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The presence and function of melatonin and structurally related indoleamines in a dinoflagellate, and a hypothesis on the evolutionary significance of these tryptophan metabolites in unicellulars

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Abstract. The bioluminescent dinoflagellate *Gonyaulax polyedra* contains various indoleamines, in particular, melatonin and 5-methoxytryptamine, as well as enzymes of their biosynthetic pathway. Melatonin exhibits a high-amplitude circadian rhythm characterized by a dramatic increase shortly after the onset of darkness. The maximum of melatonin is followed by a peak of 5-methoxytryptamine. These 5-methoxylated indoleamines seem to be involved in the mediation of the information 'darkness'. *G. polyedra* shows a short-day response, which consists in the formation of asexual cysts. Light break experiments demonstrate the photoperiodic nature of this reaction. Cells become sensitive to short days only upon exposure to a lowered temperature (< 16 °C). Melatonin mimics the short-day effect, but only at decreased temperature. 5-Methoxytryptamine is even a better inducer of cyst formation, acting also at 20 °C and in any lighting schedule, including LL. Cyst induction is associated with stimulation of bioluminescence and cytoplasmic acidification. A model on the intracellular pathway of photoperiodic information transduction assumes increased deacetylation of melatonin under cyst-inducing conditions, binding of 5-methoxytryptamine to the membrane of an acidic vacuole, proton transfer to the cytoplasm, and decreased intracellular pH as the stimulus for encystment. Melatonin shows the property of a scavenger of superoxide anions. This reaction, which is efficiently catalyzed by hemin, leads to the formation of a substituted kynuramine (AFMK). Destruction of melatonin by light-induced superoxide anions in the presence of cellular hemin may represent a property which, during evolution, has made this molecule suitable as an indicator of darkness. On the other hand, AFMK, which is formed under illumination, might have become a mediator of the information 'light'. Photoperiodism in *Gonyaulax* shows surprising parallels to that in mammals, but allows the analysis of this phenomenon at an entirely cellular level.

Key words. Circadian rhythms; *Gonyaulax*; indoleamines; kynuramines; melatonin; 5-methoxytryptamine; photoperiodism; radicals.

Introduction

Any biological rhythmicity which is related to a geophysical cycle requires a biochemical interpretation of periodical environmental changes by organisms. In particular, circadian as well as circannual rhythms have to be phased and readjusted in period length by the major Zeitgeber qualities, the cycles of light and darkness and of temperature. Moreover, temporal orientation within the year may occur on the basis of photoperiodic or thermoperiodic time measurements even in the absence of an endogenous annual periodicity. In vertebrates, the information 'darknss' is mediated by the pineal hormone, melatonin, and is, therefore, accessible to a biochemical interpretation by target cells. At least in various mammals, this chemical signal is also used for purposes of photoperiodic control of reproduction, hibernation and various other physiological functions[2, 61–63].

The occurrence of melatonin is not restricted to vertebrates. Its presence has been demonstrated in various insects[34, 66, 67] and also in planarians[69]. Circadian rhythmicity was reported especially for the face fly[67]. Moreover, several actions of melatonin were identified in non-vertebrate systems. In planarians, this indoleamine affected asexual reproduction[49], and inhibited specifically regeneration[69]. In onion roots[25] and also in the ciliate *Stentor coeruleus*[24], melatonin exerted inhibitory effects on microtubule assembly, leading to mitotic arrest and to delay of oral membranellar regeneration, respectively. The interference with cytoskeletal organization may reflect a more general phenomenon, since this is not only found in the two entirely different organisms mentioned, but also in cultured mammalian cells[26]. Nevertheless, it still seems to be difficult to detect a functional connection between an influence on the cytoskeleton and the role as a photoperiodic mediator. Prior to our studies on dinoflagellates, the presence of melatonin had not been demonstrated in unicells, although other indoleamines, in particular, serotonin, were known to occur, e.g. in ciliates[32]. The first effect of melatonin reported for dinoflagellates concerned the resetting of the bioluminescence rhythm in *Pyrocystis acuta*[56]. The phase response curve obtained upon application of melatonin in constant darkness revealed advances during late subjective day, but no sensitivity to the indoleamine in the other circadian phases and,

hence, no substantial delays, a finding resembling much recent corresponding data in mammals (Fischer, J., and Hardeland, R., unpubl.). The studies on this and other species of *Pyrocystis* led to the search for melatonin in a chrono- and cell biologically better established dinoflagellate, *Gonyaulax polyedra*. The presence of this indoleamine was demonstrated by two entirely different methods, HPLC with electrochemical or fluorometrical detections, and RIA[19,20,22,23,41,55–57]. In this unicell, it was also possible to relate melatonin to the phenomenon of photoperiodism[7,10–18,38].

