Rhythms in structures of organisms

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All things from eternity
are of like forms
and come round
in a circle

Marcus Aurelius Antonius

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Introduction

In nature many structures can be observed, which show a periodic structure. Sandy surfaces are often patterned and not just plain (figure 0.1). The shells of snails and shells (figure 4.2), annual rings of trees (figure 0.2, see Klein and Eckstein (1988)), concentric formation of spores of fungi on ripe fruit (figure 0.3) are examples from the living world. Winds in the dessert often pattern the sand. Periodic events in the environment or in the interior of organisms lead to rhythmically structured phenomena. They are wide spread, often quite obvious, but more frequently hidden and only seen after careful observation and with the aid of binoculars and microscopes.

Figure 0.1: *Large sandy areas as for example in the dessert are not uniformly flat, but due to the influence of winds wavy (see also the first illustration of the book). Likewise dunes are formed by winds: Behind a small elevation more sand accumulates, the elevation increases and according to the snow-ball principle a dune forms. This and the first image of the book was kindly supplied by Hans-Ulrich Seitz, Tübingen. First image: Algeria, Great East Erg near Fort Bir Djedid, March 2001; this image: Libya, Erg Ubari between Idri and Ubari with* Alaemon alaudipes*, March 2000*

Some of these time-structures will be described in this book. First it will be shown how the visual cells in eyes of vertebrates are renewed daily. Most cells of the body are completely replaced after longer or shorter intervalls. However, in the case of the visual cells renewal is limited to parts which carry the pigments for absorbing light. The used elements are discharged at the tip of the visual cells and decomposed. This happens at times when the visual cells are not urgently needed, thus avoiding that vision is impaired.

Green plants need light. They have to use therefore strategies for germination, growth and propagation in order to obtain light for photosynthesis. On the other hand too much light can damage the plants. We will get to know some of these strategies which some plants use to harvest light optimally. Some plants are for instance able to turn their leaves. Other plants move the

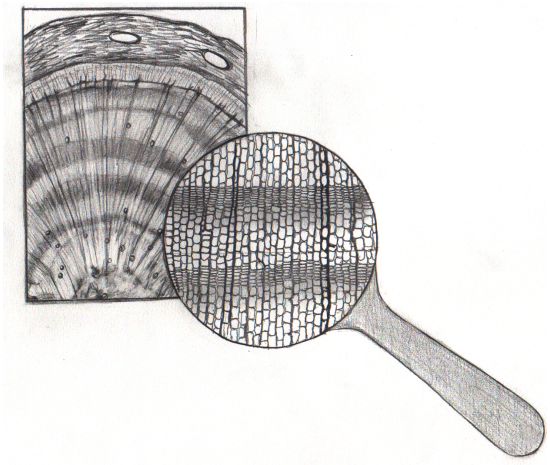

Figure 0.2: *Annual rings of a tree. At the periphery (top) bast and cork, in the interior wood. In the wood large-volumed cells of early wood and small-volumed cells of late wood form an annual ring. Under a magnifying glass the early wood (large cells with thin cell walls) and the late wood (small cells with thick walls) can be recognized bettter. The sharp border is the end of the annual ring and reflects the stop of growth during the winter*

Figure 0.3: *In the center of the first concentric ring the apple was infected by a spore of the fungus* Sclerodinia*. It germinated and the mycelium spread in all directions. After a certain time spores formed synchroneously at the ends of the mycelium. They are seen here as white structures. The mycelium continued to grow and formed again spores after the same time span. A second ring and further rings* formed

chloroplasts in their cells in such a way, that they receive light optimally. If not enough light is available, the chloroplasts move to those walls of the cells, where the irradiation is maximal. In the case of too high irradiation they move to the lateral walls of the cells.

Next the layer formation in the starch grains of chloroplasts and amyloplasts of plants are presented. During photosynthesis sugars are made which are stored in form of starch. It will be deposited during the day and the daily layers are visible in the starch grains.

Insects possess an outer armour which supports the body and protects the animals. However, after some time the old armour must be shed and a new one formed, because otherwise it would not fit anymore the growing insect. The chitin case is produced by an epidermis in daily layers, until the final thickness is reached. Sections through the chitin case show these layers. The number of layers reflects the age of the insect. Two examples are the chitin layers of a femur of a cockroach and a chitin clasp in the thorax of fruitflies. Layers can also be produced in an annual rhythm as for instance in annual rings of trees. They are brought about, because the growth conditions in our latitudes vary considerably during the course of a year. Even in dinosaurs such annual rings were found in bone structures and they allowed to find out how many years it took until they were grown up.

Fairy rings of fruiting bodies of fungi are occassionaly found on meadows and in the woods. They are formed when the mycelium of a germinating spore grows starlike in all directions and forms after a certain time (or due to favorable environmental conditions) fruiting bodies ('mushrooms'). Often concentric rings are found on fruit in the fall. If spores of certain fungi are inoculated on a solid nutrient medium in a dish, they germinate, grow radially and form after some time carriers of spores which form concentric rings. The mycelium continues to grow and forms after some time again such a ring. Finally the dish is covered with concentric rings. Depending on the fungus, the distance between the rings is exactly one day, several days or weeks. Daily rings are formed by the red bread mold *Neurospora crassa*. An internal circadian clock, which is responsible for it, has been studied thoroughly in this fungus. It is known in the meantime, how this clock works in molecular biological terms and how the rhythm of sporulation is entrained by the light-dark-cyclw of the day:

A marine amoeba from the coast of west Australia hunts for food by crawling over the bottom of the sea (mostly small pebbles) like a net taking up algae. After some time it stops moving, forms a hat-like structure and digests its bait. Afterward it starts again to hunt for food. We will see, that this is an unusual rhythmic behaviour which is adapted to the rhythmic structure of the environment by a combination of different time cues (day-night-change, temperaturee change, tides).

Some demonstrations, observations and proposals for experimentation shall evoke your interest for these interesting topics and make you do some work on it. Literature is referred to in the various chapters. The following books, which are concerned partly or completely with periodic structures, are recommended:

Bünning (1965), Bünning (1983), Engelmann (2007), **?**, Engelmann and Klemke (1983), Grell (1987), Meinhardt (2003), Neville (1975), Neville (1967), Pannella (1972), Pannella and McClintock (1968),

Runcorn (1966), Smith and Denyer (1992), Smith et al. (1997), Wada et al. (2003), Winfree (1986)

1 The daily maintenance of the eyes

The composition of a vertebrate eye and the light-sensitive retina is briefly described. Structure and function of visual cells and their connection with ganglia cells are mentioned. It will be shown how visual cells are renewed daily. This concerns on the one hand the molecules of the visual cells generally and on the other hand the membrane disks, which carry the pigments which absorb light. The used elements are discharged at the tip of the visual cells and decomposed by phagosomes. To avoid impairment with the visual function, the renewal occurs at times when these visual cells are not urgently needed. The high costs of the preventive renewal of visual cells are compared with the benefits.

1.1 Structure of the vertebrate eye

The eyes of vertebrates consist of a glass body with a lens in front and the retina in the hind part of the interior (figure 1.1). The retina contains the light-sensitive cells and a number of further structures, which are shown and described in figure 1.2. The signals received by the light sensitive cells are conducted via special neurons to the brain were they are perceived as sensory impressions. The visual cells in the eyes of vertebrates are positioned quite different from what one would expect: Instead of viewing the light in the interior of the glass body, they are positioned at the back side of the retina and the light has to pass first other cells and structures before it is absorbed by them.

1.2 Structure of visual cells

The visual cells of vertebrates consist of rods and cones. They are located in the retina of the eye. Figure 1.3 represents a rod. It consists of an outer segment, an inner segment with ellipsoid and myoid, a fiber and synapses at the terminal. The transition from the cell body to the outer segment occurs via cilia (figure 1.4). An electron microscopic view and a modell shows figure 1.5. The cilia transport material from the cell body to the outer segment. It serves to renew the membranes of the disks and their pigments and further constituents. An electron microscopic figure of a part of the outer segment with the membrane disks and one of the myoid is shown in Young (1998) (there in figure 3). They show the big differences of these structures in their consistency.

1.3 Visual cells are renewed internally

Most cells of the body are replaced by new ones after some time (which can be quite different depending on the cell type). Visual cells in the eyes, however, are preserved. They are instead internally renewed. This occurs in two ways. On the one hand, molecules are continuously renewed by those which are produced in the myoid of the inner segmentand which are

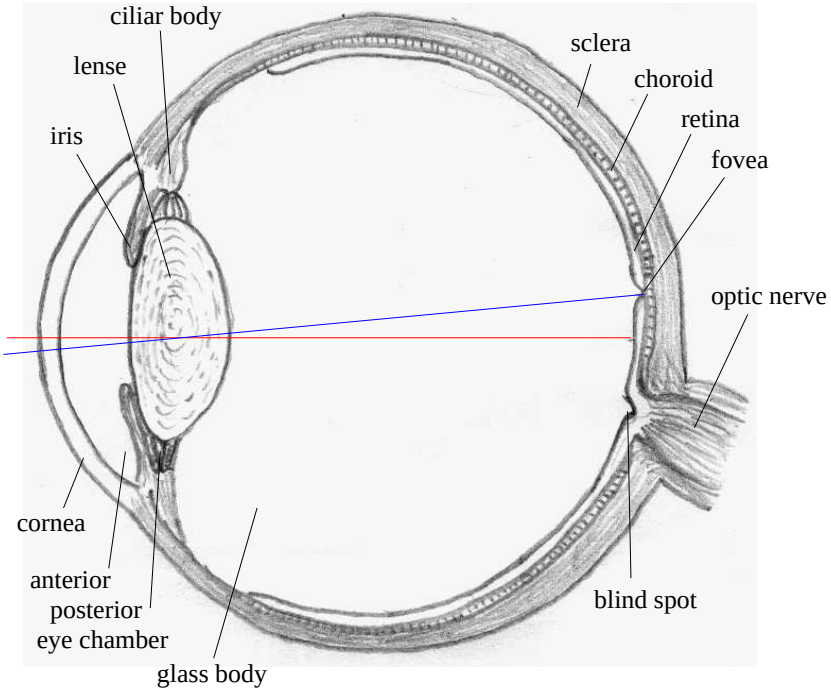

Figure 1.1: *Eye of a vertebrate with cornea, anterior eye chamber, iris, lens, ciliary body, glass body, retina, choroid, sclera, fovea, blind spot and optic nerve. Axis of eye (red) and visual line (blue) are added. Drawn by the author after a figure in Mörike and Mergenthaler (1959)*

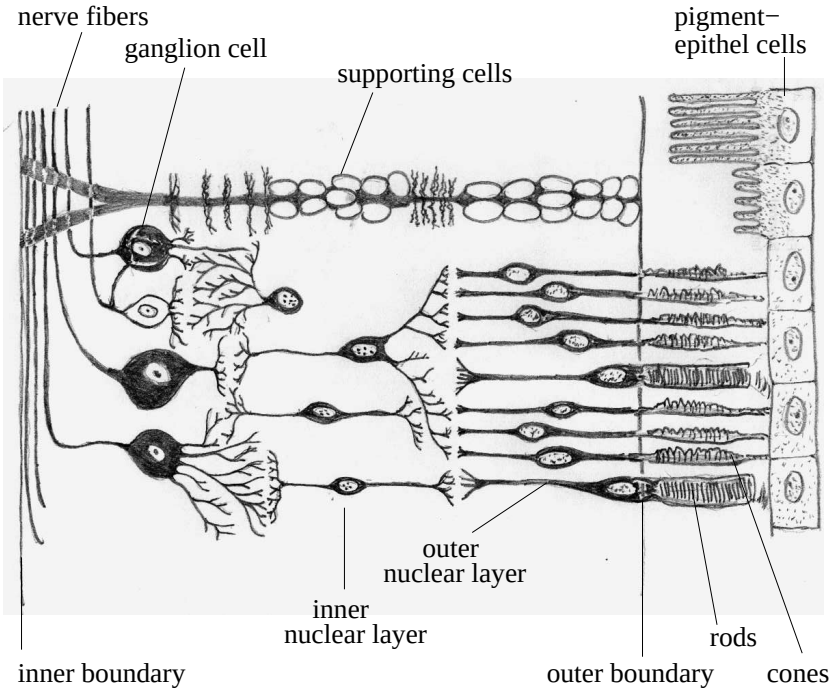

Figure 1.2: *Retina with internal border membrane, ganglia cells with processes, inner plexiform layer with bipolar and other neuronal cells, supporting cells, outer plexiform layer with nuclei of rods and cones, outer border membrane, rods and cones, pigment epithel and pigment cells. Glass body and lens would be at the left and the light would come from there. Drawn by the author after a figure in Mörike and Mergenthaler (1959)*

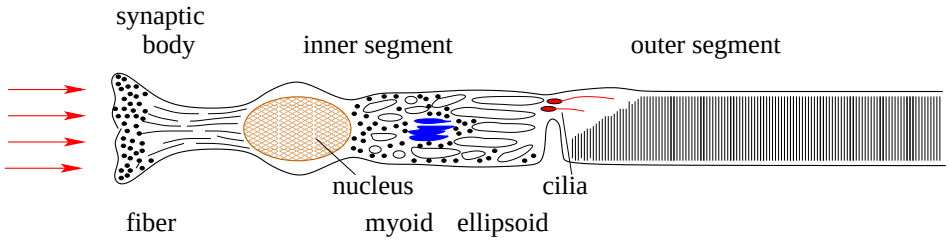

Figure 1.3: *Structure of the rod in the retina of the eye of a Rhesus-monkey. Light enters from the left (red arrow). To the right is the outer segment with numerous stacked membrane disks (electron microscopic image in figure 1.5), in which the visual pigments are found. To the left of it the inner segment with ellipsoid and myoid. Cilia (red) connect the two segments (which is shown in more detail in figure 1.4). The ellipsoid consists of closely packed mitochondria. The myoid contains the Golgi-complex (blue), free and membrane-bound ribosomes, vesicles and vacuoles. The nucleus (brown) is situated in the lower part of the myoid or in the neurit-like process with synapses, in which numerous microtubuli dominate. Small vesicles are found at the end of the synaptic bodies. Drawn by the author after an image in Young (1998)*

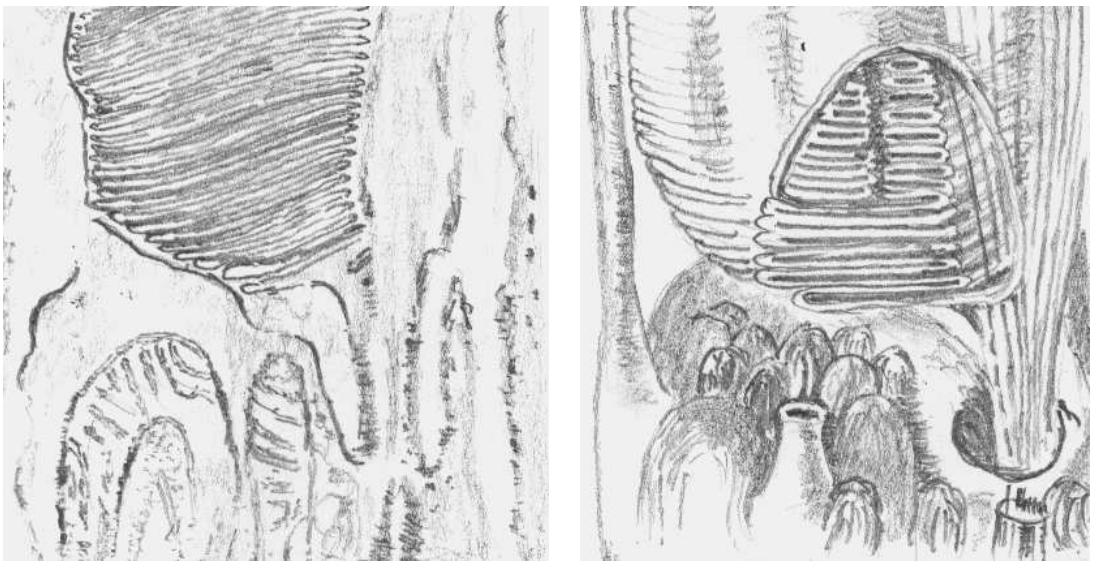

Figure 1.5: *Left: Drawing of the transition between cell body of a visual cell to the outer segment according to an electron microscopic image. Right: Part from figure* **??** *corresponding to the left part of the figure. The track with the cilia can be seen in the modell at the right. Here the lower part of the outer segment is cut open, in order to show how the outer membrane is folded and how it forms the zickzack-like membrans. Later (further up) from these foldings disks are formed which are no longer connected with the envelope membrane of the outer segments. In the membranes of these disks lie the visual pigments. Drawn by the author according to a figure in Young (1998)*

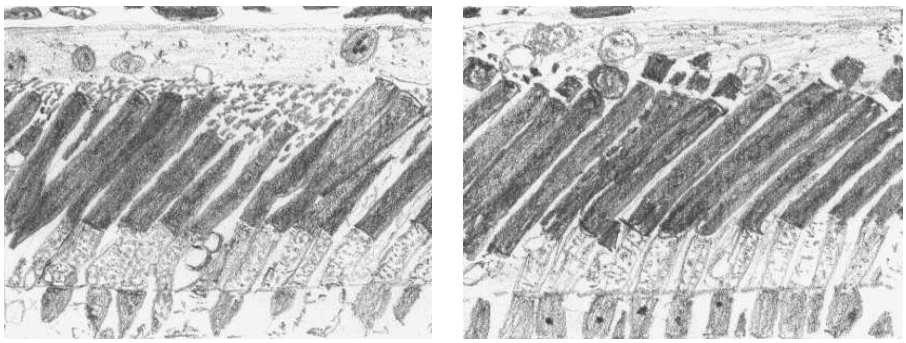

Figure 1.6: *Retina of an eye with outer segments (upper part), inner segments (loper part) and nuclei (very bottom, oval structures). Right part prepared at a time at which packages of membrane disks are shed from the tip of the outer segment layer and dismantled and disposed by phagocyts. Drawn by the author after a figure in Young (1998)*

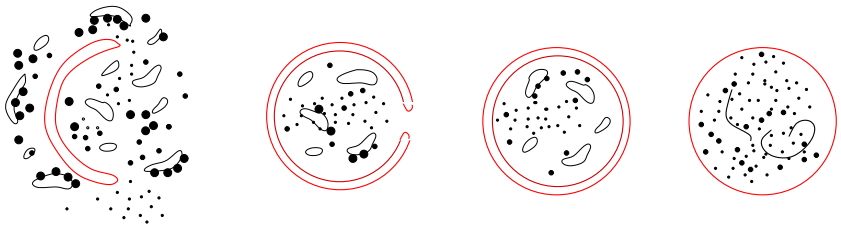

Figure 1.8: *Shed disks at the top of the rods are enclosed by membranes (sickle-moon like structure). The internal part of the double membrane together with the enclosed old disks disappears in these* phagosomes. *Drawn by the author after a figure in Young (1998)*

transported via the ellipsoid in the outer segment. On the other hand, new membrane disks are formed in the lower part of the outer segment by folding of the outer membrane into the interior (figure 1.5). Later they form new disks by separating themselfs from the outer membrane. To avoid that the outer segments of the visual cells become longer and longer, old disks are shed as packages at the tip (figure 1.6), put in phagosomes (right figure in figure 1.6), dismantled and discharged (figure 1.8). The separation of a membrane disks package is shown in electron microscopic image in figure 1.7.

But this does not occur continuoulsly, but at times, when the correcponding visual cells are not needed. In the case of rods, which are needed at night, the old disks are disposed in the morning, in the case of cones, which are used for colour vision during the day, in the evening (figure 1.9).

The phagosomes are not only rhythmically active in light-dark-cycles, but also under constant conditions of darkness, as shown in figure 1.10. It is therefore not the light condition which is responsible for the high phagocytosis of the rods disposal, but a circadian clock.

To find out, how new membrane disks are build at the lower part of the outer segments, radioactively labelled amino acids were injected into animals and waited for different lengths of time. Thereafter the animals were killed and slices through the retina of the eyes made. Using the autoradiographic method (see figure 1.12) images were obtained as shown as a drawing in figure 1.11. In the earliest prepared visual cells the radioactivity is found in the myoid of the cell body (dark dots in plasma, A). The proteins are afterward (B) accumulated at the Golgi-complex. There they are combined with carbohydrats and in this way modified. Many of these compounds pass now the small neck with the cilia and are inserted into the newly formed membrane folds (C). When these foldes are transformed to membrane disks, the proteins are labelled and move with the disks slowly further up to the tip (D), while new membrane disks are formed. Finally they reach the tip of the outer segments (E) and are discharged in small packages (F).

