



University  
of Glasgow

<https://theses.gla.ac.uk/>

Theses Digitisation:

<https://www.gla.ac.uk/myglasgow/research/enlighten/theses/digitisation/>

This is a digitised version of the original print thesis.

Copyright and moral rights for this work are retained by the author

A copy can be downloaded for personal non-commercial research or study, without prior permission or charge

This work cannot be reproduced or quoted extensively from without first obtaining permission in writing from the author

The content must not be changed in any way or sold commercially in any format or medium without the formal permission of the author

When referring to this work, full bibliographic details including the author, title, awarding institution and date of the thesis must be given

Enlighten: Theses

<https://theses.gla.ac.uk/>  
[research-enlighten@glasgow.ac.uk](mailto:research-enlighten@glasgow.ac.uk)

THE STRUCTURE AND FUNCTION OF THE PROVENTRICULUS  
AND THE ENTERIC CAECA IN THE LARVA OF THE BLOWFLY  
PROTOPHORMIA TERRAE-NOVAE, ROBINEAU-DESVOIDY  
(DIPTERA, CALLIPHORIDAE)

T H E S I S

for the

Degree of Doctor of Philosophy

in the

University of Glasgow

by

Douglas G. Cochrane, B.Sc.

November, 1958

ProQuest Number: 10656219

All rights reserved

INFORMATION TO ALL USERS

The quality of this reproduction is dependent upon the quality of the copy submitted.

In the unlikely event that the author did not send a complete manuscript and there are missing pages, these will be noted. Also, if material had to be removed, a note will indicate the deletion.



ProQuest 10656219

Published by ProQuest LLC (2017). Copyright of the Dissertation is held by the Author.

All rights reserved.

This work is protected against unauthorized copying under Title 17, United States Code  
Microform Edition © ProQuest LLC.

ProQuest LLC.  
789 East Eisenhower Parkway  
P.O. Box 1346  
Ann Arbor, MI 48106 – 1346

The Structure and Function of the Proventriculus and the Enteric  
Caeca in the Larva of the Blowfly Protophormia terrae-novae,  
Robineau-Desvoidy (Diptera, Calliphoridae)

C o n t e n t s

	<u>Page</u>
Introduction	1
Materials	3
Chapter 1: The Structure and Function of the Proventriculus in the Larva of <u>Protophormia terrae-novae</u>	
Section I: Introduction	4
Section II: Techniques and Staining Procedures	
(a) Dissection	11
(b) Fixation	12
(c) Embedding	12
(d) Methods used in anatomical studies	14
(e) Histological staining	16
(f) Histochemical staining	17
(g) Technique used for the detection of chitin	26
Section III: The Anatomy and Histology of the Proventriculus	
(a) The general anatomy of the oesophageal valve and its relation to adjacent organs	28
(b) The musculature of the proven- tricus	34
(c) The innervation of the proven- tricus	36
(d) The tracheation of the proven- tricus	38

(e) The histology of the proventriculus	39
Section IV: The Physiology of the Proventriculus	43
Section V: Discussion	53
Chapter 2: The Presence of Paraldehyde Fuchsin- Positive Material in the Cells which Secrete the Peritrophic Membrane Substance in Some Other Insects	
Section I: Materials and Methods	62
Section II: Results	63
Section III: Discussion	68
Chapter 3: The Structure and Function of the Enteric Caeca in the Larva of <u>Protophormia</u> <u>terrae-novae</u> R-D	
Section I: Introduction	70
Section II: The Anatomy and Histology of the Enteric Caeca	
(a) Materials and methods	74
(b) The anatomy of the caeca	74
(c) The histology of the caeca	77
Section III: The Physiology of the Enteric Caeca	
(a) Materials and methods	
(i) Materials	78
(ii) Staining procedures	78
(iii) Dye-feeding procedures	79
(iv) Iron saccharate feeding procedure	81
(v) Enzyme determination techniques	81
(vi) Dye injection technique	88
(vii) Ligaturing technique	88
(b) The physiology of the caeca	89
Section IV: Discussion	96
Conclusions	99

List of Figures	Facing Page
1. Partial section of the larva of <u>P. terrae-novae</u>	4
2. Unit of glycogen chain	22
3. Unit of chitin chain	22
4. The oesophageal valve and adjacent organs	29
5. A longitudinal section of the oesophageal valve	30
6. Diagram of the musculature of the proventriculus	35
7. The nerve and tracheal supply to the proventriculus	37
8. A longitudinal section of the proventricular ganglion	38
9. Part of a longitudinal section of the proventriculus showing maximum vacuolation found in epithelial cells	41
10. Drawing of portion of proventriculus illustrated in Plate 5	50
11. The oesophageal valve in <u>Forficula auricularia</u>	63
12. The oesophageal valve in a lepidopterous larva	65
13. The oesophageal valve in the larva of <u>Culex pipiens</u>	66
14. The oesophageal valve in a sawfly larva	68

## Plates

1. Musculature of the chyle ventricle in the larva of <u>P. terrae-novae</u>	36
2. Musculature of the proventriculus	36
3. Glycogen distribution in part of the proventriculus	46
4. The 'PR' ring of cells in the proventriculus	46
5. The 'PR' ring of cells stained by the paraldehyde fuchsin method	50

6.	Distribution of PF-positive material in the proventriculus of <u>F. auricularia</u>	64
7.	Enlargement of part of Plate 6	64
8.	Part of the anterior midgut of the larva of <u>Pieris brassicae</u>	65
9.	Part of the oesophageal valve in the larva of <u>C. pipiens</u>	67
10.	Enlargement of part of Plate 9	67
11.	Peritrophic membrane in the larva of <u>P. ribesii</u>	69
12.	Epithelium of anterior midgut in the larva of <u>P. ribesii</u>	69
13.	Musculature of the enteric caeca in the larva of <u>P. terrae-novae</u>	76
14.	Longitudinal section of part of an enteric caecum in the larva of <u>P. terrae-novae</u>	77
15.	Enlargement of part of Plate 14	77
16.	Part of an enteric caecum showing secretion droplets	78
17.	Enlargement of part of Plate 16	78

#### Graphs

1.	The results of two pilot experiments carried out to assess the effectiveness of the Sørensen formol titration method in detecting very small quantities of enzymes in a solution	88
2.	The results of two experiments carried out to detect the breakdown of gelatine solution by tryptase in extracts of caecal tissue	92
3.	The results of two experiments carried out to detect the breakdown of glycyl-glycine by peptidase in extracts of caecal tissue	93

#### Tables

I	47
II	53
III	80
IV & V	81

	Facing Page
VI & VII	88
VIII & IX	92
X & XI	93

## ACKNOWLEDGEMENTS

I wish to record my appreciation of the financial support afforded for this research by the Agricultural Research Council and the facilities provided to carry out the research in the Department of Zoology, University of Glasgow, by Professor C.M. Yonge, C.B.E., F.R.S.

I am deeply grateful to Dr A. Fraser of the Department of Zoology who first suggested the work, with whom it was discussed at all stages and who offered much helpful advice and criticism. I must also thank Dr A.R. Hill of the Department of Zoology for his continual advice and encouragement while the work was in progress.

Further thanks are due for technical assistance to Dr H.F. Steedman and Mr G. Owen of the staff of the University of Glasgow, to Mr A. MacKinnon and Mr W.F. Smith of the technical staff of the Department of Zoology, and to other colleagues who have offered helpful advice at different times.

Finally I am indebted to Miss I. French who checked and typed the work in its final stages.

## INTRODUCTION

Protophormia terrae-novae R-D is a member of the group of flies in the family Tachinidae which are the causal organisms of sheep "strike" in Great Britain. In comparison with the other members of this group, Lucilia spp. and Calliphora spp., the area where P. terrae-novae causes primary strike is limited, being confined to the north and north west of Scotland, but it is a significant pest in these areas (Macleod, 1943 a, b.). It has been found present, mixed with the other species, in samples taken from cases of strike in other parts of Scotland e.g. south west Scotland (Haddow & Thompson, 1937) but in proportionality very small numbers so it is doubtful if it is a significant pest in these areas.

For the work which follows P. terrae-novae was chosen as an experimental animal partly because it is a pest species about which comparatively little is known, and partly because as an experimental animal it is easily bred and maintained in the laboratory.

The work deals with the structure and function of the proventriculus and the enteric caeca in the larva of P. terrae-novae. Neither of these organs has received much attention in previous work on Cyclorrhaphan larvae although many authors make brief reference to them. Weismann (1864) and Lowne (1890) describe/

describe and figure the proventriculus in Calliphora but their observations are brief and, in the case of Lowne, somewhat inaccurate. Perez (1910) gives a brief description of the oesophageal valve in Calliphora erythrocephala which corrects the errors of Lowne but his figures show other inaccuracies and he was not concerned with the function of the structures at present under investigation. Most of the recent studies have been on Drosophila melanogaster, notably those of Strasburger (1932 and 1935 - the anatomy of the proventriculus and the enteric caeca), Siang-Hsu (1947 - cytology of the proventriculus and the enteric caeca), Bodenstein (1949 - the anatomy of the proventriculus) and Rizki (1957 - function of the proventriculus). Since it deals with two separate, though adjacent, structures this work falls into two parts. The first part deals with the anatomy and physiology of the proventriculus, special attention being paid to the origin of the peritrophic membrane. The second part deals with the anatomy and physiology of the enteric caeca.

## MATERIALS

Larvae for experimental purposes were obtained from laboratory cultures established from sources such as carrion. The cultures were maintained in 2' x 1' x 1' glass cages which were kept in a constant-temperature room at 25°C. Water, cane sugar, and portions of fresh horse liver were placed in the cages. Each cage contained a stock of about fifty adult flies, each stock being kept for about one month after which it was discarded and replaced by newly-emerged adults.

When cultures of larvae were required, portions of liver covered with damp cotton wool (to avoid desiccation of the eggs) were placed in the cages. When eggs had been laid on the portions of meat, the meat was removed and placed in Breffit jars which contained about 3" of moist sawdust. Larvae could be reared satisfactorily in these jars at 25°C. Feeding 3rd instar larvae were removed from the jars as required.

Fig. 1. A partial section of the larva of Protophormia  
terrae-novae to show the principal viscera of the  
anterior end of the body.

BR, brain; DLC, dorsal long caecum;

Oes, oesophagus; OeV, oesophageal valve,

Sal. Gl., salivary gland; VM, visceral mass,

VSC, ventral short caecum.

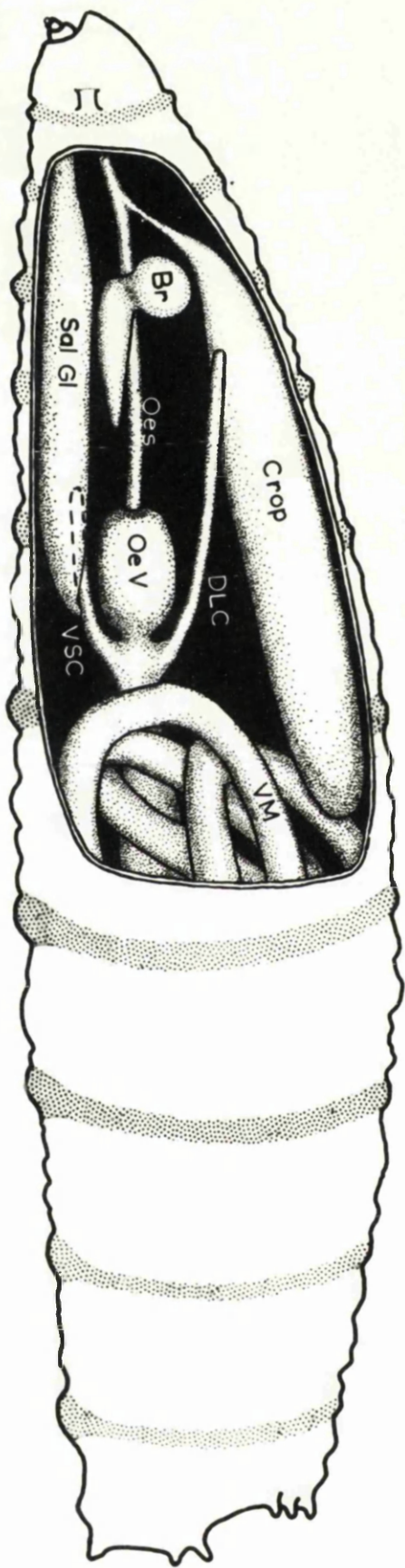


Fig. 1.

## Chapter 1

### The Structure and Function of the Proventriculus in the Larva of *Protophormia terrae-novae*, R-D.

#### Section I: Introduction

In many insects, and always in the Diptera, the gut does not pass from foregut to midgut tissue in the form of a simple straight tube. Instead, the distal end of the foregut projects down into the lumen of the midgut so that a fold is formed in the gut wall. This fold is the origin of the oval structure in the gut of Dipterous larvae (Figs 1 and 4), the outer wall of which is midgut tissue and the inner walls foregut tissue. Lowne (1890) in his work on *Calliphora erythrocephala* called this structure the proventriculus, this practice being followed by such authors as Hewitt (1914), Kruger (1926) and Wigglesworth (1930). Other authors (e.g. Imms, 1907) used the term oesophageal valve for this structure, a practice to which Wigglesworth objected on the grounds that the structure functions as a sphincter in the alimentary canal rather than as a valve. It might be argued that in a tube like the midgut of a Cyclorrhaphan larva, the walls of which are relatively thin and soft, the contents must be maintained under a pressure equal to or greater than that of the blood otherwise the gut would collapse since/

since the contents are fluid. It can be easily seen that such a pressure exists if the wall of the gut is pierced in a larva dissected in saline, the contents including the peritrophic membrane being forced out. To maintain this pressure some form of valve must be provided at each end of the gut, the valve at the anterior end having a non-return function. Reference to the Oxford English Dictionary shows that in the anatomical sense the essential difference between a sphincter and a valve is that while a sphincter may regulate the flow of material it cannot control the direction of the flow, the property of control over directional flow residing in a valve. Therefore although, as will be seen later, there is a functional sphincter muscle incorporated in the oval body some part of that body must also function as a valve. Other authors (Perez, 1910, de Boissezon, 1930, Aubertot, 1937, Rizki, 1956) employed the term proven-triculus to describe that portion of the midgut which forms the outer wall of the oval structure.. Imms (1907) called this the cardia and was followed by Wigglesworth and others. Finally, it is noted that in more primitive insect orders the proven-triculus is a muscular triturating organ in the foregut (Judd, 1948, etc.).

Rather than coin new terms which would further add to this confusion the above names have been retained, even if somewhat erroneous, in this work. The whole organ is called the oesophageal/

oesophageal valve, the invaginated portion of foregut the oesophageal invagination, and the portion of midgut tissue which forms the outer wall of the oesophageal valve, the proventriculus (Fig. 5).

No work dealing with the detailed anatomy of the proventriculus in the larvae of Cyclorrhaphan Diptera has been found. Earlier work in the field was carried out by Weismann (1864), Lowne (1890) and Perez (1910), all working on larvae of Calliphora spp., and Strasburger (1932, 1935) working on Drosophila melanogaster, but in each case emphasis has been put on the description of the proventricular epithelium and very little said about its musculature, tracheation, or innervation. However the physiology of the proventriculus in Dipterous larvae has attracted much attention.

The foregut and hindgut of insects are ectodermal in origin and are lined with a cuticle continuous with and strictly homologous to that of the outer body surface (Richards 1951). The midgut lacks a comparable cuticle. Sometimes its cells are directly exposed to the gut contents but commonly in insects and at least in some other Arthropods the midgut cells are separated from the food by one or more thin membranes. Two general types occur, separately or in the same species; a thin tube secreted by a ring of cells at the anterior end of the midgut and extending backwards, in the Orthoptera, Dermaptera, Isoptera and Diptera/

Diptera (Henson, 1930); and partial or complete sheets of membrane material delaminated from the inner surfaces of the midgut cells (in most other insect orders) which adhere to the surface of the food mass in the lumen to form a tube. A membrane thus formed surrounding the food was first given the name "peritrophic membrane" by Balbiani (1890).

Investigation of peritrophic membrane formation in insects has in most cases been incidental to the general description of the gut and has rarely been followed up in any detail. A full account of early work on the formation of the membrane can be found in Vignon (1901) and Stuhlmann (1907). By the time that these latter works were published it had become clear that in larvae of Dipterous species the peritrophic membrane was formed by the first method i.e. from a single source at the anterior end of the midgut. Van Gehuchten (1890) was the first to describe a ring of cells at the anterior end of the gut of Ptychoptera contaminata which contained vesicles which he believed were the material from which the membrane was formed.

It is now generally accepted that in Dipterous larvae the function of the proventriculus is to produce the material from which the peritrophic membrane is formed. Most of the earlier work has centred round the mechanism whereby the fluid secretion produced by the proventricular epithelium is moulded and compressed into a smooth membrane of even thickness. The out-standing/

standing papers are those of van Gehuchten (1890) noted above; Vignon (1901) who also identified a membrane-secreting ring at the anterior end of the gut of Chironomus and described the production by it of a clear colourless secretion which was consolidated into the membrane; and Wigglesworth (1929, 1930) who investigated the functioning of the oesophageal valve as an "annular mould" in forming the membrane in the larvae of Culex, Aedes, Corethra, Rhyphus, Sciara and Telmatoscopus, and in adult Glossina.

Unfortunately the studies of these workers were limited by the fact that the basic material of the peritrophic membrane is chitin (Campbell, 1929, Wigglesworth, 1921, von Dehn, 1933, Waterhouse 1953) a material about the biosynthesis of which very little is known and which was resistant to the known methods of histochemical staining. They therefore identified the cells from which the membrane material originated largely by the presence in those cells of vacuoles which appeared to release their contents through the inner cell borders into the gut lumen where the secreted material was compressed into a membrane. They were unable to identify the material in the vacuoles in any way apart from calling it "fluid chitin".

Chitin is chemically a complex polysaccharide presumably built up by the polymerisation of simpler polysaccharide molecules (Richards 1951). Lately histochemical techniques have been evolved/

evolved for the identification of polysaccharides present in cells (Hotchkiss 1948, Pearse 1953). The section on the physiology of the proventriculus in P. terrae-novae which follows is based on the hypothesis that the cells in which the chitin of the peritrophic membrane was being synthesised would contain significant quantities of polysaccharide material identifiable by these new techniques and so the source of the peritrophic membrane would be readily identifiable.

When this work had been in progress for some time a paper was published by Rizki (1956) on the function of the proventriculus in the larva of D. melanogaster. In the course of a study on the cuticle of this animal he employed one of the techniques referred to above, the periodic acid - Schiff of Hotchkiss (1948). He noticed that the tissues in the body which were PAS-positive included a band of cells in the proventricular epithelium and the peritrophic membrane. Further investigation showed that the PAS-positive cells contained a large number of small granules grouped mainly between the nucleus and the inner striated border of each cell. Waterhouse and Stay (1955) had previously noted that there were PAS-positive granules in the proventricular epithelium in the larva of Lucilia sericata but they did not identify any special band of cells or investigate the granules further. Rizki confirmed that the granules were polysaccharide in nature by various tests and since they were identical in staining/

staining characteristics with the peritrophic membrane which originated outside the granule-containing cells, he postulated that the granules were the precursors of a chitin polysaccharide in the membrane.

Although Rizki did note that there were globules in the proventricular epithelial cells he does not appear to have paid much attention to them. He believed that they might have some part in the secretion of the enzymes concerned in the polymerisation of the material secreted by the epithelial cells into a membrane. None of the staining methods employed by him stained the contents of the vacuoles.

## Section II: Techniques and Staining Procedures

### (a) Dissection

The larvae used for both the anatomical and the histological studies on the proventriculus were third instar larvae which were almost fully grown but still actively feeding. Each larva was very lightly anaesthetised with ether then pinned out in a solid watchglass containing a layer of pitch. The body wall was opened along the mid-dorsal line with a pair of scissor forceps and the halves of the wall pinned back. The tissues dorsal to the oesophageal valve were removed thus exposing the valve and adjacent organs. For the anatomical studies the larva was then immersed in physiological salt solution. If material was required for sectioning for histological purposes the larva was flooded with the appropriate fixative or, if this was impracticable (e.g. where the fixative contained osmic acid), the required tissue was dissected from the larva and dropped into a bath of fixative. It is to be noted that the enteric caeca were always fixed, embedded and sectioned along with the proventriculus for use in later studies.

The physiological saline used was that recommended by Williams (1946) namely,

Sodium chloride	7.5 gm
Potassium chloride	0.35 gm
Calcium chloride	0.21 gm
Distilled water	1000 ml

### (b) Fixation/

(b) Fixation

For the study of the histology and the histochemistry of the proventriculus, microtome sections of fixed tissue preparations were made. The fixative which gave the most satisfactory histological results was Bouin's fluid (Gray, 1954). Other investigators working on similar material have used alcoholic Bouin (Duboscq-Brasil) and Carnoy's fluid but neither of these was found to fix the epithelial cells of the proventriculus as well as Bouin's fluid. Material was left to fix in the Bouin overnight i.e. fifteen to sixteen hours.

In the histochemical investigation various fixatives were employed. These will be noted later in this section along with their appropriate histochemical method.

(c) Embedding

Initially the material was embedded in Steedman's ester wax (Steedman, 1947) by the following method.

1. Specimen to water, or the appropriate alcohol depending on the fixative, and washed for sufficient time to remove all fixative
2. Through graded alcohols to 70% alcohol 30 mins in each
3. 70% alcohol + cellosolve (equal parts) 30 mins
4. Pure cellosolve, twice 30 mins each
5. 10% graduated solutions of cellosolve and ester wax (i.e. 9 parts cell. + 1 part ester wax, 8 parts cell. + 2 parts ester wax, etc.) 15 mins each
6. Pure ester wax, 2 baths 1 hour each.

The use of such long immersions in the various reagents may seem excessive but they were made necessary by the presence of fairly impervious chitinous layers in the oesophageal valve which slowed the passage of fluids in and out of the tissues. Graduated solutions of cellosolve and ester wax were found to be necessary for the same reason. If the tissues were transferred direct from cellosolve to pure ester wax or even into a solution of equal parts of each, the delicate inner tissues of the oesophageal valve tended to collapse.

During the course of these investigations Steedman's polyester wax (Steedman, 1957) became available and thereafter this was used in preference to ester wax because the quality of the sections was somewhat better and the techniques of embedding and sectioning were simpler. The procedure was as follows:

- |    |                                                            |              |
|----|------------------------------------------------------------|--------------|
| 1. | Fixative removed and specimen to 70% alcohol as before     |              |
| 2. | 90% alcohol                                                | 30 mins      |
| 3. | 96% alcohol                                                | 15 mins      |
| 4. | 96% alcohol + eosin (1% solution)                          | 1 min.       |
| 5. | Graduated (10%) solutions of 96% alcohol and polyester wax | 15 mins each |
| 6. | Pure polyester wax, 2 baths                                | 1 hour each. |

The addition of the eosin step is necessary since, unlike ester wax, polyester wax is completely opaque and a specimen as small/

small as a proventriculus is readily lost in it.

Microtome sections were cut at 2-4 $\mu$  with ester wax and at 4-6 $\mu$  with polyester wax.

(d) Methods used in anatomical studies

In studying the musculature of the proventriculus use was made, with some modifications, of the technique of Graham-Smith (1934). Larvae were dissected in physiological saline as described above. The saline was then poured off and replaced by 50% alcohol for five seconds, sufficient to fix partially the external muscle coat of the gut. The alcohol was poured off and replaced with water. By means of an eye-scalpel the gut was pierced a short distance behind the caeca and a slit made forwards from a point between the "roots" of the dorsal caeca up to the "shoulders" of the proventriculus. The specimen was left to soak in water until the epithelial lining of the gut could be rubbed off with the point of a scalpel. The epithelium of the chyle ventricle will come off almost immediately but the epithelium of the proventriculus has to be soaked for about one hour before it comes away.

The gut wall was now cut across at the level of the anterior imaginal ring and again a short distance behind the roots of the caeca and the severed portion of gut transferred to a watchglass containing distilled water. A clean coverslip was partly immersed in the water and the tissue spread out on it with the muscle/

muscle layer against the glass. The tissue adhered closely to the coverslip and in all the subsequent manipulations it was handled on the coverslip. The water was drawn off, the specimen left until it was almost dry, then a drop of absolute alcohol was placed on it to complete fixation. The tissue was brought down through the alcohols to water and immersed in 1% iron alum for twenty minutes, washed well with water and stained with 0.1% aqueous haematoxylin solution, the progress of the staining being observed under a binocular microscope. The tissue was again washed in water, passed up through the alcohols, cleared in xylol, and mounted in canada balsam.

The innervation of the proventriculus was studied by the in vivo methylene blue method of Ludwig (1949). Larvae were dissected and the organs displayed as before. A .001 solution of methylene blue in physiological saline was added, covering the larva. The progress of the staining was watched under the microscope until satisfactory staining was obtained, the dish being agitated at intervals to mix air with the solution. When staining was complete the methylene blue was poured off and the dish waved about for ten seconds to expose the tissues to the air. Finally the larva was immersed in physiological saline. This method was fairly satisfactory for demonstrating the gross anatomy of the proventricular nervous system but the results were not as brilliant as those obtained by Ludwig and the staining of nerve endings was unsatisfactory.

No staining technique was required in the study of the tracheation of the proventriculus, the details being clear in the dissection in saline.

(e) Histological staining

The methods described in this and the following section are given in full because the standard techniques were modified to a varying degree, after much experimentation, to suit the tissue under investigation.

i). Iron haematoxylin - Orange 'G'

1. Dewax and hydrate
2. 4% ferric alum 24 hours
3. Rinse in distilled water
4. 0.5% haematoxylin 24 hours
5. Rinse in tap water
6. Differentiate in 2% ferric alum about 4 mins
7. Wash in running water
8. Take up through the alcohols to absolute alcohol
9. Counterstain with Orange 'G' in clove oil 1 hour
10. Rinse briefly in absolute alcohol
11. Xylol 10 mins
12. Mount in canada balsam

ii)./

ii). Alcian blue - Mayer's haemalum - eosin

1. Dewax and hydrate
2. 1% alcian blue (aqueous) (Steedman, 1950) 30 secs
3. Rinse in distilled water
4. Mayer's haemalum 2 mins
5. Rinse in distilled water
6. Blue in Scott's tap water substitute (Carleton & Leach 1947) 10 mins
7. Take up through the alcohols to absolute alcohol
8. Eosin in absolute alcohol 2 mins
9. Rinse briefly in absolute alcohol
10. Xylol, mount in canada balsam.

(f) Histochemical staining

Again these methods are given in full because they are modifications of the standard techniques.

i). Protein method 1 (Baker 1956)

Baker recommends that in this method the material should be fixed in a formalin - saline solution and embedded in celloidin. In the present work normal Bouin-fixed material embedded in ester wax was used with satisfactory results. Material embedded in polyester wax cannot be used since the amylopectin adhesive used with this wax is destroyed by the hot mercuric sulphate solution.

Solution A/

Solution A.	Conc. $\text{H}_2\text{SO}_4$	10 ml
	Distilled water	90 ml
	Mix together and add	
	Mercuric sulphate	10 gms

Heat until dissolved. Cool. Make up to 200 ml with distilled water.

Solution B.	Sodium nitrite	0.25 gms
	Distilled water	100 ml

#### Method

1. Dewax and hydrate
2. Put 20 ml of solution A in a petri dish. Add 2 ml of solution B
3. Place slide in dish and heat gently over asbestos gauze until fluid just boils
4. Observe until sections develop red colour
5. Remove and wash thoroughly in distilled water
6. Take up through the alcohols to absolute alcohol
7. Xylol, mount in canada balsam

By this method phenols (generally those of tyrosine protein) are coloured red.

ii). Protein method 2 (Mazia, Brewer & Alfert, 1953)

Solution A/

Solution A.	Mercuric chloride	10 gms
	Bromphenol blue	0.1 gms
	95% ethyl alcohol	100 ml

#### Method

1. Xylol 5 mins
2. Absolute alcohol 2 mins
3. Solution A 15 mins
4. 90% alcohol Wash until sections blue
5. Absolute alcohol 5 mins
6. Xylol, mount in canada balsam

Proteins are stained a deep blue by this method

#### iii). Carbohydrate method 1 (Hotchkiss, 1948)

The periodic acid - Schiff method (PAS)

Solution A.	Periodic acid	0.4 gm
	Ethyl alcohol	35 ml
	Mix. Add to these	
	0.2M sodium acetate	5 ml
	Distilled water	10 ml

#### Solution B. Schiff's reagent (Pearse 1953)

1. Weigh 1 gm basic fuchsin
2. Weigh 1 gm anhydrous sodium bisulphite
3. Boil 200 ml distilled water
4. Remove from heat, add fuchsin, and stir
- 5./

5. Cool to 50°C
6. Filter
7. Add 20 ml of N. HCl to filtrate
8. Cool to 25°C
9. Add sodium bisulphite

Store in dark in stoppered bottle. Fluid takes 1-2 days to become orange or straw-coloured when is ready for use.

