THE RIBONUCLEOPROTEIN NATURE OF LARGE PARTICLES IN THE MEIOSPORANGIA OF ALLOMYCES*

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The meiosporangia (resistant sporangia) of the watermold *Allomyces* contain particles, called chromospheres, which average 3 to 4 μ in diameter and stain with basic dyes such as methyl green and methylene blue as well as with aceto-orcein (1). Each sporangium contains several hundred chromospheres (Figs. 1 and 2). The chromospheres ultimately disappear (Figs. 3 and 4), a phenomenon which is completed in 24 to 36 hours (1–3). The sporangium is then observed to be filled with dense, granular protoplasm. The objective of this study was to establish the chemical nature of chromospheres.

Materials and Methods

Growth and Collection of Meiosporangia.—The Burma 1Db strain of *Allomyces arbuscula* was used in this study. Stock cultures were carried on YpSs (a yeast extract-soluble starch medium) nutrient agar slants (4) with periodic mycelial transfers. To obtain meiosporangia in quantity, large numbers of YpSs nutrient agar plates were inoculated at the center with mycelial blocks. After the plates became covered with mycelium they were flooded with distilled water and the sporangia scraped from the surface with the edge of a glass slide.

Coarse debris was removed, and the sporangia were dislodged from the mycelium, by forcing the suspension through a 50 mesh stainless steel screen with a jet of distilled water. The suspension was then centrifuged at approximately 3,000 R.P.M. for 5 minutes as often as necessary until a preparation of microscopically pure, intact meiosporangia was obtained; the heavier meiosporangia sedimented first, followed by the mitosporangia and mycelial fragments. The final material was dried at 40°C. under vacuum.

Extraction Procedures.—Purines, pyrimidines, nucleosides, nucleotides, RNA, and DNA were extracted by the procedure of Ogur *et al.* (8), modified in two respects. The removal of alcohol-ether soluble material was done with only three successive extractions with boiling 3:1 alcohol-ethyl ether. Secondly, the procedure was terminated after the simultaneous removal of DNA and RNA by the hot 5 per cent perchloric acid extractions. The more elaborate preliminary extractions of Ogur *et al.* (8) were found to be unnecessary, and the differential

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extraction of RNA and DNA by use of cold perchloric acid (9) did not work on whole meiosporangia where it was needed. Since there is no DNA in chromospheres, the separation of the nucleic acids was unnecessary for these analyses. In certain analyses of whole meiosporangia, the DNA and RNA extracted by hot perchloric acid were separately estimated by methods described below.

Analytical Methods.—RNA was estimated by the method of Hahn and von Euler (10) and by Mejbaum's orcinol method (11). DNA was assayed by the diphenylamine reaction (12) and Stumpf's modification of the cysteine-sulfuric acid test (13). In addition, RNA, DNA, purines, pyrimidines, nucleosides, and nucleotides were quantitatively estimated by ultraviolet absorption, using a Beckman DU spectrophotometer.

Total phosphorus was determined in material digested by sulfuric acid (14), by the Gomori method (15), and nitrogen by micro-Kjeldahl procedures (16). Free amino acids in meiosporangia were extracted (17) and estimated by a quantitative ninhydrin test (18).

The bases present in the RNA from chromospheres were identified chromatographically. After perchloric acid digestion of 25 mg. of chromospheres at 100°C. (19) and dilution with distilled water, the material was chromatographed according to Wyatt (20).

Qualitative tests for lipides were made with Sudan IV (21). A saturated 70 per cent ethanol solution of the dye was used in place of that recommended.

RESULTS

Isolation of Chromospheres.—Chromospheres are extremely fragile and fragment into many small pieces when sporangia containing them are crushed in water on a microscope slide. Numerous solutions used in the isolation of cell particles were tested by crushing sporangia in them. Intact chromospheres were obtained only in 0.75 M sucrose and the sucrose-glycerol-$CaCl_2$-phosphate buffer solution of Stanier and Philpot (22). The latter was the most satisfactory; the chromospheres not only emerged intact, but maintained their integrity for several hours. For convenience this solution will be called the SP solution, using the initials of the authors.

