THE DEVELOPMENT OF PIGMENT GRANULES IN THE EYES OF WILD TYPE AND MUTANT *DROSOPHILA MELANOGASTER*

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ABSTRACT

The eye pigment system in *Drosophila melanogaster* has been studied with the electron microscope. Details in the development of pigment granules in wild type flies and in three eye color mutants are described. Four different types of pigment granules have been found. Type I granules, which carry ommochrome pigment and occur in both primary and secondary pigment cells of ommatidia, are believed to develop as vesicular secretions by way of the Golgi apparatus. The formation of Type II granules, which are restricted to the secondary pigment cells and contain drosopterin pigments, involves accumulation of 60- to 80-A fibers producing an elliptical granule. Type III granules appear to be empty vesicles, except for small marginal areas of dense material; they are thought to be abnormal entities containing ommochrome pigment. Type IV granules are characteristic of colorless mutants regardless of genotype, and during the course of development they often contain glycogen, ribosomes, and show acid phosphatase activity; for these reasons and because of their bizarre and variable morphology, they are considered to be autophagic vacuoles. The 300-A particles commonly found in pigment cells are identified as glycogen on the basis of their morphology and their sensitivity to salivary digestion.

INTRODUCTION

Genetic control of eye pigmentation in *Drosophila melanogaster* has been carefully analyzed in the last thirty years, and much is now also known about the biochemistry of pigment synthesis. A well characterized system such as this one is ideally suited to the study of developmental consequences of gene action at the fine structure level. The purpose of this investigation was to observe the normal sequence of events during stepwise assembly of pigment granules in the eyes of wild type flies, and to interpret this process by a comparison with deviations in the sequence resulting from specific genetic mutations. Several histo- and cytochemical tests have been employed to assist in this interpretation. After the basic morphology of the process is described, several fundamental biological problems will be considered. First, how does pigment formation in the insect eye compare cytologically with other intracellular secretory processes, which are thought to involve packaging of cellular products in the Golgi apparatus for their later release into the cytoplasm? Second, by what mechanism(s) do pigment granules increase in mass and density during the process of differentiation? Thirdly, pigments as small molecules are presumably bound in some regular array by a macromolecular complex, or "matrix." What is the structural nature of this complex; does a fly genetically incapable of pigment synthesis nevertheless manufacture a normal granule matrix which would ordinarily accommo-

date pigment, or is the matrix per se a nonexistent entity, even in a wild type fly?

Wild type Oregon R flies were studied and compared with the following eye color mutants: brown (*bw*), a point mutation on chromosome II in which production of pterins is inhibited almost completely (33); vermilion (*v*), a point mutation on the X chromosome in which no ommochromes are manufactured because of blockage of the reaction tryptophan → kynurenine (2, 12); vermilion brown (*v;bw*), a fly with colorless eyes which carries both of these mutations; white (*w*), a mutant on the X chromosome blocking the production of drosopterin and ommochrome pigments (although their precursors are excreted with the meconium at eclosion) (72); Ins $w^{m4}L w^{mJR}$/Y, a colorless fly deficient for the 3C2 band which includes all or part of the *w* locus (30); Dp(w^m) 264-58a, an insertion of part of the X chromosome, including the *w* region, into the proximal heterochromatic region of the left arm of chromosome III, which produces a "variegated" eye having patches of wild type pigment against a colorless background (4).

In addition, preliminary studies have been made on wild type and white-eyed (*wa*) *Ephestia kühniella*, the corn meal moth.

For purposes of orientation a brief description of the gross morphology of the compound eye, based on the work of Bodenstein (11), is given (Fig. 1). The eye of *Drosophila melanogaster* consists of approximately 800 facets, or ommatidia. Each ommatidium is composed of a distal part (the dioptric apparatus), a proximal part (the sensory retinula), and a sheath of pigment cells surrounding these parts. The dioptric apparatus consists of the corneal lens (*c*, Fig. 1), the crystalline cone, or pseudocone (*ps*), and four cone cells (*CC*) which secrete the pseudocone material during development. Eight elongated retinular cells (*RC*) arranged radially around a longitudinal axis make up the proximal part of the ommatidium; each retinular cell possesses a specialized structure, the rhabdomere (*rh*), which is thought to be the light-sensitive portion of the ommatidium. Two primary pigment cells (*PPC*) lie adjacent to the cone cells, and nine secondary pigment cells (*SPC*) enclose the entire ommatidium. Pigment granules in these two cell types are the subject of this investigation. (In addition, small pigment granules occupy the area at the bases of the rhabdomeres.) The proximal end of each ommatidium rests on a basement membrane (*bm*) through which nerve fibers pass to the optic ganglion.

Wild type adult eyes of *D. melanogaster* contain two classes of pigment, ommochrome and four closely related drosopterins, both of which groups have been isolated chemically. The brown pigment, ommochrome, is a derivative of tryptophan by way of kynurenine (2, 8, 12, 23, 24, 39), and its synthesis is controlled by the *v* and *cn* genes (6, 7, 19–22, 62). The chemistry of the four red pigments, drosopterins, has been studied extensively (23, 25–29, 31, 41, 42, 48, 64, 71) as has the genetic control of drosopterin synthesis (26, 32, 33, 70, 73), but these interrelationships are less clear cut. (For a comprehensive review of the genetics and biochemistry of the eye pigmentary system, see Ziegler (72)). Pigments of both types are believed to be deposited on or within matrix granules composed of an RNA-protein complex (72). Several workers have described and interpreted the processes of differentiation and specialization of wild type and mutant eyes on the basis of light microscopy (15, 40, 44) and with electron optics (52, 53, 65–67, 69). With one exception, these workers have described the development of the eye as a whole, without placing emphasis on any one particular cell type or organelle. Nolte (52, 53) has discussed eye pigmentation, but has been concerned primarily with the distribution of pigment granules in adult eyes. The present author has enlarged upon this work by concentrating on the structural aspects of pigment granule formation in wild type and mutant eyes, in an attempt to correlate these morphological details with the pigment biochemistry of the different genotypes.

MATERIALS AND METHODS

Fly Culture

Flies were grown in sterile milk bottles on Carpenter's medium (13) in an incubator at 25°C. A modification of the method of Robertson (57) was used for staging. Early pupae, those with everted spiracles but whose bodies are soft when touched gently with a probe, were collected from the sides of the culture bottles and placed on damp filter paper in Petri dishes where they were incubated at 25°C ± 1° for the remainder of pupation. The time when pupae were collected was designated time zero; pupae were later selected at various representative stages, up through eclosion (96 to 100 hr), for study of pigment granule formation. There was considerable individual variation with respect to precise timing of development. Descriptions reported here are based on observations of at least five individuals

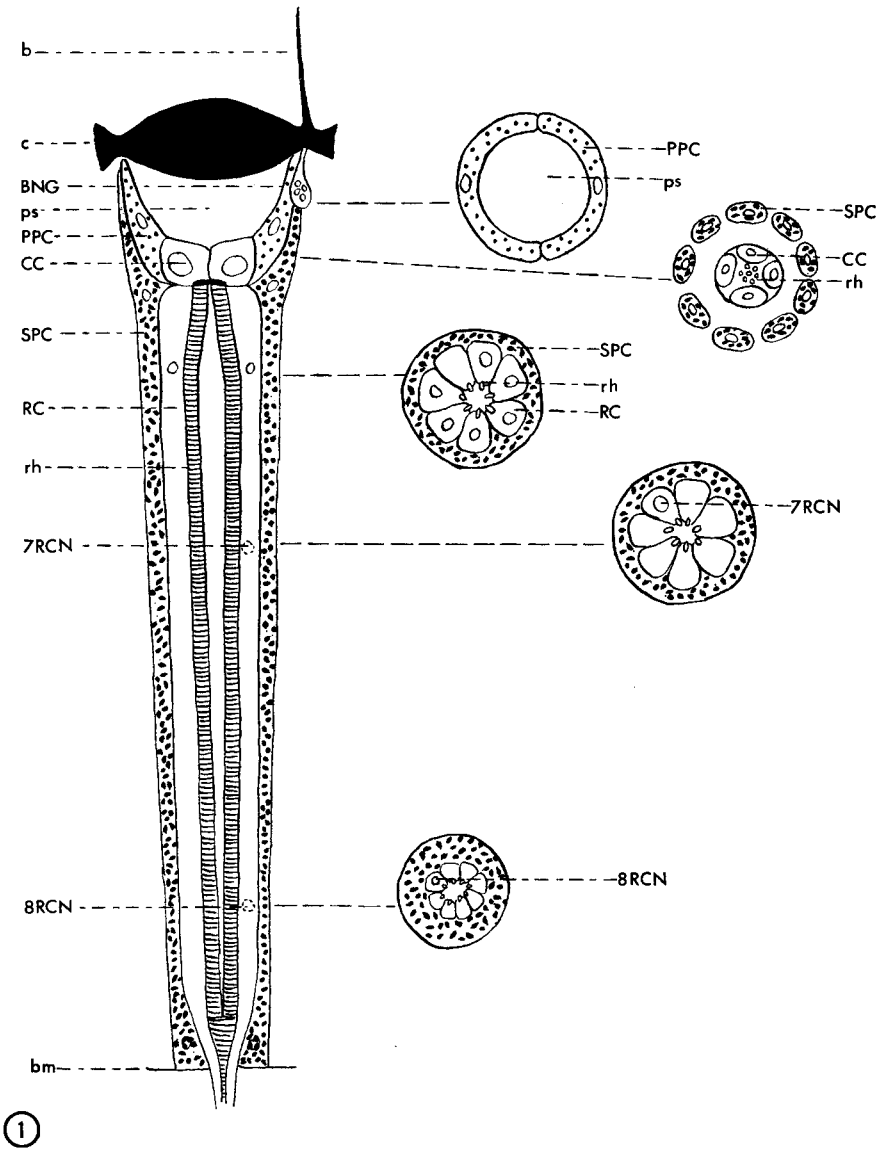

FIGURE 1 Diagram of the adult ommatidium, after Clayton (15). *b*, bristle; *bm*, basement membrane; *BNG*, bristle nerve group; *c*, cornea; *CC*, cone cell; *ps*, pseudocone; *PPC*, primary pigment cell; *RC*, retinular cell; *7RCN*, *8RCN*, nuclei of seventh and eighth retinular cells; *rh*, rhabdomere; *SPC*, secondary pigment cell.

in each time category and constitute an "average" thereof.

Histology and Cytology

Pupae at various stages were decapitated and the pupal case removed from the head. Whole heads of young pupae were fixed; in older stages, heads were bisected between the eyes. For studies of general morphology, eyes were fixed for 2 hr in cold 1% osmium tetroxide buffered to pH 7.6 with 0.2 M phosphate, or for 4 hr in cold 6% glutaraldehyde buffered to pH 6.8 with 0.2 M phosphate followed by 4 hr in 1% osmium tetroxide, pH 7.6 (58) and embedded in Epon 812. Thick sections (2 μ) for light microscopy were cut on a Porter-Blum MT-1 ultramicrotome and mounted unstained on gelatinized slides. Thin sections for electron microscopy were left unstained, or were stained in uranyl acetate or uranyl acetate and lead citrate (56).

Histochemistry and Cytochemistry

Ribonucleic Acid

White-eyed imagoes were fixed for 1 hr in cold formalin buffered to pH 7.6 with 0.2 M phosphate, treated for 1 hr at room temperature with ribonuclease (1 mg/ml) at pH 6.5, rinsed in cold 5% trichloroacetic acid, dehydrated, embedded, and sectioned for electron microscopy. Controls were incubated in water, adjusted to pH 6.5, in place of enzyme.

