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GOODMAN, Norman Loyal, 1931–
ENVIRONMENTAL STUDIES OF _HISTOPLASMA CAPSULATUM._

The University of Oklahoma, Ph.D., 1965
Bacteriology

University Microfilms, Inc., Ann Arbor, Michigan

THE UNIVERSITY OF OKLAHOMA

GRADUATE COLLEGE

ENVIRONMENTAL STUDIES OF *HISTOPLASMA CAPSULATUM*

A DISSERTATION

SUBMITTED TO THE GRADUATE FACULTY

in partial fulfillment of the requirements for the

degree of

DOCTOR OF PHILOSOPHY

BY

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Norman, Oklahoma

1964

ENVIRONMENTAL STUDIES OF *HISTOPLASMA CAPSULATUM*

APPROVED BY

DISSERTATION COMMITTEE

ACKNOWLEDGMENT

Sincere appreciation is recognized for the generous assistance extended by Dr. Howard W. Larsh in directing this study.

I am grateful to Drs. J. B. Clark, R. E. Collier, and G. C. Cozad for their criticism of the manuscript.

A note of gratitude is also extended to Dr. Carroll E. Palmer, of the U. S. Public Health Service, for his assistance in obtaining test materials and his advice concerning testing procedures.

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ENVIRONMENTAL STUDIES OF *HISTOPLASMA CAPSULATUM*

CHAPTER I

INTRODUCTION

Knowledge of the fungous disease histoplasmosis has increased considerably since it was first recognized by Darling in 1905. Extensive work has been done in the field of epidemiology and therapy. The epidemiological investigations were started in the 1940's by Christie and Peterson (11) and Palmer (31) using X-ray and skin test sensitivity studies as tools for locating individuals infected by *H. capsulatum*. These procedures were adopted as the most efficient means of determining infection and have been used extensively by many other clinicians and epidemiologists through the ensuing years. The skin testing programs by Palmer, Furcolow, Edwards, et al. (13, 15, 21, 34, 37), have been successful in delineating the endemic areas of histoplasmosis, but little progress was made in associating the

disease with the natural habitat of the etiological agent until the fungus was isolated from the soil in 1949 (17). Two years later Grayston, et al. (22), studied an epidemic of histoplasmosis in a farm family and directly linked the disease with the inhalation of the fungus by isolating the organism from a silo where the infected individuals had worked. During this same period Feller, Furcolow and Larsh (20) investigated an outbreak of histoplasmosis at Camp Gruber, Oklahoma. The fungus was isolated from the soil in a storm cellar where infected military personnel had found shelter during a rainstorm. These studies, along with others, definitely characterized histoplasmosis as a disease caused by an airborne fungus that was associated with soil.

Since this early work by Furcolow, Larsh, Loosli, et al., there have been numerous studies on this fungus and its association with soil. Ajello (2, 3, 4, 5, 6, 7) and Zeidberg (36, 37) have contributed much information about isolation of the fungus from various ecological sites. Emmons (18), Ajello (8), and Furcolow (21) have done considerable work to associate _H. capsulatum_ with the presence of chickens and birds in general. From their work, histoplasmosis has become known as a disease directly associated with areas where large concentrations of birds have been found.

Several theories have evolved from past epidemiological studies (21, 38) concerning the way H. capsulatum is spread throughout the endemic region. These theories range from how the fungus is spread by the airborne route to the type soil found in particular areas which supports good growth. All the proposed theories have plausible featues, but in general, have not been thoroughly investigated.

Epidemics of histoplasmosis outside recognized endemic areas have been reported (34). These epidemics appear to have followed the general pattern of being associated with bird roosting areas or chicken houses. Furcolow stated (21): "It is quite evident from studies in widely scattered parts of the country that the organism is present in certain areas, even of low sensitivity, but its prevalence is distinctly of a localized nature and not generalized as it is in the highly endemic areas."

It is well known that all soil microorganisms are controlled by their environmental conditions. Thus, it seems logical that the presence of H. capsulatum in a given area would be due to its ability to grow, or the inability of some antagonistic organism to grow in that particular soil type. Zeidberg observed (39): "If soil is indeed the determining factor in the occurrence of H. capsulatum in one

area and not in another it remains to be determined what particular physical, chemical and biological characteristics make it a good or poor natural habitat for the fungus."

If the soil theory is valid, it could delineate possible endemic areas of histoplasmosis in various parts of the world.

The purpose of this series of studies was to determine some of the environmental factors in soil that affect the growth of *Histoplasma capsulatum*, and thus support the soil theory. Also, this information may be helpful in finding a means of eradicating the fungus from endemic foci.

CHAPTER II

TEMPERATURE AND MOISTURE, THEIR EFFECT ON THE SURVIVAL OF *HISTOPLASMA CAPSULATUM* IN SOIL

All living organisms appear to be influenced to some degree by the temperature and moisture of their environment.

Menges, et al. (30), showed that *H. capsulatum* requires a high relative humidity and an incubation temperature of approximately 30 C for optimum growth. The map in Fig. 1 shows that in many regions of the endemic area the above optimum growth conditions exist for relatively short periods of the year. To infect the number of people that show positive skin tests to histoplasmin, the fungus must occur and remain infective for relatively long periods.

Materials and Methods

To determine the effect of temperature on the survival of H. capsulatum, two soil cultures were prepared by adding 150 g soil to a 250 ml Erlenmeyer flask. The soil

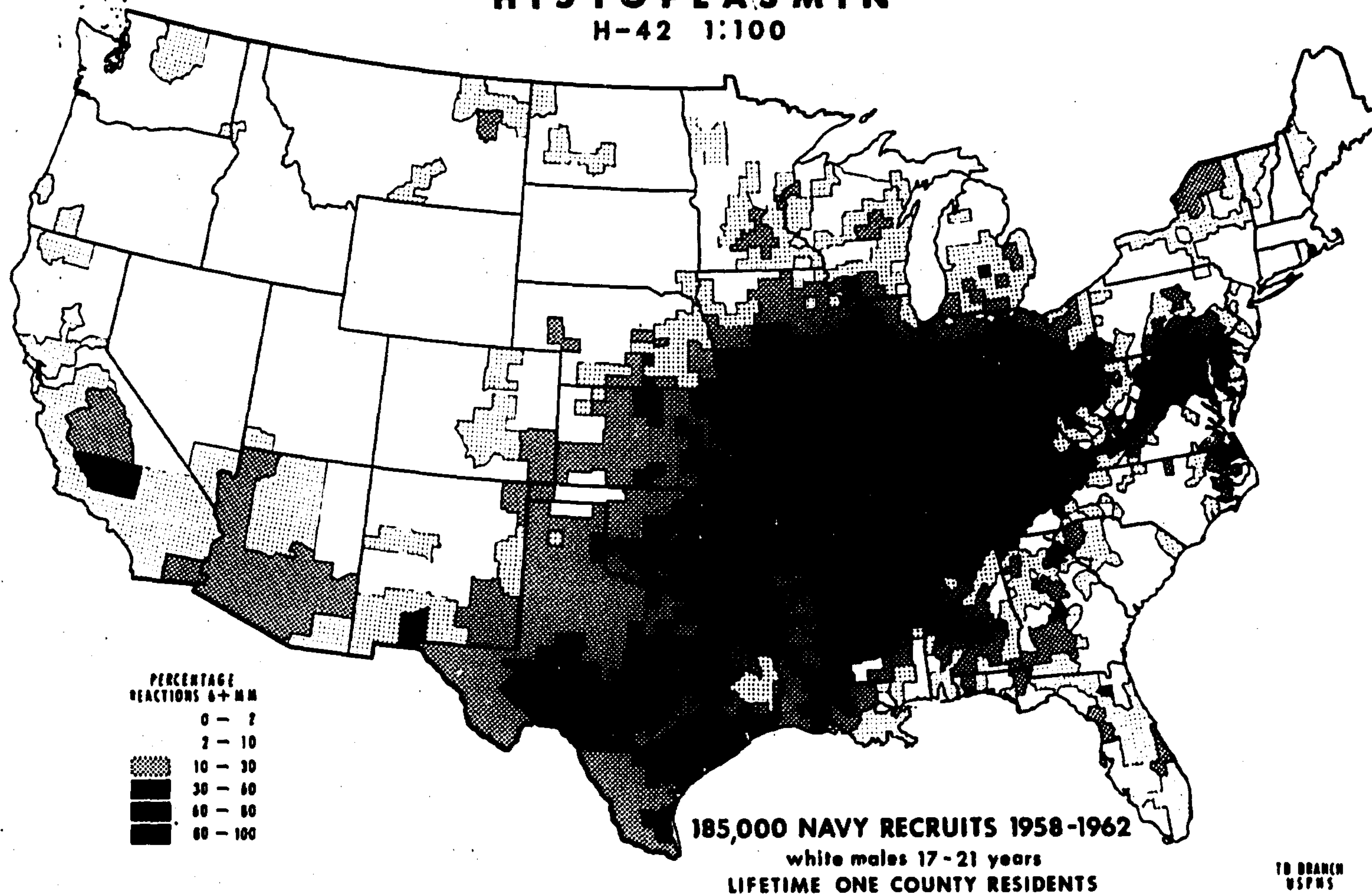

Figure 1.-- Geographic distribution of histoplasmin skin test sensitivity. (Courtesy of Dr. Corroll E. Palmer, U. S. Public Health Service).

was steam sterilized at 15 p.s.i. for two hours on two successive days. The flasks were placed into one-half gallon pickle jars containing approximately 200 ml of water. Rubber serum stoppers were placed in the pickle jar lids as inoculation ports (Fig. 2). The entire pickle jar assembly, containing the soil, was autoclaved for two hours and allowed to cool slowly in the autoclave. Immediately upon opening the autoclave, the lids were tightly sealed and the pickle jar allowed to cool at room temperature.

