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The vertebrate pineal hormone melatonin is produced by the brown alga *Pterygophora californica* and mimics dark effects on growth rate in the light

Birgit Fuhrberg[1], Ivonne Balzer[1], Rüdiger Hardeland[1], Astrid Werner[2], Klaus Lüning[2]

[1] Zoologisches Institut, Universität Göttingen, Berliner Strasse 28, D-37073 Göttingen, Germany
[2] Biologische Anstalt Helgoland, Notkestrasse 31, D-22607 Hamburg, Germany

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Abstract. Melatonin, a methoxylated indoleamine, plays a role as a mediator of darkness in animals as well as in the unicellular alga *Gonyaulax polyedra* Stein and was recently detected in higher plants. We report on the first finding of melatonin in a multicellular alga, the brown alga *Pterygophora californica* Rupr. Melatonin was identified in juvenile sporophytes of *P. californica* by two independent methods, reverse-phase high-performance liquid chromatography (HPLC) with electrochemical detection, and radioimmunoassay. Another indolic metabolite, 5-methoxytryptophol, was also indentified by HPLC. The rapid decline of growth rate upon the onset of darkness in *P. californica* is mimicked by melatonin in the light, with increasing efficiency from 5×10^{-5} M to 5×10^{-4} M, while no effect was obtained at 10^{-5} M.

Key words: Circadian rhythm (growth rate) – Dark effect – Growth rate reduction – Melatonin – 5-Methoxytryptophol – *Pterygophora* (Phaeophyta)

Introduction

The pineal hormone melatonin (N-acetyl-5-methoxytryptamine) was discovered in 1958 in search of the amphibian skin-lightening factor present in bovine pineal glands (Lerner et al. 1958, 1959). A marked dark-phase rise of pineal melatonin was soon evident in mammals (Quay 1963a, b), and numerous subsequent investigations established the central role of melatonin as a messenger of the signal "darkness" in vertebrate species, mostly mammals and birds, eliciting photoperiodic responses, and synchronizing circannual and circadian rhythms (e.g., Arendt 1986; Reiter 1991, 1993; Binkley 1993; Matthews et al. 1993; Gwinner et al. 1994). Melatonin was also found in invertebrates, e.g. in the eyes of insects, crabs and molluscs (Vivien-Roels and Pévet 1986, 1993).

Melatonin and 5-methoxytryptamine, another methoxylated indoleamine, were discovered in an unicellar alga, the marine dinoflagellate *Gonyaulax polyedra*, and the cellular concentration of melatonin exhibited a circadian rhythm, with a maximum at subjective night (Balzer and Hardeland 1991; Poeggeler et al. 1991; Balzer et al. 1993; Poeggeler and Hardeland 1994; Hardeland et al. 1995). Melatonin, when added in the light, mimicked the photoperiodic short-day effect of cyst formation in *G. polyedra* (Balzer and Hardeland 1991). These findings suggested a general function of this molecule in photoperiodism common to algae and vertebrates (Poeggeler et al. 1991; Hardeland et al. 1993a, 1995).

Melatonin and other methoxylated indoleamines are readily destroyed in plant homogenates, especially when exposed to light, and might have been easily overlooked in plant material (Poeggeler and Hardeland 1994). The recent detection of melatonin in representatives of 16 angiosperm families, including fruits such as tomatoes, banana or cucumber and cereal grains (Van Tassel et al. 1993; Dubbels et al. 1995; Kolár et al. 1995; for review see: Balzer and Hardeland 1996), supports the suggestion of Vivien-Roels and Pévet (1986) who assumed that melatonin may be an evolutionarily conserved molecule.

Moreover, melatonin is an efficient radical scavenger, the primary evolutionary function of which may have consisted of antioxidative protection (Hardeland et al. 1995). Being destroyed in the light and accumulating in the dark, melatonin may have been a suitable substance to act as an indicator of darkness, which has finally been coupled to circadian oscillators (Hardeland 1993; Hardeland et al. 1993a, 1993b, 1995; Poeggeler et al. 1993; Hardeland and Rodríguez 1995).

