INTRACELLULAR CRYSTALLINE ERGOSTEROL IN *NEUROSPORA*

SEIZO TSUDA, Ph.D., and E. L. TATUM, Ph.D.

From The Rockefeller Institute

ABSTRACT

In the fungus *Neurospora crassa*, hexagonal crystalline inclusions have been observed with both the light and electron microscopes. These crystals have been enriched by differential centrifugation and found to be identical with ergosterol by the criteria of ultraviolet spectral analysis and cytochemical analysis. Observations have been made on the distribution and fine structure of the crystalline bodies in various wild type and mutant strains of *N. crassa*.

INTRODUCTION

Hexagonal crystalline inclusions have been observed with the electron microscope in the hyphae of *Neurospora crassa* (1). It has been assumed that the crystalline bodies are metabolic storage products. However, no definitive study was done to determine the chemical nature and function of the crystals.

By means of differential centrifugation, it has been possible to isolate from *Neurospora* a subcellular fraction rich in hexagonal crystals. This report describes chemical and cytological studies on isolated and intracellular crystals, which have been shown to consist of ergosterol.

MATERIALS AND METHODS

Five strains of *Neurospora crassa* were examined. These included wild types SYR 17-3A and E5297a; two albino mutants, 4637TA (*al-1*) and 15300-13A (*al-2*); and one colonial mutant, B132A. Mycelia of all strains except B132 were harvested after 2 days' growth with aeration at 25°C in N minimal medium (2) containing 2 per cent sucrose. Strain B132, a slow growing mutant, was cultured for 4 days. For differential centrifugation, mycelia were harvested by filtration, then lyophilized, and ground to a fine powder in a mortar. 3 gm of the dry powdered material were suspended in 100 ml of 0.05 M phosphate buffer, pH 7.0, supplemented with 7.5 per cent mannitol (3). Fractions were prepared by centrifuging in a Lourdes refrigerated centrifuge. The procedure essentially involved separation, first in mannitol buffer then in distilled water, of cell wall fragments and other debris at 600 *g*, and of the crystal fractions at 1000 *g* and 3200 *g*. The crystal fractions were then lyophilized.

The ergosterol levels in two wild types of *Neurospora crassa*, SYR 17-3A and E5297a, and in the *al-2* mutant strain were determined quantitatively, using a direct solvent extraction procedure (4). The materials were cultured on the N minimal agar medium for 1 week and in minimal aqueous medium N with aeration for 48 hours at 30°C. After cultivation, the materials were harvested by filtration, lyophilized, and ground to a fine powder in a mortar. Samples of 5 gm were extracted in Soxhlet extractors for 6 hours with ether. The ether extracts were taken to dryness at room temperature. The ergosterol was dissolved in absolute ethanol, filtered, and diluted appropriately. The ergosterol levels in the extracts were determined with a Spectracord spectrophotometer by comparing the absorption at 282 mμ with that of known concentrations of pure ergosterol. The absorption in the visible spectrum by carotenoids in some extracts did not affect the characteristic ultraviolet absorption spectrum of ergosterol.

For cytochemistry, the fresh mycelia were fixed in 10 per cent neutral formalin for 1 hour and washed several times with distilled water. Two cytochemical tests for cholesterol, the Schultz method (5, 6) and

the Windaus digitonin reaction (5), were used. Fixation for electron microscopy was in 1 per cent OsO_4, buffered with pH 7.4 *s*-collidine-HCl (7), for 2 hours in an ice bath. The fixed material was dehydrated by passing it through an ethanol series of increasing concentration, and it was soaked in *n*-butyl methacrylate prior to embedding in a 1:10 mixture of methyl/butyl methacrylate. Thin sections were made on a Porter-Blum ultramicrotome and examined with the EMU-2C RCA electron microscope.

The number and distribution of the crystalline bodies in living hyphae cultured on agar plates was examined under the light microscope. Micrographs were taken with a Polaroid camera mounted on a microscope.

