JOURNAL OF BACTERIOLOGY, Nov. 1970, p. 922-932

Vol. 104, No. 2
Printed in U.S.A.

Chemical Composition of Chlamydospores of *Candida albicans*

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Received for publication 30 July 1970

A variety of analytical techniques was employed to study the composition of the chlamydospore of *Candida albicans*. The outer, thin, electron-transparent layer was found to be composed of glucan, together with a small amount of chitin. The inner, thick, electron-dense layer is proteinaceous. The central structure is composed largely of ribonucleic acid and lipid globules. In addition to being acid-fast, the chlamydospore was found to contain glycolipids and to lose the property of acid-fastness on extraction with ethanol-ether.

The previous paper (10) described the technique employed to obtain essentially uniform preparations of the chlamydospore-suspensor (C-S) cell from *Candida albicans* in amounts sufficient for chemical analyses. It was also shown that the outer layer of the chlamydospore wall is continuous with the wall of the suspensor cell. Therefore, the C-S pair may be regarded as a "unit" for analytical purposes. Furthermore, the fragility of chlamydospores precluded mechanical separation of chlamydospores from suspensor cells. In this paper we present data on the chemical composition of the C-S complex derived from cytochemical studies, sequential enzymatic hydrolysis, and chemical fractionation.

MATERIALS AND METHODS

Microbiological procedures. The strain of *C. albicans* I.H.M. 582, and culture procedures for obtaining uniform preparations of C-S complex were described in the preceding paper (10).

Staining techniques. Lipids were stained with Sudan Black B (C.I. 26150). Slides with chlamydospores were dehydrated for 3 min in ethylene glycol and stained for 1 hr in a 1% solution of Sudan Black in ethylene glycol. Differentiation was achieved by washing in 85% aqueous ethylene glycol. As counterstain, 1% alcoholic safranin was used (C.I. 50240). A solution of 0.1% aqueous auramine O (C.I. 41000), prepared in 3% aqueous phenol, was also employed as a stain for lipids. Smears were stained for 2 min and were differentiated in 0.25% aqueous safranin for 1 min (1).

Giemsa stain was used to localize nucleic acids. A stock solution was prepared by dissolving 0.5 g of the dye in 33.0 ml of glycerol at 60 C and adding 33.0 ml of methyl alcohol. A working solution was obtained by dilution (1/200) in 1 M phosphate buffer at *p*H 7.0. A 2% solution of acridine orange (C.I. 46005) in 0.02 M acetate buffer, *p*H 4.5, was also used to stain nucleic acids. Chlamydospore smears were prepared in 0.1 M acetate buffer, *p*H 4.5, hydrolyzed in 1 N HCl at 55 C for various times (5 to 120 min), and stained for 2 hr.

The procedure outlined (1) for the periodic acid-Schiff technique was used to detect polysaccharides. Chlamydospore smears were also stained with Lugol's iodine-potassium iodide solution. Two fluorescent dyes, to be detailed later, Aniline blue (C.I. 42755) and primuline (C.I. 49000), were found to become localized in the polysaccharide-containing outer wall layer of the C-S complex. Primuline, when used as a vital dye, was incorporated into chlamydospore-producing medium (1 mg/liter). In the acid-fast staining procedure, the classical Ziehl-Neelsen technique was employed without modification.

To localize protein, a 0.5% solution of Amido Black (naptha blue black; C.I. 20470) was made up in methanol-glacial acetic acid (9:1). Smears were stained for 5 min and then washed in methanol-acetic acid (9:1) for 15 min. Ponceau Xylidine (C.I. No. 16150) dissolved in 5% trichloroacetic acid (200 mg/100 ml) was also employed. Smears of chlamydospores were stained for 2 min and then differentiated in 5% acetic acid for 5 to 10 min.

Enzymatic hydrolysis. A preparation rich in β-1,3-glucanase activity was obtained by the procedure of Skujins et al. (21). *Streptomyces* species no. 3 (kindly supplied by M. Alexander, Cornell University) was maintained on a medium consisting of a 0.5% suspension of distiller's solubles (Brown, Forman Co., Louisville, Ky.) in distilled water, adjusted to *p*H 7.6, and solidified with 3% agar (Difco). For enzyme production, the organism was grown in a basal salts liquid medium (21) to which autoclaved, lyophilized cells of *C. albicans* were added (5 g/liter). This medium (25 liters) was sterilized in a 40-liter fermentor

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for 30 min at 121 C and allowed to cool. A 48-hr culture of *Streptomyces* species no. 3 grown in 500 ml of medium of the same composition served as inoculum. After 48 hr, cell-free broth was obtained by passage through a Sharples centrifuge. The clear broth was concentrated to 2 liters in a Rodney-Hunt Turbafilm evaporator and dialyzed overnight against running tap water. After adjustment to *p*H 5.5 with 0.2 N acetic acid, protein was precipitated at ambient temperature with 65% saturated $(NH_4)_2SO_4$. After dialysis against dilstilled water at 5 C, the protein-rich suspension was passed through a Sephadex G-25 column which retained a brownish impurity. The fraction eluted in the void volume was collected and stored at −20 C, and will be referred to as "glucanase." Levels of enzymatic activity were assayed with laminarin (K & K laboratories, Inc., New York, N.Y.) and pustulan (kindly supplied by B. Lindberg, Stockholm) as substrates. Reducing substances released were estimated by the method of Folin and Malmros (9).

A highly purified preparation of keratinase (EC 3.4.4.25) elaborated by *S. fradiae* was employed (lot KN). This sample of keratinase was prepared by the method of Nickerson and Durand (17).

