STUDIES ON THE GROWTH OF HISTOPLASMA CAPSULATUM[1]

I. Growth of the Yeast Phase in Liquid Media

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Received for publication May 24, 1954

Histoplasma capsulatum is a diphasic organism which grows in the yeast phase in the mammalian host or on complex organic media at 37 C and in the mycelial phase at 25 C. For use in investigations of the metabolic activities of the two phases it is desirable to have a chemically defined medium which will support a good rate of growth of either phase and ready conversion of one phase to the other. Several complex media which allow good growth of the yeast phase of *H. capsulatum* have been described (De Monbreun, 1934; Campbell, 1947; Salvin, 1947, 1950; McVickar, 1951; Zarafonetis, (1952); and Rowley and Huber, 1954, *in press*). Of these media only those solid media containing whole blood or albumin support the growth of isolated yeast cells at 37 C. Neither the growth of small inocula of the yeast phase in liquid media nor the factors necessary to obtain the maximum rate of growth in liquid media have been reported.

It is the purpose of this paper to report environmental and nutritional factors which are required for rapid growth of the yeast phase of several strains of Histoplasma following inoculation of the liquid cultures with small inocula.

MATERIALS AND METHODS

Most of the work described was done with one strain, no. 6617, of *H. capsulatum*. The results obtained with this strain were compared with those obtained with the other strains listed in table 1. The mycelial phase was maintained at 25 C on Sabouraud's agar slants and transferred once every four months. Cultures were maintained in the yeast phase on the glucose-blood-cystine agar medium of Rowley and Huber (1954, *in press*) and were transferred twice weekly. Two to four day old cultures of the yeast phase were used for inocula, and the inoculum was prepared according to the procedure of Rowley and Huber (1954, *in press*) with the exception that sterile distilled water was used to wash the cells. The number of cells in a suspension was estimated from its optical density, measured at 660 mμ in a model B Beckman spectrophotometer, by preparing a standard curve in which points were related to actual cell counts made in a Levy hemacytometer chamber. This standard curve, obtained from suspensions of the yeast phase of no. 6617, was also used for the other strains. Unless stated otherwise, an inoculum of 2.5×10^6 cells per ml of medium was used. The inoculum consisted of aggregates averaging 2.5 cells per aggregate, with a range from one to six cells per aggregate.

The basic medium used (table 2) was that of Kline and Barker (1950) with minor modifications. A ten times concentrated trace element solution was used and was prepared by mixing a 250 ml solution of 1 g $FeCl_2 \cdot 4H_2O$ plus 1 ml HCl with a 250 ml solution containing 75 mg each of $MnCl_2 \cdot 4H_2O$ and $NaMoO_4 \cdot 2H_2O$. Otherwise the medium was prepared as described by the above workers. Sterile glucose was added aseptically after sterilization of the other components. It was found initially that growth was equally good after sterilization by autoclaving or by filtration. For sterilization by filtration, the medium was passed through a Morton bacteriological filter apparatus with a fine glass filter or through a Selas no. 03 porcelain filter. Twenty-five ml amounts of the medium were placed in a 125 ml Erlenmeyer flask having an 18 by 150 mm colorimeter tube calibrated for 25 ml as a side arm. Because of the instability of cysteine the flasks were inoculated as soon after preparation of the

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TABLE 1

Strains of Histoplasma capsulatum used

Strain No.	Source of Mycelial Phase	Source Yeast Phase
6566	human	mouse passage
6567	rat	mouse passage
6571	human	mouse passage
6572	cat	mouse passage
6576	dog	conversion on blood medium
6602	soil	mouse passage
6617	skunk	mouse passage
6619	soil	mouse passage
6621	human	conversion on blood medium
6622	cat	mouse passage

