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CONTROL OF THE CIRCADIAN RHYTHM OF ACTIVITY IN THE COCKROACH

I. THE ROLE OF THE CORPORA CARDIACA, BRAIN AND STRESS

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INTRODUCTION

In a series of papers from 1954 to 1960 Harker published the results of her work on the neuro-endocrine control of the circadian rhythm of locomotor activity in the cockroach, *Periplaneta americana* (see Harker, 1964). The most important conclusions of these papers are: that the rhythm is controlled by a neurosecretory cycle occurring in the sub-oesophageal ganglion and endogenously timed therein; that the continued running of this 'clock' is maintained by a supply of neurosecretion from the corpora cardiaca; and that it has a complex, restricted ability to phase-shift related to its control by a second clock which responds directly to light signals received by the ocelli. Recently three papers have been published by Roberts (1965*a*, *b*, 1966) which cast doubt on several of these conclusions. Failing to repeat some of Harker's work Roberts finds: that it is impossible to transfer rhythms by implanting sub-oesophageal ganglia, that the rhythm continues unchanged when the corpora cardiaca are removed, and that it is not the ocelli which are the relevant photo-receptors.

The work reported here was undertaken in an attempt to reconcile some of the conflicting results of these two workers, but it must be emphasized that only a partial reconciliation was effected. The present paper concerns work on the brain, the corpora cardiaca and the possible effects of stress on the expression of the rhythm. In a second paper (Brady, 1967) investigations into the part played by the sub-oesophageal ganglion and ventral nerve cord will be reported.

MATERIAL AND METHODS

(*a*) *Culture conditions*

The *Periplaneta americana* used in this study were purchased from the Zoological Society of London and kept in a large culture tank at approximately 28° C. under an artificial light cycle of LD 12:12 (i.e. light 12 hr.: darkness 12 hr.) with the LD transition occurring at 12.00 G.M.T. They were fed on crushed whole oats and apple. Adult males were used exclusively in the experiments.

Activity recording was carried out in the light-controlled and temperature-controlled incubators designed by Brown & Unwin (1961). The light phase of the LD 12:12 cycle was at approximately 10 lux (at the level of the actographs) and the dark phase at $< 0{\cdot}05$ lux, which was also the light intensity under which the animals were held in

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'constant darkness' (DD). The timing of the LD transition was normally close to that in the stock culture tank. The daily temperature control in the incubators was approximately $\pm 0{\cdot}5°$ C. under LD, and better under DD, but the control against ambient was not perfect so that over the course of a week the mean temperature inside the incubator might fluctuate between about 27° and 30° C.

(*b*) *Activity recording*

Activity was recorded in either a photocell box (Brown & Unwin, 1961) or a running-wheel (Roberts, 1960). The Brown & Unwin apparatus consists of a 2·5 cm. diameter infra-red beam directed along the floor (7 × 13 cm.) of a plastic box at a photocell the other end. This is so arranged that a cockroach walking across the beam causes changes in the light intensity incident on the photocell which thus generates pulses to activate a pen recorder. The light-beam consists of wavelengths longer than 720 mμ (Ilford filter no. 207), *Periplaneta* being sensitive up to 650 mμ (Burkhardt, 1964). The fundamental disadvantage of this photocell box actograph is that it cannot distinguish between different forms of activity: walking, feeding and preening all produce marks on the record, which therefore tends to be rather noisy (e.g. Fig. 2*d*). Furthermore, it is very difficult to set up the actograph identically every time, so that it is impossible to compare the intensity of different records accurately.

Three running-wheel actographs were used, essentially of the design by Roberts (1960). They were 15 cm. in diameter and 5 cm. deep, mounted on a double ball-race and therefore needing no counterweight. In two wheels the switching mechanism to activate the pen recorder was a double mercury pot shorted across by a wire fork attached to the wheel spindle, and in the third a cardboard vane cutting across a light-beam/photocell arrangement. In the former, one pen-recorder mark was made per revolution, and in the latter, two. The great advantage of the running-wheel over the photocell box is that it records locomotor movements only (unless the switch just happens to be in the critical position, when feeding or drinking may produce sufficient movement of the wheel to make a record mark). Since any noise relates almost entirely to locomotor activity, rhythmic animals produce very clear records (e.g. Fig. 1). The differences between these two types of actograph are examined in the Discussion.

