in *Nitella* or to the presence of much smaller crystallites. The very great deal of amorphous scattering indicates that the wall is not highly crystalline.



TEXT-FIG. 1. Trace of infra-red absorption spectrum of a wall sample prepared, as in text, after deuteration. This shows the presence of cellulose I and the complete absence of cellulose II

In view of these (minor) differences in the detail of the X-ray diagrams of eucellulose and *Nitella* cellulose, it was thought desirable to confirm the presence of cellulose I by infra-red absorption spectrophotometry. Fresh cells, from which the protoplasm had been removed, were reduced to a fine suspension in water in a blender using a Teflon pestle, and this was smeared on to a microscope slide and allowed to dry. The quantities of water and wall material were so adjusted that the mean density of the dry film was 3 μg./mm.^{2} After the film had been dried it was removed from the slide with a sharp razor blade and examined as described by Marrinan and Mann (1954, 1956) and Mann and Marrinan (1956).[1] The results are shown in Text-fig. 1. After a vapour deuteration the material gives a cellulose I, Type B spectrum (as given by cotton, linen, &c.). The prominent band at 3,242 cm.$^{-1}$, typical of a Type A spectrum (typical of the eucellulose of *Valonia*) is absent and there is

[1] The infra-red measurements were made by Dr. J. Mann in the laboratories of The British Rayon Research Association (Manchester).

no evidence of any cellulose II. The amount of material deuterated indicates a high proportion of amorphous material, and this too is consistent with qualitative X-ray evidence.

(*b*) *Orientation of the Cellulose*

(1) *X-ray diffraction analysis*

Determination of the orientation of the crystallites in the wall by X-ray analysis should preferably be made on a single piece of wall oriented in the spectrometer with respect to some morphological axis of the cell. This would require impracticably long exposure times with *Nitella* since the wall is not highly crystalline. In this case, therefore, an accurately aligned and stacked bundle of single cell walls has been used, cut from the same cell. The beam was directed normal to the plane of the wall, with the longitudinal axis of the cell vertical (parallel to the length of the page in the figure). The material was pretreated with dilute acid to remove amorphous material and a front beam-stop[1] was used to reduce background scatter. The resulting diagram (Pl. I, Fig. 2) shows that there is some preferential orientation of the crystalline component. The (002), (10$\bar{1}$), and (101) arcs are strongest along the meridian so that the length of the crystallites must be preferentially oriented in an almost transverse direction. There is, however, a considerable angular dispersion about this mean direction.

Diagrams have also been obtained with the beam parallel to the face of the wall, (*a*) at right angles to the longitudinal axis (Pl. I, Fig. 3) and (*b*) parallel to the longitudinal axis. With the X-ray beam at right angles to the longitudinal axis, the 101 planes are represented by an equatorial arc and 10$\bar{1}$ planes are represented by a very faint meridional arc. This indicates that the 101 planes tend to be oriented parallel to the plane of the wall. The 10$\bar{1}$ reflection occurs only by virtue of the wide angular distribution of crystallites in the plane of the wall. With the beam parallel to the longitudinal axis, the position of the arcs is the same but the distribution of density around the arcs is quite different.

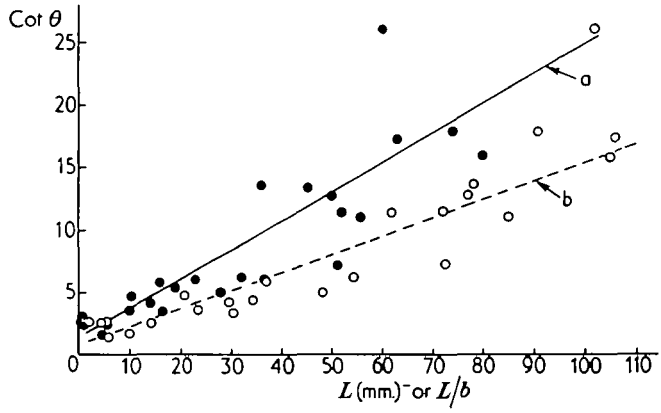
(2) *Light microscopy*

According to Green and Chapman (1955), the direction of the m.e.p.[2] in single walls of *Nitella axillaris* lies transversely to the cell axis so that the birefringence is negative. This is consistent with the above X-ray evidence. In *Nitella opaca*, however, the m.e.p. is not exactly transverse; it usually lies around the cell in a slow helix. This has already been fully documented elsewhere (Probine and Preston, 1958) and it has been accepted both by us and

[1] This is a small lead cup inserted between the specimen and film, intercepting the primary beam and therefore reducing air scatter.

[2] The 'm.e.p.' is the major extinction position of the wall when viewed between crossed nicols. It corresponds to the direction of the major refractive index and therefore to the mean direction of the cellulose crystallites.

by Green (1959) that there is in this respect a real difference between *Nitella axillaris* and *Nitella opaca*. Although the difference may be one of degree rather than of kind, it is not proposed at the moment to take this point further. As already reported by Probine and Preston (1958) there is, however, in *Nitella opaca* a change in the inclination of the m.e.p. as an internodal cell grows longer and therefore older, and this, as well as other changes also associated with length increase, will be briefly restated here. Perhaps the most noticeable, and certainly the simplest association is that between cell length and the angle (θ) which the streaming direction makes with the cell axis. It is



TEXT-FIG. 2. The relationship between cot θ (θ = angle between cell axis and streaming direction) and cell length (L) or ratio of length to breadth (L/b). Curve *a* (solid circles), L.; curve *b* (open circles) L/b.

convenient to plot the length against cot θ as in Text-fig. 2*a*. The relationship tends to be linear, with a scatter of the experimental results which is considerably reduced when the length/breadth ratio is used as abcissa in place of the length alone (Text-fig. 2*b*). It therefore follows that the streaming direction depends both upon the length and the breadth of the cell. The correlation coefficient between L/b and cot θ (0·91) is highly significant (0·62 at the 0·0001 level of significance for 23 degrees of freedom) and the regression equation is of the form

$$\frac{L}{b} = a + c \cot \theta,$$

where L and b are the length and diameter of a cell respectively and a and c are constants. A relationship of a more complex type occurs between the streaming angle (θ) and the direction (ψ) of the m.e.p. of the wall. The relationship has already been expressed graphically elsewhere (Probine and Preston, 1958) but it is more instructive to examine the numerical results themselves. These are presented in Table IV for cells of various known lengths and, therefore, ages. Although there is a highly significant correlation

