1-1-1981

Structure and function of the esophageal bulb of the apple maggot fly, Rhagoletis pomonella Walsh.

Stuart S. Ratner
University of Massachusetts Amherst

STRUCTURE AND FUNCTION OF THE ESOPHAGEAL BULB OF THE APPLE MAGGOT FLY, *RHAGOLETIS POMONELLA* WALSH

A Dissertation Presented

By

STUART S. RATNER

Submitted to the Graduate School of the
University of Massachusetts in partial fulfillment
of the requirements for the degree of

DOCTOR OF PHILOSOPHY

May 1981

Department of Entomology

Supported by Hatch Grant No. 312
to Dr. J. G. Stoffolano, Jr.

STRUCTURE AND FUNCTION OF THE ESOPHAGEAL BULB OF THE APPLE MAGGOT FLY, *RHAGOLETIS POMONELLA* WALSH

A Dissertation Presented

By

Stuart S. Ratner

Approved as to style and content by:

John G. Stoffolano Jr.
Dr. John G. Stoffolano, Jr. Chairperson of Committee

Christopher L.F. Woodcock
Dr. Christopher L.F. Woodcock, Member

Chih-Ming Yin
Dr. Chih-Ming Yin, Member

John D. Edman
Dr. John D. Edman
Acting Head
Department of Entomology

To my wife, Hilary, who sustains
me with her love, her wit, and
her own devotion to science.

ACKNOWLEDGEMENTS

I would like to express my appreciation to Dr. John G. Stoffolano, Jr., my dissertation committee chairman. This study could never have been performed without his guidance, support, and concern. His editorial advice during the composition of this dissertation was invaluable. To the members of my committee--Dr. Christopher L. F. Woodcock, of the Department of Zoology, and Dr. Chin-Ming Yin--I express sincere gratitude for their help in the formulation of research plans, and for their critical evaluation of the manuscript. I also thank Dr. Woodcock for instruction in electron microscopical techniques, and for making his microscope freely available to me. Other faculty members contributed valuable aid, most notably Dr. Henry H. Hagedorn, who helped shape this study during its early stages; Dr. Ronald Prokopy, who provided me with an ecological perspective on Rhagoletis pomonella; and Dr. John Nordin of the Department of Biochemistry and Dr. Mark Mount of the Department of Plant Pathology, in whose laboratories the radioisotopic studies were carried out. I am also grateful to Ms. Robyn Rufner of the Massachusetts Agricultural Experiment Station Electron Microscope Laboratory, for technical advice, and the use of her fine facility.

Finally, I thank my brother, Carl N. Ratner, who generously defrayed the production costs of this dissertation.

ABSTRACT

Structure and Function of the
Esophageal Bulb of the Apple Maggot Fly,
Rhagoletis pomonella Walsh

May, 1981

Stuart S. Ratner
Ph.D., University of Massachusetts

Directed by Professor John G. Stoffolano, Jr.

A characterization of the functions of the esophageal bulb (EB) of R. pomonella was developed through a histological, histochemical, and ultrastructural examination of developmental changes in the EB and its contents; and through perfusion studies performed on isolated foreguts in vitro. The ability of the EB to serve as a mycetome may stem from intima-derived fibers which accumulate in the organ's lumen. These fibers seem to provide lumen-dwelling bacteria with an anchorage against the flushing action of food flow. There is no evidence that the EB directly participates in nutrient exchange with the bacteria. The epithelium of the EB contains energy-requiring pumps for sodium, but not for rubidium or chloride. The pumps are probably confined to the columnar epithelium at the dorsum of the EB. The EB may thus play a role in maintaining the ionic equilibrium between the hemolymph and the gut contents. Ultrastructurally, the EB becomes fully

differentiated late in pupation, and undergoes no major alteration during the remainder of the life-span.

TABLE OF CONTENTS

TABLE OF CONTENTS (Continued)

LIST OF TABLES

LIST OF ILLUSTRATIONS

LIST OF ILLUSTRATIONS (Continued)

C H A P T E R I

INTRODUCTION

The apple maggot fly Rhagoletis pomonella Walsh is a major pest of apples in the United States. Consequently, many aspects of its biology, including its nutritional physiology, have been intensively studied. Still, the esophageal bulb, a dorsal evagination of the esophagus, has for the most part resisted efforts directed at the elucidation of its function. In this study, experiments were performed to determine what role the esophageal bulb may play in the alimentary processes of adult R. pomonella. This information may have practical application in the control of the fly. An understanding of the function of the esophageal bulb may "open the door" to new chemical or biological control strategies based upon the disruption of that function.

Literature Review

An understanding of the life history and symbiont transmission cycle of R. pomonella is a prerequisite for a discussion of the possible functions of the esophageal bulb (EB). The life history of R. pomonella has been described in reviews by Christenson and Foote (1960), Bateman (1972), Dean and Chapman (1973), and

Boller and Prokopy (1976). Adults emerge from mid-June to September. Their mean length is sexually dimorphic, ranging from 3.9 mm (for males) to 5.2 mm (for females). Their principle food source seems to be honeydew, although this is supplemented with insect frass, bird droppings, and the exudate of injured fruit. When flies near sexual maturity (one to two weeks after emergence) they congregate on developing host fruit (usually apples), where they mate and oviposit. The mated female punctures the fruit skin with her ovipositor and injects eggs into the flesh. Eggs hatch in about three days. The larvae tunnel through the flesh, feeding and passing through three instars, a process which takes two to three weeks. They then emerge from the fruit and penetrate one to two inches into the soil, where the third instar cuticle hardens to form a puparium. Within this structure, the prepupal and pupal stages occur. The pupae enter winter diapause, though in some areas there is a second generation in late summer. Adult longevity is estimated to be three to six weeks.

Most of the well-known tephritids have a bacterial transmission cycle associated with their life histories (Hellmuth, 1956). The tephritid symbiont cycle which has been best defined, that of the olive fly Dacus oleae (Gmel.) was first described by Petri (1910). The bacterium

Pseudomonas savastanoi is associated with all life stages of this fly. In the adult, P. savastanoi colonizes the entire length of the gut lumen. The hindgut communicates with the vagina via a longitudinal slit; eggs passing down the vaginal canal are thus smeared with an innoculum of P. savastanoi. The eggs are deposited in the flesh of olive fruit. As the larvae burrow and feed, P. savastanoi colonize their galleries. Hagen has shown (1966) that the bacteria are essential to larval survival, because they possess proteases which the larval gut lacks. They also secrete threonine and methionine, two essential amino acids not present in olive flesh. The gut of the larva contains a large population of P. savastanoi, but most of it is lost when the gut is emptied in preparation for pupation. A small innoculum, however, accumulates in the lumen of a cephalic diverticulum of the gut which forms during pupal development: the EB. Here the bacteria reproduce, eventually spreading out to colonize the entire gut lumen of the adult fly.

There is evidence that a similar symbiote transmission cycle operates in R. pomonella. Allen et al. demonstrated (1934) that the apple-rot bacterium Phytomonas (now Pseudomonas) melophthora Allen and Riker is an obligate symbiote of R. pomonella. During oviposition, the egg can be smeared with gut bacteria because a common cloaca is

formed by the union of the hindgut and vagina (Dean, 1935). When bacterial rot is not present in the apple, the larvae die during the first instar. P. melophthora synthesizes arginine and methionine, two essential amino acids not present in apple flesh (Myazaki et al., 1968). The importance of the bacteria to the adult fly is unknown. While the EB has not been proven to be the only site of symbiote residence during pupal development, histological and cultural studies (Chapter II and Appendix) show that bacterial populations achieve high densities in the EB before they are present in significant quantities elsewhere in the gut. Dean and Chapman (1973) isolated a variety of bacteria from the guts of R. pomonella captured in New York State, but failed to demonstrate the presence of P. melophthora. Thus, the role of P. melophthora may be assumed by other bacteria in some populations of R. pomonella.

Since Petri's initial description of the EB of D. oleae (1910), several studies have been published concerning the EB in many tephritid species. None, however, attempt to find a reason for the frequent association of symbiotic bacteria with the EB, nor do they determine what functions the EB may perform. These publications do, however, provide an excellent survey of some aspects of the EB: its occurrence, and its morphology and histology

in the adult fly.

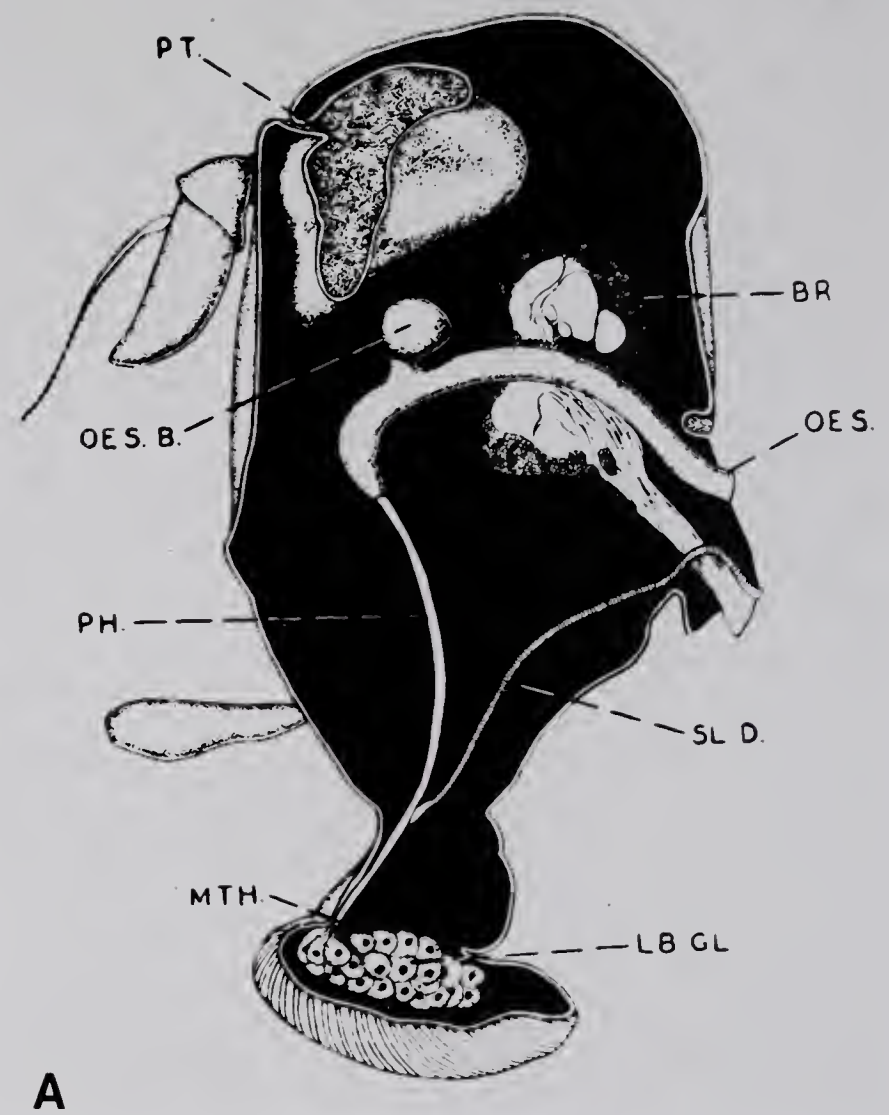
Approximately 1500 species of tephritids have been classified, and these are divided into three subfamilies: Trypetinae (which includes R. pomonella), Dacinae, and Tephritinae. The alimentary systems of 45 species have been described in some detail by Petri (1910); Stammer (1929; summarized by Buchner, 1953; and Schultz, 1970); Dean (1933); Schultz (1970); and Girolami (1973). All trypetines and dacines examined possess a cephalic organ which can be classified as an EB. Only some of the tephritines--Trypanea, Proomyxia, Sphenella, Euarestella, and Tephritis--lack an EB. The midguts of these species feature complex evaginations which may perform the mycetomal function of the EB, since they are filled with bacteria (Stammer, 1929). No esophageal modification resembling the EB has ever been found outside the Tephritidae. The dorsal esophageal diverticula of the culicids bear a superficial resemblance to the EB, but differ from it markedly in histology and ultrastructure (Clay and Venard, 1972).

A good description of the morphology and histology of the mature EB can be gleaned from the literature. The EB always appears as a simple evagination of the dorsal wall of the esophagus, immediately caudad to the pharynx, occypying a space between the ptilinal sac and the brain.

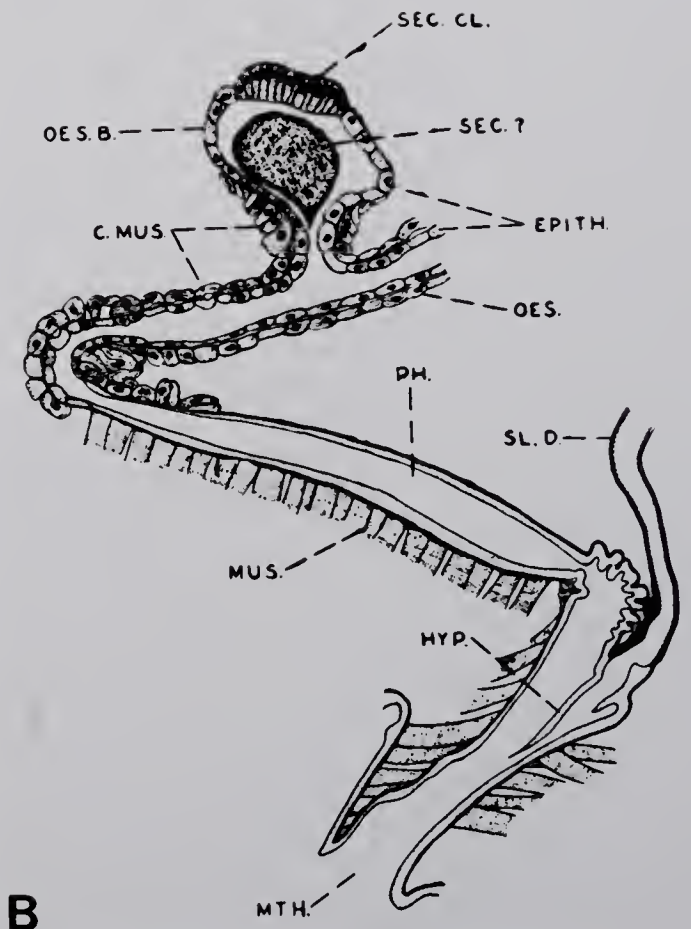
It consists of a spherical or ovoid sac attached to the esophagus by a thin stalk (Fig. 1). The only exception to this plan discovered so far is the EB of the tephritine Ensina sonchi L., which is broadly attached to the esophagus (Girolami, 1973). The maximum diameter of the EB ranges from 0.08 mm in Chaetorellis loricata Rondani (Girolami, 1973) to 0.25 mm in D. oleae (Petri, 1910). The length of the EB, including the stalk, can be as much as 0.45 mm in C. loricata or as little as 0.17 mm in E. sonchi (Girolami, 1973). The EB of R. pomonella falls in the middle of this range, having a maximum diameter of approximately 0.18 mm and a height of approximately 0.21 mm (Dean, 1933). No muscular attachments between the EB and the body wall have been reported for any of the tephritids.

The stalk and the lateral walls of the EB of R. pomonella are composed of simple cuboidal epithelium, strongly resembling the epithelium of the esophagus proper. At the distal end of the EB, however, cell height abruptly increases; a circular "cap" of tall columnar cells occupies the apex of the EB. The lumenal surface of the epithelium of the EB is lined with a cuticular intima continuous with that of the esophagus. The basal surface is covered with a thin basement membrane. The circular muscle which underlies the basement membrane of the esophagus also

Fig. 1. Reproduction of figures of Dean (1933). A. Diagrammatic saggital section through head of R. pomonella, showing location of esophageal bulb (OES.B). X26. B. Drawing of saggital histological section through esophageal bulb and pharynx (PH.). X85. C. MUS., circular muscle; EPITH., epithelium; HYP., hypopharynx; MTH., mouth; MUS., muscle; OES., esophagus; SEC. 7, possible secretory product; SEC. CL., possible secretory cells; SL.D., salivary duct.


PT.
BR
OES. B.
OES.
PH.
SL D.
MTH.
LB GL

A


SEC. CL.
OES. B.
SEC. ?
EPITH.
C. MUS
OES.
PH.
SL. D
MUS.
HYP.
MTH.

B

surrounds the stalk of the EB and extends approximately halfway up the lateral walls (Dean, 1933).

Histologically, the EB of other tephritids closely resembles that of R. pomonella. In two of the described species, however, the histological picture is considerably different. The columnar epithelium of the EB of D. oleae occurs not as an apical cap, but as a basal ring, immediately distal to the stalk (Petri, 1910). The epithelium of the EB of E. sonchi is composed only of cuboidal cells (Girolami, 1973).

A noncellular mass of unknown origin has been observed in the lumen of the EB of R. pomonella and many other tephritids (Dean, 1933; Schultz, 1970; Girolami, 1973). Although Dean describes this mass as "secretion", he allows that it may actually consist of gut contents which have been forced into the lumen of the EB.

Since the EB is not present in the tephritid larva (Dean, 1932; Knell and Stoffolano, 1973), it must form in the pupa. Unfortunately, no descriptions of the morphology or histology of the EB during pupal development have been published. Snodgrass (1924) did not include the EB in his otherwise thorough work on the metamorphosis of R. pomonella.

No histochemical study of the EB of any tephritid exists in the literature. There is no thorough description

of the ultrastructure of the epithelium of any EB. Segments of the EB of *D. oleae* appeared in electron micrographs published by Poinar *et al.* (1975), but the authors were primarily interested in describing the lumen-dwelling symbiotes.

Possible Functions of the Esophageal Bulb

In characterizing the physiological role of the EB of *R. pomonella*, a logical starting point is the compilation of all functions which the organ might be *likely* to perform. This strategy enables one to design experiments which will distinguish which of these functions, if any, are *actually* performed by the EB. From the information reviewed above, and from the results of preliminary experiments, it becomes evident that the possible functions of the EB fall into two categories: mycetomal and alimentary.

Mycetomal functions are those related to the maintenance and exploitation of symbiotic bacteria such as those found in the lumen of the EB. There is no rigorous proof that the EB is essential to the survival of a "starter culture" of the symbiotes of the newly-emerged adult *R. pomonella*. It is obvious, however, from histological preparations and Gram-stained smears of gut segments, that the microflora of the developing fly first

appear in observable quantities in the lumen of the EB (Ratner, unpublished results). Furthermore, although the bacteria are eventually found in all portions of the gut, they reach their highest densities by far in the EB (see Chapter II and Appendix). This concentration of bacteria in the lumen of the EB suggests a number of functions which might be performed by that organ. The pH of the lumen of the EB may be maintained at a value different from that of other gut regions, a value optimal for symbiote survival or reproduction. The epithelium of the EB may secrete nutrients or growth factors beneficial to the symbiotes; or it may absorb metabolites harmful to them. The reproducing bacteria may simply be impeded from leaving the EB by some physical mechanism.

Looking at mycetomal function from the host's point of view, the epithelium of the EB may provide a direct route for the absorption of symbiote-secreted nutrients. Poinar et al. (1975) speculated that the EB of D. oleae might perform this function.

Despite the narrowness of the muscular stalk, the lumen of the EB is not isolated from that of the rest of the foregut. Preliminary studies involving the feeding of vital dyes showed that ingested food normally enters the lumen of the EB. Food stored in the crop, when regurgitated up for digestion, can also gain entry to the EB

(Ratner, unpublished results). It is thus essential to consider the possibility that the EB performs an alimentary function; that is, that it plays a role in processing food ingested by the fly. It may be an absorptive organ, extracting organic or inorganic nutrients, or water, close to the site of intake. It may be an organ of digestion, secreting enzymes into the lumen to catalyze the breakdown of some dietary component. It may be a storage organ, sequestering nutrients or waste products in its epithelium. The EB does <u>not</u> appear to be a pumping organ analogous to the esophageal structures which accelerate food uptake in some Homoptera and Thysanoptera. Such structures are powered by muscles with firm anchorages to the body wall. The EB has no such musculature.

While the division of the possible fucntions of the EB into "mycetomal" and "alimentary" categories is useful in discussion, it is an artificial distinction, and overlaps are possible. For example, if the epithelium of the EB is absorptive, both bacterial products and ingested nutrients might be absorbed.

Rationale and Overview

Three experimental approaches were employed in the attempt to determine the function of the EB. The first step was a detailed histological and histochemical study

of the EB, not only in the mature fly, but throughout pupal and adult development. Histological and histochemical information can, of course, provide valuable clues about the function of an organ. The emphasis on development is important. Nothing is known about the EB of the pupa of R. pomonella or any other tephritid; its form and function may be different from that of the adult. A developmental study can also mark the milestones of the maturation of the EB: the times of its first appearance, its initiation of various activities, and its degeneration. Furthermore, if histological or histochemical changes in the EB correlate with a significant physiological event in the life of the fly--such as sexual maturation--the possibility of a relationship between the two processes is suggested, and can be pursued. The histological and histochemical studies are described in Chapter II. Flies at ages determined to be of interest in the above experiments were used in the second step of the investigation: an ultrastructural examination of the EB. The object was two-fold: to characterize the developmental changes which occur in the EB; and to find ultrastructural features which might connote specific functions. The ultrastructural study is described in Chapter III.

The results of the light and electron microscopic

studies made it clear that some of the proposed functions of the EB could be eliminated from consideration, while others merited further testing. These tests were performed on organs removed from adult flies and cultured *in vitro*. The results are reported in Chapter IV. The results of all three experimental approaches are summarized in Chapter V.

C H A P T E R I I

THE DEVELOPMENT OF THE ESOPHAGEAL BULB OF *RHAGOLETIS POMONELLA*. A MORPHOLOGICAL, HISTOLOGICAL, AND HISTOCHEMICAL STUDY

Introduction

Evidence summarized in Chapter I led to the hypothesis that the EB of *R. pomonella* executes one or more of a variety of alimentary and mycetomal functions. A study of the morphological, histological, and histochemical changes which take place during the pupal and adult development of the EB was carried out. The results are presented and discussed with respect to their bearing upon the question of the actual function of the EB.

Materials and Methods

Rearing conditions. *R. pomonella* were reared on Red Delicious apples for ca. 25 generations. The founders of this colony were of a strain reared for ca. 15 generations in the New York State Agricultural Experiment Station (Geneva). Mature larvae were allowed to pupate in moist sand. Puparia were stored at 25° ± 5°C under constant light until adult eclosion. In the stock colony, adults were maintained and exposed to apples in 35-cm

square cages of Neilson's design (1965). For developmental studies, groups of 12 adults were kept in 1-pt. cardboard ice cream containers with gauze lids; pupae were stored in Petri dishes filled with moist vermiculite. Both adults and pupae were stored in an environmental chamber at 25° ± 2°C, 50% R.H., with a 16:8 light:dark photoperiod regime. In most experiments, flies were provided with sucrose-yeast hydrolysate diet (Prokopy and Boller, 1970) coated on a filter paper strip, and a cotton wick moistened with distilled water. When gut secretions or microflora were to be histologically examined, only sucrose and water were provided. In some experiments, flies of the laboratory reared strain were compared to wild flies, which were collected as puparia from apples found in abandoned orchards in western Massachusetts.

Dissections. Flies were chilled and dissected under saline (Finlayson and Osborne, 1968). Some preparations were lightly stained with 0.04% methylene blue. The pH of various gut regions was determined by dissecting flies which had been fed sugar solutions containing one of the series of vital indicator dyes compiled by Conn (1953).

