Bio-physics. — *Notes on the structure of the wall of algae of the genus Halicystis.* By G. VAN ITERSON Jr.

(Communicated at the meeting of October 31, 1936).

In his treatise "Ueber den Bau und die Fortpflanzung von Halicystis (Areschoug) und Valonia (Ginnani)" (Botanische Zeitung **65** I, 1907, S. 137—185) P. KUCKUCK described the wall of *Halicystis ovalis*, which he had gathered at the coast of Helgoland. He observed the following about it:

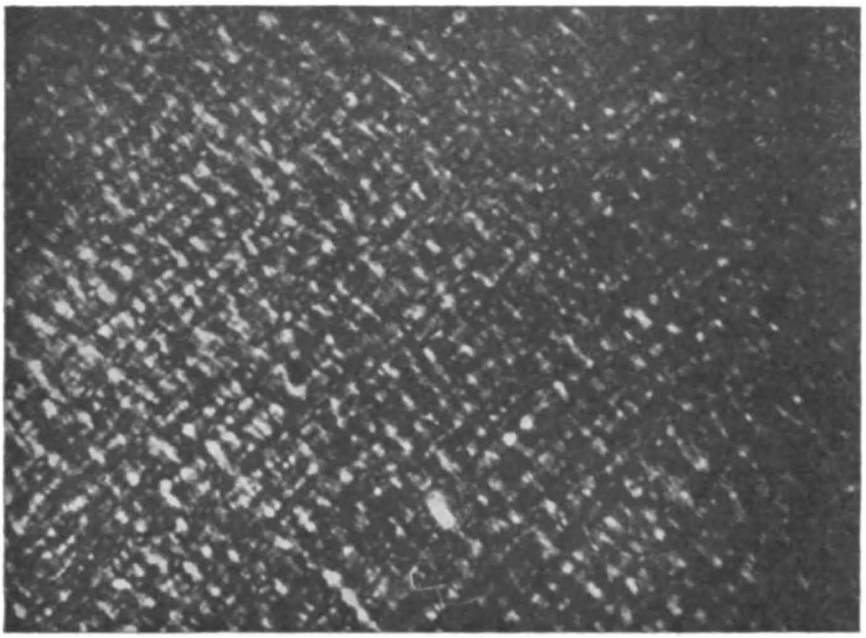
"Die Membran ist 10—12 μ dick und ohne sichtbare Struktur, wie schon Agardh angibt. Auch bei Behandlung mit verschiedenen Reagenzien und Farbstoffen bleibt sie strukturlos, nur werden beim Quellen in Kalilauge im Profil einige wenige Schichtungsstreifen sichtbar, die zeigen, dass die Membran von älteren Blasen aus etwa 2—3 Lagen bestehen kann, die durch eine zarte, weniger dichte Schicht getrennt sind. Lyngbyes Angabe, dass die Membran "sub lente minutissime punctata" sei, ist irrtümlich wie schon Areschoug zeigt. Die Membran gibt die bekannten Zellulosereaktionen".

When I attempted a few years ago to obtain some *Halicystis ovalis* from Helgoland, to study the wall more closely, I was informed that these one-celled Chlorophycea had been found there regularly for many years, but that lately they were no longer to be found. During a visit to the Hopkins Marine Station at Pacific Grove, California, in the summer of 1933, I was surprised to find there, in an aquarium, a species of *Halicystis* which was collected by Mr. HOLLENBERG near Monterey (California) and was used for physiological investigations. Presumably this species was identical to *H. ovalis*, but it might have been *Halicystis Osterhoutii*, which was reported from several stations near Monterey (vide: L. R. BLINCKS and A. H. BLINCKS, Two genera of algae new to Bermuda, Bull. Torrey Bot. Club **57**, 389—396, 1931). A very cursory investigation with the polarization microscope showed me that the wall has more interesting features than KUCKUCK had observed.

