

Factors Influencing the Production of H and M Antigen by *Histoplasma capsulatum*: Effect of Physical Factors and Composition of Medium¹

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Stagnant culture methods have permitted only limited physiological studies of the production of H and M antigens by *Histoplasma capsulatum* because, with such methods, antigen production is uncontrolled. In this investigation, a shake culture method was used to convert yeast-phase inoculum to mycelial-phase growth at 25 C. Results strongly suggest that the release of H and M antigens relates to autolysis of the cells. Among the factors influencing production of H and M antigens under shaking conditions, choice of strain was the most important. Alterations of carbon or nitrogen source or variations in amino acid to carbohydrate ratios had limited influence on antigen production. With a strain that produced both H and M antigens, however, proportions of titers of M to H antigens could be made to vary considerably by changes in the medium, the pH, and the temperature. Results suggest that the source of M antigen during autolysis is enzymatic dissolution of the cell wall. The source of H antigen is more obscure. Production of both antigens may be differentially controlled under conditions of good reproducibility by a correct choice of strain and manipulation of culture medium.

Histoplasmin with H and M antigens suitable for use in the agar gel double-diffusion test (12) and the complement-fixation test (33, 34, 39) is presently produced by use of a standardized stagnant culture method and mycelial-phase inoculum (34, 38). Although antigen production is uncontrolled in the stagnant culture (1, 9, 16, 18, 32, 33, 35, 37), the method continues in use because no better one has been available.

Efforts to improve control over antigen production by *Histoplasma capsulatum* in stagnant cultures have included studies of the effects of such factors as temperature, length of incubation, and pH, and of changes that occur in the culture medium during incubation (11, 30, 33, 35). Each of these studies has contributed to the development of the shake culture method (38). However, these contributions have been made in the presence of persistent

within-strain variation (35). Thus, detailed study of the physiology of H and M antigen production and the factors influencing production of these antigens has not been practical.

Because of these limitations, studies of the physiological events accompanying antigen production have been few (11, 30). On the other hand, the effects of such factors as pH, temperature, amounts of inoculum, culture methods, and medium composition on growth and the conversion of both the yeast phase and the mycelial phase of *H. capsulatum* have been extensively studied (2, 3, 5, 6, 10, 13-15, 19, 20, 22-29, 31, 40).

Development of a shake culture method that employed a yeast-phase inoculum which was converted to mycelial phase at 25 C (8) has made it possible to study the relationship between growth and production of H and M antigens under controlled conditions. The present studies were thus undertaken to elaborate the physiological events during growth and antigen production in shake and stagnant cultures with yeast- and mycelial-phase inocula. In addition, the effects of pH, temperature, and medium composition were studied with the shake cul-

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ture method and yeast-phase inocula. As to the effect of medium composition on antigen production, the compounds of particular interest were: casein hydrolysate, known to stimulate conversion of yeast phase to mycelial phase in minimal vitamin medium (28); glucose, which stimulates growth although it is not necessary for conversion of yeast phase to mycelial phase (28); and citrate and acetate which, when substituted for glucose in minimal vitamin medium, had greater and lesser inhibitory effects, respectively, on mycelial-phase growth (27).

Results obtained in applying the shake culture method (8) to the study of the physiology of H and M antigen production and the effects of physical factors and medium composition on antigen production are presented.

MATERIALS AND METHODS

Strains, culture methods, histoplasmin sample collection, and determination of antigen production. The strains (6617, 6623, 6624, and A811) of *H. capsulatum* used, the preparation of yeast- and mycelial-phase inocula for shake and stagnant cultures, the shake culture procedure, the collection and preparation of histoplasmin samples, the demonstration of H and M antigens in the samples by a quantitative agar gel double-diffusion micromethod, and the reporting of agar gel test results in the samples have been described (8).

Culture medium. Smith's asparagine medium (36), a synthetic culture medium used in the Biological Reagents Section procedure (34) for the production of histoplasmin, was used. In experiments to study the effects of medium composition on antigen production, selected compounds were added and deleted; the unmodified medium served as the control.

Determination of cell yield. The method for determining growth units in shake and stagnant cultures was that described previously (8). The cell yields reported are averages of duplicate or triplicate cultures. Results for individual cultures are reported where they are appropriate. Conversion of yeast phase to mycelial phase was determined by microscopic examination at each sampling interval. The amounts of yeast- and mycelial-phase growth in a sample were assessed independently and given values from an ascending scale of 5 units (24, 27). For example, a sample in which many yeast-phase cells were found but no mycelial-phase growth was seen was designated Y_5M_0 . The reverse finding was designated Y_0M_5 . Results reported are those obtained for one of the two cultures made for each strain. Major differences between duplicate or triplicate cultures are indicated.

Biochemical methods. Histoplasmin samples from cultures of the strains used in the preliminary studies were tested for reducing sugar, for carbohydrate, and for nucleic acid and protein. Determinations of pH were also made.

Glucose was determined as reducing sugar (21),

and nucleic acid and protein were determined by ultraviolet absorption at 260 and 280 nm, respectively (4, 17). The optical density values at 260 and 280 nm were converted to milligrams of nucleic acids and protein, respectively, per ml by use of a nomograph. Carbohydrates of samples that had been dialyzed against distilled water and concentrated 10 times with Polyethylene glycol 20,000 were determined by the phenol-sulfuric acid method (7).

Ultraviolet absorption determinations were made with a Beckman model DUR recording quartz spectrophotometer (Beckman Instruments Co., Fullerton, Calif.). The reducing sugar and carbohydrate determinations were made with a Beckman model B spectrophotometer.