Acid Phosphatase

Eyes of eclosed flies, 1 to 2 days old, were fixed for 2 hr in cold 6% glutaraldehyde in 0.1 M sodium cacodylate buffer at pH 6.8. After careful rinsing in buffer, the eyes were incubated in Gomori's solution for 15 min in a water bath at 37°C, according to the method of Miller and Palade (45) but omitting sucrose. After this treatment, the eyes were rinsed in cold 0.05 M sodium acetate buffer containing 4% formaldehyde, rinsed in 2% acetic acid, and rinsed again in buffer plus formaldehyde. The eyes were then postfixed for 1 hr in cold 1% osmium tetroxide in veronal-acetate buffer at pH 7.4, prior to dehydration and embedding as described above. Sections for electron microscopy were left unstained. Controls included eyes incubated in 0.05 M sodium acetate buffer instead of Gomori's solution, and mouse kidney treated as above.

Glycogen

PERIODIC ACID-SCHIFF (PAS) REACTION: To demonstrate the presence of glycogen, material that had been fixed in formalin or in formol-acetic acid-alcohol (16), embedded in Epon, and sectioned at 2 μ was subjected to the standard PAS reaction (37), with and without prior removal of Epon (43). Some sections were incubated for 1 hr in saliva at 37°C before the PAS reaction was carried out. Mouse liver treated in the same ways served as controls.

SALIVARY DIGESTION: Pupae approximately 3 days old were fixed for 1 hr in cold 6% glutaraldehyde buffered to pH 6.8 with 0.2 M phosphate, and then rinsed a number of times in cold buffer over a period of 2 hr. Excised developing eyes were in-

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FIGURE 2 Oregon R, 48 hr of pupation. Note brown pigment concentrated basally (arrows); cornea (*c*) is forming; retinular cells elongate as their rhabdomeres develop. × 1500.

FIGURE 3 Oregon R, 72 hr. Pigment extends distally and encircles retinular cells which have been cut transversely; cornea is complete. The arrow indicates the position of primary pigment cells. × 1500.

FIGURE 4 Oregon R, 96 hr. Red pigment is clearly present in proximal areas. Primary pigment cells containing brown pigment granules juxtapose the cone cells (arrows); ommatidia have elongated further. × 1500.

FIGURE 5 Oregon R, eclosed. The concentration of red pigment has increased markedly. × 1500.

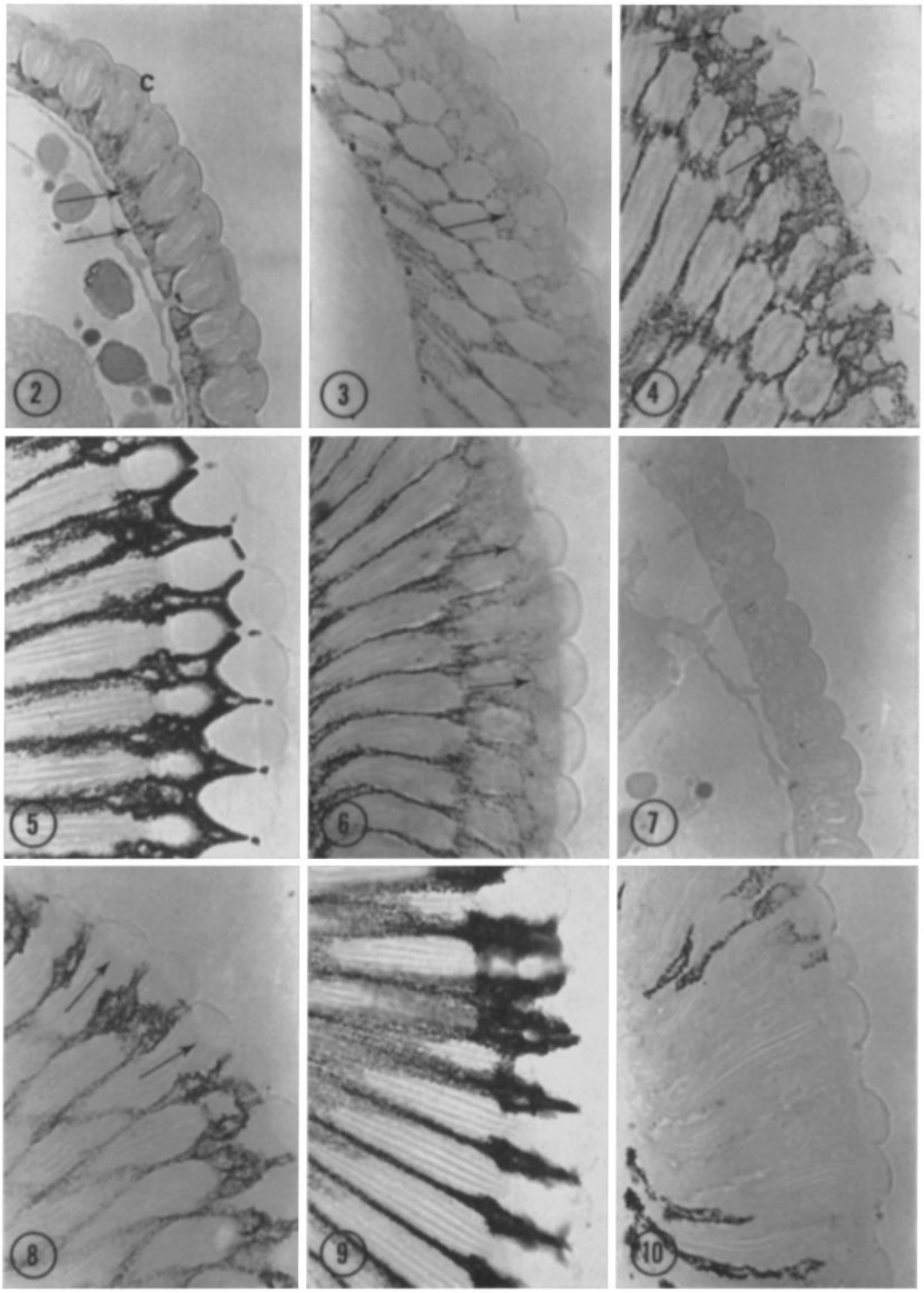
FIGURE 6 Brown mutant (*bw*), 72 hr. Brown pigment extends proximally and distally and apposes cone cells. The arrows denote the position of the primary pigment cells. × 1500.

FIGURE 7 Vermilion mutant (*v*), 48 hr. No pigment is present but the ommatidia are otherwise identical with those of wild type. × 1500.

FIGURE 8 Vermilion mutant (*v*), 72 hr. Red pigment is located proximally and distally but no pigment is seen adjacent to the cone cells (arrows) as in wild type (Figs. 3, 4) and *bw* (Fig. 6). × 1500.

FIGURE 9 Vermilion mutant (*v*), 96 hr. Red pigment has intensified. × 1500.

FIGURE 10 Position effect variegation, Dp(w^m)264-58a, 72 hr. Pigmented areas lie adjacent to colorless ommatidia; the brown pigment is well developed but no red pigment is yet visible. Ommatidia are either completely pigmented or unpigmented. Those which appear to be incompletely pigmented are merely cut obliquely. × 1500.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cubated for 2 hr in saliva at 37°C, while controls remained in buffer. After this treatment, the eyes were postfixed for 1 hr in cold 1% osmium tetroxide, pH 7.4, dehydrated, and embedded in the usual manner. Thin sections for electron microscopy were stained in uranyl acetate.

OBSERVATIONS

Developmental Stages

Light Microscopy

For purposes of orientation and comparison with electron micrographs, a series of light micrographs of wild type and mutant eye development is presented in this study, the emphasis being on pigmentation. Fig. 2 illustrates the wild type eye at 48 hr of pupation, about the earliest time at which pigment could be detected. Brown pigment (considered to be ommochrome (17, 18)) was concentrated basally, i.e. in the proximal parts of the secondary pigment cells. By 72 hr (Fig. 3), the eye structure was complete, although pigmentation was only partially developed. In Fig. 3 pigment is shown extending toward the distal surface and encircling the retinular cells in those ommatidia which are cut transversely. At 96 hours (Fig. 4), red pigments (drosopterins (17, 18)) sheathed each ommatidium but had not yet reached their maximum concentration. Thin semicircles of brown pigment in the primary pigment cells could be seen juxtaposing the cone cells proximally (as was the case a day earlier, although less clearly shown in Fig. 3). A marked increase in red pigment characterized the eclosed eye (Fig. 5), as also indicated biochemically by a steady rise in drosopterin content during the first 3 days of adult life (63).

Differentiation of the brown eye was identical with that of wild type eye except that no red pigments appeared in late pupae or adults. Fig. 6 illustrates the eye of a brown pupa at 72 hr; there was no appreciable change in color throughout the remainder of development.

In the vermilion mutant the pigmentation pattern was altered. At 48 hr of pupation (Fig. 7), there was no evidence of pigment in ommatidia otherwise identical with ommatidia of wild type (and brown mutant) pupae at the same age (*see* Fig. 2). Not until the 3rd day of pupation (Fig. 8) was drosopterin synthesis under way. Red pigments prevailed in both the proximal and distal parts of the secondary pigment cells, but a semicircle of brown pigment was not present in the primary pigment cells (arrows, Fig. 8) as it was in the primary pigment cells in wild type (Fig. 4) and brown mutant (Fig. 6) flies. Drosopterins intensified in late pupae (Fig. 9), the hue of the mature eye being bright vermilion owing to the absence of competition with brown pigment, as in wild type.

Fig. 10 illustrates position effect variegation, Dp(w^m) 264-58a, during the 3rd day of pupation. In an otherwise white eye, a patch of pigmented cells arose and differentiation proceeded at a rate similar to that in wild type eye. A short time later, drosopterins were accumulated and the adult eye had areas of wild type pigmentation adjacent to colorless ommatidia. (This genotype has been used as an internal control to illustrate concurrent processes in development of wild type and white eyes.)

Eye structure differentiation in colorless mutants was identical to that in wild type, *bw*, and *v* flies, but pigment was at no time detectable. Because of low contrast, unstained sections of unpigmented eyes had no particular value at the light microscope level and, therefore, have not been included.

Electron Microscopy

OREGON R: At about 48 hr of pupation, small dense granules appeared in the mid and proximal regions of the secondary pigment cells (Fig. 11). These granules, hereafter referred to as Type I, ranged in diameter from 0.04 to 0.13 μ, averaging 0.07 μ (*see* Table I). Golgi vesicles of medium electron opacity could often be observed adjacent to the denser, Type I granules (Fig. 12); wherever the Golgi region was observed in pigment cells, pigment granules were adjacent to it. Particles 200 to 300 A in diameter, conspicuous in both pigment cell types (Fig. 13), are probably glycogen, as demonstrated cytochemically (see below). Frequently, they were clumped together in the form of particulate aggregates not unlike the tiny pigment granules, except for their irregular surfaces. It was suggested earlier (60) that such particles were pigment precursors, but more recent evidence indicates that this aggregation process is not directly related to pigmentation. The pigment materials appear to be assembled in the Golgi region and released into the cytoplasm as membrane-bounded vesicles, which later complete their differentiation into mature pigment granules.

