A PARACRYSTALLINE INCLUSION IN *NEUROSPORA CRASSA*

Induction by Ethidium and Acridine, Isolation, and Characterization

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ABSTRACT

A paracrystal indistinguishable from the one which occurs in the mitochondrial mutant *abnormal-1* can be induced in wild-type *Neurospora crassa* after growth in either ethidium or euflavine. This paracrystal has been isolated and partially characterized. It appears to be composed of a single polypeptide (mol wt 68,000) which can be reversibly crystallized and dissociated by changes in the pH and ionic strength. When aggregated, the polypeptide forms oligomers which are arranged end-to-end into fibers.

During the characterization of the polypeptide, it was found that the polypeptide's electrophoretic and immunological properties could be used as assays. Using these methods it was found that the polypeptide normally accumulates in a soluble form in the cytoplasm of wild-type *Neurospora* at the end of the log-phase of growth.

INTRODUCTION

Garnjobst, Wilson, and Tatum (1965) noted the occurrence of abundant needle-like inclusions in the cytoplasm of two slow-growing mutants of *Neurospora crassa*: *abnormal-1* and *abnormal-2*. The slow growth of these mutants is correlated with a marked deficiency of mitochondrial cytochromes *a* and *b* (Diacumakos, Garnjobst, and Tatum, 1965; Tatum and Luck, 1967). Since the abnormal phenotype showed a maternal pattern of segregation in crosses (Garnjobst, Wilson, and Tatum, 1965) and could be transferred to wild-type cultures by the injection of purified *abnormal* mitochondria (Diacumakos, Garnjobst, and Tatum, 1965), it appears to be the result of a mutation involving mitochondrial DNA. If the presence of cytoplasmic inclusions were related to the mitochondrial defects, their study might provide some insight into the interactions between mitochondria and the rest of the cell.

We report here our studies of the correlation of the inclusion with mitochondrial abnormality, the isolation of the inclusion, and its partial characterization. We found that the inclusion is composed of a nonmitochondrial protein which is a normal product of cytoplasmic, rather than mitochondrial, protein synthesis.

MATERIALS AND METHODS

Materials

Gluculase, a snail digestive juice preparation, was obtained from Endo Laboratories, Inc., Garden City,

N. Y. Lubrol WX, a nonionic detergent, was obtained from ICI America Inc., Stamford, Conn. Sodium dodecyl sulfate (SDS)[1] was purchased from BDH Chemicals Ltd., Poole, England. Sodium silicotungstate was provided by A. D. Mackay Inc., New York. Agarose was made by Behringwerke, A. G. Marburg-Lahn, West Germany. Bovine serum albumin was obtained from Armour Pharmaceutical Co., Kankakee, Ill. and cytochrome *c* from Sigma Chemical Co., St. Louis, Mo.

L-4,5-leucine-^{3}H (6 Ci/mmole) and reconstituted protein hydrolysate-^{14}C were obtained from Schwarz Bio Research Inc., Orangeburg, N. Y. Sodium dodecyl sulfate-^{35}S was obtained from The Radiochemical Centre, Amersham, Buckinghamshire, England. Ethidium bromide (2,7-diamino-10-ethyl-9-phenyl phenanthridium bromide) B grade was purchased from Calbiochem, Los Angeles, Calif. Euflavine (3,6-diamino-10-methyl acridine) and proflavine (3,6-diaminoacridine) were purchased from Aldrich Chemical Co., Inc., Milwaukee, Wis.

Buffers

The following buffers were used: succinate, 50 mM sodium succinate-0.02% sodium azide, pH 5.3; phosphate, 10 mM sodium phosphate-0.02% sodium azide-50 mM β-mercaptoethanol, pH 7.5; phosphate-saline: 5 mM KH_2PO_4-45 mM Na_2HPO_4-140 mM NaCl.

Strains

The following strains of *Neurospora crassa* were used: Em 5256, a wild-type No. 424 from the Fungal Genetics Stock Center, Humbolt State College, Arcata, Calif., and *abnormal-1-4a*, (ABN-1), a cytoplasmic mutant with mitochondrial defects (Garnjobst, Wilson, and Tatum, 1965).

Microscopy

For electron microscopy 200- or 400-mesh grids coated with 1% Formvar and carbon were used. Whole hyphae and cell fractions were fixed for 3 hr at 4°C in the formaldehyde-glutaraldehyde fixative of Graham and Karnovsky (1966) diluted with 3 parts of 0.1 M sodium cacodylate-6.7 mM calcium chloride, pH 6.2. The sample was then rinsed four times with veronal acetate buffer (Kellenberger, Ryter, and Sechaud, 1958), the last rinse being overnight. Finally, the samples were postfixed for 2 hr at room temperature with 1% osmium tetroxide in veronal acetate and stained with 0.5% uranyl acetate for 2 hr at room temperature in the same buffer. After a graded dehydration in ethanol, the embedding procedure of Luft (1961) was used. Sections were stained with uranyl acetate and lead citrate (Karnovsky, 1961).

For negative staining, the grids were ionized just before use. The specimen was placed on the grid in succinate buffer and stained with 2% sodium silicotungstate in succinate buffer.

General Culture Conditions

All conidia were produced and handled as described by Luck (1963). Cultures were inoculated with 7×10^5 conidia per milliliter. Hyphae were grown either in 100–150-ml samples in 1 liter Florence flasks aerated by shaking as described by Luck (1963) or as 3-liter cultures in 4-liter carboys under forced aeration at 25°C (Rifkin, Wood, and Luck, 1967).

Isolation Procedure

For routine preparations of paracrystals, 25 μM ethidium bromide was added to carboys of Em 5256 4 hr after the conidial inoculation. When the paracrystal was to be radioactively labeled, leucine-^{3}H was added 20 hr after the inoculation of conidia. After 2 days of growth, the hyphae were collected by suction filtration and washed with succinate buffer containing 20% sucrose. A volume of gluculase equal to 10% of the wet weight of the hyphal pad was then added and the mixture was incubated at 32°C for 20 min to give a dispersed, frothy suspension of hyphae. The hyphae were collected by centrifugation for 5 min at 3000 rpm (7500 $g \cdot min_{ave}$) at 10°C in 50 ml Nalgene tubes in a refrigerated International Centrifuge PR2. The supernatant, which contained very few paracrystals, was discarded. The pellet was resuspended in an equal volume of succinate buffer plus 1% Lubrol WX, and the clumped hyphae dispersed with a spatula. This time the hyphae were removed by a 7500 $g \cdot min_{ave}$ centrifugation, and the supernate kept. The hyphae were usually extracted three more times until very few paracrystals were left. In some preparations a blender was used to rupture the hyphae before the last extraction.

The supernatants were pooled, and SDS added to a final concentration of 0.5% at room temperature. The mixture was then centrifuged in a Spinco No. 40 rotor for 15 min at 15,000 rpm (225,000 $g \cdot min_{ave}$) at 4°C to give a crude pellet (PI). This pellet was

[1] *Abbreviations used in this paper:* ABN-1, abnormal-1-4a; dpm, disintegrations per minute; PAMU, a mixture of phenol, acetic acid, β-mercaptoethanol, and urea; PI, the first crude pellet of paracrystals; PII, the recrystallized paracrystal preparation; PP, paracrystal protein; SDS, sodium dodecyl sulfate.

resuspended in succinate buffer and again centrifuged for 15 min at 15,000 rpm. The pellet was then resuspended in phosphate buffer to give a solution of 1–2 mg of protein/ml and dialyzed overnight at 4° against that buffer. All the particulate material left in the bag after dialysis was removed after a 225,000 $g \cdot min_{ave}$ centrifugation. The supernate was then dialyzed for 24 hr against succinate buffer at 4°C, and the floccular precipitate collected by a 225,000 $g \cdot min_{ave}$ centrifugation to give a fraction referred to as PII.

