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Development of Crystalline Inclusions ("Ergosterol Crystals") in *Neurospora crassa*

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With 12 Figures

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Summary

Development of crystalline inclusions ("ergosterol crystals") in "snowflake", a morphological mutant of *Neurospora crassa* has been examined. The inclusions which arise in membrane-bound organelles appear as electron dense deposits, increase in size, and occupy nearly all the space within the organelle at maturity. The presence of catalase activity in the organelle was not detected using cytochemical procedures employing diaminobenzidine.

1. Introduction

Hyphae of *Neurospora crassa* are commonly found to possess crystalline inclusions. These inclusions, observable with the light microscope, often appear as hexagons about 1 micron in width and up to 2–3 microns in length. They are usually close to longitudinal or septal walls and are often surrounded by mitochondria. The ultrastructure of these inclusions was first described by SHATKIN and TATUM [16] who suggested that they might have a limiting membrane. In a later study, TSUDA and TATUM [17] obtained fractions rich in these crystals and determined that they were composed of ergosterol. Moreover, they noted that the inclusions appear at an early stage of development and are well developed in the second cell from the growing tip.

During the examination of microfilaments in "snowflake", a morphological mutant of *Neurospora* [1], it was observed that hyphae from young cultures (24 hours old) possessed numerous crystalline inclusions. *Neurospora* is particularly useful for developmental studies since its pattern of growth permits the examination of cells which have been laid down in order of development. In addition, "snowflake" was used to determine the course of development of the inclusions because like many morphological mutants, it grows compactly and forms small cells, thereby permitting the examination

in sections of a larger number of cells than is possible with wild-type strains.

As will be shown, the crystalline inclusions are surrounded by an external membrane and so are similar in appearance to microbodies from other organisms. Therefore, attempts were made to determine whether catalase, an enzyme characteristic of microbodies, is present in the inclusions in addition to ergosterol.

2. Materials and Methods

The morphological mutant of *Neurospora crassa* used for this study, strain 507 ("snowflake") was obtained through the courtesy of Fungal Genetics Stock Center, Humboldt State College, Arcata, California. Cultures were grown at 22 °C in 250 ml Erlenmeyer flasks containing 100 ml of distilled water with 2% sucrose and 2% of 50 X Vogel's standard salts [20]. Cultures were harvested 24 hours following inoculation with conidia.

Hyphae were fixed in 0.1 M sodium cacodylate containing 1.75% glutaraldehyde, 1% paraformaldehyde, and 0.025% $CaCl_2$ (final pH 7.2) for 2 hours at room temperature. Following washing and post fixation (1% OsO_4 in 0.1 M sodium cacodylate for 1 hour) hyphae were dehydrated in ethanol and embedded in Epon [1]. Sections were stained with uranyl acetate and lead citrate [14] and examined with an Hitachi 11 microscope operated at 75 kV.

Localization of catalase activity using diamino benzidine (DAB) was attempted using fixed (3% glutaraldehyde in 0.1 M sodium cacodylate, pH 7.2) and unfixed tissue. The incubation media employed were: 1. 0.6% H_2O_2 in 0.1 M sodium bicarbonate buffer containing 2 mg/ml DAB (final pH 10.5) [18]; 2. 0.06% H_2O_2 in propanediol buffer containing 2 mg/ml DAB (final pH 9.0) [6]; and 3. 0.02% H_2O_2 in 0.1 M Tris-HCl buffer containing 0.5 mg/ml DAB (final pH 8.5) [5].

