EFFECT OF PHYSICO-CHEMICAL ENVIRONMENT ON SPHERULATION OF *COCCIDIOIDES IMMITIS* IN A CHEMICALLY DEFINED MEDIUM

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Coccidioides immitis undergoes a completely different type of growth *in vivo* from that *in vitro*. The parasitic form, or spherule, appears in animal tissue as a non-budding, spherical structure 20 to 80 μ in diameter with a thick, refractile, double wall, and is filled with numerous small endospores 2 to 5 μ in diameter. The saprophytic form appears in laboratory media as mycelium with branching, septate hyphae which fracture into numerous thick-walled, rectangular, spherical, or ellipsoidal arthrospores 2 to 4 μ in diameter.

Observation of the parasitic form of *C. immitis* in solid or semi-solid complex media has been reported by several authors. MacNeal and Taylor (1914) observed multiplication of spherules in infected pus when added to ascitic fluid or gelatinized horse serum containing sterile kidney slices. Lack (1938) noted development of spherules from arthrospores inoculated into Hall tubes containing glucose broth and partially coagulated egg albumin. Baker and Mrak (1941) observed the development of spherules in a variety of solid media when active growth had ceased as a result of drying of the media. Burke (1951) reported the appearance of spherules in a partially defined solid medium containing coconut milk. Conant and Vogle (1954) observed spherules in cultures transferred to Saboraud's medium following exposure to "Tween 80." Lubarsky and Plunkett (1955) were able to produce spherules in Tyrode's solution, supplemented with chick-embryo extract and serum and aerated periodically with measured amounts of oxygen and carbon dioxide. This laboratory was the first to report production of spherules in a chemically defined liquid medium (Converse, 1955).

The work reported below represents further studies on the formation of spherules in a chemically defined liquid medium (figures 1 to 6). The following factors were investigated: the total concentration of nutrients in the medium, the hydrogen ion concentration of the medium, the temperature, aeration, and exposure to light during incubation, and the preparation of inoculum.

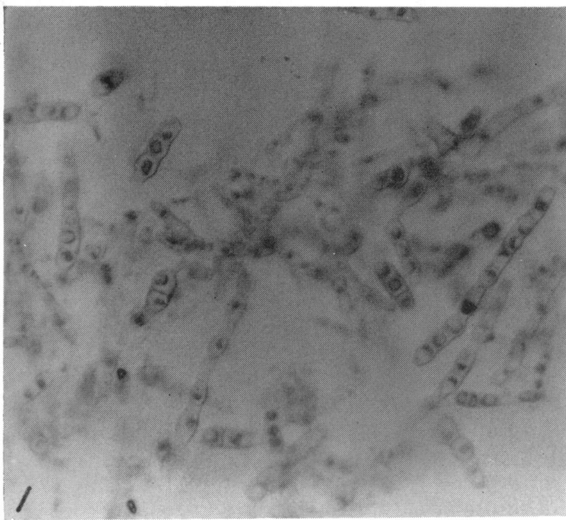
MATERIALS AND METHODS

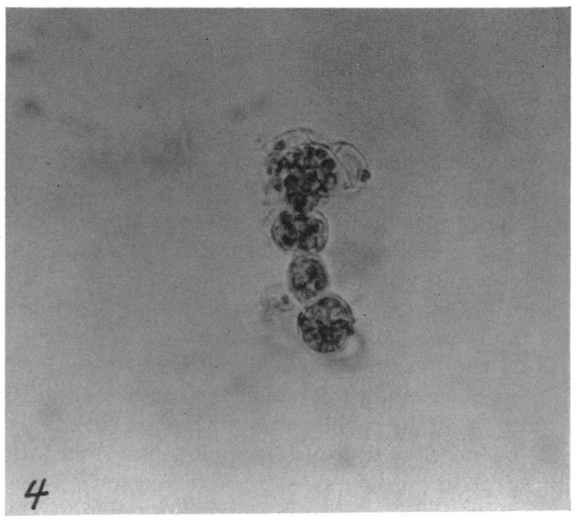
Medium. The experimental medium, unless otherwise indicated, was composed of 0.022 M glucose, 0.016 M ammonium acetate, 0.003 M each of mono-potassium and di-potassium phosphate, 0.0016 M magnesium sulfate, 1.24×10^{-5} M zinc sulfate, 2.4×10^{-4} M sodium chloride, 2×10^{-5} M calcium chloride, 1.4×10^{-4} M sodium bicarbonate, and distilled water, with a final pH of 6.6. This medium is referred to hereafter as basal spherule medium. The medium was dispensed in 50-ml volumes into 250-ml pyrex glass Erlenmeyer flasks, which were closed with rubber stoppers, each bearing a thistle tube packed with gauze-covered cotton, and was sterilized by autoclaving for 15 min at 121.5 C.

