Wilhelm Roux' Archiv 169, 216–238 (1972)

Puffs and Salivary Gland Function: The Fine Structure of the Larval and Prepupal Salivary Glands of *Drosophila melanogaster*

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Received September 3, 1971

Summary. The salivary glands of *Drosophila melanogaster* have been examined by electron microscopy for fine structural alterations occurring during larval and prepupal stages. The changes observed in the glands have been correlated with the puffing patterns of the polytene chromosomes at corresponding stages. In early third instar larvae, the lumen of the salivary gland appears empty, and no signs of secretory activity are visible in the glandular cytoplasm. From puff stages 1 to 6 the endoplasmic reticulum becomes reorganized and increases in volume. Electron dense material appears within its cisternae and subsequently within the Golgi saccules. Dense secretory granules then appear to be elaborated from the Golgi by terminal budding; these granules represent the 'glue' for adhering the pupa to its substrate, and gradually increase in size and complexity. By puff stage 6 their contents have been liberated into the glandular lumen. Following puparium formation, those granules which are not extruded coalesce to form larger granules. Other dense bodies and autophagic vacuoles, considered to be lysosomes, appear, and the 'surplus' secretory granules begin to display myelination at their peripheries; ultimately they are reduced to dense residual bodies. At puparium formation, the lumen is depleted of the glue and contains flocculent material. Histolysis commences after puff stage 11, and the cytoplasm becomes vacuolated and opaque; the nucleus becomes reduced in volume and crenated in outline. Nuclear blebbing occurs after puff stage 12, and material seemingly moves from the nucleus into the cytoplasm; the glandular lumen now becomes empty. An attempt has been made to ascertain how the chromosomal puffing activity relates to these cytoplasmic developments.

Introduction

The polytene chromosomes characteristic of certain larval tissues of the Diptera are convenient for the study of gene activity during development and its control. Active genetic sites are often distinguishable from inactive sites by the local modification of the normal banded structure of the polytene chromosome into a diffuse structure known as a puff. The hypothesis (Beermann, 1956) that puffs represent active genes is widely supported (reviewed by Clever, 1968; Panitz, 1968; Berendes and Beermann, 1969; Ashburner, 1970), yet our understanding of the phenomenon of puffing has suffered from too little consideration of its physiological significance.

The purpose of this paper is to describe the changes in ultrastructure of the larval salivary gland of *Drosophila melanogaster* which accompany the changes

in puffing activity of the salivary gland polytene chromosomes. Although morphological studies of the ultrastructure of the larval salivary glands of *Drosophila* species (Gay, 1955b; 1956; Berendes and de Bruyn, 1963; Berendes, 1965; Rizki, 1967; Tulchin and Rhodin, 1970; Smith and Witkus, 1970), of *Chironomus* (Beermann, 1961; Kloetzel and Laufer, 1969, 1970), *Simulium* (MacGregor and Mackie, 1967), *Sciara* (Phillips and Swift, 1965), *Acricotopus* (Döbel, 1968), *Smittia* (Jacob and Jurand, 1965) and *Bradysia* (Jacob and Jurand, 1963a, 1963b) have been published, few authors have attempted to correlate the changes in ultrastructure observed during development with the changes in nuclear activity. Further, no comprehensive account of the ultrastructure of the *Drosophila* salivary gland has hitherto been published.

Previous Studies on Drosophila Salivary Gland Structure and Function

The larval salivary glands of *Drosophila* are paired, rather sac-like organs connecting anteriorly to a common duct that opens into the pharynx. Their early development has been described by Sonnenblick (1939, 1950) and Poulson (1950) and the general aspects of their post-embryonic development by Ross (1939) and Bodenstein (1943, 1950).

The salivary glands are first visible at the eighth hour of embryogenesis as a pair of invaginations of the lateroventral ectodermal plate. By the eleventh hour they have differentiated into glandular and duct regions. Indications of secretory activity, such as cytoplasmic vacuolation and a haemotoxylin-staining metachromatic lumen, have been reported for the glands of 12 h. embryos (Sonnenblick, 1950) although the function of this secretion is unknown. The subsequent growth of the glands results entirely from an increase in cell size (Makino, 1938). No mitoses have been observed in the salivary glands subsequent to their first recognition in the embryo. In *D. melanogaster* the number of gland cells (excluding the duct and imaginal ring) is usually between 120 and 140 per lobe (see Altmann, 1966).

Growth of the gland continues until the late third instar and at this stage each lobe is about 1 mm in length and 0.1 mm diameter (data of Ross, 1939, from fixed material). The function of the gland during embryonic and early larval development (up to the early third instar) is unknown. It has been commonly assumed (Ross, 1939; Bodenstein, 1950; Gay, 1956; Hsu, 1948; Berendes, 1965; Tulchin and Rhodin, 1970) that it plays a role, as its name might suggest, in ingestion and digestion. We know of no evidence for this assumption (see discussion in Ashburner, 1970).

The only established function of the larval salivary gland is the secretion, during the third instar, of the puparial 'glue'. It has frequently been observed in several *Drosophila* species that, after approximately one third of the third larval instar, the most distal cells of the salivary gland show clear signs of secretory activity (Ross, 1939; Bodenstein, 1943, 1950). PAS-positive granules accumulate in the cytoplasm of these cells (Lesher, 1953; Gay, 1955b; 1956; McMaster-Kaye and Taylor, 1959; Berendes, 1965). As development proceeds, an increasing proportion of the cells contain these secretory granules, yet in *D. hydei* (Berendes, 1965) and in *D. melanogaster* at least the most anterior gland cells remain free of granules. A few hours before puparium formation the gland lumen becomes bloated with a PAS-positive secretion which the animal expectorates at puparium formation. The function of the secretion was misunderstood until Fraenkel and Brookes (1953) discovered that it serves as a 'glue' which affixes the pupal case to its substrate.

Following puparium formation the larval salivary gland commences histolysis (Ross, 1939; Bodenstein, 1943). According to the observations of Bodenstein (1950), histolysis commences about 5 h. after puparium formation in the distal cells; the gland completely disappears after pupation (see Lockshin, 1969).

Changes in Puffing Activity during the Third Instar

During the third instar dramatic changes in the pattern of gene activities, seen as changes in the pattern of puffing, occur in the polytene chromosomes of gland nuclei. In young third

instar larvae the puffing pattern of the polytene chromosomes is relatively simple; only 10 or so chromosome sites form prominent puffs. This pattern persists from the earliest age at which the chromosomes can be critically studied (80–90 h. larvae, timed from egg laying) until about 10 hours before puparium formation. Puparium formation occurs at 120 hours at 25° C. At approximately 110 hours there is initiated the sequential induction of a large number of puffs involving at least 125 specific chromosome loci (Becker, 1959; Ashburner, 1967, 1969, 1972). The induction of this sequence is the result of an increase in the haemolymph titre of ecdysone (Clever, 1961; Becker, 1962; Berendes, 1967; Ashburner, 1971) and a peak in puffing activity is reached at puparium formation. Following puparium formation there is a decline in the number of puffed sites and the period from two to six hours after puparium formation is one of relatively low puffing activity. Later there is a second very active period of puffing, in 8 hour prepupae, followed by a second reduction in puffing towards the time of pupation, at 12 hours after puparium formation.

These changes in puffing activity are continuous and at all times involve the activation and regression of specific puffs. It is convenient to divide the sequence into 21 discrete Puff Stages (PS) (Becker, 1959; Ashburner, 1967, 1972) each characterised by a particular pattern of puffed loci. The major events of the animal's development during this period are correlated with particular puff stages. The initial stage in early third instar larvae when the PAS granules are first seen in the cytoplasm is defined as Puff Stage 1 (PS 1). Following the ecdysone induction, the salivary gland lumen becomes visibly bloated with secretion at PS 5–6 and puparium formation occurs at PS 10–11. In the prepupae the abdominal air bubble is first seen in PS 14 animals and the pupal moult occurs at PS 21.

Materials and Methods

For this study the Canton-S 'wild type' strain of *D. melanogaster* was used. Larvae were cultured in 200 cc bottles at 25° C on yeast-glucose medium (Ashburner, 1967) with the addition of Nipagin to inhibit mould growth. Salivary glands of third instar larvae or prepupae were dissected in a saline medium (Becker, 1959) and the two lobes of the gland rapidly separated by cutting the common duct. One lobe was fixed for electron microscopy and its contralateral (sister) lobe for cytological examination.

