CENTRAL NERVOUS SYSTEM CONTROL OF CIRCADIAN RHYTHMICITY IN THE COCKROACH. I. ROLE OF THE PARS INTERCEREBRALIS

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Circadian rhythmicity is now well-established as a common feature of physiological systems of all levels of organization, from intact multicellular organisms to isolated organs, single (protistan) cells and individual enzyme systems. A principal aim of research at both the uni- and multi-cellular levels is to locate and characterize the nature of the self-sustaining oscillation (in control mechanisms) which causes the observed rhythmicity. In multicellular systems the first step must be to inquire whether or not a distinct group of cells functions as a pacemaker, or driver, for the rest of the system. In this series of papers we pursue the many published suggestions that some part (or parts) of the insect brain functions as that pacemaker.

The starting observation concerns decapitated insects; they may survive and move for days or weeks, but their locomotory activity loses its previous circadian rhythmicity (Eidman, 1956; Harker, 1956; Nishiitsutsuji-Uwo, unpublished data). Of the several organs in the head the corpora cardiaca and/or allata appear to have no effect on circadian locomotory rhythms (Eidman, 1956; Fingerman, Lako and Lowe, 1958; Roberts, 1966; Nishiitsutsuji-Uwo, unpublished data). On the other hand there is ample evidence that these rhythms are effected, directly or indirectly, by the brain. For instance, Dupont-Raabe (1957) and Mothes (1960) have shown that the brain exerts an endocrine control over the daily cycle of color change in *Carabus morosus*. Klug (1958) has reported a daily cycle in the number of neurosecretory cells in the brain of *Carabus nemoralis* containing secretory granules (and an associated cycle of nuclear volume change in the cells of the corpus allatum). Rensing (1964, 1966) has reported similar observations on *Drosophila melanogaster*. The suggestion arising from these latter facts is that a rhythmicity of neurosecretion from the pars intercerebralis may be underlying the locomotory rhythm; and this is certainly encouraged by the fact that extracts from corpora cardiaca, which are storage organs for this secretion (Scharrer, 1952), apparently affect the spontaneous electrical activity of isolated nerve cords (*Periplaneta*) *in vitro* (Özbas and Hodgson, 1958). Indeed Eidman (1956) has already concluded from ablation experiments that the pars intercerebralis is involved in the control of circadian rhythms of locomotion in *Carausius*, and Roberts (1966) drew a similar conclusion after making midsagittal bisections of the *Periplaneta* protocerebrum. But neither of these published observations is as yet fully compelling. Eidman's observations, for example, were limited to one day following the surgery;

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in our experience arrhythmicity is an *immediate* and transient consequence of almost any surgery of the brain.

Ingenious experiments by Lees (1964) provide conclusive demonstration that photoperiodic receptors of the aphid *Megoura viciae* are located in the protocerebrum. This protocerebral center is active even after the compound eyes have been blinded, suggesting that the neurosecretory cells in the pars intercerebralis may be implicated both as receptors and as humoral effectors.

If one regards (Bünning, 1936; Pittendrigh and Minis, 1964) photoperiodic induction as an aspect of the entrainment of circadian oscillations by light, he will be further encouraged by Lees' findings to focus attention on the pars intercerebralis as a potential pacemaker for the circadian system in insects.

Harker (1956, 1960a, 1960b) has published well-known and very important conclusions that the suboesophageal ganglion in *Periplaneta americana* is directly responsible for the circadian rhythm of locomotion in that insect. No confirmation of her finding has, however, yet been published, and Roberts (1966) describes repeated failures to obtain Harker's results from apparently identical procedures. In the meantime we conclude that the role of that ganglion remains to be fully established. In any case, Harker's work is of great importance in a quite different respect; it exemplifies the only experimental procedure which yields, in principle, unequivocal evidence of having localized the pacemaking oscillation in the system. Thus she reported transfer of the rhythm's *phase* when she implanted a ganglion into a headless host. Loss of rhythmicity following ablation of some organ is, of itself, equivocal; that organ could be indispensable to the expression of an *assayable* rhythm but in fact be only peripheral to a driving pacemaker left after the ablation, and unable to express its oscillation in terms of the assayed parameter. Indeed restoration of the rhythm by replacing the ablated part is also equivocal unless it includes (as Harker reports for *Periplaneta*) introduction with the implant of a *specific phase* different from that previously expressed by the host.

Roberts' failure to confirm the role of the suboesophageal ganglion, and the other indications noted above that the pars intercerebralis is involved have prompted our own attention, in this series of papers, to the protocerebrum of the cockroach, and to the pars intercerebralis in particular. The dependence of pars intercerebralis neurosecretion on transport by intact axons has precluded our exploitation of the technique of replacing ablated parts, and our conclusions are necessarily limited in this respect.

Materials and Methods

Two species of cockroach employed in these experiments, *Periplaneta americana* and *Leucophaea maderae*, were maintained in temperature-controlled (25° C. ± ½° C.) rooms in cycles of alternating light and dark (LD). Prior to experimental manipulation the animals were placed in monitored activity wheels (Roberts, 1960, 1962) in light-tight constant-temperature (25° C. ± ½° C.) cages equipped with a clock-controlled, water-jacketed 4W cool white fluorescent light source. They were exposed to either regularly alternating light and dark (LD), constant dark (DD), or a combination of these to test their activity rhythms. Only newly emerged adult male roaches were used. Roaches that failed to demonstrate clear rhythmicity, as judged by either a lack or low level of locomotion, or by lack of a

clear relationship between the phases of the activity rhythm (determined by the time of the onset of activity) with respect to the phase of the "entraining" LD cycle (generally 12 hours light and 12 hours dark per 24 hours, LD *12:*12) were discarded.

Methods for recording and analyzing activity data are in general use and have been frequently described (Roberts, 1960).

Surgical procedures involved anaesthetizing the roach in its activity wheel by exposing it to CO_2. The animal was then removed from the wheel and its head was wedged and firmly held in a V-shaped opening in a (3″ × 5″ × 1″) plastic box. With its head projecting out of the opening, the animal was taped across the thorax to the bottom of the box and the antennae were strapped to the top surface of the box. The box was then attached to a movable operating platform which was mounted through a universal ball-joint to a mechanical stage. The bottom of the box was equipped with an inlet for CO_2 supplied from a cylinder through a line connected to a heat exchange-coil immersed in a constant temperature (25° C. ± 0.1° C.) water bath. The head of the roach was illuminated from above on either side by focused microscope lamps equipped with heat absorbing filters. This combination of heat filters and the stream of temperature-controlled CO_2 passing over the body and around the head of the roach allowed the animal to be anesthetized and immobilized for long periods without subjecting it to temperature pulses.

