STUDIES ON THE GROWTH OF *HISTOPLASMA CAPSULATUM*[1]

II. Growth of the Yeast Phase on Agar Media

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Individual cells of the yeast phase of *Histoplasma capsulatum* grow poorly or not at all on most culture media, although a suitable medium supports good growth of a heavy inoculum. The initial paper in this series describes a liquid medium in which 100 cells of the yeast phase, after settling to the bottom of the tube, grow under conditions of stagnant incubation at 37 C (Pine, 1954). When 1.5 per cent agar is added to this medium and the inoculum is spread over approximately 20 sq cm of agar surface 10^7 to 10^8 cells are required to obtain a significant number of colonies.

Some of the factors which may be responsible for the failure of an agar medium to support growth of small inocula as yeast have been investigated and are reported here.

MATERIALS AND METHODS

Strain and culture techniques. The strain (no. 6,617) of *H. capsulatum* used, the preparation of the inoculum, the procedure for determining the growth responses in liquid media and the procedure for determining the concentration of cells in heavy suspensions have been described (Pine, 1954). In experiments using small inocula the inoculum was measured by counting cells in a Levy hemacytometer and response to added growth factors was measured by counting colonies arising from the inoculum spread on the agar surface. In experiments using large inocula the number of cells in the inoculum and the growth response were estimated by measuring light absorption of a suspension of cells with a model B Beckman spectrophotometer (Pine, 1954).

Yeast phase of the fungus was grown at 37 C in 25- or 50-ml Erlenmeyer flasks having 5 and 10 ml of medium, respectively. In preparing suspensions for growth measurements the following simple procedure was devised. To each flask, 2 ml of water and a short length (approximately 1 cm) of 17-gauge soft iron wire were added with the usual bacteriological precautions. The flask was then placed on a magnetic stirrer until, by manipulation of the flask, the rotating wire loosened and homogenized the surface growth. This usually required 15 to 30 seconds. This method of removing cells from an agar surface is rapid, it eliminates some of the hazards of conventional methods, a 1.5 per cent agar surface remains unbroken and a uniform suspension is obtained. Growth response was estimated by diluting an aliquot of the suspension and recording growth units, i.e., optical density $\times$ 1/dilution. The sample must be taken promptly because added water is absorbed rapidly by an agar medium which has been incubated at 37 C.

Media. The 3-amino acid (cysteine-glutamic-aspartic) medium containing starch or albumin previously described (Pine, 1954) was used in the preparation of agar media. Both starch and agar were purified by methanol extraction for 24 to 72 hours in a Soxhlet apparatus. Total extract from a weighed amount of each material was concentrated to 4 or 5 ml on a steam bath and was quantitatively transferred to a 12-ml graduated centrifuge tube. A sample was taken, evaporated to dryness on a weighed aluminum planchet under an infra-red lamp, and weighed. This material was soluble in ether upon acidification, gave a strong hydroxamic acid test (Feigl, 1947) for fats and fatty acids and precipitated from ether solution upon neutralization. Agar, starch, and crystalline bovine al bumin contained 1.60, 0.85, and 0.90 per cent, respectively, of methanol extractable material. When these extracts were used in a medium they were added in ether solution in amounts equivalent to those present in the crude bio-

[1] From the U. S. Department of Health, Education, and Welfare, Public Health Service, National Institutes of Health, National Microbiological Institute, Bethesda, Md.

logical materials which would have been used in the medium. The ether was then driven off by heating. In general, the liquid medium was prepared double strength and an equal volume of agar solution was added. When the 3-amino acid medium was prepared with 1.5 per cent agar, flasks were inoculated within 3 hours after preparation. When a semi-solid medium (0.3 to 0.5 per cent agar) was prepared, it was allowed to solidify overnight at room temperature before inoculation.