After cooling, the soil samples were inoculated with 15 ml of a heavy mycelial suspension of _H_. _capsulatum_. Inoculations were made through rubber ports in the pickle jar lids with a 20 ml syringe and an 18 gauge needle. To enhance diffusion through the soil, care was taken to distribute the inoculum evenly over the soil surface. The cultures were incubated at 30 C for 30 days.

Following incubation, one soil culture was removed from the pickle jar and dried in the drying chamber at 30 C until the moisture content by weight determination was two per cent. The second culture was dried, under the same conditions, to a moisture content of 12 per cent.

Wet soil was found to be very difficult to dry at temperatures low enough not to kill the organism. Prolonged

Figure 2.-- Pickle jar apparatus for growing fungal soil cultures.

exposure at 30 C caused caking of soil and a marked decrease in viability. Therefore, by keeping the temperature at 30 C and creating strong convection currents over the samples and subsequently over a dessicant, such as $CaCl_2$, the soils dried rapidly with a minimum decrease in viability.

After adequate drying, five grams of each soil culture were placed into screw-capped tubes and sealed. Six tubes of each soil sample were placed at -18, 4, 10, 37 and 40 C. Periodically, a tube for each temperature was removed, suspended in appropriate dilutions of normal saline and placed on Sabouraud's dextrose agar plates for viability studies.

All viability studies were done by thoroughly mixing the soil sample in appropriate dilutions of normal saline and plating 0.5 ml of suspension on each of ten Sabouraud's dextrose agar plates containing 20 units of penicillin and 40 units of streptomycin per milliliter of medium. All plates were incubated at 27 C for 21 days before counting. Because of large variability in pipetting soil suspensions, all viabilities were based upon the total number of viable particles per five milliliters (total volume used in 5 ml pipette), instead of the conventional mean count per plate method.

Results

The results of incubating moist soil culture (12% moisture) at various temperatures are shown in Fig. 3. The data are given as the logarithm of mean number of viable particles per gram of soil versus time. At the end of 50 weeks the logarithm of viable particles per gram of moist soil at 10 C had changed from 4.7945 to 5.7782; at 4 C from 4.7945 to 4.6990; at -18 C to 4.5478; at 37 C to 3.6902 and at 40 C to zero viability. It should be noted that the viability at 40 C dropped to zero after two weeks incubation. Results of incubating dry soil culture (2% moisture) at various temperatures are shown in Fig. 4. At the end of 50 weeks the logarithm of number of viable particles had changed from 4.5211 to: 4.1239 at -18 C; 3.8062 at 10 C; and 0 viability at 37 and 40 C. Note, however, that the death rate at 40 C was much slower in the drier soil.

Discussion

A comparison of Figs. 3 and 4 shows that the survival of *H. capsulatum* in moist soil is greater at -18, 4, 10 and 37 C but not at 40 C. Growth actually occurred in the moist soil at 10 C. Morphological studies indicated that the mycelium rapidly became dehydrated and lysed under dry

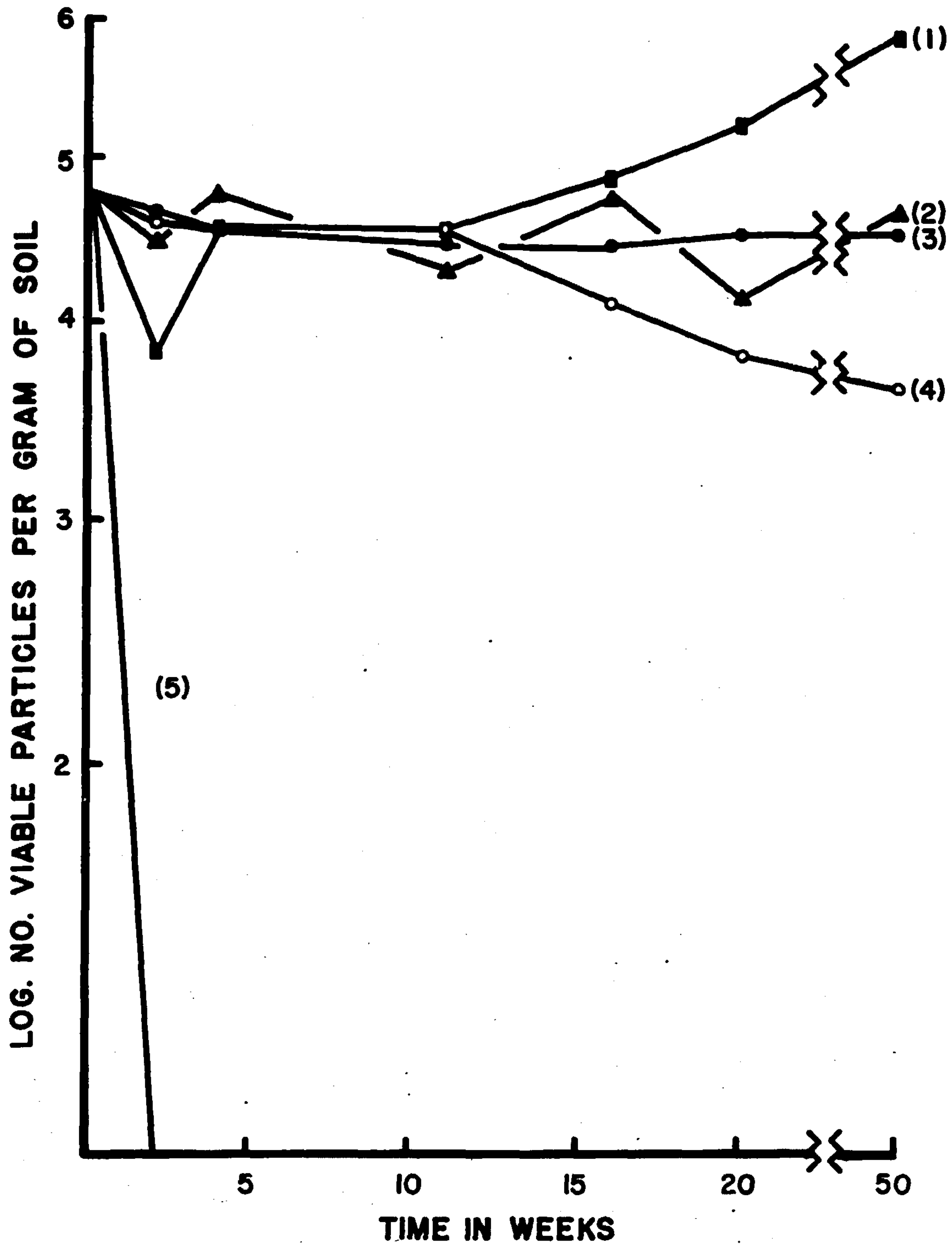

Figure 3.--The effect of temperature on the growth of Histoplasma capsulatum in soil with twelve percent moisture. (1) Viability at 10 C. (2) Viability at 4 C. (3) Viability at -18 C. (4) Viability at 37 C. (5) Viability at 40 C.