Moreover, the photoperiodic response was shown to depend on another environmental parameter, temperature[10–18,38]. This relationship gains particular importance with regard to three aspects. Firstly, many organisms take use of both photoperiodic and thermal information for identifying annual phase positions, a meaningful fact with regard to the *non-congruent* yearly time courses of length of day and temperature, and the resulting possibility of distinguishing between the same photoperiods occurring in two different seasons. Secondly, the daily temperature cycle with its resetting[4] and thermoperiodic[16] effects can influence the photoperiodic control. Thirdly, differences in temperature dependence between melatonin and its metabolite, 5-methoxytryptamine[11,13,14,17] offered a possibility of interpreting the relationship between photoperiod and temperature at a cell biological level and to develop a concept for the intracellular pathway of signal transduction starting from melatonin[18,37,39].

The unicell *Gonyaulax polyedra* allows for the first time the study of the phenomenon of photoperiodism entirely at the cellular level. Moreover, the occurrence of melatonin in this primitive eukarote leads to the question of the primary role of this molecule in ancestors of recent eucytes. Does melatonin represent a substance which is particularly suitable for being used as a chemical mediator of darkness, as it serves for this purpose in phylogenetically extremely distant organisms such as vertebrates and dinoflagellates? The answer may reside in the high sensitivity of melatonin to light, a property which is not necessarily obvious in any artificial solution of this substance, but which becomes evident in its potential biological significance when considering the *catalysis* of a light-dependent degradation reaction by cellular components[39,42,43,45,46].

Presence of 5-methoxylated indoleamines in Gonyaulax polyedra

The dinoflagellate *Gonyaulax polyedra* is a chronobiological model organism, and the many data which are available on its circadian rhythmicity made this unicell suitable for detailed investigations. During the largest part of its natural life cycle, this species exists in a motile stage, in which cells are armoured by a theca, possess two flagella, and exhibit the property of bioluminescence. Little had been known before on non-motile resting stages, although the occurrence of both sexual and asexual cysts is described for many dinoflagellates. In the laboratory, *Gonyaulax* can be cultured continuously in the free-swimming state.

The search for melatonin in *Gonyaulax* revealed a considerable instability of this substance in crude cell extracts. Particular procedures for cell sampling and preservative extraction had to be developed, which had to be carried out in darkness or dim red light, to be able to quantify this indoleamine at a sufficiently high rate of recovery[55,57]. The chemical lability was, in fact, much higher than in blood plasma or in pineal extracts, so that melatonin might have been easily overlooked in the dinoflagellate. This finding may be of particular importance for further comparative studies on material from other algae, higher plants, or extrapineal tissues, in which a rapid decay may occur, too.

In *Gonyaulax*, melatonin occurs in surprisingly high quantities; peak values attain 2.5 ng/mg protein[55,57], i.e. concentrations in the order of magnitude as found in pineals. Occasionally, even higher amounts have been measured[20,39]. Similar values were obtained by the two different methods used, HPLC with electrochemical detection and RIA[55,57].

The parallels to the situation in vertebrates extend to the circadian time pattern of melatonin: also in *Gonyaulax*, the rhythm is characterized by a prominent nocturnal maximum, low levels during photophase, and a high amplitude[55,57]. Shortly after the onset of darkness, melatonin concentration rises dramatically, and after a sharp peak about 1.5 h after lights-off, a steady decline is observed leading to minimum values already at the time of the transition from darkness to light. The rhythmicity of melatonin is circadian in the strict sense, i.e. of endogenous nature, since it persists in constant darkness[22,23].

With regard to another aspect of the role of melatonin in the *Gonyaulax* circadian system, the situation is less clear than *in Pyrocystis acuta*. By contrast with the latter organism, no substantial phase shifts were demonstrated up to now. This may turn out as a purely methodological problem, because *Gonyaulax* cells cannot be kept for many cycles in constant darkness, and therefore, summations of eventual minor effects in the range of an hour cannot be followed for a sufficiently long time span (Balzer, I., and Hardeland, R., unpubl.); with regard to the instability of melatonin under illumination, a corresponding experiment in constant light is, however, not reasonable.