Prior to cutting, the entire head was cleaned with 70% ethanol. Cutting tools, consisting of micro-scalpels made from razor blade fragments held in blade holders, and finely sharpened forceps, were sterilized in 70% ethanol. The animal was positioned with the frontal portion of the head facing the operator in such a way as to allow comfortable access to and visibility of this region through a binocular microscope.

The first step in the procedure involved exposing the protocerebrum by cutting and removing a window of cuticle directly above this portion of the brain. The piece of cuticle was carefully placed on a sterile surface and saved for later resealing of the wound with dental wax. Once the cuticle square was removed and the tracheae and fat body tissues were cleaned away, the protocerebral lobes were clearly visible and seen bathed in "blood" which was removed just prior to surgery with a sterile cotton swab (see Fig. 1). A cut was made on each side of the protocerebral lobes just lateral to the mid-sagittal point such that a wedge containing the pars intercerebralis was freed from the remainder of the brain and could be removed with forceps. At this point notation was made by the operator grading the depth of the cuts as either complete or incomplete. A complete cut was one which, in the operator's judgment, contained sufficient of the underlying brain tissue in the pars intercerebralis, in addition to the mid-lateral portions of the protocerebral lobes, to remove all neurosecretory cells present in those areas. Incomplete cuts were those which did not penetrate deeply enough to remove these areas. In no cases reported here were the cuts sufficiently deep to result in mid-sagittal bisection.

In the course of these experiments many attempts were made to burn out the pars intercerebralis with an electric micro-needle. However, any needle used was not satisfactory to destroy the pars intercerebralis only. When the pars intercerebralis was completely burned, serious damage was inflicted on a wide adjacent

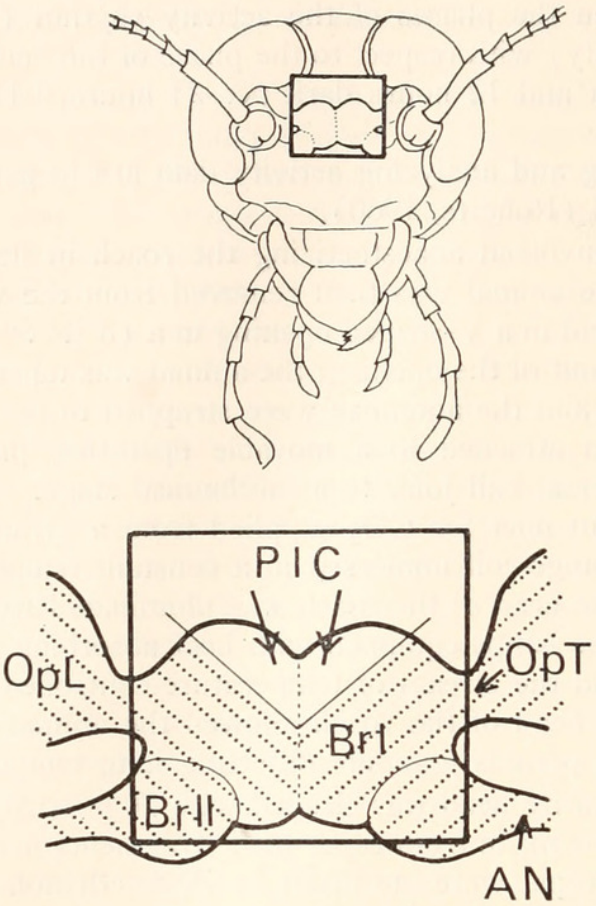

FIGURE 1. (a) A diagrammatic representation of the head of a cockroach showing the ~ 1.5-mm. square "window" made by removing a piece of cuticle from the front directly above the protocerebrum (Br I), thus allowing access to the pars intercerebralis (PIC). (b) Frontal view of the brain under the window illustrating the removal of the part of the brain containing the pars intercerebralis (white area). PIC: pars intercerebralis, Br I: protocerebrum, Br II: deutocerebrum, OpL: optic lobe, OpT: optic tract, AN: antennal nerve.

area. Since the brain is a soft, yielding structure, surgical ablation of this area was also not completely satisfactory. However, the cockroaches survived for long periods post-operatively and the extent of surgery could be easily checked by histological methods. Usually the first cut and removal of one side of the pars intercerebralis were easier than the second cut and removal of the other side. The areas immediately beneath the indentation of the pars intercerebralis, where the medial neurosecretory cells are located, were especially difficult to remove without damaging the area where the nerve tracts of the medial neurosecretory cells cross. Although notation of "complete" or "incomplete" ablation was made by the operator, histological confirmation was required, especially in those cockroaches which reestablished a normal rhythmic pattern post-operatively. Histology of the brain of cockroaches showing arrhythmicity after surgery was also important. Since some operated cockroaches remained arrhythmic for 3 weeks before they resumed normal rhythmicity, long-term observation was required to establish clearly post-operative arrhythmicity. Therefore, most of the arrhythmic animals were kept until they died. After the effect of surgery on the rhythm had been assayed by recording the animal's activity for many days, the brains of most animals, especially

those in which normal rhythms reappeared post-operatively, were fixed with Helley's fixative.

Halmi's (1952) aldehyde fuchsin-azan method as modified by Scharrer (personal communication) was used for the histological demonstration of neurosecretory materials.

Examination of the sections containing portions of the pars intercerebralis for the presence of neurosecretory cells is not difficult in brains of unoperated animals as one can use the "crotch" of the protocerebral lobes as a marker which, when present in the section, indicates to the observer that the section probably contains portions of the pars intercerebralis. Sections containing this "crotch," which also pass through the optic tracts and lobes, are the ones generally found to contain the brightly stained neurosecretory cells. In preparations where this "crotch" is absent or changed in shape because of surgical removal of the pars intercerebralis, precise serial orientation of the sections becomes a much more difficult problem. Despite these problems, there is no question about the identification of neurosecretory cells in well-stained sections when they are present. For obvious reasons, the total *absence* of neurosecretory cells in operated animals is much more difficult to discern. Control sections consisting of brains from unoperated animals were run parallel with each experimental group material in order to verify the success of the staining procedure.

TABLE I

Effect of ablation of the pars intercerebralis on the circadian locomotory rhythm in 45 operated cockroaches. Operation Grade; see text. Activity Level: Lower or Higher as compared to pre-operative normal level

Group	I	II
Post-operative rhythm	No rhythm	Rhythm
No. of animals	19*	28*
Operation grade		
Incomplete	4	9
Complete	13	10
No grade	2	9
Days of post-operative observation	35(14–78)	29(10–60)
Days until post-operative appearance of rhythm	—	9(0–24)
Activity level		
Normal	6	12
Lower	0	1
Higher	13	9
Lower then higher	0	6
Neurosecretory cells		
Present	—	11
Absent	3	8
Questionable	—	3

* Of 19 animals in Group I, 3 animals showed a questionable activity pattern and of 28 animals in Group II, 13 animals showed a rhythm but not quite a normal rhythm (see text).

