CHAPTER 6

NATURE OF CRYSTALS
6.1 INTRODUCTION

The crystal analysis is an important work to know the nature and chemical composition. Pearls have a complex structure of calcium carbonate secreted by the mantle cells. The crystals formed in the in-vitro and in-vivo experiments have to be confirmed for the presence of calcium to ensure that these were not any contaminant, which can occur during culture period. Crystal growth of shells was investigated under electron microscope and the role of mantle tissue and organic matrix in mineralization of shell was reported (Wada, 1958, 1960; Watabe, 1954). Wada (1961, 1964) investigated the crystal growth of shells by X-ray diffraction and electron microscope. Wilbur (1964) described the shell formation and regeneration of molluscan shells. Wilbur and Saleuddin (1983) explained the shell formation and Watabe (1981,1983) explained the shell repair in molluscan shells. Even though, there are lot of works on the growth of pearls in molluscan shells, the studies on in-vitro crystals were limited. Machii (1974) reported the secretion in the organ culture of mantle tissue of pearl oyster. Yano and Machii (1975) reported the amino acid composition of dark brown secretion found in organ culture of mantle tissue of pearl oyster. Machii and Wada (1989) reported the secretion in the explant culture of mantle tissue of pearl oyster. He analysed the crystals using X-ray, polarizing microscope and laser micro analyser. Samata et al. (1994) reported the SEM observation and EDS analysis of microcrystals developed over black secretion on the cultured mantle tissue of the pearl oyster, *Pinctada fucata*.

Explant and organ culture of mantle tissue of *H. varia* resulted in the formation of crystals in in-vitro condition as described in earlier chapters.
Crystals were also developed over the cover slip placed in between the mantle and shell in *in-vivo* condition of abalone as described in chapter 5. To ensure the presence of calcium in the crystals formed in *in-vivo* and *in-vitro* experiments, these were observed under scanning electron microscope (SEM), polarizing microscope and analysed by an energy dispersive X-ray microanalyser (EDS) for the purpose of comparison and confirmation.
6.2 MATERIALS AND METHODS

6.2.1 Oolitic crystals in explant cultures

Oolitic crystals developed in explant cultures were mostly attached to
the culture plate and hence found difficult to collect the crystals for the
analysis. So the crystals just appeared over the explant tissue were taken for
the EDS analysis. The tissue was washed thoroughly with distilled water and
dried prior to analysis. The free crystals were observed under polarized
microscope.

6.2.2 Rhombohedral crystals in explant cultures

The rhombohedral shaped crystals were collected from the medium of
the explant cultures and washed with distilled water by centrifuging at 1500
rpm for 10 minutes six times. The supernatant was removed and the crystals
were kept dry for the analysis. The free crystals were observed under polarized
microscope.

6.2.3 Crystals in organ cultures

The bead in the organ culture in agar base medium with pearl sac was
taken for the analysis.

6.2.4 In-vivo crystals

The coverslip placed between the mantle and shell was collected after
twelve days. The coverslip coated with crystals was washed gently in distilled
water and taken for analysis.

The samples were air-dried and were coated with gold by ion-sputtering
(JEOL-JFC1200E), and observed by an SEM (JEOL-JSM 5600LV, Tokyo,
Japan) and analysed by an energy dispersive X-ray microanalyser (EDAX, New Jersey, USA). The crystals formed on the coverslip inserted between the mantle and shell and the crystals developed in the \textit{in-vitro} cultures were observed under polarizing microscope (CENSICO, India).
6.3 RESULTS

6.3.1 Oolitic crystals in explant cultures

Explant with crystals was observed under SEM and seen that the crystals were of oolitic nature with different sizes ranging from 5 μ to 18 μ. The smaller ones joined together and formed larger crystals of irregular shape. The crystals analysed in EDS reflected the amorphous form of calcium carbonate (Plate 18). Few crystals are in hexagonal form and showed high birefringence under polarized microscope.

6.3.2 Rhombohedral crystals in explant culture

The rhombohedral crystals seen in the explant cultures were of different sizes. These crystals were observed under SEM. The observed crystals ranged from 7.5 to 12 μ in size and were seen rich in Ca with 53.97% by EDS analysis (Plate 19). These crystals showed high birefringence under polarized microscope indicating aragonite nature.

