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

HUGH-BERT EHRHARD and LEO PINE

Department of Parasitology and Laboratory Practice, School of Public Health, University of North Carolina, Chapel Hill, North Carolina 27515, and Center for Disease Control, Atlanta, Georgia 30333

Received for publication 18 August 1971

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-
ture method and yeast-phase inocula. As to the
effect of medium composition on antigen pro-
duction, 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 sub-
stituted for glucose in minimal vitamin me-
had greater and lesser inhibitory effects,
respectively, on mycelial-phase growth (27).

Results obtained in applying the shake cul-
ture 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 produc-
tion. 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 pre-
paration of histoplasmin samples, the demonstration
of H and M antigens in the samples by a quantita-
tive 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 Biolog-
ical Reagents Section procedure (34) for the produc-
tion 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 con-
trol.

Determination of cell yield. The method for de-
termining growth units in shake and stagnant cul-
tures 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 micro-
scopic 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 M : . The reverse finding was
designated Y M . Results reported are those ob-
tained for one of the two cultures made for each
sample. Major differences between duplicate or triplic-
ate cultures are indicated.

Biochemical methods. Histoplasmin samples
from cultures of the strains used in the preliminary
studies were tested for reducing sugar, for carbohy-
dr ate, and for nucleic acid and protein. Determina-
tions 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, respec-
tively (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
dia lyzed against distilled water and concentrated 10
times with Polyethylene glycol 20,000 were deter-
m ined by the phenol-sulfuric acid method (7).

Ultraviolet absorption determinations were made
with a Beckman model DUR recording quartz spec-
trophotometer (Beckman Instruments Co., Fullerton,
Calif.). The reducing sugar and carbohydrate deter-
minations 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 sam-
ple 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 1% gelatin with 1:10,000
Merthiolate.

RESULTS

Physiological responses. Some of the phys-
ilological events that accompany the produc-
tion 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 polysaccha-
ride, protein, and nucleic acids. The histo-
plasmin samples were collected from shake
cultures of yeast- and mycelial-phase inocula
and from stagnant cultures grown in aspara-
gine medium (Fig. 1).

With strain 6617, as was the case with 6624
and A811, the sequence of events during incu-
bation 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 metabo-
lized, polysaccharide had appeared, and mate-
rial 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 x 10^5 to 4 x 10^5 yeast-phase cells per ml of medium; shake culture. (B) Inoculum: 0.3 ml of mycelial-mat suspension (15 mm^2 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-mm^2 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
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.

| Days grown | 25°C | | 30°C | | 37°C |
|------------|------|---|------|---|------|
| Cells      | Titer | Cells | Titer | Cells | Titer |
| Form       | Yield | H | M | Form | Yield | H | M | Form | Yield | H | M |
| 6          | Y,M₄₄ | 0.4 | 0 | 0 | Y,M₄₄ | 0.4 | 0 | 0 | Y,M₄₄ | <0.5 | 0 | 0 |
| 13         | Y,M₄₄ | 5.5 | 1 | 0 | Y,M₄₄ | 1.6 | 2 | 4 | Y,M₄₄ | <0.5 | 0 | 0 |
| 20         | Y,M₄₄ | 12.6 | 4 | 8 | Y,M₄₄ | 1.2 | 1 | 4 | Y,M₄₄ | <0.5 | 0 | 0 |
| 27         | Y,M₄₄ | 12.6 | 16 | 16 | Y,M₄₄ | 2.0 | 4 | 8 | Y,M₄₄ | <0.5 | 0 | 0 |
| 34         | Y,M₄₄ | 9.1 | 64 | 64 | Y,M₄₄ | 3.1 | 4 | 8 | Y,M₄₄ | <0.5 | 0 | 0 |
| 41         | NR | NR | NR | NR | Y,M₄₄ | 6.7 | 4 | 8 | Y,M₄₄ | <0.5 | 0 | 0 |

* Inoculum: 9.8 × 10⁴ yeast-phase cells per ml of medium. Incubated with shaking. Results at 25 and 37°C are the averages of duplicate cultures.
* Microscopic examination: amounts of yeast phase (Y) and mycelial phase (M) present, each on an arbitrary scale of 0 to 5.
* Growth units.
* Reciprocal of dilutions.
* 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.

