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An ultrastructural analysis of the ecdysoneless *(l(3)ecd^{1ts})* ring gland during the third larval instar of *Drosophila melanogaster*

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Summary. In the late third larval instar of *Drosophila melanogaster*, the prothoracic gland, an endocrine portion of the ring gland, synthesizes ecdysteroids at an accelerated rate. The resultant ecdysteroid titer peak initiates the events associated with metamorphosis. The normal prothoracic gland displays several ultrastructural features at this developmental stage that reflect increased steroidogenic activity, including extensive infoldings of the plasma membrane (membrane invaginations) and an increase in both the concentration of smooth endoplasmic reticulum (SER) (or transitional ER) and elongated mitochondria. By contrast, the prothoracic glands of larvae homozygous for a conditional larval lethal mutation, *l(3)ecd^{1ts}*, not only fail to produce ecdysteroids at normal levels at the restrictive temperature (29° C), but also acquire abnormal morphological features that reflect the disruptive effects of the mutation. These abnormalities include an accumulation of lipid droplets presumed to contain sterol precursors of ecdysteroids, a disappearance of SER and a drastic reduction of membrane invaginations in the peripheral area of the cell. These morphological defects are observed in prothoracic glands dissected from larvae transferred from 18° C to 29° C approximately 24 h before observation and also within 4 h of an in vitro transfer to 29° C following dissection from wandering third instar larvae reared at 18° C. No ultrastructural abnormalities were noted in the corpus allatum portion of mutant ring glands. These observations further indicate the direct involvement of the *ecd* gene product in ecdysteroid synthesis and suggest a role for the gene in the proper transport of precursors to the site where they can be utilized in ecdysteroid biosynthesis.

Key words: Prothoracic gland – Ecdysteroid – Corpus allatum – Juvenile hormone – *Drosophila melanogaster* (Insecta)

Ecdysteroids and juvenile hormone regulate the course of larval development and metamorphosis in insects (Gilbert et al. 1981). Ecdysteroids are produced by the prothoracic gland and juvenile hormone is synthesized by the corpus allatum, these two tissues comprising a portion of the ring gland in higher flies, that in turn encircles the aorta dorso-anterior to the larval brain-ventral ganglion complex. Ultrastructural analysis of these endocrine glands in a variety of insects has revealed intracellular features consistently associated with hormone-producing activity (see Sedlak 1985). Furthermore, developmental changes in the frequency of certain organelles and structural membrane changes coincide with fluctuations in the titer of ecdysteroids and juvenile hormone, suggesting that these morphological characteristics reflect regulatory aspects of biosynthesis (e.g., Smith and Nijhout 1982; Sedlak et al. 1983). Nevertheless, the molecular components responsible for the regulation of ecdysteroid biosynthesis remain poorly characterized.

Several endocrine mutations exist in *Drosophila melanogaster* that reduce larval ecdysteroid levels and may, therefore, not encode the gene product necessary for normal ecdysteroid synthesis. Abnormalities in ring glands that express these mutations have provided clues about those ultrastructural features necessary and/or important for normal endocrine function as well as the primary site of action of some of these mutations (e.g., Aggarwal and King 1969; Klose et al. 1980). The *ecd^{1ts}* mutation is a conditional larval lethal that autonomously and reversibly reduces ecdysteroid synthesis in mutant prothoracic gland cells at the restrictive temperature of 29° C (Henrich et al. 1987b). Nevertheless, the mutation exerts a range of pleiotropic effects that suggest a more general and perhaps nonspecific effect upon steroidogenesis (Redfern and Bownes 1983). As part of an attempt to understand the regulatory mechanisms that underlie ecdysteroid biosynthesis, and to gain insights about a possible deficiency of the *ecd* gene and its product in this process, we have examined and compared the ultrastructure of the prothoracic gland cells of the ring gland of wild-type and mutant late third instar larvae before and after temperature upshift, both in vivo and in vitro.

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Materials and methods

Stock maintenance

A wild-type Canton-S strain and a homozygous mutant strain for a multiple marked chromosome (*ecd*^{1ts}*st red e ca*) were reared on a standard agar corn meal medium at 18° C in constant light. For comparative studies, the wild-type Canton-S strain was also reared at 25° C in constant light. To analyze the effects of prolonged upshifts to 29° C that elicit an ecdysteroid deficiency in mutant larvae (Henrich et al. 1987b), both wild-type and mutant cultures were transferred from 18° C to 29° C nine days after egg laying and sacrificed 24 h after the upshift. For in vitro experiments, wild-type and mutant larvae were reared at 18° C continuously until the onset of wandering during the third larval instar. At this time, the brain-ventral ganglion-ring gland complexes were dissected out as described previously (Henrich et al. 1987b).

Quantification of ecdysteroids

After dissection, the brain-ventral ganglion-ring gland complexes were incubated in 15 µl of pre-equilibrated Grace's insect culture medium (Gibco) at 18° C, 25° C or 29° C for 4 h in vitro. The ecdysteroids in 10 µl aliquots of medium were quantified by radioimmunoassay (RIA) by use of H-22 antiserum (Warren et al. 1984). The ecdysteroid content is expressed in ecdysone equivalents (Henrich et al. 1987b), although, in *Drosophila*, the in vitro incubation products include ecdysone, 20-deoxymakisterone and an unidentified ecdysteroid (Redfern 1984; Henrich et al. 1987a). The same glands whose ecdysteroid synthetic rates were measured were prepared for ultrastructural observation. Comparisons between wild-type and mutant ring glands were based upon observations made on a minimum of five replicates in each group. The animals for the in vivo and in vitro temperature upshift experiments were not developmentally equivalent (see above). If the same stage animals (early wandering third instar larvae) were used for the in vivo experiments as were used in vitro, the animals would immediately begin to pupariate before being sacrificed. The ecdysteroid synthetic activity of the ring gland decreases drastically at that stage (Dai and Gilbert 1991) and the animals used for the in vivo and in vitro studies would not be physiologically equivalent.

