found that model-airplane lacquer and bee's wax impregnated with carbon-black were the most effective in omitting light. For observing locomotor rhythms each cockroach was separately housed in a circular cage (*7*) which rotated freely when the animal was active. These cages were kept in temperature-controlled cabinets and their rotations were continuously monitored by a pen recorder activated by a microswitch.

All experimental animals were subjected to LD 12 : 12; the light intensity in these LD cycles never exceeded 275 lu/m^2. Figure 1 illustrates the typical response of an animal in which the compound eyes were painted but the ocelli left exposed. Before being "blinded," the roach was entrained by the LD cycle from day 1 to 19; on day 20 it was removed from its cage and its eyes painted with several coats of black lacquer. When replaced in its cage and again subjected to the LD cycle (day 20 to 50) the insect's rhythm failed to be entrained by the LD cycle. Thus, although the ocelli were exposed and could presumably "see" the light cycle, the roach's rhythm clearly changed to a "free-running" state. On day 50 the paint was removed from the compound eyes and the rhythm was rapidly re-entrained by the LD cycle. On day 68 the ocelli were surgically removed and the rhythm remained entrained until the end of the experiment. Similar tests for ocellar function were repeated on seven individual roaches with identical results. In addition, experiments with 15 animals with their compound eyes covered corroborate the result shown in Fig. 1. In seven of these insects the covering was removed after a free-running rhythm was established and subsequent re-entrainment was always observed. It should be noted that two of the 15 roaches tested never lost entrainment; subsequent examination of these animals revealed that small areas of paint had chipped away from the surface of the compound eyes.

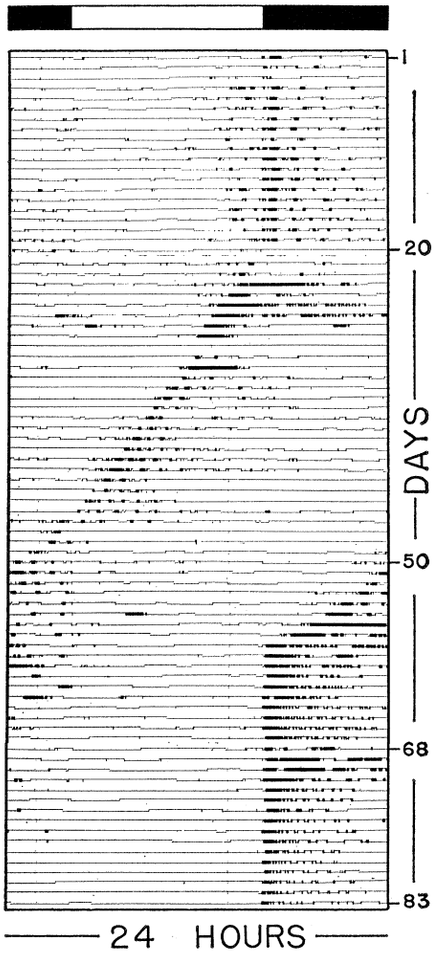

Fig. 1. Record of the rhythm of locomotor activity of a single roach, *Leucophaea*, maintained in a light-dark cycle, LD 12 : 12, for 83 days. On day 20 the compound eyes were painted with black lacquer; on day 50 the paint was peeled off; and on day 68 the ocelli were surgically removed. The position of the light and dark fractions of the LD regime is indicated at the top of the figure.

Since these results differ from those in earlier reports, several comments are warranted. First, none of the earlier experiments demonstrate that an animal's circadian rhythm can be induced to show a free-running period in an LD cycle by interference with a specific photoreceptor. This is a crucial point, since any treatment which interferes exclusively with light input to the circadian system should simply evoke a response typical of that expressed by a normal animal in constant darkness—that is, a free-running rhythm. Also, it should be emphasized that a re-examination of Harker's data reveals that entrainment was *not* lost after the ocelli were painted. Harker's data do show that in animals so treated the phase of the activity is reversed so that the onset of activity begins abnormally at the transition of dark-to-light. Although we have preliminary evidence that similar phase-reversals may occur after the brain has been damaged, no adequate explanation of Harker's observation can be given here. Finally, it must be emphasized that our findings (which strongly implicate the compound eyes as the input to the circadian system) do not rule out the possibility of direct photostimulation of the brain by light transmitted through the cuticle of the head. To ensure adequate exclusion of light in our roaches, most of the head capsule was covered as well as the compound eyes. Consequently, light was being excluded not only from the eyes, but also from much of the brain.

SHEPHERD K. ROBERTS
Department of Biology,
Temple University,
Philadelphia, Pennsylvania

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8. These observations were conducted over a period of years; they were begun in Prof. Pittendrigh's laboratory at Princeton University and were completed at Temple University. I acknowledge the following support: Porter Fellowship of the American Physiological Society; funds from the Eugene Higgins Trust allocated to Princeton University; and a research grant from Temple University.

4 March 1965

Conditional-Lethal Mutants of an Animal Virus: Identification of Two Cistrons

Abstract. *Two different temperature-sensitive conditional-lethal mutants of Sindbis virus, an animal virus that contains RNA, have been isolated. When cultured in chick fibroblast monolayers at 42°C, these mutants yield less than 0.05 percent as much virus as does the wild type, whereas at 27°C they grow normally. One mutant appears to be altered in the synthesis of a protein that is produced early in the infection and is required for viral RNA synthesis. The other mutant produces as much infectious RNA as the wild type at 42°C and appears to be altered in the synthesis of a protein produced late in the infection.*

Conditional-lethal mutants have been useful in the study of the genetics and physiology of both DNA (*1*) and RNA (*2*) bacteriophages. With the isolation of temperature-sensitive conditional-

lethal mutants of Sindbis, a group A arbovirus (*3*), the same techniques can be extended to animal virology. We describe here two mutants of Sindbis virus which are apparently blocked in different steps in the process of multiplication. Methods for the growth and titration of Sindbis virus in chick fibroblast tissue cultures have been described (*4*). Titrations, recorded as plaque forming units (PFU) per cell or per milliliter, were all done at 27°C.