The occurrence of melatonin in *Gonyaulax* finds its expression also in the presence of the typical precursors, various metabolites, and enzymes of the biosynthetic pathway. Melatonin is obviously synthesized in the dinoflagellate by the same sequence of enzymatic steps as

in vertebrates. 5-Hydroxytryptophan, serotonin, and N-acetylserotonin were demonstrated by HPLC with electrochemical and fluorometrical detections[55]. Indoleamine ('serotonin') N-acetyltransferase (NAT) and hydroxindole-O-methyltransferase (HIOMT) were determined by radioenzymatic assays[10,16,20]. The substrate specificities *in vitro* indicate higher affinities of NAT to serotonin than to 5-methoxytryptamine, and of HIOMT to N-acetylserotonin than to serotonin or 5-hydroxytryptophan[16]. The sequence of N-acetylation followed by O-methylation is further supported by the temporal appearance of 5-methoxytryptamine, which shows a peak clearly *after* that of melatonin[16,18,55]. Therefore, 5-methoxytryptamine seems to represent primarily a metabolite rather than a precursor of melatonin. Only after exposure of *Gonyaulax* to high, pharmacological dosages of serotonin, substantial concentrations of 5-methoxytryptamine may be formed by HIOMT directly[14]. Other indole metabolites that seem to be present in *Gonyaulax* according to HPLC data are 5-hydroxytryptophol, 5-hydroxyindoleacetic acid, pinoline, skatol, and a substance that eluates like N,N-dimethyl-5-methoxytryptamine[55]. The occurrence of melatonin and this spectrum of metabolites is not a general feature of primitive eukaryotes. No melatonin was found, e.g. in *Euglena gracilis*, *Astasia longa*, *Tetrahymena thermophila*, or in the fungus *Leptosphaeria michotii*, at least under usual culture conditions (Balzer, I., Bovenschulte, M., and Hardeland, R., unpubl.). Instead, these organisms contained considerable amounts of indoleacetic acid, a substance which had not been detected in *Gonyaulax*[55]. On the other hand, they all seem to differ from *Gonyaulax* by lacking photoperiodism. Nevertheless, *L. michotii* showed a metabolic response to melatonin, which consisted in a depression of the amplitude in the rhythm of aspartate aminotransferase. A similar depression was seen in the circadian periodicity of NAD^+ kinase in *Neurospora crassa*[48]. However, *Leptosphaeria* was much more sensitive to indoleacetic and 5-methoxyindoleacetic acids than to melatonin itself, so that the effect may have been mediated by the latter of these metabolites (Bovenschulte, M., and Hardeland, R., unpubl.).

Photoperiodism in Gonyaulax: *cyst induction by short-days and lowered temperature*

Cyst formation had normally been attributed to adverse environmental conditions. The possibility of a cellular photoperiodic program anticipating an unfavourable *season*, and allowing the organism to survive in a resting stage, had not been considered before for dinoflagellates, nor for unicells in general. In *Gonyaulax polyedra*, photoperiodism was demonstrated for the first time in a unicellular organism, and this mechanism was shown to control encystment[7,10–18,37,39,41,42,45,46].

The photoperiodically induced cysts of *Gonyaulax polyedra* are of the asexual type, so-called 'thin-walled' or 'temporary' cysts. The process of encystment is characterized by cessation of movements, retraction of the protoplast from the theca, loss of flagella, formation of a more or less spherical cell shape, ultrastructural rearrangements, and secretion of a cyst wall[13,16,17].