Histology and histochemistry. Unless otherwise noted, pupae were fixed under vacuum in Kahle's fluid, and adults in Carnoy's or Sinha's fluids. Tissues were dehydrated in

a graded ethanol series, cleared in t-butanol, and infiltrated and embedded in Paraplast M. Sections were cut at 8 μm and affixed to slides with gelatin adhesive (Haupt, 1930). For routine histological inspection, slides were stained with Delafield's hematoxylin and eosin Y (Humason, 1962), Nicholle's carbol-thionin (McManus and Mowray, 1960), Masson's trichrome (modified by Gurr, 1956), or the Feulgen reaction (Humason, 1962). The following histochemical tests were performed according to the procedures of Pearse (1968) unless otherwise noted; a. for carbohydrate: periodic acid-Schiff reaction (PAS); Pas after distase digestion, pyridine extraction, acetylation blockade, or acetylation-deacetylation; b. for protein: Millon's reaction (Baker, 1947); c. for lipid: Sudan black B (frozen, formalin-fixed tissue); d. for glycogen: Best's carmine; e. for glycosaminoglycans: toluidine blue; alcian blue 8GX at pH = 1.0 or 2.2; methylene blue extinction; f. for heavy metals: Timm's silver sulfide method; g. for insoluble phosphates and carbonates: Von Kossa's method; h. for uric acid: Argentaffin reaction (Humason, 1962).

Definition of developmental stages. The duration of pupal development of R. pomonella varies widely among individuals, even when temperature variation is held to

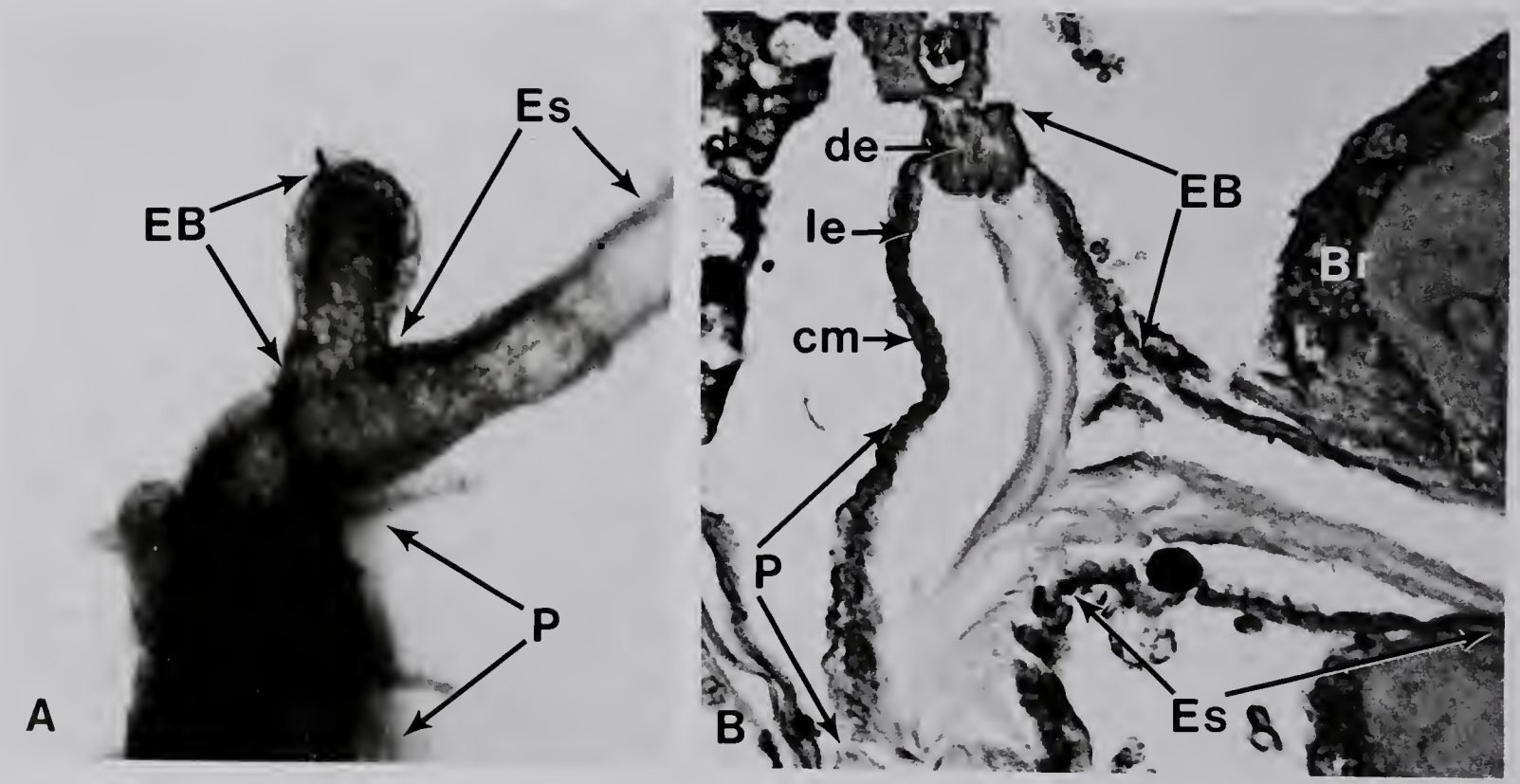
± 2°C (Ratner, unpublished results). The recording of developmental events in terms of days elapsed since pupation is thus of little value. Instead, arbitrary stages of pupal development were defined by the presence and color of pigment in the eyes and body wall, according to the criteria of Dean and Chapman (1973). Adult development is more synchronous (Ratner, unpublished results), and can be usefully defined in terms of days elapsed since emergence.

Results

Morphology and histology. During the transition between the late telomorphic and early chromoptic stages (13-20 days after pupation), the adult esophagus becomes recognizable, and the EB is present on its dorsum. At this stage it is a straight-sided, bud-like protrusion of the esophageal wall, 75-90 µm long and 60-70 µm in diameter (Fig. 2A). Histologically, the EB is at this time composed of two cell types: simple low cuboidal epithelium, ca. 3 µm high, comprising the lateral walls; and a disc of simple high columnar epithelium, 27-38 µm in diameter, which forms the dorsal "cap" (Fig. 2B). The cap contains an average of 220 (S.E. = 22.6) cells, each having a length of 22-31 µm and a diameter of only 1-2 µm. The cell nuclei are ellipsoid and basally located. The apical,

Fig. 2. Esophageal bulbs (EB) of pupae entering early chromoptic stage. A. Freshly dissected preparation (X280). B. Sagittal section. Hematoxylin and eosin (X253). Br, brain; cm, circular muscle; de, dorsal columnar epithelium of EB; Es, esophagus; le, lateral cuboidal epithelium of EB; P, pharynx.

2

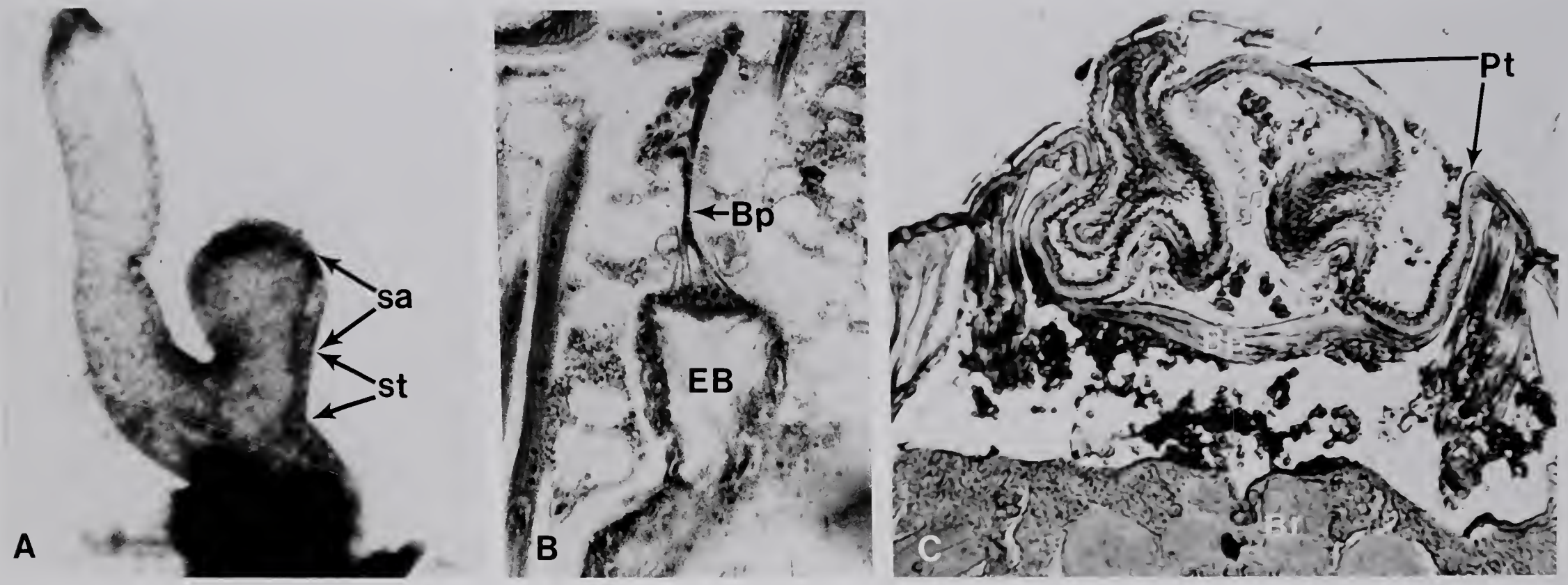


or lumen-facing side of the epithelium of the EB is covered with a cuticular intima which, being only ca. 0.5 μm thick, is only visible in some oblique sections. A basement membrane covers the basal surface, but is at this time too thin to resolve at the light microscopic level. It can be discerned in electron micrographs, however (Chapter III). The tunic of muscle which surrounds the esophagus extends dorsally to encircle the proximal 2/3 of the EB with a muscular layer ca. 3 μm thick.

By the beginning of the late chomoptic stage (15-22 days after pupation), the EB has not only increased in length (to 95-105 μm), but has also begun to differentiate into two regions: a tubular, proximal stalk, 50-60 μm in diameter; and a distal sac, 80-95 μm in diameter (Fig. 3A). The constriction of the proximal portion of the EB into a stalk coincides with an increase in the thickness of the circular muscle in this region. At the apex of the EB, another muscle has begun to form. Thin muscle fibers originate from several points on the basal surface of the apical cap and extend anterodorsally to coalesce into a single cylindrical muscle ca. 5 μm in diameter (Fig. 3B). This muscle continues anterodorsally and bifurcates, each branch inserting on the ental surface of a lateral fold of the ptilinum (Fig. 3C). The name

Fig. 3. Esophageal bulbs (EB) and bulbo-ptilinal muscles (Bp) of pupae in late chromoptic stage. A. Freshly dissected whole preparation. Note early differentiation into sac (sa) and stalk (st) (X225). B. Sagittal section, showing early development of bulbo-ptilinal muscle. Hematoxylin and eosin (X250). C. Cross section of pupal head, cut at level dorsal to EB, showing attachment of bulbo-ptilinal muscle to lateral walls of ptilinum. Masson's trichrome (X110). Br, brain; Bp, bulbo-ptilinal; Pt, ptilinum.

3

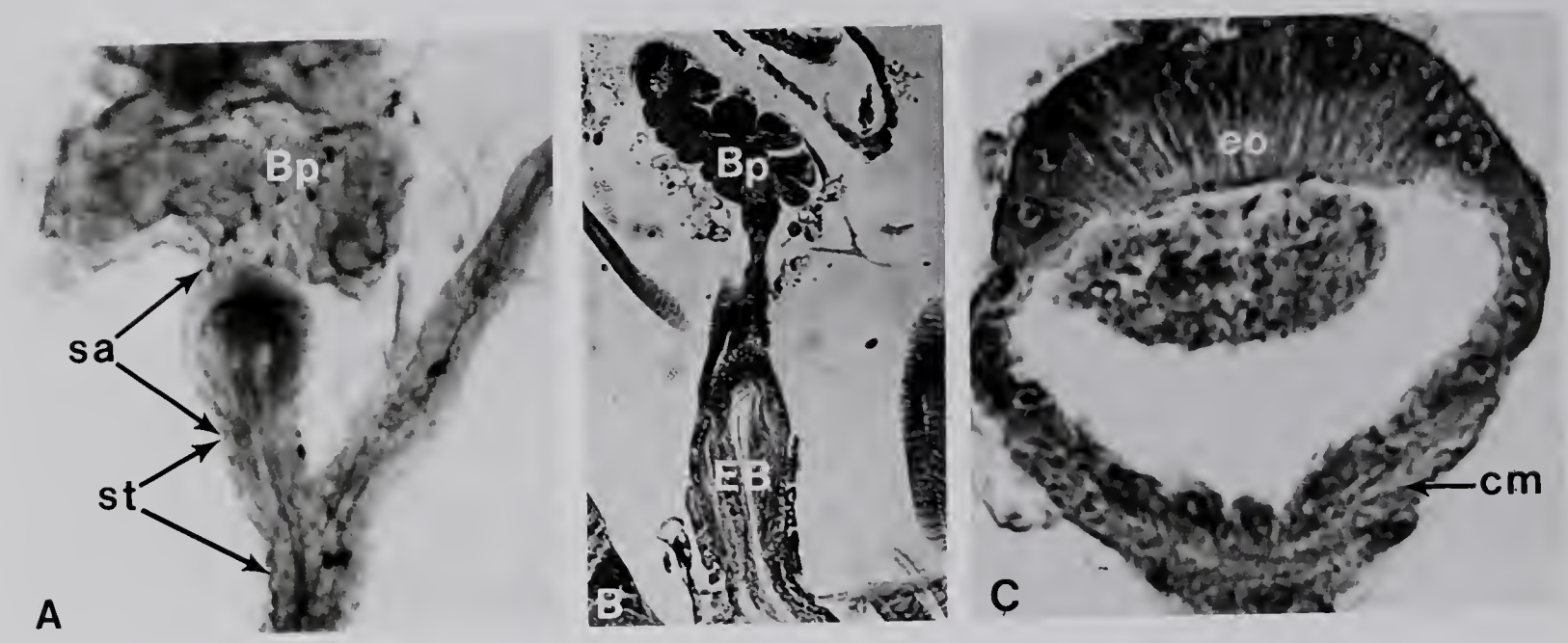


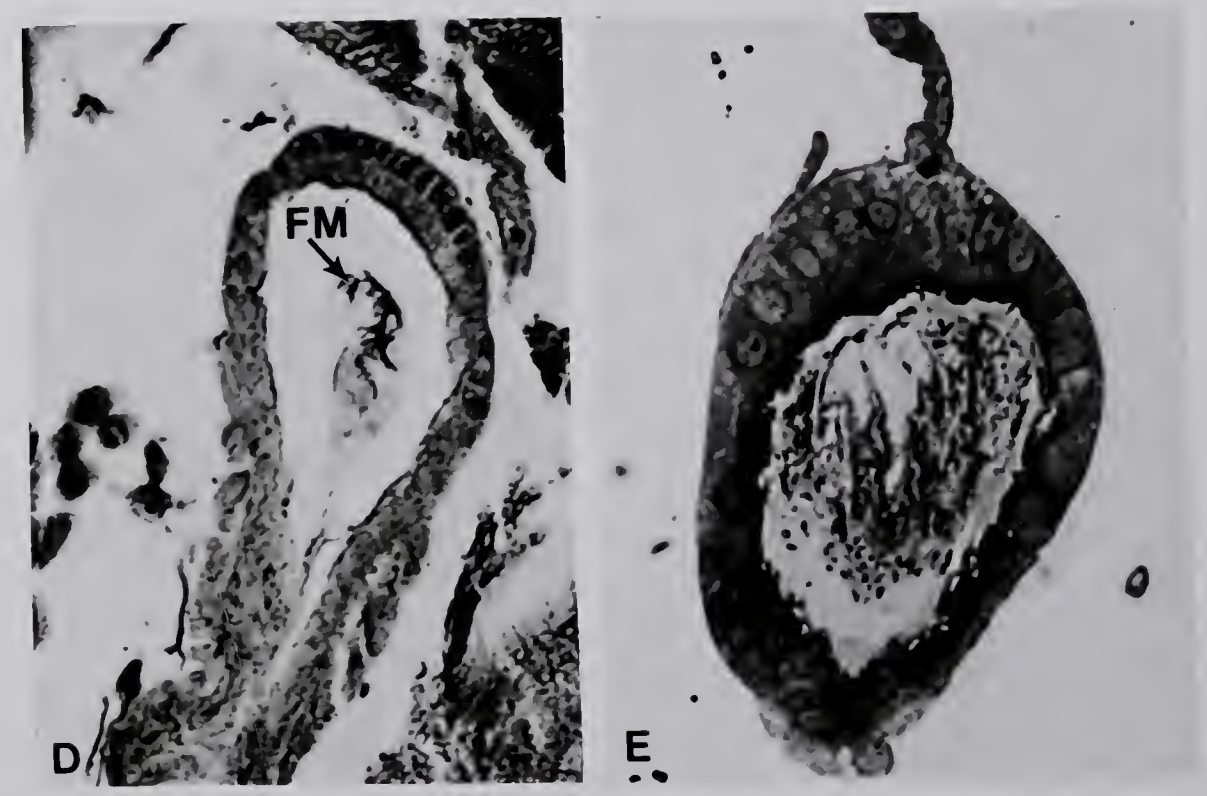
"bulbo-ptilinal muscle" was assigned to this previously undescribed structure. The cells of the dorsal cap have become lower and broader since the early chromoptic phase. They are now 14-20 μm long and 3-4 μm in diameter. The mean number of cells in the dorsal cap, however, has not significantly changed (X=216, S.E.=20.3). There has been little alteration in the epithelium of the lateral walls of the sac, or in the stalk.

As the late chromogenic stage begins (22-34 days after pupation, or 7-10 days before emergence), the diameter of the sac of the EB has increased to 105-120 μm (Fig. 4A). The muscular tunic surrounding the stalk has grown thicker (10-13 μm), and the opening of the stalk has become further constricted (10-30 μm in diameter). The bulbo-ptilinal muscle has also thickened (Fig. 4A). When pupae at this stage are fixed for histology with the ptilinum everted, the EB in the resulting sections has an elongated, tubular profile (Fig. 4B). The action of the bulbo-ptilinal muscle upon the EB is thus apparent. During emergence, the pulsations of the ptilinum bring rhythmic tension to bear on the muscle, alternately stretching it and allowing it to contract. An anterodorsally-directed force is exerted intermittantly upon the sac of the EB, first compressing, then releasing it, much as fingers pump the bulb of an eye dropper. This action

Fig. 4. Esophageal bulbs (EB) of pupae in late stage. A. Freshly dissected whole preparation, showing differentiation into sac (sa) and stalk (st). Note extensive development of bulbo-ptilinal muscle (Bp). (X136). B. Sagittal section of EB under tension from bulbo-ptilinal muscle. Hematoxylin and eosin (X119). C. Sagittal section, slightly oblique, showing eosinophilic region (eo) of dorsal columnar cells. Hematoxylin and eosin (X820). D. Sagittal section, showing small accumulation of fibrous material (FM) in lumen. Masson's trichrome (X330). E. Sagittal section, showing bacteria (ovoid, dark-staining bodies) in lumen. Carbolthionin (X304). cm, circular muscle.

4





would tend to intermix the contents of the EB and the esophagus.

The cells of the dorsal cap have not increased significantly in number since the late chromoptic stage ($\bar{X}$=223, S.E.=16.3), but they have become higher (17-23 µm) and broader (4-5 µm) (Fig. 4C). While the cytoplasm of these cells had previously been weakly and uniformly basophilic, there is now an apical zone of increased eosinophilia (Fig. 4C). The cell length of the cuboidal epithelium of the lateral walls of the sac has increased to ca. 8 µm. A gradation of cell length has appeared in the stalk. The length of the most distal cells has increased to ca. 5 µm, while that of the most proximal cells remains at ca. 3 µm.

During earlier pupal development, the EB, like the rest of the gut, contained a wispy, faintly eosinophilic material. During the late chromogenic stage, however, a fibrous, basophilic mass begins to form in the lumen of the EB. This material, which stains deep green with Masson's trichrome (Fig. 4D), is probably the "secretion" noted in the EB of the adult fly by Dean (1933). When dissected out, it proves tough and resilient. It is also during the late chromogenic phase that bacteria can first be histologically observed in the lumen (Fig. 4E). This is the only region of the gut seen to contain bacteria

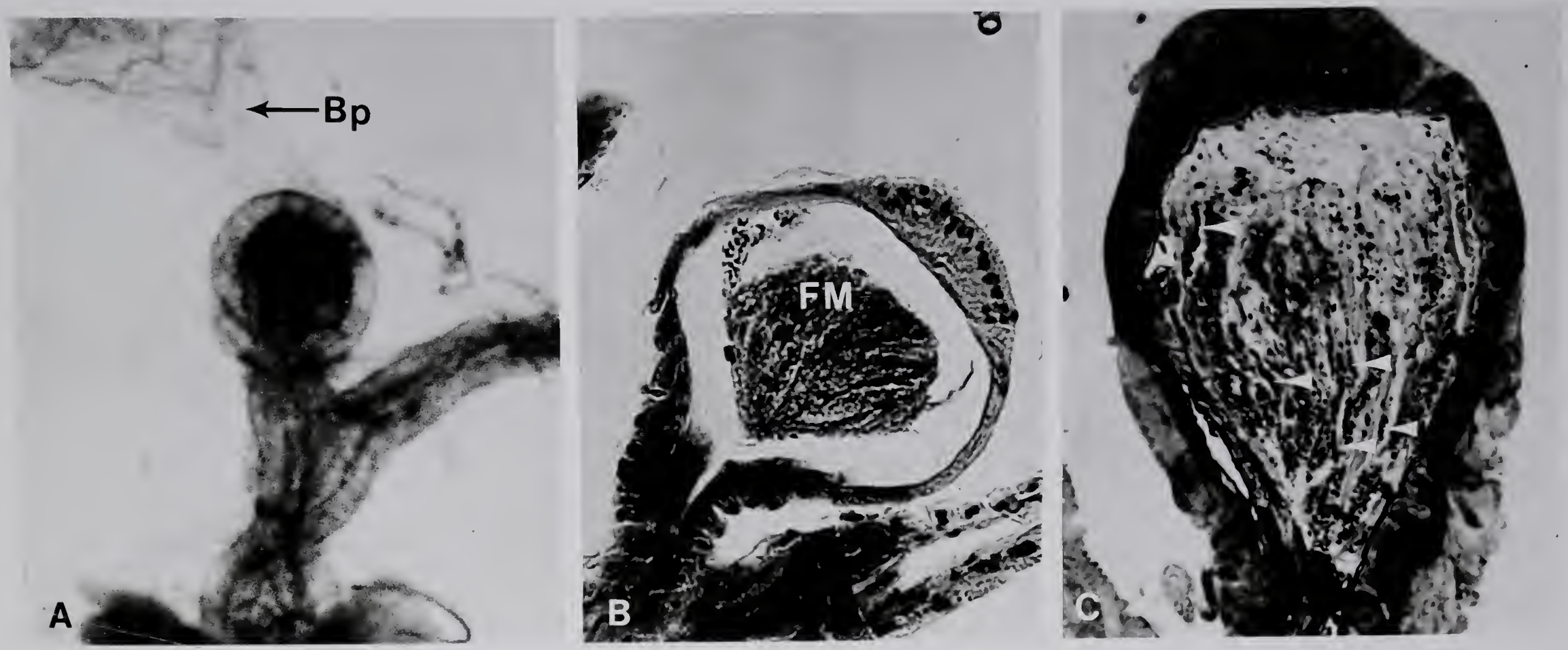
during pupal development.

