THE CRYSTAL LATTICE OF THE PYRENOID MATRIX OF PROROCENTRUM MICANS

K. KOWALLIK
Botanisches Institut, Universität Marburg/Lahn, W. Germany

SUMMARY
The pyrenoid matrix of the marine dinoflagellate Prorocentrum micans is shown to consist of regular close-packed units, which form a cubic face-centred lattice. Numerous lamellae usually consisting of two apposed thylakoids traverse the pyrenoid matrix. They normally run strikingly parallel to each other, with an average distance of 139 nm between each stack. The three-layered unit membrane of the thylakoids penetrating the pyrenoid is 70 Å thick, the same as the unit membrane of the chloroplast thylakoids. The total thickness of one thylakoid measures 190–220 Å.

The globular units of the pyrenoid matrix have a calculated mean diameter of 232 Å, forming different line and dot patterns (hexagonal and cubic arrays) due to different section angles. Hexagonal patterns on prints result from projections of superimposed close-packed layers; they do not belong to one close-packing. Line patterns parallel to the thylakoid direction are composed without exception of 11 contrasted lines (6 and 5 alternating). This fact suggests that a definite number of units is arranged between the thylakoid stacks, thus producing the constant matrix thickness. Comparable regions in the chloroplast matrix cannot be confused with the pyrenoid matrix, as they never display an ordered structure. A three-dimensional model of the matrix is presented.

Starch is stored only in the cytoplasm; there is no visible connexion with the pyrenoid. On the evidence of protein storage in similar crystal lattices both in plants and animals it is suggested that the pyrenoid may also be an organelle for protein storage in some groups of algae.

INTRODUCTION
Many attempts have been made in the past few years to resolve the fine structure of the pyrenoid. The first observations showed fibrils with a diameter of about 65–75 Å, separated by a space of the same dimensions (Gibbs, 1962a). These observations were recently confirmed in a short article (Brown, Arnott, Bisalputra & Hoffmann, 1967) describing the same fine structure as above, demonstrated with improved techniques for fixation and embedding. In an excellent series of papers concerning the fine structure of some Prasinophyceae, Manton showed impressive sections through several types of pyrenoids (Manton, 1965, 1966, 1967), but without any indication of a regular fine structure of the 'protein core' of the pyrenoids. In agreement with other authors (Sager & Palade, 1957; Gibbs, 1962a, b; Bouck, 1965; Chardard, 1965; Evans, 1966; Brown et al. 1967; Esser, 1967) Manton writes that 'the material involved usually appears finely granular and denser than the ground substance of the plastid stroma, though the ribosome-like particles and other normal stroma inclusions are absent' (Manton, 1967, p. 29).

It will therefore be of some interest to report on my observations on the pyrenoid of the marine dinoflagellate Prorocentrum micans. The present paper deals only with
the fine structure of the thylakoids penetrating the pyrenoid and with the ultrastructure of the stroma region situated in between. Observations on other constituents of the pyrenoid will be published later.

MATERIAL AND METHODS

Our clone of *Prorocentrum micans* Ehrenbg., an asulcate dinoflagellate, is a vegetative descendant of a strain collected from the North Sea which has been cultivated in our laboratory by Professor von Stosch since 1953. The culture medium used is a modified Erdshreiber solution (for further information see Manton & von Stosch, 1966) without additional vitamins. The cells were grown in culture dishes or flasks under fluorescent light of about 4000 lx (21 °C, 14:10 h light:darkness) or about 1000 lx (15 °C, 16:8 h light:darkness) and collected by gentle centrifugation when the culture was still young and growing well.

