

The Sources of Acid Hydrolases for Photoreceptor Membrane Degradation in a Grapsid Crab

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Summary. Dawn photoreceptor breakdown in the crab *Leptograpsus variegatus* is analysed at the ultrastructural level. Coated vesicles derived from microvilli are assembled as multivesicular bodies (mvbs), which degrade to multilamellar bodies (mls) and are lysed. Cytochemical markers for hydrolases were a fluoride-inhibited β -glycerophosphatase and a fluoride-insensitive p-nitrophenyl phosphatase, with indistinguishable distributions when localised at pH 5.0. These enzymes are injected into the secondary lysosomes from two sources: (i) Immediately after dawn Golgi bodies are highly active, and differentiate a trans-tubular network, from which tubules and vesicles detach, and can be seen fusing with mvbs and mlbs. (ii) Saccules derived from the rough endoplasmic reticulum (RER) provide a second source and are most often seen in association with late mlbs. Both kinds of primary lysosome rarely give AcPh-positive responses when free in the cytosol, but are seen to do so as they make contact with their secondary lysosomal targets. Lipid droplets and lipofuscin bodies are interpreted as the residual products of breakdown. These results are discussed in relation to previous findings on photoreceptor membrane breakdown in a dinopid spider. Attention is drawn to the implied diversity of organisation of lysosomal compartments in receptors which internalise membranes of similar compositions.

Key words: Photoreceptor membrane – Crab *Leptograpsus* – Acid phosphatases – Primary lysosomes – Golgi trans-tubular network.

The mass internalisation for lysis of photoreceptor membrane by a dinopid spider has been described in previous papers (Blest et al., 1978b; Blest et al.,

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* The authors thank Professor T.H. Waterman for hospitality extended to A.D. Blest at Yale University, for allowing them to see results prior to publication, and for enthusiastic encouragement of at present project during a visit to this University in 1979. We also thank Dr. Gary Hafner and Dr. D.R. Nassel for sending us results prior to publication, and others, especially Dr. Dean Bok, for discussions in correspondence. Bruce Ham helped to collect crabs. Rod Whitty and the staff of the Electron Microscope Unit provided advice and support throughout these studies. Chris Snoek prepared Fig. 1a, b

1978a; Blest et al., 1979). Secondary lysosomes were shown to be supplied with acid phosphatases (AcPhs) both by organelles assignable to GERL, and by endoplasmic reticulum via a more direct route. With rare exceptions, no AcPh is found in early lysosomes, and the major part of lysis is delayed until the membrane has been degraded to large, amorphous residual bodies (Blest et al., 1979). In crayfish, however, Eguchi and Waterman (1976) reported acid β -glycerophosphatase in multilamellar and multivesicular bodies. Its appearance coincided with the loss, presumably by degradation, of putative rhodopsin particles in freeze-etch preparations of the lysosomes.

Nassel and Waterman (1979) describe a daily cycle of rhabdomere membrane turnover, similar to that of *Dinopis*, in the grapsid crab *Grapsus*. As in the spider, membrane is synthesised at dusk and destroyed at dawn. An Australian grapsid, *Leptograpsus variegatus*, exhibits the same cycle (Stowe, 1979). The present paper describes the acid hydrolase cytochemistry of early secondary lysosomes and associated organelles in the period surrounding "dawn" in order to resolve two problems: (i) What is the origin of hydrolytic enzymes for photoreceptor membrane degradation in crustacean receptors? (ii) Are hydrolytic enzymes injected into the secondary lysosomes sufficiently early to explain the observation of Hafner and Bok (1977) that (^3H)-leucine labels crayfish multivesicular bodies (mvbs) shortly after injection into the haemolymph, and appreciably before it labels the rhabdom?

Materials and Methods

Leptograpsus variegatus (Fabricius) between 20–40 mm carapace width were collected from rocky shores at Bateman's Bay, N.S.W., They were maintained at 16°C in salt water tanks in a room illuminated by fluorescent lights controlled by a time switch to give light/dark cycle of 15/9 h. "On" at 0530h corresponded roughly to natural dawn, and "Off" at 2030h was some 3h later than natural dusk at the time of these experiments.

For observations of the daily cycle, retinæ were fixed for 1–2 h in 2.5% glutaraldehyde in 0.1 M cacodylate buffer at pH 7.2, with 0.14 M sucrose. For enzyme localisations, the same glutaraldehyde concentration and pH were employed in 0.1 M PIPES buffer with sucrose. Calcium was omitted from fixatives in all cases.

After 15–20 min of fixation, retinæ were teased into fragments each consisting of a dozen or so intact retinulae and their surrounding glial cells. When fixation had proceeded for 50–80 min the fragments were washed for 30 min in two large volumes of cold buffer, and then for 5 min in cold 0.06 M tris-maleate buffer at pH 5.0 with sucrose.

AcPh was localised as described by Blest, Price and Maples (1979) using either sodium β -glycerophosphate or p-nitrophenylphosphate as substrates. Control tissues were incubated either without substrate, or in the presence of 10 mM NaF. 1% dimethyl sulfoxide (DMSO) was added to a proportion of the media, and appeared to give sharper localisations. For aryl sulphatase, 38 mg p-nitrocatechol sulphate were dissolved in 23 ml tris-maleate buffer with sucrose and 2 ml 1% $\text{Pb}(\text{NO}_3)_2$ at pH 5.0, and total fixation times reduced, ultimately to 10–20 min.

Incubations were carried out in scintillation vials sealed with Nescofilm, in a water-bath maintained at 25°C with mechanical agitation for 1.5–2 h. Afterwards, the teased fragments were washed in tris-maleate buffer at pH 5.0 for 5 min, then in PIPES buffer at pH 7.2, and post-fixed in 1% OsO_4 in the same buffer for 0.5–1 h. Following washing for 30 min in distilled water they were dehydrated rapidly through an ethanol series and embedded in Araldite. Grey-pale gold sections were stained either in uranyl acetate alone, with lead citrate alone, or with both. Comparisons between sections of cells reacting strongly for β -glycerophosphatase stained with and without uranyl acetate did not support the possibility that reaction product might be partly removed by uranium treatment (Essner, 1973). Reactions illustrated in this paper are all for β -glycerophosphatase.

Results

General Anatomy

The retina of *Leptograpsus* is described by Stowe (1977, 1979) and by Fig. 1 a–c. Microvilli from the eight retinula cells of each ommatidium form a fused rhabdom of the grapsid type (Eguchi and Waterman, 1967, 1973), which extends for a length of 250–400 μm from the base of the crystalline cone to the basement membrane. During the day, the rhabdom consists of a thin cylinder about 2 μm in diameter. At dusk, new membrane is added to the microvilli, and the diameter of the distal end enlarges to around 5 μm . As in many decapods, one retinula of each ommatidium, R_8 , has a small, four-lobed soma and contributes only to the distal end of the rhabdom, the bulk of which is formed from alternating layers of orthogonally oriented microvilli from R_{1-7} . Four cells contribute microvilli pointing in the one direction, and three the intervening layers. The rhabdom is surrounded by a small extracellular space; a thin rind of cytoplasm, and a large intracellular palisade of enlarged ER cisternae. Reticular cell cytoplasm contains scattered pigment granules.