At 54 hr, the pigment granules appeared more

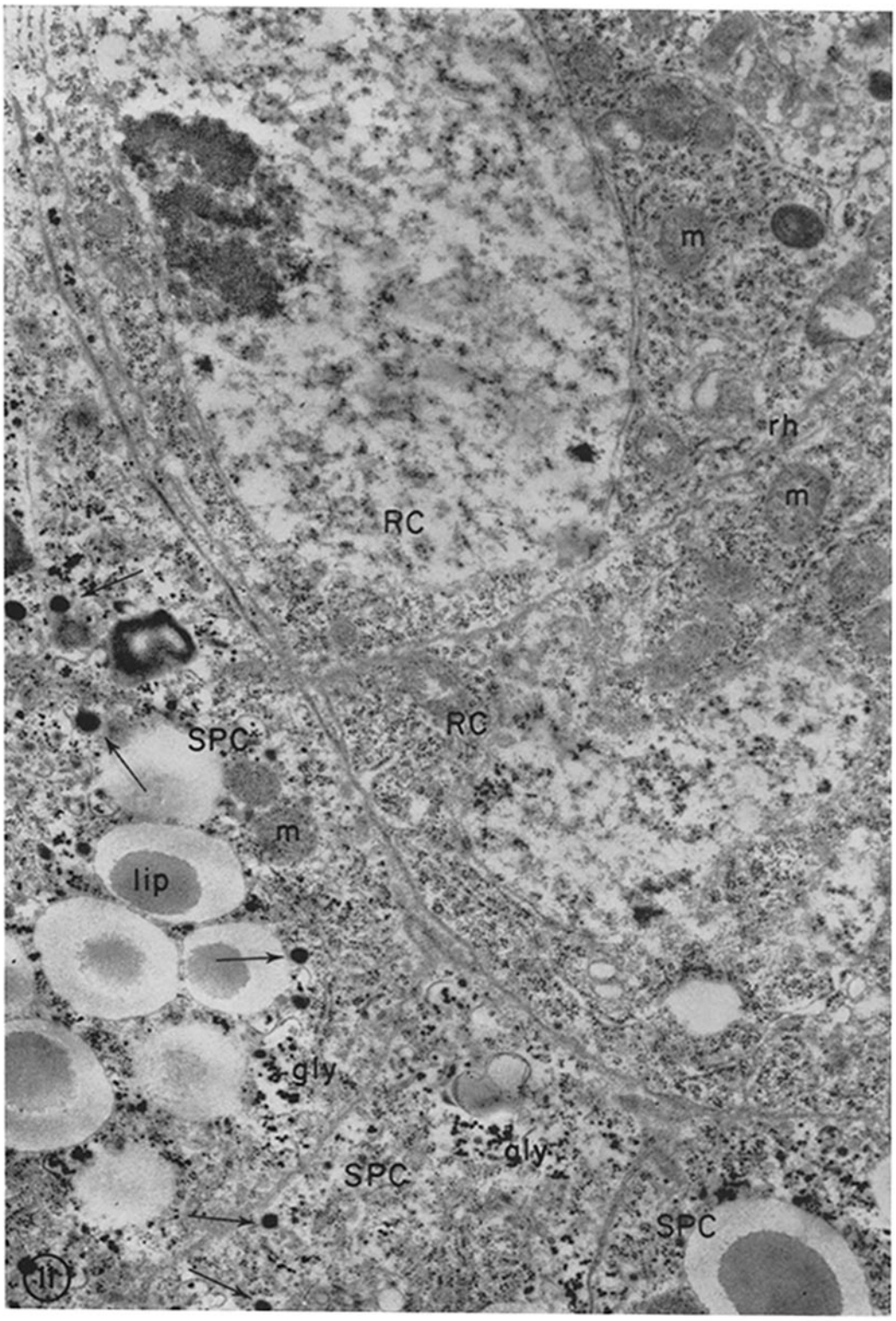

FIGURE 11 Oregon R, 48 hr. This transverse section at the level of the nuclei of retinular cells shows position of retinular cells (*RC*) with their forming rhabdomeres (*rh*) and secondary pigment cells (*SPC*) in which Type I pigment granules are beginning to form (arrows). Lipid inclusions (*lip*) and glycogen (*gly*), often clumped, are characteristic of pigment cells. Mitochondria (*m*) are rather small and inconspicuous. Uranyl acetate. × 18,000.

dense and larger in size (Fig. 13). In some specimens, Type I granules began to develop in the primary pigment cells. Higher magnifications sometimes revealed a suggestion of granular substructure, as seen in Fig. 14.

At 60 hr, the pigment granules had increased in diameter and density in the secondary pigment cells (Fig. 15), but were still quite small in the primary pigment cells. A linear increase in diameter with respect to time constituted their further development (*see* Table I). There was no evidence of a fusion of vesicles to account for this growth; the mechanism of addition of materials to the preexisting granules is as yet obscure.

At about this same time, a second type of pigment granule (Type II) first appeared in the secondary pigment cells. The exact origin could not be determined with assurance, but profiles considered to represent early stages of formation contained fibers, about 60 to 80 A across, distributed in a whorled or sometimes parallel fashion (Figs. 18 to 20, 22). The presence of a limiting membrane enclosing Type II granules was often difficult to ascertain, presumably because of poor preservation of membranes in general in this period. Maturation of these granules consisted of a filling in of material and a darkening (Figs. 21, 22). This type of granule was usually elliptical, measuring $0.75 \times 0.50\ \mu$, or round, about $0.50\ \mu$ in diameter, and there was no noticeable change in size or shape throughout the remainder of development.

Six hr later the situation was much the same. Type I granules increased in size, and Type II granules did not change appreciably in volume, but became more electron opaque. Rarely, another kind of pigment (?) granule (Type III) was also seen in this period (Fig. 17). It consisted of a clear vesicle of variable dimensions with a small accumulation of dense material at one extremity. Because it was more characteristic of *bw* and *v* flies, further details of its morphology are given later.

In the next 12 hr there was little change. By then, Type I granules were completely electron opaque, but profiles of Type II granules could still be seen in various stages of development, their constituent fibers being more, or less densely pigmented.

By 90 hr, Type II granules were often leached out in the preparative processes for electron microscopy, a phenomenon which may be related to the fact that as drosopterin-bearing pigment granules mature they become increasingly more soluble in alcohol and water (24). Fig. 34 illustrates the remnants of Type II granules in the eye of *v* mutant. Later stages were increasingly difficult to fix because the fixative could not penetrate the harder, more mature tissue, so that only rarely was well preserved material obtained in very late pupae or eclosed flies. Fig. 16 shows an area of a primary pigment cell with Type I granules in an eclosed fly.

Occasionally, a basally located cell containing pigment granules of an unidentified kind was observed in wild type pupae on or after the 3rd day (Fig. 23). These granules were bounded by a darkly staining membrane, and their substructure appeared somewhat granular. They may correspond to the ommochrome granules which Nolte

FIGURE 12 Oregon R, 48 hr. This micrograph shows a longitudinal section through the distal region of the ommatidium, including a cone cell (*CC*) and a primary pigment cell (*PPC*). Note the prominent Golgi zone (*g*) with associated Type I pigment granules (*I*). Uranyl acetate. × 32,000.

FIGURE 13 Oregon R, 60 hr. In this secondary pigment cell (*SPC*) Type I granules have increased in diameter; those enclosed in the box still show some substructure (see Fig. 14). Aggregates of glycogen particles (*gly*) are closely associated. Unstained. × 32,000.

FIGURE 14 Oregon R, 60 hr. An enlargement of the area boxed in Fig. 13 shows the fine granular appearance of developing Type I granules. Unstained. × 80,000.

FIGURE 15 Oregon R, 72 hr. This primary pigment cell (*PPC*) contains mature Type I granules. A cone cell (*CC*) lies to the right of the pigment cell. Uranyl acetate. × 32,000.

FIGURE 16 Oregon R, eclosed. Mature Type I granules are contained in this primary pigment cell (*PPC*) adjacent to the pseudocone (*ps*). Uranyl acetate. × 32,000.

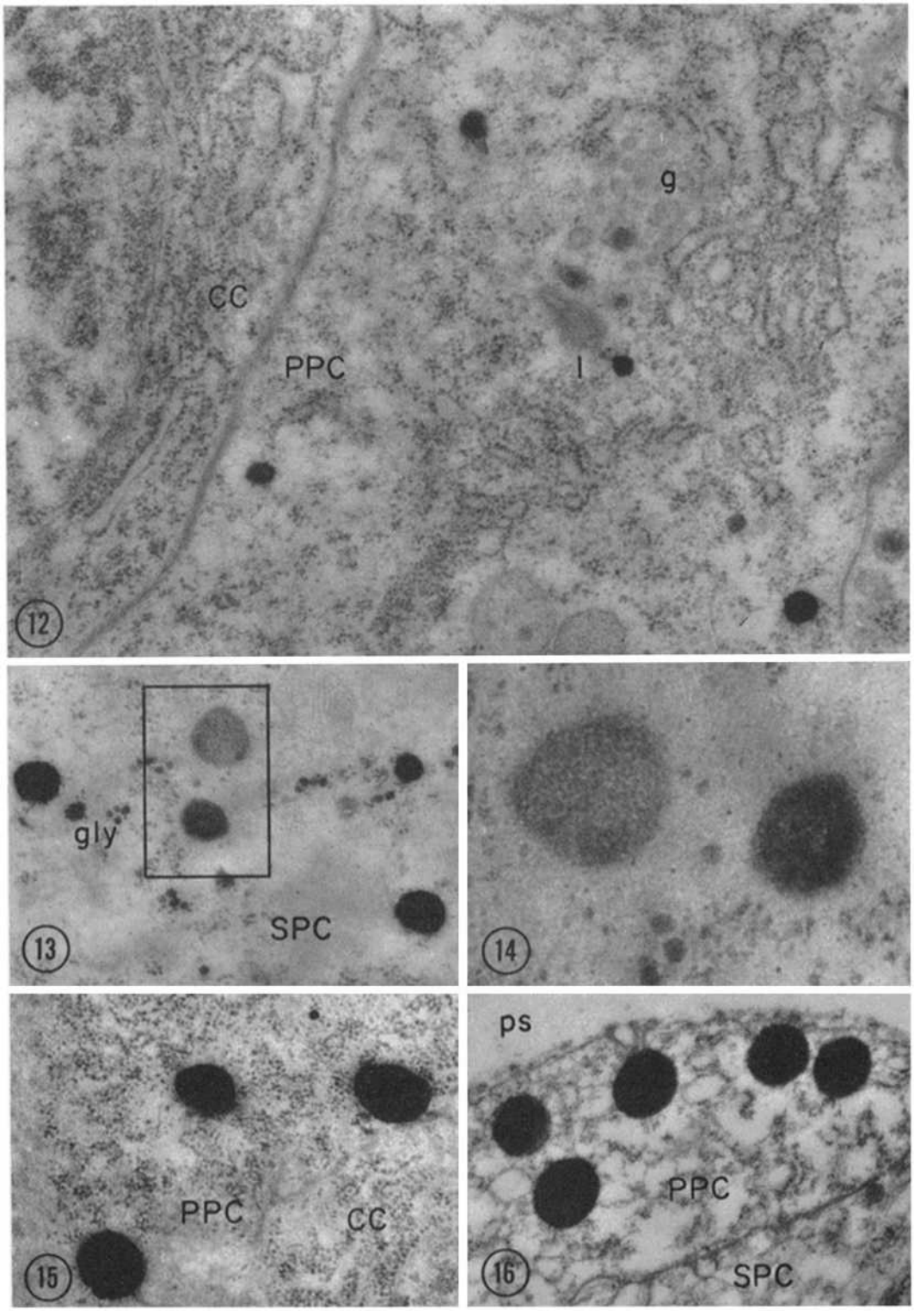
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TABLE I

*Increase in Diameter of Type I Granules through Pupation**

Age	Range	Mean	n‡
hr	μ	μ	
48	0.04–0.13	0.07	25
54	0.06–0.18	0.12	25
72	0.12–0.35	0.24	25
96	0.30–0.67	0.49	25
eclosion	0.34–0.65	0.48	25

* Measurements were made on electron micrographs of a random sample of individuals. The largest profiles in a given field were selected as representative of the maximum diameter of the granules.

‡ Number of measurements.

(52) described in the expanded bases of the retinular cells.

BROWN MUTANT: In the *bw* mutant, whose drosopterin synthesis is blocked, early development of granules was basically similar to that in Oregon R flies. At about 48 hr of pupation, and sometimes a little later, the first granules began to develop in the proximal part of the secondary pigment cells (Fig. 24). Their formation by elaboration in the Golgi region was similar to the formation of Type I granules as described above (Fig. 25). Glycogen particles were commonly observed in the pigment cells (Figs. 26, 27).

