

Phase Shifting the Circadian Rhythm of Neuronal Activity in the Isolated *Aplysia* Eye with Puromycin and Cycloheximide

Electrophysiological and Biochemical Studies

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ABSTRACT The effects of pulse application of puromycin (PURO) or cycloheximide (CHX) were tested on the circadian rhythm (CR) of spontaneous compound action potential (CAP) activity in the isolated *Aplysia* eye. CAP activity was recorded from the optic nerve in constant darkness at 15°C. PURO pulses (6, 12 h; 12–134 $\mu\text{g}/\text{ml}$) and CHX pulses (12 h, 500–2,000 $\mu\text{g}/\text{ml}$) caused dose-dependent phase delays in the CR when administered during projected night. PURO pulses (6 h, 125 $\mu\text{g}/\text{ml}$) caused phase advances when given during projected day and caused phase delays when given during projected night.

In biochemical experiments PURO (12 h, 20 $\mu\text{g}/\text{ml}$) and CHX (12 h, 500 $\mu\text{g}/\text{ml}$) inhibited leucine incorporation into the eye by about 50%. PURO (12 h; 20, 125 $\mu\text{g}/\text{ml}$) also changed the molecular weight distribution of proteins synthesized by the eye during the pulse. The effect of PURO (12 h, 125 $\mu\text{g}/\text{ml}$) on the level of incorporation was almost completely reversible within the next 12 h but the phase-shifted eye showed an altered spectrum of proteins for up to 28 h after the pulse.

In electrophysiological experiments spontaneous CAP activity and responses to light were measured before, during, and after drug treatments. In all, eight parameters in three periods were analyzed quantitatively. Of these 24 indices, only 3 showed significant changes. PURO increased spontaneous CAP frequency by 67% 0–7 h after the drug pulse and increased the CAP amplitude of the tonic light response by 23% >7 h after the pulse. CHX increased the intraburst spontaneous CAP frequency by 33% during the pulse and CAP frequency of the tonic light response by 32% 0–7 h after the pulse. The above data indicate that phase-shifting doses of PURO and CHX inhibit protein synthesis in the eye without causing adverse electrophysiological effects, and suggest that protein synthesis is involved in the production of the CR of the isolated *Aplysia* eye.

INTRODUCTION

Circadian rhythms (CRs) in intact organisms are biological rhythms of about 24 h in period that persist without environmental cues (under constant conditions), but which can be entrained to an environmental periodicity modestly different

from their natural (free-running) periods. Although the endogenous nature of the circadian timing mechanism is generally agreed on (Bünning, 1973), little is known about its biochemical components. In particular there is limited information on the nature of neuronal circadian oscillators.

In our studies, we have focused on the role of RNA and protein synthesis in the circadian mechanism of impulse generation in the eye of *Aplysia*. Past work on effects of inhibitors of macromolecular synthesis on a variety of circadian systems shows that the CRs of both unicellular and multicellular organisms are perturbed by inhibitors of macromolecular synthesis, although different CR parameters are affected in different systems. Including our work, at least seven different CRs have been shown to be abolished or modified by inhibitors of RNA or protein synthesis (Karakashian and Hastings, 1962, 1963; MacDowall, 1963; Strumwasser, 1965; Vanden Driessche, 1966; Feldman, 1967; Applewhite et al., 1973; Mergenhagen and Schweiger, 1975).

The purpose of this paper is to demonstrate the sensitivity of the CR of optic nerve activity of the *Aplysia* eye to the administration of puromycin (PURO) and cycloheximide (CHX). This work represents the first detailed account of the influence of inhibitors of macromolecular synthesis on a neuronal circadian oscillator. The isolated *Aplysia* eye is an advantageous system because it produces an endogenous, highly repeatable CR of large amplitude and low noise. The eye has been shown to produce a CR of optic nerve impulses for up to 2 wk in vitro in constant darkness (Eskin, 1971; Jacklet, 1974). Because the eye is an ensemble of neurons, it may serve as a model for the neuronal loci controlling CRs in other organisms, e.g., the suprachiasmatic nuclei of the hypothalamus (Stephan and Zucker, 1969; Moore, 1974). A foundation of studies on the ultrastructure (Jacklet et al., 1972), electrophysiological organization (Jacklet, 1969a; Audesirk, 1973), and CR (Jacklet, 1969b; Eskin, 1971; Jacklet and Geronimo, 1971; Eskin, 1972; Jacklet, 1973a; Jacklet, 1973b; Jacklet, 1974) of the *Aplysia* eye already exists and has been reviewed (Strumwasser, 1973).

PURO and CHX are known to be reversible inhibitors of protein synthesis in other systems (Mueller et al., 1962; Yeh and Shil, 1969). They were chosen for use in this study because they have very different structures and mechanisms of action. PURO inhibits protein synthesis by prematurely terminating nascent polypeptide chains, whereas CHX appears to inhibit protein synthesis by reducing the activity of transfer factor II, an enzyme that catalyzes ribosomal translocation along mRNA (Gale et al., 1972). It was hoped that the dissimilarity in the structures of these drugs would reduce the possibility of their causing similar side effects. This paper presents dose-response and phase-response studies on the effects of PURO and CHX pulses administered to the isolated *Aplysia* eye, and establishes that the drugs cause phase shifts in the CR of spontaneous CAP activity. These agents are shown to inhibit protein synthesis at doses that phase shift the CR. In addition PURO is shown to change the molecular weight distribution of proteins synthesized in the phase-shifted eye. Furthermore, the electrophysiology of the eye during and after drug treatment is shown to be near normal. These results are interpreted as indicating that ongoing protein synthesis is a part of the mechanism of the CR of the eye.

METHODS

Electrophysiological Experiments

RECORDING OF OPTIC NERVE ACTIVITY *Aplysia californica* were obtained from the Pacific Biomarine Supply Co., Venice, Calif. Animals were maintained at $14^{\circ}\text{C} \pm 0.5^{\circ}$ in a circulating seawater system of 1,500-gallon capacity. All animals were exposed to a light:dark (LD) 12:12 schedule (125 lx at the water surface) for at least 3 days before use.

Eyes were dissected with maximal (~1 cm) lengths of optic nerve attached. Connective tissue around the eye was trimmed as closely as possible to maximize penetration of drug solutions. After dissection, eyes were rinsed in seawater filtered through a $0.22\ \mu\text{m}$ Millipore filter (Millipore Corp., Bedford, Mass.) and supplemented with 100 U/ml penicillin and 100 $\mu\text{g}/\text{ml}$ of streptomycin (PS-FSW). Penicillin and streptomycin were obtained from Microbiological Associates, Bethesda, Md. The optic nerve of each eye was then sucked into a recording electrode consisting of a 27 G stainless steel hypodermic needle connected to a 2-cm length of tubing (Intramedic PE 10 or 20, Clay Adams Div. of Becton, Dickinson & Co., Parsippany, N.J.). In later experiments, a valve was employed to released suction after the eyes were in place.

Recordings were made from each eye in a separate no. 2 Nalgene beaker (Nalge Co., Rochester, N. Y.) containing 3.0 ml of PS-FSW. Up to four such eyes were kept enclosed in a light-tight box in total darkness. Impulses were amplified by a Tektronix 122 preamplifier (Tektronix, Inc., Beaverton, Ore.) and then monitored on a Grass 7B polygraph (paper speed: 10 mm/min) employing an AC EEG amplifier (Grass Instrument Co., Quincy, Mass.). Temperature in all experiments was maintained at $15^{\circ}\text{C} \pm 1^{\circ}$ (range); the temperature in any one experiment was typically $\pm 0.25^{\circ}\text{C}$.

DRUG SOLUTIONS Puromycin di-HCl (PURO) and cycloheximide (CHX) were obtained from Calbiochem, La Jolla, Calif. Puromycin aminonucleoside (PAN) was obtained from Nutritional Biochemicals, Cleveland, Ohio. PURO, CHX, and PAN were soluble in PS-FSW at the desired concentrations. Solutions of PURO were adjusted to the pH of PS-FSW (~7.8) by the addition of a small volume of 0.1 M NaOH, while solutions of PAN and CHX were adjusted to this pH by the addition of small volumes of 0.1 M HCl. The concentrations of PURO and PAN solutions were checked on a Beckman DB spectrophotometer at 276 nm (Beckman Instruments, Inc., Fullerton, Calif.). Control eyes in CHX experiments received a pulse of PS-FSW, while controls for PURO received PS-FSW or an equimolar concentration of PAN.

The solution inside each beaker was changed by means of tubing (PE 170, Intramedic) connecting the beaker to the outside of the light-tight box. When a drug or control solution was removed, each eye was rinsed with three changes of 3 ml PS-FSW, and then maintained in 3.0 ml of PS-FSW for the duration of the experiment. The three rinses were completed within 15 min.

LIGHT PULSES Each light test consisted of two 12-s light pulses separated by 12 s of darkness. Eyes received light tests before, during, and after drug administration. The light source was an incandescent bulb (Westinghouse CM47, Westinghouse Electric Corp., Pittsburgh, Pa.) operated at 6 V from a wet cell. The bulb was placed under a lucite chamber containing translucent beakers (Nalgene) for recording from eyes. Approximately 19–22 lx were measured when a detector (United Detector Technology Inc., Santa Monica, Calif., model 21A) was placed in a position approximating the eye. The light pulses did not alter the temperature of the fluid surrounding the eye as measured by a thermistor (Yellow Springs Instrument Co., Yellow Springs, Ohio, model 43TD).

