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SOME EFFECTS OF THE REMOVAL OF THE
FRONTAL GANGLION ON METABOLISM IN

Locusta migratoria migratorioides R. and F.

by

Anthony Keith CHARNLEY, B.Sc. (Dunelm)

Being a thesis submitted for the
degree of Doctor of Philosophy of the
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Hatfield College
University of Durham

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GLOSSARY

ATP	adenosine triphosphate
BSA	bovine serum albumin
C	operated control animal
ca	corpus allatum
cc	corpus cardiacum
C ₁	curie
c.p.m.	counts per minute
DGL	diglyceride
d.p.m.	disintegrations per minute
E.D.T.A.	ethylene diamine tetraacetic acid
ffa	free fatty acid
fg	foregut
g	gram
hg	hindgut
J.H.	juvenile hormone
M	molar
mg	midgut
mnc	median/medial neurosecretory cell
Na ⁺ K ⁺ Mg ²⁺ ATPase	sodium, potassium activated magnesium dependent adenosine triphosphatase (E.C. 3.1.6.3.).
ncc	nervus corporis cardiaci
PAF	paraldehyde fuchsin
pna	para nitroaniline
pnp	para nitrophenol
S	starved
SPV	sulphophosphovanillin reagent
TGL	triglyceride
tris	tris (hydroxy methyl) amino methane
w/v	weight per volume

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ABSTRACT

A study has been made on the effects of the removal of the frontal ganglion from adult Locusta migratoria migratorioides R & F on various aspects of metabolism. A decrease in lipid and carbohydrate reserves was observed in the fat body of operated animals although this was less marked when compared with starved specimens. This decrease in fat body reserves was not due to an increased release into the haemolymph nor to increased utilisation. Indeed oxygen consumption was significantly lower than in operated controls.

The fine structural appearance of the fat body cells indicated a cessation of the normal functional development. This tended to be confirmed by the observation that fat body from operated animals showed a reduced ability to incorporate C^{14} glucose into triglycerides in vitro.

Six digestive enzymes have been characterised and the optimal conditions for assay employed in determining the effects of frontal ganglion removal and starvation on the activities of these enzymes in various regions of the gut. Both treatments resulted in a reduction in amount of enzyme activity. The distribution of enzyme activity was also affected such that the foregut of starved and frontal ganglionectomised treatments had a smaller proportion of the activity than in the control.

The fact that frontal ganglion removal effects a dramatic reduction in haemolymph volume was confirmed. Ultrastructural changes in the Malpighian tubules adds support to the suggestion that this reduced blood volume was a result of chronic diuresis. However, this was shown not to be due to an effect on the activity of the $Na^+K^+ATPase$ exchange pump.

Removal of the frontal ganglion was shown to result in a cessation of growth and to reduce or prevent altogether the release of neurosecretory material from the corpus cardiacum, confirming previous work on larvae and adults.

The above observations are discussed in the light of endocrine control of metabolism and the proposal that food passage through the gut is reduced in frontal ganglionectomised animals.

CHAPTER I

GENERAL INTRODUCTION

The characteristic feature of post embryonic development of insects is the occurrence of numerous moults or ecdyses. They involve the retraction of the epidermis away from the old cuticle, secretion of a new cuticle underneath and digestion of the old exoskeleton by hydrolytic enzymes. Finally, the thin remnant of the old cuticle splits open and is cast off as the insect increases its size by swallowing air. This expands the new temporarily elastic "skin" underneath. Frequently within a few hours the new cuticle is hardened by sclerotization (WATT, 1972).

The control of growth and moulting is co-ordinated by the activity of the neuroendocrine system. A moulting hormone, ecdysone, is released from the prothoracic gland in response to ecdysiotropin from the protocerebral neurosecretory cells in the brain (WIGGLESWORTH, 1972). Both larval and metamorphic ecdyses depend on ecdysone; which type will occur is determined by the presence or absence of juvenile hormone released from the corpora allata. In the absence of the latter the animal develops to the adult stadium.

The factors responsible for initiating the growth and moulting cycle are known only in a few species of insect. In Rhodnius prolixus, the initial secretion of the ecdysiotropin is caused by the distension of the abdomen which results from feeding. The distension stimulates receptors in the wall of the abdomen and these stimulate the neurosecretory cells via the nerve cord. At the same time impulses pass down the nervi corporis cardiaci following stimulation of the sensory cells in the ventral nerve cord (WIGGLESWORTH, 1934; VAN DER KLOOT, 1961). Previously KEMPER (1931) also implicated stretching of the abdomen in the initiation of moulting in the bed bug Cimex

lecturarius L. Both these insects get a full blood meal infrequently. Initiation of moulting by a distended abdomen ensures that ecdysis coincides with the animal having an adequate food supply. There is little information about the mechanisms which ensure the continuous sequence of growth and moulting cycles in insects which normally feed more or less continuously, and which do not encounter conditions liable to interrupt their growth. An exception is the work of CLARKE AND LANGLEY (1963 a, b, c, d, e) on the control of growth and moulting in Locusta migratoria L. They found that by interrupting the pathway between the pharynx and the brain by complete removal of the frontal ganglion or severance of both the frontal connectives there was an immediate cessation of growth as indicated by body weight. The latter remained more or less constant till death some 300 hours later (CLARKE AND LANGLEY, 1963d). A similar operation on adults prevented the development of sexual maturity (CLARKE AND LANGLEY, 1963a). In another paper CLARKE AND LANGLEY (1963e) undertook an examination of the neuroendocrine system in an attempt to throw further light on mechanisms of the control of growth in Locusta. Normal third instar larvae showed no indication of a cycle of growth and moulting either in the neurosecretory cells or in the corpora cardiaca. Neurosecretory material was only infrequently visible in the nervi corporis cardiaci 1 where they enter the brain. In marked contrast, frontal ganglionectomised and starved third instar larvae exhibited an accumulation of neurosecretory material in the axons of these nerves. This accumulation of neurosecretory material was first detected in starved animals 61 hours after the start of treatment, while in frontal ganglionectomised animals it was not detected until some 200 hours after removal of the ganglion. However, subsequent work by

CLARKE AND ANSTEE (1971b) has shown an accumulation of neurosecretory material within 72 hours of frontal ganglion removal from fourth and fifth instar larvae.

As a result of their studies CLARKE AND LANGLEY (1963e) proposed the following hypothesis to explain the control exerted by the neuroendocrine system on growth and moulting in Locusta.

During the course of a normal stadium when the insect feeds, the movements of the foregut stimulate the stretch receptors that CLARKE AND LANGLEY (1963d) have shown lying on its surface. Information from these sensory receptors passes via the frontal ganglion and frontal connectives to the brain. This initiates the synthesis, transport and eventually release of neurosecretory material from the corpora cardiaca. Throughout the instar the neurosecretion is required to control the metabolic processes of the animal essential in the utilisation of food and growth. Towards the end of each stadium the animal ceases feeding prior to moulting (CLARKE, 1956). However, movements of the foregut continue and may in fact be accentuated, firstly in the process of emptying of the gut of food and secondly in the swallowing of air which is a necessary preliminary to the shedding of the cuticle. As a result of this, information continues to pass from the stretch receptors to the brain and more hormone is synthesised and released. Since the hormone is no longer required for the metabolism of food material, its titre in the haemolymph increases until it reaches a critical point at which the prothoracic glands are stimulated.

Further CLARKE AND LANGLEY (1963e) suggested that frontal ganglion removal which caused a blockage of the normal release of neurosecretion and a cessation of growth would do so primarily by an effect on protein metabolism. Subsequently, work on protein metabolism in operated and

operated control animals has tended to confirm this view. CLARKE AND GILLOTT (1967a) have shown that frontal ganglionectomised Locusta larvae fail to increase the protein concentration in the haemolymph during the course of a stadium, while the free amino acid concentration falls to 70% of that in the operated control. The operated animal also showed a slower rate of incorporation of C^{14} glycine into tissue protein. In addition, CLARKE AND GILLOTT (1967b) found that the rate of incorporation of labelled nucleotides into cytoplasmic RNA from several tissues of operated animals was lower than operated controls. To explain the above facts they proposed that neurosecretory material suppresses the inhibitory feedback 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. It would act in a way comparable to the allosteric factor of MONOD, CHANGEUX AND JACOB (1963). An in vitro study of the ability of fat body from frontal ganglionectomised adult Locusts to incorporate C^{14} valine into protein (BIGNELL, 1974) adds further support to CLARKE AND LANGLEY (1963e) hypothesis that frontal ganglion removal reduces protein synthesis.

CLARKE AND ANSTEE (1971b) found that the ultrastructure of the fat body of operated animals was consistent with a reduced capacity for protein synthesis. The endoplasmic reticulum in operated animals consisted of numerous dilated, empty rough membrane bound cisternae and the mitochondria were swollen. Similar symptoms have been recorded in rat liver following hypophysectomy (CARDELL, 1967), an operation which also results in cessation of growth and less food consumption by the animal (BARTLETT ET AL, 1956). Swelling of this type is also found following treatment with antibiotics that are known to have an effect on protein synthesis eg. puromycin and actinomycin D (JURAND AND JACOB, 1965; GAMBETTI ET AL, 1968).

In view of the evidence outlined above for the effect of frontal ganglion removal on growth and protein metabolism, it is surprising that no effect was found on oxygen consumption per mg wet weight (CLARKE AND LANGLEY, 1963d; CLARKE AND ANSTEE, 1971a). However, CLARKE AND ANSTEE (1971a) suggest that the decrease in oxygen consumption per mg seen in both operated and operated control animals come about for different reasons. In the control, oxygen consumption decreases with increasing size and age, and is attributable to the increase of non respiring tissue components common to all growth processes. By contrast operated animals do not exhibit an obvious accumulation of reserves or other non respirable material. Therefore, they conclude that frontal ganglion removal has in fact resulted in a reduced overall metabolic rate. The respiratory rate of tissues from the operated animal would appear to be much higher than expected in a non growing animal. This apparent increase may be correlated with the increased activity of succinic dehydrogenase found and thus with the swollen and presumably increased permeability of the mitochondria, allowing greater interactions between enzyme and its substrate (CLARKE AND ANSTEE, 1971a). CLARKE (1965) suggested that since the oxygen consumption of operated animals is unchanged and they appear to have normal activity then the energy cycles are largely untouched. He explained the reduced physiological state of the operated animal on the hypothesis that the normal increase in mRNA production in body cells could not occur in the absence of neurosecretory material. However, if this hypothesis were correct, then the production of all protein should be affected including the enzymes involved in carbohydrate and lipid metabolism.

In the last 10-15 years there has been a tremendous increase in our understanding of the endocrine control of metabolism (reviewed

in the introduction to Chapter 3). Endocrine factors in the brain and corpora cardiaca have been shown, that influence carbohydrate, lipid and protein metabolism (WYATT, 1972; HILL, 1972). Extracts from storage and glandular lobes of the corpora cardiaca have been shown to affect such diverse functions as heart beat and excretion (BROWN, 1965; MORDUE AND GOLDSWORTHY, 1969). Frontal ganglion removal has been shown to result in a dramatic reduction of neuro-secretory release from the storage lobe of the corpora cardiaca. However, it has not been possible to show histologically that release of endocrine factors from the glandular lobe is similarly affected. The latter lobe is the site of synthesis and storage of carbohydrate and adipokinetic hormones (HIGHNAM ET AL, 1971; GOLDSWORTHY, MORDUE AND GUTHKELCH, 1972). If frontal ganglion removal affects the release of hormones from the glandular lobes, then a direct effect on carbohydrate and lipid metabolism may be expected.

Diuretic and antidiuretic factors affecting the function of both Malpighian tubules and rectal sac have been found in the storage and glandular lobes respectively of the corpora cardiaca of Locusta (GAZAL AND GIRARDIE, 1968; MORDUE AND GOLDSWORTHY, 1969; MORDUE, 1970a). Therefore it is not surprising that removal of the frontal ganglion has been shown to affect water balance. HILL ET AL (1966) found a dramatic reduction in haemolymph volume and tissue water following frontal ganglionectomy in Schistocerca gregaria larvae. Similar observations have been recorded by ROOME (1968) and GILLOTT (1965) in operated Locusta larvae.

The present investigation has been made on adult Locusta migratoria migratorioides R & F, phase gregaria, to investigate the role of the frontal ganglion in the control of intermediary metabolism and water-balance.

GENERAL MATERIALS AND METHODS

1. Maintenance of the insects

A. Insectary

An insectary was maintained at a temperature of $28 \pm 0.5^{\circ}\text{C}$ and a relative humidity of $50 \pm 5\%$. Circulation of air was effected by two large electric fans and slight continuous air exchange was obtained with two small ventilators. A constant photoperiod of 12 hours light and 12 hours dark was maintained.

B. Stock animals

Populations of Locusta migratoria migratorioides R & F, phase gregaria (specimens of which were originally supplied by Philip Harris, Biological Supplies) were reared in perspex fronted cages, consisting of dexion angled metal framework with aluminium top and sides (43cm x 58cm x 58cm). There was a "false" floor to each cage made of perforated aluminium. This contained four holes into which paper cups filled with sand were put, and into which female locusts placed their egg pods. This false floor was separated from the true floor by a space 10cm high. The faeces from the larvae passed through the holes in the false floor into the space beneath.

Each cage was illuminated by a single 40 watt bulb. Food in the form of bran and grass was supplied daily. There was considerable local variation in temperature and humidity within the stock cages. The temperature sometimes increased to 32°C on the sides of the cage, close to the bulb. When the food was fresh, the relative humidity also increased.

Throughout their development and during experiments, animals were reared at sufficiently high density to ensure their remaining "gregarious" (JOLY AND JOLY, 1953).

C. Experimental animals

In general experimental animals were kept in cylindrical cages made of aluminium and acetate sheet (144cm² x 40cm) and provided with wooden perches. However, when daily measurements were required for individual animals, they were kept in individual plastic boxes (9cm x 9cm x 16cm) with a lid of fine gauze. Starved animals were always kept in this way, so as to prevent cannibalism. It was assumed that any change of phase occurring during the experiment was insignificant (GILLOTT, 1965).

Fresh grass was given daily but no bran; the absence of which had no apparent effect on the normal development of the insect.

No special illumination of experimental cages was provided, and although the light intensity and local temperature were, therefore, lower than in stock cages, the insects did not appear to be affected. As in the stock cages the relative humidity was rather variable.

D. Sampling technique

Cages containing late fifth instar larvae were sampled each day between 1500 - 1700 hours and newly moulted adult locusts (12 hours \pm 12 hours old) were removed and divided randomly into four groups or treatments as indicated below:

1. Newly moulted refers to adults aged 12 hours \pm 12 hours after the final ecdysis.
2. Operated animals refers to newly moulted adults that were kept in the laboratory for 24 hours without food, following removal of the frontal ganglion. A further 24 hours in the laboratory with food was followed by 3 days in the insectary.
3. Operated control animals refers to newly moulted adults which were sham operated and treated as above. They behaved essentially as normal locusts (GILLOTT, 1965; ANSTEE, 1968).

4. Starved animals refers to unoperated animals deprived of food directly after the last moult. Otherwise they were treated identically to those of groups 2 and 3 above.

Unless otherwise stated equal numbers of males and females were used for each treatment.

II. Removal of the frontal ganglion by electrical cautery

The animal was anaesthetised for two minutes by placing it in a jar containing a pad of cotton wool on which a little diethyl ether was poured.

The procedure used for exposing the frontal ganglion was that described by Langley (1962). The anaesthetised locust was placed in a perspex box with its head wedged through a hole in the top with plasticine (Fig. 1.1/1). An inverted U-shaped cut was made in the cuticle of the frons using a micro scalpel (Fig. 1.1/2). The resulting flap was folded back and held with a strip of plasticine to expose three large air sacs underneath (Fig. 1.1/3). Parting these air sacs revealed the frontal ganglion below (Fig. 1.1/4).

A Martin Electromedicine Elektrotom 60 high frequency surgery unit, was used to cauterise the ganglion. The machine was set on the lowest current and a 1 second "burst" through a fine tungsten needle touching the ganglion was sufficient to remove it without damaging any neighbouring tissues. Operated control animals were treated similarly but the gland was merely touched with the "cold" tungsten needle and not burned out.

All instruments used for the operation had been sterilised for several days in a dust free cabinet containing an ultra violet light source.

A 100% recovery from the operation was achieved, i.e. all operated and operated control animals were alive upon their return to the insectary 48 hours later.

Measurement of Growth

A permanent increase in body weight was taken as an indication that growth had occurred. Animals used were weighed at the time of sampling (1500 - 1700 hours) and immediately before being killed on a torsion balance (range 0 - 2000mg). This provided a means of checking whether an animal had grown during the course of an experiment.

From time to time a check was made on the efficiency of the cautery procedure, by sectioning heads fixed in Bouin's and looking for the remains of the frontal ganglion. None were found, nor was any regeneration observed.

III. Treatment of glassware

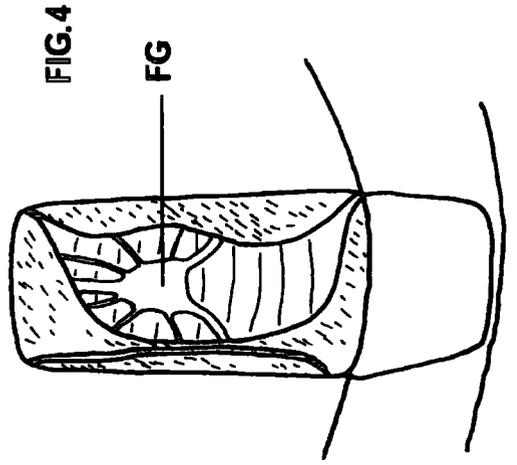
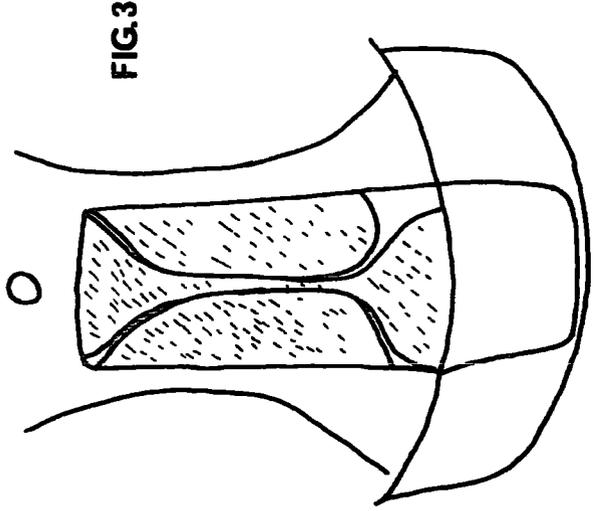
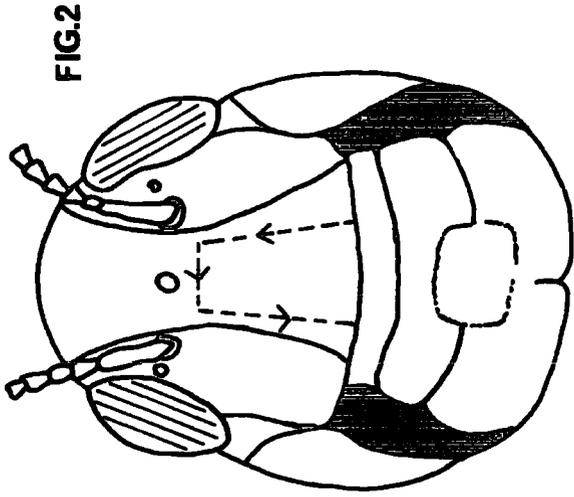
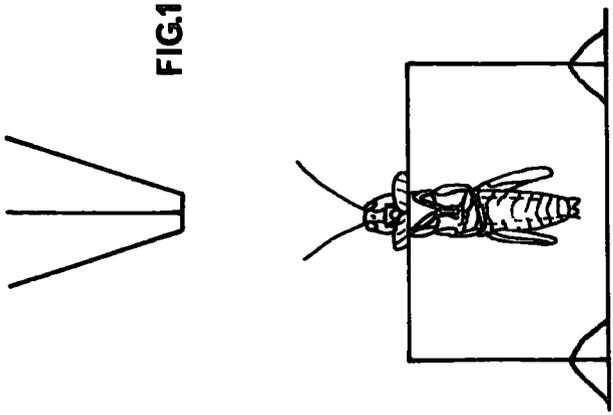
All glassware was soaked overnight in a 2% (W/V) solution of "Quadralene" laboratory detergent, rinsed six times in hot tap water and six times in distilled water. Glassware was then oven dried. Glass/Teflon homogenisers and polycarbonate centrifuge tubes were allowed to drain at room temperature.

All glassware used in lipid work was soaked in 50% aqueous HNO_3 (Anala R grade) then rinsed as above. Chromatography tanks and plates were left to drain at room temperature, while the rest of the glassware was dried in an oven.

Fig. 1.1

Procedure for exposing the frontal ganglion
prior to its removal by electrical cautery

1. An anaesthetised locust was placed in a perspex box with its head wedged through a hole in the top with plasticine.
2. An inverted U-shaped cut was made in the cuticle of the frons using a micro scalpel.
3. The resulting flap was folded back to expose three large air sacs underneath.
4. Parting these air sacs revealed the frontal ganglion underneath. (F G)



CHAPTER 2

The effect of frontal ganglion removal by electrical cautery on growth and the histology of the neuroendocrine system in young adult Locusta migratoria

Introduction

Previous work has shown that frontal ganglionectomy in larval Locusta migratoria stops normal growth and moulting (CLARKE AND LANGLEY, 1963d; ROOME, 1968). Third instar larvae subjected to the operation 12 hours and 33 hours after the previous moult lost weight initially, thereafter a slight increase was shown over the next 100 hours. This weight was maintained for a further 100 - 150 hours until the animals died. Removal of the ganglion from older third instar larvae had an increasingly more dramatic effect. Those operated on 96 hours after the moult died 60 hours later (CLARKE AND LANGLEY, 1963d). HILL ET AL (1966) and STRONG (1966) have shown a similar cessation of growth in adult Schistocerca and Locusta respectively following frontal ganglionectomy.

It has been proposed that this effect is mediated through the endocrine system, the operation greatly reducing or preventing altogether the release of neurosecretion from the corpus cardiacum (CLARKE AND LANGLEY, 1963e). More recently the removal of the ganglion has been shown to result in an abnormal appearance of the corpora allata in adult Schistocerca gregaria (HIGHNAM ET AL, 1966), adult Locusta (STRONG, 1966), and fourth and fifth instar Locusta larvae (CLARKE AND ANSTEE, 1971b).

ROOME (1968) suggested that frontal ganglion removal by surgery might remove different nerves from time to time. Hence in the present work electrical cautery was employed as a more controlled method of ablating the ganglion. Therefore, it was necessary to check that

the effects of this operation in terms of growth and appearance of the neuroendocrine system were not different to those observed after surgical removal.

Materials and Methods

Newly moulted adult locusts (12 hours \pm 12 hours after the last moult) and 2 day old locusts (36 hours \pm 12 hours after the last moult) were weighed on a torsion balance (0 - 2000mg) and then operated on. The operation and treatment of experimental animals were as described in the General Materials and Methods section (Chapter 1). All reagents used were Anala R grade or purest available and supplied by British Drug Houses and Sigma.

Histology of the stomatogastric nervous system

Animals were killed by decapitation and the heads fixed in Bouin's fluid under a vacuum (15lb per sq. inch) to facilitate penetration of the solution. The heads were then stored in fresh fixative until ready for use. At this time they were placed in 70% ethanol and the muscle and cuticle dissected away. The dissected heads were then washed clear of fixative and dehydrated by passage through an alcohol series in an Elliot Automatic tissue Processor:

1. 70% ethanol 1 hour
2. 2 washes of 95% ethanol 1 hour each
3. 2 washes of absolute ethanol 1 hour each

The dehydrated heads were cleared in chloroform:

1. Absolute ethanol: chloroform (1 : 1) 1 hour
2. 2 washes in chloroform 1 hour each

Finally they were embedded in paraffin wax. Serial sections were cut at 10 μ m on a Spencer '820' microtome. These were stained for neurosecretory material by means of a modification of Gomori's paraldehyde fuchsin technique (EWEN, 1962) see appendix (Section 2).

Measurement of faecal production

Pellets were collected daily at 1700 hours and dried for 24 hours at 100°C and weighed.

Results

The effect of frontal ganglion removal by electrical cautery on growth in young adult *Locusta migratoria*

Operated Control, Operated and Starved animals were weighed daily for 5 days after the operation. Figs. 2.1 - 2.4 show the growth curves, with weight expressed as a percentage of the original, preoperative weight. Operated and control males operated on 12 hours \pm 12 hours after the last moult both showed an initial loss of body weight in the first twenty four hours after treatment. This was due to defaecation following the operation and the imposed period of starvation during post operative care. Control animals started putting on weight as soon as feeding was resumed 24 hours after the operation and achieved a weight on day 5 which was 112% of that recorded prior to the operation. Operated males on the other hand maintained their weight at some 95% of the original. By comparison with the other two treatments, the starved animals kept losing weight until by the 5th day they were only 73% of the original weight. Death occurred following 5 - 6 days of starvation. (Fig. 2.1).

Female locusts 12 hours \pm 12 hours after the last moult responded in a similar fashion to the males to operative treatments and starvation (Fig. 2.2). However, it took control female locusts 3 days rather than the 2 days in males to recoup the initial loss of weight which occurred following the operation.

Figs. 2.3 and 2.4 show comparisons between the effect of the operation on newly moulted locusts and 2 day old locusts. Operated controls of both ages, from either sex, responded similarly to the operation. However, both 2 day old females (Fig. 2.4) and 2 day old males (Fig. 2.3) showed a more marked loss of weight following the cautery of the frontal ganglion than did newly moulted animals. This was probably due to the fact that after 2 days there was more food present in the gut and defaecation of this would result in an apparent

greater loss in weight. Male animals operated on day 2 exhibited a steady decline in weight so that 5 days after the operation they were only 81% of the preoperative weight.

In one experiment the growth of operated and operated control newly moulted adults was followed for an extended period. Fig. 2.5 shows the growth curves of one typical control and one typical operated female. 12 days after the operation only 20% of the operated animals were alive but 100% of the operated controls. This compared with 75% of operated animals up to 5 days after the operation.

It was observed that after an initial decrease in weight the control animal started to put on weight, by the 12th day it was 166% of the preoperative weight. The operated female lost weight for the first 48 hours after the operation and did not recover the loss until the 5th day. No further growth was observed and the animal maintained its weight at approximately the preoperative weight until death occurred 12 days after the operation.

The effect of removal of the frontal ganglion by cautery on faecal production

Fig. 2.6 shows the effect of the operation on faecal production by female locusts operated on 12 hours \pm 12 hours after the last moult. Both operated and operated control treatments exhibited a low faecal production until the animals were returned to the insectary 2 days after the operation. Control animals showed a dramatic increase from 20mg to 160mg dry weight of faeces between days 2 and 4. Faecal production levelled off on the 5th day after the operation. However, the operated animals had only a small increase from 10mg up to 60mg dry weight (Fig. 2.6).

Dissections of operated males and females five days after the operation revealed that extensive packing of food had taken place in

the foregut, which was consequently greatly distended (Plate 2.1). In addition a reduction was observed in the amount of material found in mid and hindguts, while in some animals there was none at all. In operated controls, although there was only a moderate amount of food in the foregut, the mid and hindguts were full. Also the midgut caecae of operated animals were small and appeared shrunken whereas in the operated control they were much larger.

The effect of frontal ganglion cautery on the histology of the neuroendocrine system of newly moulted adult male locusts

The secretory cells of the pars intercerebralis of the operated control presented a constant picture (Plate 2.2c). There were always many 'A' cells present, which contained small numbers of dark staining purple granules (paraldehyde fuchsin (PAF) positive material), while a few cells packed with this material could always be found. The presence of the PAF positive granules was taken to indicate the presence of neurosecretory material (LANGLEY, 1962).

The pars intercerebralis of operated and starved animals were not significantly different in appearance from those of the operated controls (Plate 2.2B and D).

Little stainable 'A' material was found in the nervi corporis cardiaci (ncc 1) of operated control locusts where they leave the brain or enter the corpora cardiaca (Plate 2.3A). This confirmed HIGHNAM'S (1961) observations on young adult Locusta. However, examination of these nerves from cauterised or starved animals revealed an accumulation of stainable material in their axons (Plate 2.3, B & C). The axon tracts of the ncc 1 within the brains of operated animals were also often deeply stained with PAF material (Plate 2.2A). It would appear, therefore, that as with larvae (CLARKE AND LANGLEY, 1963e) operated

control adults were continuously secreting and releasing neurosecretory material and that this release was reduced or halted in operated and starved animals.

The corpora cardiaca of operated and operated control animals were similar in appearance, large amounts of stainable material being found in the anterior lobes, particularly in that area of the gland in association with the aorta wall (Plate 2.3 A and B). However, this gland in starved animals appeared more darkly stained and dense in general appearance.

The structure of the corpora allata corresponded to that described by ODHIAMBO (1966c) for Schistocerca gregaria and CLARKE AND ANSTEE (1971b) for 4th and 5th instar Locusta. However, the corpora allata from operated control animals did not show any signs of activity (Plate 2.4A). No mitotic figures were observed and few secretory vacuoles. In contrast to the situation in larvae (CLARKE AND ANSTEE, 1971b) removal of the frontal ganglion from adults had only a small effect on the morphology of the corpora allata. In some operated animals a reduction in size of these glands was observed which was accompanied by a decrease in the cytoplasm/nucleus ratio (Plate 2.4c). However, in others there was no such decrease in size (Plate 2.4d). The operation did not lead to the gross misshapen appearance of the corpora allata seen in larvae (CLARKE AND ANSTEE, 1971b). Both small and giant nuclei were present in operated control and operated animals and no difference was observed in their distribution or appearance between treatments. In starved animals the corpora allata were consistently smaller than those found in operated controls and there was a reduction in the cytoplasm/nucleus ratio.

Fig. 2.1

Effect of the removal of the frontal ganglion
on the growth of newly moulted adult male
locusts 12 hours \pm 12 hours after the last moult

Legend: ordinate ; body weight expressed as a % of the
original weight

abscissa ; days after the operation

■ operated control animals

▲ operated animals

● starved animals

figures in parenthesis denote number
of animals

n.b. only those animals included that
survived for 5 days

vertical lines denote 1 S.E. of the mean

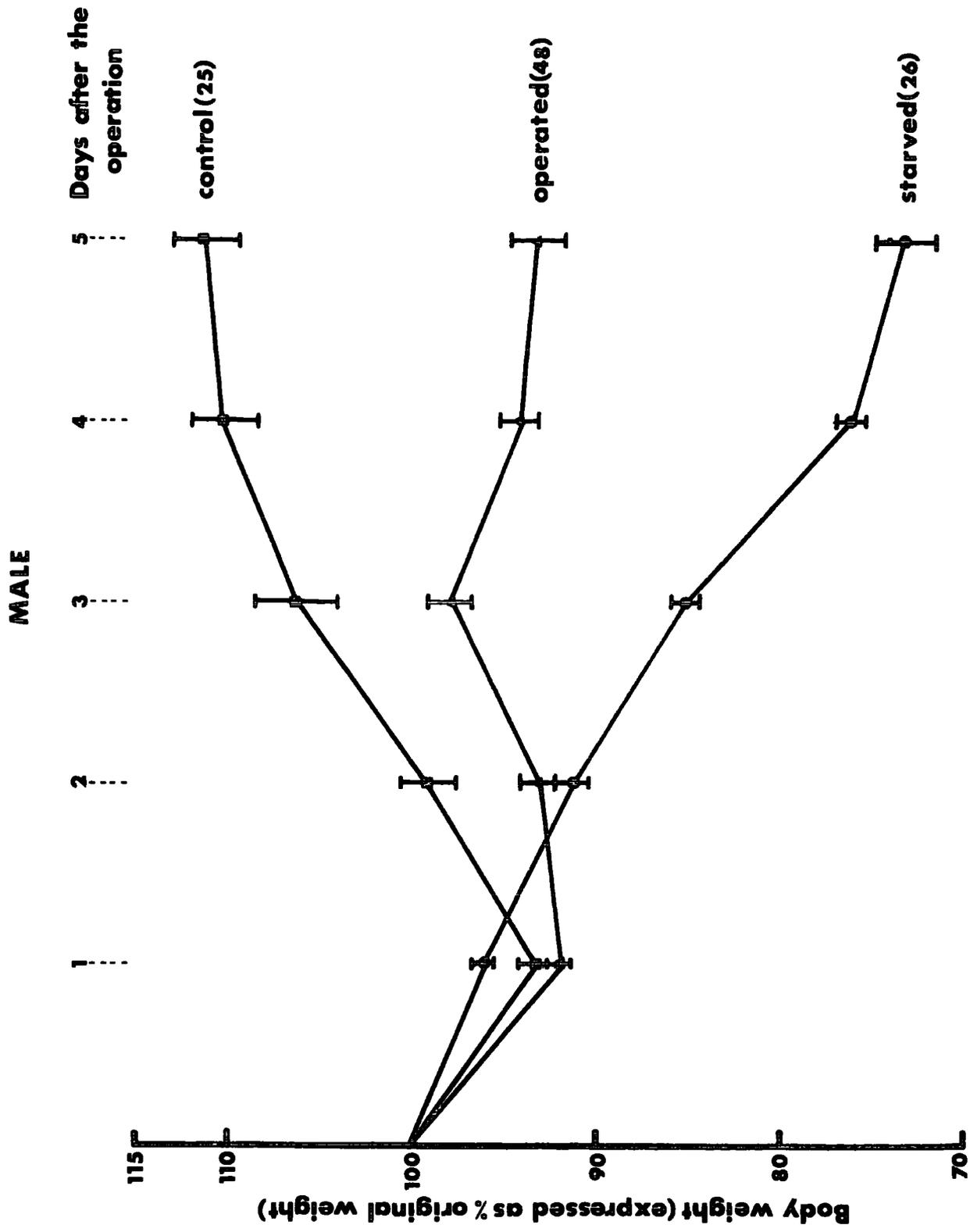


Fig. 2.2

Effect of the removal of the frontal ganglion
on the growth of newly moulted adult female
locusts (12 hours \pm 12 hours after the last moult)

Legend: ordinate ; body weight expressed as % of the
original weight

abscissa ; days after the operation

■ operated control animals

▲ operated animals

● starved animals

figures in parenthesis denote number
of animals

n.b. only those animals included that
survived for 5 days

vertical lines denote 1 S.E. of the mean

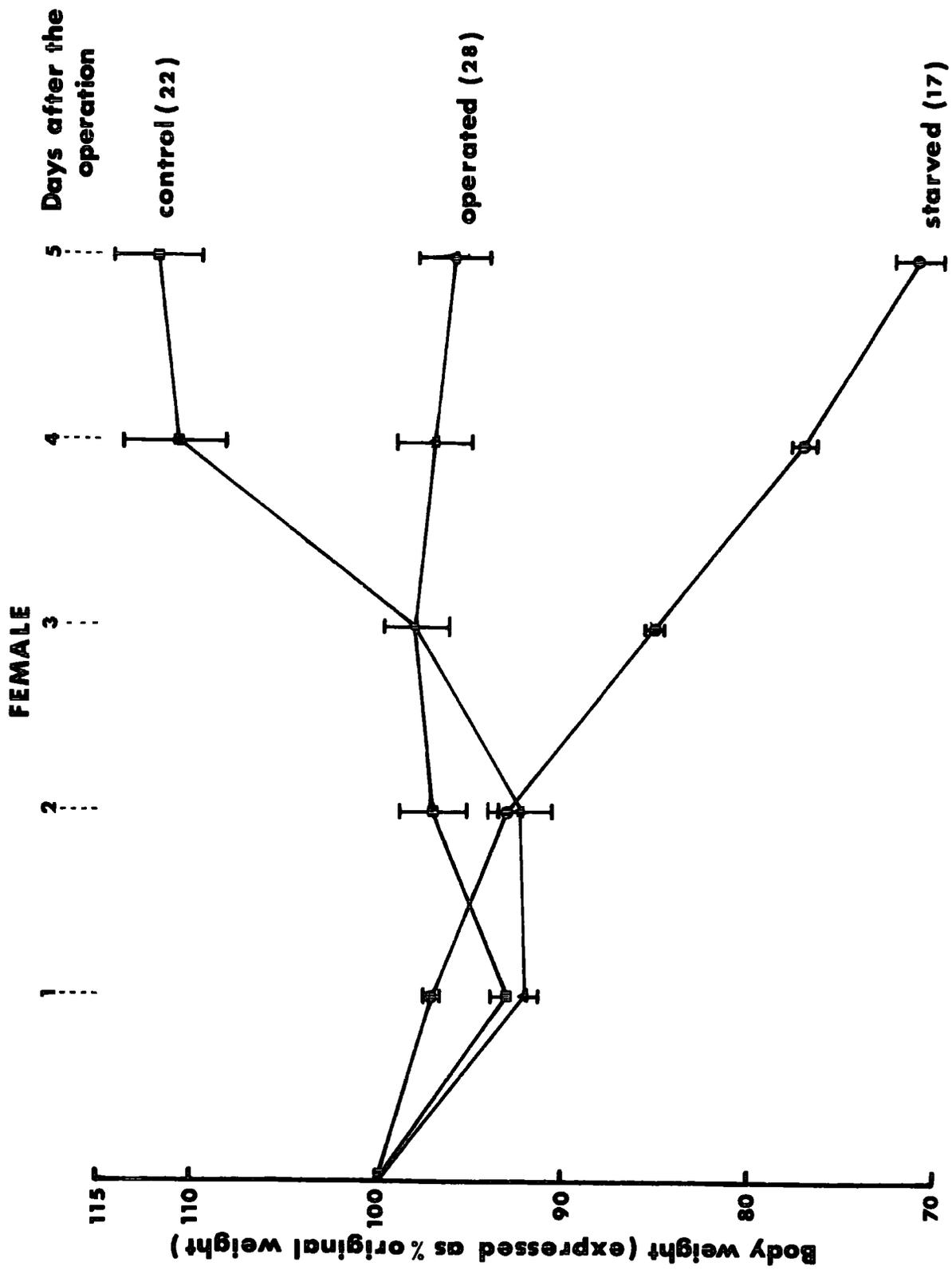


Fig. 2.3

A comparison between the effect of the removal of the frontal ganglion on the growth of newly moulted adult male locusts (12 hours \pm 12 hours after the last moult) and on the growth of 2 day old adult male locusts (36 hours \pm 12 hours after the last moult)

Legend: ordinate ; body weight expressed as a % of the original weight

abscissa ; days after the operation

○ 12 hours old \pm 12 hours

⊙ 36 hours old \pm 12 hours

figures in parenthesis denote number of animals

n.b. only those animals included that survived for 5 days

vertical lines denote 1 S.E. of the mean

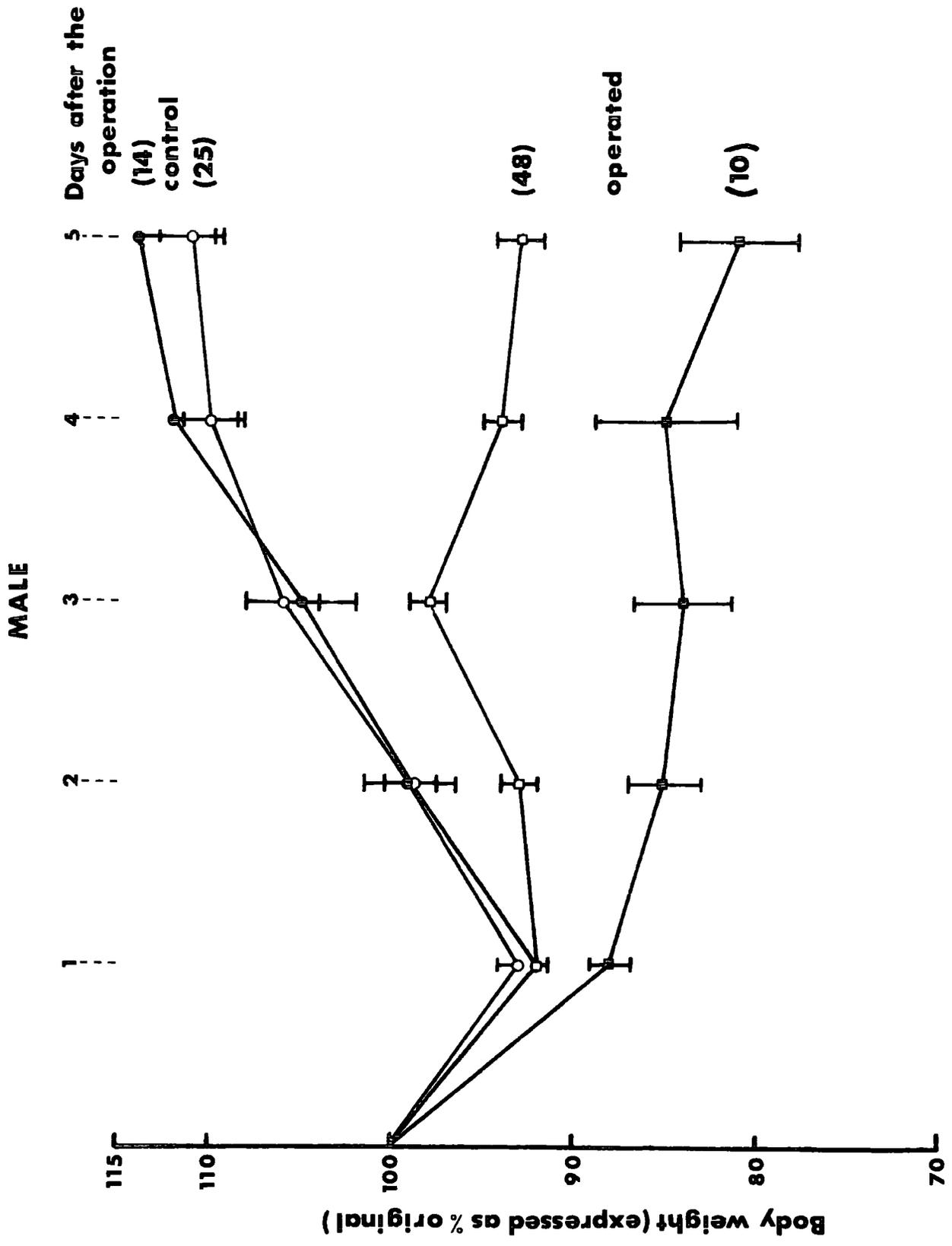


Fig. 2.4

A comparison between the effect of the removal of the frontal ganglion on the growth of newly moulted adult female locusts (12 hours \pm 12 hours after the last moult) and on the growth of 2 day old adult female locusts (36 hours \pm 12 hours after the last moult)

Legend: ordinate ; body weight expressed as % of the original weight

abscissa ; days after the operation

□
○ 12 hours old \pm 12 hours

●
■ 36 hours old \pm 12 hours

figures in parenthesis denote number of animals

n.b. only those animals included that survived for 5 days

vertical lines denote 1 S.E. of the mean

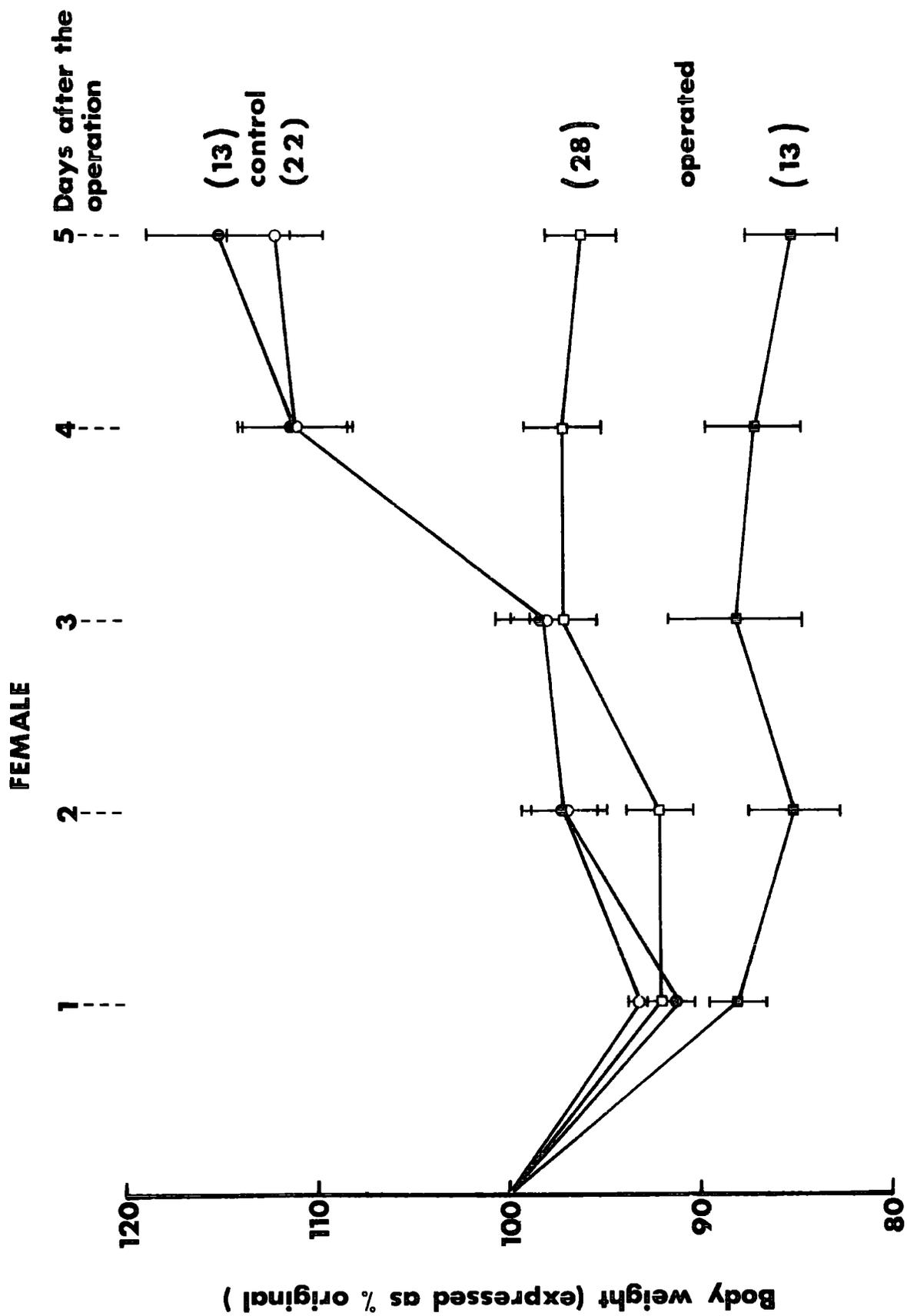


Fig. 2.5

Effect of the removal of the frontal ganglion on the growth of a newly moulted female locust (12 hours \pm 12 hours after the last moult) over an extended period of time)

Legend: ordinate ; body weight expressed as % of the original weight

abscissa ; days after the operation

■ operated control female

● operated female

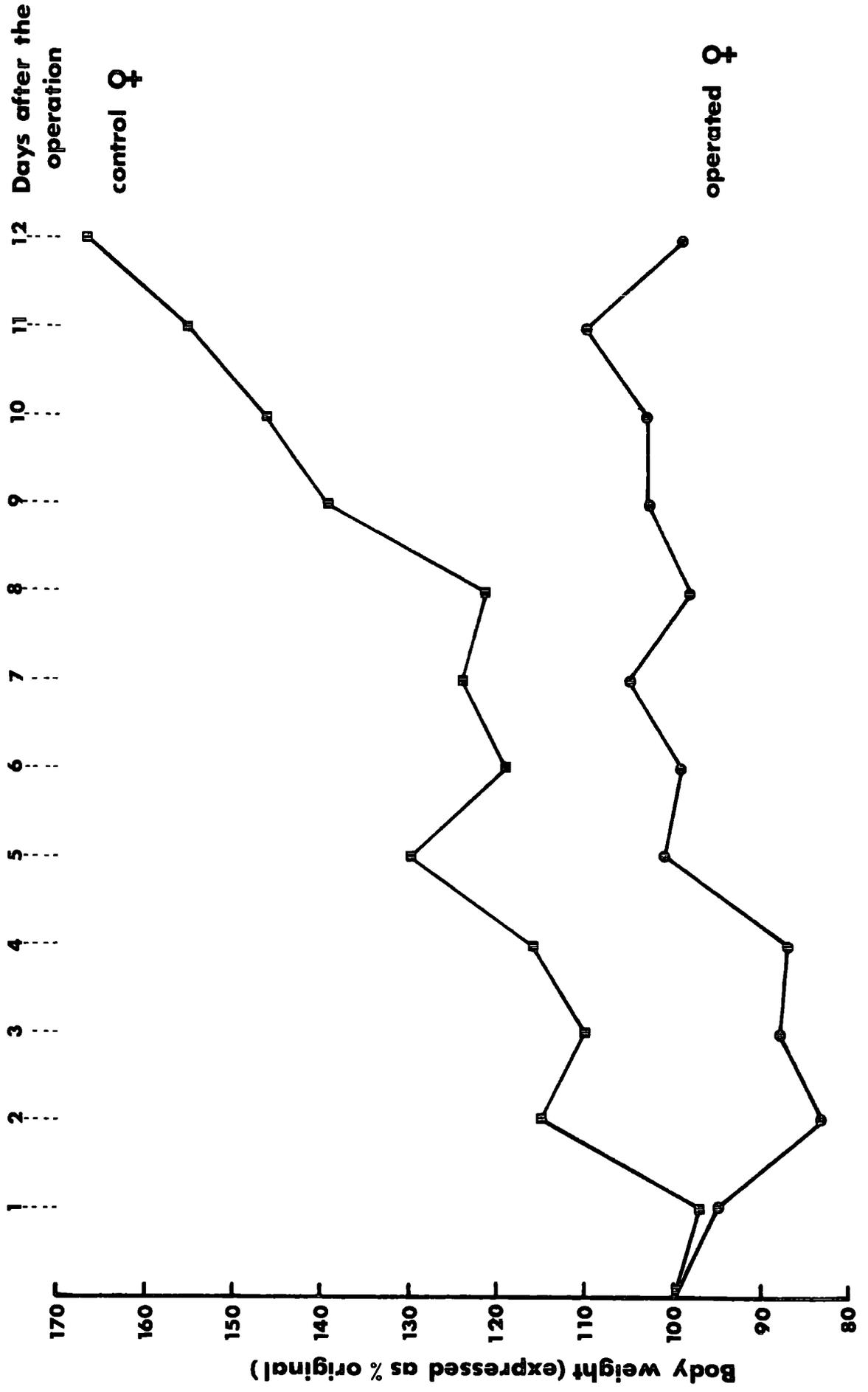


Fig. 2.6

Effect of the removal of the frontal ganglion on the
faecal production of newly moulted adult female locusts
(12 hours \pm 12 hours after the last moult)

Legend: ordinate ; faeces produced per day expressed in
mg dry weight

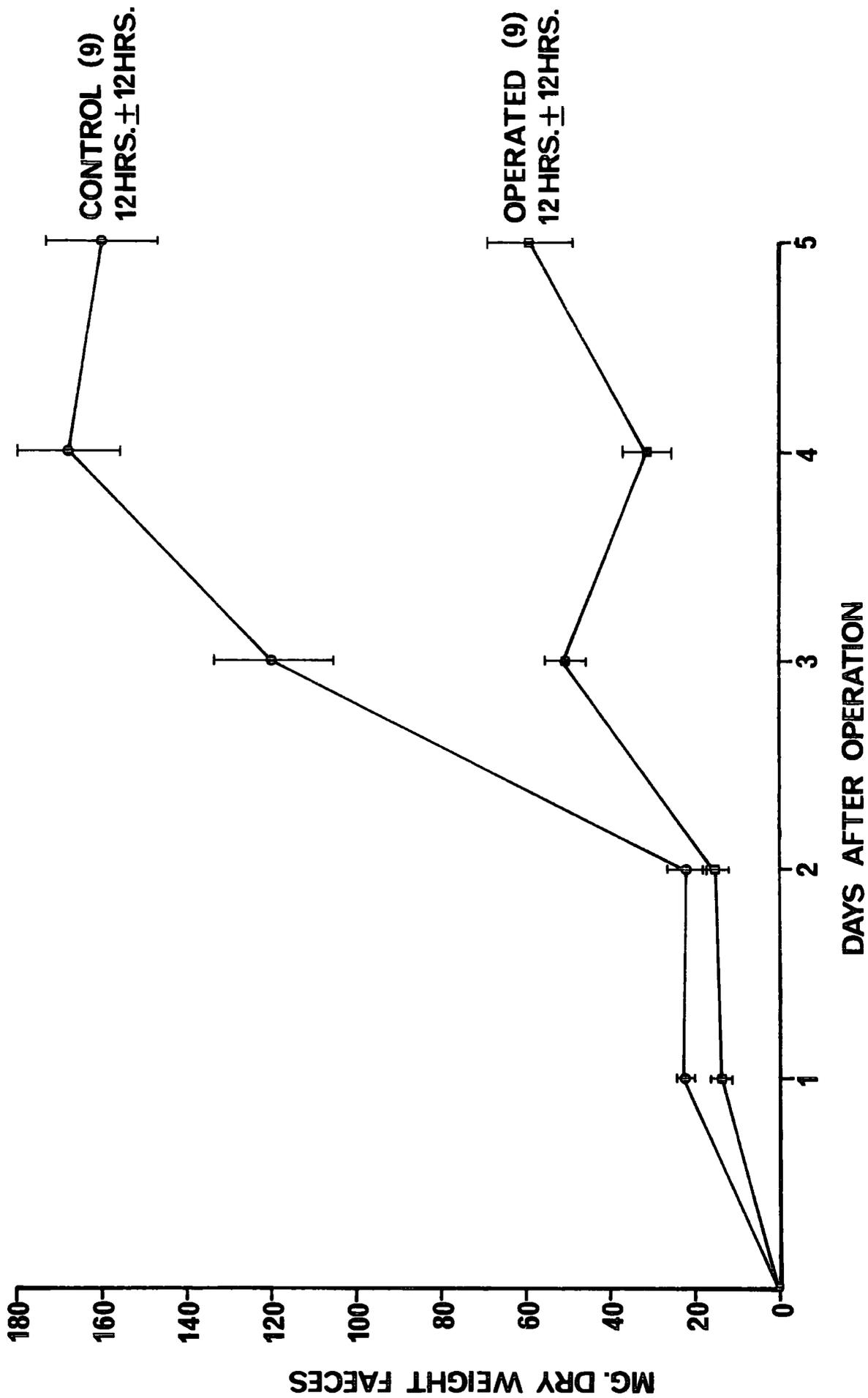
abscissa ; days after the operation

⊖ operated control animals

■ operated animals

figures in parenthesis denote number
of animals

vertical lines denote 1 S.E. of the mean



Effect of the removal of the frontal ganglion from newly moulted adult female locusts (12 hours \pm 12 hours after the last moult) on the appearance of the gut

Plate 2.1

Legend: O 1 & 2 operated animals
 C 1 & 2 operated control animals
 fg foregut
 mg midgut
 mc midgut caecae
 hg hindgut

In both the guts from operated animals the foregut is distended and packed with food by comparison with those of the operated controls. In O2 the foregut besides being distended is twisted cork screw like in appearance. The midgut caecae are small and there is little food in the midgut or hindgut. In O1 the midgut caecae are again small but although little food is present in the hindgut a lot is present in the midgut.

In both the guts from control animals the midgut caecae are large and prominent, and there is plenty of food in midgut and hindgut.

Scale: 5mm

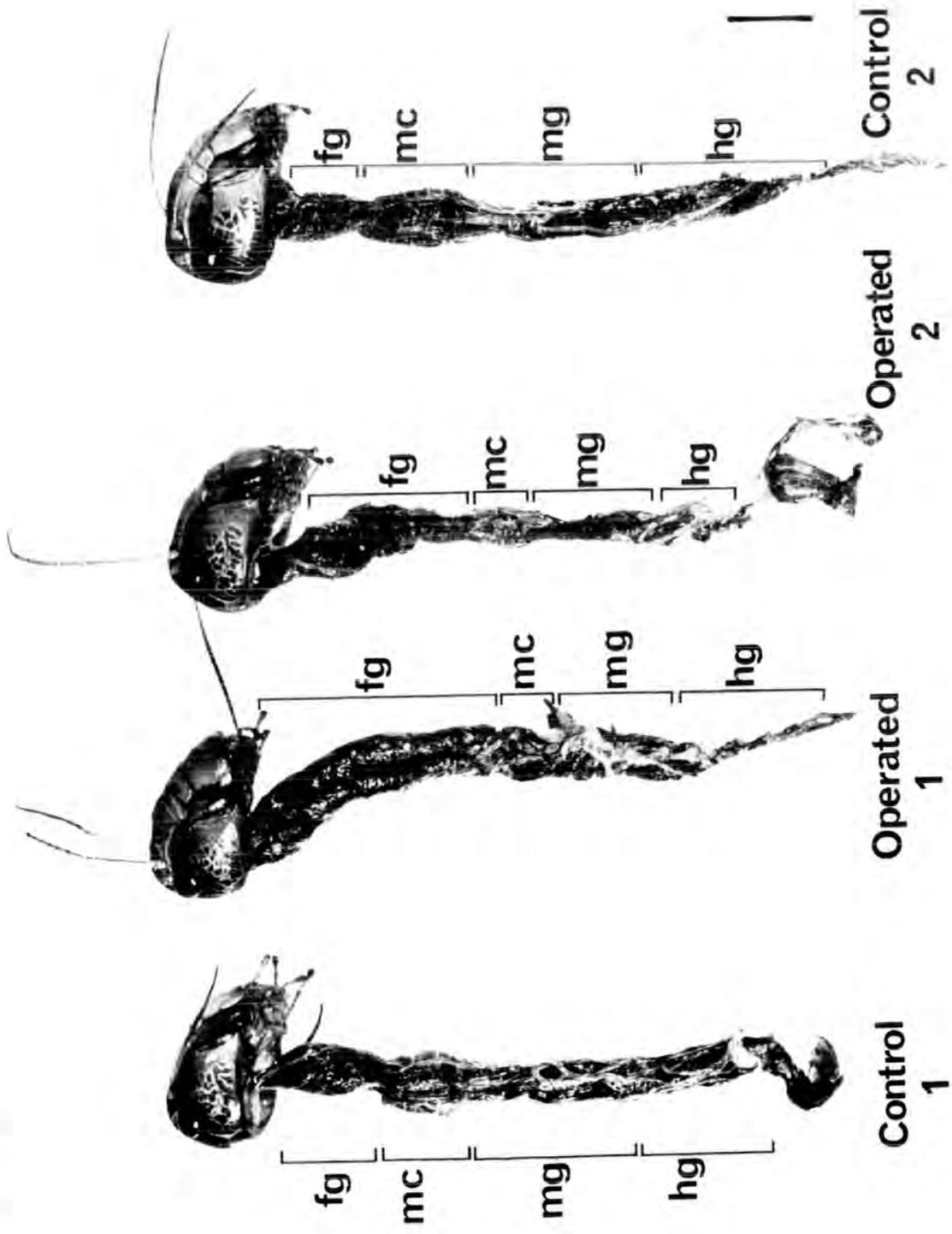


Plate 2.2

A. T.S. part of the brain of an operated adult male showing the axons of the nervi corporis cardiaci 1 (a NCC1) at the point where they cross over in the brain (chiasma). There is more stainable neurosecretory 'A' material in these axons in the operated animals than in the other treatments.

B. T.S. part of the brain of an operated adult male showing the pars intercerebralis. Note the neurosecretory 'A' material in the median neurosecretory cells (MNC). A large amount of stainable neurosecretory 'A' material is seen in the nervus corporis cardiaci 1 at the point where it leaves the brain(NCC1).

C. T.S. part of the brain of an operated control adult male showing the pars intercerebralis. Note the neurosecretory 'A' material in the median neurosecretory cells (MNC).

D. T.S. part of the brain of a starved adult showing the pars intercerebralis. Note the neurosecretory 'A' material in the median neurosecretory cells (MNC).

Scale: 100 μ m

A.



B.



C.



D.

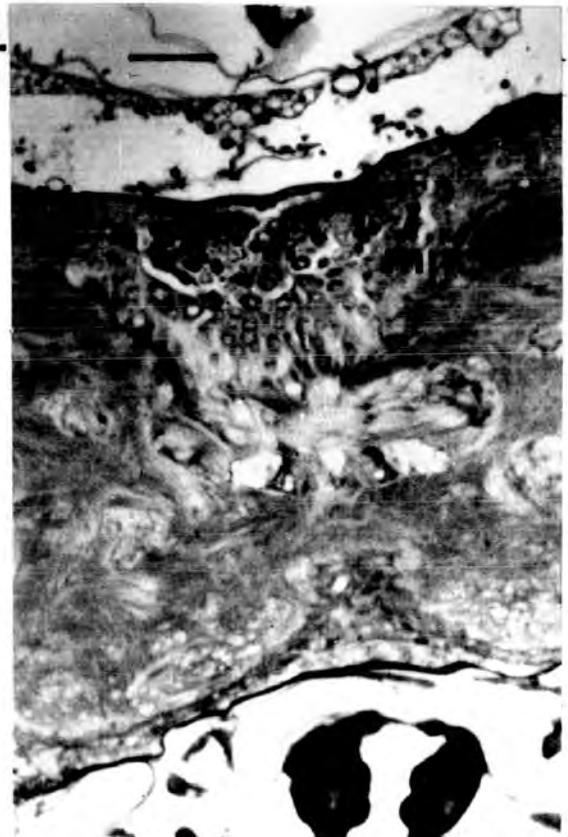


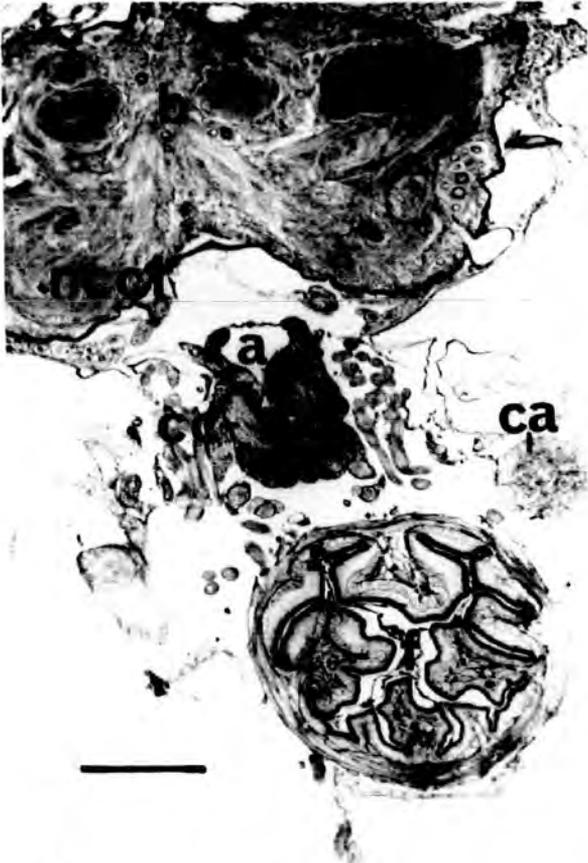
Plate 2.3

A. T.S. of the corpora cardiaca from an operated control adult male showing the absence of neurosecretory material in the nervi corporis cardiaci 1 (NCC1), note the corpus allatum (CA), aorta (a), foregut (f), corpora cardiaca (cc) which contain dark granules of neurosecretory material in their anterior lobes, brain (b).

B. T.S. of the corpora cardiaca from an operated adult male showing the accumulation of neurosecretory material in the nervi corporis cardiaci 1 (NCC1), note the corpora cardiaca (CC), brain (b), foregut (f).

C. T.S. of the corpora cardiaca from a starved adult male showing the accumulation of neurosecretory material in the nervi corporis cardiaci 1 (NCC1), note the brain (b), foregut (f), corpora cardiaca (cc).

Scale : 200µm



A.



B.



C.

Plate 2.4

Sections shown are the largest found during serial sectioning of the gland.

- A. T.S. corpus allatum from an operated control adult male, nucleus (n), giant nucleus (gn).
- B. T.S. corpus allatum from a starved adult male note: small size, with lower cytoplasm to nucleus ratio than operated control, nucleus (n), nervus corporis allati (na).
- C. T.S. corpus allatum from an operated adult male note: small size, with lower cytoplasm to nucleus ratio than operated control, nucleus (n).
- D. T.S. corpus allatum from an operated adult male note: similar size and cytoplasm/nucleus ratio to the operated control, nucleus (n), giant nucleus (gn), nervus corporis allati (na).

Scale: 50μm

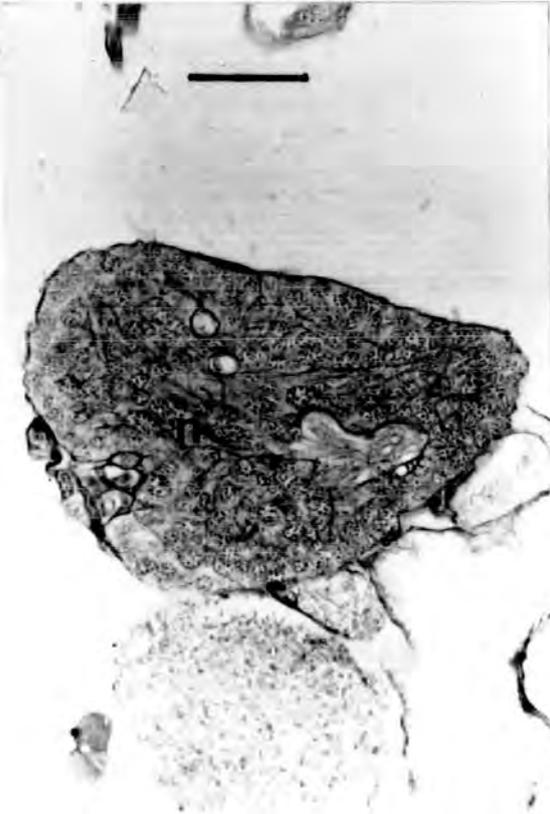
A.



B.



C.



D.



Discussion

Work presented in this chapter showed that removal of the frontal ganglion from young adult locusts by electrical cautery had the same effect on growth as has been reported for frontal ganglionectomy on larvae (CLARKE AND LANGLEY, 1963d; ROOME, 1968), and 1 - 4 day old adult males (BIGNELL, 1974).

Growth curves and faecal production of newly moulted animals (12 hours \pm 12 hours) from which the frontal ganglion had been cauterised, when compared with those of BIGNELL (1974), suggest that the animals are in a better condition following cautery than following surgical removal of the gland. 12 hours \pm 12 hours old cauterised males and females lost less weight after the operation and maintained a steadier weight later, than comparable animals that have had the gland cut out. Similarly, faecal production in cauterised females was twice that reported by BIGNELL (1974) for frontal ganglionectomised males. One may, therefore, conclude that removal by cautery is preferable to the methods previously employed. A highly localised, high frequency electric current would be more specific and less damaging than the surgical procedure employed by CLARKE AND LANGLEY (1963 a - e), ROOME (1968) AND BIGNELL (1974).

In a morphological study ALLUM (1973) has shown that many minor pharyngeal nerves pass from the frontal ganglion to the foregut while the posterior pharyngeal nerves may vary in its place of origin, sometimes coming from the recurrent nerve. Therefore, the extent to which the nervous connections between the anterior foregut and the brain are dislocated by removal of the frontal ganglion may vary from insect to insect, whether the ganglion is cut out or cauterised.

In contrast to the work on larvae (CLARKE AND LANGLEY, 1963 b - e) but in agreement with BIGNELL's study on adults (1974), removal of the frontal ganglion by cautery resulted in a number of insects failing to

empty their crops. Indeed, some operated animals had none or very little food in either their mid or hind guts (Plate 2.1). In one experiment (Fig. 2.5) a number of operated animals were found to live as long as 12 days. However, since a high mortality was observed in many experiments (25% after 5 days, 50% after 7 days) the adult Locusta would appear to be more susceptible to the operation than Schistocerca gregaria, which live for 14 days or so after frontal ganglionectomy (HILL ET AL, 1966). This may be due to a more marked effect on food passage in Locusta than Schistocerca, indicated by a lower faecal production in operated male Locusta (BIGNELL, 1974) and operated females (present work) than Schistocerca (HILL ET AL, 1966). GILLOTT (1965) and ANSTEE (1968) have also noted a reduced food consumption by frontal ganglionectomised Locusta larvae but argue that this is sufficient for operated animals which have a reduced or basal metabolism. While evidence will be presented later in this thesis which suggests that frontal ganglionectomised adults may also have a reduced metabolism, there are definite indications that the normal passage of food through the gut is interfered with in these animals.

The effects of frontal ganglion removal by cautery, and starvation, on the neuroendocrine system of young adult locusts were similar to those reported by CLARKE AND LANGLEY (1963e), ROOME (1968), ANSTEE (1968) for Locusta larvae and HIGHNAM ET AL (1966) for Schistocerca adults. The large amounts of PAF staining material in the nervi corporiscardiaci 1 of operated animals suggested that release of neurosecretion from the corpora cardiaca was at best reduced and at worst inhibited altogether.

STRONG (1966) reported that the corpora allata from adult female Locusta 14 days after removal of the frontal ganglion were only half the size of the controls. He suggested that frontal ganglionectomy stopped the normal increase in size of the corpora allata that precedes

and accompanies maturation. A similar effect has been shown in frontal ganglionectomised adult Schistocerca gregaria (HIGHNAM ET AL, 1966) and in Locusta larvae (CLARKE AND ANSTEE, 1971b). A less pronounced and more variable effect has been observed in the present work five days after the removal of the ganglion from young adult males. It is difficult to speculate upon the possible consequences of this phenomenon in young Locusta adults. Recently JOHNSON AND HILL (1975) have shown an increase in size of the corpora allata with age in young Locusta adults but have not correlated it with an increase in production of juvenile hormone. In fact JH cannot be detected in male Locusta between the 2nd and the 8th day of adult life. Sectioning of the nervi corporis allati 1 of adult male Locusta has been found to stop the normal increase in the size of the gland but it does not appear to stop the production and release of juvenile hormone, which begins on the eighth day of adult life. Therefore, it is possible that the effect of the removal of the frontal ganglion on the structure of the corpora allata of newly moulted adults has no direct physiological consequences, either because it is not normally active at this time or because the normal secretory activity is not interfered with.

CHAPTER 3

Some effects of the removal of the frontal ganglion on lipid and carbohydrate metabolism

Introduction

Removal of the frontal ganglion from Locusta larvae has been found to result in cessation of growth and moulting, the protein metabolism of the animal being held at a low maintenance level (CLARKE AND LANGLEY, 1963d; CLARKE AND GILLOTT, 1967a). The oxygen consumption of the operated animal, however, did not appear to be significantly different from operated controls. This was unexpected since it was assumed that actively growing tissues would have a higher oxygen requirement than tissues where growth was not occurring (CLARKE AND LANGLEY, 1963d). CLARKE AND ANSTEE (1971a) found that operated and operated control fifth instar locusts both showed a decrease in oxygen consumption per mg throughout the instar. They suggested that in the controls the decrease was due to an accumulation of non respirable material, whilst in the operated animal it was due to the disorganisation of metabolic processes. This would certainly be more in line with numerous reports of hormonal involvement in metabolism. For example, a drop in oxygen consumption has been found to follow the removal of the corpora allata from adult Calliphora erythrocephala (THOMSEN, 1949) and Leptinotarsa decemlineata (DE WILDE AND STEGWEE, 1958). A similar result followed the removal of the corpora cardiaca (cc) and corpora allata (ca) from female Pyrrhocoris apterus (SLÁMA, 1964a).

The injection or introduction of an endocrine factor into an insect is often accompanied by an increase in metabolic rate. However, the effect on oxygen consumption may well only be an indirect one accompanying an increase, for example, in protein synthesis. The term metabolic hormone can only be used when a growth promoting effect is

excluded (DE KORT, 1968). SLÁMA (1964 a, b) correlated a reduced oxygen consumption following allatectomy in female Pyrrhocoris apterus with a reduction in growth of the ovaries and concluded that juvenile hormone was not a metabolic hormone since allatectomy had no effect on males. However, a distinct difference in oxygen consumption between normal and allatectomised Locusta females has been observed up to the twenty-fifth day of adult life, and there was no correlation with sexual development (ROUSSEL, 1963 a, b).

A more direct effect of hormones on respiration can be shown in vitro. Isolated fat body from female Leucophaea maderae was stimulated by corpora cardiaca extract, added to the medium (LÜSCHER AND LEUTHOLD, 1965). A similar effect was shown with males of the same species, but the increase in QO_2 was attributed to a switch of metabolic substrate from carbohydrate to lipid (WIENS AND GILBERT, 1965). A stimulatory effect of cc extract on fat body metabolism has also been shown on Blaberus crangifer (RALPH AND MATTON, 1965) and Leucophaea maderae (MÜLLER AND ENGELMANN, 1968).

Changes have been shown to occur during growth and development of insects in the quantity (SILHACEK, 1967), morphology (BROSEMER ET AL, 1965; WILLIS, 1966) and oxidative activities (SILHACEK, 1967; BROSEMER ET AL, 1965) of their mitochondria. Some investigators have linked these changes in mitochondrial metabolism with endocrine control. Juvenile hormone (J.H.) has been shown to stimulate the oxidative activity of isolated mitochondria from Leptinotarsa decemlineata (STEGWEE, 1960), Locusta and Schistocerca gregaria (CLARKE AND BALDWIN, 1960), Plodia interpunctella (FIRSTENBERG AND SILHACEK, 1973). MINKS, (1967) could not stimulate oxygen consumption in isolated Locusta mitochondria but oxidative phosphorylation was increased by ca extracts. However, the in vivo importance of this finding is not known (DE KORT, 1971). Work on Blaberus discoidalis has pointed to a

hormone dependent phase in the biochemical development of adult fat body mitochondria (KEELEY, 1970; 1972). Blockage of neurosecretory release by frontal ganglion removal has been shown to affect the integrity of mitochondria from various organs of Locusta larvae (CLARKE AND ANSTEE, 1971a and b).

The relative roles played by two of the main centres of hormonal activity, the medial neurosecretory cells (mnc) and the ca, in protein synthesis and metabolism is disputed. HIGHNAM (1964) stressed the importance of neurosecretion for the regulation of protein synthesis and HILL (1963) showed that the protein level of the haemolymph is low in adult Schistocerca gregaria under conditions of an inactive neurosecretory system. But ENGELMAN (1968) and DE WILDE (1964) do not preclude an indirect role of the neurosecretory cells via the ca. Ablation of mnc in Sarcophaga bullata had no effect on protein synthesis (WILKENS, 1969) and implantation of cc into Nauphoeta cinerea did not stimulate protein synthesis (LÜSCHER, 1968). But frontal ganglionectomy reduces protein synthesis in larval Locusta (CLARKE AND GILLOTT, 1967a).

Both J.H. and brain neurosecretion influence the secretion of vitellogenins, those specific proteins that TELFER (1954) first showed were of paramount importance in yolk deposition. These proteins are not normally abundant but after ovariectomy they account for more than 50% of the total blood protein (COLES, 1965). HILL (1962, 1965) originally suggested that mnc controlled their synthesis. Obviously, there will be variation in regulatory systems in different species. However, decapitation in female Leucophaea maderae followed by implantation of brains or cc as sources of neurohormone, stimulated synthesis of certain electrophoretic protein components, whereas the vitellogenic component responds strongly and specifically to corpora allata hormone. It could be that neurosecretion is necessary for a general stimulation of protein synthesis, and J.H. then directs this

into the production of female specific protein (HILL, 1972).

Allatectomy certainly results in a lowered rate of incorporation of labelled amino acids into the fat body of locusts (HILL, 1965; MINKS, 1967). And J.H. has been implicated in the uptake of protein by the oocytes (BELL AND BARTH, 1971, DE LOOF AND DE WILDE, 1970b).

The situation is further complicated by the recent finding that a factor is produced by the hemocytopoietic tissues which may either act as a transfer molecule or a permissive factor for the effect of J.H. on protein synthesis in female Locusta migratoria (GOLTZENE AND HOFFMAN, 1974).

Lipids and carbohydrates are the two major forms of energy reserves in insects as in other animals. Therefore, it is not surprising that extensive evidence is accumulating concerning the hormonal control of the production, storage and mobilisation of these compounds; particularly in relation to that most energy demanding insect activity, flight. Insects can be divided into three broad groups with respect to the type of substrate used to provide the energy for flight (ZEBE, 1959 a and b); those that use mainly carbohydrates e.g. Diptera and Hymenoptera, those that use mainly lipids e.g. Lepidoptera and those that consume both e.g. Orthoptera. Both Schistocerca gregaria and Locusta migratoria seem to use carbohydrate at the start of flight and convert to lipid within the first half hour (KROGH AND WEIS FOGH, 1951; MAYER AND CANDY, 1969; GOLDSWORTHY, COUPLAND AND MORDUE, 1973).

Tethered flight has been shown to cause a 2 - 5 fold increase in the concentration of haemolymph diglyceride of Locusta (TIETZ, 1967; BEENAKKERS, 1965) and Schistocerca (MAYER AND CANDY, 1969). Diglycerides (DGL) have been found to be the major form of lipid in the haemolymph of a number of species of insect e.g. Locusta (TIETZ, 1967), Hyalophora cecropia, Melanoplus differentialis and Periplaneta

americana (CHINO AND GILBERT, 1965b), Oncopeltus fasciatus (THOMAS, 1974). This is in marked contrast to the situation pertaining in mammals where triglycerides (TGL) are the dominant form of lipid, and these together with free fatty acid (ffa) are transported in the blood. It is to be noted that at least in one insect Pyrrhocoris apterus the predominant lipid is TGL, although this may be present in lipid filled cells (MARTIN, 1969).

The pattern in the haemolymph is at odds with that found in the fat body where 90% of the total lipid is TGL (Hyalophora cecropia Melanoplus differentialis, Periplaneta americana (CHINO AND GILBERT, 1965b), Phormia regina (WIMER AND LUMB, 1967), Locusta (TIETZ, 1967), Diatraea grandiosella (CHIPPENDALE, 1971), Oncopeltus fasciatus (THOMAS, 1974))

Work using radioactive tracers has suggested that C¹⁴ DGL are specifically and rapidly released from "pre-labelled" fat body into haemolymph (CHINO AND GILBERT, 1965b; TIETZ, 1967). This is at variance with the findings of COOK AND EDDINGTON (1967) with Periplaneta americana. Using a similar system to CHINO AND GILBERT, they conclude that TGL is the major form of neutral lipid released. Another contradiction is the work of WLODAWER ET AL (1965, 1966, 1967) who found that ffa were released into the haemolymph of Galleria mellonella.

However, whatever the form of release it is necessary to reconcile it with the type of lipid found in the haemolymph. WLODAWER, LAGWINSKA AND BARANSKA (1966) have shown the presence of lipase activity in Galleria mellonella and proposed that it was responsible for the partial esterification of ffa released from the fat body. No lipase activity could be detected in the haemolymph of Locusta (TIETZ, 1962) or Hyalophora cecropia (CHINO AND GILBERT, 1965b). This is consistent with

the release of DGL from the fat body of these insects, since DGL is the predominant lipid in the haemolymph.

If DGL is the major form in which lipid is transported to the flight muscle, then it is necessary to show the presence of a true digestive lipase in the muscle to ensure that lipid in this form could be used as a substrate. GILBERT, CHINO AND DOMROESE (1965) demonstrated a lipase in Hyalophora cecropia fat body and muscle that had more activity against DGL than TGL. However, STEVENSON (1969) pointed out that the low activity they observed would provide only a small fraction of the activity necessary for flight. STEVENSON (1972) suggested that the low concentration of ffa in the haemolymph of Prodenia eridonia was due to a "rapid" turn over. In which case the high concentration of DGL in the haemolymph was a result of TGL being broken down to DGL and ffa.

However, most authors are in agreement concerning the close association of haemolymph neutral lipids with lipoproteins in Locusta (TIETZ, 1962; PELED AND TIETZ, 1973), in Hyalophora cecropia (CHINO AND GILBERT, 1965b), in Galleria mellonella (WLODAWER ET AL, 1966) and in Periplaneta americana (COOK AND EDDINGTON, 1967). THOMAS AND GILBERT (1968) have shown three classes which resemble the low density LD, high density HDL and very high density lipoproteins VHDL of vertebrates. The HDL are particularly prominent and it may well be that this lipoprotein actually "captures" the DGL from the fat body. PELED AND TIETZ (1973) have shown that the lipoproteins in Locusta are manufactured in the fat body. However, blocking the synthesis of these lipoproteins with cycloheximide did not affect either the synthesis of glycerides from C¹⁴ palmitate or their release. The release of glycerides from the fat body appears to be an active process (inhibited

by KCN, NaN_3) and is induced by some factor present in the haemolymph (CHINO AND GILBERT, 1965b, TIETZ, 1962).

Vertebrate hormones have been shown to elicit release from insect fat body in vitro. Cyclic 3:5 AMP and insulin induced TGL release from prelabelled larvae fat body of Danaus plexippus and Agrius cingulata (CHANG, 1974). Also epinephrine stimulated ffa release from Leucophaea maderae and Hyalophora cecropia fat body in vitro (BHAKTHAN AND GILBERT, 1968). Extracts from an insect endocrine gland, corpus cardiacum (cc), caused an increase in lipid concentration in the haemolymph of Schistocerca gregaria (MAYER AND CANDY, 1969). A similar phenomenon was observed in Locusta where cc extract caused a several fold increase in DGL concentration (BEENAKKERS, 1969). More recently this hyperlipaemic effect has been pinpointed to an "adipokinetic hormone" from intrinsic cells in the glandular lobes of the corpora cardiaca. The hormone involved is common to a number of species. Extracts from Tenebrio molitor cc will induce a response in Schistocerca gregaria and vice versa, while Periplaneta americana cc will effect an increase in haemolymph DGL concentration in Locusta (GOLDSWORTHY, MORDUE AND GUTHKELCH, 1972). Isolating the cc by cutting both nervi corporis cardiaci I and II stopped lipid mobilisation during flight. Schistocerca gregaria so treated did not increase their haemolymph lipid concentrations and had a poor flight performance on a roundabout, even though their carbohydrate levels were normal. Injections of cc extract not only produced an increase in haemolymph lipid but improved flight performance (GOLDSWORTHY, JOHNSON AND MORDUE, 1972). Changes in flight speed, perhaps correlated with a switch in the form of substrate utilized were brought about by the cc (GOLDSWORTHY, JOHNSON AND MORDUE, 1972). In vitro studies suggested that the adipokinetic hormone might be suppressing carbohydrate utilisation by the flight muscle (ROBINSON

AND GOLDSWORTHY, 1974). It is interesting to note that although ca extract from Periplaneta americana has an adipokinetic effect on Locusta it does not on the cockroach itself. In fact it appears to have a hypolipaemic effect, and induces transfer of lipid from the haemolymph to the fat body (DOWNER AND STEELE, 1972; DOWNER, 1972).

The corpora allata have been implicated in the control of lipid mobilization from the fat body, during oogenesis, but this is a long term thing in terms of days and weeks (WYATT, 1972). Lipid mobilization for flight, however, occurs in a matter of minutes (MAYER AND CANDY, 1969). Since there is no effect of allatectomy on haemolymph DGL concentrations, the corpus allatum is probably not involved with the regulation of haemolymph DGL levels (BEENAKKERS, 1969; HILL, 1972). However, allatectomy has been shown to bring about an abnormal accumulation of lipid in the fat body of a number of species of insect Periplaneta americana (VROMAN ET AL, 1965) Locusta migratoria (BEENAKKERS, 1969; STRONG, 1968) Schistocerca gregaria (ODHIAMBO, 1966a) Spodoptera littoralis (EL IBRAHMY AND BOCTOR, 1970). Paradoxically treatment of male Drosophila melanogaster with ca from females of the same species or with synthetic J.H., led to an accumulation of lipids (BUTTERWORTH AND BODENSTEIN, 1969)!. The reason for this completely opposite effect is not obvious.

The deposition of lipid following allatectomy is mainly in the form of TGL and is an accompaniment of cellular hypertrophy (VROMAN ET AL, 1965). Indirect mechanisms have been proposed to account for it. In male Schistocerca gregaria, allatectomy reduced activity and ODHIAMBO (1966b) suggested that the ca normally regulated the nervous system so that various aspects of behaviour were affected. WAJC AND PENER (1971) showed that allatectomy decreased the flight activity

of male Locusta. Both ODHIAMBO, WAJC AND PENER conclude that lipid accumulation occurs secondarily to a lethargy brought about by removal of the corpora allata. But a more direct effect of J.H. on fat body metabolism seems likely since accumulation of lipid after allatectomy has been observed in male and female insects, and in the absence of cc and gonads (WYATT, 1972).