The palisade of dilated ER separates the cytoplasm bordering the rhabdom from that of the greater part of the cell and is a single, fluid-filled cisternal compartment. Slender bridges of cytoplasm cross this compartment to join the perirhabdomeral cytoplasm to that of the rest of the cell. Synthesis of new photoreceptor membrane and its breakdown at dawn both require materials to be transported through these narrow cytoplasmic strands.

The retinulae of each ommatidium are surrounded distally by glial cells containing dark pigment granules, and more proximally by cells filled with white reflecting pigment (Stowe, 1979).

Secondary Lysosomes

Secondary lysosomes in the receptors have been classified by Eguchi and Waterman (1967, 1976) and Hafner et al. (in press). In *Leptograpsus*, we find secondary lysosomes of the same type. They are: (i) multivesicular bodies (mvbs) consisting of packed vesicles surrounded by a smooth membranous envelope; (ii) combination bodies (cbs), in which the envelope contains both vesicles and lamellae; (iii) multilamellar bodies (mlbs), which consist of irregularly concentric lamellae often indistinguishable from any envelope. They lie on the farther side of the palisade from the rhabdom, tending to surround an empty channel which contains microtubules, and in which various materials, especially lipid droplets, are transported along the length of the receptors (Fig. 2).

It is now well established that pinocytotic vesicles pinched off from the microvilli (Fig. 3) enter the envelopes of mvbs by secondary endocytosis (Eguchi and Waterman, 1976); the continuous sequence of stages between orderly mvbs and condensed or disordered mlbs and, in some arthropods, residual bodies (rbs) supports the view that each transforms in turn of the other (White, 1968; Eguchi and Waterman, 1976; Blest, 1978; Blest et al., 1978a). Hafner et al. (in press) show that after illumination of crayfish receptors, the different categories of secondary lysosome peak sequentially in the predicted order.

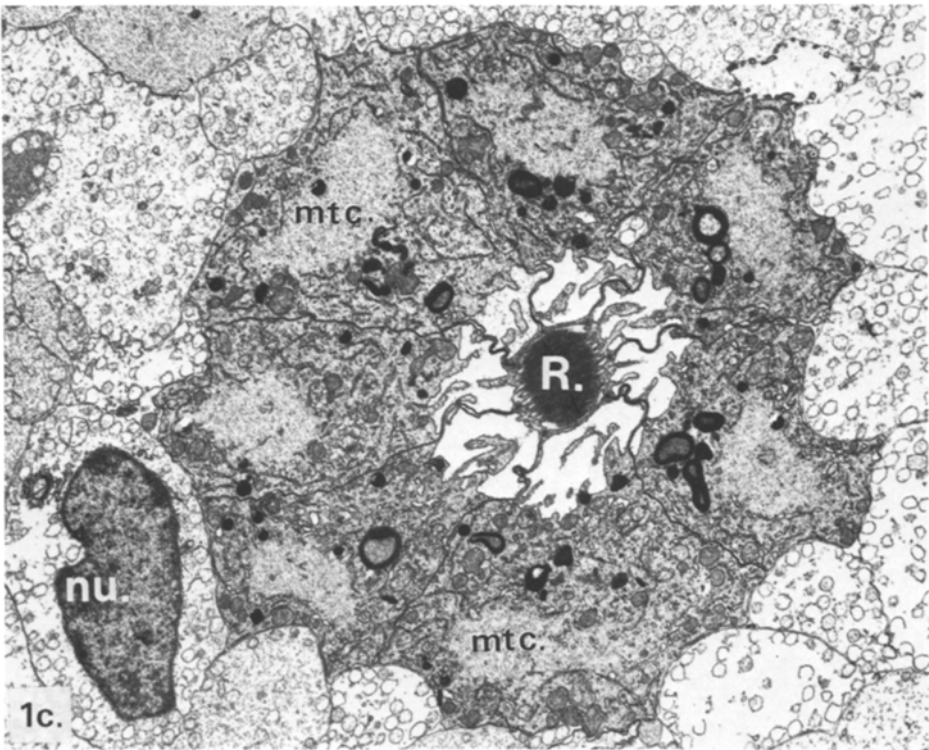
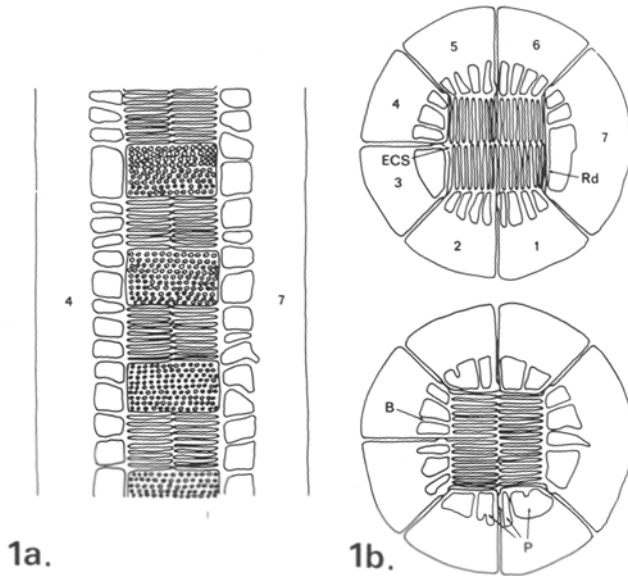


Fig. 1a-c. Structure of an ommatidium of *Leptograpsus*. **a** Diagram of longitudinal section through the retinula cell column, showing the alternating layers of microvilli. **b** Cross-sections through adjacent layers of the retinula cell column, showing the contributions of the 7 main retinula cells to the rhabdom. **c** Micrograph of a transverse section through the ommatidium of a crab fixed at mid-day at a level corresponding to the upper diagram in (b). Dark annular inclusions are late-stage multilamellar bodies. Retinula 7 lies at the bottom of the picture. $\times 6500$. *B* cytoplasmic bridge; *ECS* extracellular space; *P* palisade; *nu* nucleus of reflecting pigment cell; *mtc* microtubule-filled channel; *R* rhabdom; *Rd* rind of cytoplasm surrounding rhabdom; 1-7 retinulae

