SOME FACTORS WHICH AFFECT THE INITIATION OF GROWTH OF *CRYPTOCOCCUS NEOFORMANS*

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ABSTRACT

HOWARD, DEXTER H. (University of California, Los Angeles). Some factors which affect the initiation of growth of *Cryptococcus neoformans*. J. Bacteriol. **82**:430–435. 1961.—The observation that a strain of *Cryptococcus neoformans* failed to grow in a medium containing normal human serum led to an investigation of some of the physiological factors controlling initiation of growth by this fungus.

The data presented show that the growth of *C. neoformans* is markedly inhibited in media with initial pH values slightly above neutrality. The growth of four strains of the fungus was completely suppressed in peptone broth at pH values above 8. The growth of two strains was partially inhibited at a pH of 7.3. Two other strains grew well at 7.3, but were partially inhibited at pH 7.5. The growth in media at pH values which partially inhibited multiplication was granular in appearance. Microscopically the granules were composed of clumps of yeast cells. The possibility is suggested that pH may exert a portion of its effect on the growth of *C. neoformans* by influencing those factors which affect the stability of the cells in suspension.

At a temperature of 25 C, growth of the fungus was initiated in media with a pH which suppressed growth at a temperature of 37 C. Under the experimental conditions employed in these studies, proteose peptone no. 3 (Difco) serves as both a carbon and a nitrogen source. However, glucose, which is assimilated by *C. neoformans* in contrast to lactose, permitted the growth of the fungus at pH values which suppressed growth in the absence of the sugar.

Since weakly buffered media, containing filtered, normal human serum, have an alkaline reaction higher than that shown to suppress the growth of *C. neoformans*, it was concluded that this factor was primarily responsible for the failure of the fungus to reproduce in tissue culture media. However, it is also possible to show that even under optimal pH conditions normal human serum exerts an anticryptococcal effect. This activity of human serum was relatively heat-stable and was expressed only in high concentrations of serum. Serum adsorbed with viable cells of *C. neoformans* also inhibited the growth of the fungus, but a partially purified globulin fraction of serum was not inhibitory.

The data are discussed in relation to the work of other investigators.

Studies on the growth[1] of *Cryptococcus neoformans* in tissue cultures of mouse monocytes were undertaken in a continuing effort to evaluate the usefulness of such techniques in investigations on host-parasite relationships in the human mycoses (Howard, 1959, 1960; Howard and Herndon, 1960). But, contrary to expectations, *C. neoformans* did not grow in the medium employed for maintenance of the monocyte cultures.

Mosberg and Alvarez-de Chaudens (1951) showed that growth of certain strains of *C. neoformans* could not be initiated in media having a pH more alkaline than 7.32. Since the maintenance medium employed in the tissue culture studies had a pH more alkaline than 7.32, it was postulated that the adverse pH of the medium was probably responsible for the failure of the fungus to reproduce. In accordance with this hypothesis, the results of subsequent experiments demonstrated that the strain of *C. neoformans*, which had failed to grow in tissue culture, would not grow in a peptone medium with a pH more alkaline than 7.5.

The recognition of this striking pH sensitivity

[1] The term growth will be employed in this study to designate relative increase in numbers of cells as evidenced by visible turbidity of inoculated media.

led to further studies of physiological factors controlling the initiation of growth of *C. neoformans*; the results of these investigations are the subject of this report.

MATERIALS AND METHODS

Fungi. The strains of *Cryptococcus neoformans* used (no. 370, 373, 374, 375) were isolated from patients with cryptococcosis, and conformed to accepted criteria for pathogenic strains of this fungus (Conant et al., 1954; Littman and Zimmerman, 1956). Stock cultures were maintained on slants of a medium consisting of 2% proteose peptone no. 3 (Difco), 1% glucose (Difco), and 2% agar (Difco). The cultures were stored in a refrigerator and transferred every 2 months.

Buffer solutions. Stock solutions of the following reagents were prepared in distilled water and sterilized by Seitz filtration: 0.066 M $Na_2HPO_4 \cdot 2H_2O$; 0.066 M KH_2PO_4; and 1.4% $NaHCO_3$. Appropriate mixtures of the solutions of the phosphate salts were employed for the pH values of 5 to 7.5; appropriate volumes of the $NaHCO_3$ solution were added to a solution of $Na_2HPO_4 \cdot 2H_2O$ to obtain buffers with pH values above 7.5. The buffer mixtures were prepared in accordance with standard procedures (Gortner, 1949).