TABLE IV

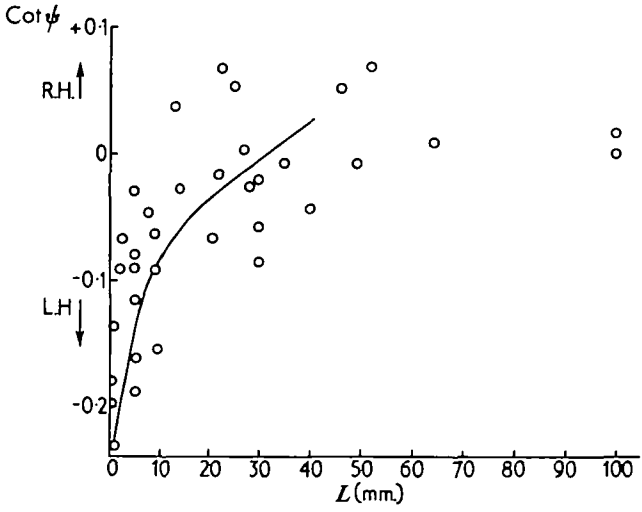
The angle between streaming direction and cell axis (θ), between the m.e.p. and the transverse plane (ψ), and the angular separation of the streaming direction and the m.e.p. (α) in internodal cells of different lengths

Cell length	θ	ψ	α
100	2·1 *r*	1·1 *r*	86·8
100	3·2	0·0	86·8
64	3·5	0·2 *r*	86·3
48	4·7	0·5 *l*	85·8
30	7·3	5·0 *l*	87·7
25	8·4	3·0 *r*	85·0
22	9·5	0·9 *l*	81·4
21	9·9	3·7 *l*	83·8
28	11·2	1·1 *l*	79·9
14	13·0	1·6 *l*	78·6
12·0	14·5	2·0 *l*	77·5
9·0	16·5	5·3 *l*	78·8
9·0	16·5	8·9 *l*	82·4
7·5	18·1	2·7 *l*	74·6
5·5	21·5	9·3 *l*	77·8
5·0	22·0	6·7 *l*	74·7
3·0	26·8	6·1 *l*	69·3
2·5	27·2	3·9 *l*	66·7
4·8	29·9	10·7 *l*	70·8
1·0	30·8	13·4 *l*	72·6
0·5	35·1	11·2 *l*	66·1
0·5	35·6	10·2 *l*	64·6

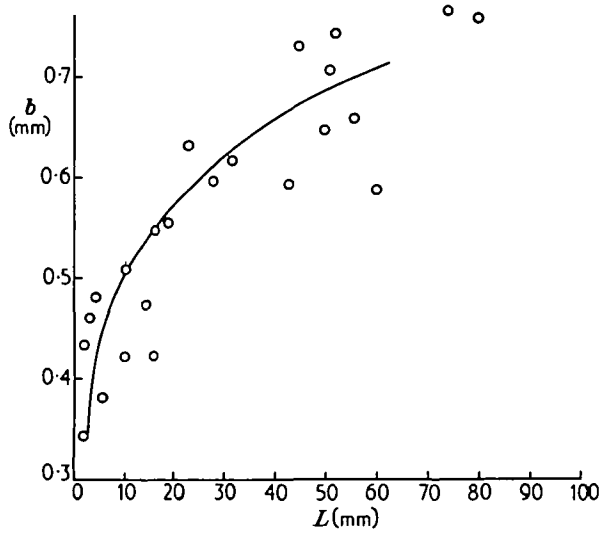
($r = 0{\cdot}85$) between streaming angle and the m.e.p. ($r = 0{\cdot}617$ at the 0·001 level of significance for 23 degrees of freedom), the fact that the angle between them (α) is not constant must imply that there is no direct geometrical connexion.

The close correlations thus described imply that there must be a correlation between m.e.p. and cell length. Such a relation is expressed graphically in Fig. 3 and is clearly complex. Up to a cell length of about 15 mm. ψ varies almost linearly with l, but in the sense that as the cell grows longer the spiral becomes flatter. Beyond this length the upward trend continues, though at a slower rate, the spiral now, however, changing sign from left-hand (S) to right-hand (Z) and becoming steeper. During the whole of the growth period illustrated in Text-fig. 3 the internodal cells are, of course, increasing in diameter as well as in length. This is demonstrated in Text-fig. 4 in which lengths and diameters are plotted for the cells used in obtaining the data in Figs. 2 and 3 and Table IV. The increase in diameter is throughout small compared with the increase in length and if changes in wall structure were determined by absolute changes in dimensions then it would be expected that length changes would be of overriding importance. This is clearly not the case. It may be significant, however, that the rate of change of diameter with length (db/dL, Text-fig. 5) is high for shorter cells and falls rapidly as the cells grow longer and therefore older. The helix defined by the m.e.p. thus becomes flatter at a time when db/dL is high and steeper when db/dL is low.

The possibility of a connexion between protoplasmic streaming and wall structure is strengthened by observation of the wall lying over the striation visible in the cytoplasm and referred to above. As already remarked upon by



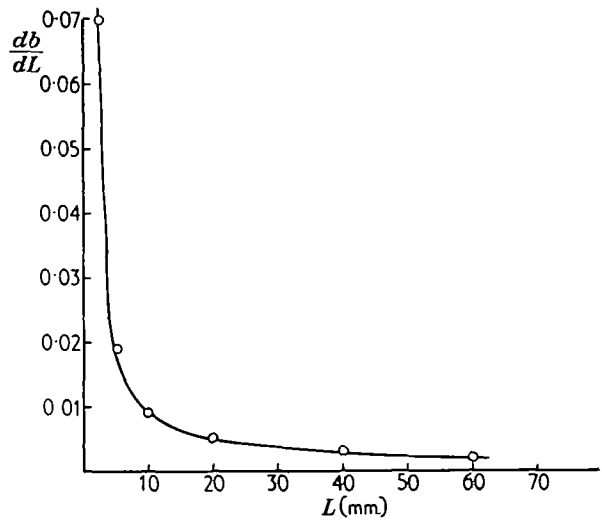
TEXT-FIG. 3. The relationship between cot ψ (ψ = angle between m.e.p. and the transverse plane) and L. R.H. = right-hand, L.H. = left-hand helix. The curve is drawn in arbitrarily.



