SOME OBSERVATIONS ON BARIUM SULPHATE IN SPIROGYRA*

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SUMMARY

The dry weight percentages of $BaSO_4$ in *Spirogyra*, reported in an earlier, preliminary paper were corrected and determined at 0.4–0.7%. Electron microscopy has shown that the $BaSO_4$ is present in the protoplasm and not in the cell wallt. It has the form of individual, mainly rectangular prisms, the majority of which is about 0.3 μ long with an average width about 0.17 μ and variations in size ranging up to about 0.9 × 0.48 μ. Presumptive evidence has indicated as their face of greatest extension the crystallographic plane containing the unit cell axes $a = 8.87$ Å and $b = 5.45$ Å. Generally, they occur at wide inter-distances, and as far as the material allowed to be concluded, mainly in the periphery of the cytoplasm. On the basis of the dry-weight content and crystallite size an estimation has been made of the distance between the crystallites in case of an even distribution in the living cell and of the degree of accumulation of $BaSO_4$ in the latter. The crystallites were found to be visible under the polarizing microscope.

1. INTRODUCTION

In a brief note on the occurrence of $BaSO_4$ in fungi and algae (KREGER 1957) the results of some experiments have been reported indicating that the $BaSO_4$ obtained by R. Frey in residues of extraction of fungi (FREY 1950) was a contamination introduced by the extraction procedure. On the other hand, the observation made originally by E. Nicolai, that samples of dried *Spirogyra* produce X-ray diffraction lines resembling those of $BaSO_4$, could be confirmed. It was shown then that $BaSO_4$ can actually be isolated from the algae, and dry weight percentages were determined.

Although the occurrence of barium in plants, probably as sulphate, has been established in other instances, in particular in certain crops (ROBINSON *c.s.* 1950) and woods (ELLIS 1965), its presence in quantities like those found in *Spirogyra* is unusual, and in algae $BaSO_4$ had not been found before. The observation, therefore, gave rise to several questions. In the first place, one would like to know something about the location of the substance in the cells and about the size of its particles. Other questions concern its role in the life of *Spirogyra* and its occurrence in other algae, which will not be considered here.

As to the first problem, it has been noted already in our earlier paper that the $BaSO_4$ would probably form part of the cell contents because it was absent in cell walls isolated by a physical cleaning procedure from a suspension of

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crushed cells, whereas it was present in the protoplasmatic fraction from the same suspension. This seemed to be contradicted, however, by the observation that cell wall preparations obtained by chemical extraction of the filaments produced X-ray diagrams with $BaSO_4$ lines. Moreover, these revealed a distinct orientation of the crystallites with reference to the tangential plane of the walls. Therefore, the particles might have to do something with the wall, from which they could have been removed in the preparations gained by physical means, *e.g.* by the shaking with glass beads involved in the procedure.

Since $BaSO_4$ is a heavy molecule and the X-ray diagrams were proof of its presence as small crystals, the latter were suspected to be easily visible in the electron microscope as electron opaque spots. This could then provide conclusive evidence concerning its presence in either the protoplasm or the wall or in both of them, as well as data about the size of the crystallites.

A research along these lines was undertaken to examine in the first instance, for the sake of simplicity, material stored in 70% alcohol. If the crystals were visible this would open the possibility to investigate, by more satisfactory fixation techniques, details of their distribution in fresh cells. The results of the former step are reported here, and will be discussed in connection with the results and experimental data of the earlier work. Since the latter data were omitted in the brief note they are fully mentioned in the present paper.

2. MATERIAL AND METHODS

Spirogyra was collected from basins of the water works of The Hague and garden ponds and ditches, mainly of the botanical gardens of the Delft Institute of Technology and those at Haren-Gr of the University of Groningen. The species were not determined and varied in filament thickness from about 165 μ (water works) to about 60 μ. The samples examined were of homogeneous filament thickness, and were stored in 70% ethanol or kept in concrete containers at a cool place outdoors.

