EFFECTS OF CULTURAL CONDITIONS ON THE DEVELOPMENT OF ANTIGENS BY *COCCIDIOIDES IMMITIS*

I. Immunodiffusion Studies†

JOHN R. ROWE, Ph.D., VICTOR D. NEWCOMER, M.D., and JOSEPH W. LANDAU, M.D.

Isolation and characterization of the antigens of *Coccidioides immitis* are essential for a better understanding of the host-parasite relationships in coccidioidomycosis. Several procedures for obtaining antigenic materials have been employed although none has proved entirely satisfactory because of poor reproducibility (1–9). Filtrates from some cultures may be found suitable as antigens in skin, complement fixation or precipitin tests, while others are of little or no value. Antigens have been prepared under identical conditions and yet exhibited as much as five-fold variations in potency (1, 2). A variety of factors appear to be important in determining the specificity and potency of these antigens, including possible strain differences, conditions of cultures and methods of extraction.

The development of antigens by *C. immitis* has been studied with the standard skin, complement fixation and precipitin tests (1–6). Another useful technic for the study of fungal antigens has been provided by the introduction of immunodiffusion technics. Under optimal conditions, these technics permit differentiation of the constituents of mixtures of antigens and identification of immunochemically related substances. They have recently been used in examination of the antigens of *C. immitis* (8–10, 24). As many as six antigens could be detected with certain sera and the existence of antigenic differences between strains was also suggested. Finally, the effects of heat, changes of pH and various enzymes suggested marked chemical differences between antigens. The purpose of this paper is to present the results of studies of various cultural factors on the development of antigens by *C. immitis* using the technique of immunodiffusion.

† From the Department of Medicine, Division of Dermatology, University of California, Center for the Health Sciences, and the Veteran's Administration Center, Los Angeles, California.

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MATERIALS AND METHODS

Preparation of antiserum

One female Dutch rabbit, weighing approximately 4 lb was infected by the intraperitoneal injection of 0.5 ml of a 50% suspension in physiological saline of fragmented mycelium, prepared from an actively growing culture of *C. immitis* (strain Silveira) in asparagine synthetic medium. Blood was drawn on the day prior to infection and 12 weeks later. After clotting, the sera were separated, divided into aliquots and stored at −20° C until used. These are referred to as pre-infection and post-infection sera.

Preparation of antigens

The Silveira strain of *C. immitis* was employed in all investigations. In addition, three other strains were used in one experiment: strains Venezuela Brown and 46 (11), and strain U.C.L.A. 58, isolated from the sputum of a patient with primary pulmonary coccidioidomycosis. The Silveira strain was cultured in the following media: Roessler's medium (12), modified to contain 0.3% ammonium acetate; asparagine synthetic medium (1), Sabouraud's medium (13); Converse's medium (14) containing 0.03% sodium fluoride and 0.05% Tamol*; Nickerson's medium (15); Kuehn's medium (16); Casamino acids medium (4% Casamino acids,** 1% dextrose) and brain heart infusion containing p-aminobenzoic acid (13). In addition, organisms were cultured in brain heart infusion containing added dextrose and in asparagine synthetic medium to which was added different amounts of streptomycin.*** All media were sterilized at 125° C for 15 minutes.

Actively growing cultures were obtained by transferring three times, at intervals of three days, in Kuehn's synthetic medium maintained at 37° C. Five milliliters of suspensions of the fungus were inoculated into 100 ml of each medium contained in 250 ml Erlenmeyer flasks and placed on a rotary action shaker operated at 160 cycles per minute. After incubation at different temperatures and for various periods of time, filtrates were passed through Seitz sterilizing pads. Portions of the growth were examined microscopically and the pH of each filtrate was determined. The filtrates were lyophilized, reconstituted to one tenth of their initial volumes in physiological saline and