For electron microscopy the glands were fixed either in Karnovsky's (1965) formaldehyde-glutaraldehyde or in 3% glutaraldehyde in 0.1 M cacodylate buffer at pH 7.4 (Sabatini, Bensch and Barrnett, 1963), with or without added 0.2 M sucrose. The tissues were fixed for 1–2 hours at room temperature, then washed in three changes of cacodylate buffer plus 0.2 M sucrose, treated in 1% osmium tetroxide in 0.1 M cacodylate buffer for one hour at room temperature, dehydrated through an ascending series of ethanol to propylene oxide, and embedded in Araldite. Sections for light microscopy were cut at 1–2 μ and stained with a 1% solution of toluidine blue in 1% borax. Ultrathin sections were cut on an LKB Ultratome III, stained with uranyl acetate (Watson, 1958) and lead citrate (Reynolds, 1963) and examined in a Philips EM 300 operated at 60 or 80 Kv.

For cytological examination the glands were fixed in propionic acid:ethanol (1:3) and their chromosomes stained with propionic-orcein-carmine (Ashburner, 1967). Puff stages of the polytene chromosomes were assigned according to the tables previously published (Ashburner, 1972). For all except the youngest glands studied the electron microscopic and cytological observations were made on sister gland pairs. The puffing patterns of the two lobes of the gland from a single individual are identical (Ashburner, 1971). Since the rate of larval development is rather variable, even under the best culture conditions, determination of puffing pattern affords a much more accurate indication of developmental stage than does age.

The youngest salivary glands studied, estimated to be from larvae approximately 70 and 80 hours after oviposition, were too small for cytological analysis and both lobes were fixed for electron microscopical examination. Except for these glands, which we call collectively 'young third instar glands', all preparations will be identified by the PS of their contralateral lobe.

For PAS staining whole salivary glands were fixed in 3:1 propionic acid:ethanol, washed with distilled water and oxidised for 2′ with 0.5% aqueous periodic acid. After a further wash in distilled water the glands were stained with Schiff's reagent and mounted in water. Sister glands were examined for their puffing acitivity.

Observations

PAS Observations on Whole Glands

Except for a few large PAS-positive granules in all cells, the salivary glands from young third instar larvae are generally PAS-negative (Fig. 1a). By 90 hours the distal cells of the gland are packed with PAS-positive material (Fig. 1b) which is granular in nature and corresponds in size to the glue secretory granules seen under the electron microscope. As development proceeds, more and more cells of the gland come to contain PAS-positive granular material until all but the most proximal 20 to 25 cells are PAS-positive (Fig. 1c). These proximal cells never develop accumulations of PAS-positive material.

After PS 5, a PAS-positive substance fills the lumen of the gland (Fig. 1d). Following puparium formation the lumen and the gland cells stain considerably less intensely with PAS. On closer examination it can be seen that all but the most proximal gland cells still contain a few large PAS-positive glue granules (Fig. 1e). In addition to their size, these granules differ from the glue granules of the larval gland by their irregular shape. These large granules persist until PS 18. After this stage of development the cytoplasm of all gland cells is generally PAS-negative, except for a few large PAS-positive bodies in the anterior gland (Fig. 1f). These appear to be similar to those granules observed in larval glands prior to glue secretion.

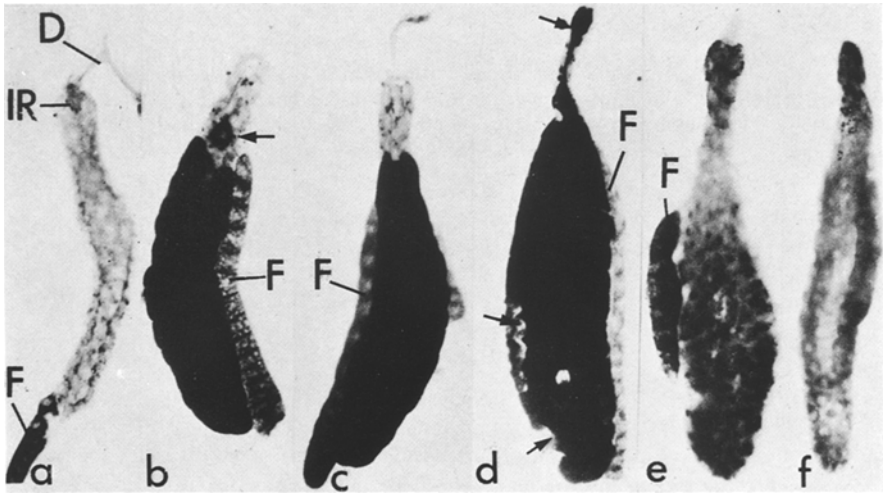


Fig. 1a–f. Light micrographs of PAS-stained whole salivary glands. (a) 80 hr. third instar larva; except for a few PAS-positive bodies the gland is PAS negative before glue granule secretion. (b) PS 1 larva. Almost all cells are packed with PAS-positive material although in the anterior of the gland a few cells surrounding the isolated PAS-positive cell (arrow) have yet to secrete. (c) Older PS 1 larva. All except the most anterior cells are now full of PAS-positive glue material. (d) PS 9–10 larva. The gland lumen is full of glue (upper arrow) and some cells in the posterior of the gland have lost most of their PAS-positive material (lower arrows). (e) PS 13, 4 hr. prepupa. The cells contain a few, large, irregular clumps of PAS-positive material that are 'left over' after glue extrusion into the lumen. (f) PS 21, 12 hr. prepupa. The cells are now again PAS-negative. The PAS-positive material in the most anterior cells, present in earlier stages, does, however, remain. Abbreviations: *F*, fat body; *IR*, adult salivary gland imaginal ring; *D*, duct. Figs. a, b, c, e, f, ×60; d, ×42

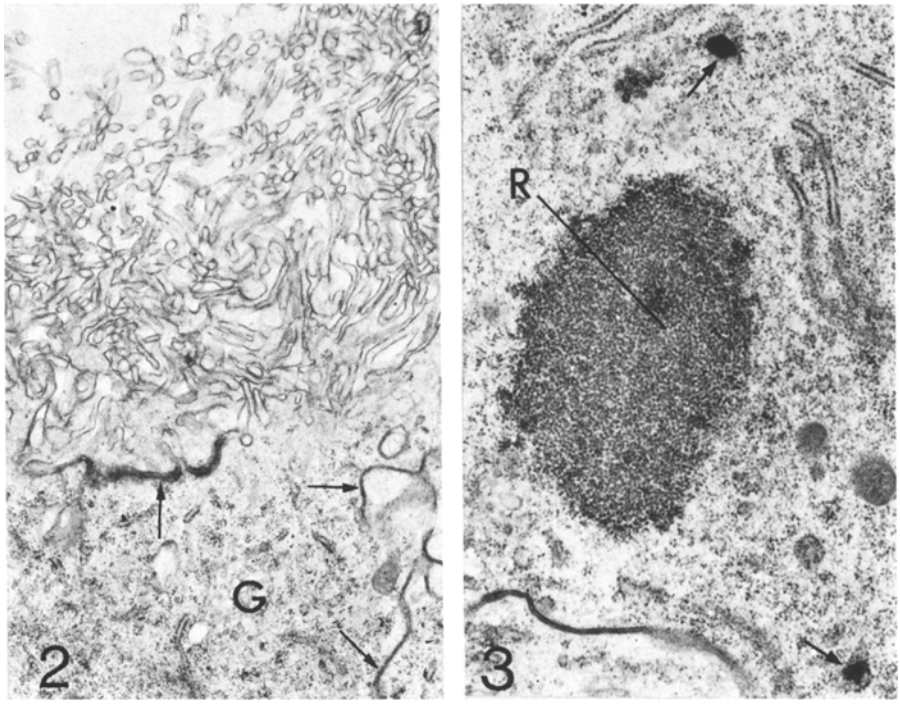


Fig. 2. Luminal surface of a gland cell from a young third instar larva in which cytoplasmic processes project into the empty lumen. Note the junctions lying laterally between adjacent cells (arrows). Ribosomes are present in abundance, although not usually associated with cisternae of endoplasmic reticulum. The Golgi complexes (*G*) are fairly inconspicuous at this stage. × 14000

Fig. 3. Gland cell from a young third instar larva, somewhat older than that of Fig. 2, containing massive aggregations of ribosomes (*R*). Cisternae of endoplasmic reticulum are more prominent than previously and may contain electron dense material (arrow). × 23400

Young Third Instar Larval Glands

In young third instar larvae, the lumina of the salivary glands appear empty (Fig. 2). The cytoplasmic processes projecting from the cells into the luminal space are less regular in length and organisation than microvilli (Fig. 2). Beneath these projections and at right angles to them lies a layer of fibrous material together with some microtubules. The basement membrane around the periphery of the gland is not highly specialised, lacks collagen fibres, and consists simply of a thin layer of acellular fibrous matrix. The plasma membrane lying adjacent to this basement membrane is thrown into a series of irregular folds which do not, however, extend to any great depth into the basal cytoplasm. The lateral junctions of adjacent cells are highly complex and interdigitated (Fig. 2), displaying zonula adherens at the luminal surface and septate desmosomes elsewhere; microtubules are commonly found in close spatial association with these cell junctions. Unlike the other intracellular organelles, these features of the salivary gland plasmalemma remain fairly constant throughout the larval stages.

