Studies on the physiology of the euglenoid flagellates.

IV. The thermal death time of *Euglena gracilis* Klebs.

By

Theo. L. Jahn[1]),
Osborn Zoölogical Laboratory, Yale University.

(With 2 figures in the text.)

Introduction.

The effect of temperature upon *Euglena* has received but little attention from most investigators in the field, and our knowledge of the subject is at best fragmentary. Previous studies of the effect of temperature have been of three types: the effect of temperature on growth, the effect of temperature on movement, and the resistance of euglenoids to extremes of temperature. The present study is an attempt to determine the thermal death time of *Euglena gracilis* Klebs at temperatures beyond the growth range of the species.

The writer wishes to thank Prof. L. L. Woodruff for his continued interest in this work and for his patient reading of the manuscript.

Historical survey.

Klebs (1883) reported that *Euglena* was able to withstand a temperature of 30° C. Zumstein (1900) found that, in cultures of *Euglena gracilis*, growth was 500 times as great at 24° C. as at 16° C. in a period of 8 days. Günther (1928) reported that *E. proxima*, *E. pisciformis*, *E. geniculata*, and *E. terricola* are positively thermo-

[1]) National Research Council Fellow in Zoology.

tropic at 22° C. to temperatures as high as 41° C., and that death takes place between 48° C. and 50° C.

BRACHER (1919) studied the burrowing movements of *E. deses* and determined the time required for burrowing when exposed to darkness at various temperatures. She found that burrowing was rapid between 10° C. and 18° C., with the optimum at 12° C. Movement was very sluggish below 5° C. and ceased completely below 2.5° C. Later experiments (BRACHER, 1929) showed that 25—30° C. is lethal to *E. deses* within four days.

MAINX (1928) found little evidence in favor of the common belief that *Euglena* can withstand freezing. He found that partially or completely frozen cultures of *E. gracilis*, *E. viridis* and *E. deses* gave rise to very few living forms, and to these only if cysts were present previously. GÜNTHER (1928), however, reports that he observed a specimen of *E. terricola* which had been frozen while in the process of mitosis. This specimen, which had been in ice at —12° C. for eight days, completed the process of division in a normal manner when the ice melted under the microscope.

Of the workers cited above, only MAINX (1928) and ZUMSTEIN (1900) used bacteria-free cultures.

Material and methods.

The bacteria free strain of *Euglena gracilis* KLEBS used in this study was obtained from Professor E. G. PRINGSHEIM of the German University of Prague and is the same as was used in a previous paper of this series (JAHN, 1931). The culture medium used was as follows:

NH_4NO_3	.50 gm.
KH_2PO_4	.50 gm.
$MgSO_4$	.25 gm.
NaCl	.10 gm.
Hydrolyzed casein	5.00 gm.
Distilled water	1000.00 cc.
Normal NaOH or HCl to the desired p_H value.	

Equal amounts (always 9 cc.) of the medium were measured directly into 16×150 mm. thin walled Pyrex test tubes by means of a Schellbach side-arm burette. The tubes were autoclaved and stored in a cool place until used. Inocula of .9 cc., measured by means of 12-inch Mohr pipettes, were taken from young (3—6 day-old) rapidly growing cultures in the same type of medium.

The experiments performed were of two types: one, a determination of the time required for sterilization of the culture, was primarily qualitative; the other, a determination of the number of survivors at various times, was quantitative in nature. In the sterilization experiments, the inoculated tubes were exposed in a water bath to the experimental temperature for the desired length of time, and the sterility or relative amount of growth was determined macroscopically at the end of a definite time. The quantitative experiments were performed in the same manner except that the initial concentration of organisms and the concentrations at the end of three days were determined by means of a counting method described previously (JAHN, 1929). These concentrations were compared with those determined for control tubes which were not exposed to extreme temperatures but were maintained under conditions otherwise identical with those to which the experimental tubes were exposed. From these concentrations it was possible, in a manner which will be described later, to determine the numbers of survivors in the different tubes.