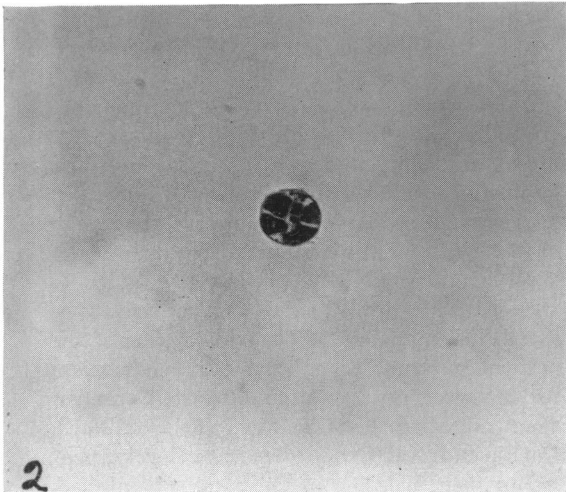
Inoculum. C. immitis strain M11, a rodent isolate from Arizona, was grown for 28 days at 34 C with shaking, in a liquid synthetic medium (Basal #2, Roessler *et al.*, 1946 + 4 ppm Zn^{++} as $ZnSO_4$) and stored at 5 C until use. Each flask of experimental medium received 0.1 ml of the inoculum or approximately 1×10^7 viable particles (short chains of arthrospores and hyphal fragments).

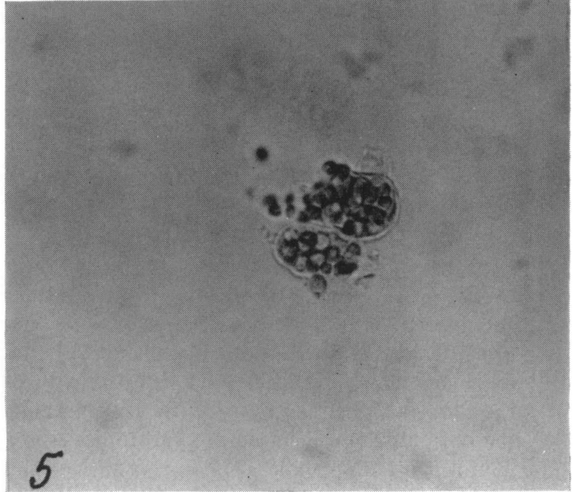
Incubation. Experimental cultures were incubated for 72 hr at 34 C on a reciprocal shaking machine operating through a 4½-in stroke at approximately 96 excursions per min.

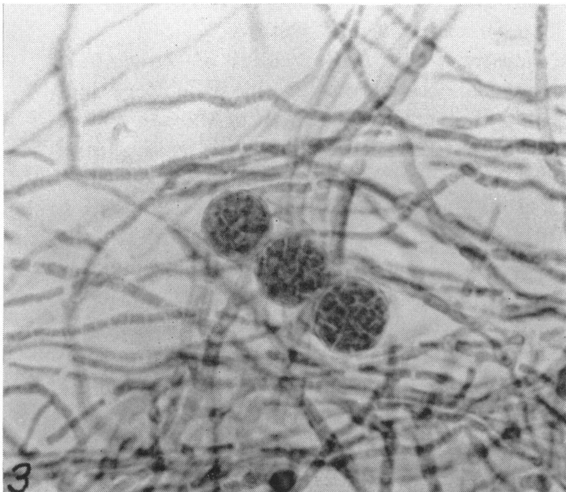
Assay procedures. Spherule yields were determined by microscopic examination of lactophenol-blue wet mounts made from culture material. Comparative rather than absolute measurements were used, due to the lack of a satisfactory method for separation of spherules from other growth structures. The relative amount of spherules to total growth was esti-