Unless otherwise specified, cell material subjected to enzymatic hydrolysis and employed for chemical determinations consisted of the C-S complex described in the previous paper (10). C-S preparations (20 mg of lyophilized material) were incubated with glucanase at 37 C in the following system: phosphate buffer, 0.2 M, *p*H 5.5, 2 ml; 2-mercaptoethanol, 1 M, 0.1 ml; ethylenediaminetetracetic acid, 2×10^{-3} M, 0.1 ml; and 0.1 ml of glucanase solution. An enzyme control was incubated without chlamydospores. Phosphate buffer was heat sterilized; all other solutions were passed through membrane filters (Millipore Corp., Bedford, Mass.) and handled aseptically. Merthiolate (1/10,000) (Lilly) was added to prevent bacterial growth during incubation. Enzymatic hydrolysis was allowed to proceed for 10 hr, whereupon the fraction solubilized was separated by centrifugation. Protein was precipitated by 5% trichloroacetic acid which, in turn, was removed by ether extraction. The acid-soluble fraction was evaporated to dryness in vacuo at 45 C and stored at −20 C. This fraction is referred to as C-S glucanase hydrolysate.

The portion of C-S resistant to glucanase was treated with keratinase in the following incubation system at 37 C. Tris(hydroxymethyl)aminomethane buffer, 0.05 M, *p*H 9.0, 2 ml; keratinase, 1 mg/ml, 0.1 ml. After incubation for 10 hr, the soluble fraction was removed by centrifugation and treated as described for glucanase hydrolysate. This fraction is termed C-S keratinase hydrolysate.

Chemical analyses. All chemical analyses were carried out on C-S complex dried to constant weight at 105 C. Lipids of lyophilized C-S were extracted by the method of Anderson as described by Peck (19). Cell material (15 mg) was shaken with 10 ml of a mixture of 95% ethanol and ethyl ether (1:1) for 24 hr at 27 C. The solvent phase was separated by filtration through defatted filter paper. Three extractions were performed, and the residue was extracted twice with chloroform. Extracts were combined and solvents were removed under vacuum. From this concentrated material, lipids were extracted into ether and dried with anhydrous Na_2SO_4, and the ether was evaporated This fraction constitutes the "readily extracted lipids.'· "Bound lipids" were extracted by subjecting the residue from the ethanol-ether and chloroform treatments to digestion in the ethanol-ether mixture 1 N with respect to HCl. Hydrolysis proceeded for 5 hr at 50 C, and was followed by two extractions with a mixture of 95% ethanol-ethyl ether (1:1) and one with chloroform; each extraction lasted 24 hr. The extracts were combined and treated as described for the readily extracted lipid fraction.

For carbohydrate analysis of C-S material, the method of Chung and Nickerson (5) was followed. Four fractions were analyzed: (i) carbohydrates soluble in 10% trichloroacetic acid were obtained by extracting cell material for 10 min at room temperature followed by two washings in distilled water. The trichloroacetic acid extract and washings were combined, and the acid was removed by shaking with ether. This fraction as well as others described below were evaporated to dryness in vacuo at 40 C. (ii) Alkali-soluble carbohydrates were extracted by heating the acid residue in 30% KOH for 30 min in a boiling-water bath. The residue was separated by centrifugation and washed in distilled water, and the washings were combined. (iii) The residue from alkali extraction was suspended in 2 N HAc, heated in a boiling-water bath for 15 min, and then washed with distilled water. (iv) The remaining carbohydrate was dissolved in H_2SO_4-water (2:1).

The total amount of polysaccharide was estimated by a modification of the anthrone procedure of Dreywood (8). Samples (1 ml) containing 20 to 200 μg of glucose equivalents were added to a colorimeter tube. The tube was immersed in an ice bath and agitated while 5 ml of anthrone (0.2% in 75% H_2SO_4) was slowly added. The tubes were heated in boiling water for exactly 10 min, cooled in ice, and read in a Klett colorimeter with a no. 54 filter. Total reducing substances present were determined by the Folin-Malmros method, and glucose content was measured with glucose oxidase (Worthington Biochemical Corp., Freehold, N.J.). The reaction mixture, prepared by following the instructions of the suppliers, was incubated for 30 min at 30 C. The reaction was stopped and color was developed with 6 N HCl. Optical density measurements were made in a Beckman DU spectrophotometer at 540 nm.

For estimation of ribonucleic acid (RNA) content, C-S complex (5 mg) was suspended in 5% perchloric acid at 5 C for 15 min, followed by extraction with the same acid at 90 C, whereupon the extracts were pooled. RNA content was measured by the method of Mejbaum (15). A standard curve was constructed by using RNA (Calbiochem, Los Angeles, Cal.).

Total phosphorus was determined by the procedure of Chen et al. (4) on 2 mg of C-S digested in concentrated H_2SO_4. Total nitrogen was estimated by Nessler's procedure (28) on C-S material digested in H_2SO_4.

Amino acid composition was quantitated in a Technicon analyzer on material hydrolyzed in 6 N

HCl for 16 hr at 105 C in ampules sealed under nitrogen. Analyses were carried out on C-S complex, the residue after hydrolysis of C-S by glucanase, and on three fractions obtained by extraction of C-S: (i) fraction soluble in cold 10% trichloroacetic acid; (ii) the residue from the previous step, suspended in 1 N KOH and heated for 30 min at 100 C; the sediment was separated by centrifugation and washed several times with distilled water; the KOH extract and washings were combined, neutralized, and evaporated to dryness in vacuo at 40 C; and (iii) the washed residue from the previous step was used.

A sample of the hot 1 N KOH extract was neutralized and dialyzed overnight at 5 C. The dialysate was concentrated to dryness in vacuo, dissolved in a minimal volume of distilled water, and divided into two parts. One was assayed on a Technicon peptide column, and the other was hydrolyzed for analysis of amino acid content.

