INFLUENCE OF TEMPERATURE AND HYDROGEN ION CONCENTRATION UPON THE SPORE CYCLE OF BACILLUS SUBTILIS.

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The investigation here reported deals with the influence of hydrogen ion concentration at different temperatures upon the spore cycle of *Bacillus subtilis*.[1] This microorganism was chosen because it is a classical spore-bearing species, which had been used in our previous investigations on the relation of the hydrogen ion concentration of the medium to proteolytic activity.

TABLE I.

*Limiting Reactions.**

Organism.	pH	Author.
B. coli	5.0	Michaelis and Marcora.†
" " (low gas ratio group)	4.3–5.3	Clark, 1915.‡
Streptococcus, Group I	4.6–4.8	Ayers, 1916.§
" " II	5.5–6.0	" 1916.§
S. erysipelatis	4.8	Itano, 1916.‖
B. subtilis (acid limit)	4.2	" 1916.‖
" " (alkali limit)	9.4	" 1916.‖

* Clark, W. M., and Lubs, H. A., *J. Bacteriol.*, 1917, ii, 1, 222.

† Michaelis, L., and Marcora, F., *Immunitätsforsch., Orig.*, 1912, xiv, 170.

‡ Clark, W. M., *J. Biol. Chem.*, 1915, xxii, 87.

§ Ayers, S. H., *J. Bacteriol.*, 1916, i, 84.

‖ Itano, A., *Massachusetts Agric. Exp. Sta., Bull. 167*, 1916, 139.

[1] The same strain of this species was used as in the previous investigations on proteolytic activity; the strain was obtained from the American Museum of Natural History.

We have found no references in the literature to the particular problem involved, but it may be interesting to present in tabular form the limiting reactions of the medium for some bacteria.

Table I indicates that these organisms survive in a certain limited range of hydrogen ion concentrations, and that slight changes in the concentration of the hydrogen ion influence the organism to a great extent.

Method of Procedure.

Preparation of Media.—The media used in this investigation had the same composition and were prepared in the same way as those described in a previous article.[2] The pH was determined electrometrically and was found to be as shown in Table II.

TABLE II.

Preparation of Media of Different pH Values.

pH desired.....	1	2	3	4	5	6	7	8	9	10	11	12	13
pH found.....	1.2	2.0	3.0	4.1	5.2	6.1	7.1	8.2	9.2	10.1	11.0	11.5	12.7

Throughout the following account, we have used the approximate figures in referring to the various pH values; *i.e.*, desired pH is used to denote the hydrogen ion concentration of the broth.

Method Used in Obtaining Free Spores.—The spores of the organism were obtained as follows: A portion of a young agar culture of *Bacillus subtilis* was emulsified in sterile 0.85 per cent salt solution. The emulsion was then transferred to a Roux flask containing standard agar, and the organisms were distributed over the surface of the medium. The flask was incubated at 30°C. for 15 days and was then kept at 25°C. for 3 weeks. At the time of the experiment the culture contained practically nothing but free spores.

Preparation of Moist Chambers.—Two moist chambers were made for each pH value for each temperature. The chambers were prepared in the usual way, using paraffin of high melting point to seal the ring to the slide. By means of a platinum loop 2 mm. in diameter,

[2] Itano, A., *Massachusetts Agric. Exp. Sta., Bull. 167*, 1916, 164.

a drop of medium of each pH value was put upon a sterile cover-slip which was then carefully sealed upon the ring of the moist chamber. The moist chamber preparations were then kept for 12 hours at the temperature to be used in the subsequent incubation.

Preparation of Dilute Emulsion of Spores.—Two drops of the medium of each pH value were put in the cells of sterile concave slides in sterile Petri dishes. With a straight needle a small inoculum of spores was put into the first drop of broth of each pH value. From this drop the second drop of the same pH value was then inoculated by use of the straight needle. The second drop was used as the source of the final inoculum.

Procedure in this manner with the medium of each pH value gave a very dilute emulsion of free spores in broth of each pH value. By this procedure we obtained for the inoculation of the hanging drops a satisfactory source for a small number of spores, well washed of all metabolic products; moreover, any of the medium carried over with the final inoculum was of the same pH value and of the same composition as the drop inoculated.

Inoculation of Drops.—The hanging drops of the moist chamber preparations were then inoculated; those of each pH value were inoculated from the dilute emulsion of spores in broth of the same pH value. By using a straight needle for transferring and by stirring the inoculated drop, a drop was obtained containing a satisfactory number of spores well distributed throughout the drop.

