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GRADUATE COLLEGE

IN VIVO GROWTH STUDIES OF *HISTOPLASMA CAPSULATUM*

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BY

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IN VIVO GROWTH STUDIES OF HISTOPLASMA CAPSULATUM

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IN VIVO GROWTH STUDIES OF HISTOPLASMA CAPSULATUM

CHAPTER I

INTRODUCTION

Considerable work has been done on the fungus Histoplasma capsulatum since the discovery of the disease histoplasmosis by Darling in 1905 (15). This organism, being diphasic, has a mycelial phase when grown at room temperature and a yeast-like budding form when grown at about 37 C on certain special media, or in tissue cells of man and some animals (11). The yeast phase is an intracellular parasitic form which mostly parasitises the reticuloendothelial system, sometimes causing chronic progressive and systemic mycosis, although usually it causes an acute benign pulmonary disease (38, 54).

The dual form of H. capsulatum has always drawn the attention of workers. DeMonbreun (16), after culturing the organism for the first time on an artificial medium, worked out details of its morphological, cultural, and growth

characteristics. He was also the first person to inject the organism into an animal and recover it after the death of the infected animal.

It is difficult to maintain the yeast phase of H. capsulatum since it grows very poorly and tends to convert into a rough mycelial form on most of the media commonly used. Pine (40), in his extensive study on growth requirements, reported that the change of yeast into the mycelial form was due to three factors. First, most of the solid media contain agar which contains fatty acids toxic to H. capsulatum yeast cells. Second, H. capsulatum cells require a constant supply of --SH groups for a long period which is difficult to maintain in most solid media (46, 48). Third, there are some unknown factors essential for smooth growth of the yeast-like cells which are difficult to provide in artificial media.

Whole blood has been widely incorporated into media for the growth of H. capsulatum yeast phase because it provides a constant supply of --SH groups and nontoxic fatty acids (40, 46). Several other complex media have been proposed for the growth of yeast phase cells of H. capsulatum by different workers (29, 45, 46).

The dimorphic behavior of H. capsulatum is primarily controlled by environmental conditions, mainly temperature,

humidity (34), and nutritional source. Although quite extensive work has been done on nutritional requirements and development of better culture media for this fastidious fungus, there still is no effective way to supply exactly the same conditions in artificial environments as are provided by its natural host.

The mouse peritoneal cavity has been widely used for the growth of H. capsulatum cells. Kotchner, et al. (28) inoculated white mice intraperitonially with mycelial suspensions and showed that H. capsulatum could be converted readily into the yeast phase, and could be recultured on artificial media. Thus the mouse can be used as a tool for in vivo growth of the organisms (19, 31). However, it is difficult to separate these organisms from tissues and blood cells since they spread from the peritoneal cavity and infect blood and internal organs. Thus due to many technical difficulties no quantitative study can be done on the number of cells inoculated and number of cells harvested from the infected whole animal.

The technique of tissue culture has been employed to a limited extent in the study of pathogenic fungi. Baroni and Friedheim in 1929 first observed the effects of fungi on in vitro cultivated cells. Dugnes inoculated rat and chicken

fibroblasts with different fungi and observed H. capsulatum intracellularly in the fibroblasts. Howard (25) reported that as few as 30 yeast cells could initiate infection of the mouse peritoneal exudate macrophage culture. He also noticed a rapid conversion of mycelial phase into yeast phase in such exudate cells (25, 27). Larsh, et al. (32) successfully converted 13 mycelial strains to the yeast phase after seven transfers in tissue culture. It was found that there was a mass destruction of mouse peritoneal exudative cells by H. capsulatum, while destruction of Earl's L-strain mouse cells and HeLa cells was negligible.

The use of tissue culture techniques introduced a new effective, but expensive way of studying the growth of the yeast phase of H. capsulatum in in vivo-like environments.

The diffusion chamber technique, introduced by Algire (2), could be another effective method of studying in vivo growth of H. capsulatum. The diffusion chamber is implanted in the mouse peritoneal cavity. The Millipore membranes on both sides of the chamber allow a free exchange of peritoneal fluid through the chamber without any entry of foreign cells into the chamber or exit of the yeast cells from the chamber.

Another method for in vivo growth of H. capsulatum is chicken embryo inoculation, first used by Goodpasture and

Moore (22, 23, 36, 37). Reports from Larsh and Cozad (30) and Larsh, et al. (33) indicated the developing chick embryo as an excellent medium for conversion of mycelial phase to yeast phase and for recovery of the organisms. The present studies using chick embryo as a living medium for cultural studies of H. capsulatum yeast cells were based upon these successes.

Isolation of pathogens from nature and their study under defined conditions in the laboratory is essential for the advancement of this phase of microbiology. Investigators (53), however, have suggested that changes in environments often alter phenotype expression, affect selection, and influence genetic transfer and mutation. Hence the difference found between microbial behavior in a natural host and that in the test tube is to be expected. These differences may also be reflected in immunological and pathogenic properties of an organism.

These investigations were initiated essentially for two purposes. One: to work out an efficient and convenient system of growing yeast cells of H. capsulatum in an in vivo environment. The conditions should be as close to natural host as possible and the system should preferably be a closed one in which the organisms could be checked and recovered at

any time for further studies. Two: to carry out a comparative study of *in vivo* grown cells recovered from this system with *in vitro* grown cells from an artificial medium.

CHAPTER II

GROWTH STUDIES OF *HISTOPLASMA CAPSULATUM* IN THE DIFFUSION CHAMBER

The diffusion chamber technique was introduced by Algire and Weaver (2, 61) for culturing immunologically active tissue cells and has been widely used since then by different workers in studies of cytomorphology of soluble antibody production (8, 18, 56), for culturing tumor cells (3) and in studies of modulation of peritoneal fluid cells (51).

It has been shown that the mouse peritoneal cavity or its exudative cells can serve as an excellent medium for the growth of *H. capsulatum*. The diffusion chamber implanted in the mouse peritoneal cavity provides a simple method of growing the organisms in an *in vivo*-like environment. Experiments were carried out to study the growth of yeast cells of *H. capsulatum* in diffusion chambers, implanted in the mouse peritoneal cavity. Cells grown this way were compared with *in vitro* grown cells.

Materials and Methods

Construction of the Diffusion Chamber

The method for construction of the diffusion chamber differs somewhat from that of other investigators. The chamber used in this study (Belco) consisted of a rubber ring with the following dimensions: outside diameter, 19 mm; inside diameter, 12 mm; thickness, 2.5 mm. A cellulose filter (Millipore Filter Corp.) of 0.1u porosity was placed on each side of this ring and a flat metal ring of the same diameter as the rubber ring was placed upon the filter membranes on both sides. The whole apparatus was sealed with three metal clips. These assembled chambers were placed in sterilizing bags and sterilized in the autoclave at 15 pounds pressure, 121 C, for 15 minutes. The chambers were used repeatedly after washing thoroughly and changing the filter membrane.

Filling the Chamber

The yeast phase of H. capsulatum, Scritchfield isolate, was grown on Cozad's medium (12) at 37 C for 3 days. The cells were harvested from the slants with cold physiological saline containing 0.01% cysteine. This suspension of yeast cells was mixed well and filtered through several layers of cheese cloth. Then the cells were washed 3 times with cold

saline, by centrifugation using International RR-2 refrigerated centrifuge. Washed cells were resuspended in the following different fluids:

1. Physiological saline (0.85% NaCl) containing 1% normal mouse serum.
2. Cysteine saline (Physiological saline with 0.01% cysteine) containing 1% normal mouse serum.
3. Tyrode's solution, TC. (Difco Labs)
4. Tyrode's solution containing 1% normal mouse serum.
5. Eagle's medium, Basal. (Microbiological Associates Inc.)

The total number of yeast cells in the suspension was checked by counting the cells in the hemocytometer counting chamber and the suspension was made up to the desired concentration of cells (Table 1-5). Prior to the filling of the chamber, dilutions of the suspension were made in cold saline and 0.5 ml of each dilution was plated out on duplicate plates of Sabouraud's dextrose agar medium and Cozad's agar medium. All the plates were incubated at room temperature and colonies were counted after three weeks.

Each assembled sterile chamber was filled with 0.2 - 0.3 ml of the test suspension just prior to implantation in the recipient animal. The suspension was introduced into the

chamber through the rubber ring by means of a syringe using a 25-27 gauge needle.

Chamber Implantation

A total of 125 normal white mice were used in this study. Prior to the implantation mice were anesthetized by injecting Nembutal intravenously. The abdominal part of the skin was shaved with a razor blade and then cleaned with alcohol. A one-half inch incision was made and the chamber was gently inserted aseptically into the peritoneal cavity. The incision was sewn with surgical gut (Ethicon Inc.). In a later part of the study, Autoclips (Clay Adams Inc., New York) also were used. For control the same procedure was followed except that the chambers were filled with sterile suspending fluid only.

Recovery and Testing of the Chambers

Chambers were taken out by sacrificing the animal, disinfecting the abdominal part with alcohol, and opening the cavity. The chambers were recovered after 1, 2, 3, 5 and 7 days and at the end of 2, 4, 8 and 12 weeks. The recovered chamber was placed in a sterile petri dish. The peritoneal cells were removed from outside the chamber by means of a brush and the chamber was washed thoroughly with sterile

saline. The filter membrane was cut open from one side and the chamber fluid was removed with a Pasteur pipette. The inside of the chambers and the filter membranes were scraped and washed thoroughly with sterile saline. All the chamber fluids and washings were collected in a sterile 5 ml volumetric flask. The volume of the fluid was made up to the 5 ml level and mixed well.

A total count was made by removing aliquots of the fluid and counting in a hemocytometer. Dilutions were made of this harvested fluid for viability testing. Duplicate plates were inoculated from each appropriate dilution and incubated at room temperature. The colonies were counted after 3 weeks. The total number of cells and total number of viable cells for each chamber were calculated.

When clot formation occurred inside the chamber, the clot was treated with 0.25% trypsin solution at room temperature for 20-30 minutes or until the clot was completely dissolved. The trypsin digested clot suspension was centrifuged, the cells resuspended in sterile cysteine saline, and transferred to the volumetric flask.

Results

The results of the chamber implantation studies are shown in tables 1 to 5.

Using physiological saline containing 1% normal mouse serum, a maximum of 1.5 times increase in the total number of cells was found on the third day of implantation. The viability of the culture started decreasing very rapidly from the first day and by the end of two weeks it was found to be less than 1% (Table 1).

When cysteine saline containing 1% normal mouse serum was used, an increase of 1.9 times the original number of cells was obtained on the third day. The viability decline was noticed from the very first recovery (24 hours) and less than 1% cells were found viable by the end of the second week (Table 2).

The results were not much different when Tyrode's solution and Tyrode's solution with mouse serum were used as the suspending fluid. The increase in total number of cells was 1.7 times on the 7th day in Tyrode's solution, and 2.6 times on the 7th day in Tyrode's solution containing mouse serum. The decrease in viability was still abrupt. The viability reached about one percent at the end of the 3rd week when Tyrode's solution was used, but when mouse serum was incorporated with it, about 2% cells were found viable up to the end of the 4th week. However, viable cells (as many as 100 cells/chamber) were recovered after 12 weeks of implantation

(Tables 3 and 4).

Eagle's medium supported the growth of the organisms somewhat better than the other suspending fluids. A maximum of 4.5 times increase in the original number of cells was obtained on the 5th day. The decrease in percent viability followed the previous pattern and was lower than 1% by the end of the 3rd week (Table 5).

It was found that the yeast cells tend to clump together inside the chamber and this made it difficult to determine a total count. To avoid discrepancy in total counts, each cell and not the whole clump was counted. Budding forms were considered as single cells. Some of the cells were found to be larger in size, with thick cell walls, when harvested 3-4 weeks after implantation.

The viability counts may be affected by clot formation, because yeast cells were subjected to harsh treatment. Teasing the clot, treating with trypsin for a prolonged time, and centrifugation and washing might have affected the viability as well as total count of the cells.