Experimental results.

Effect of temperature on sterilization time.

In this series of experiments the temperatures used were 60°, 48°, 44°, 41°, 39°, 36°, and —0.2° C., and the time intervals were 2, 4, 8, 15, 30, 45, and 60 minutes. Young, rapidly dividing stock cultures were used in which no encysted forms could be observed. The initial concentration of organisms in each tube was approximately 6 thousand per cc. The results are recorded in table 1. The relative amounts of growth were recorded at the end of 96 hours except where marked with an asterisk, in which case the asterisk denotes a slight growth not visible macroscopically before six days. It is seen that 60° and 48° C. were lethal in less than two minutes, that 44° C. produced complete sterilization in 8 minutes, that 41° C. just failed to produce sterilization in 30 minutes, and 39° C. in 60 minutes, and that 36° C. and —0.2° C. (not frozen) produced no effect noticeable macroscopically. At —4.0° C. (frozen) most, but not all, of the organisms were killed in 60 minutes. In another series of experiments (not shown in table 1) at 40.8° C. and 44.0° C. growth was positive after 20 minutes and negative after 25 minutes at 40.8° C., and positive after 4 and negative after 6 minutes at 44.0° C. In another experiment 37.5° C. produced sterilization in

48 but not in 24 hours, and 35.5° C. failed to sterilize in 96 hours. In two other experiments, also not included in the above table, —0.2° C. (not frozen) failed to sterilize, and approximately —4.0° C. (frozen) produced sterilization in 24 hours.

Table 1.

Effect of temperature on sterilization time.

Temperature	Time of exposure (in minutes). 2	4	8	15	30	45	60
60 °C	—	—	—	—	—	—	—
48 °„	—	—	—	—	—	—	—
44 °„	++++	++	—	—	—	—	—
41 °„	++++	++++	+++	++	*	—	—
39 °„	++++	++++	++++	++++	+++	+	*
36 °„	++++	++++	++++	++++	++++	++++	++++
—0.2° „	++++	++++	++++	++++	++++	++++	++++

Key:

++++ excellent growth at end of 4 days.
+++ good growth at end of 4 days.
++ slight growth at end of 4 days.
+ very slight growth at end of 4 deys.
* no growth at end of 4 days, but slight growth visible at end of 6 days.
— no growth at end of 10 days, tubes presumably sterile.

Time allowance for heating lag period:
36°—41°, 1 minute; other temperatures, 1.5 minutes.

The Q_{10} value computed from the most accurate determinations above (40.8° and 44.0° C.) is 97. Other experiments gave values ranging from 54 to 216. These indicate merely the order of magnitude of the Q_{10} value, for it might probably be affected by various unknown factors as has been described for *Paramecium caudatum* (JACOBS, 1919).

Effect of p_H on sterilization time.

In order to determine whether or not the hydrogen ion concentration affected the sterilization time, three series, at p_H values of 6.0, 7.0, and 8.0, were subjected to a temperature of 40.0° C. for periods differing by five minutes between 5 and 60 minutes. The initial concentration of organisms in each tube was about 8 thousand per cc. The relative amounts of growth in the controls were as follows: at p_H 7.0 > at p_H 6.0 > at p_H 8.0. This is in accordance with the p_H relationships observed previously (JAHN, 1931). The

sterilization times as recorded at the end of six days are as follows: p_H 6.0, 45 minutes; p_H 7.0, 35 minutes; p_H 8.0, 25 minutes. In a repetition of the experiment at p_H values of 6.1, 7.1, and 7.9, similar results were obtained. Therefore the experiments were repeated three more times with p_H ranges of 4.1 to 8.0. The results of one such series of experiments are shown in fig. 1. It is obvious from this table that resistance to 40° C. varies with p_H and that there is a maximum of resistance at about p_H 5.0. This maximal resistance is not at the same p_H value as the optimum growth rate, and the two curves are shown for comparison. The other two series showed very similar results, and for the purpose of brevity the data are omitted.