Electrophoresis

ELECTROPHORESIS IN PAMU: Electrophoresis in PAMU was performed on 7.5% polyacrylamide gels. 5.0-mm inside diameter glass tubes were filled to a height of 7 cm with the acrylamide solution described by Catsimpoolas et al., 1968. 35% acetic acid was layered over the gel solution; the tubes, sealed with Parafilm (American Can Co., Neenah, Wis.) at one end, were incubated for 1 hr in a 37°C water bath. The polymerized gels were then removed from the tubes and stirred with at least four changes of PAMU for 3 days. PAMU is the mixture of 200 g phenol, 100 ml glacial acetic acid, 100 ml 0.2 M β-mercaptoethanol, and 150 g urea. This solvent causes severe burns and should be handled only with gloves. The clear gels were then placed in 6-mm inside diameter glass tubes which were constricted at the lower end. The tubes were inserted into a Buchler polyanalyst, and the reservoirs filled with 10% acetic acid. The samples were layered on the gels in as much as 100 μl of PAM (200 g phenol, 100 ml glacial acetic acid, and 100 ml 0.2 M β-mercaptoethanol). Electrophoresis was performed at 5 mA per gel at 37°C with cytochrome *c* as a visible marker. After electrophoresis the protein was fixed overnight in 15% trichloroacetic acid. If the gels were to be stained, 0.5% amido-Schwartz in 7% acetic acid was added after which the gels were destained in 10% acetic acid. The gels were scanned with a Gilford spectrophotometer 240. Mobility was measured relative to cytochrome *c* (R_c).

DISC ELECTROPHORESIS: The method of Davis (1964) was used for disc electrophoresis. The running gel contained 5% acrylamide-0.3% *N*,N′-methylene bisacrylamide, while the stacking gel contained 2.5% acrylamide-0.85% *N*,N′-methylene bisacrylamide. The sample was loaded in sucrose, and electrophoresis was performed at 20°C. The gels were stained with bromphenol blue-mercuric chloride (100 mg bromphenol blue, 50 g mercuric chloride, 100 ml glacial acetic acid, and 850 ml water). Mobility was measured relative to the front as indicated by bromphenol blue (R_f).

Measurement of Radioactivity

Radioactivity in PAMU gels was measured after the protein had been fixed. The gels were washed with at least three 10-ml portions of succinate buffer over 24 hr, then sliced into 0.9 mm–wide discs which were digested overnight in 0.1 ml of 30% hydrogen peroxide at 50°C. Gels used for disc electrophoresis were sliced and digested without previous fixation. The digested gel discs and all other samples were made up to 0.2 ml of water and dissolved in 0.5 ml of Nuclear-Chicago solubilizer (NCS) (Amersham-Searle Corp., Des Plains, Ill.), 10 ml of toluene, and 50 mg of 2,5-diphenyloxazole (Amersham-Searle Corp.). Scintillation counting was done in a Beckman Liquid Scintillation Counter (LS-250).

Immunological Procedures

IMMUNIZATION: 2 mg of PI or 0.1 mg of the protein in the R_f 0.5–0.65 region of the disc gel in complete Freund's adjuvant (Difco Laboratories, Philadelphia, Pa.) were injected above the shoulders of New Zealand brown hares. The animals were then challenged either twice at weekly intervals with 2 mg of PI or three times at monthly intervals with 0.1 mg of the electrophoretically purified protein.

QUANTITATIVE IMMUNODIFFUSION: The amount of PP in a crude mixture of proteins was determined by immunodiffusion. Glass plates (8.3 × 10.2 cm, Kodak) were covered with 15 ml of 1% agarose, 10% glycerol, 0.02% sodium azide, and 10 mM sodium phosphate, pH 7.5. A row of wells 7.5 mm apart were punched in the gel and filled alternately with the serial dilutions of the unknown and 5 μl of a standard anti-PI serum. By using a PP preparation of known purity (shown in Fig. 6), we found that 0.2–0.4 μg of PP would give the minimum discernible immunoprecipitin line. Therefore, the dilution of the unknown which gave the last detectable line was considered to have 0.3 μg of PP in the applied volume. Wells containing a PP preparation were added to give identity reactions when identification of the immunoprecipitin could be in doubt.

Extraction of Cell Fractions

Mitochondria were isolated by differential and isopycnic centrifugation according to Luck (1963). Soluble proteins were here defined as those *Neurospora* proteins soluble in phosphate buffer. The hyphae in this case were broken by homogenizing 1 vol of filtered hyphae, 2 vol of phosphate buffer, and 6 vol of glass beads (0.11–0.12 mm, B. Braun Apparatebau, Melsungen, Germany) at 0°C for 1 min at high speed in a Sorval Omni-Mixer. The homogenate was then centrifuged for 1 hr at 40,000 rpm in a Spinco No. 40

rotor to remove all membranous organelles. The supernatant protein was termed soluble protein.

RESULTS

Induction of Inclusion Formation in Wild-Type Cultures

In order to test the hypothesis that the presence of the needle-like inclusions in abnormal-1 was associated with defects in mitochondrial function, we grew wild-type *Neurospora* in the presence of drugs known to act selectively on the mitochondrial genome. Ethidium was chosen because it was known to be the most efficient inducer of the cytoplasmic "petite" mutation in yeast (Slonimski, Perrodin, and Croft, 1968). When ethidium bromide (25 μM final concentration) was added to shaken cultures of wild-type *Neurospora*, the cytochrome content changed; and after 8 hr it resembled that of abnormal-1 mitochondria (Table I). After the culture had grown for 20 hr in ethidium, needle-like cytoplasmic inclusions were easily seen by light microscopy (Fig. 1). When viewed with the electron microscope (Figs. 2 and 3) these inclusions consisted of bundles of parallel fibers of indeterminate length, approximately 70 A wide, showing a center-to-center spacing of ~130 A in cross-sections. Because the array is periodic, but imperfect, we will refer to the inclusion as a paracrystal. The paracrystals present in ethidium-treated cells are structurally indistinguishable from those in ABN-1, and as shown in Fig. 2 occur free in the cytoplasm and occasionally within the nucleus. The mitochondria of ethidium-treated cells appear strikingly different from those of wild-type (Fig. 2) and closely resemble those of ABN-1 (Tatum and Luck, 1967).

TABLE I

Mitochondrial Cytochrome Content

The cytochrome content of mitochondria was estimated from the ox-red. difference spectra according to the method of Rifkin (1969). Dyes were added to shaken cultures 4 hr after the conidia were inoculated.

Culture	mμ moles of cytochrome/mg mitochondrial protein		
	$a + a_3$	b	$c + c_1$
Em 5256*	0.45	0.81	0.96
Abnormal-1*	0.01	0.29	2.01
Em 5256 in 25 μM ethidium	0.04	0.23	2.06
Em 5256 in 2 μM euflavine	0.02	0.15	2.48
Em 5256 in 63 μM proflavine	0.33	0.77	0.47‡

* Tatum and Luck, 1967.

‡ The low cytochrome $c + c_1$ content here may reflect loss of cytochrome c during the preparation of mitochondria. This hemoprotein is readily lost from damaged mitochondria; and since these mitochondria contain large amounts of proflavine, they may suffer damage during cell disruption and fractionation.

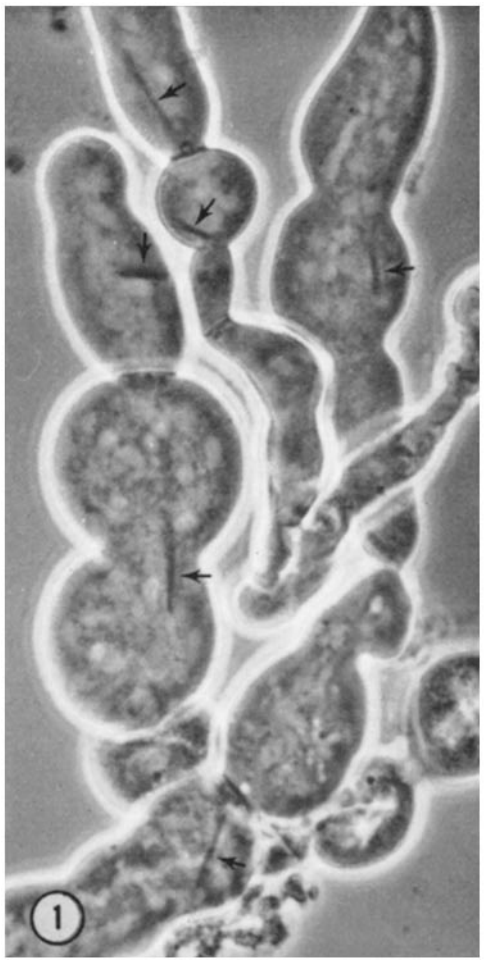


FIGURE 1 Light micrograph of an ethidium-treated culture. The culture was examined 24 hr after inoculation of conidia and 20 hr after addition of ethidium bromide (25 μM). Arrows point to the typical needle-like inclusions. Phase-contrast optics. × 2100.