These experiments began with wild-type Sindbis virus (*5*), which was able to make plaques in chick fibroblast tissue culture over the temperature range 27° to 42°C. From this virus two temperature-sensitive conditional-lethal mutants (CL-2 and CL-4) were isolated (*3*). These mutants produced normal plaques at 27°C but none at 42°C. Earlier experiments showed that although mutants were adsorbed at 42°C they were unable to carry out some essential intracellular process (*3*).

The growth patterns of these two mutants and the wild-type virus at permissive (27°C) and nonpermissive (42°C) temperatures are recorded in Table 1. The small amount of virus produced at 42°C in the mutant-infected monolayers can be accounted for by the number of wild-type revertants observed in the mutant stocks. Neither of these temperatures was optimum for the growth of the wild type; titers ten times higher were achieved at 36°C.

To test the possibility that the temperature-sensitive reaction of the mutants might be required only in the early part of the growth cycle, as observed for certain phage mutants (*6*), monolayer cultures infected with 40 PFU per cell were incubated at 27°C for 3½ hours, and then the temperature of incubation was raised to 42°C. (This time was chosen because little new virus is produced by cells during the first 3½ hours of infection.) Figure 1 shows that after such low-temperature incubation cells infected with mutant CL-4 subsequently produced virus at 42°C. In contrast, cells infected with mutant CL-2 did not profit from this early incubation at the permissive temperature. These results suggested that at 42°C mutant CL-4 was defective in the synthesis of an early viral protein, whereas mutant CL-2 was defective in the synthesis of a viral protein required late in the infection, perhaps a structural protein of the virus coat.

These suggestions were supported by the results of assay of infectious viral RNA production at 42°C (Table 2). Mutant CL-4 is defective in infectious RNA synthesis at this nonpermissive temperature. This mutant may be blocked in the synthesis of viral RNA polymerase (*7*). Mutant CL-2, in contrast, equals the wild-type virus in infectious RNA synthesis at 42°C, as would be expected if it were blocked in the formation of a "late" protein.

Since these mutants appear to be altered in different cistrons, a cell mixedly infected with both mutants at 42°C might be expected to show complementation, that is, a yield of virus greater than the sum of the yields from infection with each mutant separately. The complementation observed thus far has been very inefficient. A series of experiments has been performed in which the degree of complementation was never less than twofold and seldom higher. This low degree of complementation cannot be due to an inability to establish mixed infection, since we have observed efficient phenotypic mixing between two strains of Sindbis virus under identical growth conditions (*8*). That extensive complementation was not observed might be due to failure of good RNA polymerase to be released from its defective RNA or to failure of good coat protein to aggregate normally in the presence of defective coat protein.

Table 1. Influence of incubation temperature on growth of mutants and wild type. Chick fibroblast monolayers were drained and infected with about 40 PFU of the indicated virus per cell in 10 ml of fresh medium. After 3 hours, at the temperature noted, monolayers were rinsed once to remove unadsorbed virus and then incubated for 5 hours more; the fluids were then titrated.

Temperature during growth	Virus yield (PFU/ml)		
	Wild-type	CL-2	CL-4
27°C	1.1×10^8	1.3×10^8	6×10^7
42°C	9×10^7	1.2×10^4	4.2×10^4

Table 2. Infectious RNA production by tissue cultures at 42°C. Three and one half hours after infection (40 PFU/cell) the cells were extracted with sodium dodecylsulfate and phenol (*9*), and the infectious RNA was titrated by a plaque method in chick fibroblast tissue culture (*10*). Results from two independent experiments are recorded.

Virus	Infectious RNA: PFU per monolayer 3½ hours after infection at 42°C	
	Expt. 1	Expt. 2
Wild type	6.6×10^4	3.9×10^4
CL-2	6.6×10^4	3.3×10^4
CL-4	3.0×10^1	2.0×10^2

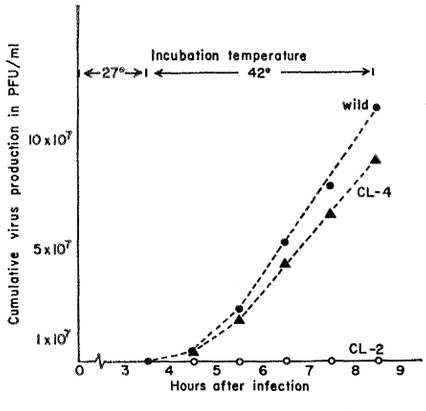

Fig. 1. Cumulative virus production during growth of the wild type and mutants CL-2 and CL-4. Chick fibroblast monolayers were infected with about 40 PFU per cell in 10 ml of fresh medium. After 2½ hours at 27°C, all monolayers were rinsed to remove unadsorbed virus. The culture medium was changed at hourly intervals thereafter, and samples were saved for titration. At 3½ hours the temperature of incubation was rapidly shifted to 42°C.

BOYCE W. BURGE
E. R. PFEFFERKORN
Department of Bacteriology and Immunology, Harvard Medical School, Boston, Massachusetts

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5. To avoid loss of infectious virus at 42°C, we used a heat resistant strain of Sindbis virus. This heat resistant strain produced plaques over the same temperature range as did the stock from which it was derived; thus, it is a wild type with respect to temperature-sensitive conditional-lethal mutations.
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