SURVEY OF FOURTEEN METABOLIC INHIBITORS FOR THEIR EFFECT ON ENDOSPORE GERMINATION IN *BACILLUS SUBTILIS*[1]

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ABSTRACT

CURRAN, HAROLD R. (U. S. Department of Agriculture, Washington, D. C.) AND GEORGES KNAYSI. Survey of fourteen metabolic inhibitors for their effect on endospore germination in *Bacillus subtilis*. J. Bacteriol. **82**:793–797. 1961. —Phase contrast microscopy was used to study the effects of metabolic antagonists upon incipient spore germination in glucose agar-film microcultures. The period of observation was 1 to 2 hr. Of 14 compounds tested, only ethanol in 10% concentration (v/v) caused almost complete inhibition. Oxamic acid and DL-ethionine were slightly stimulatory; 6-methyl thiouracil and octyl aldehyde were without effect. The inhibiting effect of the remainder depended upon the concentration of the antagonist. The stimulating effect of glucose on germination is discussed.

Germination of the endospore may be divided into two stages: The first results in a change in optical properties. Under optimal conditions, this stage lasts only a few minutes. In *Bacillus cereus* (Knaysi, 1959) it lasts only about 3 min, during which resistance of the spore to heat or drying sinks to a level perhaps even below that of the normal vegetative cell. The second stage involves growth and is terminated when the germ cell bursts through the spore coat. During this stage, which in *B. cereus* lasts more than an hour under optimal conditions, the germinating spore builds up or replenishes its supply of enzymes and undergoes some structural reorganization. In motile species, the flagella are formed during this period. Of the two stages, the first has stimulated wider interest among bacteriologists, since it includes changes peculiarly characteristic of the spore, such as appearance and resistance. Sometimes these changes differ in their requirements from those of the second stage. They do not constitute the whole process of germination, but are unmistakable signs of incipient germination, and knowledge of the reactions which lead to these changes may lead ultimately to the discovery of promoters and inhibitors of germination, the value of which in the practical control of spore-forming bacteria can not be overestimated.

The use of inhibitors in the study of biological reactions has been for many years a common practice among students of fermentation, and has been responsible for much of the progress made in this field of microbiology. There has also been some pioneering search for inhibitors of spore germination (Schmidt, 1957; Halvorson, 1959). Partial but not complete inhibition was observed for a number of compounds.

According to present knowledge, there are principally three conditions that have sufficient inhibitive power on spore germination in the genus *Bacillus* to be of practical value. These are temperature, pH, and the absence of a source of energy. This survey of 14 metabolic antagonists was undertaken in the hope of finding a substance that would sufficiently inhibit spore germination under optimal conditions of temperature and pH, and in the presence of a good source of energy, to be of practical value in the food industries.

MATERIALS AND METHODS

The organism employed in this study is an atypical strain of *B. subtilis* (15U). This strain was employed by Schmidt (1957) as no. 5230. It is able to grow at temperatures up to 55 C and its spores are quite resistant to heat. Prior to

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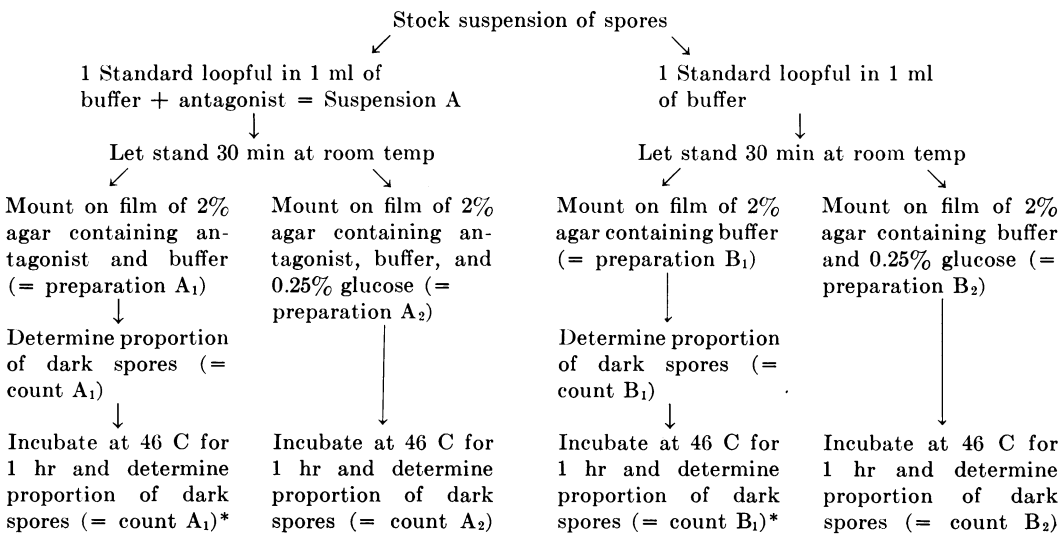
use the spores were washed 8 to 15 times with distilled water, and were free of vegetative cells. Early in the work the spores were preheated at 88 C for 10 min before testing their germination. This, however, was discontinued when it was found that nearly half of the spores germinated readily without heat treatment.

