THE ORIGIN OF SPIRAL GROWTH IN PHYCOMYCES SPORANGIOPHORES

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CONTENTS

Introduction

OORT and ROELOFSEN (1932) after having established the presence of a flat Z-spiral[1]) in the arrangement of the chitin fibrils[2]) in the wall of the growth zone of *Phycomyces* sporangiophores, did not enter into the question of how to explain the spiral growth, i.e. the rotation of the sporangium during growth. They took it for granted that the direction of greatest extensibility should be perpendicular to that of the spiral structure. Likewise, CASTLE (1942) considers that the structure of the wall is the causative factor, and also does not enter further into the question.

Starting from the assumption that a cell which possesses a spiral structure behaves during growth like a spring that is being stretched, PRESTON (1948) has developed a theory which claims to explain quantitavely the spiral growth of *Phycomyces*. On this basis he arrives at the conclusion that since normally there is a clockwise rotation of the sporangium as seen from above, there cannot be a Z-, but an S-spiral structure.

This is doubted by ROELOFSEN (1949), because PRESTON did not make any direct observations on the existence of an S-spiral structure and also because his (ROELOFSEN's) model experiment with a wire S-spiral did not show a clockwise (right-handed as seen from above), but an anti-clockwise (left-handed) rotation when stretched. Moreover he points to the fact that in addition to the stretching of Z-spirals, widening (i.e. increase in cell diameter) also would induce clockwise rotation. Finally he mentions the possibility that spiral growth might also originate from "active" intussusception of new units between the old spirally arranged fibrils.

This last factor differs essentially from the others, because here spiral growth would not be determined by the mechanical properties of the wall itself, but by properties of the protoplasm controlling the regular deposition of the microfibrils. Thereby intussusception is supposed not to follow passively the mechanical extension of the wall, but to play an active part in causing the twisting of the cell. FREY-WYSSLING and MÜHLETHALER (1950) go so far as to look upon such an explanation as the only true one,

[1]) We shall speak of a Z-spiral if the part of the spiral nearest to the observer runs in a direction parallel to the middle part of the letter Z, the main axis of the spiral being vertical.

[2]) By fibrils or microfibrils are meant interwoven threads of 150—250 Å thickness, shown recently in electron micrographs, (FREY-WYSSLING a. MÜHLETHALER 1950, ROELOFSEN 1950).

because they find spiral structures in the wall of cells in growth stage I [1]), although, as they suppose, these do not twist. However, this supposition is not quite correct, as CASTLE (l.c.) has shown. Our experiments to investigate whether intussusception does indeed take an active and not only a passive part in spiral growth, will be described in § 1.

A second point in our investigations is concerned with a possible explanation of the fact, demonstrated by CASTLE (l.c.) and by PRESTON and MIDDLEBROOK (1949, that after a stagnation in growth the rotation of the sporangium is temporarily left-handed. These experiments will be described in § 2.

Finally, in § 3, we shall give a theoretical consideration of the forces causing orientation in cell-walls in general and of spiral structure in particular, based on experiments with models.

The results of our recent investigations on wall structure of *Phycomyces* sporangiophores are published elsewhere (1950).

§ 1. Experiments on the active part taken by intussusception in spiral growth

a. Introduction

As we have described elsewhere (1950) the wall in the growth zone consist of a strongly interwoven network of threadlike microfibrils of 150—250 Å thickness, arranged with a statistical preference for a flat Z-spiral direction. For the sake of clearness we shall schematize the fibrils and represent them as short rods. The upper wall of a cell with a Z-spiral structure of 45°, is represented in fig. 1a.

Now when the intussusception of new fibrils (shaded in the diagram) occurs as in fig. 1b, there will be a clockwise rotation of the top. The original fibrils in this case separate at a right angle to their axis, surface growth taking place in the direction of the arrow. When the spiral lies flat, the growth of the cell will be chiefly lengthwise; when the spiral is steep there will be chiefly growth in girth.

When, however, the intussusception occurs as in diagram c, the rotation will take place in a reverse direction, which, when the spiral is flat, coincides with predominant lengthwise growth and when the spiral is steep, with growth in girth.

It goes without saying that the diagrams show only the exceedingly improbable theoretical extremes. All intermediate forms are possible and even probable. Given the flat spiral structure of *Phycomyces*

[1]) The denomination of *Errera* as supplemented by *Castle* (l.c.) will be followed. See also our recent publication (1950).

combined with the predominant growth in length, we arrive at the hypothesis that here the intussusception takes place chiefly in the way shown in diagram b.

If we adopt this as a working hypothesis, it still leaves unanswered

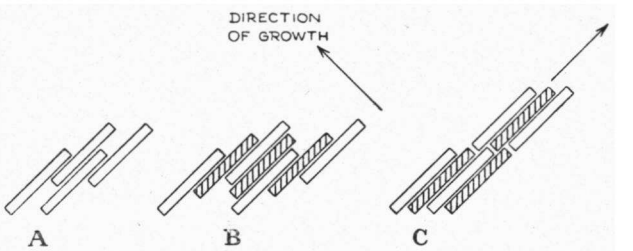

Fig. 1. Schematized diagram of the possibilities of the intussusception of new fibrils (shaded) in upper wall of a cell with *Z*-spiral structure.

the question whether spiral growth is actually regulated by the type of intussusception (active intussusception) or depends only on the mechanical properties of the wall. Of course, intussusception must also take place even if one assumes that spiral growth depends exclusively on the mechanical properties of the wall. This we call passive intussusception.

Let us suppose that the rotation of the top is the result solely of the stretching of spirals (PRESTON, 1948), or of widening of spirals. Or let us suppose that the spiral growth of *Phycomyces* is quite comparable with the rotation, due to increased pressure, of a cellophane tube with spiral structure (see ROELOFSEN, 1950). Then, intussusception must be looked upon as a secondary factor, which passively follows the extension of the wall, and in this way makes further elongation and rotation possible. Expressed simply, the process amounts to a filling up of the wall, wherever the extension has caused it to become too thin.

But we may also imagine, that it is primarily the intussusception, which, in the manner represented in the diagram, is the active cause of the spiral growth. The fact that such a wall, having thus acquired a spiral structure, gives rise to rotation, if for instance the pressure is increased, has nothing to do with spiral growth and in studying the latter, is of no importance.

It is possible that neither point of view alone is the right one, but that a combination of the two is nearer the truth.

The problem which then presented itself to us was to find out which of these possibilities lies at the root of the spiral growth of

Phycomyces. We thought that it might be possible to obtain relevant indications by seeing whether the rotation per mm elongation during growth is or is not equal to the rotation per mm elongation brought about in the same sporangiophore by varying the internal pressure, since in varying the pressure we are dealing with mechanical properties only.

If intussusception takes an active part, increased pressure may still induce rotation, for a spiral structure has become a permanent feature of the wall. But it would certainly be too remarkable a coincidence if the mechanical properties of the wall were such that the rotation per mm elongation caused by pressure change were exactly the same as the rotation per mm elongation seen during growth, i.e. presumably caused by intussusception.

If however, the intussusception is passive, an equality may certainly be expected between the rotations per mm elongation during growth and that caused by change of pressure. At least within certain pressure limits, for when the cell-wall is extended much more than normally happens through the natural turgor pressure, a different rotation per mm might arise. It was therefore necessary to work within the range of normal physiological pressures.

We shall now deal successively with: the methods applied, the determination of the natural turgor and the experiments proper concerned with rotation per mm elongation caused by natural growth and by changes in pressure.

b. Methods

VAN DEN HONERT told us privately that 20 years ago he had succeeded in fastening *Phycomyces* sporangiophores to microcapillary tubes and had been able to vary the interior pressure by pumping water into them. He stated that he had taken up these experiments again and he permitted us to use the principle of his method in our own work. Because the very delicate preparatory work required by this method seemed an obstacle to us, we resolved to apply the principle of the "iron lung"; and indeed this proved to be quite practicable.

Culture method. We first give a description of the culture method because in this work it is necessary to use long, straight and sturdy sporangiophores. A strain of *Phycomyces Blakesleeanus*, which was chosen for its relatively thick sporangiophores, was grown in small vessels on bread, which had been moistened before sterilization. These vessels stood in a deep Petri-dish. After the mycelium had developed for about three days at a temperature of 26°C., the vessels were taken out and placed under a glass bell jar in a constant

temperature dark room of 21—24°C. See fig. 2. The bell jar was supported on three points over a shallow layer of water in a flat dish. Between jar edge and water surface a space of 0.5 cm was left to allow for ventilation and to prevent the air from becoming too moist.

Measurement of rotation and elongation during growth. On the sporangium of a selected sporangiophore was placed a marker, in the shape of a 1 mm long piece of coloured rayon fiber.

The culture vessel was illuminated from above under a micros-

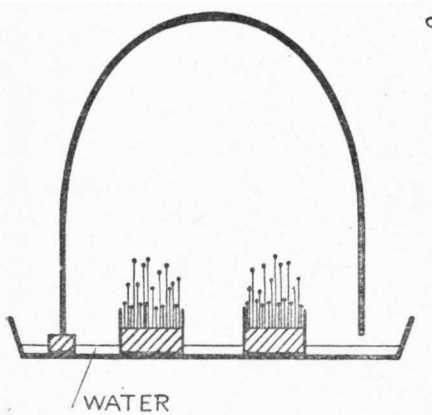

Fig. 2. Culture method.