6.3.3 Crystals in organ cultures

In organ cultures, the bead with pearl sac were observed under SEM and the crystals of calcium carbonate were seen 10 to 50 μ in width over the organic matrix here and there over the bead (Plate 20 A&B). The crystals were embedded on the organic matrix secreted by the cells over the bead. The particular crystal analysed by EDS was 40 μ in width and 70 μ in length with a rhombic shape. These crystals were rich in calcium with 48.7% (Plate 21). Aragonite tablets were seen grown on the organic matrix over the bead (Plate 22).
Plate 18. X-ray spectrum of oolitic crystals developed over explant.
<table>
<thead>
<tr>
<th>Element</th>
<th>Wt%</th>
<th>At%</th>
</tr>
</thead>
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<tr>
<td>C</td>
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<td>23.91</td>
</tr>
<tr>
<td>O</td>
<td>33.13</td>
<td>46.10</td>
</tr>
<tr>
<td>Ca</td>
<td>53.97</td>
<td>29.98</td>
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</tbody>
</table>

Plate 19. X-ray spectrum of rhombohedral crystals
Plate 20 A. Crystals with organic matrix over the bead in organ culture. B. A portion of the bead with individual crystals.
### EDS Quantitative Results

<table>
<thead>
<tr>
<th>Element</th>
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<th>At%</th>
</tr>
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<tbody>
<tr>
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<td>72.52</td>
</tr>
<tr>
<td>Ca</td>
<td>48.70</td>
<td>27.48</td>
</tr>
</tbody>
</table>

**Parameters:**
- **kV:** 15.0
- **Tilt:** 0.00
- **Tkoff:** 34.73
- **Det:** SUTW
- **Res:** 134.00
- **Amp. T:** 35.0
- **FS:** 4806
- **LSec:** 201.3
- **Prst:** None
- **Date:** 3-Dec-2002
- **Time:** 11:54:01

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**Plate 21:** X-ray spectrum of crystal over bead in organ culture
Plate 22. 1500X. A portion of bead showing aragonite tablets over the organic matrix on the bead under SEM.
6.3.4 *In-vivo* crystals

In the present *in-vivo* study, thick layers of crystals were seen on 12th day on the coverslip placed between the mantle and shell. Coverslip with layer of nacre coating was observed under SEM and seen the development or arrangement of crystal layers (Plate 23 A&B). The surface of a rhombic shaped crystal was observed over the tabular bundle (Plate 23 B). Prismatic crystals were stacked in tabular bundles. These bundles of crystals were placed horizontally and vertically to fill the vacant spaces of the coverslip. These crystals over the coverslip showed extreme birefringence and biaxial nature under polarized light. These were also rich in Ca with 49.86% (Plate 24)
Plate 23. In-vivo crystals over coverslip under scanning electron microscope.
Plate 24. X-ray spectrum of in-vivo crystals
6.4 DISCUSSION

Calcium was detected from all the crystals examined and clearly proved that the crystals developed in the \textit{in-vitro} cultures were very similar to the \textit{in-vivo} crystals in its calcium content. Morphology and size of the crystals varied in different cultures and in the same culture.

Wada (1961) pointed out that the shell substances are crystallized out after passing through the amorphous state when the solid phase separates from the liquid phases. The shell mineralization in Gastropoda and Pelecypoda is assumed to pass through the following three processes: (1) the formation of organic matrix as the basis of shell material (2) fixation of calcium in this organic matrix and (3) the deposition of calcium carbonate crystal. Wilbur (1964) reported that crystals were first seen as round or elongate crystal seeds on the matrix or on the surface of crystals. Small oolitic crystals seen over the explant in the present study were examined and found in amorphous form with less calcium content (Plate 18). The free round crystals could not be collected for the analysis as most of them were attached to the plate. However by \textit{in-situ} examination, the free crystals showed high birefringence under polarized light and were hexagonal in shape. Wada (1961) reported that the twin of aragonite exhibited regular hexagonal form, which consisted of three grains joining together on its twinning face. It exhibited the twinning types of aragonite at all times during growth of the nacre in pearl oyster shell.