Despite the similarity of ethidium-treated wild type and abnormal-1, they are not identical. The changes brought about by ethidium are only phenotypic. Unlike the situation in yeast (Slonimski, Perrodin, and Croft, 1968), they are reversed when the cultures are shifted to ethidium-free medium.

Although these results were compatible with the hypothesis that the presence of the paracrystal was related to a mitochondrial defect, it was possible that its occurrence was the result of slow growth resulting from the mitochondrial defect. A decision could be made between these two alternatives based on the effects of acridine dyes. After wild-type *Neurospora* was grown for 26 hr in 2 mM euflavine (3,6-diamino-10-methyl acridine) as described in Table I, the culture had an abnormal mitochondrial cytochrome content and numerous paracrystals identified by both light- and electron microscopy. At that time the culture had a dry mass 70% of that of untreated cultures. In contrast, 63 μM proflavine (3,6-diamino acridine) under the same conditions failed to induce paracrystals or strikingly alter the mitochondrial cytochrome content even though the dry mass accumulation was only 15% that of untreated cultures. These observations support the hypothesis that the presence of the paracrystal is related to a mitochondrial defect, rather than to slow growth itself.

Isolation of Paracrystals

Ethidium-treated wild-type cultures were used for paracrystal isolation since they provided a richer and more reproducible source than did abnormal-1. Since preliminary microdissection studies had indicated that the inclusions were fragile, they were released by osmotic shock rather than mechanical disruption. To improve the efficiency of lysis, the cell wall was partially digested with gluculase, and the plasma membrane was weakened with Lubrol. To rid the preparations of extensive contamination by nuclei-extracts were treated with SDS at a final concen, tration of 0.5%, and the paracrystals were separated from mitochondria by differential centrifugation.

The crude pellet (PI) obtained by this procedure contained needle-like, positively birefringent paracrystals when examined by light microscopy. Electron microscopy showed this pellet to be composed predominantly of paracrystals. Fig. 4 shows representative views from a thin section taken through the entire pellet. In the lower part of the pellet (Fig. 4 A) the preponderant structures were large bundles of parallel fibers with a center-to-center distance of about 130 A. At the top of the pellet (Fig. 4 B) the predominant structures were paracrystal fragments. Mitochondrial fragments and ergosterol crystals (Tsuda and Tatum, 1961) were the principal contaminants.

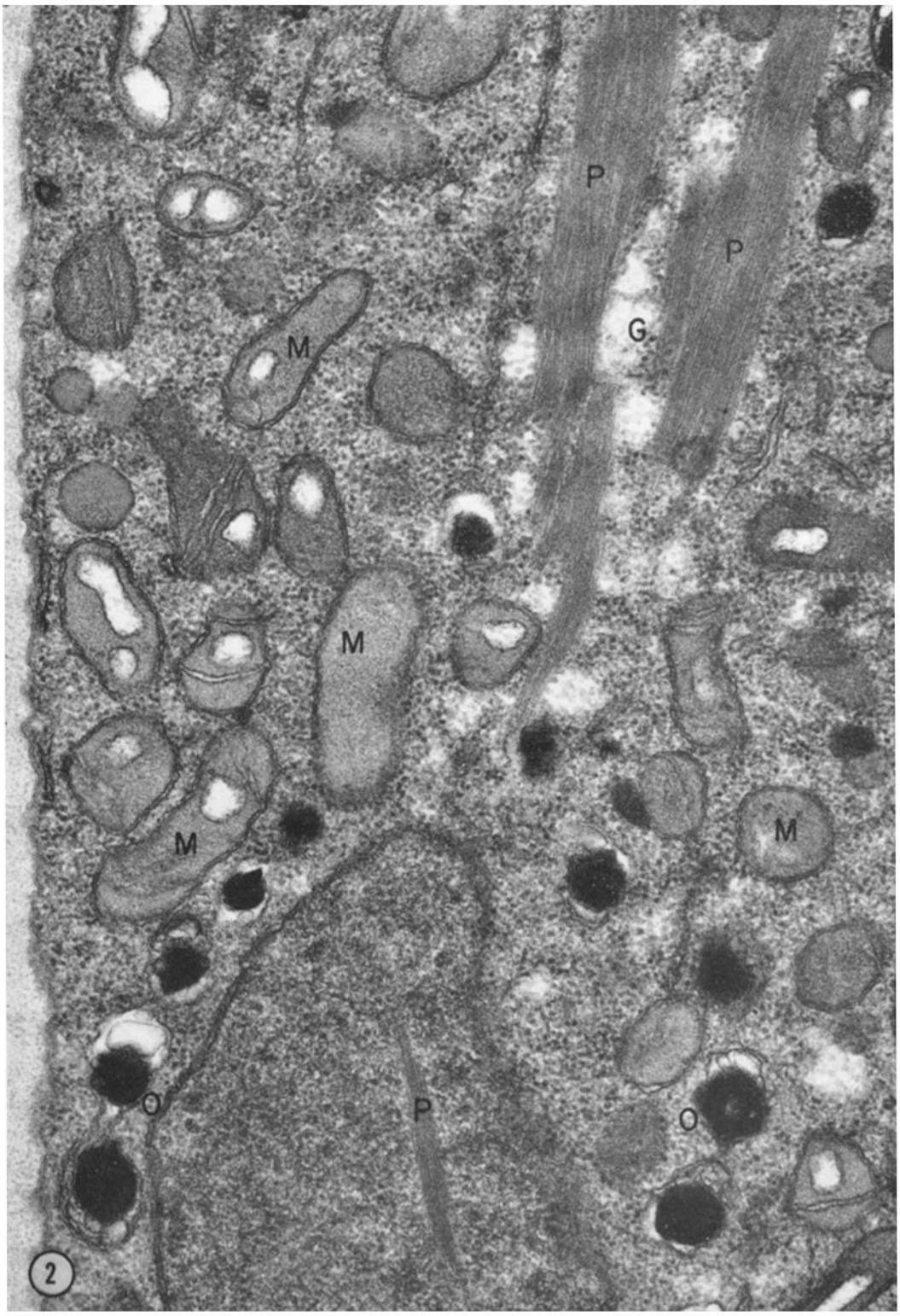
The protein content of PI estimated by the method of Lowry et al. (1541) with bovine serum albumin as a standard, accounted for 85–90% of the dry mass of the preparation. Throughout the remainder of the work the protein component of the paracrystal will be referred to as PP.

Appearance of Paracrystals in Negatively Stained Preparations

When PI was resuspended in succinate buffer and stained with silicotungstate for electron microscopy, the image shown in Fig. 5 was found. This material is certainly the paracrystal. It was present whether PI was made from abnormal-1, euflavine-treated cells, or ethidium-treated cells and was the only fibrous structure in PI. It is composed of parallel fibers (about 85 A in diameter) with a center-to-center spacing of 100–140 A. These dimensions correspond roughly to the diameter and spacing of the fibers seen *in situ*. Each fiber is composed of rectangular units (approximately 55 A long and 75 A in diameter) which are arranged end-to-end along the fiber. The distance from the center of one unit to the center of the next along the fiber is 69 ± 3 A. The units are arranged in side-to-side register. The fact that this side-to-side interaction survived cell fractionation and negative staining indicates that it is strong enough to stabilize the *in situ* paracrystalline structure.