Whole blood was obtained by heart puncture of adult rabbits, citrated, and stored at 5 C until used (maximum of 24 hours). Fractionation of the blood was done in a cold room at 5 C. The formed elements were separated from the plasma by centrifugation in graduated centrifuge tubes. The cells were then washed three times with two volumes of 0.85 per cent NaCl and resuspended in physiological saline to give a final volume equal to that of the initial whole citrated blood. Part of the first washing of the blood cells was added to the plasma fraction to make the plasma fraction equal in volume to that of the original whole blood. Red cells were lysed by resuspending in water rather than saline. Equal volumes of these preparations of red cells, lysed red cells, plasma, and whole blood were then used to compare their biological activity in stimulating the growth of *H. capsulatum*. Serum was obtained by allowing the blood to clot and was added directly without dilution. Rabbit albumin was isolated from the plasma fraction (undiluted) by the procedure of Pillemer and Hutchinson (1945). On the basis of the results obtained by Rhiel (1943) it was assumed that the rabbit albumin constituted approximately 6.85 per cent of the plasma. For use, the isolated albumin was dissolved in saline and sterilized by filtering through a Selas 03 filter.

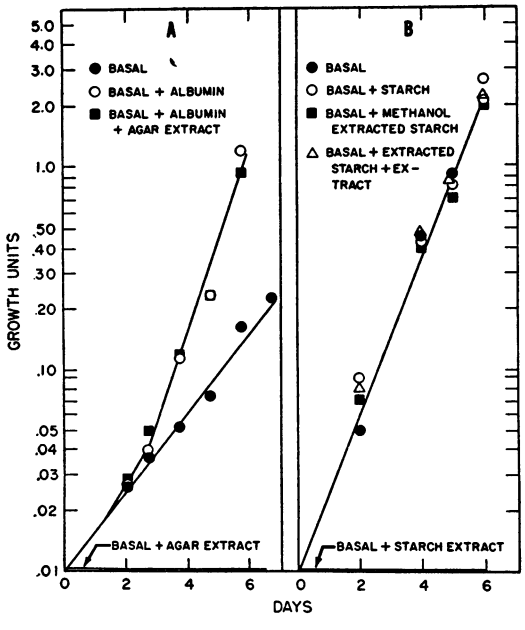

Figure 1. The effect of methanol extracts on the growth of the yeast phase of *Histoplasma capsulatum* in the cysteine-glutamic-aspartic liquid medium in the presence of albumin or starch. 6.25×10^7 cells of a 4-day-old culture of 6617 was the inoculum for 25 ml of medium. Cultures were incubated at 37 C on a rotary shaker (New Brunswick Co.) operating at 160 cycles per min. A: 24-hour methanol extract equivalent to 0.5 per cent agar was tested in the presence of 1.0 per cent crystalline bovine albumin. B: 0.05 per cent equivalent of a 72-hour methanol extract of Argo starch was tested in the presence of 0.05 per cent starch.

Throughout this work the blood-glucose-cystine agar medium, pH 7.3, described by Rowley *et al.* (1954) was used to determine the viability of the yeast inoculum and as a standard for evaluating other media, since it has consistently given the maximum rate of growth and the highest colony counts when inoculated with 100 cells.

RESULTS

The addition of many organic substances failed to stimulate growth of scattered cells on the surface of an agar medium. This suggested that the agar contained a toxic factor. Methanol extraction of agar yielded a substance having the characteristics of a fatty acid (see Methods). This extract inhibited growth of Histoplasma (figure 1A), but 1.5 per cent agar, even after extraction, did not support growth of small inocula. However, when the agar content was reduced to 0.3 to 0.5 per cent, and 0.07 per cent starch or 0.5 per cent crystalline bovine albumin was present, numerous colonies appeared from an inoculum of 100 to 1,000 cells. The use of extracted or unextracted starch or agar or any combination of these did not affect the number of colonies obtained from a small inoculum as long as 0.5 per cent agar was used.

No growth was obtained in the presence of 0.5 per cent agar when starch or albumin was omitted.

When the concentration of the extracted or unextracted agar was increased to 1.5 per cent and the amount of starch or albumin increased proportionally, an inoculum of 10^5 cells was needed to obtain a significant number of colonies. Direct comparison of the total colony counts or of growth rate in the presence of varying concentrations of starch, crystalline bovine albumin, and bovine albumin, Fraction V, showed that the optimal concentration of these substances in the 3-amino acid medium having 1.5 per cent agar was 0.2 to 0.4, 3.0, and 1.5 per cent, respectively. The corresponding figures for the semisolid media were 0.07, 1.0, and 0.5 to 0.7 per cent, respectively.