The rhombohedral crystals (Plate 19) showed high birefringence. Under polarized microscope aragonite nature was clearly reflected. Both calcite and aragonite crystals were seen in the same culture flask in the present work in some experiments. Aragonite, under ordinary conditions of temperature and pressure, is relatively unstable and changes to calcite, although the rate of
change is very slow. Crystals of aragonite have been observed which have completely changed to its stable polymorph calcite (Ford, 1932). Wada (1961) stated that the fixation of calcium and the orientation of mineral crystals were governed directly or indirectly by the elongation of epithelial cells of mantle tissue in molluscan shells and the crystals grown freely from each other exhibited rhombic, irregular or circular shape. In the present study, the cultured cells of mantle of abalone secreted oolite, hexagonal and rhombohedral crystals of calcium carbonate with extreme birefringence showing their calcite and aragonite nature. Wada (1961) reported that aragonite crystals scattered on the nacreous surface increased in size, and came to contact with one another and finally developed into a thin crystal lamella. An organic membrane was sandwiched between them when a crystal joins with adjacent ones, and is found as groove on the inner nacreous surface.

In organ cultures crystals were seen embedded in the organic matrix here and there over the bead (Plate 20). Wada (1961) found that in an early stage of formation of layer, individual crystals are scattered here and there. Wada (1964 b) reported that the small spherulite of calcium carbonate grew here and there after four days of operation. Aragonite tablets grown on the organic matrix over the bead in the present study (Plate 22) were very similar to the aragonite tablets grown on the organic matrix reported by Watabe (1981) from the nacreous layer of Haliotis during shell regeneration in Cepaea nemoralis.

Wada (1985) reported the pyramid shaped stacks of aragonite crystals growing in the spaces between large crystals. Similarly in 1968 he reported the rate of deposition of CaCO$_3$ spherulite after 11 to 25 days culture in the in-vivo preparation. In the present in-vivo study a thick layer of crystals was seen on 12$^{th}$ day on the coverslip placed between mantle and shell and was rich in
calcium (Plate 24). These crystals were stacked in peculiar manner horizontally and vertically so as to fill up the vacant spaces. The surface of a rhombic shaped crystal was observed over the tabular bundle (Plate 23 B). These crystals over the coverslip showed extreme birefringence and biaxial nature under polarized light. Crystals in twinkling form and silky lusture confirmed the presence of calcite and aragonite respectively. The surface of a rhombic shaped crystal observed over the tabular bundle, was resembled with in-vitro culture crystals. Wada (1961) reported the crystalline structures of nacres in *Haliotis discus* and *Turbo cornutus* and indicated that nacres consisted of mosaic arrangement of micro crystals having orthorhombic c-and b-axes were parallel or nearly parallel to vertical and horizontal growth directions of the layer. Due to the thick deposition of crystals, the correct size of individual crystals could not be measured in the present in-vivo study. Wada (1961) reported that initial deposition begins with the deposition of calcite rather than aragonite on the inserted glass between the mantle and shell in *P. martensii* and it is not applied to mineralization of all molluscan shells. Calcium carbonate of Haliotidae shells has one layer of calcite and one or more layers of aragonite (Boggild, 1930; Lutts et al., 1960; Wada, 1961; Wilbur, 1964). In the present study, the calcite and aragonite crystals seen over the coverslip inserted between the mantle and shell of *H. varia* supported their view. Machii and Wada (1989) also reported the strong calcium peaks in in-vitro crystals in the explant cultures of *Pinctada* with high calcium content optically shows strong birefringence. Samata et al. (1994) reported the SEM observation and EDS analysis of crystals over the black secretion and found that these types of crystals were rich in calcium. In his study sulphur was also detected along with calcium. In the present study of cultures of mantle tissue of abalone, although the black secretion was not seen mainly, the crystals were seen over
the explant tissue. These crystals were enriched with calcium. This study was
carried out to ensure the presence of calcium. So sulphur and other allied
microelements were not analysed along with calcium in the present study. The
crystals developed from the explant and organ cultures in *in-vitro* experiments
and crystals formed on the coverslip in *in-vivo* condition showed high
birefringence under polarized light as mentioned by Machii and Wada (1989)
and Samata *et al.* (1994) in their *in-vitro* experiments of *Pinctada*.

