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

I. THE EFFECT ON PROTEIN METABOLISM

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INTRODUCTION

During an investigation into the factors which initiate the growth and moulting cycle of *Locusta migratoria* L., it was found that the removal of the frontal ganglion, severance of both lateral connectives or of the nerves connecting the frontal ganglion to the foregut resulted in an immediate cessation of growth of the operated locust (Clarke & Langley, 1963*a*, *b*). This was accompanied by a very marked accumulation of neurosecretory material in the nervi corpori cardiaci I some 200 hr. after the operation and in an abnormal appearance of the corpora cardiaca. From this, and other evidence, a hypothesis was proposed that the intake of food into the gut controlled the synthesis and/or release of neurosecretory material which was utilized in the normal growth processes of the locust, most probably in protein metabolism (Clarke & Langley, 1963*d*).

If, as a result of this operation, protein metabolism is reduced in the locust, significant differences should be apparent between the concentration of proteins and amino acids of the haemolymph, and in the synthesis of proteins and enzymes in the body cells between operated and operated controls. Furthermore, if the effect of the removal of the frontal ganglion on growth is mediated through the hormones of the corpora cardiaca, then injections of corpora cardiaca extract into operated locusts should result in the resumption of growth. The present paper reports on these observations.

MATERIAL AND METHODS

Studies were made of third-, fourth-, and fifth-instar nymphs of known age of *Locusta migratoria* L. taken from a culture kept at $28 \pm 0{\cdot}5°$ C. and 70% relative humidity. The techniques for the removal of the frontal ganglion, and of the post-operational treatment of the locusts, were identical with those described by Clarke & Langley (1963*b*). All locusts, with the exception of those used in the study of the effects of the injection of corpus cardiacum extracts which were kept individually, were kept at a sufficiently high population density to ensure their remaining 'gregarispect' (Joly & Joly, 1953). No signs of phase change were apparent in the locusts kept individually.

Blood for electrophoresis and for chromatography was obtained from individual

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locusts by making a small hole in the frons, placing the animal head downwards in a centrifuge tube and by slow centrifugation forcing the blood out of the locust.

Electrophoresis of the haemolymph proteins was carried out using cellulose acetate paper as a supporting medium (Kohn, 1960); Veronal solution, pH 8·75, as a buffer, 160 V. potential difference, a current of 0·3–0·5 amp./cm., and a running time of 7 hr. at room temperature. After each run the strips were dried rapidly, stained in light green solution (0·2% in 5% acetic acid), dried, rendered transparent with Whitemor 120 oil, and scanned using a densitometer. The area under the graph so produced was cut out and weighed to provide a relative estimate of the protein concentration.

Amino acids were studied using two-dimensional paper chromatography on protein-free haemolymph samples. The proteins in the haemolymph were precipitated with 2 vol. of 80% ethanol to 1 vol. of haemolymph and then removed by centrifugation. Whatmann no. 1 paper was used as a supporting medium. The initial separation was carried out with a solution of *n*-butanol 4 parts, glacial acetic acid 1 part and water 5 parts (v/v), the second separation with phenol 4 parts water 1 part (w/v). Running time for each phase was 16 h. The positions of the amino acids were located on the chromatogram by spraying with 0·2% ninhydrin in acetone (w/v). Identification was by comparison with standard chromatograms produced from a known amino-acid mixture run under identical conditions. A semi-quantitative estimate of the amino acid concentration was made by cutting the spots from the chromatogram, eluting them with 75% ethanol containing 0·05 mg. copper sulphate (Giri, Radhakrishnan & Vaidyanathan, 1952) and measuring the optical density of the eluate using Ilford Spectrum Filter no. 605 (at 545 mμ) in a Hilger photometer. Control samples were made by eluting 1 sq. in. of ninhydrin-treated unstained chromatography paper.