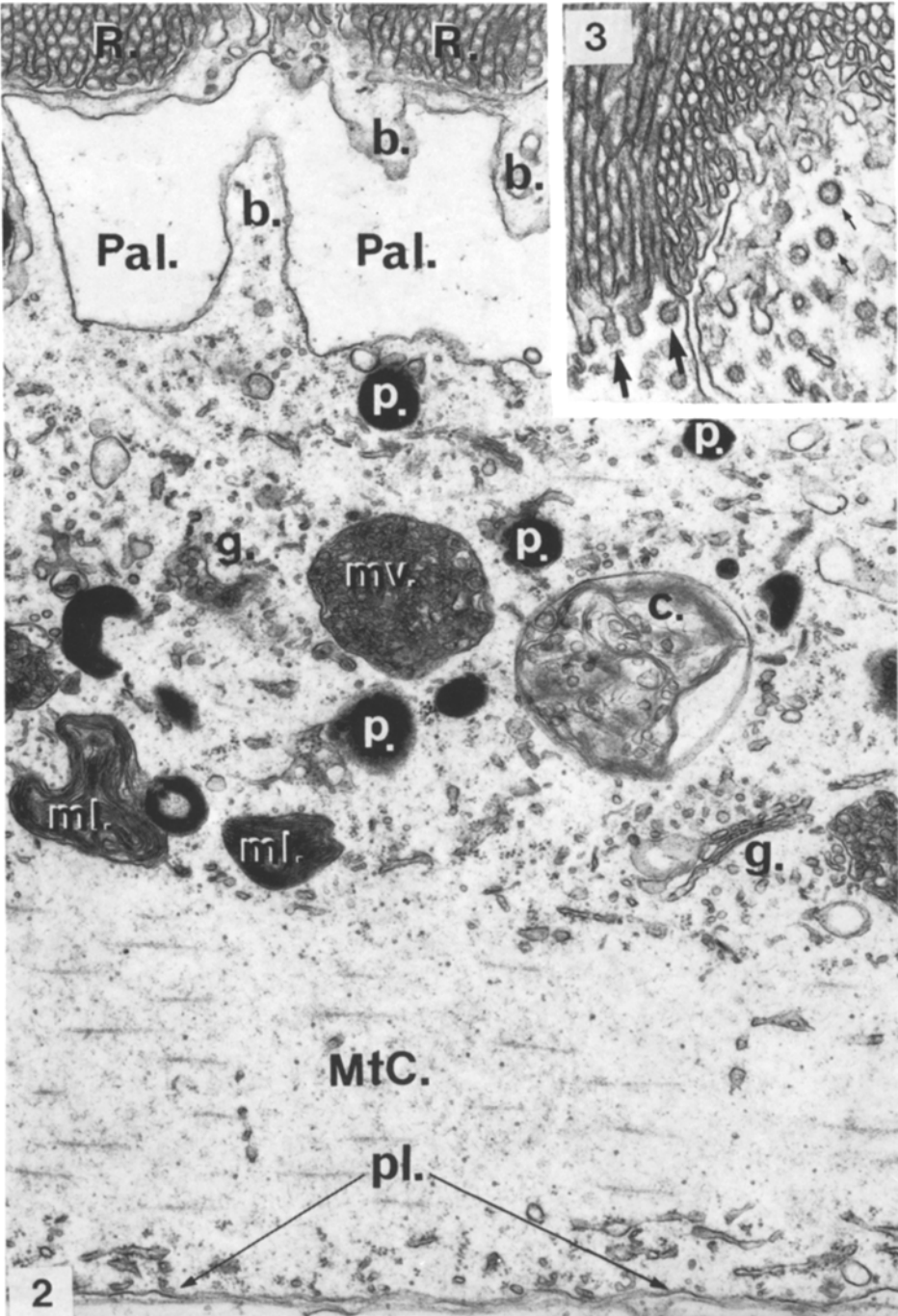


Fig. 2. Longitudinal section through a small portion of an ommatidium. *R* rhabdom; *Pal* palisade; *p* pigment granules; *b* cytoplasmic bridge; *g* Golgi body; *mv* multivesicular body; *c* combination body; *ml* multilamellar body; *Mtc* microtubule containing channel; *pl* plasma membrane of receptor. $\times 19,300$

Fig. 3. Pinocytotic vesicles budding (*thick arrows*) from rhabdomere. Detached coated vesicles lie free in the cytoplasm (*thin arrows*). $\times 38,000$