Calcium was detected from all the examined crystals and clearly proved
that these are purely calcium carbonate crystals. Rhombohedral shaped crystals
in the explant cultures; the crystals in the organ cultures and the crystals in the
*in-vivo* cultures were almost similar in its calcium content in the EDS analysis
(Plate 19, 21, 24). Morphology and size of the crystals varied in different
cultures and in the same culture. By this analysis, it was proved that the mantle
cells of *H. varia* are capable of secreting calcium carbonate crystals of high
birefringence and are more or less similar in calcium content in the *in-vitro* and
*in-vivo* conditions. The role played by the microenvironment on the formation
of aragonite and calcite crystals needs further investigations. Further
advancements in the culture technique can result in the total formation of a
nacreous layer. The crystals developed in the *in-vitro* cultures are the secretion
of an organic substance or organic pearl from the mantle tissue of *H. varia* and
the report of crystal from the *H. varia* explant and organ cultures is the first of
its kind.
Abalones are known for their rainbow coloured precious pearls and delicious meat. The mantle tissue is responsible for the multihued colour of the abalone nacreous layer and its gem quality pearls. Production of free spherical pearls from this animal becomes a rarity due to the virulent activities of its foot. Hence the mantle tissue culture was taken up in-vitro to develop the techniques and to achieve in-vitro pearl production.

Mantle tissue of abalone, *Haliotis varia* Linnaeus was taken for explant cultures. Explants of 1 mm² size were inoculated in 25ml TD flasks and petri plates (5cm dia.) and incubated in the medium 199 at 28°C. Cells proliferated from the explants were observed under phase contrast microscope after 24 hours of incubation. Cells of different morphological types such as round epithelial like cells, rectangular cells and hexagonal cells were migrated from the explant (Plate 3 A). Fibroblasts like cells were also noticed in the subsequent days (Plate 3 F). The development of pseudopodial network resulted in the formation of cell sheet (Plate 4 B, D). Round cells containing organic granules produced coloured shining crystals, which later joined together to form a shining layer (Plate 5 F). Crystals of different shapes were observed in the cultures (Plate 5, 6). The day wise and weekly cell liberation were recorded. A progressive trend was observed in cell proliferation from day one to four and it varied from experiment to experiment. On day one the average cell concentration was 7×10⁴/ml in the first experiment and it was 6×10⁴/ml in the second experiment. On day four it was around 11×10⁴/ml and 9×10⁴/ml respectively. In the first experiments the average cell concentration was 6×10⁴/ml in the first week and the cell liberation was continued up to 7th week and the total cells liberated was 56×10⁴/ml (Fig.3 of Ch.3.1). In the second experiment the average cell concentration was 10×10⁴/ml in the first
week and it continued up to 6th week and the total cells proliferated was $57 \times 10^4$/ml (Fig. 4 of Ch. 3.1). The number of cells proliferated varied from preparation to preparation. Contractive movement of explant in the medium was noticed up to 18 days. Mitosis also observed in the cultures. The general problem of contamination reported in the invertebrate marine cell cultures affected 30% of the cultures taken for the study.

Animals depurated in U.V. treated running seawater for three days and non-depurated animals were taken for the explant culture of mantle tissue of *H. varia* simultaneously to study the effect of depuration on cell proliferation. From the experiments conducted, it was confirmed that the explants from non-depurated animals liberated more cells than from the explants of depurated animals (Table 1 & 4 of Ch. 3.2). Contamination was also not more from the cultures of the non-depurated animals than from the depurated animals. The injury caused to the animal during collection might have caused secondary infections during depuration period and that might be the reason for more contamination in *in-vitro* condition in the explants from the depurated animal. Hence it was suggested that depuration of abalones for longer period may be avoided.

