and $Z = 128$ Cu_xS molecules. According to an electrochemical study by Potter (*11*), x may range from 1.934 to 1.965.

A set of intensities was measured with a Picker automatic diffractometer using Mo Kα radiation, all within an asymmetric sector in the range of 2θ from 5° to 45° (where θ is the Bragg angle). These intensities were converted to a data set of 5687 observed independent structure amplitudes, including corrections for Lorentz, polarization, absorption, anomalous dispersion, and isotropic extinction effects. The structure problem was approached from the substructure, which is based on the reported structure of high chalcocite (*7*). Phases were calculated for substructure reflections and extended to the superstructure reflections by the symbolic addition method (*12*).

In the substructure space group $P6_3/mmc$, there are three possible origins at symmetry centers for the supercell in $P2_1/n$, one of which can be ruled out as being incompatible with the hexagonal-close-packed sulfur framework and the supercell symmetry. The remaining two can each have the monoclinic a axis lying along either the + or − hexagonal a axis. The four possible orientations each led to a twofold ambiguity in the symbolic addition process, which otherwise proceeded smoothly. Thus, eight different phase models had to be tested and studied by Fourier methods. Each gave a clear image of the sulfur framework and a tantalizing image of large numbers of copper atoms in reasonable locations. Attempts to refine the eight structure models succeeded for only one, however. This model dropped to $R = 0.20$ in the first three least-squares cycles, whereas none of the others could be reduced below $R = 0.50$. The refined electron density map showed 32 sulfur and 62 copper atoms in the asymmetric unit, sharply delineated with appropriate peak heights, all in crystal chemically reasonable locations. Isotropic refinement leads to an average thermal motion for sulfur of $\bar{u} = 0.07$ Å and for copper of $\bar{u} = 0.11$ Å. The structure is currently being refined in anisotropic mode (846 parameters); at present $R = 0.12$. The latest set of atomic coordinates is given in Table 1.

No evidence has so far been found of disorder or partial occupancy in the structure. The revealed unit cell content corresponds to $x = 62/32 = 1.9375$ in the formula Cu_xS, indicating that the composition is near the low end of the homogeneity range found by Potter (*11*). If one copper atom were added to the asymmetric unit, x would be 1.9688, close to Potter's upper limit. Such an additional possible copper site has not yet been found in the crystal under study.

Of the 62 copper atoms in the asymmetric unit, 20 are in triangular coordination with sulfur in the hexagonal-close-packed sulfur layers lying normal to the a axis; the remaining 42 copper atoms are between the sulfur layers, 32 in triangular and 9 in tetrahedral coordination, often in severely distorted environments. A single copper atom, Cu(62), is in linear, twofold coordination. The structure contains two kinds of sulfur layers (S-1 and S-2), each containing 16 sulfur atoms; one contains 9 and the other 11 copper atoms. Thus, there are three different interlayer copper arrangements. The distribution of atoms is shown schematically in Fig. 1. Figure 2 shows the coordination arrangement of the structure viewed along the a axis in three sections. By contrast, the low chalcocite structure contains one type of hexagonal-close-packed sulfur layer and two different interlayer copper arrangements. So far, no extensive similarity between the two structures beyond these general features has become apparent.

Each copper atom has two, three, or four sulfur neighbors at distances ranging from 2.15 Å (for the twofold copper) to 2.8 Å. Most bond distances lie between 2.25 and 2.35 Å. Each copper atom also has two to six other copper atoms at distances less than 3 Å, some as close as 2.45 Å. These distances are most commonly in the range 2.70 to 2.80 Å. (The Cu-Cu distance in the metal is 2.556 Å.) Thus, the predominant coordination of sulfur about copper is triangular as in low chalcocite, similar to the typical triangular group in covellite (*13*) but having considerably longer average bond length (the triangular Cu-S distance in covellite is 2.191 Å). Most probably, bonding interactions between copper atoms are present and contribute to the stability of this structure and also to that of low chalcocite.

