STUDIES ON THE EFFECTS OF THE REMOVAL OF THE FRONTAL GANGLION IN *LOCUSTA MIGRATORIA* L.

II. RIBONUCLEIC ACID SYNTHESIS

By KENNETH U. CLARKE and CEDRIC GILLOTT

Department of Zoology, University of Nottingham

INTRODUCTION

In the previous paper it has been shown that the effects of the removal of the frontal ganglion on protein metabolism were mediated through a failure of the corpus cardiacum to release neurosecretory material. In the absence of this hormone the protein concentration of the haemolymph does not increase, and the incorporation of ^{14}C-glycine into protein is markedly less than in normal locusts (Clarke & Gillott, 1966) A comparison of the cellular differences between operated animals and operated controls may reveal intracellular changes that can be correlated with the presence or absence of this hormone in the haemolymph.

Hill (1963) has correlated the high cytoplasmic ribonucleic acid content of the fat-body cells with an active neurosecretory system and a low content with an inactive system. Panitz (1960) claimed that the brain hormone inhibited the activity of certain of the Balbiani rings in the salivary gland cells of the chironomid *Acricotopus lucidus*, but his preparation contained in addition to the brain the suboesophageal ganglion and the 'adherent glands'.

The present paper deals with differences between the cells of the tissues of locusts deprived of the frontal ganglion, and the cells of operated controls.

MATERIAL AND METHODS

Samples of third- and fourth-instar *Locusta migratoria* L. of known age were taken from a population maintained under crowded conditions at $28 \pm 0.5^{\circ}$ C. and 70% relative humidity. The technique for the removal of the frontal ganglion and the postoperation treatment were as described by Clarke & Langley (1963 c).

Those frontal ganglionectomized and control operated locusts used for the cytochemical study of nucleic acids were killed by decapitation. The head (since it contained a variety of tissues) and the mid-gut were immediately fixed in acetic Zenker's fluid, dehydrated, cleared and embedded in ester wax. Sections were cut at 10 μ, and stained for nucleic acids with buffered methyl green-pyronin 'Y' solution (Brachet, 1953). Sections treated with ribonuclease solution (1 mg./ml. distilled water, pH 6·0) were used as controls.

Autoradiographic studies of the uptake of tritiated uridine into the cells of frontal ganglionectomized and control-operated locusts were made on animals in the third instar aged 82 h. The locusts were injected with ^{3}H-uridine (specific activity

500 mc./mM; 2·05 mc./mg.) in aqueous solution (concentration, 2 mc./ml.) in proportion to their body weight 1 μl. to 20 mg., or 1 μl. to 50 mg. Samples of locusts were killed by decapitation, 2, 5, 20 and 60 min. after the injection. Sections were cut at 2 μ (Boyd, 1955). The sections were stained for 3 min. in Erhlich's haematoxylin which coloured the nuclei blue, leaving the cytoplasm almost colourless. After staining, sections were coated with 0·5 % celloidin. (Methyl-pyronin 'Y' stain was dissolved out by celloidin in 50:50 absolute alcohol : ether mixture.) The prepared slides were dried, coated with Kodak AR 10 autoradiographic film, and left for 14 days at 5° C. The slides were then developed for 5 min. and fixed in 15 % sodium thiosulphate for 10 min., washed for 30 min. in running tap water, dried and mounted in 'Chromogel'. The number of reduced silver grains above the nucleus and cytoplasm was counted in 12 (usually adjacent) cells in each preparation. Comparison was made with control sections treated with ribonuclease.

More precise quantitative studies were made on the rate of incorporation of uridine into operated and control operated locusts by isolating the nuclei and measuring the radioactivity of the sample. Fourth-instar nymphs were injected with ^{14}C-uridine (specific activity 251 mc./mM.; concentration 0·01 mc./0·33 ml.) in proportion to their body weight, 1 μl./40 mg., and killed by decapitation 20 min. after the injection had been given. The legs and mid-gut were removed and the carcass was quickly rinsed in ice-cold insect Ringer containing 10% unlabelled uridine. Excess fluid was removed and the carcasss was cut into small pieces and dropped into 2 ml. ice-cold 5 % citric acid (Dounce, 1955). The mixture was left at 0° C. for 40 min. to allow the nuclear membrane to harden. The tissues were then homogenized for 3 min. at maximum speed in a Waring Blender. The homogenate was filtered through four layers of fine nylon into a graduated centrifuge tube, and the residue was washed with 2 ml. of cold citric acid. The residue was thoroughly squeezed in the nylon mesh and the liquid was collected in a centrifuge tube. The filtrate was centrifuged for 10 min. at 2500 rev./min.; the larger particles including the nuclei were sedimented. The supernatant fluid was removed, the residue was re-suspended in cold citric acid and centrifuged for 2 min. at 300 rev./min., which sedimented the larger particles and left the nuclei in suspension. 0·2 ml, of nuclear suspension were pipetted onto a $\frac{7}{8}$ in. coverslip and air dried. The coverslips were fitted into 1 in. planchets and the radioactivity was measured using a thin end-window (2·1 mg./cm.³) G-M tube (G.E.C. Ltd.). The number of nuclei per unit volume of the supernatant fluid was counted using a haemocytometer (Barber, 1951). From this the mean amount of radioactivity per nucleus was calculated.