(*c*) *Operative techniques*

Corpora cardiaca were removed by the following procedure. The CO_2-anaesthetized animal was held down in plasticine with a rubber band stretched across its neck to prevent bleeding, and the head was held forward with a celluloid yoke (cut from old film). A micro-drip of saline was used and the operations performed with fine tungsten needles and forceps. Removal of the major part of the corpora cardiaca, and severance of the nerves (NCA II) connecting them to the sub-oesophageal ganglion, was effected through the dorsal side of the neck. In order to make a complete removal of all cardiaca tissue it was found necessary first to cut the three pairs of nerves (NCC I, II, III) connecting the brain to the corpora cardiaca. This was done with tungsten hooks through a hole in the frons, before the neck was opened. The corpora allata were left intact. Operational wounds were not sealed with wax: *Periplaneta* blood is extremely efficient at clotting and it was found that the application of hot wax did more harm than good.

Neurosecretory cells were destroyed *in situ* by means of a microcautery using a radio frequency oscillator (designed by J. A. Popple). This could be set to burn out areas less than 100 μ in diameter. The medial neurosecretory cells in the brain were reached through a hole cut between the eyes, the animals being held down in plasticine with a rubber ligature as above.

Autopsies were carried out on operated animals where indicated in the text. They consisted of very careful dissection combined with histological examination. Neurosecretory tissue was identified by the use of paraldehyde-fuchsin (PF), following the staining schedule of Cameron & Steele (1959) with minor modifications. Tissue was fixed in aqueous Bouin and sections were cut at 10 μ. Neurosecretory cells are here defined as being neurons which stain purple with PF.

RESULTS

Removal of the corpora cardiaca

Because of the conflict between the views of Roberts (1966) and Harker (1960) on the involvement of the corpora cardiaca in the rhythm, and because Roberts (1966, p. 475) did not perform autopsies, it was decided to reinvestigate this problem briefly. Ten trials indicated that complete removal of all corpora cardiaca tissue by a single operation through the back of the neck was almost impossible: autopsies showed that small parts of the cardiaca were left attached to the posterior ends of the nerves (NCC I, II, III) from the brain. However, in four animals the great majority of the cardiaca tissue was removed, and the two nerves (NCA II) connecting with the sub-oesophageal ganglion were severed.

In the first of these the rhythm disappeared after the operation for about 2 weeks and then suddenly reappeared. In the second animal the rhythm was clear and intense by the time it was placed in the running-wheel 19 days after the operation. Although these two animals were recorded in LD after the operation, both showed the typical gradual re-entrainment of the rhythm to the different LD transition time in the actographs (Fig. 1 *a*, *b*), for 2 days in BB.4 and for 8 in BB.5. This indicates that their activity was indeed being endogenously rhythmic and was not merely responding to the light. Histological examination of these two showed that a large haemocyte clot had formed round the cut ends of the little remaining corpora cardiaca tissue. To avoid the possible effects of regeneration that this implied (as described by Stumm-Zollinger, 1957), and any possible direct influence of a cyclical environment, the third and fourth animals were first recorded in LD and then placed in DD after the operation. In the third animal the rhythm carried over into a free-run with little break, but with a marked reduction in the amount of peak activity (Fig. 1 *c*). Autopsy showed that the great majority of the cardiaca tissue had been removed, but again that there were still small portions left, though this time with no clots round their cut ends.

A high mortality resulted from the double operation to remove the corpora cardiaca completely (see Methods), but the fourth animal survived this multiple trauma and remained clearly rhythmic in DD for 8 days between operation and sacrifice, though with an 8 hr. delay shift in the timing of the rhythm (Fig. 1 *d*). Autopsy revealed that absolutely no intrinsic cardiaca tissue remained, the NCC having been pulled out by their roots, and that the NCA II had been successfully cut distal to the corpora allata

which were left intact; the neurosecretory cells in the brain and the cut ends of the NCC contained very little material staining with PF.

Removal of the medial neurosecretory cells

The lack of effect on the rhythm of removing the corpora cardiaca left the possibility, specifically implied by Roberts (1966), that the source of activity control might centre in the neurosecretory cells of the brain. Attempts were therefore made to remove these cells, test for subsequent rhythmicity in DD and then examine the brains histologically to determine how much neurosecretory tissue had been removed.