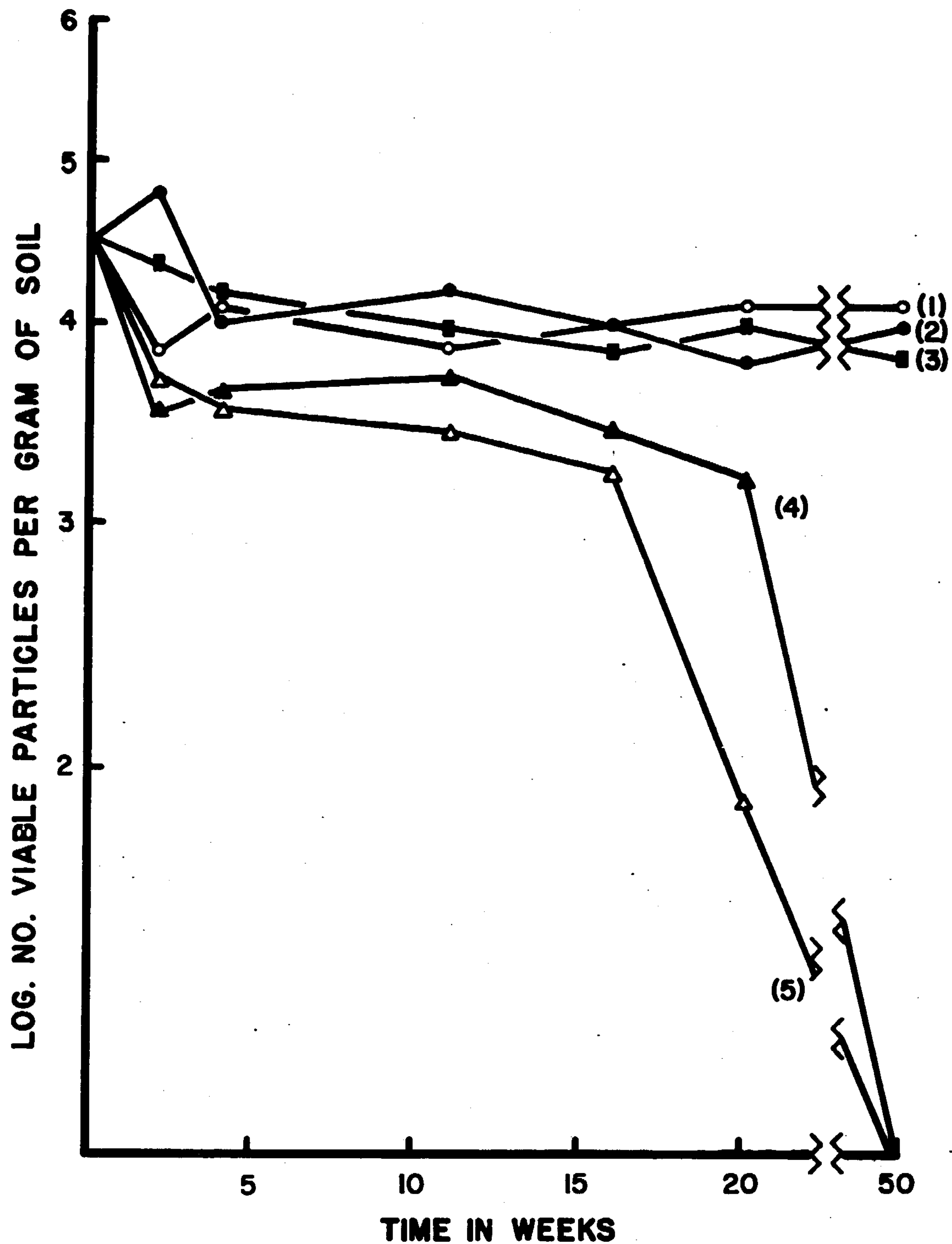

Figure 4.-- The effect of temperature on the growth of <u>Histoplasma</u> <u>capsulatum</u> in soil with two percent moisture. (1) Viability at -18 C. (2) Viability at 4 C. (3) Viability at 10 C. (4) Viability at 37 C. (5) Viability at 40 C.

conditions. However, spores remained morphologically unchanged. This accounts for the drop in viable counts in dry soils. Rapid death of the fungus in moist soil at 40 C is probably due to the inactivation of enzymes at that temperature. There appears to be a rather sharp line of heat tolerance between 37 and 40 C in moist soil.

These results indicate that _H. capsulatum_ is not greatly affected by extreme soil temperature occurring in the regions outlined as the endemic area. Soil temperatures of 40 C are not likely to occur in the endemic area; especially not in shaded, moist environments commonly associated with fungal growth. Temperatures of -18 C are quite common, and occur over prolonged periods, in many parts of the endemic area. This, however, appears to have little effect on the survival of the fungus in soil. The results of this study compare favorably with the skin test sensitivity findings in relation to the area most likely to produce growth and survival of _H. capsulatum_ in quantities sufficient to infect large numbers of people. Also, these data indicate that _H. capsulatum_ is not likely to survive in regions where the temperature rises to 40 C for prolonged periods, such as the desert Southwest or Southwestern plains.

CHAPTER III

CHICKEN MANURE AND ITS INFLUENCE ON THE GROWTH OF HISTOPLASMA CAPSULATUM IN SOIL

Histoplasmosis is a disease that has been closely associated with birds, particularly with their roosting sites where large concentrations of droppings are found. Past epidemiological studies on histoplasmosis implicated old or abandoned chicken houses as foci of infection (5, 8, 21, 36, 37, 39). Recent studies indicate that chicken houses are not unique in the ability to harbor this fungus. Bat caves, starling roosts and other areas where bird fecal material is concentrated also support growth of the fungus in concentrations large enough to allow isolation by current standard techniques (6, 7, 8, 18).

Several questions arise concerning the association of birds and isolation of H. capsulatum that are not answered in the literature. For example, were the areas under study sufficiently sampled to give an accurate generalized

picture and not merely the immediate vicinity of the bird roost? Secondly, exactly what is the effect of chicken or bird manure on H. capsulatum in soil?

According to Furcolow (21), more than one-half of the epidemics have been associated with sites in which excreta of either chickens, pigeons or bats were involved.

The purpose of this study was to determine the effect of chicken manure on growth of H. capsulatum in soil.

Materials and Methods

Soil cultures in pickle jars containing varying quantities of dry chicken manure were used for this study. The collections were made in an abandoned commercial chicken house near Norman, Oklahoma. The material had collected under the wire pens to a depth of approximately eight inches and was somewhat pulverized by animals digging for eggs. All manure was collected in large drums and mixed in 25 gal. garbage cans. Prior to use in soil cultures, it was autoclaved at 15 p.s.i. for two hours on three consecutive days. A change of 0.1 pH unit was noted after autoclaving. A much greater change in pH occurred when it was autoclaved wet. The sterilized manure was blended in a Waring blendor until all large particles had been broken.

Soil cultures containing 0, 5, 15, and 25 per cent manure were prepared by adding the appropriate amount of dry, blended manure to soil to give a final weight of 50 g. Duplicate cultures were thoroughly mixed prior to further treatment. The soil mixtures were then autoclaved for a minimum of three hours on two consecutive days. After the second autoclaving, the jars were opened and placed into large pickle jars containing 200 ml of water. The lids were placed loosely on the jars and the entire unit autoclaved for one hour. After the soil had cooled, a five milliliter inoculum of mycelial suspension of _H. capsulatum_, which had been grown on Sabouraud's dextrose agar, was distributed evenly over the soil. The pickle jar lids were tightened and the unit placed at 30 C for 30 days.

An extract of the dry manure was prepared by adding 55 liters of water to 20 Kg of dry manure. The mixture was then stirred intermittently for six hours, and filtered twice through six layers of cheesecloth. The filtrate was then poured through a layer of cotton between two layers of cheesecloth. The supernatant was centrifuged at 1000 rpm (128 X G) in a continuous flow centrifuge and the extract was filtered through #4 Whatman filter paper in a Buchner filter. The filtrate, which will henceforth be referred to as chimanex,

was then passed through a Seitz filter, pooled, and refiltered by the same process. The chimanex was refrigerated throughout the extraction process.

Soil samples for the chimanex studies were prepared as discussed in Chapter II. Dilutions of 5, 15, and 25 per cent chimanex were prepared by adding appropriate quantities of sterile distilled water to concentrated chimanex. All dilutions were passed through a Seitz filter immediately before use.

Soil cultures, using chimanex, were prepared by adding 10 ml of diluted chimanex to each 150 g of soil. The chimanex was spread evenly over the soil surface using a 10 ml syringe and 18 gauge needle. The solution diffused through the soil sample within an hour after application. A five milliliter mycelial suspension of *H. capsulatum* was distributed evenly over the soil-chimanex mixture. All cultures were incubated at 27 C for 30 days.