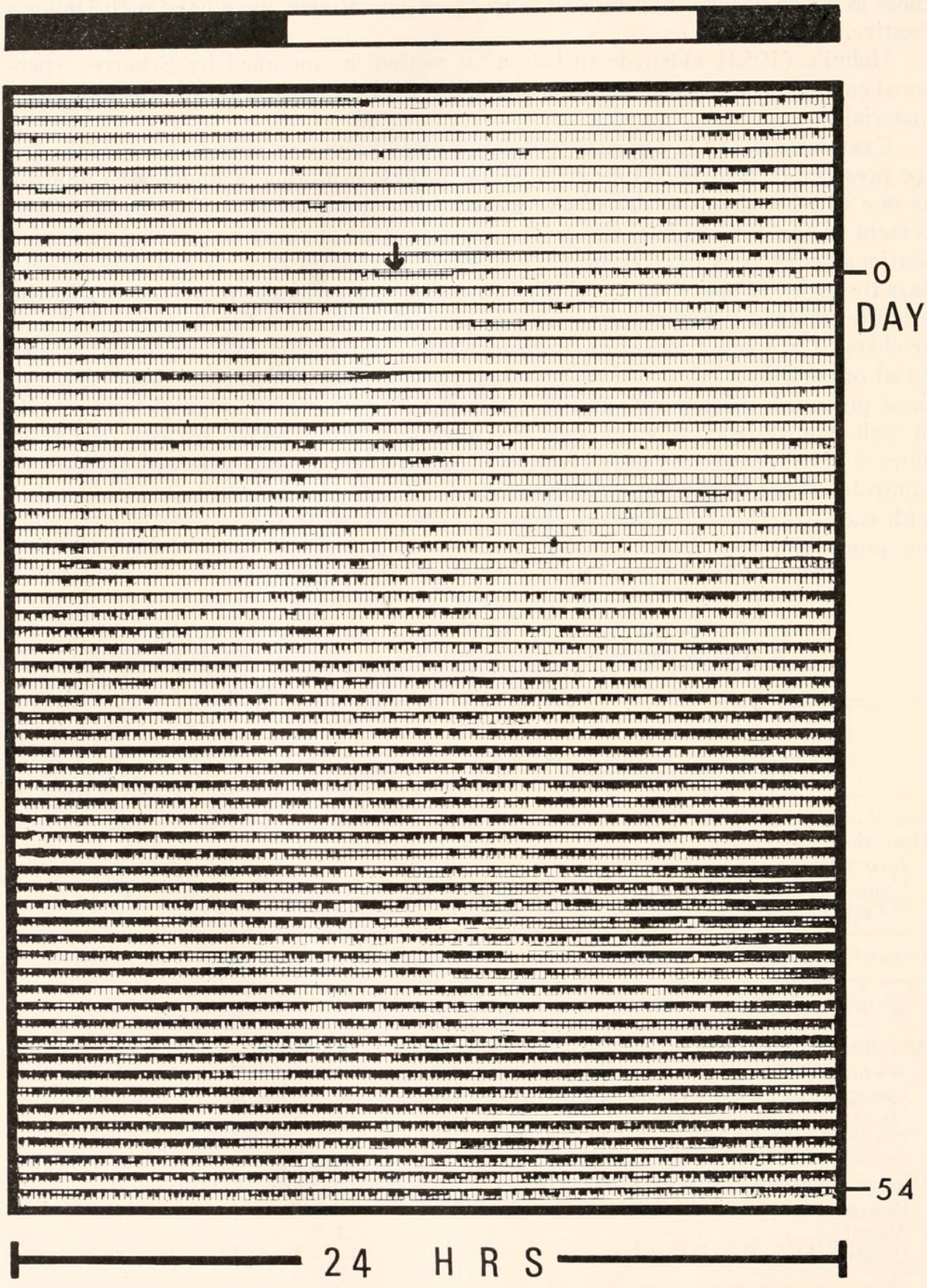

FIGURE 2.

Results

I. Effect of the removal of the pars intercerebralis

The data summarized in Table I show that a total of 47 operations on the pars intercerebralis was performed. Of these, 19 animals displayed no rhythmicity of activity post-operatively (Group I) while 28 displayed activity rhythms following removery from the operation (Group II).

1. Group I. No rhythm post-operatively (19 animals).

All the animals in Group I showed arrhythmic activity patterns following surgery (Table I). Of these, 4 were sacrificed or died within 3 weeks after surgery, which was considered the minimum observation period required to establish the absence of an activity rhythm. The average observation period for all animals in this group was 35 days, with a range of from 14 to 78 days. Only 3 arrhythmic animals served for histological examination and no neurosecretory cells were found. Thirteen out of the 19 of these operations were rated as "complete" at surgery, and 4 were rated as "incomplete."

The majority of animals in this arrhythmic group displayed abnormally high levels of post-operative activity as compared to pre-operative levels (see Fig. 2).

2. Group II. Rhythm post-operatively (28 animals)

Ten of the 28 animals in this group were rated as having "complete" removal of the pars intercerebralis at surgery; 9 were rated "incomplete"; 9 were not rated. In all of them the activity data generally showed a low level of locomotory activity for a few days after the operation, followed by a return to the normal level or to a sudden higher level. This transient post-operative suppression of activity—and hence of apparent rhythmicity—establishes clear demands on the duration of post-operative observation before reliable conclusions can be reached concerning the role of the tissue removed in the operation. In 9 cases activity was higher, in 6 cases first lower then higher, and in 1 case lower post-operatively than pre-operatively. The activity pattern became overtly rhythmic (except in 5 cases which showed unclear onsets of activity) at varying times following surgery within 2 weeks (0–15 days) (see Figs. 3, 4), but in 3 cases activity remained arrhythmic for about 3 weeks (19, 20 and 24 days), after which the rhythm became apparent. The rhythms that eventually developed in this group show atypical features in 4 respects.

First, of four animals showing an LD rhythm that were subsequently placed in DD, only 3 showed a clear circadian rhythm (Fig. 4); one of them became immediately aperiodic as though its former periodicity had been entirely imposed by the light cycle.

Second, in 5 animals the activity rhythm which developed showed clear signs of a *bimodality* which is rarely seen in normal animals (Fig. 4). A distinct peak

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Figure 2. Loss of the activity rhythm in *Leucophaea maderae* following complete ablation of the pars intercerebralis (Group I). The operation was performed on day 0 (time is indicated by arrow). Usually, animals showed high activity for 1–2 days after operation, then moderate or low activity appeared for a period (about 1 week). After this, activity increased to a very high level without showing any rhythm for 1–2 months; it decreased for a week before the animal's death.

This animal was sacrificed on day 55 and histological sections of its brain showed no neurosecretory cells. The light-dark regime is indicated at the top of the figure (open bar = light, solid bar = dark).

