THE EFFECT OF Mn^{++} AND ANTIMICROBIAL DRUGS ON SPORULATION OF *BACILLUS SUBTILIS* IN NUTRIENT BROTH

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It has been recognized recently that the addition of Mn^{++} to nutrient broth, skim milk, and other complex broth media stimulates sporulation (but not growth) of many species of the genus *Bacillus*. The requirement for Mn^{++} is specific and cannot be replaced by the addition of Na^{+}, K^{+}, Mg^{++}, Ca^{++}, Sr^{++}, Co^{++}, Ni^{++}, Cd^{++}, Zn^{++}, Cu^{++}, Sn^{++}, Ce^{++}, or low concentrations of $Fe^{++(+)}$ (Charney *et al.*, 1951; Curran and Evans, 1954).

Chlortetracycline and oxytetracycline form stable complexes with Mn^{++} (Albert, 1953), and the toxicities of these drugs toward a cell-free bacterial nitro reductase (Saz and Slie, 1954) and toward growth of cells of a pseudomonad (Weinberg, 1954) are reversed by the addition of Mn^{++}. Conversely, the toxicity of chlortetracycline toward growth of cells of *Colpoda cucullus* in the presence of alginate or chondroitin is enhanced by Mn^{++} (Little *et al.*, 1953). The presence of chlortetracycline in the diet of chicks has been found to enhance their utilization of Mn^{++} (Pepper *et al.*, 1953). The toxicities of quinacrine toward coliform bacteria (Silverman, 1948), of polymyxin toward a pseudomonad (Newton, 1953), and of streptomycin toward cell elongation of coleoptile sections of an *Avena* species (Rosen, 1954) have been observed to be reversed by Mn^{++}.

Bacterial sporulation is inhibited by many antimicrobial factors in lower concentrations than are required to inhibit vegetative growth (Wynne, 1952). In the present study an attempt was made (1) to learn if this generalization applies to the 5 antimicrobial drugs cited above and also to tetracycline, chloramphenicol, penicillin, and magnamycin; and (2) to learn if the ability of any of the 9 drugs to inhibit sporulation can be reversed by the addition of excess Mn^{++}.

MATERIALS AND METHODS

Nutrient broth. Throughout the study the nutrient broth consisted of 0.3 per cent beef extract (Difco), 0.5 per cent polypeptone (Baltimore Biological Laboratory), distilled water, pH 7.0. Many different samples of beef extract and polypeptone were employed; when incorporated in nutrient broth, the samples permitted only occasional sporulation in the absence of additional Mn^{++}. Nutrient broth, when assayed for its native content of Mn^{++} by the ammonium persulfate (Theroux *et al.*, 1943) and the periodate (Willard and Greathouse, 1917) methods, was found to contain no Mn^{++}; however, these methods are insensitive to concentrations lower than 10^{-6} M.

Addition to nutrient broth of metallic salts, organic metabolites, and antimicrobial drugs. The metallic salts employed in the study were: $MnSO_4 \cdot H_2O$, $FeSO_4 \cdot 7H_2O$, $FeCl_2 \cdot 4H_2O$, $Fe(NH_4)_2(SO_4)_2 \cdot 6H_2O$, $FeC_2O_4 \cdot 2H_2O$, anhydrous $MgSO_4$, anhydrous $CaCl_2$, $ZnSO_4 \cdot 7H_2O$, $Co(NO_3)_2 \cdot 6H_2O$, and $Na_2MoO_4 \cdot 2H_2O$. The organic metabolites consisted of L-glutamic acid, L-glutamine, glucose, and Na_3 citrate $\cdot 2H_2O$. The compounds were dissolved and diluted in distilled water, autoclaved, and aseptically added to nutrient broth.

The antimicrobial drugs employed were: tetracycline HCl, oxytetracycline HCl, chlortetracycline HCl, quinacrine HCl, dihydrostreptomycin SO_4, polymyxin B SO_4, penicillin G potassium, magnamycin, and chloramphenicol. The first 5 drugs were used as pure compounds; the latter 4 antibiotics contained citrate buffer in quantities from 1 to 3 times the concentrations of the drugs. The 9 antimicrobial compounds were individually dissolved and diluted in sterile distilled water and aseptically added to sterile nutrient broth. When not in use, the solutions of drugs were stored at -25 C.