Precondition for photoperiodical cyst induction is a moderately lowered temperature (<16 °C; cf. normal rearing temperatures of 18 or 20 °C). At 15 °C, cells become sensitive to short days[7,10–14,16–18,37,39,41]. The critical photoperiod is remarkably well-defined: in a light/dark cycle of 11:13 h (=LD 11:13), almost no cysts are formed, whereas in LD 10.5:13.5 all cells show encystment[13,38]. These narrow temporal limits for the switching between two morphological stages may be seen in relation to the high precision of the *Gonyaulax* circadian clock[39]. Under the experimental conditions used, cell populations reacted in a practically all-or-none fashion. After transfer to the experimental LD, the majority of cells is encysted after two days, all of them after three days[13,14,18]. The high synchrony in the photoperiodic response contrasts, at least under laboratory conditions, with non-photoperiodic cyst formation, which is sometimes seen in old cultures, presumably due to a lack of nutrients. In these cases, only a minor part of the cell population shows encystment, and this process extends over many days (Hardeland, R., unpubl.). Cyst induction by short days is *not* a consequence of light deficiency. Experiments with light interruptions during scotophase, using illumination schedules such as LDLD 2:2:8:12 or LDLD 8:2:2:12, clearly revealed a true photoperiodic mechanism, since cyst formation was strongly suppressed by the light breaks, although the overall amount of light was the same as in the cyst-inducing schedule of LD 10:14[7,10,13,14,16,17]. The time span from the onset of the first to the end of the second lighting phase is, therefore, interpreted by the cells as a long day. Moreover, the light requirments of *Gonyaulax polyedra* are obviously lower than applied in the photoperiodic investigations, although this species is usually regarded as a bright-light organism (as compared to other dinoflagellates such as *Pyrocystis*). In a self-selection experiment, in which cells were allowed to swim between a dark and a continuously illuminated part of a cuvette, the majority of cells stayed most of the time in darkness or dim light (Hardeland, R., and Balzer, I., unpubl.). On the other hand, high light intensities ($>1{,}000$ lx) can inhibit photoperiodic cyst induction[18], an effect which may be explained by light-dependent destruction of indoleamines involved in this response (cf. last paper in this review).

Interactions of photoperiodism and temperature, as observed in the encystment response of *Gonyaulax*, are part of a more general experience in seasonal organization of species from many different taxa, including

mammals: in the syrian hamster, e.g. low temperature and short days act synergistically with regard to gonadal atrophy; however, the temperature effect is obviously not a direct consequence of melatonin, the secretion pattern of which is not altered[54].

In ecological terms, the simultaneous requirement of short days and lowered temperature is particularly well suitable for temporal orientation within the year, as the organism takes use of two meteorological seasonal changes, and, therefore, gains a higher degree of certainty about the annual phase than with low temperature alone. Photoperiodism may, however, represent only one aspect of seasonality in *Gonyaulax*, especially with regard to natural conditions. As soon as cells have entered the dormant, amastigote stage of the cyst, they sink down to the sea bottom and are often covered by sediments. In the depths, they are efficiently shielded from exposure to light; this is, in fact, necessary for survival, since cysts would otherwise be severely bleached, an effect occurring in the laboratory already within a couple of days (Hardeland, R., and Balzer, I., unpubl.). In the absence of light, however, the termination of dormancy cannot be directly controlled by photoperiodism. The exact lighting and temperature conditions for germination of cysts have not yet been sufficiently investigated. In the laboratory, a high rate of excystment has been observed in *Gonyaulax polyedra* only after manipulations of external pH (Wolf, R., and Hardeland, R., unpubl.). In nature, two ways of time measurement could determine the end of dormancy, either an hour-glass mechanism operating for a couple of months, or an endogenous circannual rhythmicity. The latter possibility has been reported for another Gonyaulacacean species, *Alexandrium tamarense*[1]. The existence of circannual rhythmicity at the unicellular level, though hard to understand from a mechanistic point of view in organisms producing many cell generations within a year, has also been described for the growth rates of several other dinoflagellates, *Alexandrium excavatum*[30,68], *Prorocentrum micans*[30], and *Scripsiella trochoidea*[31]. In the latter species, cyst formation itself was reported to vary endogenously within the year[31]. Hence, both external photoperiodic and internal rhythmic processes may cooperate in the control of the natural annual life cycle in dinoflagellates.

5-Methoxylated indoleamines mimic short-day effects in Gonyaulax

A single addition of melatonin to *Gonyaulax*, given 1 h before the onset of darkness, at 15 °C in LD 11:13, i.e. under otherwise non-inducing conditions, provoked all cells to encyst within 3 days[7,10,12–18,41]. Again, this finding represents a parallel to the situation in mammals, since melatonin acts in both cases by mimicking a short-day effect. Moreover, it is remarkable that melatonin shows in *Gonyaulax* the same temperature dependence as the photoperiod itself: at 20 °C, the indoleamine is completely inefficient in inducing cyst formation[13,14,16–18]. With regard to temperature, the action of melatonin is entirely different from that of any other cyst-inducing indoleamine or non-indole pharmacon tested so far, an aspect being of particular importance for the understanding of the intracellular signal transduction pathway of melatonin (cf. following chapter).

