the production of congenital deformities is widely applicable. Though this communication is principally concerned with teratogenesis induced by trypan blue, it is probable that a variety of exogenous and endogenous inhibitors of enzymes could find their way into the digestive vacuoles of the fetal membranes and exert a similar action. In the rat, digestion of histiotroph is carried out by the visceral yolk-sac endoderm, but in many mammals, including man, this function is largely served by other extraembryonic membranes. Perversion of embryonic nutrition by inhibition of enzymes within lysosomes may therefore involve a variety of placental tissues in various species.

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Circadian Clock in Photoperiodic Time Measurement: A Test of the Bünning Hypothesis

Abstract. *A technique has been developed for effectively separating the direct inductive effect of a light signal from its effect on the phase of the rhythm of sensitivity to photoperiodic induction. With this technique it has been shown that a 75-minute pulse of light per day, when appropriately positioned with respect to the circadian activity cycle of the sparrow* Passer domesticus, *is sufficient to produce a response normally produced only by long days. The results cannot be interpreted in terms of a requirement of an absolute amount of either darkness or light and offer strong confirmation of Bünning's hypothesis concerning the mechanism of photoperiodic time measurement.*

Photoperiodic effects on organisms are of necessity based on the ability to distinguish one naturally occurring day length from another and to this extent imply the existence of a biological time-measuring system. The hypothesis that the photoperiodic efficiency of any given light-dark cycle depends primarily on which portion of an underlying circadian sensitivity rhythm is illuminated was first developed by Bünning (*1*). The major feature of this hypothesis is the assumption of the existence of such a circadian rhythm of sensitivity to the inductive effects of light. The circadian clock, which controls many biological rhythms (for example, activity), is assumed also to control the rhythm of photoperiod sensitivity. There is by now a body of data which is consistent with this hypothesis. Many of these data are more or less inconsistent with the somewhat vague alternative hypotheses which have in common conceptualization of the photoperiodic time-measuring system as an hourglass which times the length of the dark period (*2*). However, there are in the literature very few direct and critical tests of Bünning's hypothesis (*3, 4*).

The difficulty in testing the Bünning hypothesis arises from its prediction that light will have a dual action in photoperiodic systems (*3*). In addition to its inductive effect (for example, on flower formation or gonadal development) light, according to this hypothesis, must affect the phase of the underlying sensitivity rhythm because this rhythm is coupled to the circadian clock. This means that different light regimens will "position" the sensitivity rhythm differently with respect to the light portion of the cycle. Unless one knows the effect of a light regimen on the phase of the sensitivity rhythm, one does not know which portion of the rhythm is being illuminated and thus cannot predict the inductive effect of the regimen. Therefore, a meaningful test of Bünning's hypothesis is impossible without information concerning both effects of light. Assays of induction abound, but the sensitivity rhythm by its nature cannot be assayed independent of induction. The best one can do is to use some readily measurable overt circadian rhythm (such as locomotor activity) and make the assumption that both it and the sensitivity rhythm are controlled by the same underlying timing mechanism. Further, one must assume that these rhythms maintain a fixed relationship to one another. The behavior of the measured rhythm can then be taken to reflect the behavior of the hypothesized rhythm of sensitivity to photoperiodic stimuli. The number of cases in which a "test" of the Bünning hypothesis has included measurement of such an overt rhythm remains very small, and, as a result, much of the published discussion of this hypothesis is based on, at best, circumstantial evidence.

If some way could be found to separate the two actions of light (for example, discover a light signal which would be photoperiodically inductive but which would not affect the phase of a measured overt rhythm), various critical tests of the Bünning hypothesis would be possible. One such test arises from the prediction that for "long day" responses a single, very short daily light pulse would be inductive if it could be positioned so as to illuminate the appropriate portion of the sensitivity curve.