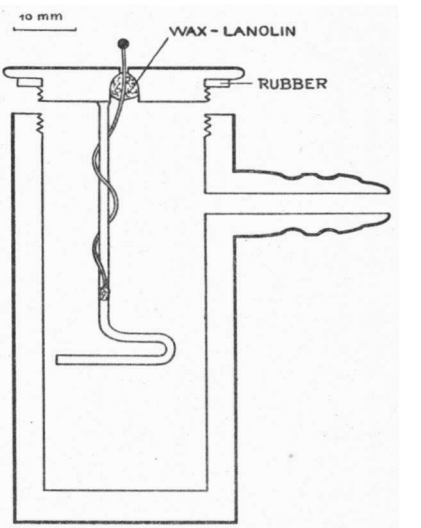

Fig. 3. The "iron lung".

cope, provided with an eyepiece with crosswires and having a pointer to indicate the rotation of the marker on a circular scale graduated in degrees. The growth in length was measured by means of a cathetometer microscope, which was provided with a 0.01 mm scale.

Measurement of rotation and elongation with change of pressure. A sporangiophore was taken between the thread covered points of a small pair of forceps and very carefully pulled out. The basal end was then pushed through a little hole of 0.5 mm diameter in the unscrewed lid of the "iron lung", (sketched in fig. 3) until this end protruded on the inside of the top. (For cells with bent basal ends a separate top was available with a hole of

0.7 mm.). The cell was then seized again on the inside of the lid and pulled further until only the sporangium and ± 3 mm of hypha protruded from the top of the lid. This part contains the entire growing zone. After this the broken basal end was sealed and at the same time glued onto the wire with a drop of a molten (70°C) mixture of 2 parts bees'wax and 1½ part lanoline. Then the cone shaped aperture on the inside of the lid was sealed of by one or two drops of this mixture.

The lid was now firmly screwed down on the small iron vessel. If necessary a new rayon fiber was attached to the sporangium. The pressure within the vessel could be increased by connecting to a compressed air cylinder provided with a regulating valve. Pressures up to 170 cm Hg could be read from a high U-shaped mercury manometer. When, in exceptional cases, higher pressures were applied, these could be read from the manometer attached to the valve. No determination was made of the bursting pressure of the cells, but this must have been above 4 atmospheres.

Measurements were most easily made when the iron lung with the sporangiophore was held upside down, the sporangium hanging down. Then the readings of the rotation were taken by means of a mirror held under 45° below the iron lung and a horizontal microscope provided with the graduated circular scale mentioned above. The extension was measured with a cathetometer microscope. It was of course necessary to start from zero turgor. This was acquired by blowing warm dry air by means of a warmed pipette against the protruding part of the cell until it wilted.

If this is done with a cell standing upright, the sporangium falls down on the lid and sticks to it. Therefore, when experiments were to be made with upright sporangiophores, zero pressure was attained first with the sporangiophore in an inverted position, then a mercury pressure of 10 cm was applied and subsequently the iron lung was returned to its proper position. Occasionally the sporangiophore was placed upright from the start and it was partially wilted, namely until the bending of the cell just became visible, which was a sign that turgor had almost completely disappeared (± 5 cm mercury pressure).

The wax and lanoline mixture at 70°C. might be thought to injure the protoplasm locally below the growing zone; but this it does not do to any great extent, because cooling follows very quickly. At any rate the communication through the sealed portion of the cell remained intact, for the protruding part reacted promptly to any change in pressure in the iron lung. Because the possibility that growth went on during the experiments had to be taken into

account, the measurements were performed as swiftly as possible. An assistant could increase the pressure very quickly and the readings were performed in a few seconds, so that passage from 10—160 cm excess pressure with 3 intermediate readings, was accomplished in one minute.

As we will see in § 2 the mechanical properties of the cell wall change rather rapidly when growth ceases. Between pulling out the cell and taking the first readings about 5 minutes elapsed and it might be possible that a small change in the wall already occurred in this interval. Therefore we also used cells in which, immediately after mounting in the lid, i.e. about one minute after removal from the culture-vessel, the growing zone was killed by running along it water at a temperature of ± 80°C. The behaviour of such partly dead cells was, however, entirely similar to that of the living cells, except that more moisture was forced out of the growing zone in the latter. The wall of cells killed with alcohol, became abnormally rigid.

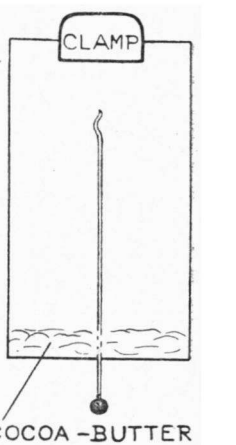

Fig. 4. Mounting of sporangiophore for determination of shrinkage with loss of turgor.

Determination of shrinkage with loss of turgor. A sporangiophore was pulled out with great care. In the broken base the cell contents appeared to be so viscous that nothing was exuded, so that the turgor may be assumed to be undiminished. Then it was immediately placed on a slide, which was provided with a narrow strip of molten cocoa-butter at one edge; (temp. ± 40°C). This was done in such a way that the upper 3 mm of the cell hung free; see fig. 4. Then the cocoa-butter was quickly hardened by applying the slide to a small cube of ice for a short time. It was fixed with the sporangium hanging down and a cathetometer microscope was swiftly focussed on the sporangium base. Subsequently the cell was cut in two with a pair of scissors, if possible, without touching the slide and the shrinkage was measured. If the shrinkage was to be expressed as % of the length of the negative doubly refractive zone, the cell was carefully taken off again and prepared as described by us elsewhere (1950).

This method can only be relied upon with long cells, which may be cut somewhere in the upper half of the cell where its contents are not viscous at all. Cells in the stages 1—3 are asa rule too short, except when they are grown in a humid atmosphere.

c. Determination of the normal turgor pressure

It stands to reason that with abnormal pressures the mechanical properties of the wall might also be abnormal. It was necessary therefore to start with a determination of the normal turgor pressure. LAURENT (1885) found an osmotic value at incipient plasmolysis in *Phycomyces* sporangiophores equivalent to 2.64% KNO_3, i.e. 8 atmospheres, viz an excess of pressure of 7 atmospheres if the cell were in equilibrium with water. It appeared, however, that in normal sporangiophores the turgor is much lower.

Actually we made only a rather rough estimate of the turgor. With the method described we measured the shrinkage of inverted turgid sporangiophores (stage 4) on loss of turgor. This varied between 0.27 and 0.56 mm; the average of 20 determinations was 0.42 mm. Similar cells from the same cultures were mounted in the iron lung and then we determined the effective pressure which had to be applied in order to get an elongation of the same amount. For the reason mentioned before, a number of these cells were killed in the growing zone, a procedure which, however, made no difference to the observed results. Due to the variable length of the extensible growing zone and perhaps also to variations in turgor pressure, a great amount of data and statistical calculations would be necessary to arrive at an accurate determination of the average turgor pressure. This was beyond the scope of our research.

However, the figures available allowed us to conclude that the turgor pressure was about two atmospheres. In the experiments to be described in the following sections, we therefore used pressures up to 160 cm Hg.

If LAURENT's data for the osmotic value are correct and applicable to our cultures, the sporangiophores will have a suction pressure of about 5 atmospheres.

d. The relation between elongation and internal pressure

Measurements were made with sporangiophores in stage 4b in both inverted and in upright positions, but with those in stages 1 and 2 only in the former position. The results of some series are tabulated in table 1 and are plotted graphically in fig. 5. It was not our aim to represent "average" cases only, but purposely to include "extreme" cases also. The elongation was followed up to an effective pressure of 160 cm Hg. Then another series of readings was made starting at zero pressure or at a pressure of 10 cm and sometimes even a third and fourth series.

In the experiments 4b E, F with the sporangium in an upright

TABLE I.

Extension of sporangiophores with increasing internal pressure. Pressure in cm Hg, extension in 0.01 mm.

stage 1 inverted		stage 2 inverted		stage 4*b* inverted		stage 4*b* upright	
press.	ext.	press.	ext.	press.	ext.	press.	ext.
A		*A*		*A*		*E*	
0— 20	10	0— 20	5	0— 10	12	10— 20	10
20— 80	6	20— 50	5	10— 20	9	20— 40	10
80—160	4	50—100	2	20— 50	17	40— 80	12
160— 0	−17	100—160	1	50—100	7	80—120	7
0— 20	10	160— 0	−11	100—160	4	120—170	10
20— 50	6	0— 20	5	160— 0	−34	170— 10	−27
50—160	4	20—160	5	0— 20	19	10— 40	11
160— 0	−17	160— 0	−11	20— 50	7	40—100	10
				50—100	4	100—170	9
B		*B*		100—160	5		
0— 20	11	0—100	11	160— 0	−33	*F*	
20— 50	4	100— 160	2			10— 44	14
50—100	3	160— 0	−10	*B*		44— 80	5
100—160	4			0— 10	11	80—170	6
160— 0	−15	*C*		10— 20	9	170— 10	−19
0— 30	7	0—100	6	20— 50	21	10— 50	10
30—160	9	100—160	1	50—100	6	50—100	5
		160— 0	−6	100—160	5	100—170	4
C				160— 10	−19	170— 10	−13
0— 10	5			10—160	19	10—170	13
10— 20	3					100—170	4
20— 50	3			*C*			
50—100	3			0— 6	5	*G*	
100—160	3			6— 20	9	0— 40	6
160— 10	−9			20— 40	9	40—120	6
10— 60	5			40— 60	3	120—160	4
60—160	4			60—120	3	160— 20	−8
				120—160	2	20—160	8
				160— 6	−16	160— 20	−6
				6— 20	6	20—160	9
				20— 40	3	160— 20	−8
				40— 60	2	20—160	9
				60—100	2		
				100—120	1		
				120—180	1		
				D			
				0— 10	8		
				10— 30	8		
				30— 60	3		
				60—120	2		
				120— 0	−17		
				0— 10	5		
				10— 30	7		
				30— 60	2		
				60—120	2		
				120— 0	−17		