RESULTS

Observations on Living Cells

The average number of hexagonal crystalline bodies per *Neurospora* cell was found to vary from ten to fifteen. Figs. 1 and 2 are light micrographs showing the crystals in *N. crassa* wild type SYR 17-3A, and in albino strain *al-2*. In the micrographs, the crystalline bodies (arrows) appear as tiny spots randomly distributed in the hyphal cell. Micrographs of wild type E5297a, of colonial mutant B132A, and of albino strain *al-1* were essentially similar. No differences have been observed in size or number of crystalline bodies in cells cultured on different media, including Fries (8) and N (2) minimal media, and glycerol complete medium (9). As to temperature, the material cultured at 25°C tends to contain more crystalline inclusions than that cultured at 30°C. In general, the mycelium cultured on the surface of agar medium contains larger bodies than the material from liquid culture. Occasionally, exceptionally large crystalline bodies have been observed in the albino-2 mutant. Figure 3 is a phase contrast micrograph of the *al-2* strain in which the crystalline bodies are large enough to be readily identified. This culture had been grown for 1 week on agar slants of N minimal medium. The crystalline inclusions appear as hexagonal plates in these micrographs. The bodies marked *a* represent planar views of a crystal, and *b* and *c* represent oblique and side views, respectively. As seen here, the crystals may attain a diameter of 3 μ and a thickness of 1 μ.

Additional observations with phase contrast microscopy were made on two respirational mutant strains, *mi-3* and *poky*. Hexagonal plates were observed in the *mi-3* strain, and hexagonal bipyramidal prisms in the *poky* strain. We have examined one strain of *Saccharomyces* (*S. fragilis*) by light microscopy for the presence of crystalline inclusion bodies. Hexagonal forms similar to those in *Neurospora* were observed. These gave a positive Schultz sterol reaction.

Electron Microscopy

Fig. 5 is an electron micrograph of a thin section of the albino-2 mutant. The left side of the micrograph contains a crystalline body (*H*) of 1 μ diameter which appears as a hexagon in this plane of sectioning. On the right, the crystal appears in cross-section as a rectangle. The crystals are composed of a dense material, and the outer surface of the crystals appears as a dense line. The micrograph also shows portions of the cell wall (*CW*), lipoid granules (*L*), several profiles of the endoplasmic reticulum (*ER*), and

FIGURES 1 AND 2

Light micrographs of different strains of *Neurospora* showing the distribution of the crystalline inclusions (arrows) in living hyphae. × 2000.

Fig. 1, wild type strain SYR 17-3A.