| Days grown | pH 4.40 | | pH 6.41 | | pH 6.78 |
|------------|---------|---|---------|---|--------|
| Cells      | Titer   | Cells | Titer   | Cells | Titer |
| Form       | Yield   | H | M | Form | Yield | H | M | Form | Yield | H | M |
| 6          | Y,M₄₄  | <0.5 | 0 | 0 | Y,M₄₄ | 0.4 | 0 | 0 | Y,M₄₄ | <0.5 | 0 | 0 |
| 13         | Y,M₄₄  | 8.7 | 0 | 0 | Y,M₄₄ | 5.5 | 1 | 0 | Y,M₄₄ | 6.3 | 1 | 0 |
| 20         | Y,M₄₄  | 15.5 | 16 | 16 | Y,M₄₄ | 12.6 | 4 | 8 | Y,M₄₄ | 17.4 | 8 | 16 |
| 27         | Y,M₄₄  | 17.0 | 8 | 32 | Y,M₄₄ | 12.6 | 16 | 16 | Y,M₄₄ | 11.5 | 16 | 64 |
| 34         | Y,M₄₄  | 11.3 | 32 | 64 | Y,M₄₄ | 9.1 | 64 | 64 | Y,M₄₄ | 7.4 | 32 | 128 |

* Inoculum: 9.8 × 10⁴ yeast-phase cells per ml of medium. Incubated with shaking at 25°C.
* Microscopic examination: amounts of yeast phase (Y) and mycelial phase (M) present, each on an arbitrary scale of 0 to 5.
* Growth units.
* 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
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-asparagine were tested with strains 6623 and 6624 (Tables 7 and 8). Less than the standard amount of inoculum was inadvertently used, necessitating reincubation of the cultures 4 days later.

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

| Medium | Days grown | Cells | Titer | H | M | Form | Yield | Cells | Titer | H | M | Form | Yield |
|--------|------------|-------|-------|---|---|------|-------|-------|-------|---|---|------|-------|
| Unmodified | 7 | Y,M<sub>s</sub> | 0.4 | 0 | 0 | Y,M<sub>s</sub> | 8.6 | 1 | 1 | Y,M<sub>s</sub> | <0.5 | 0 | 0 | Form<sup>a</sup> | Yield<sup>b</sup> |
|          | 13 | Y,M<sub>s</sub> | 5.5 | 1 | 0 | Y,M<sub>s</sub> | 14.2 | 8 | 16 | Y,M<sub>s</sub> | 2.0 | 0 | 0 | |
|          | 20 | Y,M<sub>s</sub> | 12.6 | 4 | 8 | Y,M<sub>s</sub> | 7.9 | 8 | 32 | Y,M<sub>s</sub> | 9.6 | 1 | 2 | |
|          | 27 | Y,M<sub>s</sub> | 12.6 | 16 | 16 | Y,M<sub>s</sub> | 7.1 | 16 | 16 | Y,M<sub>s</sub> | 17.3 | 8 | 32 | |
|          | 34 | Y,M<sub>s</sub> | 9.1 | 64 | 64 | Y,M<sub>s</sub> | 7.2 | 32 | 32 | Y,M<sub>s</sub> | 10.5 | 8 | 32 | |
| Modified | 6 | Y,M<sub>s</sub> | 0.5 | 0 | 0 | Y,M<sub>s</sub> | 7.7 | 1 | 1 | Y,M<sub>s</sub> | 0.5 | 0 | 0 | Form<sup>a</sup> | Yield<sup>b</sup> |
|          | 13 | Y,M<sub>s</sub> | 5.2 | 1 | 0 | Y,M<sub>s</sub> | 10.8 | 8 | 16 | Y,M<sub>s</sub> | 2.4 | 1 | 0 | |
|          | 20 | Y,M<sub>s</sub> | 8.2 | 2 | 8 | Y,M<sub>s</sub> | 10.0 | 16 | 32 | Y,M<sub>s</sub> | 9.7 | 1 | 4 | |
|          | 27 | Y,M<sub>s</sub> | 12.1 | 8 | 32 | Y,M<sub>s</sub> | 8.8 | 32 | 32 | Y,M<sub>s</sub> | 16.6 | 16 | 8 | |
|          | 34 | Y,M<sub>s</sub> | 10.5 | 8 | 32 | Y,M<sub>s</sub> | 6.2 | 32 | 64 | Y,M<sub>s</sub> | 9.6 | 16 | 16 | |

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

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

<sup>c</sup> Growth units.