Recrystallization of PI

The paracrystalline fibers dissociated when PI was dialyzed against a low–ionic strength buffer with a pH above 7 (for example: Tris, phosphate, or borate). After the insoluble contaminants were removed by centrifugation, a white precipitate could be formed by dialyzing the solution against succinate buffer. Electron microscopy of this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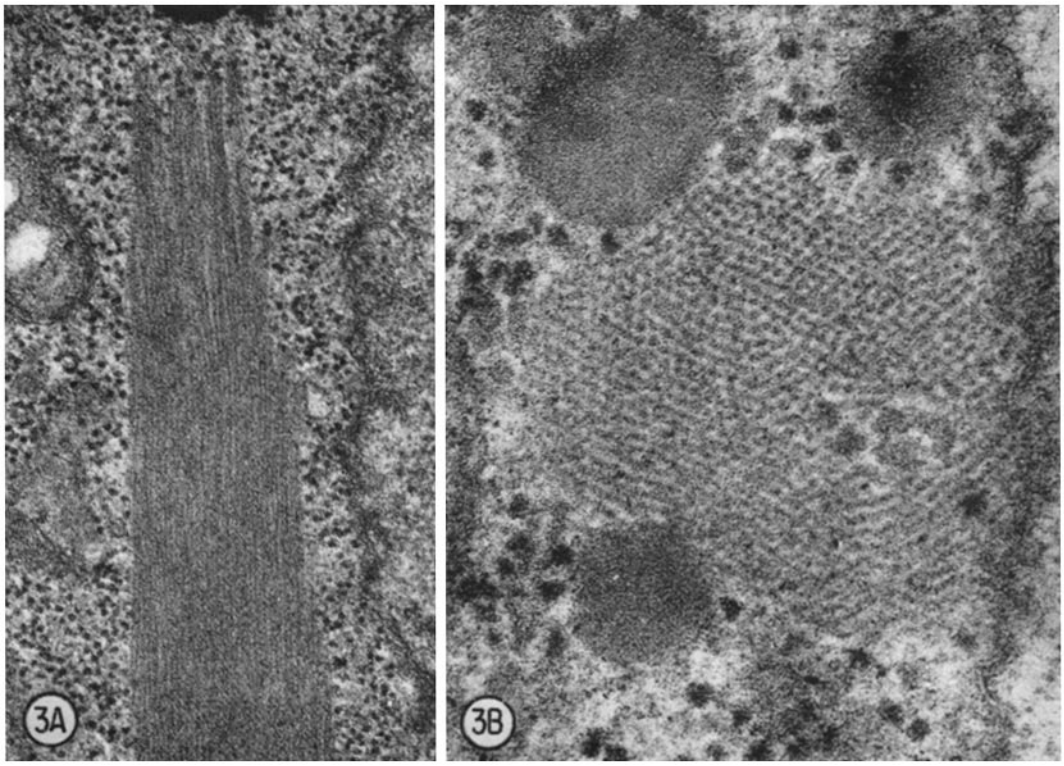
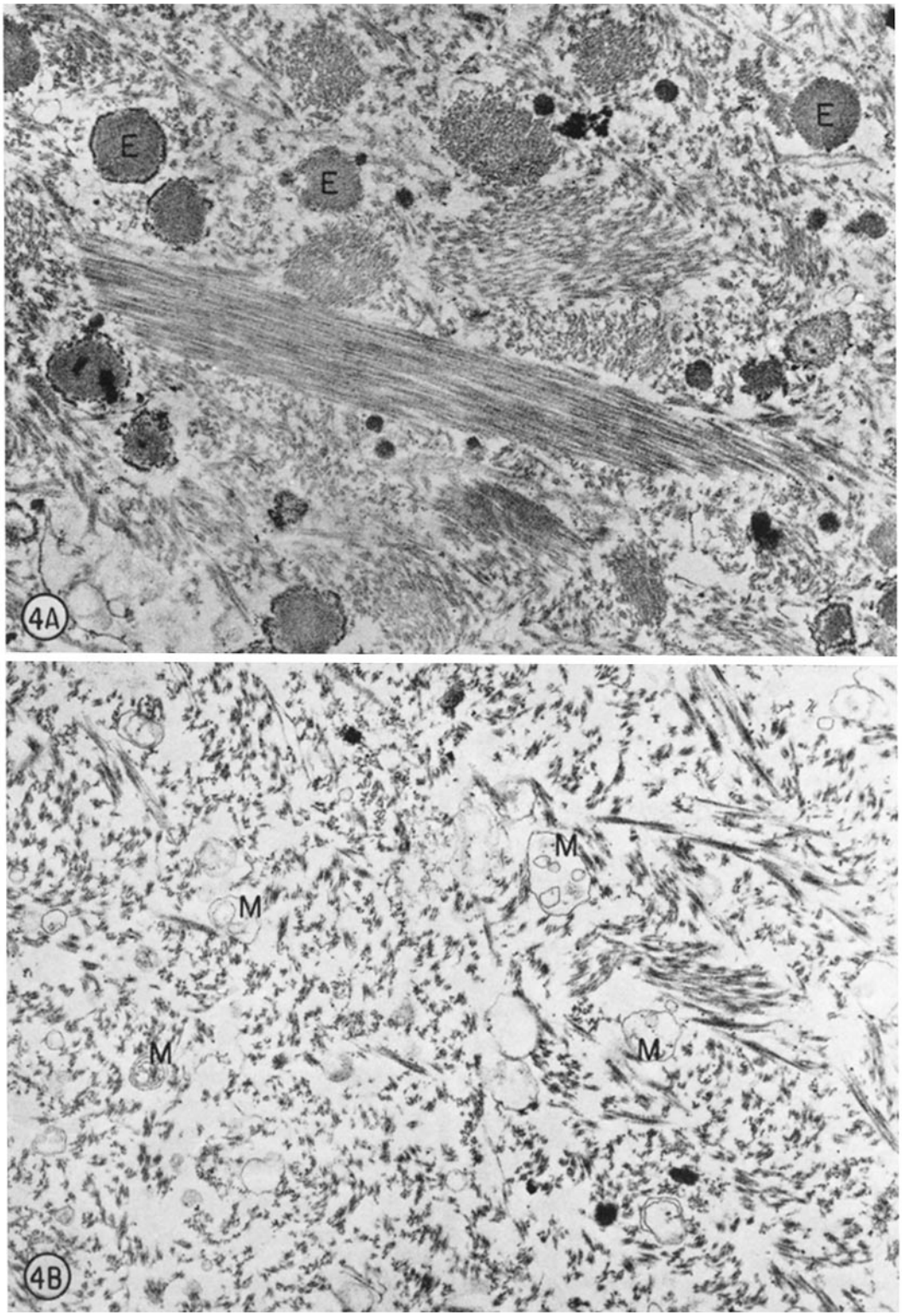


FIGURE 3 Electron micrographs of paracrystalline inclusions. A: Longitudinal section through a paracrystal in an ethidium-treated culture (as in Fig. 2). Fibers of indeterminate length are arranged in parallel. From micrographs at higher magnification the fibers are estimated to be approximately 70 A wide. × 56,000. B: Cross-section through a paracrystal inclusion in abnormal-1. The fibers are seen here as dots where the section is truly perpendicular. Although a very small region of the upper segment of the paracrystal gives the appearance of having an hexagonal lattice it may not accurately describe the true structural arrangement. The pattern is not always encountered and its appearance may represent a compression artifact from sectioning. × 100,000.

material (PII) showed that it had the typical fibrous structure of the PP.