TABLE 2

Composition of basic medium

	g per 100 ml		mg per 100 ml
Glucose	1.0	Thiamin·HCl	0.20
KH_2PO_4	0.2	Pyridoxine·HCl	0.10
$(NH_4)_2SO_4$	0.2	Pyridoxamine·2HCl	0.05
$MgCl_2 \cdot 6H_2O$	0.02	Pyridoxal·HCl	0.05
$CaCl_2 \cdot 2H_2O$	0.002	Ca pantothenate	0.20
$FeCl_2 \cdot 4H_2O$	0.004	Riboflavin	0.20
$MnCl_2 \cdot 4H_2O$	0.00015	Nicotinic acid	0.20
$NaMoO_4 \cdot 2H_2O$	0.00015	Nicotinamide	0.10
Cysteine·HCl	0.1	*p*-Aminobenzoic acid	0.10
Casein hydrolyzate*	0.2	Biotin	0.01
		Pteroylglutamic acid	0.005
		Inositol	0.005
		Adenine sulfate	0.1
		Guanine·HCl·$2H_2O$	2.0
		Uracil	2.0
		Xanthine	2.0
		Thymine	2.0

* Nutritional Biochemicals Corporation "vitamin-free" casein hydrolyzate (acid).

medium as possible and were then placed at 37 C on a rotary shaker (New Brunswick Scientific Co.) which was set to operate at 160 oscillations per min. Due to evaporation it was necessary to add sterile distilled water frequently to bring the fluid volume to the 25 ml mark.

Since growth usually became too heavy for direct optical density readings, appropriate dilutions were made, and the growth was recorded in growth units (GU), i.e., OD $\times \frac{1}{\text{dilution}}$. The maximum growth obtained was usually between 7 and 8 GU (estimated 10^8 cells ml). Because the growth of strain 6617 is granular in nature, it was necessary to homogenize the cell aggregates to obtain a relatively uniform suspension for optical density readings. This was done by drawing the suspension into and expelling it 7 to 10 times from a 5 ml serological pipette equipped with a rubber bulb. Determinations of growth as measured by OD were found to parallel total cell volume as measured in a graduated conical centrifuge tube.

Cysteine-cystine was determined by the method of Sullivan and Folk (1952), free —SH compounds by the method of Grunert and Phillips (1951), relative reducing values of the medium by the standard Thunberg technique employing methylene blue (Umbreit *et al.*, 1945), and protein by the biuret procedure of Weichselbaum (1946). Starch (commercial Argo) was made "fat-free" by methanol extraction in a Soxhlet apparatus for 72 hours; 0.85 per cent of the initial dry weight was extracted by this procedure. Fats were removed from crystalline bovine albumin by the acetone extraction procedure of Oyama *et al.* (1953) with the exception that two preliminary extractions were made with 75 per cent methanol. Methanol has been reported to be a more effective solvent for the salts of fatty acids (Schoch and Williams, 1944). In two separate experiments 0.85 and 0.95 per cent of the dry weight of the albumin were obtained as extractable material. The extracts of the above materials gave strong hydroxamic acid tests for free fatty acids and esters (Feigl, 1947) and were completely soluble in ether upon acidification.

The amino acids used were obtained from Nutritional Biochemicals Corp.; crystalline bovine serum albumin and bovine serum albumin, Fraction V, were obtained from Armour Co.

RESULTS

Oxygen requirements. In preliminary experiments growth of *H. capsulatum* was found to occur only in the presence of oxygen. Since growth of the yeast phase under anaerobic conditions has been reported by Salvin (1947), the

oxygen requirements were carefully reinvestigated.

The 10 strains of *Histoplasma* listed in table 1 were tested by inoculating duplicate tubes of Sabouraud's medium with mycelium and spores and incubating at 25 C. After 12 days, when growth was well established, one tube of each pair was made anaerobic with a pyrogallol, K_2CO_3 seal and all tubes were reincubated for 14 days. In all strains, growth continued normally in the tubes which remained aerobic. Growth, as estimated by visual examination, stopped abruptly in all sealed tubes and was not resumed so long as the tubes remained anaerobic.