Tissue preparation and microscopy

For electron microscopy, brain-ventral ganglion complexes with the ring gland attached were fixed in 2.5% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4) for 2 h at 4° C and then postfixed in 2% OsO_4 (1:1, 4% OsO_4:0.2 M cacodylate buffer) for 1.5 h at 4° C. Samples were embedded in vinyl cyclohexane dioxide (Spurr 1969) and sections were cut with a Sorvall MT-2 ultramicrotome. For ultrastructural orientation, 1-µm-thick serial sections were cut and stained with toluidine blue solution while ultrathin sections (50–60 nm) were stained with 5% uranyl acetate in a 70% ethanol solution for 10 min at room temperature and post-stained with lead citrate (Reynolds 1963). Examination was conducted with a Zeiss EM-10 electron microscope operating at 60–80 kV. Ultrastructural comparisons between wild-type and mutant glands were based upon observations made on a minimum of five replicates of EM samples in each group and a minimum of 80 partially serial sections of each sample.

Results

General ultrastructural features of prothoracic gland cells from early wandering third instar larvae

Previous studies on whole larvae and pupae demonstrated that a peak of 20-hydroxyecdysone occurs 4–6 h prior to puparium formation and persists until pupation (Borst et al. 1974; Hodgetts et al. 1977). Our RIA data reveal that the ring glands of wild-type wandering third instar larvae reared at 25° C possess a slightly greater capacity for ecdysteroid biosynthesis in vitro than do glands from larvae reared at 18° C (Fig. 1). Developmental studies indicate that the prothoracic glands are most active in the in vitro synthesis and secretion of ecdysteroids during the late third larval instar (Dai and Gilbert 1991). The correlated ultrastructural features of wild-type prothoracic gland cells are shown in Fig. 2, and although similar observations have been made previously (King et al. 1966; Aggarwal and King 1969), a re-examination was necessary for subsequent comparison with mutant glands, both in terms of ecdysteroid production and ultrastructure. Numerous elongated mitochondria and smooth endoplasmic reticulum (SER) were the most prominent characteristics, as well as a high frequency of deep plasma-membrane invaginations which form intercellular channels in the periphery of prothoracic gland cells, increasing the surface area of the cell, probably for secretion purposes. It should be noted that in some specialized eukaryotic cells, e.g., most secretory cells, SER is really a small ribosome-free region of the rough endoplasmic reticulum (RER). Such regions are usually called transitional ER (rather than SER), and they represent the specialized region of ER from which the vesicles carrying newly synthesized protein or lipids bud off for intracellular transport (see De-Pierre and Dallner 1975). In *Drosophila* prothoracic gland and corpus allatum cells, the SER examplifies this transitional ER. The relative ratio between SER and RER reflects the fluctuations in cell-secretion activity during development (King et al. 1966; Dai and Gilbert 1991). Since there is no accepted definition of the size of these small ribosome-free regions, i.e., the distance between two neighboring ribosome-attached regions, it is sometimes difficult to clearly distinguish SER from RER. In addition, since the transitional ER is mainly

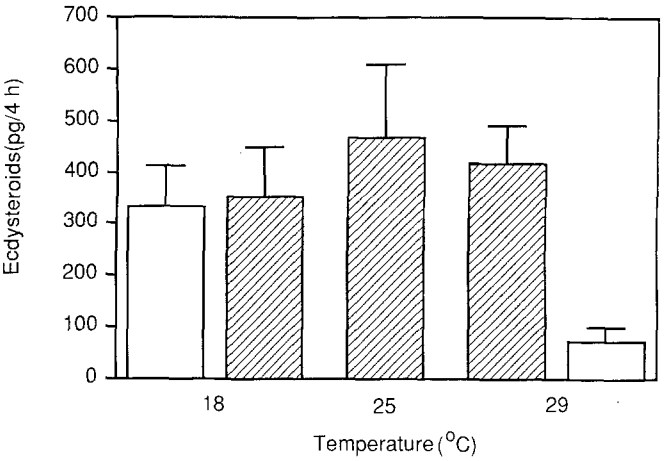


Fig. 1. Ecdysteroid biosynthesis in vitro by the brain-ventral ganglion-ring gland complexes of *D. melanogaster* wild-type (Canton-S) and mutant (*ecd*^{1ts}) larvae at different incubation temperatures for 4 h as measured by RIA. Ecdysteroid levels expressed in ecdysone equivalents (see Materials and methods). ▨ Canton-S; □ *ecd*^{1}*st red e ca*