The PAS method was first used to stain glycogen in the epithelial cells of the proventriculus. Material used in glycogen work was fixed in ordinary Bouin, chilled to 0°C, and left in the fixative for twenty-four hours at 0°C. Carleton and Leach (1947) state that this method is adequate for fixing glycogen and Gomori (1953) corroborates this. Since periodic acid attacks glycogen readily (Pearse 1953) it was necessary to coat the sections with celloidin.

Only ester wax sections can be used with this method since the adhesive (amylopectin) used with polyester wax, being itself a polysaccharide could interfere with the staining results.

The method used was as follows.

- |                                                               |         |
|---------------------------------------------------------------|---------|
| 1. Dewax in xylol                                             | 5 mins  |
| 2. Absolute alcohol                                           | 2 mins  |
| 3. 1% celloidin in equal parts of absolute alcohol and ether  | 5 mins  |
| 4. Wipe back of slide dry quickly and plunge into 70% alcohol | 15 mins |

5./

5. Solution A. (periodic acid) 3 mins
6. Rinse in distilled water
7. Solution B. (Schiff) 30 mins
8. Wash in running water 10 mins
9. Blot dry, rinse briefly in three changes of absolute alcohol
10. Xylol, mount in canada balsam

Hotchkiss recommended an intermediate reducing rinse between steps 5 and 6 but Purves and Griesbach (1951) state that this is unnecessary since the Schiff reagent contains a large excess of sulphurous acid which reduces any normal amount of periodates remaining in the sections.

The method used to detect insoluble polysaccharides was the same as the above except that steps 3 and 4 were omitted. Instead, the dewaxed sections were taken to water then treated with diastase for thirty minutes to remove all the glycogen from the sections. By this method glycogen is stained a vivid scarlet and non-labile polysaccharides a darker purple-red. Other substances are a pale pink. Chitin, although a polysaccharide, is not PAS-positive. Some explanation of this important fact must be given. The success of the PAS method is dependent upon the presence in the units of the polysaccharide, or other, molecule of 1-2 glycol groups ( $-\text{CHOH}-\text{CHOH}-$ ) such as are found, for instance, in the units of glycogen chains (Fig. 2). The periodic acid ruptures the bonds between the second and third/

Fig. 2. Unit of glycogen chain (Richards, 1950)

Fig. 3. Unit of chitin chain (Richards, 1950)

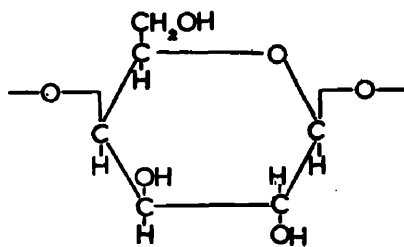


Fig. 2.

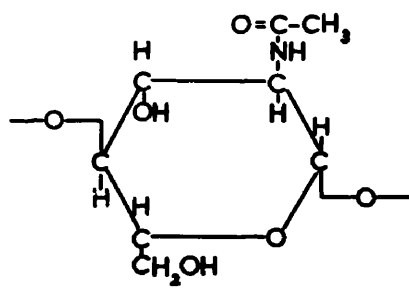


Fig. 3.

third carbon atoms, converting the two alcohol groups to aldehydic groups. These aldehydic groups then combine with the leucofuchsin in the Schiff reagent to form a purple compound. However the acetyl-glucosamine residues which are the units of the chitin molecules (Fig. 3) have an acetamide group in place of the hydroxyl group on the second carbon atom. The substitution of this latter group blocks the PAS reaction since acyl-amino derivatives of glycols are stable in the presence of periodic acid (Brunet, 1953).

iv). Carbohydrate method 2 (Fraser, 1957)

The paraldehyde fuchsin method of Gomori (1950) as adapted by Gabe (1953) with counterstaining recommended by Clark (1956).

Solution A.	Potassium permanganate	3 gms
	Sulphuric acid (conc.)	3 ml
	Distilled water	1000 ml
Solution B.	Sodium bisulphite	2.5 gms
	Distilled water	100 ml

Solution C.

1. Add 1 gm basic fuchsin to 200 ml of boiling distilled water. Boil for 1 minute
2. Cool. Filter
3. Add to filtrate 2 ml conc. HCl, 2 ml paraldehyde. Leave in laboratory in stoppered bottle
- 4./

4. Spot test at intervals\*
5. Filter when mature
6. Dry filter paper and put in bottle with 200 ml of 70% alcohol  
This is the stock solution.

To make up the paraldehyde fuchsin stain, mix 25 ml of stock, 75 ml of 70% alcohol and 1 ml of glacial acetic acid.

Solution D. Groat's haematoxylin

Ferric alum	1 gm
Sulphuric acid (conc.)	0.8 ml
Distilled water	50 ml
Mix together.	Add to this
90% alcohol	50 ml
Haematoxylin	0.5 gm

Shake until dissolved. Filter. Keeps six months without refiltering.

Solution E. Picric acid, aq.sat.sol.	100 ml
Indigo carmine	0.25 gm

Either ester wax or polyester wax sections can be used with this/

---

\* Spot test. Drops of the solution as prepared in 3 are placed on filter paper. At first an appreciable quantity of basic fuchsin appears in the centre of the blot but this gradually diminishes until it disappears in four days or less. The solution is filtered immediately the basic fuchsin colour disappears.

this method although it is a staining method for aldehydes. It is not clear why the paraldehyde fuchsin does not stain the amylopectin since permanganate oxidation should release aldehydes from this polysaccharide adhesive.

#### Method.

1. Dewax and hydrate
2. Oxidise in Solution A 45 secs
3. Rinse in water
4. Decolourise in Solution B 45 secs
5. Wash in running water 30 secs
6. Stain in Solution C 2½ mins
7. Differentiate in acid alcohol
8. Rinse in tap water
9. Stain nuclei in Solution D 3 mins
10. Wash in Scott's tap water substitute 15 secs
11. Wash in distilled water 10 secs
12. Stain muscle tissue in Solution E 1 min
13. Blot dry and dehydrate rapidly in three changes of absolute alcohol
14. Clear in xylol, mount in canada balsam.

Some difficulty was experienced in obtaining consistent results by this method. Although care was taken to ensure that the paraldehyde fuchsin stain was always prepared in exactly the same way it was found that different batches of stain gave varying intensities of colouration. A much more vivid colouration/

colouration could be obtained if freshly-prepared solutions were used.

v). Lipid method (Pearse 1953)

Material was fixed either in Flemming without acetic (Gray 1954) or in the following (McManus, 1946)

Cobalt nitrate	1 gm
10% calcium chloride	10 ml
40% formaldehyde	10 ml
Distilled water	80 ml

Tissues were fixed in this for one week and then postchromed in 3% potassium dichromate for twenty-four hours.

Embedding was in either ester or polyester wax. The staining procedure was as follows.

1. Dewax and take down to 70% alcohol
2. Stain for 30 minutes at room temperature in a saturated solution of Sudan Black 'B' in 70% alcohol
3. Remove excess dye by rinsing quickly in 70% alcohol
4. Wash in running water
5. 1% aqueous neutral red 1 min.
6. Wash in running water, mount in glycerine jelly

v)./

v). Method for demonstrating metachromasia (Pearse, 1953).

Material was fixed in Bouin and embedded in either ester or polyester wax. The method was as follows.

1. Dewax and hydrate
2. 0.5% aqueous toluidine blue 4 hours
3. Rinse in distilled water
4. Examine immediately in water.

(g) Technique used for the detection of chitin

This was a slightly modified version of the van Wisselingh test as given by Campbell (1929) and Wigglesworth (1953).

The peritrophic membranes used in this test were obtained by slitting up the wall of the midgut of P. terrae-novae larvae and withdrawing the membrane with fine forceps. The membranes from twenty guts gave sufficient material with which to work.

The dissected membranes were washed and then placed in a  $\frac{3}{4}$ " x 3" "Pyrex" glass tube containing 3 ml of saturated potassium hydroxide. The tube was closed with a rubber stopper furnished with a Bunsen valve and then supported by a clamp in a 250 ml beaker full of glycerine. The glycerine was heated slowly through a period of fifteen to twenty minutes to 160°C, held at that temperature for three minutes, and then allowed to cool to room temperature.

When cold the contents of the tube were poured into a watchglass/

watchglass. The insoluble material was transferred to another watchglass containing 90% alcohol, washed thoroughly, then transferred through 70%, 50% and 30% alcohol to water. The water was poured off and the material covered with a drop of 0.2% iodine in potassium iodide solution which coloured it a deep brown. The iodine solution was drawn off and replaced by a drop of 1% sulphuric acid under which the brown colour was changed to a deep scarlet. This result, a positive chitosan test (chitosan being the substance formed when chitin is heated in concentrated alkali), was confirmed on another portion of material obtained by heating a further twenty membranes in alkali. This was placed in a watchglass and covered with a drop of 3% acetic acid. The material dissolved. The addition of a drop of 1% sulphuric acid caused a white precipitate to be thrown down. This confirmed that the insoluble material left after the membranes were treated with alkali was chitosan and, therefore, the membranes contained chitin.

In a control experiment run at the same time as the above in which midguts from which the peritrophic membranes had been removed were subjected to treatment with hot alkali there was no insoluble material left to test for chitosan.

### Section III: The Anatomy and Histology of the Proventriculus

#### (a) The general anatomy of the oesophageal valve and its relation to adjacent organs

After it passes below and between the cerebral hemispheres the gut, in the larva of P. terrae-novae, traverses a comparatively open space in the body cavity before entering the main visceral mass. This small space is bounded by the crop above, the salivary glands below and the malpighian tubules and main tracheal trunks at the sides. It contains the oesophageal valve and the enteric caeca (Fig. 1.).

As was stated in the introduction, the proventriculus forms the outer wall of the oesophageal valve. Before examining the proventriculus in detail a short description of the anatomy of the oesophageal valve is necessary since both the form and the function of the former are bound up with the form of the latter. The description which follows was worked out partly by dissection of anaesthetised larvae but mainly from stained serial sections of the valve.

The valve is formed by a folding in the gut wall and as a result has three distinct parts. Those parts are (Fig. 5.), from the inside out, a descending portion of the foregut, an ascending, reflected, portion of the foregut (these two portions of the foregut together forming the oesophageal invagination), and a descending portion of midgut, the proventriculus.

Fig. 4. The oesophageal valve, the enteric caeca and their adjacent organs in the larva of P. terrae-novae.

Ao, aorta; Br, brain; CG, cell garland;  
CV, chyle ventricle; DLC, dorsal long caecum;  
MT, malpighian tubules; Oe, oesophagus; OeV,  
oesophageal valve; PvG, proventricular ganglion;  
RN, recurrent nerve; Sal. Gl, salivary gland;  
TG, thoracic gland; TT, tracheal trunk; VSC,  
ventral short caecum.

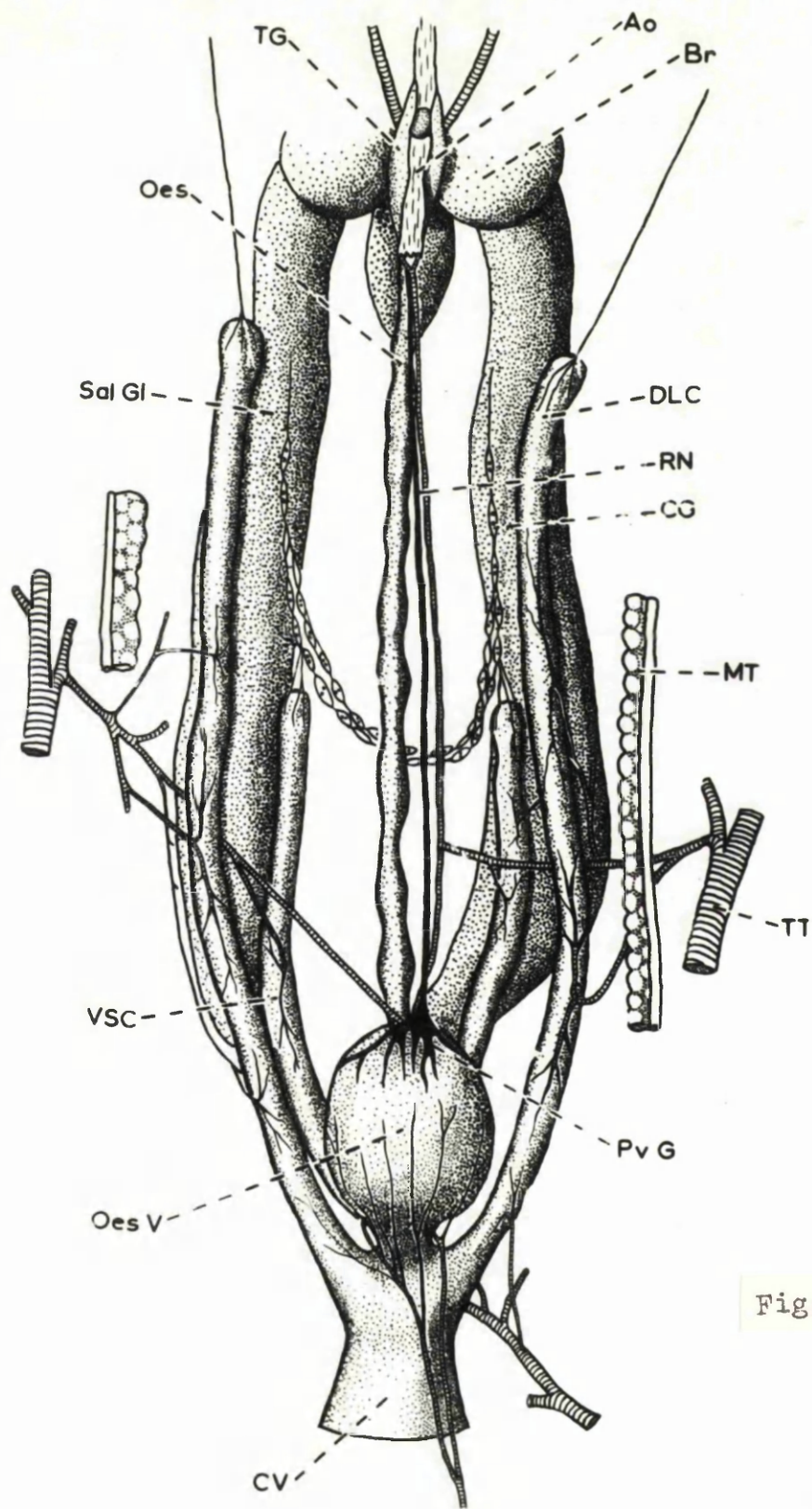


Fig. 4.

The descending portion of foregut which forms the core of the valve is for the most part anatomically indistinguishable from the oesophagus anterior to the valve. It has a simple structure consisting of an external muscle coat covering a basement membrane upon which rests a flattened epithelium, the latter secreting a thin tough intima which is pleated longitudinally.

Unlike that of the midgut, the muscle coat of the oesophagus is not clearly differentiated into longitudinal and circular muscle layers. Instead there is a close irregular meshwork of muscle fibres, these fibres branching in all directions so that the longitudinal and the circular components are completely mixed although there is a distinct predominance of circular fibres. In the valve the muscle layer is fairly thick but no thicker than that of the rest of the oesophagus. In other words there is no evidence of the development of a thick annular sphincter muscle such as is figured for by Wigglesworth (1930) as being found in other, more primitive, Diptera. Nevertheless, as will be described later, the muscles at this point do function as a sphincter. A short distance from the point of inflection of the gut the muscle layer becomes greatly attenuated and from here to the junction with the midgut it is almost indistinguishable.

The epithelium of the inner portion of foregut is made up of flat/

Fig. 5. A longitudinal section of the oesophageal valve in the larva of P. terrae-novae. (Camera lucida; Alcian blue - Mayer's haemalum - eosin).

AIR, anterior imaginal ring; BS, blood sinus;  
Bu, air bubble in food mass; EC, enteric caecum;  
ECV, epithelium of the chyle ventricle; En,  
entonnair; OH, oesophageal hypodermis; OI, oesophageal intima; OM, oesophageal muscle coat,  
PG, oblique section of the proventricular ganglion;  
PM, peritrophic membrane; Prov, limits of the proventriculus; TC, tangential section of the caecum.



flat polygonal cells greatly elongated along the axis of the gut. These cells have indistinct cell walls and flattened disc-like nuclei and they carry a fairly thick chitinous intima. Both this epithelium and its intima are thrown into longitudinal folds which allow for expansion of the lumen as the latter becomes filled with food. A short distance before the point of reflection the nature of the epithelium changes, the elongated flat cells giving way to shorter, deeper cells with distinct cell boundaries. These small cells form the "hinge" of the inflection. The intima is greatly thickened over these cells forming what Aubertot (1937) and others have termed the "entonnoir" (Fig. 5. En.). The function of this thickening of the intima is obscure since, as Wigglesworth (1930) points out, this plug of intima is too far from the source of the peritrophic membrane to take any part in the "annular mould" and moreover, as can be seen from Figure 5, it is unlikely that this ring of thick intima could be apposed to any adjacent part of the midgut to form a press. Inspection of sections also clearly demonstrates that the membrane is fully consolidated before it reaches this point in the gut. No hooks can be discerned on the intima at this point such as have been described in larvae of more primitive Diptera and said to draw the peritrophic membrane down as it forms.

Some distance beyond the point of inflection the shape of the epithelial cells begins to change again, the cells becoming larger/

larger and more elongated until the typical cells of the middle wall of the valve appear. These are very large ( $140\mu \times 100\mu$ ) rectangular cells with large round, or oval, central nuclei. They have distinct cell walls and an almost homogeneous non-granular cytoplasm. None of the staining methods employed, histological or histochemical, coloured this cytoplasm or demonstrated any secretory activity in it. The intima on the cells of the middle wall is thick for some distance beyond the inflection but over the large clear cells it is thinner.

The inner and middle walls together form the bell-shaped oesophageal invagination. On dissection this invagination is found to be a remarkably rigid structure, presumably due to a combination of high turgor in the large clear cells and a strong intima. Other investigators (vide Wigglesworth 1930) have described in larvae of other species a blood sinus between the two layers of the oesophageal invagination in which the pressure of the blood helps to maintain the rigidity of the invagination. In the larva of P. terrae-novae very little of this sinus is discernible (Fig. 5, B.S.) although blood cells can occasionally be seen trapped in it. It is this rigid invagination plus the form of the circular muscle layer on the proventriculus which gives the oesophageal valve its regular and constant shape.

A short distance before the second point of inflection of the gut wall there is an abrupt change in the form of the epithelium. Tightly/

Tightly packed against the last of the large cells is a compact mass of tiny cells, the anterior imaginal ring (Fig. 5. AIR). Perez (1910) investigated the function of this ring in the larva of Calliphora vomitoria and concluded that it was the imaginal rudiment from which the adult foregut was formed at metamorphosis. In this ring a large number of nuclei are visible but the cell membranes are barely discernible. The foregut intima is continued over these cells but it is extremely thin and at the distal extremity of the ring it appears to pass down to the basement membrane where it ends. This point marks the junction of foregut and midgut and lies almost at the apex of the reflected portion of the gut. At this point the proventriculus begins.

The proventriculus is composed of a thin outer layer of regularly arranged longitudinal and circular muscles covering a basement membrane on which rests a typical columnar midgut epithelium the cells of which carry a distinct striated border. The proventricular epithelium is pressed tightly against the intima of the middle wall cells. That this is the case in life and not merely a result of shrinkage during fixation was confirmed by dissection. A more detailed account of the tissues of the proventriculus is given later.

If the oesophageal valve is observed through the skin of a living larva or in one dissected in physiological saline the sphincter function of the valve can be seen. At short irregular intervals/

intervals food boli leave the crop and travel down the oesophagus to the valve. Here they are stopped at some point in the oesophageal invagination until a number, usually four or five, have built up then they are released into the chyle ventricle.

When the anterior midgut of a dissected larva is opened by a cut, made with a sharp eye scalpel, from a point behind the caeca to the anterior extremity of the proventriculus (the "shoulders" of the valve) the muscular wall of the gut will curl back to reveal the bell-shaped invagination at the anterior end with, hanging from it, the clear colourless cylinder of the peritrophic membrane. The membrane adheres closely to the invagination so that it is difficult to discern exactly how far up the latter it is present since it is so similar in appearance and texture to the intima of the invagination. It appears, however, to be present over at least half of its length. The cells of origin of the membrane material must therefore be situated somewhere about the lumen between the oesophageal invagination and the proventriculus.

(b) The musculature of the proventriculus

As has already been noted, the fairly constant shape and the rigidity of the oesophageal valve can be ascribed partly to its internal structure and partly to the unusually dense and regular arrangement, in comparison with the rest of the alimentary canal, (Plate 1) of the circular muscle layers of the proventriculus.

In common with the rest of the midgut the musculature of the proventriculus consists of a regular coat of circular muscles overlaid with a very few longitudinal muscles (Fig. 6).

The longitudinal muscles are evenly distributed round the circumference of the proventriculus. There are about twelve of these muscles present. They are continuous with, and similar to, the longitudinal muscles of the chyle ventricle, each consisting of a small number of muscle fibres within a sarcolemma. There are generally two nuclei within the length of each muscle over the proventriculus. These are flattened disc-shaped nuclei lying in expansions of the sarcolemma, the expansions being found at irregular intervals along the bands. The longitudinal muscles are striped but the striations are very indistinct.

Unlike the longitudinal muscles the circular muscles cover the proventriculus with a close dense coat. They are arranged in regular bands (Fig. 6 and Plate 2). Each band contains a small number of bundles of muscle fibres with numerous anastomoses between/

Fig. 6. Diagram of the musculature of the proventriculus  
in the larva of P. terrae-novae.

CM, circular muscle bands; CMN, circular muscle  
band nuclei; LM, longitudinal muscle bands;  
LMN, longitudinal muscle band nuclei.

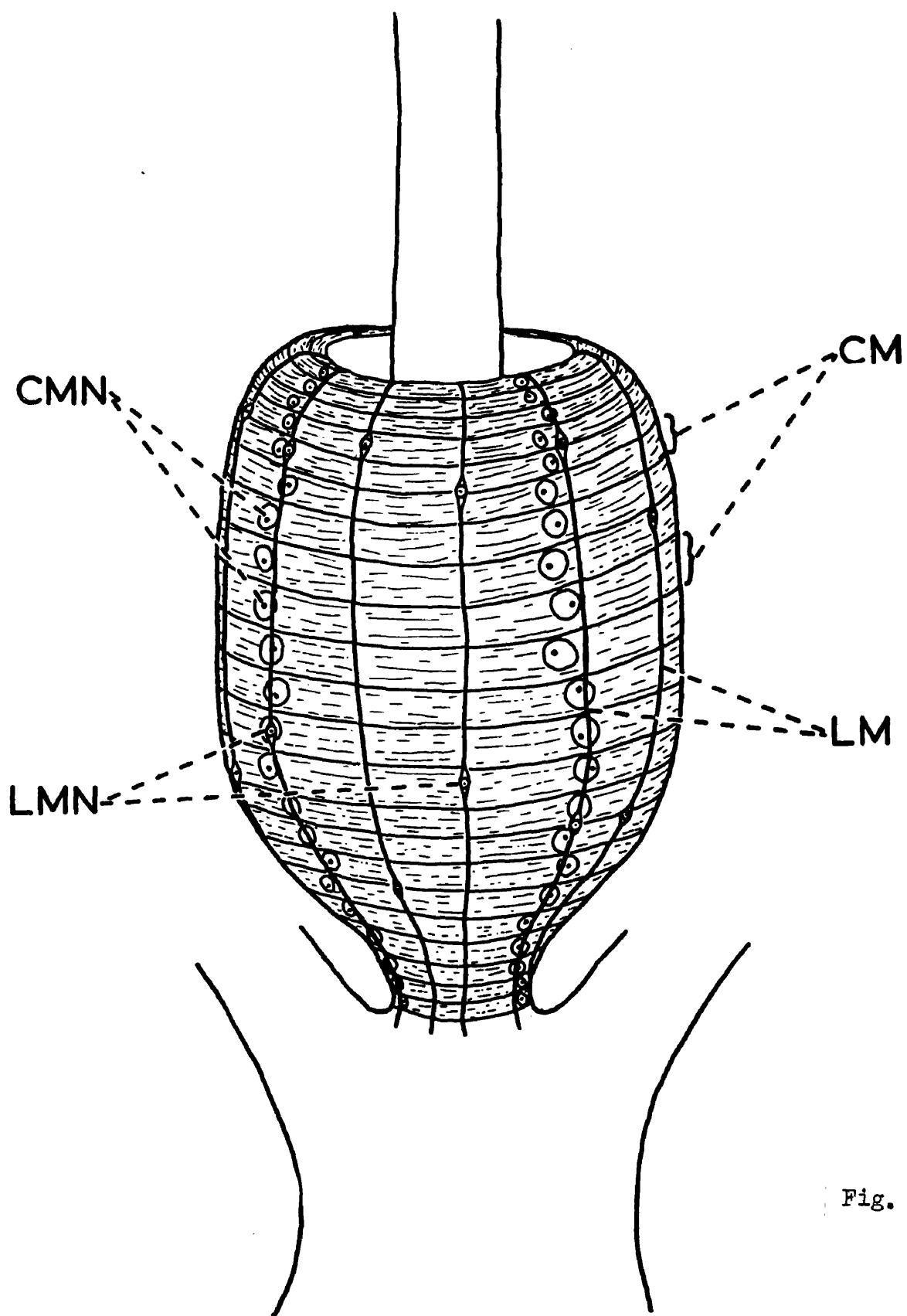


Fig. 6.

between the bundles within the bands but only very rarely do anastomoses occur between individual bands. The circular bands are more clearly striped than the longitudinal bands (although not as clearly as the circular muscles of the chyle ventricle). Each band is contained in a sarcolemma. Within this sarcolemma are four nuclei evenly distributed around the circumference of the proventriculus, these nuclei being in approximately the same positions in all the bands with the result that the nuclei are arranged in four rows along the length of the proventriculus. (Fig. 6). The nuclei are fairly large, in comparison with nuclei in other parts of the gut musculature, and disc-shaped.

In larvae dissected in saline the circular muscles of the proventriculus exhibit slow regular contractions. These are greatest in magnitude at the anterior end over the "shoulders" and least at the posterior end. The contractions are not synchronous with the peristaltic contractions of the oesophagus or the chyle ventricle. Sectioning of the recurrent nerve to isolate the proventricular ganglion does not alter or stop the contractions. They are presumably related to the functioning of the oesophageal valve as a mould for the peritrophic membrane. If the sinus between the oesophagus and the reflected end of the foregut is functional then the greater magnitude of contraction at the anterior end may be a mechanism to constrict the neck of the sinus where it opens into the haemocoel thereby increasing the pressure in the sinus and increasing the rigidity of the oesophageal/

Plate 1. The musculature of the chyle ventricle in the larva  
of P. terrae-novae.

Lettering as in Figure 6.

Plate 2. The musculature of the proventriculus in the larva  
of P. terrae-novae.

Lettering as in Figure 6.

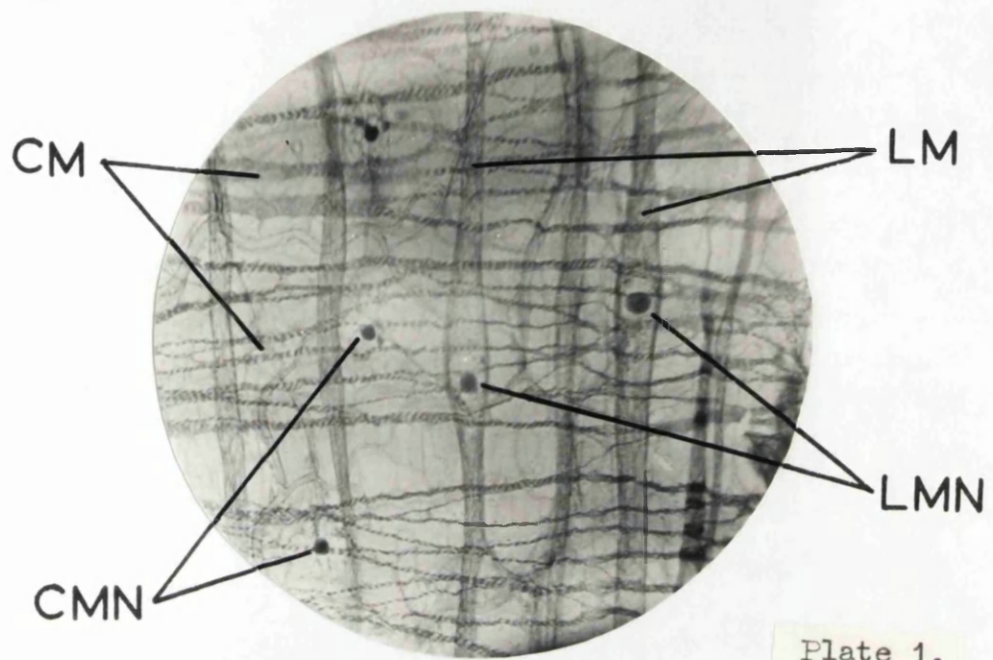


Plate 1.