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9. I thank Ozark Lead Co. and R. W. Potter, II, of the U.S. Geological Survey for their generous help in obtaining this material.
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7 September 1978

Intraventricular Carbachol Mimics the Effects of Light on the Circadian Rhythm in the Rat Pineal Gland

Abstract. *Environmental lighting regulates numerous circadian rhythms, including the cycle in pineal serotonin* N*-acetyltransferase activity. Brief exposure of rats to light can shift the phase of this enzyme's circadian rhythm. Light also rapidly reduces nocturnal enzyme activity. Intraventricular injections of carbachol, a cholinergic agonist, can mimic both of these effects. Light and carbachol presumably act on the suprachiasmatic nucleus of the hypothalamus. These experiments demonstrate the feasibility of using a neuropharmacologic approach to the mechanisms underlying mammalian circadian rhythms.*

The enzyme serotonin *N*-acetyltransferase, which is involved in the synthesis of the hormone melatonin (*1*), displays a circadian rhythm in the rat pineal gland (*2*). At night, there is 30- to 50-fold more activity in the gland than during the day. The endogenous nature of this rhythm is indicated by its persistence in constant darkness or in blinded animals (*2*); that is, the rhythm can "free run." Enzyme activity rises and falls with a period slightly longer than 24 hours (*3*). Although the rhythm is endogenous to the organism, it does not appear to be endogenous to the rat pineal itself. Rather, like other circadian rhythms, the oscillations are determined by the central nervous system.

The rat pineal gland is innervated by sympathetic nerve fibers with cell bodies in the superior cervical ganglia (*4*). These fibers release the neurotransmitter norepinephrine. During the night, more norepinephrine is released than during the day (*5*). This rhythm in adrenergic stimulation drives the rhythm in enzyme activity. If the connection between the gland and the brain is cut, for example, by bi-

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lateral ganglionectomy, the rhythm in serotonin *N*-acetyltransferase is abolished (*6*); enzyme activity remains at its low daytime level. The mechanism by which norepinephrine increases serotonin *N*-acetyltransferase activity is partially understood (*1, 7*). The neurotransmitter interacts with β-adrenergic receptors on the pinealocytes, resulting in the synthesis of adenosine 3′,5′-monophosphate (cyclic AMP). Cyclic AMP, in turn, causes the induction of enzyme activity by a process involving RNA and protein synthesis.

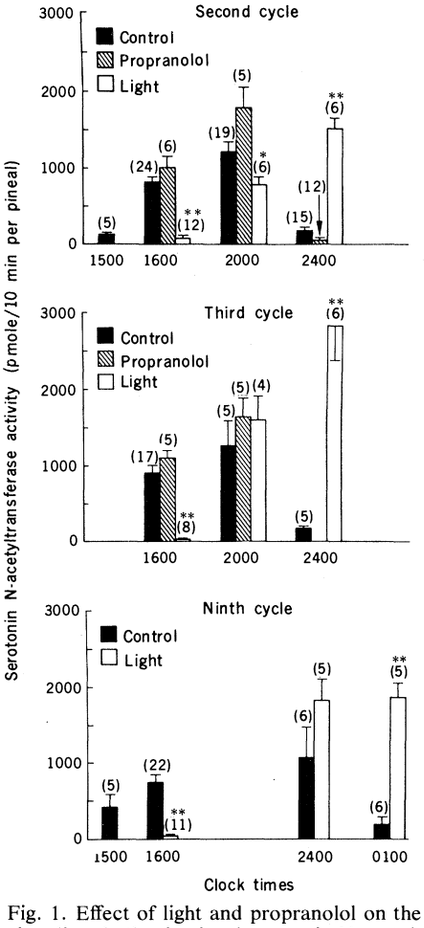

Fig. 1. Effect of light and propranolol on the circadian rhythm in pineal serotonin *N*-acetyltransferase activity. All animals were entrained to LD 12:12 for at least 7 days prior to the experiment. Control animals were blinded at 0800 of the "first cycle." Light-treated animals were exposed to environmental lighting for 90 minutes (1500 to 1630) after 3 hours of darkness and then blinded. Propranolol-treated animals were blinded at 0800 and injected with *dl*-propranolol (40 mg/kg, intraperitoneally) at 1500. Serotonin *N*-acetyltransferase was assayed, as in (*18*), at various times during the second, third, and ninth cycles. Values shown represent mean and the standard error for the number of pineals indicated in parentheses. Determinations were serially independent. (*)Differs from corresponding control with $P < .05$ by Student's *t*-test. (**)Differs from corresponding control with $P < .001$.