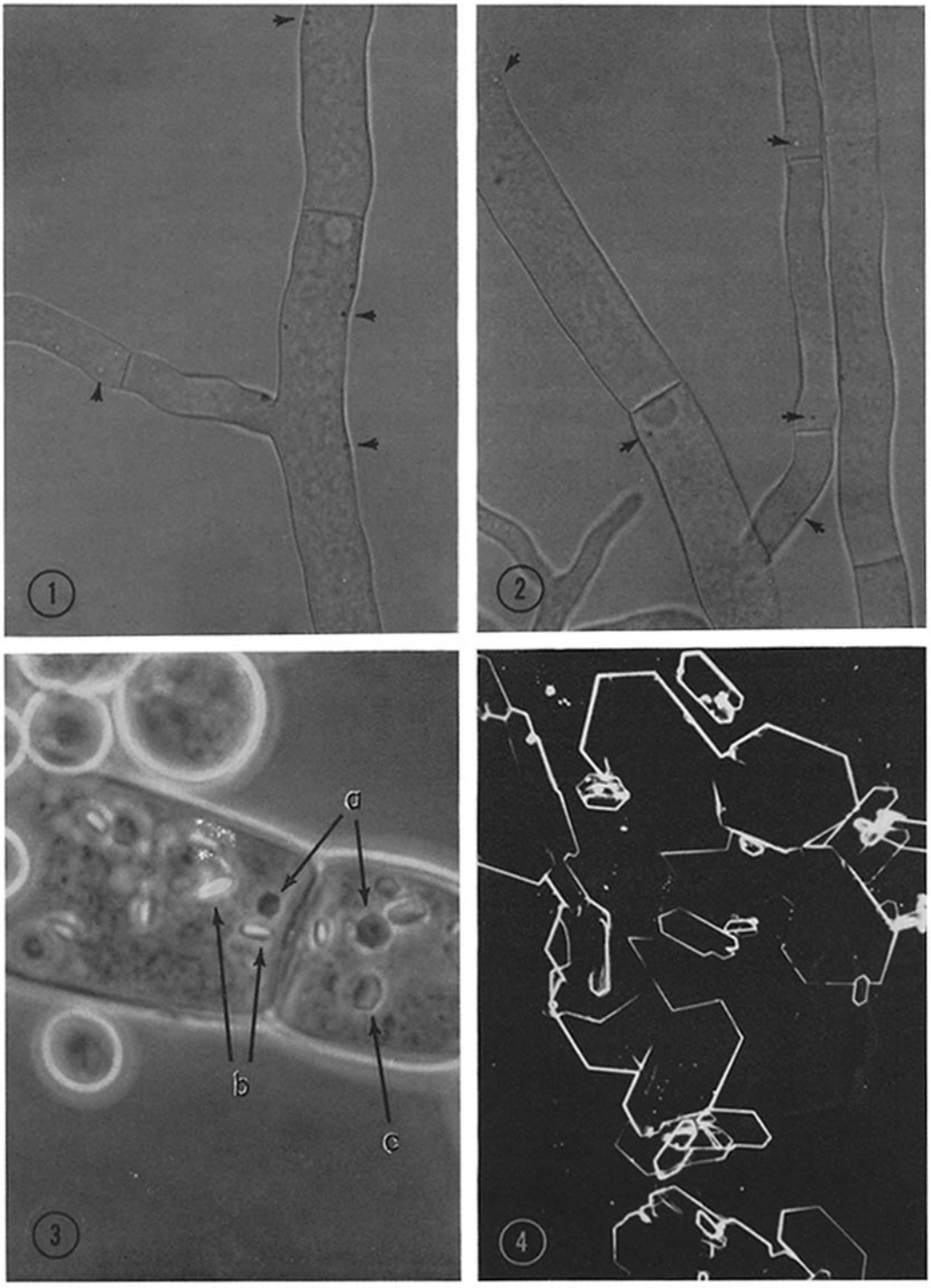
Fig. 2, albino strain 15300-13A (*al-2*).

FIGURE 3

Phase contrast micrographs of mature hyphae (1-week-old culture on minimal solid medium) of the albino (*al-2*) strain of *Neurospora*. Large hexagonal crystalline bodies can be seen in planar (*a*), oblique (*b*), and side (*c*) views. × 2000.

FIGURE 4

Dark field micrograph of authentic ergosterol crystallized from ethanol. × 200.



mitochondria (*M*). Many light spaces relatively free of RNP particles appear in the cytoplasm. Based on the PAS staining reaction, these light areas probably are regions of carbohydrate storage.

Fig. 6 is an electron micrograph of the colonial mutant B132A. A cross-section of the homogeneous, dense crystalline body can be seen in the center of the micrograph. Profiles of endoplasmic reticulum (*ER*) as well as lipoid granules (*L*) also are visible.

Identification of the Crystals as Ergosterol

In order to obtain an enriched preparation of crystals for identification, mycelia were fractionated by differential centrifugation as described under Materials and Methods. The final sedimented fractions contained a relative concentration of crystalline bodies sufficient for qualitative chemical tests. After lyophilization, the crystal-containing pellets were extracted with pure heptane. The ultraviolet absorption of the heptane-soluble material was determined on a Cary spectrophotometer. Absorption maxima were found at 272 mμ, 282 mμ, and 294 mμ for samples from all strains of *Neurospora* examined. These maxima are in excellent agreement with those of authentic ergosterol determined in the same manner.

Two additional lines of evidence support the finding that the crystal bodies in *Neurospora* hyphae consist of free ergosterol. Authentic ergosterol recrystallized from ethanol has been examined. Fig. 4, a dark field micrograph of such a preparation, shows that the typical hexagonal plates of ergosterol closely resemble the crystalline inclusion bodies of *Neurospora* hyphae. Two cytochemical tests for sterols, that of Schultz (5, 6) and the digitonin reaction of Windaus (5), gave positive reactions when applied to the crystalline inclusions in intact hyphal cells of *Neurospora*.