Fixation was carried out according to Manton's procedure for sea-water organisms (Manton, 1965), i.e. 4 % glutaraldehyde neutralized with CaCO₃ and made up in 0.05 M cacodylate buffer, pH 7.0. In order to obtain isotonic conditions the final mixture contained 0.25 M sucrose. The fixation time ranged from 1 to 5 h at 4 °C or room temperature, followed by several rinsings in buffer with decreasing sucrose content. Post-fixation in 1 % or 2 % OsO₄ in cacodylate buffer pH 7.0 was applied for periods ranging from 2 h to overnight, either at 4 °C or room temperature. Dehydration in ethanol and embedding in Epon were carried out according to the usual procedure. For certain purposes the cells were fixed overnight in a mixture containing 3:1 methanol:formic acid (von Stosch & Drebes, 1964) and then directly transferred to 90 % alcohol without postosmication. The subsequent procedure was the same as above. The material was sectioned either with glass knives or a DuPont diamond knife mounted on a Reichert Ultramicrotome OM-U 2; the sections were picked up on carbon-coated copper grids and double-stained with uranyl acetate (20 min), followed by lead citrate (10 min), and finally examined in a Siemens electron microscope, Elmiskop I A. For complete serial sections the blocks were carefully trimmed to produce sections covering exactly one square plus one bar of the grid. Using this method a given part of the sections recurs at the same point of each following square. It was necessary to use only high-resolution micrographs; therefore the astigmatism of the lenses was corrected very carefully. Most of the negatives were photographed at magnifications of ×40000 according to the indication of the microscope. All measurements were done with dividers and a millimetre rule on prints enlarged with calibrated factors (mainly ×5 enlargement of the negatives) and the resulting data checked on models made of wadding balls. From about 400 measurements mean values and corresponding scatterings could be calculated.

RESULTS

*Prorocentrum micans* usually possesses two large pyrenoids, which can easily be seen under the light microscope. These are normally situated opposite each other approximately in the middle of the almost symmetrical valves of the almond-shaped cell, occupying part of the space between the arms of the U-shaped nucleus. Figure 4 shows a cross-section with a general view of the cell components. The pyrenoids occupy a strikingly large space. They can reach a length of 12–15 μm, which corresponds to a quarter of the cell length. That part of the pyrenoid which is nearest to the cell wall is covered by the chloroplast, while the inner portion generally extends nakedly towards the cell interior. According to its outer morphology the pyrenoid of *Prorocentrum* may be compared with the bulging pyrenoids of some lower brown algae (Evans, 1966). The photographs of *Prorocentrum* showed no evidence of stalked
pyrenoids as discovered by Dodge (1968) in two species of sulcate marine dinoflagellates. The considerable size of the pyrenoid of *Prorocentrum* and its characteristic inner morphology would preclude this anyway, as demonstrated in Figs. 4 and 5.

Some of the chloroplast thylakoids end at the transition point from the chloroplast to the pyrenoid; the majority traverse the pyrenoid remarkably parallel to each other. Usually the pyrenoid is clearly distinguished from the chloroplast by its characteristic matrix, even in a transition zone, which can extend over a larger area, so that in these cases the pyrenoid is closely dovetailed with the chloroplast (Fig. 9).

![Graph showing data](image)

Fig. 1. Diagram of the data obtained from the prints. The first peak belongs to the 116-Å distances between lines in cross-sections, the second one to the 135-Å distances between dots arranged in hexagonal patterns. The third group corresponds to the broad distances between lines in cross- and oblique sections, ranging from about 180 Å to 225 Å. All values were obtained from enlarged photographs using dividers and a millimetre rule.

High-resolution micrographs show that the pyrenoid matrix consists not of an irregular fibrillar or granular substance but of more strongly contrasted particles forming regular patterns. In all the pyrenoids examined dot patterns and line patterns are to be observed, which up to now have been established in similar form only in virus crystals (Morgan, Howe, Rose & Moore, 1956; Bergold, 1963), seed proteins (Perner, 1965), protein crystals in chloroplasts (Shumway, Weier & Stocking, 1967; Newcomb, 1967; Manton, 1967) and yolk crystals (Karasaki, 1963, 1967).