The oxygen requirement of the yeast phase was tested in paraffin and petrolatum sealed tubes of Salvin's Y. P. medium (Salvin, 1947) and on Rowley's glucose-blood-cystine agar (Rowley and Huber, 1954, *in press*) incubated in evacuated desiccators or in desiccators evacuated and filled 3 times with 100 per cent N_2 or a mixture of 95 per cent N_2 and 5 per cent CO_2. Each desiccator contained a tube of 0.02 per cent cysteine plus dilute methylene blue as an indicator for the presence or absence of oxygen. Additional blood agar tubes were incubated with a pyrogallol, K_2CO_3 seal. All tubes of the Y.P. and blood media were inoculated with one loopful of the total growth from a 4 day old blood agar slant suspended in two ml of water and were incubated for 5 days at 37 C.

When strict anaerobic conditions were maintained, there was no case where growth was apparent by gross observation. Growth did not occur in evacuated desiccators or in desiccators containing nitrogen or nitrogen and carbon dioxide as long as the methylene blue indicator remained reduced. No growth was observed in agar "deeps" sealed with paraffin as long as the seal between the glass and paraffin remained intact. Under aerobic conditions in unsealed tubes containing ten ml of the semisolid Y.P. medium, growth appeared at or very near the surface of the medium whether the inoculations were by stab or by suspension of the inoculum. (In this medium, however, as growth progressed, the cell aggregates settled to the bottom if the gel state of the medium was broken.) In all cases, control tubes incubated in the presence of oxygen exhibited good growth. It was therefore concluded that *H. capsulatum* requires oxygen for growth whether it is in the mycelial or yeast phase. For this reason growth studies were carried out under conditions which allowed the maximum aeration possible.

Size of inoculum. To obtain growth of strain 6617 on the basic medium, large inocula (10^9 to 5×10^5 cells per ml) were required. Growth did not occur when an inoculum of 5×10^4 cells per ml of medium was used and incubated for two weeks. The rate of growth on the basic medium was found to be slow, the generation time being 18 to 24 hours as estimated by increase in optical density. Since observations of the growth rates on blood agar plates showed that the organism had a generation time of approximately 12 hours, attempts were made to increase the rate of growth in liquid media.

Effect of changes in the composition of the basic medium. Increasing the phosphate concentration or casein hydrolyzate concentration from 0.2 to 1.0 per cent did not affect the rate of growth nor did the addition of yeast extract, boiled yeast phase Histoplasma cells, or tryptophan. Deletion of the purine-pyrimidine or trace element supplements had no effect. The concentration of cysteine was not increased since cystine crystallized from the medium. Substitution of the casein hydrolyzate (acid hydrolyzed) by an equal concentration of enzymatically digested casein hydrolyzate (vitamin-free, Nutritional Biochemicals Corp.) completely inhibited growth, as did the addition of 0.1 per cent acid hydrolyzed yeast extract, 10 μg of oleate per ml of medium, or culture filtrate (sterilized by filtration) at a 60 per cent concentration.

When 0.3 per cent glutathione was substituted for 0.1 per cent cysteine in the presence of 0.2 per cent casein hydrolyzate, no significant growth appeared during a 9 day incubation period. Analyses for cysteine-cystine in a medium to which cysteine had been added initially showed that virtually all of the cysteine (0.1 per cent) had disappeared from the medium when growth had reached its maximum.

Substitution of the glucose by fructose, galactose, sucrose, maltose, cellobiose, arabinose, ribose, glycerol, starch, or glycogen showed that growth was most rapid when glucose or glycerol was the substrate, with glucose being the better of the two. Addition (0.1 per cent) of various organic acids (table 3) did not increase the rate of growth. However, fumarate and pyruvate inhibited growth.