Large quantities of intact chromospheres were obtained by grinding sporangia in SP solution with an A. H. Thomas Company No. B-188 tissue homogenizer equipped with a teflon pestle. It was necessary to keep the speed below 30 R.P.M., otherwise the chromospheres were broken. A homogenate of chromospheres prepared as described was centrifuged at 10,000 *g*. The chromospheres, instead of sedimenting, collected at the interphase between the SP solution and the layer of fat derived from the sporangia. The fat appeared to protect the chromospheres against disintegration. Advantage was taken of this observation by adding oleic acid to the SP solution during homogenization. As a result of the increased stability of the chromospheres, the homogenizer could be run at moderately high speeds without disintegration of the particles.

The resultant mixture of chromospheres, SP solution, and oleic acid was centrifuged at 10,000 *g* at 0°C. By the end of the run, the oleic acid was solidified and was removed as a plug with the chromospheres embedded in the lower end of the plug. The purification was continued by slicing off the end of the plug, resuspending in the mixture of SP solution and oleic acid, and recentrifuging. This process was repeated up to eight times.

Subsequently, a more effective and much faster purification procedure was devised. The sporangia were homogenized as described above. The homogenate was then passed very slowly into a column (6 inches long and 0.5 inch in diameter) of fine quartz sand saturated with oleic acid. After the homogenate had penetrated into the top of the column, the chromospheres were selectively eluted off by passing into the column alternate small quantities of SP solution and oleic acid. The chromospheres passed through the column into the collecting flask, while the sporangial debris was retained. Neither SP solution alone nor oleic acid alone were satisfactory eluants.

The preparations obtained were examined microscopically for cellular debris and found to be almost, but not completely, free of wall material. The RNA content of the preparations was not increased by more than five successive centrifugations of homogenate or by more than one passage through the column

TABLE I

The Nucleic Acid Composition of Chromospheres

(per cent dry weight)

Test*	Isolated by column (No. of passages)		Isolated by centrifugation (No. of centrifugations)	
	1	2	5	8
RNA-UV absorption	12.7	10.4	10.7	10.2
RNA-orcinol	15.5	14.1	10.5	12.4
RNA-phosphorus	15.6	12.5	12.1	13.6

* Tests made on the hot 5 per cent perchloric acid extract (see text). No DNA was detected by either the cysteine or diphenylamine tests.

(Table I). After isolation, the chromospheres were washed three times under acetone to remove the oleic acid, and then dried under vacuum at 40°C.

Analysis of Chromospheres.—As shown in Table I, about 12 per cent of the dry weight of chromospheres is RNA with no DNA present. When chromospheres were digested with perchloric acid and the digest chromatographed (as much as 5 mg. of digested chromosphere material was spotted), only guanine, cytosine, adenine, and uracil were found; thymine was absent. The absence of DNA in chromospheres is in agreement with the lack of nuclear contamination indicated by the microscopic examination of the material. The results also indicate that the chromosphere RNA, grossly speaking, is of typical composition.

Before proceeding with the presentation of the chemical balance sheet on the composition of chromospheres, the question of the presence of lipides requires attention. These would be lost during the acetone washing except for phospholipides. When sporangia were crushed in Sudan IV, however, the

chromospheres did not stain. It would appear, therefore, that they contain very little or no lipides.

Three separate samples of chromospheres were analyzed, with the results as tabulated in Table II. The values given are averages, there being good agreement among the samples. They have been calculated on the basis of 10 mg. dry weight of chromospheres for ease of conversion to percentages.