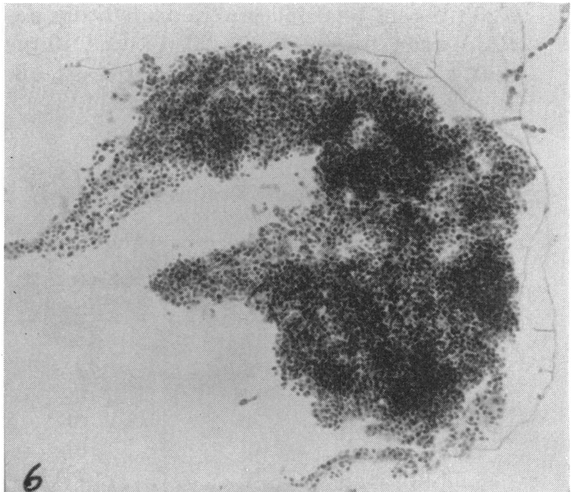












Figures 1 to 6 show development of *Coccidioides immitis* spherules from arthrospores and release of endospores into substrate.

Figure 1. Arthrospore inoculum. × 300. *Figure 2.* Young spherule showing start to cleavage. × 300. *Figure 3.* Spherules in later stage of development showing an increase in cleavage planes. × 435. *Figure 4.* Spherules with maturing endospores in evidence. × 300. *Figure 5.* Spherules with fractured cell-walls. Endospores are being released into substrate. × 435. *Figure 6.* Appearance of culture following endospore release. × 167.

mated for each culture and assigned a number on an arbitrary scale of 0 to 8, with increasing numbers indicating increased spherule formation. With this method, comparative measurements could be made among cultures.

Total growth (hyphae, arthrospores, spherules, and endospores) of cultures was determined either by macroscopic appearance of cultures, using comparative estimations on a scale of 0 to 8, or by viable plate counts. In the latter method, the pour-plate technique was used with a plating medium composed of peptone, 1 per cent; glucose, 2 per cent; yeast autolysate, 0.1 per cent; and agar, 2 per cent; with final pH of 6.9 to 7.2. Plates were incubated for 48 hr at 34 C.

Light intensities were measured in foot candles with a General Electric exposure meter (PH 77 C, Type DW-48).

Hydrogen ion concentrations were determined with the glass electrode.

RESULTS

Dilution of medium. Previous observations (Converse, 1955) indicated that the total concentration of nutrients in the chemically defined medium influenced the *in vitro* growth of the parasitic phase of *C. immitis*. To determine the optimum concentration, duplicate flasks of a liquid synthetic medium (Basal #2, Roessler *et al.*, 1946, plus 4 ppm of Zn^{++} as $ZnSO_4$) having 3.36 per cent total solids were diluted with distilled water to 90, 80, 60, 20, 15, and 10 per cent of the specified total solids content and inoculated with *C. immitis* strain M11.

TABLE 1

Effect of dilution of medium on growth and spherulation

Per Cent Solids in Medium (Total Nutrient Content)	Viable Fragments*	Spherule Yield*
	× 10^6 *per ml*	
3.36	113	0
3.03	74	0
2.69	72	0†
2.02	49	0†
0.67	16	2
0.48	14	4
0.34	6	5

* Reported as averages of duplicate flasks.
† Occasional spherules noted (very scarce).

