CORRELATION OF ENVIRONMENTAL AND BIOLOGICAL CHANGES OCCURRING IN A COMPLEX MEDIUM DURING GROWTH AND SPORULATION OF *BACILLUS* SPECIES

NORMAN G. ROTH,¹ DAVID H. LIVELY,² AND S. NICHOLAS METCALFE, JR.

Chemical Corps, Fort Detrick, Frederick, Maryland

Received for publication October 28, 1957

The extensive literature on environmental factors which affect formation of endospores by members of the genus *Bacillus* has been adequately reviewed by Knaysi (1945, 1948) and more recently by Ordal (1956). In most cases the effect of variation of environmental conditions on total spore production or per cent sporulation was studied. Factors considered include type and initial concentration of nutrients, molecular oxygen, pH, accumulation of by-products, and desiccation.

Knaysi (1945) pointed out that these various factors were not independent variables, but in reality were so interrelated that the role of one could not be elucidated without considering its relation to all of the others.

In the course of studies on the influence of environmental factors on sporulation, measurements were made of many of the changes occurring in the environment during vegetative growth and spore formation. These changes are reviewed in the articles previously mentioned. However, of the measurements reported, many were made under conditions in which sporulation was incomplete or total growth was of a low order of magnitude. Reports of extensive measurements under conditions which favored both essentially complete sporulation of all vegetative cells and a high final spore count could not be found.

In this work, a series of concomitant measurements of biological, physical, and chemical changes were made at 2-hr intervals during growth and sporulation of *Bacillus anthracis* and *Bacillus subtilis* var. *niger*. Growth conditions were rigidly controlled so that greater than 95 per cent sporulation and a final count of greater than two billion spores per ml was achieved in all cultures.

¹ Present address: Whirlpool Corp., St. Joseph, Mich.

² Present address: University of Texas, Austin, Texas.

MATERIALS AND METHODS

Organisms. The test organisms were a typical, chain-forming, virulent *Bacillus anthracis* which was a sib-progeny of the Vollum strain and the Fort Detrick strain of *Bacillus subtilis* var. *niger* (previously identified by reports from this laboratory as *Bacillus globigii*).

Method of growing cultures. Cultures were grown in a series of 2-L Erlenmeyer shake flasks containing 160 ml of medium. Each flask was inoculated with a 5 per cent (by volume) heat-shocked, triple-washed spore suspension containing 1.5 to 2.0×10^9 spores/ml. The cultures were incubated at 34 C on a reciprocating shaker adjusted to 99 3-in strokes/min. Previous work by this laboratory has shown that this ratio, medium volume/flask size, was optimum for growth and sporulation of *B. anthracis* under these conditions (Roth *et al.*, 1955; Auro *et al.*, 1957). At 2-hr intervals a flask was removed from the shaker and the various tests were performed. All curves reported are averages of at least three independent replications performed in this manner.

The growth medium employed in the experiments consisted of the following: H₂SO₄ partial hydrolyzate of casein, 1.2 mg Kjeldahl nitrogen per ml; and, in per cent, autolyzed yeast, 0.5; K₂HPO₄, 0.5; glucose, 0.75; thiamin·HCl, 0.0025; MgSO₄·7H₂O, 0.005; MnSO₄·4H₂O, 0.003; FeSO₄·7H₂O, 0.001, made up in tap water; pH 7.5 ± 0.1.

Procedure for physical tests. Turbidity was measured in a Coleman spectrophotometer using a wave length of 650 mμ. Equal volumes of culture and 40 per cent formaldehyde solution were mixed in a dilution bottle, agitated by swirling 10 to 15 times, and allowed to stand for 30 min. A 1:10 aqueous dilution of this suspension was used for turbidity measurements.

Total solids were determined on a 5-ml sample, diluted 1:1 with 40 per cent formaldehyde. Sus-

pensions were dried to a constant weight in a vacuum oven at 80 C.

Using undiluted culture, pH was determined with a Beckman pH meter using a glass-calomel electrode system; specific gravity was measured by means of a Fisher hydrometer. Viscosity was measured with a calibrated Ostwald Viscosimeter, series 200. The viscosity of the cultures was obtained by measuring the efflux time for the samples at 30 ± 1 C. The efflux times for the samples were measured by 5 readings of flow with a variation of not more than 0.1 sec in 3 determinations.

Volume of sediment was measured using a 10 ml sample in a calibrated 15-ml centrifuge tube after 10 min centrifugation at 2500 rpm in a Clay-Adams angle centrifuge with a maximum radius of 12 mm.

Procedure for chemical tests. At 2-hr intervals, cultures were removed from the shaker and immediately centrifuged at 2500 rpm for 10 min. The supernatant was decanted and autoclaved for 20 min at 121 C. In a preliminary experiment using *B. subtilis* var. *niger*, it was shown that there was no difference in the carbohydrate or nitrogen values before and after autoclaving.