On the 3rd day, Type I granules continued to increase in size and density; after this time their average diameter did not enlarge significantly, so that at stages of late pupae and young adults, the granules were usually a little smaller than in wild type pupae, i.e. about 0.35 μ.

In late pupae, usually just 6 to 10 hr before eclosion, Type III granules were fairly common in the distal regions of the secondary pigment cells (Fig. 29). These granules, rarely present in wild type pupae as mentioned above, were round or elliptical vesicles in which dense material was accumulated at one side. For reasons which will be described later, these vesicles are believed to be abnormal Type I granules.

Also in late pupae, a fourth kind of cytoplasmic granule (Type IV) was sometimes observed. As early as 72 hr, but usually 12 to 24 hr later, agglomerations morphologically equivalent to lysosomes appeared in the secondary pigment cells (Figs. 28, 29). These inclusions were of medium electron opacity and sometimes contained glycogen and/or ribosomes. Type IV granules were more numerous in *w* flies, and are discussed in greater detail below.

FIGURE 17 Oregon R, 72 hr. The secondary pigment cell (*SPC*) contains abnormal Type III pigment granules, which are clear vesicles with accumulations of dense material often with fibers attached (arrows). The primary pigment cell (*PPC*) contains a normal Type I granule. Unstained. × 32,000.

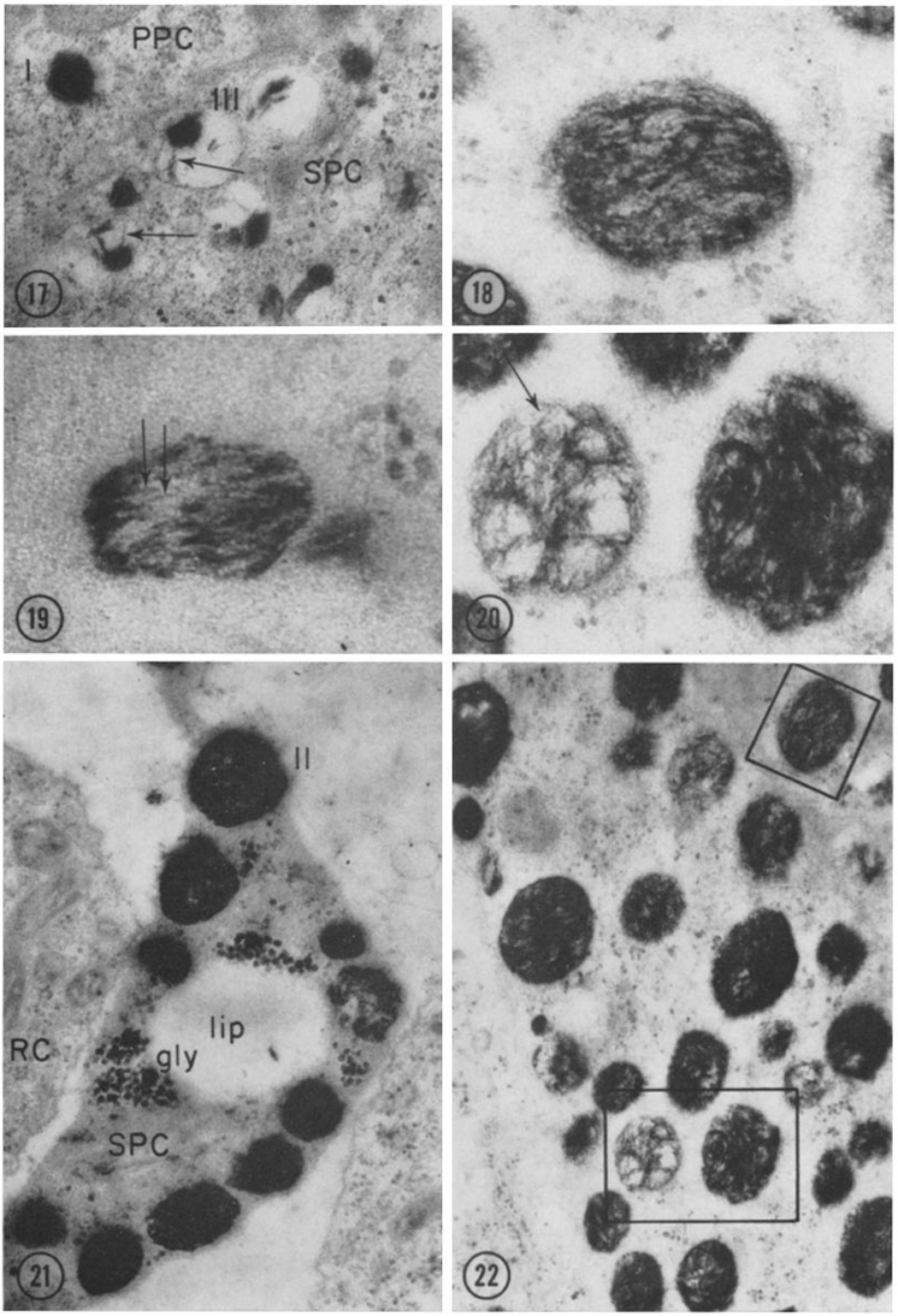
FIGURE 18 Oregon R, 96 hr. This is an enlargement of the upper boxed area of Fig. 22 showing fibrous substructure of a Type II granule. Uranyl acetate. × 80,000.

FIGURE 19 Oregon R, 72 hr. Fine fibers 60 to 70 A across, characteristic of Type II granules, are indicated by the arrows. Note the parallel orientation of the substructure of the granule. Unstained. × 80,000.

FIGURE 20 Oregon R, 96 hr. The irregular or whorled pattern of fibrous material is evident in this enlargement of the lower boxed area of Fig. 22. The arrow designates an 80 A fiber. Uranyl acetate. × 80,000.

FIGURE 21 Oregon R, 82 hr. Type II granules (*II*) are almost completely electron opaque in this section. Glycogen (*gly*) lies near a lipid inclusion (*lip*). A retinular cell (*RC*) with numerous mitochondria is at the left. Uranyl acetate. × 32,000.

FIGURE 22 Oregon R, 96 hr. This secondary pigment cell contains Type II granules in various stages of pigmentation. The boxed areas are enlarged in Figs. 18 and 20. Uranyl acetate. × 32,000.


PPC
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In the eclosed fly, Type I granules in the primary and secondary pigment cells were uniformly electron opaque but slightly smaller than in Oregon R flies (Fig. 29), as also reported by Nolte (52). Type II granules were never observed. Type III granules were more common than in wild type flies, but not invariably present. Type IV granules were also fairly numerous in secondary pigment cells.

VERMILION MUTANT: The *v* mutant, in which ommochrome synthesis is impeded, showed no evidence of pigment formation until midway through pupation. Prior to this time, structural development of the eye proceeded normally but no pigment granules of any kind were present (Fig. 30).

At 66 hr of pupation, Type III granules were often present (Figs. 31, 32). Occasionally, a Golgi profile was detected with moderately dense vesicles adjacent to it, as mentioned above with respect to the formation of Type I granules (Figs. 31, 32). Since mature Type I granules were never observed, these inclusions apparently were incapable of normal development into Type I granules.

About the 3rd day of pupation, Type II granules began to appear in the secondary pigment cells (Fig. 33). Their formation conformed to that described for these granules in wild type pupae.

By 90 hr, Type II granules were lost in preparation, as in the case of wild type pupae. Occasionally found were lysosomelike inclusions (Type IV) containing ribosomes and glycogen particles (Fig. 34).

WHITE MUTANT: In the *w* mutant the eye pigment was absent in early stages, and highly abnormal "pigment granules" developed in mature pupae. At 48 hr, the eye in *w* pupae was indistinguishable from the eye in *v* pupae, i.e. there was no evidence of pigment granules (Fig. 35).

Sometimes as early as 54 hr, but usually 6 to 12 hr later than this, large, irregular, lysosomelike granules (Type IV) appeared in the secondary pigment cells (Fig. 36). They were variable in appearance but generally consisted of a medium-dense "ground substance" and often an accumulation of 300-A particles (glycogen) or 150-A particles (ribosomes) (Figs. 39, 41), which appeared to have been sequestered from the surrounding cytoplasm. In young pupae, a limiting membrane around the granule was often hard to distinguish, not necessarily because of its absence, but because membranes in general were not well preserved at this stage, as has been mentioned previously.

By the 3rd day (72 hr), lysosomelike granules of increasingly bizarre morphology were fairly numerous and their limiting membranes were more easily demonstrable (Figs. 39 to 41). They frequently contained ferritinlike particles, usually arranged in a crystalloid pattern (Figs. 37, 38) Near and after eclosion, myelinlike membranes were often observed within Type IV granules (Fig. 40), and the matrix material tended to have a more uniform density.

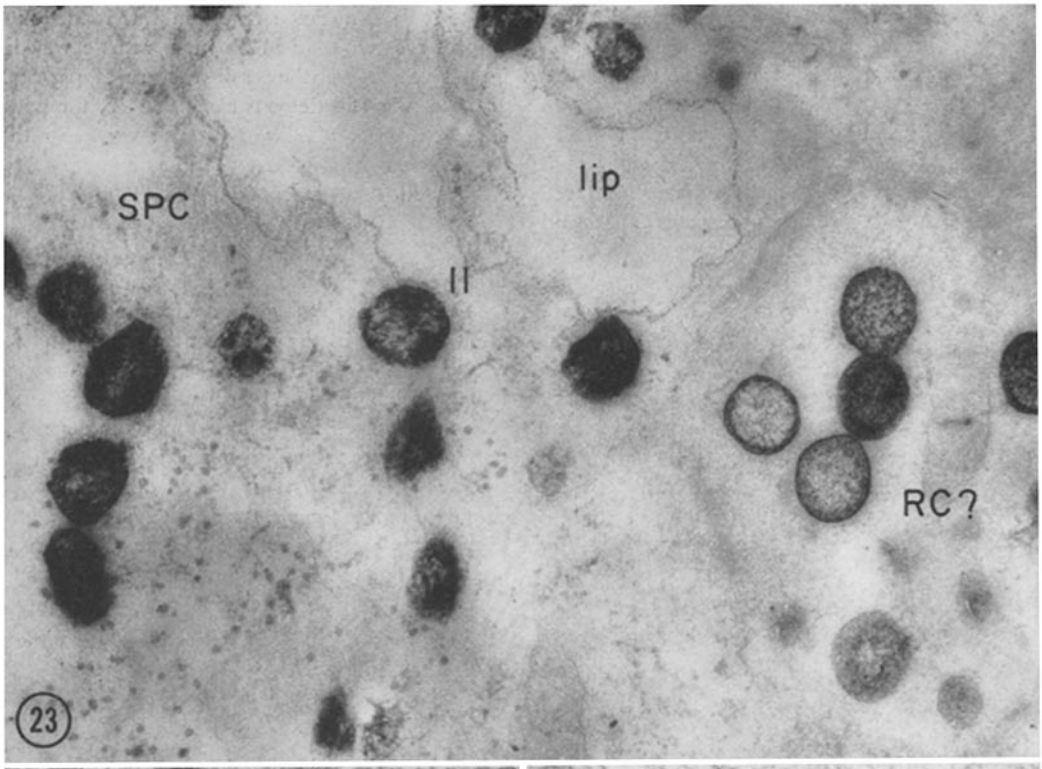
OTHER MUTANTS AND ANOTHER SPECIES: The eyes of colorless mutants of other genetic constitution, e.g. *v;bw*, were indistinguishable morphologically from those of *w* mutants. Typical lysosomelike granules (Type IV) were produced in *v; bw* mutants (Fig. 42), as well as in flies with the 3C2 deficiency (Fig. 43).