The germination test was generally carried out in the following manner: A standard loopful (about 0.02 ml) of the stock suspension of spores was diluted with 1 ml of a solution of the antagonist in potassium phosphate buffer (pH 7). Neutral antagonists were usually dissolved in 0.01 M buffer. Acid antagonists were dissolved in distilled water and neutralized with 1 M K_2HPO_4 before use. Concentration of the antagonist was often determined by its solubility. These secondary suspensions were left at room temperature for about 30 min to allow penetration and action of the antagonist. At the end of this period a small loopful was placed on the surface of a film of 2% agar (Difco Laboratories, Detroit, Mich.) 0.30 to 0.35 mm thick and containing the desired concentration of the antagonist (preparation A_1). Antagonists of low solubility were added in excess. Another small loopful was placed on a similar agar film containing, in addition to agar and antagonist, 0.25% glucose (preparation A_2). The excess liquid was withdrawn with a capillary glass tube and the agar film covered with a cover glass and sealed with vaspar. A control secondary suspension containing an equal concentration of buffer but no antagonist was run simultaneously. A small loopful of this was placed on a film of 2% agar (preparation B_1) and another on a 2% agar film containing 0.25% glucose but no antagonist (preparation B_2). Here also the excess liquid was removed and the films covered with glasses and sealed. Preparations A_2 and B_2 were immediately incubated at 46 C. Preparations A_1 and B_1 were examined in dark contrast with the phase microscope to determine the proportion of dark and bright spores and then incubated at 46 C. Incubation was usually for 1 hr; when growth was to be observed, it was for 2 hr. In the latter case, a richer medium, usually Difco brain heart agar, was used, and the preparation was modified to insure gaseous exchange between the microculture chamber and the outside atmosphere (Knaysi, 1952). At the end of incubation, all preparations were examined with the phase microscope, in dark contrast, and the proportion of dark spores determined. Sometimes the proportion of dark spores showing a definite increase in volume was also determined. The change of a spore from bright to dark marked the beginning of germination (first stage); a definite increase in the size of a dark spore indicated growth (second stage). A general schema of the procedure is represented in Fig. 1.



* Counts A_1 and B_1 are not reported in the tables.

FIG. 1. *General schema of the test for incipient germination*

TABLE 1. *Effect of metabolic inhibitors on incipient spore germination*

Antagonist	Presumed to be against	Concentration*	Dark spores	
			In presence of antagonist	In control
		%	%	%
Dimethyl pyruvic acid	Glucose	0.1	51.0	51.1
		0.3	25.5	45.6
Oxamic acid	Lactic dehydrogenase	0.05	77.6	74.3
Sorbic acid	SH enzymes	0.01 or 0.10	88.7	88.8
		0.1†	43.7	52.6
Benzimidazole	Purines, pyrimidines, and nucleic acids	0.1	75.5	84.4
8-Azaguanine		0.1 of saturation	81.1	86.5
2-Amino-4-methyl pyrimidine		0.1	42.1	50.1
6-Methyl thiouracil		Saturated	47.3	47.3
DL-Ethionine	Methionine	0.16	49.0	47.7
Diisopropyl fluorophosphate	Sulfhydryl proteases	0.018	47.7	50.0
DL-Desthiobiotin	Biotin	0.11	44.5	46.0
Octyl aldehyde	Probably nonspecific	Saturated	88.1	88.0
Octyl alcohol		0.1	52.9	81.2
Ethanol		10 by volume	0.8	44.2
l-Glucose	*d*-Glucose	0.25	39.5	43.1

* Refers to concentration of the antagonist in the incubation medium. Before this, the spores were soaked in a solution of the antagonist, often more concentrated.

