CYTOLOGICAL OBSERVATIONS ON THE SPORULATION PROCESS OF CLOSTRIDIUM PERFRINGENS

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Cytological studies on the sporulation process of Clostridium perfringens have been limited by the fact that the organism sporulates very poorly on most laboratory media. Attempts to promote extensive sporulation of this organism have met with little success in the past (Simmonds, 1915; Adamson, 1918; Jahbons, 1920; Headlee, 1931; Bethge, 1947). Some investigators (Hobbs et al., 1953) have had to resort to procedures lasting up to seven weeks in order to obtain spores. Recently, however, a medium was described (Ellner, 1956) in which sporulation of C. perfringens takes place rapidly and quantitatively. This medium readily permits serial observations on the cytological changes which occur during sporulation of this organism. This paper summarizes cytological observations made on C. perfringens during its sporulation cycle.

MATERIALS AND METHODS

The organism used was a strain of C. perfringens obtained from the Department of Bacteriology of the Walter Reed Army Institute of Research, Washington, D. C.

Two media were employed during this investigation: (1) brain heart infusion broth (Difco) and (2) Ellner’s medium, a peptone yeast extract starch MgSO4 formula buffered at pH 7.8. Anaerobic conditions for work with C. perfringens were established in both media by placing deep tubes of the media in a boiling water bath for 10 to 15 min and then rapidly cooling the tubes to room temp prior to inoculation. Prior to the withdrawal of inocula or samples, cultures were mildly agitated by pipette to insure the mixing of settled and suspended cells. The infusion broth (vegetative medium) was employed to establish the actively growing vegetative phase of the organism from which the inoculum for the sporulation medium was obtained. The sources of inoculum for the production of the vegetative cells were stock cultures maintained in fluid thioglycolate medium (BBL) or spore suspensions in Ellner’s medium. When 0.1 ml of either stock culture was introduced by pipette into the bottom of a tube of the vegetative medium, there was rapid and heavy growth within 6 hr at 37 C. For induction of sporulation, 0.5 ml of a 12-hr vegetative culture was introduced by pipette into the bottom of a tube of the sporulation medium. Incubation during sporulation was at 37 C.

Specimens for microscopy were prepared by the following technique. At hourly intervals 0.2 to 0.3 ml of the culture was transferred from the sporulation medium to a relatively dry agar plate and spread evenly over about one-half the surface with a glass spreader. After absorption of the medium had taken place, impression smears were made by cutting out agar blocks and inverting them on 22 mm square, 0 thickness cover glasses. The inverted blocks were removed and the impression smears allowed to air dry without further fixation. The impression smears then were treated in a mordant of 0.2 per cent KMnO4 at pH 7.0 for 5 min, after which they were washed in running distilled water and then stained in 1.0 per cent safranin, pH 8.5, for 3 min. The stained smears were mounted in distilled water. A previous reference to the permanganate safranin sequence is to be found in Lee’s Vade Mecum (1937, p. 184). Parallel preparations were made in which the air dried smears were hydrolyzed in N HCl at 60 C for 5 min prior to the mordanting and staining steps.

A silver impregnation technique was devised in order to visualize the sporangium wall. This consisted of submerging the impression smears for 15 min in 10.0 per cent formalin, followed by

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15 min in 0.5 per cent KMnO₄ and finally exposing to Fontana's ammoniacal AgNO₃, 5.0 per cent (Conn and Darrow, 1943), for 15 min. Distilled water washes were applied between steps.

Specimens were observed by means of a Bausch and Lomb research microscope, model CCTB, equipped with a 90 X, 1.4 N.A. oil immersion apochromatic objective. For phase microscopy the long working distance phase contrast condenser for use with individually removable annular diaphragms was employed. The film employed in photomicrography was "kodak panatomic X", and the light source was a 300-watt zirconium arc lamp. Contrast was heightened by using a 550 mp interference filter. The Wratten E filter was used during phase contrast photomicrography.

RESULTS

Figure 1 shows the appearance of cells taken from a 1-hr-old sporulation culture. Cells from such cultures were almost homogeneously basophilic and showed little cytological detail. At 2 hr, figure 2, it was noted that cells from the culture were swollen and showed considerable enlargement in contrast to those sampled at 1 hr. In addition, there was a relative loss of cytoplasmic basophilia. Yet, no forespore region was obvious. Even hydrolysis of 2-hr cells did not reveal any nuclear component which appeared on cytological grounds to be associated with, or destined for, the nuclear apparatus of the forespore, figure 3. Three hr after transfer to the sporulation medium, the cells were predominantly ellipsoid in shape and for the first time there was seen a basophilic spherical structure terminally located. This appeared to be the forespore (spore primordium), figure 4. Hydrolysis of such a preparation, figure 5, revealed this forespore structure to be composed of a densely basophilic nucleus surrounded by a less basophilic membrane. Occasionally, the above components of the forespore were seen in unhydrolyzed cells. Chapman (1956) shows an analogous structure of the spore primordium in his ultrathin sections of Bacillus megaterium and Bacillus cereus.

