FACTORS WHICH AFFECT THE GROWTH OF A COLORLESS FLAGELLATE, ASTASIA KLEBSII, IN PURE CULTURES*

HERMAN VON DACH
Mary S. Muellhaupt Scholar in Zoology,
The Ohio State University

INTRODUCTION

This paper is the first of a series of studies on various factors affecting the growth and decline of populations of a protozoan, *Astasia klebsii*, in pure clone culture. In the past, a number of similar investigations have been made on pure cultures of bacteria and yeasts, but very few on the protozoa. The great importance of working with *pure* (i. e., bacteria-free) cultures under controlled conditions is now generally recognized (see Phelps, 1935), and need not be elaborated upon here. In the present paper growth is considered in relation to hydrogen ion concentration, type and concentration of food material, and oxygen tension.

This work has been done under the supervision of Professor W. J. Kostir, of The Ohio State University, who suggested the general problem and supplied the clone culture of *Astasia klebsii*. I am grateful to him for suggestions and criticisms.

THE ORGANISM

The species used in these experiments was *Astasia klebsii*, first described by Lemmermann in 1910, and further studied and described by Pringsheim (1936). The shape of this colorless flagellate is at times altered by protoplasmic contractions, known as euglenoid movements or metaboly. Nutrition is saprozoic. Food reserves of paramylum, a starch-like polysaccharide which does not respond to the usual iodine test for starch, are stored as small granules in the cell. No thick-walled resting stage is known. The cells of the strain used in this investigation measure 40 to 50 microns in length by 10 to 15 microns in width; these dimensions conform more closely to Pringsheim's description than to Lemmermann's.

*Contributions from the Department of Zoology and Entomology, Number 114.
Most of the material included in this paper forms part of a dissertation submitted to the graduate school of The Ohio State University in partial fulfillment of the requirements for the degree of doctor of philosophy, June, 1939.

The delicate striation of the pellicle, mentioned both by Lemmermann and by Pringsheim, is not easily seen, but is definitely demonstrable with the best optical equipment.

This species has not previously been studied in bacteria-free culture.

MATERIALS AND METHODS

Cells from a stock clone culture of *Astasia klebsii* were washed bacteria-free, employing the "migration-pipette" method of Glaser and Coria (1930), and pure cultures were established.

The following inorganic solution was used as the basis of all culture media:

KNO_3	0.5 g.
KH_2PO_4	1.5 g.
$MgSO_4 \cdot 7H_2O$	0.1 g.
NaCl	0.1 g.
$CaCl_2$	0.01 g.
$FeCl_3$	trace
Triple-distilled water (Distilled in Pyrex still)	1 liter

The following culture media were used:

Medium B-1: 1.0 g. Bacto-Tryptone per liter of inorganic solution.

Medium B-5: 5.0 g. Bacto-Tryptone per liter of inorganic solution.

Medium B-25: 25.0 g. Bacto-Tryptone per liter of inorganic solution.

Medium C: 2.0 g. anhydrous sodium acetate per liter of Medium B-5.

The pH of each medium was adjusted to the desired level by addition of N/1 HCl or N/1 NaOH, colorimetric determinations being made with a Hellige comparator. 9.3 cc. portions of medium were measured into 20 x 150 mm. Pyrex culture tubes which were then plugged with cotton and autoclaved.

A single flask culture was used as the source of the inoculum in an experiment. Equal volumes of inoculum were transferred to dilution flasks of the various types of media employed; from each dilution flask in turn 1.0 cc. portions were transferred to the culture tubes of the corresponding series. In this way the original inoculum was greatly diluted (from 50 to 1000 volumes in different experiments).

The initial number of cells per cc. was determined. After varying periods of incubation (at 25° C. ± 1° unless otherwise noted) the final number of cells per cc. was determined. Cell counts were made by the Sedgwick-Rafter counting method as described by Hall, Johnson, and Loefer (1935). In each instance the mean count of several samples from three cultures was computed. Increase in number of cells was the only criterion of growth studied.

The customary bacteriological techniques and tests for purity of the cultures were employed. Contaminated cultures were discarded.