Role of antibiotics such as streptomycin and penicillin on cell proliferation and contamination was studied by incorporating them in washing solution for tissues prior to culture. Testing of three concentrations of these antibiotics was attempted in experiment I to evaluate appropriate dose for processing the mantle tissue. Washing of tissue in 1000 µg/ml of streptomycin and 2000 IU/ml of penicillin yielded more cell proliferation with less contamination than the lower dosage of streptomycin and penicillin, 250 µg/ml and 500 IU/ml and the higher dosage of 4000 µg/ml and 8000 IU/ml respectively (Table 3 & 5 of Ch. 3.3). Another experiment was conducted by
incorporating fungizone along with streptomycin and penicillin to minimize the fungal contamination and found that the combination of streptomycin, penicillin and fungizone at a rate of 1000 μg/ml, 2000 IU/ml and 200 μg/ml yielded better cell proliferation and less contamination than the lower dosage of 250 μg/ml, 500 IU/ml and 50 μg/ml and the higher dosage of 4000 μg/ml, 8000 IU/ml and 800 μg/ml of streptomycin, penicillin and fungizone respectively (Table 7&9 of Ch. 3.3).

The effect of different media viz., M-199, L-15, Ham’s F12 was studied with 10% FCS and also with 10% tissue extracts of gonad, mantle and whole body of abalone, H. varia on the initiation and proliferation of cells in mantle explant culture of H. varia. Number of cells proliferated were counted to determine the best media and the best extract for cell proliferation and growth. Eventhough cultures with L-15 media proliferated more cells numerically, significant difference was not observed statistically between media L-15, F-12 and M-199. Cultures with medium 199 showed better anchorage and thus helpful in the formation of pearl sac. It was proved that the medium 199 was the best suited for cell adherence (Plate 10). Addition of mantle extract to all media enhanced the cell proliferation to a maximum followed by whole body extract (Fig.5 of Ch. 3.4). Addition of whole body extract increased the cell adherence followed by the addition of mantle extract, which in turn helped in the formation of pearl sac and then in in-vitro pearl formation.

Cryopreservation experiments were conducted to preserve the abalone mantle cells in liquid nitrogen. It facilitated the long-term storage of cells with out contamination. For preservation in liquid nitrogen, two types of cryoprotectants namely DMSO and glycerol were used in different concentrations to find out the most suitable concentration. Cryoprotectant toxicity at different temperatures was studied at five concentrations from 5-
15%. Toxicity of glycerol and DMSO tended to increase as the concentration increased at physiological temperatures of 30°C and 5°C. At subzero temperatures (-70°C, -180°C and -196°C) viability of cells increased up to 10% concentration and then declined. At 5% concentration, the viability was 74% and at 10% concentration maximum viability of 88% was obtained and it decreased to 73% with 15% DMSO at -196°C. The viability was 72%, 86% and 68% respectively with glycerol. Survival rate at physiological temperatures was more with glycerol than DMSO (Fig.1& 2 of Ch. 3.5). At 30°C at 5% concentration the survival rate was 85 and 82% with glycerol and DMSO respectively. At sub zero temperatures the survival rate was better with DMSO than glycerol (Fig. 3, 4&5 of Ch. 3.5). 10% concentration was found most suitable with both the cryoprotectants DMSO and glycerol. The survival rate at -196°C at 10% optimum concentration was 88 and 86% with DMSO and glycerol respectively.

Effect of freezing with different concentrations of cryoprotectants and duration of preservation on viability in liquid nitrogen was studied. Freezing with different periods, it was found that survival rate decreased with the increase in number of days in the freezing condition. Extension of duration from 0 to 70 days decreased the viability from 72 to 38 % at 5% concentration of glycerol and it decreased from 74 to 46 % at 5% concentration of DMSO. With all five concentrations the survival rate decreased from 0 to 70 days in both cryoprotectants glycerol and DMSO. At end of the experiment after 70 days it was noticed that 10% concentration of DMSO and glycerol was the most suitable for the highest survival rate of more than 50% (Fig.8 of Ch. 3.5).