The dry chromospheres contain 12.7 per cent nitrogen and 1.78 per cent phosphorus. The alcohol-ether extractions removed 6.5 per cent of the dry weight, but none of the nitrogen or phosphorus, thus indicating the absence of phospholipides. The combined hot and cold perchloric acid extracts accounted

TABLE II

*Analysis of Chromospheres**

Fraction	N	P	Dry weight
	μg.	μg.	mg.
Isolated chromospheres	1,270.0	178.0	10.00
Alcohol-ether extract	0.0	0.0	0.65
Cold 2 per cent perchloric acid extract	45.0‡	24.0	3.20§
Hot 5 per cent perchloric acid extract	190.0	128.0	
Final residue	960.0	0.0	6.15

* Average of analyses of three samples of chromospheres.

‡ Highest of three values (see text).

§ Calculated as the difference.

for 32 per cent of the weight (by difference), with the residue constituting 61.5 per cent by direct weighing.

The cold perchloric acid extract contained 15.8 per cent of the total phosphorus and the equivalent of 200 to 250 μg. RNA based on UV absorbance at 260 mμ. The agreement between these two measures indicates the phosphorus to be primarily nucleotide phosphorus. The nitrogen estimation on the cold perchloric acid extracts was unreliable because of the small amount present. The figure given, 45 μg., was the highest of the three and probably the most dependable. The remaining phosphorus (to the extent of an 85.4 per cent total recovery) was found in the hot perchloric acid extract. Both this and the nitrogen value agree well with the RNA content. The perchloric acid extracts are presumed to contain most of the impurities in the chromosphere preparation, since the weight of these fractions is only partially accounted for by nucleotides and RNA.

The residue, after perchloric acid extractions, constituted 61.5 per cent of the weight of the chromospheres and contained 75.6 per cent of the total nitrogen, but no phosphorus. The residue was soluble in hot strong acid and strong alkali and was equivalent to 6.0 mg. protein (N × 6.25). The actual weight of the residue was 6.15 mg. The nitrogen and phosphorus found in the fractions accounted for 94.5 per cent and 85.4 per cent, respectively, of the total amounts present in the chromospheres.

In summary, 72 per cent of the weight of the chromospheres was accounted for as RNA (12.2 per cent) and protein (60.0 per cent). The alcohol-ether ex-

TABLE III

Analyses of Meiosporangia with and without Chromospheres

	Amounts, *mg. per 100 mg. sample*	
	Sporangia with chromospheres	Sporangia without chromospheres
Cold 2 per cent perchloric acid extract in RNA equivalent:		
UV absorbance	0.285	0.435
Orcinol	0.03–0.05	0.25–0.32
Cysteine (DNA test)	*	*
Hot 5 per cent perchloric acid extract:		
UV absorbance (RNA)	3.75–4.18	3.85–4.15
Orcinol (RNA)	3.74–4.07	3.70–3.85
Phloroglucinol (RNA)	3.63–4.02	3.95–4.18
Cysteine (DNA)	0.33–0.47	0.47–0.50
Free amino acid amino nitrogen:	0.165–0.168	0.207–0.230

* Too little to measure.

tractible material amounted to 6.5 per cent, leaving about 21 per cent of the dry weight unknown and presumed to be wall fragments. Such fragments were observed in the preparations and could not be eliminated.

Chromosphere Disappearance.—The disappearance of the chromospheres described previously and shown in Figs. 3 and 4 was initially ascribed to hydrolysis of the chromosphere ribonucleoprotein. To test this possibility, sporangia with and without chromospheres were analyzed for free amino acids, cold 2 per cent perchloric acid extractibles, RNA, and DNA, with the results listed in Table III. Although the free amino acids, cold perchloric acid extractibles, and DNA all increased upon disappearance of chromospheres, the absolute changes were extremely small. At the same time, there was no detectable change in RNA. In making these analyses it was necessary, before harvesting, to dip the meiosporangia in 95 per cent ethanol for 60 seconds to

prevent germination of the mature sporangia upon contact with water. Suitable tests showed the alcohol treatment not to affect the analyses. Sporangia without chromospheres contain many small granules. These were found to stain strongly with methyl green, methylene blue, and aceto-orcein, as do the chromospheres.

