Reversible Blockage of Membrane Retrieval and Endocytosis in the Garland Cell of the Temperature-sensitive Mutant of *Drosophila melanogaster, shibire^{ts1}*

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ABSTRACT Temperature-induced structural changes in the cortical region of the garland cell, which is considered to be active in endocytosis, were investigated in a temperature-sensitive, single gene mutant of *Drosophila melanogaster, shibire^{ts1}* (*shi*) and wild-type (Oregon-R). At 19°C, both *shi* and wild type showed similar structural features: an irregularly extended network of labyrinthine channels, coated pits and vesicles, tubular elements and alpha vacuoles. Tannic acid (TA) impregnation showed that coated pits comprised ~20–25% of the total coated profiles at 19°C in both *shi* and wild-type. When flies were incubated in a horseradish peroxidase (HRP) solution for 5 min, organelles such as coated profiles, tubular elements, and alpha vacuoles were labeled.

In wild-type at 30°C, minor changes were observed—mainly a decrease in the distribution of the labyrinthine channels and an increase in HRP uptake. On the other hand, in *shi* at 30°C, the labyrinthine channels were much elongated and their network became far more complex, indicating the expansion of the surface area of the cell. Also, the coated profiles were increased in number while the number of tubular elements was decreased considerably. The TA method showed that almost all of the coated profiles were coated pits, coated vesicles being almost completely absent at 30°C in *shi*. Furthermore, HRP uptake activity was considerably decreased at 30°C. These structural changes, as well as the reduced HRP uptake activity, were reversible when the temperature was lowered to 19°C. The observations suggest that in the garland cell of *shi* the conversion of coated pits to coated vesicles, that is, membrane pinch-off, is blocked at high temperature.

The temperature-sensitive single gene mutant of *Drosophila melanogaster, shibire^{ts1}* (*shi*),[1] behaves normally at 22°C but shows a reversible paralysis at 29°C (6). Physiological studies have shown that a progressive diminution of its neuromuscular excitatory junction potentials occurs as the temperature is raised above 28°C with moderate stimulation (0.5 Hz) (11, 23). This effect appears to be presynaptic, since the muscle fibers respond normally to direct stimulation (11), the sensitivity of the postsynaptic membrane to iontophoretically applied l-glutamate (putative transmitter, reference 9) remains unaltered (Koenig, J. H., and K. Ikeda, manuscript submitted for publication), and a reversible synaptic vesicle depletion at neuromuscular junctions (12, 15, 19) has been observed. The transmitter release mechanism (exocytosis) is apparently unaffected at high temperature, since normal-appearing miniature excitatory junction potentials and a normal-sized response to stimulation are observed if activity at the terminal is suppressed while the temperature is raised (14, 20). These observations suggest that a step in the recycling of synaptic vesicles might be blocked in this mutant, resulting in vesicle depletion as exocytosis proceeds at high temperature. This possibility is further suggested by the observation that many pitlike structures accumulate on the presynaptic membrane of various types of synaptic terminals of *shi* at high temperature (16).

These observations suggest that the process of endocytosis

[1] *Abbreviations used in this paper:* DAB, 3,3′-diaminobenzidine-tetrahydrochloride; HRP, horseradish peroxidase; *shi*, *shibire^{ts1}*; TA, tannic acid.

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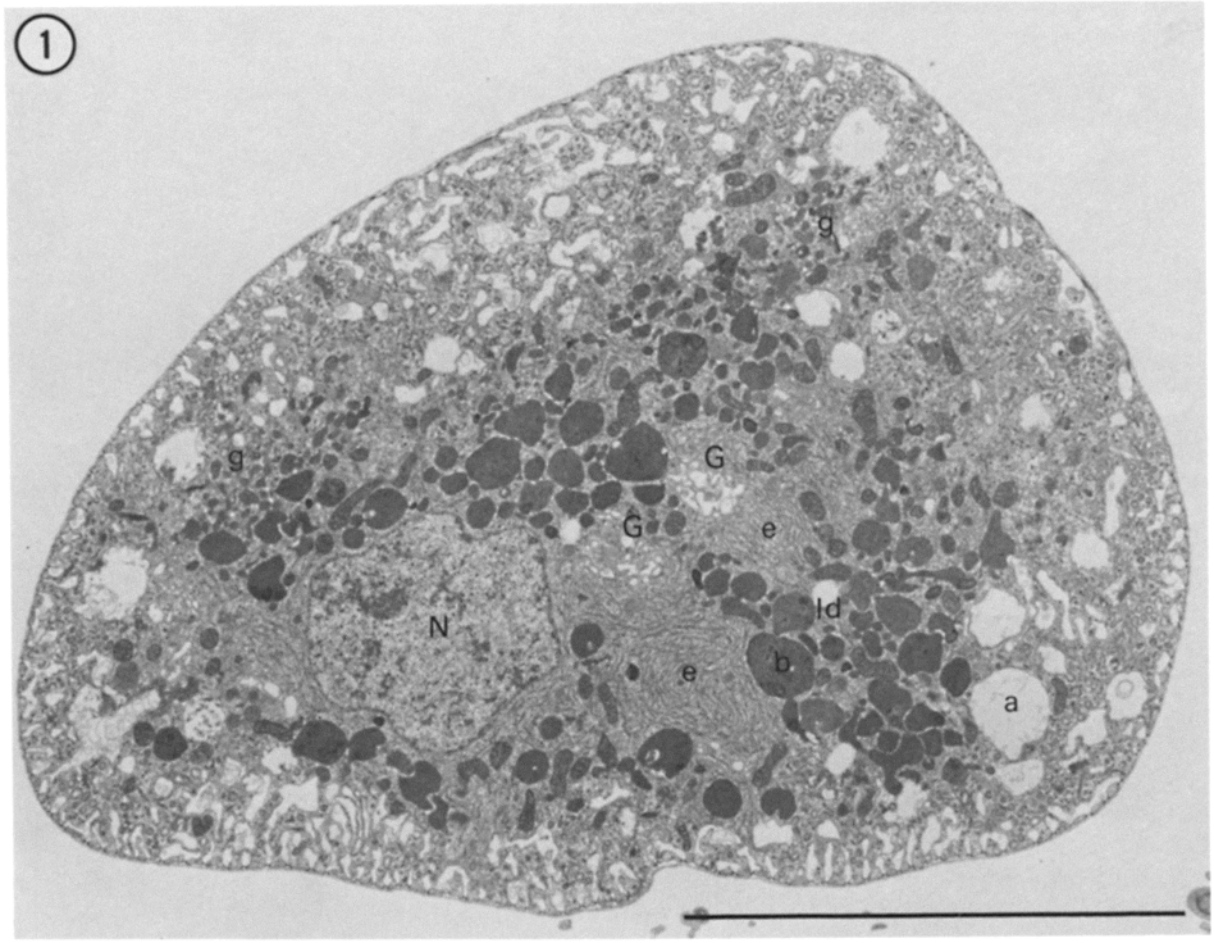