<div align="center">RESULTS</div>

<div align="center">(1) The cytochemical investigation of nucleic acids</div>

The general pattern found in the control locusts was consistent with current ideas on the relationship between nucleic acids and protein synthesis. The mid-gut, fat body and (at times) the epidermis, which synthesize much protein, were rich in ribonucleic acid (RNA)—that is, the cytoplasm was strongly basophilic and the nucleoli were very large and prominent. The cells of tissues which do not synthesize much protein, for example the brain, showed only weak pyronin staining. In control sections treated with ribonuclease the cytoplasm appeared transparent and the nucleoli were no

onger visible. The epidermis, brain and mid-gut of the locusts subjected to the various treatments (normal, frontal ganglionectomized, control-operated and starved) were examined at various times during the instar.

A large proportion of the epidermal cells contained a dark brown pigment, probably an insectorubin (Goodwin, 1952). In the pigment-free cells the control animals showed a cytoplasm well stained with puronin and one, often two and three, very prominent nucleoli. Towards the end of an instar, with the formation of the new cuticle, the size of the epidermal cells increased and this was accompanied by an increase in basophilia and in nucleolar size.

In locusts from which the frontal ganglion had been removed the epidermal cells were small, the nuclei were closely packed, the cytoplasm was weakly basophilic, and the nucleoli, if visible at all, were very small. There was no sign of the formation of a new cuticle at the end of the instar. The epidermis of starved locusts was similar to that of the operated animals; both were reminiscent of the resting epidermis of *Rhodnius* as described by Wigglesworth (1957).

The fat body in control locusts was quite extensive, particularly at the end of a stadium. Its appearance varied considerably even in one individual. At extremes, it appeared either highly vacuolated or quite dense; all intermediate stages could be found. The cytoplasm which was visible in the less vacuolated cells was quite pyronophilic; in all cells the nuclei and the nucleoli were relatively large. In operated animals it was very difficult to find any fat-body tissue. In the very small amounts which did occur the cytoplasm was weakly basophilic and the nucleoli were very small or invisible. Air sacs filled the space normally occupied by the fat body (Clarke, 1956). The observations correlate with those of Hill (1963) who showed cytochemically that the RNA content of the fat body was high at the time that the neurosecretory system was known to be active. Starved locusts were similar in appearance to the operated ones. In control locusts the nerve cells of the brain stained only slightly with pyronin, and the nucleoli were not visible. The large neurosecretory cells were markedly basophilic, and in their nuclei prominent nucleoli were visible. No variation of intensity in staining with time was observed in control animals which supports Clarke & Langley's (1963e) suggestion that there is continuous synthesis of brain hormone. The neurosecretory cells of operated and starved locusts did not show any pronounced differences from those of control animals in their affinity for pyronin. The nucleoli were possibly smaller, but the effect was certainly not so obvious as in the fat body and epidermis.

The cells of the mid-gut epithelium stained more strongly with pyronin than did those of any other tissue. In control locusts the mature enzyme producing cells at the top sides of the crypts (Day & Powning, 1949) were extremely rich in RNA. The intensity of the stain was greatest around the nuclei, and in the cells the nucleoli were very prominent. No cycle of staining intensity was observed in these cells suggesting that protein synthesis was a continuous process. This supports the earlier observation on protease activity in the mid-gut tissue and contents (Clarke & Gillott, 1966). In operated animals the mature mid-gut cells were very much less basophilic than those of control locusts; the nuclei were much smaller and the nucleoli usually invisible. (Fig. 4b). In starved animals the cells were similar to those of operated animals. However, 24 hr. after a meal the cells of starved locusts were much enlarged, the nucleoli

were again prominent, and the cytoplasm was intensely stained with pyronin, especially around the nucleus.

No changes were visible in the DNA content of the nuclei in any of the tissues.