The results of experiments obtained in liquid culture (figure 1) suggested that one function of the starch or albumin in an agar medium is to detoxify fatty acids present in the agar. When a methanol extract of starch or agar was added to the liquid 3-amino acid medium, growth was completely inhibited. The addition of starch or albumin with such extracts reversed the inhibition. Albumin appeared to have an additional stimulating effect (figure 1A) and therefore it was preferentially used to detoxify fatty acids in solid and semisolid media.

The effect of pH on the growth of a 1,000-cell inoculum in the semisolid medium containing bovine albumin, Fraction V, is given in table 1. At pH 4.5 the albumin coagulated. The resultant opacity of the medium and the large number of yeast colonies obtained in a small area made the colony counts at this pH inaccurate. Since the albumin very often coagulated at pH 5.0, the medium was adjusted to pH 5.2 or 5.3 in further experiments. Table 2 shows the colony counts obtained when certain organic additions were made to the medium at pH 5.2. The addition of glutathione, beef extract, hemin, and fumarate all stimulated the growth of inoculum to the point where the total number of colonies obtained was equal to that obtained with the blood medium. Furthermore, the rate of growth obtained when glutathione or beef extract was added was equal to that obtained on the whole blood medium.

TABLE 1

Effect of pH on the growth of a 1000-cell inoculum on the cysteine-glutamic-aspartic acid medium containing 0.5 per cent methanol extracted agar and 0.5 per cent crystalline bovine albumin

pH	Colony County per Flask	Average
4.5	250, 180, 156, 150, 160, 180, 150, 150	170
5.0	115, 96, 105, 147, 105, 100	111
5.5	29, 46, 35, 43, 47, 33, 82, 75	40
6.0	2, 20, 39, 49, 38, 40	33
6.5	1, 0, 10, 0, 0, 0, 0	2
Blood agar*	205, 200, 205, 175, 100, 200, 154, 125, 150	168

* Medium of Rowley, Haberman, and Emmons (1954).

TABLE 2

*Effect of various organic additions on the growth of small inocula of Histoplasma capsulatum on the cysteine-glutamic-aspartic acid medium containing 0.5 per cent bovine serum albumin, Fraction V, and 0.5 per cent agar, pH 5.2**

	Size of Inoculum		
	1000 cells	500 cells	100 cells
	Colonies†		
Control media			
Casamino acid medium, 25 C‡	>200	135	36
Blood agar	>200	25	8
Additions to basal medium			
Carbon dioxide§	0	0	0
Glutathione	>200	78	13
Liver extract	19	2	0
Beef extract	>200	76	3
Autolyzed yeast	2	0	0
Hemin	137	45	5
Egg yolk	7	0	0
Acid hydrolyzed yeast extract	31	3	0
Potassium fumarate	>200	55	2
Sodium pyruvate	4	1	0

* All additions were tested at a concentration of 0.1 per cent and at 37 C unless designated otherwise.

† Average of 10 plates.

‡ This medium at 25 C has been shown to give a reliable count of viable yeast cells which grow as mycelia (Rowley and Huber, 1955).

§ A small pellet of dry ice was added to the desiccator containing the flasks.

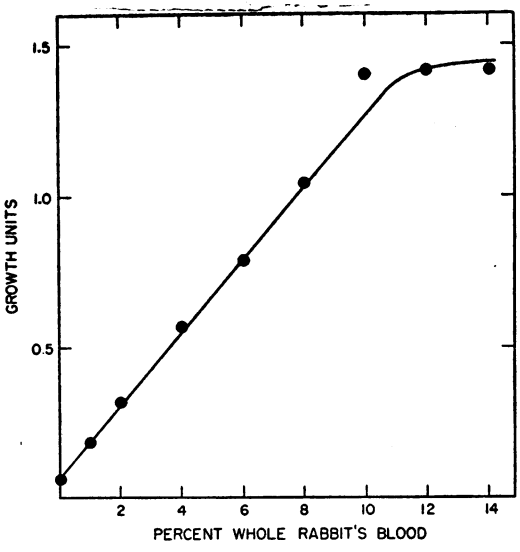

Figure 2. The effect of whole rabbit blood on the growth of the yeast phase of *Histoplasma capsulatum*. The basal medium was the cysteine-glutamic-aspartic acid medium containing 1.5 per cent agar. 25-ml Erlenmeyer flasks containing 5 ml of medium were inoculated with 10^5 cells of a 4-day-old culture of strain 6,617, and incubated 5 days at 37 C.