DATA ANALYSIS For each eye the value of each light response parameter measured during and after drug administration was divided by the value of that parameter measured just before drug treatment. This resulted in a "normalized parameter." Because there was usually no spontaneous CAP activity occurring at the beginning of the PURO and CHX pulses, the values of its amplitude and frequency parameters were normalized to their values occurring at the peak of the first activity cycle. The difference between each normalized parameter value for an experimental eye, and the corresponding value of its control was calculated. Differences between normalized experimental and control measurements for each pair of eyes were averaged according to parameter and evaluated by a paired *t*-test.

Biochemical Experiments

LABELING OF EYES After dissection, a surgical thread with an identifying tag was tied to the optic nerve of each eye. Two eyes prepared in this way were suspended by their threads in 1.0 ml of PS-FSW in a Nalgene no. 00 beaker. Eyes remained in this prelabel medium for about 1.5 days at $14^{\circ}\text{C} \pm 0.5^{\circ}$ (range) in constant darkness. After the appropriate drug or control treatment, eyes were labeled in a medium that contained [^3H]- or [^{14}C]leucine. In addition to the label, the medium contained (mM): 423 NaCl, 13.3 CaCl_2 , 49 MgCl_2 , 28 Na_2SO_4 , 5 Tris:HCl buffer, pH 7.7–7.8; 100 U/ml penicillin and streptomycin, and 20 $\mu\text{l/ml}$ 0.05 M NaOH. The base was added to neutralize the 100 μl of 0.01 M HCl in which the labeled leucine was dissolved. Eyes were labeled for a period of 5 or 8 h.

Two experimental eyes, each from a different animal, were labeled together in 0.5 ml of medium containing 3.2 nM/ml L-[4,5- ^3H]leucine (2.9 Ci/mM), which was obtained by diluting L-[4,5- ^3H]leucine (46 Ci/mM, Schwarz/Mann, Div. of Becton, Dickinson & Co., Orangeburg, N. Y.) with nonradioactive L-leucine. Two control eyes from the same two animals were labeled together in 0.5 ml of medium containing 3.2 nmol/ml of L-[U- ^{14}C]leucine (312 mCi/mM, Schwarz/Mann). All solutions were changed in the dark. In biochemical studies the dissection technique gave eyes with much shorter optic nerves than in electrophysiological experiments.

PREPARATION OF GEL SAMPLES After labeling, eyes were rinsed for about 5 min in three changes of 1.0 ml PS-FSW. A ^3H -labeled experimental eye and its ^{14}C -labeled control were freed of optic nerve and any remaining connective tissue, and ground together in a glass homogenizer at 14°C . Each pair of eyes was ground together in 20 μl of a solution which consisted of 0.04 M sodium phosphate buffer, pH 7.2, containing 10% glycerol, 0.2% sodium dodecyl sulfate (SDS), 0.0015% bromphenol blue, and 0.2% 2-mercaptoethanol. The grinder was rinsed with $2 \times 20\text{-}\mu\text{l}$ aliquots of grinding solution and the rinses were pooled with the homogenate. The homogenate was stored frozen for as long as 1 wk. Before being applied to gels, samples were heated at 65°C for 30 min, and then centrifuged at $8,000g$ (4°C) for 15 min. The supernate was separated from the pellet, and the pellet frozen until it was washed and counted for radioactivity at a later time. In all experiments, the ^3H - or ^{14}C -radioactivity remaining in the pellet was at most 7% of the total incorporated radioactivity in the supernate.

GEL TECHNIQUES The general procedure for gel electrophoresis was that of Shapiro et al. (1967). The gel solution consisted of 0.1 M sodium phosphate buffer, pH 7.2, containing 5% acrylamide, 0.135% methylene bisacrylamide, 0.2% SDS, 0.075% ammonium persulfate, and 0.00075% tetramethylethylenediamine (TEMED). The gel solution was pipetted into glass tubes of 3-mm ID to a height of 6 cm. After the gel solution hardened, a 20- μl aliquot of sample supernate was placed in the tube to cover the top of the gel. The tube was then filled to the top with reservoir buffer (0.1 M sodium phosphate

buffer, pH 7.2, containing 0.2% SDS). Gels were electrophoresed at room temperature at 20 V for 4–5 h. The position of the tracking dye (bromphenol blue) and length of each gel were recorded. Gels were removed from their glass tubes and fixed for 2 days in three changes of 12.5% trichloroacetic acid (TCA). Each gel was cut into about 50 slices of about 1.25 mm in width by means of an "egg slicer." Each slice was extracted overnight in a scintillation vial containing 5 ml of a toluene 2,5-diphenyloxazole/1,4-bis[2-(5-phenyloxazolyl)]benzene (PPO-POPOP) scintillation fluid supplemented with 10% NCS tissue solubilizer (Amersham/Searle Corp., Arlington Heights, Ill.) and 2% 4 M NH₄OH, as described by Ward et al. (1970). Sample pellets were counted in 5 ml of the same fluid.

Radioactivity was assayed on a Beckman LS 200B liquid scintillation counter at 32% ³H and 57% ¹⁴C counting efficiencies. ³H Crossover into the ¹⁴C channel was 4%, and ¹⁴C crossover into the ³H channel was 19.6%. All samples were counted for 10 min.

RESULTS

Circadian Rhythm

NORMAL EYES In constant temperature and darkness the isolated eye of *Aplysia californica* produces a circadian rhythm (CR) (Jacklet, 1969*b*) of compound action potentials (CAPs) which are propagated down the optic nerve to the cerebral ganglion (Jacklet, 1969*a*). For animals entrained on an LD 12:12 schedule, the maximum frequency of CAP activity in the isolated eye is at projected dawn, circadian time 0 (CT 0), while the minimum of CAP activity is reached near projected dusk, CT 12 (Fig. 1, upper left). The correspondence of activity cycles to the entrainment schedule of the donor animal becomes less strict as more cycles are recorded (Fig. 1, lower left), since in constant darkness in PS-FSW eyes free run with a period usually less than 24 h (see next section). If a pair of eyes is taken from the same animal, and each eye is maintained in a separate beaker under identical conditions, a striking similarity in their CRs is observed, as in Fig. 1, lower left.

QUANTIFICATION OF CR PARAMETERS To quantitate their similarities, the CRs of six pairs of eyes were compared for amplitude of activity (CAP frequency), time of maximum activity, and CR waveform. In addition, the free-running period and damping coefficient of the CR were determined. The results of this analysis are presented in Table I. These data indicate that the CRs of eyes from the same animal are very similar in phase, amplitude, and waveform. This property furnishes the basis of using one eye of each pair as a valid control for the other. In addition the analysis of free-running period and damping coefficient of the CR presented in Table I provides a statistical basis for determining significant changes in the CR parameters in drug experiments.

THE EFFECTS OF PUROMYCIN AND CYCLOHEXIMIDE PULSES The spectrum and range of effects on the CR were determined with 12-h pulses of PURO (5–134 μg/ml) or CHX (10–2,000 μg/ml). All pulses began on the second projected night after dissection, at CT 17. The effects on CAP activity were classified as: (a) those occurring during the drug pulse; (b) those occurring 0–7 h after the end of the pulse; and (c) those occurring from 7 h after the pulse to the end of the experiment (2–6 days later). The nature and dose-dependence of the changes during each period are discussed below.

CAP Activity during the Pulse The amplitude and waveform of the CR were changed during pulses of PURO or CHX at high doses. The average CAP activity during the pulse was depressed by 40% to almost 100% in eyes given PURO at concentrations ranging from 50–134 $\mu\text{g/ml}$ compared to controls (Figs. 2, 3a). Some eyes given lower doses of PURO (12–18 $\mu\text{g/ml}$) showed increased

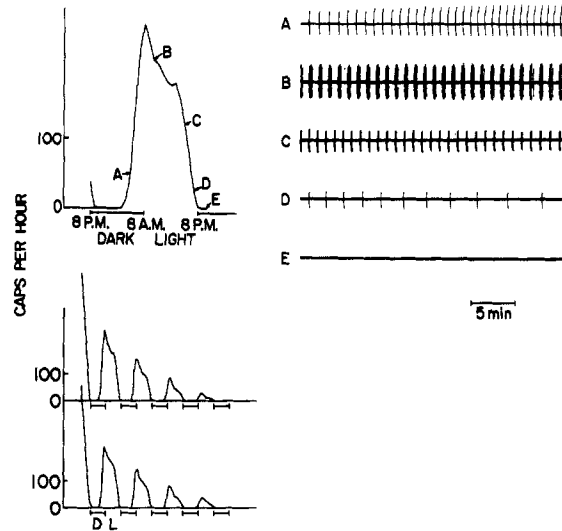


FIGURE 1. *Above*, Plot of spontaneous CAP activity vs. time for the first activity cycle recorded in constant darkness from an isolated *Aplysia* eye. The value of CAP activity is plotted at each hour of Pacific daylight time and represents the total number of CAPs fired for 1 h starting at the beginning of the hour plotted. This convention is used in all plots of CAP activity. Dissection occurred 4 h before the beginning of the record. The donor animal was entrained to an LD 12:12 schedule with light onset at 8 A.M. (CT 0) and light offset at 8 P.M. (CT 12). A complete record of this eye's CAP activity is plotted below. *Right*, Samples of spontaneous CAP activity recorded at 5 A.M. (CT 21) (A), 10 A.M. (CT 2) (B), 5 P.M. (CT 9) (C), 7 P.M. (CT 11) (D), and 10 P.M. (CT 14) (E). *Below*, A complete record of spontaneous CAP activity, plotted as CAP frequency vs. time, for two eyes taken from the same animal. Bars represent the projected dark periods (D), and the spaces between them the projected light periods (L) of the donor animal's entrainment schedule. This convention is used in all following CAP activity plots. The periods of the CRs are 25.1 h and 25.3 h, respectively.