Since most bird manure is known to contain large amounts of uric acid, an investigation was made to determine the effect of this material on *H. capsulatum* in soil. Studies have been made, using artificial media, to determine the effect of uric acid on the growth of some systemic fungi (33, 35). No stimulatory activity was reported.

To study the effect of uric acid on the growth of _H. capsulatum_ in soil, uric acid (Fisher c.p.) was added to the soil in quantities of 0.1, 0.5 and 1.0 mg/g soil. Since uric acid has a very low solubility in water (0.06 g/liter), uric acid crystals were mixed into the sterile dry soil with a Waring blendor. After mixing, one milliliter of water was added to each 10 g of soil. The mycelial inoculum was added and the culture incubated at 27 C for 30 days.

A standard procedure was used for quantitating all soil studies. The soil was removed from each jar and placed in a sterile, gauze-covered jar and dried in the drying chamber. Cultures were then blended for five seconds. Triplicate one gram samples were removed from each culture and placed into 100 ml sterile normal saline. Additional dilutions were made from this suspension and 0.5 ml of each dilution was pipetted onto each of 10 Sabouraud's dextrose agar plates. Sterile glass rods were used to spread the suspension evenly over each plate. All plates were incubated at 27 C for 21 days before viability counts were made. An untreated culture was used as a positive control.

Results

The results of the study on the effect of sterile, dry chicken manure on the growth of _H. capsulatum_ in sterile

soil are given in Fig. 5. When the per cent dry chicken manure was plotted against the logarithm of the number of viable particles per gram of soil, a significant drop in viability was found as the concentration of manure increased. When zero per cent concentration of manure was used as 100 per cent viability, a five per cent concentration of manure decreased the viability by 23 per cent; a 15 per cent concentration by 87 per cent and a 25 per cent concentration by 98.4 per cent.

An analysis of variance test on the data from Fig. 5 showed a significant difference, at the five per cent level, in viability between positive control, zero per cent manure, and the concentrations of manure used. Also, there was a significant difference between the zero per cent and five per cent; the five per cent and 15 per cent; but not between the 15 per cent and 25 per cent level; however, the values were so close to being significant that it can be predicted that a significant level would have been reached at a 30 per cent concentration of manure. This would indicate that a manure concentration of 25 per cent is not the maximum concentration needed to completely inhibit the growth of *H. capsulatum.* Related studies with pure chicken manure confirmed this assumption in that the fungus would not grow in

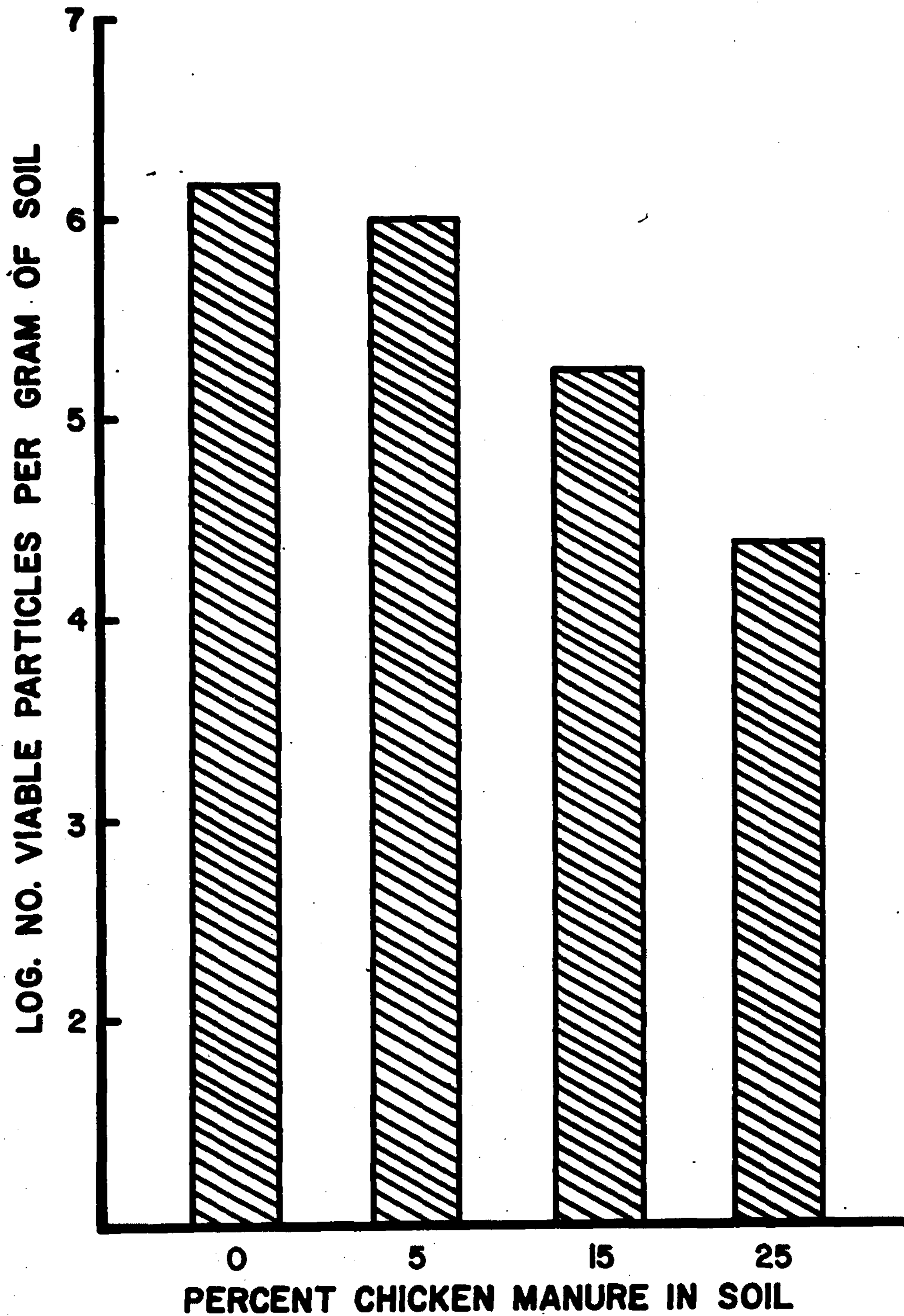

Figure 5.--The effect of chicken manure on the growth of *Histoplasma capsulatum* in soil.

moist or dry concentrated chicken manure.

Figure 6 shows the results of the effect of chimanex on the growth of H. capsulatum in sterile soil. The per cent chimanex used in the soils is plotted against the logarithm of the mean number of viable particles per gram of soil culture. The mean number of viable particles for each concentration of chimanex was determined from counts on 100 Sabouraud's dextrose agar plates. Since 0.5 ml of suspension was placed on each of 10 plates for each dilution, the counts were made for each five milliliters of suspension to offset any pipetting error that might occur from using heavy soil suspensions.

Counts from soil treated with uric acid showed that when acid was added in a concentration of 0.1 mg/g of soil, a decrease of 54 per cent in growth occurred. A concentration of 0.5 and 1.0 mg acid per gram of soil gave a 56 per cent and 70 per cent decrease in growth, respectively. Further work in this laboratory has affirmed these findings.

These data indicate that chicken manure is inhibitory to the growth of H. capsulatum in soil. An extract of the manure also gave an inhibitory effect, although less than the dry manure. Uric acid, a major component of chicken manure, also inhibited H. capsulatum in soil cultures.

