EFFECTS OF THE PHYSICAL ENVIRONMENT ON SOME LIPOPROTEIN LAYER SYSTEMS AND OBSERVATIONS ON THEIR MORPHOGENESIS

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Plates 74 and 75

The sensitivity of lipoprotein layer systems to their physical environment is well known. One may cite for instance the occurrence of "myelin forms," which arise under certain conditions from a number of paracrystalline lipoprotein layer systems. It is a common observation that lipoprotein systems such as mitochondria, chloroplasts, and similar organelles (including the endoplasmic reticulum (ER)) swell rapidly when isolated in hypotonic media (and even in so called physiological media), and in view of the intensive effort currently directed toward biochemical investigation of such systems, it is of importance to determine the effects of this isolation on their fine structure.

During the course of several investigations[1] on the structure (1, 2) and development (3) of chloroplasts and of the endoplasmic reticulum in the plant cell in relation to the isolated microsome fraction (4), a number of observations have been made which seem to throw some light on the structural changes accompanying swelling phenomena. It will be shown that such membrane systems exhibit a generalized swelling response, *viz.*, a strong tendency, understandable in general physical chemical terms, to round up into the form of spherical vesicles. Concerning developmental aspects, the evidence suggests that the endoplasmic reticulum of *Nitella*, which is characterized in the mature cell by a moderately complex arrangement of tubules, cisternae, and other elements, arises by fusion or coalescence of small spherical vesicles (3). Similarly, the lamellae of *Zea* chloroplasts (2) appear to arise in the first instance by

[1] The results described here have been derived from a number of investigations carried out in collaboration with Dr. R. K. Morton, Miss M. Branster, and Mr. E. M. Martin of the Biochemistry Department, University of Melbourne, and Dr. F. V. Mercer and Mr. J. D. McLean of the Botany Department, University of Sydney. The results will be described in detail elsewhere.

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fusion of minute vesicles (150 to 250 A in diameter) giving rise to closed double membranes, which later became elaborated into the characteristic compound structure already described (2). In general, the small vesicles appear to represent a mechanism by which lipides (particularly structural lipides) may be transported within the cell from the sites of synthesis to the regions of lamellar growth.

Materials and Methods

Materials used included mouse liver, blowfly flight muscle, mature cells and young apical cells of *Nitella cristata*, root tips of germinating wheat, and leaves of *Zea mays* grown under various conditions. Buffered osmium tetroxide was used as a fixative in all cases. The details of preparation and fixation are outlined in the appropriate figure legends. Following dehydration in an ethanol series, specimens were embedded in *n*-butyl methacrylate (sometimes with admixtures of up to 10 per cent of the methyl monomer) and polymerized by heating (usually at 45–60°C.). Sections were cut with a rotary cantilever microtome (5) and examined in a modified RCA-EMU1 electron microscope.

RESULTS AND DISCUSSION

Endoplasmic Reticulum

Parenchymal cells of mouse liver when suitably fixed (short fixation in the cold) exhibit a well developed endoplasmic reticulum (8) of the ergastoplasmic[2] type (*i.e.* membranes + dense RNA granules) and numerous mitochondria with the usual double membrane structure (6, 7). While investigating isolated liver cells,[3] it was observed that in some preparations many of the cells showed evidence of considerable swelling and disorganization (Fig. 1). The mitochondria are rounded up into crescent forms, which appear to arise by swelling of material in the perimitochondrial space (*i.e.* the space between the inner and outer components of the double limiting membrane). The endoplasmic reticulum is in process of rounding up into spherical vesicles (in some cells, this process is complete). The dense RNA granules (9) are still associated with the membranes throughout such changes. Swelling has also occurred in the perinuclear space. The outer membrane (studded with granules) is widely separated from the inner membrane in many places, and the picture appears to confirm the continuity of the nuclear membrane with the endoplasmic reticulum described by Watson (10). The disintegration of the endoplasmic reticulum often proceeds further to give a population of spherical vesicles indistinguishable from those seen in preparations of the microsome fraction. Palade (11) by use of 30 per cent sucrose and Novikoff (12) by use of polyvinyl pyrollidone (PVP) have shown that it is possible to obtain microsome fractions in which the con-

[2] This terminology was suggested by Dr. K. R. Porter during the present Conference. Ergastoplasm is thus construed to mean that part of the ER characterized by the presence of RNA granules.