As will be discussed later, this demonstration of additional growth requirements suggested that the —SH form of a growth factor such as coenzyme A was required. Therefore, a crude preparation of coenzyme A[2] and beef extract were compared for their effect on growth of a 1000-cell inoculum in the 3-amino acid medium containing 1.5 per cent agar and bovine serum albumin, Fraction V. When 0.5 per cent beef extract was added the colonies appeared approximately two days later than those on the blood medium but gave comparable colony counts. The addition of 0.1 per cent beef extract or 5 Lipmann units/ml of the coenzyme A were less effective, since the number of colonies which appeared was less than that on the blood medium and the colonies appeared approximately 3 to 4 days later. However, the 0.1 per cent beef extract medium and the coenzyme A medium were equal in their ability to promote growth of the inoculum. When neither the beef extract or crude coenzyme A preparation was added, the number of colonies which appeared were few and were visible 7 days later than those on blood.

[2] The author is indebted to Dr. Simon Black for this preparation which was 20 per cent pure.

Fractionation and testing of blood. Throughout this investigation, whole rabbit blood added to the synthetic medium or in the medium of Rowley *et al.* (1954) supported the maximum rate of growth observed and promoted the growth of small inocula of the yeast phase in a medium containing 1.5 per cent agar. The following experiments were done to obtain information regarding the growth stimulating factors in whole blood.

The addition of whole citrated rabbit blood to the 3-amino acid medium containing 1.5 per cent agar resulted in a rate of growth proportional to the amount of blood added until a

TABLE 3

*Effect of blood fractions or treatments of blood fractions on the growth of the yeast phase of Histoplasma capsulatum on solid media**

Experiment	Fraction	Per Cent Activity
1	10 per cent whole blood	100
	Serum	28
	Whole blood, heated 15 min at 100 C	0
	Whole blood, heated 60 min at 60 C	16
2	Plasma	33
	Formed elements	51
	Plasma + 2 per cent formed elements	54
	Plasma + 6 per cent formed elements	92
	Plasma + 10 per cent formed elements	90
	Plasma + 2 per cent formed elements, lysed	16
	Plasma + 6 per cent formed elements, lysed	16
	Plasma + 10 per cent formed elements, lysed	24
3	Formed elements	52
	Plasma	40
	Plasma + formed elements	93

* All fractions added to the cysteine-glutamic-aspartic acid medium, pH 7.3, containing 1.5 per cent unextracted agar in volumes equivalent to 10 per cent whole blood unless designated otherwise. 25-ml flasks containing 5 ml of medium were inoculated with 10^5 cells of a 3-day-old culture of the yeast phase of strain 6617 and incubated 5 days at 37 C.

concentration greater than 10 per cent was reached (figure 2). Further addition of blood showed no significant increase in the rate of growth. The effects of various treatments of the blood or blood fractions are given in table 3. Heating the blood greatly decreased its growth-stimulating properties. Although the plasma fraction contained considerable activity, a greater amount of stimulation was obtained from the formed elements. Recombination of the two fractions had an additive effect and the recombined blood had approximately 90 per cent of the activity of whole citrated blood. That the activity of the formed elements is due primarily to the presence of the intact red cell is suggested by the fact that use of lysed red cells resulted in reduction of growth to the level obtained with the plasma fraction alone. Figure 3 indicates that the activity of the plasma fraction is due to the albumin or some contaminant associated with it, inasmuch as the addition of isolated rabbit albumin gave approximately 90 per cent of the stimulation obtained with the entire plasma. Addition of 0.5 per cent crystalline bovine albumin caused an immediate lysis of the rabbit red cells and a definite inhibition of growth was observed. The lytic effect could be due to the toxic effect of the fatty acids present in the crystalline bovine albumin (Davis and Dubos, 1947). In this experiment the total amount of growth obtained with the individual addition of 10 per cent equivalents of plasma, 0.5 per cent rabbit albumin, or 0.5 per cent crystalline bovine albumin in the absence of the cellular components was 0.42, 0.22, and 0.05 growth units, respectively.