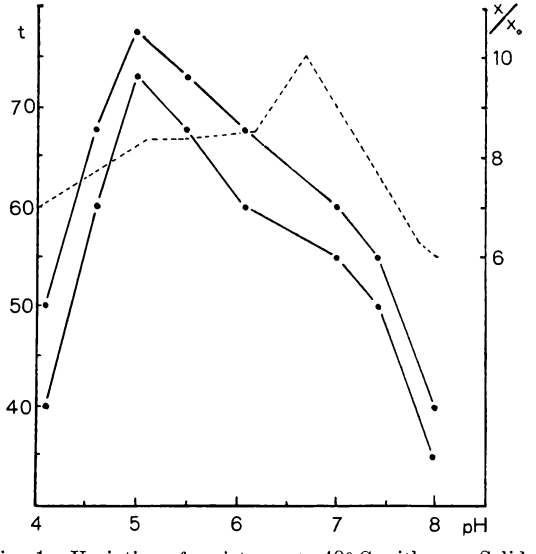

Fig. 1. Variation of resistance to 40° C with p_H. Solid lines: variation of resistance measured by time (t) in minutes necessary to produce sterilization in tubes containing about 8 thousand organisms per cc. (ordinate on left). Upper curve shows the shortest exposure which produced sterilization. Lower curve shows longest exposure that did not produce sterilization. Dotted line: relative amounts of growth at different p_H values (data of JAHN, 1931) shown for comparison (ordinate on right). The controls of the present experiment showed the same p_H growth relationship.

The effect of concentration on sterilization time.

In preliminary experiments it had been observed that the concentration of organisms in a tube seemed to affect the sterilization time, the concentrated cultures requiring a longer time for sterilization. Therefore, a series of experiments was performed in order to determine the relation of concentration to sterilization time. A very densely populated stock culture was examined microscopically for cysts. None were found, and a 20 cc. inoculation was made into 50 cc. of fresh medium. Three days later this was diluted by four serial 10 cc. inoculations into 50 cc. volumes of fresh medium. Twelve hours later 76 experimental tubes (19 from each of the

dilutions) were inoculated. Initial concentration of the highest dilution was .11; that of the lowest, 25.0 thousand per cc. The p_H was $6.9 \pm .1$ in all cases. At the end of another twelve hours all tubes were placed in a water bath at 40° C. Every five minutes one tube of each concentration was withdrawn and cooled. The results as recorded at the end of seven days incubation at 27° C. under constant light are shown in table 2. It is seen that in the least concentrated cultures (.11 thousand per cc.) apparent sterilization was produced in 45 minutes, but that in the most concentrated culture (25.0 thousand per cc.) apparent sterilization was not produced until after 75 minutes of exposure.

Table 2.

Effect of concentration on sterilization time.
Results at end of 8 days.

Conc. in thousands per cc.	Exposure (in minutes) at 40° C.															
	15	20	25	30	35	40	45	50	55	60	65	70	75	80	85	90	95
25.0	+	+	+	+	+	+	+	+	+	+	+	+	—	—	—	—	—
4.1	+	+	+	+	+	+	+	+	+	+	—	—	—	—	—	—	—
.7	+	+	+	+	+	+	+	+	—	—	—	—	—	—	—	—	—
.11	+	+	+	+	+	+	—	—	—	—	—	—	—	—	—	—	—

+ denotes growth; — denotes no growth.

Table 3.

Effect of concentration on sterilization time.
Results at end of 15 days.