3. Results

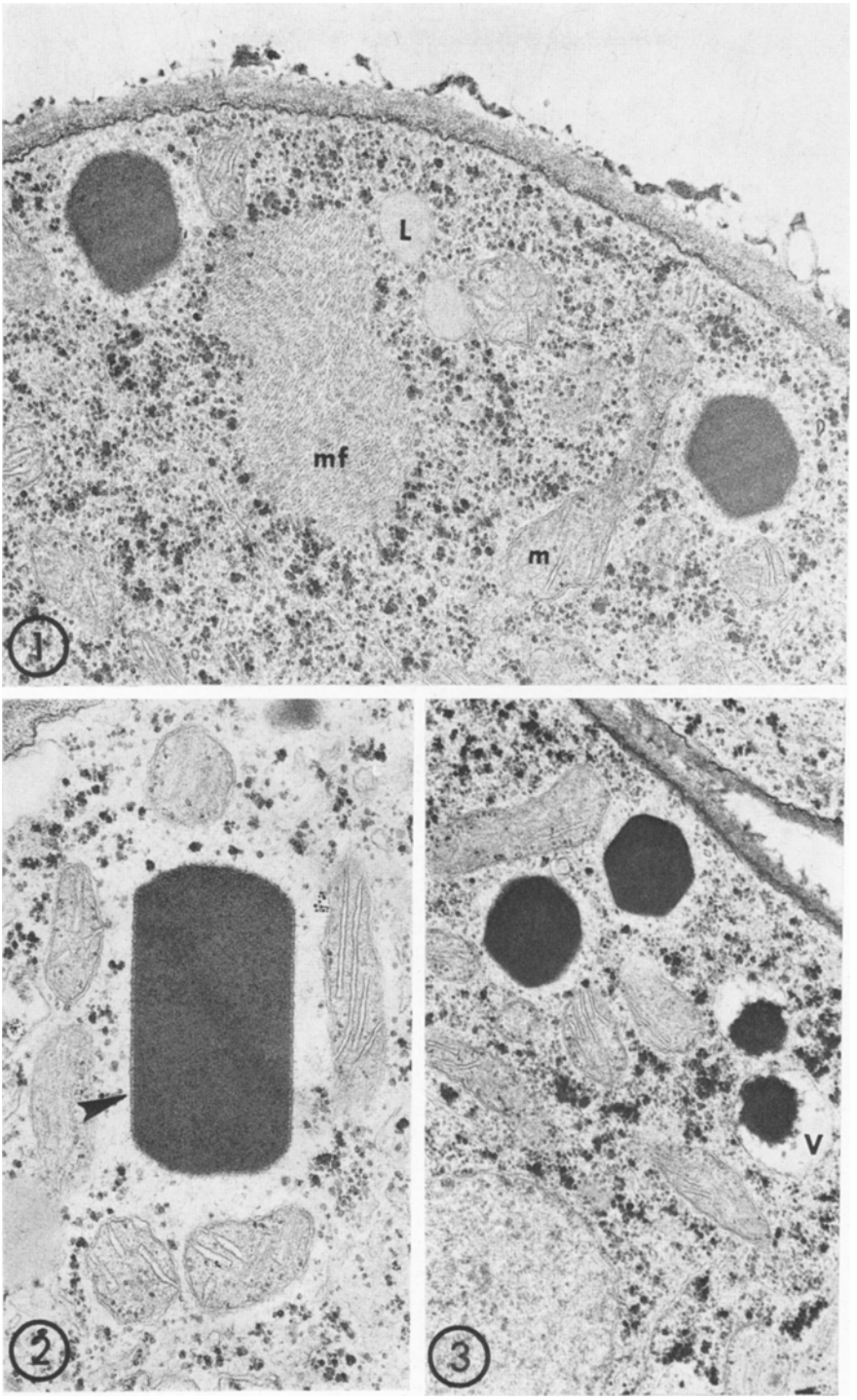
Well developed crystalline inclusions like those shown in Figs. 1–3 are found in cells away from the apical cell. The inclusions often occur close to longitudinal or cross walls (Figs. 7–9) and are ovoid, rectangular, or hexagonal and usually surrounded by mitochondria.

That crystalline inclusions possess a limiting membrane can be seen in Figs. 2 and 4. A halo-like region with few contents which appears less dense than the background cytoplasm often surrounds the organelle (Figs. 1–3). The contents of the organelles usually appear amorphous although a crystalline subunit structure can be seen in a few (Fig. 4).