Protein synthesis was compared in operated and control-operated locusts by following the incorporation of ^{14}C glycine into the body proteins. Third-instar locusts were injected with ^{14}C-glycine (specific activity 8·0 mc.mM., 106·7 μc./mg.) in distilled water (concentration 0·1 mc./ml.) either as a fixed volume (5 μl.) or as a proportion of the body weight (1 μl./20 mg.). The locusts were killed at known times following the injection, the body was slit open ventrally, the contents of the alimentary canal were removed and the legs were cut off. The carcass was then weighed, and the protein was extracted from it following the technique of Kemp (1956) with the following modifications. After removal of the lipids and the trichloroacetic-acid-soluble fractions the remaining protein was extracted and hydrolysed with 5% potassium hydroxide at 80° C. for 20 min. The hydrolysate was then centrifuged to remove the cuticle fragments. The ^{14}C activity of the supernatant fluid was then estimated by taking a sample of 0·1 ml. of the hydrolysate and pipetting it on to a planchet with two drops of very weak Teepol to ensure even spread of the sample. The sample was air-dried overnight, drying being completed by placing the planchet under a hot lamp for 30 min. before counting. The radioactivity of each sample was measured by counting with a thin end-window (2·1 mg./cm.^{2}) G.M. tube for three periods of 5 min. Since the investigation was purely comparative, and the weight of the samples on the planchet were found to be almost constant, no corrections for self absorption were made. Locusts in which the mid-gut protease activity was to be estimated were killed by decapitation, the abdomen was slit open ventrally, and the mid-gut excluding the mesentric caeca was removed. This was freed from adherent matter by rolling it

gently on filter paper. The mid-gut was slit longitudinally and the contents were washed into phosphate buffer solution (pH 8·0). The mid-gut tissue was freed from excess buffer by blotting gently with filter paper and then weighed. The mid-gut tissue and the contents were homogenized separately to give either one mid-gut wall or one mid-gut contents per 0·5 ml. of buffer. These homogenates were left at room temperature until required, when they were centrifuged and the protease activity of the supernatant fluid was measured using the methods of Day & Powning (1949), the enzyme substrate mixture being incubated at 37° C for 21 h. The volume of N/20 alcoholic potassium hydroxide necessary to neutralize the amino acids liberated was converted into arbitrary enzyme activity units, 100 units equalling the volume of N/20 KOH previously found necessary to neutralize the amino acids produced by the protease activity of two whole mid-guts per ml. of supernatant fluid prepared from normal locusts.

The effect of injections of corpus cardiacum extract into locusts from which the frontal ganglion had been removed 4 days previously was studied on three groups of animals. One group was injected daily with 10 μl. of freshly prepared corpus cardiacum extract, one with 10 μl. of distilled water, and one control group left untreated. The locusts were weighed daily immediately before the injections were given. The effects of the injections were estimated by comparing the growth curves of the three groups studied.

The corpus cardiacum extract was freshly prepared each day from third- and fourth-instar locusts of the same age as the experimental insects. The corpus cardiacum was approached through the dorsal surface of the head, freed from the hypocerebral ganglion and surrounding tissue, and removed. It was homogenized in 100 μl. of distilled water and left to stand at 0° C. for 30 min., then centrifuged; and the supernatant fluid was used for injection. Injections (10 μl. per animal) were made into the haemocoele, laterally, through the intersegmental membrane of the third and fourth abdominal segments.

RESULTS

Electrophoresis of haemolymph proteins

In unoperated locusts the protein concentration of the haemolymph was low immediately after an ecdysis and increased to about four times the initial value when the locusts commenced feeding. This level was maintained throughout the stadium until just before ecdysis when it rapidly decreased (Fig. 1). Operated controls showed a similar pattern, but the increase at the beginning of the instar lagged some 48 hr. behind that of normal locusts, presumably due to the effects of postoperative treatment. The total protein concentration in the haemolymph of locusts deprived of the frontal ganglion remained more or less constant throughout the instar (Fig. 1). The slight rise that occurred on the day following the operation was attributed to a wound effect (Highnam, 1962). In starved locusts the protein concentration in the haemolymph decreased throughout the period of their survival.