Conc. in thousands per cc.	Exposure (in minutes) at 40° C.															
	15	20	25	30	35	40	45	50	55	60	65	70	75	80	85	90	95
25.0	+	+	+	+	+	+	+	+	+	+	+	+	+	+	—	—	—
4.1	+	+	+	+	+	+	+	+	+	+	+	+	—	—	—	—	—
.7	+	+	+	+	+	+	+	+	+	—	+	—	—	+	—	—	—
.11	+	+	+	+	+	+	+	+	—	+	—	—	+	—	—	—	—

+ denotes growth: — denotes no growth.

However, when the cultures were allowed to remain undisturbed for eight more days, growth appeared in certain other tubes as shown in table 3. At the end of seven more days (twenty-two days since exposure to heat) no more growth appeared. From these results it seems possible that there might have been present in the stock culture a few organisms which were capable of withstanding exposure to 40° C. for 80 minutes. In the concentrated cultures

these highly resistant organisms were presumably present in numbers sufficient to produce visible growth in seven days. In the more dilute cultures (some were only 1/216 as concentrated) these resistant forms were perhaps present in very small numbers, and some tubes might have contained none at all. These latter tubes remained sterile after exposures of 55 minutes. Some dilute cultures, presumably those containing a few resistant organisms, developed visible growth at the end of fifteen days. Thus the variation of sterilization time with concentration of organisms in this case may be due, in part at least, to the random distribution of a few highly resistant organisms. This same phenomenon was observed for bacteria by ESTY and MEYER (1922), who termed it the "skip-stop" phenomenon, and explained it as being due to differences in the individual resistance of cells.

Repetitions of this experiment gave the same positive correlation of sterilization time and concentration of organisms, but the "skip-stop" phenomenon was not always so definite. This can be explained as being due to a relative scarcity of highly resistant organisms.

Effect of time of exposure on number of survivors.

The number of viable cells in a culture of bacteria or yeasts can be determined by platting or staining methods respectively. However, these methods are not so applicable to studies on Euglena, and the following indirect method has been devised.

When a culture of *Euglena gracilis* contaminated with bacteria is heated just beyond the thermal death time of the Euglenas, the organisms wich are killed are cytolyzed in a relatively short time by the action of bacterial enzymes. In the case of bacteria-free cultures, however, the cells do not undergo cytolysis for at least a week, and if only part of the cells are killed and the culture is examined, both living and dead cells may be seen. If the initial concentration of organisms and the concentrations at the end of a definite period of time (4—6 days) are known for heated cultures and also for control (unheated) cultures, the approximate number of organisms not killed by the heat may be computed. The method is as follows:

Let x_o = concentration of organisms in all tubes at beginning of the experiment, that is, the initial concentration,

and x_c = concentration in control tubes at end of the experiment.

Then the amount of growth may be measured by the ratio x_c to x_o, which, assuming growth to be logarithmic for the period

under consideration, is equal to 2^n where n is the number of generations that have occured during the experiment. That is,

$$\frac{x_c}{x_o} = 2^n$$

Then let x_h = concentration of organisms in a heated tube at end of the experiment,

and x_{oh} = concentration of viable cells in heated tube at end of heating period.

Then $(x_o - x_{oh})$ = concentration of non-viable cells at end of heating period and also at end of experiment.

Assuming that the division rate of the survivors in the heated tubes is approximately the same as that of the controls, the value of n, the number of generations, should be approximately the same in both heated and control tubes. Therefore,

$2^n x_{oh}$ = concentration of living cells in heated tubes at end of the experiment,

and the total number of cells (x_h) will be equal to the sum of the living and the dead. That is,

$$x_h = 2^n x_{oh} + (x_o - x_{oh})$$

From this,

$$x_{oh} = \frac{x_h - x_o}{2_n - 1}$$

and

$$\frac{100\ x_{oh}}{x_o} = \text{percentage of survivors}$$

Using this method it was possible to compute the approximate number of survivors after various periods of heating. In this series thirty-two cultures of equal volumes and concentrations were used. The initial concentration (14.5 thousand per cc.) was determined for four tubes. Then twenty-four tubes were heated in a water bath at 40° C. At successive intervals groups of four tubes were removed from the bath and cooled. When the last set of tubes had been removed, the heated tubes and the unheated controls were placed at 28° C. under conditions of constant light. At the end of 72 hours all of the cultures were killed by heating to 60° C., and the concentrations were determined. The survivors at various times were as follows: 15 minutes, 95.9%; 22 minutes, 95.8%; 30 minutes, 88.3%; 40 minutes, 51%; 50 minutes, 31.0%; 60 minutes, 6.9%. Each concentration given is the average of two samples from each