A comparative study on the cyst-inducing potency of different indole compounds revealed that N-acetylserotonin, the direct precursor of melatonin, was completely ineffective, at both 20 and 15 °C[13,14,39]. However, serotonin, and also bufotenin, showed a certain cyst-inducing capacity, although only in high concentrations (10^{-4} M)[12,14,39]. At the first glance, this seems to be a discrepancy; but it can be resolved by considering the conversion of the two substances to 5-methoxytryptamine, or N, N-dimethyl-5-methoxytryptamine, respectively. In fact, these two amines represent the most potent cyst inducers within the class of indole compounds[7,10–14,16–18,22,37,39]. Both of them are effective at 20 °C, in any lighting schedule including LL; 5-methoxytryptamine elicits cyst formation down to 5×10^{-6} M[13,14,39]. Therefore, sufficient amounts of the cyst-inducing 5-methoxylated indoleamines will be formed via HIOMT, if one of the two low affinity substrates is given in high concentrations[14]. Degradation products which may be formed by MAO, 5-hydroxytryptophol and 5-methoxytryptophol, at least one of which is present in *Gonyaulax*[55], showed no effect on encystment (Balzer, I., and Hardeland, R., unpubl.). Another catabolite which is produced from melatonin by cleavage of the pyrrole ring, a substituted kynuramine (cf. last chapter), also did not lead to encystment (Fuhrberg, B., and Hardeland, R., unpubl.). Since 5-methoxytryptamine is present in *Gonyaulax* in concentrations roughly half as high as melatonin[23,55], its efficiency being higher than that of melatonin by more than an order of magnitude, and since products from other catabolic pathways are inefficient, one should conclude that the photoperiodic information carried first by melatonin is transduced by its metabolite, 5-methoxytryptamine, which finally elicits the cellular response of encystment.

Assuming a role of 5-methoxytryptamine in a dinoflagellate does not necessarily mean referring to a very particular situation existing only in this group of primitive organisms. Also in mammals and other vertebrates, 5-methoxytryptamine has been shown to occur[27,29,36] and to exert physiological effects[59,60], so that it has been discussed as another pineal hormone[52,53]. Moreover, 5-methoxytryptamine can be formed by deacetylation of melatonin in *Xenopus*[29] as well as in the rat[27].

A crucial point for the understanding of the photoperiodic encystment response appears to be the difference

between melatonin and 5-methoxytryptamine with regard to the requirement of low temperature. Since the deacetylated compound is effective already at the higher temperature of 20 °C, the assumption of its formation from melatonin, which induces encystment only below 16 °C, would imply that, a) at the higher temperature, concentrations of 5-methoxytryptamine remain below the threshold for encystment, and b) at the lower temperature, the concentration or the efficiency of 5-methoxytryptamine is increased. As there is no indication for an enhanced degree of potency, the most likely explanation would be that of a negative correlation between temperature and 5-methoxytryptamine concentration. This may be either due to a progressive decline of degradation of 5-methoxytryptamine with decreasing temperature, or to a stimulation of deacetylation by aryl acylamidase (AAA)[16,18,39]. The second possibility is by no means less likely, since overexpression of certain chronotypic genes at low temperature has already been demonstrated in *Gonyaulax*[44].

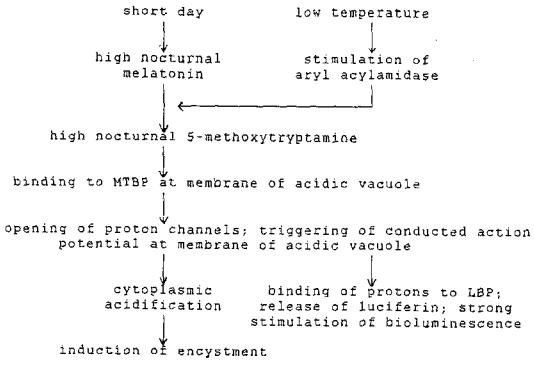
Parallelism of effects on bioluminescence and on encystment: role of proton transfer in the signal transduction chain from melatonin to encystment