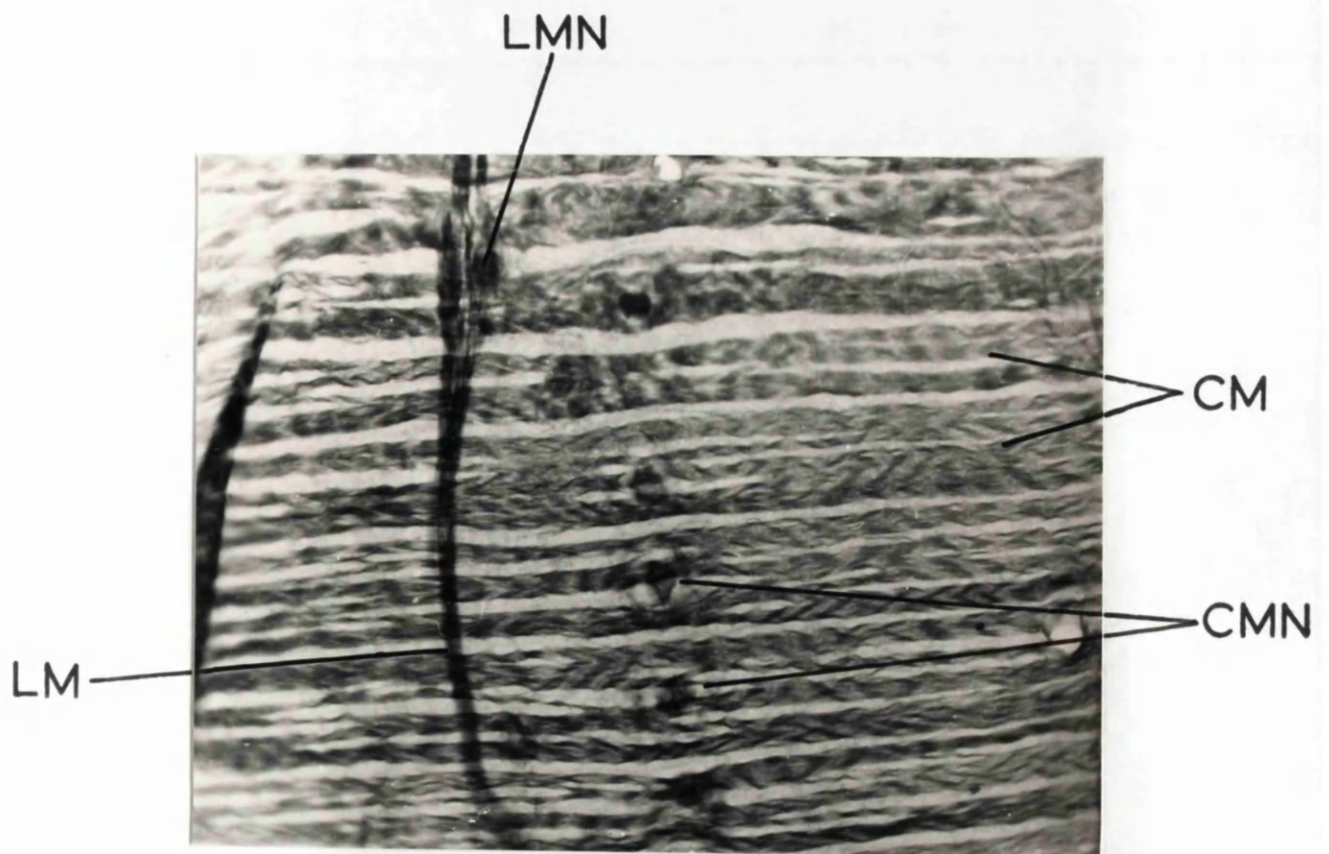


Plate 2.

oesophageal invagination.

(c) The innervation of the proventriculus

The proventriculus appears to be innervated only by the sympathetic nervous system. The arrangement of nerves is similar to that described by Lowne (1890) in the larva of C. erythrocephala and by Bhatia (1939) in the larva of the Syrphid Platychirus scutatus Meig.

The description given by Lowne is brief. He refers to a large crutch-shaped ganglion at the end of the recurrent nerve from which ganglionated fibres pass into the wall of the proventriculus, the chyle stomach, and the salivary glands but he does not provide detailed figures of this distribution.

Bhatia gives a more detailed description of the proventricular innervation in P. scutatus. In this species the nerve bifurcates and the two branches, each containing a small ganglion, pass round opposite sides of the oesophagus and ultimately reunite so that a "nerve-ring" is formed round the proventriculus. At intervals around the ring smaller branches are given off over the surface of the proventriculus (and, according to his illustration, to the chyle stomach).

Hewitt (1914) working on the larva of Musca domestica states briefly that the recurrent nerve forms a posterior ganglion on the proventriculus/

Fig. 7. The nerve and tracheal supply to the proventriculus in the larva of P. terrae-novae.

ATS, right anterior tracheal supply; DLC, dorsal long caecum; GB, branches of the recurrent nerve making up the proventricular ganglion; Oes, oesophagus; Prov, proventriculus; PTS, posterior tracheal supply; RN, recurrent nerve.

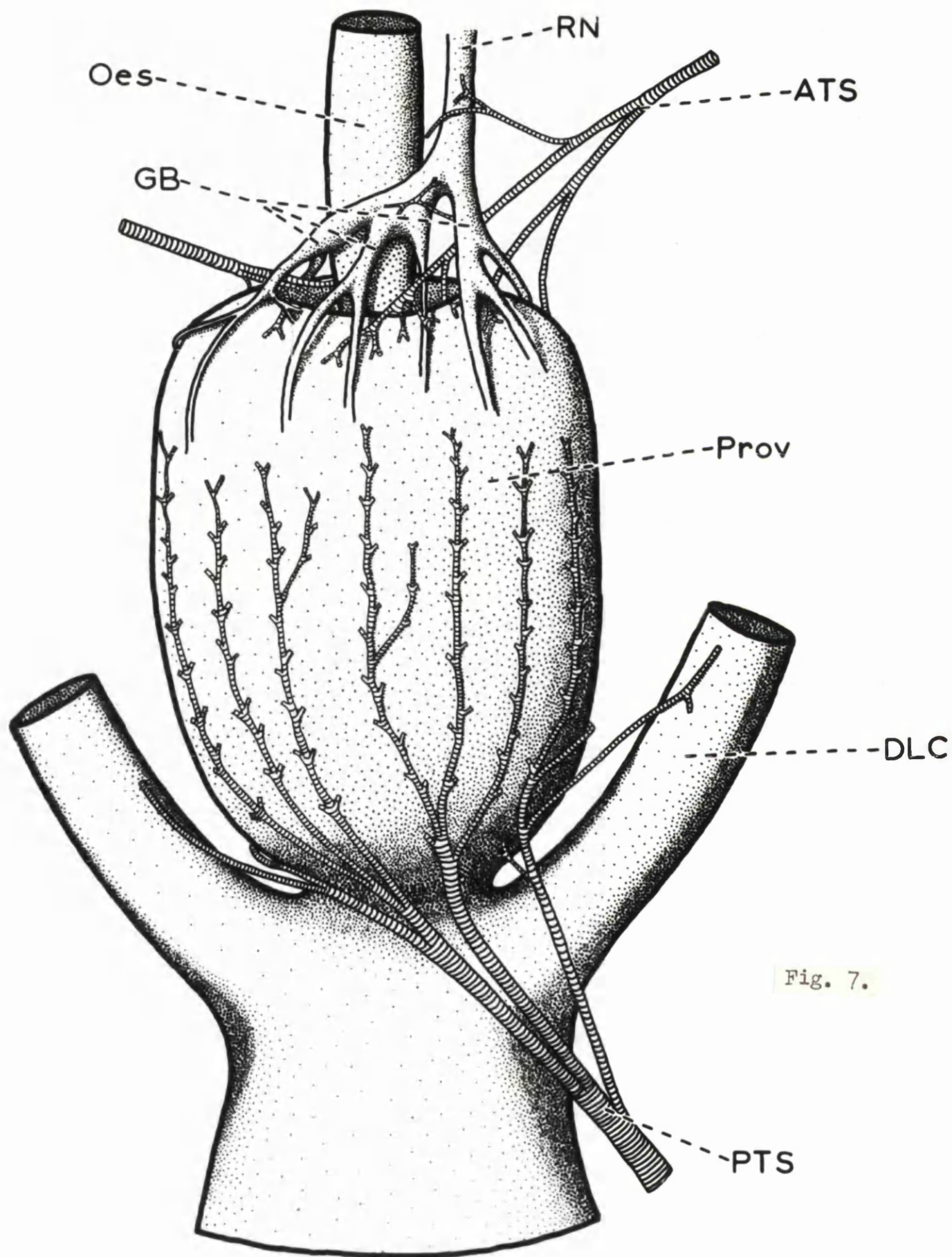


Fig. 7.

proventriculus from which five nerves run to the anterior end of the proventriculus.

In the larva of P. terrae-novae the recurrent nerve passes back alongside the oesophagus from the hypocerebral ganglion. It does not usually divide until it reaches the oesophageal valve where it divides once, the two branches again dividing several times. In a few larvae the recurrent nerve was found to divide into two separate nerves about half-way along its length but the halves reunite again before reaching the proventricular ganglion. The distal end of the recurrent nerve, its first two branches and the thickened beginnings of the subsequent branches together form the proventricular ganglion (Fig. 7). These various parts of the ganglion are packed with a large number of bipolar and multipolar nerve cells (Fig. 8). The finger-like processes of the branched ganglion pass down over the face of the proventriculus, their number and distribution being similar to that of the longitudinal muscle fibres although there is no obvious relationship between the two. These processes become very indistinct after they have travelled about one-third of the distance down the proventriculus and it is only with difficulty that the nerve fibres from them can be traced beyond this point. In some preparations a few extremely fine nerves were seen forming a thin network over the proventriculus. These were entirely superficial and almost certainly innervated only the muscles.

Fig. 8. A longitudinal section of the proventricular ganglion in the larva of P. terrae-novae. (Camera lucida; Alcian blue - Mayer's haemalum - eosin)

BPG, branch of the proventricular ganglion; CM, circular muscle; LMN, longitudinal muscle nucleus; LPE, anterior limit of the proventricular epithelium; NC, nerve cells; PG, proventricular ganglion; PEC, proventricular epithelial cell.

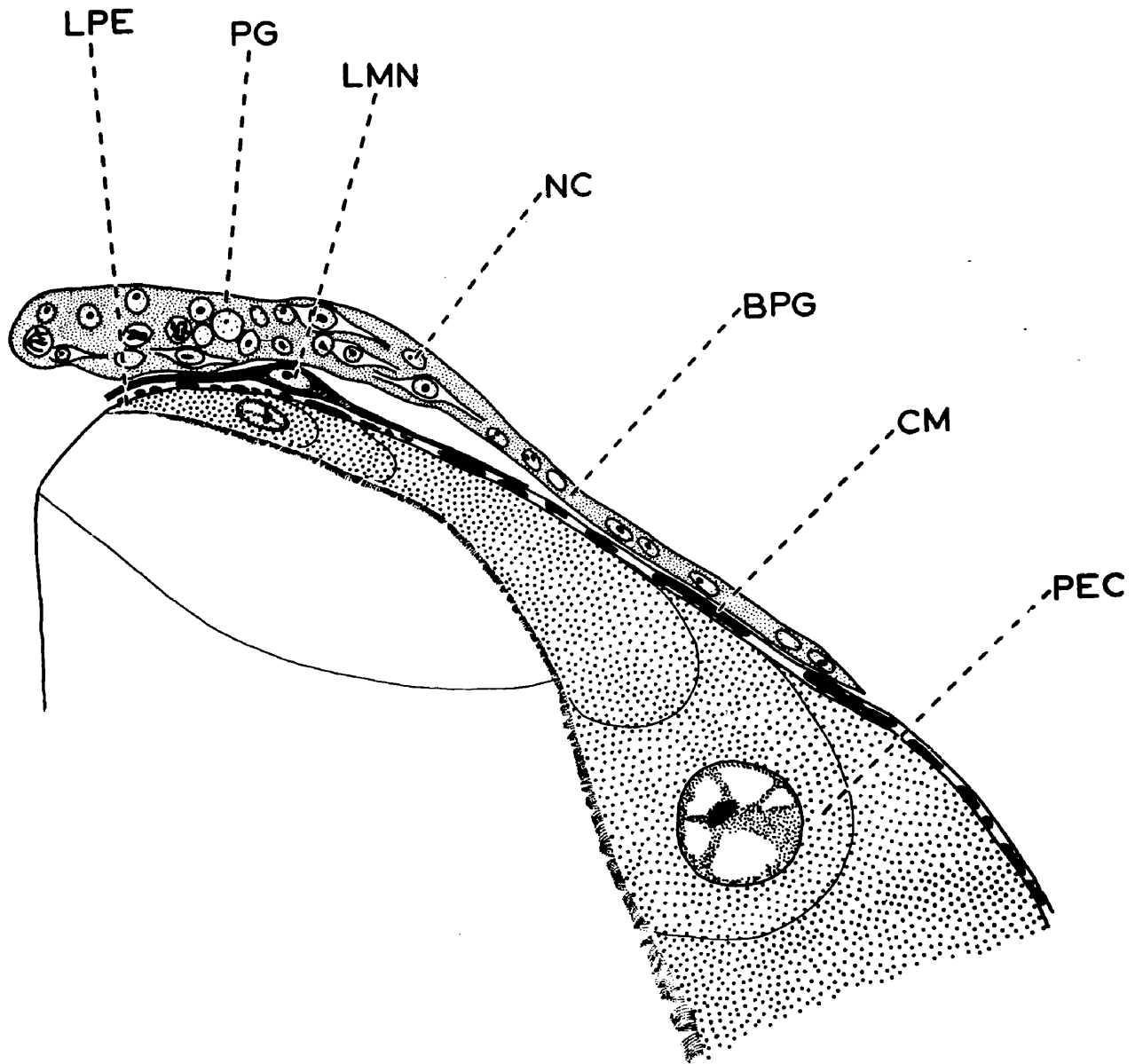


Fig. 8.

In none of the specimens examined was any branch of the proventricular ganglion seen to innervate the salivary glands as was observed by Lowne in the larva of C. erythrocephala nor were any of the nerve fibres from the proventriculus observed to pass down on to the chyle ventricle or to the enteric caeca.

Although the proventricular ganglion appears to innervate the muscles of the proventriculus, sectioning of the recurrent nerve does not obviously affect the functioning of these muscles. It must be admitted however that these observations were made in dissected larvae in saline. It proved technically impossible for reasons given below (p. 93) to section the recurrent nerve through a small incision in the skin so that any resulting changes might be observed in a semi-entire larva.

(d) The tracheation of the proventriculus

The proventriculus receives tracheae from four sources, two anterior and two posterior.

The anterior sources are symmetrical, originating from the main left and right tracheal trunks in the region of the fifth body segment (Fig. 4). The branch from the left trunk at this point gives off tracheae to the oesophagus, the malpighian tubules, the salivary glands and the enteric caeca, as well as to the proventriculus. The branch from the right trunk as well as supplying these same tissues on its respective side also supplies tracheae/

tracheae to the sympathetic nervous system lying behind the brain including the unusual tracheation of the thoracic gland described in this species by Fraser (1957a).

The two anterior sources supply only a very limited area of the anterior end of the proventriculus (Fig. 7). The majority of the proventricular tracheae come from the posterior sources which are, unlike the anterior, asymmetrical, both originating from the right main tracheal trunk. There is some variation between individual larvae but usually the posterior supply branches to provide twelve to fourteen main longitudinal tracheae which in turn branch freely along their length providing a dense network of tracheoles over four-fifths of the proventriculus.

The tracheation appears to be restricted to the muscles and the basement membrane. Neither in dissected specimens nor in stained sections were tracheoles observed to be penetrating the basement membrane to enter the cells of the proventricular epithelium.

(e) The histology of the proventriculus

The histology of the proventriculus was studied by means of stained serial sections of whole oesophageal valves. The staining methods employed have been described.

In structure the epithelium of the proventriculus is a cylinder/

cylinder of cells about twenty-four cells in circumference and twenty-four cells in length, limited anteriorly by the anterior imaginal ring and posteriorly by the abrupt commencement of the heavily-secreting digestive tissue of the midgut. The cells at the anterior end of the epithelium are mainly square or oblong in section, the largest cells ( $22-27\mu$  deep  $\times$   $17-21\mu$  long) being just anterior to the centre of the wall (Figs 5 and 9). Towards the posterior end of the proventriculus the cells become very shallow and elongated along the axis of the gut ( $4-6\mu \times 22-35\mu$ ).

All the cells in the epithelium carry a very distinct striated border on the adaxial face (Fig. 9). This border varies in height from  $2-3\mu$  at the extremities of the proventriculus to  $6-7\mu$  on the deeper cells in the centre of the wall (in comparison with a normal height of  $3.5-5\mu$  on the digestive cells in the chyle ventricle). The border is most clearly seen in sections stained with iron haematoxylin - Orange "G" in which it stands out a clear golden-yellow. The "bristles" of the striated border show both types of elements described by Wigglesworth (1953). The majority of the cells of the proventriculus back to about the fifteenth row from the anterior end carry a "brush border" the elements of which are obviously of a flexible nature since in some sections they were seen to be clumped so that the elements of adjacent cells overlapped or diverged from each other. Each element would also appear to be independent of its neighbours since there were/

Fig. 9. Part of a longitudinal section of the proventriculus in the larva of P. terrae-novae showing maximum vacuolation found in proventricular epithelial cells.

BB, "brush" border; HB, "honeycomb" border; PM, limits of the PM ring of epithelial cells.

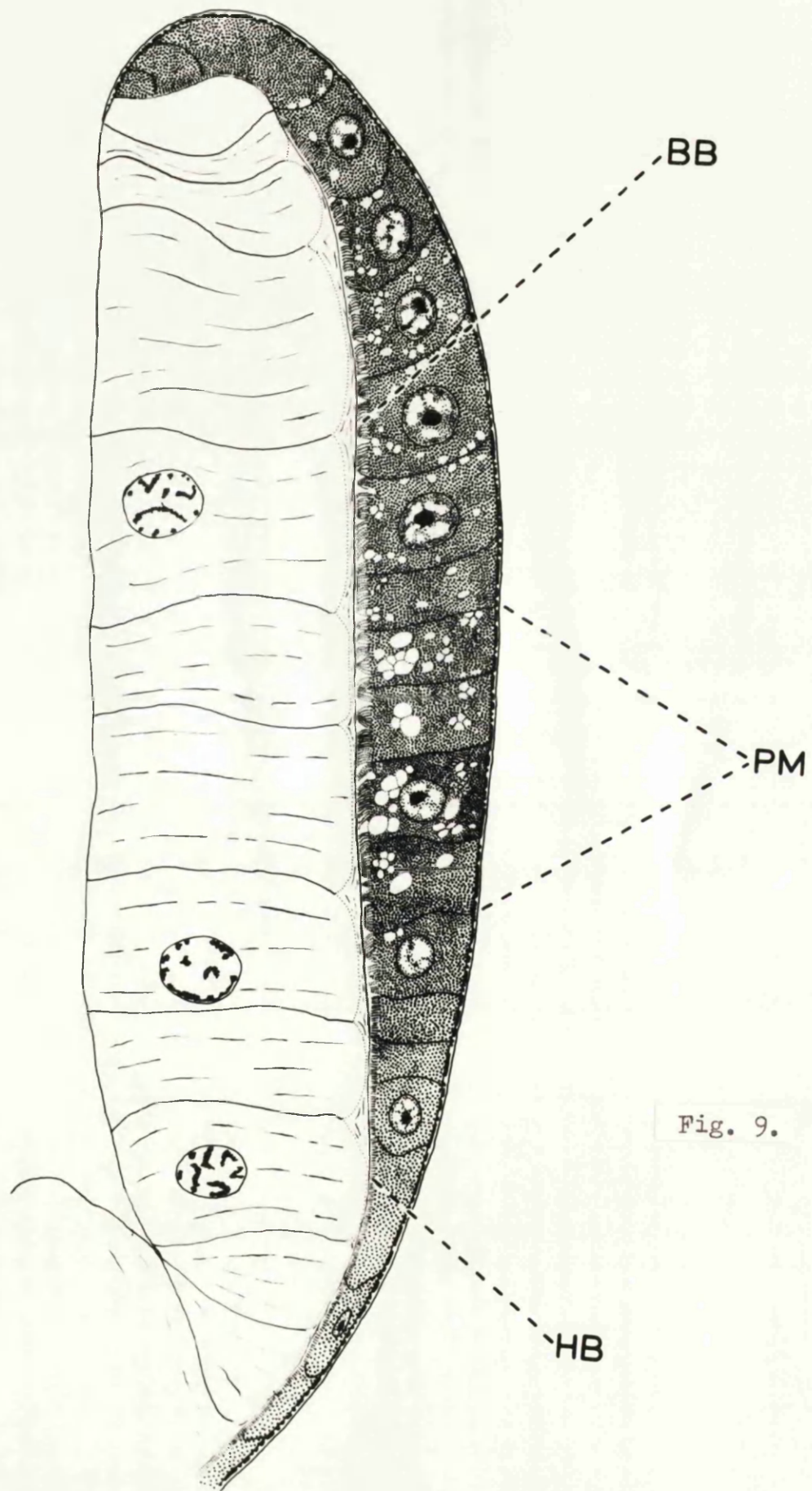


Fig. 9.

were similar overlappings and divergences among the elements of the border of a single cell. Beyond about the fifteenth row the cells appear to carry a "honeycomb border" in which the elements are shorter and apparently rigid since no overlapping of elements was observed. This latter type of striated border is also found on the cells of the caecal epithelium and that of the chyle ventricle.

The nuclei of the epithelial cells vary in size and shape with the cells, being flat and platelike (mean size =  $8.9\mu \times 3.2\mu$ ) in the cells at the extremities and large and round (mean size =  $9.0\mu \times 8.6\mu$ ) in the deep central cells.

All the cells of the epithelium contain a dense, granular, chromatophil, acidophil cytoplasm. The anterior fourteen rows of cells usually show vacuoles in their cytoplasm but the cells posterior to these have a homogeneous cytoplasm. In occasional proventriculi all the cells of the epithelium had a homogeneous cytoplasm the vacuoles being absent from those at the anterior end.

The vacuoles in the cells in the anterior half of the proventriculus are of two distinct types. In the anterior nine or ten rows of cells the vacuoles are small ( $3\mu$ - $5\mu$  in diameter) and scattered throughout the cytoplasm with some concentration between adjacent cells. These small vacuoles are erratic in their occurrence/

occurrence, sometimes large numbers being present but more often very few are scattered through the cytoplasm of these cells. In the remainder of the vacuolated cells, a band of cells about four cells deep between the tenth and the fourteenth cell rows (Fig. 9 PM), there are much larger vacuoles ( $7\mu$ - $10\mu$  in diameter) as well as a few small vacuoles. The large vacuoles are largely confined to the cytoplasm between the nucleus and the brush border, there being a distinct relationship between them and the nucleus, as if they were forming on the periphery of the latter (Figs 9 and 10). The smaller vacuoles are confined mainly to the distal poles of these cells away from the lumen.

In a few sections the lumen outside the epithelial cells was filled with a granular material. This was stained a bluish-grey by haemalum and appeared very similar to the cytoplasm of the adjacent epithelial cells. This could have been taken as evidence of merocrine secretion by these cells but it was thought more likely to be a fixation artifact.

#### Section IV: The Physiology of the Proventriculus

As has been described in Section III (a), the peritrophic membrane is formed at some point in the lumen between the oesophageal invagination and the proventriculus. There are three possible sources of the membrane material around this lumen; the large clear cells of the oesophageal invagination; the cells of the anterior imaginal ring; and the cells of the proventricular epithelium.

It is unlikely that the large clear cells secrete the material of the membrane. They show no evidence of any secretory activity, neither vacuoles nor granules of any sort being observed in the cytoplasm. Moreover they carry the fairly thick intima described in Section III (a). The reason why, on dissection, the peritrophic membrane appears to be attached to these cells is that, as will be seen later, the membrane material is secreted on to the surface of the intima. This smooth rigid surface acts as a mould to form the membrane which naturally has the same circumference as the outside circumference of the invagination and so remains over the latter like a sleeve when the gut is opened.

As was noted in Section III (a) the function of the anterior imaginal ring has been investigated and satisfactorily explained by Perez (1910) who found that it was the rudiment from/

from which the imaginal oesophageal valve and part of the oesophagus are developed at metamorphosis.

The only remaining source of the peritrophic membrane material was therefore the proventricular epithelium. An attempt was therefore made to identify, mainly by histochemical staining, the cells in this epithelium which secreted the membrane material and to identify the material in these cells.

From the histological study described in the previous section it is evident that the cells of the anterior half of the proventriculus are secreting fairly actively. Unfortunately the staining methods used in this earlier part of the work did not differentiate the peritrophic membrane from adjacent tissues in sections of the proventriculus so that its exact point of origin could not be determined but it was thought likely that the vacuolate cells in the epithelium were the source of the membrane material.

In common with other insects, the peritrophic membrane in the larva of P. terrae-novae contains chitin, this fact being ascertained by the van Wisselingh test. A search was therefore made for a source of chitin in the proventriculus.

According to Richards (1950) chitin can be defined as a high molecular weight polymer of N-acetyl glucosamine residues, these residues being formed into long unbranched chains by linkage/

linkage between carbon atoms one and four. Nothing is known of the precursors of chitin. There is much evidence in the literature that it originates from the transformation of glycogen but Richards after reviewing the evidence for this belief, is not convinced. However it seemed logical to assume that chitin is built up from simpler polysaccharides and that cells in which chitin was being synthesised would contain a significant quantity of polysaccharide, the precursor of chitin in the process of synthesis.

The first staining method employed was the periodic acid - Schiff (PAS) of Hotchkiss (1948). By this process 1-2 glycol groups in polysaccharide molecules are oxidised by the periodic acid to form aldehydes which then combine with the fuchsin of the Schiff reagent to give a scarlet product. McManus (1956) notes that the PAS method is fairly certainly specific for carbohydrates if oxidation is carried out with an 0.5% periodic acid solution for no longer than five minutes. This precaution was taken in the present case.