of three tubes, or an average of six different samples. The p_H was 6.9 ± .1 in all cases.

In another experiment at 40.8° C. with an initial concentration of 30 thousand per cc., similar results were obtained. The survivors at various times were as follows: 10 minutes, 82.0 %, 20 minutes, 69.3 %; 30 minutes, 43.3 %; 45 minutes, 7.7 %; 55 minutes, 3.6 %; 65 minutes, none. Survivors' curves for these experiments are plotted in fig. 2, and they are definitely not logarithmic but show an increasing rate of death. In view of the results of a previous study of a lower growth rate in cultures of higher concentration (JAHN, 1929), it seems probable that these percentages as calculated are subject to some inaccuracies, but the results seem to be accurate enough for the present purpose.

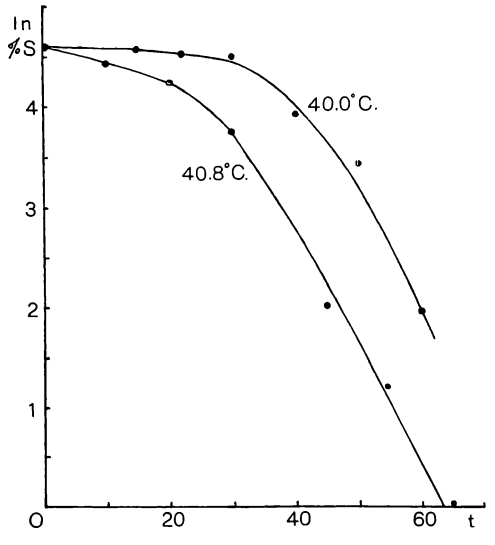

Fig. 2. Survivors' curves for 40° and 40.8° C. The logarithm of the percentage of survivors (S) is plotted against time (t) in minutes.

Morphological effects of heat.

When *E. gracilis* is killed at 40° C. the cytoplasm becomes vacuolated, and the body is shortened so that it resembles a short spindle. The chromatophores are contracted, and the nucleus may be seen as a granular structure. At p_H 5.6 to 7.6 the chloroplasts remain bright green, but at p_H 5.0 they become olive green, and below p_H 5.0 they become a brownish olive green. This is probably due to the acid decomposition of chlorophyll at and below p_H 5.0 to form pheophytin (WILLSTÄTTER and STOLL, 1928). No tendency for the organisms to encyst as a result of exposure to lethal and semi-lethal temperatures was observed.

Discussion.

The lethal temperatures as here determined for *E. gracilis* differ somewhat from those previously recorded for Euglena. GÜNTHER (1928) gave 48—50° C. as the lethal temperature for *E. proxima*,

E. pisciformis, *E. geniculata*, and *E. terricola*, but he states this more as the result of casual observation than of definite experiments. BRACHER (1929) reports, as the result of several experiments, that 25—30 ° C. will kill *E. deses* in four days. Therefore, *E. deses* and *E. gracilis* must differ decidedly in temperature relationships, since cultures of *E. gracilis* have been maintained within this range (usually at 28—29 ° C.) for more than a year. The present experiments indicate that *E. gracilis* has approximately the same resistance to heat as *Paramecium caudatum* (JACOBS, 1919; CHALKLEY 1930) and a somewhat higher resistance than *P. aurelia* (WOODRUFF and BAITSELL, 1911); however, the different methods used for these organisms do not allow accurate comparisons to be made. The experiments in which *E. gracilis* was frozen show that the organism is not nearly so resistant to freezing as is *E. terricola* according to GÜNTHER'S observations.