In marine macroalgae, the growth rate of the laminarian species *Pterygophora californica* is controlled by circadian rhythmicity (Lüning 1994) and also exhibits a circannual rhythm (Lüning 1991). A typical and rather fast

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Abbreviations: ANOVA = analysis of variance; DMSO = dimethyl sulfoxide; LD = light-dark cycle

Correspondence to: K. Lüning; FAX: 49 (40) 89693115

dark response in *P. californica*, as in other brown algae, is the decline of growth rate after the onset of darkness (Lüning 1991, 1994). The objectives of our investigation were to test for the presence of melatonin in *P. californica*, and for the possibility that the rapid decline of growth rate observed upon the onset of darkness is mimicked by melatonin in the light.

Materials and methods

Algal material and culture conditions. Juvenile sporophytes of *Pterygophora californica* Rupr. (Bamfield, Vancouver Island, Canada; isolated from zoospores) were obtained by fertilization of red-light-grown gametophytes in white light (Lüning and Dring 1975) and cultured for four to six months up to a blade length of 5–10 cm at 16 h light per day (Osram-L 65W/25S, Universal White; Osram, München, Germany; $30 \pm 5\ \mu mol \cdot m^{-2} \cdot s^{-1}$; light phase 2200–0600), at 10°C (growth experiments) or 7°C (indole determinations), in aerated 10-1 wide-mouth bottles, in Provasoli's enriched seawater (PES; Starr and Zeikus 1978). The seawater medium was replaced once a week, and the sporophytes were trimmed to about 5 cm blade length on this occasion, leaving the 2- to 3-cm long stipe. Prior to automatic growth measurements, the stipe of each plant was trimmed to about 5 mm length.

Determination of melatonin by reverse-phase HPLC with on-line electrochemical detection. Pieces of blades were cut from the plants and immediately shock-frozen in liquid nitrogen. The steps from freezing to extraction were carried out in darkness or in red light. The material was ground in a mortar, in the presence of liquid nitrogen, to give a powder which was stored at −80°C (Poeggeler et al. 1991; Poeggeler and Hardeland 1994). A 300-mg aliquot of powder was extracted with 300 µL of 0.4 N perchloric acid for 15 min at 4°C, centrifuged (15 min at $12000 \cdot g$) to sediment precipitated macromolecules, and diluted with HPLC eluent mixture to give a concentration appropriate for the detection range. The eluent consisted of 20% methanol, $3.75\ g \cdot L^{-1}$ NaH_2PO_4, $11.25\ g \cdot L^{-1}$ citric acid, $320\ mg \cdot L^{-1}$ octanesulfonic acid, and $32\ mg \cdot L^{-1}$ EDTA. An aliquot of 200 µL of extract/eluent mixture was injected into the HPLC. The flow rate was $1\ mL \cdot min^{-1}$. The HPLC equipment consisted of a Gynkotek HPLC pump (M480 HD; Gynkothek, Germering, Germany) equipped with a pre-column of Perisorb RP-18 and a separation column of Spherisorb ODS 1, a column thermostat (STH 585) operated at 40°C, a pulsation reducer (PD-1), and an electrochemical detector (EP 30). In contrast to previous investigations with other equipment (Poeggeler et al. 1991), an oxidation potential of 840 mV was applied.

Determination of melatonin by radioimmunoassay (RIA). Powder prepared as described above was extracted with acetone and lyophylized; the lyophylizate was dissolved in RIA buffer and the melatonin content was measured by RIA according to the procedures described by Poeggeler et al. (1991), with the modifications that azide was omitted from the buffer, and that a charcoal assay (Poeggeler and Hardeland 1994) was used instead of the scintillation proximity assay.