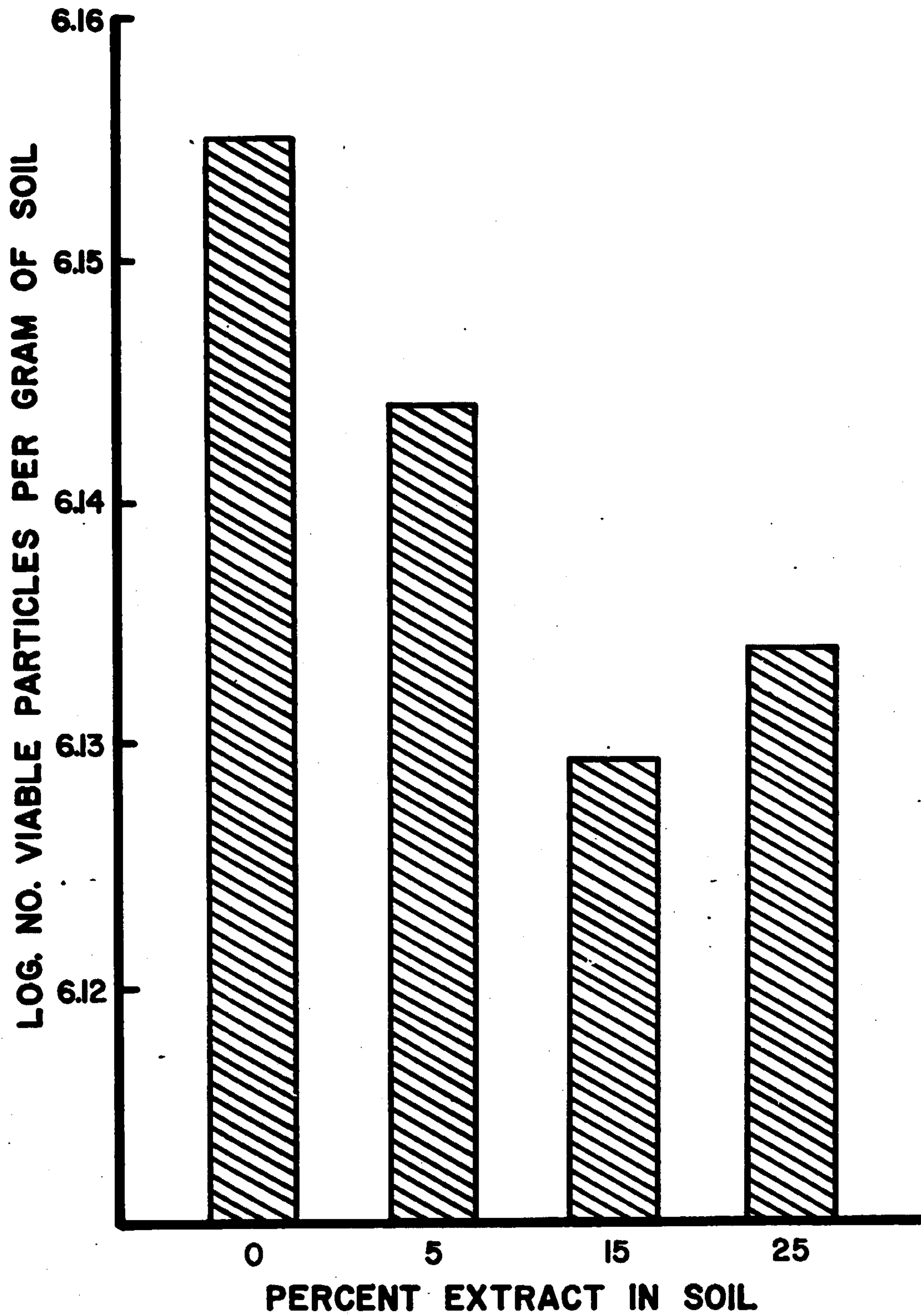

Figure 6.-- The effect of chicken manure extract on the growth of *Histoplasma capsulatum* in soil.

Discussion

The results of this study raise the question of why *H. capsulatum* is associated with bird excreta, especially chicken manure, when this appears to be inhibitory. Natural material is desirable for this type study; however, because of the inability to successfully work with mixed cultures, sterilized manure and soil were used. Autoclaving these may change their properties. The criterion used in this study to indicate the absence of such changes was the relatively small drop in pH on autoclaving (0.1 to 0.2 pH units). One explanation for the association of *H. capsulatum* with chicken manure, or bird manure in general, is the apparent tendency for epidemiological investigators to over-sample those areas where birds have roosted and under-sample adjacent areas where the fungus could grow under relatively optimum conditions.

These results are corroborated by the fact that chimanex, when incorporated into agar, or other media, inhibits the mycelial growth while increasing conidial production. With an increase of spore production, an increased possibility of inhalation and subsequent infection could be expected.

Additional research in this field is needed to fully answer the question of the relationship between bird manure and histoplasmosis.

CHAPTER IV

PHYSICAL AND CHEMICAL FACTORS OF SOIL AFFECTING THE GROWTH OF *HISTOPLASMA CAPSULATUM*

The fungus *H. capsulatum* is considered to be a soil organism, but it has not been isolated from all soils. The exact nature of its environmental requirements has not been completely determined. The soils of the endemic area of histoplasmosis are quite variable in all respects. A study of the soil conservation maps of the states in the endemic region reveals soil types ranging from fine sandy loam to hard clay. These different soil types have varied chemical and physical features which undoubtedly influence the type microflora present.

Zeidberg, et al. (40), in a study of the chemical composition of soils from which *H. capsulatum* was isolated, showed that the only constant factor observed was that all positive soils had a pH somewhat more acidic than the negative soils. This is the only physical-chemical study of *H.*

capsulatum in soil reported in the literature. Emmons (18) has stated that the pH of soils from which H. capsulatum has been most frequently isolated has been acidic. Elconen, Egeberg and Egeberg (16) have shown that an increased concentration of soluble salts in the soil increases the growth of Coccidioides immitis. It is recognized that soil fungi are more abundant in acid soil than in basic soil (9). Some workers explain this as being due to the decrease in bacterial competition in the acidic soils. Others think this is due to physiologic constitution of the fungi. Generally H. capsulatum has been found to grow over a wide pH range on synthetic media (26).

This study was done to observe the effects on H. capsulatum of some physical and chemical factors occurring in natural soils. Also, major emphasis was placed on determining the effect of various pH values on the growth of H. capsulatum.

Materials and Methods

Soil for the pH study was collected from the old corn plot near the University of Oklahoma botany greenhouse. It will be referred to as soil batch no. 6. Soil samples of different pH values were prepared by adding 30 ml of varying

concentrations of NaOH and HCl to 60 g soil. A second study was made using the same volumes of NH_4OH and H_2SO_4. The soil slurry was thoroughly mixed in a beaker with a glass stirring rod. The beaker was covered and allowed to stand, with frequent stirring, for 48 hours. The supernatant was decanted and the soil allowed to dry at 30 C. The dried soil was broken into small, homogeneous particles and divided into two 30 g samples in four ounce wide-mouthed, screw-capped specimen jars. They were then autoclaved for a total of four hours on two successive days; the caps removed and the samples placed into one pint Ball jars containing approximately 100 ml water. This entire unit was autoclaved for two additional hours and allowed to cool. Upon cooling, five grams of soil were aseptically removed from each jar for pH determination and the remaining 25 g were inoculated with five milliliters of a mycelial suspension of _H_. _capsulatum_. All cultures were incubated at 30 C.

Soil samples collected from several sites were sterilized as described above and placed in one-half gallon pickle jars containing 200 ml of water. After autoclaving, a portion of each sample was removed for pH determinations. The remaining samples were inoculated with an equal volume of a mycelial suspension of _H_. _capsulatum_.

pH determinations on the five grams of soil from each sample were made by mixing the soil with five milliliters of neutral deaminized water. The slurry was allowed to stand approximately 15 minutes, then the pH read on a Beckman zeromatic pH meter. The pH readings are shown in Table 1.

After four weeks incubation, the soil cultures were removed from the pickle jars and covered with a sterile cover of gauze wrapped cotton. The soil was dried at 30 C in a drying chamber. After drying, large soil particles were broken with a sterile spatula; sterile caps were placed on the containers and the soil was thoroughly mixed by manual shaking. Duplicate one gram samples were removed from each culture and each sample placed into 10 ml sterile normal saline. The suspensions were thoroughly shaken. To assure that all soils were in suspension for the same period, only two suspensions were made at a time. Dilutions of 10^{-2} and 10^{-3} were made from the original suspension. One-half milliliter of each dilution was pipetted onto each of 10 Sabouraud's dextrose agar plates. Five milliliter open-end pipettes were used for pipetting soil suspensions. All calculations were based on the count per five milliliters of suspension and converted to viable particles per gram of

Table 1. pH of Soils Treated with NaOH, NH_4OH, H_2SO_4 and HCl

Normality of NaOH Added	pH	Normality of HCl Added	pH	Normality of NH_4OH Added	pH	Normality of H_2SO_4 Added	pH
Untreated	6.9	Untreated	6.9	Untreated	6.9	Untreated	6.9
.01	7.3	.01	6.6	.01	7.2	.01	6.8
.05	8.8	.05	5.7	.05	7.8	.05	5.1
.10	9.3	.10	4.2	.10	8.2	.10	3.3
.50	10.1	.50	2.5	.50	8.2	.50	1.7
1.0	11.3	1.0	1.7	1.0	8.2	1.0	1.2

soil culture.