For the analysis of glucose, culture filtrates that had been neither dialyzed nor concentrated were used. Carbohydrate was determined in samples that had been dialyzed against 25 volumes of distilled water for 48 hr, with a change of water at 24 hr, and then concentrated 10 times with Polyethylene glycol 20,000. Nucleic acid and protein estimations were made by ultraviolet absorption measurements at 260 and 280 nm. An untreated sample diluted 10-fold was used; the zero-time sample was the blank.

Photomicrography. Examinations were made by medium dark-phase microscopy; photomicrographs were made with Polaroid 4 by 5 inch (10.2 by 12.7 cm), black and white, type 55 P/N film. To obtain maximal contrasts, a green filter was used.

For photography, the packed cells from the samples were resuspended in an equal volume of 0.01 M phosphate-buffered saline (PBS), pH 7.40 (potassium salts), and, to prevent Brownian movement, wet mounts were prepared in 10% gelatin with 1:10,000 Merthiolate.

RESULTS

Physiological responses. Some of the physiological events that accompany the production of H and M antigens were determined to obtain information about the physiology of antigen production. *H. capsulatum* strains 6617, 6624, and A811 were used for these determinations. Culture filtrates taken at various intervals were examined for changes in glucose concentration and for formation of polysaccharide, protein, and nucleic acids. The histoplasmin samples were collected from shake cultures of yeast- and mycelial-phase inocula and from stagnant cultures grown in asparagine medium (Fig. 1).

With strain 6617, as was the case with 6624 and A811, the sequence of events during incubation was similar irrespective of the kind of inoculum used or the conditions of growth (Fig. 1). At the time of maximum cell yield, the glucose was essentially completely metabolized, polysaccharide had appeared, and material absorbing at 260 to 280 nm was released. At this time or just before, H antigen or H and M antigens were first observed. Later, the

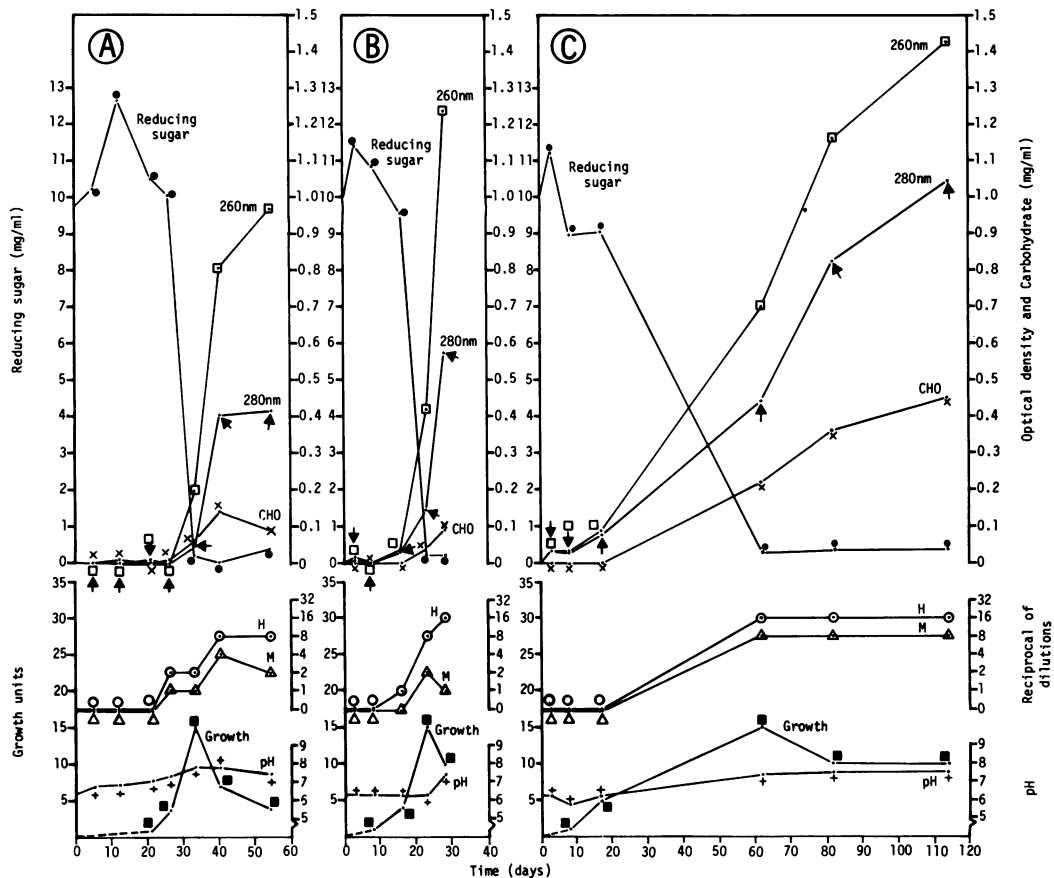


FIG. 1. *H* and *M* antigen production and physiological data from cultures of *H. capsulatum* 6617 in Smith's asparagine medium at 25 C. (A) Inoculum: 1.1×10^7 to 4×10^7 yeast-phase cells per ml of medium; shake culture. (B) Inoculum: 0.3 ml of mycelial-mat suspension (15 mm² of mat from a slant broken up with a pipette in 1.0 ml of sterile distilled water) per 100 ml of medium; shake culture. (C) Inoculum: Three 3- to 5-mm² pieces of mycelial mat from a slant placed on the surface of 100 ml of medium; stagnant culture.

amount of cells decreased, in many instances precipitously, while the values for polysaccharide, antigens, and 260 to 280 nm readings increased.