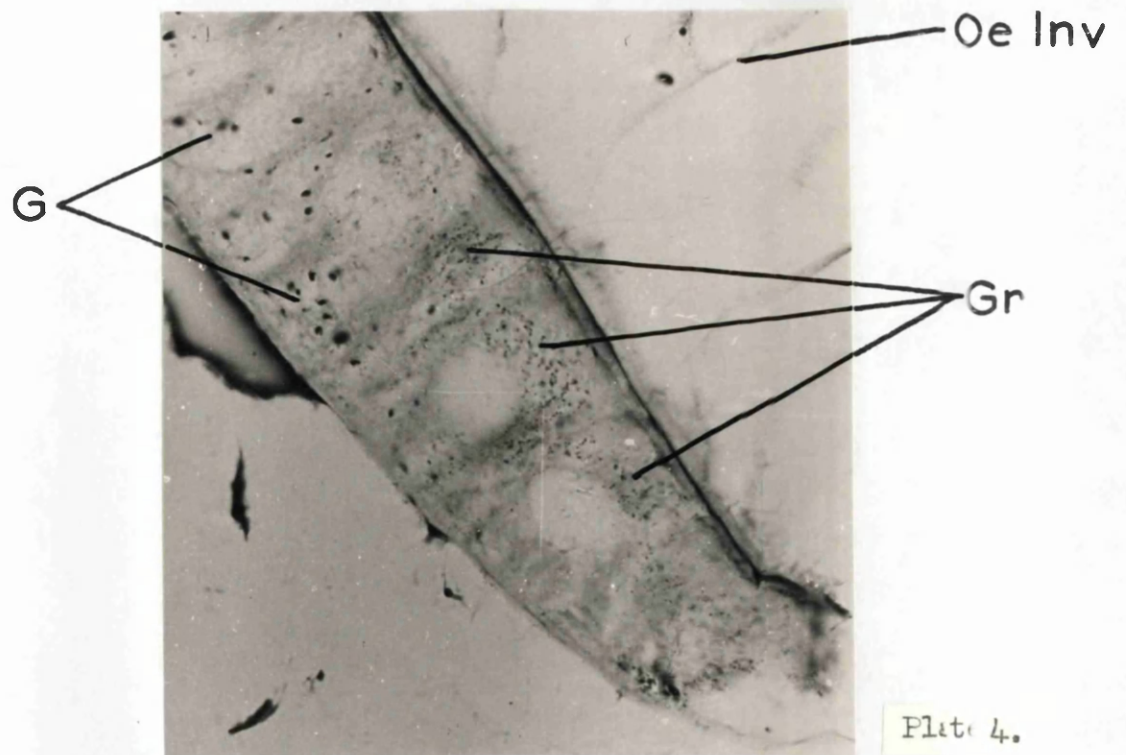
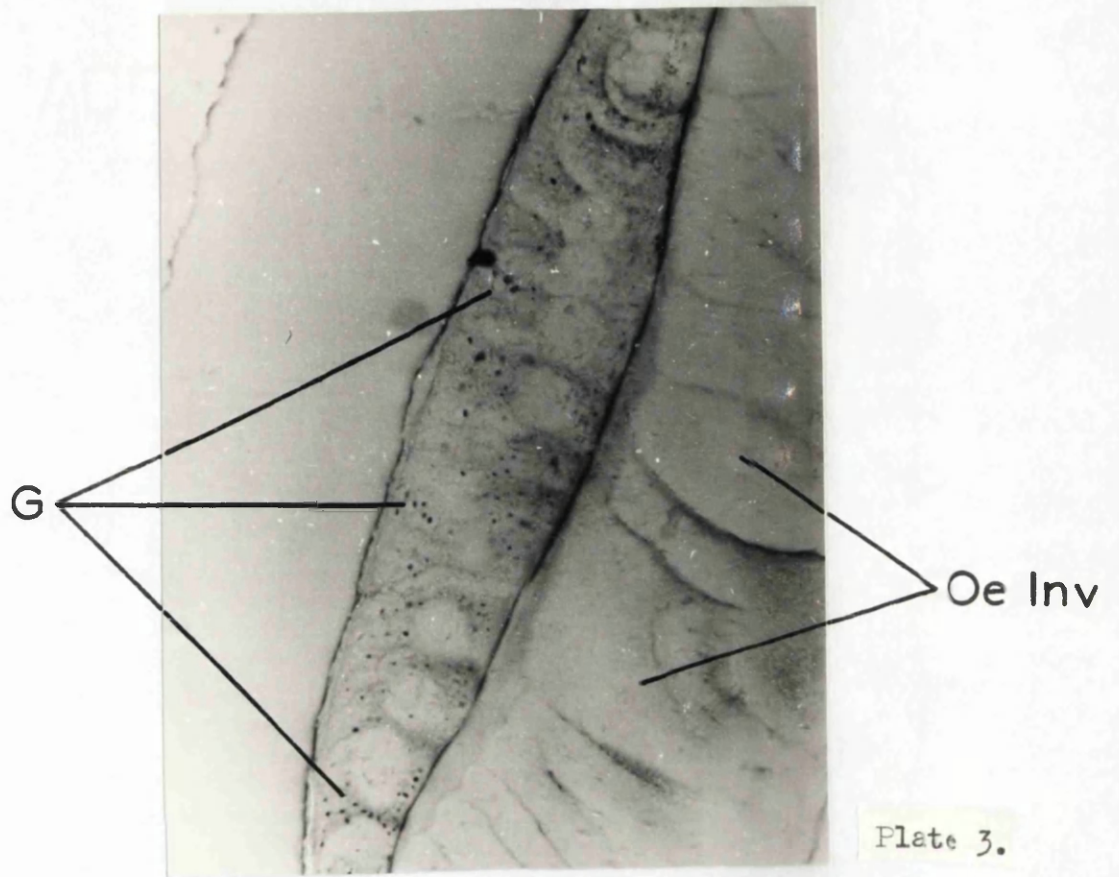
The distribution of glycogen in the proventricular epithelium was investigated first. Sections of material fixed in Bouin at 0°C for twenty-four hours were protected with celloidin to prevent leaching of the glycogen and stained by the PAS method. Glycogen in the cells stained a deep purple colour by this method. It was precipitated in the form of small/

Plate 3. Glycogen distribution in the deep central cells of the proventricular epithelium in the larva of P. terrae-novae. (Longitudinal section; PAS after celloidin)

G, glycogen; Oe Inv, cells of the oesophageal invagination.

Plate 4. The PR ring of cells in the proventriculus of the larva of P. terrae-novae. Note the comparative scarcity of glycogen in them and the presence in them of PAS-positive granular material. (Longitudinal section; PAS after celloidin).

G, glycogen; Gr, granules; Oe Inv, cells of the oesophageal invagination.



small pieces spread fairly evenly through the cytoplasm of the cells without a clear concentration in any of the cells (Plate 3). There was however a marked scarcity of glycogen in the band of cells about four cells deep which have already been described as containing large vacuoles (hereafter referred to as "the PM cells"). What glycogen there was in these cells was restricted to the abaxial part of the cells between the nucleus and the basement membrane. Attention was then turned to the other polysaccharides. The PAS method was again employed but the sections were not protected with celloidin and before being stained they were treated with diastase for thirty minutes to remove the glycogen. After this treatment the only PAS-positive material in the proventriculus was the basement membrane and a quantity of small granules in the PM cells. The vacuoles were not differentiated by this staining method. These granules were distributed throughout the cytoplasm of the cells with some concentration between the nucleus and the striated borders (Plate 4).

Being PAS-positive these granules can be placed according to the classification of Pearse (1953) in the groups of substances containing polysaccharides, mucopolysaccharides, mucoproteins, glycolipids, and lipoproteins (Table I). Further cytochemical tests were undertaken following the procedure detailed by Pearse to determine to which of these groups the granules belonged.

Table I. Classification of some PAS-positive substances (Pearse, 1953, modified)

Classification	Protein tests	Sudan Black 'B'	PAS	Diastase	Metachromasia	Alcian blue
Simple polysaccharide and glycogen	-	-	+	Labile	-	-
Acid mucopolysaccharide	+	-	+	Fast	gamma	+
Mucoprotein or neutral mucopolysaccharide	+	-	+	Fast	beta or -	Faint or -
Simple basic protein	+	-	-	Fast	-	-
Glycolipid	-	+	+	Fast	-	-
Lipoprotein	+	+	+	Fast	-	-

The tests already carried out on diastase-treated sections had showed that the granules were not labile and therefore could not be simple polysaccharide or glycogen.

Protein was tested for by the methods of Baker (1956) and of Mazia, Brewer and Alfert (1953). Both these methods were somewhat simpler than the Millon method advocated by Pearse or Pollister's (1950) modification of the Millon used by Rizki (1956). The granules were stained a faint pink by Baker's method, sufficient to differentiate them from the yellowish colour of the cytoplasm of the PM cells. With the method of Mazia et.al. the cytoplasm of all the proventricular epithelial cells took the stain so strongly that it was impossible to differentiate the granules from the rest of the cytoplasm. This latter result was attributed to the chromophil nature of the cytoplasm of the epithelial cells rather than evidence of a concentration of protein in all the cells since a similar colouration could be found in other cells in the gut with dense cytoplasm e.g. the basal non-vacuolated portion of the cells of the digestive epithelium.

Lipids were tested for by the Sudan Black 'B' method of McManus as given by Pearse (1953), using tissue fixed in Flemming - without-acetic or McManus' cobalt nitrate fixative with post-chroming. The granules were not stained by this method. No concentration of lipids was observed in any of the/  
the/

the cells of the proventriculus, a result which was in marked contrast to that from the epithelial cells of the chyle ventricle immediately following the proventriculus which were packed with lipid inclusions. This result agrees with that of Waterhouse and Stay (1955) who stated that lipids were absent from the proventricular epithelium of the closely-related larva of Lucilia sericata.

As can be seen from Table I, these results indicated that the granules were of a mucopolysaccharide nature, acid or neutral.

Acid mucopolysaccharide was tested for by using alcian blue 8 GS (Steedman, 1950). This coloured the cytoplasm of all the epithelial cells a faint reddish-blue without differentiation of the PAS-positive granules. The granules were therefore probably of a neutral mucopolysaccharide nature. This result was checked by investigating the metachromasia of the granules by the standard toluidine blue method Pearse (1953). The only metachromatic material in the proventriculus was in the nucleoli of all the nuclei of the epithelium. These showed gamma (red) metachromasia. The cytoplasm of the cells was a strong violet-blue colour, again as with the protein test, so strong that the granules were not differentiated.

These tests, while not conclusive, indicate that the granules/

granules are of a neutral mucopolysaccharide nature.

While these investigations were being carried out a colleague working on a neurophysiological problem in the larva of Lucilia caesar (Fraser, 1957b) noticed that one of his staining methods, a modification of Gomori's (1950) paraldehyde fuchsin method, acting on sections of pupal material was staining material at points where chitin was being synthesised and laid down in new imaginal structures, e.g. in new tracheae, or broken down by moulting fluid. It was suggested that this staining method might be tried on proventricular material.

At first the results were disappointing, nothing being differentiated in the cytoplasm of the epithelial cells except the faint outlines of the vacuoles in the anterior half. It was found however, that if a freshly-prepared solution of the paraldehyde-fuchsin stain (hereafter termed "the PF stain") was employed a better result was obtained. The PM cells which had been shown to contain large vacuoles and diastase-fast PAS-positive granules were now shown in some cases to contain a complete secretion picture rather like that described by Siang-Hsu (1947) in D. melanogaster. At the abaxial poles of some of the cells were a few minute crescent-shaped chondriosomes, the arms of the crescents pointing outwards towards the basement membrane (Fig. 10 and Plate 5). These crescents were stained an intense purple-black. Into the curve/

Fig. 10. A drawing of the portion of a longitudinal section of the proventriculus illustrated in Plate 5. (Camera lucida; paraldehyde fuchsin).

BB, "brush" border; CB, crescent bodies; IOI, intima of the oesophageal invagination; LM, longitudinal muscle; LV, large vacuoles containing PF-positive material; N, epithelial cell nucleus.

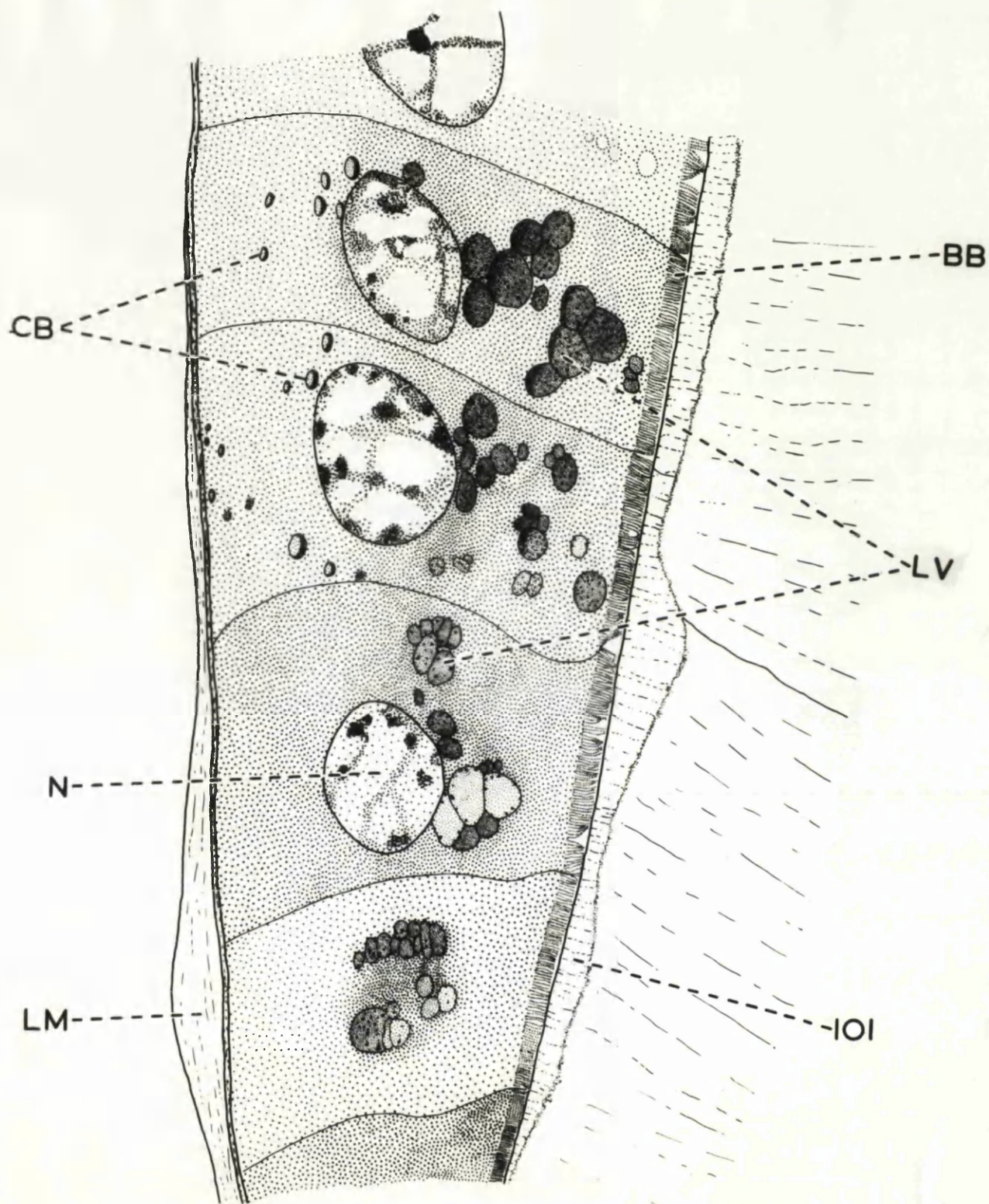


Fig. 10.

Plate 5. The PR ring of cells in the proventriculus stained  
by the paraldehyde fuchsin method.

(For details see Figure 10).



Plate 5.

curve of each crescent was fitted a tiny vacuole the contents of which appeared homogeneous and stained a faint violet. As they approached the nucleus these bodies increased in size evenly and equally until at the nucleus the whole body, crescent plus vacuole, measured as much as  $3\mu \times 1.8\mu$ .

At the level of the nucleus a change was observable, the crescent-shaped bodies being no longer visible and the vacuoles becoming considerably larger. These are the large vacuoles seen in the histologically stained sections. There was a mass of these vacuoles between the nucleus and the striated border some as much as  $10\mu$  in diameter. Most of them had granular contents which stained a deep purple and some had almost entirely homogeneous contents which stained a light violet. There was no significant distribution of these two types of vacuole contents, the two being mixed together randomly, so no conclusions can be drawn about why the contents should differ.

This picture of globules of secretion being formed initially in the vicinity of mitochondria was only seen in some cells. In a majority of the cells the crescent bodies and their vacuoles were absent or in the abaxial regions of the cells there were only a few minute irregular darkly-stained bodies. But even when the crescent bodies were absent the vacuoles were usually present between the nucleus and the striated/

striated border. In a few cases, as was noted during the histological investigation, occasional proventriculi showed no vacuoles in the PM cells, the cytoplasm being homogeneous.

The paraldehyde fuchsin did not stain the granules in the PM cells. The contents of the vacuoles in the cells anterior to the PM ring were not stained by any of the staining methods employed.

It would have been useful if these histochemical tests had thrown some light on the nature of the peritrophic membrane and the point in the lumen at which it is formed but unfortunately this was not the case. After the membrane has reached the point where there is a space between the oesophageal invagination and the proventriculus and is free in the gut it is possible to observe the effects of the various histochemical tests on it. However since it is positive to protein, lipid and carbohydrate tests alike, no conclusion can be drawn since it is as likely to be food contaminants which are staining as the components of the membrane. Above the point where the two tissues part the membrane is impossible to distinguish from the surrounding tissue. Only the PAS and the PF stains colour it strongly and unfortunately the outer layer of the intima over the oesophageal invagination cells is also coloured by these stains. This is true of the entire intima from the thickened part at the first point of inflection right forward to the anterior imaginal ring so the point at which the membrane forms cannot/

cannot be seen. (The layer stained in the intima is presumably the epicuticle. Richards (1952) claims that this layer is PAS-positive in the integument although Brunet (1953) questions this on the grounds that no polysaccharide is known to be present in the epicuticle.)

The results obtained with the histochemical tests employed are summarised in Table II.

In a very few proventriculi it was noted that one or two cells were entirely and homogeneously filled with material that was diastase-fast and strongly PAS- and PF-positive. These cells were usually found immediately below the posterior cells of the PM ring. There was not a complete ring of them but only two or three distributed about the circumference of the proventriculus. The appearance of this phenomenon could not be related to any particular physiological condition in the larvae in which it was found. It remains unexplained.

Other tissues which were PF-positive in the oesophageal valve were a thin layer of the oesophageal intima immediately against the hypodermis (and possibly, though faintly, the pore canals in the intima) and the sclerotised outer layer of the entonnoir. This latter layer was intensely PF-positive.

Table II. Staining characteristics of cytoplasmic inclusions in the proventricular epithelium and of the peritrophic membrane.

Material stained	Protein	S.B.B.	PAS	Metachromasia	Alcian blue	PF
Small vacuole contents	-	-	-	-	-	-
Large vacuole contents	-	-	-	-	-	+
Granules	±	-	+	-	-	-
Peritrophic membrane (free in gut)	+	+	+	gamma	-	+

## Section V: Discussion

It is apparent that the components of the oesophageal valve in the larva of P. terrae-novae have three distinct functions in the gut.

1. They provide the material for the peritrophic membrane and the mechanism whereby this material is moulded into a solid membrane.
2. They provide a valve at the anterior end of the midgut to prevent reflux of the food in the chyle ventricle.
3. They provide a sphincter muscle at the posterior end of the functional oesophagus. There is no enlargement of the muscles at this point into a distinct annular muscle such as was described by Thompson (1905), Imms (1907) or Wigglesworth (1930) in other Dipterous larvae but nevertheless these muscles were observed to be functioning as a sphincter in controlling the entry of food into the chyle ventricle.

The development of a complex oesophageal invagination must be concerned with the first function since a much less complex structure would suffice for a valve and the sphincter could function in the absence of any invagination as it does in insects which lack a fold in the gut wall at the junction of the fore- and midgut. Too much cannot be made of this latter point however since some insects which do not produce a membrane possess an oesophageal invagination. Nevertheless, to quote Waterhouse (1953), "A well-developed and often complex invagination is characteristic of insects which produce a Type I (single source) peritrophic membrane although this occurs also in groups in which no membrane has been recorded and in insects which/

which produce only Type II (multiple source) membranes.

However, a poorly-developed invagination is possessed by most insects producing Type II membranes".

The anatomy and histology of the proventriculus demonstrate that while it is clearly midgut tissue there is sufficient difference between it and the remaining digestive parts of the midgut to make it obvious that it is functionally a separate tissue. While both tissues consist basically of a fairly thin muscle coat, longitudinal muscles over circular muscles, overlying a columnar epithelium the cells of which carry a striated border on their inner face, each of these components shows some difference in each tissue.

The regular and closely-set circular muscles give to the proventriculus a constant shape and a rigidity not found elsewhere in the midgut. This is no doubt necessary for it to function as a part of the mould for the peritrophic membrane in the manner described by Vignon (1901) and Wigglesworth (1930).

The epithelial cells of the proventriculus differ from those in the adjacent digestive tissue in the type of striated border they possess, at least on the vacuolated cells, and in their secretory activity. They do not show vacuolation comparable with that produced by the intense secretory activity of the digestive tissue. There seems little doubt that the function of/  
1

of the proventriculus is to produce the material from which the peritrophic membrane is formed. The membrane definitely originates in the lumen between the oesophageal invagination and the proventriculus and the tissues other than the proventriculus which lie about this lumen have been eliminated as sources of membrane material. Although Bradfield (1950) claims that silk is secreted through an intima in the silk glands of Cossus cossus, the presence of a thick intima on the cells of the oesophageal invagination makes it highly improbable that these cells could be secreting a chitin-like substance. This fact, together with the absence of any demonstrable secretory activity in the cells would seem to rule out the oesophageal invagination. The anterior imaginal ring has been satisfactorily explained by Perez (1910) as an imaginal rudiment.

It was possible that the membrane material was produced by all the cells of the proventricular epithelium but the preliminary histological investigation demonstrated that cells which showed evidence of secretory activity in the form of vacuoles in their cytoplasm were confined to the anterior half of the proventriculus. The histological stains did not reveal any other definite evidence of secretory activity apart from the brush borders of the vacuolate cells being disarranged as if globules of material were passing through them. A picture of clear droplets of material passing through the border and being visible/

visible outside the cells such as was described by Wigglesworth (1929) or von Dehn (1933) could not be obtained in this case since the close proximity of the proventricular brush border to the intima of the invagination cells meant that any secretion passing through the brush border would immediately impinge on the intima so that droplets could not form.

At this point it may be pertinent to question whether the methods of fixation employed by previous workers were entirely satisfactory. In descriptions of other species investigated, including, for example, the closely-related larva of C. vomitoria, the illustrations given show a distinct, and in some cases fairly large, space between the adjacent walls of the oesophageal invagination and the proventriculus (e.g. vide Lowne, 1890). Dissection of living material of P. terrae-novae indicated that these two tissues lie closely apposed to each other in life but much experimentation with methods of fixation and embedding was required before this condition was obtained in sections (Fig. 5), due apparently to the extremely delicate nature of the cells of the oesophageal invagination which tended to collapse during the embedding process. This in turn raises doubts as to whether the "droplets of secretion" mentioned above were not, in fact, artifacts.

Earlier workers who noted that vacuoles were present in the proventricular epithelium postulated that they contained the precursors/

precursors of the membrane material in fluid form. Further attempts to identify the material in the vacuoles were frustrated by the absence of satisfactory histochemical tests. The main difficulty lay in the fact that the only substance which had been established as being present in peritrophic membranes in general was chitin, a substance which is very resistant to stains. The standard chitosan test obliterates all cellular detail so is useless in detecting cellular chitin and, due to its chemical nature, chitin is not PAS-positive although it is a polysaccharide. There is some evidence of an inconclusive nature that a protein component is also present, based on protein tests (Wigglesworth, 1929; Waterhouse, 1953) and on protein-removing treatments which resulted in changes in the membranes visible with the electron microscope (Richards and Korda, 1948)

Nothing is really known about the intracellular precursors of chitin. In the present work the search for the cells of origin of the membrane material was based on the assumption that the precursor material of the chitin of the membrane would be of a simpler polysaccharide nature than chitin itself and would be identifiable in the proventricular epithelium, probably in the vacuoles described above. For the identification of this simpler polysaccharide material the PAS method was employed.

In spite of the doubts of Richards (1950) that glycogen could be a precursor of chitin it was hoped that the PAS test would/

would reveal a concentration of glycogen in the cells where chitin was being synthesised. In support of this, for example, Paillot (1938) had found that glycogen accumulated in quantity in the epidermal cells of the silkworm immediately prior to the laying down of the new cuticle, Day and Powning (1949) found a marked concentration of glycogen in the proventricular epithelium of the cockroach gut, and Wigglesworth (1956) found glycogen in the epidermal cells of Rhodnius prolixus during the deposition of the cuticle. No such concentration was found in any of the cells of the proventricular epithelium of P. terrae-novae. In fact there was a marked absence of glycogen from the PM cells but in these cells the PAS test showed that there was a mass of small granules grouped mainly between the nucleus and the brush border which were shown by further histochemical tests to be probably of a neutral mucopolysaccharide nature.

On the whole this result is in accord with that found by Rizki (1956) in D. melanogaster. The granules are histochemically identical with those found by Rizki and they are limited to a similar narrow band of cells in the proventricular epithelium, although in D. melanogaster this band lies nearer to the anterior end of the proventriculus and the cells appear to be somewhat more closely and homogeneously packed with granules. The presence of vacuoles in the cytoplasm of some of the epithelial cells was noted by Rizki but he was unable to determine the nature of/

of their contents by any of the histochemical tests he employed.

In the present work the contents of the large vacuoles in the same band of cells as the granules were differentially stained by a modified version of Gomori's (1950) aldehyde fuchsin stain for elastic fibres. Because of the uncertainty about what, histochemically speaking, the paraldehyde fuchsin method stains the histochemical significance of this result is uncertain. Gomori used this stain on elastic fibres and it has since been used by Gabe (1953) and others to stain the product of neurosecretory cells. The potassium permanganate oxidant used in this method will presumably attack glycol linkages to yield aldehydes in the first stages of oxidation and the undecolourised fuchsin found in this stain is then able "to combine with aldehydes and yield purplish dyes" (Gomori, 1953). In spite of this apparent parallelism between PAS and PF staining effects the results do not necessarily correspond. In the present work the PAS-positive granules in the PM cells are not PF-positive. Likewise Fraser (1957b) found that granules in neurosecretory cells which were PF-positive were not PAS-positive. Finally the polysaccharide amylopectin which was used as an adhesive with Steedman's polyester wax is PAS-positive but not PF-positive.

It seems therefore that the PF method must have somewhat different aldehyde-staining properties to the PAS. For the purpose of the present work however a clear indication of one of the/  
the/

the histochemical properties of the PF stain was provided by Fraser's (1956) observation that there was PF-positive material at a point where chitin was being synthesised or degraded in a pupating insect.

In P. terrae-novae the peritrophic membrane could not be distinguished from the adjacent tissue at its point of formation. Because of this the relationship between the membrane and the secretory products of the PM cells could not be established. However Rizki's observation that the membrane, in D. melanogaster, if formed at the level of the rearmost cell of the PM ring is not disputed since the only distinct evidence of secretory activity in the epithelial cells of the proventriculus in the P. terrae-novae larva is found in this band of cells.

Since the contents of the vacuoles in the cells of the PM ring were not stained by any of the histochemical methods employed other than the PF method they cannot consist of any of the normal aldehyde-yielding polysaccharides. It is postulated that the material in these vacuoles is a late stage in the synthesis of chitin. At this stage the chitin units may have been acetylated or polymerised in such a way that they resist the PAS method but they are still at a sufficiently early stage of polymerisation to be vulnerable to the PF staining method. This would, to some extent, explain the resistance of the material to other stains.

A further step can therefore be added to the process of secretion of membrane material as described by Rizki. The virtual absence of glycogen from the PM cells in comparison with the other cells of the proventricular epithelium indicates that glycogen is not accumulated in these cells as it is in the others. From this evidence it can be concluded that it is being utilised as soon as it passes into the cells from the blood so that it is only present in the PM cells against the basement membrane. The progress of the synthesis is presumably from glycogen to simple PAS-positive polysaccharide, to a more complicated PF-positive polysaccharide, which is finally polymerised to chitin. The appearance of this PF-positive stage in chitin synthesis would explain why PF-positive material has been found where chitin is either being formed or broken down in parts of the insect body.

In fixed material this process of chitin synthesis appears in the proventricular epithelium as glycogen being converted into a granular product from which are formed droplets of fluid material which coalesce to form the contents of the vacuoles. These vacuoles discharge their contents through the brush border of the epithelial cells into the lumen where the discharged material is moulded and polymerised into the thin homogeneous peritrophic membrane.

## Chapter 2

### The Presence of Paraldehyde Fuchsin-Positive Material in the Cells which Secrete the Peritrophic Membrane Substance in Some Other Insects.

#### Section I: Materials and Methods

In order to obtain some confirmation of the results obtained with the paraldehyde fuchsin method in the larva of P. terrae-novae sections were prepared of the guts of a number of other common insect species. These sections were treated with the paraldehyde fuchsin in the same manner as before. The species treated were:

<u>Forficula auricularia</u>	(imago)	Dermaptera
<u>Pieris brassicae</u>	(larva)	Lepidoptera
<u>Culex pipiens</u>	(larva)	Diptera, Nematocera
<u>Pteronidea ribesii</u>	(larva)	Hymenoptera, Tenthredinidae

In each case, except that of C. pipiens, a portion of the gut including the region where the foregut and midgut meet, was dissected out and dropped into Bouin. In the case of C. pipiens, larvae were punctured and dropped entire into the fixative. The specimens were embedded in polyester wax, sectioned longitudinally at 4 $\mu$ -6 $\mu$ , and stained with paraldehyde fuchsin.

