INTRAMITOCHONDRIAL YOLK-CRYSTALS OF FROG OOCYTES

I. Formation of Yolk-Crystal Inclusions by Mitochondria during Bullfrog Oogenesis

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ABSTRACT

Electron microscope examination of thin sections of bullfrog (*Rana catesbeiana*) ovarian oocytes has shown the presence of mitochondria containing yolk-crystal inclusions in oocytes of all sizes, from 160 to 1500 μ mean diameter. The hexagonally shaped yolk-crystals have major periodicities of 73.8 $\pm$ 10.7 A (n = 100). Several forms of modified mitochondria, observed in the smaller oocytes, may be arranged into a series of structurally intermediate forms between standard oocyte mitochondria and the typical mitochondria with yolk-crystal inclusions. The observation of such intermediate forms is consistent with proposals that the yolk-crystal inclusions arise within a limited portion of the oocyte chondriome by a complex process of mitochondrial differentiation.

INTRODUCTION

Since their discovery in *Rana esculenta* embryos by Lanzavecchia (10, 13), and in *Rana pipiens* oocytes by Ward (26, 27), intramitochondrial yolk-crystals have been observed in many other amphibian oocytes and embryos. They have been reported in the frogs *Rana esculenta* (4, 11, 12, 31), *R. pipiens* (7, 9, 29, 30), *R. catesbeiana* (14, 22), *R. japonica* (23), *R. nigromaculata* (9, 18, 19), *R. ornativentris* (24), *R. clamitans* (W. H. Massover, unpublished observations), and possibly also in a urodele, *Triturus helveticus* (17). These hexagonally shaped crystals have been widely assumed to be a variant form of the crystals found (8) within each of the proteid yolk organelles, the yolk platelets. Previous investigators have proposed both a morphogenetic transformation of endomitochondrial yolk-crystals into the single membrane-limited yolk platelets (11, 29, 30), and a conversely progressing biogenesis of mitochondria from the yolk platelets via intramitochondrial yolk-crystal intermediate stages (10, 11, 13).

Direct evidence in support or denial of either of these hypotheses has remained scanty. The observation of intermediate stages in the formation of intramitochondrial yolk crystals in developing oocytes of the mollusc *Planorbis* (3) would indicate that structurally intermediate forms should also be present in frog oocytes if similar mitochondrial activities were indeed occurring; such observations do not appear to have been previously made. The several types of altered mitochondria observed during this study may represent such structurally intermediate forms, and further increase our knowledge of the morphological heterogeneity of the chondriome in these cells.

MATERIALS AND METHODS

Small clumps of ovarian oocytes obtained from post-metamorphic bullfrogs (*Rana catesbeiana*) of a wide range of body sizes throughout the year (The Lemberger Company, Oshkosh, Wis.) were fixed by immersion in ice-cold 1% OsO_4 buffered with 0.1 M potassium phosphate at pH 7.4–7.5 for 1–1½ hr. After dehydration with graded ethanols and embedment in Epon 812, the prepared thin sections were doubly stained with aqueous uranyl acetate and lead citrate (15, 25), and were examined with a Siemens Elmiskop I electron microscope.

Oocytes from 163 to 1530 μ in mean diameter were examined; oocyte "mean diameter" was calculated as the average of the measured largest and least diameters of the embedded oocyte before it was trimmed and sectioned. Electron microscope magnification was routinely determined by coordinately photographing a cross-hatched carbon grating replica of 2160 periods/mm (Ernest F. Fullam, Inc., Schenectady, N. Y.).

To distinguish the mitochondrial inclusions discussed in this paper from the crystalline inclusions found in mitochondria from other tissues and from other organisms, the term intramitochondrial "yolk-crystals" will be used (note the hyphen); this usage also reflects the previous designations by other investigators who have considered these crystals to be identical to the crystals found in the yolk platelets. It is important for readers to recognize that "yolk-crystal" is being used in the present study as a descriptive morphological term, and is to be distinguished from the chemically defined "crystalline yolk" or "yolk crystals" of the yolk platelets.

RESULTS

Bullfrog oocytes contain two structural classes of mitochondria, those with crystalline inclusions and those without them. Smaller oocytes (those less than 500 μ in mean diameter) have very small numbers of mitochondria which contain yolk-crystal inclusions; these mitochondria are scattered throughout the entire cytoplasm, but have a slight peripheral concentration (Fig. 1). Larger oocytes harbor moderate numbers of yolk-crystal–containing mitochondria among their peripheral layer of mitochondria (Fig. 2); in these oocytes, mitochondria with the crystal inclusions are present only infrequently within the internal mass of yolk platelets. While mitochondria containing the yolk-crystal inclusions are present in all oocytes irrespective of oocyte size (e.g. the oocyte 250 μ in diameter in Fig. 1, and that 1150 μ in diameter in Fig. 2), the single membrane–bounded yolk platelets have been observed only in bullfrog oocytes of mean diameter greater than about 500 μ (e.g. Fig. 2).

The "Standard Mitochondrion" of Bullfrog Oocytes

The mitochondria of bullfrog oocytes have a diameter of about 0.3 μ, and have a slightly curving or sinuously elongated shape (Figs. 3 and 4); many fine-structural features of these mitochondria are qualitatively similar to those of the mitochondria of most other eucaryotic cells (see reference 5). The inner mitochondrial membrane is infolded to form a moderate number of cristae extending perpendicularly to the longitudinal axis of the mitochondrion (Fig. 3); cristae usually appear to extend at least three-quarters of the distance across the mitochondrial diameter (Fig. 3). The cristae generally have a tubular profile, although flattened saccular forms may also be observed (Figs. 3 and 4). As in other mitochondria, the inner membrane system encloses the matrix compartment, whose contents are a homogeneous or finely granular material within which a small number of variably sized, dense granules averaging approximately 350 A in diameter are often visible.

Modified Mitochondria

In oocytes whose mean diameter was less than 400 μ, many organelles were observed which have structures different from those of either the "standard mitochondria" or the "mitochondria with yolk-crystal inclusions," but which also have some features of one or the other of these two structurally distinct classes of mitochondria; in all cases a portion of these formed elements retains the usual and characteristic mitochondrial fine-structural features, thus establishing the identity of these organelles as modified mitochondria. The description of these modified mitochondria is presented in an arbitrarily ordered series below; consideration of the possible relations of these forms to each other will be given in the discussion section.