The late chromogenic stage ends with adult emergence. Throughout the entire life span of the adult (up to 58 days in these experiments), few additional changes are noted in the morphology and histology of the EB. The bulbo-ptilinal muscle degenerates during the first five days after emergence (Figs. 5A and 5B). Basophilic material continues to accumulate in the lumen; although it becomes partially obscured by food particles, its fibrous nature remains clearly distinguishable (Fig. 5B). The bacterial population of the lumen becomes extremely dense by the second week of adult life (Fig. 5C), although the rate and extent of increase cannot be determined from sectioned material. Often, the bacteria, trapped between adjacent strands of fibrous material, grow in parallel, linear arrays.

In other respects, the EB of adult R. pomonella of any age differ little from that of the pupa in late chromogenic stage. There is no change in the length of the EB, or in the number or size of the cells of the dorsal cap. A slight increase in the maximum diameter is occasionally noted (up to 150 μm). There is no histological evidence of absorption or vacuolar secretion, whether the flies are starved or fed. There are no signs of degeneration at any age.

Fig. 5. Esophageal bulbs of adult flies of various ages. A. Freshly dissected EB of 5-day old female, showing degeneration of bulbo-ptilinal muscle (Bp) (X165). B. Sagittal section of EB of two-week old male fly, showing accumulation of fibrous material (FM) in lumen. Masson's trichrome (X357). C. Sagittal section of EB of two-week old female fly, showing dense bacterial population in lumen. Arrowheads indicate bacteria in linear arrays between parallel strands of fibrous material. Carbol-thionin (X632).

5



The preceding description holds true for females of both the laboratory and wild strains of R. pomonella. The development of the EB in the male fly follows the same course, but its dimensions at each stage are 25-35% smaller. This is not surprising, since male flies have a length and wingspan ca. 25% smaller than those of females (Dean and Chapman, 1973).

Histochemistry. Histochemical characterizations were made of the EB of pupae at the early and late chromoptic and late chromogenic stages; and of adults at 2, 14, and 50-55 days after emergence. In all stages, the epithelium of all regions of the EB exhibits weak, diffuse staining in tests for protein and carbohydrate (mainly glycogen). Lipid droplets are only occasionally seen. There are no concretions of heavy metals, phosphates, carbonates, or uric acid. No mitotic figures are seen at any of the stages examined.

The histochemical reactions of the intima and the fibrous material in the lumen are summarized in Table 1. Both structures react identically in these tests, an indication that they are similar, if not identical, in chemical nature. The component responsible for their shared histochemical properties is probably chitin. Although chitin theoretically should be unreactive in the PAS tests (it contains no 1,2 glycol or amino groups), in

Table 1. Histochemical reactions of the fibrous material and intima of the EB of R. pomonella.

Method	Reactions* Fibrous material	Intima	Interpretation
Millon reaction	+	+	protein
Sudan black B	0	0	lipids absent
Schiff's reagent	0	0	free aldehydes absent
PAS	++	++	1:2 glycol or amino-hydroxyl groups
PAS after acetylation	0	0	
PAS after acetylation and saponification	++	++	1:2 glycol groups
PAS after pyridine extraction	++	++	glycol groups on carbohydrate
PAS after malt distase	++	++	glycogen absent
toluidine blue	+++ (metachromatic, ethanol-labile)	+++	acid glycosaminoglycans, possibly sulfated
alcian blue			
pH 2.2	+	+	acid glycosaminoglycans, probably non-sulfated
pH 1.0	0	0	
methylene blue			
pH 5.5	++	++	
pH 5.0	+	+	acid glycosaminoglycans, probably non-sulfated
pH 4.5	0	0	

* 0 = no reaction; + = weak reaction; ++ = moderate reaction; +++ = strong reaction

actual practice it has been shown to react positively (Runham, 1961; Sundman and King, 1964). Runham (1961) proposed a mechanism to account for this anomalous result. Chitin also stains with alcian blue, possibly because of chelation of the dye's copper ions between adjacent polysaccharide chains (Runham, 1961).

The only other class of compounds which might account for the histochemical identity between the intima and the fibrous material is the acid glycosaminoglycans. The main evidence for the presence of these compounds, metachromatic staining with toluidine blue, is ambiguous, because the metachromasia is eliminated by ethanol post-treatment. The significance of alcohol-labile metachromasia is in some doubt (for discussion, see Pearse, 1968). If acid glycosaminoglycans are present, they must bear only weakly acidic groups, such as carboxyls. The failure of staining with methylene blue at pH 4.5-5.0, and the lack of alcianophilia at pH 1.0 preclude the presence of strong acid groups, such as sulfates.

pH. The pH of the lumen of the EB was determined by indicator dyes to be 6.8-7.4, regardless of the age or sex of the adult fly. The other regions of the foregut fell within the same range. The anterior midgut was slightly more alkaline (pH 7.2-7.8). Protein deprivation had no effect on pH.

Discussion

The EB develops as an integral part of the esophagus; both appear 2-3 weeks after pupation. By the time the pupa reaches the late chromoptic stage, 9-14 days later, the EB has taken on all the morphological, histological, and histochemical properties which it will display in adult life. (An additional feature, the bulbo-ptilinal muscle, is lost soon after emergence.) The transition between these two stages involves only the enlargement of epithelial cells and the development of musculature around the stalk. On the basis of structure and histochemistry, then, there is no evidence that the EB is specialized to perform different functions in the pupa and the adult fly. Throughout adult life, the epithelial cells of the EB show no significant change in size, number, vacuolation, or integrity correlated with development or feeding. There is no indication that the EB undergoes cycles of protein or lipid secretion; there is only the slow, steady build-up of fibrous material in the lumen. The EB does not appear to be a site of nutrient or waste storage.

The presence of a bulbo-ptilinal muscle has never been reported in Tephritidae, probably because it degenerates early in adult life. Similar temporary muscular connections between the foregut and ptilinum have been

noted in Drosophila and Calliphora (Miller, 1965), where they evidently act as ptilinal retractors. Perhaps this is the primary function of the bulbo-ptilinal muscle as well. The forces exerted upon the EB through the bulbo-ptilinal muscle during emergence would also tend to force some bacteria from the sac of the EB into the esophagus, an action which might be important in the establishment of gut flora in the adult fly.

The high density of bacteria in the lumen of the EB does not appear to be attributable to a favorable local pH. The pH of the esophagus differs from that of the EB by no more than 0.6 units. While it is possible that the epithelium of the EB secretes nutrients, or absorbs bacterial wastes, there is no evidence of such activities at the light microscopic level. The explanation for the bacterial concentrations in the EB may lie, at least in part, in the fibrous material which accumulates there. This material is histochemically identical to the intima which lines the EB, and its fibers are not randomly oriented, but lie parallel to the intima's surface (Chapter III). These findings strongly support Girolami's suggestion (1973) that the material is derived from the intima, possibly by a process of delamination. Being composed of chitin, and possibly acid glycosaminoglycan, the material would not be of much use to the lumen bacteria. It might,

however, be a physical factor favoring their presence in the EB. Bacteria enmeshed in--or perhaps attached to--the chitinous fibers would be resistant to the flushing action of food movement. Motility-restricting interactions between symbiotic bacteria and the chitin or keratin linings of the gut wall have been reported in the roach Blaberus posticus Erichson (Fogelsong et al., 1975), and in some mammals (Savage and Blumershine, 1974; McCowan et al., 1978). The fibrous material might cause net concentration of bacteria in the EB by holding the rate of bacterial outflow to a level lower than that of bacterial division. The EB, then, might act as a "feeder organ", containing a population of actively dividing bacteria, only a portion of which is released into the main body of the gut. The value of such an organ becomes obvious when one considers the symbiont transmission cycle of R. pomonella. Only the larvae can ingest the symbiotes, because they are pathogens of apple flesh. The symbiotes which survive through the pupal stage must be maintained in the gut of the adult fly, which cannot obtain a fresh supply. Yet the adult constantly loses symbiotes through defecation, and through the fecal innoculation of the apples into which it oviposits. A meal traverses the gut in 1-2 hr (Dean and Chapman, 1973), so bacterial reproduction in the gut

proper must be minimal. An organ which gradually releases symbiotes into the gut would seem to be a necessity. Since R. pomonella lacks other centers of bacterial proliferation (eg. rectal crypts), the EB is a likely candidate for this function. Unfortunately, because of the small size and inaccessibility of the EB, a direct experimental test is not presently feasible.

The entire epithelium of the EB is capable of producing intima and fibrous material. The cells of the dorsal cap, with their increased volume and apical eosinophilic region, appear to be specialized for some additional function. They are prime targets for an ultrastructural investigation.

C H A P T E R I I I

THE DEVELOPMENT OF THE ESOPHAGEAL BULB OF FEMALE *RHAGOLETIS POMONELLA*. AN ULTRASTRUCTURAL STUDY.

Introduction

The object of this study was to determine whether the subcellular organization of the EB yields clues to its function; and whether that organization changes in response to the physiological demands of eclosion, feeding, and sexual maturation.

Materials and Methods

The techniques of rearing *R. pomonella* were described in Chapter II, as were the criteria used in defining the stages of pupal development. Wild flies, which were compared to individuals of the laboratory strain in some experiments, were obtained as pupae from abandoned orchards in Western Massachusetts. For developmental studies, the EB was obtained from pupae in the early and late chromoptic, and early and late chromogenic stages; and from adults aged 1, 7, 14, 21, 30, and 50-60 days post-eclosion. Only females were used in this study. For 6-12 hr prior to excision of the EB, the flies were held at 25° $\pm$ 2° C, 90-100% R.H., but were allowed no

food or water. In experiments designed to detect short-term changes in the EB in response to meals of carbohydrate, amino acids, lipids, or inorganic salts, groups of 6-9, 2-week old females were allowed only water for two days, then fed to satiation on one of the following: powdered glucose; a 4:1 mixture of powdered glucose and yeast hydrolysate (ICN, Cleveland, Ohio); a 200:1 mixture of powdered glucose and KCl; or a 1:8 mixture of glycerol and olive oil. The EB was excised at 0.12, 0.25, 0.5, 1.0, 2.0, 4.0, 24.0, and 72.0 hr after feeding (one group at each time). A control group was allowed only water. In studies of fluid transport by the EB, groups of 6-9 flies were allowed neither food nor water for 12-18 hr, then fed to satiation on 0.1%, 5.0%, or 15% glucose in 3 mM KCl; or were left unfed as controls. The three glucose solutions are, respectively, hypotonic, approximately isotonic, and hypertonic to the hemolymph of most terrestrial insects (Stobbart and Shaw, 1964). The EB was excised at 0.12, 0.5, 0.75, 1.0, 1.25, and 1.5 hr after feeding. Excision of the EB was always performed in ice-cold primary fixative (2.5% glutaraldehyde buffered at pH 7.2 with 0.05 M sodium cacodylate). The tissue was placed in fresh fixative for 3 hours, then washed for approximately 18 hours in ice-cold buffer. A 1.5-hour secondary fixation was carried out in 1.0% OsO_4

in the same buffer as the primary fixative. Both fixatives, and the intermediate wash, contained glucose at a final concentration of 0.5 M. The tissue was dehydrated in a graded ethanol series and embedded in Epon 812, with propylene oxide as the transitional solvent. Silver sections were cut on a Sorvall Porter-Blum MT2B ultra-microtome and mounted on Parlodion-coated grids. Sections were stained for 20 minutes with 5% uranyl acetate in 50% methanol, followed by 5 minutes in Reynold's (1963) lead citrate diluted 5X with 0.02 N NaOH. Electron micrographs were taken on a Zeiss EM 9 S-2 or an RCA EMU-4 microscope, operated at 60 and 75 kV, respectively. For light microscopy, 1-µm thick sections were stained with 0.5% toluidine blue in 1% sodium tetraborate.

Results

As described by Dean (1933), the EB of adult R. pomonella consists of a proximal tubular stalk and a distal ellipsoid sac ("st" and "sa", respectively, in Fig. 6). The stalk and the lateral walls of the sac are composed of cuboidal epithelium. The dorsal wall of the sac is a concave disc of columnar epithelium (Fig. 7). The lumen of the EB is lined with a cuticular intima continuous with that of the esophagus.

The ultrastructure of the dorsal columnar epithelium

Fig. 6. Light micrograph of longitudinal section of EB of adult R. pomonella. Esophagus (Es) is seen in cross section. Dotted line denotes transition between sac (sa) and stalk (st) of EB. cm, circular muscle; de, dorsal columnar epithelium; l, lumen of EB; le, lateral cuboidal epithelium; Ne, nerve tract. Semi-thin (1 µm) section stained with toluidine blue (X340).

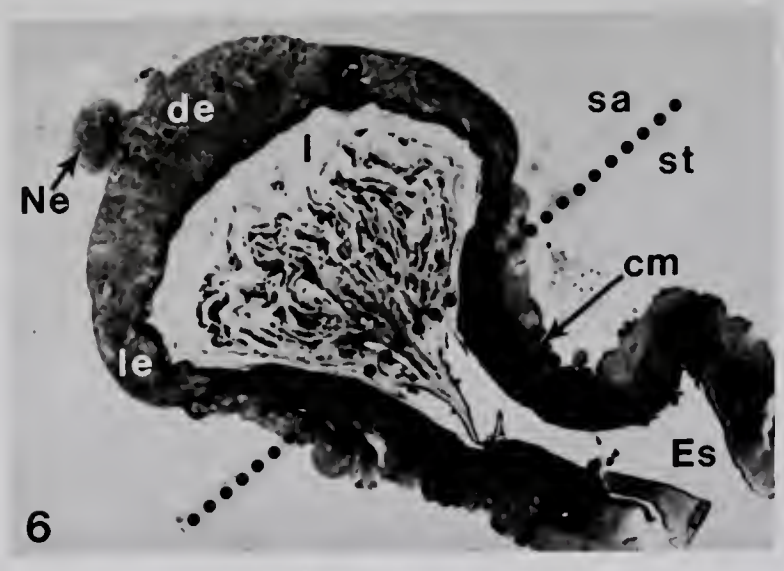
Fig. 7. Longitudinal section of dorsal columnar epithelium of EB of pupa in late chromoptic stage. FB, fat body; H, hemocoel; i, intima; l, lumen of EB; n, nucleus of dorsal columnar cell. Light micrograph of semi-thin section stained with toluidine blue (X780).

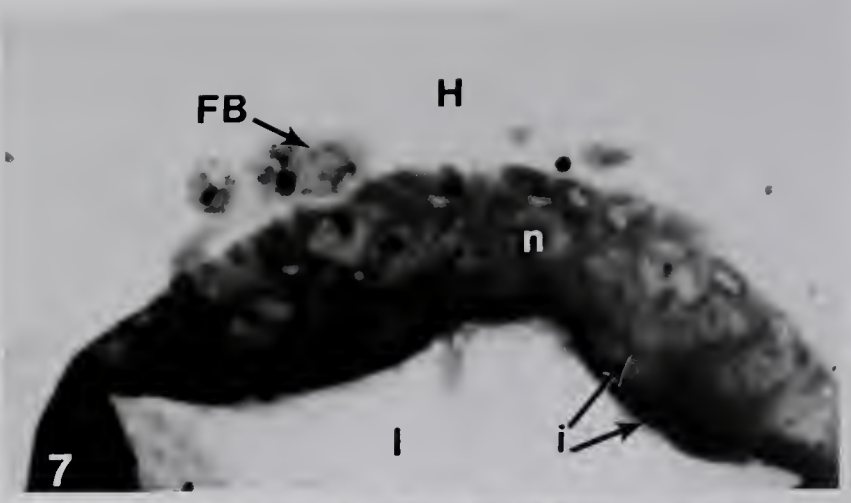
Fig. 8. Longitudinal section of basal region of two adjacent predifferentiated dorsal columnar cells (X23,750), bm, basement membrane; ib, invaginated basal plasma membrane; n, nucleus.

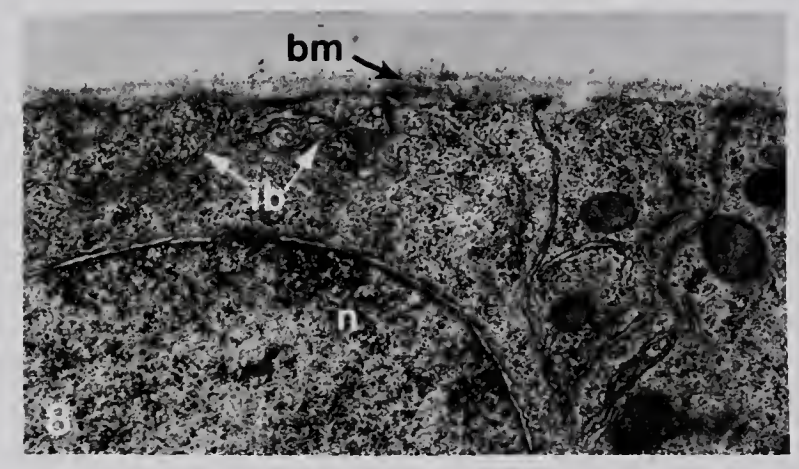
Fig. 9. Longitudinal section of apical region of predifferentiated dorsal columnar cell (X22,800). cv, coated vesicle; i, intima; ia invaginated apical plasma membrane; lm, lateral plasma membrane.

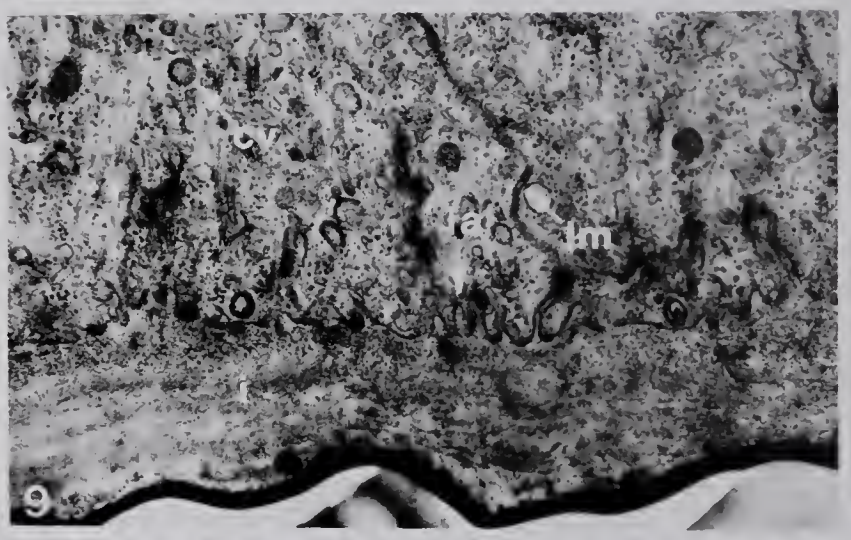
Fig. 10. Higher magnification of apical region depicted in Fig. 9. Note fusion of one of the coated vesicles (asterisk) with plasma membrane (X38,000). cv, coated vesicle; i. intima; lm, lateral plasma membrane.

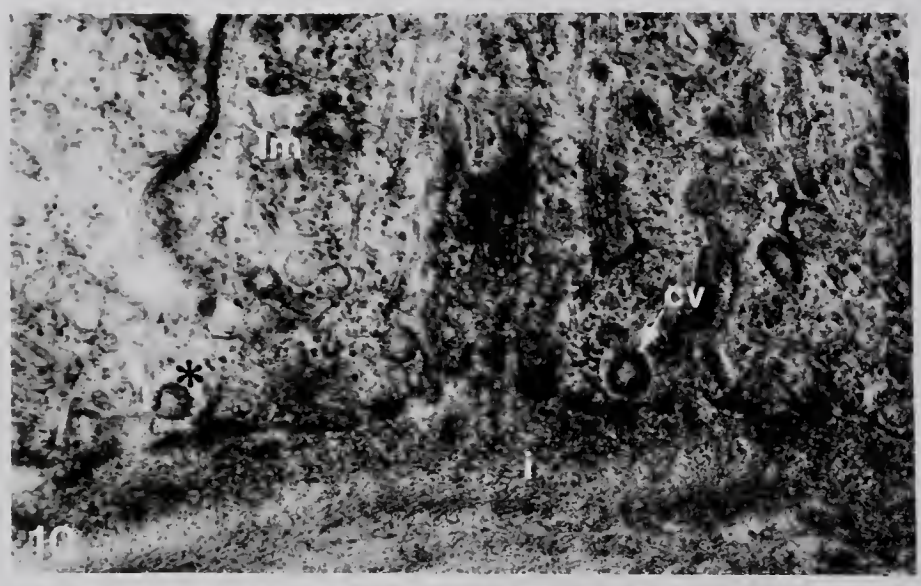
Fig. 11. Longitudinal section of perinuclear regions of two adjacent dorsal columnar cells. Note transition point between basal zone of open intercellular space (is) and apical zone of septate junction (sj) (X30,875). g, Golgi region; m, mitochondrion; mt, microtubules; n, nucleus; rer, rough endoplasmic reticulum.

