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EXPERIMENTAL PRODUCTION OF MIDGUT TUMOURS IN *PERIPLANETA AMERICANA* L.

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(With Plate 8)

INTRODUCTION

In earlier papers (Harker, 1955; 1956) it has been shown that the sub-oesophageal ganglion of *Periplaneta americana* L. undergoes a 24 hr. rhythm of secretory activity to which is related the 24 hr. rhythm of locomotor activity. Furthermore, the sub-oesophageal ganglion retains its secretory rhythm even when it is implanted into the abdomen of another animal, and the animal into which it is implanted will take up a locomotor activity rhythm in phase with the secretory rhythm of the implant, provided that the animal is not already showing a strong rhythm.

Now that this autonomous 'clock' has been found it may be possible to upset the cycle of 24 hr. to which the activity rhythm seems bound, and this might be done by continuous stimulation to activity through the ganglion secretion. Since the factors which control the secretory rhythm are change from light to darkness and the length of time spent in each of these environments within 24 hr. cycles, it is not possible to bring about continuous stimulation through the animal's own sub-oesophageal ganglion; but implanted ganglia can be used to provide a secretion during the inactive phase of the animals.

In the following pages the effect of this secondary secretion on the tissues of the alimentary canal is described. The effect on the locomotor activity rhythm will be discussed in another paper.

METHODS

Adult *P. americana* of both sexes were used in the experiments. Animals were kept in alternating light and darkness, of periods of 12 hr. each, for at least 3 weeks before the experiments were begun, and the locomotor activity rhythms were recorded to ensure that the timing of the phases of the rhythm was known. Cockroaches hereafter described as having a normal 24 hr. activity rhythm were kept in darkness from 10 p.m. to 10 a.m., those described as having a reversed rhythm were kept in darkness from 10 a.m. to 10 p.m.

Prior to the implantation of sub-oesophageal ganglia a small polythene ring was sealed with wax around a circular incision on the dorsal surface of the abdomen of each cockroach; the haemolymph flowed into the ring. The sub-oesophageal ganglia were then inserted, as rapidly as possible after dissection, and the ring was sealed with a fragment of cover-slip. It was found that the cuticle grew up the sides of the

ring rather than across the wound, so that renewal of the implant did not necessitate damaging the cuticle repeatedly. Great care was taken in making the cuticular incision to ensure that it did not penetrate the muscle layer beneath the cuticle.

EFFECT ON THE GUT OF IMPLANTED SUB-OESOPHAGEAL GANGLIA

Sub-oesophageal ganglia removed from cockroaches known to have a reversed activity rhythm were implanted, one each, into thirty-five cockroaches showing normal activity rhythms. The implants were replaced by fresh ganglia every 24 hr. at 10.0 a.m., that is at the time of change of light conditions. The implanted ganglia were sectioned after removal and it was found that they had not been encapsulated by blood cells and that the neurosecretory cells appeared to be in a normal condition.

In this series of experiments the cockroaches receiving implants were supposedly receiving secretions from the sub-oesophageal ganglia in two phases, in one phase from their own ganglion, and in the other from the implant.

The cockroaches were divided into groups of five animals, and implantation was continued for from 1 to 4 days in different groups. The treatment and the time of killing are given in Table 1. The alimentary canal of each animal was examined, and it was found that a small swelling appeared in the midgut of many. The guts of all animals were sectioned and the swellings were found to be small tumours; detailed descriptions of these are given in the next section.