PII was 90% protein by the procedure of Lowry et al. (1951), 14.6% nitrogen by micro-Kjeldahl, and 3.5% ash. The average amount of protein recovered was 1.5% of that in the original lysate and 60% of that in PI. The cycle of dissociation and recrystallization could be repeated

FIGURE 2 Electron micrograph of an ethidium-treated culture. Samples were taken 48 hr after inoculation of conidia and 44 hr after addition of ethidium bromide (25 μM). Typical paracrystals (*P*) are visible in the nucleus and much more frequently in the cytoplasm. Here the cytoplasmic paracrystal is surrounded by glycogen deposits (*G*) and ribosomes. The intranuclear structure is smaller and appears to consist of only a few fibers. The mitochondrial profiles (*M*) have a strikingly different appearance from wild-type organelles. The number of cristae per profile is small and in some cases no cristae are seen. When present, the cristae often show a fixation artifact not encountered in wild type; namely a dilatation of the intracristal space. In structural form these mitochondria closely resemble those seen in abnormal-1 (Tatum and Luck, 1967). A number of osmophilic inclusions (*O*) are visible. In many cases (lower left corner of the micrograph for example) it is clear that they are within mitochondria. These large osmophilic inclusions are seen in ethidium and euflavine-treated cultures but not abnormal-1. × 45,000.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many times; but because of the substantial losses of protein during the recrystallization step, the cycle was usually done only once.[2]

PII contained little of the SDS used in the initial stages of preparation. Using SDS-^{35}S, we found less than 380 ng of SDS/mg of PII. Since the molecular weight of the PP as estimated by electrophoresis in SDS is 66,000–70,000 (Wood, 1970), the SDS content is less than 1 mole per 10 moles of PP.

Electrophoresis of PP in PAMU

In view of our interest in identifying the PP as a possible component of mitochondria, we tested the behavior of the PP during electrophoresis on polyacrylamide in phenol-acetic acid-β-mercaptoethanol-urea (PAMU). This method provides sharp resolution of many water insoluble proteins on the basis of both charge and molecular weight (Work, 1964; Takayama, et al., 1964; Catsimpoolas, et al., 1968; Wood, 1970). Fig. 6 shows the profile obtained when a leucine-^{3}H–labeled PII preparation was analyzed by electrophoresis in PAMU. 88% of the radioactivity in the load penetrated the gel. Of the protein penetrating, 79% of the radioactivity and 69% of the amido-Schwartz staining (OD 540) was located in a band with a mobility relative to cytochrome *c* of 0.31 ± 0.02 (R_c = 0.31). The remainder of the protein was located in minor discrete bands, none of which accounted for more than 7% of the load. Some of these minor bands appear to be degradation products of the R_c 0.31 band because, when the preparation shown in Fig. 6 was reanalyzed after 6 months of storage in phosphate buffer at 4°C, the R_c 0.31 band was reduced to 25% of its initial value with a commensurate increase in the minor bands and the background. These results led us to the tentative conclusion that the R_c 0.31 band was the PP.

Preparation of Antibodies to the PP

We anticipated that a second approach to the identification of the PP in cell fractions would make use of specific antibodies. Rabbit antisera prepared against PI from ethidium-treated cultures gave a single precipitin line by immunodiffusion when assayed against PII from other ethidium-treated cultures. Furthre, this precipitin line gave complete fusion with the lines produced by PII from euflavine-treated cultures or abnormal-1. Although the results suggested that the antiserum reacted only with PP, the major component of the test antigen, the presence of other antibody specificities, could not be excluded.

To obtain a more homogeneous antigen for antiserum production, PII was subjected to disc electrophoresis. Using the same leucine-^{3}H–labeled preparation analyzed in Fig. 6, we found that only 52% of the radioactivity penetrated the disc gel and of that 45% migrated as a single band with a mobility relative to the front of 0.55 ± 0.03 (R_f = 0.55) (Fig. 7). The remaining fraction of the penetrating radioactivity was found as a poorly resolved haze of low mobility. Additional evidence suggests that the protein which did not enter the gel and the low mobility material was rich in PP in the form of large aggregates.[3]

[2] The loss of protein is in part accounted for by its solubility in succinate buffer. Even though the pH of the succinate buffer was chosen to correspond to the isoelectric point of the PP as determined by isoelectric focussing (Wood, 1970), it was estimated by quantitative immunodiffusion and by concentration and reprecipitation of the succinate supernate that succinate buffer can dissolve 150–190 μg of PP/ml.

[3] An analysis of the electrophoresis data shows that 69.5% of the PII migrated as a single band in dissociating PAMU system but only 23.4% of the same preparation did so in the nondenaturing disc system. This leaves 50% of the protein unaccounted for. When a PII preparation was subjected to centrifuga-

FIGURE 4 Electron micrograph of a thin section through PI. PI, obtained from an ethidium-treated culture, was fixed, and oriented in an embedding block so that the entire depth of the pellet was represented in each thin section (Luck, 1962). A: A representative field taken from the bottom of the pellet. Paracrystal bundles are seen in longitudinal, oblique, and cross-section. The predominant contaminants in this zone are ergosterol crystals (*E*) (Tsuda and Tatum, 1961). × 20,000. B: A representative field taken from the top of the pellet. The paracrystal appears mainly in the form of fragments. The major contaminants in this zone are membrane vesicles (*M*) which appear to be derived from mitochondria. × 20,000.

When anti-PI serum was allowed to immunodiffuse against a gel from disc electrophoresis, a very distinct immunoprecipitin line was formed with the protein migrating with an R_f of 0.55 (Fig. 8 A). On the basis of these results, we assumed that the R_f 0.55 band represented a purified preparation of the PP. When the R_f 0.50–0.65 region of the gel was used to immunize rabbits, an antiserum was obtained which gave a single immunoprecipitin line against PII (Fig. 8 B). As expected this line showed complete fusion with the one produced by the cruder anti-PI serum.

Consolidation of Techniques

We have described several independent characteristics of the paracrystal preparation (paracrystalline structure, antigenicity, and electrophoretic behavior) which we have assumed to be characteristics of the paracrystal protein itself. We will now show that these characteristics are indeed all dependent on a single protein. The R_f 0.55 band from disc gel electrophoresis could be eluted in phosphate buffer and precipitated with succinate buffer. When examined in the electron microscope with negative staining, typical paracrystalline structures (see Fig. 5) were found. The R_f 0.33 and 0.80 regions of the gel failed to yield these structures when treated in the same way. Hence, the major disc gel band, the antigen used for the production of specific antibody, could be clearly identified as a paracrystal forming protein.

Making use of the anti-R_f 0.55–0.65 serum, we were able to establish the identity of the major band in PAMU electrophoresis. The antibody was mixed with PII; the immunoprecipitate was collected, solubilized in PAMU, and analyzed by electrophoresis in PAMU (Fig. 9). The major band at R_c 0.31 was enriched in this precipitate suggesting that it was indeed the PP. Two minor bands were also enriched. As noted before these minor bands are likely to be degradation products of the PP.

PP as a Mitochondrial Component

Following the hypothesis that the presence of the paracrystalline inclusion was correlated with mitochondrial defects, we examined the mitochondria of wild-type *Neurospora* for the presence of PP. This was first attempted by dialyzing purified mitochondria against phosphate buffer for 1 wk and then precipitating the solubilized protein with succinate buffer. The resulting pellet showed no paracrystalline structure in the electron microscope; nor, when assayed by quantitative immunodiffusion, did it show any antigenic activity characteristic of the paracrystal. In this case the assay could have detected PP present as 0.3% of the mitochondrial protein.

Because lipids may have interfered with the solubilization of the PP by phosphate buffer, mitochondria were extracted with 90% acetone and the precipitated protein tested for PP by quantitative immunodiffusion. Although the assay again was capable of detecting the PP present as less than 0.3% of the mitochondrial protein, none was detected. In a sample of mitochondria to which PII had been added before acetone extraction, full antigenic activity was recovered. These results indicated that if the PP were associated with mitochondria, its properties were masked.

As a final test for the PP in mitochondria, we analyzed authentic PP and solubilized mitochondrial proteins together by PAMU electrophoresis. For this experiment mitochondria were labeled by growth for six generations in medium supplemented with a mixture of amino acids-^{14}C. These mitochondria were mixed with leucine-^{3}H–labeled PP and solubilized in 10% SDS. To simplify the electrophoretic pattern, the mixture was passed over Bio-Gel P300 (Bio-Rad Laboratories, Richmond, Calif.) in phosphate buffer with 1% SDS. Several fractions around the elution peak of PP (K_d 0.20) were then further analyzed by electrophoresis in PAMU. The distribution of radioactivity in these gels gave no indication that the mitochondria contained PP. Instead the ^{14}C counts which superimposed on the tritium peak appeared to represent the leading or trailing edges of nonsuperimposing mitochondrial proteins. Nevertheless, even if the superimposed

tion on a sucrose density gradient buffered as is the stacking gel for disc electrophoresis, the protein spread across the gradient. When the gradient fractions were analyzed for PP by quantitative immunodiffusion, it was found that about 50% of the PP sedimented with a velocity greater than that expected for its molecular weight. Probably the polymeric PP detected by centrifugation is the same protein which appeared as a low mobility haze and a nonpenetrating fraction during disc electrophoresis.

