ON THE SUBMICROSCOPIC STRUCTURE OF CUTICULAR CELL WALLS

BY

P. A. ROELOFSEN

Laboratory for Technical Botany, Techn. University, Delft, Netherlands

Received June 1951

With plates I—IV

INTRODUCTION

Mainly as a result of studies on double refraction before and after extraction of waxes, Miss M. MEYER (1938) concluded that the cuticular layers of leaves from *Clivia nobilis*, *Gasteria*, *Dasylirion* and *Yucca* consisted of two layers, i.e. an outer layer with cutin and wax and an inner layer containing in addition cellulose and pectin.

The double refraction of the outer layer was always negative with reference to the cuticular surface; the highest refractive index was radial. However, after extraction of waxes with boiling [1] pyridine for 120 hours or after melting them at 100° C, this layer became isotropic provided the section was mounted in benzene (n = 1.50) to eliminate the form double refraction of the pores in the cutin skeleton structure (Gerüstsubstanz). This proved that the original double refraction was due to extractable wax molecules.

Since a solution of friedelin (from cork wax) in *m*-cresol showed positive streaming double refraction, it was assumed that the cutin wax molecules would behave similarly [2]. Accordingly the negative birefringence of the outer cuticular layer could only be explained by assuming a radial orientation of these molecules. Presumably the wax molecules are crystallized in tangentially oriented platelets, since a positive rodlet form double refraction was discernable when extracted sections were mounted in liquids with refractive indexes higher or lower than 1.50. It was assumed, but in our opinion not proven, that these platelets were equidimensional in surface view.

The inner parts of the cuticular layers of the plants mentioned, differed in one respect from the outer parts: they contained in addition cellulose and pectin. In *Dasylirion* the double refraction of

[1] In MEYERS' article no reference as to the temperature of extraction was made, but Prof. A. FREY-WYSSLING privately informed the author, that the extractions were performed at the boiling point.

[2] WEBER (1942) has shown, that the streaming double refraction of wax solutions may be positive or negative, dependent on the orientation of the molecules in anisotropic molecular aggregates which are usually present in solutions at normal temperature. Strictly speaking therefore, the observations of positive flow birefringence of a wax solution did not fully permit MEYER to conclude that wax molecules are positively birefringent. However, the long aliphatic chains of most plant waxes, with little or no polar side groups certainly exclude negative molecular birefringence.

fugation of the tube contents while still hot and by consecutive washing in hot alcoholic alkali, alcohol and water.

The iodine dichroism (fig. 2) and the double refraction of Congo red coloured specimens suggest a fibril orientation as indicated in fig. 3a.