MODIFICATION 1. LOCALIZED INCREASE IN THE MATRICAL COMPARTMENT WITH CONCOMITANT SHORTENING OF ADJACENT CRISTAE: A localized enlargement of the matrix in some mitochondria (Figs. 5 and 6) results in a two- to threefold increase of the mitochondrial diameter at the involved region. Concomitantly,

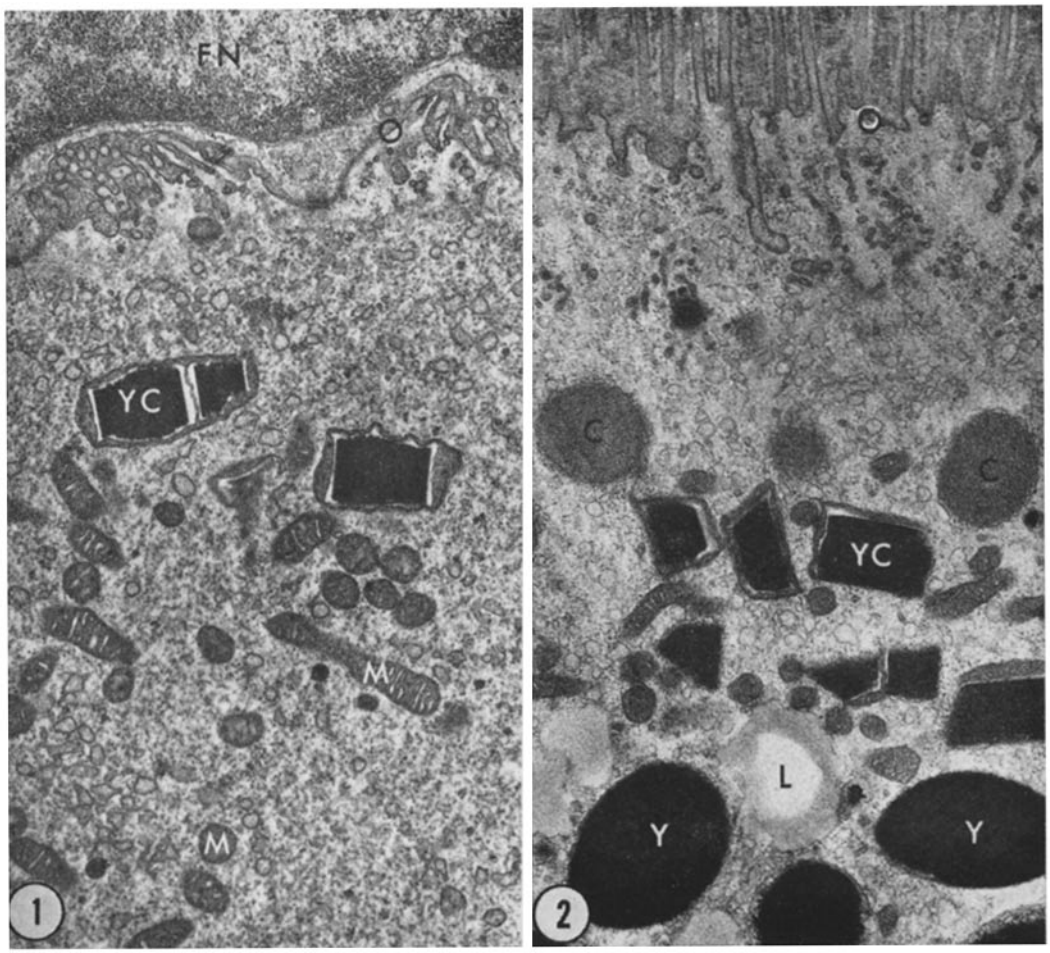

FIGURE 1 Peripheral zone of a 250 μ oocyte. Intramitochondrial yolk-crystal (*YC*); mitochondrion (*M*); oolemma (*O*); follicle cell nucleus (*FN*). ×19,000.

FIGURE 2 Peripheral zone of a 1150 μ oocyte. Intramitochondrial yolk-crystal (*YC*); yolk platelet (*Y*); lipid droplet (*L*); cortical granule (*C*); oolemma (*O*). ×12,500.

the cristae within this region assume a very diminished size.

MODIFICATION 2. PRESENCE OF A SPHERICAL MASS WITHIN A REGION OF ENLARGED MATRIX: A single spherical body up to 0.4 μ in diameter and composed of an entangled meshwork of very fine (about 20–30 A in diameter) fibrillogranular material is present within the locally enlarged matrix in some altered mitochondria (Fig. 7). This matrical mass has never been observed in mitochondria which do not also appear to have an area of enlarged matrix and shortened cristae.

MODIFICATION 3 DILATATION OF ONE OR MORE CRISTAE WITHIN A REGION OF EXPANDED MATRIX: A marked dilatation of one or more cristae is present in some altered mitochondria (Fig. 8) within a region of expanded matrix and shortened cristae; the cisternæ of the dilated cristae appear to be without any contents of appreciable electron opacity. The three membranes (i.e. cristal membrane, and inner and outer mitochondrial membranes) situated between the intracristal space and the surrounding ooplasm preclude the possibility that such formations are invaginations of cytoplasm into these mitochondria. Dilatation of cristae has never been observed in mitochondria appearing to