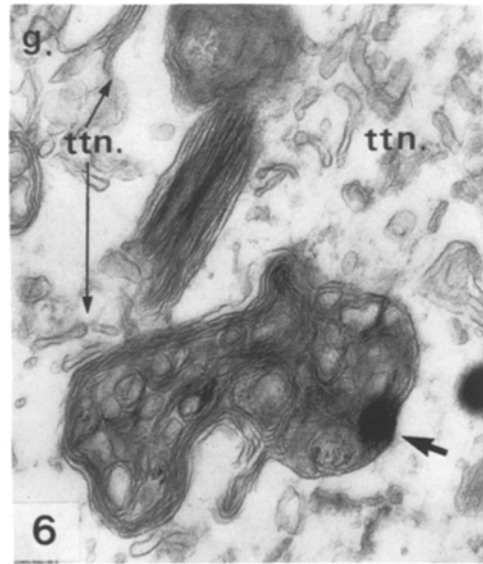
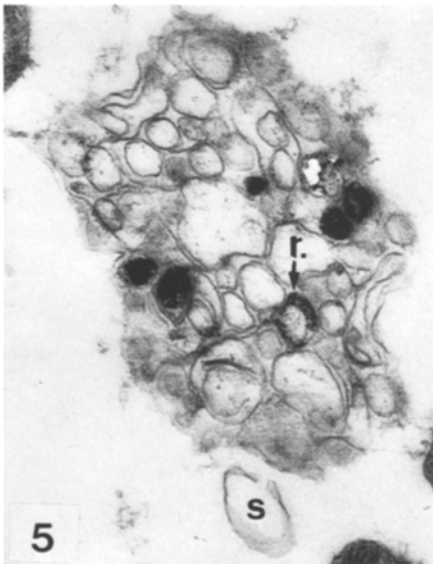
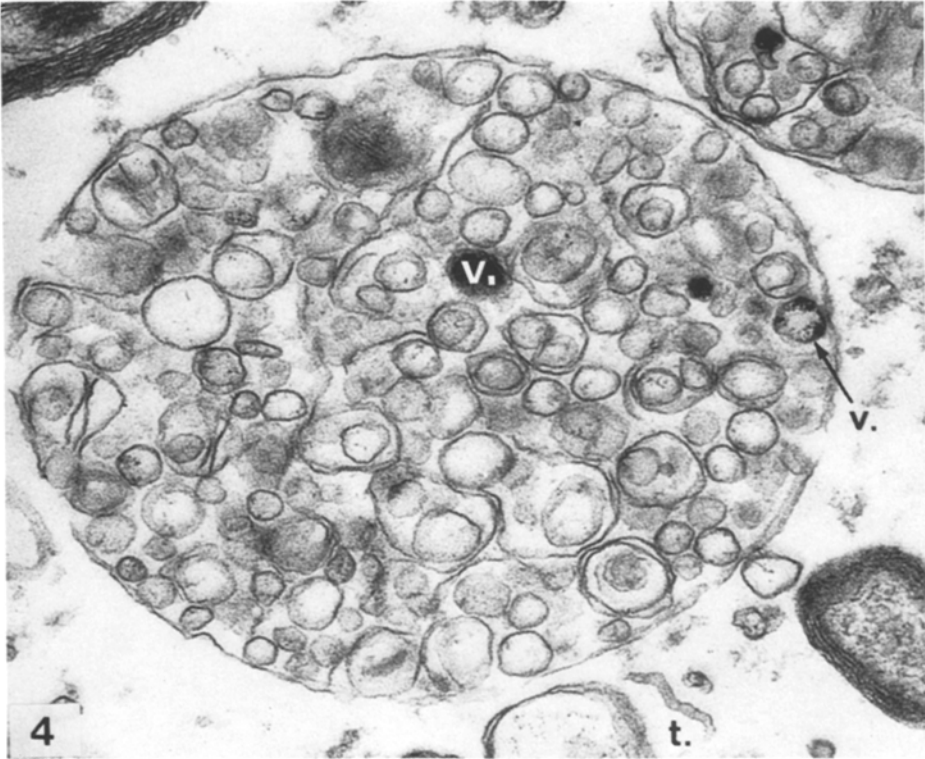
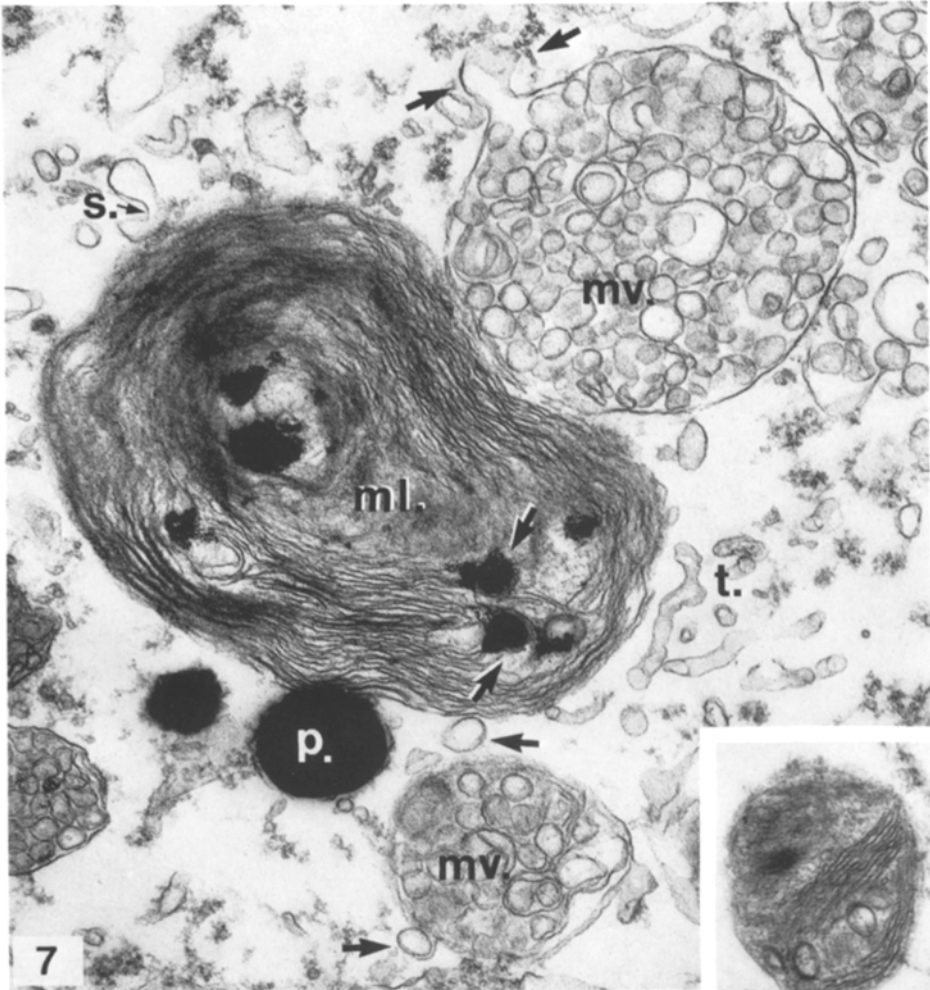


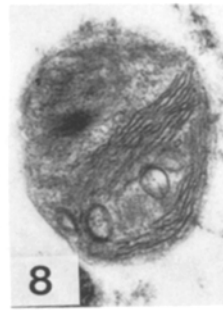
Fig. 4. Multivesicular body (mvb) with AcPh reaction in two vesicles (*v*). A small tubule (*t*) is seen at bottom of field. $\times 57,000$

Fig. 5. An irregular mvb with AcPh reaction in vesicles and also in the matrix (*r*). Note closely adjacent saccule (*s*). $\times 44,000$

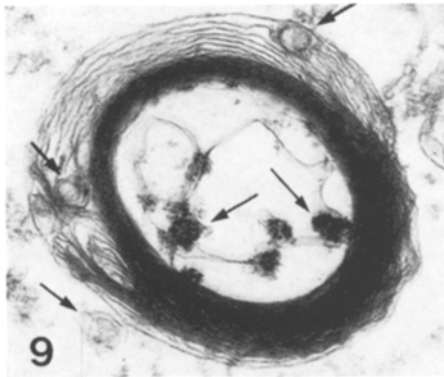
Fig. 6. Combination body showing AcPh reaction (*thick arrow*) surrounded by fragmented trans-tubular network (*ttn*), some elements at edge of a Golgi body (*g*). $\times 44,000$



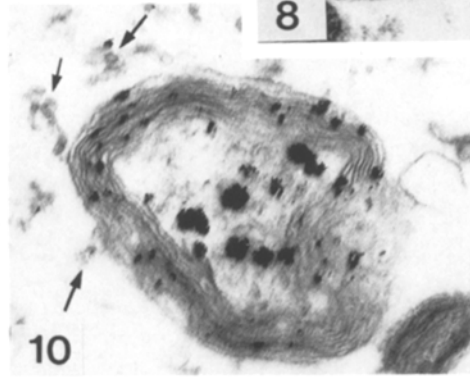
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Fig. 7. Large mlb (*ml*) with vesicular sites of internal AcPh reaction (*arrows*). Also arrowed are elements of RER in continuity with an mvb (*mv*) and small double-walled vesicles presumed to be primary lysosomes. A saccule (*s*) is in contact with the mlb. There are many tubules (*t*) of the trans-tubular network. $\times 46,000$

Fig. 8. Combination body with enclosed lamellae and vesicles. $\times 40,000$

Fig. 9. AcPh responses in mlb, with unreactive vesicles of similar size at periphery (*arrows*). Stained with lead only. $\times 40,000$

Primary Lysosomes

Primary lysosomes are identified by their relationships with secondary lysosomes and their occasional AcPhase reactions (both are described in the next sections). They offer much variety of form. Smooth, saccular vesicles, sometimes with double walls, tubules with a considerable range of diameters, and quite large saccules are all seen in the process of being incorporated into the secondary lysosomes (Figs. 11, 12, 20–22).

Tubular Primary Lysosomes

The tubular organelles are derived from Golgi bodies; the trans-face differentiates “explosively” immediately after dawn to produce a fragmenting transtubular network of the kind described by Rambourg et al. (1979) for Sertoli cells of rat testis (Figs. 13, 14). The tubules may branch irregularly, as in Fig. 14, and can reach considerable lengths. The unbranched tubule shown in Fig. 15 is roughly 1 μm long.

