

CHEMICAL PRESERVATIVES IN FOODSTUFFS.

V. THE EFFECT OF BENZOIC ACID ON THE SPORULATION OF *BACILLUS MEGATERIUM*

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Although the bacterial spore has attracted the interest of investigators since the beginning of microbiology, the biochemistry of spore formation and germination has so far remained a matter largely for conjecture. The bulk of the investigations reported in the rather abundant literature in this field are concerned with the germination of the spores, whereas less attention has been devoted to spore formation. It has been generally stated (9) that conditions which are optimal for sporulation are approximately the same as the optimal conditions for reproduction, although in the former case the range of tolerance is narrower. Qualitative differences in the requirements concerning the medium were first recognized when investigators learned how to prepare media which were chemically fully defined. A review of these has been made by ORDAL (9) and CURRAN (3). These statements have no direct significance for food microbiology, since food normally contains sufficient materials essential for sporulation. Again, investigations relating to the study of substances inhibiting sporulation have been rather few in number. HARDWICK, GUIRARD & FOSTER (6) stated that some saturated fatty acids may act as inhibitors of sporulation. GOLLAKOTA & HALVORSON (4) observed that compounds which form metal chelates, such as EDTA, inhibit the spore formation of bacteria by binding cations essential for the process. The same authors also stated that α -picolinic acid may prevent sporulation in *Bacillus cereus*. In a study which is of interest from the standpoint of food microbiology, VAS & PROSZT (11) investigated the heat resistance of two *Bacillus* species in the brine of canned peas, to which had been added various preservatives and antibiotics. These substances reduced to various extents the thermal resistance of the experimental organisms. Other studies on

the influence of different chemicals on the heat resistance of spore-forming bacteria were carried out by MICHENER, THOMPSON & LEWIS (7). Furthermore, mention may be made of the experiments made by CAMPBELL, SNIFF & O'BRIEN (2) where subtilin and nisin were successfully used in canning with aim of reducing the severity of heat sterilization. In these experiments, two *Bacillus* species were used as experimental organisms.

The purpose of the studies mentioned above was to reduce the heat resistance of spores already formed. In the present study, based on the observation made in preliminary experiments of the authors (unpublished), that benzoic acid and esters of p-hydroxybenzoic acid inhibit sporulation much more effectively than the vegetative growth of some *Bacillus* species (*B. megaterium*, *B. cereus* and three unidentified species), attention has been mainly directed to the effect on sporulation of the preservatives mentioned.

Materials and methods

The experimental organism selected was a strain of *Bacillus* which had been isolated from canned food. The organism grew well on ordinary peptone meat broth agar. It was identified according to Bergey's Manual of Determinative Bacteriology, 7th ed., as *B. megaterium*, although its spore formation was more rapid than in the basic type described in the Manual. The vegetative cells grew singly or in pairs, without forming pellicle or cell chains.

The medium utilized, unless otherwise stated below, was ordinary peptone meat broth, or peptone meat broth agar (pH 7). In all experiments, the incubation temperature was 34°C.

For mass production, the bacteria were grown for 18 hrs. at 34°C. The mass was separated by centrifugation for 30 min. (4,600 r.p.m.); both this and the later steps were performed aseptically. The mass was washed with sterile saline by centrifuging 3 to 5 times for 30 minutes on each occasion. After the last washing, the mass was mixed in a small volume of sterile saline to give a homogeneous suspension, from which the inoculation samples were taken by means of a pipette. In such a bacterial mass, the amount of spores varied between 0.6—10.5 % of the total number of cells. The bacterial count was effected by the plate method. Incubation lasted for 24 hrs. at 34°C, following which the colonies were stained with 2, 3, 5-triphenyltetrazolium chloride and counted.

The relative quantities of spores and vegetative cells were determined in such a way that on the one hand plating was done with bacterial suspension as such, and on the other with a suspension which had been heat-treated (20 minutes at 75°C). The difference between the total number of colonies and the number of colonies formed by the heat-treated bacteria was taken as the amount of vegetative bacteria. Microscopical examination showed that the method was suitable for the purpose, with the exception of a few instances in which it seemed that the heat resistance of the spores was somewhat reduced after long periods of incubation. This phenomenon was observable in old cultures irrespective of whether the medium contained preservative or not.

