THE INFLUENCE OF IRON OR MANGANESE UPON THE FORMATION OF SPORES BY MESOPHILIC AEROBES IN FLUID ORGANIC MEDIA

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There is growing evidence that many complex organic substrates, including most of the commonly used bacteriological fluid media, although supporting heavy vegetative growth, yield relatively few or no aerobic spores unless fortified with certain minerals. The salts which, when added to a particular substrate, promote the formation of spores depend upon its composition, upon the organism used, and other factors; thus, Fabian and Bryan (1933) noted that the addition of monovalent but not bi- and trivalent salts increased the percentage of mesophilic spores formed in peptone solution. Knaysi (1945) observed that added magnesium sulfate had a beneficial effect on the sporulation of *Bacillus mycoides* in glucose broth but only if the oxygen supply was limited. Foster and Heiligman (1949) found that a deficiency of potassium in the usual organic media was limiting in the sporulation of *Bacillus cereus*. More recently Charney *et al.* (1951) have pointed to a specific effect of manganese in trypticase and nutrient broths on the sporulation of *Bacillus subtilis* and certain other mesophiles.

In the course of observations on the germination of spores in milk, we found that certain salts, notably iron and manganese, although without effect upon the germination of the spores, transformed the spore generating potential of the medium for certain species. Since normal milk is a notoriously poor medium for the production of aerobic spores, the apparent specific effect of the two salts led us to study this reaction in some detail. The results are presented in this paper.

MATERIALS AND METHODS

The principal organisms used were: *Bacillus subtilis* (15u) American Can Company and *B. brevis* Temple University. Limited observations were made on *B. subtilis* (9499), *B. stearothermophilus* (1518), and *B. thermoacidurans* (*B. coagulans*) (43P, 2353) from National Canners Association; *B. subtilis* (4149), *B. stearothermophilus* (CM2), and unidentified culture (3679) from American Can Company; *B. subtilis* (6051, 6634), *B. brevis* (8185), *B. mycoides* (*B. cereus* var. *mycoides*) (6462), and *B. circulans* (4513) from American Type Culture Collection; *B. cereus* (401), *B. macerans* (277), *B. polymyxa* (354), *B. laterosporus* (314), and *B. megaterium* (753) from the N. R. Smith Collection.

The spores were produced, collected, and prepared for use as described previously (Curran and Evans, 1945) except that dispersion of the clumps was accomplished by shaking with small glass beads.

The basal media were autoclaved skim milk, usually unadjusted, and nutrient broths containing the following ingredients per liter, each adjusted to a final pH 7.0:

Nutrient broth—beef extract (Difco), 3 g; peptone (Difco), 5 g.

Yeast broth—yeast extract (Difco), 10 g; peptone (Difco), 10 g; K_2HPO_4, 5 g.

Pork infusion—1 lb lean pork, 200 ml; peptone (Difco), 5 g; tryptone (Difco), 1.6 g; K_2HPO_4, 1.25 g.

Peptone broth—peptone (Difco), 1 g.

Chemically pure salts were used, solutions of which were passed through fritted glass filters and tested for sterility before use. Except as noted the iron salt used was $FeCl_3 \cdot 6H_2O$, the manganese salt was $MnCl_2 \cdot 4H_2O$.

In general, the spores were seeded in the test media and the suspensions heated at 85 or 95 C for 10 minutes, cooled, and incubated at 30 or 37 C. After incubation, the samples were heated at 85 C for 15 minutes, cooled, and vigorously shaken with sterile sand or small glass beads sufficiently to disperse the clumps. Each sample was plated in triplicate after heating, both before and after incubation. The plating medium was