During rapid post-dawn differentiation, the cis-saccules are always grossly swollen. They are supplied with abundant transition vesicles by the rough endoplasmic reticulum (RER), which usually appears to be no more than co-extensive with the cis-faces (e.g., Fig. 13).

Association between ER and the trans-faces of the Golgi complex has not been consistently observed. RER fragments (Fig. 13, bottom), or saccules (Fig. 14) are often quite distant from the trans-tubular profiles, so that the evidence does not favour the possibility of continuity between them. Rambourg et al. (1979) query whether the trans-tubular network should be classified as GERL for the same reason, with added weight from their failure to see continuities under high-voltage TEM examination.

By 4–5 h after dawn the activity of the Golgi complex is less marked (Figs. 16, 17). Fewer tubules are seen peeling off from the trans-face, and these and other compartments appear less electron dense. Profiles are sometimes seen in which much-disorganised Golgi bodies are so closely in apposition to late multilamellar bodies as to suggest that they are being incorporated into them. It is exceptional for any part of the trans-tubular network to show AcPh positive responses when still attached; a rare example is given in Fig. 16.

Saccular Primary Lysosomes

Saccular structures with characteristic profiles often suggestive of concentric paired membranes are also seen, sometimes abundantly, in the post-dawn period (Figs. 11, 12). They fuse with and are incorporated into multilamellar bodies. Indeed, they may be so abundant as to suggest that they contribute notably to the lamellae of the secondary lysosomes. Their origin is not certain. They occasionally show AcPh-positive responses (Fig. 12), and so also do segments of unequivocal ER (Fig. 17). Alternatively, some may be saccular trans-Golgi components sectioned *en face*.

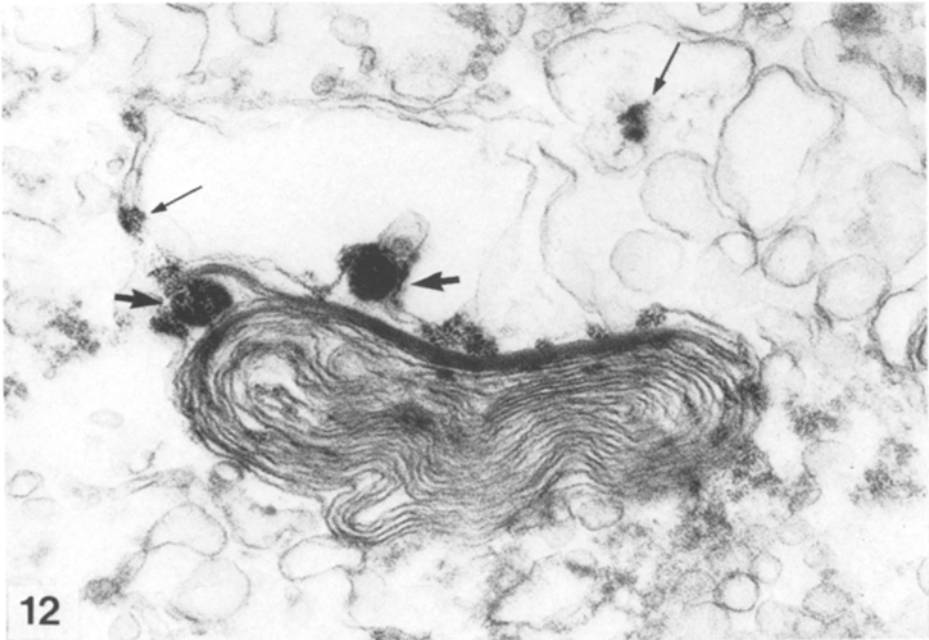
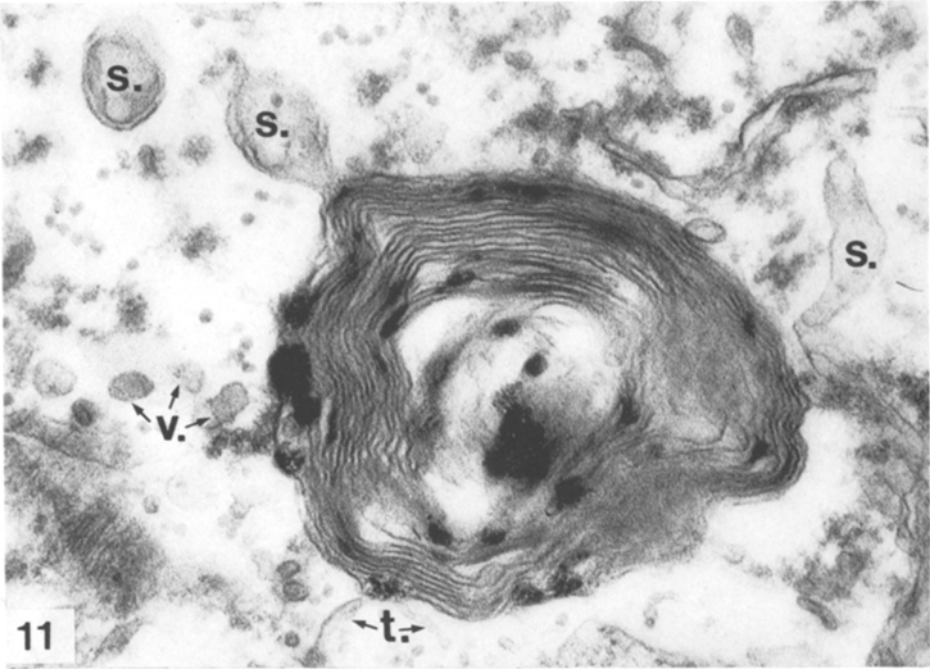
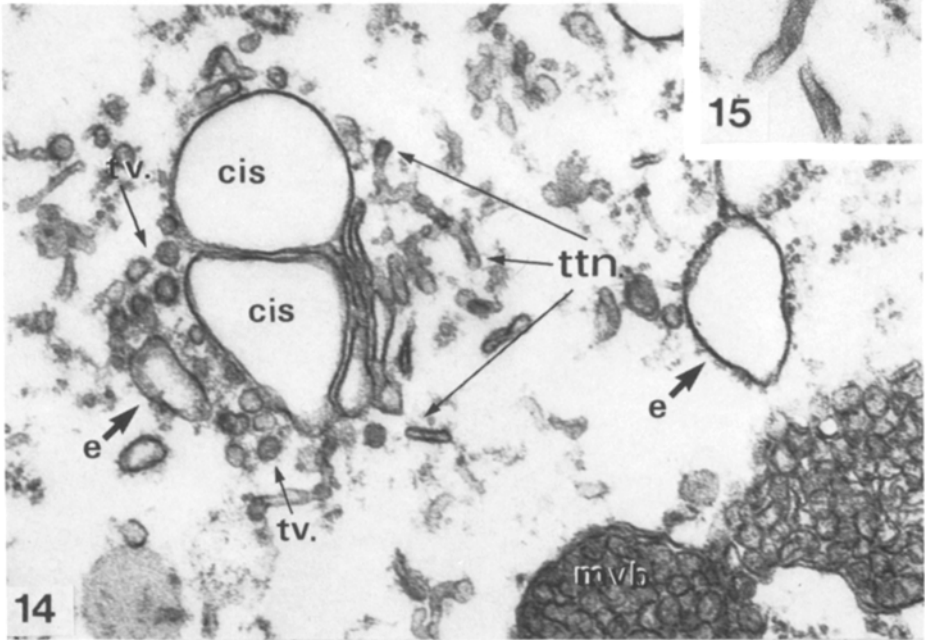
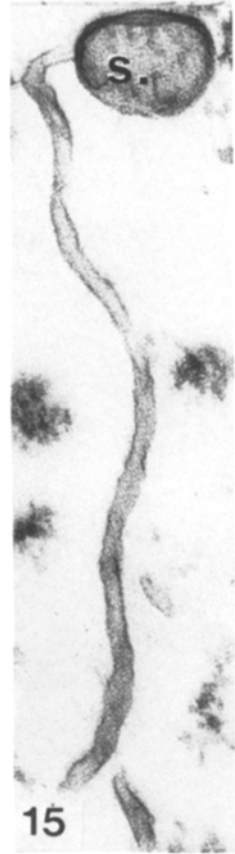
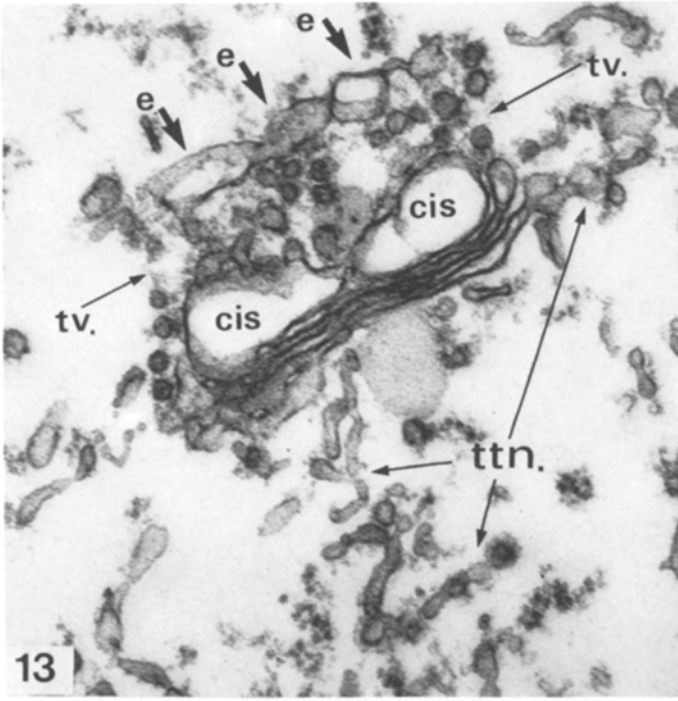