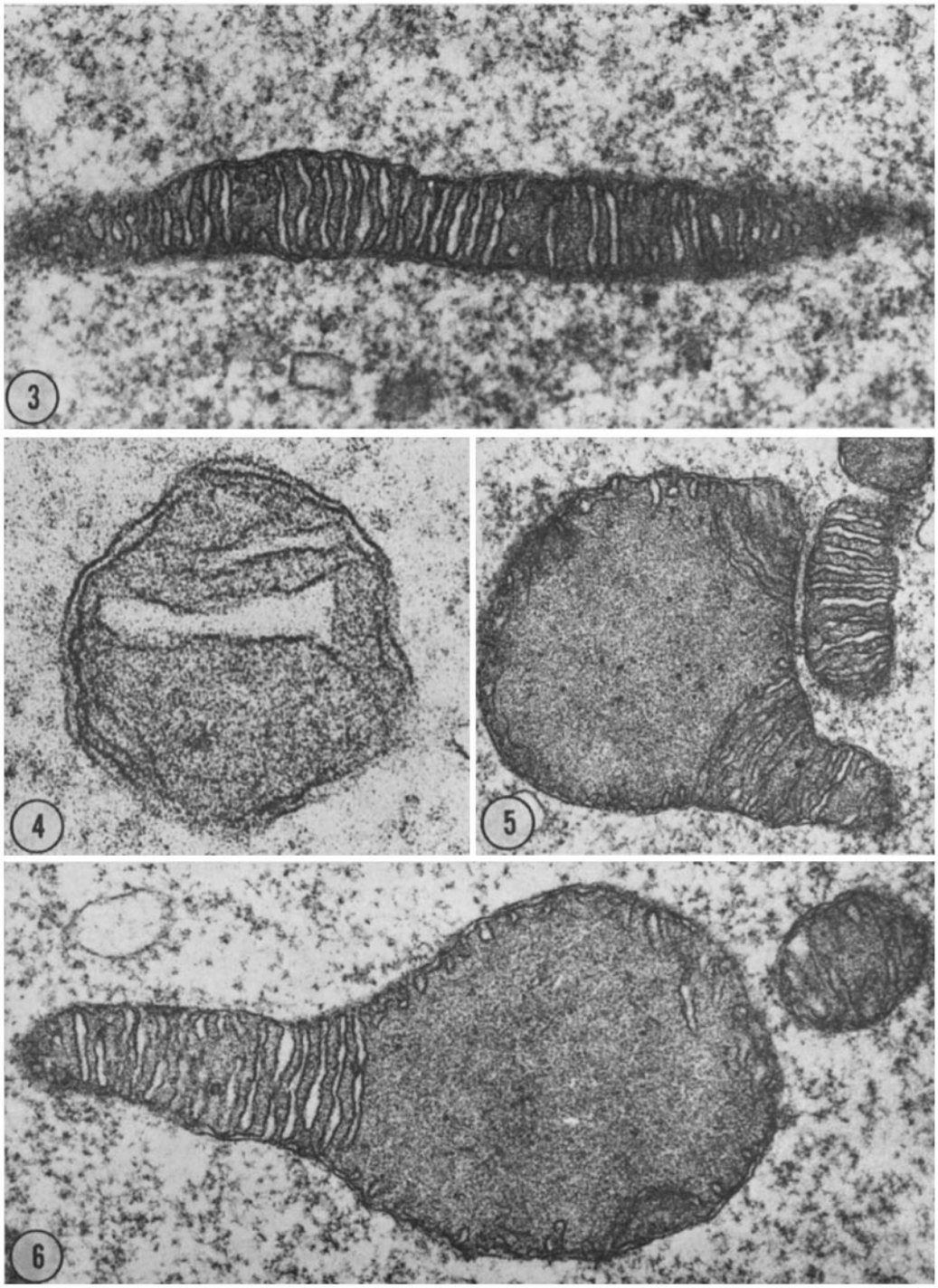

FIGURE 3 Standard bullfrog oocyte mitochondrion in longitudinal section. ×38,000.

FIGURE 4 Standard bullfrog oocyte mitochondrion in cross-section. Note the two types of cristal profiles. ×94,000.

FIGURE 5 Modified mitochondrion of type 1. Enlargement of matrix compartment is localized to an intermediate segment of this mitochondrion. Compare with adjacent standard mitochondrion. ×38,000.

FIGURE 6 Modified mitochondrion of type 1. Cristae shortened in localized region of matrix enlargement. Standard mitochondrion is adjacent. ×37,500.

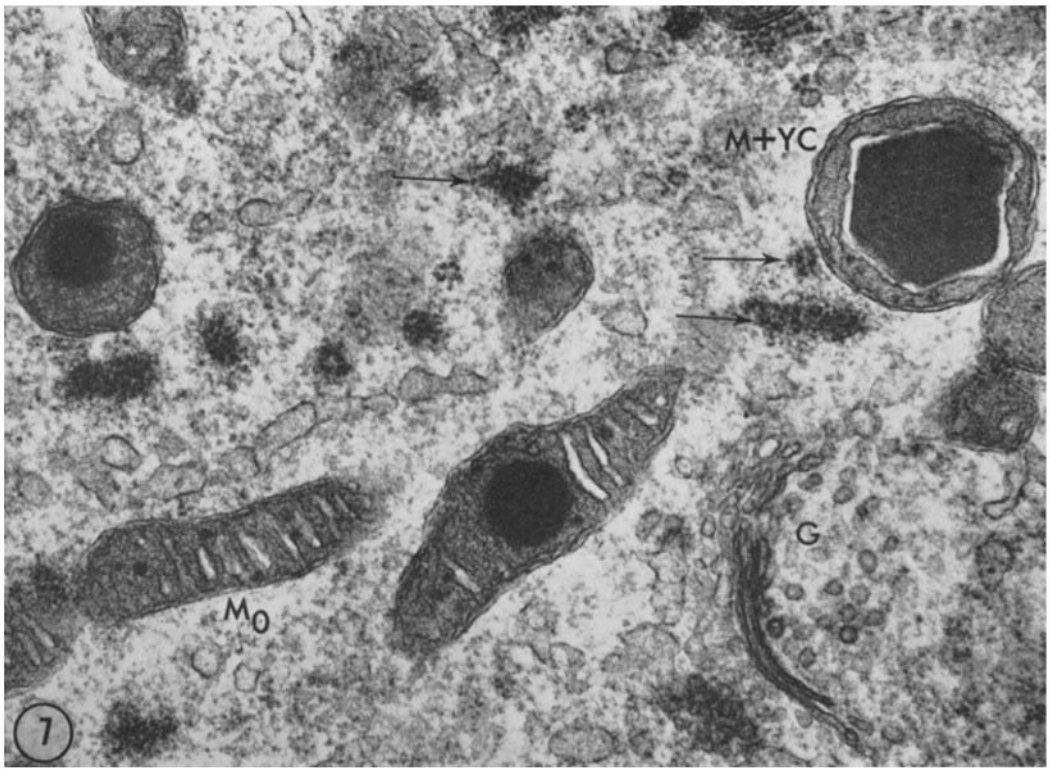

FIGURE 7 Modified mitochondria of type 2. Mitochondria with spherical matrical inclusions are in very close proximity to standard mitochondria (M_o) and mitochondria having yolk-crystal inclusions ($M + YC$). Polygranular bodies (arrows) may be an unusual form of cytoplasmic cylinders (14). Golgi complex (G). ×38,500.

have the usual 0.3 μ diameter throughout their length.