† The spores were not preheated, in contrast with the preceding line.

RESULTS

A summary of the results is given in Table 1. The data of columns 4 and 5 are averages from two or more experiments. Figures of 80 or above in column 5 indicate preheating of the spores; those of 55 or below indicate that the spores were not preheated. Intermediate figures are averages from experiments with and without preheating. The principal difficulties encountered in running these tests were the low solubility, or instability toward heat, or both, of some of the antagonists. In instances of very low solubility, an excess of the antagonist in the form of an emulsion or a suspension in the incubation medium (e.g., octyl aldehyde) was used. Instability (e.g., dimethyl pyruvic acid) usually caused a drop in pH and made necessary the use of more concentrated buffer solutions. In experiments with diisopropyl fluorophosphate, the samples were incubated in a liquid medium to avoid the heating necessary in the preparation of the agar film. Since this compound is said to be inactive at pH 7, tests with this antagonist were run at pH 5.7 to 6, a range in which incipient germination can still take place readily. With this exception, all the antagonists were tested at approximately pH 7.

TABLE 2. *Effect of ethanol on incipient spore germination*

Pre-heating	Ethanol by vol	Period of incubation	Dark spores		
			Initial	Final	Net
	%	*hr*	%	%	%
−	0	1	0.5	44.2	43.7
−	1	1	0.5	36.2	35.7
−	0	1	6.1	49.2	43.1
−	2	1	5.2	36.3	31.1
−	0	1	3.1	53.1	50.0
−	5	1	3.2	20.9	17.7
−	0	1	0.5	44.2	43.7
−	10	1	0.3	0.8	0.5
−	10	1	3.3	3.5	0.1
+	10	1	5.6	7.4	1.7
−	10	1	2.9	3.8	0.9
+	10	1	2.8	8.9	6.1
−	10	19	3.4	2.4	−0.9
+	10	19	5.7	5.2	−0.4

The data of Table 1 show that the efficacy of

an antagonist may depend not only on its nature but also on its concentration (e.g., dimethylpyruvic acid), and the lack of inhibition by substances like 6-methyl thiouracil may be due to their low solubility. The data also show, at least in tested instances, that preheating of the spores may reduce their sensitivity to the antagonist (e.g., sorbic acid). Oxamic acid and DL-ethionine, contrary to expectation, caused a slight, but consistently observed stimulation. Octyl aldehyde, in contrast with octyl alcohol, was without effect. With the exception of ethanol, all effective substances tested showed only retardation or partial inhibition. Three of four antagonists of purines, pyrimidines, and nucleic acids revealed a definite inhibiting action, which is in agreement with the previous observation (Knaysi and Baker, 1947), suggesting an active role for ribonucleic acid in spore germination. Of the alcohol concentrations used, complete inhibition was approached only when the ethanol concentration was as high as 10%. Table 2 shows that a very small proportion of the spores turned dark during the first hour of incubation in the presence of 10% ethanol. When the spores were preheated before incubation, the proportion reached a few percentage units. However, these as well as the spores which turned dark during preheating gradually disappeared, so that after 19 hr of incubation the proportion of dark spores was found to be less than existed initially.

DISCUSSION

The procedure used in this work is highly sensitive, reproducible, and flexible, in spite of its simplicity.

Selection of incubation medium for testing incipient germination was determined by a desire to use the simplest possible medium, since complex media may contain alternate substrates and obscure the effect of the antagonist. The use of glucose-phosphate, with or without agar, was based on previous observations (Knaysi, 1945, 1948) that the endospore of *B. mycoides* contains an excellent source of nitrogen and would germinate and grow in a solution with a good source of energy such as glucose. It was also noted that the efficacy of glucose was several times greater when freshly autoclaved, or when phosphate was present. Both agents activate the reducing power of glucose, although the phosphate exerts its effect more slowly. The data of Table 3 show that glucose also stimulates germination of the endospore in the strain employed and that phosphate has an appreciable stimulating effect, even within the incubation period of 1 hr, in the presence of glucose, but little or no effect in its absence (a slight effect is noted in the presence of agar). The glucose was not replaceable by potassium pyruvate—of passing interest in view of the role attributed to pyruvic acid in spore germination by some bacteriologists. Hills (1950)