Lipid accumulation in Schistocerca gregaria is not inhibited but prolonged by the removal of the ca prior to the period of intense feeding. If males are allatectomized after this period then no lipid accumulation occurs (WALKER AND BAILEY, 1971a). The operated animal has twice the lipogenic activity during the period of intense feeding (WALKER AND BAILEY, 1971c) and a higher content of lipogenic enzymes than the controls (WALKER AND BAILEY, 1971b).

J.H. can be detected in the haemolymph shortly before the end of the somatic growth period when the period of intense feeding begins to decline (JOHNSON AND HILL, 1972) and this is associated with a decrease in lipogenesis. Also HILL AND GOLDSWORTHY (1968) have shown that in the final instar of Locusta, when the corpora allata are inactive, the fat body contains a higher proportion of lipid and a lower proportion of glycogen than the fat body of the fourth larval instar where it is active. STEPHEN AND GILBERT (1970) explain the action of J.H. as blocking the desaturation of palmitic acid and stearic acids. They suggest that this would retard the synthesis of TGL and direct saturated fatty acids into DGL for transport.

As mentioned earlier the energy for flight in Diptera comes mainly from carbohydrates (CLEMETS, 1955). Trehalose is present in the haemolymph of insects (WYATT AND KALF, 1957; EVANS AND DELTHIER, 1957) and provides the major portion of the energy for the flight of the blowfly, Phormia regina (CLEGG AND EVANS, 1961). Evidence exists for the homeostatic regulation of haemolymph trehalose levels and as with

lipid mobilization, a controlling factor from the corpora cardiaca is implicated. STEELE (1961) showed that the concentration of haemolymph trehalose increased and fat body glycogen went down following injection of cc extract into Periplaneta americana. It was subsequently shown to have an effect on fat body phosphorylase and possibly on the permeability of the flight muscle membrane as muscle glycogen increased in response to the haemolymph trehalose level. Similar effects have been shown by McCARTHY AND RALPH (1962) on Periplaneta americana, BOWERS AND FRIEDMAN (1962) on Blaberus discoidalis, WIENS AND GILBERT (1967 a,b) on Leucophaea maderae. WIENS AND GILBERT showed that the cc factor accelerated the rate of trehalose synthesis, by increasing the rate of glycogen breakdown through the action of phosphorylase. The phosphorylated sugars thus formed were shunted into haemolymph trehalose, while the endogenous metabolism of the fat body became more dependent on lipid reserves.

It has been suggested that the active factor is manufactured in the mnc and stored in the cc, because glycogen accumulates in a number of insects following mnc cautery (VAN HANDEL AND LEA, 1965; LEA AND VAN HANDEL, 1970; WYATT, 1967). However, besides a hyperglycaemic factor in the storage lobes, a more potent factor has been found in the glandular lobes of the cc of Locusta migratoria (MORDUE AND GOLDSWORTHY, 1969; HIGHNAM AND GOLDSWORTHY, 1972) and Pyrrhocoris apterus (DIVAKAR AND NEMEC, 1973). Only during the period of intense feeding is there sufficient glycogen stored in the fat body of Locusta to respond to extracts of cc. However, a potent response to Locusta cc extract is obtained in Periplaneta americana (GOLDSWORTHY, 1969; MORDUE AND GOLDSWORTHY, 1969).

It is interesting that similar hyperglycaemic and adipokinetic hormones appear to be produced by Locusta and Periplaneta. However, the response that they elicit in these two insects is very different

and geared to the lipid orientated flight metabolism in Locusta and carbohydrate orientated flight metabolism in the cockroach (GOLDSWORTHY, MORDUE AND GUTHKELCH, 1972).

Denervation or removal of the cc stops the maintenance of the trehalose level in haemolymph of flying Calliphora erythrocephala (VEJBJERG AND NORMANN 1974). Control of the release of the humoral factor involved from the cc has been shown to come from the mnc because electrical stimulation of the brain causes an increase in trehalose concentration in the haemolymph (NORMANN AND DUVE, 1969).

As far as the corpora allata are concerned no hyperglycaemic effect has been demonstrated, and such possible effects in certain species may be due to the presence of neurosecretion within the corpora allata, rather than to the J.H. itself (HILL, 1972).

The metabolic control exerted by the corpora cardiaca/medial neurosecretory cell system is far from being completely understood. In 1962 McCARTHY AND RALPH showed that saline extracts of cc from Periplaneta americana sometimes caused a hypoglycaemic rather than the usual hyperglycaemic effect. They come to the conclusion that there must be two types of factor, the one with the greatest amount of activity at the time of extraction determined the kind of response. A similar contradiction has been found by NORMANN (1975). He surprisingly found that removal of the head from Calliphora erythrocephala caused a hyperglycaemic response. This operation should have been equivalent to mnc cautery which causes the reverse. He reported that a number of effects were caused by mnc cautery; hyperglycaemia, blood hypertonicity, polydipsia, lipid catalysis, and fatigue i.e. decreased and slow motor activity, resulting perhaps from impeded uptake of trehalose by the muscles. The problem came in deciding which was the primary effect.

Since the early work on the role of the frontal ganglion in Locusta migratoria by CLARKE AND LANGLEY (1963 a-e), there has been a dramatic increase in our knowledge of the control exerted by the neuroendocrine system on metabolism. As a result of these more recent studies Langley's statement (LANGLEY, 1963) that frontal ganglion removal has no effect on lipid and carbohydrate metabolism at least initially, seems unlikely. The object of the work presented in this chapter was to study some of the effects of blocking neuroendocrine release, by removal of the frontal ganglion, on the intermediary metabolism in young adult Locusta migratoria.

Materials and Methods

The animals employed, the operation and treatment of experimental animals were as described in the general materials and methods section (Chapter 1). All reagents used were Anala R grade or purest available and supplied by British Drug Houses, Sigma, Nuclear Enterprises or Amersham.

Measurement of Oxygen Consumption (the method employed was similar to that described by CLARKE (1957)).

The oxygen consumption was measured by the direct method of carbon dioxide absorption with potassium hydroxide using the constant volume type of Warburg respirometer. The insect was placed in a 50ml flask where it was held down by a small wad of cotton wool in such a way as not to interfere with abdominal movements. The central well was filled with 20% KOH and a small roll of filter paper (Whatman No. 40), such that it projected 5 mm above the top of the well. The flask was attached to the manometer and immersed in a water bath at 28°C. A period of 30 minutes was allowed for equilibration prior to readings being taken. This also allowed for the locust to overcome any effects caused by handling. Readings were taken every five minutes for 40 minutes and a thermobarometer was used to correct for changes in temperature and pressure during the course of the experiment.

The volume of the locust was large compared to the amount of air the flask contained, so that the amount of air available to the animal had to be measured each time. Calculations See Appendix (Section 5).

Extraction of carbohydrates and lipid

Haemolymph was collected from animals by the method described by STERNBURG AND CORRIGAN (1959). A small hole was made in the vertex

of the head and the exposed air sacs removed. The tarsi were cut off each leg producing an "open system" through the insect. The animal was fixed with a wad of plasticine over a centrifuge tube in such a way that the hole in the vertex was directly over the top of the tube and the mouth was shielded from it by the plasticine, to exclude contamination by regurgitated gut contents. Two animals were treated similarly and placed to balance each other in a Gallenkamp, "junior" bench centrifuge and spun at three hundred r.p.m. for 5 minutes. The haemolymph was thrown down as a clear serum with most of the haemocytes massed at the bottom of the tube. There was no visible contamination from gut contents. A micro-cap was used to transfer the haemolymph to the homogenisation tube.

Fat body was collected from the thorax and abdomen. The neck membrane was broken by twisting the head first to one side and then to the other. A ventral incision was made from the "neck" to the anus and the head, with gut attached, pulled away from the thorax and abdomen exposing the fat body in the abdomen and over the muscles of the thorax. All readily visible fat body tissue was removed and blotted gently on filter paper to remove surface water and attached haemolymph and then weighed.

Tissue was pooled from equal numbers of male and female animals to give 250 μ l of haemolymph and 30mg of fat body and each was extracted by the method described by VAN HANDEL (1965b). Each tissue immediately after removal from the animal was put into 4 ml of chloroform-methanol (1 : 1, V/V) with two drops of saturated sodium sulphate and homogenised for 2 minutes in a Potter Elvehjem homogeniser with a Teflon pestle (clearance 0.1 - 0.15mm) rotating at approximately 3,000 r.p.m. on a Vortex Waring Blender (MSE Ltd.). The homogenate

was centrifuged for 10 minutes at 1,000 r.p.m. on a Mistral 2L centrifuge (MSE Ltd.) to spin down insoluble material, Procedure (A). The supernatant was then decanted into a boiling tube. The pellet was washed twice, first with 1ml of chloroform:methanol (1 : 1) and then with 4 ml of chloroform, and treated as in Procedure (A) above. The pooled extract was made up to 12.5ml with chloroform. The lipids in organic solution were mixed with 1.25ml of distilled water by pouring the mixture back and forth between two tubes to extract non lipid contaminants (including some carbohydrate). Then the emulsion was poured into two 10ml centrifuge tubes. Complete separation of the organic and aqueous phases was ensured by centrifuging at 1,500 r.p.m. for 1 hour on a Mistral 2L centrifuge. The aqueous phase was removed quantitatively with a Pasteur pipette and placed in a separate tube Procedure (B). Once again the organic phase was made up to 12.5ml with chloroform and washed with 2.5ml of water as before, and the aqueous phase added to the tube from Procedure (B). The washed lipid extract was reduced in volume by evaporation in a water bath at 40°C by passing pure dry nitrogen over it, and stored in sealed glass vials under an atmosphere of pure nitrogen in the dark at -20°C until required.

The pellets formed in Procedure (A) above were each re-suspended in 2ml of 66% ethanol saturated with sodium sulphate by mixing on a "whirly-mix" (Vortex mixer, Fison's Laboratory apparatus). This was poured into an homogeniser with a 1ml "wash" of the tube with the same solution. The suspension was homogenised for 2 minutes at 2,000 r.p.m. and the homogenate spun on a Mistral 2L centrifuge for 10 minutes. The supernatant liquid containing the non glycogen carbohydrate was added to the washings from the lipid extract in Procedure (B). A further 2ml of 66% ethanol was added to the pellets which were again suspended by "whirly-mixing" and centrifuged as before.

The supernatant was added to the tube from Procedure (B).

The residual pellets left after the last procedure with fat body extracts were heated in a water bath at 100°C to drive off residual ethanol and prevent foaming at the next step. The pellet was then suspended in 1ml of 30% KOH and heated to 100°C for 15 minutes in a water bath. 4ml of absolute ethanol were added to the digest which precipitated glycogen adsorbed on sodium sulphate. The latter was spun down by centrifuging at 1,000 r.p.m. in a Mistral 2L centrifuge for 10 minutes. The supernatant was taken off with a Pasteur pipette and glycogen redissolved in distilled water.

The non-glycogen carbohydrate and glycogen extracts prepared in the above way were stored at 4°C in glass stoppered tubes until quantified (up to 4 days after extraction).

66% ethanol was used to separate non-glycogen carbohydrate from glycogen as the latter is extremely insoluble in it (VAN HANDEL, 1965a). Several workers have suggested that electrolytes accelerate the precipitation of the glycogen by ethanol from an alkali digest. Na_2SO_4 is a more efficient co-precipitant than other salts and is very soluble in water and only slightly soluble in 66% ethanol. It crystallizes readily on addition of ethanol and it does not interfere with the anthrone reaction (VAN HANDEL, 1965a). Carbonate in KOH also causes precipitation of glycogen and decanting of the supernatant could lead to a substantial loss of glycogen in a supernatant carbonate layer (VAN HANDEL, 1965a). Assay of the supernatant fraction suggested that there was no such interference in the present work.

Efficiency of the extraction procedure

Efficiencies of recovery of pure samples of glucose, glycogen and lipid (BOEHRINGER standard lipid) from tissue homogenates were 90 - 95%, 80 - 85% and 90 - 95% respectively.

Quantification of lipids

1. Total lipid; on extracts of lipid from fat body as prepared above and small aliquots of haemolymph ($\approx 10 \mu\text{l}$) without prior extraction. The method employed was essentially that described by ZÖLLNER AND KIRSCH (1962).

Reagents: concentrated H_2SO_4 , sulphophosphovanillin reagent SPV (4 parts phosphoric acid + 1 part 0.6% aqueous vanillin solution), lipid standard (BOEHRINGER C^0 $100 \mu\text{g}/10 \mu\text{l}$).

An amount of lipid extract or haemolymph with 10 - 120 μg of lipid was digested with 1ml of concentrated sulphuric acid in a water bath at 100°C for ten minutes. The tubes were cooled and 5 ml of the sulphophosphovanillin reagent added to an aliquot of the digest. After standing at room temperature for 40 minutes, the absorbance was measured at 530nm in a Pye Unicam SP 1800 spectrophotometer.

The reaction was linear up to 140 μg of lipid (See Figure 3.1A). A standard tube was estimated at each determination.

Separation and quantification of Neutral Lipids

Thin layer chromatography

(a) Preparation of the plates and application of the sample

The method employed was essentially that described by SKIPSKI ET AL (1965). Glass plates (200mm x 200mm x 3mm) were placed in a "Unoplan" holder (Shandon Scientific Ltd.) using plastic gloves, and thoroughly washed with chloroform. 40g of silica gel H (MERCK) without CaSO_4 binder, was suspended in 90ml of water by vigorous stirring. The slurry so formed was poured into a "Unoplan" adjustable spreader with the aperture set at 250 μm using a feeler gauge. The spreader was moved smoothly and without interruption across the

surface of the glass plates. The plates were briefly vibrated against a whirlmix mixer to ensure that an absolutely smooth surface was obtained. Finally the plates were dried overnight in a horizontal position. The slurry was sufficient to coat four plates.

Initially the plates were "pre-washed" with chloroform-methanol (4 : 1, V/V) in the manner of ascending thin layer chromatography (see later) for several hours. This was to remove organic material present in the silica gel to the uppermost edge of the plate. Subsequently this was found to be unnecessary. Plates were activated at 110°C for 1 hour before use. All subsequent operations were performed under dry nitrogen gas. After the plate was cooled, lipids were applied as a chloroform solution using either a 10 or 25 μ l Terumo micro syringe (Shandon Scientific Co. Ltd.) with a squared needle tip. The syringe was mounted on a micro-manipulator. Up to 200 μ g of total lipid was routinely applied as a series of spots (20 x 1mm) 15 mm above the lower edge of the plate, taking care not to disturb the silica gel.

(b) Solvents and development of chromatoplates:

Plates were developed by ascending chromatography in large moulded glass tanks (SHANDON) lined with Whatmann No.1 filter paper. The tanks were fully saturated with solvent vapour prior to development by vigorously shaking the tank and solvent, and allowing it to stand for 30 minutes at room temperature. The developing solvents were present as a shallow layer approximately 10mm from the bottom of the tank and were allowed to rise up the chromatoplate until the solvent front was approximately 20mm from the top of the plate.

Neutral lipids were separated using the system of MANGOLD AND MALINS (1960) (petroleum ether 80 : diethyl ether 20: acetic acid 1). The plates were then removed from the tank and dried in a stream of pure dry nitrogen.

Lipids were detected on thin layer chromatograms in two ways (i) non destructive - by means of Iodine Vapour (MANGOLD AND MALINS, 1960; SIMS AND LAROSE, 1962). Plates were placed in a glass chamber containing iodine crystals. All lipids took up iodine and became brown spots on a pale brown background. Iodine was sublimed off after marking the position, by warming at 105°C (ii) destructive - by means of FeCl₃ reagent (LOWRY, 1968) (50mg FeCl₃ · 6H₂O in 90ml of H₂O, 5ml glacial acetic acid, 5ml concentrated sulphuric acid. The solution is stable for 3 months at room temperature). The plates were sprayed with the reagent using a glass spray (T.W. Wingent & Co. Ltd.) powered by compressed air and heated at 105°C for 2-3 minutes. Cholesterol develops a blue/purple, fatty acids yellow/brown, triglycerides brown, cholesterol ester red/purple.

Identification of neutral lipid spots was made by comparison with authentic standards run at the same time.

Quantification of Neutral Lipids separated by thin layer chromatography

10 - 20 μl aliquots of the neutral lipid extracts were separated on thin layer chromatograms using the method of MANGOLD AND MALINS (1960), and visualised with Iodine vapour. The position and extent of the diglyceride (DGL), free fatty acid (ffa) and triglyceride (TGL) fractions were marked with a scalpel blade. Following removal of the iodine by sublimation the lipids were scraped into centrifuge tubes using a microscope slide.

The lipids were estimated using a charring technique with concentrated sulphuric acid as described by MARSH AND WEINSTEIN (1966) and KRITCHEVSKY ET AL (1973). The acid dichromate technique of AMENTA (1964) was found to be inconsistent; highly erratic blanks

being obtained despite extensive washing of the silica gel with chloroform/methanol/formic acid to remove inorganic contaminants.

2ml of concentrated sulphuric acid was added to the lipid spot in the centrifuge tube and whirlymixed. The mixture was heated in an aluminium block (10cm x 7cm x 7cm with 12 holes drilled in it) to $200^{\circ}\text{C} \pm 2$ for 15 minutes. The tubes were cooled at room temperature for 10 minutes then put on ice. 3ml of cold distilled water were added and the solution mixed. The silica gel was removed by centrifugation at 2,000 r.p.m. in the Mistral 2L centrifuge for 15 minutes and the absorbance of the supernatant determined at 375nm in an SP 1800 Pye Unicam spectrophotometer. Silica gel blanks, comparable to each spot, were taken from each plate. Standard curves were constructed for the three neutral lipids to be assayed (viz. diglyceride, free fatty acid and triglyceride) by processing known amounts of standard lipids (SIGMA) as above after running them on TLC plates (Fig. 3.1B).

Standard amounts of cholesterol were assayed directly and after running on TLC to check the efficiency of recovery off the plate, which was 98%.

Quantification of carbohydrate extracts using an anthrone reagent

The method employed was that described by ROE (1955). The anthrone reagent was 0.05% anthrone, 1% thiourea in 66% by volume sulphuric acid, made up fresh each week. (Thiourea was used as an anti-oxidant, to prevent the oxidation of anthrone in sulphuric acid and so help storage). Anala R glucose was dried in a vacuum oven and dissolved in saturated benzoic acid, 100 mg (100ml to give a standard carbohydrate solution.

Aliquots of carbohydrate extracts with less than $150\mu\text{g}$ were made up to 1ml with distilled water. 10ml of anthrone reagent was added to the extract in a boiling tube and well mixed. This gave a final sulphuric acid concentration of 60%. The tubes were placed in a boiling water bath (in a metal tray to cut down direct light) for 15 minutes, then cooled in a cold water bath and stored in the dark for 30 minutes. The absorbance was read at 620nm in an SP1800 spectrophotometer.

Trehalose was isolated from other non glycogen carbohydrates by virtue of its extreme stability to acid and alkali (WYATT AND KALF, 1957). Appropriate aliquots of nonglycogen carbohydrate extract were evaporated to dryness and redissolved in 0.2ml of 0.1M sulphuric acid. The tubes were capped with foil and heated to 100°C for 10 minutes to hydrolyse sucrose. Then the solution was made alkaline by addition of 0.15ml of 6N sodium hydroxide and again heated to 100°C for 10 minutes to destroy reducing sugars. The sample was tested with anthrone as above. The efficiency of the hydrolysis of non trehalose carbohydrates by the above procedure was investigated (Fig. 3.2 A & B). 100% hydrolysis of glucose and sucrose was observed while trehalose was unaffected.

Estimation of Haemolymph Protein

Haemolymph was collected from animals by the method of STERNBURG AND CORRIGAN (1959) described above. The protein content of these samples was estimated using a micro Biuret method (ITZHAKI AND GILL, 1964) See Appendix (Section 4.2).

In vitro incubation of fat body with C^{14} labelled glucose and its incorporation into fat body lipids

50mg of fat body were dissected out from equal numbers of males and females (see above). It was placed in 1ml of 0.025M p.H 7.2 potassium

phosphate buffered 0.9% sodium chloride (TIETZ, 1962) in a specimen tube with $5\mu\text{Ci}$ of C^{14} glucose (specific activity 283m Ci/m mol) in a shaker water bath at 30°C . Incubation took place for various periods of time, see Results section. The fat body was then removed, washed in fresh saline, blotted on Whatman No. 1 filter paper and weighed. Lipids were extracted and separated as described previously. Initially the lipid was extracted from the silica gel by placing the spot on a scintered glass funnel and eluting with 3 aliquots of 5ml of chloroform under the pressure of a flow of pure dry nitrogen gas. The filtrate was collected in a scintillation vial and the chloroform evaporated off with nitrogen. The lipid was taken up in 10ml of N.E. 216 scintillator and the incorporation into the DGL, ffa and TGL fractions was found by counting the samples in a Beta/Gamma Scintillation counter (NE 8312, Nuclear Enterprises). Using this procedure a 95% recovery of cpm off the plate was achieved. Subsequently it was found that an equivalent recovery could be attained by suspending each silica gel spot directly in the scintillator. Therefore, prior separation of lipid from the silica gel was dispensed with. Counts per minute were converted to disintegrations per minute by reference to a quench correction curve (Fig. 3.3).

Statistical analysis of results

Statistical comparisons of data were performed using conventional techniques as described by SNEDECOR AND COCHRAN (1967). Where appropriate, reference was made to the statistical tables of FISCHER AND YATES (1963). Values of $p \leq 0.05$ were taken as being significant.

Fig. 3.1

A. Standard calibration curve for the determination of total lipid using the sulphophovanillin reagent

Legend: ordinate ; absorbance nm
 abscissa ; μ g lipid standard

B. Standard calibration curve for the determination of three key neutral lipids after separation by thin layer chromatography

Legend: ordinate ; absorbance nm
 abscissa ; μ g of neutral lipid

● TGL tristearin

■ FFA palmitic acid

▲ DGL distearin

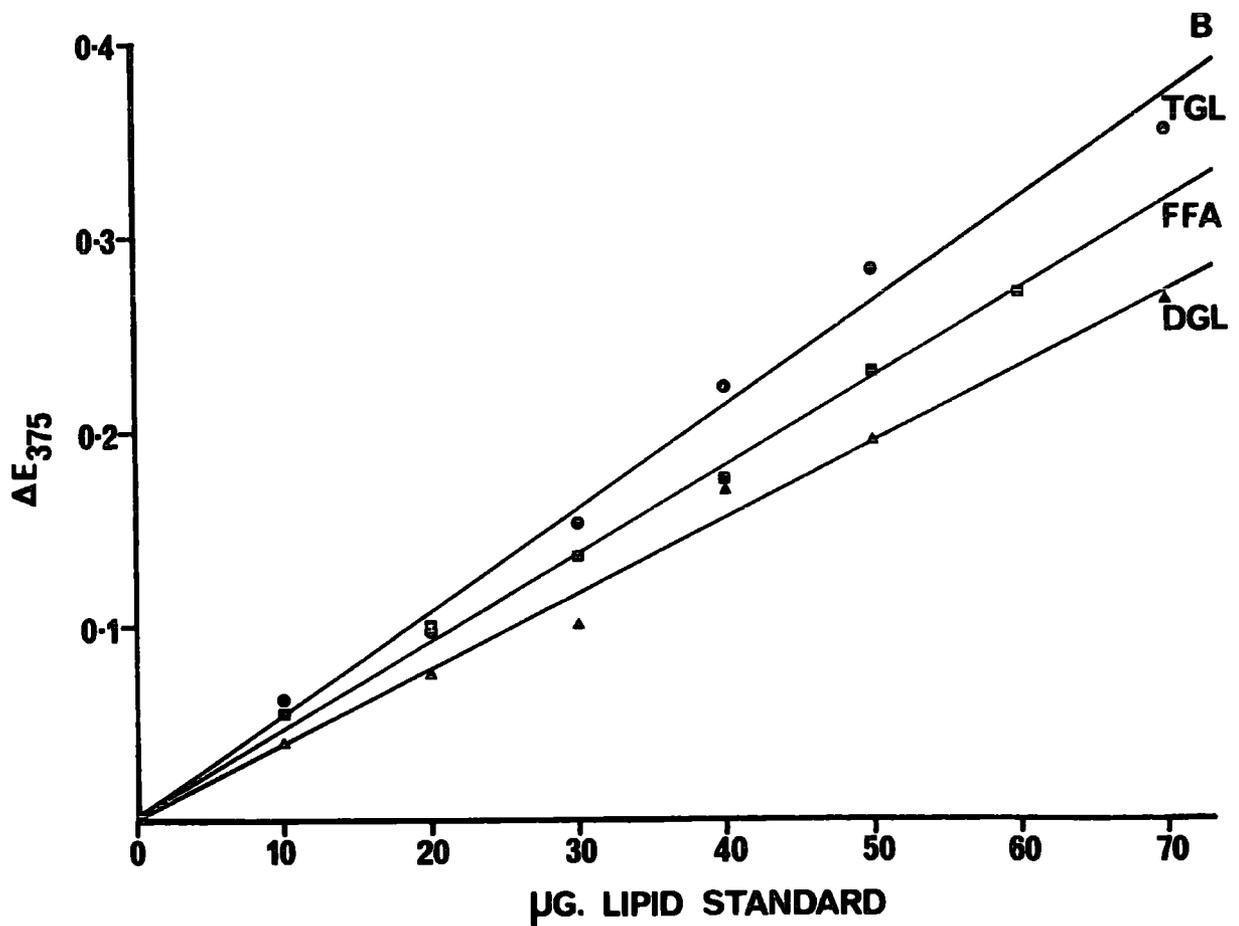
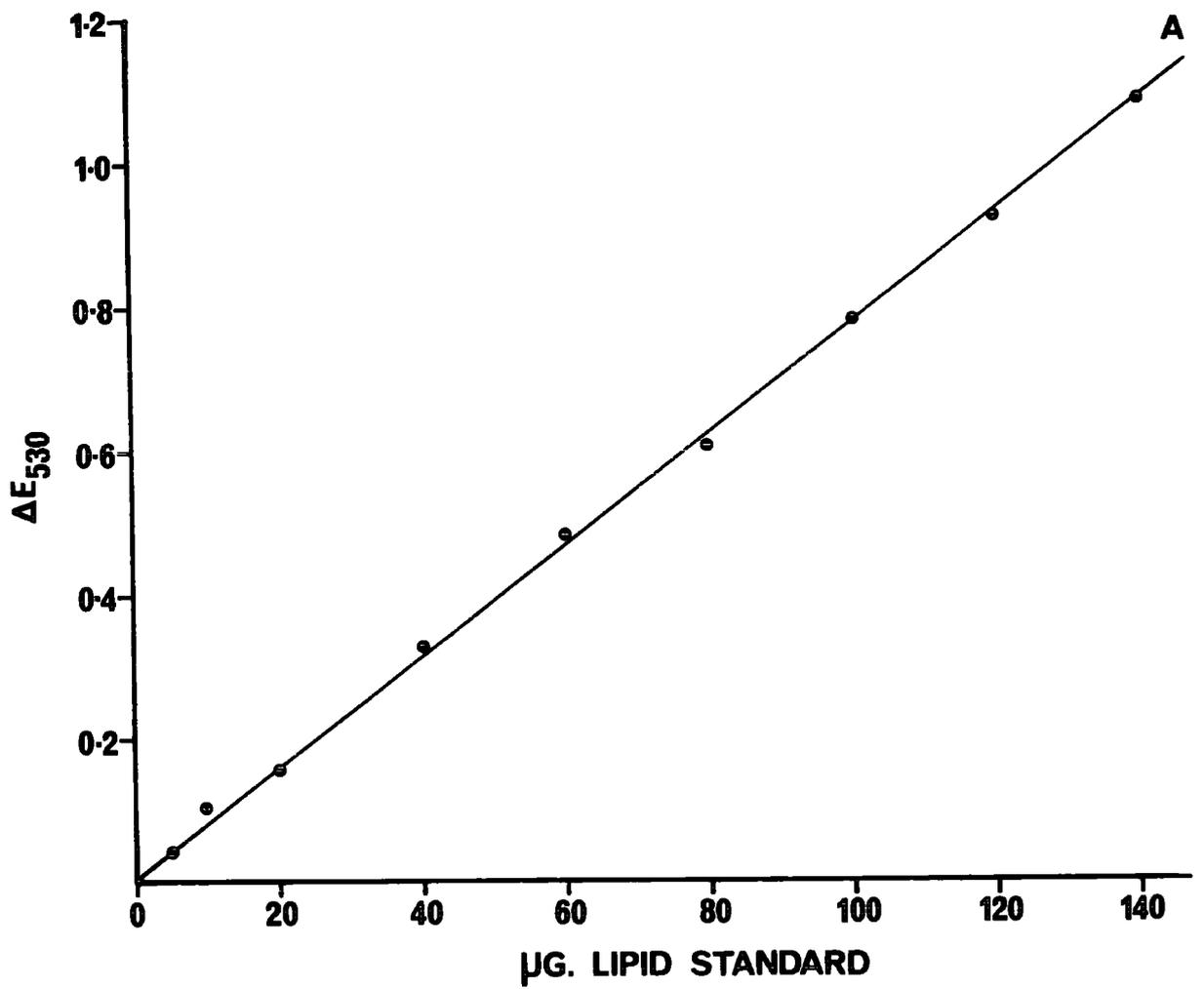


Fig. 3.2

A. Effect of acid/alkali hydrolysis on reducing sugars and trehalose

Legend: ordinate ; absorbance nm
 abscissa ; treatment

● ● 20 μ g glucose] with hydrolysis
● +
○ ○ 20 μ g sucrose] without hydrolysis
○

● ● 20 μ g trehalose with hydrolysis
●

○ ○ 20 μ g trehalose without hydrolysis
○

B. Effect of acid/alkali hydrolysis on trehalose

Legend: ordinate ; absorbance nm
 abscissa ; μ g of trehalose

● with hydrolysis
■ without hydrolysis

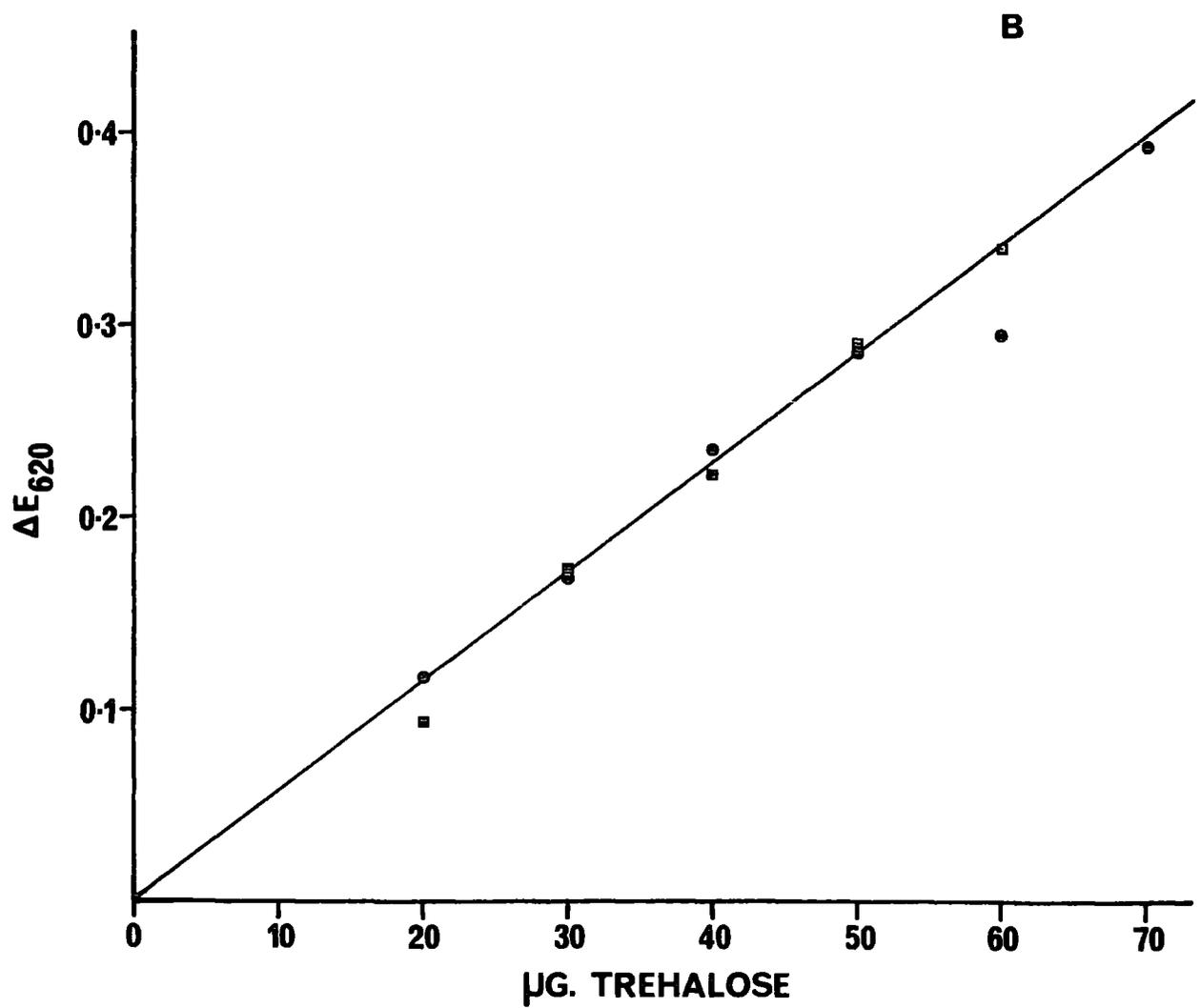
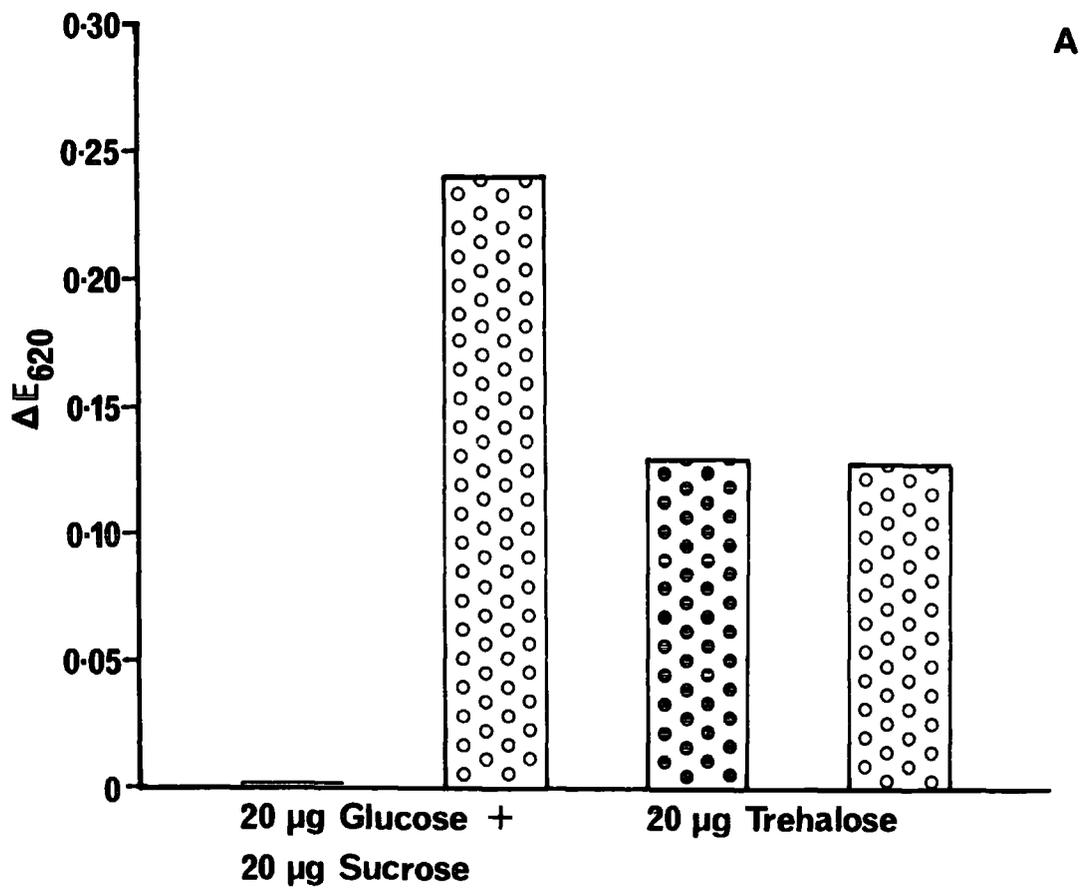
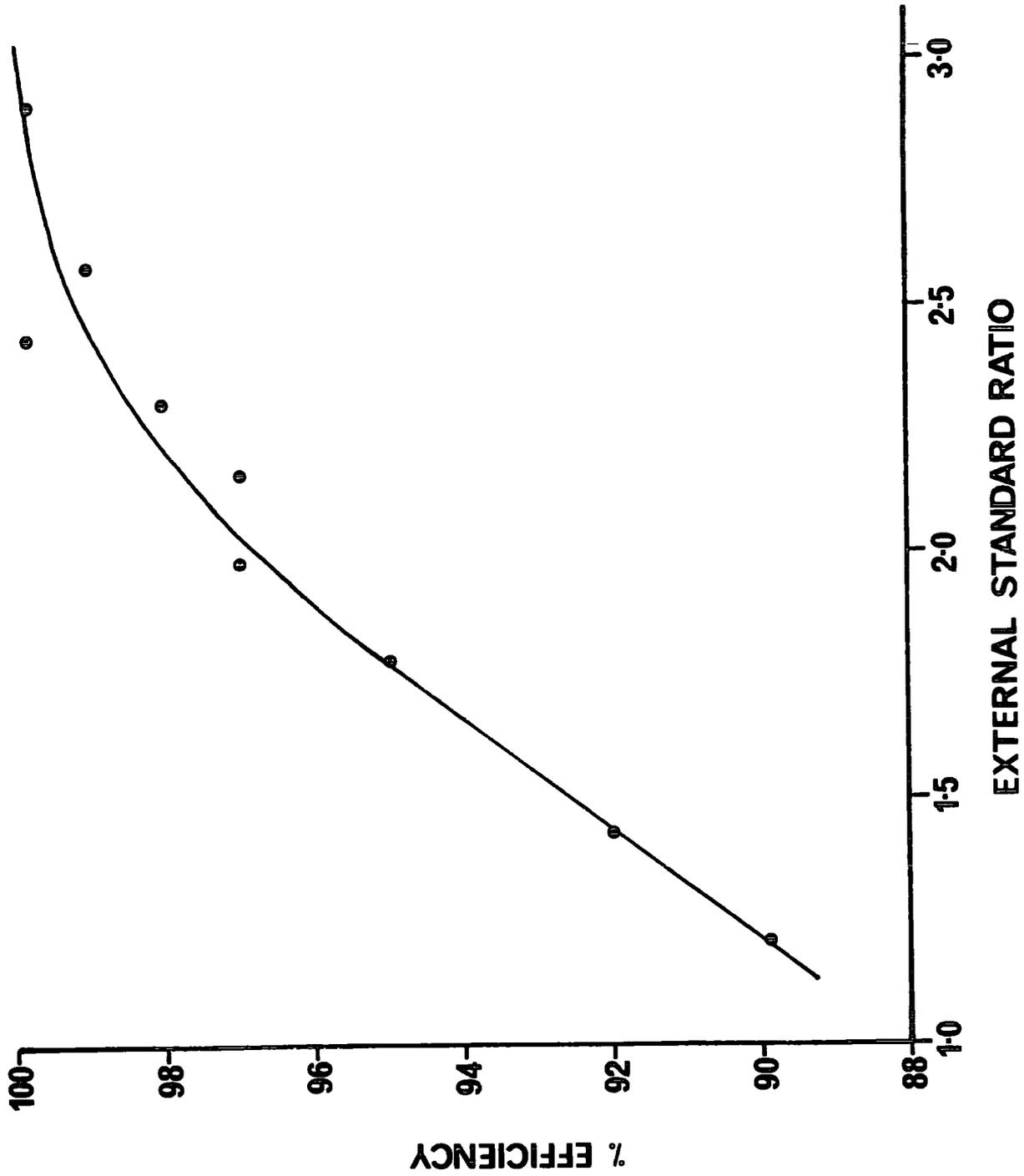


Fig. 3.3

Quench correction curve

Effect of silica gel as quenching agent on the efficiency of counting of β particles from a C^{14} source by a β/γ scintillation counter.

Legend: ordinate ; efficiency %
 abscissa ; external standard ratio



Results

1. Oxygen consumption of the whole animal

The oxygen consumption of operated controls increased as the animals grew, so that newly moulted locusts which prior to the operation consumed $661.7\text{mm}^3/\text{hour}$ had increased their consumption five days later to $764\text{mm}^3/\text{hour}$. The difference was not statistically significant owing to the large variation in size, and hence in oxygen consumption, of the control animals. When expressed in terms of $\text{O}_2/\text{mm}^3/\text{g}/\text{hour}$ the oxygen consumption changed from 669.14 to 696 (see Table 3.1). Although body weight was greater five days after the control operation than it was prior to it, the respiratory rate/g of tissue was not less. This was contrary to the work of BUTLER AND INNES (1935) who found that growth in adult Locusta was accompanied by a decrease in oxygen consumption/gm. In operated animals, the oxygen consumption 5 days after the operation was $559.2\text{mm}^3/\text{hour}$ which was not significantly different from the 12 hour old animal. However, when expressed in terms of $\text{O}_2/\text{mm}^3/\text{g}/\text{hr}$ the oxygen consumption decreased from 669.14 to 581, which was significant ($p = 0.05$). In starved animals oxygen consumption dropped to $352.3\text{mm}^3/\text{hr}$ or $420\text{mm}^3/\text{g}/\text{hr}$. The starved clearly used less oxygen per animal and per gram than did operated control or newly moulted adults ($p \ll 0.01$). So in contrast to the situation found in larvae (CLARKE AND LANGLEY, 1963d; CLARKE AND ANSTEE, 1971a), the operated adult not only used less oxygen per animal than did the operated control but also less oxygen per gram. ($p \ll 0.05$).

Levels of stored Energy reserves in the fat body

Since the activities of the fat body are an important part of the metabolic life of the young adult, it was decided to determine to what extent the reduced oxygen consumption reflected a mal-functioning of this organ. To this end the levels of stored sugar and lipid reserves in the fat body were determined.

The quantities of total non-glycogen anthrone-positive carbohydrate, glycogen, and acid-alkali stable carbohydrate in the fat bodies of the four treatments are shown in Table 3.2. Following the procedure of WYATT AND KALF (1957) the acid-alkali stable carbohydrate is designated trehalose. No significant difference was found between the quantity of total anthrone-positive carbohydrate (apc) and the quantity of trehalose in operated, operated control or starved animals.

Newly moulted Locusta had only a small glycogen store. The level in the operated animal did not rise above this, while the starved animal had a similar level that had not been further depleted. Operated controls increased their store some four fold in the five days following the operation to a level that was significantly larger than operated and starved animals ($p = 0.02$) Table 3.2.

apc was the most prominent carbohydrate in the fat body. Operated controls did not show a build up of apc in the first five days following the operation (Table 3.2); the levels remained more or less constant over this period. By contrast, over the same period operated animals showed a decrease in their apc reserves to a level that was significantly different from both the 12 hour old and operated control animals ($p < 0.001$). The starved animals exhibited an even greater drop, which was significantly lower than either of the other three treatments ($p \leq 0.001$).

Table 3.3 shows the amount of total lipid in the fat bodies of operated control, operated, starved and twelve hour old treatments. The picture is similar to that found for the carbohydrates. The operated control did not increase the size of its lipid store, whereas that of the operated dropped significantly and the starved even more so ($p < 0.001$). A significant difference was found between operated control and operated, between operated control and starved and between

operated and starved animals ($p \leq 0.01$).

Concentration and quantity of lipid and carbohydrate in the Haemolymph

The above study showed that the lipid and carbohydrate reserves stored in the fat body of frontal ganglionectomised and starved animals were significantly lower than in the operated control. This might have been caused by an increased release of the reserves into the haemolymph. To investigate this possibility the levels of haemolymph lipids and carbohydrates were determined.

The concentrations of trehalose and total anthrone-positive carbohydrates in the haemolymph from the four treatments are listed in Table 3.4, together with the total quantity of anthrone positive carbohydrate calculated from the haemolymph volumes (Chapter 7, Table 7.2 male and female). As in the fat body there was no significant difference between the acid-alkali stable fraction and the total anthrone positive carbohydrate Table 3.4. This confirmed GOLDSWORTHY'S (1969) observation on normal 1 day old adult Locusta.

The concentration of apc in the haemolymph of operated control animals in the haemolymph was not significantly different from the 12 hour old animal. The concentration in the operated animal fell and in the starved animal this fall was even more marked ($p \leq 0.001$). As with other parameters measured in relation to frontal ganglion removal, the operated animal holds a position intermediate between operated control and starved animals, the differences being highly significant ($p \leq 0.01$).

The total quantity of carbohydrate in mg shows a similar pattern to the concentrations (Table 3.4). Although standard errors have been put on values for the total quantities of carbohydrate and lipid; their significance is questionable due to the fact that mean haemolymph volumes were used to calculate them.

The total lipid content of the haemolymph of the operated control showed a dramatic increase in both concentration and total quantity in the five days following the operation (Table 3.5). The operated animals did not show this increase while animals starved for five days exhibited a significant decrease, although after only 2 days' starvation the concentration was not significantly lower than the 12 hour old animal. Once again the operated control had a significantly greater concentration and total quantity than operated which was similarly greater than the starved ($p < 0.01$).

It would seem, therefore, that the reduction in energy reserves in the fat body of operated animals was not due to an increased release into the haemolymph.

Concentration and quantity of protein in the Haemolymph

Since frontal ganglionectomy has been shown to have a dramatic effect on protein metabolism in larval Locusta (CLARKE AND GILLOTT, 1967a; CLARKE AND ANSTEE, 1971a; ROOME, 1968). It was decided to look at the level of haemolymph protein to see if there were any indications of disruption of protein metabolism in the frontal ganglionectomised adult (Table 3.6).

The newly moulted adult exhibited a concentration of protein that was not maintained by the control at least up to five days after the operation. The total amount showed an even greater reduction. Similarly the operated and starved animals showed a distinct fall in both concentration and total amount of protein ($p < 0.001$). As with lipid and carbohydrate the total quantity of protein in the operated had an intermediate value between control and starved ($p < 0.001$). There was no significant difference between protein concentration in operated control and operated animals ($p = 0.1 - 0.05$), but they were both significantly different from the starved.

Table 3.7 shows that both sexes respond similarly to the treatments. No significant difference was found between the concentration of protein in males and females, in any of the treatments.

Relative amounts of key neutral lipids found in the fat body

Results presented previously in this chapter have shown that both removal of the frontal ganglion and starvation reduced the levels of starved energy reserves in the fat body. These procedures also decreased the amounts of lipid and carbohydrate transported in the haemolymph.

It was decided to see if three key neutral lipids (TIETZ, 1967) Diglyceride (DGL), Free fatty acid (ffa) and Triglyceride (TGL) were equally affected by the treatments. Table 3.8 shows the neutral lipids from the fat body expressed as a percentage of their combined weight. In six day old normal adults 96.7% of the combined weight of ffa, DGL and TGL from an aliquot of fat body lipid (see Materials and Methods section) was in the form of TGL. Only 2.33% was DGL and 1% ffa. In 12 hour old animals there was a significantly larger proportion in DGL 5.7%, with 93.56% in TGL and 0.93% in ffa. In comparison with the first six days in the life of a normal adult locust, the effect of the operation and starvation was to decrease the proportion of TGL with a concomittant increase in the ffa and DGL fractions (Table 3.8). Once again the effect was greatest on the starved, least on the operated control with the operated in between. However, there is a certain amount of variability in the results as indicated by the standard errors. This combined with the interdependence of the DGL, ffa and TGL results expressed in this way has meant that many of the differences are not statistically significant.

The reduced levels of energy reserves in the fat body of operated animals did not appear to be due to increased release into the haemolymph.

The results of the study presented in this section suggested that the reduction of lipid reserves affected TGL more than DGL or ffa.

In vitro incorporation of C¹⁴ glucose into the neutral lipid of the fat body

The low level of lipid in the fat body of operated animals must be due to either a reduction of necessary intermediates made available to the fat body or to reduced capacity of this organ for synthesis. To test the second possibility it was decided to look at the ability of operated fat body to synthesize neutral lipids from radio active precursors, in vitro. Fatty acids in the lipid stores of plant feeding insects in the main are derived from carbohydrate and protein (CHINO AND GILBERT, 1965). Therefore, C¹⁴ labelled glucose was supplied as the precursor in this study. Figs. 3.4 and 3.5 show the results obtained when fat body was incubated with 5 μ Ci of C¹⁴ glucose for various time periods, as indicated, lipids extracted and C¹⁴ incorporation determined. Only a small amount of labelled glucose was found to be converted to lipid (at best 0.4%). A great deal of variation was found in the amount of incorporation and it was not possible to show that the amount of incorporation was linear with time within each treatment.

These preliminary experiments indicated that the level of incorporation of C¹⁴ into neutral lipid of operated fat body appeared to be greatest into DGL, far less into TGL and only a small amount in ffa. The situation was the same in the starved animal. In the operated control, on the other hand, after the first fifteen minutes the level of incorporation was greatest in TGL.

Table 3.1

O₂ consumption (data from equal numbers of males and females)

Treatment	<u>O₂mm³/hr/animal</u>		<u>O₂mm³/g/hr</u>		Number of animals	
	Mean	SE	Mean	SE		
Normal 12 hours † 12 hours	661.7	46	669.14	32	14	
Operated	559.2	46.1	581	27.6	10	
Operated Control	764	75	696	47	10	
Starved	352.3	53	420	42.7	8	
	t	df	p	t	df	p
12:O	1.529	22	0.1	1.983	22	0.05
12:C	1.231	22	0.2	0.484	23	0.6
12:S	4.246	20	< 0.001	4.687	20	< 0.001
O:C	2.336	18	0.02	2.057	19	0.05
O:S	2.961	16	0.01	3.287	16	0.01
C:S	4.288	16	< 0.001	4.172	17	< 0.001

12 Normal 12 hours † 12 hours

O Operated

C Operated Control

S Starved

t indicates the values obtained from applying Student's t-test

P indicates values of probability

Carbohydrate levels in the Fatbody

Treatment	Total nonglycogen, anthrone positive carbohydrate µg/mg wet weight of fatbody			glycogen µg/mg wet weight of fatbody			trehalose (acid-alkali stable carbohydrate) µg/mg wet weight fatbody		
	Mean	SE	Number of determinations	Mean	SE	Number of determinations	Mean	SE	Number of determinations
Normal, 12 hours - 12 hours	17.7	1.05	9	1.45	0.5	9			
Operated	9.46	1.33	13	0.82	0.14	9	6.7	0.8	10
Operated Control	22.88	3.31	10	6.24	2.09	8	16.1	1.72	7
Starved	3.80	0.78	11	0.84	0.46	7	3.3	1.19	4
12:O	t 4.503	df 20	p < 0.001	t 1.196	df 16	p 0.3	* C _{TO} :C _{TRE} 1.6 15 0.2-0.1 O _{TO} :O _{TRE} 1.633 21 0.2-0.1 S _{TOTAL} : 0.388 13 0.8-0.7 S _{TREHALOSE}		
12:C	1.425	17	0.2	2.362	15	0.05			
12:S	10.865	18	< 0.001	0.872	14	0.4			
O : C	4.114	21	< 0.001	2.759	15	0.02			
O : S	3.508	22	0.001	0.034	14	> 0.9			
G : S	5.870	19	0.001	2.370	13	0.05			

*Comparison between Total non-glycogen CHO and trehalose

Table 3.2

Table 3.3

Total lipid content of the Fat body

μ g/mg wet weight of fat body

<u>Treatment</u>	<u>Mean</u>	<u>SE</u>	<u>Number of determinations</u>
Normal (aged 12 hours \pm 12 hours)	38.38	3.56	10
Operated	13.13	1.85	8
Operated Control	37.43	5.53	8
Starved	5.98	0.53	5

	<u>t</u>	<u>df</u>	<u>p</u>
O : C	4.167	14	< 0.001
O : S	2.959	11	0.01
C : S	4.43	11	< 0.001
12 : O	5.837	16	< 0.001
12 : C	0.151	16	0.9 - 0.8
12 : S	6.304	13	< 0.001

O = Operated

C = Operated control

S = Starved

12 = 12 hours \pm 12 hours, normal.

Carbohydrate levels in the Haemolymph

Treatment	Total anthrone positive carbohydrate total quantity mg				Total anthrone positive carbohydrate concentration g/100ml				Acid-alkali stable carbohydrate (trehalose) concentration g/100ml				
	haemolymph volume μ l	Mean	SE	No. of determinations	Mean	SE	No. of determinations	Mean	SE	No. of determinations	Mean	SE	No. of determinations
Normal (aged 12 hours - 12 hours)	329	5.81	0.34	9	1.767	0.14	9						
Operated	241	2.15	0.27	14	0.892	0.113	14	0.798	0.13	10			
Operated Control	308	4.78	0.38	12	1.552	0.122	12	1.329	0.142	8			
Starved	224	0.83	0.15	7	0.371	0.067	7	0.435	0.055	4			
12:O	t 8.386	df 21	p < 0.001		t 5.330	df 21	p < 0.001		C TO:C	t 1.18	df 18	p 0.3-0.2	
12:C	1.956	19	0.1-0.05		1.279	19	0.2		O TO:O	0.792	22	0.5-0.4	
12:S	12.056	14	< 0.001		10.515	14	< 0.001		S TO:S	0.650	9	0.6-0.5	
O : C	5.781	24	< 0.001		3.973	24	< 0.001						
O : S	3.268	19	0.01-0.001		3.096	19	0.01						
C : S	7.734	17	< 0.001		6.974	17	< 0.001						

Table 3.4

Total lipid content of the Haemolymph

Treatment	Total quantity mg				Concentration g/100ml			
	haemolymph volume μ l	Mean	SE	Number of determinations	Mean	SE	Number of determinations	
Normal (aged 12 hours - 12 hours)	329	0.75	0.08	10	0.228	0.0223	10	
	241	0.59	0.06	12	0.244	0.0244	12	
Operated	308	1.282	0.09	12	0.416	0.03	12	
Starved 5 days 2 days	224	0.247	0.04	15	0.11	0.014	15	
	-	-	-	-	0.205	0.014	8	
12:O	1.720	df 20	p 0.2-0.1		t 0.463	df 20	p 0.7-0.6	
12:C	4.177	20	< 0.001		4.706	20	< 0.001	
12:S	6.592	23	< 0.001		4.356	23	< 0.001	
O:C	6.193	22	< 0.001		4.374	22	< 0.001	
O:S	5.155	25	< 0.001		4.785	25	< 0.001	
C:S	11.149	25	< 0.001		9.511	25	< 0.001	
					2S:12	16	0.5-0.4	
					2S:O	18	0.3-0.2	
					2S:C	18	< 0.001	
					2S:S	21	< 0.001	

Protein content of the Haemolymph
data from equal numbers of ♂ and ♀ combined

Treatment	Quantity mg				Concentration g/100ml			
	Haemolymph volume μ l	Mean	SE	Number of determinations	Mean	SE	Number of determinations	
+ 12 hours	329	12.57	0.64	16	3.83	0.17	16	
- 12 hours	241	4.53	0.31	10	1.88	0.13	10	
Operated	308	7.19	0.49	18	2.30	0.15	20	
Operated Control	224	2.98	0.18	10	1.33	0.08	20	
O:C	t 3.809	df 26	P < 0.001		t 1.809	df 28	P 0.1-0.05	
O:S	4.291	18	< 0.001		3.596	18	0.001	
O:12	9.407	24	< 0.001		8.201	24	< 0.001	
C:S	6.259	26	< 0.001		4.393	28	< 0.001	
C:12	6.759	32	< 0.001		6.810	34	< 0.001	
S:12	11.540	24	< 0.001		11.143	24	< 0.001	

Table 3.6

Protein content of the Haemolymph
(males and females separately)

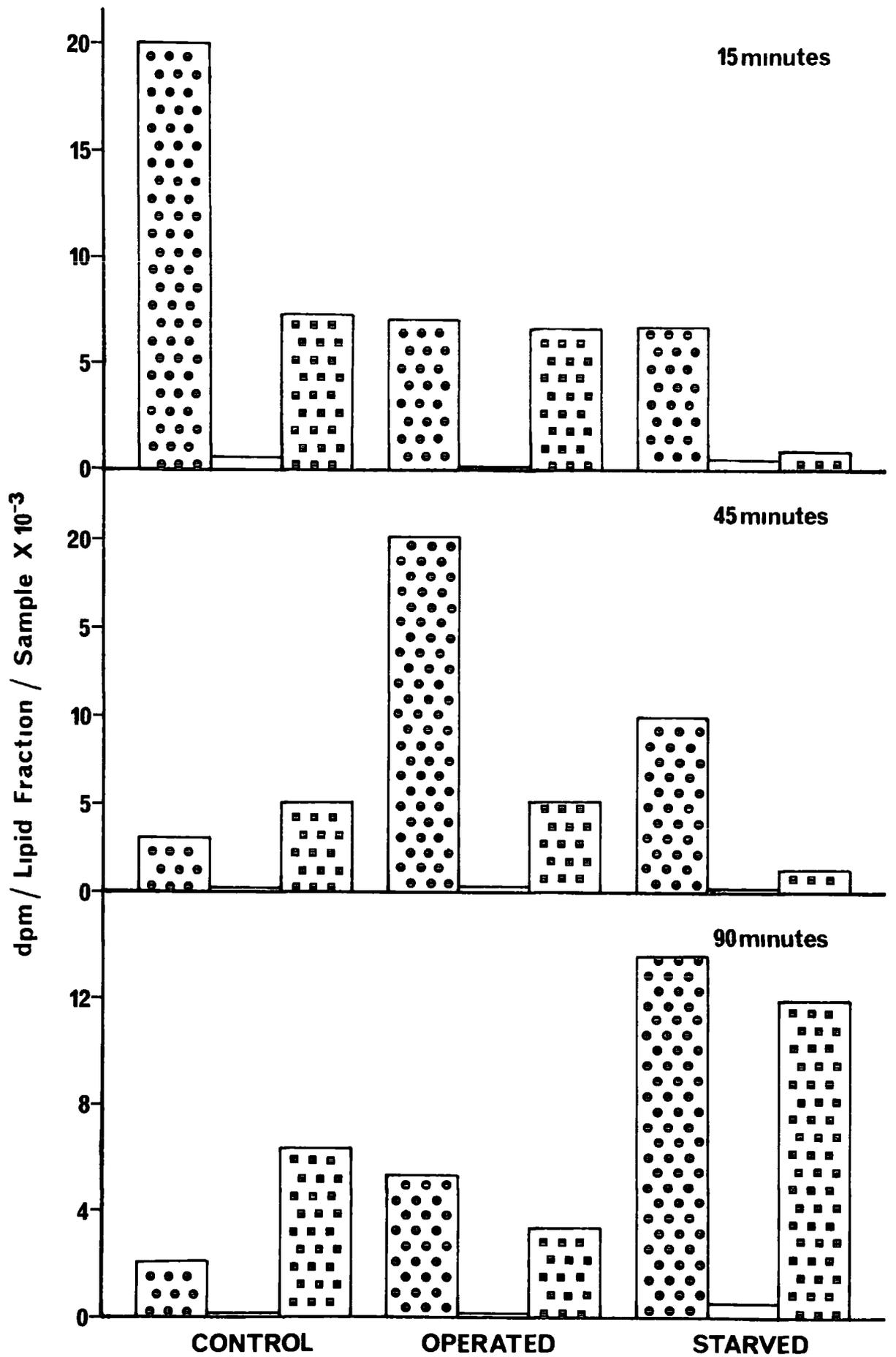
Treatment	Male concentration g/100ml				Female concentration g/100ml		
	Mean	SE	Number of determinations		Mean	SE	Number of determinations
12 hours +	3.85	0.17	8		3.87	0.2	13
12 hours -	1.77	0.11	6		2.0	0.22	5
Operated	2.12	0.21	10		2.48	0.2	10
Operated Control	1.26	0.09	5		1.28	0.12	8
Starved							
12♂ : 12♀ 0♂ : 0♀ C♂ : C♀ S♂ : S♀	t	df	p				
	0.055	19	0.9				
	0.972	9	0.4				
	1.231	18	0.2				
	0.123	11	0.9				

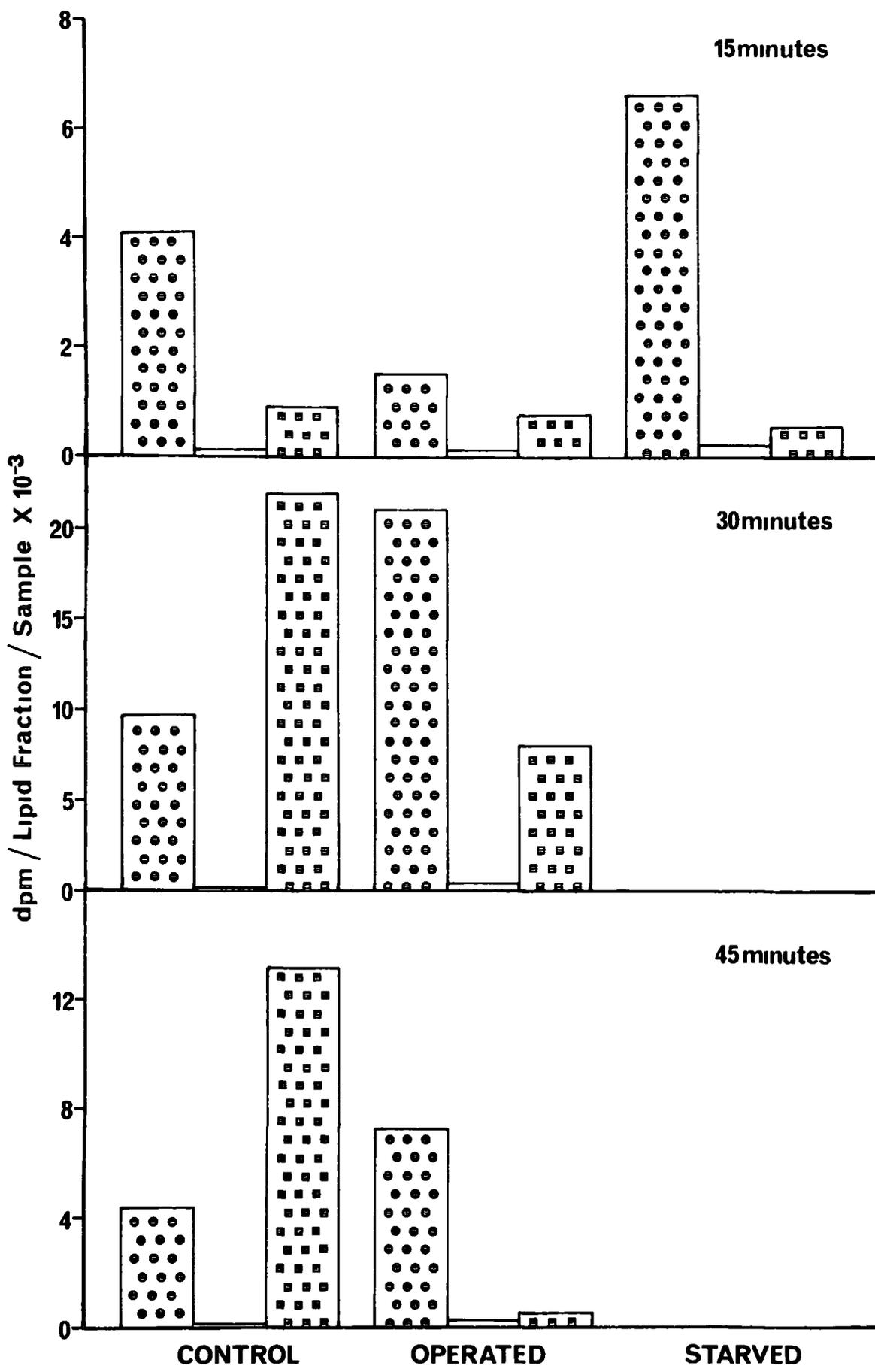
Table 3.7

Fat body neutral lipids (expressed as a percentage of their combined weight)

Lipid fraction	6 day old Normal n = 7			12 hours old Normal - 12 hours n = 9			Operated n = 13			Operated Control n = 12			Starved n = 10		
	t	df	p	FFA	t	df	p	TGL	t	df	p	Control	t	df	p
DGL	2.33	23	0.02	5.7	2.102	23	0.05	O:C	14.59	23	0.05	9.71	17.9	23	0.01
FFA	1.00	21	0.4	0.93	1.115	21	0.2	O:S	6.58	21	0.2	2.97	9.2	21	0.2
TGL	96.7	20	0.02	93.56	3.079	20	0.01	O:12	79.45	20	0.01	87.18	72.6	20	0.001
DGL	3.4	18	0.001	0:6	2.682	18	0.02	O:6		18	0.02			18	0.001
O:C	2.266	20	0.05	C:S	3.314	20	0.01	C:S		20	0.01			20	0.001
O:S	1.344	19	0.2	C:12	2.160	19	0.05	C:12		19	0.05			19	0.05
C:12	2.351	17	0.05	C:6	1.879	17	0.1	C:6		17	0.1			17	0.01
C:6	3.831	17	0.001	S:12	4.148	17	<0.001	S:12		17	<0.001			17	<0.001
S:12	4.648	15	0.001	S:6	3.650	15	0.01-0.001	S:6		15	0.01-0.001			15	<0.001
12:6	3.882	13	0.001	12:6	0.189	14	0.9	12:6		14	0.9			14	0.01

Table 3.8





Discussion

CLARKE (1957) made a general observation that O_2 consumption/mg/hour fell with increasing size and age in larval Locusta, and attributed this to the increase of non respirable tissue components common to all growth processes. However, work presented in this chapter suggested that this was not the case in days 1 - 6 of adult life. Operated control animals, although they increased in weight (Fig. 2.1 and 2.2) and consumed more oxygen than newly moulted locusts (Table 3.1), had the same O_2 consumption per g as animals of the latter treatment. A possible explanation for this difference with CLARKE (1957) may be an increase in respirable material in the early days of adult life. BÜCHER (1965) showed that in the first five days of adult life the longitudinal dorsal muscles double in weight. Concomittant with this was a large increase in mitochondrial volume and activity of enzymes associated with aerobic respiration. So that the increase in weight in the first five days may be matched by an increased capacity for aerobic respiration in the flight muscle resulting in approximately the same oxygen consumption per g as at day 1. There was certainly no accumulation of non respiring energy reserves in the fat body of operated control animals (Tables 3.2, 3.3).

As in the larvae (CLARKE AND LANGLEY, 1963d; CLARKE AND ANSTEE, 1971a), the operated adult had a significantly lower O_2 consumption than the operated control when the results were expressed per animal. However, in larvae because the O_2 consumption per mg fell as the operated control grew there was no significant difference between the O_2 consumption per mg in operated control and operated animals (CLARKE AND ANSTEE, 1971a). In the adult the operated animal exhibited a decrease in oxygen consumption but the operated control did not and therefore there was a significant difference between the two. So it would appear that the frontal ganglionectomised adult exhibited a

reduction in metabolic rate as was suggested but not shown by CLARKE AND ANSTEE (1971a) for larvae.

Frontal ganglionectomy has been shown to cause a blockage of release of neurosecretion from the median neurosecretory cells and from the corpora cardiaca, and to bring about "degenerative" changes in the structure of the corpora allata in larval Locusta (CLARKE AND LANGLEY, 1963e; CLARKE AND ANSTEE, 1971b). Similar phenomena have been observed in adult Locusta (Present work chapter 2; STRONG, 1966). Many workers have attempted to show a direct response of hormones from these glands on oxygen consumption e.g. SLÁMA (1964) on Pyrrhocoris apterus, THOMSEN (1949) on Calliphora erythrocephala. However, the effect observed in the present work was probably an indirect one.

The normal development of the fat body of Galleria mellonella needs undisturbed hormonal conditions during the first three days after the larval-pupal ecdysis (DUTKOWSKI AND SAZALA-DRABIKOWSKA, 1973). Also KEELEY (1972) has shown a hormone dependant phase in the development of fat body mitochondria from young adult Blaberus discoidalis. It is possible that a similar prerequisite is essential for the normal development of adult Locusta fat body (see chapter 6). Certainly CLARKE AND ANSTEE (1971b) showed that frontal ganglionectomy affected quite dramatically the integrity of the mitochondria from a number of tissues including the fat body.

The reduction in protein synthesis which has been shown to follow frontal ganglion removal in larvae (CLARKE AND GILLOTT, 1967a) and adult fat body (BIGNELL, 1974; present work) may well affect not only the development of adult fat body but also of the respiratory capacity of the flight muscle. A direct endocrine involvement in the development of insect flight muscle has been shown by EDWARDS (1967) in Dysdercus

intermedius and DE KORT (1968) in Leptinotorsa decemlineata.

It is possible then that a reduction in metabolic rate in frontal ganglionectomised adults, may be a consequence of the disruption of metabolic processes throughout the body. This would reduce the normal synthesis of energy reserves within the fat body. Such an effect would be particularly important to a young growing locust. WALKER AND BAILEY (1970a) have shown a dramatic increase in lipogenesis in Schistocerca gregaria during the phase of somatic growth with an optimum between five and seven days after emergence. The results displayed in Tables 3.2, 3.3 would suggest that the operated animal is laying down less energy reserves than the operated control.

It is difficult to make a direct comparison between the energy reserves found in the present study and those found in the normal animal by HILL AND GOLDSWORTHY (1968) and GOLDSWORTHY (1969). In the present work the results are expressed per mg wet weight of fat body while HILL AND GOLDSWORTHY expressed their results per fat body. However, energy reserves in newly moulted and operated control animals appear to be lower than in the animals used by the above authors. HILL AND GOLDSWORTHY (1968) found 20mg of lipid in newly moulted adult Locusta, which was approximately 60% of the dry weight of the fat body. Newly moulted animals used in this thesis had only some 6mg wet weight of fat body (see Appendix Section 3, Table 3.1). Since 80% of the fat body was water (personal observation) this meant that only 12% of the dry weight was lipid.

As with other studies on the effect of the removal of the frontal ganglion from a locust, the level of energy reserves in the fat body of operated animals was intermediate to those found in operated control and starved animals. That this was not due to increased release was shown by examination of the amounts of carbohydrate and lipid in the haemolymph.

The dramatic drop in carbohydrate and lipid levels in the fat body and haemolymph of newly moulted animals starved for five days was similar to the situation found when 4 day old adults (GOLDSWORTHY, 1969) or 5th instar Locusta (HILL AND GOLDSWORTHY, 1970) were starved.

The amount of anthrone positive carbohydrate in the haemolymph of operated controls agrees with values reported by other workers for adult Locusta of the same age (HANSEN, 1964; MINKS, 1967), although they were somewhat lower than GOLDSWORTHY (1969). Nearly all the carbohydrate in the haemolymph of operated, operated control and starved animals was trehalose which confirmed the findings of GOLDSWORTHY (1969). MINKS (1967) suggested that the concentration of trehalose increased with age. However, in the present work the level of total anthrone positive carbohydrate five days after the control operation was found to be similar to that in newly moulted Locusta. This was in agreement with GOLDSWORTHY (1969) who found no difference in the first fifteen days of adult life. MORDUE AND GOLDSWORTHY (1969) suggested that there might be a regulatory mechanism to maintain this concentration.

The fat body of Phormia regina is geared to a maximum production of trehalose and control is exerted from outside by the endocrine system. However, in Blaberus discoidalis the synthetic process is limited by phosphorylase activity (FRIEDMAN, 1967). In Locusta the fat body can respond to extracts of corpora cardiaca by an increase in activity of the phosphorylase system (GOLDSWORTHY, 1969, 1970). This can only result in an increased trehalose level in the haemolymph when the stored glycogen is high enough to respond namely at six days after the last moult (GOLDSWORTHY, 1969, 1970). Therefore, it is not clear how haemolymph carbohydrate is maintained. JUNGREIS AND WYATT (1972) suggested that the haemolymph carbohydrate level in Hyalophora cecropia was regulated primarily by the rate of production rather than utilization. A similar hypothesis may explain the situation in the

frontal ganglionectomised adult. In the operated animal the respiratory rate was found to be lower than the operated control (Table 3.1) which would indicate a reduced utilisation of energy reserves. However, at odds with this was the fact that the level of haemolymph carbohydrates in operated animals was below that found in controls. This suggests that synthesis is reduced to such an extent that haemolymph carbohydrate can not be maintained at the normal level. The low carbohydrate store in the fat body would support this contention.

The rate limiting step in trehalose production by Hyalophora cecropia fat body is that catalysed by trehalose -6- phosphate synthetase, which is inhibited by high concentrations of trehalose:



(MURPHY AND WYATT, 1965).

If such an enzyme is of similar importance in Locusta then the inability of the enzyme to respond to low trehalose concentrations in the haemolymph of operated and starved animals must be due to either a reduced level of activity of this enzyme, or to an inadequate supply of intermediates. The second possibility may be particularly important in the starved animal but it is not obvious which is more important in the case of the operated animal (see chapters 2 and 5).

BEENAKKERS (1973) found a drop in the protein content of the haemolymph following the final ecdysis in Locusta. By the fifth day of adult life both the concentration and total quantity of protein had returned to the levels found at ecdysis. In the present work the operated controls had a significantly lower protein content in the haemolymph than the newly moulted animals. The period of post operative care (see chapter 1) delayed the return of the haemolymph protein in the operated control to the premoult level.

BIGNELL (1974) found that fat body from frontal ganglionectomised adults had a reduced ability to synthesis protein in vitro and a lower level of protein than operated controls. This may account for the reduced amount of protein in the haemolymph in the operated animal in comparison with the operated control (Table 3.6). CLARKE AND GILLOTT (1967a) found that frontal ganglionectomised Locusta larvae failed to show the normal increase in protein concentration in the haemolymph during the course of an instar. They explained this (CLARKE AND GILLOTT, 1967 a and b) as the failure of operated animals to release a brain hormone that controlled protein synthesis. The dramatic drop in protein content of starved haemolymph is similar to that found in Locusta larvae (HILL AND GOLDSWORTHY, 1970).

It has been suggested that the frontal ganglionectomised locust has a basal metabolism which it can maintain but not increase (CLARKE AND LANGLEY, 1963d; CLARKE AND GILLOTT, 1967a). If this is true then the initial drop in carbohydrate level in the haemolymph which would result from the 24 hours' starvation during post operative care (GOLDSWORTHY, 1969) may become the normal level in the operated animal, which it can maintain but not improve upon. However, the operated animals managed to maintain the same concentration and total amount of lipid as the newly moulted locust. Although the latter did not have large lipid reserves in the fat body (Table 3.3) they may have been enough to maintain the haemolymph lipid over the period of post operative care. Even 48 hours' starvation produced only a small decrease in lipid concentration (Table 3.5). In the wax moth Galleria mellonella, the haemolymph lipid was maintained over long periods of starvation (WLODAWER AND WISNIEWSKA, 1965). Whereas carbohydrate can be converted by the fat body into lipid the reverse does not appear to be possible in Locusta (CLEMENTS, 1959) or Hyalophora cecropia (CHINO AND GILBERT,

1965a). Therefore, although carbohydrate reserves could be used to maintain the lipid level the lipid reserves could not be diverted into maintaining the haemolymph carbohydrate level. However, it is to be noted that after five days of starvation there was a significant fall in both concentration and total quantity of lipid in the haemolymph in comparison with other treatments.

Once again the operated animal displayed a condition midway between the starved and the operated control. The latter increased both the concentration and total amount of lipid in the haemolymph in the five days following the operation. TIETZ (1967) found that the concentration of diglyceride doubled between 4 - 8 and 8 - 15 days of adult life in Locusta. However, the nature of the increase in haemolymph lipid in the first five days noted in the present work is not known. But a similar phenomenon has been observed in adult female Schistocerca gregaria (HILL AND IZATT, 1974). The concentrations of total lipids in operated control animals were similar to those reported by GOLDSWORTHY, MORDUE AND GUTHKELCH (1972) for Locusta. However, the animals they used were of an unstated age but capable of flight and, therefore, likely to have been somewhat older.

GOLDSWORTHY, MORDUE AND JOHNSON (1973) found a fall in the adipokinetic hormone content of the glandular lobes of the corpora cardiaca following the final ecdysis, and suggested that the hormone may be released during the first few days of adult life. This may explain the increase in haemolymph lipid observed in the present work. The inability of the operated animals to follow suit may be due to a reduced release of the hormone from the corpora cardiaca and/or a reduction in lipid synthesis necessary to effect it. Certainly synthesis rather than use of stored reserves would be necessary, as the newly moulted locust hardly has sufficient reserves to bring about the

0.5mg increase in the level of haemolymph lipid in the operated control (Tables 3.3, 3.5). However, it must be noted that JUTSUM AND GOLDSWORTHY (1975) found that cardiectomy produced no major long term changes in the haemolymph lipid levels and concluded that the adipokinetic hormone played no major role in the homeostasis of haemolymph lipid in resting Locusta.

The relative proportions of the three major neutral lipids in the fat body of 6 day old normal adults (Table 3.8) confirmed the observations of TIETZ (1967). She found that 97% of the total body lipids were in the form of TGL, 2% DGL, whilst ffa and phospholipids were present only in small amounts.

By comparison with the increase in the proportion of TGL found in the neutral lipid fraction in the first 6 days of normal adult life, the operated showed a significant decrease and concomittant increase in DGL and ffa. This trend was most marked in the starved and least marked in the operated control. Therefore, although the difference in lipid reserves found between the three treatments cannot be attributed to decrease in one particular fraction of neutral lipid, it is evident that the effect was more marked with respect to TGL.

TIETZ (1967) found that despite the release of considerable amounts of DGL from "prelabelled" fat body into haemolymph, the specific activity of labelled DGL within the fat body stayed the same. This suggested that the DGL release was synchronized with the formation of new diglyceride from a low specific activity source, namely the TGL. Therefore, under conditions of release of DGL from the fat body it might be expected that the TGL would decrease to a relatively greater extent than DGL, particularly if the level of synthesis was low.

The biochemical study discussed above showed that energy stores in frontal ganglionectomised young adult Locusta were significantly lower than the controls, but significantly higher than the starved animals.

This could be due to an inability of operated animals to digest and absorb food, in which case essential metabolites would not be made available to the fat body (see chapter 5). A similar situation may be brought about if the operated animal has a reduced food intake due to mechanical injury arising from the operation (see chapters 1 and 8). Alternatively, substrate supply may be adequate but the synthetic "machinery" of the fat body may be defective, i.e. it may have a structural basis (see chapter 6). Certainly it is possible that there is a relative inactivity of those anabolic enzymes in the fat body necessary for the synthesis of lipids and sugars.

A number of preliminary experiments were performed to look at the ability of fat body from operated control, operated and starved animals to incorporate C^{14} labelled glucose into glycerides in vitro. For periods of incubation greater than 15 minutes, the operated control animals had more dpm in the TGL fraction than in the DGL. The reverse was true for the operated and starved treatments. TIETZ (1969) has shown that synthesis of di and triglycerides in fat body homogenates proceeds by similar pathways to those already shown in liver and adipose tissue of rats and chickens by KENNEDY (1961) viz:-

1. Fatty acid + ATP + CoA \longrightarrow fatty acyl CoA + AMP + pyrophosphate.
2. 1α glycerophosphate + 2 fatty acyl CoA \longrightarrow phosphatidic acid + 2 CoA.
3. Phosphatidic acid + H_2O \longrightarrow $D \alpha, \beta$ diglyceride + phosphate.
4. $D \alpha, \beta$ diglyceride + fatty acyl CoA \longrightarrow triglyceride + CoA.

The lower level of incorporation of C^{14} into TGL of operated and starved fat body suggests that these animals have reduced activities of those enzymes necessary to effect the conversion of DGL to TGL (step 4 above) or that insufficient fatty acyl CoA is made available.

The Characterisation of six digestive enzymes from the midgut

Introduction

In the last chapter, frontal ganglion cauterization was shown to result in a decrease in the energy reserves of the fat body. It was suggested that this may be due to an inability of the operated animal to digest food and hence important metabolites are not being made available to the fat body. To test this possibility it was decided to look at the activity of some of the enzymes in the digestive tract of the operated animal (Chapter 5).

Since estimation of enzyme activity is usually performed under optimal conditions (DIXON AND WEBB, 1964) and relatively little is known about the properties of digestive enzymes from Locusta. A study was undertaken of the characteristics of five carbohydrate and one proteolytic enzyme from the midgut of this insect.

Because of the small size of insects and the difficulty of purifying enzymes from the amount of material available; much of the work on digestive enzymes of insects has comprised simply the detection of enzyme activities in crude homogenates by the hydrolysis of selected substrates (GILMOUR, 1961). Attempts have been made to correlate the kinds of enzymes detected in this way with the dietary constituents (DAY AND WATERHOUSE, 1953). However, positive results are sometimes obtained in experiments involving substrates which would seem unlikely to form part of the natural diet of the insect concerned. Active carbohydrases have been detected in the midgut of larval Calliphora erythrocephala yet the diet contains little carbohydrate (EVANS, 1956). Similarly carbohydrases such as β galactosidase and β glucosidase have been reported in Tribolium molitor, yet substrates for these enzymes have not been reported to occur in its natural environment (KRISHNA AND SAXENA, 1962).

This problem was emphasized by FRAENKEL (1940) who preferred to explain his results in terms of the bond specificity hypothesis of WEIDENHAGEN. This postulates the existence of five basic carbohydrases that could account for the hydrolysis of say maltose and α methyl glucoside and sucrose without the necessity for supposing the animals have a specific enzyme for each of these α D glucosidases. It has been criticised many times (GOTTSCHALK, 1950; EVANS, 1956; SAXENA, 1958). Evans showed that in adult Calliphora erythrocephala five α D glucosides could be hydrolysed and presumably by the one enzyme, whereas in the larva only four of these were viable substrates. The one negative result must preclude the idea of one common enzyme. However, more recently BONGERS (1970) and WENZL (1969) have used the idea of an unspecific α D glucoside to explain their results from Oncopeltus fasciatus and Calliphora erythrocephala respectively. Because of this it was felt valid in the present work to assay the activities of the four main classes of oligosaccharidases (α and β D glucosidase, α and β D galactosidase) using "generalised" artificial p nitrophenol substrates (see materials and methods section).

However, having shown the presence of an enzyme in the gut, this does not guarantee complete digestion of the corresponding substrate and subsequent absorption and utilization. Enzyme concentration, rate of passage of food and the pH of the gut are all important (KRISHNA AND SAXENA, 1962). In relation to this it is interesting to note the marked degree of correspondence that occurs between the pH optima of the carbohydrases and the pH of the gut they are found in (DROSTE AND ZEBE, 1974; EVANS, 1956). The pH of the fore and hindguts may show a variation with change of food, but the midgut appears to have a buffering action as the pH is not markedly affected by the food given to it (SRIVASTAVA AND SRIVASTAVA, 1956).

The bond specificity exhibited by the four main oligosaccharidases is found to be at odds with the enzyme that splits trehalose. Although there is some evidence to suggest that α D glucosidase can hydrolyse trehalose (RETIEF AND HEWITT, 1973); there appears to be a highly specific trehalase of which two types have been shown. A "soluble" enzyme which when purified from several species had a pH optimum in the region of 5.5 and a substrate Km near or below 10^{-3} M (KALF AND REIDER, 1958; FRIEDMAN, 1960; YANAGAWA, 1971). Also a structure or "membrane" bound trehalase with pH optimum and Km somewhat higher. The soluble enzyme is concentrated in the intestinal tissues and the structure bound form in the muscle (GUSSIN AND WYATT, 1964). The "soluble" form of the trehalase enzyme was the fifth carbohydrase enzyme investigated in the present study.

Insect proteases are active at neutral or alkaline pH and thus resemble more closely mammalian trypsins than pepsins (POWNING ET AL, 1951; LIN AND RICHARDS, 1955). However, several of the artificial substrates used for their assay are split for example by plant proteases and since most of the work has been done on crude homogenates it is not clear to what extent the insect enzymes are really trypsin like. Work done on more purified preparations of Tenebrio molitor (APPLEBAUM ET AL, 1964; PFLEIDERER AND ZWILLING, 1966; ZWILLING, 1968) has shown enzymes with active centres similar to bovine trypsins and the same cleavage pattern against bovine insulin B, as well as being active against specific trypsin substrates (GIEBEL ET AL, 1971; HAGENMAIER, 1971). Although the protease assayed for in the present work was active against benzoyl arginine pnitranilide, because of the "crude" nature of the preparation employed the enzyme is termed a "trypsin-like" protease.