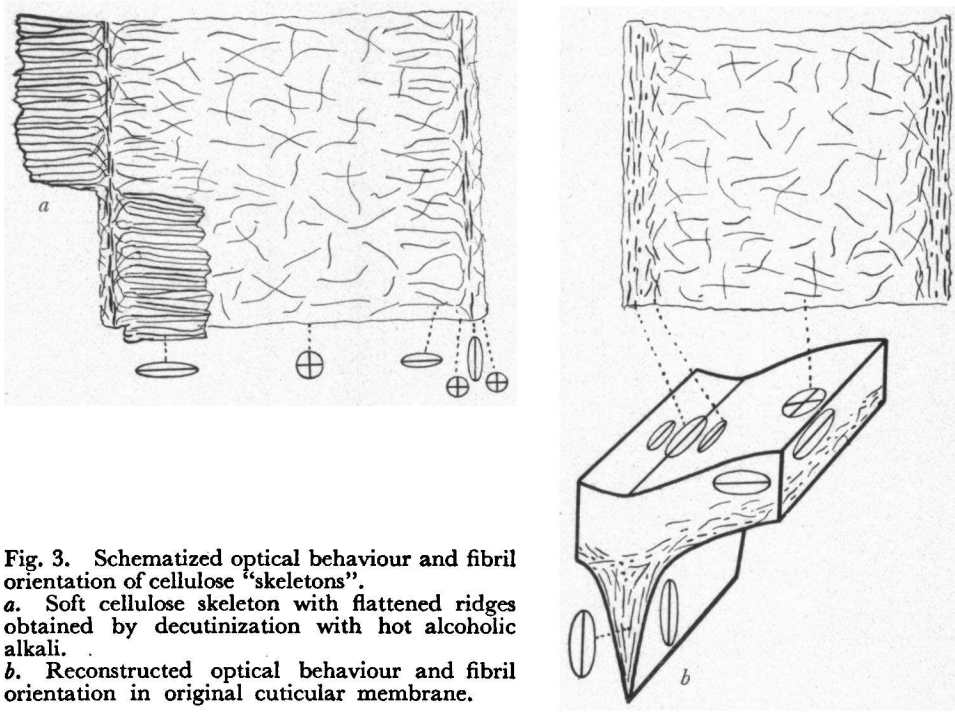

Fig. 3. Schematized optical behaviour and fibril orientation of cellulose "skeletons".
a. Soft cellulose skeleton with flattened ridges obtained by decutinization with hot alcoholic alkali.
b. Reconstructed optical behaviour and fibril orientation in original cuticular membrane.

Most conspicuous are the ridges. The cellulose fibrils in them show a high degree of orientation in radial direction. They are usually flattened on the slide, demonstrating their loss of rigidness on decutinization. Where the ridges are attached to the tangential part of the membrane, i.e. on the cell boundaries, a thin strip with axial orientation is present. This is bordered by two narrow isotropic strips and two somewhat broader zones with predominant transverse cellulose orientation. The central parts of the "cells" are isotropic both in long and in short cells.

This applies to the cellulose skeleton as it is lying flat on the slide, but in the cutinized original membrane it will be somewhat curved near the ridges (fig. 1b). As a result, on surface view, the situation near the ridges will be changed. A probable reconstruction of the optical behaviour as far as the cellulose skeleton is concerned, is depicted in fig. 3b. In accordance with MEYERS' suggestion (l.c. p. 567) the cellulose is supposed to be mainly located in the inner parts of the membrane. However, additional proof of this seems desirable.

§ 2. Optical behaviour of cuticular membranes of *Clivia nobilis*

a. Non-extracted membranes

M. Meyer studied both sections and isolated cuticular membranes. The optical properties she described were presented schematically as in fig. 4a.

We were not able to corroborate certain details of this picture as will become apparent on studying fig. 4b and c.

