STUDIES ON SPORE GERMINATION

II. Effect of Caramels from Sugars and Other Carbon Sources on Spore Germination

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In the preceding publication (Hachisuka *et al.*, 1955) we reported that spore germination of *Bacillus subtilis* (PCI 219) occurred in a short time in glucose-phosphate medium, or in a nitrogen-free synthetic medium (containing inorganic salts and glucose) in the presence of L-asparagine, DL-isoleucine, DL-valine, DL-serine, or DL-phenylalanine as a nitrogen source. In glucose-free media germination was not observed. From these results we have paid attention to effect of glucose on the germination.

In further research we have found that stimulation of germination occurs when the above mentioned media are autoclaved, while without autoclaving the stimulation does not occur. Concerning this phenomenon we have supposed that the change of glucose to caramel in the autoclaved media is the cause of this difference.

This paper is a report of some investigations on (1) whether our supposition is true, (2) effect of caramels made from some sugars, (3) effect of other carbon sources, and (4) effect of inhibitors of glycolysis and tricarboxylic acid cycle on the germination.

MATERIALS AND METHODS

Organism and strain. Bacillus subtilis (PCI 219) was used.

Spore suspensions. Spores were harvested in distilled water from a five day culture on meat extract agar, heated for 15 minutes at 80 C, washed repeatedly by centrifugation with distilled water, and resuspended in phosphate buffer.

Medium. As shown in each table.

Caramel. Caramels from glucose, fructose, galactose, mannose, sucrose, maltose, and lactose were tested. These sugars were heated until they were brownish yellow. Concentration of caramel is shown as content of glucose before heating throughout the present experiments.

Other carbon sources. Acetic, lactic, pyruvic, citric, α-ketoglutaric, succinic, fumaric, and malic acids were tested. The pH of media in which the acids were added was adjusted to 7.2 with sodium hydroxide.

Inhibitors of glycolysis and tricarboxylic acid cycle. Monoiodacetic acid, malonic acid, sodium fluoride, potassium cyanide, and arsenous acid were tested. Moreover 2,4-dinitrophenol (DNP) was tested. The pH of media in which these substances were added was adjusted to 7.2 with sodium hydroxide or hydrochloric acid.

Criterion of germination. Same as in the previous report (Hachisuka *et al.*, 1955).

Incubation period. Rate of spore germination was determined in a 2 hour culture throughout the present experiments.

RESULTS

Difference between effect of glucose and that of caramel on the germination. In the previous report (Hachisuka *et al.*, 1955) we used media containing one of the effective amino acids and glucose after autoclaving of the media for five minutes at 120 C. These autoclaved media proved to have a remarkable stimulative effect on germination. In practice, the autoclaving seemed to be unnecessary if we consider a few hours culture, so we tried an experiment using unautoclaved media, with expectation that the same results would be obtained. But unexpectedly, these unautoclaved media did not show stimulation. In further experiments it was decided that to show the stimulative effect on the germination, the glucose-phosphate buffer media containing L-asparagine, DL-isoleucine, or DL-serine would have to be autoclaved. These results are shown in table 1. The data of this table show that in the autoclaved media the germination is remarkable while in the unautoclaved ones the germination occurs very scarcely. Then to decide whether autoclaving affected glucose, we have compared the effect of glucose with that of its caramel which was made by heating glucose until it became a

TABLE 1

Effect of autoclaving of media on spore germination

Medium		Autoclaving for 5 Minutes at 120 C	Rate of Germination in 2 Hour Culture
			%
Buffer + L-asparagine + glucose		Unautoclaved	18
Buffer + L-asparagine + glucose		Autoclaved	97
Control	Buffer + L-asparagine	Unautoclaved	5
	Buffer + L-asparagine	Autoclaved	10
Buffer + DL-isoleucine + glucose		Unautoclaved	44
Buffer + DL-isoleucine + glucose		Autoclaved	93
Control	Buffer + DL-isoleucine	Unautoclaved	21
	Buffer + DL-isoleucine	Autoclaved	20
Buffer + DL-serine + glucose		Unautoclaved	6
Buffer + DL-serine + glucose		Autoclaved	99
Control	Buffer + DL-serine	Unautoclaved	5
	Buffer + DL-serine	Autoclaved	6

Buffer was phosphate buffer of M/50 and pH 7.2.

Each amino acid was added in 0.5 per cent as final concentration.

Glucose was added in 1 per cent as final concentration.

It was autoclaved after amino acid and glucose or amino acid separately was added to phosphate buffer.

In the case of unautoclaving, each amino acid, glucose, and phosphate buffer was not autoclaved separately or mixed.

brownish yellow color. The results are shown in table 2.