Total nitrogen was determined on the supernatant by the standard Kjeldahl method and amino nitrogen by the Van Slyke method (AOAC, 1945). A standard Kjeldahl analysis for total nonprotein nitrogen was performed on the supernatant remaining after removal of the protein with 8 per cent trichloroacetic acid.

Total carbohydrate and reducing sugar in the supernatant were determined colorimetrically; the former by the Anthrone test and the latter by the copper reduction method (Neish, 1952).

Procedure for biological tests. Per cent sporulation, average number of cells per chain, and per cent autolysis of vegetative material were determined by microscopic examination of smears stained by the Schaeffer-Fulton technique (1933).

RESULTS

Sporulation, chain-formation, and autolysis.

Under the growth conditions used in these experiments, the first microscopic evidence of sporulation occurred at about 12 hr of incubation in the case of *B. anthracis* and at about 14 hr in the case of *B. subtilis* var. *niger*. Sporulation proceeded at a very rapid rate after initiation and, with both organisms, 90 to 95 per cent of

the cells had sporulated within 6 hr after the first sign of sporulation (figures 1 and 2). Cultures of both organisms reach viable spore counts of 2 to 3×10^9 at the peak of sporulation.

The curves of average chain length for the two organisms were quite different. In the case of *B. anthracis*, chain formation was quite apparent after 4 hr of incubation (figure 1). Chain formation increased throughout the vegetative growth phase until an average chain length of approximately 20 cells was achieved after 12 hr. At this point, vegetative growth apparently ceased, spore production began, and chain length remained unchanged. Shortly after sporulation began (i. e., after 12 to 14 hr incubation), the vegetative chains began to break up. The rate of chain break-up proceeded at about the same rate as that of chain formation and vegetative chains essentially were completely broken up by the time sporulation was complete.

After sporulation and vegetative chain break-up of *B. anthracis* was essentially complete (i. e., after about 20 hr of incubation), the effect of autolytic enzymes became visibly apparent; vegetative material disappeared rapidly. When per cent autolysis of vegetative material was

Figure 1. Microscopic appearance, *Bacillus anthracis*.

Figure 2. Microscopic appearance, *Bacillus subtilis* var. *niger*.

plotted against incubation time, an "S" shaped curve similar to the curve for sporulation rate resulted (figure 1). Autolysis was essentially complete within 6 hr. It should be noted that appreciable visible evidence of autolysis did not occur until the chains had broken to the one- or two-cell length. Chain break-up and sporulation seemed to be complete at about the same time; autolysis of the vegetative material then began. These data indicate that the chain break-up was either completely independent of the autolytic enzymes or that the enzymes attacked the linkage between vegetative cells before attacking the rest of the vegetative material.

In an attempt to determine if the autolytic enzymes of *B. anthracis* were responsible for breaking the linkage, a technique similar to that used by Greenberg and Halvorson (1955) with *B. terminalis* was employed. Vegetative cells of a 6-hr culture averaging 12 cells per chain were shaken with cell free filtrate of a completely autolyzed 22-hr culture. Suspensions were shaken using the same aeration and temperature conditions as those employed during growth and sporulation. Smears were made on cultures after 0, 0.5, 1, 2, 4, 8, 24, 48, and 96 hr of shaking

under these conditions. The smears were stained by the Schaeffer-Fulton technique and average number of cells per chain determined microscopically. The initial average level of 12 cells per chain was maintained after incubation periods of up to 96 hr. Practically no autolysis was observed and approximately 75 per cent of the cells had sporulated within 24 hr. No conclusions could be made on the mechanism of chain break-up; however, it appeared that, under the experimental conditions, extracellular autolytic enzymes were either not produced or inactive.

The degree of autolysis as observed under the ordinary oil immersion lens of the microscope seemed to indicate that the spores were entirely free; however, electron microscope studies reported elsewhere (Roth *et al.*, 1956) showed that some of these spores actually were linked together in pairs or short chains by plasmodesmoid extensions of the spore coat.

In the case of *B. subtilis* var. *niger*, no chain-formation occurred (figure 2). The cells remained in pairs throughout a greater portion of the growth cycle. The pairs broke up again to the single cell stage after sporulation was complete. With this organism, autolysis was initiated at about the same time as that with *B. anthracis*; however autolysis proceeded at a lower rate with less than 50 per cent of the vegetative material autolyzed after 24 hr.

Physical changes. Changes in the physical characteristics of growth media are shown in figures 3 through 7. All curves shown represent the average of at least three independent repli-

Figure 3. Viscosity

Figure 4. Volume of sediment

Figure 5. Turbidity

cations. In general, the physical changes observed in the cultures were very consistent between replications.