CAP activity (Fig. 3a). In contrast to the depressed CAP activity seen during the larger PURO pulses, the level of CAP activity during CHX pulses was not changed except at 2,000 $\mu\text{g/ml}$, where it was increased by about 40% (Figs. 2, 3a). Both agents accelerated the falling phase of the activity cycle.

Rebound Excitation of CAP Activity 0–7 h after the Pulse Within 7 h of the removal of PURO (50–134 $\mu\text{g/ml}$) or CHX (10–2,000 $\mu\text{g/ml}$) CAP activity that had been decreasing during the drug pulse increased from a relative minimum to a relative maximum and then began to decline again (Fig. 2c–h). Analysis of the average CAP activity expressed by each eye during the “rebound” period

showed that CHX-treated eyes produced about two times more CAP activity than control eyes (Fig. 3*b*). Similar results were obtained with larger doses of PURO (125, 134 $\mu\text{g/ml}$) (Fig. 2*d*). Some eyes given low doses of PURO (12–18 $\mu\text{g/ml}$) also showed increased levels of CAP activity during the rebound period (Fig. 3*b*), although the CR waveform was normal (not shown).

Phase Delays in the CR Subsequent to the Rebound The magnitude of the phase shift caused by either drug was dependent on the dose administered. Phase changes were considered significant if they were greater than 2 h (2σ) since the CR maxima in pairs of control eyes occurred within 2 ± 50 min (Table I). Phase delays were caused by both drugs when applied as a 12-h pulse at CT 17. The degree of phase delay caused by different doses of PURO or CHX is presented in Fig. 3*c*. With increasing PURO and CHX dosage the phase of the CR was increasingly delayed (slope = 11.4 h and 8.5 h per decade drug concentration, respectively).

TABLE I
CIRCADIAN RHYTHM PARAMETERS OF NORMAL EYES

Similarities between eyes from the same animal ($n = 24$ cycles)*	
Differences in time of maximum CAP frequency (0.5 h)‡	02 min \pm 50 min (SD)
Differences in maximum CAP frequency (1 h)	16% \pm 33%
Correlation coefficient§ (1 h)	0.96 \pm 0.06
Other parameters ($n = 36$)	
Period (0.5 h)	23.4 \pm 1.5 h
Damping coefficient (1 h)	0.67 \pm 0.17

* For six pairs of eyes, each of four activity cycles of one eye was compared to the corresponding cycle of its mate.

‡ Number in parentheses indicates resolution of analysis.

§ Linear correlation coefficient (Pearson product-moment) of corresponding hourly CAP totals for each pair of cycles.

|| The free-running period of the CR was determined by averaging the 36 time intervals between successive half-hourly CAP activity peaks (12 eyes). The damping coefficient of the CR was determined by averaging the ratios of maximum hourly CAP frequencies between successive cycles.

The CRs expressed subsequent to the rebound period were also examined for changes in waveform, period, and damping. The level of activity minima was raised in most eyes treated with 10–134 $\mu\text{g/ml}$ PURO pulses (Fig. 2*b–d*) or 500–2,000 $\mu\text{g/ml}$ CHX pulses (Fig. 2*g, h*); whereas it was unaffected by lower doses of either drug (Figs. 2*a, e, f*). In addition, CR activity cycles were less smooth (Fig. 2*a–d, g, h*). The period of the CR after drug treatment was not changed by PURO or CHX. The damping of the CR after drug treatment was significantly decreased in a few cases (details in Table II).

The Effect of Varying the Duration of the PURO Pulse Decreasing the length of the PURO pulse caused the subsequent rebound peak and CR maxima to occur at earlier times. The effects of 125 $\mu\text{g/ml}$ PURO pulses begun at CT 17 of the second projected night and lasting 12 ($n = 1$), 9.5 ($n = 1$) or 6 h ($n = 3$) were compared. When computed as in dose-response experiments (see legend, Fig. 3*c*), the phase delays caused by the PURO pulses averaged 15.5, 11.9, and 5.0 h, respectively, for the decreasing pulse durations. The rebounds peaked at CT

5:30, 4:30, and 0:30, respectively, and thus occurred within 2 h after the pulse was washed out.

PHASE-RESPONSE RELATIONSHIPS OF EYES GIVEN PURO PULSES The dependence of the effects of PURO on the phase of its administration was tested on a population of 15 experimental and 15 control eyes. In each experiment, a pair

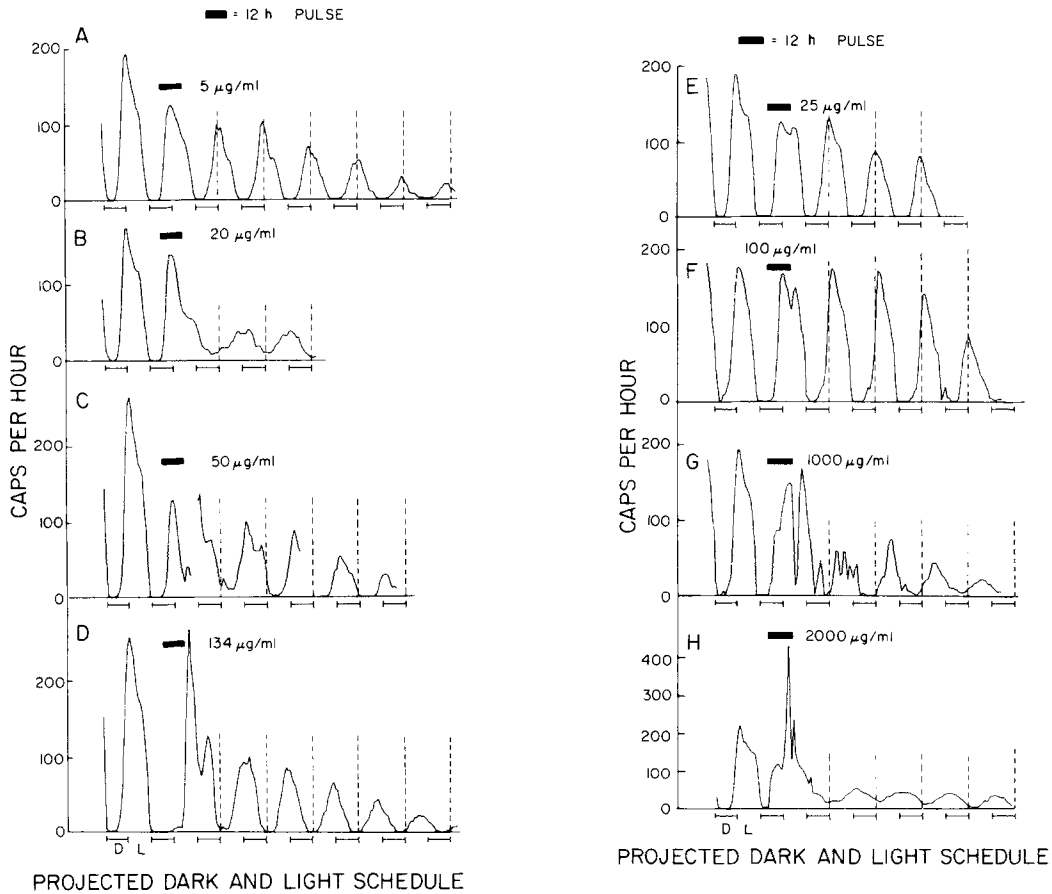


FIGURE 2. Spontaneous CAP activity of eyes receiving various doses of PURO or CHX. Drugs were administered as a 12-h pulse (■) beginning at CT 17 of the second projected night. Records A, B, C, D, F, and G were obtained from a pair of eyes taken from the same animal. Vertical dashed lines are drawn at projected dawn of the donor animal's LD 12:12 entrainment schedule, which is represented by the bars (dark period) and spaces (light period) along the abscissa.

of eyes dissected from the same animal between CT 5 and CT 8 was used. One eye received a 6-h PURO pulse at a concentration of $125 \mu\text{g/ml}$ (0.23 mM) while its mate received an equimolar ($64 \mu\text{g/ml}$) pulse of PAN at the same time. PURO and PAN pulses were delivered at seven different phase points of the CR,

during the 46-h period beginning at CT 10, a few hours after dissection. PAN was chosen as a control agent because it is a cleavage product of PURO, lacking the *O*-methyl tyrosine moiety, that does not inhibit protein synthesis (Bondeson et al., 1967). Eyes treated with PAN expressed normal CRs (Fig. 4).