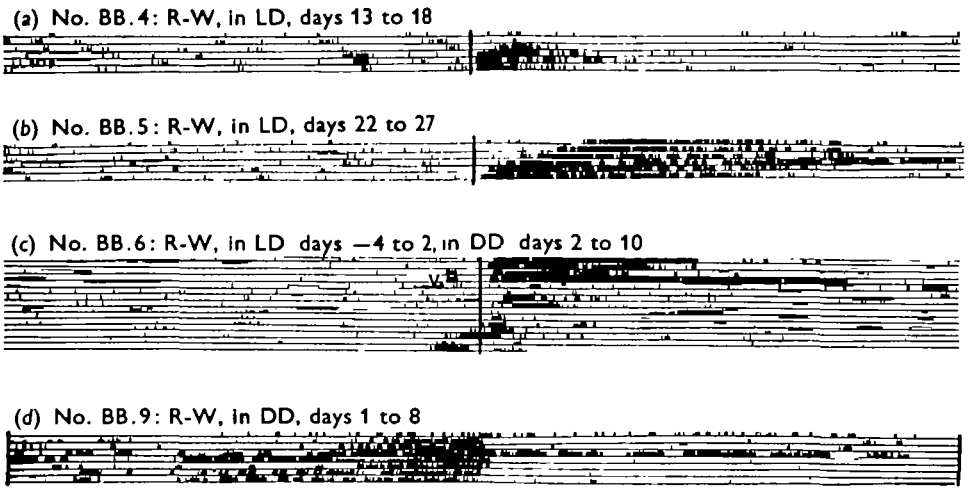

Fig. 1. Activity records of *Periplaneta* whose corpora cardiaca have been removed: (*a–c*) with the majority of the cardiaca removed, (*d*) with the cardiaca removed in entirety. Thick vertical line at LD transition (in LD) or previous transition (in DD). R-W, running-wheel actograph. Day 0 = day of operation (at V). Each horizontal line represents 24 hr. activity, successive days are displayed in order one beneath the other.

The cells were destroyed *in situ* by micro-cautery in 37 *Periplaneta*; 2 died soon after, 7 were made apparently arrhythmic, 12 were left possibly rhythmic and 16 clearly rhythmic. Of these 16 rhythmic animals 13 were examined histologically; 2 had the majority of their medial neurosecretory cells left intact, the remaining eleven are described in Table 1, and examples of their post-operative records in DD are illustrated in Fig. 2.

The medial neurosecretory cells were counted in the pars intercerebralis of eight healthy intact male *Periplaneta* and the mean number found to be 310 (S.E. ± 14). The counting was done with care on specimens stained with PF, and re-counts on five of the eight came to within 5 % of the original count. The great majority of the neurosecretory cells in the pars intercerebralis of cockroaches normally contain very much PF-positive material and their classification as neurosecretory is therefore easy. Some, however, are always difficult to classify because their faint PF reaction may lead to confusion with the many other brain cells which have small amounts of PF-positive material in them. Such cells in the pars intercerebralis were counted as neurosecretory if they contained more than twice the amount of PF-positive material found in neurons from parts of the brain which are generally presumed to be non-neurosecretory.

A further complication arises in cauterized animals when the brain becomes much invaded by haemocytes. These are frequently PF-positive (Willey, 1961), but considerable experience in the examination of healthy brains stained with PF showed that they are probably distinguishable from neurosecretory cells by their smaller size and by the form of PF-positive material which they contain. These considerations, plus the individual variability indicated by the standard error, make the figures in Table 2 only approximate except in the last two cases where, to the best of the author's belief, there remained intact only two and one cells, respectively.

Table 1. *Post-mortem examinations on the brains of* Periplaneta americana *remaining rhythmic after cauterization of their medial neurosecretory cells (MNC)*

Animal no.	No. of days between operation and sacrifice	Type of actograph*	No. of MNC left apparently intact	Approx. percent of normal no. of MNC
BB 13	13	R-W	c. 75	25
BB.38	6	R-W	50–60	20
B.24	9	R-W	50–60	20
A.90	6	P-B	40–50	15
BB.11†	9	R-W	c. 30	10
B.51	6	P-B	10–20	5
BB.12†	7	R-W	10–20	5
C 42†	6	P-B	c. 15	5
AA.79	6	R-W	c. 10	3
CC.28†	23	P-B	2	< 1
CC.31	6	R-W	1	< 0·5

All classes of neurosecretory cell in the pars intercerebralis have been totalled. The mean number of these cells in normal healthy *Periplaneta* is 310 ± 14 (see text).

