OBSERVATIONS ON THE NUCLEUS OF RESTING AND GERMINATING SPORES OF BACILLUS MEGATERIUM

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Received for publication September 8, 1952

Information on nuclei in bacterial spores was both scanty and inconsistent until the Feulgen technique, in the hands of Stille (1937), Delaporte (1939), Schaede (1939) and others, revealed Feulgen positive matter in the shape of a granule or rodlet at the periphery of the majority of mature, resting spores of many well known Bacillus species, including Bacillus megaterium. A more detailed picture was obtained when hydrolysis with N/1 HCl at 60 C, the initial step of the Feulgen procedure, was followed by staining with Giemsa solution, hematoxylin, or crystal violet in the place of the conventional SO4-fuchsin (Schiff reagent). Numerous concordant observations made with this useful, if less specific, technique have confirmed and extended the older findings of peripheral chromatinic bodies in resting, viable bacterial spores (Robinow, 1942, 1945, 1951; Klieneberger-Nobel, 1945; Flewett, 1948; Delaporte, 1950, Preuner, 1951; Bisset and Hale, 1951).

In most of these investigations, e.g., those of Delaporte (1939, 1950) or Robinow (1942, 1945), the spores first had been fixed with osmium tetroxide vapor or by some other conventional procedure before they were hydrolyzed and stained. Later it was found that hydrolysis need not be carried out at the standard temperature of 60 C but is effective already at room temperature; that the result of hydrolysis is the same with living or variously fixed spores and that the peripheral nuclear element which becomes stainable during hydrolysis is visible clearly already in the unstained condition. In other words, the complex procedures used in the earlier work proved reducible to one single essential treatment—acid hydrolysis.

With these facts well established by numerous experiments, it is surprising to find that DeLamater and Hunter (1952) and Hunter and DeLamater (1952), using a technique which includes hydrolysis, should have obtained a very different result. Writing of the spore of B. megaterium they note:

"The nucleus at all times is observed to be central in the cell, and at no time has been seen to be eccentric. It seems likely that Robinow and others have induced the eccentric position of the spore nucleus by the drastic nature of the procedures which they have used. It may be emphasised in this connection which the methods herein utilised, in which a rapid freezing-dehydration process is used, probably constitute a much more gentle procedure."

These remarks seem unreasonable when it is realized that Hunter and DeLamater actually have relied on the very same method of fixation with osmium followed by acid hydrolysis that has given the clearest pictures of eccentric nuclei in the hands of those whom they criticize. The novel features of the authors' procedure, a new stain and dehydration in deep cooled alcohol, are irrelevant to the controversy because in the procedure followed by Hunter and DeLamater the morphology of the spore is determined already irreversibly by the hydrolysis which precedes staining and dehydration. After all, if hydrochloric acid induces the eccentric position of spore nuclei when used by Stille, Delaporte, Robinow, and others, it is unlikely that it will forebear to do so when used by Hunter and DeLamater in the same way!

These considerations dispose of differences in technique as the cause of Hunter and DeLamater's failure to find peripheral nuclei in resting spores, but they do not account for the central nuclei which they found instead. The explanation of this finding lies in the rapidity with which the resting spore's characteristic response to hydrolysis, i.e., the appearance of peripheral nuclei, is lost at the onset of germination. Under favorable conditions this change is seen a few minutes after spores have been placed on a nutrient medium. In the changed spores a central nucleus of the...
kind described by Hunter and DeLamater is found. It follows that in studies on resting spores the latter must be taken directly from the culture in which they were formed and that contact with a nutrient medium before fixation must be avoided lest the cytological picture be confused by the onset of germination changes.

DeLamater and Hunter (1952) have neglected to take these precautions. Thus, their account of spore formation ends with a picture of two cells fixed six hours (!) after transfer to fresh nutrient medium, and the two "resting" spores whose picture opens the author's account of spore germination apparently were fixed (precise details are not given) after 4 to 5 hours' incubation on yeast extract or casein hydrolyzate agar. Pictures or descriptions of mature spores freed from the sporangium and examined directly, without prior contact with nutrient medium, are not given. There then is no evidence that DeLamater and Hunter have studied resting spores.

The same difficulties, though with the time scale reversed, are encountered during spore formation. It is only in mature, fully formed spores that hydrolysis reveals a peripheral nucleus. Earlier stages, in the same preparation, show different nuclear configurations. Unless the structure of the mature resting spore is known beforehand, some uncertainty will be experienced in tracing the course of spore development to its proper end.