Results

Cultures in nutrient medium

The experimental organism reproduced in a medium to which 1 per cent of sodium benzoate had been added (initial concentration 1.1×10^{-4} -m undissociated benzoic acid/l; the dissociation constant of benzoic acid used in calculations was 6.3×10^{-5}). During the course of culturing the pH changed from 7.0 to approx. 7.8. In media which contained 1 % sodium benzoate, only vegetative cells were observed after 2 to 3 days, irrespective of the age of the inoculated bacterial suspension and its spore content at the time of inoculation. As one part of the preliminary experiments was carried out with pure spore suspensions as inocula, it is evident that benzoic acid did not inhibit germination of the spores, but prevented the formation of new ones.

For most of the experiments, the concentration of sodium benzoate chosen was 0.5 % (5.5×10^{-3} -m undissociated benzoic acid at pH 7), which is thus clearly a sublethal concentration. The composition of the medium was as follows:

Meat extract (Difco)	0.6 %
Peptone (Witte)	1.0 %
Phosphate buffer (Sørensen)	1/15-m
Tap water	ad 100 %

In general, the period of culturing was 2 to 4 weeks.

Fig. 1 presents the typical result of a culturing experiment in nutrient solution at initial pH 7. After 5 to 6 days, complete sporulation had occurred in the benzoate-

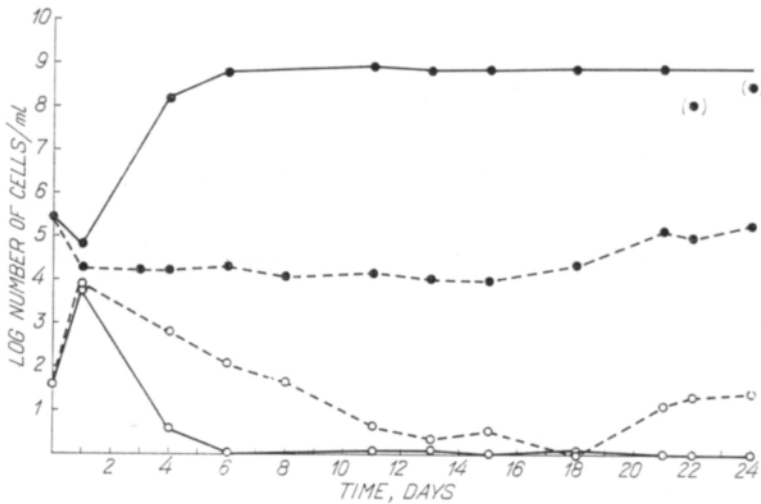


Fig. 1. Effect of benzoic acid on the amounts of spores and vegetative cells of *B. megaterium* in nutrient solution.

Initial pH of the nutrient solution 7.0.

- ——— ○ = vegetative cells in nutrient solution without benzoate
- - - - - ○ = " " " " with 0.5 % sodium benzoate
- ——— ● = spores in nutrient solution without benzoate
- - - - - ● = " " " " with 0.5 % sodium benzoate

free medium. In the medium with benzoate, the amount of spores was somewhat decreased during the first day and then remained unchanged for approximately 2 weeks. During this time, the amount of vegetative cells gradually decreased. After an incubation period of about 2 weeks, new spores began to be formed in the benzoate-containing medium, and there was also an increase in the amount of vegetative cells. The formation of spores in the benzoate-containing medium after a long lag period is not the result of an adaptation, or at least not of a permanent adaptation, since the organism which was derived from the spores formed in the benzoate-containing medium, even after pure culturing, always displayed behaviour similar to that described above.

In the experiment reported above (Fig. 1) the bacteria were inoculated in 200 ml samples of medium in 750 ml conical flasks, which were shaken twice a day during the experiment. Since it was assumed that the result obtained was based on the change of pH during the experiment, an additional experiment was made to check this. *B. megaterium* was grown in the same medium as above. The experi-

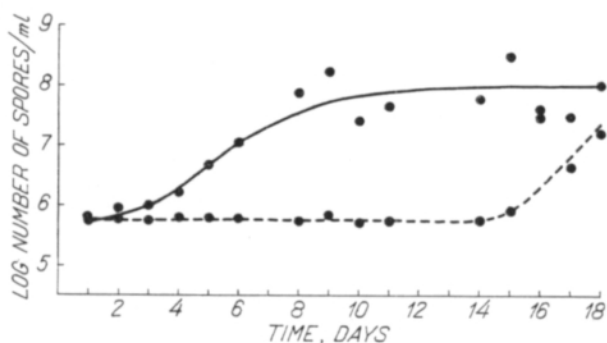


Fig. 2. Effect of benzoic acid on the spore formation of *B. megaterium* in nutrient solution.