Discussion

The formation of a clot in the chamber was a hindrance in these studies. The clot was of gelatinous character and sometimes it became a tough membrane difficult to tease off.

Digestion of the clot to separate the cells was attempted with 0.5% E.D.T.A. (Ethylene diamine tetracetate acid, sodium salt). This treatment was unsatisfactory. However, 0.25% trypsin solution was quite effective in digestion of the clot. Clot formation usually started about the fifth day and clots were found tougher and darker in color when chambers were left for longer periods inside the peritoneal cavity. When chambers were left inside the cavity longer than five days a tough, thick membrane was formed around the outside of the chamber.

The increase in cell number was not very significant inside the chamber. Of the five suspending fluids, none showed any significant enhansive effect on growth of the organisms, or maintenance of viability as it should be in in vivo conditions. The increase was highest in Eagle's medium and lowest in physiological saline. The slight increase in total count may be explained by the fact that some of the cells, when placed in the chamber, were about to multiply, or that some of the buds grew during the incubation. As each single cell was counted it affected the total number of the population. Since Eagle's medium was richer in nutritional supply, this medium resulted in somewhat greater increase in total population as well as maintenance of viability for a longer period. The rapid decrease in viability may be indicative of lack of proper

nutrition for the organisms.

The possibility of antibodies interfering with the growth of the organisms is very improbable. First, the mouse is not a very good antibody producer against Histoplasma; second, there is no exit of the organisms from the chamber, except that some of the cells may have lysed allowing cell proteins to diffuse out; third, according to Algire and Amos (2, 3) the Millipore filter acts as a partial barrier for antibodies.

Although the chamber technique has been used very successfully in culturing tissue and peritoneal cells, it was not greatly successful in culturing the yeast cells of H. capsulatum. This could be attributed to some of the following reasons:

Histoplasma is an intracellular parasite and since the chamber was cell-free, the organisms did not find suitable conditions to grow in the chamber fluid. This problem can possibly be solved by using spleen or peritoneal cells in the chamber, along with the yeast cells in suspending fluid.

The process of chamber implantation and the presence of the chamber inside the cavity caused a constant irritation around that area. This might have caused production by the body of some toxic materials harmful to the organisms.

Clot formation could be detrimental to the growth of the organisms because the cells were trapped in the tough gelatinous material and it might have blocked the free exchange of the fluids. So far no technique has been developed to prevent this clot formation.

In this study the most significant finding was the enlargement of the cells and the maintenance of viability of some of the organisms for a long period of time. It was found that after two weeks some of the yeast cells started increasing in size, and they had definite halos, or capsules, around their thick cell wall. It is possible that the enlarged cells had developed a tough cell wall, thus becoming dormant and remaining viable for as long as 12 weeks.

CHAPTER III

IN VIVO GROWTH STUDIES OF *HISTOPLASMA CAPSULATUM* IN THE YOLK SAC OF CHICK EMBRYO

The use of chick embryos for culturing organisms goes back as far as 1905 when Levaditi experimented with spirillum of fowls by chick embryo inoculation. The chick embryo has been widely used in the study of viruses since 1920 when Juan and Staub used it for the first time to culture avian pest virus.

Goodpasture (22, 23) was the first to use chick embryo in the study of fungi, and showed that certain pathogenic fungi could multiply in it. Moore (36, 37) reported the use of chorioallantoic membrane of chick embryos for cultivation of different pathogenic fungi, including *Histoplasma capsulatum*. He used 12-14 day-old embryos and described the macroscopic lesions of the membrane along with reversion of certain fungi into the form seen in human lesions. He described the chick embryo technique as a more significant one, since it is time consuming and less expensive than the use of laboratory animals. Brueck and Buddingh (6) inoculated some pathogenic

fungi, including H. capsulatum, into the yolk sac of embryonated eggs and found it a good site for their growth. Raftery and Hartman (43) proved that in the yolk sac the contaminating fungi could not grow. Later Pileggi and Sherwood (42) reported that H. capsulatum disseminated after inoculation of yolk sac and it was possible to demonstrate organisms in the spleen or liver of the embryo. Larsh, Hinton and Cozad (30) used H. capsulatum spores for the yolk sac inoculation of fertile eggs and recovered organisms from 75% of the yolk sacs, 25% of liver, and 13% of the spleens. They reported the chick embryo as an excellent medium for recovery and conversion of filamentous cultures into yeast forms. Their results showed that 33 C temperature was suitable for conversion of mycelial forms into yeast forms and the infection was not highly fatal to the embryos since 72% of the inoculated embryos lived to the termination of the experiment. Larsh, et al. (33) used single-particle inoculation of H. capsulatum mycelial fragments and spores and reported that 8% of the embryos became infected. The use of chick embryo for culturing H. capsulatum since then has been neglected, and no further report concerning studies of this type is found in the literature.

Vogel and Conant (59, 60) inoculated the yolk sac of

chick embryos with arthrospores of Coccidioidis immitis and found it to be a good method of obtaining spherules. These investigators recovered the spherules by centrifugation after treating the harvested yolk with four parts of 10% NaCl and allowing it to stand overnight.

It was demonstrated by Howitt in 1930 and Chaeffer and Brebner in 1933 that lipoidal and some proteinaceous materials could be removed from saline virus extract by adding an equal volume of ether with little or no damage to the virus (10). Craigi (14) did an extensive study on the use of ethyl-ether-water interface effects on the separation of some organisms, especially rickettsiae, from yolk sac. It was shown that rickettsiae, like some viruses and bacteria, were repelled from the ethyl-ether water interface, while insoluble tissue or medium constituents were selectively attracted to the interface. When crude yolk sac preparations of rickettsiae were shaken with ethyl-ether, a complex physical system was created in such a way that rickettsiae could be separated in almost pure form from the water layer.

This method has been used widely for the recovery of a variety of viruses and rickettsiae. Surprisingly, no one has ever tried this system for recovery of pathogens like H. capsulatum from the yolk sac. Possibly the large size of the

organisms was thought to be a hindrance to separation by the ether-water system.

Studies were initiated for further work on the growth of H. capsulatum in the yolk sac and its effect on the mortality rate of the embryo, using yeast-phase cells as the inoculum. An extensive investigation was also carried out to develop a method of separation of the yeast cells of H. capsulatum from infected yolk and yolk sac. These studies facilitated further comparative studies of in vivo grown cells, since the organisms could be recovered from the yolk sac conveniently at any time.

Materials and Methods

Preparation of Inoculum

Histoplasma capsulatum (Elliot strain) was used throughout this study. The yeast phase of this fungus was maintained on brain heart infusion agar (Difco), to which 5% blood was added. The smooth yeast-like growth from this medium was transferred to the surface of Cozad's medium (12), incubated at 37 C for 3 days, and then the cells were harvested and washed in sterile, cold physiological saline. The washed cells were suspended in cold sterile saline and 40 units of Streptomycin and 20 units of Penicillin were added to each ml of the suspension.

After adjusting the total count to the desired number, dilutions were made from this suspension for viability count. One-half ml of each dilution was plated on Sabouraud's agar plates (2% glucose, 40 units of Streptomycin and 20 units of Penicillin/ml of medium). The plates were incubated at room temperature and colonies were counted after 3 weeks.

Inoculation of the Yolk Sac

Fertile eggs from white leghorns obtained from a private hatchery were used in this study. The eggs were incubated at 37 C in a regulated high humidity egg incubator (Sears, Roebuck Co.), and were rotated every 12 hours.

After 5 to 6 days incubation, the eggs were candled, and the dead embryos were discarded. The surviving embryos were used for the inoculation. A total of 2,140 embryonated eggs were used in this study. The egg shell over the air sac was disinfected with tincture of iodine, and a hole was made in the center by means of a thumbtack. The egg shell around the puncture was once again disinfected and then the test suspension was inoculated into the yolk sac with a syrine, using a 20-gauge 1½-inch needle. In most of the experiments different dilutions of H. capsulatum yeast cells were used as the inoculum, except in preliminary studies where a few

batches of eggs were inoculated with mycelial suspensions. Each embryonated egg received 0.5 ml of the test suspension. After inoculation the site was again wiped with tincture of iodine, and the hole was sealed with melted vaspar. Airplane glue was also used but was found lethal to the embryo. The inoculation was done under the inoculation hood to reduce possible contamination.

The inoculated eggs were re-incubated at the same temperature and humidity. In the preliminary work some of the inoculated eggs were incubated at 33 C in a regular incubator with a pan of water and a blower for regulated high humidity. The eggs were checked after 3, 5, 7, 10, 12, and 14 days of inoculation, and number of embryos dead at each interval was recorded. The dead embryos were discarded or checked for the presence of yeast cells by making direct yolk sac smears. Control eggs were inoculated with sterile saline and checked at the same intervals.

Harvesting the Yolk and Yolk Sac

At different intervals the yolk and yolk sac were harvested by the following method. The egg shell around the air sac was cleaned with alcohol and the shell was removed around the air sac. The whole egg contents were emptied into

a sterile petri dish. Care was taken not to break the yolk sac. The yolk sac was carefully separated from the egg white and the embryo and lifted out intact with the yolk and placed in a sterile Omni mixer cup (Ivan Sorvall Inc., Norwalk, Conn.). The harvested yolk sacs, along with the yolks, were weighed and the weight of yolk-yolk sac/egg was determined. Sterile saline was added to make a final 1:20 dilution (w/v) of yolk and yolk sac. This suspension was ground using the Omni mixer.

At first, different speeds and timings of the grinding were tried. It was found that destruction of some of the yeast cells occurred at high speeds and longer times. A speed of 8,000 rpm for 5-10 minutes was found to be satisfactory. With longer incubation periods a longer grinding time was required because the yolk sac became tough and granulated. The suspension of yolk and yolk sac thus obtained was used for making dilutions for viability determinations. One-half ml of each dilution was plated out on Sabouraud's agar plates supplemented with antibiotics. The plates were incubated at room temperature and colonies were counted after 3 weeks. Smears also were made from the harvested yolk sac and were stained by crystal violet method described below.

Staining of the Smears

Thin smears were made from the infected yolk sacs, along with some yolk, by teasing the yolk sac and smearing on the slide. The smear was air dried and fixed in absolute alcohol for 15 minutes. The following different staining procedures were tried for staining the fixed smears: Gram staining, Giemsa staining, Wright's staining, and Methanamine silver staining.

Since the above mentioned staining methods did not provide good results, a special staining method was developed. The fixed smear was oxidized by leaving the slide in 5% chromic acid solution for about one hour. The slide was washed in distilled water for 5 minutes and then immediately stained with Gram's crystal violet for 1-2 minutes depending on the thickness of the smear.

Yolk Sac Passages of H. capsulatum

H. capsulatum yeast cell passages in yolk sac were performed by harvesting the infected yolk and yolk sac after 5-6 days of inoculation, making a 1:2 suspension of it and reinoculating a fresh batch of 5-6 day-old embryonated eggs. Penicillin (20 units/ml) and Streptomycin (40 units/ml) were added to the yolk-yolk sac suspension prior to inoculation

and 0.5 ml of the suspension was inoculated into each egg. Six days after the second passage the yolks and yolk sacs were harvested and the organisms were recovered.