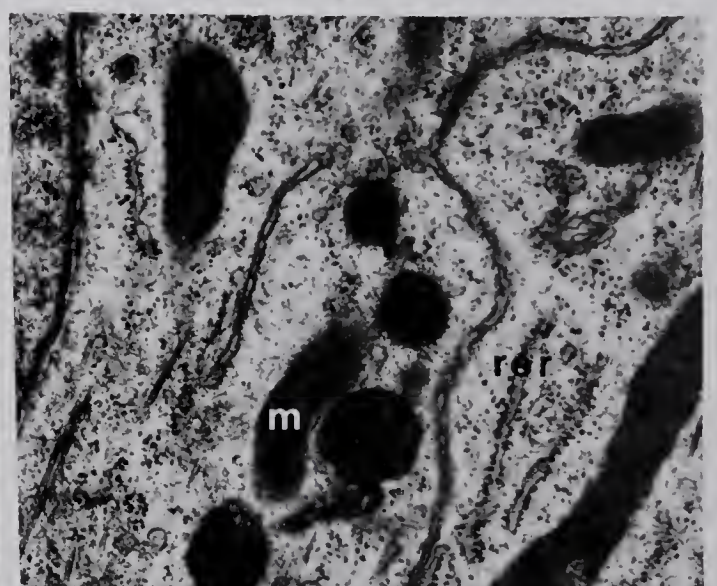

de
sa
st
l
Ne
cm
le
Es
6

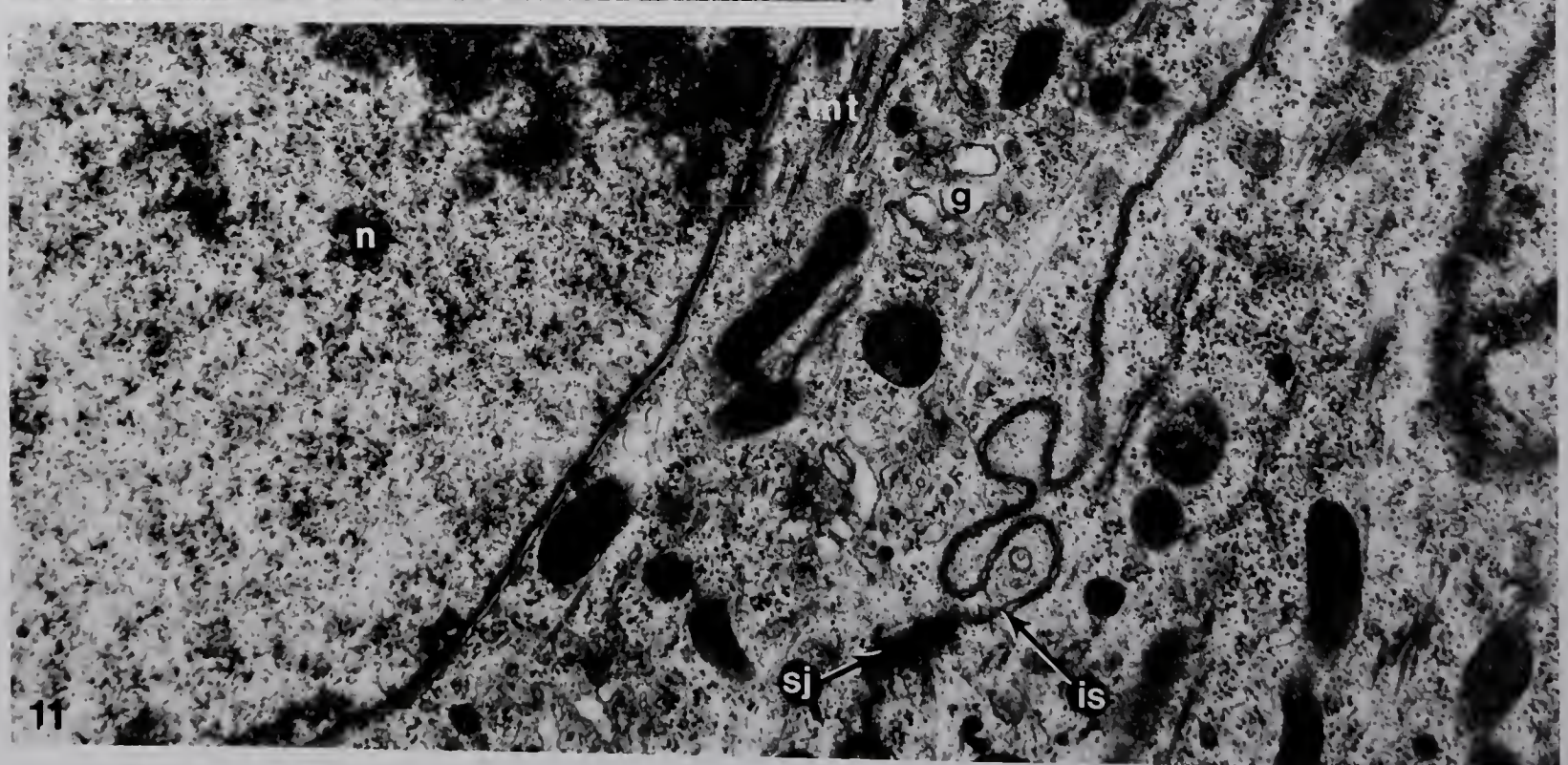

FB
H
n
l
i
7


bm
lb
n
8


m
9


m
cv
*
i
10


rer
m


mt
g
n
sj
is
11

will be considered in detail in this report, since it undergoes significant modification during development. The epithelia of the stalk and lateral walls will be treated briefly, since they remain ultrastructurally unspecialized throughout the life of the fly.

Dorsal columnar epithelium. The development of the dorsal columnar epithelium can be divided into three phases. As the pupa of R. pomonella enters the early chromoptic stage of development (13-20 days post-pupation, under laboratory conditions), the EB becomes recognizable as an evagination of the newly-formed esophagus. From this point until the commencement of the early chromogenic phase (7-11 days later) the dorsal columnar epithelium passes through the first, or pre-differentiated, phase of development. Early in this phase, the roughly hexagonal columnar cells which comprise the epithelium are extremely long and thin, having a length of 22-31 μm and a diameter of only 1-2 μm. Toward the end of the phase, the cells become lower and broader (14-20 μm in length, 3-4 μm in diameter) (Chapter II). Despite the alteration in cellular shape, the major ultrastructural features of the epithelium remain unchanged over the course of the predifferentiated phase. The basal, or hemocoel-facing side of the epithelium is

underlain with a basement membrane approximately 0.1 μm thick. The basal plasma membranes are closely applied to the basement membrane, but occasionally invaginate to form convoluted channels which extend no more than 0.5 μm into the cells (Fig. 8). The apical surface of the epithelium is covered with a cuticular intima approximately 0.75 μm thick. No pores or ducts penetrate this intima. The apical plasma membrane is thrown into small infoldings not more than 0.2 μm long. Spherical vesicles approximately 0.1 μm in diameter are plentiful in the apical region (Figs. 9 and 10, "cv"). These vesicles, which often possess a fuzzy coat, are sometimes observed to fuse with the apical plasma membrane (Fig. 10). Abundant mitochondria are evenly distributed throughout the cytoplasm. Many of the mitochondria are elongate, with the long axis parallel to that of the cells (Fig. 11). A system of microtubules is oriented along the same axis (Fig. 11). Rough endoplasmic reticulum (rer) and Golgi regions are found mainly in the perinuclear area. The rer is sparsely distributed, appearing in sections as widely separated tubules, rather than laminar arrays of cisternae (Figs. 8 and 11).

The boundary between adjacent columnar cells is organized into two structurally distinct regions. From the basement membrane to a point approximately 2/3 of

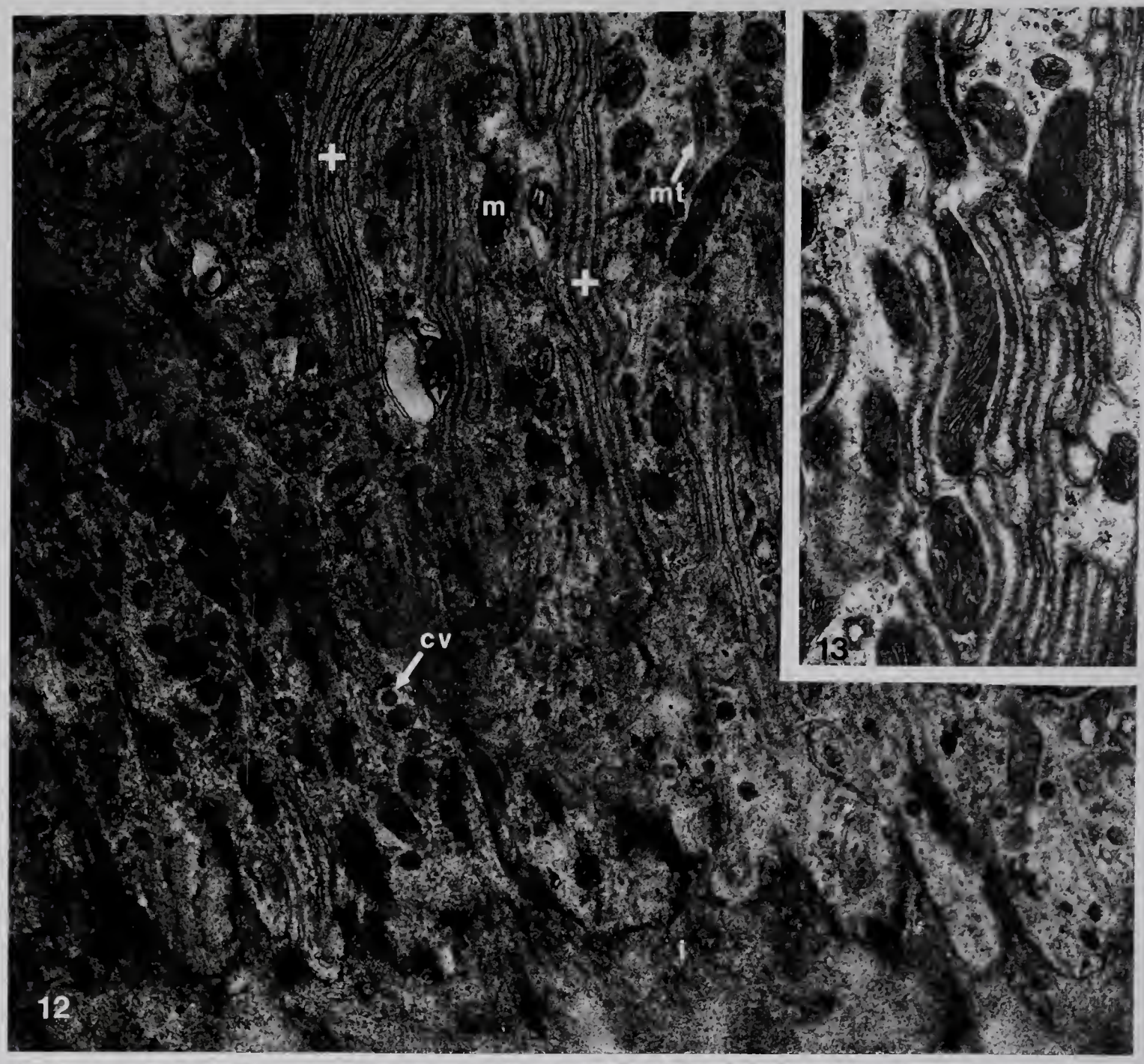
the way along its length, the lateral plasma membranes are separated by an electron-lucent space 10-30 nm wide (Fig. 11). The apical 1/3 of the intercellular boundary is a region of septate junctions approximately 30 nm wide, interrupted by short segments of clear intercellular space (Figs. 9 and 11).

The predifferentiated phase lasts 7-11 days, ending during the latter part of the late chromoptic or the beginning of the early chromogenic stage (i.e. 22-26 days post-pupation). At this point, the columnar epithelium undergoes a rapid transition to the second, or differentiated phase. This phase is marked by an increase in cell length (to 17-23 μm) and diameter (to 4-5 μm). The most striking change, however, occurs in the apical region of the cells. Sheets of plasma membrane have become invaginated and repeatedly folded to form tightly packed leaflets which extend up to 6 μm into the cell (Fig. 12). Each leaflet is densely surrounded by mitochondria, many of which are less than 75 nm from the inner surface of the infolded membrane (Figs. 12 and 13). In cross section, it can be seen that the sheets of membrane within a leaflet are not mutually parallel, but undulate, like pleated drapery. At many points, the membrane pleats fold back upon themselves, isolating cylindrical segments of cytoplasm (Fig. 14). The region of invaginated plasma

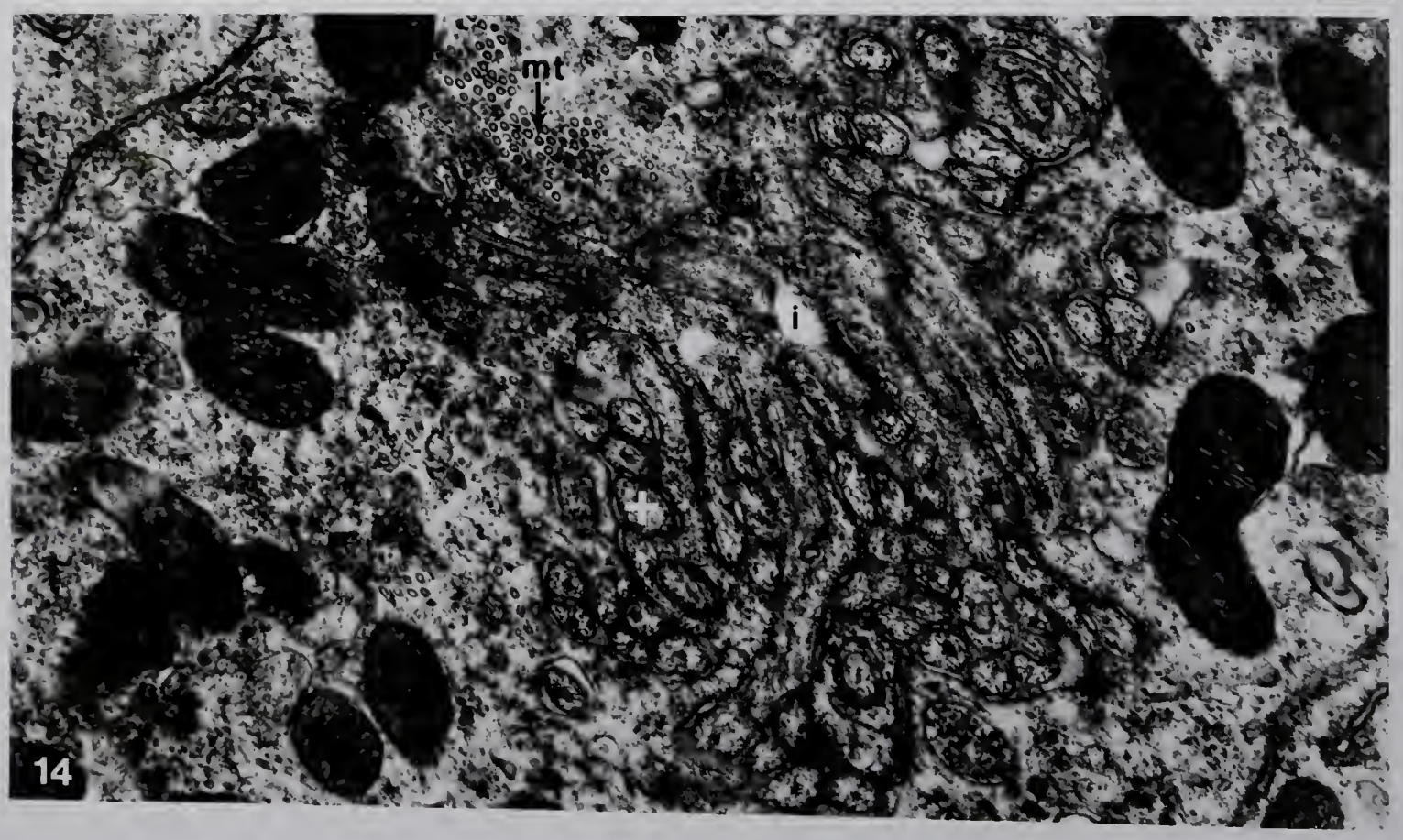
Fig. 12. Apical region of differentiated dorsal columnar cell, seen in longitudinal section. Note leaflets of invaginated apical plasma membrane (White crosses) (X29,575). cv, coated vesicle; i, intima; m, mitochondrion; mt, microtubules.

Fig. 13. Leaflets of invaginated plasma membrane are often closely associated with mitochondria (X34,200).

Fig. 14. Invaginated apical plasma membrane of dorsal columnar cell, seen in cross section. Pleated configuration of membrane sheets can be discerned. Segments of cytoplasm can be completely enfolded by the pleats (e.g. at white cross) (X41,800). i, intima; mt, microtubules.


m
mt
v
cv
12

13


mt
i
14

membrane occupies the apical 1/3 of each columnar cell, and corresponds to the eosinophilic region observed in light microscopic preparations (Chapter II).

Pronounced changes have also occurred at the boundary between adjacent columnar cells. As in the predifferentiated phase, there is a basal region of open intercellular space and, apical to this, a region of septate junctions. In the differentiated phase, a third region has appeared, apical to the septate junctions. Here, the plasma membranes of adjacent cells are extensively interdigitated, but always separated by an intercellular space of 15-32 nm. The interdigitations are closely associated with mitochondria (Fig. 15). The interdigitated region comprises the most apical portion of the intercellular boundary, extending for a distance of 6-8 um. It is thus coextensive with the leaflets of invaginated apical plasma membrane.

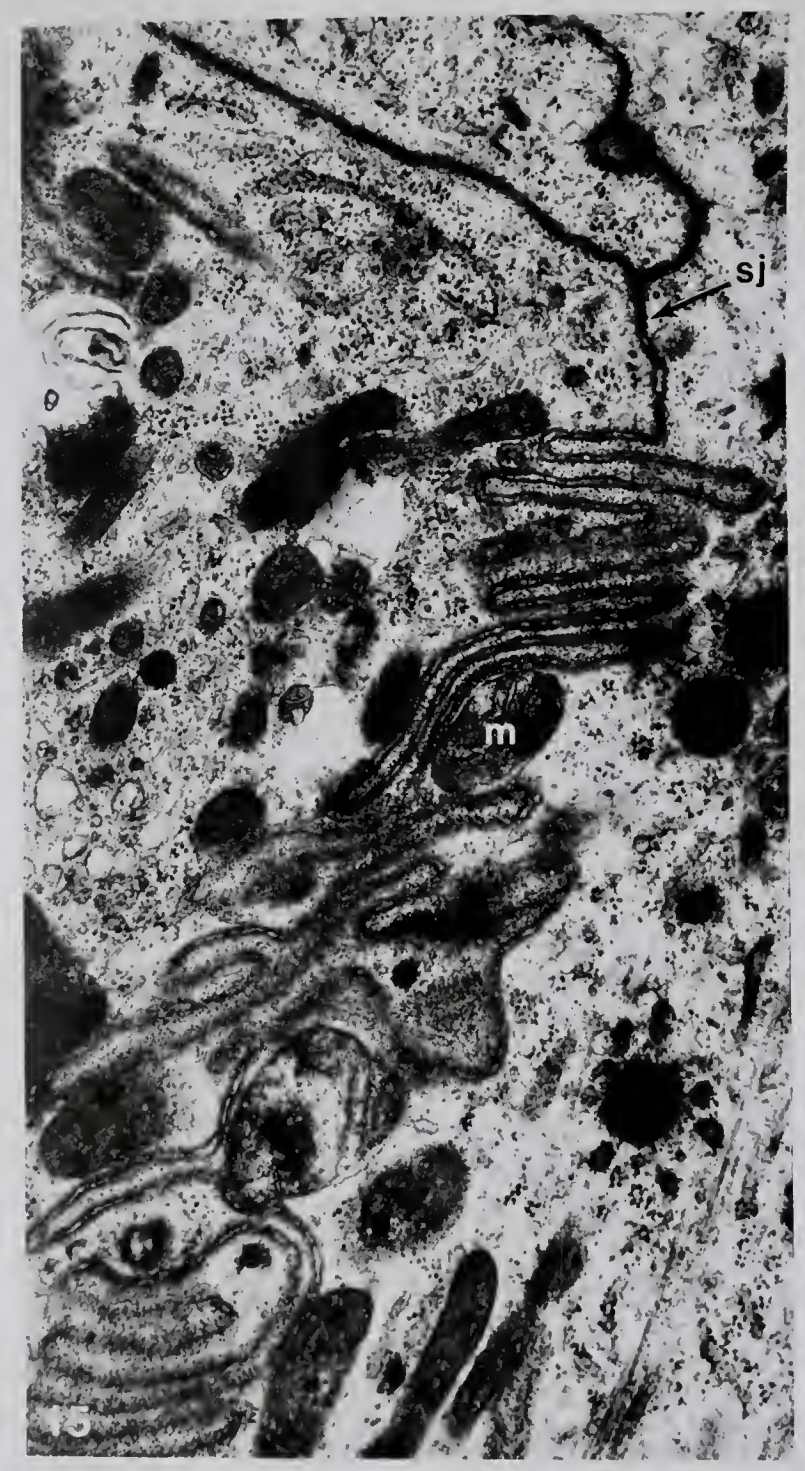
The basal plasma membrane has undergone a less drastic change. At irregular intervals of 0.1-0.6 um, it forms slit-like invaginations which extend up to 3 um into the cell (Figs. 16 and 17). These basal invaginations enclose an extracellular space about 50 nm in diameter. Although they traverse an area rich in mitochondria, the invaginations seldom come into close contact with these organelles.

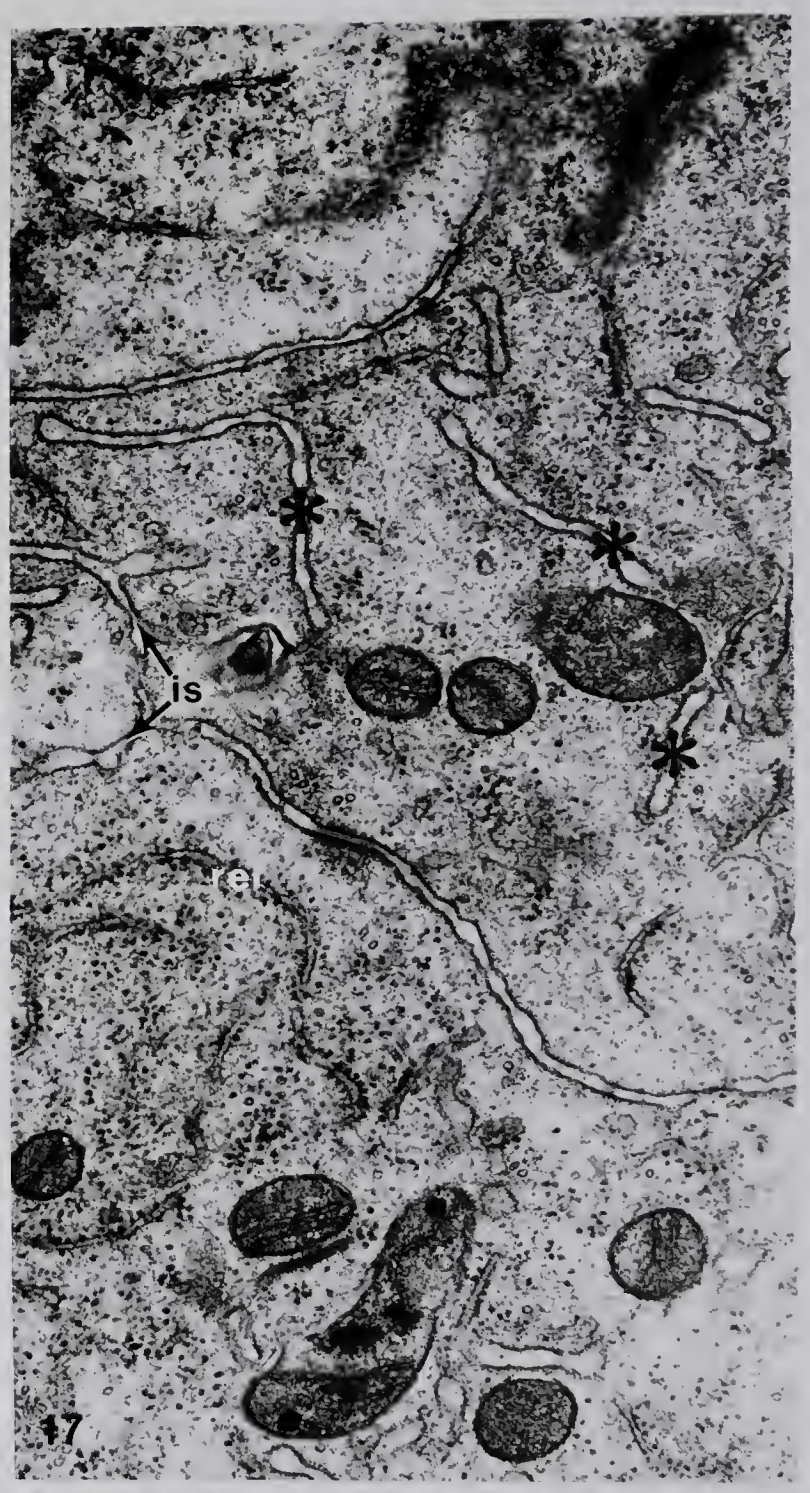
Fig. 15. Convoluted intercellular boundary typical of apical region of dorsal columnar epithelium. Note open intercellular space and close association of mitochondria (m) with lateral plasma membranes. In upper part of figure, more medial zone of septate junction (sj) can be seen. Longitudinal section (X30,875).