Table 1. *Sub-oesophageal ganglia taken from cockroaches with reversed 24 hr. locomotor activity rhythms and implanted into cockroaches with normal rhythms.*

Groups of five animals	Days									
	1	2	3	4	6	8	10	12	14	16
A	Implanted	Implanted	Implanted	Implanted	—	Killed, no tumour	—	—	—	—
B	Implanted	Implanted	Implanted	Implanted	—	—	Killed, small tumour in two animals	—	—	—
C	Implanted	Implanted	Implanted	Implanted	—	—	—	Killed, small tumour in two animals	—	—
D	Implanted	Implanted	Implanted	Implanted	—	—	—	—	Killed, tumour in all animals	—
E	Implanted	Implanted	Implanted	Implanted	—	—	—	—	—	Killed, tumour in all animals
F	Implanted	Implanted	—	—	—	—	—	—	—	Killed, tumour in three animals
G	Implanted	—	—	—	—	—	—	—	—	Killed, no tumours

It can be seen from Table 1 that when implantation was performed from two to four times the recipient cockroaches, in all but one case, formed tumours by the sixteenth day from the beginning of the experiment. The tumours all appeared in the midgut, irrespective of the region of the abdomen in which the implant was made. To confirm the lack of dependence of the position of the tumour on the

position of the implant, implantation into the thorax was performed daily for 4 days on three cockroaches; at the end of 18 days a tumour was found in the midgut of each animal.

As a control similar implantations were made into two groups of five animals each, but, instead of sub-oesophageal ganglia, brains were implanted in one group and corpora allata in the other. Implantation was repeated for 8 days in each case. There was no sign of tumour formation, after 28 days, in any of the animals.

Sub-oesophageal ganglia taken from cockroaches showing normal activity rhythms were implanted into ten cockroaches which also had normal activity rhythms. In this series of experiments the recipient cockroaches were supposedly receiving secretions from both sub-oesophageal ganglia at the same time, that is, during only part of the 24 hr. Implantation was continued daily for 4 days in five animals, and for 8 days in five animals. None of the animals showed any sign of tumour formation, even after 28 days from the beginning of the experiment.

Extracts of sub-oesophageal ganglia were prepared by grinding a single ganglion in 0·05 ml. of Ringer solution, and a fresh extract was injected into the abdomen of each of five cockroaches during the inactive phase. This was repeated daily for 12 days. No tumours were formed in any of these animals. Similar extracts were prepared using five ganglia in 0·05 ml. of Ringer solution, and these were injected daily for 5 days into five other animals. After 21 days three of the cockroaches were found to have a small tumour in the midgut. An extract of this strength (five ganglia in 0·05 ml. Ringer) injected into five cockroaches every day during their active phase, and repeated for 5 days, did not produce tumours in any of the animals.

Previous experiments (Harker, 1956 and unpublished) have shown that, although phases of the locomotor activity rhythm of *Periplaneta* can be determined by the phases of the light:dark rhythm to which they are subjected, not all the processes in the animal which undergo a 24 hr. rhythm are controlled in this way. For instance, there is a variation over 24 hr. in the type of reaction of headless cockroaches to injections of sub-oesophageal ganglia extracts, and the type of reaction appears to be related to the time of day and not to the light:dark treatment, nor to the resultant locomotor activity rhythm of the animal before decapitation. It seems likely then that, although the phases of the locomotor activity rhythm are reversed in cockroaches which have been subjected to reversed light and darkness, the phases of some metabolic rhythms persist in the normal form. If this is so, cockroaches might show some variation at different times of day in their sensitivity to implanted sub-oesophageal ganglia; the following experiment was performed to test this hypothesis.

Five cockroaches were kept in reversed light and darkness for 3 weeks; their locomotor activity rhythms were found to be reversed. Into these cockroaches were implanted sub-oesophageal ganglia taken from animals with normal activity rhythms; implantation was repeated daily for 4 days. No tumours had formed in these animals by the twentieth day. The experiments were repeated, this time the treatment was continued for 8 days. Two of the animals had formed tumours in the midgut by the twentieth day. In this experiment the secretory activity of the

sub-oesophageal ganglia is supposedly similar to that in the previous experiments, yet the incidence of tumours is lower and treatment had to be continued for a longer period before tumours were formed. It seems likely that either the gut shows a rhythm of sensitivity to sub-oesophageal secretion, a rhythm which is not reversed by reversed light:dark conditions, or that some other organ affected by the sub-oesophageal ganglia secretion and involved in tumour production has a rhythm independent of light:dark conditions. In either case it appears that *Periplaneta* is not as sensitive to the sub-oesophageal ganglion hormone at the time when the hormone would normally be present as at the time when normally none would be present.