Automatic growth measurements and statistics. All measurements were performed in temperature-controlled rooms at 10°C. During measurement, an experimental sporophyte of 3–4 cm blade length was kept in a Plexiglas measuring chamber supplied with flowing seawater of constant temperature. The algal image was recorded by a charged coupled device (CCD) camera and evaluated for thallus area every 30 min or 10 s by an on-line computer, as described in detail by Lüning (1992). The algal measuring chamber was placed in a light-tight housing, and six algal chambers, each equipped with a surveying camera, were run in parallel. The experimental algae were illuminated from above by white light from circular fluorescent- tubes (Osram L 22 W/25C, Universal White) at 30–40 $\mu mol \cdot m^{-2} \cdot s^{-1}$. Photon fluence rates were measured with an LI-190SA flat quantum sensor (LI-COR, Lincoln, Neb., USA). Growth was also followed in darkness, since infrared radiation produced by Linestra tungsten lamps combined with 3 mm Schott RG 850 filters (transmitted wavelengths > 850 nm; Schott, Mainz, Germany) were used for producing the algal image. Relative growth rate per hour (R) was computed from the successive differences of the natural logarithms of algal area and was expressed as percentage increase per hour (100 × R; see Lüning 1992 for discussion of growth rate terminology). For experiments on the effects of melatonin, thallus area was recorded at intervals of 10 s, linear regression was calculated for successive 30-min portions of the area records using Excel 5.0 (Microsoft), and growth rates were calculated from these successive linear regression segments. Growth rates of replicate experiments were compared by single classification analysis of variance (ANOVA; Sokal and Rohlf 1981) using the program InStat (GraphPad Software, San Diego, Cal., USA).

Melatonin treatment procedures. One day before melatonin treatment, the experimental algae were removed from their PES medium and mounted in measuring chambers supplied with flowing seawater. Each chamber was connected by black plastic tubes to a Plexiglas reservoir (110 mm of diameter, 300 mm high) containing 1.5 L of plain seawater. The reservoir was placed 0.7 m lower in dim white light ($0.1–0.15\ \mu mol \cdot m^{-2} \cdot s^{-1}$) and supplied with a centrifugal plastic pump (Eheim, Deizisau, Germany) transporting the seawater at a rate of $0.2\ L \cdot min^{-1}$. The PES medium was not used for melatonin treatments because of possible interference of melatonin with PES metal-mix ions, especially iron-EDTA (Poeggeler and Hardeland 1994). It had been ascertained previously that the sporophytes grew sufficiently fast for a few days also in plain seawater. Melatonin was dissolved directly before use in dimethyl sulfoxide (DMSO), at a concentration of 0.2 M, and then added to the seawater reservoir to give the desired molarity. Controls were run with DMSO at the highest concentration used to dissolve melatonin, i.e. 2.0 mL DMSO per 1.0 L of seawater, or 28 mM DMSO, for 5×10^{-4} M melatonin. A constant DMSO concentration of 7 mM in seawater was used for melatonin molarities of 10^{-4} M, 5×10^{-5} M or 10^{-5} M. Six replicate experiments were performed for each treatment condition.

Results

Detection of melatonin. Melatonin was formed by *Pterygophora* in high quantities, as shown by HPLC with electrochemical detection (Fig. 1). The melatonin peak was well separated from other constituents of the extract. The presence of melatonin was, moreover, confirmed by RIA, i.e. by an entirely different method requiring another extraction procedure. The amount of melatonin detected in the run shown was 1.46 ng per mg protein. The protein concentration in juvenile sporophytes of *Pterygophora californica* was a little higher than 1% of fresh weight (dry weight/fresh weight ratio is around 0.15; 75% of the dry weight is organic matter, the protein fraction in a laminarian sporophyte is of the order of 10% of the organic dry weight; Lüning 1990). The overall concentration of melatonin in the *Pteryophora* tissue is, therefore, in the range of 7×10^{-8} M; assuming a cytoplasmic protein concentration around 10% and a predominantly cytoplasmic localization of the indoleamine, the level of cytoplasmic melatonin would be one order of magnitude higher. No substantial difference was seen between the meristematic and mature zones of the blade (data not shown).