Initial pH of the nutrient solution 7.0.

● — ● = spores in nutrient solution without benzoate
 ● - - - ● = spores in nutrient solution with 0.5 % sodium benzoate

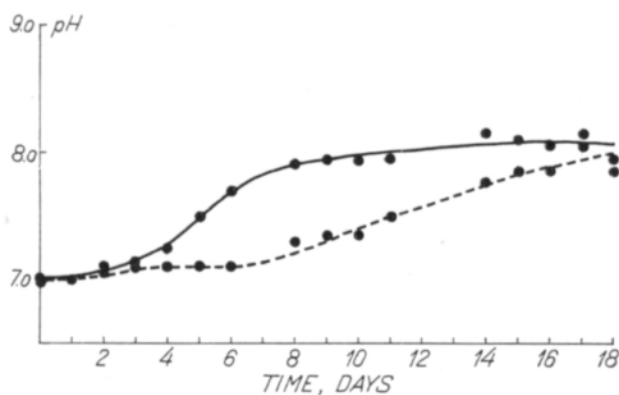


Fig. 3. Changes in pH of the nutrient solutions of Fig. 2.

● — ● = pH of nutrient solution without benzoate
 ● - - - ● = pH of nutrient solution with 0.5 % sodium benzoate

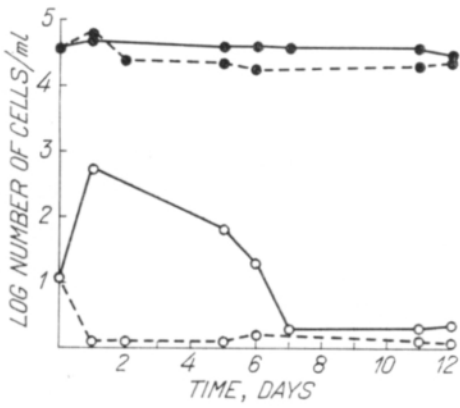


Fig. 4

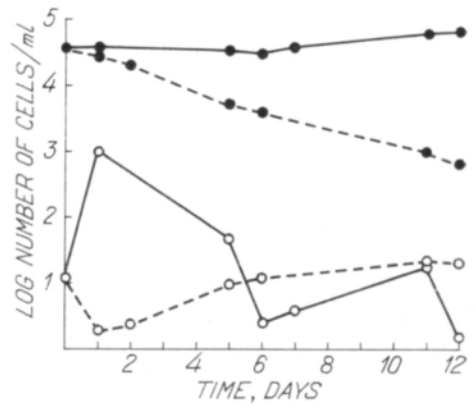


Fig. 5

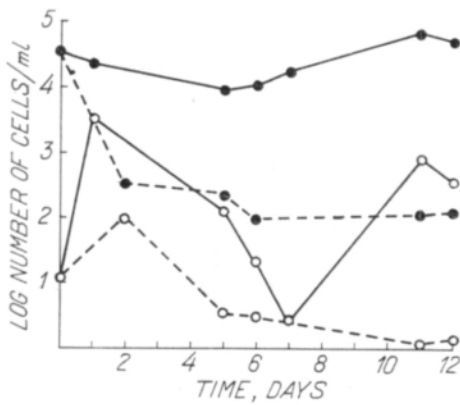


Fig. 6

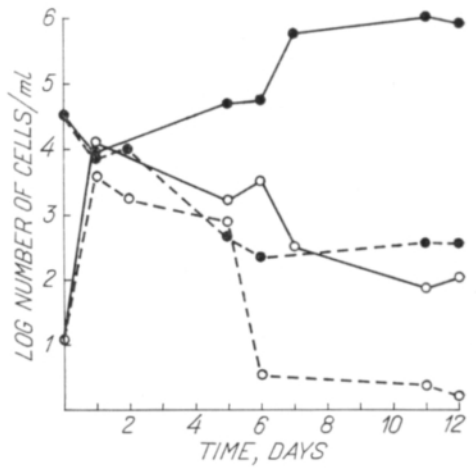


Fig. 7

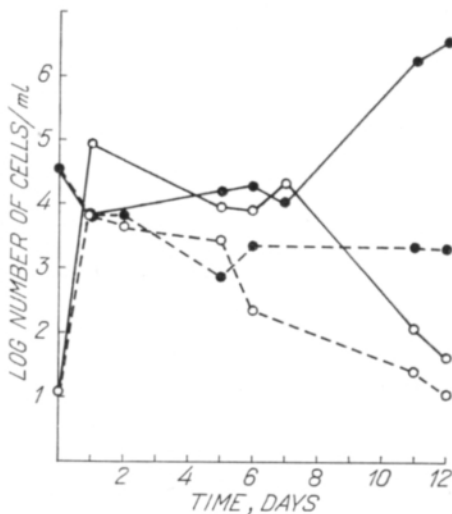


Fig. 8

Figs. 4, 5, 6, 7, and 8: Effect of benzoic acid on the amounts of spores and vegetative cells of *B. megaterium* in nutrient solution at different pH values.

Fig. 4: Initial pH of the nutrient solution 5.0

Fig. 5: — — — — — 5.5.

Fig. 6: — — — — — 6.0.

Fig. 7: — — — — — 6.5.

Fig. 8: — — — — — 7.0.

- ———— ○ = vegetative cells in nutrient solution without benzoate
- - - - - ○ = vegetative cells in nutrient solution with 0.3% sodium benzoate
- ———— ● = spores in nutrient solution without benzoate
- - - - - ● = spores in nutrient solution with 0.3% sodium benzoate

ment was performed so that the medium was distributed into tubes which were inoculated; one tube was taken for every plating. Fig 2 illustrates that in this experiment the spore formation in the benzoate-free medium was initially somewhat slower than in the preceding experiment, but that in principle the sporulation was similar to that described above. In the medium which contained benzoate the spore formation started after 2 weeks. In Fig. 3 can be seen the changes in pH in the second experiment. The form of the pH curve in the benzoate-free medium is very much like the curve of the amount of spores. In the medium containing benzoate, the pH increased rather evenly — evidently as a result of the proteolytic metabolism of the vegetative cells — and after 18 days arrived at approximately the same value (pH 8) as that of the benzoate-free medium. At the beginning of the sporulation in the benzoate-containing medium, its pH was approx. 7.75. Owing to the increase in pH, the concentration of undissociated benzoic acid had then decreased from 5.5×10^{-5} molar to 9.6×10^{-6} molar. According to the results of this experiment, *B. megaterium* is able to form spores in benzoate-containing medium only when the concentration of undissociated benzoic acid is lower than 10^{-5} moles/l.