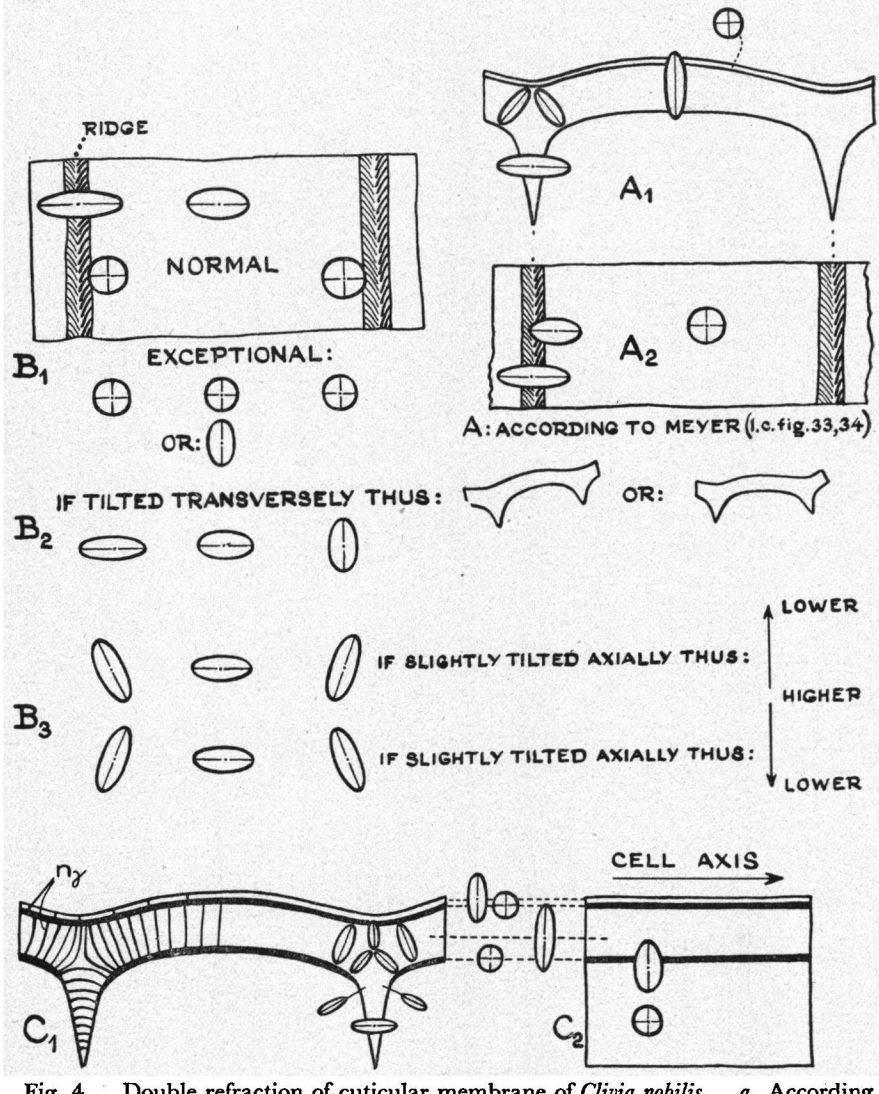

Fig. 4. Double refraction of cuticular membrane of *Clivia nobilis*. *a.* According to Meyer; a_1 transverse section and a_2 surface view. b_1 normal surface view, $b_{2,3}$ view with membrane tilted as indicated. c_1 transverse and c_2 axial section.

leaf surface) as the radial cellulose orientation lead one to expect, but stayed positively birefringent, just like those which were not extracted.

In thin (6 μ) axial and transverse sections of extracted membranes, observed with high magnification (fig. 7c), three layers could be discerned in the cuticular membrane, viz. a thin one on the outer and on the inner sides and a thick central layer. The two thin layers were faintly positive ($\pm$ 4.5 mμ in 6 μ transverse sections); the central part was distinctly negative ($\pm$ 7 mμ) and apparently outweighed the influence of the two thin layers when a lower magnification was used. The cut ridges also, whether axial or transverse, showed three layers. At first we considered this three layer structure to be a demonstration of progressive extraction from the outside inwards, but the image did not change on further extraction. Hence we may assume this to be caused by differences in structural or chemical properties. The outer thin layer obviously conforms to the cuticle *sensu stricto*, already distinguishable in non-extracted sections (fig. 6) and the inner thin layer might be that part of the cuticular layer which contains relatively much cellulose and a small quantity of cutin and wax.

In a radial view of extracted cuticular membranes mounted in benzene or methylbenzoate, the tangential parts are usually isotropic, sometimes faintly positive or negative (fig. 8). In the middle of the cell boundaries there is a thin negatively birefringent strip, obviously due to the ridge (fig. 7c). This is bordered by two isotropic strips and two broader zones with positive double refraction.

When observed with low magnification the central negative part in the axial ridges was dominated by the two outer positive ones, whereas in the transverse ridges usually the central part predominated.

Having observed a residual negative double refraction in extracted sections, we subsequently tried to see whether this also occurred when sections were heated to temperatures of 220—240° C, a procedure said to cause irreversible disappearance of all double refraction. Extracted and non-extracted sections were put into a small glass tube and this was kept in a sand bath at 220—240° C for 15 min. Alternatively, sections lying under a thick cover glass on a slide were held between a pair of forceps in the hot sand. After cooling, benzene was applied under the coverslip. Even without bleaching a similar faint negative double refraction was visible as was seen with extracted specimens.

c. Form double refraction in extracted membranes

In confirmation of MEYERS' observations we observed a form double refraction in the cuticular layers of sections and in membranes which previously had been extracted, bleached and dehydrated as described earlier.