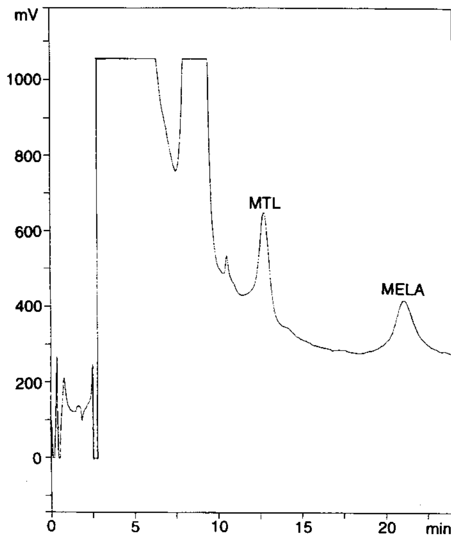

Fig. 1. High performance liquid chromatography chromatogram with electrochemical detection demonstrating the presence of melatonin (*MELA*) and 5-methoxytryptophol (*MTL*) in an extract from *Pterygophora californica*. Ordinate: mV; abscissa: retention time (min)

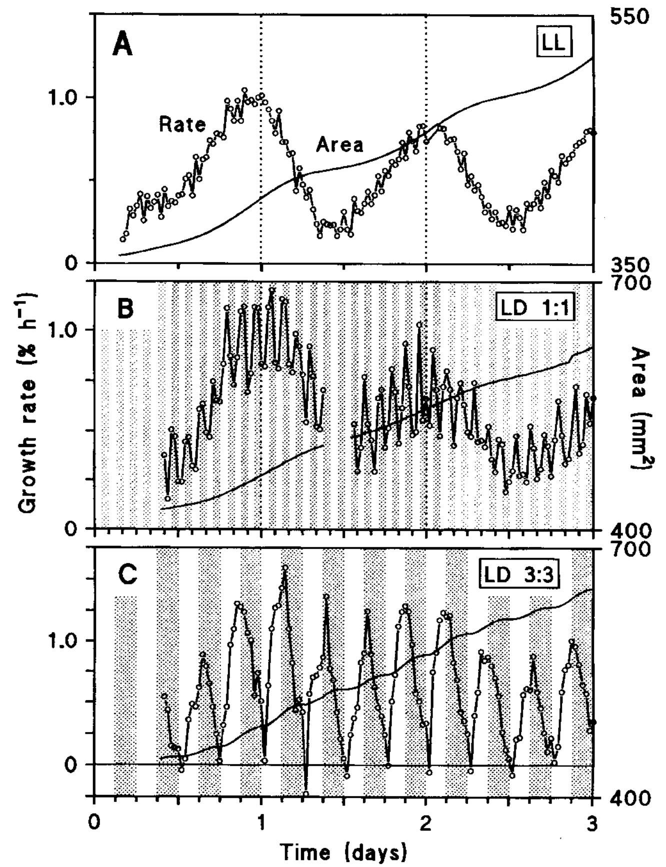

Fig. 2A–C. Effects of dark intervals on the growth rhythm of *Pterygophora californica*. **A** continuous white light from fluorescent lamps (*LL*; 30 μmol·m^{-2}·s^{-1}; 10°C); **B** light-dark cycle (*LD*) of 3 h light, 3 h dark (*LD* 3:3) **C**; as in **B** but *LD* 1:1. *Shaded areas* represent dark phases. Thallus area measured at 30-min intervals (——); relative growth rate derived from thallus area measurements (o—o). Day 0 represents the day of transfer from LD 16:8 during preculturing to measuring conditions. Numbers on abscissa refer to 0600 hours and start of light phase during pre-cultivation

In addition to melatonin, another 5-methoxyindole was detected in the HPLC runs, namely, 5-methoxytryptophol. Although located at the descending part of the chromatogram curve, the peak was fairly well separated from other substances. In the run shown in Fig. 1, the concentration of 5-methoxytryptophol attained a value of 0.4 ng·mg^{-1} protein. 5-Methoxytryptamine, on the other hand, was not demonstrable under the conditions used.