After 4 hr, sporulating cells showed a considerably enlarged forespore, and in some instances encystment of the forespore was evident, figure 6. Figure 7 shows that by 5 hr the encyst-

Figures 1-16. All figures are of cells of Clostridium perfringens taken from Ellner's medium at various times during the process of sporulation. Unless otherwise stated specimens were mordanted in 0.2 per cent KMnO₄ for 5 min and then stained in 1.0 per cent safranin for 3 min. Magnification is 2600 X.

Figure 1. 1 hr. Cells are homogeneously basophilic and show little cytological detail.

Figure 2. 2 hr. Cells are swollen and show a loss of cytoplasmic basophilia. No forespore region is obvious.

Figure 3. 2 hr. Specimen was pretreated in N HCl at 60 C for 5 min and then stained as above. No nuclear component is seen which cytologically appears destined for the nucleus of the forespore.

Figure 4. 3 hr. Cells are predominantly ellipsoid in shape. In some the basophilic forespore (spore primordium) is seen, terminally located.

Figure 5. 3 hr. Specimen was pretreated in HCl as in figure 3 above. The forespore structure shows a central basophilic nucleus within a less basophilic membrane.

Figure 6. 4 hr. Sporulating cells show an enlarged forespore which, in some instances, is undergoing encystment.

Figure 7. 5 hr. The encystment process is predominant in sporulating cells.

Figure 8. 5 hr. Specimen was pretreated in HCl as in figure 3 above. The encysted forespore is resistant to hydrolysis.

Figure 9. 6 hr. The sporangia are contracting and forespores are present which are not mordanted by KMnO₄.

Figure 10. 7 hr. Various stages in the contraction of the sporangium are illustrated.

Figure 11. 8 hr. The proportion of forespores centrally or subterminally located and resistant to mordanting by KMnO₄ is greatly increased.

Figure 12. 9 hr. As in figure 11.

Figure 13. 8 hr. Specimen was pretreated in HCl as in figure 3 above. At this stage the forespore core still is rendered basophilic by hydrolysis.

Figure 14. 10 hr. Forespores are encased within drastically contracted sporangia.

Figure 15. 11 hr. As in figure 14.
ment process was well advanced and was evident in the majority of cells. Moreover, encystment of the forespores was accompanied by increased resistance of these structures to hydrolysis, as figure 8 reveals. At this stage the forespore appeared to consist of a basophilic core surrounded by a relatively nonbasophilic cortex and this in turn was encased within a basophilic spore wall.

By 6 hr further changes were evident. The sporangium appeared to be contracting and forespores appeared which were refractory to the mordanting effect of KMnO₄. The core in such forespores was even less basophilic than its surrounding cortex. In addition, the forespore appeared to be retracting from its extreme terminal position, figure 9. In figure 10, 7 hr, various stages in the contraction of the sporan-

Figures 16–22. See legend, figures 1–15

Figure 16. 12 hr. As in figure 14.

Figure 17. 24 hr. Mature spores, untreated and unstained as seen by brightfield microscopy.

Figure 18. 24 hr. Same as in figure 17, but viewed by phase contrast microscopy. The sporangium wall is visualized.

Figure 19. 25 days. Specimen was treated as follows: 15 min each in 10.0 per cent formalin, followed by 0.5 per cent KMnO₄, finally, ammoniacal AgNO₃. The sporangium wall is visualized by silver impregnation.

Figure 20. 48 hr. Mature spores. Specimen was pretreated in HCl for 20 min prior to mordanting and staining. Spores are quite resistant to hydrolysis. Some show increased basophilia of core.

Figure 21. 48 hr. Mature spores. Specimen treated as in figure 20. Examples of the extrusion phenomenon are seen.

Figure 22. 48 hr. Mature spores. Specimen treated as in figure 20. At a spores appear to have lost their basophilic components completely.
gum are demonstrated. Occasionally, spores which appeared mature on morphological grounds were seen at this time. From this point on, samplings showed an ever increasing proportion of forespores located centrally or subterminally within progressively contracting sporangia; the proportion of forespore cores resistant to the mordanting effect of KMnO₄, likewise, continued to increase, figures 11 and 12, 8 and 9 hr, respectively. However, forespores at this stage still showed some response to hydrolysis in that the nonbasophilic were rendered basophilic, figure 13.

Sampling from the culture at 10, 11, and 12 hr support the thesis that the spore of *C. perfringens* is not released from, but is encased within, its drastically contracted sporangium, figures 14, 15, and 16. By 12 hr, hydrolysis of the forespores showed the core, in most cases, to be unaffected by such treatment. This is in contrast to the results obtained with forespores at 8 hr (see figure 15).