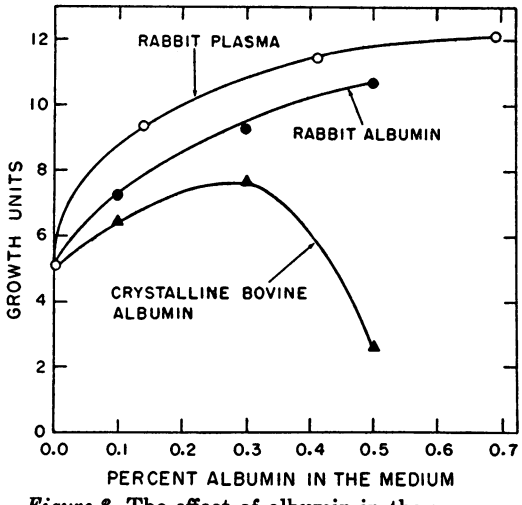

Figure 3. The effect of albumin in the presence of red cells on the growth of the yeast phase of *Histoplasma capsulatum*. The basal medium was the cysteine-glutamic-aspartic acid medium containing 1.5 per cent agar and 10 per cent whole blood equivalents of washed formed elements. Based on the results of Rhiel (1943) it is assumed that the rabbit plasma contains 6.85 per cent albumin. 25-ml Erlenmeyer flasks containing 5 ml of medium were inoculated with 10^5 cells of a 4-day-old culture of strain 6,617, and incubated 5 days at 37 C.

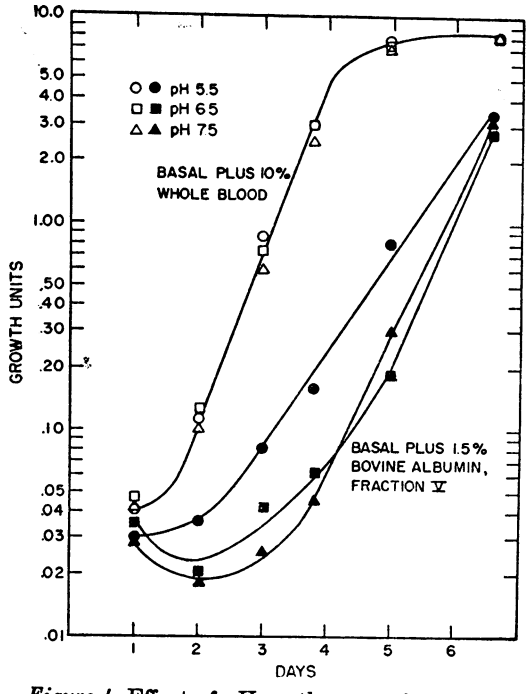

Figure 4. Effect of pH on the rate of growth on agar media in the presence and absence of whole blood. The basal medium was the cysteine-glutamic-aspartic acid medium containing 1.5 per cent agar. 10 per cent whole rabbit blood was used. In the absence of whole blood, 1.5 per cent bovine serum albumin, Fraction V, was added. 50-ml Erlenmeyer flasks containing 10 ml of medium were inoculated with 10^5 cells of a 4-day-old culture of strain 6,617 and incubated at 37 C. Points represent the average of 3 flasks, harvested at each time.

It was further noted that, in the presence of whole blood, the rate of growth of *H. capsulatum* was constant from pH 5.5 to pH 7.5. In the absence of whole blood, but in the presence of bovine albumin, the rate of growth increased with an increase of pH to the point where the

rate at pH 7.5 appeared to equal that obtained with the whole blood medium except that a 4-day lag period occurred (figure 4). These results are in accord with the results obtained in liquid media (Pine, 1954). However, on the solid 3-amino acid medium containing bovine albumin, the lag period is definitely shorter at pH 5.5 than that obtained at pH 7.5 (figure 4). This might be expected from the effects, reported above, of low pH on the growth of small inocula. It would therefore appear that the pH independence of this strain in a whole blood medium is due to the presence of the whole red cell since the cysteine-glutamic-aspartic acid medium contained added albumin.