* Rohm and Haas Co., Philadelphia, Pennsylvania

** Difco Laboratories, Detroit, Michigan

*** Squibb and Sons, New York, New York

stored at −20° C until used. These were designated culture filtrate concentrates. Packed mycelia were washed with water, dried by passing air through them while still on the filter pads and weighed. Ethylene glycol extracts were prepared from the packed mycelia as described previously (9). Samples of each uninoculated medium were lyophilized and reconstituted to one-tenth volume for use as culture media controls. Double diffusion in agar gel was performed according to the method of Ouchterlony (17) with some modifications. Glass lantern slides 3¼″ x 4″ were coated with a 0.2% solution of Bacto-agar,** dried at 90° C for 30 minutes, then covered with 15 ml of 1.3% agar containing 0.03% sodium azide as a preservative, allowed to cool and then stored at 5° C until used. Wells, 2 mm in diameter, were cut in the agar in two patterns. The first pattern, used in screening, consisted of wells at intervals of 8.0 mm in rows 6.0 mm apart (Fig. 1). Alternate rows were offset 4 mm. In the second pattern, used in titrations, the wells were also cut at intervals of 8.0 mm in rows 6.5 mm apart (Fig. 2). Approximately 5 μl of either serum or solution of antigen was added to the wells using capillary tubes. The amount of the antigen in a filtrate was determined by adding successive dilutions of the culture filtrate concentrate to the wells in one row and post-infection serum to the wells in the adjacent row (18). (Fig. 1). The slides were then stored in a moist atmosphere at room temperature. All slides were examined daily for five days, although in most instances, precipitin lines appeared within 48–72 hours. The slides were then washed in physiological saline and stained with Amido Schwarz. All studies were carried out at least in duplicate, and in those instances where differences occurred, all results are recorded.

Immunoelectrophoresis was performed essentially in the manner described by Scheidegger (19).

RESULTS

Precipitin lines formed by the various culture filtrate concentrates are illustrated in Fig. 1. No precipitin lines were formed by the pre-infection serum when tested against either culture media controls, culture filtrate concentrates or ethylene glycol extracts. None was formed by the post-infection serum when tested against the culture media controls. When culture filtrate concentrates diluted to contain comparable amounts of antigen were tested in a row of wells, the precipitin lines were observed to meet and form a continuous line, indicating that a common antigen was developed in all media.

Immunoelectrophoresis

Electrophoresis of post-infection serum and diffusion against culture filtrate concentrates resulted in arcs only in the gamma region, identifying the reacting component in the serum as a gamma globulin (Fig. 3).

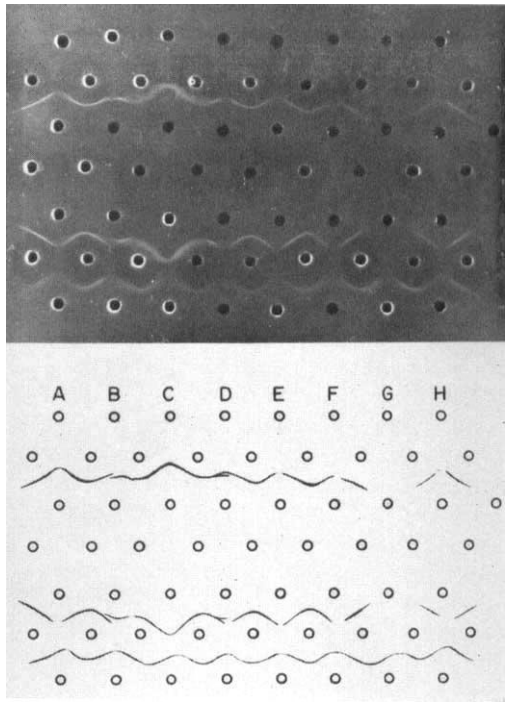

FIG. 1. Formation of precipitin lines by *C. immitis* culture filtrate concentrates. Top row, culture media controls; 2nd row, post-infection serum; 3rd row, culture filtrate concentrates from 45 day cultures at 30° C, 4th row, pre-infection serum; 5th row, culture filtrate concentrates from 45 day cultures at 37° C; 6th row, post-infection serum; 7th row, 37° C culture filtrate concentrates diluted to contain comparable amounts of antigen. Culture media in the wells are identified by letters: A, Sabouraud's; B, asparagine; C, Roessler's; D, Converse's; E, Nickerson's; F, Kuehn's; G, Casamino acids; H, brain heart infusion. In the 7th row, well G contained culture filtrate concentrate from a culture in asparagine medium with 2.0% streptomycin added.