1.4 Costs and benefits of renewal of the visual cells

The visual cells are thus continuously renewed internally. This is much more ex-

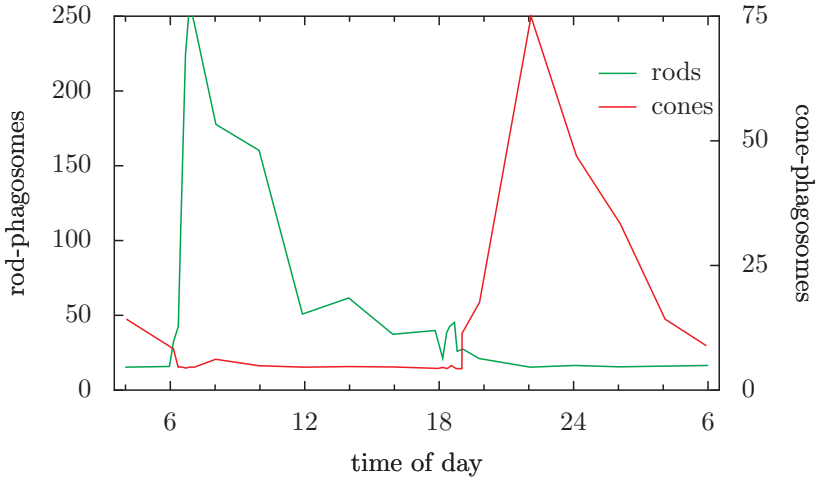

Figure 1.9: *The membrane disks of visual cells are shed in packages from the tip of the outer segment. At the same time new disks are formed close to the cilia. This does not happen continuously, but at times only, when the corresponding visual cells are not needed. In the case of rods, which are needed at night, the old disks are disposed in the morning (green curve), in the case of cones, which are used for colour vision during the day, in the evening (red curve). After Herman (1983)*

pensive than the costs of viewing. At least the most frequently used visual cells in the fovea can rest during the sleep several hours per day. The processes of renewing run, however, continuously, 24 hours per day. Without any interruption material is produced, transported, build in and dismantled. It is an enormous task. In the eye of Resus monkeys each day 80 to 90 disks of the rods are renewed in the outer segments. With 900 disks per rod it takes 9 to 12 days, until the visual cell is renewd. This occurres 35 times per year. I man the figures are probably similar. That means, that in a 80-year old person the rods would have renewed threethousand times. This corresponds to 90 million membrans of the disks.[1]

Why is so much energy put into the renewal of the visual cells? Actually it would not be necessary to replace all the time those parts which are still functioning. However, it was apparently of essential advantage during the evolution of the vertebrates to renew the constituents of the visual cells before they were worn out. This *preventive renewal* is comparable with the exchange of wear out parts in important technical equipments and vehicles. In the case of airplanes one does not wait until the wheels are worn out, but exchanges them in regular intervals.

In the case of visual cells new membrane disks are produced. But in addition molecules are replaced continuously in the whole visual cell including the outer segmentes. These two renewal processes are schematically shown in figure 1.14.

[1] This kind of renewal occurs also in some other organs. In the digestive tract of the rat in 20 to 30 days the same amount of cells is replaced as the body of the rat possesses cells (Leblond (1965)).

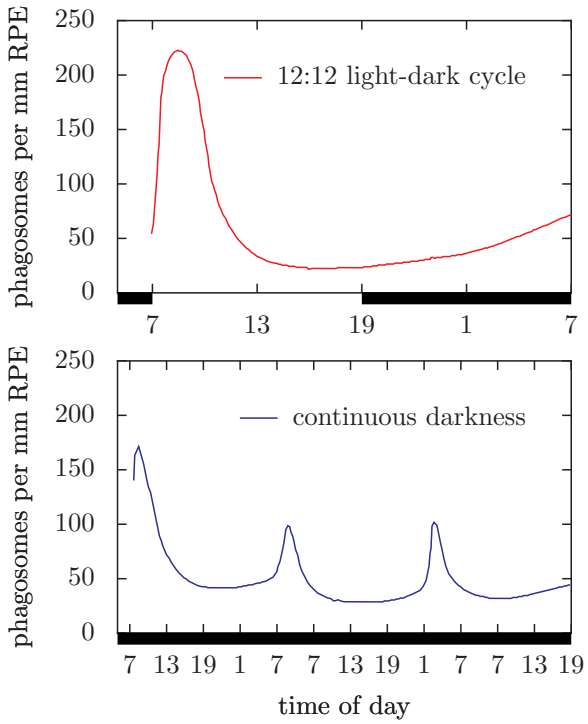

Figure 1.10: *Disposal of used membrane disks of rod visual cells in the retinal pigment epithelium RPE by phagosomes in a light-dark change (top) and in continuous darkness (two and a half days measured, below). Note the different length of the x-axis!). It is therefore not the onset of light which is responsible for the high phagozytosis (top), but a circadian clock (below). After Young (1998)*

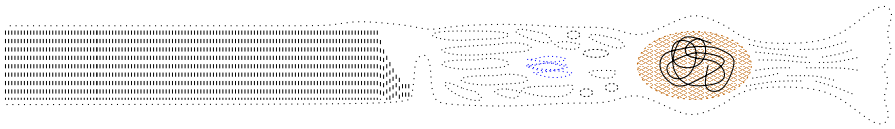

Figure 1.13: *In contrast to many other cells of the body the visual cells* are renewed internally. *Only the DNA (dark structures) in the nucleus are maintained. All other parts of the cell are renewed in different lengths of time and at various time points (shown punctured). From Young (1998)*

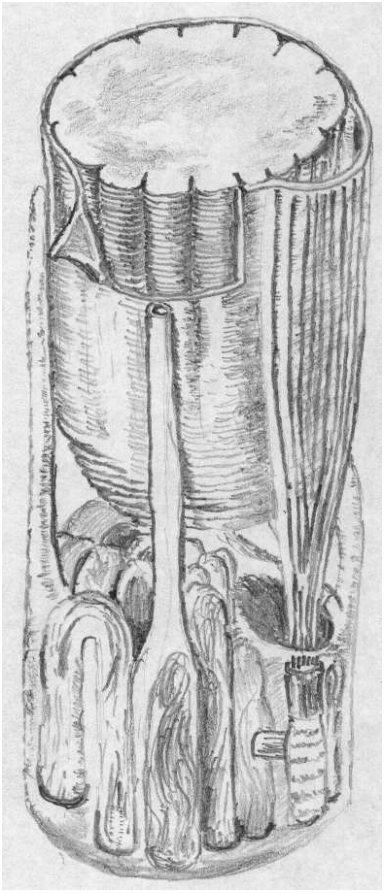

Figure 1.4: *Transition from cell body of a visual cell to the outer segment. The tract with the cilia can be seen at the right. It originates from the basal body, and a striped root is situated underneath it. The cilia are the only connection between cell body (below) and outer segment (top) and serve to supply the densely staggered disks and the visual pigments. A part of the outer segment is cut open, in order to show the arrangement of the membranes. Around the basis of the cilia in the ellipsoid are a number of mitochondria and three processes reaching uopward. Their function is unknown. Drawn by the author after an image in Young (1998)*

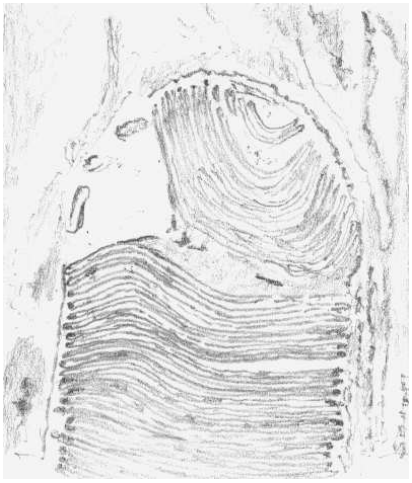

Figure 1.7: *This electron microscopic image shows, how a package of membrane disks of the outer segment of a rod is shed. Drawn by the author after a figure in Young (1998)*

Independent of the age of the animals a visual cell consists all the time of new parts. By this renewal program it protects also elder animals from reducing or loosing the functioning of visual cells. The functional efficiency of visual cells exceeds by far the life expectency of a vertebrates (Friedenwald (1942)). This is understandable in respect to the importance of the sight in vertebrates (and many other animals) and also in aged animals. Visual cells become old without getting aged.

If material for renewal of visual cells is becoming tight or lacking, problems arise. The body is not able to produce fatty acids, vitamin A and taurin. If they are lacking in the food, the visual cells degenerate. In the case of the RCS stem of rats a renewal path is disturbed: The pigment epithelium is not able to distroy the shedded upper parts of the outer segments. They accumulate at the tip of the rods and the rods degenerate.

In the case of the ‘Refsums-syndrom’ the

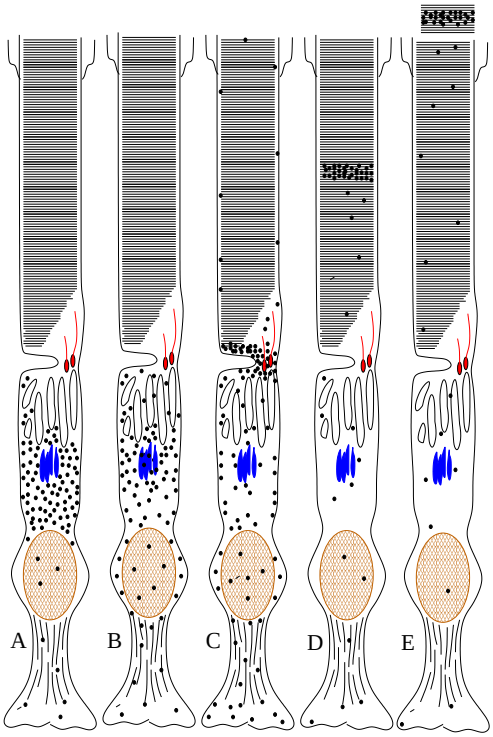

Figure 1.11: *Radioactively labelled amino acids were injected into animals and waited for different lengths of time. Thereafter the animals were killed and slices through the retina of the eyes made. Using the autoradiographic method (see figure 1.12) images were obtained as shown here as a drawing. In the earliest prepared visual cells the radioactivity is found in the myoid of the cell body (dark dots in plasma, A). The proteins are afterward (B) accumulated at the Golgi-complex. There they are combined with carbohydrats and in this way modified. Many of these compounds pass now the small neck with the cilia and are inserted into the newly formed membrane folds (C). When these foldes are transformed to membrane disks, the proteins are labelled and move with the disks slowly further up to the tip (D), while new membrane disks are formed. Finally they reach the tip of the outer segments (E) and are discharged in small packages (F). After Young (1998)*

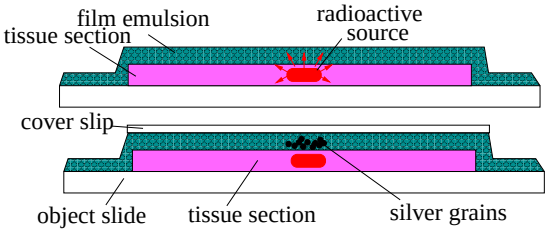

Figure 1.12: *The silver grains in figure 1.11 were obtained and established by autoradiography. For thos purpose a radioactivly labelled preparation was put on a microscope slide, covered with a thin film emulsion and exposed in the dark for a certain time to the radioactive β-rays. The film is developed and the silver grains are found on top of the radioactive material. From Young (1998)*

renewal of fatty acids in the visual cells is interrupted and they the. In the 'Bassen-Kornzweig-syndrom' the liver does not produce β-Lipoprotein. Therefore no fatty acids are transported to the visual cells and they the. In the case of *Retinitis pigmentosa* the life expectance of the visual cells is reduced. Apparently this mutation has affected the renewal system of the visual cells.

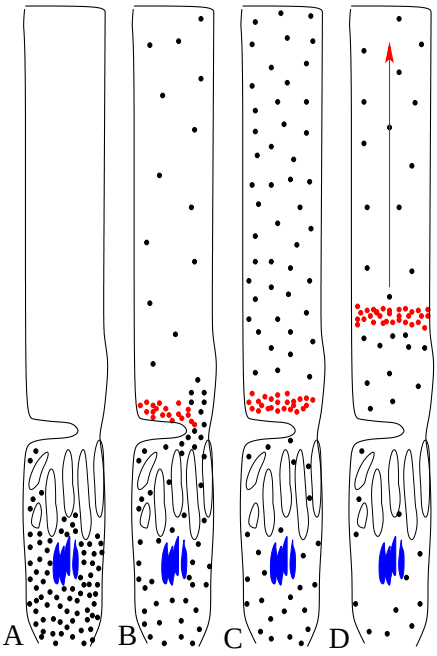

Figure 1.14: *The scheme shows the difference between renewal of membranes (red) and replacement of molecules in the visual cells. Radioactive labeling with amino acids and autoradiography demonstrate how the substances are made in the myoid (A), transported to the outer segment (B) and there used for new membrane disks (C, red dots), which finally reach the tip of the segments (D, red arrow). How the molecules in the whole cell are replaced is visible from the distribution of black dots of the labelled amino acids. From Young (1998)*

2 Light harvesting

Light is essential for green plants. However, too strong light might lead to damage. Different mechanisms prevent those damages. Thus, in some plants chloroplasts, in which photosynthesis takes place, are able to move from the strongly irradiated places of the cell to the lateral walls. This was for instance studied in Selaginella*, but occurs also in many higher plants and numerous algae.*

Green plants need light for living. They are therefore forced to use for germination, growth and propagation strategies in order to obtain light optimaly. It serves as a source of energy for producing sugar compounds from carbon dioxid in the air and water in the soil. This photosynthesis occurs in special organelles of the plant cells, the chloroplasts.

Plants, which do not get enough light, because they grow, for instance, in the shadow of trees, are able to orient their leaves in such a way that light can be harvested optimally. You can observe this nicely in the case of Wood sorrel. With the help of a magnifying glass you can also see joints between the leaflet stalks and the leaflets. They can turn the leaflets in the right position allowing to receive much light.

However, a plant can also be damaged by too much light. To avoid it, the wood sorrel lowers its leaflets. In this way less light hits the leaflets. Other plants use another stratagem for avoiding light damages. The chloroplasts are transported from positions, where light falls perpendicular on the cells, to the lateral walls of the cells. They furthermore change their form. Instead of being spheric they become flat. This you can observe in *Selaginella serpens* (figure 2.1).

Figure 2.1: *Spike moss* Selaginella serpens*. Botanical garden Tübingen*

There are other algae which are able to change the form of their chloroplasts or to move to other places in the cell. Some examples will be described.

2.1 Chloroplasts of *Selaginella* at day and night

Numerous plants change the shape of the chloroplasts in their cells diurnally. in the morning they are flat, in the evening spheric. One of these plants is the *Selaginella serpens*. It is a Westindian shadow plant. Its funnel-like epidermis cells in the leaves contain just one chloro-

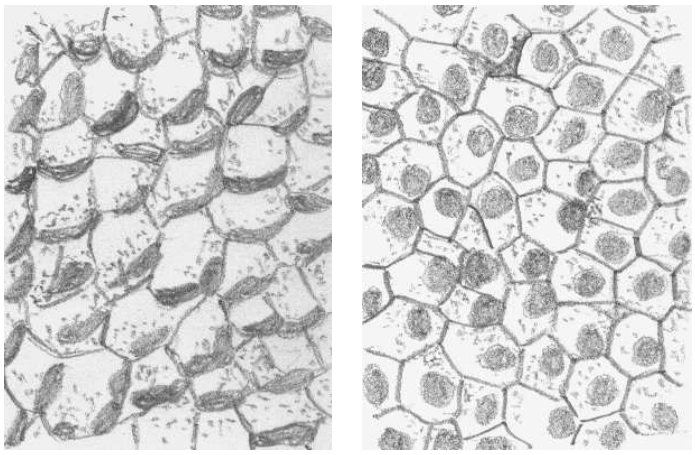

Figure 2.2: *The chloroplasts in the epidermis cells of a* Selaginella*-leaf are flat during the day (left) and spheric in the night (right). Each cell has just one large chloroplasts.* Drawing of the author after a *figure in Busch (1953)*

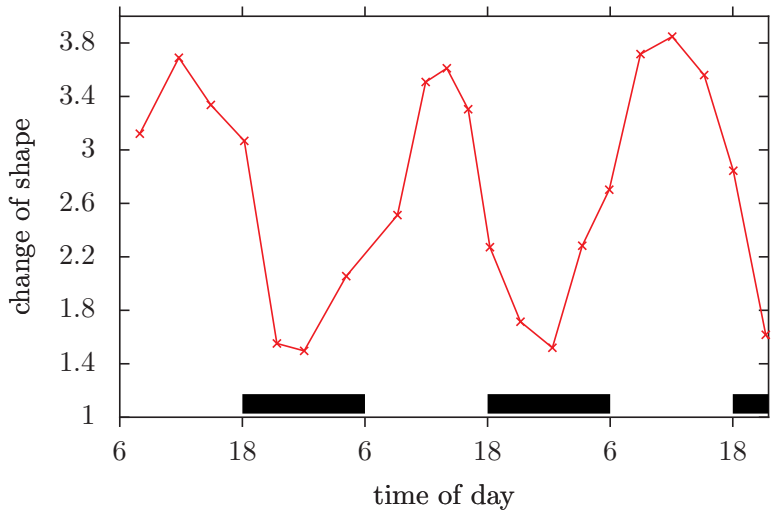

Figure 2.5: *Change in shape of chlorplasts of* Selaginella *at different times of the day under 12:12 hour light-dark changes (light from 6 to 18 o'clock, darkness from 18 to 6 o'clock, black lines). The ordinate is the length/width index, a measure of the change in shape of the chloroplasts. After Busch (1953)*

plast.[1] It is large and can change its shape and position during the day (figure 2.2). Busch (1953) has studied these changes in form and translocations of chloroplasts. For this purpose she measured the chloroplasts of the upper epidermis cells of leaves at certain times of the day at the same position in the leaf (red square in figure 2.3) and showed the results as a figure (figure 2.4) and diagram (figure 2.5). During the day

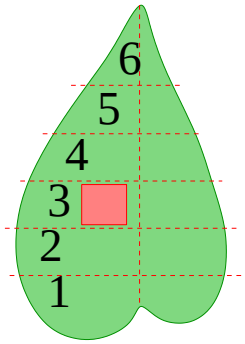

Figure 2.3: *The chloroplasts in the epidermis cells of a* Selaginella*-leaf do not change their shape in all regions of the leaf (1-6) simultaneously at the different times of the day. Instead, the transition from the day- to the night-shape starts from below and continues to the top. For this reason always the region in the third section which is labelled red was chosen. After Busch (1953)*

the chloroplasts are flat and broad, during the night spheric. The shape in between is oval (figure 2.4).

If the pH-value of the cytoplasm is determined at different times of the day, it varies. At night it is lower (pH 5.6, acid) as compared to day measurements (pH 5.9,

[1] The epidermis cells of the lower side of the leaves show the same specialty. However, the chloroplasts of cells, which lie in the interior of the leaf (mesophyll), do not change their shape during the course of a day.

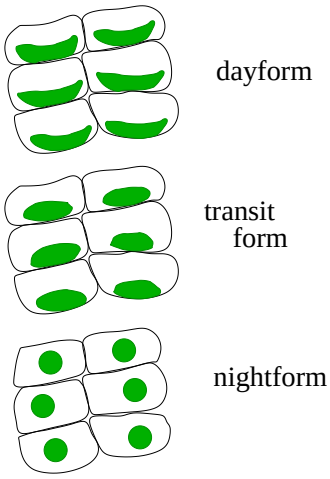

Figure 2.4: *Chloroplasts of* Selaginella *at different times of the day. During the day the chloroplasts are flat and broad, during the night spheric. The shape in between in oval. After Busch (1953)*

figure 2.6). If an acid buffer is added to the leaf praparation, the shape of the chloroplasts can be changed. Buffer with a pH of 5 to 6 lead to the flat day form, buffer with either lower (pH 4-5) or higher (6.5-7) pH to the night form. More extreme values denaturate the chloroplasts or make them crack (figure 2.7).

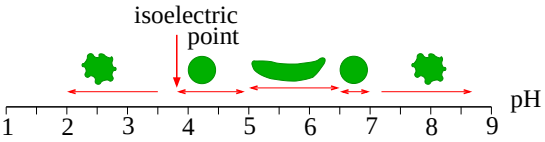

Figure 2.7: *Change in chloroplast shape induced by acid buffer. Buffer with a pH of 5 to 6 induce the flat day form, buffer with lower (pH 4-5) or higher (6.5 to 7) pH-values the day form. More extreme values denaturate the chloroplasts or make them crack. From Busch (1953)*

The chloroplasts are negatively charged. It is possible to make them move to the

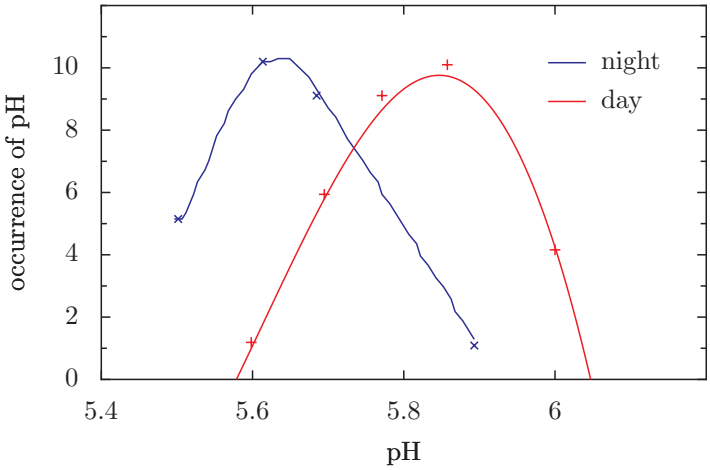

Figure 2.6: At *12 o'clock and at midnight the pH-values in the cytoplasm of the upper epidermis cells of* Selaginella *were measured and plotted. The values (blue) are lower during the night (more acid; they fluctuate around a pH of 5.6) as compared to day values (red, values fluctuate around 5.9). Nach Busch (1953)*

cathode (negatively charged electrode of the electric field) by applying an electric field. It is noteworth that all chloroplasts are positioned with their convex (bend) side to the shoot of the twig.