MODIFICATION 4. PRESENCE OF A DENSE FIBRILLOGRANULAR INTRACRISTAL BODY: A single spherical body of up to 0.3 μ diameter is present within a dilated crista in some altered mitochondria (Fig. 9). This body is composed of a relatively dense fibrillogranular material, the individual elements of which (about 30 A in diameter) bear no apparent ordered relation to each other. Such intracristal bodies have never been observed within undilated cristae or within mitochondria which do not also have an increased diameter due to a localized expansion in their matrix compartment.

MODIFICATION 5. PRESENCE OF A CRYSTALLINE INCLUSION WITHIN AN EXPANDED CRISTA: Within the dilated cristae of some altered mitochondria, a hexagonally shaped crystal is present (Figs. 10 and 11); the major periodicities of these crystals are identical with those that are characteristic of yolk-crystals (see next paragraph). Neither the fibrillogranular bodies described under modification 4, nor any other component has been observed within the same dilated cristal cistern when a crystal inclusion is present. The mitochondria containing the structural modifications characterizing this form are often of immense size (Fig. 10). In any given section, such altered mitochondria may also appear to harbor cristae containing a fibrillogranular body, and also cristae which are dilated but without any apparent intracristal inclusion (Fig. 10); similarly, the matrical mass described under modification 2 is observed in some enlarged mitochondria which contain crystalline inclusions (Fig. 10, inset). That the yolk-crystals are inside expanded cristae and not inside large vesicles situated within the matrix is made evident by observations (Fig. 11) of a continuity of the

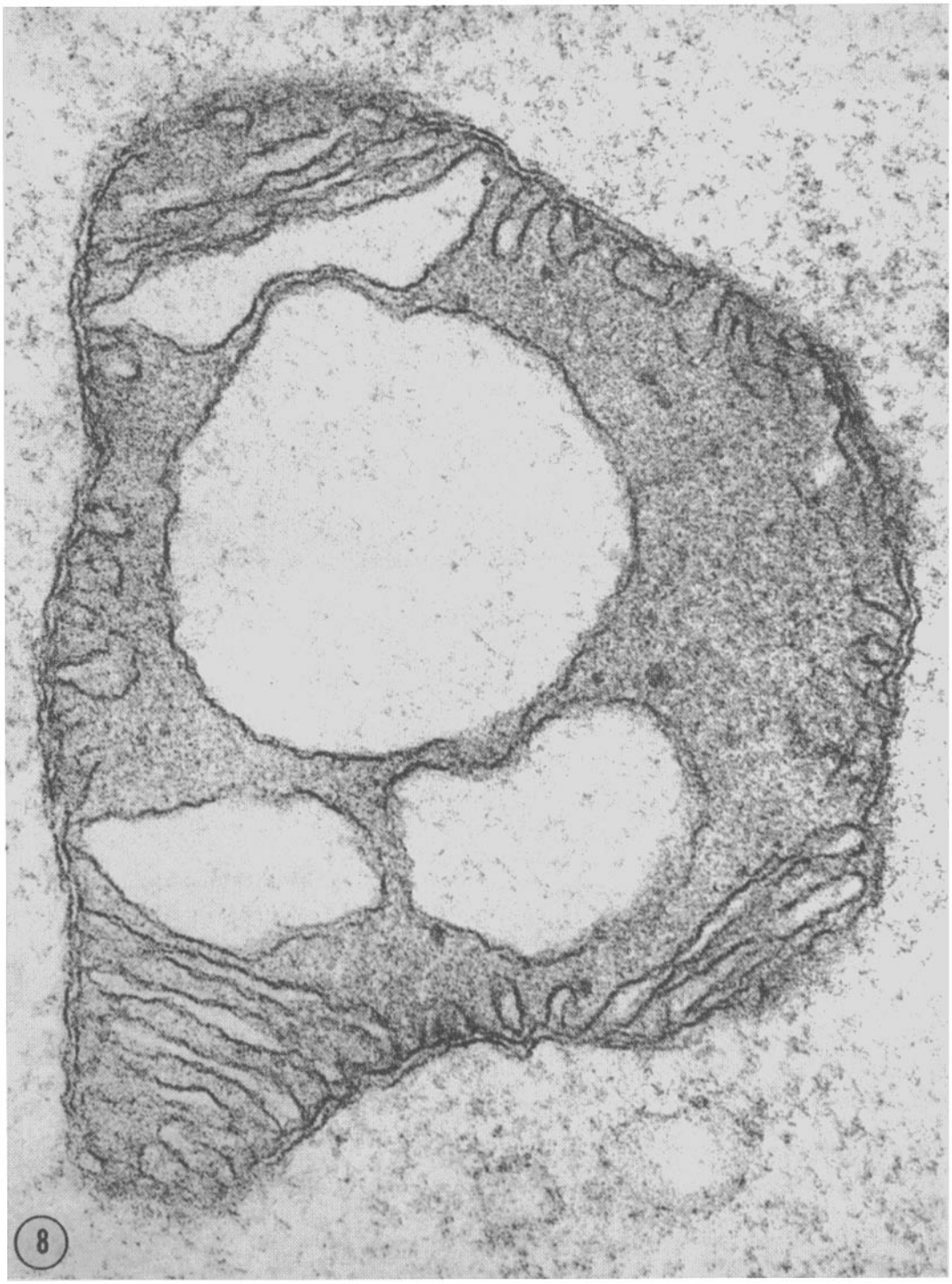

FIGURE 8 Modified mitochondrion of type 3. Several dilated cristae are present in this enlarged mitochondrion. ×85,500.