Thick (25 μ) transverse and axial sections or the membranes were mounted in absolute alcohol and then retardation at certain selected points was measured with the elliptic compensator, using white light. Then, without touching the coverglass, the preparation was

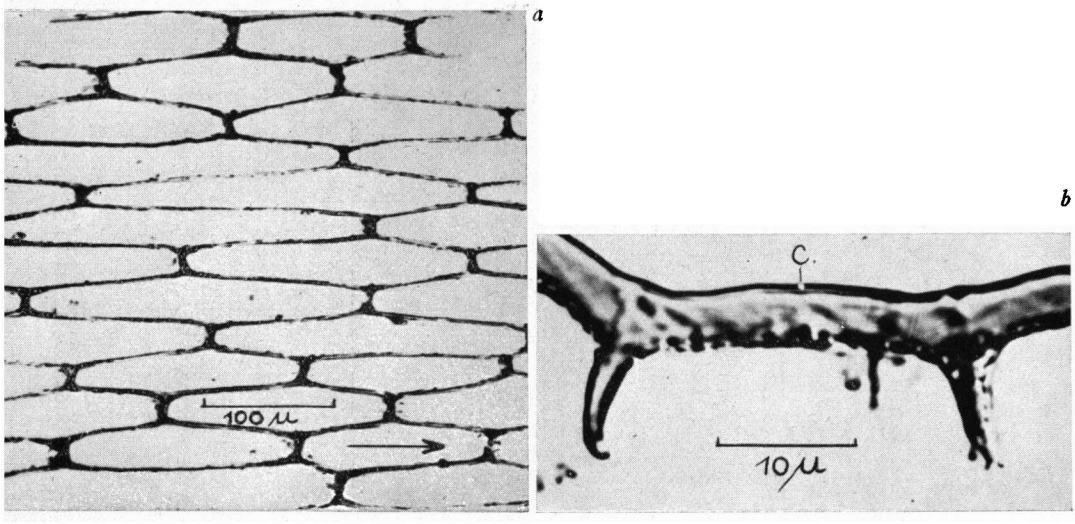

Fig. 1. *a.* Surface view of cuticular membrane of upper leaf surface of *Clivia nobilis*; leaf axis indicated.
b. Transverse section through a similar membrane, coloured with malachite green. Note cuticle *sensu stricto* (c) and more strongly coloured inner layer.

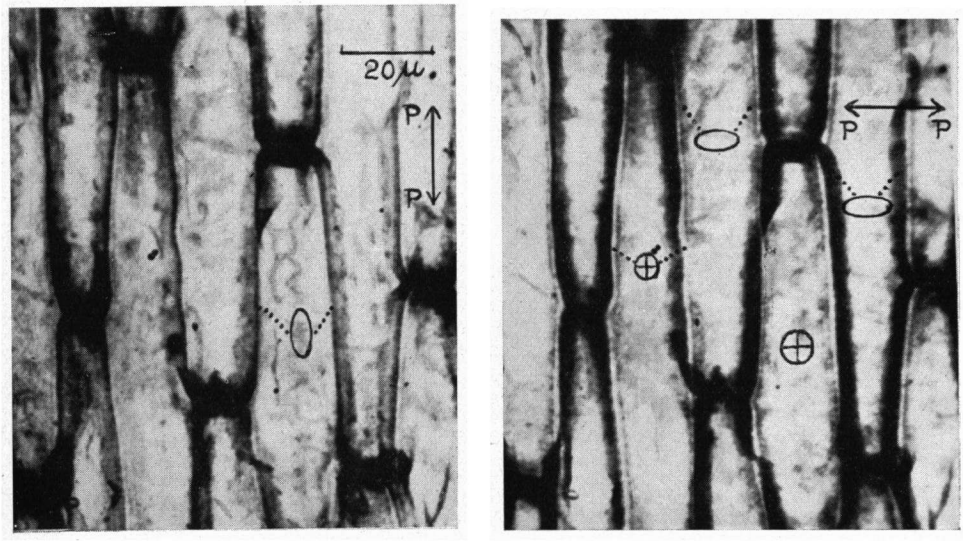

Fig. 2. Cellulose "skeletons" of cuticular membranes coloured with chlor-zinc iodide and photographed in polarized light. Vibration plane of polarizer indicated. Note the soft flattened "ridge skeletons" and the index ellipses indicating fibril orientation.