FIGURE 1 Electron micrograph of a garland cell of *shi* at 19°C. A thin layer of basement membrane covers all of the cell surface. In the cortical region, many labyrinthine channels and alpha vacuoles (*a*) are seen. In the central region, dense beta vacuoles (*b*), small dense granules (*g*), lipid droplets (*ld*), cisternae of rER (*e*), and Golgi apparatus (*G*) surround a nucleus (*N*). Bar, 10 μm. × 6,640.

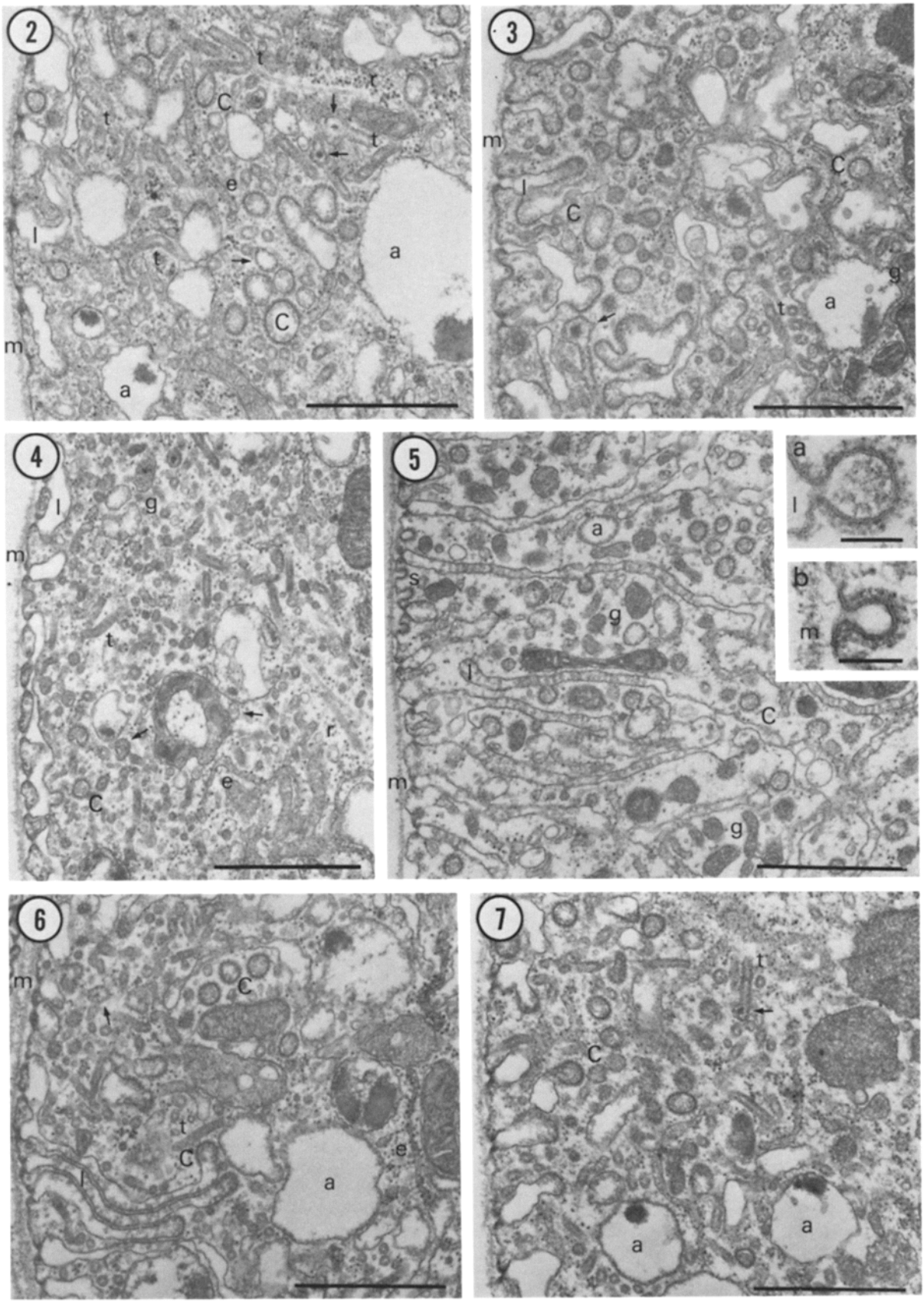
in general may be blocked in *shi* at high temperature. Therefore, the garland cells (or wreath cells), which are considered to be very active in endocytosis via coated vesicles (25), were observed. These cells are very similar to pericardial cells (21) in structure and function and are classed together with the latter as "nephrocytes", since they appear to function in the segregation and storage of waste products (25). We report here temperature-induced structural changes in these garland cells in adult *shi* flies. These changes suggest that the endocytotic activity of the garland cell is blocked at high temperature at the step that converts coated pits to coated vesicles.