As it may be seen, caramel made from glucose has a stimulative effect while the effect of intact glucose is very little. Without one of the above mentioned effective amino acids, phosphate buffer medium containing only caramel from glucose had no effect on germination. Moreover, spores did not so germinate in the media containing one of the intact stimulative amino acids, autoclaved glucose, and phosphate buffer. The same results were obtained in the media containing autoclaved glucose, one of the autoclaved stimulative amino acids, and phosphate buffer. Therefore, the spores germinated better in the medium made up of materials autoclaved together than in the medium made up of materials autoclaved separately.

TABLE 2

Difference between effect of glucose and that of its caramel on spore germination

Amino Acid (Concentration: 0.5%)	Carbon Source (Concentration: 1%)	Rate of Germination in 2 Hour Culture
		%
L-Asparagine	None	5
	Glucose	20
	Caramel	100
DL-Isoleucine	None	21
	Glucose	40
	Caramel	85
DL-Valine	None	67
	Glucose	57
	Caramel	92
DL-Phenylalanine	None	0
	Glucose	0
	Caramel	70
DL-Methionine	None	0
	Glucose	0
	Caramel	38
None	Caramel	0

The basal medium was phosphate buffer solution (M/50, pH 7.2). All of these media, and each of amino acid, glucose, and caramel were not autoclaved.

The fact that these materials are glucose and one of the stimulative amino acids makes us think that there are two possibilities concerning the appearance of a substance or substances effective on spore germination. First, by Maillard reaction, autoclaving produces a new effective substance from amino acid and glucose. Second, by the addition of amino acid, autoclaving produces caramel from glucose better than no addition of amino acid.

TABLE 3

Relation between effect and color of caramel of glucose on spore germination

Color of Caramel	Rate of Germination in 2 Hour Culture
	%
No color	83
Faint yellow	85
Brownish yellow	94
Brown	76
Intact glucose	18

The basal medium was phosphate buffer (M/50, pH 7.2) containing 0.5 per cent L-asparagine without autoclaving. Each caramel was added into the basal medium in 1 per cent concentration. The colors of caramel were determined subjectively.

In our experiments caramel from glucose stimulated spore germination in a sufficient medium without autoclaving.

The relation between effect and color of caramel made from glucose and the minimal effective concentration. Caramel used in the preceding experiments was made by heating glucose until it became a brownish yellow color after being melted. But various degrees of its color can be obtained by heating, and it is of interest to know what degree of caramel is most effective on germination. For solving this, various degrees of caramel were made from glucose, and their effects on the germination examined. The results are shown in table 3. From the data in the table it is clear that the brownish yellow caramel is most effective, while the brown faint yellow and the only melted ones are less effective. These facts suggest that an unknown effective substance or substances contained in caramel appear and then disappear in proportion to the time of heating. The minimal effective concentration of brownish yellow caramel of glucose was 0.1 per cent concentration.

Effect of caramels made from some other sugars on the germination. From the preceding experiments it is clear that caramel from glucose has a remarkable effect on the germination. It has been examined to learn whether caramels from other sugars, that is, fructose, galactose, mannose, maltose, lactose and sucrose, have the same effect. The results are shown in table 4. As may be seen, caramel from fructose, maltose, lactose, and sucrose has almost the same effect as caramel from glucose, while intact sugars have very little effect except maltose. The color of the caramel has also the same relation with the effect on the germination as described in the caramel from glucose. Mannose and galactose, both intact and caramel, have only little effect.

Effect of other carbon sources on germination. It is important to know whether energy sources are necessary for spore germination. If caramels from some sugars are used as energy sources for the germination, can the fatty acids which are the intermediates of tricarboxylic acid cycle substitute for the energy sources? To clear this problem experiments were carried out. Acetate, pyruvate, lactate, citrate, α-ketoglutarate, succinate, malate, and fumarate were tested. The results are shown in table 5. The data indicate that these substances have almost no effect on germination. Then it is apparent that effect of caramels of some sugars cannot be substituted by these fatty acids.

Effect of some inhibitors of glycolysis and tricarboxylic acid cycle on germination. The inhibitors tested were monoiodacetic acid, malonic acid, arsenite, sodium fluoride, cyanide, and 2,4-

TABLE 4

Effect of caramels of various sugars on spore germination

Kind of Sugar		Rate of Germination in 2 Hour Culture
		%
Fructose	Intact	2
	Caramel	84
Maltose	Intact	50
	Caramel	75
Lactose	Intact	8
	Caramel	75
Galactose	Intact	6
	Caramel	24
Mannose	Intact	26
	Caramel	23
Sucrose	Intact	10
	Caramel	81
None		2

The basal medium was same as in table 3. Concentration of sugars was 1 per cent. Color of caramels of these sugars was brownish yellow. The media were not autoclaved.