2.1. Preparation of clean cell walls

2.1.1. Native walls

Clean, native walls were prepared from living material which was treated in a blendor until a suspension was obtained containing pieces of no longer than two or three whole cells and broken cells. These were separated on the centrifuge from cell contents and then crushed further by shaking in a Mickle shaker with glass beads about 230 μ diameter. Subsequently, the wall material was collected by repeated washing and centrifuging, in which the use of slightly alkaline water promoted the removal of plasma debris. Not all samples could be fully freed from the latter.

2.1.2. Walls cleaned by extractions

Another method started with material stored in 70% ethanol. The threads were heated in 96% ethanol at 60 °C for 1 hour, after which the alcohol was decanted

and replaced by a mixture ethanol-acetone 1:1, in which the material was boiled on reflux for 15 minutes. This was sometimes repeated, dependent on the degree of bleaching attained, until it was light yellow to white. A further treatment with 3% NaOH or KOH solution at 100°C on a water bath gave nearly empty cells, usually still containing some granular material.

2.2. Determination of $BaSO_4$ content

The weighed, dry material was ashed in a porcelain crucible, washed with warm water, dried, mixed with aqua regia and evaporated on a steam bath. Water was added and evaporated, after which it was washed several times with warm water until neutral. It was then melted with equal quantities of Na_2CO_3 and K_2CO_3 in a platinum crucible. The melt, after extraction with water and washing until free of sulphate, was dissolved in HCl from which $BaSO_4$ was precipitated by addition of H_2SO_4. (After ARTIS & MAXWELL 1916, slightly modified.)

The accuracy of this procedure was determined by treating in the same manner two samples prepared by mixing 17.7 and 16.6 mg of $BaSO_4$ respectively with $CaCO_3$, $Ca_3(PO_4)_2$, $MgSO_4$, 10 mg of each, and starch and peptone in the proportion 1:1 to make 3 g. The yield of $BaSO_4$ was 12.0 mg in both cases, an average therefore of 70%.

In determining the dry weight content of $BaSO_4$ in *Spirogyra* stored in alcohol, the fact had to be accounted for that part of the protoplasm dissolved in the alcohol. In flasks standing for some weeks the ratio of the dry weights of the filament fraction and the material obtained from the alcohol by evaporation was about 2:1. Therefore, dry weight percentages of $BaSO_4$ in material from alcohol had to be corrected for fresh material and for losses during the quantitative analysis. The two correction factors, 2/3 and 10/7 respectively, nearly neutralize each other.

2.3 X-ray diffraction and electron microscopy

X-ray diffraction was carried out with a flat-film camera and Ni-filtered CuKα radiation on specimens 0.5 mm thick in the beam direction, using pinhole collimators 40 mm long and 0.5 or 0.25 mm diameter. The specimen-to-film distance was 40 mm.

The specimens for electron microscopy were embedded in Vestopal W according to Ryter and Kellenberger and sectioned on an LKB-Ultrotome with glass knives cut at 45°, in which a clearance angle of 4°, a speed of 1.0 mm/sec. and a feed of 900 Å were found to be favourable. With thinner sections most crystallites were pushed out of the section. Generally, no metal staining was applied because the crystallites were expected to be highly electron opaque by themselves, and introducing heavy metal might obscure their perceptibility.

3. RESULTS

3.1. X-ray diffraction

X-ray diagrams demonstrating the presence of $BaSO_4$ in *Spirogyra* are shown in

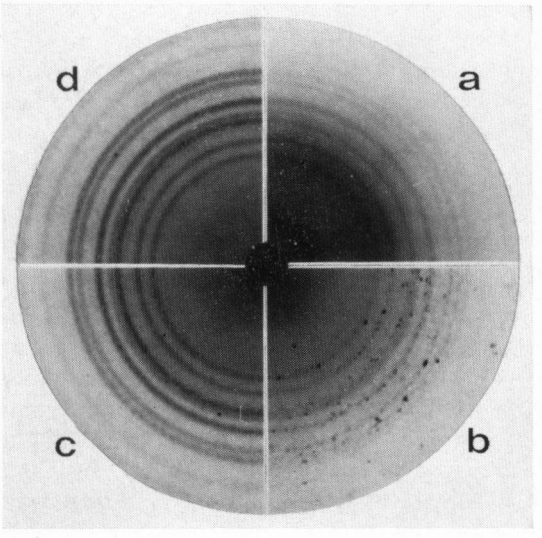

Fig. 1. Quadrants of X-ray powder diagrams of *Spirogyra* in several stages of treatment, demonstrating the presence of $BaSO_4$.
a. Extracted with alcohol and acetone,
b. Ashed sample after extraction with aqua regia,
c. Precipitate with H_2SO_4 in HCl solution of carbonate melt,
d. Commercial sample of $BaSO_4$.