On cross-sections (sections perpendicular to the thylakoid direction) three different matrix configurations can be observed: (a) line patterns parallel to the thylakoid direction; (b) dot patterns with hexagonal arrays; and (c) line patterns perpendicular to the thylakoid direction. It is well known that the direction of the section has a decisive influence on the appearance of lattice structures. As demonstrated in the following, the three-layered structure of the unit membranes is a sensitive indicator
for exact cross-sections. For this reason Fig. 2 gives the dimensions of a thylakoid. The unit membrane of the thylakoid is composed of two electron-scattering lines approximately 20 Å thick with an uncontrasted layer of approximately 30 Å between. With a thickness of 70 Å it has the same dimension and structure as the unit membrane of the chloroplast thylakoids (Fig. 7) and may be compared with thylakoids from other sources (cf. Mühlethaler, 1960). The total thickness of one thylakoid is 190–220 Å, as the space between the unit membranes can vary from 50 to 80 Å. Assuming an average section thickness of 600 Å (Peachey, 1958), a maximum departure of ±3° from the exact cross-section may be allowed until the two dark lines of the unit membrane merge. If this condition for perpendicularity is fulfilled, each of the three lattice structures can be plainly associated with cross-sections.

(a) This pattern, a ‘fibrillar’ striation of the pyrenoid matrix running parallel to the thylakoid direction, is quite common. The interthylakoidal area is filled with 11 dense lines with a thickness of about 80 Å, having a centre-to-centre distance of 110–120 Å (mean value 116 Å) (Fig. 1). Corresponding to the 11 dark lines there are 12 light interspaces, by which a matrix thickness of 130–145 nm with an average value of 139 nm is produced (Fig. 6). It should be pointed out that in spite of several hundred measurements on various cross-sections not a single deviation from the 11 dense lines was found. This provides strong evidence for a definite number of units between the thylakoids.

(b) The dot pattern arises directly from pattern (a) in the same matrix region, the rows of dots forming angles of 60° or 120° to the thylakoid direction. As a mean value an angle of 121.5° with a scattering of ±5° was calculated. The dots, with a diameter of about 80 Å, are arranged in hexagonal patterns (Fig. 6), the mean distances between the dots (centre-to-centre) being 135 Å (scattering 125–142 Å, Fig. 1). This mean value is almost exactly 2/\sqrt{3} times the distance between the lines of pattern (a), i.e. the side of an equilateral triangle with a height of 116 Å.

(c) The line pattern perpendicular to the thylakoid direction connects with both the 116-Å line pattern and the 135-Å dot pattern, usually separated by narrow intermediate zones without defined structure (Fig. 6). The distances between these lines range from 190 to 225 Å (Fig. 1). In many cases it is apparent that these line patterns can be resolved as rows of dots with once again a mean distance of 116 Å. The blurred lines or rows of dots, the first visible after the unresolvable intermediate zone, are usually broader than the following ones. This is due to identical rows of dots situated behind each other being partially hidden by those in front. The increasing clarity of the following rows suggest that those rows situated behind each other are covered with progressive exactness by the uppermost rows (Fig. 6).

In an almost exact cross-section a dot pattern with 90° angles between the rows of dots is demonstrated in Fig. 11. Oblique sections reveal a greater number of lattice structures due to an additional degree of freedom. In Fig. 10 three different but common arrangements of dots may be observed. These different configurations are due to various angles with respect to the thylakoid plane. The first matrix region shows a dot pattern with dots having in this figure a mean centre-to-centre distance of 139 Å (this may correspond to the 135-Å distances of the hexagons in cross-sections) and forming
angles of about 105° or 75° between each other. The adjacent matrix region is characterized by distinct zig-zag lines, always arranged perpendicularly or at a slight angle to the thylakoid direction. The centre-to-centre distance between these lines ranges usually from about 190 to 225 Å (Fig. 1); in Fig. 10 a mean value of about 205 Å can be measured. In the third matrix region a line pattern almost parallel to the thylakoid direction is apparent, in which the distance between the lines is 135 Å. The striking similarity to the broad line pattern of the cross-sections suggests that we are dealing here with the same kind of lattice planes, which because of different section angles appear in different patterns.