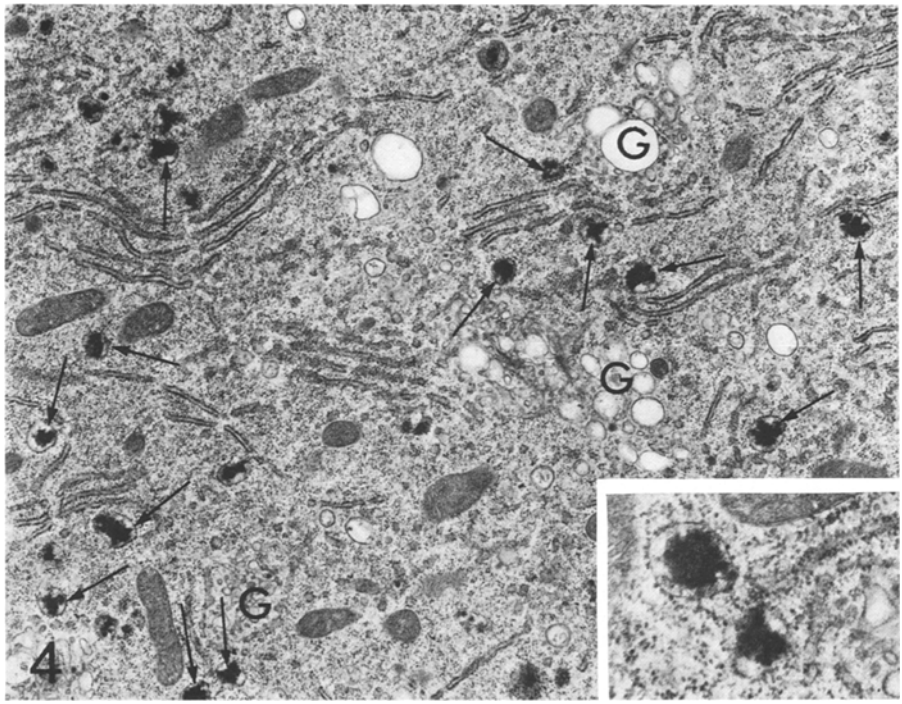
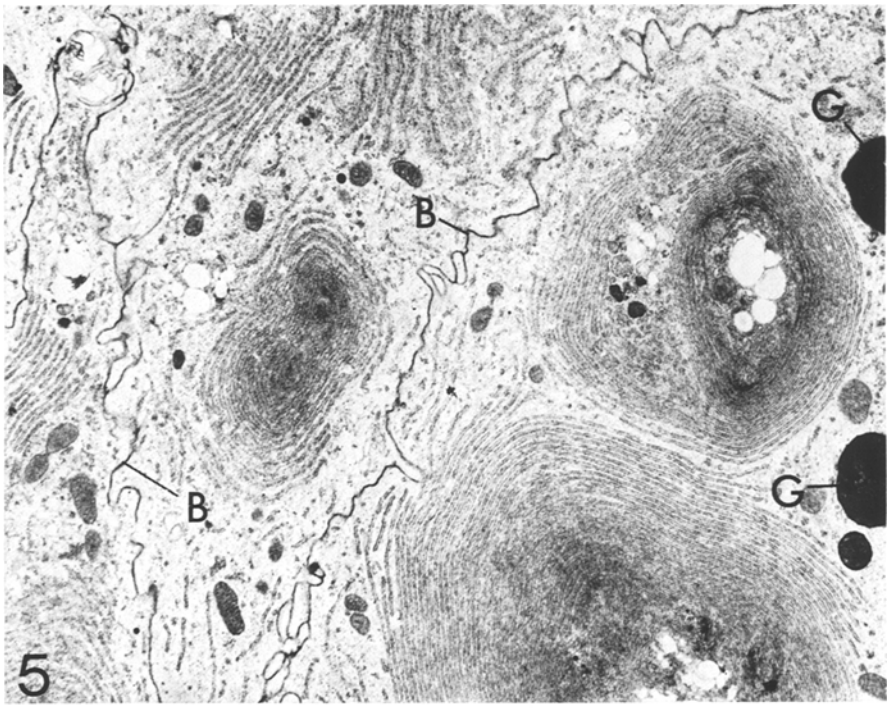
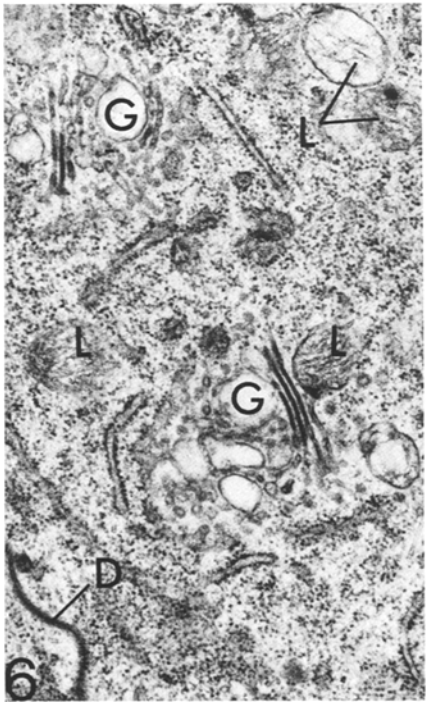
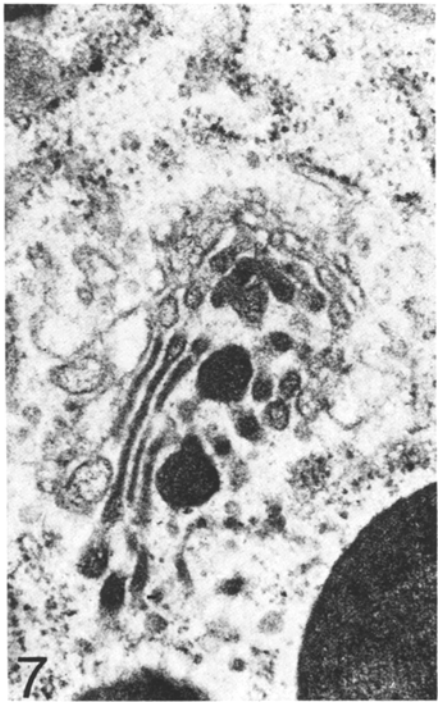


Fig. 4. Gland from a young third instar larva, older than those of Figs. 2 and 3, showing an increased volume of endoplasmic reticulum, in some cases arranged in stacks, or containing electron opaque material (arrows). The Golgi apparatus (*G*) is also prominent. × 15380. *Insert:* A portion of this gland enlarged to show the ribosome-studded membranes of endoplasmic reticulum around the dense, intra-cisternal material. × 37600

In these young third instar larvae the salivary gland cell nuclei contain large granular nucleoli, which are highly irregular in outline. The cytoplasm contains many large, irregularly-shaped mitochondria and numerous free ribosomes. There are some ribosome-studded cisternae of endoplasmic reticulum, but these are relatively sparse and scattered at random throughout the cell. However, in localised regions of the cytoplasm, that tend to be near the Golgi complex, some cisternae of the endoplasmic reticulum are distended into vesicles which may contain a fibrous component. Of the profiles of this dilated endoplasmic reticulum, some of the membranes are only partly ribosome-studded, the smooth surface facing the Golgi. In some cases the dilated vesicles of rough endoplasmic reticulum contain clumped electron dense material (Fig. 4); these also appear most frequently in the vicinity of the Golgi. Small smooth-surfaced bodies containing fibrous material are also encountered (Fig. 6); these may represent lysosomes or lysosome precursors. At this stage the Golgi complex is extensive, and is localised in the form of 'dictyosomes' throughout the cytoplasm (Fig. 4). It consists of smooth vesicles and large vacuoles as well as some associated fenestrated membranes. Two to three stacks of flattened saccules become apparent later, when one or two of the Golgi saccules and some small Golgi vesicles begin to contain