Decline of growth rate due to darkness, or melatonin treatment in the light. The growth rate of *Pterygophora californica* started to decline around 30 min after the onset of darkness, when measured at 30-min intervals in light-dark (LD) regimes of LD 1:1 or LD 3:3 (Fig. 2). The circadian rhythm of growth rate, as evident in continuous white light (Fig. 2A), was still obvious as an underlying oscillation in algae subjected to the two light-dark schedules (Fig. 2B, C).

Treatments with melatonin in continuous white light (Figs. 3,4) were placed close to the peak of the circadian growth rhythm, when the growth rate exhibited only minor changes for several hours (see Fig. 2A; note that measuring points are 30 min apart). In continuous light, thallus area increased linearly during the experimental period of 2.5 h as illustrated by an example in Fig. 3A. Growth-inhibiting effects due to darkness (Fig. 3F) or added melatonin (Fig. 3B-D) were observed as a negative deviation of the area record compared to the projected linear regression of area increase before the start of the treatments. The growth rates calculated from six replicate experiments for each melatonin concentration indicated a significant ($P < 0.001$; ANOVA) reduction of growth rate in comparison to untreated controls in continuous light 60 min after addition of 5×10^{-4} M or 10^{-4} M melatonin (Fig. 4A, B), or after the onset of darkness (Fig. 4F), or 120 min after addition of 5×10^{-5} M melatonin (Fig. 4C). No effect was obtained with 10^{-5} M melatonin (Fig. 4D), or with 28 mM DMSO, i.e. the highest concentration used to dissolve melatonin (Fig. 4E). A reduction of growth rate by about 50% was achieved after a 2-h treatment in darkness (Fig. 4F), or by 10^{-4} M melatonin (Fig. 4B).

The time required for the first visible effect on thallus area increase was 15–20 min after addition of 5×10^{-4} M melatonin and can be seen from the short-term recordings of thallus area at intervals of 10 s (Fig. 3B). Similarly, 15–20 min were required for the same effect after the onset of darkness (Fig. 3F). Initial minor reductions of thallus area occurred within 3 min after addition of 28 mM DMSO, either alone (Fig. 3E) or together with 5×10^{-4} M melatonin (Fig. 3B). As to the optimal DMSO molarity to be used for a given melatonin concentration, it had been found that 10^{-4} M melatonin dissolved in 28, 14 or 7 mM DMSO produced a similar growth-inhibiting effect, while 10^{-4} M melatonin dissolved in 3.5 mM DMSO was not growth-inhibiting (data not shown).