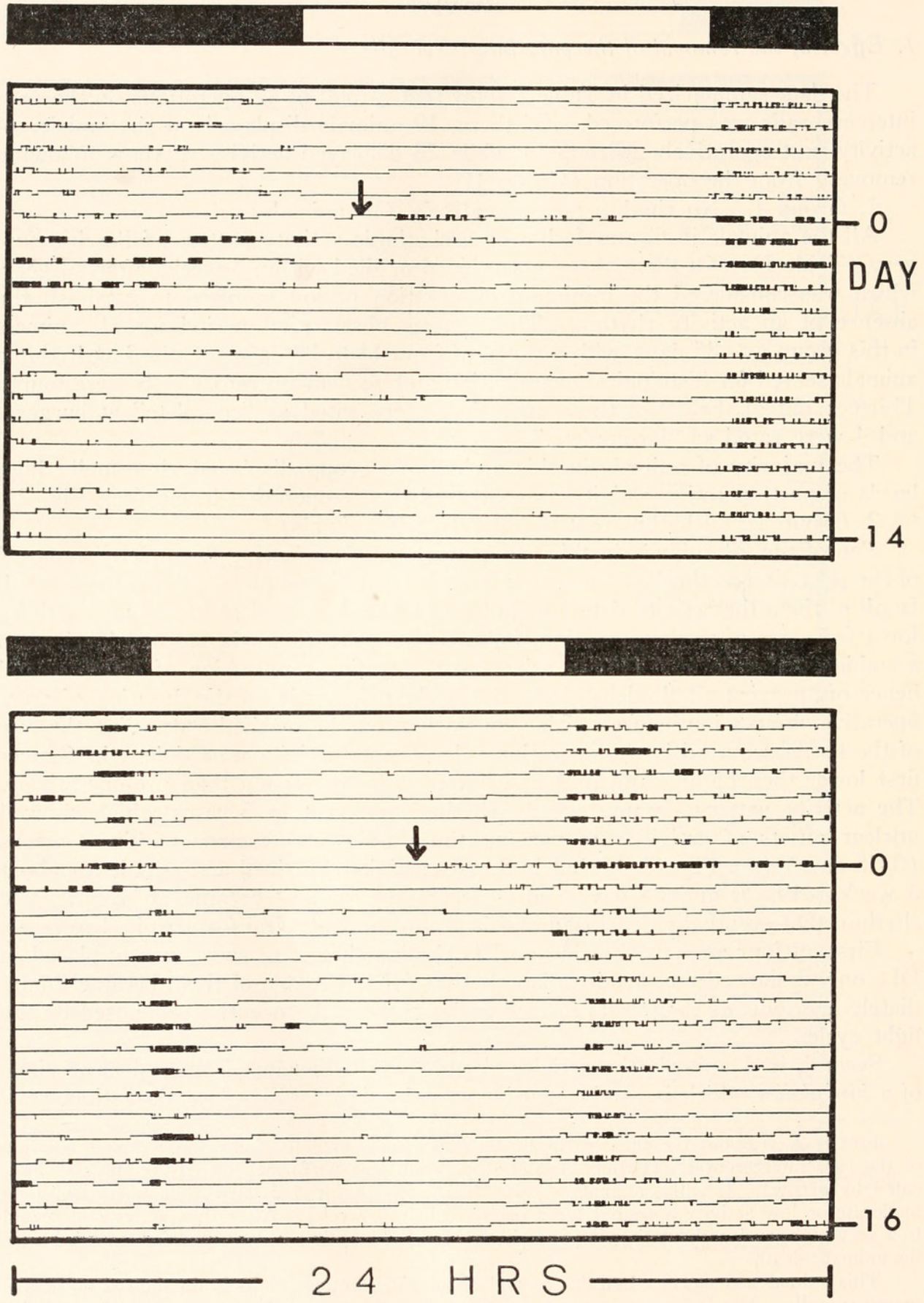

FIGURE 3.

of activity immediately *follows* dawn. Figure 3b shows one of the rare cases of bimodality in a normal animal; after operation its dawn peak switched from anticipating dawn to following dawn.

Third, as seen in Figure 5a, the operation caused the activity period, which is normally restricted to darkness, to extend as a single peak far into the end of the dark period.

Fourth, one animal remained unimodal but its activity maximum switched from the dark to the light part of the entraining LD cycle (Fig. 5b).

Most animals were sacrificed after the normal rhythm of locomotion was established post-operatively, in order to examine the brain histologically. The brains appeared normal except for a flat angle of indentation ("crotch") of the protocerebrum. In general, cut surfaces of the brain were covered with a neural lamella and perilemma which, although continuous with the uncut surface, were only about $\frac{1}{3}$ or $\frac{1}{2}$ the thickness of the intact layers.

Eleven of the 22 post-operative rhythmic animals which served for histological observation (Table I) revealed the presence of neurosecretory cells, many of which were filled with neurosecretory material. In 3 of 11 cases, only lateral neurosecretory cells of one side of the protocerebrum were found. An additional 3 cases showed questionably positive evidence, *i.e.,* very weakly stained cells, and in 8 cases, no neurosecretory cells were found.

II. Effect of actinomycin D on the pars intercerebralis

Since the brain hormone produced in the neurosecretory cells of the pars intercerebralis has been shown to be a protein-like substance (Ichikawa and Ishizaki, 1963), chemicals which block such synthesis, such as actinomycin D, might be expected to have an effect on the circadian rhythm of locomotion if this portion of the brain does indeed function as a humoral effector in controlling activity. We attempted to block synthesis in the pars intercerebralis using chips of gel containing actinomycin D. These chips were inserted from the top surface of the protocerebrum by making a small cut in the brain sheath and forcing the chips into the mid portion of the pars intercerebralis bilaterally.

The gel was prepared in the following way: 1 mg. actinomycin D was placed in a pre-heated (40° C.) petri dish (5 cm. diameter) to which were added 2 or 3 drops of acetone and then several drops of 2% Bacto agar solution (40° C.). This mixture was left at room temperature in the dark until the acetone and water had completely evaporated. The resulting dried disc of agar gel was cut into small pieces of $0.005 \sim 0.014$ mm.^{2} which contained approximately $0.02 \sim 0.05$ μg. of

FIGURE 3. Persistence of the activity rhythm in *Periplaneta americana* following the operation (Group II). The operation was performed on day 0 (time indicated by arrow) and the rhythm persisted in LD to day 14 (a) and day 16 (b). The animals were sacrificed on day 15 (a) and 17 (b), respectively, and histological sections of their brains showed the presence of neurosecretory cells. The light-dark regime is indicated at the top of the figures (open bar = light, solid bar = dark). (a) Typical example of persistence of the activity rhythm. (b) Biphasic rhythm: The activity rhythm which developed showed clear signs of a bimodality. This animal is one of the rare cases of bimodality in a normal animal (see pre-operative record); after operation its dawn peak switched from anticipating dawn to following dawn.