Unless otherwise stated in the results, the additions of salts, metabolites, and drugs to nutrient broth were made immediately prior to inoculation.

Strain, inoculum, and conditions of incubation.

Cells of the Marburg strain of *Bacillus subtilis* were grown at 37 C in 25 ml of nutrient broth contained in a 250 ml Erlenmeyer flask in a New Brunswick shaker (180 strokes per minute). At 24 hr the culture contained 6×10^8 viable cells per ml. One ml was diluted 10^{-4} in distilled water and 0.05 ml of the diluted sample, which contained approximately 3,000 viable cells, was added to each 25 ml sample of nutrient broth to be used in the actual experiments. All experimental flasks were then shaken at 37 C for 48 hr and, in some cases, for 72 hr. In a few experiments, incubation was continued for 7 days.

Determination of pH, cell growth, and sporulation. Determinations of the pH reaction were made at the beginning and after 12, 24, and 48 hr of incubation by testing aliquot portions of the flasks with a Beckman pH meter. Determinations of cell growth and sporulation were made at frequent intervals during the 48 or 72 hr period; the intervals are stated in the section on results. Cell growth was measured by spreading 0.05 ml samples of various dilutions of the broths on the surfaces of nutrient agar contained in petri plates followed by 48 hr incubation and counting of visible colonies. Sporulation was observed by preparing stained specimens from the contents of the flasks. Two methods of staining were employed: a simple stain using 0.1 per cent crystal violet and the malachite green spore stain described by Bartholomew and Mittwer (1950). Results of the 2 methods were in close agreement. One hundred vegetative cells, cells containing spores, and isolated spores were counted independently by each of 2 persons; the counts were averaged, and the percentages of cells in the populations that formed spores are presented in the results.

EXPERIMENTAL RESULTS

Growth, pH change, and sporulation in nutrient broth with or without added Mn^{++}. The extent of cellular multiplication and the change in pH during the first 48 hr of incubation are indicated in figure 1. Neither growth nor the alkaline pH shift was affected by initial or subsequent addition to the broth of 10^{-9} M to 10^{-3} M Mn^{++}. The ability of the cells to sporulate, however, was considerably influenced by the presence of added Mn^{++}. The lowest initially added concentration of the cation that consistently yielded maximum sporulation (40–60 per cent) was 10^{-6} M (see table 1); accordingly, a control flask of nutrient