Harvesting the Organisms from the Yolk and Yolk Sac

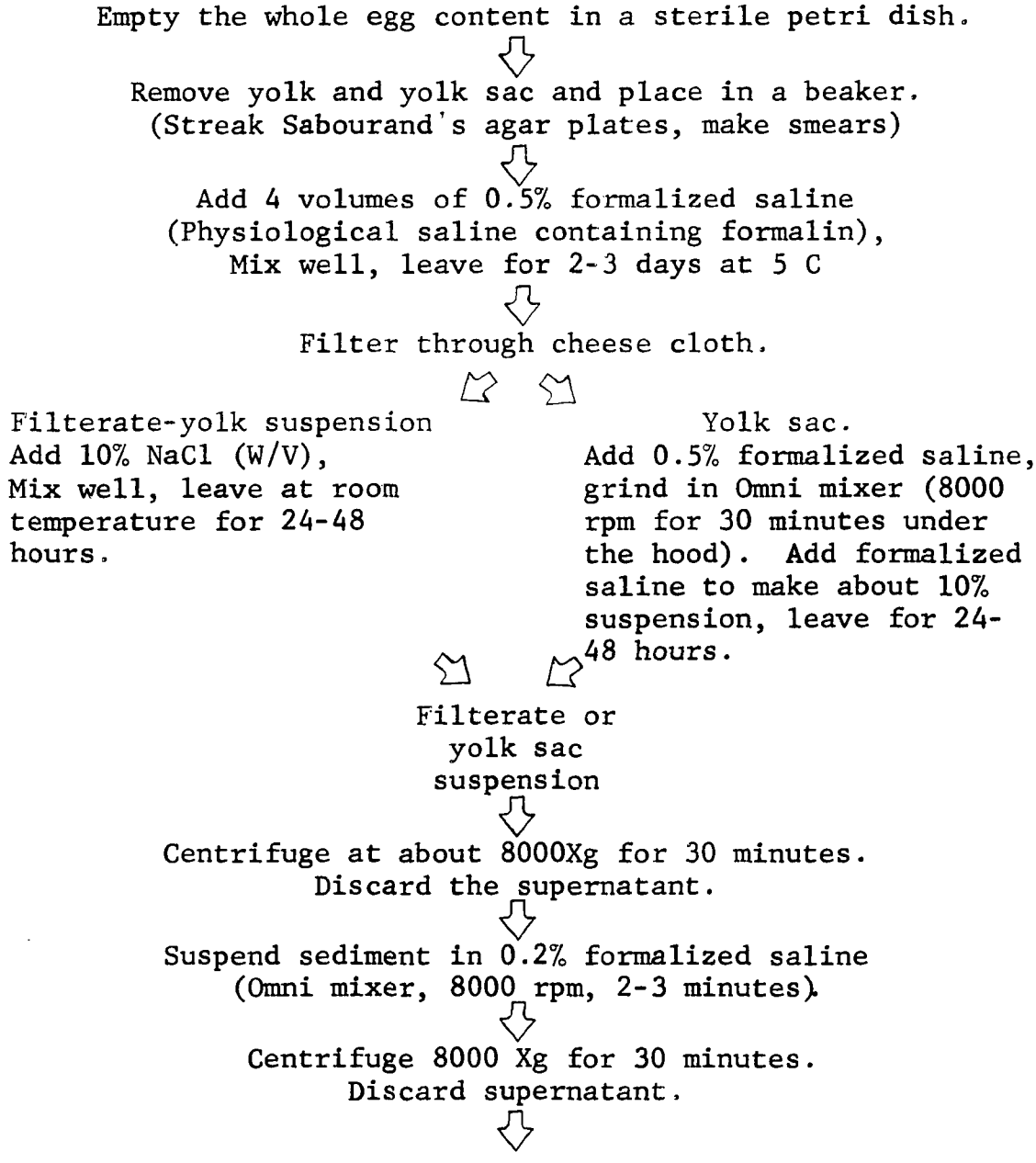
At first trypsin enzyme was tried to free the yeast cells from the yolk sac. Later the ethyl-ether-water interface system which has been used for separation of viruses and rickettsiae was attempted, and this proved to be the method of choice. After some modifications a successful procedure was adopted to separate the yeast cells from the yolk and yolk sac. A complete procedure of harvesting the organisms is outlined in the flow diagram.

Morphological Studies of *In vivo* Grown Cells

Smears which were prepared from the infected yolk sacs were stained with the special method of staining. A total of 100 cells were measured for each 5, 10, and 14 day period. In two batches, a few infected eggs were allowed to hatch, and the yolk sacs from the hatched chickens were removed (17 days after inoculation). Smears were made and cells were measured.

Wet mounts were made from the yeast cell suspension

Flow Diagram 1. Method of Harvesting the Yeast Cells of *Histoplasma capsulatum* from Infected Yolk and Yolk Sac



Suspend sediment in 0.1M Phosphate buffer pH 6-7.
(Omni mixer 8000rpm, 2-3 minutes.)

⇩

Centrifuge 8000 Xg, 30 minutes.
Discard supernatant.

⇩

Resuspend sediment in Phosphate buffer.
(Omni mixer)

⇩

Centrifuge 8000 Xg, 30 minutes.
Discard supernatant.

⇩

Suspend sediment in Citric acid-phosphate or Acetic acid-acetate buffer, 0.1M, pH 4.5
(Omni mixer 8000 rpm, 2-3 minutes).

⇩

Centrifuge 8000 Xg, 30 minutes.
Discard supernatant.

⇩

Resuspend in the same buffer (Omni mixer).
Make a 3-4% suspension.

⇩

Add ½ volume of ethyl ether (anhydrous) with the suspension in a separatory funnel. Shake for 5 minutes vigorously. Allow to stand for 15-20 minutes.

Remove the aqueous portion

Interface and ether layer.
Add ½ volume buffer.
Shake 10-15 minutes.
Allow to stand for 15 minutes.

⇩

Remove the aqueous portion

Interface and ether layer.
Add ½ volume buffer and ¼ volume ether.
Shake vigorously for 5 minutes.
Allow to stand for 15 minutes.

Remove the aqueous portion

Add ½ volume ethyl ether.
Shake vigorously for 5 minutes.
Allow to stand for 15-30 minutes.

⇩

Collect the aqueous portion.



Bubble air through this suspension at
37 C to eliminate ether.
Leave overnight in refrigerator.



Centrifuge at 12100 Xg for 30 minutes.



Supernatant
Filter through millipore filter (0.45μ porosity), discard the filtrate,
Suspend the residue in physiological saline.

Sediment
Suspend in physiological saline.



Add preservative
Count the cells and make the suspension according to desired concentration.

obtained from ether separation, and 100 cells were measured for each incubation period of 5 days, 5 days following 2 passages, and 10 days.

As a control, a yeast culture of H. capsulatum, grown at 37 C on brain heart infusion blood agar slant, was harvested after 3, 5, 10, 14, and 17 days of incubation. The cells were suspended and killed in 0.5% formal saline, washed 2 times, suspended in citrate buffer (pH 4.5) and then were shaken for 5 minutes with an equal volume of ethyl ether. The water layer was removed and the treatment was repeated once more. The resulting aquous suspension was centrifuged and the sedimented yeast cells were resuspended in physiological saline. Wet mounts of these preparations were used for size measurements.

A 100x oil immersion objective and a 10x ocular equipped with micrometer were used in all measurement studies. The ocular micrometer was calibrated in microns with a standard stage micrometer.

To avoid bias in measurements, the slide was moved longitudinally on the microscope stage and any cell which superimposed the ocular micrometer was measured. Exceptionally large cells, if seen in the field out of reach of the micrometer axis, were measured separately.

Animal Inoculation

Antigens: The following different antigens were used for the immunological studies.

1) formalin killed H. capsulatum yeast cells, grown in vitro for 3 days at 37 C.

2) Same as above but treated with ethyl-ether.

3) H. capsulatum, grown in vivo, harvested after 5 days of yolk sac inoculation by ether-water interface system.

4) Same as above but harvested after 10 days of inoculation.

5) Same as above but harvested after 5 days of the second passage.

6) 2% suspension of infected yolk and yolk sac harvested after 10 days of inoculation (no ether extraction).

7) Control. 2% suspension of yolk and yolk sac of a 16-day-old embryonated normal egg.

Animals: 22 white leghorn hens, 4-6 months old were used. The chickens were obtained from a private farm in Oklahoma. Three chickens were used for each of the seven antigens and one chicken was used as the animal control. The chickens were bled by heart puncture prior to the first

injection, and at weekly intervals after each injection. Each chicken received a total of four intravenous injections into the wing vein, each injection one week apart and containing 1 x 10^8 cells/kilogram body weight. In the case of antigent 6 and 7, 1 ml/kilogram body weight was given. The blood samples were allowed to stand at room temperature and then centrifuged to separate the serum. All the sera were frozen.

Serological Studies

Capillary Tube Agglutination (CTA)

Antigen for CTA was prepared according to the procedure described by Cozad and Larsh (13). Two different concentrations of antigen, 25% transmission and 35% transmission at 5900 Å (Bausch and Lomb Spectronic '20') were used. Sera were diluted two fold up to 1:2048 dilution in physiological saline and were tested against the antigen for CTA. In the latter part of the study higher salt concentrations of the diluent were also tried. Some test samples were heated at 50 C for 30 and 60 minutes and others were heated at 60 C for 30 minutes. These heat treated sera were again checked by the capillary tube agglutination test.

Tube Agglutination

Testing the Chicken Sera. The procedure for the

agglutination test described by Cozad (12) was followed. Two fold dilutions of the test sera were made in such a way that each tube contained 0.5 ml quantity. To each tube 0.5 ml of the yeast cell antigen was added. The tubes were incubated at the following temperatures and times, and then centrifuged at 2400 rpm for 7 minutes (Int. centrifuge no. 1).

25 C for 2 hours ---- centrifuge

25 C for 2 hours; 5 C overnight ---- centrifuge

37 C for 2 hours ---- centrifuge

37 C for 6 hours ---- centrifuge

Readings were made by gently tapping the bottom of the tubes.

Tube Agglutination

Testing the Antigens. Different antigens, in vivo and in vitro grown, were tested against known positive anti H. capsulatum rabbit serum (CTA titer 1:1024). Dilutions of 1:300, 1:500, 1:1000 and 1:2000 of in vitro grown yeast cells were made. The cell counts in these dilutions were determined and dilutions of in vivo grown cells were made in such a way that each dilution would have the same number of cells as the corresponding suspension of in vitro grown cells. The rabbit antiserum was serially diluted two fold in physiological saline in 0.5 ml quantities. To each serum dilution 0.5 ml of the test antigen was added and the tubes were shaken

vigorously. The same incubation times and temperatures were used as in the previous experiment.

Precipitation Tests

Agar Gel Precipitation. The Ouchterlony plate method was employed. Seven tenths to 1% agar was used in the plates. Two concentrations (5X and 10X) from two different batches of histoplasmin were used. The plates were incubated at 5 C, 25 C, and 37 C for two weeks.

The micro Ouchterlony method (20) also was tried. Two concentrations of histoplasmin (5X, 10X) were used as the antigen. Salt concentrations of 0.85%, 1.7%, 5%, 8%, and 10% were used in the agar. It was found that in higher salt concentrations agar should be melted first before the addition of the salt; otherwise, the agar would not go into solution. The slides were left at room temperature in a humidity chamber and were checked for up to two weeks for the presence of precipitation bands.

Immunoelectrophoresis: The method described by Adamson and Cozad (1) was followed. Two concentrations (5X and 10X) from two different batches of histoplasmin were used. First, histoplasmin was added to the side wells and, after electrophoresis, the test chicken serum was added in the center trough.

In another experiment the above procedure was reversed, i.e., electrophoresing the test serum first and then adding histoplasmin in the center trough.

In still another set of experiments the antigen was electrophoresed and the slides were immediately bathed in the following salt solutions for 1-4 minutes: 1.7%, 5%, 8%, 10%, and 15%. The serum was then added to the center trough and allowed to diffuse. In the last set of tests the above procedure was reversed.

Capillary Precipitation

The capillary precipitation test described by Salvin and Furcolow (47) was adapted for checking the presence of antibody in the chicken sera. Histoplasmin (10X) was used as an antigen. Starting with undiluted antigen for the first tube two fold dilutions in physiological saline were made up to a 1:512 dilution. The capillary tubes were incubated at 5 C, 25 C, and 32 C for different periods of time.

In another set of experiments higher salt concentrations, 1.7%, 5%, 8%, and 10%, were also used as the antigen diluent. Some of the capillary tubes were incubated as long as two weeks and periodically checked for the formation of precipitate.