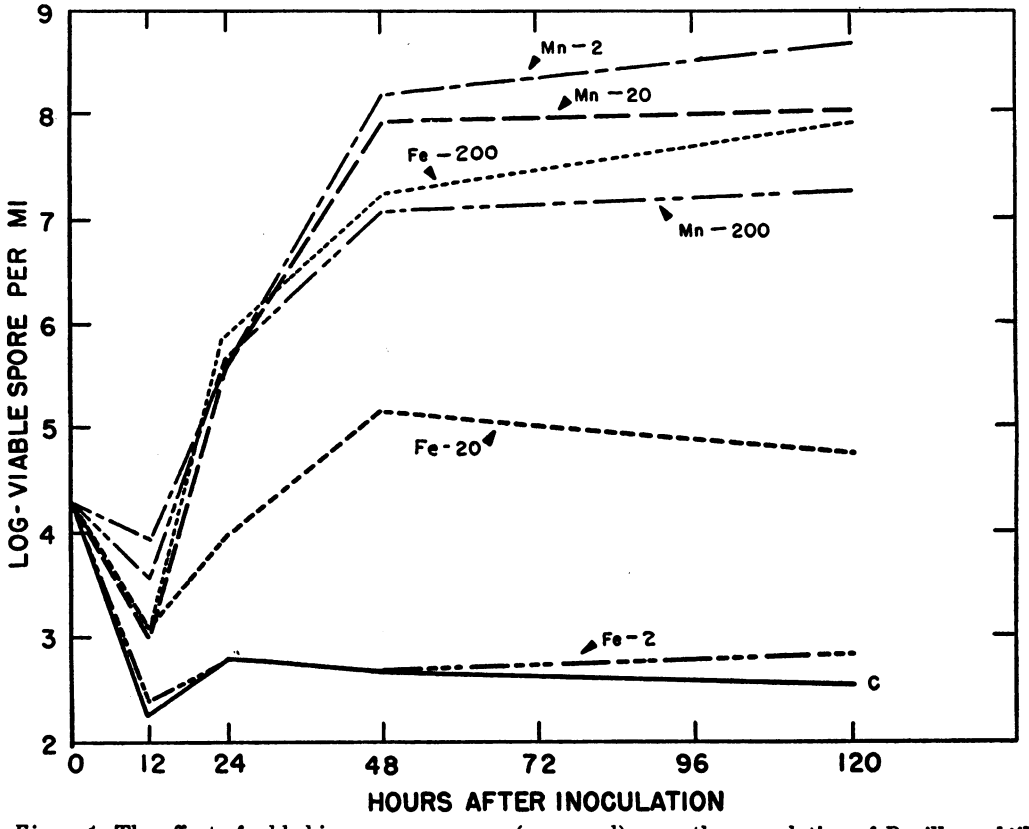

Figure 1. The effect of added iron or manganese (μg per ml) upon the sporulation of *Bacillus subtilis* (15u) in milk—iron as $FeCl_3$, manganese as $MnCl_2$. Temperature of incubation 37 C.

glucose nutrient agar containing 0.1 per cent soluble starch. The plates were counted after 48 hours of incubation and occasionally recounted after 48 hours of additional incubation; colonies developing on the plates were assumed to have originated from spores.

EXPERIMENTAL RESULTS

Sporulation in milk. Sterile skim milk to which had been added different quantities of iron or manganese was seeded uniformly with spores in Erlenmeyer flasks, heated at 95 C for 10 minutes, cooled, and incubated as still cultures at 37 C. At intervals from 0 to 120 hours, spore counts were made by plating well mixed suspensions after heating at 85 C for 15 minutes. The results obtained with a strain each of *B. subtilis* and *B. brevis* are shown in figures 1 and 2. Since vegetative growth was abundant in both the treated and untreated milk, it is clear that added iron or manganese provided something essential for rapid sporulation. In their absence, spore synthesis, after rapid and almost complete germination, was negligible in 5 days although it occurred promptly and at high levels when the milk was fortified suitably with these minerals. It is noteworthy that manganese was effective in low concentration while approximately 100 times as much iron was required to produce a comparable result—a fact which probably explains why the spore promoting activity of this element has been previously overlooked. Increasing the concentration of added iron in milk to 400 and 600 μg per ml resulted usually in a further increase of the spore crop when the pH was adjusted to 6.4. Omission of the pre-incubation heating did not greatly affect the results. Iron or manganese added directly to agar plates did not increase the spore count.

Examination of stained preparations made at the plating periods after incubation revealed visible spores in the presence of the added minerals but no discernible spores in the controls; these preparations also indicated that the iron

