PRELIMINARY OBSERVATIONS ON GERMINATION OF THE SPORES OF BACILLUS MYCOIDES IN A NITROGEN-FREE MEDIUM AND CERTAIN PROPERTIES OF THE TRANSPARENT CELLS

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Received for publication February 18, 1948

The ability of the endospores of *Bacillus mycoides* to germinate in a medium free of nitrogen, but containing a source of energy, was recently shown by Knaysi (1945). This was confirmed by Knaysi and Baker (1947), who also observed germination in solutions of glucose and sodium acetate, showing that the endospore contains a source of both nitrogen and phosphorus. This and other considerations (Knaysi, 1946) indicated that the spore contains a relatively large quantity of ribonucleic acid. Search of the literature to ascertain whether spore germination under similar conditions had previously been observed led only to a parenthetical statement by Eijkman (1918) indicating that he observed endospore germination in solutions of glucose, lactose, and mannitol, but not in solutions of glycerol. In the case of *Bacillus mycoides*, Knaysi (1945) showed that lactose is inactive and pointed out that germination is induced only by substances which can be utilized as a source of energy. He also pointed out the importance of buffering the solution. It should be emphasized, however, that the proportion of spores which germinate under these conditions is variable and usually small, and that many of the vegetative cells observed develop as a result of growth. In view of the value of the vegetative cells which develop in such media for cytological investigations, and of their potential value in other fields of theoretical and practical bacteriology, it was found desirable to study the conditions which control spore germination in nitrogen-free media.

SCOPE, STRAIN, AND TECHNIQUE

In the present work we did not study the effect of the medium in which the spores are formed. In all experiments the spores of *Bacillus mycoides*, C_2, were harvested from slant cultures on the medium: meat infusion, $\frac{1}{4}$ strength, 100 ml; tryptone, 0.25 g; glucose, 0.25 g; and agar, 1.5 g. The growth was very carefully scraped off two 7-ml slants and washed three times, each time in 10 ml of sterile, distilled water, and finally suspended in 10 ml of sterile, distilled water. When the cultures were 6 or more days old at 27 to 28 C, the suspension was practically free of vegetative cells (<1 per cent). Since germination in the media used was slow, we recorded the proportions of vegetative cells and spores in cultures 16 to 24 hours old at 33 C. The media were inoculated heavily (4×10^6 to 20×10^6 spores per ml).

The medium used most in this work was a solution of 0.2 g of glucose and 0.2 g of sodium acetate in 100 ml of distilled water. This medium was often supple-

mented with small amounts of an equimolar mixture of monopotassium and dipotassium phosphates varying from 1 to 10 parts in 2,000 parts of the medium. In a few experiments the medium was supplemented, in addition, with other mineral salts (0.1 ml of Speakman's mixture B which had been diluted 100-fold to 5 ml of the medium), or with biotin (3 μg per ml of medium).

RESULTS

The results of numerous experiments led to the following conclusion: The number of vegetative cells that develop in the glucose, acetate solution depends within wide limits, on the age of the culture from which the spores were harvested and, particularly, on the age of the spore suspension. In one typical experiment, for instance, a spore suspension freshly prepared from a slant culture 24 days old at 25 to 27 C gave 20 per cent of vegetative cells after 16 hours of incubation; 23 days later the same suspension gave 2.4 per cent. In both cases the inoculum was 15×10^6 spores per ml. The addition of ammonium sulfate to the medium did not increase the number of vegetative cells when the medium was inoculated from the old suspension.

We also made the interesting observation that the number of vegetative cells that develop in the glucose, acetate medium may be increased when the stock solutions from which the medium is prepared (2 g of glucose and 2 g of sodium acetate, each in 100 ml of distilled water) are freshly autoclaved. The effect of autoclaving persists for a number of hours. In a typical experiment, a freshly prepared suspension gave 23 per cent of vegetative cells in 16 hours when the stock solutions were autoclaved on the preceding day, and 65 per cent when the stock solutions were autoclaved on the same day.

The number of vegetative cells may also be increased when the glucose, acetate solution is supplemented with as little as 1 part of potassium phosphate mixture in 2,000 parts of the solution. The effect is particularly noticeable when the stock solutions are not freshly autoclaved or when the spore suspension is relatively old. With a fresh suspension and stock solutions which were autoclaved 1 week previously, the proportion of vegetative cells obtained in 16 hours may be raised by the phosphate from about 12 to 20 per cent; with freshly autoclaved solutions and a suspension 23 days old, the proportion of vegetative cells may be raised from 8.9 to 28 per cent. Supplementing the glucose, acetate solution with Speakman's salts B or with biotin has no beneficial effect.