<sup>d</sup> Reciprocal of dilutions.

| Medium | Days grown | Cells | Titer | H | M | Form | Yield | Cells | Titer | H | M | Form | Yield |
|--------|------------|-------|-------|---|---|------|-------|-------|-------|---|---|------|-------|
| Unmodified | 6 | Y,M<sub>s</sub> | 16.8 | 1 | 0 | Y,M<sub>s</sub> | 19.2 | 2 | 0 | Y,M<sub>s</sub> | 17.4 | 2 | 0 | Form<sup>a</sup> | Yield<sup>b</sup> |
|          | 13 | Y,M<sub>s</sub> | 11.9 | 16 | 0 | Y,M<sub>s</sub> | 13.3 | 8 | 0 | Y,M<sub>s</sub> | 14.8 | 16 | 0 | |
|          | 20 | Y,M<sub>s</sub> | 6.5 | 32 | 0 | Y,M<sub>s</sub> | 5.8 | 16 | 0 | Y,M<sub>s</sub> | 8.7 | 64 | 0 | |
|          | 27 | Y,M<sub>s</sub> | 6.3 | 16 | 0 | Y,M<sub>s</sub> | 6.3 | 32 | 0 | Y,M<sub>s</sub> | 6.4 | 32 | 0 | |
|          | 34 | Y,M<sub>s</sub> | 5.7 | 32 | 0 | Y,M<sub>s</sub> | 5.6 | 32 | 0 | Y,M<sub>s</sub> | 5.6 | 64 | 0 | |
| Modified | 6 | Y,M<sub>s</sub> | 8.4 | 1 | 0 | Y,M<sub>s</sub> | 15.1 | 2 | 0 | Y,M<sub>s</sub> | 12.2 | 2 | 0 | Form<sup>a</sup> | Yield<sup>b</sup> |
|          | 13 | Y,M<sub>s</sub> | 7.2 | 4 | 0 | Y,M<sub>s</sub> | 10.7 | 16 | 0 | Y,M<sub>s</sub> | 11.7 | 16 | 0 | |
|          | 20 | Y,M<sub>s</sub> | 7.5 | 16 | 0 | Y,M<sub>s</sub> | 11.3 | 32 | 0 | Y,M<sub>s</sub> | 9.9 | 32 | 0 | |
|          | 27 | Y,M<sub>s</sub> | 9.7 | 32 | 0 | Y,M<sub>s</sub> | 11.3 | 64 | 0 | Y,M<sub>s</sub> | 9.1 | 32 | 0 | |
|          | 34 | Y,M<sub>s</sub> | 9.8 | 64 | 0 | Y,M<sub>s</sub> | 9.5 | 64 | 0 | Y,M<sub>s</sub> | 7.6 | 64 | 0 | |

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

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

<sup>c</sup> Growth units.

<sup>d</sup> 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.

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

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

* Inoculum: 9.8 × 10⁴ 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.

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

* Growth units.

* 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)*

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

* Inoculum: 9.8 × 10⁴ 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.

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

* Growth units.

* 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.
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

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

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 0.4% casein hydrolysate. Inoculum: $9.8 \times 10^6$ yeast-phase cells per ml of medium; cultures shaken at 25°C.

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: $9.8 \times 10^6$ 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**

| Sample                  | Age (days) | $H. capsulatum$ 6623 | $H. capsulatum$ 6624 |
|-------------------------|------------|-----------------------|-----------------------|
|                         |            | $pH$                  | Cell yield            | $pH$                  | Cell yield            | Titer   |
|                         |            |                       | $H$ | $M$ |                       |                       |         |
| Culture                 | 14         | 6.60                  | 7.1 | 2  | 4  | 6.20                  | 7.0 | 4  | 0  |
| Mycelial suspension     | 0          | 7.40                  | 7.1 | 0  | 0  | 7.40                  | 7.0 | 0  | 0  |
| in PBS                  | 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  |

* 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.

