Influence of microgravity on crystal formation in biomineralization

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Becker, Wilhelm, Julia Marxen, Matthias Epple, and Oliver Reelsen. Influence of microgravity on crystal formation in biomineralization. J Appl Physiol 89: 1601–1607, 2000.—Biomineralized tissues are widespread in animals. They are essential elements in skeletons and in statocysts. The function of both can only be understood with respect to gravitational force, which has always been present. Therefore, it is not astonishing to identify microgravity as a factor influencing biomineralization, normally resulting in the reduction of biomineralized materials. All known biominerals are composite materials, in which the organic matrix and the inorganic materials, organized in crystals, interact. If, during remodeling and turnover processes under microgravity, a defective organization of these crystals occurs, a reduction in biomineralized materials could be the result. To understand the influence of microgravity on the formation of biocrystals, we studied the shell-building process of the snail Biomphalaria glabrata as a model system. We show that, under microgravity (space shuttle flights STS-89 and STS-90), shell material is built in a regular way in both adult snails and snail embryos during the beginning of shell development. Microgravity does not influence crystal formation. Because gravity has constantly influenced evolution, the organization of biominerals with densities near 3 must have gained independence from gravitational forces, possibly early in evolution.

Biomphalaria glabrata; mollusk; aragonite; snail shell; evolution

Biominerals are widespread, essential elements of skeletons, as well as of gravity receptors. They are composite materials of organic matrix and inorganic crystals. Calcium phosphates in bones of vertebrates or calcium carbonates in mollusk shells and in statoliths of vertebrates and mollusks are well-known examples (17).

The evolution of the skeleton for supporting the body of an animal and of statocysts for orientation in a gravitational field can only be understood with respect to the presence of gravitational force, which has existed since the beginning of life on Earth. It is therefore hypothesized that gravity has influenced and continues to influence the organization of biominerals with high densities of around 3.

To prove this hypothesis, we studied the shell-building process of the pulmonate snail, Biomphalaria glabrata, which has several advantages compared with other models.

First, the inorganic material consists only of calcium carbonate in one of three possible modifications (calcite, aragonite, or vaterite) compared with vertebrate bones, which are composed of dahlite (carbonated apatite), with anions like chloride or fluoride in varying relations and even other calcium phosphates (8, 14, 20, 31).

Second, more than 99% of the shell is composed of inorganic material (18). The biominerals in Biomphalaria glabrata are organized as 250-nm-wide needles in a highly ordered cross-lamellar structure (18). In contrast, crystals in vertebrate bones have smaller dimensions, only about 4 nm (31), resulting in only a diffuse X-ray diffraction pattern. On a dry weight basis, bone is roughly 60–70% mineral embedded in an organic matrix (24).

Third, the shell is built from the mantle epithelium across the extrapallial space. Therefore, the product of biomineralization can be separated easily from the mantle epithelium without any adherent tissue.

Fourth, snails lay spawn packs with ~20–40 eggs every day. Shell building starts at 25°C, the condition of our study, as soon as 40 h later. Therefore, even under short-time orbital missions, the beginning of the shell-building process can be studied under microgravity.

For these reasons, we studied biomineralization in Biomphalaria glabrata during two space flights; the snails were flown together with 11 other experimental models as an integrated component of the “closed equilibrated biological aquatic system” (CEBAS) (5).

MATERIALS AND METHODS

Microgravity experiments. The microgravity experiments were performed on space shuttle flights STS-89 (January 1998, 9 days) and STS-90 (Neurolab, April 1998, 16 days). CEBAS is a fresh water habitat that allows the controlled incubation of various aquatic species in a self-stabilizing, artificial ecosystem. Xiphophorus helleri, Biomphalaria glabrata, and microorganisms are present as consumers, and...
Ceratophyllum demersum and microalgae are present as producers in the system (5). The experiment module (OHB, Bremen, Germany) offers four separate tanks for the adult and larval stages of the fish and snails and a tank for the plants and the microbiological filter system in a total water volume of 8.6 liters. A support module controls water flow, thermal conditions, and the illumination cycles, as well as the monitoring and storage of the water parameters (temperature, pH, and oxygen concentration).

Twenty-four adult and nine juvenile snails were introduced to the system. The temperature was regulated to 25 ± 1°C. A 16:8-h light-dark cycle was used. When the oxygen fell below 4.5 mg/l, a light was switched on in the plant tank to produce oxygen by photosynthesis. If the oxygen fell below 2.5 mg/l, a gas exchanger, as an emergency device, was available. The pH fluctuated between 7.2 and 8.8. All of these parameters are within the range of what occurs in the natural habitats of Biomphalaria glabrata.