TABLE I

EFFECT OF pH ON GROWTH IN 0.5% TRYPTONE
Medium B-5 used in all series.
Inoculations from a Medium B-5 culture at pH 5.8.
Initial cell concentration = 420 cells per cc.

Culture Series	No. Cells per cc. at 4 Days	No. Cells per cc. at 8 Days	Final pH
Series A—Initial pH 3.2	1,400	10,360	3.2
Series B—Initial pH 4.2	4,220	29,330	4.2
Series C—Initial pH 5.1	12,110	29,980	5.2
Series D—Initial pH 6.0	13,940	31,830	6.0
Series E—Initial pH 7.0	3,830	13,310	7.0
Series F—Initial pH 7.9	1,890	3,770	7.8
Series G—Initial pH 8.4	1,720	2,540	8.2

EFFECT OF HYDROGEN ION CONCENTRATION

Hydrogen ion concentration has long been recognized as an important ecological factor affecting the growth of protozoa. Various workers have determined the pH relationships of a number of protozoa in pure culture, including several species of *Euglena;* these results were summarized by Loefer (1935). The first two experiments of the present study dealt with the effects of this factor in two different types of medium: 0.5% tryptone (Medium B-5), and acetate-tryptone (Medium C).

In 0.5% tryptone at eight days there was optimum growth at pH 6.0, a range of nearly equal growth between pH 4.2 and 6.0, less growth at pH 3.2 and 7.0, and very little growth at

pH 7.9 and 8.4. (See Table I.) The limits of the pH range of growth were not revealed by this experiment. Comparison of these results with those given for other euglenoids in Loefer's (1935) table of pH relationships shows that *Astasia klebsii* has a wider pH range of growth and a lower optimum pH than most previously-studied members of the group.

In acetate-tryptone, maximum growth was at pH 5.1 and 5.9, with good growth occurring over the range pH 4.4 to 6.7, much less growth at pH 7.4 and 8.2, and no growth at all at pH 3.8. (See Table II.)

TABLE II

EFFECT OF pH ON GROWTH IN ACETATE-TRYPTONE
Medium C used in all series.
Inoculations from a Medium C culture at pH 5.9.
Initial cell concentration = 100 cells per cc.
Incubation at 26.5° C. (±0.5°).

Culture Series	At 4 Days		At 8 Days	
	No. Cells per cc.	pH	No. Cells per cc.	pH
Series A—Initial pH 3.8	60	3.8	50	3.8
Series B—Initial pH 4.4	1,460	4.4	310,600	4.7
Series C—Initial pH 5.1	6,600	5.1	764,000	7.6
Series D—Initial pH 5.9	6,920	5.9	748,000	8.2
Series E—Initial pH 6.7	4,320	6.7	228,200	7.5
Series F—Initial pH 7.4	1,580	7.4	11,600	7.4
Series G—Initial pH 8.2	550	8.2	960	8.1

Comparison of Table I and Table II shows that addition of acetate to 0.5% tryptone medium greatly increases growth over the range pH 4.4 to 6.7, has little effect above pH 7.0, and completely inhibits growth at pH 3.8.

In the foregoing experiments, the tryptone cultures and the acetate-tryptone cultures had been inoculated from different source cultures and had been run at slightly different temperatures. As a further check on these general results, another experiment was performed to compare growth at pH 5.9 in 0.5% tryptone and in acetate-tryptone, making inoculations

from a single source culture. The results, presented in Table III, clearly show the great increase in growth produced at this pH by addition of acetate to the tryptone medium.

In Table III the marked increase in pH in the acetate-tryptone cultures is in contrast to the constancy of pH observed in the tryptone cultures. The same phenomenon was observed in the other experiments included in this paper. In general, the pH of tryptone cultures remained practically constant; on the other hand, in vigorously-growing cultures in acetate-tryptone medium there was a marked increase in pH. The following tentative explanation is suggested: Probably the sodium

TABLE III

Growth in Tryptone and in Acetate-Tryptone at pH 5.9
Inoculations from a Medium C culture of pH 5.9.
Initial cell concentration of cultures = 1700 cells per cc.