PLATE II

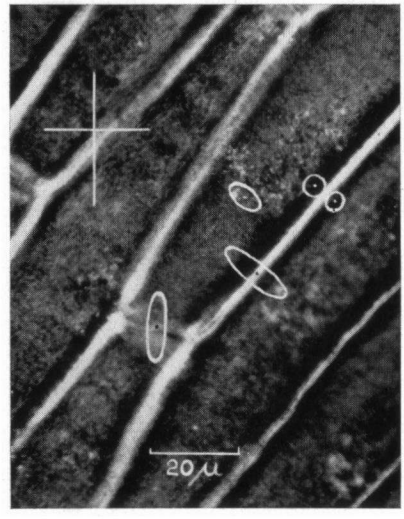

Fig. 5. Radial view of cuticular membrane between crossed nicols. Vibration planes and index ellipses indicated.

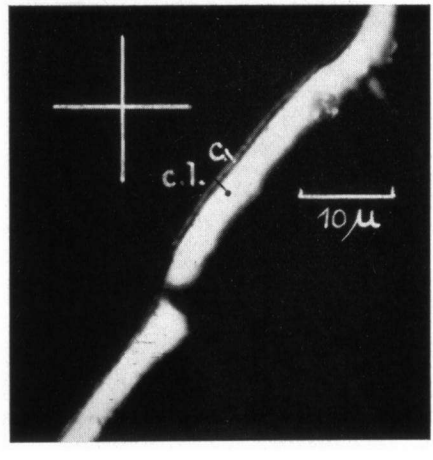

Fig. 6. Thin transverse section (3 μ) through non extracted cuticular membrane, seen between crossed nicols. Note the doubly refractive cuticle (c) and the isotropic zone between cuticle and cuticular layer (c.l.).

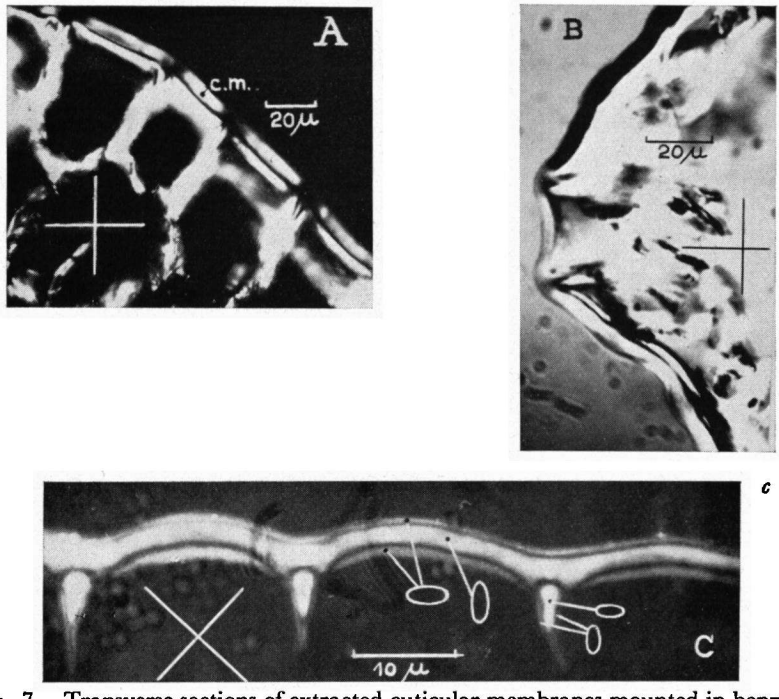

Fig. 7. Transverse sections of extracted cuticular membranes mounted in benzene and observed between crossed nicols.
a. Thick transverse section observed with low magnification, showing conspicuous negative residual double refraction in cuticular membrane (c.m.).
b. Similar section with compensated double refraction in the cuticular membrane of the upper part.
c. Thin section observed with high magnification showing three layers with different optical properties (indicated by ellipses).

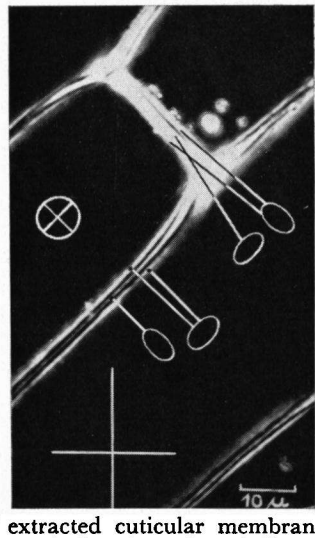

Fig. 8. Radial view of extracted cuticular membrane between crossed nicols. Vibration planes and direction of $n\gamma$ indicated.