In the majority of electrophoretic patterns three protein fractions could be distinguished. Occasionally four appeared, the fourth being due to the separation of the middle band into two fractions. Of the three fractions found in operated controls the first and third bands remained at approximately the same concentration throughout

the instar. The changes manifest in the total concentration were due almost entirely to a change in the concentration of the second band. In locusts deprived of the frontal ganglion the second band remained at its original low concentration throughout the instar.

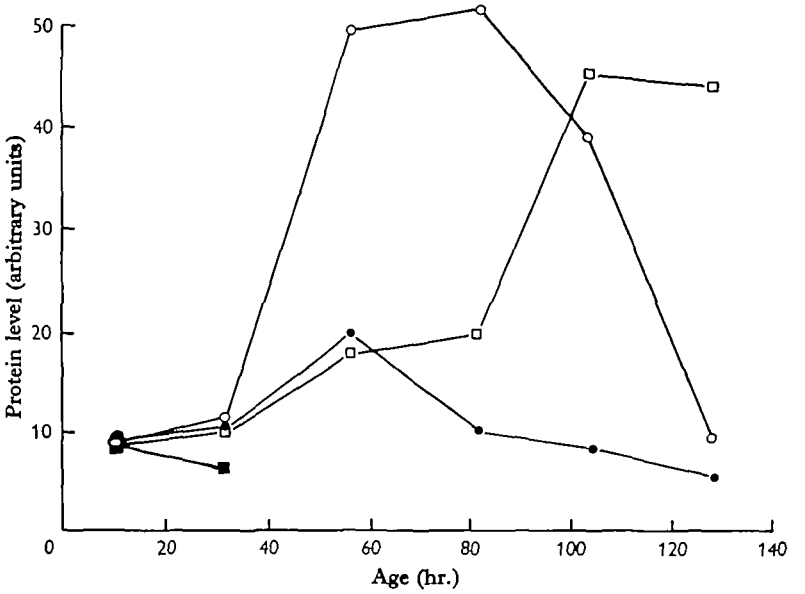

Fig. 1. Changes in the concentration of the haemolymph proteins in normal, ○——○; control operated, □——□; starved, ■——■; and frontal ganglionectomized animals, ●——●. Third-instar locusts during the third stadium. Each point is the mean of three observations.

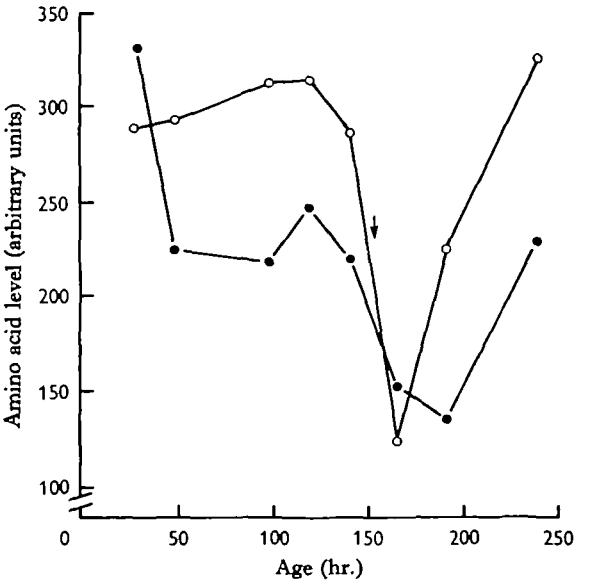

Fig. 2. Changes in the concentration of the haemolymph amino-acids in control-operated, ○——○; and frontal ganglionectomized animals, ●——●. Third and fourth-instar locusts during the third and fourth stadia. The arrow indicates the time of moulting of the control-operated animal.

Chromatography of the free amino acids

In control-operated animals there was a high concentration of free amino acids in the haemolymph throughout most of the stadium. At the onset of ecdysis the concentration fell to about 50% of its mid-stadial level; after ecdysis it increased rapidly to its original value (Fig. 2). In locusts from which the frontal ganglion had been removed, the total amino acid values, which were originally as high as those of the operated controls, decreased to about 70% of this figure. At the time at which the control locusts moulted there was a corresponding decrease in the total amino acid level in the haemolymph of the operated locusts, although moulting and ecdysis did not occur in these insects.