Effect of temperature on antigen production

The maximum dilutions of culture filtrate concentrates in which antigen was detectable following growth of *C. immitis* in the various media at different temperatures for 45 days are presented in Table I. Culture filtrate concentrates obtained from 10 days growth in Roessler's and Sabouraud's media contained antigens in dilutions of 1:1 or 1:2. No antigen was detectable in culture filtrate concentrates after 10 days growth in any of the other media. In two of the three in-

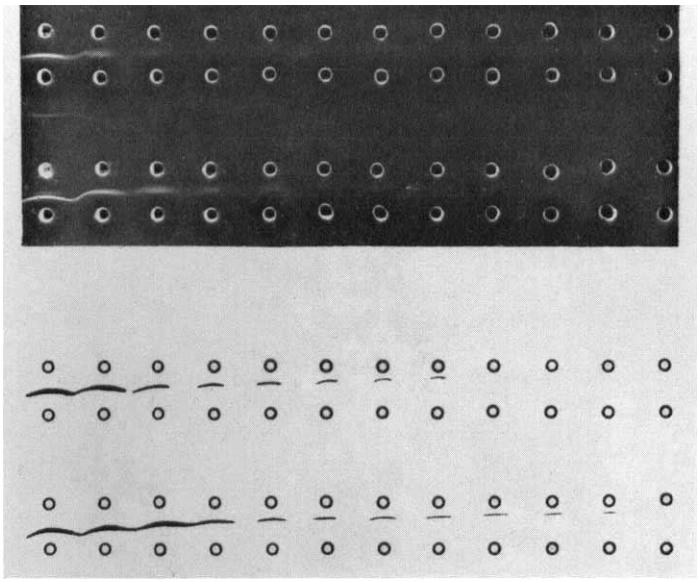

FIG. 2. Titrations of antigen in culture filtrate concentrates. Wells in the top and 3rd rows contained increasing dilutions of culture filtrate concentrates; wells in the 2nd and 4th rows contained post infection serum.

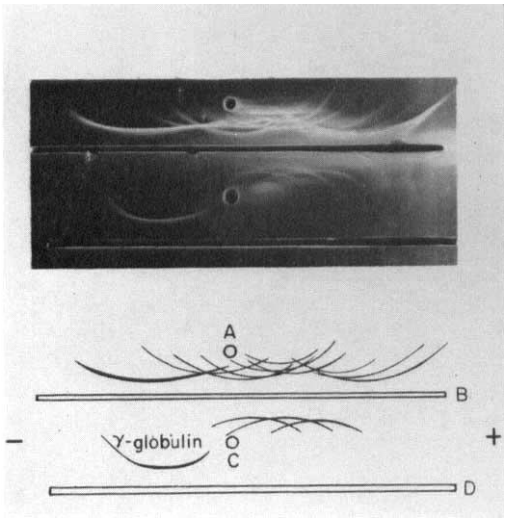

FIG. 3. Immunoelectrophoresis of immune sera. A, human coccidioidomycosis serum; B, goat anti-human serum; C, post infection rabbit serum; D, *C. immitis* culture filtrate.

TABLE I

Antigen production by C. immitis *in various media after 45 days growth at different temperatures*

Medium	Room Temperature	30° C	37° C
Roessler's	1:32*	1:64	1:64
			1:128
Asparagine	1:1	1:8	1:8
			1:16
Sabouraud's	1:16	1:16	1:8
			1:16
Converse's	Not done	1:8	1:4
			1:8
Nickerson's	Not done	1:4	1:4
		1:8	1:8
Kuehn's	Not done	1:2	1:1
Casamino acids	Not done	None	None
Brain heart infusion	Not done	1:1	None
		1:2	1:2

* Maximum dilutions of culture filtrate concentrates producing detectable precipitin lines.

stances where culture filtrate concentrates were obtained at room temperature and 37° C, greater amounts of antigen were obtained from cultures grown at higher temperatures. No significant differences were observed in antigen production if only cultures at 30° and 37° C were compared. The highest dilutions containing detectable antigen were obtained from cultures in Roessler's medium. Maximum dilutions permitting detection of antigen were approximately the same in filtrates from cultures in Sabouraud's, Converse's, Nickerson's and asparagine media. The smallest

amounts of antigen were obtained from cultures in Kuehn's and brain heart infusion media and no antigen was found in filtrates from cultures in the Casamino acids medium. At 45° C, the fungus failed to produce visible growth in any of the media and no antigen could be detected.