Recently I received from Dr. L. R. BLINCKS a few dried cells of *Halicystis Osterhoutii* which he had gathered in Bermuda. The alga is found there, namely, washed up on some of the coasts, as beautiful transparent cells up to two or three centimeters in diameter, and known as "sea bottles". The Bermudan species reaches a larger size than *H. ovalis*. The cells received from Dr. BLINCKS indeed were much larger than those I studied in Hopkins Marine Station. With the walls of the dry cells after they were boiled I

found the same optical phenomena as with the smaller bladders from the Pacific. Although my studies are far from complete (I lacked the necessary material), I think it is well to publish a few of my observations.

On viewing the cell walls of *Halicystis* between crossed nicols, I found that the wall shows a very striking aspect. We refer here to the included micro-photo prepared by my assistant, Miss Dr. ALIDA C. SLOEP. One will observe the lightening of the field of vision in spots. These spots are in



reality short light-giving band sections which alternate with dark sections. It is remarkable that many band sections are placed in line, which produces intermittent stripes. These stripes occur in two directions, perpendicular to each other. The directions halve the angles between the vibrational directions of the polarizer and the analyzer, viz. in a normal position of the nicols form angles of 45° and 135° with a vertical. Especially at the points of intersection of two lighting bands the light is strong. I was able to observe further, by alternately focusing exactly on various heights in the wall, that the two systems of band sections are not situated in the same plane, but in two different planes very close above each other.

When the preparation is rotated over 360°, the two stripe systems are most clearly visible in four mutually perpendicular positions, and disappear, almost but not completely, in four positions in between. In the photo they are shown in the most clear position. During the rotation the stripes do not change their direction, but only their intensity. Inserting a $\frac{1}{4}\lambda$-plate when the stripe systems are in the most clear position, or a gypsum plate red 1st order, produces an image that is still more clear. With gypsum

red 1st order, in one direction blue band sections appear and in the other, yellow; both band sections are alternated by red stripes.

The image, however, is not regular at all, and it changes also, of course, on rotating the object. Moreover, the whole field of vision becomes coloured slightly blue in two positions, and in the two positions perpendicular to the former, slightly yellow.

The images obtained by exactly focusing on a single layer with band sections reminded me strongly of the images which I obtained some time ago when I smeared a solution of the cadmium salt of glyceric phosphoric acid on a slide, and observed the result some time afterwards between crossed nicols, with a gypsum plate red 1st order inserted (vide "Some remarkable properties of a double refracting liquid", Proc. Royal Acad. Amsterdam, **37**, 367—376, 1934). I assume from this that the double-refracting matter is present as a series of crystallites placed in waves, and that there are two layers, with crystallites so placed, present closely above each other, while the directions of the waves in these two layers are perpendicular to each other. This supposition, however, is not more than a working hypothesis.

Also at the folds of folded walls of *Halicystis* a structure is visible, when viewed between crossed nicols, and I have used such folds in the beginning to enlighten myself further about this structure. In this manner I have especially investigated a few young walls. To investigate older walls, however, I have made use of cross-cuts, since I suspected that on the folds of thicker walls double refraction would occur which must be ascribed to tensions. I prepared these cross-cuts of cell walls which I had mounted in paraffin. The slices of paraffin obtained by cutting with the microtome were not pasted to the slide, but I dissolved the paraffin in chloroform, filtered the chloroform in which the cuts were floating through a black micro-filter paper (diam. approx. 8 mm), onto which I collected the cuts. I used for this a special micro-filtration apparatus which need not be described here; suffice it to state that the small filter paper is placed as a flat disk on the apparatus and is not folded. The cuts could be loosened easily by moving the reversed filter paper, with a pair of pincers, in a drop of water on the slide. In such a way I could bring tens of cuts under one cover glass, they could be viewed under the microscope, and be subjected to the action of chemicals.

It now appeared that at the outer side of the walls a very thin cuticula is present (naturally it is not proved that this cuticula can be considered as identical to the cuticula of higher plants), and that under this thin cuticula a strongly double-refracting thin layer is present which is followed by a much thicker, practically non-double-refracting layer in older walls. The longest axis of the index ellipse of refraction of the double-refracting layer is placed parallel to the surface of the wall.