TABLE 3. *Effect of phosphate and of the concentration of glucose on the rate of incipient germination*

Concentration of glucose	Concentration of phosphate	Concentration of agar	Dark spores in 1 hr at 46 C
%	M	%	%
0.00	0.01	2	10.4
0.01	0.01	2	35.5
0.05	0.01	2	40.3
0.10	0.01	2	38.5
0.25	0.01	2	37.7
0.01	0.01	0	30.1
0.02	0.01	0	34.5
0.10	0.01	0	36.5
0.20	0.01	0	35.2
0.00	0.00	0	3.2
0.25	0.00	0	29.2
0.00	0.001	0	3.1
0.25	0.001	0	31.4
0.00	0.00	2	3.5
0.25	0.01	2	39.5
0.00	0.001	2	5.1
0.25	0.001	2	42.8

TABLE 4. *Rate of incipient germination on glucose-phosphate and brain heart agar media*

Medium	Preheating at 89 C for 10 min	Concentration of glucose in 0.01 M phosphate	Dark spores in 1 hr at 46 C
		%	%
Glucose-phosphate	+	0.25	82.8
Brain heart	+		82.3
Glucose-phosphate	+	0.25	82.9
Brain heart	+		84.2
Brain heart	−		33.4

also found that alanine is not replaceable by pyruvic acid. Table 3 shows that glucose has a maximum efficacy at a surprisingly low concentration, between 0.05 and 0.1%. Table 4 shows that a complex medium like brain heart infusion, which also contains some glucose, has no advantage over a dilute solution of glucose and potassium phosphate for the stimulation of spore germination in the strain employed.

In view of the efficacy of *d*-glucose in stimulating germination, it is surprising to note that the inhibiting action of *l*-glucose, in equal concentration, amounted only to about 3.5 percentage units. Perhaps a higher concentration of the *l* form would have exerted a more significant effect, but this form is not available commercially and our supply did not allow further experiments with this substance.

Oxamic acid is believed to inhibit lactic dehydrogenase, which promotes the oxidation of lactic to pyruvic acid. The lack of inhibiting power in oxamic acid, presuming it can permeate the spore, might suggest something less than a major role for pyruvic acid in the first stage of germination. Our results with 8-azaguanine are at variance with those of Kawasaki, Nishio, and Shinagawa (1959), who failed to note any effect of this substance on the beginning of germination in *B. subtilis*.

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LITERATURE CITED

HALVORSON, H. O. 1959. Symposium on initiation of bacterial growth. IV. Dormancy, germination and outgrowth. Bacteriol. Revs. **23**:267–272.

HILLS, G. M. 1950. Chemical factors in the germination of sporebearing aerobes. Observations on the influence of species, strain and conditions of growth. J. Gen. Microbiol. **4**:38–47.

KAWASAKI, N., M. NISHIO, AND T. SHINAGAWA. 1959. Effect of 8-azaguanine on the germination of spores of *Bacillus subtilis*. Osaka Shiritzu Daigaku Igaku Zasshi **8**:1315–1319.

KNAYSI, G. 1945. Investigation of the existence and nature of reserve material in the endospore of a strain of *Bacillus mycoides* by an indirect method. J. Bacteriol. **49**:617–622.

KNAYSI, G. 1948. Preliminary observations on germination of the spores of *Bacillus mycoides* in a nitrogen-free medium and certain properties of the transparent cells. J. Bacteriol. **55**: 753–757.

KNAYSI, G. 1952. The structure, division, and fusion of nuclei observed in living cells of *Mycobacterium thamnopheos*. J. Bacteriol. **64**:859–864.

KNAYSI, G. 1959. Optical density of the endospore of *Bacillus cereus* and its relation to germination and resistance. J. Bacteriol. **78**:206–216.

KNAYSI, G., AND R. F. BAKER. 1947. Demonstration, with the electron microscope, of a nucleus in Bacillus mycoides grown in a nitrogen-free medium. J. Bacteriol. **53**:539–553.

SCHMIDT, C. F. 1957. Activators and inhibitors of germination, p. 56–71. *In* H. O. Halvorson, [ed.] Spores. Publ. No. 5, American Institute of Biological Sciences, Washington, D. C.