A DETERMINATION OF THE TEMPERATURE CHARACTERISTIC OF SPORE GERMINATION IN A PUTREFACTIVE ANAEROBE

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Variation of the equilibrium constant of biological processes with changes in temperature has commonly been assumed to conform, at least over a considerable range of temperature, to the Arrhenius equation (Lardy, 1949), which may be expressed as

$$\frac{d \ln k}{dT} = \frac{\mu}{RT^2} \tag{1}$$

where k = velocity constant of limiting reaction,
T = absolute temperature,
R = gas constant = 1.987 calories per degree per mole, and
μ = temperature characteristic.

The term μ is employed rather than E, the "energy of activation," which is generally employed in similar expressions applied to chemical reactions. Integration of equation (1) and conversion to Briggsian logarithms gives

$$\log k = \frac{-\mu}{2.3R}\left(\frac{1}{T}\right) + C \tag{2}$$

Since this equation is in the slope intercept form $y = mx + b$, it allows the determination of the temperature characteristic by plotting log k vs. $1/T$.

Various investigators have determined μ for such phenomena as yeast fermentation, bacterial growth, and various measurements of bacterial metabolism (Lardy, 1949). To the authors' knowledge, however, no such determinations have been made for the germination of bacterial spores. The present investigation represents chiefly a determination of μ for spore germination in the well-known food spoilage organism commonly designated as putrefactive anaerobe no. 3679.

METHODS

The strain of putrefactive anaerobe no. 3679 was obtained from the Department of Bacteriology of the University of Texas in Austin. It was repurified by the isolation of colonies from serial shake tubes, and spore suspensions were prepared from 15-day cultures in Difco brain heart infusion broth with BBL thioglycolate supplement added. The cultures were heated to 75 C for 30 minutes to destroy vegetative cells, washed three times with sterile distilled water, and

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finally resuspended in sterile distilled water containing glass beads. The suspension was then shaken for 1 hour on a rotary shaking machine and stored at 4 C. By comparisons of counts obtained by plating procedures with direct microscopic counts using the Petroff-Hausser chamber, it was shown by Wynne and Foster (1948*a*) that such a shaking procedure results in a fairly efficient homogenization.

The following general method was utilized in germination experiments. Approximately 1,000 viable spores per ml were inoculated into Difco brain heart infusion broth with BBL thioglycolate supplement added. The pH of the medium was 7.2. The tubes were then heated to 75 C for 20 minutes to remove the dissolved oxygen and to ensure any possible heat activation of the spores (Wynne and Foster, 1948*a*). All tests were run in triplicate and the tubes were incubated in an atmosphere of natural gas. A separate vacuum desiccator was used for each incubation period in order to allow removal of the tubes for counting purposes without disturbing the anaerobiosis of other tubes being incubated for longer periods. The time intervals between successive determinations of the number of germinated spores varied from 2 hours at temperatures above 35 C to 3 hours at temperatures around 20 C. Tubes were stored in a refrigerator at 4 C until all incubation intervals at a given temperature had been completed. Triplicate counts of residual spores were then made on each tube after heating at 75 C for 20 minutes to destroy vegetative cells. The counting medium was Yesair's pork infusion agar with 0.1 per cent soluble starch added (see Wynne and Foster, 1948*a*). An anaerobic seal was obtained by placing 4 ml of 2 per cent agar-agar containing BBL thioglycolate supplement over the solidified counting medium.

RESULTS AND CALCULATIONS

It was observed by Wynne and Foster (1948*b*) that the logarithm of the numbers of residual *Clostridium botulinum* spores in a germinating spore suspension plotted against time gives a straight line until 95 per cent or more of the spores have germinated. Their data appeared to fit the following general equation for a first-order reaction:

$$.434k = \frac{1}{t} \log \frac{I}{I - G} \tag{3}$$

in which k = the velocity constant of germination,
t = time elapsing since beginning of germination (t_o),
I = number of spores per ml at beginning of germination, and
G = number of germinated spores at time t.

In the present investigation, t_o appeared to have values of zero or close to zero, in contrast to the rather pronounced lag phase of germination noted for *Clostridium botulinum* (Wynne and Foster, 1948*b*).

If we let $I - G = S$[2] (residual spores at any given time t), then the germination process may be visualized quantitatively by plotting log S against t.

[2] The symbol S is used in this paper in place of R as employed by Wynne and Foster (1948*b*) to avoid confusion with the gas constant of equation (1).