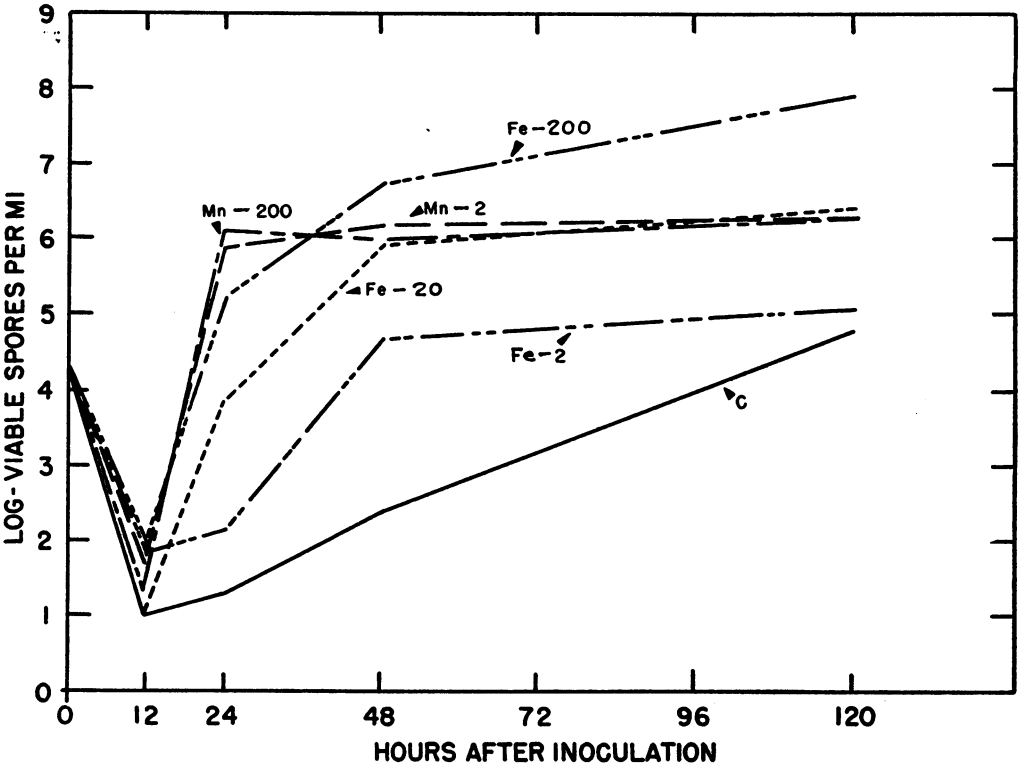

Figure 2. The effect of added iron or manganese (μg per ml) upon the sporulation of *Bacillus brevis* (Temple) in milk—iron as $FeCl_3$, manganese as $MnCl_2$. Temperature of incubation 37 C.

(280 μg per ml) increased the vegetative population an estimated 2 to 4-fold during comparable periods of incubation; manganese (2.7 μg per ml) produced little or no increase in the vegetative population.

Essentially similar results were obtained with six other strains of *B. subtilis* including (6051) (Marburg) and 6634 (Ford), *B. brevis* (8185), and *B. laterosporus* (314). Negative results or very slight enhancement of sporulation was noted with *B. megaterium* (753), *B. cereus* (401), *B. mycoides* (6462), *B. macerans* (277), *B. circulans* (4513), *B. polymyxa* (354), *B. stearothermophilus* (1518, CM_2), *B. thermoacidurans* (43P, 2353). The one anaerobe tested, 3679, was negative to iron and manganese.

A large number of other metal salts used in the same and lower concentrations either reduced the spore crop or had no significant influence; these included the chlorides of potassium, sodium, magnesium, calcium, cadmium, zinc, cobalt, nickel, strontium, tin (stannous), copper (cupric), and cerium (cerous).

Sporulation in broth media. In view of the results obtained with milk, it was of interest to ascertain if the spore promoting function of iron and manganese could be demonstrated similarly in other complex organic media, particularly those used in the laboratory for the growth of sporogenic species. Figures 3 and 4 show the development of spores in several broth media supplemented with iron or manganese. The methods were similar to those used in the previous experiment except that after adding the iron the pH was readjusted to 7.0. Under the test conditions the normal, untreated broth media like milk were not conducive to spore formation,[1] a deficiency which was corrected by suitable additions of either iron or manganese. Each of the several unfortified media supported heavy vegetative growth but produced few or no new spores. Thus, manganese or iron was critical

[1] Unfortified broths made from different batch lots of ingredients have shown differences in their spore promoting capacity possibly due to variations in their iron and manganese content.