TABLE 3

Effect of certain organic acids on the growth of strain 6617*

Acid Added	Growth Units
None	2.25
Citrate	2.00
α-Ketoglutarate	2.35
Succinate	2.25
Malate	1.80
Fumarate	0.20
Pyruvate	0.23
Lactate	2.00
Gluconate	1.40

* All acids added as the sodium salts at 0.1 per cent concentration. Medium was sterilized by autoclaving at 15 lb pressure for 20 minutes. Values given are the averages of duplicate flasks. Flasks were harvested on seventh day before maximum growth was reached.

Investigations of the amino acid requirements of the organism were then undertaken. No growth occurred in the absence of the casein hydrolyzate and cysteine, and growth of strain 6617 in the presence of cysteine alone was extremely meager and slow. The results of the additions of single amino acids to cysteine are given in table 4. Of the combinations tested, cysteine plus glutamic acid gave a growth response equal to that previously obtained with cysteine plus casein hydrolyzate, while the combination of cysteine plus aspartate gave a somewhat lower rate of growth.

As shown by figure 1, the combination of the three amino acids, cysteine, aspartic acid, and glutamic acid, each at a 0.1 per cent concentration, gave a greater rate of growth than that obtained with any two of the amino acids. D-Aspartic acid was found to substitute equally well for the L-aspartic acid. Strains 6571, 6576, 6621, and 6622 responded to these amino acids in a similar manner.

In the presence of glutamic and aspartic acids, the rate of growth of strain 6617 on cysteine was 15 times that obtained with methionine. Furthermore, the addition of 0.1 per cent methionine to a medium containing 0.1 per cent each of cysteine, glutamic, and aspartic acids completely inhibited growth during a 6 day incubation period. No further attempts were made to increase the rate or amount of growth by addition of other amino acids, and all subsequent experiments were carried out in a medium containing 0.1 per cent each of cysteine, glutamic, and aspartic acids.

TABLE 4

*Growth of strain 6617 on various amino acids**

Amino Acid	Growth Units
None	0.00
L-Cysteine	0.71
L-Cysteine + casein hydrolyzate (acid)	5.60
L-Cysteine + L-glutamic acid	5.55
L-Cysteine + glycine	0.96
L-Cysteine + DL-aspartic acid	3.50
L-Cysteine + DL-serine	0.86
L-Cysteine + DL-alanine	0.37
L-Cysteine + L-histidine	0.16
L-Cysteine + DL-isoleucine	0.34
L-Cysteine + L-leucine	0.11
L-Cysteine + L-lycine	0.76
L-Cysteine + DL-phenylalanine	0.00
L-Cysteine + DL-threonine	0.00
L-Cysteine + L-tryptophan	1.05
L-Cysteine + DL-valine	0.20
L-Cysteine + arginine	1.37
L-Cysteine + L-proline	1.38
L-Cysteine + β-alanine	0.98

* All amino acids present at 0.1 per cent concentration; casein hydrolyzate was at 0.4 per cent concentration. Cysteine and leucine added as the hydrochlorides. Incubation time was 7 days.

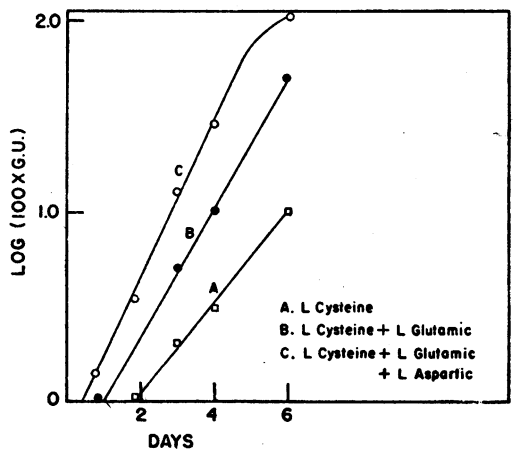

Figure 1. Growth of yeast phase of *Histoplasma capsulatum*, strain 6617, on amino acids. Media were inoculated with 2.5×10^6 cells/ml of 3 day old culture.