* P-B = photocell box; R-W = running-wheel.

† Activity record illustrated in Fig. 2.

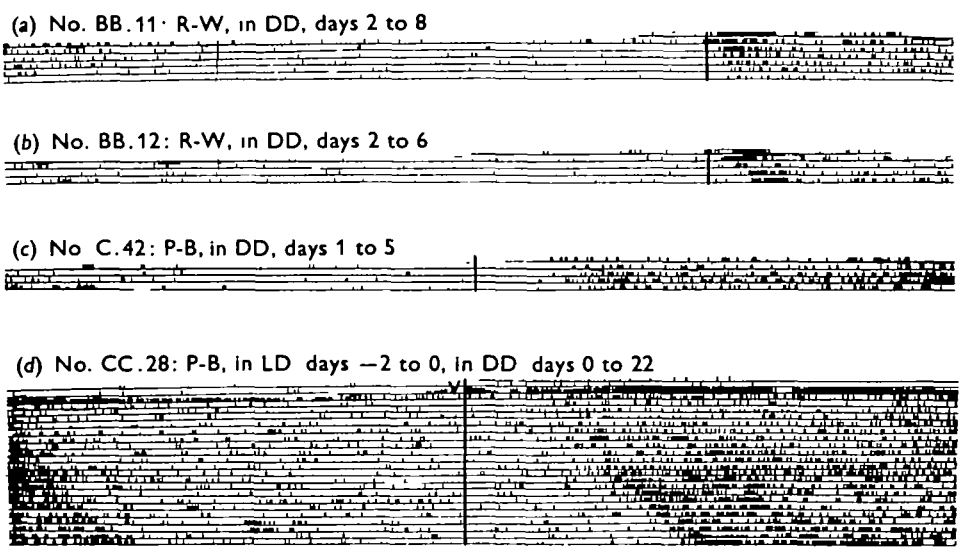

Fig. 2. Activity records of *Periplaneta* remaining rhythmic in DD after cauterization of the majority of the medial neurosecretory cells in their brains. R-W, running-wheel; P-B, photocell box. Other details as in Fig. 1. See Table 2 for details of autopsies.

The effect of stress on the expression of the activity rhythm

It was found that removing cockroaches from their actographs and subjecting them to very short periods of enforced activity, by chasing them round the inside of a large glass jar with one's fingers for 1–2 min., markedly increased subsequent peak activity. Eight trials of this were performed on four animals. In seven trials the animals were in the entrained steady-state (though two had experienced a 4 hr. delay in LD transition 5 days previously), but in one case the animal was phase-shifting to an earlier LD transition. The amount of peak activity was assessed as the number of pen marks on the first 2 hr. of record after the LD transition for the seven entrained animals, and on 2 hr. of record centred on the apparent peak for the shifting animal. In each case a photocell box recorder was used.

The results, expressed as a percentage of the mean activity during the 7 days' peaks for each individual, are shown in Fig. 3. It will be seen that the activity on day 0 (= day stressed) is considerably greater than on either the previous or the subsequent 3 days (using a t test with Bessel's correction the least difference, between day 0 and day +3, is probably significant; $P < 0{\cdot}05$). There is some indication that activity was still increased on the day after stress, since the mean on day +1 is statistically greater than the mean on day −1 ($P < 0{\cdot}05$). It also appears that peak activity is more