TEXT-FIG. 4. The relationship between cell diameter b and cell length, L, in growing cells. Each point refers to a *different* cell.

Green and Chapman (1955) the position of the striation—associated with a discontinuity in flow—is marked by a linear region of the wall with optical properties different from those of the rest of the wall. Each striation is visible as a linear region which appears to extinguish, in the polarizing microscope,

when its long axis, rather than that of the cell, is parallel to the vibration plane of either polaroid. Movement of a striation through the extinction position reveals changes in brightness which are somewhat difficult to interpret. Many striations show a very narrow isotropic 'line' down the centre. While their properties are difficult to determine, there is no question that the striations differ structurally from the rest of the wall. The results of the



TEXT-FIG. 5. The relationship between the relative rate of breadth increase (db/dL) and cell length.

present investigation are so far in general agreement with those of Green and Chapman (1955), but there are some further observations which are relevant. In *Nitella opaca* the structural difference which occurs in the region of the striation is certainly a difference in microfibril arrangement, i.e. it is a discontinuity of arrangement of the crystalline component and not of the amorphous material. This is clear from the fact that, after a wall has been chemically treated,[1] lamellae stripped from it in the region of the striations and observed in the electron microscope show microfibrils with little or no amorphous material encrusting them (see below). When cells which have been treated in this way are examined in the polarizing microscope, however, the optically different striations are still clearly visible. Not all striations, however, are structurally similar. Some show the very narrow isotropic line down the centre mentioned by Green and Chapman (1955) but the common appearance between crossed nicols is as shown in Pl. I, Fig. 4. In these cases the striation shows up as a central bright 'line' bounded on each side by a dark 'line' when viewed with the microscope stage considerably displaced

[1] The cells were treated with hot 2 per cent. HCl for 30 minutes, washed, treated with hot 4 per cent. KOH, and washed. This cycle was repeated three times. They were then given a chlorite treatment as described by Cronshaw, Myers, and Preston (1958).

from the wall extinction positions. The presence of these dark lines, which do not extinguish sharply, suggests that, in these localized areas, the structure differs from the rest of the wall in one or more of the following ways:

(*a*) The wall might be much thinner, or be less crystalline. The fact that the wall separates into lamellae which are continuous sheets, and which do not appear to fail preferentially in these regions, does not reinforce this view.

(*b*) The microfibrils might be considerably disoriented about their mean preferred direction in these regions. This seems to be the more likely explanation. It is difficult to measure the retardation of these dark areas accurately, but such measurements as have been made indicate that the retardation is indeed considerably lower than in the rest of the wall.

The variation in birefringence across the striation is illustrated in Pl. II, Fig. 5, a composite photograph of a striation and the wall on either side of it, built up in the following way. A piece of wall was set to extinction, and the stage then turned through 45° so that the wall appeared bright. An elliptic compensator was inserted and rotated until the wall was extinguished. A photograph of wall and striation was taken in this position, and at 2° steps on either side of the extinction setting. Narrow strips of the same portion of wall cut from each photograph, at right angles to the striation, were then assembled side by side in order and rephotographed. The result is Pl. II, Fig. 5. The effect can best be seen by viewing the figure at a low angle to the plane of the page in the direction of the striation. It will be seen that the wall on either side of the striation extinguishes at a compensator setting (shown on the side) which is different from that at either the centre of the striation or in the bands on either side. These latter appear to have a lower birefringence than the rest of the wall, and the centre a slightly higher birefringence. This piece of wall was chosen because the striation and wall extinguished together between crossed nicols and the birefringence was of the same sign so that this is not likely to be an artifact due to these regions having different m.e.p.'s.

The nature of the structural discontinuity which occurs in the region of the striation appears to be somewhat variable and, therefore, difficult to define with precision. This topic will be discussed further after the electron microscopic evidence has been presented. At this stage it is merely noted that a definite structural discontinuity occurs at the boundary of the two cytoplasmic streams, consistent with the possibility that direction of streaming and microfibril direction might be linked. One further point might be made, however, before leaving this topic. It has been tacitly assumed in the foregoing discussion that the flow boundaries are more or less fixed in relation to the cell surface. There is good evidence that they do indeed occupy the same region of the cell surface throughout most of the life of the cell (Probine, 1959).

(3) *Wall swelling in water*

Anisotropy of swelling was used early as an indication of anisotropy of structure (Schwendener, 1874); and, more recently, attempts have been made to relate anisotropy of swelling to orientation in man-made fibres, see, e.g.

Hermans, 1949. In the present investigation measurements of the degree of swelling in the radial, transverse, and longitudinal directions in the cell wall of *Nitella* have yielded valuable information on the structure of the wall.

The increase in thickness of the wall (radial direction) due to wetting has been measured in two ways. In the first method a Philips displacement transducer (GM 5537) was used as a thickness gauge. In this instrument, linear displacement of a lightly loaded measuring stylus is converted into an electrical signal, and the magnitude of the displacement is indicated directly on the output meter of a direct-reading measuring bridge (GM 5536). For this application the transducer was held rigidly above the surface of a good precision-ground and lapped surface plate. A cell, from which the contents had been removed, was opened out flat and dried down on to a glass plate which was rigidly fixed to a precision-ground vee block movable over the surface of the plate in such a way that the stylus rested either on the glass reference surface, or on the specimen. The difference in the readings obtained in these two positions represented the dry thickness of the specimen. In practice, a reading was taken in the middle of the specimen and on the glass reference surface on either side of the specimen, these two latter readings being averaged to give the 'zero' reading. The procedure was repeated at several points along the length of the specimen. A drop of water was then placed on the specimen with the stylus in position and, after swelling had ceased, the bridge output meter indicated the increase in thickness directly. The results obtained from a number of specimens indicated that the increase in wall thickness on wetting was of the order of 100 per cent. The dry and wet thickness of a strip of wall was also measured directly by an optical method. This measurement was much more difficult and inconvenient and served only as a check on the transducer measurements. The increase in thickness was again of the order of 100 per cent.