Examination of Preparations and Conditions of Observation.—Immediately after inoculation the preparations were placed under a microscope and examined for absence of vegetative forms and for even distribution of the spores. After examination the preparations were placed at the temperature of incubation. Observations were made at intervals of 30 minutes for the first 5 hours and then at intervals of 1 hour.

RESULTS.

Series I (5°C.).—This series (pH 1 to 13) was incubated at 5°C. for 20 days.[3] No apparent change took place except a slight swelling of the spores in all hydrogen ion concentrations. This temperature

[3] The cold storage room of the Dairy Department of this institution was used.

seems to be near the limiting temperature for spore germination of *Bacillus subtilis*. Schreiber[4] reports the germination of spores of this organism at 8°C. after 7 days, but states that the spore cycle is not completed at this temperature.

Series II (25°C.).—This series was kept at room temperature, which varied during the time of the experiment from 23.5–25°C. The observations and results are given in Table III.

TABLE III.

Time in Hours Required by B. subtilis to Reach Various Stages of Development at Different pH Values. (Temperature Approximately 25°C.)

pH	Spore.		Vegetative cell.				Spore.	
	Swollen.	Germination begins.	Single.	Chains.	Granule.	Refractive body.	Endospore.	Free spore.
	hrs.	*hrs.*	*hrs.*	*hrs.*	*hrs.*	*hrs.*	*hrs.*	*hrs.*
1	S							
2	"							
3	"							
4	"							
5	2.5	7.5	10.0	12.0	25.0	30.0	32.0	50.0
6	2.5	6.0	8.0	9.0	22.0	30.0	31.0	46.0
7	2.0	4.0	5.0	6.0	20.0	26.0	29.0	39.0
8	2.0	3.0	4.0	5.0	16.0	41.0	51.0	65.0
9	3.0	7.0	10.0	13.0	20.0	44.0	52.0	66.0
10	3.5	8.0	11.0	15.0	22.0	25.0	31.0	39.0*
11	S							
12	"							
13	"							

Figures represent average of four experiments.

S indicates no apparent change except slight swelling and a certain loss of refrangibility.

* Few spores germinated; chains are short; not all cells formed spores.

The results in Table III show that at 25°C. the spores of *Bacillus subtilis* germinated in broth at pH values from 5 to 10, while higher and lower concentrations of hydrogen ions inhibited their development. At pH 7 and pH 8, which are nearest the optimum hydrogen ion concentration for this organism, germination took place in the shortest time and multiplication was most rapid.

[4] Schreiber, O., *Centralbl. Bakteriol., 1te Abt.*, 1896, xx, 432.

An interesting phenomenon was observed in the broth at pH 5, 6, and 9. At the time given in Table III only a few spores had germinated. These first vegetative cells did not exhibit the characteristic motility of this species, neither did rapid multiplication begin at once. Several hours later, however, after most of the spores had germinated the vegetative cells became actively motile and multiplication became much more rapid, indicating that by the life processes of the organisms

TABLE IV.

Time in Hours Required by B. subtilis to Reach Various Stages of Development at Different pH Values. (Temperature 37°C.)

pH	Spore.		Vegetative cell.				Spore.	
	Swollen.	Germination begins.	Single.	Chains.	Granule.	Refractive body.	Endo-spore.	Free spore.
	hrs.	*hrs.*	*hrs.*	*hrs.*	*hrs.*	*hrs.*	*hrs.*	*hrs.*
1	S							
2	"							
3	"							
4	"							
5	2.0	11.0	13.0	15.0	20.0	24.0	27.0	32.0
6	1.5	8.0	9.0	10.0	14.0	19.0	22.0	28.0
7	1.5	5.0	7.0	9.0	12.0	17.0	19.0	26.0
8	1.5	3.5	6.0	8.0	10.0	12.0	13.0	20.0
9	1.5	4.0	5.0	7.0	8.0	10.0	14.0	23.0
10	1.5	11.0	12.0	14.0	15.0	18.0	25.0	35.0
11	S							
12	"							
13	"							

Figures represent average of four experiments.

S indicates no apparent change except slight swelling and a certain loss of refrangibility.

the reaction of the medium was approaching the optimum. In view of facts shown in a previous publication,[5] this behavior may be explained as a manifestation of the beginning of the automatic adjustment of the medium.