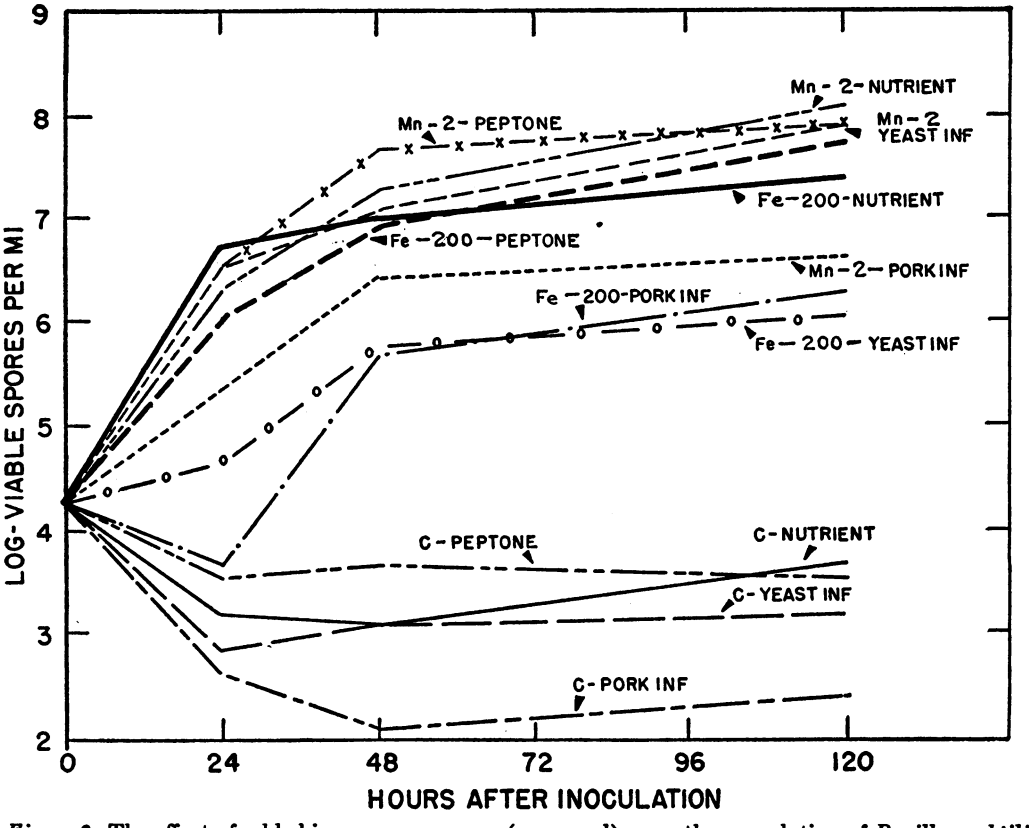

Figure 3. The effect of added iron or manganese (μg per ml) upon the sporulation of *Bacillus subtilis* (15u) in broth media—iron as $FeCl_3$, manganese as $MnCl_2$. Temperature of incubation 37 C.

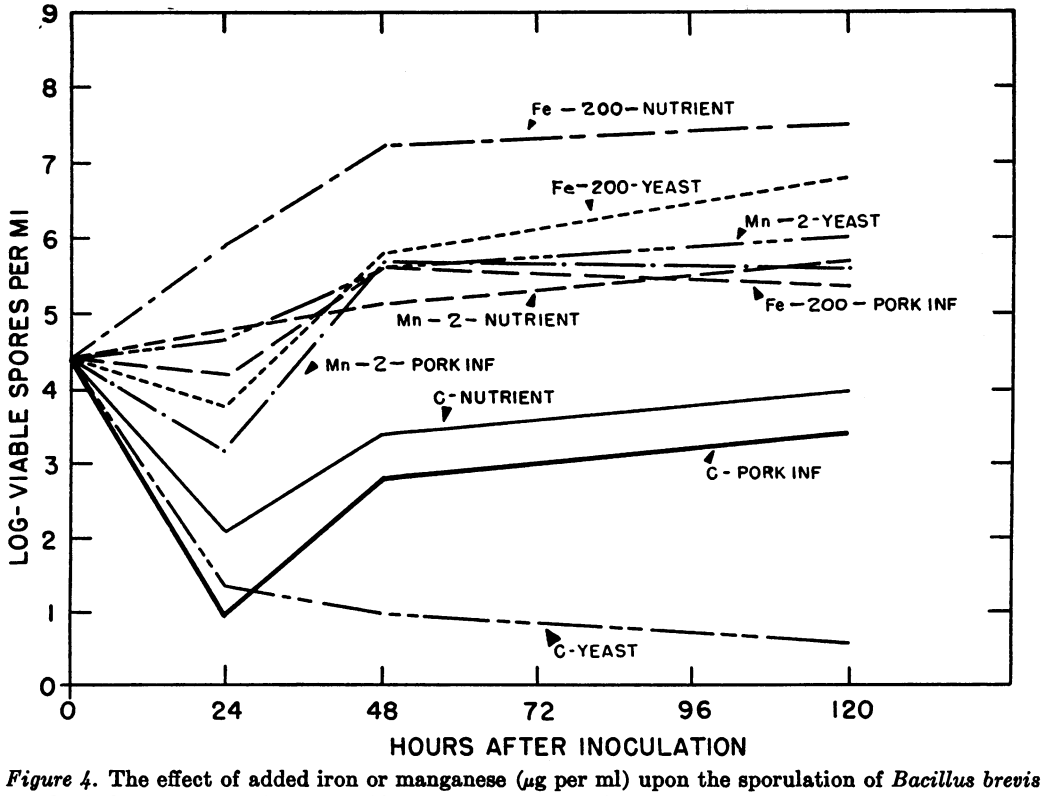

Figure 4. The effect of added iron or manganese (μg per ml) upon the sporulation of *Bacillus brevis* (Temple) in broth media— iron as $FeCl_3$, manganese as $MnCl_2$. Temperature of incubation 37 C.