Standardization of inocula. The fungi were grown at 37 C for 72 hr in a medium consisting of 2% proteose peptone no. 3. The cells were centrifuged, washed three times in saline, and resuspended in 5 ml of saline. The density of the suspension was determined in a Coleman Junior spectrophotometer model 6A at a wavelength of 4,000 A. The optical density of the suspension was related to actual cell counts made in a hemocytometer chamber.

Preparation of inocula. The fungi were grown, washed, and resuspended in saline as before. The number of cells in the suspension was estimated spectrophotometrically. Inocula containing the desired number of cells were prepared by appropriate dilution of the suspensions with saline.

Experimental procedure and analysis of results. The experimental medium employed consisted of equal volumes of the appropriate buffer mixture and a 2% solution of proteose peptone no. 3 (Difco). In those experiments in which the effect of carbohydrates was studied, an appropriate amount of a 10% stock solution of the sugar (sterilized by Seitz filtration) was added to the buffer-peptone mixtures to achieve a final concentration of 1%. The initial pH of each combination was recorded before inoculation.

Screw cap tubes (16 × 125 mm) were employed as the culture vessels. The total volume of medium in the culture vessels was 5 ml. The inoculum consisted of 0.2 ml of a suspension containing 1×10^6 yeast cells per ml. Immediately after inoculation, the caps of the tubes were replaced with nontoxic rubber stoppers in order to minimize changes in pH occasioned by loss of gasses during incubation. The inoculated tubes were incubated at 25 C or 37 C for 120 hr. In a few

TABLE 1. *Growth of four strains of Cryptococcus neoformans in media[a] at different initial pH values[b]*

Initial pH	Strain of *C. neoformans*							
	370		373		374		375	
	Final pH	Growth[c]	Final pH	Growth	Final pH	Growth	Final pH	Growth
8.40	8.45	0	8.45	0	8.45	0	8.45	0
8.00	8.10	0	8.00	0	8.00	(±)G	8.00	(±)G
7.60	7.45	1+ G	7.50	0	7.45	1+ G	7.50	1+ G
7.35	7.30	2+ G	7.25	2+ G	7.25	3+	7.30	3+
7.05	7.00	2+	7.00	3+	6.95	3+	7.00	3+
6.00	6.10	2+	6.00	3+	6.00	3+	6.10	3+

[a] Medium consisted of equal volumes of 2% proteose peptone no. 3 (Difco) and appropriate phosphate buffer mixture (*see* "Materials and Methods").

[b] Results recorded after incubation at 37 C for 120 hr.

[c] Growth recorded as relative visual turbidity. 3+ = maximal turbidity; 2+ = moderate turbidity; 1+ = slight turbidity; ± = detectable turbidity; 0 = no turbidity; G = turbidity granular in appearance (*see* text).

experiments the incubation period was extended to 3 weeks. Uninoculated tubes of media were included in each series of experiments.

After incubation, the degree of turbidity in each tube was estimated visually. The cultures were placed in an Arnold sterilizer (at approximately 100 C) for 30 min and the pH of the medium in each culture vessel was determined, after it had cooled to room temperature. Determinations of pH were made with a Beckman pH meter, model G. Since the primary object of these investigations was to study factors controlling the initiation of growth, no attempt was made to measure the total amount of growth in precise quantitative terms.

RESULTS

Preliminary experiments revealed that the pH of buffered, uninoculated media was not markedly altered, either by the technique employed to sterilize the cultures or during the two periods of incubation at 37 C. The same observation was made in all subsequent experiments. Thus, the data concerning uninoculated media are omitted from ensuing tables.

The results of studies on the effect of pH on the initiation of growth of four strains of *Cryptococcus neoformans* are shown in Table 1. Growth of all strains was completely suppressed at pH values above 8. The growth of strains no. 370 and 373 was partially inhibited at a pH value of 7.3; strain no. 374 and 375 grew well at pH 7.3, but were partially inhibited at pH 7.5. Even in those tubes showing maximal turbidity, there was little change in pH over the 120-hr period of observation.

The growth in tubes of media at an initial pH which partially inhibited multiplication was granular. Microscopically, the granules were composed of clumps of yeast cells. Occasionally, short chains of elongated blastospores were seen. Marked granularity was invariably associated with those turbidities recorded as ± and 1+. Slight granularity was often seen with turbidities recorded as 2+, whereas turbidities recorded as 3+ were never granular in appearance. Thus, suppression of the growth of the fungus was accompanied by aggregation of the cells.