Concentration of Ergosterol in Neurospora

Ergosterol in the intact lyophilized mycelia of wild types SYR 17-3A and E5297a and of the *al-2* mutant was determined quantitatively by ultraviolet spectrophotometry. The results are given in Table I.

It may be noted from the table that relatively high concentrations of ergosterol were found in the *Neurospora* strains examined. The results indicate that the wild types contain more sterol than the albino mutant under either culture condition. In the production of ergosterol, the surface culture on agar exceeds the submerged culture. It should be noted that cultures of SYR 17-3A and *al-2* grown on agar medium contained well developed conidiating hyphae as well as conidiospores.

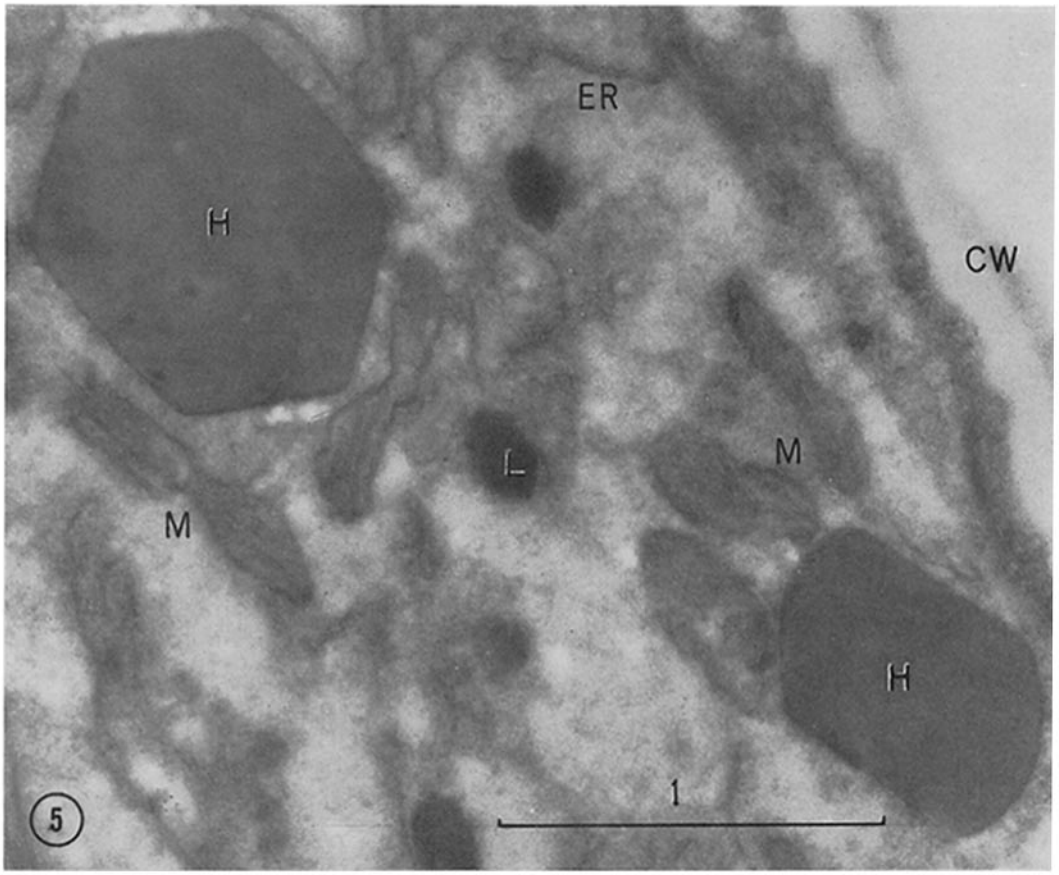
The ergosterol concentration in the crystalline body fraction of *al-2* mycelium obtained from differential centrifugation was determined by the digitonin method. This fraction, which had a dry weight of 32 mg, was obtained from 3 gm of dry mycelium. By calculation, the dry crystalline fraction contained about 20 per cent of ergosterol. This represents the recovery, in the crystal fraction, of ergosterol corresponding to 0.213 per cent of the dry mycelium. By spectrophotometric analysis the crystalline fraction contained 17.4 per cent ergosterol, equivalent to 0.185 per cent of the dry mycelium. As observed in Table I, the albino mutant cultured in the aqueous medium contains 0.73 per cent ergosterol. Therefore, by differential centrifugation more than one-fourth of the total ergosterol was recovered in the crystalline body fraction.