DISCUSSION

The Purification Method.—The separation of the chromospheres from the other sporangial components by passage through a column of oleic acid-saturated sand appears to be theoretically similar to the technique independently developed by Albertsson (23) for the purification of *Chlorella pyrenoidosa* and several of its components. The procedure is based on the partition between two liquid phases previously agitated with the particles to be separated. Albertsson explains the separation on the basis of J. N. Brönsted's theory (as described in Albertsson (23)), which indicates that if a liquid-liquid system is shaken with a suspension of high molecular weight particles, all the particles should be found in one phase or the other or at the interphase. Albertsson attributes the separation to differences in interfacial tension between the liquids, their interphase, and the particles. In the column used in the present study the chromospheres appear to collect at the oleic acid-SP solution interphase and are carried rapidly through the stationary sand column. The debris, mostly sporangia walls, presumably adheres to the oleic acid-coated sand particles and, therefore, moves slowly through the column. This method, with suitable choice of liquid, should be adaptable to the separation of other kinds of particles.

The Chromospheres.—Although the preparations of chromospheres analyzed in this study were as free of extraneous matter as the methods allowed, it is, nevertheless, true that careful microscopic inspection always revealed an occasional fragment of sporangial walls. It appears reasonable, therefore, that the unidentified portion of the samples was wall material and that the chromospheres are pure or nearly pure ribonucleoprotein. Compared to other ribonucleoprotein particles, chromospheres are unprecedented in size. The individual chromosphere, when examined carefully with a phase microscope, appears to be one homogeneous particle.

Sporangia from which chromospheres have disappeared contain as much RNA as those with chromospheres. Although both the cold perchloric acid extract and the free amino acid pool increased with chromosphere disappearance, the absolute amounts of the increases were small. These data eliminate the possibility that chromosphere disappearance is a hydrolysis of the nucleoprotein to its constituent amino acids and nucleic acid components. The chromosphereless sporangia are heavily populated with granules (Fig. 4) which take the same stains as the chromospheres. It seems very likely that the disappearance of the chromospheres is a process of fragmentation.

Relation of Chromospheres to Postmeiotic Chromospheres and Nuclear Caps.—Chromospheres disappear from meiosporangia after periods of a few days to several weeks from the time of development of the sporangia. The exact period depends on the strain of the organism and the cultural conditions (1–3). The sporangia will not germinate (when placed in water) until the chromospheres have disappeared. As the chromospheres disappear, the diploid nuclei are seen to be in the prophase of meiosis I. Meiosis is arrested here for as long as the meiosoporangia are kept dry—a period which can be many years. Wetting initiates the completion of meiosis with the formation of about four dozen haploid nuclei. There then appear in the meiosporangia, particles, labelled postmeiotic chromospheres (1), which collect around the nuclei and ultimately form cup-shaped structures which become the nuclear caps of the spores, one for each spore. The protoplast then cleaves, the sporangium splits, and the motile spores are released, thus completing the cycle (1–7). While this work was in progress, Turian (24) showed cytochemically and with the use of ribonuclease that the nuclear cap contains RNA. Some years earlier Hatch (25) found the nuclear cap to be Feulgen-negative and thought it to be formed by the aggregation of short rods which he called chondriosomes. More recently Turian and Kellenberger (29) have shown the mitochrondria to be quite distinct from the nuclear cap. Hatch (25) and Emerson (4) have both noted that when swarmers germinate, the nuclear caps disintegrate. The resulting numerous granules cause difficulties in nuclear studies because they stain very deeply with most of the usual nuclear stains (4). These observations strongly suggest that nuclear caps, and in meiosporangia, their predecessors the postmeiotic chromospheres, are also ribonucleoprotein like the chromospheres. Further, it seems likely that postmeiotic chromospheres are reassembled chromospheres.