When *Gonyaulax* is treated with cyst-inducing indoleamines, the first detectable reaction consists in a considerable stimulation of bioluminescence, up to 90-fold[39] (exceptionally even 200-fold[19]). There exists a strong parallelism in the potency to induce cysts and to enhance light emission. For instance, N-acetylserotonin hardly affects bioluminescence, except for some very short responses which are not consistently seen, whereas 5-methoxytryptamine again proves to be the substance with the highest efficiency and lowest threshold[6,9,39]. Strong effects were also exerted by N,N-dimethyl-5-methoxytryptamine[6,19,21,39], whereas serotonin, bufotenin[39], 5-methoxy- and 5-hydroxytryptophols[6,9,19,20,56] were less potent. The parallelism mentioned becomes particularly obvious in the case of melatonin: at 15 °C, light emission was stimulated by 23-fold, whereas at 20 °C, only a short increase by two-fold was observed[39].

All effects on bioluminescence vary within the circadian cycle, partly because of considerable changes in the capacity of the bioluminescent system, partly because of a cyclicity in the sensitivity to 5-methoxylated indoleamines[6,9,19–21].

The absolute concentrations required for stimulation of bioluminescence are generally much lower than those for cyst induction[39]: effects on light emission are obtained with 5-methoxytryptamine already at 2×10^{-8} M[6,9,19,20,39]. These results indicate that a cell physiological process which regulates light emission at low levels of indoleamines initiates cyst formation at a considerably higher level. This assumption is supported by several other results. Firstly, the same relationship between bioluminescence and cyst induction was observed with various, structurally different MAO inhibitors such as pargylin, tranylcypromine, *p*-benzoquinone, amitriptyline, noreleagnine, and harmaline[8,9,39,40]. The same was found with kynuramine, a substance possibly acting also *via* inhibition of MAO when administered in higher concentrations[3,5,8,9,39,40], and with a quinoline carrying a methoxy group in the position homologous to that of melatonin, 1,2-dihydro-4-hydroxy-6-methoxy-N-methyl quinoline[17,39,48]. Especially the effects of MAO blockers may be of significance for elucidating the different roles of melatonin and 5-methoxytryptamine in *Gonyaulax*, since the former is resistent to MAO, whereas the latter is usually a good substrate[36,60], another fact indicating the necessity for deacetylation of melatonin in the *Gonyaulax* encystment response. Unfortunately, direct measurements of MAO activity in material from this dinoflagellate have not been satisfactory when applying the usual substrates such as benzylamine or kynuramine (Behrmann, G., and Hardeland, R., unpubl.), and should be repeated with 5-methoxytryptamine itself.

Further experiments supporting an interrelationship between light emission and encystment have been performed with special reference to the mechanism of bioluminescence, as described by the so-called scintillon model[33,35,50]. Like other bioluminescent dinoflagellates, *Gonyaulax* contains a highly acidic vacuole; its membrane is excitable and conducts intracellular action potentials, which lead to an efflux of protons from the vacuole into the cytoplasm. Scintillons represent intrusions into the vacuole, which are enriched in the components of the bioluminescent system, i.e. luciferin, a luciferin-binding protein (LBP), and luciferase. After the opening of proton channels, protons bind to the LBP, which undergoes a conformational change, thereby releasing luciferin, which now becomes available as a substrate to the luciferase. Complete dissociation of LBP and luciferin occurs at pH 6[50]. Rapidly increased bioluminescence is, therefore, always an indication of stimulated proton transfer from the acidic vacuole and, hence, cytoplasmic acidification. This may be restricted to the scintillons and to the vicinity of the membrane as long as bioluminescence is stimulated only moderately. However, a stronger acidification, as indicated by the enormous light emissions in the beginning of the encystment response, can substantially decrease the intracellular pH in the entire cell. This view is confirmed by experiments with two electroneutral protonophores, the Na^+/H^+ antiporter monensin, and the K^+/H^+ antiporter nigericin, which stimulate bioluminescence and induce cyst formation, already at concentrations slightly above 10^{-7} M[11,17,39]. Similar results were obtained with a less specific protonophore, gramicidin D, and also by manip-



Scheme 1. Model of the signal transduction pathway for photoperiodic cyst induction in *Gonyaulax polyedra*. MTBP: 5-methoxytryptamine-binding protein; LBP: luciferin-binding protein.

ulating the extracellular pH (Wolf, R., and Hardeland, R., unpubl.). Moreover, measurements of intracellular pH by means of dicyanohydroquinone fluorescence showed that 5-methoxytryptamine led to a cytoplasmic acidification, whereas this effect was extremely weak with melatonin at 20 °C[37].