Experiments with the 3 amino acid medium. When the concentration of cysteine was decreased from 0.1 per cent to 0.05 per cent, the

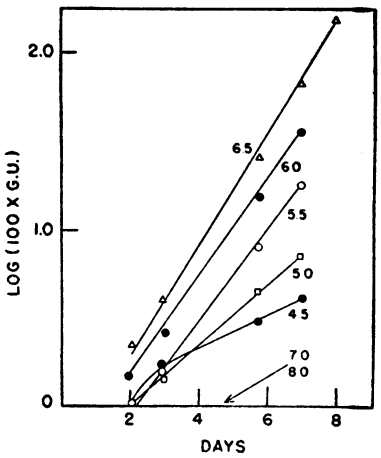

Figure 2. Effect of pH on the growth of the yeast phase of *Histoplasma capsulatum*. Media were inoculated with 10^6 cells/ml of 3 day old culture of strain 6617.

rate of growth and the final amounts of cells formed by strain 6617 remained virtually the same. However, when the initial concentration of cysteine was decreased to 0.025 per cent, no growth occurred. Furthermore, the addition of 0.1 per cent fumarate or pyruvate to the cysteine-glutamic-aspartic acid medium again completely inhibited growth if these additions were autoclaved with the medium. If, however, the fumarate were sterilized and added separately, growth was identical to that obtained in the cysteine-glutamic-aspartic acid medium alone. When pyruvate was added and sterilization was by either autoclaving or filtration, growth was inhibited. Increasing the pH of the medium from 4.5 to 8.0 resulted in increased rates of growth until pH 6.5 was reached (figure 2). Increasing the pH to 7.0 resulted in the complete inhibition of growth; at pH 8.0 crystallization of cystine from the medium occurred.

Since it is known that cysteine is rapidly oxidized to cystine at pH 7.0, and since it also is known that mercaptans react with ketones and compounds having double bonds (Schubert, 1935; Posner, 1907), it appeared that under the above conditions growth was being limited by the depletion of free —SH groups. Experiments were therefore carried out to determine the effect of pyruvate, fumarate, cysteine concentration, and pH on the survival time of the —SH group in the medium or in model systems, under the conditions of aeration used for growing the organism. In all cases the conditions under which no growth occurred corresponded with a rapid depletion of —SH groups or reducing power of the medium.

TABLE 5

*Effect of albumin and sodium oleate on the growth of strain 6617**

Additive	Total Number of Cells Added				
	10^5	10^4	10^3	10^2	10
	Growth units				
Basal medium (cysteine-aspartic-glutamic acids)	0.0	0.0	0.0	0.0	0.0
+ albumin	0.16	0.06	0.03	0.0	0.0
+ extracted albumin	0.04	0.0	0.0	0.0	0.0
+ extracted albumin + extract	0.17	0.08	0.05	0.02	0.0
+ extracted albumin + 0.001 μg sodium oleate/ml	0.03	0.02	0.01	0.0	0.0
+ extracted albumin + 0.01 μg sodium oleate/ml	0.09	0.05	0.02	0.0	0.0
+ extracted albumin + 0.1 μg sodium oleate/ml	0.08	0.05	0.02	0.0	0.0
+ extracted albumin + 1.0 μg sodium oleate/ml	0.17	0.08	0.03	0.02	0.0

* The 3 amino acid medium made to 5/3 concentration, and 5 per cent albumin added to give a final concentration of 0.5 per cent. Albumin extract was added as an ether solution to give a final concentration equivalent to 0.5 per cent albumin. The ether was volatilized off by warming. The final media were sterilized by filtration and were tubed (4 ml) in 20 by 150 mm cotton stoppered test tubes at 5/4 concentration. Tubes were inoculated with one ml of washed cells of a 2 day old culture and incubated at 37 C for 14 days. Growth units were determined by combining duplicate tubes, adjusting the volume to 10 ml, homogenizing the aggregated cells, and reading the OD at 660 mμ. Values given are corrected for uninoculated controls.