The Function of Chromospheres and Nuclear Caps.—Previous speculation on the function of nuclear caps has related them to motility (1), to growth (26), to food reserves (27), and to metabolism (28). The chromosphere cycle, on the other hand, has been related to the dormancy of the meiosporangia. Emerson and Wilson (6) believed that the disappearance of the chromospheres marked the end of the dormancy of the sporangia. Later, Machlis and Ossia (2) found that although sporangia do not germinate as long as chromospheres are present, the disappearance of chromospheres did not, in itself, assure meiosporangia capable of germination. Thus in one experiment in which about 90 per cent of the sporangia were free of chromospheres, only about 50 per cent germinated when placed in water.

A possible role of both chromospheres and nuclear caps is suggested by both their composition and time of occurrence in the life cycle. These nucleoprotein bodies are found in the meiosporangia and in the swarmers—structures released from the parent hyphae. The free meiosporangium when it germinates increases its nuclei four times by meiosis; the swarmers, after they become immobile, begin vegetative growth, giving rise to the mycelium. Preceding meiosis in the meiosporangium and vegetative development by the swarmers, the nucleoprotein bodies fragment into granules which have the same staining characteristics as the nuclear caps and chromospheres. We suggest that the granules derived from chromospheres and nuclear caps are concerned with the synthesis of proteins (by analogy to the role of other nucleoprotein bodies in protein synthesis) which must take place during both meiosis and spore germination. The speculation that the granules have synthetic activity is supported by no evidence at present and must await proof in the form of their isolation and testing for biosyn-

thetic capacity. An alternative speculation is that chromospheres and nuclear caps are functionally similar to nucleoli on the basis that all three particles fragment into granules prior to nuclear divisions.

There would appear to be at least three reasons why germination of meiosporangia must be preceded by fragmentation of the chromospheres. First, the granules presumed to be necessary for the metabolism accompanying meiosis are formed. Second, the fragmentation makes possible the reassembling of the nucleoprotein into the nuclear caps. Finally, it would appear (Figs. 1 and 2) that as long as chromospheres are present, there is no room for meiosis to take place. Rather than considering chromospheres and their fragmentation as the cause and termination of the dormancy of meiosporangia, respectively, we suggest that dormancy is independently controlled and that the mechanism includes a process which initiates chromosphere fragmentation. To test this speculation external means for causing the granulation of chromospheres at will need to be discovered.

SUMMARY

Particles averaging 3 to 4 μ in diameter, which are called chromospheres and fill the immature meiosporangia of the watermold *Allomyces*, were isolated and analyzed. The preparations were obtained by repeated centrifugations or by passage of the homogenate into a column of sand saturated with oleic acid, followed by selective elution of the chromospheres with alternate layers of oleic acid and an aqueous solution.

The chromospheres contain approximately 12 per cent RNA, no DNA, and 60 per cent protein. It was concluded that they are pure or nearly pure ribonucleoprotein. Analysis of meiosporangia with chromospheres and after the chromospheres have disappeared showed no significant change in RNA or free amino acids. It was concluded that chromosphere disappearance is a fragmentation into small granules.

The relation of chromospheres to postmeiotic chromospheres and nuclear caps is discussed. Speculation as to the function of these bodies is presented.