Interfacial Precipitation Method

Histoplasmin (10X) was diluted two fold in Durham tubes using saline with salt concentrations of 0.85%, 1.8%, 5%, 8%, 10%, and 15%. Equal quantities of undiluted serum were layered on top of the antigen. The tubes were left at room temperature and precipitation bands were checked at different intervals.

Results and Discussion

The results of preliminary studies on the effect of incubation temperature on H. capsulatum cells in the embryonated egg disagree with the findings of some previous workers (30, 33) who reported 33 C as optimum temperature for the growth of yeast phase in the yolk sac. It was found in this study that when yeast cells were inoculated into the yolk sac and the eggs were incubated at 33 C, the cells had a tendency to convert into the mycelial form. Chain formation and short mycelia were observed on the slides made from infected yolk sac at different intervals. When the mycelial suspension was used as inoculum, the results were the same. A mixed population of yeast cells and mycelial fragments were obtained. However, eggs which were incubated at 37 C had perfect yeast phase, and no mycelial fragments were observed.

Difficulties arose in staining the smears made from

the infected yolk sac. Most of the staining methods provided poor results because of the thickness of the smear and the presence of lipoidal material from the yolk. The nuclei of the chicken red blood cells were sometimes difficult to differentiate from the yeast cells. The special staining method using crystal violet after oxidizing the smear with chromic acid was found quite satisfactory. By this method the cytoplasm of the cell took an uneven stain, but the cell wall was stained very prominently, revealing a very distinct halo around it.

Effect of Dose of Inoculum on the Death Rate of Embryo

Table 6 shows the effect of size of H. capsulatum yeast cell inoculum on mortality rate of the embryos. As indicated by Figs. 1 and 2, the death rate of the embryos after inoculation was proportional to the number of organisms inoculated. The death rate was found to be quite variable in different batches of embryos receiving the same dose. When an average was estimated it was found that most of the embryos died 5 to 7 days after the inoculation. Figure 1 indicates that at higher doses the peak mortality rate was on the 5th day. However, upon decreasing the number of organisms in the inoculum the peak shifted toward the 7th and 10th day. Figure

2 indicates accumulative percentage of dead embryos at different intervals. The percentage of embryos hatched in each case is also shown in Figure 1. The number of embryos hatched increased as the inoculum was decreased.

The lethal effect of H. capsulatum on the chicken embryo has not been reported by previous workers. Welsh and Guidary (64) reported a study on the infection of chorioallantoic membrane of chicken embryos by H. capsulatum. They observed reactive plaques and ulcerative lesions. The plaques consisted of extracellular yeasts overlying a non-ulcerated area of reactive ectoderm. They found that the infection was not progressive, and peaked on the 5th day, followed by abrupt subsidence with degeneration of intracellular yeast and ultimate calcification and return of the membrane to normal thickness by the 12th day. They suggested an immune response as a possible explanation for the self-limited infection. The feasibility of antibodies overcoming infection during the developing stage seems improbable due to lack of immune response in chick embryo (7). Moreover, according to this report infection was not progressive, but, on the contrary, positive cultures were obtained almost every time in this study when liver and spleen of the embryo from an infected egg were plated. These findings and the findings of

previous workers (30) indicate that the infection in an embryonated egg is not localized and yeast cells can be obtained from almost any part of the embryo or the egg. Very recently Guidary and Spence (24) observed yeast cells in the subcutaneous tissue and feathers of inoculated embryo which also indicates a generalized infection.

Considering these facts, it can be stated that the death of chick embryos was due to the infection by H. capsulatum. On the contrary, according to some reports (35, 50), adult chickens are immune to Histoplasma infection and the organisms are destroyed in the tissues as soon as they enter into the body. Although H. capsulatum has been frequently isolated from chicken manure which gives an indication that chickens may be associated in some way with this fungus, search for H. capsulatum in chickens has been unsuccessful. Skin testing by Menges (35) showed negligible positive results. A thorough study of normal chicken guts (50) showed no trace of these organisms. So far experimental infection of chickens with Histoplasma has been unsuccessful (35, 50). In a recent report Guidary and Spence (24) reviewed the work of Tewari and Campbell (55) for histologic and cultural evidence of association of chickens with H. capsulatum, and the isolation of H. capsulatum from a feather pillow by Campbell,

Hill, and Falgout (9). On the basis of these reports and their own work they supported the working hypothesis that maintenance of H. capsulatum in nature might involve passage of the fungus through chicken with the feather pulp as the portal of exit.

The other possible reason for the death of embryo besides infection could be the toxicity of the organisms growing inside the egg. There was considerable growth and increase in the cell number, and during this growth toxic materials deleterious to the chicken embryo may be produced.

Viability of the Organisms Inside the Egg at Different Periods of Incubation

Variable results were obtained when whole yolk sac along with the yolk was harvested, ground, and its dilutions plated. It was found that different numbers of viable cells could be obtained from different batches of eggs inoculated with the same dose. When an average was calculated, it was found that the maximum number of viable cells could be obtained around 5-7 days after inoculation. Table 7 shows the average number of viable cells obtained at 3, 5, 7, 10, and 14 days after inoculation. A growth curve was obtained when the log of the total number of viable cells harvested was plotted against time of incubation (Fig. 3). This curve

shows a gradual rise until the peak is obtained on the 7th day. This part of the curve can be compared with the log or exponential phase of a typical bacterial growth curve. After 7 days there was a sudden decrease up to 10 days followed by a slower decrease. This portion is comparable to the stationary phase of a typical bacterial growth curve. In a recent report by Reca and Campbell (43), it was shown that when the yeast phase of H. capsulatum was grown on a synthetic medium, an increase in the number of viable cells was obtained up to the 4th day of incubation, after which there was a marked decrease in the number of new cells. When growth was followed by optical density determinations, the exponential phase extended up to the 6th day, after which the stationary phase started. The difference in the growth curve reported by these workers and the growth curve obtained in this study is probably due to differences in cultural conditions. Reca and Campbell (43) used a liquid medium (in vitro), while in these studies the cells were grown under in vivo conditions.

The use of a larger number of eggs and more frequent checking on the viability of the organisms in the eggs would possibly give better results for satisfactory statistical analysis.

A problem was encountered in this study when the yolk

sac was ground in the Omni mixer. Due to the high speed of the Omni mixer blades and agitation of the suspension, destruction of the yeast cells was inevitable. The consistency of the yolk sac changed on prolonged incubation and sometimes the yolk was found to be tough, requiring higher speed for making an even emulsion. This might have influenced the viability of the cells. In this study only yolks and yolk sacs were checked for the viability studies. The yeast cells were also disseminated in the embryo and checking different parts of eggs and embryos may possibly give a somewhat different picture in regard to the increase in cell numbers.

It was found that when dilutions of the ground yolk-yolk sac suspension were plated out on brain heart infusion blood agar and incubated at 37 C, yeast colonies were obtained. The number of yeast colonies obtained was the same and sometimes greater than the number of mycelial colonies obtained on Sabouraud's agar plates at the same dilution. These yeast colonies probably appeared because the yolk and egg proteins were present in the diluted suspension in sufficient quantity to supplement brain heart infusion blood agar medium for the support of yeast phase growth.

Prolonged viability of the organisms in the frozen yolk and yolk sac was another significant observation. When

yolks and yolk sacs, frozen at -20 C for as long as one year, were checked, viable cells were found which grew very well on Sabouraud's agar plates.

Harvesting of the Cells

Separating the yeast cells of H. capsulatum from the yolk and yolk sac was one of the most difficult problems in this study. An attempt was made to use trypsin for digestion of the yolk sac tissues. The organisms could be separated to some extent by the use of higher than 0.25% trypsin solution for prolonged treatment, but this treatment ruptured the cell wall of the organisms. Moreover, cells present in the yolk material still could not be separated by this method.

Other physio-chemical methods were attempted to find a suitable procedure for separation of the organisms. In a preliminary study an ethyl-ether-water separation system was found promising. Different methods of ethyl-ether-water separation as reported by Craigie (14) were investigated for this separation. The efficiency of the method in separation of the cells from suspension was found to be dependent on the following factors:

a) Nature and concentration of the buffer system: Different buffers like citric acid-phosphate buffer, acetate-acetic acid buffer, and phosphate buffer

were tried with molarities varying from 0.1 to 0.5.

b) Hydrogen ion concentration: This effected the development and separation of the emulsion from the aqueous portion. Buffers of varying pH from 3 to 8 were used.

c) Quantity of the buffer and the test suspension: Change in quantity of either component controlled the interface and aqueous layer development.

After working with the above variables, a procedure was adopted which resulted in the best yield of organisms from yolk-yolk sac suspensions (Flow diagram-1).

The main problem in this separation was the presence of small egg protein particles which adhered to the yeast cells in the final harvested suspension. The association of yeast cells with the particles was so strong that they could not be separated by centrifugation. The harvested suspension was shaken with phosphate buffers of varying pH. It was observed that 0.1M phosphate buffer at pH 6-7 facilitated the separation of the organisms from the egg protein.

Results obtained from the use of citric acid-phosphate buffer and from acetic acid-acetate buffer (0.1M) were almost the same and either one of these two buffers could be used in this separation. The pH, however, was very critical and best

results were obtained at pH 4.5.

It was noticed that a better yield was obtained if fresh yolk-yolk sac were used in comparison with the frozen one. Moreover, better yields were obtained if yolk was separated from the yolk sac and both were treated separately. The separated yolk was treated with high 10% salt, as suggested by Vogel (60), to break down some of the yolk proteins. After high salt treatment the yolk lost its viscosity and the yolk material was eliminated to a certain extent by centrifugation.

Some of the egg material was always present with the harvested yeast cell suspension, although a second shaking of this suspension with ½ volume ether helped in removal of some of these undesirable particles. A pure suspension of H. capsulatum yeast cells free of contaminating egg materials could not be obtained.

When the interface which was supposed to contain egg proteins and tissue cells was checked microscopically, numerous yeast cells were found adhering to the protein particles. Some of these cells were recovered when this interface emulsion was shaken with an additional quantity of buffer and ether, but still a great number of organisms, mostly the larger ones, could not be recovered and were lost in the

interface emulsion.

An accurate estimation of the total number of organisms found per egg was not possible because of the loss of the cells during the ether harvesting process. A rough estimation, however, indicated a maximum increase of 25 times the original number on the 10th day of inoculation.

When the cell suspension harvested by the ether-water system was centrifuged, it was found that some of the cells remained in suspension and did not spin sediment even at a speed as high as 10,000 rpm (12100 Xg). This was probably due to the light weight of the cells and also because of the presence of buffer at acidic pH. The only successful way to wash these cells was to filter the suspension through a Millipore filter and resuspend them in physiological saline. Centrifugation of the cell suspension at high speed (10,000 rpm, 12100 Xg) followed by filtration of the supernatant, was found to be the most convenient and rapid method.

Cell Size at Different Intervals

One of the most significant findings in this study was the presence of giant yeast cells after prolonged incubation of infected fertile eggs. It was found that the increase in size was related to the period of incubation; the longer the incubation time, the greater the size of the

organisms (Plate 1).

Table 8 shows average size of the cells and the size distribution at different intervals. Figure 4 indicates the cell size increase upon incubation. The average size of cells in the inoculum (3 day old culture), when measured after shaking the cells with ether, was 3.15μ. After 5 days of incubation the average cell size on blood agar (in vitro) was found to be 3.17μ, which is not a very significant increase. Cells grown in the yolk sac (in vivo) became larger with an average size of 3.6μ from ether harvested suspensions, and 3.95μ from stained yolk-sac smears (Fig. 5A). By the 10th day the increase was greater. The average control in vitro grown cell measurement (ether treated) was 3.65μ while in vivo grown cells harvested with ether had an average size of 4.82μ. The average cell size measured from yolk sac stained smears after 10 days' incubation was 4.83μ (Fig. 5B). Ether harvesting was not done on cultures older than 10 days, but when smears were made from yolk-yolk sacs after 14 and 17 days of incubation, averages of 5.06μ and 6.0μ were obtained. The cell size on blood agar (after ether treatment) was 3.7μ and 3.77μ, after 14 and 17 days' incubation, respectively. This shows that the increase in in vivo grown cells was significantly higher than in in vitro grown cells. Especially in 14

to 17 day cultures there was not much increase in the cell size of *in vitro* grown cells while *in vivo* grown cells showed a tremendous increase in size. In two batches of embryos when the infected eggs were hatched (17 days) and the yolk sac was harvested, cells as large as 17.6μ were observed; the majority of the cells were found to be in the larger size range (Fig. 5C, Plate 1).