Requirements for growth of small inocula. During the progress of this work, separate investigations regarding the growth of the yeast phase of *H. capsulatum* on solid media drew our attention to the beneficial effect of crystalline bovine albumin. Albumin not only stimulated the rate of growth of various strains but allowed growth of small inocula. In shake flasks of the cysteine-glutamic-aspartic acid medium, the presence of 0.5 per cent albumin supported the growth of 4×10^4 cells per ml which represents a 12-fold decrease in the

TABLE 6

The effect of various additions to the cysteine-aspartic-glutamic acids medium on the growth of small inocula

Additions*	Strain								
	6571		6576		6617		6621		6622
	Size of inoculum (cells)								
	10^4	10^2	10^4	10^2	10^4	10^2	10^4	10^2	10^4
Control (no addition)	—	—	—	—	—	—	—	—	—
Albumin (crystalline bovine, Armour)	—	—	—	—	—	—	—,— 2,—	—	—
Extracted albumin (crystalline bovine, Armour)	—	—	—	—	—	—	—	—	—
Albumin (Fraction V, bovine serum, Armour)	—	—	4,4 5,5	1,— —,—	1,2 3,2	—	3,3 3,3	1,— —,—	—
Gelatin	—	—	—	—	—	—	—	—	—
Starch (Argo)	3,4 3,4	—	1,2 4,4	—	2,2 3,4	—	4,4 4,4	—	—
Extracted starch (Argo)	—	—	—	—	1,— 2,2	—	1,1 1,2	—	—
Versene	—	—	—	—	—	—	—	—	—

* Relative growth given in values from 1 to 5; — signifies no growth. All additions of albumin and gelatin were added to give a final concentration of 0.5 per cent, additions of versene and starch to give a final concentration of 0.05 per cent. Four ml media (5/4 strength) were contained in 20 by 150 mm pyrex test tubes covered with aluminum caps. Tubes were inoculated with 1 ml of washed cells of a two day old culture and incubated for 17 days at 37 C.

size of inoculum usually required. Since one function of albumin could be as a carrier of oleic acid (Davis and Dubos, 1947), experiments were carried out to determine if oleic acid was required for growth of small inocula of various strains of the yeast phase. Of the five strains tested initially, only strain 6617 showed a requirement for oleic acid or the extract of albumin (table 5). Three strains showed a requirement for the presence of albumin and growth was obtained after 14 days with a 1,000 cell inoculum; the extracted albumin in these cases was as effective as the unextracted albumin. Strain 6622 showed no growth with a 10^5 cell inoculum and presumably has additional growth requirements. The slow rates of growth obtained under the conditions of the experiment may be attributed to the decreased amount of oxygen supplied in the stagnant culture tubes.

These results would indicate that the requirement for albumin by strains 6571, 6576, and 6621 either is not related to an oleic acid requirement by these strains or that contamination of the medium with fatty material, presumably from the cotton stoppers, has occurred. To test the latter possibility, simultaneous experiments were done in carefully washed and rinsed test tubes having aluminum caps, and the results were compared with those obtained with the cotton stoppered, hot air sterilized test tubes. It was found that not only was contamination from the cotton sufficient to stimulate growth in the presence of extracted albumin, but in some cases it was at such a concentration that growth was inhibited.