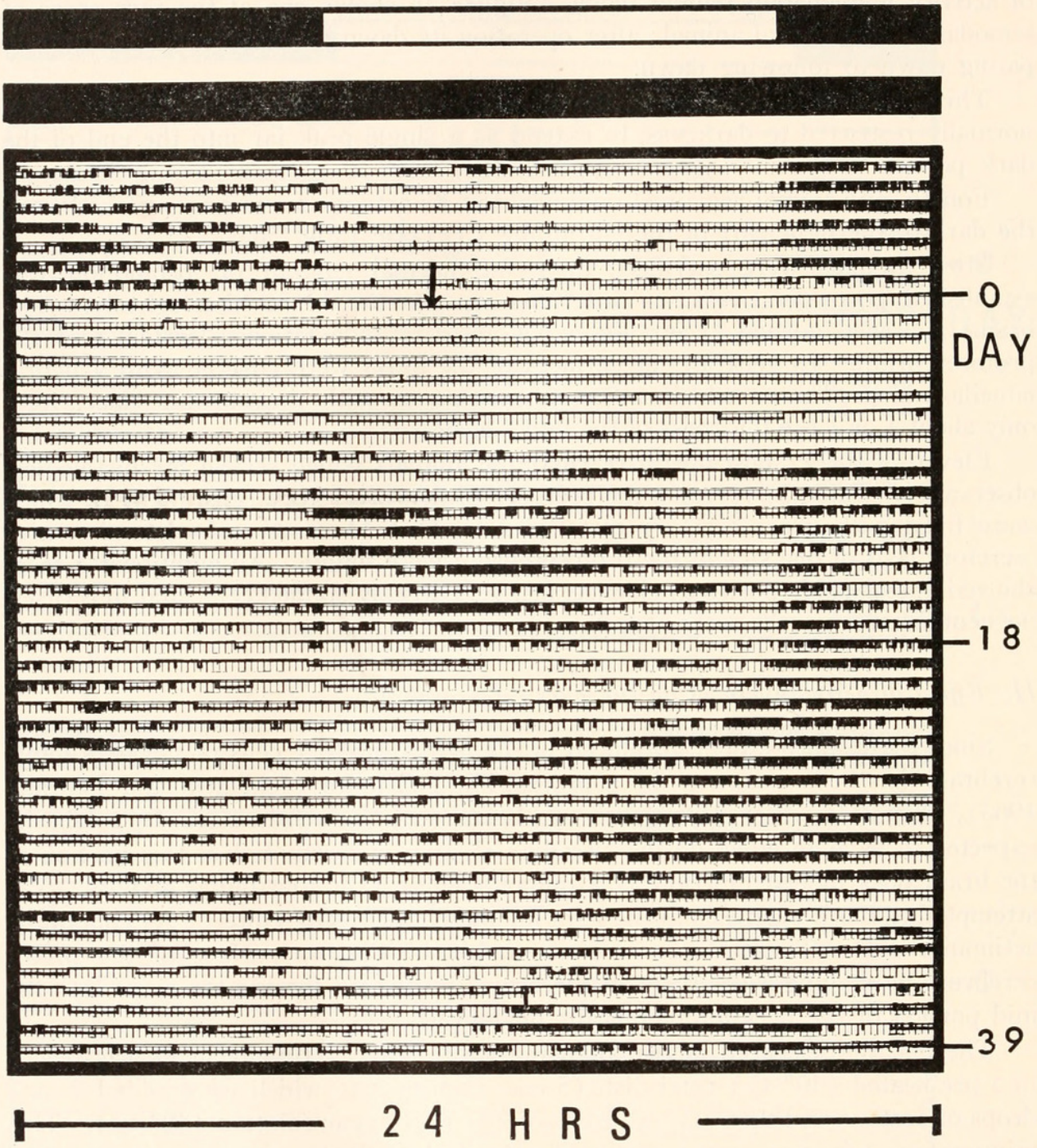

FIGURE 4. Biphasic rhythm in *Leucophaea maderae* following recovery from the operation. Within a week after the operation, the animal showed very low levels of activity; then the activity level suddenly increased and a biphasic rhythm, with the onsets of activity clearly phased to dawn and dusk, appeared. From days 19–39, the animal showed a free-running rhythm in DD. The animal was sacrified for histology on day 40 and neurosecretory cells were found.

actinomycin D. Control chips were made in the same way using eosin instead of actinomycin D.

As seen in Table II, 7 animals which received actinomycin D gel were observed for 35 days (21 ~ 57 days) post-operatively. Activity levels were generally normal or lower post-operatively than pre-operatively. None of these animals recovered

TABLE II

Effect of exposing the pars intercerebralis to actinomycin D on the circadian locomotory rhythm in cockroaches

Experimental procedure	No. of animals	Post-operative observations			
		Days of observation	Activity level	Rhythm	Days until appearance of rhythm
Act. D → PIC	7	35(21–57)	Normal 4 Other 3	None	—
Eosin → PIC	5	41(13–61)	Normal 4 Other 1	Normal	3(1–7)
Act. D → 5AG	2	43, 48	Normal	Normal	0*

Act. D. = Actinomycin D gel; PIC = Pars intercerebralis; 5AG = 5th abdominal ganglion.
* 0 means immediately after implantation.

locomotory rhythm after operation. Four out of 7 operated animals were dead at the end of the observation period (average days of survival was 38 days) and the others were sacrificed for further observation of the brain. Usually, the protocerebrum was partially (upper half or pars intercerebralis only) histolyzed and there was always a large pigmented tumor-like mass in the place of degenerated brain tissue. The optic lobes and tracts appeared to be intact. Histological preparations were unfortunately not made.

On the other hand, eosin gel-implantation did not affect activity or locomotory rhythms except that temporarily low arrhythmic activity persisted for 2 to 6 days post-operatively. All animals (5) survived until they were sacrificed for observation of the brain which showed no changes in shape nor pigmentation. Histological observations confirmed that the brains were normal except that some parts of the pars intercerebralis had been damaged. None of the implanted gel could be detected.

As an additional control, an actinomycin D gel chip (2 × the usual size) was inserted into the 5th abdominal ganglion in 2 animals. Both showed normal level of activity and rhythms immediately after the implantation.

From these experiments it was evident that although the implantation of the gel caused some physical damage to the pars intercerebralis, normal locomotory rhythmicity resumed shortly after the operation unless the gel contained actinomycin D. We conclude that actinomycin D was responsible for loss of the rhythm. But it is equally clear that our original goal—the local blockage of m-RNA synthesis—was not attained; the amounts of actinomycin D in the chips were too great and the positive results we obtained reflected an extensive "chemical surgery" of the dorsal section of the protocerebrum.