The zig-zag lines frequently merge into hexagonal patterns, in which the same distances as in cross-sections may be measured. There is, however, one difference: compared with the hexagons of the cross-section each hexagon of the oblique section is turned through an angle of 30°. In addition to this, the zig-zag lines occur predominantly when the distance between the obliquely sectioned thylakoids increases to 300 nm. Since this distance can be used to calculate the section angle to the normal to the thylakoid plane, further information of the lattice structure and the position of the crystal lattice between the thylakoids can be obtained.

If the angle to the cross-section plane increases still further, the oblique sections merge gradually into sections in which the thylakoids are touched tangentially. It is obvious that the transition is a continuous one, and therefore no sharp distinction can be made as between cross- and oblique sections. It is also obvious that true tangential sections are found only partially, as the thylakoids are not flat over large distances (even if they are strikingly parallel in the cross-sections). The distances between lines in those sections agree closely with the corresponding distances between lines in cross- and oblique sections; the lowest values were about 185 Å, and the maximum values about 230 Å. The values differ from pyrenoid to pyrenoid, and also slightly within the same pyrenoid due to changing section angles (Fig. 1). The individual groups of lines meet at very different angles, though angles of about > 120° or < 60° and angles of 90° are predominantly observed (Fig. 12).

Broad matrix regions, often detectable within the chloroplast (Figs. 5, 8), cannot be confused with the pyrenoid, as they never display a crystalline form.

Obviously in the interpretation of ultramicroscopical observations the fixation problem plays a decisive role, especially when high magnifications are required. On the one hand structures may be altered during fixation or disappear; on the other hand, structures may deceptively appear to be there which do not exist in vivo (Shumway, Weier & Stocking, 1967). Therefore it was of interest to apply a fixation used otherwise only in light microscopy. Due to the properties of the fixation medium, methanol-formic acid, it was to be expected that proteins and proteids would be preserved by the alcohol and the nucleic acids by the acid. These expectations were confirmed, as proved by Fig. 13. The lipid-containing thylakoids were not fixed and were therefore dissolved by the alcohol, whereas the pyrenoid matrix showed the same lattice structures as with normal glutaraldehyde fixation. In addition, well-preserved ribosome-like particles were found within the pyrenoid matrix (compare the glutaraldehyde-fixed pyrenoids in Fig. 5) which will be dealt with in detail in a forthcoming
paper. Since the pyrenoid structure is preserved even by such a crude fixative, it is to be assumed that it will not be altered by the more gentle glutaraldehyde fixation.

**DISCUSSION**

In any analysis of ultramicroscopical photographs one of the main difficulties is that the two-dimensional prints present projections of three-dimensional structures. In this case serial sections are of little help, since the units forming the crystal lattice are certainly considerably smaller than the thickness of the sections. They prove merely that the lattice structures observed on one section recur in the same manner on subsequent sections.

The first indications of the specific type of lattice are provided by the hexagonal pattern observed in cross- and oblique sections. Hexagonal packing of equally sized units in a plane (each is surrounded by 6 others) could correspond in the three-dimensional lattice to the basal layer of the orthorhombic body-centred lattice or to the octahedral layer in the cubic face-centred lattice (Correns, 1949; Frey-Wyssling & Mühlethaler, 1965). It is of importance to our considerations that in the former lattice the fourth close-packed layer once again corresponds to the first; in the latter this would be the third (see Perner, 1965). In both lattices the units occupy a volume of 74%.

The first factor suggesting a cubic face-centred lattice in our case is the frequent occurrence of 90° angles of cross- and oblique sections; the most significant indications, however, are provided by indirect evidence arising from the supposed position of the crystal lattice between the thylakoids. It should be stated first of all that the crystal units must be at least as large as the largest distances measured. Since these distances range between 185 and 225 Å the hexagonal patterns in cross- and oblique sections cannot be formed of 135-Å particles (the diameter of the particles was equated to the centre-to-centre distance of the units forming the hexagonal patterns). A model constructed according to such assumptions does not show in any of the possible section planes distances exceeding 135 Å. Moreover, a maximum section thickness of not more than 150 Å may be calculated to avoid the hexagonal patterns of one crystal layer being obliterated by those situated behind it. This applies to the cubic face-centred as well as to the orthorhombic lattice.