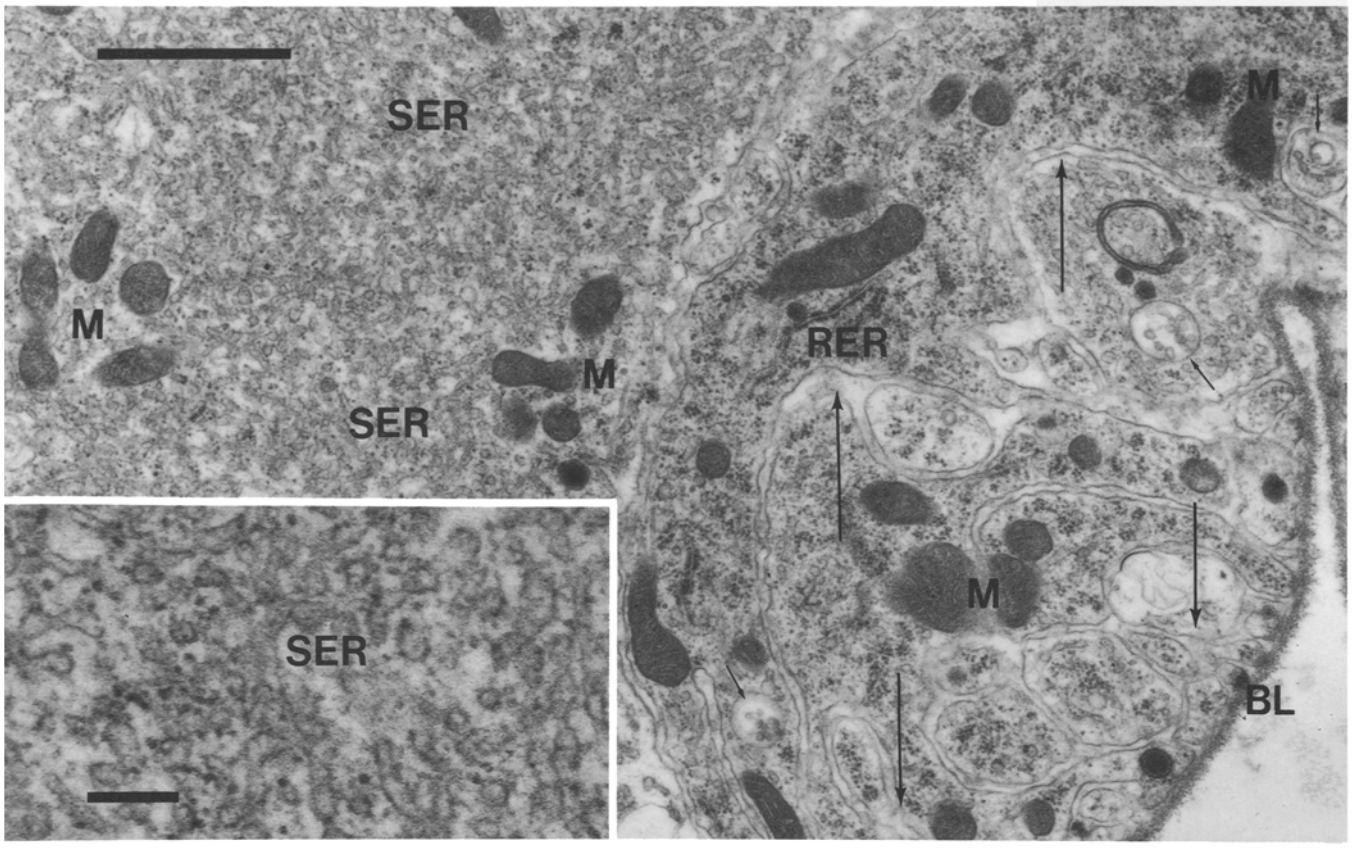


Fig. 2. Electron micrographs of prothoracic gland cell from a wild-type, early wandering, third instar larva of *Drosophila* reared at 25° C. *BL* Basal lamina; *M* mitochondrion; *RER* rough endoplasmic reticulum; *SER* smooth endoplasmic reticulum; *long arrow* membrane invagination; *short arrow* multivesicular body. *Bar*: 1 μm; ×25000. *Inset: bar*: 0.2 μm; ×64000

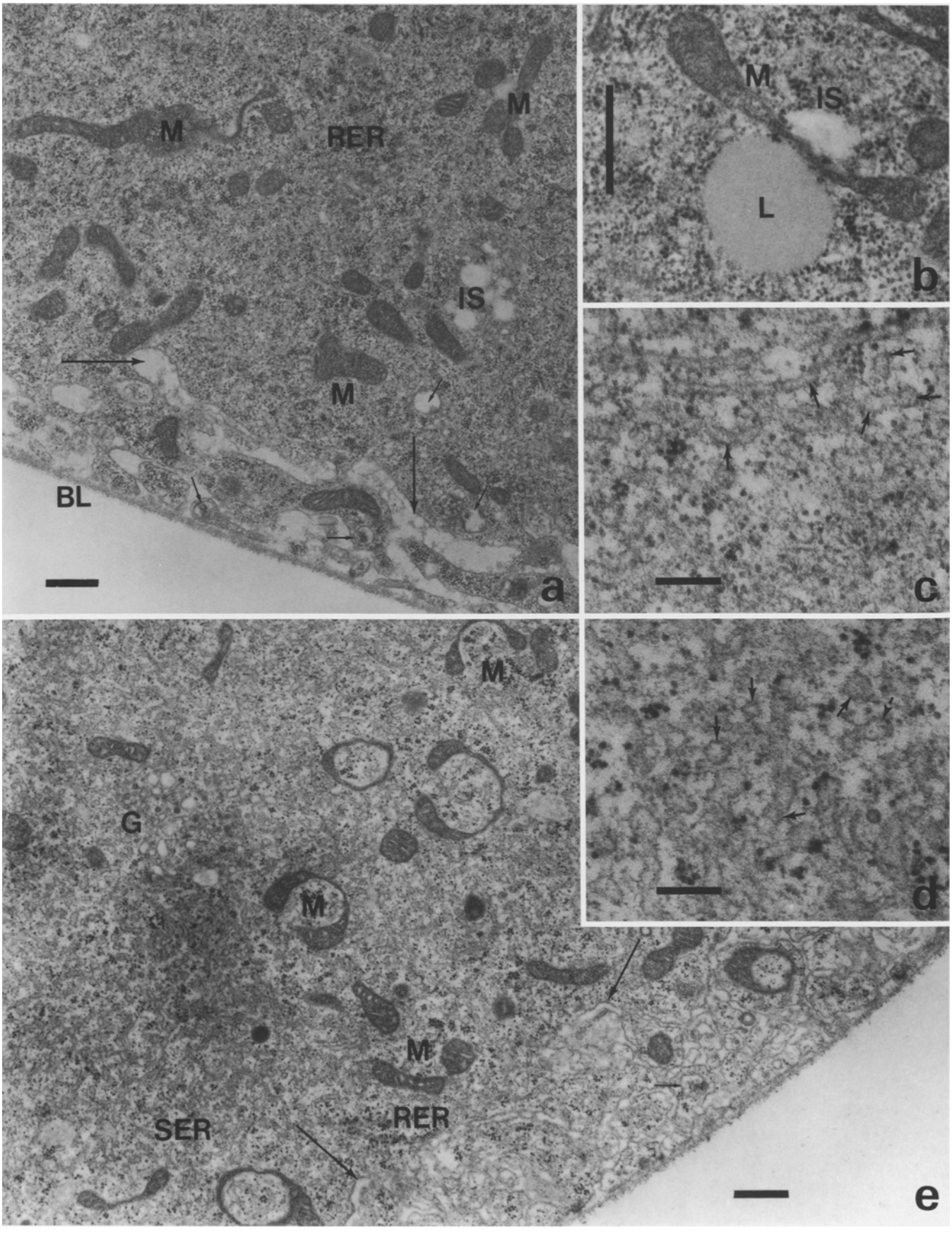
classified as SER and conventional SER terminology has been used previously to describe *Drosophila* ring glands (King et al. 1969; Aggarwal and King 1969; Klose et al. 1980; Dai and Gilbert 1991), we will use the term SER here, but it is actually transitional ER. Multivesicular bodies were also found in the cytoplasm, especially at the cell periphery and consisted of some small vesicles which were enclosed within a membrane and were usually surrounded by SER (Fig. 2). In addition, numerous multivesicular body remnants were present in the membrane invaginations. The internal vesicles of these remnants disappeared, and only the outer bounding membrane remained (data not shown), as also described by Sedlak et al. (1983) for the cells of a lepidopteran prothoracic gland. Their function in the prothoracic gland cell is not known. Although lipid droplets may be storage sites for ecdysteroid precursors (Romer 1971; Rees et al. 1980), each cell in a section contained only a few lipid droplets, approximately 0–10 droplets with an average diameter of 0.67 μm, and usually surrounded by RER and mitochondria (Fig. 3b). Nevertheless, dynamic fluctuations in the frequency of lipid droplets may be important indicators of prothoracic gland activity as will be seen subsequently.