TABLE 2

Effect of pH of medium on growth and spherulation

pH		Visual Estimation of Total Growth*	Spherule yield*
Initial	Terminal		
3.0	3.35	0	0
4.0	4.27	0	0
5.0	6.23	0–1†	0
6.0	7.20	4	2
6.6	7.40	4	2
8.0	7.63	4	2
9.0	7.70	4	1–2
10.0	7.93	3–4	1

* Reported as averages of 2 to 4 flasks.
† No sporulation of any type in these cultures.

As shown in table 1, spherule formation increased progressively with dilution of the medium, beginning at the 80 per cent level and becoming very apparent at the 20 per cent level. However, a proportionate decrease in growth of the cultures also resulted. Consideration of these opposing effects on spherule yield led to the selection of a medium with 20 per cent of the specified total solids (a five-fold dilution of the original medium) as a basal spherule medium for further experimentation. Repeated observations indicating the stimulatory effects of NaCl, $CaCl_2$, and $NaHCO_3$ on spherule development led to their inclusion in the basal spherule medium at the concentrations reported in the section on materials and methods.

Hydrogen ion concentrations of the medium. Previous observations in this laboratory indicated that optimum growth of the saprophytic or mycelial phase of *C. immitis* in a glucose-ammonium acetate-inorganic salts medium was obtained if the initial pH was adjusted to between 7.0 and 7.5. It was also noted that the final pH of the cultures was always approximately 7.7 regardless of the initial pH of the medium. The optimum pH range for growth of the parasitic phase was established by inoculation of duplicate flasks of basal spherule medium adjusted to pH values of 3.0, 4.0, 5.0, 6.0, 6.6, 8.0, 9.0, and 10.0 before sterilization.

The effect of initial pH of the medium on growth and spherule production is summarized in table 2. No growth occurred below pH 5.0 and no sporulation of any type (arthrospores, chlamydospores, or endospores) occurred below pH

6.0. Maximum spherule yields were obtained at 6.0, 6.6, and 8.0, with no significant difference among the three levels. A few spherules were noted in media with initial pH values as high as 10.0. Total growth of the cultures was not significantly different at any pH level between 6.0 and 10.0.

Temperature of incubation. The importance of incubation temperature in the *in vitro* growth of the parasitic phase of other dimorphic fungi, *Histoplasma capsulatum* and *Blastomyces dermatitidis*, has been noted by Salvin (1949*a*, *b*), who was able to grow the yeast phase of both organisms at 37 C, but not at 25 C. It was thought that an incubation temperature close to that in normal animals might also stimulate *in vitro* spherule formation of *C. immitis*. To determine the effect of incubation temperature on spherulation of this organism, duplicate flasks of basal spherule medium were inoculated and incubated with shaking at 25, 26, 30, 34, 37, and 41 C.

Microscopic examination of the cultures after 72 hr of incubation indicated (table 3) that an increase in temperature from 26 and 37 C was paralleled by an increase in spherule formation. No spherules were formed at 25 or 26 C. Approximately equal growth was obtained at all temperatures except at 41 C, which inhibited germination of the inoculum. No spherules were evident in the 41 C cultures at 72 hr, but continued incubation at this temperature for 10 to 14 days resulted in almost 100 per cent conversion of the arthrospore inoculum to spherules, without intermediate mycelial growth.

Aeration of cultures. Observation of Mader (1943), Henry and Anderson (1948), Barnett and Lilly (1950), and others, indicated that oxygen tension, carbon dioxide content, and accumulation of volatile metabolic products in the medium were important factors in the sporulation of various fungi. Conant (1941) observed the yeast-like or parasitic phase of *H. capsulatum* in sealed tubes of blood agar, but not in unsealed tubes. Bullen (1949) was able to grow the yeast-like or parasitic phase of the dimorphic, pathogenic fungi, *H. capsulatum*, *B. dermatitidis*, and *Sporotrichum schenckii* on peptone agar by controlling the carbon dioxide content of the atmosphere above the cultures. Since all of these factors may be influenced by changes in aeration of cultures during incubation, an attempt was made to establish the effect of aeration on development of the parasitic phase of *C. immitis* in synthetic medium.