Hexagonal patterns may, however, also occur as projections of superimposed close-packed layers, as shown in Fig. 2. This is only possible, however, in the cubic face-centred lattice, since here it is only the projection of the fourth layer that coincides with the first. Therefore the orthorhombic lattice can be excluded. If these considerations are applied to the micrographs, the visible hexagonal patterns with the 135-Å distances do not belong to one single close-packed layer. Each particle is then surrounded by 6 others, 3 in each of the neighbouring layers, as demonstrated in Fig. 2. The visible distances of 116 Å between lines in cross-sections must then correspond to half of the true particle diameter, which thus amounts to 232 Å. The 80-Å particles therefore represent only the dense core of the 232-Å units (Fig. 2). The assumption of an opaque core is supported by studies of very different protein bodies,
in which an electron-scattering inner part is surrounded by a less-dense sheath, e.g. ferritin molecules (Kuff & Dalton, 1957), virus crystals (Sjöstrand & Polson, 1957; Morgan et al. 1956) and seed proteins (Perner, 1965). The consequence for the crystal lattice model is that some of the octahedral layers of the cubic face-centred lattice must be arranged perpendicular to the thylakoid direction (Fig. 3). Likewise, octahedral layers must also occur in oblique sections, which is in fact the case in the model. The hexagons of these octahedral layers are turned by an angle of 30° towards the hexagons of the octahedral layers perpendicular to the thylakoid direction, in the same manner as observed in cross- and oblique sections.

Fig. 2. Two-dimensional representation of the crystal lattice as observed in cross-sections. The left-hand side shows projections of three superimposed close-packed layers, which can be seen singly, and differently shaded, on the right. For better understanding, only the dense core of the units belonging to the second (stippled) and third (white) layer are presented on the right side, whereas the units of the first (black) layer are demonstrated in their entirety. Note the position of the hexagons of one close-packed layer for comparison with the hexagons as seen on the left-hand side of the diagram or on the photomicrographs.
With the 232-Å units the broader distances between lines of approximately 185–225 Å can now be easily interpreted. Distances of 190 Å in cross-sections occur on the model if one octahedral layer is turned through an angle of approximately 20° (more exactly, \( \cos x = \frac{3}{\sqrt{2}} \)) towards the neighbouring octahedral layer. Angles larger or smaller than 20° produce broader lines or dot rows such as occur at the margins of the 190-Å lattices. The mathematical representation is \((d/3)\sqrt{6}\), where \(d = 232\) Å. Corresponding observations may be made of the oblique and tangential sections; the zig-zag lines and the broader distances between lines usually connected with them are also demonstrated on the model. However, close analysis of the crystallographic aspects will be made in a separate paper.

![Figure 3](Image)

**Fig. 3.** Three-dimensional model of the crystal lattice. Two close-packed layers (front and right side) and one cubic layer (left side) are apparent. The thylakoids must be considered as attached to the fourth visible surface (top).

Electron-microscopical studies to date give little indication as to the origin, reproduction, and function of the pyrenoids. *Prorocentrum* sometimes shows separate pyrenoids situated closely together (Fig. 5), suggesting a division of the pyrenoid before cytokinesis (see also Manton, 1966; Evans, 1966). Nevertheless light-microscopical studies would be more successful in clarifying this question (cf. Drawert & Mix, 1962). The functional nature of ‘the pyrenoid’ is also not clear. The convenient conception of it as a centre for the synthesis of reserve starch, which is subsequently deposited on the periphery, can surely no longer be postulated exclusively in this form, since starch deposited on the pyrenoid (which is, moreover, usually found together with normal stroma starch) is generally observed only in the chlorophycean and related groups of algae. Brown algae (Bouck, 1965; Evans, 1966) as well as diatoms (Drum, 1963; Drum & Pankratz, 1964) definitely do not accumulate starch, although distinct pyrenoids are present. *Prorocentrum* does possess starch as a reserve product, but this has no visible connexion with the pyrenoids, as in most of the red algae and Cryptomonadales. The starch is always deposited in the cytoplasm, as Fig. 4 demonstrates. Geitler (1926), however, showed for a species of *Peridinium* that a distinct starch shell
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closely appressed to a pyrenoid is obvious. Geitler described this pyrenoid as bulging nakedly into the cytoplasm.