The low level of activity of such proteases in the midgut epithelium (KHAN, 1963; DADD, 1956) suggests that they may be secreted in an inactive form. A positive effect of mammalian enterokinase on insect proteases has been reported (SCHLOTTKE, 1937) but not confirmed, and no activity mechanism has been shown in insects. Attempts to increase the level of protease activity in a gut preparation of Locusta by preincubation with an extract of gut tissue, were to no avail (KHAN, 1963).

Materials and Methods

The animals employed were 2-10 day old adult locusts, which had been reared and maintained as described in the general materials and methods section (Chapter 1). All reagents used were Anala R grade or purest available and supplied by British Drug Houses or Sigma.

Preparation of homogenates

The guts from one male and one female locust were quickly dissected out under ice cold distilled water, Malpighian tubules, adhering fat body and gonads were removed. The midgut and midgut caecae with their contents were then transferred to 10ml of ice cold distilled water in an homogenisation tube standing on ice.

Homogenisation of the preparations was carried out in a Potter Elvehjem homogenizer with a Teflon pestel (clearance 0.1 - 0.15mm) with 15 passes of the plunger at 2,000 r.p.m., on a vortex Waring blender (M.S.E. Ltd.). The homogeniser was surrounded by ice throughout this procedure. Each homogenate was spun at 2,000 r.p.m. on a Mistral 2L centrifuge (M.S.E. Ltd.) for 20 minutes. The supernatant was decanted and stored at -20°C . Experiments showed that there was no significant drop in activity of the homogenate towards the six substrates over the maximum period of storage which was 7 days. This property of insect digestive enzymes has been noted by other authors (BROOKES, 1961; ENGELMANN, 1968). Prior to use they were thawed at room temperature, appropriately diluted with distilled water and kept on ice.

Assay of four oligosaccharidases

1. α D glucosidase and β D glucosidase; two x 2ml aliquots of McIlvaine's buffer (citric acid 0.1M, Na_2HPO_4 0.2M) (DAWSON ET AL (1969) at pH 5.4 and 5.2 were equilibrated at 30°C with 0.5 ml of 51.3mM p nitrophenol α D glucoside and p nitrophenol β D glucoside respectively

for 5 minutes. The reaction was started by the addition of 0.2 ml of homogenate and experiments were incubated routinely for 15 minutes.

2. α D galactosidase and β D galactosidase; two x 2ml aliquots of McIlvaine's buffer (as in 1 above) at pH 5.2 and 4.8 were equilibrated at 30°C with 0.5 ml of 35.1mM p nitrophenol α D galactoside and p nitrophenol β D galactoside for five minutes respectively. The reaction was started by the addition of 0.2ml of homogenate and experiments were incubated routinely for 15 minutes.

The activities of the above four enzymes were determined by estimation of the p nitrophenol liberated from the substrates during the reaction. The development of the yellow colour of the p nitrophenol was subsequently found to be dependent on an alkaline pH, with an optimum at pH 8.5 (see Fig. 4.1A). Consequently the oligosaccharidases have been characterised at both optimal and suboptimal pH. The above characteristic has also been noted by JERMYN (1955 a, b, c) and AIZAWA (1939).

The enzyme reactions were terminated by the addition of 3.0ml of 75% K_2HPO_4 , this also brought the solutions to the required pH (JERMYN, 1955b). Following the addition of the K_2HPO_4 , mixing was effected by corking the tubes and inverting them several times. Shaking was avoided because it caused air bubbles to form, which in the highly viscous solution took a considerable time to disperse. Substrate blanks were performed by adding the enzyme after the K_2HPO_4 . It was not necessary to spin down the protein. An allowance was made for a possible contribution to the absorbance from the homogenate by having an homogenate blank, with distilled water replacing the substrate. The yellow colour produced was found to be stable at room temperature over the period of 30 minutes it took to read the absorbance on a Pye Unicam SP 1800 dual beam spectrophotometer at 405nm. The p nitrophenol released was determined by reference to a calibration graph prepared by assay of standard p nitrophenol.

solutions serially diluted from a stock solution containing 10μ moles/ml and made up to 5.7ml with 3ml of 75% K_2HPO_4 (see Fig. 4.1B).

Assay of trehalase

The method employed was that described by DEFR AND RANDALL (1966). 2ml of 0.1M sodium hydrogen maleate/NaOH buffer pH5.2 (DAWSON ET AL, 1969) were equilibrated at $30^{\circ}C$ with 0.5ml of diluted homogenate for 5 minutes. The experiment was started by the addition of 0.5ml of 0.14M trehalose and the experiments were run routinely for 30 minutes.

Trehalase activity was determined by estimation of the glucose liberated from the trehalose during the reaction, using the glucose oxidase method of WERNER ET AL (1970), (reagents were supplied by Boehringer). The reaction was stopped by heating the tubes to $100^{\circ}C$ in a boiling water bath for 10 minutes. After cooling insoluble material was spun down by centrifuging for 15 minutes at 1500 r.p.m. on a Mistral 2L M.S.E. centrifuge. 5ml of glucose oxidase reagent was added to 1ml of the supernatant, mixed thoroughly on a Gallenkamp Vortex mixer and incubated at $30^{\circ}C$ for 15 minutes. The glucose oxidase enzyme in the above reagent oxidises glucose to gluconolactone which is converted to gluconic acid and H_2O_2 . The latter in the presence of peroxidase oxidises the chromogen "Perid" with the formation of a dye. The intensity of the colour of the dye is proportional to the glucose concentration. The absorbance of the solution was read against a distilled water blank at 560nm on a Pye Unicam SP 1800 Dual Beam spectrophotometer and the glucose content was calculated by reference to a standard solution of glucose (BOEHRINGER; 9.1mg glucose/100ml). Substrate and homogenate blanks were prepared by the addition of distilled water instead of homogenate in the case of the former and substrate in the case of the latter.

The assay of a "Trypsin-like" protease

The method employed was essentially that described by ERLANGER ET AL (1961). 2ml aliquots of 0.2M glycine/NaOH buffer pH 9.1 containing 25mM CaCl_2 were equilibrated with 0.2ml of appropriately diluted homogenate for 5 minutes. The experiment was started by the addition of 0.2ml substrate, 36mM D.L. benzoyl arginine p nitranilide BAPN in dimethyl formamide. The experiments were incubated routinely for 15 minutes.

ERLANGER ET AL (1961) used 1% dimethylsulfoxide detergent to solubilize the BAPN. NAGEL ET AL (1965) used 1.86% dimethyl formamide while BOEHRINGER (1975) found that 8% dimethyl formamide was necessary to solubilize high substrate concentrations. It was decided to follow Boehringer and use 8% dimethyl formamide and accept the 10% inhibition of activity which ensues. The substrate, made up in dimethyl formamide was stored in a dark bottle at room temperature and used within a week.

'Trypsin' activity was determined by estimation of the amount of p nitro aniline released from DL BAPN during the reaction. The optical density OD_1 , was measured before incubation and after incubation OD_2 ,

$\Delta E = OD_2 - OD_1$, being a measure of the p nitro aniline released during the reaction. This procedure obviated the need to spin down the protein. However, it meant that the reaction could not be stopped by the addition of 30% acetic acid (ERLANGER ET AL, 1961). Strict adherence to the 15 minute incubation time followed by prompt reading of the absorbance OD_2 on a Pye Unicam SP 1800 Dual Beam Spectrophotometer at 405nm ensured accurate assays. Alternatively, for certain experiments, incubations were performed in Bausch and Lomb "spectronic 20" tubes and changes in absorbance followed in a "spectronic 20" spectrophotometer. The amount of p nitro aniline released was determined by reference to a calibration graph of standard p nitro aniline solutions serially diluted from a stock solution containing $1.2 \mu\text{moles/ml}$ of p nitro aniline (see Fig.4.2A).

Possible effect of bacterial contamination during enzyme assays

EVANS (1956) showed how important it is to prevent bacteria from interfering with enzyme assays. Bacteria may remove the products of the enzymes' action. Thus it has been common practice to use a bactericidal agent such as toluene. However, more modern enzyme assay procedures, such as used in the present work, do not require long incubation periods, 10-30 minutes instead of 24 hours or more. So the possibility of bacterial action is very much reduced. To check this, the gut homogenate was tested for its action on trehalase and trypsin over a period of $\frac{1}{2}$ hour, with and without toluene (see Table 4.1). Since no significant difference was found between those determinations with toluene and those without, it was decided that a bactericide was unnecessary.

Temperature stability of the buffers used for enzyme assays

All the buffers employed were found to be stable over the temperature ranges used (see results section), apart from glycine/NaOH (see Fig. 4.2B). In this instance the buffer was made up and the pH measured at each temperature used for assay.

Method of incubation for enzyme assays

Enzyme activities were assayed in glass centrifuge tubes. All temperatures of assay were controlled to $\pm 0.1^{\circ}\text{C}$ by a 500 watt immersion heater connected by a hotwire vacuum switch relay (Sunvic Controls Ltd.) to a 'Jumos' electrical contact thermometer (A. Gallenkamp & Co. Ltd.).

For experiments on the temperature characteristics of enzymes, temperature gradients were set up using a thick aluminium bar ($1.2 \times 0.1 \times 0.06\text{m}$) with a series of holes drilled at short intervals along its length, to accommodate the assay tubes. A crushed ice bath at one end and a hot water bath at the other provided a gradient of temperatures between 10°C and 65°C .

Protein estimations

The protein content of enzyme preparations was determined by the method of LOWRY, ROSEBROUGH, FARR AND RANDALL (1951) using bovine serum albumin (BSA) fraction V as standard (Sigma Chemical Co. Ltd.). See Appendix (Section 4.1).

Enzyme activities were then expressed as μ moles or μ g product/mg protein/min.

Statistical techniques

1. Statistical comparisons of data were performed using conventional techniques as described by SNEDECOR AND COCHRAN (1967). Where appropriate reference was made to the statistical tables of FISCHER AND YATES (1963). Values of probability ≤ 0.05 were taken as significant.

2. Treatment of saturation kinetics data

The Michaelis Menton equation as modified by Lineweaver and Burk (1934) permits the graphical representation of the kinetic parameters K_m and V_{max} (DIXON AND WEBB, 1964).

Kinetic constants were determined by the method of least squares (SNEDECOR AND COCHRAN, 1967). Regression analysis provides a best estimate fit of the regression line for the available data. V_{max} was calculated from $\frac{1}{\text{intercept}}$ on the Y axis and $K_m = V_{max} \times \text{slope}$.

The double reciprocal plot is the most often used of the several straight line conversions available (DIXON AND WEBB, 1964) but it may be the least reliable source of estimate of V_{max} and K_m . DOWD AND RIGGS (1965) found that if used without adequate weighting it can result in large errors in the kinetic constants since the smallest value of V plays an inordinately important role in determining the position of the fitted line. They concluded that more consistent

estimates of V_{max} and K_m are given by plotting V against $(\frac{V}{S})$ EADIE (1952), HOFSTEE (1952). Once again these parameters, V_{max} and K_m , were calculated by regression analysis as indicated previously for Lineweaver-Burk plots. Estimates of V_{max} and K_m calculated from both Lineweaver-Burk and Eadie-Hofstee transformations of the Michaelis Menton equation are quoted.

3. Estimation of Arrhenius activation energy

Arrhenius plots of $\log V$ against $\frac{1}{T} K$ were prepared and the slope (A) of the equation $\log V = -\frac{A}{T} + B$ was determined by regression analysis (SNEDECOR AND COCHRAN, 1967). Arrhenius activation energy was computed from the equation $E_a = R \times 2.303 \times A \text{ K joules mole}^{-1}$ where $R =$ gas constant, $8.314 \text{ K joules/mole/}^{\circ}\text{K}$

$E_a =$ activation energy.

Fig. 4.1

A. The effect of pH on the development of the yellow colour of p nitrophenol

Method

25 μ l of 10 μ moles/ml standard solution of p nitrophenol was added to 3ml of McIlvaine's buffer (see text) at the pH indicated.

Note, above pH 8.0 colour development was not substantially increased.

Legend:

Ordinate ; absorbance at 405nm

Abscissa ; pH

B. Standard calibration graph for the determination of p nitrophenol released from artificial substrates by carbohydrases

Method

A standard p nitrophenol solution was serially diluted from a stock solution containing 10 μ moles/ml with 3ml of McIlvaine's buffer (see text) and brought to pH 8.5 with 3ml of 75% K_2HPO_4 .

Legend:

Ordinate ; absorbance at 405nm

Abscissa ; μ moles p nitrophenol.

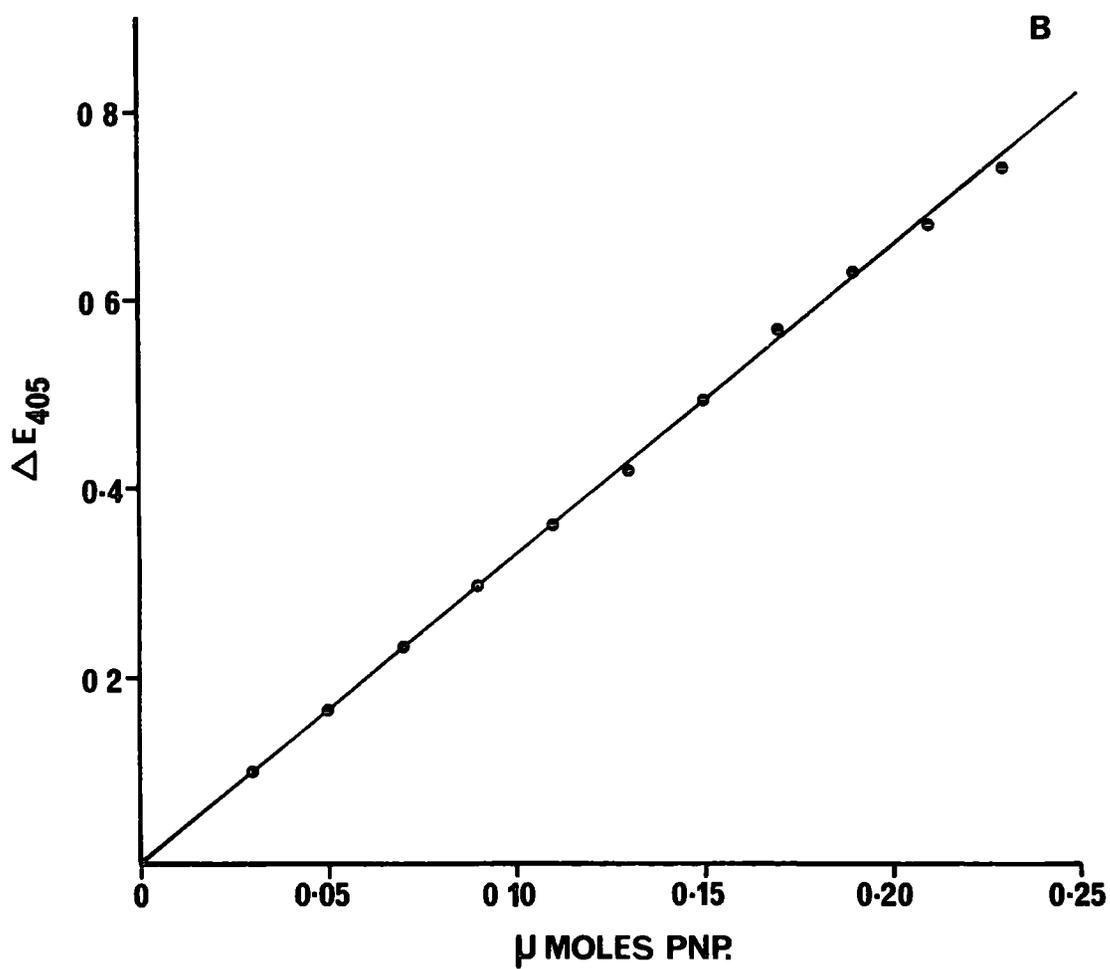
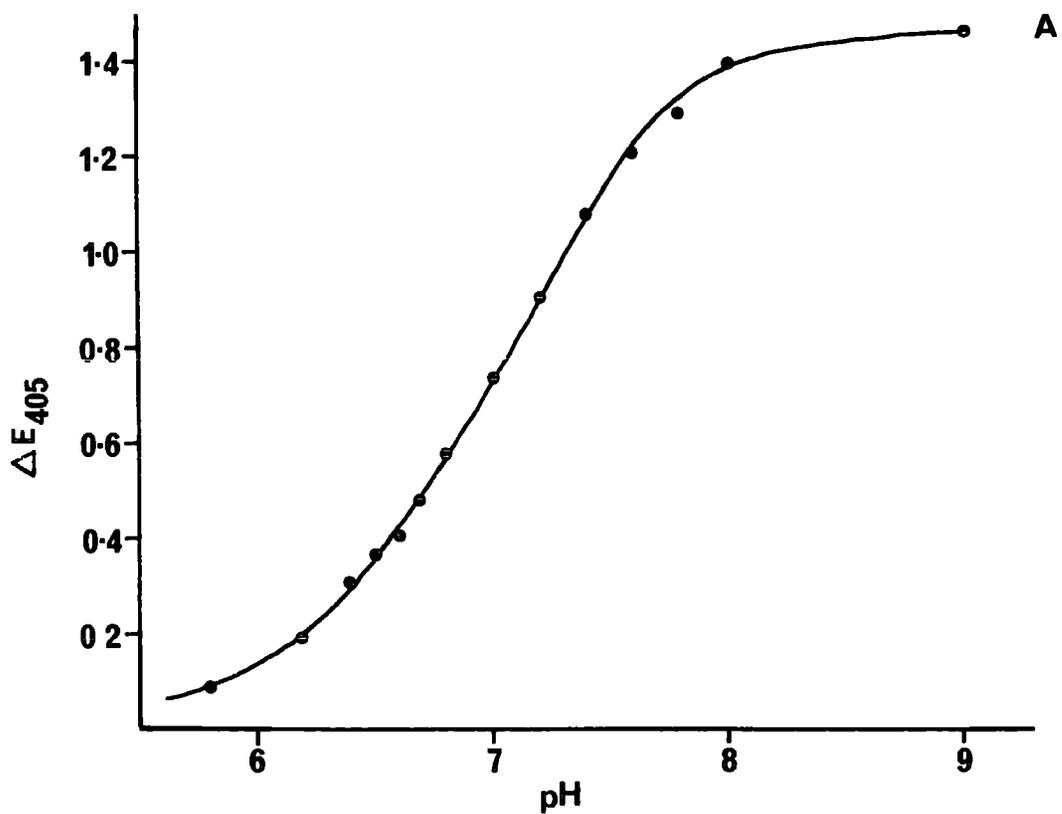


Fig. 4.2

A. Standard calibration graph for the determination of p nitroaniline released from benzoyl arginine p nitranilide

Method

A standard p nitroaniline solution was serially diluted from a stock solution containing 2μ moles/ml of p nitro aniline in dimethyl formamide. 0.2ml of the standard was diluted with 2ml of glycine/NaOH buffer (see text) and 0.2ml distilled water.

Legend:

Ordinate ; absorbance at 405nm

Abscissa ; μ moles p nitroaniline

B. The effect of temperature on the pH of glycine/NaOH buffer used for assaying "Trypsin"

Legend:

Ordinate ; pH

Abscissa ; temperature $^{\circ}$ C.

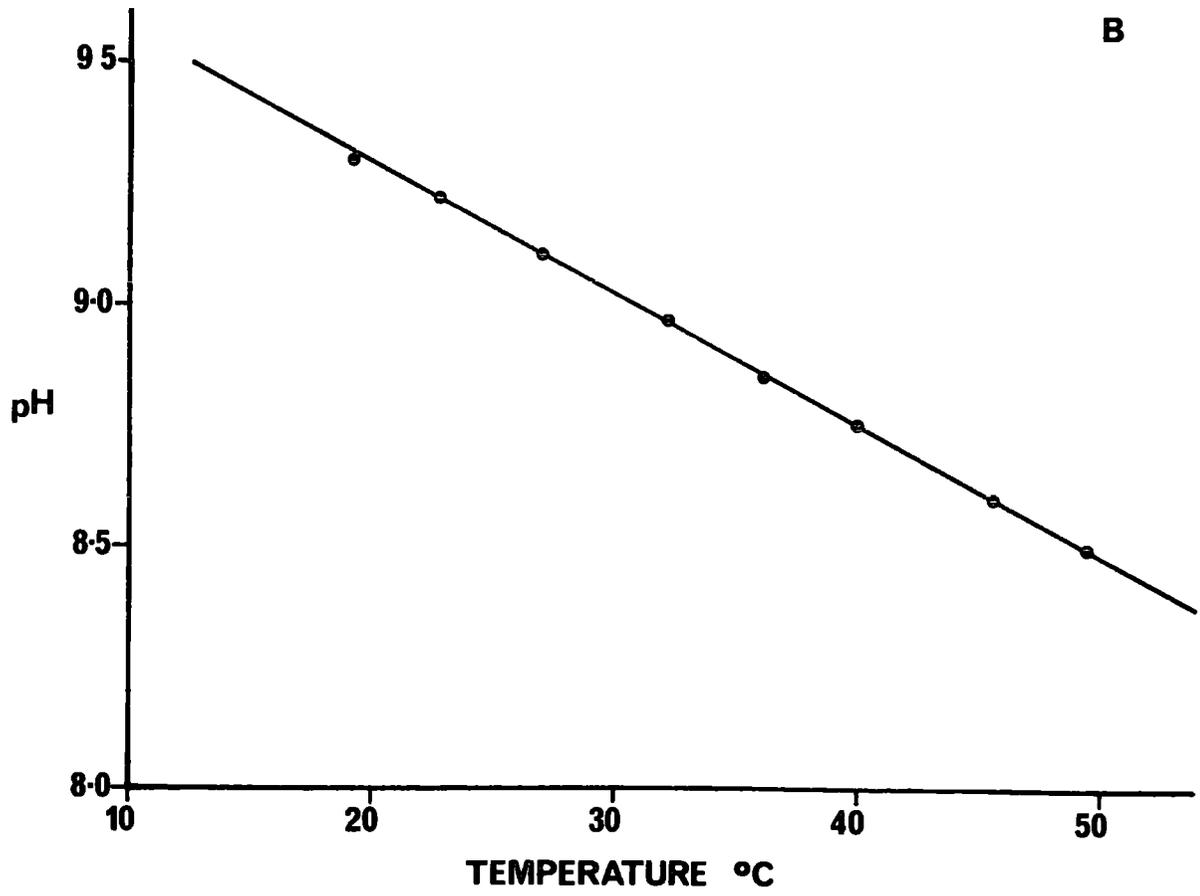
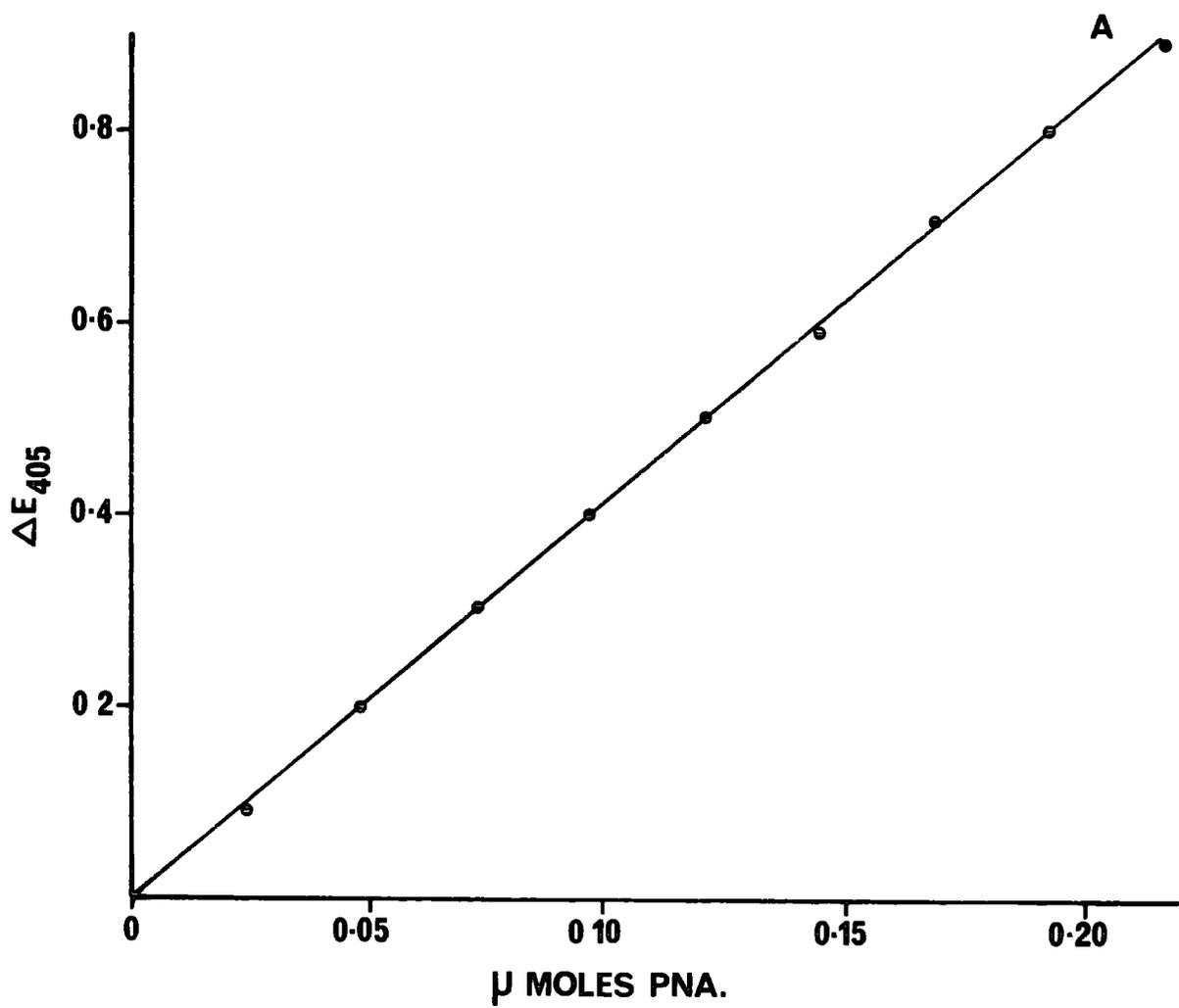


Table 4.1

Possible effect of bacterial contamination during enzyme assays

Method

The standard assay procedure for these enzymes was employed.

1. trehalase

activity ; μg glucose/mg protein/min

Presence of 1ml of
the bactericide,
toluene

No toluene

24.47

24.47

25.30

24.39

22.65

23.81

24.20

22.46

2. 'trypsin'

activity ; μ moles p nitro aniline/mg protein/min

Presence of 1ml of
the bactericide
toluene

No toluene

0.086

0.086

0.085

0.088

0.089

0.089

Results

1. α D glucosidase

The effect of pH on enzyme activity

The effect of pH on the activity of α D glucosidase is shown in Fig. 4.3A. The two buffers used did not appear to alter the pH profile. And the greatest activity was observed between 5 and 5.8, with an optimum at pH 5.4.

The effect of substrate concentration upon enzyme activity

Over a period of 50 minutes there was a linear relationship between enzyme activity and time with substrate concentrations greater than 0.1mM (see Fig. 4.3B).

The effect of substrate concentration on the catalytic rate of α D glucosidase was determined under sub optimal (pH 6.45) and optimal conditions (pH 5.4) of pH. The kinetic constants K_m and V_{max} were calculated from regression analysis of the data using both Lineweaver-Burk and Eadie-Hofstee transformations of the Michaelis Menton equation see Appendix Table (1.1). There was no significant difference between K_m and V_{max} calculated in either of the two ways. Since this is true for all six enzymes the values quoted in the text are those calculated by the Lineweaver-Burk method. Figs. 4.4A and B are representative graphs of Lineweaver-Burk plots performed under suboptimal and optimal conditions of pH.

The mean K_m at pH 6.45 was found to be 2.67 mM/litre and V_{max} was 0.43μ moles p nitrophenol mg protein⁻¹ min⁻¹. At the optimal pH (5.4) the mean K_m was lower, 1.57 ± 0.08 mM/litre while V_{max} was higher, $1.05 \pm 0.03 \mu$ moles p nitrophenol mg protein⁻¹ min⁻¹ see Table 4.2.

The effect of temperature on the activity of the enzyme

The effect of temperature on the activity of α D glucosidase was determined under conditions of optimal and suboptimal pH in the ranges 15 - 55°C, 5.4 and 15-70°C at pH 6.45. The optimum temperature at pH 6.45 was found to be 55°C and that at pH 5.4 was 50°C. Arrhenius plots prepared from this data exhibited breaks at approximately 30.5°C (pH 6.45) and 32.3°C (pH 5.4). (Representative graphs are shown in Fig. 4.5). The mean activation energy under suboptimal conditions of pH was 52.52 ± 2.84 K joules mole⁻¹ between 47.5°C and 37.5°C, while between 30.5°C and 16.8°C it was 68.85 ± 2.12 K joules mole⁻¹. Under optimal pH conditions the mean activation energy was found to be 33.22 ± 4.08 K joules mole⁻¹ between 48.4°C and 32.3°C, while between 32.3 and 18.5°C it was 64.2 ± 2.91 K joules mole⁻¹. See Table 4.2 and Appendix Table (1.1).

These values were calculated by the method indicated in the materials and methods.

2. β D glucosidase

The effect of pH on enzyme activity

The effect of pH on the activity of β D glucosidase is shown in Fig. 4.6A. The two buffers used did not appear to alter the pH profile. And the greatest activity was observed between pH 5 and pH 5.5, with an optimum at pH 5.2.

The effect of substrate concentration upon enzyme activity

Fig. 4.6B shows the relationship between enzyme activity and time. The activity of the enzyme was found to be linear for concentrations above 0.1mM, over the 60 minute period studied.

The effect of substrate concentration on the catalytic rate of β D glucosidase was determined under suboptimal conditions of pH (6.75) and optimal conditions of pH (5.2). A Lineweaver-Burk plot

of the results obtained at pH 6.75 reveals the expected straight line relationship as predicted from the Michaelis Menton equation (for a representative graph see Fig. 4.7A). However, a complex graph consisting of two straight lines intersecting at approximately 1.98mM p nitrophenol β D glucoside is obtained at pH 5.2 (for a representative graph see Fig. 4.7B). For analytical purposes the two lines were taken to represent separate enzyme systems. K_m and V_{max} for each line were calculated separately.

The kinetic constants K_m and V_{max} were calculated using both Lineweaver-Burk and Eadie-Hofstee transformations of the Michaelis Menton equation, see Appendix Table (1.2). The mean K_m under sub-optimal conditions of pH was found to be 4.65mM/litre and mean V_{max} was 0.1μ moles p nitrophenol mg protein⁻¹ min⁻¹. Under optimal conditions of pH the mean K_m at high substrate concentrations was 4.13 ± 0.57 mM/litre and mean V_{max} was $0.52 \pm 0.08 \mu$ moles p nitrophenol mg protein⁻¹ min⁻¹. At low substrate concentrations the mean K_m was 1.59 ± 0.34 mM/litre and the mean V_{max} $0.34 \pm 0.07 \mu$ moles p nitrophenol mg protein⁻¹ min⁻¹. (See Table 4.3).

The effect of temperature on the activity of the enzyme

The effect of temperature on the activity of β D glucosidase was determined under suboptimal conditions of pH (6.75) and optimal conditions of pH (5.2) in the temperature ranges 19 - 66°C and 10-56°C respectively.

The optimum temperature at pH 6.75 was approximately 56°C but none was recorded at pH 5.2 up to a temperature of 56°C. This enzyme only exhibited a slight break in the Arrhenius plots at either of the two pH regimes it was assayed under. These breaks occurred at approximately 35.8°C under suboptimal and 29°C under optimal

conditions of pH (Representative graphs are shown in Fig.4.8).

The mean activation energy under suboptimal conditions of pH was found to be 51.69 ± 1.71 K joules mole⁻¹ between 53°C and 35.8°C, and 66.23 K joules mole⁻¹ between 35.8°C and 19°C. Under optimal conditions the mean activation energy was found to be 49.47 K joules mole⁻¹ between 51°C and 29°C, and 53.38 K joules mole⁻¹ between 29°C and 13.3°C (See Table 4.3 and Appendix Table 1.2).

3. α D galactosidase

The effect of pH on enzyme activity

The effect of pH on the activity of α D galactosidase is shown in Fig. 4.9A. The two buffers used did not appear to alter the pH profile. A broad peak of activity was observed between pH 5 and 5.4 with an optimum at pH 5.2.

The effect of substrate concentration upon enzyme activity

Over a period of 50 mins there was a linear relationship between enzyme activity and time for substrate concentrations greater than 0.1mM See Fig. 4.9B.

The effect of substrate concentration on the catalytic rate of α D galactosidase was determined under suboptimal conditions of pH (7.0) and under optimal conditions of pH (5.2). The kinetic constants Km and Vmax were calculated using both Lineweaver-Burk and Eadie-Hofstee transformations of the Michaelis Menton equation, See Appendix Table (1.3).

The mean Km under suboptimal conditions of pH was found to be 0.73mM/litre while the mean Vmax was 0.014 μ moles p nitrophenol mg protein⁻¹ min⁻¹. Under optimal conditions of pH the mean Km was found to be 1.26 ± 0.12 mM/litre while the Vmax was 0.16 μ moles p nitrophenol mg protein⁻¹ min⁻¹ (See Table 4.4 and for representative graphs Fig. 4.10A and B).

The effect of temperature on the activity of the enzyme

The effect of temperature on the activity of α D galactosidase was determined under conditions of optimal and suboptimal pH in the ranges 22 - 66°C at pH 7.0 and 22 - 65°C at pH 5.2. The optimum temperature at pH 7.0 was found to be 52°C while none was measured at pH 5.2 up to an assay temperature of 65°C. Breaks were observed in the Arrhenius plots at each pH, at approximately 32°C for pH 7.0 and 33°C for pH 5.2 respectively. (Fig. 4.11 shows representative graphs).

The mean activation energy under conditions of suboptimal pH was found to be 43.41 ± 2.32 K joules mole⁻¹ between 53°C and 32°C, while between 32°C and 22°C it was 64.07 ± 2.75 K joules mole⁻¹. Under optimal pH conditions the activation energy was found to be 50.53 K joules mole⁻¹ between 54°C & 32.5°C while between 32.5°C and 19°C it was 88.49 K joules mole⁻¹ (see Table 4.4 and Appendix Table (1.3)).

4. β D galactosidase

The effect of pH on enzyme activity

The effect of pH on the activity of β D galactosidase is shown in Fig. 4.12A. The two buffers used did not appear to alter the pH profile. And the highest activity was observed between pH 4.6 and pH 5.0, with an optimum at pH 4.8.

The effect of substrate concentration upon enzyme activity

Over a period of 60 minutes there was a linear relationship between enzyme activity and time for substrate concentrations greater than 0.1mM. See Fig. 4.12B.

The effect of substrate concentration on the catalytic rate of β D galactosidase was determined under suboptimal conditions of pH (6.6) and optimal conditions of pH (4.8) Lineweaver-Burk plots of the

results obtained under both pH regimes show complex graphs consisting of two straight lines intersecting at approximately 1.36mM p nitrophenol β D galactoside at pH 6.6 and 2.46mM at pH 4.8 (representative graphs are shown in Fig. 4.13A and B). For analytical purposes the two lines were taken to represent separate enzyme systems. K_m and V_{max} for each line were calculated separately.

The kinetic constants K_m and V_{max} were calculated using both Lineweaver-Burk and Eadie-Hofstee transformations of the Michaelis Menton equation, see Appendix Table (1.4). Under suboptimal conditions of pH the mean K_m at substrate concentrations greater than 1.36mM β D galactoside was found to be 1.0mM/litre and mean V_{max} was 0.058 μ moles p nitrophenol mg protein⁻¹ min⁻¹. At substrate concentrations below 1.36mM β D galactoside the mean K_m was found to be 0.19 mM/litre and the mean V_{max} 0.038 μ moles p nitrophenol mg protein⁻¹ min⁻¹. Under optimal conditions of pH the mean K_m at substrate concentrations in excess of 2.46mM β D galactoside was found to be 1.78 \pm 0.19mM/litre and V_{max} 0.139 \pm 0.05 μ moles p nitrophenol mg protein⁻¹ min⁻¹. Below a 2.46mM substrate concentration a mean K_m of 0.71 \pm 0.09mM/litre was found and a mean V_{max} of 0.089 \pm 0.03 μ moles p nitrophenol mg protein⁻¹ min⁻¹ (See Table 4.5).

The effect of temperature on the activity of the enzyme

The effect of temperature on the activity of β D galactosidase was determined under suboptimal conditions of pH (6.6) and optimal conditions of pH (4.8) in the temperature ranges 19 - 65°C and 15 - 65°C respectively. The optimum temperature at pH 4.8 was found to be 58°C but none was measured at pH 6.6. The Arrhenius plots under both pH regimes exhibited breaks at approximately 30°C at pH 4.8 and 38°C at pH 6.6 (representative graphs are shown in Fig. 4.14). The mean activation energy under conditions of sub-

optimal pH was found to be 67.52 ± 2.47 K joules mole⁻¹ between 58°C and 38°C, while between 38°C and 19°C it was 99.02 ± 16.2 K joules mole⁻¹. Under optimal pH conditions the mean activation energy was found to be 50.74 ± 2.74 K joules mole⁻¹ between 53 and 30°C, whereas between 30 and 15°C the activation energy was found to be 77.88 ± 3.88 K joules mole⁻¹. (See Table 4.5 and Appendix Table 1.4).

5. Trehalase

The effect of pH on enzyme activity

The effect of pH on the activity of trehalase is shown in Fig. 4.15A. The three buffers used did not appear to alter the pH profile. A broad peak of activity was observed with an optimum between pH 4.5 and 5.7.

A separate experiment was performed using only one buffer (0.1mM McIlvaine's buffer). This was to check that under the standard assay conditions (see materials and methods section) only the "soluble" trehalase was assayed and that centrifugation of the gut homogenate at 2,000 r.p.m. during the preparation of the extract removed the "membrane" bound form of the enzyme. Previous work has shown that the two forms have quite different pH optima, the "soluble" enzyme exhibited an optimum of about pH 5.0 and the "membrane" bound form about pH 6.0 (GILBY ET AL, 1967; GUSSIN AND WYATT, 1964).

The shape of the pH curve was found to be the same whether the homogenate was centrifuged at 1,000, 2,000 or 40,000 r.p.m. (Fig. 4.15B). There were no indications of two peaks of activity in the supernatant fractions of homogenates centrifuged at any of these three speeds. Therefore, it would seem that the membrane bound form, which is only 10% of intestinal trehalase in other insect species (GILBY ET AL, 1967) was removed by the standard extraction procedure.

Broad peaks of activity were found with each of the three homogenates. An optimum between pH 4.5 and 5.5 was found for gut homogenates centrifuged at 2,000 and 40,000 r.p.m. and between pH 3.9 and 5.5 for the gut extract centrifuged at 1,000 r.p.m. (Fig. 4.158).

The effect of substrate concentration upon enzyme activity

Over a period of 60 minutes a linear relationship was found between enzyme activity and time for substrate concentrations greater than 1.0mM (See Fig. 4.16A).

The effect of substrate concentration on the catalytic rate of the trehalase was determined under optimal conditions of pH (5.1) only. The kinetic constants K_m and V_{max} were calculated using both Lineweaver-Burk and Eadie-Hofstee transformations of the Michaelis-Menton equation (See Appendix Table 1.5). The mean K_m was found to be 0.92 ± 0.06 mM/litre and the mean V_{max} was 13.43 ± 3.33 μ g glucose mg protein⁻¹ min⁻¹ (See Table 4.6, a representation graph is shown in Fig. 4.16B).

The effect of the temperature on the activity of the enzyme

The effect of temperature on the activity of trehalase was determined under optimal conditions of pH in the range 16 - 65°C. The enzyme was found to have a temperature optimum of approximately 58°C. In contrast to the other enzymes studied in this work trehalase exhibited no break in the Arrhenius plot (for a representation graph see Fig. 4.17). The mean activation energy was found to be 45.62 K joules mole⁻¹ between 55 and 16°C (see Table 4.6 and Appendix Table 1.5).

6. "Trypsin-like" protease

The effect of pH on enzyme activity

The effect of pH on the activity of 'trypsin' is shown in Fig. 4.18A. The glycine/NaOH buffer used at high pH appeared to depress the activity enzyme somewhat with respect to that observed with Tris buffer. Therefore the activities of the enzyme assayed with glycine/NaOH buffer were expressed as a 100% of the activity with tris buffer at pH 9.3. A broad peak of activity was observed between pH 8.7 and 9.7, with an optimum pH of 9.1.

The effect of substrate concentration upon enzyme activity

Over a period of 50 minutes a linear relationship was found between enzyme activity and time for substrate concentrations greater than 0.17mM (see Fig. 4.18B).

The effect of substrate on the catalytic rate of the "soluble" trehalase was determined under optimal conditions of pH (9.1) only. The kinetic constants K_m and V_{max} were calculated using both the Lineweaver-Burk and Eadie-Hofstee transformations of the Michaelis-Menton equation (see Appendix Table 1.6).

The mean K_m was found to be $0.88 \pm 0.07\text{mM/litre}$ and mean V_{max} was $0.16 \pm 0.04 \mu \text{ moles p nitroaniline mg protein}^{-1} \text{ min}^{-1}$. (See Table 4.7 and representative graph shown in Fig. 4.19).

The effect of temperature on the activity of the enzyme

The effect of temperature on the activity of the 'trypsin' was determined under optimal conditions of pH in the temperature range 17 - 60°C. A temperature optimum of approximately 47°C was found. Arrhenius plots exhibited a break at approximately 27.7°C (for a representative graph see Fig. 4.20).

The mean activation energy between 45.2 and 27.7°C was found to be 33.31 ± 3.28 K joules mole⁻¹, while between 27.7 and 16.3°C it was found to be 66.0 ± 3.67 K joules mole⁻¹ (see Table 4.7 and Appendix Table 1.6).

Effect of ovomucoid trypsin inhibitor on the active of "trypsin-like" protease

The proteolytic activity was inhibited by an ovomucoid type 11-0 trypsin inhibitor (Sigma Co.) (see Fig. 4.21). Small amounts of inhibitor had an effect but a concentration was reached in excess of which a relatively small increase in inhibition took place, $50 \mu\text{g}/0.086 \mu\text{ moles p nitroaniline mg protein}^{-1} \text{ min}^{-1}$.

In the presence of $100 \mu\text{g}$ of inhibitor, an enzyme activity of $0.085 \mu\text{ moles p nitroaniline mg protein}^{-1} \text{ min}^{-1}$ was inhibited 70%. The 30% of the activity that remained could have been due to a number of factors:

1. The inhibitor was progressively broken down by the enzyme. To test this possibility the inhibitor was preincubated with the enzyme for 15 minutes. If the above hypothesis had been correct a marked reduction in inhibition would have been observed. However, no such phenomenon was seen (Fig. 4.21).
2. The inhibitor was itself inhibited by some constituent of the homogenate. Six sets of reactants were equilibrated for 10 minutes (see Table 4.8), and the reactions started by the addition of the substrate and incubated for a further 20 minutes. The combined activities of tube 5 (containing locust 'trypsin' + inhibitor) and tube 6 (containing pure trypsin + inhibitor) were similar to tube 1 (containing locust 'trypsin' + trypsin + inhibitor). If the gut homogenate had an inhibitory action on the inhibitor then activity in tube 1 would have been less than the activities of tube 5 and tube 6 combined.

3. The inhibitor also split the substrate. For the latter to be substantiated, a decrease in the period of contact between the substrate and the inhibitor must be shown to result in a reduction in enzyme activity and a concomittant increase in inhibition. This set of conditions was achieved by preincubating the inhibitor and enzyme and starting the reaction by addition of the substrate. However, no increase in inhibition was observed (Fig. 4.21).

The effect of ovomucoid trypsin inhibitor on the activity of pure bovine pancreatic trypsin type III (Sigma) was for more dramatic than its effect on the 'trypsin' from Locusta gut (Fig. 4.21). 100% inhibition was observed at a concentration of $11\mu\text{g}$ of inhibitor/ $2\mu\text{g}$ of pure enzyme.

Table 4.2

Kinetic constants of α D glucosidase

pH	<u>Plot</u>	<u>Mean Km</u> ml./litre	<u>SE</u>	<u>mean Vmax</u> μ moles pnp/mg protein/min	<u>SE</u>	<u>Number of</u> <u>determinations</u>
6.45	L/B	2.67		0.43		2
	E/H	2.61		0.42		2
5.4	L/B	1.57	0.08	1.05	0.03	4
	E/H	1.53	0.08	1.04	0.06	4
pH	<u>average</u> <u>temperature</u> <u>range °C</u>	<u>mean activation energy</u> <u>Ea, K joules mole⁻¹</u>		<u>SE</u>	<u>Number of</u> <u>determinations</u>	
6.45	47.5 - 30.5	52.52		2.84	3	
	30.5 - 16.8	68.85		2.12	3	
5.4	48.4 - 32.3	33.22		4.08	3	
	32.3 - 18.5	64.20		2.91	3	

μ moles pnp

μ moles p nitrophenol

S.E.

standard error of the mean

L/B

Lineweaver-Burk

E/H

Eadie-Hofstee

Fig. 4.3

A. The effect of pH on the activity of α D glucosidase

Legend:

ordinate ; μ moles p nitrophenol/mg protein/min

abscissa ; pH

- pH 3.4 - 6.2 0.1M McIlvaine's buffer
- pH 5.8 - 7.0 0.1M sodium phosphate buffer

B. To demonstrate the linearity of α D glucosidase activity with time

Legend:

ordinate ; μ moles p nitrophenol/mg protein

abscissa ; time in minutes

- 3.07 mM p nitrophenol α D glucosidase
- 1.0 mM " "
- ▲ 0.1 mM " "

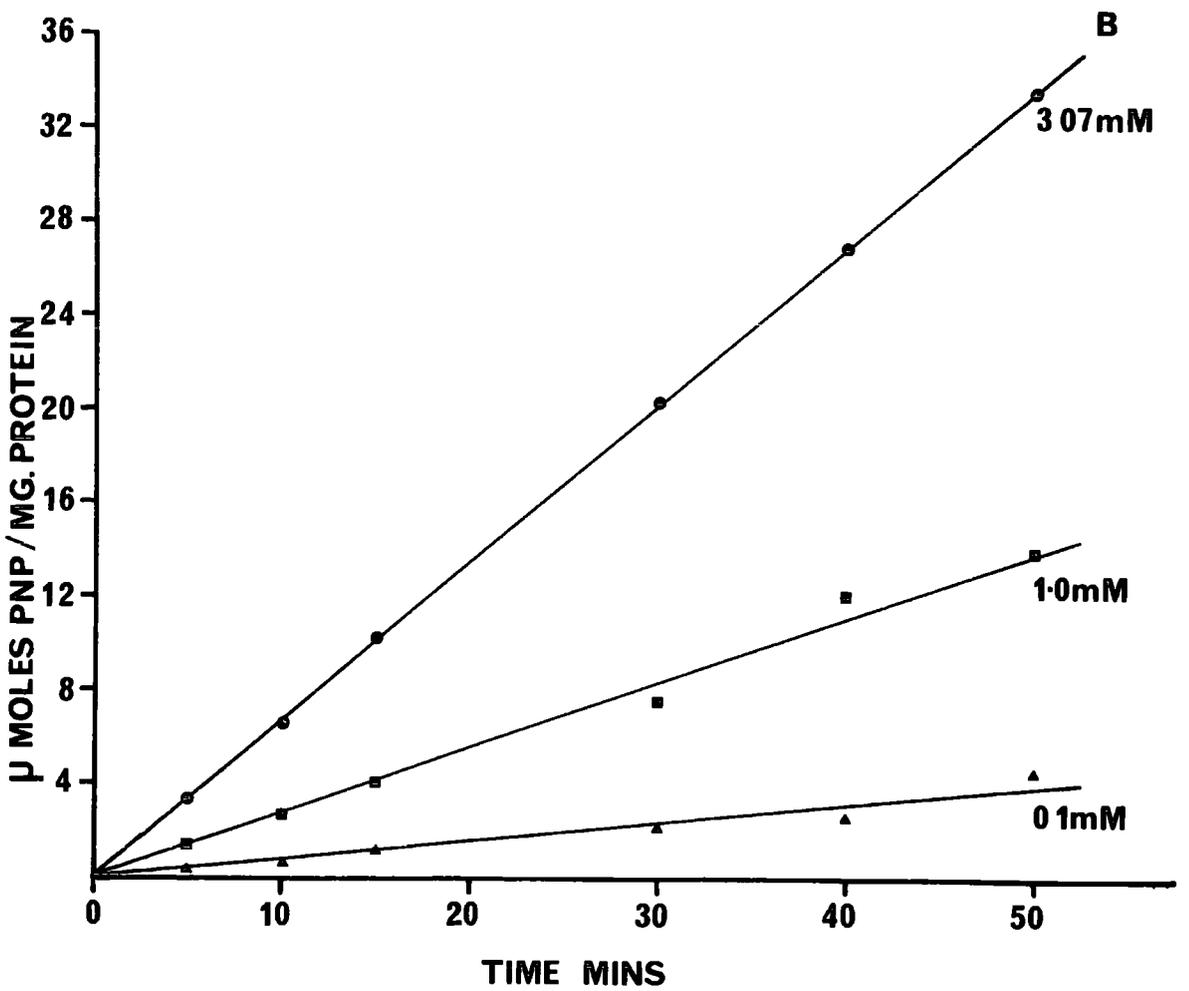
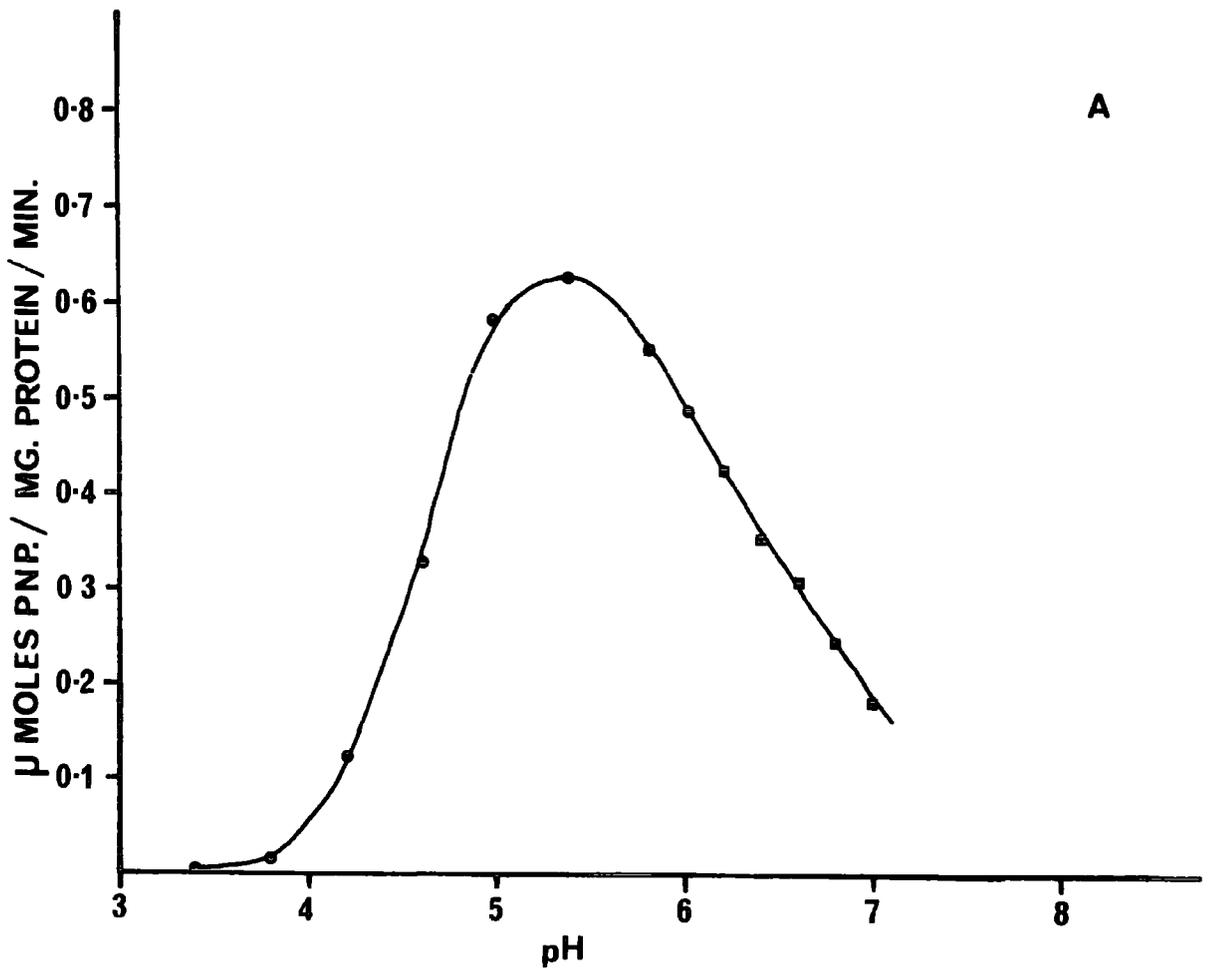


Fig. 4.4

The effect of substrate concentration on the activity
of α D glucosidase

A. under suboptimal conditions of pH
0.1M McIlvaine's buffer pH 6.45

Legend:

ordinate ; $\frac{1}{V}$ (μ moles p nitrophenol/mg protein/min)⁻¹
abscissa ; $\frac{1}{S}$ p nitrophenol α D glucoside mM⁻¹

B. under optimal conditions of pH
0.1M McIlvaine's buffer pH 5.4

Legend:

ordinate ; $\frac{1}{V}$ (μ moles p nitrophenol/mg protein/min)⁻¹
abscissa ; $\frac{1}{S}$ p nitrophenol α D glucoside mM⁻¹

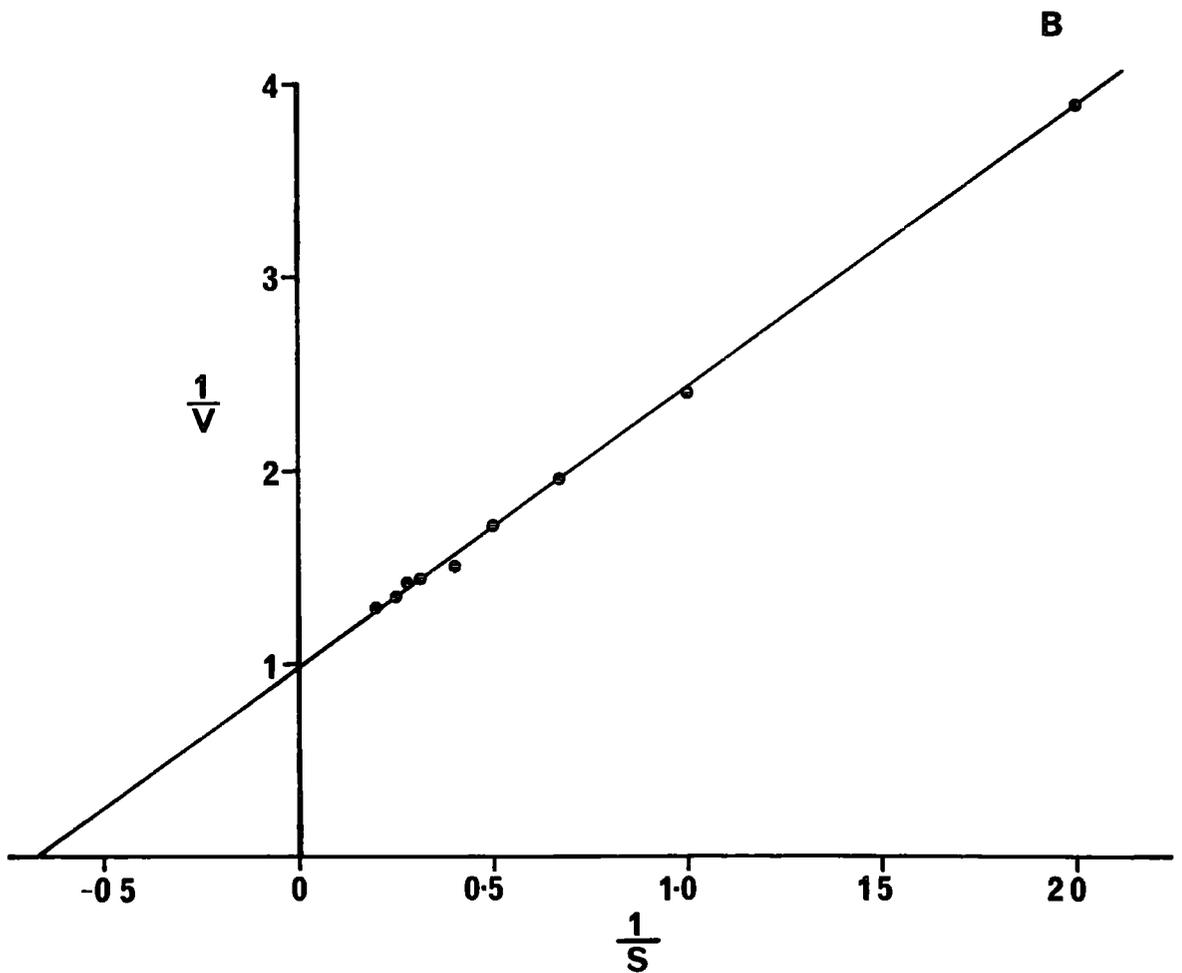
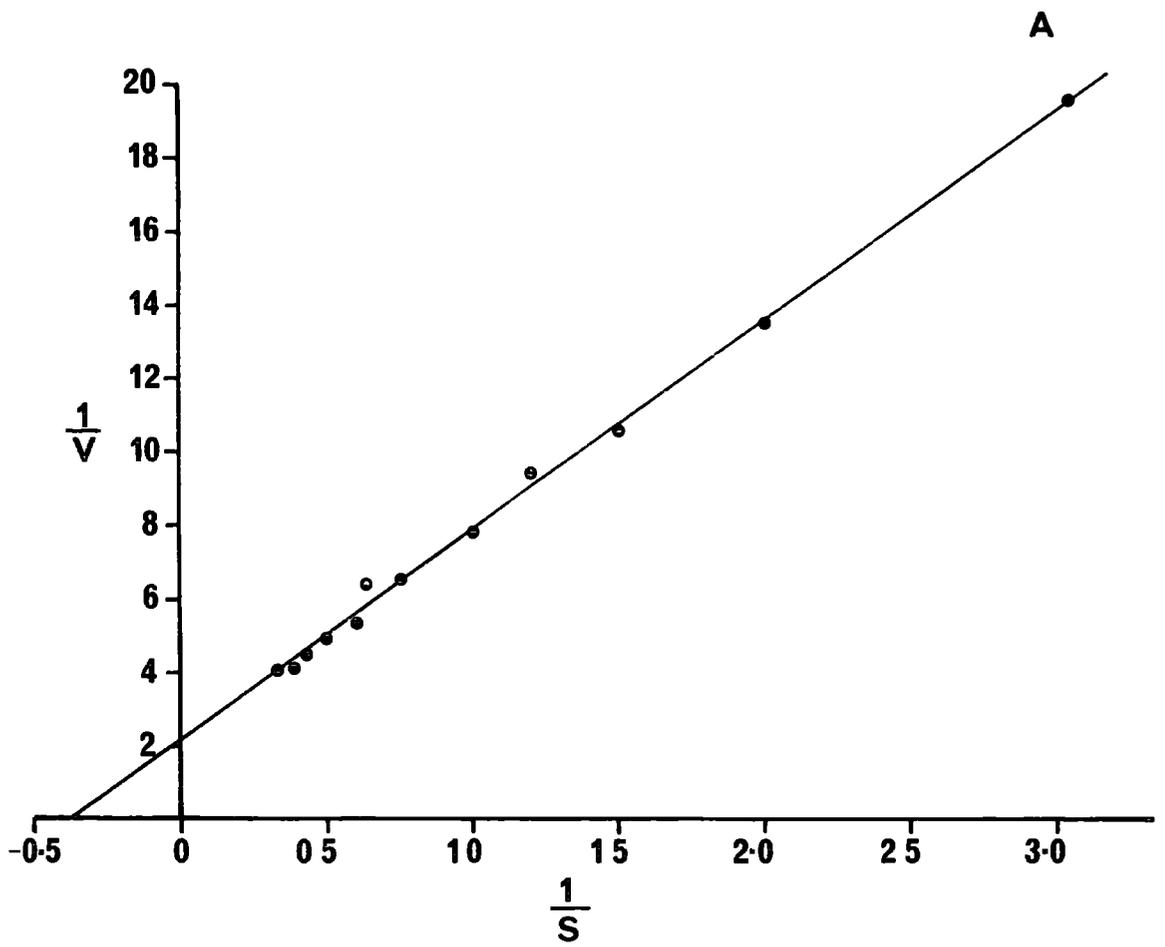


Table 4.5

The effect of temperature on the activity of
 α D glucosidase

Legend:

ordinate ; activity (μ moles p nitrophenol/mg protein/min)

abscissa ; $\frac{1}{\text{temperature}^{\circ}\text{A}} \times 10^3$

■ suboptimal conditions of pH, 6.45

● optimal conditions of pH, 5.4, activity $\times 10^{-1}$

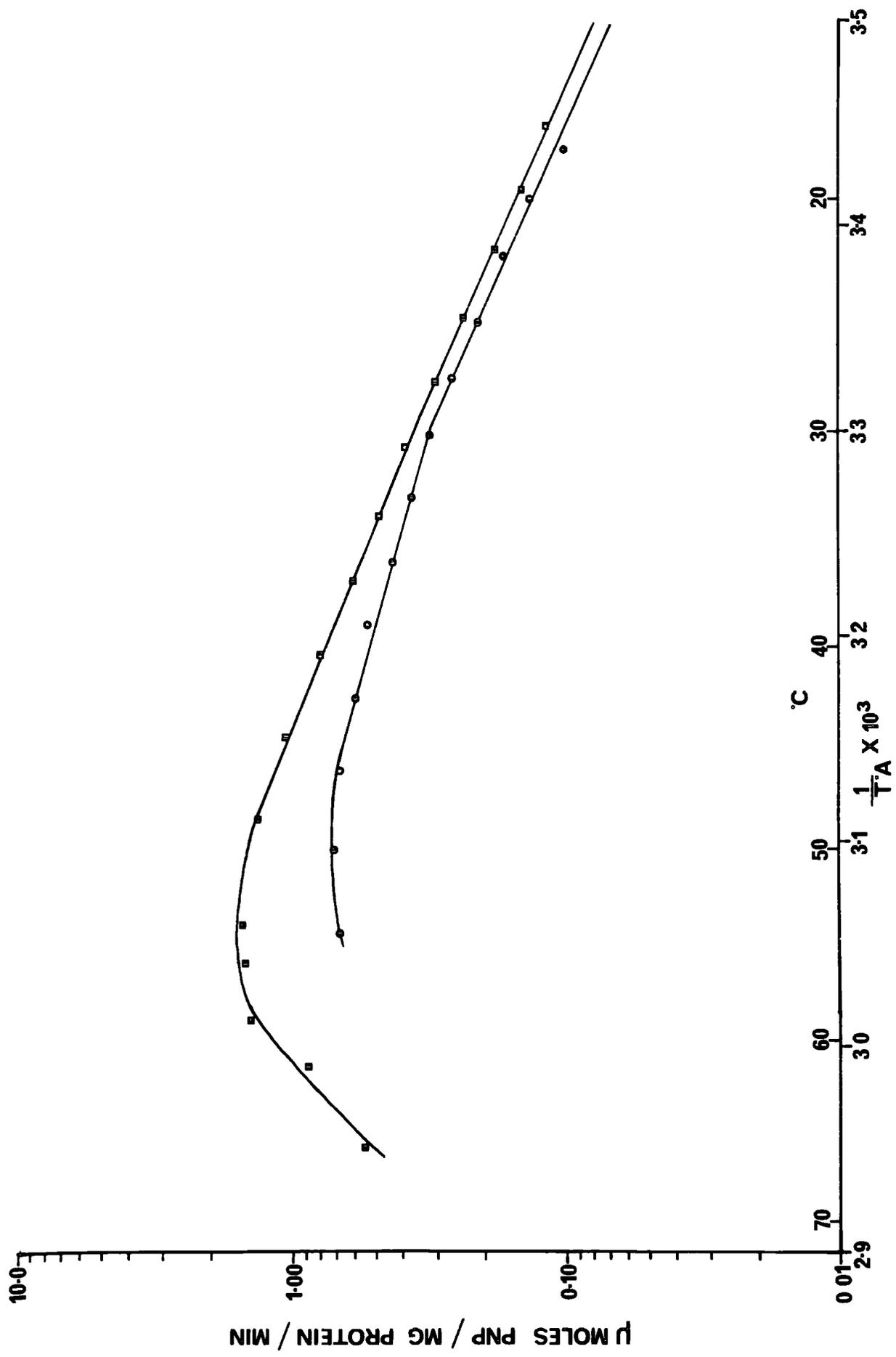


Table 4.3

Kinetic constants of β D glucosidase

<u>pH</u>	<u>range of substrate concentration mM</u>	<u>Plot</u>	<u>Mean Km mM/litre</u>	<u>SE</u>	<u>mean Vmax μ moles pnp/ protein/min</u>	<u>SE</u>	<u>Number of determinations</u>
6.75	0.6 - 5.0	L/B	4.65		0.1		2
		E/H	4.38		0.1		2
5.2	1.98- 8.6	L/B	4.13	0.57	0.52	0.08	4
			1.59	0.34	0.34	0.07	5
	0.33- 1.98	E/H	3.88	0.30	0.5	0.06	4
			1.63	0.34	0.34	0.07	5

<u>pH</u>	<u>average temperature $^{\circ}$C</u>	<u>mean activation energy Ea, K joules mole $^{-1}$</u>	<u>SE</u>	<u>Number of determination</u>
6.75	53 - 35.8	51.69	1.71	3
	35.8 - 19	66.23		2
5.2	51 - 29	49.47		2
	29 - 13.3	58.38		2

μ moles pnp μ mole p nitrophenol

S.E. standard error of the mean

L/B Lineweaver-Burk

E/H Eadie-Hofstee

Fig. 4.6

A. The effect of pH on the activity of β D glucosidase

Legend:

ordinate ; μ moles p nitrophenol/mg protein/min

abscissa ; pH

- pH 3.4 - 6.2 0.1M McIlvaine's buffer
- pH 5.8 - 7.0 0.1M sodium phosphate buffer

B. To demonstrate the linearity of β D glucoside activity with time

Legend:

ordinate ; μ moles p nitrophenol/mg protein

abscissa ; time in mins

- 7.38mM p nitrophenol β D glucoside
- 2.0mM " "
- ▲ 0.1mM " "

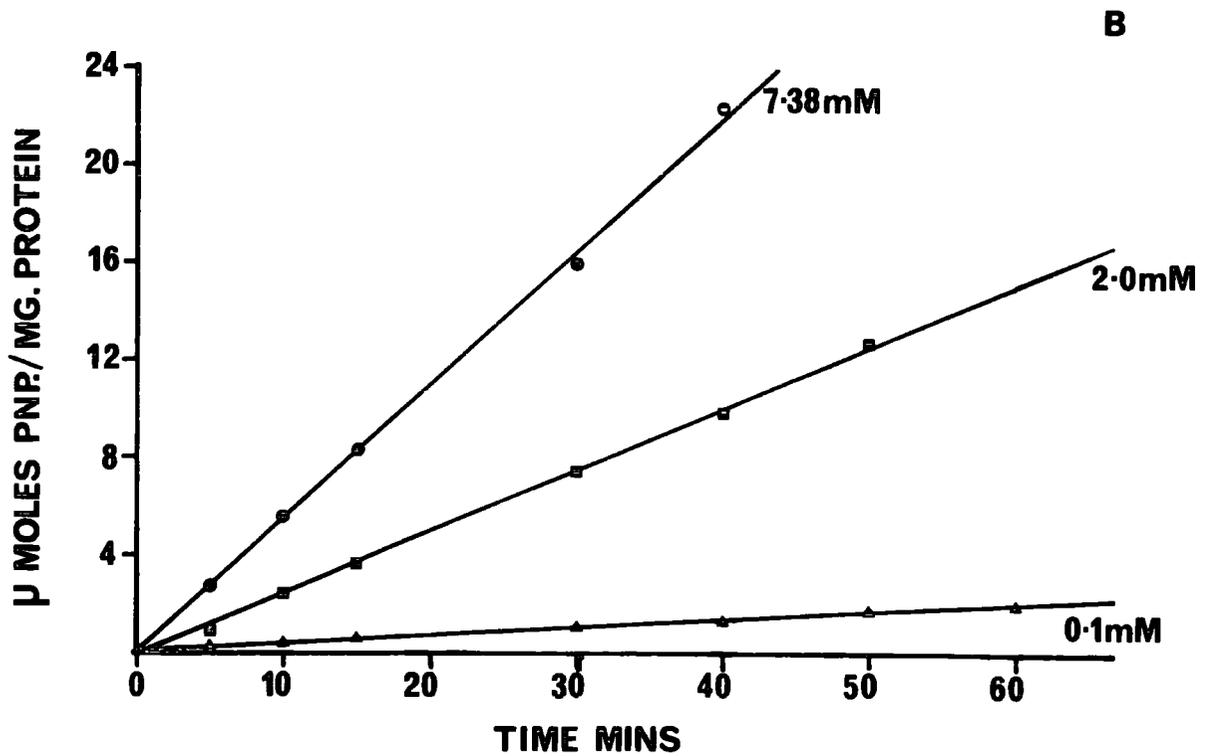
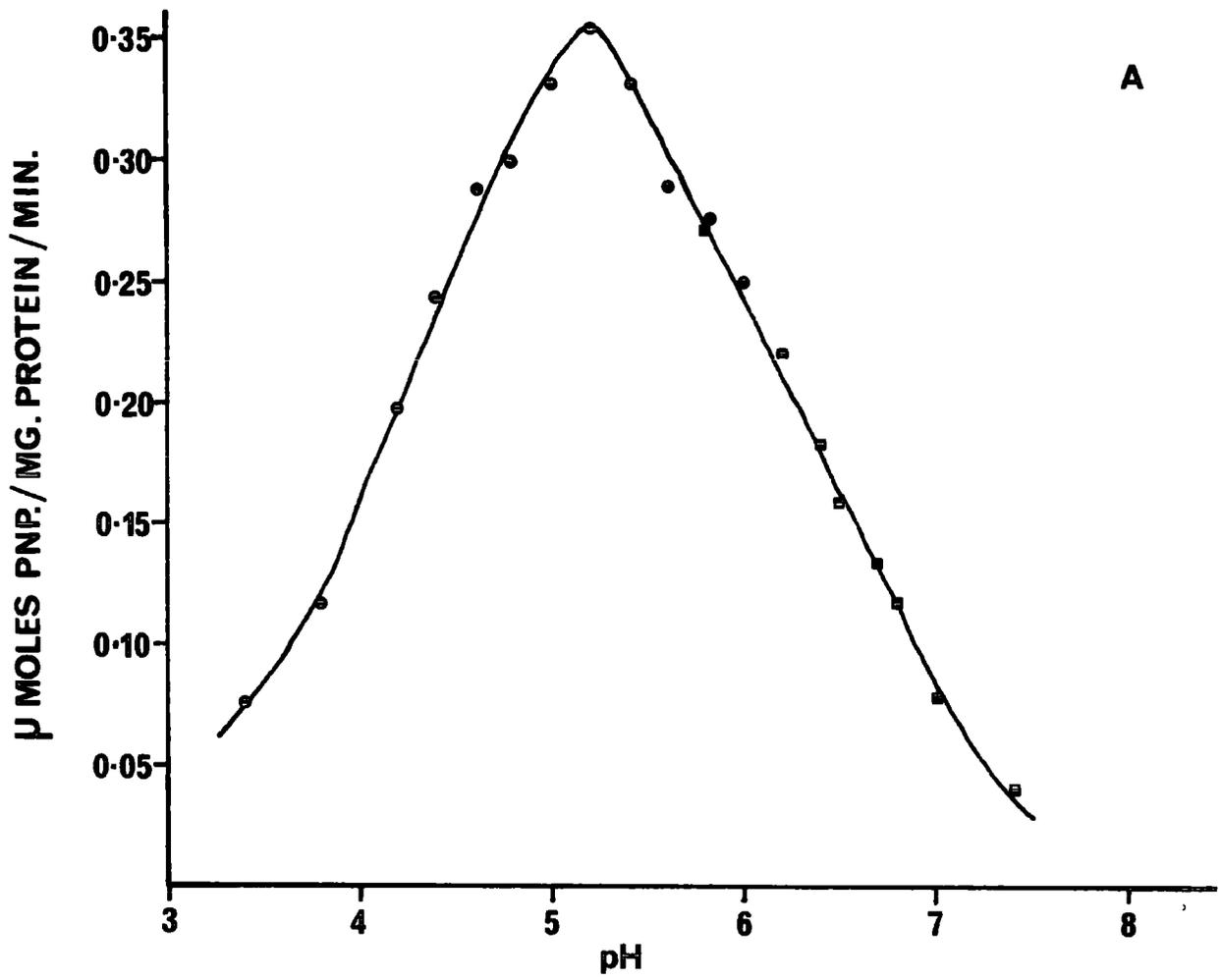


Fig. 4.7

The effect of substrate concentration on the activity
of β D glucosidase

A. under suboptimal conditions of pH

0.1M McIlvaine's buffer pH 6.75

Legend:

ordinate ; $\frac{1}{V}$ (μ moles p nitrophenol/mg protein/min)⁻¹

abscissa ; $\frac{1}{S}$ p nitrophenol β D glucoside mM⁻¹

B. under optimal conditions of pH

0.1M McIlvaine's buffer pH 5.2

Legend:

ordinate ; $\frac{1}{V}$ (μ moles p nitrophenol/mg protein/min)⁻¹

abscissa ; $\frac{1}{S}$ p nitrophenol β D glucoside mM⁻¹

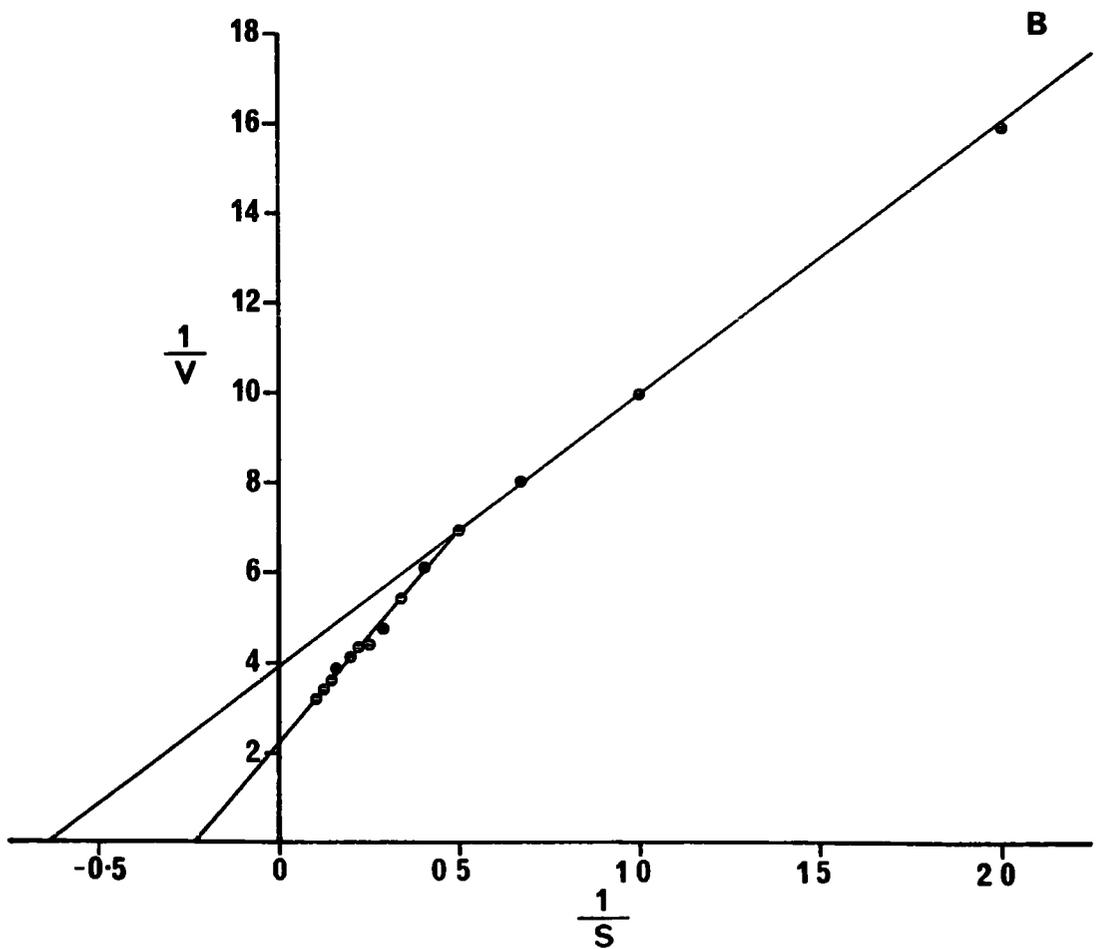
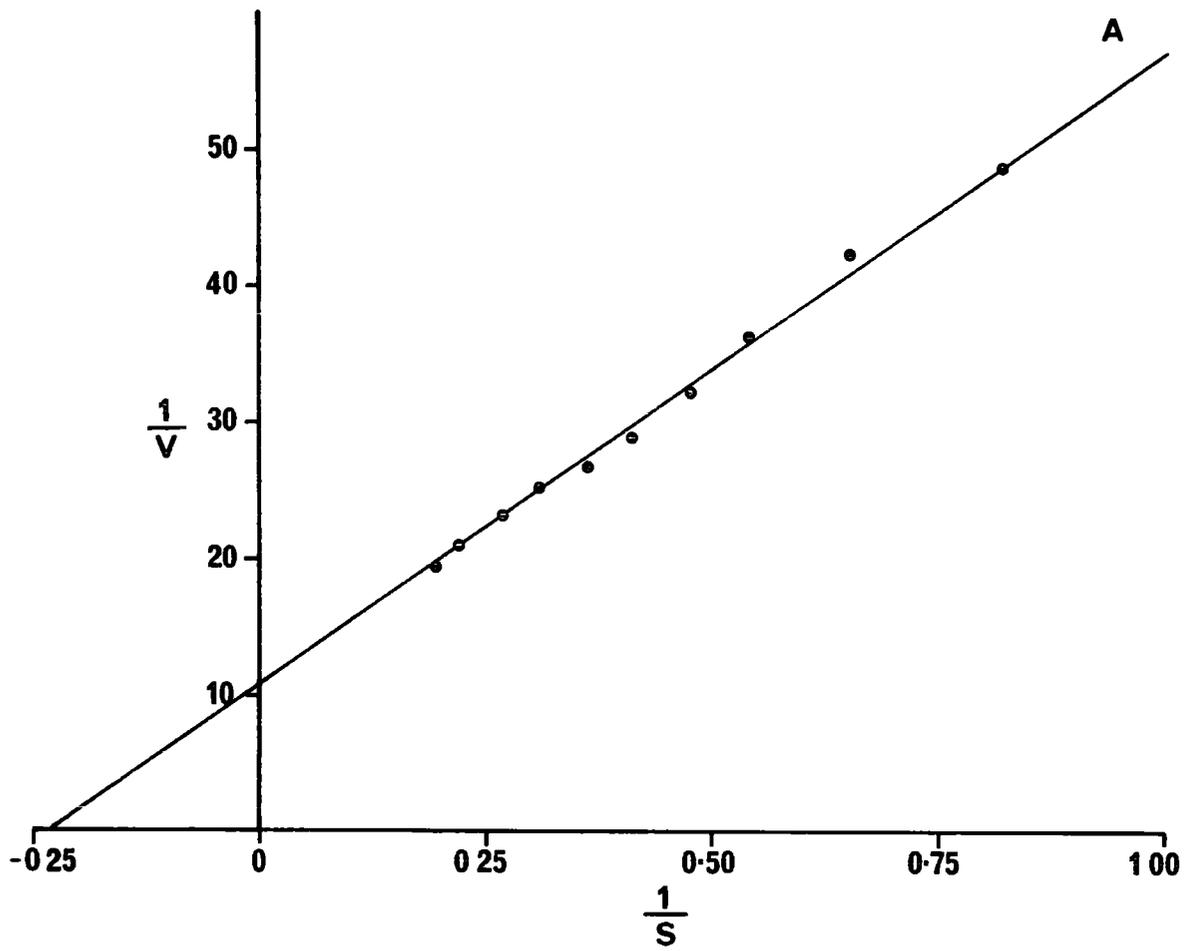


Fig. 4.8

The effect of temperature on β D glucosidase activity

Legend:

ordinate ; activity (μ moles p nitrophenol/mg protein/min)

abscissa ; $\frac{1}{\text{temperature } ^\circ\text{A}} \times 10^3$

■ suboptimal conditions of pH, 6.75

● optimal conditions of pH, 5.2, activity $\times 10^{-1}$

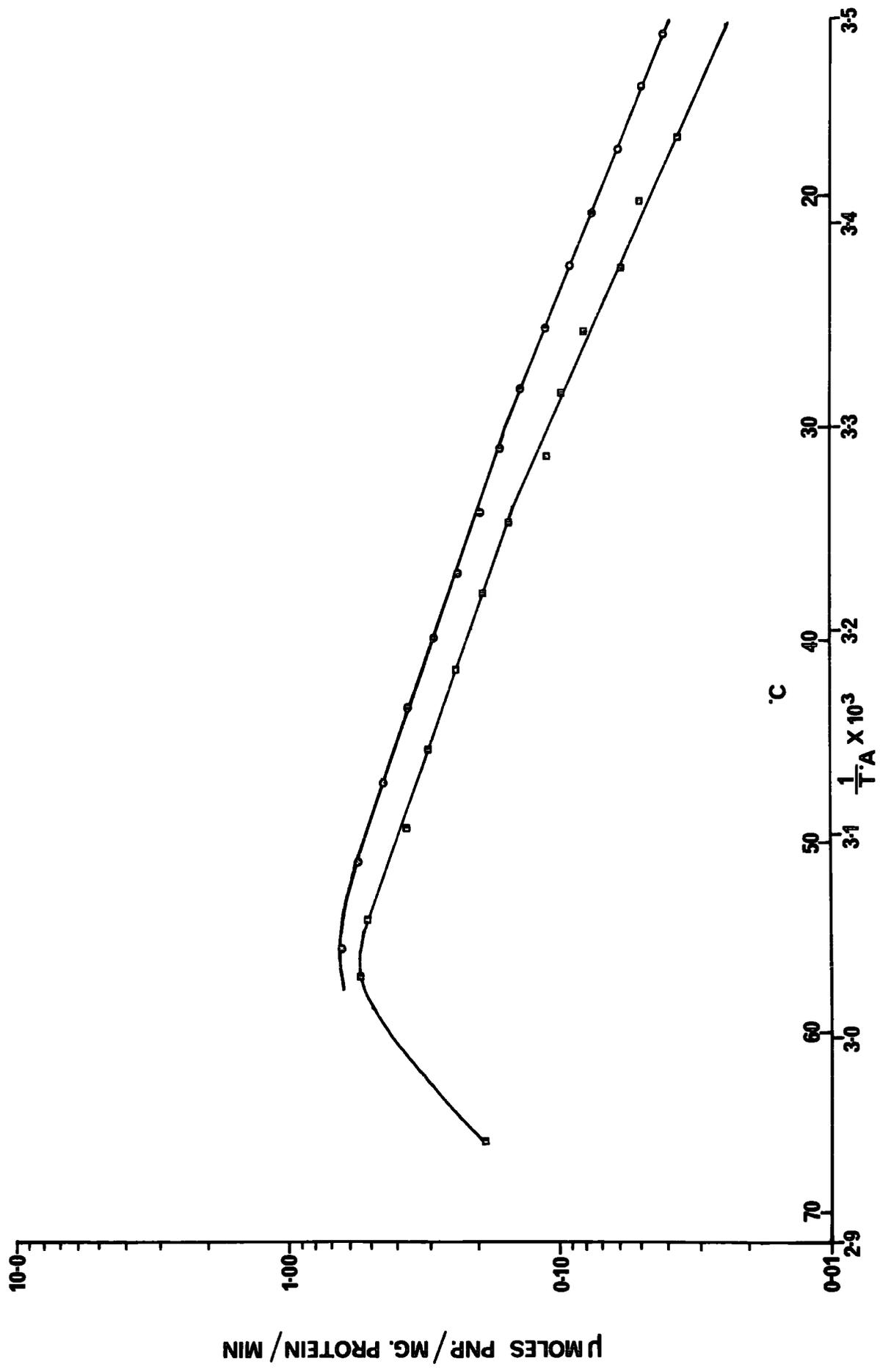


Table 4.4

Kinetic constants of α D galactosidase

pH	<u>Plot</u>	<u>Mean Km</u> mM/litre	<u>SE</u>	<u>mean Vmax</u> μ moles pnp/ mg protein/min	<u>SE</u>	<u>Number of</u> <u>determinations</u>
7.0	L/B	0.73		0.014		2
	E/H	0.73		0.014		2
5.2	L/B	1.26	0.12	0.16	0.04	4
	E/H	1.23	0.12	0.16	0.04	4

pH	<u>Average</u> <u>temperature</u> <u>range</u> °C	<u>Mean activation energy</u> <u>Ea, K joules mole⁻¹</u>	<u>SE</u>	<u>Number of</u> <u>determinations</u>
7.0	53-32	43.41	2.32	3
	32-22	64.07	2.75	3
5.2	54-32.5	50.53		2
	32.5-19	89.49		2