Strain no. 373, shown to be the most sensitive to alkaline pH, was selected for further experiments designed to evaluate the combined influence of pH, incubation temperature, and length of incubation on initiation of growth. The results (Table 2) indicate that, at a temperature of 25 C, the fungus initiated growth in media with a pH which suppressed growth when the temperature was 37 C. It was obvious that further growth took place during the extended incubation period, even n those tubes in which a marked pH change did not occur.

Since the growth of *C. neoformans* on most common culture media is improved by the addition

TABLE 2. *Combined effect of length of incubation time, temperature, and pH on growth of Cryptococcus neoformans (strain no. 373)*[a]

Initial pH	Incubation temperature							
	25 C				37 C			
	120 Hr		3 Weeks		120 Hr		3 Weeks	
	Final pH	Growth[b]	Final pH	Growth	Final pH	Growth	Final pH	Growth
8.30	8.50	0	8.70	0	8.50	0	8.00	0
8.10	8.10	(±)G	7.65	2+ G	8.20	0	7.85	0
7.50	7.40	2+	7.20	2+ G	7.35	0	7.30	1+ G
7.30	7.25	2+	7.10	3+	7.20	1+ G	7.10	2+ G
7.00	7.00	2+	6.90	3+	6.90	2+	6.85	3+
6.00	6.20	2+	6.20	3+	6.05	2+	6.10	3+

[a] Medium consisted of equal volumes of 2% proteose peptone no. 3 (Difco) and appropriate phosphate buffer mixture (*see* "Materials and Methods").

[b] Growth recorded as relative turbidity. 3+ = maximal turbidity; 2+ = moderate turbidity; 1+ = slight turbidity; ± = detectable turbidity; 0 = no turbidity; G = turbidity granular in appearance (*see* text).

TABLE 3. *Effect of pH and incubation temperature on growth of Cryptococcus neoformans (Strain no. 373) in media containing carbohydrates*[a]

Carbohydrate[b]									
Glucose					Lactose				
Initial pH	Final pH	Growth	Final pH	Growth	Initial pH	Final pH	Growth	Final pH	Growth
	25 C		37 C			25 C		37 C	
8.90	8.75	1+ G	8.80	0	8.90	8.75	0	8.80	0
8.40	8.10	2+ G	8.55	0	8.35	8.10	± G	8.15	0
7.80	7.35	3+	7.70	0	7.80	7.60	1+ G	7.70	0
7.40	7.30	3+	7.35	1+ G	7.40	7.60	2+	7.40	(±)G
7.00	6.85	3+	7.05	3+	7.00	7.20	2+	7.05	2+
6.00	5.95	3+	6.00	3+	5.85	6.15	2+	6.10	2+

[a] Results recorded after 120 hr of incubation. Growth recorded as relative turbidity. 3+ = maximal turbidity; 2+ = moderate turbidity; 1+ = slight turbidity; ± = detectable turbidity; G = turbidity granular in appearance (*see* text).

[b] Medium consisted of equal volumes of 2% proteose peptone no. 3 (Difco) and appropriate phosphate buffer mixture. Sugars added to a final concentration of 1%.

TABLE 4. *Effect of filtered normal human serum on growth of Cryptococcus neoformans*

Medium			Initial pH	Final pH[d]	Growth[e]
Human serum[a]	BSS[b]	2% PP no. 3[c]			
%	%	%			
40	60		6.00	6.30	(±)
40[f]	60		6.00	6.15	(±)
40	40	20	6.20	6.20	(±)
40[f]	40	20	6.25	6.35	(±)
	60	40	5.90	5.50	3+

[a] Normal human serum (pooled) filtered by Seitz filtration.

[b] Hanks Balanced Salt Solution (Hanks, 1955).

[c] Proteose peptone no. 3 (Difco) prepared in 2% solution and sterilized by autoclaving.

[d] Tubes not heat-sterilized before final pH reading because of serum content.

[e] Growth recorded as relative turbidity. 3+ = maximal turbidity; ± = detectable turbidity. Incubation at 37 C for 120 hr.

[f] Serum inactivated by heating at 56 C for 30 min.

of a utilizable carbohydrate (Littman and Zimmermann, 1956), studies were conducted to examine the effect of carbohydrates on the growth of the fungus in broth buffered at different pH values. The results recorded in Table 3 show that glucose, which is assimilated by *C. neoformans*, in contrast to lactose, which is not assimilated, permitted the fungus to initiate growth at higher pH values. However, such stimulation was obvious only at an incubation temperature of 25 C. Incubation of the cultures at 37 C prevented the expression of the effect of glucose.