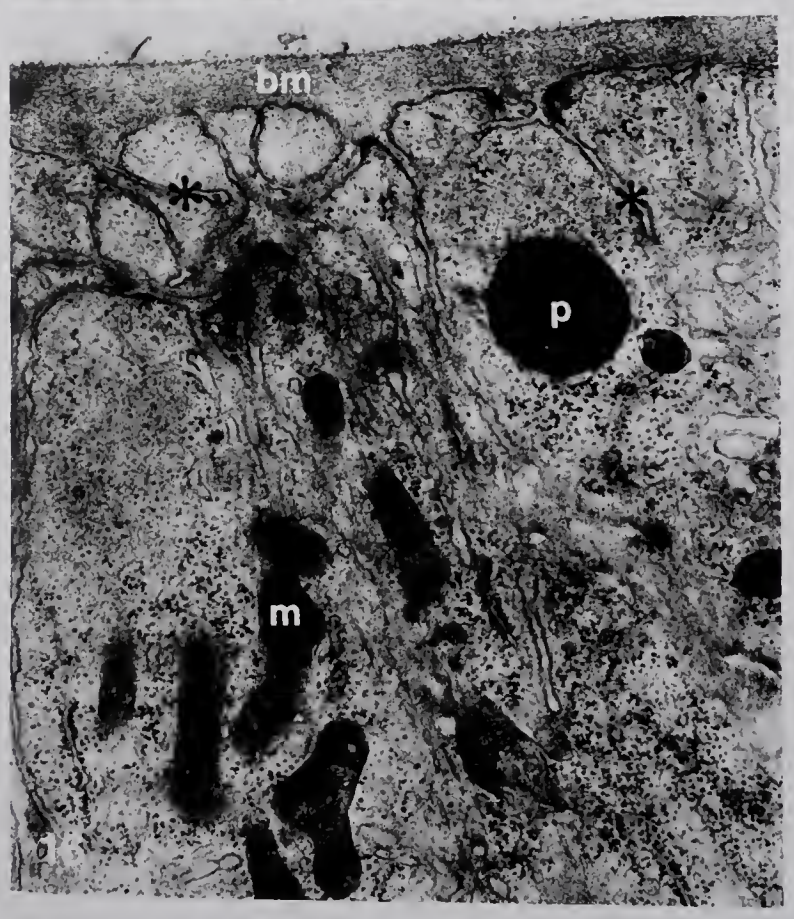
Fig. 16. Longitudinal section of basal region of differentiated dorsal columnar cell, showing simple invaginations of basal plasma membrane (asterisks) (X30,825). bm, basement membrane; m, mitochondrion; p, possible pigment deposit.

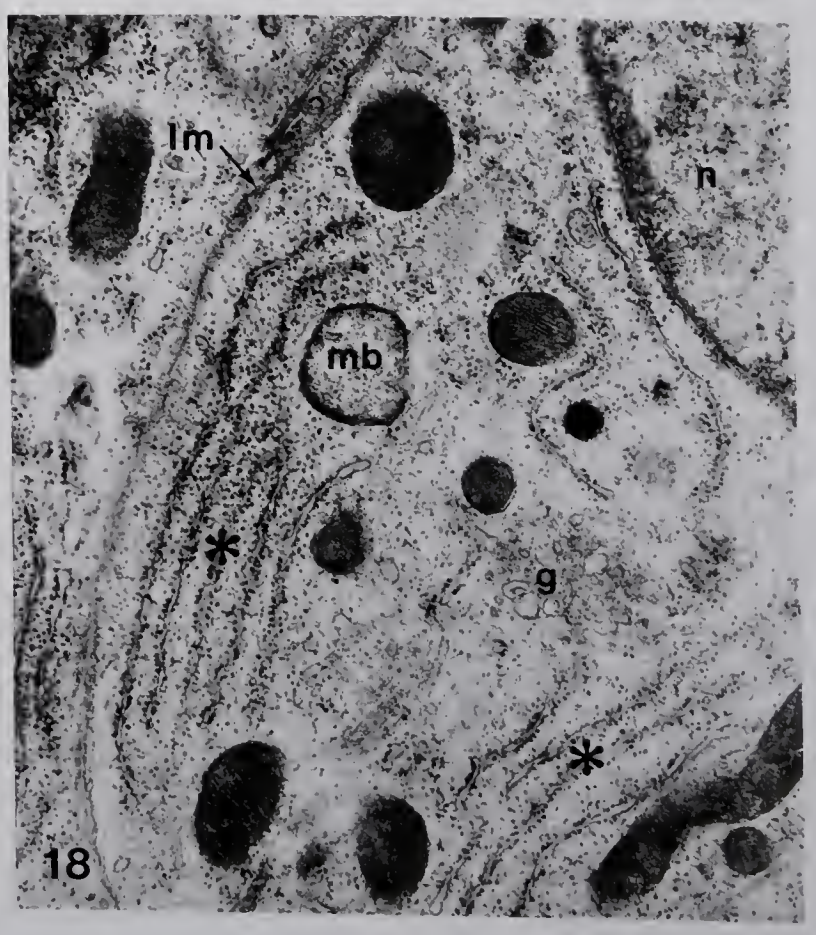
Fig. 17. Cross section of basal regions of four differentiated dorsal columnar cells. Slit-like nature of basal membrane invaginations (asterisks) is apparent in this orientation (X38,000). is, intercellular space; rer, rough endoplasmic reticulum.

Fig. 18. Perinuclear region of differentiated dorsal columnar cell in longitudinal section. Note parallel arrays of rer (asterisks) (X26,200). g, Golgi region; lm, lateral plasma membrane; mb, mycoplasma-like body; n, nucleus.


sj
m


is
17


bm
p
m


lm
n
mb
g
18

The transition to the differentiated phase brings little change in the population density of cytoplasmic organelles and inclusions. Cisternae of rer are occasionally seen in parallel arrays (Fig. 18), but they are still sparsely distributed and found mainly in the perinuclear area. No evidence of pinocytosis or exocytosis is observed at the basal or lateral plasma membranes. Coated vesicles are still seen in the apical region, mainly in the areas between the leaflets of invaginated membrane (Fig. 12). The apical region also contains evidence of pinocytotic activity: the presence of multivesicular bodies, generally regarded to be aggregations of pinocytotic vesicles and primary lysosomes (DeDuve and Wattiaux, 1966; Becker et al., 1967). The multivesicular bodies, which are approximately 0.5 µm in diameter, are membrane-bound clusters of electron-lucent spheres embedded in an electron-dense matrix. In some sections, an electron-lucent core, containing a single central vesicle, is revealed (Fig. 19).

Two other types of inclusion appear in the differentiated columnar epithelium. Spherical deposits of electron-opaque, amorphous material, which may be membrane-bound, are infrequently seen in the basal and medial cytoplasm (Fig. 16). These may be pigment granules, or droplets of highly unsaturated lipid (Smith, 1968).

Fig. 19. Multivesicular body (mvb) as seen in longitudinal section of dorsal columnar cell (X42,750).

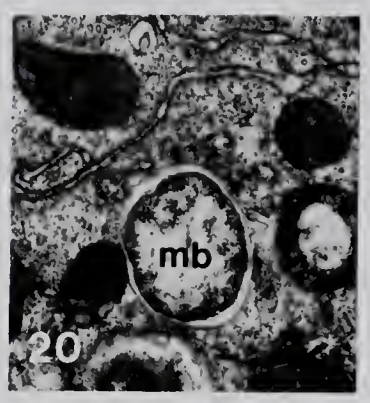
Fig. 20. Cross section of basal region of differentiated dorsal columnar cell, showing mycoplasma-like body (mb) (X47,500).

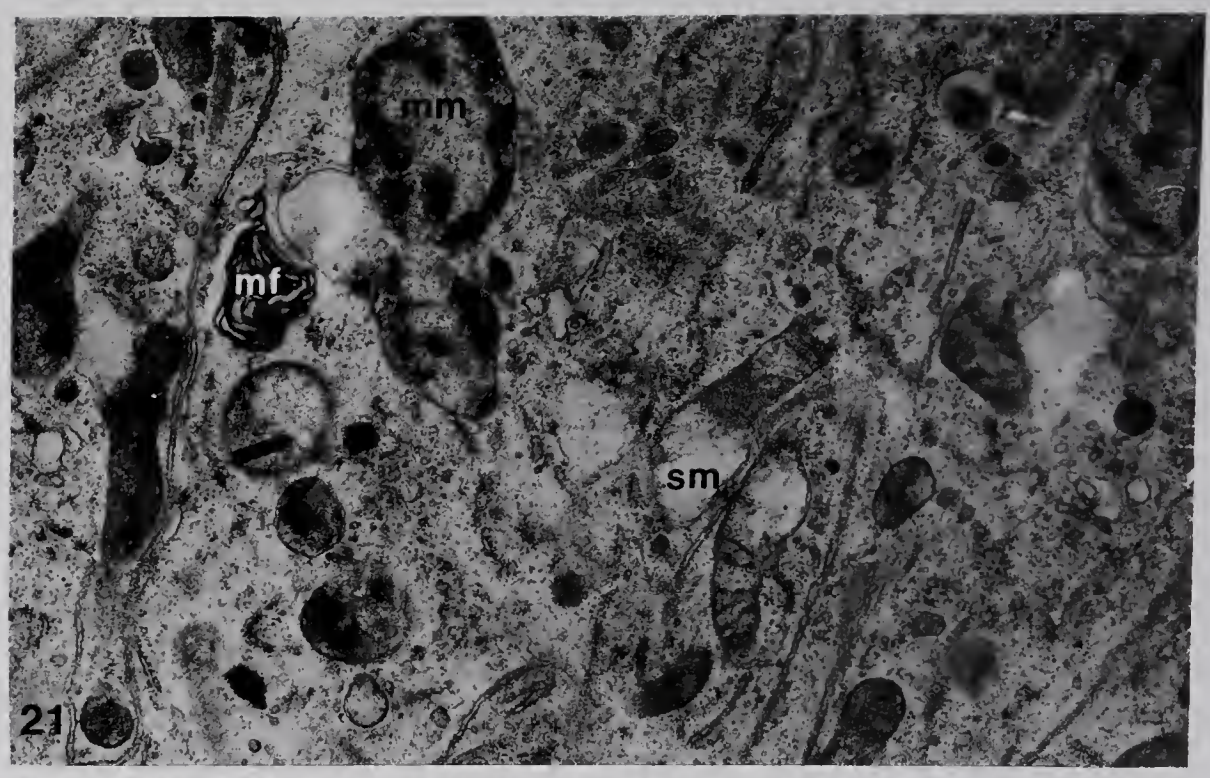
Fig. 21. Mitochondrial degeneration, as seen in longitudinal section of senescent dorsal columnar cell (X29,150). mf, myeloid figure, mm, maculate mitochondrion; sm, swollen mitochondrion.

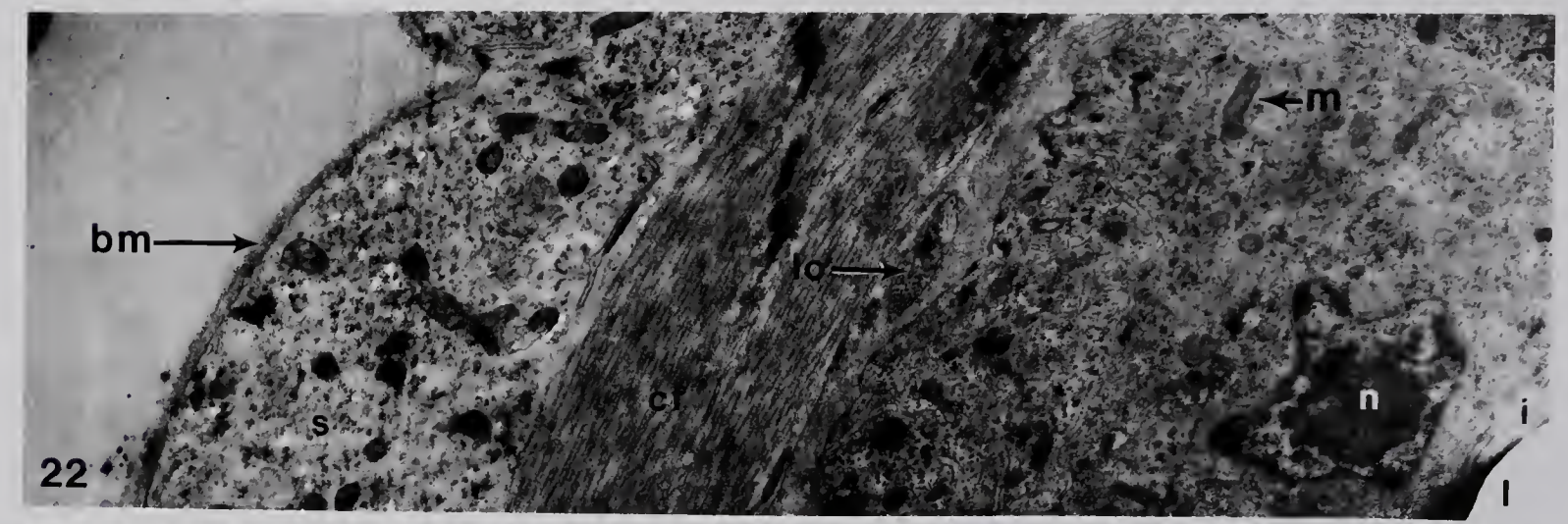
Fig. 22. Cross section of segment of stalk epithelium of EB of one-day old adult (X13,440). bm, basement membrane of stalk epithelium; cf, fibrils of circular muscle; i, intima; l, lumen of EB; lo, longitudinal muscle; m, mitochondrion; n, nucleus of stalk cell; s, sarcoplasm of circular muscle.

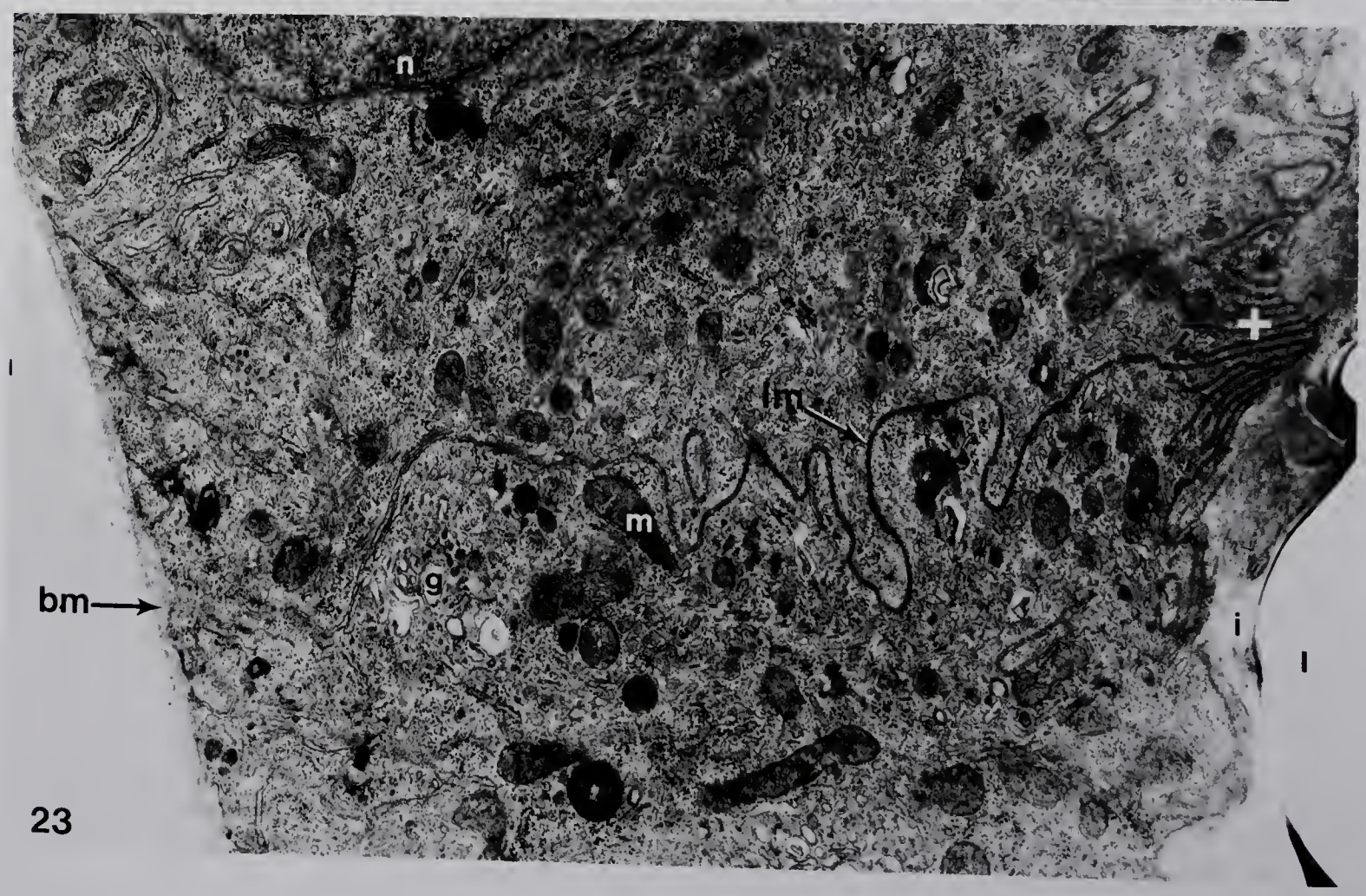
Fig. 23. Cross section of segment of lateral wall of sac of EB of one-day old adult. Note small, simple invaginations of apical plasma membrane (white cross) (X22,750). bm, basement membrane; g, Golgi region; i, intima; l, lumen of eb; lm, lateral plasma membrane; m, mitochondrion; n, nucleus.


mvb
19


mb
20


mm
mf
sm
21


bm
m
lo
s
c
n
i
l
22


n
+
lm
m
g
bm
i
l
23

Inclusions resembling mycoplasma are observed in about 10% (n=57) of specimen inspected (Fig. 20). Their significance is unknown.

The third developmental phase, senescence, is characterized by a gradual accumulation of changes of a type usually associated with age-related degeneration (see Rockstein and Miquel, 1973; Ciampor and Líbíková, 1975). These changes begin to appear during the second or third week of adult life, and become progressively more obvious through the remainder of the life span (i.e. 3-6 weeks under laboratory conditions). Degenerative changes in the mitochondria are particularly conspicuous, and include bloating, disorganization and loss of cristae, and maculate densification (Fig. 21). Myeloid figures, which probably represent an advanced stage of mitochondrial deterioration (Kjaerheim, 1968), become common during senescence. In the 50-60-day old fly, nearly all mitochondria exhibit some abnormalities. It is possible that these abnormalities are only an indirect indication of age-related change. For example, they might be a fixation artifact caused by an increase in the internal tonicity of the mitochondria. On the basis of available evidence, then, it cannot be determined whether respiratory impairment accompanies the senescent phase.

Stalk and wall epithelia. The epithelia of the stalk and the walls of the EB secrete an intima which is continuous with, and of about the same thickness as, that of the dorsal columnar epithelium. The stalk is composed of a single layer of cuboidal cells whose length ranges from 2-3 μm in the early chromoptic pupa to 2-5 μm in the late chromoptic pupa and adult. The length of the cells decreases along a distal-to-basal gradient. The basal surface of the epithelium is covered with a basement membrane within which are embedded longitudinal muscle fibers (Fig. 22). Underlying the basement membrane is a much thicker layer of circular muscle. The combined thickness of the muscular layers is about 1-4 μm in the early chromoptic pupa, increasing to 6-13 μm in the early chromogenic pupa and adult. The broad ranges of these measurements are probably caused by differences in muscular contraction among specimens during fixation. The plasma membranes of the stalk cells show no infolding or other modification. The mitochondrial population is far more sparse than that of the columnar cells.

The lateral walls of the sac of the EB lack a muscular tunic. In some respects, the epithelium which comprises these walls seems to represent a transition between the low cuboidal epithelium of the stalk and

the columnar epithelium of the dorsum of the EB. The length of the cells ranges from 2.0-3.5 μm in the early chromoptic pupa to 5.0-8.0 μm in the early chromogenic pupa and adult. The apical plasma membranes are somewhat invaginated, but the folds are not nearly as numerous, deep, or complex as those of the dorsal columnar epithelium. They extend no more than 0.6 μm into the cells (Fig. 23). Mitochondria are more plentiful than in the stalk epithelium, but not as densely distributed as in the dorsal columnar epithelium.

In the epithelia of both the stalk and the lateral walls, the boundary between adjacent cells is divided into two regions. From the basement membrane to a point approximately halfway along its length, the boundary is free of junctions, and the intercellular space is 20-30 nm wide. The remaining apical portion is an almost continuous band of septate junction. Both the stalk and wall epithelia are poor in rer and Golgi regions. Except for the above mentioned increase in size, and a small amount of mitochondrial degeneration during senescence, neither epithelium undergoes significant developmental change.

Contents of lumen. It was suggested in Chapter II, on the basis of histological and histochemical evidence,

that the tough, fibrous material which accumulates in the lumen of the EB is derived from the intima. It was also suggested that the high population density of bacteria in the lumen is at least partially attributable to the entrapment of actively dividing bacteria in this material (since this would render the bacteria less susceptible to the flushing action of food movement). Ultrastructural evidence now supports both suggestions. Many actively dividing microorganisms, which appear to be Gram-negative bacteria (Costerton et al., 1974) are found in the lumen of the EB of the adult fly (Fig. 24). They occur in clumps or rows in spaces between undulant fibers (Figs. 24 and 25). The bacteria are closely appressed to the fibers, and sometimes completely enmeshed in them. The fibers cannot be bacterially derived; they first appear during the early chromoptic stage of pupal development, when few bacteria are present. On the basis of their orientation and density, the fibers appear instead to be shed from the intima (Fig. 26).