TABLE 3

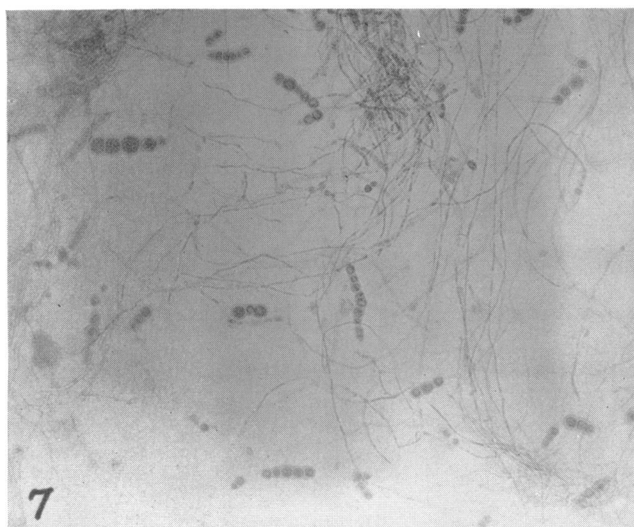
Effect of temperature of incubation on spherulation

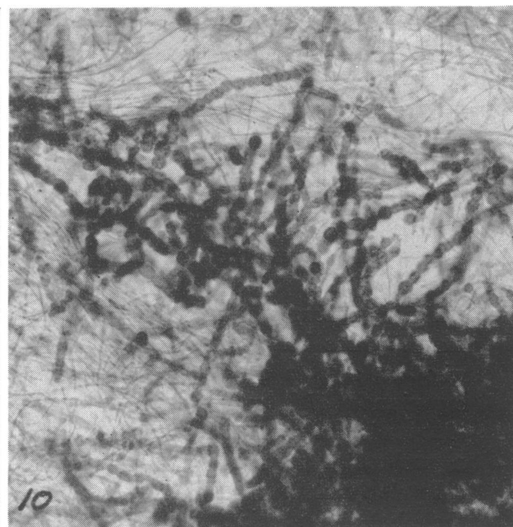
Temperature of Incubation	Spherule Yield*
C	
25	0
26	0
30	1–2
34	3
37	6
41	0

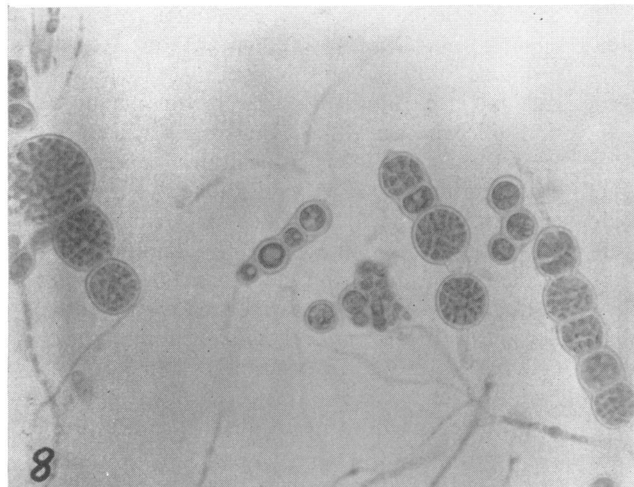
* Reported as averages of duplicate flasks.