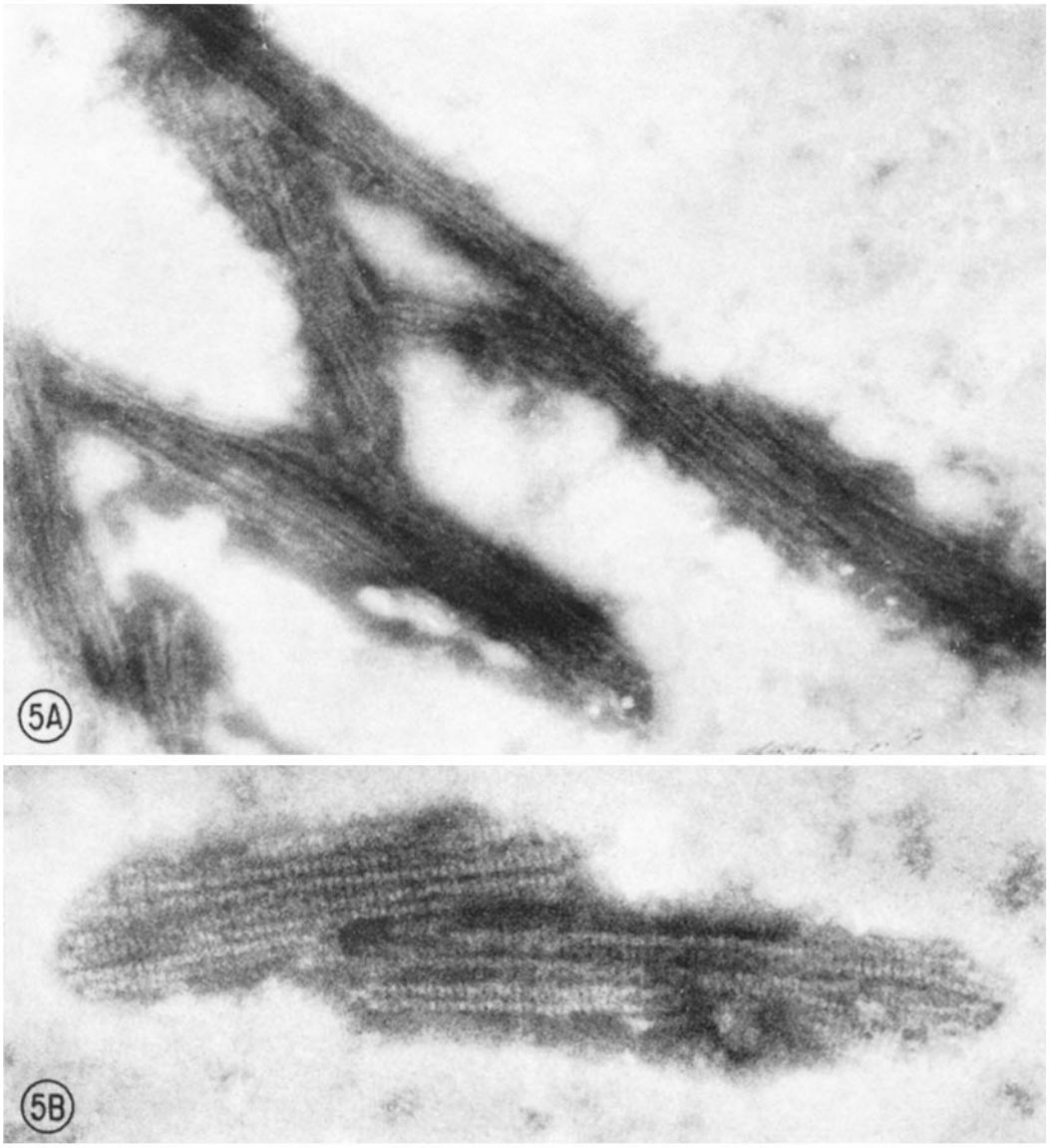

FIGURE 5 Electron micrograph of negatively stained paracrystal. A: A sample of the PI fraction shown in Fig. 4 was resuspended in succinate buffer and stained with sodium silicotungstate. In this representative field the fibrous nature of the material is apparent. × 100,000. B: The R_f 0.5–0.65 regions of several disc gels (see Consolidation of Techniques in Results) were pooled and eluted with 10 mM sodium phosphate, pH 7.5. The precipitate obtained by dialysis of the eluate against succinate buffer was stained with sodium silicotungstate. At this magnification the detailed subunit structure typical of all paracrystal preparations is apparent. The fibers appear to be composed of subunits ~66 A long. The subunits appear to be in register and the center-to-center spacing of the fibers is ~110 A. × 200,000.

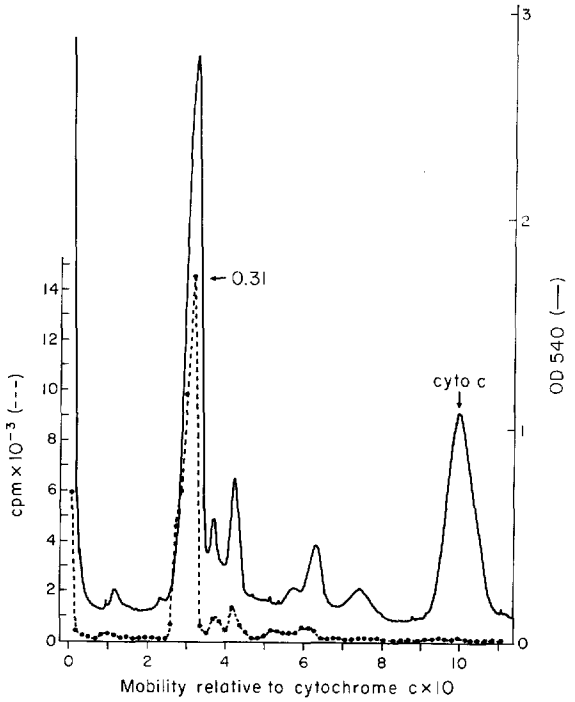

FIGURE 6 Electrophoresis of PII in PAMU. 150 μg (1.4×10^5 disintegrations per minute [dpm]) of a leucine-^{3}H–labeled PII preparation from an ethidium-treated culture were analyzed by electrophoresis on each of two polyacrylamide gels containing PAMU. One was stained with amido-Schwartz, and the optical density of the gel at 540 mμ scanned. The distribution of radioactivity was measured in slices taken from the other. The distribution of slices and the OD scan were matched at the origin and at the cytochrome *c* peak. The peaks of OD which contain no label are the cytochrome *c* monomer (R_c 1.0) and dimer (R_c 0.75). The background in the OD 540 tracing is due to light scattering by the gel.

^{14}C counts were considered to represent mitochondrial PP, it would constitute less than 1.7% of the mitochondrial protein.

Localization of PP in the Soluble Fraction

The three methods used to detect PP in mitochondria failed to give positive evidence of its presence. If present, the PP is a minor component of mitochondrial protein. This was not the case for the nonparticulate cell fraction obtained after homogenization of wild-type cultures. When the "soluble" fraction was assayed by quantitative immunodiffusion, the amount of PP rose from an undetectable amount to 4% of the total soluble protein during the time that the culture was moving from end log phase to stationary phase (Fig. 10). Using the same technique, cultures treated with ethidium or euflavine showed a PP content of ~1% at 20 hr and ~12% at 52 hr. At both times the dye-treated cultures contained abundant crystals and the values reflected the total, crystalline plus soluble, PP content.