Figs. 4 to 8 show the results of culture experiments with *B. megaterium* in media containing 0.3 % sodium benzoate, and with initial pH values between 5 and 7, the differences being 0.5 pH units. The concentration of undissociated benzoic acid decreased in these solutions from 2.8×10^{-3} moles (pH 5.0) to 3.3×10^{-5} moles (pH 7.0), and thus in all solutions the initial concentration was greater than the critical concentration of the spore formation (10^{-5} moles). As controls, cultures were grown in similar nutrient solutions but without benzoic acid.

At pH 5.0 (Fig. 4), all vegetative cells were rapidly killed by the benzoic acid. In the benzoate-free medium, a rather limited reproduction of vegetative cells took place initially, but practically speaking they were all killed in one week. As regards the spores, no essential changes were noted in a medium as acid as this. Evidently the low pH prevented the germination of the spores, which again protected them from the effect of benzoic acid.

At pH 5.5 (Fig. 5), the organism in the benzoate-free medium behaved in principle in the same way as at pH 5.0. In the medium containing benzoate, the amount of spores (i.e. cells resistant to the heat treatment) slowly and evenly decreased, while the number of vegetative cells, after a sharp decrease, started a slow increase, in which the rise nearly corresponded to the loss in the number of spores. This experiment was repeated, and on this occasion the experimental period was prolonged to 28 days. In this checking experiment, the behaviour of the organism was almost similar to that observed in the preceding one, and after about 10 days the amount of both spores and vegetative cells slowly dropped and approached zero. However, this was not reached by the end of the experiment. Microscopic observations indicated that the increase in the vegetative cells was due to germination of the spores, but that the germination process itself had been slowed and was possibly incomplete.

