X-ray and Dissolution Studies of Paramylon Storage Granules from *Euglena*

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**Summary**

Paramylon is the \(\beta-1,3\) glucan storage carbohydrate in the euglenoid algae. Mature paramylon granules are highly crystalline, fibrillar, and have a complex substructure. X-ray diffraction was used to demonstrate that mature paramylon granules are much more crystalline than immature granules. Freeze-etch electron microscopy showed that in mature granules, the microfibrils are organized in highly ordered arrays while the microfibrils of immature granules are less organized. The data suggest that the high crystallinity of paramylon is due to higher-order aggregates of microfibrils and the interaction of water with the microfibrils. The dissolution of paramylon was recorded by darkfield videomicroscopy. In a 0.5 N NaOH solution, paramylon dissociates in a regular manner into its constituent 4 nm microfibrils, and the central region of the granule is the last remaining refractile area during the dissolution process.

**Keywords:** \(\beta-1,3\) Glucan; *Euglena*; Freeze-etch; Microfibril; Paramylon; X-ray diffraction.

**Abbreviation:** LN liquid nitrogen

1. **Introduction**

One of the characteristic features of the euglenoid algae is paramylon, their storage carbohydrate (Kiss and Triemer 1988). Paramylon is a \(\beta-1,3\) glucan that occurs as membrane-bound granules in the cytoplasm. The membrane surrounding the granule has been implicated in paramylon synthesis (Kiss et al. 1988). The granule is composed of microfibrils and has a complex substructure (Kiss et al. 1987).

Several approaches have been used to further elucidate the structure of paramylon. X-ray diffraction (Kreger and Meeuse 1952, Clarke and Stone 1960, Picciolo 1964, Leedale et al. 1965, Marchessault and Deslandes 1979), electron diffraction (Booy et al. 1965) and polarization microscopy (Deflandre 1949, Kampfner 1952, Pochmann 1956, 1958) have been used to further resolve the substructure of the paramylon granule. Numerous reflections in X-ray diffraction patterns of paramylon demonstrate that it is highly crystalline. Chemical degradation studies also have been used to examine paramylon structure (Clarke and Stone 1960, Picciolo 1964, Barber et al. 1966, Barras and Stone 1968), and dilute alkali solutions have been shown to disrupt paramylon granules (Barber et al. 1966, Barras and Stone 1968). In the present study, we examine various forms of isolated paramylon granules from *Euglena gracilis* by X-ray diffraction and freeze-etch. We use sodium hydroxide to demonstrate the regular, organized dissociation of paramylon into its elemental microfibrils.

2. **Materials and Methods**

2.1. **Cell Culture**

*Euglena gracilis* Klebs strain Z (Pringsheim, University of Texas Culture Collection, no. 753) was grown in a modified Hutner's medium. This medium and the system to regulate paramylon synthesis have been described (Kiss et al. 1986). "Immature" paramylon granules are defined as those granules isolated from cells at 0 hours in the experimental time course protocol. "Mature" paramylon granules are those granules isolated from cells at 24 hours or later in the experimental protocol (Kiss et al. 1986).

2.2. **Paramylon Isolation and Preparation**

In order to isolate paramylon, cells were heated in 2\(^{\circ}\) (w/v) sodium dodecyl sulfate in 0.125 M Tris buffer (pH 6.8). After 10 minutes, the paramylon was collected from the bottom of the tube and washed with distilled water.
Paramylon was dissociated by placing isolated granules in 0.5 N NaOH for 10 minutes and then washing in distilled water. Granules that were left in 0.5 N NaOH for longer than 2 hours or granules placed in a greater than 0.5 N NaOH solution were completely dissolved. Paramylon granules were dried by lyophilization. Granules were rehydrated within several minutes of the addition of distilled water (at room temperature).

2.3. Videomicroscopy

Isolated paramylon granules in distilled water were placed on a microscope slide, and a drop of 0.5 N NaOH was placed on the edge of the cover slip. As the NaOH diffused across the slide, the dissolution of paramylon was observed with a Zeiss Universal microscope using darkfield optics and was recorded with a videocamera. (Differential-interference-contrast and polarization microscopy also were used, however, darkfield optics were found to give the best image of the dissolution sequence.) Photographs of the dissolution process were taken directly from a videomonitor.

2.4. Transmission Electron Microscopy

In preparation for freeze-etch, paramylon granules and dissociated paramylon granules were frozen by the supercooled liquid nitrogen (LN) technique (Mueller and Brown 1980). LN was supercooled by placing the liquid into a Styrofoam container which was pumped to a pressure of 1-2 mm Hg in a Denton vacuum evaporator. After the nitrogen solidified, it was brought to atmosphere, and the uncryoprotected specimens on gold specimen discs were plunged into it. The samples were fractured and etched for 45 seconds-1 minute at -100°C in a Balzers BAF-301 freeze-etch apparatus equipped with electron guns and a quartz crystal thickness monitor. The specimens were shadowed with 2.5 nm of platinum-carbon and 25 nm of carbon at a vacuum of 1.5 x 10^-6 mm Hg. The replicas were cleaned in commercial bleach and sulfuric acid and then mounted on 100-mesh Formvar coated grids.