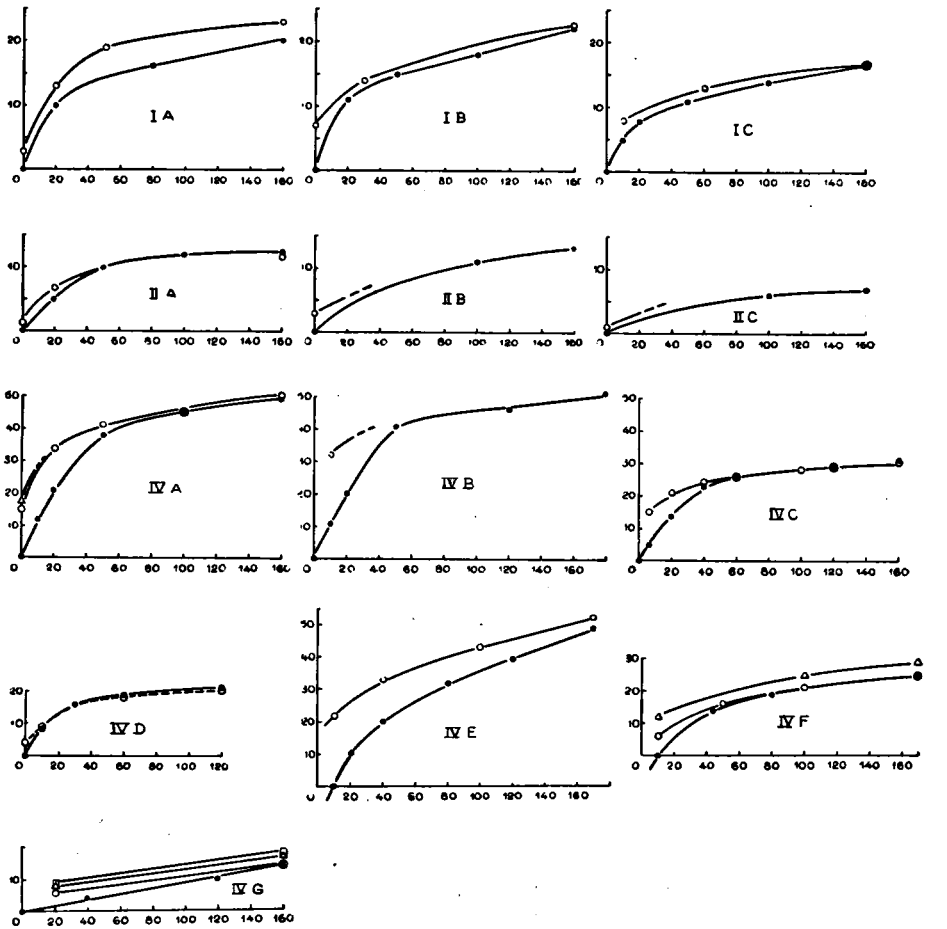

Fig. 5. Elongation of sporangiophores at stages 1, 2 and 4*b* in 0.01 mm (ordinate), with varying effective pressure (cm Hg, abscissa). Data in table 1. In experiments E, F and G cells in upright position, others inverted. Exp. D with a cell killed in growth zone. First, second, etc., applications of pressure indicated by •, O, Δ and □ respectively.

position the initial pressure was 10 cm, because otherwise the sporangium would have wilted and collapsed. In experiment 4b G the pressure at the beginning was recorded as zero, but there was still some natural turgor left. In such experiments initially more rectiliniar stress-strain diagrams are obtained. Later on no natural turgor remains, because there is loss of moisture through evaporation.

These experiments, together with others not recorded here, allow us to make the following conclusions. With the sporangiophore upside down the stress-strain curves in all growth stages are always concave to the pressure -axis; there is a high initial extension. The differences in extensibility of different cells can be explained by unequality in length of the growing zones, as will be seen later on. In experiments with upright sporangiophores the elongation is lower, especially with low pressures, so that as a rule more rectiliniar curves are obtained. The weight of the sporangium is bound to reduce the elongation.

It is a remarkable fact that after the first applications of pressure the elongation becomes partly irreversible. Sometimes there is a slight recurrence of this phenomenon at the second application of pressure. The irreversible extension cannot be due to growth only, for in that case the rate of growth must have been exceedingly high. Besides, the difference in length of the sporangiophore after the first and the second application of 160 cm pressure should then be equal to the non-recoverable extension appearing after the first application, which is not the case. Sometimes this difference is nil, but usually it is positive though small; in other words, the cell continues to extend a little with each subsequent pressure application. Perhaps this is growth, but it might also be stretching beyond the elastic limit, due to the application of too high a pressure.

This was somewhat disturbing, because the average shrinkage with loss of natural turgor (0.42 mm) and the average pressure, necessary to elongate the cell to that extent, had indicated that a pressure of 160 cm Hg was within the physiological range. We feared therefore that in the interval of five minutes elapsing between the pulling out of the cell and the measurement of the extension with applied pressure, the cell wall had become more rigid. In § 2 it will be shown that this phenomenon is very obvious after immersion of the cell in water for half an hour. However, special experiments showed that our fear was unfounded. The stress-strain diagrams were determined for cells that had been killed in the growth zone with hot water immediately after having been mounted in the lid of the "iron lung", i.e. about one minute after pulling out. Their extensibility proved to be equal to that of normal cells from the

same culture. Graph IV D illustrates such a case (the control also showed a rather low extensibility). The actual reason for the continued extension observed, cannot then be a stretching beyond the elastic limit.

Similar stress-strain diagrams as were found here were established by OPPENHEIMER (1930), who determined the diameter of various parenchyma cells immersed in different concentrations of cane-sugar solutions. Similar diagrams can also be found with many materials, e.g. cellulose-gels and cellophane. We are aware of the fact that critical stress-strain diagrams can only be achieved with constant rates of loading. This, however, was unnecessary for our aims.

e. Distribution of cell wall extensibility in the growth zone

We studied the distribution of extensibility in the growth zone as follows. A sporangiophore (stage 4b) was mounted in the, "iron lung". Then, under a binocular microscope, it was marked with *Lycopodium* spores at regular distances. Subsequently a pressure of 10 cm Hg was applied, a photograph was made of the protruding part and a second one on the same plate at a pressure of 170 cm. See fig. 6, (this was a case where extension was small and rotation nil). With the help of a traversing microscope the distances from the base of the sporangium to the centres of the *Lycopodium* spores were measured on an enlarged print. These distances, reduced to

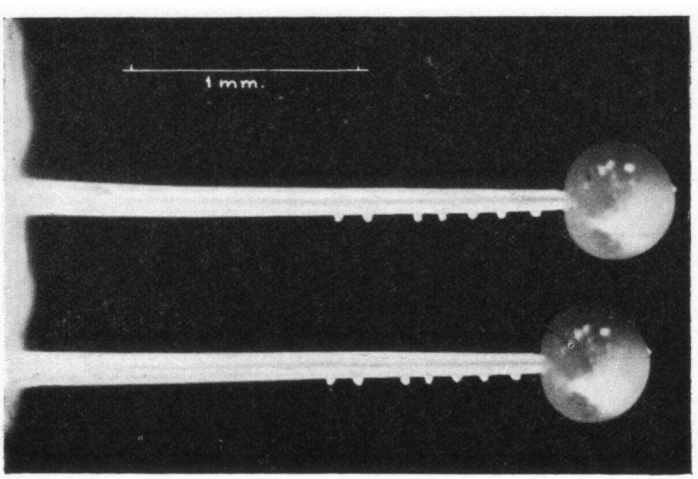

Fig. 6. Photograph (reduced) of inverted sporangiophore with markers in growing zone, at 10 and 170 cm pressure.

TABLE II. Distribution of extensibility in the growth zone.

Column 1. Distance in 0.01 mm from base of sporangium to subsequent markers at 10 cm pressure.
,, 2. Same at 170 cm pressure.
,, 3. Increase of distance between subsequent markers in % of that distance at 10 cm pressure.
,, 4. Dislocation of a marker in % of dislocation of sporangium, recalculated as described in text.

exp. I				exp. II				exp. III				exp. IV			
1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4
0	0		100	0	0		100	0	0		100	0	0		100
18.7	19.8	5.5	91	10.8	12.1	12.0	90	20.6	25.0	15.0	85	15.7	18.1	15.0	89
36.5	38.9	8.0	82	24.6	27.0	8.5	81	37.1	42.8	16.0	72	33.5	39.0	17.0	76
55.7	59.5	7.5	70	36.7	39.8	5.5	76	50.7	57.3	6.0	68	40.7	46.8	9.2	74
77.3	82.0	4.0	63	49.2	53.0	7.0	69	54.8	61.6	6.5	67	61.8	70.7	13.0	61
96.0	102.0	4.0	57	59.3	63.7	5.5	65	73.5	82.3	10.5	57	87.1	99.5	10.3	47
122.0	130.0	8.0	42	79.8	85.5	6.0	56	86.3	96.5	12.5	50	102.0	113.5	3.5	45
145.0	154.0	7.0	28	92.2	98.8	8.0	48	100.0	111.0	5.5	46				
								124.0	137.0	8.8	36				
								177.0	195.0	8.0	16				
								185.0	203.0	5.0	14				
								206.0	225.0	7.5	6				
								218.0	239.0	10.0	0				

exp. V				exp. VI				exp. VII			
1	2	3	4	1	2	3	4	1	2	3	4
0	0		100	0	0		100	0	0		100
23.4	27.8	18.0	80	14.1	18.1	25.5	82	13.1	14.5	11.4	85
38.1	45.5	20.0	66	24.6	28.4	2.0	81	23.1	25.3	5.0	80
61.2	70.3	7.0	58	47.3	52.7	7.0	74	34.6	36.8	1.5	78
67.7	87.0	8.5	53	58.5	66.4	23.0	61	42.6	45.3	6.0	74
86.5	97.8	11.0	47	92.3	104.0	4.5	53	59.8	63.3	5.5	65
113.5	127	8.0	38	106.0	121.0	20.0	40	79.7	84.1	5.0	55
137.0	150.0	4.5	32	116.0	132.0	5.0	37	93.7	99.0	5.5	48
158.0	174.0	8.0	24	141.0	159.0	7.5	28	103.0	109.0	5.0	43
174.0	192.0	10.0	16	158.0	177.0	1.9	26				
189.0	208.0	6.5	12	172.0	191.0	7.5	21				
				195.0	212.0	9.5	10				

0.01 mm units, are to be found in columns 1 (10 cm pressure) and 2 (170 cm pressure) of table 2 ,where the results of 7 experiments are recorded. When the elongation between subsequent markers was expressed in percentages of the length of that zone under a pressure of 10 cm, we obtained the figures of column 3.