The outer surface of the wall is not flat, but slightly waved; sporadically, distinct conical protuberances appear. It is remarkable that the double-

refracting matter seems usually to be more concentrated along the troughs of the waves than near the crests. Sometimes it may be observed that this matter penetrates also deeper into the wall, often as triangular figures of which the bases are placed along the cuticula and the tops directed toward the interior, but thin walls may be also spotted irregularly, which hints at another distribution of the double-refracting matter. Always, however, the most intense double refraction is observed immediately under the cuticula. I estimate the continuous layer of this matter at approximately twice the thickness of the cuticula; the latter I estimate at approximately 1.5 μ. The non- or weakly double-refracting layer, which follows on that of the double-refracting matter in the direction of the interior, may become, in the case of large cells, as thick as 30 μ.

If swelling agents are applied on cross-cuts, it appears that the layer containing the double-refracting matter swells very easily; the wider layer under this swells less, yet increases to two or three times its thickness. This has been found for the swelling in 50 % alkali, cupric oxide ammonia, 55 % sulphuric acid and chlorozinc solution; the swelling in chloral hydrate is somewhat more difficult. Chloro-zinc iodine solution, especially, gives clear images.

On swelling, the original double refraction disappears practically instantaneously; the systems of bands soon cannot be seen any more in a top view, and in the cross-cuts the double-refracting sub-cuticular layer too almost immediately disappears. It is notable that the inner part of the wall may then become strongly double-refracting; the longest axis of the index ellipse of refraction is perpendicular to the surface of the wall. I consider it probable that this double refraction is tensional, due to tensions produced by the swelling.

It is of importance that a lamellae-structure becomes visible in the last mentioned part of the swollen cross-cuts; with older cell walls I counted from 50 to 60 lamellae, of which some were apparent than others. Occasionally I received the impression that a structure of coarser layers must be accepted aspresent (in KUCKUCK's sense), but often this was not to be observed, and I do not consider this structure as real.

On swelling, the cuticula is lifted up and in the part of the swollen wall situated between the cuticula and the wall layer containing the lamellae there is no structure visible. Especially with the swelling in chloral hydrate it may be observed how in the beginning the cuticula removes itself from the wall in lens-shaped bubbles; the blisters formed in this way are filled with non-double-refracting matter. The outer wall lamellae of the thick layer do not remain unbroken in the case of the heavily swollen walls, but are split by wedgeshaped cuts across the lamellae penetrating through ten or more of these, towards the interior. I consider these cracks as artifcial ones (supported by the fact that lamellae which are most conspicuous continue on the other side of the cracks), which may be compared with the transverse cracks I described some time ago (Biologische Inleiding tot het Cellulose-symposium,

Chem. Weekblad **30**, 2—19, 1933) in the case of fibres of which cross-cuts have been prepared along chemical lines, according to the method of J. WIESNER (Unt. üb. die Organisation der veget. Zellhaut, Sitz. ber. Akad. Wiss. Wien, Abt. I, **93**, 17—81, 1886) and M. A. EL KELANEY and G. O. SEARLE (The Chemical Sectioning of Plant Fibres, Proc. Roy. Soc. London **106**, 357—363, 1930). The fact that the cracks in the swollen wall of *Halicystis* occur only on the outer side indicates, to my mind, that the outer lamellae have been stretched by the growth of the wall to a larger extent than is the case with the inner lamellae, since in swelling agents a great stretch is followed by a great shrinkage (as I shall demonstrate elsewhere).

As far as the chemical nature of the wall substances is concerned, the following may be communicated.