Dissociated paramylon granules (paramylon shells) were placed on Formvar coated grids and stained with 2% (w/v) uranyl acetate for 1 minute. In some cases, the shells were allowed to dry down on the grids for 15 minutes before staining (to allow for visualization of the microfibrils). All preparations were examined and photographed with a Philips 300 electron microscope operating at 80 kV.

2.5. Scanning Electron Microscopy

After dehydration through an ethanol series, dissociated paramylon granules were critical point dried, sputter-coated with gold-palladium, examined and photographed with a Hitachi S 450 scanning electron microscope operating at 25 kV.

2.6. X-ray Diffraction

X-ray powder patterns of intact and dissociated paramylon granules were obtained. Samples in glass capillary tubes (diameter 0.5 mm) were placed in a Debye-Scherrer camera and inserted into a Philips PW 1729 X-ray generator (operating at 35 kV and 25 milliamps).

For dried paramylon samples, the capillary tubes were sealed by flaming the end of the tube. The ambient room humidity was 58% R.H.

3. Results and Discussion

We examined various forms of isolated paramylon from Euglena gracilis by X-ray diffraction. Hydrated granules have an X-ray diffraction pattern with many discrete reflections which indicates that the granules are crystalline (Fig. 1). Our diffraction pattern is similar to the one published by Kreger and Meeuse (1952). The d-spacings for hydrated granules (Table 1) correspond closely to two previous X-ray studies of Euglena paramylon granules (Clarke and Stone 1960, Kreger and Van der Veer 1970).

<table>
<thead>
<tr>
<th>Wet paramylon</th>
<th>Freeze-dried paramylon</th>
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Figs. 5 and 6. Freeze-etch electron micrographs of paramylon granules. Scale bars = 0.5 μm

Fig. 5. Mature (wet) paramylon has highly ordered arrays of microfibrils.

Fig. 6. Immature (wet) paramylon does not have such organized arrays of microfibrils.
Freeze-dried paramylon granules have fewer reflections in the X-ray diffraction pattern than wet granules (Fig. 2: d-spacings given in Table 1) indicating that dried granules are less crystalline than wet granules. Rehydrated granules have a diffraction pattern identical to that of wet (never dried) granules (Fig. 3). We found that dried paramylon granules readily rehydrated within several minutes even though Marchessault and Deslandes (1979) reported that it was difficult to rehydrate dried paramylon. Taken together, these results indicate that water plays a role in paramylon structure and that it contributes to the high crystallinity of paramylon granules. A previous report demonstrated that the addition of water changed both the morphology (as seen with the scanning electron microscope) and the electron diffraction pattern of paramylon (Booy et al. 1981). In addition, water molecules have been shown to be part of the crystal lattice in curdlan, another β-1,3 glucan (Marchessault et al. 1977) and in the β-1,3 xylan of algal cell walls (Atkins and Parker 1969).

The above X-ray diffraction patterns were results of mature paramylon granules (Figs. 1–3). Paramylon levels in Euglena can be regulated (Kiss et al. 1986), and the granules from cells at the early stages of paramylon synthesis are referred to as “immature” granules. The X-ray diffraction pattern of immature paramylon granules is dramatically different from the pattern of mature granules (Fig. 4). The lack of discrete reflections in the diffraction pattern of immature paramylon indicates
that this paramylon is less crystalline than mature paramylon. This report is the first to consider the X-ray diffraction of paramylon granules at various stages of development since previous X-ray studies have been reports only of mature paramylon granules (Kreger and Meuse 1952, Barras and Stone 1960, Marchessault and Deslandes 1979). We can conclude from the X-ray diffraction results that the crystallinity of paramylon depends on its developmental state. The dramatic crystallinity changes in paramylon are not typical of most biological systems, but in one study, Chanzy et al. (1978) demonstrated crystallinity differences between the cellulose of the primary and secondary walls of cotton.

After having established the change in crystallinity between mature and immature paramylon, we now can ask what accounts for this difference. Freeze-etch electron microscopy is a useful method to study the microfibrils and fibrils (aggregates of microfibrils) of the paramylon granule (Kiss et al. 1987). In freeze-etch micrographs, the microfibrils of mature paramylon granules are in highly organized arrays (Fig. 5). In contrast, although immature granules are composed of microfibrils, these microfibrils are not found in such organized arrays (Fig. 6). The above data suggest that as paramylon granules mature, the microfibrils line up to form discrete arrays. The numerous discrete reflections in X-ray diffraction pattern of mature granules probably are due to higher order groupings of microfibrils rather than to individual microfibrils themselves.