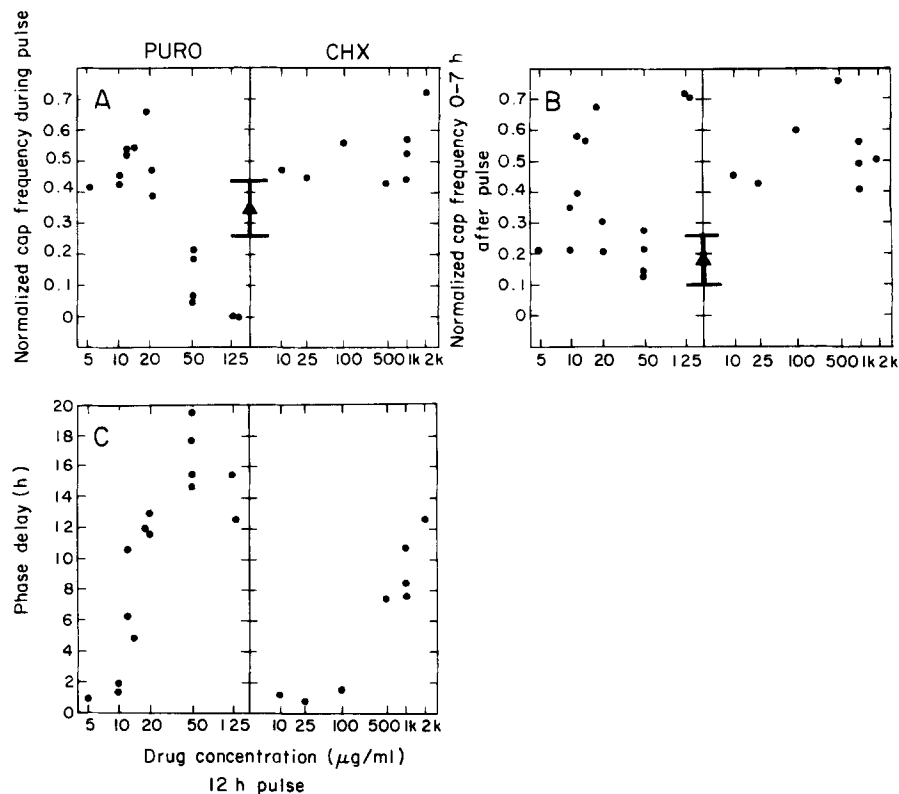


FIGURE 3. Dose-response data derived from experiments exemplified in Fig. 2. The results from 15 PURO and 8 CHX experiments are displayed, each point (\bullet) representing the data derived from one eye. Drug doses are plotted on a logarithmic scale along the abscissa. A, Plot of normalized hourly CAP frequency during a drug pulse vs. drug concentration. CAP activity for each eye was quantitated by computing the average hourly CAP frequency during the 12-h pulse divided by the peak hourly CAP frequency for the first activity cycle. This procedure allowed the CAP activity that occurred during the pulse to be normalized to the level of previous CAP activity. B, Plot of normalized CAP frequency during the period from 0 to 7 h after a drug pulse vs. drug concentration. Hourly CAP activity during this period was averaged and normalized as above. C, Average phase delay in the CR subsequent to the drug pulse vs. drug concentration. The phase delay of each activity cycle occurring on or after the third projected dawn was estimated relative to a CR projected from the peak of each eye's first activity cycle assuming a 23.4-h free-running period. Triangle (\blacktriangle) and heavy bars ($=$) on central ordinate represent the mean and ± 1 SD, respectively, of data derived from 12 control eyes in panels A and B.

More than 7 h after the PURO Pulse At the end of the rebound period, 7 h after the termination of the pulse, the CAP activity of PURO-treated eyes began to follow a CR that was phase shifted. The magnitude and direction of the phase shift in each experiment were determined by comparing the CR of the PURO-treated eye with that of its PAN-treated control. The example in Fig. 4*a* represents a phase delay of 4 h, and the example in Fig. 4*b* represents a phase advance of 4 h.

The relationship between the phase of PURO pulse administration and the magnitude and direction of the resultant phase shift is plotted with solid lines in Fig. 5. These data reveal that PURO pulses administered entirely during projected day caused phase advances, while those given slightly before (CT 10-

TABLE II
CHANGES IN CR PARAMETERS CAUSED BY DRUG
TREATMENT

Drug	Percent Δ control*	P‡
I. Puromycin		
A. Damping coefficient		
20 μ g/ml (N = 6)	+44	0.01
B. Period	NSD§	
II. Cycloheximide		
A. Damping coefficient		
25 μ g/ml (N = 3)	+32	0.05
100 μ g/ml (N = 4)	+31	0.05
B. Period	NSD	

* Controls in Table I.

‡ Determined by *t*-test.

§ No significant difference.

10:30) or entirely during projected night caused phase delays. Projected dawn separates the phases at which maximal phase delays and advances occurred. Phase shifts caused by PURO pulses administered at times about 24 h apart are similar in magnitude and direction.

The phase response data plotted in solid lines in Fig. 5 are in good agreement with those plotted in dashed lines. The latter data are a partial representation of those obtained by administering 4-h high potassium (107 mM) artificial seawater pulses to *Aplysia* eyes at different phases of their free-running CR (Eskin, 1972). The similarity of the phase response curves to PURO and high potassium pulses will be discussed later.

During the PURO Pulse The effects of PURO during the pulse were analyzed by comparing the CAP activity of PURO-treated eyes with that of their PAN-treated controls.¹ PURO pulses occurring during activity cycles depressed

¹ Before comparison, the average hourly CAP rate of each eye during a 6-h PURO or PAN pulse was normalized to its peak hourly CAP rate during the first activity cycle, except for eyes given pulses at the two earliest phase points (CT 10, CT 17:30 of the day of dissection). Since pulses beginning at these times occurred before the first activity cycle peak, the CAP activity taking place between CT 9 and CT 10 on the day of dissection was used to normalize CAP activity during a PURO or PAN pulse.

CAP activity by 13–70% ($n = 8$) (Fig. 4b) while those occurring at the very end of an activity cycle (Fig. 4a), or during the inactive part of the CR tended to have little effect on CAP activity (PURO/PAN control = 81–127%, $n = 6$).

0–7 h after the PURO Pulse An analysis of CAP activity from 0 to 7 h after the termination of a PURO pulse showed that 13 out of 15 eyes produced a rebound (Fig. 4b) although there was no obvious relationship between the phase of PURO administration and the magnitude of the rebound. No PAN-treated controls showed a rebound.

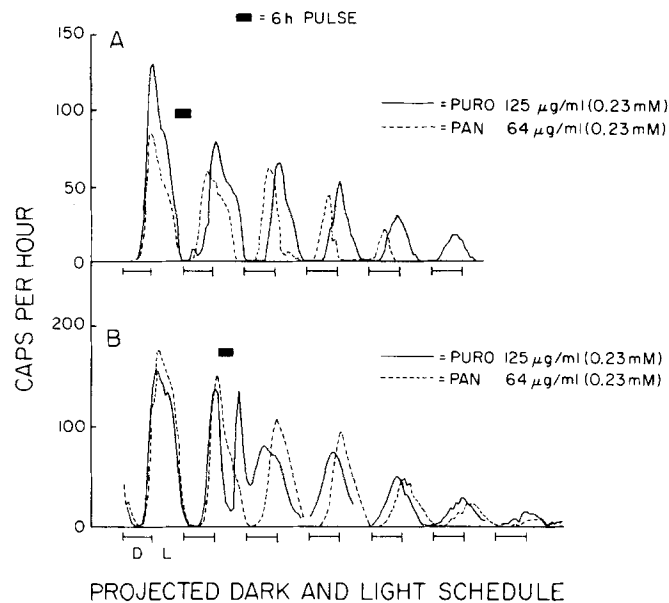


FIGURE 4. Spontaneous CAP activity of eyes receiving a 6-h pulse of PURO (125 µg/ml) or PAN (64 µg/ml). A, An example of a 4-h phase delay caused by a PURO pulse begun at CT 10:30 on the 1st projected day after dissection. B, An example of a 4-h phase advance caused by a PURO pulse begun at CT 1:30 on the 2nd projected day after dissection. Box (■) represents the PURO or PAN pulse.

Effects on Leucine Incorporation and Spectrum of Proteins Synthesized

CONTROL EYES An SDS-polyacrylamide gel system was used because 93% of the radioactivity incorporated into the eye could be solubilized (see Methods) and this material separated according to molecular weight. A calibration of the gel system used in these studies showed a linear relationship between the log molecular weight of four marker proteins (12,000–68,000 daltons) and their mobility relative to bromphenol blue tracking dye (Fig. 6 *inset*). The linear relationship does not hold for proteins below 12,000 daltons in weight.

The gel patterns of label incorporated into eyes taken from the same animal were compared to see if one eye was a valid control for the other. One eye was labeled for 8 h with [^3H]leucine, while the other was labeled with [^{14}C]leucine. Each pair of intact eyes was then ground together, and electrophoresed on the

SDS-polyacrylamide gel system. The ^3H - and ^{14}C -gel patterns derived from one of two such experiments are presented in Fig. 6a.

The pyramid-shaped gel pattern, peaking at about 40,000 daltons, and showing little incorporation below 12,000 daltons is typical of the *Aplysia* eye. The gel patterns of the ^3H -labeled and the ^{14}C -labeled eyes were almost identical. This finding is reflected in Fig. 6b, where the ratio of ^3H - to ^{14}C -counts in each gel slice is presented.

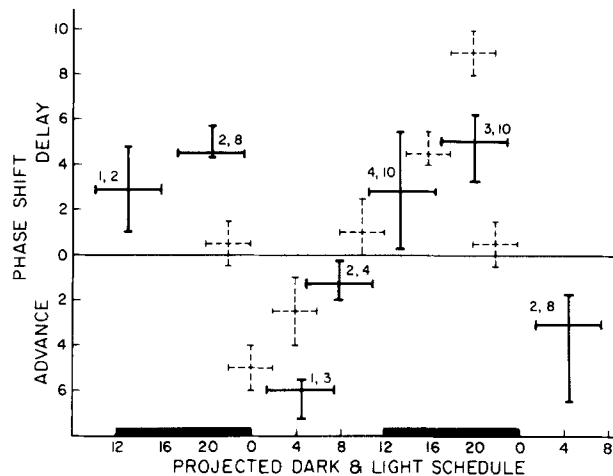


FIGURE 5. Solid lines, Phase-response curve for eyes given 6-h pulses of PURO (125 $\mu\text{g}/\text{ml}$). Position of horizontal bars relative to the projected dark and light schedule (abscissa) shows phase at which 6-h PURO pulse was applied. Position of horizontal bar relative to the ordinate represents the average phase shift caused by PURO pulses. Phase differences between corresponding half-hourly cycle peaks were pooled and averaged for eyes given a PURO pulse at the same time. At each data point, the first number of each pair indicates the number of eyes from which data were obtained. The second number represents the number of phase differences averaged together. The vertical bar spans the range of phase differences. Dashed lines, Partial representation of data collected by Eskin (1972) who administered 4-h pulses of high potassium (107 mM) seawater to isolated *Aplysia* eyes at different phases of their free-running CR. Eskin's data are plotted in the same manner as those of PURO-treated eyes except that phase shifts are first averaged for each eye, and then mean phase shifts are averaged together for a population of similarly treated eyes.