When examining these data, or when plotting them in a graph, there appeared a tendency to a greater extensibility in the upper 3mm, but the figures were so variable that it was impossible to arrive at a reliable conclusion in this manner. The reason for this is presumably to be found in the fact that we measured the distances between the centres of the Lycopodium spores; but the points where these spores, which are more or less polygonal, were attached to the cell might have been located away from their centres, without this being visible in the photograph. To acquire more accurate figures much smaller markers, e.g. small starch grains, should be used.

It was impossible to determine an arithmetical average of all series, because the distances between the markers as well as the lenghts of the extensible zones were different in every case. Therefore the figures were recalculated as follows. First the total elongation of the zone localized between the sporangium in experiment III and 2.18 mm below it (lowest marker), was calculated. This amount was called 100%. Then the elongation of the zone between the first marker and the marker at 2.18 mm was expressed in % of this: 85%. The same was done with the other spaces between the marker at 2.18 mm and the markers above it. These data were plotted against the distance from the sporangium and a curve was drawn through them: fig. 7, curve III.

Subsequently the same figures were calculated for the other series. As an example curve II is drawn through these figures for experiment II as shown in fig. 7. As these data apply to a shorter distance the slope of curve II is steeper. In order to compare it with curve III, the zero point of curve II should be brought onto curve III and the other points should be recalculated. Then the points marked as open squares (II') are arrived at. As an example the recalculation for experiment II is given in table 3.

The result of this recalculation is that all the curves are brought to the same level, but that no changes are effected in the original slope of each curve. Therefore the curve A in fig. 8, drawn through all the points obtained in this manner, gives an average representation of the shape of all the curves. It is obvious that the extensibility in the growing zone is higher the smaller the distance from the sporangium base. This becomes more evident if one differentiates

TABLE III.
Recalculation of data of experiment II.

marker nr:	dislocation with regard to marker 7 in 0.01 mm	the same in % of 6.7	× 52/100 [1])	plus 48 [1])
0 (spor.)	6.7	100	52	100
1	5.5	81	42	90
2	4.3	64	33	81
3	3.6	54	28	76
4	2.7	40	21	69
5	2.2	33	17	65
6	1.0	15	8	56
7	0	0	0	48

[1]) see fig. 7 for deduction of correction factor $\times \frac{52}{100} + 48$.

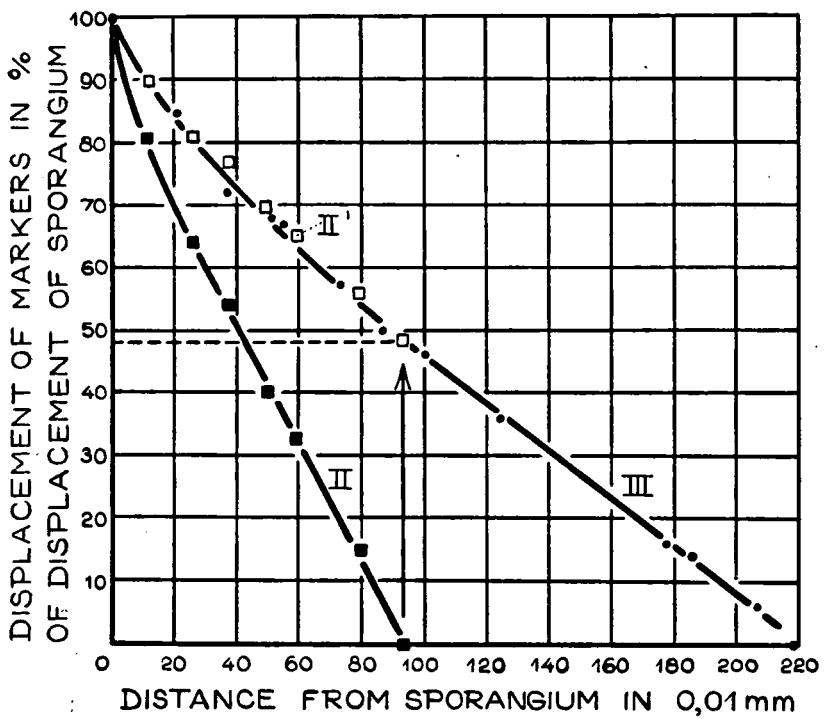

Fig. 7. Movement of markers in growing zone expressed in % of movement of sporangium. Curve III data for exp. III. Curve II for exp. II. Curve II': data of II recalculated as indicated in text.

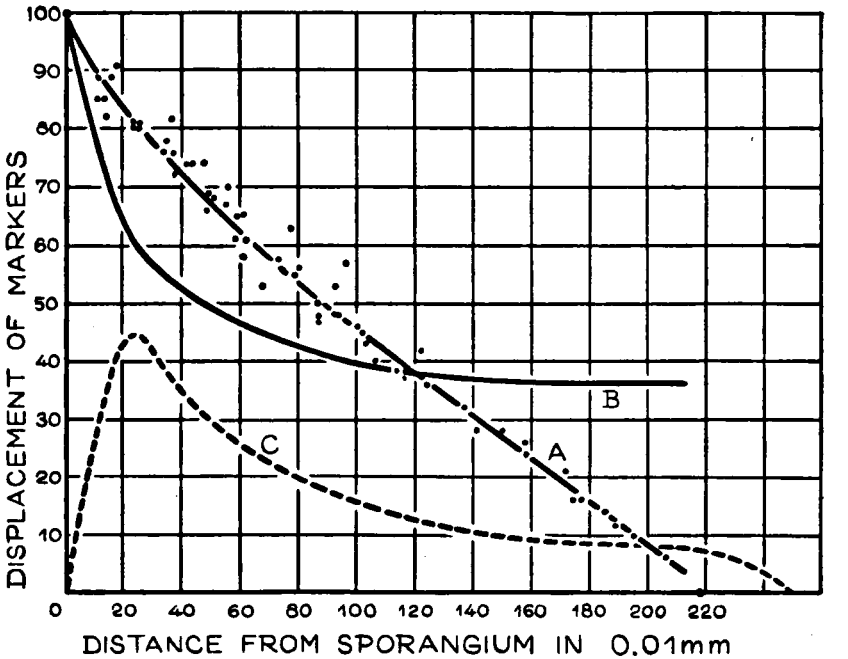

Fig. 8. Distribution of extensibility and growth in the growing zone.
Curve A: Movement of markers in growing zone expressed in % of movement of sporangium; data from several experiments recalculated as indicated in text.
Curve B: Curve A differentiated with respect to distance from sporangium. This curve represents the part played by each 0.1 mm of the sporangiophore in the total displacement of the sporangium (expressed in %).
Curve C: Similar to curve B, but applying to growth rate, as deduced by CASTLE (1937*b*). Abscissa units are arbitrary and therefore the curve is only comparable qualitatively.

curve A with respect to the distance from the sporangium: curve B. Here the extension of each 0.1 mm is plotted as a % of the total extension.

The shape of the dotted curve C indicates qualitatively (!) the distribution of growth rate in the growing zone as determined by CASTLE (1937b).

The essential difference between curves B and C is that there is an optimum rate of growth at 0.2 — 0.3 mm below the sporangium, which optimum is absent in the extensibility-curve. The optimum may be caused for example by a varying growth hormone production,

or by varying sensitivity to it. Of course such factors are eliminated during the rapid extension caused by increased pressure. The reason for the decrease of extensibility as the distance from the sporangium increases lies probably in the increasing thickness of the wall. As a matter of fact, CASTLE (1938) demonstrated an increase of the negative birefringence from the sporangium to 1.8 mm below it followed subsequently by a decrease, presumably as a result of deposition of the secondary wall, the fibrils of which are arranged lengthwise.

f. The rotation per mm elongation with change of pressure and during growth

In the introduction to this section it was explained how measurements of the rotation per mm elongation with change of pressure and also during growth might decide whether intussusception plays an active or a passive role in spiral growth. In table 4 are to be found the figures we obtained on this subject. Because great variability was to be expected, we measured the rotation of the same cells under both these conditions.

TABLE IV.