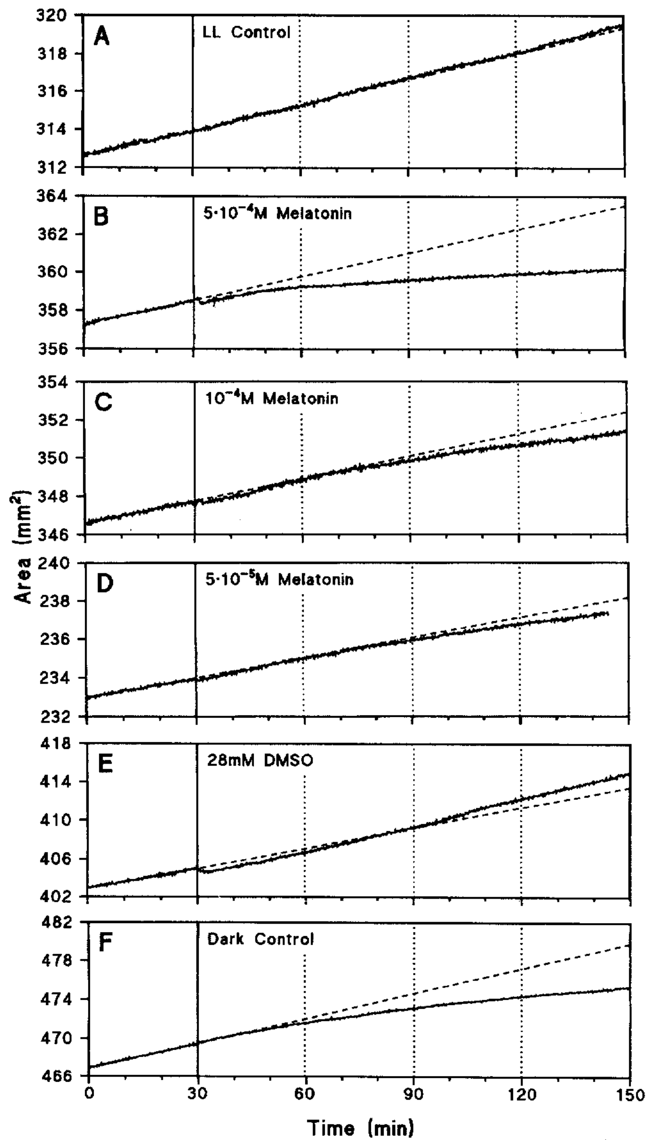

Fig. 3A–F. Original tracings of thallus area measurements at 10-s intervals (*continuous lines*) of *Pterygophora californica* in continuous white light from fluorescent lamps (A), or after treatments with melatonin (B–D), or DMSO (E), or darkness (F). Treatments were started 30 min after the start of the area record. *Broken lines* indicate linear regression of the 30-min area record prior to the start of treatments