Chromatography. Two solvent systems were employed in descending chromatography: *n*-butanol–pyridine–HAc–water (60:40:3:30) and isopropanol-water-NH_4OH (160:40:1). Whatman no. 1 and no. 3 MM papers were employed as well as thin layers of MN cellulose (Macherey, Nagel & Co., Duren, Germany) prepared as described by Stahl (23). Sugars were detected with alkaline silver nitrate (27) or aniline hydrogen phthalate (2); 6-deoxy sugars were located with meta periodate-Rimini spray (29). The relative proportions of identified monosaccharides were determined by spraying paper chromatograms twice with aniline hydrogen phthalate in water-saturated *n*-butanol, allowing the solvent to evaporate, and heating at 105 C for 6 min. The spots were cut out and eluted with 0.7 N HCl in 80% ethanol for 1 hr with frequent shaking. The optical density at 350 nm of the eluted material was measured with a Beckman DU spectrophotometer. Identical spots were cut out of the background and eluted to serve as blanks. Standard preparations of sugars were used simultaneously.

RESULTS

Cytochemical reactions of chlamydospores. To survey the types of substances present in the C-S complex and, when possible, their cytological distribution, various staining techniques were employed. It is well known that, in many cases, staining is not specific and the chemical basis of the reaction may be poorly understood.

To detect lipid material, C-S preparations were stained with Sudan Black B, auramine O, and osmic acid. The latter stained the entire spore intensely; no differentiation was attempted. Sudan Black B dissolved in ethanol (3) did not penetrate the spore. A similar finding was reported by Weyman-Rzucidlo (30). However, when ethylene glycol was employed as solvent for the stain, black globules were localized inside the spore. Granules which leave the chlamydospore when the cell wall is broken by slight mechanical pressure (Fig. 1) also stained deeply with the dye. After staining with auramine O, slides were differentiated in 3% ethanolic HCl until clearly localized intracellular areas of bright yellow fluorescence could be distinguished. In fully developed cultures, only the center of the spore was fluorescent. In younger cultures, in which chlamydospores were in the process of formation, auramine O stained intracellular material located in the tips of the suspensor cells. A similar pattern was observed when chlamydospores were treated with Ziehl-Neelsen carbol fuchsin. Acid fastness was lost after extraction for 14 hr in ethanol-ether (1:1), as seen in Fig. 2. It has been reported that ether-hydrochloric acid removes an acid-fast staining lipid fraction from *Mycobacterium* (22). Lartigue and Fite (13), however, state that exhaustively defatted tubercle bacilli remain acid-fast.

With the cytochemical procedures for detection of carbohydrates, *p*-aminosalicylic acid stained the culture uniformly; both suspensor cells and spores were bright red. Chlamydospores developed in the presence of primuline showed differential incorporation of the dye. As observed in a fluorescence microscope, the outer layer of the cell wall and peripheral granules were brightly fluorescent, whereas the center of the spores remained unstained (Fig. 3). A similar pattern was obtained by staining C-S with aniline blue. Primuline has been utilized to stain bud scars in yeast (24) where it supposedly binds to the glucan protein of the cell wall. Aniline blue has been used previously to localize aligned carbohydrate material in plants (6) and the bud scar region in yeast (18).

Amido Black and Ponceau Xylidine, two stains utilized extensively to localize proteins after electrophoretic separations, were found to stain chlamydospores. Amido Black-treated smears were inspected by bright field microscopy, and Ponceau Xylidine was found to be a strongly fluorescent dye. A well-delineated zone of dark orange fluorescence was obtained between the central globule (which fluoresced yellow) and the outer wall. This reaction could be abolished by extraction with hot 1 N KOH. Weyman-Rzucidlo (30) also found that the inner part of the chlamydospore wall had a greater affinity for Amido Black than the outer.

When stained with acridine orange (a cytochemical reagent for nucleic acids) and examined under fluorescence, chlamydospores exhibited metachromasy, being greenish yellow to deep orange. The orange fluorescence was removed by hydrolysis in 1 N HCl at 60 C before staining. Bleaching of the orange fluorescence was also