PLATE IV

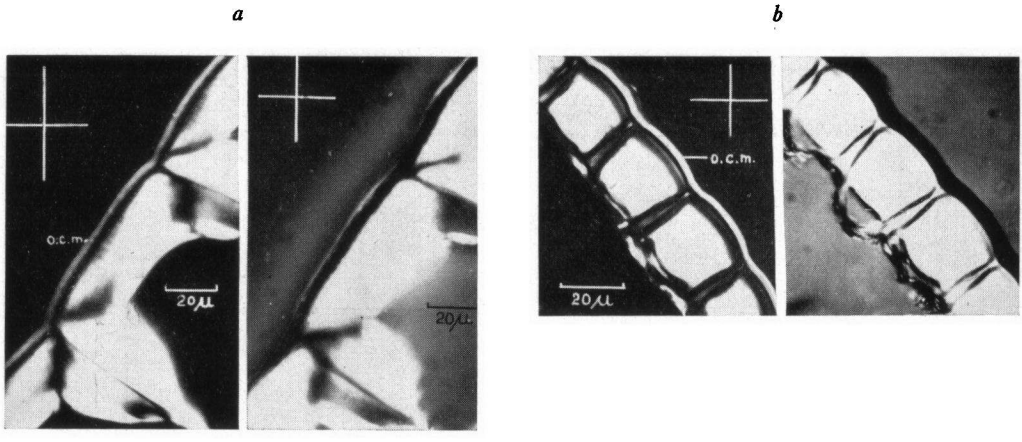

Fig. 12. On the left: extracted sections of cuticular membranes between crossed nicols. On the right the same spot compensated for the negative double refraction of the outer layer (o.c.m.).
a. Gasteria trigona.
b. Dasylirion serratifolium.

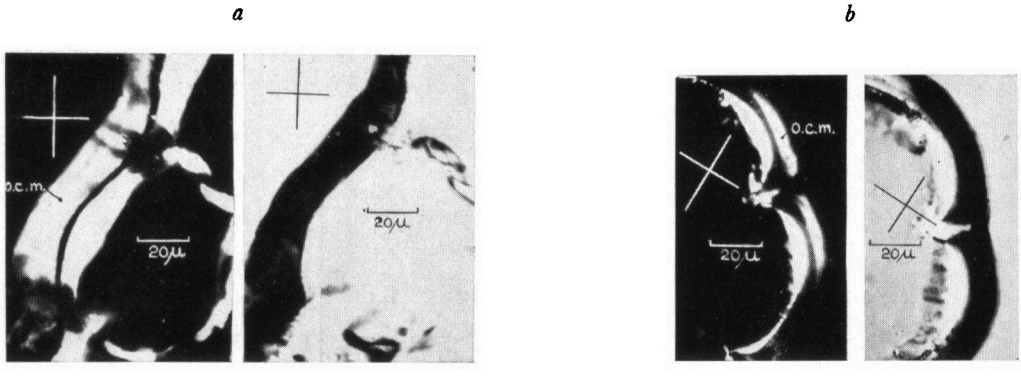

Fig. 13. On the left: extracted sections of cuticular membranes between crossed nicols. On the right: the same spot compensated for the negative double refraction of the outer layer (o.c.m.).
a. Yucca aloifolia.
b. Aloe glauca.

dried at 50° C for several hours. Then benzene was applied under the coverglass and the retardation was determined again at the same points. At first we thought that it would take some time to attain complete penetration of the mounting liquid, but the retardation almost immediately attained its final value. This is understandable since cutin is lipophilic as are the liquids themselves. Finally the retardation was measured after impregnation with CS_2. The same procedure was followed with 6 μ sections obtained from paraffin-embedded extracted membranes. In such sections the three layers as depicted in fig. 7c were studied separately.

The results are given in table 1.