[3] This technique will be described in a forthcoming publication by M. Branster and R. K. Morton.

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stituent particles are fragments of the ER which retain their characteristic *in situ* morphology. It seems clear that the enzymatic and chemical properties of the microsome fraction are mainly those of the ER.

The microsome fractions of *Beta vulgaris* and wheat root tip have been described in terms of enzyme and chemical properties by Martin and Morton (13), and it has been shown (4) by thin-sectioning that they comprise mainly thin-walled spherical vesicles studded with small dense granules (presumably the RNA component). A well developed endoplasmic reticulum has been observed in many of the plant cells investigated (*e.g.* Figs. 3 and 4) and there can be little doubt that as in animal cells the microsome fraction is derived from this system.

Development of Endoplasmic Reticulum in Nitella

As already indicated by Mercer *et al.* (1), the mature *Nitella* cell possesses a fairly complex endoplasmic reticulum. Fig. 4 illustrates some of the profiles observed in such cells. However, the cytoplasm of young apical *Nitella* cells appears to be devoid of cisternae and tubules such as characterize the cytoplasm of mature cells. Rather it contains numerous spherical vesicles. In some regions, one observes structures such as those shown in Figs. 5 and 6. Clusters of the small vesicles appear to be in process of fusing or coalescing to yield closed double membrane structures (flattened sacs or cisternae). In the center of each cluster, fusion is apparently complete. Surrounding this is a zone of incomplete fusion (note the holes in Fig. 5), and further out still, the vesicles remain separate. It seems reasonable to interpret the micrographs as representing morphogenesis of ER elements by a fusion process.

Chloroplast Structure and Swelling

The structure and swelling properties of *Nitella* chloroplasts have been described by Mercer *et al.* (1). They contain numerous closely packed lamellae within a well defined limiting membrane (Fig. 4), the interlamellar spacing being as low as 70 A. We have since found that each dense lamella is actually a double membrane structure, a result which is in better agreement with our morphogenetic findings (3). In our experience, isolation of the chloroplasts has always resulted in some degree of swelling. Initially, swelling results in a simple increase of interlamellar spacing. However, as the tonicity is reduced, progressive vacuolization occurs until a mass of spherical vacuoles or vesicles results (1), often still enclosed by an intact chloroplast limiting membrane.

The fine structure of *Zea* chloroplasts has been described recently (2). When fixed *in situ* the individual chloroplast lamellae exhibit a characteristic compound structure (Fig. 7). They comprise a dense P zone (identified as protein) interposed between two less dense layers (L zones, identified as oriented mixed lipide layers). Within the grana, the lamellae are closely packed (spacing *ca.*

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120 A) to give a myelin-like structure with definite intermediate lines (I zones) appearing midway between adjacent P zones. We have since found that the P zone comprises two dense lines, usually in close apposition, but sometimes, as in Fig. 8, easily resolvable as a pair of membranes. It is of interest that a similar doubling has been found in the corresponding dense zones of nerve myelin sheath (14).

When *Zea* leaves are sliced thinly in fixative (identical in composition with that used to fix the leaf portions from which Figs. 7 and 8 were obtained) and the chloroplasts isolated by gentle shaking, thin sections generally show some degree of disorganization as compared with *in situ* preparations. Usually this takes the form of some degree of swelling, the interlamellar separation being twice or more the normal value. When the chloroplasts are isolated in a medium similar to that used in recent photosynthetic studies (15) prior to fixation, more severe swelling and disorganization are apparent (Fig. 9). The grana have swollen by increasing the interlamellar separation (*ca.* 400 A in Fig. 9). The lamellae of the intergrana regions often (as in Fig. 9) round up to form closed vesicles. The swelling effects are much more severe in "less physiological" media.

The above results clearly demonstrate the "plasticity" of the lipoprotein lamellae of the chloroplast in response to their environment, and suggest strongly that detailed electron microscopic control studies will be essential if biochemical function is to be precisely correlated with fine structure in cytoplasmic organelles such as mitochondria and chloroplasts.