The data thus far presented support the original contention that the inhibitory effect of serum on *C. neoformans* was related to the alkaline pH brought about in media to which this ingredient is added. The results recorded in Table 4 reveal, however, that a fungistatic effect of serum was still operative, even in media buffered at pH 6, and, further, that the effect was not altered by heating the serum at 56 C for 30 min or by the addition of an extraneous nitrogen source in the form of peptone. The effect of serum was not noted at concentrations of serum which were less than 10% of the total volume of a medium. The inhibition of growth of *C. neoformans* in serum-containing media was not accompanied by visible or microscopic agglutination of cells. Serum adsorbed[2] with viable cells of *C. neoformans* also inhibited the growth of the fungus, but a partially purified globulin fraction prepared from serum by the method of Thurston, Rheins, and Buehler (1957) was not inhibitory.

DISCUSSION

The data presented confirm the observation by Mosberg and Alvarez-de Chaudens (1951) that

[2] Three volumes of serum were adsorbed with one volume of packed cells of *C. neoformans*. Adsorption was conducted at 37 C for 2 hr, and the product was sterilized by Seitz filtration.

the growth of *C. neoformans* is inhibited in media with pH values slightly above neutrality. The pH effect was also related to the temperature of incubation. The optimal temperature of growth of *C. neoformans* has been reported to be approximately 29 C (Kuhn, 1939). In keeping with this observation, the data from the present study show that, at a temperature of 25 C, growth of the fungus was initiated in media with a pH which suppressed growth at a temperature of 37 C.

The effect of glucose in overcoming an adverse initial pH was expressed at 25 C but not at 37 C; this again emphasizes that the optimal growth temperature for *C. neoformans* is closer to 25 C than to 37 C. Alterations of pH occasioned by the production of acids from the fermentation of the sugar do not satisfactorily explain the glucose effect, because the organism grew in glucose-containing media at a higher final pH than would have permitted growth in the absence of the sugar. Apparently, a utilizable carbon source stimulates the growth of *C. neoformans* in media with an organic nitrogen source, even under optimal conditions of pH (Littman, 1958; Littman and Zimmerman, 1956).

In confirmation of the original observations reported in abstract form by Allen and Evans (1955), normal human serum was found to inhibit the growth of *C. neoformans* under optimal pH conditions. The anticryptococcal activity of human serum, as well as that of other mammalian sera and chicken serum, has been studied in some detail by Allen (Allen, 1955), but the results of his observations have not been published. A similarity of the anticryptococcal activity of serum to the inhibition of growth of dermatophytes by serum (Roth et al., 1959) is suggested by the fact that both effects are relatively heat-stable and both are expressed only in rather high concentrations of serum. Gale and Welch (1961) recently reported the inhibition of growth of *Rhizopus oryzae* by human serum. The inhibitory effect described by these workers is similar to the anticryptococcal effect in that it was relatively heat-stable and not expressed in partially purified globulin fractions of serum. During the preparation of the manuscript of this paper for publication, Baum and Artis (1961) reported on growth inhibition of *C. neoformans* by human serum. The limited amount of information given by these workers clearly suggests that they have observed the same effect originally reported by Allen and Evans (1955) and described in the present report.

It is well known that pH is important in controlling initiation of growth by microorganisms (Lichstein, 1959). Suggested mechanisms of action of pH include effects upon (i) production and activity of enzyme systems controlling growth and division, (ii) solubility of essential nutrients, and (iii) permeability of cells to substances essential for growth (Lichstein, 1959; Cochrane, 1958; Mitchell, 1951). The data from the present study do not permit an explanation of the marked influence of slightly alkaline pH on the growth of *C. neoformans*. However, it was observed that the partial suppression of growth of the fungus in media with an adverse pH was accompanied by a noticeable aggregation of the cells. Indeed, quite apart from considerations of growth or reproduction, suspensions of *C. neoformans* are unstable and agglutinate spontaneously in phosphate buffer at pH 7.6, and such instability is more marked in suspensions maintained at 37 than in those maintained at 25 C. Although instability of suspensions and inhibitions of growth may not be directly related, pH may exert a portion of its effect on the growth of *C. neoformans* by influencing those factors which affect the stability of the cells in suspension.

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