The mottled eye resulting from position effect variegation, Dp(w^m)264-58a, had patches of wild type pigment adjacent to colorless areas. Its fine structure at several representative stages exhibited

FIGURE 23 Oregon R, 72 hr. This section through a basal area of the ommatidium includes a secondary pigment cell (*SPC*) with normal Type II granules and lipid inclusions (*lip*). Another cell, possibly the expanded base of a retinular cell (see 52), contains unidentified pigment granules having darkly staining membranes and a granular substructure. Unstained. × 32,000.

FIGURE 24 Brown mutant (*bw*), 48 hr. The secondary pigment cells (*SPC*) adjacent to retinular cells (*RC*) contain small Type I pigment granules similar to those in early wild type pupae. Uranyl acetate. × 12,000.

FIGURE 25 Brown mutant (*bw*), 54 hr. Type I granules (*I*) in a primary pigment cell are forming adjacent to Golgi areas (*g*) which contain vesicles of high electron opacity. Note the microtubules (*mt*). Uranyl acetate. × 32,000.


SPC
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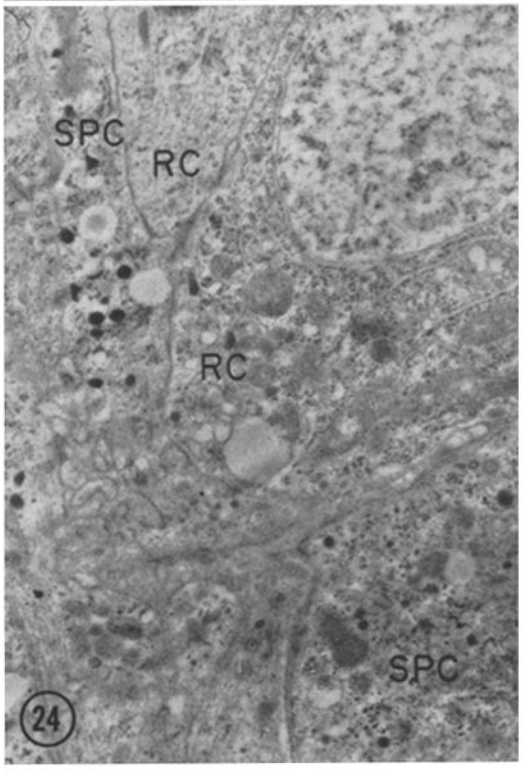
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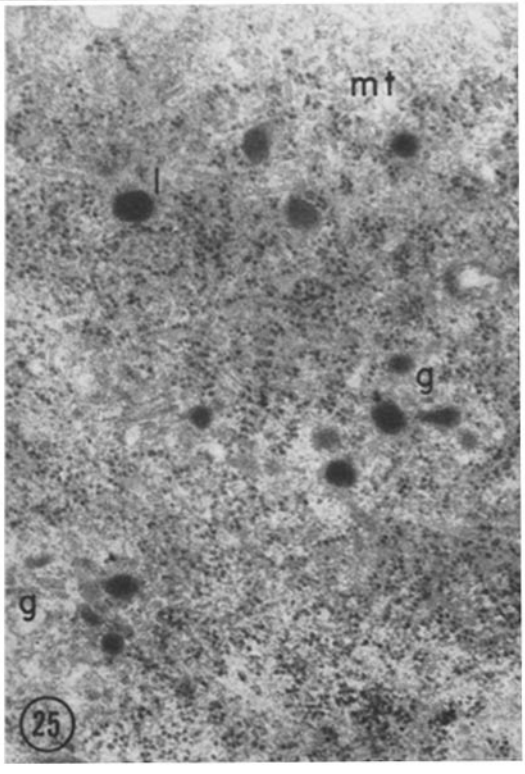
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g
25

no apparent deviation from the fine structures described above for Oregon R and *w* eyes, respectively. The position effect variegation pattern was demonstrated to be ommatidium specific, i.e. an ommatidium is either colorless or wild type. In Fig. 44, a white ommatidium is adjacent to a wild type area. Type IV granules were produced in the former, and apparently normal Type I and Type II granules in the latter.

In the corn meal moth, *Ephestia kühniella*, the situation with respect to the presence of pigment granules in the white-eyed mutant was different from that in *Drosophila*. Fig. 45 shows a wild type moth eye with pigment granules of the secondary pigment cells adjacent to a retinular cell. In the white-eyed mutant (*wa*) (Fig. 46), no pigment granules whatsoever are seen in a comparable area.

Histochemistry and Cytochemistry

Ribonucleic Acid

In colorless eyes, small ribosomelike particles were frequently observed in Type IV granules, as has been mentioned above. To confirm their identification as ribosomes, formalin-fixed, eclosed *w* eyes were subjected to ribonuclease digestion and stained for nucleic acids with uranyl acetate at pH 3.8. Stainability of the particles was effectively removed (Figs. 47, 48). Subsequent to this observation, it was of interest to determine the relation of RNA to normal developing pigment granules. Spatially, there was no evidence for direct involvement of ribosomes in the formation of Type I and Type II granules. Unfortunately, fixation appropriate for ribonuclease digestion was inadequate to preserve structure in an interpretable way in immature stages, when substructure of pigment granules was still demonstrable. Hence, the presence or absence of RNA within or very near the forming normal pigment granules could not be established.

Acid Phosphatase

Because "pigment granules" in colorless eyes have such bizarre morphology in comparison with pigment granules in the eyes of other mutants and wild type flies, it seemed reasonable to suppose that these bodies could be classified as autophagic vacuoles engaged in "focal cytoplasmic degradation" similar to those in degenerative mammalian tissue (61). As such, they would be expected to contain, at some time in their existence, hydrolytic enzymes characteristic of lysosomes, e.g. acid phosphatase. To test this hypothesis, the classical Gomori method for determination of acid phosphatase was applied to eyes of adult *w* mutants a day or two after eclosion. It was found that some of the pigment granules gave a strong reaction for acid phosphatase (Fig. 49), whereas many others, often those containing ribosomes or glycogen particles, did not. The latter presumably represented stages in the sequestration process before enzymes were present or stages subsequent to autodigestion.

Glycogen

PERIODIC ACID-SCHIFF REACTION: The 300-A particles abundant in both primary and secondary pigment cells possessed the morpho-

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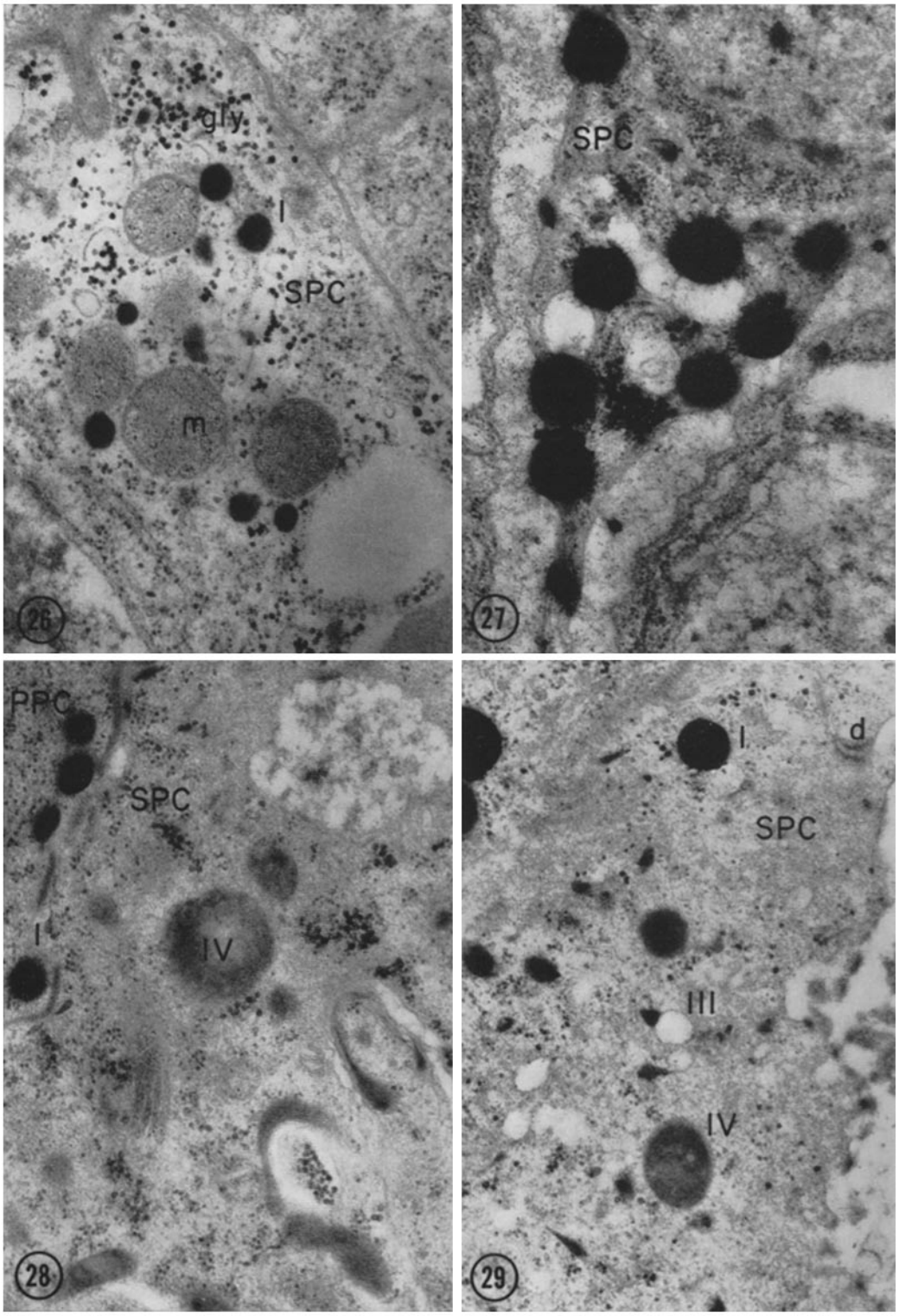
FIGURE 26 Brown mutant (*bw*), 54 hr. Glycogen particles (*gly*) are numerous in this section through a secondary pigment cell which contains forming Type I granules. A few mitochondria (*m*) are present. Unstained. × 32,000.

FIGURE 27 Brown mutant (*bw*), 72 hr. Dense Type I granules in the secondary pigment cell have reached their mature size. Note the clump of glycogen. Uranyl acetate. × 32,000.

FIGURE 28 Brown mutant (*bw*), 72 hr. Type I granules in the primary pigment cell (*PPC*) are small in size as compared to those at the same stage in wild type pupae. The secondary pigment cell contains a lysosomelike granule (*IV*) and several myelinated profiles suggestive of a somewhat degenerative cell. One of them includes glycogen particles. Uranyl acetate. × 32,000.

FIGURE 29 Brown mutant (*bw*), 96 hr. Type III and Type IV granules are shown in this secondary pigment cell (*SPC*) along with normal, but small, Type I granules. A desmosome (*d*) is located along the microvillous border. Uranyl acetate. × 32,000.


gly
I
SPC
m
26
SPC
27
PPC
SPC
I
IV
28
I
d
SPC
III
IV
29

logical characteristics of glycogen, although their natural, unstained electron opacity was greater than that reported in most other species (1, 55). Because at stages midway in the development of pigment granules, rather large accumulations of these particles were present in the eyes of all genotypes examined, a histochemical determination of glycogen at the light microscope level was carried out on 2-μ Epon sections of wild type and white eyes at about 60 hr of pupation. Epon sections of formalin or formol-acetic acid-alcohol-fixed material, from which Epon had been removed, gave a negative reaction after 1 hr of treatment in saliva (Figs. 50, 51). From this, it was concluded that glycogen was, indeed, present in the pigment cells.

SALIVARY DIGESTION: Identification of the 300-A particles as glycogen was demonstrated conclusively by incubating the developing wild type eyes in saliva for 2 hr. In the untreated controls, the particles were numerous and darkly staining (Fig. 52). In the experimental eyes subjected to treatment with saliva, no particles could be detected in any of the ommatidia (Fig. 53).