Organ culture was undertaken to develop the technology on in-vitro pearl production by understanding the aspects on cell dissociation, cell behaviour, cell migration and cell survival in the organ culture of the mantle
tissue of abalone, *H. varia* by retaining its tissue architecture. The investigation formed a stepping-stone for advanced research on *in-vitro* pearl production in abalone. Different methods were tried to find out the suitable one. Cultures were conducted in cell well and TD flasks using liquid medium 199 and in TD flasks with agar based medium. The shell beads and cover slips were used. The cells grew over it and formed a pearl sac.

In cell well culture soon after incubation the explant released numerous cells and occupied the surface of the bead. Different types of cells such as round, rectangular and fibroblast-like were observed which later formed the pearl sac with pseudopodial network over the bead. Some round crystal-like structures developed on day 8 over an organic matrix and it increased in number in the subsequent days (Plate 11). Crystals were also seen on the coverslip on a matrix like formation (Plate 13).

The agar medium provided better substratum for outgrowth of explant, proliferation of cells, formation of pearl sac and growth process-like structures. Coloured granules were observed in the cells of this culture, which later became the coloured crystals (Plate 14). Better growth was observed on the shell bead and formed a pearl sac that was considered to be a precursor of *in-vitro* pearl production. In liquid medium only few cells were attached over the cover slip and the rest of the cells liberated were migrated into the medium and formed organic granules and crystals there. Most of them occupied the periphery of the TD flask and seen in groups with shining crystals.

Studies were carried out on the cells and crystals of *in-vivo* condition and compared the cells and crystals of *in-vitro* condition. A piece of cover slip was inserted between the shell valve and mantle of the abalone. The cover slip was taken out everyday and examined the development under the phase
contrast microscope. The sequence of development of cells and crystals was photographed. The study provided the basic information on cell proliferation, pattern of crystal deposition, the formation of nacre layer and the appearance of nacre colour similar to the abalone shell. A high degree of similarity was observed between in-vivo and in-vitro crystal formation.

Crystals developed in the in-vitro and in-vivo conditions were observed under scanning electron microscope (SEM), polarized microscope and analysed in the energy dispersive X-ray microanalyser (EDS) to study the nature and chemical composition of crystals. The presence of calcium in all the crystals examined proved that these are calcium carbonate crystals. Rhombohedral shaped crystals in the explant cultures, the crystals in the organ cultures and the crystals in the in-vivo cultures were almost similar in its calcium content in the EDS analysis (Plate 19, 21, 24). Morphology and size of the crystals varied in different cultures and in the same culture. High birefringence and biaxial nature was noticed under polarized light showing its calcite and aragonite nature. It was proved from this present study that, the mantle cells of H. varia are capable of secreting calcium carbonate crystals of high birefringence and are more or less similar in calcium content in the in-vitro and in-vivo conditions. The role played by the microenvironment on the formation of aragonite and calcite crystals needs further investigations. The total formation of a nacreous layer needs further advancements in the culture techniques. The crystals developed in the in-vitro cultures are the secretion of an organic substance or organic pearl from the mantle tissue of H. varia and the report of crystal from the H. varia explant and organ cultures is the first of its kind.

The cell characteristics and its development in in-vitro condition, the effect of depuration on cell proliferation, the role of antibiotics in washing
solution and its effect on cell proliferation, the effect of different media on cell proliferation and the preservation of cells of the mantle cells of *H. varia* were studied in the present work. The studies on cryopreservation of mantle cells will create an opportunity to utilize the cells from *in-vitro* cultures for genetical and biochemical studies. The study on cryopreservation of cells caused long-term storage for future use. The medium with agar base formulated for organ cultures, were found suitable for better pearl sac formation. The cells and crystals formed in the *in-vitro* cultures were compared and confirmed by *in-vivo* studies. The crystals developed in the cultures were analysed and clearly proved that these are purely calcium carbonate. In the present study, success has been achieved in the production of *in-vitro* crystals or an ‘organic pearl’ which is a pioneer report in abalone. The analysis of *in-vivo* and *in-vitro* crystal formation indicated a close similarity. The present *in-vitro* studies on the mantle tissue of abalone formed a stepping-stone for *in-vitro* pearl production.