Table 1. *Differences in the haemolymph concentrations of individual amino acids between control-operated and frontal ganglionectomized locusts in the third instar*

Amino acid	Mean value (arbitrary units) Control locusts (C)	Operated locusts (O)	O/C × 100
Leucine/isoleucine	21·1	6·8	32·2
Phenylalanine	2·3	1·6	69·6
Valine	26·4	7·9	29·9
Tyrosine	9·7	3·8	39·2
Proline	4 0	3·9	97·5
Alanine	15·0	10·5	70·0
Threonine	6·0	1·4	23·3
Aspartic acid	0·5	0·3	60·0
Glycine	28·0	25 8	92 1
Glutamic acid	41·5	52·6	126·7
Lysine	26·4	20·0	75·8
Histidine	95·3	60·6	63·6
Arginine	28·4	27·8	97·9
Total	304·6	225·9	74·5

The mean value for each acid is that for five locusts observed at ages 48, 99, 120, 142 and 240 hr. during the stadium.

There was some variation in the day-to-day concentration of individual amino acids in the haemolymph of operated controls, but the relative concentration of the different amino acids tended to remain constant. Histidine was always present in high concentration (95·3 units), glutamic acid (41·5 units), arginine (28·4 units), glycine (28·0 units), valine and lysine (both 26·4 units), leucine/isoleucine (21·1 units), and alanine 15 units). All the remaining amino acids were below 10 units in concentration. These proportions are in broad agreement with those given by Duchâteau, Florkin & Sarlet (1952); the major variations such as the high value for histidine were attributed to dietary differences.

Following the removal of the frontal ganglion the proportions of amino acids to each other changed (Table 1). With the exception of glutamic acid all the amino acids fell in concentration, but the amount by which the concentration decreased varied greatly. Of the five amino acids whose values fell by more than 40%, four (leucine, isoleucine,

threonine and valine) have been shown to be essential acids for *Tribolium* (Lemonde & Bernard, 1951) and for *Drosophila* (Rudkin & Schultz, 1947).

The incorporation of ^{14}C-glycine into protein

The rate of incorporation of ^{14}C-glycine into protein in control-operated locusts was high for an initial period of 5 h. following the injection, then followed a gradual decrease in rate until a maximum value for radioactivity was found in samples 7 hr. after the injection. Thereafter the level remained constant for 16 hr. and then began to decrease slightly (Fig. 3).

In locusts from which the frontal ganglion had been removed the rate of incorporation was slower than that of the controls but the equilibrium plateau, although lower, was reached after a similar time interval (Fig. 3).

Starved animals showed a similar response to operated locusts (Fig. 3).

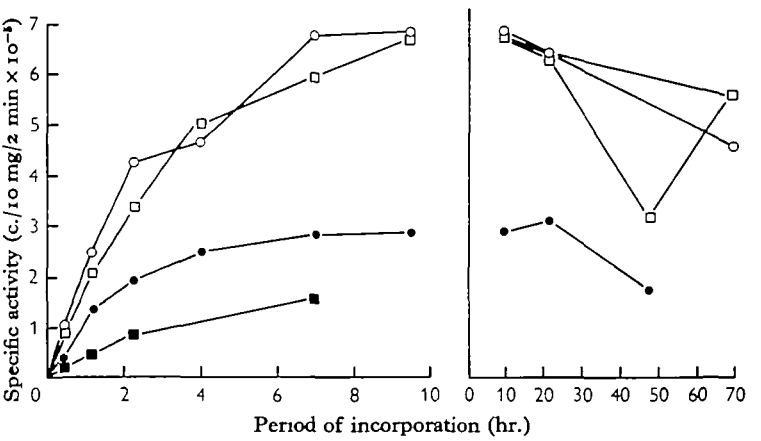

Fig. 3. The rate of incorporation of ^{14}C-glycine into protein in normal, ○——○; control-operated, □——□; starved, ■——■; and frontal ganglionectomized animals, ●——●. All in third-instar locusts.