Clockwise rotation in degrees per mm elongation with increase of pressure and during growth of the same sporangiophores in stage 4b.

during growth	with increased pressure up to 160 cm Hg in the position:	
	upright	upside down
30	6	
90	30	
100	10	
130	70	
170	40	
170	6	
200	80	
200	70	
500		125 [1])
350		100 [1])
100		80
180		90
250		110
390		280
average rotation/mm in % of that during growth	19%	64%

[1]) cells in which the growth zone was killed with hot water ± 1 minute after pulling them out.

It appears that the rotation per mm elongation brought about by increased pressure is greater in cells hanging down than in cells held upright. Obviously the weight of the sporangium causes the difference, but how is not clear. However, the question as to which, of these positions can best be compared with growth is unimportant, for in both cases the rotation with change of pressure is always less than during previous growth. The differences are very considerable and leave no room for doubt about this phenomenon. This cannot possibly be the result of changes in the wall after the cells have been pulled out, for it also occurs in cells which were killed with hot water immediately after having been pulled out.

From this the conclusion may be drawn that rotation during normal growth cannot be explained, at least not completely, in terms of the mechanical properties of the wall, but that intussusception of the fibrils during growth is an active agent in bringing about rotation, presumably in the manner schematized in fig. 1b.

At first sight it seems that plausible objections may be advanced against this conclusion; but on closer scrutiny they will be found to be beside the mark.

In the first place there is the fact that the rotation with increase of pressure was measured over the pressure range of 0—160 cm of mercury, whereas growth is accompanied by a relatively constant turgor pressure. We therefore measured the rotation/mm during step-wise increases of pressure over the whole pressure range, the size and number of the steps varying widely from experiment to experiment.

Some of these series are to be found in table 5.

Great irregularity is found here, partly because the method was not accurate enough to measure very small rotations. This is very evident in the series 9—14 with upright cells. It may be said, however, that no consistent differences in rotation/mm occur at different levels up to pressures of 2 atmospheres. This objection need not therefore be considered.

A second objection which might be advanced against our conclusion is the following. The different shape of curves B and C in fig. 8 proves that in the growth zone the distributions of growth-rate and of the extensibility do not coincide. Now if we assume that the pitch of the structural spiral also varies in the growth zone, and is such that in the zone of maximum growth a stronger rotation occurs than elsewhere (both during growth and during elastic extension), then this might be presented as the explanation of the above difference in rotation per mm during growth and during

TABLE V.

Rotation in °/mm elongation in reponse to pressure changes over varying ranges (indicated by line), 1—8 inverted cells, 9—14 upright cells.

exp. nr	pressure in cm Hg. 0 10 20 30 40 50 60 70 80 90 100 110 120 130 140 150 160
1	93 — 88
2	215 — 137 — 200 — 200 — 70 — 100
3	100 — 75 — 70
4	115 — 100 — 185 — 124 — 70 — 33
5	63 — 100 — 300
6	240 — 260 — 310 — 330 — 300
7	100 — 166 — 122 — 132 — 200 — 150
8	116 — 123 — 60
9	60 — 90 — 100
10	30 — 0 — 80 — 150
11	25 — 17
12	14 — 25 — 77 — 80 — 25
13	0 — 41 — 0 — 18
14	80 — 100 — 50

pressure changes, and not our assumption that intussusception is an active agent in causing twist.

This reasoning however may be refuted, because Castle (1937b) proved that during growth the rotation per mm elongation is equal in every part of the growing zone.

Our conclusion, that intussusception plays an active part in causing spiral growth, may therefore be upheld. However, there is no reason to believe that the mechanical properties of the wall play no part at all in spiral growth. The data in table 4 give an indication of the maximal part they can play, but it is not in any way certain that they play any part at all.

§ 2. Direction of rotation in relation to wall structure during and after stagnation of growth

a. Stagnation of growth of sporangiophore during growth of sporangium

The direction of rotation during growth was always the same as that due to increase of pressure. This could be studied quantitatively with cells mounted in the "iron lung" but with the following simple

method many more cells could be studied qualitatively at one and the same time.

The sporangiophores were pulled out and mounted in an inverted position on a slide, as described for the determination of shrinkage with loss of turgor. After this the sporangia (or the acute tips in stage 1) were marked with several Lycopodium spores. After having cut the cell in two, the slide and the cell were taken between finger and thumb and while pressure was varied gently, the displacement of the markers was followed by means of a horizontal microscope.

Actively growing sporangiophores with yellowish brown or brown sporangia used to rotate in a clockwise direction with increase of pressure (seen from above). We seldom found a cell rotating either in an anti-clockwise direction or not at all (fig. 6). When we did, some abnormal circumstance could be assumed to be causing this behaviour.

Cells without sporangia (stage 1) also used to rotate clockwise, but this could only be seen at the extreme tip and even there the motion was so slight that with the coarse method used, it could not be recognised at all in many cases. This tallies with the fact that during the growth of such cells CASTLE (1936a) could not see any rotation at first, but later on (1942) he did notice clockwise rotation: "if pains are taken to attach markers to the extreme tip".

Sometimes also, with cells having very small yellow sporangia (stage 2), our method did not allow us to distinguish any rotation, but as a rule it was anti-clockwise (left-handed) with increasing pressure. This proved that the left-handed rotation, which CASTLE (1942) observed when the longitudinal growth was resumed (stage 4a), corresponds with the structure of the wall soon after growth has stopped at the end of stage 1.

CASTLE (1942) supposed, as an explanation of the left-handed rotation on growth-resumption, that during the stagnation in growth in stages 2 and 3, a thin layer of the more or less axially oriented secundary wall was deposited in areas which were bound to resume growth later on. The secondary wall has thus a steep S-spiral structure.

To investigate whether this explanation could be upheld, we proceeded to measure the negative doubly refractive (n.d.r.) zone in cells of various stages of growth. CASTLE (1938) had already prooved that the growing zone is only slightly shorter than this n.d.r. zone. Before doing this the direction of rotation with increasing pressure and the shrinkage on loss of turgor were determined in the same cells. These data are given in table 6.

TABLE VI.

Length of negative doubly refractive zone, direction of rotation with increasing pressure and shrinkage with loss of turgor, in cells at different growth stages.

growth stage	1	2 long cells	2 short cells	large yellow sporangium with rotation: left-handed	large yellow sporangium with rotation: Nil.	large yellow sporangium with rotation: right-handed	brown sporangium
rotation with increasing pressure	right-handed	left-handed	left-handed	left-handed	Nil.	right-handed	right-handed
average lenght of n.d.r. zone in 0.01 mm, corrected for the average skrinkage through loss of turgor	230	100	50	70	80	130	260
Mean shrinkage (0.01 mm) with loss of turgor. Number of data given between ()		unreliable (see § 1*b*)		10 (27)	14 (16)	28 (12)	42 (20)

Obviously, there is a positive correlation between the length of the n.d.r. zone and the extensibility of the cell as expressed in the shrinkage on loss of turgor. Because it was possible (although improbable) that the turgor pressure varied in different growth stages and because the shrinkage on loss of turgor could not be determined accurately in cells of stage 2 (see section b) we also compared the extensibility of cells in different stages using the "iron lung" method. The expected correlation was obtained from many data we collected on this point. It is another demonstration of the well known fact that extensibility is greatest perpendicular to the fibril orientation. Extension and shrinkage appear to take place almost exclusively in the n.d.r. zone, for if we express them in % of the n.d.r. zone-lengths, we obtain the same figures with cells differing greatly in this respect. In table 7 some data are given to demonstrate this.

Furthermore, table 6 shows that left-handed rotation is seen in cells of stage 2 and also in some cells having full-grown yellow sporangia, namely those which have a relatively short n.d.r. zone and consequently a low extensibility (stage 3). Apparently growth is resumed (stage 4a) while the sporangium is still yellow. Indeed this was confirmed by direct observation of living cells.

TABLE VII.

Extension in % of length of n.d.r. zone with increase of pressure from 2 to 120 cm Hg of cells in stage:

2	4b
10	12
11	11
13	11
13	12
7	10
8	8
	9
	11
	10
av. 10.3	av. 10.4

As can also be seen in table 6, the "growing" zone is reduced in stage 2 to less than half of its length in stage 1, so there must have been deposited enough secondary wall material in its lower part to alter the sign of its d.r. When growth is resumed later on, the growing zone becomes longer than before, but to establish this, of course, it is not necessary that growth should be renewed in areas where secondary wall material has already been deposited. Growth might be resumed e.g. just below the sporangium and the newly formed areas might retain their meristematic properties long enough to reproduce a long growing zone.

CASTLE (1942) found that in stage 4a growth is first resumed at ± 0.6 mm below the sporangium base. In cells which had resumed growth less than 15 min. previously, we found n.d.r. zones of 0.8 —1.2 mm (see also table 6 in column "large yellow sporangium"). We may therefore assume with some probability that growth starts in the lower part of the n.d.r. zone and now the question arises as to whether secondary wall material can be demonstrated there.

Although he did not use it as a support for his assumption, CASTLE himself (1938) was aware of the fact that in the lower 0.9 mm of the n.d.r. zone the birefringence diminished steadily to zero in the compensation point. This points to the presence of a very thin wedge-shaped axially-oriented secondary wall-layer with its thin edge at a considerable distance from the compensation point. As a matter of fact one can see between crossed nicols a fine lengthwise striation in the wall in this area (figure 16 of our publication; 1950). Because the secondary wall is known to have a steep S-spiral structure and growth starts here, the temporary left-handed rotation is clear. As this is only temporary, the growth in the upper parts of the growing zone apparently soon predominates.