Fig. 11. The oesophageal valve in Forficula auricularia  
(from Wigglesworth, 1930) 4, cells secreting the  
peritrophic membrane.

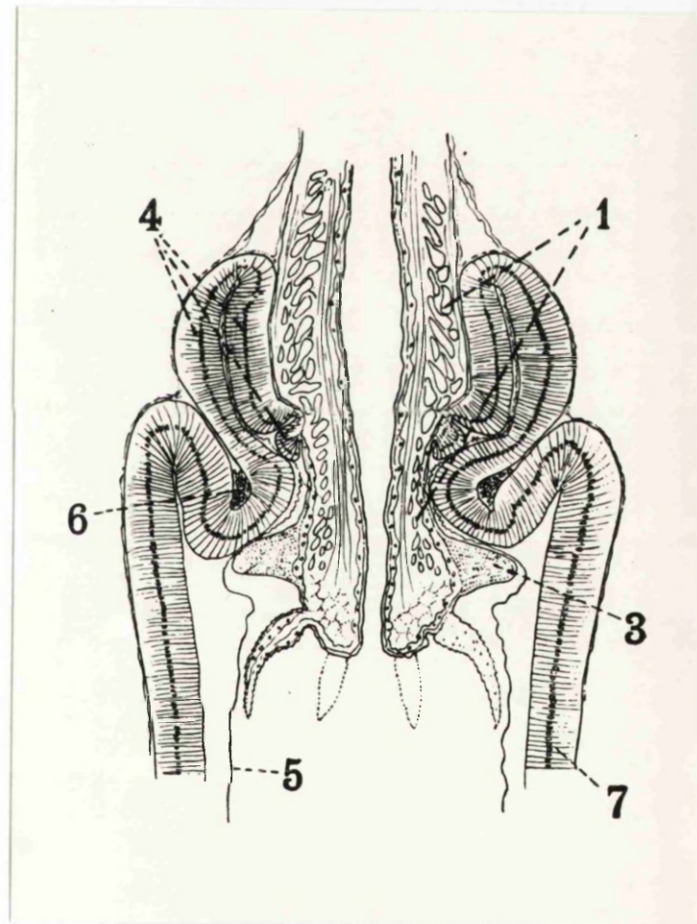


Fig. 11.

## Section II: Results

Forficula auricularia: The formation of the peritrophic membrane in this insect was described by Cuenot (1896). He stated that the membrane was a product of the "caecum annulaire", otherwise the proventriculus. This was confirmed by Wigglesworth (1930) although his figures of the anatomy of the oesophageal valve differ somewhat from those of Cuenot.

The structure of the oesophageal valve was found to conform to that figured by Wigglesworth (Fig. 11). The peritrophic membrane was clearly being formed in the vault of the proventriculus but it was difficult to determine which of the epithelial cells were responsible for the secretion of the material of the membrane. Both Cuenot and Wigglesworth were of the opinion that all the cells contributed to the membrane.

In sections of the proventriculus stained with paraldehyde fuchsin it was found that only some of the epithelial cells contained PF-positive material (Plates 6 and 7). These cells were only a short distance from the junction of the fore- and midguts. The PF-positive material was mainly confined to the adaxial region of the cells between the nucleus and the striated border. This material had a peculiar appearance. It would appear to have been precipitated in the form of thin laminae by the /

Plate 6. The distribution of PF-positive material in the proventriculus of Forficula auricularia. The portion of gut in the photograph is that marked '4' in Wigglesworth's illustration (Fig. 11). Poor fixation has caused the various parts of the "annular mould" to separate with consequent tearing off of the striated border of the epithelial cells. (Longitudinal section; paraldehyde fuchsin).

PF, PF-positive material; PM, peritrophic membrane.

Plate 7. Part of Plate 6 enlarged. Note the laminar condition in which the PF-positive material in the epithelial cells is precipitated by fixation.

PM, peritrophic membrane; SB, detached striated border.

PF

PM



Plate 6.

SB

PM

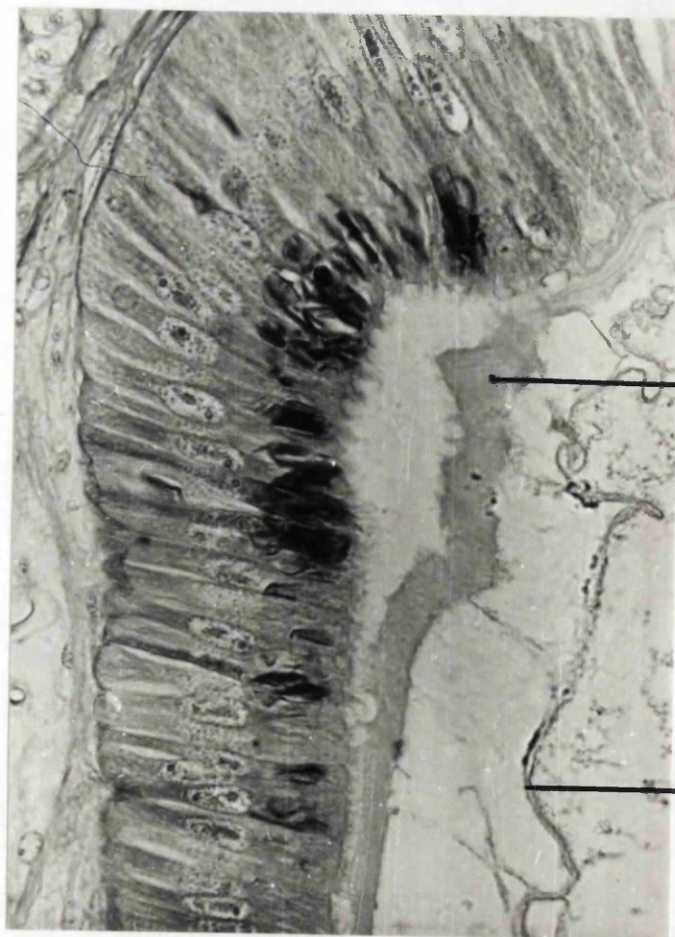


Plate 7.

the fixative, these laminae becoming distorted and somewhat displaced by subsequent treatments.

No other PF-positive material was stained in the proventricular epithelium or in the peritrophic membrane.

Pieris brassicae: Previous views on the formation of the peritrophic membrane in lepidopterous larvae are conflicting. Bordas (1911) stated that the membrane was secreted by a ring of cells in the epithelium at the anterior end of the midgut. Wigglesworth (1930) described the press mechanism by which this secretion was moulded to a membrane. However Vignon (1901) stated that in the silkworm the membrane was formed from a secretion produced by the epithelial cells of the entire midgut. Henson (1931) made the general statement that in all Lepidoptera the membrane was mainly secreted by the whole of the anterior midgut with some additions further down the gut.

The anatomy of the proventriculus in a lepidopterous larva was described by Wigglesworth (1930) (Fig. 12).

In the present investigations on the larva of P. brassicae the peritrophic membrane was seen to be closely applied to the surface of the epithelium of the anterior midgut for a short distance after which it leaves the epithelium and lies free in the gut (Plate 8). It appears to be formed from laminae as described by Henson. There was no obvious ring of cells secreting membrane/

Fig. 12. The oesophageal valve in a lepidopterous larva  
(from Wigglesworth, 1930) 3, cells secreting the  
peritrophic membrane; 4, peritrophic membrane.

Plate 8. Part of a longitudinal section of the anterior  
midgut of the larva of Pieris brassicae immediately  
posterior to the oesophageal valve. (paraldehyde  
fuchsin).

Ep, midgut epithelium; EPF, epithelial cells with a  
distinct PF-positive layer on the outer margin of their  
striated border; PM, peritrophic membrane.

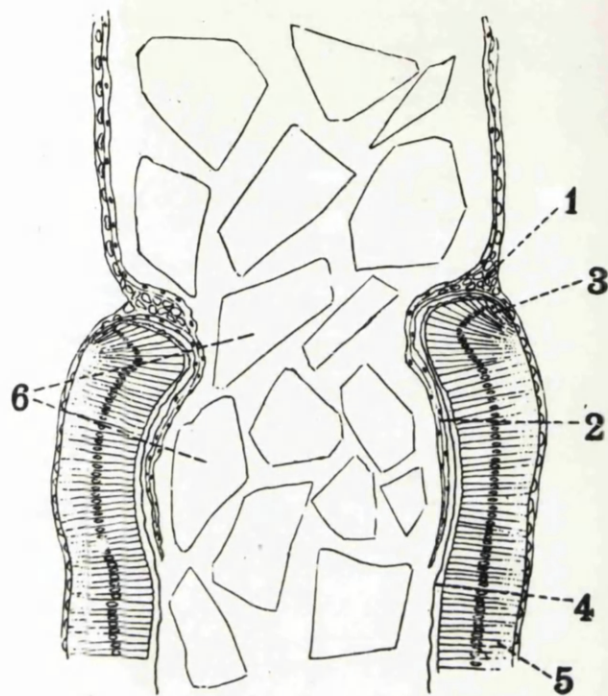


Fig. 12.

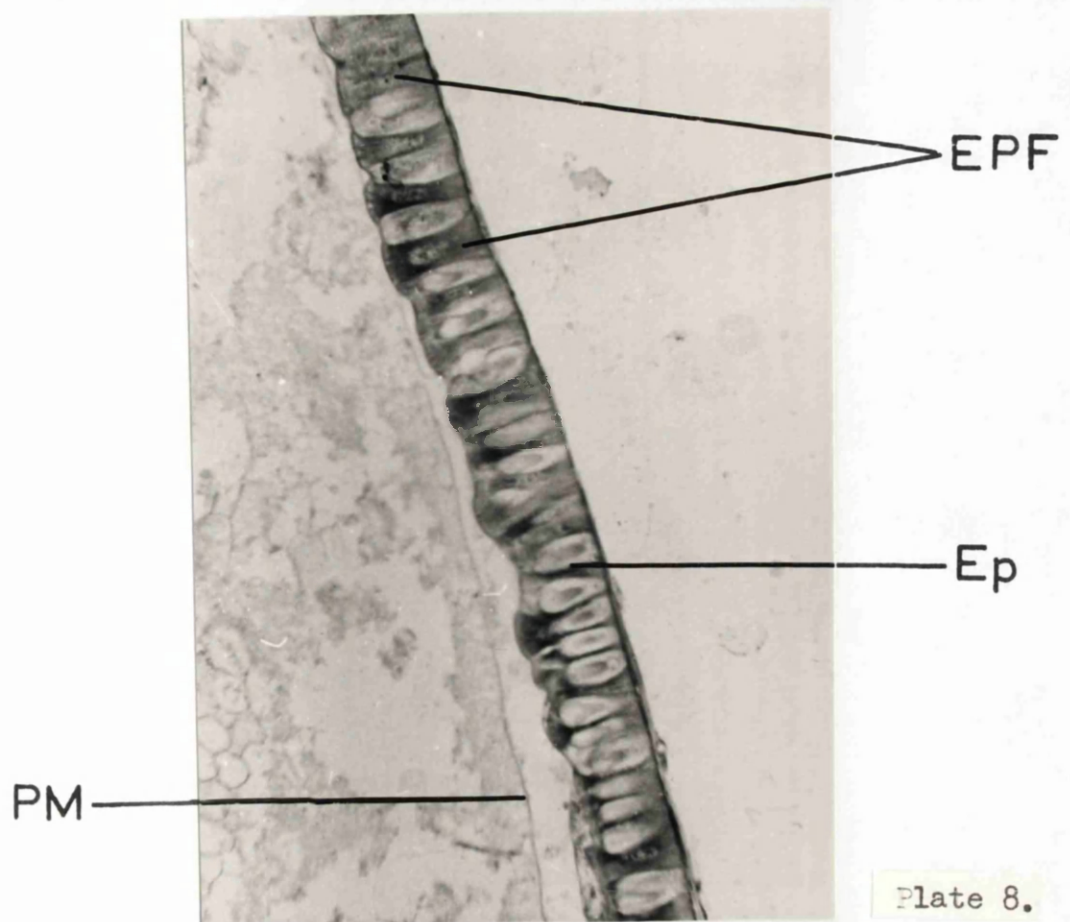


Plate 8.

membrane material or containing PF-positive material at the anterior end of the midgut nor was there any PF-positive material in the cytoplasm of the cells from which the component layers of the membrane were delaminated. But at the outer limit of the striated border of these cells where the laminae were presumably being laid down there was a distinct layer of moderately PF-positive material.

The peritrophic membrane was not PF-positive.

Culex pipiens: Thompson (1905) described the anatomy of the oesophageal valve in the larva of C. pipiens and Imms (1907) working on the larva of Anopheles sp., which is closely related and anatomically similar to C. pipiens, identified the source of the peritrophic membrane as a ring of deeply-staining cells in the proventriculus. Wigglesworth (1930) corroborated these observations and, as has already been noted, stated that globules of secretion could occasionally be seen in the proventricular cells. His illustration of the anatomy of the proventriculus is given in Figure 13.

In the sections stained with paraldehyde fuchsin no globules were visible in the cells of the proventricular epithelium. There was an extremely thin but distinct layer of PF-positive material along the outer limits of the striated border on most of the epithelial cells (Plate 9) with a very distinct concentration of material at a point about two-thirds of the length of the proventriculus/

Fig. 13. The oesophageal valve in the larva of Culex pipiens  
(from Wigglesworth, 1930).

1, sphincter muscle; 2, blood sinus; 3, chitinous  
thickening; 4, proventriculus; 5, peritrophic  
membrane.

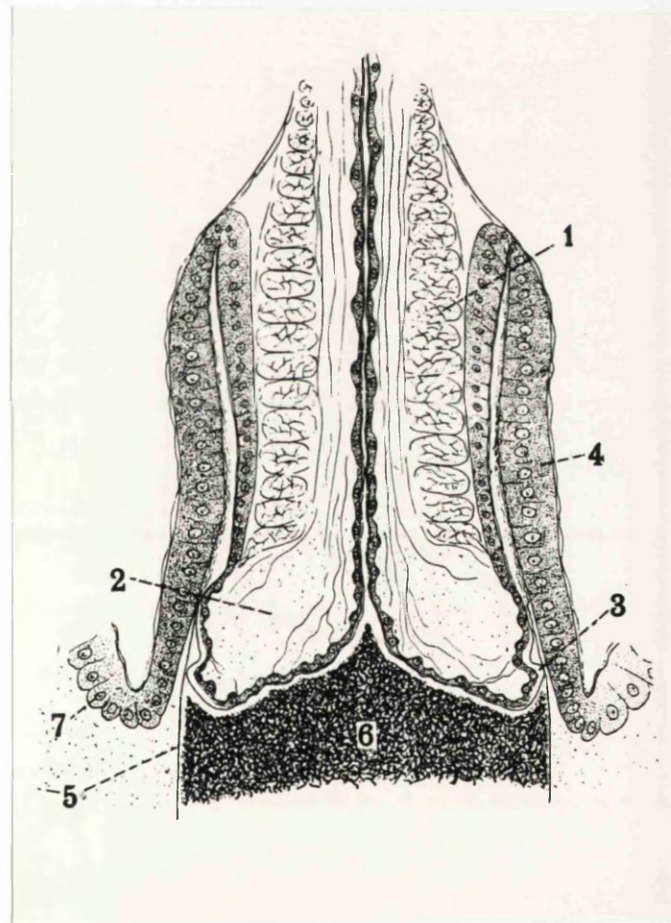


Fig. 13.

proventriculus from the anterior end. The concentration of material is strongly PF-positive. It appears to take the form of folded curtains of material, several of these curtains being compressed together into a compact mass (Plate 10). Beyond this point of concentration the peritrophic membrane appears. It is considerably thicker than the layer of PF-positive material anterior to the point of concentration and very strongly PF-positive.

Pteronidae ribesii: The structure of the oesophageal valve and the anterior midgut in this larva was found to be similar to that illustrated by Wigglesworth (1930) in an unnamed sawfly larva (Fig. 14). Wigglesworth stated that the peritrophic membrane in this insect arose from a ring of special cells in the proventriculus ("cardia" of Wigglesworth). In P. ribesii this ring of cells could not be distinguished from the remainder of the proventricular epithelium.

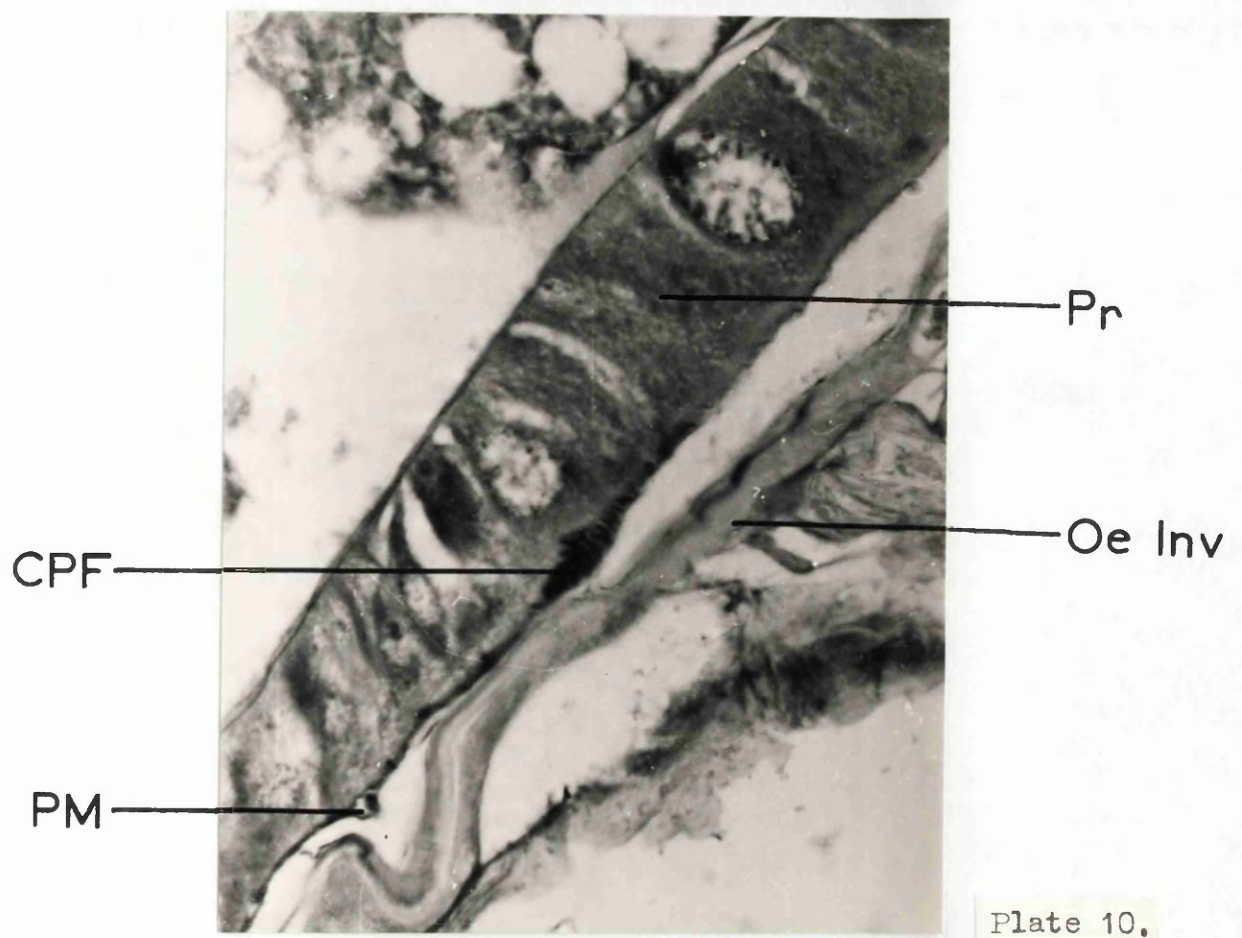
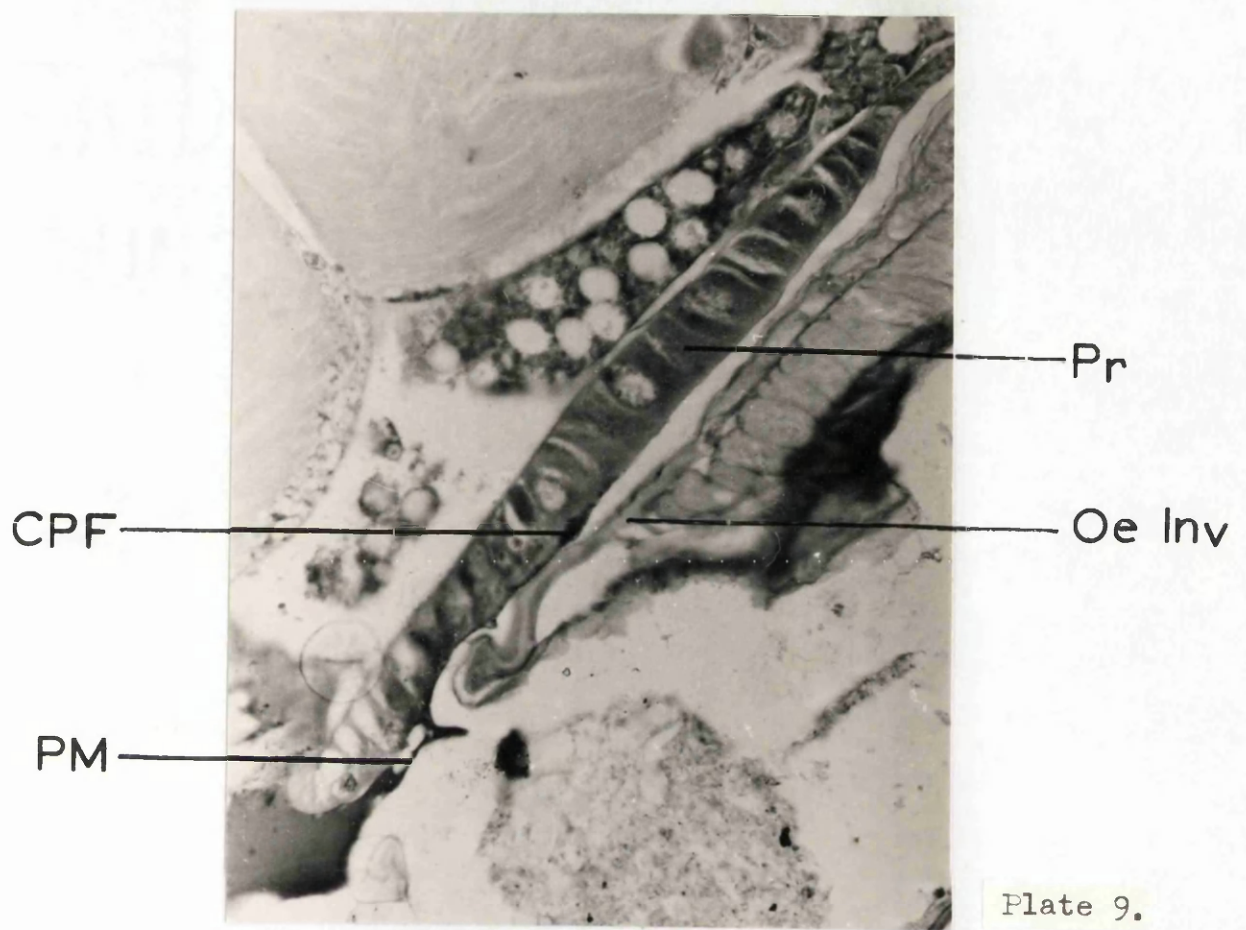
It was, however, not at all certain that the peritrophic membrane had a single source. The food mass in the gut was surrounded by a thick ( $36\mu$ ) membrane which was clearly multi-laminate (Plate 11). In some places granular material was seen to be trapped between the layers of the membrane. It appears therefore that the membrane, in this Tenthredinid at least, is formed from laminae contributed by the cells of at least the anterior midgut.

Plate 9. Part of the oesophageal valve in the larva of Culex pipiens. (Longitudinal section; paraldehyde fuchsin).

CPF, concentration of PF-positive material;  
Oe Inv, oesophageal invagination; PM, peritrophic membrane; Pr, proventriculus.

Plate 10. Part of Plate 9 enlarged. Note the laminar condition of the PF-positive material concentrated in the lumen between the proventriculus and the oesophageal invagination.

Lettering as in Plate 9.



The striated border on the cells of the anterior midgut is remarkably thick ( $24\mu$ ) in comparison with the depth of the cells themselves ( $60\mu$ ). The inner limit of this border is clearly demarcated from the granular cytoplasm of the epithelial cells by a dark line. At short intervals along the striated border against this line is a row of tiny vacuoles containing PF-positive material (Plate 12) but there was no PF-positive layer in the border such as was found in the larva of P. brassicae.

The peritrophic membrane was not PF-positive.

Fig. 14. The oesophageal valve in a sawfly larva (from Wigglesworth, 1930).

3, cells secreting the peritrophic membrane;

5, peritrophic membrane.

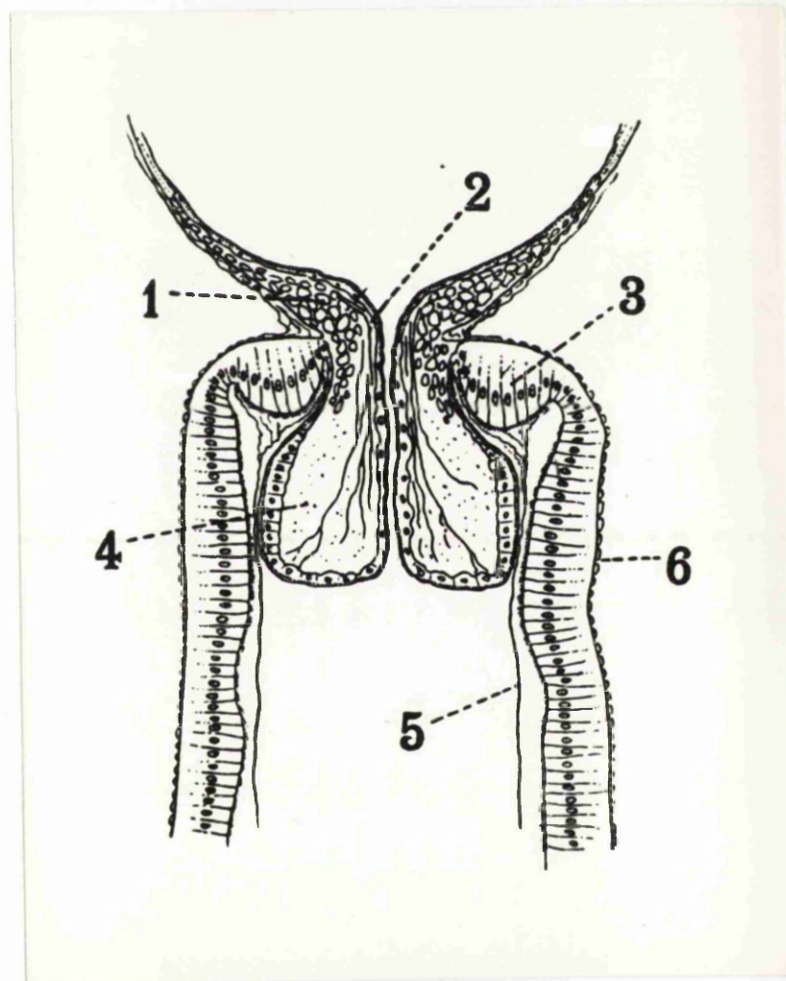


Fig. 14.

### Section III: Discussion

The results obtained by treating the anterior midgut of F. auricularia with the paraldehyde fuchsin stain provide striking corroboration of the results obtained in P. terrae-novae. Again a narrow band of cells in the proventriculus was found to contain PF-positive material. This material was precipitated by the fixative in a somewhat different state in F. auricularia to that found in P. terrae-novae but the material was again situated in the cells between the nucleus and the striated border. No other cells either in the foregut or in the anterior midgut exhibited this phenomenon.

Although the evidence was not so clearcut in the other species examined it was possible to detect some PF-positive material at the site of secretion of the membrane material in every case. In P. brassicae this material was only moderately PF-positive but the compressed laminae of material in C. pipiens and the droplets in the striated border of P. ribesii were both strongly positive.

This evidence provides good confirmation of the postulate advanced in the previous chapter, that there is a PF-positive step in the formation of the material for the peritrophic membrane in insects. It demonstrates that the result obtained in P. terrae-novae was not an isolated phenomenon.

Comment/

Plate 11. The peritrophic membrane in the larva of the sawfly Pteronidea ribesii. Note the distinct lamination.

