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# *Circadian Disc Shedding in* Xenopus *Retina In Vitro*

John G. Flannery and Steven K. Fisher

**To further examine the endogenous rhythm of disc shedding and phagocytosis observed in several species, adult Xenopus were entrained to a 12 hr light/12 hr dark cycle and then placed in constant darkness. At various times during a 3-day period of constant darkness, eyes were explanted and placed into culture medium, then processed for light and electron microscopy. A clear rhythmicity of disc shedding was observed, with pronounced peaks at the times light onset occurred in the original entrainment cycle. Modification of the $HCO_3^-$ ion concentration in the medium was found to raise the amplitude of the peak of endogenous disc shedding. Explants maintained in culture medium containing deuterium oxide (a compound known to perturb circadian oscillators) were found to shed with a longer interval between peaks. The addition of the protein synthesis inhibitor, anisomycin, to this preparation suppressed the shedding rhythm. The action of anisomycin was investigated by autoradiographic examination of the pattern of $^3$H-leucine uptake and protein synthesis by the explant. The findings suggest the presence of a circadian oscillator for rhythmic disc shedding within the amphibian eye. Invest Ophthalmol Vis Sci 25:229–232, 1984**

LaVail[1] was the first to show that most disc shedding from rod photoreceptors occurs within 1 to 2 hrs of the onset of light in animals entrained to a daily lighting cycle. More recently, he has shown that, in rats, this peak in phagosome number within the RPE has characteristics of a circadian rhythm, with light acting as a zeitgeber.[2] While earlier work showing that reserpine diminished this daily rhythm suggested that input from the pineal may be important in cyclic disc shedding,[1] more recent studies have shown that the rhythm occurs unimpaired after removal of the pineal, superior cervical ganglia, hypophysis, and thyroid-parathyroid glands.[3,4] In albino rats in which one eye is patched and the optic nerve is transected, constant light blocks rhythmic shedding only in the unpatched eye,[5] suggesting that the circadian oscillator controlling rhythmic shedding resides within the eye.

In the African clawed frog (*Xenopus laevis*), this shedding response is largely evoked by the onset of light; but there is evidence that rhythmic shedding continues in constant darkness, although at a greatly reduced level when compared with animals maintained under cyclic lighting.[6] We have previously demonstrated that *light-induced* disc shedding proceeds normally in Xenopus eyes kept in vitro.[7] It seemed to us that the most direct approach to determining whether a circadian oscillator that controls disc shedding exists within the eye would be to look for evidence of rhythmic disc shedding in Xenopus eyes placed in culture and kept in constant darkness. In this report, we demonstrate that explanted eyes show a rhythmic increase in rod disc shedding that persists in darkness,