(2) *Autoradiographic studies*

In the control locusts labelled uridine appeared in the nucleus within 2 min. of its being injected into the haemocoele. At this time practically none was present in the cytoplasm (Pl. 1 a). The amount of ^{3}H-uridine in the nucleus increased for 20 min.
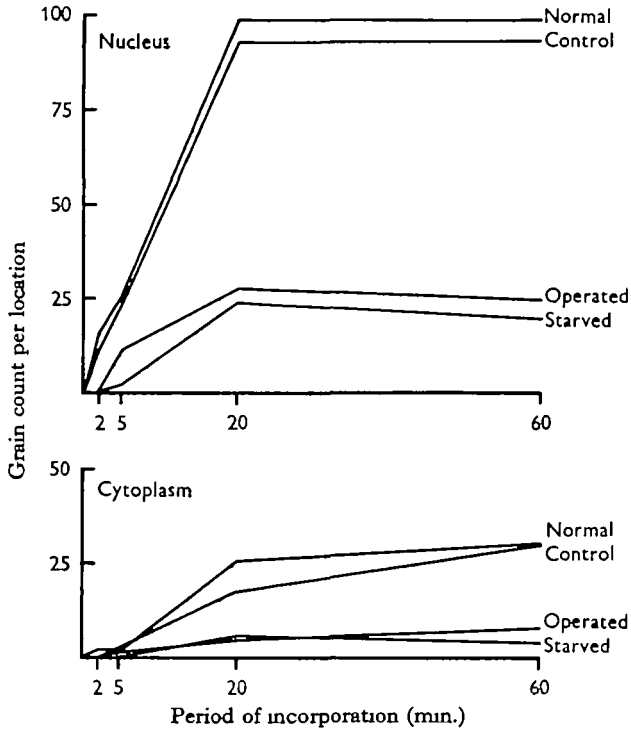
Text-figure 1. The incorporation of ^{3}H-uridine into the nucleus and cytoplasm of mid-gut epithelial cells following an injection of labelled uridine into the haemocoele of third-instar locusts.

following the injection. Its concentration was then maximal (Pl. 1b); no further significant changes were seen 60 minutes later (Pl. 1c). In the cytoplasm there was little ^{3}H-uridine to be seen in the cytoplasm at 2 min. and at 5 min. following the injection, but after 20 min. the amount had increased tenfold; after 60 min. only a slight further increase was seen. In operated locusts, after 2 min., no labelled uridine was detected in either the nucleus or the cytoplasm. After 5 min. some was present in both nucleus and cytoplasm and this increased to a maximum some 20 min. later (Pl. 1c); no further increase had occurred 60 min. later. The maximal amount accumulated in operated animals was never more than 25% of that found in the controls. Starved animals gave similar results to the operated locusts (Pl. 1d). The rate of accumulation of uridine in the nucleus and cytoplasm of control, operated and starved animals is shown in Text-fig. 1.

(3) *The accumulation of* ^{14}*C-uridine into the nuclei of control and operated locusts*

These experiments were performed to confirm that different amounts of uridine accumulated in the nuclei of control and operated locusts. Studies were made on male and female fourth-instar nymphs injected with ^{14}C-uridine 4 days after the operations had been performed. The results demonstrated that much less ^{14}C uridine was accumulated in operated and starved locusts than in operated controls (Table 1).

Table 1. *The incorporation of* ^{14}*C-uridine into the nucleus of control, frontal ganglionectomized, and starved third-instar locusts*

		Treatment		
Experiment	Sex	Control	Frontal ganglionectomized	Starved
1	Male	2 05	0·49	0·87
	Female	1·45	0·80	0·52
2	Male	1·20	0·22	0 54
	Female	0·76	0·23	0·27

Each figure is the mean of the counts per nucleus per second × 10^7, for six individuals.

DISCUSSION

The results of the present investigation show that the removal of the frontal ganglion markedly and adversely affects the ability of many tissues to synthesize RNA in the nucleus, and thus to synthesize protein. The resumption of growth in an operated locust brought about by daily injections of freshly prepared corpus cardiacum extract indicates that the failure of these glands to secrete the appropriate hormones is an important link in the chain connecting the two events. The question arises whether this is due to a direct action of these hormones on the tissue cells, or whether there is a further link, the hormones affecting the prothoracic glands whose hormone ecdysone then influences the tissue cells. In a previous paper (Clarke & Gillott, 1966) reasons have been given for assuming that the active hormone from the corpus cardiacum is in fact the brain hormone or neurosecretory material, and this identification is accepted in the discussion that follows.

The following evidence favours the view that there is a direct action of the hormone on the tissue cells. In adult insects tissue growth continues despite the absence of the prothoracic glands which degenerate after the last moult (Carlisle & Ellis, 1959). The removal of the frontal ganglion has the same effect in adult locusts as it does on nymphs (Clarke & Langley, 1963 a). The prothoracic glands of locust nymphs show cycles of activity (Clarke & Langely, 1963 b) while the neurosecretory cells of the brain appear to produce material continuously (Clarke & Langley, 1963 d). The results of the present investigations indicate that, in the presence of abundant food, synthesis of RNA and protein is more or less continuous and does not show changes that can be correlated with prothoracic gland activity. An obvious exception to this is the epidermis in which cycles of synthesis of RNA and protein can be demonstrated. In this tissue these can clearly be correlated with the cyclical activity of the prothoracic gland.