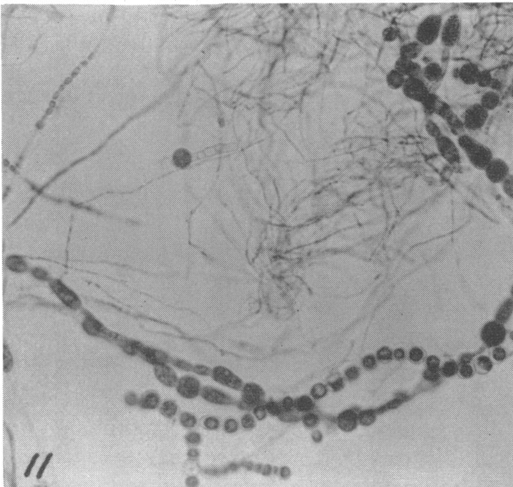
Preliminary experiments, in which aeration of the medium was varied by changing either the diameter of the flask opening or the surface-to-volume ratio of the medium, resulted in a relative increase of spherule formation with decreased aeration. Stationary incubation at 34 or 37 C resulted in improved spherule production over that of incubation with shaking at either of these temperatures. Stationary cultures contained twice the number of spherules found in shaken cultures, but the total growth was reduced by 50 per cent. Also the length of incubation for maximum spherule production was 9 to 12 days in stationary cultures, as compared with 3 to 5 days in shaken cultures. Spherules in stationary cultures occurred in extremely long chains (figures 7 to 9), in contrast to the short chains and single spherules noted in shaken cultures (figures 10 to 12).

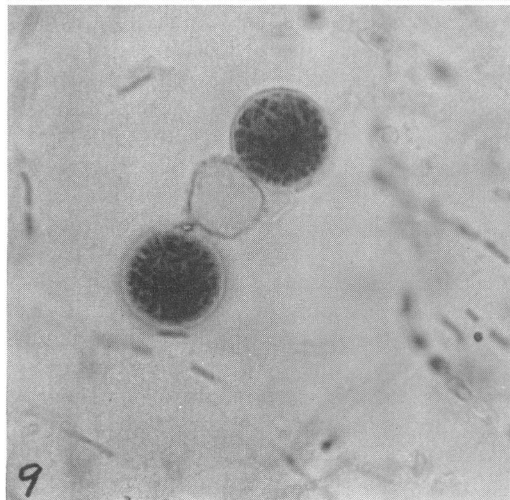
Attempts were made to increase spherule formation and at the same time maintain normal growth levels by initiating growth with stationary incubation and completing the growth cycle on the shaker or by reversing this procedure. Two sets of replicate cultures were incubated at 34 C, one set in a stationary position and the other on the shaker. At 24-hr intervals throughout a 4-day period duplicate flasks were removed from the shaker and placed in a stationary position. At 48-hr intervals over a 12-day period duplicate flasks were removed from the stationary position and placed on the shaker. The latter time intervals were longer since growth of stationary cultures was much slower. All flasks were examined microscopically at 24-hr intervals between the third and fourteenth day of incubation. Optimum spherule development was obtained in

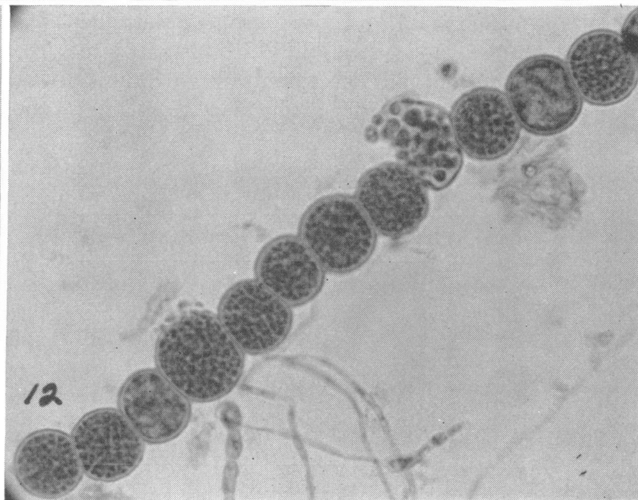












FIGS. 7–12

cultures completing a stationary period of incubation of 7 to 9 days during the initial stage of growth. Cultures remaining on the shaker for more than 3 days, initially, were unsatisfactory, due to the gradual disappearance of spherules after the 3rd or 4th day. However, it was necessary to incubate cultures on the shaker for the first 2 or 3 days in order to maintain normal growth levels.