μ moles pnp

μ moles p nitrophenol

S.E.

standard error of the mean

L/B

Lineweaver-Burk

E/H

Eadie-Hofstee

Fig. 4.9

A. The effect of pH on the activity of α D galactosidase

Legend:

ordinate ; μ moles p nitrophenol/mg protein/min

abscissa ; pH

- pH 3.4 - 6.2 0.1M McIlvaine's buffer
- pH 5.8 -7.0 0.1M sodium phosphate buffer

B. To demonstrate the linearity of α D galactosidase activity with time

Legend:

ordinate ; μ moles p nitrophenol/mg protein

abscissa ; time in minutes

- 6.5 mM p nitrophenol α D galactoside
- 4.5 mM " "
- ▲ 0.225mM " "

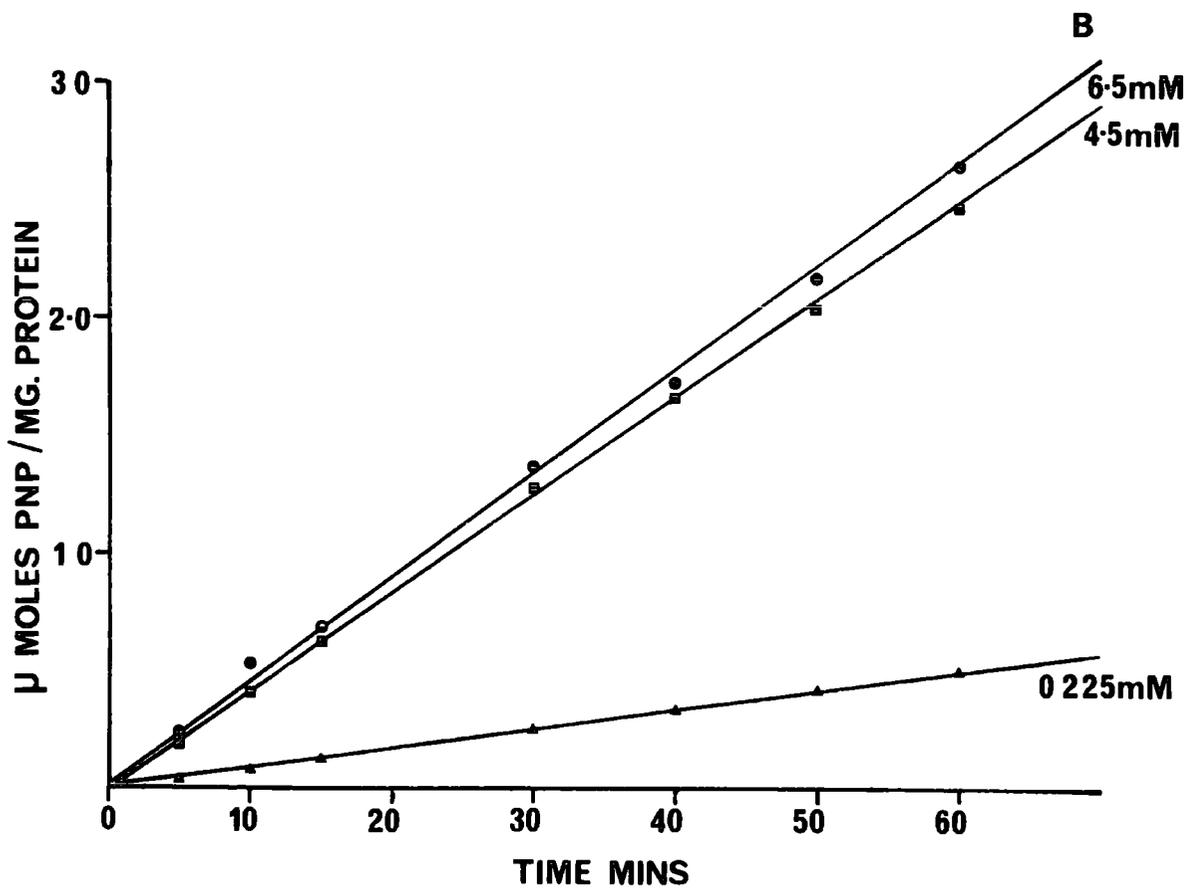
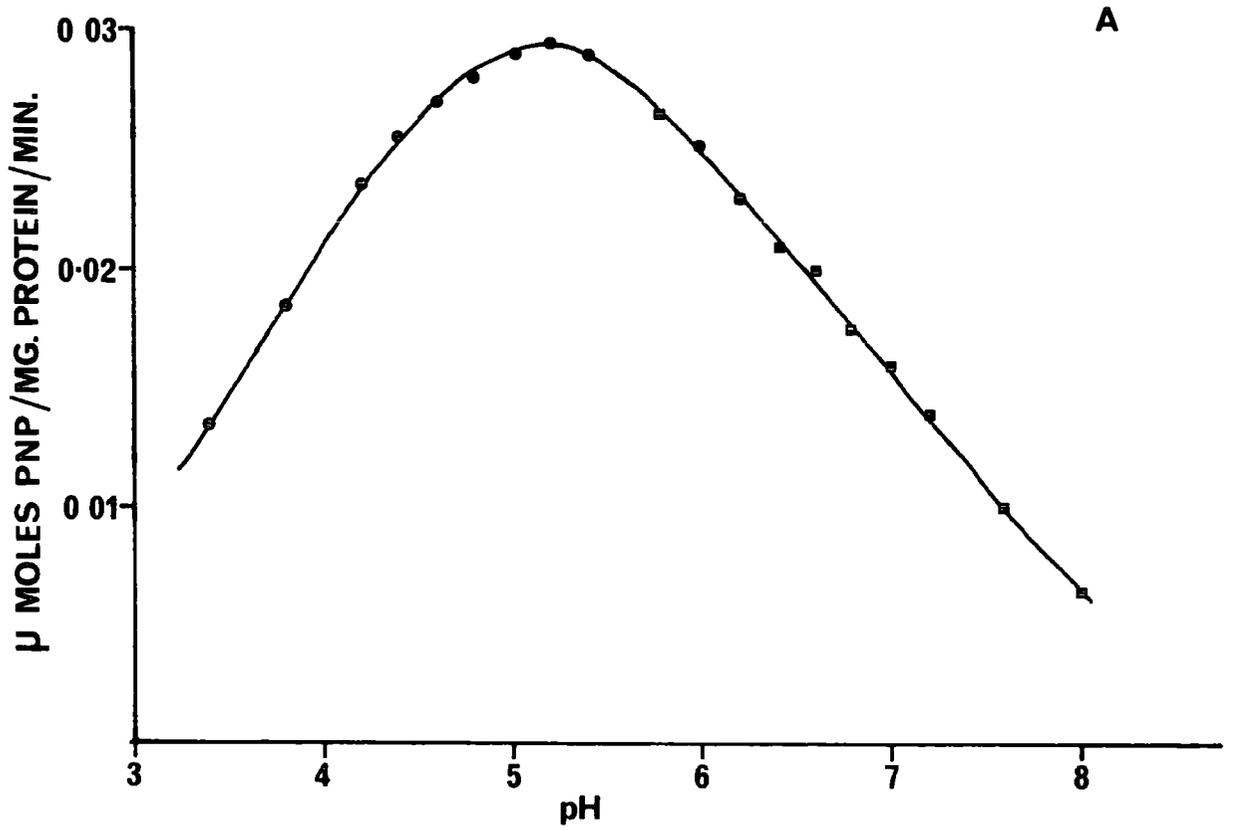


Fig. 4.10

The effect of substrate concentration on the activity
of α D galactosidase

A. under suboptimal conditions of pH
0.1M McIlvaine's buffer pH 7.0

Legend:

ordinate ; $\frac{1}{V}$ (μ moles p nitrophenol/mg protein/min)⁻¹
abscissa ; $\frac{1}{S}$ p nitrophenol α D galactoside mM⁻¹

B. Under optimal conditions of pH
0.1M McIlvaine's buffer pH 5.2

Legend:

ordinate ; $\frac{1}{V}$ (μ moles p nitrophenol/mg protein/min)⁻¹
abscissa ; $\frac{1}{S}$ p nitrophenol α D galactoside mM⁻¹

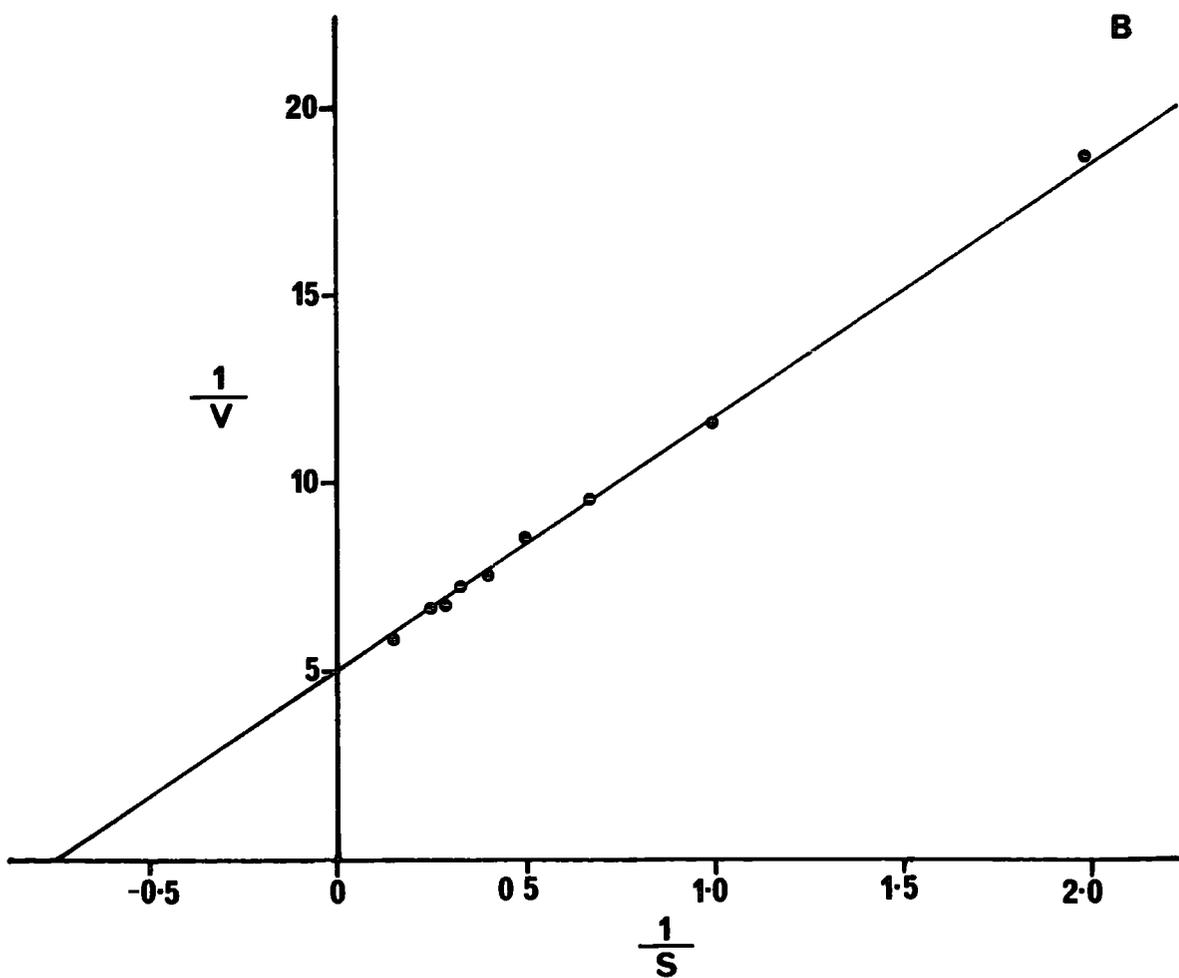
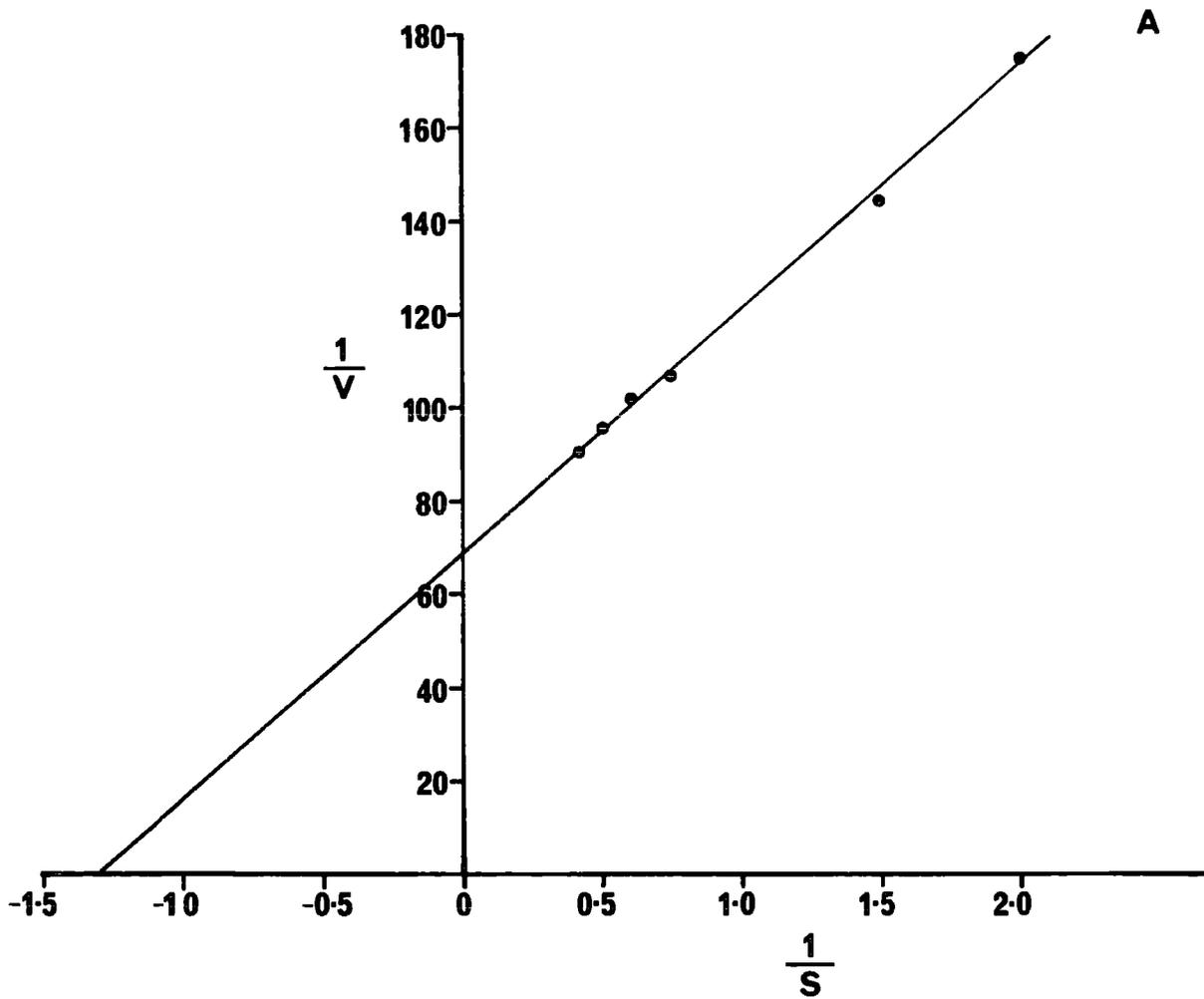


Fig. 4.11

The effect of temperature on the activity of
 α D galactosidase

Legend:

ordinate ; activity (μ moles p nitrophenol/mg protein/min)

abscissa ; $\frac{1}{\text{temperature}^{\circ\text{A}}} \times 10^3$

- suboptimal conditions of pH, 7.0, activity $\times 10$
- optimal conditions of pH, 5.2, activity $\times 10$

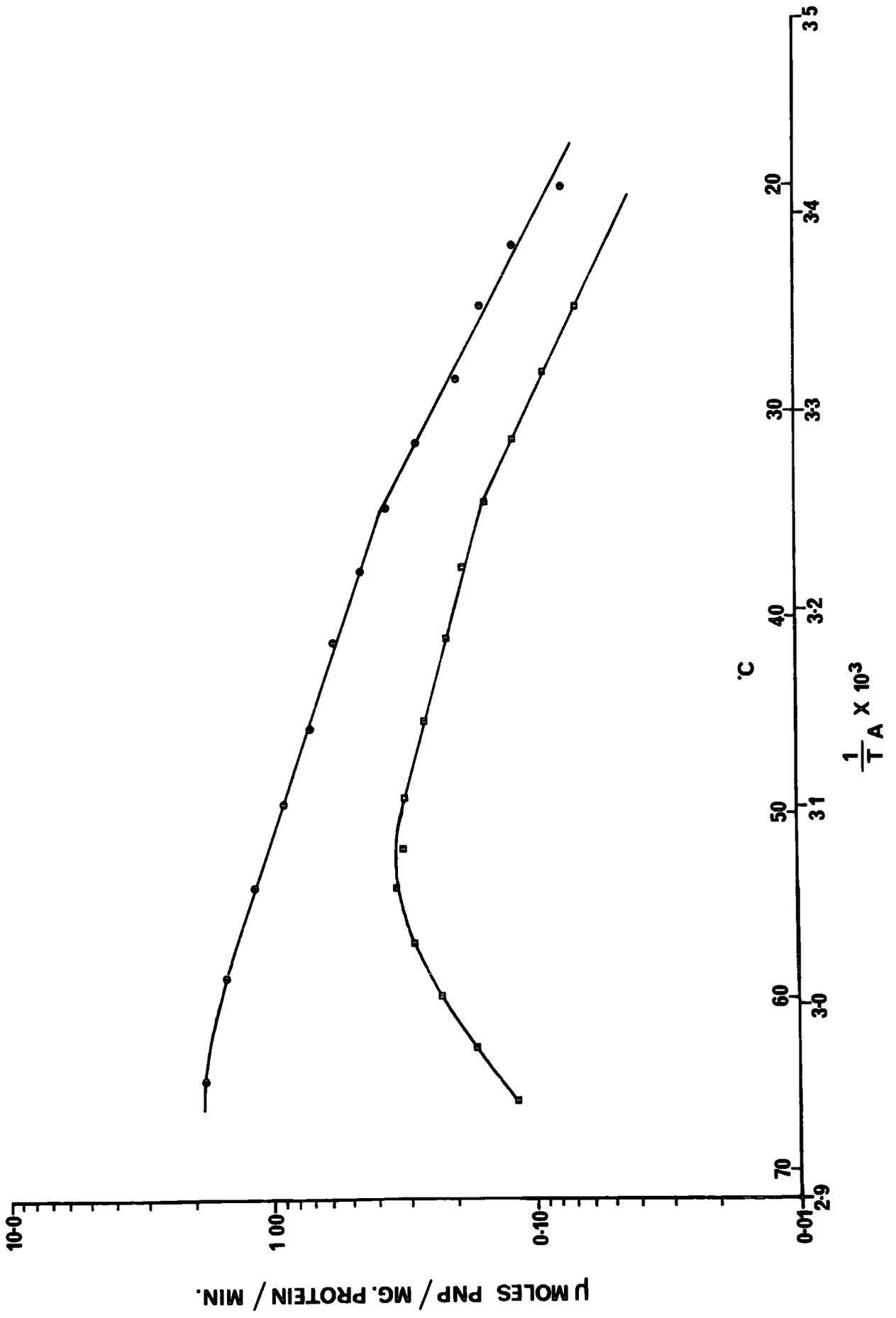


Table 4.5

Kinetic constants of β D galactosidase

<u>pH</u>	<u>range of substrate concentration mM</u>	<u>Plot</u>	<u>Mean Km mM/litre</u>	<u>SE</u>	<u>mean Vmax μ moles pnp/mg protein/min</u>	<u>SE</u>	<u>Number of determinations</u>
6.6	1.36-3.2	L/B	1.00		0.058		2
	0.3 -1.36		0.19		0.038		2
	1.36-3.2	E/H	0.97		0.057		2
	0.3 -1.36		0.20		0.038		2
4.8	2.46-6.5	L/B	1.78	0.19	0.139	0.05	4
	0.13-2.46		0.71	0.09	0.089	0.03	5
	2.46-6.5	E/H	1.68	0.17	0.127	0.05	4
	0.13-2.46		0.72	0.09	0.090	0.03	5
<u>pH</u>	<u>average temperature range $^{\circ}$C</u>	<u>mean activation energy Ea, K joules mole$^{-1}$</u>		<u>SE</u>	<u>Number of determinations</u>		
6.6	58-38	67.52		2.47	3		
	38-19	99.02		16.62	3		
4.8	53-30	50.74		2.74	3		
	30-15	77.88		3.88	3		

μ moles pnp μ moles p nitrophenol
 S.E. standard error of the mean
 L/B Lineweaver-Burk
 E/H Eadie-Hofstee

Fig. 4.12

A. The effect of pH on the activity of β D galactosidase

Legend:

ordinate ; μ moles p nitrophenol/mg protein/min

abscissa ; pH

- pH 3.4 - 6.2 0.1M McIlvaine's buffer
- pH 5.8 - 7.0 0.1M sodium phosphate buffer

B. To demonstrate the linearity of β D galactosidase activity with time

Legend:

ordinate ; μ moles p nitrophenol/mg protein

abscissa ; time in minutes

- 6.4mM p nitrophenol/ β D galactoside
- 2.0mM " "
- ▲ 0.1mM " "

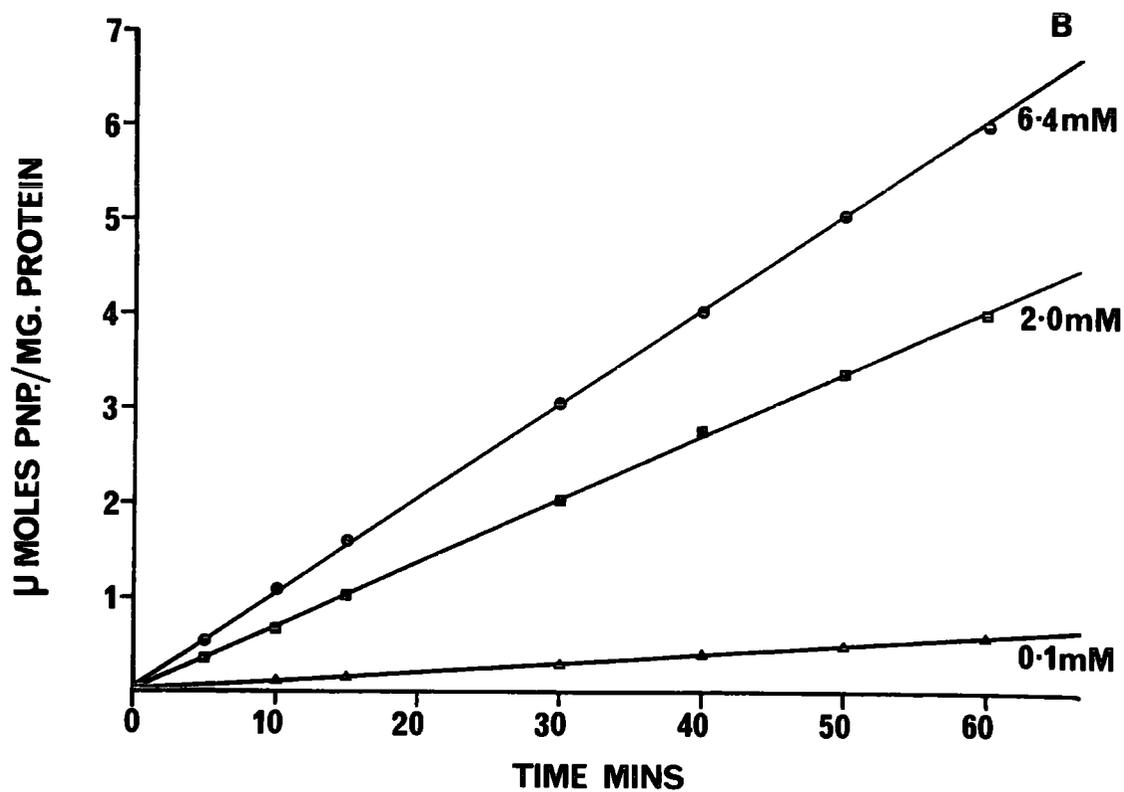
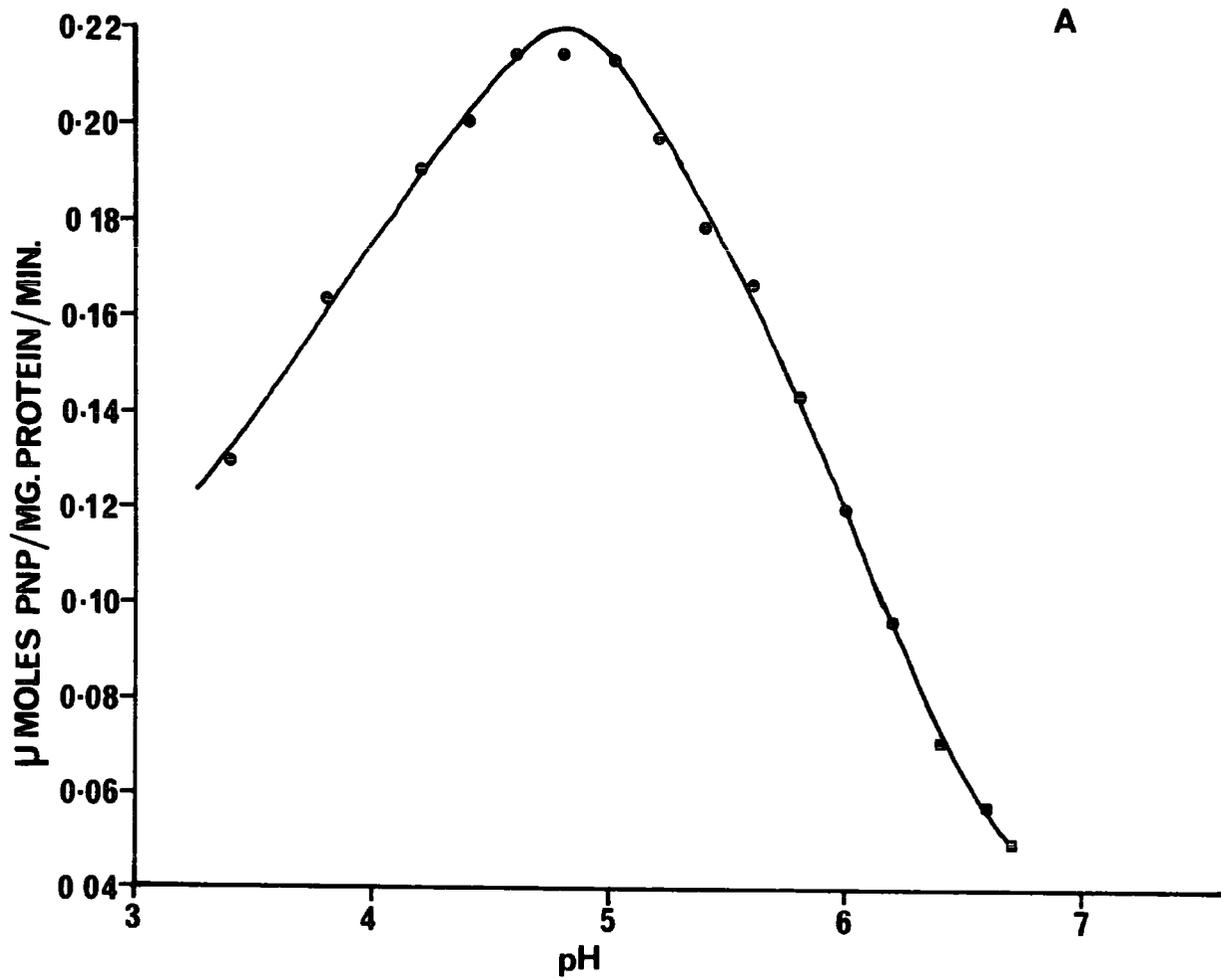


Fig. 4.13

The effect of substrate concentration on the activity
of β D galactosidase

- A. Under suboptimal conditions of pH
0.1M McIlvaine's buffer pH 6.6

Legend:

ordinate ; $\frac{1}{V}$ (μ moles p nitrophenol/mg protein/min)⁻¹
abscissa ; $\frac{1}{S}$ p nitrophenol β D galactoside mM⁻¹

- B. Under optimal conditions of pH
0.1M McIlvaine's buffer pH 4.8

Legend:

ordinate ; $\frac{1}{V}$ (μ moles p nitrophenol/mg protein/min)⁻¹
abscissa ; $\frac{1}{S}$ p nitrophenol β D galactoside mM⁻¹

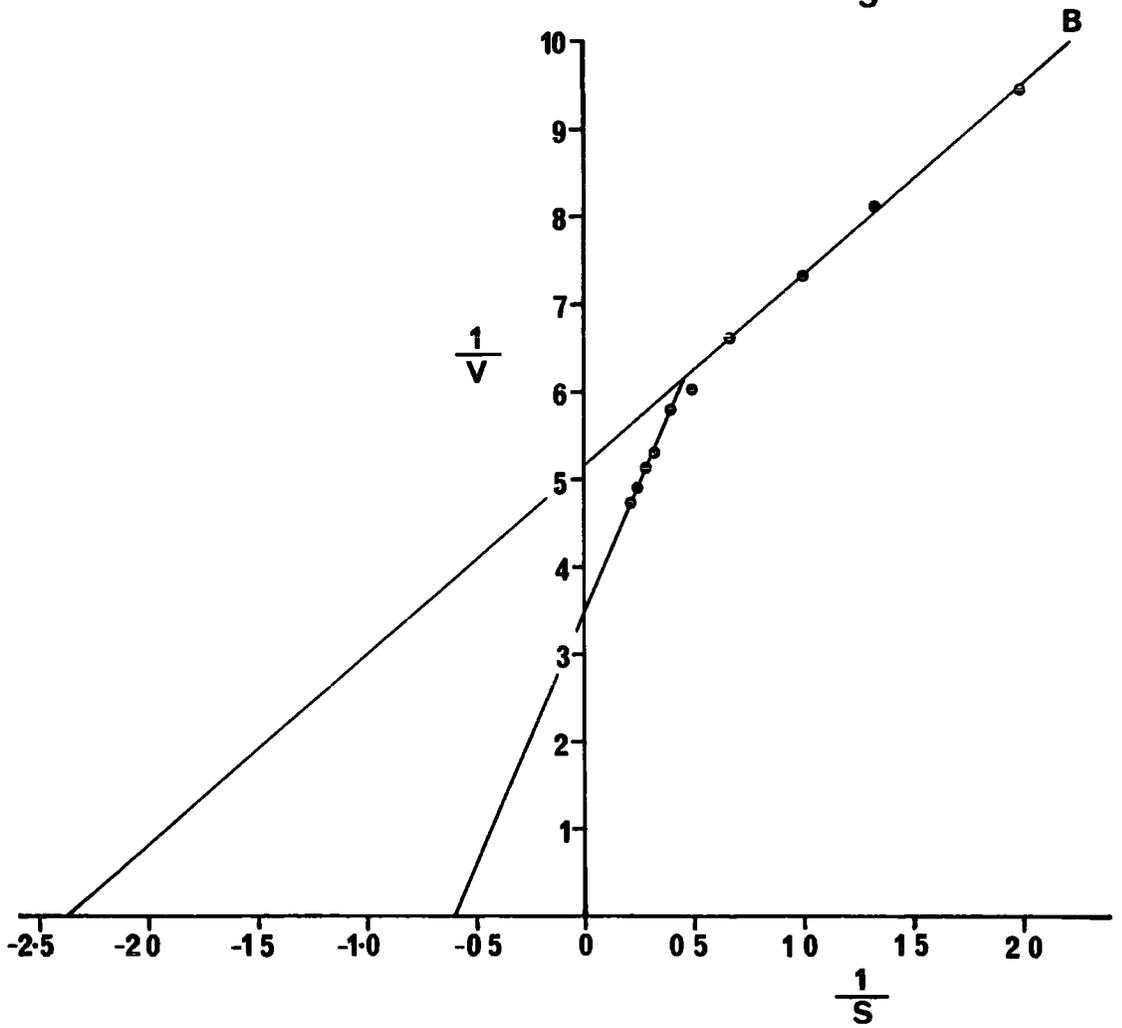
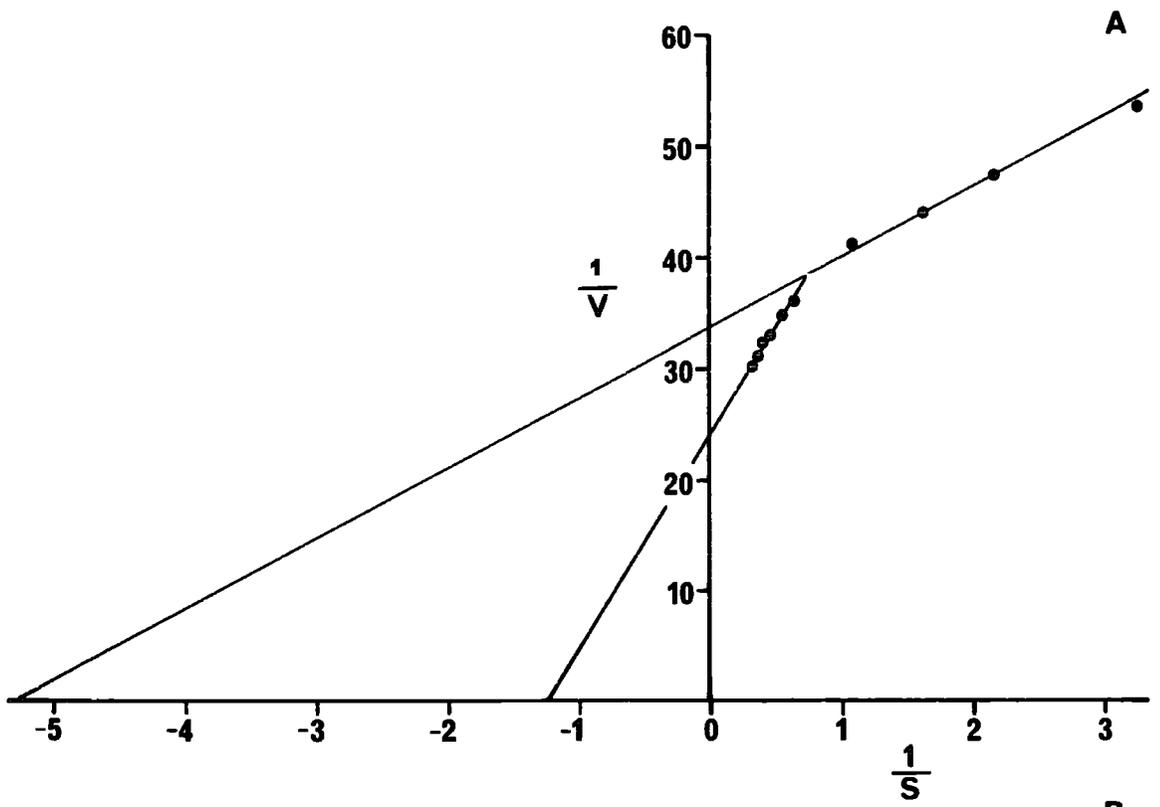


Fig. 4.14

The effect of temperature on the activity of
 β D galactosidase

Legend:

ordinate ; activity (μ moles p nitrophenol/mg protein/min)

abscissa ; $\frac{1}{\text{temperature } ^\circ\text{A}} \times 10^3$

- suboptimal conditions of pH 6.6
- optimal conditions of pH, 4.8, activity $\times 10$

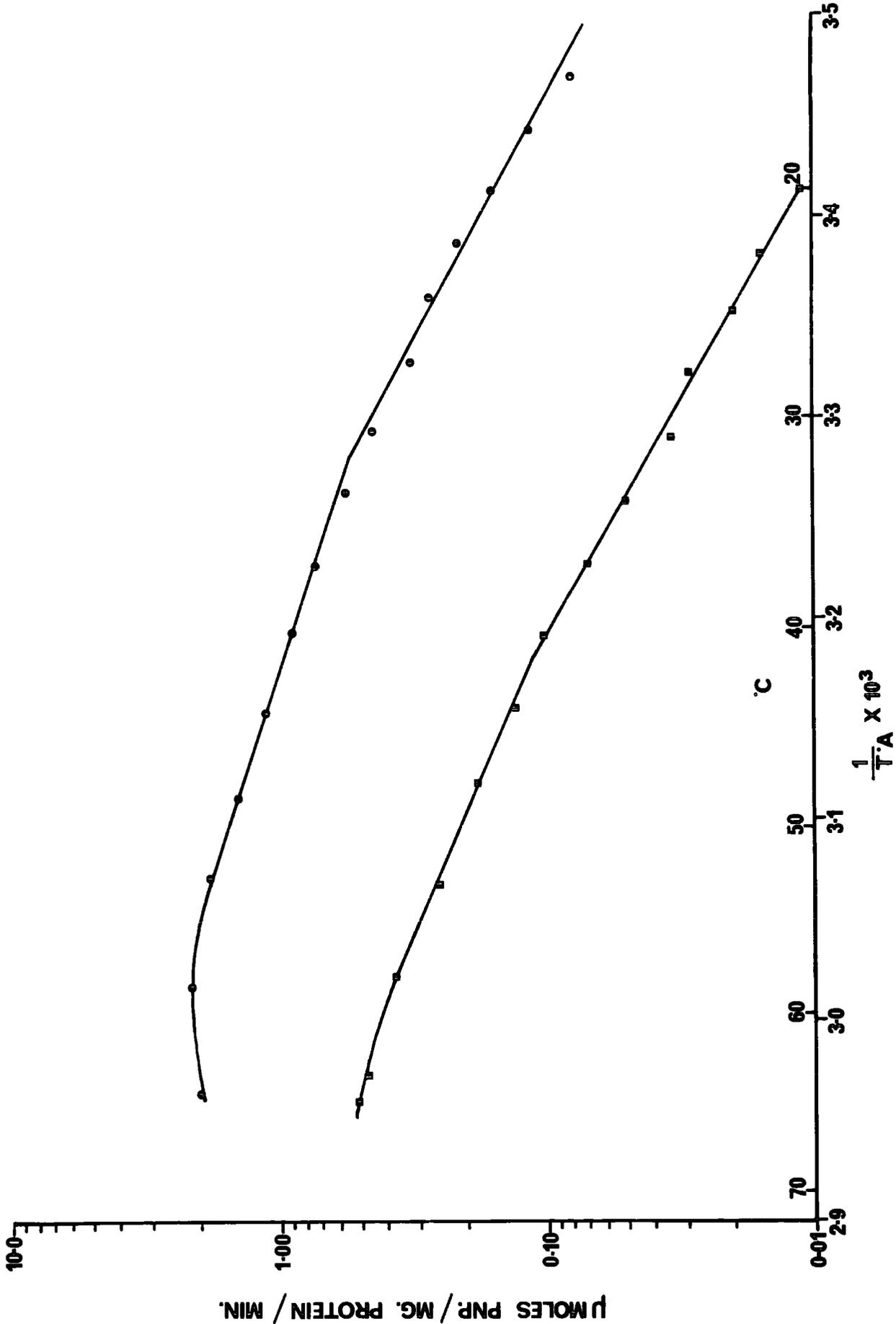


Table 4.6

Kinetic constants of trehalase

<u>pH</u>	<u>Plot</u>	<u>Mean Km</u> <u>mM/litre</u>	<u>SE</u>	<u>mean Vmax</u> <u>µg glucose/mg</u> <u>protein/min</u>	<u>SE</u>	<u>Number of</u> <u>determinations</u>
5.1	L/B	0.92	0.06	13.43	3.33	4
	E/H	0.90	0.07	13.29	3.27	4

<u>pH</u>	<u>Average</u> <u>temperature</u> <u>range °C</u>	<u>Mean activation energy</u> <u>Ea, K joules mole⁻¹</u>	<u>SE</u>	<u>Number of</u> <u>determinations</u>
5.1	55-16	46.52		2

S.E. Standard error of the mean
L/B Lineweaver-Burk
E/H Eadie-Hofstee

Fig. 4.15

The effect of pH on the activity of trehalase

- A. Using a gut homogenate prepared in the standard way (see Materials and Methods Section).

Legend:

ordinate ; μ g glucose/mg protein/min

abscissa ; pH

- pH 3 - 6 0.1M McIlvaine's buffer
- ▲ pH 5.6-6 0.1M sodium phosphate buffer
- pH 6-8.5 0.1M bis tris propane buffer

- B. Using a gut homogenate prepared by centrifugation at different speeds.

Legend:

ordinate ; μ g glucose/mg protein/min

abscissa ; pH

0.1M McIlvaine's buffer

- 1. supernatant from the homogenate spun at 1,000 r.p.m.
- 2. supernatant from the preparation in 1 above spun at 2,000 r.p.m.
- 3. supernatant from the preparation in 2 above spun at 40,000 r.p.m.

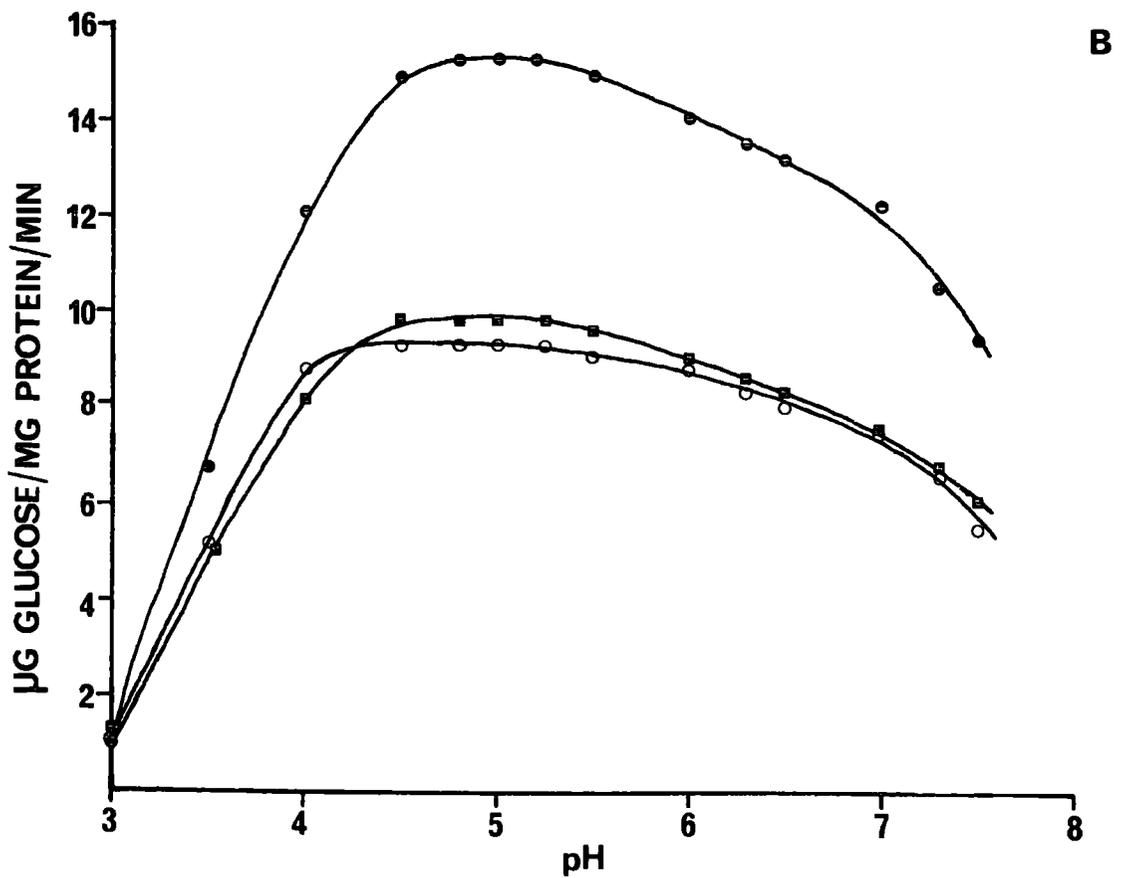
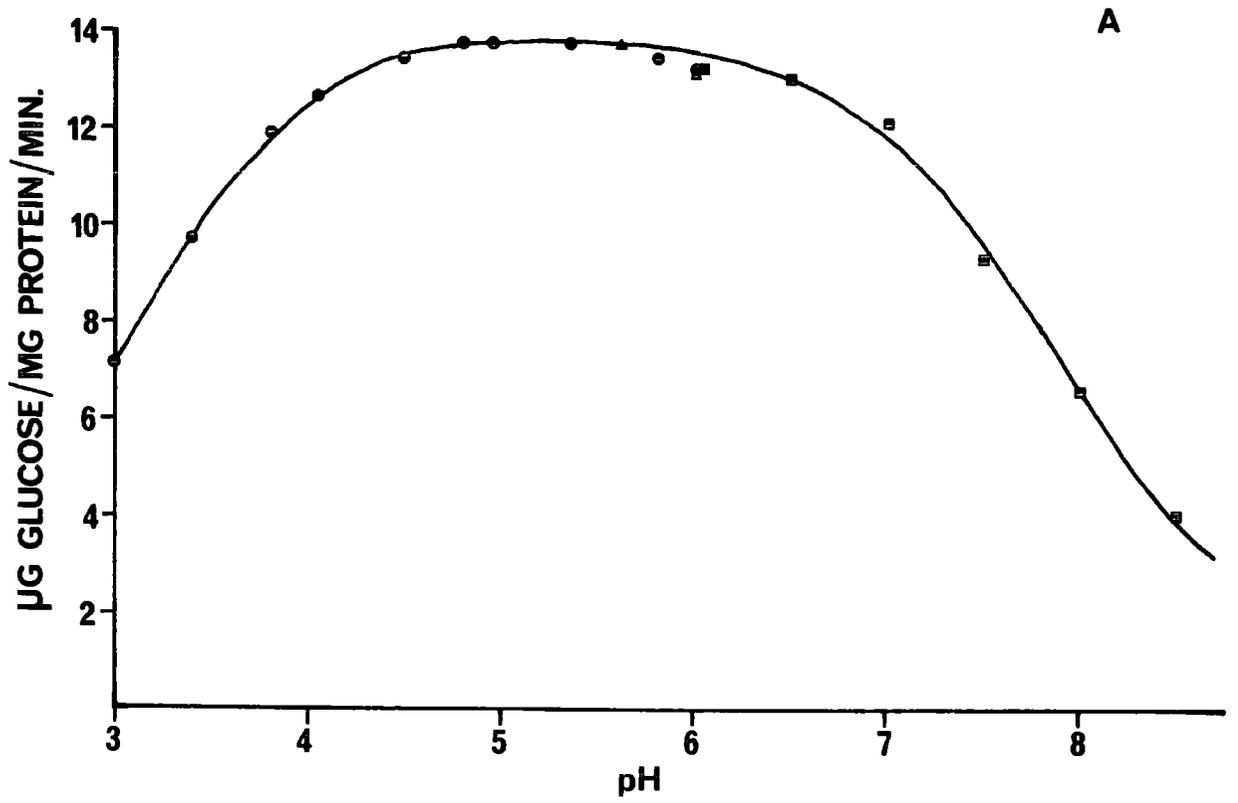


Fig. 4.16

A. To demonstrate the linearity of trehalase activity with time

Legend:

ordinate ; μ g glucose/mg protein

abscissa ; time in minutes

- 23mM trehalose
- 6.0mM trehalose
- ▲ 1.0mM trehalose

B. The effect of substrate concentration on the activity of trehalase

Legend:

ordinate ; $\frac{1}{V}$ (μ g glucose/mg protein/min)⁻¹

abscissa ; $\frac{1}{S}$ trehalose mM⁻¹

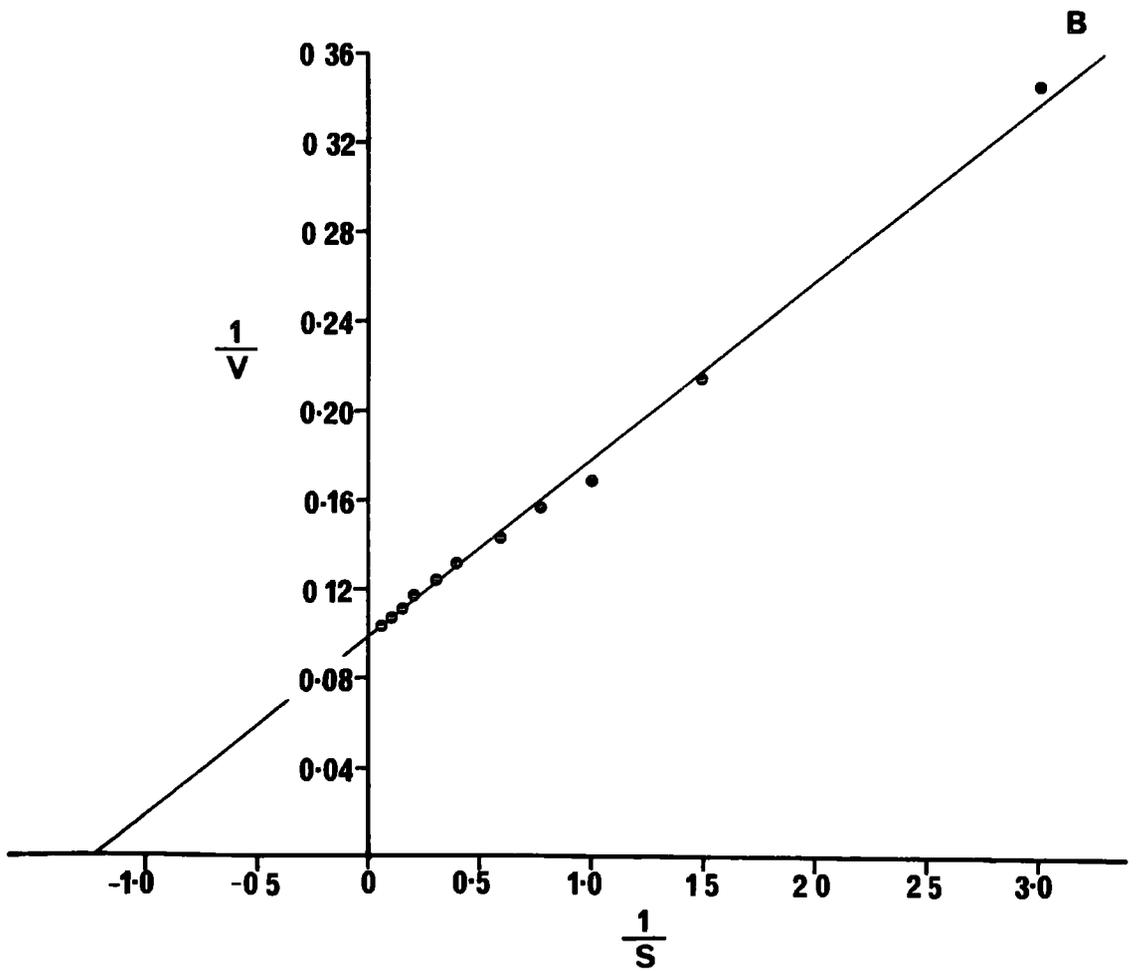
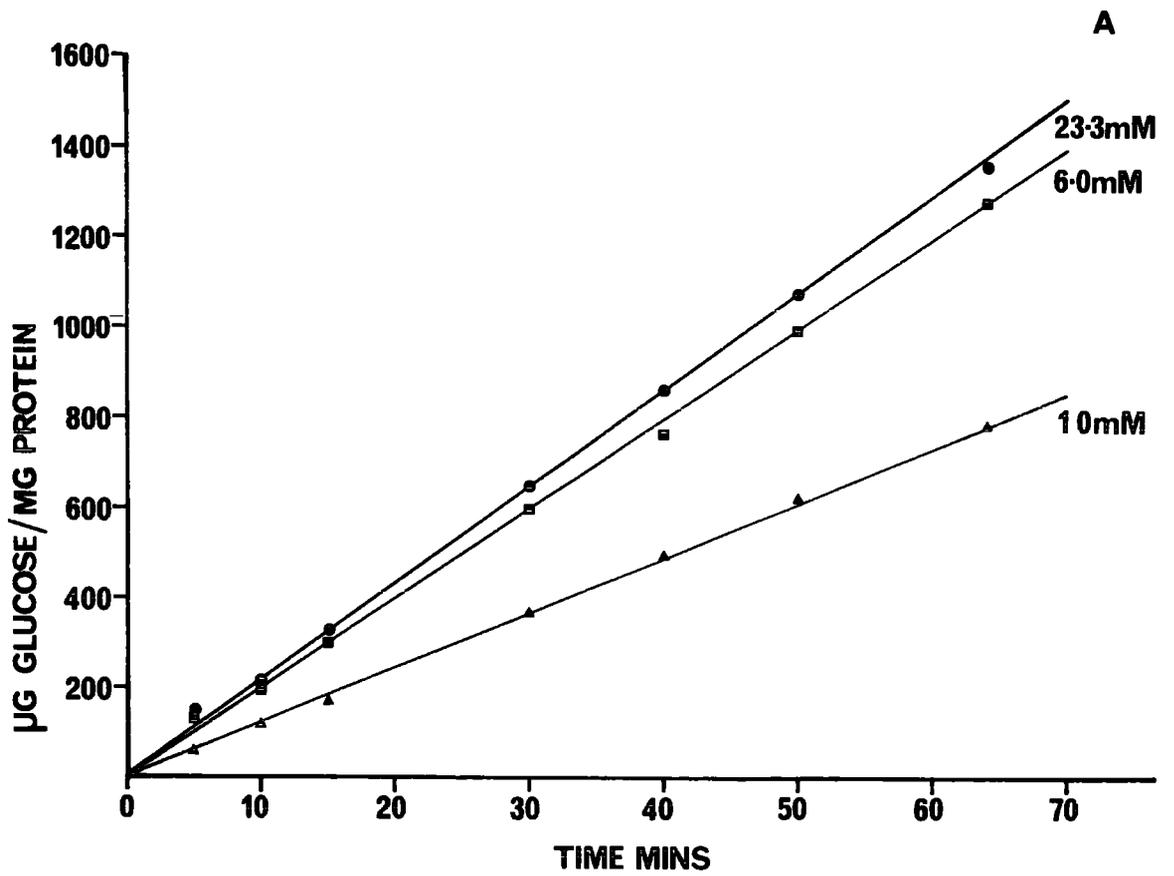


Fig. 4.17

The effect of temperature on the activity of
trehalase

Legend:

ordinate ; (μ g glucose/mg protein/min) $\times 10$

abscissa ; $\frac{1}{\text{temperature}^{\circ}\text{A}} \times 10^3$

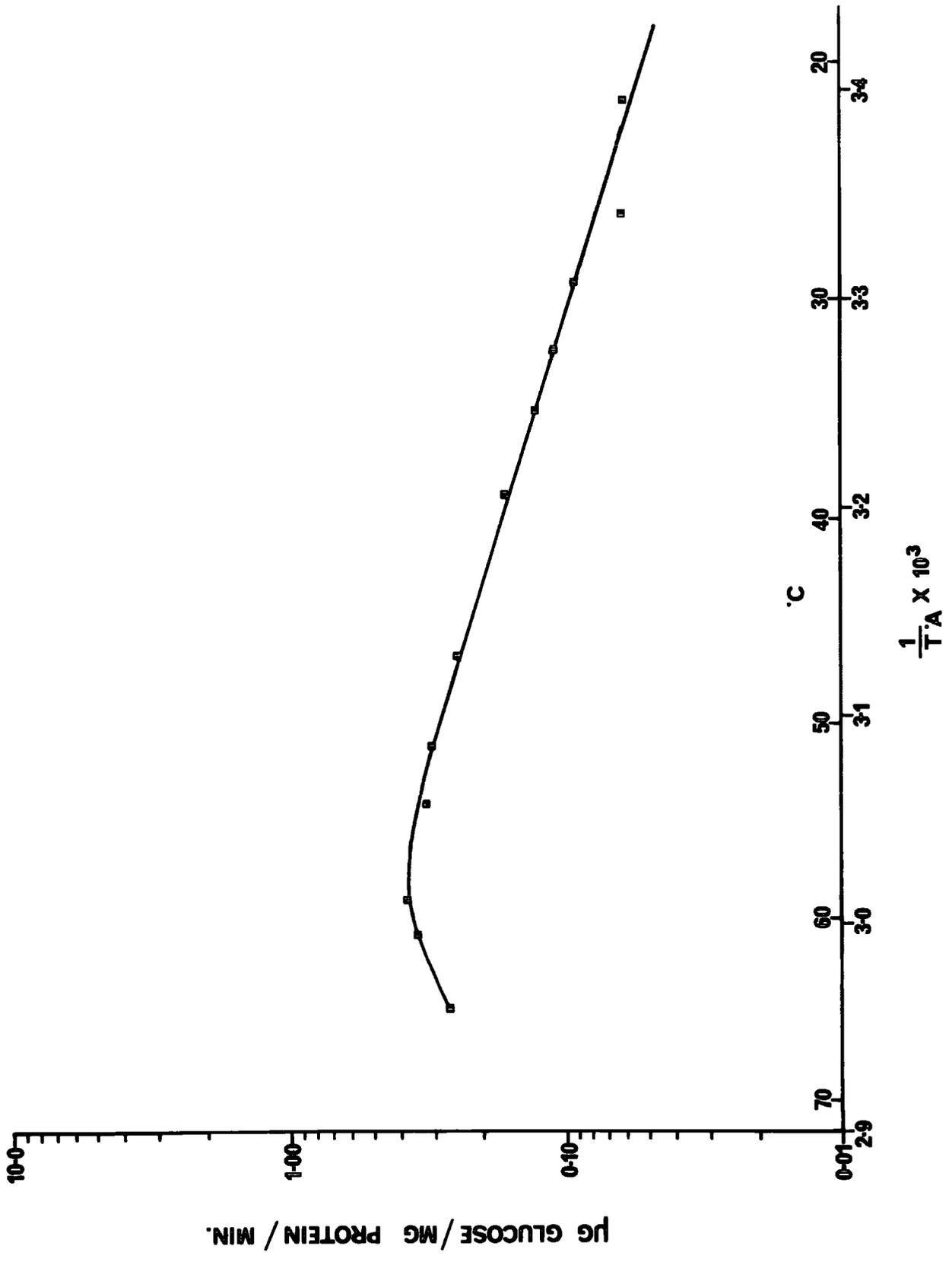


Table 4.7

Kinetic constants of "trypsin"

<u>Plot</u>	<u>mean Km</u> <u>mM/litre</u>	<u>SE</u>	<u>mean Vmax</u> <u>μ moles pna/mg</u> <u>protein/min</u>	<u>SE</u>	<u>Number of</u> <u>determinations</u>
L/B	0.88	0.07	0.16	0.04	3
E/H	0.88	0.08	0.16	0.04	3

<u>Average temperature</u> <u>range °C</u>	<u>mean activation energy</u> <u>Ea, K joules ⁻¹</u>	<u>SE</u>	<u>Number of</u> <u>determinations</u>
45.2 - 27.7	33.31	3.28	3
27.7 - 16.3	66.00	3.67	3

μ moles pna μ moles p nitroaniline
 S.E. Standard error of the mean
 L/B Lineweaver-Burk
 E/H Eadie-Hofstee

Fig. 4.18

A. The effect of pH on the activity of "trypsin"

Legend:

ordinate ; μ moles p nitroaniline/mg protein/min

abscissa ; pH

- pH 7 - 9.3 0.2M tris buffer
- pH 8.7 - 11 0.2M glycine/NaOH buffer

B. To demonstrate the linearity of "trypsin" activity with time

Legend:

ordinate ; μ moles p nitroaniline/mg protein

abscissa ; time in minutes

- 3.0mM benzoyl arginine p nitroanilide
- 1.0mM "
- ▲ 0.5mM "
- 0.17mM "

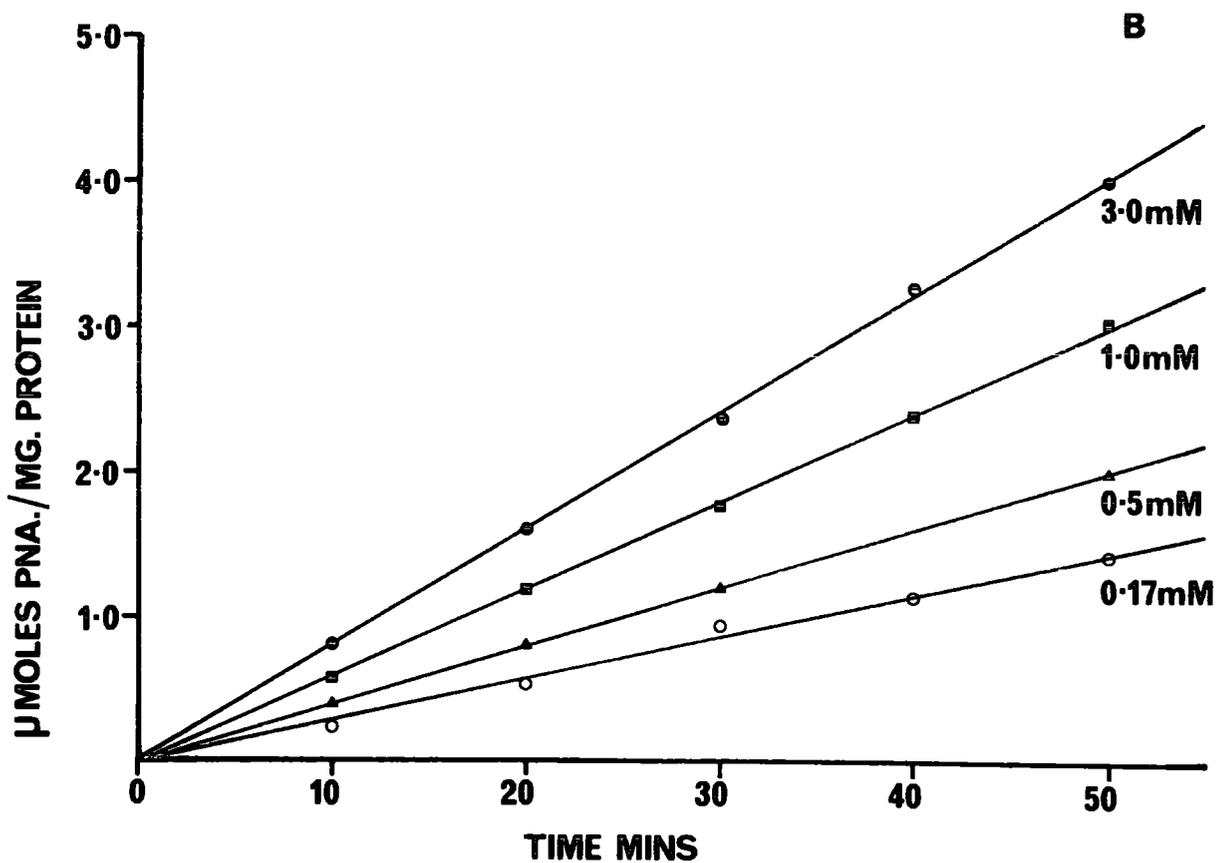
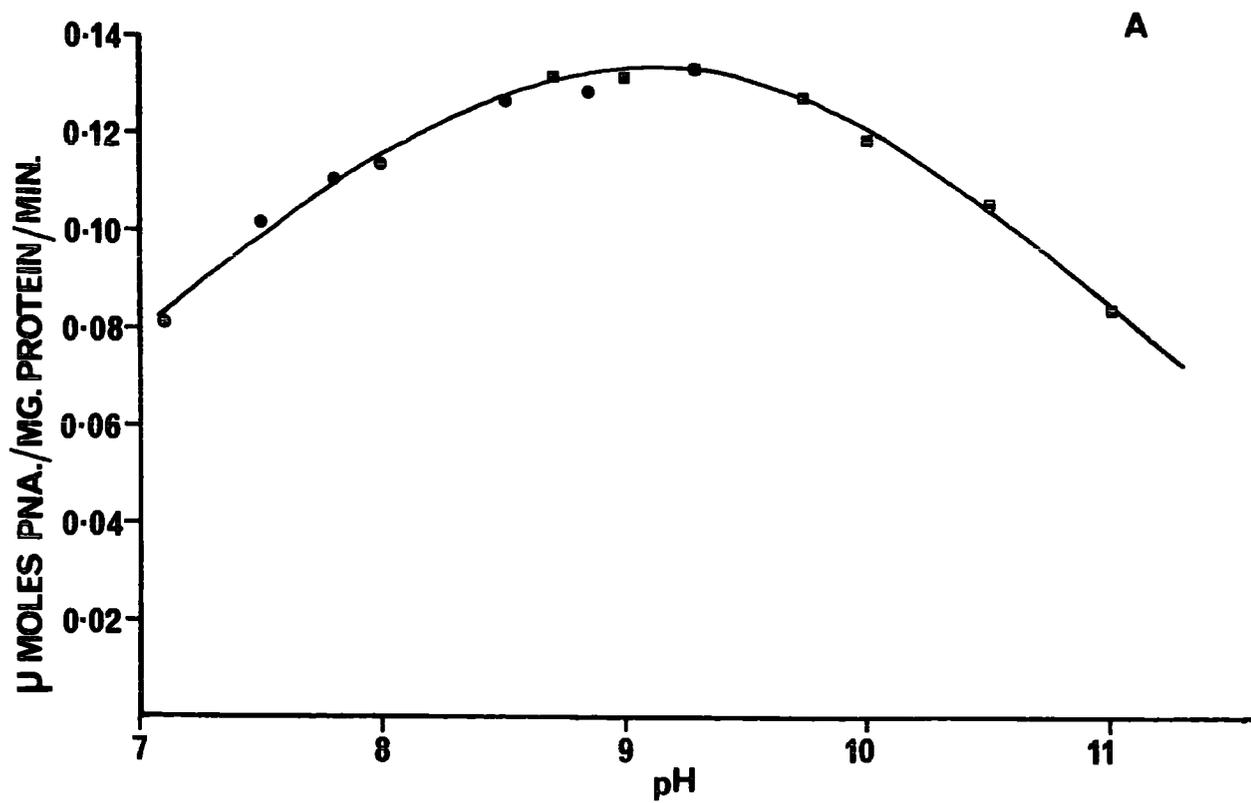


Fig. 4.19

The effect of substrate concentration on the activity
of "trypsin"

Legend:

ordinate ; $\frac{1}{V}$ (μ moles p nitroaniline/mg protein/min)⁻¹
abscissa ; $\frac{1}{S}$ benzoyl arginine p nitroanilide mM⁻¹

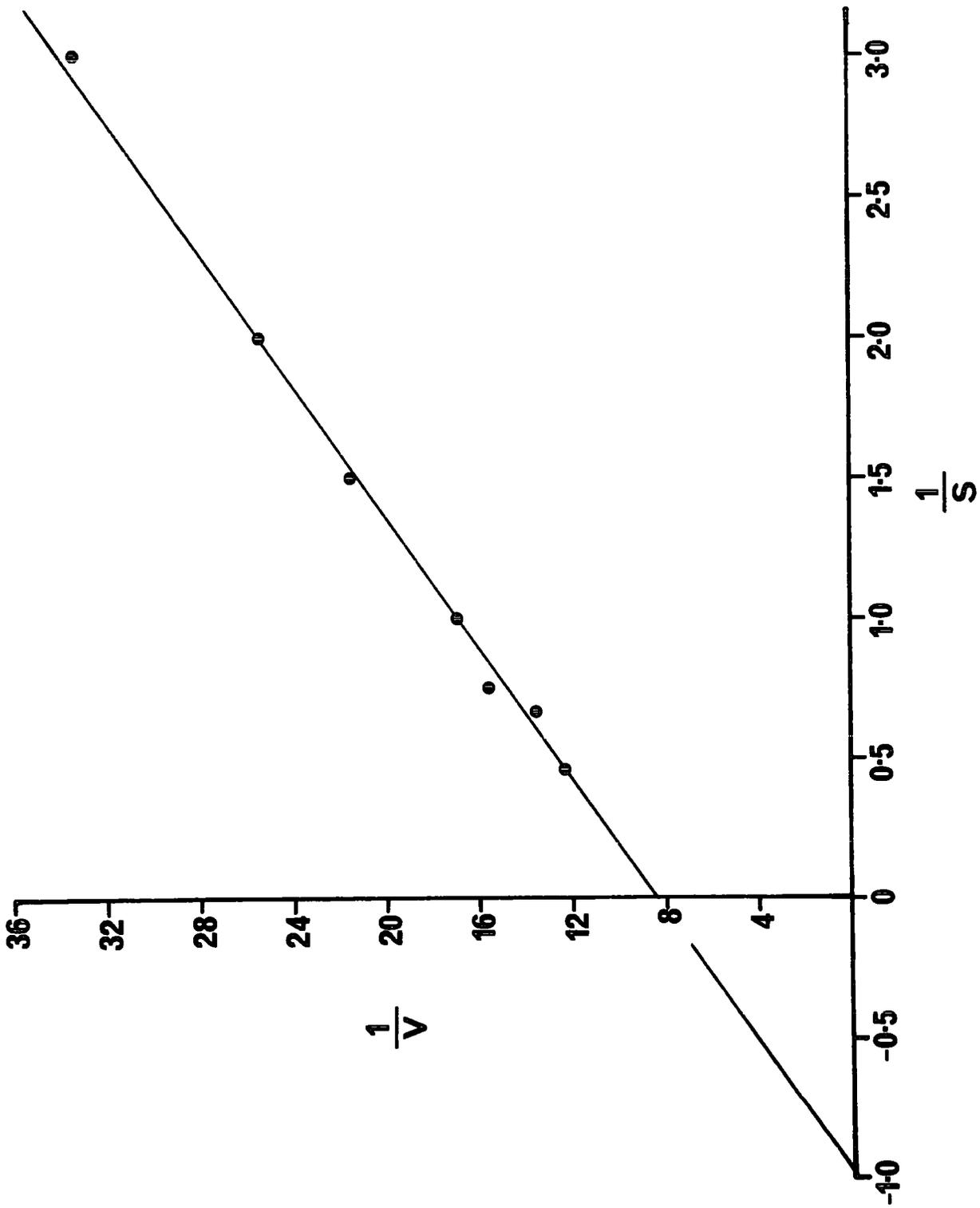


Fig. 4.20

The effect of temperature on the activity of "trypsin"

Legend:

ordinate ; (μ moles p nitroaniline/mg protein/min) $\times 10^3$

abscissa ; $\frac{1}{\text{temperature}^{\circ}\text{A}} \times 10^3$

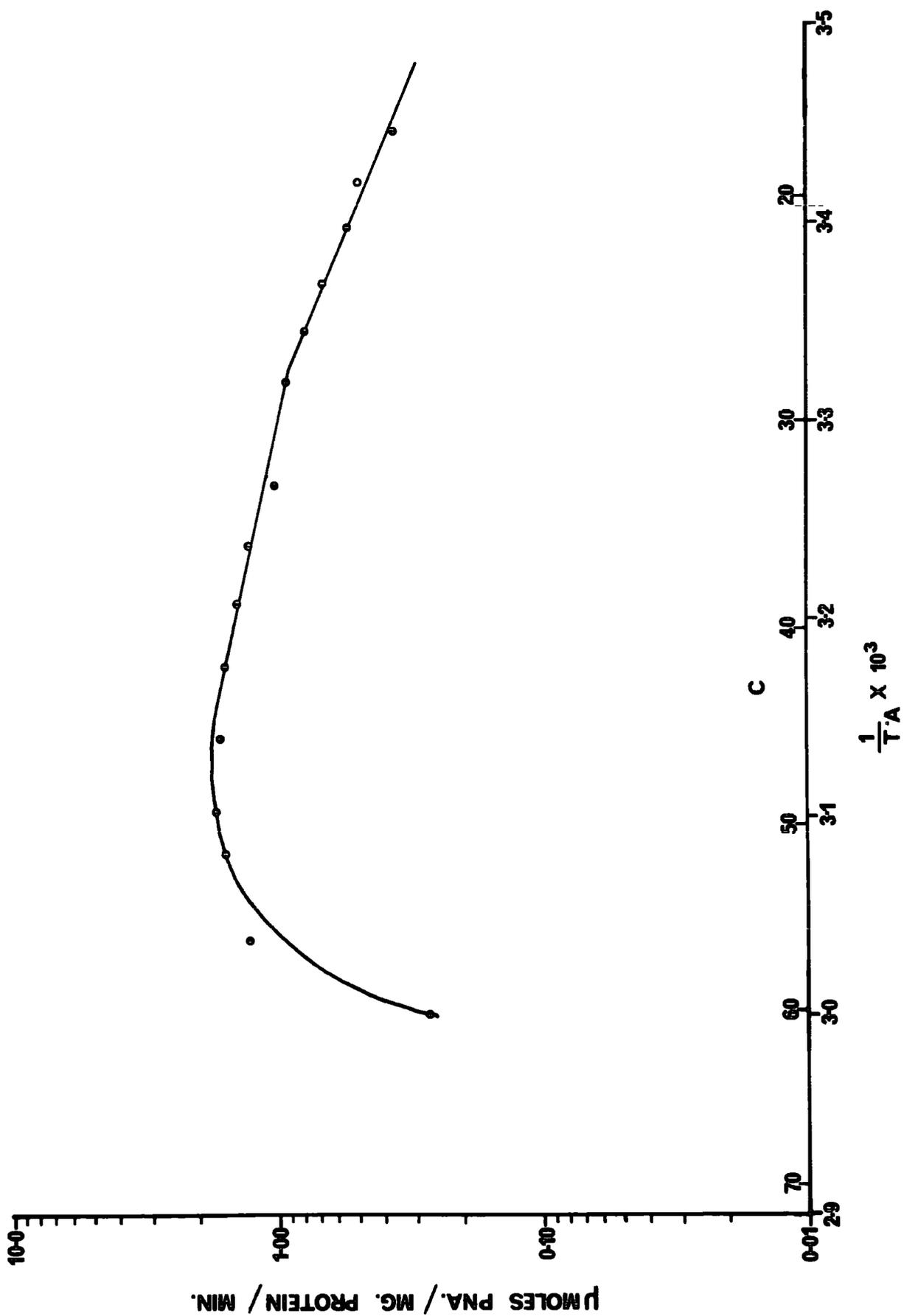


Fig. 4.21

The effect of ovomucoid inhibitor on the activity
of "trypsin"

Legend:

ordinate ; % inhibition of "trypsin" activity

abscissa ; μ g of ovomucoid inhibitor

▲ effect of the inhibitor without
preincubation with the homogenate

■ effect of the inhibitor with
preincubation with the homogenate

● effect of the inhibitor on pure
pancreatic trypsin

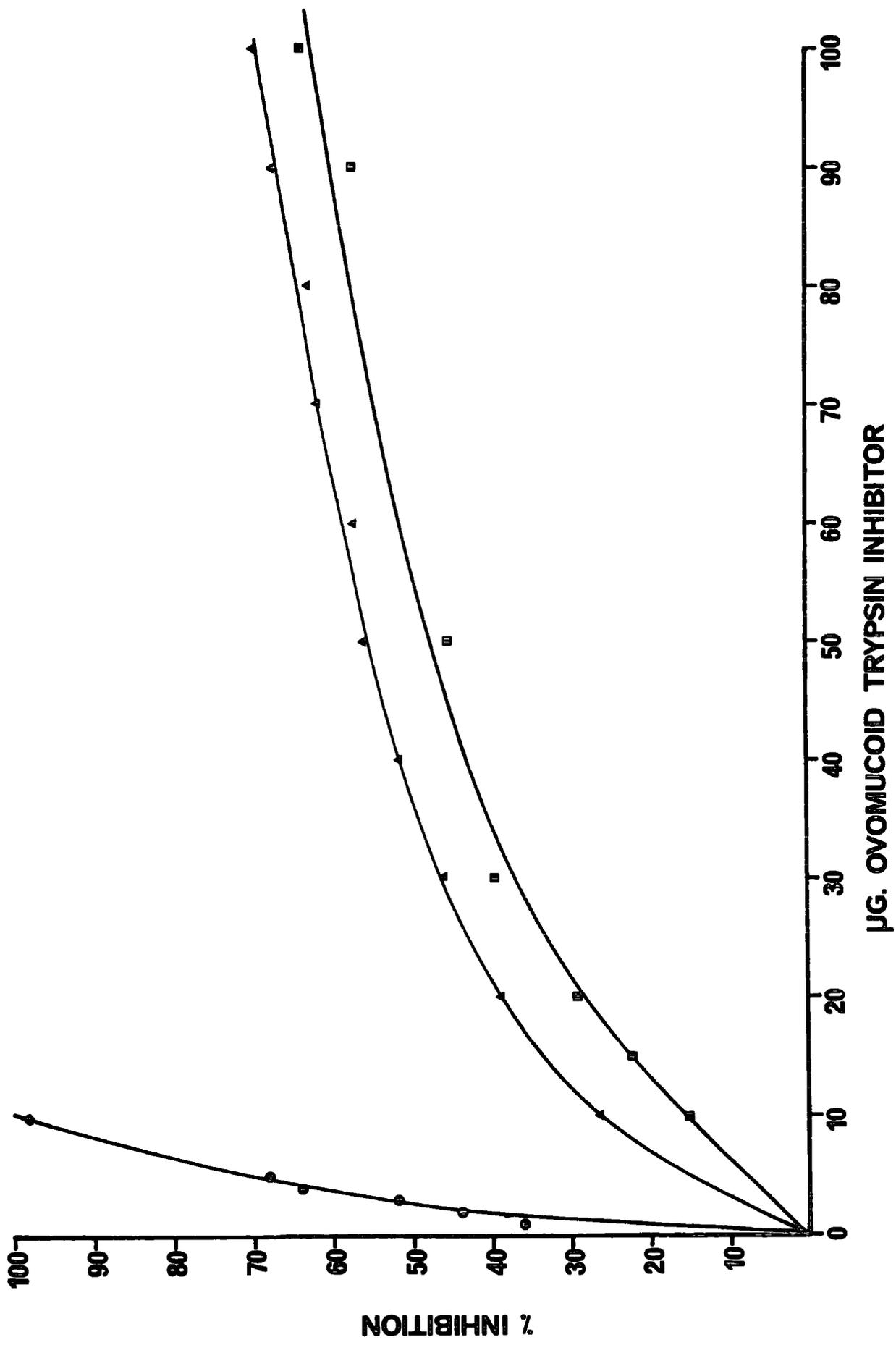


Table 4.8

The effect of ovomucoid inhibitor on the activity
of "trypsin"

<u>Tube number</u>	<u>Contents</u>	<u>Activity</u> <u>μ moles p nitroaniline/mg</u> <u>protein/min</u>
1	Locust "trypsin", bovine trypsin, inhibitor (7μ g)	0.074
2	Locust "trypsin", bovine trypsin	0.122
3	Locust "trypsin"	0.0785
4	Bovine trypsin	0.0415
5	Locust "trypsin", inhibitor (7μ g)	0.068
6	Bovine trypsin, inhibitor (7μ g)	0.0045

Discussion

The pH optima of the six enzymes studied were consistent with values published for the digestive enzymes of other species of insect (Table 4.9), and corresponded to the pH of gut contents found in several species of Orthoptera including Locusta (DROSTE AND ZEBE, 1974; EVANS, 1956). DROSTE AND ZEBE (1974) found a quite marked discrepancy between the pH optima of Locusta oligosaccharidases obtained using artificial nitrophenol substrates and those from natural substrates. A pH optimum of 7.2 was found for α D galactosidase using p nitrophenol α D galactoside whereas an optimum of 5.3 was obtained when melibiose and raffinose were used as substrates. In the present work high pH optima were found for all four oligosaccharidases assayed when the absorbance of p nitrophenol liberated was not measured under alkaline conditions (see materials and methods). Under these conditions α D glucosidase appeared to have a pH optimum of 6.5 instead of 5.4, β D glucosidase pH 6.7 instead of 5.2, α D galactosidase pH 7.0 instead of 5.2 and β D galactosidase 6.6 instead of 4.8 (unpublished observations).

The substrate affinities of the six enzymes compared well with values obtained for similar enzymes in other species (see Table 4.10). However, once again there is a discrepancy between the present work and that of DROSTE AND ZEBE (1974). Assay of α D glucosidase under suboptimal conditions of pH resulted in a K_m similar to that obtained by DROSTE AND ZEBE (1974) using the same substrate. Assay at a pH the present work would suggest was optimal resulted in a significantly lower K_m . Therefore, the discrepancies between the pH optima and K_m values of oligosaccharidases recorded in the present work and those found by DROSTE AND ZEBE (1974), using the same insect and substrate, may be due to a failure of these authors

to recognise the dependence of the colour development of p nitrophenol on a highly alkaline pH.

The activities of β D glucosidase and β D galactosidase did not show simple Michaelis Menton kinetics. This does not appear to have been shown before in insect digestive enzymes. However, biphasic Lineweaver-Burk plots have been reported elsewhere (COSSINS, 1974). A number of alternative explorations of this phenomenon must be considered. There may be two different enzymes or isoenzymes working within the system, each with a different Km value. Hence each enzyme becomes saturated at different concentrations of substrate (DIXON AND WEBB, 1964; RUIZ-HERRERA ET AL, 1972). This situation could be confirmed only with the chromatographic or electrophoretic separation of two separate enzymes. A second possibility is that the same enzyme is responsible for each type of activity, and that there is some change in the kinetic parameters of the system in response to different concentrations of the substrate. The substrate may be acting not only upon the enzyme as a substrate but also as a regulator which controls the binding of substrate and rate of decomposition of the enzyme-substrate complex (YAMAMOTO AND TONOMURA, 1967). An alternative hypothesis is that the enzyme may require two substrate molecules and the attachment of one facilitates the rate of reaction of the second molecule (NEILANDS AND STUMPF, 1958). But, without further data it is not possible to make conclusions about the nature or physiological significance of each type of activity. However, ROBINSON (1964) found three β glucosidase components active against 4 methyl umbelliferyl β glucoside in the crop fluid of Locusta. POWNING AND IRZYKIEWICZ (1962) also found that the activity of β D glucosidase against p nitrophenol β glucosidase in gut extracts from Periplaneta americana suggested a mixture of enzymes. In

contrast to the above work MORGAN (1975) has reported only a single component of β D glucosidase activity in a preparation of Locusta gut using cellobiose as substrate with a K_m similar to that found in the present work at low concentrations of pnp β glucoside. DROSTE AND ZEBE (1974) used a preparation and substrate similar to that employed by MORGAN (1975) but obtained a K_m higher than MORGAN and comparable to that obtained in the present work at high concentrations of substrate (See Table 4.10). A direct comparison between the present work and DROSTE AND ZEBE (1974), MORGAN (1975), however, is impossible because the latter workers used a different substrate.

The trypsin-like protease activity found in the present study was not inhibited 100% by the specific ovomucoid trypsin inhibitor. POWNING, DAY AND IRZYKIEWICZ (1951) using gelatin as a substrate found that they could only inhibit the protease activity in a crude homogenate from Locusta with an ovomucoid inhibitor by 57%. BROOKES (1961) working on a semi-purified extract of a proteolytic enzyme from Phormia regina extracted from regurgitated saliva during feeding, found that a concentration of Soybean inhibitor could be achieved above which no further inhibition could be detected. This left some 25% of the activity when benzoyl arginine ethyl ester was used as substrate. The protease activity assayed in the present study may be due to several enzymes or isoenzymes which differ in their susceptibility to inhibition by ovomucoid trypsin inhibitor. This may result in the residual 30% of activity in the presence of the inhibitor. Certainly FREEMAN (1967) working on the regurgitated crop fluid of Locusta separated several protein fractions on cellulose acetate paper that differed in their activity towards benzoyl arginine p nitranilide.