In the transverse direction in the plane of the wall the increase in dimension on wetting, measured microscopically, was of the order of 4 per cent., and, in the longitudinal direction, 6 per cent.

The large percentage increase in the thickness of the wall on wetting suggests that there is little reinforcement of the wall in the radial direction, such as would occur, for instance, if the microfibrils were interwoven and entangled throughout the thickness of the wall. The observed behaviour of the wall is consistent with the idea that the microfibrils are arranged in discrete layers with pectic substances providing an amorphous matrix between microfibrillar reinforced laminations, or, at very least, that the microfibrils lie strictly in the plane of the wall. That the first of these models is substantially correct is shown by the fact that, when the wall is treated with hot 0·5 per cent. ammonium oxalate for 30 minutes to remove pectic substances, it can be separated into thin lamellae using a needle and fine forceps. In Pl. II, Fig. 6 a photograph is reproduced of a piece of wall which had been torn across and had failed in such a way that five separate wall layers are visible. The photograph was taken between crossed nicols so that differences in optical thickness show up clearly

and reveal the boundaries of the laminations. This is a new observation for *Nitella*. Green and Chapman (1955) recorded consistent failure of attempts to flake or tear the wall of *Nitella axillaris* into thin lamellae and concluded that the wall was uniform in composition. Apart from its structural importance and its bearing on the elastic symmetry of the wall, the fact that the wall can be separated into thin lamellae opens up new possibilities for more precise structural examination in the electron microscope.

(4) *Electron-microscopic examination*

Further chemical treatment is necessary, however, before the lamellae are sufficiently free of amorphous material for detailed study. The following treatment, which is a slight modification of one used by Kreger (1957), has been found satisfactory. The wall was treated successively with hot 2 per cent. HCl and hot 4 per cent. KOH and washed. This cycle was repeated three times. The wall was then given a chlorite treatment as described by Cronshaw, Myers, and Preston (1958). It could then easily be stripped under water into lamellae free of amorphous material when examined in the electron microscope.

An electron micrograph of a piece of wall so treated is reproduced in Pl. II, Fig. 7. In spite of the high magnification, the microfibrils show up quite clearly and cleanly. The microfibrils are unusually narrow, the finest being of the order of 50 Å. wide, compared with the 200 Å. or so thick microfibrils of *Valonia* (Preston *et al.*, 1949; Preston, 1951; Preston and Cronshaw, 1958). Among the other algae for which figures have been published, *Cladophora rupestris* has microfibrils 300–250 Å. wide and the alga with the narrowest microfibrils in the group listed by Cronshaw, Myers, and Preston (1958), was *Ulva lactuca* (180–90 Å.). Microfibrils 50–100 Å. wide have been recorded for conifer tracheids by Hodge and Wardrop (1950), and Rånby (1952) lists widths of 73–80 Å., 87–90 Å., and 108–160 Å. for wood cellulose, cotton cellulose, and tunicin respectively. *Nitella* microfibrils appear, therefore, to be on the lower limit of microfibril size. The small size of the crystallites was one of the reasons advanced above for the diffuse nature of the X-ray diagram of *Nitella*, and this electron-microscope evidence is qualitative confirmation of this suggestion.

Examination of stripped material also enables some estimate of the microfibril orientation to be made. The mean direction of the microfibrils with respect to the longitudinal axis cannot be determined with any very great accuracy by this means (within $\pm 20°$, say), as it is not always possible to orient accurately wall strips on the specimen grid. Reasonably reliable estimates can be made, however, of the distribution of microfibrils about the mean fibril direction—subject to the proviso that the act of stripping has not caused misalignment at the interface as the strip was pulled away.

An example of the appearance of an inner layer stripped from a young cell (length about 10 mm.) is shown in Pl. III, Fig. 8. The *approximate* direction

of the longitudinal axis is shown. It is apparent that the bulk of the microfibrils lie approximately at right angles to the longitudinal axis, in agreement with the X-ray and polarizing microscope evidence. There is, however, a fairly large number of microfibrils lying roughly at right angles to the main direction. It is almost as if this is a very imperfect example of a crossed-fibrillar structure. The possibility that the 'longitudinal' microfibrils are artifacts due to fibril displacement in the stripping process is unlikely for two reasons. First, there appear to be fibrils with longitudinal orientation lying deep within the wall with transverse fibrils overlying them. Secondly, Green (1958) has published replicas of the wall which show microfibrils considerably displaced from the transverse direction—particularly in his Pl. 247, Fig. 6. His replica technique was not likely to cause displacement of the microfibrils from the transverse direction, so that it must be concluded that, in some circumstances, the microfibrils can assume this peculiar orientation. Green notes that this particular photograph is of an internode approaching the end of its elongation. Pl. IV, Fig. 10, which is from an unknown location within the wall, also shows evidence of a crossed fibrillar pattern, but with considerable disorientation about both directions.

The angular frequency distribution of the microfibrils has been obtained in the following way. The central area of the micrograph shown in Pl. III, Fig. 8, was enlarged twofold and the enlarged print was repeatedly and randomly pierced with a pin all over the surface. The direction of the microfibril nearest each pin-point was determined and a histogram constructed showing the number of microfibrils having directions within the angular ranges, 0–10°, 10–20°, 20–30°, &c. relative to some fixed direction. The angular frequency distribution obtained in this way is shown on the diagram overlying the photograph. It is evident that there are two main directions. The method clearly tends to cause a bias in favour of the 'longitudinal' fibrils which appear, in general, to be overlying the 'transverse' fibrils. It will be recalled that the X-ray diagrams do not show the characteristic 'crossed-fibrillar' pattern which could therefore be expected. It seems likely, however, that the 'longitudinal' microfibrils occur very much less frequently than do the transverse. Further work remains to be done before this feature of the wall is completely understood. At this stage, attention is merely drawn to the apparent presence of a crossed-fibrillar structure.

In some cases the wall shows a series of disturbances (Pl. III, Fig. 9) such as have been described by Green (1958).

In Pl. IV, Fig. 11, an electron micrograph is reproduced of the extreme outside layer of an immature cell. This layer was notable in that, after the wall had been prepared for stripping, there was very little evidence of bonding between it and the rest of the wall and it was separated with very little manipulation indeed. In this case the microfibrils are aligned longitudinally and this was the case for all grids examined on which this particular layer was mounted. This agrees very well with what one would expect on the basis of the multinet theory of Roelofsen (1951) and Roelofsen and Houwink (1953).