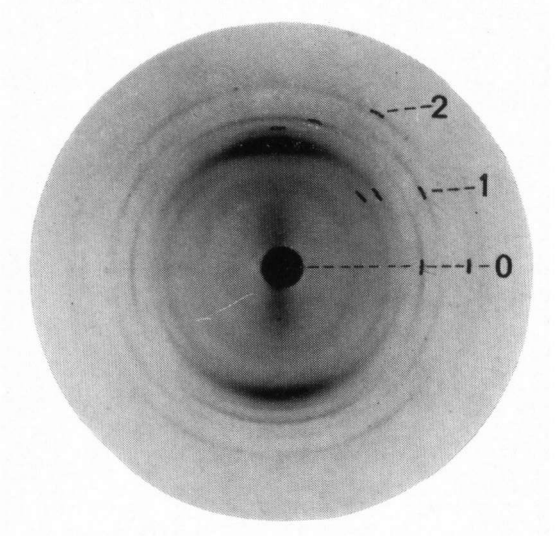

Fig. 2. X-ray diagram of a stack of flattened cell walls from which the contents had been removed by extraction. Beam parallel to the walls, the latter parallel to equator and nornormal to page. Layer-lines are indicated in one quadrant, with the corresponding positions of the centres of the arcs of enhanced intensity in the $BaSO_4$ rings. The broad arcs are cellulose reflections.

fig. 1. It is evident that the diffraction rings in the various stages of treatment correspond with those of a sample of commercial $BaSO_4$.

Fig. 2 represents an X-ray diagram of *Spirogyra* filaments cleaned according to 2.1.2. and irradiated in a direction parallel to the walls. For that purpose the filaments were suspended in water and dried on a glass surface to form a thin, coherent layer, in which the cell walls flatten out on to the glass. Several of such layers were heaped with some gum arabic as an adhesive. A strip, about 1 mm wide, was then cut out of the stack and irradiated in a direction perpendicular to the direction of cutting and parallel to the leaflet faces. The equator in the diagram of *fig. 2* is parallel to the longitudinal direction of the strip.

The diagram shows diffraction rings all of which exhibit arcs of distinctly increased intensity, with a symmetrical position with reference to the equator, thus revealing a definite orientation of the crystallites in the specimen. The sharp diffraction rings correspond to $BaSO_4$ spacings, while those which are somewhat broadened come from cellulose micelles. The position of the arcs in the latter indicates that the *Spirogyra* species in question has in its walls cellulose with a so called uniplanar orientation of crystallites in which the plane (002) is parallel to the wall surface (KREGER 1957).

The orientation of the $BaSO_4$ crystallites can be determined similarly from the position of the intensified arcs and the known crystallographic data of $BaSO_4$ mentioned in the A.S.T.M. data file (SMITH 1960). The crystals are indicated to be orthorhombic with axes $a = 8.87$ Å, $b = 5.45$ Å and $c = 7.15$ Å. The data provided by the diagram from which the orientation can be derived are mentioned in *table 1*. They are indicated as follows: $2f_{40}$ = ring diameters in mm of rings with distinct arcs, d = corresponding spacing in Å, hkl = Miller indices of the crystallographic plane with spacing d (from SMITH 1960), n = layer-line position of the arcs.

Table 1. Data from *fig. 2* used to determine the crystallite orientation of $BaSO_4$ in the specimen in question. Explanation in the text.

$2f_{40}$	d	hkl	n
29.7	4.35	011	1
33.5	3.90	111	1
37.0	3.58	002	2 (merid.)
38.4	3.44	210	0 (equat.)
40.4	3.32	102	2
43.6	3.10	211	1
48.9	2.83	112	2
51.4	2.73	020	0

The correspondence of the layer-line number n with the Miller-index l allows of the interpretation that the axis c has a preferential orientation normal to the incident beam and the equator of the diagram, *i.e.* normal to the leaflet plane of the specimen or the cell wall surface. Hence, since the crystallites are orthorhombic, they are oriented with the a, b-plane parallel tot the wall.

