TRANSPORT OF GLIAL CELL ACID PHOSPHATASE BY ENDOPLASMIC RETICULUM INTO DAMAGED AXONS

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SUMMARY

Earlier observations indicated that cutting the photoreceptor (R) cell perikarya in the retina of the flies Musca domestica and Calliphora erythrocephala resulted in an extremely rapid degeneration of the R-cell axons. The process manifested itself within minutes and secondary lysosomes appeared early on in the degenerating axons. In this study, biochemical assays of the classical lysosomal marker enzyme acid phosphatase (AcPase) showed a rapid increase in activity upon cutting the retina, reaching a peak around 5 min, followed by a drop and a subsequent slow steady rise in activity between about 4 and 24 h after the cut. Inhibitor studies indicated that at least 2 different acid phosphatases were involved, which showed identical activity changes. EM cytochemical studies with either β-glycerophosphate or cytidine monophosphate as substrates indicated that the degeneration-dependent AcPase activity originated exclusively in one specific glial cell type in the distal lamina, the satellite glia. These cells are contiguous with R-cell axons in the pseudocartridges, directly proximal to the basement membrane of the retina. In normal uncut flies, reaction product indicative of AcPase activity was found in satellite glial cells in both rough ER (and perinuclear cisternae) and in smooth ER, the 2 types of cisternae being clearly in continuity. The electron-dense reaction product resulting from AcPase activity often showed continuity between the smooth ER of satellite glial cells and extracellular sites between the satellite glia and R-axons. This may result from the low levels of AcPase being synthesized in rough ER, and then exported to extra-cellular sites. Within minutes of cutting the retina the frequency of satellite glia smooth ER staining for AcPase increases significantly. Increases in AcPase activity were subsequently found sequentially in extracellular sites, in axons in the distal lamina, and finally in the axon terminals. The time course for these increases in AcPase activity correlated well with the time course of the fine-structural pathological changes in the axons.

A significant feature of the satellite glia cells is the massive packing of unidirectional microtubules (MTs), running perpendicular to the projections of the R-axons — in the same direction as AcPase transport. The MTs terminate at the glial membrane directly adjacent to the axon. The smooth ER cisternae interdigitate among the MTs and may be structurally connected to them by thin fibrils.

Thick-section cytochemistry and stereology were used to reveal the 3-dimensional extent of the AcPase-reactive smooth ER system. Evidence is presented which suggests that this system represents GERL, a specialized region of smooth ER involved in lysosome formation, that in this glial cell appears to function also to export hydrolytic enzymes.

INTRODUCTION

When the photoreceptors in the retina of the fly are cut the axons degenerate in an extremely short period of time, the first signs of the process appearing in a matter of minutes (Griffiths & Boschek, 1976). The morphological symptoms are indistin-
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guishable from symptoms of classical Wallerian degeneration in vertebrates, a process that usually manifests itself only after many hours or days. It was shown that the first signs of degeneration, including shrinkage, increase in electron density and clumping of synaptic vesicles, occurred within 2–5 min following retina lesions. Between approximately 5 and 15 min further shrinkage and increase in electron density occurred. From this period up to 24 h further changes were, by comparison, rather small and occurred slowly. In an earlier study Campos-Ortega & Strausfeld (1972) had shown that between 1 and 6 days post-operation the most striking observation was the proliferation of glial cells around the degenerating terminals of the photoreceptor axons. A similar observation was made in the lobster eye where the degenerating terminals were still not completely destroyed after 15 days post-operation (Hamori & Horridge, 1966). Both of the latter groups suggested, on morphological grounds, that the surrounding glial cells played an active role in the degeneration process.

Due to the slowness of the degeneration process in general it has proved very difficult to pinpoint the exact sequence of events that occur prior to complete dissolution of an axon. The bulk of available evidence now indicates that degeneration proceeds centrifugally from the site of damage and is probably associated with the processes of axonal flow that are, in the normal axon, responsible for movement of required materials from the synthetic sites in the perikaryon to the active, distal parts, including the synaptic areas (see Joseph, 1973, for review). Damaging the axon blocks the route from the perikaryon to the terminals and it is suggested that when depletion of materials due to this blockage becomes severe intra-axonal lysosomes release their complement of hydrolytic enzymes which then effect the degenerative changes (Joseph, 1973). So far, while there is good evidence for an important role of lysosomes in the process, the evidence that they are the primary trigger of degenerative events is only indirect. For example, the morphological appearance of secondary lysosomes associated with increases in activities of various hydrolytic enzymes coincides temporarily with the first signs of degeneration (Joseph, 1973; Porcellati, 1970; Holtzmann & Novikoff, 1965).

Over the past 15–20 years much attention has been focussed on the problems of lysosome formation and the packaging and distribution of lysosomal enzymes. There is now strong ultrastructural and cytochemical evidence that lysosome formation in the Golgi region is a widespread phenomenon (Novikoff, 1973; Holtzmann, 1975). In the last few years increasing evidence has been presented in a variety of cell types for the existence of an alternative, more direct route from rough endoplasmic reticulum (rough ER) to a specialized smooth ER region where lysosome formation occurs. This region, termed GERL by Novikoff (1964), although situated close to the inner ‘trans’ aspect of the Golgi apparatus is thought to be topographically distinct from Golgi (Novikoff, 1976). GERL contains cytochemically demonstrable acid phosphatase (AcPase) (as well as aryl sulphatase and esterase (Decker, 1974)) but no thiamine pyrophosphatase (TPPase) activity, whereas the converse is true for the Golgi apparatus, which shows TPPase (and nucleoside diphosphatase) activity on its inner (trans) cisterna but no AcPase activity. First described in rat dorsal root ganglia (Novikoff, 1964), it has now been described, using the above morphological and
cytochemical criteria, in a variety of cell types including hepatocytes, pancreatic exocrine cells, thyroid epithelial cells (Novikoff, 1976; Novikoff & Novikoff, 1977), alveolar macrophages (Essner & Haimes, 1977), lacrimal glands (Hand & Oliver, 1977) and most recently in plant root meristems (Marty, 1978). A GERL-like system was also described in locust and snail ganglia by Lane & Swales (1976). Although AcPase and TPPase cytochemistry was not used in this latter study a fortuitous staining by the zinc iodide/osmium technique, which is often used to stain synaptic vesicles, selectively impregnated elements close to the inner Golgi cisterna as well as secondary lysosome structures. In the rat dorsal root ganglia smooth surfaced tubules of the GERL system extend into each polygonal compartment of the trans Golgi compartment, suggesting a close functional relationship between the 2 organelles but evidence in support of this hypothesis is, until now, lacking (Novikoff, 1976).