FIGURE 5

Electron micrograph of the albino (*al-2*) mutant strain of *Neurospora* showing crystalline bodies (*H*) in the cytoplasm. The cell wall (*CW*), mitochondria (*M*), endoplasmic reticulum (*ER*), and lipoid granules (*L*) are also indicated in the micrograph. × 50,000. Scale indicates 1 μ.

FIGURE 6

High magnification electron micrograph of colonial mutant strain B132A of *Neurospora* showing fine detail of the crystalline body (*H*). Also indicated are endoplasmic reticulum (*ER*) and lipoid granules (*L*). × 135,000. Scale indicates 0.2 μ.



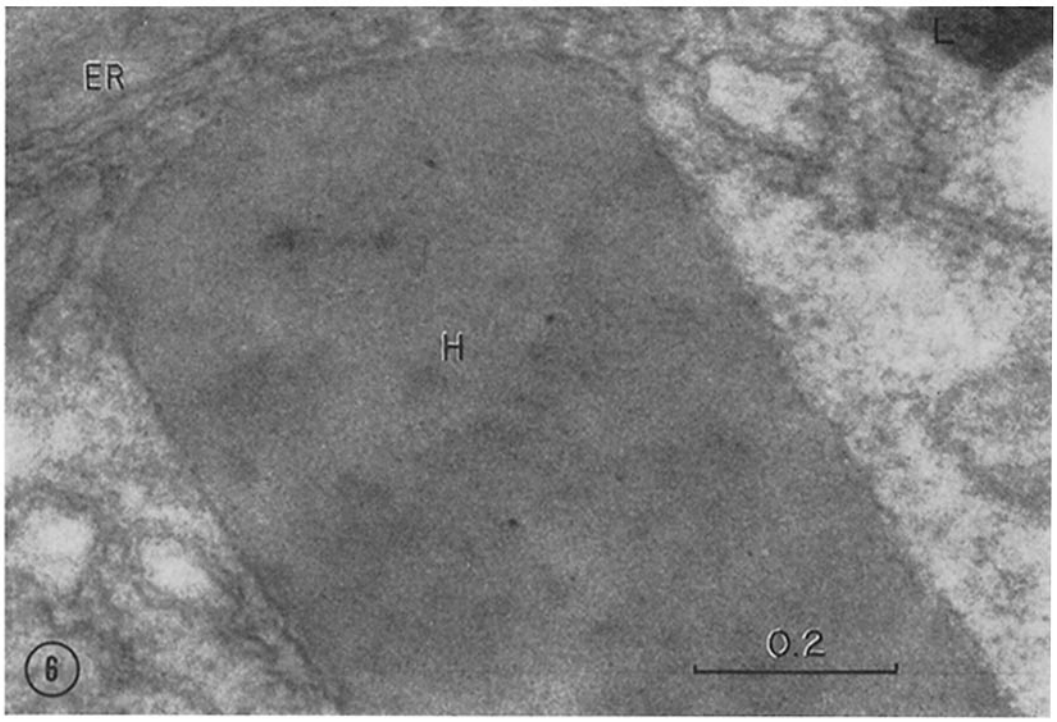


TABLE I

Ergosterol Content of Neurospora crassa

Strain	Grown 1 week on N minimal agar medium	Grown 48 hours on N liquid medium with aeration
	%	%
SYR 17-3A	1.39	0.95
E5297a		0.94
al-2	1.06	0.73

DISCUSSION

The chemical and cytochemical evidence given in the present study shows that the crystalline inclusions observed in the hyphae of various strains of *Neurospora crassa* consist of ergosterol. This sterol, typical of most fungi, was first demonstrated in *Neurospora* by Ottke (10), who reported that wild type *Neurospora crassa* mycelia (strain 4540A) contained 0.13 per cent ergosterol. Appleton *et al.* (11) found that their wild type strain of *Neurospora crassa* produced 1.07 per cent ergosterol when cultured under shaking conditions in complete medium. This value is similar to that found in the present study.