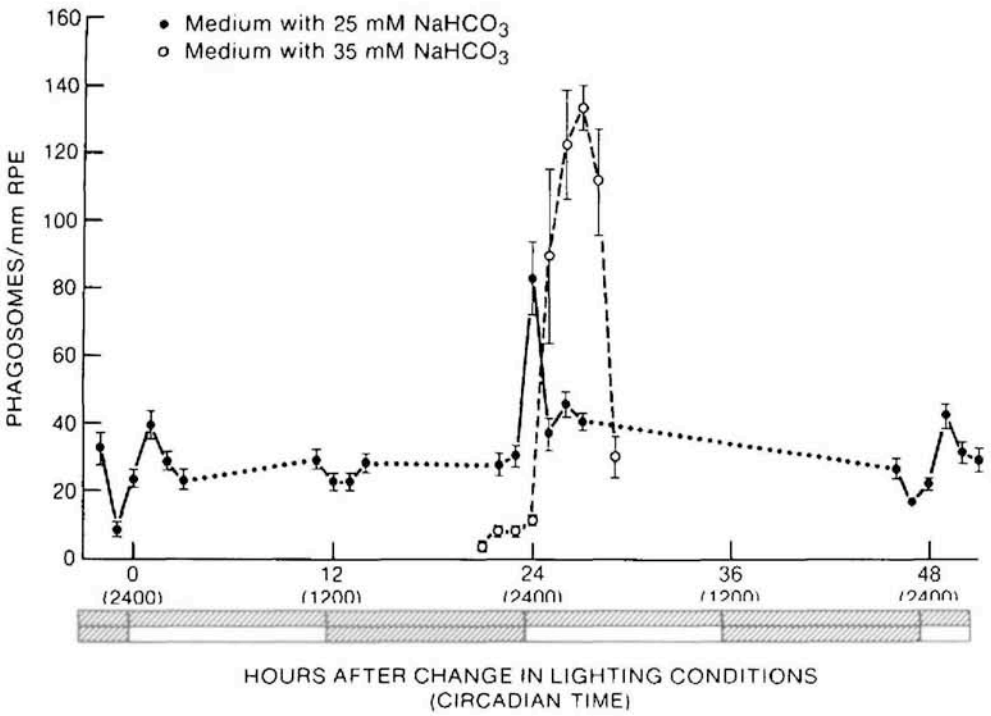


**Fig. 1.** Mean phagosome counts from the RPE of eye explants incubated in culture medium containing either 25 mM or 35 mM $NaHCO_3$. Each data point is the mean of counts from three eyes, error bars equal +/− 1 standard error. Lighting conditions are indicated by the bars below the graph; the upper bar indicates experimental conditions (darkness), while the lower bar shows the light and dark periods of the cycle to which the animals were entrained.

and we show that this system is perturbed by chemicals known to affect circadian oscillators.

***Materials and Methods.*** Young adult *Xenopus* were entrained to a 12:12 light/dark cycle at 25°C for several months. At the beginning of each experiment, a group of frogs was maintained in constant darkness from the end of the normal 12 hrs of dark in the entrainment cycle. The amount of rod disc shedding was then assayed in eyes that were explanted, maintained in vitro, and histologically processed at times clustered around the following timepoints: 0, 12, 24, and 48 hrs as measured from subjective light onset in the cycle to which the frogs were entrained. The hours chosen for analysis correspond to the times of three light-to-dark transitions and one dark-to-light transition in the original entrainment cycle (Fig. 1). For the 0 hr group, whole eyes were removed under the dim illumination of a sodium vapor safelight (<2 lux.) 3 hrs before light onset occurred in the entrainment cycle. These explants were placed into oxygenated Wolf and Quimby amphibian culture medium (Gibco) containing 25 mM sodium bicarbonate. At each hr, eyes were removed from the medium and fixed in buffered glutaraldehyde. The timepoints represented, therefore, are "−2," 2 hrs before subjective light onset, to "+3," three hrs following subjective light onset (Fig. 1). Eyes then were fixed in buffered glutaraldehyde. Tissue fixation and processing procedures have been described previously.[7]

The longest period during which we have maintained eye explants while preserving structural integrity of the photoreceptor and retinal pigment epithelial cells is 24 hrs. Therefore, to examine timepoints in the second light/dark cycle, (the 24 hr group), eyes were explanted at 21 hrs, placed in medium, and then fixed hourly between 22 and 27 hrs. Similarly, the 48-hr group consists of eyes explanted at 45 hrs, placed in medium, and fixed hourly at 46 through 51 hrs. The light-to-dark transition in the first light/dark cycle was studied by placing eyes from animals treated as above in vitro 10 hrs into the experimental dark period and then fixing them hourly between 11 and 14 hrs. A simpler experimental design (attempted unsuccessfully) was to explant a large number of eyes at the beginning of the "subjective dark" period and maintain them in vitro for 80 hrs of continuous darkness. Unfortunately, our methods of organ culture did not permit such extended periods.

The strong endogenous response occurring at 24 hrs was utilized in further experiments to test the effect of several pharmacologic agents on photoreceptor disc shedding. The 24-hr group of timepoints was reexamined in four distinct media containing: 35 mM sodium bicarbonate, ($NaHCO_3$); 35 mM $NaHCO_3$ and 25% deuterium oxide (Aldrich Chemical Co.); 35 mM $NaHCO_3$ and $10^{-3}$ M anisomycin (Pfizer Diagnostics); and 35 mM $NaHCO_3$ and $10^{-5}$ M anisomycin, respectively. In these last four conditions, eyes were explanted at 20 hrs and fixed between 21 and 29 hrs. Each hourly data point represents the mean count of phagosomes (2 $\mu$M or greater) from 1 mm lengths of retina (10 from each retina) in each of three eyes.