The wall in a top view shows a strong blue colouration with a not too diluted solution of iodo-potassium iodide (with a diluted solution it becomes yellow). The wall, therefore, contains a compound which may be called *amyloid*, but I am not convinced that the "amyloid" of *Halicystis* is identical to the amyloid of higher plants. On the contrary, I consider it very probable that we have to deal here with another matter. It is interesting that H. ZIEGENSPECK, who made a special study of the occurrence of amyloid (see especially: Ueber Zwischenprodukte des Aufbaues von Kohlenhydrat-Zellwänden und deren mechanische Eigenschaften, Bot. Arch. **9**, 297—376, 1925), mentions three cases of the occurrence of this substance in the cell walls of algae, viz. in *Microspora*, in *Conferva*, and in the ring of *Oedogonium* (the latter instance ZIEGENSPECK derived from NÄGELI and SCHWENDENER: „Das Mikroskop"). As far as is known to me, there occurs no amyloid in the walls of *Siphonales*, and I have ascertained once more that no amyloid can be found in the wall of *Valonia*, of *Chaetomorpha* and of *Cladophora* (these were the only *Siphonocladales*[1]) which were at my disposal besides *Halicystis*).

I have also treated cross-cuts of *Halicystis* walls with iodo-potassium iodide, and was able to demonstrate that the thick inner wall built up of lamellae gives a strong amyloid reaction. The cuticula does not give this, naturally, but I got the impression that the double-refracting matter present under this cuticula too does not show the reaction in the beginning. However, as soon as the concentration of the iodine becomes somewhat high, there also, an intensive colouration appears.

It will be clear that also a solution of chloro-zinc-iodide causes a blue colouration, but the action of this solution produces a marked swelling very quickly, especially of the wall matter under the cuticula, which after the swelling is no longer coloured blue. In every case it not allowed to conclude from this reaction at the presence of cellulose, as probably has been done by KUCKUCK.

[1]) I am following here the nomenclature of H. PRINTZ in "Die natürlichen Pflanzenfamilien" 2. Aufl., **3**. Bd., Leipzig (1927).

With ruthenium red only a faint red colouration may be observed. Reaction on cross-cuts shows that this colouration is restricted to the cuticula: I could not make out whether the colouring substance (which must be considered, presumably, as a pectinous matter or as another cell wall matter derived from a poly-uronic acid) is present in or immediately under the cuticula. With certainty I could determine that the material which shows the strong double refraction does not give the reaction with ruthenium red, for this material lies slightly deeper in the walls, toward the interior, than the constituent which may be coloured with ruthenium red.

With coralin soda solution, and also with a solution of reso-blue (acc. to TSWETT) and with the acetic acid solution of marine blue (acc. to L. MANGIN) I could observe that the wall gives a strong so-called *callose* reaction. I wish to state, however, that the conception "callose" has been very insufficiently defined, and I doubt seriously whether all cell wall substance which shows these reactions is chemically the same matter. I even consider it possible that the same material which above has been indicated as amyloid will give also the callose reactions. In the cross-cuts I was not able to observe any difference in intensity of the callose reactions between the various wall layers.

Further, I let a mixture of dyes react, which mixture is used for "metachromatic" colourations, namely a solution consisting of the following: benzobrown 0.5 g, oxamineblue 0.5 g, sodium carbonate 0.5 g, in 100 cm^3 of water. A distinct difference in colouration was to be seen here: the layer with the many lamellae became blue, the cuticula and also the strongly refracting layer underneath, turned brown.

Finally I treated the wall for three days with a strong solution of cupric oxide ammonia, after that with diluted acetic acid, and finally washed it with water. As was to be expected from the above, no double refraction was visible when the specimen was now viewed from the side; the band system under the cuticula, observed between crossed nicols, seemed to have disappeared. On the folds of wall parts folded double, double refraction could certainly still be observed; I ascribe these to tensions in the folds. The cuticula was visible as a separate layer; the pectin reaction was as weak as that on the untreated wall. It appeared further that the callose reactions, as well as the amyloid reactions, gave positive results with the wall treated with cupric oxide ammonia. The first mentioned reactions showed nothing of importance. On the reaction of iodine solution (I received the impression that much iodine was necessary to make it start) a remarkable structure became visible; the blue colouring appeared to be concentrated in nodal points from which small blue bands radiated, which connected the nodes to each other. I wish to call attention to the fact, however, that in the foregoing it was stated that cross cracks occurred in the wall, perpendicular to the wall surface, after intense swelling. On viewing swollen walls from the side, one will therefore look through layers of different thickness. Presumably the just ment-

ioned distribution of the amyloid has something to do with this, but I dare not decide whether there are no other reasons why this structure became visible.