MATERIALS AND METHODS

The experimental animals were the wild-type strain, Oregon-R, and the single gene mutant, (*shi*), of *Drosophila melanogaster*. 4–10-d old adult female flies that had been raised at 17°C were used.

For dissection, a fly was mounted laterally (right side up) with Tackiwax over an opening in a plastic tube in a dish, so that its left side remained exposed to air. Then it was covered with modified Bodenstein saline solution (10), and

FIGURES 2–7 Fig. 2: Wild-type at 19°C. Coated profiles (*C*), tubular elements (*t*) labyrinthine channels (*l*), alpha vacuoles (*a*), cisternae of rER (*e*), and many ribosomes (*r*) are seen. Arrows indicate bulbous swellings of tubular elements. *m*, basement membrane. Bar, 1 μm. × 26,200. Fig. 3: *shi* at 19°C. Organelles appear similar to those of wild-type. Small dense granule (*g*) shown here is also seen in wild-type. Bar, 1 μm. × 26,200. Fig. 4: Wild-type at 30°C. Labyrinthine channels (*l*) decrease in extent and are confined to an area close to the plasma membrane. The structure of the openings of the labyrinthine channels is unchanged, showing extracellular dense material and a cytoplasmic dense undercoating of the membrane. Coated profiles (*C*) appear to be decreased in number and size, while tubular elements (*t*), small dense granules (*g*) and other vesicular profiles (arrows) appear to be increased in number. Bar, 1 μm. × 24,700. Fig. 5: *shi* at 30°C. Labyrinthine channels (*l*) are elongated. Coated profiles (*C*) and small dense granules (*g*) are increased, while tubular elements (*t*) are decreased. Many coated pits of the small type (*s*) are seen at the cell surface. Presumable small alpha vacuoles (*a*) with periodically arranged dense lining are seen. Bar, 1 μm. × 26,200. (*Inset a*) A coated pit of the large type (containing dense material) arising from a labyrinthine channel (*l*). Bar, 0.1 μm. × 107,200. (*Inset b*) A coated pit of the small type (containing no dense material) arising from the cell surface opposing the basement membrane (*m*). Bar, 0.1 μm. × 107,200. Fig. 6: Wild-type after 30-min recovery. The structural features have returned to a condition very similar to that of the controls. Bar, 1 μm. × 26,200. Fig. 7: *shi* after 30-min recovery. Complete recovery is seen. Labyrinthine channels are shortened. Tubular elements and alpha vacuoles have reappeared and are similar to those of the control. Bar, 1 μm. × 26,200.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the episternum, epimeron, and scutum were removed. The temperature was controlled by a thermoelectric unit placed under the dissecting dish and monitored by a thermistor in the saline solution. Four flies, two wild-type and two *shi*, were processed together to insure that these four flies were exposed to exactly the same conditions.

Light and Electron Microscopy: For low temperature control experiments, the flies were dissected in saline at 19°C, kept at that temperature for 5–20 min, and fixed at the same temperature with a mixture of 2% paraformaldehyde and 2% glutaraldehyde in 0.1 M phosphate or cacodylate buffer (pH 7.2–7.4).

For high temperature experiments, the flies were dissected at 19°C, kept at that temperature for 5 min, and then the temperature was raised to 30°C at a rate of about 1–2°C/min. After 10-min exposure to 30°C, the flies were fixed at 30°C with the same aldehyde mixture.

For recovery experiments, flies were exposed to 30°C for 10 min in the same way as that described above, then the temperature was lowered to 19°C at a rate of ~1–2°C/min and kept at that temperature for 10 min (10-min recovery) or 30 min (30-min recovery) before fixation.

Specimens were postfixed in 2% osmium tetroxide (OsO_4) in 0.1 M cacodylate buffer (pH 7.2), block-stained in 1% aqueous uranyl acetate, dehydrated in a graded series of ethanol, and embedded in Epon 812. Semithin (0.5 μm) sections were cut with glass knives and stained with 1% toluidine blue. Two to four series of partially serial semithin sections of the anterior portions of ventral halves of the thorax were made from each of four different categories, i.e., wild-type at 19° and 30°C, and *shi* at 19° and 30°C. Thin sections with white interference color were cut with Dupont diamond knives in a LKB ultramicrotome, double-stained with uranyl acetate and Millonig's lead hydroxide (18), and examined with a Philips 301 electron microscope.

Tannic Acid Impregnation (TA) Method: Following the method of Bungaard, Frøkjar-Jensen, and Crone (3), tannic acid (powder: Matheson Coleman and Bell Manufacturing Chemists, Norwood, OH) was used to distinguish the organelles continuous to the extracellular spaces from intracellular organelles. After osmification, specimens were rinsed twice briefly with 1% tannic acid in 0.1 M cacodylate buffer (pH 7.2) and immersed in the same tannic acid solution for 1 h at room temperature. Then they were dehydrated in ethanol and embedded in Epon 812. Thin sections were double-stained as described above. The impregnation was very capricious. In a single specimen, some cells were well impregnated while others were not. Furthermore, even a single cell could have both well and poorly impregnated portions. For analysis, only well impregnated portions were selected.

Horseradish Peroxidase (HRP) Uptake Experiments: To test the endocytotic activity of the garland cells, a 0.7% solution of horseradish peroxidase (type VI, Sigma Chemical Co., St. Louis, MO) in modified Bodenstein saline solution was used.