Time of appearance of antigen

The time of appearance of antigen in culture filtrates of *C. immitis* grown in Roessler's, Sabouraud's and asparagine media at 37° C was investigated (Table II). Antigen was detected in Roessler's and Sabouraud's media as early as the 10th day and in all 3 media by the 15th day. Dilutions of culture filtrate concentrates with which precipitin lines could be detected increased with the period of growth.

Effect of concentration of dextrose on antigen production

Increasing amounts of antigen were produced by *C. immitis* cultured in brain heart infusion, supplemented with 1% and 4% dextrose, compared to cultures in the unmodified medium, containing 0.2% dextrose (Table III).

Effect of streptomycin on antigen production

The addition of streptomycin in concentrations of 0.2, 1.0 and 2.0 mg/ml to asparagine synthetic medium did not alter the growth or the amounts of antigen in culture filtrates.

Ethylene glycol antigens

Ethylene glycol antigen was extracted from packed mycelia of *C. immitis* obtained from cultures grown in all media at 30° C and 37° C for 10 days and 45 days. Precipitin lines were produced with undiluted extracts of all mycelia including those whose culture filtrates did not contain detectable amounts of antigen.

TABLE II

Times of appearance of antigen in cultures of C. immitis *at 37° C*

Medium	Day 5	Day 10	Day 15	Day 20	Day 30	Day 45
Roessler's	None*	1:2	1:16	1:32	1:16	1:64 1:128
Asparagine	None	None	1:2 1:4	None 1:2	1:4 1:8	1:8 1:16
Sabouraud's	None	1:1	1:4	1:8	1:8	1:8 1:16

* Maximum dilutions of culture filtrate concentrates producing detectable precipitin lines.

TABLE III

Effects of dextrose on antigen production by C. immitis *cultured in brain heart infusion at 30° C and 37° C*

Total dextrose concentration	30° C	37° C
0.2%	1:1*	None
	1:2	1:2
1.2%	1:8	1:16
4.2%	1:16	1:32
	1:32	

* Maximum dilutions of culture filtrate concentrates producing detectable precipitin lines.

Antigen production by different strains

The production of culture filtrate antigen by four different strains of *C. immitis* grown in Sabouraud's medium for 30 days at 37°C was studied. Antigen was detected in equal concentration when prepared from strains Silveira and U.C.L.A. 58. The amounts produced in cultures of strains 46 and Venezuela Brown were approximately equal and in all instances lower than in the other two strains (Table IV).

Changes of mycelial weights and pH of media during growth

The weights of packed mycelia and pH of filtrates from one series of cultures of *C. immitis* are presented in Table V. No general correlation was found between the weights of packed mycelia from the cultures in different media and the amounts of antigen in culture filtrate concentrates. The weights of packed mycelia obtained from cultures in Roessler's medium increased to a maximum at 10 days, then decreased, while the amounts of antigen increased. The weights of packed mycelia and the amounts of antigen in concentrated filtrates from cultures in Sabouraud's medium and asparagine medium increased until the 30th day, with little change at the 45th day. The addition of dextrose to brain heart infusion produced increases in both weights of packed mycelia and amounts of antigen in culture filtrates on the 45th day.

The pH of uninoculated media ranged from

5.3 to 7.5. After 45 days, all inoculated culture media had become alkaline with pH's generally between 8.0 and 8.7. These changes had occurred by the 10th day in all except Roessler's and Nickerson's media. There was no apparent correlation between the volumes of sodium hydroxide required to adjust the pH's of uninoculated media to 8.5 and the amounts of antigen produced. No correlation could be established between either pH and weights of mycelia in different cultures or between pH and the amounts of antigen they contained.

Morphology of cultures

Microscopic examination of portions of most cultures revealed that the fungus grew in the mycelial phase without the production of arthrospores. Cultures in Roessler's medium, however, contained a few arthrospores and many round, thick-walled bodies after the 10th day. No spherules containing endospores were observed in any of the cultures.