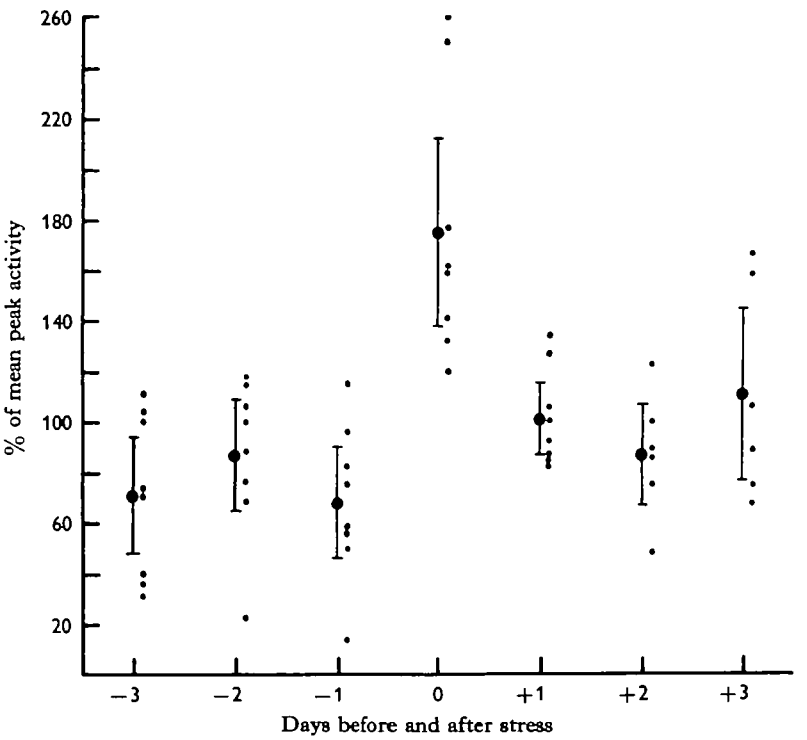

Fig. 3. The increase in peak activity following mild stress. Cockroaches were subjected to brief periods of enforced activity (see text) between 2½ and 19 hr. before the LD transition on day 0. Peak activity is assessed as the number of recorder-pen marks during the first 2 hr. after the LD transition, and is expressed as a percentage of the mean of the seven peaks measured for each individual. Vertical lines show the means for each day (plus twice their standard errors) of the eight sets of individual data shown as dots.

affected than background activity, since the activity during the peak 2 hr. on day 0 was 175 ± 19 % of mean peak, whereas the background activity during the remaining 10 hr. of darkness on day 0 was 120 ± 8 % of mean background.

A precisely contrary effect is produced by the presumably more severe stress involved in the collection of a small blood sample—as in a haemocyte count, for example. When a cockroach thus suffers a minor wound by having its coxa pricked and losing 10–20 μl. of blood, the subsequent activity peak is usually greatly reduced. Some sixty trials of this were carried out on sixteen animals during a study of cockroach blood components. Nearly all the records were taken in running-wheels and the density of pen marks was far too great to permit accurate quantitative estimates. However, simple visual assessment indicated that, although about twenty cases showed relatively little change in activity following the collection of a blood sample, about forty showed a clear decrease; in only two cases did the activity increase. In eight out of nine cases where the record was sufficiently light to permit reasonable quantitative estimates of the decrease, the activity was also reduced on the second day after wounding—in much the same way as the reverse occurred after mild stress. Also, in the same eight cases out of the nine, the amount of decrease in the peak activity was greater than the amount of decrease in the background.

It seems reasonable to conclude that (*a*) stress has a distinct affect on activity, (*b*) this effect is complex and, according to its nature, can either reduce or increase the amount of activity, (*c*) the effect is prolonged and may still affect activity after an interval of more than 24 hr., (*d*) the effect is greatest on that part of activity which is expressed rhythmically, i.e. peak activity.

DISCUSSION

The involvement of the corpora cardiaca

Harker states (1960) that the pair of nerves connecting the corpora cardiaca to the sub-oesophageal ganglion (NCA II) are an 'integral part of the timing mechanism'. My own four results have confirmed Roberts's more extensive data (1966) showing that cockroaches exhibit unimpeded rhythmicity for weeks in the absence of these nerves. Clearly, although it may be involved in some aspects of activity, this pair of nerves is not integral to the timing thereof in otherwise intact animals. The difficulty experienced in removing the cardiaca completely, plus the fact that Roberts did not perform autopsies, suggests that it may be slightly premature to conclude that the corpora cardiaca play no part in the control of the rhythm. But in a single animal, however, it has been possible to show categorically that the rhythm continued in the absence of any cardiaca tissue. The most likely answer to this conflict with Harker's results seems to lie in the influence of the type of actograph used.

Harker used photocell boxes in which, in the author's experience, the majority of cockroaches show a much more marked activity peak during the first day or two than subsequently. After about a week the record frequently suggests that peak activity is only slightly higher than background noise, but when placed in running-wheels such animals usually immediately revert to indicating a clear rhythm. A similar effect has been observed by Harker in *Blaberus* (1964). This fading of the record in photocell boxes thus apparently indicates a reduction in the *amount* of peak activity but not a

change in the *periodicity* of it. The running-wheel as used by Roberts rarely indicates such fading; he has published many long-term records of largely unchanging rhythms, one of which (1960) lasted for at least 13 weeks in DD. In a running-wheel the intensity of peak activity relative to background is usually ten or more times greater than in a photocell box; perhaps an element of positive feedback from mild stress caused by the moving substratum enforcing the activity is involved in this.