These experiments do not only establish a functional relationship between bioluminescence and encystment, but they also characterize the cyst as a cellular stage which is entered by lowering the intracellular pH. This assumption is in accordance with the general observation that, in various organisms, from bacteria and yeasts to arthropods and amphibia, resting stages including unfertilized eggs have a lower intracellular pH than activated cells[28,51].

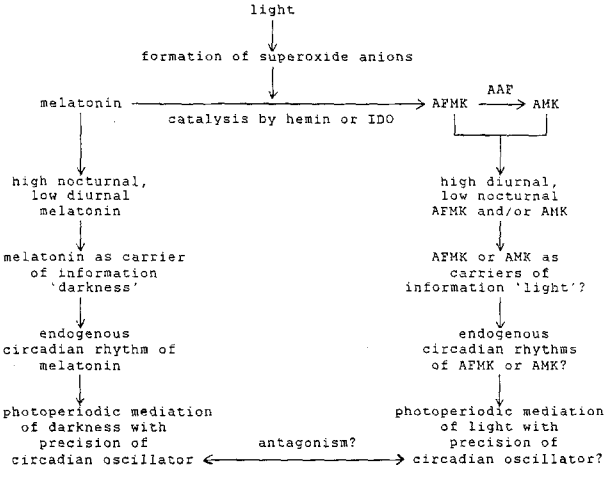
The analysis of the actions of melatonin and 5-methoxytryptamine, as exerted at different temperatures, together with the data on the role of intracellular pH, lead to the following model (scheme 1): Melatonin exhibits a circadian cycle with nocturnal maximum, which may represent the chemical signal for darkness, but which normally does not elicit a photoperiodic response. In short days, the overall amount of melatonin may be larger, but nocturnal values of 5-methoxytryptamine still remain below the encystment threshold. This is, however, surpassed after a decrease in temperature, either by stimulation of AAA or reduced degradation of 5-methoxytryptamine. The deacetylated indoleamine binds to an intracellular receptor protein, which is located in the membrane of the acidic vacuole, and which opens, eventually in cooperation with a GTP-binding protein, a proton channel. High concentrations of 5-methoxytryptamine lead to increased proton efflux, which is seen in the augmented bioluminescence, and which triggers the encystment response.

The destruction of melatonin by light: origin of photoperiodism?

The extreme instability of melatonin in extracts of some biological materials, e.g. homogenates from *Gonyaulax*[55,57], and the sensitivity of melatonin to light, as observed in certain solutions[43,46], seem to be caused by the same chemical reaction: an iron-catalyzed oxidative cleavage of the pyrrole ring by a species of highly reactive oxygen radicals, the superoxide anions, which can be induced either by light or by other kinds of energy transfer occurring during homogenization. The product formed is a substituted kynuramine, N^1-acetyl-N^2-formyl-5-methoxykynuramine (AFMK). This type of reaction is known to be catalyzed by an enzyme, indoleamine 2,3-dioxygenase[47]. In *Gonyaulax*, however, and also in suitable artificial solutions, AFMK is also formed non-enzymatically at high rates, due to an efficient catalysis by hemin[42,43,45,46]. Both protein-bound and free hemin may be involved in this reaction, but the importance of free iron-porphyrin became obvious when investigating light sensitivity in *Gonyaulax* extracts in which protein had been precipitated[46].

Already at the moderate intensity of 240 lx, visible light containing only negligible amounts of UV caused a substantial production of AFMK, whereas the reaction did not occur either in darkness or in the absence of *Gonyaulax* extract. In artificial solutions, the efficacy of hemin as a catalyst was demonstrated[43]. The formation of AFMK was followed either fluorometrically or by monitoring light emission from an excited state generated by an intermediate peroxide[43]. Chemiluminescence as a means for investigating peroxidation of indoleamines by superoxide anions had already been used by Uemura and Kadota[64], who generated the radicals by a xanthine/xanthine oxidase system. A product formed in this reaction mixture from serotonin was isolated and identified as a β-carboline[65]. In our systems, however, peroxidation of melatonin led to AFMK, not to a homologous β-carboline[42,43,45,46], a difference which may be explained by prevention of cyclization by the acetyl group in melatonin and AFMK. Neverthess, a β-carboline has also been detected in homogenates from *Gonyaulax*, namely, 6-methoxytryptoline (=pinoline)[55], which may derive from the non-acetylated analogue, 5-methoxytryptamine.