Why the tumor-like change happened after implantation of actinomycin D is a puzzle. Although there is a report that subcutaneous administration of actinomycin S and L to various stocks and strains of mice produced sarcomas (Kawamata, Nakabayashi, Kawai, Fujita, Imanishi and Ikegami, 1959), there is as yet no information on their effects in invertebrate tissues.

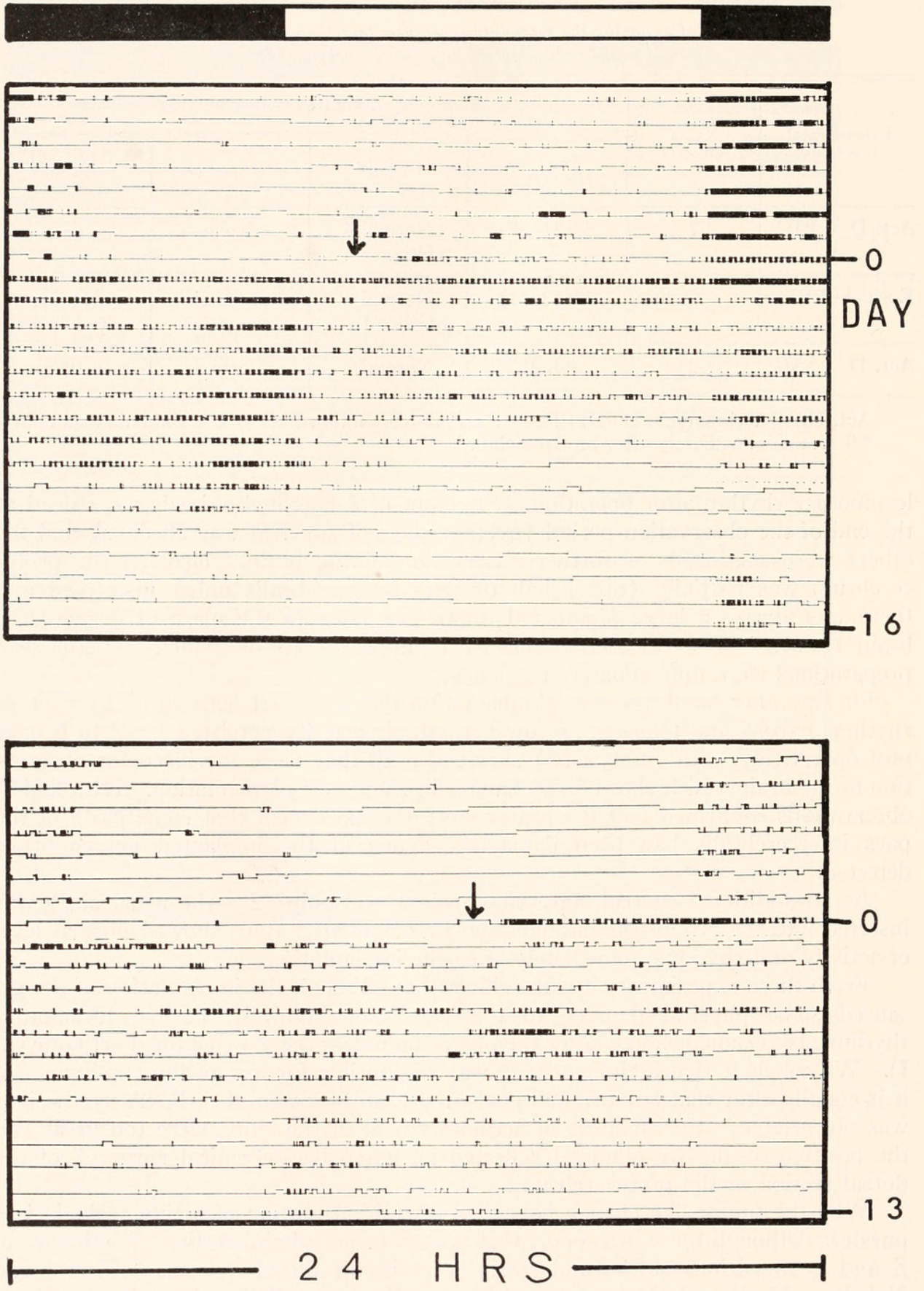

FIGURE 5.

Discussion

The data presented here are interpreted as evidence demonstrating a relationship between the neurosecretory cells of the pars intercerebralis and the circadian locomotory rhythm in the cockroach. Removal of this portion of the brain together with the neurosecretory cells results in a loss of rhythmicity provided that all neurosecretory cells, including the lateral and medial groups, are removed or incapacitated during surgery. In over one-half of those cases studied in which rhythmicity "regenerated" post-operatively, neurosecretory cells have been demonstrated to be present, and most of them were presumably functioning (as judged by the presence of neurosecretory material in the cells). As noted earlier, failure to demonstrate neurosecretory cells by the presence of stained neurosecretory substance is not a clear demonstration that they were not present in the specimen; it merely means they could not be found or were not functional. Thus, in light of this, the 11 positive cases and the 3 questionable cases out of 22 brains studied are even stronger evidence than the figures alone would suggest of a correlation between the presence of neurosecretory cells in the pars intercerebralis and the persistence or reappearance of rhythmic locomotory patterns in operated animals. This, together with the demonstration of a permanent loss of rhythmic activity in 19 of the 47 animals studied, further points up the important relationship between the pars intercerebralis and circadian rhythms of locomotion in roaches.

The nature of this relationship remains unclear; however, evidence presented here is suggestive of a system in which the neurosecretory material elaborated by the neurosecretory cells of the pars intercerebralis, in some way acts as a suppressor of locomotory activity. Thus, surgical removal or incapacitation of the productive sites or routes of dispersal of this material "releases" the animal from the inhibitory control of the brain, and its general level of activity is raised. This would explain the relatively higher level of activity post-operatively in animals rendered arrhythmic as compared to those in which rhythms "regenerated," since in the former the sites producing the locomotory suppressor have been removed or incapacitated whereas in the latter they have been only temporarily inactivated or partly removed. This interpretation is compatible with the evidence of Özbas and Hodgson (1958) who demonstrated that extracts of the corpora cardiaca believed to be a storage organ of brain neurosecretory material (Scharrer, 1952) of the cockroach cause a decrease in the spontaneous electrical activity of isolated ventral nerve cords *in vitro*.

To explain the loss of rhythmicity resulting from the surgical removal of these neurosecretory cells would presumably require that the neurosecretory material is either rhythmically produced, stored or released. The evidence of Klug (1958), demonstrating a correlation between the activity cycle of *Carabus nemoralis* M. with changes in the volume of the nuclei of the cells of the corpora allata and with changes in the number of neurosecretory cells in the brain which contain secretory granules, together with reports of similar findings in *Drosophila* (Rensing,

Figure 5. Abnormal activity rhythms in *Periplaneta americana* following removal of the pars intercerebralis (Group II). The operation was performed on day 0. The animals were sacrificed on day 17 (a) and 14 (b), respectively, and no neurosecretory cells were found in their brains. (a) The operation caused the activity period to extend as a single peak far into the end of the dark period. (b) This animal remained unimodal but its activity maximum switched from the dark to the light part of the entraining LD cycle.