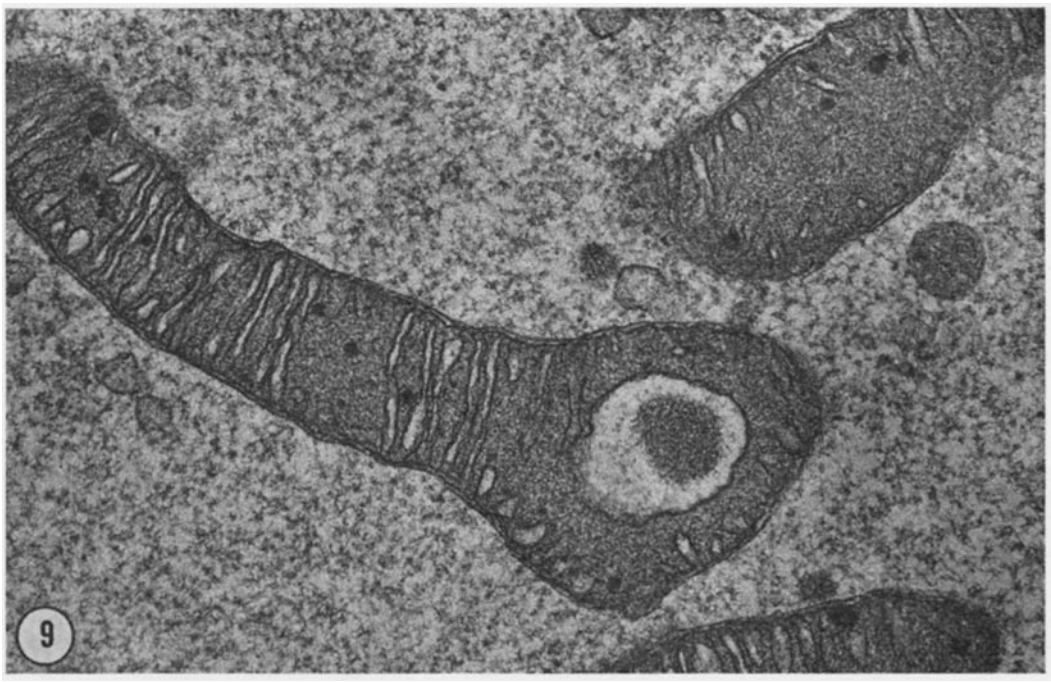

FIGURE 9 Modified mitochondrion of type 4. Spherical inclusion within a dilated crista. ×62,000.

membrane enclosing a crystal with the inner mitochondrial membrane.

THE "TYPICAL MITOCHONDRION HAVING YOLK-CRYSTAL INCLUSIONS": Intramitochondrial yolk-crystals in bullfrog oocytes have major periodicities of 73.8 ± 10.7 A (n = 100) (Fig. 12). Most observations of mitochondria which contain such yolk-crystals show these altered mitochondria to appear to be without any of the features of the mitochondrial modifications just described, beyond a localized increase in mitochondrial and cristal diameter which accommodates the size of the inclusions (Fig. 12; see also Figs. 1, 2, and 7). Semiquantitative estimates of the maximum proportion of the chondriome which has yolk-crystal inclusions do not exceed a figure of 20% in any size class of oocyte.

Other Morphological Observations

All forms of standard and altered mitochondria may be observed side-by-side (Figs. 1, 2, 5–7, 9, 11, and 12). An intermitochondrial body (Fig. 11) composed of two layers or segments of electron-opaque material separated by an electron-lucent region is occasionally present within the smaller oocytes. One member of the associated pair of mitochondria, either of standard or modified types in many combinations, may be indented by its neighbor in the sharply delimited region where both adjoin the intermitochondrial body (Fig. 11).

DISCUSSION

Genesis of the Intramitochondrial Yolk-Crystal Inclusions

Observations in smaller bullfrog oocytes of a number of forms of modified mitochondria with varied fine-structural characteristics necessitate the recognition of a complex, morphologically defined heterogeneity of frog oocyte mitochondria. Previously, the frog oocyte chondriome was considered to be divided into only two structurally distinct subpopulations, those mitochondria with, and those without, the yolk-crystal inclusions. Since some of the less numerous forms share one or another of the structural features characterizing the two main types, the possibility that these forms may represent intermediate stages of a dynamic reorganization of one type into the other must be considered.

Lanzavecchia originated proposals (10, 13)

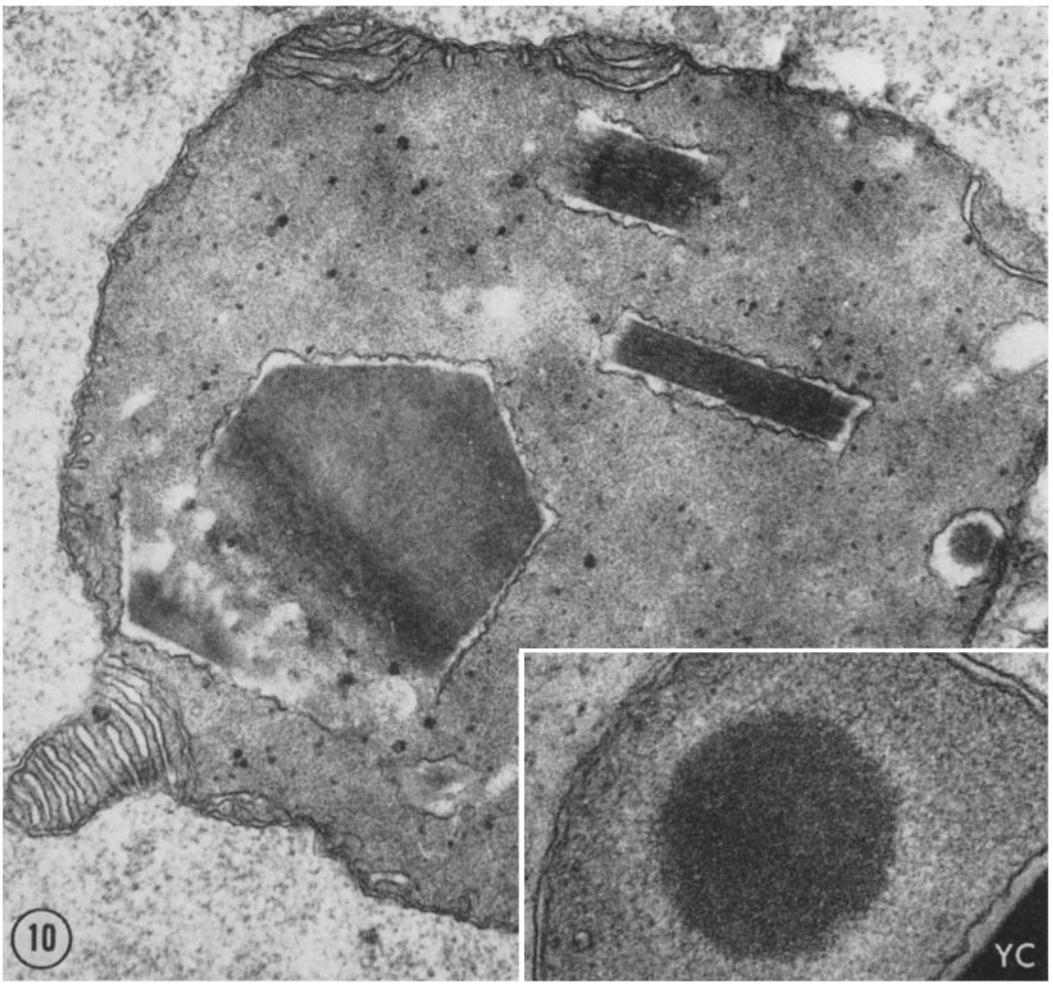

FIGURE 10 Modified mitochondrion of type 5. In addition to the crystalline inclusions characterizing this form of modified mitochondria, this very enlarged mitochondrion also appears to have cristae with simple dilatation, and some with dilatation plus an intracristal body. Note prominence of numerous matrical granules. *Inset*: spherical matrical mass within another modified mitochondrion of type 5; yolk-crystal (*YC*). ×33,500; *Inset:* ×69,000.