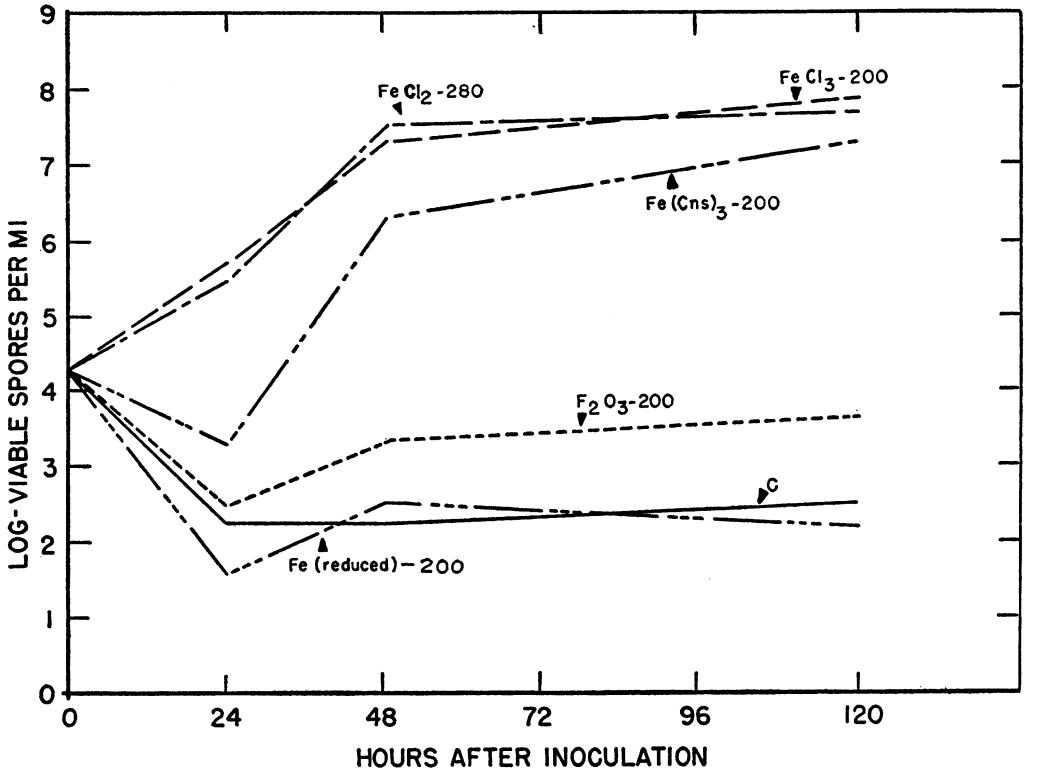

Figure 5. The effect of different iron compounds (μg per ml of iron) upon the sporulation of *Bacillus subtilis* (15u) in milk. Temperature of incubation 37 C.

for the formation of spores under the test conditions. It is apparent that the more complex infusion and yeast media were less favorable to spore production than the simpler nutrient extract or peptone broth; the latter supplemented with iron or manganese gave the best spore yields. The observed response of *B. subtilis* to added manganese in nutrient broth is in agreement with the findings of Charney *et al.* (1951).

Sporulation as influenced by the form in which iron is supplied. In this experiment sporulation of *B. subtilis* (15u) was measured in milk in response to a variety of iron containing compounds. The results are plotted semilogarithmically in figure 5. It may be seen that iron was effective in promoting the formation of spores only when it was present in a soluble form since neither the oxide nor the reduced iron significantly influenced the production of spores, whereas all soluble iron salts were sporogenically active. The results obtained with iron thiocyanate suggest that not only the ions but the iron in the nonionic form contribute to the formation of spores since this compound is feebly ionized. The rate at which spores became labile to heat was unchanged by the addition of iron or manganese, indicating that their effect is primarily upon a specific phase of cell metabolism—that is, sporulation.

Sporulation in milk with added rust or prior contact with rusting container. The effects which extraneous iron may induce in sporogenic organisms in milk suggest that contamination with iron may play a role in certain problems in milk preservation; the wide distribution of this element in soil and the many routes by which it may enter milk lend substance to this belief. Of special interest is the effect upon sporulation of rust contamination in milk handling equipment. To obtain some information on this point, a sample of sterile skim milk was treated with sterile rust scraped from a used rusting dairy utensil while a second portion from the same source served as control; the two samples were inoculated similarly with spores