The behavior in pH 10 was also interesting. Only a few of the spores germinated and these passed into the spore stage in a comparatively short time without much further multiplication.

[5] Itano, A., *Massachusetts Agric. Exp. Sta., Bull. 167*, 1916, 174.

Series III (37°C.).—This series was kept in the incubator at 37°C. The results are given in Table IV.

In general Table IV shows the same results as Table III, except that the rate of completion of the cycle was accelerated at the higher

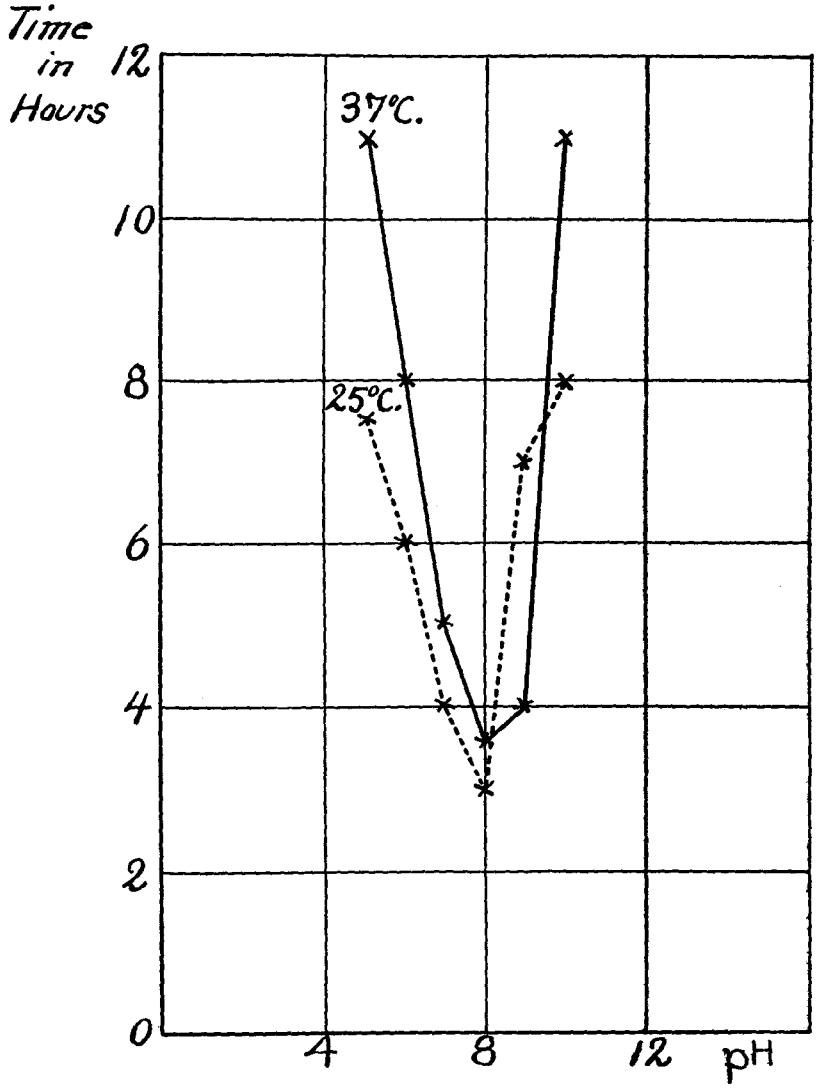

FIG. 1. Curves showing the time required for the germination of spores of *B. subtilis* in broth of different pH values at 25° and 37°C.