Using the house sparrow *Passer domesticus* we have made an effective, though far from absolute, separation of the two actions of light and have used this separation in a critical test of the hypothesis. The feasibility of

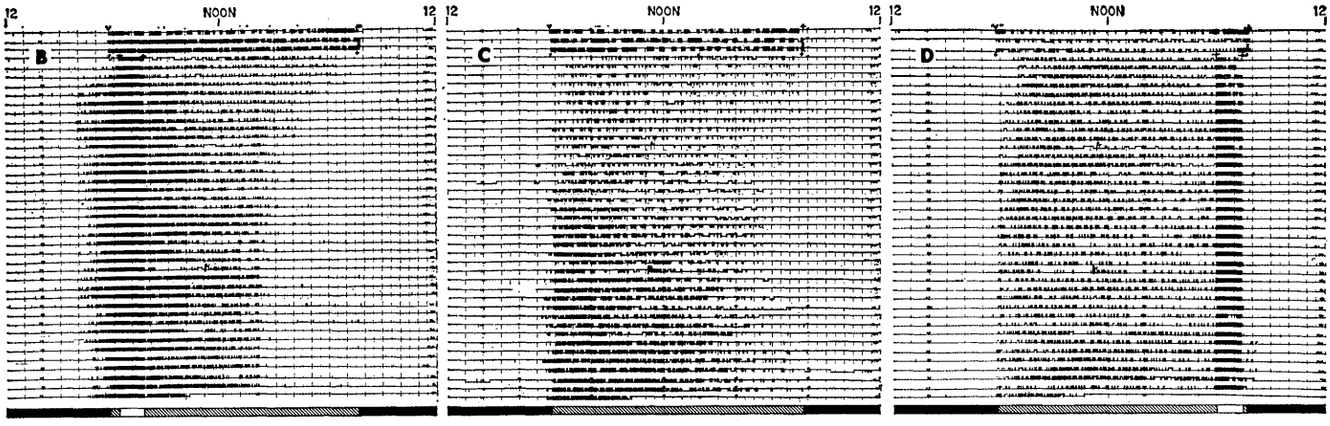

Fig. 1. Continuous perching records of three representative birds, one each from groups B, C, and D. The lighting regimen, which is different for each group, is diagrammed at the bottom of each record (solid black, darkness; cross hatched, dim green light; white, bright white light). Each line is 1 day's record (midnight to midnight), and each day's record has been pasted under the record of the day preceding it. The solid black areas of the activity record indicate almost continuous perch-hopping (note how this corresponds with the bright illumination), and the intermittent pen strokes indicate less vigorous use of perches. For all birds the first 3 days were spent in LD 14:10 (white light only). The experimental light regimens began on day 4 of the records.

such a separation was suggested by the long-established facts that, in birds, very dim light is relatively noninductive (*5*), green light is less inductive than red or white light (*6*), and very dim light signals are sufficient to entrain the locomotor rhythm (that is, to control its phase and period).

Sparrows were presented with a cycle of 14 hours of very dim green light and 10 hours of darkness each day. This cycle was sufficient to entrain the locomotor rhythm when given alone (Fig. 1C). There are at least two locations within the dim green cycle at which 75 minutes of bright white light (*7*) can be superimposed with very little effect on the phase of the locomotor rhythm. One of these regions lies around the beginning of the bird's daily activity, shortly after the dark-green transition, and another lies about 12 hours later, shortly before the green-dark transition. With white light at these positions, one has effectively separated the phasing action of white light from its photoperiodic effectiveness. The green light cycle controls the phase of the activity rhythm and presumably the phase of the rhythm of sensitivity to induction. The Bünning hypothesis makes the clear prediction that white light falling on different portions of the sensitivity rhythm will have different effects on the photoperiodically mediated testis weight.

Adult male sparrows were used. Eight birds (aviary controls) were killed at the beginning of the experiment. The remaining 56 birds were housed in individual activity cages, each in a light-tight box. Food and water were provided ad libitum and were replenished every 2 weeks (*4*). The experimental birds were divided into four groups, each group receiving a different lighting regimen, as diagrammed in Figs. 1 and 3. The experiment began on 9 July, when all the birds were transferred from outdoor aviaries to their individual cages and given 3 days of LD 14 : 10 (white light only), that is, 14 hours of light (L) and 10 hours of dark (D). The experimental light regimens began on 12 July and were continued without interruption until 19 August, when the experiment was terminated.

Figure 1 shows the complete activity records from three representative birds from groups B, C, and D. By comparing the phase of the onset of locomotor activity in B and D with that in C, one can see that in the presence of 14 hours of dim green light the addition of 75 minutes of white light per day at the positions shown in B and D have very little effect on the phase of the overt rhythm. Figure 2 has been included only to show that 75 minutes of white light may have a great effect on the phase of the overt rhythm if presented in some other relationship to the 14 hours of dim green illumination.