3.2. Electron microscopy

At first sight the sections did not show the crystallites to be expected, but careful searching revealed in some of them the presence of individual, small and opaque crystallites, measuring about 3000 Å, with a distribution in the order of one or two in a field about 20 × 20 μ. Sometimes they appeared as undamaged prisms, but in other cases as trapezia, or there was a small hole containing remnants of a crystallite fractured by the knife. Also empty holes were observed

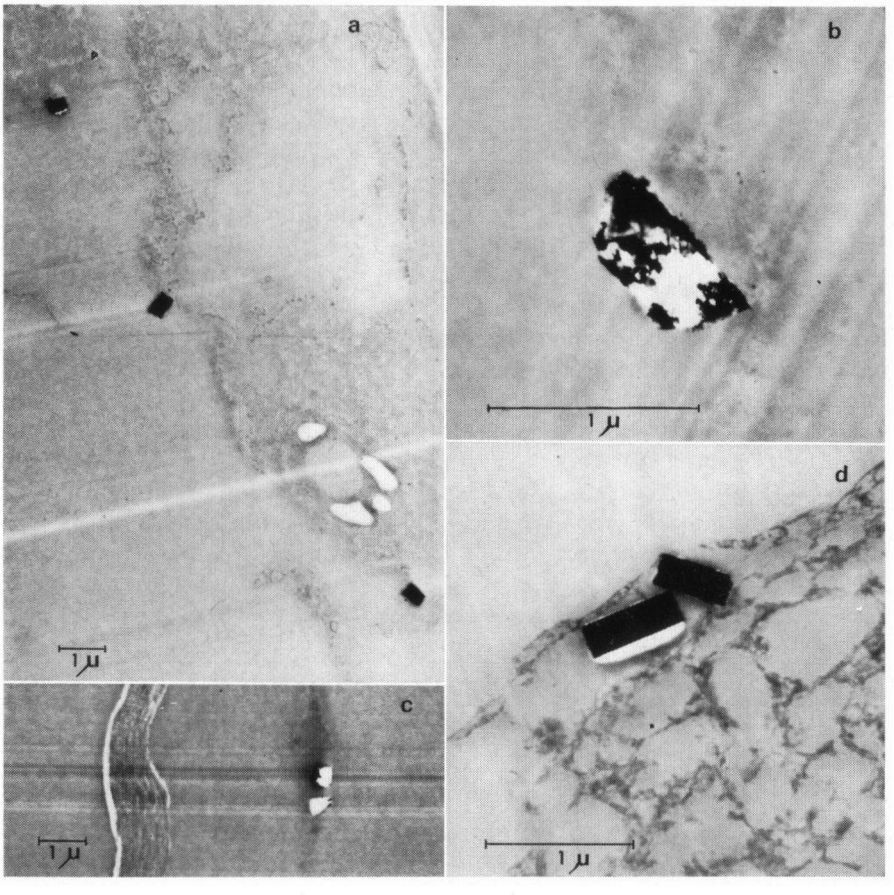

Fig. 3. Electron micrographs of some $BaSO_4$ crystallites.

a. Part of a section showing 4 crystallites. Note their connection with the periphery of shrunken protoplasm. Top right: part of the cell wall. Bottom right: cross section of a chloroplast with electron transparant core and empty holes which presumably have contained starch.

b. Fractured crystallite at high magnification. Note contact with plasma membrane.

c. Two empty holes, with scratches due to knife damage caused by the crystals that have been pushed out.

d. Two well formed prisms. Plasma in this case stained with uranyl acetate.

where crystallites had been fully pushed out of the section. Most sections were heavily scratched, and where a crystallite or its remnants was found, it was frequently situated in a scratch as proof that the scratches were caused by damage of the knife edge on the crystals. Some examples are shown in *fig. 3.*

As to the location of the crystallites it may be noted that they were all present in the cell contents. In no instance were any crystallites observed in or on the wall.