Culture Series	At 3 Days		At 8 Days	
	No. Cells per cc.	pH	No. Cells per cc.	pH
Series A Medium C Initial pH 5.9	141,990	6.7	702,520	8.3
Series B Medium B-5 Initial pH 5.9	53,390	5.9	57,840	5.9

acetate partly hydrolyzes to form sodium hydroxide and acetic acid, the acetic acid being oxidized by the cells to CO_2 and water; the accumulation of sodium bicarbonate and sodium hydroxide results in a gradual alkalinization.

According to the review by Hall (1939), it has been shown that acetate increases growth in a number of saprozoic flagellates, including three other species of *Astasia.* The toxic effect of acetate on protozoan cultures at low pH was discussed by Jahn (1934). He suggested that at low pH much of the acetate would be in the form of undissociated acetic acid, and that only the undissociated acetic acid molecule was toxic.

EFFECT OF OXYGEN TENSION

It has been shown that cultures in acetate-tryptone at pH 5 or 6 develop dense concentrations of cells (700,000 cells per

cc.); this suggested that this species may be anaerobic or nearly so. As Rahn (1932, p. 80), speaking of bacteria cultures, says: "There can be no doubt that in a test-tube culture or flask culture of aerobes, all cells will exist under practically anaerobic conditions except those in the very top surface layer." Likewise, Pringsheim (1936) had grown bacteria-containing cultures of *Astasia klebsii* under films of paraffin oil, which points to the same possibility. To test this point, the experiment summarized in Table IV was performed, employing acetate-tryptone of initial pH 5.9 as the culture medium.

TABLE IV

EFFECTS OF OXYGEN TENSION ON GROWTH IN ACETATE-TRYPTONE

Medium C (initial pH 5.9) used in all series.
Inoculations from a Medium C culture of pH 5.9.
Initial cell concentration of cultures = 1700 cells per cc.

CULTURE SERIES	AT 3 DAYS		AT 8 DAYS	
	No. Cells per cc.	pH	No. Cells per cc.	pH
Series A—Control	141,990	6.7	702,520	8.3
Series B—Nearly Anaerobic	41,040	6.2	549,400	7.6
Series C—Constant Aeration	41,140	6.2	638,960	8.4

In this experiment the cultures of one series (B) were treated as follows: Each newly-inoculated culture tube was fitted with a rubber stopper equipped with a glass inlet tube extending almost to the bottom of the culture tube and an outlet tube which came only to the bottom of the rubber stopper. Tank nitrogen was shaken in a pressure bottle containing strong pyrogallate solution to absorb almost all the oxygen present. The treated gas was sterilized by passing through cotton, saturated with water vapor and then bubbled vigorously through the cultures for 12 minutes, after which the gum rubber connections on inlet and outlet tubes were clamped off. Culture tubes so treated presumably contained only minute amounts of oxygen; but they could not be regarded as completely oxygen-free, for pyrogallate solution does not absorb every trace of oxygen, and also oxygen can diffuse through rubber tubing.

In another series (C) the culture tubes were fitted with rubber stoppers and glass tubing as in Series B, and a constant stream of sterilized water-saturated air was bubbled through the cultures during the entire period of incubation.

The oxygen tension in the cotton-plugged controls (Series A) was presumed to be more or less intermediate between that in Series B and that in Series C.

It will be seen from Table IV that both the constantly-aerated cultures (Series C) and the nearly anaerobic cultures (Series B) showed lower growth rates, but also showed longer periods of comparatively vigorous growth, than the controls of Series A. As a result, at eight days the population levels in the former two series were not greatly less than that of the controls. Jahn (1936), working with the ciliate *Glaucoma pyriformis* and the flagellate *Chilomonas paramecium*, studied growth in aerated cultures and in non-aerated cultures; his results agree on the whole with those of the corresponding part of the present experiment.