It is of some interest to note that lipoprotein systems are not immune to severe structural alterations after fixation in osmium tetroxide solution. Fig. 2 shows the effect of low pH (produced by 1 per cent phosphotungstic acid) on the structure of insect flight muscle mitochondria. The characteristic closely packed double lamellae normally seen in osmium-fixed material have been transformed into a mass of tubules which may be regarded as micromyelin forms.

Chloroplast Development

The compound lamellae characteristic of *Zea* chloroplasts appear to arise initially by a fusion mechanism similar to that described for the ER of *Nitella*. This development will be described in detail elsewhere (3). Suffice it to say here that during normal development, minute vesicles accumulate to form the prolamellar body[4] which often exhibits an orderly "crystalline" arrangement (Fig. 10) similar to that already described by Leyon (16) and Heitz (17). Double membranes "grow out" from this prolamellar body. The morphogenetic process is more easily followed in etiolated plants during their recovery following illumination. In etiolated leaves, the plastids accumulate large numbers of

[4] We prefer this term to "primary granum" used by several other authors since the structure in question is clearly not a granum in the accepted specialized sense of the word. The term "prolamellar body" also has the advantage that it implies that lamellae arise from it.

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the minute vesicles, but no lamellae are formed. Relatively slight exposure to light is sufficient to initiate formation of double lamellae (Fig. 11) which always appear to be growing out from the prolamellar body. Within 48 hours, the prolamellar body has vanished, indicating its complete conversion into lamellae. The results suggest that the absence of chlorophyll in the etiolated plant may be responsible for the failure of the vesicles to form lamellae since the plastids of several lethal chlorophyll-deficient mutants of *Zea* are similarly devoid of lamellae and contain comparable accumulations of vesicles (3). Our results further suggest that the vesicles are formed in a specialized peripheral zone of the chloroplast immediately underlying the double limiting membrane. Long after the prolamellar body has disappeared, active vesicle formation by fusion appears to be proceeding in this zone (*e.g.* Fig. 12) and lamellae are constantly being added to the existing system. The presence or absence of a prolamellar body and its size appear to depend on the ratio between the rate of formation of vesicles and the rate of their conversion to double lamellae by fusion (apparently limited by the availability of chlorophyll).

The vesicles and the double lamellae formed by fusion are much less dense than the P zones of "mature" compound lamellae (see Fig. 12). If our identification of the P and L zones with protein and lipide respectively (2) is correct, this suggests that the vesicles may be exclusively lipoidal (presumably mainly phospholipides radially oriented so as to present hydrophilic surfaces), and that the protein moiety is laid down later.

General Concepts

The above concept is attractive since the small vesicles would represent stable micelles suitable for the transport of lipides within the cell. Palade (18) has suggested that such vesicles may play a part in fluid transport across cell membranes in capillaries, and it is possible that some such mechanism could account in general for cell permeability and ion transport.

The plasticity of cellular membrane systems (*e.g.* mitochondria, and the cell membrane) has been impressively demonstrated by phase contrast motion pictures. The results described here show another aspect of this property. Indeed, it appears to be just this plasticity which is responsible on the one hand for the marked structural changes accompanying swelling, and on the other, for the proposed fusion mechanism by which lamellar systems may be formed.

The general concept of morphogenesis of lamellar structures by fusion of vesicles seems plausible. It receives some support from the work of De Robertis on the morphogenesis of the retinal rod (19), in which similar vesicles precede the appearance of lamellar structure in the rod outer segment. It seems therefore that fusion may represent a general mechanism for the increase or replacement of membrane area. Vesicles could presumably coalesce as easily with a cell surface membrane as with one another.

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The myelin-like structure of the chloroplast granum is of considerable interest in relation to morphogenesis. In the case of peripheral nerve myelin, the multiple layer structure is formed by invagination of the Schwann cell membrane and a rotation of the Schwann cell relative to the axon (20). As has been shown here, constituent lamellae of the chloroplast granum appear to arise directly by fusion of vesicles. In view of this, it seems not unreasonable to postulate that myelinogenesis may represent a specialized form of a general fusion mechanism, and that vesicles fuse with the Schwann cell membrane prior to this being incorporated into the developing myelin sheath. If this be the case, and the fusion process in the chloroplast is the archetype of a general mechanism, then it is to be expected that various other elaborations of the same fundamental process will be found.