In media with an initial pH between 6.0 and 7.0 (Figs. 6—8), the growth curves are largely similar. In benzoate-free solutions the growth of the organism was

stimulated with a rise in pH (at pH 7.0 in this particular experiment, the lag phase was unusually long). In benzoate-containing media the amount of vegetative cells first showed an increase; this increase was least marked at pH 6.0, and greatest at pH 7.0. Subsequently there was a drop in the amount of vegetative cells, the number approaching zero. The amount of spores fell at first, but at 6 days, definite levels were reached which were not subject to further decrease during the course of the experiment.

On consideration of these results, it is observable that in media with a pH below 6.0, the addition of 0.3 % sodium benzoate introduced no noteworthy decrease in the total bacterial count, although the concentration of the undissociated benzoic acid was greatest in these media. In all probability, this is explained by the high acidity itself already inhibiting the growth of the organism to such an extent that benzoic acid, which evidently influences the vegetative cells alone, makes no great difference on the final result. Benzoic acid displays the maximum effect on the total bacterial count within the pH range 6.0—6.5, whereas the effect is somewhat less at pH 7.0. The latter phenomenon is evidently a result of the decrease in the concentration of undissociated benzoic acid. Since pronounced sporulation in benzoate-free medium occurs only when the pH is higher than 6.0, the effect of addition of benzoate on the final spore numbers is greatest in those media with a pH of 6.5 or 7.0. As was shown by the preceding experiments, however, the sporistatic effect of benzoate depends on the buffer capacity of the medium. In a medium to which 0.3 % benzoate has been added, spore formation is possible if the pH has been raised above 7.5, following which the concentration of undissociated benzoic acid passes below the limit of 10^{-5} moles.

Corresponding experiments were carried out using media to which had been added various amounts of p-hydroxybenzoic acid ethyl ester. The length of culturing was 3 to 4 weeks, and the initial pH of the media varied from 5.0 to 7.0. It was shown that 0.05 % (approx. 3×10^{-3} -m) p-hydroxybenzoic acid ethyl ester exerts an effect on the sporulation which is almost the same as the effect of undissociated benzoic acid in a concentration of 10^{-5} -m; moreover, the former does not inhibit reproduction of the bacteria.

Experiments in nitrogen-free media

A study was also made of the effect of the addition of benzoate on *B. megaterium* in media with no nitrogen sources. In most of the experiments, either 1/15 m phosphate buffer alone or a similar buffer with 0.3 % glucose was utilized. The pretreatment and incubation, together with the counting of spores and vegetative cells, was effected in the same way as that described for previous experiments.

Fig. 9 shows the typical results obtained with *B. megaterium* suspension, incubated in two 0.3 % phosphate buffer solutions containing glucose at pH 7.0, with one solution containing 0.5 % sodium benzoate in addition. In these experiments, spores were rapidly formed in the benzoate-free solution, and consequently the number of vegetative cells showed a corresponding loss. Under these conditions, the essential changes took place during about the two first days, and subsequently

practically no further change took place during the culture period (usually three weeks). In media containing 0.5 % sodium benzoate, the spore number did not change during the course of the experiment. The amount of vegetative cells decreased more slowly in benzoate-containing media than in benzoate-free ones, in which they formed spores. In the benzoate-containing solution, again, the loss of vegetative cells was caused by their death, which attained 100 % only after a prolonged period (2—3 weeks).

In media containing glucose, the results on each occasion were invariable, but in phosphate buffer alone the organism showed two different modes of behav-