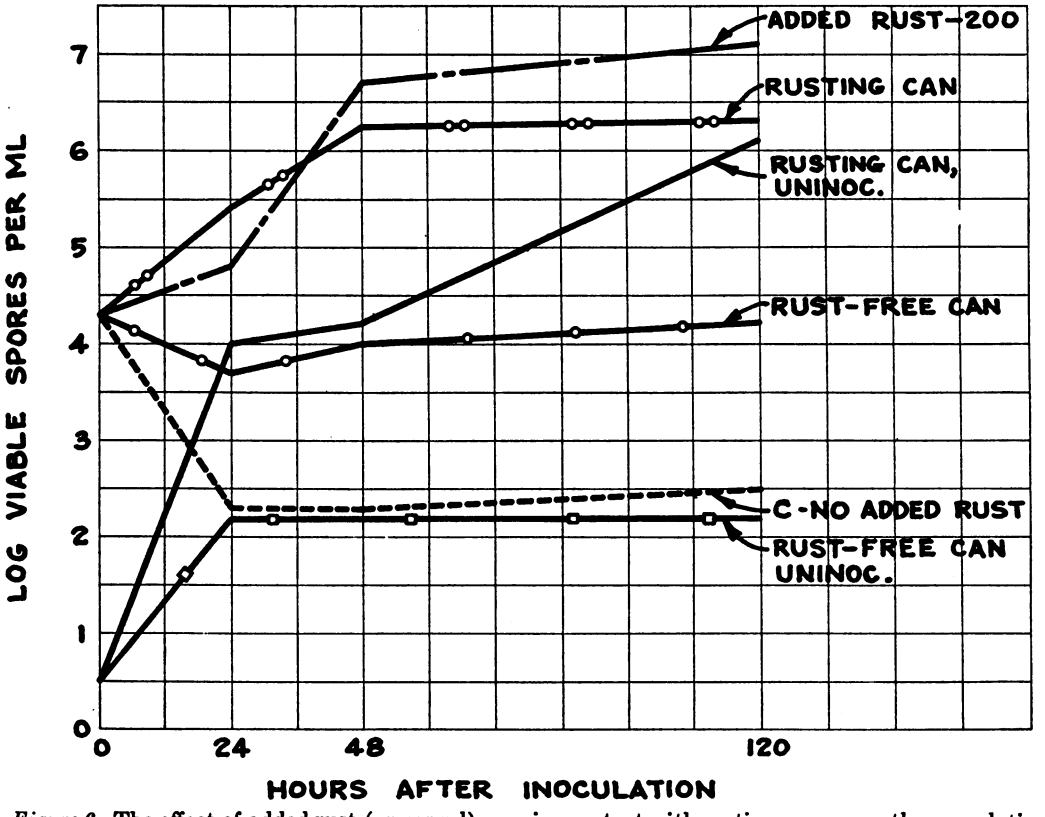

Figure 6. The effect of added rust (μg per ml) or prior contact with rusting cans upon the sporulation of *Bacillus subtilis* (15u) in raw milk. Rust scraped from rusting cans was added directly to milk or milk was held in rusting or rust-free cans at 0 C for 24 hours after which 10 ml samples were inoculated with spores, incubated at 37 C, and plate (spore) counts made as indicated. The spore count after refrigeration before inoculation was: rusting can 0.5, rust-free can 2.5 per ml. Temperature of incubation 37 C.

of *B. subtilis*, and plate (spore) counts were made at zero hours and after the usual periods of incubation as detailed. In a second experiment equal volumes of well mixed fresh raw milk were stored at 0 C for 24 hours in each of two similar sterile milk cans, one of which was visibly rust-free while the other contained on its inner surface large areas of rust. After preliminary storage at 0 C, aliquots of each sample were incubated at 37 C with and without previous inoculation with spores of *B. subtilis*. Spore counts at the usual periods provided the desired data. The results of each experiment are presented in figure 6. First, it may be seen that the data obtained in milk with added rust (figure 6) were comparable with those observed previously with purified soluble iron salts; also, it is evident that previous contact of milk with rusting surfaces even at low temperature actively promoted the formation of new spores whereas aliquots of the same bulk lot similarly refrigerated in a rust-free container produced no significant new spores at 37 C in 5 days. It may be noted that the iron effects were produced in both the sample in which *B. subtilis* spores were added and in the uninoculated sample, indicating the presence of an iron responsive sporogenic mesophile in the natural milk flora. The spore promoting activity of iron was pronounced in milk diluted with water 1:10.

The effect of added iron or manganese in aerated and in sealed cultures. The mechanism by which iron or manganese promotes the formation of spores in milk and broth solutions is not known. E_h measurements of milk showed the expected shift to a more positive potential when chlorides of iron or manganese were added; the magnitude of this change (0.02 to 0.06 v) in the used con-