Figs. 5–7

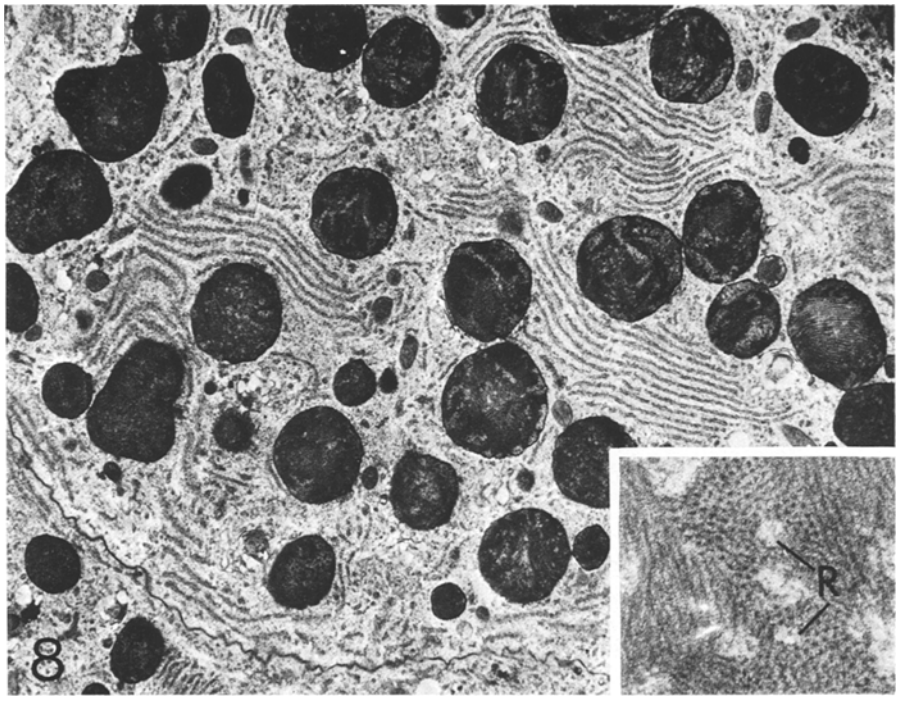


Fig. 8. Gland cell typical of PS 1–6 containing numerous dense glue granules of varying sizes. Stacked cisternae of endoplasmic reticulum also occur. × 6400. *Insert:* Portion of a secretory granule from a PS 2 larva illustrating the two component parts, paracrystalline arrays and 'rosettes' (*R*). × 51000

electron dense material (Fig. 6). In some cells, usually near the lumen, areas containing what appear to be masses of free ribosomes are found (Fig. 3).

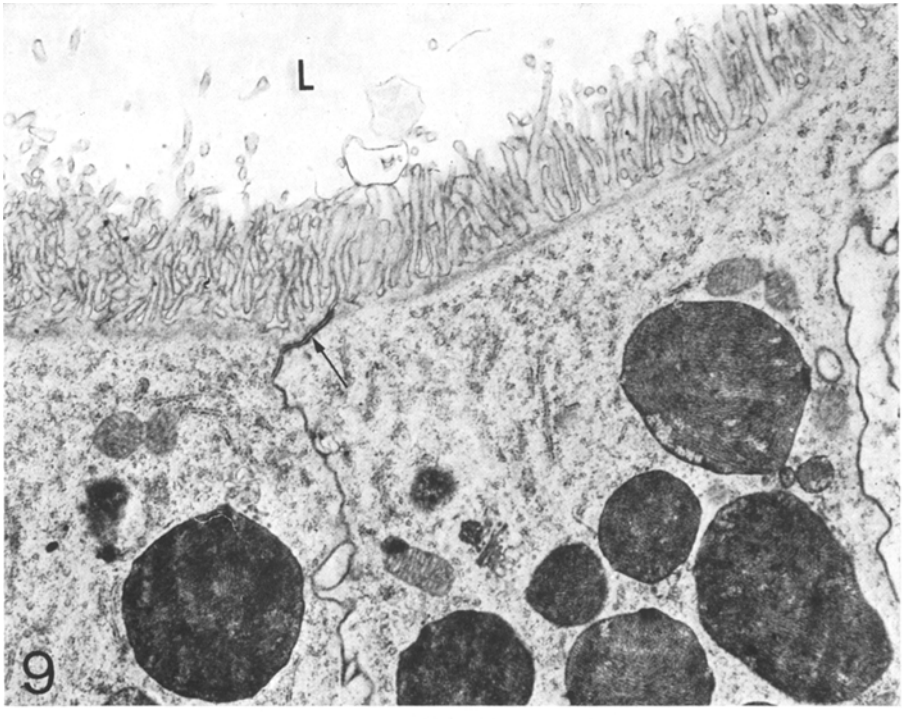
PS 1 to PS 5 Glands

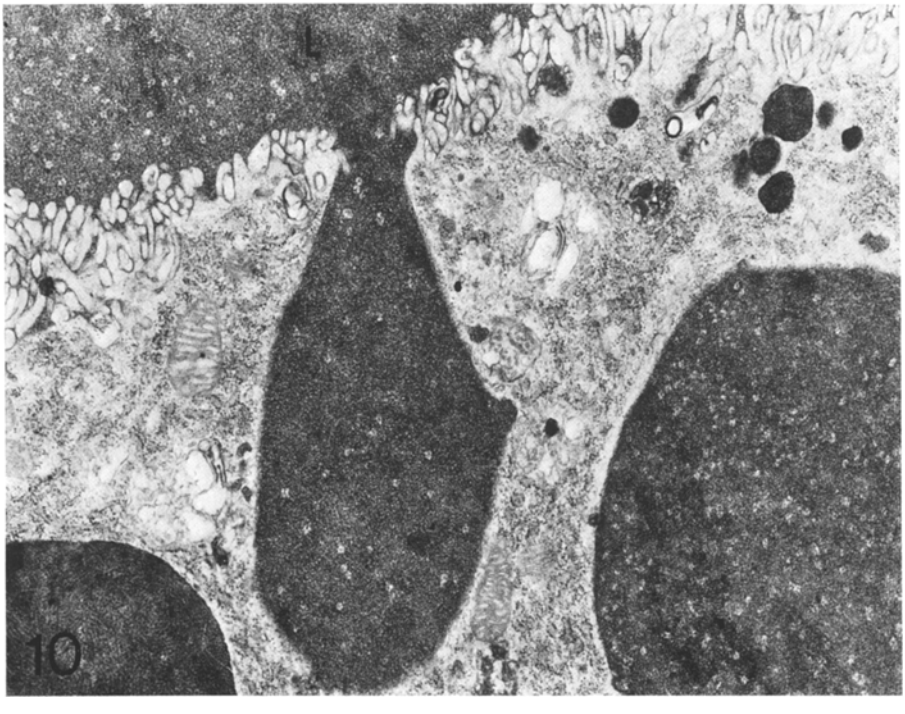
In slightly more mature glands, from puff stage 1 larvae, the cells are radically altered in appearance. The nuclei are large and contain recognizably banded polytene chromosomes. The rough endoplasmic reticulum is abundant and is present in one or two forms, either as parallel-arranged stacks of cisternae, or as concentric whorls of cisternae (Fig. 5). In these whorls the inner cisternae are more closely

Fig. 5. Gland in PS 1 showing the extensive reorganization of the endoplasmic reticulum in the form of whorls and stacks. By this time some dense secretory granules have already been formed (*G*). *B*, cell borders where desmosomes occur. × 8000

Fig. 6. Golgi complexes (*G*) from young third instar larva containing dense material within their saccules prior to granule formation. Fibrous granules (*L*), which may be an early form of lysosome, are present. *D*, Septate desmosome between adjacent cells. × 26500

Fig. 7. Golgi complex from a PS 1 gland. The saccules contain electron dense material and appear to be the site of condensation of the opaque secretory granules. A larger granule, elaborated earlier, lies nearby. × 49900





Figs. 9 and 10

packed than the peripheral ones; polysome-like arrays are frequently seen in association with their surfaces. The whorls contain one or more of a variety of organelles in their core; these range from mitochondria, vacuoles and dense droplets, probably lipid in nature, to masses of ribosomes and polysome-like arrays.