TABLE 1

Form double refraction and intrinsic double refraction (benzene) of extracted cuticular membranes

Imbibition liquid	nD	Average retardation in mμ of cuticular membrane in:				Surface view of central part of "cell"	Character of predominant double refr. in radial view of ridges:	
		25 μ sections		layers of 6μ section				
		axial	transverse	outer	middle		axial	transverse
Abs. alcohol	1.363	+10	+(0.8?)	+14.3	−4.5	−2.5	+	+
Benzene	1.500	−19	−48	+ 4.5	−7.0	+1.7	+(0.−)	−(0.+)
CS_2	1.623	−11	−42	+ 7.3	−5.1	+	+(0.−)	−, 0, +
		+ or − with resp. to leaf surface				+ or − referring to cell axis	+ or − referring to ridge direction	

If we disregard the intrinsic double refraction, seen when mounted in benzene and only consider the character and the magnitude of the changes induced by mounting in alcohol or CS_2, we are studying the WIENER form double refraction effect. Apparently there is a WIENER effect in all cases, viz. in axial and transverse sections, in the layers of the sections, on surface view of the central parts and in the ridges. This proves that there are non-equidimensional pores everywhere in the cutin structure. The orientation of the major pore dimension may be deduced from the index ellipsoid of the form double refraction.

In axial as well as in transverse sections the higher index is tangential and the shorter one ($n\alpha$) radial. Hence the shortest pore dimension must be the radial one. Because the WIENER effect is greater in transverse than in axial sections of equal thickness the pores (or the projection of the pores) must be longer in a transverse than in an axial direction. This is confirmed by the character of the WIENER effect in radial view, which allows one to conclude that $n\gamma$ of the index ellipsoid of form double refraction is transverse and $n\beta$ axial. This leaves two possibilities: either the pores are tangentially oriented oblong platelets with their longest dimension transverse, or they are tangentially oriented rodlets having a predominantly transverse orientation.

In the ridges the minor pore dimension must be oriented perpendicular to the surface of the ridge. Bearing in mind the cellulose orientation in the ridge, it seems very probable that the major pore

ellipses marked W in fig. 9). This cannot be due to cellulose double refraction, because the cellulose skeleton is isotropic when observed in a radial direction (fig. 3). Neither can it be due to form double refraction because it does not disappear when the membrane is mounted in benzene. It must therefore be due to a special orientation of wax molecules. It is self-evident that not all of these will be oriented strictly radially. Many will deviate slightly from this position, inclining to one direction or another. Such an inclined molecule (or group of molecules: crystals) will produce some double refraction of its own when the membrane is viewed in a strictly radial direction. The negative double refraction in question shows that molecules inclined to the transverse direction are more numerous than those inclined to the axial direction, or the inclinations are more pronounced or both.

Thus far there is little theory in this. But how is this phenomenon to be explained? As we saw, the pores may be imagined as transversely oriented rulers. It seems obvious that these rulers will tend to incline in axial direction (as depicted with the three "rulers" near the ridge in fig. 9) rather than in transverse direction. If the pores would each contain one big wax crystal filling up the whole pore space, then the preference for axial inclination would produce positive double refraction whereas a negative double refraction is observed. However, it is rather unlikely that the pores, which in reallity of course have a very irregular shape, will each contain one big wax crystal. They will be filled with aggregates of many small crystalline wax platelets, also irregularly shaped, but for the sake of convenience to be regarded as square platelets. If these crystallites are not too small compared with the pore space, they will more readily incline along the length of the oblong pore space than across its breadth. This state of affairs is demonstrated in fig. 10 [1], which of course is entirely theoretical, merely illustrating the effect in question. Although admittedly, this picture is rather hypothetical, as far as we can see it is the most likely explanation, which fits all the known facts.

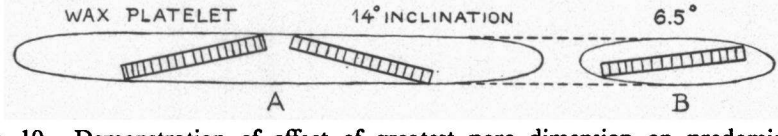

Fig. 10. Demonstration of effect of greatest pore dimension on predominant direction of inclination of wax platelets located in it. *a.* lengthwise; *b.* breadthwise section through same hypothetical pore space.