Ground-based experiments under 1 G, but otherwise identical conditions, were carried out for comparison with the space experiments. Deposition of individual spawn packs on a screen were registered by video monitoring to calculate the time of oviposition. Animals were available 4 h after landing.

The maximum diameters of the shells were measured with a precision sliding caliper to an accuracy of ±0.05 mm. The shells were crushed, and all parts of the soft body were removed. All pieces of each shell were washed three times with 0.9% NaCl solution and five times with bidistilled water and then dried at 30°C until weight constancy was achieved. The weight was determined with the use of a Sartorius electronic balance to ±10 μg accuracy at 20°C and 50% relative humidity.

**Scanning electron microscopy.** Intact shell edges were broken parallel to the sagittal plane, and the fractured surfaces were coated with gold. Embryos were removed from the egg capsules and washed twice with fresh water of the type used in the experimental tanks. They were then fixed for 2 h in 2% glutaraldehyde in a 0.02 M cacodylate buffer (pH 7.4). After this, embryos were washed three times with buffer, contrasted 1 h with 1% osmium tetroxide-1.5% potassium hexacyanoferrat in buffer, and washed three times with buffer, all at 4°C. They were dehydrated in a series of 30, 50, 70, 85, 95, and 100% ethanol, critical point dried, and coated with gold.

All scanning specimens were visualized with the use of a Camscan DV 4.

**Transmission electron microscopy.** Mantle edges were fixed and contrasted as described for scanning electron microscopy. They were washed three times with 0.05 M maleate buffer (pH 5.2), contrasted with 1% uranylacetate in 0.05 M maleate buffer (pH 5.2) for 2 h, and washed three times with

Fig. 1. A: from STS-89 flight module [scanning electron microscopy (SEM)] showing embryo of Biomphalaria glabrata; age by video = 66 ± 4 h. The praeveliger larva possesses a well-developed shell field (arrows); the central shell-field invagination (arrowhead) is covered with the embryonic periostracum. Scale bar = 10 μm. B: from STS-89 ground control (SEM) showing embryo of B. glabrata; age by video = 66 ± 4 h. The praeveliger larva shows a shell field (arrows); the central shell-field invagination (arrowhead) is covered with the embryonic periostracum. Scale bar = 10 μm. C: from STS-89 flight module (SEM) showing embryo of B. glabrata; age by video = 81 ± 1 h. Dorsal view of the veliger larva shows the vaulting embryonic periostracum of the shell (S, arrows). Scale bar = 30 μm. D: from STS-89 ground control (SEM) showing embryo of B. glabrata; age by video = 80 ± 0 h. Dorsal view of the veliger larva shows the vaulting embryonic periostracum of the shell (S, arrows). F, foot. Scale bar = 30 μm.
maleate buffer. Specimens were dehydrated in a series of 30, 50, 70, 85, 95, and 100% ethanol. Mantle edges were embedded in spurr, cut with a diamond knife (70 nm), contrasted with lead citrate, and visualized with a Zeiss EM 902 A electron microscope.

**X-ray analysis.** High-resolution X-ray powder diffraction was carried out at Hamburg Synchrotronstrahlungslabor (HASYLAB) at Deutsches Elektronensynchrotron. The samples were measured at room temperature in transmission mode on Mylar foils at a wavelength of 120.76 pm. The incoming beam was selected from the white beam with a $^{111}$Ge double-crystal monochromator placed between the sample and detector. Data were collected from positions of 8.7–41.22° 2θ in steps of 0.01°, with a counting time of $5\text{ s}$ at each point (scintillation counter). Rietveld refinement was done with the program Fullprof (23), based on the known crystal structure of aragonite (13), vaterite (16), and calcite (19).

**Statistical analysis.** A statistical comparison of the shell weight, dependent on shell diameter, was performed between STS-89 and STS-90 flight and ground control animals. From all data of the compared groups, a combined linear regression line was constructed, and the residual of the single data to the regression line was calculated.

**RESULTS**

Nineteen adult snails and 298 embryos from STS-89 flight module and 19 adult snails and 176 embryos from the ground control module, respectively, were used for our experiments. From STS-90, we used 15 adult snails and 6 embryos from the flight module and 15 adult snails and 13 embryos from the ground control module.