Other outer layers showed this tendency to longitudinal orientation but this particular case provided the most notable example.

For electron microscopical observation of the wall in the region of the striation fairly mature cells (i.e. length 30 mm.) were chosen because optical studies suggested that the striation might be more easily recognized in these cells. The protoplasm was removed as previously described and each cell was cut into 2-mm. lengths. These were chemically treated for stripping and each cylinder was then opened out to form a flat sheet. A thin layer of wall was removed from the inner surface of the cell, and placed on a Philips grid so that the direction of the cell length was at right angles ($\pm 15°$) to the grid slot.

It was found that a striation always showed up as two electron-dense bands close together and easily recognizable (Pl. IV, Fig. 12). The identification of these bands with the striation region was based on the grounds that (*a*) the bands were always at right angles to the grid slot within the limits given, (*b*) where two pairs of such bands were visible they were parallel to each other and separated by a distance of the same order as half of the cell circumference, (*c*) on the one occasion when grid slot and longitudinal cell axis were parallel, a pair of electron-dense bands, present by chance, ran the whole length of the specimen, (*d*) the bands did not have the appearance of folds, which are easily distinguished, and (*e*) the separation of the electron-*dense* bands agreed very well with the separation of the *low* birefringence areas visible in the polarizing microscope.

In the bands there sometimes appear to be a number of microfibrils running parallel to the striation, but apart from this there seem to be no particular associated structures. On either side of the 'striation' area the wall appears quite uniform over some considerable distance from it and within this the microfibrils seem to lie roughly at right angles to the striation. The 'striation' areas of a number of cells were examined and in each case the electron-dense regions were easily distinguishable.

There is therefore very strong evidence that a discontinuity of wall structure does exist in the region of the striation. It would appear that the 'electron-dense' areas are regions of greater disorder than found either in the rest of the wall or in the middle of the striation. Electron-microscope investigation of the disturbances has not been as carefully related to cell age as was Green's investigation but the observations are generally in harmony with Green's conclusions.

DISCUSSION

X-ray diffraction analysis, chemical analysis, and infra-red spectrophotometry together, therefore, confirm that the skeletal material in the wall of *Nitella opaca* is a cellulose. This material, comprising about 17 per cent. of the wall by weight, yields on hydrolysis mannose and xylose as well as glucose, resembling in this respect the cellulose of many of the marine algae studied by Cronshaw, Myers, and Preston (1958). All examination procedures demonstrate the presence of amorphous incrusting substances and the chemi-

cal analysis presented in Table II shows that these include compounds of the polyuronide type. The diffuse appearance of the X-ray diagrams suggests that the cellulose crystallites are unusually narrow, which is well understandable since the microfibrils are only about 50 Å. wide.

The wall is lamellated and the high degree of swelling in thickness, coupled with the easy separation of the lamellae after extraction with ammonium oxalate, shows that the polyuronides contribute toward the binding together of the lamellae. The lamellae themselves may be stripped as coherent sheets even after such chemical treatments, presumably because the microfibrils, though tending toward a preferred orientation, show considerable scatter about this direction with some degree of entanglement. The microfibrils of an innermost wall lamella tend toward an orientation which is approximately transverse whereas those of an outermost wall lamella tend toward longitudinal orientation, a difference which is quite clear-cut and decisive. While transitions between the one orientation and the other in intermediate lamellae have not been observed, this is precisely the kind of evidence which led Roelofsen (1951) and Roelofsen and Houwink (1953) to their multinet-growth hypothesis for other cell types and in part confirms the conclusions reached by Green (1960) by more indirect methods. The high degree of angular dispersion of the microfibrils in the innermost lamella and their apparent entanglement shows, however, that if the structure of the outermost lamella has been produced by the longitudinal extension of a lamella initially resembling the innermost lamella, then the microfibrils must be free to slide over each other. This is a feature common, in greater or less degree, to all cell walls to which the multinet-growth hypothesis has been applied and it is common experience that carelessness during the stripping of a wall can lead to a pulling out of individual microfibrils from a lamella within which they have appeared to be hopelessly entangled. Multinet growth may well therefore be involved in the extension of this particular cell wall.

In considering both the mechanism of cellulose orientation and the changes in the orientation arising as a consequence of growth, note must of course be taken of the fact that the orientation of the microfibrils of the innermost—and therefore most recently deposited—layer is far from universally transverse. On the contrary, microfibrils can already be observed lying displaced at all azimuths from the transverse with a tendency, indeed, to favour the longitudinal as a second preferred orientation less marked, but no less certain, than the approximately transverse. It seems unlikely that this innermost lamella could already have been subjected to a longitudinal extension sufficient to cause a major reorientation, and the conclusion seems inescapable that the lamellae are laid down with this diversity of orientation. The structure recalls that already reported in the S1 layer of the secondary wall of a conifer tracheid (Frei *et al.*, 1957). It is not at the moment clear whether this is to be regarded as expressing an imperfection in a mechanism imposing predominantly transverse orientation or whether this wall represents a crossed fibrillar structure differing only in perfection from that shown by some other algae (Preston

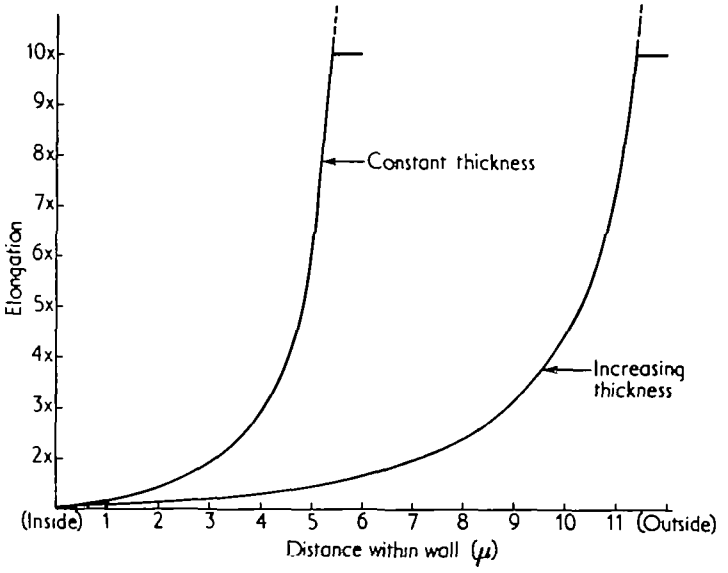
and Astbury, 1937; Astbury and Preston, 1940; Nicolai and Frey-Wyssling, 1938; Steward and Muhlethaler, 1953; Cronshaw and Preston, 1958; Frei and Preston, 1961).