We know from numerous light-microscopical studies (for literature see Thaler, 1966) and electron-microscopical studies, especially of higher plants (Schneepf, 1964; Perner, 1965; Shumway et al., 1967; Newcomb, 1967; Manton, 1967) that protein (mostly reserve protein) is often stored in a crystalline form. If one bears in mind that pyrenoids occur predominantly in primitive representatives of the individual algal groups (the same is true of the primitive liverwort Anthoceros) the conclusion that the pyrenoid of at least some algal groups could be regarded as a reserve organ for proteids rather than a place of synthesis for reserve starch (cf. Manton, 1967) is justified. The absence of pyrenoids in the advanced representatives of the algal groups could be explained by pointing out that these organisms have separate, differentiated areas for storing of reserve substances (this is, of course, true to an even greater extent of all higher plants).

In a recent short communication Holdsworth (1968) demonstrated a crystalline pyrenoid matrix of the diatom Achnanthes breviceps. This suggests that there might be similar pyrenoids in other algal groups, too. It will therefore be of interest to concentrate observations on those pyrenoids which either have no starch shell or which belong to algal groups without solid reserve carbohydrates. In conjunction with biochemical research a systematic study with improved preparation techniques of pyrenoid-containing representatives of different algal groups will doubtless lead to a more sophisticated view of the function of the pyrenoids.

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REFERENCES


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Fig. 4. Cross-section through *Prorocentrum micans* to show the main components. (c, chloroplast; chr, chromosome; cw, cell wall; er, endoplasmic reticulum; g, Golgi body; l, lipid body; m, mitochondrion; n, nucleus; nf, nucleolus; p, pyrenoid; s, starch; t, trichocyst; to, trichocyst opening.) Approximately x 6000.
Fig. 5. Two cross-sectioned pyrenoids situated close together. The chloroplast matrix (cm) can be clearly distinguished from the pyrenoid matrix by its lower contrast and irregular structure. Ribosome-like particles appear in groups within the pyrenoid. (Abbreviations as on Fig. 4.) $\times 45000$. 
Fig. 6. Cross-section through a pyrenoid to show the strikingly parallel thylakoids and the crystalline pyrenoid matrix in between. Dot patterns with hexagonal arrays are to be observed, as well as line patterns of constantly 11 dense lines parallel to the thylakoid direction, and line patterns with broad distances perpendicular to the thylakoid direction. ×120000. Inset: part of the matrix region of another pyrenoid, highly magnified. The dots arranged in hexagonal patterns are encircled. The unit membrane above belongs to the thylakoid envelope. ×250000.
Fig. 7. Part of two stacks of chloroplast thylakoids, demonstrating the resolved unit membranes. \( \times 150000 \).

Fig. 8. Section through a chloroplast; a large matrix region, which is not traversed by thylakoids, cannot be confused with the pyrenoid because of its irregular structure and lower contrast. \( \times 60000 \).

Fig. 9. Transition zone between chloroplast and pyrenoid. Note the matrix between parallel thylakoids; these regions already belong to the pyrenoid. \( \times 80000 \).
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Fig. 10. Oblique section through the pyrenoid, showing three different matrix configurations: dot pattern (left), zig-zag lines (middle) and line pattern (right). × 150000.

Fig. 11. Cubic arrangement of dots in an almost exact cross-section. The encircled dots represent the pattern of the basal layer of the cubic face-centred lattice. The arrows indicate the projections of units belonging to the adjacent cubic layer. × 150000.

Fig. 12. Tangential section through the pyrenoid, touching a thylakoid (left); the matrix shows line patterns and different angles between groups of lines. × 60000.

Fig. 13. Part of a pyrenoid in oblique section, fixed with methanol-formic acid. As expected, the thylakoids are not preserved (light spaces), whereas the matrix region shows the same pattern as with glutaraldehyde fixation. × 60000.