To obtain further information regarding the function of albumin, substitutions were made for the crystalline bovine albumin using substances which could function in the several ways reported for albumin. Extracted crystalline albumin, bovine serum albumin (Fraction V), starch, and methanol extracted starch were used as sources of and as binding agents of fatty acids. Versene was used as a chelating agent, and gelatin was used as a possible source of stimulating peptides or amino acids. The results are given in table 6. No growth occurred in the media having gelatin, versene, crystalline bovine albumin, or extracted crystalline bovine albumin. Excellent growth with a 10^4 cell inoculum occurred in the media having either bovine albumin (Fraction V) or starch; less growth occurred in the medium if

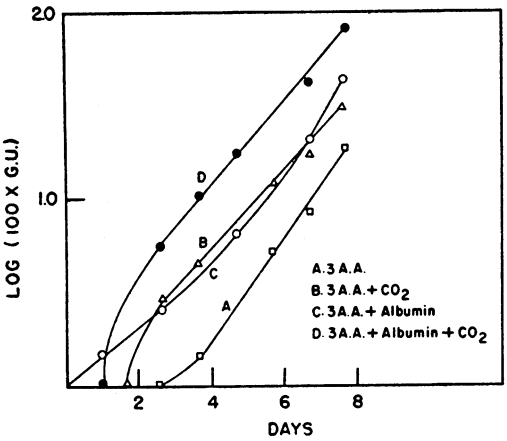

Figure 3. Effect of carbon dioxide on the growth of the yeast phase of *Histoplasma capsulatum*, strain 6617. Media were inoculated with 10^6 cells/ml of a 4 day old culture.

the extracted starch rather than the unextracted starch was added.

It was shown in separate experiments that, as measured by the biuret reaction, albumin rapidly disappears from the medium during the growth of the yeast phase of strain 6617. Simultaneously, determinations for free —SH groups showed that the presence of albumin did not prolong the existence of these groups in the medium.

Requirement for carbon dioxide. It has been demonstrated for other organisms that a requirement for carbon dioxide may be satisfied by using large inocula (Rahn, 1941). Although the requirement for a high inoculum to obtain growth of *H. capsulatum* could be explained on the basis of a requirement for fatty acids, a requirement for carbon dioxide was also a possibility. Such a requirement was shown in the following manner. Four shake flasks were prepared with a large glass bulb attached to the side in addition to the colorimeter tube. Five-tenths of a ml of 20 per cent KOH was added to each of the bulbs of two flasks, and sterile cotton stoppers were inserted at the mouths of the bulbs to prevent contamination of the medium with the alkali during shaking. Two of the flasks contained no alkali in the side bulb. The flasks were sealed with glass stoppers after inoculation and incubated in the usual manner. When 2.5×10^6 cells per ml were transferred to the flasks containing the cysteine-glutamic-aspartic acid medium, growth occurred during a 7 day incubation period only in the flasks having no alkali in the side arm. In a second series of experiments, the pH of the cysteine-glutamic-aspartic acid medium was adjusted to pH 5.0, and 1 ml of 10 per cent $KHCO_3$ was placed in the side bulb of the flasks. After inoculation of the flasks with 10^6 cells per ml, the flasks were stoppered and the bicarbonate solution was tipped in, giving a final pH of 6.5. The stimulating effect of such an increased carbon dioxide pressure within the flask in the presence and absence of albumin is shown in figure 3.

DISCUSSION

The results of the experiments on the growth of the yeast phase of *H. capsulatum*, strain 6617, have shown it to be a strict aerobe. The fact that in a synthetic medium containing cysteine, glutamic acid, and aspartic acid, no growth is obtained with an inoculum of 2.5×10^6 cells per ml if the pH is greater than 6.5 or if substances, such as pyruvate or fumarate, which are known to react with —SH compounds (Schubert, 1935, 1936; Posner, 1907) are present in the medium indicates that —SH must be present to initiate growth. That the requirement for cysteine was not a requirement for —SH groups alone was suggested by the fact that it could not be replaced by glutathione. Under the conditions of growth (i.e., strong aeration) no significant amount of —SH groups could be demonstrated in the medium 4 to 6 hours after inoculation. It therefore appears probable that the requirement for the —SH group is to maintain the viability of the organism until growth commences. Since growth from the inoculum used was seldom measurable prior to 48 hours' incubation, it is assumed that the rate of growth in a medium to which cysteine has been added is a response to cystine rather than cysteine. That cysteine or cystine is a required amino acid is suggested by the fact that an insignificant amount of growth occurred when glutathione was substituted for cysteine in the casein hydrolyzate medium. Salvin (1949) has reported requirements for sulfur containing compounds under different conditions. Under his conditions organic sulfides and mercaptans were equally effective in maintaining the growth of the yeast. The requirement for cysteine by the yeast phase of *Candida albicans* has been reported by Nickerson and Mankowski (1953).