EFFECT OF DIFFERENT CONCENTRATIONS OF TRYPTONE UPON THE GROWTH CURVE

Among the bacteria and yeasts considerable work has been done on the determination of the various phases of the growth curves, and on the factors affecting these phases. In much pure-culture work on the protozoa, however, the general practice has been to determine relative amounts of growth after only one or two arbitrarily-chosen periods of incubation. Sometimes it is assumed that logarithmic growth-rates are being determined, but usually no attempt is made to differentiate between the various phases of the growth curve. As a result, such work on the protozoa has lacked completeness and precision. The results summarized in Tables I and II of the present paper illustrate the shortcomings of this procedure. A fuller and more satisfactory picture of the growth of a culture is afforded by growth curves—especially logarithmic curves, in which the various phases of growth can readily be seen at a glance.

The only published detailed study of the growth curve of a protozoan in pure culture is that on the ciliate *Glaucoma pyriformis* by Phelps (1935, 1936). He compared his findings on this form with the data on the growth curves of yeasts and bacteria as summarized by Buchanan and Fulmer (1928) and by Rahn (1932).

The obvious desirability of ascertaining the rate of growth at various stages in the development of astasia cultures, and thus constructing true growth curves, led to the following procedure. An experiment was undertaken to determine the course of growth in tryptone media of 0.1%, 0.5%, and 2.5% concentrations. Inoculations were made from a 7-day-old culture in 0.5% tryptone which was probably in the logarithmic growth phase. Initial cell concentrations of the cultures were 400 cells per cc. The hydrogen ion concentration of all cultures remained constant at pH 5.8. The results of this experiment are presented in Figure 1.

Buchanan and Fulmer (1928) divide the growth curve of bacteria into the following phases: (1) the initial stationary phase, during which no cell division occurs; (2) the lag phase, during which the division rate increases with time; (3) the logarithmic growth phase, during which the rate of cell division is constant and at a maximum; (4) the phase of negative growth acceleration, during which the division rate decreases with time; (5) the maximum stationary phase, during which the population is constant and at a maximal level; (6) phase of accelerated death, during which the rate of decrease in the number of living cells increases with time; (7) so-called logarithmic death phase, during which the rate of decrease of living cells is constant. In this paper only the first five phases of growth will be discussed.

In the present experiment, though counts were made at rather wide intervals, the general trends of the growth curves are fairly clear. (See Fig. 1.)

No initial stationary phase is evident from the data at hand.

The growth curve of the 0.5% tryptone cultures showed a very brief and ill-defined lag phase which went quickly into the logarithmic growth phase. The 0.1% tryptone cultures showed a longer and more well-defined lag phase, which was still more pronounced in the 2.5% tryptone cultures.

Concerning a similar situation, Phelps (1936) states: "Animals [*Glaucoma*] taken from the stock cultures containing 0.1% yeast extract, upon being placed in much higher concentrations suffered a shock, and failed to divide for sometimes as much as 30 hours." In the present experiment it may be postulated that astasias from the 0.5% tryptone inoculum suffered some shock on transfer to medium of a different tryp-

tone concentration, whether higher or lower, and this shock resulted in a well-defined lag phase of growth.