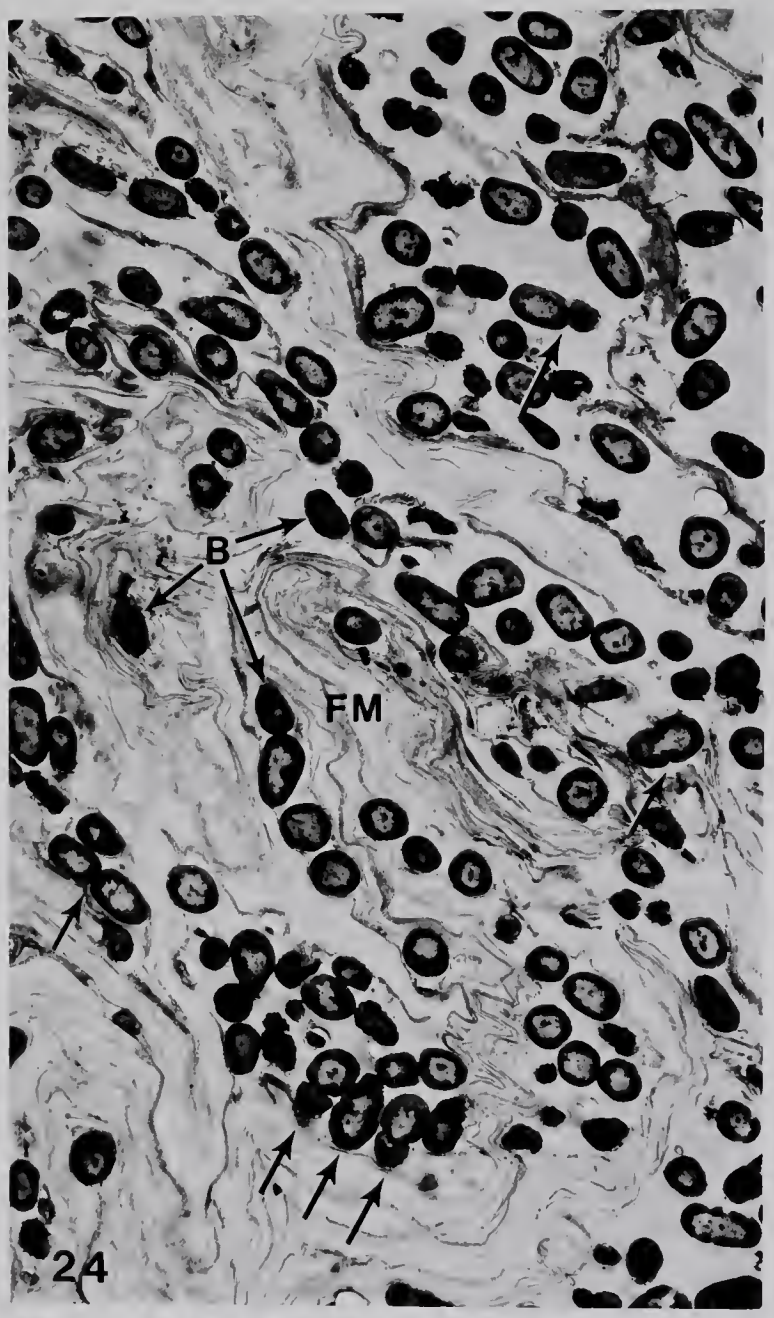
Effects of feeding. The ultrastructure of the EB is not appreciably altered after the ingestion of yeast hydrolysate, olive oil, or dry glucose plus KCl. The inbibation of glucose-KCl solutions, however, often

Fig. 24. Bacteria (B) and fibrous material (FM) in the lumen of the EB of two-week old adult. Note frequent occurrence of bacteria in process of division (arrows) (X5,225).

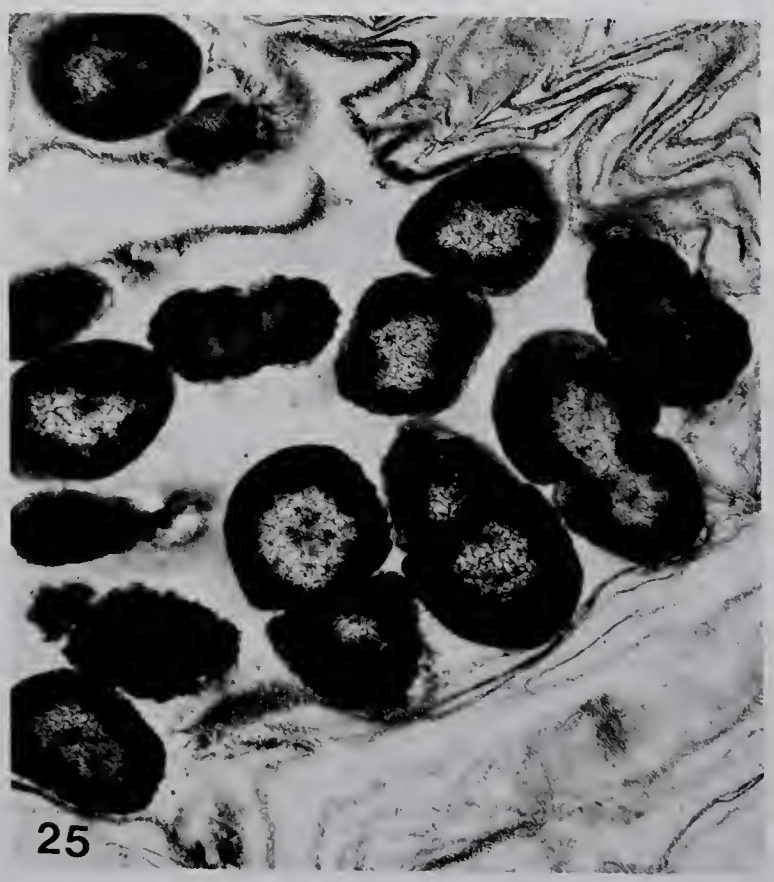
Fig. 25. Higher magnification of portion of area shown in Fig. 24. Group of bacteria is enmeshed in fibrous material in lumen (X19,000).

Fig. 26. Intima of dorsal columnar epithelium of early chromoptic pupa, in longitudinal section. Fibrous material (FM) which seems to be delaminating from intima (i) is similar in appearance to that seen in Figs. 24 and 25 (X15,840). L., lumen.

Fig. 27. Longitudinal section of apical region of dorsal columnar epithelium, 0.25 hr after ingestion of 5% glucose-3 mM KCl solution. Note distension of extracellular spaces (asterisks) enclosed by infolded apical plasma membrane (X42,750). i, intima; m, mitochondrion.

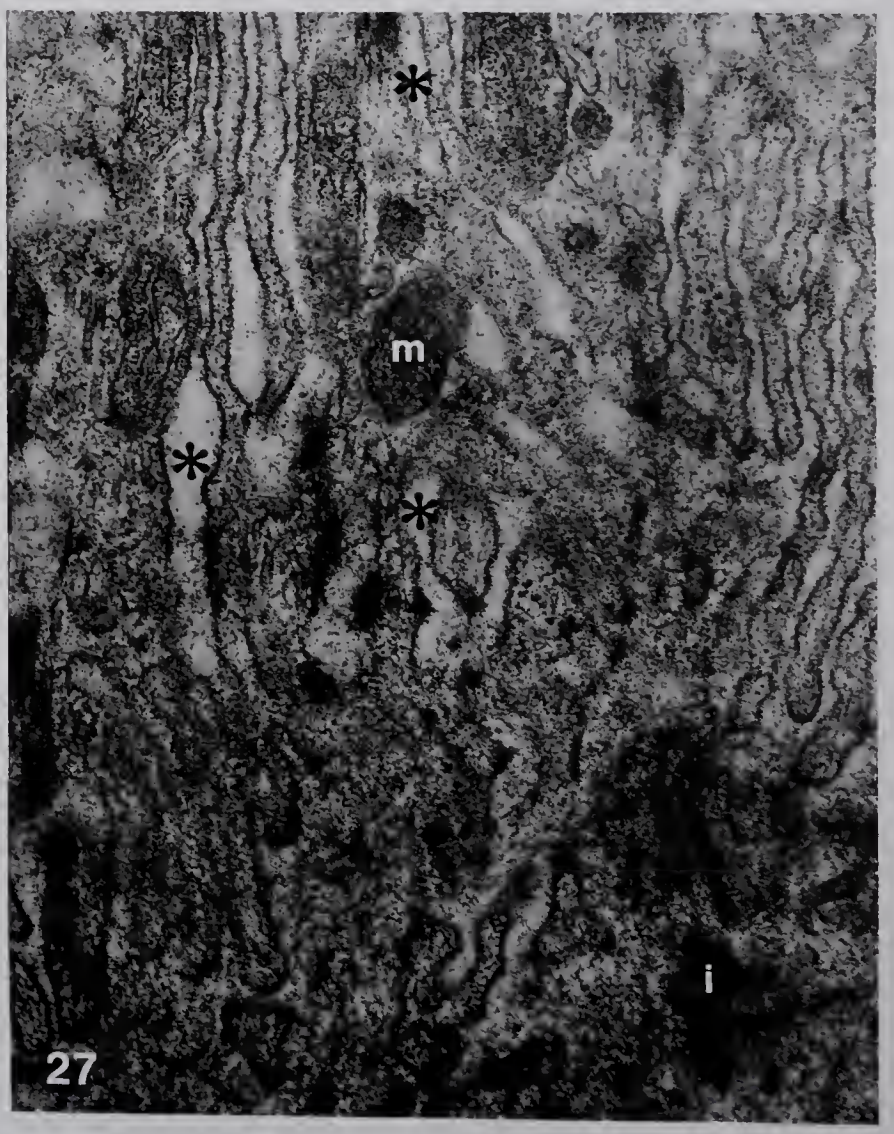


24



25

26



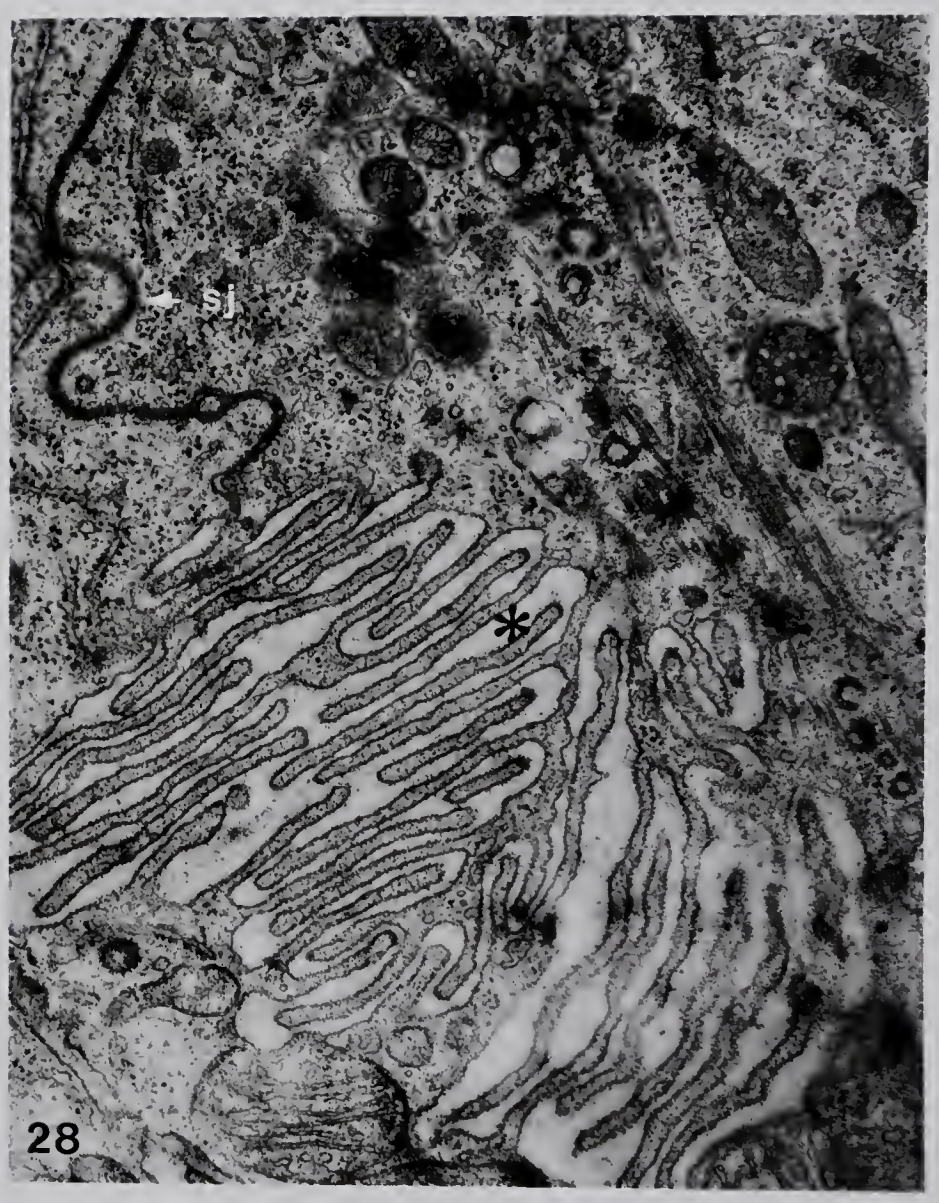
27

brings about a striking change: an electron-lucent substance causes distension of the extracellular spaces defined by the folds in the apical, apicolateral, and basal plasma membranes of the dorsal columnar cells. These changes occur less than 0.12 hr after the cessation of feeding, and persist for as long as 1 hr. The infoldings of the apical plasma membrane distend to a maximum diameter of 140 nm (Fig. 27). In the unfed fly, the diameter is never more than 40 nm (Fig. 13). In the apical, interdigitated region of the lateral plasma membranes, the diameter of the intercellular space increases to as much as 180 nm (Fig. 28), as opposed to 32 nm in the unfed fly (Fig. 15). This swelling does not extend into the region sealed by septate junctions (Fig. 28). The invaginations of the basal plasma membrane dilate to a maximum diameter of 150 nm (Fig. 29), in contrast to 60 nm in the unfed fly (Fig. 17). The mitochondria, Golgi regions, and perinuclear spaces of the columnar cells exhibit no distension.

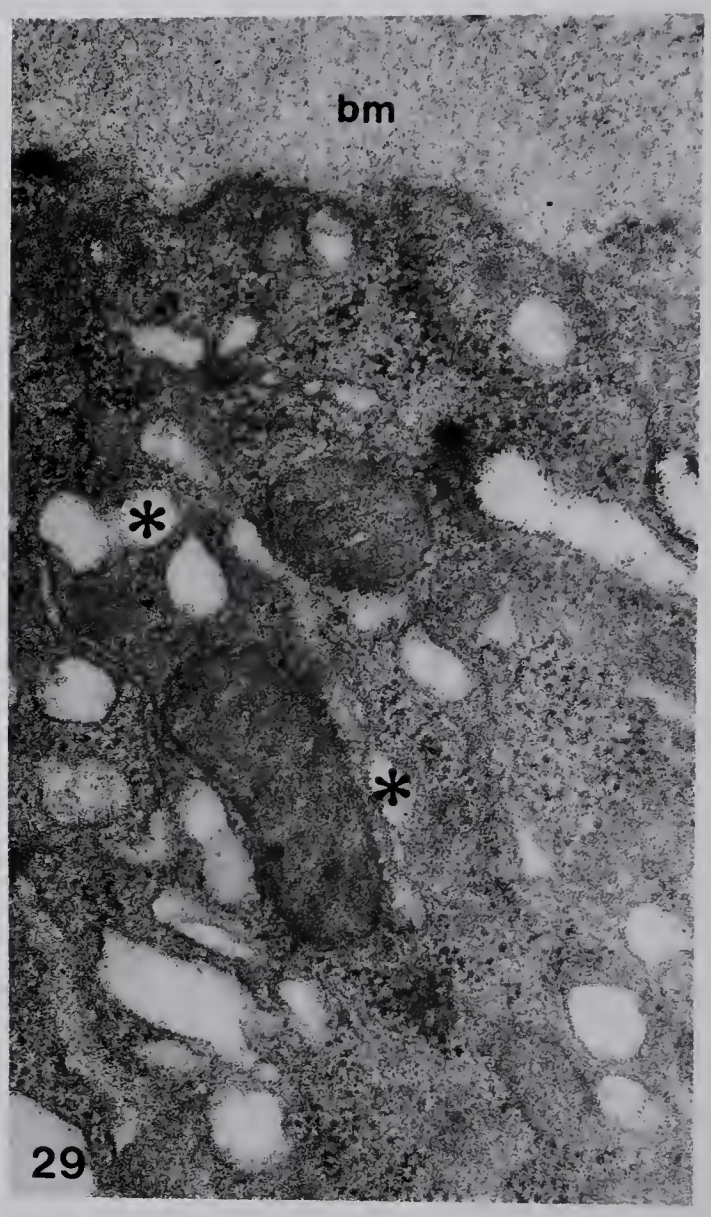
Distended extracellular spaces are not observed in all flies which have imbibed glucose-KCl solutions. The glucose tonicity of the solutions seems to have no relationship to the incidence of these changes. Distension of the extracellular spaces of the dorsal

Fig. 28. Longitudinal section of apicolateral border between two dorsal columnar cells, 0.5 hr after ingestion of 0.1% glucose-3 mM KCl solution. Asterisk denotes swollen intercellular space (X31,825). sj, septate junction.

Fig. 29. Longitudinal section of basal region of dorsal columnar cell, 0.5 hr after ingestion of 5% glucose-3 mM KCl solution. Basal invaginations (asterisks) are distended. Some invaginations appear to be vesicular because they lie at angles to the plane of section (X53,805). bm, basement membrane.



28



29

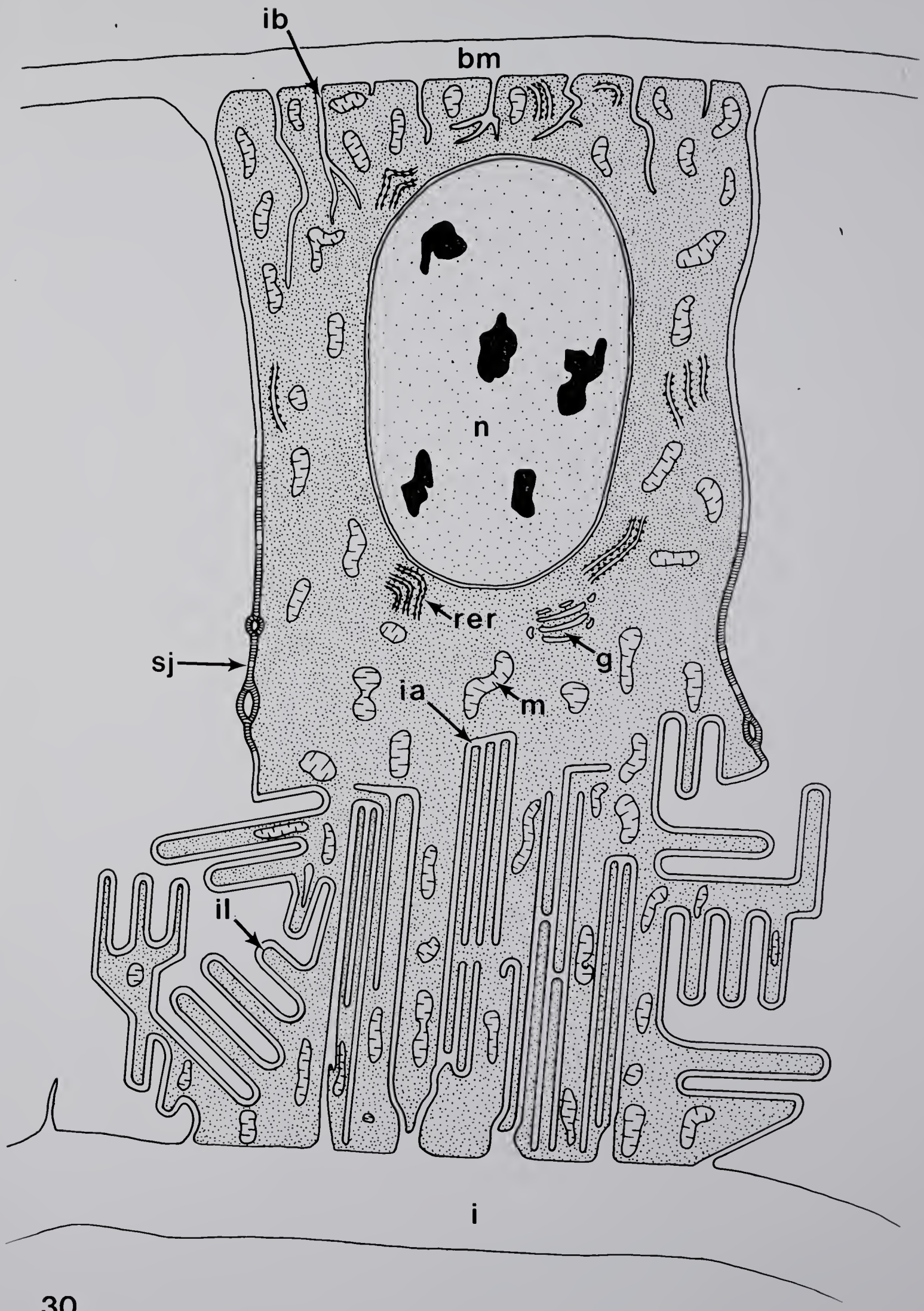
columnar epithelium occurred in 56% (n=9) of flies fed 0.1% glucose; 36% (n=8) of those fed 5.5% glucose; and 57% (n=7) of those fed 15% glucose. These results would seem to indicate that the distension occurs independently of the osmotic gradient across the epithelium; but this conclusion must be treated cautiously. It was discovered subsequent to the experiment that, after a liquid meal, crop fluid is regurgitated into the esophagus. Some of this fluid can enter the EB, diluting its contents to an unpredictable extent (Ratner, unpublished results). The osmolarity of the contents of the EB cannot, therefore, be directly related to that of a test meal. As a result, no conclusion can presently be drawn concerning the effect of transepithelial osmotic gradient upon the distension of extracellular spaces.

Discussion

In their lack of ultrastructural evidence of specialization, the stalk and lateral walls of the EB are reminiscent of the esophageal epithelia of R. pomonella and other Diptera (Smith, 1968; Ratner, unpublished results). It would appear that these structures confine, but do not otherwise interact with, the food which passes in and out of the EB. The dorsal columnar epithelium, on the other hand, exhibits ultrastructural

features which indicate that it is more than a passive extension of the esophageal wall. The ultrastructure of a representative cell is presented semi-schematically in Fig. 31. Its most striking feature, mitochondrion-associated folds of plasma membrane, is one often found in transport epithelia (for reviews, see Oschman and Berridge, 1971; Wall, 1971; Phillips, 1977). On the basis of structural evidence alone, it is impossible to determine definitely whether transport across the dorsal columnar epithelium actually takes place; and, if so, the nature and direction of this transport. Nevertheless, some tentative, testable conclusions can be drawn from the results of the feeding studies. The swelling of extracellular spaces is presumably caused by the uptake of aqueous fluid, since it is observed after the ingestion of glucose solution, but not dry glucose. Fluid uptake in the rectal pads of Calliphora is characterized by similar electron-lucent swellings (Berridge and Gupta, 1967). The fluid uptake observed in the dorsal columnar epithelium is not an artifact of osmotic stress caused by the entry of water into the lumen of the EB. The subcellular organelles of the epithelium show no evidence of such stress. Furthermore, the epithelia of the stalk and lateral walls exhibit no intercellular swelling, although they are exposed to the same milieu. It is

Fig. 30. Semi-schematic representation of ultrastructure of differentiated dorsal columnar cell. bm, basement membrane; g, Golgi region; i, intima; ia, invaginated apical plasma membrane; ib, invaginated basal plasma membrane; m, mitochondrion; rer, rough endoplasmic reticulum; sj, septate junction.



30

therefore proposed that the swellings observed in the dorsal columnar epithelium are evidence of true transepithelial fluid movement.