From the foregoing it follows that the thick inner wall layer of the larger cells of *Halicystis*, which are built up of lamellae, does not consist of cellulose, but presumably of a mixture of amyloid and callose, or of a cell wall substance which shows both the reactions of amyloid and callose. Besides the lamellae structure nothing particular was shown by this thick layer. The lamellae are presumably visible because of a difference in content of water on the outer and the inner side of a lamella, but the layer is further probably completely homogeneous and optically isotropical when not under tension.

The nature of the strongly double-refracting substance which is present as a thin layer under the cuticula could not be ascertained; we shall return to this further on.

In any case, it becomes clear now why the mechanical nature of the wall of *Halicystis* differs completely from the one of *Valonia*. In a soaked condition the wall of *Halicystis* is slightly elastical and extremely supple; the wall of *Valonia* is coarse, very slightly elastic and paper-like.

The mechanical properties of the wall of *Halicystis* may be regarded as a consequence of the uniform condition of the thick inner wall layer. We call to mind here that the wall of *Valonia* consists of numerous lamellae built up of cellulose fibrillae which course, in two successive lamellae, along directions that cross each other, which structure must cause a great stiffness (see, among others, what I communicated about the wall of this one-celled alga in my lecture: "Introduction to the cellulose symposium", Chem. Weekbl. **30**, 2—19, 1933).

Finally I wish to state that on diaphragming strongly and focusing sharply on the various wall layers of *Halicystis*, also in regular light, the impression is given that the wall has still another structure. This structure is best designated as "fine grained", and I can subscribe LYNGBYE's statement, notwithstanding KUCKUCK's denouncement.

If I connect the previous results with what is known of related algae, the following may be stated.

In the first place the poverty in pectin is striking, compared, for instance, with the richness of the walls of *Siphonales* in this cell wall matter. I refer here to R. MIRANDE, "Recherches sur la composition chimique de la membrane et le morcellement du thalle chez les Siphonales" (Ann. Sc. nat., botanique 9e sér., **18**, 147—264, 1913). I should like to observe, however, that I consider it very probable that the "composés pectiques" of MIRANDE were partly compounds of poly-uronic acids, which we, at this time, should not call pectinous material any more. An investigation of the cell wall material of *Siphonales* in the light of recent observations in this field (I refer here to the thesis of my pupil H. A. FRANKEN: The presence, preparation,

and properties of uronic acids, and some related acids found in nature. Delft, 1934) promises really important results.

The poverty in pectinous material in the wall of *Halicystis* is especially interesting since *Valonia* too is poor in this material. With *Valonia* I found the material in, or directly under, the cuticula, thus in the same place as in *Halicystis*, but *Valonia* is somewhat richer in this cell wall material.