Exposure to light. The effect of light on sporulation of various fungi often has been reported in the literature. A stimulatory effect was noted by Christenberry (1938), Yarwood (1941), Timnick *et al.* (1951), and others. However, it was suspected that the opposite may obtain with the parasitic phase of *C. immitis*, since absence of light during incubation would duplicate more nearly the normal conditions of spherule development in animal tissue. The effects of simulated daylight, twilight and darkness on spherule formation and the time at which these conditions exerted their greatest effect were determined in the following manner.

For the daylight condition, a reflector containing two 15-watt daylight fluorescent bulbs was suspended 12 in above a shaker tray, to give a light intensity of 63 foot candles at the base of the flasks. Artificial twilight was maintained by positioning a second tray on the shaker so that the reflected light from the fluorescent bulbs resulted in a light intensity of 4.5 foot candles. Complete darkness was maintained by placing a loosely fitting metal cover over a third tray, which excluded all light but did not interfere with aeration of the cultures.

Duplicate flasks of basal spherule medium were inoculated with strain M11 and incubated at 34 C for 72 hr under conditions of continuous light, darkness, and twilight. Other flasks were alternated between light and darkness and *vice versa* at the end of each 24-hr interval of the 72-hr incubation period. Data from microscopic examination of the cultures are recorded in table 4. Complete absence of light was most favorable for spherule formation. Under conditions of intermittent illumination, spherule formation was increased when darkness occurred during the initial part of the incubation period. The rate of growth, or total growth of the cultures as estimated macroscopically, was not affected by varied illumination.

TABLE 4

Effect of light on spherulation

Light Exposure in Foot Candles during 72-hr Incubation Period			Spherule Yield*
0–24 hr	24–48 hr	48–72 hr	
63	63	63	0–1†
63	63	0	1–2
63	0	63	1–2
63	0	0	1–2
4.5	4.5	4.5	3
0	63	63	3–4
0	63	0	4
0	0	63	4–5
0	0	0	4–5

* Reported as averages of 4–10 flasks.

† Occasional spherules noted (very scarce).

Preparation of inoculum. The occasional failure of *C. immitis* to produce spherules under optimum conditions led to an investigation of the inoculum as a possible cause. Replicate flasks of basal spherule medium were inoculated with cultures of strain M11 grown for 28 days and stored at 5 C for either 1 month or 4 months. Other flasks of the medium were inoculated with fresh cultures grown for 4, 7, 14, or 28 days. All flasks were incubated with shaking at 34 C for 72 hr and examined microscopically for spherule production.

Figures 7 to 9, and 10 to 12, show the appearance of spherules in shaken cultures, and stationary cultures, respectively.

Figure 7. Representative field of shaken culture. × 167.

Figure 8. Enlarged view of culture in figure 7 showing detailed morphology of spherules in all stages of development. × 300.

Figure 9. Mature spherules. Note shrunken empty spherule wall in center following endospore release. × 435.

Figures 10 and 11. Representative fields showing extreme length of spherule chains in stationary cultures. × 167.

Figure 12. Enlarged view of culture in figures 10 and 11 showing detailed morphology of spherule chain. Note beginning of endospore release in two of the spherules. × 435.

TABLE 5

Effect of age of inoculum on spherulation

Age of Inoculum		Spherule Yield
Growth at 34 C	Storage at 5 C	
days	*mo*	
28	4	0†
28	1	2
28	0	2
14	0	4
7	0	5
4‡	0	0

* Reported as averages of 2 to 4 flasks.

† An occasional spherule seen in these cultures.

‡ No arthrospores present in this inoculum.

As indicated in table 5, extended storage at 5 C was detrimental to the development of spherules. Inocula grown for 7 and 14 days were superior to those grown for either 4 or 28 days. A complete lack of arthrospores in the 4-day inoculum was noted on microscopic examination of the various inocula, which suggested that, although younger inocula were more effective in spherule formation, the presence of arthrospores in the inoculum was necessary.