SUMMARY

Lipoprotein membrane systems such as chloroplasts and the endoplasmic reticulum exhibit a generalized swelling response. The initial effect is an increase in interlamellar spacing, but as swelling proceeds, the membranes are transformed into closed thin-walled spherical vesicles.

Available evidence suggests that morphogenesis of the endoplasmic reticulum of *Nitella* and the lamellar system of the *Zea* chloroplasts involves fusion of small spherical vesicles to yield closed double membrane structures, which subsequently undergo further differentiation.

It is suggested that the vesicles comprise a convenient "micellar" form by which lipides may be transported within the cell from the sites of lipide synthesis to regions of lamellar growth. The characteristic formation of vesicles in swelling and the apparent fusion of vesicles in morphogenesis appear to represent two aspects of a fundamental plasticity of lipoprotein layer systems.

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EXPLANATION OF PLATES

PLATE 74

FIG. 1. Isolated liver cell showing considerable disorganization of the endoplasmic reticulum and mitochondria (*m*). Fixation for 2 minutes at 0°C. with 1 per cent OsO_4 in Locke's medium (pH 7.4). The ER is in process of rounding up to form spherical vesicles. Note RNA granules associated with membranes, also the apparent continuity of the outer nuclear membrane with the membranes of the ER. The mitochondria (*m*) are of crescent form, with swelling of the perimitochondrial space between the inner and outer limiting membranes. × 12,500.

FIG. 2. Transverse section of blowfly flight muscle fixed in buffered OsO_4 (pH 7.4), then stained heavily with PTA. Both the myofibrils (*mf*) and mitochondria are severely disorganized. The regularly packed internal double membranes seen in osmium-fixed material have become disorganized into a mass of tubules (micromyelin forms). × 30,000.

FIG. 3. Part of the cytoplasm of a cell from wheat root tip fixed for 10 minutes, at 0°C. in 1 per cent OsO_4 in Locke's medium, one-third saturated with dextrin (pH 7.4), showing the numerous densely packed profiles of the ER and the Golgi zones (*gz*). × 30,000.

FIG. 4. *Nitella cristata* fixed as in (1) showing part of a chloroplast with closely packed lamellae (*cl*), the chloroplast limiting membrane (arrow), and various profiles of the ER in the cytoplasmic layer between the chloroplast membrane and the tonoplast (*t*). × 75,000.

FIGS. 5 and 6. Young apical cell of *Nitella cristata* fixed as in (1), showing two clusters of vesicles apparently in process of fusion and sectioned in different orientations with respect to the plane of the section. Fig. (5) cut essentially in plane of lamellae; note the holes in the subperipheral zone of incomplete fusion. Fig. (6) cut essentially normal to lamellar plane showing the double membranes, apparently formed by fusion of the vesicles. × 46,000 and × 60,000 respectively.