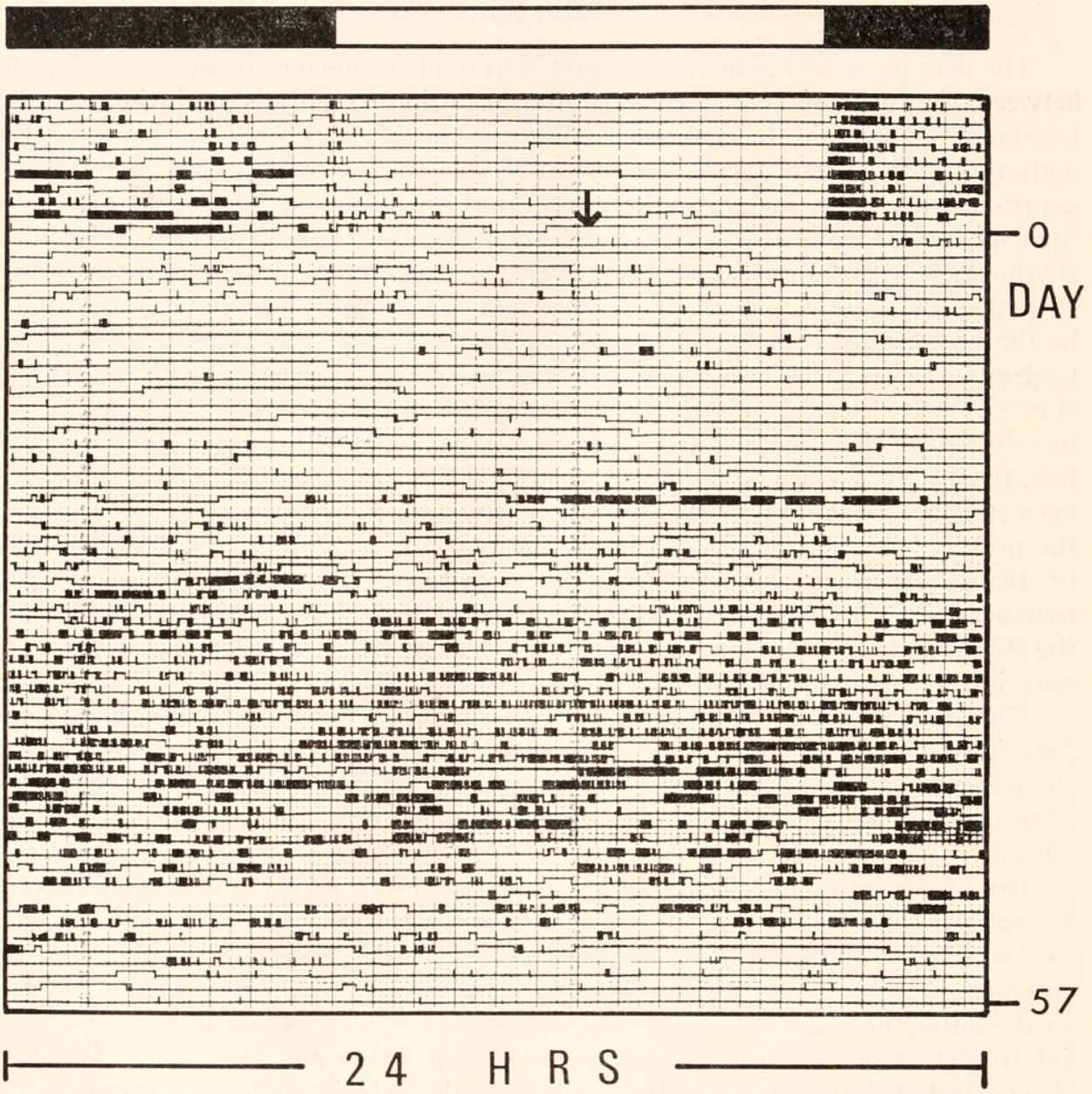

FIGURE 6. Effect of actinomycin D on the pars intercerebralis of *Leucophaea maderae*. Actinomycin D gel was inserted bilaterally into the pars intercerebralis on day 0. The animal showed low activity for 19 days after the operation, then activity increased to a very high level without showing any rhythm for 5 weeks. This activity pattern is similar to that of animals in which the pars intercerebralis was ablated (*cf.* Fig. 2). The animal died on the 57th day following gel insertion.

1964, 1966), suggests that rhythmic neurosecretion may be the mechanism operating in the cockroach. This mechanism is also favored on the basis of evidence in which no role could be demonstrated for the corpora allata and corpora cardiaca in the locomotory rhythm of cockroaches (Roberts, 1966), *Carausius* (Eidman, 1956) and *Romalea* (Fingerman *et al.*, 1958).

Roberts (1966) has concluded that a relationship exists between the neurosecretory cells of the pars intercerebralis and the locomotory rhythm in the cockroach on the basis of his midsagittal bisection through the pars intercerebralis, which caused a loss of the locomotory rhythm. Midsagittal bisection between the