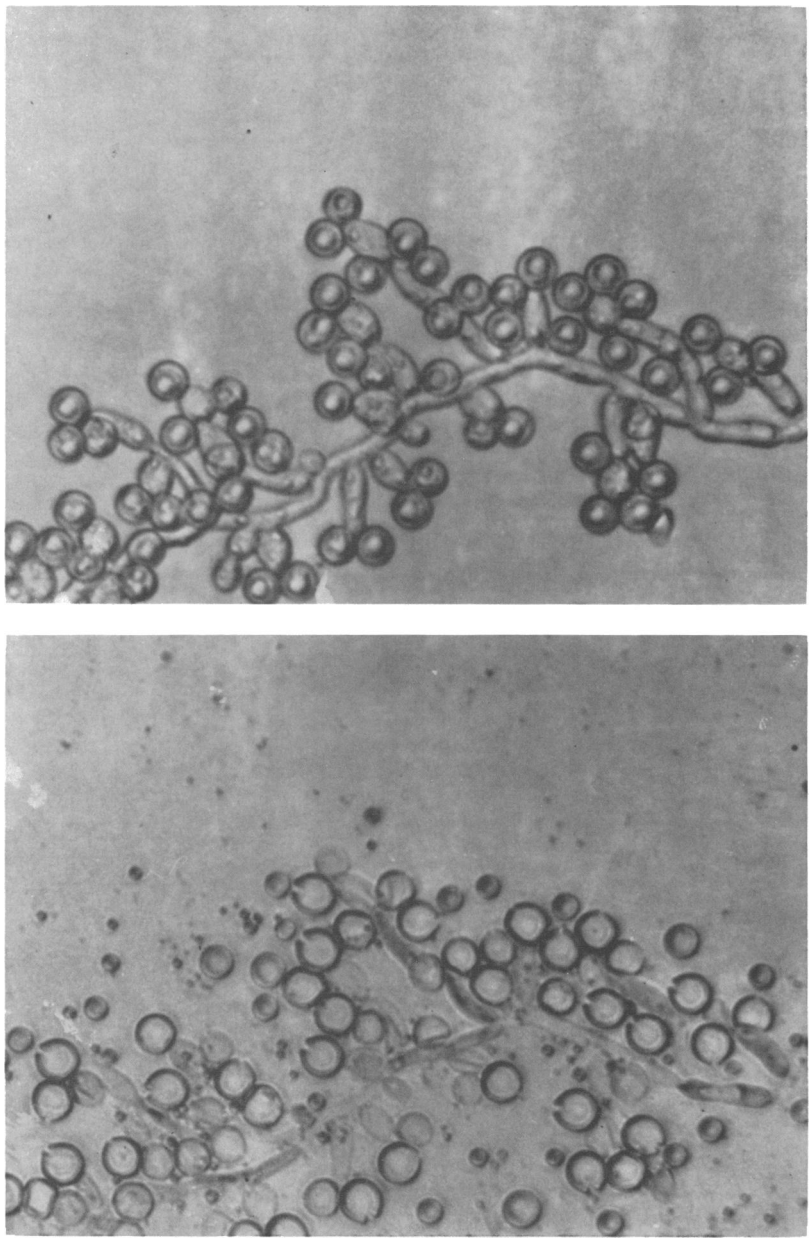

FIG. 1. *Fragility of the chlamydospore wall. Before (top) and after (bottom) applying slight pressure on the coverslip, ×340.*

observed upon exposure to ultraviolet light, a phenomenon previously described (11). However, it was not possible to discern any structures that could be identified as nuclei. Giemsa stain has also been employed to reveal chromatin material; with this technique, Robinow (20) studied the mitotic phases of *Lipomyces*. By following his procedure, we were unable to localize a nucleus in the chlamydospore.

Enzymatic hydrolysis of C-S complex. Early in this work, it was observed that the snail gut enzyme, glusulase (Endo Laboratories, New York), rapidly hydrolyzed the outer layer of the chlamydospore wall and the supporting fila-

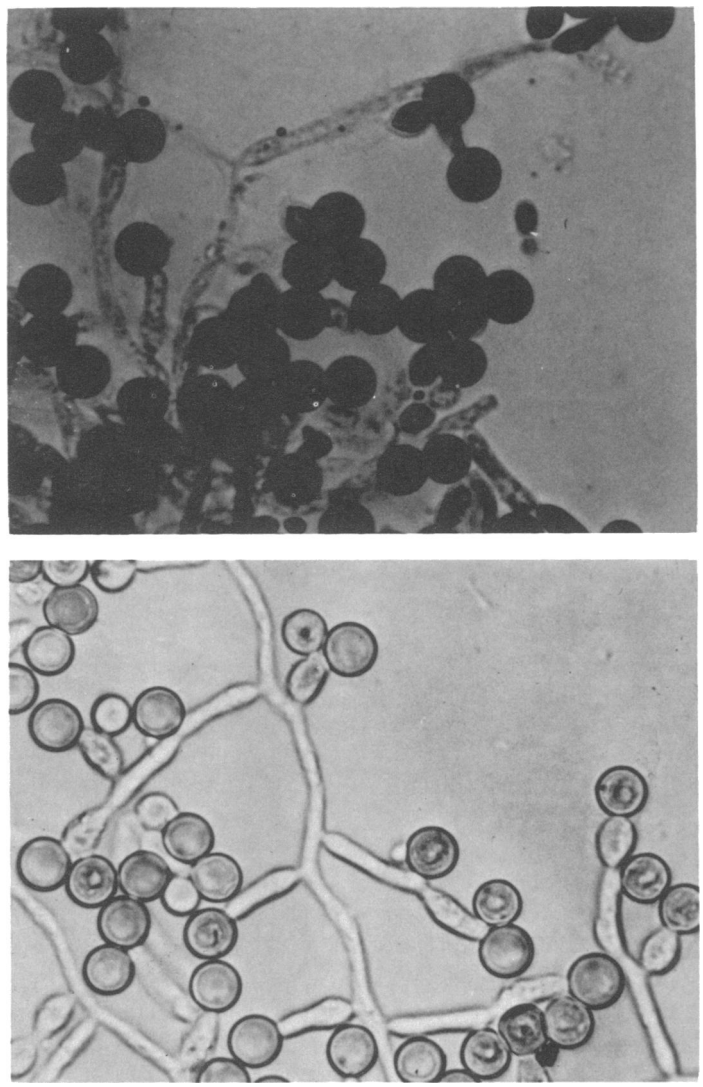

FIG. 2. *Acid-fastness of chlamydospores; Ziehl-Neelsen stain (top). Chlamydospore preparation extracted for 14 hr with ethanol-ether (1:1) prior to staining (bottom), ×645.*

mentous structures. As the snail gut preparation is rather complex and uneconomical, a β-1,3-glucanase was prepared from *Streptomyces species* no. 3.

Incubation of *Streptomyces species* no. 3 with autoclaved *C. albicans* cells resulted in complete digestion of the yeast cells within 48 hr. Since *Streptomyces* grows in the form of pellets, the fermentation broth became clear. The glucanase preparation promoted rapid hydrolysis of the β-1,3-linked polymer laminarin. The number of reducing groups exposed was 10-fold greater than that obtained under similar conditions with pustulan, a β-1,6-linked glucose polymer. When methyl cellulose was incubated under similar conditions, no activity could be demonstrated.