Mid-gut protease activity

In control-operated locusts the protease activity of both the mid-gut wall and its contents were very variable, and no consistent pattern of release and synthesis was found. The activity of the mid-gut wall was always low (in many instances no activity at all was detected). Activity of the mid-gut contents was much greater; even at ecdysis a high activity could be demonstrated.

In locusts deprived of the frontal ganglion the activity of protease prepared from the mid-gut wall was low and no significant difference from that of the controls was detected. Protease activity could still be detected in the gut contents, but the protease content per gut was always much less than that of the operated controls (Fig. 4). Starved locusts showed a similar pattern to that of operated animals. When expressed as total activity per unit weight of mid-gut there was no significant difference between values for normal, starved and operated locusts.

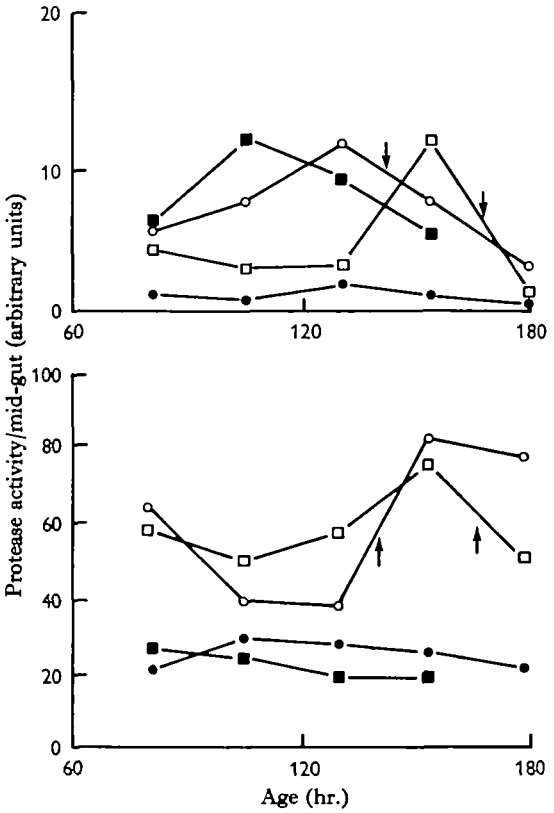

Fig. 4. Changes of protease activity expressed in arbitrary units of: (*a*) the mid-gut wall, (*b*) the mid-gut contents in normal, ○——○; control-operated, □——□; starved, ■——■; and frontal ganglionectomized animal, ●——●. All third and fourth instar locusts. The vertical arrows mark the third ecdysis of the normal and control-operated animals.

Table 2. *The body weight of operated animals (males) injected with corpus cardiacum extract*

(Expressed as a percentage of that at the time of operation (14 hr.))

Treatment		Age from emergence (hr.) 83	107	131	154	178	207	227	255	274
U	Mean	88·9	95·2	105·0	110·1	114·6	112·4	121·9	115·2	—
	S.E.	4·9	2·2	3·7	1·6	5·0	6·1	8·7	—	—
		(10)	(8)	(6)	(5)	(5)	(4)	(3)	(1)	
	Comparison of means *P*	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.	—
W	Mean	85·9	100·7	115·9	114·5	121·9	126·0	126·2	115·5	116·4
	S.E.	8·5	4·6	4·9	6·8	8·9	8·8	6 4	6·6	5·3
		(10)	(9)	(8)	(8)	(6)	(6)	(5)	(3)	(2)
	Comparison of means *P*	N.S.	N.S.	N.S.	< 0·02	< 0·02	< 0·01	< 0 01	< 0·05	< 0·05
E	Mean	84·3	102·7	128·5	145·1	151·6	159·9	161·7	193·8	192·0
	S.E.	4·3	6·9	8·2	8·0	5·5	4·9	6·4	25·5	21·8
		(11)	(9)	(7)	(7)	(7)	(6)	(6)	(2)	(2)

U = untreated operated animals. W = operated animals injected with distilled water only. E = operated animals injected with hormonal extract. N.S. = not significant. Figures in ( ) indicate number of animals in sample.

Table 3. *The body weight of operated animals (females) injected with corpus cardiacum extract*

(Expressed as a percentage of that at the time of operation (14 hr.))