The ratios of total ^3H -cpm (corrected for ^{14}C -crossover) to ^{14}C -cpm found in the two control gels are presented in Table III. The two ratios are about 20% below the ratio of 5.22 predicted on the basis of ^3H and ^{14}C specific activities and counting efficiencies, assuming equimolar incorporation of [^3H]leucine and [^{14}C]leucine. This could readily be due to impurity or decomposition of the [^3H]leucine (Rothman, 1975).

THRESHOLD STUDIES Determinations were made on the effects of 12-h PURO (20 $\mu\text{g}/\text{ml}$, 37 μM) and 12-h CHX (500 $\mu\text{g}/\text{ml}$, 1.8 mM) pulses on the gel

pattern of label incorporated into eyes. Eyes received drug pulses about 1.5 days after dissection, from CT 17 to CT 5. These doses, when applied as 12-h pulses at the same phase in dose-response experiments (Fig. 3c) phase delayed the eye CR by 12 (PURO) and 7 (CHX) h. Experimental eyes received a 12-h drug pulse with label present during the last 5 h. Control eyes received a 7-h pulse of PS-FSW followed by a 5-h pulse of medium containing [^{14}C]leucine.

Incorporation of label into PURO- and CHX-treated eyes was inhibited by about 50% (Table III) and there were small changes primarily in the pattern of the PURO gel. Because the changes in the incorporation patterns were small, only the ratio patterns of the gels are presented in Fig. 7a, b. There is a pronounced decrease in the ratio values of the gel at molecular weights above 75,000 daltons in PURO-treated eyes. A less steep decrease is seen in CHX-treated eyes.

THE KINETICS OF PURO INHIBITION A series of experiments was designed to test the reversibility of changes in the gel pattern of incorporated [^3H]leucine caused by administration of PURO pulses (125 $\mu\text{g}/\text{ml}$) to eyes. The pulse was administered from CT 17 to CT 5, 1 day after dissection. Two experimental eyes were labeled with [^3H]leucine during the last 5 h of the PURO pulse; two were labeled for 8 h, beginning 2 h after the end of the pulse; two were labeled for 8 h, beginning 12 h after the pulse; and two were labeled for 8 h, beginning 20 h after the pulse. Control eyes were labeled with [^{14}C]leucine at the same time as their corresponding experimental eyes were labeled with [^3H]leucine.

The effect of 125 $\mu\text{g}/\text{ml}$ PURO pulses on the gel pattern was very striking compared to the pattern of control eyes. As seen in Fig. 8a, [^3H]leucine incorporation was strongly inhibited at molecular weights above 12,000 daltons. Furthermore, the ratio patterns revealed that the inhibition of [^3H]leucine incorporation increased with the apparent molecular weight of the labeled material. During the 125 $\mu\text{g}/\text{ml}$ PURO pulse, incorporation was only 15% of that computed for control gels (Table III).

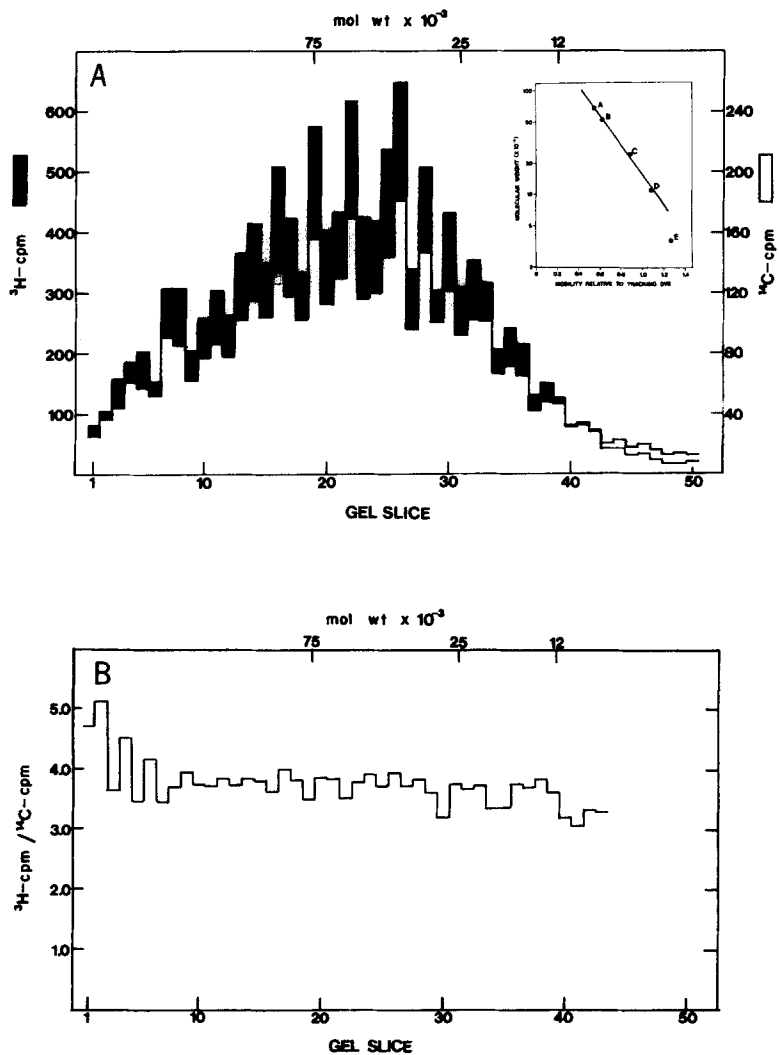
Eyes labeled from 2 to 10 h (that is, primarily in the rebound period) after the removal of a PURO pulse showed a dramatic recovery of [^3H]leucine incorporation. The ratio patterns (Fig. 8b) showed two prominent peaks between about 72,000 and 109,000 molecular weights, and a minor peak at about 20,000. The decreasing incorporation seen at high molecular weights during PURO pulses was no longer present at this time. In addition, total incorporation returned to a level close to that of control eye pairs (Table III). Eyes labeled from 12 to 20 h (Fig. 8c) after the end of the PURO pulse, and from 20 to 28 h (Fig. 8d) after the pulse, had ratio patterns that showed a peak at about 20,000 daltons, but that no longer had peaks between 72,000 and 109,000 daltons. There was little additional change in total incorporation compared to eyes labeled 2–10 h after the end of the PURO pulse.

Electrophysiology of Drug-Treated Eyes

DESCRIPTION OF TESTS APPLIED TO THE EYE The electrophysiological properties of eyes given 12-h PURO or CHX pulses were tested to see if the phase shifts induced by these drugs could have been caused by electrophysiological

damage to the eye. Spontaneous CAP activity as well as phasic and tonic light responses to test light pulses were extracellularly recorded. All three activities produce CAPs that are conducted down the optic nerve (Jacklet, 1969*a*). Light-evoked CAPs result from interactions among receptors which appear to electrically synapse on follower cells in the eye. Spontaneous CAPs are thought to be generated by pacemaker cells which chemically synapse on follower cells (Aude-sirk, 1973). Hence phototransduction, synaptic transmission, and action potential conduction could be assayed by applying light pulses to the eye.

Each light test consisted of two 12-s pulses of light (19–22 lx) separated by 12 s of darkness. A phasic light response (Jacklet, 1969) elicited during the first half of the first light pulse gradually adapted into a tonic response by the end of this pulse. The second light pulse caused a tonic light response with little or no phasic component.



Data derived from recordings of light responses and spontaneous CAP activity from experimental and control eyes were compared quantitatively. Eight parameters were measured: the latency, maximum peak-to-peak CAP amplitude, and CAP frequency were measured for both the phasic and tonic light responses; in addition, the maximum peak-to-peak amplitude and frequency of spontaneous CAP activity were measured every hour for the entire length of each experiment. Figs. 9 and 10 show examples of phasic and tonic light responses and spontaneous CAP activity waveforms recorded at various times before, during, and after treatment of eyes with PURO or CHX pulses. The controls for each PURO- and CHX-treated eye received a pulse of PS-FSW coincident with the drug pulse.

ELECTROPHYSIOLOGICAL EFFECTS The doses of PURO (20 $\mu\text{g/ml}$) and CHX (500 $\mu\text{g/ml}$) used in these experiments caused significant phase delays in dose-response experiments (Fig. 3c). Six eyes were administered a PURO pulse, and four eyes were administered a CHX pulse beginning at CT 17 of the second projected night.