In the shake cultures, nucleic acid increased, but protein did not increase concurrently (Tables 1 and 2). However, in the stagnant culture of strain 6617, calculation of protein and nucleic acid concentration in the 17-day sample showed 0.5 and 0.03 mg/ml, respectively. By 114 days, these values were 5.3 and 0.53 mg/ml, respectively. In general, the nucleic acids increased to comparable levels in both stagnant and shake cultures irrespective of the length of incubation or the strain used. The final level of nucleic acid was somewhat higher for the culture of strain A811 than for the other cultures (Table 2).

Polysaccharide was produced in similar

amounts by the various shake cultures, but stagnant cultures produced higher final levels.

Although production of *H* antigen alone or of *H* and *M* antigens together could not be related directly to production of polysaccharide, protein, or nucleic acid, the *H* antigen appeared first with release of nucleic acid and carbohydrate. *M* was observed in stagnant cultures when major amounts of protein were released into the culture medium. This relationship of *M* antigen and protein released was not observed in shake cultures. However, antigen titers always increased just before growth ceased and often continued to increase when cell yields were decreasing. Thus, the data indicate that the appearance of both antigens was related, most probably to cell autolysis. This indication was supported by a concomitant, although not always parallel, increase in

TABLE 1. *H and M antigen production and physiological data from H. capsulatum 6624 in Smith's asparagine medium, shake culture*^a

Days grown	Cell yield ^b	Titer ^c		Reducing sugar (mg/ml)	Carbohydrate ^d (mg/ml)	Protein (mg/ml)	Nucleic acid (mg/ml)
		H	M				
0	0.0	0	0	9.89	<0.01	0.00	0.00
2	5.0	1	0	11.47	<0.01	0.35	0.01
11	30.0	32	0	0.19	0.04	0.00	0.32
17	20.0	128	0	0.18	0.09	0.00	0.49
22	15.0	256	0	0.21	0.19	0.00	0.51

^a Inoculum: three loopfuls of yeast-phase growth transferred from a slant to 100 ml of medium. Incubated with shaking at 25 C.

^b Growth units.

^c Reciprocal of dilutions.

^d Nondialyzable.

TABLE 2. *H and M antigen production and physiological data from H. capsulatum A811 in Smith's asparagine medium, shake culture*^a

Days grown	Cell yield ^b	Titer ^c		Reducing sugar (mg/ml)	Carbohydrate ^d (mg/ml)	Protein (mg/ml)	Nucleic acid (mg/ml)
		H	M				
0	0.0	0	0	9.89	<0.01	0.00	0.00
2	2.0	0	0	12.24	<0.01	0.00	0.00
11	8.0	1	0	3.85	0.03	0.00	0.47
17	7.0	2	0	2.74	0.08	0.00	0.72
22	6.0	4	0	1.44	0.13	0.00	0.88

^a Inoculum: three loopfuls of yeast-phase growth transferred from a slant to 100 ml of medium. Incubated with shaking at 25 C.

^b Growth units.

^c Reciprocal of dilutions.

^d Nondialyzable.

the 260 to 280 nm readings and in the production of nonreducing carbohydrate.

In asparagine medium, strain 6623 produced H and M antigens early and at good titers; strain 6624 produced only H antigen (8), but it appeared early and was produced to a high titer. For these reasons, these two strains were chosen for further study.

Temperature of incubation. To study the effect of temperature, strain 6623 was grown in duplicate cultures at 25, 30, and 37 C, and morphology, cell yields, and antigen production were observed (Table 3).

Growth at the three temperatures was markedly different. It was much delayed at 30 C, and essentially no growth and no antigen production occurred at 37 C. At 30 C, log-phase mycelial growth was not only delayed, but it was also initiated at different times in the duplicate cultures, a variation that did not occur at 25 and 37 C. In general, antigen production at 30 C was about equal to that at 25 C. The antigens appeared in the 13-day sample at both temperatures, but titers were

higher in samples from the 30 C cultures. Antigen production by the cultures at 30 C was surprising, because of the small amount of mycelial-phase growth. By the 34th day, culture b at 30 C had begun log-phase growth. Maximum cell yields and antigen titers at 41 days corresponded to those of the 25 C cultures at 27 and 34 days, respectively. Culture a at 30 C had begun log-phase growth by 41 days, and 1 week later was much like culture b in gross appearance. Thus, at 25 C the high antigen titers appeared earlier and were less subject to variation than at the other temperatures tested.

pH of the culture medium. Strain 6623 was used to determine the effect of pH on the production of antigens in the asparagine medium. The pH values used were 4.40, 6.41, and 6.78 (Table 4). The last was the highest pH that could be used without precipitate formation.

Conversion of yeast phase to mycelial phase was somewhat slower at pH 4.40 and 6.78 than at 6.41, but cell yields were greater. Antigen production was improved only at pH 6.78; M

TABLE 3. *Effects of incubation temperature on growth and antigen production of H. capsulatum 6623 at 25, 30, and 37 C in Smith's asparagine medium^a*

Days grown	25 C				30 C								37 C			
					Culture a				Culture b							
	Cells		Titer ^d		Cells		Titer		Cells		Titer		Cells		Titer	
	Form ^b	Yield ^c	H	M	Form	Yield	H	M	Form	Yield	H	M	Form	Yield	H	M
6	Y ₅ M ₂	0.4	0	0	Y ₅ M ₁	0.4	0	0	NR ^e	0.4	0	0	Y ₅ M ₀	<0.5	0	0
13	Y ₁ M ₅	5.5	1	0	Y ₅ M ₁	1.6	2	4	NR	1.2	2	4	Y ₄ M ₊	<0.5	0	0
20	Y ₀ M ₅	12.6	4	8	Y ₅ M ₁	1.2	1	4	NR	1.2	8	16	Y ₃ M ₊	<0.5	0	0
27	Y ₀ M ₅	12.6	16	16	Y ₅ M ₂	2.0	4	8	NR	2.4	4	16	Y ₃ M ₊	<0.5	0	0
34	Y ₀ M ₅	9.1	64	64	Y ₄ M ₂	3.1	4	8	Y ₄ M ₄	11.8	4	8	Y ₃ M ₂	<0.5	0	0
41	NR	NR	NR	NR	Y ₄ M ₃	6.7	4	8	Y ₃ M ₄	14.1	32	64	Y ₃ M ₊	<0.5	0	0

^a Inoculum: 9.8×10^6 yeast-phase cells per ml of medium. Incubated with shaking. Results at 25 and 37 C are the averages of duplicate cultures.