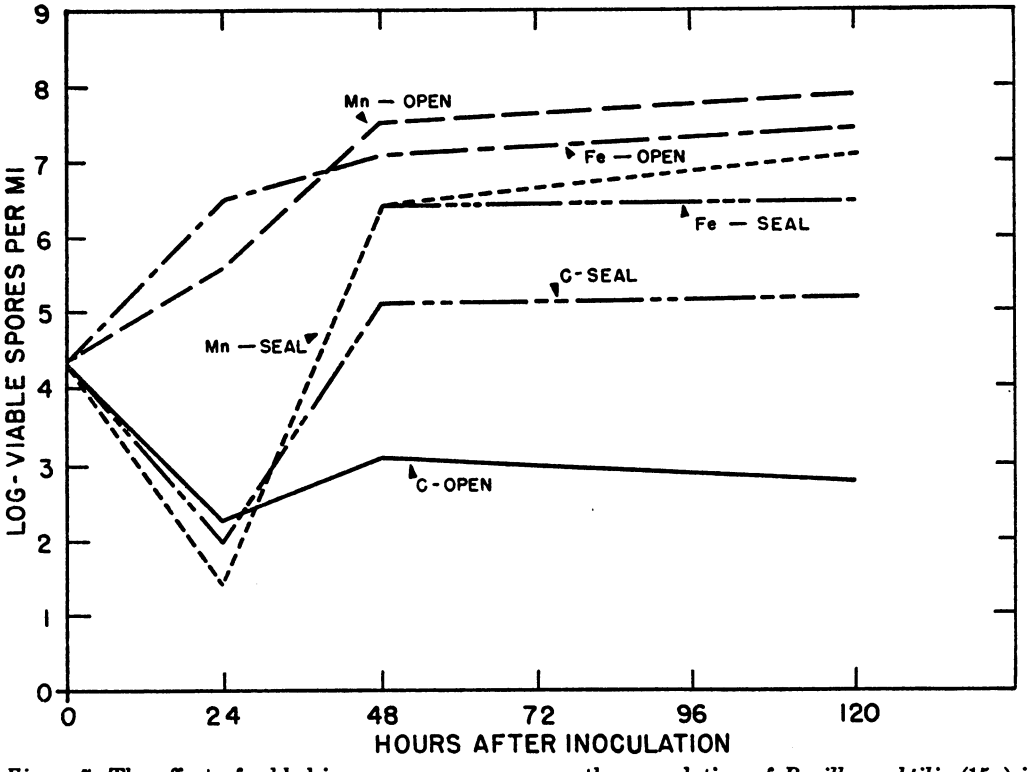

Figure 7. The effect of added iron or manganese upon the sporulation of *Bacillus subtilis* (15u) in milk contained in open and sealed tubes—iron (200 μg per ml) as $FeCl_3$, manganese (2 μg per ml) as $MnCl_2$. Temperature of incubation 37 C.

centrations would seem to exclude a *per se* E_h effect. The possibility that soluble iron or manganese salts might produce their effects by increasing the available molecular oxygen was considered. Two experiments were carried out to provide information on this point.

The effect of iron upon sporulation under conditions of reduced oxygen concentration was observed as follows: Eight ml volumes of sterile milk in standard tubes with and without added iron were inoculated with spores and the samples preheated and cooled; a portion of the tubes was sealed quickly with agar containing thioglycolate, and the sealed and unsealed samples incubated at 37 C, the plate (spore) counts made at the usual periods. In the samples under seal (figure 7), iron or manganese increased the total number of spores, but the level so attained was much less than in the open tubes similarly fortified. This result may be attributed to the very limited oxygen supply of the sealed cultures, which by materially restricting growth militated against the more effective utilization of the salts. Of interest in figure 7, is the higher yield of spores in the control under seal than in the absence of the seal.

The effect of added minerals upon sporulation in aerated milk cultures was studied next. Forty ml volumes of well mixed milk were sterilized in 100 ml glass bottles. Individual samples were treated then with iron or manganese solution or an equivalent volume of distilled water. After preliminary heating the samples were cooled to 30 C and shaken continuously on a reciprocating shaking machine; unshaken samples treated similarly in all other respects served as still controls. Plate (spore) counts were made at the usual periods. The results (figure 8) show that vigorous and continued aeration increased the spore level of the unfortified samples, did not greatly affect the spore promoting activity of manganese, and measurably reduced the spore level in the presence of added iron. The reason for the apparent fall in spore concentration in the shaken samples, evident in 5 days, is not clear but may be due to the slow