The immunologically active material detected in Fig. 10 could be shown to be indistinguishable from PP. If the soluble protein from a stationary phase wild-type culture was dialyzed against succinate buffer, 80% of the immunologically active material precipitated. This precipitate contained a large amount of protein with an R_c of 0.31, characteristic of the PP. Finally, the immunoprecipitate formed by adding anti-R_f 0.5–0.65 antiserum to the soluble fraction of a 52 hr wild-type culture and analyzed by electrophoresis in PAMU was found to contain a major band at R_c 0.31.

DISCUSSION

Paracrystalline inclusions have been shown to occur in wild-type *Neurospora* after treatment with ethidium bromide and euflavine. Reviewing the earlier euflavine studies of Freese-Bautz (1957), it seems likely that the structures she identified by light microscopy to be abnormal mitochondria were in fact paracrystals. We have isolated these

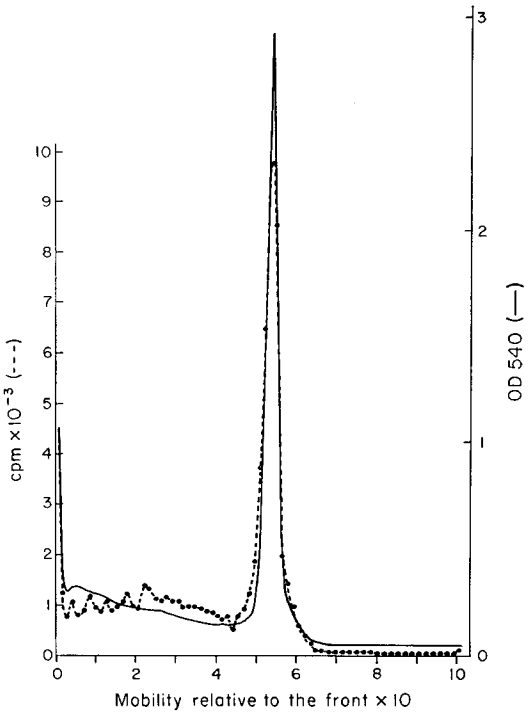

FIGURE 7 Disc electrophoresis of PII. Approximately 400 μg (370,000 dpm) of the same leucine-^{3}H-labeled PII preparation shown in Fig. 6 was analyzed on each of two disc gels. One was stained with bromphenol blue-mercuric chloride and scanned at 540 mμ. The distribution of radioactivity was measured in the other. The two patterns were superimposed by matching the origin and the front.

inducible paracrystals and shown that in ultrastructure, by electrophoretic properties, and by immunological cross reactivity they are identical to the inclusions of abnormal-1. Using the isolated paracrystals, we have been able to draw several conclusions concerning their structure and function.

Structure

The simplest model of the paracrystal which is compatible with our data is one in which a single type of polypeptide undergoes self-aggregation into tetramers which in turn arrange to form fibers. We believe that the protein of the paracrystal is a single polypeptide chain because in the PAMU system a single electrophoretic band (R_c = 0.31) can account for 70% of the protein present in the PII preparation. In this preparation no other band accounted for as much as $\frac{1}{10}$ of that amount. In other words, any additional polypeptide would have to be either of low-molecular weight or be present in less than stoichiometric amounts with the R_c = 0.31 polypeptide. Probably four molecules of PP aggregate to form the units seen by negative staining in the electron microscope. These units exclude the stain from a volume 55 A long and 75 A in diameter. Since the PP has a molecular weight of 68,000 and a density of 1.29 g/cc (Wood, 1970), it follows that each unit is composed of about 4.6 PP.

We believe that the correct number is 4 rather than 5 on geometric grounds. These units arrange end-to-end to form fibers and side-to-side to arrange those fibers into a lattice. The nature of this lattice is uncertain; however, it appears to be either square or hexagonal. If it is assumed that the number of PP in each unit is reflected in the structure of the lattice, then it is to be expected that the fiber lattice might be square.

Function

The evidence presented in this paper suggests that the PP is a nuclear gene product which can crystallize as a result of a mitochondrial defect. The strongest evidence for this conclusion came from our study of drug induction of the paracrystal. We found that euflavine could induce the paracrystal formation while proflavine, which differs from euflavine only in the absence of a methyl group, could not. Euflavine and proflavine kill *Neurospora* at the same concentration; however, while proflavine shows a monophasic inhibitory effect on growth, euflavine shows a diphasic effect the first part of which occurs at $\frac{1}{10}$ of the concentration of proflavine needed to produce the same degree of inhibition (Wood, 1970). This suggests that euflavine and proflavine share nonspecific toxic properties and that euflavine has an additional specific effect. Since this specific effect is probably the inhibition of reactions involving mitochondrial DNA with little effect on reactions involving nuclear DNA (Meyer, et al., 1969; Meyer and Simpson, 1969; South and Mahler, 1968; Kellerman, Biggs, and Linnane, 1969), it seems likely that the PP is a nuclear gene product which crystallizes because of the lack of a mitochondrial gene product. The fact that ethidium also appears to have a specific effect on mitochondria (Knight, 1969; Zylber, Vesco, and Penman, 1969; Zylber and Penman, 1969) adds weight to this conclusion.

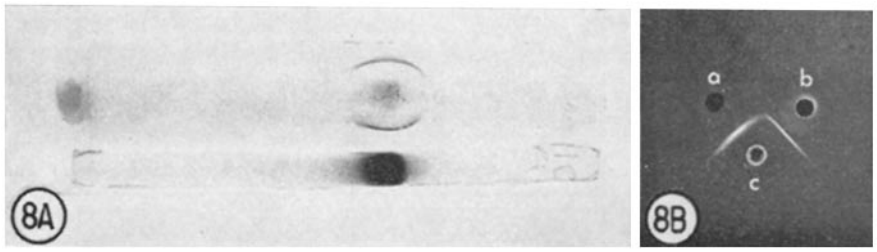

FIGURE 8 A Disc immunoelectrophoresis of PII. A PII preparation from an ethidium-treated culture was analyzed by disc electrophoresis. The gel was sliced longitudinally, and one slice embedded in agar (as pictured here the electrophoretic origin is at the left). Two troughs were cut parallel to, and 5 mm from the gel slice. In the two troughs were placed 60 μl (lower) and 180 μl (upper) of anti-PI serum. After 2 days at 4°C soluble proteins were removed with phosphate-saline and the immunoprecipitate was stained with bromphenol blue-mercuric chloride. In the lower part of the photograph a second gel slice stained immediately provides a marker for the distribution of protein. Electrophoretic migration was toward the cathode.

FIGURE 8 B Comparison of immunological activity of anti-PI and anti-R_f 0.5–0.65 sera. Well *a* contained anti-PI serum, well *b* contained anti-R_f 0.5–0.65 serum, and well *c*, PII from an ethidium-treated culture. Immunodiffusion was allowed to procede for 2 days in the agar medium described for quantitative immunodiffusion and the unstained preparation was photographed.

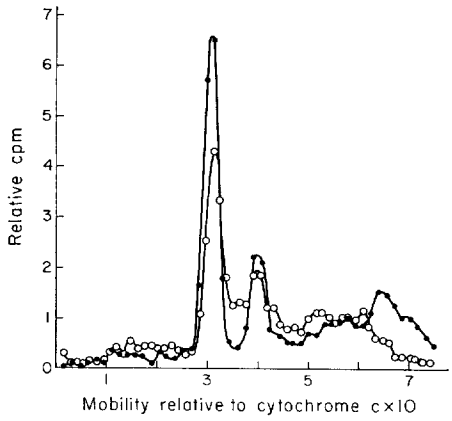

FIGURE 9 Electrophoresis of an immunoprecipitate in PAMU. Anti-R_f 0.50–0.65 serum was added to tritium-labeled PII, and the resulting immunoprecipitate collected by centrifugation. The pellet was washed once with phosphate-saline and taken up in PAM. The immunoprecipitate (●) was analyzed on one gel, and the original PII (○) on another. The gels were then sliced and counted. The distribution of radioactivity has been plotted so that the area under the two curves is the same.