2.2 Chloroplast movements and change in shape in other plants

The shape of chloroplasts varies during the course of a day also in other plants. This was described for example by Bünning (1942) for water lilies, tobacco and bean plants. In other plants and algae the chloroplasts move to the irradiated side of the cell (pericline wall) or the lateral walls, which are irradiated only from above (anticline walls), depending on the light intensity or the time of day.

This photomovement has been studied in detail in the fern *Adiantum*, bei the höheren plant *Arabidopsis* and the moss *Physcomitrella*. Figure 2.8 shows an example for reactions of accumulation respectively avoidence of the chloroplasts of *Adiantum*. In the shadow the accumulation reaction promotes photosynthesis, because the flat chloroplasts are directly hit by light. In strong light damage is prevented, because the chloroplasts lie at the lateral walls which are less exposed to the light. This type of reaction is especially important for plants, which grow on locationa with extreme variations in the light conditions (for example under trees).

As light receptors serve phototropines. They are protein kinases, which are activated by blue light. Flavin-mononucleotid FMN is the chromophore (pigment carrier). Phototropin 1 leads to accumulation at low light intensities. Phototropin 2 induces likewise accumulation at low light intensities, but additionally an avoidance reaction at high light intensities. Phototropines are involved as photoreceptors for the opening of stomata.

The speed of the chloroplasts is aroundb 0.3 μm per minute. They seem to move along actin filaments, calcium-ions play also a role.

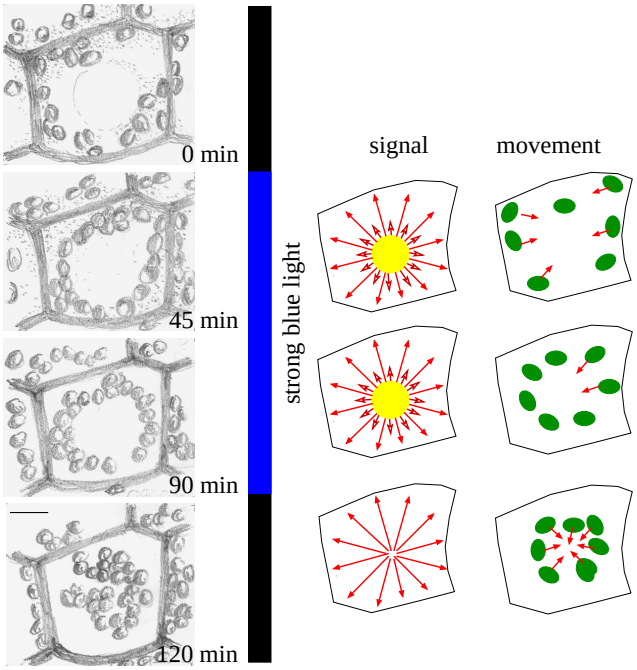

Figure 2.8: *Light reaction of chloroplasts of a prothallus in the fern* Adiantum. *In darkness the chloroplasts move to the lateral walls (anticline walls, upper left figure '0 min'). The cell was irradiated with a strong blue micro-light beam (white spot). This makes the chloroplasts move towards the irradiated area (top right and left '45 min'). They do, however, not enter the illuminated spot, because the light zis too strong ('90 min'). If the light is switched off, the chloroplasts move into the area which was illuminated before ('120 min'). The signal 'accumulate' (middle vertical row) can apparently travel long distances, but the avoidance signal not. From Wada et al. (2003)*

2.3 Chloroplast movements in algae

The chloroplasts of many algae can be translocated in the cells. As in higher plants, this increases the yield in absorbed light and prevents damage by strong light.

In several *Ulva*-species the light absorption of the thallus was measured at various times of the day. There were large differences between day and night. Blue and red light is absorbed (Britz and Seliger (1973)). The thallus of *Ulva* consists of two cell layers only. Each cell contains a single large cup-like chloroplast. During the day the chloroplast is spread out at the outer wall of thea cell, thus allowing to take up much light. During the night it moves to the profile side of the cell. This leads to the observed differences in absorption.

Parallel to the absorption the photosynthesis was recorded. Both measurements showed large differences between the day- and night phase, but also under constant conditions. This shows, that a circadian clock is involved (Britz and Briggs (1976), Britz et al. (1976)).

In the brown alga *Dictyota dichotoma* the chromatophores do also move (Nultsch et al. (1984), figure 2.10). Here the thallus consists of three cell layers, the upper and lower epidermis and an unpigmented layer in between. The phaeoplasts (several per cell) are positioned differently during the day as compared to the night. This change continues during constant darkness as a circadian rhythm (figure 3.9). The rhythm can still be observed in the *isolated* upper or lower cortical cell layer. It is assumed, that the algae are protected in this way from high light intensities during low tide.

The distribution of the the chloroplasts varies especially pronounced in large siphonophor green algae such as *Caulerpa* (Dawes and Barilotti (1969)) and *Halimeda* (Drew and Abel (1992)). In these algae the individual cells form a composed organism without cell walls in the interior. They are imbedded in a calciumcarbonate-structure and possess primary utricles at the surface (figure 2.11). During the day the chloroplasts accumulate in these utricles and absorb light for photosynthesis. In the dark period they retract in the medullar-filaments in the interior of the calcearious structure. The algae have a white appearance, whereas during the day they possess a strong green colour. Here too the translocation of the chloroplasts is controlled by a circadian rhythm. The period has the same length at various water temperatures (Drew and Abel (1995), figure 2.12).

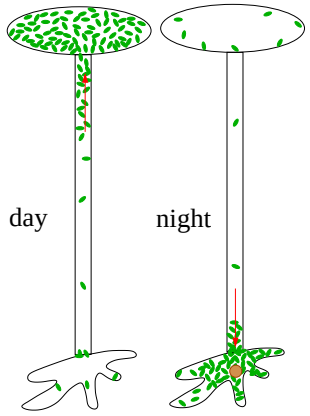

Figure 2.13: *Circadian chloroplast movements of* an Acetabularia mediterranea *cell. During the day they are accumulated in the hat and upper stalk (left), during the night in the rhizoid and lower part of the stalk. After Schweiger (1984)*

Another alga, in which the chloroplasts move in a diurnal rhythmus, *is Acetabularia* (Koop et al. (1978)). The chloroplasts migrate during the night to the rhi-

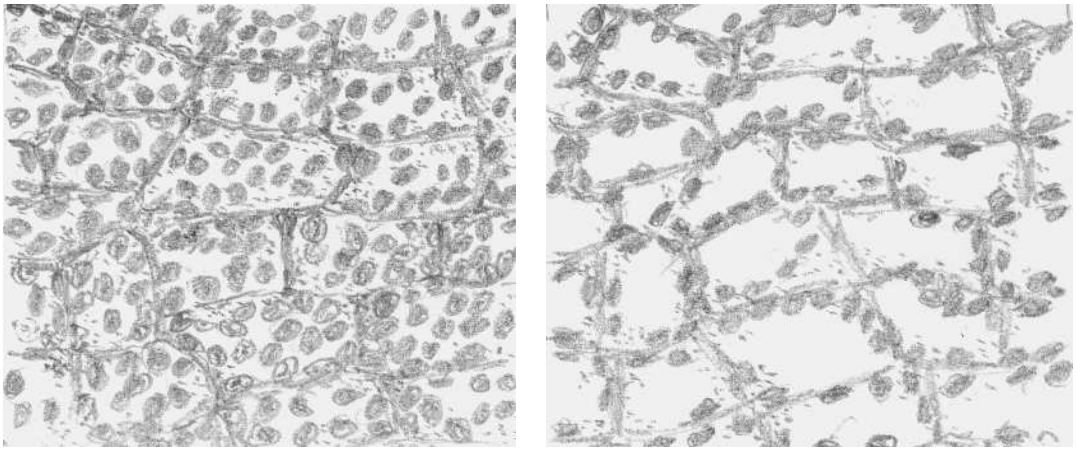

Figure 2.9: *Day- and night position of phaeoplasts in the thallus of* Dictyota. *Left: Day position with phaeoplasts spread out on the illuminated wall. Right: in the night position with phaeoplasts mainly at the lateral walls. Drawn by the author after a figure in Nultsch et al. (1984)*

zoid at the foot of the alga and during the day to the upper parts (figure 2.13). This movement is controlled by a circadian clock. The movement is observable under the microscope and can be recorded automatically with the aid of light beams photoelectrically (Koop et al. (1978), Broda et al. (1979)). Furthermore the number, shape and ultrastructure of the chloroplasts fluctuates diurnally (spheric during the dark period, oval in the light period, see Vanden Driessche et al. (1976)).

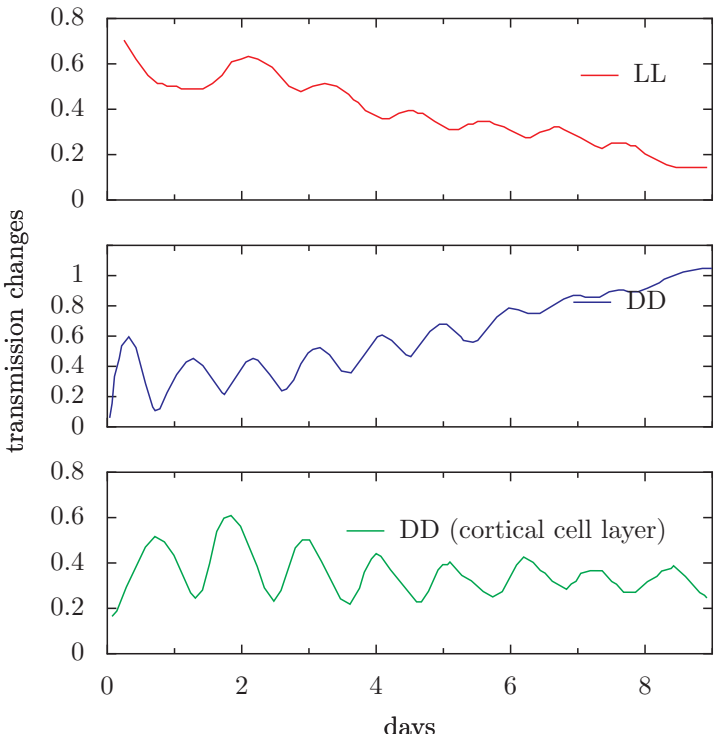

Figure 2.10: *Circadian changes of transmission in the thallus of* Dictyota dichotoma. *The transmission of light was recorded with a microphotometer using light of* $10^{-4}Wm^{-2}$ *at a wavelength of 439 nm. The transmission is a measure for the more anticline or pericline position of the chromatophores. The upper curve (red) shows the rhythm im continuous light, the curve in the middle (blue) at physiological darkness (weak blue light bei* $10^{-4}Wm^{-2}$*) and the lowest curve (green) the rhythm of an isolated cortical cell layer. After Nultsch et al. (1984)*

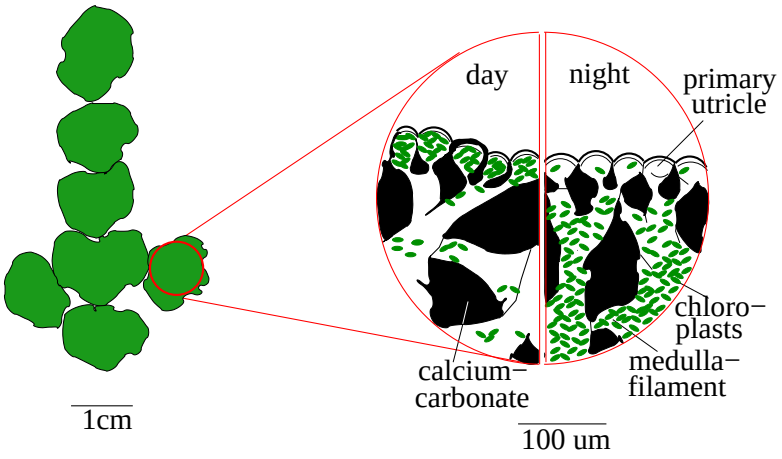

Figure 2.11: Halimeda *is a siphonophore gree alga, in which the individual cells are combined to a single body without internal walls (left illustration). They sit in a structure out of calcium carbonate and possess primary* utricle*s at the surface. The distribution of the chloroplasts varies considerably between day (left part of right illustration) and night (right part of right illustration). As a consequence the algae appear green during the day and white during the night. After Drew and Abel (1992)*

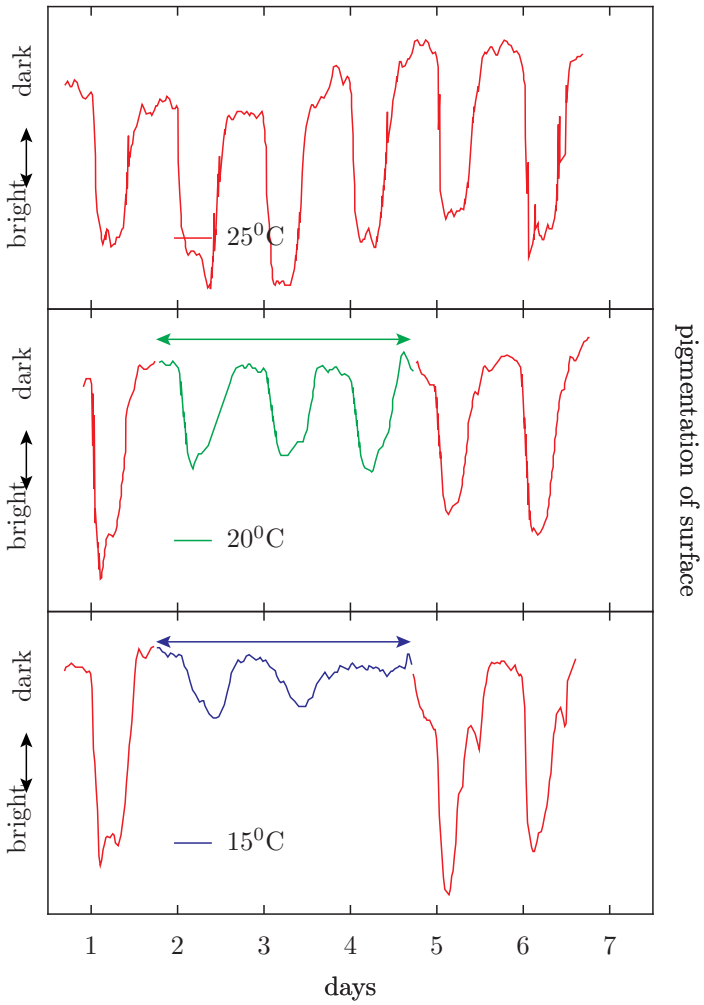

Figure 2.12: *The chloroplast movement of* Halimeda *varies stronger at higher water temperatures (compare upper diagram, 25° C, with the diagram in the middle at 20°), but the period length changes only slightly. After Drew and Abel (1995)*

3 Starch-depositions in time-layers

As a result of photosynthesis plants synthesize sugars and store them partly as starch in the starch grains of the chloroplasts and in amyloplasts during the night. Daily deposition layers are recognizable in the starch grains.

Light is the source of energy for green plants. They use in their cells special organells, the chloroplasts for the energy conversion. In the chloroplasts sugar and ATP is synthesized during photosynthesis. ATP is the energy currency of the cell, sugar the basic substance for compounds which are needed for life, growth and propagation of the plants.

During the course of a day usually more sugar is produced than used. The surplus is stored as starch. This has two important advantages: Firstly, starch needs much less space as compared to sugar, since the sugar molecules in the starch are densely packed. Secondly, starch has a very low turgor: In contrast to sugar it does not attract water.

The starch is stored in starch grains, which are either in the chloroplasts or in the amyloplasts of tubers and other storage organs. In a number of plants daily depositions of layers can be seen in the starch grains.

In this chapter we will learn, how starch is composed and structured and how it is produced and decomposed by the plants. Next we will have a closer look at the starch grains of the chloroplasts and amyloplasts and their layers of starch-depositions. Finally we will see how plants are able to find with the aid of starch grains the plumb line and use it for growing under gravity.

For deposition layers in starch grains see Wunder (1988). More detailled overviews are given by Smith et al. (1997) and Ball and Morell (2003).

3.1 Starch formation and starch disintegration

Starch grains consist mainly of starch. Besides they contain about 1% protein in form of enzymes, 3% phorphoric acid, 1% minerals and 20% water. The starch consists -depending on the plant and organ- of 15 to 30% amylose[1] and 70 to 85% amylopectin[2]. The content of amylose and amylopectin varies a lot in the different species. Starch of red algae, for instance, consists only of amylopectin. On the other hand, there are new pea varieties with an amylose content of up to 85%.

How the sugar is converted to starch during photosynthesis is shown in figure 3.1. This dimer is decomposed to monomeric sugars by UDP-sucrose-pyrophosphorylase. After phosphorylation the three produced sugar phosphates glucose-6-phosphate, glucose-1-phosphate and ADP-glucose are transported by special transporters from the cytoplasm (cytosol) to the plastids. In the plastids amylose is made with starch-synthase, and amylopectin with starch-synthase and

[1] amylon is the greek word for starch and stays for 'obtained without a mill' (a-mylon)

[2] pectin comes from the latin word for comb. It refers to the braching of the molecule

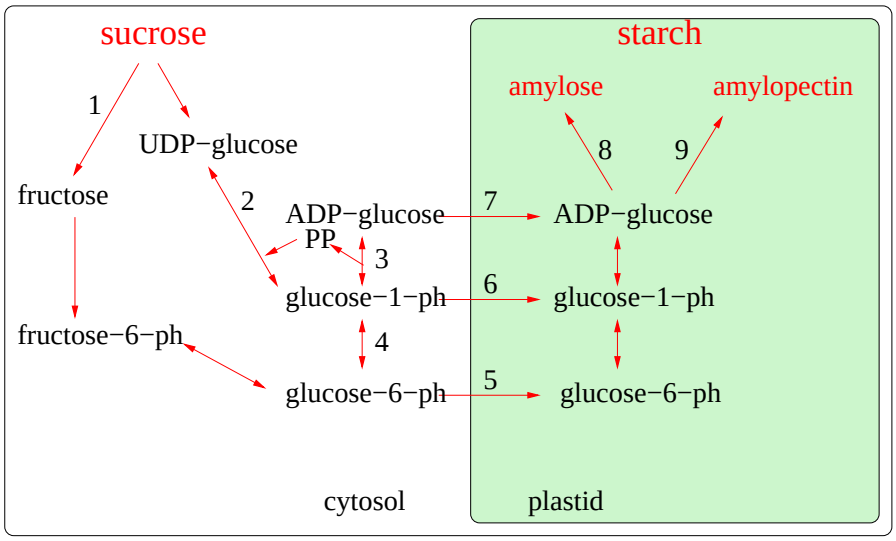

Figure 3.1: *The starch is synthesized by plants in chloroplasts or amyloplasts (plastids). It consists of amylose and amylopectin. Starting point of synthesis ist sucrose, which is produced during the photosynthesis. Three different sugar phosphates are transported by special transporters (5 and 6: hexose-phosphate-transporter; 7: ADP-glucose-transporter) from the cytoplasm (cytosol) to the plastids: glucose-6-phosphate, glucose-1-phosphate and ADP-glucose. In the plastids amylose is made with starch-synthase (8) and amylopectin with starch-synthase and starch-branching enzyme (9). Other enzymes which are involved in the transformation of sugar in the cytosol are sucrose-synthase (1), UDP-sucrose-pyrophosphorylase (2), ADP-sucrose-pyrophosphorylase (3), phosphoglucomutase (3). From Smith et al. (1997)*

starch-branching enzyme.

The chemical structure of glucose, fructose and sucrose is shown in figure 3.2. Sucrose consists of fructose and glucose. The figure illustrates also, how glucose molecules are linked to starch (or in the case of β-bindings to cellulose). Figure 3.3 shows, how glucose molecules are connected to amylose and amylopectin. Amylose consists of about one thousand to three thousand glucose molecules, which are connected by α-1,4-bindings and form a helix. In amylopectin there are additionally 1,6-bindings. They allow branching of the molecule. Mit 6000 to 50000 monomeres (individual glucose-molecules) amylopectin belongs to the largest polymers. A modell of amylopectin from Robin (1974) is shown in figure 3.4. It explains also, how starch layers might be produced. Amylopectin is synthesized by a soluble and by a starch grain bound starch-synthase. Additionally several branching enzymes and branch-dissolving enzymes (debranching enzymes such as pullulanase) are found. More in Ball and Morell (2003). In this way strongly crystalline and densely packed osmotically ineffective carbohydrate is stored. It is, however, available any time as a source of carbohydrate compounds and as fuel for the metabolism of the plants.