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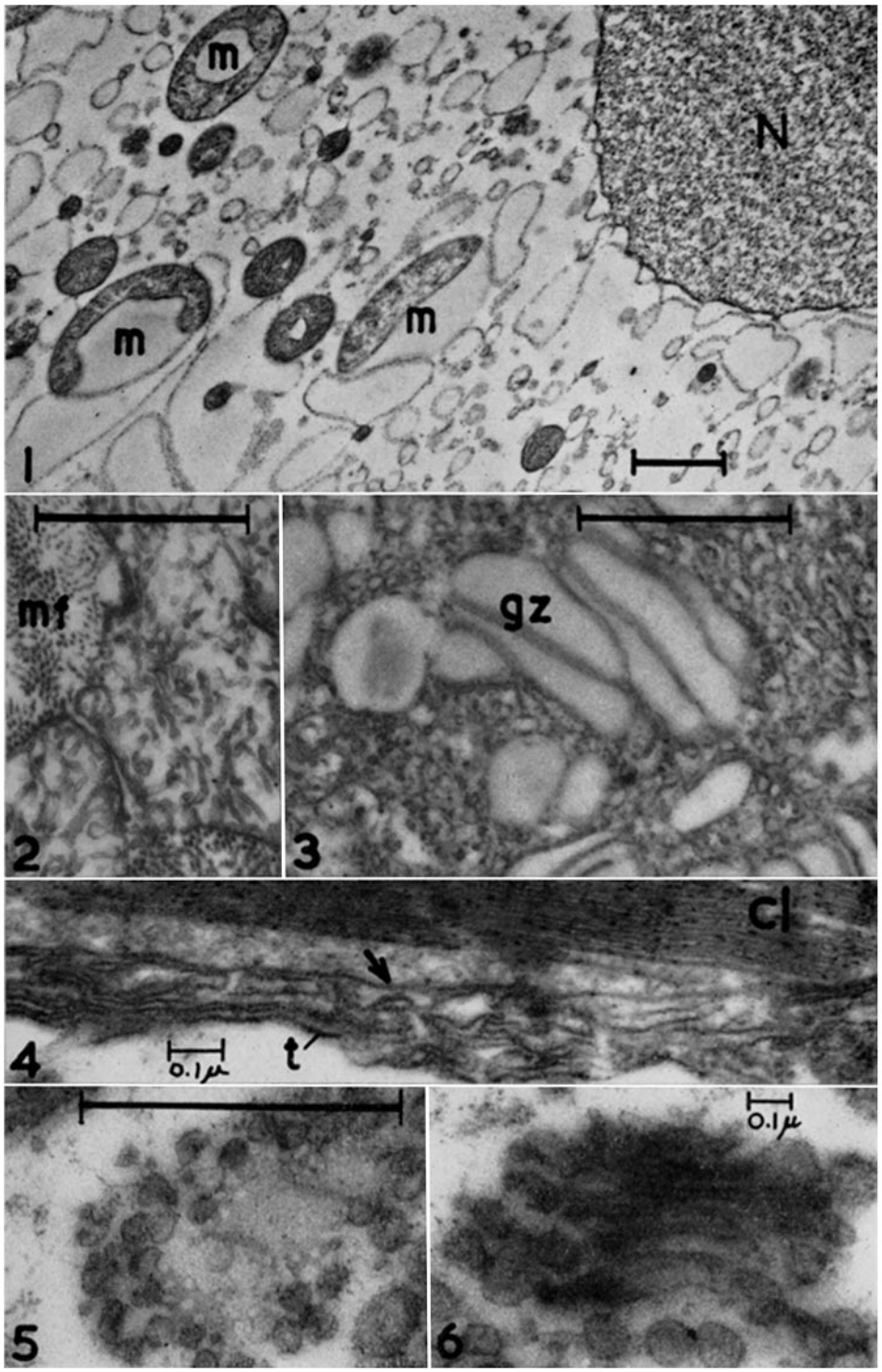

(Hodge: Lipoprotein layer systems)

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PLATE 75

FIG. 7. Part of a chloroplast of 3 week old *Zea mays* leaf fixed as in (2) showing the compound layer structure of the individual lamellae (dense P zones interposed between less dense L zones) and the close packing of the compound lamellae in the grana (*g*). Note the thin dense intermediate lines (I zones) situated midway between adjacent P zones. × 155,000.

FIG. 8. Granum of a yellow-green non-lethal mutant of *Zea mays* (fixation as in Fig. 7), showing that the individual P zones are often resolvable into two thin dense lines (arrow) separated by a clear space. × 205,000.

FIG. 9. Chloroplast of *Zea mays* isolated in 0.5 M glucose in phosphate buffer prior to fixation in 1 per cent OsO_4 in 0.2 M NaCl. Note the swollen grana (*g*) (interlamellar spacing *ca.* 400 A) and the rounding-up of the intergrana lamellae into spherical vesicles (compare with Fig. 7). × 45,000.

FIG. 10. Young chloroplast of *Zea mays* from a seedling, showing the "crystalline" prolamellar body and radiating double lamellae within a well defined limiting membrane. Fixation as in (2). × 42,500.

FIG. 11. Plastid from an etiolated leaf of *Zea mays* after several hours' exposure to daylight, fixed as in (2), showing accumulation of minute vesicles in the prolamellar bodies (*PB*) and the formation of double membranes, apparently by fusion of these vesicles. × 57,500. Inset to show vesicular structure of the prolamellar body. × 80,000.