The rapidity of the degeneration process in the fly photoreceptor axons seemed to provide a good experimental system with which one could ask specific questions about the role of lysosomal enzymes in the process. In this paper the results of combined biochemical and cytochemical studies of the classical lysosome marker enzyme acid phosphatase (AcPase) are presented along with correlated cytochemical studies of the Golgi marker TPPase.

Materials and Methods

flies

Four- to six-day-old Musca domestica and Calliphora erythrocephela were taken from stock institute cultures. In all experiments flies were secured with wax on to a Petri dish and the dissecting fluid (either 0.9 % NaCl or 1 % glutaraldehyde) was poured over them. The retina, lamina and medulla on both sides of the head were dissected out as a complex (referred to here, for simplicity, as the visual complex (see inset, Fig. i); in fact the 2 parts of the third optic ganglion, the lobula and lobular plate, not included here, are also part of the true visual complex). This complex was free of all contaminating fat body, tracheoles and connective tissue.

degeneration

The 2 retinae of the fly were cut using a vibrating razor blade device (Kirschfeld, 1967) as described previously (Griffiths & Boschek, 1976). For the biochemical studies it was important to make one straight cut (as opposed to a number of small cuts as in the earlier work) through the retina as near to the peripheral cuticle as possible: the eye was left intact in one corner (see inset Fig. 1) so that the peripheral section would remain on the eye. In this way the cut was identical from fly to fly and the total amount of tissue would be equivalent in the cut and in the control, uncut eye. There were no noticeable differences between the morphology of the degeneration process in flies cut in this manner and that in flies in the earlier experiments where the cut was complete. It follows from such a cut that at the periphery of the eye, R1-7 will be damaged; only in the central parts will R8 also be damaged (see Fig. 1). Following the operation the flies were left in moist chambers for the various degeneration times required. For the biochemical experiments, Calliphora, because of its larger size, was used exclusively. Both Calliphora and Musca were used for the cytochemical studies.

biochemistry

The main assay used for acid phosphatase was that using p-nitrophenol phosphate (PNPP) as substrate, as developed by Schin & Laufer (1973) for Chironomus salivary glands. At various times following the retinal lesions the flies were covered with ice-cold 0.9 % NaCl solution and
the visual complexes dissected out, a process taking 5 min. For the control, the flies were left uncult. Four pairs (from 2 male and 2 female flies) were thoroughly homogenized in a tight-fitting 1-ml glass homogenizer using 0-2 ml of 0-9 % NaCl solution. The homogenate was incubated for 60 min at 38 °C in a test tube also containing 1 ml of 0-05 M citrate buffer, pH 5-0 and 8 mg PNPP (Sigma) (final concentration 25 mM). The reaction was terminated with 4 ml of 0-1 M NaOH and the tubes were centrifuged at 2000 g for 10 min. Aliquots of the supernatant were read on a Zeiss PMQ 3 spectrophotometer at 410 nm, the peak of p-nitrophenol, against a blank containing a similar reaction mixture that was stopped at time 0. Initially, the optimum pH for activity of the enzyme was determined using a series of different pH citrate buffers, 0-05 M, from pH 3-6 to 6-4. The assay, at the optimum pH 5-0, was linear with time, at least up to 3 h incubation time.

Fig. 1
In order to have some direct comparison with the cytochemical experiments where β-glycerophosphate (GP), the classical lysosomal acid phosphatase substrate, was used as substrate, this compound was also used in preliminary biochemical experiments, up to 4 h degeneration time. The set-up was similar to that described above for PNPP except that 25 mM GP dissolved in 1 ml 0.05 M acetate buffer, pH 5.0 was used, and the reaction, incubated for 1 h at 38 °C, was terminated with 4 ml of cold, 8 % trichloroacetic acid. Following centrifugation, phosphate content of the supernatant was determined by the method of Fiske & Subbarow (1925). This assay, which is a variation of that described by Bowers, Finkelstaedt & DeDuve (1967), was also linear with time, at least up to 3 h.

For inhibitor studies the effect of 0.01 M sodium fluoride on both β-glycerophosphatase (GPase) and p-nitrophenol phosphatase (PNPPase) was studied. This was added to the incubation tubes from a 1 M standard NaF solution. The effect of glutaraldehyde in cacodylate buffer on activities was also examined. Following fixation in 1 % glutaraldehyde in cacodylate buffer and washings in the same buffer (as for the cytochemical studies – see below), the tissue was washed in either acetate or citrate buffers and assayed for GPase or PNPPase respectively.

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**Fig. 1.** Highly schematic drawing to show the relevant parts of the retina and lamina of the fly. The proportions of the lamina are greatly exaggerated with respect to the length of the retina in order to show clearly the relevant connexions and position of the three glial cell types in the lamina.

- **BM** basement membrane; this separates the retina from the lamina.
- **CB** cell body of retinula (photoreceptor) cell.
- **C** cartridge: this is a subperiodic element composed primarily of 6 different R-cell endings (2 are shown) from 6 different ommatidia plus 5 second-order monopolar cells (one is shown) with which the R-cells are in synaptic contact.
- **EG** epithelial glial cells. These are intimately associated with the R-cell endings in the cartridges.
- **L** monopolar cell; cell body is shown distally, above the cartridge.
- **LAM** lamina.
- **LS** lens of one ommatidium.
- **MG** marginal glial cells.
- **OC** outer chiasma.
- **PS** pseudocartridge. This is an anatomical unit only, consisting of the axons of 8 R-cells from one ommatidium. In the pseudocartridge the 6 peripheral (R1-6) axons twist in a manner that allows them to end in 6 different cartridges.
- **RET** peripheral retina. In the fly this consists of approximately 3000 functional units – ommatidia.
- **Rh** rhabdomere, the specialized light gathering, microvillar apparatus of the photoreceptor cell. The 8 rhabdomeres together make the rhabdom.
- **SG** satellite glial cells: these are intimately associated with the R-cell axons in the pseudocartridge area.