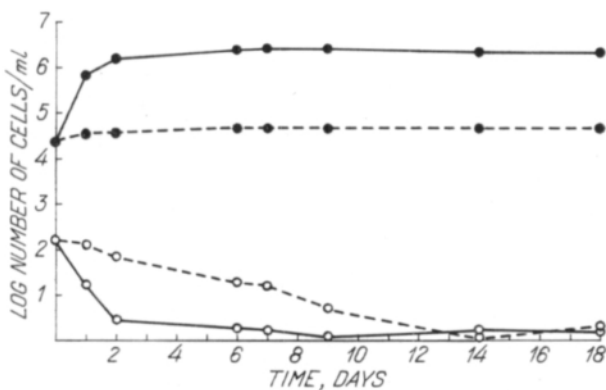


Fig. 9

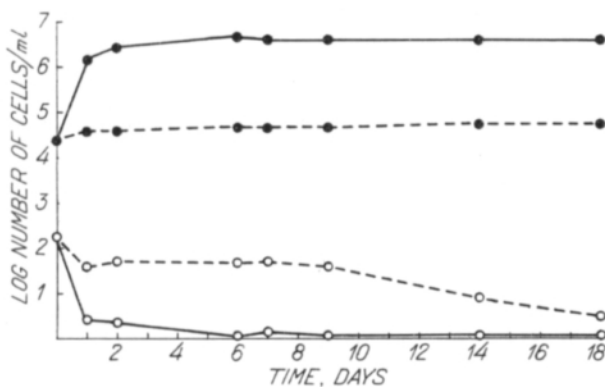


Fig. 10

Fig. 9, and 10. Effect of benzoic acid on the amounts of spores and vegetative cells of *B. megaterium* in nitrogen-free medium. Fig. 9 with glucose, Fig. 10 without glucose.

Nitrogen-free basal medium:

Phosphate buffer 1/15 m (pH 7.0)

Glucose 0.3 %

- = vegetative cells in basal medium without benzoate
- - -○ = " " " " " " with 0.5 % sodium benzoate
- = spores in basal medium without benzoate
- - -● = " " " " " " with 0.5 % sodium benzoate

ious. If the flasks were shaken during the first day of incubation, the curves were almost the same as those of the media containing glucose (Fig. 10). But if the flasks were left untouched during the first days (up to 3 to 5 days), in general the spore number remained unchanged, and the number of vegetative cells decreased only slowly, in the same way as in the solutions containing benzoate. A probable explanation for this is that sporulation depends on some oxidation processes, for which the organism contains enough hydrogen donors, but which can take place only under sufficiently aerobic conditions (TINELLI) (10). Such conditions are created by shaking the flasks. Further, the presence of glucose is enough to raise the redox potential to such a level that spore formation can occur. When sodium benzoate was added to the phosphate buffer, no sporulation took place, and the curves took on a similarity to those obtained when glucose-phosphate buffer solutions were used. Thus, it can be concluded from these observations that benzoic acid acts as an inhibitor of the oxidative reactions necessary for sporulation.

In other experiments, an attempt was made to eliminate the sporulation-inhibiting effect of benzoic acid by adding to different bacterial suspensions containing benzoate certain metal ions which have been mentioned as important for sporulation (Ca^{++} , Mn^{++} , Zn^{++} and Mg^{++}) (3). However, no definite effect of the metals was observable. Similarly, the addition of dipicolinic acid was unable to eliminate the effect of benzoic acid.

When glucose was replaced with pyruvic acid, whereby the pH was adjusted to 7, the effect of benzoic acid was quite similar to that in the solutions containing glucose. In benzoate-free solution, spores were formed, although to a somewhat less extent than in glucose-phosphate buffer solutions.

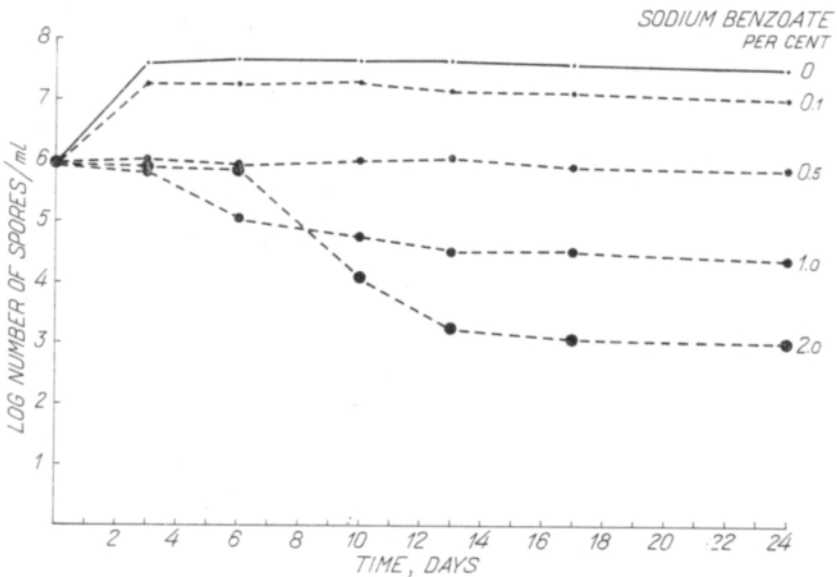


Fig. 11. Effect of various concentrations of benzoic acid on the amounts of spores of *B. megaterium* in nitrogen-free medium containing glucose.