Judging from the locations of the swellings, it is evident that the fluid follows a primarily intracellular path across the epithelium; intercellular channels are not involved. In this respect, the dorsal columnar epithelium differs from many of the known transport epithelia, such as the mammalian gall bladder or the rectal pads of the cockroach. In these tissues, fluid follows a paracellular path from the organ lumen to the bloodstream or hemolymph: first into the cells, then through intercellular channels into which solute has been pumped to create a favorable osmotic gradient (Diamond and Tormey, 1966; Wall, 1977). This gradient can be maintained because the intercellular channels are isolated from the organ lumen by either tight or septate junctions, both of which are thought to act as barriers against back-diffusion of ions (Lord and DiBona, 1976; Noirot and Noirot-Timothee, 1976). In the dorsal columnar epithelium, no such channels exist. Elaborate intercellular spaces are present, but they cannot be isolated from the lumen because they are on the apical side of the septate junctions (Fig. 30). These spaces may serve simply to increase the surface area of membrane facing the lumen.

The direction of fluid movement across the dorsal columnar epithelium cannot be determined at this time. In this study, extracellular spaces seemed to swell or shrink simultaneously in the apical and basal poles of the cells. A time course study with finer resolution might prove otherwise.

Fluid movement across the EB may be the result of passive diffusion, or of an energy-requiring process. The latter possibility seems the more likely one, because of the close association of mitochondria with the infoldings of the plasma membrane. In a model proposed by Berridge and Oschman (1969), fluid transport can be driven by the active generation of high osmotic pressure within such infoldings. Still, concrete proof of active transport can come only from metabolic inhibition studies.

Transepithelial water movement can be accompanied by net movement of solutes. Any solutes which might travel across the epithelium of the EB would be expected to be species of low molecular weight, such as inorganic salts, amino acids, or monosaccharides. This is because a nonporous intima can act as a molecular sieve. In the locust rectum, for example, the intima permits the passage of molecules with radii no larger than 6.5 Å (Treherne, 1962).

The possibility of a vesicular transport system is

raised by the presence of coated vesicles in the apical region of the dorsal columnar epithelium (Figs. 10 and 12). One function proposed for these vesicles is the uptake and release of macromolecules at the plasma membrane. It is improbable, however, that macromolecules can successfully traverse the ductless intima in any direction. A more likely explanation for the vesicular activity is the continuous addition of new material to the intima (which, despite the delamination of fibers, becomes gradually thicker during the life of the fly, reaching a maximum of 1.6 μm). Ultrastructural studies by Locke (1976) indicate that, in the developing insect epidermis, coated vesicles transport Golgi-derived material to the cuticle.

At present, the physiological role of the EB is open to speculation. Many tissues architecturally similar to the dorsal columnar epithelium of the EB have been implicated in the maintenance of water and salt balance in arthropods (Sohal and Copeland, 1966; Mills, et al., 1970; Coons and Axtell, 1971; Marshall and Cheung, 1973; Dallai, 1977). It is unusual to find such a tissue situated in the foregut, which is generally regarded to be impermeable to water and solute (Treherne, 1962). This situation is not without precedent, however: The foregut of the land crab Gecarcinus lateralis is permeable

to sodium, chloride, and water (Mantel, 1968).

C H A P T E R I V

IN VITRO MEASUREMENT OF MOVEMENT OF WATER AND SOLUTES OUT OF THE ESOPHAGEAL BULB OF FEMALE RHAGOLETIS POMONELLA.

Introduction

The esophageal bulb (EB) of the adult apple maggot fly, Rhagoletis pomonella Walsh, contains ultrastructural features which indicate that it may transport fluid into or out of the foregut (Chapter III). A technique has now been developed by which the EB can be isolated and perfused in vitro. This makes possible a partial characterization of the direction of fluid movement across the EB, and the nature of solutes dissolved in this fluid.

Materials and Methods.

Insects. Rhagoletis pomonella were reared as described in Chapter II. Adult females 12-14 days old were used in this study.

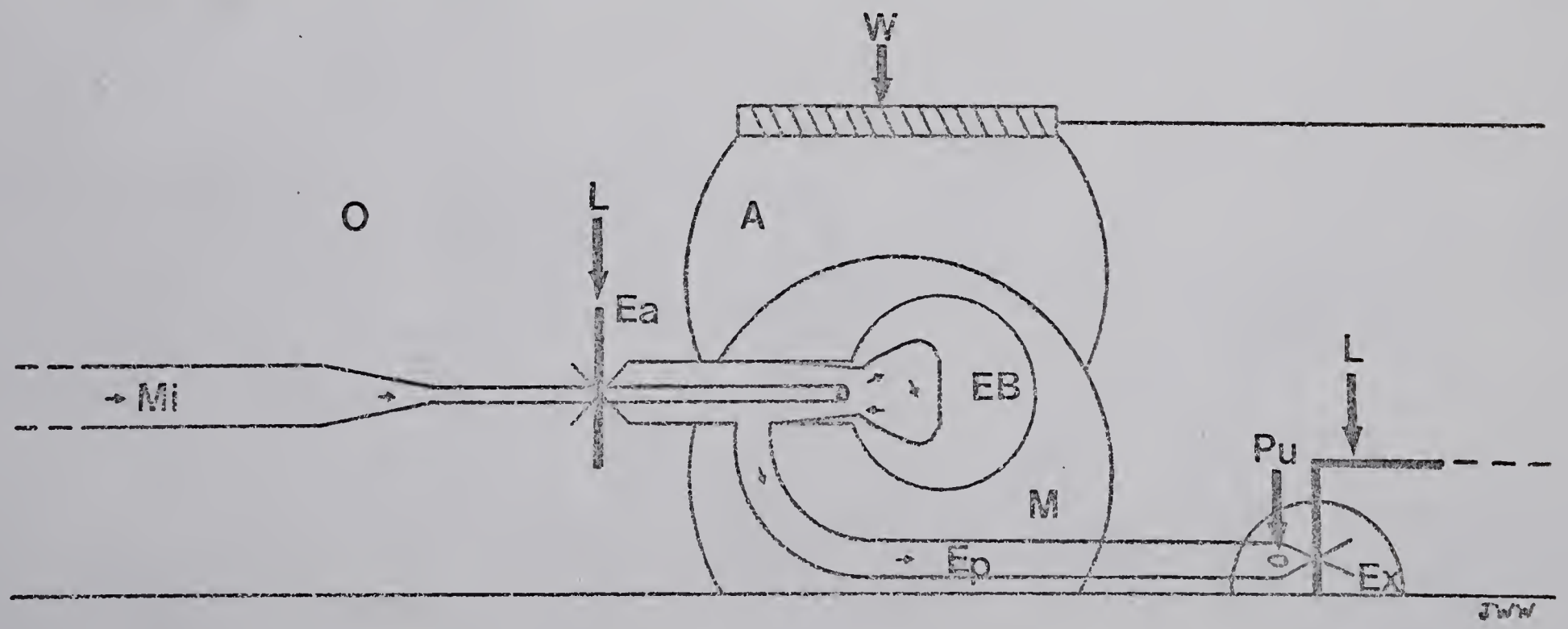
Culture medium. Modified Berridge and Patel (1968) medium was used. The final concentration of each constituent is given in Table 2.

Constituent	Concentration (mM)
NaCl	263
NaH_2PO_4	2
Na_2HPO_4	3
$CaCl_2$	4
$MgCl_2$	20
trehalose	11
glucose	20
glutamine	10
α-alanine	9
glycine	13
fumaric acid	9
malic acid	7
citric acid	5

Table 2. Concentrations of constituents of Berridge and Patel medium (Berridge and Patel, 1968).

In vitro organ culture and cannulation of the EB. The procedure used is a modification of that employed by Ramsay (1954) in his study of Malpighian tubule secretion. Cold-anesthetized, 2-week-old female flies were submerged in culture medium, and their heads were opened along the ptilinal suture. The EB and a segment of attached esophagus were excised by cutting the esophagus immediately posterior to the pharynx and immediately anterior to the cervix. The tissue was transferred to a fresh droplet of medium on the surface of a 10-cm Falcon petri dish. A glass micropipette, mounted on a Narashige micromanipulator, was inserted into the anterior segment of the esophagus until its tip reached past the stalk of the EB. It was then ligatured firmly into place with a nylon monofilament. The segment of esophagus posterior to the EB was ligatured shut at its distal end. The free end of the ligature was attached to a second micromanipulator. This allowed the posterior segment of esophagus to be pulled partially out of the droplet of medium (see Fig. 31 for a diagram of this arrangement). The dish was then flooded with paraffin oil to prevent evaporation. To permit gas exchange, an air bubble was trapped in the oil with a wire loop and brought into contact with the droplet of medium. At the start of an experiment, the micropipette was attached to a

Fig. 31. Schematic diagram of esophageal bulb prepared for in vitro perfusion. A, air bubble; Ea, anterior segment of esophagus; EB, esophageal bulb; Ep, posterior segment of esophagus; Ex, exudate; L, ligature; M, droplet of culture medium, Mi, micropipette; O, paraffin oil; Pu, puncture in posterior segment of esophagus; W, wire loop. Arrows indicate direction of flow of perfusion medium.


W
O
L
A
Ea
Mi
EB
L
Pu
M
Ep
Ex
JWW

microsyringe, and the posterior segment of the esophagus was punctured with a microneedle proximal to the ligature (Pu in Fig. 31). Perfusing medium could thus be pumped from the microsyringe, through the EB preparation, to exude from the punctured esophagus and collect as a droplet (which will be referred to as "exudate") under the oil. Both this exudate and the bathing medium could be sampled with a microsyringe while an experiment was in progress. After 12 hr under this regime, the EB retains its normal ultrastructure and Trypan blue exclusion properties. It was therefore assumed that the tissue remains viable during the 4-5-hr course of an experiment.

Bathing and perfusion media. Berridge and Patel medium, which was the basis for both the bathing and perfusion media, was modified according to the needs of each experiment. Isotopically-labeled solutes (New England Nuclear, Boston MA) were added to the perfusion medium. The composition of the bathing medium was altered in some experiments to provide favorable diffusion gradients for specific solutes. These modifications are all summarized in Table 3.

Measurements of water and solute movement. In all experiments, perfusing medium was pumped through the EB

Table 3. Modifications of Berridge and Patel culture medium for tests on water and solute movement across the EB *in vitro*.

Test	Modification of perfusion medium	Modification of bathing medium	Comments
Movement of water	Added [carboxyl-^{14}C-inulin (spec. activ. 2.5 μCi/mg). Final concentration: 80mM.	Added unlabeled inulin. Final concentration: 80mM.	Isoosmolarity of perfusion and bathing media maintained.
Movement of glucose	Added D-[6-^{3}H]-glucose (spec. activ. 25 Ci/mmole). Final concentration: 100mM Total [glucose]: 120mM.	Increased [glucose] to 40mM.	3X concentration difference created outward diffusion gradient.
Movement of neutral amino acid.	Added L-[4,5-^{3}H(N)]-leucine (spec. activ. 5.0 Ci/mmole). Final concentration: 0.05mM. Also added unlabeled leucine. Total [leucine]: 330mM	None.	Created outward leucine diffusion gradient.

Test	Modification of perfusion medium	Modification of bathing medium	Comments
Movement of a basic amino acid.	Added L-[^{3}H(G)]-lysine (spec. activ. 5 Ci/mmole). Final concentration: 0.05mM. Also added unlabeled lysine. Total [lysine]: 330mM.	None.	Created outward lysine diffusion gradient.
Movement of an acidic amino acid.	Added L-[2,3-^{3}H(N)]-aspartic acid (spec. activ. 15 Ci/mmole). Final concentration: 0.25mM. Also added unlabeled aspartic acid. Final [aspartic acid]: 330mM.	None.	Created outward aspartic acid diffusion gradient.

Test	Modification of perfusion medium	Modification of bathing medium	Comments
Movement of a saturated lipid.	Added [9,10-^{3}H(N)]-palmitic acid (spec. activ. 5.6 Ci/mmole) as olive oil emulsion. Final concentration: 0.167 mM. Also added unlabeled palmitic acid. Final [palmitic acid]: 330 mM.	Added 2% bovine serum albumin.	Created outward palmitic acid diffusion gradient. Bovine serum albumin is a lipid carrier.
Movement of Na^{+}.	Added ^{22}NaCl (spec. activ. 5.8-6.2 Ci/gNa). Final concentration: 1.4mM. Total [Na^{+}]: 272.4	Reduced [Na^{+}] to 90.8mM. Compensated with choline chloride.	3X concentration difference created outward sodium diffusion gradient.

Test	Modification of perfusion medium	Modification of bathing medium	Comments
Movement of Rb^+.	Added $^{86}RbCl$ (spec. activ. 6.2-7.8 Ci/gRb). Final concentration: 1.4mM. Also added unlabeled RbCl Total $[Rb^+]$: 272mM.	Added RbCl. Total $[Rb^+]$: 91mM.	3X concentration difference created outward Rb^+ diffusion gradient. Rb^+ is an active transport analog of K^+.
Movement of Cl^-.	Added $H^{36}Cl$ (spec. activ. 8.1 mCi/gCl). Final concentration: 300mM.	Reduced [NaCl] to 59mM. Compensated with Na_2SO_4. Total $[Cl^-]$: 107mM.	3X concentration difference created outward Cl^- diffusion gradient.
Inhibition of possible active transport.	Added NaCN. Final concentration: 1mM.	Added NaCN. Final concentration: 1mM.	NaCN is an inhibitor of aerobic respiration.

at the rate of 3 µl/hr. After 2 hr of equilibration the bathing medium was changed, the droplet of exudate was removed, and the experiment was begun. In experiments on water movement the perfusion medium was loaded with ^{14}C-inulin. The exudate was collected hourly for 3 hr. The dried residues of 1-µl samples of perfusing medium and exudate were compared. Differences in concentration were taken to be due to water movement, since inulin cannot move across cell membranes (Guyton, 1961) and the EB contains no unsealed intercellular spaces (Chapter III).

In experiments on solute movement, a 2-hr equilibration period and a 3-hr experimentation period were again used. At the end of each hour, the entire drop of bathing medium was collected and replaced with a fresh drop. The collected medium was dried. The concentration of tritiated solutes and ^{36}Cl in this residue was measured by scintillation counting; ^{23}Na and ^{86}Rb were quantitated on a Searle Model 1185 gamma counter.

In all the above experiments, ^{14}C-inulin was present in the perfusing medium as a check for leaky preparations. If ^{14}C-inulin appeared in the bathing medium, the preparation was discarded.

Results and Discussion

Water movement across the EB. When perfusion medium passes through an EB preparation, its inulin concentration increases significantly, by an hourly amount which remains nearly constant over the 3-hr duration of the experiment (Table 4, "Normal preparation"). The degree of inulin concentration indicates that 10.4-12.4% of the water originally present in the perfusion medium is removed. The ultimate destination of this water must be the bathing medium, since the cells of the EB do not become swollen after water imbibition (Chapter III).

The *in vitro* preparation includes not only the EB, but also the adjacent segments of esophagus and pharynx. In order to determine which components of the preparation were responsible for the removal of water from the perfusion medium, a second set of experiments was performed. ^{14}C-inulin was once again perfused through the preparation, but this time the EB was ligatured shut. In these preparations, the perfusing medium did not become significantly more concentrated (Table 4, "EB sealed"). These results prove that the EB itself is the site of water movement across the preparation.

Table 4. Comparisons of concentrations of ^{14}C-inulin solutions before (i.e. "Perfusion medium") and after passage (i.e. "Exudates") through normal and modified esophageal bulbs *in vitro*; and the changes in water content indicated by these comparisons. Each value is the mean of 6 replicates. For each type of esophageal bulb preparation, means followed by different superscripts are significantly different at $P < 0.05$, as determined by Student's t-test. Changes in water content were calculated using the formula

$$\Delta\ \%\ H_2O = 100X\ \ 1- \frac{[\text{inulin}]_{\text{perfusion medium}}}{[\text{inulin}]_{\text{exudate}}}$$

(after Phillips, 1964).

Sample	Mean ^{14}C cpm/µl (± S.E.)	Mean [^{14}C-inulin] (mmoles/L)	Mean % H_2O, with respect to perfusion medium
	Normal preparation		
Perfusion medium	22,258.3±198.5	78.8^{a}	------
1-hr exudate	24,633.0±315.1	87.2^{b}	-10.4%
2-hr exudate	25,043.5±298.7	88.7^{b}	-12.4%
3-hr exudate	24,913.7±290.4	88.2^{b}	-11.3%
	EB sealed		
Perfusion medium	26,543.8±314.6	94.0^{a}	--
1-hr exudate	27,317.3±503.2	96.7^{a}	NS
2-hr exudate	26,699.8±257.9	94.5^{a}	NS
3-hr exudate	27,004.6±257.9	95.6^{a}	NS
	Inhibited preparation (1.0mM NaCN)		
Perfusion medium	23,511.8±241.7	83.2^{a}	--
1-hr exudate	24,424.5±324.7	87.0^{b}	-4.6%
2-hr exudate	24,643.7±755.2	87.3^{b}	-4.6%
3-hr exudate	24,165.8±352.3	$85.6^{a,b}$	NS

Movement of water across the EB in the absence of an osmotic gradient implies that an active transport system is operating. The energy dependence of water movement was confirmed in experiments in which 1 mM NaCN was added to both perfusion and bathing media. Water movement was greatly diminished (Table 4, "Inhibited preparation"). The movement which did occur may have been driven by anaerobic respiration. Since water movement fell to insignificant levels only during the final hour of the experiments, it is also possible that aerobic metabolism was only slowly and progressively inhibited by the NaCN.

In the normal preparation, perfusing medium flowed through the EB at a rate of 3 µl/hr, and the average hourly reduction in water content was 11.7%. This translates to 0.35 µl H_2O/hr. Assuming that the region of the EB most likely to be engaged in fluid transport is the dorsal columnar epithelium (Chapter III), the rate of transport per unit of absorptive surface area is 0.103 nl/μ^2/hr. This is within the range of fluid transport rates calculated for other absorptive epithelia of insects. For example, the rectal pads of Calliphora-transport approximately 0.064 nl/μ^2/hr (figures derived from data of Phillips, 1969, and diagram of Graham-Smith, 1934). Maddrell and Gardiner (1974), testing a variety

of Malpighian tubules, found rates ranging from 0.3 to 1.0 nl/μ^2/hr.

No confirmed case of direct active transport of water has ever been found in an animal; rather, water movement can be osmotically driven by simultaneous active transport of solutes (Phillips, 1977a; Goh and Phillips, 1978). It is therefore important to know what type of solute transport might be associated with the active water movement observed across the EB.

Solute movement across the EB. In perfusion studies, no detectable amounts of labeled glucose, leucine, lysine, or aspartic acid moved across the epithelium of the EB into the bathing medium, despite the fact that concentration gradients had been set up to favor such movement. It is unlikely that the negative results are a reflection of net inward transport of the solutes in question, since the outcome was not altered when NaCN was added to the preparation. It is more likely that the chitinous intima of the EB blocks the passage of the solutes. The molecular sieving action of such intimas has been well documented in the insect hindgut (for review, see Maddrell, 1971). It has been suggested that one function of the EB might be the direct uptake of nutrients secreted by the symbiotic bacteria which

reside in its lumen (Poinar et al., 1975; Ratner, Chapter I). In light of the results of these experiments, such a function now appears to be unlikely, at least as far as sugars and amino acids are concerned.

Tests on palmitic acid movement also yielded negative results in both normal and inhibited preparations. This is unlikely to be a case of intima impermeability, since lipids have often been found to pass freely through the crop walls of several insect species (Eisner, 1955; Hoffman and Downer, 1976; Conner et al., 1978). While it is possible that the intima of the EB possesses unusual lipid-blocking properties, it is also possible that the bovine serum albumin included in the bathing medium was insufficient, in affinity or concentration, to solubilize any fatty acid which may have passed out of the EB.

The intima certainly presents no barrier to the 3 inorganic ions tested; they moved freely across the epithelium of the EB. The case of Na^+ is especially interesting, since it appears that this ion is actively transported from the EB lumen to the bathing medium (Table 5). In the normal preparation, Na^+ left the EB at an average rate of 242 nmoles/hr. When 1mM NaCN was added to the preparation, the rate was significantly

reduced, to 81 nmoles/hr. When absorptive area is taken into account, the rate of sodium movement across the EB--0.707 μmoles/cm^2/hr--is comparable to that determined for most other active ion transporting epithelia. In both vertebrates and invertebrates, this value generally falls between 1 and 6 μmoles/cm^2/hr (Phillips, 1977b; Ahearn et al., 1977). Actually, the EB in vitro is probably a poorer transporter of sodium than this comparison would suggest. In the cited studies, the ion of interest was present in equimolar concentrations on both sides of the epithelium. In the EB preparation, the concentration of Na^+ in the perfusion medium was kept 3% higher than that in the bathing medium. Assuming that the sodium movement observed in the inhibited preparation (0.237 μmoles/cm^2/hr) was purely diffusive, this value can be subtracted from the sodium movement in the normal preparation to yield the component of movement due to active transport: 0.470 μmoles/cm^2/hr.

Rb^+ and Cl^-, when present in the same relative concentrations as Na^+ (i.e. 3X more concentrated in the perfusion medium than the bathing medium), move out of the EB at a slower rate (Table 5). Taking absorptive area into account, these rates are 0.056 and 0.109 μmoles/cm^2/hr, respectively. As Rb^+ and Cl^- movement

Table 5. Solute movement out of EB *in vitro*. Each value is the mean of 6 replicates. For each solute, means followed by different superscripts are significantly different at $P < 0.05$, as determined by Student's t-test.

Solute	Condition of EB	Perfusion period (hr)	Mean cpm in bathing medium (± S.E.)	Mean solute in bathing medium (moles)
Na^+	Normal	0-1	84,046.7±1,628.6	234[a]
		1-2	85,256.3±1,818.5	253[a]
		2-3	82,339.8±1,257.2	238[a]
	Inhibited	0-1	24,891.7±2,307.9	73[b]
		1-2	28,982.2±1,554.3	84[b]
		2-3	29,067.3±1,351.7	86[b]
Rb^+	Normal	0-1	60,962.3±1,701.5	18[c]
		1-2	61,524.7±1,404.4	19[c]
		2-3	61,874.8±1,511.0	19[c]
	Inhibited	0-1	58,602.0±2,740.9	21[c]
		1-2	59,884.3±2,687.9	21[c]
		2-3	57,861.2±2,512.2	20[c]
Cl^-	Normal	0-1	2,895.7± 137.9	34[d]
		1-2	3,225.0± 123.3	39[d]
		2-3	2,982.3± 177.8	35[d]
	Inhibited	0-1	2,950.7± 190.0	35[d]
		1-2	3,017.3± 175.9	36[d]
		2-3	3,101.0± 160.8	36[d]

were not significantly reduced in the presence of NaCN (Table 5), this movement can be attributed solely to passive diffusion.

As stated earlier, active water transport is not an independent process, but rather is osmotically coupled to active solute transport. The solutes most commonly found to be implicated in this process are Na^+, K^+, and amino acids (Phillips, 1977a). Of all the solutes tested in this study, only Na^+ is actively transported out of the EB, and is thus the only one which may account, at least in part, for the outward, energy-requiring movement of water.