* With respect to the 3 glial cell types, only the approximate positions of the cells are shown. In reality their structures are far more complex and to some extent overlap one another (i.e. the SG partly overlaps the EG which itself interdigitates with the MG).

**Inset.** Structure of the ‘visual complex’ of a cut fly retina after complete dissection. The arrow indicates the retinal cut. Dotted lines indicate the cuts made with a razor blade prior to incubation for AcPase activity. d, distal part cut retina; L, lamina; M, medulla; p, proximal part cut retina; R, retina.
Electron microscopy and cytochemistry

At various times following the retinal lesions (degeneration control flies left uncut) the visual complex was dissected (5 min) in one of two fixative solutions:

(a) Ice-cold 1% glutaraldehyde in 0.08 M cacodylate buffer, with 5% sucrose, pH 7.4. The tissues were left in this solution for a total of 20–60 min.

(b) A slight modification of the mixture published by Luftig, McMillan, Weatherbee & Weihing (1977) with the aim of preserving microtubules. This consists of 1% glutaraldehyde in 0.1 M PIPES pH 7.4 containing 5% sucrose, 1 mM GTP, 2 mM EGTA and 1 mM MgSO4. The fixation was carried out at room temperature for 30 min and then on ice for a further 30 min.

Following fixation the tissue was rinsed for 0.5–2 h in the appropriate buffer.

AcPase incubation

Following a brief (1-min) rinse in 0.05 M acetate buffer, pH 5, the tissue was incubated in one of two media:

(a) Gomori's AcPase medium which consists of 0.25 ml of 12% Pb(NO3)2 dissolved in 25 ml 0.05 M acetate buffer, pH 5, to which 2.5 ml of a 3% β-glycerophosphate solution was added dropwise. The solution was immediately filtered, left at 60 °C for 1 h, cooled and then filtered again before use. The tissue was incubated for 15, 30 or 60 min at 20, 30 or 38 °C: optimal conditions were 30 °C for 30 min.

(b) Novikoff's (1963) CMP medium. This consists of 25 mg of cytidine monophosphate (CMP), Na salt, 12 ml H2O, 10 ml 0.5 M acetate buffer, pH 5, to which is added, dropwise, 3 ml of 1% Pb(NO3)2.

Control solutions for both incubation media contained either no substrate or 10 mM sodium fluoride. In one experiment, the tissue was placed in a 95 °C waterbath for 3 min prior to incubation. Any effect of GTP on the incubation medium was ruled out by omitting this compound from the primary fixative solution in some experiments.

Thiamine pyrophosphatase (TTPase)

The method of Novikoff & Goldfisher (1961) was used. Following a brief rinse in Tris maleate buffer, pH 7.2, the tissue was incubated in a medium consisting of 25 mg TPP (HCl salt), 7 ml H2O, 10 ml 0.2 M Tris maleate buffer, pH 7.2, and 3 ml 1% Pb(NO3)2. Incubation was carried out for 30 or 60 min at 30 °C. Following incubation, the tissue was rinsed 3 times (5 min) in ice-cold Tris maleate buffer. Control tissue was incubated in medium lacking substrate.

Postfixation was carried out on ice using either 1% OsO4 in veronal acetate buffer or in Karnovsky's OsO4-ferricyanide mixture (Karnovsky, 1971) (15–30 min). The tissue was dehydrated using acetone or ethanol/proplylene oxide and embedded in Epon-812. Thin and thick sections were either viewed unstained or stained lightly with uranium acetate and lead citrate prior to viewing on a Zeiss EM9 or a Philips 400 (80–100 kV) which is equipped with a tilting stage.

Preliminary studies were made on 1- to 2-μm sections at 1000 kV of AcPase-incubated, operated flies using the AEI EM 7 Mark II at Madison, Wisconsin.

RESULTS

Biochemistry

With PNPP as substrate the maximum activity of acid phosphatase occurred at pH 5.0, as one would expect for a lysosomal acid hydrolase (Fig. 2).

When the activity of acid phosphatase at different degeneration times was plotted the results are as shown in Fig. 3. It is clear that up to 5 min following the cut there is a highly significant increase in enzyme activity (P < 0.005). This is followed by a
Transport of glial AcPase into axons

Fig. 2. Relative activity of AcPase (P-nitrophenyl phosphatase) for normal fly head homogenates at different pHs as measured by monitoring O.D. 410, the peak of nitrophenol.

Fig. 3. Change in activity of AcPase with different degeneration times for fly visual complexes. Means and standard errors of means of 4 experiments. Because of the need to show the degeneration time on a log scale what is in fact a peak around 5 min degeneration time appears more of a plateau.

decrease down to 60 min degeneration time (negative slope in linear regression curve, \( P < 0.02 \)). Between 1-4 h and 24 h there occurs a significant secondary increase in activity (\( P < 0.01 \)).

A preliminary experiment using GP as substrate up to 4 h degeneration time showed a similar result. In their studies on the breakdown of Chironomus salivary glands during
larval-pupal transformation, Schin & Laufer (1973) noted a sharp increase in acid PNPPase and a similar increase in acid GPase. Clear indications that 2 enzymes were involved in the salivary glands, however, came from studies of their reaction to fluoride inhibition: GPase was much more sensitive to inhibition by fluoride than PNPPase. A similar finding was made by Aidells, Lockshin & Cullin (1971) in wax moth silk glands. For this reason the effect of 0.01 m sodium fluoride on the activity of AcPase using both PNPP and GP as substrates was studied. Table 1 shows, in good agreement with the earlier workers, a large difference between the 2 substrates.

Table 1. Percentage inhibition of GPase and PNPPase activities by fluoride and fixative solution

<table>
<thead>
<tr>
<th>Glutaraldehyde</th>
<th>NaF</th>
<th>Cacodylate</th>
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<tbody>
<tr>
<td>GPase</td>
<td>83.6 ± 3.5*</td>
<td>3.4 ± 6.2</td>
</tr>
<tr>
<td>PNPPase</td>
<td>38.8 ± 5.6</td>
<td>5.1 ± 3.5</td>
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* Means and standard errors of means of 3 experiments.
† Difference between means highly significant ($P < 0.005$).
§ Difference between means significant only at 10% level ($P < 0.1$).