Typical Golgi complexes were commonly found in prothoracic gland cells, often associated with electron-dense coated vesicles. Since there has been no evidence supporting a correlation between the appearance of these complexes and biosynthetic activity in Orthoptera and Lepidoptera, it has been assumed that this organelle is not directly involved in the synthesis and/or secretion of ecdysteroids (Gersch et al. 1975; Birkenbeil 1983; Sedlak et al. 1983). Prothoracic gland cells of the *Drosophila* ring gland are innervated by axons containing various electron-dense granules that penetrate into the basal lamina or indent the cells. What appear to be a presynaptic density and omega profile typical of exocytotic events are also visible in these axon endings (data not shown).

As shown in Fig. 1, the amount of ecdysteroids secreted by wild type, Canton-S and ecd^{1ts} mutant glands at 18° C were almost identical, but less than those of wild type glands at 25° C. Both wild-type (Fig. 3a–c) and mutant glands (Fig. 3d, e) at 18° C displayed similar ultrastructural features except that glands at 18° C contained less and smaller SER and membrane invaginations than glands at 25° C. Nevertheless, these characteristics generally suggest that both wild-type and mutant ring glands at 18° C and wild-type ring glands at 25° C function normally.

Effects of shift to the restrictive temperature in vivo on the ultrastructure of the ecd^{1ts} prothoracic gland

In order to compare the possible effects of the ecdysoneless (ecd^{1ts}) mutation on ring-gland morphology, wild-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type and mutant mid-third instar larvae were transferred from 18° C to 29° C 24 h prior to ring gland dissection. Mutant individuals subjected to this upshift acquire an ecdysteroid deficiency that stems from a reduction in ecdysteroid synthesis by the ring gland, and ultimately die after experiencing a delayed and abnormal tanning of the larval cuticle (Henrich et al. 1987b). Nevertheless, the larvae attain essentially full size and exhibit normal wandering behavior. The prothoracic glands of wild-type larvae transferred to 29° C show extensive invaginations of the plasma membrane, consistent with the onset of increased ecdysteroid secretion that normally occurs during the wandering period of the third larval instar (Fig. 4a). Under higher magnification, it was apparent that the cytoplasm contained a certain amount of SER mixed with RER and free ribosomes (Fig. 4a, inset).

By comparison, mutant prothoracic gland cells displayed several abnormal features after upshift to the restrictive temperature. These include a decrease in the extensive invaginations of the plasma membrane as well as a striking accumulation of lipid droplets in the cytoplasm (Fig. 4b), i.e., ~20–30 droplets in each cell of a section with an average diameter of 1.35 μm. This correlates with a drastic reduction in the ecdysteroid biosynthetic capacity of the ring gland (Henrich et al. 1987). Highly electron-dense mitochondria were also noted in prothoracic gland cells from mutant larvae. In addition, the concentration of RER appeared to be much higher in these cells, while no SER was observed (Fig. 4b, inset).

In summary, the prolonged upshift affected the prothoracic gland cells of the mutant by eliciting the accumulation of lipid droplets, caused the enhancement of mitochondrion density, and resulted in a preponderance of RER at the expense of the SER as well as a decrease in the depth of the membrane invaginations.

Effects of shift to the restrictive temperature in vitro on the ultrastructure of the ecd^{1ts} prothoracic gland

If the morphological features associated with active prothoracic gland cells are causally related to the ability of the glands to synthesize ecdysteroids, then ring glands from ecd^{1ts} larvae should differ from their wild-type counterparts when subjected to 29° C in vitro. In this experiment, after incubation in vitro for 4 h, mutant ring glands produced and/or secreted substantially less ecdysteroids into the incubation medium than wild-type ring glands. As determined by RIA, the glands from ecd^{1ts} larvae at 29° C produced only about 16% the amount of ecdysteroids synthesized by the glands from wild-type animals at 29° C (Fig. 1).

The prothoracic gland cells of both Canton-S and ecd^{1ts} early wandering third instar larvae displayed similar ultrastructural features at 18° C. However, within 4 h after the temperature upshift to the mutant's restrictive temperature, there were dramatic changes (Fig. 5). Whereas there were almost no lipid droplets in the prothoracic gland cells of wild-type glands (Fig. 5a), numerous lipid droplet deposits were present in the cytoplasm of the ecd^{1ts} prothoracic gland cells (Fig. 5b) as described for the experiment in vivo. Wild-type cells contained abundant SER (Fig. 5a, inset) and deeper membrane invaginations and more intracellular spaces that were not membrane bounded and probably derived from lipid droplets as occasionally seen in mutant cells in vivo (Figs. 4b, 5a). Intracellular spaces are commonly considered to be fixation artifacts as a consequence of samples not being fixed rapidly enough. In our experiments, however, since all ultrastructural comparisons were temporally consistent, the same EM preparation procedures were used, and the differences in intracellular space frequency between wild-type and mutant were consistent and reproducible, our data may indicate the utilization of lipid droplets or other electron-lucent substances. In contrast to the lipid droplets, more RER than SER, numerous free ribosomes, shorter and narrower membrane invaginations and fewer and smaller intracellular spaces characterized the prothoracic gland cells of the mutant after the temperature upshift (Fig. 5b, inset). However, the mitochondria of the glands from mutant animals appeared more electron dense than those from wild-type larvae (Fig. 5a, b). Moreover, after the upshift, the glands from ecd^{1ts} larvae contained a relatively high level of lipid droplets, RER and considerably less SER and membrane invaginations than either glands from wild type or mutant larvae that were kept at 18° C (Figs. 5b, 3a, c–e). The effects noted here did not appear to be a consequence of developmental changes resulting from the action of the mutant gene product, but rather to be associated with the disrupted physiology of the cell, since all appeared within a 4 h period following the temperature upshift.