The fact that melatonin acts as a radical scavenger[42,43,45,46,64] reflects a fundamental property of potential importance for the understanding of its role as a chemical mediator of darkness. During evolution, melatonin may have been used originally as a part of the antioxidative protection system[39,42,46]. Indoleamines in general can be utilized for this purpose[46]. As compared to serotonin, melatonin represents, however, the more efficient substance at physiological pH[64], and may have been preferred by the ancestors of dinoflagellates. The mechanism of radical scavenging has, moreover, been optimized by introducing the catalysis by hemin. As a consequence, melatonin will be destroyed in the light, and can remain comparably stable in the dark. This may be regarded as the primary cause for the suitability



Scheme 2. A hypothesis on the evolutionary origin of melatonin as a mediator of the information 'darkness' and the possible role of melatonin-derived kynuramines. AFMK: N^1-acetyl-N^2-formyl-5-methoxykynuramine; AMK: N^1-acetyl-5-methoxy-kynuramine; AAF: arylamine formamidase; IDO: indoleamine 2,3-dioxygenase.

of melatonin as a carrier of the information 'darkness' (scheme 2). The light/dark cycle leads to the consequence of a corresponding high-amplitude cycle in the occurrence of superoxide anions[46], and any light-exposed cell is subjected to this strong exogenous periodicity. This can be assumed for the ancestors of both recent unicells and vertebrates, in which retinal and pineal photoreceptor cells had to be protected from aggressive oxygen radicals. It is, therefore, possible that the role of melatonin has evolved polyphyletically, on the basis of the particular suitability of this molecule. In a later evolutionary step, melatonin formation may have been coupled to the circadian oscillator, a connection providing the advantage that the rhythm of melatonin concentration becomes independent of changing irradiation intensities due to weather and, moreover, subject to cellular control. This might represent the precondition for a sufficient precision in the daily pattern of melatonin, as required for purposes of photoperiodic time measurement[39,46].

This hypothesis on a common evolutionary basis for melatonin as a mediator of scotophase, a hypothesis which is founded on a molecule's property, not necessarily on phylogenetical relationships, may even be extended to the question of the additional existence of a chemical mediator of light. Does a chemical mediator of darkness suffice for the photoperiodic time measurement, as is normally assumed, or is there a necessity for an antagonistic carrier of the information 'photophase'? With regard to the generation of AFMK by a light-dependent process, this substance may appear to be a candidate for such a role (scheme 2). In fact, substituted kynuramines have proved to possess a considerable spectrum of biological activities[5], and represent a class of scarcely investigated biogenic amines with poorly understood physiological significance. Moreover, when screening for potential mediators of light, also other kynuramines such as the deformylated AMK may be considered – since formylated kynuramines (like N-formyl kynurenine) are usually rapidly cleaved by arylamine formamidase[43] – as well as the non-acetylated analogues deriving from 5-methoxytryptamine, N^2-formyl-5-methoxykynuramine (FMK) or 5-methoxykynuramine (MK), or even the corresponding β-carboline, pinoline. The non-substituted kynuramine, which may derive from either tryptamine or kynurenine, is certainly no light mediator in *Gonyaulax*, since it stimulates bioluminescence, preferentially in the dark, and can induce cyst formation[3,5,8,9,39,40]. These effects may, however, be explained by inhibition of MAO. On the other hand, we obtained completely different results when we studied the effects of AFMK/AMK which did not stimulate bioluminescence even at high concentrations, and sometimes showed tendencies to suppress this cellular function (Fuhrberg, B., and Hardeland, R., unpubl.). These investigations do not yet allow definitive conclusions and are being extended. In earlier experiments, however, we had detected in extracts from *Gonyaulax* a substance with spectral properties like AFMK; this compound showed a circadian rhythmicity, both in LD and LL, with a maximum during (subjective) photophase[58].

Evolutionary origins of physiological mechanisms are difficult to prove directly. Hence, the ancient roles of melatonin and kynuramines may remain a matter of hypotheses. Nevertheless, we have to recognize that entirely different, phylogenetically very distant organisms such as dinoflagellates and mammals utilize the same molecule for photoperiodic time measurement, a fact that has broadened the biochemical and cell physiological basis of photoperiodism considerably.

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