Of course the inclination of the wax crystals will also be affected by the inclination of the pore spaces themselves. Probably this effect comes into play in the isotropic zones adjacent to the cell boundaries, which are visible when wax-containing membranes are viewed in a radial direction between crossed nicols (fig. 5). Because in these areas

[1] The wax molecules are drawn perpendicular to the crystal surface since natural waxes crystallize mostly in the rhombic form (KREGER, 1948). In fatty acids and soaps the molecules are often obliquely oriented.

the membrane is somewhat curved, the $n\gamma$ lines are also inclined (fig. $4c_1$). This should merely increase the already negative double refraction. Instead, the double refraction disappears. If the membrane is tilted in a transverse direction so that the membrane in one of the two curved isotropic cell parts is now strictly perpendicular to the direction of view this part becomes positively doubly refractive (fig. $4b_2$). What is the explanation of this?

First we thought that the double refraction of the cellulose skeleton as depicted in fig. 3b could explain it. Such an explanation was given by MEYER (l.c. pag. 582) for the occurrence of similar positively birefringent zones in surface view of non-extracted membranes of *Gasteria*. We have no reason to reject her explanation, but it does not apply to the problem in question with *Clivia*.

Firstly the cellulose double refraction as seen in the cellulose skeletons is much lower than the positive double refraction seen in the upper parts of the slightly tilted membranes. Moreover the cellulose can only produce positive double refraction in the highly curved parts at the base of the ridges (fig. 3b), for if the cellulose skeleton is lying flat as in fig. 3a, the double refraction near the ridges is negative.

Secondly, if the wax is extracted, the isotropic zones in question do not become broader and do not change into positive zones, as would be expected. Admittedly, small positive zones are produced, which are very probably due to cellulose double refraction (fig. 8), but these are less broad than the isotropic zones in the wax-containing membranes (compare fig. 5) and are too faint.

Hence we must assume that near the cell boundaries the predominant inclinations of the wax molecules are axial instead of transverse as in the central parts. It is only owing to the fact that the membrane is somewhat curved and therefore observed in a slightly inclined position that isotropism occurs. By eliminating the inclination, positive double refraction appears, as indicated in fig. $4b_2$, right part.

What might be the reason for the predominant axial inclination in these zones? The character of the form double refraction (see F ellipses in fig. 9) excludes the presence of axially elongated or equidimensional pores. We can see only one more possibility, viz. that the inclinations of the "ruler-like" pores themselves are more marked here than in the central part. This is depicted schematically in fig. 9. In reality of course there are no ruler-like pores; it is better to say that the pore-space is less flattened tangentially, but more irregular than in the central cell parts.

RESIDUAL INTRINSIC DOUBLE REFRACTION. It has been shown in section 2b, that extracted or heated sections and membranes show a residual negative double refraction when mounted in benzene to eliminate form double refraction. As depicted in fig. 7c there are three layers, differing in character, but the middle layer predominates. The resulting effect is indicated in fig. 9 by the ellipses marked R.

It cannot be due to the only crystalline substance already known to occur in extracted membranes, viz. cellulose. Therefore we must assume the presence of another non-extractable and non-melting

double refraction of the inner layer is increased when the outer layer is compensated, which proves that their doubly refractive character is opposite. A similar explanation as given for *Clivia nobilis*, must be accepted here.

SUMMARY

By dissolving the cutin in hot alcoholic alkali, the cellulose "skeleton" from the cuticular layer of *Clivia nobilis* leaves has been isolated (fig. 2) and the fibril orientation therein has been determined (fig. 3).

The form double refraction of wax-free cuticular membranes of *Clivia nobilis* indicates the presence of emptied oblong flat pores, tangentially oriented and with their major axis transversal (fig. 9). The wax platelets are mainly tangentially oriented too, but these probably have a preference for slight transverse inclinations (fig. 10). thus producing a corresponding double refraction in surface view. The cuticle *sensu stricto*, up till now considered isotropic, is birefringent too (fig. 6).

After extraction or heat-destruction of true waxes the major part of the cuticular membrane still shows a faint double refraction with a radially oriented major refractive index (fig. 7). This is tentatively ascribed to chemically bound oriented wax molecules, lining the pore space (fig. 11). A similar state of affairs occurs in the cuticular membranes of *Gasteria*, *Yucca*, *Dasylirion* and *Aloe*.

ACKNOWLEDGEMENT

We are greatly indebted to Prof. L. J. Audus for correction of the English text.

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