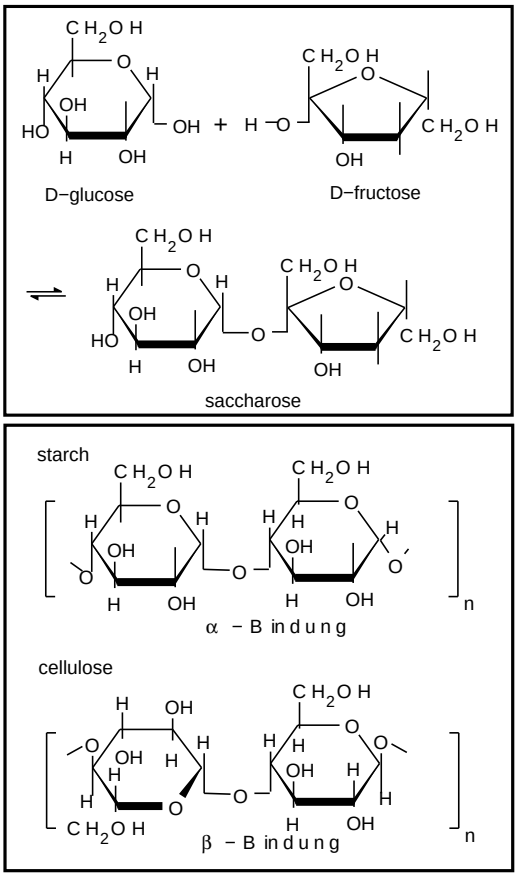

Figure 3.2: *Sucrose is made from glucose and fructose (top). Starch as well as cellulose is synthesized from numerous glucose-molecules (bottom). In the case of starch the glucose-molecules are connected with α-bindings, in the case of cellulose with β-bindings. Due to the different spacial arrangement helix-like twisted polymeres are formed in the case of starch, whereas in the case of cellulose the polymeres* are *like fibers*

It is not yet known, how the polysaccharide-synthesis is started. In animals, where glycogen is stored instead of starch, glycogenin serves as a starter. A corresponding amylogenin was assumed, but not found. It is also unknown, how the starch grain is initiated.

Number, size and shape of the starch grains are under genetic control. It is not known, which genes are responsible for it.

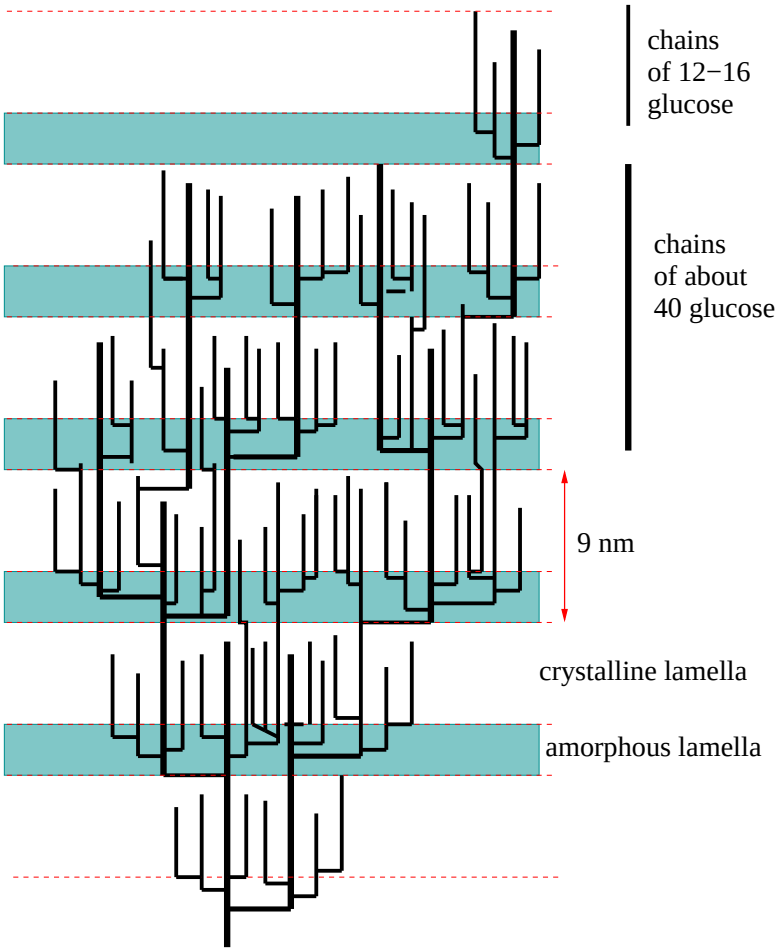

Figure 3.3: *Structur of amylopectin. From Smith et al. (1997)*

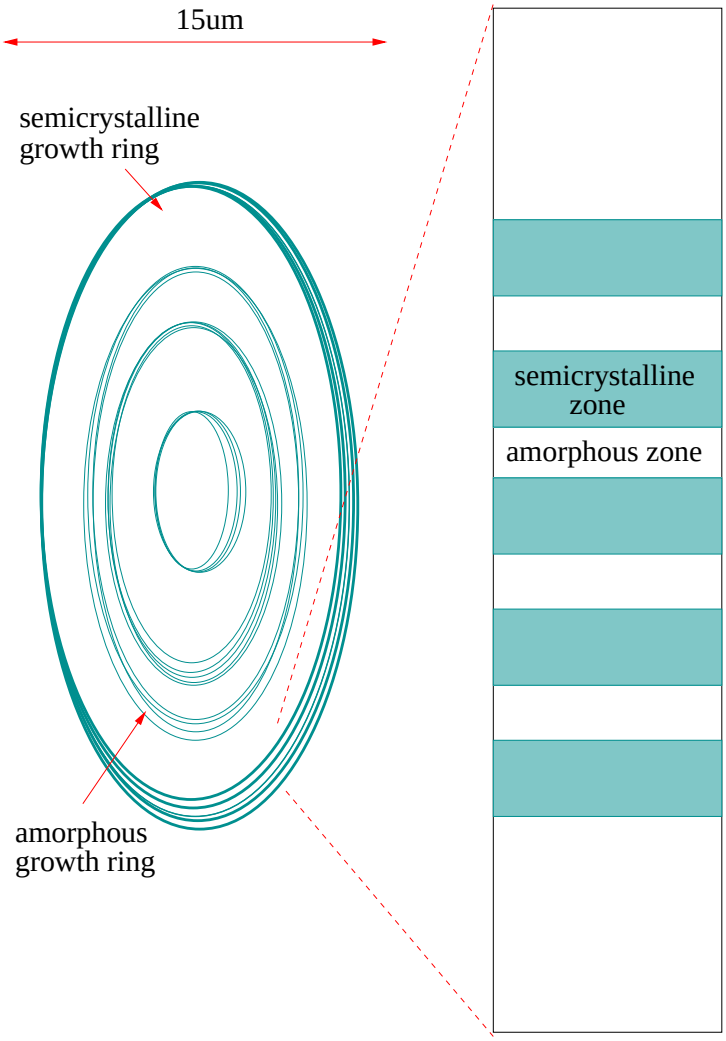

Figure 3.4: *Structure of amylopectin. Aus Smith et al. (1997)*

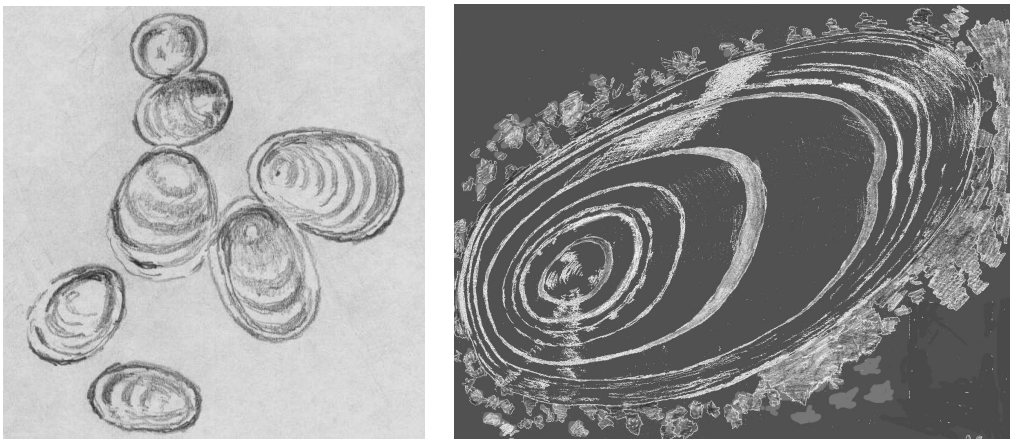

Figure 3.5: *Left: Light microscopic view of a starch grain of potatoe. Daily deposition layers are recognizable, although the plant was kept under constant temperaturee and in continuous light. Right a starch grain under the same conditions as in the left figure, but viewed under the electron microscope. Drawn by the author after a figure in Buttrose (1962)*

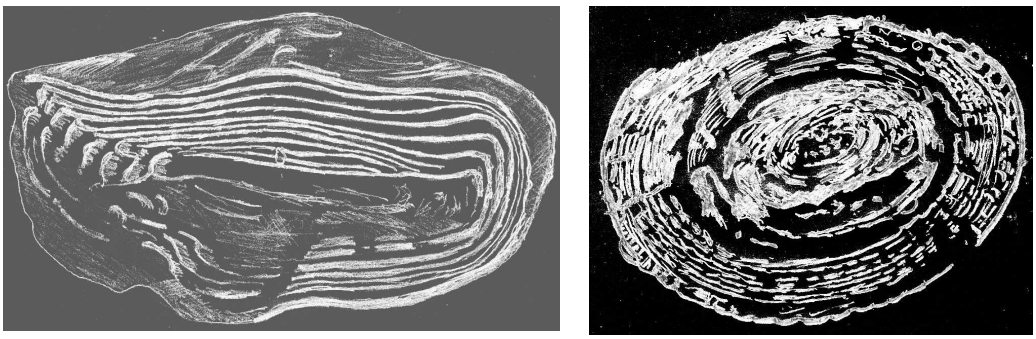

Figure 3.6: *Left scanning electron microscopic image and right electron microscopic image of a starch grain of barley. In the right figure the starch was slightly digested, which allows to see the layered structure more easily. Drawn by the author after a figure in Ball and Morell (2003)*

3.2 The layers of the starch grains

Flour consists of starch grains. They are about 50μm thick in the case of cereals and up to 100μm in the case of potato tubers. Starch grains in pollen are especially small (0.5μm). Under the light-, polarization- or electron microscope layers can be recognized in the starch grains (figure 3.6 and 3.5). They are produced every day in the case of wheat and potatoes. If the plants are kept in continuous light instead of light-dark-cycles, in contrast to potatoes no layers are found in wheat starch grains (figure 3.5). Thus, in potatoes an endogenous clock seems to control the layer formation (Buttrose (1962)).

3.3 Starch grains show plants up and down

Starch grains do not only serve plants as a means of storing carbon hydrates. They can also produce signals which help plants to orientate themself in space. A sprout growth usually upward, the main root downward into the soil. If a plant is turned to the side, one can observe, that after a certain time the amyloplasts in the cells of the tip of the root move from the original tip position in the cells to the lateral walls (figure 3.7). They induce bending of the root tips in direction of the plumb line after a sequence of reactions.

For this reaction the gravity-stimulus has first of all to be perceived. According to the statolite theory specific cells, the statocytes, are responsible for the perception (figure 3.8). They contain amyloplasts with starch grains[3] (‘statiliths´). The statiliths

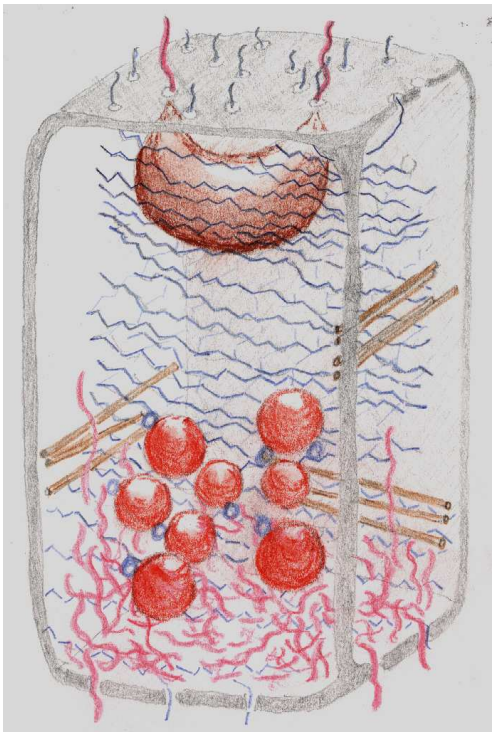

Figure 3.8: *Scheme of the effect of the amyloplasts (red spheres in the lower part) in a statocyte. They interact with a net of actin-filaments (brown), pull receptors in the plasmalemma (blue) and the endoplasmc reticulum (red threads, see figure 3.9). They perceive the gravity (direction of the plumb line) and make the root tips grow into the soil. Nucleus brown. Drawn by the author after a figure in Driss-Ecole et al. (2003)*

[3] or in the case of *Chara* vesicles with $BaSO_4$

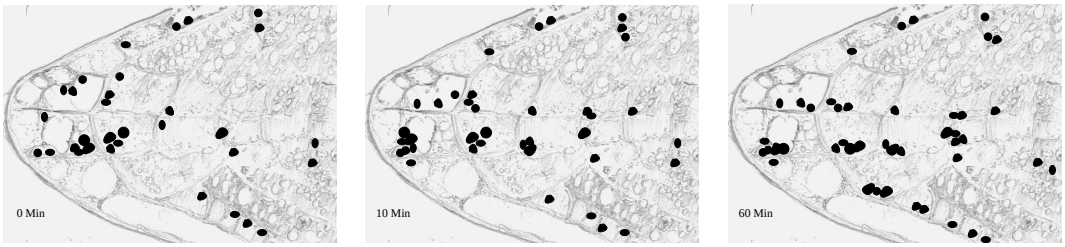

Figure 3.7: *The cells in the root tips are statocytes, which are able to perceive gravity with the aid of amyloplasts (dark grains). The left image was taken immediately after turning the root tips to the side (O min). 10 minutes later the amyloplasts had begun already to move towards the new lower side (image in the middle). After 60 minutes they have accumulated at the lower side (right image). They induce bending of the root tips in direction of the plumb line. Drawn by the author after a figure in MacCleery and Kiss (1999)*

possess a higher density as compared to the cytoplasm. It amounts to 1.3 in the case of starch, whereas the density of the cytosol and the nucleus is only 1.0. But the size of the particle is also important. Too small and too lightweight particles show Brownian movements and are therefore not suited as gravity-receptors. In favour of the statiliths-hypothesis speak a number of arguments, which are discussed by Sievers et al. (1996).

It was claimed, that mutants, which are not able anymore to produce starch grains, do still react to gravity. However, these mutants still contain amyloplasts. And they can still work even without starch grains (Kiss et al. (1989)), since their density is beyond 1.

A detailled model of the statiliths-hypothesis was put forward by Yoder et al. (2001) (tensegrity-based model). It is schematically shown in figure 3.9. According to this model the gravity-sensitive cells are supposed to consist of an actin-net in the cytoplasm. It is denser in the interior as compared to the periphery and is connected with signal-receptors in the plasma-membrane. These receptors are sensitive towards pulling forces. The statiliths (amyloplasts) are not directly connected with the cytoskeleton-net. They are able to activate or inactivate the receptors locally by destroying the net locally and thus influencing the pulling forces in the net. Assymetrically organized nodale endoplasmic reticulum could supply the system with a direction vector by protecting the plasma membrane from this direct contact with the statiliths. That actin-filamentes are present in statocyts is shown in figure 3.10.

After the gravitational stimulus has been perceived, it has to be transferred into a signal, which controls growth in such a way that the appropriate reaction occurs (vertical growth upward in the case of the shoot, downward in the case of the root, see figure 3.9). A lateral re-distribution of auxin by the gravitational stimulus (Cholodny (1926), Went (1926)) or changes in the sensitivity towards auxin (Salisbury et al. (1988), Evans (1991)) could lead to this reaction. Gravitropic experiments with coleoptils by Edelmann (2001) are in favour of another model: On that side, which will bend the organ, a factor is produced which softens the cell walls. At the opposite side

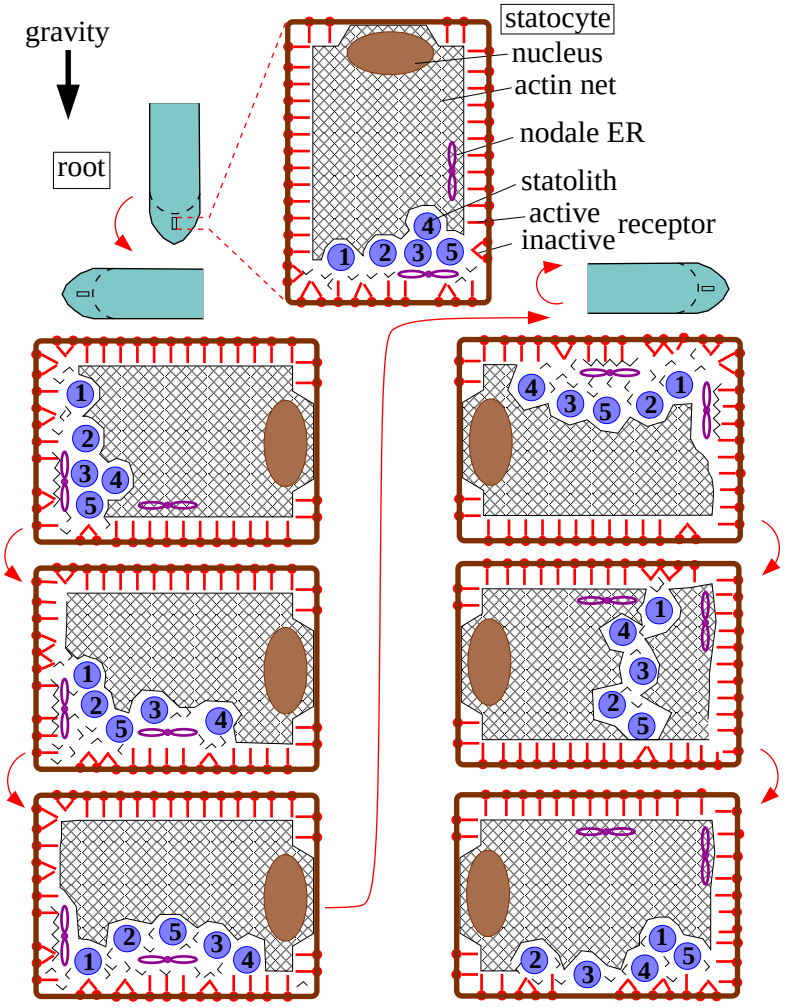

Figure 3.9: *In the root tips (columella) cells (statocytes) are present, which can perceive gravity with the aid of amyloplasts (here:1-5, blue). An actin-net (net-like marked) is denser in the interior of the cytoplasm as compared to the periphery. It is connected with a signal-receptor (red) in the plasma membrane (brown). These receptors are sensitive towards pull. The amyloplasts are not directly connected with the cytoskeleton-net. They are able to activate or inactivate the receptors locally by destroying the net locally and thus influencing the pulling forces in the net. Assymetrically organized nodale endoplasmic reticulum (ER, magenta) could supply the system with a direction vector by protecting the plasma membrane from this direct contact with the statiliths. Nucleus brown. The top image shows the situation in the statocyst of a vertically growing root tip. If the plant is bend, so that the root lies horizontally (left row of images), the amyloplasts fall, because of their higher density, down in the cytoplasm and inactivate partly the pull-receptors. If the root is turned (right row of images), the amyloplasts are dislocated to the upper side. They are now dislocated to the lower side by gravity and will make a channel through the actin net. Here too pull-receptors are partly inactivated. After Yoder et al. (2001)*

this factor is retained by the cells. By this means the shoot bends upward.