Eleven soil samples collected in: Milan, Michigan; Dalton, Georgia; Mexico, Missouri; Grand Island, Nebraska; Williston, North Dakota and Cleveland County, Oklahoma, were sent to the soils laboratories of Oklahoma State University and Noble Foundation at Ardmore, Oklahoma, for chemical analyses. These samples were representative of the most common type soil found during studies for the isolation of *H. capsulatum*. Soils from Cleveland County, Oklahoma were chosen for chemical analysis on the basis of their ability to support growth of *H. capsulatum.*

The ability of the above soils to support growth of the fungus was determined by sterilizing samples of each and inoculating them with mycelial suspensions as described for the pH study.

Results and Discussion

Results of the pH study are shown in Table 2. The results indicate that *H. capsulatum* will grow within a wide pH range. The range for growth of the fungus was between pH 6 and 10. Undoubtedly, in nature there are many exceptions to these findings, because of the micro-environment involving other microorganisms. To determine this presents special

Table 2. Growth of *H. capsulatum* and Soil pH

NaOH	HCl	NH_4OH	H_2SO_4	Natural Soils		
pH	VP/g x 10^3	pH	VP/g x 10^3	Source	pH	VP/g x 10^3
11.3	0			Grand Island Nebr.	7.5	1.3
10.1	0			Dalton Georgia 096	7.0	213
9.3	+261 287.2	8.2	92	Dalton Georgia 041	6.9	647
8.8	219.4	7.8	102.4	Dalton Georgia 039	6.7	131
7.3	194.3	7.2	166.1	Mexico Mo. 5	6.7	60.7
untreated 6.9	256.8	untreated 6.9	90	Dalton Georgia 030	6.6	605
6.6	198.8	6.8	95	Dalton Georgia 033	6.1	302
5.7	0	5.1	0	Norman Okla. 4	6.1	68.3
2.5	0	3.3	0	Mexico Mo. 1	2.8	0
Inoculum size: 1,098 VP/g soil		Inoculum size: 226 x 10^2 VP/g soil		Inoculum size: Not available		

VP = Viable particles

problems requiring techniques for working with mixed cultures.

The study of soils collected from various areas indicated that pH was not a sole factor determining the growth of *H. capsulatum*. Soil collected from different parts of Dalton, Georgia showed marked differences in ability to support growth of the fungus, even when the pH values were similar. Other striking examples are shown with Dalton, Ga. sample no. 039 and Mexico, Mo. sample no. 5. These soils had a pH of 6.7 but there was more than a two-fold viability per gram of Dalton soil. Another example was Dalton, Ga. no. 033 and Norman, Okla. no. 4. These soils were pH 6.1. The Dalton soil gave almost a five-fold greater viability. There are obviously other factors involved that need to be considered to fully understand the environmental conditions needed for growth of *H. capsulatum* in natural soil.

One observation from these results may be of significant value in continuing work with this organism. At no time during this series of studies has *H. capsulatum* been grown on soil at pH values below 5.0 or above 10.0. In addition, this organism has never been isolated, in this laboratory, from natural soil within these pH ranges. Since this organism may not survive such pH values, this may be a means of eradicating the fungus in small endemic foci. Perhaps by adjusting the

soil pH to 5.0, or below, the growth of the organism can be reduced to a point below an infectious state.

Results of the chemical and viability studies are given in Table 3. Histoplasma capsulatum has been isolated from three of the soils shown in Table 3; Milan, Mexico and Grand Island. The fungus was not recovered from the Dalton, Georgia or Williston, North Dakota soils, even though skin test sensitivity patterns indicated a high incidence of histoplasmosis in those areas.

This study failed to show any clear-cut differences in chemical composition that could be associated with an increased growth of H. capsulatum. Of the Norman, Okla. soils used, nos. 2 and 6 were most suitable for the growth of the fungus; however, nos. 1, 3, 4 and 5 did not show any pattern in chemical or physical characteristics that would explain their failure to support growth of the fungus. Obviously, additional research is needed to determine why some soils will not support the growth of H. capsulatum while others of similar composition will support abundant growth.

Table 3. Chemical Analyses of Soils on which H. *capsulatum* Has Been Grown

Source	pH	Phosphorus lb/A	Per Cent Organic Matter	Potassium lb/A	Per Cent Nitrogen	Color	Texture	Viable Particles /g x 10^3
Milan, Mich. 474	6.3	735	---	650	0.88	dark brown	loamy sand	182
Norman, Okla. 2	7.8	135	2.6	470	0.19	dark red brown	sandy loam	450
Norman, Okla. 3	7.6	19	2.0	300	0.12	dark red brown	sandy loam	1.7
Dalton, Ga. 039	6.6	300	4.6	620	0.29	red brown	loam	131
Norman, Okla. 5	7.4	---	1.3	230	0.13	red brown	sandy loam	2.2
Norman, Okla. 6	6.8	98	3.8	1,140	0.20	red brown	sandy loam	500
Mexico, Mo. 5	7.0	170	6.6	490	0.26	gray brown	silt loam	61
Grand Island Nebr. 1	8.0	140	3.2	420	0.18	gray brown	sandy loam	1.3
Williston, N.D. 6	7.8	110	6.6	1,180	0.39	gray brown	sandy loam	113
Norman, Okla. 1	8.0	156	1.1	404	0.07	gray brown	sandy loam	0.1

CHAPTER V

MORPHOLOGIC STUDIES OF HISTOPLASMA CAPSULATUM IN SOIL

The morphologic characteristics of *H. capsulatum* have been described by many investigators since De Monbreun published his detailed study in 1934 (12, 24, 25, 26, 32). It is general knowledge that this organism occurs in soil in its saprophytic or mycelial form; however, little work has been done to study the morphological characteristics of the organism in its natural habitat.

Materials and Methods

This study was done in conjunction with all other studies requiring soil cultures. Several clean glass microscope slides were imbedded vertically in each soil sample (10). Care was taken to replace the soil around the slide by gently shaking the soil so that no packing occurred. The soil samples were then sterilized and processed as described in Chapter II. When the soil samples were inoculated, care

was taken to distribute the inoculum evenly around each slide.

After the soil cultures had been incubated for four weeks, the slides were gently removed with sterile forceps and carefully shaken to eliminate all large clumps of soil.

Because of its pathogenicity, the organism had to be killed before the slide could be observed for growth. This procedure presented a problem because the usual agents, such as formalin and mercuric chloride, would leave residual crystals or completely deform mycelial and spore structure. A satisfactory system was devised by immersing the slides in water for a few minutes to permit the excess soil to go into suspension, leaving the mycelium intact; then making a standard lactophenol blue wet mount.

Results

The most significant finding was the growth of this fungus in the upper portion of the soil. The major mycelial growth and sporulation occurred within the upper 1-2 centimeters. Figure 7 shows the difference in growth at the upper level of soil and at the 1.5 cm level. Note the large numbers of microconidia and tuberculated macroconidia at the upper level of growth.

Figure 7.-- Morphology of Histoplasma capsulatum in soil culture. (a). Type of growth at the surface level of the soil culture. (b). Type of growth at 1.5 cm below soil surface.

At the lower level of growth, usually down to 2.5 cm, there was occasionally relatively good vegetative growth of mycelium, but no sporulation. Sporulation was evident within the upper 0.5 to 0.75 cm of soil.

Figure 8 shows the difference in morphology of H. capsulatum in soil in the presence and absence of chicken manure and an extract of chicken manure. These photographs were made on the upper level of the soil sample where best mycelial growth and sporulation were most likely to occur.

Note in Fig. 8 there was increased sporulation on the slide from soil containing five per cent chicken manure. The increase in sporulation was generally accompanied by an apparent decrease in mycelium.