Another useful method to examine paramylon structure is the use of chemical degradation studies. Paramylon granules have been shown to be disrupted by dilute alkali solutions (Barber et al. 1966, Barras and Stone 1968). We recorded the process of disruption of mature granules by an NaOH solution with a videocamera (Figs. 7-12). Using darkfield optics, we demonstrate that paramylon, before NaOH disruption, is visible as refractile, oval-shaped granules (Fig. 7). A few seconds after dissociation begins, the granules rotate and elongate (Fig. 8, taken 2 seconds, Fig. 9, taken 4 seconds after dissociation begins). By 7 seconds, two large refractile spots are positioned near the center of the granule, perpendicular to the long axis (Fig. 10). At 11 seconds, the two refractile spots become three smaller, less intense, spots (Fig. 11), and by 15 seconds, the refractile area is no longer present (Fig. 12). The remaining paramylon shell is about twice as long as the original paramylon granule (compare Figs. 7 and 12). The fact that paramylon granules always rotate and elongate during dissociation indicates that the microfibrils in intact granules may be under tension. The NaOH disrupts the bonding that maintains paramylon structure. Consequently, the microfibrils unwind somewhat like a clock spring under tension, which results in the formation of the paramylon shells.

What is the structure of these paramylon shells (dissociated granules) that remain after NaOH treatment? The central area of paramylon shells stained with uranyl acetate is more electron dense than the other parts of the shell (Fig. 13). The periphery of the shell has numerous fibrils (which appear to be composed of microfibrils, see Kiss et al. 1987) attached to it. The central area also is visible in scanning electron micrographs of paramylon shells (Fig. 14). The central area corresponds to the central region present in intact, in vivo granules (Kiss et al. 1987). During the dissolution process, the central region changes its orientation relative to the long axis of the paramylon granule. The fact that the central region is the last refractile area remaining suggests that its organization is somehow different from the other parts of the granule and that it may contain a tighter packing of microfibrils.

After the paramylon shells settle for several minutes on a grid (and then are stained with uranyl acetate the individual, elemental microfibrils are visible (Fig. 15). These microfibrils are in a loose network.

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Fig. 13. Transmission electron micrograph of paramylon shell stained with uranyl acetate. The central region (C) as well as the fibrils (►) on the periphery of the shell, are present. Scale bar = 1 μm

Fig. 14. Scanning electron micrograph of paramylon shell. Note the expanded central region (C). Scale bar = 1 μm

Fig. 15. Transmission electron micrograph of fully dissociated paramylon shell stained with uranyl acetate. Individual 4 nm microfibrils are present in a loose network. Scale bar = 0.2 μm

Fig. 16. Freeze-etch electron micrograph of paramylon shell. The 4 nm microfibrils are visible. Scale bar = 0.2 μm

Fig. 17. X-ray diffraction of paramylon shells (wet) demonstrating the loss of crystallinity following alkali treatment. (Same scale as Figs. 1-4)

Fig. 18. Freeze-etch electron micrograph of paramylon shell. A portion of the intact granule (with regular arrays of microfibrils) has dissociated microfibrils (►) attached to it. Scale bar = 0.2 μm
rather than the organized arrays present in mature, intact granules (Kiss et al. 1987). Freeze-etch also permits the visualization of the microfibrils present in paramylon shells (Fig. 16). In some cases, the loose network of microfibrils emerges from a portion of the granule with arrays of microfibrils (Fig. 18). In both freeze-etch and uranyl acetate stained micrographs, the smallest microfibrils measure 4 nm, which is the same as intact, in vivo granules (Kiss et al. 1987). Microfibrils of fungal β-1,3 glucan synthesized in vitro measure between 3–4 nm (Wang and Bartnicki-Garcia 1976). Two reports have suggested that these microfibrils of paramylon are composed of triple-helices of glucan chains (Marchessault and Deslandes 1979, Booy et al. 1981).

X-ray diffraction patterns of paramylon shells (that were wet) are rather diffuse, which indicates that the shells are not very crystalline (Fig. 17). Again, this result suggests that higher-order arrays of microfibrils rather than individual microfibrils themselves account for the high crystallinity of paramylon.

In summary, we have demonstrated that water molecules contribute to paramylon’s high crystallinity. Immature paramylon is much less crystalline than the mature form. Under dilute alkaline conditions, paramylon is dissociated into its constituent, elemental microfibrils, which measure 4 nm. The central region of the paramylon granule is organized in a different manner than the rest of the granule and may be the key to maintaining the complex structure of paramylon.

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