At this stage, both saccules and granules of the Golgi complex contain dense material (Fig. 7); the saccules appear to be engaged in elaborating the small electron dense granules by terminal budding. These small granules seem to be the precursors of larger dense ones as graded series of such organelles can be found in the cells, ranging from very small ones, associated spatially with the smooth membranes of the Golgi, to intermediate sized and large granules, up to 3 microns in diameter, lying scattered through the cytoplasm (Figs. 8 and 9). These dense granules contain a characteristic internal structure consisting of crystalline-like material randomly arranged in small aggregations with a periodicity of approximately 10 nm; this material can be seen cut in longitudinal, tangential or cross section (see Fig. 8 insert). In cross section a single strand measures 10–14 nm. They also contain a second component, which is more prominent at later stages in the development of the gland. This second structural constituent is a rosette-like or star-shaped material which is electron lucent, but which can be seen outlined against the relatively denser backround material comprising the rest of the granular matrix. That these granules are a secretory product for storage and subsequent release is shown by the fact that after puff stage 5, the lumen of the gland, previously empty (Fig. 9), contains dense material with 'rosettes' of the same nature as the granules' 'rosette' material (Fig. 10). Indeed, granules containing this material appear to fuse with the luminal plasma membrane, seemingly extruding their contents by exocytosis (Fig. 10).

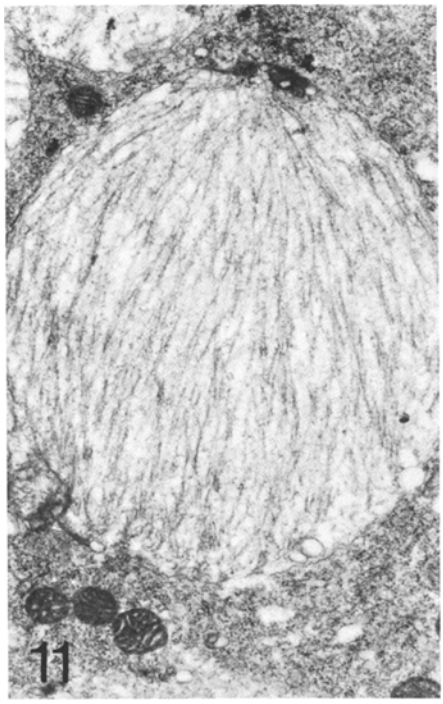
In stained preparations, the cells during these stages appear either 'light' or 'dark' in that their ground cytoplasm stains lightly or heavily with heavy metals. However, no functional significance can be attached to this differential staining. A similar observation was made by Wiener, Spiro and Loewenstein (1964) in salivary glands of *D. flavorepleta*.

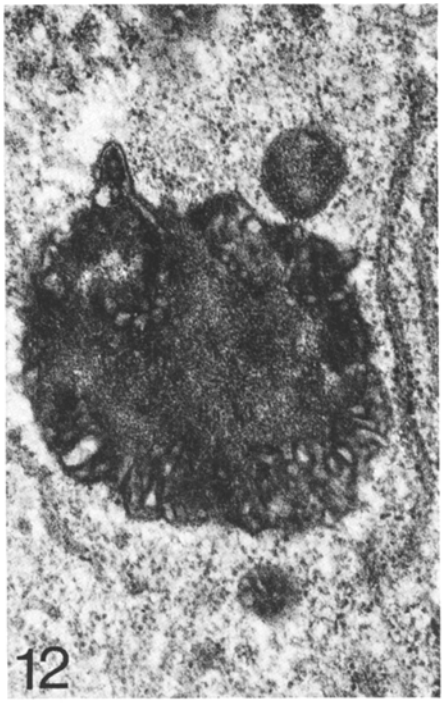
Late Larval Stages (PS 6–PS 10)

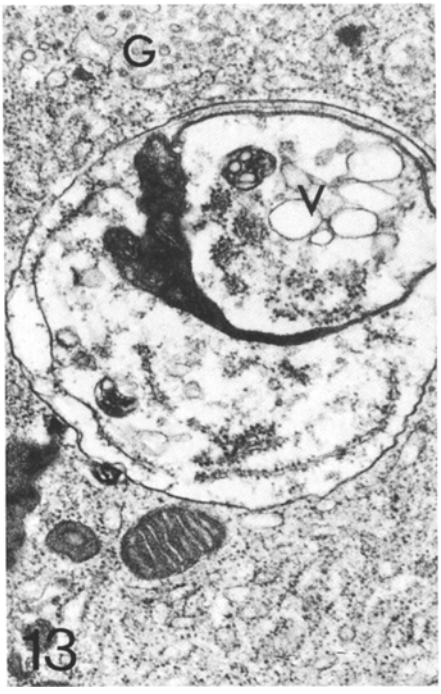
As the insect approaches puparium formation the secretory granules grow larger. By this stage the lumen is filled with dense material presumed to be glue secretion. Those granules that are not extruded into the lumen appear to lie in such intimate contact with one another that they may deform each others' outline; ultimately they appear to fuse with one another. In some cases, one or two giant granules come almost to fill a cell and they become very much less electron opaque in appearance.

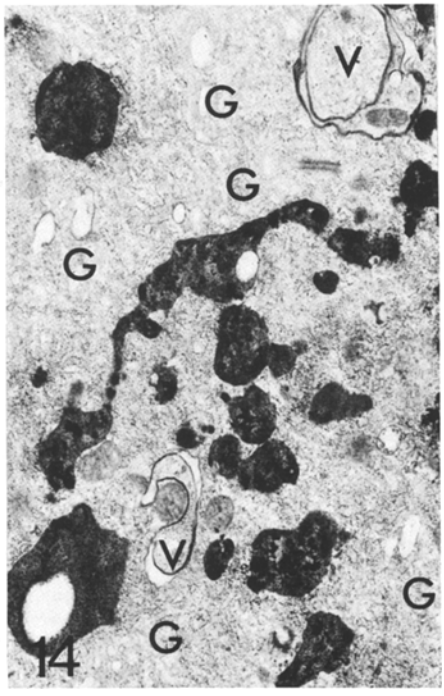
Fig. 9. Secretory granules near the luminal surface of the gland prior to extrusion of the granular contents. Note the junctions between adjacent cells near the lumen (arrow). The lumen (*L*) at this stage is empty. × 14700

Fig. 10. Gland from a PS 5 larva with a secretory granule apparently in the process of extruding its contents, by exocytosis, into the lumen (*L*), now full of electron opaque material containing 'rosettes'. × 18750









Figs. 11–14

By this stage the endoplasmic reticulum has also become more scattered, although in some cases it assumes the form of whorls of cisternae arranged concentrically; these might, however, represent residual whorls left over from the early stages. In some cases dense material still appears to be associated with the lumen of the vesiculated endoplasmic reticulum.

Prepupal Stages

The glue secretion is released from the lumen of the gland by expectoration at puparium formation. From PS 10 or 11 onwards, the cells of the salivary gland show further changes. The cytoplasm gradually becomes dense and vesiculated (Fig. 18); this presumably represents degeneration. Different degrees of vesiculation can be found in adjacent cells of the same gland (Fig. 18).

Autophagia commences as the large granules begin to show accumulation of myelin figures on their periphery (Fig. 15). Golgi vesicles are prominent near these regions and it seems that the large granules are becoming converted to lysosomes, possibly by transport of lysosomal acid hydrolases to them via the Golgi vesicles. Other lysosome-like granules begin to appear in these stages as well; membrane-delimited cytoplasmic areas, often containing recognizable mitochondria or ribosomes, are frequently encountered (Figs. 13 and 14). Moreover, other sorts of granules become apparent at this stage. These include 1) fibrous granules which we term spindle granules; these occur infrequently, (Fig. 11) and 2) smaller dense granules (Fig. 12), rather like electron-dense multivesicular bodies; these have a somewhat vacuolated appearance at their periphery. In addition, around PS 10 to 15, vacuoles containing a dense flocculant material begin to appear in the cells, especially near the luminal surface (Fig. 19). Ultimately any recognizable dense secretion granules disappear and the number of residual-body type of lysosome increases markedly (Figs. 14 and 15), presumably formed from the degraded secretion granules (Fig. 15) or by membrane sequestering of regions of cytoplasm. The remaining cytoplasm becomes more vacuolated and in some cases lipid globules can be seen to accumulate.