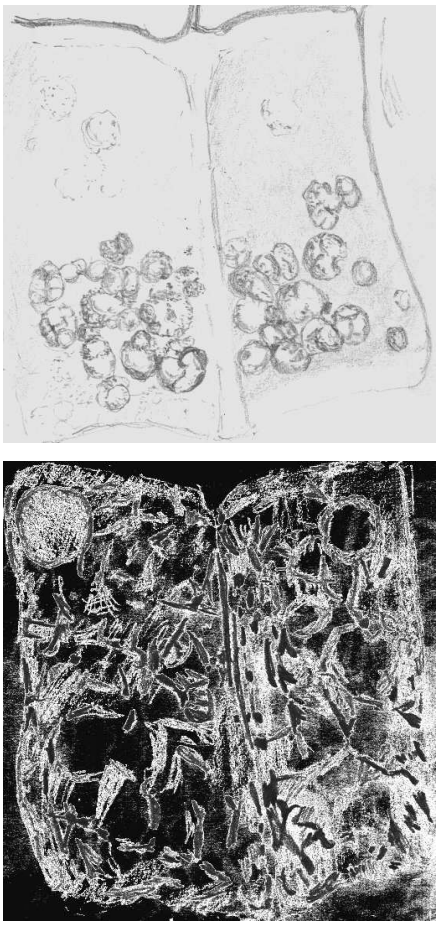

Figure 3.10: *Statocyte in the root tips with amyloplasts (top) and actin filaments (bottom). Drawn by the author after a figure in Collings et al. (2001)*

4 How snails doll up

The mantle of snails and shells form the cases. Differences in the condition of day and night show up in layers. They can furthermore be influenced by the tides, low- and high tides and differences in growth during the course of a year. The patterns can be simulated successfully by models.

Meinhardt has written a book with the German title 'Wie Schnecken sich in Schale werfen'[1]. A recent edition is in English (Meinhardt (2003)). This book describes, how the patterns of snails and shells are produced. It contains a large number of simulations and programs, which one can use and change. I recommend this book highly.

Shells and snails show often a conspicuous structure of its shells (figure 4.2). During growth the shell is enlarged by deposing new shell material at the seam of the shell by the mantle (figure 4.1). Since the depositions differ during day and night, concentric layers are the result. Tides and the spring tides can also be reflected in the pattern of the shells of sea shells. Finally annual rhythms, which are for instance the result of varying temperatures of the seawater during summer and winter, can be seen as permanent patterns in the shells. Thus, molluscs can reflect in the pattern of their shells the events of long ago aeras.

How these structures and the additonal patterns and colours of the shells are formed, was described by Meinhardt in his book and his publications. We will have a

[1] A play with words. 'Wie Schnecken sich in Schale werfen' is in English 'How snails doll up'

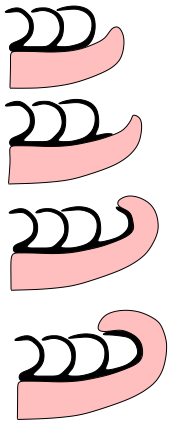

Figure 4.1: *The shell of shells are produced by the seam of the mantle. It bends upward, secrets the shell material (mainly calcium carbonate) and an additional shell chamber is formed (bottom image)*

Figure 4.2: *Structure of the shell* Macoma baltica. *The various concentric rings and sub-rings are the result of tidal, daily and monthly differences in the deposition of the calcium carbonate. From Rensing and Deutsch (1988)*

look at one of these models. It is based on the principle of positive and negative feedback.

4.1 Pattern formation by positive and negative feedback

To understand the pattern formation in a shell (or any other object), the processes have to be described which lead to the depositions. These depositions might result in coloration of the shell or to a change in structure (different thickness, ring formation), or to both. We restrict ourself here to colouration. For the involved substances three factors play an important role: The production rate of the pigment in a cell of the seam of the mantle, the dissociation rate and the interaction with the neighboring cells. All the cells of the mantle seam form the pattern of the shell during growth.

Gierer and Meinhardt (1972) showed, that in a homogenous situation a local self-amplification together with an antagonistic long-distance effect can lead to a pattern. The local self-amplification induces a constantly increasing deviation from the homogenous starting situation. This is called positive feedback. The antagonistic long-distance effect restricts the positive feedback and restricts it. The principle of this activator- inhibitor-system is shown in figure 4.3.

How such a local pattern can arise is shown in the four images of figure 4.4.

By a small local disturbance the concentration of the activator increases spontaneously. Autocatalysis amplifies this effect. With some time delay more of the inhibitor is produced, since (see figure 4.3) the activator does not only activate its own production, but also that of the inhibitor. A cloud of inhibitor substance forms around

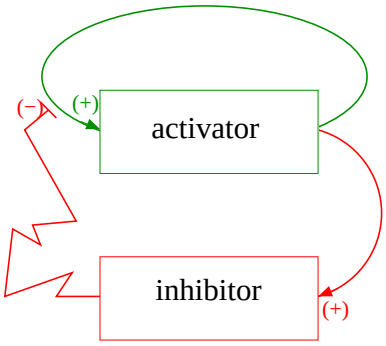

Figure 4.3: *Pattern formation by autocatalysis of an activator (green with green (+) and arrow) and* long-distance *inhibitor (red zickzack-line with red (-) as a sign for inhibition). After Meinhardt (2003)*

the activator. The inhibitor spreads, however, faster laterally (at least 7 times as fast as the activator). Furthermore the inhibitor is more quickly degraded as compared to the activator. As a consequence the activator is only locally amplified.

The events can be described by the two following differential equations:

$\partial a/\partial t = s(a^2/b + b_a) - r_a + D_a\partial^2 a/\partial x^2$
$\partial b/\partial t = sa^2 - r_b b + D_b\partial^2 b/\partial x^2$

where t is time, x the spacial coordinate (the cells are arranged side by side), $D_a$and D_b the diffusion coefficients, and r_a and r_b the degradation rate of a and b. The various terms are:

sa^2/b production rate. The activator a influences the system non-linearly and autocatalytical. The inhibitor b slows the production. s describes the capability of the cells, to act autocatalytically.

$-r_a a$ degradation rate. It is proportional to the number of the existing molecules

$D_a\partial^2 a/\partial x^2$ exchange by diffusion

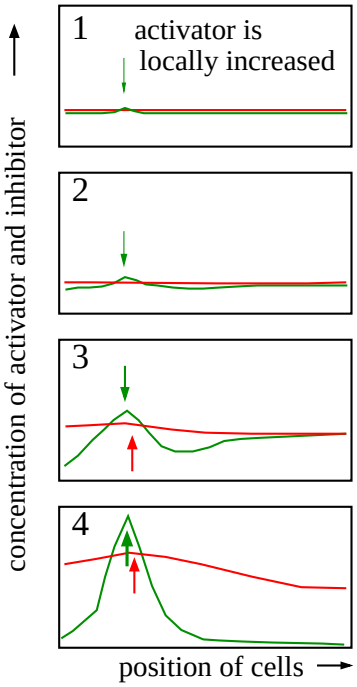

Figure 4.4: *Simulation of pattern formation by activator (green) and inhibitor (red). On the horizontal axis the cell row is plotted which is responsible for the pattern formation (cells in the seam of the mantle). On the vertical axis the concentration of the activator and des inhibitors are plotted. In the upper image 1 the concentration of the activator increases spontaneously (marked by a green arrow). Autocatalysis amplifies this effect (images 2-4, the green arrow increases in thickness). With some time delay the amount of inhibitor increases too (image 3), since the activator does not only activate itself, but also the inhibitor. The inhibitor inhibitor spreads, however, faster laterally and consequently the activator is amplified only locally. After Meinhardt (2003)*

b_a original production of the activator. Needed for the start of the system at lower concentration of the activator, for the regeneration of the pattern, for new maxima during growth and for self excited oscillations

b_b original production of the inhibitor

In nature this can lead to a striped pattern in snails, as shown for example in *Meinhardt (2003)* with the striped shells of the marine snail *Lyria planicostata taiwanica*. Such a striped pattern is also seen on the bulge of the Davidsharp *Harpa* ventricosa; however, in this case further pigment-patterns of time-dependent processes between the bulges occur (figure 4.5). How such a striped pattern develops as a function of time is shown in figure 4.6. Stripes

Figure 4.5: *Striped bulge in the shell of the indo-pacific* marine snail Harpa ventricosa. *It shows a stable spatial pattern. Between the bulges the pigment pattern is determined by other time-dependent processes. Thanks to Hans Meinhardt, Tübingen*

parallel to the seam of the mantle occur, if the activator is periodically active in all cells of the seam. More in the book of Meinhardt (2003).

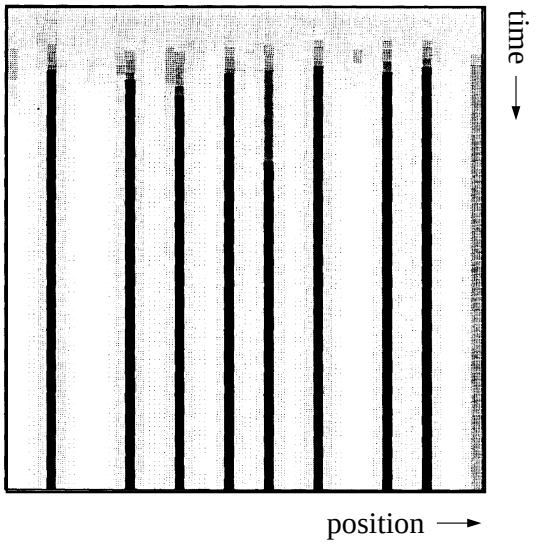

Figure 4.6: *Simulation of the pattern formation in the marine shell* Lyria planicostata taiwanica *by the model described in the text and figure 4.4. From Meinhardt (2003)*

5 Coral clocks and daylength

The deposition of calcium-carbonate in the foot of corals is another example for pattern formation. Fossil corals show, that 400 million years ago a year consisted of 400 days.

Corals are widespread in warmer oceans. They belong to the phylum of *Cnidaria* and the class *of Anthozoa*. Hard corals secret a foot (*epithek*) out of limestone (calcium carbonate $CaCO_3$). Each night a new layer is laid down (figure 5.1). There are 20 to 30 layers per millimeter, and they can be measured with a *microdensitometer*. The tides modulate the deposited layers. Furthermore annual changes are recognizable. They are caused by varying seawater temperatures during the summer and winter. If the daily deposition layers of a year are determined, it amounts to 365 in present-day corals; they reflect the number of days per year nowadays.

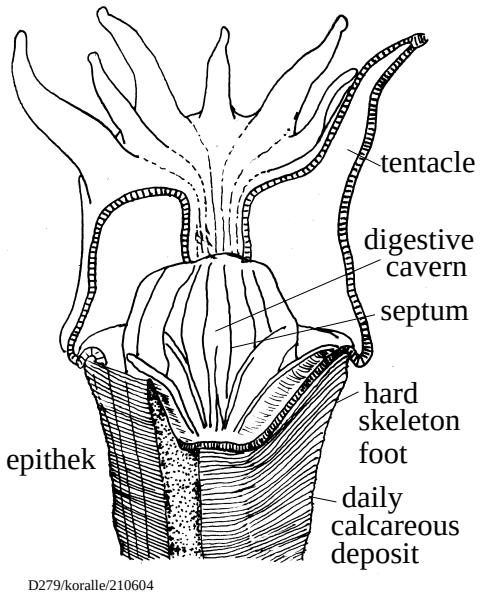

Figure 5.1: *Coral with hard skeleton foot (*epithek*). It consists of daily layers of calcium carbonate (*$CaCO_3$*). On top of the foot is the coral. With tentacles it catches food, ingests it via the gullet and digests it in the digesting cavern covered with septa. Drawn by the author after a figure in Runcorn (1966)*

Fossile corals show these layers too (figure 5.3). Astonishingly, however, 400 daily layers per year are found in corals, which lived 400 million years ago during the Devon age. Accordingly the year 400 of that time consisted of 365 days (Pannella et al. (1968)).

How can this happen? It is known that the moon moves away from the earth due to tidal frictions. If an ice skater draws a pirouette, she can slow down her turning speed by streching out the arms. The same occured earth-moon-system: If the moon moves away from the earth, the rotation of the earth slows down. Observations and calculations showed, that a day lengthens

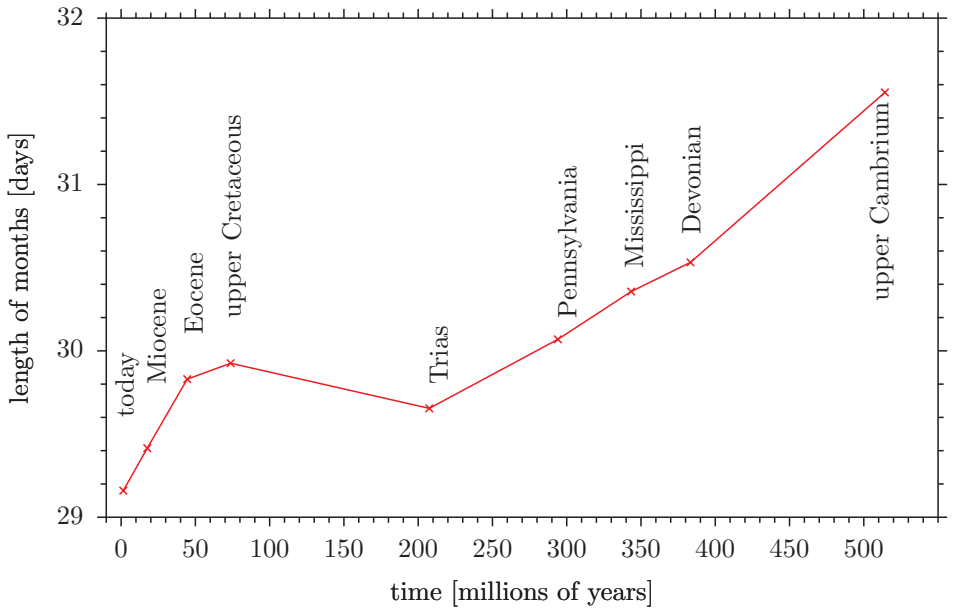

Figure 5.2: *Number of daily* $CaCO_3$*-layers (left y-axis) in the foot (epithek) of fossil corals per year from various aeras of the earth history (upper x-axis: age of the earth). Lower x-axis: Name of the aera of the earth). The y-axis shows the length of the day during the particular aera of the earth. After Rosenberg and Runcorn (1975)*

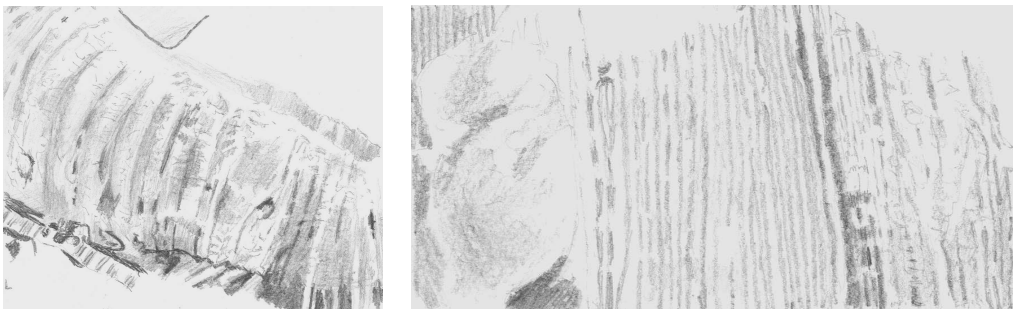

Figure 5.3: *Annual (left) and daily (right) pattern formation in the foot (epithek) of a fossil coral. Drawn by the author after a figure in Runcorn (1966)*

by 2 seconds in 10 000 years. 400 million years ago a day had therefore only 22 hours. Since the revolution of the earth around the sun has not changed, the earth year consisted of 400 days. And indeed one finds more layers per year in the epithek of fossil corals as compared to living ones [1].

Tidal and lunar rhythms can also be recognized in fossils. If one compares these 'imprints' of fossil corals with the physiological processes, which lead to time structures in pattern formation, with organisms of today, one finds in corals from the middle Devon 13 monthly bands per year instead of 12 in living corals. A month is thus today longer as compared to the time 400 million years ago.

Such geochronometer were described already by Whitfield (1898) and intensively studied by Wells (1963). There is an interesting article by (Runcorn (1966)) and a book (Rosenberg and Runcorn (1975)) on this topic. Shells, cephalopoda and stromatoliths (algae, *Conophyton*, see figure 5.4) show likewise such depositions. Recently periodic depositions was found also in bones of fossil dinosaurs (annual: Curry (1999), daily: Ricqlès (1983)), which lived about 150 million years ago. More about it in the next chapter.

Figure 5.4: *Fossil Stromatoliths from Marocco, Hamada du Guir south-southeastern Erfoud; kindly supplied by Hans-Ulrich Seitz, Tübingen*

[1] The lunar tidal friction slows the revolution of the earth by 18.1 seconds per one million years, the tidal sun-earth-interaction by 5 seconds. That makes 23 seconds per one million years Johnson (1975)

6 How old is a fly and when was a Dino grown up?

Layers are formed in various insects as diurnal depositions during the formation of the chitin armour. Cockroaches add each day a new chitin layer to their outer skeleton thus increasing its strongness. In fruitflies chitin layers were found in the clasp of the thorax where the wing muscles adhere. The age of insects can be determined by counting the number of chitin lamellae.

Insects are wraped by an outer skeleton which gives the body support and protects the animals. However, after a while the old armour has to be shed and replaced by a new one, because otherwise the growing insect would have not enough space. The chitin case is produced by an epidermis, until the final thickness is reached. In some insects it was found, that these chitin layers change their structure during the course of a day. This can be recognized for example in cross sections under the polarization microscope as bright and dark layers. The number of double layers reflects thus the age of the animals. As an example a part of a foot of a cockroach and the chitin clasp in the thorax of fruitflies is presented.

Cockroaches are ill-reputed as irksome varmints. In zoology, however, they are often used as experimental animals, because they can easily be bred. Because of their big size students learn from them the morphology and anatomy of insects. Seldom is, however, a clock in the structure of the chitin skeleton shown, which can be used to determine the age of the sexually mature animal (*imago*) (figure 6.1). More about it in the following section.

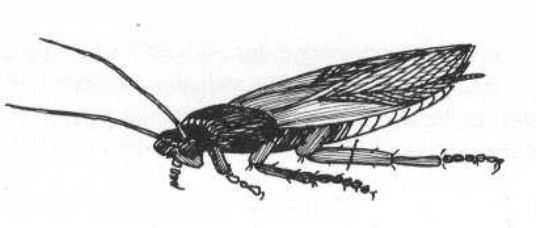

Figure 6.1: *If the femur of the hind leg of a cockroach is cross sectioned with a razor blade (see the line), bright and dark layers are recognizable under the polarization microscope in the chitin case (figure 6.3). They reflect the age of the adult animals after the last moult. After Engelmann and Klemke (1983)*

6.1 Rhythmic cuticle depositions in the outer skeleton

Immediately after the moult the cuticle of an insect is still colourless, soft and thin. After having expanded to its final size, it becomes pigmented and sturdy in a few hours. It might take, however, still several days for thickening. Often the epidermal cells secret the endocuticle not continuously spread over the day, but in a daily rhythm (figure 6.2 and 6.3). During the night Chitin is deposited in specially organized lamella as cristallite. During the day chitin is secreted in equal amounts, but not as lamella. In this way two layers

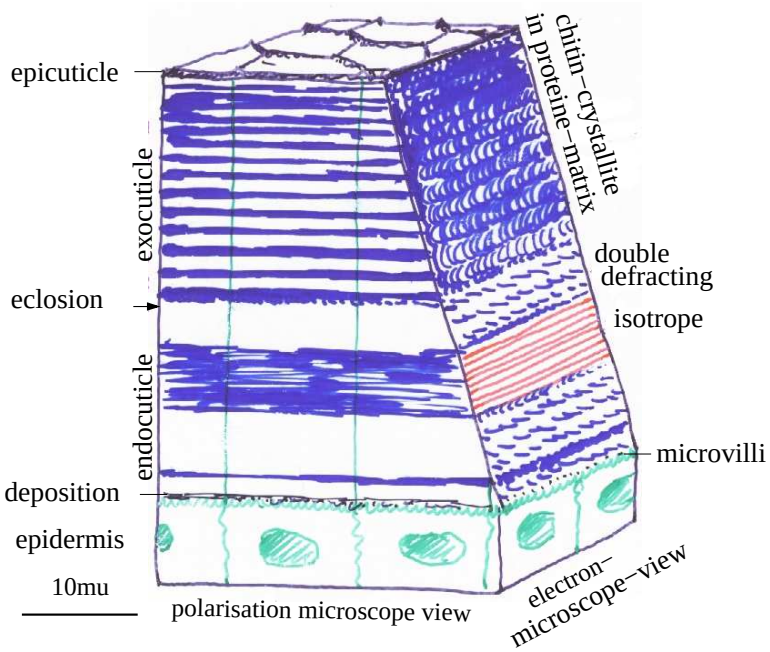

Figure 6.2: *The epidermis cells of insects (cell row below, green, each cell about 10 µm in length) produce the chitinous outer skeleton by secretion. The polygonal part of the epicuticle (very top) and the layers of the exocuticle and endocuticle (underneath) originate from corresponding epidermis cells. The front section shows the view under the polarization microscope, the right slanted section the electron microscopic view. Here you can recognize the changing orientation of the chitin-crystallite in the staggered layers. The kristallites are imbedded in a proteine matrix. Due to their orientation dark and bright layers can be seen under polarized light. If they are oriented towards the front, the layer appears dark, if they run parallel to the cut, they appear bright. First the epicuticle and the exocuticle is formed. Afterward the animal emerges (*eclosion*). Then the endocuticle is build, often in diurnal depositions. Each day a bright and a dark layer are produced. The example given thus shows the cuticle (of a new mealworm) one and a half days after eclosion (arrow). 1 (bright) and 2 (dark) layer of the first day, 3 bright layer of the second day and 4 the onset of the dark layer of the second day. Drawn by the author after a figure in Neville (1975)*

are produced each day, which appear differently under the polarization microscope: A double defracting (lamellated) and a dark (non-lamellated) growth layer (figure 6.3, Neville (1975)).