Much less is known about the oscillatory mechanisms in the brain, which generate the rhythm in norepinephrine release. Anatomical experiments have implicated the suprachiasmatic nucleus of the hypothalamus in the central regulation of pineal and other circadian rhythms (*8*). Studies of the effects of temperature and, particularly, of light have provided insight into the formal properties of the oscillatory mechanisms (*9*). Environmental lighting regulates the phase of the pineal rhythm (provides a zeitgeber), as it does for other circadian rhythms. This is most readily demonstrated by the reversal of the manifest cycle following the reversal of the light-dark (LD) cycle. Environmental lighting has an additional effect upon the pineal, however, which it does not have on several other circadian rhythms. Brief exposure of rats to light, after the nocturnal rise in enzyme activity has occurred, causes a precipitous fall in enzyme activity to daytime levels (here termed the acute effect) (*10*). Constant exposure to light abolishes the circadian rhythm in pineal serotonin *N*-acetyltransferase activity. Enzyme activity remains at its low daytime level.

We have used this phenomenon to attempt a pharmacological approach to the central oscillatory mechanism which regulates the pineal, and presumably other, circadian cycles. The only measurable properties inherent in the oscillatory mechanism are the period and phase of a cycle. Thus, a change in the period or phase of a circadian rhythm reflects a change in the oscillatory mechanism that drives the cycle. These properties (unlike amplitude) have proved remarkably resistant to pharmacological manipulation (*9, 11*). Since, in the rat, environmental lighting affects the oscillatory mechanism via the retinohypothalamic projection to the suprachiasmatic nucleus (*12*), it should be possible to mimic the effects of light with neuropharmacological agents. Several pharmacological agents, infused near the suprachiasmatic nucleus, do mimic the ability of light to rapidly reduce pineal serotonin *N*-acetyltransferase activity. One of these, carbachol, a cholinergic agonist, was shown to also mimic the ability of light to cause a shift in the phase of the pineal rhythm.

Male Sprague-Dawley rats (200 g; Zivic-Miller) were housed in our facilities, under a LD 12:12 lighting schedule with free access to food and water, for at least 7 days prior to experiments. Most animals were blinded by bilateral enucleation under light ether anesthesia at approximately 0800 to initiate a "free-running" rhythm. Blinding had no significant effect on the amplitude of the serotonin *N*-acetyltransferase rhythm. Clock times refer to the original LD cycle with lights on from 0000 to 1200 and off from 1200 to 2400, by definition.

Maintenance of the elevated nocturnal serotonin *N*-acetyltransferase activity requires continued stimulation of the gland (*10, 13*). This is illustrated by the effect of propranolol, a β-adrenergic receptor blocker. Injection of propranolol rapidly reduces enzyme activity (Table 1). This drug acts directly on the rat pineal gland; it has a similar effect in organ culture. Exposure of intact animals to

