INVESTIGATION OF THE EXISTENCE AND NATURE OF RESERVE MATERIAL IN THE ENDOSPORE OF A STRAIN OF BACILLUS MYCOIDES BY AN INDIRECT METHOD

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Received for publication February 9, 1945

Because of its high density and low permeability, the bacterial endospore is not suitable for direct cytological investigations, and little is known with certainty about its internal structure. In this and similar cases it is desirable to resort to indirect methods which, although not including tinctorial and microchemical tests, should, nonetheless, be considered as legitimate methods of cytology. As an example we may refer to the brilliant work of Mudd and Mudd (1927) on the wetting property of the cell surface of *Mycobacterium tuberculosis*. Their conclusions were later confirmed for the human strain by the direct microchemical studies of the author (Knaysi, 1929).

Of considerable practical and scientific importance is whether the endospore contains reserve material and, if it does, what the nature of that material is. There is nothing in the literature to supply that much-needed information; one finds only statements that the reserve material of the sporangium gradually disappears during the maturation of the endospore, and that it may serve as nutritive material for the building of the spore (Meyer, 1899; Preisz, 1904); Lewis (1934) considered also the possibility that it may be utilized by the sporangium in its own metabolic processes.

The present work was inspired by the observation that endospores of strain C_2 of *Bacillus mycoides* washed several times in distilled water and resuspended in distilled water have no tendency to germinate. On the other hand, the addition of very small quantities of food, in the form of tryptone or meat infusion, to the suspension resulted in normal germination of most of the endospores within the expected time (Knaysi, 1945).

These early observations indicated clearly that endospores germinate only in the presence of utilizable food, and led us to the obvious corollary that an endospore containing reserve material both for energy and the synthesis of protoplasmic substances would tend to germinate when suspended in water, although the absence of an extraneous supply of food may make further growth impossible. The fact that endospores of the strain investigated had no tendency to germinate without an extraneous supply of utilizable food could mean either that they did not contain any reserve material or, if they did, that the reserve material was not suitable both as a source of energy and for the building of protoplasmic substance.

METHODS

Stock suspensions of the endospores of the strain used in this investigation (strain C_2 of *Bacillus mycoides*) were prepared from 3-day-old cultures of the

organism at 33 C. The cultures were grown in large test tubes each containing 5 ml of the following medium: 100 ml of meat infusion diluted 4 times with distilled water + 0.25 g of tryptone + 1.5 g of bacto-agar, pH 7.0–7.2; or on that medium + 0.25 g of glucose. The endospores from a given culture were suspended in about 10 ml of sterile, distilled water, centrifuged down, resuspended in about the same volume of sterile, distilled water, recentrifuged down, etc. This process of washing was repeated 5 times over a period of 2 days. The final suspension was usually free of vegetative cells and could be kept in the laboratory for several weeks without significant change in the appearance and numbers of the endospores.

Numbers of the cellular elements per ml of the various media used were counted by the cover-glass method recently described (Knaysi, 1945).

In view of the fact that the organism investigated is able to utilize glucose as a source of energy and carbon, and KNO_3 as a source of nitrogen, and that its washed endospores can germinate normally and grow into a culture when heavily inoculated into the medium (100 ml of distilled water + 0.2 g of glucose + 0.2 g of KNO_3 + 0.23 g of an equimolar mixture of KH_2PO_4 and K_2HPO_4), it was decided to use the components of this medium separately and in various combinations. We also used solutions in which lactose, a sugar not fermented by the organism, was substituted for glucose. The various solutions and concentrations used are recorded in the table.

Stock solutions of the various components were sterilized separately and mixed aseptically just before use. All test tubes used were thoroughly recleaned with dichromate cleaning solution and, after thorough rinsing with tap water then with distilled water, were plugged with fresh cotton and sterilized in the inverted position, care being taken that the plugs did not get soaked in water during sterilization. The chemicals used were of the grade "Baker's Analyzed, C.P." or equivalent brands, and the volume of each suspension was 10 ml. All suspensions were incubated at 33 C.

The pH was determined by the "spot plate" method, using the indicators of Clark and Lubs.

RESULTS

The data recorded in the table show that the washed endospores of strains C_2 of *Bacillus mycoides* have *no tendency to germinate* in distilled water or in the following aqueous solutions of the indicated strengths: an equimolar mixture of KH_2PO_4 and K_2HPO_4; potassium phosphate + KNO_3; or lactose + potassium phosphate + KNO_3. In addition to the lack of tendency to germinate in these media, one observes no change in the pH of the suspensions containing phosphate, and no *significant* change in the suspensions not containing a buffer. The pH of the latter suspensions was so unstable that we had difficulty in making accurate determinations.