^b Microscopic examination: amounts of yeast phase (Y) and mycelial phase (M) present, each on an arbitrary scale of 0 to 5.

^c Growth units.

^d Reciprocal of dilutions.

^e Not read.

TABLE 4. *Effects of pH on growth and antigen production of H. capsulatum 6623 at pH 4.40, 6.41, and 6.78 in Smith's asparagine medium^a*

Days grown	pH 4.40				pH 6.41				pH 6.78			
	Cells		Titer ^d		Cells		Titer		Cells		Titer	
	Form ^b	Yield ^c	H	M	Form	Yield	H	M	Form	Yield	H	M
6	Y ₅ M ₁	<0.5	0	0	Y ₅ M ₂	0.4	0	0	Y ₃ M ₁	<0.5	0	0
13	Y ₀ M ₅	8.7	0	0	Y ₁ M ₅	5.5	1	0	Y ₁ M ₅	6.3	1	0
20	Y ₀ M ₅	15.5	16	16	Y ₀ M ₅	12.6	4	8	Y ₀ M ₅	17.4	8	16
27	Y ₀ M ₅	17.0	8	32	Y ₀ M ₅	12.6	16	16	Y ₀ M ₄	11.5	16	64
34	Y ₀ M ₅	11.3	32	64	Y ₀ M ₅	9.1	64	64	Y ₀ M ₅	7.4	32	128

^a Inoculum: 9.8×10^6 yeast-phase cells per ml of medium. Incubated with shaking at 25 C.

^b Microscopic examination: amounts of yeast phase (Y) and mycelial phase (M) present, each on an arbitrary scale of 0 to 5.

^c Growth units.

^d Reciprocal of dilutions.

antigen appeared to be differentially increased over H antigen.

Effects of added casein hydrolysate and citric acid in media with L-asparagine or DL-asparagine. The effects of casein hydrolysate and citric acid were tested by using a single inoculum in the asparagine medium with either L- or DL-asparagine (Tables 5 and 6).

Adding casein hydrolysate to the two media caused earlier growth and antigen production by both strains. When citric acid was added, growth and production of H and M antigens by strain 6623 were depressed in both media; H antigen was more severely limited by citric acid in medium with L-asparagine. Adding casein hydrolysate had little effect on strain

6623 in the L-asparagine medium; however, in the DL-asparagine medium, it increased the M antigen titer fourfold. DL-Asparagine, by itself, had little effect on growth or on antigen production by strain 6623 (Table 5). Conversely, casein hydrolysate and citric acid had little effect on antigen production by strain 6624 in either L- or DL-asparagine medium. Growth of strain 6624 was decreased by 50% when the DL-asparagine medium was used. The decrease indicates that this strain could not use the D isomer of asparagine. This limitation of cell yields was relieved by adding casein hydrolysate (Table 6). Although H antigen production was delayed by DL-asparagine, the final titer was equal to or greater than that observed with the L-asparagine media. M antigen production

TABLE 5. Effects of casein hydrolysate and citric acid on growth and antigen production: shake cultures of *H. capsulatum* 6623 in unmodified and modified (DL-asparagine) Smith's asparagine medium^a

Medium	Days grown	Additions to medium											
		None				Casein hydrolysate, 0.4%				Citric acid, 1.0%			
		Cells		Titer ^d		Cells		Titer		Cells		Titer	
		Form ^b	Yield ^c	H	M	Form	Yield	H	M	Form	Yield	H	M
Unmodified	7	Y ₅ M ₂	0.4	0	0	Y ₁ M ₅	8.6	1	1	Y ₅ M ₁	<0.5	0	0
	13	Y ₁ M ₅	5.5	1	0	Y ₁ M ₅	14.2	8	16	Y ₁ M ₅	2.0	0	0
	20	Y ₀ M ₅	12.6	4	8	Y ₀ M ₅	7.9	8	32	Y ₀ M ₅	9.6	1	2
	27	Y ₀ M ₅	12.6	16	16	Y ₀ M ₅	7.1	16	16	Y ₀ M ₅	17.3	8	32
	34	Y ₀ M ₅	9.1	64	64	Y ₀ M ₅	7.2	32	32	Y ₀ M ₅	10.5	8	32
Modified	6	Y ₅ M ₁	0.5	0	0	Y ₁ M ₅	7.7	1	1	Y ₅ M ₁	0.5	0	0
	13	Y ₂ M ₅	5.2	1	0	Y ₁ M ₅	10.8	8	16	Y ₁ M ₅	2.4	1	0
	20	Y ₁ M ₅	8.2	2	8	Y ₀ M ₅	10.0	16	64	Y ₀ M ₅	9.7	1	4
	27	Y ₁ M ₅	12.1	8	32	Y ₀ M ₅	8.8	32	128	Y ₀ M ₅	16.6	16	8
	34	Y ₀ M ₅	10.5	8	32	Y ₀ M ₅	6.2	32	64	Y ₅ M ₅	9.6	16	16

^a Inoculum: 9.8×10^6 yeast-phase cells per ml of medium. Incubated with shaking at 25 C.