C-S complex was incubated with the glucanase preparation as described in Materials and Methods. Complete hydrolysis of the suspensor cells and outer layer of the chlamydospore wall was observed after incubation for 10 hr (Fig. 4). No further hydrolysis could be observed on prolonged incubation, or after treatment with fresh enzyme-buffer preparation.

Since staining reactions had suggested the presence of proteinaceous material in the wall,

several proteolytic enzymes were tried: crystalline trypsin, pepsin, and papain at appropriate *p*H values and with activators, when necessary. The results were negative; however, purified keratinase (17) actively hydrolyzed the next layer of the chlamydospore wall. A prolonged incubation period (10 to 12 hr at 37 C) was necessary for this reaction. The action of keratinase resulted in the appearance of "spheroplast-like" round bodies which originally had the diameter of the central part of the chlamydospore but which gradually enlarged (Fig. 4). It is difficult to decide on the basis of present observations whether the stability of the "spheroplast" is due to a thin, incompletely hydrolyzed inner wall layer, or to a separate membrane-like structure that is somewhat resistant to osmotic pressure. On prolonged incubation, lysis occurred even in the presence of mannitol or KCl in concentrations reported to stabilize protoplasts (12, 25).

Chemical composition of chlamydospores. The lipid content of chlamydospores is reported in Table 1. The free lipid content was 12% of the dry weight of chlamydospores; this is a higher value than that reported (19) for yeastlike cells of *C. albicans*. The bound lipid contents of yeastlike cells and of chlamydospores were similar. The alcohol-ether extract of chlamydospores was hydrolyzed with 1 N HCl for 16 hr at 100 C, and then chloroform was added. The water phase was assayed for the presence of reducing substances; three compounds were found with R_F values identical to those of glucose, mannose, and rhamnose.

The total amount of carbohydrate (expressed as glucose) in chlamydospores was 40% of the dry weight. The results obtained with the fractionation procedure employed are summarized in Table 2. Most carbohydrate was found in the hot KOH fraction and in the glucan residue. No precipitate was obtained upon treatment of the KOH fraction with Fehling's solution. When 6 N HCl hydrolysates were tested for the presence of glucosamine by the Elson-Morgan method (26), the results were negative. However, small amounts of glucosamine were found when the hydrolysate was assayed on an amino acid analyzer.

Total nitrogen was found to be 7.7% of the dry weight, 65% of which was accounted for as amino acid nitrogen. Based on the latter figure, protein content was estimated to be 32% of the dry weight. When a 6 N HCl hydrolysate of chlamydospores was analyzed on an amino acid analyzer, 19 peaks of ninhydrin-positive material were obtained; 18 of them were recognized and quantitated (Table 3), but the identity of 1 (unknown a) is still in doubt. One of the ninhydrin-positive compounds was identified as glucosamine. Among the amino acids identified, aspartic acid, glutamic acid, lysine, and glycine predominated.

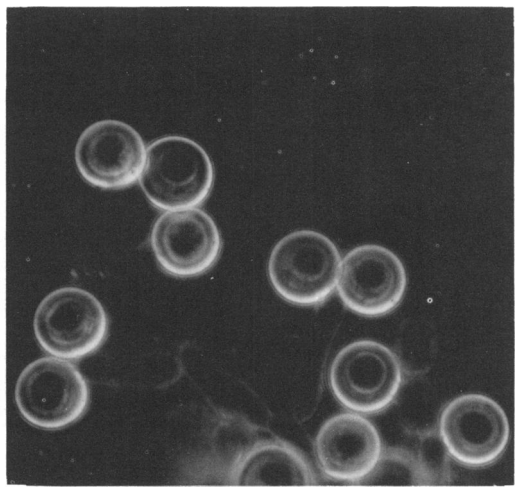

FIG. 3. *Fluorescence of chlamydospores after vital staining with primuline. Note localization of dye in outer wall of the chlamydospore,* ×*1250.*

Of the total amount of amino acids present in the chlamydospore, 5% was extractable with cold 10% trichloroacetic acid. A comparison between the amino acid composition of the trichloroacetic acid extract before and after hydrolysis (Table 3) indicates that some of the acid-extractable material was other than free amino acid; 5.03 μmoles of glutamic acid was recovered after hydrolysis compared to 3.64 μmoles in the unhydrolyzed trichloroacetic acid extract, and the quantity of glycine increased fourfold after hydrolysis. Since trichloroacetic acid was removed by extraction with ether, loss of lipid-bound amino acids is possible. Ether extracts were not analyzed for the presence of amino acids.

The hot 1 N KOH fraction contained nearly all of the amino acids present in the hydrolysate of whole C-S (Table 3). However, only small amounts of cysteic acid, threonine, serine, and arginine were found. The percentage of recovery of these amino acids was also low, indicating loss (ether extraction discussed in previous paragraph) or destruction. All other amino acids were present in very nearly the same amounts as in the hydrolysate of whole chlamydospores, except with glutamic acid, 14.3% of which was found in the trichloroacetic acid extract.