The incubation period also had a great influence on the size distribution of the organisms (Table 8). Figures 5A, B, and C show the distribution of size of *in vitro* and *in vivo* grown cells at 5, 10, and 17 days. Most of the cells in the original inoculum lie between 2-4μ in diameter (long axis) with a very sharp and high peak in 3-4μ area. On further incubation (5 days, Fig. 5A) the peak begins to flatten and spreads out. *In vitro* grown cells are mostly in the range of 2-4μ while *in vivo* grown cells are mostly in the range of 3-5μ and the curve extends up to 6-7μ (Fig. 5A). The peak of the cells measured on the 10th day shifts toward a higher range of size (Fig. 5B). The majority of *in vitro* grown cells are in the range of 2-4μ but cells up to 7-8μ are found. The majority of *in vivo* grown cells measured on the 10th day of incubation are in the 3-5μ range, but the distribution curve extends up to the 12-13μ range. On the 14th day of

incubation after inoculation the range of cell sizes is greater. In vitro grown cells are found in the range of 2-9u with the majority of them in the range of 2-5μ. The majority of the in vivo grown cells at the same time are in the range of 3-6μ with the curve extending up to 12-13μ. The difference in in vivo and in vitro grown cell sise is more prominent on the 17th day. In vitro grown cells (Fig. 5C) have a wider peak with the majority of the cells lying in the range of 2-5μ and the curve extends up to the 10-11μ range, while peak of in vivo grown cells also flattens and the curve is flatter, extending up to 13-14μ category. The majority of the cells in this case are found in the range of 3-6μ (Fig. 5C).

Some complications were encountered in the cell size studies due to the presence of several variables. The size of the cells differed among different embryos of the same age. Furthermore, change in size of the cell was inevitable due to shrinkage of cells during drying, oxidizing and staining of the yolk sac smear. When the cells were harvested with ether, the loss in the interface of cells representing the larger population was unavoidable. Moreover, cells which were recovered from the aqueous phase had undergone violent shaking with ether, which affects the size. A decrease of 4.5% was obtained in the average cell size when formalin killed in

vitro grown cells were treated with ether. The control cells (*in vitro* grown cells) conld not be measured by simple stained smears on slide because the cell shrinkage and change in the shape of the cell was too great to be overlooked. Cells grown on blood agar, killed by formal saline, and shaken with ether were used as a control. These variables did not, however, influence the significance of the data obtained from cell size studies because the above mentioned variables had a shrinking effect on the cells and the presence of large cells would still be significant.

The presence of giant cells in the embryonated egg once again opened a controversial question concerning *H. capsulatum* and *H. duboisii*. "Is *H. duboisii* a mere morphological variation of *H. capsulatum* or is it a separate species?" (17, 49, 57, 58). *H. capsulatum* and *H. duboisii* are identical in the mycelial phase, grossly and microscopically. The primary basis of separating the two is the presence of large yeast cells in addition to classical small forms in *H. duboisii* infection.

In 1953 Schwarz (49) noticed giant forms of *H. capsulatum* in tissue explants. He believed that giant forms were simply a growth in size in the original small yeast-like cell. Binford (5) reported large extracellular forms of *H. capsulatum*

in some necrotic tissues. Large forms have been occasionally observed in tissues of infected animals as well as in the tissues of American patients (5, 52). Drouhet and Schwarz (17) studied 18 strains of Histoplasma including four strains of H. duboisii. They observed large forms in almost all cases of H. capsulatum and H. duboisii. They concluded from their findings that "The presence of large forms in tissues of non-African patients and the experimental production of such forms indicate the great variability of the parasitic phase of H. capsulatum." Weed (62) also agreed with the idea that the dual form infection might represent just variation in size of Histoplasma.

Vanbreuseghem (57, 58), who originated the idea of separate species, infected guinea pigs with H. duboisii. After 8-15 days the parasite looked so much like H. capsulatum that he named these forms the capsulatum form of H. duboisii. Later the organisms enlarged and he called these mature forms duboisii forms. Okudaria and Schwarz (39) in a later study reported that H. capsulatum could be differentiated from H. duboisii by the size in the parasitic yeast phase, by the difference in pathogenicity, and by particular tissue response in hamsters and mice. They also reported some characteristics of H. duboisii, mainly simultaneous occurrence of small,

middle-sized and large forms of yeast cells in tissue, and a double contoured cell wall.

Pine and Drouhet (41) suggested that the ability to form the duboisii form varied with strain, with the experimental animal and with the form of the yeast culture used in the inoculum. They concluded from their results that the capsulatum form was a transient stage in the morphogenesis of the parasite. The duboisii form, once formed, reproduced only duboisii daughter cells, even though some of the latter might fall within the size range of the mature capsulatum form. The very large adult duboisii-like cell in necrotic tissue, according to Pine, represented a terminal phase of growth. These cells, which sometimes had a multi-layered thick wall, were probably a degenerated form (41).

Although most of the workers have agreed that H. duboisii can be differentiated from H. capsulatum on the basis of cell size and thus possibly can be placed as a separate species, the presence of large cells (so-called duboisii form) in the yolk sac makes this concept more deceptive. It has been claimed that large forms of H. capsulatum are found in necrotic and dead tissues or in in vitro conditions, but these large cells were harvested from living infected embryonated eggs, the conditions of which are obviously not in vitro.

Whether the yolk sac should be considered as necrotic tissue is questionable.

The morphological characteristics of the large forms of H. capsulatum obtained from the yolk-yolk sac also were very conspicuous. The giant cells had a thick cell wall which appeared to have a double contour with a refractile halo around it, all of which are characteristic of H. duboisii (Plate 1). Some of the cells had an occasional notch in the capsule-like layer on the narrower side, which is also a characteristic of H. duboisii, as described by Vanbreusegham (57).

At this point no definite statement can be made about the possible relationship of H. capsulatum and H. duboisii, since H. duboisii was not used in these studies. A more thorough study is required to reveal some of the facts about the large size of H. capsulatum. A study involving the use of H. duboisii and H. capsulatum is suggested with inoculation of yolk sac of chick embryo and measurement of organisms at different intervals for comparison. A study of the cells recovered from liver and spleen of the infected chick embryo would help also in resolving the question.

Immunological Studies

Chickens were used in this study as the test animal for hyperimmunization because of previous reports about high

antibody production in this animal against certain antigens. Precipitin production in chickens has been extensively studied (4, 21, 65, 66) and titers as high as $1:32 \times 10^6$ have been reported by Wolfe.

In a preliminary work three one month old chickens, hatched from the control eggs, were bled and checked for the CTA titer. No agglutination was observed. Later these chickens were immunized by giving four injections, each one week apart. When the serums taken after one week of the last injection were tested, a CTA titer of 1:256 was obtained. On the other hand, when infected eggs were hatched and sera of two of these newly hatched chickens were tested, a titer of 1:32 was obtained by the CTA method. These findings stimulated the use of chickens as the test animal.

It was found in later studies that adult normal chicken sera agglutinated the antigen for CTA with a great variation of titer ranging from 1:4 to 1:256. The agglutination was found weak and the results were very inconsistent. This finding raised a new question about the chicken serum: a) do chickens have natural agglutinins against histoplasma or it is the property of the chicken serum to agglutinate non-specifically with antigen used in CTA? and, b) at what age do chickens start producing substances which agglutinate CTA

antigen?

To solve the first question the normal chicken sera were heated at 56 C for 30 and 60 minutes and 65 C for 30 minutes and then tested again. Agglutination was still observed in these heated sera when tested with the CTA method. The second problem was investigated by bleeding newly hatched normal chickens. The chickens were bled at the age of 6, 24, 48, and 72 hours, and at 1, 2, 3, 4, 5, 6, 7, 9, and 15 weeks. The sera were frozen and were tested later with the CTA method. No reaction was observed until the age of 4 weeks, after which slight agglutination was observed in the first tube (1:2 dilution of the serum). This persisted until the 6th week, after which some agglutination showed up in the second tube also (1:4 dilution). On the 7th week a 1:2 dilution showed definite agglutination, while slight agglutination was observed at a 1:4 and 1:8 dilution. The results were the same up to the 15th week when definite agglutination was observed up to a 1:4 dilution with a 1:8 dilution showing only slight agglutination.

No increase in the CTA titer was observed when sera were tested from chickens hyperimmunized against <u>in vivo</u> and <u>in vitro</u> grown cells. The same inconsistency was observed and the titers varied from 1:16 to 1:256. No noticeable

difference was found in the sera from chickens receiving different kinds of test antigens. In some instances the titer was higher in the serum from initial bleeding prior to first injection than in the titer after hyperimmunization. Due to these discrepancies the CTA results were inconclusive and could not be interpreted.

Higher salt solutions for the dilution of the serum were also used, but the results were found to be as inconsistent and inconclusive as before. When normal mouse, guinea pig and rabbit sera were tested against the test CTA antigen, no agglutination was observed.

The presence of precipitins in these chicken sera was also investigated. No bands were obtained when the sera from hyperimmunized chickens were tested against histoplasmin antigen by Ouchterlony plate method and micro-Ouchterlony slide method. Some workers (4, 21, 63) reported that higher precipitin titers were obtained by using higher salt concentrations in the diluent. Salt concentrations from 8-18% have been recommended in the literature (4, 63). No success was achieved by using higher salt concentrations in Ouchterlony diffusion methods. By micro-Ouchterlony technique in one instance light bands were obtained with one normal and three immune chicken sera with the use of 8% salt in the agar.

These bands could be due to precipitation of the serum proteins because high salt concentration was used and the bands appeared after one week.

Immunoelectrophoresis results were also negative even when the slides were bathed in 5, 8, 10, and 15% salt solution as recommended by Benedict (4).

Capillary precipitation tests were also attempted using the method described by Salvin (47). Slight precipitation was observed in the first 3 tubes of normal as well as immune sera when the capillary tubes were refrigerated overnight. Higher salt concentrations for the diluent did not give any better results.

Wolfe (65, 66) reported a strange phenomenon in the chicken precipitin titers. He observed that the precipitin titer increased considerably if the serum was left in the refrigerator for 7-10 days. No improvement was observed in this study when the immune chicken sera were left in the refrigerator for the recommended period and then tested.

Tube agglutination was found to be unsuccessful in differentiating between the titers of normal and hyperimmunized chicken sera. Normal as well as hyperimmune sera gave agglutination up to the second or third tube (1:5 - 1:20 dilution). Incubation at different temperatures and times

did not help in the results.

In vivo grown cells also were compared with *in vitro* grown cells by the tube agglutination method using known immune rabbit serum against *H. capsulatum*. Different times and temperatures were tested. The results showed less agglutination in the *in vivo* grown cells than in *in vitro* grown cells. This could be due to the presence of egg proteins in the suspension of *in vivo* cells or due to the loss of some antigenic properties of the yeast cells during the process of ether separation.

The results of immunological and serological studies did not lead to any definitive conclusions. Although morphological differences of *in vivo* and *in vitro* grown cells are quite evident and also significant, the differences in antigenicity of the two kinds are still to be revealed. Presence of giant cells makes the situation especially interesting and one wonders if these large forms would have some significance in pathogenicity and antigenicity or in the progressive chronic nature of the disease. A further extensive study is required to find out the difference in pathogenicity and antigenicity between *in vivo* grown cells and *in vitro* grown cells. The use of other animals and further purification of the ether harvested cells could be helpful in these studies.