^b Microscopic examination: amounts of yeast phase (Y) and mycelial phase (M) present, each on an arbitrary scale of 0 to 5.

^c Growth units.

^d Reciprocal of dilutions.

TABLE 6. Effects of casein hydrolysate and citric acid on growth and antigen production: shake cultures of *H. capsulatum* 6624 in unmodified and modified (DL-asparagine) Smith's asparagine medium^a

Medium	Days grown	Additions to medium											
		None				Casein hydrolysate, 0.4%				Citric acid, 1.0%			
		Cells		Titer ^d		Cells		Titer		Cells		Titer	
		Form ^b	Yield ^c	H	M	Form	Yield	H	M	Form	Yield	H	M
Unmodified	6	Y ₀ M ₅	16.8	1	0	Y ₀ M ₅	19.2	2	0	Y ₀ M ₅	17.4	2	0
	13	Y ₀ M ₅	11.9	16	0	Y ₀ M ₅	13.3	8	0	Y ₀ M ₅	14.8	16	0
	20	Y ₀ M ₅	6.5	32	0	Y ₀ M ₅	5.8	16	0	Y ₀ M ₅	8.7	64	0
	27	Y ₀ M ₅	6.3	16	0	Y ₀ M ₅	6.3	32	0	Y ₀ M ₅	6.4	32	0
	34	Y ₀ M ₅	5.7	32	0	Y ₀ M ₅	5.6	32	0	Y ₀ M ₅	5.6	64	0
Modified	6	Y ₁ M ₅	8.4	1	0	Y ₀ M ₅	15.1	2	0	Y ₀ M ₅	12.2	2	0
	13	Y ₁ M ₅	7.2	4	0	Y ₁ M ₅	10.7	16	0	Y ₀ M ₅	11.7	16	0
	20	Y ₀ M ₅	7.5	16	0	Y ₀ M ₅	11.3	32	0	Y ₀ M ₅	9.9	32	0
	27	Y ₀ M ₅	9.7	32	0	Y ₀ M ₅	11.3	64	0	Y ₀ M ₅	9.1	32	0
	34	Y ₀ M ₅	9.8	64	0	Y ₀ M ₅	9.5	64	0	Y ₀ M ₅	7.6	64	0

^a Inoculum: 9.8×10^6 yeast-phase cells per ml of medium. Incubated with shaking at 25 C.

^b Microscopic examination: amounts of yeast phase (Y) and mycelial phase (M) present, each on an arbitrary scale of 0 to 5.

^c Growth units.

^d Reciprocal of dilutions.

was not stimulated in strain 6624 by the various parameters of media tested.

Effects of acetate, glycerol, and glucose in modified asparagine medium (DL-asparagine). The individual effects of adding 0.2% sodium acetate and of deleting the glucose or glycerol of asparagine medium having DL-as-

paragine were tested with strains 6623 and 6624 (Tables 7 and 8). Less than the standard amount of inoculum was inadvertently used, necessitating reinoculation of the cultures 4 days later.

Sodium acetate depressed the growth of strain 6623 and lowered the titers for H and M

TABLE 7. Effects of various carbohydrates on growth and antigen production of *H. capsulatum* 6623 in modified Smith's asparagine medium (D,L-asparagine)^a

Days grown	Addition to or deletion from modified asparagine medium															
	None				+Na acetate, 0.2%				-Glucose				-Glycerol			
	Cells		Titer ^d		Cells		Titer		Cells		Titer		Cells		Titer	
	Form ^b	Yield ^c	H	M	Form	Yield	H	M	Form	Yield	H	M	Form	Yield	H	M
7	Y ₅ M ₁	<0.5	0	0	Y ₄ M ₂	0.4	0	0	Y ₅ M ₁	<0.5	0	0	Y ₅ M ₁	0.5	0	0
14	Y ₁ M ₄	4.7	0	0	Y ₂ M ₅	3.6	1	0	Y ₄ M ₂	0.8	0	0	Y ₁ M ₄	3.1	0	0
21	Y ₂ M ₅	7.5	4	16	Y ₂ M ₅	5.1	2	1	Y ₃ M ₃	1.0	0	0	Y ₀ M ₅	8.9	4	1
28	Y ₂ M ₅	7.9	16	16	Y ₂ M ₅	5.6	4	2	Y ₃ M ₃	1.6	1	0	Y ₀ M ₅	8.2	16	2
35	Y ₀ M ₅	8.6	16	32	Y ₂ M ₅	7.0	2	8	Y ₃ M ₃	1.2	1	1	Y ₀ M ₅	7.0	16	8

^a Inoculum: 9.8×10^6 yeast-phase cells per ml of medium in two steps: 16.7% on day 0, 83.3% on day 4. Incubated with shaking at 25 C.

^b Microscopic examination: amounts of yeast phase (Y) and mycelial phase (M) present, each on an arbitrary scale of 0 to 5.

^c Growth units.

^d Reciprocal of dilutions.