The sporangium wall was perceptible in brightfield preparations as a thin enveloping membrane, which remained refractory to the KMnO₄ safranin staining procedure throughout the sporulation process. However, it was visualized readily by phase contrast microscopy and by the silver impregnation technique. Attempts to increase its visibility by applying conventional cell wall staining procedures failed. Figures 17 and 18 illustrate what was seen by brightfield observation versus phase contrast observation of untreated, unstained, mature spores. The core, the core membrane, the cortex, and the spore wall were seen by brightfield. By phase contrast these same structures were seen plus the enclosing sporangium wall. The latter observation was substantiated by the results obtained with the silver impregnation technique, figure 19.

Mature spores proved to be quite resistant to hydrolysis, as figure 20 illustrates. Here spores from the culture at 48 hr were treated in the hydrochloric acid bath for 20 min and the most obvious effect, usually, was an increase in the basophilia of an occasional core. However, sometimes the effects of hydrolysis were more devastating and these results are shown in figures 21 and 22. In the former, in addition to instances of increased basophilia of the central core, there are examples showing the breakdown of the core and extrusion of its basophilic substances to a peripheral position. In figure 22, not only is the extrusion phenomenon illustrated, but at a spores are seen which appear to have lost their basophilic components completely.

**DISCUSSION**

It appears from the observations presented that the sporulation cycle of *C. perfringens* involves four rather distinct cytological stages. First, a terminal portion of the vegetative cell's chromatin forms the nucleus of the forespore. This did not become obvious until about 3 hr after vegetative cells were introduced into the sporulation medium. The forespore remains localized in an extreme terminal position until well along toward maturation. Second, once the forespore is formed it enlarges considerably and becomes encysted. Third, following encystment the once ellipsoid sporangium begins to contract, causing a displacement of the forespore to a more centralized position. The sporangium progressively contracts until, finally, fourth, the cytoplasmic membrane of the sporangium appears to envelop the forespore wall by a process of lamination. Thus, the wall of the mature spore is composed of at least two layers. These may be analogous to the *exine* and *intine* referred to by Knaysi *et al.* (1947) for the spores of the C₂ strain of *Bacillus mycoides*. The sporangium wall, unlike the sporangium cytoplasmic membrane, does not appear to laminate, but remains separate and distinct. The more overt stages observed in the sporulation cycle of *C. perfringens* are presented diagrammatically in figure 23.

Under the environmental conditions of the above investigation, cells that are going to sporulate appear to form the spore primordium within 3 to 4 hr. After this, cells entering the early phases seldom are seen. Represented in each of the figures are the predominant morphological types of cells observed at a given stage in the sporulation cycle of *C. perfringens*. Since the cells when inoculated into the sporulation medium are not synchronized with regard to their phases of cytokinesis and metabolism there is an overlapping of the different stages in samples taken during the sporulation cycle.

The extrusion phenomenon seen in the acid treated mature spores occurs readily among species of the aerobic spore formers. It has led some workers to the conclusion that the nucleus of the resting spore was peripheral to the spore cytoplasm (Robinow, 1945, 1951; Delaporte, 1950). Bisset and Hale (1951) suggested that
peripherally located chromatin was an artifact of the hydrolysis procedure and this contention was later proved by Robinow (1953) who showed, in addition, that the core of resting spores was composed of both ribose and deoxyribose nucleoprotein complexes. These components help to account for the strong basophilia of the spore’s core. The susceptibility of the artifactual peripheral structure to protease action was studied by Robinow (1950). Keigler and Smith (1954) recorded its response to the action of proteases and nucleases.

The sporulation cycle as represented in figure 23 would seem to lend itself favorably as a model whereby both cytological and metabolic studies could be made of the sporulation cells at various stages in the maturation process. Such studies would be facilitated greatly if a degree of synchronization were attainable and maintained throughout the cycle. Then, experiments designed to take advantage of the various stages in the cycle could lead to a correlation of structure and physiology in the sporulating bacterial cell.

**SUMMARY**

Cytological observations were made on the sporulation cycle of *Clostridium perfringens* using a peptone yeast extract starch MgSO₄ medium which promoted rapid and abundant sporulation.

The cycle of sporulation was observed by sampling a culture at hourly intervals and preparing impression smears which were then treated with KMnO₄ as a mordant and stained with safranin. A parallel series of preparations was hydrolyzed in HCl prior to the mordanting and staining steps.

Observations over a 12-hr period showed the following series of changes taking place during the transformation of the vegetative cell to the mature spore. The forespore arose terminally, enlarged, and became encysted within a 4- to 5-hr period. Following this, the sporangium progressively contracted until it encased the forespore.

The mature spore is composed of a central core surrounded by a cortical layer. These are enclosed by a laminated spore wall. The mature spore does not appear to be released from the sporangium, but remains enclosed within the shrunken sporangium wall. The latter is refractory to the KMnO₄ safranin staining technique and to conventional cell wall staining procedures. It is seen by phase contrast microscopy and can be visualized by a silver impregnation technique.
REFERENCES


BETHGE, J. 1947 The sporulation of Clostridium welchii Type A in artificial culture. Z. Hyg. Infektionskrankh., 127, 452–484.

BISSET, K. A. AND HALE, C. M. F. 1951 Observations upon the bacterial spore nucleus. J. Hyg., 49, 201–204.