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EXPLANATION OF PLATE 288

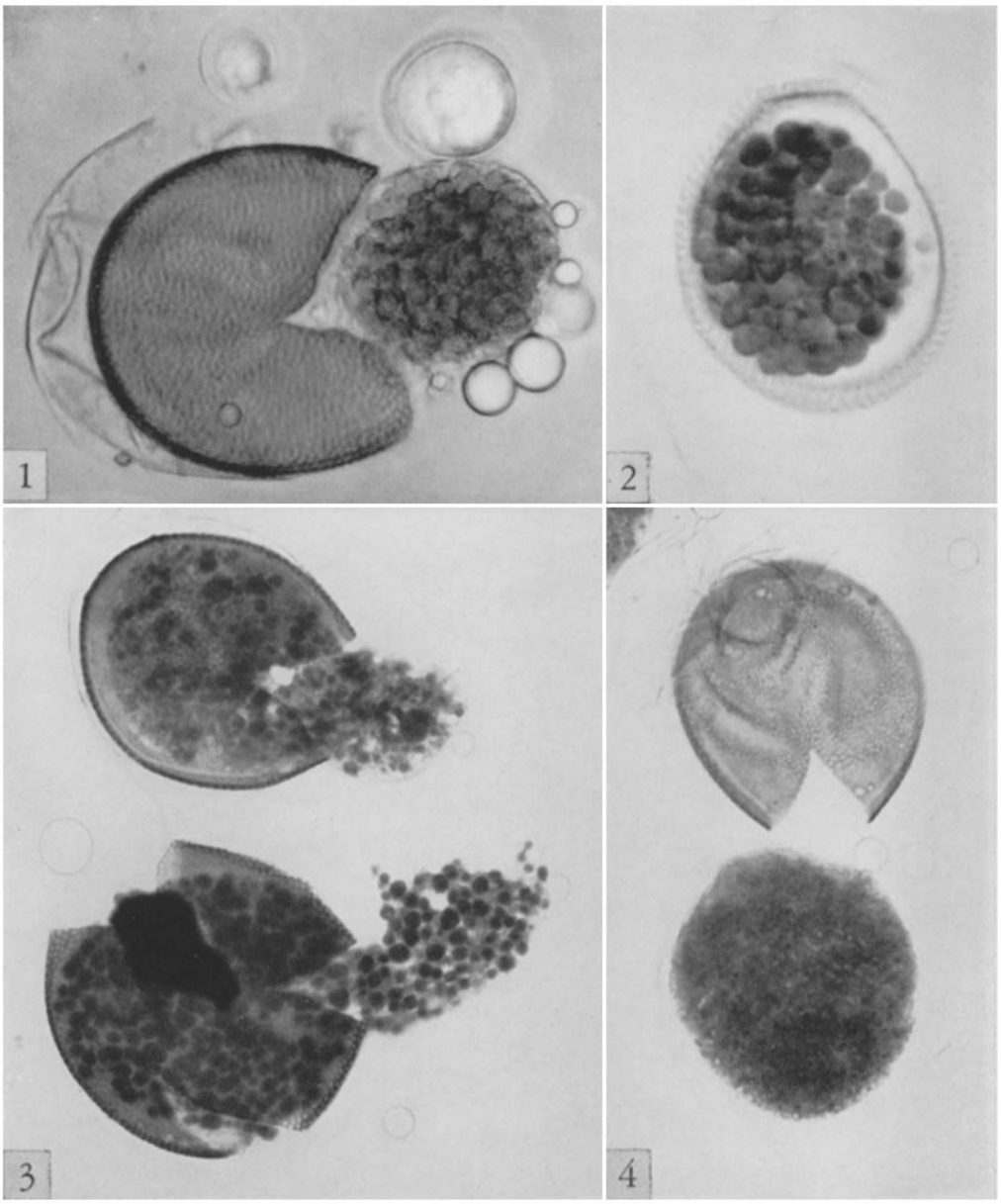
FIGS. 1 to 4. Meiosporangia with chromospheres, with chromospheres in the process of fragmentation, and after fragmentation of chromospheres. The meiosporangia are all approximately 37 × 43 μ.

FIG. 1. A crushed fresh sporangium stained with methyl green. Note the fat globules formed from natural fatty materials present in meiosporangia.

FIG. 2. A 5 μ section through a sporangium stained with safranin after fixation, dehydration, and embedding.

FIG. 3. Crushed fresh sporangia stained with methylene blue, in which the chromospheres are disintegrating. Note the eroded appearance of the chromospheres, particularly in the upper sporangium.

FIG. 4. Fresh sporangia, after chromosphere disappearance, stained with methylene blue and crushed. Note the densely granular appearance of the protoplasm.



(Rorem and Machlis: Ribonucleoprotein in meiosporangia)