DISCUSSION

Table II and Fig. 54 summarize the observations of pigment granule formation in the eyes of wild type flies and three eye color mutants.

Two common types of pigment granules (Types I and II) were clearly demonstrable. Type I granules are interpreted as the carriers of ommochrome pigment, for the following reasons. At 48 hr of pupation these granules, as seen in thin sections in the electron microscope, correspond spatially to the brownish eye pigmentation of pupae in serial sections from the same block observed in the light microscope. A number of investigators have reported the appearance at this time of brown pigment identified chemically as ommochrome (17, 18, 34, 49). In addition, the pigment granules produced in the primary and secondary pigment cells of the *bw* mutant, which manufactures ommochromes only, are morphologically identical to the above granules.

TABLE II

Summary of Pigment Granule Types and Their Cellular and Genotypic Distribution

Pigment granule type	Pigment	Pigment cell type	Genotype
I	Ommochrome	PPC, SPC	$\frac{+}{+}$, *bw*
II	Drosopterin	SPC	$\frac{+}{+}$, *v*
III	Abnormal ommochrome?	SPC	*bw*, *v*, $\frac{+}{+}$ (rarely)
IV	Abnormal pigment precursors?	PPC, SPC	*w*, *v*;*bw*, 3C2 deficiency, et cetera

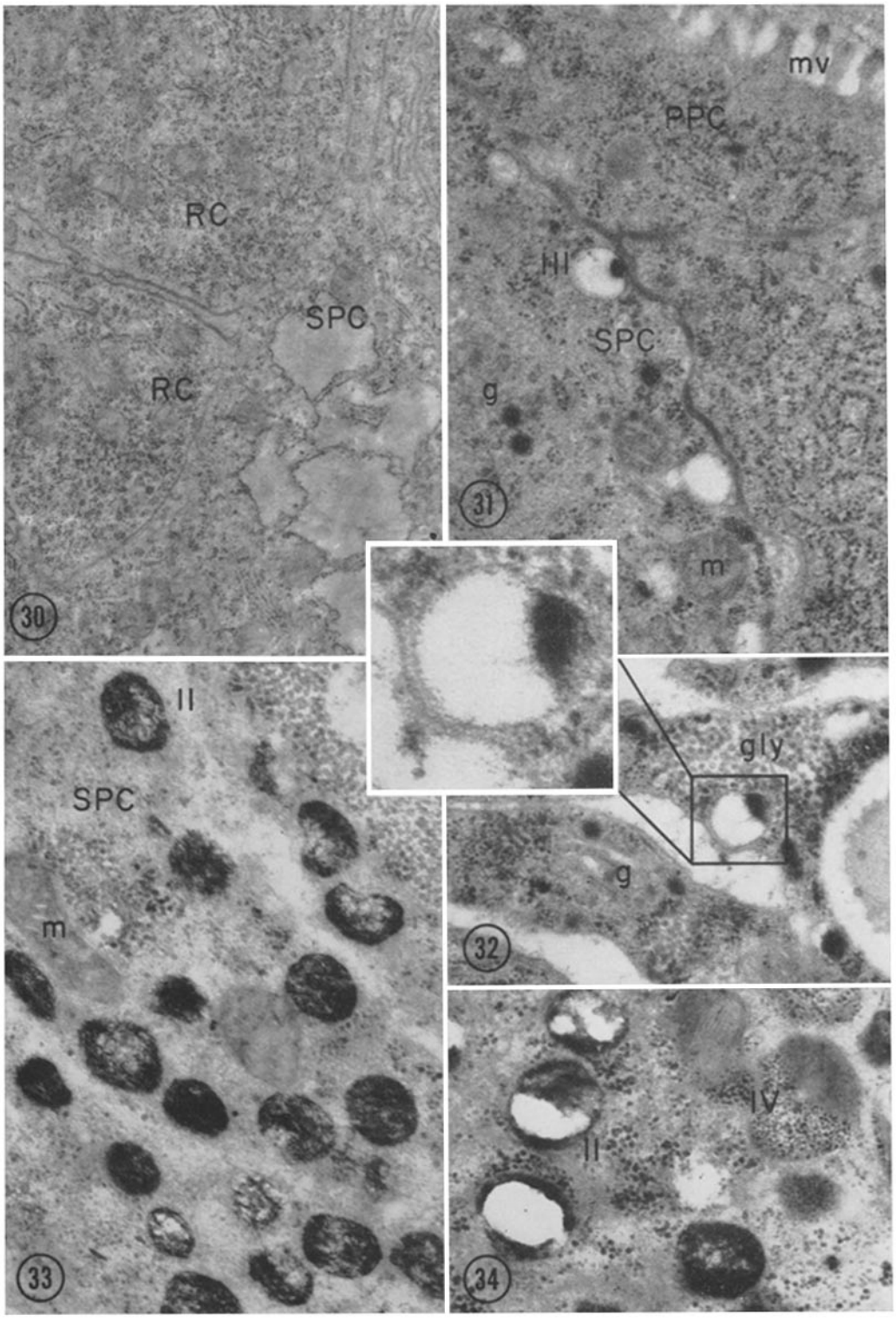
FIGURE 30 Vermilion mutant (*v*), 48 hr. The secondary pigment cell (*SPC*) shows no evidence of pigmentation. Retinular cells (*RC*) are developing at a rate similar to that of wild type pupae. Uranyl acetate. × 12,000.

FIGURE 31 Vermilion mutant (*v*), 66 hr. The secondary pigment cell has a Type III granule and a Golgi area (*g*) with electron-opaque vesicles reminiscent of Type I granules. Several mitochondria (*m*) are present. The primary pigment cell with its microvillous border (*mv*) contains no pigment granules. Uranyl acetate. × 32,000.

FIGURE 32 Vermilion mutant (*v*), 72 hr. Another example of a Type III granule in a secondary pigment cell. Note the glycogen particles (*gly*) and Golgi area (*g*) with dense vesicles. The inset is an enlargement of the Type III granule. Uranyl acetate. × 80,000.

FIGURE 33 Vermilion mutant (*v*), 72 hr. Type II granules in a secondary pigment cell are in various stages of development. Fibrous substructure is still detectable in most of them. Uranyl acetate. × 32,000.

FIGURE 34 Vermilion mutant (*v*), 96 hr. Type II granules are usually lost during fixation because of their solubility in water and alcohol. A Type IV granule containing ribosomes is also shown here. Uranyl acetate. × 32,000.


RC
SPC
RC
30
mv
PPC
III
SPC
g
31
m
II
SPC
m
33
gly
g
32
IV
II
34

Type II granules are believed to contain drosopterins, for the following reasons. It has been reported in the literature that chemically identifiable drosopterins begin to appear in eyes on the 3rd day of pupation (17, 18, 34). Also, Throckmorton (63), using more sensitive techniques, has demonstrated a quantitative increase in all four drosopterins in wild type eyes during the first 10 days after eclosion. No measurements were made on the eyes of pupae, but the curves could be extrapolated to a zero point about 2 days before eclosion. In other words, the combined evidence suggests that drosopterin synthesis begins no later than 60 to 70 hr of pupation. Furthermore, in the *v* mutant, whose biochemical machinery is limited to the production of drosopterins, the pigment granules occurring in the secondary pigment cells are identical to the ones described above.

In addition to these normal granules, inclusions of two other types were found in the eyes of the mutants studied. Type III granules, because of their occasional proximity to Golgi profiles with darkening vesicles reminiscent of early Type I granules, may possibly carry an abnormal ommochrome pigment incapable of normal development. This interpretation is not inconsistent with the postulated interdependence between ommochrome and drosopterin syntheses (72). Nolte (50), noting that most eye color genes affect both pigments at least to some extent, proposed the existence of a common substrate during some early stage of their formation (51). Forrest (24) has suggested that the two pigments may compete for some precursor material, or that one of the products in the pteridine sequence functions as a cofactor for one of the oxidative steps in ommochrome synthesis. The appearance of abnormal pigment granules would not be unexpected in the eyes of either *bw* or *v* flies. In eyes of *v* mutant, abnormal ommochrome could be attributed to "leakage" of the *v* gene, which, in fact, is not infrequently encountered. Abnormal ommochrome granules and normal but smaller than wild type Type I ommochrome granules in *bw* mutant is in accord with the Forrest cofactor hypothesis. Another possible explanation for small Type I granules in *bw* mutant is that when Type I granules are mature they normally contain some drosopterin as well as ommochrome pigment. There is no evidence for or against this interpretation. The observation of Type III granules in wild type flies is unexplained.

Type IV granules, characteristic of all colorless mutants examined, are believed to be related to pigment formation, though of an abnormal kind because they were restricted to pigment cells. They did not conform to earlier descriptions by Yasuzumi and Deguchi (69) and Ziegler (71). It is my suggestion that Type IV granules which first appear in the immature pupa represent accumulations of pigment precursors which, because of genetic defects, are prevented from normal development. As maturation of the ommatidia proceeds, the pigment cells begin to undergo catabolic processes resulting in the formation of autophagic vacuoles in which cytoplasmic components are sequestered and later hydrolyzed, in much the same way as described in degenerating mammalian tissues or in response to experimental stimuli (61).

Particles 200 to 300-A in diameter frequently encountered in pigment cells of all genotypes examined were identified by histo- and cytochemical tests as glycogen. Their role, if any, in pigment granule formation is obscure at the present time.

The processes of pigment granule formation involve several problems of general biological interest. First, how do the processes of insect eye pigmentation compare with other intracellular secretory systems? In the typical secretory tissue,

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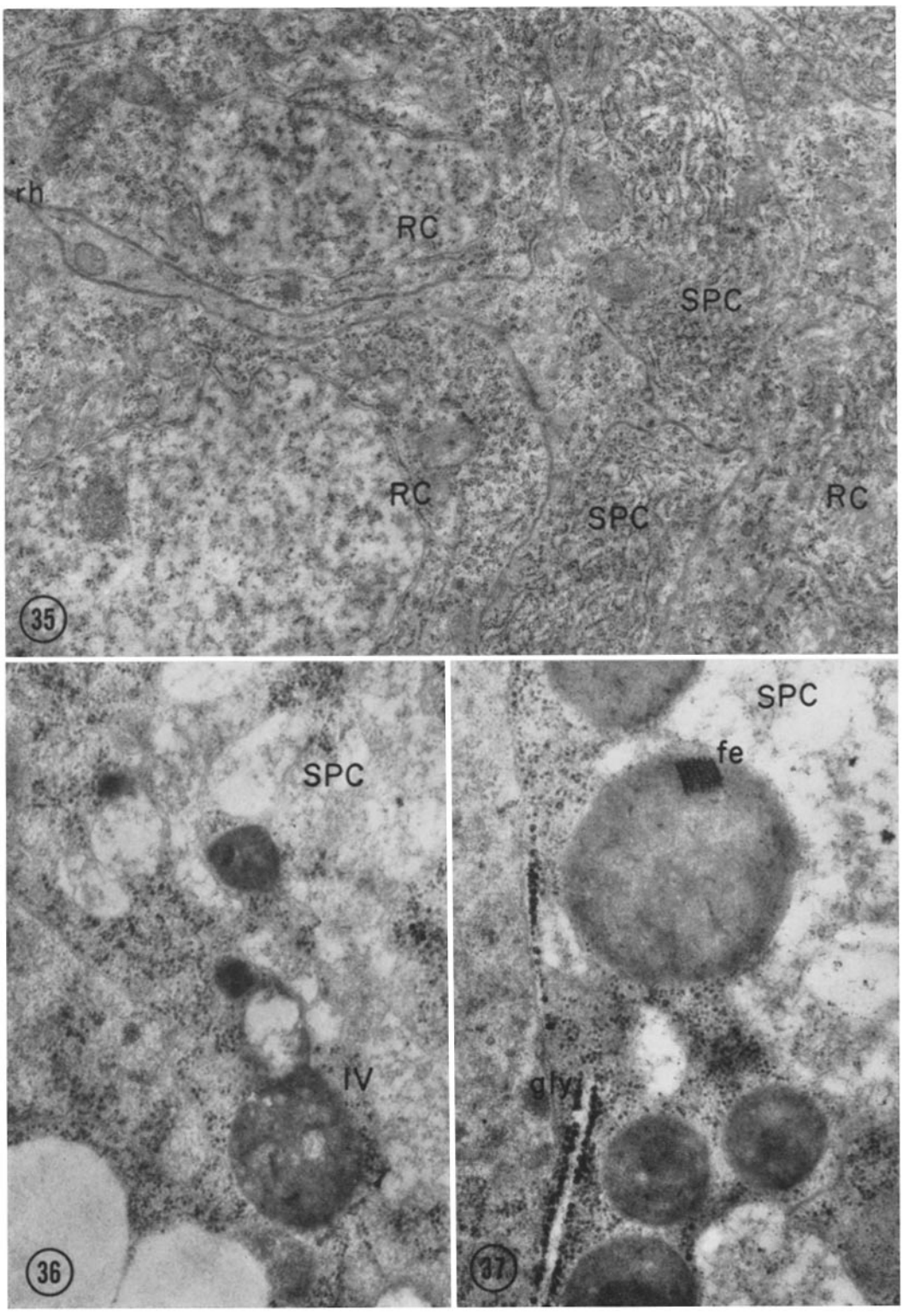
FIGURE 35 White mutant (*w*), 48 hr. Normal cell types are developing in this eye but no pigment can be detected. Note the forming rhabdomeres (*rh*) in the retinular cells (*RC*). Uranyl acetate. × 12,000.