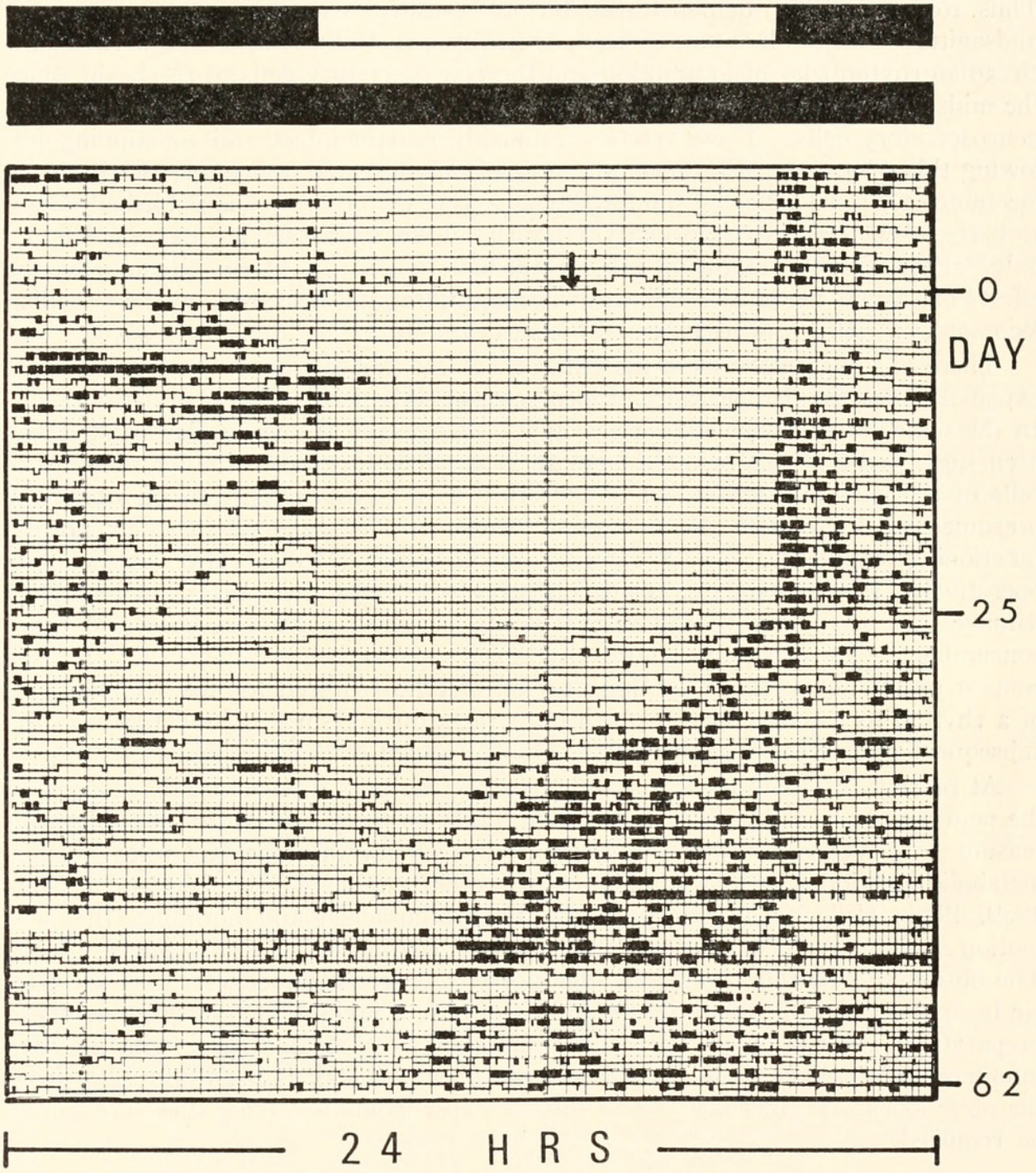

FIGURE 7. Effect of eosin control gel on the pars intercerebralis of *Leucophaea maderae*. Gel containing eosin instead of actinomycin D was inserted bilaterally into the pars intercerebralis on day 0. Within a week after the operation, the animal resumed its pre-operative rhythm and showed a free-running rhythm in DD (on day 25–62). Histological observation confirmed (on day 68) that the brain was normal except that some parts of the pars intercerebralis had been damaged and that none of the implanted gel could be detected.

two lobes of the protocerebrum severs the nerve tracts which emanate from the medial neurosecretory cells at the point where they cross to opposite sides. We have found that in post-operative animals in which only some lateral neurosecretory cells remained intact, normal rhythmicity could be re-established post-operatively.

Thus, Roberts' (1966) demonstration of post-operative arrhythmicity resulting from midsagittal bisection does not, of itself, constitute proof of a relationship between the circadian rhythmicity of locomotion and the neurosecretory cells of the brain, since the midsagittal bisection does not sever the nerve tracts emanating from the lateral neurosecretory cells. These tracts presumably remain intact and functioning following this operation. Furthermore, most of our operated animals showed normal locomotor rhythms following midsagittal bisection of the brain (unpublished data). Roberts, also, has shown normal rhythmicity in some cases. He has emphasized only "arrhythmicity" following midsagittal bisection of the brain. Results on the effects of various nerve sectionings, including midsagittal bisection, on the circadian locomotory rhythm will be reported in the near future.

There is a suggestion from the data in Table I that there may be an additional explanation for the reappearance of locomotory rhythmicity in the operated animals. In the majority of animals, rhythms appeared between 0 and 15 days and it has been suggested that this resulted from an incomplete removal of the neurosecretory cells of the pars intercerebralis. The return to a rhythmic pattern of activity is presumed to occur after the remaining neurosecretory cells regain their normal functioning capacities. In 3 cases, rhythms regenerated several weeks post-operatively (19, 20, and 24 days) which suggests that (in these cases) more drastic damage had been done to the system which required more elaborate and time-consuming repair processes before rhythmicity could be re-established. In this context one might attribute the long period of time required for the reappearance of a rhythm to damage done to the nerve tracts during surgery, and the need for subsequent regeneration of the tracts before a return of the rhythmicity.

At present, it is still unclear whether or not the pars intercerebralis—especially the neurosecretory cells—*directly* controls the locomotory rhythm for the following reasons: (1) Neurosecretory cells in the brain play several important roles in other metabolic functions; such as stimulation of protein synthesis (Thomson and Møller, 1959, 1963; Hill, 1962), triggering of the prothoracic gland hormone, the promotion of water retention and stimulation of oviposition (*cf.* Van der Kloot, 1960). The observed arrhythmicity might, therefore, be a secondary effect. (2) To date, we have examined histologically the brains of only 3 arrhythmic animals. In these preparations, most of the corpora pedunculata has been ablated along with the pars intercerebralis. Since these carry important fiber connections including those from the optic lobes (*cf.* Horridge, 1965) further experiments involving these areas may be required.

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Summary

1. Ablation of the region of the pars intercerebralis of the cockroach brain induced arrhythmicity of locomotory activity in the animal. Evidence is presented which demonstrates a relationship between the neurosecretory cells of the pars intercerebralis and the circadian rhythm.

a. Surgical removal of the pars intercerebralis, including the lateral and medial neurosecretory cells, results in arrhythmicity and an increased level of activity.

b. In a large number of cases where normal activity and rhythms regenerated post-operatively, neurosecretory cells could be demonstrated histologically to be present and presumably functioning.

2. The suggestion is made that the pars intercerebralis acts as a rhythmic suppressor of general locomotory activity resulting in rhythmic locomotory behavior.

3. In animals where rhythms "regenerate" several weeks post-operatively, the speculation was made that biological regenerative processes, such as regeneration of several nerve tracts of a few remaining neurosecretory cells, in addition to the general recovery from and adjustment to the brain surgery, are the time-consuming processes which must occur before rhythms manifest themselves.

4. Insertion of a gel containing actinomycin D into the pars intercerebralis induced arrhythmicity in the animal. However, when actinomycin D gel was implanted into the fifth abdominal ganglion normal activity and rhythm continued unchanged; when gel containing eosin instead of actinomycin D was inserted into the pars intercerebralis, rhythm regenerated post-operatively.

5. It is still unclear if the corpora pedunculata play some role in activity rhythms, and if secondary effects following ablation of the neurosecretory cells are responsible for inducing arrhythmicity in the animal.

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