It is probably for these reasons that DeLamater and Hunter's (1952) account of spore formation concludes arbitrarily with rather indistinct pictures of cells which, though they may represent developmental stages, certainly are not mature, resting spores.

If my interpretations of the findings of DeLamater and Hunter are correct, then it should be possible, using their method of fixation, hydrolysis, and staining, to demonstrate eccentric nuclei in mature resting spores and central nuclei during early germination.

MATERIALS AND METHODS

A freshly isolated strain of B. megaterium was grown on potato extract agar plates (Robinow, 1951) for four days or slightly longer. This sufficed for ample and complete sporulation. The spores were spread in distilled water on grease-free coverslips, fixed for 2 to 5 minutes in osmium tetroxide vapor, dried, placed for a few minutes in 70 per cent alcohol, and hydrolyzed in N/HCl at 60 C for 8 minutes. Early stages in spore formation obtained from 18 hour old cultures were prepared in the same way. To study germination, spores from the same cultures were spread directly on freshly poured and lightly dried Difco heart infusion agar plates and incubated at 37 C. Small slabs of spore covered agar were removed at intervals and, spores up, fixed in osmium vapor. Impression films were made of the fixed growth and processed as above. Germination was complete usually at the end of the first hour in agreement with earlier experience of the behavior of other strains of B. megaterium under the same conditions. Hunter and DeLamater's figures of 3 and 4 hours required for germination indicate that they were not working under the most favorable conditions.

Hydrolyzed preparations were stained in Columbia dishes for coverslips for 2 to 4 hours in 10 ml of a 0.25 per cent solution of thionin (National Aniline Division) to which one drop of thionyl chloride had been added according to DeLamater and Mudd (1951). Stained films, invariably covered by a heavy precipitate of dye crystals, were rinsed with and mounted in tap water. The optical equipment was as used by Robinow (1951). Photomicrographs were taken with the light from a Baird Associates interference filter with a transmission peak at 5390 A, illuminated by a tungsten ribbon lamp.

RESULTS

Our findings are illustrated in figures 1 to 6. Figure 1 shows how several developmental stages, i.e., polar granules, clear rings, and hydrolysis resisting basophilic forespores and mature spores with eccentric nuclei, may be found together in a single preparation. In this strain of B. megaterium the spores reach morphological maturity while still in their mother cells. In other strains it was found that the nucleus remained central as long as the spore was not entirely free from remains of the vegetative phase. But here too, eccentric nuclei were revealed in fully mature spores after acid hydrolysis.

Figure 2, a small sample of many entirely uniform preparations, shows the familiar aspect of eccentric nuclei in resting spores, closely resembling pictures of Feulgen preparations published by Stille (1937) and Delaporte (1939). Acid hydrolysis has shown the same eccentric nuclei in all of the many other strains of B. megaterium which the writer has examined. The small size
of the nuclei is due to the temperature of 60°C at which hydrolysis was carried out. The nuclei of spores of *B. megaterium* hydrolyzed at room temperature appear larger (Bisset and Hale, 1951; Robinow, 1951).

**Figure 1.** Various stages in the formation of endospores in *Bacillus megaterium* from an 18 hr culture on potato extract agar. A bacillus containing a finished spore with eccentric nucleus is seen in the left bottom corner, another one is indicated near the center. Further explanation in the text. OsO₄, HCl, SO₂-thionin after DeLamater. Photographed in water. Magnified × 3,600.

Figures 3, 4, and 5 show how the configuration of the nucleus changed during the first ten minutes after transfer to heart infusion agar. In most of the spores the nucleus is now found in the center. At first the nucleus has a clear interior, surrounded by a thin layer of chromaticin matter; later the center becomes deeply stainable. A further picture, figure 6, shows examples of the first division of the nucleus, closely resembling comparable stages in the germination of *B. cereus* described and documented by Delaporte (1950).