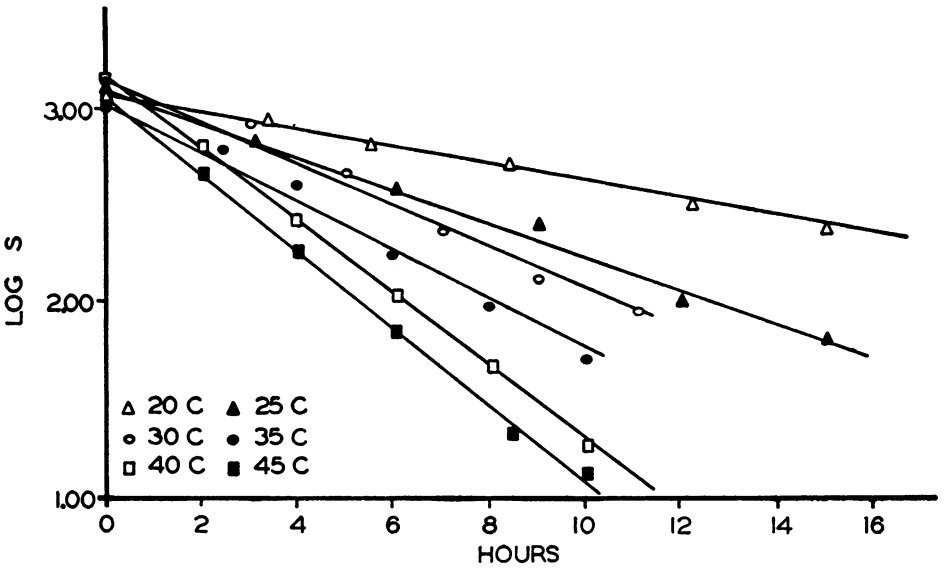

Figure 1. Germination curves of putrefactive anaerobe no. 3679.

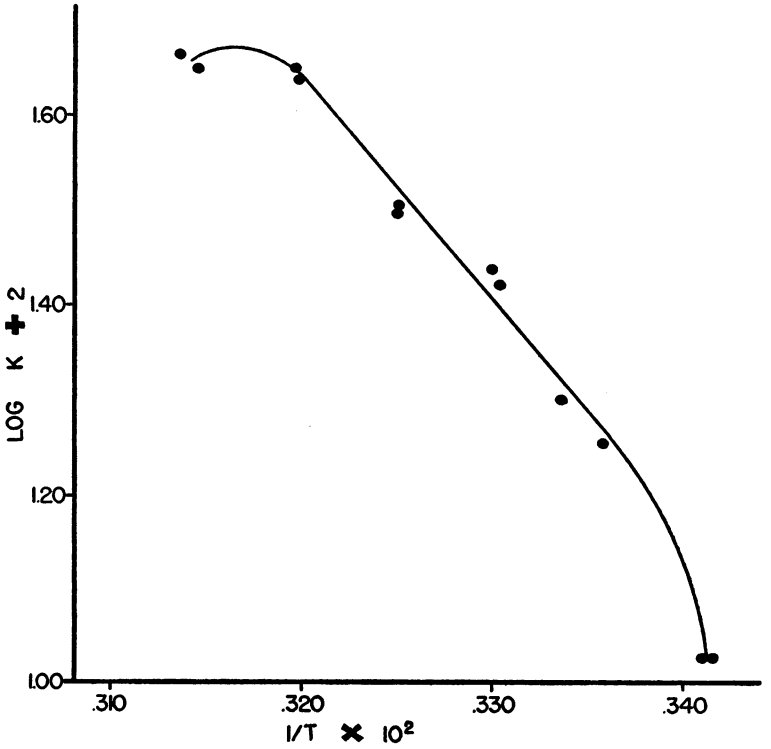

Figure 2. The variation in the rate of germination of putrefactive anaerobe no. 3679 with temperature.

Germination curves for putrefactive anaerobe no. 3679 are shown in figure 1. Actually duplicate determinations were made at temperatures very close to each temperature plotted, but these are omitted from the graph since the results were quite similar.

If equation (3) is expressed in the slope intercept form

$$\log (I - G) = \log S = -.434\ kt + \log I \tag{4}$$

then

$$k = \frac{-m}{.434} \tag{5}$$

where m is the slope of the line obtained by plotting log S vs. t, as in figure 1.

TABLE 1

Velocity constants of germination of spores of putrefactive anaerobe no. 3679 at various temperatures

TEMPERATURE, C	k	TEMPERATURE, C	k
20.0	0.107	34.8	0.324
20.0	0.107	35.0	0.318
25.0	0.182	39.9	0.436
26.0	0.203	40.0	0.445
30.0	0.266	45.0	0.451
30.1	0.277	46.0	0.464

TABLE 2

Values of the temperature coefficient of spore germination in putrefactive anaerobe no. 3679

TEMPERATURE RANGE, C	Q_{10}
20–30	2.49
25–35	1.75
30–40	1.67

Values of m for the lines in figure 1 and for duplicate plots not shown were determined by the method of least squares.