With *B. anthracis* as the test organism, viscosity (figure 3) and volume of sediment (figure 4) paralleled the average chain length. Both viscosity and volume of sediment increased as chain length increased and then, as chains broke up, these values decreased at approximately the same rate. With *B. subtilis* var. *niger*, in which chain formation did not occur, viscosity increased directly with growth and leveled off after sporulation took place. Contrary to the *B. anthracis* data, there appeared to be no measurable decrease in viscosity after sporulation was complete. A slight decrease in volume of sediment occurred with *B. subtilis* var. *niger* at the point where pairs broke up to single cells; however, it was of a much lower magnitude than that occurring with chain-forming *B. anthracis*. With both organisms, any decrease in viscosity

or volume of sediment appeared to be associated with break-up of chains rather than with visible autolysis of vegetative material.

Turbidity (figure 5) in both cases increased with growth and reached a peak shortly before sporulation began. There was a slight decrease in turbidity during the period of almost complete autolysis of vegetative material which occurred with *B. anthracis*; essentially no decrease in turbidity accompanied partial vegetative autolysis occurring with *B. subtilis* var. *niger*.

The pH of the growth medium of *B. anthracis* showed initially a slight drop after germination; a slight rise in pH occurred during the vegetative and presporulation stages; a sharp increase in pH accompanied sporulation with a peak slightly above pH 8.0 at the time the sporulation process

Figure 6. pH

Figure 7. Total solids

was complete; pH was constant after sporulation (figure 6). With *B. subtilis* var. *niger* the pH change was similar but more extreme; a greater drop occurred during the early, postgermination stage and a higher pH was attained at the end of the cycle. With both organisms, the sharp pH rise appeared to parallel or slightly precede sporulation and seemed to reach a constant value when sporulation was complete.

With both organisms, total solids of culture (i. e., cells and medium) decreased sharply before spore production and then leveled off after sporulation had begun (figure 7). These determinations indicated that the combined total solids of culture medium ingredients and organisms had its greatest loss in the presporulation period when ingredients were actively being metabolized and a portion of the material was volatilizing away. The total solids markedly paralleled the sugar utilization curves.

Specific gravity values of cultures of the test organisms remained relatively constant in a range of between 1.010 and 1.020 throughout the entire cycle.

Chemical changes. With both organisms, there was a rapid and almost complete utilization of available carbohydrate during the vegetative or presporulation phase (figures 8 and 9). Between 2 and 4 hr before any sporulation was apparent, essentially all of the carbohydrate had been utilized. A slight decrease in rate of utilization of carbohydrate was noted in the case of *B. an-*

Figure 8. Carbohydrate utilization, *Bacillus anthracis*.

Figure 9. Carbohydrate utilization, *Bacillus subtilis* var. *niger*.

Figure 10. Nitrogen utilization, *Bacillus anthracis*.

thraxis after this point; this decrease may have been due to the action of trace enzymes released into the culture medium or it may indicate a low exogenous energy requirement during sporulation and autolysis. Since no utilization of carbohydrate by *B. subtilis* var. *niger* was noted after the initial drop, the former explanation is most likely. The difference between the total carbohydrate values and reducing values was constant and negligible.

Results of the nitrogen analysis indicated a pattern similar to carbohydrate values (figures

Figure 11. Nitrogen utilization, *Bacillus subtilis* var. *niger*.

10 and 11). A large portion of the utilizable nitrogen was assimilated 2 to 4 hr before sporulation took place. A relatively sharp increase in total nitrogen and nonprotein nitrogen values occurred during autolysis of *B. anthracis*; a more gradual increase took place during incomplete autolysis which occurred with *B. subtilis* var. *niger*. This increase undoubtedly was accounted for by the nitrogen released into the culture medium during autolysis. During autolysis of *B. anthracis*, there was no apparent increase in the amino nitrogen content of the culture medium; this observation indicated that the autolytic products probably were polypeptide in nature. As indicated by the constant difference between the total and nonprotein-nitrogen curves, unhydrolyzed protein nitrogen apparently was not utilized.

DISCUSSION

The data presented in this paper include a series of measurements of certain biological, physical, and chemical characteristics occurring during the process of spore production of certain bacilli in liquid media. The cultures were incubated under conditions which resulted in essentially complete sporulation of all vegetative cells and yielded greater than 2 billion spores per ml. All measurements at each time interval were made on samples taken from the same culture.

It is apparent from observation of these data that utilization of both carbohydrate and nitro-

genous material is complete before sporulation begins. As first pointed out by Hardwick and Foster (1952, 1953) it appears that the nutritional requirements for sporulation must be satisfied before sporulation occurs. Even though there is considerable nitrogenous material available in the medium after sporulation has been initiated, none of the exogenous nitrogen appears to be used in the sporulation process. This has been illustrated quite conclusively by the work of Foster and Perry (1954) who studied the uptake of radioactive methionine. Our results are in complete agreement with their conclusions.