The nuclei appear smaller than in previous stages and their normally circular outline becomes folded and crenated in appearance (Figs. 16 and 17). From PS 12 to 21, the polytene chromosomes come to lie close to the nuclear envelope. The outer, ribosome-studded, membrane of the nuclear envelope begins to protrude out into the cytoplasm and dense material, in the form of circular masses, can be seen apparently being extruded from the nucleus into these protrusions (Figs. 16

Fig. 11. 'Spindle' granule, of considerable size, occurring in the glandular cytoplasm at PS 13. × 18100

Fig. 12. Electron dense body, with a vacuolated periphery, typical of the gland cells at PS 11. × 48700

Fig. 13. PS 18 when the gland cytoplasm contains an autophagic vacuole (*V*) in this case enclosing some endoplasmic reticulum. Note the Golgi vesicles lying nearby (*G*) . × 31250

Fig. 14. Dense bodies of irregular outline which occur in great numbers in the glandular cytoplasm during the prepupal stages. Some autophagic vacuoles (*V*) also occur, and Golgi complexes (*G*) are present in the near vicinity. (PS 19.) × 11200

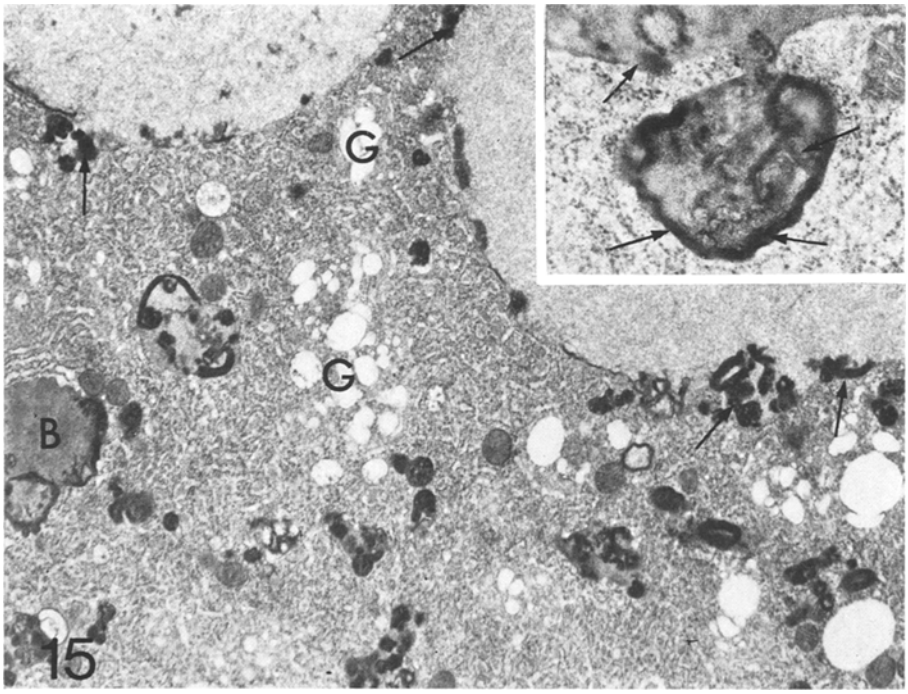


Fig. 15. Beginning of histolysis at PS 16. Note the enlarged, less opaque, secretory granules with myelinated peripheries (arrows) that have been "left over" and the dense lysosomes scattered through the cytoplasm. The Golgi complexes (*G*) have become highly vacuolated. The smaller myelinated bodies (*B*) may represent reduced secretory granules. × 9200. *Insert:* Portion of a degenerating secretory granule from a PS 16 prepupal showing the 'myelin' (arrows) of the peripheral border. The budding protuberance on the edge of the larger granule may represent a region which has degraded more speedily than the remainder or it could be a lysosomal body attached to the granule's periphery. × 56000

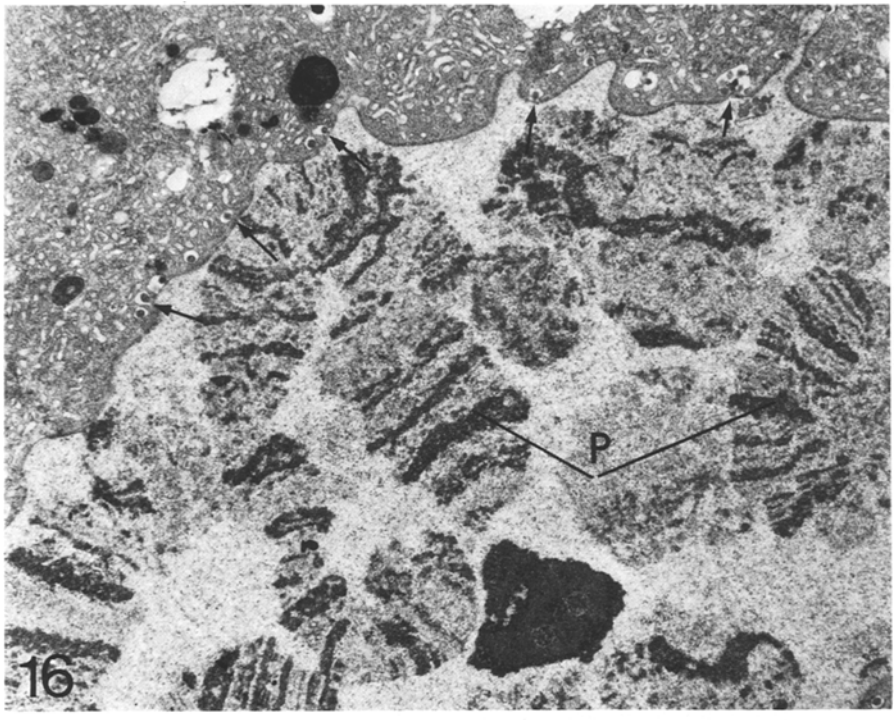
and 17). Dense masses bordered with a ribosome-studded membrane are also present, apparently free in the cytoplasm, suggesting that they may have budded off the nuclear membrane and moved out into the cytoplasm (Fig. 17); alternatively they may simply represent tangential sections through the protruding envelope. This nuclear blebbing is much more extensive than that which is occasionally encountered in earlier stages.

In young prepupae a dense flocculent material fills the lumen (Fig. 19). In some cases dense material can be found between the cytoplasmic processes at the border of the lumen (Fig. 19); this may represent extrusions of some of the lysosomes or cellular contents. Towards the approach of pupation, near PS 21, the lumen of the gland is again empty except for cell remnants and debris (Fig. 20).

Discussion

1. 'Glue' Secretion

The secretion of the puparial mucoprotein 'glue' (Fraenkel and Brookes, 1953) is the most obvious function of the salivary gland of *Drosophila* third instar larvae.