We may therefore conclude that CASTLE's explanation is true, but, as we shall see later on, an additional role might be ascribed to another circumstance. In fact, under certain conditions the outermost layer of the wall also seems to acquire an axial orientation, but it is not yet known whether this also happens under normal conditions of growth.

Although left-handed rotation with resumption of growth clearly occurs in stage 4a, it is not yet plain why increase of pressure also should cause left-handed rotation in cells of stages 2 and 3. For, as was shown in fig. 8, not only the basal part of the growth zone but also the apical part of it chiefly participates in the extension. This apical part does not show any change in optical properties which might point to the deposition of secondary wall material. In fact there is no visible difference between cells in stage 2 and those in stage 4b, except that the n.d.r. zone is considerably shorter in the former. Probably the difference in rotation is caused by the fact that in the short n.d.r. zone of stage 2 the part near the sporangium, being still free from any structure which might cause a left-handed rotation, is relatively short and is bound to play a minor part, while in cells of stage 4b its part is a major one.

This is reasonable, but it does not tally with the fact that the extensibility of the n.d.r. zone as a whole is the same in cells of both stages (table 7). The extensibility should have been lower in stage 2, because presumably most of the lower part of it, having axial structures, would be more rigid. However, the data for stage 2 in table 7 are apt to be too high, for, although extension will be localized chiefly in the n.d.r. zone, the part of the cell below the compensation point will, as far as it protrudes from the iron lung, also partake in it. The share it takes in extension will be higher in stage 2 than in stage 4b, because it is expressed as % of a smaller n.d.r. zone. If the extension had been determined as % of the n.d.r. zone proper, in all probability a difference would have been found.

b. Induced stagnation and stagnation in mature cells

OORT(1931)drew attention to the fact that left-handed or abnormally low rotations seem to occur in stage 4b due to unfavourable circumstances. CASTLE (1936c) did not see a reversed but only a diminished right-handed rotation per mm elongation, when growth started again after a repression brought about by traces of ethyl ether or by lack of oxygen. PRESTON and MIDDLEBROOK (1949) did see left-handed rotation after cessation of growth in stage 4b, induced by immersion in water for half an hour.

In an investigation of this phenomenon, the following experiments were made. A number of sporangiophores in stage 4b were pulled out and immersed in water. After half an hour the shrinkage on loss of turgor was considerably reduced (from about 0.4 mm to about 0.08 mm). This might have been the result of a reduced turgor, as the cells were clearly limp as compared with fresh ones. However, using the "iron lung method", it could be proved that the wall had indeed grown more rigid, see table 8. Furthermore the right-handed rotation (per mm elongation) had diminished in every case and in some cases was even nil or left-handed, thus confirming the observation by PRESTON and MIDDLEBROOK (l.c.) of left-handed spiral growth.

We then proceeded to measure the distance between the sporangium base and the compensation-point with ten normal sporangiophores and ten similar ones which had been immersed in water for half an hour. To increase their birefringence, all of them were cleaned with alkali and coloured with congo red in the usual way.

TABLE VIII.

Extension and rotation with pressure increase from 0—160 cm, of immersed cells compared with normal ones. Growth stage 4b, inverted position.

	extension in 0.01 mm	rotation direction + = right-handed	rotation o/mm
immersed for ½—1 hr	10	—	40
	17	+	60
	12	—	30
	10	+	80
	15	—	60
	15	—	75
	11	+	86
	14	+	65
	17	0	0
	12	—	17
	8	0	0
	6	—	20
	10	+	40
	18	—	50
mean:	14		
controls	35	+	100
	35	+	120
	52	+	125
	26	+	110
	31	+	90
mean:	36		

We expected a decrease as in cells in stage 2 as compared with those stage 1; (scheme A of fig. 9). In contrast however an increase of this distance was found (mean of 5 determinations: controls 0.21 mm, immersed 0.25 mm). So an upward displacement of the compensation point due to deposition of secondary wall material in the lower part of the n.d.r. zone, was out of question.

Nevertheless, everything pointed to the establishment in the wall of some structure having axially oriented material (rigidity), with orientation particularly in a steep S-spiral (left-handed rotation with pressure). Indeed it was plain that the wall in the original n.d.r. zone had changed. For whereas normally the area between sporangium base and the isotropic compensation point was plainly negatively birefringent with respect to the cell axis, with immersed cells this area had become wholly or partly isotropic or even positively birefringent, as schematized in fig. 8, B and C.

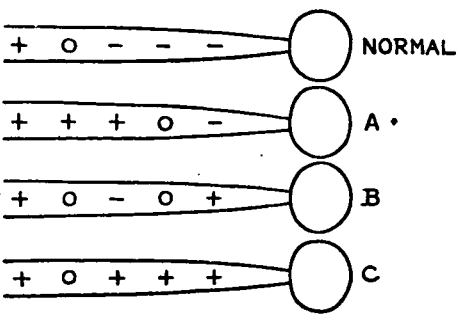

Fig. 9. Scheme of change in optical properties of the growth zone in stage 4b after immersion of the cells in water. A: expected; B, C: found.

It is significant that the same optical pnehomena, and also decreased extensibility and reduced rotation, could be found in normal mature sporangiophores which had almost ceased their growth. So here too the compensation point does not rise, but the zone above it changes as a whole its sign of double refraction. Curiously enough therefore, the compensation point is not the first but the last area to acquire a positive double refraction; the area immediately below the sporangium changes first. It seems probable that the explanation of both cases should be similar.

One obvious and simple explanation is the deposition of secondary wall material in the entire growth zone at the same time.

The second possibility is that, due to stretching of the wall beyond the elastic limit (in particular the outermost layer of it, see later), the orientation of the chitin fibrils has been reversed from a predominantly transverse to a predominantly axial direction, in particular to a steep S-spiral.

This requires some explanation. We have stated elsewhere (1950) that the transverse orientation in tubular meristematic walls

can easily be changed to an axial one by pulling the cell lengthwise. Extension through increased pressure, we argued, is quite another thing, because then the stress in the wall in the transverse exceeds that in the axial direction. Such an extension therefore is not bound to reverse the orientation, even if deposition of new wall material is excluded, as was demonstrated by VAN ITERSON (1937a) in *Tradescantia* staminal hairs and by FREY-WYSSLING and SCHOCH-BODMER (1938) in stamens of *Anthoxanthum* (see also SCHOCH-BODMER, 1939). In these cases the fibrils in the wall seem to be connected so loosely that they can be re-arranged so that longitudinal growth in surface occurs at the expense of wall-thickness without the fibrils changing their transverse orientation.

But the wall must be in a special condition to show this, for the *Anthoxanthum*-stamens do show, at the end of their growth period, reversal of the sign of birefringence which was reversible on loss of turgor pressure (FREY-WYSSLING, l.c.). The authors proposed strain birefringence as the explanation; in our opinion reversal of fibril orientation is more likely. But how can either of these accord with the fact that the transverse stress in the wall must have predominated over the axial one throughout the whole process. As far as we can see, reversal of orientation in the *whole* wall is theoretically impossible.

Reversal in part of the wall however, e.g. the outermost layer, is rather self-evident. It is evident that the inner layer, being near the protoplasm, is in a more favourable position for the intussusception of new fibrils than the outer layer. Now if we assume that at the end of the growth period of the Anthoxanthum stamens intussusception is still possible in the inner layer, whether by deposition of new fibrils or by re-arrangement of old ones at the expense of wall-thickness, but is impossible or slight in the outer layer, it is clear that the fibril-orientation in the latter will be reversed and may ultimately produce positive d.r. of the compound cell wall. Then the (high) transverse stress will be taken up chiefly by the inner layer and the (lower) axial one by the outer, reversed layer.

Such a phenomenon might also occur in sporangiophores of *Phycomyces* which have ceased growth either temporarily or permanently. But here the reversal appears to be irreversible, for, as we have seen, the sign of the birefringence remains positive after loss of turgor. In stages 2 and 3 this phenomenon apparently does not occur, at least to any extent, so that the birefringence is not changed. During immersion of the cells, reversal is moreover enhanced by their temporary acquisition of an exceedingly high turgor pressure, for, although these cells are usually limp after immersion for half an

hour, one frequently finds cells which have burst in the upper part. Thus the reason for the somewhat increased length of the upper part is clear, (see earlier).

This theory is corroborated by the electron micrographs of the normal *Phycomyces* primary wall, which show that the outer layer has an apparently isotropic structure, whereas the inner layer has a transverse structure (FREY-WYSSLING & MÜHLETHALER 1950; ROELOFSEN, 1950)[1]). Moreover, on shrinkage, transverse folds appear mainly on the inside, thus demonstrating its looser structure. This makes it probable that during normal growth a slight reversal of fibril orientation occurs in the outer layer. The steep S-spiral pattern of the cuticle in the growth zone of turgid growing cells (ROELOFSEN l.c.) leaves no doubt as to the direction of orientation the outermost layer will acquire if it is reversed. This will be an S-spiral and as soon as this layer is mechanically dominant, a left handed rotation with increased pressure follows naturally.