TABLE IV

Production of antigen by different strains of C. immitis *in Sabouraud's medium at 37°*

Strain of *C. immitis*			
Silveira	46	Venezuela Brown	U.C.L.A. 58
1:16*	1:1 1:2 1:8	1:1 1:4 1:8	1:8 1:16 1:16

*Maximum dilutions of culture filtrate concentrates producing detectable precipitin lines.

DISCUSSION

A single precipitin line was observed in immunodiffusion studies with culture filtrates and the post-infection serum. The detection of a single precipitin line can be attributed to the antiserum used in these studies since the same culture filtrates when tested against human sera or certain other rabbit sera produced additional lines. The possibility that different antigens determined the maximum dilutions producing detectable precipitin lines was excluded since a continuous line was formed when the various culture filtrate concentrates were tested in adjacent wells. The specificity of the precipitin line is stressed by the fact that none developed between pre-infection serum and any of the culture media concentrates or between post-infection serum and any of the culture media controls. Further, the identification of the reactive component in the rabbit serum as a gamma globulin essentially eliminates the possibility that the precipitin line observed in these studies was non-specific (20, 21). The failure of culture filtrate concentrates from Casamino acids medium to

TABLE V

Changes of mycelial weights in grams and pH of filtrates from cultures of C. immitis *at 37° C*

Medium	pH prior to inoculation	Day 5		Day 10		Day 15		Day 20		Day 30		Day 45		Ml. 0.25 N* Na OH
		Wt.	pH.	Wt.	pH.	Wt.	pH.	Wt.	pH.	Wt.	pH.	Wt.	pH.	
Roessler's	5.8	2.3	7.4	5.6	6.8	5.0	7.3	3.5	8.0	2.4	8.1	1.5	8.0	15.0
Asparagine	6.5	0.2	7.2	1.8	8.3	1.8	8.3	2.0	8.3	4.2	8.2	4.5	8.2	13.0
Sabouraud's	5.8	0.5	7.3	3.5	8.4	3.9	8.3	3.8	8.3	5.0	8.2	4.8	8.2	1.9
Converse's	6.0	—	—	1.1	8.2	—	—	—	—	—	—	1.5	8.0	4.0
Nickerson's	6.2			6.7	6.5	—	—	—	—	—	—	2.3	7.5	9.0
Kuehn's	7.0	—	—	5.7	9.2	—	—	—	—	—	—	3.0	9.0	10.0
Casamino acids	5.3	—	—	1.3	8.3	—	—	—	—	—	—	1.0	8.0	12.5
Brain heart infusion	7.5	—	—	3.7	9.2	—	—	—	—	—	—	1.4	8.6	6.3
BHI + 1% dextrose	7.4	—	—	3.4	7.9	—	—	—	—	—	—	2.9	8.4	6.5
BHI + 4% dextrose	7.3	—	—	2.2	8.2	—	—	—	—	—	—	6.9	8.7	8.0

* Volume required to adjust pH of uninoculated media to 8.5.

produce precipitin lines was not the result of the solubility in excess antigen as has been reported with rabbit serum (22) since lines also failed to develop when the culture filtrate concentrate was diluted.

The relationship of the antigen-antibody combination producing a precipitin line in agar gel to that in the standard precipitin and complement fixation tests has not been elucidated. Preliminary studies suggest that multiple precipitin lines in agar gel are produced with sera containing high titers of complement fixation antibodies (9, 23). To date, precipitin lines have not been found in sera from patients without coccidioidomycosis, although sera from some patients with the disease have failed to produce precipitin lines.

The effect of temperature on the development of antigens *in vitro* has been considered to be of some importance (3). The highest amounts of complement fixing antigen have been found to be produced in cultures maintained at 30°C. In the present study, it was found that cultures in Roessler's and asparagine synthetic media generally developed higher amounts of antigen with increasing temperature. There were no significant differences in the amounts of antigens in filtrates from cultures in other media incubated at 30°C and 37°C.

Antigens were first detected in culture filtrates as early as the 10th day and increased in quantity during the succeeding periods of growth. This supports previous observations that maximum antigen development is achieved by extending the period of incubation (1–3, 5).