TABLE 8. Effects of various carbohydrates on growth and antigen production of *H. capsulatum* 6624 in modified Smith's asparagine medium (D,L-asparagine)^a

Days grown	Addition to or deletion from modified asparagine medium															
	None				+Na acetate, 0.2%				-Glucose				-Glycerol			
	Cells		Titer ^d		Cells		Titer		Cells		Titer		Cells		Titer	
	Form ^b	Yield ^c	H	M	Form	Yield	H	M	Form	Yield	H	M	Form	Yield	H	M
7	Y ₂ M ₄	1.7	0	0	Y ₂ M ₄	3.2	0	0	Y ₅ M ₁	<0.5	0	0	Y ₂ M ₄	8.7	1	0
14	Y ₀ M ₄	3.5	2	0	Y ₀ M ₃	4.0	1	0	Y ₁ M ₂	2.9	0	0	Y ₀ M ₃	9.8	8	0
21	Y ₀ M ₅	4.1	8	0	Y ₀ M ₅	6.7	2	0	Y ₂ M ₄	2.5	1	0	Y ₀ M ₅	5.9	16	0
28	Y ₀ M ₅	5.4	16	0	Y ₀ M ₅	5.9	16	0	Y ₂ M ₄	1.6	1	0	Y ₀ M ₅	5.1	16	0
35	Y ₀ M ₅	6.1	16	0	Y ₀ M ₅	7.2	8	0	Y ₃ M ₃	0.8	2	0	Y ₀ M ₅	3.9	8	0

^a Inoculum: 9.8×10^6 yeast-phase cells per ml of medium in two steps: 10.5% on day 0, 89.5% on day 4. Incubated with shaking at 25 C.

^b Microscopic examination: amounts of yeast phase (Y) and mycelial phase (M) present, each on an arbitrary scale of 0 to 5.

^c Growth units.

^d Reciprocal of dilutions.

antigens from, respectively, 1:16 and 1:32 to 1:2 and 1:8. In the absence of glycerol, strain 6623 grew slightly better than in the basal medium. H antigen was produced to the same level, a titer of 1:16, but an M titer of only 1:8 was produced in the medium without glycerol compared to a titer of 1:32 in the basal medium.

Adding sodium acetate improved the growth of strain 6624 slightly; H antigen production was affected very little. Deletion of glycerol improved the growth and H antigen production of strain 6624, but the final H antigen titer was the same as that reached in the basal medium.

Growth and antigen production were greatly

diminished for both strains in the absence of glucose. Thus, the importance of glucose to growth and antigen production was clearly indicated. The response of both strains to the presence and absence of glycerol indicates that it depressed growth and antigen production when glucose was present.

Effects of ratio of casein hydrolysate to glucose. Two asparagine media were prepared, one with 4.0% glucose and 0.4% casein hydrolysate, and one with 0.4% glucose and 4.0% casein hydrolysate. Unmodified asparagine medium was used as a control. Because the two strains behaved differently (Fig. 2 and 3), the effects of these media are considered separately.

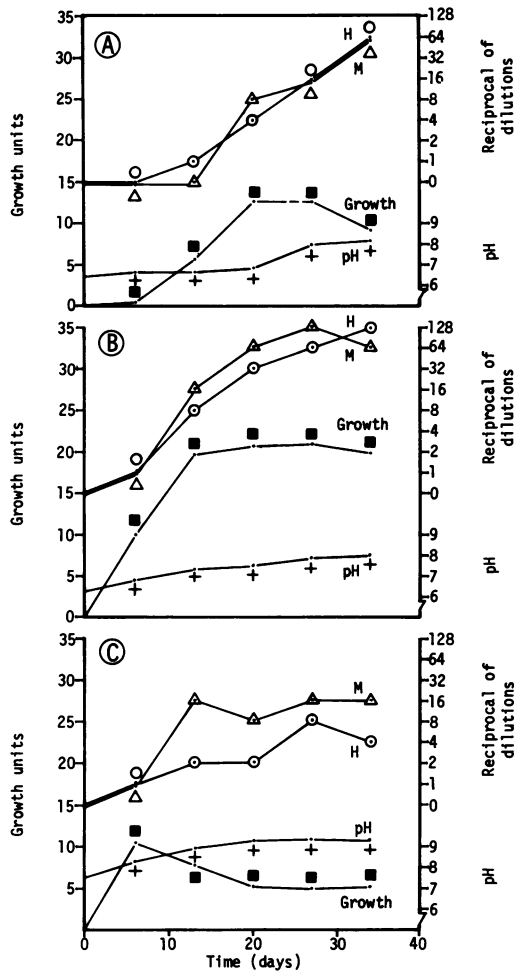


FIG. 2. H and M antigen production from cultures of *H. capsulatum* 6623 in unmodified and modified Smith's asparagine medium. (A) Unmodified Smith's asparagine. (B) Smith's asparagine + 4.0% glucose and 0.4% casein hydrolysate. (C) Smith's asparagine + 0.4% glucose and 4.0% casein hydrolysate. Inoculum: 9.8×10^6 yeast-phase cells per ml of medium; cultures shaken at 25 C.

For strain 6623, the high glucose-low amino acid combination markedly increased cell yields, but it did not affect the total production of either H or M antigen when compared with that of the unmodified medium. The low glucose-high amino acid combination, however, markedly decreased both cell yields and H and M antigen production. However, M antigen titers were markedly higher than the H antigen titers. Although the high glucose-low amino acid combination showed high cell yields (27 units) and the low glucose-high amino acid combination showed somewhat

lower cell yields (20 units), strain 6624 had maximal H antigen production (titers of 1:128) with both combinations of glucose and amino acid. Conversely, the unaltered asparagine medium showed low H titers even though 17 units of cells were attained (Fig. 3). No medium induced strain 6624 to produce M antigen.