The phenomenon of chain formation and chain break-up in *B. anthracis* is of special interest. Reports of *B. anthracis* growing *in vitro* without forming chains are lacking. Personal communications with many individuals who have worked on this organism indicate that such a phenomenon has not been observed. However, it is well known that this organism, when grown *in vivo*, forms neither chains nor spores. The observations reported here indicate that *in vitro* chain formation accompanies vegetative growth and precedes spore formation. Vegetative chain break-up accompanies sporulation and precedes other evidences of autolysis. Whether or not the vegetative chains break up because of the autolytic enzymes or because of some other factor is uncertain. In fact, the data presented here merely describe the process of chain formation and break-up of *B. anthracis* in relation to other changes occurring in the culture but throw no light on the mechanism. The mechanism of chain formation as it occurs *in vitro* vs. lack of chain formation as observed *in vivo* is worthy of further investigation.

ACKNOWLEDGMENT

The authors wish to express their appreciation to Mr. Jule Dews, Chemical Corps, Fort Detrick, for assistance with some of the chemical tests

SUMMARY

A series of concomitant measurements of biological, physical, and chemical changes occurring during growth and sporulation of *Bacillus anthracis* and *Bacillus subtilis* var. *niger* under conditions which favor essentially complete sporulation and a high final spore count are reported. With chain-forming *B. anthracis*, viscosity and volume of sediment increases as

chain length increases and then decreases as chains break up; with nonchain-forming *B. subtilis* var. *niger*, these values increase directly with growth and do not decrease measurably after growth is complete. In both cases turbidity increases with growth and reaches a peak shortly after sporulation begins; a drop in turbidity is associated with autolysis of vegetative material. The pH of the growth medium of both organisms decreases slightly from neutrality shortly after germination occurs, rises slightly during the vegetative stage, increases sharply to a pH greater than 8.0 during sporulation, and levels off after sporulation. Combined total solids of medium plus cells decrease sharply during vegetative growth and level off after sporulation has begun. No measurable changes in specific gravity of cultures occurs. Rapid utilization of carbohydrate and nitrogen occurs during the vegetative phase and is essentially complete 2 to 4 hr before sporulation is apparent. Increase in extracellular nitrogen occurs during autolysis.

REFERENCES

- Association of Official Agricultural Chemists (A.O.A.C.) 1945 Official and Tentative Methods of Analysis, 6th Ed. Washington D.C.
- AURO, M. A., HODGE, H. M., AND ROTH, N. G. 1957 Correlation of oxygen absorption rates in shaken flasks. *Ind. Eng. Chem.*, (in press).
- FOSTER, J. W. AND PERRY, J. J. 1954 Intracellular events occurring during endotrophic sporulation in *Bacillus mycoides*. *J. Bacteriol.*, **67**, 295-302.
- GREENBERG, R. A. AND HALVORSON, H. O. 1955 Studies on an autolytic substance produced by an aerobic sporeforming bacterium. *J. Bacteriol.*, **69**, 45-50.
- HARDWICK, W. A. AND FOSTER, J. W. 1953 Enzymatic changes during sporogenesis in some aerobic bacteria. *J. Bacteriol.*, **65**, 355-360.
- HARDWICK, W. A. AND FOSTER, J. W. 1952 On the nature of sporogenesis in some aerobic bacteria. *J. Gen. Physiol.*, **35**, 907-927.
- KNAYSI, G. 1945 A study of some environmental factors which control endospore formation by a strain of *Bacillus mycoides*. *J. Bacteriol.*, **49**, 473-493.
- KNAYSI, G. 1948 The endospore of bacteria. *Bacteriol. Revs.*, **12**, 19-77.
- NEISH, A. C. 1952 Analytical methods for bacterial fermentation. National Research Council. Can., Report No. 46-8-3.
- ORDAL, Z. J. 1956 Environmental and nutritional factors affecting sporulation. Conference on Bacterial spores. Allerton Park, Ill. Oct. 11-12, 1956.
- ROTH, N. G., LIVELY, D. H., AND HODGE, H. M. 1955 Influence of oxygen uptake and age of culture on sporulation of *Bacillus anthracis* and *Bacillus globigii*. *J. Bacteriol.*, **69**, 455-459.
- ROTH, N. G., WOLFE, V. L., AND MARCUS, L., 1956 Studies on the Plasmodesm-like Linkage between Spores of *Bacillus anthracis*. *J. Bacteriol.*, **72**, 344-348.
- SCHAEFFER, A. B. AND FULTON, MAC D. 1933 A simplified method of staining endospores. *Science*, **77**, 194.