Fig. 1. Section showing two crystalline inclusions near the cell wall. Microfilaments (*mf*), mitochondria (*m*) and lipid granules (*L*) are also shown. ×44,800

Fig. 2. Rectangular shaped inclusion surrounded by mitochondria. The presence of an outer limiting membrane can be seen (arrow). ×39,200

Fig. 3. Section showing mature inclusions as well as vesicles (*v*) containing electron-dense deposits. ×29,600



Figs. 1–3

Observations of hyphae suggest that the inclusions probably originate in membrane-bound organelles containing a fine granular amorphous material which are closely associated with the endoplasmic reticulum, as shown in Figs. 5 and 6. A very early stage in the process giving rise to the mature crystalline inclusion may be found as a small amorphous electron-dense deposit in organelles containing the fine granular material (Fig. 7). The stages depicted above are seen only in the first or second cell of a growing hyphae. As the inclusion increases in size it occupies a larger area of the organelle in which the fine granular material decreases (Figs. 7–9). As additional material is deposited, the crystal takes shape and occupies a major portion of the organelle as may be seen in Figs. 7–9. When mature, the crystalline inclusion fills most of the space within the membrane, the remainder being occupied by a small amount of granular material (Figs. 10–12). It may be seen in these figures that some of the mature organelles still have small portions of membrane attached. Whether the connection with the membrane remains in all cases after development and is observed only when the organelle is sectioned in a particular plane is not known.

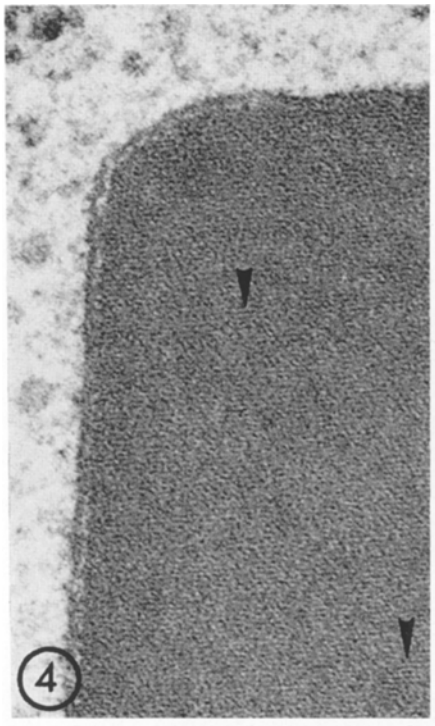
The only other organelle in *Neurospora crassa* which appears as electron dense as the crystalline inclusions are vesicles such as those seen in Fig. 3. These vesicles commonly are seen at the tip of apical cells and may aid in the synthesis of walls in the growing cells [8]. It can be seen that the contents of these vesicles are less compact than those of the crystalline inclusions, and the vesicles contain areas void of stainable materials.

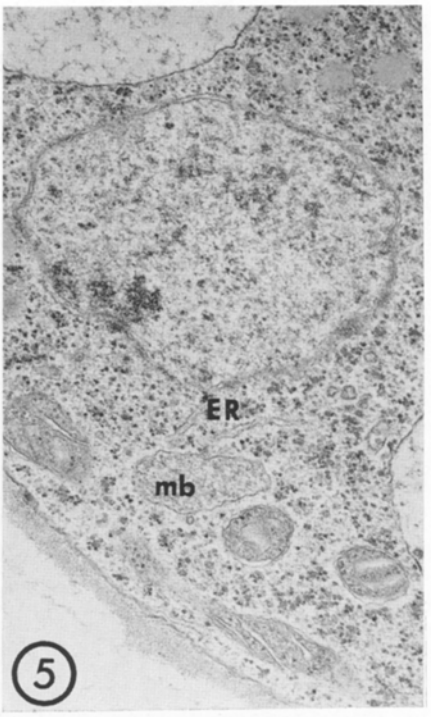
Since the crystalline inclusions arise in organelles possessing a limiting membrane and, in this respect at least, are similar in appearance to the inclusions observed in microbodies, attempts were made to determine whether they possess catalase in addition to ergosterol. Hyphae incubated in DAB-media for localization of catalase activity gave a negative reaction. Although a few crystalline inclusions appeared to show a reaction product, its electron-density was very low when compared to the positive reactions obtained by others with microbodies from other organisms treated under similar conditions [5, 6, 18]. Furthermore, even within the same cell not all crystalline inclusions showed the slight positive reaction.