Although most workers in bird photoperiodism have used the induction of testis growth as an assay, maintenance of testis weight is photoperiodically mediated in the house sparrow (*4*). Our experiment was performed toward the end of the sparrow's seasonal reproductive period, and maintenance of testis weight was used as the assay of the photoperiodic effectiveness of the various experimental light regimens. The birds were killed after 39 days of exposure to the experimental regimens. Weights of the fresh testes were plotted (Fig. 3) with reference to the light cycle which each group experienced. Statistical analysis reveals no significant differences among groups A, B, and C. Fourteen hours of dim green light is thus no more effective in maintaining the testis than is complete darkness and further, the addition of 75 minutes of white light beginning about 1.5 hours after the onset of daily activity brings

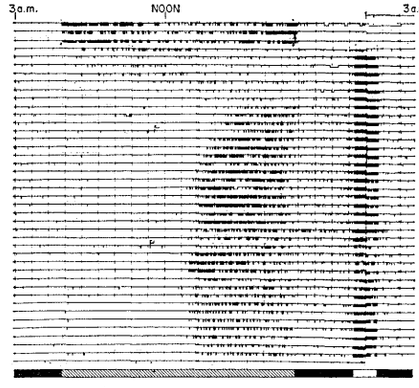

Fig. 2. The complete activity record of one of several birds which were given a daily 75-minute pulse of white light beginning 17½ hours after the dark-green transition. The data have been treated as in Fig. 1 except that the entire record has been cut vertically at 3 a.m., and the first 3 hours of data have been transposed from the left to the right side of the figure to facilitate interpretation. Note that white light in this position has a marked effect on the phase of the activity rhythm (that is, compared with Fig. 1D). The testis weights of these birds are not included in the paper. The data are complex and will be discussed in a later publication.

about no increase in effectiveness. However, groups A, B, and C are all significantly different from group D (*8*). The most interesting comparison is clearly that of B with D. These two regimens are identical with respect to absolute amounts of darkness, dim green light, and white light. The only difference between them is the position of the white light relative to the other features of the cycle (*9*). The dependence of photoperiodic effect on position of the signal confirms the existence of a rhythm of sensitivity to the inductive effects of light, which is the major assumption of Bünning's hypothesis.

In addition to offering strong support for the Bünning hypothesis, the experiment described above makes it clear that at least some of the physiological processes involved in maintenance of testis weight are dependent on light. The difference in testis weights between groups C and D (in Fig. 3) is clearly due to the additional 75 minutes of white light at the appropriate phase in group D. The data leave no room for an interpretation involving a dark requirement for this aspect of the photoperiodic response of house sparrows.

The aviary controls (group E in Fig. 3) are not significantly different from group D. This suggests that light regimen D may have been completely effective in maintaining the testes of those birds exposed to it at their initial weights. If this is true, then the wide variance in the testis weights of the birds in group D may be understood as a reflection of an initial variance in the population rather than a variance in response to the experimental treatment. This interpretation appears reasonable in view of the facts that regression in the field population in Austin begins in the last weeks of July and that the experimental birds had been held in outdoor aviaries for periods of up to a month before the beginning of the experiment.

The results clearly support our original assumptions. The photoperiodic efficiency of the 75-minute light pulse depends only on its position in the birds' daily cycle of activity. Though only two portions of the sensitivity rhythm have been probed, the experimental results allow no doubt of its existence. The difference between groups B and D can be explained only on the basis that under the conditions of the experiment, the activity rhythm bears a determinate phase relationship to the rhythm of sensitivity to photoperiodic induction.