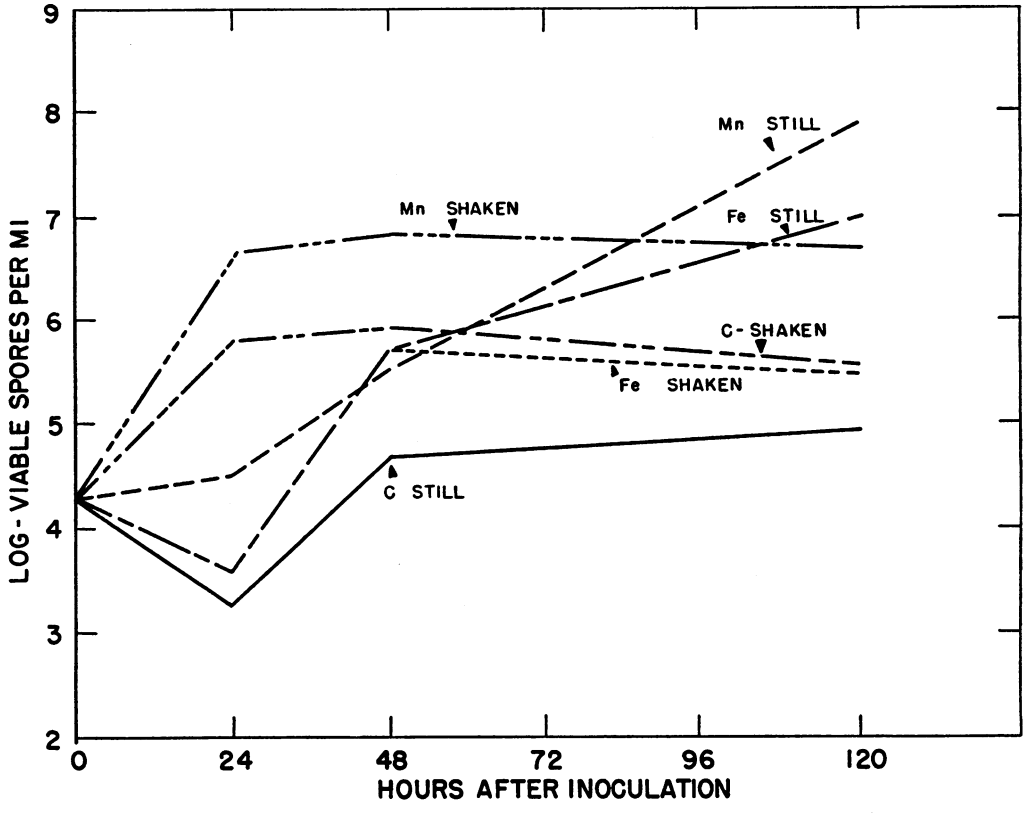

Figure 8. The effect of added iron upon the sporulation of *Bacillus subtilis* (15u) in aerated (shaken) milk—iron (200 μg per ml) as $FeCl_3$. Temperature of incubation 30 C.

germination of some spores whose development is influenced favorably by oxidation of growth inhibiting, metabolic by-products. The lower temperature of incubation and the altered volume-surface ratio of the medium as compared with the previous tube cultures changed somewhat the development of spores in the still cultures.

DISCUSSION

This study is concerned with the effect of certain mineral supplements upon the total number of spores formed in complex fluid organic substrates. The effects produced by added iron or manganese in substrates which otherwise produced abundant vegetative cells but few spores invite speculation as to their mechanism of action.

Since the addition of 0.1 per cent soluble starch to the substrates did not appreciably affect the spore levels, antisporulation factors are excluded from consideration.

A theory of sporogenesis has been advanced recently by Hardwick and Foster (1952) based upon the observed influence of various nutrients upon the sporulation of washed vegetative cells in distilled water; they conclude that "sporulation results from the endogenous degradation of pre-existing vegetative (enzyme) proteins which occurs in the absence of readily utilizable exogenous sources of energy and ex- or endogenous nitrogen". Since manganese seems to be essential for substantial proteinase production by *B. subtilis* in complex organic media (Stockton and Wyss, 1946) and has an apparent specific effect upon the sporulation of this organism over a wide range of oxygen concentration, this element may promote the formation of spores by contributing to the reserve of intracellular enzyme proteins believed by Hardwick and Foster to be the basic material for spore synthesis. It is of interest in this connection to note that the manganese content of milk is extremely low (0.002 mg per 100 ml,

Bulletin National Research Council, 1950), and that Difco peptone and beef extract are reportedly deficient in manganese for proteinase production by *B. subtilis* (Stockton and Wyss, 1946).

Iron in contrast to manganese considerably increased the vegetative population; however, the increment increase of spores was of a different order of magnitude. Since, for its maximal activity, relatively large quantities of iron were required in a medium of limited oxygen concentration, it might be supposed that ferric iron increased significantly the amount of available oxygen in the medium; however, this view seems to be contradicted by the results obtained with ferric and ferrous chloride and cyanate (figure 5). The action of iron in promoting the formation of spores is apparently not direct and therefore not specific to ferric or ferrous ions or to undissociated iron compounds but resides, perhaps, in the common capacity of iron compounds to create for the responding organism a physicochemical environment suited to the synthesis of spores.

The relationship between contamination of milk with iron and the development of resistant spores is of manifest interest to the commercial food canner. It may well explain the occasional, sudden appearance in condensery milk of large numbers of spores which make sterilization difficult and uncertain; such outbreaks frequently are not explicable by external contamination and yet are difficult to reproduce when inoculation is made into different milk. Emphasis thus is directed to the elimination of rust-prone equipment in food handling establishments, and since complete exclusion of iron from food is difficult, new importance is attached to rapid heating and cooling and the maintenance of the product at subgrowth temperatures.

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SUMMARY

Suitable additions of soluble iron or manganese salts to fluid organic media were found to increase greatly the total spore yields of *Bacillus subtilis*, *Bacillus brevis*, and *Bacillus laterosporus* (one culture)—a variety of other salts was without significant effect or reduced the spore crop. Manganese actively promoted spore synthesis in relatively low concentration and was effective through a wide range of oxygen concentration. Comparable increases in spore yield were obtained by iron supplements, but about 100 times the concentration of manganese was required and the stimulation did not occur when cultivation was carried out in an excess of air (oxygen).

Exposure of milk to rusting utensils was found to stimulate the subsequent development of spores comparable in degree to that obtained with purified iron salts. It is concluded that the relationship between iron-rust and spore development has practical significance in the commercial canning of milk and other food products.

Possible mechanisms of the action of iron and manganese are discussed.

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