The effect of temperature on insect digestive enzymes has been little studied apart from proteases and trehalases (see Table 4.11).

In the present work no consistent pattern was observed in the temperature optima of the enzymes studied, they varied from 'trypsin' which had an optimum of 47°C to α D galactosidase for which no optimum was recorded up to an assay temperature of 65°C. Similarly variation of the conditions of pH had different effects on the temperature optima of the four oligosaccharidases. At a suboptimal pH of 6.45 α D glucosidase had a higher temperature optimum than at the optimal pH of 5.4 (Fig. 4.5). While at the suboptimal pH of 7.0 α D galactosidase had a temperature optimum of 54°C, when none was recorded at the optimal pH of 5.2 up to an assay temperature of 65°C. A common feature observed in the effect of temperature on the digestive enzymes apart from trehalase, was an apparent discontinuity in the Arrhenius plot, which occurred around 30°C. The interpretation of enzymes that display two different activation energies is not straight forward and various explanations have been offered (DIXON AND WEBB, 1964). CROZIER ET AL (1925) suggested that this change in activation energy represented a shift from one rate limiting step to another. However, DIXON AND WEBB (1964) argued that two simultaneously occurring processes of this type would not yield a sharp inflexion in Arrhenius plots but would produce a smooth curvilinear transition. They suggested that the sharp discontinuity was the result of incorrectly drawing the intercepts of tangents to smooth curves. More recent evidence from studies on membrane bound enzymes suggests that these sharp breaks are real and brought about by a phase change in the non polar hydrocarbon environment of the enzyme causing a conformational change in it (INESI ET AL, 1973; RAISON ET AL, 1971). However, interpretation of the observed phenomenon in digestive enzymes in terms of the above effect is invalid as they are soluble in form rather than membrane bound. MEYER ET AL (1953) have shown a similar discontinuity in the Arrhenius plot of another

soluble enzyme, β amylase from wheat (Table 4.11). But they made no comment about its significance, biological or otherwise. HEWITT ET AL (1974) observed a break at 31°C in the Arrhenius plot of a β glucosidase found in the head of Trinervitermes trinervoides. However, MORGAN (1975) has not found a break in a cellobiase from Locusta gut. It is possible that the enzymes studied in the present work exist in two forms of differing activities. If these two forms are in equilibrium with one another and have different activation energies, and the conversion of one form to another is markedly affected by temperature, then an abrupt discontinuity will be seen (FLORKIN AND STOTZ, 1964). Although largely speculative, this would lend support to CLARKE (1965) hypothesis concerning temperature acclimation in Locusta. In this paper CLARKE proposed that the enzymes in Locusta (perhaps only certain key enzymes) occurred in two forms which were identical in specific chemical action but differed in their chemical characteristics, such that the activity of one was shifted on the temperature scale relative to the activity of the other. Under the influence of humoral factors the animal would produce more of one form than the other depending on the temperature of the environment it was living in and thus maintain metabolic efficiency. The effect of pH on the activities of the digestive enzymes studied in the present work did not indicate the presence of two forms or isoenzymes. However, at least in two enzymes, β D glucosidase and β D galactosidase, breaks were seen in Km plots which may also be interpreted as indicating two forms of these enzymes active against the substrates employed (see earlier).

Caution must be shown in interpreting the effect of temperature on enzyme preparations in physiological terms. At high temperatures the activity must be a balance between the denaturation of the enzyme and the high velocity at such temperatures. So the optimum

must depend on the length of the incubation period amongst other factors. EVANS (1956) showed a temperature optimum for a protease from Caliphora erythrocephala of 44°C yet the animal in its natural environment will not be exposed to more than 29°C or so, above which it is sterile anyway. However, carbohydrases from Schistocerca and Locusta have been shown to be markedly temperature resistant, (EVANS AND PAYNE, 1964; DROSTE AND ZEBE, 1974). EVANS AND PAYNE suggested that the absence of denaturation when α D glucosidase was kept at 45°C for 12 hours was a clear adaptation to the mode of life of the desert locust. Locusts usually feed in the early hours of the morning and since the food takes about four hours to pass through the gut, digestion takes place at the hottest period of the day. Body temperature is close to the environment and this may well be in the region of 50 - 55°C near ground surfaces (POPOV, 1958). It is interesting to note that a number of enzymes in the present work showed optima within this region.

Table 4.9

pH optima of some insect digestive enzymes

<u>Enzyme</u>	<u>Source</u>	<u>pH optimum</u>	<u>Substrate</u>	<u>Reference</u>
α glucosidase	<u>Locusta</u>	6.0	saccharose	Droste & Zebe (1974)
		6.6	maltose	" "
		7.2	pnp glucose	" "
	<u>Schistocerca gregaria</u>	5.4	pnp glucoside	Present work Evans & Payne (1964)
		5.4	methyl glucoside	
β glucosidase	<u>Locusta</u>	5.5	cellobiose	Morgan (1975)
		5.4	4 methylumbelliferone	Robinson (1956)
		5.6	cellobiose	Droste & Zebe (1974)
	<u>Bombyx mori</u>	5.2	pnp glucoside	Present work Ito & Tanaka (1959)
		5.0 - 6.4	salicin	
	<u>Blaberus craniifer</u>	5.0	p nitrophenol glucoside	Fisher (1964)
	<u>Periplaneta americana</u>	5.4	4 methylumbelliferone	Robinson (1956)
	<u>Schistocerca gregaria</u>	5.1 - 5.3	4 methylumbelliferone	Price & Robinson (1966)
α galactosidase	<u>Locusta</u>	5.3	raffinose	Droste & Zebe (1974)
		5.3	melibiose	" "
		7.2	pnp galactoside	" "
	<u>Calliphora erythrocephala</u>	5.2	pnp galactoside	Present work Fraenkel (1940)
		5.4	melibiose	
<u>Trogoderma</u>	5.4	raffinose	Krishna (1958)	
β galactosidase	<u>Locusta</u>	6.0	lactose	Droste & Zebe (1974)
		5.1 - 5.4		Price & Robinson (1966)
		4.8	pnp galactoside	Present work Hori (1959)
	5.8 - 6.3	lactose		
<u>Schistocerca gregaria</u>	5.0		Price & Robinson (1966)	

<u>Enzyme</u>	<u>Source</u>	<u>pH optimum</u>	<u>Substrate</u>	<u>Reference</u>
trehalase (soluble form)	<u>Locusta</u>	5.6	trehalose	Droste & Zebe (1974)
		5.0 - 5.2	"	Present work
	<u>Melanoplus differentialis</u>	5.6	"	Derr & Randall (1966)
	<u>Hyalophora cecropia</u>	5.7	"	Gussin & Wyatt (1965)
	<u>Blaberus discoidalis</u>	5.0	"	Wyatt (1967)
Protease	<u>Locusta</u>	8.5	gelatin	Powning, Day & Iryzkiewicz (1951)
		9.0 - 9.3	benzoyl arginine p nitranilide	Present work
	<u>Phormia regina</u>	7.9 - 8.3	casein	Brookes (1961)
	<u>Calliphora erythroce- phala</u>	7.6 - 7.8	azocasein	Evans (1958)
	<u>Gryllulus taiwanema</u>	8.3	casein	Nakashima et al (1965)

Table 4.10

Substrate affinities of some insect digestive enzymes

<u>Enzyme</u>	<u>Source</u>	<u>Km</u> mM/litre	<u>Substrate</u>	<u>Reference</u>
α glucosidase	<u>Locusta</u>	33	saccharose	Droste & Zebe (1974)
		5.8	maltose	" "
		2.8	pnp glucose	" "
		1.57	pnp glucoside	Present work
	<u>Bombyx mori</u>	1.67	maltose	Hori (1959)
β glucosidase	<u>Locusta</u>	1.95	cellobiose	Morgan (1975)
		5.5	cellobiose	Droste & Zebe (1974)
		1.59	low concentra- tions pnp	Present work
	4.13	glucose high		
	<u>Bombyx mori</u>	13	salicin	Ito & Tanaka (1959)
		13	salicin	Hori (1959)
<u>Blaberus craniifer</u>	16.6	p nitrophenyl glucoside	Fisher (1964)	
α galactoside	<u>Locusta</u>	1.6	raffinose	Droste & Zebe (1974)
		3.4	pnp galactose	" "
		1.26	pnp galactoside	Present work
	<u>Bombyx mori</u>	8.9	melibiose	Hori (1959)
β galactosidase	<u>Locusta</u>	83	lactose	Droste & Zebe (1974)
		0.2		Price & Robinson (1966)
		1.78	high concentra- tions pnp	Present work
		0.71	galactoside	
		low		
	<u>Schistocerca gregaria</u>	0.25		Price & Robinson (1966)
<u>Bombyx mori</u>	0.51	lactose	Hori (1959)	
Trehalase (soluble form)	<u>Locusta</u>	3.4	trehalose	Droste & Zebe (1974)
		0.92		Present work
	<u>Bombyx mori</u>	0.5		Yanagawa (1971)
	<u>Hyalophora cecropia</u>	0.4		Gussin & Wyatt (1965)
	<u>Blaberus discoidalis</u>	0.5		Gilby, Wyatt & Wyatt (1967)
Protease	<u>Locusta</u>	0.876	benzoyl arginine p nitronilide	Present work
	<u>Gryllulus taiwanemma</u>	1.25	casein	Nakashima et al (1965)
	<u>Calliphora erythroce- phala</u>	0.703	azocasein	Evans (1958)
	<u>Manduca sexta</u>	0.017	benzoyl arginine ethyl ester	Miller et al (1974)
	Pure trypsin (bovine)	1.2	chymotrypsin	Green & Newarth (1953)

Table 4.11

Activation energies of some insect digestive enzymes

<u>Enzyme</u>	<u>Source</u>	<u>Temperature range °C</u>	<u>Activation energy K joules/mole⁻¹</u>	<u>Substrate</u>	<u>Reference</u>
glucosidase	<u>Schistocerca gregaria</u>	20-40	61.6	methyl glucoside	Evans & Payne (1964)
	<u>Locusta</u>	48 - 32.3	33.2	pnp glucoside	Present work.
glucosidase	<u>Locusta</u>	51 - 29	49.47	pnp glucoside	Present work.
		29 - 13	58.38	cellobiose	Morgan(1975)
		10 - 70	45.2	pnp glucoside	Fisher (1964)
	<u>Blaberus craniifer</u>		39.3	amylose	Meyer et al (1953)
	Wheat	50 - 20	38.97		Hewitt et al (1974)
		20 - 0	54.47		
	<u>Trinervitermes trinervoides</u>	31 - 12	58.8		
		45 - 31	24.9		
galactosidase	<u>Locusta</u>	54 - 33	50.5	pnp galactoside	Present work
		33 - 19	89.49		
galactosidase	<u>Locusta</u>	53 - 30	50.74	pnp galactoside	Present work
		30 - 15	77.88		
amylase (soluble form)	<u>Locusta</u>		46.52		Present work
	<u>Blaberus discoidalis</u>		63.2		Gilby, Wyatt & Wyatt (1967)
	<u>Bombyx mori</u>		35.6		Yanagawa (1971)
	<u>Melanoplus differentialis</u>		30.15		Derr & Randall (1966)
protease	<u>Locusta</u>	45 - 27.7	33.3	benzoyl arginine p nitranilide	Present work
		27.7 - 16.3	66.0	azocasein	Evans(1956)
	<u>Calliphora erythrocephala</u>	5 - 35	54.7		
	<u>Musca domestica</u>	10 - 45	62.8		Lin & Richards (1956)
	<u>Trypsin</u>	0 - 40	62.4	benzoyl arginine amide	Butler (1941)

CHAPTER 5

The effect of the removal of the frontal ganglion on the activities of six digestive enzymes

Introduction

The production of digestive enzymes is probably a continuous process in many insects since the alimentary canal especially in larvae is often kept full of food, except at the moult. However, some means of control may be expected particularly in insects which feed intermittently, for example predators (WATERHOUSE, 1957). Three possible mechanisms have been suggested for the stimulation of secretion of digestive enzymes in insects: (a) secretagogue, the foodstuff or its products stimulate secretion; (b) nervous, the act of feeding, the detection of food or the presence of food may set up a nerve reflex to which secretory cells respond; (c) hormonal, like the nervous mechanism except that the feeding results in the production of a hormone that reaches the digestive tract via the haemolymph (HOUSE, 1974).

The possibility of a direct nervous stimulus does not appear to have been seriously considered since DAY AND POWNING (1949) observed that the nerves innervating the midgut of Blattella germanica appear to be motor and to supply only the musculature. However, their evidence was purely anatomical.

On the other hand many authors have cited that the level of proteolytic enzymes in the gut lumen increases after food ingestion, in various species of Diptera (FISK, 1950; FISK AND SHAMBAUGH, 1952; YANG AND DAVIES, 1968), in Tenebrio molitor (DADD, 1956), Periplaneta orientalis (SCHLOTKE, 1936) and Locusta migratoria (KHAN, 1963).

A qualitative or quantitative relationship has been shown between the two processes but it is difficult to establish whether the effect of feeding is direct (secretagogue) or indirect (endocrine). Evidence for a secretagogue mechanism comes from various sources. It has been shown that feeding stimulates the secretion of some enzymes but not others in several species (SAXENA, 1955; SRIVASTAVA, 1961). The amount of protein in the posterior midgut of Aedes aegypti is correlated with protease activity (GOODING, 1974). This is at odds with work by LANGLEY (1966, 1967) on another species of mosquito Glossina morsitans. LANGLEY's work points to a relationship between meal size and protease activity and he proposed that meal size controls liberation of hormones that control the production of enzyme precursors. These are activated in the lumen of the gut by some factor in the blood serum. However, ENGELMANN (1969) and CHAMPLAIN AND FISK (1956) have shown that only certain proteins will increase the proteolytic activity in Leucophaea maderae and Stomyxs calcitrans respectively. Perhaps even more interesting as evidence in support of the secretagogue theory are those studies in which the removal of the centres of hormonal influence has no effect on levels of digestive enzymes. Ablation of brain medial neurosecretory cells of blood fed mosquitoes does not affect the midgut protease activity (FOSTER, 1972; LEA, 1967). A similar operation performed on Blatella orientalis did not significantly affect the total activity of the insect's midgut proteases (GORDON, 1970). ENGELMANN AND WILKENS (1969) removed the medial neurosecretory cells from Sarcophaga bullata and found that food intake dropped by half, but the amount of protease activity was proportional to the liver juice injected in both operated and operated control groups, while feeding on a sucrose solution did not cause an elevated enzyme level.

Further evidence for the secretagogue theory was provided by ENGELMANN (1966). He starved newly moulted female Leucophaea maderae for 4-5 days and then fed them for one hour. Removal of the brain, corpora cardiaca and corpora allata from such animals had no effect on the activity of protease in the gut twenty four hours after the operation. However, DOGRA AND GILLOTT (1971) have pointed out that feeding of previously starved insects brings about a massive, rapid release of neurosecretion. Therefore, if the amount of protease activity were dependent on the level of hormone circulating in the haemolymph, the failure of the operation described above to reduced enzyme activity may be due to the fact that twenty four hours is insufficient time to allow for degradation of hormone circulating at the time of the operation. Indeed GILLOTT ET AL (1970) have proposed that ENGELMANN's work is best explained by a mechanism similar to that proposed by CLARKE AND LANGLEY (1963e) (see later in this introduction).

The first indications of a hormonal influence on digestion were the observations by WIGGLESWORTH (1936, 1948) that the stimulation of egg development in Rhodnius prolixus by the corpora allata was followed by a more rapid digestion of the intestinal contents. More recently evidence has come from work on Tenebrio molitor. DADD (1961) showed that protease activity in the midgut of Tenebrio failed to develop in adults decapitated one day before emergence, but developed if decapitation followed emergence. If blood from fed animals was injected into starved animals the protease values were found to be higher in the recipients than if starved animals were used as donors. However, similar attempts to stimulate enzyme secretion by injection of haemolymph from fed to unfed mosquitoes was without success (FISK AND SHAMBAUGH, 1952). The protease activity in adult female Calliphora erythrocephala on the first five days after emergence are

highly influenced by diet, one including meat having a far greater effect than one without, while the mean protease activity of females deprived of their medial neurosecretory cells by cautery was only a quarter or a third of maximum values for the meat fed flies (THOMSEN AND MÖLLER, 1960). Implantation of the corpora cardiaca - corpora allata complex partially restored the protease activity (THOMSEN AND MÖLLER, 1963; THOMSEN AND LEA, 1968). Similarly STRANGWAYS-DIXON (1961, a, b, and c) found that in Calliphora erythrocephala medial neurosecretory cells are necessary for ingestion and digestion of protein, while allatectomy resulted in selection of low carbohydrate food.

Attempts have been made to correlate changes in other hormonally controlled systems with the production of digestive enzymes. The concentration of trypsin in mated female Nauphoeta cinerea was found to be high when there was a demand for yolk protein (RAO AND FISK, 1965). This concentration increased during ovarian development and was dramatically reduced when the oocytes passed down the oviducts and were retained in the uterus. Both THOMSEN AND MÖLLER (1963) and RAO AND FISK (1965) suggested that the apparent control exerted by the medial neurosecretory cells on the proteases of the midgut as well as on ovarian development were just two examples of a general control on protein synthesis exerted by these cells. HILL (1965) and OSBORNE ET AL (1968), however, have demonstrated that neurosecretion may be acting in a much more specific manner, by stimulating blood protein synthesis in the fat body.

The removal of the frontal ganglion from larval Locusta migratoria resulted in a general failure to increase the concentration of haemolymph protein during an instar and a lowering of protease synthesis in the midgut (CLARKE AND GILLOTT, 1967a). They used the

hypothesis proposed by CLARKE AND LANGLEY (1963e) to explain their results. Removal of the frontal ganglion stops the relay of nerve impulses from the stretch receptors on the foregut to the brain. This in turn reduces release of neurosecretory material from the median neurosecretory cells and corpora cardiaca which controls protein synthesis.

In the present work (Chapter 3), the removal of the frontal ganglion from young adult Locusta migratoria has been shown to result in a reduction in lipid and carbohydrate reserves. It was suggested that this might be due to a reduced synthetic ability of the fat body or an inability to digest and/or absorb food. The experiments recorded in this chapter were carried out in order to investigate the possibility that there was a reduction in activity of digestive enzymes which could be responsible for a low efficiency of digestion.

Using the optimal conditions for assay determined in Chapter 4, the distribution of six digestive enzymes α D glucosidase, β D glucosidase, α D galactosidase, β D galactosidase, "soluble" trehalase and a "trypsin-like" protease were determined quantitatively in four regions of the gut.

Materials and Methods

The animals employed, the operation and treatment of experimental animals were as described in the general materials and methods (Chapter 1). All reagents used were Anala R grade or the purest available and supplied by British Drug Houses and Sigma.

Preparation of homogenates

The method described applies to each of the three treatments investigated viz. operated control, operated and starved. The guts from one male and one female locust were quickly dissected out under ice cold distilled water, Malpighian tubules, adhering fat body and gonads were removed. Then the foreguts and their contents, the midgut and caecal tissues, midgut and caecal contents, hindgut and contents were washed quantitatively into separate Potter Elvehjem homogenisers with 10ml, 5ml, 5ml and 5ml respectively of ice cold distilled water. The rest of the procedure was as described in the materials and methods section of Chapter 4.

Assay of six digestive enzymes

The optimal conditions for assay determined in Chapter 4 were employed. These conditions and the method of assay were as described in the materials and methods section in Chapter 4.

The activities of α D glucosidase, β D glucosidase, α D galactosidase, β D galactosidase, soluble trehalase and "Trypsin-like" protease were determined as follows:

- (a) in the foregut lumen (tissue and lumen contents)
- (b) midgut lumen contents (including midgut caecae)
- (c) midgut tissue (including midgut caecae)
- (d) hindgut (tissue and lumen contents).

Previous work has shown that the midgut and midgut caecae are the main sites of synthesis of digestive enzymes (EVANS & PAYNE, 1964;

DAY AND POWNING, 1949; WIGGLESWORTH, 1972). Therefore, no attempt was made to differentiate between the luminal contents and tissue of other regions of the gut. The activities in the regions other than the midgut were taken to represent those of the lumina only. Each determination was made on an homogenate of material from one male and one female. The results were expressed in two ways, product formed/gut region/min and product formed/mg protein/min.

Statistical techniques

Statistical comparisons of data were performed using conventional techniques as described by SNEDECOR and COCHRAN (1967). Where appropriate reference was made to the statistical tables of FISCHER AND YATES (1963). Values of probability ≤ 0.05 were taken as being significant.

Results

1. Distribution of the enzyme activity between the four regions of the gut

(1) activity expressed in terms of product formed/gut region/min.

A pattern emerged which was followed to a greater or lesser extent by all the enzymes and was exemplified by the most prominent one α D glucosidase (Table 5.1, Fig. 5.1). The foregut of the control animal had far greater activity than either the operated or the starved ($p < 0.001$) which were not significantly different from each other. In contrast the midgut lumina of control and operated treatments had similar activities and both were significantly different from the starved ($p < 0.01$). In the hindgut considerable variation in activity was found with all the enzymes. As a result of this it was not possible to establish differences between the three treatments. This variability in the hindgut is to be expected, since the presence of digestive enzymes in this region will depend on supply from the

midgut and midgut caecae, and they will be voided periodically with the faeces. The midgut and caecal tissues mirrored the situation found in the foregut, namely that the control had significantly more activity than operated or starved animals ($p < 0.001$).

The effects of frontal ganglion removal and starvation on the activity of β D glucosidase were similar to those on α D glucosidase activity described above (see Table 5.3 and Fig. 5.1).

α D galactosidase activity in the midgut tissue was very low and no significant difference could be demonstrated between the three treatments (Table 5.5, Fig. 5.2). However, the other regions of the gut followed the pattern set by the glucosidases.

Table 5.7, Fig. 5.2 show the distribution of β D galactosidase activity. Once again the pattern was similar to that observed with α D glucosidase. The control foregut contained some three fold more activity than either operated or starved. In the midgut contents the β D galactosidase activities of operated and operated control treatments were similar and both greater than the starved. For this enzyme the latter differences were not significant owing to large standard errors. The activity in the midgut tissue of the control suggested that the latter was synthesising significantly more β D galactosidase than either operated or starved ($p < 0.01$). Once again there was no significant difference between treatments in the hindgut.

The distribution of trehalase follows the pattern set by the other carbohydrases (Table 5.9, Fig. 5.3). Here the deviations were in the hindgut where the starved animal had a significantly lower level of activity than the control and in the midgut tissue which showed no significant differences between the three treatments.

The 'trypsin' may be expected to deviate from the pattern in the midgut tissue, since tryptic enzymes have been shown to be stored and secreted as inactive precursors (NEILANDS AND STUMPF, 1958). It might

be that the low levels of activity in the 'trypsin' in the midgut tissue found in the present work were due to it being stored in an inactive form. This would explain the absence of a difference between the three treatments in this tissue. The above result agrees with the findings of CLARKE AND GILLOTT (1967a) who reported no differences between the midgut tissue protease activity of frontal ganglionectomised and control Locusta larvae.

By comparison with the carbohydrases, 'trypsin' activity in the hindgut was very low (Table 5.11, Fig. 5.3). This suggests that an efficient inhibitor or inactivator was present similar to that found by ENGELMANN (1969) in Leucophaea maderae and RAO AND FISK (1965) in Nauphoeta cinerea.

(11) activity expressed in terms of product formed/mg protein/min

An attempt was made to express the results in terms of the amount of protein in the gut extract. The results obtained were more varied and more difficult to interpret than 1(1) above. The foregut of control animals had greater enzyme activity than those of operated and starved animals as in 1(1) but the differences are not always significant (see Tables 5.2, 5.4, 5.6, 5.8, 5.10, 5.12 and Figures 5.1, 5.2, 5.3). The activities of the enzymes in the midgut contents showed an inverse relationship to those found when the results were expressed per gut region, namely that the operated control animals had significantly less activity than either operated or starved. In the midgut tissue no pattern emerged at all, and in the hindgut the picture was similar to that found in section 1(1).

2. Total activity of each enzyme in the gut lumen

The activities of each enzyme from the three regions of the gut lumen were summed to give the total luminal activity.

(1) activity expressed in terms of product formed/gut lumen/min

Examination of Table 5.13 reveals a situation shown to be characteristic of the effect of the removal of the frontal ganglion from Locusta larvae (CLARKE AND GILLOTT, 1967; CLARKE AND ANSTEE, 1971a). The operated controls had the greatest activity, starved the least and operated in between for each of the six enzymes studied. The differences between the levels of activity of the three treatments were subjected to a 't' test and shown to be significant ($p \leq 0.05$). The exceptions were the differences between operated and starved β D glucosidase and operated and starved β D galactosidase which were not significant.

(ii) activity expressed in terms of product formed/mg protein/min

When the results from Table 5.13 were re-expressed in terms of mg protein of enzyme extract a different picture altogether was obtained (Table 5.14). There were no significant differences between the three treatments with any of the six enzymes. The problem found in section 1(11) when the activities of the enzymes in each region of the gut were expressed per mg protein was not apparent when the total luminal activity of each enzyme was expressed in this way.

3. Enzyme activity in the foregut, midgut luminal contents and hindgut expressed as a percentage of the total gut lumen activity

As a third approach to studying the effect of the removal of the frontal ganglion and starvation on the activities of enzymes in the gut, it was decided to look at the relative distribution of the six enzymes under investigation in three regions of the gut lumen.

(i) results expressed in terms of the percentage of the total luminal activity/gut lumen/min

The five carbohydrases showed the same relative percentage distribution (Table 5.15). In the operated control animal the foregut

had the most activity with some 57 per cent, the midgut 38 per cent and the hindgut only 5 per cent. Removal of the frontal ganglion and starvation not only reduced the total activities of the enzymes, but there appeared to be a significant redistribution of what activity there was (Tables 5.15, 5.16). By comparison with the control, the operated animal showed an increase in the percentage of the carbohydrase activity found in the midgut from 38 up to some 63 per cent and in the starved from 38 up to some 53 per cent. This was due to a reduction in the percentage amount in the foregut ($p \leq 0.01$) as there were no significant differences between the percentages of the activities found in the hindguts of the three treatments.

In the operated control treatment a majority of the 'trypsin' activity is found in the midgut (54.5 per cent) and there was very little activity in the hindgut (0.73 per cent). Removal of the frontal ganglion and starvation not only caused a reduction in the total amount of 'trypsin' activity but brought about a redistribution similar to that found for the carbohydrases. The drop in the proportion of the 'trypsin' activity found in the foregut was more marked in the operated than in the starved animal. The difference between the two treatments was highly significant ($p = 0.01-0.001$). However, there were no significant differences between the proportions of carbohydrase activity in the operated and starved foreguts (see Table 5.16).

The increase in the proportion of enzyme activity found in the midgut was greater in the operated than in the starved animals ($p < 0.05$) apart from the galactosidases.

(11) Results expressed in terms of the percentage of the total luminal activity/mg protein/min

Due to an increased share of the activity in the hindgut when the results were expressed in this way 7-17% instead of the 5% in the control), the proportion of carbohydrase activity in the foregut

appeared somewhat lower (some 47% instead of 57%). However, frontal ganglion removal and starvation had similar effects on the distribution of enzyme activity as they did in 3(1) above. There was a significant increase in the proportion of enzyme activity found in the midgut of operated and starved treatments. Once again this appeared to be due to a reduction in the percentage of activity in the foregut. (Tables 5.17, 5.18).

4. The activities of the five carbohydrate enzymes in the gut lumen and midgut tissue expressed as a percentage of the total carbohydrase activity in these two regions

α D glucosidase appeared to be the most important of the carbohydrate enzymes studied as it contributed 42% of the carbohydrase activity measured in the gut lumen of the operated control animal. β D glucosidase contributed 29% of the activity, β D galactosidase 12.6%, Trehalase 12.4% and α D galactosidase 4%. Frontal ganglion removal and starvation had no effect on this pattern (Table 5.19, Fig. 5.4). The importance of α D glucosidase became even more apparent when the relative activities of the enzymes were looked at in the midgut tissue. In the operated control it represented 51.39% of the carbohydrase activity. β D glucosidase was the next most active (19.77%) followed by Trehalase (15.48%), β D galactosidase (12.35%) and finally α D galactosidase which represented only 1.01% of the total carbohydrase activity in the midgut tissue. Trehalase activity in operated and starved midgut tissue assumed a greater proportion of the total activity than in the control. One might tentatively suggest that either the production of this enzyme was relatively less affected by these procedures or that its release into the lumen was reduced (Table 5.20, Fig. 5.4).

Table 5.1

The effect of the removal of the frontal
ganglion and starvation on the activity
of α D glucosidase

activity is expressed in terms of μ moles p nitrophenol/gut region/
min $\times 10^2$

Region of the gut	Treatment	Number of determinations	Mean activity $\times 10^2$	SE		t	P
Foregut	O	7	102.37	11.64	O:C	7.917	< 0.001
	C	8	269.98	16.94	O:S	1.147	0.3-0.2
	S	7	80.63	14.96	C:S	8.988	< 0.001
Midgut Lumen Contents	O	7	206.25	6.41	O:C	0.546	0.7-0.6
	C	8	196.36	15.92	O:S	4.421	< 0.001
	S	7	113.95	19.87	C:S	3.273	0.01-0.001
Midgut Tissue	O	7	31.71	1.79	O:C	4.261	< 0.001
	C	8	45.34	2.54	O:S	1.918	0.03-0.2
	S	7	27.43	1.33	C:S	5.973	< 0.001
Hindgut	O	7	8.267	1.93	O:C	1.440	0.2-0.1
	C	8	24.953	10.66	O:S	1.066	0.4-0.3
	S	7	11.54	2.39	C:S	1.150	0.3-0.2

O = operated

C = operated control

S = starved

SE indicates the standard error of the mean

t indicates the values obtained from applying student's t-test

P indicates values of probability

Table 5.2

The effects of the removal of the frontal ganglion and starvation on the activity of α D glucosidase in the gut

activity is expressed in terms of μ moles p nitrophenol/mg protein/min $\times 10^2$

Region of the gut	Treatment	Number of determinations	Mean activity $\times 10^2$	SE		t	P
Foregut	O	7	24.80	2.74	O:C	2.768	0.02-0.01
	C	8	38.76	4.05	O:S	0.865	0.5-0.4
	S	7	28.20	2.82	C:S	2.076	0.1-0.05
Midgut Lumen Contents	O	7	49.401	4.9	O:C	2.585	0.05-0.02
	C	8	36.196	2.15	O:S	0.095	> 0.9
	S	7	50.01	4.07	C:S	3.116	0.1-0.001
Midgut Tissue	O	7	7.324	1.47	O:C	1.236	0.3-0.2
	C	8	9.213	0.64	O:S	1.218	0.3-0.2
	S	7	9.173	0.39	C:S	0.05	> 0.9
Hindgut	O	7	4.493	1.05	O:C	1.380	0.2-0.1
	C	8	10.933	4.25	O:S	2.341	0.05-0.02
	S	7	8.421	1.31	C:S	0.532	0.7-0.6

O = operated

C = operated control

S = starved

SE indicates the standard error of the mean

t indicates the values obtained from applying student's t-test

P indicates values of probability

Table 5.3

The effects of the removal of the frontal ganglion
and starvation on the activity of β D glucosidase
in the gut

activity is expressed in terms of μ moles p nitrophenol/gut region/min
 $\times 10^2$

Region of the gut	Treatment	Number of determinations	Mean activity $\times 10^2$	SE		t	P
Foregut	O	7	72.14	8.17	O:C	5.763	< 0.001
	C	8	196.44	18.8	O:S	0.449	0.7-0.6
	S	7	65.19	13.14	C:S	5.561	< 0.001
Midgut Lumen Contents	O	7	154.29	19.1	O:C	1.007	0.4-0.3
	C	8	130.00	15.17	O:S	2.717	0.02-0.01
	S	7	88.55	14.85	C:S	1.940	0.1-0.05
Midgut Tissue	O	7	9.673	1.92	O:C	3.881	0.01-0.001
	C	8	17.420	0.84	O:S	0.639	0.6-0.5
	S	7	8.424	0.38	C:S	9.308	< 0.001
Hindgut	O	7	15.576	3.08	O:C	1.831	0.1-0.05
	C	7	23.481	3.02	O:S	0.499	0.7-0.6
	S	7	18.106	4.02	C:S	1.068	0.4-0.3

O = operated

C = operated control

S = starved

SE indicates the standard error of the mean

t indicates the values obtained from applying student's t-test

P indicates values of probability

Table 5.4

The effects of the removal of the frontal ganglion
and starvation on the activity of β D glucosidase
in the gut

activity is expressed in terms of μ moles p nitrophenol/mg protein/
min $\times 10^2$

Region of the gut	Treatment	Number of determinations	Mean activity $\times 10^2$	SE	t	P	
Foregut	O	7	16.98	1.24	0:C	3.539	0.01-0.001
	C	8	27.54	2.56	0:S	1.935	0.1-0.05
	S	7	22.56	2.61	C:S	1.357	0.2-0.1
Midgut Lumen Contents	O	7	34.89	2.26	0:C	4.035	0.01-0.001
	C	8	23.57	1.73	0:S	1.000	0.4-0.3
	S	7	39.01	3.44	C:S	4.168	0.01-0.001
Midgut Tissue	O	7	2.161	0.51	0:C	2.472	0.05-0.02
	C	8	3.580	0.3	0:S	1.251	0.3-0.2
	S	7	2.831	0.15	C:S	2.157	0.1-0.05
Hindgut	O	7	8.850	1.87	0:C	0.859	0.5-0.4
	C	7	10.963	1.6	0:S	0.661	0.6-0.5
	S	7	11.31	3.23	C:S	0.097	> 0.9

O = operated

C = operated control

S = starved

SE indicates the standard error of the mean

t indicates the values obtained from applying student's t-test

P indicates values of probability

α D GLUCOSIDASE

β D GLUCOSIDASE

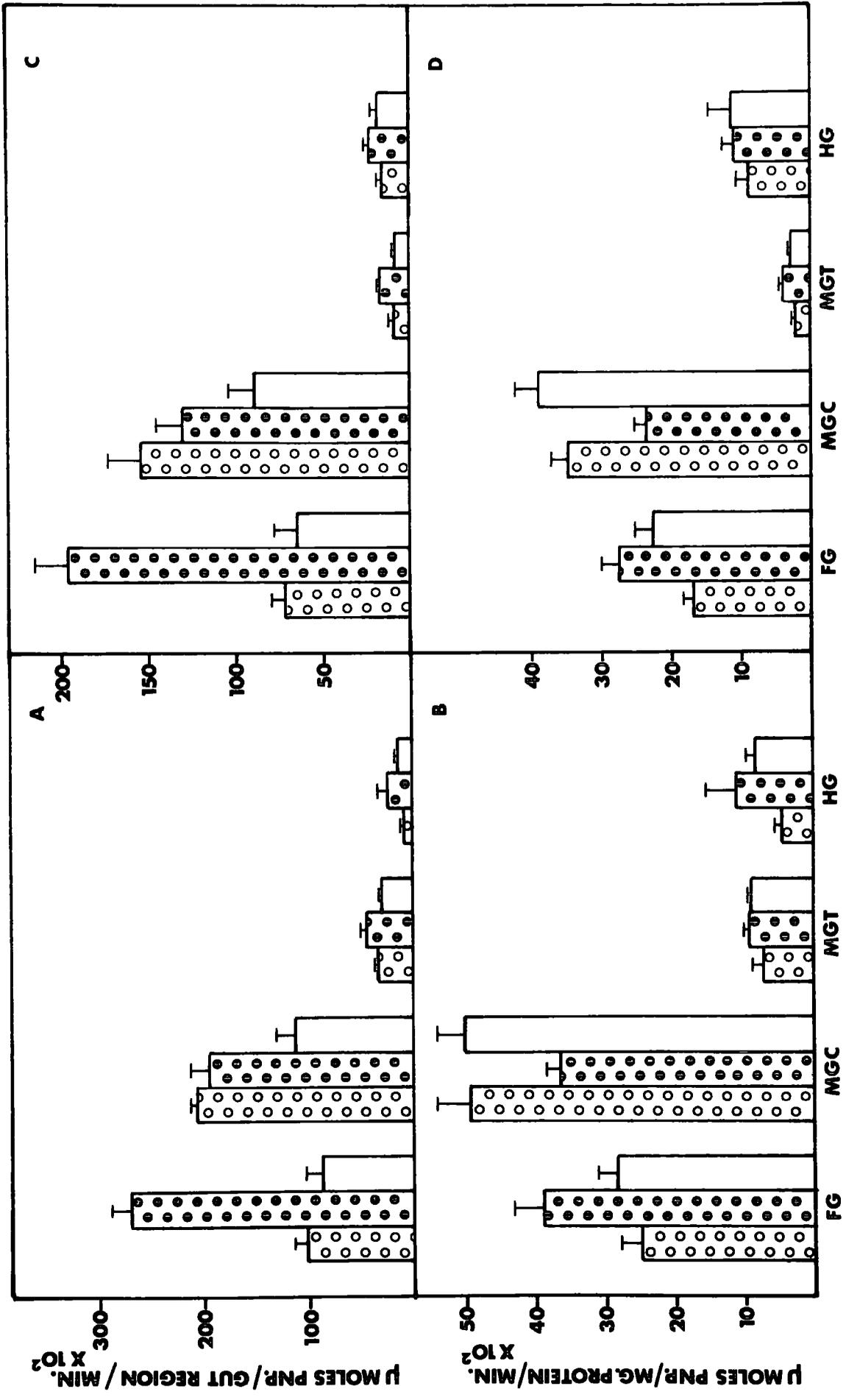


Table 5.5

The effect of the removal of the frontal ganglion
and starvation on the activity of α D galactosidase
in the gut

activity is expressed in terms of μ moles p nitrophenol/gut region/
min $\times 10^2$

Region of the gut	Treatment	Number of determinations	Mean activity $\times 10^2$	SE		t	P
Foregut	O	7	8.41	1.91	O:C	4.984	< 0.001
	C	8	31.99	4.08	O:S	0.841	0.5-0.4
	S	7	6.37	1.5	C:S	5.566	< 0.001
Midgut Lumen Contents	O	7	17.141	1.89	O:C	0.469	0.7-0.6
	C	8	15.843	1.99	O:S	3.281	0.1-0.001
	S	7	8.946	1.64	C:S	2.626	0.02-0.01
Midgut Tissue	O	7	0.634	0.16	O:C	1.492	0.2-0.1
	C	8	0.899	0.09	O:S	1.057	0.4-0.3
	S	7	0.873	0.16	C:S	0.146	0.9-0.8
Hindgut	O	7	0.600	0.19	O:C	1.953	0.1-0.05
	C	7	1.243	0.27	O:S	1.260	0.3-0.2
	S	7	1.046	0.3	C:S	0.495	0.7-0.6

O = operated

C = operated control

S = starved

SE indicates the standard error of the mean

t indicates the values obtained from applying student's t-test.

P indicates values of probability

Table 5.6

The effects of the removal of the frontal ganglion
and starvation on the activity of α D galactosidase
in the gut

activity is expressed in terms of μ moles p nitrophenol/mg protein/
min $\times 10^2$

Region of the gut	Treatment	Number of determinations	Mean activity $\times 10^2$	S.E.		t	P
Foregut	O	7	2.237	0.24	O:C	3.690	0.01-0.001
	C	8	4.456	0.52	O:S	1.251	0.3-0.2
	S	7	2.63	0.2	C:S	3.111	0.01-0.001
Midgut Lumen Contents	O	7	3.910	0.28	O:C	3.17	0.01-0.001
	C	8	2.82	0.22	O:S	0.14	0.9-0.8
	S	7	4.01	0.65	C:S	1.84	0.1-0.05
Midgut Tissue	O	7	0.127	0.042	O:C	1.183	0.3-0.2
	C	8	0.181	0.02	O:S	2.412	0.05-0.02
	S	7	0.287	0.05	C:S	2.054	0.1-0.05
Hindgut	O	7	0.333	0.11	O:C	1.465	0.2-0.1
	C	7	0.55	0.100	O:S	1.477	0.2-0.1
	S	7	0.85	0.33	C:S	0.858	0.5-0.4

O = operated

C = operated control

S = starved

SE = indicates the standard error of the mean

t = indicates the values obtained from applying student's t-test

P = indicates value of probability

Table 5.7

The effects of the removal of the frontal ganglion
and starvation on the activity of β D galactosidase
in the gut

activity is expressed in terms of μ moles p nitrophenol/gut region/
min $\times 10^2$

Region of the gut	Treatment	Number of determinations	Mean activity $\times 10^2$	SE		t	P
Foregut	O	6	24.38	4.51	O:C	6.758	< 0.001
	C	8	87.96	7.36	O:S	0.076	> 0.9
	S	7	24.94	5.55	C:S	6.677	< 0.001
Midgut Lumen Contents	O	7	53.76	7.4	O:C	0.016	> 0.9
	C	8	53.59	6.43	O:S	1.517	0.2-0.1
	S	7	37.86	7.42	C:S	1.612	0.2-0.1
Midgut Tissue	O	7	7.223	0.64	O:C	3.490	0.01-0.001
	C	8	10.904	0.81	O:S	2.134	0.1-0.05
	S	7	5.663	0.36	C:S	5.612	< 0.001
Hindgut	O	6	5.86	1.3	O:C	1.900	0.1-0.05
	C	7	9.21	1.2	O:S	0.456	0.7-0.6
	S	7	6.80	1.56	C:S	1.221	0.3-0.2

O = operated

C = operated control

S = starved

SE indicates the standard error of the mean

t indicates the values obtained from applying student's t-test

P indicates values of probability

Table 5.8

The effects of the removal of the frontal ganglion
and starvation on the activity of β D galactosidase

activity is expressed in terms of μ moles p nitrophenol/mg protein/
min $\times 10^2$

Region of the gut	Treatment	Number of determina- tions	Mean activity $\times 10^2$	SE	t	P	
Foregut	O	6	6.213	1.13	O:C	4.658	< 0.001
	C	8	12.166	0.72	O:S	1.561	0.2-0.1
	S	7	8.844	1.22	C:S	2.424	0.05-0.02
Midgut lumen contents	O	7	12.723	0.7	O:C	2.582	0.05-0.02
	C	8	9.780	0.87	O:S	1.680	0.2-0.1
	S	7	17.103	2.51	C:S	2.908	0.02-0.01
Midgut Tissue	O	7	1.493	0.29	O:C	2.240	0.05-0.02
	C	8	2.20	0.15	O:S	1.302	0.3-0.2
	S	7	1.891	0.09	C:S	1.694	0.2-0.1
Hindgut	O	6	3.405	0.79	O:C	0.893	0.4-0.3
	C	7	4.274	0.6	O:S	0.778	0.5-0.4
	S	7	4.503	1.11	C:S	0.181	0.9-0.8

O = operated

C = operated control

S = starved

S.E. indicates the standard error of the mean

t indicates the values obtained from applying Student's
t-test

P indicates values of probability

Fig. 5.2

The effects of the removal of the frontal ganglion
and starvation on the activities of α D galactosidase
and β D galactosidase in the gut

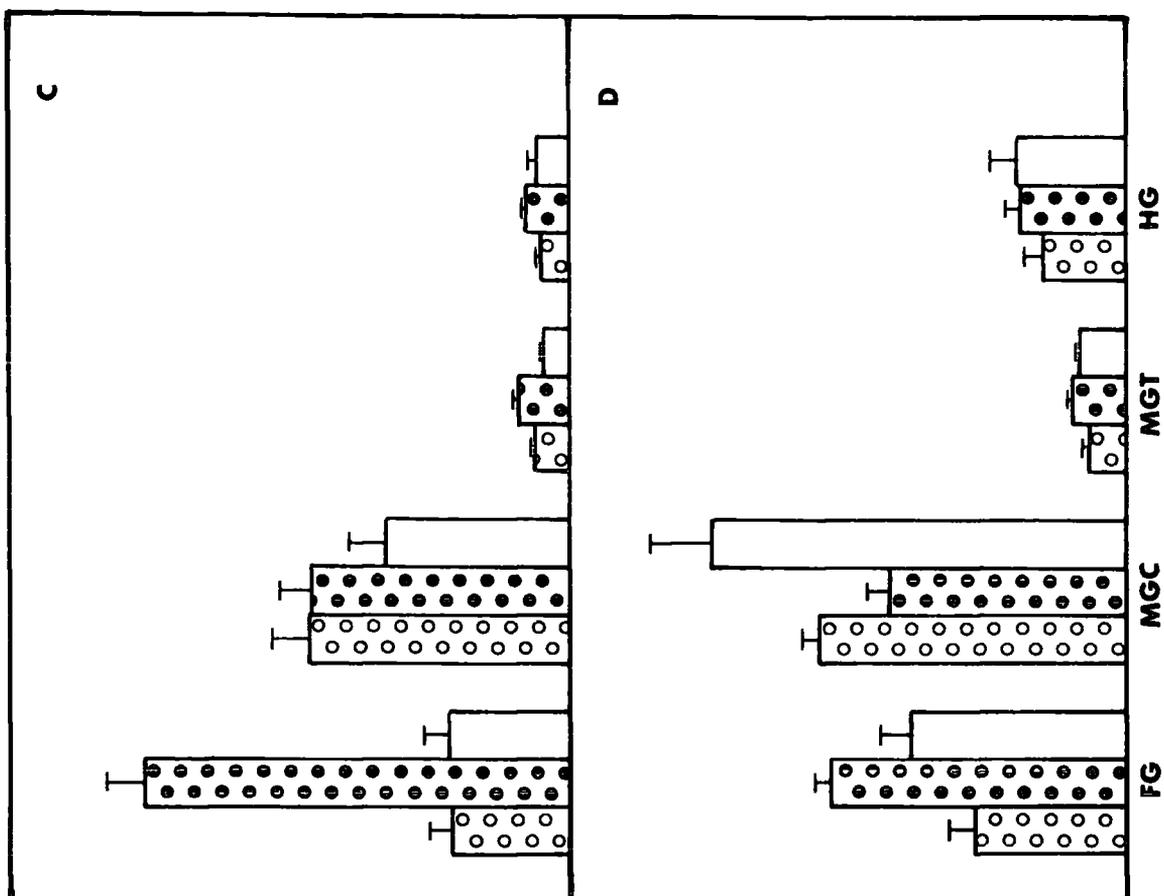
Legend:

FG = foregut
MGC = midgut lumen contents
MGT = midgut tissue
HG = hindgut

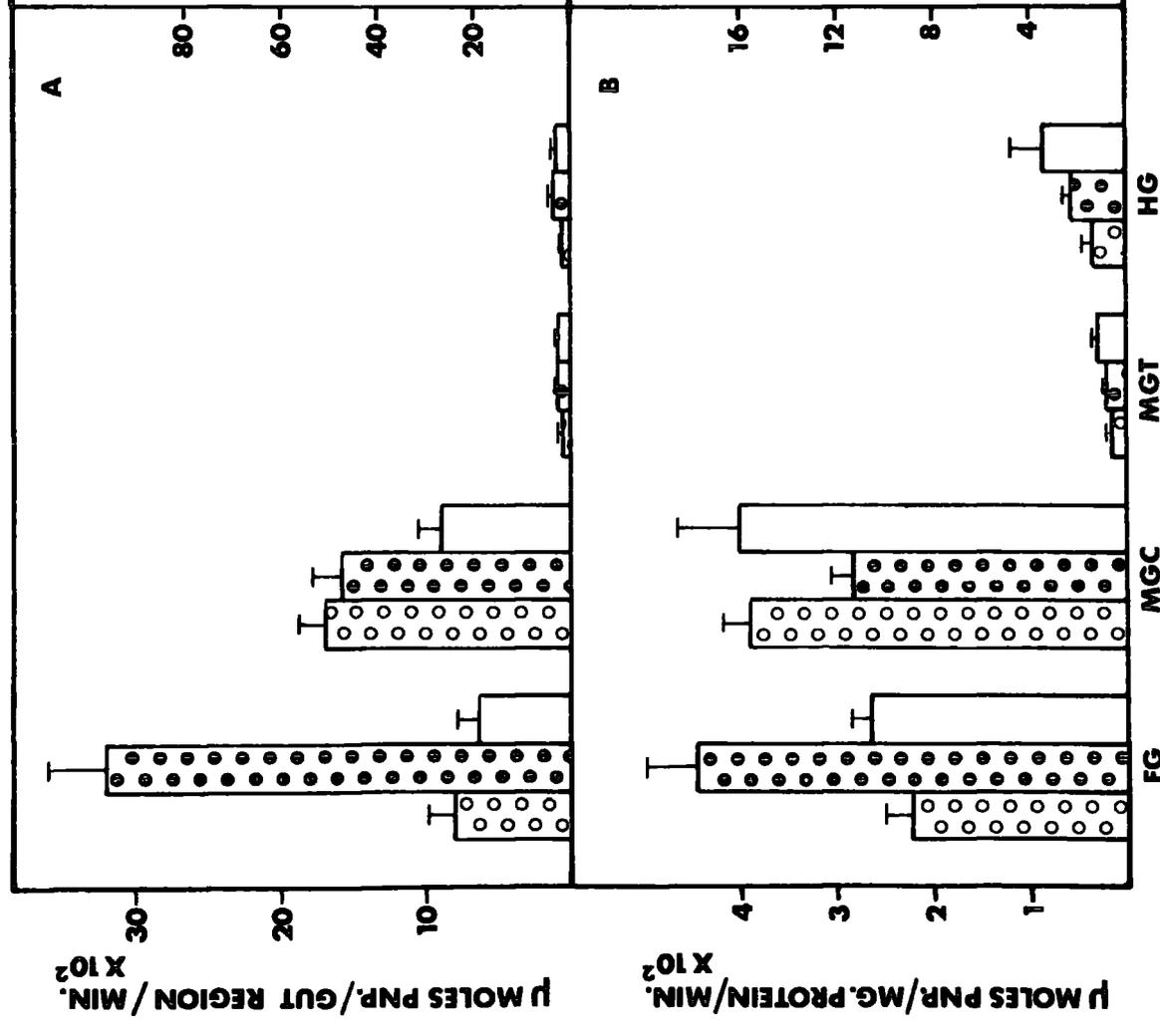
○ ○ operated
○
● ● operated control
●
starved

D galactosidase A. ordinate ; μ moles p nitrophenol/gut region/
region/min $\times 10^2$
abscissa ; region of the gut
B. ordinate ; μ moles p nitrophenol/mg protein/
min $\times 10^2$
abscissa ; region of the gut
D galactosidase C. ordinate ; μ moles p nitrophenol/gut region/
min $\times 10^2$
abscissa ; region of the gut
D. ordinate ; μ moles p nitrophenol/mg protein/
min $\times 10^2$
abscissa ; region of the gut

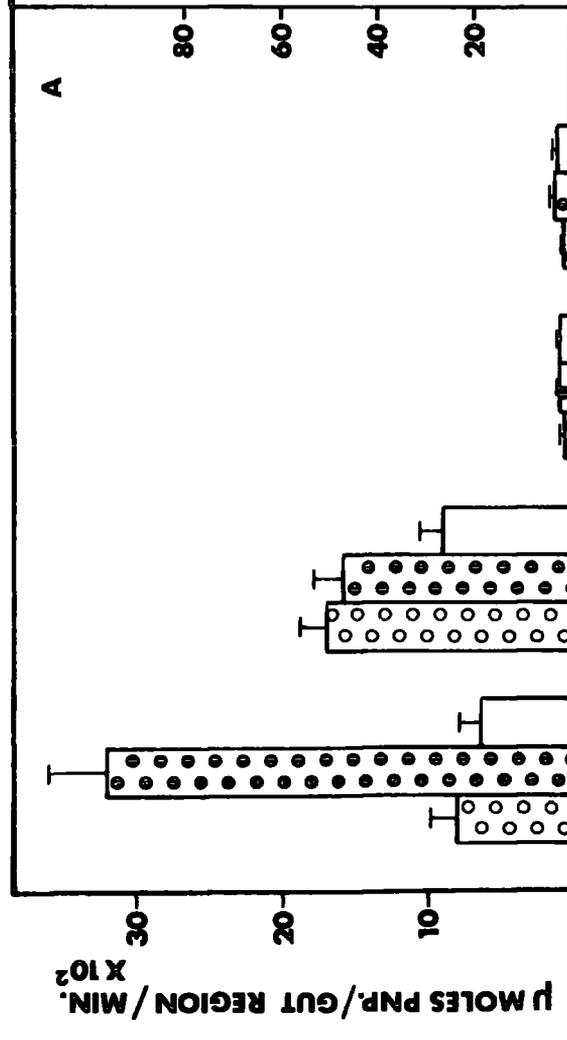
β D GALACTOSIDASE



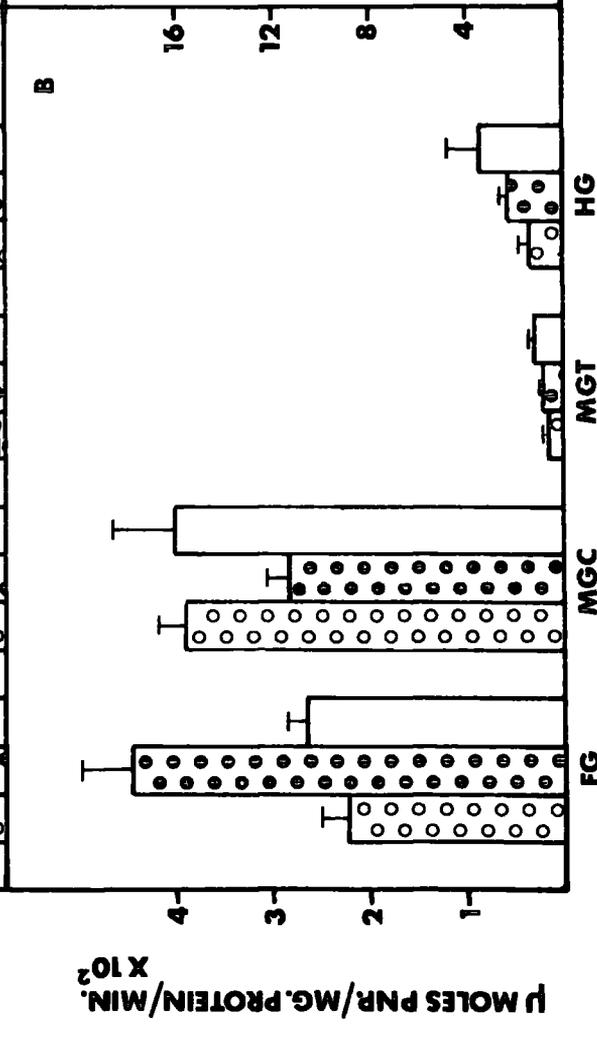
α D GALACTOSIDASE



A



B



μ MOLES PNR/GUT REGION/MIN. X 10²

μ MOLES PNR/MG.PROTEIN/MIN. X 10²

Table 5.9

The effects of the removal of the frontal ganglion
and starvation on the activity of trehalase in the
gut

activity is expressed in terms of μ g glucose/ gut region/min

Region of the gut	Treatment	Number of determinations	Mean activity	SE	t	P	
Foregut	O	8	73.65	6.65	O:C	6.749	< 0.001
	C	8	143.92	8.02	O:S	1.774	0.1-0.05
	S	7	52.60	10.18	C:S	7.135	< 0.001
Midgut Lumen Contents	O	8	124.79	10.80	O:C	1.243	0.3-0.2
	C	8	105.80	10.80	O:S	3.784	0.01-0.001
	S	7	66.29	10.99	C:S	2.555	0.05-0.02
Midgut Tissue	O	8	19.75	1.55	O:C	1.915	0.1-0.05
	C	8	24.61	2.02	O:S	0.544	0.6-0.5
	S	7	20.94	1.55	C:S	1.409	0.2-0.1
Hindgut	O	8	9.419	1.88	O:C	0.846	0.5-0.4
	C	8	11.39	1.38	O:S	1.406	0.2-0.1
	S	7	5.52	2.04	C:S	2.440	0.05-0.02

O = operated

C = operated control

S = starved

SE indicates the standard error of the mean

t indicates the values obtained from applying student's t-test

P indicates values of probability

Table 5.10

The effects of the removal of the frontal ganglion
and starvation on the activity of Trehalase

activity is expressed in terms of μ g glucose/mg protein/min

Region of the gut	Treatment	Number of determinations	Mean activity	SE		t	P
Foregut	O	8	17.38	1.28	O:C	1.295	0.3-0.2
	C	8	20.78	2.29	O:S	0.398	0.7-0.6
	S	7	18.30	2.0	C:S	0.801	0.5-0.4
Midgut Lumen Contents	O	8	28.01	1.77	O:C	4.332	< 0.001
	C	8	19.35	1.25	O:S	0.352	0.8-0.7
	S	7	29.04	2.36	C:S	3.769	0.01-0.001
Midgut Tissue	O	8	4.395	0.66	O:C	0.743	0.5-0.4
	C	8	5.008	0.49	O:S	2.931	0.02-0.01
	S	7	7.061	0.61	C:S	2.664	0.02-0.01
Hindgut	O	8	5.200	1.05	O:C	0.137	0.9-0.8
	C	8	5.360	0.49	O:S	0.793	0.5-0.4
	S	7	3.849	1.37	C:S	1.094	0.3-0.2

O = operated

C = operated control

S = starved

SE indicates the standard error of the mean

t indicates the values obtained from applying student's t-test

P indicates values of probability

Table 5.11

The effects of the removal of the frontal ganglion and starvation on the activity of "trypsin" in the gut

activity is expressed in terms of μ moles p nitroaniline/gut region/
min $\times 10^2$

Region of the gut	Treatment	Number of determinations	Mean activity $\times 10^2$	SE		t	P
Foregut	O	8	21.764	3.3	O:C	6.456	< 0.001
	C	8	82.32	8.78	O:S	0.755	0.5-0.4
	S	7	25.87	4.4	C:S	5.484	< 0.001
Midgut Lumen Contents	O	8	109.36	13.69	O:C	0.326	0.8-0.7
	C	8	102.99	13.95	O:S	3.545	0.1-0.001
	S	7	53.38	6.18	C:S	3.091	0.1-0.001
Midgut Tissue	O	8	5.016	0.96	O:C	0.671	0.6-0.5
	C	8	4.31	0.43	O:S	0.013	> 0.9
	S	7	5.00	0.45	C:S	1.110	0.3-0.2
Hindgut	O	8	0.052	0.04	O:C	2.383	0.05-0.02
	C	8	1.165	0.47	O:S	1.747	0.2-0.1
	S	7	1.063	0.62	C:S	0.133	0.9-0.8

O = operated

C = operated control

S = starved

SE indicates the standard error of the mean

t indicates the values obtained from applying student's t-test

P indicates values of probability

Table 5.12

The effects of the removal of the frontal ganglion
and starvation on the activity of "trypsin" in the
gut

activity is expressed in terms of μ moles p nitroaniline/mg protein/min $\times 10^3$

Region of the gut	Treatment	Number of determinations	Mean activity $\times 10^3$	SE		t	P
Foregut	O	8	49.83	6.63	O:C	4.055	< 0.001
	C	8	130.46	18.75	O:S	1.686	0.2-0.1
	S	7	76.2	14.98	C:S	2.215	0.05-0.02
Midgut Contents	O	8	238.8	18.3	O:C	1.887	0.1-0.05
	C	8	187.7	19.95	O:S	0.538	0.6
	S	7	216.5	39.37	C:S	0.677	0.6-0.5
Midgut epithelium	O	8	11.56	2.54	O:C	1.043	0.4-0.3
	C	8	8.71	1.03	O:S	1.696	0.2-0.1
	S	7	16.8	1.57	C:S	4.428	0.01-0.001
Hindgut	O	7	0.274	0.27	O:C	2.506	0.05-0.02
	C	8	5.636	2.12	O:S	1.892	0.1-0.05
	S	7	7.459	4.06	C:S	0.413	0.7-0.6

O = operated

C = operated control

S = starved

SE indicates the standard error of the mean

t indicates values obtained from applying student's t-test

P indicates values of probability

Fig. 5.3

The effects of the removal of the frontal ganglion
and starvation on the activities of trehalase and
"trypsin" in the gut

Legend:

FG = foregut
MGC = midgut lumen contents
MGT = midgut tissue
HG = hindgut

○ ○ operated
○
● ● operated control
●
starved

trehalase A. ordinate ; μ g glucose/gut region/min
abscissa ; region of the gut

B. ordinate ; μ g glucose/mg protein/min
abscissa ; region of the gut

"trypsin" C. ordinate ; μ moles p nitroaniline/gut region/min $\times 10^2$
abscissa ; region of the gut

D. ordinate ; μ moles p nitroaniline/mg protein/min $\times 10^3$
abscissa ; region of the gut

Table 5.13

The effect of the removal of the frontal ganglion and starvation on enzyme activity in the gut

activity is expressed in terms of product formed/gut lumen/min

<u>Enzyme</u>	Treatment	Number of determinations	Mean	SE	t	P	
1. α D glucosidase	O	7	316.89	15.5	0:C	5.011	< 0.001
	C	8	491.29	29.47	0:S	3.194	0.01-0.001
	S	7	206.12	31.01	C:S	6.658	< 0.001
2. β D glucosidase	O	7	242.00	26.45	0:C	2.399	0.05-0.02
	C	7	339.47	30.84	0:S	1.891	0.1-0.05
	S	7	171.85	26.00	C:S	4.155	0.01-0.001
3. α D galactosidase	O	7	26.15	3.42	0:C	3.553	0.01-0.001
	C	7	45.79	4.34	0:S	2.218	0.05-0.02
	S	7	16.36	2.78	C:S	5.708	< 0.001
4. β D galactosidase	O	6	82.96	10.79	0:C	4.453	< 0.001
	C	7	147.98	9.86	0:S	0.819	0.5-0.4
	S	7	69.60	11.92	C:S	5.066	< 0.001
5. Trehalase	O	8	207.86	15.62	0:C	2.281	0.05-0.02
	C	8	261.11	17.35	0:S	3.395	0.01-0.001
	S	7	124.41	19.3	C:S	5.281	< 0.001
6. Trypsin	O	8	131.20	15.9	0:C	2.322	0.05-0.02
	C	8	186.47	17.71	0:S	2.608	0.05-0.02
	S	7	80.32	10.14	C:S	4.998	< 0.001

O = operated
 C = operated control
 S = starved

gut lumen = foregut + midgut lumen contents + hindgut

activity 1 - 4 μ moles p nitrophenol/gut lumen/min $\times 10^2$

activity 5 μ g glucose/gut lumen/min

activity 6 μ moles p nitroaniline/gut lumen/min $\times 10^2$

SE indicates the standard error of the mean

t indicates the values obtained from applying student's t-test

P indicates values of probability

Table 5.14

The effect of the removal of the frontal ganglion and starvation on enzyme activity in the gut lumen

activity is expressed in terms of product formed/mg protein/min

Enzyme	Treatment	Number of determinations	Mean	SE		t	P
1. α D glucosidase	O	7	78.70	6.61	O:C	0.681	0.6-0.5
	C	8	85.89	8.0	O:S	0.858	0.5-0.4
	S	7	86.63	6.46	C:S	0.071	> 0.9
2. β D glucosidase	O	7	60.72	3.79	O:C	0.499	0.7-0.6
	C	7	63.36	3.70	O:S	1.542	0.2-0.1
	S	7	72.89	6.92	C:S	1.214	0.3-0.2
3. α D galactosidase	O	7	6.48	0.41	O:C	1.71	0.2-0.1
	C	7	7.84	0.68	O:S	1.431	0.2-0.1
	S	7	7.49	0.58	C:S	0.390	0.8-0.7
4. β D galactosidase	O	6	22.41	2.12	O:C	2.159	0.1-0.05
	C	7	26.98	0.76	O:S	1.711	0.2-0.1
	S	7	30.45	3.93	C:S	0.867	0.5-0.4
5. Trehalase	O	8	50.59	2.9	O:C	1.210	0.3-0.2
	C	8	45.49	3.06	O:S	0.125	> 0.9
	S	7	51.19	3.94	C:S	1.159	0.3-0.2
6. Trypsin	O	8	28.9	2.28	O:C	1.027	0.4-0.3
	C	8	32.34	2.45	O:S	0.228	0.9-0.8
	S	7	29.90	3.91	C:S	0.543	0.6-0.5

O = operated

C = operated control

S = starved

gut lumen = foregut + midgut lumen contents + hindgut

activity 1-4 μ moles p nitrophenol/mg protein/min $\times 10^2$

activity 5 μ g glucose/mg protein/min

activity 6 μ moles p nitroaniline/mg protein/min $\times 10^2$

SE indicates the standard error of the mean

t indicates the values obtained from applying student's t-test

P indicates values of probability

Table 5.15

The effect of the removal of the frontal ganglion and starvation on the distribution of enzyme activity in the gut lumen

the activity in each region of the gut lumen as a percentage of the activity in the whole gut lumen expressed in terms of product formed/gut lumen/min

(see Table 5.13)

Regions of the gut lumen

Treatment	Foregut			Midgut lumen contents			Hindgut			Enzyme
	Mean	SE	n	Mean	SE	n	Mean	SE	n	
O	31.86	2.08	7	65.57	2.17	7	2.57	0.61	7	α D glucosidase
C	55.31	2.42	8	40.09	2.18	8	4.60	1.73	8	
S	38.7	3.9	7	54.43	3.18	7	6.87	2.25	7	
O	30.29	2.37	7	63.42	2.22	7	6.29	1.15	7	β D glucosidase
C	54.71	2.21	7	38.29	2.25	7	7.00	0.87	7	
S	37.57	4.59	7	51.14	2.35	7	11.29	2.88	7	
O	30.00	4.64	7	67.57	5.11	7	2.43	0.95	7	α D galactosidase
C	63.14	3.31	7	33.86	3.13	7	3.00	0.79	7	
S	36.28	5.91	7	55.86	7.50	7	7.86	2.79	7	
O	31.5	5.58	6	61.67	5.41	6	6.83	0.83	6	β D galactosidase
C	57.97	4.41	7	36.86	4.00	7	6.07	0.56	7	
S	36.14	4.71	7	53.00	2.89	7	10.86	2.79	7	
O	35.5	1.60	8	60.12	1.97	8	4.38	0.78	8	Trehalase
C	55.5	2.01	8	40.25	1.86	8	4.25	0.53	8	
S	41.29	3.76	7	52.86	2.06	7	5.87	2.79	7	
O	16.45	2.07	8	83.5	2.09	8	0.05	0.038	8	Trypsin
C	44.77	3.86	8	54.5	3.93	8	0.73	0.3	8	
S	30.94	2.87	7	67.14	1.93	7	1.92	1.38	7	

Table 5.16

Statistical significance of the differences between the distribution of enzyme activities in the gut lumens of the three treatments

(see Table 5.15)

Enzyme	Foregut			Midgut lumen contents		Hindgut	
		t	P	t	P	t	P
α D glucosidase	O:C	7.591	< 0.001	8.239	< 0.001	1.045	0.4-0.3
	O:S	1.542	0.2-0.1	2.892	0.02-0.01	1.842	0.1-0.005
	C:S	3.800	0.01-0.001	3.798	0.01-0.001	0.811	0.5-0.4
β D glucosidase	O:C	7.541	< 0.001	7.941	< 0.001	0.491	0.7-0.6
	O:S	1.409	0.2-0.1	3.792	0.01-0.001	1.614	0.2-0.1
	C:S	3.363	0.01-0.001	3.943	0.01-0.001	1.427	0.2-0.1
α D galactosi- dase	O:C	5.812	< 0.001	5.629	< 0.001	0.463	0.7-0.6
	O:S	0.835	0.5-0.4	1.290	0.3-0.2	1.839	0.1-0.05
	C:S	3.965	0.01-0.001	2.705	0.02-0.01	1.673	0.2-0.1
β D galactosi- dase	O:C	3.641	0.01-0.001	3.759	0.01-0.001	0.778	0.5-0.4
	O:S	0.640	0.6-0.5	1.474	0.2-0.1	1.289	0.3-0.2
	C:S	3.244	0.01-0.001	3.271	0.01-0.001	1.684	0.2-0.1
Trehalase	O:C	7.780	< 0.001	7.336	< 0.001	0.138	0.9-0.8
	O:S	1.484	0.2-0.1	2.541	0.05-0.02	0.547	0.6-0.5
	C:S	3.457	0.01-0.001	4.551	< 0.001	0.610	0.6-0.5
Trypsin	O:C	6.464	< 0.001	6.521	< 0.001	2.245	0.05-0.02
	O:S	4.169	0.01-0.001	5.700	< 0.001	1.460	0.2-0.1
	C:S	2.800	0.02-0.01	2.756	0.02-0.01	0.902	0.4-0.3

Table 5.17

The effect of the removal of the frontal ganglion and starvation on the distribution of enzyme activity in the gut lumen

the activity in each region of the gut lumen as a percentage of the activity in the whole gut lumen expressed in terms of product formed/mg protein/min

(see Table 5.14)
Regions of the gut

Treatment	Foregut			Midgut lumen contents			Hindgut			Enzyme
	Mean	SE	n	Mean	SE	n	Mean	SE	n	
O	32.42	1.66	7	62.72	1.92	7	4.86	1.16	7	α D glucosidase
C	45.3	2.42	8	43.38	2.85	8	11.33	3.35	8	
S	32.43	1.86	7	57.86	2.08	7	9.71	1.27	7	
O	28.43	1.91	7	57.71	2.44	7	13.86	2.77	7	β D glucosidase
C	43.29	2.45	7	39.57	2.20	7	17.14	2.13	7	
S	31.57	3.03	7	54.14	2.04	7	14.29	3.77	7	
O	33.86	1.74	7	60.42	2.39	7	5.72	2.17	7	α D galactosidase
C	54.86	2.91	7	38.14	3.15	7	7.00	1.23	7	
S	36.00	2.90	7	52.29	5.78	7	11.71	4.96	7	
O	27.5	3.6	6	58.17	3.04	6	14.33	2.89	6	β D galactosidase
C	46	2.88	7	38.29	2.81	7	15.71	2.08	7	
S	30.29	3.89	7	55.57	3.05	7	14.14	3.15	7	
O	34.25	1.05	8	55.50	1.92	8	10.25	2.04	8	Trehalase
C	45.12	2.17	8	42.88	2.01	8	12.00	1.15	8	
S	36.10	2.54	7	56.86	1.93	7	7.04	2.55	7	
O	16.76	1.81	8	83.13	1.85	8	0.11	0.09	8	Trypsin
C	40.12	4.1	8	58.03	4.44	8	1.85	0.72	8	
S	29.7	8.73	7	67.73	8.66	7	2.57	1.15	7	

Table 5.18

Statistical significance of the differences between the distribution of enzyme activities in the gut lumens of the three treatments

(see Table 5.17)

Enzyme		Foregut		Midgut lumen contents		Hindgut	
		t	P	t	P	t	P
α D glucosidase	O:C	4.253	< 0.001	5.449	< 0.001	1.719	0.2-0.1
	O:S	0.004	> 0.9	1.718	0.2-0.1	2.820	0.02-0.01
	C:S	4.113	0.01-0.001	3.998	0.01-0.001	0.427	0.7-0.6
β D glucosidase	O:C	4.787	< 0.001	5.520	< 0.001	0.937	0.4-0.3
	O:S	0.876	0.4-0.3	1.123	0.3-0.2	0.092	> 0.9
	C:S	3.009	0.02-0.01	4.851	< 0.001	0.659	0.6-0.5
α D galactosidase	O:C	6.200	< 0.001	5.633	< 0.001	0.513	0.7-0.6
	O:S	0.632	0.6-0.5	1.301	0.3-0.2	1.107	0.3-0.2
	C:S	4.591	< 0.001	2.151	0.1-0.5	0.922	0.4-0.3
β D galactosidase	O:C	4.059	0.01-0.001	4.806	< 0.001	0.395	0.8-0.7
	O:S	0.519	0.7-0.6	0.600	0.6-0.5	0.044	> 0.9
	C:S	3.246	0.01-0.001	4.171	0.01-0.001	0.324	0.8-0.7
Trehalase	O:C	4.517	< 0.001	4.542	< 0.001	0.746	0.5-0.4
	O:S	0.707	0.5-0.4	0.498	0.7-0.6	0.992	0.4-0.3
	C:S	2.719	0.02-0.01	4.975	< 0.001	1.852	0.1-0.05
Trypsin	O:C	5.239	< 0.001	5.220	< 0.001	2.226	0.05-0.02
	O:S	1.550	0.2-0.1	1.856	0.1-0.05	2.162	0.05-0.02
	C:S	1.130	0.3-0.2	1.036	0.4-0.3	0.545	0.6-0.5

Table 5.19

The effect of the removal of the frontal ganglion and starvation on the relative activities of five carbohydrate enzymes in the gut lumen

activity is expressed in terms of μ moles product/gut lumen/min

Treatment	Enzyme	Mean gut lumen activity	SE	% of the total luminal carbohydrate activity
Operated	α D glucosidase	316.89	15.5	40.5
	β D glucosidase	242.00	26.45	30.9
	β D galactosidase	82.96	8.67	10.59
	Trehalase	114.7	10.79	14.65
	α D galactosidase	<u>26.15</u>	3.42	3.36
	TOTAL	<u>782.7</u>		
Operated Control	α D glucosidase	491.29	29.47	42
	β D glucosidase	339.47	30.84	29
	β D galactosidase	147.98	9.86	12.6
	Trehalase	144.93	4.07	12.4
	α D galactosidase	<u>45.79</u>	4.34	4.0
	TOTAL	<u>1169.46</u>		
Starved	α D glucosidase	206.12	31.02	38.7
	β D glucosidase	171.85	26	22.2
	β D galactosidase	69.60	11.92	13.1
	Trehalase	69.00	5.16	12.9
	α D galactosidase	<u>16.36</u>	2.78	3.1
	TOTAL	<u>532.93</u>		

Table 5.20

The effect of the removal of the frontal ganglion and starvation on the relative activities of five carbohydrate enzymes in the midgut tissue

activity is expressed in terms of μ moles product/midgut tissue/min

Treatment	Enzyme	Mean mid-gut tissue activity	SE	% of the total luminal carbohydrate activity
Operated	α D glucosidase	31.71	1.79	53
	β D glucosidase	9.67	1.92	16.06
	β D galactosidase	7.22	0.64	11.69
	Trehalase	10.96	0.86	18.2
	α D galactosidase	<u>0.63</u>		
	TOTAL	<u>60.2</u>		
Operated Control	α D glucosidase	45.34	2.54	51.39
	β D glucosidase	17.42	0.84	19.77
	β D galactosidase	10.90	0.81	12.35
	Trehalase	13.66	1.12	15.48
	α D galactosidase	<u>0.899</u>	0.09	1.01
	TOTAL	<u>88.219</u>		
Starved	α D glucosidase	27.43	1.33	50.78
	β D glucosidase	8.42	0.38	15.59
	β D galactosidase	5.66	0.36	10.48
	Trehalase	11.62	0.86	21.51
	α D galactosidase	<u>0.87</u>	0.16	1.64
	TOTAL	<u>54.01</u>		

Fig. 5.4

The effect of the removal of the frontal ganglion and starvation on the relative activities of five carbohydrate enzymes in the gut lumen and in the midgut tissue

(see Tables 5.19 and 5.20)

Legend:

ordinate ; the percentage of the total carbohydrate activity in the gut lumen or midgut tissue

abscissa ; carbohydrate enzyme

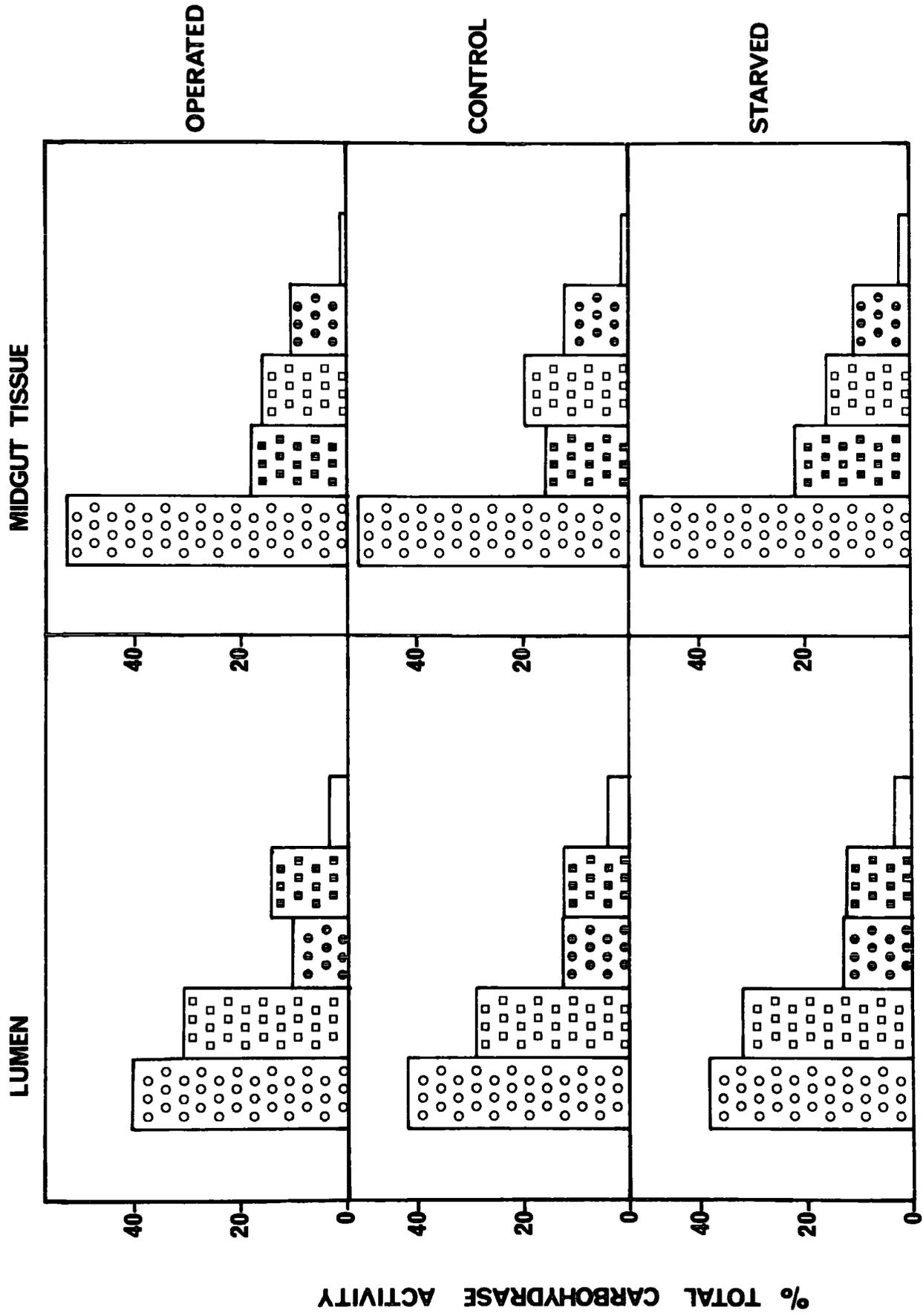
○ ○ α D glucosidase

□ □ β D glucosidase

α D galactosidase

● ● β D galactosidase

■ ■ trehalase



Discussion

It was seen in the results section that removal of the frontal ganglion from young adult Locusta dramatically reduced the activities of digestive enzymes in the lumen of the digestive tract, when the activity was expressed per gut lumen per minute (Table 5.13). As with other effects of the removal of the frontal ganglion from larvae (CLARKE AND GILLOTT, 1967a, b; CLARKE AND ANSTEE, 1971a), it tends to produce an effect which is intermediate between control and starved treatments.

DADD (1956) hypothesised a uniform "digestive juice" in Tenebrio molitor. He suggested that in this insect and in other insects whose normal diet always requires a full complement of enzymes, differential secretion would effect no improvement in digestive efficiency. This was supported by the present work where both starvation and frontal ganglion removal caused a significant drop in the activities of all the six enzymes assayed, from the level found in the operated control animal.

When the total activities in the gut lumen were re-expressed per mg protein per minute no differences were observed between the three treatments (Table 5.14). The soluble protein content of the gut, which made up the homogenate used as a source of the digestive enzymes came from a number of sources. If it were purely an extract of soluble protein from Locusta tissue then it may be expected that the ratio of the amount of enzyme protein (measured as enzyme activity) to the total amount of protein would remain constant. That is, although there was a reduction in enzyme activity per gut lumen per minute this would be matched by a similar reduction in total soluble protein, resulting in no difference in total activities per mg protein per min between the three treatments. However, soluble plant protein made up a considerable but variable contribution to the homogenate. Therefore

the fact that the ratio of enzyme activity to total soluble protein was constant between the three treatments suggests that there was a relationship between the amount of soluble plant protein and the level of enzyme activity. This would appear to lend support to the secretagogue theory of control of enzyme secretion (ENGELMANN, 1969; GOODING, 1974).

Neither starvation nor frontal ganglion removal has any effect on the relative proportions of the five carbohydrases in the lumen (Table 5.19, Fig. 5.4). A gradient of activity α D glucosidase > β D glucosidase > β D galactosidase = trehalase > α D galactosidase is found in all three treatments and is similar to that found in normal animals (DROSTE AND ZEBE, 1974).

Since the midgut and caecal tissues are the main site of synthesis, the distribution of digestive enzymes throughout the gut lumen will depend on movements of the gut to carry enzymes forward into the crop and back into the hindgut (EVANS AND PAYNE, 1964; DROSTE AND ZEBE, 1974). The bulk of digestion takes place in the foregut of Orthoptera (WIGGLESWORTH, 1972). And this was reflected in the distribution of digestive enzymes in the gut lumen of operated control animals, approximately 57% of the luminal carbohydrase activity when it is expressed/gut lumen/min was found in the foregut, 38% in the midgut and 5% in the hindgut. This is similar to the situation found in Schistocerca gregaria (EVANS AND PAYNE, 1964). However, in the operated locust the midgut had some 63% of the activity and only 32% was in the foregut, the same trend was shown in the starved animal with approximately 53% of the activity in the midgut and 37% in the foregut. A similar situation was found when the results were expressed/mg protein/min. It has been suggested that interference with the stomatogastric nervous system including removal of the frontal ganglion causes a decrease in the ability of the crop to pass food into the midgut

(ENGELMANN, 1968; ROOME, 1968; HIGHNAM ET AL, 1966, present work Chapter 2). If this is true then the drop in the proportion of enzyme activity found in the foregut contents could be due to an inability of the operated animal to regurgitate midgut contents forward into the crop. But it would be difficult to explain the situation in the starved animal on this basis, unless gut movements are reduced during starvation. Studies on such diverse insects as Phormia regina (KNIGHT, 1962) and Schistocerca gregaria (CLARKE AND GRENVILLE, 1960) have suggested that gut movements are myogenic. However, movement of food through the crop may be under nervous control.

An alternative explanation is that blockage of neurosecretory release caused by both starvation and frontal ganglion removal stops release from the corpora cardiaca of those humoral factors that have been shown to induce movement of the gut (CAMERON, 1953; ROOME, 1968). Hence it may be seen that it is impossible to get a clear picture of the effect of the removal of the frontal ganglion on enzyme activity, simply by reference to the midgut lumen and tissue in the way that CLARKE AND GILLOTT (1967a) attempted to.

It is reasonable to expect that the amount of activity in each of the three regions of the lumen (foregut, midgut and hindgut) should have a similar pattern to that of the total activity, namely that the operated control has more than the operated which in turn has more than the starved. However, the amount of enzyme activity/gut region/min in the midgut lumen of operated and control were similar and significantly greater than the starved. Yet in the foregut the operated control had significantly greater activity than both operated and starved treatments (Tables 5.1, 5.3, 5.5, 5.7, 5.9, 5.11 and Figs. 5.1, 5.2, 5.3). Once again the discrepancy might be explained if there was a restriction of movement of the midgut contents back into the foregut. This would cause an abnormal amount of activity in the midgut

of an operated animal in relation to the total activity present in the gut lumen. Treating the results in this way emphasises that the two regions, foregut and midgut cannot be discussed separately.

When the specific activities of the enzymes in the gut lumen were considered, that is the activity expressed per mg protein per minute, no significant difference was found between the three treatments. It was reasoned that this suggested a relationship between the amount of soluble protein in the gut and the enzyme activity. However, when the enzyme activities in the three regions of the gut lumen were expressed separately in this way a different picture was seen (see Tables 5.2, 5.4, 5.6, 5.8, 5.10, 5.12 and Figs. 5.1, 5.2, 5.3). The midgut of operated and starved animals had greater enzyme activities than the controls while the reverse was true in the foregut. This can be explained if the amount of enzymes secreted into the midgut is related to the plant protein/food material ingested into the gut rather than to the amount of this material in the midgut lumen. The normal distribution of the enzymes secreted into the midgut is prevented by the mal-function of the gizzard in the operated treatment and reduction of gut movement in the starved treatment.