For low temperature experiments, flies were incubated at 19°C with the HRP solution for 5 min after dissection, and then fixed at this temperature. For high temperature experiments, flies were dissected in saline solution and the temperature was raised as described previously. After 4-min of exposure to 30°C, the saline solution was replaced with the HRP solution. After a 5-min incubation with HRP at 30°C, the flies were fixed with the aldehyde mixture at 30°C and kept at that temperature for 10–15 min, then returned to room temperature.

For recovery experiments, the flies were incubated with the HRP solution in the same manner as that described for the high temperature experiments, then the temperature was lowered and kept at 19°C for 10 min or 30 min. The flies were fixed at 19°C with the same aldehyde mixture.

After 1-h fixation in the aldehyde mixture, all flies were further dissected, with removal of the dorso-ventral muscles, the tergotrochanter muscle, and the upper five dorsal longitudinal muscles on both sides. The total duration of the aldehyde fixation was 2–3 h. Specimens were rinsed overnight in 0.1 M phosphate buffer (pH 7.2) while being kept in the refrigerator. They were preincubated at room temperature for 30 min in a 0.05% solution of 3,3'-diaminobenzidine-tetrahydrochloride (DAB; grade II, Sigma Chemical Co.) in the same phosphate buffer and were subsequently incubated for 30 min in a 0.5% DAB solution in the same phosphate buffer containing 0.01% H_2O_2 (5, 22). The specimens were rinsed in the same phosphate buffer and postfixed in 2% OsO_4 in 0.1 M cacodylate buffer (pH 7.2). After block-staining in uranyl acetate and dehydration in ethanol, the specimens were embedded in Epon 812. Thin sections were usually observed without staining. For controls, either the incubation with HRP solution or the incubations in DAB and DAB + H_2O_2 solutions were omitted. In these controls, small dense granular structures, ~40–50 nm in diameter, were observed that were easily distinguishable from HRP-labeled structures.

Measurements: Experimental changes in the numbers of coated profiles and tubular elements were estimated by counting numbers of profiles per 1 μm perimeter of the cell surface. For this purpose, electron micrographs were taken at a primary magnification of 10,000 or 13,000 (calibrated by a Pelco's carbon grating replica) and printed with a 2.5 magnification. Portions of garland cells cut perpendicular to the cell surface were selected. Profiles showing no clearly defined limiting membranes were not counted. The length of the surface perimeter was measured with a thread along the layer of the basement membrane. 5–22 photographs of cortical regions with 45–165 μm of total surface perimeter were analyzed from each fly.

The numbers of coated vesicles and pits were counted by using electron micrographs of TA materials, and the percentage of coated vesicles in the total number of coated profiles was calculated. ~200–600 coated profiles were analyzed from each fly.

For the measurements of the outer diameter of coated vesicles and pits, one TA material was selected for each condition. ~15–20 electron micrographs were taken from each fly at a primary magnification of 16,700 and enlarged 2.5 times. The final magnification of the prints was 41,700 (calibrated by a Pelco's carbon grating replica). The outside diameters of the coated vesicles and pits were measured on the prints using calipers. The diameter of the coated vesicles of elongated or irregular shape was determined by $\sqrt{ab}$, where a and b were distances along the shortest and longest axes, respectively.