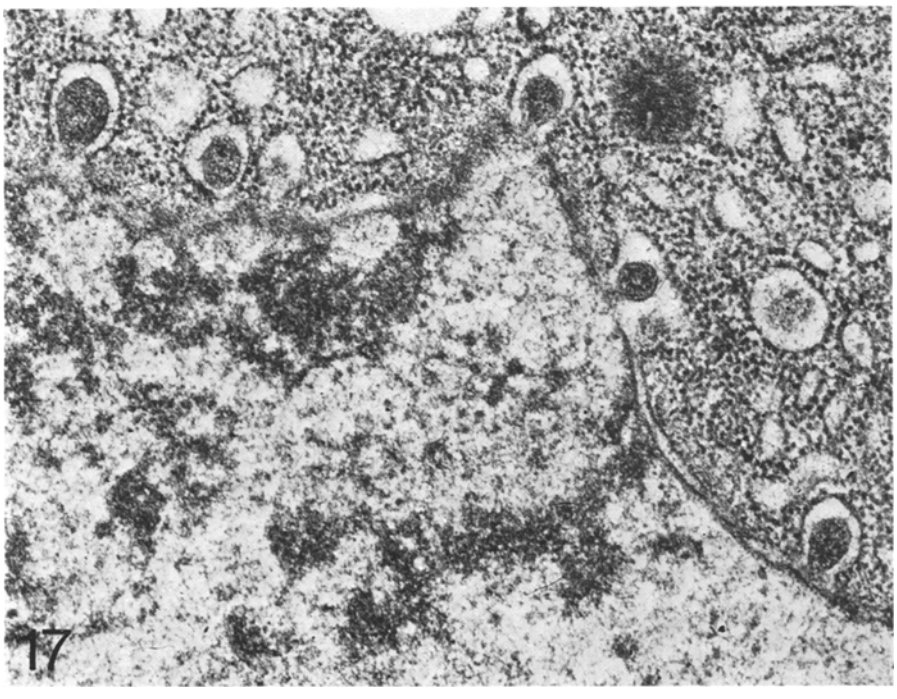
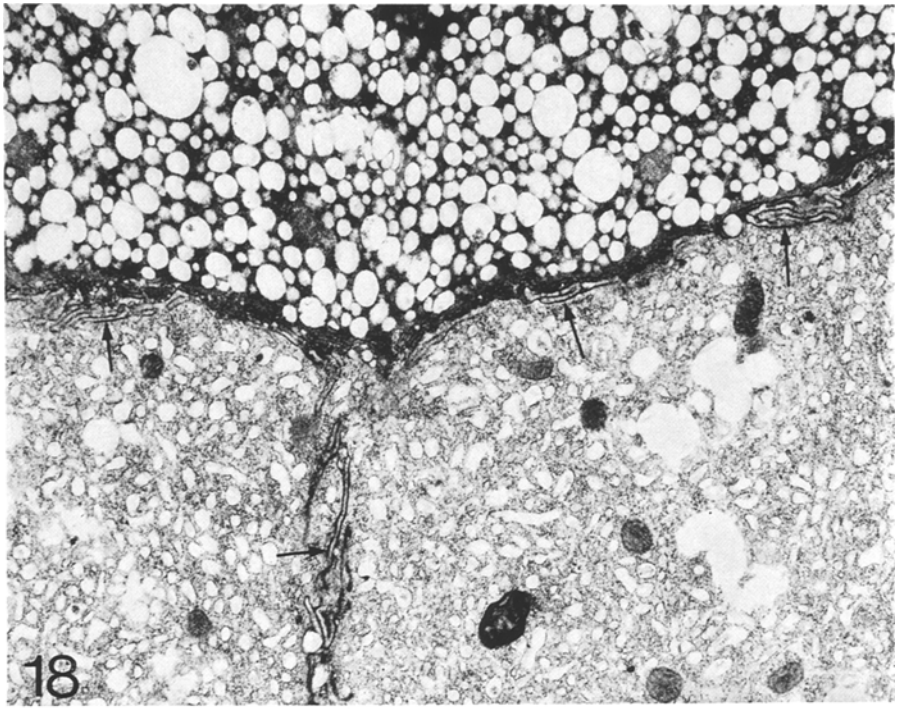
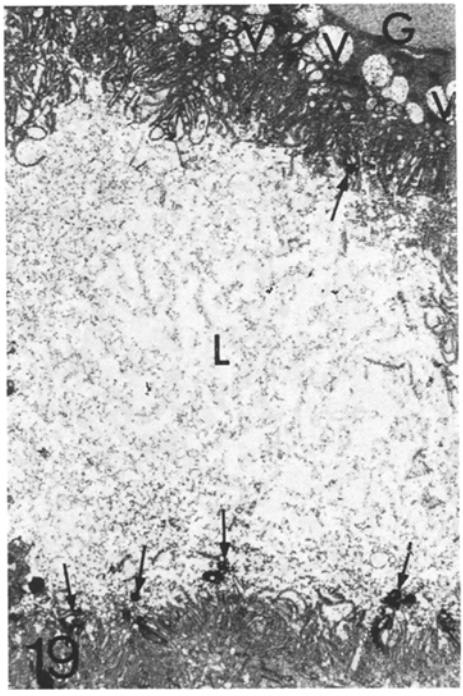
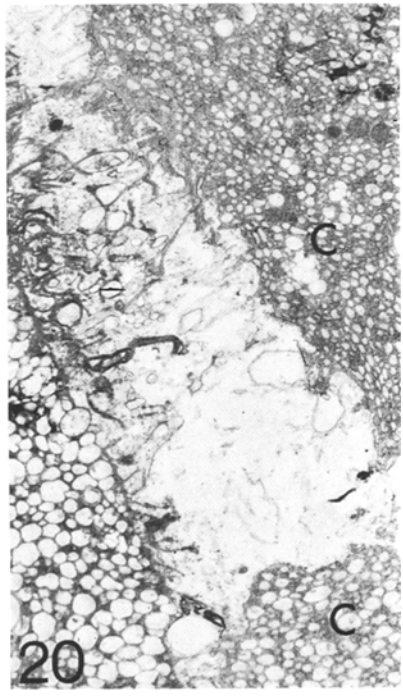


Fig. 16. PS 21, when nucleus has become reduced in volume and its surface becomes crenated. *P* banded polytene chromosomes; arrows indicate nuclear blebbing. × 6900

Fig. 17. Nuclear blebbing showing dense material within the membranous outpocketing in a prepupal nuclear envelope just prior to moulting. (PS 21.) × 50000







Figs. 18–20

In the youngest larvae examined in this study there is no evidence of a secretory function. The first indication of secretion is the appearance of electron dense material within some vesicles of the rough endoplasmic reticulum and subsequently within the Golgi saccules; this is accompanied by an increase in amount, and the extensive reorganisation, of the rough endoplasmic reticulum as evidenced by the presence of extensive stacks and whorls. Later, terminal swelling of the Golgi saccules suggests that they bud off electron dense material as numerous small dense granules are found in the cytoplasm near the Golgi complex. It seems possible, therefore, that part or all of the granular mucoprotein substance is synthesized in the endoplasmic reticulum and condensed into granules by the Golgi complex. Alternatively, the dense material observed within the cisternae of the rough endoplasmic reticulum need not necessarily represent a precursor of the mucoprotein; it could be membrane protein being synthesized in readiness for the massive membrane increase that occurs just before granule formation. The time of appearance and the size of the dense granules correspond to that of the PAS-positive granular material observed in the gland cells under the light microscope. The granules increase in size as development proceeds (as observed by McMaster-Kaye and Taylor (1959) for the PAS-positive granules of *D. repleta*) and are seen to possess the characteristic para-crystalline internum described by Rizki (1967), although the dimensions of the component strands are different. Later during development, but still prior to the ecdysone-induced puffing cycle, the second component, electron lucent rosettes, appears within the granules. This component was not described in previous ultrastructural studies of the secretory granules of *Drosophila* salivary glands, perhaps due to differences in the fixatives employed (Gay, 1955b, 1956; Kaufmann and Gay, 1958; Swift, 1962; Berendes and de Bruyn, 1963; Wiener, Spiro and Loewenstein, 1964; Berendes, 1965; Rizki, 1967).

By the time ecdysone initiates the puffing cycle in late third instar larvae the cytoplasm of the distal gland cells contains numerous, larger secretory granules whose size ranges up to 3 microns. Extrusion of the granular contents into the previously empty gland lumen takes place at PS 5. In *Drosophila*, the relationship between the synthesis of the glue by the salivary gland and the puffs, if indeed any such relationship exists, remains to be elucidated. It is clear that in the only two Drosophila species closely studied, *D. melanogaster* and *D. hydei* (Berendes, 1965; Poels, 1970), synthesis precedes by many hours the ecdysone-induced changes in gene activity.

Fig. 18. Three gland cells during the late prepupal stage (PS 21) showing different degrees of cytoplasmic degradation. The cytoplasmic vacuolation is more marked in the uppermost cell. Note that very few dense bodies are present at this stage. The lateral cell borders are highly convoluted (arrows). × 9800

Fig. 19. Lumen of the gland (*L*) at PS 13. Note the flocculent material within it and the cytoplasmic vacuoles (*V*) seemingly moving towards the lumen, bearing a similar substance. Note the dense bodies, possibly being extruded into the lumen (arrows). The edge of a large degenerating secretion granule (*G*) is present. × 4300

Fig. 20. Lumen of the gland at PS 21. It is now empty except for cytoplasmic debris, presumably arising from the vacuolated degenerating cells of the gland (*C*). × 5600

In other Diptera, notably the Chironomids, there is evidence of a causal relationship between the activity of particular puffs and the synthesis of particular protein components of the secretion in the salivary gland. In *Acricotopus lucidus* the production of a secretion containing hydroxyproline by the main and side lobes of the salivary gland is correlated with the activity of a particular large puff (BR2) (Baudisch, 1967; Baudisch and Panitz, 1968). In *Chironomus tentans* and *C. pallidivittatus* there is good evidence for the involvement of the large puffs (Balbiani Rings) in the synthesis of certain polypeptide components of the secretion (Grossbach, 1968, 1969; see Beermann, 1961; Wobus, Panitz, and Serfling, 1970).

Physiologically there is one major difference between the salivary gland secretion of Chironomids and that of *Drosophila*. In *Chironomus* the salivary gland secretion is a silk-like protein used by the larva to coat the tube in which it lives and to form a net used to trap its food (Walshe, 1947). Moreover, the gland secretes throughout larval life. In *Drosophila*, on the other hand, secretion is a transitory event occupying only a limited period of larval development and the secretory product is used to attach the pupa to its substratum.