It is unfortunate that the minuscule capacity of the lumen of the EB made the measurement of the inward movement of ions impossible. It must also be noted that directions and rates of water and ion movement determined *in vitro* may not accurately reflect the *in vivo* situation. These uncertainties do not alter the conclusion that the EB probably plays some role in regulating the water and/or salt balance of *R. pomonella*. I propose that, of these two possible functions of the EB, maintenance of salt balance is the primary one (with water movement occurring as an unimportant side effect of ion transport). The foregut of *R. pomonella* is an unsuitable site for an organ of water conservation or

excretion. Any water-conserving dehydration of gut contents would soon be undone in the freely permeable midgut. Not surprisingly, all documented gastric water-conserving organs of insects are located in the hindgut (for review, see Maddrell, 1971). As for water excretion, there is simply no direct exit route for any water which may be extracted from the foregut by the EB. Besides, the natural diet of R. pomonella, which probably consists mainly of honeydew and fruit exudates (Dean and Chapman, 1973), would seem to require no preliminary concentration. Instead, the problem presented by such a plant-derived diet would be that of ionic incompatibility with dipteran hemolymph. The importance of the midgut and hindgut in maintaining hemolymph ionic equilibrium in the face of dietary insult is well known (for review, see Stobbart and Shaw, 1964). There is no reason why a foregut structure with salt-transporting properties cannot serve a similar function. It is toward the exploration of such a function that future research should be directed. A logical first step would be the in vivo ligation or ablation of the EB, with subsequent determination of effect on salt or water balance.

C H A P T E R V

FINAL DISCUSSION

To return to the question originally posed in the introduction to this work: What mycetomal and alimentary functions does the EB perform in Rhagoletis pomonella? Treating the mycetomal function first, it was not possible to determine whether the EB is solely responsible for the maintenance of the adult phase of the symbiont transmission cycle. Such a determination would require an experiment which, from a technical standpoint, is presently impossible: a characterization of changes in the cycle brought about by destruction or ligation of the EB, beginning with the early pupa. On the basis of histological evidence (Chapter II) and the results of a preliminary cultural experiment (Appendix), it is clear that the EB is far more hospitable to bacteria than any other segment of the gut. This hospitality does not appear to be mediated through nutrient secretion, waste absorption, or pH adjustment by the EB. Instead, from the available evidence, the most likely reason for the high bacterial densities is a physical one: bacterial entrapment in, or adhesion to, the fibers shed from the intima of the EB. Even if the entrapment gives each bacterium only a slight or

temporary resistance to the flushing action of food movement, the EB would still serve as a constant reservoir of bacteria as long as the rate of reproduction exceeds the rate of egress. There is no indication that bacteria in any other part of the gut are protected from being swept out as excreta; that is, there are no other accumulations of fibers or concentrations of bacteria. It would be useful to determine whether a particular species of bacterium is represented disproportionately in the EB; and to study in closer detail the possible adhesive action between the lumen fibers and the bacteria.

From the results of the *in vitro* study, the alimentary function of the EB seems to be the transport of sodium and water (and, possibly, any of a host of ions which were not tested). EB-ligation studies designed to ascertain the direction of transport *in vivo* proved to be technically too difficult to perform. The only conclusion that can be drawn at present, then, is that the EB plays some role in maintaining the sodium and/or water balance of *R. pomonella*. It is not surprising that *R. pomonella* might require a sodium transporting organ. The hemolymph ion composition of this insect has never been determined, but it would be expected to have a high Na^+/K^+ ratio, since this is the usual case for Diptera

(Florkin and Jeuniaux, 1964). The principal diet of R. pomonella, honeydew, would be expected to have a low Na^+/K^+ ratio, as it is a little-modified form of plant sap [and, while the aphid honeydew has never been ionically analyzed, the urine of another group of sap-feeding homopterans, the cicadas, conforms to this assumption (Cheung and Marshall, 1973)]. This imbalance between hemolymph and gut contents would cause the insect to become depleted in sodium if uncorrected. Perhaps the EB has a role to play in this correction. As a further, and technically feasible, step in the investigation of the importance of the EB, it would be interesting to determine whether sodium pumps are found in any other portion of the gut.

The finding that the EB of R. pomonella is an ion-transporting organ may have no immediate practical implications concerning the control of the pest, but it may prove important in the future. It is beginning to become apparent that the ion transport systems of at least some insects are based upon mechanisms different from those of vertebrates. Jungreis and Vaughan (1977) have found evidence that ion transport in the Lepidopteran midgut, unlike that of vertebrates, is insensitive to ouabain. The differences in mechanism may someday be exploited in the creation of insect-specific transport

inhibitors. When such control measures become a reality, the EB may become a vulnerable point in the biology of *R. pomonella*.

B I B L I O G R A P H Y

AHEARN, G.A., MAGINNIS, L.A., SONG, Y.K., and TORNQUIST, A., 1977. "Intestinal water and ion transport in freshwater Malacostracan prawns (Crustacea)." In Water Relations in Membrane Transport in Plants and Animals (Jungreis, A.M., Hodges, T.K., Kleinzeller, A., and Schultz, S.G., eds.), pp. 129-142. Academic Press, New York.

ALLEN, T.C., PINCKARD, J.A., and RIKER, A.J., 1934. "Frequent association of Phytomonas melophthora with various stages in the life cycle of the apple maggot, Rhagoletis pomonella." Phytopathol. 24, 228-238.

BAKER, J.R., 1947. "Histochemical recognition of phenols, especially tyrosine." Quart. J. Microsc. Sci. 88, 115-121.

BATEMAN, M.A., 1972. "The ecology of fruit flies." Annu. Rev. Entomol. 17, 493-518.

BECKER, N.H., NOVIKOFF, A.B., and ZIMMERMAN, H.M., 1967. "Fine structure observations on the uptake of intravenously injected peroxidase by the cat choroid plexus." J. Histochem. Cytochem. 15, 160-165.

BERRIDGE, M.J., and GUPTA, B.L., 1967. "Fine structural changes in relation to ion and water transport in the rectal papillae of the blowfly, Calliphora." J. Cell Sci. 2, 89-112.

BERRIDGE, M.J., and OSCHMAN, J.L., 1969. "A structural basis for fluid secretion by Malpighian tubules." Tissue and Cell 1, 247-272.

BERRIDGE, M.J., and PATEL, G., 1968. "Insect salivary glands: Stimulation of fluid secretion by 5-hydroxy-tryptamine and adenosine-3'5'-monophsphate." Science 162, 462-463.

BOLLER, E.F., and PROKOPY, R.J., 1976. "Bionomise and management of Rhagoletis." Annu. Rev. Entomol. 23, 223-246.

BRAY, G.A., 1960. "A simple, efficient liquid scintillator for counting aqueous solutions in a liquid scintillation counter." Anal. Biochem. 1, 279-285.

BUCHNER, P., 1965. Endosymbiosis of Animals with Plant Microorganisms, revised English edition. Interscience, New York.

CHEUNG, W.W.K., and MARSHALL, A.T., 1973. "Water and ion regulation in cicadas in relation to xylem feeding." J. Insect Physiol. 19, 1801-1816.

CHRISTENSON, W.D., and FOOTE, R.R., 1960. "Biology of fruit flies." Annu. Rev. Entomol. 5, 171-192.

CIAMPOR, F., and LÍBÍKOVÁ, H., 1975. "Ultrastructure of in vitro aged chick embryo cell cultures in relation to viral infection." In Cell Impairment in Aging and Development (Cristofalo, V.J., and Holečová, E., eds.), pp. 481-485. Plenum Press, New York.

CLAY, M.E., and VENARD, C.E., 1972. "The fine structure of the cesophageal diverticula in the mosquito Aedes triseriatus." Ann. Entomol. Soc. Amer. 65, 964-975.

CONN, H.J., 1953. Biological Stains, sixth edition. Biotech, Geneva (New York).

CONNOR, W.E., WILKINSON, C.F., and MORSE, R.A., 1978. "Penetration of insecticides through the foregut of the honeybee (Apis mellifera L.)." Pest. Biochem. Physiol. 9, 131-139.

COONS, L.B., and AXTELL, R.E., 1971. "Ultrastructure of the excretory tubes of the mite Machrocheles muscadomesticae (Mesostigmata, Macrochelidae) with notes on altered mitochondria." J. Morphol. 133, 319-338.

COSTERTON, J.W., INGRAM, J.M., and CHENG, K.J., 1974. "Structure and function of the cell envelope of Gram negative bacteria." Bacterial Rev. 38, 87-110.

DALLAI, R., 1977. "Comparative analysis of the hindgut in the species of Protura (Insecta, Apterygota)." J. Morphol. 151, 165-185.

DEAN, R.W., 1932. "The alimentary canal of the apple maggot, Rhagoletis pomonella Walsh (Dipt:Trypetidae)." Ann. Entomol. Soc. Amer. 25, 210-223.

DEAN, R.W., 1933. "Morphology of the digestive tract of the apple maggot fly, Rhagoletis pomonella Walsh." N.Y. State Agric. Expt. Sta. Bull. 215, 17 pp.

DEAN, R.W., 1935. "The anatomy and postpupal development of the female reproductive system in the apple maggot fly, Rhagoletis pomonella Walsh." Tech. Bull. N.Y. State Agric. Expt. Sta. 229, 7 pp.

DEAN, R.W., and CHAPMAN, P.J., 1973. "Bionomics of the apple maggot in eastern New York." Search Agriculture (N.Y. State Agr. Expt. Sta.) 3, 64 pp.

DEDUVE, C., and WATTIAUX, R., 1966. "Functions of lysosomes." Annu. Rev. Physiol. 28, 435-492.

DIAMOND, J.M., and TORMEY, J. MCD., 1966. "Role of long extracellular channels in fluid transport across epithelia." Nature 210, 817-820.

EISNER, T., 1955. "The digestion and absorption of fats in the foregut of the cockroach, Periplaneta americana L." Ann. Entomol. Soc. Amer. 44, 159-181.

FINLAYSON, L.H., and OSBORNE, M.P., 1968. "Peripheral neurosecretory cells in the stick insect (Carausius morosus) and the blowfly larva (Phormia terraenovae (sic.))." J. Insect Physiol. 14, 1793-1801.

FLORKIN, M., and JEUNIAUX, CH., 1964. "Hemolymph: composition." In The Physiology of Insecta (Rockstein, M., ed.), Vol. 3, pp. 110-152. Academic Press, New York.

FOGELSONG, M.A., WALKER, D.H., PUFFER, J.S., and MARKOVETZ, A.J., 1975. "Ultrastructural morphology of some prokaryotic organisms associated with the hindguts of cockroaches." J. Bacteriol. 123, 336-345.

GIROLAMI, V., 1973. "Reperti morfo-histologici sulle batteriosymbiosi del Dacus oleae Gmelin e di altri Tripetidi in natura e negli allevamente su substrati artificiali [Morphohistological findings on the bacteriosymbiosis of Dacus oleae Gmelin and other Trypetids in the field and artificial media rearing]." Redia 54, 269-294.

GOH, S., AND PHILLIPS, J.E., 1978. "Dependence of prolonged water transport by in vitro locust rectum on ion transport." J. Exp. Biol. 72, 25-41.

GRAHAM-SMITH, G.S., 1934. "The alimentary canal of Calliphora erythrocephala L. with special reference to its musculature, proventriculus, and its rectal valve and papillae." Parasitol. 26, 176-248.

GURR, E., 1956. A Practical Manual of Medical and Biological Staining Techniques. Leonard Hill, London.

GUYTON, A.C., 1961. Textbook of Medical Physiology, Second edition. Saunders, Philadelphia.

HAGEN, K.S., 1966. "Dependence of the olive fly, Dacus oleae, larvae on symbiosis with Pseudomonas savastanoi for the utilization of olive." Nature 209, 423-424.

HAUPT, A.W., 1920. "A gelatin fixative for paraffin sections." Stain Technol. 5, 97.

HELLMUTH, H., 1956. "Untersuchungen zur Bakteriensymbiose der Trypetiden (Diptera) [Investigation of the bacterial symbiotes of Trypetidae (Diptera)]." Z. Morphol. Okol. Tiere 44, 483-517.

HOFFMAN, A.G.D., and DOWNER, R.G.H., 1976. "The crop as an organ of glyceride absorption in the American cockroach, Periplaneta americana L." Can. J. Zool. 54, 1165-1171.

HUMASON, G.L., 1962. Animal Tissue Techniques. L.H. Freeman, San Francisco.

JUNGREIS, A.M., and VAUGHAN, G.L., 1977. "Insensitivity of lepidopteran tissues to ouabain: absence of ouabain binding and Na^+-K^+ ATPases in larval and adult midguts." J. Insect Physiol. 23, 503-509.

KJAERHEIM, A., 1968. "Studies of adrenocortical ultrastructure. 4.. Effects of ACTH on interrenal cells of the domestic fowl." J. Microscopie 7, 715-738.

KNELL, J., and STOFFOLANO, J.G., 1973. "Histology of the third instar of the apple maggot, Rhagoletis pomonella." Ann. Entomol. Soc. Amer. 66, 1221-1238.

LOCKE, M., 1976. "The role of plasma membrane plaques and Golgi complex vesicles in cuticle deposition during the moult-intermoult cycle." In The Insect Integument (Hepburn, H.R., ed.), pp. 237-258. Elsevier, Amsterdam.

LORD, B., and DIBONA, D.R., 1976. "Role of septate junction in the regulation of paracellular transepithelial flow." J. Cell. Biol. 71, 967-972.

MADDRELL, S.H.P., 1971. The mechanisms of insect excretory systems." Adv. Insect Physiol. 8, 199-331.

MADDRELL, S.H.P., and GARDINER, B.C.C., 1974. "The passive permeability of insect Malpighian tubules to organic solutes." J. Exp. Biol. 60. 641-652.

MANTEL, L.M., 1968. "The foregut of Gecarcinus lateralis as an organ of salt and water balance." Am. Zoologist 8, 433-442.

MARSHALL, A.T., and CHEUNG, W.W.K., 1973. "Studies in water and ion transport in homopteran insects. Ultrastructure and cytochemistry of the cicadoid and cercopoid hindgut." Tissue and Cell 5, 671-678.

MCCOWEN, R.P., CHENG, K.-J., BAILEY, C.B.M., and COSTERTON, J.W., 1978. "Adhesion of bacteria to epithelial cell surfaces within the reticulo-rumen of cattle." Appl. Environ. Microbiol. 35, 149-155.

MCMANUS, J.F.A., and MOWRAY, R.W., 1960. Staining Methods, Histologic and Histochemical. Hoeber, New York.

MILLER, A., 1965. "The internal anatomy and histology of the imago of Drosophila melanogaster." In Biology of Drosophila (Demerec, M., ed.), pp. 420-534. Hafner, New York.

MILLS, R.R., WRIGHT, R.D., and SAUER, J.R., 1970. "Midgut epithelium of the American cockroach: Probable mechanisms of water secretion." J. Insect Physiol 16, 417-427.

MIYAZAKI, S., BOUSH, G.M., and BAERWALD, R.J., 1968. "Amino acid syntheiss by Pseudomonas melophthora, bacterial symbiote of Rhagoletis pomonella (Diptera)." J. Insect Physiol. 14, 513-518.

NEILSON, W.T.A., 1965. "Culturing the apple maggot, Rhagoletis pomonella." J. Econ. Entomol. 58, 1056-1057.

NOIROT, C., and NOIROT-TIMOTHEÉ, C., 1976. "Fine structure of the rectum in cockroaches (Dictyoptera): General organization and intercellular junctions." Tissue and Cell 8, 345-368.

OSCHMAN, J.L., and BERRIDGE, M.J., 1971. "The structural basis of fluid secretion." Federation Proc. (Wash.) 30, 49-59.

PEARSE, A.G.E., 1968. Histochemistry, Theoretical and Applied, third edition. Little, Brown, Boston.

PETRI, L., 1910. "Untersuchungen uber die Darmbakterien der Olivenfliege [Investigation of the gut bacteria of the olive fly]." Zentralbl. Bakteriol. Parasitenk. Infekt. II. 26, 357-367.

PHILLIPS, J.E., 1964. "Rectal absorption in the desert locust, Schistocerca gregaria Forskal. I. Water." J. Exp. Biol. 41, 15-38.

PHILLIPS, J.E., 1969. "Osmotic regulation and rectal absorption in the blowfly, Calliphora erythrocephala." Can. J. Zool. 47, 851-863.

PHILLIPS, J.E., 1977a. "Excretion in insects: function of gut and rectum in concentrating and diluting the urine." Federation Proc. (Wash.) 36, 2480-2486.

PHILLIPS. J.E., 1977b. "Problems of water transport in insects." In Water Relations in Membrane Transport in Plants and Animals (Jungreis, A.M., Hodges, T.K., Kleinzeller, A., and Schultz, S.G., eds.), pp. 333-354. Academic Press, New York.

POINAR, G.O. JR., HESS, R.T., and TSITSIPAS, J.A., 1975. "Ultrastructure of the bacterial symbiotes in the pharyngeal diverticulum of Dacus oleae (Gmelin) (Trypetidae; Diptera)." Acta Zoolog. 56, 77-84.

PROKOPY, R.J., and BOLLER, E.F., 1970. "Artificial egging system for the European cherry fruit fly." J. Econ. Entomol. 63, 1414-1417.

RAMSAY, J.A., 1954. "Active transport of water by the Malpighian tubules of the stick insect, Dixippus morosus (Orthoptera, Phasmidae)." J. Exp. Biol. 32, 183-199.

REYNOLDS, E.S., 1963. "The use of lead citrate at high pH as an electron-opaque stain in electron microscopy." J. Cell Biol. 208-212.

ROCKSTEIN, M., and MIQUEL, J., 1973. "Aging in insects." In The Physiology of Insecta (Rockstein, M., ed.), Vol. 1, pp. 371-478. Academic Press, New York.

RUNHAM, N.W., 1961. "Investigations into the histochemistry of chitin." J. Histochem. Cytochem. 9, 87-92.

SAVAGE, D.C., and BLUMERSHINE, R.V.H., 1974. "Surface-surface associations in microbial communities populating epithelial habitats in the murine gastrointestinal ecosystem. Scanning electron microscopy." Infec. Immun. 10, 240-250.

SCHULTZ, G.A., 1970. "A comparative morphological study of five species of tephritids, with special emphasis on possible mycetomes and sex pheromone producing glands." M.S. Thesis, University of Wisconsin.

SMITH, D.S., 1968. Insect Cells. Their Structure and Function. Oliver and Boyd, Edinburgh.

SNODGRASS, R.E., 1924. "Anatomy and metamorphosis of the apple maggot, Rhagoletis pomonella Walsh. J. Agric. Res. 38, 1-36.

SOHAL, R.S., and COPELAND, E., 1966. "Ultrastructural variations in the anal papillae of Aedes aegypti (L.) at different environmental salinities." J. Insect Physiol. 12, 429-439.

STAMMER, H.J., 1929. "Die Bakteriensymbiose der Trypetiden (Diptera) [Bacteriosymbioses of the trypetids (Diptera)]." Z. Morphol. Okol. Tiere 15, 481-523.

STOBBART, R.H., and SHAW, J., 1964. "Salt and water balance: excretion." In The Physiology of Insecta (Rockstein, M., ed.), Vol. 3, pp. 190-258. Academic Press, New York.

SUNDMAN, J.A., and KING, D.R., 1964. "Morphological, histological, and histochemical studies of the alimentary canal and Malpighian tubules of the adult boll weevil, Anthonomus grandis." Ann. Entomol. Soc. Amer. 57, 89-95.

TREHERNE, J.E., 1962. "The physiology of absorption from the alimentary canal in insects." In Viewpoints in Biology (Carthy, J.D., and Duddington, C.L., eds.), Vol. 1, pp. 201-243. Butterworth's, London.

WALL, B.J., 1971. "Local osmotic gradients in the rectal pads of an insect." Federation Proc. (Wash.) 30, 42-48.

WALL, B.J., 1977. "Fluid transport in the cockroach rectum. In Transport of Ions and Water in Animals (Gupta, B.L., Moreton, R.B., Oschman, J.L., and Wall, B.J., eds.), pp. 599-612. Academic Press, New York.

A P P E N D I X

NUMERICAL CHANGES IN POPULATIONS OF GUT BACTERIA DURING THE DEVELOPMENT OF *RHAGOLETIS POMONELLA*

Histological results (Introduction; Chapter II) indicated that the EB is the site of the earliest detectable appearance of gut microflora in the pupa of *R. pomonella*, and of the heaviest bacterial concentrations throughout life. These findings were tested in a cultural study.

Late chromogenic pupae, and adults aged 24 hr, and 1, 2, or 4 weeks were selected for this study. They were surface-sterilized with 1% Zephiran chloride and opened under sterile saline, in a laminar-flow hood. Segments of the gut (EB, esophagus, ventriculus, crop, or hindgut) were tied off with nylon monofilament and removed. Each segment was surface-sterilized with 0.5% $HgCl_2$ and placed in a tube containing 1 ml of sterile nutrient broth (Difco). If the tube showed no turbidity after 24 hr at 25°C, the gut segment was broken and thoroughly crushed with a sterile glass rod. The suspension was diluted 1:10 and 1:100 with warm, sterile nutrient agar (Difco) and poured into disposable, 10 cm Petri dishes (Fisher). After 24-36 hr further incubation,

colonies were counted. No attempt at identification of species was made. Six replicates were performed for each gut segment at each developmental stage. The results are displayed in Table 6.

At the earliest developmental stage at which cultures could be successfully performed (late chromogenic pupa), bacteria are already present in all portions of the gut. Hence, it was not possible to test the hypothesis that they appear first in the EB. Nor is the EB the site of the largest bacterial population, in absolute terms [except, to a small degree, during the late chromogenic stage (Table 6A)]. It is clear, however, that the highest bacterial densities (i.e. bacteria per unit volume) are indeed to be found in the EB throughout the developmental period studied. Densities exceed those in other gut segments by a factor of at least 7 (Table 6B).

It should be noted that this experiment did not consider anaerobic bacterial populations, nor those bacteria with special nutritional requirements. As far as aerobic bacteria with general nutritional requirements are concerned, the EB seems to be a particularly supportive environment.

Table 6. Numerical changes in populations of bacteria in five segments of the gut of R. pomonella from late pupal development to senescence. A. Absolute numbers of bacteria in each segment, estimated from numbers of single colonies cultures. B. Population densities of bacteria for each segment.

Gut Segment	A. Number of Bacteria Present (S.E.) Late Chromogenic	24-hr Adult	1-week Adult	2-week Adult	4-week Adult
EB	86.2 23.1 ±	71.8 26.8	118.9±39.4	159.2± 50.3	244.7± 73.6
Esophagus	39.9 14.5 ±	57.0 10.9	84.1±32.2	123.0± 50.9	162.6± 32.4
Ventriculus	33.7 8.0 ±	41.2 13.6	125.8±39.4	163.7± 42.2	302.4±577.1
Crop	21.4 7.5 ±	86.7 33.1	211.0±64.6	376.5± 70.5	785.8±181.2
Hindgut	17.8 6.2 ±	395.8 30.8	325.5±86.7	410.2±106.8	653.1±109.8

B. Number of Bacteria/Estimated Volume (mm^3)

Gut Segment	Late Chromogenic	24,hr Adult	1-week Adult	2-week Adult	4-week Adult
EB	28,733.3±7,708.0	25,666.7±8933.5	39,600.0±15,113.3	53,066.2±16,766.7	81,566.7±24,533.6
Esophagus	2,216.7± 805.6	3,166.7± 607.5	4,672.2± 1,788.9	6,833.7± 2,827.8	9,055.3± 4,022.2
Ventriculus	139.8± 33.2	170.9± 56.4	521.9± 163.5	679.3± 211.2	1,253.1± 236.9
Crop	48.3± 16.9	195.7± 74.7	476.3± 145.8	839.9± 159.1	1,772.0± 409.0
Hindgut	370.8± 129.2	1,995.8± 641.7	6,802.1± 1,806.3	8,592.9± 2,225.0	13,583.3± 2,285.4