HISTOLOGY AND THE DEVELOPMENT OF THE TUMOURS

The histology of the normal midgut of *P. americana* has been described by Day & Powning (1949). There is an outer layer of scattered strands of longitudinal muscle, inside of which run the circular muscles. Between the inner epithelium and the circular muscles is a distinct region of connective tissue. The epithelial cells are separated into groups, more clearly so at the anterior end of the midgut, by the presence of nidi of regenerative cells; the cells of the nidi replace degenerating epithelial cells, and these degenerating cells are always those furthest from the nidi (Pl. 8 (i)). In the normal midgut the nidi are evenly distributed and the number of epithelial cells between nidi is practically constant. The number of cells in each nidus in well-fed animals is also fairly constant. Counts were made of these cells and of the number of mitoses per twenty-five nidi in the normal midgut; the results appear in Table 2.

Table 2

	Normal midgut	Midgut of treated animals		
		2 days after implantation	4 days after implantation	8 days after implantation
Mitoses per 25 nidi, means of 10 counts	8·1	18·0	18·3	Nidi not clearly defined
Cells per nidus, means of 50 counts	8 (8–10)	16 (16–18)	16 (16–18)	Nidi not clearly defined
No. epithelial cells between nidi, means of 50 counts	16	28	30	30

Histological examination was made of the midguts of cockroaches fixed at 24 hr. intervals from the beginning of experiments in which sub-oesophageal ganglia from insects with reversed rhythms were being implanted into insects with normal rhythms. From 2 days after the beginning of the experiments areas of tissue could be seen in which the number of mitoses had increased and in which the clear pattern of cells had become slightly distorted (Pl. 8 (ii–iv)). The following descriptions apply only to these regions. Counts were made of the number of mitoses per twenty-five nidi, the number of cells in each nidus, and the number of epithelial cells between neighbouring nidi. It is difficult to delimit with any certainty the region

affected, and the numbers given in Table 2 cannot be taken as accurate; where they err it will be because normal cells have been included and so the numbers will be low.

From the results appearing in Table 2 it can be seen that the number of mitoses increases rapidly after treatment, more than doubling after 2 days; the number of cells in each nidus also increases, but it appears that some cells are moving up into the epithelium. Seven days after the beginning of treatment, in the majority of cases, there is a double line of cells in the epithelium (Pl. 8 (ii), (iv)); in determining this, it is necessary to ensure that sections are cut at right angles to the main axis of the gut, and that the region of suspected tumour formation is not at either end of the midgut, where a double line of epithelial cells may normally be present.

At the same time as the mitotic activity increases, that is from the second day, cells from the nidi are seen to have moved into the connective tissue layer adjacent to the muscle layer; it appears to be these cells which form the tumour. After 14–18 days the epithelial cells begin to break down (Pl. 8 (iv), (v)); the tumour then consists of small cells with very little cytoplasm and which stain densely with haematoxylin; these cells form a whorled pattern and a number of tracheae run between them.

Small metastases have been found in eight cockroaches after 24–30 days; they appear in other regions of the midgut, the foregut and hindgut and in the salivary glands (Pl. 8 (vii), (viii)). The metastases may arise from cells moving in the connective tissue layer of the gut as well as in the haemocoele, for cells appear to migrate in both.

Transplants of small portions of the tumours, about 0·5–1·0 mm.^{2}, were placed in the haemocoele of twenty cockroaches. The implants from two animals were examined after 2 days and were seen to be surrounded by a sheath of blood cells. Two cockroaches were killed every 2 days for 14 days, and it appeared that the transplanted portions had increased in size; however, after 4 days it is difficult to differentiate between the surrounding blood cells and the transplanted cells. Of the remaining cockroaches, two were examined at 20, 24 and 28 days respectively; three of these six animals had developed tumours in the midgut, and one in the hindgut.