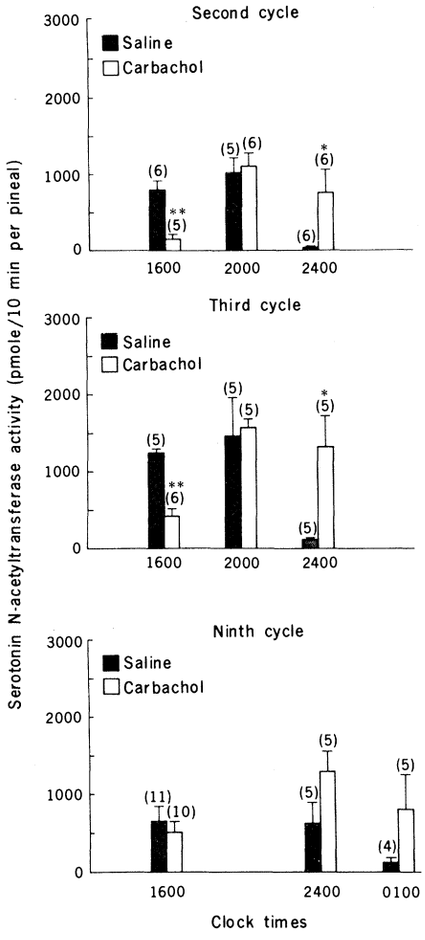

Fig. 2. Effect of intraventricular carbachol on the circadian rhythm in pineal serotonin *N*-acetyltransferase. All animals were entrained to LD 12:12 for at least 7 days prior to the experiment. Animals were blinded at 0800 of the "first cycle." Saline (5 μl) or carbachol hydrochloride (5 μl, 0.02*M*) was injected into the right lateral ventricle, under light ether anesthesia, between 1500 and 1600. Serotonin *N*-acetyltransferase was assayed (*18*) at various times during the second, third, and ninth cycles. Values shown represent mean and the standard error for the number of pineals indicated in parentheses. Determinations were serially independent. (*)Differs from saline control with $P < .05$ by Student's *t*-test. (**)Differs from saline control with $P < .001$.

light also rapidly reduces enzyme activity (*10*) (Table 1). However, the effect of light is indirect; it acts centrally, ultimately reducing the release of norepinephrine from the sympathetic nerve endings in the gland. A lesion which cut the caudal projections of the suprachiasmatic nucleus mimicked the acute effect of light on nocturnal enzyme activity (data not shown). Similarly, infusion of a local anesthetic adjacent to the nucleus also rapidly reduced nocturnal serotonin *N*-acetyltransferase activity (Table 1). These experiments suggest that light acts to inhibit neuronal activity in the suprachiasmatic nucleus.

We infused a number of putative neurotransmitters adjacent to the suprachiasmatic nucleus and measured their effect on nocturnal enzyme activity. Serotonin, dopamine, thyrotropin-releasing hormone, and substance P (a total of 250 pmole each), and glycine and γ-aminobutyric acid (25 nmole) each had no effect on enzyme activity under the conditions tested. Infusion of carbachol, however, rapidly reduced serotonin *N*-acetyltransferase activity (Table 1). Intraventricular injections of carbachol could also rapidly reduce enzyme activity, but required 200 times greater amounts of the drug. Similar amounts of methacholine had no effect.

In order to determine whether carbachol could affect the oscillatory mechanism, we assessed its ability to cause a phase shift in the circadian rhythm of serotonin *N*-acetyltransferase activity. For comparison, we examined the effects of a pulse of light and of propranolol (Fig. 1). Since phase delays are more readily and rapidly obtained than are phase advances (*11*), we used conditions designed to elicit a phase delay. Experimental manipulations were performed during the "first cycle," and the temporal pattern of enzyme activity was assessed on subsequent nights. During the second and third cycles serotonin *N*-acetyltransferase activity in the controls was significantly elevated by 1600, remained elevated at 2000, and had fallen by 2400 (Fig. 1).

In contrast, animals exposed to 90 minutes of light (starting 3 hours after lights-off) during the first cycle showed a different pattern (*14*). Enzyme activity was not yet elevated at 1600, had risen by 2000, and remained elevated at 2400. This shift to the right in the temporal pattern reflects a phase delay in comparison to the controls. In contrast, injection of propranolol had no effect on the pattern of enzyme activity in subsequent cycles (Fig. 1). Thus, this drug mimics the acute effect of light by acting directly on the pineal, not by acting on the central oscillatory mechanism. After 9 days, the control pattern had changed somewhat. Enzyme activity fell about an hour later, and there was increased variability in the values obtained. These changes reflect a period slightly longer than 24 hours and individual variations in the free-running period.

Injection of saline into the cerebral ventricles of blinded rats had no effect on the subsequent pattern of enzyme activity. These animals provided controls for the effect of intraventricular carbachol. Like light, carbachol caused a shift to the right in the temporal patterns of serotonin *N*-acetyltransferase activity during the second and third cycles; there was a clear phase delay relative to controls (Fig. 2). The effect of carbachol appeared "smaller," however, than that of 90 minutes of exposure to light (*14*). Increased variability within groups obscured the effect after 9 days.