If it is assumed that the secretion of the puparial glue by the *Drosophila* salivary gland is under the control of the nuclei of the gland, there would appear to be four possibilities as to the relationship between its synthesis and the puffing activity of the polytene chromosomes:

i) The two processes may be unrelated. As a consequence this would mean that the genes that code for glue synthesis do not, when active, form morphologically visible puffs. There can be no doubt that the spectrum from a large puff to an unpuffed band active in RNA synthesis is continuous and that many chromosome sites are active but not scored as puffs on morphological criteria (see Pelling, 1964; Ashburner, 1970).

ii) The secretory proteins may be translated from RNA species transcribed at puffs during the puffing cycle accompanying the moult from the second to third instar. In *Drosophila melanogaster* the chromosomes are too small for the detailed analysis of this puffing cycle although Becker (1959) was able to discern puffs in second instar larval salivary glands. In *Chironomus tentans* the puffing cycles accompanying the moult from third to fourth larval instar and the moult from the fourth larval instar to pupa are similar (Clever, 1962, 1963). This suggestion implies that the RNA species necessary for glue synthesis are stable; this is supported by the fact that in *Chironomus tentans* and *C. pallidivittatus* the synthesis of the secretion by the salivary gland is insensitive to actinomycin D applied at the time of secretion production (Clever, 1969; Doyle and Laufer, 1969). Putatively stable RNA species have also been implicated in other insect systems, notably for the synthesis of cocoonase by the silkmoth galea cells (Kafatos, 1969).

iii) The synthesis of puparial glue may be coded by the 10 or so puffs active at the time of appearance of the secretory granules. We have no estimate of the number of genes that might be needed to code for the puparial glue. Preliminary investigation of the chemical nature of the glue by Ashburner and Blumenthal (unpublished) confirmed earlier observations (Kodani, 1948; Gay, 1955b; Perkowska, 1963) that it is a mucoprotein; its composition was determined as approximately 70% protein and 30% carbohydrate. The protein fraction of the glue may be heterogeneous and in addition its synthesis would certainly require the presence

of enzymes not only to synthesize the modified (e. g. amino and N-acetyl amino) hexoses of the carbohydrate moiety, but also to link these sugars into their correct groupings and to join the carbohydrates to specific amino acids of the protein. It might reasonably be predicted therefore that the activity of several genes is necessary for glue synthesis.

iv) Finally, and this suggestion is not incompatible with those previously discussed, the products of the puffs in late third instar larvae may be involved in the modification of the glue subsequent to its initial synthesis and storage in the secretory granules.

2. Possible other Secretory Functions

Before PS 5 the lumen of the gland appears to be empty, at least of solid content. We have no evidence that the gland is involved in the secretion of any material other than the glue during the third instar larval stages. The small (0.2–0.4 μ) electron dense granules observed near the luminal cell border of second instar glands by Tulchin and Rhodin (1970) were not present in our preparations of young third instar glands. According to these authors the lumen of the salivary gland during the first and second instars is empty.

Following the release of the glue by the larva at puparium formation, the lumen contains a rather loose flocculent material of unknown origin or function. It is PAS-negative but may possibly represent remnants of the glue; this material disappears during the prepupal period. Cytoplasmic vesicles containing a similar material are found in prepupal glands near the lumen which suggests that during this period material may be extruded into the lumen.

3. Histolysis

The larval salivary gland, in common with many other larval tissues and organs, is completely histolysed during prepupal and pupal development, the salivary gland of the adult fly differentiating from the imaginal ring located in the anterior of the gland (Ross, 1939) (see Fig. 1 a).

In an important series of experiments Bodenstein (1943) laid the foundation for an analysis of salivary gland histolysis in *Drosophila*. He discovered that both gland growth and histolysis are under hormonal control from the ring gland. Furthermore, the gland has to reach a certain stage in its growth before becoming competent to react to the hormonal stimulus to histolyse; competence is only acquired early in the third instar larval stage. The hormonal stimulus for histolysis of the salivary gland is almost certainly ecdysone.

Current views on cell histolysis centre on the concept of the lysosome (see Dingle and Fell 1969, for discussion) and there have been several previous reports of lysosomes, identified either ultrastructurally or cytochemically as such, occurring in Dipteran larval salivary glands (Misch, 1962; Rasch and Gawlick, 1964; Jacob and Jurand, 1965; Schin and Clever, 1965, 1968; Döbel, 1968; Lockshin, 1969).

In *Chironomus tentans* Schin and Clever (1965, 1968) found a dramatic increase in the types of acid phosphatase-containing bodies during the metamorphosis of the larval salivary gland. In the present study only Golgi vesicles and Golgi-associated fibrous bodies could represent lysosomes in the glands of early third

instar larvae. After puparium formation, however, this situation changes dramatically and, as is clear from Figs. 13 and 14, dense bodies and autophagic vacuoles, interpreted as lysosomes, become increasingly common in the cytoplasm as prepupal development progresses. The autophagic vacuoles appear to develop from smooth membranes surrounding areas of cytoplasm for focal degradation. These membranes may originate either from cisternae of smooth endoplasmic reticulum as suggested for the autophagic vacuoles found in rat nerves (Novikoff, 1967), or from Golgi elements, as suggested for those found in the degenerating prothoracic glands of insects (Osinchak, 1966). By PS 10 to 15 the cytoplasm of some cells of the gland becomes increasingly vacuolated. As can be seen from Fig. 18 the rate of cell histolysis varies considerably within salivary glands even between neighbouring cells. A similar lack of synchrony in the rate of cell death has been described by Scharrer (1966) in the prothoracic gland of cockroaches.

There are other indications of salivary gland histolysis after puparium formation. Not all of the glue secretory granules of the larval gland are emptied into the lumen (see Painter, 1946); a few remain behind. Soon after puparium formation these granules may fuse with each other to form vast granules with a decreased electron opacity. Around their peripheral limiting membrane, electron dense myelin figures begin to appear. The small vesicles found in the vicinity of myelinated areas of the granules suggest the possibility that Golgi vesicles, bearing acid hydrolases, and/or some of the dense lysosomal bodies (Fig. 15, insert), are converging on the granules leading to the destruction of their substance save for the lipid moiety represented by the myelin figures. This may represent an example of 'crinophagy' which is said to occur when lysosomes fuse with secretion granules (Dingle and Fell, 1969). The observations in PAS-treated preparations parallel those made with the electron microscope; large PAS-positive granules are visible in the cytoplasm of prepupal glands but after PS 18 the cytoplasm is PAS negative, suggesting a change in the chemical nature of these granules, or, as is more likely, their destruction or loss by this stage. In *Chironomus*, 'surplus' secretory granules also remain in the salivary gland after pupation; these aggregate and become enclosed within autophagic vacuoles (Kloetzel and Laufer, 1970).

4. Nuclear Behaviour

Although attention has been concentrated in this study in the cytoplasmic components of the salivary gland, certain aspects of nuclear behaviour merit discussion. In common with the observations of previous authors, notably the work of Gay (1954, 1955a, 1955b, 1956; Kaufmann and Gay, 1958) we have observed slight blebbing of the nuclear membrane in the larval salivary glands engaged in secretory granule production. Nuclear blebbing has also been described in insect oenocytes during autolysis before the pupal moult (Locke, 1969). However towards the end of prepupal development in salivary glands, a stage not previously studied, we find that the nuclear membrane undergoes a dramatic change. By PS 12 the nucleus is no longer regular in outline, but has become crenated. Moreover, it appears to be extruding electron dense material surrounded by nuclear membrane into the cytoplasm (as in Figs. 16 and 17). The origin of this dense material is not clear. We suggest four possible interpretations of this phenomenon. It may be a process concerned with nuclear histolysis, although it

is unlike the examples of nuclear histolysis previously described from metamorphosing insect tissues (Matsuura *et al.*, 1968; Scharrer, 1966; Lockshin and Williams, 1965). It may be that the nuclear envelope, as part of the endoplasmic reticulum, is engaged in synthetic activity, the electron dense material in the perinuclear space representing the product. Alternatively, it may be that this material represents hydrolytic enzymes, synthesized in the cytoplasm and fusing with the nucleus as a stage in its degradation. Finally it may be concerned with the packaging and storage of nuclear material destined to survive histolysis. It has been previously suggested (Ashburner, 1967, 1970) that a function of the prepupal salivary gland may be the elaboration of macromolecules to be used by other tissues during histogenesis. The cytoplasmic sequestering of electron dense material from the nuclei of the late prepupal salivary glands may be a visible manifestation of the temporary storage of these substances prior to macrophage engulfment (Whitten, 1964; Perez, 1910; see Crossley, 1968; Barritt and Birt, 1971). Their precise origin and fate deserve further study.

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