The culture medium has been demonstrated to be important in determining the potency and specificity of the resulting antigens (3, 4, 6). Currently asparagine medium (1, 2) and various media containing peptone (6, 24) are most frequently employed in the production of culture filtrate antigens of *C. immitis* for use in diagnostic tests. Antigens satisfactory for use in immunodiffusion tests were produced in five media, including Sabouraud's and asparagine media. The highest amounts of antigen were obtained from cultures in Roessler's medium. Others have found this medium unsatisfactory for the production of complement fixing antigens, although these results are not comparable since cultures were maintained at room temperature and for shorter periods of time (6).

The addition of dextrose to cultures in brain heart infusion increased the amounts of antigen produced. Particular attention has been directed toward the high polysaccharide content of most fungal antigens, including coccidioidin (8, 25). Dextrose is intimately involved in the biosynthesis of polysaccharides, although whether the addition of dextrose produced an increase in extracellular polysaccharides was not determined. The concentration of dextrose in the various culture media did not correlate with the amounts of antigen. Satisfactory development of antigen was obtained from cultures in Sabouraud's medium which contained 4% dextrose, Roessler's medium, which contained 2% and asparagine medium, which contained 1%. In addition, reduction of the level of dextrose in a synthetic medium from 4% to 1% has been found to enhance growth of *C. immitis*, although no investigations of antigen development were made (12).

The addition of streptomycin to cultures of *C. immitis* has been reported both to enhance (26) and to be without effect (27) on growth. No effect on either growth or the development of antigen was observed in the present studies.

The possibility has been considered that antigenic differences exist between strains of *C. immitis*. For this reason, strains derived from a variety of sources are currently employed in the production of commercial coccidioidin, although autogenous coccidioidin has not been shown to elicit stronger cutaneous reactions than the stock antigen (1). Recent studies using gel diffusion technics suggest that some antigenic differences may exist between strains (9). Variations in virulence of different strains in mice have been demonstrated, but these cannot be correlated with the extent of the disease in the patients from whom the organisms were isolated (11). Strain Silveira is highly virulent for white mice while strain 46 has low virulence and Venezuela Brown is intermediate. Using standard precipitin tests, the formation of antigen was found to be most rapid with Venezuela Brown and least rapid is strain 46 cultured in asparagine synthetic medium (5). In the present study, with the immunodiffusion technic and using cultures in Sabouraud's medium, the largest amounts of antigen were found to be developed in cultures of strains Silveira and U.C.L.A. 58 and lower amounts in cultures of Venezuela Brown and strain 46.

Microscopic examinations revealed the usual mycelial phase morphology of *C. immitis* in most cultures. In Roessler's medium, modifications of which have been used to produce spherules (28),

round, thick-walled bodies were observed and may have represented either chlamydospores or early spherule formation.

Some fungi produce increases and others decreases of pH in culture media (29). *C. immitis* has been demonstrated in this and other studies to increase pH to 8.0 and above in most media (3–5). The release of extracellular antigens by the fungus in culture media has been attributed to this increase in pH, possibly by producing greater permeability of the fungal cell wall (3). This concept is supported by the fact that the increase in pH precedes the development of antigen. This increase in pH occurred whether or not there was subsequent development of antigen. The increase in pH in cultures of *C. immitis* and other fungi has been attributed to the release of ammonia into the medium (30). It might be anticipated that the readiness with which pH of the medium were increased influenced the production of antigens. However, there was no correlation between the development of antigen and the amount of sodium hydroxide required to increase pH to an arbitrarily chosen value of 8.5.

No consistent relationship was discernible between the amounts of antigen produced and either the weights of packed mycelia on any day or the sequence of weight changes. Antigen could be extracted from packed mycelia from cultures in all media at all stages of growth including those instances where no antigen could be detected in the culture filtrate. Previous evidence has suggested that these ethylene glycol antigens are not identical with those released into culture media and it is conceivable that they are precursors.

Autolytic degeneration of mycelia has been proposed as the mechanism involved in the production of soluble extracellular antigens (3–5, 7). This theory is supported by the successful preparation of complement fixing antigens by lysis of young mycelia of *C. immitis* (7) and by the observation that the highest titers of antigen in standard precipitin tests were found in culture filtrates of the Venezuela Brown strain at a time when the mycelial weights were lowest (5). The presence of antigens in all ethylene glycol extracts and the observation that weights of mycelia from cultures in Roessler's medium decrease after 10 days while the amounts of antigen showed continued increase are consistent with the autolysis theory. In other instances, however, the increase in weights of mycelia paralleled the production of antigen in culture filtrates. While this is also consistent with the autolysis theory, the possibility must be considered that soluble antigens may be released without cellular destruction.