Small portions of normal gut tissue approximately equal in size to the transplants of tumour tissue were implanted into ten control animals. None of these animals showed any sign of tumour formation after 28 days.

The sequence of tumour formation was examined in three groups of four animals into which tumour tissue had been transplanted 8, 14 and 28 days previously. The sequence appeared to differ in some details from that of primary tumour formation. Eight days after the transplantation had been made cells were seen to be invading the connective tissue layer below the nidi of the midgut; the width of the connective tissue layer increased about fourfold in the next 5 days (Pl. 8 (ix)), and it was not until this stage was reached that the epithelial cells (in all but one animal) showed any abnormal appearance. In the one exception the epithelial cells had begun to degenerate by the eighth day. The nidi had lost their normal form by the eighth day in two animals, and in all four of the animals killed on the fourteenth day. The nidi of the surrounding tissue showed no increase in mitosis until after the epithelium

had begun to degenerate; in this type of tumour formation it appears that the nidi do not supply cells to the tumour, but only carry out their normal function of replacing epithelial cells. After the epithelium had begun to break down, that is, between the eighth and fourteenth day, blood cells began to accumulate along the muscle layer and these added superficially to the size of the swelling. The swelling at this stage is invaded by tracheae.

In insects swellings due to wound healing have something of the appearance of malignant growth (Day, 1952; Wigglesworth, 1937), but that neither the primary nor transplanted tumours are swellings of this type appears evident (i) from the control experiments, (ii) from the fact that in the formation of primary tumours the site of implantation is not related to the region in which the tumour is formed, and (iii) from the evidence of increase in mitotic activity of the cells of the nidi.

TEMPORARY CONTROL OF THE TUMOURS

Ten groups of three cockroaches were implanted daily for 4 days. Sub-oesophageal ganglia taken from cockroaches with reversed activity rhythms were implanted daily for 4 days into ten groups of three cockroaches each. Implantation was discontinued from day 4 to day 10. On day 10 one group was killed; tumours were found to be developing in all three animals, and had reached the stage in which the number of epithelial cells had increased, but there was no breakdown as yet of the epithelial tissue (see Table 3). Daily implantations were made into the remaining groups from day 10 until day 20, using sub-oesophageal ganglia from animals whose rhythms were normal and in phase with those of the recipient cockroaches. Every second day one group of animals was killed and the guts sectioned. The sections showed that tumours had not developed beyond the stage already reached when the second series of implantations began on day 10. The number of mitoses in the nidi had apparently returned to normal, but the affected region was recognizable by the increased number of cells. On day 20 implantation was discontinued and the examination of one group every 2 days continued. By day 24 the tumour size had increased considerably, the epithelial layer had been broken down, and the general appearance was that of a mass of small cells in whorled formation. This stage is referred to as 'stage 14 days' in Table 3; the term is only one of convenience and it is not claimed that certain stages are closely correlated with the number of days from the beginning of the treatment.

In this experiment the presence of excess sub-oesophageal ganglion hormone at the time when some secretion would normally be present appears to control the growth of the tumours. That the cells of the tumour have undergone an irreversible change, however, is shown by the increased growth as soon as the hormone level returns to normal.

The number of animals used in this experiment, as in those previously described, has had to be limited because of the time which every operation takes, and because of the large numbers of cockroaches necessary to supply the sub-oesophageal ganglia for implantation.