TABLE 5

Effect of other carbon sources on the germination

Kind of Carbon Sources	Rate of Germination in 2 Hour Culture
	%
Acetate	0
Pyruvate	4
Lactate	0
Citrate	0
α-Ketoglutarate	0
Succinate	6
Malate	10
Fumarate	7
Caramel of glucose	98
None	2

The basal medium was same as in table 3. pH of the medium was adjusted to 7.2 with NaOH before the inoculation of spores. Concentration of these carbon sources was 1 per cent. The media were not autoclaved.

TABLE 6

Effect on some inhibitors of glycolysis and tricarboxylic acid cycle on germination of spores of Bacillus subtilis

Basal Medium	Kind of Inhibitors	Concentration	Rate of Germination in 2 Hour Culture
			%
Buffer + L-Asparagine (0.5 per cent) + Caramel of glucose (1 per cent)	Monoiodacetate	M/50	96
	Malonate	M/100	88
	Arsenite	M/100	84
	Fluoride	M/100	95
	Cyanide	M/100	92
	DNP	M/1,000	90
	None		98

The media were not autoclaved.

dinitrophenol (DNP). The results are shown in table 6. As may be seen, all the inhibitors have no inhibitory effect on the germination as long as the germination is determined by the decrease of optical density of culture media. But electron microscopically, the germination may be unaffected by the inhibitors at least until the first phase which is the appearance of translucent areas in spore (Hachisuka *et al.*, 1955) has finished.

In order to decide to which phase the germination can develop in the media containing the inhibitors, experiments were carried out. For this purpose the sufficient medium which supported both the development of all phases of germination and vegetative growth had to be used as the basal medium. Therefore, a synthetic medium composed of 1 per cent caramel from glucose, 0.5 per cent L-asparagine, 0.6 per cent $Na_2HPO_4 \cdot 12H_2O$, 0.2 per cent KH_2PO_4, 0.1 per cent $MgSO_4 \cdot 6H_2O$, 0.01 per cent $CaCl_2 \cdot 2H_2O$, and 25 mg per 100 ml $FeSO_4 \cdot 7H_2O$ was used. The rate of germination was determined in two hour cultures, the degree of the development of the germination was determined in 48 hour cultures by electron microscope, and the vegetative growth was determined from whether surface pellicle formed or not in 48 hour cultures. The material for the electron microscope was obtained by centrifuging culture media and washing repeatedly by centrifugation with distilled water to omit the constituents of media.

As may be seen in table 7, malonate and sodium fluoride do not inhibit germination and vegetative growth, and the other inhibitors do not inhibit germination (strictly speaking, the beginning of the germination) but inhibit completely the vegetative growth. In the case of monoiodacetate and DNP all spores have translucent areas, but the formation of germ pore and the appearance

TABLE 7

Effect of some inhibitors of glycolysis and tricarboxylic acid cycle on both germination of spores and vegetative growth of Bacillus subtilis

Kind of Inhibitors	Concentration	Rate of Germination in 2 Hour Culture	Growth in 48 Hour Culture
		%	
Monoiodacetate	M/100	98	−
Malonate	M/100	94	+*
Arsenite	M/100	89	−
Fluoride	M/100	86	+*
Cyanide	M/100	92	−
DNP	M/1,000	90	−
Control		90	+*

The composition of the basal medium was 1 per cent caramel of glucose, 0.5 per cent L-asparagine, 0.6 per cent $Na_2HPO_4 \cdot 12H_2O$, 0.2 per cent KH_2PO_4, 0.1 per cent $MgSO_4 \cdot 6H_2O$, 0.01 per cent $CaCl_2 \cdot 2H_2O$, and 25 mg per 100 ml $FeSO_4 \cdot 7H_2O$.

* Shows that surface pellicle was formed.

Each inhibitor was added aseptically to the previously autoclaved medium.

of vegetative bacilli were not observed although in the case of arsenite one or two spores have a germ pore. Then it may be possible to say that in media containing monoiodacetate, arsenite, and DNP separately, germination stopped at the first phase. But in the case of cyanide there are vegetative bacilli with spores which have translucent areas and a germ pore. This may show that cyanide does not inhibit the development of the germination until the emergence of new bacilli has finished but does inhibit their further growth.

DISCUSSION

It has been shown that autoclaving affects glucose.

The caramel made from glucose has a remarkable effect on the beginning of the germination in phosphate buffer medium containing one of the effective amino acids. Moreover caramels of fructose, maltose, lactose, and sucrose have almost the same effect as that of caramel of glucose. The mechanism of the action of these caramels on spore germination has not yet been solved.