The residue after the previous extractions still retains the shape of an intact chlamydospore (Fig. 5). Hydrolysis of this fraction showed it to

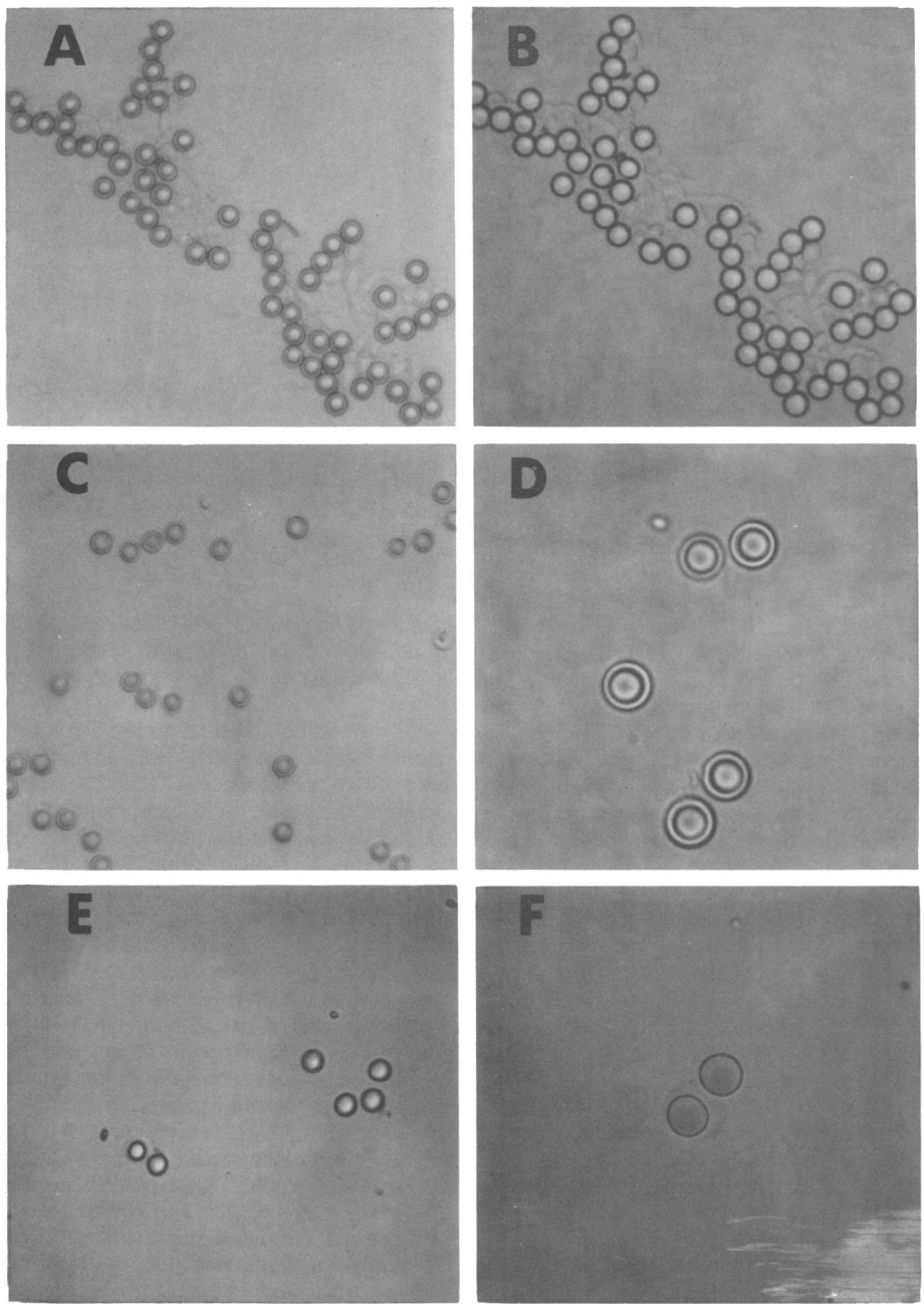

FIG. 4. *Sequential hydrolysis of chlamydospores by glucanase and keratinase. A. Chlamydospores and supporting structures before hydrolysis, ×300. B. Same as A; optical sectioning to emphasize supporting structures, ×300. C. After incubation with glucanase, ×300. D. Same as C, ×645. E. After further incubation with keratinase, ×300. F. Same as E, ×645. Note loss of supporting structures and slight decrease in size of chlamydospores in C, and loss of thick proteinaceous layer in E and F.*

be practically free of amino acids; none of the identified compounds accounted for more than 5%; in many instances, the amounts were less. The only ninhydrin-positive compound present in 10-fold higher amounts than all the others and found only in the residue has the same elution characteristics as glucosamine in the autoanalyzer; it superimposed on a glucosamine standard.

When analyzed by the procedure of Mejbaum (15), RNA was found to be 6.2% of the dry weight of chlamydospores. Phosphate, expressed as P_2O_5 was 3.6% of the dry weight of chlamydospores. Chemical composition of chlamydospores is summarized in Table 4.

Chemical analysis of enzymatic hydrolysates. The fraction solubilized by glucanase was rich in carbohydrate; of the total carbohydrate content of chlamydospores, 81% was released by glucanase (Table 5). A predominant component of this fraction was free glucose, comprising 90% of the total when assayed by the glucose oxidase procedure. Other monosaccharides identified by chromatography were mannose and rhamnose, 2% and <1% of the glucose content, respectively. The keratinase hydrolysate contained only a small amount of carbohydrate, and the only monosaccharide identified was glucose.

The residue after glucanase hydrolysis was washed several times by centrifugation, and then subjected to 6 N HCl hydrolysis. As shown in Fig. 4, the chlamydospore is still surrounded by a thick wall layer after hydrolysis by glucanase. The amino acid composition of the "residual chlamydospore" is given in Table 6. A full spectrum of amino acids was found, mostly ranging from 24 to 50% of the quantities present in acid

TABLE 1. *Lipid content of chlamydospores and yeastlike cells of C. albicans*[a]

Form of growth	Free lipids	Bound lipids	Total
Chlamydospores (C-S)	12	8	20
Yeastlike cells[b]	5.3	8.6	13.9

[a] Expressed as per cent of dry weight.
[b] Data from Peck (20).