Further though less convincing, indication that we are dealing with at least 2 different enzymes comes from the effect of glutaraldehyde/cacodylate on activities. This was initially studied in order to gain an approximate indication of the efficiency of the cytochemical method for this system. With approximately 35% inhibition of β-glycerophosphatase by the fixative combined with a certain inherent (unknown) inefficiency of the lead capture reaction it is clear that the cytochemical reaction is far from quantitative.

**Satellite glia cell ultrastructure**

Since the cytochemical activities described below are primarily associated with the satellite glia cells in the first optic neuropile, the lamina, the important fine-structural features of these cells will be briefly described. Their position in the lamina is shown schematically in Fig. 1, p. 364. They are long, relatively flattened cells which cover the distal region of the lamina from the basement membrane of the retina distally to the areas occupied by the monopolar (L) cell bodies proximally. The processes of these cells are thus intimately associated with the receptor cell axons in the region of the pseudocartridges. Large tracheoles are characteristically found associated with these cells. Septate and osmiophilic desmosomes are often found connecting

Fig. 4. Low-magnification micrograph of a sagittal section though the distal region of the lamina of *Calliphora*. The axons (a) of photoreceptor cells are cut transversely. Two distinct regions of satellite glia cells are apparent: (1) ‘translucent’ area with massive packing of unidirectional microtubules (mt) associated with ER cisternae, mostly of the smooth variety (arrows); (2) more dense part of the cell where the MTs are less well organized. *Calliphora*. $\times 17200$. 
Transport of glial AcPase into axons
Transport of glial AcPase into axons

Contiguous satellite glial cells and septate desmosomes are also occasionally found between satellite glia and R-axons.

These satellite glial cells have large nuclei which allow them to be distinguished from the small nuclei of the monopolar cell bodies even at the light-microscope level in semi-thin sections. The most characteristic and outstanding feature of these cells, however, is the massive packing of unidirectional microtubules (MTs) found within the cytoplasm. ('Unidirectional' here means parallel orientation.) Conventional electron-microscope fixation methods, with glutaraldehyde in phosphate or cacodylate buffer on ice, allowed a satisfactory preservation of this tissue in general but resulted in very poor fixation of the satellite glial cells. While occasionally parts of these cells showed a close packing of MTs there were often large areas which were 'empty', clearly areas where cytoplasmic constituents had been removed during fixation. Using the fixation technique of Luftig et al. (1977) this problem was eliminated. This fixation mixture contains GTP, Mg²⁺ and EGTA (to remove Ca²⁺) and the initial fixation is carried out at room temperature. In their paper, Luftig et al. reported a 2-fold increase on MTs in cells fixed by this method compared with cells fixed by more conventional techniques. With this method the overall tissue preservation was improved slightly over conventional fixation; but in the satellite glia a distinct improvement was seen. Fig. 4 shows a low-magnification micrograph of a sagittal section (i.e. cut perpendicularly to the R-axon projections) through the distal region of the lamina in the region of the pseudocartridges. Such a section allows the MTs to be visualized in their long axes. The close packing and relative parallel projections of the MTs is apparent (Figs. 5-7). In a horizontal section, i.e. when the MTs are cut transversely, areas can often be observed where groups of 2, 3 or 4 or more MTs appear to be 'structurally attached' (Fig. 25). The MTs appear to terminate contiguous with the glial membrane adjacent to the axonal membrane (Fig. 6).

Closely associated with the MTs is an extensive system of smooth ER which in thin sections appears to run in thin cisternae either parallel to the MTs or perpendicularly to them (Figs. 4-9). Rough ER is also present in some parts of the cells (Figs. 4-10), mostly in the perpendicular regions; the smooth ER is, however, far more extensive. Rough and smooth ER are continuous (Fig. 10). Smooth ER is often

Fig. 5. This section, slightly thicker (70-80 nm) than a normal thin section shows the satellite glia smooth ER cisternae clearly (arrows) due to their thick, densely-stained membranes. The unidirectional nature of MTs is apparent. a, R-cell axons; g, Golgi apparatus. x 32 600.

Fig. 6. 70 to 80-nm section showing the satellite glia MTs extending very close to the glial membrane (arrows) directly adjacent to R-axon (a) membrane. s, smooth ER. Calliphora. x 52 400.

Fig. 7. Thin section showing part of ‘dense’ region of satellite glia cell (equivalent to 2 in Fig. 4) showing the close packing of unidirectional MTs closely associated with ER. a, R-cell axons; m, mitochondrion. Calliphora. x 28 500.

Fig. 8. Higher magnification of satellite glia cell showing part of a smooth ER cisterna that appears to weave in and out of the MTs. Arrow indicates possible cross-linking fibre between adjacent MTs. Calliphora. x 76 500.
Transport of glial AcPase into axons

The Golgi apparatus is localized both in perinuclear regions (Figs. 31, 32, 35) and in areas far removed from the nuclei (Figs. 5, 33, 36) but its identification in the latter is often more difficult due to the mass of smooth ER cisternae. Both smooth and rough ER are found in close proximity to the Golgi apparatus (Fig. 34). The MTs often have fibrillar-like processes on their periphery (Figs. 8, 9). Some of these may be crossbridges between adjacent MTs but there are also hints of connexions between MTs and smooth ER membranes (Figs. 8, 9).

Cytochemistry

Incubations in the cytochemical media for AcPase and TPPase activities led to depositions of electron-dense reaction product at specific sites. These were always absent in control incubation. It is therefore concluded that the localizations of reaction products described below correspond to localizations of enzyme activities.

AcPase in normal flies

Reaction product indicative of AcPase activity was consistently found in the optic neuropiles of normal, unoperated flies. This activity was mostly associated with glial cells. In general, there was very little indication of activity in neuron perikarya, axons or terminals. In the lamina, where most of these studies were focused, reaction product was especially evident in the 2 major classes of glial cells: the satellite and epithelial glia (schematically shown in Fig. 1). The latter showed reaction product in perinuclear cisternae, rough ER, smooth ER and secondary lysosome structures. Reaction product was also present in small amounts in extracellular sites between the glial plasma membranes and that of R-axons (Fig. 11).