FIG. 12. Plastid from etiolated leaf of *Zea mays* 48 hours after exposure to daylight, showing the well developed lamellar system of dense grana (*g*) and intergrana lamellae, and the specialized peripheral zone containing vesicles (apparently in process of fusing together) immediately beneath the double limiting membrane (arrow) of the chloroplast. × 110,000.

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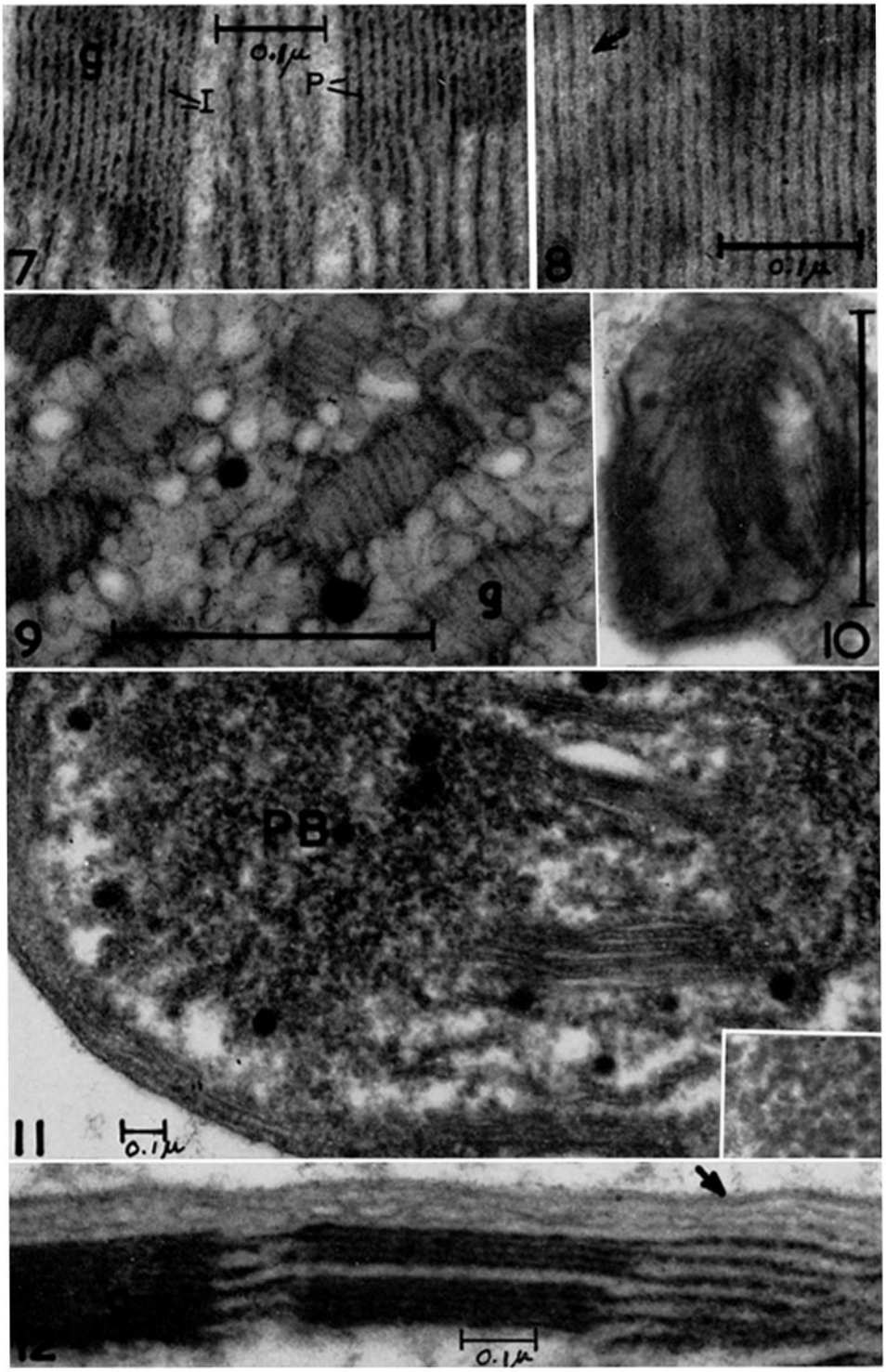

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