Nitrogen-free basal medium:

Phosphate buffer 1/15 m (pH 7.0).

Fig. 11 presents changes in the amount of spores in glucose-phosphate buffer solutions containing various amounts of sodium benzoate (0 %, 0.1 %, 0.5 %, 1.0 % and 2.0 %). By reason of the phosphate buffer, the initial pH 7 did not change in these experiments to any notable degree, and thus the concentration of undissociated benzoic acid remained almost the same until the end of the experiment (24 days). When the pH of the solution is 7, the concentration of undissociated benzoic acid in 0.1 % solution is very near to 10^{-5} moles/l, which has been shown in nutrient solutions to be the limiting concentration of spore formation. In this experiment, spores were formed in 0.1 % sodium benzoate solution, although clearly not as many as in the benzoate-free solution. In 0.5 % sodium benzoate solution, the amount of spores remained unchanged, as in the previous experiment (cf. Fig. 9). In solutions with sodium benzoate concentrations of 1.0 and 2.0 %, the amount of spores initially remained unchanged, following which it dropped rather steeply to reach definite levels which evidently depended on the concentration of benzoic acid; subsequently, a further slow decline seemed to occur.

Discussion

BOSUND (1) has stated that apart from other effects, benzoic acid strongly prevents oxidation processes connected with respiration in some bacteria. According to TINELLI (10), the initial stages of spore formation imply high respiratory activity. Again, according to NAKATA (8), the initial step in the sporulation process is a strong oxidation of acetic acid, a process which according to BOSUND (1) cannot start in the presence of benzoic acid.

The inhibition of spore formation by benzoic acid stated in the present investigation is evidently connected with the above observations. As there is still a lack of knowledge of the spore formation in bacteria, there is no reason to draw sweeping conclusions, especially since benzoic acid is also able to disturb metabolical processes other than respiration (1).

Nevertheless, the central role of the inhibition of oxidation is indicated by the observations that the organism is able to form spores in glucose solution alone, but not in a similar solution containing benzoic acid, nor in a buffer solution alone under relatively anaerobic conditions.

According to the investigations of HARDWICK & FOSTER (5), glucose inhibits spore formation in *Bacillus mycoides*, whereas spores are formed in plain water. This is in apparent disagreement with the results of the present work. The explanation may be found in the differences in experimental methods, of which the most essential is evidently that HARDWICK & FOSTER made short-term experiments (up to about 1 day) using shake culture, whereas in the present study, where food technological points of view have been given preference, the experiments lasted for many days, and cultures were stationary. The redox conditions were accordingly made very different.

Summary

In *Bacillus megaterium*, benzoic acid does not inhibit the reproduction or germination of already-formed spores when the concentration of undissociated benzoic

acid is 10^{-5} -m, but it prevents the formation of new spores in this concentration.

It was not possible to eliminate this sporulation-inhibiting effect of benzoic acid by the addition of nutrients containing nitrogen or various metal ions.

Ethyl ester of p-hydroxybenzoic acid in the concentration 3×10^{-3} -m showed an effect on the sporulation of *B. megaterium* similar to that of benzoic acid.

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SELOSTUS:

ELINTARVIKKEIDEN KEMIALLISTA SÄILÖNTÄÄINEISTÄ

V. Bentsoehapon vaikutus sporulationiin *Bacillus megaterium*illa

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*Bacillus megaterium*illa on tutkittu bentsoehapon vaikutusta itiöihin. Kun dissosioitumattoman bentsoehapon väkevyys on 10^{-5} -m, se ei estä k.o. bakteerin lisääntymistä eikä olemassaolevien itiöiden itämistä, mutta sensijaan uusien itiöiden muodostuminen estyy tässä rajaväkevydessä.

Bentsoehapon sporistaattinen vaikutus lienee selitettävissä itiönmuodostukseen liittyvien hapestusreaktioiden estymisen kautta.

Bentsoehapon itiönmuodostusta estävää vaikutusta ei voida kumota lisäämällä tyypipitoisia ravintoaineita tai Ca^{++} , Mn^{++} , Zn^{++} tai Mg^{++} ioneja.

*Bacillus megaterium*in itiönmuodostukseen vaikuttaa p-hydroksibentsoehapon etylesteri konsentraatiossa 3×10^{-3} -m samalla tavalla kuin bentsoehappo.