Measurements of width and length of some 60 crystallites yielded distribution curves showing a peak at 1700 Å for the widths and at 2800 Å for the lengths, in which the half peak numbers for the widths were found at 1400 Å and 3000 Å, and for the lenghts at 2000 Å and 4500 Å. In addition there were a number of bigger crystals around 4500 × 7500 Å, representing about 10% of the total. About 55% of the crystals appeared in the sections as rectangles, 20% as trapezia, 15% as squares, 8% as rhombs and a few were round.

The plasma, despite the absence of any staining, stands out reasonably well and shows a strong plasmolysis and retraction around the chloroplasts. Most crystals were observed on the inner side of the membrane surrounding these clusters or in contact with plasma clusters and membranes elsewhere in the cell.

Although outside the scope of this paper, it may be noted that the chloroplasts are characterized in the sections by a ring of large, very electron transparant spots in their periphery, which on account of their shape are reminiscent of starch grains. By metal shadowing of some sections they were shown to be empty holes. It is supposed that the embedding medium had not been able to penetrate the starch, and that this, by swelling, had disappeared when the sections were floated on a water surface to come free from the knife. The chloroplasts have a nearly round core which is slightly more electron transparant than its surroundings.

3.3. Dry weight percentages of $BaSO_4$

A survey of the most representative determinations of the $BaSO_4$ content is given in *table 2.*

Table 2. Dry weight percentages of $BaSO_4$ found in some samples of *Spirogyra.*

sample	time in alcohol	dry weight (in g)	$BaSO_4$ (in mg) precipitated	in % of dry weight	in % corrected
1	2 weeks	4.55	20.6	0.45	0.43
2	2 months	4.08	18.00	0.44	0.42
3	fresh	1.03	3.0	0.29	0.415
4	2 weeks	1.35	10.5	0.78	0.75

Samples 1, 2 and 3 in *table 2* refer to a *Spirogyra* species with filaments about 165 μ wide, collected from water works basins. No. 3 had been kept for three months in an outdoor container, and the sample consisted of two fractions: 198 mg clean walls prepared as indicated under 2.1.a. in the methods, and 836 mg cell contents obtained by evaporation of the collected washings. The

$BaSO_4$ content was determined in the two fractions separately, in which only a negligible quantity was found in the walls. Sample 4 refers to a different species with much thinner threads, collected from a ditch in the Delft botanical gardens. It contains more $BaSO_4$ than do the other samples, and the X-ray diagram of *fig. 2* refers to material from the same batch.

4. DISCUSSION

Since $BaSO_4$ crystallites in the cell wall, or against the wall, were never observed in the electron micrographs, and the cell wall fraction of sample 3 in *table 2* was free of $BaSO_4$, we may conclude that it does not form part of the wall. The only reason for the presence of $BaSO_4$ crystallites in cell wall specimens obtained by extraction, as shown in the X-ray diagram of *fig. 2*, and in fact by the diagrams of a number of similarly prepared specimens, has to be sought therefore in their insolubility, by which they remain captured in the empty cells.

The reason of the orientation of the crystallites might be twofold. In the first place, their plane of orientation, *i.e.* the *a*, *b*-plane or crystallographic plane (001), might correspond to the crystal face of greatest extension, on account of which the crystals would orientate themselves during the process of drying with this plane parallel to the wall. In the second place, there might be some physico-chemical interaction between the oriented cellulose micelles, or the interfibrillar matrix substance of the wall, and the $BaSO_4$, which predominates in (001) and, hence, would evoke the orientation. Since $BaSO_4$ is chemically very inert we prefer the former possibility.

The data about the dry weight content of $BaSO_4$ in the cells and the size of the crystallites enable a rough calculation to be made of their mean distribution in the cell. For that purpose the crystallites may be devided into two size-groups, one ranging in length about 3000 Å and with a square basal plane about 1700 × 1700 Å, *i.e.* with a contents about 0.009 μ^3, the other with a crystallite length about 7500 Å and a square basal plane of 4500 × 4500 Å, *i.e.* with a contents about 0.15 μ^3. If the latter group comprises, as is indicated by the measurements, about 10% of the total number of crystals, the numbers of the two types are in the proportion 9:1, and the quantities of $BaSO_4$ represented by each group in the proportion 9 × 0.009:0.15, *i.e.* about 8:15 or roughly 1:2. Therefore, only 1/3 of the 0.45% $BaSO_4$ of the cells is present in the form of the smaller crystals, while these represent about 90% of the total number of crystals. Neglecting the large crystals this enables an estimate to be made of the mean cell volume containing one small crystal and of the mean distance between these crystallites.