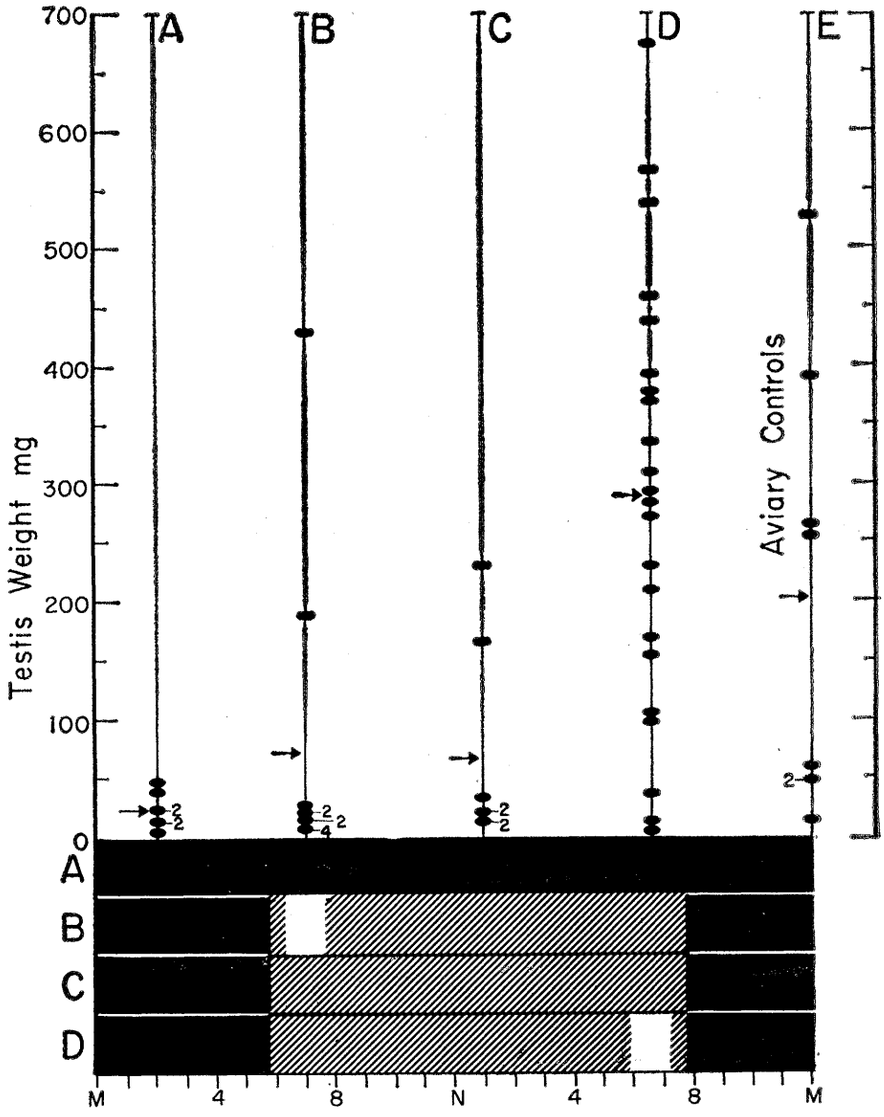

Fig. 3. Each point represents the combined fresh weights of the testes from a single bird. The numbers next to some of the points indicate the number of birds whose combined testes weights overlapped at this value and were consequently plotted as a single point. Groups A, B, C, and D are plotted with reference to the light regimen (see Fig. 1) which they received as diagrammed in the lower part of the figure. Groups B, C, and D are the groups from which representative samples are shown in Fig. 1. The mean of each group is designated by the arrow to the left of the line on which the points are plotted. The points for Group E are the values for eight "aviary control" birds killed at the beginning of the experiment. During the experiment, one bird in constant darkness died. [Four birds each from groups A and C were killed after only 23 days of exposure to the experimental regimens (data not included in this figure). The mean combined testis weights for the birds killed early were 153 mg for group A, and 177 mg for group C. These are slightly lower than, but not significantly different from, the aviary controls, indicating that under these conditions, most of the regression occurred during the last 16 days of experimental treatment.]

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7. Green light was produced by electroluminescent Sylvania panelescent night lights (one per box, approximately 0.1 lux). White light (approximately 500 lux) was produced by Ken Rad 4-watt fluorescent bulbs (cool white F4T5/CW).
8. Students *t*-tests were performed and yielded the following *P* values: A and D, $P < .001$; B and D, $P < .001$; C and D, $P < .005$. The small differences between A and B and between A and C may reflect either chance variation or a very slight effect of the dim green light. Final body weights were very similar in all groups and showed no correlation with testis weights.
9. With the onset of activity taken as hour 0, the birds in group B received the white light beginning at hour 1.5 ± 46 minutes (mean and standard deviation of the 11 birds), whereas those in group D received the white light beginning at hour 11.6 ± 46 minutes (mean and standard deviation of the 22 birds).
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Electrical Recordings from Meningioma Cells during Cytolytic Action of Antibody and Complement