* Growth units.

* Reciprocal of dilutions.

* Original culture used for the production of washed mycelium.
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 asparagine 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.

ACKNOWLEDGMENTS

Training was provided by the Laboratory Directors' Program, which is supported by Public Health Service Training Grant 5 T01 GM 00567 from the National Institute of General Medical Sciences. The laboratory research was performed at the Laboratory Division, Center for Disease Control, under the supervision of the junior author.

LITERATURE CITED

1. Abernathy, R. S., and D. C. Heiner. 1961. Precipitation reactions in agar in North American blastomycosis. J. Lab. Clin. Med. 57:604–611.
2. Anderson, K. L., and S. Marcus. 1968. Sporulation characteristics of Histoplasma capsulatum. Mycopathol. Mycol. Appl. 36:179–187.
3. Artis, D., and G. L. Baum. 1963. Tuberculate spore formation by thirty-two strains of Histoplasma capsulatum. Mycopathol. Mycol. Appl. 21:29–35.
4. Chase, M. W., and C. A. Williams. 1968. Ultraviolet spectroscopy for protein determination, p. 278–282. In C. A. Williams and M. W. Chase (ed.), Methods in immunology and immunoochemistry, vol. 2. Academic Press Inc., New York.
5. Cross, F. W. 1948. The effect of hydrogen-ion concentration on the yeastlike phase of Histoplasma capsulatum (Darling). Pub. Health Rep. 63:739–746.
6. De Monbreun, W. A. 1934. Cultivation and cultural characteristics of Darling's Histoplasma capsulatum. Amer. J. Trop. Med. 14:93–125.
7. Dubois, M., K. A. Gilles, J. K. Hamilton, P. A. Rebers, and F. Smith. 1956. Colorimetric method for determination of sugars and related substances. Anal. Chem. 28:350–356.
8. Ehrrhard, H.-B. and L. Pine. 1972. Factors influencing the production of H and M antigens by Histoplasma capsulatum: development and evaluation of a shake culture procedure. Appl. Microbiol. 23:236–249.
9. Emmons, C. W., B. J. Olson, and W. W. Eldridge. 1945. Studies in the role of fungi in pulmonary disease. I. Cross reactions of histoplasmin. Pub. Health Rep. 60:1383–1394.
10. Gilbert, B. E., and D. H. Howard. 1970. Incorporation of cystine by yeast cells of Histoplasma capsulatum. Infect. Immunity 2:169–174.
11. Goodman, N. L., R. F. Sprouse, and H. W. Lash. 1968. Histoplasmin potency as affected by culture age. S. bournaudia 6:273–284.
12. Heiner, D. C. 1958. Diagnosis of histoplasmosis using precipitin reactions in agar gel. Pediatrics 22:616–627.
13. Howell, A., Jr. 1939. Studies on Histoplasma capsulatum and similar form species. I. Morphology and development. Mycologia 31:191–216.
14. Howell, A., Jr. 1940. Studies on Histoplasma capsulatum and similar form species. II. Effect of temperature. Mycologia 32:671–690.
15. Howell, A., Jr. 1941. Studies on Histoplasma capsulatum and similar form species. III. Effect of hydrogen ion concentration. Mycologia 33:103–117.
16. Howell, A., Jr. 1947. Studies of fungus antigens. I. Quantitative studies of cross-reactions between histoplasin and blastomycin in guinea pigs. Pub. Health Rep. 62:631–651.
17. Kabat, E. A., and M. M. Mayer. 1961. Experimental immunochrometry. Charles C Thomas, Publisher, Springfield, Ill.
18. McDearman, S. C. 1962. A modified histoplasmin in the hemagglutination test for histoplasmosis. Amer. J. Med. Technol. 28:146–160.
19. McVeigh, L., and K. Morton. 1965. Nutritional studies of Histoplasma capsulatum. Mycopathol. Mycol. Appl. 25:294–308.
20. Mahy, T. A., and H. W. Lash. 1969. Nutritional study of the mycelial phase of Histoplasma capsulatum. Mycopathol. Mycol. Appl. 38:231–241.
21. Park, J. T., and M. J. Johnson. 1949. A submicrodetermination of glucose. J. Biol. Chem. 181:149–151.
22. Pine, L. 1954. Studies on the growth of Histoplasma capsulatum. I. Growth of the yeast phase in liquid media. J. Bacteriol. 65:671–679.
23. Pine, L. 1955. Studies on the growth of Histoplasma capsulatum. II. Growth of the yeast phase on agar media. J. Bacteriol. 70:375–381.
24. Pine, L. 1957. Studies on the growth of Histoplasma capsulatum. III. Effect of thiamin and other vitamins on the growth of the yeast and mycelial phases of Histoplasma capsulatum. J. Bacteriol. 74:239–245.
25. Pine, L. 1970. Growth of Histoplasma capsulatum. VI. Maintenance of the mycelial phase. Appl. Microbiol. 19:413–420.
26. Pine, L., and E. Drouhet. 1963. Sur l'obtention et la conservation de la phase levure d'Histoplasma capsulatum et d'H. duboisii en milieu chimiquement défini. Ann. Inst. Pasteur (Paris) 108:788–804.
27. Pine, L., and C. Peacock. 1968. Studies on the growth of Histoplasma capsulatum. IV. Factors influencing conversion of the mycelial phase to the yeast phase. J. Bacteriol. 75:167–174.
28. Rowley, D. A., and L. Pine. 1955. Some nutritional factors influencing growth of yeast cells of Histoplasma capsulatum to mycelial colonies. J. Bacteriol. 69:695–700.
29. Salvin, S. B. 1949. Cysteine and related compounds in the growth of the yeastlike phase of Histoplasma capsulatum. J. Infec. Dis. 84:273–283.
30. Salvin, S. B., and G. A. Hotle. 1948. Factors influencing histoplasmin formation. J. Bacteriol. 56:541–546.
31. Schell, G. J. 1946. Biochemical and immunological properties of Histoplasma capsulatum No. 650. Yale J. Biol. Med. 18:41–54.
32. Schubert, J. H., L. Ajello, J. S. Cooper, and L. C. Runyon. 1955. Evaluation of histoplasmin and yeast phase antigens derived from a single strain of *Histoplasma capsulatum* in the complement fixation test. J. Bacteriol. 69:558-562.