The Q_{10} values for the death of *E. gracilis* (54—216) are much higher than most Q_{10} values for the death of bacteria. However, some of the non-heat-resistant bacteria have high Q_{10} values which are in the same range as these for Euglena. In a very careful series of studies, CHICK (1910) obtained Q_{10} values of 50—320 for *Bacterium typhosum* at 49—53 ° C. JACOBS (1919) reported still higher Q_{10} values and much greater variations than these for *Paramecium caudatum* at 36—43 ° C. He states that the death time of *P. caudatum* is "subject at all times to considerable and sometimes inexplicable fluctuations."

The variation of thermo-resistance with p_H as found for *E. gracilis* seems to be the first observation of this type to be recorded for the euglenoids. However, a similar variability has been described for bacteria (ESTY and MEYER, 1922) and for Paramecium (CHALKLEY, 1930). ESTY and MEYER (1922) found one maximum of resistance, the position of which varied (6.5 to 7.8) with the buffer mixture used. CHALKLEY (1930) found two maxima, one in the acid and one in the alkaline range, whose positions varied with the buffer. In *E. gracilis* there is one maximum at about p_H 5.0, under conditions of the present investigation.

The data given in tables 2 and 3 show that the sterilization time of *E. gracilis* is sometimes longer when the concentration of organisms is higher. Similar phenomena have previously been reported in the case of many bacteria, and it has been explained in different ways by various authors. The more usual explanations are as follows: (1). The cells are not all equally resistant, but the

resistance differs in different cells of the same strain. In a large inoculum, the chances are greater that some of the cells with a very high resistance will be included, and therefore the sterilization time may be increased (summary, BUCHANAN and FULMER, 1929, 1930). (2). The velocity constant for death will be constant (logarithmic death rate) or will increase with time, depending upon whether there is one or more molecules in the cell which must be destroyed in order to cause death (RAHN, 1929, 1929 a, 1931). In either case a larger number of organisms will necessitate a longer time for complete sterilization to occur. (3). Large numbers of cells exert a "mass protective action", perhaps by secreting a protective substance into the medium (summary, ALLEE, 1931).

Evidence for both the first and second theories is available if results obtained by various workers with bacteria and other organisms are analyzed according to the methods of RAHN (1929 a). He shows that increasing values of the velocity constant denote the presence of more than one molecule which must be destroyed in order to cause death, and that a decreasing velocity constant denotes a variation in resistance. However, it is at present impossible to detect an unequal distribution of resistances in cases of increasing velocity constants by this method. A form of the third theory, that of "mass protective action" was first proposed by EIJKMAN (1908) to explain the phenomenon in bacterial cultures. His experiments showed that liquid in which cells had died protected other cells against heat, and he suggested that a protective substance was liberated by dead cells. A modified form of this theory, involving the liberation of a protective secretion by living cells was used by ROBERTSON (1921) to explain the resistance of numbers of Enchelys to heat, and it has been expanded by ALLEE (1931). In ROBERTSON'S experiments the substance thrown into the medium was supposed to be the so-called "autocatalyst", but the presence of such an autocatalyst of growth has since been shown to be mathematically inconsistent with the growth equations (JAHN, 1930). Also, ROBERTSON'S results may be explained in a different manner (D. RAFFEL, unpublished observations). In view of later knowledge of the effect of salts (ESTY and MEYER, 1922; VILJOEN, 1926; LEVINE, BUCHANAN, and TOULOUSE, 1927) and of variations in other components of the medium (BROWN and PEISER, 1916) on death rates, it seems probable that the results of EIJKMAN (1908), as well as results of other workers, might be explained without postulating a protective secretion. Diffussion of materials from dead cells might easily explain his results.