F, food material; GM, granular material trapped between laminae of the peritrophic membrane; PM, peritrophic membrane.

Plate 12. The epithelium of the anterior midgut in the larva of the sawfly P. ribesii. (Longitudinal section; paraldehyde fuchsin).

EC, epithelial cell; PL, possible potential laminae of the peritrophic membrane in the striated border; SB, striated border; VPF, vacuoles containing PF-positive material.

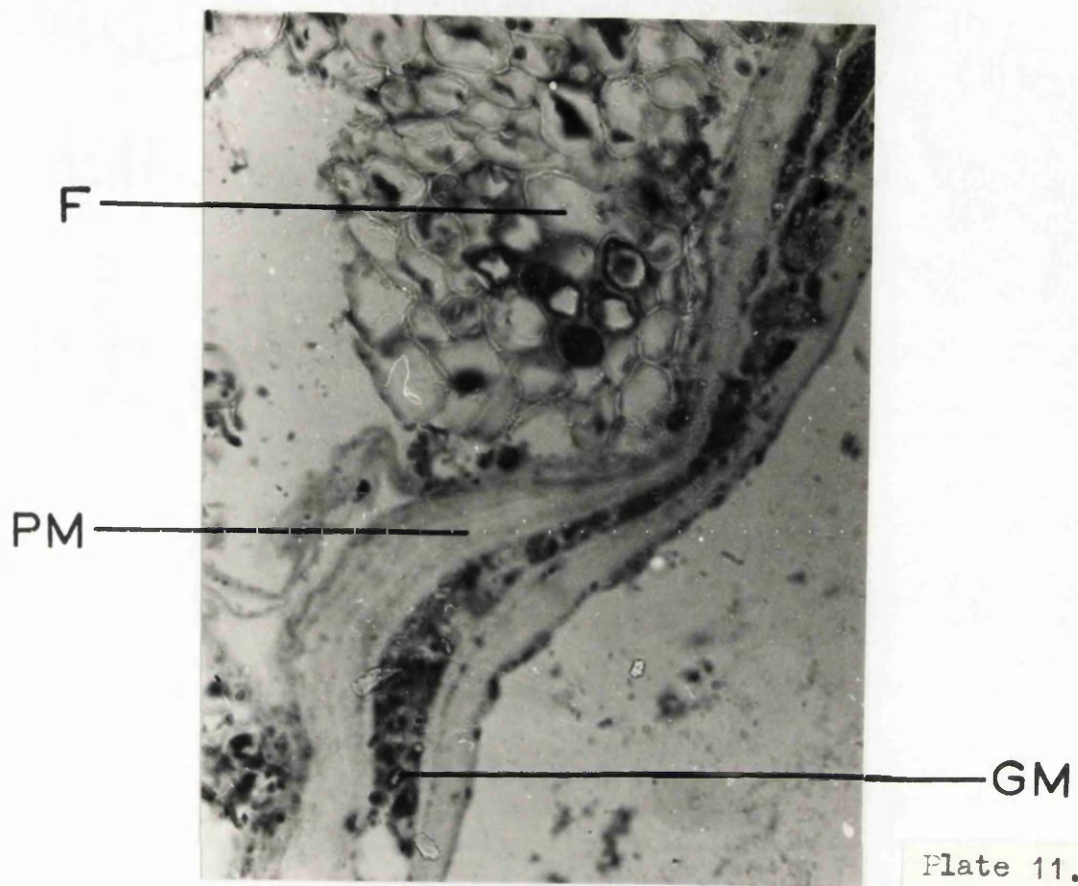


Plate 11.

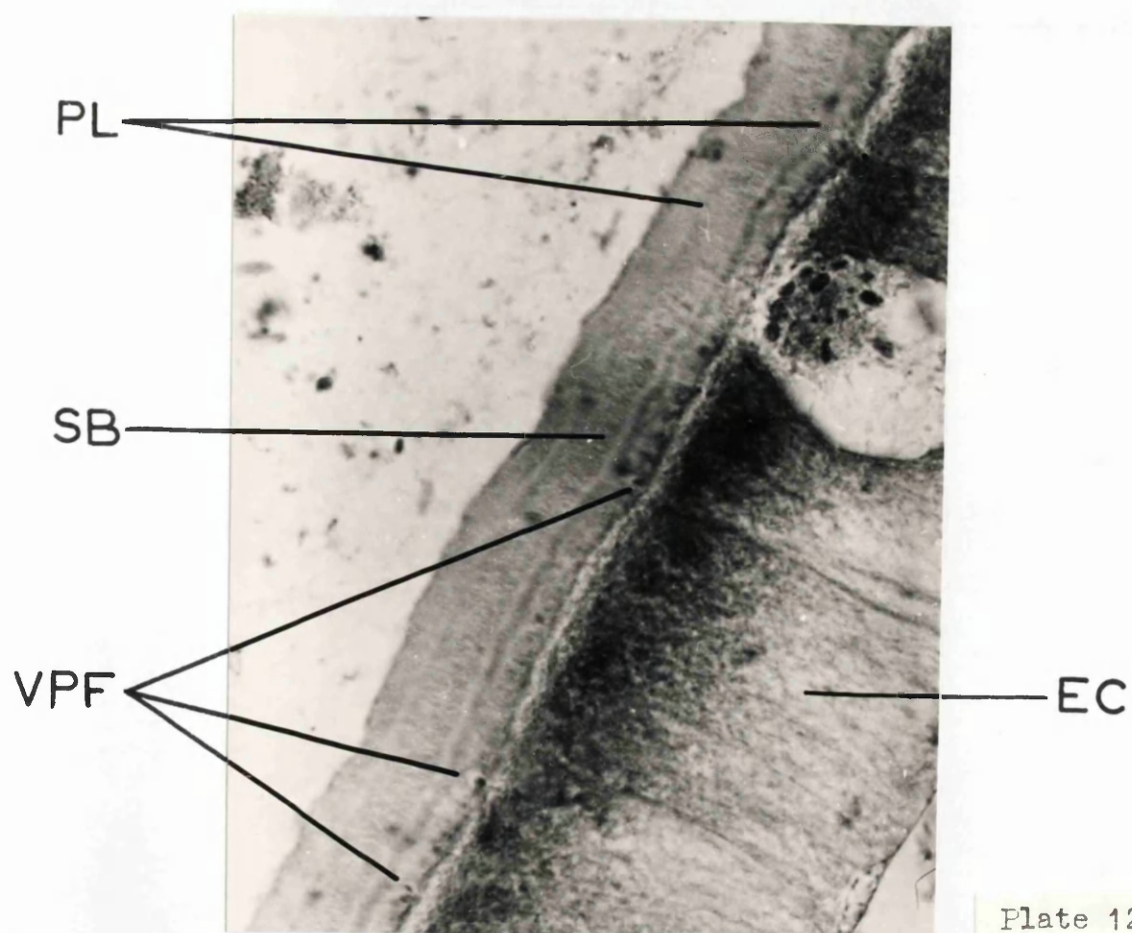


Plate 12.

Comment should also be made on the fact that of the four species examined in only one, C. pipiens, was the peritrophic membrane PF-positive. This bears out the earlier assumption that the PF-positive property of the membrane in P. terrae-novae was due to a food contaminant since it would seem unlikely that the membrane has a different chemical constitution in different insects.

### Chapter 3

#### The Structure and Function of the Enteric Caeca in the Larva of *Protophormia terrae-novae*, R-D.

##### Section I: Introduction

Enteric caeca, or gastric caeca - diverticulae of the midgut of insects - are generally regarded as means whereby the total surface area of midgut epithelium, available for secretion or absorption, is increased (Imms, 1957). In most insects which possess them they closely resemble the adjacent midgut tissue anatomically and appear to absorb and secrete in the same manner as that tissue so that they are merely simple pouches in the gut wall.

All the previous studies on the caeca known to the author have depended for their conclusions either on the detection of enzymes or food material in the caeca or on the detection of secretory structures in the cytoplasm of the caecal epithelial cells.

Swingle (1925) studied the caeca in *Blatta orientalis* and, in a classical paper on the methods of enzyme detection in the insect gut, concluded that they were secretory because he detected a proteinase in them.

Gresson (1934) worked on the caeca in Periplaneta americana using histological staining of sections of fixed material. He found that some of the cells of the caeca contained secretory granules and some cells contained food material so concluded that the caeca were both secretory and absorptive. Schlottke (1937) found a proteinase in the caeca of crickets and concluded that they were secretory. Day and Powning (1949) also concluded that the caeca in B. orientalis and P. americana were secretory.

The caeca in larval Diptera are mentioned frequently in descriptions of gut anatomy and histology. De Boissezon (1930) observed that in Culex pipiens the histology of the caeca is much like that of the midgut. The epithelial cells are strongly vacuolate and emit drops of secretion. Strasburger (1932, 1935) described the anatomy and histology of the caeca in Drosophila melanogaster but did not study their physiology. <sup>Siang</sup> Hsu (1947) found some evidence that the caeca in D. melanogaster were secretory. Although he found that most of the cells in the caeca appeared to be quiescent a few were found to contain secretory granules distributed evenly through the cytoplasm. None of the work carried out so far on the caeca in Dipterous larvae has included enzyme determinations, possibly because of the extremely small size of these organs in these larvae.

There is no record of any work having been carried out on the structure and function of the caeca in blowfly larvae.

Occasional

Occasional authors (e.g. Weismann, 1864) have made slight reference to their anatomy but no detailed studies have been made. Previous workers on the gut of blowfly larvae appear to have accepted that the caeca were functional expansions of the midgut epithelium and have made no attempt to investigate them separately.

There are five possible explanations for the presence of enteric caeca in the gut of these larvae, viz.:

1. They serve as extensions of the area of absorptive epithelium in the midgut.
2. They serve as extensions of the area of secretory epithelium in the midgut.
3. They are organs of excretion akin to the malpighian tubules. This is unlikely since their position at the anterior end of the midgut would mean that any excreted material would become mixed with the food and possibly reabsorbed.
4. They are imaginal rudiments. This possibility can be discarded since they are broken down with the rest of the larval midgut at metamorphosis and do not reappear in the adult (Perez, 1910).
5. They are functionless i.e. vestigial. This is unlikely in such a highly evolved animal which has disposed of all other unnecessary organs and appendages.

Only explanations 1 and 2 are worthy of further examination.

The anatomy and histology of the caeca was studied by dissection of supra-vitally stained larvae and by means of the sections of the caeca prepared with the proventriculi in the previous sections of the work.

The/

The investigation of the physiology of the caeca centred on whether they were absorptive or secretory (or possibly both) in function. The absorptive function was investigated by the feeding of dyes and iron saccharate mixed with the food. The secretory function was investigated by a variety of techniques for the detection of enzymes, by the introduction of dyes into the haemocoel, and by other means. Again use was made of the sections of caeca prepared with the proventriculi. The various histologically stained sections prepared with the proventriculi were inspected for evidence of absorption or secretion.

The studies on enzymes in the gut of blowfly larvae which are relevant to this work are those of Hobson (1931) and Fletcher and Haub (1933). The work of other authors (Guyenot, 1906, 1907, Weinland, 1906, Wollmann, 1922) is conflicting. Both Hobson and Fletcher and Haub treated the anterior midgut and the caeca as, functionally, one tissue. Hobson found tryptase, peptidase, and a possible lipase in the anterior midgut and caeca of the larva of Lucilia sericata, these in fact being the only enzymes which he found in the entire gut apart from a doubtful amylase in the salivary glands. In the anterior midgut and caeca of the larva of Phormia regina Fletcher and Haub found invertase, trypsin and a doubtful lipase.

## Section II: The Anatomy and Histology of the Enteric Caeca

### (a) Materials and methods

The anatomy of the caeca was studied by the same combination of in vivo staining with methylene blue (Ludwig, 1949) and whole-mount staining with iron haematoxylin (Graham-Smith, 1934) as was employed for the proventriculus. Due to their extremely small size it proved impossible to obtain a satisfactory whole mount of the caeca to display the musculature.

As has already been noted, caeca were dissected along with the other material which was being prepared for the investigation of the proventriculus. Material for the study of the histology of the caeca was therefore available fixed in Bouin's fluid, embedded in ester or polyester wax and stained with iron haematoxylin - Orange 'G' or Alcian blue - Mayer's haemalum - eosin.

### (b) The anatomy of the caeca

There are four caeca in the midgut of the larva of P. terrae-novae. They are long, narrow, finger-like organs projecting from the wall of the anterior midgut immediately behind the proventriculus (Figs 1 and 4). They are of two different lengths. The dorsal pair of caeca are long (mean length = 2.82 mm), and taper from a mean diameter of 0.17 mm at the root to a mean diameter of 0.11 mm at the tip. The ventral pair of caeca are shorter (mean length = 1.54 mm) and only taper slightly (mean diameter/

diameter of 0.14 mm at the root, 0.13 mm at the tip). (Means are of measurements taken in ten larvae.) The dorsal caeca appear to be somewhat stretched in the usual dissection so the lengths given here may be too great.

The caeca extend forward in the haemocoel on either side of the oesophageal valve. The long dorsal caeca extend almost to the cerebral hemispheres of the brain. They are suspended at their tips by thin muscle strands inserted into the anterior fat body beside the brain and by tracheae. The short ventral caeca lie along the dorso-lateral surface of the salivary glands to which they are attached by muscle strands. Both sets of caeca are also maintained in position by numerous tracheae.

Apart from their differing external dimensions the dorsal and the ventral pairs of caeca are anatomically identical. Both have a similar structure to the anterior midgut from which they arise. The muscle coat consists of a few longitudinal muscles overlying a sparse coat of circular muscles. Beneath these is a basement membrane carrying an epithelium of large cells, polygonal in section and with large round central nuclei.

At the root of each caecum where it joins the anterior midgut there are between ten and twelve longitudinal muscles (Plate 13) which originate as branches of the longitudinal muscles of the anterior midgut. Along the length of the caecum these muscles decrease in number by anastomosis until at the extremity of the  
 ✓ caecum/

Plate 13. The musculature of the enteric caeca in the larva of P. terrae-novae. (Whole mount; iron haematoxylin. Since these organs were very small a more satisfactory mount could not be obtained.)

CMB, circular muscle bundle; CMN, circular muscle nucleus; LM, longitudinal muscle; LMN, longitudinal muscle nucleus.

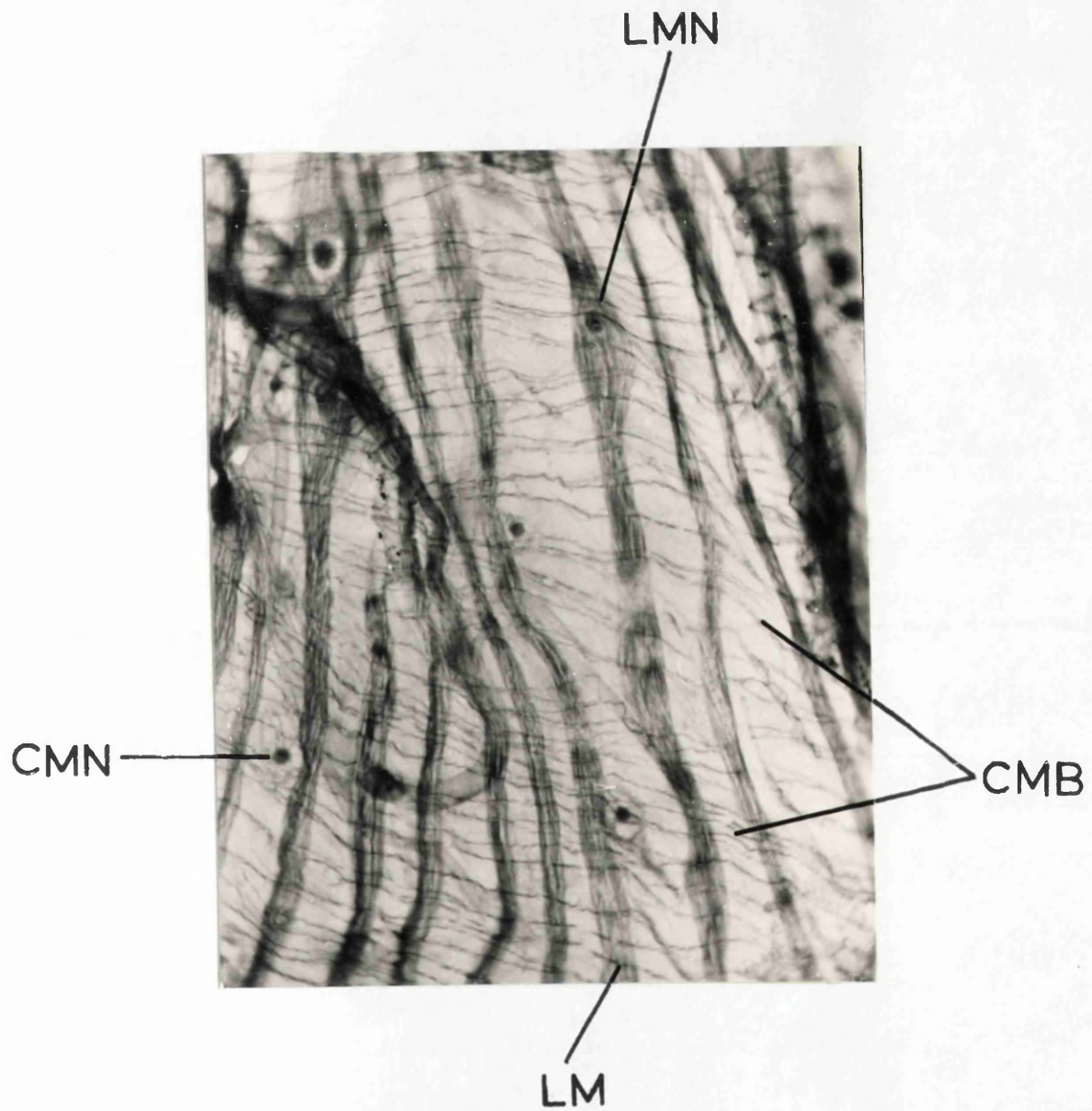


Plate 13.

caecum there are only five or six muscles. These muscles are carried on over the tip of the caecum where they are joined in a single bundle to form the suspensory muscle strand which attaches the caecum either to fat body or salivary gland. The longitudinal bands are narrow and contain nuclei in swellings at irregular intervals along their length.

The circular muscle coat is a thin network of muscles roughly arranged into circular bands with many anastomoses between adjacent bands (Plate 13). The arrangement is similar to the circular muscle coat of the anterior midgut but the bundles of fibres within the bands are very much thinner than those on the midgut so that the circular muscle coat is very sparse. The fibres are very clearly striped.

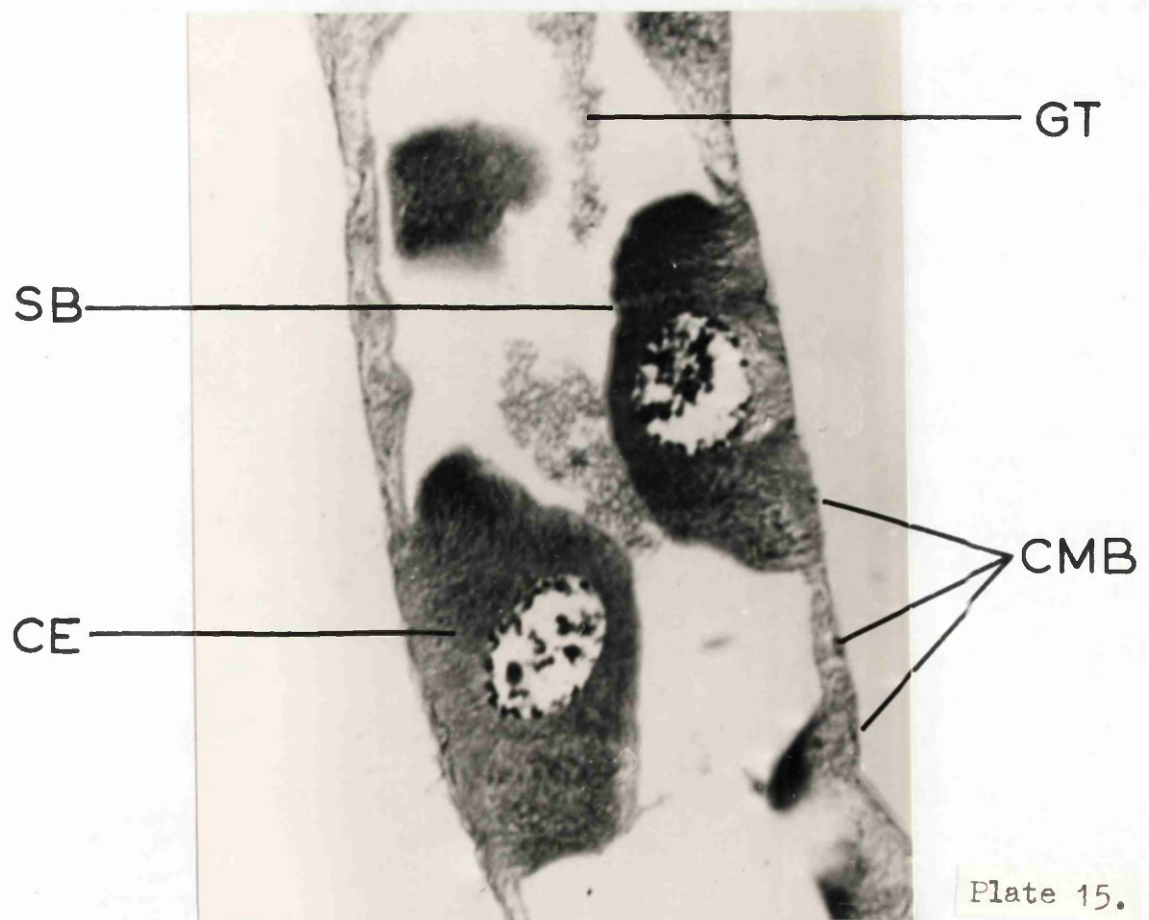
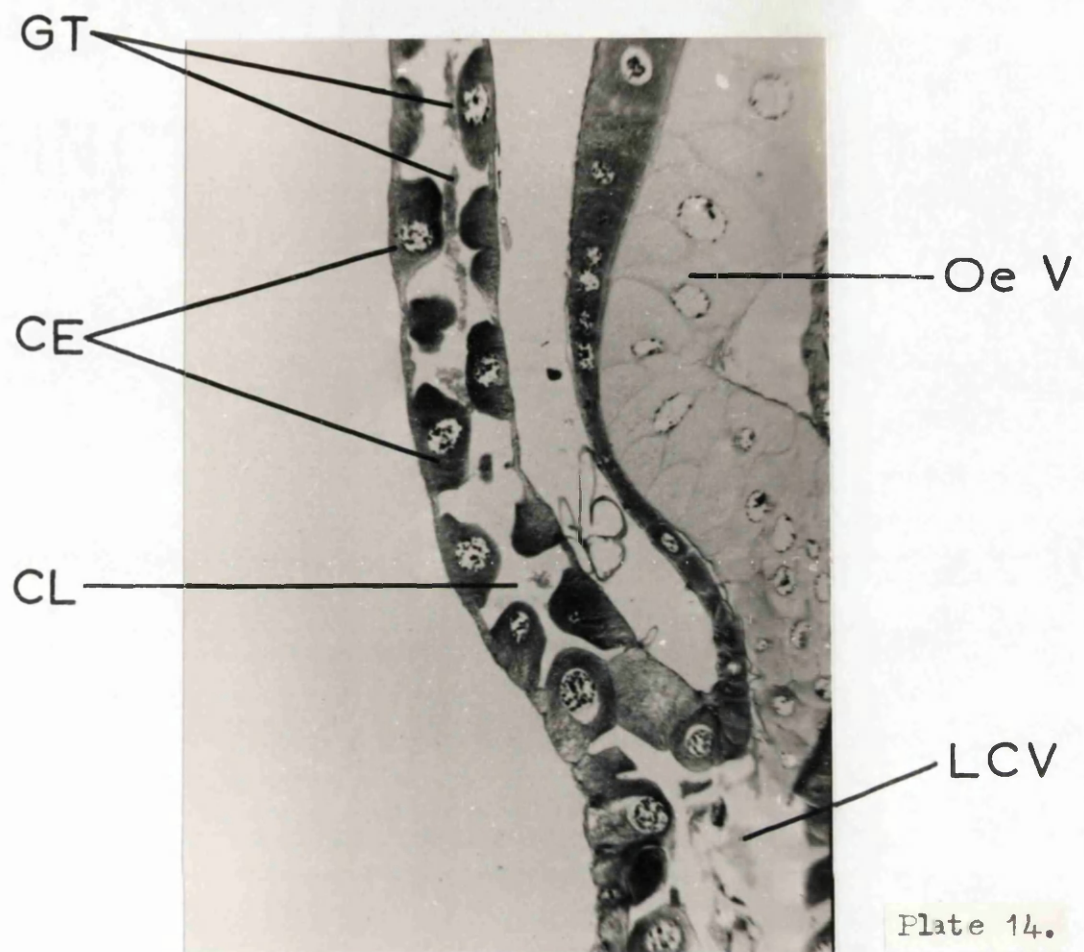
The caeca are tracheated from three different sources. The greater part of each caecum receives tracheae from the branches of the main tracheal trunks which serve the anterior limits of the proventriculus (Fig. 4). The roots of the caeca are tracheated from the same source as the main body of the proventriculus (Fig. 7). The tips of the caeca are supplied with fine tracheae which originate in the main trunks at the level of the brain. The tracheation is very light in comparison with the proventriculus but very similar in density to the part of the midgut from which the caeca arise. The tracheoles penetrate the muscle coat to the basement membrane but do not penetrate/

Plate 14. The proximal half of one of the enteric caeca in the larva of P. terrae-novae. (Longitudinal section; iron haematoxylin-orange 'G').

CE, caecal epithelial cells; CL, caecal lumen;  
GT, granular traces of secretion; LCV, lumen of  
the chyle ventricle; Oe V, oesophageal valve.

Plate 15. Part of Plate 14 enlarged.

CMB, circular muscle bundles; SB, striated border;  
other lettering as in Plate 14.



penetrate into the cells of the epithelium.

The nervous supply to the caeca was not detected.

(c) The histology of the caeca

The epithelium of the caeca is made up of large polygonal cells containing a round or ovoid central nucleus. The lumen face of each cell carries a thin, indistinct striated border. The cytoplasm of the cells is moderately granular and contains no structures whatsoever apart from some variations in density of granulation in different parts of the cells (Plates 14 and 15).

The lumen of the caeca is very narrow. In fixed material it contains traces of a diffuse, faintly granular secretion (Plate 15).

In a few cases the epithelial cells were observed to carry one or two of what appeared to be large globules of secretion on their lumen face (Plates 16 and 17), the presence of which could not be related to any physiological condition. It is doubtful if these are true secretory globules. It is more likely that they are fixation artifacts.

Plate 16. Part of one enteric caecum in the larva of  
P. terrae-novae showing "secretion droplets", which  
are probably fixation artifacts, on the lumen faces  
of the epithelial cells. (Longitudinal section.  
Alcian blue Mayer's haemalum - eosin)

Oe V, oblique section of the oesophageal valve.

Plate 17. Part of Plate 16 enlarged.



OeV

Plate 16.

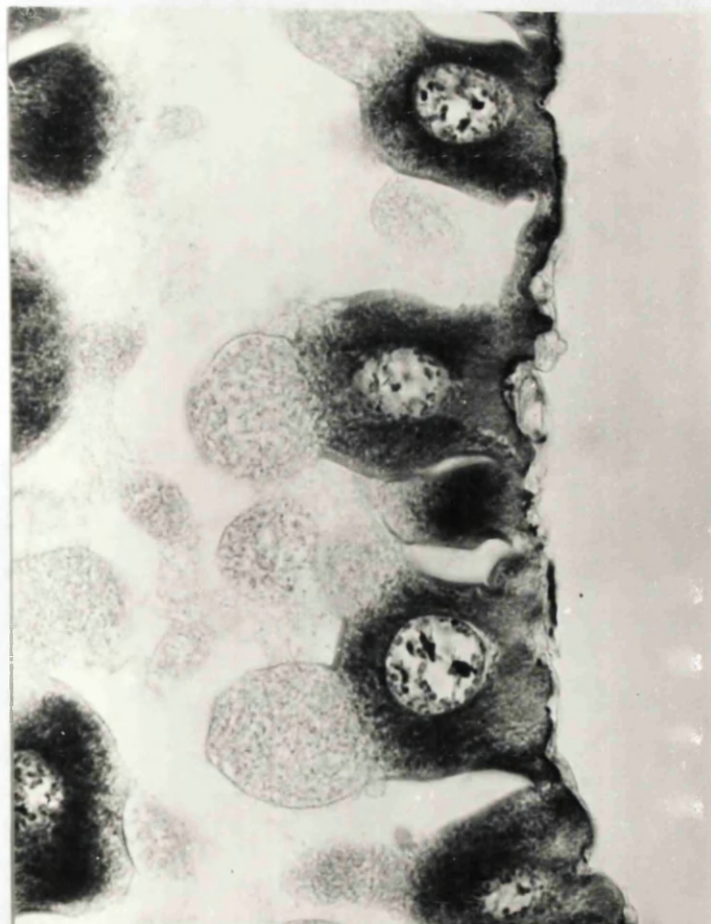


Plate 17.