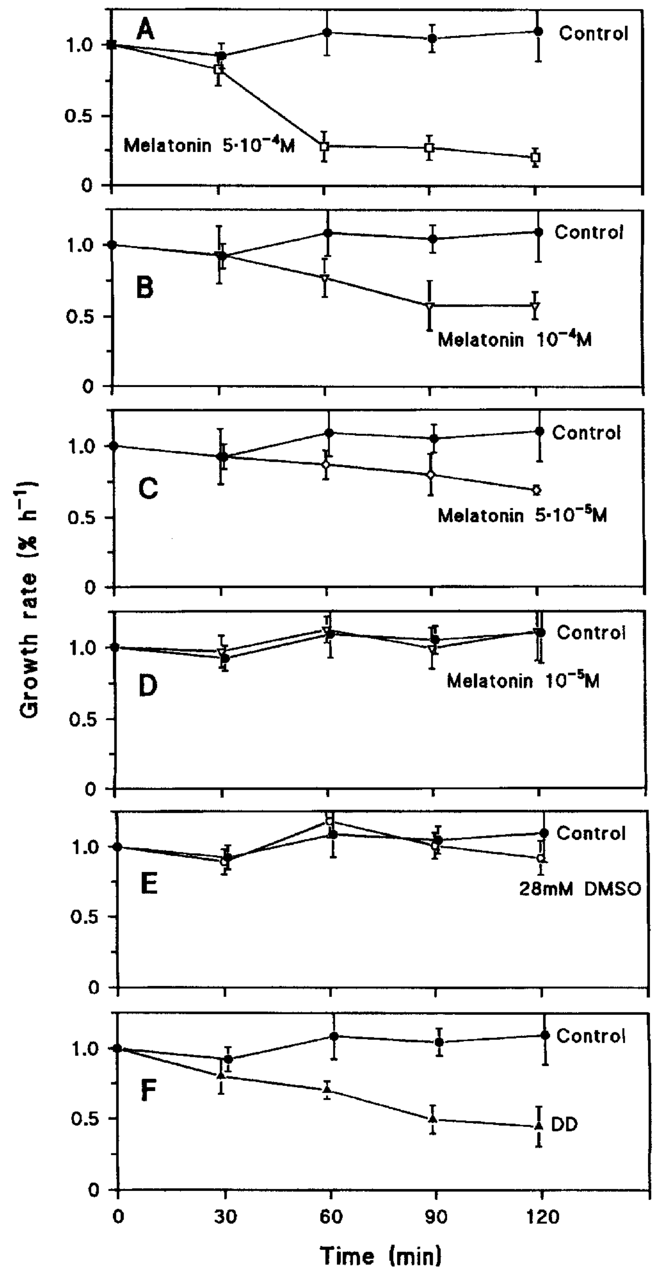

Fig. 4A–F. Effects of melatonin (A–D) or DMSO (E) in continuous white light from fluorescent lamps, as well as dark treatment (F, *DD*), on the growth rate of *Pterygophora californica*. Treatments started at 0 min. Growth rates were calculated from thallus area measurements at 10-s intervals during successive 30-min intervals. *Control*, continuous light (with slightly displaced symbols). Growth rates were normalized to 1.0 for the 30-min interval preceding the start of treatment. Vertical bars indicate standard deviation ($n = 6$)

Discussion

The detection of melatonin for the first time in a multicellular alga strengthens the view of melatonin as an evolutionarily conserved molecule. A search for this molecule in other brown, and also in red or green algae, may be successful in view of the wide distribution of melatonin in the animal kingdom (Vivien-Roels and Pévet 1986, 1993) and in higher plants (Van Tessel et al. 1993; Dubbels et al. 1995; Kolár et al. 1995). If melatonin does convey the signal "darkness" in marine macroalgae, too, as in the animal kingdom, at least 50 known macroalgal species with short-day reactions (Dring 1984; Lüning 1990) would be appropriate candidates for testing whether melatonin occurs in these algae and mimicks the photoperiodic reaction when externally supplied in the light, as was achieved in the dinoflagellate *Gonyaulax polyedra* (Balzer and Hardeland 1991). Another research field would be that of circannual growth rhythms of laminarian species (Lüning 1991), which may be synchronized by melatonin as in animals.

The concentration range of more than 1 ng melatonin · mg^{-1} protein in *Pterygophora californica* may be compared to a range of 100–800 pg melatonin · mg^{-1} protein in higher plants (Dubbels et al. 1995), or to the nocturnal maximum value of 2.5 ng melatonin · mg^{-1} protein in the dinoflagellate *Gonyaulax polyedra* (Poeggeler et al. 1991). Cytoplasmic concentrations of melatonin have not yet been determined in higher plants. In *Gonyaulax*, the nocturnal maximum attains a cytoplasmic level of about 1 μM. In *Pterygophora californica*, the exact concentration of melatonin in the cytosol is difficult to determine, for several reasons. On the one hand, it is known that the cytosol fraction of a juvenile laminarian sporophyte makes up 20% of the fresh weight, the cell walls 30% and the vacuoles 50% (B. Kloareg, Station Biologique, Roscoff, France, and C. Wiencke, Alfred-Wegener-Institut für Polar- und Meeresforschung, Bremerhaven, Germany, personal communication). However, the partition of melatonin between the compartments of the tissue, i.e. cell wall, vacuoles, and cytoplasm, is still unclear. Moreover, saturatable nuclear binding sites, as found in animal tissues (Menendez-Pelaez and Reiter 1993; Menendez-Pelaez et al. 1993), may complicate the intracellular distribution. Another uncertainty relates to the problem of recovery during extraction. Melatonin can be largely destroyed during homogenization of unicellular algae or higher plants, and, therefore, special preservative procedures have to be applied (Poeggeler and Hardeland 1994).

The levels of melatonin detected in *Pterygophora* may still represent underestimations. For this reason, we have not extended this study to determinations of temporal patterns and statistical variability of melatonin concentration, because further investigations on the stability of methoxyindoles during extraction are required. Nevertheless, the presence of high concentrations of melatonin in *Pterygophora* is beyond any doubt. The – perhaps still underestimated – melatonin level of 7×10^{-8} M, as an overall concentration in the *Pterygophora* tissue, is by two to three orders of magnitude higher than in the blood and most organs of vertebrates, with a nocturnal maximum of 10^{-9} M or less, and general levels down to 10^{-11} M. Even the pineal gland as the major source of circulating melatonin usually does not contain more than 10 ng · mg^{-1} protein (roughly 4×10^{-6} M) at nighttime (Vaughan and Reiter 1986; Huether 1993).