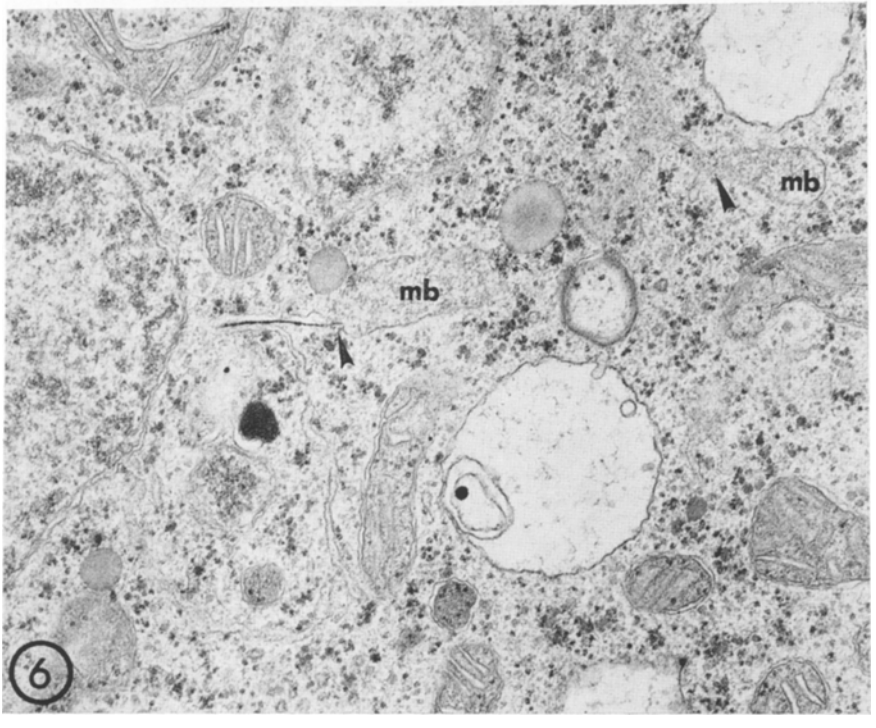
Fig. 4. Higher magnification of inclusion shown in Fig. 11. The fibrillar subunit structure of the inclusion can be seen (arrows). ×223,200

Fig. 5. Section showing microbody-like organelle (*mb*) closely associated with smooth endoplasmic reticulum (*ER*). ×31,000

Fig. 6. Microbody-like organelles (*mb*) showing possible attachment to smooth endoplasmic reticulum (at arrows). It is within these organelles that the inclusions are believed to originate. ×36,900







Figs. 4–6

4. Discussion

These data suggest that the crystalline inclusions (ergosterol crystals) are the final stage in a developmental sequence. As such they are similar to microbodies from several organisms and appear to follow a similar pattern of development [19, 21].