that the intramitochondrial crystals are an intermediate stage in the transformation of yolk platelet crystals into mitochondria; with reference to bullfrog oocytes smaller than 500 μ in mean diameter, this type of proposal (6, 11, 34) would have to involve yolk platelets which were still present from the embryonic period of possibly years before. Some indirect evidence against the validity of these hypothetical suggestions has already been established. First, contrary to the postulated derivation of mitochondria from yolk platelets, the vast majority of biochemical and morphological investigations on mitochondrial biogenesis in diverse cell types has concluded that mitochondria arise from mitochondrial progenitors (evidence reviewed in reference 21). Secondly, such proposals would require that processes of yolk platelet formation and yolk platelet utilization must take place simultaneously in the same intracellular environment of the growing oocytes; however, none of the published fine-structural studies of frog oogenesis (e.g. 2, 22–24, 29, 31) has reported any observations of multiple, concentric, laminated membranes surrounding oocyte yolk platelets similar to those formations seen

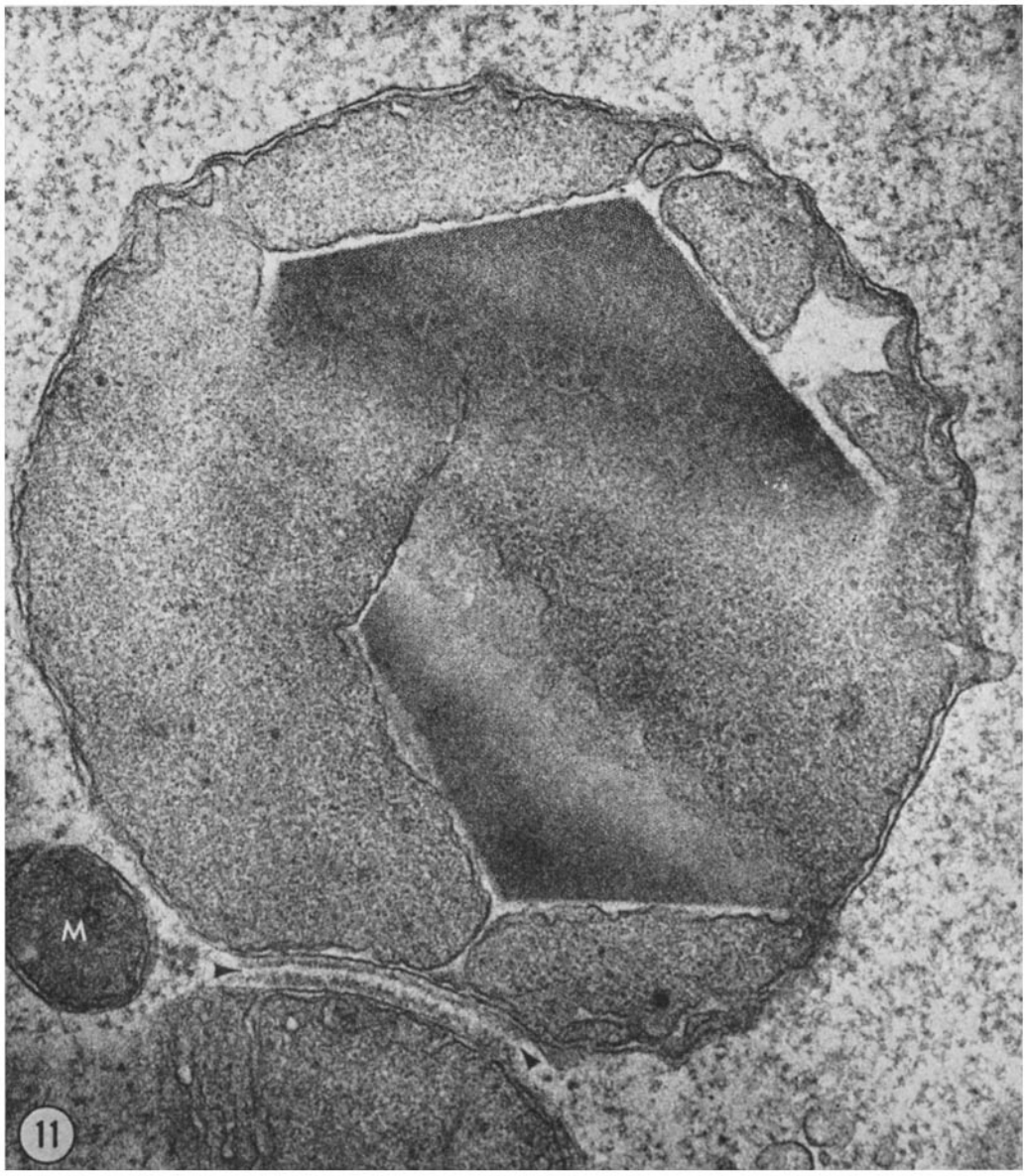

FIGURE 11 Modified mitochondrion of type 5. Two hexagonal yolk-crystal inclusions are present in this enlarged mitochondrion. Note the continuity between the membranes surrounding the crystals and the inner mitochondrial membrane. Intermitochondrial body is shown between arrowheads; standard mitochondrion (*M*) is adjacent. ×51,000.

about embryonic yolk platelets during their yolk utilization (9, 10, 13, 19).

