**237. Reconstitution of Vesicular Membrane from
Degraded Subunits of Pteridine Granule
Membrane in the Silkworm^{*)}**

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As we described in our previous papers, mature pteridine granules produced in the hypodermis of a normal larva have such a high density that they can be separated by procedures devised by us (Tsujita and Sakurai 1963, '66). Using those isolated granules, experiments on reconstitution of vesicular membrane from degraded membrane subunits were carried out.

Materials and methods. C124 and YD₄ strains were used as normal strains, and larvae homozygous for *w^b* or *ol* gene, allelic to *w-3* locus on the 10th chromosome, as mutant strains, producing abnormal and defective pteridine granules. Mature granules were separated from the larval hypodermis on the 4th day of 5th instar by a procedure shown in Fig. 1. Those isolated granules were dissolved in 0.2 mol phosphate buffer (pH 8.0) and heated at 95°C for 3 minutes. Non-degraded granules and granule fragments were sedimented using Spinco L type ultracentrifuge at 34,000×g for 40 minutes.

As it has been proved by us that granule contents, i.e. secretion products can be dialysed against Visking dialysing membrane, the supernatant was overnight dialysed against distilled water. Thus a solubilized membrane solution was prepared.

For the formation of granule membranes from degraded subunits, 1 volume of 0.156 mol sodium chloride solution containing 0.01 mol magnesium chloride was mixed with the supernatant. PH of the mixture which was about 8, was converted to 5 by adding acetic acid and the preparation was incubated at 5°C for 12 hours. The produced white sediment was separated by centrifugation at 3,000 r.p.m. for 10 minutes and examined in electron microscope.

Materials were fixed in 2% osmic acid containing Millonig's phosphate buffer. After fixation, the materials were dehydrated and embedded in Epon resin. Sections were stained with uranyl acetate and lead citrate.

Experimental results i). Reconstitution of vesicular membrane: After production of a white turbid substance when a solubilized

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Fig. 1. Separation procedure of pteridine granules.

granule membrane preparation was mixed with metal ion (Mg^{++} , Ca^{++} , Na^{+}) solution, it was centrifuged and examined in electron microscope. Aggregates of particles 40–50 Å in diameter, or of linearly arranged particles, could be observed in the sediment (Fig. 2). On the contrary, a number of granule vesicles surrounded by a thin membranes which were formed from self-assembling of membrane subunits were found in the white sediment produced by incubation at 5°C for 12 hours after the pH change of the mixture from 8 to 5 by acetic acid (Fig. 3).

It was observed that more or less large particles were scattered on the vesicular membrane, although their nature remains to be determined (Fig. 4).

Thin sections of reconstituted membrane materials showed a similar pattern to that of the original membranes of pteridine granules. For the formation of membrane structure, the presence of divalent cation (MgCl_2) gave the best results but CaCl_2 was as effective.

The granule membrane subunits from which the lipid has been extracted by treatment with chloroform and methanol no longer showed membranous structure; reintroduction of a lipid into the extracted subunits restored the property of vesicular formation.

Using abnormal pteridine granules separated from hypodermis of larvae homozygous for w^b , the same reconstitution experiments as above were carried out. However, it seems that the vesicle membrane is incompletely formed by degraded membrane subunits of w^b

mutant granule; aggregates of abnormally shaped masses of particles or fibrous substances were produced (Fig. 5).

ii) Fine structure of granule membrane: Using pteridine granules separated from the hypodermis of a normal larva (C124) the following electron microscopic studies were carried out.

After heating a suspension of granules in phosphate buffer (pH 8.0) at 95°C for 3 minutes the degrading granules were centrifuged and the sediments were examined in electron microscope. Fibrous substances (called "string") about 40 Å in thickness, sometimes bundles of them protruding from broken parts of granule membranes were observed. In a positively stained granule section, linear arrangement of 30–50 Å thick particles could be observed in the granule membranes (Fig. 6).

Especially when a frozen sample was thawed those fibrous substances could be easily observed (Fig. 7).

The fibrous structure was clearly observed in abnormally shaped pteridine granules produced in hypodermis of *ol* mutant larvae. In these mutant granules an abnormally thick membrane (Fig. 8) in which entangled short fibers, 0.2–0.3 μ in length, were observed. In addition short fibers, 0.2–0.4 μ in length, composed of linearly arranged particles (Fig. 9) or masses of them (Fig. 10) were scattered here and there around the granules. They seemed to be defective fibrous substances which failed to form vesicle membranes.