Also, the supposed protective action of higher organisms against toxic agents may be due to adsorption of the toxic substance or to inactivation by the usual excreta of the organism, and in carefully investigated cases this has been demonstrated (ALLEE and BOWEN, 1932).

The presence of a protective secretion as yet remains unproven, and it seems probable that variations in individual resistances, the order of the death reaction (fig. 2, this paper; and RAHN, 1929, 1929 a, 1931), and perhaps diffusion of substances from killed cells may explain all of the results obtained in the present experiments on *Euglena gracilis*, but the relative importance of these factors has not been determined.

If interpreted according to the method of RAHN (1929, 1929 a, 1931), the increasing death rates shown in fig. 2 indicate that there is more than one molecule which must be destroyed in order to cause death of the cell. RAHN also shows that in the case of only a single vital molecule, if the resistances of the cells are unequal, the velocity constant of death will be decreasing. However, the fact that the velocity constant increases (as in fig. 2) is no evidence that the resistances are equal, for an unequal distribution of resistances would serve only to decrease the curvature of the convex form of the curve in such a way as to be undetectable by the present experimental methods. Moreover, it seems most unnatural to assume that the individuals of a pure culture of *Euglena* which differ from each other in size, in chlorophyll content, in the amount of reserve materials, and in stage of the division-interdivisional cycle, should offer exactly equal resistances to heat. It seems more probable that such a collection of individuals, even though the descendants of a single organism and kept under identical conditions, would offer at least an unequal, and perhaps a very wide distribution of resistances.

It might be argued that the "skip-stop" phenomenon as recorded for very dilute cultures of *Euglena* was due to presence of a few encysted forms and not necessarily to individual resistances of the flagellated forms, but careful examination of the cultures revealed no cysts. The fact that the original stock culture showed no cysts and that the organisms later underwent two transplants, one just before and one just after dilution, makes encystment seem unlikely. Furthermore, ESTY and MEYER (1922) obtained this same type of result with suspensions of *spores* of *Clostridium botulinum*. Lack of data for other protozoa precludes a comparison with more closely related forms.

Summary.

1. The temperature necessary for the thermal sterilization of cultures of *E. gracilis* is 37.5 to 44.0 ° C., depending upon the length of exposure, and the Q_{10} values obtained are 54 to 216. *E. gracilis* will not withstand prolonged freezing.

2. The resistance of cultures of *E. gracilis* to a temperature of 40 ° C. is shown to vary with the p_H of the medium, and the maximum of resistance is at about p_H 5.0.

3. It is shown that the sterilization time sometimes varies with the concentration of organisms, and that *E. gracilis* shows the "skip-stop" phenomenon described for bacteria. This may explained on the basis of variations in individual resistances.

4. An indirect method for determining the number of survivors after various periods of heating is described. By this method it is demonstrated that the survivor's curve is not logarithmic but shows an increasing velocity constant.

Literature cited.

Allee, W. C. (1931): Animal Aggregations. Univ. of Chicago Press.

Allee, W. C. and Bowen, Edith S. (1932): Studies in Animal Aggregations: Mass Protection Against Colloidal Silver Among Goldfishes. Journ. Exper. Zool. Vol. 61 p. 185.

Bracher, R. (1919): Observations on Euglena deses. Ann. of Bot. Vol. 33 p. 93.

— (1929): The Ecology of the Avon Banks at Bristol. Journ. Ecology Vol. 17 p. 35.

Brown, C. W. and Peiser, K. (1916): A Study of the Factors Which Influence the Resistance of Lactic Acid Bacteria to Heat. Tech. Bull. Vol. 30, Mich. Agr. Col. Expt. Sta.

Buchanan, R. E. and Fulmer, E. I. (1929—1930): Physiology and Biochemistry of Bacteria. Vols. I and II. Baltimore.

Chalkley, H. W. (1930): On the Relation Between the Resistance to Heat and the Mechanism of Death in Paramecium. Physiol. Zool. Vol. 3 p. 425.