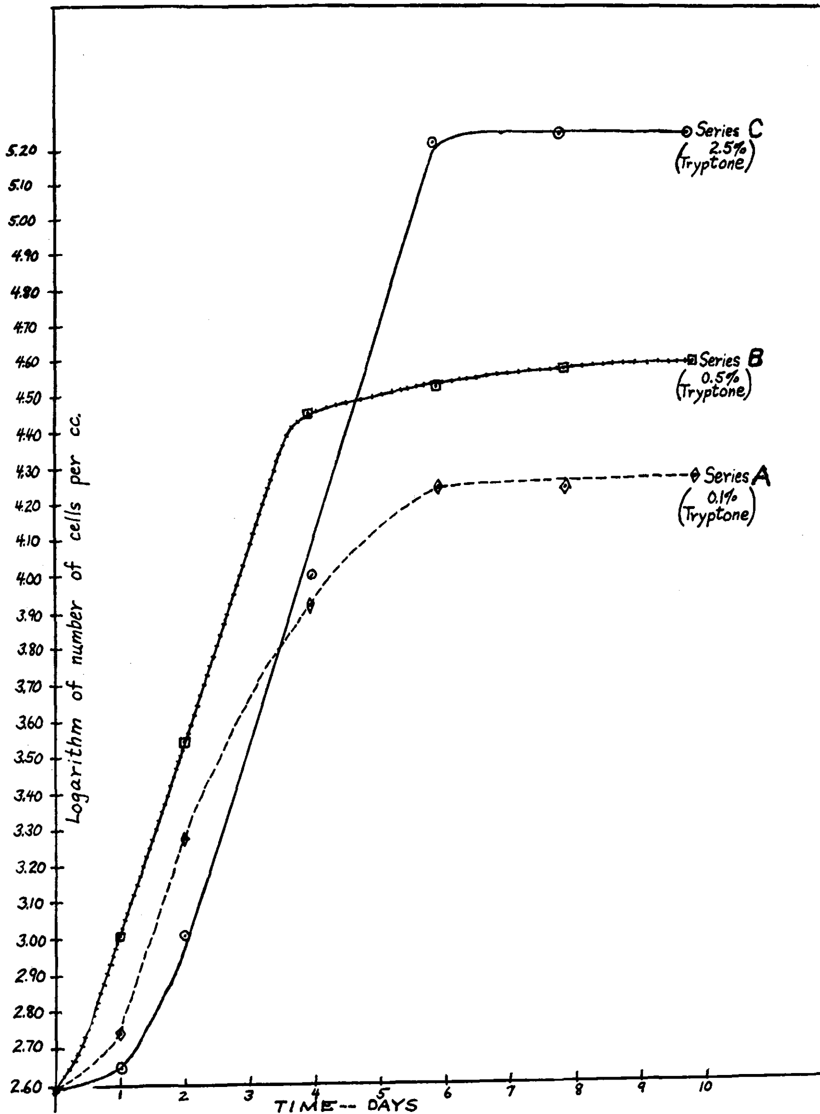

Fig. 1. Growth of *Astasia klebsii* in different concentrations of tryptone.

Logarithmic growth phase. When the logarithm of the number of cells per cc. is plotted against time, this part of the growth curve will be an ascending straight line. (See Fig. 1.)

The time required for the number of cells in a culture to double (presumably by each cell dividing into two cells) is called generation time (g). During the logarithmic growth phase g is a constant. According to Rahn (1932) the standard formula for generation time is

$$g = \frac{t \log 2}{\log b - \log a}$$

where a = initial number of cells, and b = number of cells after time t. Applying this formula to the logarithmic growth periods of the cultures in this experiment, the values given in Table V were obtained.

TABLE V

GROWTH RATES IN LOGARITHMIC PHASE IN DIFFERENT CONCENTRATIONS OF TRYPTONE

Culture Series	Portion of Logarithmic Growth Period Taken in Calculation of g	g Generation Time of Logarithmic Phase
Series A—0.1% tryptone	1 day to 2 days	13.6 hours
Series B—0.5% tryptone	1 day to 2 days	13.4 hours
Series C—2.5% tryptone	2 days to 6 days	13.1 hours

Table V shows that in the logarithmic growth phase the generation time is practically constant in tryptone concentrations between 0.1% and 2.5%. However, the duration of the logarithmic growth phase increased with increasing food concentration, as Figure 1 shows.

At the end of the logarithmic growth phase, the growth curves of Series B and C showed abrupt decline in division rate as the phase of negative growth acceleration began; while the curve of Series A showed a prolonged gradual decline in growth rate.

Maximum stationary phase. In each series of cultures, the cell concentration at 20 days and at 22 days was very slightly less than that at 10 days. Consequently the cell concentration at 10 days may be regarded as the maximum yield in each case.

According to Rahn, when the maximum populations are proportional to the concentrations of food in the medium, then food is the sole limiting factor of the growth of the populations,

and accumulation of metabolic wastes plays no part in such limitation.

In Table VI, Series B and C show such a proportionality between yields of cells and concentrations of medium, indicating that possibly food supply is here the limiting factor. Yet this relationship between food and cell yields breaks down when the weaker concentrations of food are compared (Series A and Series B).