For autoradiography, two groups of explants (nine

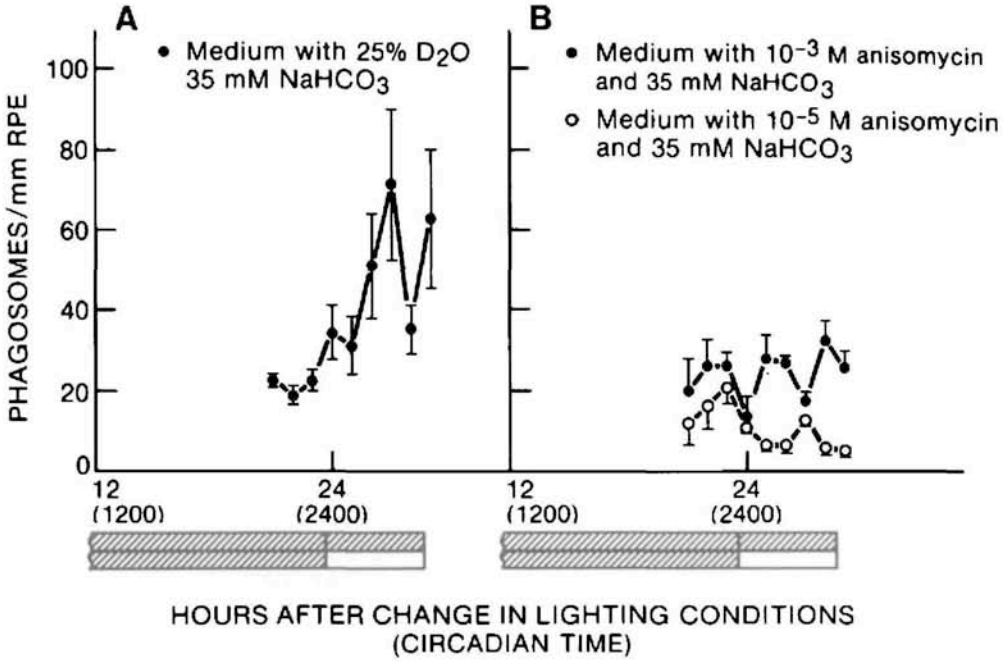


**Fig. 2A.** Mean phagosome counts from eye explants incubated in medium with 25% $D_2O$ and 35 mM $NaHCO_3$. **B.** Mean phagosome counts from eye explants incubated in medium with either $10^{-3}$ M or $10^{-5}$ M anisomycin and 35 mM $NaHCO_3$. In these experiments, eyes were explanted from frogs that had been in darkness for a total of 32 hrs. Each data point is the mean of counts from three eyes, error bars equal +/− 1 standard error. The upper (dark) bar indicates the experimental lighting conditions; the lower bar shows the time of the dark-to-light transition in the cycle to which the frogs were entrained.

eyes each) were placed in culture medium containing 27 $\mu$Ci of 1-4,5-$^3$H-leucine (New England Nuclear). $10^{-3}$ M anisomycin was added to the medium of one group. Eyes were removed hourly and fixed in 2% formaldehyde in 0.10 M sodium cacodylate buffer (formaldehyde was used in place of glutaraldehyde to avoid binding of free amino acid). The eyes were embedded, 2 $\mu$M thick sections were cut, placed on glass slides, and dipped in Kodak NTB-2 nuclear track emulsion diluted 1:1 with distilled water. After 4 wks exposure at 4°C, autoradiograms were developed in Kodak D-19 developer at 20°C for 2 min. Area measurements and grain counts from the ellipsoid, myoid, nucleus, and outer segment areas of the rod photoreceptors were made with a Zeiss MOP-3 digital plotting tablet.

***Results and Discussion.*** The number of phagosomes within the RPE show a clear rhythmic variation over the time course of this experiment (Fig. 1). Within one hr after each of the three times of expected dark-to-light transition, phagosome numbers peak, and then, within two hrs, return to the basal level. A particularly large shedding response occurs in all eyes fixed during the 22-to-27-hr interval (Fig. 1), while on day 3, the shedding level is comparable to that on day 1. Within the RPE of intact Xenopus kept in darkness, the number of phagosomes observed within the RPE rises only slightly during the first 2 days, but on the third day increases significantly.[6,8] Another difference between the in vivo and the in vitro shedding responses seems to be the time at which the shedding reaches its peak. At all three time intervals sampled, the peak occurred within an hour after the time of expected light onset (Fig. 1). In Xenopus kept in darkness, the peaks were delayed for several hrs. Thus, it appears that the eye explants did not show a phase shift in the peak shedding response as is shown in rats kept in constant darkness.[1] No increase in phagosome number occurred in the 11–14 hr interval (the light-to-dark transition, Fig. 1).