The activities of the carbohydrase enzymes expressed per gut region per min in the midgut and midgut caecal tissues of operated and starved animals were significantly lower than the controls. This suggests that the synthesis of these enzymes is reduced in operated and starved treatments. However, this view is not supported by consideration of the specific activities of the enzymes. No consistent pattern is seen when the results are considered in this way. This may be due to the problem highlighted by DAHLMAN (1972). He suggested that the state of an enzyme's activity in the type of preparation used in this study may be masked by the change in concentration of other proteins or compounds which react with the Folin's reagent.

The relative proportions of the five carbohydrases in the midgut tissue are somewhat different to those found in the gut lumen (Tables 5.19, 5.20, Fig. 5.4). A gradient of activity α D glucosidase $>$ β D glucosidase $>$ Trehalase $>$ β D galactosidase $>$ α D galactosidase was found in the operated control. The effect of removal of the frontal ganglion and starvation was to increase the relative proportion of trehalase activity, such that trehalase and β D glucosidase reverse their positions on the above gradient of activity. The reason for this is not obvious but it might be that the production of this enzyme was relatively less affected by these treatments than the other carbohydrases.

The relationship between the enzyme activity in the gut lumen and in midgut tissue is difficult to interpret. DADD (1956) studied two insects Tenebrio molitor and Dytiscus marginalis and termed them a continuous feeder and an intermittent feeder respectively, using DAY AND POWNING (1949) nomenclature. He showed that enzyme secretion in Tenebrio was a continuous process initiated at a moult or emergence and accelerated after a feed resulting in high protease activity in the midgut lumen. In Dytiscus on the other hand the independence of discharge from synthesis, by allowing the intra epithelial accumulation of enzymes during starvation, results in secretion being markedly intermittent.

The present work suggests that neither starvation nor frontal ganglion removal result in an accumulation of digestive enzymes within the midgut tissue, as the percentage of the total (lumen + tissue) carbohydrase activity found in the midgut tissue of starved animals, 9%, is only slightly greater than that found in operated and operated control animals, 7%. This is at variance with DROSTE AND ZEBE (1974) who found a dramatic drop in the proportion of carbohydrase activity

in the midgut tissue as opposed to the lumen when starved adult Locusta were fed, 35% down to 2%. This is supported by histological evidence. HEINRICH AND ZEBE (1973) showed that the release of material from midgut cells followed refeeding after starvation. However, DROSTE AND ZEBE (1974) do not give the results of the experiments from which they derive the figures they quote, while DAY AND POWNING (1949) showed how difficult it is to explain histological evidence in terms of secretion. They convincingly showed that a similar pattern of midgut secretion in Blatella germanica following a period of starvation was due to cell breakdown rather than an indication of secretory activity.

KHAN (1964) found a rise in the activity of invertase enzyme in the tissue of the midgut of Locusta following the moult to adult. This happened whether the animals were fed or starved, during the first three days of adult life. If fed the increase in activity was maintained, if starved for longer than three days there was a slow decline. Several days after the start of feeding, the starved animals showed an increase in invertase activity. KHAN interpreted his results as indicating an endogenous rate of enzyme secretion that is hormonally controlled, upon which a secretagogue form of control is imposed. He supported this hypothesis with some preliminary experiments which suggested that ligation of newly moulted adults within the first 24 hours halved the normal increase in invertase activity normally seen in the first three days of life.

In the present work the enzyme activity in the whole gut lumen appeared to be related to the amount of ingested plant protein (Table 5.14). However, this relationship did not hold when the enzyme

activities of the different regions of the gut lumen were considered separately (Tables 5.2, 5.4, 5.6, 5.8, 5.10, 5.12). Since the midgut is the site of secretion of digestive enzymes, if a secretagogue mechanism were operative, then a relationship between activity and protein content in the midgut contents would be expected, resulting in no differences between the three treatments. This was not the case. Therefore, the amount of enzyme secreted by the midgut must be related to the total quantity of food ingested. Perhaps a nervous pathway is involved bringing information concerning the distention of the foregut to the brain and effecting release of a hormone controlling enzyme secretion. This is a similar mechanism to the one proposed by CLARKE AND LANGLEY (1963e) to explain the control of neurosecretory release in Locusta (see General Introduction, Chapter 1). However, this pathway and the secretion of the hormone would both have to remain untouched by the removal of the frontal ganglion.

In conclusion, the present work showed that removal of the frontal ganglion had a number of effects on the activities of digestive enzymes in the gut of adult Locusta.

1. It reduced the activities of the enzymes studied in the whole gut lumen in comparison with the control animals, but not by as much as in the starved animals. Also, both operated and starved animals had less activity in the midgut tissue than the control.
2. It did not have any effect on the specific activities of the enzymes in the whole gut lumen and it was reasoned that this showed a relationship between enzyme activity and the amount of ingested plant material.
3. From 1 and 2 above, therefore, it would seem that the reduction in enzyme activity exhibited by the operated animal parallels a reduced food intake (see Chapter 2).

4. The distribution of digestive enzyme activity in the three regions of the gut lumen point to a mal-function of the gizzard in operated animals.

It was suggested in Chapter 3 that a reduced efficiency of digestion was responsible for the low energy reserves in operated animals. No inordinate drop in enzyme activity was observed in the present Chapter to support this contention. However, a marked reduction in the proportion of the enzyme activity found in the foregut of operated animals may well reduce the efficiency of digestion which would otherwise be made possible by the enzyme activity available.

CHAPTER 6

The effect of the removal of the frontal ganglion on the ultrastructure of the fat body

Introduction

In chapter 3, the removal of the frontal ganglion was shown to have a dramatic effect on the levels of energy reserves in the fat body. It was suggested that this might be due to an inability of the fat body in operated animals, to make use of available metabolites. The present study, described in this chapter, was carried out to determine what structural evidence there was to support this suggestion.

The ultrastructure of fat body has been studied in a number of species of insect Bombyx mori (BISHOP, 1958), Philosamia cynthia (ISHIZAKI, 1965), Periplaneta americana (WALKER, 1965). Investigations on locust fat body have been carried out by ODHIAMBO (1967) on Schistocerca gregaria and CLARKE AND ANSTEE (1971b) on Locusta larvae.

Materials and Methods

The animals employed, the operation and treatment of experimental animals were as described in the general materials and methods section (chapter 1). All reagents used were Anala R grade or purest available and supplied by British Drug Houses or Sigma.

Electron Microscopy

Animals were killed by decapitation and the fat body dissected out under ice cold buffered glutaraldehyde (5% in 0.1M sodium cacodylate pH 7.3). The material was then placed in fresh fixative overnight. Thereafter, it was washed for 10 minutes in 0.1M sodium cacodylate buffer containing 0.2M sucrose (pH 7.3) prior to post osmication with 1% OsO₄ in 0.1M sodium cacodylate (pH 7.3) for 2 hours. A further wash was performed in 0.1M sodium cacodylate buffer (pH 7.3) for 30 minutes. The material was dehydrated by passage through an alcohol series; 10 minutes in each of 50%, 70%, 95% ethanol and two changes of 30 minutes in absolute ethanol. Throughout these treatments the material was kept at 0 - 4°C, thereafter all procedures were carried out at room temperature.

After two 10 minute rinses in propylene oxide, the material was left in a 50 : 50 mixture of Epon resin (see below) and propylene oxide overnight. Following infiltration in Epon resin for 8 hours, the material was embedded in fresh Epon and polymerisation was effected at 60°C for 48 hours.

Epon resin ; equal parts of	<u>A</u> Epon 812	(62 volumes)
	ODSA	(100 volumes)
	and <u>B</u> Epon 812	(100 volumes)
	MNA	(89 volumes)

Silver/silver-gold sections were cut on a Reichert NK ultratome, expanded with diethyl ether vapour and mounted on uncoated copper grids.

Sections were stained with uranyl acetate and lead citrate (REYNOLDS, 1963) prior to their examination in an AEI EM 801 electron microscope.

Results

The results presented below are representative observations made during the course of two experiments involving 4 male locusts of each treatment. During the course of this investigation several hundred cell profiles were examined of each treatment.

Ultrastructure of fat body from newly moulted male locusts (12 hours \pm 12 hours after the last moult)

Cells of fat body from newly moulted animals possessed a cytoplasm which was characterised by the presence of numerous osmiophilic droplets (Plate 6.1A, Plate 6.2B). Similar inclusions in Schistocerca gregaria have been identified as lipid in nature (ODHIAMBO, 1967). The area of "free" cytoplasm was small in comparison with the area taken up by the lipid droplets. Each cell contained a nucleus that was surrounded by the characteristic double membrane (Plate 6.1A). In places the lipid droplets seemed to press against the nucleus giving it an irregular outline (Plate 6.1 A & B). Throughout the cytoplasm elements of the rough endoplasmic reticulum were seen although it was not well developed (Plate 6.2 A & B). There were many free ribosomes and polyribosomal clusters. Mitochondrial profiles were seen but few Golgi bodies. A few glycogen granules were found in particular around the lipid droplets (Plates 6.2A & B).

Ultrastructure of fat body from operated control male locusts

Fat body cells from operated controls were far larger than those from newly moulted animals and intercellular spaces were more highly developed (Plate 6.3 A & B). The presence of an extensive network of such spaces was noted in larval fat body by ANSTEE (1968). The suggestion was made that they were brought about by a lack of cohesion between cells due to the absence of desmosomes. Observations made in the present work agreed with this. No desmosomes were found in the fat body from any of the four treatments.

Although the cytoplasm was again characterised by many osmiophilic/lipid droplets, the "free" cytoplasm was more highly developed (Plate 6.3 A & B) than in newly moulted locusts. In the cytoplasm many more Golgi bodies were seen and rough endoplasmic reticulum was more highly developed and in places was arranged in parallel rows (Plate 6.4 A & B). Small vesicles were frequently found in association with the lateral plasma membrane (Plate 6.4 A). Similar vesicles (pinosomes) have been observed in the fat body of young Schistocerca gregaria adults. It has been suggested that they enlarged to form granular cytoplasmic bodies containing protein or lipid precursors (ODHIAMBO, 1967).

Glycogen granules were more frequently encountered in operated controls than in newly moulted animals. They were found scattered throughout the cytoplasm but congregated in particular around the lipid droplets (Plates 6.4B, 6.5A).

Numerous lysosomes were seen in the cytoplasm (Plate 6.4 A & B). Occasionally, groups of lysosomes were found together within a fat body cell (Plate 6.5 A & B). The cytoplasm of such cells gave the appearance of being highly disorganised with little discernable structure (Plate 6.5 B).

Ultrastructure of the fat body from frontal ganglion cauterised male locusts

The operated animals had very much less fat body than either the operated control or the newly moulted treatments. A reduction was also seen in the size of the cells which contained few osmiophilic droplets (Plate 6.6 A & B, Plate 6.7 A & B). As in operated control animals groups of lysosomes were found within some operated fatbody cells (Plate 6.7 B, Plate 6.8 B). Once again the cytoplasm of such cells was disorganised.

The appearance of the cells containing the lysosomes in both operated and operated control fat bodies suggests that the latter are involved in some form of autolysis of the cells concerned. However, the lysosomes do not correspond to any of the cytolysosome types described by DE DUVE AND WATTIAUX (1966).

The cytoplasm possessed little rough endoplasmic reticulum, little glycogen and few Golgi bodies (Plate 6.7A, Plate 6.8A). In places one or two mitochondria appeared swollen or enlarged (Plate 6.8 A & B) by comparison with mitochondria from operated control fat body cells (Plate 6.4 A & B). Occasionally rough endoplasmic reticulum showed signs of dilation (Plate 6.8 A & B). The latter may be an early indication of the osmotic disruption of organelles in several tissues including the fat body noted by CLARKE AND ANSTEE (1971b) in frontal ganglionectomised Locusta larvae. The nuclei were no different in appearance to those of operated control or newly moulted animals.

Ultrastructure of fat body from starved male locusts

In animals that had been starved the fat body was extremely reduced in amount and the cells were small in comparison with the other treatments. The tissue appeared as a mass of empty lacunae surrounded by strands of cytoplasmic material (Plate 6.9A). The intercellular spaces were very prominent. Cytoplasmic structure was very difficult to make out because of its dense appearance. Mitochondria were small and reduced in number (Plate 6.9B, Plate 6.10B). There was little rough endoplasmic reticulum, no glycogen and no osmiophilic droplets occurred (Plate 6.9B, Plate 6.10B). Although the nucleus had a normal appearance it was irregular in outline, as in the newly moulted locust, due to the lacunae that pressed in against it.

PLATE 6.1

A. Low power electron micrograph of fat body from a newly moulted male.

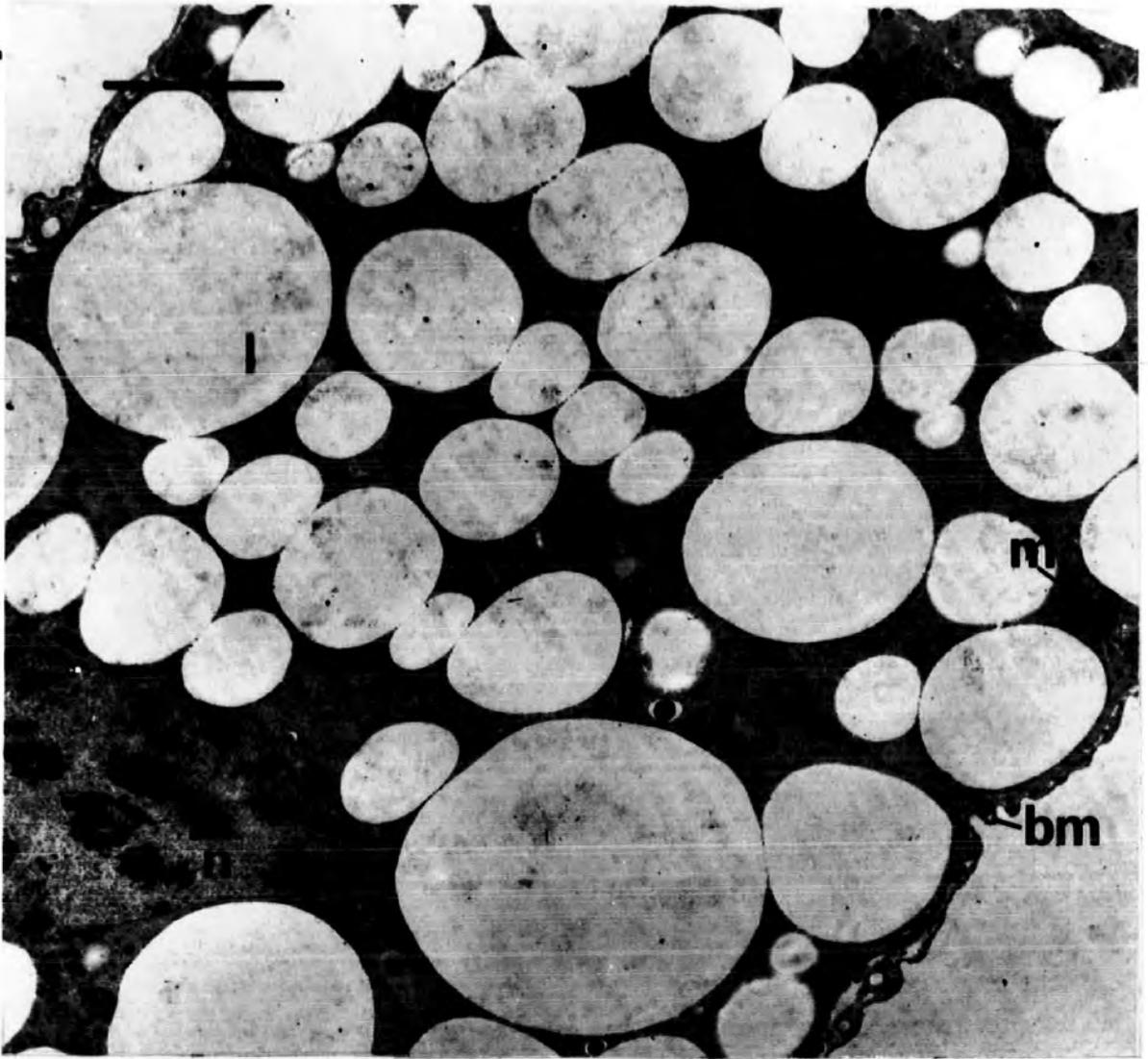
Note: basement membrane (bm), small amount of "free" cytoplasm (cy), numerous lipid droplets (l), mitochondrion (m), irregularly shaped nucleus, with a double membrane, containing densely staining blocks of chromatin material (n).

B. Low power electron micrograph of fat body from a newly moulted male.

Note: basement membrane (bm), lipid droplets (l), mitochondrion (m), nucleus (n), tracheole (t).

Scale: 5 μ m

A.



B.

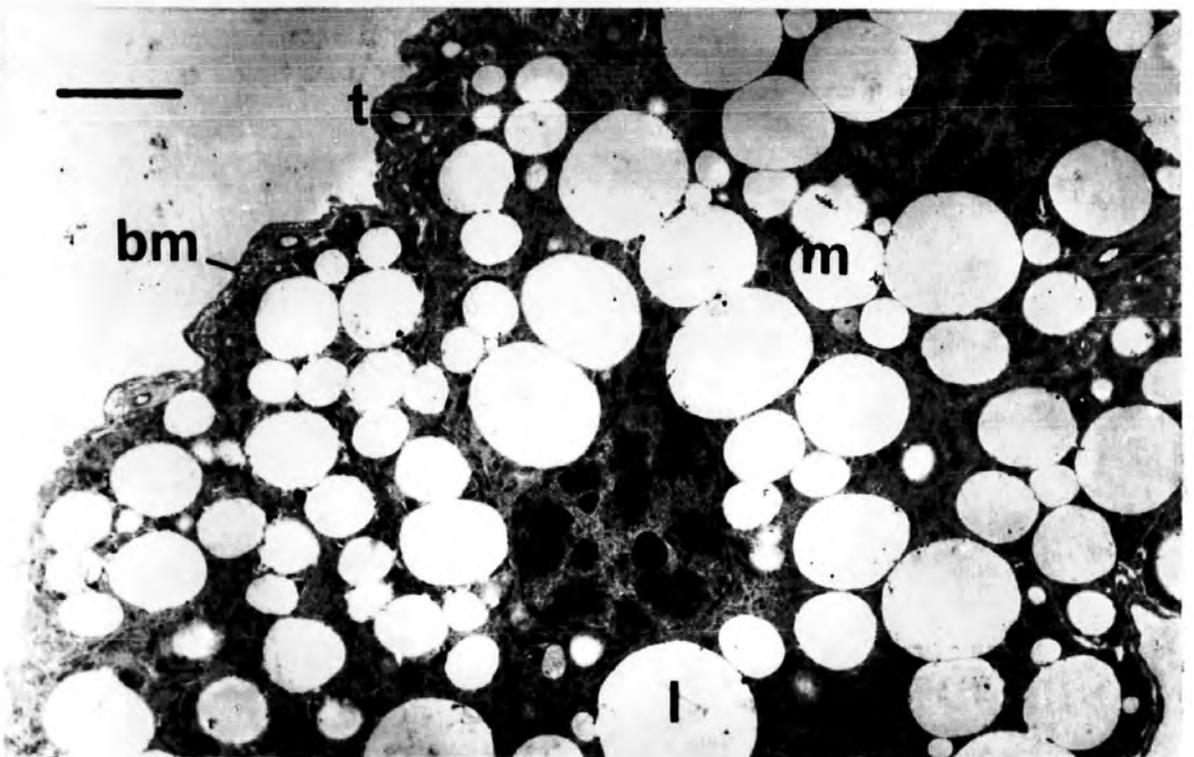


PLATE 6.2

A. Electron micrograph of fat body from a newly moulted male.

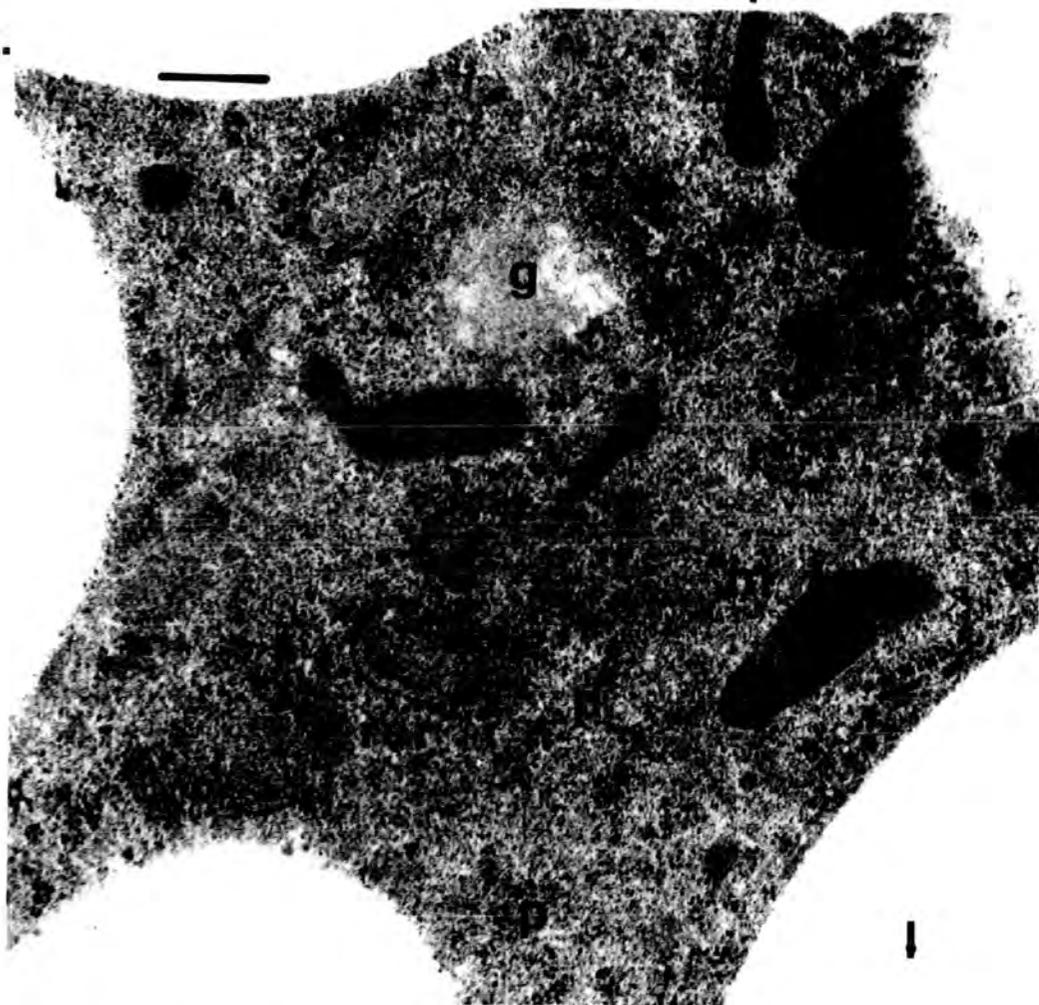
Note: Golgi body (g), glycogen granules (gl), lipid droplet (l), mitochondrion (m), polyribosomal clusters (p), many free ribosomes (r), rough endoplasmic reticulum (rer).

B. Electron micrograph of fat body from a newly moulted male.

Note: glycogen granules (gl), osmiophilic/lipid droplets (l), lysosome (ly), mitochondrion (m), many polyribosomal clusters (p), rough endoplasmic reticulum (rer).

Scale: 0.5 μ m

A.



B.

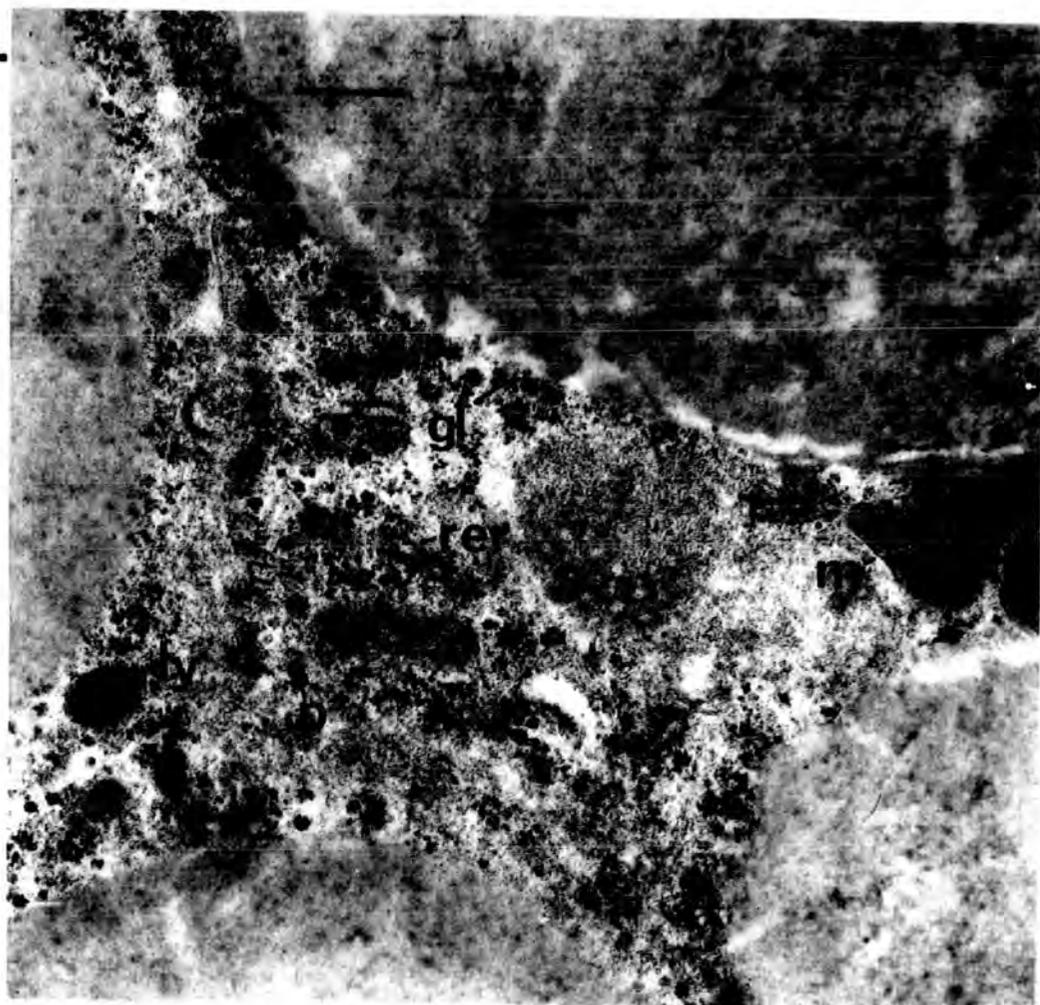


PLATE 6.3

A. Low power electron micrograph of fat body from an operated control male.

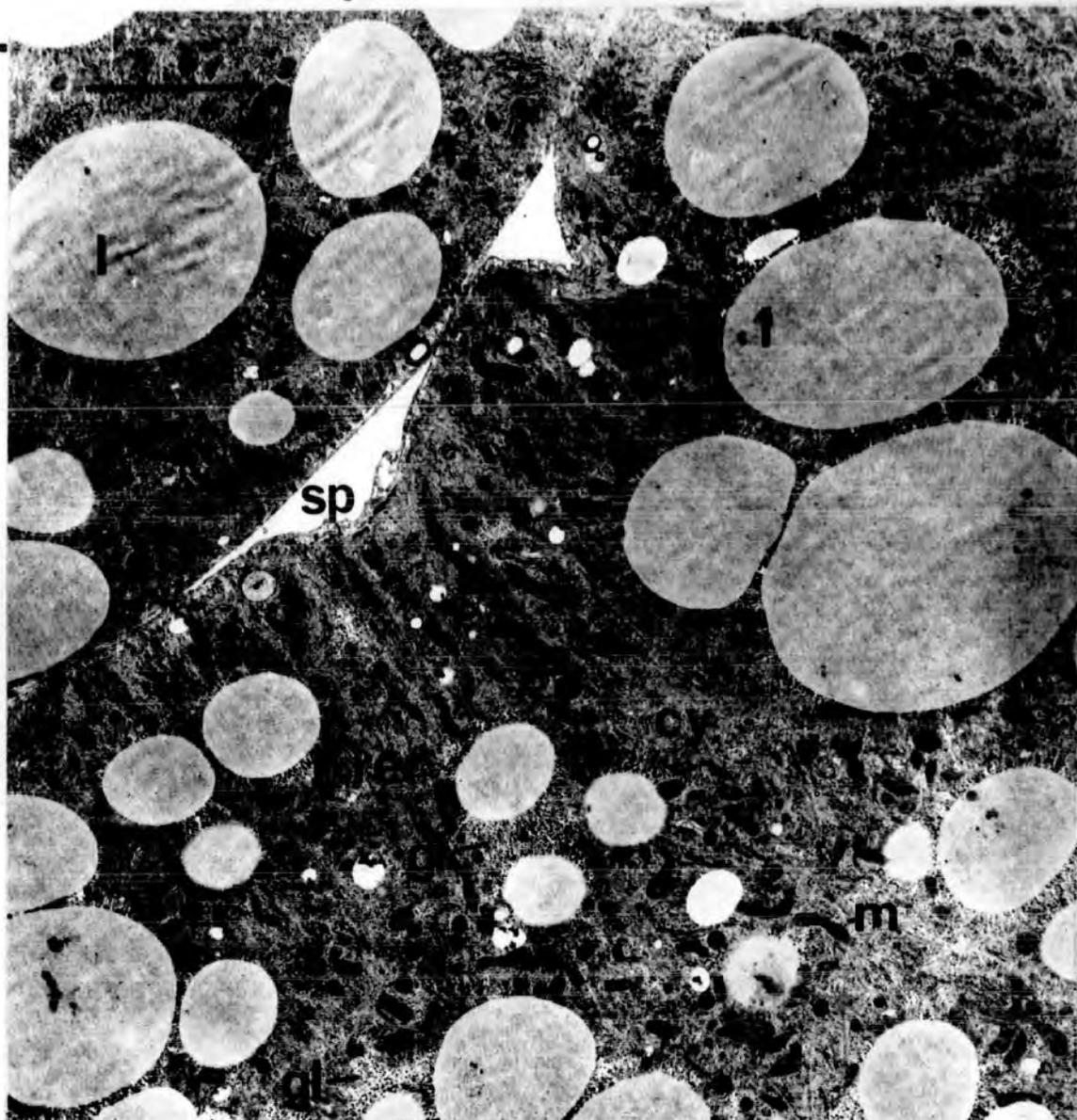
Note: extensive "free" cytoplasm (CY) i.e. that part of the cytoplasm other than the osmiophilic droplets, glycogen granules (gl) congregated in particular around the osmiophilic/lipid droplets (l), mitochondrion (m), rough endoplasmic reticulum (rer), intercellular space (sp).

B. Low power electron micrograph of fat body from an operated control male.

Note: the glycogen granules (gl) congregated in particular around the osmiophilic/lipid droplets (l), nucleus (n), intercellular space (sp).

Scale: 5 μ m

A.



B.

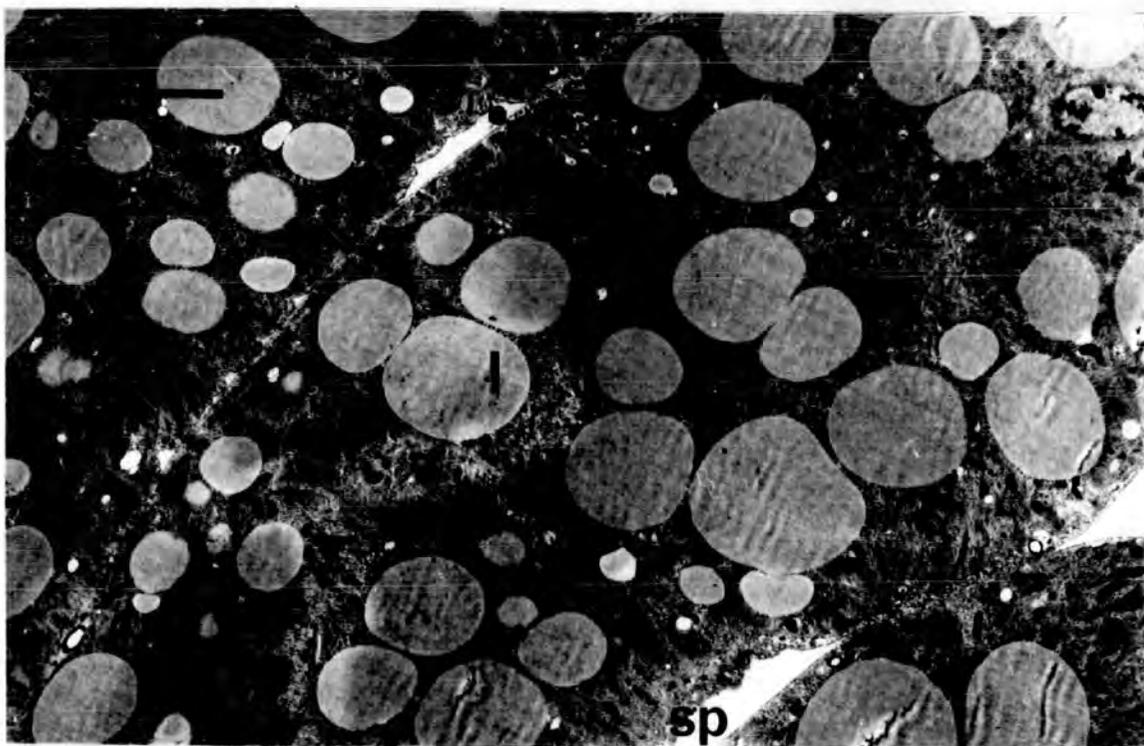


PLATE 6.4

A. Electron micrograph of fat body from an operated control male.

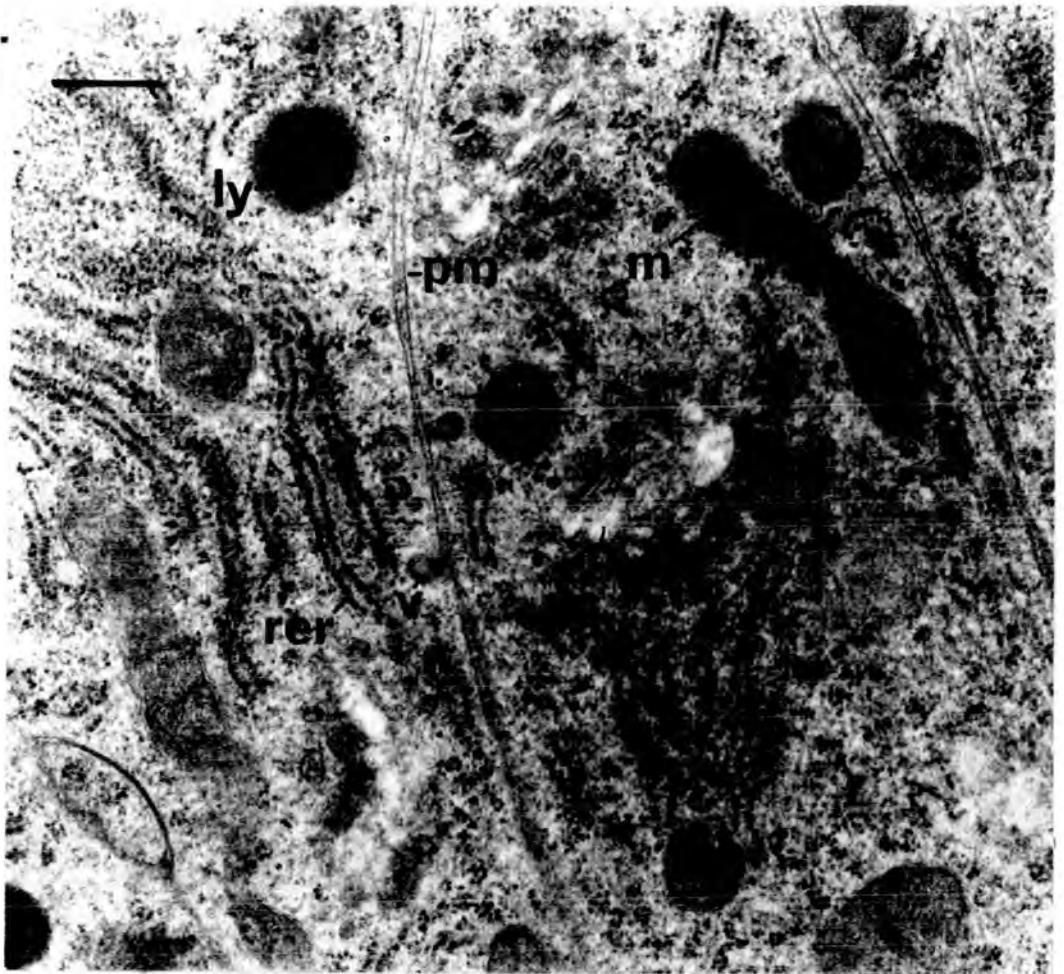
Note: Golgi body (g), lysosome (ly), mitochondrion (m), the small vesicles like pinosomes (v) on the lateral plasma membrane (pm), free ribosomes (r) parallel rows of rough endoplasmic reticulum (rer).

B. Electron micrograph of fat body from an operated control male.

Note: Golgi body (g), the glycogen granules (gl) in evidence in small clusters throughout the cytoplasm but in particular around the lipid droplets (l), lysosome (ly), mitochondrion (m), nucleus (n), rough endoplasmic reticulum (rer).

Scale: 0.5 μ m

A.



B.

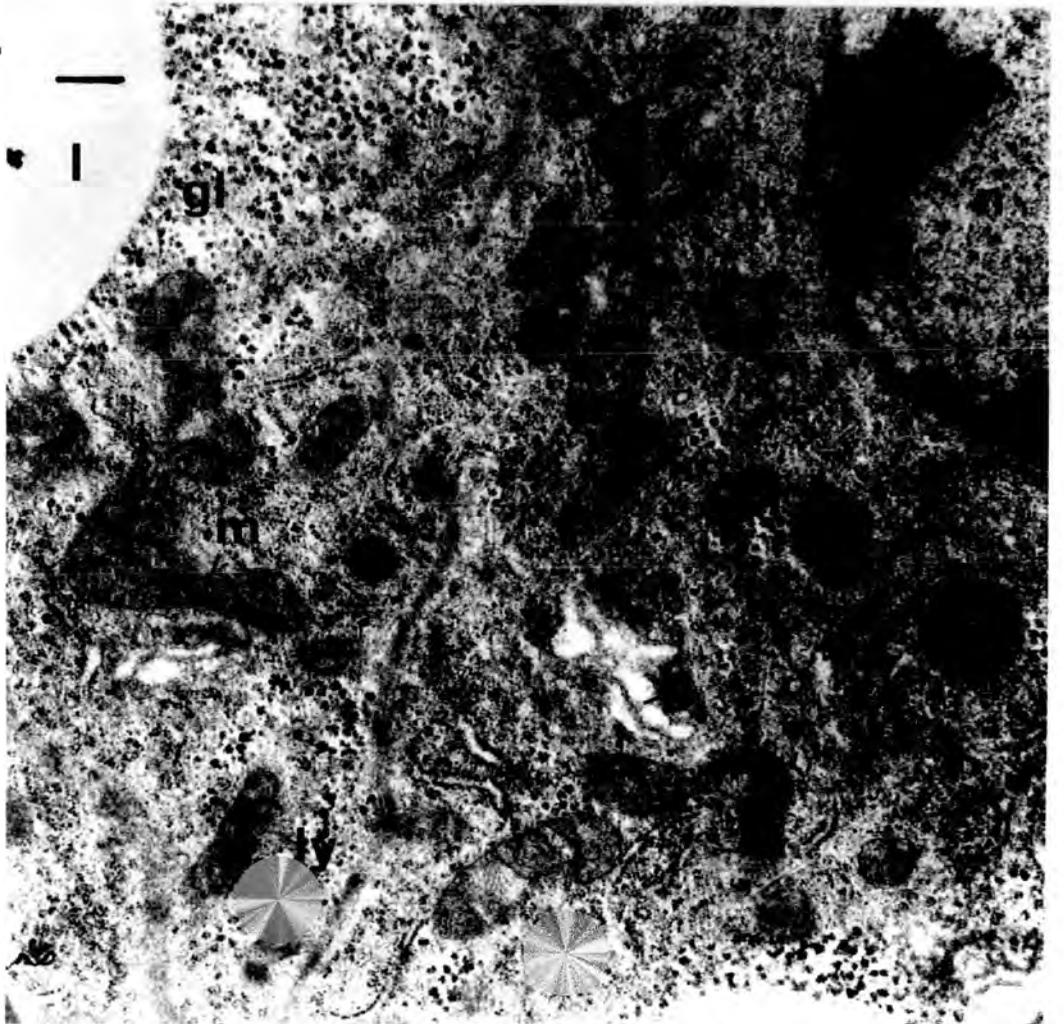


PLATE 6.5

A. Electron micrograph of fat body from an operated control male.

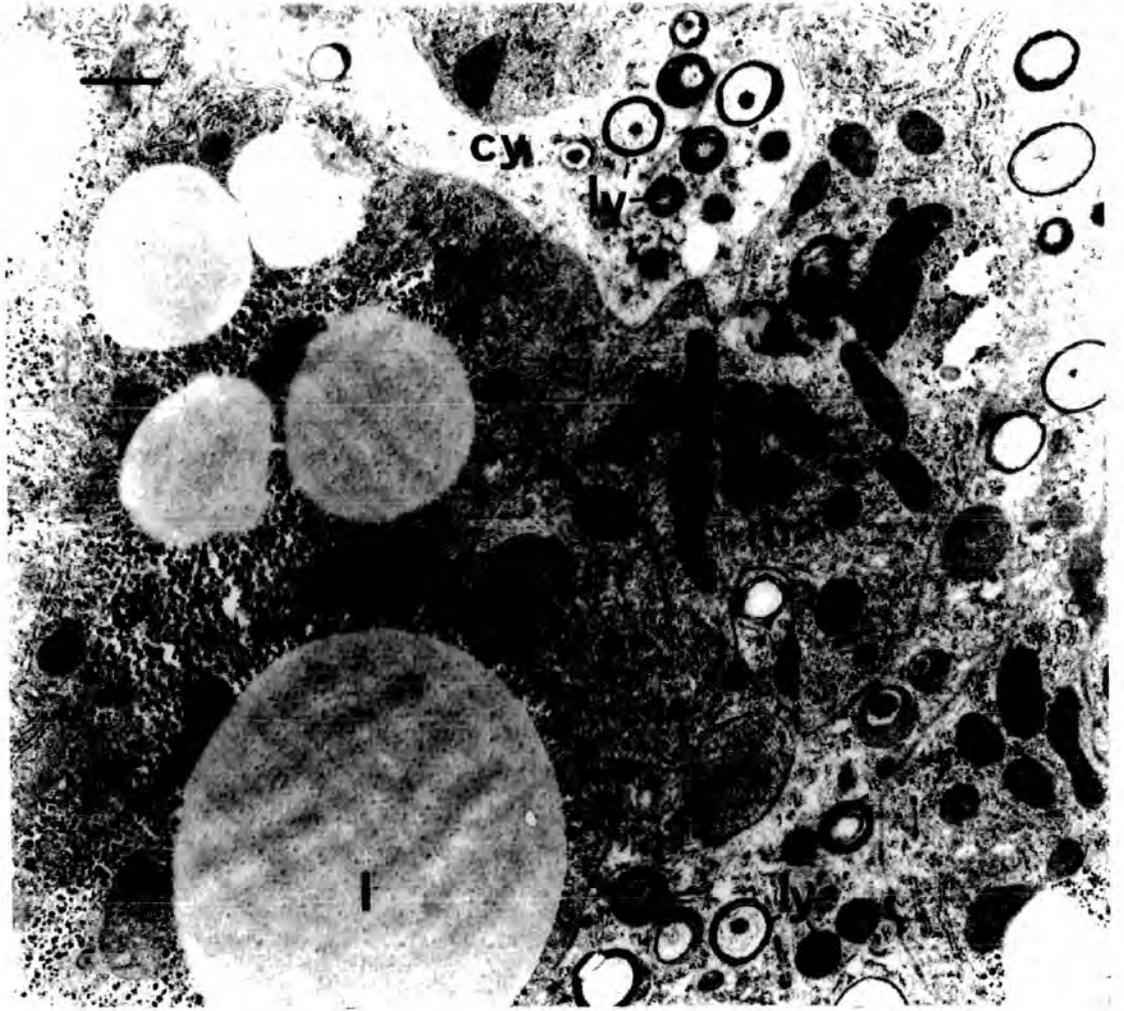
Note: glycogen granules (gl), groups of lysosomes (ly) and the disrupted nature of the cytoplasm of the cells they are in (cy), osmiophilic/lipid droplet (l), mitochondrion (m), rough endoplasmic reticulum (rer).

B. Electron micrograph of fat body from an operated control male.

Note: the lysosomes, which appear in a number of different forms (ly 1, 2, 3) and the disrupted nature of the cytoplasm of the cell they are in (cy), nucleus (n).

Scale: 1 μ m

A.



B.

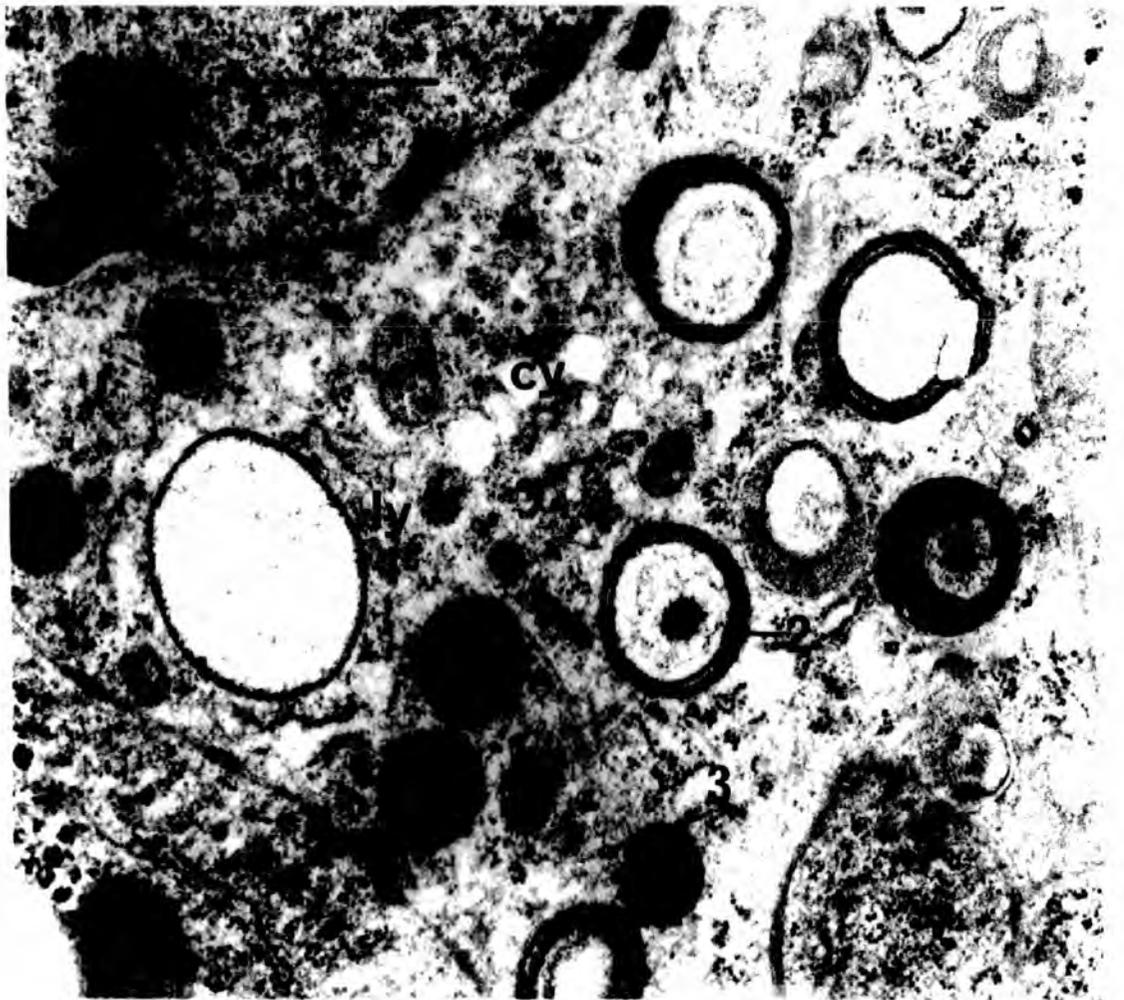


PLATE 6.6

A. Low power electron micrograph of fat body from an operated male.

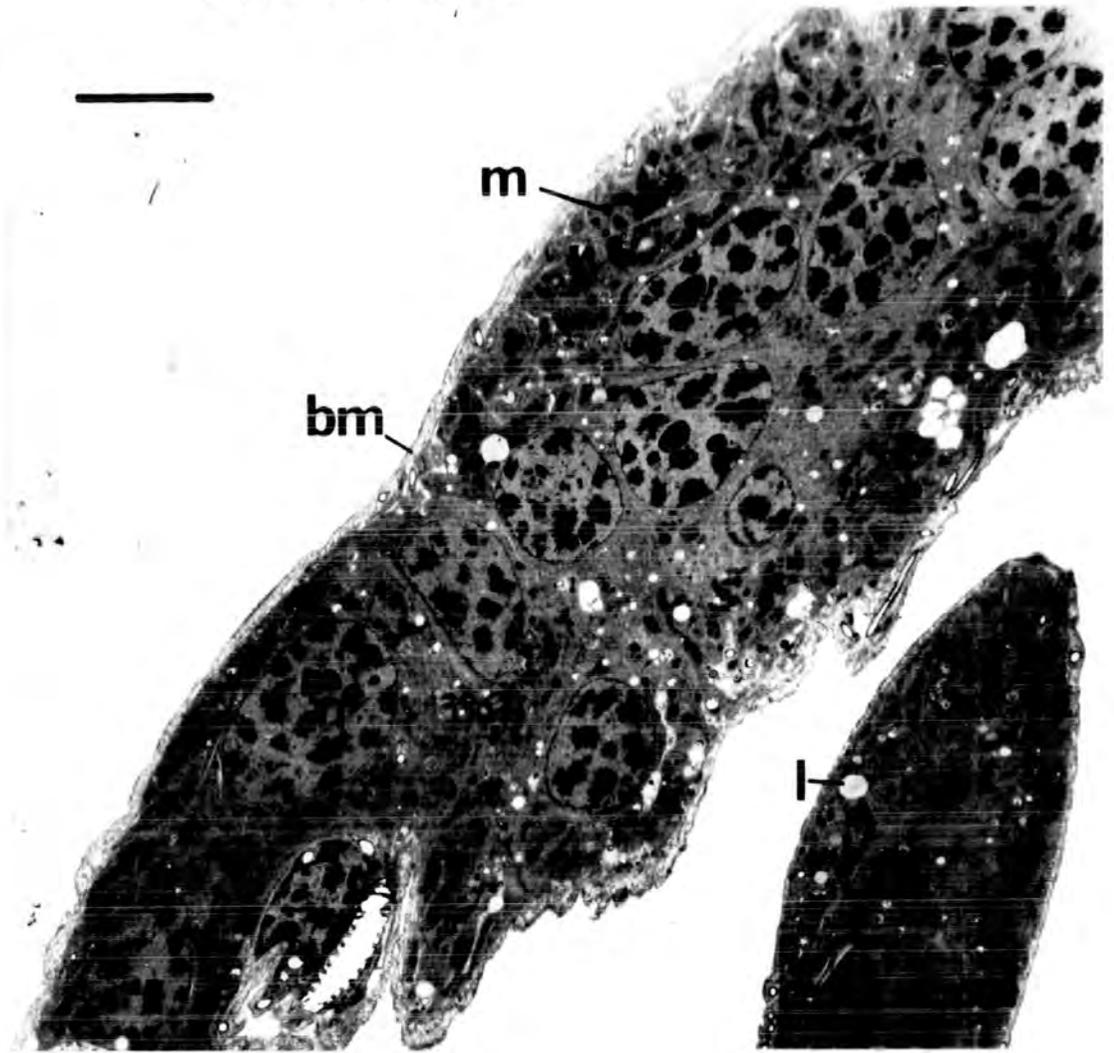
Note: basement membrane (bm), small size of the cells and the dramatic reduction in number of osmiophilic/lipid droplets (l), mitochondrion(m), nucleus (n).

B. Low power electron micrograph of fat body from an operated male.

Note: basement membrane (bm), small size of the cells and dramatic reduction in number of osmiophilic/lipid droplets (l), mitochondrion (m), nucleus (n), rough endoplasmic reticulum (rer), tracheole (t).

Scale: 10 μ m

A.



B.

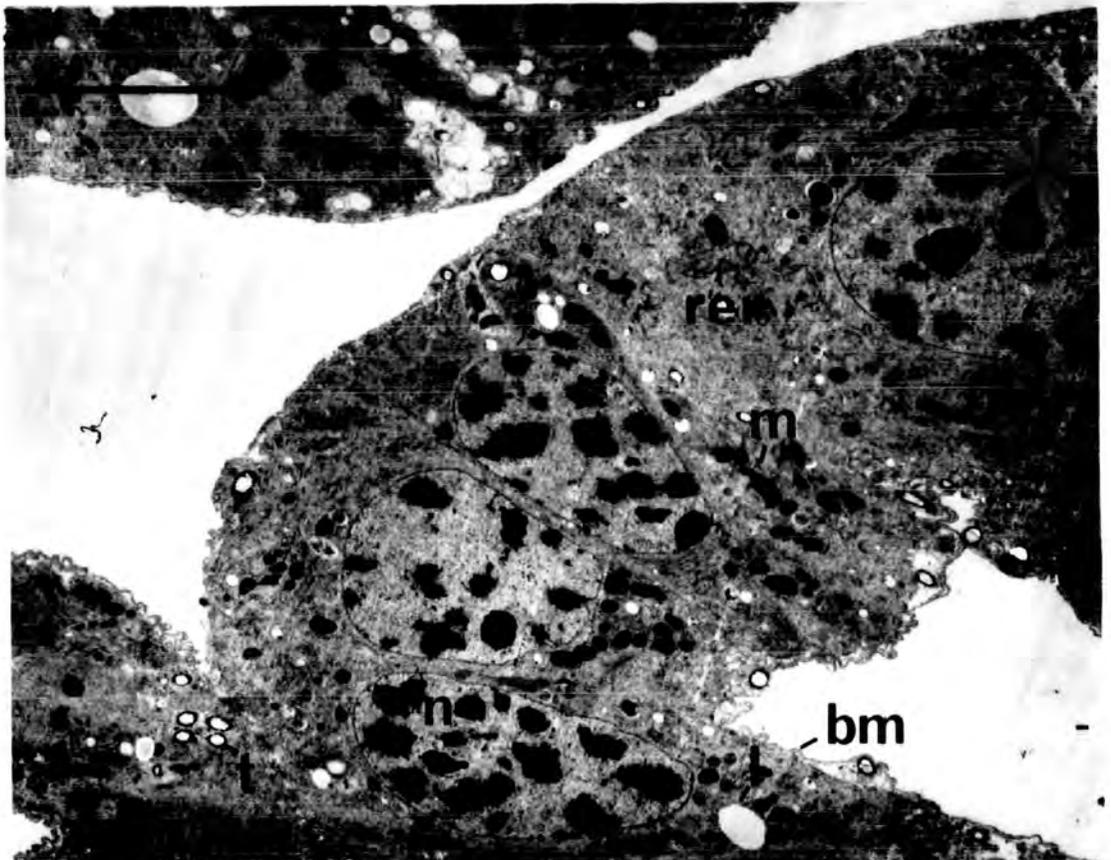


PLATE 6.7

A. Low power electron micrograph of fat body from an operated male.

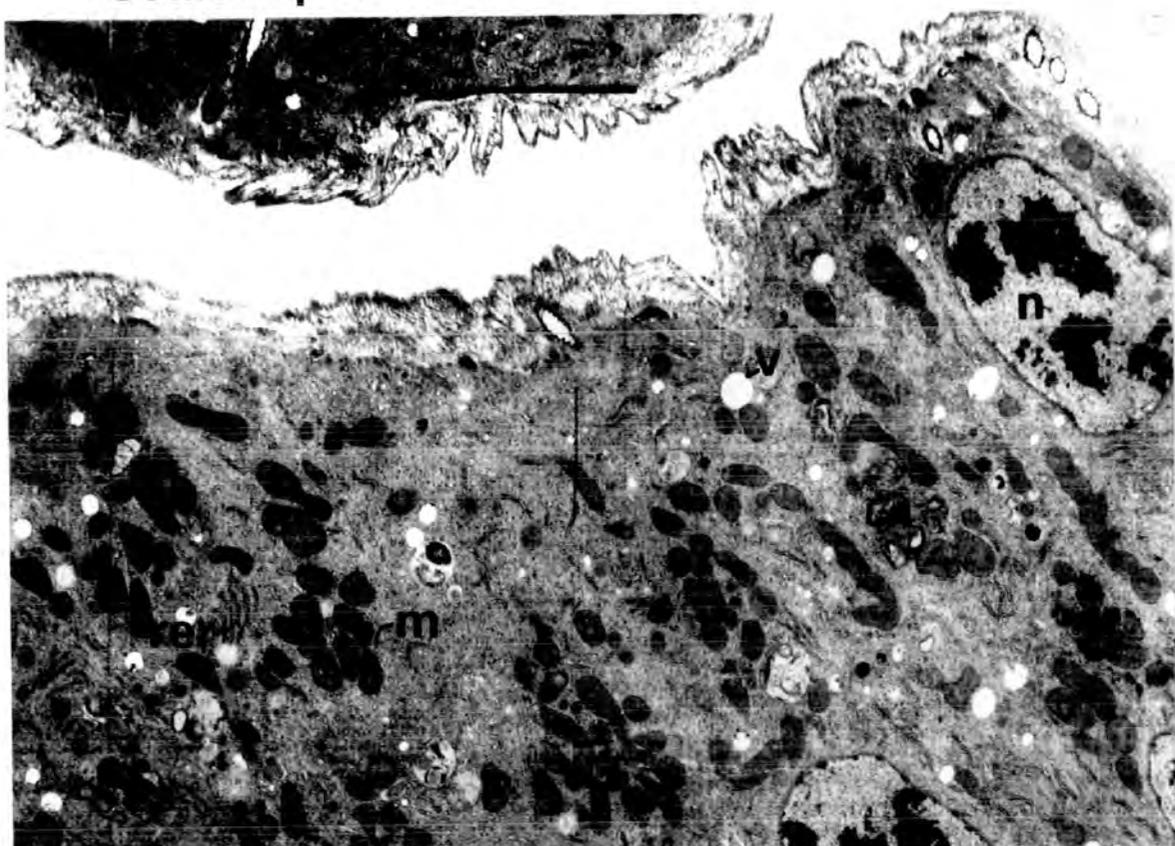
Note: large area of cytoplasm with no osmiophilic/lipid droplets but a few small vacuoles (v), enlarged mitochondria (m), nucleus (n), little rough endoplasmic reticulum (rer).

B. Low power electron micrograph of fat body from an operated male.

Note: in this region a small number of osmiophilic/lipid droplets are seen (l), groups of lysosomes (ly), nucleus (n), mitochondrion (m).

Scale: 5 μ m

A.



B.

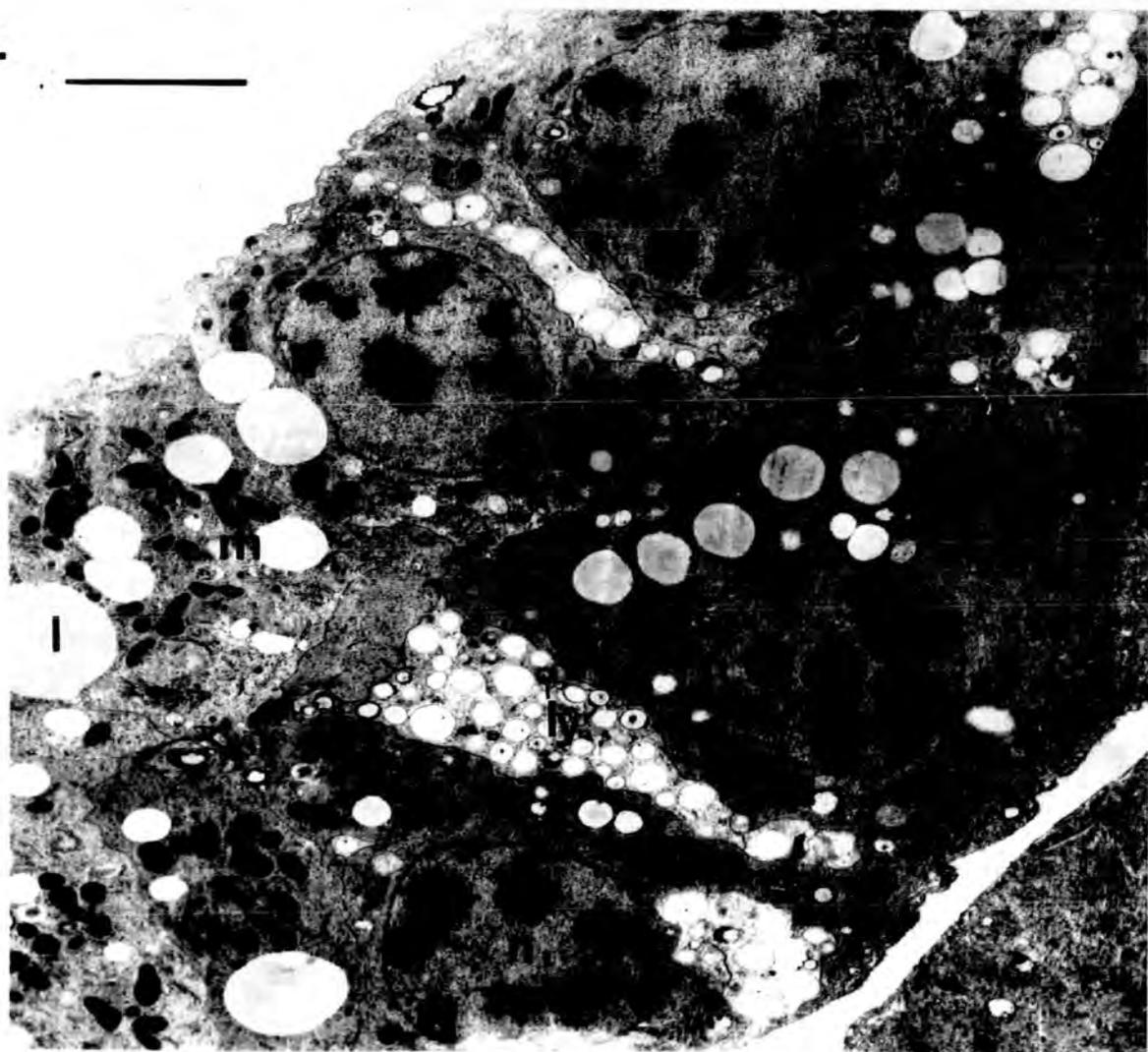


PLATE 6.8

A. Electron micrograph of fat body from an operated male.

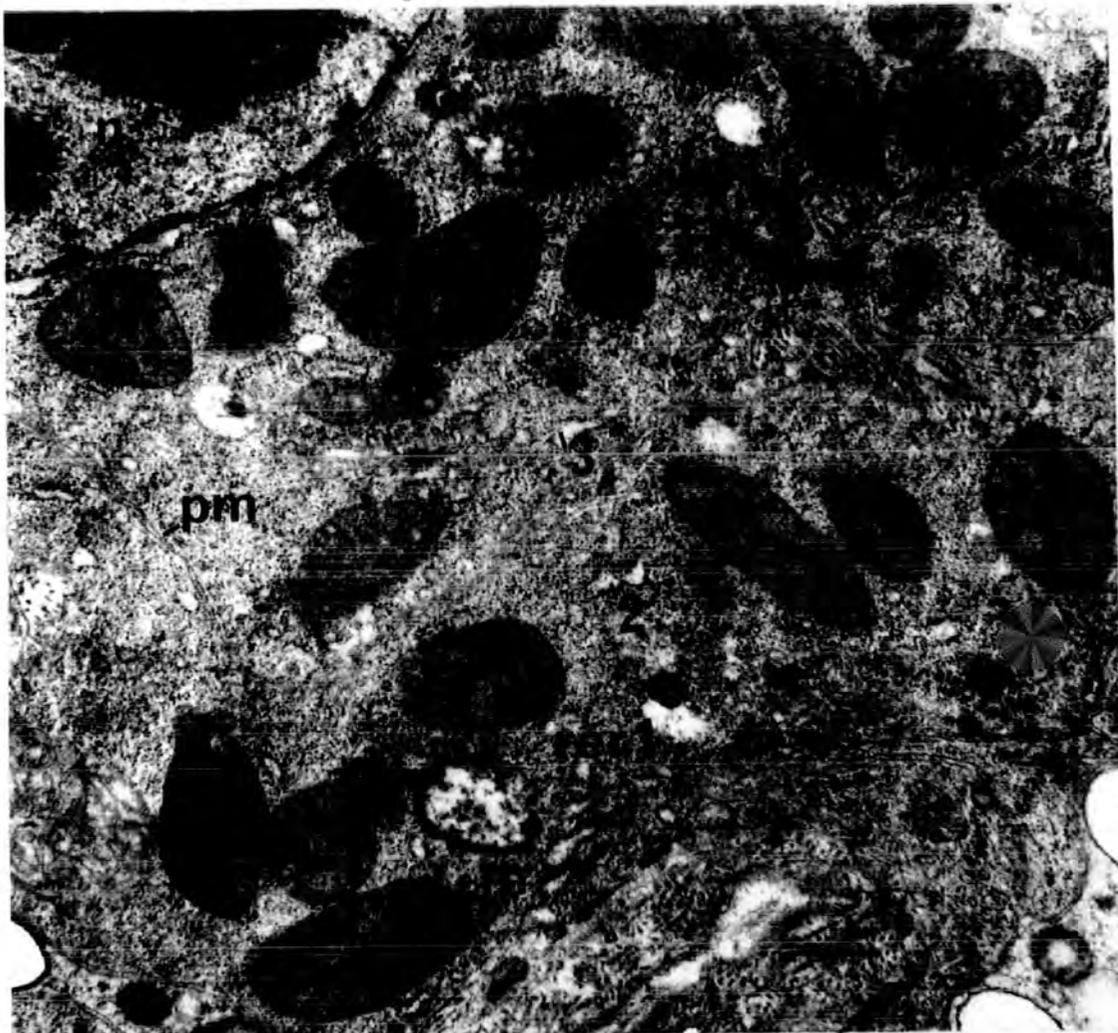
Note: mitochondria (m) which by comparison with those of operated control fat body cells are enlarged (cf. Plate 6.4 A & B), the upper mitochondria of the two labelled is burst, nucleus (n), plasma membrane (pm), indications of dilation of the rough endoplasmic reticulum (rer 1, 2, 3).

B. Electron micrograph of fat body from an operated male.

Note: lysosomes similar to those found in operated control fat body cells (cf Plate 6.5 A & B) (ly), enlarged mitochondria with cristae clearly visible (m), plasma membrane (pm), once again signs of dilation of rough endoplasmic reticulum (rer 1, 2, 3).

Scale: 0.5 μ m

A.



B.

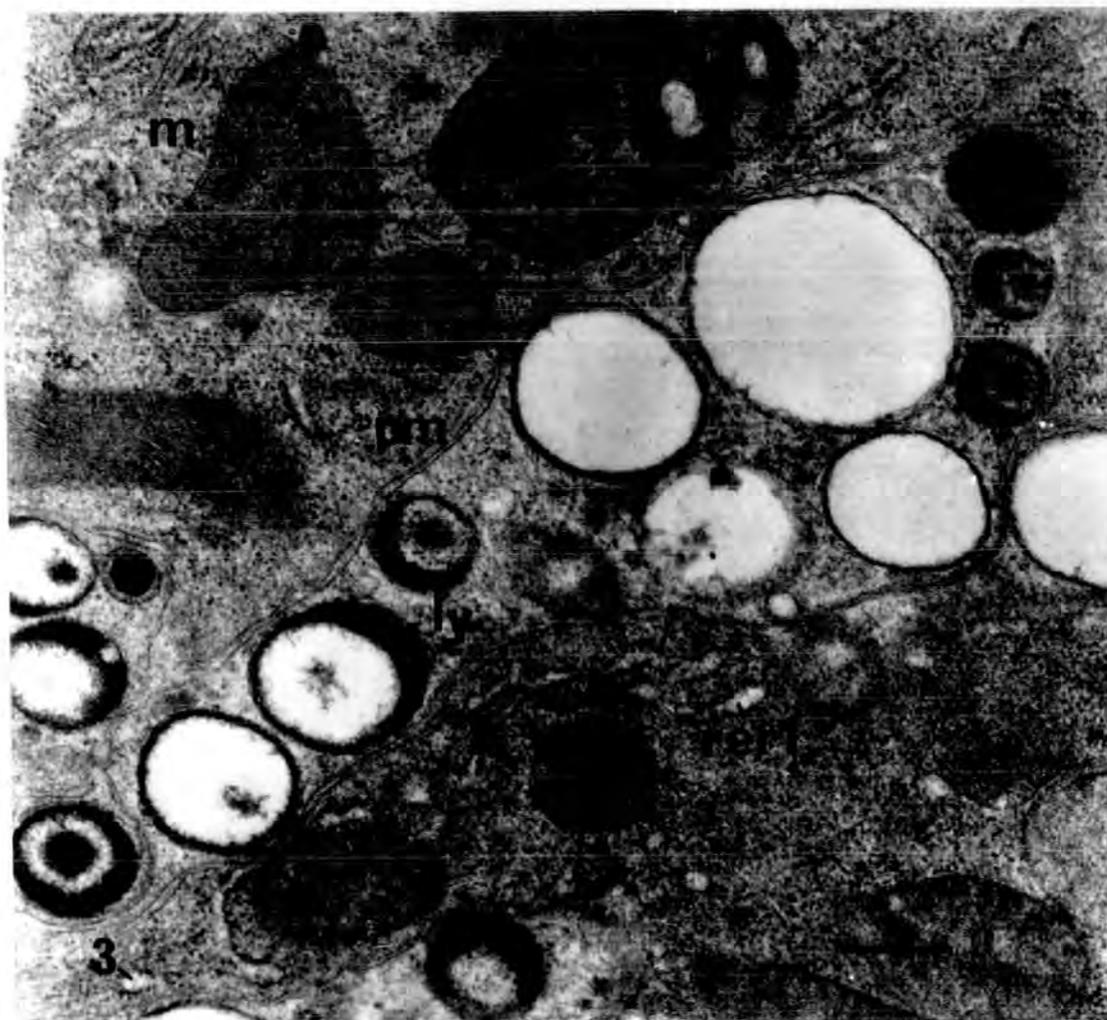


PLATE 6.9

A. Low power electron micrograph of fat body from a starved male.

Note: basement membrane (bm), dense, sparse cytoplasm (cy), empty lacunae (l), mitochondrion (m), nucleus (n), tracheole (t).

B. Low power electron micrograph of fat body from a starved male.

Note: dense cytoplasm (cy), empty lacunae (l), mitochondrion (m), extensive intercellular spaces (sp).

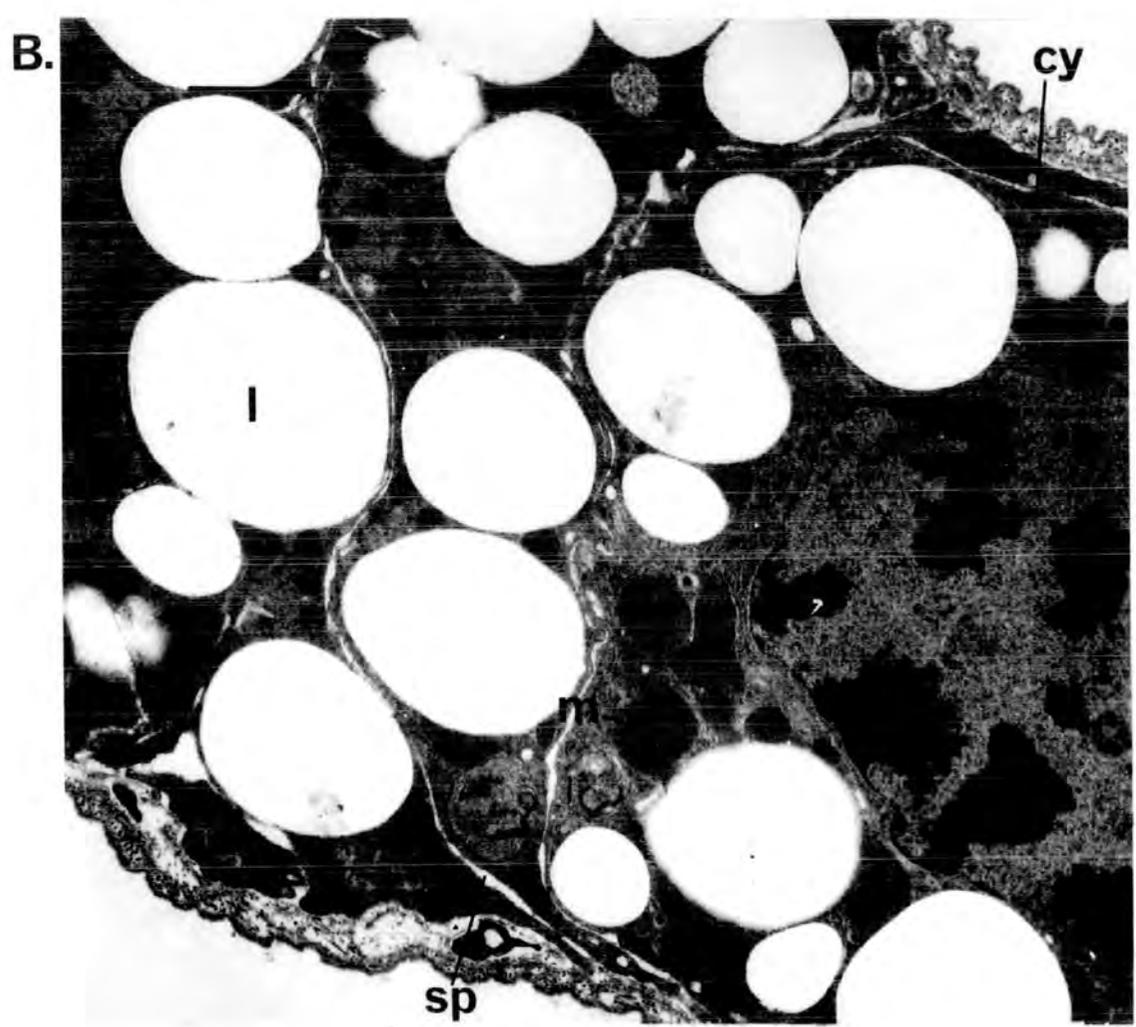
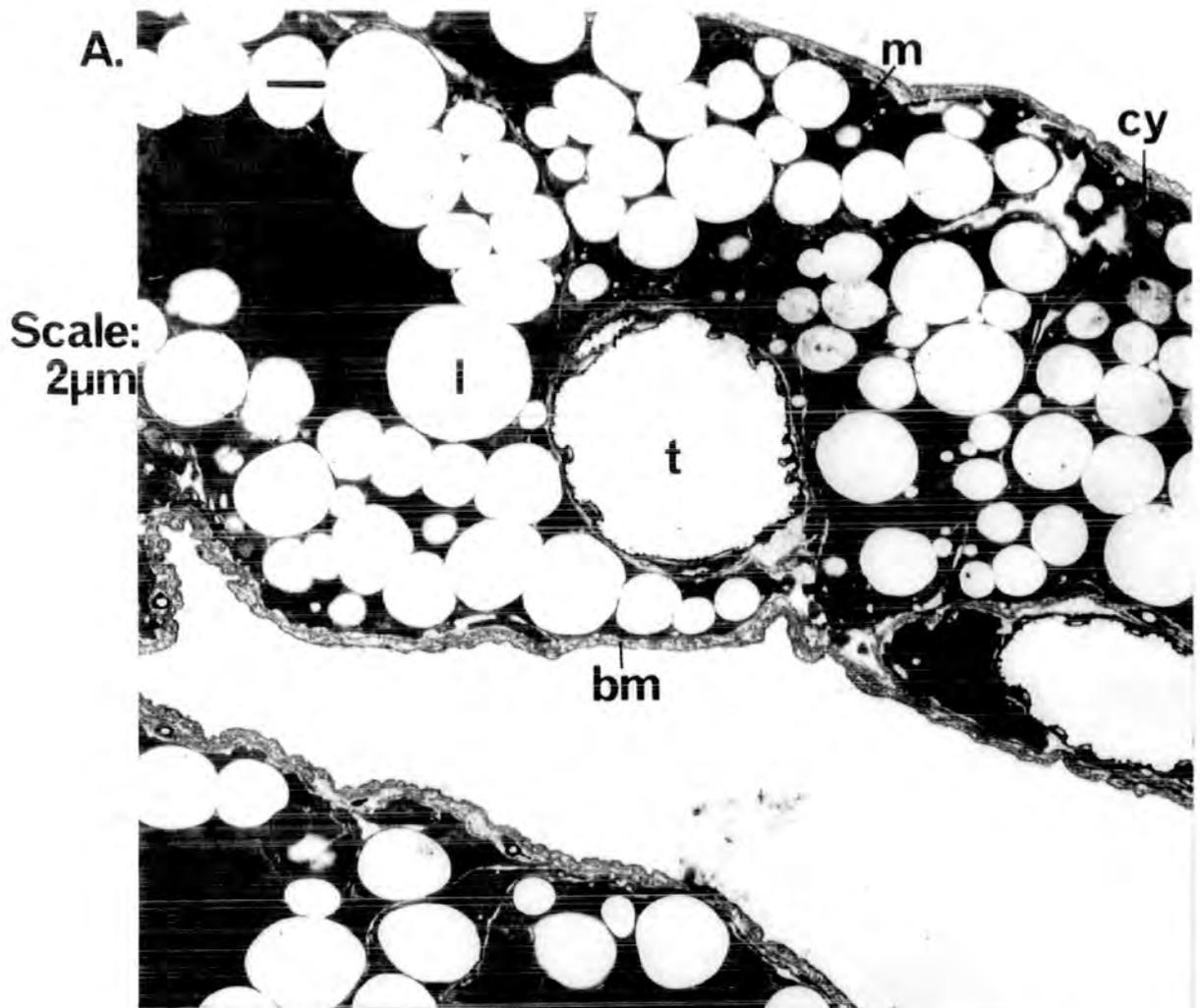


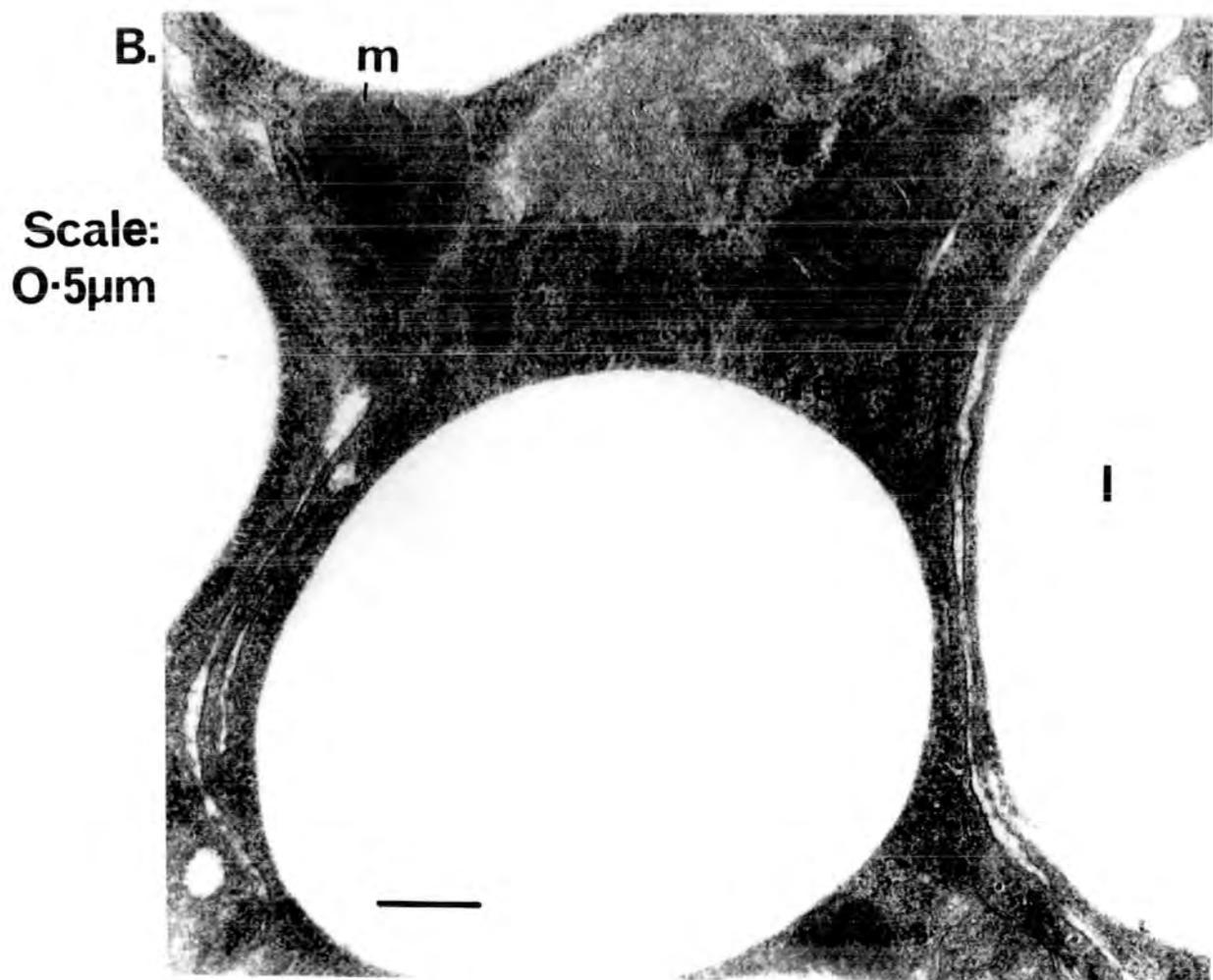
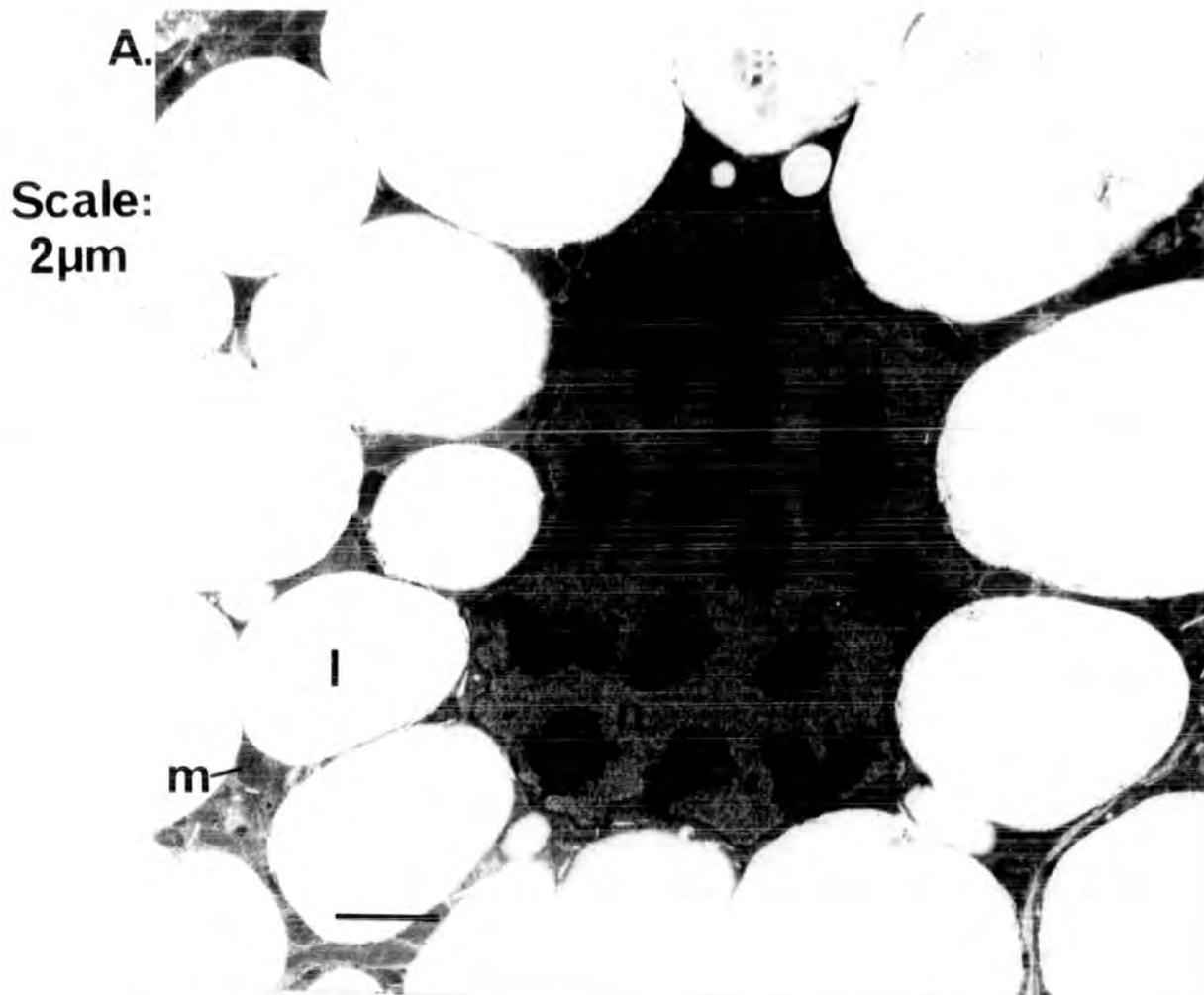
PLATE 6.10

A. Electron micrograph of fat body from a starved male locust.

Note: lacunae (l), mitochondrion (m), nucleus (n) appears similar to those of other treatments but the lacunae appear to press against it producing an irregular outline.