Abbreviation Rp: repeating particles; St: string.

Fig. 2. Aggregates of particles and fibrous substances produced in the mixture of solubilized granule membrane preparation and metal ion solution. $\times 20,000$.

Fig. 3. Reconstitution of membrane vesicles from degrading membrane subunits of the normal pteridine granules. $\times 20,000$.

Fig. 4. Reconstitution of membrane vesicles from degraded membrane subunits of the normal pteridine granules. More or less large particles are scattered on the vesicle membrane. $\times 14,000$.

Fig. 5. Incomplete formation of vesicle membrane from degraded membrane subunits of *w^b* mutant granules. $\times 20,000$.

Fig. 6. Section of the membrane of the *w^b* mutant granule. $\times 40,000$.

Fig. 7. Section of isolated pteridine granules which were previously freezed and thawed. A number of fibrous substances showing linear arrangement 40–50 Å thick particles can be observed. $\times 40,000$.

Fig. 8. Abnormally shaped pteridine granules with thick membrane produced in hypodermis of *ol* mutant larva. $\times 20,000$.

Fig. 9. Mass of fibrous substances which were scattered here and there around the granules. These also seem to be defective fibrous substances which failed to form vesicle membrane. $\times 20,000$.

Fig. 10. Fibrous substances showing linear arrangement of particles produced in hypodermis of *ol* mutant larvae. These seem to be defective fibrous substances which failed to form vesicle membrane. $\times 27,000$.

Figs. 2-10.

The fibrous structure was also confirmed in the reconstitution experiments with vesicular membranes; fibers about 40 Å thick, 0.3–1.2 μ long, running closely and almost parallel to each other in one layer were observed in newly formed membranes. Furthermore, in each of the fibers linearly arranged particles, 30–50 Å in diameter, were observed.

It seems from above mentioned observations that the membrane of pteridine granules is made up of rows of strings composed of linearly arranged particles.

Discussion. It was reported that protein and lipid are the principal components of pteridine granule membrane (Tsujiita and Sakurai 1966) and that their membrane protein consists of a homogeneous single protein (unit membrane protein) (Sakurai and Tsujita 1967).

Based on the formation of membranes by purified cytochrome oxidase (McConnell *et al.* 1966, Tzagoloff *et al.* '67) and on other many informations, Green and Perdue (1966) define the membrane of vesicular or tubular systems, as a continuum made up of repeating lipoprotein units (one layer thick) which are the only structural elements. As it is considered that their repeating units correspond to particles linearly arranged in strings in the pteridine granule membrane, the following diagrammatic representation of the vesicular membrane construction is given in Fig. 11.

Fig. 11. Diagrammatic representation of the membrane of pteridine granules as a fused continuum of repeating particles. It seems that particles are connected by a lipid.

Rp: Repeating particles; St: String.

Vesicular membrane formation from subunits confirmed by our reconstitution experiments may be regarded as a case of vesicularization by repetition of units, and strings which may be an intermediate form of vesicle membrane are characteristic in this case. It seems that the particles are connected by a lipid.

Generally speaking, organelles have two or more component membranes, each of them having entirely different repeating units and functional attributes and are rather systems of membranes than single membrane. However, it seems from our observations that the

membrane of pteridine granules produced in larval hypodermis of normal silkworm is a monolayer membrane consisting of a homogeneous single protein. Rarely, partly double membranes which are perhaps overlapping parts of the monolayer could be seen. It is highly probable that the membrane of pteridine granules is an example of a single membrane structure.

It was noted in our previous paper that there is clear-cut difference in electrophoretic pattern of the membrane protein between normal and *w^b* mutant granules. According to our other experimental results concerning amino acid analysis (unpublished data), slight difference can be seen in the amount of glutamic acid and lysine between the membrane protein of normal granules and *w^b* mutant granules. It may be assumed that these abnormalities of the membrane protein may be the cause of incomplete formation of vesicular membranes by the degraded subunits of *w^b* mutant membranes.

Summary. In the mixture of a solubilized membrane preparation and metal ion solution aggregates of particles 40–50 Å in diameter or filamentous substances showing linear arrangement of particles were produced. When pH of the mixture was converted from 8 to 5 and incubated at 5°C for 12 hours, a number of vesicle membranes were produced.

By the same experimental procedure using pteridine granules of *w^b* mutant larvae incomplete formation of vesicle membranes was observed which may be due to the abnormality of their membrane protein.

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