Although this evidence suggests that the paracrystal formation can result from the lack of a mitochondrial gene product, not every mitochondrial mutant contains the paracrystal. For example, *poky* has no paracrystal although it has a mitochondrial cytochrome content indistinguishable from that of euflavine or ethidium-treated wild type of abnormal-1 (Rifkin, 1969). We do not know the reason for the absence of the paracrystal in poky. It could be that poky simply has the crucial gene product which the other cultures presumably lack or, since poky is known to recover a normal cytochrome spectrum with time (Rifkin, 1969; Haskins, et al., 1953), it could be that the PP is never concentrated enough to crystallize before recovery starts.

There is also at least one case in which the paracrystal is known to appear in a wild-type culture growing in the usual sucrose supplemented minimal medium. Beck, Decker, and Greenawalt, 1970, reported the presence of paracrystal in the wild-type SY7A between 4 and 10 hr after inoculation of conidia. Interpretation of this finding is difficult because no cytochrome analyses are given and no biochemical data are provided. In these experiments the paracrystal was observed early in the growth of SY7A, a time when comparable Em 5256 cultures contain no immunologically detectible PP. The paracrystal disappeared from SY7A by 20 hr when the accumulation of PP was beginning in Em 5256. We do not know the reason for these differences, but it is interesting to note that SY7A spontaneously gave rise to abnormal-2, which is phenotypically related to abnormal-1. The occurrence of the paracrystal in SY7A may be related to genetic differences between SY7A and Em 5256.

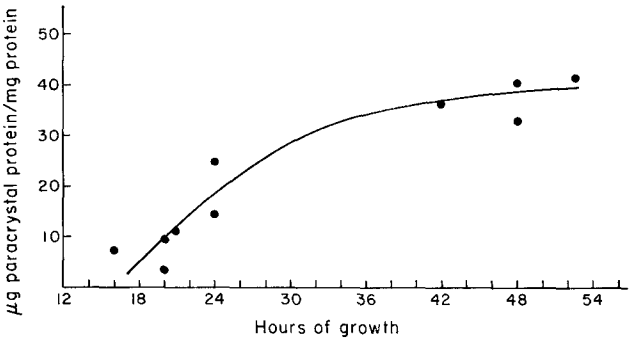

FIGURE 10 Paracrystal protein in the soluble fraction of homogenates of wild type *Neurospora*. A soluble fraction was obtained from wild-type cultures as described in Methods. The total protein was determined by the method of Lowry et al. (1951) and the amount of paracrystalline protein measured by quantitative immunodiffusion. No PP was detected between 1 and 12 hr.

The conclusion that the PP is a nuclear gene product frequently occurring as a result of a mitochondrial defect lead us to assay for the PP in two cell fractions of wild-type *Neurospora*: the mitochondria and the soluble proteins. By immunological techniques and by dissociation and chemical assay, we found no detectable PP in the mitochondria although it was present as 4% of the soluble proteins. Since there is about 10 times as much soluble protein as mitochondrial protein in the cell, our estimates indicate that at late stages of growth there is at least 20 times more soluble PP then mitochondrial PP. We feel that such an excess makes it unlikely that the soluble PP is serving as a precursor pool for insoluble mitochondrial protein. However it must be noted that in our experiments, soluble protein is not only cytoplasmic protein but also includes all proteins lost from organelles upon disruption of cells and dilution in 10 mM phosphate, pH 7.5. The possibility that PP is an easily detached component of mitochondria or other cell organelles cannot be excluded.

In an attempt to identify the PP, we studied several of its properties and compared them with the properties of known proteins (Wood, 1970). Because of the ability of the PP to form fibers, we wondered if it might be an actin-like protein; however, very little nucleotide could be released from PII by perchloric acid, and PII bound no nucleotide triphosphates when solubilized in phosphate buffer.

As previously described the PP had a molecular weight of about 68,000, an isoelectric point of about 5.3, and a crystal structure composed of rows of subunits each of which is probably composed of four polypeptide chains. Since these properties are shared by bovine catalase (Hruban and Swift, 1964), we wondered whether the PP might be the apoenzyme of *Neurospora* catalase; however, this was not the case. In *Neurospora*, catalase activity is found in the soluble fraction Treatment of the soluble protein from a 52 hr culture with anti-R_f 0.5-65 antiserum precipitates all the immunologically active PP without decreasing the catalase activity.

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BIBLIOGRAPHY

BECK, D. P., G. L. DECKER, and J. W. GREENAWALT. 1970. *J. Ultrastruct. Res.* **33**:245.

CATSIMPOOLAS, N., C. EKENSTUM, D. A. ROGERS, and E. W. MEYER. 1968. *Biochim. Biophys. Acta.* **168**:122.

DAVIS, B. J. 1964. *Ann. N.Y. Acad. Sci.* **121**:404.

DIACUMAKOS, E. G., L. GARNJOBST, and E. L. TATUM. 1965. *J. Cell Biol.* **26**:427.

FREESE-BAUTZ, E. 1957. *J. Cell Comp. Physiol.* **50**:203.

GARNJOBST, L., J. F. WILSON, and E. L. TATUM. 1965. *J. Cell Biol.* **26**:413.

GRAHAM, R. C., and M. J. KARNOVSKY. 1966. *J. Histochem. Cytochem.* **14**:291.

HASKINS, F. A., A. TISSIERES, H. K. MITCHELL, and M. B. MITCHELL. 1953. *J. Biol. Chem.* **200**:819.

HRUBAN, Z., and H. SWIFT. 1964. *Science (Washington).* **146**:1316.

KARNOVSKY, M. J. 1961. *J. Biophys. Biochem. Cytol.* **11**:729.

KELLENBERGER, E., A. RYTER, and J. SECHAUD. 1958. *J. Biophys. Biochem. Cytol.* **4**:671.

KELLERMAN, G. M., D. R. BIGGS, and A. W. LINNANE. 1969. *J. Cell Biol.* **42**:378.

KNIGHT, E., JR. 1969. *Biochemistry.* **8**:5089.

LOWRY, O. H., N. J. ROSEBROUGH, A. L. FARR, and R. J. RANDALL. 1951. *J. Biol. Chem.* **193**:265.

LUCK, D. J. L. 1962. Ph.D. Thesis. The Rockefeller University, New York.

LUCK, D. J. L. 1963. *J. Cell Biol.* **16**:483.

LUFT, J. H. 1961. *J. Biophys. Biochem. Cytol.* **9**:409.

MEYER, R. R., E. BIKOFF, C. C. TEITZ, and S. A. BIEDENBACK. 1969. *J. Cell Biol.* **43**:92 a.

MEYER, R. R., and M. V. SIMPSON. 1969. *Biochem. Biophys. Res. Commun.* **34**:238.

RIFKIN, M. R. 1969. Ph.D. Thesis. The Rockefeller University, New York.

RIFKIN, M. R., D. D. WOOD, and D. J. L. LUCK. 1967. *Proc. Nat. Acad. Sci. U.S.A.* **58**:1025.

SLONIMSKI, P. P., G. PERRODIN, and J. H. CROFT. 1968. *Biochem. Biophys. Res. Commun.* **30**:232.

SOUTH, D. J., and H. R. MAHLER. 1968. *Nature (London).* **218**:1226.

TAKAYAMA, K., D. H. MACLENNAN, A. TZAGOLOFF, and C. D. STONER. 1964. *Arch. Biochem. Biophys.* **114**:223.

TATUM, E. L., and D. J. L. LUCK. 1967. *In* Control Mechanisms in Developmental Processes. M. Locke, editor. Academic Press Inc., New York. 32.

TSUDA, S., and E. L. TATUM. 1961. *J. Biophys. Biochem. Cytol.* **11**:171.

WOOD, D. D. 1970. Ph.D. Thesis. The Rockefeller University, New York.

WORK, T. S. 1964. *J. Mol. Biol.* **10**:544.

ZYLBER, E., and S. PENMAN. 1969. *J. Mol. Biol.* **46**:201.

ZYLBER, E., C. VESCO, and S. PENMAN. 1969. *J. Mol. Biol.* **44**:195.