With the low glucose-high amino acid combination, strain 6623 rapidly produced a greenish-black pigment rather than the usual lavender-to-purple pigment. In comparison, the gross appearance of strain 6624 was not unusual; that is, the culture was tan and the

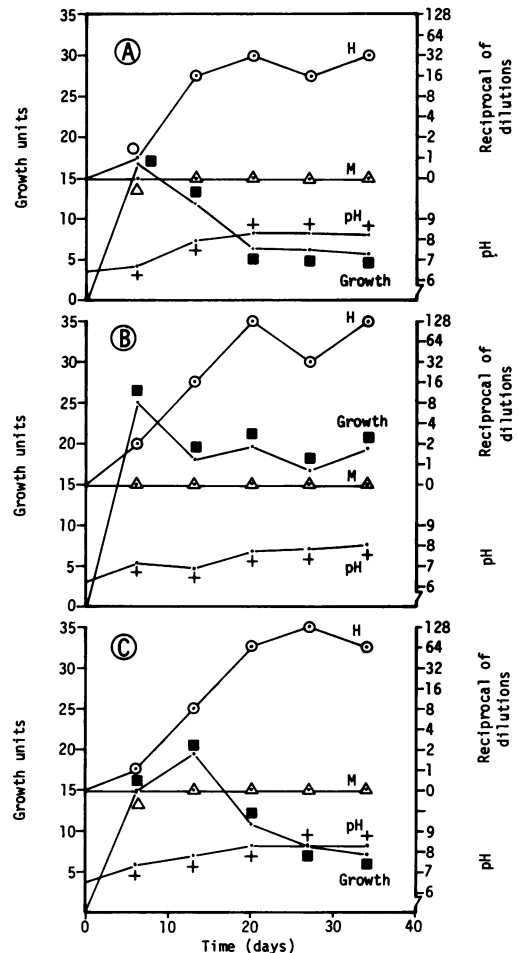


FIG. 3. H and M antigen production and physiological data from shake cultures of *H. capsulatum* 6624 in unmodified and modified Smith's asparagine medium. (A) Unmodified Smith's asparagine. (B) Smith's asparagine + 4.0% glucose and 0.4% casein hydrolysate. (C) Smith's asparagine + 0.4% glucose and 4.0% casein hydrolysate. Inoculum: 9.8×10^6 yeast-phase cells per ml of medium; culture shaken at 25 C.

black pigmentation of mycelium did not develop.

Release of H and M antigens by autolysis. Because antigen production by the standardized shake culture method appeared to be related to autolysis in strains 6617, 6624, and A811, release of antigen was attempted by shaking mycelium in buffer for extended periods. Strains 6623 and 6624 were grown by the standardized shake culture method in Smith's asparagine medium (36) and harvested at 14 days. Cultures of this age were used, because antigen production would have just begun and autolysis would be limited.

The mycelial-phase growth was collected by the usual method, and was washed three times by centrifugation in 0.01 M PBS at pH 7.40. The volume of culture medium removed was replaced with PBS (potassium salts), and the mycelium-phase suspension was returned to the shaker at 25 C. Samples were collected in the usual manner at 4 and 11 days, concentrated 10 times with Polyethylene glycol 20,000, and tested quantitatively for H and M antigens (Table 9).

Antigens were released from the mycelia of both strains after 11 days in amounts exceeding those in the 14-day cultures. Strain 6623, which normally released relatively equal amounts of both H and M antigens, released M antigen with very low levels of H antigen. H antigen released by strain 6624 exceeded the amount in the 14-day cultures, and M antigen was not observed.

Morphological examination of the two strains (Fig. 4) before and after incubation in the buffered saline showed marked alterations in the physical aspects of the mycelium. These alterations were reflected by changes in the

packed-cell volumes, particularly for strain 6623. The entire mycelium of strain 6623 became soft, devoid of internal content, and flexible; it formed twisted clumps when the medium was swirled (Fig. 4B). In contrast, strain 6624 showed little change in cell volume and retained, in general, its structural rigidity, although many of the hyphae were either empty or without internal structure (Fig. 4D, arrows). Whether a direct relationship exists between the structural changes observed in the mycelium of the two strains and the particular antigen released remains to be determined.

DISCUSSION

A correlation of results of growth and antigen production reported earlier (8) with those results reported here, in which antigen production was related to growth, glucose utilization, and release of carbohydrate, protein, and nucleic acids, strongly suggests that the release of H and M antigens results from autolysis of the cells. Virtually without exception, maximum H and M production occurred after maximum cell yields, and, in general, antigen production remained constant or increased during a decline in the mycelial mass. These results, plus those of the direct experiment in which the mycelium was incubated in buffer with continued shaking, support the conclusion that autolysis releases H and M antigens even though limited growth under these latter conditions was probable.

Among the factors found to influence the production of H or M antigens under shaking conditions, choice of strain was the most important. Of the three media tested initially (8), there was no doubt that the more complex media elicited the strongest production of M

TABLE 9. Release of H and M antigens from washed mycelium incubated at 25 C under shaking conditions^a

Sample	Age (days)	<i>H. capsulatum</i> 6623				<i>H. capsulatum</i> 6624			
		pH	Cell yield	Titer ^c		pH	Cell yield	Titer	
				H	M			H	M
Culture	14	6.60	7.1	2	4	6.20	7.0	4	0
Mycelial suspension in PBS	0	7.40	7.1	0	0	7.40	7.0	0	0
	4	7.40	3.4	1	0	7.22	6.8	1	0
	11	7.75	1.9	1	8	7.50	6.2	8	0

^a Mycelium from 14-day shake cultures of *H. capsulatum* 6623 and *H. capsulatum* 6624 in Smith's asparagine medium at 25 C were centrifuged and washed in 0.01 M phosphate-buffered saline (PBS) at pH 7.40 (potassium salts), and were resuspended in the PBS at original concentration.

^b Growth units.

^c Reciprocal of dilutions.

^d Original culture used for the production of washed mycelium.