In the further calculation we have to take into consideration that the dry weight of algae is no more than about 5% of the fresh weight, and therefore 0.15% $BaSO_4$ for the dry weight becomes 0.0075% for the fresh weight. Since the specific weight of $BaSO_4$ is 4.48, for which we take 4.5, and the specific weight of the fresh cells will not be far from 1, we find for the volume V containing one crystallite of 0.009 μ^3:

$$V/100 \times 0.0075 = 4.5 \times 0.009\ \mu^3, \text{ or } V = 540\ \mu^3.$$

The above outcome would mean that evenly distributed crystallites would occupy the centre of a cube about $8 \times 8 \times 8\ \mu$, and the mutual distance between the crystallites would then be $8\ \mu$.

The chance of hitting crystallites of a similar distribution and size by sectioning at 1000 Å feed in a section field of $18 \times 18\ \mu$, *i.e.* a field corresponding to a normal field of view on a 18×18 cm photograph at $10.000 \times$ magnification, can be calculated at about 1 in 5. This picture of the ease of detecting the crystallites does not seem to be far from what was experienced in practice.

From the above data it is furthermore easily calculated that a *Spirogyra* cell of average dimensions, say $80\ \mu$ diameter and $500\ \mu$ long, would contain – with a dry weight content of $BaSO_4$ around 0.5% – a number of crystallites in the order of 5000.

A last point of interest to consider within the scope of these calculations forms the question of the degree of accumulation of the $BaSO_4$ in the cells as compared with the concentration in the environment. Since the solubility of $BaSO_4$ in water of 20 °C is indicated as 2 mg/liter or 2^{-4}%, and conversion of the data of *table 2* for fresh *Spirogyra*, *i.e.* with a water content of 95%, leads to a content of 0,022 – 0,037% $BaSO_4$ in the cells, there is 110–185 times as much of the substance per unit of volume in the living cell as can be maximally present in the environment.

Because the size of the crystallites is near, or somewhat above the resolution limit of the light microscope, the question might be raised whether they are visible by this means. Our experience in this respect was that a *Spirogyra* containing $BaSO_4$ according to X-ray evidence, when examined under the polarizing microscope, showed many, very small, highly birefringent particles in Brownian movement. They were concentrated in the lower part of the cells and gradually reassumed this position after rotation of the filaments. Once knowing where to look we could also find them under phase contrast. There is hardly any reason for doubt that these particles were the heavy $BaSO_4$ crystallites. At the same time this observation seems to indicate that they are freely moving in the cell and are not connected to any organelle system.

REFERENCES

ARTIS, B. & H. L. MAXWELL (1916): Barium in tobacco and other plants. *Chem. News* **114**: 62.

ELLIS, E. L. (1965): Inorganic elements of wood. In: W. A. CÔTÉ Jr., ed.: *Cellular Ultrastructure of Woody Plants*. Syracuse University Press, Syracuse, N. Y., p. 181–189.

FREY, R. (1950): Chitin und Zellulose in Pilzzellwänden. *Ber. Schweiz. Bot. Ges.*: **60**: 199–230.

KREGER, D. R. (1957a): X-ray interferences of $BaSO_4$ in fungi and algae. *Nature (London)* **180**: 867–868.

— (1957b): New crystallite orientations of cellulose I in Spirogyra cell walls. *Nature (London)* **180**: 914–915.

ROBINSON, W. O., R. R. WHETSTONE & G. EDGINGTON (1950): The occurrence of barium in soils and plants. *U.S. Dept. Agric. Tech. Bull.* **1013**: 1–34.

SMITH, J. V. ed. (1960): *X-ray powder data file*, Sets (1–5), Revised. Am. Soc. Test. Mat., Philadelphia.