Corresponding values of k for all experimental determinations are given in table 1.

From equation (2) it may be shown that

$$\mu = -4.57\ m' \tag{6}$$

where m' is the slope of the line obtained by plotting log k vs. $1/T$. In figure 2 values for log $k + 2$ and $1/T \times 10^2$ were plotted for convenience. It will be noted that the germination rate appeared to be depressed at temperatures around 45 C and 20 C; therefore in the determination of m' by the method of least squares, the four experimental points at these temperatures were not included. The corresponding value of μ obtained by this method was 10,300 calories.

Values of Q_{10} for spore germination over three temperature intervals are given in table 2.

DISCUSSION

We are well aware of the difficulties, both theoretical and practical, of attempting to determine μ for complex physiological processes such as spore germination. The accuracy of the spore-counting method employed in this work was reported to be $\pm$ 9 per cent for *C. botulinum* (Wynne and Foster, 1948*a*). Although the accuracy of the method was not precisely determined in the case of putrefactive anaerobe no. 3679, it is believed to be at least as high. Values of k can be considered as accurate at best to two significant figures. It is thus conceivable that a continuous variation of log k with $1/T$ might be masked by the innate experimental error, with an apparent straight line resulting on plotting these quantities. In this connection it is perhaps pertinent to cite the warning of Buchanan and Fulmer (1930) that even the method of plotting may give such spurious results if, e.g., the unit chosen for plotting log k is sufficiently small.

Crozier (1924) has offered an intriguing explanation for the nonlinearity at times observed in plots of variables analogous to log k and $1/T$. According to his view such biological processes as respiration, fermentation, and growth are resultants of a series of catenary reactions, with different members of the series becoming the limiting factor in the velocity of the over-all reaction at different temperatures. If the reactions in such a series have different temperature characteristics, then the value of μ for the over-all process will change abruptly at definite "critical temperatures." Inspection of figure 2 reveals that such a critical temperature may exist between 20 C and 25 C in spore germination in putrefactive anaerobe no. 3679.

According to Wynne and Foster (1948*a*), spore germination may be regarded as a change from a thermoresistant spore to a thermolabile entity, which may not necessarily be a true vegetative cell. It was claimed that the appearance of turbidity cannot be a valid quantitative measure of the rate of germination. This conclusion was confirmed in the present work, since at 20 C turbidity appeared when only 50 per cent or less of the spores had germinated, but at 40 C and 45 C almost complete germination was accomplished before the appearance of turbidity.

This observed lack of correlation between the amount of germination and the time of appearance of turbidity at various temperatures would seem to indicate that the rate of germination and the rate of vegetative cell increase are apparently affected differently by temperature changes. However, a comparison of temperature characteristics for vegetative cell growth measured as an increase in cell mass or numbers with the temperature characteristic of spore germination would be of little or no significance, since values of μ for vegetative cell growth may apparently vary even within a given species, depending upon the criterion of growth utilized (see Buchanan and Fulmer, 1930).

SUMMARY

Spore germination in putrefactive anaerobe no. 3679 was apparently logarithmic at all temperatures studied. The general method of Wynne and Foster (1948*a*, *b*) was employed for the determination of rate constants of germination at var-

ious temperatures, followed by substitution in the Arrhenius equation for the calculation of μ. A value of 10,300 calories was found for the temperature characteristic of spore germination.

The optimum temperature for germination appeared to lie between 40 C and 45 C. At temperatures of approximately 20 C, germination was somewhat retarded, indicating the possible occurrence between 20 and 25 C of a "critical temperature" in the sense of Crozier (1924).

The rate of spore germination and the rate of subsequent vegetative development were apparently affected differently by temperature changes.

REFERENCES

BUCHANAN, R. E., AND FULMER, E. I. 1930 Physiology and biochemistry of bacteria. Vol. II. Williams & Wilkins Co., Baltimore, Md.

CROZIER, W. T. 1924 On biological oxidations as a function of temperature. J. Gen. Physiol., **7**, 189–216.

LARDY, HENRY A. 1949 Respiratory enzymes. Burgess Publishing Co., Minneapolis, Minn.

WYNNE, E. S., AND FOSTER, J. W. 1948*a* Physiological studies on spore germination, with special reference to *Clostridium botulinum*. I. Development of a quantitative method. J. Bact., **55**, 61–68.

WYNNE, E. S., AND FOSTER, J. W. 1948*b* Physiological studies on spore germination, with special reference to *Clostridium botulinum*. II. Quantitative aspects of the germination process. J. Bact., **55**, 69–73.