Effects of shift to the restrictive temperature in vivo on the ultrastructure of the corpus allatum

Several studies have demonstrated that the ecd^{1ts} mutation exerts a range of pleiotropic effects upon development (Redfern and Bownes 1983; Sliter 1989), suggesting that the disruption of ecdysteroid synthesis is only one of several consequences of the mutation. Therefore, the corpus allatum portion of the larval ring gland was also analyzed after treatment of larvae undergoing the aforementioned experimental regimes, as a control for the studies of the prothoracic glands.

Fig. 3a–e. Prothoracic gland cells from early wandering, third instar larvae of wild-type and ecd^{1ts} mutant reared at 18° C. Same abbreviations as in Fig. 2, plus *G* Golgi complex; *IS* intracellular space; *L* lipid droplet; *long arrow* membrane invaginations; *short arrow* multivesicular body. **a** Wild-type cell; *bar*: 0.5 μm; ×20000. **b** Mitochondrion associated with lipid droplet and intracellular space in cell of wild-type; *bar*: 0.5 μm; ×40000. **c** Smooth endoplasmic reticulum (*arrow*) in cell of wild-type; *bar*: 0.2 μm; ×64000. **d** Smooth endoplasmic reticulum (*arrow*) in cell of ecd^{1ts} mutant; *bar*: 0.2 μm; ×64000. **e** Cell of ecd^{1ts} mutant; *bar*: 0.5 μm; ×20000

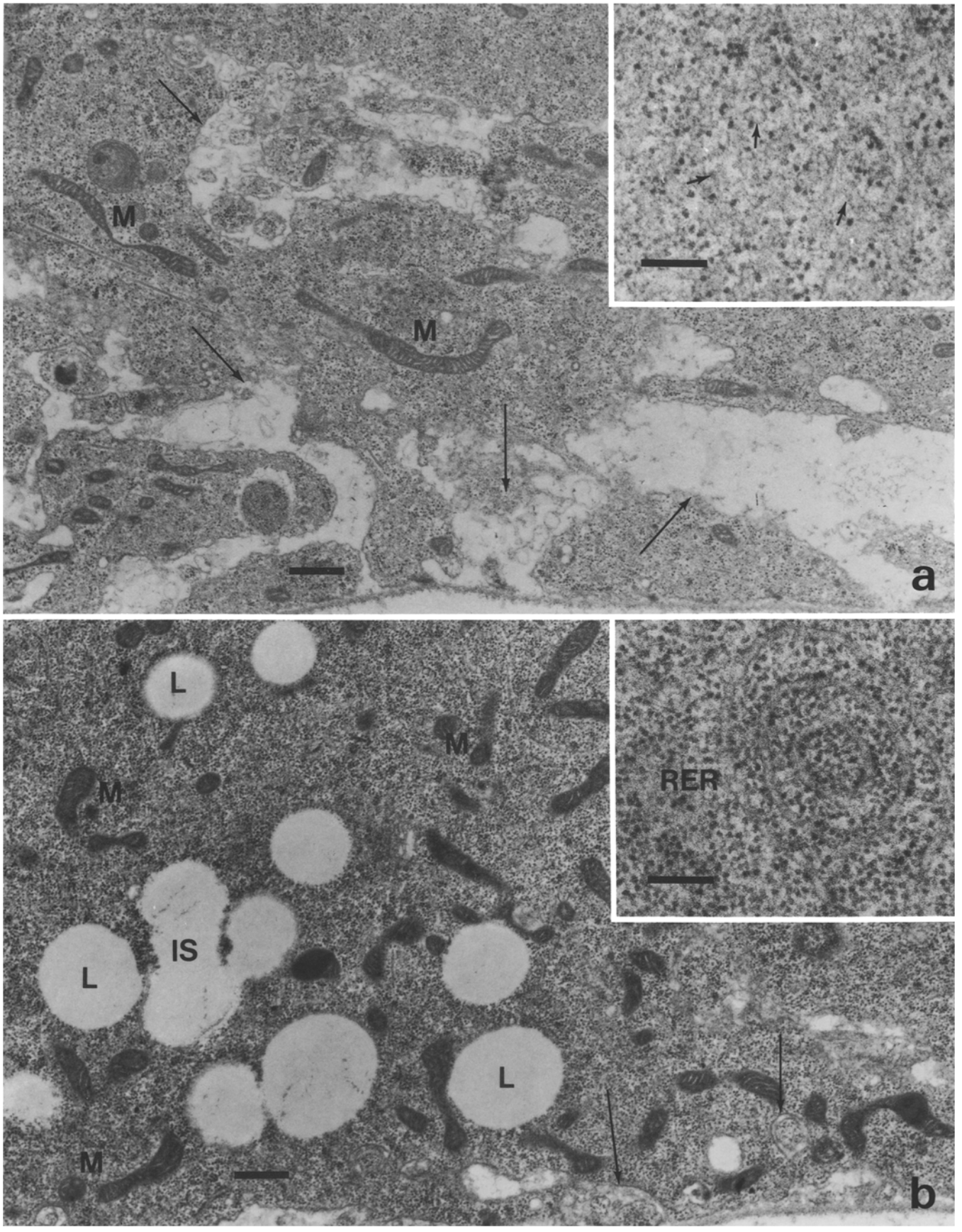

Fig. 4a, b. Prothoracic gland cells from larvae transferred nine days after egg laying from 18° C to 29° C for 24 h prior to observation. Same abbreviations as in Fig. 3. *Long arrow* Membrane invagination; *short arrow* SER. **a** Cell of wild-type; *bar*: 0.5 µm; ×20000. *Inset: bar*: 0.2 µm; ×64000. **b** Cell of ecd^{1ts} mutant; *bar*: 0.5 µm; ×20000. *Inset: bar:* 0.2 µm; ×64000