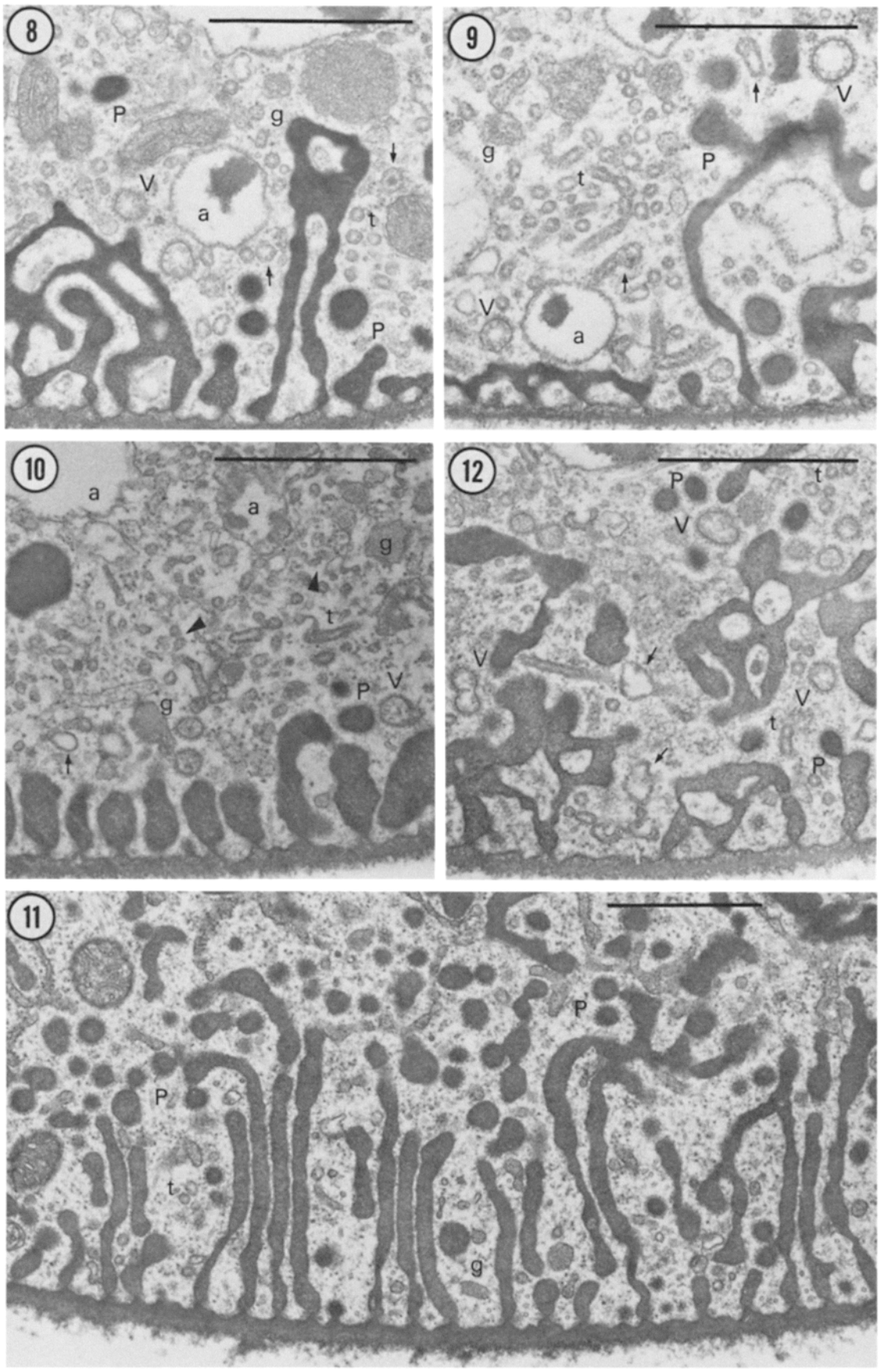
RESULTS

The structural features of garland cells and pericardial cells have been well investigated both light and electron microscopically and are considered to be very similar in various insects (1, 2, 4, 7, 8, 13, 17, 21, 24). Our observations were consistent with these previous descriptions.

In adult *Drosophila*, the garland cells usually cluster near the junction between the ventriculus and the esophagus. There are ~10 garland cells on each side, which are ovoid in shape (~28–35 μm by 10–17 μm) and have two subcentral spherical nuclei ~4 μm in diameter (Fig. 1). No consistent differences in the distribution, size, or shape of the garland cells between wild-type and *shi* at either low or high temperatures were observed.

The cytoplasm of the garland cell consists of two concentric regions, the central region and the cortical region (Fig. 1), as

FIGURES 8–12 Fig. 8: Wild-type at 19°C, tannic acid impregnation. Two unimpregnated coated vesicles (*V*) are clearly distinguishable from impregnated coated pits (*P*). Labyrinthine channels are impregnated. Arrows indicate bulbous swellings of tubular elements, one of which shows a small dense granular aggregate. Bar, 1 μm. × 33,800. Fig. 9. *shi* at 19°C. Structural features are almost the same as those of wild-type. Two coated vesicles (*V*) are seen, one of which appears to be partially coated. Many tubular elements appear to cluster. Arrows indicate bulbous swellings of tubular elements. Bar, 1 μm. × 33,800. Fig. 10. Wild-type at 30°C. Labyrinthine channels are shortened. Coated vesicles (*V*) and pits (*P*) are distinguishable by tannic acid. Many vesicular or tubular profiles (arrowheads) are seen. Small dense granules (*g*) are increased in number. Bar, 1 μm. × 33,800. Figure 11. *shi* at 30°C. Labyrinthine channels are elongated and appear to run parallel to one another. All coated profiles in this figure are impregnated, i.e., they are coated pits. Note: Disappearance of coated vesicles. Tubular elements have greatly decreased in number—only two presumable tubular elements (*t*) can be seen here. Bar, 1 μm. × 26,200. Fig. 12. *shi* after 30-min recovery. After 10 min exposure to 30°C, the temperature was lowered to 19°C and maintained for 30 min. The features of the labyrinthine channels, coated vesicles (*V*), coated pits (*P*), and tubular elements appear to return to those of the controls. Arrows indicate structures appearing intermediate between bulbous swellings of tubular elements and small alpha vacuoles. Bar, 1 μm. × 33,800.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described previously in pericardial and garland cells of various insects (1, 2, 4, 13, 21, 24). Here, we will focus on the structure of the cortical region, where a major part of the endocytotic process takes place.

Effect of Temperature

WILD-TYPE AND *SHI* AT 19°C: At 19°C, no obvious difference in the fine structure of the cortical region of the garland cells was observed between *shi* and wild-type flies. A layer of basement membrane, ~25–30 nm thick, covered the whole surface of each garland cell but never penetrated into the labyrinthine channels (Figs. 1–3). The cytoplasm surrounding the channel openings contained a dense material, while a thin bridge of extracellular material spanned the openings. Within some parts of the channels, an extracellular material that sometimes showed periodicity was observed (Figs. 2 and 3). Almost all of the labyrinthine channels were located near the surface of a cell and did not penetrate into the central region (Figs. 1–3).

Many coated profiles were seen in the cortical region. They have been classified into two groups (2, 4), one ~60–90 nm in diameter, the other ~120–180 nm in diameter. The smaller coated profiles were usually found near or at the surface opposing the basement membrane and did not contain any material in them (see inset *b* in Fig. 5). The larger ones were usually seen to arise from or lie between labyrinthine channels and contained material similar to that in the channels (Figs. 2 and 3; also, for higher magnification see inset *a* in Fig. 5). The latter were far more prevalent, representing ~95% of the total coated profiles in *shi* and wild-type at 19°C.