SUMMARY

1. *Coccidioides immitis* (strain Silveira) was cultured in Roessler's, asparagine, Sabouraud's, Converse's, Nickerson's, Kuehn's, Casamino acids and brain heart infusion media. The development of a single antigen in culture filtrates was studied with immunodiffusion technics.

2. Various amounts of antigen were found after incubation for 45 days at 30°C and 37°C in culture filtrates from all media except Casamino acids.

3. In Roessler's, asparagine and Sabouraud's media, the amounts of antigen developed increased as the temperature of incubation was raised from room temperature to 37°C.

4. The amount of antigen found in culture filtrates increased with the period of incubation.

5. Increased amounts of antigen were produced in brain heart infusion medium supplemented with dextrose compared with the unmodified medium.

6. An antigen could be extracted from washed mycelia from all media at all stages of growth, regardless of whether antigen was developed in the culture filtrate.

7. Slightly larger amounts of antigen were produced by cultures of strains Silveira and U.C.L.A. 58 in Sabouraud's medium compared with cultures of strains Venezuela Brown and 46.

8. The pH in all media increased to 8.0–8.7, but no correlation was discernible between the amount of antigen in culture filtrates and either the pH on any day or the rate of increase of pH.

9. No correlation was observed between the amounts of antigen produced and either the initial pH or the buffer properties of the medium in which the fungus was cultured.

10. No consistent relationship was discernible between the amounts of antigen produced and either the weights of packed mycelia on any day or the sequence of weight changes.

REFERENCES

1. Smith, C. E., Whiting, E. G., Baker, E. E., Rosenberger, H. G., Beard, R. R. and Saito, M. T.: The use of coccidioidin. Amer. Rev. Tuberc., **57**: 330, 1948.
2. Smith, C. E., Saito, M. T., Beard, R. R.,