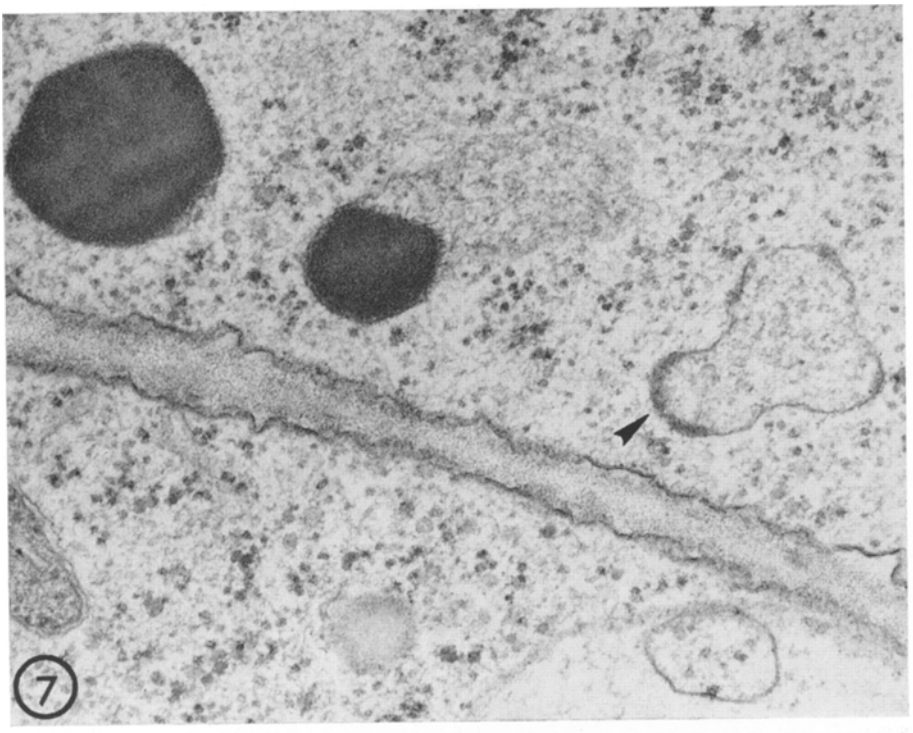
It has been assured in this work that the large inclusions represent the last stage in a developmental process but an alternative to this interpretation is that the crystalline inclusions are an early stage and then broken down and their contents utilized during various stages depicted. However, this seems unlikely because of the location of the various stages of the inclusion in relation to the growth pattern of *Neurospora.* The organism advances by tip growth with the youngest cell being at the apex and older cells extending behind. Mature crystalline inclusions are not observed in the apical cell but become numerous as one examines cells away from the tip. Reciprocally, the "younger" stages (those showing organelles with small amounts of electron dense material) regularly are seen in apical cells but are not observed away from the growing tips.

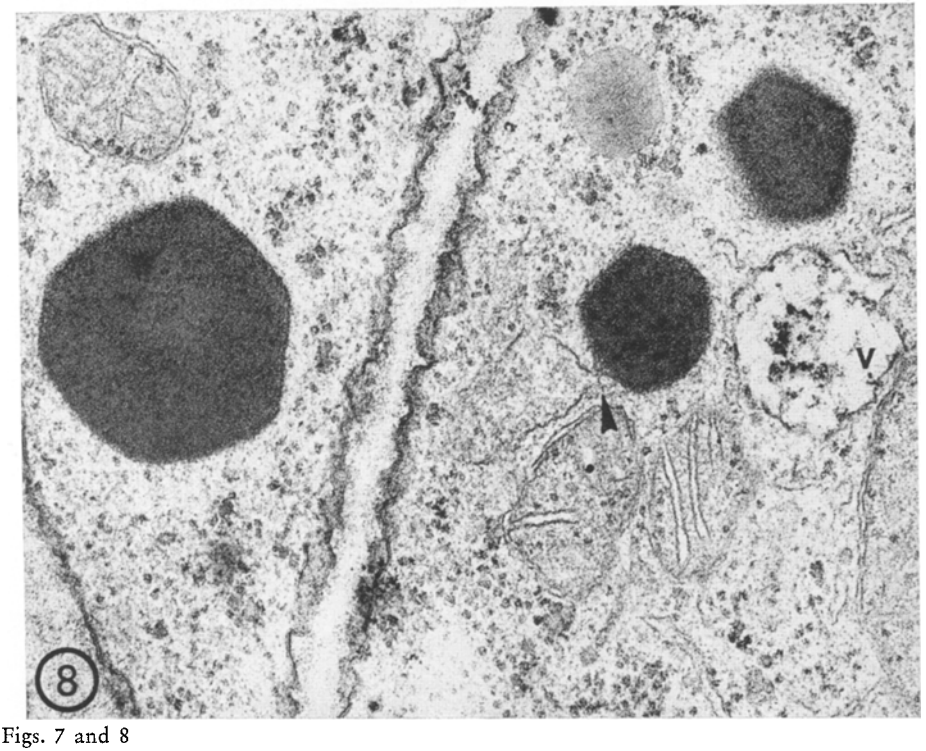
For convenience, VIGIL [19], defines a plant microbody as an organelle surrounded by a single limiting unit membrane that may be associated with the endoplasmic reticulum. Microbodies possess a moderately dense, finely granular matrix in which may be found various inclusions. This definition includes organelles such as peroxisomes and glyoxysomes which possess known enzyme systems [2, 3] as well as others whose enzymes are unknown [11].