Abstract. *Resting membrane potential and total cell resistance of human meningioma cells in tissue culture have been measured with fine microelectrodes. Addition of either antiserum inactivated with heat or control serum from normal rabbits produced small depolarizations (2 to 4 millivolts) with no discernible (< 5 percent) change in resistance. Addition of antiserums with complement, however, produced larger depolarizations and decreases in resistance before any changes in cell morphology were visible with light microscopy; as cytoplasmic swelling progressed, membrane potential dropped close to zero, and resistance decreased five- to tenfold. The electrical recording technique may be useful in the study of sublethal as well as lethal damage to immune cells and, in particular, may permit temporal resolution of damaging events and repair mechanisms in a single cell.*

A method which could assess the initiation and time course of cell damage and subsequent repair mechanisms in individual isolated living cells would be an important addition to present methods of evaluating cell membrane pathology and might be of particular application to a number of immunological problems involving sublethal as well as lethal types of cell membrane damage. We have used the intracellular microelectrode recording technique to study the cytolytic action of antibody and complement.

Human meningioma cells were directly explanted after surgical removal and were then maintained in tissue culture through six subcultures for use as antigen. The cells were cultivated in monolayer on a glass surface in a fluid consisting of 80 percent F_{10} medium (a modified Eagle's medium) (*1*) and 20 percent fetal calf serum, and containing penicillin (100 mg/ml) and streptomycin (100 μg/ml).

After treatment with trypsin and centrifugation in the cold, ghost cells were prepared by Haughton's technique (*2*) and stored in Earle's balanced salt solution at −70°C until ready for use as antigen. We prepared rabbit antibody against these cell membranes by injecting subcutaneously in the left mid-dorsal area 60,000 meningioma ghost cells in 0.7 ml of balanced salt solution with 0.3 ml of complete Freund's adjuvant. A second injection was made in 10 days, and serum was collected at 20 days.

Preliminary reactions were observed on cultured cells of the same line on cover slips (50 by 10.5 mm) in Leighton tubes (small deep-well chambers for holding the coverslip and media). The antiserum to meningioma cells produced a cytolytic reaction in meningioma cells, characterized by swelling and bleb formation. For purposes of comparison human glioblastoma multiforme cells (grade IV astrocytoma) were tested with antiserum to meningioma under identical conditions. No morphological changes were noted even after 24 hours, and growth continued unchanged. As controls, serums were also collected and pooled from rabbits before inoculation. Portions of antiserum to meningioma were inactivated with heat at 56°C for 30 minutes. Control serums, those inactivated with heat and unheated serums, were stored at −70°C. Experiments were performed in a room kept at 30°C.

Cells that appeared viable and which had distinct cytoplasmic boundaries were selected for microelectrode study 3 days after subculture. Selected cover slips were transferred to a small chamber on a microscope substage and immersed in 1 to 2 ml of fresh F_{10} media. Isolated (nonsyncytial) cells were viewed through a microscope (× 320) and impaled with micromanipulator-directed fine glass micropipettes filled with 3*M* KCl [direct-coupled (DC) resistances 15 to 30 megohm] (Fig. 1). Cells which adhered well to glass were easily impaled. Electrical activity was led to a PICO-metric amplifier (Instrumentation Laboratories, Inc.) by a short chlorided silver wire and then to a DC input of a Tektronix-502 oscilloscope, a similar wire immersed in the bath being used as reference. A Wheatstone bridge permitted simultaneous stimulation through and recording from the electrode. Rectangular constant current pulses, 30 to 100 msec in duration, were delivered through a 10^9-ohm resistor in series with the electrode, and current intensity was calculated as the voltage drop across the resistor. The microelectrode techniques have been described fully (*3*), and our method for measurements of membrane properties of Betz cells has been further described elsewhere (*4*).

In all, 28 meningioma cells were satisfactorily impaled; in ten cases membrane properties alone were studied; in 18 cases studies were made before and then after addition of control, heat-inactivated, or unheated serum. Resting membrane potentials ranged from −10 to −20 mv and were remarkably stable (< 1 mv change) during the control period of 5 to 10 minutes. Total cell or "input" resistances ranged from 10 to 40 megohm, the higher values being obtained in the smaller cells. Time constants [times for voltage to reach $(1 - e^{-1})$ or 63.2 percent of the final values] ranged from 5 to 30 msec; the six largest values (20 to 30 msec) were obtained in rounded meningioma cells with large nuclei. The steady-state changes of the voltage with applied current were linear over a range of ± 40 mv from the resting value (Fig. 2, A and B).

Addition of one to two drops of inactivated antiserum caused a 2- to 4-