**Discussion**

The evidence presented shows that DeLamater’s procedure of osmium fixation, acid hydrolysis, and staining with SO₂-thionin reveals the same eccentric nuclei in resting spores of *B. megaterium* which in the past had been demonstrated repeatedly in the spores of this species and other bacilli by essentially the same procedure. No nuclei are visible in normal, resting, untreated spores, and on the basis of evidence presented so far it is not possible to decide whether in the untreated spore the nucleus is at the periphery or even outside the cytoplasm, as I have argued repeatedly, or whether, as Bisset and Hale (1951) speculate, it is extruded to the periphery from its normal central position under the influence of hydrolysis. Until the question of the true localization of the spore nucleus is settled, one has to agree with Preuner (1951) and Hunter and DeLamater (1952) that eccentric nuclei are “induced” by our treatments. But it must be clearly understood that this situation is not the fault of this or that cytological technique. We have no choice in the matter! Essentially the same result is obtained regardless of whether spores are hydrolyzed directly or after exposure to any one of a large number of “good” conventional fixatives. N/3 HNO₃, a relatively weak reagent, used at room temperature, may replace the customary hydrolysis with N/1 HCl at 60°C, and eccentric nuclei are revealed very effectively by mere extraction of spores with N/1 HClO₄ at 4°C (P. C. Fitz-James, unpublished data). It follows that it is not our methods which are drastic, but that it is in the nature of normal resting spores to respond drastically to certain accepted cytological procedures (e.g., the Feulgen technique) not known to have serious morphological effects on other kinds of cells. This behavior deserves further exploration. Our knowledge of spores is not advanced by ignoring it.

Comparison of figure 2, where the nuclei are peripheral, with figures 3 and 4, where they are mostly central, establishes for *B. megaterium* what had previously been shown in *B. cereus* (Robinow, 1942, 1945; Flewett, 1948) and *B. subtilis* (Preuner, 1951), namely that the peculiar responsiveness of resting viable spores to acid hydrolysis, resulting in peripheral nuclei, is lost very rapidly after spores have been placed on a
Figure 2. Resting spores of Bacillus megaterium with eccentric nuclei.

Figures 3, 4, and 5. Samples fixed 10 minutes after transfer of resting spores of Bacillus megaterium to heart infusion medium. In many spores central, ring shaped, or solidly stained nuclei are now seen. Figures 4 and 5 are from the same experiment. Figure 3 is from a separate, less advanced specimen.

Figure 6. Various stages in the first division of the nucleus in germinating megaterium spores. Fixed 60 minutes after transfer of resting spores to heart infusion agar.

All figures from preparations fixed with OsO₄ vapor, hydrolyzed with N/1 HCl at 60 C, and stained with DeLamater's SO₂-thionin. Photographed in water. The magnification is the same for all figures and is indicated by a scale underneath figure 3.
suitable nutrient medium. The change from the resting stage to spores with central, ring shaped nuclei is accompanied by loss of the spore’s characteristic refractivity and the acquisition of a strong affinity for basic dyes. These are clear signs of beginning germination and support my contention that the long incubated spores with central nuclei which DeLamater and Hunter (1952) and Hunter and DeLamater (1952) have presented as “ungerminated” or “resting” had, in reality, already suffered initial germination changes.

In the present study the DeLamater stain has proved delicate and very suitable for photomicrography of nuclear detail. Hydrolyzed, stained spores also have been examined after dehydration by DeLamater’s method. Such spores still showed peripheral nuclei but had shrunk and had acquired angular contours. Further attempts probably will show that this can be avoided, but I believe, nevertheless, that the advantages to be gained from dehydration, apart from permanency of the specimen, have been exaggerated. DeLamater and Mudd (1951) believe that “fine detail is obscured by . . . non-optimal refractive indices of cells mounted in a water medium”, but other factors besides maximum transparency have to be considered. Two passages from C. P. Shilla-ber’s text on photomicrography (1944) may be quoted usefully in this connection.

“The medium must differ in index from the subject sufficiently to give the necessary visibility . . . .” and “The histologist usually prefers a medium of the same index as the specimen. His specimens are usually stained and he wants absorption images only . . . . (But) The belief that the maximum transparency of the specimen ensures the maximum visibility of stained detail is a matter to question . . . .”

Decision on the relative merits of wet mounts and dehydrated specimens must await the demonstration, not yet given, that there really is a significant difference in the amount of detail visible in the same SO₄-thionin stained specimen examined first in water and once more after dehydration according to DeLamater.

SUMMARY

The method of fixation, hydrolysis, and staining employed by DeLamater and Hunter regularly induces the appearance of eccentric nuclei in resting spores of Bacillus megaterium.

Hunter and DeLamater’s criticism of descriptions of the structure of resting spores by Robinow and others is invalid because the observations on which it is based were not made on resting spores.

REFERENCES

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