Thus, carbachol, a cholinergic agonist, mimics the effects of light on the oscillatory mechanism regulating pineal serotonin *N*-acetyltransferase activity. Light and carbachol presumably act on the suprachiasmatic nucleus. We cannot yet say, however, that acetylcholine mediates the effects of light. This would require, at least, the demonstration that a cholinergic antagonist can block the effects of light. It appears most likely, however, that the neurotransmitters mediating the effects of light and carbachol have an inhibitory effect on the output of the suprachiasmatic nucleus. Studies with radioactive 2-deoxyglucose indicated an increase in metabolic activity in the suprachiasmatic nucleus caused by light (*15*). This may have reflected an increase in inhibitory neurotransmission within the nucleus.

The underlying assumption in these experiments, which remains unproved, is that light rapidly reduces serotonin *N*-acetyltransferase activity via the same mechanism by which it provides a zeitgeber for the enzyme's circadian rhythm. Since the effect of light on the oscillatory mechanism is mediated by a known neuroanatomical pathway, it should be possible to mimic or block the effects of light by neuropharmacologic agents. These, in turn, might provide some insight into the mechanisms by which circadian oscillations are generated or regulated. This approach, which is similar to that used recently in the *Aplysia* eye (*16*), implies a role for the plasma membrane (*17*) in photoentrainment of the circadian oscillator. Most of the agents that have been found to affect circadian rhythms have profound effects on neuronal plasma membranes.

The acute effect of light on nocturnal serotonin *N*-acetyltransferase provides a rapid screening test for drugs that might affect the circadian oscillatory mechanism. Drugs that mimic the acute effects of light on the pineal can then be tested for effects on the phase or period of the circadian rhythm. Such an approach might be useful in studying the basis of other circadian rhythms in addition to that of pineal serotonin *N*-acetyltransferase.

Table 1. Reduction of nocturnal pineal serotonin *N*-acetyltransferase (NAT) activity by light or drugs. All animals were entrained to LD 12:12 for at least 7 days prior to the experiment. Except for animals to be exposed to light, rats were blinded at 0800. They were killed between 1730 and 2130. Control enzyme levels were stable over this period. The control groups for the various experiments did not differ and have been pooled. *dl*-Propranolol (40 mg/kg) was injected intraperitoneally, and rats were killed after 20 minutes. Intact animals were brought into the light after 6 hours of darkness and killed 15 minutes later. Nupercaine (25 mg/ml in 0.9 percent NaCl) was infused for 2 minutes (0.125 μl/min) through two 30-gauge needles (2 mm apart) placed adjacent to the suprachiasmatic nucleus (R7.6, L1, V8.3, 5° nose down). Pineals were removed after 15 minutes. Carbachol hydrochloride (0.1 m*M*) or serotonin (0.1 m*M*) were similarly infused for 10 minutes (0.25 μl/min), and the pineals were removed 5 minutes later. In another experiment, various concentrations of carbachol hydrochloride were injected into the right lateral ventricle (5 μl), and pineals were removed after 15 minutes. Results shown are for 0.01*M* carbachol. Data shown are means ± the standard error for the number of animals indicated in parentheses.

Item	Serotonin NAT (picomoles per 10 minutes per pineal)
Controls	1180 ± 97 (23)
Intraperitoneal propranolol	69 ± 10 (5)*
Environmental light	79 ± 21 (20)*
Infused Nupercaine	118 ± 47 (5)*
Infused carbachol	336 ± 91 (5)*
Infused serotonin	1152 ± 188 (5)
Intraventricular carbachol	306 ± 59 (8)*

*Differs from control with $P < .001$ by Student's *t*-test.

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28 June 1978; revised 24 October 1978

Mammary Cancer: Selective Action of the Estrogen Receptor Complex

Abstract. *Progesterone receptors in the autonomous rat mammary tumor MTW-9B are reduced 80 to 90 percent after ovariectomy, but are not reduced if ovariectomized animals are given estrogen. Tumor growth, however, is independent of estrogen status and insensitive to pharmacological doses of estradiol. This represents an unusual system characterized by a selective action of an inducing agent on the genome.*

Approximately 55 percent of breast cancer patients whose tumors contain estrogen receptors respond to endocrine ablative or additive steroid therapy (*1*). The failure of this therapy in patients positive for the estrogen receptor implies that the estrogen receptor is not functionally competent or that the tumor cells or their nuclei no longer respond to hormonal stimulation (*2*). Other possibilities have been summarized by McGuire *et al.* (*3*).