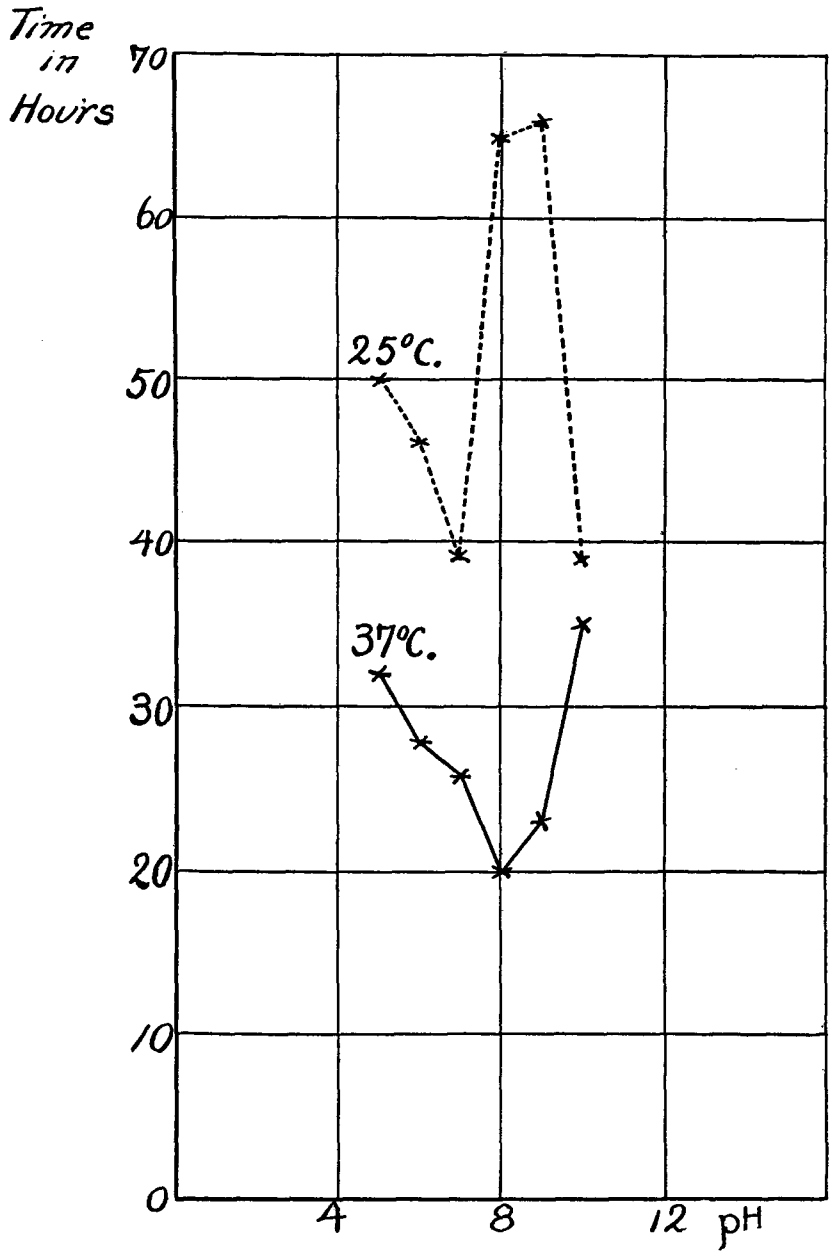

FIG. 2. Curves showing the time required for completion of the spore cycle of *B. subtilis* in broth of different pH values at 25° and 37°C.

temperature. The time required for germination does not show this acceleration, as is evident from Fig. 1. This indicates that 37° is decidedly above the optimum for germination.

The time required for the completion of the spore cycle is shown graphically in Fig. 2, which indicates that the spore cycle was completed only at pH 5 to 10 and that the rate of completion is accelerated by the rise in temperature. The acceleration, however, is irregular and does not maintain any uniformity throughout the series. The irregularity in the curve may be due to the previously mentioned automatic adjustment of the reaction of the media during the period of active growth which is included in the spore cycle. In broth of pH 5, 6, and 9, the hydrogen ion concentration would be gradually approaching the optimum during the period of active growth.

The apparent discrepancy in pH 10 of the 25°C. is probably due to the formation of spores because of the unfitness of medium of this pH value for growth and multiplication. The slight difference in time required for spore formation in broth of this pH value at 25° and 37° indicates that spore formation in this medium is probably not induced by products of metabolism.

SUMMARY AND CONCLUSIONS.

1. At 5°C. no germination took place.

2. At 25°C. and at 37°C. germination occurs if the hydrogen ion concentration of the broth is kept between pH 5 and pH 10, but not at higher or lower pH values.

3. The completion of the spore cycle likewise requires a hydrogen ion concentration between pH 5 and pH 10.

4. The spores can germinate when the pH value is 10, although after germination the vegetative cells multiply only to a very slight extent and soon pass into spores.

5. The slight growth and multiplication of vegetative cells in broth of pH 10 suggest that the formation of endospores in this medium must be caused largely by the unfavorable reaction of the medium rather than by the accumulation of metabolic products.

6. Automatic adjustment of the medium seems to play a rôle in the completion of the spore cycle.

7. The results are not only of theoretical importance but they have a practical application to the preservation of food by canning and by other methods.