Fig. 11. Large mlb with sites of AcPh reaction. Saccules (*s*), tubules (*t*) and vesicles (*v*) are in close association with this body. $\times 88,000$

Fig. 12. An mlb late in lysis with associated vesicles and saccules reactive for AcPh (*arrows*) and also associated with smooth ER. $\times 88,000$



Figs. 13 and 14. Golgi bodies one hour after “dawn”, showing highly active transtubular network (*ttn*). *Cis* cis-saccules; *e* endoplasmic reticulum; *tv* transition vesicles. $\times 63,000$

Fig. 15. Isolated, long tubules from trans-network in association with ovoid saccular profile. $\times 87,000$

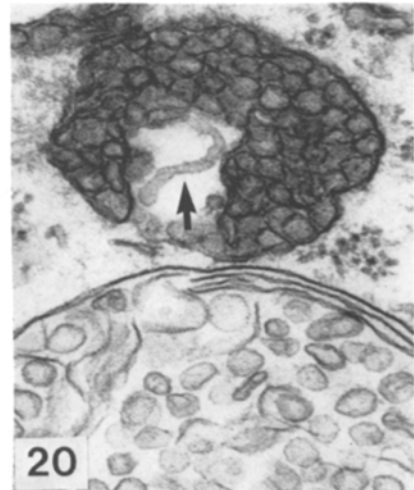
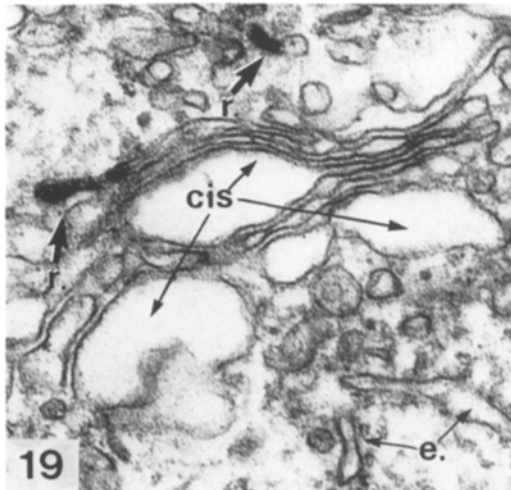
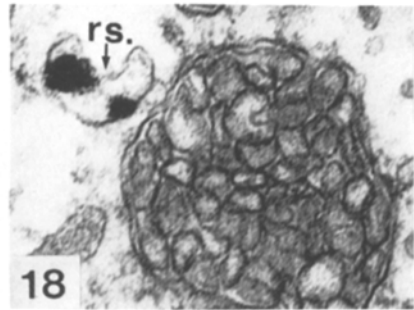
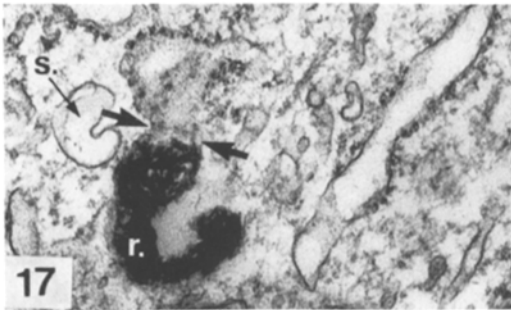
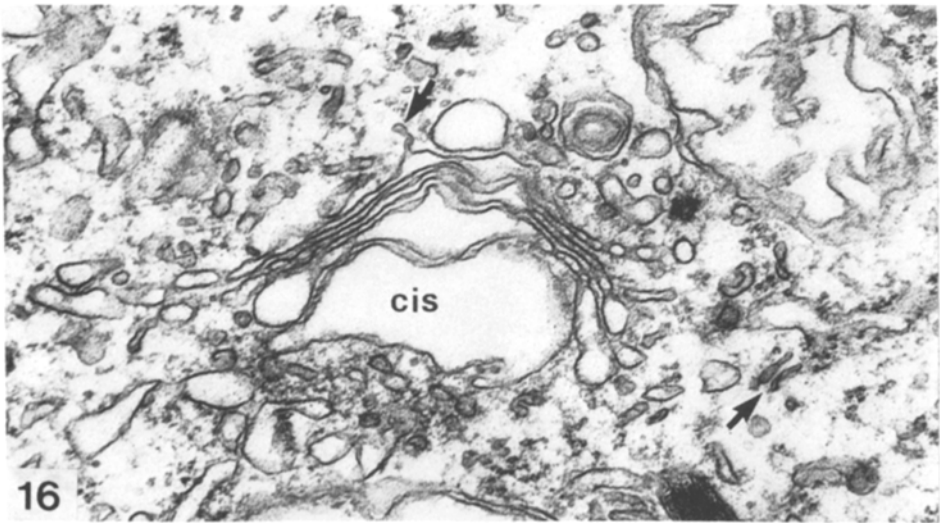