FIGURE 6 *inset*. (*Opposite*) Plot of molecular weight (logarithmic scale) vs. mobility on SDS-polyacrylamide gels relative to bromphenol blue for protein standards of known molecular weight. Protein standards represented by each point (●) are: bovine serum albumin, molecular weight 68,000 daltons (Castellino and Barker, 1968) (A); human gamma globulin, heavy subunit, molecular weight 50,000 daltons (Edelman et al., 1972) (B); human gamma globulin, light subunit, molecular weight 23,500 daltons (Edelman et al., 1972) (C); horse heart cytochrome *c*, molecular weight 11,700 daltons (Weber and Osborn, 1969) (D); glucagon, molecular weight 3,500 daltons (Bromer et al., 1957). The position of points A–D relative to the abscissa represents the average of four mobility determinations. Point E is the average of three determinations. The line is a least-squares fit to all 16 mobility determinations for points A–D, and is described by the equation:

$$Y = -1.397X + 2.602,$$

where Y is the logarithm (base 10) of molecular weight in units of 1,000 daltons, and X is mobility relative to bromphenol blue. The correlation coefficient of the correspondence between Y and X is 0.998 (Pearson product-moment). A, Gel incorporation pattern for a pair of control eyes labeled from CT 6:30 to CT 14:30, 2 days after dissection. Both eyes were taken from the same animal; one eye was labeled in a medium containing [^3H]leucine while the other was labeled in a medium containing [^{14}C]leucine. Eyes were homogenized together and electrophoresed on a gel, which was then fixed and sliced. Raw ^3H (■) and ^{14}C (□) radioactivity in each slice was then determined. Molecular weight scale was determined from the mobility of protein standards (*inset*). Slice no. 1 is at the top of the gel. Total cpm in gel corrected for background and crossover were 11,810 (^3H) and 3,338 (^{14}C). B, Histogram of the ^3H cpm to ^{14}C cpm ratio computed for each slice of the gel represented in part A. Background radioactivity (11.8 cpm and 11.4 cpm, respectively) was subtracted before the ratios were computed. However, crossover of ^{14}C radioactivity into the ^3H channel was not subtracted, causing the ratio histogram to be uniformly raised by a value of 0.196. No ratio has been computed for slices having fewer than 25 raw ^3H cpm or ^{14}C cpm.

Differences between the corresponding normalized values of each experimental and control eye measurement were averaged according to the time at which the data were collected. Thus, the electrophysiological data for each parameter were averaged according to those collected during the pulse, those collected 0–7 h after the pulse (rebound period), and those collected more than 7 h after the pulse. Examples of light response and spontaneous CAP waveforms are presented in Figs. 9 and 10.

The *mean* differences between each pair of eyes (control vs. drug treated) were averaged together in each of the 24 categories (eight parameters \times three time slots). Of all 24 categories for each of the two drugs, significant differences between experimental and control eyes were found in only 3 categories (Table IV). First, there was a 23% increase in the CAP amplitude of the tonic light response of PURO-treated eyes measured more than 7 h after the end of the drug pulse. Second, there was a 67% increase in the spontaneous CAP frequency of PURO-treated eyes during the period from 0 to 7 h after the pulse (Fig. 9, spontaneous CAP activity trace 5). Third, there was a 32% increase in the tonic

TABLE III
INCORPORATION OF ^3H - AND ^{14}C -LEUCINE INTO PURO-TREATED AND CHX-TREATED EYES DETERMINED BY TOTAL RADIOACTIVITY IN SDS-POLYACRYLAMIDE GELS

Experiment	Hours of label	Ratios*	Percent control
1. Control vs. control	8	3.54, 4.82	100
2. During PURO pulse (20 $\mu\text{g}/\text{ml}$)	5	1.90, 2.01	47
3. During CHX pulse (500 $\mu\text{g}/\text{ml}$)	5	1.66, 2.06	44.5
4. During PURO pulse (125 $\mu\text{g}/\text{ml}$)	5	0.59, 0.67	15
5. 2–10 h after PURO pulse (125 $\mu\text{g}/\text{ml}$)	8	3.83, 4.37	98
6. 12–20 h after PURO pulse (125 $\mu\text{g}/\text{ml}$)	8	4.04, 4.11	97
7. 20–28 h after PURO pulse (125 $\mu\text{g}/\text{ml}$)	8	3.47, 4.77	99

* Ratio = Total ^3H cpm in gel/Total ^{14}C cpm in gel (corrected for ^3H and ^{14}C background of 11.8 and 11.4 cpm/slice, respectively; and for 19.6% crossover of ^{14}C into ^3H channel). Experimental eyes were labeled with [^3H]leucine while controls were labeled with [^{14}C]leucine.

light response frequency in CHX-treated eyes during the period from 0 to 7 h after the pulse (Fig. 10, tonic light response trace 4).

Qualitative examination of the waveforms of CAPs from drug-treated eyes did not reveal any systematic changes in the light responses compared to controls. The only other effect that could be observed was that the frequency of CAP activity during *bursts* was increased by about 33% during CHX pulses (Fig. 10, spontaneous CAP activity trace 3). Soon after the CHX pulse was removed, the burst duration began to increase (Fig. 10, spontaneous CAP activity trace 4) and returned to normal by 3 h.

DISCUSSION

Nature of the Phase Shift Caused by PURO and CHX

The data presented in this study clearly show that PURO and CHX pulses (Figs. 2, 4) phase shifted the CR of spontaneous CAP activity in the *Aplysia* eye. The

phase shift in the CR of each eye caused by a drug pulse persisted for the duration of the experiment (two to six cycles) and showed no sign of reversal. This finding is upheld by the fact that the free-running period did not change in phase-shifted eyes (Table II).

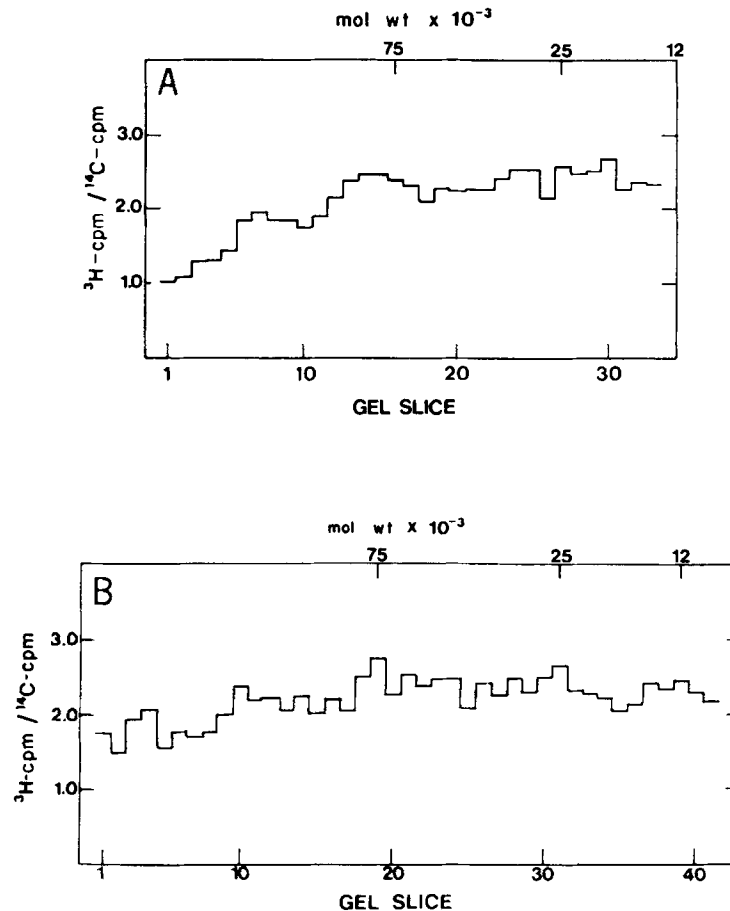


FIGURE 7. A, Ratio histogram derived from the gel incorporation pattern of a pair of eyes from the same animal. One eye was treated with a 20 $\mu\text{g}/\text{ml}$ pulse of PURO from CT 17 to CT 5, 1.5 days after dissection, and labeled with [^3H]leucine during the last 5 h of the PURO pulse. The other eye was treated with PS-FSW from CT 17 to CT 0, and then labeled with [^{14}C]leucine from CT 0 to CT 5. Gel had a total of 47 slices and a total cpm of 4,396 (^3H) and 2,191 (^{14}C). Other details are presented in Fig. 6. B, Ratio histogram derived from gel of ^3H -labeled experimental eye treated with 500 $\mu\text{g}/\text{ml}$ CHX, and ^{14}C -labeled control. Gel had a total of 50 slices and total cpm of 4,697 (^3H) and 2,281 (^{14}C). Other details as in part A.

DOSE-RESPONSE AND DURATION-RESPONSE STUDIES In dose-response studies, where 12-h PURO (5–134 $\mu\text{g}/\text{ml}$) or CHX (10–2,000 $\mu\text{g}/\text{ml}$) pulses were begun at CT 17, all phase shifts were interpreted as phase delays. For CHX- and PURO-pulsed eyes, this interpretation is supported by the fact that increasing

drug concentrations caused increasing delays in the occurrence of CR maxima subsequent to the pulse (Figs. 2, 3*c*). In addition, the results of experiments in which the length of PURO pulses beginning at CT 17 was varied demonstrated that activity maxima appeared at earlier times after shorter-duration pulses.