FIGURE 36 White mutant (*w*), 54 hr. Lysosomelike Type IV granules are first appearing in this secondary pigment cell. Uranyl acetate. × 32,000.

FIGURE 37 White mutant (*w*), 60 hr. Type IV granules often include ferritin crystalloids (*fe*). Glycogen particles (*gly*) are arranged in rows in this section. Uranyl acetate. × 32,000.


rh
RC
SPC
RC
SPC
RC
35
SPC
IV
36
SPC
fe
glyj
37

cellular products are thought to be packaged in the Golgi region and released to the surrounding cytoplasm as membrane-bounded droplets. Melanin pigments are generally believed to be synthesized in this way (3, 5, 9, 10, 54, 59, 68), although other mechanisms have been suggested (46, 47). Recently, Kessel and Beams have reported a continuity between Golgi elements and immature forms of a chemically unidentified pigment in the tunicate *Styela* (38). The findings in *Drosophila* presented here suggest no significant deviation from the classical hypothesis in the accumulation of totally different kinds of pigment. It is quite clear that Type I granules arise from vesicles. The origin of Type II granules is not known. They were first detected as elliptical bodies composed of pigmented fibers which increased in density during development. At present, however, there is no evidence that these profiles represent the earliest stages in drosopterin granule formation. The fact that no increase in the size of these granules has been observed would suggest that immature profiles have been undetected.

Second, how is increase in mass and density in pigment granules accomplished? Evidence bearing directly on this question is lacking, but experiments designed specifically to answer it are a next step in the study of this system. However, it is interesting to note that the small Golgi-like vesicles are already electron opaque, so that the formation of mature Type I granules apparently involves only the accumulation of already formed pigment substance. This accumulation could arise through concentration of materials from the cytoplasm or through fusion of smaller vesicles. No evidence for fusion has been obtained to date. By contrast, Type II granules do not increase in size during their demonstrable development, but do undergo a marked change in electron opacity, progressing from fine, non-opaque fibers to an alcohol- and water-soluble condition in the adult. These morphological changes are very likely indicative of alteration in chemical state during development, suggesting synthesis on the granule. A similar mechanism characterizes the development of melanin granules (59). The lamellar pattern of low-density pro-pigment granules (premelanosomes) gradually becomes obscured as the amorphous melanin granule is formed. The process of melanogenesis is associated with a chemical change involving the incorporation of tyrosine.

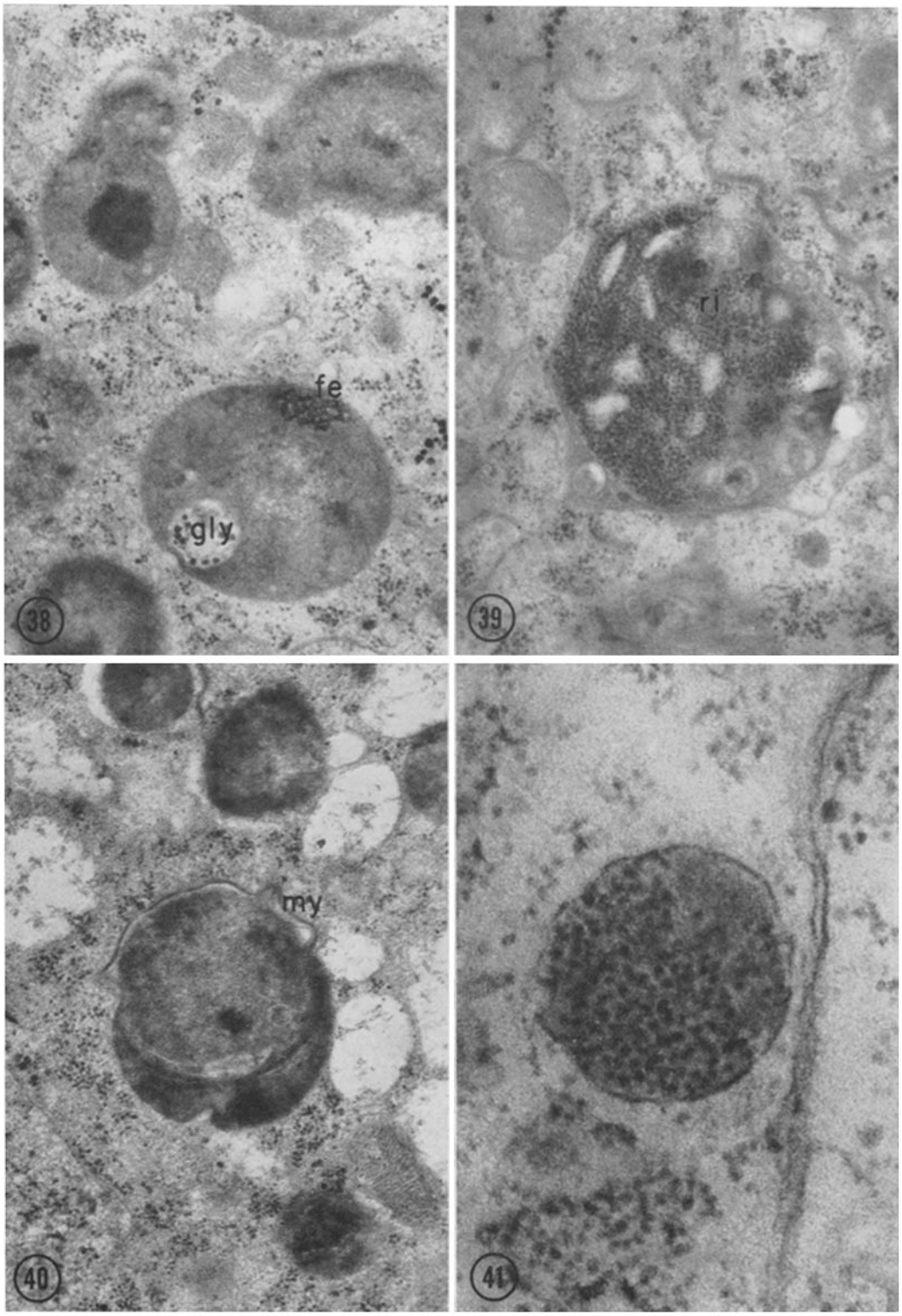
Third, is the term "matrix" biologically meaningful, and if so, what is a realistic definition? It has been stated that ommochromes and pteridines in arthropod eyes are normally bound to core, or matrix, granules. In studies on wild type *Ephestia*, Hanser (35, 36) found that the granules contain protein. Caspari and Richards (14) demonstrated the presence of ribonucleoprotein in *Ephestia* matrix granules by means of ribonuclease digestion and pyronine staining. In wild type and *w Drosophila*, Ziegler-Gunder and Jaenicke analyzed the chemical content of isolated "core granules" and found their protein:RNA ratios to be identical (74). However, they did observe some differences in enzyme content between the two (tyrosinase was higher and succinic acid dehydrogenase lower in *w* flies than in wild type flies), from which they concluded that the function of the "core granules" in the terminal synthesis of eye pigments is associated with an energy-requiring reaction (74). Ziegler, in an electron microscope description of isolated "core granules," reported a number of "subgranules" (maximum diameter 0.4 μ) interspersed with osmiophilic grains, which "seem to reflect the natural site of ommochromes and

FIGURE 38 White mutant (*w*), 60 hr. This Type IV granule contains ferritin (*fe*) and glycogen (*gly*). Uranyl acetate. × 32,000.

FIGURE 39 White mutant (*w*), 72 hr. Another common inclusion of Type IV granules is ribosomes (*ri*). Note also the myelinlike membranes at the periphery of the granule. Uranyl acetate. × 32,000.

FIGURE 40 White mutant (*w*), 96 hr. Myelinlike membranes (*my*) are associated with this Type IV granule. Uranyl acetate. × 32,000.

FIGURE 41 White mutant (*w*), eclosed. Ribosomes in a Type IV granule are shown here at high magnification. Uranyl acetate, lead. × 96,000.


fe
gly
38
rl
39
my
40
41

pterins" (71, 72). She reported that "core granules" of *w* mutants lacked these subunits and grains and contained only an osmiophilic ball, possibly representing the blocked ommochrome and drosopterin precursors. The present observations on intact eyes of wild type flies and *w* mutants do not confirm the presence of such "core granules" per se. The relationship between Ziegler's isolated "core granules" and the autophagic vacuoles reported here in *w* mutants is unclear, and careful biochemical and morphological analysis is required before a meaningful interpretation of their content can be made.

If the pigment granule matrix is a real, genetically independent entity, i.e. related to pigment in a functional, but not synthetic, way, we might expect colorless mutants to have unpigmented, but otherwise normal, matrices. This, clearly, is not the case. The pigment granules of three genetically unrelated, colorless mutants were all highly abnormal and resembled the autophagic vacuoles of degenerating tissue. We must conclude, then, that pigment and its "matrix" granule are interdependent, if not indistinguishable, substances. Presumably, pigments as small molecules are held in place by a macromolecular complex. This complex would properly represent the "matrix." The accumulation of pigment would give the matrix stability, and Type IV granules of colorless mutants would be remnants of precursor molecules, which were incapable of organization into normal macromolecular complexes, i.e. matrices. Evidence from *Ephestia* is also in accord with this interpretation of "matrix." The white-eyed mutant, *wa*, first described by Hanser (35, 36), produces no pigment granules, in contrast to colorless mutants in *Drosophila*. Transplantation experiments have shown that normal pigment manufacturing machinery is present, however, and that pigment precursors are produced. Presumably, the defect in *wa* mutant involves formation of the macromolecular complex, i.e. normal pigment molecules are not packaged in a stable, ordered array; and probably for this reason they are not detected as visible pigment.

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FIGURE 42 Vermilion brown mutant (*v*; *bw*), 72 hr. The ommatidia of this colorless mutant also contain lysosomelike granules. Note the ferritin particles (*fe*). Uranyl acetate. × 32,000.

FIGURE 43 Ins $w^{aL}w^{mJR}$/Y, containing a 3C2 deficiency, eclosed. Typical Type IV granules here contain myelinlike membranes (top) and ribosomes. Uranyl acetate. × 32,000.