Table 3. *Sub-oesophageal ganglia taken from cockroaches with reversed activity rhythms, R; taken from cockroaches with normal activity rhythms, N. Recipient cockroaches all had normal activity rhythms*

Groups of three animals	Days											
	1–4	4–10	10	12	14	16	18	20	22	24	26	28
A	Implanted, R	—	Killed, tumours in all at 'stage 10 days'*	—	—	—	—	—	—	—	—	—
B	Implanted, R	—	Implanted, N	Killed, tumours at 'stage 10 days'	—	—	—	—	—	—	—	—
C	Implanted, R	—	Implanted, N	Implanted, N	Killed, tumours at 'stage 10 days'	—	—	—	—	—	—	—
D	Implanted, R	—	Implanted, N	Implanted, N	Implanted, N	Killed, tumours at 'stage 10 days'	—	—	—	—	—	—
E	Implanted, R	—	Implanted, N	Implanted, N	Implanted, N	Implanted, N	Killed, tumours at 'stage 10 days'	—	—	—	—	—
F	Implanted, R	—	Implanted, N	Implanted, N	Implanted, N	Implanted, N	Implanted, N	Killed, tumours at 'stage 10 days'	—	—	—	—
G	Implanted, R	—	Implanted, N	Implanted, N	Implanted, N	Implanted, N	Implanted, N	—	Killed, tumours at 'stage 12 days'	—	—	—
H	Implanted, R	—	Implanted, N	Implanted, N	Implanted, N	Implanted, N	Implanted, N	—	—	Killed, tumours at 'stage 14 days'	—	—
I	Implanted, R	—	Implanted, N	Implanted, N	Implanted, N	Implanted, N	Implanted, N	—	—	—	Killed, tumours at 'stage 16 days'	—
J	Implanted, R	—	Implanted, N	Implanted, N	Implanted, N	Implanted, N	Implanted, N	—	—	—	—	Killed, tumours large

* For explanation of term 'stage *x* days' see text.

DISCUSSION

The three main conclusions which might be drawn from these results are that the presence of excess amounts of sub-oesophageal ganglion secretion brings about formation of transplantable tumours in the gut of *Periplaneta*, that the midgut is the region most frequently affected, and lastly, the most striking conclusion, that the *timing* of the presence of the secretion is of first importance in tumour formation.

The importance of timing, both in the production of tumourous cells and in the growth of the tumours, apparently results from the interaction of 24 hr. rhythms of several processes. Although the introduction at any time of day of large excesses of hormone is followed by the formation of tumourous cells, at a low level of concentration not only is the 24 hr. rhythm of secretion concerned, but also the sensitivity of the gut, which itself shows a 24 hr. rhythm. The hormone continues to act when the cells have become tumourous and again it is not just the presence of the hormone which is involved, but the timing of its presence, for this same hormone which was responsible for the formation of the tumour can control its growth, if present at the normal time. Perhaps it is also significant that the cells which give rise to the tumour are cells which probably show a 24 hr. rhythm of mitosis under normal conditions; this has not been shown, but it appears likely, since the regeneration of the epithelium is connected with feeding, and feeding follows the 24 hr. rhythm of locomotory activity.