### Section III: The Physiology of the Enteric Caeca

#### (a) Materials and methods

i). Materials. Actively-feeding third instar larvae were again used in this investigation. In his work on the enzymes secreted by the gut of the larva of L. sericata Hobson (1931) used larvae which had been starved in running water for twenty-four hours because their faeces contained tryptase the re-ingestion of which could have given false results. It was not thought necessary to use starved larvae in the enzyme determinations in the present work since the dye-feeding experiments demonstrated that none of the contents of the midgut made their way up into the caeca.

ii). Staining procedures. As before the sections of caeca employed in this part of the work were obtained from caeca fixed, embedded and sectioned along with the proventriculi. Material was therefore available stained by the following methods:

Baker's protein method

The protein method of Mazia, Brewer  
and Alfert

The periodic acid-Schiff

Paraldehyde fuchsin

Toluidine blue

and after fixation in McManus' fixative with post-chroming,

Sudan Black 'B'

The slides stained by the two protein methods, by toluidine blue and by Sudan Black 'B' were impermanent so observations were made on the caeca at the same time as those on the proventriculi and the observations laid aside for future reference.

iii). Dye-feeding procedures. Before carrying out the dye-feeding experiments a pilot experiment was undertaken to determine which dyes would be most suitable since the dyes used had to be capable of passing readily through the peritrophic membrane and in sufficient concentration to give a colour visible through the wall of the caecum. The following dyes were tested, the principle source of information on the nature of these dyes being Palm (1952)

<u>Dye</u>	<u>Nature</u>
Eosin	Acid, crystalloid, very diffusible
Indigo carmine	Acid, crystalloid, slightly less diffusible than eosin
Methylene blue	Basic, crystalloid
Neutral red	Basic, crystalloid
Trypan blue	Acid, colloidal
Congo red	Amphoteric, colloidal

Small pieces of horse liver were dipped in aqueous solutions or suspensions of these dyes and placed on a layer of moist cotton wool in 4" x 1<sup>1</sup>/<sub>4</sub>" glass tubes. Larvae which had been starved/

Table III. Results of preliminary tests of ability of various dyes to pass through the peritrophic membrane.

Dye	Passed peritrophic membrane	Absorbed by epithelium	In other body tissues	Colour in caeca	Effect on tissues
Eosin	+	+	+	-	Faintly present in blood. Absorbed by malpighian tubules, other tissues faintly coloured.
Indigo carmine	±	-	-	-	None.
Methylene blue	+	+	+	-	Present in fat body, malpighian tubules, pericardial cells, garland cells and salivary glands.
Neutral red	+	+	+	-	All tissues coloured except brain, ectodermal tissue in general, caeca, oesophageal valve.
Trypan blue	-	-	-	-	Pericardial cells and garland cells coloured.
Congo red	+	+	+	-	

starved for twenty-four hours to encourage them to feed readily were put on the meat at a rate of ten per tube. The larvae were left on the dyed meat for twenty-four hours then dissected and the distribution of the dyes in the tissues noted. The results are given in Table III. The dyes selected for use from this group were methylene blue and neutral red. Of the other crystalloid dyes tested, eosin gave too faint and diffuse a colour to be useful and only a small fraction of the indigo carmine passed through the peritrophic membrane, this small fraction neither being absorbed by the epithelial cells of the gut nor finding its way through the gut wall into the blood. Of the colloids, trypan blue did not pass the membrane and only a small fraction of the congo red did, possibly an impurity. None of these discarded dyes was detected in the lumen of the caeca or in the caecal epithelium at any time.

For the final experiments cultures were set up on meat dyed with methylene blue and neutral red. Each culture contained only five starved larvae so that the larvae would disturb each other as little as possible and would commence feeding immediately. Twenty cultures were set up for each dye. The first two hours after the larvae were put on the meat were divided into ten-minute periods and in each period a culture was selected at random from the group and two larvae taken from it. After the second hour larvae were only inspected at two-hour intervals. The results of these experiments are given in Tables IV and V.

iv)./

Time after start	Position of dye in gut	If present in caeca	Tissues coloured other than gut
10 mins	Mid-midgut	-	None
" "	Anterior midgut	-	"
20 "	Mid-midgut	-	"
" "	" "	-	"
30 "	Hind-midgut	-	"
" "	" "	-	"
40 "	" "	-	"
" "	" "	-	"
50 "	" "	-	"
" "	" "	-	"
1 hour	" "	-	"
" "	Just entered hindgut	-	"
70 mins	Hind-midgut	-	"
" "	" "	-	"
80 "	" "	-	"
" "	" "	-	"
90 "	" "	-	"
" "	Weak in hindgut	-	"
100 "	Hind-midgut	-	"
" "	Hindgut	-	"
110 "	"	-	Blood faint blue
" "	"	-	" " "
2 hrs	"	-	Malpighian tubules blue
" "	"	-	" " faint blue
4 "	In entire gut	-	Blood, malpighian tubules, fat body
" "	" " "	-	" " " " "
6 "	Hereafter very dense	-	" " " " "
" "	in epithelial cells	-	" " " " "
8 "	of hind-midgut, fore-	-	" " " " "
" "	midgut much lighter	-	" " " " "
10 "	coloured, mid-midgut	-	" " " " "
" "	not coloured at all	-	" " " " "
12 "		-	" " " " "
" "		-	" " " " "

Time after start	Position of dye in gut	If present in caeca	Tissues coloured other than gut
10 mins	Anterior midgut	-	None
" "	Beginning of mid-midgut	-	"
20 "	Hindgut	-	Blood pink
" "	In all of gut	-	" " Garland cells faint pink
30 "	"	-	Garland cells, pericardial cells, Malp'n tubules
" "	"	-	" " , " "
40 "	"	-	" " , " "
" "	"	-	Fat body faint pink, G.C., P.C., M.T's
50 "	"	-	G.C., P.C., M.T.
" "	"	-	" " "
1 hour	"	-	" " " + fat body
" "	"	-	" " "
70 mins	"	-	Hereafter it became apparent that
" "	"	-	only mesodermal tissue was coloured
80 "	"	-	since neither foregut nor hindgut
" "	"	-	wall, muscles of body wall, salivary
90 "	"	-	glands, nervous system nor brain
" "	"	-	were taking up the colour. An
100 "	"	-	odd anomaly was that neither the
" "	"	-	proventriculus nor the enteric
110 "	"	-	caeca took up the colour from the
" "	"	-	blood.
2 hrs	"	-	
" "	"	-	
4 "	"	-	
" "	"	-	
6 "	"	-	
" "	"	-	
8 "	"	-	
" "	"	-	
10 "	"	-	
" "	"	-	
12 "	"	-	
" "	"	-	

iv). Iron saccharate feeding procedure (Yonge, 1927). A group of six starved larvae were placed on meat which had been sprinkled with iron saccharate. After two hours these larvae were removed and the anterior midgut and caeca dissected from each. These tissues were dropped into the following fixative;

5% ammonium sulphide in 95% alcohol	25 ml
Bouin's fluid	25 ml

The two fluids were mixed immediately before use. The tissues were washed in 70% alcohol and embedded in Steedman's polyester wax. The sections obtained from this material were taken down the alcohols to water, treated first with 10% aqueous potassium ferrocyanide for five minutes and then with dilute hydrochloric acid for five minutes to demonstrate iron by the Prussian Blue test. They were thereafter dehydrated, counterstained lightly with eosin and mounted in canada balsam.

v). Enzyme determination techniques. A different dissection technique had to be evolved for the enzyme determinations in order that large numbers of caeca could be dissected out in the shortest possible time. Each larva was secured by pins in an extended attitude on a layer of pitch in a solid watchglass. A cut was made right round the body wall at the level of the fifth segment. The two severed portions of the wall moved back exposing the viscera between the brain and the gut mass. The tissues/

tissues surrounding the caeca were cleared away then each caecum in turn was grasped at its proximal end with fine forceps and severed proximally to the forceps with fine scissors. In this manner a large number of caeca with their contents could be removed quickly and with a minimum of dissection.

For each enzyme determination a fresh tissue extract was prepared to a standard formula. This was termed a Standard Caecal Extract (S.C.E.) and was prepared in the following manner. Caeca were removed from one hundred larvae (about four hundred caeca) into 0.2 ml of extracting fluid (equal parts of glycerine and distilled water; Wigglesworth, 1928) in an agate mortar. A few grains of sand were added and the mixture ground thoroughly. A further 1 ml of extracting fluid was added and the mixture left standing at room temperature for about one hour. It was then centrifuged for fifteen minutes at 3000 r.p.m. after which 1.0 ml of clear supernatant fluid could be drawn off. This was divided into two equal parts, one of which was placed in a test-tube and immersed in boiling water for fifteen minutes to provide a control solution.

The following techniques for the detection of enzymes were employed:

Lipase: the method of George (1952). This makes use of the fact that when droplets of a neutral fat are hydrolysed to fatty acid the reduced droplets take on a blue colour in the presence of/  
of/

of nile blue sulphate, whereas droplets of unhydrolysed fat remain unchanged in colour.

An olive oil suspension was prepared by adding a few drops of olive oil to 50 ml of distilled water and shaking vigorously until a fine suspension of oil was obtained.

The buffers employed throughout this work were the standard citric acid/sodium phosphate buffers of McIlvaine as given by Clark (1925). The pH of 7.6 was chosen for these experiments because this is the approximate pH of the anterior midgut of these larvae where any enzyme which is secreted by the caeca will presumably operate. The pH of the anterior midgut was found by feeding larvae on meat treated with phenol red and inspecting the gut by dissection. It was also noted that Champlain and Fisk (1956) state that the optimum pH of many insect trypsins lies near pH 7.8.

The following solutions were prepared in 1 oz bottles; each in duplicate:

1. 0.2 ml of S.C.E. + 0.5 ml of oil suspension + 0.4 ml of buffer (pH 7.6) + 1 drop toluol.
2. 0.2 ml of S.C.E. (boiled) + 0.5 ml of oil suspension + 0.4 ml of buffer + 1 drop toluol.

The bottles were placed in a water bath at 37°C for twenty-four hours with automatic shaking at intervals.

After twenty-four hours had elapsed a drop of the mixture was/

was taken from each of the bottles in turn and mixed with a drop of aqueous nile blue sulphate on a glass slide. The result was inspected through a microscope.

Invertase: the method of Fletcher and Haub (1933). The following solutions were prepared in 1 oz bottles; each in duplicate:

1. 0.2 ml of S.C.E. + 0.5 ml of 15% sucrose solution + 0.4 ml of buffer + 1 drop toluol.
2. 0.2 ml of S.C.E. (boiled) + 0.5 ml of 15% sucrose solution + 0.4 ml of buffer + 1 drop toluol.

The bottles were placed in a water bath at 37°C for seventy-two hours with automatic shaking at intervals.

The solutions were tested by adding 5 ml of Benedict's solution to each.

Tryptase: two different methods were used in attempts to detect this enzyme. The first, the congo red - fibrin test, was a gross qualitative test. When this was found to give a negative result with an extract of midgut tissue which was certain to contain this enzyme, as well as with the S.C.E., it was decided to use the much more sensitive formol titration method.

Congo red - fibrin test: this test depends upon the fact that when the virtually insoluble protein fibrin is impregnated with some dyes these dyes cannot be washed out again even in acids or alkalis. They will only pass into solution again if the fibrin is digested. If dyed fibrin is dropped into a solution it can be taken as evidence of the presence of a protease if dye is released/

released into the solution. The method of preparation of congo red - fibrin is standard and can be found in any physiological textbook.

The following solutions were prepared in 1 oz bottles; each in duplicate:

1. 0.2 ml S.C.E. + 0.1 ml of 5%  $\text{Na}_2\text{CO}_3$  + 3 small flakes of congo red - fibrin + 1 drop toluol.
2. 0.2 ml S.C.E. (boiled) + 0.1 ml of 5%  $\text{Na}_2\text{CO}_3$  + 3 small flakes of congo red - fibrin + 1 drop toluol.

The bottles were placed in a water bath at  $37^\circ\text{C}$  for twenty-four hours with automatic shaking after which the solutions were inspected for released dye.

Sørensen formol titration method: the production of amino acids as a result of enzymatic action on proteins cannot be detected by direct titration with standard alkali solutions because of the amphoteric properties of the amino acids. If, however, they are first treated with neutral formaldehyde solution methylene compounds are formed and the basic  $\text{NH}_2$  groups in the amino acids are replaced by neutral  $\text{CH}_2\text{:N}$  groups. Consequently the carboxyl groups can be titrated directly with standard  $\text{NaOH}$  solution.

Since formaldehyde solutions usually contain formic acid and since amino acids are seldom exactly neutral both the formaldehyde solution and the amino acid solution must, before mixing, be brought to the same pH. This was done by making each solution just/

just alkaline to phenolphthalein with dilute NaOH solution.

To prepare the neutral formaldehyde solution 50 ml of 40% formaldehyde were taken in a flask and to this were added ten drops of 0.5% phenolphthalein. To this solution 0.1 N NaOH was added drop by drop until a faint pink colour was obtained (This solution gradually became acid again so a further drop of NaOH was added from time to time to bring it back to neutrality).

A standard colour solution at pH 8.4 for comparative purposes was prepared by measuring 5 ml of pH 8.4 buffer into a 2" x 1" glass tube and adding five drops of 0.5% phenolphthalein.

The substrate was a 3% aqueous solution of bacteriological gelatine brought to neutrality by adding dilute sodium hydroxide.

The following solutions were prepared in 1 oz bottles in triplicate:

1. 0.1 ml of S.C.E. + 0.5 ml of 3% neutral gelatine + 0.4 ml of buffer + 1 drop toluol.
2. 0.1 ml of S.C.E. (boiled) + 0.5 ml of 3% neutral gelatine + 0.4 ml of buffer + 1 drop toluol.

The bottles were placed in a water bath at 37°C with automatic shaking at intervals.

At the start of each experiment and at twenty-four hour intervals thereafter 0.1 ml samples were drawn off from each bottle and run into 2" x 1" glass tubes. To each sample was added/

added 5 ml of distilled water and five drops of 0.5% phenolphthalein. The samples were then titrated with  $N/100$  NaOH to a faint pink colour and readings taken. 0.1 ml of neutral formaldehyde was added and the solutions again titrated with  $N/100$  NaOH until they reached the colour of the standard colour solution, i.e. pH 8.4, and a reading again taken. The difference between the two readings in each case was the measure of the quantity of amino acids in the solutions. This difference is termed the "formol acidity" (Wigglesworth, 1929).

The experiment was repeated using a caecal extract prepared from the caeca of larvae from a different culture of flies reared under exactly the same conditions as the first.

The tests for a dipeptidase in the caeca were carried out in exactly the same fashion except that the substrate was a 1% aqueous solution of glycyl-glycine as used by Hobson (1931).

Before the experiments detailed above were performed, a pilot experiment was carried out using material which was almost certain to contain tryptase, i.e. the anterior midguts of P. terrae-novae larvae. This was done to ensure that the procedure was effective in detecting small dilute quantities of enzyme. An extract was prepared from the anterior midguts of seventeen larvae. Calculation showed that this number of guts gave approximately the same quantity of tissue as that obtained from four hundred caeca.

The/

Table VI. First pilot experiment carried out to assess the effectiveness of a modified version of the Sørensen formol titration method in detecting very small quantities of enzymes in a solution.

Experiment 1

Time	Start	17 hours	27 hours	42 hours	67. hours
Sample					
1	0.17	0.25	0.40	0.47	0.52
2	Mean = 0.17	0.23	0.41	0.54	0.60
3	0.12	0.11	0.12	0.13	0.13
4	Mean = 0.12	0.12	0.13	0.17	0.16
Control solutions containing boiled extract					

Values are 'formol acidity' and are given in millilitres of  $N/100$  NaOH.

Table VII. Second pilot experiment carried out to assess the effectiveness of a modified version of the Sørensen formol titration method in detecting very small quantities of enzymes in a solution.

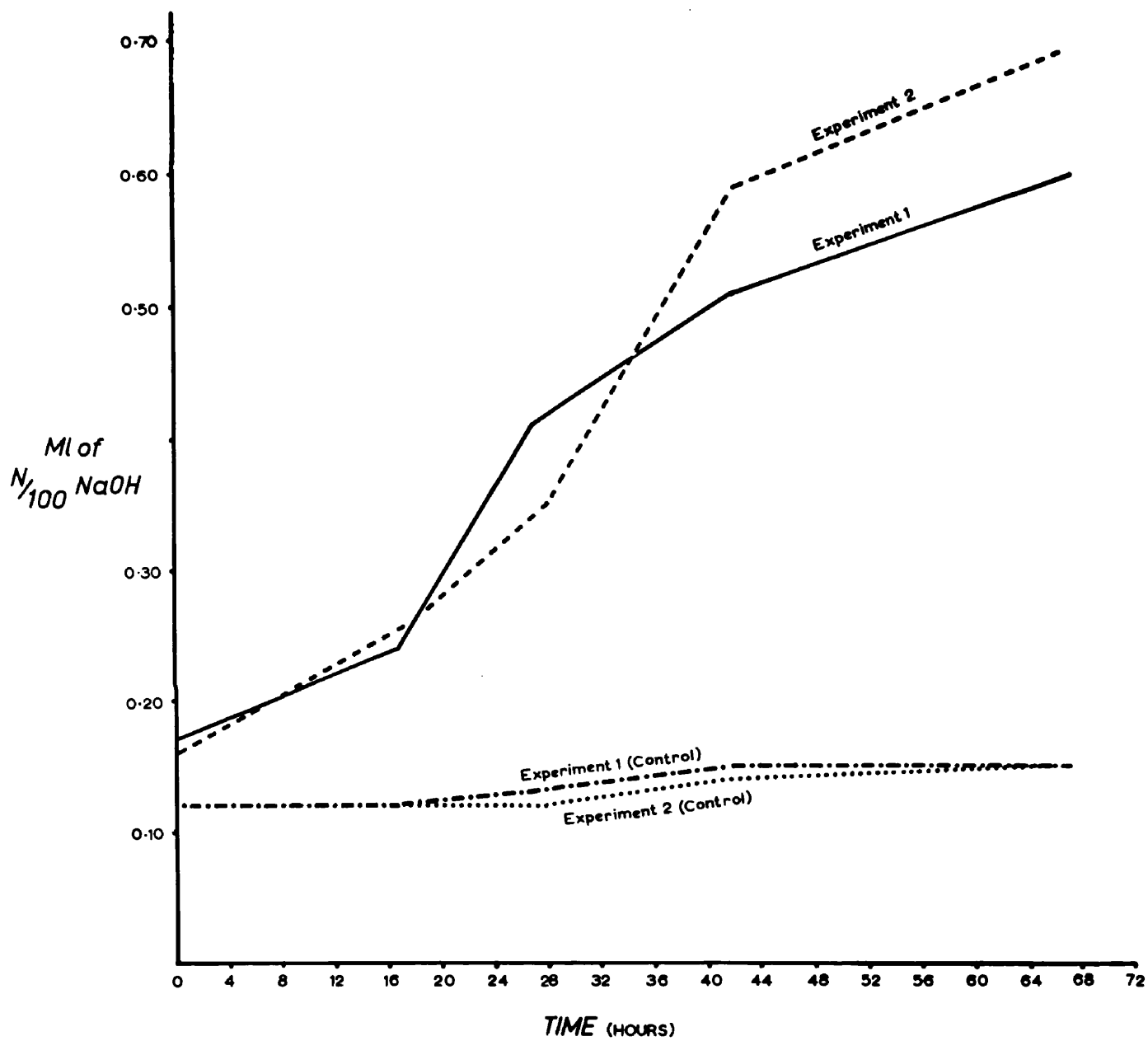
Experiment 2

Time	Start	18 hours	28 hours	42 hours	66 hours
Sample					
1	0.15	0.25	0.34	0.60	0.71
2	Mean = 0.16	0.27	0.36	0.57	0.66
Control solutions containing boiled extract					
3	0.12	0.12	0.12	0.15	0.14
4	Mean = 0.11	0.12	0.12	0.12	0.15

Values are 'formol acidity' and are given in millilitres of  $N/100$  NaOH.

Graph 1. The results of two pilot experiments carried out to assess the effectiveness of the Sørensen formol titration method in detecting very small quantities of enzymes in a solution.

The values plotted are the mean values given in Tables VI and VII.



Graph 1.

The procedure detailed above was followed exactly and the experiment performed in duplicate. The readings obtained are given in Tables VI and VII and summarised in Graph 1. It can be seen from these that the method is effective in detecting tryptase. The results of the experiments carried out using caecal extracts are given in Tables VIII, IX, X and XI and Graphs 2 and 3.

vi). Dye-injection technique. A range of dyes were injected into the haemocoel of the larvae after the fashion of Woodruff (1933) and Palm (1952). These dyes were made up in 1 : 10,000 solution or suspension with physiological saline. Sets of ten larvae were injected with each dye, each larva receiving approximately 0.005 ml of fluid. This was injected through the dorsal wall of the ninth body segment lateral to the heart by means of a micrometer syringe fitted with a 30 gauge  $\times \frac{5}{8}$ " needle. When the syringe was withdrawn a small piece of fat body was usually extruded through the wound thus effectively preventing any haemorrhage. Larvae which showed signs of haemorrhage were discarded. The injected larvae were kept in moist peat moss. Two larvae were withdrawn from each set at half-hour intervals, dissected, and the caeca inspected. (Larvae which were obviously moribund were discarded.)

vii). Ligaturing technique. The ligatures were single strands of silk drawn from pieces of bolting-silk. They were tied immediately/

immediately above the proximal ends of the caeca, one caecum being ligatured in each larva. Of the larvae which were treated, in half the dorsal caeca were ligatured and in half the ventral caeca.

(b) The physiology of the caeca

When living larvae were dissected in physiological saline to display the viscera the caeca showed very little visible evidence of being physiologically active. No food material was visible in the caecal lumen nor were there any iridescent droplets in the epithelial cells such as were visible in the cells of the adjacent midgut epithelium. The only visible sign of their functioning was a slight peristalsis at erratic intervals.

Caeca were opened lengthwise so that the walls curled back to expose the inner faces of the epithelial cells. The opened caeca were excised and quickly transferred to a drop of saline on a glass slide. Under the microscope it was observed that there were no globules of secretion on the inner cell faces. This agrees with the observations made on a majority of the fixed tissue preparations noted in the previous section. It is fairly certain therefore that the globules observed on the lumen faces of the epithelial cells in some preparations are fixation artifacts. The secretion of which granular traces were found in the lumen of the caeca in fixed tissue preparations was not distinguishable in dissections of fresh material.

Neither/

Neither histological nor histochemical staining of sections of fixed material revealed any further evidence of absorption or secretion. There were no concentrations of fat, protein or carbohydrate in the epithelial cells nor was food material of any kind shown to be present in the lumen.

Absorption of dissolved food material by the caeca would require a flow of material from the lumen of the gut up into the caeca. To establish whether such a flow was actually taking place soluble dyes which were known to pass through the peritrophic membrane were mixed with the food of the larvae. These dyes were methylene blue and neutral red. It is clear from the results given in Tables IV and V that both of these dyes passed freely through the peritrophic membrane therefore the contents of the anterior midgut outside the membrane should be coloured by them. Since no colour was observed in the caeca at any time with either dye it is quite evident that none of the material in the anterior midgut is finding its way into the enteric caeca.

Confirmation of this latter observation was obtained from the iron saccharate feeding experiment. The result of this was negative, no iron being subsequently detected in the lumen of the caeca or in the caecal epithelium.

If the caeca are secretory the most likely secretory products are enzymes. It is however unlikely that there would be/

be different enzymes in the caeca to those found in the anterior midgut. The absence of any flow of material up into the caeca means that enzymes of caecal origin must operate in the midgut proper and would be detected there. Hobson (1931) and Fletcher and Haub (1933) detected lipase, invertase, trypsin and peptidase in the anterior midgut of closely related blowfly larvae. These enzymes were therefore sought in the caeca.

Before the enzyme determinations were begun an abortive attempt was made to find out the pH in the lumen of the caeca. Attempts were made using micro-injection apparatus to introduce indicators into the lumen of severed caeca but the lumen was so narrow that this proved to be impossible. Nor was it possible to obtain a pure sample of the contents of a caecum because the very small quantity of fluid which was exuded from the end of a severed caecum contained cytoplasm from damaged cells. Attempts to introduce indicators in the food were frustrated by the absence of any flow of material from the gut up into the caeca. However since any enzymes which are produced by the caeca must operate in the anterior midgut where the pH had been found to be in the region of 7.6 the enzyme determinations were carried out at this latter pH

The caecal extracts employed were prepared from whole caeca since pure samples of the caecal contents could not be obtained.

The/

Table VIII. Results of formol titrations carried out to detect the breakdown of gelatine solution by tryptase in extracts of caecal tissue.

Experiment 3

Time	Start	24 hours	49 hours	72 hours
Sample				
1	0.12	0.10	0.16	0.14
2	0.15	0.15	0.16	0.11
3	0.10	0.09	0.12	0.11
Control solutions containing boiled extract				
4	0.14	0.14	0.15	0.12
5	0.11	0.10	0.12	0.13
6	0.10	0.09	0.13	0.12

Values are 'formol acidity' and are given in millilitres of  $N/100$  NaOH.

Table IX. Results of formol titrations carried out to detect the breakdown of gelatine solution by tryptase in extracts of caecal tissue.

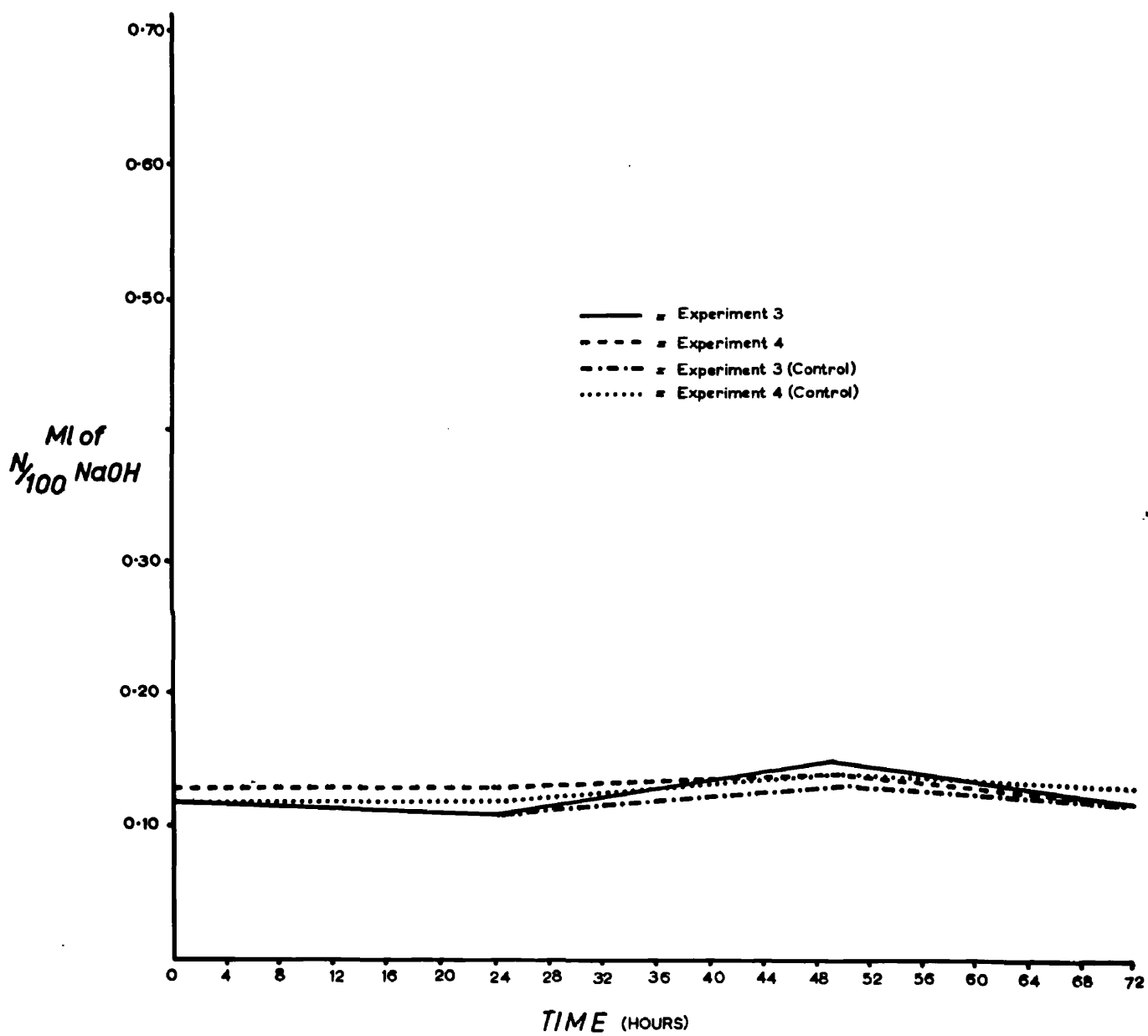
Experiment 4

Time	Start	24 hours	49 hours	72 hours
Sample				
1	0.12	0.12	0.14	0.11
2	0.14	0.13	0.13	0.12
3	0.12	0.14	0.15	0.12
Control solutions containing boiled extract				
4	0.13	0.12	0.16	0.14
5	0.14	0.14	0.14	0.13
6	0.10	0.12	0.13	0.13

Values are 'formol acidity' and are given in millilitres of  $N/100$  NaOH.