CHAPTER IV

SUMMARY

Two methods, chamber implantation and yolk-sac inoculation of chicken embryo, were employed to grow Histoplasma capsulatum for studies of growth characteristics and differentiation of in vivo and in vitro grown cells.

No significant increase in cell numbers was obtained when yeast cells of H. capsulatum were placed in the diffusion chambers implanted in the peritoneal cavities of mice. The decrease in the number of viable cells was rapid, and the different test suspending fluids for the yeast cells had little effect on the maintenance of viability. Significantly, some of the cells when recovered from chambers 12 weeks after implantation were still viable. Large forms of the yeast cells with thick cell walls were obtained 2-4 weeks after implantation. This is one possible explanation for maintenance of viability by organisms for such a long time. The inability of the organisms to reproduce inside the implanted chamber can be attributed to two possible factors: cell-free environments of the chamber, and the formation of gelatinous tough clot inside and around the chamber which could stop

free exchange of peritoneal fluid through the millipore diffusion membrane.

Yolk sac of chicken embryo was found to be an excellent site for the growth of H. capsulatum. Studies on the effect of temperature on the growth showed that 37 C temperature was required to grow yeast phase in the yolk sac. The growth was found to be lethal to the embryo and most of the embryos died within 5-7 days after inoculation. The death rate was found to be directly proportional to the dose of inoculum of H. capsulatum yeast cells; the greater the inoculum, the faster was the death rate.

When the yolk and yolk sac of the infected eggs were harvested, ground in an Omni mixer and dilutions plated, the maximum number of viable cells was obtained 5-7 days after inoculation. After this the viability started decreasing slowly.

A modified ethyl-ether-water interface system was worked out for the separation of the yeast cells from the yolk and yolk sac suspension. When the yolk and yolk sac suspension was made on 0.1M citric acid phosphate or acetic acid acetate buffer of pH 4.5 and was shaken with ethyl ether, a complex physical system resulted in such a way that the yeast cells remained in the aqueous portion, almost in

pure form.

Morphological studies revealed significant differences between the sizes of *in vivo* and *in vitro* grown cells. Infected yolk sac smears were made at different intervals and a special method was developed to stain these smears. The data obtained from the measurement of cells on 5, 10, 14 and 17 days on stained smears and in the ether harvested suspension were compared with the *in vitro* grown cells on blood agar for the same periods. *In vivo* grown cells increased in size considerably and giant cells were found commonly 10 to 17 days after inoculation. It was found that the average cell size was proportional to the period of incubation. Average cell size as well as the majority of cell population shifted toward the larger size range on the increase of incubation period. The giant cells had a typical thick cell wall and some characteristics similar to those of *H. duboisii*. A possible relationship of the so-called two species is discussed.

Chickens were used for immunological studies of the *in vivo* cells. It was found that normal chicken serum will agglutinate antigen for CTA nonspecifically and thus no differentiation between normal and hyperimmune chicken serum could be established. No success was achieved when capillary

tube agglutination, tube agglutination, precipitation and immunoelectrophoresis procedures were employed to assay for presence of antibodies in the sera of injected and hyperimmunized animals. Due to discrepancies of the immunological and serological results, no satisfactory comparison of *in vivo* and *in vitro* grown cells could be made at this point and further studies are required.

Table 1. Total Counts and Viable Counts Obtained from Chambers Using Physiological Saline Containing 1% Normal Mouse Serum

Time after Implantation	Number of Chambers Recovered	Total Number of Yeast Cells/Chamber	Total Viable Cells/Chamber	% Viability
0 day	-	1.0×10^7	3.6×10^6	36
1 day	2	1.4×10^7	3.5×10^6	25
2 days	2	1.4×10^7	1.4×10^6	10
3 days	2	1.5×10^7	1.9×10^6	13
5 days	2	7.5×10^6	6.0×10^5	8
7 days	2	9.5×10^6	2.9×10^5	3
2 weeks	2	6.0×10^6	7.7×10^4	1
3 weeks	2	6.2×10^6	3.2×10^4	<1

Table 2. Total Counts and Viable Counts Obtained from Chambers Using Cysteine Saline Containing 1% Normal Mouse Serum

	Time after Implantation	Number of Chambers Recovered	Total Number of Yeast Cells/Chamber	Total Viable Cells/Chamber	% Viability
	0 day	-	1.0×10^7	3.8×10^6	38
	1 day	2	1.4×10^7	4.2×10^6	30
	2 days	2	1.8×10^7	4.6×10^6	25
	7 days	2	2.7×10^6	-	-
	10 days	2	6.8×10^7	3.4×10^6	5
*	0 day	-	1.0×10^7	3.8×10^6	38
	1 day	2	1.4×10^7	4.3×10^6	31
	2 days	2	2.0×10^7	5.4×10^6	27
	3 days	2	2.2×10^7	4.6×10^6	21
	5 days	2	1.9×10^7	2.1×10^6	11
	7 days	2	8.7×10^6	5.2×10^5	6
	10 days	2	1.8×10^7	5.5×10^6	3
	2 weeks	2	6.2×10^6	3.1×10^4	< 1
	3 weeks	1	6.8×10^6	4.2×10^4	< 1

* Same experiment repeated.

Table 3. Total Counts and Viable Counts Obtained from Chambers Using Tyrode's Solution

Time after Implantation	Number of Chambers Recovered	Total Number of Yeast Cells/Chamber	Total Viable Cells/Chamber	% Viability
0 day	-	1.0×10^7	7.1×10^6	71
1 day	2	1.1×10^7	6.4×10^6	58
2 days	2	1.3×10^7	3.6×10^6	28
3 days	2	1.5×10^7	2.3×10^6	15
5 days	2	1.4×10^7	2.1×10^6	14
7 days	2	1.7×10^7	2.1×10^6	7
2 weeks	2	9.8×10^6	1.8×10^5	2
3 weeks	2	8.0×10^6	7.0×10^4	1
4 weeks	2	2.6×10^6	1.8×10^3	<1
8 weeks	2	2.8×10^6	1.8×10^2	<1
12 weeks	2	1.4×10^6	1.0×10^2	<1

Table 4. Total Counts and Viable Counts Obtained from Chambers Using Tyrode's Solution Containing 1% Normal Mouse Serum

Time after Implantation	Number of Chambers Recovered	Total Number of Yeast Cells/Chamber	Total Viable Cells/Chamber	% Viability
0 day	-	1.0×10^7	5.8×10^6	58
1 day	2	1.2×10^7	-	-
2 days	2	1.5×10^7	5.4×10^6	36
3 days	2	1.6×10^7	4.3×10^6	27
5 days	2	2.6×10^7	4.7×10^6	18
7 days	2	1.2×10^7	1.2×10^6	10
2 weeks	2	6.2×10^6	5.0×10^5	8
3 weeks	2	7.1×10^6	1.4×10^5	2
4 weeks	2	5.0×10^6	1.3×10^5	2.5
6 weeks	2	5.3×10^6	7.3×10^3	<1
8 weeks	2	6.0×10^6	4.8×10^2	<1
12 weeks	2	8.7×10^6	1.1×10^2	<1

Table 5. Total Counts and Viable Counts from Chambers Using Eagle's Medium

Time after Implantation	Number of Chambers Recovered	Total Number of Yeast Cells/Chamber	Total Viable Cells/Chamber	% Viability
0 day	-	1.0×10^7	4.2×10^6	42
1 day	-	-	-	-
2 days	2	2.1×10^7	5.0×10^6	25
3 days	2	4.0×10^7	9.6×10^6	24
5 days	2	4.5×10^7	8.1×10^6	18
7 days	2	2.3×10^7	2.3×10^6	10
10 days	2	1.3×10^7	1.4×10^5	11
2 weeks	2	1.4×10^7	7.0×10^5	5
3 weeks	2	8.0×10^6	7.4×10^4	<1
* 0 day	-	1.3×10^6	4.5×10^5	36
1 day	2	2.3×10^6	5.2×10^5	23
2 days	2	2.3×10^6	4.6×10^5	20
3 days	2	3.9×10^6	4.5×10^5	11
5 days	2	3.5×10^6	6.1×10^5	18
7 days	2	2.3×10^6	2.7×10^4	12
10 days	2	1.3×10^6	2.4×10^4	<2
3 weeks	2	2.5×10^6	2.0×10^5	<1
4 weeks	1	2.0×10^6	8.0×10^3	<1
6 weeks	1	9.1×10^5	3.0×10^3	<1

* Same experiment repeated.

Table 6. Effect of Dose of *Histoplasma capsulatum* Yeast Cells on the Mortality of Chick Embryos

Total Number of Cells Inoculated/Egg	Total Number of Eggs Used	Percent of Embryos Dead at Each Interval						Number of Embryos Hatched
		3 day	5 day	7 day	10 day	12 day	14 day	
3.5×10^8	61	21.3	57.4	82.0	96.7	100	-	-
1.0×10^8	151	22.5	54.3	82.1	96.0	100	-	-
5.0×10^7	165	13.3	40.0	81.8	95.1	100	-	-
5.0×10^6	127	12.6	31.5	66.9	88.2	96.9	99.2	0.8
5.0×10^5	58	10.3	24.1	55.1	84.4	93.0	93.0	7
5.0×10^4	42	11.9	21.4	42.8	69.0	83.3	88.1	11.9
Control	42	7.1	16.6	23.7	35.7	42.8	47.6	52.4

Table 7. Number of Viable Cells of H. capsulatum Obtained from Yolk and Yolk Sac at Different Intervals

Average Total Number of Cells Inoculated/Egg	Average Total Viable Cells Inoculated/Egg	Days after Inoculation	Number of Eggs Harvested	Average Viable Cells Harvested/Egg
1×10^7	4.4×10^6	3	56	3.40×10^7
"	"	5	56	2.86×10^8
"	"	7	54	8.50×10^8
"	"	10	52	1.54×10^8
"	"	14	47	9.60×10^7

Table 8. Size Distribution of Yeast Cells of H. capsulatum at Different Incubation Periods*

Type of Cell**	Days of Incub.	Aver. Cell Size μ	Size Distribution Percent of Cells in Each Size Group												
			1-2 μ	2-3 μ	3-4 μ	4-5 μ	5-6 μ	6-7 μ	7-8 μ	8-9 μ	9-10 μ	10-11 μ	11-12 μ	12-13 μ	13-14 μ
A	3	3.15	0	32	58	10	0	0	0	0	0	0	0	0	0
	5	3.17	5	44	40	11	0	0	0	0	0	0	0	0	0
	10	3.65	2	34	36	14	10	3	1	0	0	0	0	0	0
	14	3.72	0	33	37	19	7	3	0	1	0	0	0	0	0
	17	3.77	1	29	30	25	9	2	1	1	1	1	0	0	0
B	5	3.6	1	21	39	35	1	3	0	0	0	0	0	0	0
	10	4.82	0	3	26	36	13	12	3	5	0	1	0	1	0
C	5	3.95	0	4	50	39	6	1	0	0	0	0	0	0	0
	10	4.83	0	2	23	48	10	10	1	1	1	3	0	1	0
	14	5.06	0	2	22	43	15	9	1	1	1	3	1	2	0
	17 (Hatched)	6.0	0	3	19	28	13	9	7	8	3	3	3	3	1

* Diameter of the long axis.

** A - In vitro grown cells, formalin killed, ether treated, wet mount.
B - In vivo grown cells, formalin killed, harvested with ether, wet mount.
C - In vivo grown cells, yolk sac smear, stained.