Properties of the Vegetative Cells

The vegetative cells that develop in the glucose, acetate solution have several interesting properties. In 70 to 90 per cent of these cells the cytoplasm becomes transparent to electrons at normal voltages and loses its property of staining with methylene blue at low pH (Knaysi and Baker, 1947). It also becomes gram-negative, and the nuclei, which remain definitely gram-positive, may be demonstrated in these cells by gram staining. In the present work we used Burke's method modified by omitting the sodium bicarbonate. The electric charge of the surface of these cells and its relation to pH and cationic detergents are similar

to those of cells of the same organism grown on ordinary media (Dyar and Knaysi, 1947). On the other hand, these cells seem to have undergone a considerable change in permeability. They are highly permeable to neutral red in extremely dilute solutions (e.g., 1 to 10 mg per liter).

Although the proportion of the spores which germinate in the glucose solution is, usually, relatively small, a high proportion of the spores which do not germinate acquire vegetative characteristics, becoming readily permeable to dyes in dilute solutions. Of these, many stain deeply and uniformly, but many others show a faintly stained cytoplasm containing two or more deeply stained nuclei, indicating loss of ribonucleic acid without germination. All gradations between these two extremes may be observed.

DISCUSSION

The present work confirms that of Knaysi (1945) and of Knaysi and Baker (1947) that the endospore of *Bacillus mycoides* contains a source of both nitrogen and phosphorus, but not of energy. Since microscopic observations reveal that the vegetative cells that develop in the glucose, acetate solution contain at least as much diffuse lipoid material as those that develop in ordinary media, it is unlikely that the source of nitrogen and phosphorus would be a phospholipid. Since this source has a low isoelectric point (about pH 2), it is unlikely that it is protein. On the other hand, the low isoelectric point of this material is similar to, and its physiological behavior is identical with, that of volutin (Knaysi, 1946), which, according to Delaporte (1939), is chiefly ribonucleic acid. The present work further shows that when this material is used up, the cell becomes gram-negative, just as it becomes gram-negative when ribonucleic acid is removed by means of enzymes or chemicals (Dubos and MacLeod, 1938; Henry and Stacey, 1943). This conclusion is further confirmed by the work of Vendrely and Lipardy (1946), who attributed the poor stainability of cells hydrolyzed with hydrochloric acid to the loss of ribonucleic acid.

The present work also confirms that of Ruehle (1923) and others who demonstrated a number of enzymes, and that of Curran, Brunstetter, and Myers (1943), who demonstrated several minerals, in the endospores of various bacteria. Indeed, the endospore of *Bacillus mycoides* seems to be fully equipped to begin and carry on normal metabolic processes provided a source of energy is supplied. The coenzymes and minerals which may be necessary are probably held within the spore because of the low permeability of its coat. A spore which had lost its supply of coenzymes or minerals by leakage or in which these were somehow inactivated would not be able to germinate in the glucose, acetate solution. Analysis of the effect of aging of the spore suspensions indicates that aging is due to gradual oxidative processes that at first inactivate and later destroy something, in the spore, essential for metabolic activities, probably certain coenzymes. In the early stages, the process may be reversed by the addition of traces of phosphate or by the use of freshly autoclaved stock solutions; both heat and phosphate are known to activate solutions of sugar and of other complex biological material. The effect of autoclaving on dye-containing, complex culture media is common

knowledge among bacteriologists; the effect persists for hours. Dubos (1929) showed that autoclaved bouillon became more reducing and more suitable for the initiation of growth of small inocula. Wurmser (1930) reported that, below pH 9, the potential of sugar solutions is more positive when they are buffered with borate than when they are buffered with phosphate. Knaysi (1935) found that the potential of meat infusion was more quickly established and more definite and reproducible at 60 C than at room temperature; this potential was not affected by NaCl, but became more negative when phosphate was added at all pH values above 5.75. The beneficial effect of sublethal heat on spore germination observed by Evans and Curran (1943) and Curran and Evans (1945) appears very likely to be an effect of potential.

The present investigation does not favor the concept of dormancy as a significant biological phenomenon.

SUMMARY

The number of vegetative cells that develop in a solution of glucose and acetate inoculated with endospores of strain C_2 of *Bacillus mycoides* decreases with the age of the culture from which the spores were harvested and, particularly, with the age of the spore suspensions. When the spore suspension is moderately old, the number of vegetative cells may be considerably increased when the stock solutions of glucose and acetate are freshly autoclaved or when traces of potassium phosphate are added. When the spore suspension is too old, autoclaving and the addition of phosphate lose their effect. Aging is attributed to oxidative processes which, in the early stages, are reversed by the activated sugar.

The vegetative cells that develop in the glucose, acetate solution are gram-negative and highly permeable to neutral red. Many spores that do not germinate acquire vegetative characteristics.

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