**Embryonic development.** Microgravity does not influence shell building in snail embryos, when measured from the beginning of development, including during spawning and the first cleavages. The shell building occurs in a regular manner (4) (Fig. 1, A–D). The shell is formed with no differences shown between flight and control animals, as compared to the ground control animals.

**Fig. 2.** A and B: Shell weight, dependent on shell diameter, of flight vs. control snails in STS-89 (A) and STS-90 (B), presented as box-plots. For STS-89, $n = 19$ in the flight module (FM) as well as in the ground control module (LM). For STS-90, $n = 15$ in flight module and in ground control module, respectively. Residuals, which may have positive or negative values, are presented for each group in the following way: the box represents 50%, the vertical lines 100%, and the bold horizontal line the mean of the residuals. A tendency of shelf weight reduction in flight animals can be stated.

**Fig. 3.** A: from STS-90 flight module (SEM) showing piece of adult shell of *B. glabrata*, broken in the sagittal plane near the shell edge. Beneath the periostracum (P), the outer (I) and the inner (II) cross-lamellar layers stand rectangularly on each other. Scale bar = 30 $\mu\text{m}$. B: from STS-89 ground control (SEM) showing piece of adult shell of *B. glabrata*, broken in the sagittal plane near the shell edge. Beneath the periostracum (P), the outer (I) and the inner (II) cross-lamellar layers stand rectangularly on each other. Scale bar = 20 $\mu\text{m}$.
ground control animals up to the time of hatching. No indication of defects in embryogenesis due to microgravity could be demonstrated.

**Adult snails.** There was no significant difference in shell weight, with respect to the shell diameter, between flight and control snails (Fig. 2). A tendency of shell weight reduction in flight animals in contrast to the ground control group can be seen.

Under microgravity conditions, the cross-lamellar structures are built in the usual manner, with two lamellar layers perpendicular to each other (Fig. 3, A and B). The distance between the beginning of the first and the beginning of the second cross-lamellar layer shows continuous shell growth under microgravity.

Transmission electron microscopy revealed no significant differences between flight and control snails with respect to the shell-forming tissue (Fig. 4, A and B). All cell types described in standard snails (3) can be identified in flight and ground control animals. Periostracal units are secreted and organized during the periostracum formation. However, fewer units were observed in flight animals compared with controls.

Shell material built during experiments in adult snails was ground to a powder. High-resolution X-ray powder diffraction demonstrated aragonite with good crystallinity in flight and ground control animals (Fig. 5). If vaterite was present, its mass fraction in the shell was below 0.1%. The same was true for calcite. No indication of any other substance could be found.

**DISCUSSION**

Organic matrix with <1% wt/wt (18) and inorganic material (calcium carbonate in the aragonite modification) are the two components that interact during shell building in *Biomphalaria glabrata*.

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**Fig. 4.** A: from STS-89 flight module [transmission electron microscopy (TEM)] showing mantle groove of *B. glabrata* cut in the sagittal plane, proximal side on the left. A coat cell (CC), a mucous cell (MC), a secretion cell (SC), lamellar cells (LC) with lamellar vesicles (arrow), and periostracum cells (PC) are identified. Lamellar units are visible on the cells of zone 1 (Z1; arrowheads). Scale bar = 2.5 μm. B: from STS-89 ground control (TEM) showing mantle groove of *B. glabrata* cut in sagittal plane, proximal side on the left. A coat cell (CC), a secretion cell (SC), two lamellar cells (LC) with lamellar vesicles (arrows), and periostracum cells (PC) are identified. Lamellar units are visible on the cells of zone 1 (Z1; arrowheads). Scale bar = 2.5 μm.
The main question addressed in this paper is whether microgravity influences the organization of biominerals and, in a further step, the shell-building process. Both questions can clearly be answered. As shown by high-resolution synchrotron X-ray determinations, the biocrystals are built as aragonite in the same way under both 1 G and microgravity conditions. This is the first time that the formation of mineralized biocrystals can be demonstrated. The organization of the crystals in a highly ordered cross-lamellar structure is also not influenced in a detectable way under microgravity. The gravity dependency of convectional streams or sedimentation processes in cells, in the extracellular and, especially, in the extrapallial fluid, does not influence biomineralization in such a way that crystal formation is altered, although the inorganic material has a density near 3. The calcium carbonate modification remains constantly as aragonite (density = 2.95) (26). Calcite and vaterite, with lower densities, 2.72 and 2.64, respectively (26), and thermodynamically higher (calcite) or lower (vaterite) stability, are not formed within the extrapallial fluid.