B. Electron micrograph of fat body from a starved male locust.

Note: lack of definition of structure in contrast to the other treatments, lacunae (l), mitochondrion (m), rough endoplasmic reticulum (rer).



Discussion

Much of the cytoplasm of the fat body cells from newly moulted locusts was filled with stored lipid droplets. What "free" cytoplasm there was, possessed a poorly developed, rough endoplasmic reticulum and Golgi bodies were infrequently encountered. Therefore, it may be expected that synthesis of energy reserves would take place at a low rate. The presence of many polyribosomal clusters suggested the start of an upsurge in protein synthesis (WHITE ET AL, 1964). This would be necessary to bring about the dramatic increase in size of the fat body cells, seen in operated control fat body. The free cytoplasm of the operated control, besides being greater in amount than that in newly moulted fat body, had a well developed rough endoplasmic reticulum system and there were many Golgi bodies. There was also much evidence of pinocytotic activity on the lateral plasma membranes. One must conclude from the structure of these cells that they are highly active in synthesis. This agrees well with the observations of WALKER AND BAILEY (1970a) who have shown that between the 5th and 8th days of adult life in Schistocerca gregaria lipogenesis is at a maximum.

By comparison with the operated control and newly moulted animals, the fat body of operated animals was very different. Although operated fat body cells had not increased in size since they were 12 hours \pm 12 hours old, there was far more "free" cytoplasm. Stored lipid and glycogen were very much reduced and judging by the sparsity of both rough endoplasmic reticulum and Golgi bodies and the lack of pinocytotic activity on lateral plasma membranes, one must conclude that operated fat body was synthetically quiescent.

The cells of the fat body from starved animals were much smaller than those of newly moulted animals. They had very little structure to the cytoplasm which contained few mitochondria and little rough

endoplasmic reticulum. Most of the fat body cell was occupied by empty lacunae. These presumably arose due to the use of lipid reserves originally present in the newly moulted adult and to autolysis of cytoplasmic tissue.

The effects of starvation on the ultrastructure of adult fat body were similar to those found in larvae by CLARKE AND ANSTEE (1971b). However, the effects of the removal of the frontal ganglion on the ultrastructure of adult fat body were somewhat different from those of a similar operation performed on larvae (CLARKE AND ANSTEE, 1971b). In adults, as in larvae, there was a reduction in the amount of rough endoplasmic reticulum. However, there were only occasional indications of the considerable dilation of the rough endoplasmic reticulum and mitochondrial swelling/bursting seen in larvae. While very few lipid droplets or vacuoles were seen in the operated adult, the operated larva contained many vacuoles and in less affected cells, fat droplets. It may well be that five days after the removal of the frontal ganglion from adults, only the early stages in cellular breakdown observed in larvae are seen, and that several days later the situation in the two stages would be comparable.

One may conclude, therefore, that the removal of the frontal ganglion from newly moulted adult male Locusta stops the normal growth and development of the fat body cells. The absence of glycogen granules and lipid droplets is in agreement with the biochemical evidence (chapter 3) that stored energy reserves in the fat body of operated and starved animals are very low.

CHAPTER 7

Some effects of the removal of the frontal ganglion on ion and water balance

Introduction

A number of authors have suggested that the frontal ganglion might be involved in the control of water balance (ROOME, 1968; PENZLIN, 1971; BERNAYS AND CHAPMAN, 1962). ROOME (1968) showed that frontal ganglionectomy in larval Periplaneta americana resulted in a dramatic shrinkage of the abdomen which was greater than that observed in starved animals. Removal of the frontal ganglion also reduces the size and possibly the activity of the corpora allata in Locusta larvae (CLARKE AND ANSTEE, 1971a) and adults (STRONG, 1967; present work Chapter 2). Therefore, ROOME (1968) suggested that frontal ganglion removal from Periplaneta americana might decrease the activity of the corpora allata to such an extent that feeding is inhibited and a reduction in water content of the body occurs. PENZLIN AND STOLZNER (1971) have found that larval Periplaneta americana lose considerable weight following frontal ganglion removal. This occurred more slowly, and the animals survived longer, the higher the relative humidity of the surroundings. An operated animal and a dead animal lost the same amount of weight following 24 hours in a dessicator above CaCl_2 . Two hypotheses have been put forward to explain this phenomenon. PENZLIN (1971) proposed a failure of those active processes that maintain the cuticular water at a low level. Normally there is a 14 atm pressure difference between haemolymph and cuticle (WINSTON & BEAMENT, 1969). PENZLIN AND STOLZNER (1971) proposed that normally the frontal ganglion receives impulses from osmoreceptors and exerts an inhibitory effect

on neurosecretory centres in the brain which have been shown by BERRIDGE (1966), HIGHNAM ET AL (1965) to be responsible for the release of a diuretic factor. Cutting the frontal connectives results in a loss of inhibition and consequently in an increased release of the diuretic factor from the brain. LANGLEY (1962) found a layer of dust on anterior segments of frontal ganglionectomised Locusta larvae and attributed this to lack of replacement of the water proof layers of the cuticle and leakage of fluid from the membrane. However, subsequent work has shown that the dust is caused by dried regurgitated fluid from the foregut (ROOME, 1968; ANSTEE, 1968).

An increasingly large volume of literature over the last twenty years has shown the Malpighian tubules and rectal sac to be the main sites of osmoregulation and excretion. A primary "urine" is produced by the Malpighian tubules which resembles in many ways a filtrate of the blood (MADDRELL AND GARDINER, 1974; RAMSAY, 1958). This flows into the gut and accumulates in the rectum where a second, reabsorptive phase takes place (reviewed by PHILLIPS, 1970; MADDRELL, 1971; WALL AND OSCHMAN, 1975). Here substances that are required for the metabolism of the animals are reabsorbed into the haemolymph while wastes are retained in the rectal lumen, concentrated and voided with the faecal matter.

As a result of studies by RAMSAY (1953, 1954, 1955, 1956), MADDRELL (1969, 1971), PILCHER (1970), a model has been proposed to explain fluid transport across Malpighian tubules (BERRIDGE AND OSCHMAN, 1969; MADDRELL, 1971). The main features of this include active transport of ions at both basal and apical surfaces of the cells and water movements osmotically linked to ion movements. Potassium is generally considered to be the "prime mover" (BERRIDGE, 1968). Movement of which is brought about by a electrogenic pump

on the apical membrane. Potassium entry from the haemolymph into the tubule cell is brought about by a Na^+ dependent pump on the basal membrane (BERRIDGE AND OSCHMAN, 1969; MADDRELL, 1972). Since fluid secreted by the Malpighian tubules is slightly hypertonic over a wide range of osmotic concentrations of bathing fluid, and rate of fluid secretion is inversely related to the osmotic concentration of the bathing fluid, water transport like anion transport must be passive (MADDRELL, 1971).

The ultrastructure of the Malpighian tubules lends support for the above model, which is based on a model proposed by DIAMOND AND BOSSERT (1967, 1968) to explain fluid transport in rabbit gallbladder. The basal membrane has extensive infoldings and the apical membrane many microvilli which will increase the surface area and, therefore, increase the rate of fluid transport (BERRIDGE AND OSCHMAN, 1969). Also the dimensions of the spaces between infoldings and microvilli are similar to those proposed by DIAMOND AND BOSSERT (1967) as being necessary to allow the development of local standing osmotic gradients.

A $\text{Na}^+ \text{K}^+$ activated Mg^{2+} dependent ATPase E.C.3.6.1.3 (SKOU, 1965) has been implicated in ion and water transport across various epithelia from a large variety of different species (WHITTAM AND WHEELER, 1970; SKOU, 1969). Active transport of ions is an essential part of the mechanisms proposed to explain fluid transport in both Malpighian tubules and Rectal sac. Therefore, it is not surprising that a Mg^{2+} activated ATPase has been localized histochemically in the rectal sac (BERRIDGE AND GUPTA, 1968) and Malpighian tubules (BERRIDGE, 1967) of Calliphora erythrocephala. Recently an $\text{Na}^+ \text{K}^+$ activated Mg^{2+} dependent ATPase has been demonstrated in microsomal preparations from Jamaicana flava, Homorocoryphus nitidulus vicinus (PEACOCK ET AL, 1972) and Locusta (ANSTEE AND BELL, 1975). The latter have shown

that ouabain inhibits secretion by Malpighian tubules at a similar concentration to that which inhibits the $\text{Na}^+ \text{K}^+$ ATPase. In every case so far studied the ATPase activity has been shown to consist of two components (SKOU, 1969). One is only dependent for its activity on the presence of Mg^{2+} ions and is not inhibited by the cardiac glycoside ouabain (the Mg^{2+} ATPase). The second is Mg^{2+} and $\text{Na}^+ \text{K}^+$ dependent, and is inhibited by ouabain (the $\text{Na}^+ \text{K}^+$ ATPase). It is the latter enzyme that has been implicated in active transport of the monovalent cations Na^+ and K^+ .

Control of the Malpighian tubule/Rectal sac system may well reside in the cerebral neurosecretory cells. HIGHNAM ET AL (1965) have shown that cauterization of these cells in immature male Schistocerca gregaria results in water retention. Similar phenomena have been observed in Schistocerca paranensis (STRONG, 1965 a and b) Locusta (GIRARDIE, 1963). HIGHNAM ET AL (1966) have shown a gradient of neurosecretory activity. Operated control Schistocerca gregaria have the most, there is less in allatectomised animals, while frontal ganglionectomised and median neurosecretory cell (mnc) cauterised have the least. It is possible to show a direct relationship between an increasing titre of neurosecretion and an increasing rate of excretion, and a decreasing titre of neurosecretion and an increasing haemolymph volume (MORDUE, 1969). Starved animals have very low neurosecretory activity and very low levels of excretion. Feeding of starved animals brings about release of neurosecretion and an increase in rate of excretion.

Injection of extracts of storage lobes of corpora cardiaca increase the excretion rate in mnc cauterised Locusta (MORDUE AND GOLDSWORTHY, 1969). CAZAL AND GIRARDIE (1968) found not only a diuretic factor in mnc but an antidiuretic factor in the corpora

cardiaca of Locusta. MORDUE (1970a) showed that this factor was produced only in the glandular lobes. While diuretic and anti-diuretic factors have both been shown to be effective on rectal function (MORDUE, 1969; CAZAL AND GIRARDIE, 1968), an anti-diuretic factor active against tubules in Locusta hardly seems necessary since the basal rate is so low (MORDUE, 1969). Indeed a decrease in tubule secretion seems to be brought about by the disappearance from the haemolymph of diuretic hormone rather than the presence of an anti-diuretic hormone.

A link has been shown in several insects between feeding and release of diuretic factor Rhodnius prolixus (MADDRELL, 1964), Dysdercus fasciatus (BERRIDGE, 1966), Carausius morosus (PILCHER, 1970). Also BERNAYS AND CHAPMAN (1972) have shown that information on foregut distention in Locusta leading to changes in palp resistance and diuresis proceeds along the posterior pharyngeal nerves to the frontal ganglion and thence to the brain via the frontal connectives.

WALL AND RALPH (1964) found that excretion in Periplaneta americana was reduced by extracts of corpus allatum and suggested an antidiuretic factor stored here from the brain. It is interesting that POELS AND BEENAKKERS (1969), BEENAKKERS AND VAN DEN BROECK (1974) have reported that juvenile hormone promotes water retention in Locusta. However, an indirect effect on the mnc system cannot be ruled out.

In the light of the work reviewed above it was decided to look at the following:

1. The effect of frontal ganglion cautery on haemolymph volume and Na^+ and K^+ content of the haemolymph, and on the activity of microsomal preparations of the Na^+ K^+ activated Mg^{2+} dependent ATPase from Malpighian tubule/rectal sac. This was in order to monitor a possible

effect on the ion and waterbalance of the operated animal as a consequence of a change in activity of the $\text{Na}^+ \text{K}^+$ exchange pump.

2. The effect of synthetic juvenile hormone in vivo on the levels of Na^+ and K^+ in the haemolymph and in vitro on the activity of microsomal preparations of the $\text{Na}^+ \text{K}^+$ activated Mg^{2+} dependent ATPase from Malpighian tubule/rectal sac; to determine one route by which juvenile hormone may figure in the control of ion and water balance in Locusta.

3. The effect of frontal ganglion removal on the ultrastructure of the Malpighian tubules. This was in order to obtain ultrastructural evidence for a possible mal-function of the ion and water balance system.

Materials and Methods

The animals employed for experiments on the removal of the frontal ganglion, the operation and the treatment of experimental animals were as described previously (see general materials and methods section Chapter 1). However, mature locusts were used for the study of the effect of two insect hormones on the activity of a microsomal preparation of $\text{Na}^+ \text{K}^+ \text{Mg}^{2+}$ ATPase while 7 day old adult animals were used to study the effect of J.H. on Na^+ and K^+ ions in the haemolymph. All reagents used were Anala R grade or purest available and supplied by British Drug Houses, Sigma, Cal Biochem, Nuclear Enterprises, Amersham and I.C.I.

Measurement of haemolymph volume using C^{14} labelled carboxy inulin

The method employed was essentially that described by LEVENBOOK (1958).

Animals were cooled in a 4°C cold room for eight minutes. This slowed them down and reduced the chance of haemolymph being lost during injection, due to violent abdominal movement. 440,000 cpm of carboxy inulin in $5\mu\text{l}$ of distilled water (from a stock of $50\mu\text{Ci}$ freeze dried C^{14} carboxy inulin in 1.25ml of distilled water) were injected laterally in between the tergum and sternum of the fourth abdominal segment into the haemocoel. Care was taken not to pierce the gut.

The animals were put in individual containers with grass and water in a temperature cabinet at 28°C for 30 minutes to allow dispersion of the inulin. There was no significant excretion of inulin within this time. Three samples of $5\mu\text{l}$ of haemolymph (where possible) were withdrawn by capillarity into microcaps from a small hole made by a fine tungsten needle in the corium of the coxa of the right hind leg.

The duplicate samples were transferred by microcap to scintillation vials and solubilised with 0.5ml of hyamine hydroxide and 0.5ml acetic acid (1.0N in toluene) (NICOLL AND EWER, 1972). 10ml of a toluene based scintillator were added (Nuclear Enterprise, N.E.233) and the samples were counted on an automatic Beta-Gamma Spectrometer (N.E.8312).

Quenching was corrected for using the external standard ratio method (Fig. 7.1).

The following formula was used to calculate the haemolymph volume (hv)

$$hv = \frac{(\text{dpm}/\mu\text{l } C^{14} \text{ carboxy inulin injected}) (\mu\text{l } C^{14} \text{ inulin solution injected}) - (\mu\text{l } C^{14} \text{ carboxy inulin solution injected})}{(\text{dpm}/\mu\text{l haemolymph sample after 30 minutes equilibration})}$$

Determination of Na⁺ and K⁺ ions in haemolymph

A small hole was made in the corium of the right hind coxa and 5 μ l/10 μ l of haemolymph withdrawn and put in 3ml/6ml of deionised water and shaken. The experimental samples were compared to standard curves. Sodium ion and potassium ion standards were prepared by serially diluting BDH volumetric solutions of Na OH and K OH with deionised water. They were read by emission on a Pye Unicam SP 90 atomic absorption spectrophotometer.

The effect of synthetic juvenile hormone on the concentration of the above ions was determined by injecting 10 μ l of spectroscopically pure ethanol containing 1.69×10^{-5} moles of J.H. into each animal and sampling the haemolymph an hour later. Control animals were injected with just 10 μ l of ethanol.

Electron Microscopy

The preparation of material, sectioning and processing of sections were as described in Chapter 6.

Microsomal preparation of a Na^+ K^+ activated Mg^{2+} dependent

ATPase (E.C.3.6.1.3) (SKOU, 1965) from rectal sac and Malpighian tubules

The method employed was that described by PEACOCK, BOWLER AND ANSTEE (1972).

Reagents

Homogenisation medium : 40mM histidine/HCl (pH 7.2) 250mM mannitol, 5mM EDTA, 0.1% sodium deoxycholate.

Sodium iodide medium : 4 mM Na I, 5mM MgCl_2 , 10mM EDTA (pH 7.2).

Washing medium : 5mM Na Cl, 5mM EDTA (pH 7.2)

Buffered ionic reaction medium for estimation of Na^+ K^+ ATPase and Mg^{2+} ATPase activity together ("High Ions") : 6mM MgCl_2 , 200mM Na Cl, 40mM K Cl, 50mM histidine/HCl pH 7.2.

Buffered ionic reaction medium for estimation of Mg^{2+} ATPase activity only ("Low Ions") : 6mM MgCl_2 , 50mM histidine/HCl pH 7.2.

ATP : disodium ATP was converted to its acid form by shaking with Dowex H^+ resin. The H^+ ATP was recovered from the supernatant by centrifugation of the resin. H^+ was converted to its tris salt by titration with 2M Tris to pH 7.0 and stored until required at -20°C .

W-Lubrol or Cirrasol ALN-F : equal volumes of either 1% lubrol or cirrasol in distilled water and 1% ammonium molybdate in 1.8N sulphuric acid.

Preparation of microsomes

The rectal sac and Malpighian tubules were quickly dissected out from 8-12 animals of each treatment. The guts were cut open longitudinally and washed free of gut contents with homogenisation medium. They were transferred to a fresh 10ml of homogenisation medium. Homogenisation was carried out in a Potter-Elvehjem homogeniser with a Teflon pestle (clearance 0.1 - 0.15mm) with 10 passes of the plunger at 2,000 r.p.m., on a Vortex Waring Blender (M.S.E. Ltd.).

An equal volume of NaI medium was added to the homogenate and left to stand on ice for 30 minutes, following the method of NAKAO ET AL (1965). This extract was then spun at 50,000g for 30 mins at 0°C using an M.S.E. automatic superspeed 40 centrifuge, head No. 2409. The pellet was discarded and the supernatant centrifuged at 100,000g for 60 minutes. The resulting pellet was resuspended in 20ml of washing medium and re-centrifuged at 100,000g for 60 minutes. This washing procedure was repeated. After the final spin the pellet was resuspended in deionised water and resuspended by homogenisation for five passes.

ATPase assay

Enzyme activities were assayed in Pyrex glass centrifuge tubes. The reaction was started by the addition of 0.5ml microsomal preparations to 1.0ml of reaction medium ("high" or "low" ions) and 0.5ml of 12mM ATP, which had been previously equilibrated for 5 minutes at the incubation temperature of 30°C. All experiments were run for 30 minutes (unless indicated otherwise) in a water bath whose temperature was controlled to $\pm 0.1^\circ\text{C}$ by a 500 watt immersion heater connected by a hot wire vacuum switch relay (Sunvic Controls Ltd.) to a "Jumos" electrical contact thermometer (A. GALLENKAMP AND CO. LTD.)

Enzyme activity was determined using the ATKINSON, GATESBY AND LOWE (1973) modification of the method of FISKE AND SUBBAROW (1925).

The reaction was stopped by the addition of 4ml of a freshly prepared Lubrol or Cirrasol solution. A reagent blank was prepared by addition of the Lubrol before the enzyme was introduced. The tubes were left at room temperature (18 - 20°C) for 10 minutes to allow the yellow colour to develop and then transferred to crushed ice for storage. Precipitated protein was removed by centrifugation at 1000g for 15 minutes at 4°C in a "Mistral 2L" Centrifuge (M.S.E.Ltd.)

The optical density of the supernatant was read against a distilled water blank at 390nm in a Pye Unicam SP 1800 Dual beam Spectrophotometer. Inorganic phosphate content was determined by reference to a calibration graph prepared by assay of standard phosphate solutions serially diluted from a stock solution containing 20 μ g phosphorus (as KH_2PO_4 /ml see Fig. 7.2). The response characteristics of assay mixtures prepared with Lubrol and Cirrasol are identical (COSSINS, 1974).

Protein estimations

The protein content of enzyme preparations was determined by the method of LOWRY ET AL (1951) using bovine serum albumin (BSA) fraction V as standard (Sigma Chemical Co. Ltd.). See Appendix (Section 4.1). Enzyme activities were then expressed as n moles P_i liberated/mg protein/min.

Calculation of the Na^+ K^+ component of the ATPase activity

The Na^+ K^+ component of the ATPase activity, ion "pump", was calculated as being the difference between the activity in the reaction tube containing "High Ions" (Mg^{2+} , Na^+ , K^+) and the activity in the reaction tube containing "Low Ions" (Mg^{2+}).

Both Na^+ K^+ and Mg^{2+} components of the ATPase were active in the presence of all three ions but only the Mg^{2+} component in the presence of the single ion Mg^{2+} .

In vitro effect of two hormones on the activity of a microsomal preparation of Na^+ K^+ activated Mg^{2+} dependent ATPase

The first of the hormones tested was a synthetic juvenile hormone (from Cal Biochem), mixed farnesenic derivatives produced by bubbling HCl through farnesenic acid (WIGGLESWORTH, 1969; LAW ET AL, 1966)

M.W = 294.12. The second was β ecdysterone (from Schwarz Mann)

MW = 492.

Cholesterol was also tested as a control. As it is a steroid molecule with a large molecule weight (MW = 386.64) without hormonal effect but related to the hormones under test.

10^{-5} - 10^{-9} moles of the above reagents dissolved in 10μ l of either absolute spectroscopically pure ethanol (J.H. and cholesterol) or 50 : 50 mixture of absolute spectroscopically pure ethanol and distilled water (β ecdysterone) were preincubated with ATP and ionic reaction medium for ten minutes. The enzyme activities were assayed as described previously.

Statistical analysis of results

Statistical comparisons of data were performed using conventional techniques as described by SNEDECOR AND COCHRAN (1967). Where appropriate, reference was made to the statistical tables of FISCHER AND YATES (1963). Values of $p \leq 0.05$ were taken as being significant.

Ultrastructural study of Malpighian tubules

The results presented below are representative observations made during the course of two experiments involving 4 male locusts of each treatment. During the course of this investigation several hundred cell profiles were examined of each treatment.

Fig. 7.1

Quench Correction curve

The effect of hyamine hydroxide and acetic acid on the efficiency of Beta particle counting in a β/γ Scintillation counter.

Haemolymph in the amounts used had no effect on the efficiency of the count or on the external standard ratio.

Legend :

ordinate ; % efficiency

abscissa ; external standard ratio

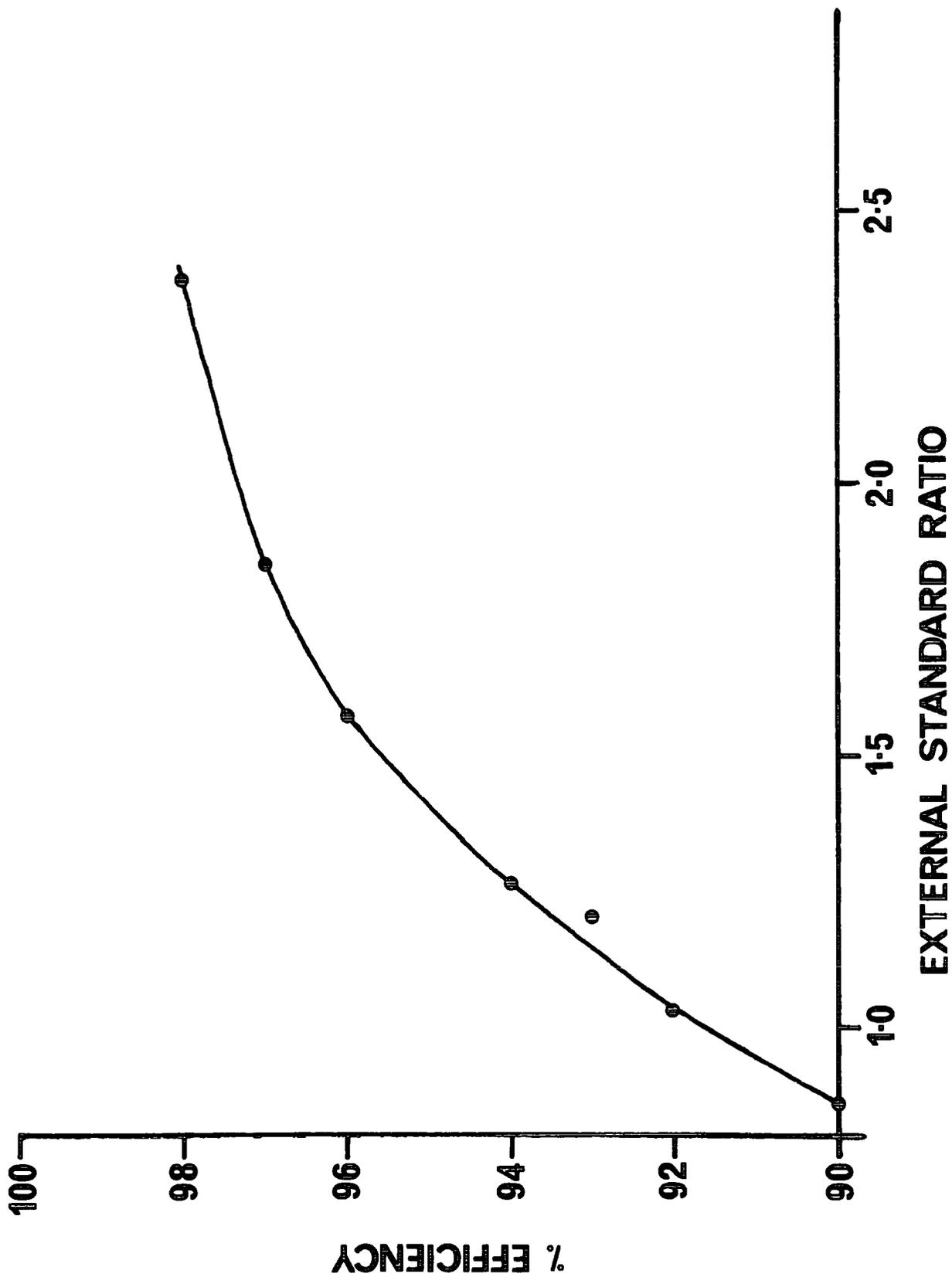


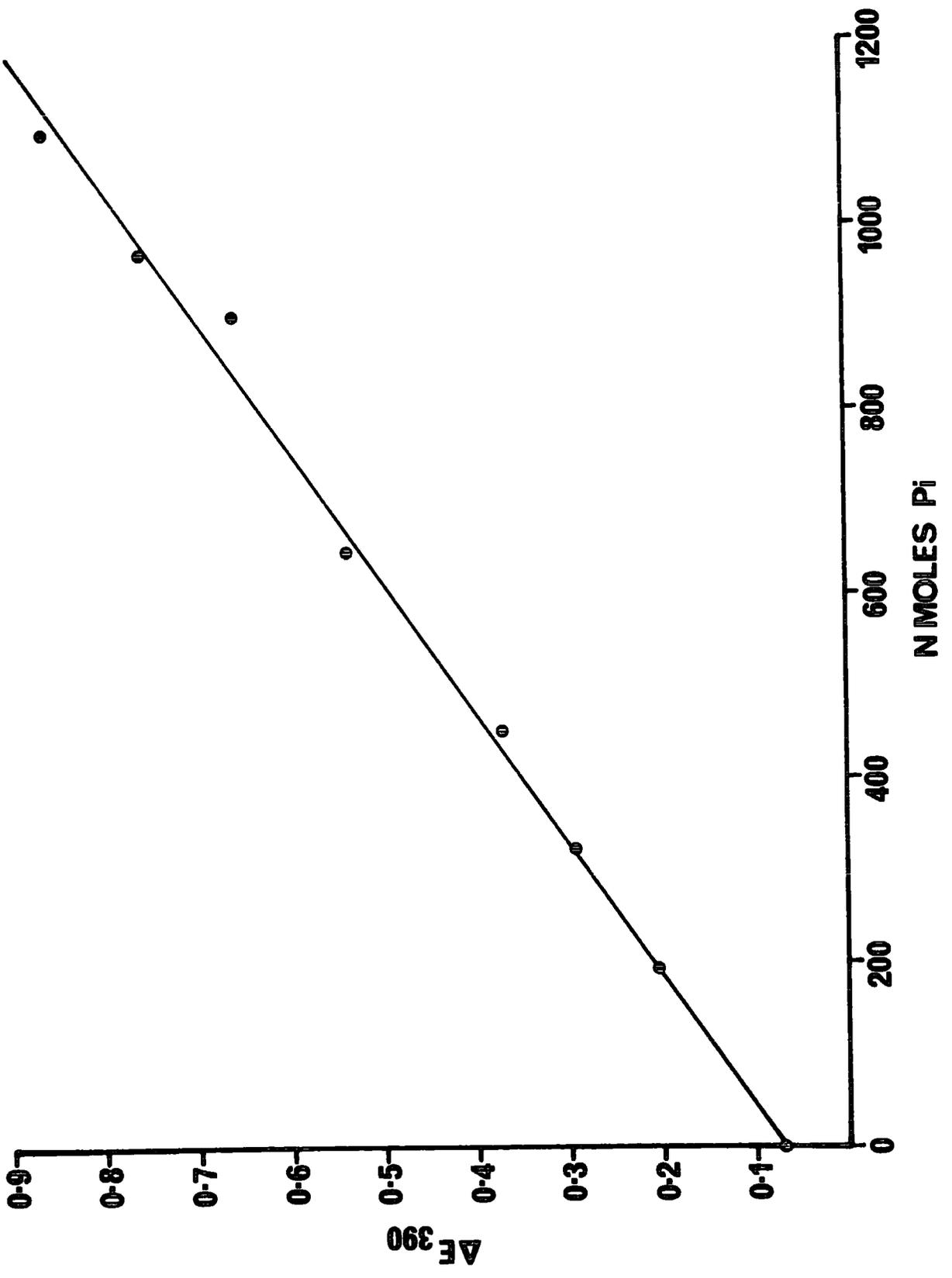
Fig. 7.2

Standard Phosphate Curve

Legend:

ordinate ; absorbance nm

abscissa ; n moles inorganic phosphate Pi



Results

The effect of the removal of the frontal ganglion on the haemolymph volume of young adult locusts

The effect of the removal of the frontal ganglion on haemolymph volume was more dramatic than would appear from direct measurements using carboxy inulin. A marked increase was observed in operated animals in the number of individuals that appeared to be devoid of haemolymph. Table 7.1 shows the availability of haemolymph from animals used in a number of experiments including the haemolymph volume analysis. While haemolymph could be collected from 96% of the 12 hours \pm 12 hours old animals and 88% of the operated controls, only 77% of the starved and 64% of the operated animals had extractable haemolymph.

Haemolymph volumes expressed per animal and per gm wet weight are shown in Tables 7.2, and 7.3 (a statistical treatment of these results is recorded in Table 7.4). These mean values are based on biased samples, that is only those animals are included from which haemolymph could be extracted.

It is apparent that only those animals with extractable haemolymph could be used to study the carbohydrate and lipid content of the haemolymph (Chapter 3). Therefore, the present approach to haemolymph volume was thought to be valid, in order to relate the analysis of energy reserves to the volume.

Newly moulted adult males (12 hours \pm 12 hours old) had 299 \pm 14.8 μ l/animal of haemolymph, while females of the same age had 358 \pm 18.4 μ l/animal. It was observed that animals that had just moulted had larger haemolymph volumes than those that had moulted earlier in the 12 hours \pm 12 hours period. 5 days after the operation the

haemolymph volume of the operated control was not significantly different from the preoperated 12 hours \pm 12 hours old animal, male $302 \pm 35.2 \mu\text{l}/\text{animal}$ and female $310 \pm 21.2 \mu\text{l}/\text{animal}$. By comparison the starved had dropped to a significantly lower level, 181 ± 30.5 in the male and $248 \pm 15.7 \mu\text{l}/\text{animal}$ in the female ($p < 0.001$). The operated animal too had a significantly lower haemolymph volume than prior to the operation, male $205 \pm 15.8 \mu\text{l}$ (animal and female $280 \pm 30.4 \mu\text{l}/\text{animal}$ ($p < 0.001$). For both sexes, operated and starved animals had significantly lower haemolymph volumes than controls ($p < 0.05$). However, there was no significant difference between operated and starved treatments.

Since females were larger and heavier than males of the same treatment it was not surprising that the males had a significantly lower haemolymph volume than the females ($p < 0.05$). The exception was the operated control treatment, where the P value for the difference between the mean haemolymph volumes of the sexes was $0.1 - 0.05$.

When the haemolymph volume results were expressed per gm wet weight the haemolymph volumes of the four treatments appeared very similar (Tables 6.2 and 6.3).

The effect of the removal of the frontal ganglion on the concentration of Na^+ and K^+ ions in the haemolymph

Neither frontal ganglion cautery nor starvation had any effect on the concentration of Na^+ and K^+ ions in the haemolymph. Operated, Operated Control and starved treatments all showed a similar significant increase in Na^+ K^+ concentration and in the ratio Na^+/K^+ , in the five days following the operation. No significant difference was observed between males and females in any of the four treatments (See Table 7.5).

The effect of a synthetic juvenile hormone on the concentration of Na⁺ and K⁺ ions in the haemolymph of 7 day old normal adults

Both J.H. treated and ethanol treated (Control) animals had a significantly lower K⁺ concentration than the 7 day old normal animals (p < 0.001) (See Table 7.6).

Therefore, it must be concluded that the ethanol solvent produced the effect rather than the J.H. The concentration of Na⁺ ions in the J.H. treated animals was significantly lower than in the 7 day old normal animals (p = 0.01 - 0.001) but not significantly different from the controls. There was no significant difference between the Na⁺ concentration in the ethanol control treatment and the 7 day old normal animals.

The ratios of the two ions Na⁺/K⁺ in control and J.H. treated animals were significantly lower than in the normal animals (p < 0.001). But there was no significant difference between the Na⁺/K⁺ ratios of control and J.H. treatments. Once again this may be explained as effect brought about by the ethanol rather than the J.H.

The in vitro effect of a synthetic juvenile hormone on the activity of a microsomal preparation of a Na⁺ K⁺ activated Mg²⁺ dependent ATPase from the Malpighian tubules and rectal sac of mature adults

Fig. 7.3A shows the results obtained from one of several experiments. An inhibitory effect by J.H. was observed on the Na⁺K⁺ component of the activity, but little effect was seen on the Mg²⁺ ATPase. The percentage inhibition of the Na⁺K⁺ "pump" found in three experiments are plotted in Fig. 7.4. A threshold of inhibition occurred at 1.69×10^{-9} moles J.H. and there was a peak at 1.69×10^{-5} moles J.H. with 56% inhibition.

The in vitro effect of β ecdysterone and Cholesterol on a microsomal preparation of a Na⁺ K⁺ activated Mg²⁺ dependent ATPase from Malpighian Tubules and rectal sac of mature adults

It was decided to see if another hormone, β ecdysterone had a similar effect to J.H. observed above (Fig. 7.3B). A slight inhibitory

effect was observed on both the $\text{Na}^+ \text{K}^+$ and Mg^{2+} components of the activity with a maximum of 11% inhibition occurring with 10^{-7} moles of β ecdysterone.

Cholesterol was also tested for its effect on the ATPase as a control, being a large steroid molecule related to the other compounds employed in this study. It was found to have no significant effect on either component of the activity (Table 7.7).

The effect of a synthetic juvenile hormone on the development of the yellow colour produced during the estimation of inorganic phosphate

The apparent effect of J.H. on the activity of the $\text{Na}^+ \text{K}^+$ ATPase may have been due not to an inhibitory effect on the enzyme itself but on the development of the yellow colour during the determination of the inorganic phosphate (see materials and methods section). An experiment was performed to investigate this possibility (see Fig. 7.5).

1.69×10^{-7} moles - 1.69×10^{-9} moles of J.H. had little effect on the development of the colour. However, 1.69×10^{-6} moles of J.H. increased the absorbance of the inorganic phosphate determination. This was probably due to a cloudiness observed in tubes containing 1.69×10^{-6} moles and 1.69×10^{-5} moles J.H. However, this particular effect would have been compensated for by the controls. It may be concluded from this experiment that the inhibitory effect of J.H. on the $\text{Na}^+ \text{K}^+$ ATPase was not due to an effect on the colour development.

The effect of frontal ganglion removal on the activity of microsomal preparations of $\text{Na}^+ \text{K}^+$ activated Mg^{2+} dependent ATPase from the Malpighian tubules and rectal sac

The results presented in Fig. 7.6A demonstrate the linearity of the $\text{Na}^+ \text{K}^+$ activated component of the ATPase activity of two microsomal preparations from operated animals and one from operated control animals over the period of time employed for incubation.

The two preparations from operated animals had similar activities and these were greater than that of the controls.

In a second experiment (Fig. 7.6B) involving one group of control and one group of operated animals a similar linearity was observed. However, in this case the preparation from control animals had a greater activity than that of the operated animals.

The effect of frontal ganglion removal on the ultrastructure of the Malpighian tubule

The Malpighian tubules from young adult Locusta showed a uniform morphological appearance like those of Melanoplus differentialis (BEAMS ET AL, 1955) and Dissoteria carolina (TSUBO AND BRANDT, 1961), but unlike those of Schistocerca gregaria (SAVAGE, 1966) and Rhodnius prolixus (WIGGLESWORTH, 1931) in which the tubules are morphologically differentiated into distal and proximal regions. In Rhodnius prolixus the distal and proximal regions have been shown to carry out secretory and absorptive functions respectively (WIGGLESWORTH, 1931).

Two types of cell were found in the Malpighian tubules in the course of the present study. A large cell similar in structure to the cells of the distal region of Rhodnius prolixus (Plate 7.1 A,B,C) and a smaller secretory cell (Plates 7.6 and 7.7). The latter were only infrequently encountered in electron micrographs presumably because they presented narrow profiles in transverse section due to their small size.

Lower power electron micrographs of T.S. of the primary tubule cells from operated, operated control and starved all displayed the same basic organisation that has been observed in the tubules of many insects Gryllus domesticus (BERKALOFF, 1959), Calliphora erythrocephala (BERRIDGE AND OSCHMAN, 1969), Periplaneta americana (WALL ET AL, 1975) and in many epithelia noted for ion and water transport including

mammalian gall bladder (KAYE ET AL, 1966) and mammalian kidney (RHODIN, 1958).

The basement membrane consisted of two layers (Plate 7.1 A,B,C, Plate 7.2A, B, Plate 7.3). Basal Infoldings of the cell membrane formed deep channels that extended perpendicular to the cell surface. The cytoplasmic processes between these infoldings had electron dense tips which were similar to the hemidesmosome junctions described by BERRIDGE AND OSCHMAN (1969) in the Malpighian tubules of Calliphora erythrocephala (Plate 7.2 A,B, Plate 7.3). The apical border of the tubule cell was extensively folded into microvilli many of which had long thin mitochondria along their length (Plate 7.1 A,B,C).

There was no marked polarisation of the distribution of mitochondria at apical and basal borders in any of the three treatments, as was seen in Calliphora erythrocephala (BERRIDGE AND OSCHMAN, 1969). Instead they were spread throughout the intervening cytoplasm. The latter contained many free ribosomes but few Golgi bodies and little rough endoplasmic reticulum (Plate 7.1 A,B,C, Plate 7.4 A & B, Plate 7.5 A).

A feature common to the intermediate zone of cytoplasm of all three treatments was the presence of many vacuoles of different sizes, some containing granules or other inclusions and large lamellated concretions. The latter comprised concentric shells of densely staining material (Plate 7.1 A,B,C, Plate 7.4 A & B). These structures are common in insect Malpighian tubules (GOURANTON, 1968, WIGGLESWORTH AND SALPETER, 1962). An early study of SRIVASTAVA (1962) suggested that these structures contained ureates but GOURANTON (1968) was unable to detect uric acid. More recently WALL ET AL (1975) have suggested a role in calcium phosphate storage for such structures in Periplaneta americana.

The plasma membranes between adjacent cells were characterised by septate junctions which had a series of thin diaphragms across them. This sort of junction between cells was first described by LOCKE (1965) and designated comb or septate desmosomes by DANILOVA ET AL (1969) (Plate 7.4A and Plate 7.5A). Near the apical border there were regions of the plasma membrane that had no junctional structures and then a different form of desmosome the so called, Macula adhaerans was observed close to the apical membrane (SMITH, 1968) (Plate 7.4A and Plate 7.5B).

The tubules from operated animals showed several features that distinguished them from operated control and starved animals. The extracellular spaces between the basal infoldings in operated tubules appeared much wider than those of operated control and starved treatments (Plates 7.1, 7.2 and 7.3). At the apical surface the tips of the microvilli were considerably swollen (Plate 7.1A, Plate 7.5B) on which many vesicles occurred. While in the cytoplasm there were many more vacuoles and concretions than in the other two treatments (Plate 7.1 A,B,C).

There were no indications of mitochondrial swelling or disruption of the rough endoplasmic reticulum as reported in certain other tissues of Locusta larvae following frontal ganglionectomy (CLARKE AND ANSTEE, 1971b).

The second type of cell that has been observed in the Malpighian tubules was found infrequently. A study of the structure of these cells has been made in normal animals but no investigation was carried out into the effect of frontal ganglion removal on its structure. The appearance of this cell was characteristic of a secretory cell (Plate 7.6A). There were many well developed Golgi bodies that were associated with large vesicles or vacuoles containing a granular

material (Plate 7.6B, Plate 7.7A). These were similar to the secretory vacuoles found extensively throughout the cytoplasm of these cells. The rough endoplasmic reticulum unlike that seen in the primary cells of the Malpighian tubule was very well developed; numerous parallel stacks of membranes being visible (Plate 7.7A). In contrast to the primary cells, the secretory cells had four vacuoles and no concretions. The nucleus was small, and there were few, small, mitochondria (Plate 7.6A). The infoldings of the basal surface and the microvilli of the apical surface were poorly developed by comparison with those of the primary cell (Plate 7.7 A & B).

Table 7.1

The availability of haemolymph from
treated animals

	12 hours ⁺ 12 hours	Operated Control	Operated	Starved
Number of animals from which haemolymph could be obtained	52	30	27	34
Number of animals from which haemolymph could not be obtained	2	4	15	10
% success	96	88	64	77

Table 7.2

The effect of the removal of the frontal ganglion on haemolymph volume $\mu\text{l}/\text{animal}$

where it was possible to collect haemolymph

Treatment	mean μl haemolymph/ animal	S.E.	Number of determina- tions	Sex
1. 12 hours \pm 12 hours	209	14.8	19	Male
Operated	202	15.8	7	
Operated Control	302	35.2	6	
Starved	181	30.5	7	
2. 12 hours \pm 12 hours	358	18.4	19	Female
Operated	280	30.4	7	
Operated Control	310	21.2	8	
Starved	248	15.7	12	
3. 12 hours \pm 12 hours	329	12.6	38	Male + Female
Operated	241	19.7	14	
Operated Control	308	21.6	12	
Starved	224	20.9	14	

NOTE: mean values based on biased samples, that is only those animals were included from which haemolymph could be extracted.

Table 7.3

The effect of the removal of the frontal ganglion
on haemolymph volume μ l/gm wet weight

where it was possible to collect haemolymph

Treatment	mean μ l haemolymph/ gm	S.E.	Number of determinations	Sex
12 hours \pm 12 hours	277	11.54	19	Male
Operated	227	16.9	6	
Operated Control	258	32.5	6	
Starved	225	33.96	7	
12 hours \pm 12 hours	266	12.4	19	Female
Operated	250	22.8	7	
Operated Control	221	11.6	8	
Starved	226	9.0	12	

NOTE: mean values based on biased samples, that is only those animals were included from which haemolymph could be extracted.

Table 7.4

Statistical treatment of the haemolymph
volume data

Treatments compared	Volume/animal			Treatments compared	Volume/gm		
	t	df	p		t	df	p
00♂ : 00♂	2.732	11	0.02-0.01	00♂ : 00♂	0.888	11	0.5-0.4
00♂ : 80♂	0.613	12	0.6 -0.5	00♂ : 80♂	0.042	12	> 0.9
00♂ : 120♂	3.688	24	0.1-0.001	00♂ : 120♂	2.316	24	0.05-0.02
00♂ : 0♀	2.276	12	0.05-0.02	00♂ : 0♀	0.797	12	0.5-0.4
0♀ : 00♂	0.203	12	0.1-0.05	0♀ : 00♂	1.209	12	0.3-0.2
00♂ : 8♀	2.408	18	0.05-0.02	0♀ : 8♀	0.318	18	0.8-0.7
0♀ : 12♀	1.520	25	0.2-0.1	0♀ : 12♀	2.206	25	0.05-0.02
0♀ : 0♀	0.832	13	0.5-0.4	0♀ : 0♀	1.172	13	0.3-0.2
00♂ : 80♂	2.614	11	0.05-0.02	00♂ : 80♂	0.690	11	0.6-0.5
00♂ : 120♂	0.081	23	> 0.9	00♂ : 120♂	0.7	23	0.5-0.4
8♀ : 0♀	1.042	17	0.4-0.3	8♀ : 0♀	1.161	17	0.3-0.2
80♂ : 8♀	2.165	17	0.05-0.02	80♂ : 8♀	0.003	17	> 0.9
8♀ : 12♀	4.189	29	< 0.001	8♀ : 12♀	2.375	29	0.05-0.02
80♂ : 120♂	3.883	24	< 0.001	80♂ : 120♂	1.871	24	0.1-0.85
12♀ : 120♂	2.484	36	0.02-0.01	12♀ : 120♂	0.644	36	0.6 -0.5
12♀ : 0♀	2.208	24	0.05-0.02	12♀ : 0♀	0.682	24	0.6-0.5

combined male and female volume/animal

Treatment compared	t	df	p
0:C	2.305	24	0.05-0.02
0:S	0.571	26	0.6 -0.5
C:S	2.772	24	0.02-0.01
0:12	3.666	50	< 0.001
C:12	0.806	48	0.5 -0.4
S:12	4.283	50	< 0.001

Table 7.5

The effect of the removal of the frontal ganglion
on the Concentration of Na⁺ and K⁺ in the haemolymph
expressed as m moles/litre

Treatment	Ion	Sex	Mean day 1	SE	Number of determina- tions	Mean day 6	SE	n
Operated	Na ⁺	♂	65.73	1.69	11	90.09	4.07	11
		♀	72.33	6.16	6	88.83	5.4	6
	K ⁺	♂	10.64	0.34	11	14.45	0.91	11
		♀	10.17	0.6	6	13.0	0.82	6
Ratio	Na ⁺ /K ⁺	♂	6.47	0.36	11	6.46	0.48	11
		♀	6.88	0.48	6	6.0	0.7	6
Control	Na ⁺	♂	75.67	4.79	9	89.67	3.32	9
		♀	74.17	4.91	6	95.00	2.9	6
	K ⁺	♂	12.22	0.68	9	12.33	0.53	9
		♀	10.83	0.6	6	13.0	1.03	6
Ratio	Na ⁺ /K ⁺	♂	6.33	0.51	9	7.38	0.43	9
		♀	6.95	0.49	6	7.48	0.5	6
Starved	Na ⁺	♂	69.91	1.74	11	93.91	3.35	11
		♀	70.75	3.13	8	92.38	2.73	8
	K ⁺	♂	10.91	0.31	11	13.64	0.81	11
		♀	10.75	0.31	8	14.13	0.91	8
Ratio	Na ⁺ /K ⁺	♂	6.53	0.38	11	7.05	0.36	11
		♀	6.58	0.27	8	6.78	0.55	8

Table 7.6

Effect of juvenile hormone on the concentration of Na⁺ and K⁺ ions in the haemolymph of seven day old adults

(equal numbers of males and females were employed)

Treatment	Number of determinations	Ion	Mean	SE	Mean Ratio	SE
Juvenile hormone (JH)	12	Na ⁺	81.46	3.22	3.76	0.37
		K ⁺	23.26	1.6		
Ethanol Control (CO)	12	Na ⁺	89.23	2.91	4.23	0.30
		K ⁺	21.84	1.16		
7 day normal (7)	18	Na ⁺	93.83	2.88	6.52	0.35
		K ⁺	14.85	0.58		

Statistical comparison

	Na ⁺			K ⁺			Ratio		
	df	t	p	df	t	p	df	t	p
7:JH	28	2.806	0.01-0.001	28	5.669	< 0.001	28	5.237	< 0.001
7:CO	28	1.079	0.3-0.2	28	5.952	< 0.001	28	4.581	< 0.001
CO:JH	22	1.789	0.1-0.005	22	0.716	0.5-0.4	22	0.988	0.4-0.3

Table 7.7

The in vitro effect of cholesterol on the activity of a microsomal preparation of a Na⁺ K⁺ activated Mg²⁺ dependent ATPase from Malpighian tubules and rectal sac

Amount of cholesterol (Moles)	Activity in n Moles P _i /mg protein/min			% Inhibition of Na ⁺ K ⁺ ATPase
	Presence of Mg ²⁺ only	Presence of Na ⁺ K ⁺ Mg ²⁺	Na ⁺ K ⁺ ATPase	
10 ⁻⁵	100	736.7	636.7	0
10 ⁻⁶	100	716.7	616.7	3
10 ⁻⁷	100	730	630	0
10 ⁻⁸	110	740	630	0
10 ⁻⁹	100	730	630	0
Ethanol Control	100	736.7	636.7	

Fig. 7.3

A. The in vitro effect of synthetic juvenile hormone on the activity of a microsomal preparation of an Na⁺ K⁺ activated Mg²⁺ dependent ATPase from the Malpighian tubules and rectal sac

Legend:

ordinate ; n moles Pi/mg protein/min

abscissa ; amount of juvenile hormone
1.69 x 10⁻⁹ - 1.69 x 10⁻⁵ moles

control, absolute ethanol

- ○
○ 1. Na⁺ K⁺ activated Mg²⁺ dependent ATPase
○ 2. Mg²⁺ activated ATPase

percentages quoted = inhibition of Na⁺ K⁺ ATPase
(1 minus 2 above)

B. The in vitro effect of β ecdysterone on the activity of a microsomal preparation of an Na⁺ K⁺ activated Mg²⁺ dependent ATPase from the Malpighian tubules and rectal sac

Legend:

ordinate ; n moles Pi/mg protein/min

abscissa ; amount of β ecdysterone
1.0 x 10⁻⁹ - 1.0 x 10⁻⁶ moles

control, 50:50 ethanol/water

- ○
○ 1. Na⁺ K⁺ activated Mg²⁺ dependent ATPase
○ 2. Mg²⁺ activated ATPase

percentages quoted = inhibition of Na⁺ K⁺ ATPase
(1 minus 2 above)

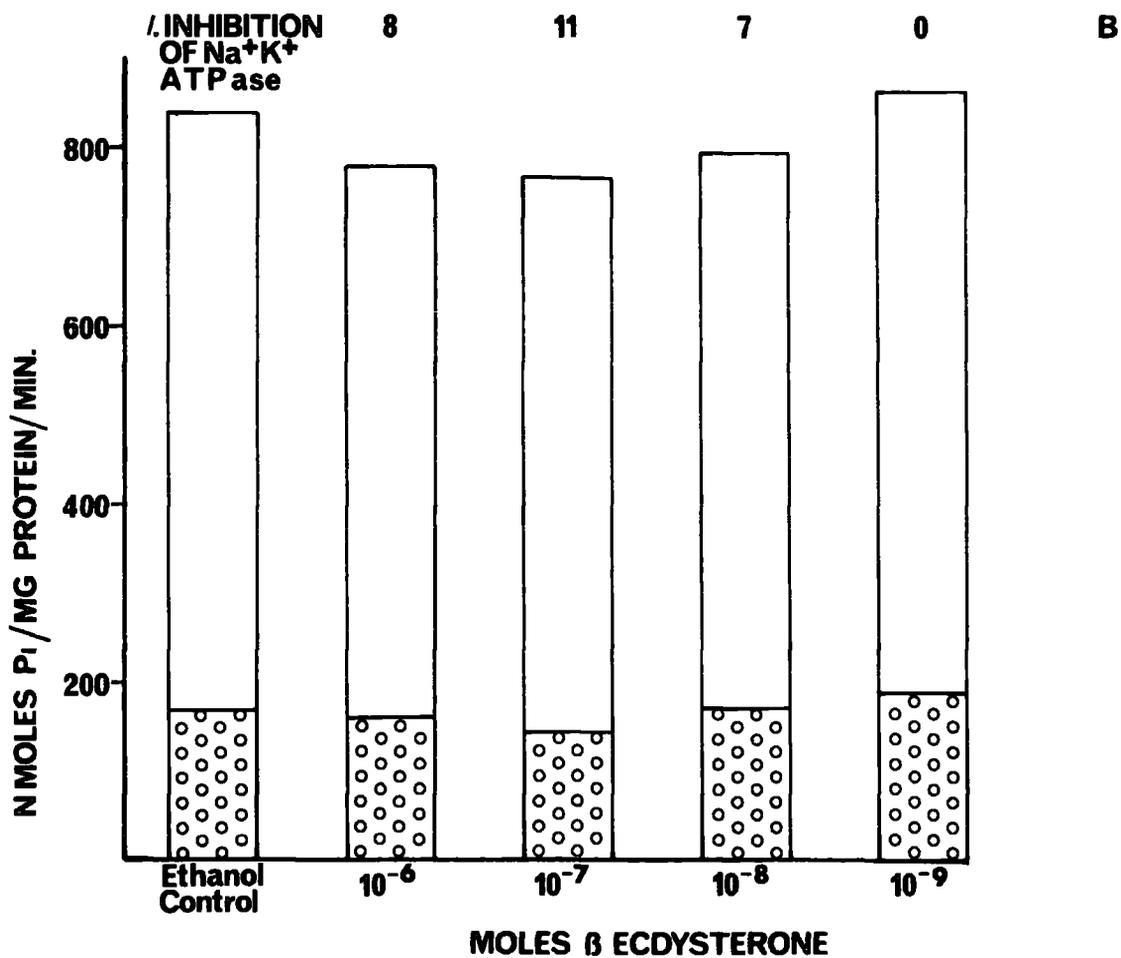
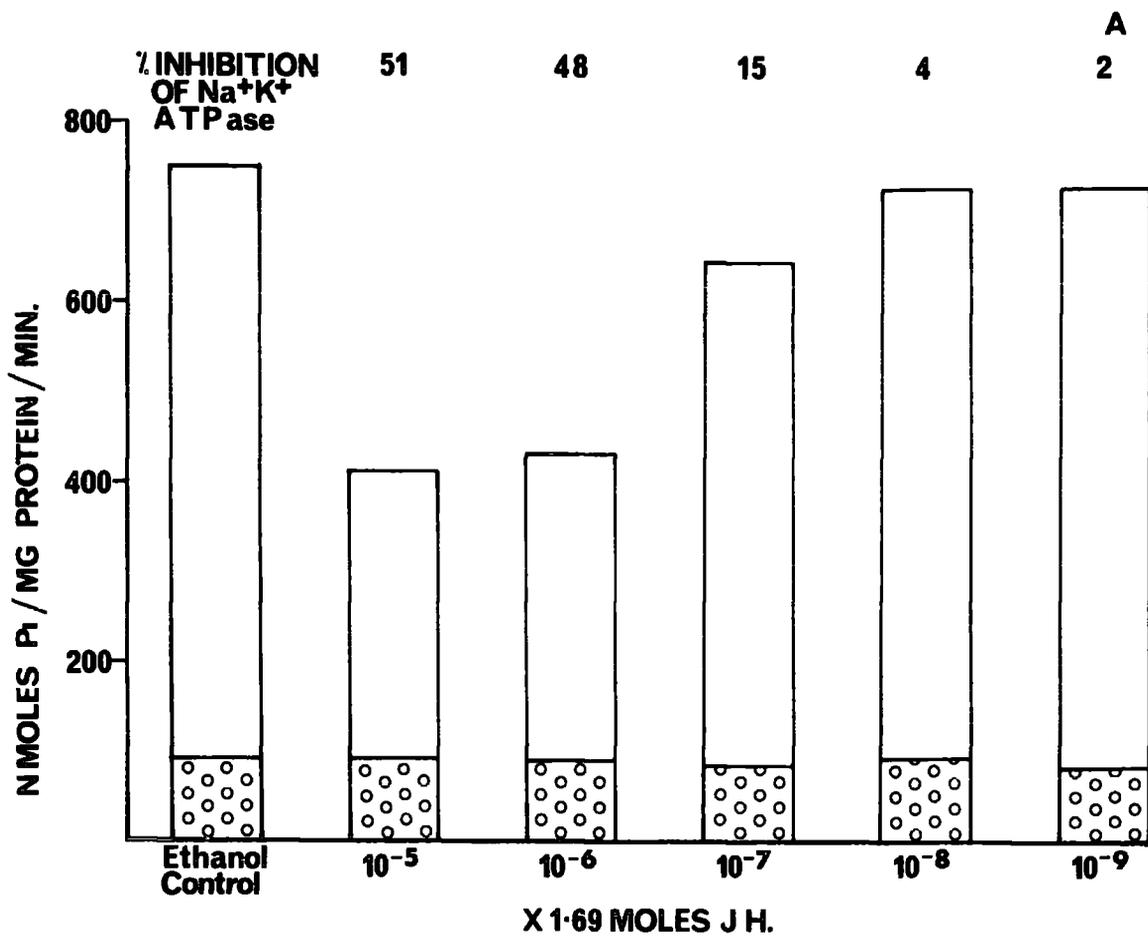


Fig. 7.4

The inhibitory effect of synthetic juvenile hormone
on the activity of a microsomal preparation of a
Na⁺ K⁺ ATPase

Legend:

ordinate ; % inhibition of the Na⁺ K⁺ ATPase

abscissa ; amount of juvenile hormone
- log (1.69 x 10⁻⁹ - 1.69 x 10⁻⁵ moles)

▲ experiment 1

■ experiment 2

○ experiment 3

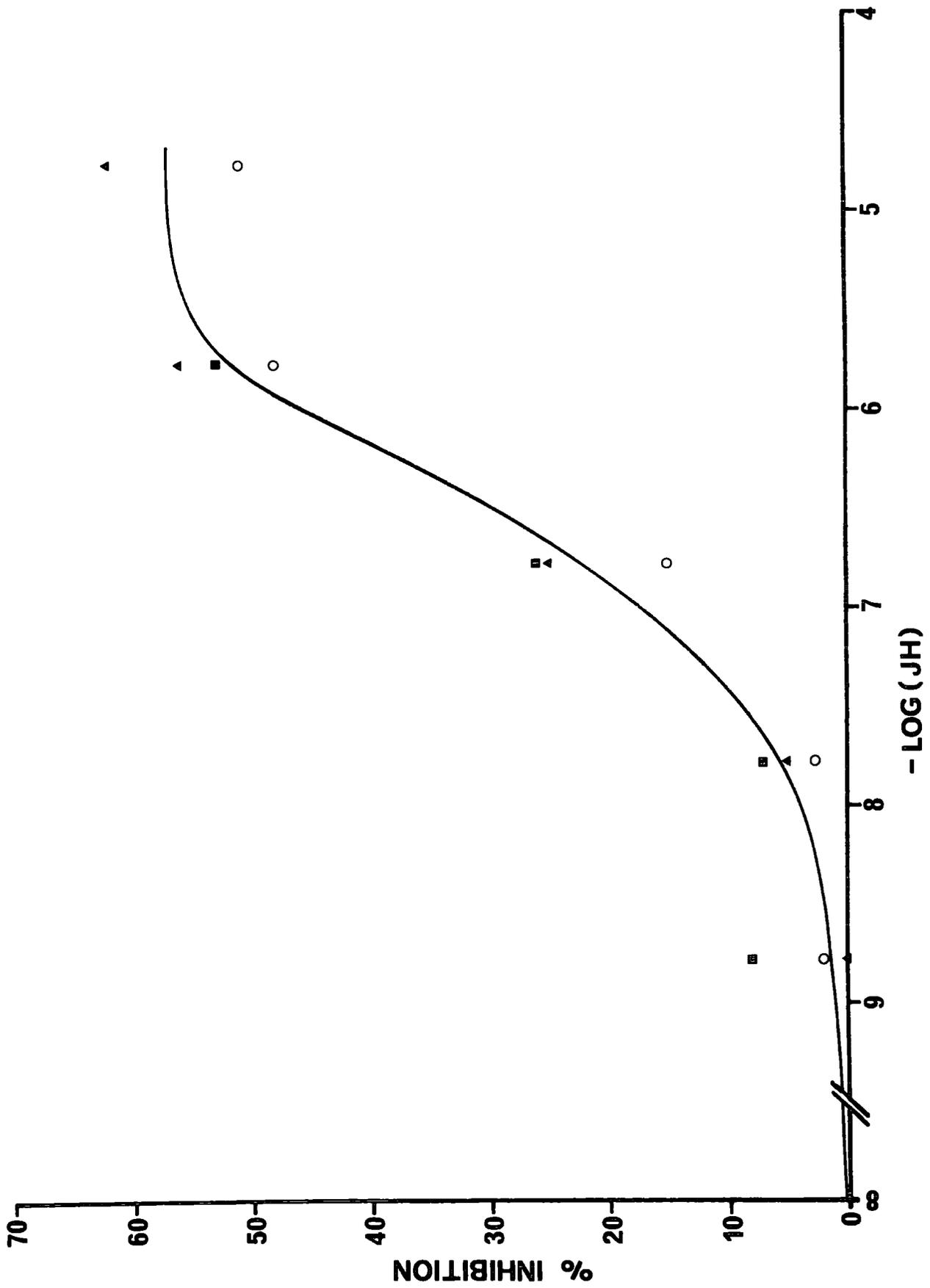


Fig. 7.5

The effect of synthetic juvenile hormone on the development of the yellow colour produced during the estimation of inorganic phosphate

Method

Standard phosphate solutions were made up by serial dilution of a stock solution (Sigma) containing $20\mu\text{g}$ phosphorus as KH_2PO_4 . $10\mu\text{l}$ of J.H. at different concentrations was added to aliquots of the above followed by 4ml of Lubrol reagent. Absorbance was measured as described previously.

Legend:

ordinate ; absorbance nm

abscissa ; n moles P_i

- Control
- 10^{-6} moles J.H.
- 10^{-7} moles J.H.
- ▲ 10^{-8} moles J.H.
- 10^{-9} moles J.H.

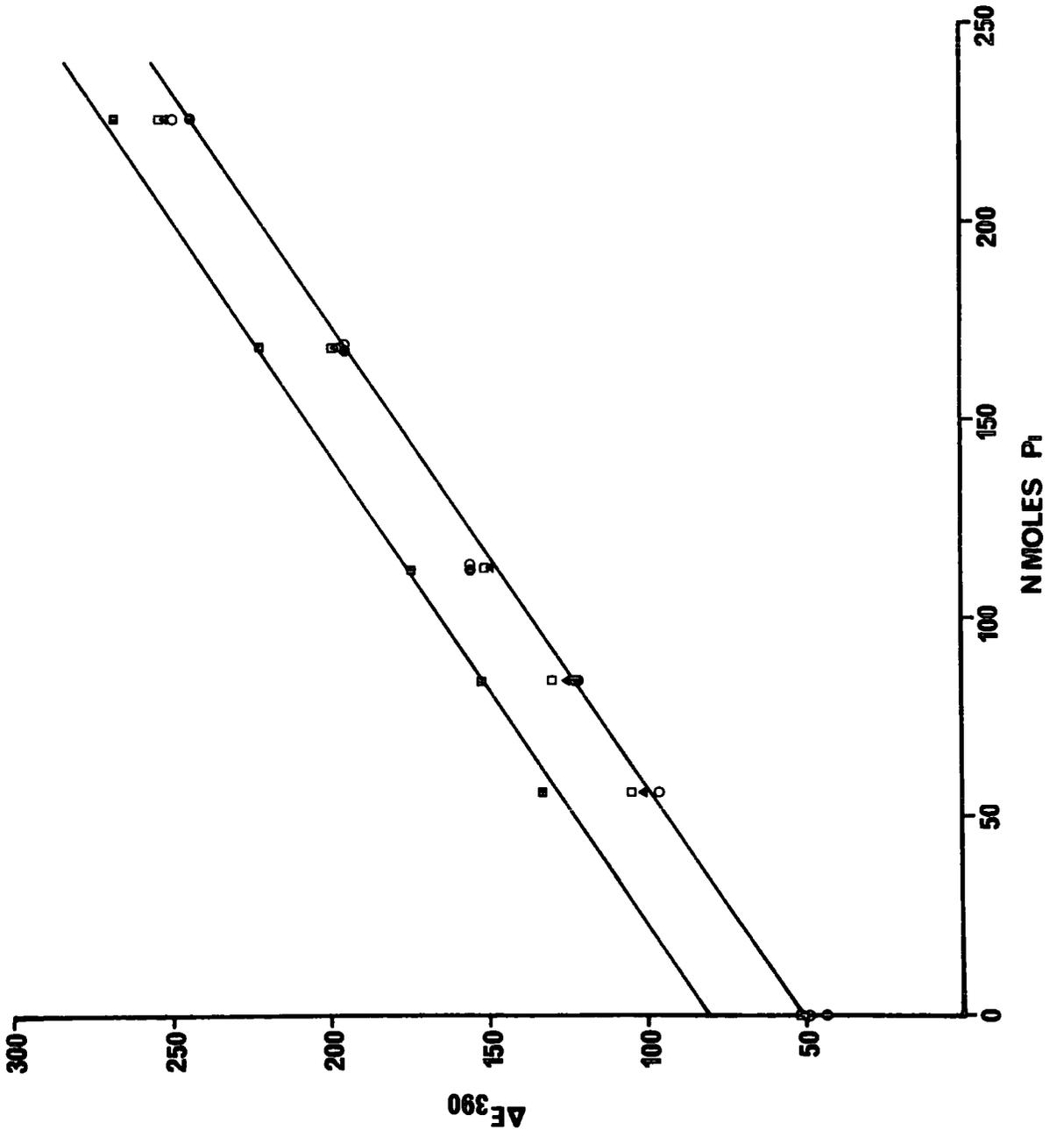


Fig. 7.6

The effect of the removal of the frontal ganglion on the activity of a microsomal preparation of a Na⁺ K⁺ activated Mg²⁺ dependent ATPase from the Malpighian tubules and rectal sac

A. Legend:

ordinate ; μ moles P_i/mg protein

abscissa ; time in minutes

- 1. Preparation from operated animals
- 2. Preparation from operated animals
- 3. Preparation from operated control animals

B. Legend:

ordinate ; μ moles P_i/mg protein

abscissa ; time in minutes

- 1. Preparation from operated animals
- 2. Preparation from operated control animals

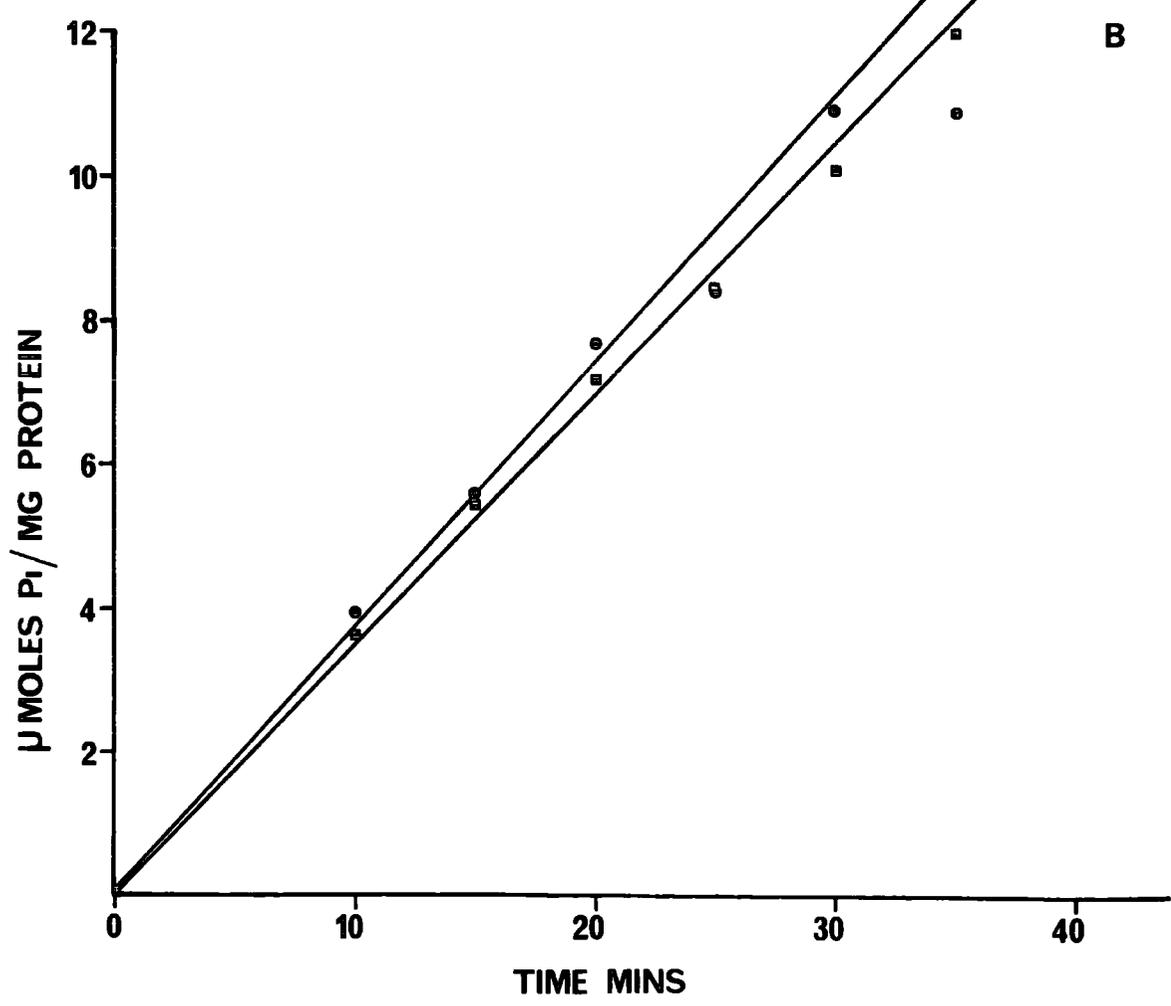
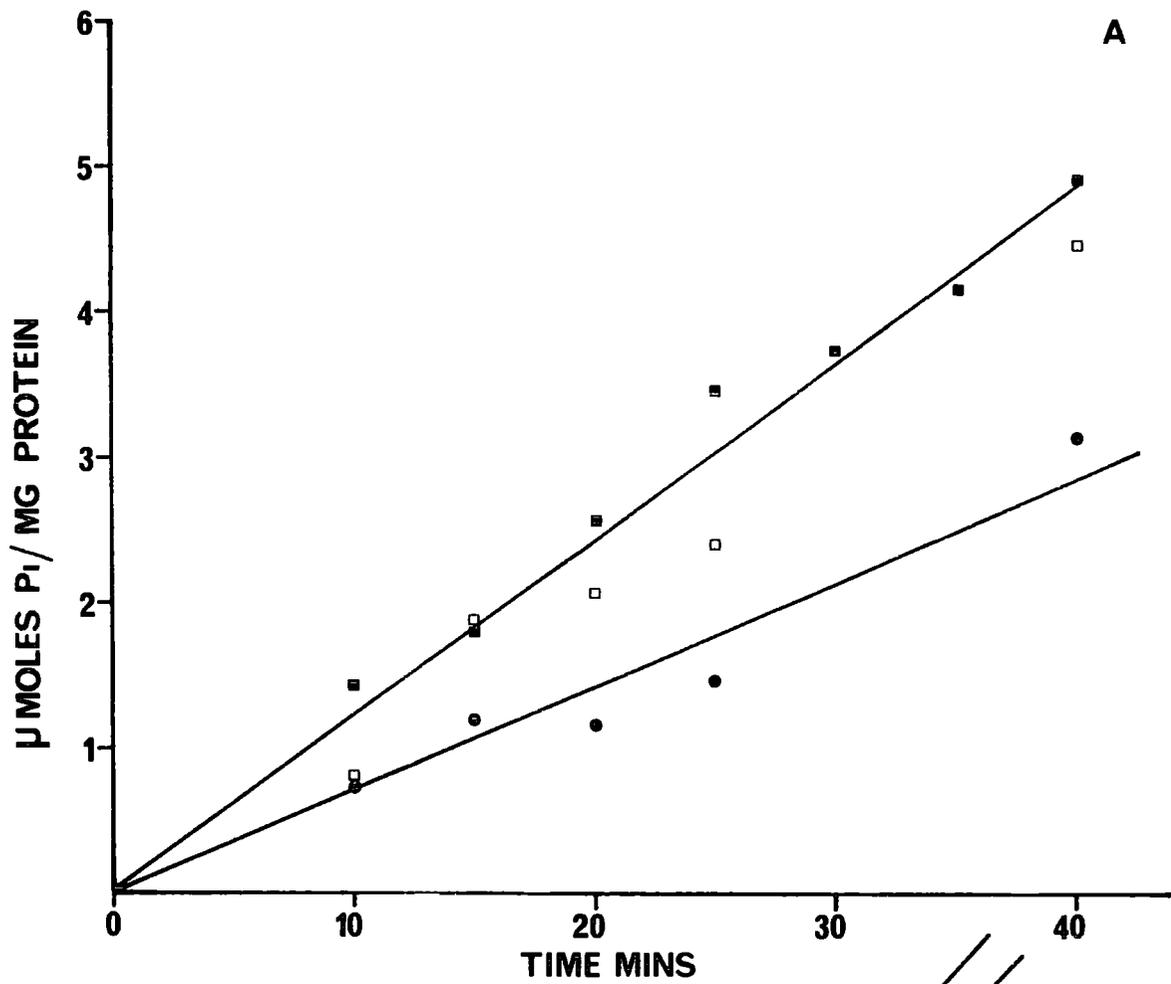


Plate 7.1

A. Low power electron micrograph of a T.S. of a Malpighian tubule from an operated control male.

Note, apical membrane (am), basement membrane (bm), basal infoldings (bf) have little space between them, no concretions, large tubule lumen (lu), mitochondria (m), absence of swollen tips to the microvilli (mv), plasma membrane (pm), few large vacuoles (v).

B. Low power electron micrograph of a T.S. of a Malpighian tubule from an operated male.

Note, apical membrane (am), basement membrane (bm), basal infoldings (bf), numerous concretions (c), large tubule lumen (lu), mitochondria (m), presence of microvilli with swollen tips (mv), nucleus (n), tracheole (t), many large vacuoles (v), some containing fine granular material.

C. Low power electron micrograph of a T.S. of a Malpighian tubule from a starved male.

Note, apical membrane (am), basement membrane (bm), basal infoldings (bf), few small concretions (c), small lumen (lu), straight microvilli some with swollen tips (mv), nucleus (n), plasma membrane (pm), a few large vacuoles containing granular material (v).

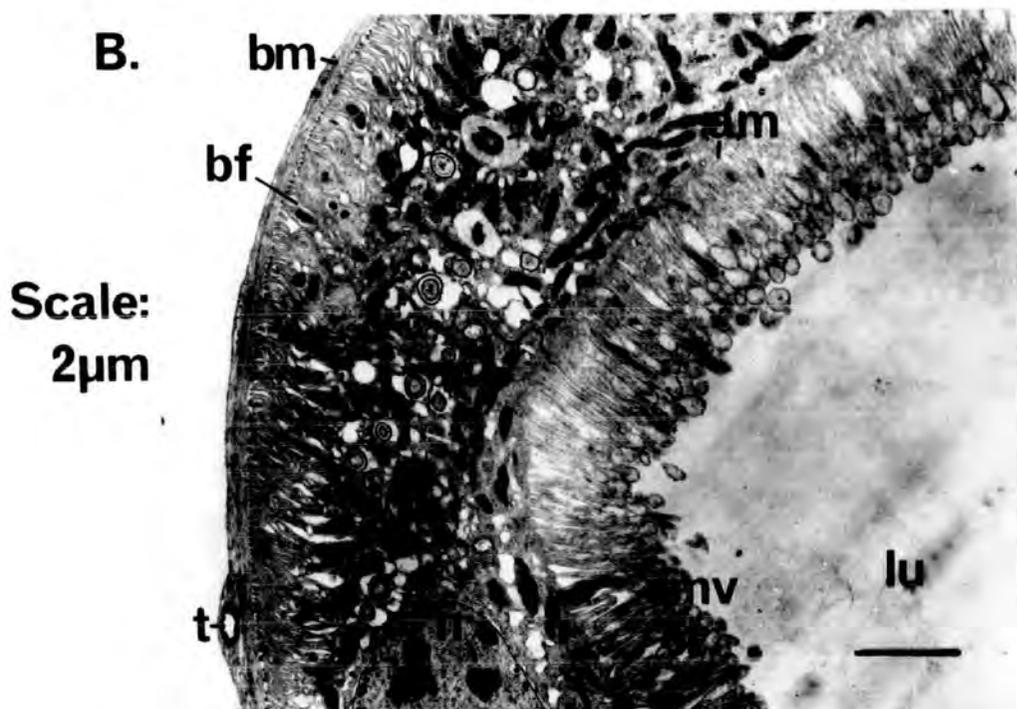
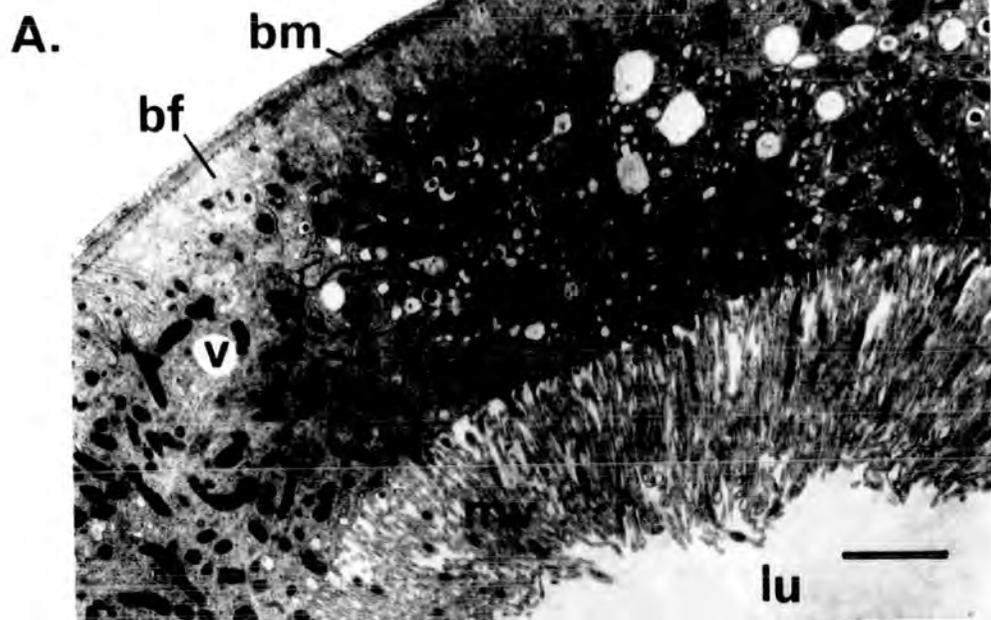


Plate 7.2

A. An electron micrograph of a T.S. of a Malpighian tubule from an operated control male showing the basal surface.

Note, the basement membrane (bm), basal infoldings with little extra cellular space (bf), electron dense tips (et), mitochondrion (m), small muscle on the surface of the tubule (mu), few vacuoles (v).

B. An electron micrograph of a T.S. of a Malpighian tubule from an operated male showing the basal surface.

Note, the basement membrane (bm), basal infoldings with dilated extra cellular spaces between (bf), concretions (c), electron dense tips (et), mitochondrion (m), many vacuoles (v).

Scale: 0.5 μ m

A.



B.

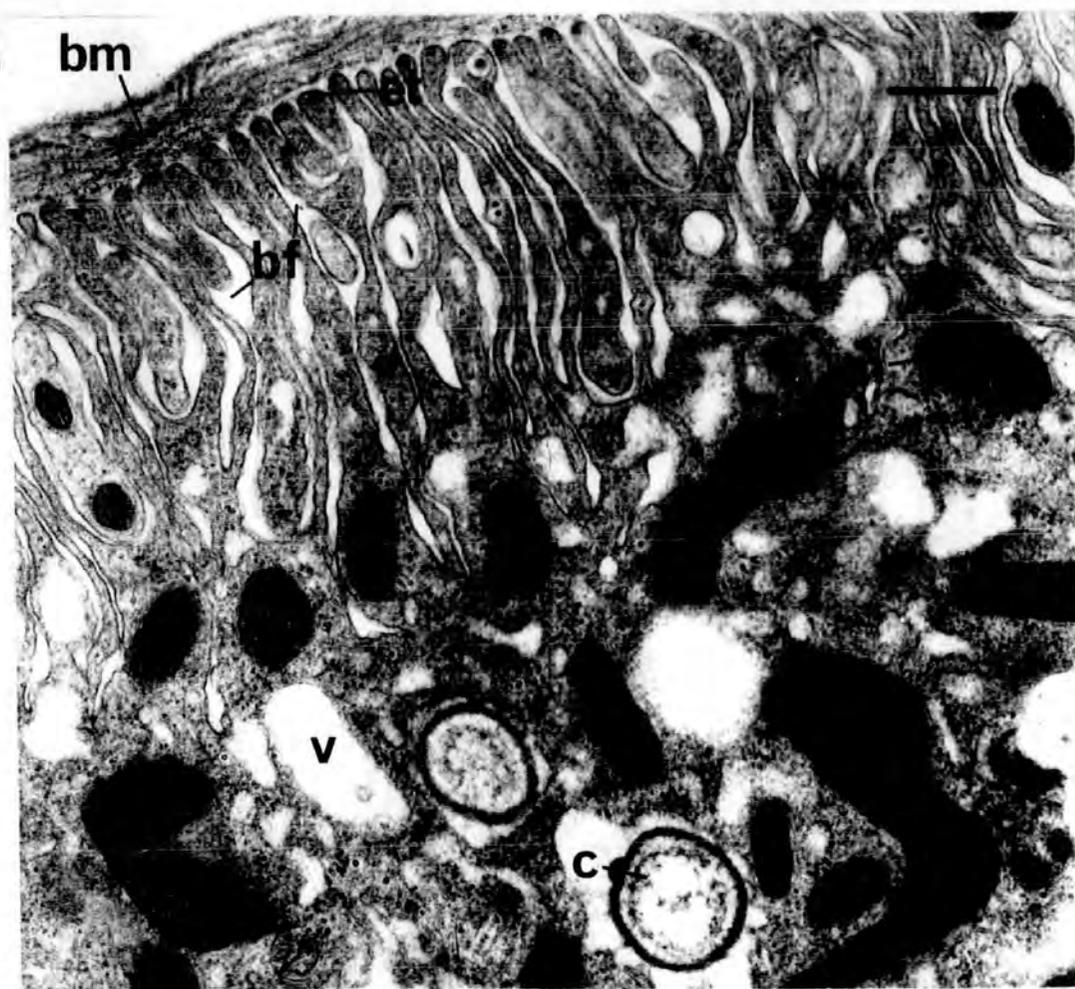


Plate 7.3

An electron micrograph of a T.S. of a Malpighian tubule from a starved male showing the basal surface.