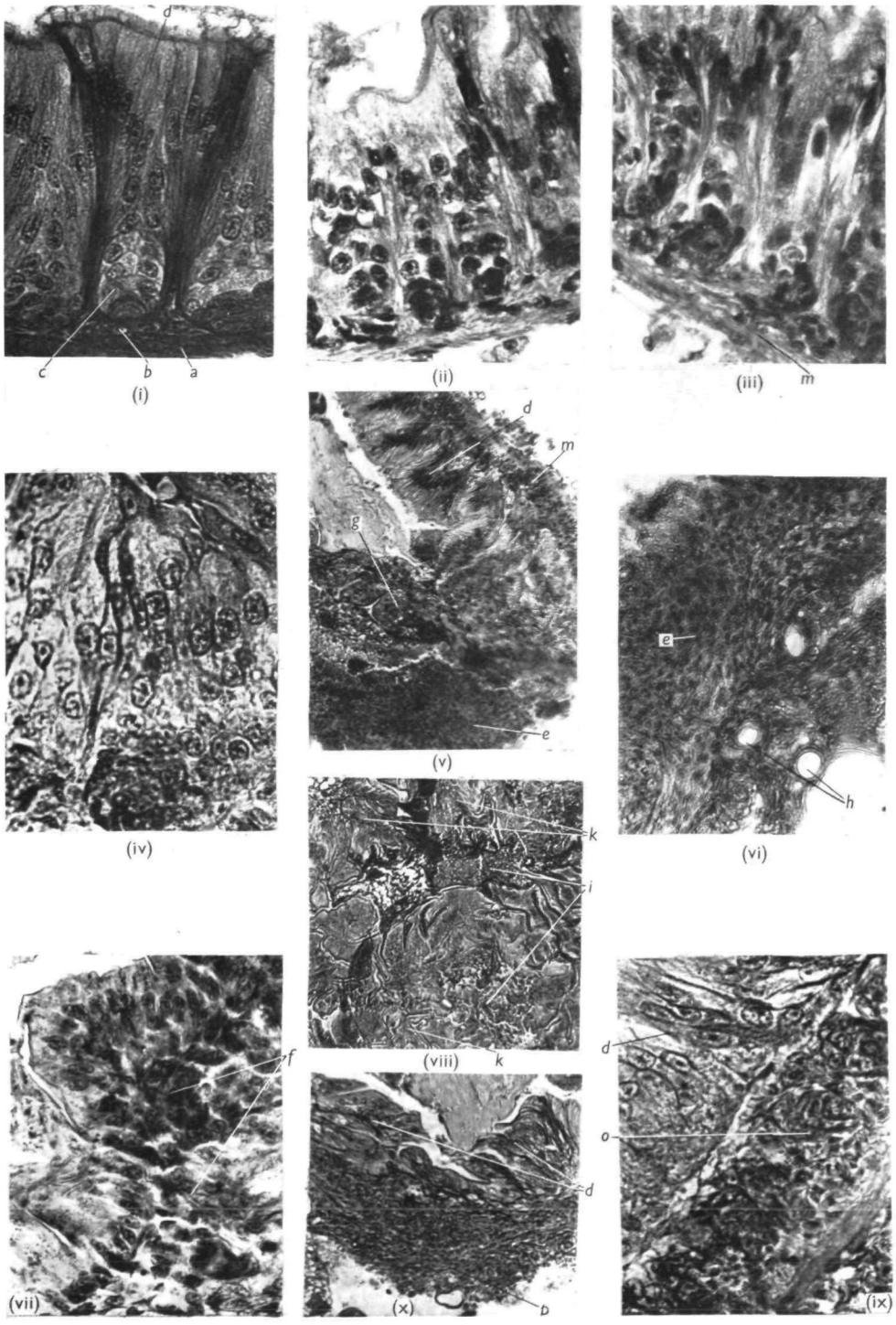
Nothing is known of the chemical nature of the sub-oesophageal ganglion secretion, nor of the way in which it acts on the animal in regulating the locomotor activity rhythm. Whether it acts through one or several systems, and whether it is the secretion itself or a secondary system which is responsible for the formation of tumours, is unknown, but, however tumour formation is brought about, the essential feature appears to be the continuous presence of the hormone. The normal animal seems to be well safeguarded from continuous secretion; even if the sub-oesophageal ganglion could maintain a continuous supply, the secretory phase appears to be bound to a 24 hr. rhythm which has, as yet, proved unalterable by experimental means.

SUMMARY

1. Sub-oesophageal ganglia taken from *Periplaneta americana* L. which had been kept in reversed conditions of light and darkness have been implanted into the abdomens of cockroaches living in normal light: dark conditions. When implantation was continued for 4 days transplantable tumours appeared by the eighteenth day in the midgut of the majority of cockroaches.

2. No tumours appeared in the guts of cockroaches when the implanted sub-oesophageal ganglia had been taken from animals kept in normal conditions of light and darkness, nor when brains or corpora allata were implanted.

3. Injection of an extract of five sub-oesophageal ganglia in 0·05 ml. Ringer solution produced tumours only when injection took place during the inactive phase of the 24 hr. locomotory rhythm.



HARKER—EXPERIMENTAL PRODUCTION OF MIDGUT TUMOURS IN *PERIPLANETA AMERICANA* L.

(*Facing p.* 258)