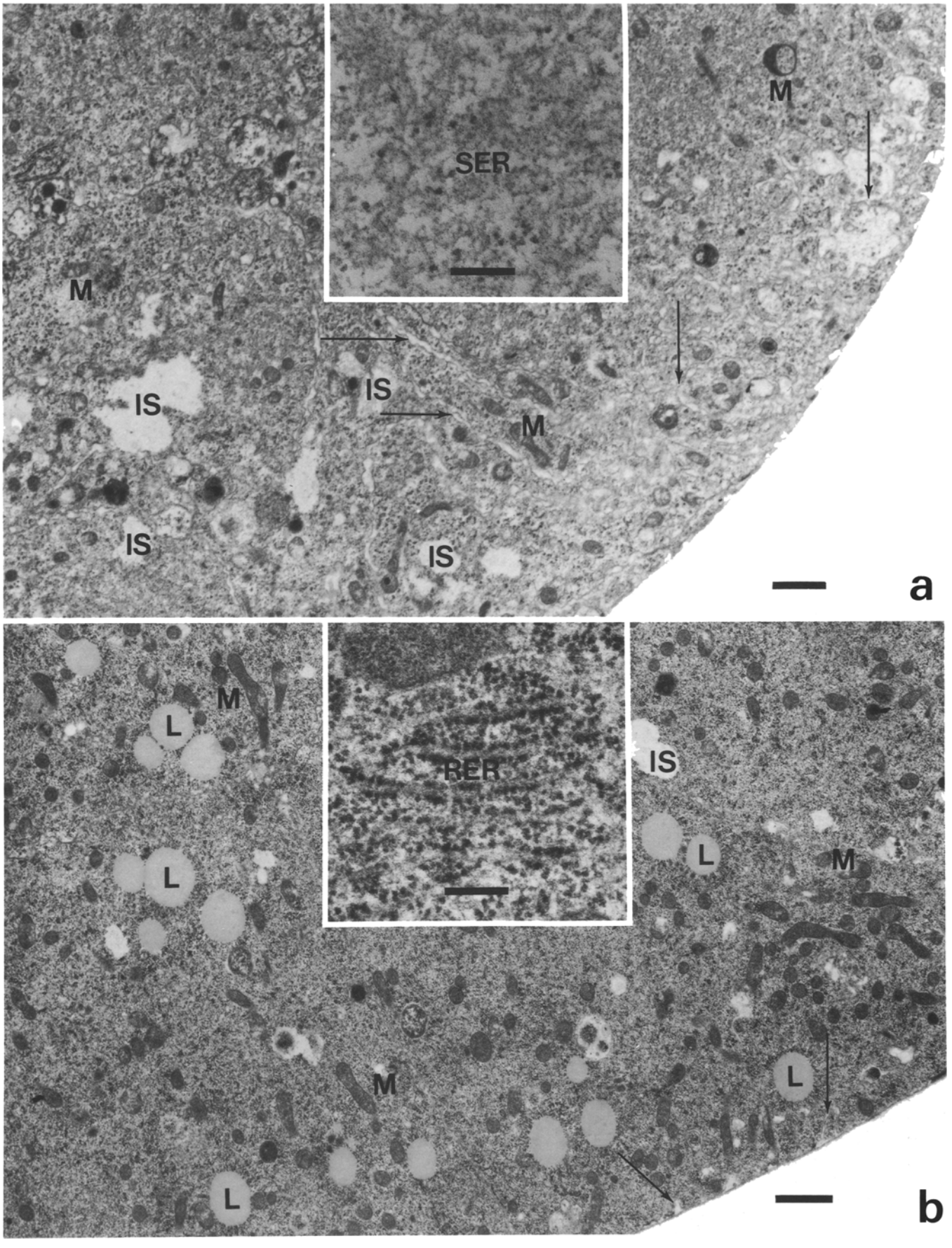

Fig. 5a, b. Prothoracic gland cells from ring glands incubated at 29° C in vitro for 4 h after dissection from larvae reared at 18° C. Same abbreviations as in Fig. 3. *Arrow* Membrane invagination. **a** Wild-type cell from a gland producing 438.15 pg ecdysteroids/4 h in vitro; *bar*: 1 μm; ×10000. *Inset: bar*: 0.2 μm; ×64000. **b** ecd^{1ts} mutant cell from a gland producing 75.45 pg ecdysteroids/4 h in vitro; *bar*: 1 μm; ×10000. *Inset: bar*: 0.2 μm; ×64000