Treatment		Age from emergence (hr.) 83	107	131	154	178	207	227	255	274	298	327
U	Mean	86·3	98·0	109·0	115·1	118·1	109·0	127·9	124·2	140 4	117·7	115 7
	S.E.	3·2	3·5	5·9	7·8	7·8	2·1	13·4	—	—	—	—
		(12)	(11)	(8)	(7)	(5)	(4)	(2)	(1)	(1)	(1)	(1)
	Comparison of means *P*	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.	< 0·05	N.S.	N.S.
W	Mean	84·6	92·5	97·5	108·0	104·8	116·2	124·0	120·7	118·8	117·1	104 0
	S.E.	3·4	3·7	2·6	3·2	3·0	6·8	3·8	11·1	3·2	3·2	11·0
		(11)	(9)	(9)	(8)	(7)	(5)	(3)	(3)	(2)	(2)	(2)
	Comparison of means *P*	N.S.	< 0·01	< 0·001	< 0·001	< 0·001	< 0·01	< 0·001	< 0·05	< 0·01	< 0·001	< 0 02
E	Mean	90·7	110·9	130·1	138·7	146·2	143·5	158·3	158·4	167·3	169·2	175·7
	S.E.	2·7	5·0	6·0	5·3	2·6	2·0	2·8	5·4	6·9	2·5	9 7
		(12)	(11)	(8)	(8)	(8)	(8)	(6)	(3)	(3)	(3)	(2)

U = untreated operated animals. W = operated animals injected with distilled water only. E = operated animals injected with hormonal extract. N.S. = not significant. Figures in ( ) indicate number of animals in sample.

Injection of corpus cardiacum extract

During the first 2 days following the removal of the frontal ganglion all the locusts decreased in weight, but upon return to warmer conditions in the insectary 48 hr. after the operation the insects became fully active, commenced feeding and their body weight returned to its pre-operational value (see Tables 2 and 3).

The operated locusts were divided into three batches; the first was left untreated and these locusts remained at approximately constant weight until they died; the second

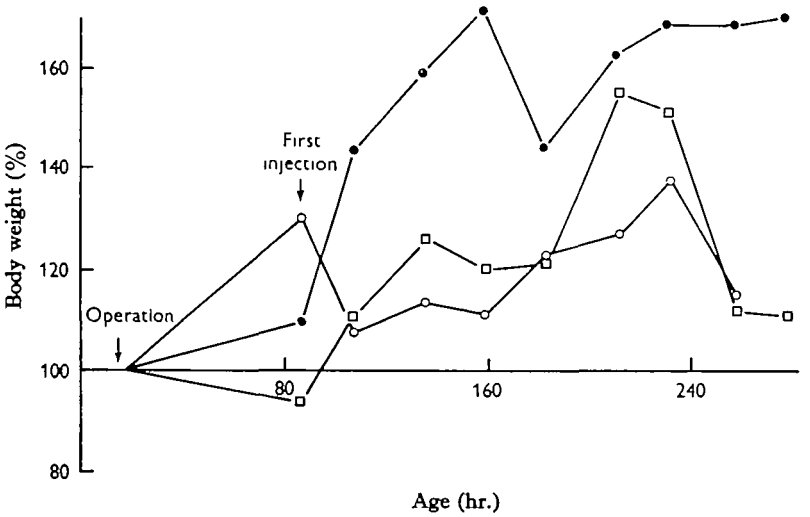

Fig. 5. The growth in weight of male fourth instar locusts from which the frontal ganglion had been removed and which were: given daily injections of distilled water, O——O; given daily injections of freshly prepared corpus cardiacum extract, ●——●; untreated, □——□.