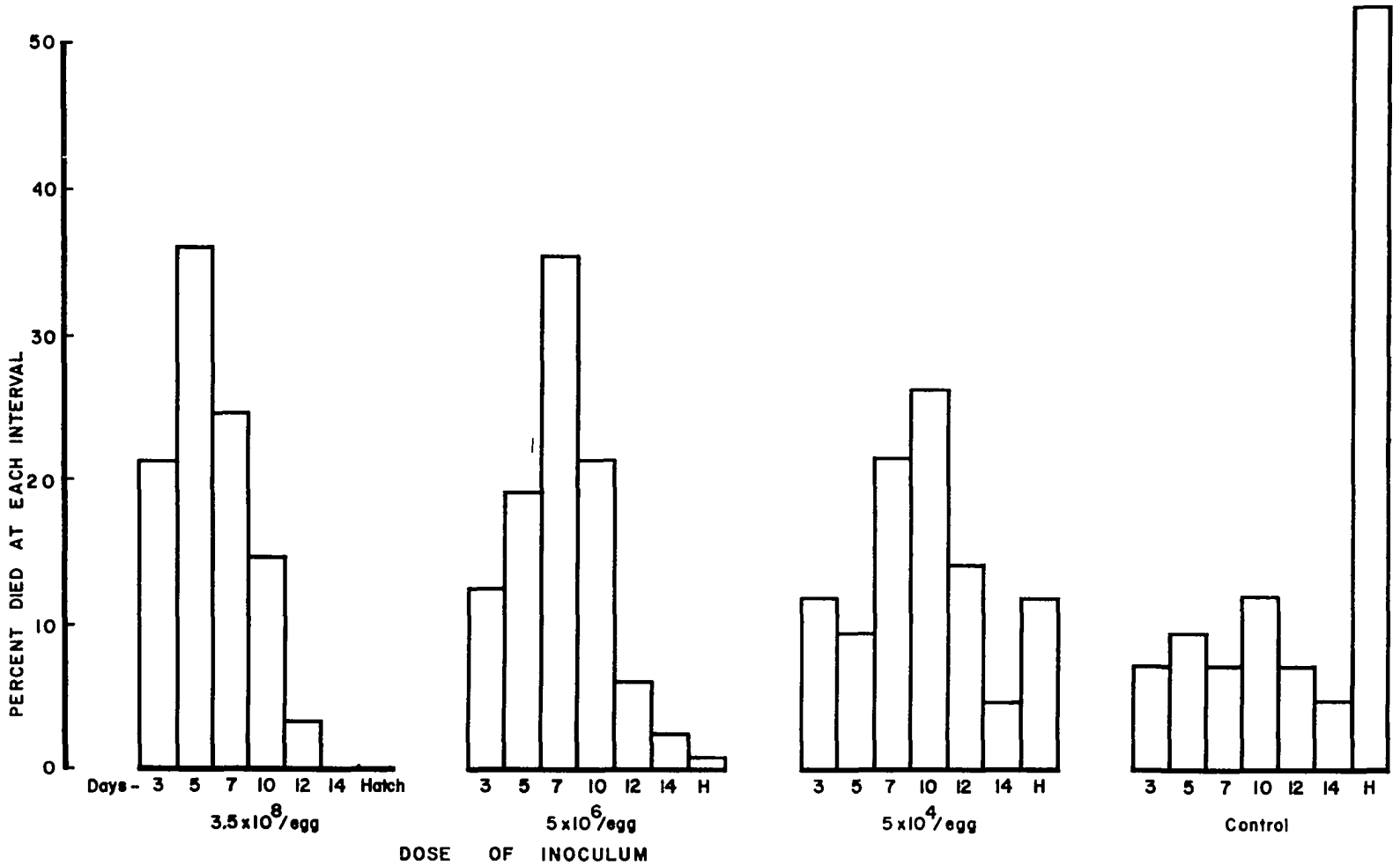


Figure 1. Effect of dose of inoculum of Histoplasma capsulatum yeast cells on the death rate of chick embryos.

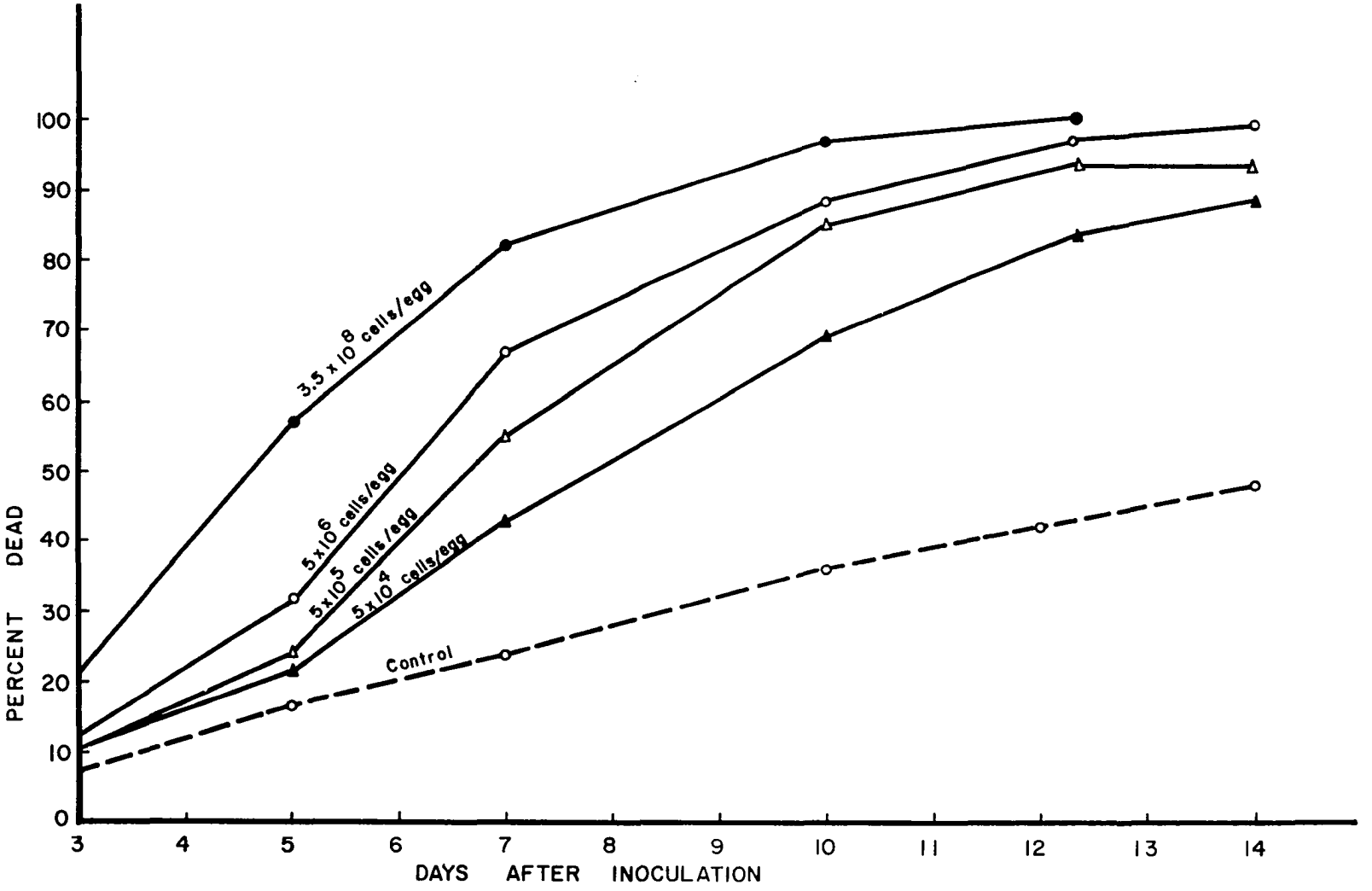


Figure 2. Effect of dose of Histoplasma capsulatum yeast cells inoculum on the death rate of chick embryos .

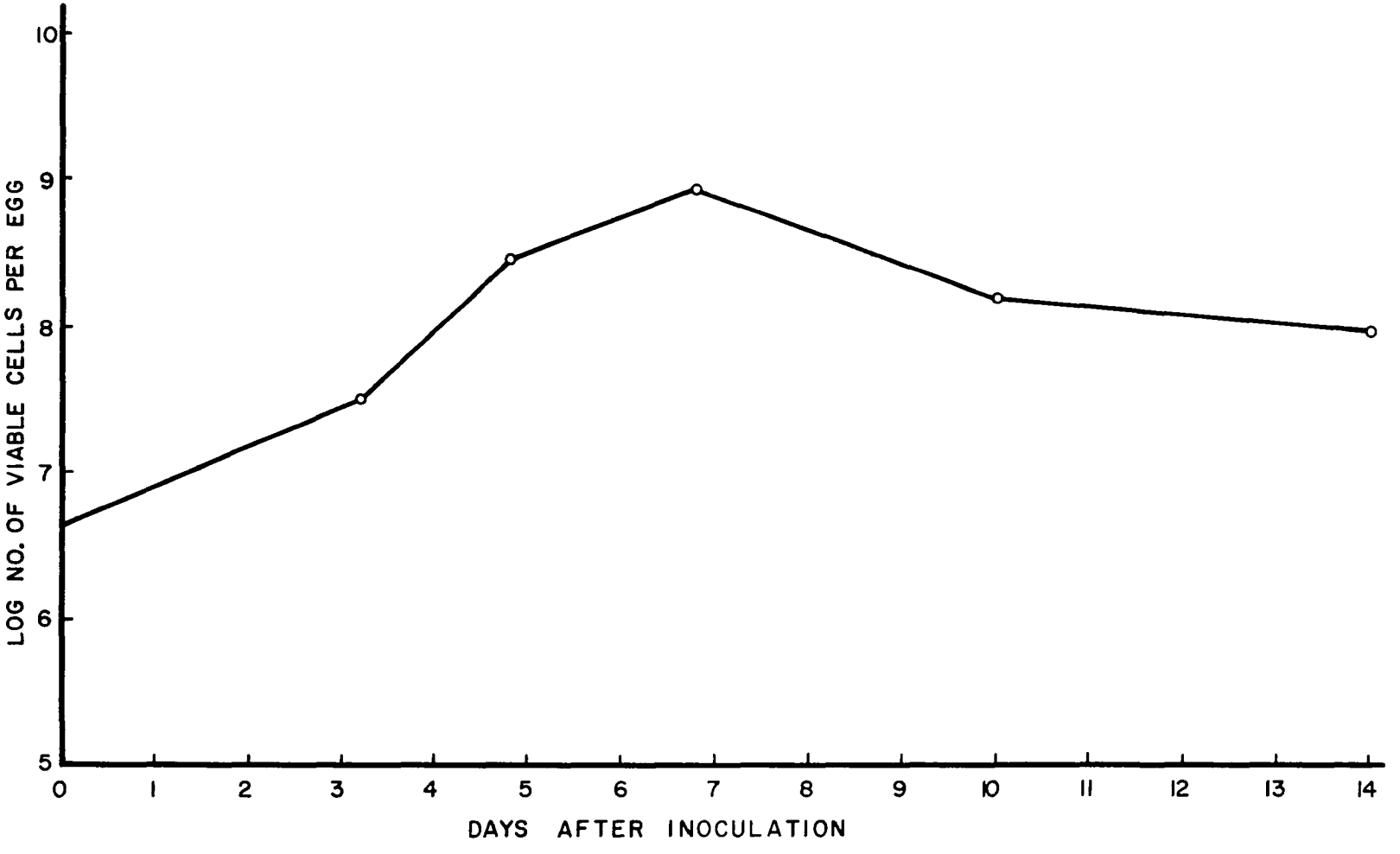


Figure 3. Number of viable cells of Histoplasma capsulatum obtained from yolk and yolk sac at different intervals.