Note, basement membrane (bm), narrow extra cellular spaces between the basal infoldings (bf), electron dense tips (et), mitochondrion (m), few vacuoles (v).

Scale: 0.5 μm

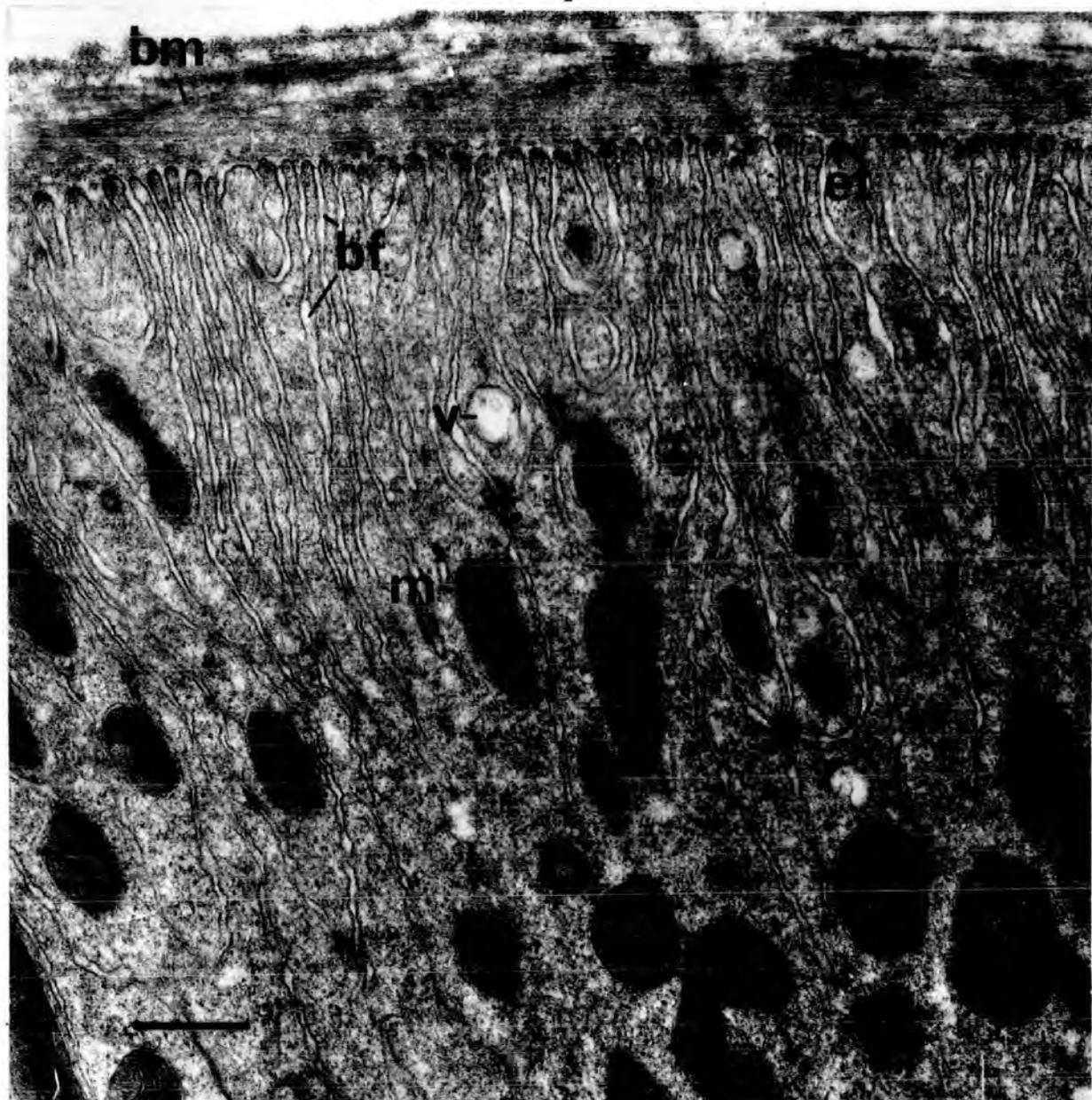


Plate 7.4

A. An electron micrograph of a T.S. of a Malpighian tubule from an operated male showing the apical surface.

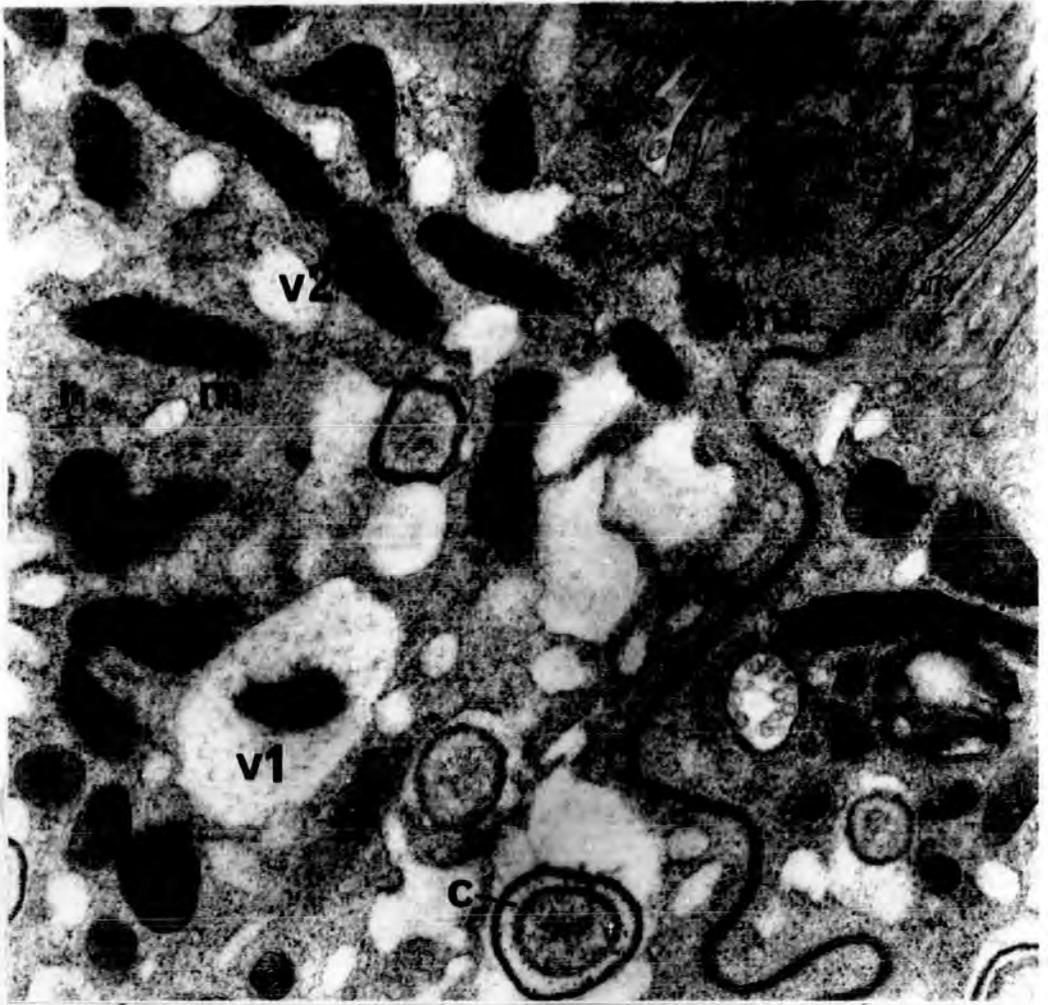
Note, concretions (c), septate desmosome on the plasma membrane (sd), a short distance on the plasma membrane without a desmosome and then the macula adhaerens desmosome (ma), mitochondrion (m), microvilli (mv), many free ribosomes (r), two types of vacuole (v1) with inclusion bodies possibly related to the "formed bodies" of Riegel (1966 a, b) and vacuoles without (v2).

B. An electron micrograph of a T.S. of a Malpighian tubule from an operated control male showing the apical surface.

Note, microtubules (mt), microvilli (mv), mitochondrion (m), many free ribosomes (r), two types of vacuole (v1) with inclusion bodies possibly related to the "formed bodies" of Riegel (1966 a, b) and vacuoles without (v2).

Scale: 0.5 μm

A.



B.

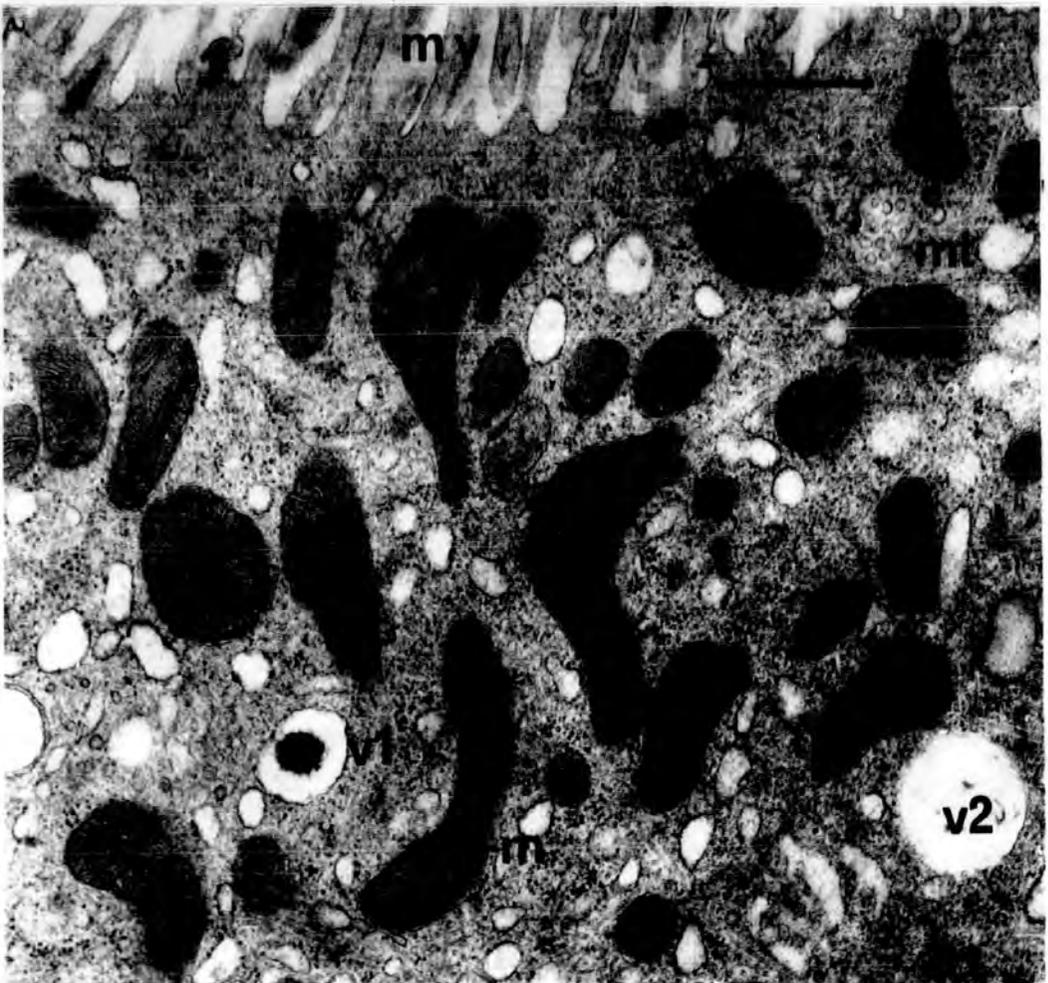


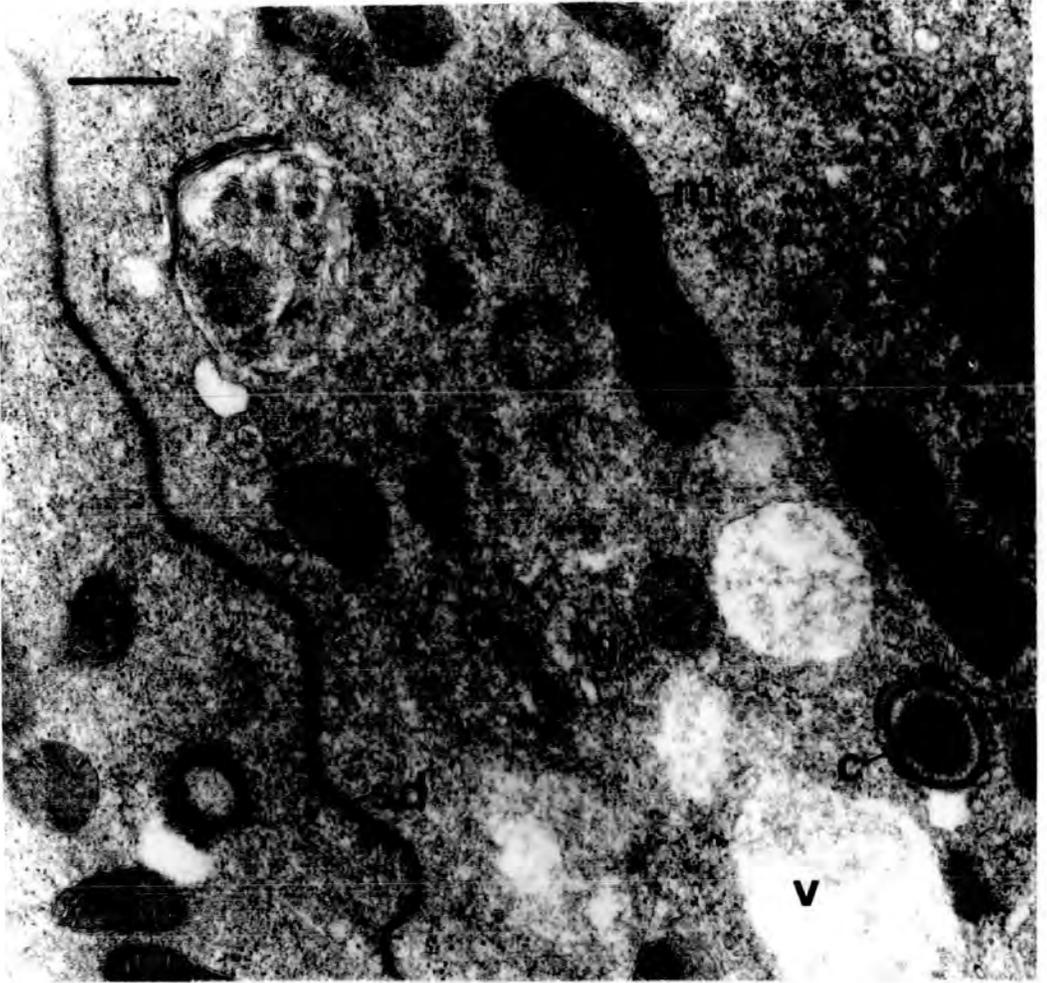
Plate 7.5

A. An electron micrograph of a T.S. of a Malpighian tubule from a starved male showing the cytoplasm just below the apical membrane. Note, concretion (c), Golgi body (g), mitochondrion (m), many free ribosomes (r), septate desmosome on the plasma membrane (sd), vacuoles without inclusion bodies but containing some granular material (v).

B. An electron micrograph of a T.S. of a Malpighian tubule from an operated male showing the microvilli (mv) of the apical membrane. Note, long thin mitochondrion in the tubule (m), swollen tips of the microvilli with pinocytotic-like vesicles (t), macula adhaerens desmosome on the plasma membrane (ma).

Scale: 0.5 μ m

A.



B.

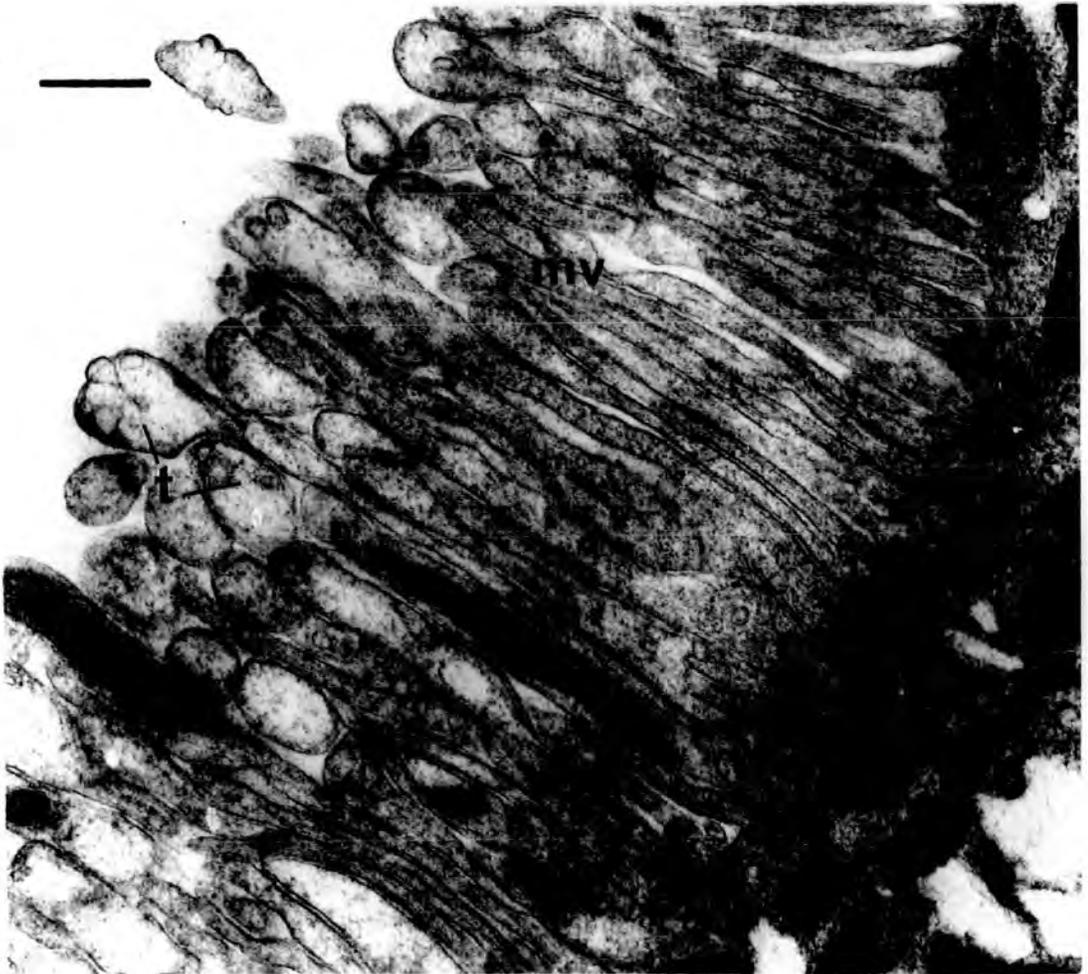


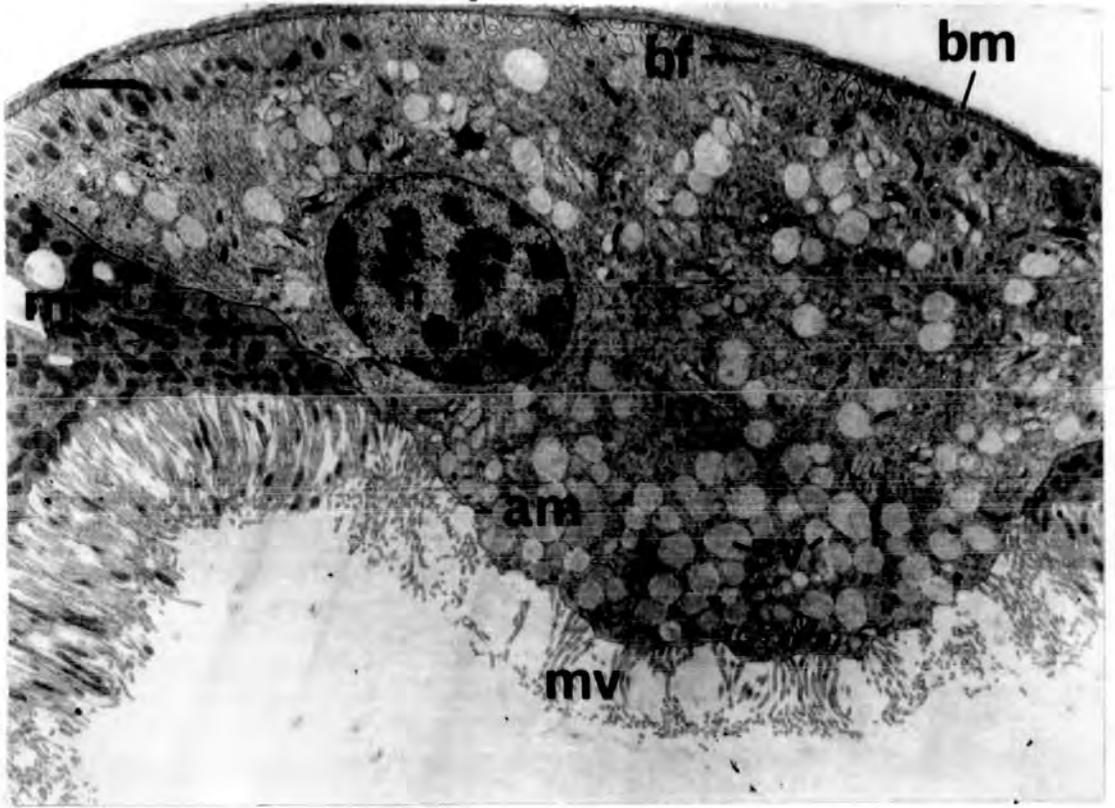
Plate 7.6

A. Low power electron micrograph of T.S. of a Malpighian tubule from a normal adult male showing a secondary or "secretory" cell. Note, apical membrane (am), basement membrane (bm), basal infoldings (bf), few small mitochondria (m), few microvilli (mv), "secretory" vacuoles (sv).

B. An electron micrograph of a T.S. of a Malpighian tubule from a normal adult male showing a large Golgi body (g). Note, many vacuoles associated with the Golgi body similar to the secretory vacuoles (sv) found throughout the cytoplasm, the secretory vacuoles contain a granular material, transversely cut Golgi vesicles (gv), mitochondrion (m), rough endoplasmic reticulum (rer).

Scale: 2 μ m

A.



Scale: 0.5 μ m

B.

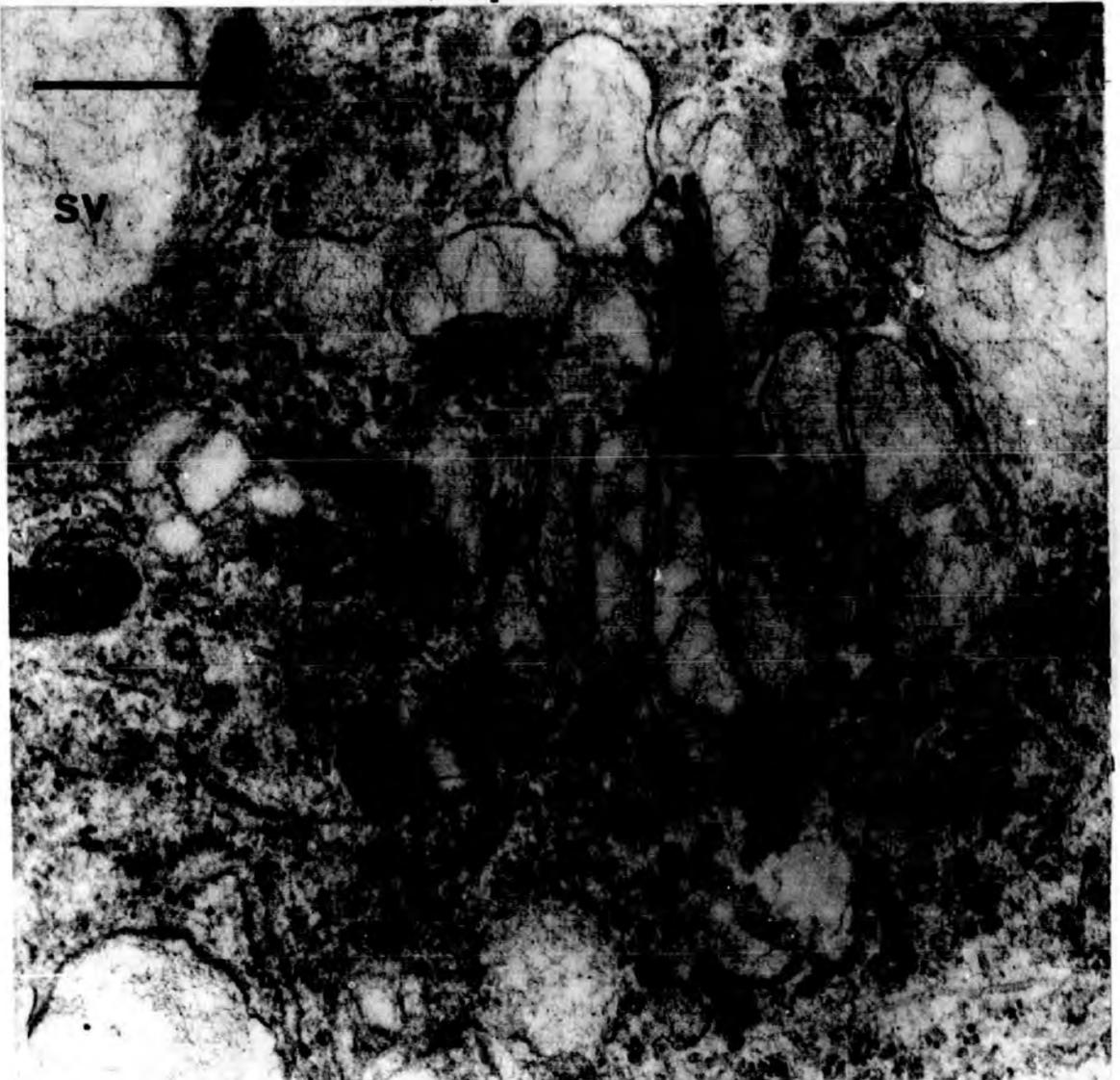


Plate 7.7

A. An electron micrograph of a T.S. of a Malpighian tubule from a normal adult male showing the basal region of a "secretory" cell.

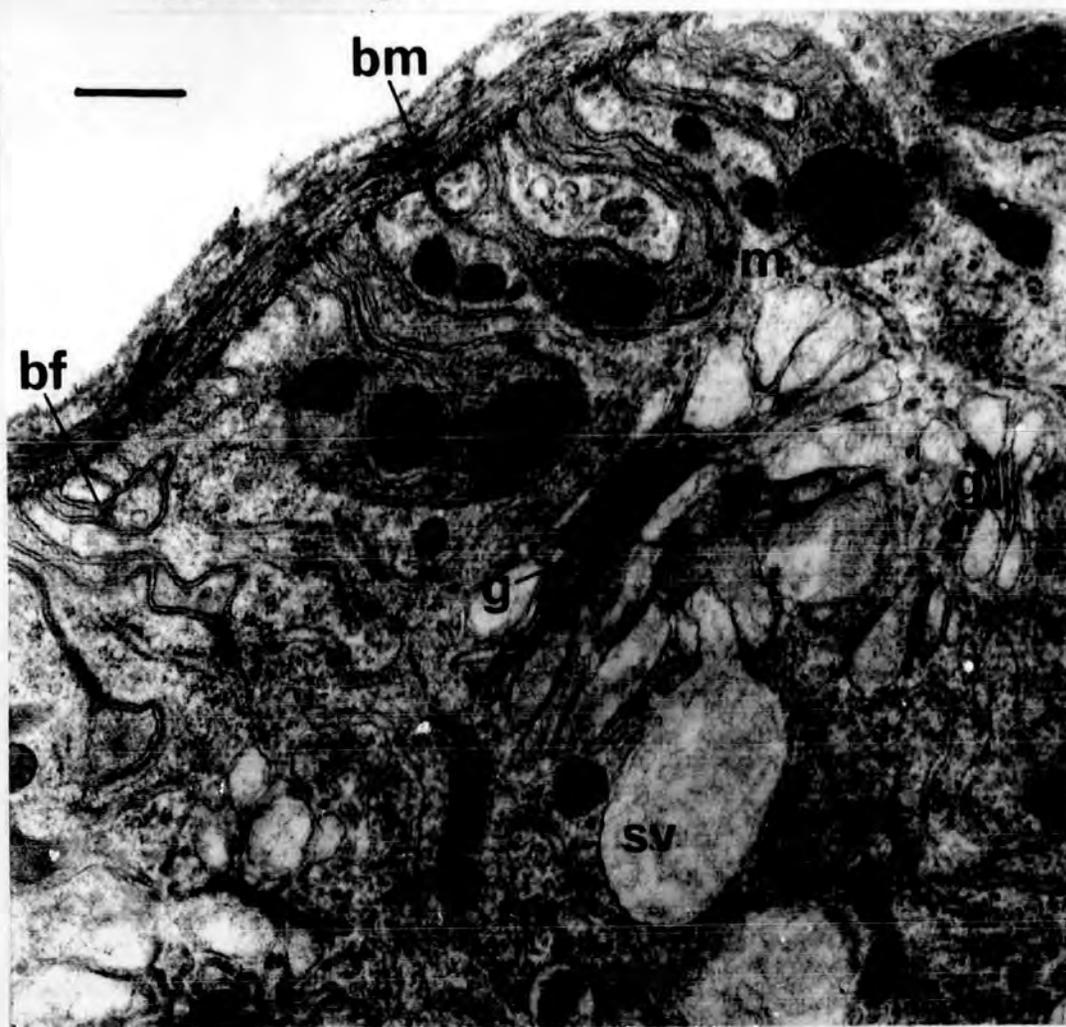
Note, basement membrane (bm), few basal infoldings that do not penetrate very far into the cell (bf), highly developed Golgi bodies (g), small mitochondrion (m), rough endoplasmic reticulum (rer), secretory vacuoles (sv), associated with the Golgi body.

B. An electron micrograph of a T.S. of a Malpighian tubule from a normal adult male showing the apical region of a secretory cell.

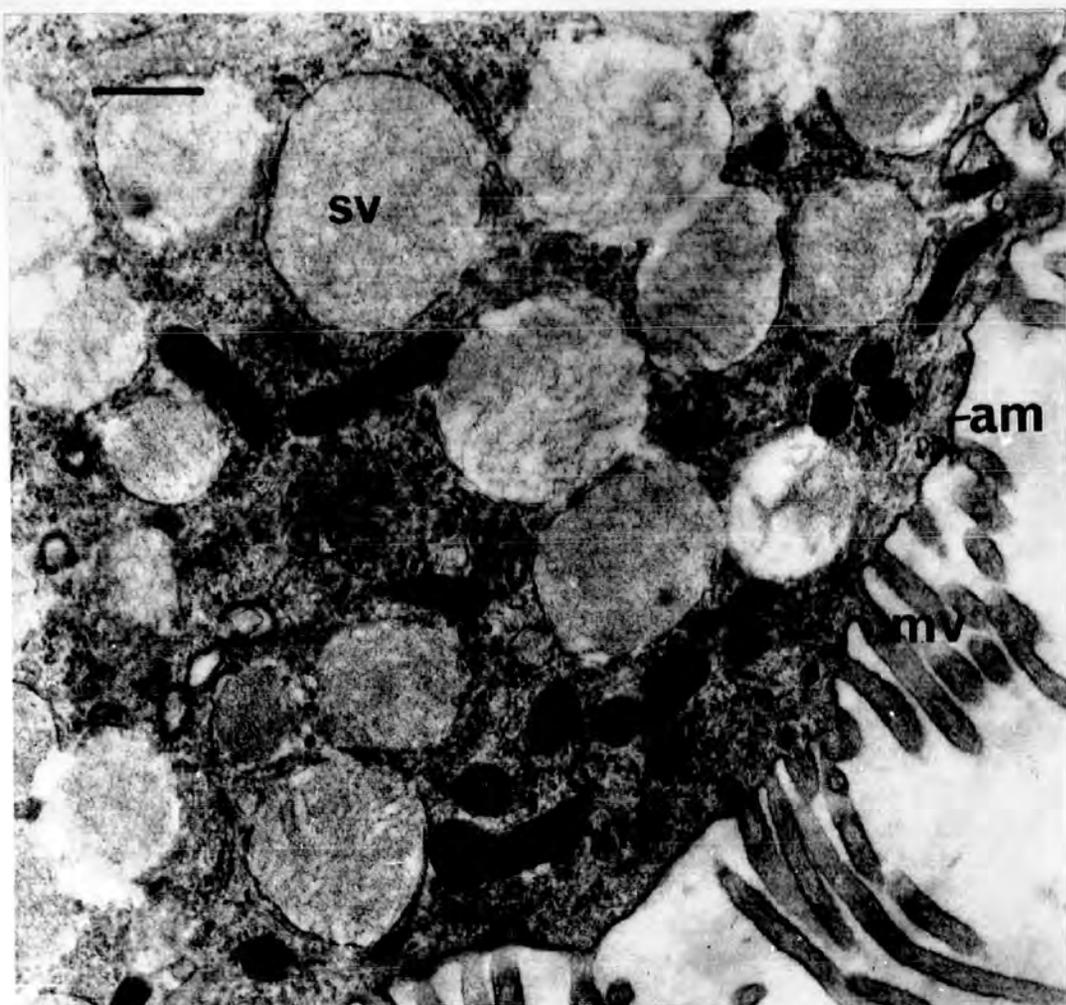
Note, apical membrane (am), transversely cut Golgi vesicles (gv), small mitochondrion (m), secretory vacuoles (sv) containing granular material.

Scale: 0.5 μ m

A.



B.



Discussion

There are great discrepancies in the literature between values quoted for the haemolymph volumes of both Locusta and Schistocerca. Using a C¹⁴ inulin method similar to the one employed in the present study, HILL ET AL (1968) found that the haemolymph volume of newly moulted adult male Schistocerca gregaria was 373 μ l which increased to 569 μ l by the 6th day of adult life. However, LEE (1961) found a volume of 305 μ l in newly moulted Schistocerca adult males that decreased to 176 μ l on day 4. BEENAKKERS (1973) also found a high haemolymph volume on the 1st day of adult life in Locusta and a decline over the next few days. The haemolymph volumes reported in the present work were somewhat higher than those of animals of similar age in BEENAKKERS (1973) work. However, a similar situation was found to prevail; namely that 5 days after the operation, control animals had a smaller haemolymph volume than just after the last moult. LEE (1961) suggested that the increase in haemolymph volume/unit weight preceding ecdysis occurred at the expense of cellular water and was maintained for 24 hours after the moult to enable the cuticle to harden at maximum body volume. This allowed for later growth.

The reduction in haemolymph volume noted in operated animals was consistent with a similar phenomenon observed in frontal ganglionectomised Schistocerca by HILL ET AL (1966). They suggested that a release of diuretic and other neurosecretory hormones still occurred in frontal ganglionectomised Schistocerca gregaria (MORDUE, 1969; HIGHNAM ET AL, 1966) and that death was brought about by dehydration, due to a restricted intake of water into the midgut and a continued release of diuretic hormone. CLARKE AND LANGLEY (1963e), CLARKE AND ANSTEE (1971b), however, have suggested that a greatly reduced release of neurosecretion is a consequence of frontal ganglion removal in Locusta larvae, and a

similar situation would appear to hold in the adult (see Chapter 2). Therefore, it seems unlikely that the dramatic reduction of haemolymph volume in operated adults was caused by continued release of diuretic hormone from the corpora cardiaca.

Apparently at odds with the above work are the findings of BERNAYS AND CHAPMAN (1972). They found that following distension of the foregut of 5th instar Locusta with agar there was a dramatic increase in the water content of the faeces. No such increase was observed when the frontal connectives were cut. They suggested a hypothesis similar to that employed by CLARKE AND LANGLEY (1963e) to explain their results, viz. information from stretch receptors on the foregut passes via the frontal ganglion and the frontal connectives to the brain, where it elicits release of diuretic hormone from the corpora cardiaca. This would suggest an anti-diuretic effect following the removal of the frontal ganglion. Discussion on possible reasons for the apparent dichotomy between BERNAYS AND CHAPMAN (1972) and the work of HIGHNAM ET AL (1966) and the present work, is reserved for the general discussion (Chapter 8).

The concentrations of Na^+ and K^+ in the haemolymph of operated and starved animals were not significantly different from the operated controls. OKASHA (1973) similarly found little effect of starvation and desiccation on these ions in Thermobia domestica, while WALL AND OSCHMAN (1970) found only a slight increase in Na^+ K^+ concentrations in dehydrated Periplaneta americana. However, contrary to the present work, HOYLE (1954) found a significant drop in the K^+ concentration of adult Locusta haemolymph after only 15 hours of starvation.

The concentration of Na^+ and K^+ ions found in the present work falls within the range reported by other workers for Locusta.

	Na ⁺	K ⁺
Hoyle (1954) fed	64-154 m moles/litre	12-29 m moles/litre
starved	73-156	7-16
Ramsay (1953) fed	74-102	15-33
Duchâteau (1953) fed	60	12

Although there was a dramatic reduction in haemolymph volume of frontal ganglionectomised animals this did not result in an increase in the concentration of Na⁺, K⁺ ions, which contribute 27% of the osmotic pressure of the haemolymph (calculated from SUTCLIFFE, 1963). This, of course, does not preclude an effect on the osmotic pressure. PENZLIN (1971) suggested a link between osmoreceptors and release of diuretic hormone, and found a 25% increase in osmotic pressure three days after the removal of the frontal ganglion from Periplaneta americana. However, diuretic stress, leading to a reduction of haemolymph osmolarity would account for the observations of CLARKE AND ANSTEE (1971a, b). They found that frontal ganglion removal from larval Locusta resulted in mitochondrial swelling and disruption in the fat body and midgut, with a consequent sharp increase in whole body succinic dehydrogenase activity.

PEACOCK (1975) has reported a decrease in activity of microsomal preparations of Na⁺ K⁺ activated Mg²⁺ dependent ATPase from hindgut and Malpighian tubules of larval Locusta that have had the frontal connectives severed. This may hinder the capability of the rectal sac to reabsorb water and ions from the primary "urine" of the Malpighian tubules and bring about a reduction in haemolymph volume. However, despite the fact that frontal ganglionectomy and frontal connective severance have similar effects (CLARKE AND LANGLEY, 1963d), frontal ganglion cautery in adult Locusta does not bring about a reduction in the activity of Na⁺ K⁺ Mg²⁺ ATPase from microsomal preparations of Malpighian tubules and rectal sac. One must, therefore, conclude that enzyme function has not been impaired and that if any effect on ATPase

ion pump is involved it is likely to be a direct one and not effected by means of synthesis.

Evidence which indicates that the Malpighian tubule system may be responsible to some extent for the observed reduction in haemolymph volume in operated animals is forthcoming from ultrastructural studies. Tubules from operated animals exhibited features that have been interpreted as indicative of gross movement of water in other tissues involved in transport, rectal sac in Calliphora erythrocephala (BERRIDGE AND GUPTA, 1967), gall bladder epithelium (WHITLOCK ET AL, 1965). In particular the extra cellular spaces between the basal infoldings were distended. A similar dilation of the subcuticular spaces of rectal sacs from Calliphora erythrocephala was observed by BERRIDGE AND GUPTA (1967) when they injected hypotonic solutions into ligated recta of starved flies. In contrast WIGGLESWORTH AND SALPETER (1962) found little change in the appearance of Malpighian tubules of Rhodnius prolixus within the first few hours after feeding, when there must have been a rapid flow of water across the cytoplasm of the tubules. They did, however, observe the appearance of swellings at the tips of the microvilli similar to those observed with tubules of operated animals in the present work. The nature of the concretions found in Malpighian tubule is disputed, but it is possible that they contain waste products (WALL ET AL, 1975). Therefore, an increasing incidence of these structures in tubules from operated animals may also be an indication of an increased movement of water across the tubules. A similar interpretation may perhaps be placed upon the many large vacuoles found in the cytoplasm of the Malpighian tubule cells of such animals.

By comparison with operated animals, starved animals had very narrow extra cellular spaces between the basal infoldings and the lumina of the tubules were almost completely excluded. Once again a

parallel is to be found in the work of BERRIDGE AND GUPTA (1967). They found that the subcuticular spaces of the rectal sacs from starved Calliphora erythrocephala were almost completely obliterated.

BERRIDGE AND OSCHMAN (1969) suggested an application of DIAMOND AND BOSSERT (1967, 1968) standing gradient hypothesis to explain the almost isosmotic urine production of the Malpighian tubules. Isotonic fluid enters the basal channels of the tubules and becomes progressively hypotonic towards the closed ends of the channels, because solute is transported into the cell by an ATPase ion pump. Hypotonicity within the channel provides the osmotic gradient for passive movement of water into the cell. The cytoplasmic compartments between basal infoldings may also contain standing osmotic gradients which favour entry into the cell. Similar osmotic gradients within the microvilli and the narrow channels between the microvilli may be involved in osmotic equilibration across the apical surface also. Based on this hypothesis, it is difficult to see how an increased movement of water across the Malpighian tubules of operated animals occurs without an increase in the activity of the ATPase ion pump. However, the hypotonicity within the channels of the basal infoldings required for the passive movement of water into the cell may be more easily achieved in the operated animal if the osmotic pressure of the haemolymph is very low. This is likely because although the concentrations of Na^+ and K^+ ions are unaffected (present Chapter) there are significant reductions in the concentrations of carbohydrates, proteins and lipid (Chapter 3). What is clear is that if the operated animal is losing water through the Malpighian tubule/gut system, it must be as much a mal-function of reabsorption by the rectal sac as it is to an increased production of urine by the Malpighian tubules.

The results presented in this Chapter confirm previous work which suggests a breakdown of the normal water balance system in frontal ganglionectomised locusts (ROOME, 1968; HILL ET AL, 1966; LANGLEY, 1962). ROOME,(1968) implicated a mal-function of the corpora allata in a similar derangement of the water balance system in frontal ganglionectomised Periplaneta americana. However, the corpora allata are not active in the adult life of Locusta from the 2nd day till after the end of the somatic growth period (JOHNSON AND HILL, 1975). Rather than a specific hormone or lack of hormone effect on the Malpighian tubule/rectal sac excretory system, the failure of the water balance system may well prove to be due to a general failure of water conservation.

The second type of cell found in the Malpighian tubules, which has not been considered in relation to frontal ganglion removal, for reasons outlined above was similar in appearance to cells reported in Malpighian tubules of other species. BERRIDGE AND OSCHMAN (1969) have observed stellate cells intermingled with larger epithelial cells in the tubules of Calliphora erythrocephala, so have WALL ET AL (1975) in Periplaneta americana. TAYLOR (1971) has also shown two types of cell in the Malpighian tubules of Carausius morosus. Two main functions have been suggested for these secondary cells, the secretion of mucous (MARTOJA, 1959, 1961) and the absorption of ions and water from the lumen (BERRIDGE AND OSCHMAN , 1969). BERKALOFF (1960) and MARTOJA (1961) have identified, by histochemical methods, cells in the Malpighian tubules of various Orthoptera that contain acid mucopolysaccharides. The cells described in the present work, judging by the development of the Golgi bodies and endoplasmic reticulum, are secretory in function and are more like those identified by MARTOJA (1959, 1961). The function of mucous secreted by these cells could as TAYLOR (1971) suggested, be

to provide a lubricant for passage of faeces along the gut. It is also possible that it serves as a lubricant for the movements of the tubular microvilli.

Juvenile hormone from the corpora allata may be an important diuretic factor in mature locusts. BEENAKKERS AND VAN DEN BROEK (1974), POELS AND BEENAKKERS (1969) have shown that J.H. promotes water retention in adult Locusta. They found that implantation of corpora allata resulted in loss of water, while STRONG (1968) observed that allatectomised male locusts had a reduced water content in comparisons with controls. WALL AND RALPH (1964) have shown the presence of an anti-diuretic factor in the corpora allata of Periplaneta americana.

In the light of the above work it is interesting to report that a synthetic J.H. at physiological concentrations has an inhibitory effect in vitro on the $\text{Na}^+ \text{K}^+$ ATPase exchange pump from the Malpighian tubule/rectal sac system (Fig. 7.3A and Fig. 7.4). This may well prove to be a specific effect since it is not mimiced to any great extent by related substances such as β ecdysterone and cholesterol (Fig. 7.3B, Table 7.8). The highest two concentrations of J.H. employed, produced a certain amount of cloudiness in the reaction medium due to precipitation of insoluble hormone. Therefore, the maximum of 56% inhibition was probably due to an inability to get sufficient J.H. into solution.

MINKS (1967) reported an inhibitory effect of J.H. on Mg^{2+} and DNP stimulated ATPase in mitochondria extracted from adult Locusta flight muscle. Mitochondrial ATPase is principally concentrated in the membrane and is of special interest for its role in active transport (SIEKEWITZ ET AL, 1958). MINKS suggested that J.H. had an effect on $\text{Na}^+ \text{K}^+$ pump which resulted in a decrease in the ATPase activity. This has certainly been confirmed using the present system.

CHAPTER 8

GENERAL DISCUSSION

During the course of the present investigation the removal of the frontal ganglion by electrical cautery has been shown to have dramatic effects on growth and the histology of the neuroendocrine system of young adult Locusta migratoria (Chapter 2). The observed cessation in growth and build up of neurosecretion in the nervi corporis cardiaci 1 confirmed the work of CLARKE AND LANGLEY (1963b - e), CLARKE AND GILLOTT (1967a, b), CLARKE AND ANSTEE (1971a, b), ROOME (1968) on larvae and STRONG (1966) and BIGNELL (1974) on adults of the same species. STRONG (1966) showed that newly moulted frontal ganglionectomised adult females survived at least 14 days. However, subsequently HILL AND STRONG (1966) expressed the view that a mean constant weight was only maintained by the fact that the feeding activity was reduced in some animals which lose weight and die, while other animals increased in weight. This was not upheld by the present work (Chapter 2). Although a high mortality was found, those that lived showed a constant low weight.

Contrary to the findings of CLARKE AND LANGLEY (1963d), ROOME (1968) and BERNAYS AND CHAPMAN (1973) have found that removal of the frontal ganglion reduced food intake and faecal output of larvae. Similar observations were made by HILL ET AL (1966) on frontal ganglionectomised Schistocerca gregaria larvae and on Locusta adults by BIGNELL (1974) and in the present work (chapter 2). Two schools of thought have grown up over the interpretation of these phenomena. CLARKE & GILLOTT (1965) maintained that operated animals showed no difference in feeding behaviour or in ability to defaecate in comparison with controls. The metabolic rate was low, and the food

intake was reduced because the hunger stimulus was reduced (ANSTEE, 1968). However, ANSTEE (personal communication, 1975) has accepted that a reduction in food intake may occur through mechanical damage but proposes that this would be sufficient for an animal with a low metabolic rate. In support of this contention a comparison has been made between frontal ganglionectomy in the locust and hypophysectomy in the rat. In both growth stasis occurs, protein synthesis and food intake are reduced and mitochondria swell and lose their integrity (CLARKE AND ANSTEE, 1971b). On the other hand the other school of thought holds the view that operated animals are in a state of semi starvation due to mechanical damage following the removal of the ganglion (HILL ET AL, 1966; HIGHNAM ET AL, 1966). However, CLARKE AND ANSTEE (1971a) using semi starvation diets of mixtures of cellulose and grass. found similar, but less severe, effects to starvation on the succinic dehydrogenase activity in 5th instar larvae. This involved a decrease in activity of this enzyme. Yet by comparison the operated animal exhibited an increase in the activity of this enzyme.

The possibility that operational trauma may be responsible for the effects of the removal of endocrine glands has been recorded many times. ENGELMANN (1968) found a 50% reduction in food consumption following cardiactectomy in Leucophaea maderae. KEELEY (1975) also showed that 50% less food was consumed when corpora allata and corpora cardiaca were removed from Blaberus discoidalis. However, in the latter case, efficiency of conversion of food material to dry weight increased two fold over operated controls and KEELEY suggested that a gross nutritional deficiency was unlikely in operated animals.

The frontal ganglion has been implicated in the control of gut movement. ROUSSEL (1966) found that removal of the frontal ganglion

from Gryllus bimaculatus blocked food passage. Movements of the gut appear to be myogenic in Locusta (CLARKE AND GRENVILLE, 1960) and Phormia regina (JONES, 1960). But passage of food through the crop into the midgut is dramatically reduced by severance of the nerves supplying the crop in a number of insects, Periplaneta americana (DAVEY AND TREHERNE, 1963), Gryllus bimaculatus (ROUSSEL, 1966). A possible mal-function of the ^{gizzard} is indicated in frontal ganglionectomised adult locusts. In the present work (Chapter 2) and BIGNELL (1974) the foregut of operated animals was found to be highly distended and in a number of animals there was a marked reduction in the amount of food in the mid and hindguts. A reduction in both food intake and faecal production would result from a dramatic slowing down of the movement of food from the crop into the midgut. The distribution of the activities of digestive enzymes in the gut lumen of operated animals also tends to support the contention that there is a mal-function of the gizzard (Chapter 5). A significant reduction was observed in the amount of enzyme activity in the foregut of operated animals, but not in the midgut. The presence of digestive enzymes in the foregut is dependant on regurgitation from the midgut (CHAPMAN, 1969). The activities of the six digestive enzymes investigated in the whole gut expressed in terms of product formed per gut region per minute were significantly lower in operated animals than control, but no difference existed between the two treatments when the results were expressed in terms of product formed per mg protein per minute. Therefore, it was proposed that a direct relationship existed between the amount of plant protein present in the gut and enzyme activity, in both operated and operated control animals. This might indicate that a reduction in enzyme activity in operated animals was related to a reduced intake of food rather than to a reduced synthesis per se.

ENGELMANN AND WILKENS (1969) found that a reduced protease activity in the midgut of Sarcophaga bullata following median neurosecretory cell cauterization was the result of a decreased food intake caused by operational trauma rather than a neuroendocrine deficiency.

Therefore, the possibility exists that the reduction in metabolic rate in operated adult locusts indicated by the lower oxygen consumption per gram (Chapter 3) was a response to a poor nutritional state as suggested by HILL ET AL (1966) for Schistocerca gregaria larvae. However, that the effects of the removal of the frontal ganglion on the physiology of young adult Locusta cannot simply be explained as a result of semi starvation is shown by an ultrastructural study of the fat body (Chapter 6). The normal development and increase in size of the fat body cells in the first few days of adult life seen in operated control animals did not occur in operated animals. Rough endoplasmic reticulum was reduced and in places showed indications of the swelling noted by CLARKE AND ANSTEE (1971b), while there was a dramatic reduction in lipid droplets and glycogen granules. The picture was that of a quiescent organ. On the other hand, the fat body of starved animals was very different, mitochondria were very few in number and very dense, and there was very little structure in the cytoplasm which appeared as a mass of lucinae. Therefore it may be expected that the observed reduction in metabolism in operated and starved treatments followed different courses.

One result of a lack of neurosecretion and its effect on growth may, therefore, be a cessation of the normal development of the fat body. KEELEY (1972) has shown a hormone dependent phase in the development of the fat body mitochondria from Blaberus discoidalis. Cardiectomy in this animal results in a lack of development of the metabolic capability of the fat body. Similarly if adult

Blaberus discoidalis were prevented from chewing or swallowing by oral blockage, the neuroendocrine dependent phase in the maturation of the fat body mitochondria was inhibited. Lack of food or water did not prevent development. KEELEY (1974) proposed that a stretching of the pharynx or crop caused neural stimuli which resulted in the release of neurosecretion. This is similar to the mechanism proposed by CLARKE AND LANGLEY (1963e) for the control of the release of neurosecretion in Locusta. It is possible that removal of the frontal ganglion from newly moulted adult Locusta, which also blocks release of neurosecretion (Chapter 2), inhibits a neuroendocrine controlled development of the fat body, independent of any effect of a reduced nutrition.

The above hypothesis, as with the general case outlined by CLARKE AND LANGLEY (1963e), is dependant upon the interpretation of a build up of neurosecretory 'A' material within the nervi corporis cardiaci 1 as being due to a dramatic drop in its release. This is a view that has gained wide acceptance since it was first put forward by HIGHNAM (1961). However, HIGHNAM ET AL (1966) proposed that whereas this was true for starved Schistocerca gregaria, the reverse was true for frontal ganglionectomised animals. They suggested that while the rate of release of neurosecretion in operated animals was similar to that in controls, the rate of synthesis was much greater, resulting in a build up of neurosecretion. As they themselves state, interpretation of a static histological picture in dynamic terms is difficult. However, it is conceivable that the operation has different effects on Locusta and Schistocerca. HIGHNAM AND HASKELL (1964) have shown marked differences in the neuroendocrine control of maturation between the two species. But HIGHNAM ET AL (1966) interpretation of the effects of frontal ganglion removal on the endocrine system of Locusta

would appear to be incorrect, particularly in the light of more recent work by BERNAYS AND CHAPMAN (1972). They demonstrated that frontal connective severance, an operation shown to be similar to removal of the frontal ganglion (CLARKE AND LANGLEY, 1963d), dramatically reduced the release of a hormonal factor from the storage lobes of the corpora cardiaca that controls the chemosensillae on the maxillary palps. They found that the normal increase in electrical resistance across the sensilla, seen when the foregut is distended, can be brought about by injections of corpora cardiaca extract. Severance of the frontal connectives stopped the normal increase.

The study of the effects of frontal ganglion removal on the ultrastructure of the fat body of young male adult locusts confirmed biochemical observations (Chapter 3) that energy reserves were dramatically reduced. Determination of glycogen, nonpolysaccharide carbohydrate and total lipid showed that these substances were all present in significantly smaller amounts in operated than operated control animals. This was not due to an increased release into the haemolymph. Therefore the reduced levels of energy reserves in operated fat body could have been due to either an inability of the under developed fat body to make use of metabolites made available and/or a reduction in available precursors. Although the activity of digestive enzymes when expressed in terms of product formed per gut region per min were lower in the operated than the operated control, this was related to a reduced plant protein content in the gut. Therefore, a reduction in efficiency of digestion is unlikely. But a reduction in food intake would itself reduce the available precursors.

Extensive evidence has built up for the hormonal control of the mobilization of energy reserves (see introduction to Chapter 3). The adipokinetic hormone (GOLDSWORTHY, MORDUE AND GUTHKELCH, 1972) and hyperglycaemic factor (GOLDSWORTHY, 1969) are found in glandular lobes of the corpora cardiaca of Locusta. However, while frontal ganglion removal has been shown to block release of neurosecretion from the storage lobe of the corpora cardiaca (CLARKE AND LANGLEY, 1963e; CLARKE AND ANSTEE, 1971b; present work Chapter 2), there is no evidence to show a similar effect on the glandular lobe, as there is no secretory 'A' material present there. It was suggested earlier in the present work (Chapter 3) that the failure of the operated animals to show the increase in haemolymph lipid concentration seen in the operated control in the five days following the operation was possibly due to a lack of adipokinetic hormone. However, the possibility cannot be excluded that the hormone is available but that the fat body cannot respond to it.

JUNGREIS AND WYATT (1971) have proposed that the trehalose concentration in the haemolymph of Hyalophora cecropia larvae is controlled by the rate of production rather than utilisation. This also seems to be the case in the frontal ganglionectomised Locusta adult. Since the oxygen consumption of operated animals is reduced, utilisation of reserves must also be less. Such a reduced utilisation should not stress a synthetic machinery that has normal capacity. Yet the haemolymph carbohydrate concentration of the operated animal was lower than the operated control indicating that the rate of synthesis was reduced. Preliminary experiments on the ability of operated fat body to incorporate C^{14} glucose into neutral lipid suggested a reduced capacity for triglyceride synthesis. Therefore, it would appear that both the structure and enzyme complement of

the fat body from frontal ganglion cauterised adults was underdeveloped. The observed reduction in the quantity of energy reserves may thus have been a secondary effect brought about by a direct action on the protein synthesis. This supports the hypothesis of CLARKE AND GILLOTT (1967b). They propose that neurosecretory material suppresses the inhibitory feedback of a number of different metabolic products onto their respective genes. Removal of the frontal ganglion dramatically reduces the release of neurosecretion and, therefore, reduces mRNA and protein synthesis. Evidence to support this hypothesis has come from the work of CLARKE AND GILLOTT (1967a, b), ROOME (1968), CLARKE AND ANSTEE (1971a, b) on Locusta larvae which has already been discussed in the General Introduction. In the present work operated adults were found to have a reduced protein concentration in the haemolymph in comparison with operated controls. This may be a result of reduced synthesis by the fat body, as BIGNELL (1974) has shown that fat body from a young adult Locusta has a decreased ability to incorporate C¹⁴ valine into protein in vitro.

BIGNELL (1974) found a certain amount of overlap between the amounts of protein and rate of incorporation of labelled amino acids into operated and operated control fat bodies. He accounted for this using the observations made by ALLUM (1973) during the course of a morphological study. ALLUM found that the posterior pharyngeal nerve sometimes joins the recurrent nerve rather than entering the frontal ganglion. In the latter case, the removal of the frontal ganglion would not destroy the pathway from the stretch receptors on the foregut to the brain. However, in the present work a significant difference was observed between operated and operated control treatments in relation to the biochemical parameters investigated viz,

the levels of carbohydrate and lipid in fat body and haemolymph. It may be that the locusts used in the present study had less variability in the position of the nerves innervating the frontal ganglion.

A dramatic reduction in haemolymph volume was observed in operated adults (Chapter 7). In a number of insects there appeared to be none at all. This confirmed observations by HILL ET AL (1966) on Schistocerca gregaria and PENZLIN AND STOLZNER (1971) on Periplaneta americana. The latter workers suggested that cutting the frontal connectives resulted in a loss of inhibition and consequently in an increased release of diuretic hormone (BERRIDGE, 1966; HIGHNAM ET AL, 1966) from the brain. HILL ET AL (1966) also implicated a release of a diuretic factor to explain the reduction in haemolymph volume and tissue water observed in frontal ganglionectomised Schistocerca larvae. It has been argued elsewhere in this thesis (Chapter 7) that this is unlikely to account for the observed water loss in operated Locusta adults as frontal ganglion removal causes a decrease in neurosecretory release. This is supported by BERNAYS AND CHAPMAN (1972) who found a decrease in water content of the faeces following the severance of the frontal connectives in Locusta larvae and proposed, as expected, a reduction in the release of diuretic hormone. However, a reduction in diuresis would appear to be at odds with the signs of chronic loss of water observed in operated Locusta adults (present work Chapter 7) and Schistocerca gregaria (HILL ET AL, 1966). It is conceivable that although the initial effect of frontal ganglion removal is to reduce water loss, in the long term there is a general breakdown of water balance due to a drastic reduction in protein synthesis. The experiments performed by BERNAYS AND CHAPMAN employed animals 26 hours after the operation, whereas in the present work experimental animals were used 5 days after treatment.

CLARKE AND GILLOTT (1965), ANSTEE (1968) have argued that the reduced intake of food by frontal ganglionectomised Locusta larvae is adequate for animals which have a reduced or basal metabolism. However, in young adult Locusta removal of the frontal ganglion has been shown to affect the normal passage of food from the foregut into the midgut (present work Chapters 2 and 5; BIGNELL, 1974). Therefore, it is impossible to tell whether the observed reduction in oxygen consumption and energy reserves in young operated adults was a result of reduced food intake or reduced release of metabolic hormones. This is inspite of the fact that the operated insects had significantly larger levels of carbohydrate and lipid, in haemolymph and fat body, than comparable starved animals. Also evidence has been put forward to indicate that in certain respects young adult Locusta migratoria respond differently to frontal ganglion removal and starvation, in particular the ultrastructure of the fat body (Chapter 6) and Malpighian tubules (Chapter 7). Much evidence has accumulated implicating the frontal ganglion in the control of growth and protein metabolism in larvae (CLARKE AND LANGLEY, 1963d; CLARKE AND GILLOTT, 1967a, b; CLARKE AND ANSTEE, 1971a, b). This includes experiments by CLARKE AND GILLOTT (1967a) in which a permanent 100% increase in weight was achieved in operated animals by injecting extracts of corpora cardiaca. However, from the work presented in this thesis it must be concluded that in young adult Locusta the possibility that semi-starvation is involved in the effects of frontal ganglion removal cannot be ruled out.

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APPENDIX

Section 1

Table 1.1

The Kinetic constants of α D glucosidase

Lineweaver-Burk					Eadie-Hofstee				
pH	Km	Vmax	Slope	r	Fig	Km	Vmax	Slope	r
6.45	2.649	0.385	6.877	0.997	4.4A	2.505	0.374	-2.504	0.985
	2.694	0.468	5.761	0.999		2.723	0.472	-2.723	0.972
5.4	1.478	1.019	1.450	0.995	4.4B	1.449	1.010	-1.449	0.995
	1.578	1.046	1.509	0.999		1.489	1.023	-1.489	0.992
	1.417	1.126	1.258	0.999		1.414	1.126	-1.414	0.998
	1.797	1.009	1.782	0.999		1.783	1.006	-1.783	0.993
pH	temperature °C	range	activation energy ₋₁ Ea, K joules mole ⁻¹		Slope	r	Fig		
6.45	48.5	- 30.8	58.19		-3.039	0.998	4.5		
	30.8	- 17	64.62		-3.375	0.998			
	49	- 28.8	49.97		-2.610	0.997			
	28.8	- 17	71.15		-3.716	0.996			
	44.9	- 32	49.39		-2.580	0.999			
	32	- 16.3	70.79		-3.697	0.998			
5.4	48.75	- 34.8	25.62		-1.338	0.916	4.5		
	34.8	- 19.5	66.88		-3.493	0.999			
	50.5	- 32.1	34.43		-1.798	0.994			
	32.1	- 18.3	61.10		-3.191	0.997			
	46	- 30	39.60		-2.068	0.995			
	30	- 17.75	64.62		-3.375	0.995			

Table 1.2

The Kinetic Constants of β D glucosidase

Lineweaver-Burk						Eadie-Hofstee					
pH	range of substrate concentration	Km	Vmax	Slope	r	Fig	Km	Vmax	Slope	r	
6.75	1.0 - 5.0	4.746	0.0986	48.13	0.998	4.7A	4.489	0.095	-4.489	0.983	
	0.6 - 2.6	4.545	0.1018	44.65	0.998		4.264	0.097	-4.264	0.970	
5.2	2.63- 7.5	5.691	0.745	7.639	0.977	4.7B	4.586	0.666	-0.459	0.898	
	0.5 - 2.63	2.208	0.437	5.052	0.999		2.192	0.436	-0.219	0.976	
	2.15- 9.0	4.311	0.453	9.516	0.994		4.179	0.447	-0.418	0.978	
	0.5 - 2.15	1.555	0.258	6.029	0.999		1.622	0.264	-0.162	0.988	
	1.69-9.0	3.292	0.496	6.637	0.998		3.426	0.506	-0.343	0.993	
	0.5 - 1.69	0.855	0.243	3.517	0.999		0.871	0.245	-0.087	0.994	
	1.44- 9.0	3.24	0.391	8.289	0.998		3.327	0.396	-0.396	0.990	
	0.5 - 1.44	0.848	0.192	4.415	0.998		0.866	0.194	-0.087	0.989	
	-										
	0.165-2.0	2.502	0.564	4.436	0.999		2.574	0.577	-2.574	0.992	
pH	temperature range °C	activation energy Ea, K joules mole ⁻¹			Slope	r	Fig				
6.75	54 - 39.5	49.28			-2.574	0.997	4.8				
	39.5 - 20.3	69.26			-3.617	0.998					
	54 - 34	50.80			-2.653	0.998					
	34 - 18	63.20			-3.303	0.991					
	51 - 34	54.99			-2.872	0.997					
5.2	51 - 30	48.00			-2.507	0.998	4.8				
	30 - 13.3	58.93			-3.078	0.999					
	51 - 28	50.93			-2.660	0.999					
	28 - 13.3	57.82			-3.020	0.999					

Table 1.3

The Kinetic Constants of α D galactosidase

Lineweaver-Burk					Eadie-Hofstee				
pH	Km	Vmax	Slope	r	Fig	Km	Vmax	Slope	r
7.0	0.720	0.0141	51.09	0.992	4.10A	0.709	0.014	-0.709	0.971
	0.757	0.0145	52.23	0.998		0.744	0.014	-0.744	0.993
5.2	0.932	0.166	5.616	0.996	4.10B	0.960	0.167	-0.960	0.985
	1.443	0.228	6.329	0.994		1.513	0.232	-1.513	0.978
	1.447	0.208	6.958	0.999		1.483	0.210	-1.483	0.994
	1.222	0.0416	29.365	0.987		0.964	0.037	-0.964	0.907
pH	temperature range °C		activation energy ₁ Ea, Kjoules mole ⁻¹		Slope	r	Fig		
7.0	53	- 33.1	46.16		-2.411	0.995	4.11A		
	33.1	- 20.3	62.67		-3.273	0.991			
	54	- 34.3	38.79		-2.026	0.997			
	34.3	- 25.2	69.37		-3.623	0.999			
	52	- 27.9	45.28		-2.365	0.988			
	27.9	- 19.6	60.16		-3.142	0.979			
5.2	59	- 34.5	48.94		-2.556	0.999	4.11B		
	34.5	- 19.75	76.65		-3.999	0.994			
	50	- 30.6	52.12		-2.722	0.998			
	30.6	- 18	102.32		-5.344	0.996			

Table 1.4

The Kinetic constants of β D galactosidase

Lineweaver-Burk

Eadie-Hofstee

pH	range of substrate concentration	Km	Vmax	Slope	r	Fig	Km	Vmax	Slope	r
6.6	1.39 - 3.69	0.841	0.0418	20.108	0.985	4.13A	0.825	0.0416	-0.825	0.97
	0.308-1.39	0.191	0.030	6.34	0.999		0.196	0.0303	-0.196	0.977
	1.33 - 2.67	1.16	0.0737	15.73	0.982		1.121	0.0728	-1.121	0.961
	0.167- 1.33	0.191	0.0456	4.18	0.995		0.199	0.0462	-0.199	0.986
4.8	2.17 - 4.6	1.662	0.286	5.67	0.991	4.13B	1.58	0.284	-0.158	0.981
	0.5 - 2.17	0.449	0.199	2.26	0.998		0.459	0.201	-0.046	0.995
	2.32 - 6.0	2.263	0.104	21.76	0.991		2.167	0.103	-2.167	0.973
	0.4 - 2.32	0.930	0.076	12.24	0.996		0.864	0.074	-0.864	0.983
	3.0 - 6.0	1.848	0.0535	34.54	0.890		1.602	0.0515	-1.60	0.775
	0.4 - 3.0	0.790	0.0420	18.81	0.998		0.787	0.042	-0.79	0.991
	2.35 - 6.5	1.381	0.0708	19.51	0.988		1.387	0.071	-1.387	0.976
	0.4 - 2.35	0.544	0.054	9.91	0.998		0.554	0.055	-0.554	0.991
	0.125- 2.0	0.827	0.071	11.65	0.999		0.921	0.076	-0.921	0.992

pH	temperature range °C	activation energy ₁ Ea, K joules mole	Slope	r	Fig
6.6	58 - 39.5	69.79	-3.645	0.997	4.14
	39.5 - 16.4	82.62	-4.315	0.999	
	58 - 33.1	70.19	-3.666	0.998	
	33.1 - 20.2	132.36	-6.913	0.999	
	58 - 42.2	62.59	-3.269	0.999	
	42.2 - 20	82.18	-4.295	0.997	
4.8	53 - 33.1	46.74	-2.441	0.996	4.14
	33.1 - 15	82.93	-4.331	0.997	
	53.25- 32.0	49.50	-2.585	0.999	
	32.0 - 15.25	79.02	-4.127	0.999	
	51.25 -27.3	55.97	-2.923	0.999	
	27.3 - 14	69.85	-3.648	0.978	

Table 1.5

The Kinetic constants of trehalase

Lineweaver-Burk					Eadie-Hofstee				
pH	Km	Vmax	Slope	r	Fig	Km	Vmax	Slope	r
5.1	1.045	10.142	0.103	0.998	4.168	1.079	10.192	-1.079	0.997
	0.836	10.299	0.081	0.998		0.776	10.069	-0.776	0.993
	0.808	9.867	0.0819	0.999		0.792	9.819	-0.792	0.997
	0.993	23.419	0.0424	0.999		0.960	23.084	-0.960	0.997

pH	temperature range °C	activation energy Ea, K joules mole ⁻¹	Slope	r	Fig
5.1	57.7 - 12	46.28	-2.417	0.996	4.17
	53 - 21	46.75	-2.442	0.991	

Table 1.6

The Kinetic Constants of "trypsin"

Lineweaver-Burk					Eadie-Hofstee				
pH	Km	Vmax	Slope	r	Fig	Km	Vmax	Slope	r
9.1	0.957	0.243	3.937	0.997		0.987	0.247	-0.987	0.984
	0.932	0.113	8.244	0.998	4.19	0.943	0.114	-0.943	0.978
	0.739	0.127	5.817	0.995		0.720	0.126	-0.720	0.983
pH	temperature range °C		activation energy _a Ea, K joules mole ⁻¹		Slope	r	Fig		
9.1	44.6 - 26		29.30		-1.530	0.978			
	26 - 14.8		71.78		-3.749	0.994			
	45.6 - 28.4		30.81		-1.609	0.974	4.20		
	28.4 - 17.2		59.20		-3.092	0.992			
	45.5 - 28.8		39.81		-2.079	0.994			
	28.8 - 17		67.01		-3.500	0.999			

Section 2

Ewen's (1962) modification of Gomori's paraldehyde fuchsin technique for staining neurosecretory material.

1. Paraffin wax was removed from the sections by immersing them in xylene for 3 minutes.
2. Sections were hydrated by 1 minute in each of, absolute ethanol, 95% ethanol, 70% ethanol, 50% ethanol and distilled water.

The staining procedure was as follows:

3. Oxidize 1 minute, in acid permanganate
KMnO₄ 0.15gm
Concentrated H₂SO₄ 0.1ml
Distilled water 50ml
4. Rinse in distilled water.
5. Decolorize in 2.5% sodium bisulphite.
6. Pass through rinses of distilled water, 30% and 70% ethanol to aldehyde fuchsin. Stain 2 - 10 minutes.
7. Wash in 95% ethanol.
8. Differentiate, 10 - 30 seconds, in acid-alcohol:
Absolute ethanol 100ml
Concentrated ethanol 0.5ml
9. Pass through rinses of 70% and 30% ethanol, and distilled water.
10. Mordant, 10 minutes, in phosphotungstic-phosphomolybdic acid.
phosphotungstic acid 4.0gm
phosphomolybdic acid 1.0gm
distilled water 100ml
11. Rinse in distilled water.
12. Counterstain 1 hour:
distilled water 100ml
light green SF yellowish 0.4gm
orange G 1.0gm
chromotrope 2R 0.5gm
glacial acetic acid 1.0ml
13. Dehydrate rapidly through absolute ethanol; clear in xylene; mount in Canada balsam.

Results: cytoplasm, light green

corpus cardiacum, green with secretion dark purple

corpus allatum, pale green with small amounts of orange in the nuclei.

Section 3

Table 3.1

The effect of the removal of the frontal ganglion on the quantity of fat body available from the thorax and abdomen

Treatment	Mean wet weight of fat body mg	S.E.	Number of determinations	Number of animals per determination (equal numbers of males and females)
12 hours ⁺ 12 hours (normal)	6.6	0.65	13	2 - 4
Operated Control	8.5	1.21	9	2 - 4
Operated	4.2	0.49	11	4 - 6
Starved	3.81	0.32	9	4 - 6

Section 4

Methods employed for the estimation of protein

1. The protein content of enzyme preparations was determined by the method of Lowry, Rosebrough, Farr and Randall (1951), using bovine serum albumin (BSA) fraction V as standard.

Reagents : solution 1 2% (W/V) sodium carbonate in
0.1N sodium hydroxide

solution 2 0.5% copper sulphate

solution 3 1% sodium potassium tartrate

Folin's reagent (solution A) was prepared by diluting 4 volumes of Folin and Ciocalteu's phenol reagent with 6 volumes of distilled water. Solution B was made by mixing equal volumes of solutions 2 and 3 and adding 50 volumes of solution 1 to each volume of the mixture.

Standard protein solutions were made up ranging from 0 to 400 μ g BSA/ml. The enzyme preparations were diluted if necessary to give protein levels within this range. 0.2ml of protein solution was added to 3ml of solution B and left for 30 minutes at room temperature. 0.3ml of solution A was then added and the mixture was left for 60 minutes at room temperature. After this time the absorbance of the mixture was measured at 500nm in a Unicam SP1800 spectrophotometer. From the standard protein solutions a calibration graph was plotted and the unknowns determined from this graph. A typical standard graph is shown in Appendix Fig. 4.1A. A new standard graph was constructed for each determination.

2. The amount of protein in haemolymph was estimated using the micro-Biuret method of Itzhaki and Gill (1964).

Reagents : 1. 0.21% copper sulphate in 30% sodium hydroxide
(to prevent precipitation of copper hydroxide

the copper sulphate was added as a dilute solution to the alkali).

2. 30% sodium hydroxide.

Standard protein solutions were made up to give concentrations of between 0.1 - 1.2mg of BSA fraction V/2ml.

A1 20 μ l of haemolymph was suspended in 2ml distilled water and 1ml of reagent 1 added. The optical density ODA1 was measured 15 minutes later in quartz cuvettes at 310nm in a Unicam SP1800 spectrophotometer using a deuterium lamp.

A2 2ml + 20 μ l of distilled water and 1ml of reagent 1
ODA2 was measured as above.

B1 2ml of distilled water + 20 μ l haemolymph and 1ml of reagent 2,
ODB1 was measured as above.

B2 2ml + 20 μ l of distilled and 1ml of reagent 2, ODB2 was
measured as above.

$$\Delta OD = (ODA1 - ODA2) - (ODB1 - ODB2)$$

From the standard protein solutions a calibration graph was plotted and the unknowns determined from this. A typical standard graph is shown in Appendix Fig. 4.1B. A fresh set of standards was made up for each determination.

Fig. 4.1

Standard calibration curves for the determination of protein

- A. Protein was estimated using the Folin Ciocalteu phenol reagent (ROSEBROUGH, FARR AND RANDALL, 1951).

Legend:

Ordinate ; absorbance at 500 nm

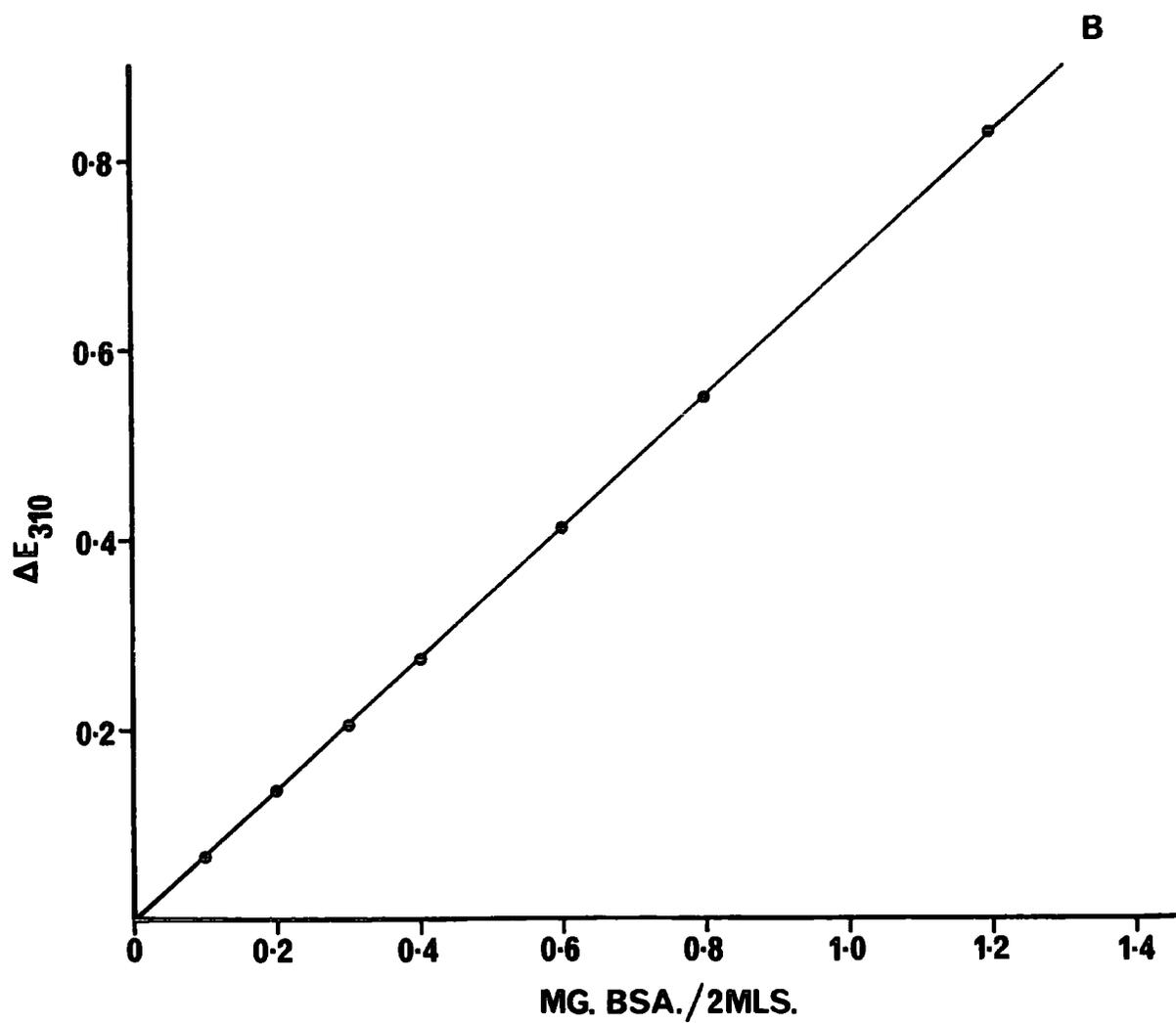
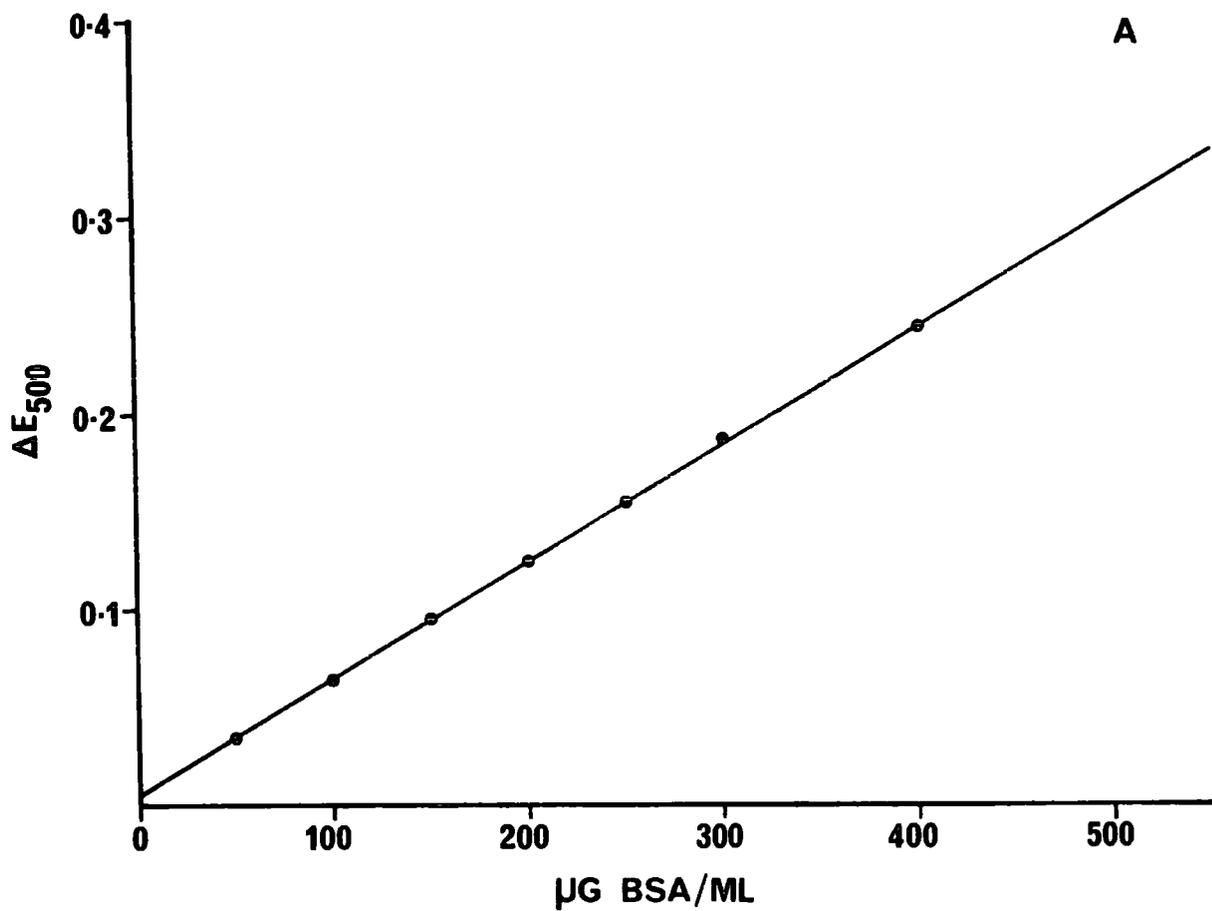
abscissa ; μg BSA (fraction v)/ml

- B. Protein was estimated using the micro-Biuret method of ITZHAKI AND GILL (1964).

Legend:

Ordinate ; absorbance at 310 nm

abscissa ; mg BSA (fraction v)/2ml



Section 5.

Calculation of the O₂ consumed by a locust using the Warburg apparatus

See Chapter 3.

In order to express the volume of the gas respired in terms of mm³ of oxygen, it was necessary to calculate the manometer constant K. This requires that the volume of gas in the flask as far as the fluid surface in the manometer limb be known. Since the volume of the insect was unknown it was not possible to calculate this volume in the standard way (DIXON, 1943), and the volume of the insect relative to the amount of air in the flask was too great to be ignored. This gas volume, V_g, was therefore measured in the way described by (CLARKE, 1955). The procedure was as follows:

At the end of the experiment the cock on the manometer was opened and the fluid in the limbs raised to the upper limit of the scale. The volume of gas which was now above the right hand limb was designated V₁. The cock was then closed and the fluid lowered until the right hand meniscus was set at zero. This increased the volume V₁ by an amount, ΔV, which was a known quantity since the manometer had been calibrated. When the cock was opened the volume V₁ was at atmospheric pressure, P₁. This increase in volume just described was accompanied by a decrease in pressure which, in mm of Brodie's fluid, was equal to the difference between the level of fluid in the left and right hand limbs of the manometer, a value designated ΔP. Thus by applying Boyle's law:

$$P_1 V_1 = P_2 V_2$$

where P₁ = atmospheric pressure ; V₁ = initial

volume (ie. the volume of gas in the flask and manometer arm as far as the upper limit of the manometer scale) ; P₂ = the pressure of the final gas volume, in this case P₁ - ΔP ; and V₂ = the initial volume plus the increase in volume brought about by movement of the fluid from the

upper limit of the scale to the zero mark; ie. $V_1 + \Delta V$

$$\begin{aligned}\text{Thus, } P_1 V_1 &= P_2 (V_1 + \Delta V) \\ &= P_2 V_1 + P_2 \Delta V\end{aligned}$$

$$P_1 V_1 - P_2 V_1 = P_2 \Delta V$$

$$V_1 (P_1 - P_2) = P_2 \Delta V$$

$$\text{and } V_1 = \frac{P_2 \Delta V}{(P_1 - P_2)}$$

Since all the values on the right hand side of the equation were known, it was possible to calculate the volume of gas in the flask as far as the upper limit of the manometer scale. Then by adding ΔV to this value, the volume of gas as far as the zero mark can be obtained:

$$V_g = V_1 + \Delta V$$

With the value of V_g known, the amount of oxygen consumed by each locust was calculated in mm^3 per hour at N.T.P. according to the formula given by DIXON (1943) except that the expression for the volume of fluid in the flask and the solubility of the gas evolved was ignored.

$$\text{Thus, } K = \frac{V_g + \frac{273}{T}}{P}$$

Where K is the manometer constant; V_g is the gas volume; T is the bath temperature in $^{\circ}\text{A}$ and P is the normal pressure of the atmosphere in mm of Brodie's fluid.

$$\text{Then } x = h \cdot K$$

Where x is the amount of gas used; h is the manometer reading and K is the manometer constant.