Similar considerations have to be made with regard to 5-methoxytryptophol. This substance, representing another putative signal molecule in vertebrates, has also been detected in the dinoflagellate *Gonyaulax* (Fuhrberg and Hardeland 1995; Fuhrberg et al. 1995). In this unicell, the indole alcohol is formed by catabolism of 5-methoxytryptamine via monoamine oxidase and alcohol dehydrogenase. 5-Methoxytryptamine is mainly produced as a metabolite of melatonin in *Gonyaulax* and represents the active signal molecule in the encystment response (Balzer and Hardeland 1991; Balzer et al. 1993; Hardeland 1993). In *Pterygophora*, 5-methoxytryptophol may be synthesized via several pathways, either, as in *Gonyaulax*, by deacetylation of melatonin and subsequent catabolization, an assumption which would require high activities of monoamine oxidase. Another possibility would imply that 5-methoxy-tryptamine is synthesized directly from serotonin by *O*-methylation and would be rapidly converted by monoamine oxidase, so that it would practically not enter the melatonin pool. A third possibility would consist of *O*-methylation of 5-hydroxytryptophol. All these alternatives imply that melatonin has to be synthesized in the classical sequence of enzymatic steps, i.e. by *N*-acetylation of serotonin followed by *O*-methylation of *N*-acetylserotonin, since otherwise higher concentrations of 5-methoxytryptamine should be detectable.

Another relevant question is that of whether the melatonin levels detected in *Pterygophora* are sufficiently high to be compatible with the assumption of physiological actions by melatonin in this brown alga. In this study, exogenous concentrations of more than 10^{-5} M were required to obtain effects. Cytosolic levels are presumably much higher than the overall tissue concentration, and, also with regard to the likelihood of incomplete preservation during extraction, effective cytosolic levels may already be in the micromolar range. On the other hand, externally administered melatonin will not easily equilibrate with the cytosol. Although the plasma membrane is usually not a barrier to this lipophilic substance (Hardeland et al. 1993b; Hardeland and Rodríguez 1995; Costa et al. 1995), carbohydrate polymers as found in the cell wall largely prevent penetration and retain much of the melatonin given (data not shown). It seems plausible that exogenous concentrations of melatonin much higher than those in the cytosol are required for influencing *Pterygophora*.

A dark-mimicking effect of melatonin may be seen in the rapid decline of growth rate of *Pterygophora californica* in the light after administration of melatonin. Dimethyl sulfoxide used for dissolving melatonin was clearly not the agent inducing this effect since growth rate did not decline after addition of an appropriate concentration of DMSO in the light. The initial, minor reduction of thallus area occurring within 3 min after administration of 28 mM DMSO most probably indicates an osmotic effect, since osmotic changes of thallus area are usually completed within a few minutes after molarity changes (Kirst 1990).

A decline in growth rate upon the onset of darkness, and the opposite process, light-stimulated cell enlargement, seem to be common phenomenona in brown and red algae (Lüning 1991), and are known for leaves of several higher-plant species such as silver birch (Taylor and Davies 1985) and bean (Van Volkenburgh and Cleland 1990). The decisive factors under photocontrol are thought to be a light-induced rise in cell wall extensibility and a decline in cell surface pH resulting in breakage of acid-labile bonds, as found in experiments with birch leaves (Taylor and Davies 1985). Photosynthesis is not involved in light-stimulated cell enlargement and the necessary acidification of epidermal walls of bean leaves, and both processes are probably under control of photomorphogenetic pigments such as phytochrome and a separate blue-light receptor (Van Volkenburgh and Cleland 1990; Van Volkenburgh et al. 1990). If similar cellular processes control the dark-induced decline of cell enlargement in the brown alga *P. californica*, one may hypothesize that endogenously produced melatonin is part of the signal chain,

and exogenously administered melatonin entering a cell in the light would start the reaction chain leading to dark-stimulated decline in cell expansion by circumvention of the step of light perception. Further experiments are required to test this hypothesis, and it might also be interesting to investigate this possibility in those higher plants that were recently found to produce endogenously melatonin.

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