Other organelles observed in the cortical region were tubular elements, small dense granules, alpha vacuoles, free ribosomes, and small cisternae of rough endoplasmic reticulum. The tubular elements were membrane-bound and cylindrical, ~60–75 nm in diameter. A layer of dense material that appeared to be made of subunits (2, 4, 21) was seen to occupy the inside of the tubular elements. Some of the tubular elements had swollen bulbs on one end (Figs. 2 and 3), which also contained dense materials. Tubular elements that appeared to be connecting to alpha vacuoles were often observed. As reported previously (2, 4, 21), no tubular elements showed a connection with the plasma membrane. Alpha vacuoles (4), which were usually located slightly away from the cell surface, were very prominent structures in the cortical region (Figs. 1–3). They were round or ovoid in shape and varied considerably in size, from ~0.2 to 3 μm in diameter. The inside of the vacuoles was lined with a dense material and usually contained one or more aggregates of a dense granular material that appeared to adhere to the limiting membrane. Small dense granules of various shapes containing dense materials similar to those in the beta vacuoles were also observed (4). The beta vacuoles were mainly localized in the central region (Fig. 1).

WILD-TYPE AT 30°C: The structural features of the cortical region in wild-type flies at 30°C were fairly similar to those of the low temperature controls, but there seemed to be minor quantitative changes (Fig. 4). The labyrinthine channels appeared decreased in length and were usually confined to the peripheral 0.5 μm of cytoplasm, although some channels extended close to the nucleus. The number of coated profiles stayed almost the same, but the size appeared to be smaller. Alpha vacuoles appeared to decrease in size and become irregular in shape, and the number of tubular elements and small, dense granules appeared to be increased.

SHI AT 30°C: In contrast to the minor changes seen in wild-type, *shi* showed consistent, major structural changes at 30°C (Fig. 5). One prominent change was an elongation of the labyrinthine channels. In striking contrast to wild-type, the majority of the labyrinthine channels of *shi* extended radially 2–3 μm or more from the surface. They ran relatively parallel to one another and appeared to make a wide channel layer of regular width at the periphery. Thus, the surface area of the labyrinthine channels was increased considerably. A second prominent change was an increase in the number of coated profiles, particularly coated pits. Small coated profiles were encountered very frequently near or at the surface of the cell, while large coated profiles were frequently observed to arise from the elongated channels. Many of these coated profiles could be recognized as coated pits arising from the cell surface or the labyrinthine channels. A third prominent change was a considerable decrease in the number of tubular elements and alpha vacuoles. In addition to the three major changes described above, the small dense granules appeared to increase in number and were frequently observed between elongated labyrinthine channels.

RECOVERY: The temperature-induced structural changes in the cortical region of the garland cell in *shi* were reversible when the temperature was lowered to 19°C. After a 30-min recovery, the structural features of the cortical region were very similar to those at 19°C (Figs. 6 and 7). The distribution of labyrinthine channels and the number of coated profiles and tubular elements appeared to return to their control levels. The minor structural changes observed in wild-type also appeared to be reversible.

TA Impregnation

TA-impregnated materials were used to distinguish between those structures that were continuous with the extracellular space (e.g., labyrinthine channels and coated pits) and those that were not (e.g., coated vesicles, tubular elements).

WILD-TYPE AND *SHI* AT 19°C: The distribution of the labyrinthine channels, which appear as darkly impregnated structures, is well demonstrated by this technique. Furthermore, coated profiles can now be clearly distinguished as impregnated coated pits or unimpregnated coated vesicles (Figs. 8 and 9). Thus, it could be observed that there were no significant differences between wild-type and *shi* at 19°C in the number of coated profiles per 1 μm surface perimeter or in the percentage of coated vesicles in the total number of coated profiles (20–25%) (Table I). Tubular elements were not impregnated with TA. Therefore, they do not connect with the extracellular space.

WILD-TYPE AT 30°C: The labyrinthine channels were confined to the cortical region near the surface as shown in Fig. 10. Both coated pits (impregnated) and coated vesicles (unimpregnated) are seen in this figure. The number of coated profiles per μm of surface perimeter decreased and the percentage of the coated vesicles in total coated profiles increased compared with those at 19°C (Table I).

SHI AT 30°C: The labyrinthine channels in *shi* at 30°C showed a striking change from those at 19°C. This is clearly demonstrated in Fig. 11. The channels were extended radially toward the center of the cell, forming a 2–3-μm-wide impregnated layer along the cell surface. This temperature-induced

elongation is apparent when this is compared with either *shi* at 19°C (Fig. 9) or wild-type at 30°C (Fig. 10). Another prominent characteristic revealed by TA impregnation was that almost all of the coated profiles were impregnated (Fig. 11). Thus, they represent coated pits, not coated vesicles. On the other hand, coated vesicles (unimpregnated) were very rare (Fig. 11 and Table I). Thus the percentage of coated vesicles in the total coated profiles was drastically reduced (Table I). Large coated pits were seen to arise from the elongated labyrinthine channels. Since the total number of coated profiles was considerably increased in addition to the decrease in the percentage of coated vesicles in the total coated profiles, a large increase in the number of coated pits occurred in *shi* at 30°C. Besides the increase in the number of large coated pits, small coated pits, which were rare in *shi* at low temperature or in wild-type, were observed frequently near or at the surface of the cell (Fig. 11). ~10% of the total coated profiles were small coated pits. It was also observed that the number of tubular elements decreased considerably (Fig. 11 and Table I).

RECOVERY: After exposure to high temperature (30°C) for 10 min, the temperature was lowered to the control level (19°C). The reversibility of the temperature effect was observed after 30 min at 19°C. The distribution of the labyrinthine channels and the number of coated profiles and tubular elements returned to the original level (Fig. 12). Also, the percentage of coated vesicles in the total coated profiles returned to the original level (Table I). Thus, the temperature effect was reversible.

The temperature effect on the number of coated profiles and tubular elements and on the percentage of coated vesicles in total coated profiles is summarized in Table I. This table depicts clearly the structural changes in the *shi* garland cells, i.e., the increase in the number of coated pits and the drastic decrease of the number of coated vesicles and tubular elements. Table II shows the sizes of the coated vesicles and pits in various conditions. The sizes of the coated pits and vesicles seemed to be the same in *shi* and wild-type at 19°C. At 30°C, a decrease in the size of the coated pits was apparent in *shi*.