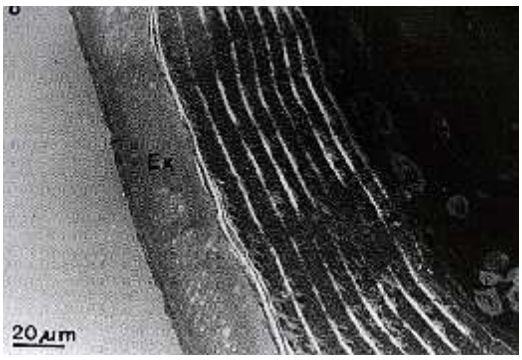

Figure 6.3: *Chitin lamella in the outer skeleton of the tibia of a leg of a cockroach (*Leucophaea maderae*). Eight bright layers (and a weaker nineth) are recognizable with dark layers in between. They are achieved, because the epidermis cells of the endocuticle are differently organized during day and night. In this way two growth layers per day are produced, which appear differently under the polarization microscope: a bright double defracting (lamellated) and a dark (non-lamellated) one. After Wiedenmann (1978)*

These rhythmic patterns are formed also under constant conditions. They are therefore not a direct reaction towards the light-dark-change or towards other environmental factors which change daily (for example temperature differences between day and night), but are instead driven by an internal clock (Weber (1994)). If migrotory locusts are kept in continuous darkness, chitin is rhythmically deposited in the cuticle for more than two weeks.

The period amounts to 23 hours. It is hardly affected by temperaturee (between 22 and 30° C the Q_{10}[1] is 1.04). In continuous light of 100 lux the rhythm damps in the course of a day. The lamella formation of the chitin is than decoupled from the internal clock. This rhythm can be synchronized by a light-dark-change. No normal photoreceptors are used, and the signal is not transfered by the neuroendocrine system, because a light-shielded leg deposits under continuous light still chitin rhythmically. The epidermis cells are thus not directly sensitive to light. The threshold of the sensitivity lies between 1 and 10 lux, the wavelengths with the strongest effect are between 435 and 520 nm.

A rhythm of chitin deposition in continuous darkness was found also in the cave locust *Dolichopoda hinderi* at 13^0C (Neville (1965)). At higher temperatures the layers were thicker, but the final thickness was not changed. That means, that at higher temperatures fewer layers were deposited.

A further example is the Weta *Hemideina thoracica,* a cricket (Orthoptera: Stenopelmatidae) from New Zealand. The pattern formation in this insect is controlled by an oscillator, which differs from the one controlling locomotor activity (Waddel et al. (1990)).

6.2 Rhythmic depositions in thorax clasps of flies

Daily growth layers were found also at the internal attachements *(Apodeme*) of the muscles of flies and mosquitoes (figure 6.4). They can be used to determine the age *of Drosophila* flies which were caught outdoors (Johnston and Ellison (1982) and figure 6.5).

[1] the Q_{10} shows, how much faster or slower a reaction proceeds at a 10° higher temperaturee. At a Q_{10} of 2 the reaction is twice as fast at a 10°C higher temperaturee, at a Q_{10} of 1 the reaction has the same speed. At a Q_{10} smaller than 1 the reaction is *slower* at higher temperatures.

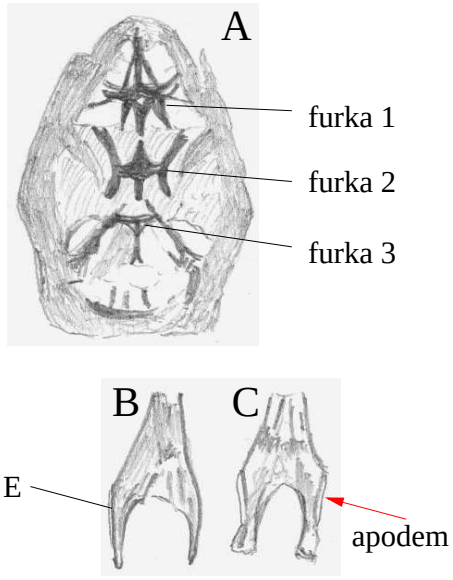

Figure 6.4: *A: View from underneath at the thorax of a* Drosophila*-fly. The muscle clasps 1 to 3 are labelled (furka 1 bis 3); Furka 3 has been dissected out and is shown in the lower part (B and C). In B the furka 3 of a recently eclosed animal is shown. The* attachements of *the muscles (Apodem) have not grown yet. Therefore only the endodermis layer E is visible at the edge. The image C to the right is from the furka 3 of an 8 day old fly. The apodem (arrow) has grown on top of the endodermis and shows layers, which are magnified in figure 6.5. Drawn by the author after a figure in Johnston and Ellison (1982)*

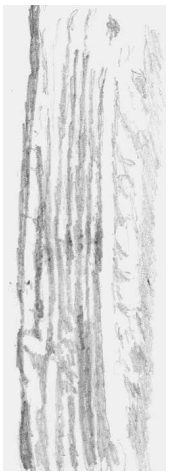

Figure 6.5: *Chitin lamella at the attachment of the muscles (*Apodem*) in the thorax clasp 3 (furka, see figure 6.4) of fruitflies (*Drosophila mercatorum*). At the right is the layer immediately after eclosion of the fly, the layers to the left were produced on the following days. The line at the bottom right corresponds to 10 µm. Eight double layers can be recognized. They exist, because the epidermis cells of the endocuticle deposit the chitin lamella during the night differently organized as those deposited during the day. In the polarization microscope a bright and a dark double defracting layer (lamellated) is observable for each day. Drawn by the author after a figure in Johnston and Ellison (1982)*

6.3 The age of young Dinos

Layers are produced also in an annual rhythm, as demonstrated by the annual rings of the trees (figure 0.2). They exist, because the growth conditions in our latitudes vary strongly during the course of a year.

Those annual rings were found also in dinosaurs. They show up in the bone structure during growth. It was assumed so far, that a giant dinosaur as for example the *Apatosaurus* (figure 6.6) needed many years before reaching adulthood. However, Curry (1999) could show annual depositions on shoulder bones which proof that such a hulk was grown up already after 8 years. This makes surely sense, because a young dinosaur lived constantly under the danger, to be stamped on by its mother, if it or she did not pay attention. Physiologically such a fast growth is indeed possible. If the bones of a goose of today would grow in the same speed for eight years instead of just one, until grown up, the product would be an animal of the size of a dinosaur.

Why the dinosaurs grew so large and what kind of advantage it had is currently intensively discussed. Presumable at the time of the dinosaurs the climatic conditions were such that they had to migrate long distances in order to have enough food throughout the year. From their physique it is known that they were not especially fast. But due to their large size they made considerable headway, because each step brought them quite a bit forward.

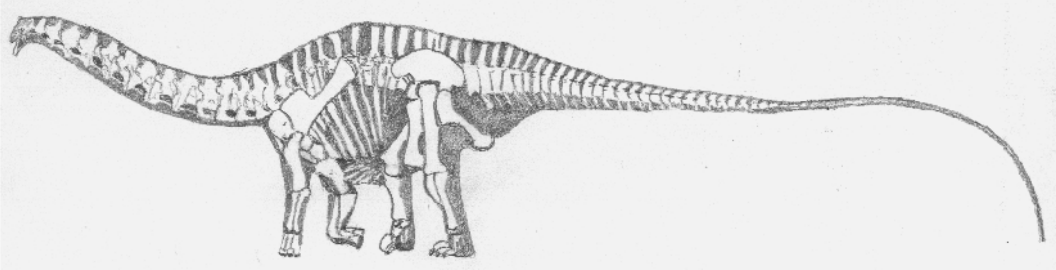

Figure 6.6: *Skeleton and profile of a giant dinosaur* Apatosaurus. *In the bladebones growth layers were found which show, that these animals were grown up in 8 to 11 years. The adult animals lived probably several hundred years and had a weight of 30 tons. Drawn by the author after a figure in Curry (1999)*

7 Of fairy rings and spore stripes: Fungal growth and propagation

Fairy rings of mushrooms originate, if the fungal mycelium extends radially and forms fruiting bodies after a certain time. Fairy rings are also found on fruit of orchard trees. Daily rings are produced by the red bread mould Neurospora crassa. *They are controlled by an internal clock. It has been studied, how these clocks function on a molecular biological level and how the rhythm of sporulation is synchronized by the light-dark-change of the day. In other fungi the rings are separated from each other exactly one day, or several days, or weeks.*

Fairy rings of fruiting bodies of mushrooms are occassionaly seen on meadows or in the woods (figure 7.1). They originate by a radial growth of the fungal mycelium and the formation of fruiting bodies ('mushrooms') after a certain time (or are induced by favorable environmental conditions.

Often one can find fairy rings on fruit in the fall (figure 0.3). If spores of certain fungi are inoculated on a solid nutrient medium in a dish, they grow like a fairy ring radially and form after a certain time spore carriere which form a ring. The mycelium continues to grow and forms after some time again such a ring. Finally the dish is covered with concentric rings. Depending on the fungus, the rings are separated from each other by exactly one day, several days, or weeks.

Daily rings are formed by the red bread mould *Neurospora crassa* (figure 7.5). The internal clock, which is responsible for it, has been studied intensively in this fungus. Today it is known how this clock works on a molecular biological level and how the rhythm of the sporulation is synchronized by the day. For these studies growths tubes were used, in which a hot nutrient agar medium is poored. After cooling down a few spores can be inoculated at the entrie of the tube (figure 7.2). The spores germinate and grow in growths tubes containing a nutrient medium. Daily conidial bands are formed. If the growths front is marked each day at for instance 12 o'clock with a bar on the glass tube, the time between two conidial bands can be measured with a ruler. Since the growth is uniform, the distance can be calibrated in 'time' and the period length of the rhythm determined.

Light suppresses this rhythm. Already $4.2\,erg/cm^2sec$ suffice (figure 7.3).

The period length of the rhythm is at different temperatures the same, although the growth rate depends heavily on it (at 10°C higher temperature the mycelium grows twice as fast, figure 7.4). This property is typical for true circadian rhythms.

7.1 The time history of the mould *Neurospora*

If a spore is placed at the edge of a Petri disk with an adequate agar-medium, it germinates and the mycelium grows over the agar surface to the rim of the disk. Daily rings of spores are produced, which at-

Figure 7.1: *Fairy ring of chestnut mushrooms in a clearing. Drawn by the author*

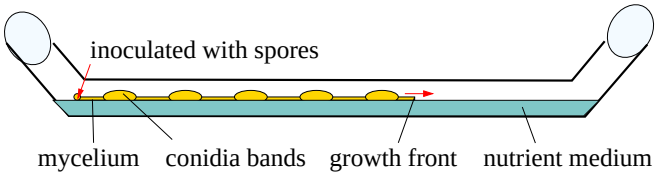

Figure 7.2: *Spores of* Neurospora crassa *are inoculated on agar containing nutrients (left). The spores germinate and grow in the growth tube (red arrow). Daily conidial bands are formed (see figure 7.6). If the growths front is marked each day at for instance 12 o'clock with a bar on the glass tube, the time between two conidial bands can be measured with a ruler. Since the growth is uniform, the distance can be calibrated in 'time' and the period length of the rhythm determined*

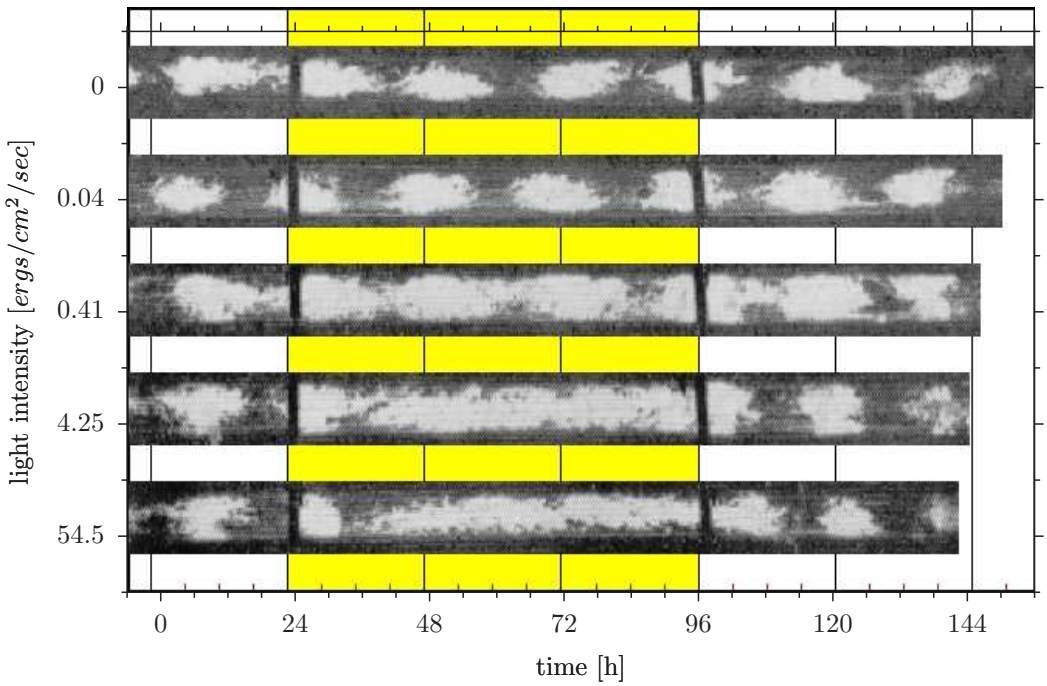

Figure 7.3: *Growths tubes with mycelium of* Neurospora *were transferres from the dark for 72 hours (yellow background) in continuous light of various intensities (shown on the y-axis). The rhythmic conidia formation is suppressed allready at intensities of* $4.2\,erg/cm^2 sec$. *After Paietta and Sargent (1983)*

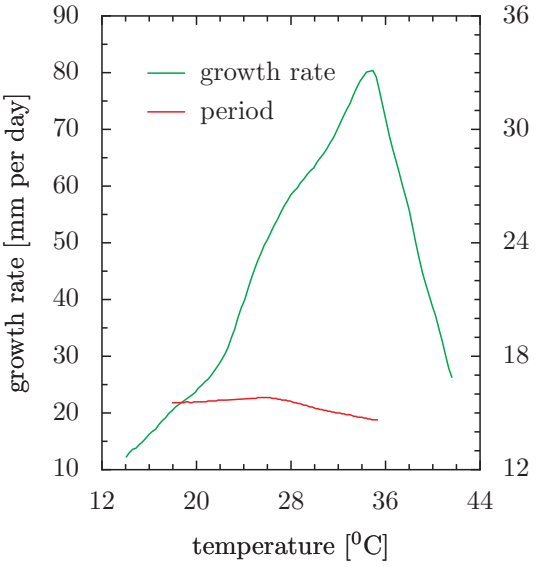

Figure 7.4: *The period length of the circadian conidia formation in* Neurospora crassa *does not depend on the temperature between* 18^0 *and* 30^0 *(red curve, right y-axis), whereas the growths rate is strongly dependent (green curve, left y-axis). After (Gardner and Feldman (1981))*

tract more attention as compared to the mycelium because of their yellowish coloration (figure 7.5). Seen from the side the

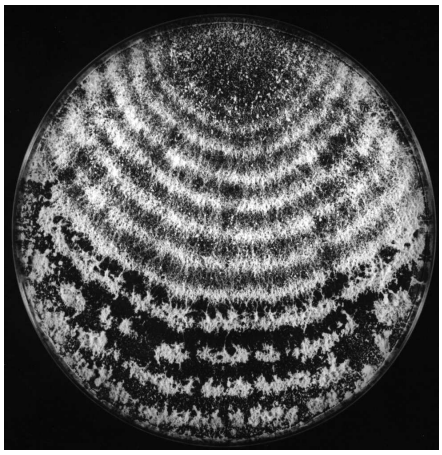

Figure 7.5: *Conidiospores of* Neurospora crassa *were inoculated on an agar medium and began to germinate from this place under continuous darkness, to start with. Rings of conidia were formed in a circadian rhythm. A 12:12h light-dark-cycle synchronized the conidia formation. The formation of bands begins briefly before lights-on and is completed a few hours afterward*

mycelium starts to grow on the mycelium after germination of the spores. After a certain time the mycelium grows upward towards the surface and aerial hyphae are produced. At the aerial hyphae conidiophores are made with conidia (macrospores) at the tips. This occurs each day (figure 7.6).

7.2 How the circadian clock of *Neurospora* works

Neurospora is especially well suited to find out the clock-mechanism, which controls the timing of conidia formation. The fun-

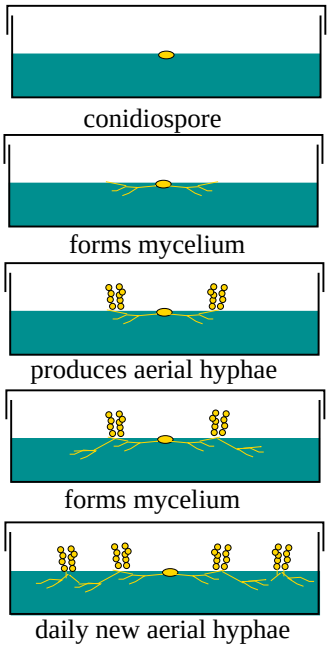

Figure 7.6: *From top to bottom: Conidiospores of* Neurospora crassa *germinate. They grow as hyphae on a substrate forming a mycelium. After some time aerial hyphae are produced by growing upward out of the substrate and conidiophores are formed at the tips. Afterward the mycelium grows again normal in the substrate, until the next conidial bands are made. After Rensing (1993)*

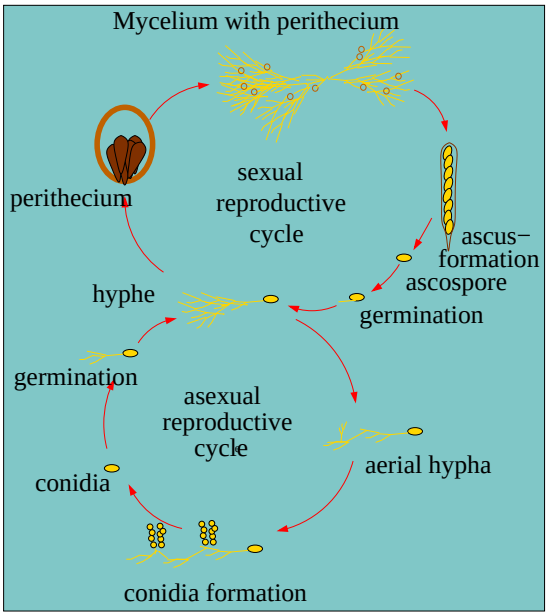

Figure 7.7: *Developmental and generation* cycle of Neurospora. *Sexual (top) and asexual (bottom) reproductive cycle* of Neurospora. *Top: After germination the ascospores germinate and form a mycelium (coenocytic, that is, many nuclei share a common cytoplasm). Via properithecia perithecia are formed. In a perithecium asci are produced in which ascospores develop again. The asexual cycle of propagation has closed. Bottom: In the asexual reproductive cycle aerial hyphae are formed, which produce later conidia (‘macroconidia´). They germinate and form new mycelia. The switch between undifferentiated mycelium and aerial hyphae is controlled by a circadian clock. After Russo (1986)*

gus is easy to rear, can be crossed and many mutants are available. The recording of the circadian rhythm of conidia formation is simple.

To find out, how the clock works, it was tried first to add substances and inhibitors which interfere with certain parts of the metabolism. It was checked, whether the circadian clock was influenced by the treatments: Did the period of the clock change or did the clock stop running? If the substances were administered for a certain time only, they might shift the rhythm. This type of experiments showed, that membranes and fatty acids are important. Inhibitors of protein synthesis did also affect the clock.

More effective were, however, experiments with mutants. Known biochemical mutants were, for instance, studied in order to find out whether the rhythm had changed. If not, the affected metabolic path is not essential for the clock. Mutants can furthermore be used, to change the properties of the clock. The next step is, to find out how the mutant differs from the wild type.

More than 5000 mutants of *Neurospora crassa* are known. In some of them the rhythmic conidia formation is affected (overview Lakin-Thomas et al. (1990)). Some of the clock-mutants have lost the temperature compensation and in others the sensitivity towards light is affected (Loros et al. (1986), Loros and Feldman (1986), Gardner and Feldman (1981), Dharmananda (1980)). There are also mutations in which the period length is changed. These mutants are especially interesting, because the properties of the clock have changed.