In the satellite glial cells, which are of central importance to this paper, AcPase activity was found in rough ER (Fig. 37) and the continuous perinuclear cisternae and in smooth ER cisternae (Figs. 18–20, 24). The presence of direct continuities between rough and smooth ER was given further support in incubated tissue due to the high contrast of the reaction product within the cisternae (Fig. 18). The extent of reaction product deposition within the extensive smooth ER cisternae was quite

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Fig. 9. High-magnification micrograph showing details of satellite glia smooth ER cisterna running parallel to MTs. Arrows indicate possible connecting fibres either between adjacent MTs or between MTs and SER. Calliphora. × 108,000.

Fig. 10. Apparent continuity between satellite glia rough and smooth ER. Arrow indicates ribosomes. Calliphora. × 84,000.

Fig. 11. Control, uncut Calliphora. Reaction product for AcPase activity localized in small amounts in extracellular sites (arrows) between satellite glia cell processes and R-axons (a). × 23,800.

Fig. 12. Calliphora, 3 min following retina lesion. Note increased AcPase reaction product in satellite glial processes close to axons (a) and in extracellular spaces (arrowheads). Arrows indicate contiguities between reaction product in ER and glial/axon membranes. × 26,400.

Fig. 13. Calliphora, 5 min degeneration. This shows increased AcPase activity in ER satellite glia cell (sg) and especially in glial processes either around R-axons or extracellular sites (arrows). × 17,200.
variable from one preparation to the next: Figs. 18, 20 and 24 all show fairly representative images. However, the amount of reactive smooth ER was always less than that found following retinal lesions (see below). As stated previously, in unincubated material the smooth ER is often contiguous with the glial plasma membrane adjacent to the R-axon membrane. AcPase cytochemistry strengthens this observation by delineating the smooth ER. Images of contiguities of smooth ER reaction product and glial plasma membrane were commonly seen (Fig. 18). Less frequent but unequivocal was the continuity of reaction product in ER and extracellular space (Fig. 19). This suggests that the small amounts of reaction product that are consistently found in the extracellular sites in normal flies (Fig. 11) may have originated in the satellite glial cell ER. AcPase activity is often associated with various lysosomal structures (Fig. 27). An instance of continuity of an autophagic vacuole and smooth ER, both showing AcPase activity, is seen in Fig. 38.

The Golgi apparatus is devoid of any significant deposits of reaction product, although parts of the smooth ER close to the inner, trans aspect of the Golgi apparatus often show AcPase activity (Fig. 33). This will be discussed below in more detail.

**AcPase activity in operated flies**

Although in unincubated tissue no fine-structural changes between unoperated and operated flies were apparent in the satellite glial cells, AcPase incubation at various times following retinal lesions revealed a distinct pattern of change. Large increases in reaction product deposition were found sequentially in the following

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**Fig. 14.** In this preparation of *Calliphora*, 5 min after cutting the retina, reaction product indicative of AcPase activity is found within the R-axons especially close to the axonal membrane (arrows). No increase in axonal electron density is apparent at this stage in this preparation. *m*, mitochondria. × 17700.

**Fig. 15.** *Musca*, 10 min degeneration time. Increase in electron density of axoplasm clearly apparent in longitudinal section. Reaction product for AcPase activity seems to have filled parts of the axonal MT central cores (arrows). Conventional fixation. × 76900.

**Fig. 16.** *Calliphora*, 10 min degeneration time showing R-axon, slightly oblique, clearly degenerating. Electron-dense reaction product of AcPase activity scattered diffusely in the cytoplasm. × 22600.

**Fig. 17.** *Musca*, 5 min degeneration. Reaction product for AcPase activity present in peripheral axoplasm of degenerating R-cell cut transversely. Arrow indicates reaction product apparently within MT central cores. Conventional fixation. × 20800.

**Fig. 18.** Two instances showing contiguity between AcPase-reactive smooth ER and glial membrane directly adjacent to R-axon (a) membrane (arrows). Note also continuity between AcPase reaction product in rough ER (r) and smooth ER (s) of satellite glial cell (sg). Uncut, control *Calliphora*. × 42000.

**Fig. 19.** The electron-dense AcPase reaction product here is continuous from satellite glia smooth ER (t) to extracellular space between R-axon (a) and glia (tg) (arrow). Arrowheads indicate the unit membranes of the axon and glia. *Calliphora*, uncut control. × 50700.

**Fig. 20.** Control uncut *Calliphora* showing part of satellite glia cell where only part of one smooth ER cisternum shows reaction product for AcPase activity (arrow). Compare with Figs. 21-23. × 36600.
Transport of glial AcPase into axons

sites: (1) Satellite glial cell cytoplasm (> 2 min); (2) Satellite glia/R-axon extracellular spaces (> 3 min); (3) R-cell axoplasm in the vicinity of satellite glia (> 4–5 min); (4) R1–6 axon terminals in the lamina (> 5 min); and (5) R7 and R8 axon terminals in the medulla (> 8–10 min) The times given are those found for Musca; at every step there was a delay of a few minutes in Calliphora. The times are approximate and variable from one preparation to the next but this pattern, based on a total of about 10 repeated experiments, was consistently found.

Satellite glia. At least up to 10 min post operation the increases in AcPase reaction product in these cells were associated with the smooth ER only. Within minutes of cutting the retina this organelle ‘stained’ extensively (Figs. 12, 13, 21–23, 25, 26, 28–30). After about 10 min it was estimated that the proportion of AcPase-reactive rough ER was increased compared to uncut flies but during the early periods after cutting the deposition of reaction product in the rough ER was never as extensive as in the smooth ER. After about 3 min, the localization of reaction product in smooth ER in the satellite glial processes surrounding the axons is especially evident (Figs. 12, 13). The selective deposition of reaction product in the smooth ER allowed the extent of its structure to be appreciated. By studying both thin (50–70 nm) and thick (150–250 nm) sections of AcPase-incubated, operated flies it became clear that the smooth ER in the satellite glial cells represented an extensive labyrinth of interconnected tubes and cisternae which were continuous with rough ER cisternae and perinuclear space. The spaces between the ER cisternae were filled with the MTs. Figs. 25 and 26 show the close proximity of the 2 organelles in thick and thin sections respectively. The MT subunit structure becomes more apparent in the thicker sections (Fig. 25, inset). Stereo pairs of thick-section images photographed at different tilt angles give a firmer impression of the 3-dimensional structure of the labyrinth (Fig. 29). Fig. 30, in stereo, indicates continuity between AcPase reaction product in satellite glial perinuclear cisternae and smooth ER. Thick section cytochemistry, especially using different tilt angles, has further reinforced the observations made in thin sections of continuities between reaction product in glial ER and extracellular space.