No explanation is available for this discrepancy. Phelps (1936) found the same general sort of situation in *Glaucoma:* namely, that there was a proportionality between yields of animals and concentrations of medium, which however did not hold true for very weak food concentrations.

TABLE VI

FOOD CONCENTRATIONS AND MAXIMUM POPULATIONS

Culture Series	Food Concentration Ratio (tryptone)	Maximum Populations (No. Cells per cc.)	Ratio of Maximum Populations
Series A	0.1%	18,330	0.48
Series B	0.5%	38,100	1.0
Series C	2.5%	173,100	4.54

Besides the points already mentioned, the following conclusions from the present experiment are in substantial agreement with Phelps' results on *Glaucoma:* (1) The generation time during the logarithmic growth phase is approximately the same at several concentrations of food; (2) there is an increase in duration of the logarithmic growth phase with increasing food concentration.

SUMMARY

Bacteria-free clone cultures of *Astasia klebsii* in 0.5% tryptone medium after 8 days at 25° C. showed optimum growth at pH 6.0, a range of nearly equal growth between pH 4.2 and pH 6.0, less growth at pH 3.2 and pH 7.0, and very little growth above pH 7.0.

Addition of acetate to the tryptone medium greatly increased growth over the range pH 4.4 to 6.7, had little effect above pH 7.0, and completely inhibited growth at pH 3.8.

In tryptone cultures pH remained practically constant, while in vigorously-growing acetate-tryptone cultures there was a marked increase in pH.

In acetate-tryptone at initial pH 5.9, both nearly-anaerobic cultures and constantly-aerated cultures showed slower growth than the controls, but the yields of all three series of cultures after eight days were fairly comparable.

Growth curves were determined at 25° C. for three different concentrations of tryptone (0.1%, 0.5%, 2.5%). During the logarithmic growth phase the generation time was slightly greater than 13 hours at all three food concentrations; duration of this phase increased with increasing concentration of tryptone. In the case of the 0.5% and 2.5% tryptone cultures the maximum populations were roughly proportional to food concentration, but this proportionality did not hold for the 0.1% tryptone cultures.

LITERATURE CITED

Buchanan, R. E., and **Fulmer, E. I.** 1928. Physiology and biochemistry of bacteria. Vol. I. Williams and Wilkins Co., Baltimore.

Glaser, R. W., and **Coria, N. A.** 1930. Methods for the pure culture of certain protozoa. J. Exp. Med. 51: 787–806.

Hall, R. P. 1939. The trophic nature of the plant-like flagellates. Quart. Rev. Biol. 14: 1–12.

Hall, R. P., Johnson, D. F., and **Loefer, J. B.** 1935. A method for counting protozoa in the measurement of growth under experimental conditions. Trans. Amer. Micr. Soc. 54: 298–300.

Jahn, T. L. 1934. Problems of population growth in the protozoa. Cold Spring Harbor Symposia on Quant. Biol. 2: 167–176.

Jahn, T. L. 1936. Effect of aeration and lack of CO_2 on growth of bacteria-free cultures of protozoa. Proc. Soc. Exp. Biol. and Med. 33: 494–498.

Lemmermann, E. 1910. Algen I. Schizophyceen, Flagellaten, Peridineen. (III. Bd., Kryptogamenflora der Mark Brandenburg.) Borntraeger, Leipzig.

Loefer, J. B. 1935. Relation of H-ion concentration to growth of *Chilomonas* and *Chlorogonium*. Arch. Protist. 85: 209–221.

Phelps, A. 1935. Growth of protozoa in pure culture. I. Effect upon the growth curve of the age of the inoculum and of the amount of the inoculum. J. Exp. Zool. 70: 109–130.

Phelps, A. 1936. Growth of protozoa in pure culture. II. Effect upon the growth curve of different concentrations of nutrient materials. J. Exp. Zool. 72: 479–496.

Pringsheim, E. G. 1936. Zur Kenntniss saprotropher Algen und Flagellaten. I. Mitteilung. Über Anhäufungskulturen polysaprober Flagellaten. Arch. Protist. 87: 43–97.

Rahn, O. 1932. Physiology of bacteria. P. Blakiston's Son and Co., Philadelphia.