In other fungi, ergosterol values for 24 molds studied by Pruess *et al.* (12) ranged from 0.25 to 1.70 per cent of the dry weight. For several species of *Penicillium* and *Aspergillus,* values of 0.13 to 2.20 per cent have been reported by Appleton *et al.* (11). For various yeasts values of somewhat less than 0.1 per cent ergosterol have been reported by Dulaney *et al.* (13), although for *Saccharomyces* the values reached 7 to 10 per cent.

The hexagonal crystalline bodies have been observed in the hyphae of all strains of *Neurospora* used in this study, including two wild type strains and several mutants. The hexagonal crystals have also been observed in yeast. Few comparable instances of crystalline cellular inclusions have been described, and the present report is apparently the first instance of the occurrence of crystalline ergosterol in living cells. No information is yet available on the function, if any, of these crystals in *Neurospora.* They appear to be distributed randomly throughout the cytoplasm, although they may at times appear to plug the pore in the transverse cell wall. Since most plugs do not appear to be crystalline, however, crystals may merely be caught mechanically as a result of flow of protoplasm through the pore.

Ergosterol crystalline body formation in the *Neurospora* cell has not been studied extensively from the cytological viewpoint. However, it was observed that the ergosterol crystalline bodies appeared at an early stage of development. It is usually difficult to identify crystalline bodies in the tips of rapidly growing hyphae. However, in the second cell from the tip one can observe well developed crystalline bodies.

Ergosterol crystalline bodies have not been observed in conidiating hyphae or in young conidiospores. These materials probably contain at least as much ergosterol as older hyphae, since the level of ergosterol in heavily conidiating cultures grown on solid media is somewhat higher than that in cultures grown on liquid media.

The initial observation that more and larger crystals were present in albino than in pigmented hyphae suggested a reciprocal relationship between ergosterol and carotenoid biosynthesis. Such a relationship has been inferred between cholesterol and carotenoid biosynthesis, with mevalonic acid as a key common precursor (14). Although this relationship probably exists in *Neurospora,* it is apparently not related to the greater number of ergosterol crystals in albino hyphae, since the wild type contained more ergosterol. Rather, it might be suggested that more ergosterol is present in non-crystalline form in the lipid-pigment granule inclusions which are more plentiful in wild type conidiating hyphae and conidia.

Two morphologically distinct types of crystalline bodies may be observed with the electron microscope: hexagonal plates and hexagonal bipyramidal prisms. The hexagonal plate form has been observed in the wild type SYR 17-3A, in the mutant strains B132A and albino 15300-13A, and in the respirational mutant strain *mi-3* 2543-2A. In these strains the crystalline body appears as a hexagon when it is sectioned parallel to the large crystal face. In cross-section it appears as a rectangle, and in oblique sections as a triangle or as a pentagon. On the other hand, the hexagonal bipyramidal prism, which has been seen in the wild type strain E5297a and in the respirational mutant strain *poky* 3627-2a, always appears as a hexagon when sectioned. Apparently, the plates and bipyramids are alternate crystalline forms of ergosterol, and the crystallization may be influenced in some way by the internal environment of the strain in which the sterol is being deposited.

This work was supported in part by Research Grant RG-6972 from the National Advisory Health Council, United States Public Health Service.

The authors wish to thank Drs. E. Reich and A. J. Shatkin for their generous advice and technical assistance, Drs. K. R. Porter and G. E. Palade for the use of their electron microscope, and Dr. H. Jaffe for determining the absorption spectra.

Received for publication, May 4, 1961.

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