Horwitz *et al.* (*4*) suggested that the simultaneous presence of the progesterone receptor with the estrogen receptor would be a more reliable indicator of hormone responsiveness in breast cancer. This proposal is supported by studies wherein the synthesis of the progesterone receptor was shown to depend on the presence of estradiol in uterus (*5*), oviduct (*6*), the hormone-dependent mammary tumor induced by 9,10-dimethyl-1,2-benzanthracene (DMBA) (*7–9*), and the human breast cancer cell line MCF-7 (*10*). In the last case, progesterone receptor synthesis was shown to be mediated by the estrogen receptor system (*10*). Presumably, synthesis of the progesterone receptor, as a gene product, would be indicative of a functioning estrogen receptor. Indeed, when both receptors are present, an improved clinical response rate is observed, although about 17 percent of patients with both receptors do not respond, and, conversely, some patients whose tumors have receptors for estrogen but not progesterone do respond (*11*). This suggests that there might not always be an association between estrogen-induced tumor growth and progesterone receptor regulation; the results reported herein show that in the estrogen-independent MTW-9B mammary tumor system, these two parameters are completely dissociated.

The transplantable mammary tumor MTW-9B, in Wistar/Furth rats, is estrogen-independent as evidenced by its equal growth rate in syngeneic males and females and also by the lack of effect of ovariectomy or hypophysectomy on its growth rate (*12*). In addition, this tumor does not regress in animals treated with pharmacological doses (1 mg per kilogram of body weight) of estradiol benzoate (*13*). Both estrogen and progesterone receptors are present (Fig. 1) and in amounts comparable to those observed in the hormone-dependent DMBA tumor (*9*, *14*). Specific binding of [^{3}H]estradiol and the synthetic progestin [^{3}H]R5020 to tumor cytosol is saturable and of high affinity. Binding of [^{3}H]-progesterone to tumor cytosol is similar to that observed with [^{3}H]R5020; however, the K_d is about threefold higher.

The presence of the progesterone receptor in this autonomous tumor suggests that the concept advanced by Horwitz *et al.* (*4*) requires further critical evaluation. The data in Table 1 indicate that the synthesis of the progesterone receptor in the MTW-9B tumor is under estrogen regulation. Thus, in tumors obtained from rats ovariectomized 2 weeks earlier, progesterone receptor levels were decreased to 10 to 20 percent of those in the sham-operated controls. This represents a true decrease in binding capacity since the binding affinity, measured by Scatchard analysis (*15*), did not change. That this decrease was due to a reduction in circulating estradiol is suggested by the fact that when estradiol benzoate (25 μg/kg) was given to rats daily for 2 weeks, starting 1 day after ovariectomy, progesterone receptor levels in the tumor remained in the normal range. In fact, when R5020 was used as the tritiated ligand, an apparent "superinduction" was observed. These changes occurred in the absence of any effect on tumor growth. It is not known whether the differences between binding observed with tritiated progesterone and R5020 are real, or whether they simply reflect variation from one group of animals to another. It is also possible that in comparison to progesterone, R5020 binds to additional components, such as serum albumin, although this binding has been reported to be nonspecific (*16*).

The concentration of estrogen receptor showed a slight but not significant increase after ovariectomy (Table 1). Similar results have been reported for the R3230AC mammary adenocarcinoma (*17*) and for hormone-independent DMBA-induced mammary tumors (*8*), although both a decrease (*7*, *8*, *18–20*) and an increase have been noted for the more common hormone-dependent DMBA tumors (*9*). The decrease in estrogen receptors observed after estradiol treatment may not reflect a true decrease in receptor concentration but rather an increase of receptors in the nucleus or of occupied receptors in the cytosol, since the assay used here measured only available cytosol sites. Kelly *et al.* (*18*) also showed a decrease in cytosol estrogen receptor levels in DMBA tumors from intact rats after long-term administration

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