Graph 2. The result of two experiments carried out to detect the breakdown of gelatine solution by tryptase in extracts of caecal tissue.

The values plotted are the mean values given in Tables VIII and IX.



Graph 2.

The tests for the presence of lipase and of invertase in the caecal extracts were both negative.

The first method employed for the detection of proteases, the congo red - fibrin test, was found to be too crude to detect such small quantities of enzymes as might be found in the caeca since it gave a negative result with midgut extract. The more sensitive Sørensen formol titration method proved to be quite satisfactory for the detection of proteases in midgut extract and was used in the major effort to detect such enzymes in caecal extract. The version of the Sørensen method given by Wigglesworth (1928) was modified and adapted to suit the tissue under investigation.

It is obvious from the results of the tryptase detection tests (Tables VIII and IX, Graph 2) that this enzyme is not produced by the enteric caeca nor is there any evidence that a peptidase is being secreted (Tables X and XI, Graph 3). Thus none of the enzymes which had previously been shown to be present in the anterior midgut of blowfly larvae could be detected in the caeca. It was not thought advisable to attempt to use more sensitive methods of enzyme detection since these might merely have detected the tissue enzymes from the macerated caecal epithelial cells.

Any secretory activity in the caeca would require a passage of material in through the walls of the caeca. It seemed likely/

Table X. Results of formol titrations carried out to detect the breakdown of glycyl-glycine by peptidase in extracts of caecal tissue.

Experiment 5

Time	Start	24 hours	48 hours	72 hours
Sample				
1	0.31	0.33	0.33	0.33
2	0.29	0.32	0.31	0.32
3	0.30	0.32	0.35	0.34
Control solutions containing boiled extract				
4	0.29	0.32	0.31	0.32
5	0.28	0.30	0.30	0.30
6	0.29	0.29	0.33	0.34

Values are 'formol acidity' and are given in millilitres of  $N/100$  NaOH.

Table XI. Results of formol titrations carried out to detect the breakdown of glycyl-glycine by peptidase in extracts of caecal tissue.

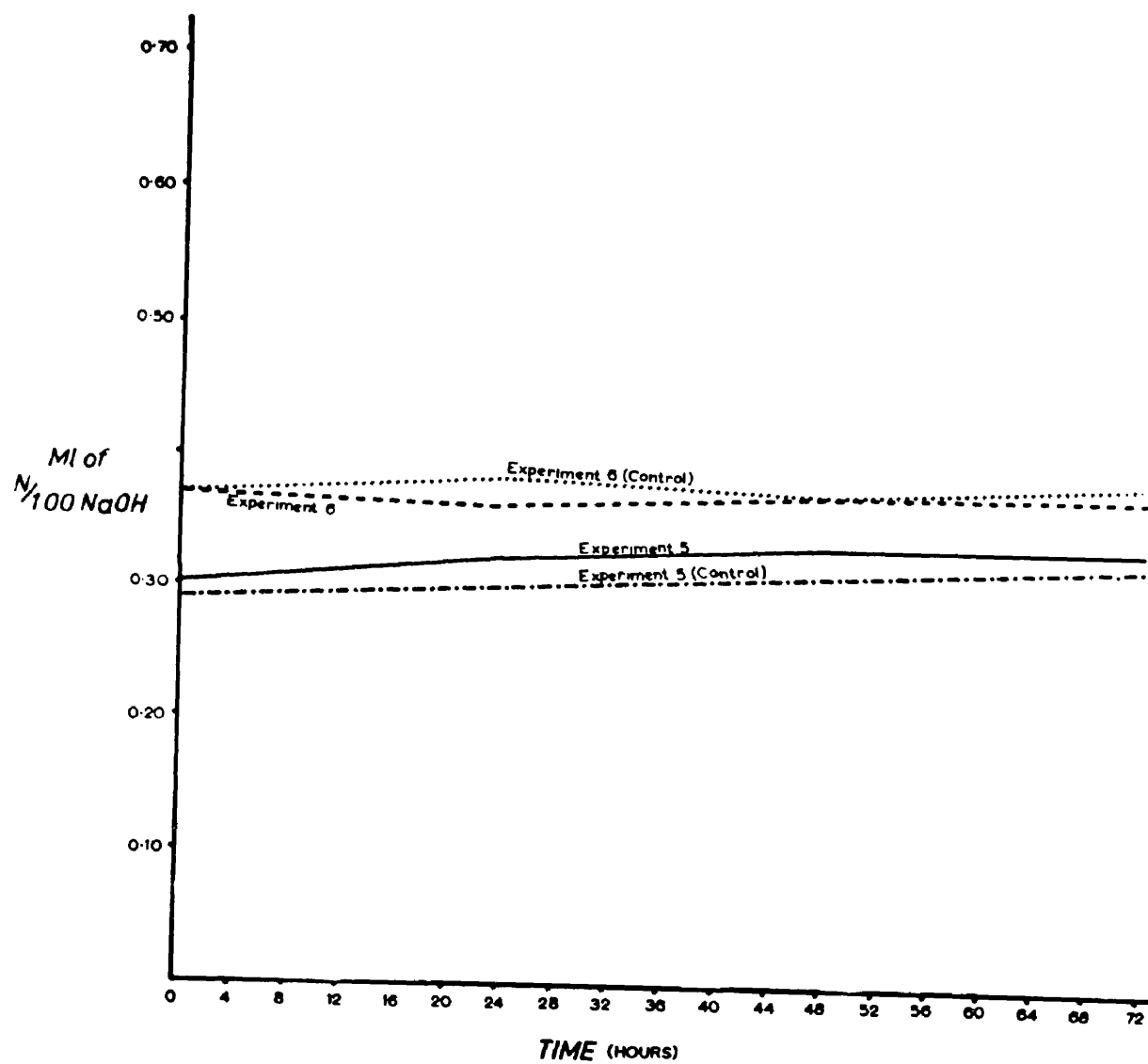
Experiment 6

Time	Start	24 hours	48 hours	72 hours
Sample				
1	0.38	0.38	0.39	0.38
2	0.36	0.36	0.36	0.37
3	0.36	0.35	0.36	0.36
Control solutions containing boiled extract				
4	0.36	0.36	0.35	0.36
5	0.37	0.40	0.39	0.40
6	0.37	0.37	0.38	0.38

Values are 'formol acidity' and are given in millilitres of  $N/100$  NaOH.

Graph 3. The results of two experiments carried out to detect the breakdown of glycyl glycine by peptidase in extracts of caecal tissue.

The values plotted are the mean values given in Tables X and XI.



Graph 3.

likely that such a flow of material could be detected by injection of various dyes into the haemocoel. The procedure adopted was largely empirical since the caecal cells presumably absorb selectively so a range of dyes was employed. The method used was adapted from the work of Woodruff (1933) and Palm (1952). The dyes injected were as follows:

Acid fuchsin	Methylene blue	Indigo carmine
Basic fuchsin	Trypan blue	Neutral red
Alcian blue	Azocarmine	Cyanosin
Celestin blue	Congo red	Gentian violet
Chlorantin blue	Eosin	Indian ink

Only two of the dyes, eosin and gentian violet, appeared in the caeca. The others appeared to be treated as waste products, the oenocytes in the pericardial bands and the inter-segmental membranes scavenging them from the blood.

The eosin was visible at the distal ends of the caeca within the first half hour after injection, there being an apparent concentration of the dye at these points, but throughout the remainder of the length of the caeca the colour was absent. If the dye was present it was in too low a concentration to be visible.

The gentian violet was clearly visible throughout the length of the caeca within the first half hour after injection. The colour was stronger in the lumen of the caeca than in the epithelial/

epithelial cells i.e. the dye was apparently being passed through the cells and concentrated in the lumen. It was also apparent that the dye was being carried out of the caeca into the lumen of the anterior midgut since a concentration of dye was seen in the midgut beside the mouth of each caecum and for a very short distance down the gut dye could be observed lying against the epithelium possibly trapped between it and the peritrophic membrane.

The caeca are therefore very selective in the substances which they absorb since they have been shown to accept only two of the crystalloid dyes offered to them, rejecting the remainder of the crystalloids and all the colloids. The accepted dyes were passed through into the lumen where the gentian violet, and presumably the eosin, was carried down and out of the caeca thus demonstrating that there was in fact a flow of material down the lumen of each caecum.

Corroboration of the latter observation was sought by ligaturing the caeca so that any fluid flowing down the lumen might build up behind the ligature thus causing a visible swelling. It was not possible to ligature the caeca through a small opening in the skin of a living larva since the turgor of the blood in such a soft-skinned creature causes the blood to rush out and the animal to collapse immediately the body is opened. Nor is it possible to employ the operating methods of Fraser/

Fraser (1957) since the bulk of the viscera at this level in the body is such that the body cannot be adequately compressed on either side of the wound to keep the blood away from the wound without crushing the viscera. It was only possible therefore to perform the rather unsatisfactory operation of ligaturing the caeca of larvae dissected in saline. The ligatures, made of single silk strands tied round the roots of the caeca did cause a distension on the distal side of the ligature in the majority of cases, providing slight but positive evidence of a buildup of fluid behind the ligature and hence corroborating the observation that there was a flow of fluid down the caeca.

#### Section IV: Discussion

At the beginning of this work it was decided that of the two probable functions of the caeca, absorption and secretion, the latter was the more likely. On the basis of their position in the gut and their morphology it could be reasoned that the caeca have no absorptive function. Since they are located at the anterior limit of the normal digestive tissue any food which entered them would be largely undigested except by the possible amylase found by Hobson (1931) in the crop or by extra-intestinal digestion by the tryptase found by the same author in the excreta. Their long and very narrow lumen precludes, in the absence of cilia, the possibility of a circulation of material in them. As Day and Powning (1949) remarked of the caeca of Periplaneta which are morphologically similar to those in the larva of P. terrae-novae, "(they) are ill-fitted for the rapid diffusion of materials from the gut lumen to their closed ends".

The dye-feeding experiments and those in which indicators or iron saccharate were fed to larvae prove conclusively that the caeca cannot be absorptive. Since these substances all pass freely through the peritrophic membrane they must be mixed with the midgut contents outside the membrane. If, therefore, any part of the midgut contents was moving up into the caeca these substances would be carried up as well and would be visible in the lumen in the case of the dyes and indicators or in appropriately/

appropriately treated microtome sections of the caeca in the case of the iron saccharate. None of these substances were detected in the caeca. Therefore in the absence of any flow of material up into the caeca from the gut absorption can not take place.

If the caeca are secretory the most likely products are enzymes. Hobson (1931) showed that tryptase and dipeptidase were present in the anterior midgut of the larva of L. sericata. He established that these enzymes could have been secreted only by the anterior midgut (with which he included the caeca) since they were absent from the crop and virtually absent from the mid-midgut. If, therefore, the caeca are functional expansions of the anterior midgut enzymes should be detectable in them in a feeding larva. No enzymes were found. Thus all possibilities of the caeca in this larva functioning as digestive diverticulae or accessory sites of enzyme production can be dismissed.

That there is a flow of secreted material through the caeca towards the midgut is, however, certain. Gentian violet which passed readily from the haemocoel into the lumen of the caeca was carried in this flow out of the caeca into the lumen of the gut. Additional evidence of such a current of fluid was provided by the ligaturing experiments.

The histological study of the caeca confirmed the above observations/

observations. Food material was never detected in any sections of caeca nor were there any concentrations of proteins, fat or carbohydrate demonstrable in the epithelial cells. The heavy concentration of fat globules, presumably ingested from the food, which is so distinct in the cells of the anterior midgut epithelium was absent in the caeca. There was no evidence of the type of secretory activity, in the form of vacuoles, such as is so apparent in sections of anterior midgut cells. The globules of granular secretion on the lumen face of the caecal cells seen in many sections are probably an artifact. However a diffuse faintly granular material frequently observed in the lumen is assumed to represent the fixation residue of the secretion whose flow has been detected.

The nature of this secretion is obscure. The rate of flow indicated by the rapid transfer of dye from caecum to gut shows that a fair volume of fluid is being secreted. It is possible that this fluid is simply some fluid fraction of the blood which serves to lubricate the passage of food in the gut.

## CONCLUSIONS

The following original conclusions can be drawn from this work:

1. That in the larva of P. terrae-novae the principal modification of the normal midgut structure to be found in the proventriculus lies in the musculature. This modification takes the form of a circular muscle coat which is much denser and more regularly arranged than that found elsewhere in the midgut. This gives to the proventriculus the rigid outline and the regular shape required for it to fulfil its function as part of the mould for the peritrophic membrane.
2. That within the proventriculus of the larva of P. terrae-novae, already known from previous work to be the source of the peritrophic membrane, the ability to produce chitin which is the basic material of the membrane resides only in a narrow band of cells. The process of chitin synthesis within these cells has been demonstrated histochemically to be from the simple polysaccharide glycogen to a more complicated polysaccharide which appears in granular form in fixed material and then to a fluid polysaccharide, possibly very close, chemically, to chitin itself. The use of the paraldehyde fuchsin staining method to identify this latter step in chitin formation is entirely novel. Some corroboration of this work was therefore necessary and this was obtained/

obtained by treating guts obtained from four other insect species with this stain and inspecting the sites of secretion of peritrophic membrane material in each. Very satisfactory corroboration was obtained in two of the species treated, Forficula auricularia and Culex pipiens. Further elucidation of the problem of intracellular chitin formation must await the emergence of some knowledge about the precursors of chitin and the development of suitable histochemical staining methods which would stain these precursors and, more especially, would specifically stain chitin itself.

3. That the enteric caeca in the gut of the larva of P. terrae-novae are not simple expansions of the digestive tissue of the anterior midgut. Anatomically they are very similar to the anterior midgut, the only distinct difference being in the circular muscle coat of the caeca which is much thinner and less regularly arranged than that on the anterior midgut. Physiologically they are completely separate from the anterior midgut in that neither food absorption nor enzyme secretion takes place in them. It is clear that there is no flow of material from the midgut lumen into the caeca so that no absorption of any kind can be taking place. The caeca do however show evidence of producing some form of secretion which passes from them into the anterior midgut. The nature of this secretion, in the absence from it of any detectable enzymes, remains obscure.

## References

- AUBERTOT, M. 1937. Sur les membranes peritrophiques des insectes.  
Arch.Zool.exp.gen., 79, 49-57.
- BAKER, J.R. 1956. The histochemical recognition of phenols, especially tyrosine.  
Quart.J.micr.Sci., 97, 161-164.
- BALBIANI, E.G. 1890. Etudes sur le tube digestif des Cryptops.  
Arch.Zool.exp.gen., 2 (8), 1-82.
- BHATIA, M.L. 1939. Biology, morphology and anatomy of aphidophagous syrphid larvae.  
Parasitology, 31, 78-129.
- BODENSTEIN, D. 1949. The postembryonic development of Drosophila.  
Biology of Drosophila (ed. M. Demerec).  
New York: John Wiley & Sons.
- BOISSEZON, P. de 1930. La biologie et de l'histophysiologie de Culex pipiens. I.  
Arch.Zool.exp.gen., 70, 281-431.
- BORDAS, L. 1911. Les tubes de malpighi des larves des Lepidopteres.  
Ann.Sci.nat., 13-14, 193-273.
- BRADFIELD, J.R.G. 1950. Phosphatases and nucleic acids in silk glands.  
Quart.J.micr.Sci., 92, 87-112.
- BRUNET, P.C.J. 1952<sub>a</sub>. The formation of the ootheca by Periplaneta americana. II. The structure and function of the left colleterial gland.  
Quart.J.micr.Sci., 93, 47-69.
- BRUNET, P.C.J. 1952<sub>b</sub>. Periodic acid - Schiff reaction of the insect cuticle.  
Science, 116, 126.
- CAMPBELL, F.L. 1929. Detection and estimation of chitin in the roach.  
Ann.ent.Soc.Amer., 22, 401-426.

- CARLETON, H.M. & LEACH, E.H. 1947. Histological Technique, Oxford: Oxford University Press.
- CHAMPLAIN, R.A. & FISK, F.W. 1956. The digestive enzymes of the stable fly Stomoxys calcitrans. Ohio J.Sci., 56, 52-62.
- CHATTON, E. 1920. Les membranes peritrophiques des Drosophiles (Dipteres) et des Daphnies (Cladoceres); leur genese et leur role a l'egard des parasites intestinaux. Bull.Soc.Zool.Fr., 45, 265-280.
- CLARK, W.M. 1925. The Determination of Hydrogen Ions (2nd edn.), Baltimore: Williams & Wilkins Co.
- CLARK, R.B. 1956. Personal communication.
- CUENOT, L. 1895. Etudes physiologiques sur les Orthopteres. Arch.Biol.Paris, 14, 293-333.
- DAY, M.F. 1949. The occurrence of mucoid substances in insects. Aust.J.sci.Res., B2, 421-427.
- DAY, M.F. & POWNING, R.F. 1949. Processes of digestion in certain insects. Aust.J.sci.Res., B2, 175-215.
- DEHN, M. von. 1933. Untersuchungen über die Bildung der Peritrophischen Membran bei den Insekten. Z.Zellforsch., 19, 79-105.
- FLETCHER, F. & HAUB, J.G. 1933. Digestion in blowfly larvae, Phormia regina, Meigen, used in the treatment of osteomyelitis. Ohio J.Sci., 33, 101-109.
- FRASER, A. 1956. Personal communication.
- FRASER, A. 1957a. The retrocerebral endocrine organs of the larva of Protophormia terrae-novae R-D. Proc.R.ent.Soc.Lond., A.32, 40-46.

- FRASER, A. 1957b. An Investigation of the Phenomenon of Diapause in the Larva of Lucilia caesar L. Glasgow. Glasgow University, Ph.D. Thesis.
- GABE, M. 1953. Sur quelques applications de la coloration par la fuchsine - paraldehyde. Bull.Microsc.appl., 3, 154-162.
- GEHUCHTEN, A. van 1890. Recherches histologiques sur l'appareil digestif de la larve de la Ptychoptera contaminata. La Cellule, 6, 185-289.
- GEORGE, W.C. 1952. The digestion and absorption of fat in Lamellibranchs. Biol.Bull., Wood's Hole, 102, 118-127.
- GOMORI, G. 1950. Aldehyde fuchsin; a new stain for elastic tissue. Amer.J.clin.Path., 20, 665-666.
- GOMORI, G. 1953. Microscopic Histochemistry. Chicago: The University of Chicago Press.
- GRAHAM-SMITH, G.S. 1934. The alimentary canal of Calliphora erythrocephala L. with special reference to its musculature and to the proventriculus, rectal valve and rectal papillae. Parasitology, 26, 176-248.
- GRAY, P. 1954. The Microtommists Formulary and Guide. London: Constable & Co.
- GRESSON, R.A.R. 1934. Cytology of the midgut and hepatic caeca of Periplaneta orientalis. Quart.J.micr.Sci., 77, 317-334.
- GUYENOT, E. 1906. Sur le mode de nutrition de quelques larves de mouches. C.R.Soc.Biol., Paris, 61, 634-635.
- GUYENOT, E. 1907. L'appareil digestif et la digestion de quelques larves de mouches. Bull.Biol., 41, 353-370.
- HADDOW, A.J. & THOMSON, R.C. 1937. Sheep myiasis in South-west Scotland, with special reference to the species involved. Parasitology, 29, 96-116.

- HENSON, H. 1931. The structure and post-embryonic development of Vanessa urticae. I. The larval alimentary canal. Quart.J.micr.Sci., 74, 321-360.
- HEWITT, C.G. 1914. The Housefly. Cambridge: Cambridge University Press.
- HOBSON, R.P. 1931. Studies in the nutrition of blowfly larvae. J.exp.Biol., 8, 109-123.
- HOTCHKISS, R.D. 1948. A microchemical reaction resulting in the staining of polysaccharide structures in fixed tissue preparations. Arch.Biochem., 16, 131-14.
- IMMS, A.D. 1907. On the larval and pupal stages of Anopheles maculipennis, Mg. J.Hyg.,Camb., 7, 291-398.
- IMMS, A.D. 1957. A General Textbook of Entomology (Revised by Richards, O.W. & Davies, R.G.) London: Methuen & Co., Ltd.,
- JUDD, W.W. 1948. A comparative study of the proventriculus of Orthopteroid insects with reference to its use in taxonomy. Canad.J.Res., 26, 93-161.
- KRAWKOW, N.P. 1893. Uber verschiedenartige Chitine. Z.Biol., 29, 177-198.
- KRUGER, F. 1926. Biologie und Morphologie einiger Syrphidenlarven. Z.Morph.Okol.Tiere, 6, 83-149.
- LOWNE, B.T. 1890. The Anatomy, Physiology, Morphology and Development of the Blowfly (Calliphora erythrocephala). Vol. 1. London: R.H. Porter.
- LUDWIG, C.E. 1949. Embryology and morphology of the larval head of Calliphora erythrocephala. Microentomology, 14, 75-111.
- MACLEOD, J. 1943a. A survey of British sheep blowflies. Bull.ent.Res., 34, 65-88.

- MACLEOD, J. 1943b. A survey of British sheep blowflies. II. Relation of strike to host and edaphic factors. Bull.ent.Res., 34, 95-111.
- McMANUS, J.F.A. 1946. J.Path.Bact., 58, 93-98. Reference in Pearse (1953).
- McMANUS, J.F.A. 1956. Factors favouring restriction to 1,2 glycols of materials coloured by the periodic acid - Schiff reaction. Nature, Lond., 178, 914-915.
- MAZIA, D., BREWER, P.A. & ALFERT, M. 1953. The cytochemical staining and measurement of protein with mercuric bromphenol blue. Biol.Bull., Wood's Hole, 104, 57-67.
- PAILLOT, A. 1939. Contribution a l'etude cytologique et histophysiologique du Bombyx du murier pendant la mue. Ann.Epiphyt., N.S.5, 339-386.
- PALM, N.B. 1952. Storage and excretion of vital dyes in insects. Ark.Zool., 3, 195-272.
- PEARSE, A.G.E. 1953. Histochemistry, Theoretical and Applied. London: Churchill.
- PEREZ, C. 1910. Recherches histologiques sur la metamorphose des muscides (Calliphora erythrocephala Mg.) Arch.Zool.exp.gen., 4, 1-274.
- POLLISTER, A.W. 1950. Quelques methodes de cytologie chimique quantitative. Rev.Hemat., 5, 527-554.
- PURVES, H.D. & GRIESBACH, W.E. 1951. The site of thyrotrophin and gonadotrophin production in the rat pituitary studied by McManus - Hotchkiss staining for glycoprotein. Endocrinology, 49 (2), 244-264.
- RICHARDS, A.G. 1951. The Integument of Arthropods. London: Oxford University Press.
- RICHARDS, A.G. 1952. Studies on arthropod cuticle. VII Patent and masked carbohydrate in the epicuticle of insects. Science, 115, 206-208.

- RICHARDS, A.G. & KORDA, F.H. 1948. Electron microscope studies of extracted cuticle. Biol.Bull., Wood's Hole, 94, 212-235.
- RIZKI, M.T.M. 1956. The secretory activity of the proventriculus of Drosophila melanogaster. J.exp.Zool., 131, 203-218.
- ROEDER, K.D. 1953. Insect Physiology. London: Chapman & Hall.
- SCHLOTKE, E. 1937. Untersuchungen über die Verdauungsfermente von Insekten. Z.vergl.Physiol., 24, 422-450.
- SHINODA, O. 1927. Comparative histo-cytology of the midgut of various insects. Z.Zellforsch., 5, 278-292.
- SIANG-HSU, W. 1947. On the cytoplasmic elements in the midgut epithelium of the larvae of Drosophila melanogaster, Meigen. J.Morph., 80, 161-193.
- STEEDMAN, H.F. 1947. Ester wax: a new embedding medium. Quart.J.micr.Sci., 88, 123-133.
- STEEDMAN, H.F. 1950. Alcian blue 8 GS: a new stain for mucin. Quart.J.micr.Sci., 91, 477-479.
- STEEDMAN, H.F. 1957. Polyester wax. Nature, Lond., 179, 1345.
- STRASBURGER, E.H. 1935. Drosophila melanogaster. Eine Einführung in den Bau und die Entwicklung. Berlin: Julius Springer.
- STRASBURGER, M. 1932. Bau, Funktion, und Variabilität des Darmtractus von Drosophila melanogaster Z.wiss.Zool., 140, 539-649.
- STUHLMANN, F. 1907. Beiträge zur Kenntnis der Tsetsefliege (Glossina fusca und G. tachinoides) Arb.Gesundheitsamt, 26, 301-383.
- SWINGLE 1925. Digestive enzymes of an insect. Ohio J.Sci., 25, 209-218.

- THOMPSON, M.T. 1905. Alimentary canal of the mosquito.  
Proc.Boston Soc.Nat.Hist., 32, 145-202.
- VIGNON, P. 1901. Recherches sur les epitheliums.  
Arch.Zool.exp.gen., 3(9), 371-715.
- WATERHOUSE, D.F. 1953. The occurrence and significance of the peritrophic membrane, with special reference to adult Lepidoptera and Diptera.  
Aust.J.Zool., 1, 299-318.
- WATERHOUSE, D.F. & STAY, B. 1955. Functional differentiation in the midgut epithelium of blowfly larvae as revealed by histochemical tests.  
Aust.J.biol.Sci., 8, 253-277.
- WEINLAND, E. 1906 Ueber die Ausscheidung von Ammoniak durch die larven von Calliphora und uber eine Beziehung dieser Tatsache zu dem Entwicklungs-stadium dieser Tiere.  
Z.Biol., 47, 232.
- WEISMANN, A. 1864. Die Entwicklung der Dipteren. Leipzig Wilhelm Engelmann.
- WIGGLESWORTH, V.B. 1928. Digestion in the cockroach. III The digestion of proteins and fats.  
Biochem.J., 22, 150-161.
- WIGGLESWORTH, V.B. 1929. Digestion in the Tsetse-fly: a study of structure and function.  
Parasitology, 21, 288-321.
- WIGGLESWORTH, V.B. 1930. The peritrophic membrane in insects with special reference to mosquito larvae.  
Quart.J.micr.Sci., 73, 593-616.
- WIGGLESWORTH, V.B. 1948. The insect cuticle.  
Biol.Rev., 23, 408-451.
- WIGGLESWORTH, V.B. 1953. The Principles of Insect Physiology. London: Methuen & Co., Ltd.
- WIGGLESWORTH, V.B. 1956. The haemocytes and connective tissue formation in an insect.  
Quart.J.micr.Sci., 97, 89-98.
- WILLIAMS, C.M. 1946. The physiology of insect diapause.  
Biol.Bull., Wood's Hole, 90, 234-243.

- WOLLMAN, E. 1922. Biologie de la mouche domestique et des larves de mouche a viands en elevages aseptiques.  
Ann.Inst.Pasteur, 36, 784-788.
- WOODRUFF, B.H. 1933. Studies of the epithelium lining the caeca and midgut in the grasshopper.  
J.Morph., 55, 53-80.
- YONGE, C.M. 1927. Structure and physiology of the organs of feeding and digestion in Ostrea edulis.  
J.Mar.biol.Ass., 14, 295-386.