On the other hand, Ward has proposed (26–29) that the crystals within mitochondria are intermediate stages during a production of yolk platelets by normal mitochondria, as one part of a dual mechanism of yolk platelet formation in frog oocytes (30; see also 22–24, 35). If the intracristal inclusions do indeed develop in mitochondria previously containing no inclusions, then structural intermediates between the progenitor and fully matured forms, analogous to

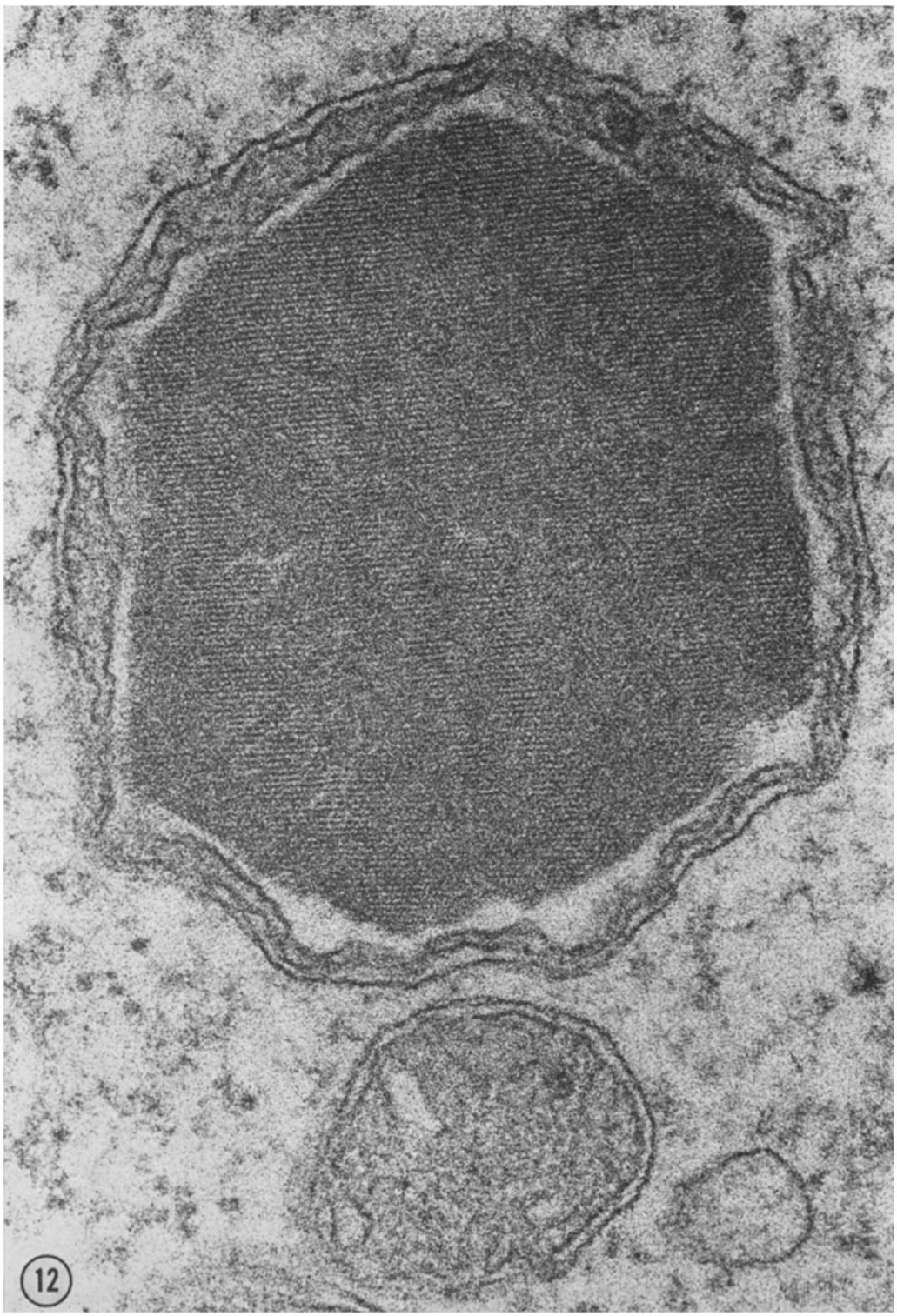

FIGURE 12 Typical mitochondrion with a yolk-crystal inclusion. Standard mitochondrion is immediately adjacent. ×130,000.

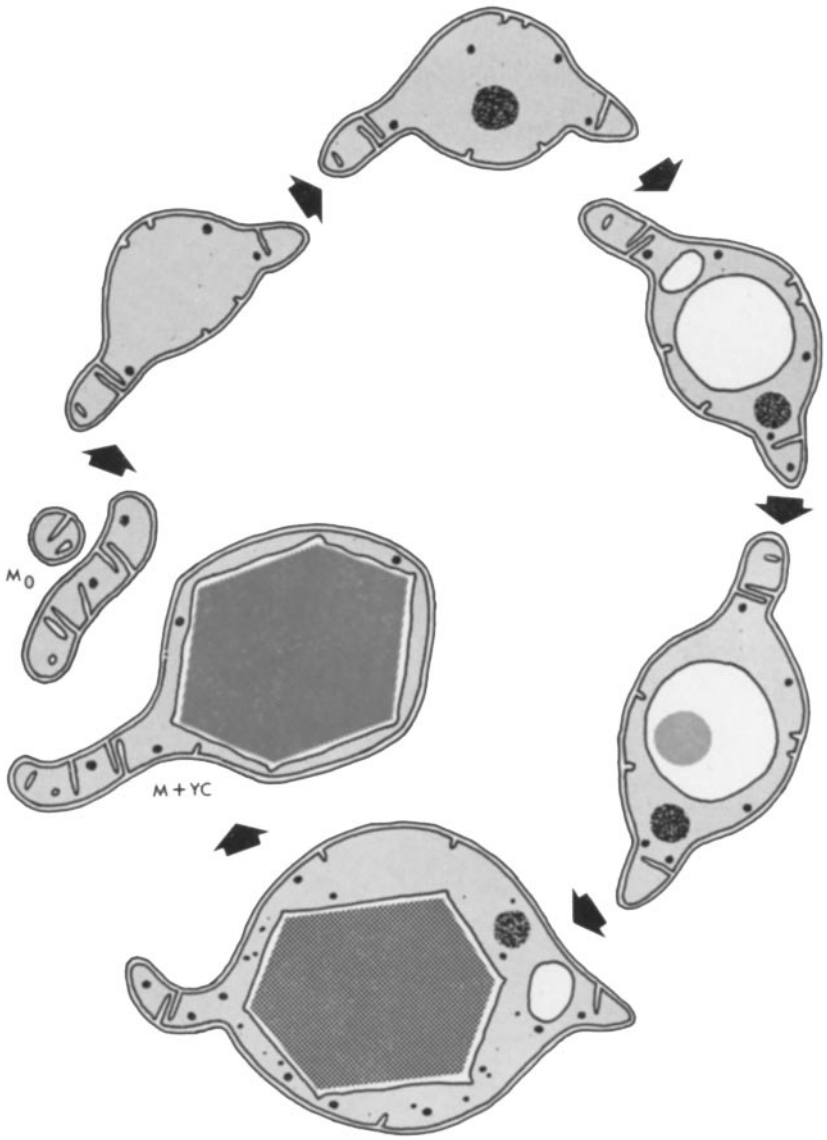

FIGURE 13 Diagrammatic representation of the proposed sequence of organelle modifications and structural additions during the formation of a yolk crystal inclusion within a standard mitochondrion (M_o). Typical mitochondrion having a yolk-crystal inclusion ($M + YC$).