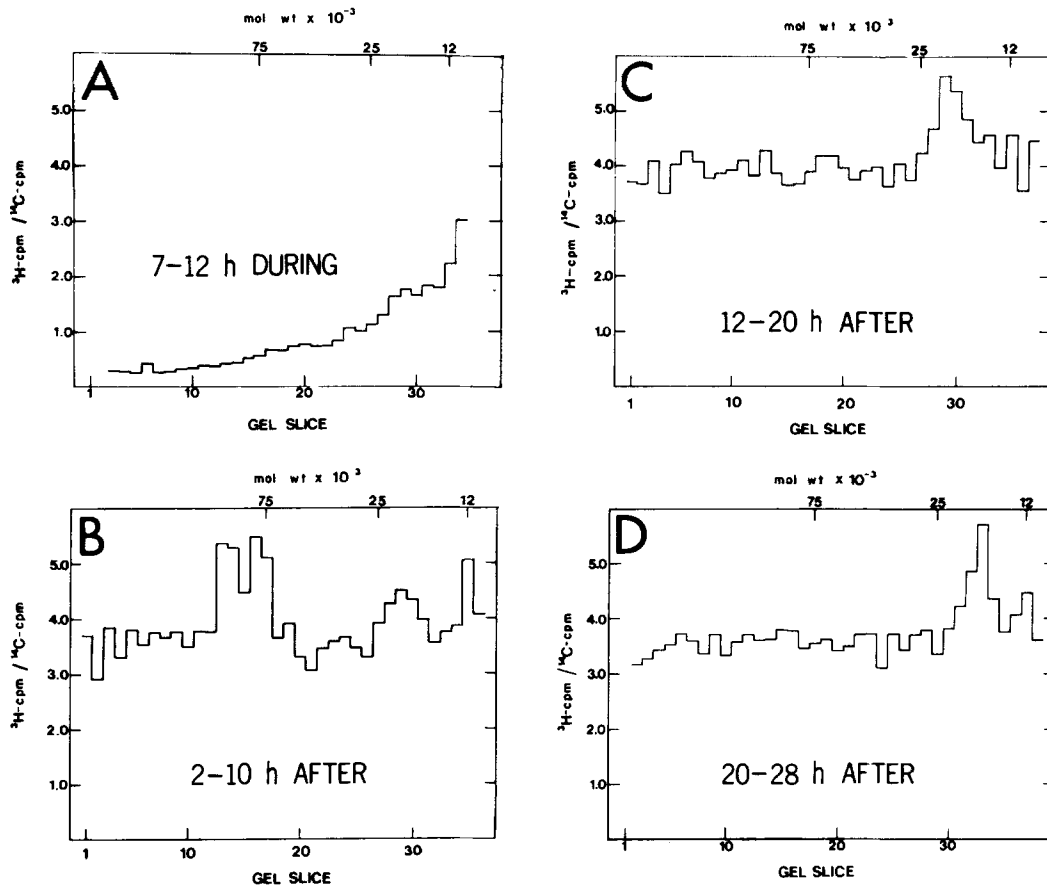


FIGURE 8. Ratio histograms derived from gel incorporation patterns of a pair of eyes labeled 7–12 h during (A), 2–10 h after (B), 12–20 h after (C), or 20–28 h after (D) a PS-FSW and 125 $\mu\text{g}/\text{ml}$ PURO pulse. Pulses were administered from CT 17 to CT 5, 1.5 days after dissection. One eye of each pair was treated with PURO, and labeled with [^3H]leucine. The other eye was treated with PS-FSW, and labeled with [^{14}C]leucine. The gels each had a total of 49, 49, 48, and 48 slices, respectively. Total cpm in each gel were: A, 1,966 (^3H), 3,354 (^{14}C); B, 13,640, 3,566; C, 15,059, 3,724; D, 6,866, 1,981. Other details as in Fig. 6 B.

These data support the interpretation that PURO and CHX pulses caused phase delays when begun at CT 17, a time when the CR of the eye is in its inactive phase.

PHASE-RESPONSE STUDIES The clock was clearly not stopped at all phase points by PURO or CHX. In phase-response experiments, it should be remem-

bered that the direction of the phase shift was interpreted as that which yielded the smaller magnitude shift. A replot of Fig. 5, assuming all phase shifts were delays, reveals a nonlinear function, so that delays as large as 23 h or as small as 3 h were caused by a 6-h PURO pulse, depending on its position in the 24-h day. The assignment of direction of phase shift as that which gave the smaller magnitude shift is further supported by the dose-response studies (see above).

A striking aspect of the phase response curve (PRC) to PURO pulses was its general similarity to other PRCs based on the effects of light on intact organisms (Pittendrigh, 1965). In addition, the PRC to PURO pulses mimicked the PRC to high K^+ pulses (Eskin, 1972) and was reasonably close to the PRC to 1-h, 600-lx light pulses (Jacklet, 1974) in the *Aplysia* eye.

MECHANISMS OF DRUG-INDUCED PHASE SHIFTS At present, the mechanisms by which high potassium and PURO pulses phase shift the circadian clock remain unresolved. However, both treatments seem to have two general properties in common. First, they seem to depolarize neurons. High potassium treatment does this by lowering the potassium gradient across the cellular membrane. PURO probably causes depolarization of neurons by increasing the permeability of their membranes (Cohen et al., 1966; Bondeson et al., 1967; Dahl, 1969; Paggi and Toschi, 1971; Burka et al., 1975).

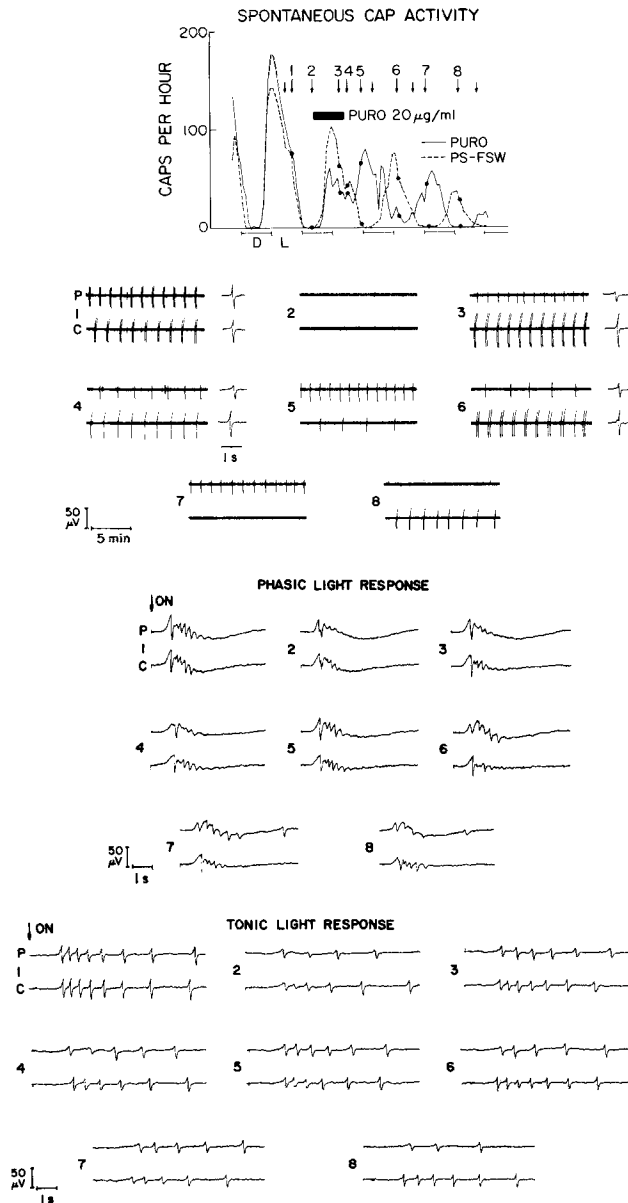
Second, both treatments inhibit protein synthesis. PURO interrupts protein synthesis by prematurely terminating growing polypeptide chains (Gale et al., 1972). The manner in which high potassium pulses affect protein synthesis is not clearly understood. However, Ram (1974), in this laboratory, showed that leucine incorporation into the isolated parietovisceral ganglion of *Aplysia* was reduced by 50% during 4-h pulses of high potassium (90 mM) medium.

Of the two effects listed above, it seems likely that both contribute, perhaps additively, to the inhibition of CAP activity and phase shifting of the CR of the eye at high doses of PURO (see next section). Arguments that PURO can act without depolarizing eye cells include the observations that: (a) 20 $\mu\text{g/ml}$ PURO pulses phase delayed the CR without significantly depressing spontaneous CAP activity during the pulse and without causing rebounds; and (b) both PURO and CHX pulses phase shifted the CR while CHX did not produce similar electrophysiological effects; both PURO (20 $\mu\text{g/ml}$) and CHX (500 $\mu\text{g/ml}$) lowered leucine incorporation in the *Aplysia* eye by about 50%.

Biochemistry of Drug-Treated Eyes

THRESHOLD STUDIES Minimal doses of PURO (20 $\mu\text{g/ml}$) and CHX (500 $\mu\text{g/ml}$) capable of phase shifting the CR of the eye also influenced the level and gel pattern of leucine incorporation. Both drug treatments caused the level of the ratio pattern to drop to about half that of control eyes at molecular weight regions below 75,000 daltons, and to decline to even lower values at molecular weight regions above 75,000 daltons. Preferential inhibition of higher molecular weight protein synthesis is consistent with the mechanism of PURO action, while CHX would be expected to equally inhibit leucine incorporation at all molecular weights. The mechanism of PURO action, however, does not provide an explanation for the flat ratio pattern seen at molecular weights below 75,000 daltons in our studies.

KINETICS OF PURO INHIBITION The effects of PURO (125 $\mu\text{g}/\text{ml}$, 12 h) on leucine incorporation of eyes were almost completely reversible some 12 h after the drug's removal. Eyes labeled from 12 to 20 h, or from 20 to 28 h after the end of the PURO pulse had ratios of total ^3H cpm to total ^{14}C cpm that were close to the ratios in control vs. control experiments. Furthermore, their ratio patterns showed a peak at 20,000 daltons, which was the only feature that differed from the control vs. control experiments.



The existence of the 20,000 dalton peak in the ratio pattern suggests two alternative interpretations. First, the peak may represent an effect of PURO on protein synthesis that is not readily reversible. More interestingly, the 20,000 dalton peak may be due to a difference in the spectrum of proteins synthesized by eyes whose CRs are out of phase. This interpretation is based on the finding that eyes given 125 $\mu\text{g}/\text{ml}$ PURO pulses in dose-response experiments expressed CRs that were phase delayed by about 12 h.

During the rebound period the pattern of proteins synthesized may also be correlated with the electrophysiological state of the eye. Eyes labeled 2–10 h after the end of the PURO pulse had incorporation ratios that were close to control levels, and ratio patterns that showed major peaks between 72,000 and 109,000 daltons. It is tempting to speculate that this peak is related to the rebound in spontaneous CAP activity seen from 0 to 7 h after the end of 125 $\mu\text{g}/\text{ml}$ PURO pulses in dose-response experiments.