KEPP, R. MCF., CLARK, R. W. AND EDDIE, B. U.: Serological tests in the diagnosis and prognosis of coccidioidomycosis. Amer. J. Hyg., **52:** 1, 1950.
3. JOHNSON, D. E.: The growth of *Coccidioides immitis* in relation to production of antigenic material. Doctoral Dissertation, University of California, Los Angeles, 1955.
4. RAMSDELL, F. W.: Studies on antigen production by *Coccidioides immitis*. Doctoral Dissertation, University of California, Los Angeles, 1957.
5. PAPPAGIANIS, D. AND KOBAYASHI, G. S.: Production of extracellular polysaccharides in cultures of *Coccidioides immitis*. Mycologia, **50:** 229, 1958.
6. AJELLO, L., WALLS, K., MOORE, J. C. AND FALCONE, F.: Rapid production of complement fixing antigens for systemic mycotic diseases. I. Coccidioidin: Influence of media and mechanical agitation on its development. J. Bact., **77:** 753, 1959.
7. PAPPAGIANIS, D., SMITH, C. E., KOBAYASHI, G. S. AND SAITO, M. T.: Studies of antigens from young mycelia of *Coccidioides immitis*. J. Infect. Dis., **108:** 35, 1961.
8. PAPPAGIANIS, D., PUTMAN, E. W. AND KOBAYASHI, G. S.: Polysaccharide of *Coccidioides immitis*. J. Bact., **82:** 714, 1961.
9. ROWE, J. R., NEWCOMER, V. D. AND WRIGHT, E. T.: Studies of the soluble antigens of *Coccidioides immitis* by immunodiffusion. J. Invest. Derm., **41:** 225, 1963.
10. ABERNATHY, R. S. AND HEINER, D. C.: Precipitation reactions in agar in North American blastomycosis. J. Lab. Clin. Med., **57:** 604, 1961.
11. FRIEDMAN, L., SMITH, C. E., ROESSLER, W. G. AND BERMAN, R. J.: The virulence and infectivity of twenty-seven strains of *Coccidioides immitis*. Amer. J. Hyg., **64:** 198, 1956.
12. ROESSLER, W. G., HERBST, E. J., MCCULLOUGH, W. G., MILLS, R. C. AND BREWER, C. R.: Studies with *Coccidioides immitis*. I. Submerged growth in liquid mediums. J. Infect. Dis., **79:** 12, 1946.
13. Difco Manual of Dehydrated Culture Media and Reagents, Difco Laboratories, Detroit, Michigan. 9th Edition, 1953: 79, 200.
14. CONVERSE, J. L. AND BESEMER, A. R.: Nutrition of the parasitic phase of *Coccidioides immitis* in a chemically defined liquid medium. J. Bact., **78:** 231, 1959.
15. KESSLER, G. AND NICKERSON, W. J.: Glucomannan-protein complexes from the cell walls of yeasts. J. Biol. Chem., **234:** 2281, 1959.
16. KUEHN, H. H.: Nutritional requirements of *Arthroderma tuberculatum*. Mycopathologia, **14:** 123, 1962.
17. OUCHTERLONY, O.: Antigen-antibody reactions in gels. V. Types of reaction in co-ordinated systems of diffusion. Acta. Path. Microbiol. Scand., **32:** 231, 1953.
18. VAN OSS, C. J. AND HECK, Y. S. L.: Qualitative and quantitative interpretation of double diffusion. Z. Immunitaetsforsch., **122:** 44, 1961.
19. SCHEIDEGGER, J. J.: Une micro-méthode de l'immuno-electrophorèse. Int. Arch. Allerg., **7:** 103, 1955.
20. METZGAR, R. S. AND GRACE, J. T., JR.: Agar precipitation between human serum albumin and hemoglobin in human tissue extracts. J. Immun., **86:** 578, 1961.
21. DECKERS, C. AND MAISIN, J.: Non-specific precipitin patterns obtained in double diffusion between serum and extracts of normal organs and tumours. Nature (London), **197:** 397, 1963.
22. GRABAR, P.: Immunoelectrophoretic analysis. *Methods of Biochemical Analysis*, Vol. 7, 1–38. New York, N. Y., Interscience, 1959.
23. NEWCOMER, V. D., LANDAU, J. W., LEHMAN, R. AND ROWE, J. R.: The local cellular response in coccidioidomycosis. Arch. Derm. (In press).
24. SCHUBERT, J. H. AND HAMPSON, R. C.: An appraisal of serologic tests for coccidioidomycosis. Amer. J. Hyg., **76:** 144, 1962.
25. HASSID, W. Z., BAKER, E. E. AND MCCREADY, R. M.: An immunologically active polysaccharide produced by *Coccidioides immitis*. Rixford and Gilchrist. J. Biol. Chem., **149:** 303, 1943.
26. CAMPBELL, C. C. AND SASLAW, S.: Enhancement of growth of certain fungi by streptomycin. Proc. Soc. Exp. Biol. Med., **70:** 562, 1949.
27. ROESSLER, W. G., HERBST, E. J., MCCULLOUGH, W. G., MILLS, R. C. AND BREWER, C. R.: Studies with *Coccidioides immitis*. II. *In vitro* effects of streptothricin and streptomycin. J. Infect. Dis., **79:** 23, 1946.
28. CONVERSE, J. L.: Growth of *Coccidioides immitis* in a chemically defined liquid medium. Proc. Soc. Exp. Biol. Med., **90:** 709, 1955.
29. GOLDFARB, N. J. AND HERRMAN, F.: A study of pH changes by molds in culture media. J. Invest. Derm., **27:** 193, 1956.
30. BUMP, W. S.: Observations on growth of *Coccidioides immitis*. J. Infect. Dis., **36:** 561, 1925.

DISCUSSION

DR. HERBERT MESCON (Boston, Mass.): Our bacteriologist insisted, when we were using fungi as antigens, that we grow them on liquid medium rather than agar medium. Is it your experience that you tend to get false titers or differences in titers, using liquid versus agar medium?

DR. JOHN R. ROWE (in closing): We have not extracted antigens from *Coccidioides immitis* grown on agar. In a recent publication, we reported the extraction of antigens from the fungus grown on the surface of Sabouraud's broth and found that they formed precipitin lines in double diffusion. The relative specificity of these antigens was not investigated, however.