TABLE 2. *Carbohydrate composition of chlamydospore (C-S) fractions*[a]

Fraction	(μg/mg amt of dry wt)	Per cent distribution
10% Trichloroacetic acid	13.2	3.8
Hot 30% KOH	139.2	40.2
2 N Acetic acid	5.0	1.5
Concn. H_2SO_4	188.8	54.5
Total	346.2	100

[a] Expressed as glucose.

TABLE 3. *Amino acid composition of chlamydospores*[a]

Amino acid	Hydrolysates[b]				Cold trichloroacetic acid extract before hydrolysis
	Whole chlamydospores	Cold trichloroacetic acid extract	Hot KOH (1 N) extract	Residue	
Cysteic acid	5.00	0.64	Traces		
Aspartic acid	41.94	1.91	40.00	0.50	1.55
Threonine	10.55	0.39	1.97	0.19	0.89
Serine	16.67	1.36	4.45	0.69	1.33
Glutamic acid	35.00	5.03	20.70	0.33	3.64
Proline	7.22		7.22	Traces	
Glycine	22.50	1.61	26.10	0.72	0.47
Alanine	14.15	0.88	13.85	0.50	0.88
Valine + Cystine	9.43		9.70		
Unknown a	Some		Some		
Glucosamine	5.28		Traces	5.55	
Isoleucine	7.22		5.28	0.39	
Leucine	16.05		16.90		
Tyrosine	9.70		10.25		
Phenylalanine	9.43		10.00		
Ammonia	44.26	2.71	40.00	15.25	6.71
Lysine	25.55	0.58	26.70	Traces	0.96
Histidine	7.78		7.78	Traces	Traces
Arginine	9.18	0.89	2.95		1.08

[a] Micromoles per 100 mg of dry weight (C-S).
[b] Hydrolysis in 6 N HCl for 10 hr at 105 C.

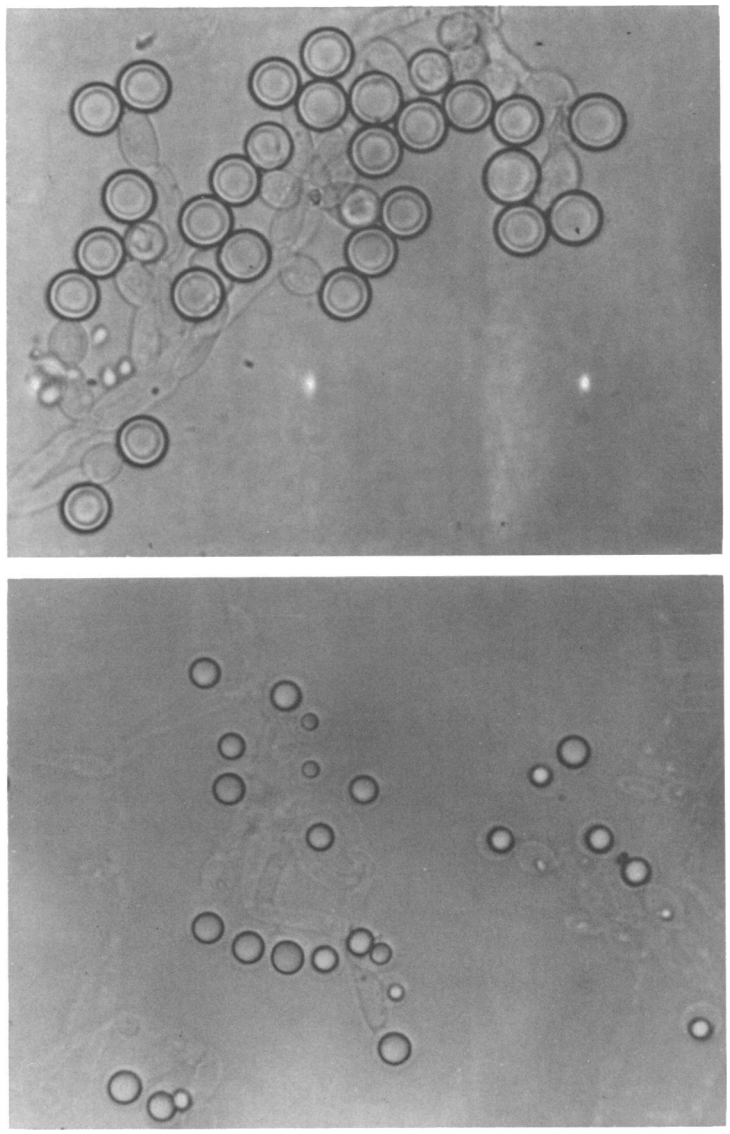

FIG. 5. *Appearance of chlamydospore of Candida albicans before (top) and after (bottom) extraction with 1 N KOH for 30 min at 100 C, ×645.*

TABLE 4. *Chemical composition of chlamydospores (C-S)*

Classes of compounds	Measured as	Per cent of dry weight
Carbohydrates	Glucose	35–40
Protein	Amino acids	32
Lipids	Weight	20
RNA	Ribose	6.2
Phosphate	P_2O_5	3.6

TABLE 5. *Carbohydrate composition of enzymatic hydrolysates of chlamydospores (C-S)*

Hydrolysate	Per cent of total carbohydrate	Monosaccharides identified
Glucanase	81	Glucose, mannose, rhamnose
Keratinase	7	Glucose

TABLE 6. *Amino acid composition of the "residual chlamydospore" after hydrolysis with glucanase*[a]

Amino acid	Amt (μmoles/100 mg of dry wt)	Per cent of amino acid present before enzymatic hydrolysis
Cysteic acid	3.9	79.8
Aspartic acid	17.1	40.6
Threonine	2.8	26.6
Serine	4.5	27.5
Glutamic acid	11.1	31.8
Proline	1.7	24.3
Glycine	8.0	35.5
Alanine	3.7	26.5
Valine + Cystine	3.1	32.9
Methionine	Undecided	
Isoleucine	1.9	26.9
Leucine	6.7	41.4
Tyrosine	3.6	39.4
Phenylalanine	4.3	46.0
Lysine	10.5	41.0
Histidine	4.0	50.7
Arginine	10.5	100

[a] Residue was hydrolyzed in 6 N HCl for 12 hr at 105 C.

hydrolysates of whole chlamydospores. It is interesting to note that the glucanase hydrolysis did not remove any arginine and little cysteic acid. The predominant amino acids in the "residual chlamydospore" were: aspartic acid, glutamic acid, lysine, and arginine.