HRP Experiments

The three drastic structural changes in *shi* at 30°C that were described in the previous section suggest that endocytotic activity is affected at high temperature in this mutant. This was confirmed by experiments on HRP uptake.

At 19°C, no differences were observed between wild-type and *shi* in HRP uptake. After a 5-min incubation with HRP, coated profiles, tubular elements, and alpha vacuoles were labeled (Figs. 13 and 14).

When flies were incubated with HRP for 5 min at 30°C, wild-type showed an HRP uptake similar to or greater than that at 19°C. The structures labeled with HRP were the same as those labeled at 19°C, i.e., coated profiles, tubular elements, and alpha vacuoles (Fig. 15). On the other hand, in *shi* at 30°C, only alpha vacuoles of reduced size with dense granular aggregates were labeled (Fig. 16). Since the number and the size of alpha vacuoles were decreased as described before, the overall uptake of HRP was considerably decreased at this temperature.

The suppression of HRP uptake in *shi* at 30°C was reversible. After exposure to 30°C for 5 min, the temperature was lowered to 19°C and the fly was allowed to recover for 10 min. After that, HRP was applied for 5 min. Coated profiles, tubular elements, and alpha vacuoles were labeled in the same way as those of the low temperature controls.

DISCUSSION

The results present four major observations on the effect of high temperature on the garland cells of the mutant, *shi*: (*a*) The surface area of the plasma membrane increased drastically through the elongation and increase in complexity of the labyrinthine channels. (*b*) The number of coated pits increased while the number of coated vesicles decreased. (*c*) The number of tubular elements and alpha vacuoles decreased. (*d*) The uptake of HRP was greatly reduced.

What do these observations suggest about the nature of the *shi* defect? The increase in the labyrinthine channels, which represents a large increase in the surface area of the plasma membrane, suggests that an imbalance in the rates of exo- and endocytosis has occurred. This increase could be due either to a relative increase in the rate of exocytosis, which would add intracellular membrane to the plasma membrane,

TABLE II

Outside Diameters of Coated Pits and Vesicles

Genotype	Temperature	Coated pit	Coated vesicle
		nm	*nm*
Wild-type	19°C	150 ± 23 (130)	160 ± 27 (40)
Wild-type	30°C	134 ± 22 (172)	137 ± 22 (115)
shi	19°C	146 ± 23 (134)	158 ± 22 (45)
shi	30°C	117 ± 20 (168)	
shi	19°C (30 min recovery)	133 ± 20 (146)	128 ± 19 (48)

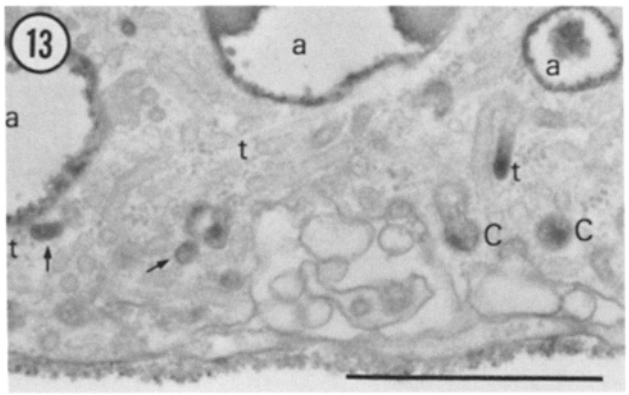
Values are expressed as mean ± standard error nm. Values in parentheses are numbers of profiles measured. One tannic acid-impregnated material was used for measurement of both coated pits and vesicles in each condition. Small coated profiles near the cell surface are excluded. Not enough coated vesicles in *shi* at 30°C were obtained.

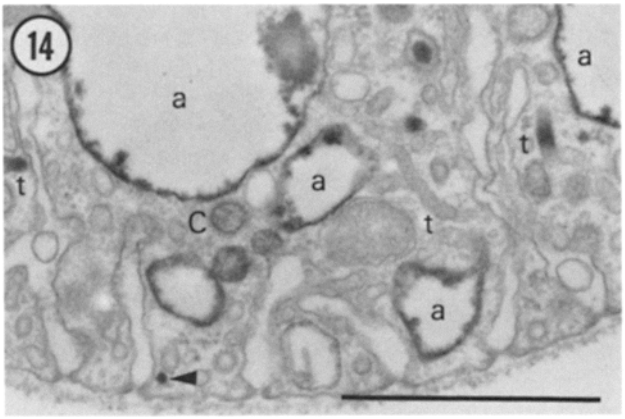
TABLE I

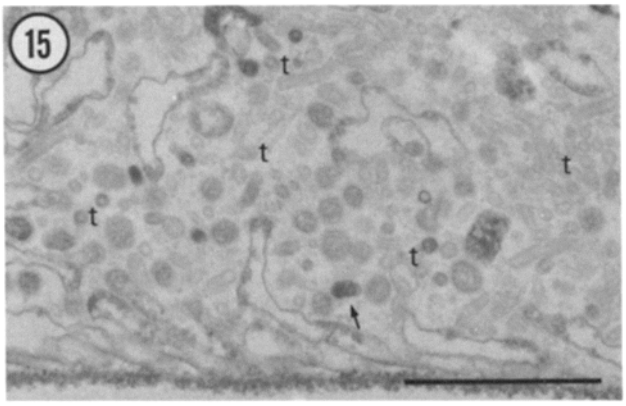
Temperature Effects on Coated Profiles and Tubular Elements