The predominant color of H. capsulatum in soil culture was grayish-white. Even after two or three years incubation the mycelium remained white. Old soil cultures of the fungus did not appear to contain more spores than a culture 30-60 days old. In general, mycelial growth and sporulation appeared to increase very rapidly within 10-14 days after inoculation. From 10-20 days after inoculation, relatively large numbers of non-tuberculated macroconidia were present. Few non-tuberculated macroconidia were found 30-45 days following inoculation. The mature tuberculated

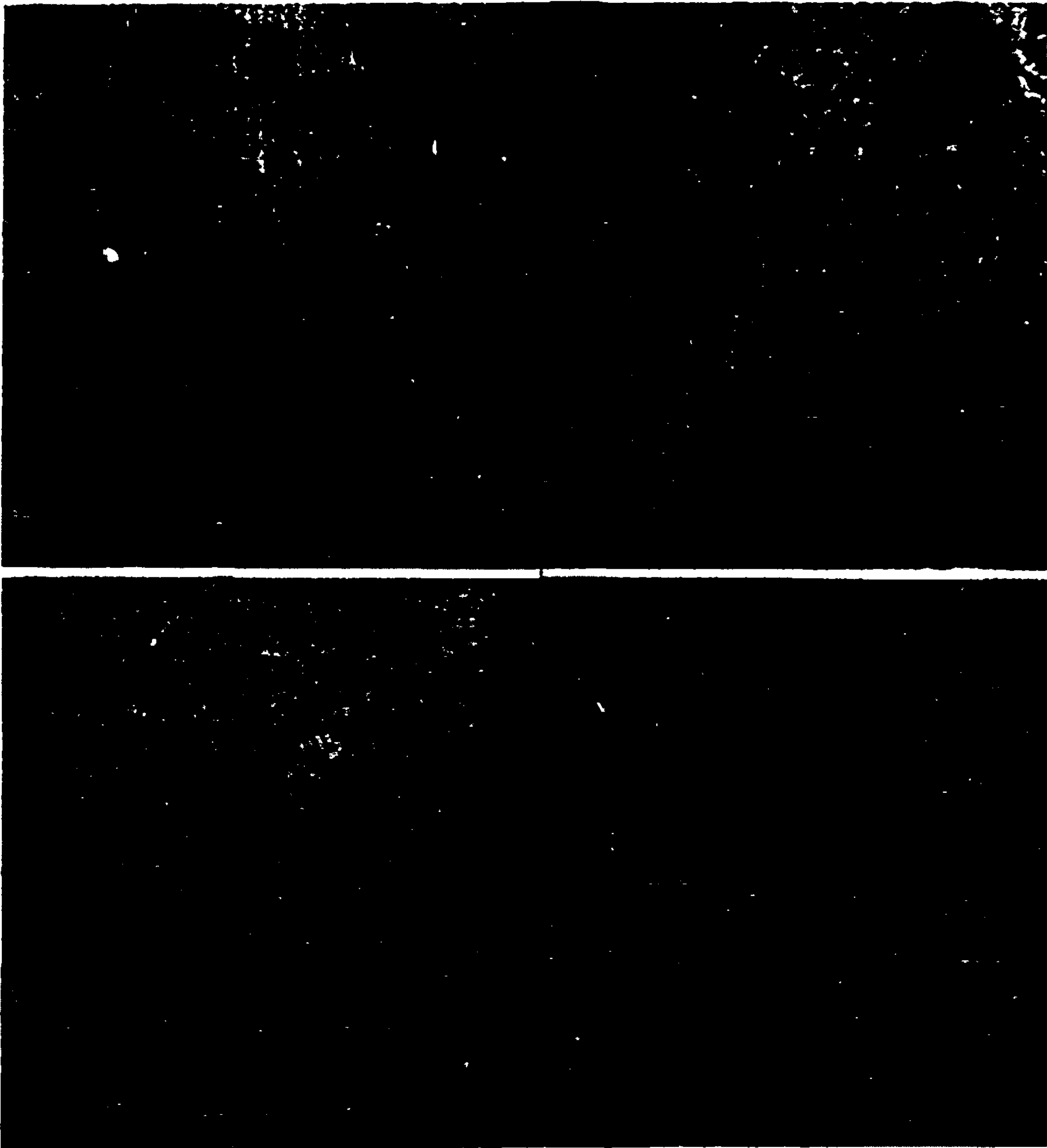

Figure 8.-- The effect of chicken manure on the morphology of Histoplasma capsulatum in soil culture. (a). Untreated soil culture. (b). Soil containing 5% dry chicken manure. (c). Soil containing 5% extract of chicken manure. (d). Soil containing 15% extract of chicken manure.

macroconidia were the predominant type.

Discussion

It was pointed out in Chapter IV that loose, loamy type soil was most favorable for the growth of *H. capsulatum*. The loose upper layer of soil contained most of the spores found on Cholodny slides. Apparently, this was due to the availability of oxygen. Available moisture would not be a factor in this type culture because the moisture added diffused throughout the soil sample.

The addition of five per cent chicken manure to soil caused an increase in spore production of the fungus. There was an increase of both type spores. At present there is no explanation for this increase in sporulation and decrease in mycelium. Perhaps this is a simple case of inhibition, since a definite inhibition of overall growth did occur.

Since the spores are easily aerosolized on agitation of the soil, increased sporulation by chicken manure supplement may account for the higher frequencies of infection from old chicken houses or areas where a large concentration of chicken manure is present. The increased number of spores, especially macroconidia, would increase the chances of recovery of the fungus from soil because of their resistance to drying and isolation processing procedures.

CHAPTER VI

INFECTIVITY OF *HISTOPLASMA CAPSULATUM* IN SOIL

Extensive X-ray and skin test sensitivity studies show that several million people in the United States have been infected with *H. capsulatum*. Furcolow (21) estimates that fifty thousand new infections occur each year. It is generally accepted that histoplasmosis results from inhalation of spores or mycelial particles of *H. capsulatum* in soil or dust. The exact nature of infection is not known; however, the infectious particles are considered to enter the alveoli and subsequently reach the lymphatic or blood system.

Ajello (1) reported that a single macroconidium of *H. capsulatum*, when inoculated intraperitoneally on an agar block, was sufficient to infect a mouse. Larsh, et al. (29) showed that small numbers of either mycelial fragments, microconidia or macroconidia, are required to infect mice, when inoculated intraperitoneally. Hinton, Larsh and Silberg (23) showed that mice exposed to soil containing *H.*

capsulatum developed histoplasmosis and the fungus could be recovered by culturing the liver and spleen of the exposed mice.

There are several theories as to how this fungus infects so many people (21). Some consider the fungus to be a common soil organism present throughout the endemic areas, the spores being airborne and inhaled during the normal activity of the population. Others postulate that there is a point source of growth and the infected individuals have had intimate contact with these sources.

Several studies have been undertaken to determine the number of particles of H. capsulatum in air within various regions of the endemic areas (19, 27). Results showed that the fungus was not commonly found in air, and when present, it was in small quantities. In view of Ajello's work, showing that one spore is sufficient to cause infection, small numbers of spores in air would not preclude infection of large numbers of individuals, considering the quantity of air inhaled. However, if an individual required multiple fungous particles for infection, and the findings concerning the number of spores found in air are correct, the probability of being exposed to sufficient numbers of particles would seem low. On the other hand, if there are point sources

where large numbers of infectious particles are found, such as deeply shaded areas, chicken houses, etc., and these point sources are areas where large numbers of people frequent, the point source theory seems plausible.

The purpose of this study was to determine if very low numbers of infectious particles, in soil, will cause infection in mice which have been exposed directly to the soil under simulated natural conditions.

Materials and Methods

Soil cultures of *H. capsulatum* were dried at 30 C and blended in a Waring blendor for two five second intervals, with hand mixing between the two intervals. Sterile, blended soil was used to prepare dilutions of soil culture. All dilutions were prepared in triplicate and blended to assure homogeneity. Triplicate one gram samples were removed from each dilution and each one gram sample sprinkled evenly over the medium in ten Sabouraud's dextrose agar plates. All plates were incubated at 30 C for three weeks before being read. At the end of three weeks the colonies were counted and the mean number of viable particles per gram of soil culture calculated.

The mean number of viable particles per gram of soil

culture was used as a basis for preparing 100 gram soil dilutions containing progressively larger numbers of viable particles per gram. Ten to thirteen soil dilutions were used for each study. A negative control, using sterile soil, and a positive control, using undiluted soil culture, were included in each study. Triplicate samples were removed from each dilution and the mean number of viable particles per gram was determined as described above. The remaining soil was placed into clean mouse cages which were covered with filtering material to prevent cross contamination. Screen wire was placed under the filter to prevent the mice from destroying it. The cages were placed inside a completely enclosed hood. A few pellets of mouse food were added directly to the soil, and twelve mice placed into each cage. The cages were then sealed inside the hood. The mice were not given water or additional food during the 24 hour exposure period.

After 24 hours, the mice were removed from the soil and placed into clean cages containing fresh litter. These cages were left inside the hood for another 24 hours to facilitate decontamination of the mice. Following decontamination, the mice were removed to a third clean cage and housed in a room for infected animals for two weeks, at which

time they were autopsied. The spleen and liver from each mouse were pooled, ground in a teflon grinder and 0.5 ml of the homogenate pipetted onto each of four Sabouraud's dextrose agar plates. All plates were incubated at 30 C and held for four weeks before a final reading was made. The plates were read as being either positive or negative for the growth of *H. capsulatum*.