The use of diaminobenzidine (DAB) for the localization of catalase in microbodies of higher plants and animals is well documented [5, 6, 19], but this procedure has yielded negative results with the putative "microbodies" of fungi [4, 10, 21]. An exception to this has been in yeasts, especially those grown on methanol in which large numbers occur. Isolated microbodies from yeast have been shown to contain catalase and alcohol oxidase [2, 7] as well as D-amino acid oxidase [7]. Baker's yeast grown on lactose possesses microbodies (peroxisomes) with the above enzymes as well as urate oxidase and L-α hydroxy acid oxidase (glycollate oxidase) [13]. Also catalase has been demonstrated in *Hansenula polymorpha* [18] and *Kloeckera* sp. no. 2,201 [7] grown on methanol. Similar findings have been observed in *Candida tropicalis* pK 233 grown on n-alkane [12] and *C. boidinii* grown on methanol [15].

Fig. 7. Appearance of crystalline inclusions at various stages of development. The organelle indicated (by arrow) represents one of the earliest stages in which electron-dense material has been observed. ×52,800

Fig. 8. Three crystalline inclusions, one of which shows a membrane continuity to a microbody-like organelle (arrow). Also shown in the figure is a vesicle (*v*) containing electron-dense material. ×64,500





Figs. 7 and 8

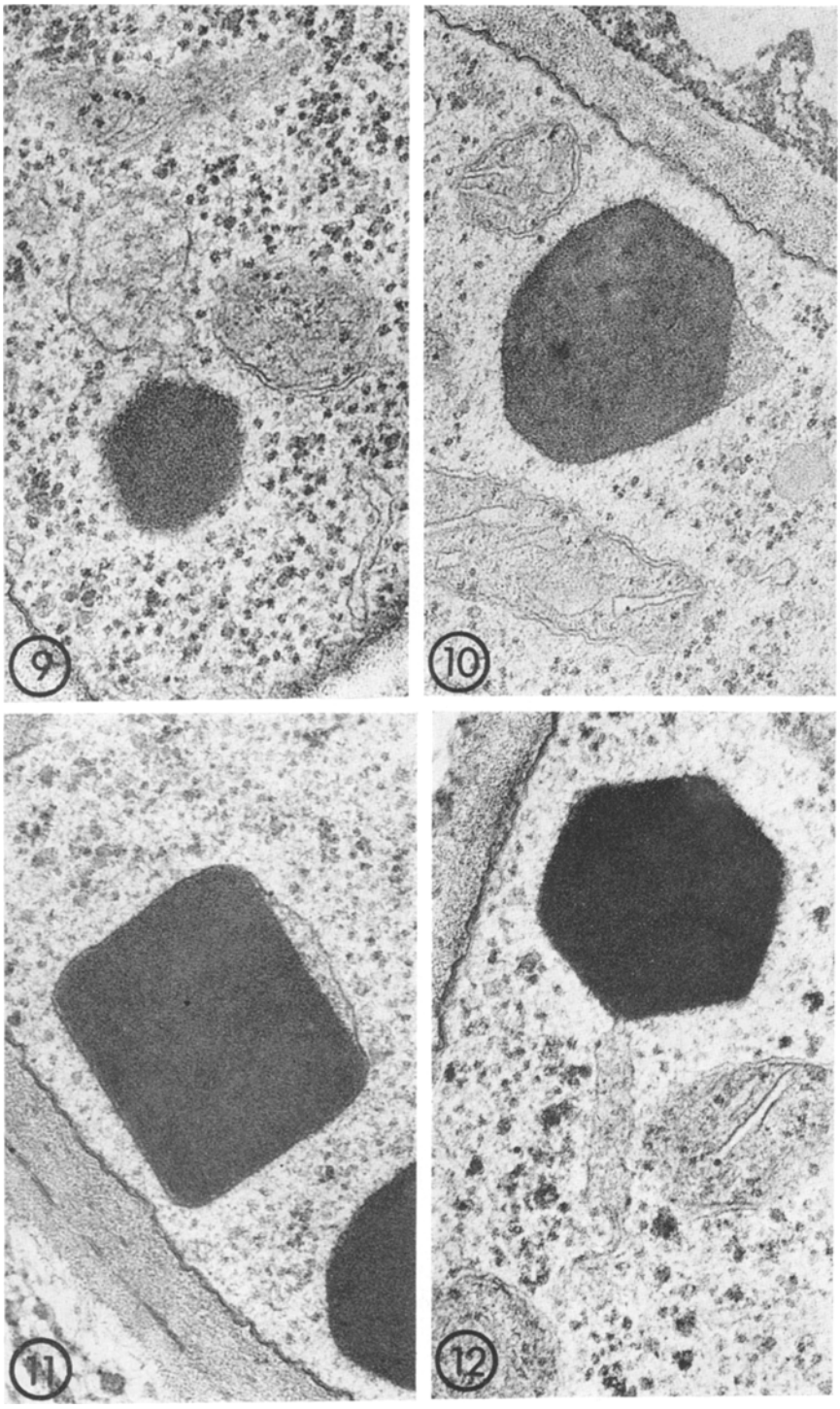


Fig. 9. Developing inclusion showing membrane continuity to microbody-like organelle. ×68,000

Figs. 10–12. Developed crystalline inclusions showing membranes extending away from the inclusion. ×60,100 (Fig. 10); ×55,600 (Fig. 11); ×67,000 (Fig. 12)