The most obvious difference between the photocell box and the running-wheel is that the former provides a small, enclosed, static environment, whereas the latter provides a moving one which is limitless in the sense that it is impossible to come to the end of a treadmill. Clearly these two types of actograph are both very artificial environments, and the cockroach presumably derives widely differing sensory information from them. It may well be that when the animal's clock mechanism dictates a commencement of activity the first few steps in a photocell box provide it with virtually no new stimuli so that no further behaviour patterns are initiated. In the running-wheel, on the other hand, the first few steps must provide stimuli from the movement of the wheel, and these may initiate a chain of running sequences each of which is commenced in an attempt to reach a static substratum.

Other workers have shown that the corpora cardiaca of the cockroach produce secretions which can be released by enforced locomotor activity, and which influence impulse discharge frequency in the nerve cord, the general locomotor behaviour, and the heart-beat frequency (Cameron, 1953; Ozbas & Hodgson, 1958; Hodgson & Geldiay, 1959; Milburn & Roeder, 1962; Davey, 1963). Taken together with Harker's observations on the effect of severing the only direct neurosecretory fibre connection between the corpora cardiaca and the ventral nerve cord, these results may be taken as good *prima facie* evidence for the implication of the cardiaca in the control of at least some aspects of activity.

Hodgson (1962) suggests that stress, hyperactivity and subsequent quiescence may be interlinked via a feedback mechanism involving the brain, a corpora cardiaca hormone, and the central nervous system. Such a hypothesis receives possible support from the observations reported above on the differential effects on activity of mild and more severe stress. Since these effects appear to last for at least two activity cycles, it suggests that a hormonal mechanism may be involved (see Fig. 3). More direct evidence of corpora cardiaca involvement in activity control comes from recent unpublished observations on the locust by Ellis and Carlisle. These workers have found that injecting the equivalent of 2 corpora cardiaca per animal into well-fed hoppers stimulates marching, whereas injection of 4 corpora cardiaca per hopper results in an inhibition of marching (Dr Peggy E. Ellis, personal communication). It is of interest that in the cockroach the effect of stress is more marked on the typically rhythmic part of activity, the peak, than on the background noise. If the corpora cardiaca are involved in the changes of activity following stress, then the normal route for their influence may be via the nerves, NCA II.

It is perhaps apposite here to emphasize that an operation which causes a loss of apparent rhythmicity is essentially a negative result and cannot by itself prove that a vital part of the circadian system has been directly affected. Harker states (1960) that when she cut the NCA II the rhythm faded in about a week. If one of the normal functions of these nerves is to deliver some sort of activity-augmenting substance to

the ventral nerve cord, then the apparent fading of the rhythm which Harker observed might merely have been the result of a loss of activity in a photocell box, as described above, rather than a loss of rhythmicity. Such a loss of activity in the absence of these nerves would not be expected to occur in a running-wheel if the sensory input was sufficiently intense to override any lack of endogenous stimulus. The nature of this hypothetical activity-augmenting secretion (if such it is) is quite unknown, but its axonal delivery to the sub-oesophageal ganglion via the NCA II is clearly not necessary for the normal expression of both activity and rhythm in a running-wheel. This is not to suggest that the corpora cardiaca play no part in the control of activity; on the contrary, the results of stress and the work of other authors (see above) imply that they probably do. The evidence does suggest, however, that they play no part in the clock mechanism itself.

The involvement of the medial neurosecretory cells of the brain

Roberts's experiments to show that the neurosecretory cells of the pars intercerebralis are involved in the control of the rhythm took the form of bisecting the brain through this area. The result of this operation was to make the animals arrhythmic and, judging by his three published records, hyperactive. The operation must have severed all the nerve tracts linking the two halves of the brain and in particular the central body which is known to play an important role in integrating the bilateral co-ordination of locomotion (Huber, 1965). Those fibres to the ventral nerve cord which cross over from one side of the brain to help co-ordinate the reflexes of the other side of the body must likewise have been cut. If there is any cerebral part of the clock mechanism, it is perhaps not surprising that such a comprehensive operation might upset it. It is interesting to note that cutting the circumoesophageal commissures has a similar overt effect on activity (Brady, 1967). Roberts's control operation, on the other hand, only involved bisecting the brain to one side of the pars intercerebralis. This would have left the entire system on one side intact, with its links to the central body and to both sides of the ventral nerve cord uninterrupted. The relevance of this operation as a control seems to be rather limited.