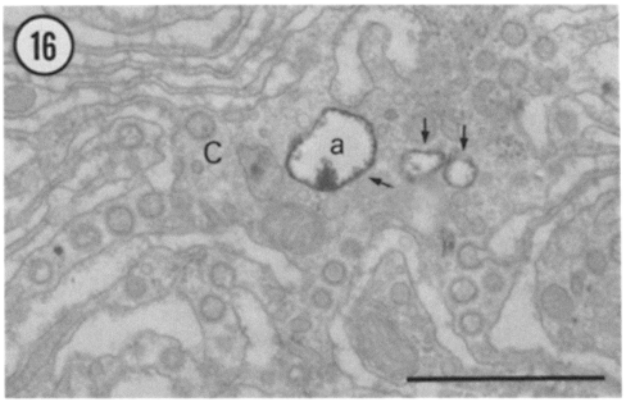
Genotype	Temperature	Number of coated profiles/1 μm surface perimeter	% Coated vesicles in total coated profiles	Number of tubular elements/1 μm surface perimeter
Wild-type	19°C	3.1 (6; 2,135)	20.4 (3; 1,100)	13.8 (3; 1,890)
Wild-type	30°C	2.5 (4; 746)	37.1 (1; 299)	12.7 (1; 955)
shi	19°C	3.5 (7; 2,444)	24.0 (5; 2,454)	11.9 (2; 1,177)
shi	30°C	6.2 (5; 2,181)	0.4 (4; 1,590)	0.7 (5; 242)
shi	19°C (30 min recovery)	3.7 (3; 1,073)	26.2 (1; 580)	10.1 (3; 2,501)

Values are expressed as mean.
Number of flies and profiles observed are shown in parentheses.









FIGURES 13–16 Figure 13. HRP uptake. Wild-type, 5-min incubation at 19°C. Coated profiles, tubular elements, and alpha vacuoles are labeled. Bar, 1 μm. × 33,800. Fig. 14. HRP uptake. *shi*, 5-min incubation at 19°C. Almost the same labeling pattern as wild-type is seen, i.e., labeling of coated profiles, tubular elements and alpha vacuoles. A dense particle (arrowhead) is observable without HRP. Bar, 1 μm. × 33,800. Fig. 15. HRP uptake. Wild-type, 6-min incubation at 30°C. Coated profiles, tubular elements and alpha vacuoles are labeled. The number of labeled structures appears to be increased compared with that at 19°C. Bar, 1 μm. × 26,200. Fig. 16. HRP uptake. *shi*, 6-min incubation at 30°C. The number of labeled structures is decreased considerably. Only one alpha vacuole and three presumable small alpha vacuoles are labeled. None of the coated profiles are labeled. Bar, 1 μm. × 26,200.

or to a relative decrease in the rate of endocytosis, which would cause a build-up of plasma membrane as exocytosis proceeded uninhibited. The considerable decrease in HRP uptake at high temperature suggests that the second possibility, i.e., a decrease in endocytosis, may be responsible.

If, indeed, the endocytotic process is blocked (or slowed down), then at what step in this process does the block occur? TA impregnation showed that at 30°C the number of coated pits increased while the number of coated vesicles and tubular elements drastically decreased. These observations suggest that the conversion of coated pits to coated vesicles, i.e. the membrane pinch-off mechanism, is blocked in *shi* at high temperature.

The mechanism responsible for this block in pinch-off appears to be common to various types of cells. For example, in neurons it has been observed that very similar morphological changes occur at the terminal in *shi* at high temperature, i.e., an increase in the surface area of the plasma membrane, an increase in pitlike structures, and a decrease in synaptic vesicles (16). The temperature-dependent depletion of synaptic vesicles was suggested to be the result of a block in endocytosis at the step of membrane pinch-off (15, 16).

Although most investigators seem to agree that coated vesicles in pericardial and garland cells take up protein from the hemolymph, the intracellular pathway of these absorbed proteins is not clear. Sanger and McCann (21) showed that organelles such as coated vesicles, alpha vacuoles, and tubular elements of the pericardial cell were labeled by ferritin. On the basis of these results, they presented a scheme of the pathway of protein uptake, in which they postulated the transformation of coated vesicles to tubular elements via smooth tubular vesicles (Fig. 9 in Sanger and McCann [21]). On the other hand, Crossley (4) reported that tracer molecules did not enter the tubular elements, suggesting that tubules are not involved in the endocytotic pathway. The present results, however, suggest that the tubular elements are, indeed, involved in the endocytotic pathway. Our HRP experiments showed labeling of tubular elements as well as coated profiles. Secondly, a decrease in the number of these elements occurred, along with a decrease in coated vesicles, when the temperature was raised to 30°C. These observations are consistent with Sanger and McCann's (21) suggestion that coated vesicles are transformed into tubular elements, although the pathway through smooth tubular vesicles remains to be determined. In addition, our observations suggest that the tubular elements may give rise to alpha vacuoles. Thus, it was often observed that tubular elements are connected to a bulb that contains electron-dense material reminiscent of a small alpha vacuole. Furthermore, these organelles were labeled with HRP. Therefore, it may be that, in the garland cell, the endocytotic pathway occurs in the following order; coated pit—coated vesicle—tubular element—bulb—alpha vacuole. Of course, much experimentation must still be performed before a determination of the pathway can be made. The fact that the endocytotic process can be reversibly controlled by temperature in this mutant should prove an invaluable tool

for studying the pathway. Such a study using this mutant is presently underway in our laboratory.

The above results, as well as those on synaptic terminals (15, 16), are compatible with the possibility that the membrane pinch-off mechanism is blocked in *shi* at high temperature. This possibility must be further investigated in these tissues and in other tissues as well and is being pursued in our laboratory at the present time. Hopefully, this mutant will offer some important new clues as to how membrane pinch-off occurs.

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