DISCUSSION

The mechanism responsible for the development of either the parasitic or the saprophytic phase of *C. immitis* under different environmental conditions is not fully understood, nor have the physiological conditions necessary for development of the parasitic phase *in vitro* been fully established. However, the data presented above provide evidence that spherulation in a chemically defined medium must take place at a critical period early in the development of the cultures, beyond which growth of the parasitic phase will be unsuccessful. The rate of growth of cultures may be of prime importance in establishing this critical point. This may explain the failure of inocula devoid of arthrospores to produce spherules (the extra time required for hyphal conversion to arthrospores and thence to spherules).

Aeration had a marked effect on rate of growth. Stationary incubation resulted in slower development of the cultures and approximately 50 per cent of the total growth compared to incubation with shaking, but the relative spherule yield was doubled. It was necessary to harvest spherules from shaken cultures not later than the fourth or fifth day of incubation, as a gradual disappearance of the parasitic form obtained thereafter, unless development was halted by storage at 5 C. Conversely, cultures could be incubated in a stationary position for 14 days or longer without ill effects. Disappearance of the parasitic form in shaken cultures was probably due to a selective change in environment, such as exhaustion of specific nutrients or accumulation of metabolic products, which favored mycelial development and inhibited spherule development. The occurrence of these effects would be a function of the rate of growth.

The amount of oxygen or carbon dioxide available to the organism during growth was probably an important factor in spherule development and could be directly affected by either rate of growth or aeration. This is supported by observations by Conant (1941) and Bullen (1949) on the growth of the so-called yeast phase of other dimorphic pathogenic fungi under controlled carbon dioxide atmospheric conditions. Bullen was able to separate the effects of oxygen from those of carbon dioxide and show the oxygen tension was unimportant, but increased carbon dioxide content favored the growth of the parasitic or yeast phase. Lubarsky and Plunkett (1955) were able to show favorable effects of increased carbon dioxide on production of the parasitic phase of *C. immitis* in complex medium. All of this evidence agrees with our observations that reduced aeration results in an increase of spherule formation, which may be due to an accumulation of carbon dioxide in the flask, and that the addition of sodium bicarbonate to the medium can be substituted for reduced aeration with the same results. Additional information on this point is expected from experiments being conducted to determine the effects of continuous passage of an inert gas, containing various concentration ratios of oxygen and carbon dioxide, over cultures incubated in chemically defined medium.

Total growth of cultures was not always affected similarly by factors enhancing spherule development. A decrease in total growth always accompanied an increase in relative spherule yield due to variation of either the aeration or the total concentration of nutrients in the medium,

whereas total growth remained constant when relative spherule yields were increased by changes in pH, temperature, or reduced illumination. This would seem to rule out adverse growth conditions as a stimulant for *in vitro* growth of the parasitic phase of the organism. It appears more likely that inhibition of arthrospore germination, at a specific time during the incubation period, is the causative factor.

It was noted that spherules may develop from secondary arthrospores following an initial mycelial growth or that they may be converted directly from the arthrospores in the inoculum. The number of spherules present in the cultures greatly exceeds the number of arthrospores originally present in the inoculum, indicating intermediate mycelial development. Also the length of spherule chains, shown in figures 10, 11, and 12, is far greater than that of the arthrospore chains introduced in the inoculum. However, microscopic examinations of cultures incubated at 41 C indicated a direct conversion of the arthrospores in the inoculum to spherules, with complete absence of arthrospore germination at this temperature.

Production of spherules in chemically defined medium would materially enhance physical, biochemical, and nutritional studies of the parasitic phase of *C. immitis*. It would also permit the accumulation of large amounts of antigenic material, free from foreign protein.

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SUMMARY

Studies were made on some physico-chemical environmental factors affecting the growth of the parasitic phase (spherule) of *Coccidioides immitis* in a chemically defined liquid medium. Most satisfactory production was obtained from young (7- to 14-day) well sporulated inocula, in a medium containing 0.67 per cent solids (total nutrient content) with initial pH values between 6.0 and 8.0, incubated at 37 C in complete darkness for 72 hr under reduced aeration.

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