It would appear to be risky to draw the inference from this experiment that Roberts suggests, namely that the neuro-secretory cells of the pars intercerebralis play a 'critical role in the maintenance of insect locomotor rhythms'. In support of his hypothesis that it is these cells which are involved he quotes Rensing's findings (1964) that there are daily changes in the medial neurosecretory cells of *Drosophilia*, but he does not mention that later Rensing, Thach & Bruce (1965) also showed daily changes in the corpora allata which he himself had just shown to be not involved. Nor does he mention that they even found daily changes in the fat body cells.

The examples of post-operative records illustrated in Fig. 2, when examined in conjunction with the data in Table 1, show that cockroaches are capable of retaining a normal rhythm after a massive reduction in the number of the medial neurosecretory cells in the pars intercerebralis. Most animals were sacrificed within about a week of cautery to prevent confusion from possible regeneration, and it is conceivable that sufficient extrinsic neurosecretion could have been stored in their corpora cardiaca to tide them over this period. In CC.28, however, the rhythm was improving, in the sense that the peak activity was increasing, up to 23 days after the operation (Fig. 2*d*),

and this animal retained only two intact neurosecretory cells at this stage. This suggests that storage of secretory material in the corpora cardiaca is not important.

If many of the medial neurosecretory cells are involved in controlling the rhythm, then a reduction in their numbers to less than 5% of normal might be expected to affect the post-operative activity, at least to the extent of increasing noise. This did not occur to any significant degree (e.g. Fig. 2*b*, *c*). Alternatively, if only one or two specific cells are involved, it seems extraordinarily unlikely that the same ones should have remained undamaged in both CC.28 and CC.31. It therefore appears probable that *Periplaneta* can remain rhythmic in the absence of all of its medial neurosecretory cells.

The micro-cauterization was performed with care and only on that part of the protocerebrum where it was possible to see the medial neurosecretory cells in the living tissue. They occur in two groups, about $100 \times 300\ \mu$ across, one each side within the pars intercerebralis. When the part burnt out was extended beyond these two small areas the result was frequently a loss of rhythm. One of the seven animals thus made arrhythmic was autopsied. It showed every sign that large areas of the brain were degenerating and were invaded by haemocytes. These findings emphasize once again the dangers involved in assuming that a vital part of the clock has been revealed when the result of an operation is to cause a loss of rhythm. Roberts reports (1965*a*) unpublished observations by Nishiitsutuji-Uwo that cautery of the pars intercerebralis of *Leucophaea maderae* causes a loss of rhythm. Unless the neuro-anatomy of this species is radically different from that of *Periplaneta americana* it seems likely that the explanation of her results may be sought along the lines just indicated, as may also the results of Roberts's bisection experiments.

Finally it must be pointed out that it was only the medial neurosecretory cells which were destroyed. Neither the lateral neurosecretory cells nor the 'C cells' of the tritocerebrum (Raabe, 1963), or any of the many other brain cells containing sparse PF-positive inclusions, were investigated. If a hormone from the brain is involved in the control of the rhythm then all of these secretory cells must remain possible candidates for its source. But apart from the work of Raabe, which does not strictly concern rhythm control, there is in any case no direct evidence that the brain is hormonally involved in the rhythm (Harker, 1956).

SUMMARY

1. Harker has reported (1960) that the locomotor rhythm in the cockroach is maintained only if a secretion from the corpora cardiaca is delivered to the sub-oesophageal ganglion via the pair of small nerves connecting these two organs. Roberts has counterclaimed (1966) that neither this nerve nor the corpora cardiaca themselves are concerned in the control of the rhythm.

2. It has been possible to confirm Roberts's findings that these nerves can be severed, and much (possibly all) of the corpora cardiaca removed, without affecting the periodicity of the rhythm.

3. Brief periods of enforced activity are shown to increase peak activity in the rhythm, whereas stress in the form of minor wounding depresses it.

4. Cockroaches are found to maintain rhythmicity after the almost complete elimination of the medial neurosecretory cells from their brains.

5. It is suggested that the differences between the views of Harker and Roberts concerning the influence of the corpora cardiaca may be explained in terms of the type of actograph used and the possibility that a cardiacum secretion controls the *amount* of activity but not its *periodicity*.

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