33. Schubert, J. H., L. Ajello, S. Stanford, and V. Q. Grant. 1953. Variation in complement fixation antigen production by different strains of *Histoplasma capsulatum* grown on two media. J. Lab. Clin. Med. 41:91-97.

34. Schubert, J. H., H. J. Lynch, Jr., and L. Ajello. 1961. Evaluation of the agar-plate precipitin test for histoplasmosis. Amer. Rev. Resp. Dis. 84:845-849.

35. Schubert, J. H., and G. L. Wiggins. 1966. Additional studies of histoplasmin formation. Mycopathol. Mycol. Appl. 30:81-91.

36. Smith, C. E., E. G. Whiting, E. E. Baker, H. G. Rosenberg, R. R. Beard, and M. T. Saito. 1948. The use of coccidioidin. Amer. Rev. Tuberc. 57:330-360.

37. Tenenberg, D. J. 1960. The serology of histoplasmosis, p 168-188. In H. D. Sweany (ed.), Histoplasmosis. Charles C Thomas, Publisher, Springfield, Ill.

38. U.S. Department of Health, Education and Welfare. 1970. Preparation of *Histoplasma capsulatum* (Histoplasmin) CF antigen, p. 83-84. In Procedural manual for production of bacterial, fungal, and parasitic reagents. Public Health Service, Center for Disease Control, Atlanta, Ga.

39. Wiggins, G. L., and J. H. Schubert. 1965. Relationship of histoplasmin agar-gel bands and complement-fixation titers in histoplasmosis. J. Bacteriol. 89:589-596.

40. Yen, C. M., and D. H. Howard. 1970. Germination of blastospores of *Histoplasma capsulatum*. Sabouraudia 8:242-252.