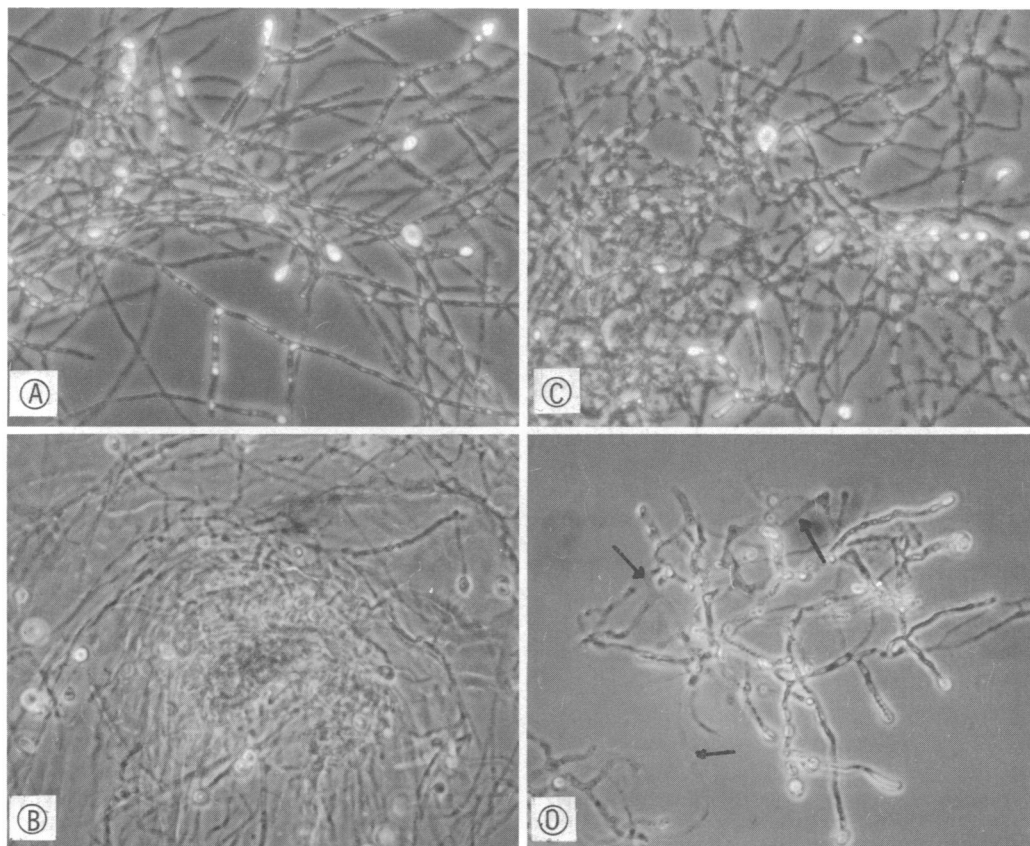


FIG. 4. Morphology of mycelial-phase *H. capsulatum* before and after incubation in phosphate-buffered saline (PBS). (A) *H. capsulatum* 6623 shake culture at 25 C in Smith's asparagine medium: day 14, mycelium and young macroconidia; $\times 600$. (D) *H. capsulatum* 6624 mycelium incubated at 25 C in 0.01 M PBS, pH under shaking conditions for 11 days; mycelium from 14-day shake culture in Smith's asparagine medium at 25 C; $\times 645$. (C) *H. capsulatum* 6624 shake culture at 25 C in Smith's asparagine medium: day 14, mycelium and young macroconidia; $\times 600$. (D) *H. capsulatum* 6624 mycelium incubated at 25 C in 0.01 M PBS, pH 7.40, under shaking conditions for 11 days; mycelium from 14-day shake culture in Smith's asparagine medium at 25 C; $\times 645$.

antigen, but such increases in M production were only within the range of titers from 0 to 1:4 for all of the strains except *H. capsulatum* 6623. Strain 6623 consistently produced high titers (1:64 to 1:256) of M antigen and was little influenced by any of the three media. With the exception of strain 6623, changes of carbon source or nitrogen source or variation in amino acid to carbohydrate ratios, though affecting the total antigen produced somewhat, did little to alter the basic characteristics of antigen production by the various strains. With strain 6623, the proportions of M to H antigen titers could be caused to vary considerably by changes in the medium. Thus, in the presence of acetate, the respective titers were 8:2; in the absence of the glucose of the aspar-

agine medium, they were 8:16; with the use of DL-asparagine instead of the L-asparagine of the asparagine medium, they were 32:8. In contrast, in the unmodified medium the respective titers were 64:64. Finally, adding 0.4% casein hydrolysate to the DL-asparagine medium produced M to H antigen ratios of 128:32. Similarly, these ratios in strain 6623 could be influenced by pH and temperature.

One might ask about the source of these antigens during autolysis; that is, were the antigens released from within the cell or from the dissolution of the cell wall? The rather acute disintegration of hyphae with the concomitant release of M antigen when the mycelium of strain 6623 was incubated in PBS strongly suggests that M antigen is derived from enzy-

matic destruction of the cell wall. The origin of H antigen is more obscure. Pine and Bradley (*unpublished data*) have observed protein to carbohydrate ratios in several purified preparations of M and H antigens to be 0.64 ± 0.16 and 1.19 ± 0.33 , respectively. These ratios suggest a covalent linkage of carbohydrate to protein in the M antigen.

One might also ask what distinguishes strain 6623 from the remaining strains in its ability to form the M antigen. Inasmuch as six of the strains demonstrated the ability to form M antigen (8) in titers of 1:4 or 1:8, the antigen may be present in relatively equal amounts in these strains, but only in strain 6623 do we have the enzymatic breakdown necessary for its release. Certain data obtained with *H. capsulatum* strains Ven-6 and 6617 under stagnant conditions suggest that these conditions are more suited for the differential production of M antigen. Regardless of these fundamental considerations, production of both antigens may be differentially controlled under conditions of good reproducibility by a correct choice of strain and manipulation of culture medium.

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