Recently, 1–2 μm sections of the AcPase-incubated operated flies were examined at 1000 kV using the high voltage EM at the University of Wisconsin (courtesy of Dr H. Ris). Although this study was brief and preliminary the selective deposition

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Figs. 21–23. Three different images showing extensive smooth ER ‘staining’ for AcPase activity in Calliphora, 5–10 min following cutting the retina. In Fig. 23 the contrast is partially reversed simply by illuminating the partially developed print while still in the developer (solarization). In some micrographs this technique helped to show up the MTs which now appear white against a dark background. The reactive smooth ER and nuclear envelope (arrow) retains normal (black) contrast, n, nucleus; m, mitochondria. x 29360, 26700, and 26300, respectively.

Fig. 24. Higher-magnification micrograph showing detail of smooth ER staining for AcPase in normal uncut Calliphora. Arrows indicate areas where the smooth ER is cut transversely showing its tubular nature. m, mitochondrion; mt, microtubules. x 55100.
of reaction product in the ER of the satellite glial cells allowed one, even in such thick sections, to resolve its structure clearly. One stereo pair of such material is shown in Fig. 41.

As was the case for the unoperated flies, the Golgi apparatus in satellite glial cells of operated flies was unreactive to AcPase. The occasional trace deposits of lead in some Golgi cisternae were probably insignificant. The AcPase 'staining' of smooth ER close to the inner aspect of the Golgi apparatus, described earlier for normal flies, became even more distinct in operated flies. Many images were obtained of AcPase activity in cisternae close to one aspect of the Golgi apparatus. When the plane of the section allowed unequivocal identification of the Golgi cisternae, the smooth ER was mostly found on the concave, 'cis', aspect of the Golgi apparatus. Often, such interpretation was helped by tilting the specimen. However, in no single image was the orientation of both the Golgi apparatus and AcPase-reactive smooth ER optimal to allow unequivocal identification of the smooth ER as GERL. Figs. 33 and 36 show 2 representative images suggestive of GERL elements. Fig. 34 shows, in unincubated tissue a more typical appearance of GERL (i.e. in better section orientation) at the 'cis' aspect of the Golgi apparatus: the rough ER appears to be directed towards this region. As mentioned above, continuities exist between rough and smooth ER, most clearly seen in AcPase incubated tissue; this is further support for the GERL-like nature of the smooth ER. Fig. 35 shows continuity between AcPase-reactive GERL-like element and rough ER. Fig. 39 indicates a commonly seen phenomenon in AcPase-incubated operated flies, namely a hexagonal pattern of the smooth ER (see also Fig. 29); this is also reminiscent of GERL. The AcPase-reactive smooth ER is often enlarged or bulbous in some areas (Figs. 28, 29, 40) and autophagic vacuole-like structures can often be seen within or close to these cavities (Fig. 40). This effect may occasionally be seen in satellite glia of unoperated flies. Such observations, in addition to the continuities with AcPase-containing autophagic vacuoles/residual bodies, strengthens the idea that the smooth ER represents a large lysosomal system.

_Extra cellular space._ After about 3 min, increased AcPase activity is evident not only
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in the smooth ER of the satellite glial cells but also in the glial cell processes around the R-axons and especially in the extracellular spaces between satellite glia and axons (Figs. 12, 13). As mentioned above, continuities in reaction product between the ER of satellite glial cells and extracellular space were found.

*R-cell axoplasm.* Following the appearance of AcPase reaction product in the extracellular space it was subsequently found in the R-cells axoplasm close to the satellite glia firstly in patches close to the R-axon membrane (Figs. 13, 39) and then throughout the axoplasm (Figs. 14, 16). Reaction product was usually found in the axoplasm before any increases in electron density were observed. The typical darkening of the degenerating axons made it increasingly difficult to identify reaction product clearly (Fig. 14). After about 5 min degeneration time reaction product was found in the degenerating R1–6 terminals in the lamina and a few minutes later was apparent in the R7, and, when cut, R8 terminals (see Fig. 1 for explanation).

Within the axons on a few occasions a striking localization of reaction product was found in the axonal MT central spaces or 'cores' (Figs. 15, 17). The significance of this localization is unclear.

*Thiamine pyrophosphatase activity*

Incubations for TTPase activity led to deposits of reaction product in parts of rough ER, perinuclear cisternae and in one cisternae of the Golgi apparatus, namely the inner, cis, aspect (Figs. 31, 32). There were no differences in localization between unoperated (Fig. 31) and operated flies (Fig. 32). There was no TPPase activity in the smooth ER cisternae on the inner aspect of the Golgi apparatus which showed AcPase activity.

In some but not all preparations there was significant deposition of reaction product in secondary lysosome-like structures; images similar to Fig. 28, which shows AcPase incubation, were obtained.

In the R-cell axons there was significant deposition of reaction product in the ER cisternal 'sieve plates' (see Boschek, 1971).

**DISCUSSION**

The biochemical and cytochemical studies presented here agree well with the earlier morphological description of degenerative events that result from retinal lesions in the fly (Griffiths & Boschek, 1976). The biochemical data show an early peak of AcPase...
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activity around 5 min after the cut, followed by a lag and subsequent secondary rise in activity between 4 and 24 h. The cytochemical data indicate that this degeneration-dependent increase in activity originated exclusively in one glial cell type in the distal lamina – the satellite glia. These cells are contiguous with R-cell axons in the pseudo-cartridges (i.e. where bundles of axons from one photoreceptor enter the lamina) directly proximal to the basement membrane of the retina. In normal, uncut flies, reaction product indicative of AcPase activity was found in satellite glia cells in both rough ER (and perinuclear cisternae) and in smooth ER, the 2 types of cisternae being clearly continuous. There were also suggestions of continuities between reaction product in smooth ER and extracellular sites between glia and R-axons. Hence, it appears that relatively low levels of AcPase synthesized in rough ER cisternae were exported, via smooth ER channels, to extracellular sites.