It is noteworthy, however, that *Valonia* is rich in cellulose, and that the presence of this in *Halicystis* is doubtful. It is certain that cellulose fibrillae which form the principle structural constituent of the walls of *Valonia* (see especially C. CORRENS: Zur Kenntn. der inneren Struktur einiger Algenmembranen, ZIMMERMANN's Beitr. Morph. u. Physiol. Pflanzen. **1**, 260—305, 1893, and F. BRAND, Ueb. die Faserstruktur der Cladophora-Membran, Ber. d. D. bot. Ges. **24**, 64—71, 1906), are lacking in *Halicystis*. The thick inner wall, rich in lamellae, which for the larger cells of *Halicystis Osterhoutii* forms 90 % of the cell wall, certainly does not consist — as I have said above — of cellulose. Yet I consider it not excluded that the double-refracting material, which we found under the cuticula, consists of cellulose crystallites which are imbedded in such a way in the material capable of swelling that with the swelling a disarrangement of the crystallites occurs, owing to which the double refraction disappears. This hypothesis is especially tempting, since with it a certain analogy with the cell wall of *Valonia* can be indicated. With *Halicystis* as well as with *Valonia* there should lie, inside the cuticula, an extremely thin layer which is rich in pectins, thereupon there should follow two lamellae with cellulose crystallites, which in the two lamellae should be arranged in directions which are practically perpendicular to each other. I note here that I sometimes found images, in viewing the wall of *Valonia* between crossed nicols, which reminded me of the photo shown above, which images I ascribe to the fact that in *Valonia* (also in the direction parallel to the wall) waves may be present in the fibrillae. The difference in the wall of the species of both genera should then be found herein: with *Halicystis* no new identical lamellae follow the two cellulose-containing lamellae, but numerous lamellae of amyloid should be found deposited on them from the inside; with *Valonia*, on the contrary, many wall lamellae with cellulose crystallites should be found next to the first two lamellae. One could call attention here to the fact that amyloid is considered by ZIEGENSPECK as an in-between product in the building up of cellulose, and it could be accepted, therefore, that in the lamellae of *Valonia*, which are situated more towards the interior, the synthetic process of cellulose formation goes one step further than is the case in the corresponding lamellae of *Halicystis*.

However attractive this explanation may be, I must state that I have not succeeded in settling for certain whether the double-refracting matter of *Halicystis* really consists of cellulose. It makes up only a small percentage of the wall, which is rich in amyloid, and which makes reacting for cellulose impossible. In this connection we do not refrain from warning the reader

that our photo may cause a flattering impression of the significance of the double-refracting matter; this significance can be seen only in the cross-cuts.

It is my conviction that a closer study of the wall of *Halicystis* and of its development may lead still to important points of view. For instance, I have already determined that the layer built up of lamellae of amyloid with young specimens is developed to a much smaller extent than with older ones, and above I called attention to it that the older lamellae are in all probability strongly stretched when the cells become larger; the deposition of the lamellae occurs therefore very probably by *apposition*. It is further noteworthy that the "stripe structure" appeared to be much finer with young walls than with older ones. This probably means that the waves of the series of crystallites become longer and higher as the wall grows older, from which, however, must be concluded that a uniform growth of these waves takes place by *intussusception*.

Finally I wish to state that also a chemical study of the wall of *Halicystis* will be found very promising, since in that wall a material is found which gives strong amyloid and callose reactions, while cellulose and pectinous matter are practically absent.

In any case it will be clear that the cell wall of *Halicystis*, which algae have been the subject of such interesting physiological investigations during the last years (among others, those of W. J. V. OSTERHOUT and M. J. DORCAS and of L. R. BLINCKS), is worth a closer study.

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Delft, July 1936.

Plantkunde. — *De grenzen der bloeibaarheid en het groeien van den Iris-bol.* IIB. (with summary). Door A. H. BLAAUW, IDA LUYTEN en ANNIE M. HARTSEMA. (Mededeeling N°. 50 van het Laboratorium voor Plantenphysiologisch Onderzoek te Wageningen.)

(Communicated at the meeting of October 31, 1936).

In het voorafgaande nummer der Proceedings werden (onder IIA) de bloei-resultaten na de proeven van 1934—'35 en 1935—'36 gegeven. Moeilijker is het bij een vermijden van den bloemaanleg tevens een behoorlijken groei te bewerken. De uitkomsten van dien groei worden hier nader besproken onder verwijzing naar de cijfers, die reeds in de 5 tabellen van IIA zijn opgenomen.

Groei.

In de cultuur worden de bollen gezeefd en aldus gesorteerd naar den omtrek der bollen. Dit sorteeren van bollen en het uitdrukken van de