Fig. 16. Golgi body ca. 5 h after “dawn”, showing very fine tubules produced at trans-face (*arrows*). *Cis* cis-saccules. $\times 50,000$

Fig. 17. AcPh reaction (*r*) in saccule in full continuity with RER. Note adjacent smooth saccule. $\times 50,000$

Fig. 18. Smooth saccule with sites of AcPh reaction (*rs*) close to mvb. $\times 65,000$

Fig. 19. Golgi body, 5 h after “dawn”, with small foci reactive for AcPh in trans-components (*r*, *arrowed*). *Cis* cis-saccules; *e* ER. $\times 63,000$

Fig. 20. Tightly-assembled mvb with contained trans-tubule (*arrow*). It lies above the edge of a combination body. $\times 63,000$

Preservation of Ultrastructure After Cytochemical Procedures

Careful comparisons were made between retinal fragments fixed for 1–2 h in glutaraldehyde in cacodylate buffer and those fixed for cytochemistry and subsequently washed for longer periods and incubated. No evidence was found to suggest that any of the reacting structures considered to be lysosomal compartments are artefactual. Rhabdomeral microvilli are well-preserved after incubation, and the only structures which consistently suffer damage are the “bridges” across the palisade. A proportion of bridges are always found to have broken and re-organised as myelin figures after incubation.

The possibility that some mlbs may be artefactual myelin figures can be excluded. In both sorts of material they are seen to be in identical relationship to the organelles identified as primary lysosomes. Intracellular whorls which resemble those derived from collapsed bridges are seen only occasionally, and are readily distinguished from mlbs. They are never in orderly relationship to primary lysosomes.

Types of Acid Phosphatases

Two acid phosphatases with indistinguishable distributions are present during membrane breakdown: (i) A fluoride-inhibited β -glycerophosphatase (GPase), and (ii) a fluoride-insensitive p-nitrophenylphosphatase (PNPPase). Incubation for GPase gives even less background precipitate than for PNPPase in this material, and was used throughout most of the study.

Distribution of AcPh-Positive Reactions

AcPh reactions are seen only infrequently in primary lysosomes outside their target organelles. Rarely, small portions of the trans-tubular network of the Golgi complex respond either before or after they have peeled off from the stack (Fig. 16). Reactions are also seen in saccular components (Fig. 18). Both these classes of presumptive primary lysosomes are seen to be reactive where they touch secondary lysosomes, or as they are incorporated into them (Figs. 12, 21, 22).

Within secondary lysosomes, reactions are either confined to clearly distinguishable vesicles, corresponding roughly in size range to the saccular primary lysosomes, or they are more dispersed around foci which are of comparable size. In peripheral zones to which tubules can be seen to be attached, reactions are often weak, granular and diffuse (Fig. 22).

Despite the amount of material which is incorporated into the secondary lysosomes, and the extent of their degradation from mvbs, reaction product does not usually occupy a major part of the area of a given profile. Positive reactions are found in relatively early mvbs, usually occupying single vesicles or saccular compartments, but sometimes in mvb matrices. Although pinocytic vesicles presumably enter the mvbs by secondary endocytosis, as they do in crayfish (Eguchi and Waterman, 1976), this cannot be the principal mode of entry for primary

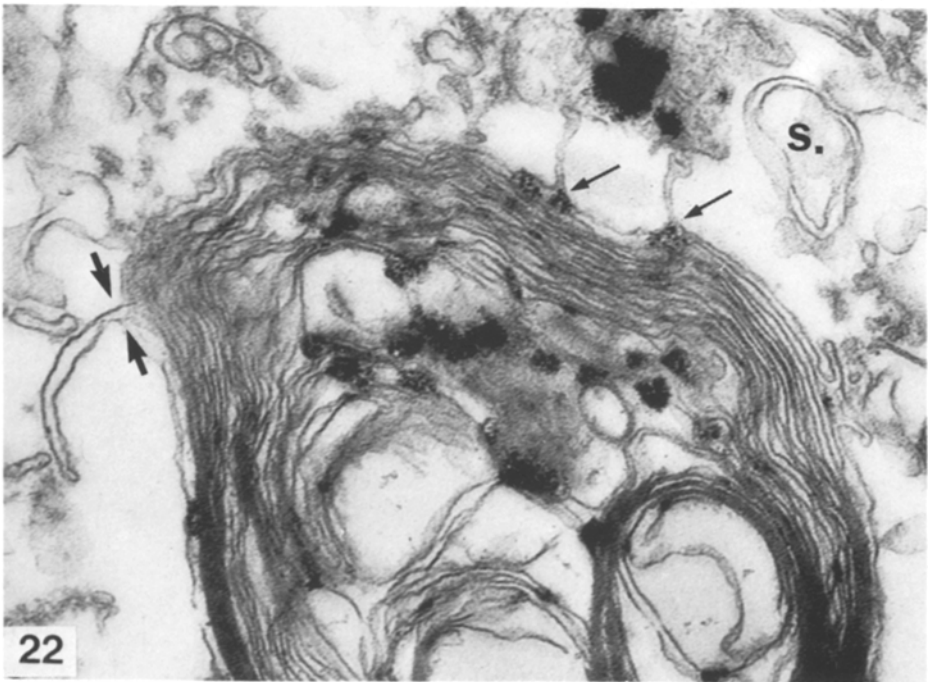
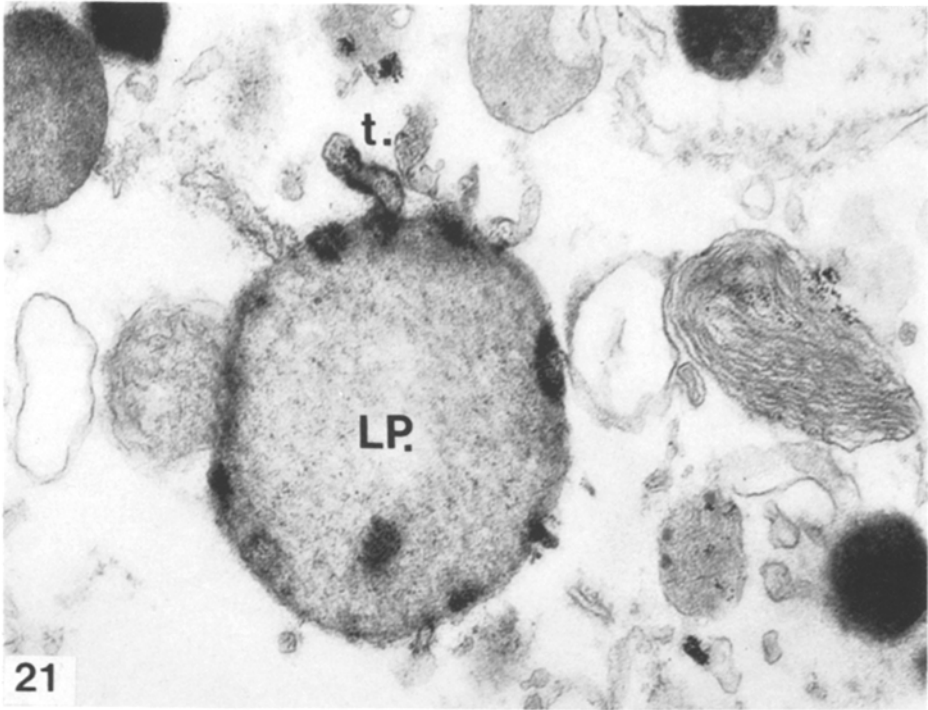