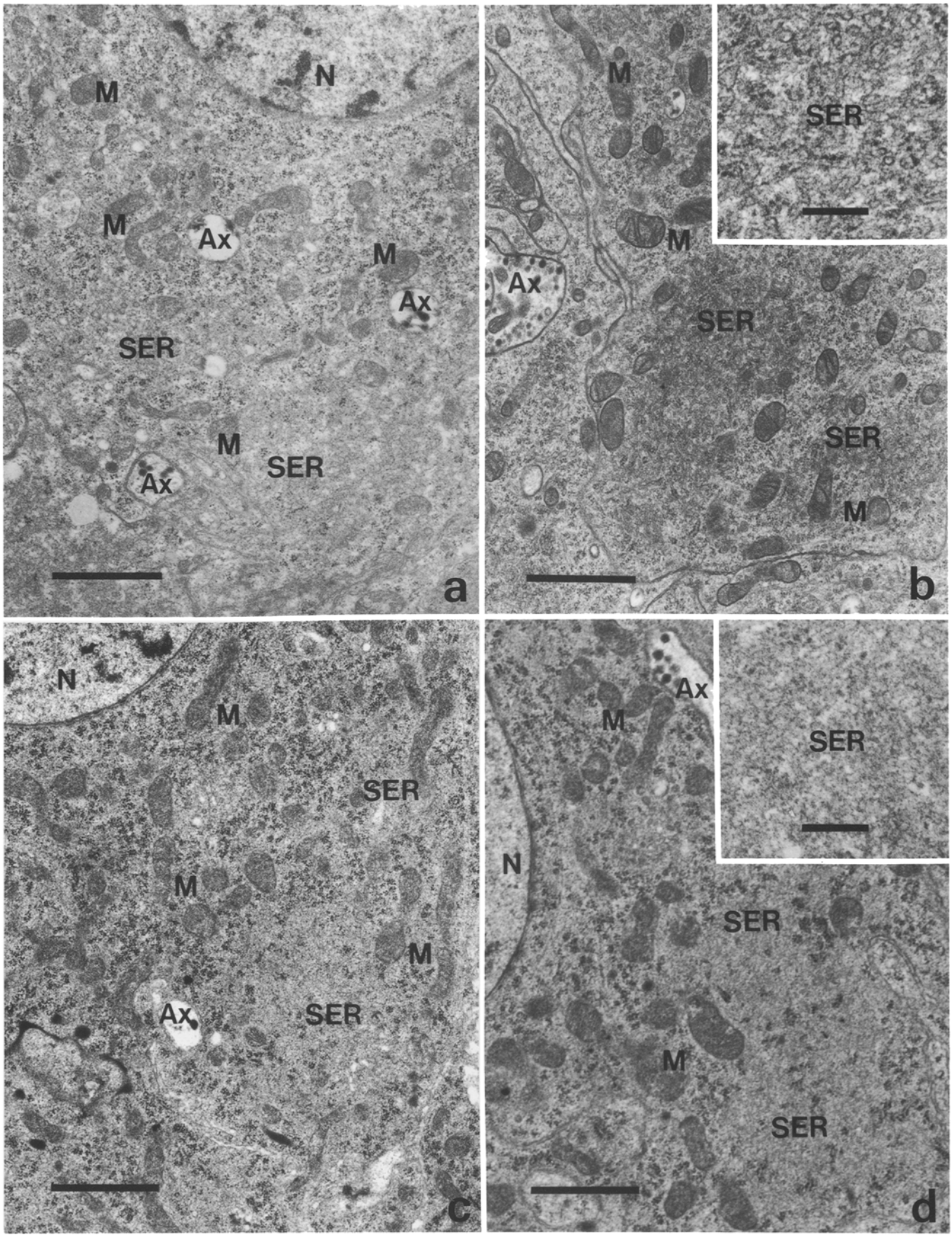

Fig. 6a–d. Corpus allatum cells from wild-type (**a**) or a *ecd*^{1ts} mutant (**b**) early wandering, third instar larvae, reared at 18° C, and the corpus allatum cell from wild-type (**c**) or *ecd*^{1ts} mutant (**d**) early wandering, third instar larvae, transferred nine days after egg laying from 18° C to 29° C for 24 h prior to observation. Same abbreviations as in Fig. 3, plus axon (*Ax*) ending on cell. *Bar*: 1 μm; ×18600. *Inset: bar*: 0.2 μm; ×64000

The corpus allatum is the site of elevated juvenile hormone bisepoxide III (JHB_3) synthesis through the wandering phase of the third larval instar (Richard et al. 1989). No apparent ultrastructural differences were observed between corpus allatum cells from wild-type (Fig. 6a, c) and ecd^{1ts} (Fig. 6b, d) larvae either before (Fig. 6a, b) or after (Fig. 6c, d) the upshift to the restrictive temperature. Like the corpus allatum from wild-type larvae reared at 25° C, cells from both the mutant and wild-type animals were devoid of whorls of SER (presumably the equivalent of allatum bodies, Aggarwal and King 1969; see Dai and Gilbert 1991) which may be SER precursors associated with corpus allatum inactivity. These cells contained extensive SER structures and numerous mitochondria of various shapes that are indicative of high corpus allatum activity (see Sedlak 1985). No evidence of the accumulation of lipid droplets or of the abnormalities of membrane invaginations, mitochondria or SER was found in the mutant corpus allatum after the temperature upshift. These observations suggest that the ecd^{1ts} mutation does not directly exert effects on endocrine tissues other than the prothoracic glands.

Discussion

Both the corpus allatum and the prothoracic gland of the *Drosophila melanogaster* ring gland undergo ultrastructural changes during the late third instar that are consistent both with the concomitant fluctuations in hormone titers and with developmental changes observed in previous studies of insect endocrine organs. The active prothoracic gland cell is characterized by increased amounts of SER, mitochondria of various shapes, and cell surface area, the latter by the formation of extensive infoldings of the plasma membrane. Lipid droplets, presumably the depositories of sterol precursors for ecdysteroid biosynthesis, occur in limited quantities during the normal wandering stage when whole body ecdysteroid titers and biosynthetic activity increase. The corpus allatum of the ring gland accumulates lipid droplets after pupation when juvenile hormone synthetic activity is negligible (Dai and Gilbert 1991).

Little is known about the molecular and cellular processes involved in ecdysteroidogenesis (see Rees 1985). By analogy with vertebrate steroidogenic pathways (Nussdorfer and Mazzocchi 1983; Lehoux et al. 1987; Simpson and Waterman 1988; Kappler et al. 1989), at least five important steps should occur: (1) the transport of precursors (cholesterol, β-sitosterol) to the prothoracic gland cells and their storage in subcellular sites; (2) the conversion and subsequent transport of these precursors to biosynthetic sites in the mitochondria; (3) the biosynthesis of ecdysteroids by P450 enzymes in the SER and mitochondria; (4) the packaging and subsequent secretion of these products from the cell; and (5) the regulation of one or more of these components by the action of one or more tropic neurohormones. It should be noted that among the five steps noted, only the biosynthesis of ecdysteroids (step 3) would be a process confined to steroidogenic organs (Waterman et al. 1986; Simpson et al. 1987; Simpson and Waterman 1988). The genetic approach available in *Drosophila melanogaster* allows for the identification of discrete gene products that, when structurally altered, could cause a failure in any of these steps. Ultrastructural analysis of ring glands from mutant larvae homozygous for lethal giant larva (*l(2)gl*) (Aggarwal and King 1969) suggested that the well-studied ecdysteroid deficiency of this mutant (Hadorn 1937) may arise from a failure of prothoracicotropic hormone release caused by extensive abnormal proliferation of surrounding tissues, and not as the consequence of some intrinsic failure of the prothoracic gland (i.e., step 5). Later, *lgl* was found to be a tumor-promoting mutation that affects imaginal disc growth (Gateff 1978).