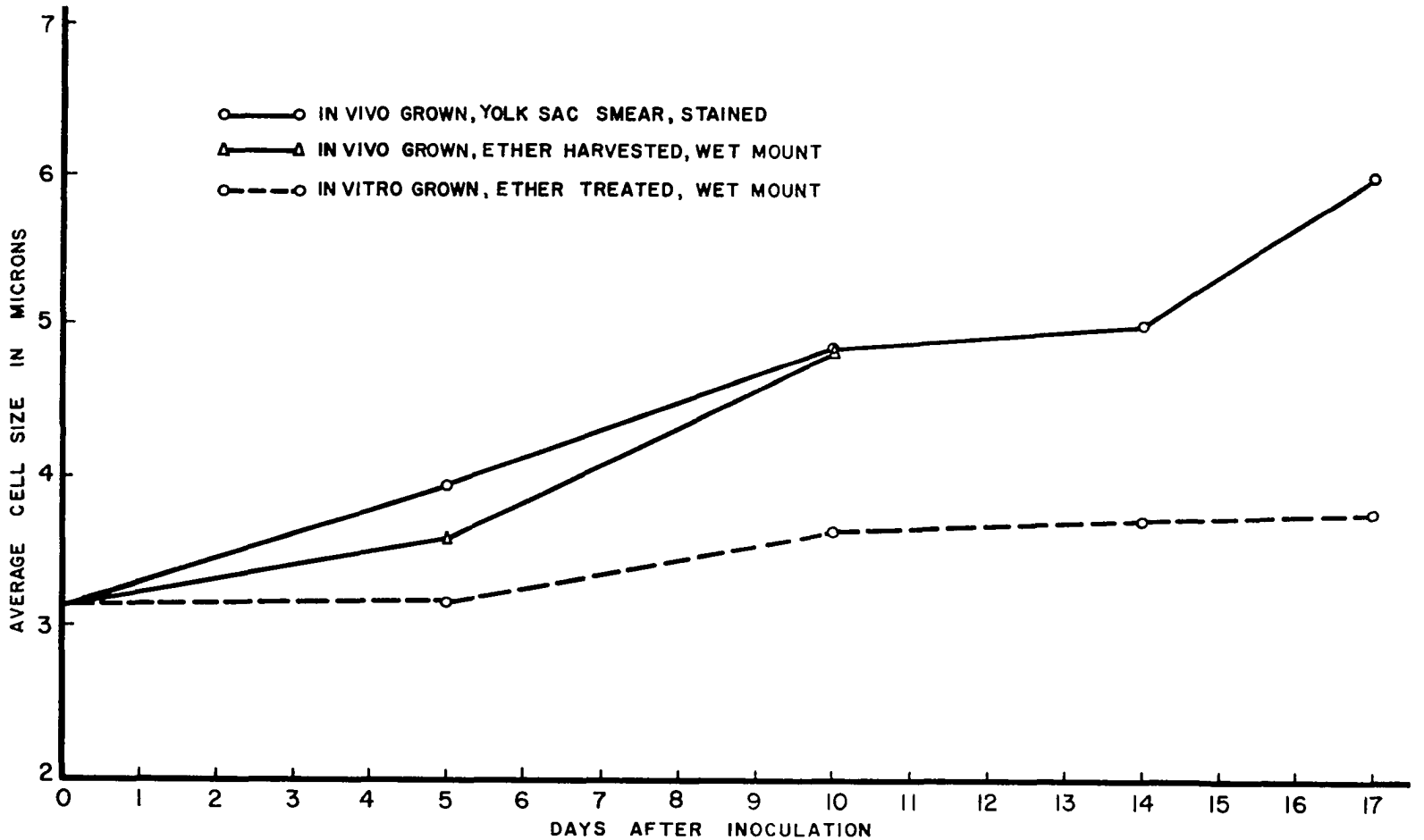


Figure 4. Increase in cell size. Average size of Histoplasma capsulatum yeast cells at different intervals.

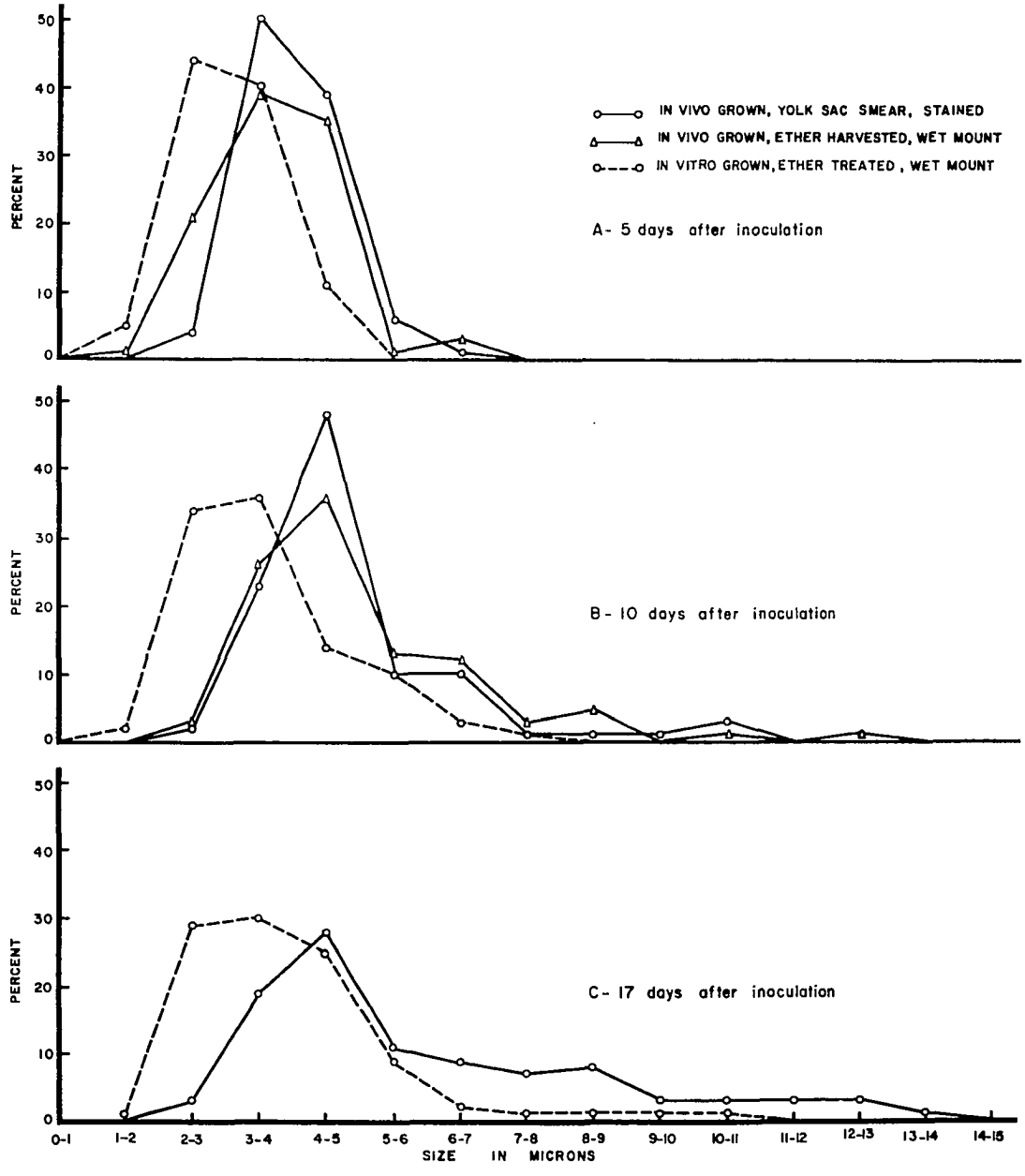


Figure 5. Size distribution of Histoplasma capsulatum yeast cells at different intervals.

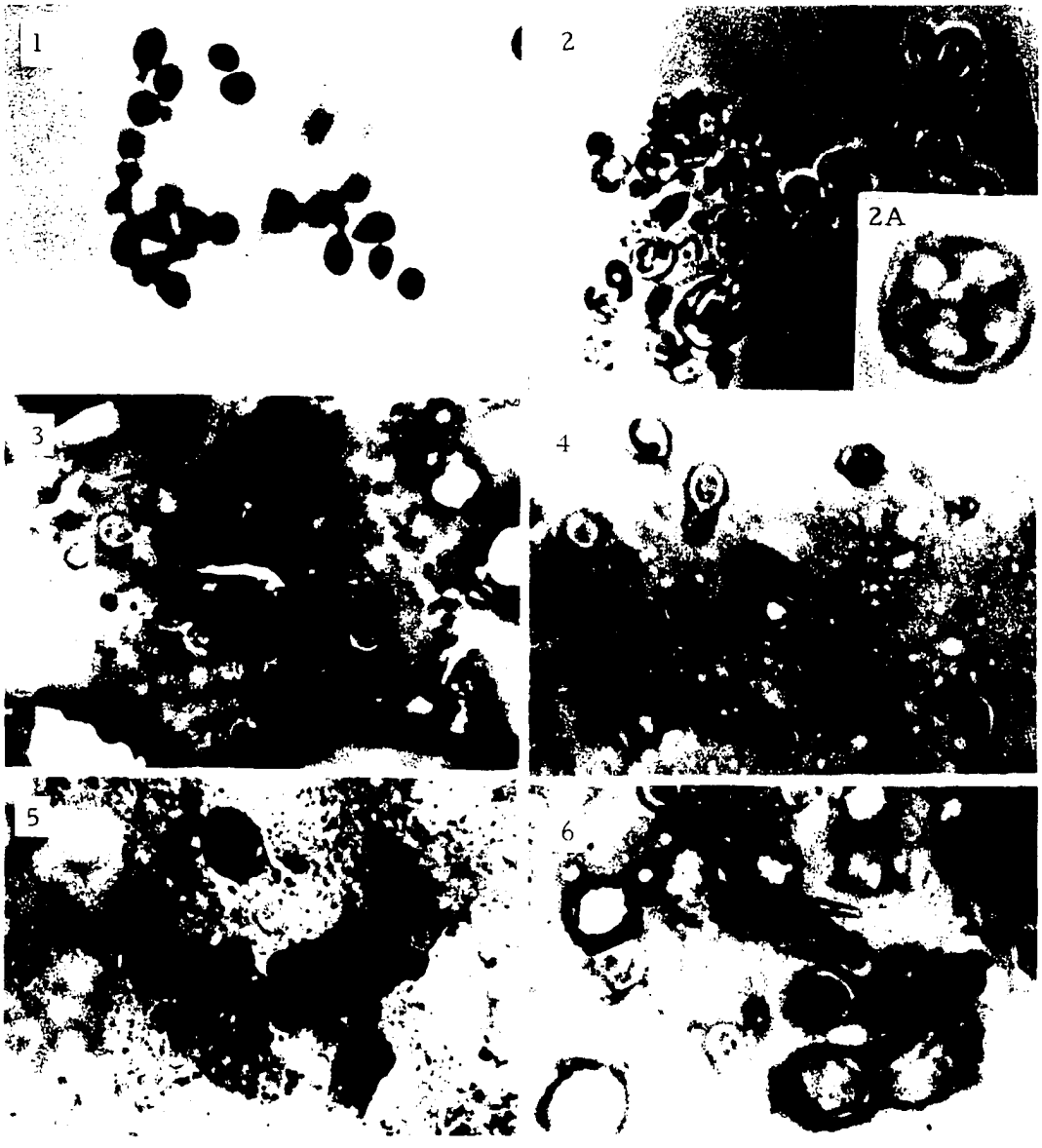


Plate 1 - Histoplasma capsulatum yeast cells grown in yolk sac for different intervals. 1 - In vitro grown 3 day old cells, ether treated (wet mount). 2 - Grown in yolk sac, ether harvested 10 days after inoculation (wet mount). 2A - A single cell from the same preparation, enlarged to show double contoured cell wall. 3 - Infected yolk sac smear, 5 days after inoculation (special crystal violet staining). 4 - Infected yolk sac smear, 10 days after inoculation, cells with prominent halo and thick cell wall (special crystal violet staining). 5 - Infected yolk sac smear 14 days after inoculation (methanamine silver staining). 6 - Infected yolk sac smear, 17 days after inoculation (special crystal violet staining).

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