Possible changes in orientation of the microfibrils as a cell elongates have been followed only through measurement of the m.e.p., i.e. by determining the net orientation throughout the wall thickness in cells of various lengths. The mutual correlations between cell length, m.e.p., and the direction of streaming in the cytoplasm appear to be rather complex. This makes it unlikely that the direction of the m.e.p. can causally be connected with cell length. Moreover, it can be shown that the process of multinet growth and extension need not produce major changes in the overall fibril distribution and, therefore, in the m.e.p.[1] In this type of growth the whole wall is considered, at any stage, to stretch passively and therefore becomes proportionally thinner. This reduction in thickness is counterbalanced, to a greater or less degree, by simultaneous apposition of new wall lamella on the inner face of the wall, which then comes in turn under stress. Suppose, therefore, a young cell, with a wall 6 μ thick, increases in length ten times. For the sake of simplicity imagine that first the cell, of length l, increases in length to $6/5{\cdot}5l$ and then lays down a new lamella 0·5 μ thick so that the wall remains 6 μ thick. By repetition of this process, a state can be reached at which the cell has become ten times as long while the wall has maintained its thickness of 6 μ. Though cells do not grow in this stepwise fashion the resultant elongations, after deposition, of the lamellae successively laid down will give a fair approximation to the truth. Fig. 6 shows the amount by which each lamella of the wall has extended, when a cell has increased in this way 10× in length for the condition of constant wall thickness outlined above and for the condition in which the wall increases in thickness by 0·5 μ at each step. In either case the outermost lamella, 0·6 μ thick, represents the original 6 μ-thick wall. Remembering that the original cell itself would already have extended, then the actual curve would extend steeply as shown by the dotted line. Now it is clear that in a cell at any stage of growth the distribution of strain through the wall will be much as exemplified in Text-fig. 6. Hence, after any subsequent elongation the fibril distribution (and the m.e.p.) will tend to be the same. This argument assumes that the newly deposited innermost lamella has, at each stage, the same structure, and assumes that the proportion of longitudinal to diametral extension is similar throughout growth (i.e. $\delta l/l = k\,\delta l/b$, where k is a constant). The fact that the birefringence remains almost constant during growth is experimental evidence in support of this suggestion.

It does not therefore seem probable, either on these theoretical grounds or on the basis of the results presented in this paper, that the observed correlation between cell length and wall structure can imply a direct causal connexion. The change in the m.e.p. must rather be associated with changes in the microfibrillar orientation in the successive innermost lamellae deposited

[1] This was pointed out to us independently by Dr. Barber of the Dominion Physical Laboratory, New Zealand.

as a cell grows. These changes must in turn be a consequence of some property of the cytoplasm which changes with cell length and the only property known so far to change is the direction of streaming. It could therefore be that the direction of streaming—which changes as a cell elongates—is involved in the orientation of microfibrils. Such a connexion can hardly be direct since



TEXT-FIG. 6. The distribution of strain at various points within a wall which, initially uniform, has extended overall by 10 times while either maintaining its thickness at 6 μ or increasing it. For further explanation, see text.

presumably the layer of cytoplasm nearest to the wall is stationary. If a connexion does exist, it is rather to be sought through an orientation of protein chains or chain aggregates in the stationary layer, the microfibrils being oriented at right angles to the protein chains. The variation in the angle α of Table IV could then be due to differing rates of cell elongation, relative to the amount of wall deposited, as a cell grows.

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PLATES

FIG. 1. X-ray sector diagram of *Cladophora* cellulose (top right and bottom left), and *Nitella opaca* wall after treatment with 2% H_2SO_4 (top left and bottom right). Note the correspondence between the rings in the two diagrams.

FIG. 2. X-ray diagram of a stack of aligned strips of untreated *Nitella opaca* wall: beam normal to wall surface, cell axis parallel to longer edge of page. The white shadow is due to the support of the front beam-stop. Note that the rings are most intense along the meridian.

FIG. 3. As in Fig. 2, but beam parallel to wall surface and perpendicular to the long axis of the cell.

FIG. 4. Photomicrograph of a single wall of *Nitella opaca* between crossed polaroids, showing a striation passing from lower left to upper right. An elliptic compensator was inserted in the light path and displaced slightly from the position in which the wall was extinguished.

FIG. 5. Composite photograph of a striation between crossed polaroids at various settings of an elliptic compensator. Compensator settings shown at the side of each photographic strip. For further explanation, see text.

FIG. 6. Photomicrograph of torn piece of wall between crossed polaroids showing about 5 lamella, and one striation passing obliquely upwards.

FIG. 7. Electron micrograph of a wall lamella shadowed Pd-Au, magnification 120,000×.

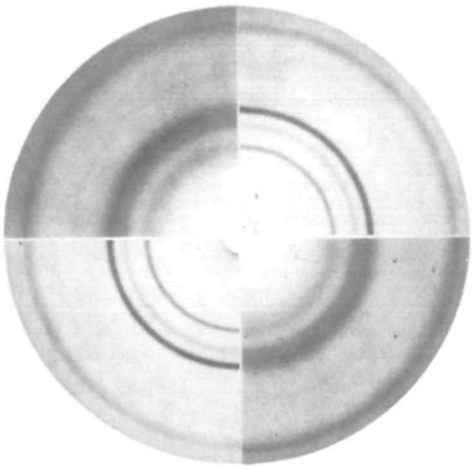
FIG. 8. Electron micrograph of the innermost wall lamella of a young internodal cell; shadowed Pd-Au; magnification 27,900×. Axis of cell parallel (approx.) to longer edge of page. Note that most microfibrils run almost transversely though many lie at other azimuths. The polar curve drawn on the lower right gives a measure of the angular distribution of the microfibrils, the length of line joining any point on the curve to the centre being proportional to the number of microfibrils lying in the direction of the line. There is therefore a marked tendency toward a crossed microfibrillar structure.