It should be emphasized that the satellite glia represent a class of cells that are structurally complex in their relationships with the R-axons and it is impossible in the absence of extensive serial sectioning to follow the contours of any one cell. Some parts, for example, are much denser than others due to a great mass of ribosomes and may have a less well organized MT system (shown in Figs. 4, 7), while one large,

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Fig. 31. Calliphora, uncut incubated for TPPase. Reaction product apparent in inner Golgi cisterna (g) of satellite glial cell and in perinuclear cisternum (arrows), ge, possible GERL elements (unreactive). n, nucleus. x 43520.

Fig. 32. TPPase activity in inner Golgi cisternae (arrow) of satellite glial cell of Calliphora 10 min after retinal lesion. Also staining of perinuclear cisternae (arrows). ge, possible GERL elements (unreactive); n, nucleus. x 42300.

Fig. 33. AcPase activity in smooth ER adjacent to Golgi apparatus which is suggestive of GERL. Note that the Golgi apparatus itself (g) is completely devoid of reaction product. Calliphora, control uncut. x 92600.

Fig. 34. In an unincubated, normal Calliphora this section shows more characteristic appearance of typical GERL elements (ge) on the inner aspect of the Golgi apparatus (g) that is cut transversely. Note that the rough ER (r) is oriented towards the GERL region and may be continuous with it (arrowhead). av, autophagic vacuole. x 48700.

Fig. 35. AcPase incubation, 6 min degeneration Calliphora showing continuity between reactive ER (er) and typical GERL-like element (ge). mt, microtubules. x 47700.

Fig. 36. Calliphora, 3 min degeneration showing AcPase reaction product in smooth ER elements resembling GERL (ge) or one aspect of an obliquely cut Golgi apparatus (g) which is unreactive. x 56400.

Fig. 37. Calliphora, control uncut showing AcPase reaction product in some portions of rough ER cisternae. x 39000.

Fig. 38. AcPase activity is seen in a large autophagic vacuole/residual body which is continuous (arrow) with partially reactive smooth ER cisternae. That a continuity existed was confirmed by 3 separate tilt images of this thick (150 to 200-nm) section. Control, uncut Calliphora, x 66000.

Fig. 39. Calliphora, 5 min degeneration incubated for AcPase showing reaction product in hexagonal array of smooth ER suggestive of GERL. MTs are positioned in the hexagon centres. Reaction product is seen directly beneath the axonal plasma membrane (arrow) as well as in extracellular space (e). a, axon. x 76400.

Fig. 40. Calliphora, normal, uncut – after AcPase incubation showing light deposits of reaction product in a distended area of the smooth ER. av, autophagic vacuole. cv, possible coated vesicle budding off the ER cisterna. x 49000.
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characteristic part of the cell (shown in Figs. 4, 8) is easy to recognize, even with the light microscope, due to its translucent nature and the distinct arrangement of MTs and smooth ER. It is far from clear whether we are dealing with one or more types of glial cell in this area, although, if different, these cells respond similarly to axon lesions. While the question could be solved by serial sectioning, it does not change the basic tenet of the cytochemical results.

Fig. 41. Approximately 1.5-μm section of Calliphora at 6 min degeneration time showing extensive network of smooth ER with AcPase reaction product. Large dense bodies are reactive lysosomal structures. Tilt 10°. 1000 kV - HVEM. × 20000.

Within minutes of cutting the peripheral retina, the frequency of satellite glial smooth ER ‘staining’ for AcPase increases significantly (followed subsequently by increases in rough ER ‘staining’). Increases in AcPase activity were then found sequentially in extracellular sites, in axons in the distal lamina, and finally in the terminals (suggesting that in the early stages, at least, axonal flow is still occurring). The time course for these increases in AcPase activity correlates well with the time course of the morphological changes described earlier (Griffiths & Boschek, 1976). This evidence strongly suggests that AcPase is being transported from the satellite glial cells into the cut axons. It is postulated that upon cutting the R-cell perikarya some ‘injury signal’ is communicated from the axons into the neighbouring satellite glia where it activates previously synthesized, but biochemically and cytochemically undetectable, AcPase (and associated acid hydrolases) in the smooth ER. These enzymes are exported, along pre-existing channels, into the extracellular space and then enter only those axons whose membranes have somehow been made ‘leaky’ by the act of cutting.

With respect to the degeneration times given it must be emphasized that the absolute time values have little meaning in these experiments: at the end of the allotted time
following cutting, the eye of the fly is removed from its holder and dissected under fixative. It takes approximately 5 min to take out the 2 sets of 'visual ganglia' from each fly. During this time, fixation is continually occurring and it is assumed that all cellular reactions are 'frozen' when the fixative reaches the tissue. Parallel experiments have shown that it is possible to get complete fixation (albeit not optimal) within 10 min of starting the dissection. This gives us an approximate upper limit of time within which further reactions could occur following the allotted degeneration time. Hence, for example 3 min degeneration may really mean 13 min degeneration but no more. The relative times are, however, meaningful (a 10-min degeneration time means that all reactions will have continued 5 min longer than in a 5-min degeneration time) and the above pattern, based on a total of about 10 repeated experiments was consistently found.

The biochemical activity of AcPase during the degeneration process (at least up to 4 h) is identical whether GP or PNPP is used as substrate. However, with respect to the 2 substrates, the AcPase activity shows a very different sensitivity to fluoride (and to a lesser extent glutaraldehyde/cacodylate) inhibition. This, in conjunction with data of earlier workers (Aidells et al. 1971; Schin & Laufer, 1973) supports the notion that 2 different AcPases are involved, both behaving identically. DeDuve (1969) has pointed out that AcPase (i.e. GPase) distribution has been found to be a fairly reliable indicator of lysosomal hydrolase activity in a wide range of species and tissues tested. Hence it is a reasonable assumption (although clearly still an assumption) that the satellite glia release a whole battery of lysosomal enzymes which effect the degenerative changes.