A total of five studies were completed on the infectivity of *H. capsulatum* in soil. Three were made using filtered mouse cages in a hood; two were made in a closed ventilation instrument devised by personnel at the Army Biological Service, Fort Detrick, Md. A photograph of this apparatus appears in Fig. 9. In this system the soil and mice were enclosed in a cage with filtered inlet and exit air. The exit air was exhausted through a second absolute filter. All procedures for processing the soil and animals were the same for both methods of exposure.

Results and Discussion

The results for the inhalation study are shown in Tables 4, 5, 6, and 7. The data indicate that there is a tremendous variability in the infectious dose of *H. capsulatum* required to infect mice by the inhalation route. Of 238 mice

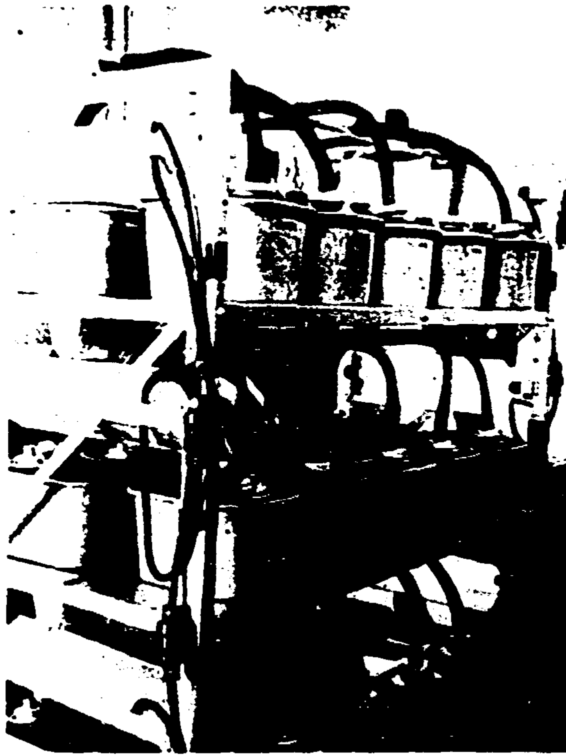

Figure 9.--Closed ventilation apparatus for exposing animal directly to infected soil.

Table 4. Inhalation Studies on the Infectivity of *H. capsulatum* in Mice Exposed to Soil

Number Viable Particles Per Gram of Soil	Number Mice Exposed	Number Mice Autopsied	Number Mice Pos. for *H. capsulatum*	Per Cent Positive
Study I				
0	12	12	0	0
0.5	12	10	0	0
0.9	12	8	0	0
3.1	12	8	0	0
6.6	12	11	0	0
12.0	12	10	0	0
70.0	12	10	0	0
248.0	12	12	1	8
800.0	12	11	2	18
Pos. Cont.	12	10	10	100
Study II				
0	12	12	0	0
0.3	12	12	0	0
0.4	12	12	0	0
0.8	12	12	0	0
2.8	12	12	0	0
5.8	12	12	0	0
19.0	12	12	0	0
31.0	12	12	0	0
67.0	12	12	0	0
270.0	12	12	0	0
485.0	12	12	1	8
Pos. Cont.	12	12	5	42

Table 5. Inhalation Studies on the Infectivity of *H. capsulatum* in Mice Exposed to Soil

Study III

Number Viable Particles Per Gram of Soil	Number Mice Exposed	Number Mice Autopsied	Number Mice Pos. for *H. capsulatum*	Per Cent Positive
0	12	11	0	0
22	12	11	0	0
38	12	12	0	0
54	12	12	0	0
82	12	12	0	0
250	12	12	0	0
528	12	8	0	0
560	12	12	1	8
634	12	12	3	25
p.c.	12	12	12	100

Study IV

Number Viable Particles Per Gram of Soil	Number Mice Exposed	Number Mice Autopsied	Number Mice Pos. for *H. capsulatum*	Per Cent Positive
0	12	12	0	0
25	12	12	0	0
59	12	12	1	8
88	12	12	0	0
106	12	12	0	0
131	12	12	3	25
225	12	11	3	27
287	12	12	0	0
304	12	12	4	33
305	12	11	3	27
529	12	12	3	25
p.c.	12	12	12	100

Table 6. Inhalation Studies on the Infectivity of *H. capsulatum* in Mice Exposed to Soil

Study V

Number Viable Particles Per Gram of Soil	Number Mice Exposed	Number Mice Autopsied	Number Mice Pos. for *H. capsulatum*	Per Cent Positive
0	10	10	0	0
237	10	10	0	0
369	10	10	0	0
648	10	10	1	10
1098	10	10	1	10
1356	10	9	2	22
1380	10	10	0	0
1880	10	8	1	13
1910	10	10	0	0
2360	10	10	3	30
3040	10	10	1	10
4560	10	10	1	10
5240	10	10	2	20
5470	10	10	7	70
6360	10	10	1	10
6740	10	9	5	62
p.c.	10	9	9	100

Table 7. Summary of Inhalation Studies on the Infectivity of *H. capsulatum* in Mice Exposed to Soil

Number Viable Particles Per Gram of Soil	Number Mice Exposed	Number Mice Autopsied	Number Mice Pos. for *H. capsulatum*	Per Cent Positive
0	58	57	0	0
1- 100	264	238	1	0.4
100- 200	24	24	3	13
200- 300	70	67	4	6
300- 400	34	33	8	24
400- 500	12	12	1	8
500- 600	36	32	4	12
600- 700	22	22	4	18
700- 800	12	11	2	18
800- 900	--	--	-	--
900-1000	--	--	-	--
1000-1100	10	10	1	10
1100-1200	--	--	-	--
1200-1300	--	--	-	--
1300-1400	20	19	2	10
1400-1800	--	--	-	--
1800-1900	10	8	1	13
1900-2000	10	10	0	0
2000-3000	10	10	3	30
3000-3000	10	10	1	10
4000-5000	10	10	1	10
5000-6000	20	20	9	45
6000-7000	20	19	6	32
10,000 or <	58	55	48	89

exposed to 1-100 viable particles of *H. capsulatum* per gram soil only one mouse, or 0.4 per cent, showed infection. From this viable count upward, the rate of infection increased, but not directly with an increase in viable particles; instead, the rate varied considerably, even as the count reached in the thousands per gram. Only 89 per cent of the mice autopsied following exposure to 10,000 or more viable fungal particles per gram soil showed infection.

It was not possible to determine exactly how many particles each mouse inhaled during the exposure period. But it seems plausible to assume that more than one particle would be inhaled within a 24 hour exposure period from soil containing three to four thousand particles per gram. If this assumption is acceptable, the theory that one infectious unit will cause disease in mice will not hold for inhalation.

There are several variables in this type study. First, each mouse cannot be expected to come into contact with the same number of spores even though the particles were dispersed by blending. However, neither would an individual necessarily under natural conditions. Secondly, each mouse would probably have a different degree of susceptibility; in spite of the fact that all the mice were of the same sex, age and approximate weight. Thirdly, the activity of the mice

themselves would influence direct contact with the soil and thus influence their possibility of inhaling fungal particles.

These results indicate that relatively large numbers of infectious particles must be present to cause a significant degree of infectivity in mice which have been exposed to a point source of infectious material.

Extrapolation of these results from mice to humans would be pointless, and perhaps misleading. However, these results may be used for further epidemiological studies with experimental animals.

CHAPTER VII

SUMMARY

Environmental factors influencing the growth of *Histoplasma capsulatum* in soil have been studied. The role of temperature and moisture in the growth of the fungus was found to be critical. The fungus can tolerate very low temperatures if the soil moisture content is high, but cannot withstand temperatures of 40 C or above for an extended period.

Dry, sterile chicken manure and an extract of unsterile chicken manure showed an inhibitory effect on the growth of the fungus. However, the relationship between bird manure and *H. capsulatum* has not been satisfactorily clarified.

The growth of *H. capsulatum* in soil is markedly affected by soil pH above 10 and below 5. No growth was observed on soil cultures outside these values. There was no definite pH range within these values in which the fungus

grew more abundantly. A chemical analysis of natural soils from which *H. capsulatum* had been isolated failed to show any common chemical factors that would explain the presence of the fungus in the soils.

Morphological studies of *H. capsulatum* in soil showed that the fungus grows within the upper two inches of the soil, and a majority of sporulation occurs within the upper one-half inch of soil. Morphological studies of the fungus, in the presence of chicken manure and chicken manure extract, showed an increased number of macroconidia and microconidia and decreased mycelial production.

When white Swiss mice were exposed to dry soil containing known quantities of viable mycelial particles, soil containing large numbers of particles were necessary to infect the mice. Approximately 85 per cent of the mice were infected when exposed to soil containing 10,000 viable particles per gram.

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