In this context it is interesting to note that Wigglesworth (1963) has for more than 30 years held the opinion that the prothoracic gland hormone 'is not a necessary ingredient for the growth of insect cells in general'.

Although the probable production of several hormonal factors by the brain (Gersch, 1962) is acknowledged it is perhaps significant that the two best known effects of the brain hormone, protein synthesis and activation of the prothoracic gland, can be explained by attributing a common function to the brain hormone, namely that of promoting synthesis of messenger RNA within the cell. The present investigation has shown that the brain hormone affects the synthesis of nuclear RNA within the cell, and Oberlander & Schneiderman (1963) have shown that within 12 hr. exposure to brain hormone the prothoracic gland cells are engaged in active synthesis of nuclear RNA.

Throughout the present investigation an 'all-or-none' effect following the removal of the frontal ganglion has never been noted. For example, there was *some* synthesis of mid-gut protease, *some* incorporation of uridine into RNA within the nucleus, and *some* incorporation of ^{14}C-glycine into protein. Hill (1963) also found *some* incorporation of ^{14}C glycine into protein following cautery of the neurosecretory cells in *Schistocerca*. Thomsen & Møller (1963) also found that after cautery of the neurosecretory cells in *Calliphora* some mid-gut protease synthesis took place. In saturniid moths in diapause when neurosecretory material is absent from the haemolymph (Williams, 1946) some incorporation of radioactive uridine and hence some RNA synthesis still occurs (Krishnakuman & Schneidermaran, 1963; Schneiderman & Gilbert, 1964; Krishnakumaran, Oberlander & Schneiderman 1965). It seems clear therefore that some synthesis of RNA protein can and does occur in insect tissues in the absence of the brain hormone. Conversely, Clarke (1966) has shown that in *Locusta*, under conditions in which a rapid turnover of protein might be expected, the neurosecretory system is almost depleted of this material.

These facts can be accounted for if it is assumed that the production of proteins from genes follows the model proposed by Jacob & Monod (1961) for bacteria and considered valid at least in outline for higher organisms (Sirlin, 1963). Then the action of neurosecretory material would be analogous to that of the 'allosteric' factor suggested by Monod, Changeux & Jacob (1963). That is, the neurosecretory material suppresses the inhibitory feed-back of a number of different metabolic products onto their respective genes, thus exercising a general control over the rate of metabolic activity within the cell. If the hormone was to activate the genes directly, then in its absence the gene would be inactive and no protein synthesis whatsoever would occur. By accepting the Jacob & Monod hypothesis and thus postulating that there must always be some autonomous gene activity, and assuming that the brain hormone acts as an 'anti-de-accelerator' then the steady-state conditions found in its absence as well as the types of response produced by its presence can be explained (Clarke, 1965).

The results of this and previous investigations show how the intake and utilization of food in the locust are related. By means of this mechanism whereby the intake of a given quantity of food will cause the release of a proportional amount of brain hormone, protein metabolism and indeed the complete process of growth and development can be facilitated with maximum efficiency.

SUMMARY

1. A cytochemical investigation was made of the RNA content of the cells of the mid-gut, fat body and epidermis in third- and fourth-instar *Locusta migratoria* L. from which the frontal ganglion had been removed, in control operated and in starved animals. In operated locusts the nucleus was smaller, the nucleoli small or absent, and the cytoplasmic RNA much less than that found in operated controls. No differences were observed in the DNA content of the cells.

2. Autoradiograph studies were made of the uptake of ^{3}H-uridine into the cells of operated and control operated locusts. In operated locusts the appearance of labelled uridine in the nucleus was delayed, the rate of uptake slower, and the total amount incorporated less (never more than 25%) than in the controls.

3. Studies were made of the uptake of ^{14}C-uridine into the nuclei of operated and control-operated locusts. Nuclei were isolated from locusts killed at known times after the isotope had been injected and their radioactivity was measured. The results confirmed those found in the radioautographic studies.

4. The significance of these results is considered in the light of the Jacob & Monod model of the control of protein synthesis.

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EXPLANATION OF PLATE

PLATE 1

Autoradiographs of the mid-guts of third-instar locusts aged 82 hr. following an injection of ^{3}H-uridine into the haemocoele.

(*a*) Normal animal killed 2 min. following the injection.

(*b*) Normal animal killed 20 min. following the injection.

(*c*) Frontal ganglionectomized insect killed 20 min. following the injection.

(*d*) Starved locust killed 20 min. following the injection.

The camera is focused in the plane of the grains. × = 1100.