The fixation method of Luftig et al. (1977) proved to be invaluable in revealing the fine structure of the satellite glial cells. The rationale for this method is based on sound in vitro studies of tubulin polymerization (see Snyder & McIntosh, 1976, for review). The sheer mass of MTs in these cells might suggest artificial polymerization by the fixative, a possibility argued against by Luftig et al. (1977) but, apart from the difficulty of visualizing such a process occurring in the presence of glutaraldehyde, the possibility tends to be ruled out by the fact that a similar distribution of MTs could, on occasion, be observed in some parts of these cells following conventional fixation methods; similar observations have been made by a number of earlier workers on insect glial cells (see Lane, 1974, for references). In the absence of controlled experiments it is difficult to comment on the various contributions played by the GTP, Mg, EGTA, PIPES buffer or room temperature fixation: clearly all these factors may not be necessary for glial cell fixation and preliminary experiments suggest that GTP may not be essential. The MTs may be structurally linked to the smooth ER (and to one another) but the evidence for this is admittedly weak. The mere fact that the MTs in such large numbers are oriented parallel to the direction of AcPase transport is a strong suggestion that they may play some role in the transport. A number of workers have shown that the specific antimicrotubular agents, such as colchicine, block intracellular transport of cell materials and organelles (e.g. Malaisse et al. 1975; Malawista, 1975; Le Marchand et al. 1975; Ginsel, Debets & Daems, 1975). From such experiments has grown the idea that MTs play an important role.
in intracellular transport: this is supported by a diverse array of experiments. These include morphological studies showing that a significant correlation exists between the positions of MTs and those of neighbouring pigment granules in teleost chromatophores (Murphy & Tilney, 1974; Byers & Porter, 1977) or between MTs and lipid particles in intestinal cells (Reaven & Reaven, 1977) or biochemical studies, e.g. showing selective binding of MTs to pituitary secretory granules (Sherline, Lee & Jacobs, 1977). In very few cases have clear-cut structural connexions been shown between the organelles in question and MTs (e.g. Allen, 1975). Hence, although there is now an extensive list of papers indicating an important role of MTs in intracellular transport and secretion, these papers all fall short when it comes to concrete evidence for specific mechanisms (see Roberts, 1974, for review). In keeping with this tradition, these studies, as mentioned, are suggestive of a role of MTs in AcPase transport but, based on this evidence alone, this remains speculation. It does seem probable, however, that the MTs are important in maintaining structure and asymmetry in these glial cells; there are more established functions of MTs (Roberts, 1974).

The ‘structural connexions’ described between adjacent MTs in transverse section is clearly an interesting phenomenon (Fig. 25). The images suggest that the MTs with complete sets of subunits (approximately 13, but not unequivocally established) are contiguous, as opposed to a sharing of subunits such as that seen in outer doublets and triplets of sperm, cilia and flagella. In normal EM preparations single MTs are almost invariably surrounded by an electron-transparent ‘space’ that separates them from their neighbours. The reason for the absence of such a ‘space’ in some of the satellite glial cells MTs is unknown; we cannot, at this time, rule out the possibility that it is artefactually induced by the fixative solution.

It is likely, but not unequivocally established by the observations recorded above, that the labyrinthine system of smooth ER with demonstrable AcPase activity is part of GERL (for reviews see Novikoff, 1976; Novikoff & Novikoff, 1977). More definite documentation is desirable for the restriction of TPPase activity to the inner (trans) element of the Golgi stack and for the continuity of the AcPase-active cisterna lying at the trans aspects of the Golgi stacks with rough ER. The status of GERL and rough ER continuity in other cell types is discussed in the above references. The labyrinthine nature of what we tentatively consider to be GERL in the fly satellite glial cell most resembles the large and cage-like GERL described by Marty (1978) in cells of the plant *Euphorbia*.

When the peripheral retina was cut, no change was apparent in the TPPase localization in the Golgi stack (Figs. 31, 32). In contrast, a dramatic increase in AcPase activity in GERL is apparent within minutes of cutting the retina. This increase parallels the biochemical data. Changes in cytochemically demonstrable hydrolases of GERL and associated ER in physiological or pathological states have been described by Holtzmann & Novikoff, 1965; Lane & Novikoff, 1975; Decker, 1974; and Paavola, 1976.

TPPase, while localized within the inner Golgi cisternae and rough ER (i.e. ‘classical’ sites) also ‘stains’ secondary lysosome structures in the satellite glial cells in some preparations. Similar localizations of TPPase were shown by Lane (1968)
Transport of glial AcPase into axons in grasshopper thoracic ganglion neurons. While it is possible that some insect cell lysosomes are different from vertebrate ones in containing a neutral-pH-optimal TPPase in addition to the acid hydrolases, it is perhaps more conceivable that the localization in these structures is due to the activity of a non-specific acid phosphatase with a 'significant tail' of activity at pH 7.2 i.e. the localization is artefactual. Lane (1968) also found, for example, that both ATP and ADP substituted for TPP could result in reaction product localization in the secondary lysosomes. In the absence of specific inhibitors of TPPase (which do not appear to exist) or of biochemical data on the pH-activity range of the enzyme, it is difficult to make definitive conclusions at this time.

It has recently been suggested that, in crayfish peripheral nerves, the rate of the degeneration process, which is quite variable from one neuron to the next, is primarily dependent on the reaction of the surrounding glial cells (Bittner & Mann, 1976). From morphological evidence these workers speculated that glial cells could be classed as either 'destructive' or 'supportive' with respect to a cut axon; this in turn depended on whether the glial cell had a 'destructive' or 'supportive' function to play in the normal axon. As mentioned earlier, there is also morphological evidence for a destructive role of glial cells, at least in the later stages of the degeneration process in the fly and lobster photoreceptors (Campos-Ortega & Strausfeld, 1972; Hamori & Horridge, 1966). Morphological evidence alone cannot, however, distinguish between a glial cell that is itself responsible for destroying a damaged axon and one that is reacting to changes induced within the axons themselves (e.g. intra-axonal lysosomes). The evidence presented here shows that the satellite glia plays a destructive function with respect to R-cell axons, hence supporting Bittner & Man's (1976) concept of a destructive class of glial cells. With respect to the unusually fast rate of degeneration of the R-cells it seems important that the inactivated acid hydrolase(s) and the cellular channels for its (their) release are already present on the normal fly; cutting the R-cell activates the enzyme(s) and the export process is hence merely accentuated.

Finally it is tempting to speculate that this destructive role of some glial cells could also play an important role in the development of the nervous system, where cell death of many specific nerve cells is known to occur at relatively early stages (Speij, 1971). Hence, the low level of AcPase that is exported to extracellular sites on the surface of the satellite glial cells of normal flies may simply represent the vestigial remains of a process that was highly active during the early developmental stages of the fly.

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