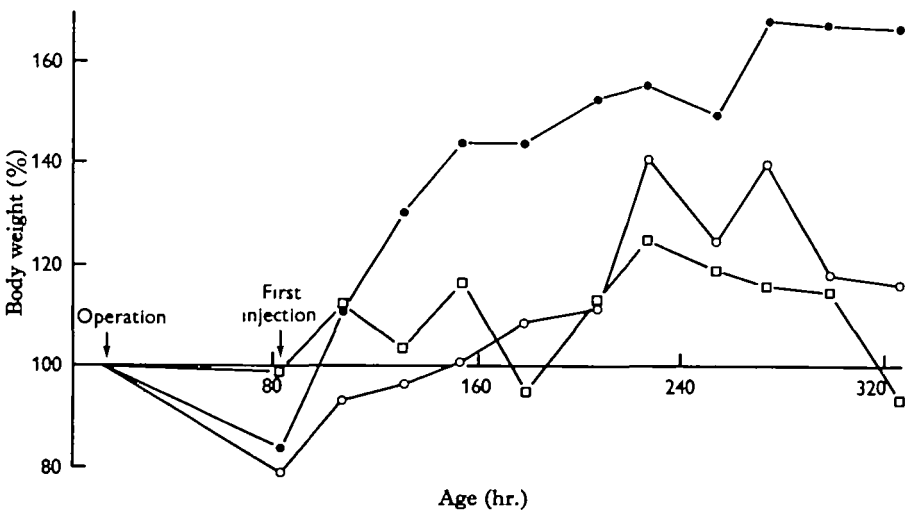

Fig. 6. The growth in weight of female fourth-instar locusts from which the frontal ganglion had been removed and which were: given daily injections of distilled water, O——O; given daily injection of freshly prepared corpus cardiacum extract, ●——●; untreated, □——□.

batch were given injections of distilled water, their mean body weight was not significantly different from that of the untreated locusts; a third were given daily injections of corpus cardiacum extract and in these the body weight was significantly higher than that of either of the other two groups (Tables 2, 3).

The weight changes of the locusts were followed for each individual treated (Figs. 5, 6). Those injected with distilled water showed increases of as much as 30% from day to day. This increase, which was due to the intake of food, was only temporary, the body weight over a long period remaining more or less the same as the pre-operational weight. In animals injected with corpus cardiacum extract the increase in weight was permanent and reached values as high as 119% above the pre-operational level.

DISCUSSION

That the removal of the frontal ganglion from individuals of *Locusta migratoria* L. has a definite effect on protein metabolism is shown by a comparison of the electrophoretic patterns of the haemolymph proteins from both operated and control-operated locusts. In operated locusts the second band, which in the normal animal increases in concentration during the stadium, showed no increase at all. Correlated with this was the observation that the fat body, a major site of haemolymph protein synthesis (Shigematsu, 1958; Faulkner & Bheemeswar, 1960; Hill, 1963) was very under-developed and rudimentary in operated locusts. Gillott (1964) has noted that in operated locusts there was an accumulation of dust, particularly at the intersegmental membranes which was due to exudation of moisture through the cuticle. It is possible that this breakdown in water balance in the locust may result from the decrease in concentration of proteins and amino acids in the haemolymph.

The failure in operated locusts to show the normal increase in concentration of haemolymph proteins and amino acids could be due to either (*a*) a specific failure of the mid-gut cells to synthesize or release proteolytic enzymes (Thomsen & Moller, 1959), or (*b*) the general failure of the body cells to utilize for protein synthesis amino acids made available to them in the haemolymph. A comparison of the proteases in the mid-guts of operated and control-operated animals together with the utilization in protein synthesis of a labelled amino acid injected into the haemolymph, thus by-passing the mid-gut, helps to decide between these two alternatives.

In all cases the protease activity of the mid-gut wall was found to be very low and often could not be detected at all. No differences were therefore apparent between locusts from the different experimental treatments. The protease activity of the mid-gut contents was appreciable, and in normal locusts activity occurred throughout the stadium and at the time of ecdysis. The low protease activity of the mid-gut wall and the relatively high activity of the mid-gut contents indicates that the synthesis and release of proteolytic enzymes must be almost simultaneous. In locusts deprived of the frontal ganglion the protease activity of the mid-gut contents was less than that of the operated controls, but appreciable activity was still present. The release and synthesis of proteolytic enzymes was not therefore stopped in the absence of the frontal ganglion. It is interesting to note that there was less variation in the amount of enzyme activity per gut in operated animals than there was in the operated controls.