FIG. 9. Electron micrograph of a wall lamella in the area between two striations; shadowed Pd-Au; magnification 15,000×. Note the areas of disturbed orientation.

FIG. 10. Electron micrograph of a wall lamella of unknown location in the wall of an internodal cell; shadowed Pd-Au; magnification 27,900×. Axis of cell parallel (approx.) to longer edge of page. Note clear signs of a tendency toward a crossed fibrillar structure.

FIG. 11. As in Fig. 10, except that the lamella is the outermost coherent lamella of a young cell. Note the tendency toward axial orientation of microfibrils.

FIG. 12. Electron micrograph of a wall lamella at a striation; shadowed Pd-Au; magnification 7,200×.



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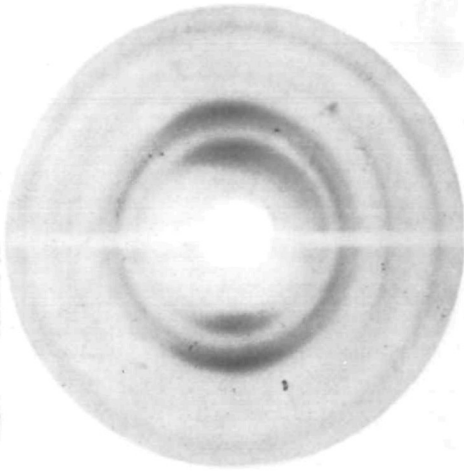

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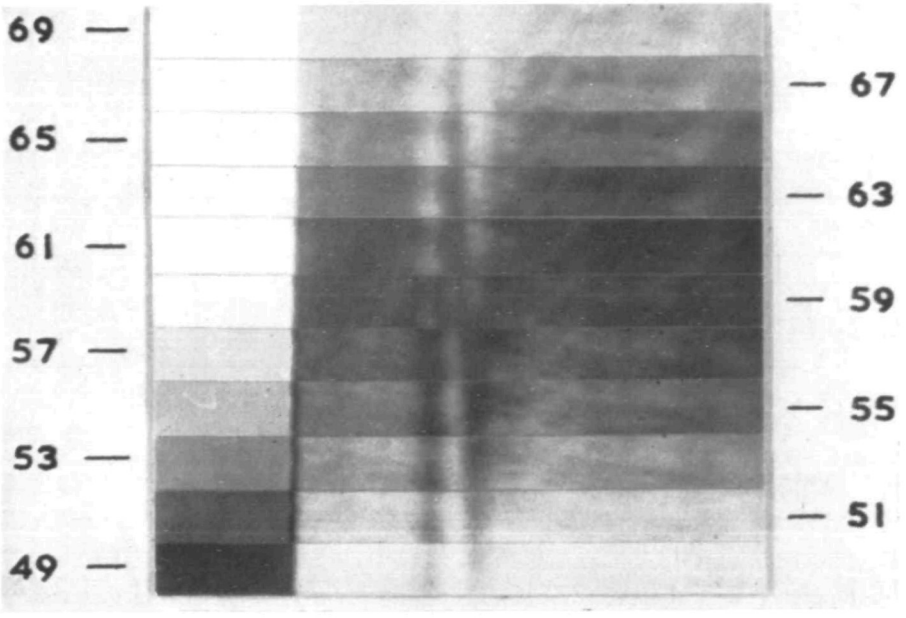
M. C. PROBINE AND R. D. PRESTON—PLATE I



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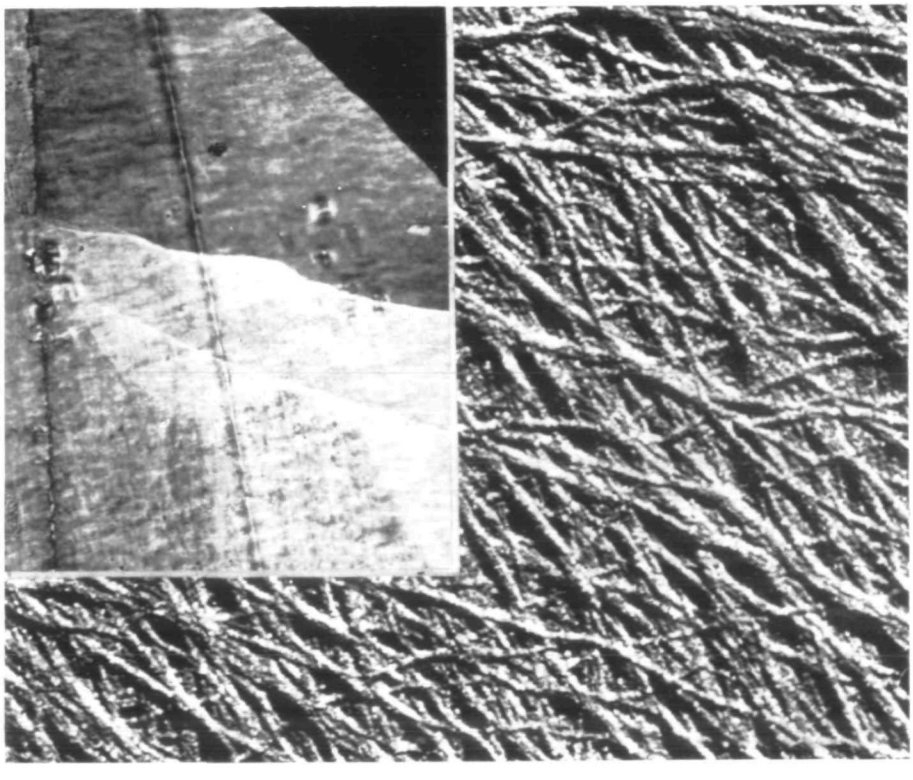

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M. C. PROBINE AND R. D. PRESTON—PLATE II