Typical germination of the endospores into normal, rodlike vegetative cells and further growth of these cells were observed in the solutions containing glucose + potassium phosphate mixture, or glucose + potassium phosphate mixture + KNO_3, of the concentrations indicated in table 1. Indeed, in the first of the

TABLE 1

Behavior of the washed endospores of Bacillus mycoides, strain C_2, when suspended in the indicated media at 33 C

COMPOSITION OF MEDIUM	AGE OF SUSPENSION		VEGETATIVE CELLS PER ML × 10^6	NORMAL ENDOSPORES PER ML × 10^6	NONREFRACTIVE ENDOSPORES PER ML × 10^6	GERMINATING ENDOSPORES PER ML × 10^6	pH	REMARKS
	Hr	Min						
Distilled water	2	20	0.0	21.2	9.1	0.0	7.0	
	22	5	0.0	19.7	11.3	0.0	7.6	
	70	10	0.0	27.7	13.0	0.0	7.8	
Potassium phosphate mixture, 0.23 g % + KNO_3, 0.2 g %	5	0	0.0	33.6	0.0	0.0	6.8	
	29	0		29.0	0.2	0.0	6.8	
Potassium phosphate mixture, 0.23 g %	5	15	0.0	24.5	1.2	0.0	6.9	
	29	30	0.0	32.4	0.7	0.0	7.0	
	81	30					7.0	Microscopic appearance unchanged
KNO_3, 0.25 g %	5	30	0.0	54.5	2.4	0.0	8.2	
	24	10	0.0	67.6	2.9	0.0	7.6	
	0	0	0.0	41.0	1.2	0.0	7.6	
	5	0	0.0	39.6	0.7	0.0	7.2	
Lactose, 0.5 g %	3	5	0.0	24.5	11.0	0.0	7.8	
	5	50	0.0	24.5	12.7	0.0	7.8	
	23	50	0.0	25.7	17.5	0.0	7.8	
Lactose, 0.2 g % + phosphate, 0.23 g % + KNO_3, 0.2 g %	18	25	0.0	27.6	11.8	0.0	6.8	Observations previous to this time revealed no change in microscopic picture
	93	40	0.0	28.3	10.3	0.0	6.8	
Glucose, 0.2 g %	0	0	0.0	60.5	4.6	0.0	8.0	Typical germination
	5	30	0.0	74.2	4.1	1.4	7.2	
	29	30	0.0	50.2	5.8	2.1	6.3	
	81	30	No change in microscopic picture				4.8	
Glucose, 0.2 g % + KNO_3, 0.2 g %	5	15	0.2	41.6	4.1	2.9	7.2	Typical germination
	29	30	0.0	28.3	21.6	2.9	4.4	Nonrefractive spores are mostly shells
	81	30	No vegetative cells. Besides normal spores, many shrunken, refractive ones, apparently without exine or, occasionally, attached to an exine. Numerous shells and shell fragments.				4.0	Germination not typical, characteristic of low pH

TABLE 1—*Continued*

COMPOSITION OF MEDIUM	AGE OF SUSPENSION		VEGETATIVE CELLS PER ML × 10^6	NORMAL ENDOSPORES PER ML × 10^6	NONREFRACTIVE ENDOSPORES PER ML × 10^6	GERMINATING ENDOSPORES PER ML × 10^6	pH	REMARKS
	Hr	Min						
Glucose, 0.2 g % + phosphate, 0.23 g %	5	0	0.0	16.8	0.0	7.2	6.9	Typical germination
	28	45	8.1	21.6	12.0	2.4	6.6	
	77	30	Nonmotile cocci, mostly large and normal, motile rods, and many sporangia.				6.4	
Glucose, 0.2 g % + phosphate, 0.23 g % + KNO_3, 0.2 g %	0	0	0.0	50.4	10.2	0.0	6.9	Typical germination
	5	0	3.1	24.0	0.0	5.5	6.9	
	77	30	Typical culture with pellicle and many spores that did not germinate. No sporangia yet.				6.0	

Note. % = per 100 ml of water.

the two solutions numerous sporangia were observed in about 3 days; the suspension containing KNO_3 in addition to glucose and phsophate contained no sporangia at the end of that period. This is additional evidence to that recently presented (Knaysi, 1945) indicating that endospores are formed by healthy cells facing starvation.