Thus the *frq* mutants has been studied intensively. They possess faster or slower circadian clocks in respect to the wild type, but a normal growths rate. The *frq* gene is located on chromosome IV R. The gene and its product plays a decisive role for circadian rhythms (overview of genetic studies: Feldman and Dunlap (1983); overview of the molecular biological studies Dunlap (1993), Aronson et al. (1994), Loros (1995), Liu et al. (1997), Dunlap et al. (1998), Lakin-Thomas (1998), Loros and Dunlap (2001)).

In the *Neurospora*-system circadian control, control by light, control of the metabolism and developmental controls interact with each other. The mechanism is therefore quite complicated.

We will briefly talk about the circadian control of the clock mechanism, its control by light and its function on a molecular level. We will get to know the player, to start with, than the play (the interactions between the players), and finally the rules and goals of the game.

7.2.1 The player and the stage

The product FRQ of the *frq*-mRNA of the *frq*-gene is one of the main players in the circadian game of *Neurospora crassa*. The *frq* gene was cloned and sequenced. It is a 7.7 kb DNA with two transcripts (4 and 4.5kb). All *frq*-mutations are point mutations: Only one single amino acid is changed in the proteine.

There are two more important players, White Color WC-1 and WC-2. They are expressed by the *wc*-1 and *wc*-2 genes. They were cloned and are transcription factors, which play a role during signal transduction and perception of light .

A further player in the circadian system of *Neurospora* is the vivid gene (vvd). It transcribes VVD, a recently discovered member of the PAS proteins. It was cloned and characterized (Heintzen

et al. (2001)). It affects input *and* output of the clock without being a part of the clock-mechanism (vvd null-mutants are still rhythmic).

Other players must be involved in the circadian system *of Neurospora*, since *frq*-null mutants are still rhythmic (although not circadian). These players are so far not yet known (see subsection 7.2.2).

7.2.2 The play

Especially by molecular biological studies of the group of Dunlap a model for the circadian clock of *Neurospora* was developed (figure 7.8). According to it the product FRQ of the *frq* gene is an essential component of the circadian oscillator. The mRNA and the FRQ proteine of the *frq* gene are parts of the feedback system. In it FRQ controls its own expression via the *white color* complex WCC (Lee et al. (2000), Aronson et al. (1994)). The proteine synthesis is important for the transfer of the blue light-signal to the circadian clock. FRQ is more and more phosphorylated with time, especially by a calcium/calmodulin-dependent phosphokinase. Phosphorylated FRQ is, however, decomposed, and therefore its concentration decreases with increasing phosphorylation (Yang et al. (2001)).

Light influences the circadian system by making the *frq* gene work: It is activated by the WCC-Komplex (figure 7.8, and figure 7.9). It interrupts the negative feedback of FRQ on its own synthesis. The model explains the effect of single light pulses on the rhythm of conidia formation in continuous darkness, the behaviour in light-dark-cycles and in photoperiods consisting of only two short light periods per day. It explains furthermore, how a light signal advances or delays the rhythm depending on the phase, at which it was administered. Independent on the effect on the clock WCC transfers light signals als to light-sensitive and clock-controlled genes. There are other genes, which are controlled by the clock as well as directly by light.

Finally the vivid gene (vvd) is involved in the play. It influences entrance points and outputs of the clock. It is induced by light, but independently controlled also by the circadian clock, without being a part of the clock-mechanism.

Temperature

Like light pulses temperature pulses are also able to shift the circadian rhythm of *Neurospora* (Francis and Sargent (1979), Liu et al. (1998), Gooch et al. (1994)). Up to certain limits the period length is only marginaly affected by the environmental temperature. The effcts of temperature pulses as well as temperature compensation of the clock can be explained by the molecular-biological model.

Temperature compensation is achieved, because -depending on the temperature- different amounts of two distinct species of FRQ are produced (Liu et al. (1997)). At higher temperatures more sFRQ is made, at lower temperatures more lFRQ. Thus the ratio of the two FRQ's depends on the temperature. The two FRQ-species determine the period length in different ways: With more sFRQ the period length is shorter, with more lFRQ longer.

FLO oscillator The role of FRQ was recently newly interpreted in two respects: One group doubts, whether it is indeed an essential constituent of the circadian clockwork (that is, a wheel in the clockwork). They claim, that FRQ is only involved in processes before the actual oscillator and (via lipid-signals?) act on the real oscillator

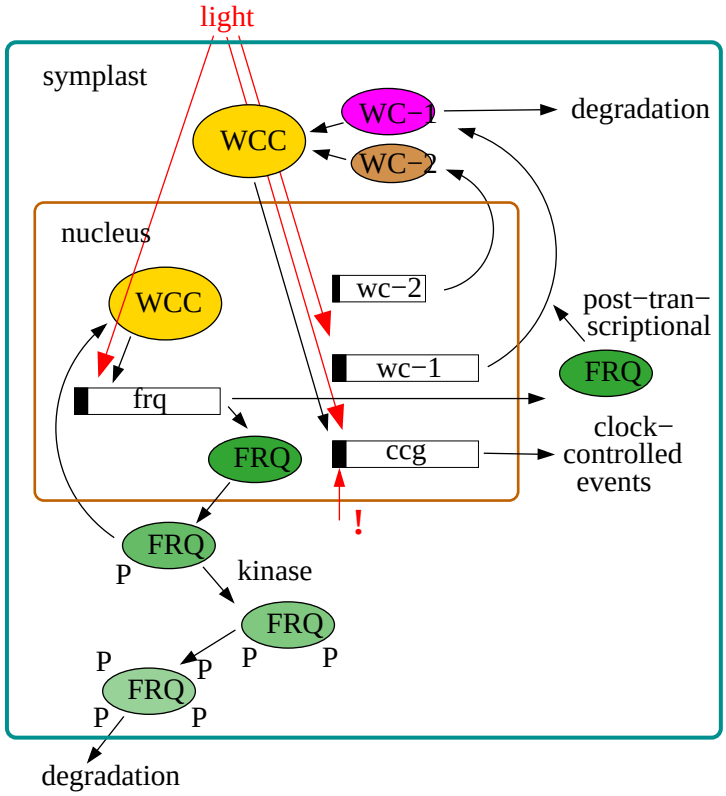

Figure 7.8: *Model of the feedback oscillator* of Neurospora crassa. *The production of mRNA and FRQ protein of the* frq*-gene is part of the feedback system in the circadian clockwork. FRQ plays several roles. It regulates the* frq*-mRNA via trans-acting factors of circadian controlled elements (CCRE's) and induces in this way a specific transcription at certain times of the day. It furthermore activates directly or indirectly genes, which are thus controlled by the circadian clock. They are therefore called 'clock controlled genes' (ccg's).*
Light influences transcription of the frq*-gene. The protein WC-1 relays the light signal. Light influences furthermore the clock-controlled genes (ccg's) also directly. Likewise the wc-1-gene is influenced by light directly. (After Dunlap et al. (1998))*

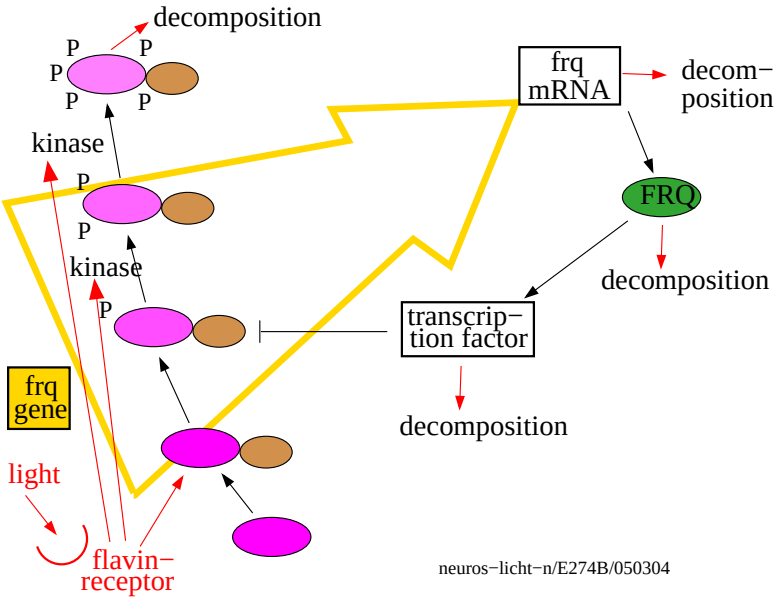

Figure 7.9: *How light affects the molecular feedback-oscillator of* Neurospora*: WC-1 (magenta) and WC-2 (brown) play a decisive role. WC-1 is expressed constitutively in the dark (below). Under light it forms together with WC-2 the WCC complex. This newly synthetized WCC (second from below) is in light as well as in darkness phosphorylated by kinases (P) and thereby inactivated and dismantled. Light is perceived by a flavin-receptor and activates (red arrow at flavin receptor) the kinases stronger. This amplifies the* frq*-transcription (frq-gene yellow, yellow hemed arrow, frq-mRNA in box top right). In this way the phosphorylation of the WC-1 in WCC is increased (fourth from below). More FRQ (green oval) is formed. FRQ produces a transcription factor (box), which inhibits the formation of weakly phosphorylated WCC (third from below) in the dark (inhibition sign). In light the transcription factor is not able any more to inhibit the phosphorylated WCC.*
Result: The clock-protein FRQ inhibits its own transcription, but light suppresses this inhibition. After Ruoff et al. (1999)

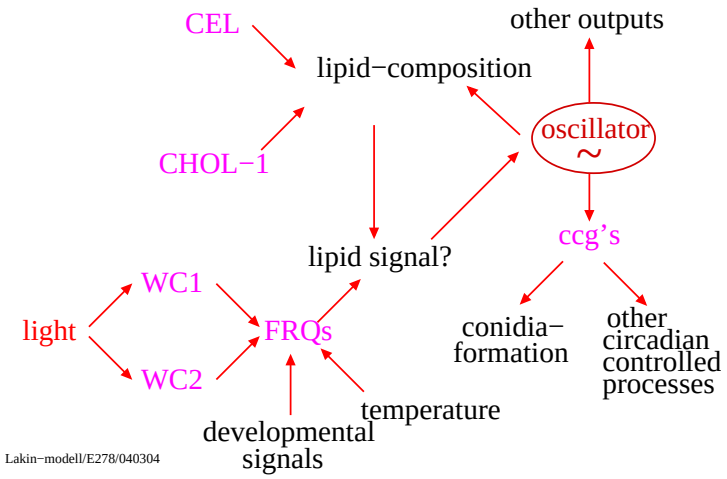

Figure 7.10: *According to Lakin-Thomas FRQ is not a direct constituent of the circadian oscillator, but instead a component before the oscillator. Light affects FRQ via WC-1 and WC-2 (temperature and developmental signals affect FRQ additionally). FRQ affects the circadian oscillator via lipid-signals. Experiments with the mutant* cel *and* chol-*1 are in favour of this interpretation. CEL and CHOL-1 influence the lipid-composition and at the same time the lipid signal and the oscillator. The circadian oscillator controlls 'clock controlled genes' (ccg's), the conidia formation and other processes. The circadian oscillator has outputs and one of them feeds back on the lipid composition. After Lakin-Thomas (1998)*

(Roenneberg and Merrow (1998), Lakin-Thomas (2000)). It was proposed (Roenneberg and Merrow (1998)), to take out transcription and feedback of the proteins on its own mRNA-formation from the real oscillator (figure 7.10).

The other group adds another oscillator (or perhaps several?) to the FRQ-oscillator (so called FRQ-less oscillator FLO). Although the FRQ oscillator is needed for the circadian rhythm, it is probably not sufficient (Iwasaki and Dunlap (2000)).

Reasons for assuming an additional oscillator are earlier reports on the frq9 mutant (Loros and Feldman (1986), Loros et al. (1986)). According to these reports these mutants show still a rhythm, although several of the typical attributes of a true circadian rhythm are lacking. Thus, the rhythm is found only in a part of the cultures in the growths tubes, the period length is quite variable (12 to 35 hours), the rhythm can not be synchronized by light, and it is not temperature-compensated and not independent on the nutrients. It reminds of the peculiar rhythm of *Thalassomyxa australis* (see section 8) and represents perhaps a developmental rhythm.

7.2.3 Goals of the play

We got to know in broad outline the molecular biological mechanism of the *Neurospora*-clock. It is now time, to ask for the meaning of the game. It has the following goals:

A reliable clock: The mechanism of a circadian oscillator, which consists of positively and negatively acting feedback loops, does not only affect the period length of the circadian clock, but confers also robustness and reliability. The strength of the FRQ oscillation and with it the robustness of the rhythm increases with the amount of WC-1 and WC-2 zu (Yang et al. (2001)).

Synchronization by light: A circadian clock is able to run also under continuous light (or continuous darkness) and under constant temperature conditions and to control clock-dependent events. In nature, however, it has to be synchronized to the 24 hour day. Otherwise it would quickly loose measure with the day-night-cycle and could not serve any more as a reliable clock. We have seen, that for this purpose photoreceptors and transduction pathes exist.

Synchronization by temperature: Temperature cycles are in *Neurospora* even stronger time cues as the light-dark cycles is. This could be important for a fungus which often grows on substrate which is not exposed to the day light.

Temperature compensation: Additionally the circadian clock mechanism of *Neurospora* is temperature compensated, which is important for a reliable clock.

Photoperiodism in *Neurospora*? It was recently discussed, whether the annual sporulation rhythm, which is often found in fungi, exists also in *Neurospora* and whether it is controlled photoperiodically (Roenneberg and Merrow (2001)). If so, it has to be presumed that the day length is measured by a circadian clock, as known from other photoperiodically reacting organisms.

7.3 Outputs of the clock and control of its hand

The circadian system of *Neurospora* and most likely of other organisms is thus much more complicated as assumed before. In the penultimate section we were dealing with the clock mechanism and the way to study it. Since the mechanism is so far not well known, we have to use the hand of the clock in order to deduce the properties of the clock or have to use mutants which influence the clock. However, the outputs of the clock and the way, in which the observed rhythms originate are also important constituents of the circadian system and it is worthwhile to study them. It should furthermore help also to understand the underlying mechanism, if the events between the peripheric rhythms are followed back to the clock.

The best studied of these events in *Neurospora* is the switch between the growth in the medium and the growth of the hyphens into the air where conidia are produced. This is under control of the circadian clock. Rhythmic conidia formation occurs only at the front of the growing mycelium on the agar-medium. There it is decided, whether aerial hyphae or normal hyphae are made.

Many biochemical rhythms are connected with this switch in the development: Events, that decide how many normal hyphae, aerial hyphae, hyphal branches are made, what happens, if in the aerial hyphae walls (*septum*) are produced, if ripe conidia are discharged, the nuclei divide. Glycolysis, lipid metabolism, the glyoxalat cycle, the tricarbonic acid cycle, the storage of lipids are also affected. Carbohydrates, CO_2-production, the activity of a number of enzymes are rhythmic.

It has, of course, to be checked first whether these events are rhythmic only, because they depend on the conidia formation. This is apparently not the case. Rather, the circadian clock seems to control especially those enzymes, which control decisive points in the metabolism. This was found not only in *Neurospora*, but also in other organisms and seems to be a general principle of circadian control.

7.3.1 Clock-controlled genes

Genes, which are expressed even under constant conditions in a circadian way, are called *clock-controlled genes* ('ccgs´). If these genes do not function anymore, the *clock* is not influenced, but only the *output* of the clock via these genes. How are these genes controlled by the clock? There must be factors, which pass phase-specific time-informations of the clock to the target-genes (details in Loros and Dunlap (2001)). In the meantime quite a number of ccgs are known and many will be added by using new methods (differential screening, mikroarray-analysis).

How this time is read from the clock is not yet well understood. As mentioned already, transcriptional and translational steps are involved.

An effective methode to find out is the subtractive hybridization of morning- versus evening-mRNA with day-specific cDNA-libraries (see Bell-Pederson et al. (1996) and the *Neurospora* cDNA sequencing project http://www.genome.ou.edu/fungal.html).

In the next step the promotors of the ccgs will be characterized. These clock control regulatory elements (CCRE´s) define clock-boxes. Trans-operating factors, which bind and control CCRE´s, have to be isolated. If this cascade is trailed backward, factors can finally be isolated which interact with components of the clock-mechanism. Some of these steps might be specific for the individual organisms, others are perhaps conservative and found in many organisms.

ccgs are often additionally controlled by light

and by developmental steps (see table 2 in Loros and Dunlap (2001)). Thus there must be besides clock controlled regions other specific regions, which transfer control by development and by light to the gene expression.

Whether one has really to differentiate between ccgs and genes of the clock strictly has become somewhat questionable. Thus the expression *of the frq* gene is not only clock-controlled, but additionally controlled directly by light

8 The hat-game of an ancient clock

A marine amoeba from the coast of West Australia crawles over the surf of the sea in a reticular state hunting for -mainly algal- food. After some time it converts to a hat-like state and digests its food. After a while it starts again to hunt for food. We will see, that this is an unusual rhythmic behaviour which is adapted to the rhythmic structure of the environment by a combination of various time cues (day-night-change, temperature change, tides).

(Grell (1985))[1] discovered at the west coast of Australia a new marine amoeba which he named *Thalassomyxa australis*. It lives in rocky cavities in the surf of the sea and alternates rhythmically between a resting phase and an active phase (figure 8.1). During the rest it looks like a flat hat. Afterward many pseudopodia are formed which crawl over the substrate, take up unicellular algae and digest them during the following resting phase. A movie about these animals can be obtained by the Institut für den wissenschaftlichen Film in Göttingen (Grell (1987)). It shows the life of these amoeba and its alternation between rest and movement.

Originally Grell thought, that these rhythmic alternations between an immobile form and a reticular active form occurs periodically in a tidal rhythm. We have found, however, in a number of experiments, that the alternation between rest and movement occurs at 22°C every 25 hours. That is almost the length of a day and we assumed first, that it is a daily rhythm. Organisms with a true circadian rhythm show very similar period lengths, if studied at various environmental temperatures. The rhythm is said to be temperature compensated.

To check it in *Thalassomyxa*, we recorded the alternations in shape at 10, 15, 20 and 26°C with a video camera and evaluated the time lapse movie. We noted down the times at which the resting phase ended and began and calculated from it the period lengths. To our surprise we did deal with a rhythm, which was not temperature-compensated. At higher temperature (26°C) the period length of the alternation in shape was only 19 hours, at 10°C it was slowed to almost 90 hours (figure 8.2).

Furthermore, organisms with a circadian rhythm are synchronizable by the 24-hours time structure of the environment such as light and temperature cycles. Wie tried therefore in further experiments to test for these typical properties. Again a surprise: The amoeba could not be synchronized by light-dark-cycles (figure 8.3).

Next we tried to synchronize the rhythmic alternation in shape by using a temperature cycle alternating between 15 and 23°C. This did not work either. Since the amoeba live at the shore of the sea which is influenced by low and high tides, we tried next to simulate high tides by periodically shaking the cultures in an interval of 6 hours. Again wrong.