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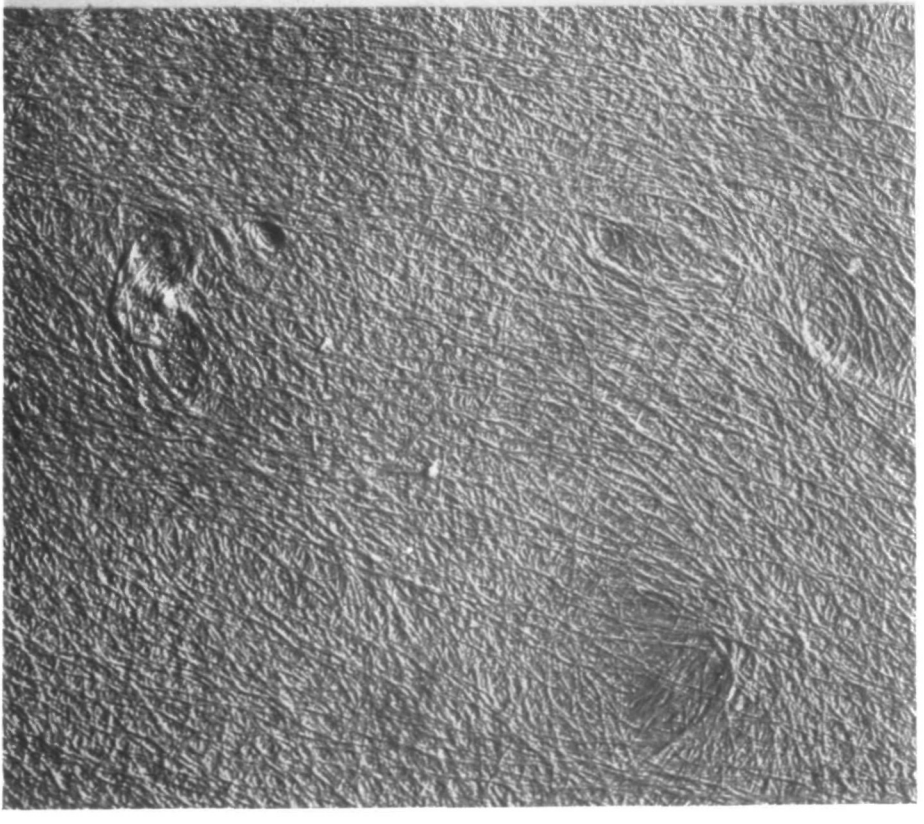

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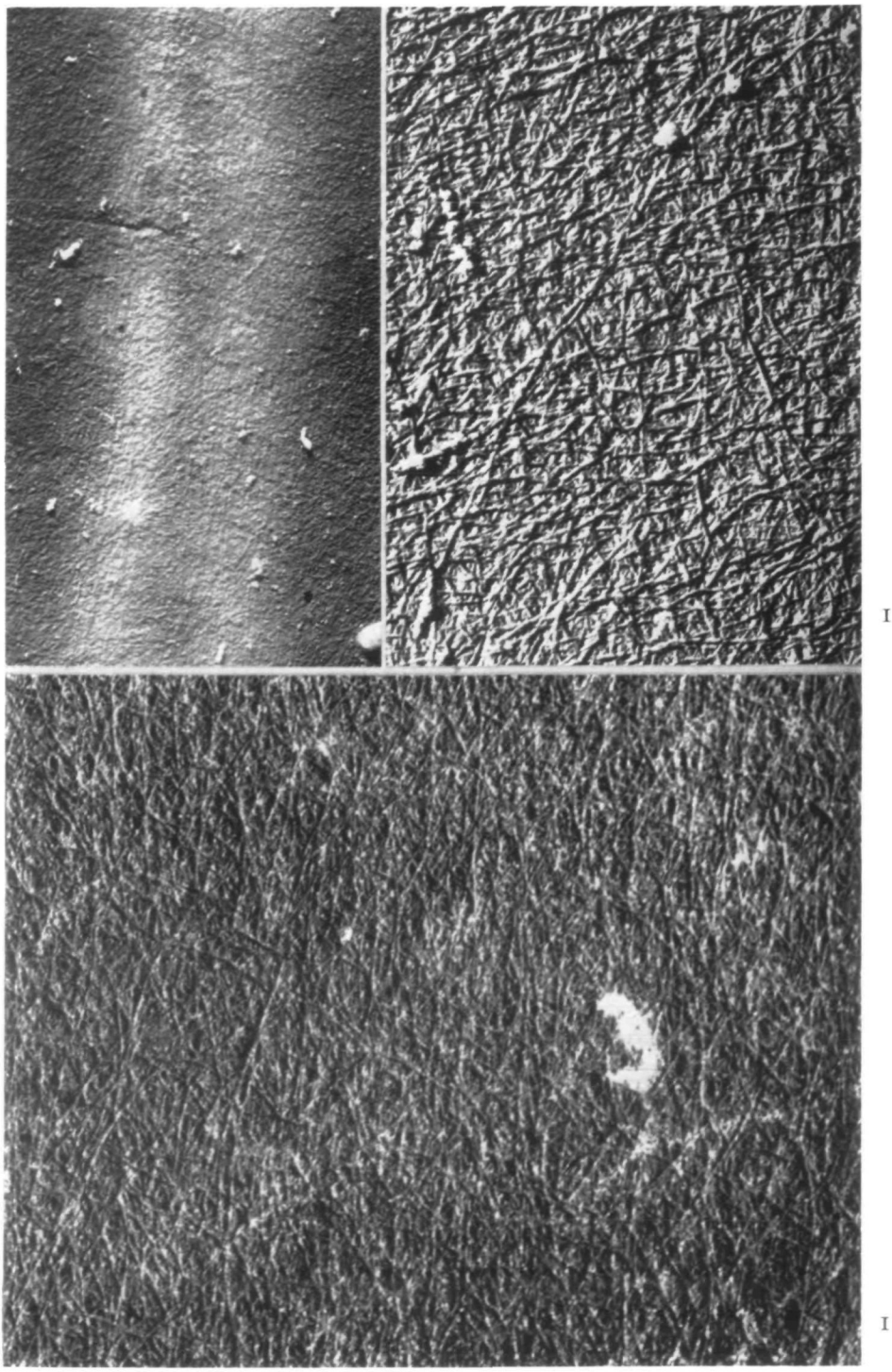
M. C. PROBINE AND R. D. PRESTON—PLATE III



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M. C. PROBINE AND R. D. PRESTON—PLATE IV