The behavior of the endospores in the solutions of glucose or of glucose + KNO_3 (the concentrations are recorded in table 1) needs special consideration. In both solutions the endospores have a tendency to germinate; this is to be expected on the basis of the results reported in the preceding section. However, typical germination is observed only until the pH drops to a value somewhere between 5 and 6. Below this pH, the vegetative cells already formed are unable to survive, and typical germination is inhibited probably because of the destruction of potential cells in the early stages of germination. Finally, the cellular elements in such suspensions consist of a reduced number of unchanged spores with pairs and clumps of various sizes in which some still refractive, but apparently shrunken, endospores are found with nonrefractive spores, shells, and shell fragments; occasionally, a refractive body may be seen attached to a cracked exine. This phenomenon is particularly noticeable in the suspension containing glucose + KNO_3 where the pH reaches a minimum of 4 or slightly below, and we have come to consider it as typical of a tendency to germinate at a pH too low for the survival of the organism. It also confirms our previous conclusion (Knaysi, 1945) that, in the presence of nutrients, endospores tend to germinate even in an environment where the germ cell cannot survive.

Before concluding this report of our observations, we wish to describe the behavior of certain endospores in suspensions where there is no tendency for

normal germination and growth. In those suspensions, a few endospores may very slowly become nonrefractive without a noticeable change in size. Although most of these spores may still be surrounded by an exine, their frequent presence in pairs and small groups, sometimes as tetrads, indicates a softening of the exine. Occasionally, the exine seems to have disappeared; these are virtually vegetative cells, and have a tendency to divide without significant growth, appearing as diplococci. This phenomenon needs further study, although we are now inclined to believe that the energy necessary for the slight change in the endospores may have been derived from a breakdown of the exine.

DISCUSSION AND CONCLUSIONS

The data presented in the preceding section and in the table show that endospores of the organism investigated are able to germinate typically into normal cells which are able to grow and form endospores in a medium containing only glucose and potassium phosphate, without a source of nitrogen. This indicates that the endospore contains a relatively considerable amount of a nitrogen-containing reserve material. The lack of tendency for normal germination and growth in solutions containing KNO_3, a suitable source of nitrogen, with or without phosphate, indicates that this reserve material is not suitable as a source of energy, and that the endospore contains no other reserve material suitable for that purpose. In view of the fact that the vegetative cells of the investigated organism are able to deposit fat as reserve material, the results of the present investigation mean that the reserve fat of the sporangium is not absorbed by the endospore, and that the endospore forms its own reserve material.

In comparing, at the end of 5 hours, the number of germinating spores in a glucose solution with the number of germinating spores in a solution of glucose + potassium phosphate, one is impressed by the accelerating effect of the phosphate; obviously, the phosphate does not act only as a buffer (see the review by Barron, 1943).

A question often asked in connection with the formation of endospores is: Is there a limiting concentration of nutrients below which endospore formation is not possible? In a previous report (Knaysi, 1945) we have shown that endospores can be formed on bacto-agar to which no nutrients have been added. The present investigation permits us to be more precise and state that, in the presence of a suitable source of energy, the nitrogenous reserve material present in the endospores of the strain investigated is sufficient for a completion of the cycle from spore to spore.

Of considerable interest is the minimum pH reached when endospores are suspended in unbuffered solutions of glucose (4.8) or, particularly, glucose + KNO_3 (4.0 or slightly below). Suspensions of vegetative cells of the same strain in the latter solution reach a minimum pH of only 5.0 to 5.2. The difference must be sought not only in the low permeability of the exine, but also possibly in a greater concentration of nondiffusible substances in the endospore, as would be expected from a Donnan equilibrium. In any case, it is probable

that the internal pH of the vegetative cell in the solution of glucose + KNO_3 at pH 5 is about the same as that of the endospore in a similar solution at pH 4.0. The value of that internal pH is of considerable importance and should correspond to the level of acidity at which the enzymes which take part in the fermentation of glucose are inhibited. The use of such solutions for testing sugar fermentation by the usually proteolytic organisms of the genus *Bacillus* recommends itself in preference to the complex media commonly used.

SUMMARY

The endospores of strain C_2 of *Bacillus mycoides* washed five times in distilled water are able to germinate normally in a solution of glucose (0.2 g in 100 ml of water) without the addition of a source of nitrogen. When this glucose solution is buffered with potassium phosphate (0.2 g) at a pH of about 7, germination is followed by growth and sporulation. There is no tendency for germination in a solution of KNO_3 (0.2 g + 100 ml of water) with or without potassium phosphate (0.23 g) unless glucose is added. It is concluded that endospores of the strain investigated contain relatively large amounts of a nitrogen-containing reserve material not suitable as a source of energy, and that they contain no other reserve material for that purpose.

It is shown that endospore suspensions in solutions of glucose or, particularly, glucose + KNO_3 reach a minimum pH much below that reached by vegetative cells in similar solutions. New evidence is given to show that endospores are formed by a healthy cell facing starvation, and the question of a minimum concentration of nutrients for sporulation is discussed.

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