In the previous report (Hachisuka *et al.*, 1955) it has been suggested that the action of the effective amino acids may be regarded as a sparking factor for the beginning of spore germination rather than as materials for the synthesis of cell substances. Also it seems likely that these caramels are used in another way than as energy sources for spore germination because it may be supposed that energy sources from external media are unnecessary for the development of spore germination until the first phase has finished. The evidence of this supposition is as follows: the first phase of almost all spores inoculated in phosphate buffer media containing one of the effective amino acids and one of the effective caramels is able to finish, while the development of spore germination is scarce if intact glucose is used in the place of caramels; the effect of caramels cannot be substituted by some fatty acids which are the intermediates of tricarboxylic acid cycle; and DNP and monoiodacetate do not affect the development of the first phase of spore germination. From the fact that monoiodacetate, which is a blocking agent of glycolysis, does not inhibit the development of the first phase of spore germination but does inhibit the further development, it seems likely that energy derived from glycolysis may be unnecessary for the development of the first phase. Similarly, from the fact that DNP, which is an uncoupling agent in the synthesis of energy-rich phosphate link, does not inhibit the development of the first phase but does inhibit the further development, it may be possible to say that the energy derived from oxidative phosphorylative mechanism is unnecessary for the development of the first phase of the germination, while the energy from that mechanism may be required for the further development of the germination. From these facts, two possibilities may be suggested; one is that the spores have a reserve material which is immediately available as an energy source for the development of the first phase of the germination, and if sufficient external conditions are given, the development begins to occur; the other is that the development of the first phase does not require energy and controlling factors of the development but only proper physicochemical conditions.

It has been found that spores which have finished the first phase of the germination show a marked respiratory activity (Hachisuka *et al.*, *unpublished data*). Considering both this fact and the above supposition, it may be probable that from the point of view of the energy requirements from the external media the process of spore germination is divided into two phases—the first nonenergy source requiring phase and the latter energy source requiring phase. In this case the first phase may correspond to the appearance of translucent areas in spore and the latter phase to the further phases until new bacilli emerge and develop to vegetative cells.

From the fact that cyanide does not inhibit the development of the germination until the emergence of new bacilli but does inhibit further growth, it may be supposed that aerobic respiration catalyzed finally by cytochrome oxidase is blocked. Therefore it seems likely that the metabolic processes in the phase of the emergence of new bacilli are somewhat different from those in the vegetative growth. Monoiodacetate, DNP, and arsenite do not inhibit the development of the first phase of spore germination but do inhibit further development. As arsenite has been proved to inhibit oxidative decarboxylation of α-ketoglutarate in tricarboxylic acid cycle (Baldwin, 1949), the significance of the inhibitory action of arsenite on the further development of spore germination may involve the inhibition to the tricarboxylic acid cycle.

Knaysi (1945) has reported that spores of strain C_2 of *Bacillus mycoides* are able to germinate normally in a solution of glucose, and he concluded that the spores of the strain contain a relatively large amount of a nitrogen containing reserve material not suitable as a source of energy and that they contain no other reserve material for energy. The difference between Knaysi's conclusion and ours may be due to the difference of species investigated. Amaha and Sakaguchi (1952) have also reported that in synthetic media without glucose spores of *B. mycoides*, *B. megaterium*, *B. natto*, *B. cereus*, and *B. subtilis* do not grow to vegetative cells, and surface pellicles are not formed while growth occurs if glucose is added. However, from their experiments it is not clear whether energy sources are required for spore germination.

Previous workers studying the nutrition of spore germination have never considered the phases of the process of spore germination. The results presented here may suggest that the nutritional requirements of spores differ in accordance with their phase. It should be emphasized that to study the nutritional requirements and the appearance of enzymic activities in spore germination, "the phases of spore germination" have to be taken into consideration. Further studies of the problems are being conducted.

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SUMMARY

Almost all spores of *Bacillus subtilis* (PCI 219) begin to germinate in a short time when they are inoculated in phosphate buffer media containing caramel made from glucose, and L-asparagine, DL-isoleucine, DL-valine, or DL-serine separately.

Caramels of fructose, maltose, lactose, and sucrose have almost the same effect of caramel of glucose.

The effect of these caramels cannot be completely displaced by the intact sugars and some fatty acids belonging to the tricarboxylic acid cycle.

The significance of caramels on spore germination has not yet been decided, but they seem not to be used as an energy source for the development of the first phase of spore germination.

Monoiodacetate, 2,4-dinitrophenol, and arsenite do not inhibit the development of the first phase of spore germination but do inhibit further development of spore germination after the second phase. Cyanide does not inhibit the emergence of new bacilli but does inhibit their further growth.

Possible explanations on the significance of these inhibitors have been discussed.

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