In light of the findings of TSUDA and TATUM and those described herein, the crystalline inclusion observed in *Neurospora* probably is part of a microbody, whose contents include ergosterol. In fact, the organelle appears identical to microbodies containing hexagonal-shaped inclusions observed in the filamentous fungus, *Whetzelinia sclerotiorum* [1]. In agreement with the findings in *Neurospora*, microbodies of *W. sclerotiorum* and other filamentous fungi [4, 10, 21] show no catalase activity. The absence of a reaction product using cytochemical methods, of course, does not rule out the presence of the enzyme so further proof is necessary. The presence of ergosterol in the crystalline inclusions of *Neurospora* raises the point as to whether enzymes related to steroid metabolism are also present. A further question is whether the ergosterol crystals develop in organelles containing the usual enzymes found in microbodies of higher plants and animals. In *Neurospora*, isolated particles similar to glyoxysomes of higher plants have been shown to possess isocitrate lyase, malate synthase, malate dehydrogenase and possibly NAD-isocitrate dehydrogenase [9]. Also in *Neurospora*, evidence for two classes of peroxisomes containing urate oxidase and isocitrate lyase respectively exists [13]. However, the latter work cited has only been discussed in a personal communication, so its significance remains to be evaluated. At present, the relationship between the ergosterol crystals and such organelles is unknown.

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