DISCUSSION

Evidence has been presented which suggests that the fatty acids present in agar inhibit the growth of a small inoculum of *H. capsulatum*. The toxicity of fatty acids for *H. capsulatum* has been reported (Pine, 1954). Ley and Mueller (1946) reported the isolation from agar of fatty acid inhibitors of *Neisseria gonorrheae* and, with use of methanol-extracted agar, growth of the organism occurred in their medium. Our extracted agar at a level of 0.5 per cent did not support growth of 10^2 to 10^3 cells unless starch or albumin were added. The albumin or starch presumably functions to detoxify and to supply required fatty acids (Pine, 1954). McVickar (1951) has also shown that albumin detoxifies substances in peptone media used for growing the yeast phase of *H. capsulatum*. When the concentration of agar was increased to 1.5 per cent, the addition of starch or albumin was no longer sufficient to permit growth and other organic additions were required. A simple explanation for the inhibitory effect of solid agar is not readily apparent. Salvin (1947) has also reported the beneficial effect of a semisolid agar medium.

The general requirement of *H. capsulatum* for —SH groups has been suggested previously (Salvin, 1949; Pine, 1954). Much of the data might be interpreted on the basis of such a requirement. For example:

(a) Better growth of small inocula occurs at a low pH. At a low pH the oxidation of cysteine is minimized.

(b) The rate of growth is stimulated by the whole red cell and not by a red cell lysate or heated whole blood. Evidence supporting a hypothesis that the "living" red cell can supply a constant source of —SH groups may be obtained from the papers of Meldrum (1932), Meldrum and Tarr (1935) and Francoeur and Denstedt (1954).

(c) Growth and growth rate of the yeast phase are not affected by a change of pH from 5.5 to 7.5 in the presence of the red cell. In the absence of the red cell, the initial growth of the yeast phase is inhibited at pH 7.5, and the rate of growth is decreased at pH 5.5. Presumably, then, the red cell can maintain a sufficient —SH concentration at either pH.

(d) The addition of glutathione, beef extract, or a preparation of coenzyme A stimulates growth of small inocula in the presence of added cysteine. These additions may serve to either maintain or supply —SH forms of growth factors. Brown and Snell (1954) have presented evidence that required growth factors for the strict aerobic *Acetobacter suboxidans* are the —SH form of coenzyme A or its precursors, not the corresponding disulfides.

The presence of —SH groups cannot be shown in liquid shake cultures after several hours' incubation or in liquid stagnant deeps after 2 days. It would appear that the presence of —SH is required merely to initiate growth or that extremely small amounts are needed continuously. It is also possible that, once the organism is actively growing, it is capable of reducing the disulfide to —SH and could maintain the concentration of the sulfhydryl group in its immediate vicinity. Therefore, growth of a small inoculum in a stagnant liquid culture may be stimulated by environmental conditions at the bottom of the tube where the total inoculum is aggregated or by growth factors carried over in the cells of the inoculum. When the same inoculum is spread on an agar surface not only are the cells exposed to toxic substances in the agar, but the mutual protection or stimulation of adjacent cells is lost. In addition, the use of the solid medium creates the problem of supplying and maintaining the required sulfhydryl group on the agar surface for periods sufficiently long to initiate growth.

SUMMARY

Evidence is presented which suggests that:

(1) Agar contains fatty acids which inhibit

growth of the yeast phase of *Histoplasma capsulatum*.

(2) The use of solid media creates a technical problem of supplying and maintaining —SH groups for periods sufficiently long to initiate the growth of the organism under aerobic conditions.

(3) Unknown growth factors are required to promote the growth of a small number of cells when they are separated on the surface of the agar-containing medium.

(4) The results of experiments with blood and blood fractions show that the stimulating effect of whole blood is due to the presence of the whole red cell and the albumin fraction. It is considered possible that the red blood cell functions to maintain a constant source of —SH groups required for the growth of the organism. Secondly, the presence of albumin of the whole blood presumably supplies and maintains a nontoxic source of fatty acids.

(5) A simple, safe, and rapid procedure for the removal of the growth of certain pathogenic microorganisms from the surface of agar media is described.

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