FIGURE 44 Dp(w^m)264-58a, 82 hr. The white ommatidium (upper left) contains Type IV granules. The wild type facet has numerous Type II granules. A retinular cell (*RC*) probably belonging to the latter ommatidium lies in the middle of the micrograph. Uranyl acetate. × 12,000.

FIGURE 45 *Ephestia kühniella*, wild type, eclosed. Dense pigment granules occupy the secondary pigment cell, which also contains numerous tiny microtubules (*mt*). A retinular cell (*RC*) with characteristic granular structure lies to the left. Uranyl acetate. × 32,000.

FIGURE 46 *E. kühniella*, *wa*, eclosed. An area comparable to that in Fig. 45 contains no pigment granules whatsoever. Uranyl acetate, lead. × 32,000.

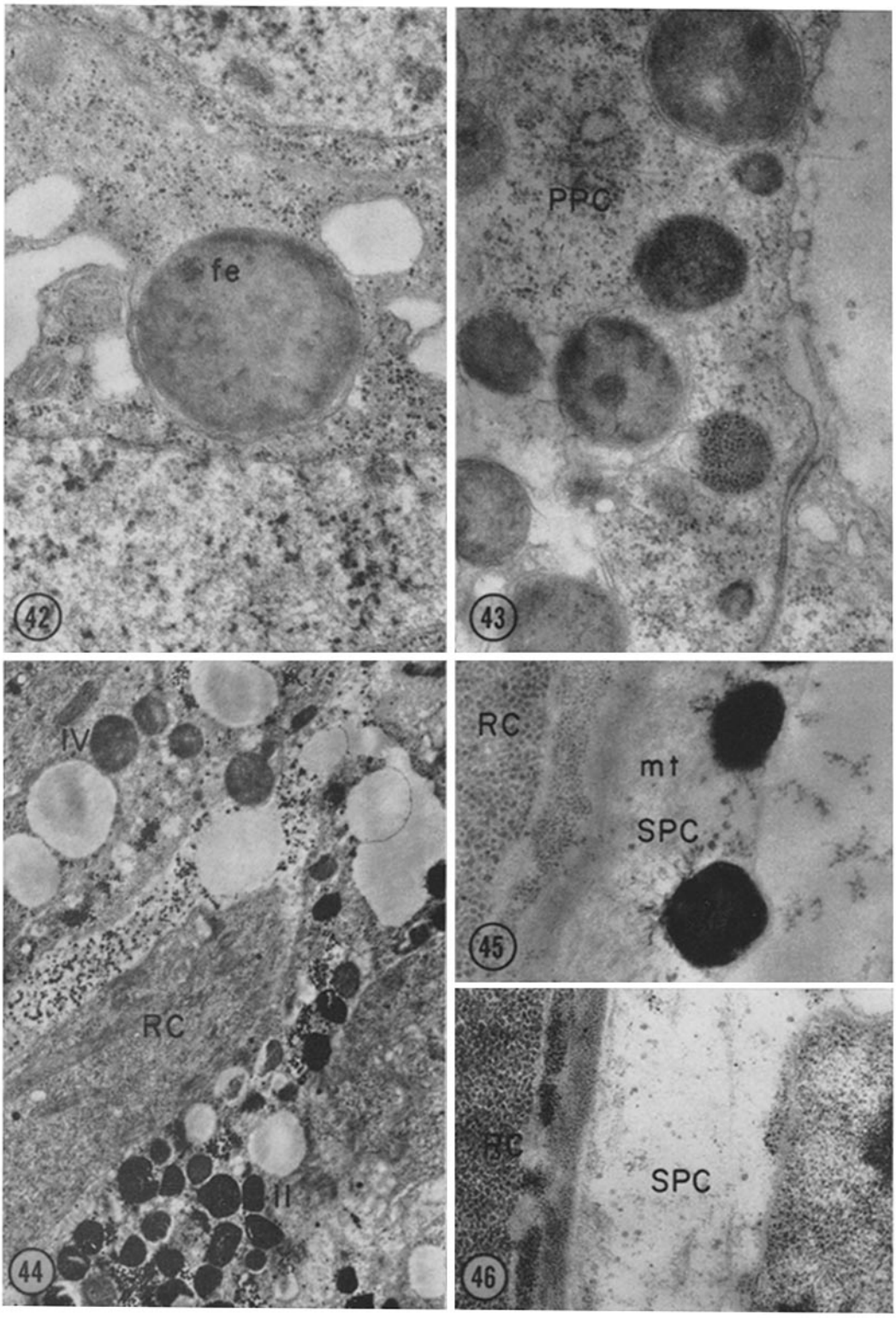
fe
42
PPC
43
IV
RC
II
44
RC
mt
SPC
45
RC
SPC
46

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FIGURE 47 White mutant (*w*), eclosed. This is a control eye fixed in formalin, showing ribosomes in Type IV granules. Uranyl acetate. × 32,000.

FIGURE 48 White mutant (*w*), eclosed. Type IV granules in this eye, treated for 1 hr in ribonuclease, have lost their stainability. Uranyl acetate. × 32,000.

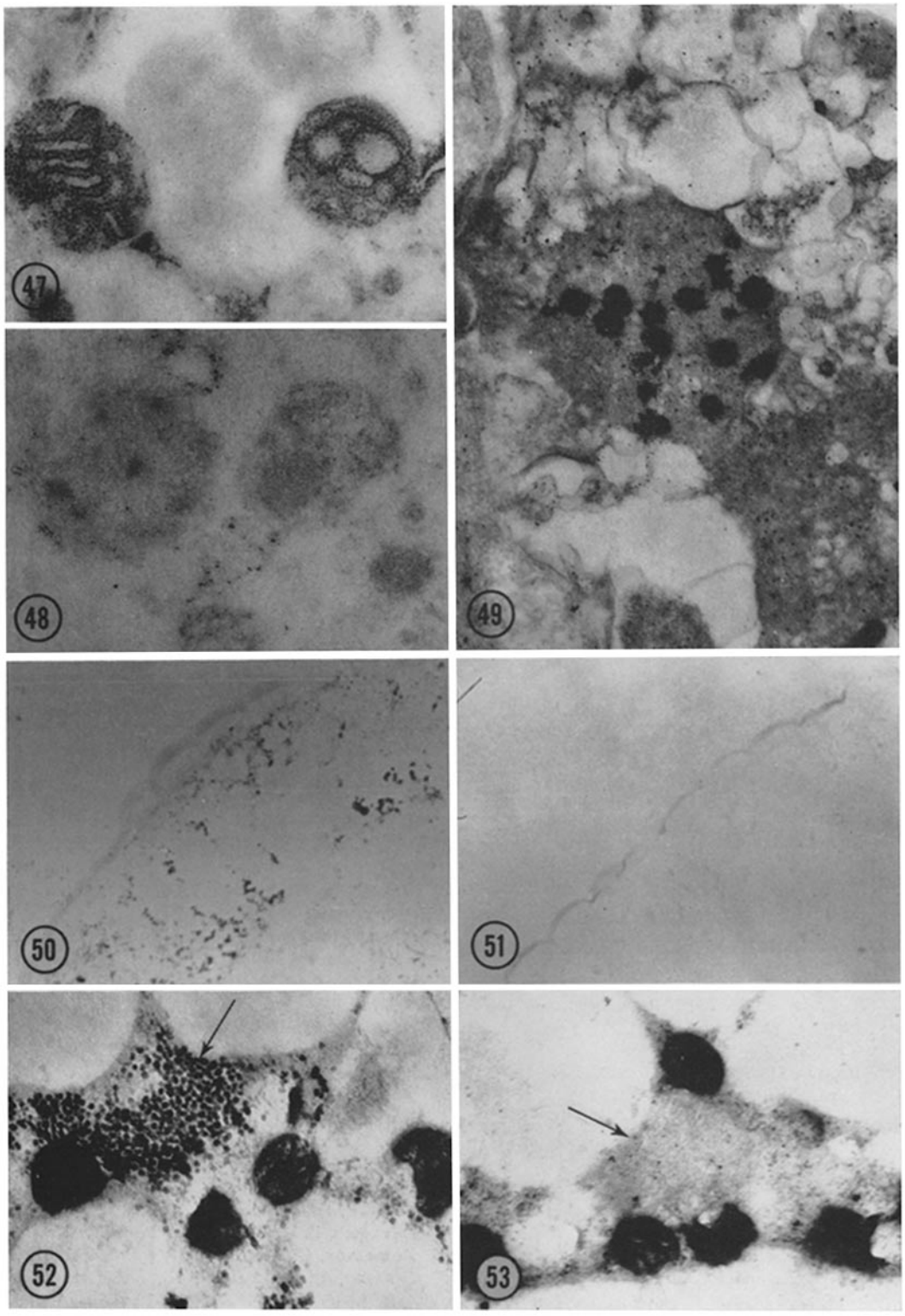
FIGURE 49 White mutant (*w*), eclosed. A fly incubated in Gomori's medium for determination of acid phosphatase shows heavy deposits of lead phosphate in some of its Type IV granules. Unstained. × 32,000.

FIGURE 50 Oregon R, 60 hr. An eye fixed in formol-acetic acid-alcohol and subjected to the PAS reaction showed intense staining of the ommatidia in the areas of the pigment cells. × 1500.

FIGURE 51 Oregon R, 60 hr. When the eye fixed as above was incubated in saliva prior to the PAS reaction, stainability was lost completely. × 1500.

FIGURE 52 Oregon R, 72 hr. Glycogen particles in the secondary pigment cell of the control eye are numerous and intensely staining. Uranyl acetate. × 32,000.

FIGURE 53 Oregon R, 72 hr. After incubation for 3 hr in saliva, stainability of the particles is completely lost. Uranyl acetate. × 32,000.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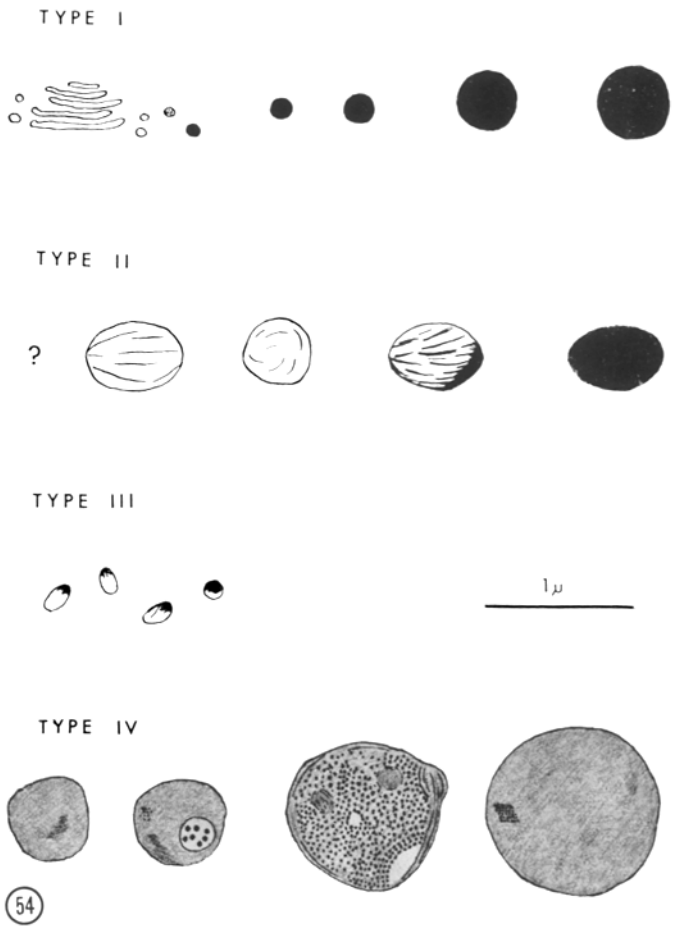

FIGURE 54 Diagram of proposed mechanisms of pigment granule formation (see text).

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