Chick, H. (1910): The Process of Disinfection by Chemical Agencies and Hot Water. Journ. Hyg. Vol. 10 p. 237.

Eijkman, C. (1908): Die Überlebungskurve bei Abtötung von Bakterien durch Hitze. Biochem. Zeitschr. Bd. 11 p. 12.

Esty, J. R. and Meyer, K. F. (1922): The Heat Resistance of Spores of B. botulinus and Allied Anaerobes. Journ. Infect. Diseases. Vol. 31 p. 650.

Günther, Franz (1928): Über den Bau und die Lebensweise der Euglenen, besonders der Arten E. terricola, geniculata, proxima, sanguinea und lucens nov. spec. Arch. f. Protistenk. Bd. 60 p. 511.

Jacobs, M. H. (1919): Acclimatization as a Factor Affecting the Upper Thermal Death Points of Organisms. Journ. exper. Zool. Vol. 27 p. 427.

Jahn, Theo. L. (1929): Studies on the Physiology of the Euglenoid Flagellates. I. The relation of the density of population to the growth rate of Euglena. Biol. Bull. Vol. 57 p. 81.

— (1930): Studies on the Physiology of the Euglenoid Flagellates. II. The autocatalytic equation and the question of an autocatalyst in growth of Euglena. Ibid. Vol. 58 p. 281.

— (1931): Studies on the Physiology of the Euglenoid Flagellates. III. The effect of hydrogen ion concentration on the growth of Euglena gracilis Klebs. Ibid. Vol. 61 p. 387.

— (1931): The Thermal Death Time of Euglena gracilis Klebs. Anat. Rec. Vol. 51 p. 81.

Klebs, Geo. (1883): Über die Organisation einiger Flagellatengruppen und ihre Beziehungen zu anderen Infusorien. Unters. Bot. Inst. Tübingen Bd. 1 p. 233.

Levine, M., Buchanan, J. H. and Toulouse, J. H. (1927): Influence of Sodium Chloride, Sodium Carbonate, and Tri-Sodium Phosphate on Germicidal Efficiency of Sodium Hydroxide. Iowa State Col. Journ. Sc. Vol. 2 p. 19.

Mainx, Felix (1928): Beiträge zur Morphologie und Physiologie der Eugleninen. I. Teil. Morphologische Beobachtungen, Methoden und Erfolge der Reinkultur. II. Teil. Untersuchungen über die Ernährungs- und Reizphysiologie. Arch. f. Protistenk. Bd. 60 p. 305.

Rahn, Otto (1929): The Size of Bacteria as the Cause of the Logarithmic Order of Death. Journ. Gen. Physiol. Vol. 13 p. 179.

— (1929a): The Non-Logarithmic Order of Death of Some Bacteria. Ibid. Vol. 13 p. 395.

— (1931): The Order of Death of Organisms Larger Than Bacteria. Ibid. Vol. 14 p. 315.

Robertson, T. B. (1921): Experimental Studies on Cellular Multiplication. I. The multiplication of isolated infusoria. Biochem. Journ. Vol. 15 p. 595.

Viljoen, J. A. (1926): Heat Resistance Studies. II. The protective effect of sodium chloride on bacterial spores heated in pea liquor. Journ. Infect. Diseases Vol. 39 p. 286.

Willstätter, R. and Stoll, A. (1928): Investigations on Chlorophyll, Methods and Results. Eng. trans. by F. M. Schertz and A. R. Merz. Sc. Press. Lancaster, Pa.

Woodruff, L. L. and Baitsell, G. A. (1911): The Temperature Coefficient of the Rate of Reproduction of Paramecium aurelia. Amer. Journ. Physiol. Vol. 29 p. 147.

Zumstein, H. (1900): Zur Morphologie und Physiologie der Euglena gracilis. Jahrb. f. wiss. Bot. Bd. 34 p. 149.