An increase in the sodium bicarbonate concentration of the culture medium to 35 mM greatly increases the magnitude of the disc shedding response (Fig. 1). This finding is consistent with reports showing that light-evoked shedding is enhanced in Xenopus posterior eyecup preparations (explanted eyes from which the cornea, lens, and iris are removed) kept in medium containing 35 mM $NaHCO_3$.[9] Thus, the photoreceptors appear to respond in the same manner to increased bicarbonate, whether the stimulus for disc shedding is internal (circadian clock) or external (light). The increase in $NaHCO_3$ causes two ways in which more outer segment membrane is shed in our experiment: not only does the number of phagosomes increase, but the mean phagosome size also increases from 3.8 +/− 0.2 $\mu$m to 7.0 +/− 0.5 $\mu$m in diameter.

In other circadian systems, deuterium oxide ($D_2O$) produces an increase in the free-running period or a loss of entrainment to the light-dark cycle.[10] Eyes maintained in medium containing 25% $D_2O$ show a much greater variability in the phagosome counts at times on either side of the expected time of peak shedding (Fig. 2). In fact, the overlap in the error bars of mean phagosome counts between 21 and 19 hrs makes it difficult to define an exact peak, even though the mean counts do show rises at 27 and 29 hrs.

Anisomycin, an antibiotic protein synthesis inhib-

**Table 1.** Mean grain counts per 100 $\mu m^2$ in autoradiograms

| *Hours* | *Wolf and Quimby medium* | *Wolf and Quimby medium + $10^{-3}$ M anisomycin* |
|---|---|---|
| 1 | 12.4 | 6.0 |
| 2 | 24.0 | 28.0 |
| 3 | 11.1 | 40.0 |
| 4 | 54.4 | 54.6 |
| 5 | 80.8 | 45.0 |
| 9 | 120.5 | 114.5 |

Each measurement represents the mean of counts from a total area of 10,000 $\mu m^2$ evaluated in 10 autoradiograms.

itor, appears to act selectively on the circadian clock.[11] Concentrations of $10^{-6}$ M result in 75% inhibition of protein synthesis in the isolated Aplysia eye and up to 90% inhibition in Aplysia central neurons.[12] Although in the isolated Aplysia eye, lower concentrations cause significant phase shifts in the rhythm of compound action potentials, $10^{-6}$ M anisomycin causes a complete suppression of the rhythm of action potentials.[12] Both $10^{-5}$ M and $10^{-3}$ anisomycin completely abolish any peak of shedding in the eye explants between 22 and 27 hrs (Fig. 3). The drug does not completely inhibit disc shedding, but higher concentrations do result in a level of shedding below baseline levels of cultures without the drug (Fig. 3).

While anisomycin had a profound effect on the peak shedding response, it did not appear to significantly decrease protein synthesis in the cultured eyes. Grain counts from autoradiograms prepared from eyes incubated with $^3$H-leucine for 1 to 9 hrs in the presence or absence of $10^{-3}$ M anisomycin show that amino acids are incorporated to about the same extent in both cases (Table 1). Since we were using whole eye explants, it is possible that the concentration of the drug within the eye is great enough to affect the circadian clock but not high enough to affect protein synthesis to the degree detectable by simple grain counts in autoradiograms. There is also a high degree of variability in the levels of protein synthesis inhibition by this drug in the eye, central nervous system, and abdominal ganglia of Aplysia,[12] although in these cases there is clear inhibition in comparison to controls.

Our experiments show that amphibian eyes continue to shed discs rhythmically in constant darkness and in the absence of any extraocular input, although the peak number of phagosomes is reduced by comparison with that occurring in light-induced shedding.[7,8] In eyes explanted from frogs kept in constant darkness, there are peaks of shedding near the time of light onset in the cycle to which the frogs were entrained. Two reagents known to affect circadian oscillators also exert effects on these shedding peaks. These results reinforce the hypothesis that a circadian oscillator that controls disc shedding resides within the eye of *Xenopus laevis*.

**Key words:** photoreceptors, pigment epithelium, phagocytosis, tissue culture, circadian rhythms, deuterium oxide, anisomycin

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