those found by Favard and Carasso (3) during the formation of intramitochondrial yolk inclusions in a mollusc oocyte, should probably be present in frog oocytes; we must thus consider whether the several forms of modified mitochondria now observed in immature oocytes of bullfrogs are such forms or whether they are unrelated components of a complex, intracellular, mitochondrial heterogeneity. Some cases of the presence of several apparently unrelated forms of altered mitochondria and mitochondrial inclusions within a single cell type are certainly known to occur (e.g. 20, 33). That the altered mitochondria in bullfrog oocytes are related stages in a complex process of organelle development and specialization seems a more likely interpretation, owing to the fact that each of the individual elements characterizing the different modified mito-

chondria is always observed not in isolation but in conjunction with some other elements *including the yolk-crystals*; e.g., the matrical mass seen in modified mitochondria of the second type occurs only in mitochondria which also have a region of enlarged matrix and shortened cristae. Similarly, the intracristal body occurs only in mitochondria with dilated cristae, and in turn, dilated cristae are only observed in mitochondria which have a region of enlarged matrix and shortened cristae. Much of the sequence of events which may be logically formulated on the basis of such considerations may be deduced a priori on the basis of dimensional requirements: the presence of an intracristal body having a diameter of 0.3 μ necessitates the conclusion that the usual mitochondrial diameter of 0.3 μ must have been enlarged at the involved area. In the case of the intracristal body, the possibility that the cristae are passively expanded by the enlarging inclusion is ruled out by the observed presence of large, empty regions of intracristal space around these bodies; similar arguments against the possibility that the mitochondrial diameter is passively expanded by an enlarging matrical mass or a dilating crista may also be made, thus imposing a certain order in the appearance of these structural alterations upon any proposed sequence of events.

One such sequence of organelle morphogenesis, leading from a standard oocyte mitochondrion to a typical mitochondrion with a yolk-crystal inclusion, which is consistent with the observed patterns of occurrence of mitochondrial alterations, is depicted in Fig. 13; only one structural modification at a time has been added for diagrammatic simplicity. It should be noted that the proposed sequence of events is specific to both the whole mitochondrion and to a given crista in this mitochondrion; thus, Fig. 10 shows several different cristae of the same mitochondrion appearing to be simultaneously at different stages of the proposed sequential process. Other observations may be interpreted consistently with the proposed sequence, e.g., the observed mutual exclusion of the fibrillogranular and crystalline inclusions within dilated cristae would be expected if the material of the former subsequently became ordered and formed the yolk-crystal.

The possibility of the real occurrence of the postulated sequence must be well tempered by the fact that observations were made upon sections of an object that may not have intersected a given smaller part of the entire object. Thus, a mitochondrion which had an enlarged matrical region, a matrical mass, and a dilated crista containing an intracristal body, when sectioned in an appropriately tangential manner, could appear to be a mitochondron only with an enlarged matrix, or only with an enlarged matrix plus an empty dilated crista. The coordinate occurrence of certain of the additional structural elements could be produced equally well by randomly distributed sections through a single type of altered mitochondrion which contained all of them; this single type of mitochondrion would be equivalent to those described under modification 5. Altered mitochondria of this type would remain distinguishable from the typical yolk-crystal–containing mitochondria, owing to the absence of such elements as matrical masses and the dense fibrillogranular intracristal bodies in the latter.

Altered mitochondria in oocytes of *Triturus helveticus* were interpreted by Sentein and Humeau (17) as being morphological intermediates in the production of crystalline yolk inclusions within mitochondria of these oocytes. The location of the crystals within the matrix of mitochondria of this urodele amphibian is clearly very different from the intracristal location of frog oocyte yolk-crystals; differences in the structural events comprising the proposed intermediate stages may be related to the difference in the locus of the final product.

Mitochondrial Structural Plasticity in Relation to Mitochondrial Specializations

The mitochondrial specializations just described in bullfrog oocytes, and those occurring during the mitochondrial modifications in mouse oocytes (32) and during the mitochondrial changes in oospheres of the plant *Selaginella* (16), are most strikingly similar. The cristal dilatations in mitochondria of developing mouse oocytes are of unknown function and significance; the plant mitochondria sequentially develop cristal dilatations, spherical intracristal inclusions, and finally crystalloid inclusions within their cristae, all apparently in response to a series of localized cytoplasmic stimuli. In addition to these cases, the matrical masses present within some mitochondria in liver cells of the slender salamander (5) appear similar to the spherical masses seen

within the enlarged matrix regions of some modified mitochondria in bullfrog oocytes. The remarkable similarities of these alterations in the usual mitochondrial form within very diverse cellular situations are probably a reflection of the universality of these modes of mitochondrial structural plasticity (see review in reference 1). It seems noteworthy that new structural components are acquired by the frog oocyte organelles during the proposed sequence of mitochondrial differentiation resulting in the formation of yolk-crystal inclusions, whereas those changes of mitochondrial plasticity alone are usually reflected in reorganizations in the form or dimensions of structural elements already present. The fact that the several morphologically distinct classes of mitochondria in bullfrog oocytes exist side by side in the same cytoplasmic environment makes it reasonable to speculate that some portion of the genetic specificity for their structural distinctions might reside in the organelles themselves.

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Note Added in Proof: T. B. Aizenshtadt has reported (1969. *Dokl. Akad. Nauk SSSR.* **189**:1367.) the presence of flattened giant mitochondria in physiologically manipulated oocytes of *Rana temporaria* which were treated with puromycin or a hypophyseal suspension in saline; a translated version of this article has recently been published by the Consultants Bureau of the Plenum Publishing Corporation, New York, (1969. Giant Mitochondria in Frog Oocytes. *Dokl. Biol. Sci.* **189**:801.).

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