INTERPRETATION OF INCORPORATION EXPERIMENTS The most serious problem in assaying the state of protein synthesis in the eye is the fact that it is not a homogeneous tissue. The possibility that incorporation in the whole eye may not accurately reflect incorporation in the structures that produce the CR is supported by the fact that 80% of the neurons in the *Aplysia* eye are claimed to be photoreceptor cells (Jacklet and Geronimo, 1971) that most probably do not act as circadian oscillators (Audesirk, 1973). However, preliminary autoradiographic experiments indicate that with 1–2-h labeling periods ($[^3\text{H}]$ leucine), photoreceptors are considerably less labeled than second-order neurons and neurosecretory cells.²

The Electrophysiology of Drug-Treated Eyes

THE THREE EFFECTS OF PURO The increase in spontaneous CAP frequency in the period 0–7 h after the 20 $\mu\text{g}/\text{ml}$ drug pulse seems likely to be part of a CAP

² Strumwasser, Alvarez, and Greengard, unpublished observations.

FIGURE 9. (*Opposite*) Top, Plot of spontaneous CAP activity of an eye that received a 12-h pulse of PURO (20 $\mu\text{g}/\text{ml}$) beginning at CT 17 of the second subjective night (—) and a control eye that received a 12-h pulse of PS-FSW at the same time (----). Both eyes were taken from the same animal. Eyes were maintained in separate beakers housed in the same cooling dish and light-tight box. Both eyes received light tests at various times (indicated by arrows) during the experiment. Numbered arrows refer to optic nerve recordings shown below. Box (■) shows when PURO and PS-FSW pulses were administered. The CR of the PURO-treated eye was phase delayed by about 14 h compared to its control. Below, Examples of spontaneous CAP activity, phasic light responses, and tonic light responses recorded before (1, 2), during (3), and after (4–8) the administration of a PURO (P) or control (C) pulse. Recordings from each eye were made by means of a single suction electrode attached to the optic nerve. Signals were amplified by a Tektronix 122 preamplifier, fed into an EEG-type amplifier of a Grass 7B polygraph, and then recorded. Half-amplitude band-pass filters were set at 0.3 Hz (low) and 250 Hz (high). Down is negative voltage.

activity cycle during this period since it was in phase with the next activity cycle (Fig. 9 top, under arrow 5, and Fig. 2 B). The second effect of PURO, that is the increase in the maximum in the CAP amplitude by 23% during the tonic light response, at times more than 7 h after the end of the pulse, may reflect an

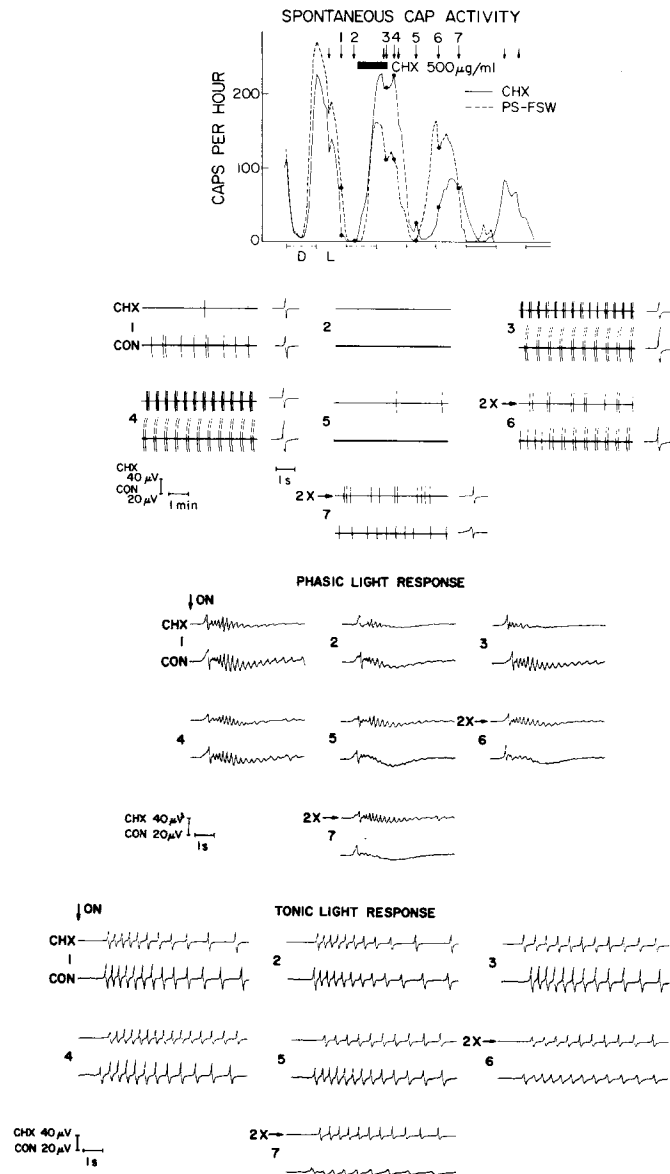


FIGURE 10. Top, Plot of spontaneous CAP activity of an eye that received a 12-h pulse of CHX (500 $\mu\text{g}/\text{ml}$) beginning at CT 17 of the second subjective night (—) and a control eye that received a 12-h pulse of PS-FSW at the same time (-----). Box (■) represents CHX and PS-FSW pulses. The CR of the CHX-treated eyes was phase delayed by about 7 h compared to its control. Other details are the same as in Fig. 9.

increased excitability of PURO-treated eyes. Alternatively, it is difficult to rule out the possibility that this small change is entirely due to the phase delay in the CR of CAP amplitude or even may reflect greater preservation of PURO-treated eyes compared to controls. The third and largest effect of PURO was to depress spontaneous CAP activity when used at high concentrations. At 125 $\mu\text{g/ml}$ PURO concentration it appears likely that direct membrane effects, at a minimum, contaminate those effects due to inhibition of protein synthesis. Thus it becomes important to compare these electrophysiological effects with those of CHX.

CHX EFFECTS Eyes receiving a 12-h pulse of CHX (500 $\mu\text{g/ml}$) in electrophysiological experiments showed a 32% increase in the tonic light response frequency during the period from 0 to 7 h after the end of the pulse (Fig. 10,

TABLE IV
SIGNIFICANT CHANGES IN THE ELECTROPHYSIOLOGY OF DRUG-TREATED EYES

Parameter	Period	Percent Δ Control	n	P*
I. Puromycin (20 $\mu\text{g/ml}$)				
Spontaneous CAP frequency	0-7 h after pulse	+67	6	0.05
CAP amplitude (Tonic light response)	>7 h after pulse	+23	4	0.02
II. Cycloheximide (500 $\mu\text{g/ml}$)				
Spontaneous CAP burst frequency	During pulse	+33	4	‡
CAP frequency (Tonic light response)	0-7 h after pulse	+32	4	0.05

* Determined from paired *t*-test.

‡ Indicates a qualitative difference.

tonic light response trace 4), and a 33% increase in the frequency of spontaneous CAPs during each burst in the presence of the drug (Fig. 10 spontaneous CAP activity trace 3). These findings suggest that CHX caused an increase in the excitability of the *Aplysia* eye. However, as with the case of PURO it is unclear whether the small increase of light responsiveness is due to better preservation of the eye in the presence of CHX. The increase in CAP burst frequency coincident with the CHX pulse, and the increase in the tonic light response CAP frequency during the period from 0 to 7 h after the pulse may both reflect transient electrophysiological changes capable of causing a phase shift in the CR. However, because CHX and PURO had different electrophysiological effects it seems more likely that these effects did not cause the phase shifts in the CR common to both drugs.

Relationship of Electrophysiological and Biochemical Effects to the Modification of the CR

The results of these studies are summarized in Table V. We have shown that: (a) PURO and CHX pulses were capable of phase shifting the CR of the eye; (b) the rapid and long-lasting phase shifts caused by PURO and CHX imply that these agents are affecting a process within the clock mechanism itself rather than the transduction of the clock; (c) doses of PURO and CHX that could phase shift the

CR inhibited the level and modified the pattern of leucine incorporation into the eye; (d) the drugs did not appear to modify the CR by causing electrophysiological side effects. It is also unlikely that these drugs, with very different structures, have common biochemical side effects.

These studies strongly suggest that the production of the CR of the isolated *Aplysia* eye is dependent on protein synthesis. However, these studies do not elucidate the manner in which macromolecular synthesis is involved in the

T A B L E V
SUMMARY OF THE EFFECTS OF PURO AND CHX ON THE CR,
ELECTROPHYSIOLOGY AND BIOCHEMISTRY OF THE *APLYSIA* EYE

Drug	Dose	Hours	CR	Electrophysiology	Biochemistry
PURO	20 μ g/ml	12 (CT 17-05)	Phase delay 12-14 h	(TLR)* up 23%; spontaneous CAP frequency up 67%	Inhibited leucine incorporation by 53%
	125 μ g/ml	12 (CT 17-05)	Phase delay 16 h	Depressed spontaneous CAP activity almost completely; caused rebound excitation after removal	Inhibited leucine incorporation by 85%
		6	Phase delays in projected night, phase advances in projected day	Not determined	Not determined
CHX	500 μ g/ml	12	Phase delay 6 h (CT 17)	Spontaneous CAP burst frequency up 33%; CAP frequency (TLR)* up 32%	Inhibited leucine incorporation by 56%

* Tonic light response.

production of the CR. The solution to this problem awaits the identification of the specific neurons in the eye that generate the CR and the creation of specific biochemical lesions that cause changes in the underlying properties of the CR.

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