Specific organic matrix material can determine the phase, the morphology, and the orientation of deposited calcium carbonate crystals (2, 7). From our results, we can state that the production of organic matrix and the allocation of inorganic material are not influenced under microgravity in such a way that inappropriate formation of the shell occurs. This is supported by the findings of our electron microscopic studies, in which no effect of microgravity on cell growth and cell differentiation can be demonstrated in the mantle edge tissue of adult snails and in embryos, where shell building occurs under microgravity from the very beginning.
We, of course, cannot completely ignore alterations in the organic matrix due to microgravity. However, if they occur, they are unimportant with respect to crystal formation and organization in the shell.

Several studies have indicated that microgravity affects cell growth and differentiation (15, 22), and increasing information is available that demonstrates the influence of microgravity on cell metabolism (6, 30). Even skeletal muscle fibers are directly responsive to microgravity (27).

Cell metabolism is believed to be influenced directly via a mechanosensory system within cells, for example, via actin-microfilament systems (6) or indirectly from the cell’s environment by contact stresses (12, 30). All of these possible influences, however, do not result in altered crystal formation in our model.

It has been well known for many years that altered gravity, hyper- as well as hypogravity, influences mineralized structures. Statoliths in Aplysia californica decrease in size under hypergravity (21). Also, for chilid fish larvae, hypergravity results in a significantly smaller size of otoliths compared with controls in 1 G. No morphological differences with respect to the general shape of the otoliths and the overall pattern of daily incremented ring layers could be found (1). Microgravity shows the opposite effect in snails reared on board CEBAS (32). Similar results are reported for Xenopus laevis larvae reared in space. However, results of studies on the influence of space flights on otoliths are controversial (21).

Many studies deal with the influence of hypergravity and especially microgravity on bone formation and bone mineralization. Bone loss and negative calcium balance have been documented in cosmonauts as well as in rodents (29, 30, 34). An impairment of both osteoblastic and osteoclastic functions appeared to be responsible for the bone loss; the loss of bone during spaceflight could be the result of both impaired mineralization as well as increased resorption (11, 28).

The impairment of mineralization may be caused by a defective organization of biominerals. There have been no studies dealing with this aspect, either in vertebrates or invertebrates. Only in one case was a decrease in apatite crystal size and/or perfection under microgravity estimated, using X-ray determinations in rat bones (25).

From our results, it is shown that a defective organization of biominerals under microgravity (during remodeling or turnover processes) is not necessarily the reason for the reduction of mineralized materials. We can find no differences between 1 G and microgravity conditions. In both cases, the crystals in our model are built in pure aragonite modification, and they are organized in the same highly ordered cross-lamellar structure.

However, these findings do not exclude that, under a quantitative aspect, altered gravity may influence the amount of mineralized material deposited in mineralized structures. Reductions of bone material in weight-bearing as well as in non-weight-bearing bones and of statoliths are well-known examples, especially in longer-lasting flight experiments (10, 30, 33). This may be the result of hormonal (9) and/or neuronal (1) regulation mechanisms influenced by altered gravity.

Such influences can also not be excluded for Biomphalaria glabrata. Our experiments lasted only 9 and 16 days. We could not demonstrate a significant difference in shell weight, with respect to shell diameter, between flight and control snails. However, a tendency for shell-weight reduction in flight animals vs. that shown in the ground control group can be seen, with an even slightly lower mean of residuals in the 16-day compared with the 9-day flight group. Peristomal units are secreted and organized during the periostracum formation, but they seem to be reduced for the STS-89 flight group and even more for the STS-90 flight group. Longer-lasting experiments in the International Space Station are necessary for final conclusions.

Gravity is the only factor that constantly influences organisms during evolution, and the development of biominerals for skeleton elements and statocysts is believed to be connected to this fact. It is astonishing that cessation of this factor in the flight experiments does not influence the organization of biominerals, although influences on cell metabolism are demonstrated unequivocally (6, 30). This means that the organization of biominerals seems to have gained independence from gravitational forces, possibly during early phases of evolution. The only other explanation would be that gravitational forces in the range of 10^-3 to 10^-4 G, which occur in orbit, are still enough to organize biominerals in a proper manner.

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