The *ecd* mutation studied here is typified by a reduction in the ecdysteroid titer after prolonged maintenance of larvae at the restrictive temperature of 29° C, and disruption of imaginal disc development (Garen et al. 1977; Redfern and Bownes 1983; Sliter 1989). This has led to the suggestion that the mutation interferes with a broad range of cellular functions, including ecdysteroidogenesis. Although the ecdysoneless mutation causes cellular degeneration and death after some weeks at its restrictive temperature, it does not block ongoing morphogenetic processes or reduces general life sustaining processes, such as feeding and respiration, and therefore may play a specific regulatory role in steroidogenesis.

In this study, the prothoracic gland cells from the mutant showed no signs of degeneration, such as the proliferation of lysosome-like structures that accompanies the normal degeneration of the prothoracic gland component of the ring gland during metamorphosis (Dai and Gilbert 1991). Nor, in fact, did the mutation appear to alter the morphology of the corpus allatum cells. Both in vivo and in vitro, an upshift to 29° C caused an accumulation of lipid droplets, the disappearance of SER, enhancement of electron-dense mitochondria, and a decrease in membrane invaginations concomitant with a measured reduction in ecdysteroid secretion in mutant prothoracic gland cells.

The basic question concerns the specific step that the *ecd* mutation disrupts in the prothoracic gland. It is unlikely that the mutation alters a biosynthetic enzyme (step 3), because it is autonomously expressed in nonendocrine tissues (Sliter 1989). The abnormally high accumulation of lipid droplets, if in fact they contain precursors (Romer 1971; Rees et al. 1980), also suggests that the cells retain the ability to sequester cholesterol, i.e., cholesterol reaches the prothoracic gland (step 1) and, therefore, ecdysteroid deficiencies do not arise from an inadequate supply of precursors to the cell. It should be noted that the incubation medium contains no cholesterol, so the droplets, if truly sterol-containing, must form from substrates already in the cell. Furthermore, glands from ecd^{1ts} larvae incubated in vitro do not accumulate ecdysteroids, as measured by radioimmunoassay, ruling out a failure of secretion (step 4).

Thus, two possibilities remain for explaining the ecd^{1ts} phenotype: interference with cholesterol conver-

sion and transport, and/or the activation of heightened synthetic levels via a neurohormonally modulated pathway, as in *Manduca* (see Gilbert et al. 1988). The ring gland of *Drosophila* also responds to neural extracts that presumably contain PTTH (Henrich et al. 1987a) and the response of glands from ecd^{1ts} larvae to these extracts is impaired. However, the interpretation of this observation is complicated because the mutation only partially disrupts its gene product (Henrich et al. 1987b). The reduction in cell surface area alone in glands from ecd^{1ts} animals at the restrictive temperature could account for the diminished response by reducing the number of available PTTH receptors. Brains from ecd^{1ts} larvae contain abnormally high levels of PTTH-like activity, eliminating the possibility that the *ecd* gene encodes a PTTH, but suggesting that a failure to release PTTH could be among the mutation's developmental consequences.

Little is known about the cellular storage of cholesterol, its conversion and subsequent intercellular transport in insects. Among vertebrates, chronic treatment with adrenocorticotropic hormone (ACTH) elicits a notable time-dependent enhancement of steroidogenesis accompanied by dramatic ultrastructural changes in adrenocortical cells both in vivo and in vitro (e.g., an accumulation of lipid droplets and mitochondrial hypertrophy), whereas acute exposure of isolated cells to ACTH provokes a striking depletion of lipid droplets that is proportional to the extent of corticosterone secretion (Nussdorfer et al. 1971, 1974; Andreis et al. 1989). These droplets contain cholesterol (Moses et al. 1969; Frühling et al. 1973) which is then converted to the steroid hormone (Boyd et al. 1983). In addition, chronic ACTH exposure stimulates the de novo synthesis of various enzymes that participate in steroidogenesis and are located mainly in the mitochondria and SER (Tamaoki 1973; Brown and Goldstein 1980; Nussdorfer and Mazzocchi 1983; Mazzocchi et al. 1986; Nussdorfer 1986; Singer et al. 1988). Moreover, the activity of cholesterol ester hydrolase in the bovine adrenal cortex is increased via ACTH stimulation of adenylate cyclase (Boyd and Gorban 1980). If an analogous situation exists in *Drosophila*, then a mutationally induced impairment of neurohormonal receptors, a transduction enzyme, or cholesterol ester hydrolase could elicit the subcellular disappearance of SER, the appearance of electron-dense mitochondria (Kaiser 1980; Haget et al. 1981; Johnson et al. 1985) and an abnormal accumulation of lipid droplets such as observed here.

Any explanation of the effects of the ecd^{1ts} mutation must account for the qualitative and quantitative decrease in intercellular channels found in glands from mutant larvae at the restrictive temperature. This phenotype does not simply arise because of a retardation of gland development. When upshifted to 29° C in vitro, at a time when glands from mutant larvae at the permissive temperature show normal morphology, ecd^{1ts} glands lose their SER and membrane invaginations, and accumulate lipid droplets, despite the fact that the medium supplies no exogenous cholesterol or lipid. This observation suggests that ultrastructural abnormalities arise from, or cause, the disrupted flow of sterols from storage sites in the cell to sites of utilization. When the surface area of the plasma membrane increases or the level of ecdysteroidogenesis increases, one would expect a reduction in the size and number of lipid droplets, as seen in the active, wild-type prothoracic gland. However, the glands from ecd^{1ts} animals exhibit a decreased rate of ecdysteroid synthesis, decreased quantities of SER and plasma membrane and an increased abundance of lipid droplets. The mutation may cause the sequestration of sterols in an insoluble form in the lipid droplets so that the effects of the ecd^{1ts} mutation would be most severe in an ecdysteroid-producing organ and in cells undergoing a rapid increase in size and/or number.

Thus, at its restrictive temperature, the *ecd* mutation directly or indirectly blocks the formation of SER, disturbs mitochondrial function, and reduces ecdysteroid biosynthesis, presumably by interrupting the normal cycle of intracellular conversion of cholesterol to ecdysteroids. The latter compounds are presumably synthesized in the mitochondria and SER as is the case for mammalian steroids.

The unequivocal demonstration of the *ecd* gene's role in steroidogenesis can only be elucidated by studing new alleles of the mutant and, ultimately, sequencing the gene. Nevertheless, ultrastructural observations of this and other endocrine mutations in *Drosophila melanogaster*, can lead to testable hypotheses concerning the cellular and developmental regulation of steroidogenesis.

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