Although sparse growth occurs in the presence

of cysteine alone, or aspartate plus glutamate, the high rate of growth is apparently not obtained unless all three amino acids are present. This has been found to be true for five strains tested.

The requirement of various organisms for oleic acid has been reported by several workers (Broquist and Snell, 1951; Oyama *et al.*, 1953; Davis and Dubos, 1947). A similar requirement apparently exists in the yeast phase of Histoplasma, and the effect of added albumin, as a carrier of fatty acids, may be explained on this basis. Contamination of test tubes by fatty material from cotton stoppers has been reported by Pollack (1948), Wright (1934), and Drea (1942). However, removal of this source of fatty acids and using "fat-free" albumin have on several occasions resulted in good growth of small inocula of the yeast phase. In such cases the origin of the fatty acid necessary to initiate growth may be from the inoculum itself, the function of the albumin being to regulate concentrations of the fatty acid. Of the five strains tested, all are completely inhibited by concentrations of oleic acid in the range of 1 to 10 μg per ml.

Albumin could possibly function in other ways since it is known to have metal binding properties (Tanford, 1952), to react with mercaptans (Huggins *et al.*, 1950), or to serve as source of —SH groups (Huggins *et al.*, 1951; Weissman *et al.*, 1950). That the primary function of albumin to promote growth of the yeast phase of Histoplasma is to supply and bind excess fatty acids is suggested by the following results. Starch has been shown to substitute for albumin, and the fatty acid binding nature of starch is known (Schoch and Williams, 1944; Ley and Mueller, 1946). Extraction of the starch with hot methanol decreased its growth promoting properties. Albumin was shown to disappear from the medium during the growth of the yeast. However, the direct determination of the free —SH groups in the medium indicated that the albumin did not increase the free —SH groups significantly or preserve those added as cysteine. Actually a more rapid disappearance of —SH groups was observed in the presence of albumin. The increased rate of growth obtained in the presence of albumin may be due to stimulatory peptides formed from it or unknown contaminants.

Since versene may be inhibitory in the concentration used, no conclusion may be reached regarding the possible chelating action of albumin in promoting the growth of the yeast. From the existing evidence, it appears that such a function of albumin for promoting the growth of Histoplasma is unlikely. The use of albumin to remove factors in peptone toxic to *H. capsulatum* has been reported by McVickar (1951). Zarafonetis (1952) has reported the use of albumin in liquid media to maintain yeast phase growth.

The general requirement for a large inocula to obtain growth of the yeast phase may be due in part to the filling of fatty acid requirement or to the possible protective action by dead cells against an excess of fatty acids. Direct evidence for such an activity of cells of mycobacteria has been reported by Klip *et al.* (1952). In addition, results of the studies with *H. capsulatum* suggest that large inocula may function to supply carbon dioxide which is required under conditions in which fatty acids are available. The relative differences in the rates of growth of the strains tested and the sizes of the inocula required to initiate growth indicate that certain strains have growth requirements which are not completely satisfied by the medium described.

SUMMARY

Growth of the yeast phase of *Histoplasma capsulatum* has been studied in liquid media. The organism gave the maximum rate of growth obtained in a medium containing glucose, cysteine, aspartic acid, and glutamic acid. In addition, oxygen, carbon dioxide, oleic acid, and apparently —SH groups are required for growth. Growth can be inhibited by the concentrations of fatty acids derived from cotton stoppers, and this toxicity can be reversed by additions of starch or albumin.

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