The rate of incorporation into protein of ^{14}C-glycine injected into the haemocoele was followed in operated and control-operated animals. In operated locusts the rate

was slower and the amount incorporated was less than in the control animals. In both groups the maximum amount incorporated was found 7 hr. after the injection had been given.

These results indicate that the failure of operated locusts to show increases in protein concentration in the haemolymph was correlated with a general breakdown in protein metabolism and not specifically with a failure of the synthesis and the release of proteolytic enzymes.

The effect of daily injections of corpus cardiacum extract in causing the resumption of growth in insects which have been deprived of the frontal ganglion clearly indicates that the effects of this operation are mediated through this endocrine organ. They are not due to the failure of the animal to ingest food in consequence of neuromuscular injury.

It is possible to make a reasonable identification of the hormone involved. It had previously been noted (Clarke & Langley, 1963*d*) that neurosecretory material accumulated in the nervi corpori cardiaci I in operated locusts, indicating the retention of this material. Further identification of neurosecretory material as the hormone(s) affected, rests on the similarity of the effects of this operation with those of other works in which neurosecretion was specifically involved. Nuñez (1956) and Altman (1956) have both demonstrated that injections of neurosecretory material promote water retention in insects. Hill (1963) has shown that the protein level of the haemolymph is low in adult *Schistocerca gregaria* under conditions of an inactive neurosecretory system. Thomsen & Møller (1959, 1963) have demonstrated that extirpation of medial neurosecretory cells of *Calliphora erythrocephala* has a profound effect on the synthesis of proteases in the mid-gut of this insect, but suggest that a general effect on protein metabolism has occurred and may be reversed by injections of corpus cardiacum extract. Protein synthesis has been shown to be in a steady state in diapausing pupae of *Cercopia samia*, a condition in which the neurosecretory system is 'silent' (Williams & Telfer, 1960).

The injections of corpus cardiacum extract into locusts deprived of the frontal ganglion cause growth in weight to be resumed and continued, but none of the animals show any signs of the formation of a new cuticle. This may argue against the principal factor being neurosecretion, as one of this hormone's most important effects is to activate the prothoracic glands. It is possible that the amount injected was not enough to stimulate these organs, perhaps because as Clarke & Langley (1963*d*) have suggested, the prothoracic gland is relatively insensitive to this hormone, the need for protein synthesis in the general body cells having the greater priority.

SUMMARY

1. Studies were made on the third, fourth-, and fifth-instar nymphs of *Locusta migratoria* L. from which the frontal ganglion had been removed, on control-operated animals, and on starved animals.

2. The effects of this operation on protein metabolism were observed by study of: electrophoresis of haemolymph proteins, chromatography of haemolymph amino acids, production of protease in the midgut, and the incorporation of ^{14}C-glycine into protein by the body cells.

3. The total protein concentration in the haemolymph of operated locusts did not increase with time as did that of the controls, in which the increase was almost entirely due to changes in the second of the three bands which normally separate out.

4. The concentration of the free amino acids in the haemolymph fell to about 70% of that found in the operated controls. In operated locusts the proportions of the amino acids relative to one another changed.

5. The incorporation of ^{14}C-glycine into protein was slower and the equilibrium concentration less in operated as compared with control-operated animals. The time taken to reach equilibrium was the same in both cases.

6. In both operated and control-operated animals the protease activity of the mid-gut wall was very low; no difference could be detected between them. The protease activity of the mid-gut contents expressed per mid-gut was lower in operated than in control-operated animals. The protease activity expressed per mg. was found to be the same in operated, control-operated and starved animals.

7. The hypothesis that the effects of the removal of the frontal ganglion were mediated through changes in the secretion of hormones from the corpus cardiacum was tested by giving daily injections of freshly prepared corpus cardiacum extract to locusts from which the frontal ganglion had been removed, and observing the growth in weight of these animals. A permanent increase in weight amounting to 100% of their initial weight was found. Animals injected with distilled water showed a temporary increase amounting to 30% of their initial weight. Uninjected animals maintained approximately constant weight.

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