DISCUSSION

The fragility of the chlamydospore wall precluded use of mechanical techniques for separation of intact spores from the supporting structures. Therefore, chemical analyses were carried out by using both the spores and the attached suspensor cells. Since the suspensors bearing mature chlamydospores did not stain with protein-specific dyes, acridine orange, iodine, or lipid stains, it may be assumed that most of the cytoplasmic material concentrated in the chlamydospore. Since the condition for chlamydospore production entailed limitation of growth, only small quantities of spores could be harvested at any given time. Thus, separate batches of spores were used for the various chemical analyses. An indication that the procedure for obtaining C-S is reproducible was supplied by amino acid analyses. When different batches of chlamydospores were employed to determine the total quantity of each amino acid and their distribution in the various fractions, nearly uniform recovery values were obtained.

A striking difference in the chemical composition between yeastlike cells of *C. albicans* and chlamydospores was the high lipid content of the spore. Of course, the lipid content of different yeasts is very variable, ranging from 2.3 to 63% (16), and the amount of lipid produced may depend not only on the organism but also on the composition of the culture medium, *p*H, and temperature (7). The finding that the free lipid fraction of chlamydospores contains sugars is interesting when compared with work on *Mycobacterium tuberculosis* (14). Upon subjection of the free lipid fraction of tubercle bacilli to acid hydrolysis, methyl ethers of rhamnose and fucose were found. Another parallel between the chlamydospore of *C. albicans* and *Mycobacterium* is acid fastness.

The cytological distribution of protein in chlamydospores was suggested by staining reactions. The thick inner wall layer of the spore is removed by alkaline extraction, and accounted for a major fraction of all amino acids present in the chlamydospore. The residue of the chlamydospore after KOH (1 N) extraction was devoid of protein, but still retained the shape of an intact chlamydospore (Fig. 5). Another approach to the study of chlamydospore structure involved enzymatic hydrolysis. It was shown that the chlamydospore wall contains at least two structurally different layers. The outer layer was hydrolyzed with an enzyme system containing pre-

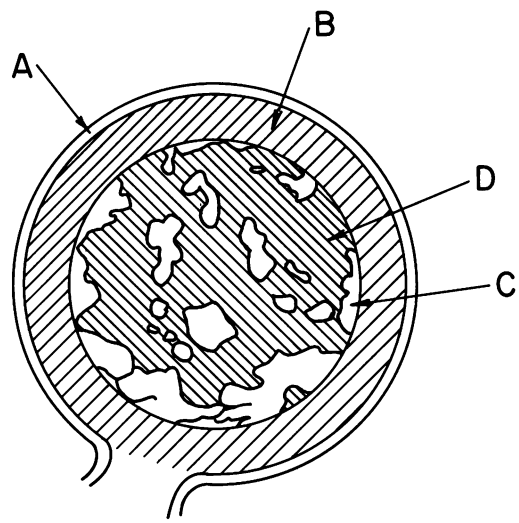

FIG. 6. *Schematic representation of compartmentalization of components of chlamydospore of Candida albicans. A, outer β-glucan layer that is continuous with wall of suspensor cell; B, inner, electron-dense protein layer; C, ribonucleic acid; and D, electron-dense lipid material. Based on Fig. 5 of preceding paper (11), and analyses described in text.*

dominantly β-1,3-glucanase activity, and the inner layer, resistant to the action of glucanase, was hydrolyzed by keratinase.

On the basis of the results obtained with cytochemical reagents, chemical analyses, and sequential enzymatic hydrolysis, the composition of the chlamydospore of *C. albicans* may be interpreted as depicted in Fig. 6. The regions of the chlamydospore in the drawing correspond to those seen in electron micrographs of thin sections (Fig. 5 of preceding paper, 10). The thin outer wall is composed of a glucan, presumably linked β-1,3-, together with a small amount of an alkali-resistant polymer containing glucosamine. The thick, electron-dense, inner layer is comprised largely of protein. The inner core of the chlamydospore is rich in lipid material and RNA, and is bounded by an unusual "membrane" that maintains the integrity of the center core after the outer layers have been removed.

In the preceding paper (10), it was shown that the chlamydospore-suspensor complex arose from well-nourished vegetative yeast cells, in the absence of added nutrients, at the expense of endogenous metabolism. Glycogen and mannan, characteristic of the yeast cell, are essentially absent in the C-S complex. Most likely these polymers were metabolized and converted in some manner to materials characteristic of the chlamydospore. This process, accompanied by extensive compartmentalization of protein into an electron-dense inner wall layer, and of lipid plus RNA into a central core, gives rise to the structure of the chlamydospore.

ACKNOWLEDGMENTS

This investigation was supported by Public Health Service grants AI-00251 and TI AI-43 from the National Institute of Allergy and Infectious Diseases.

We thank Susan C. Durand of the Institute of Microbiology for conducting the quantitative amino acid analyses.

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