Now the following facts show that the second possibility, although being the less obvious one at first sight, is the true one. CASTLE (1938) has stated that the birefringence of the growth zone increases considerably after cleaning the wall with hot dilute acid in addition to the usual treatment with alkali. Using this method, we saw that the original growth zone of the immersed cells as well as of the nearly mature ones, no longer showed the isotropic or positively birefringent areas seen so strikingly after cleaning with alkali only, but had become brightly negative. Now it is well known (WÄLCHLI 1945) that the penetration into cell walls of the congo red particles which enhance the double refraction is slow, so that one may assume that the outer layers are coloured more intensely than the inner ones. After additional cleaning with acid the congo red will penetrate more deeply. If therefore the layer causing the positive double refraction lies on the inside of the wall, an increase of the positive d.r. would be expected to follow cleaning with acid. However, what happens is an appearance of negative d.r., apparently because the axially oriented layer lies on the outside and the transversely oriented layer beneath it, being better coloured, now takes its full share of the d.r. This is corroborated by the layer structure of the normal cells as shown in electron micrographs. The acid treatment always displaces the compensation point away from the sporangium both in normal cells and in immersed ones. This is not due to elongation of the growth zone during the acid treatment for, as a

[1]) It is significant that also young cotton hair electron micrographs have shown an axial orientation in the outer layer and a transverse in the inner one. This will be published elsewhere.

matter of fact, this part of the cell is thereby shortened by ± 6%. The shift of the compensation point varies between 0.2 and 0.6 mm, (average 0.3 mm.). It is only slight, for on the inside the axially orientated secondary wall layer is also present and its d.r. is enhanced too by the acid treatment.

Another phenomenon, supporting the second possibility is that the positively birefringent areas appearing after cleaning with alkali only, always do so first just below the sporangium (fig. 9b), where, as fig. 8 shows, the extension is greatest. The compensation point, where the extensibility is very low because the secondary wall material has already been deposited and because the outermost layer has probably already been somewhat reversed, remains isotropic This is to be expected if our second explanation is valid, but is very strange if a deposition of secondary wall material is accepted as the cause, for this is likely to occur first where secondary thickening has already begun, viz. in and above the compensation point.

At first sight the fact that in cells, which were burst by squeezing, the growth zone does not show any change of the n.d.r. zone (see ROELOFSEN 1950), seems to contradict our explanation. But it is clear that reversal requires time and cannot take place in a flash.

Electron micrographs of the primary walls of cells which have ceased to grow and further experiments with cells subjected to high pressures for a long time should be taken to confirm the tentative conclusions drawn here.

For the cell wall as a whole, FREY-WYSSLING (1936) has postulated reversal of fibril orientation, but rightly discarded this hypothesis after a short time. It is theoretically possible with simple stretching only, not with increase of internal pressure. As far as we know, a reversal of part of the wall has not been described or postulated earlier. We wish to call attention to this modification of FREY-WYSSLING's theory, for we imagine that the same phenomena, which have led to our assumption of the theory, will be found in many instances.

In summarizing this section, we arrive at the conclusion that there is much to be said for an explanation of the left-handed rotation in stage 4a being the result of growth starting in the lower part of the growth zone, where already some secondary wall material has been deposited on the inside of the wall, but not enough to compensate its negative double refraction. Probably the junction points between the fibrils are loosened by growth hormone or by a substance produced as a result of the presence of hormone. Being arranged in a steep S-spiral fashion, extension and intussusception

will then produce left-handed rotation. Whether intussusception too plays an active part here remains an open question.

However, we must state that the outermost layer of the wall in this lower part of the growth zone might have reversed its orientation and might also take part in causing left-handed rotation. In sporangiophores which have ceased growth temporarily or permanently, and also in immersed ones, the latter circumstance is very probably the chief agent causing rigidity of the wall and left-handed rotation with pressure. The first-mentioned factor will play a minor part because it is restricted to the area near the compensation point. Which of these factors becomes the chief one in causing left-handed rotation when growth is resumed in such cells, depends on the position of the area where growth is first resumed, a fact which is as yet unknown.

§ 3. Considerations of the causes of fibril orientation in cell walls

a. Transverse and axial orientation of primary and secondary wall layers

It is well known that in growing tubular cells the cellulose or chitin microfibrils are always oriented more or less transversely. This was found to be the case in many instances,e.g.in growing points of roots and stem-tips, in coleoptiles, in cambium, in the seta of Pellia, in the pith of *Juncus*, in bast and leaf fibres, in cotton and kapok (*Ceiba*) seed hairs, in stamens of grasses and in the growth zone of Phycomyces sporangiophores. This was established by means of the study of double refraction, dichroïsm, X-ray diagrams and particularly electron micrographs. It was also deduced from the transverse orientation of oval pits in meristematic walls, from anisotropic shrinkage or swelling, from mechanical properties, etc.

Notwithstanding very intensive stretching, this transverse structure is maintained, even in those cases where the deposition of wall material is entirely inadequate to meet the surface growth, which therefore takes place at the expense of wall thickness (stretching by endosmosis of Tradescantia staminal hairs, rapid growth with decreasing wall-thickness of grass-stamens and of the seta of *Pellia*). In the mature, secondarily thickened wall, the outer layer, i.e. the original meristematic wall, is still transversely oriented. This was demonstrated in *Phycomyces* by OORT and ROELOFSEN (1932) and confirmed by CASTLE (1938). Furthermore it was shown by WERGIN (1937) in mature cotton hairs, and by PRESTON (1947) in conifer

tracheids, whereas electron micrographs made by MÜHLETHALER (1949) showed it in the fibres of flax, rameh and cotton.

Thus the transverse orientation remains unimpaired while the cell grows to thousands, even millions of times its original length (rameh fibre). The only possible explanation for this, and one which is generally accepted at present, is that the points of junction between the existing microfibrils are loosened so that they separate transversely. New fibrils are deposited parallel to the older ones, because the open spaces are oriented that way and because the existing fibrils exercise a directive influence.

While it was clear how transverse orientation could be maintained in the growing primary wall, the reason for the initial transverse orientation was rather obscure before 1937. The old hypothesis that protoplasmic streaming accounted for fibril orientation in general could not be applied here, because in meristematic cells no preponderant transverse streaming had ever been discovered. Then independantly, CASTLE (1937a) and VAN ITERSON (1937a, see also DIEHL c.s. 1939) developed the theory that the cause of the initial transverse orientation is to be found in the tension of the wall, which in a tubular cell is always greater transversely than it is axially. If therefore in a short tubular cell just formed by division, the wall should have an isotropic net-structure, this would soon become transverse as a result of the anisotropic tension and extension. The plasma membrane would acquire a similar orientation and would exert a directive influence on new fibrils from then on, in addition to the similar influence of the wall itself. As no other explanation has been advanced since then and no facts produced to disprove it, we are of opinion that its correctness may be taken for granted.

VAN ITERSON (1936b, 1942) was of the opinion that the origin of the usual axially oriented *secondary wall* might also be explained by anisotropic extension. His reasoning is based as follows upon the presence of transverse orientation in the meristematic wall. Because transverse orientation increases and more fibrils are laid down in that direction, a moment will arrive when the axial extension exceeds the transverse one, notwithstanding the lower axial stress. This imparts an axial orientation to the plasma membrane, which subsequently produces an axially orientated secondary wall. As soon as the extension in the axial direction drops again below the transverse extension, a more or less transversely orientated tertiary layer would be deposited. This process is supposed to repeat itself many times in *Valonia*, and in this way the peculiar structure of that wall could be explained.

Several objections were raised to VAN ITERSON's supplementary

theory (FREY-WYSSLING, 1948; PRESTON, 1947; PRESTON and WARDROP, 1949). The theory does not take into account the well known fact that a cell may extend lengthwise for a long period without losing its transverse structure. Furthermore, the fact that the same kind of cambium cells in wood produce fiber tracheids and libriform fibres with steep spiral structure in the secondary wall as well as wood-vessels with a transverse structure is also unexplainable. The investigators mentioned decline to accept a purely mechanical explanation, and assert that it is necessary to assign a structure-determining role to a protoplasmic factor, of which, however, they have failed to give a definite description up to the present.

Fully recognizing these objections, we venture to propose the following hypothesis concerning this protoplasmic factor. As was already pointed out by van ITERSON, a mainly transverse extension will produce a transverse orientation in the wall and in the plasma membrane as well, for this also is a gel in which fibrillar macromolecules or micelles are joined into a network. Like the wall during stretching, the plasma membrane is supposed to be able to grow in surface by means of intussusception, thereby preserving its transverse orientation. So this is nothing but the accepted intussusception theory extended to the plasma membrane.

In order to explain the formation of an axially oriented secondary layer we may now assume that at a certain moment the growth of the plasma membrane lags behind the growth of the wall. Consequently its protein net-structure is reversed. One may imagine for instance a lack of fibrillar protein material. One may also imagine a fixing of the junction points in the protein-network, thus impairing separation of the fibrils and preventing transverse intussusception.

This moment might be determined by factors as yet unknown and originating for example from the nucleus. The essential point is that the reversion is supposed to happen in the plasma membrane only and not in the wall, because here surface-growth might occur at the expense of wall-thickness, or because here the junction points in the fibrillar network are not fixed.

The orientation of the plasma membrane having been reversed, it is now clear that the formation of an axially oriented secondary wall is to be expected and that consequently the lengthwise growth will have to stop. If the protoplasm is going to stream, the streaming will very likely be parallel to the direction of orientation of the fibrillar network.

Thus the stratifications of walls of cells with fibre texture (FREY-WYSSLING's "*Faserstruktur*") would be explained. Cells with tube

texture ("*Röhrenstruktur*") in the secondary wall, as e.g. wood vessels, sieve tubes and latex vessels, may be assumed to owe their structure to the simple fact that the velocity of the growth of the plasma membrane has always been sufficient to prevent its orientation from being reversed. The formation of cells with different wall textures within the same tissue now may be ascribed to factors regulating the rate of plasma-growth, which in the nature of things, may be different for each cell.

In order to explain the wall structure of the more or less isodiametric *Valonia* cell, one would have to presuppose a periodically varying rate of intussusception growth in the plasma membrane, which would then cause a periodical reversion of itself, e.g. during periods of rest or of low growth rate.