Fig. 21. AcPh-reactive tubules (*t*) fusing with large lipoprotein body (*LP*). $\times 51,000$

Fig. 22. Mlb with foci of AcPh reaction, tubules reacting at fusion points (*thin arrows*), a double-walled saccule (*s*), and a thick tubule in process of incorporation (*thick arrows*). $\times 65,000$

lysosomes, for they would void their enzymes into the mvb matrices as they turn inside out. In the figures of Eguchi and Waterman (1976) showing reactive mvbs this appears to have happened, and the reactions are, indeed, in the mvb matrices. The disparity between the two sets of results is probably explained by the much tighter organisation of crayfish mvbs. In crabs, it is by no means certain that the mvb saccule maintains its integrity, for profiles such as those in Fig. 20, in which parts of mvbs are open to the cytosol, are usual; it shows a trans-tubule enclosed in a space within an mvb, next to a discontinuity via which it may have entered. Tightly-assembled mvbs of this kind are as common as loosely-assembled mvbs, resembling the situation in *Dinopis* (Blest, 1978), and raise the same question as to whether all mvbs are assembled in identical ways.

The Fate of Secondary Lysosomes

Lysis of mlbs is rapid, and most have disappeared from the receptor cytoplasm by 1200 h. They are not replaced by the large, amorphous residual bodies typical of *Dinopis* (Blest et al., 1978a; Blest et al., 1979), but some typical lipofuscin granules of complex appearance and lipid droplets remain in the receptors. Their subsequent fate will be described separately; ER-derived lysosomal systems were shown to play a role in their disposal.

Aryl Sulphatase

Convincing positive reactions for aryl sulphatase were not demonstrated in any part of the lysosomal system, even with short (10–20 min) fixation times.

Discussion

The present results, taken in conjunction with those obtained on spiders (Blest, Price and Maples, 1978) illustrate an unanticipated diversity of lysosomal systems evolved to deal with the same materials. The compositions of rhabdomeral membranes of different invertebrates are closely similar (e.g., octopus, Akino and Tsuda, 1979; squid, Anderson et al., 1978; the insects *Deilephila*, *Calliphora* and *Ascelaphus*, Zinkler, 1975), so that differences in the biochemical requirements for degradation are unlikely to be involved. Yet, in *Dinopis*, the Golgi complex makes little if any contribution to the primary lysosomal compartments which consist of a grossly hypertrophied system of GERL type (Novikoff and Novikoff, 1977), differentiated from a massive store of RER (Blest et al., 1978b). In contrast, the RER-derived lysosomes of *Leptograpsus* are far less in evidence, but there is an explosive differentiation of the trans-tubular network of the Golgi bodies at dawn. Vesicles are associated with the network, although they are not abundant. Thus, it seems that the primary lysosomal mechanisms of even one cell type, the arthropod photoreceptor, cannot be discussed in terms of endoplasmic reticulum and its derivatives alone, as was suggested by Blest et al. (1979). However, this conclusion still rests on our failure to see any connection or even

juxtaposition between the trans-tubular network and ER, and the apparent equivalence between the networks in *Leptograpsus* and the Golgi complex in rat Sertoli cells (described by Rambourg et al., 1979), where the three-dimensional geometry is better understood. The rather modest dimensions and lack of complexity of arthropod Golgi bodies make reliable classification of their cisternae difficult, as for example in the study of *Galleria* haemocytes of Rowley and Ratcliffe (1979) where it is clear that Golgi bodies or Golgi-associated compartments contribute AcPh-positive primary lysosomes, but uncertain from which part of the system they are derived. Holtzman (1977) has noted that some of the controversies which have arisen over the design of lysosomal compartments may merely reflect the evolutionary lability of a basic set of relationships. Some components of the overall ER-Golgi system may in a particular cell type be emphasised at the expense of others. Our results so far tend to confirm this view.

The problem posed by the study of Hafner and Bok (1977) on crayfish (see Introduction) seems capable of resolution in terms of the behaviour of lysosomal systems. Direct comparison with our results is not possible, because their experiments, in which (^3H)-leucine was injected into crayfish, were not designed in relation to the transition points of the 12 h cycle of illumination, and the extent and temporal localisation of turnover in crayfish are not known. However, lysosomal systems are likely to be most active during "day" when the experiments were performed, and labelling of mvbs before labelling of rhabdoms 5 min after injection can be explained in two ways: (i) the provision of bounding membranes for the mvbs from RER (Blest et al., 1979, and above) may involve rapid manufacture of both membrane and membrane proteins, and (ii) hydrolases are likely to be synthesised with equal rapidity, as Hafner and Bok (1977) have pointed out. It would be of interest to see whether early labelling is also obtained with (^3H)-mannose; if the interpretation suggested here is correct, specificity of mannose to the oligosaccharide core of rhodopsin should ensure that the rhabdom is labelled first.

AcPh responses in both *Dinopis* and *Leptograpsus* are never very strong when implicated in the sequence of membrane breakdown, although massive reactions can be found associated with autophagic vacuoles and isolation membranes in the spider (Blest et al., 1979), and in *Leptograpsus* large compartments of the pigmented glial cells may, on occasion, be filled with reaction product. A few experiments in which incubation with β -glycerophosphate was carried out at pH 9.0, in order to test the possibility that responses in secondary lysosomes at pH 5.0 might represent the tail of alkaline phosphatase reactions, gave no deposits at these sites. AcPh reactions in both species are only seen regularly as primary lysosomes make contact with their targets. Instances are well-known from arthropod material in which pre-synthesised AcPhs are activated with great rapidity as they encounter their substrates (Locke, 1976) at a particular developmental stage (Schin and Laufer, 1973), or in response to cellular insult (Griffiths, 1979; Griffiths and Boschek, 1976). Since it is clear from our observations that enormous amounts of primary lysosomal material are incorporated into the secondary lysosomes, some explanation is needed for the failure of AcPh to build up in them, for profiles wholly occupied by reaction product are almost never observed. It is probable that they are either de-activated or destroyed by an appropriate regulatory process after a limited effective life span.

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