[1] Professor Grell was full professor for protozoology at the University of Tübingen from 1957 onward until his retirement

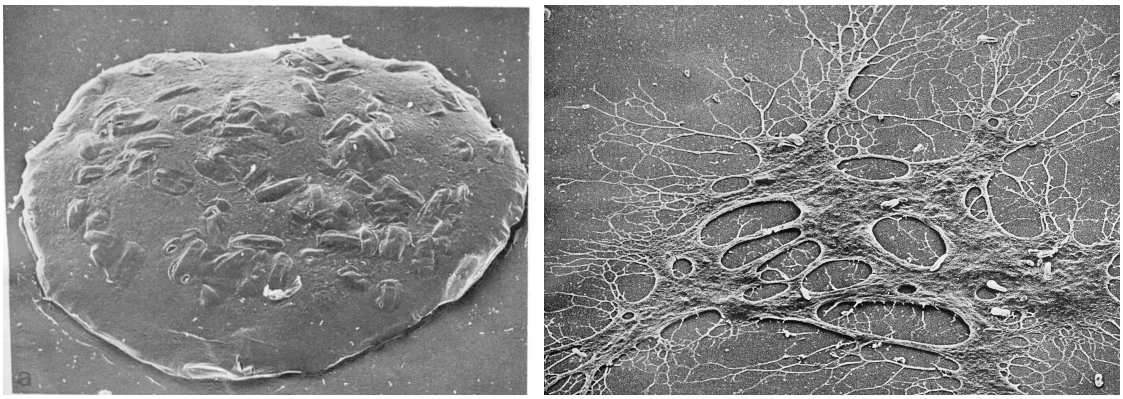

Figure 8.1: *Alternation of shape of a marine amoeba: Left the hat-like resting phase during the digestion of unicellular algae, right in the active phase, in which the amoeba crawl with a net of pseudopodia over the substrate and take up food. Images from Grell (Tübingen)*

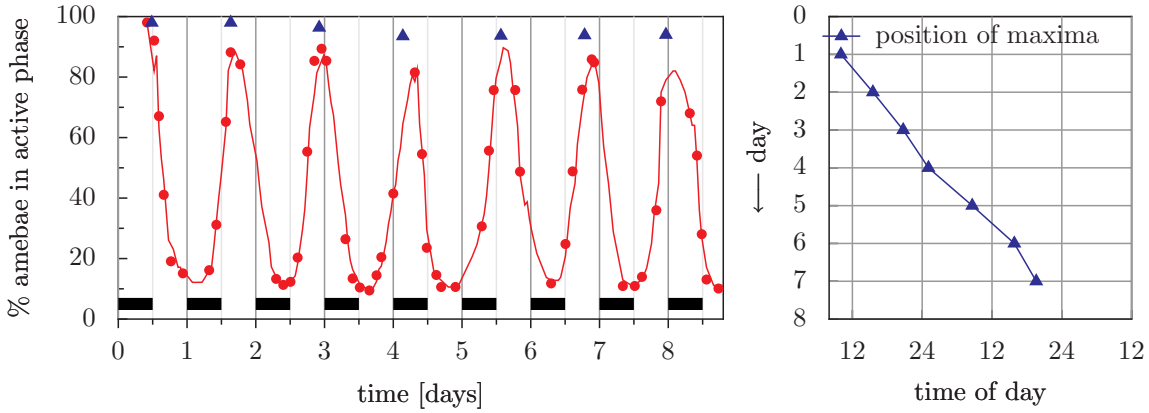

Figure 8.3: *Circadian rhythms are normally synchronized by a light-dark-cycle ('clocked'). This is, however, not the case in the amoeba* Thallasomyxa australis, *as shown by the diagram. In the left part the percentage of active amoeba of the recorded amoeba-populations are shown as a red curve during the course of eight days under a 12:12-hour light-dark-cycle (bright: light period, grey: dark period). The highest values (blue triangles) occur each day a few hours later. This is better recognizable in the right diagram. Here only the maxima are plotted as blue triangles and connected with a blue curve. If the rhythmic alternation in shape of the amoeba would be synchronized with the light-dark-cycle, the maxima would lie beneath each other in a vertical line. Instead they occur each day 7 hours later. The period length of the rhythm amounts thus to 24+7= 31 hours. In spite of the light-dark-cycle the amoeba show a* 'freerun'

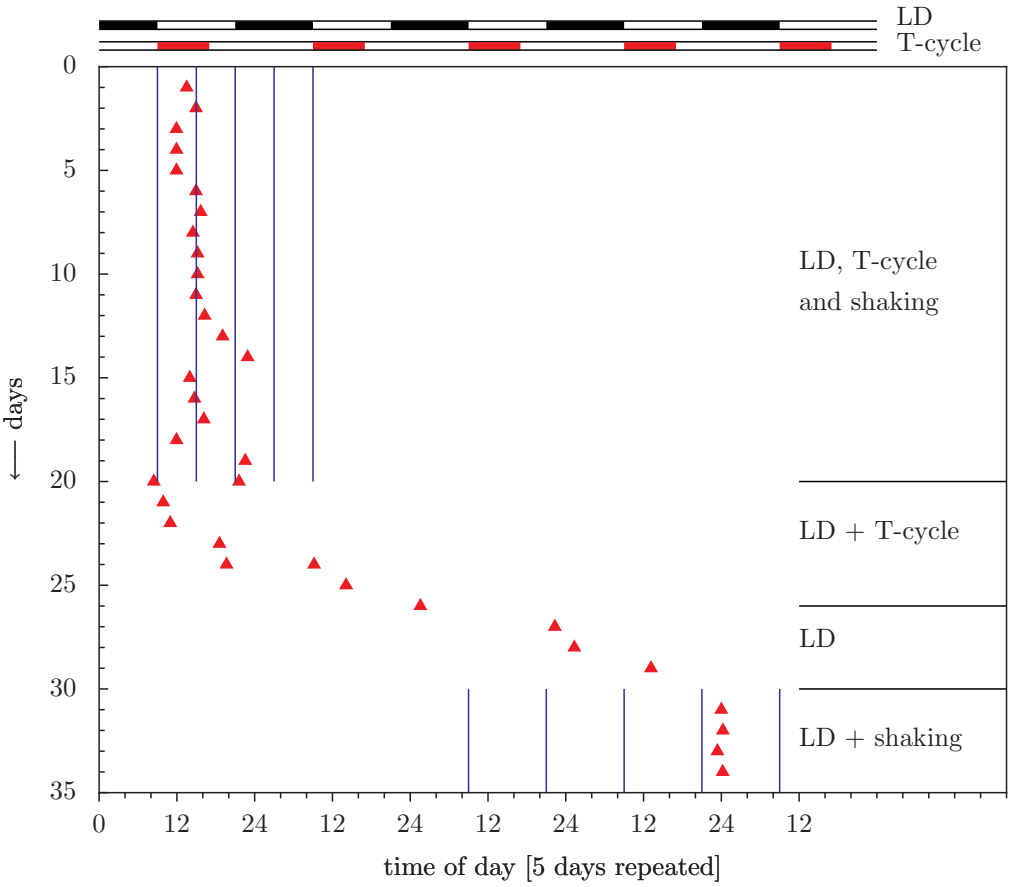

Figure 8.4: *The marine amoeba* Thallasomyxa australis *was kept in a 12:12-hour light-dark-cycle and a cyclic alternation between 12 hours higher temperature and 12 hours lower temperature. Additionaly the cultures were shaken every 6 hours for 15 minutes, which simulated the onset and end of the high tide. During the twenty days of this treatment the amoeba were during most of the days synchronized to 24 hours. A 12:12-hour light-dark-cycle combined with alternating 12 hours higher and lower temperatures did not suffice to synchronize the rhythm (day 20 to 27). From figure 8.3 we know already that a light-dark-cycle by itself does not synchronize. That is confirmed here (day 28 to 31). A light-dark-cycle combined with shaking every 6 hours does, however, synchronize* the rhythm

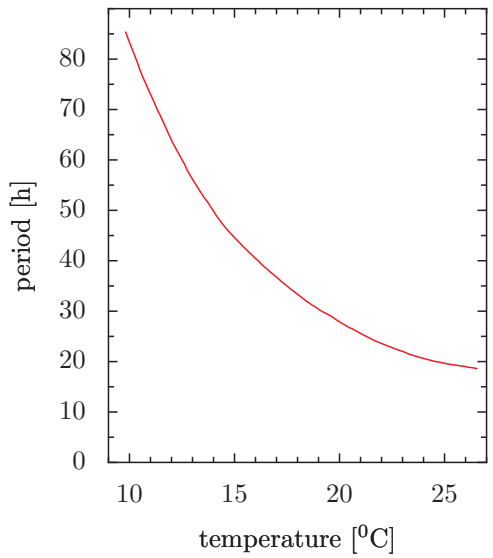

Figure 8.2: *Alternation in shape of a marine ameoba at different temperatures. At the vertical axis the time is plotted in hours, in which the amoeba have attained again the original shape (for example the hat shape) (the so called period length)*

We had to combine several time cues (temperature cycle, light-dark-cycle, periodic shaking), until we were able to synchronize the alternation in shape of the amoeba (figure 8.4).

This organismus posses thus quite an excentric kind of rhythm in its alternation in shape. In contrast to normal circadian rhythms it is temperature-dependent and not synchronizable by a light-dark-cycle. Furthermorea temperature cycle is also not able to synchronize it to 24 hours. Neither does shaking in tidal intervals synchronize. It needs the combination of several environmental rhythms for synchronization to occur.

We might deal with here a kind of precursor of a circadian clock, a kind of ancient clock (Ur-Uhr in German), which does not yet possess all the typical properties of 'modern' circadian clocks. The scheme in figure 8.5 shows, how circadian clocks with temperature compensation and the ability to synchronize might have developed from precursors, which were not yet temperature compensated and could not be synchronized. Most of the 'fast clocks' (called ultradian clocks, that is clocks, which ran faster then circadian clocks) have period lengths which depend heavily on temperature. There are, however, a few cases in which the ultradian rhythm is not influenced by the environmental temperature. In the scheme it is indicated, how a typical modern circadian clock with temperature compensation and the ability to be synchronized might have developed from these precursor clocks. This is, however, pure speculation.

Anyway, circadian rhythms have evolved much earlier as assumed so far, since they were found in the meantime even in cyanobacteria (bluegreen algae), which belong to the prokaryonts. Cyanobacteria

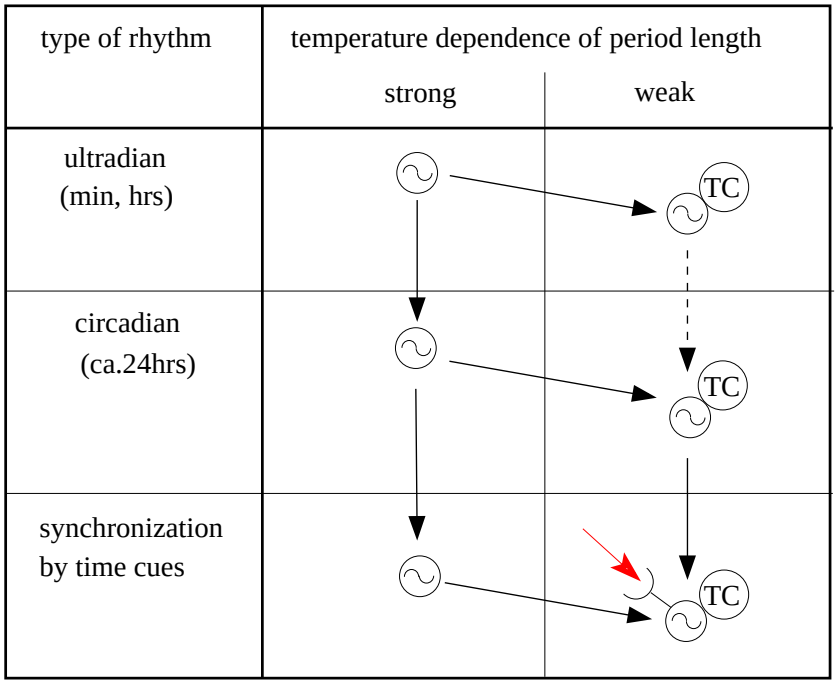

Figure 8.5: *How circadian clocks might have originated. See text for details*

were found as fossils in the Gunflint in Ontario and lived already 2 billion years ago. They are able to take up and use nitrogen from the air. There is, however, a problem: The oxygen which arises during photosynthesis, inhibits the enzyme for nitrogen fixation. Two different strategies were developed in order to circumvent this dilemma: Some cyanobacteria separate photosynthesis and nitrogen fixation in space by taking up the nitrogen of the air in specialized cells called heterocysts. They possess especially thick and impermeable walls. Other cyanobacteria use a separation in time: During the night nitrogen is taken up from the air, and during the day photosynthesis occurs. This is controlled by a circadian clock, which can separate even under constant conditions (weak continuous light) successfully the two incompatible processes.

9 Demonstrations, simulations and experiments

How a mould forms bands, how the wood sorrel lifts and drops its leaves, the chloroplasts in the leaves of a mossfern changes its shape and how potato starch-grains form layers can be studied. Simulations lead to patterns, which can be observed also in certain chemical reactions.

Some demonstrations, observations and proposals for experiments shall motivate you to deal with more intensively with these interesting topics. Detailled instructions for the experiments are found in Engelmann and Klemke (1983) and **?**.

9.1 Demonstrations

***Neurospora*-conidial bands:** A few spores *of Neurospora crassa* are inoculated on an agar-nutrient in a disk. The spores germinate and form each day a concentric ring consisting of conidia carrier with numerous orange conidiospores (see figure 7.5). A short time-lapse movie of the growth and the conidial band formation of *Neurospora crassa* can be found on the home page of J. Dunlap and/or downloaded:
http://www.dartmouth.edu/~biochem-/dunlap/
or also at V. Gooch
http://www.northwestern.edu/ccbm

Wood sorrel *Oxalis acetosella*: In coniferous forests the Wood sorrel *Oxalis acetosella* is often found. In the shadow the three parted leaflets turn in such a way, that they can absorb as much light as possible. If the light is very strong, they drop their leaflets. They are also dropped during the night. To observe it, the best way is to take a plant with soil home in a pot and observe it there.

Mossfern *Selaginella*: Changes in shape of the chloroplasts in the epidermal cells can be observed under the binocular microscope. See figure 2.2.

Starch grains of potatoes: The layers of the starch grains of a potato tuber can be observed under a microscope.

9.2 Simulations

Cellular automates are often used in physics, mathematics, life sciences and economic sciences in order to simulate certain events. Such a model was proposed by Martin Gerhardt and Heike Schuster from the University of Bielefeld (see Dewdney (1988)). It is called the Mischmasch-machine. More under http://surf.de.uu.net/zooland!htm. There is also a demonstration (http://www.geocities.com/SiliconValley/Ridge-/2628/mischmasch/maschine.html) and the C-program for the simulation for download.

9.2.1 The Mischmasch-machine

The Mischmasch-machine uses a matrix of cells, for example 500*500 (number of the columns w, number of the rows h). At time t each cell is in a certain state. In state 0 the cell is healthy, in state n sick. Between 0 and n the cell is infected. The closer the in-between state is to n ist, the stronger the cell is infected.

The state of the cells one time step later t+1 (a 'tick') depends on its own state and on the state of the neighbouring cells.[1] The following rules are valid: If the cell is healthy (state 0), its state depends one tick later on the number of the infected (A) and sick neighbor cells (B). Furthermore two parameters k1 and k2 play a role. They weight the healthy cells. The state of each cell is determined by the following equation:

$[A/k1] + [B/k2]$

where the squared brackets mean, that the values are rounded down. Thus, if $A/k1 = 2.34$, it is changed to 2. How strongly a cell is infected at a certain time, depends on two quantities: How strongly the neighbor cells are infected and how fast the infection spreads (determined by g). The degree of infection is calculated by dividing the sum S of the state numbers of the cell and its neighbors by the number A of the infected neighbor cells. In addition the value g is added, which tells how fast the infection spreads. The state of a cell is thus in the next time step t+1

$[S/A] + g$

A cell can, however, not become sicker then n. Finally there is a rule, that a sick cell (that is in the state n) becomes healthy again a tick later (the infection is so to speak overcome by the immune system).

In a simulation the matrix can for example consist of $20 * 20 = 400$ cells, n could be 100, k1 2 and k2 3. The speed of the infection g is specified and the number of calculations (=ticks, for example t, t+1, t+2 ... t+10000).

Depending on the chosen value of g four different types arise, which are shown one-dimensional as a function of the play time in figure 9.1. At very low values of g not much happens. Most cells stay boringly healthy. If g is somewhat increased, most cells are infected and stay so, although healthy cells occur irregularly and by chance. That is type 1.

At even higher g a regular series of infection rings occurs, which last for about 30 cycles. Occassionally all 400 cells might be healthy, before a new infection wave begins. This is type 2.

In type 3 g is further increased. About every twentieth cycle infected and healthy cells alternate with each other.

Finally at a still higher g type 4 is found. A few cycles after begin of the game the number of infected cells fluctuates with high regularity around a saturation value of about 75%.

One example for each of the four different types is shown in figure 9.2. Here $100 * 100$ or $500 * 500$ matrices (plural of matrix) were used and the results color coded. Waves of type 1 spread over short distances only, before they vanish. In type 2 waves migrate outward in circles. The width of the waves fluctuates strongly. In type 3 the waves are also in circles, but more regular. They reflect the regular coming and vanishing of infected cells. In type 4 the waves follow a spiral pattern, which spreads from the center of the matrix.

It is recommended to perform the sim-

[1] Neighbors can either be the cells in the lateral neighborhood ('Neumann neighborhood') or additionally the cells at the four corners ('Moore-neighborhood').

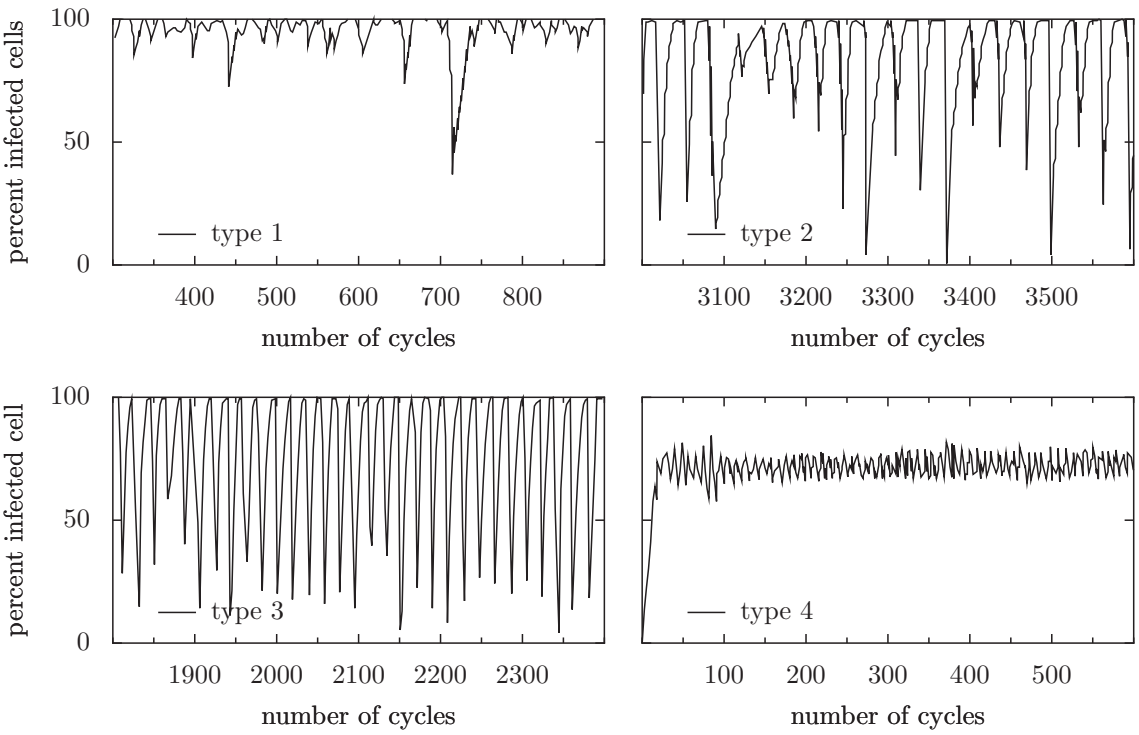

Figure 9.1: *Depending on the chosen infection speed g, four different types are found which are shown here onedimensionally as a function of the duration of the game.*
Type 1 is the result of low values of g: Most cells are infected and stay so, although healthy cells occur irregularly *and by chance.*
Type 2 is found at a higher g: A regular series of infection rings occurs, which last for about 30 cycles. Occasionally all cells become healthful, until a new wave of infection begins.
Type 3 with still higher g: About every twentieth cycle infected and healthy cells alternate with each other.
Type 4 at very high g: A few cycles after the begin of the game the number of infected cells fluctuates regularly around a saturation value of about 75%. From Dewdney (1988)

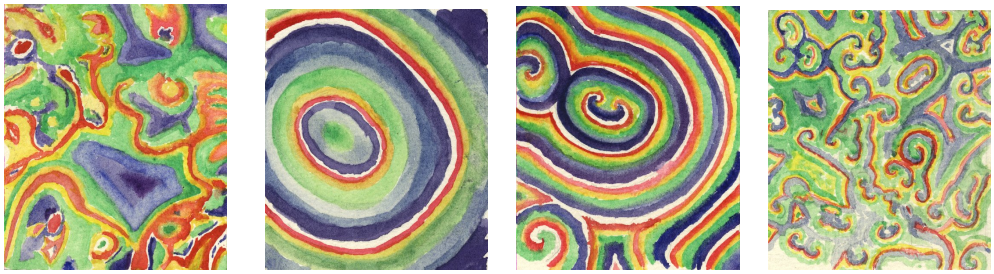

Figure 9.2: *One example for the four various types of the Mischmasch-machine are presented. Here* $100 * 100$ *and* $500 * 500$ *cells were used and the results colour coded. From left to right: Type 1: Waves spread over short distances only, before they vanish. Type 2: Waves migrate outward in circles. The width of the waves fluctuates strongly. Type 3: The waves are also in circles, but more regular. They reflect the regular coming and vanishing of infected cells. Type 4: The waves follow a spiral pattern, which spreads from the center of the matrix. Drawn by the author after a figure from Dewdney (1988)*

ulations yourself. Click for this purpose at http://surf.de.uu.net/zooland/#H and choose there the *Hodgepodge machine* by Jörg Heitkötter or choose alternatively the *Mischmasch-machine* by Claus Claves.

9.3 Experiments

Experiments concerning the topics treated in this book are found in the following books:

Engelmann and Klemke (1983), Engelmann (2007)

I have written some more books or am in the process of writing. They are also concerned with topics which have to do with rhythmic events in organisms - my special field as a scientist (Engelmann (2007), Engelmann (2004c), Engelmann (2009a), Engelmann (2009b), Engelmann (2009c), Engelmann (2009d), Engelmann (2008), Engelmann (2004a), Engelmann (2004b)). They contain also some experiments.

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