This theory requires that the formation of a spirally orientated secondary wall be assigned either to spiral growth, or to a twist imparted by external forces during growth. In the first case such cells must have a meristematic wall with a spiral structure, but orientated in the opposite direction to that in the corresponding secondary wall. As far as we know, this has been found in *Phycomyces* and in conifer tracheids (PRESTON, 1947), whereas spiral structures of the same sense in both primary and secondary wall have never been found.

Supplemented in this way, we see in the theory of van ITERSON an attractive working hypothesis, which explains very plausibly not only the wall stratification, but at the same time the orientation of plasma streaming and in several cases the direction of cuticular striations (*Tradescantia* hairs; *Phycomyces*, see ROELOFSEN, 1950).

However, in some cases, an autonomous orientation of the plasma membrane must be assumed. A theory based on the stretching of the plasma can never explain how, for example around a bordered pit, the protoplasm and the fibrils in the wall can be circularly oriented, or how in xylem elements with a steep spiral structure in the wall, rings or spiral bands or ladderlike thickenings can arise with similarly oriented fibrils and protoplasmic streaming coinciding in direction. Neither is it possible to explain on the basis of wall extensions how, without any connection with the wall, the neatly arranged cellulose spirals with highly orientated fibrils can be formed within the epidermis cells of *Cobaea scandens* seed coats (AMBRONN, 1925). Similar difficulties arise with the tubular spirals in the seed coats of *Cuphea*, which are only connected to the wall at one end. In all such cases and probably in many less obvious instances, autonomous orientation of the protoplasm must be admitted.

b. The origin of spiral structure

The transverse orientation in meristematic cell walls in general, could not be explained by protoplasm streaming. It is not surprising therefore that the spiral structure in the meristematic wall of *Phycomyces* also could not be explained in this way (OORT and ROELOFSEN 1932).

However, POP (1938) stated that the protoplasm in the growth zone showed a tendency to move in an axial direction and, supposing that orientation arose from protoplasmic streaming, pointed out that the acetylamine groups protruding from the glucosamine monomers of the chitin chain molecules might be the reason for the formation of a spiral structure instead of an axial one. Obviously, however, a flat spiral cannot be explained in this way and furthermore the close parallism of fibril orientation and plasma streaming in the secondary wall would also be unexplainable.

CASTLE (1936b) designed a model demonstrating the development of a spiral structure out of an originally axial one. He admitted, however, that it could be applied neither in Phycomyces nor in most other instances.

HEYN (1936, 1939) supposed that the spiral structure in *Phycomyces* was due to slipping planes in the crystal structure of chitin. His theory is unacceptable, as VAN ITERSON (1943) and others have pointed out.

This investigator (v. I.) described a model which was meant to show the principle of the formation of spiral structure in general. It consisted of a number of separate straight bamboo rods, arranged to form a cylinder like the staves of a barrel. Wire hoops kept these rods in place. The top and bottom of this "barrel" consisted of wooden cones with their apices towards each other. When these cones are pushed inwards, the rods aquire an oblique position (spiral structure) while the cones turn in relation to each other (rotation). According to the designer, it is essential that the pressure of the cones is brought to bear on the inner edges of the rods. By analogy he supposes, that if in the wall of a growing cell the outside presents less resistance to stretching than the inside, it would also result in a spiral structure.

In our opinion this model also is not satisfactory. We do not see clearly why the cause of the displacement of the rods should lie in the difference of pressure on their inner and outer sides. Wood is so rigid a material that such differences of strain in the ends of the rods can only exist over a very small distance. The experiment would therefore also have been successful if the material had been stil more rigid, e.g. steel rods. In our opinion therefore the model does

not demonstrate that strain differences in the rods are the cause of their acquiring oblique positions. It demonstrates something else however, namely the simple fact that the rods are in labile positions and, because their sides touch each other, must all fall in the same direction like books on a bookshelf.

However, it seemed to us that the fundamental idea, viz: that spiral structures might be the result of differences in extensibility of the outer and the inner layers of the wall, could be an acceptable hypothesis, particularly since electron micrographs in fact have shown up a difference in structure of the inner and outer side of the wall (ROELOFSEN 1950, see also § 2b). It is apparent that the inner side is the more extensible, while VAN ITERSON's theory is based on greater extensibility of the outer side, but in principle this is irrelevant.

We have tested this hypothesis with the following model-experiments.

a. A piece of rubber bicycle tyre was pulled over a wooden cylinder and fastened down at both ends in an extended condition. A second piece of tyre was pushed over this without stretching and then was glued onto the inner tube with rubber adhesive. They were then removed together from the wooden stick. The inner tube of course shortened, but was counteracted by the outer one. Transverse folds showed up but no twist was visible and this was also the case when the outer tube was the stretched one.

b. The second experiment was based on the same principle. As is well-known, wetted paper on drying shrinks most perpendicular to the so-called machine-direction, because the paper fibres are orientated parallel to it. Two wet pieces of paper were glued to each other with crossed machine directions. On drying strips cut out of this double layer of paper a twist developed, but only, if the sides of the strips did not coïncide with the machine-direction of either component, otherwise only a bending occurred, (STEINBRINCK, 1906). Now tubes were made out of the double layer of paper while this was still wet. The machine-directions were placed exactly transverse and axial. On drying, stresses will occur as with the rubber tubes of the former experiment and these paper-tubes similarly showed no twist.

c. As in paper, commercial cellophane also has a machine direction, the extensibility being least in this direction. Out of such cellophane we made similar double tubes with transverse and axial machine directions. They were made in two kinds, one with the inner and outer tubes loose from each other, the other with the two tubes stuck together. These double tubes were wetted and put under

pressure. No twist ever resulted, not even if "assistance" was given by twisting the tube slightly.

We therefore were forced to draw the conclusion that this hypothesis should be rejected. We then sought for another which would be in harmony with the known facts.

In § 1*f* we arrived at the conclusion that the spiral growth of a cell having spiral structure could not be explained by the mechanical properties of that wall only, but that "active" intussusception took place. Consequently it is logical to suppose that the origin also of spiral structure is to be assigned to intussusception and not to mechanical properties. How this happens might be imagined hypothetically as follows.

Electron micrographs of the primary wall of *Phycomyces* suggest that a chitin fibril, which is being deposited in the wall, is first fixed by one end only, the other end hanging loose in the protoplasm (ROELOFSEN 1950). This free end is influenced by two directive forces. The one, which issues from the existing transversely built wall and the similar plasma membrane, tends to make the fibril become transversely orientated too. This is based on a common physico-chemical directive effect, also to be found for instance in macromolecular solutions.

The second directive force may be found in the fact that, since the top of the cell grows, the protoplasm steadily moves in an axial direction with regard to the wall. POP (1938) in fact postulated a gross protoplasmic flow towards the top. Admittedly the plasma membrane will be fixed to the wall, but the loose end of the fibril might protrude into plasm-layers which are free to move. However it is not at all necessary to accept a direct mechanical influence like this. Cellulose is known to be negatively charged and very likely chitin will be electrically charged too. The moving protoplasm containing many charged particles, will therefore exert by induction a directive influence on the loose ends of the fibrils.

The result of these two directive forces, one of them transverse and the other axial, will be that the fibril will come to lie in an oblique position in the wall, either in an S-spiral or in a Z-spiral. The slope may of course vary in every fibril as the influence of the two directive forces likewise vary.

If we can explain how the first mentioned directive influence may show a preference for either right- or left-handed orientation of the loose ends of the fibrils, the origin of a spiral structure will also become apparent. This problem of explaining preference for either Z- or S-spiral structure is a very old one and many suggestions have been made as to its possible explanation, e.g.

electrical polarity and circular magnetic lines of force, (SCHMUCKER, 1925), stereo-isomerism of the molecules of the cell wall material, etc. None of these seem very likely to us and as far as we know, no experimental facts have ever been produced on this point. So this question must be left open.

It is obvious that in laying down the secondary wall the forces mentioned here may have lost their influence wholly or partly, because other factors, such as the conspicuous longitudinal protoplasm streaming, will exercise a paramount effect. It is also clear that the conditions will vary both in different cells and during the growth of each cell, so that the spiral structure will likewise vary considerably in different cells and within the same cell.

SUMMARY.

§ 1. Methods are described for the study of extension and rotation of sporangiophores of *Phycomyces* on varying the internal pressure and for determining the shrinkage on loss of natural turgor (fig. 3, 4). The latter is about 2 atmospheres. Within this pressure range the stress-strain diagrams were determined (fig. 5). The distribution of extensibility in the growth zone is different from the distribution of growth rate, the zone of maximum extensibility being nearer to the sporangium than the zone of maximum growth rate, (fig. 8).

The rotation per mm elongation with increased pressure is considerably lower than during growth (table 4) which shows that spiral growth must be due not only to the mechanical properties of the wall, but also to some special aspect of intussusception, (fig. 1b).

§ 2. In mature cells and in cells where cessation of growth is induced by immersion in water, the wall becomes very rigid; its optical properties change and the direction of rotation with increased pressure is reversed. This is very probably due mostly to irreversible reversion of fibril orientation in the outermost layer of the primary wall. Reversal of orientation in certain layers of growing cell walls (not in such walls as a whole) is supposed also to be of importance in other cases.

Reversed rotation also occurs when growth is resumed after the natural stagnation in growth in stages 2 and 3. This can be ascribed mainly to resumption of growth in areas where secondary wall material has already been laid down.

§ 3. Theories as to the cause of fibril orientation in the meristematic and secondary cell wall, in particular those on the origin of spiral structure, are considered and supplemented.

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