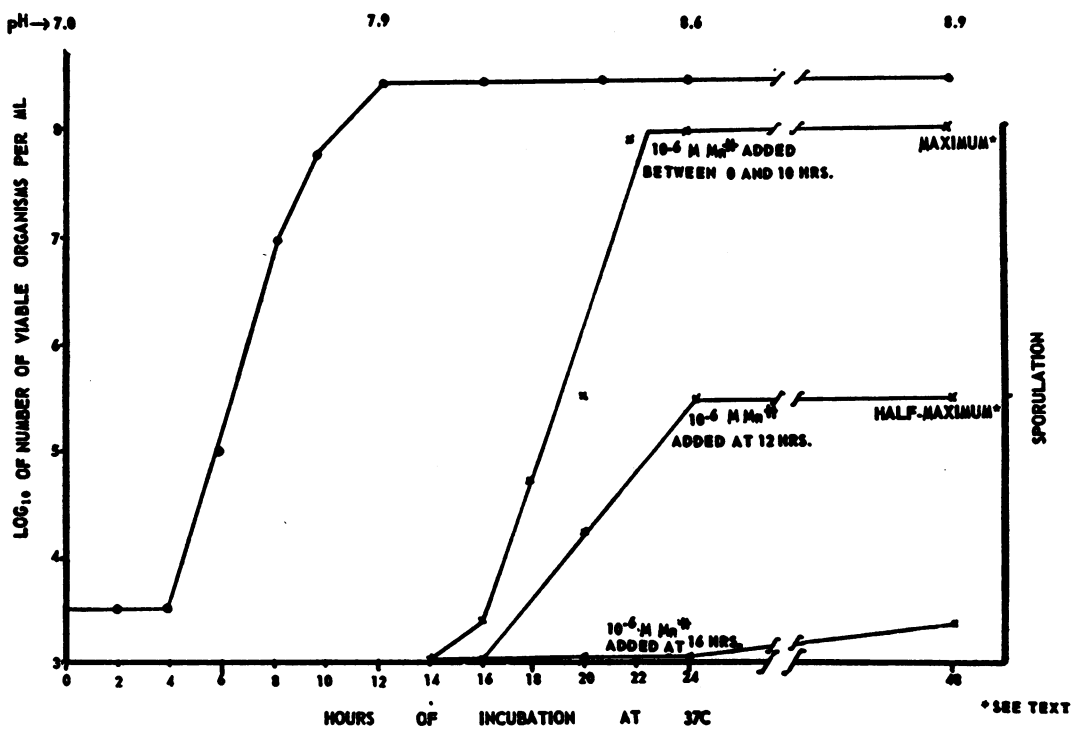

Figure 1. Cellular multiplication, pH shift, and sporulation in nutrient broth.

broth containing the addition of this amount of the cation, as well as a control flask containing no added Mn^{++}, was included in all subsequent experiments.

If 10^{-6} M Mn^{++} was added to the broth at any time prior to the cessation of logarithmic growth, spores appeared at the normal time: from 4–10 hr after the maximum stationary phase had been reached. Addition of the cation during the 4 hr period subsequent to cessation of logarithmic growth permitted half-maximum sporulation; however, the spores were delayed in time of appearance (see figure 1). Addition of Mn^{++} at 16 hr of incubation permitted only slight sporulation, and the spores did not appear until between 24 and 48 hr of incubation. If the cation was not supplied until 24 hr (or was withheld entirely), only occasional spores (< 1 per cent) appeared during the six subsequent days of incubation. Cultures were not kept beyond 7 days. If, at 10 hr, the young broth cultures were refrigerated at 5 C and at 24 hr replaced on the shaker and supplied with 10^{-6} M Mn^{++}, maximum sporulation occurred within the next 12 hr.

TABLE 1

Effect on sporulation of initial addition of Mn^{++} to nutrient broth

	Final Concentration of Added Mn^{++} (M)		
	0, 10^{-9}, or 10^{-8}	10^{-7}	10^{-6}, 10^{-5}, 10^{-4}, or 10^{-3}
Per cent of vegetative cells that sporulated:	0 to 1 (occasional)	10–20 (partial)	40–60 (maximum)

TABLE 2

Effect on sporulation of initial addition of metallic ions or organic metabolites to nutrient broth

Final Concentration of Added Substance (M)	No Mn^{++}	10^{-6} M Mn^{++}
10^{-4} Fe^{++}, 10^{-4} Ca^{++}, 10^{-4} Mg^{++}, 10^{-5} Co^{++}, 10^{-5} Zn^{++}, or 10^{-5} $MoO_4^{=}$	Occasional	Maximum
2×10^{-3} Na_3 citrate, 7.5×10^{-3} L-glutamic acid, or 7.5×10^{-3} L-glutamine	Occasional	Maximum

Effect of inorganic ions, metabolites, or drugs on growth, pH and sporulation in nutrient broth with or without added Mn^{++}. The effect of the initial addition of inorganic ions or organic metabolites to nutrient broth on sporulation is summarized in tables 2 and 3. It may be noted that, except for high concentrations of Fe^{++}, these substances neither replaced nor antagonized the Mn^{++} requirement for sporulation. Also these substances, in the concentrations used, had no effect on vegetative growth or the alkaline pH shift. In table 4 is summarized the effect of initially added glucose on pH and sporulation in the presence of Mn^{++}. Of interest is the observation that in the presence of the larger concentration of glucose, the alkaline pH shift was merely suppressed for 12

TABLE 3

Effect on sporulation of initial addition of ferrous salts to nutrient broth

Salts	Source	Grade	Per cent of Mn^{++} Contamination		Sporulation in Presence of Final Concentration of Fe of:	
			Stated on label	Determined by ammonium persulfate assay	10^{-6}, 10^{-5}, or 10^{-4} M	10^{-3} M
				%		
$FeSO_4 \cdot 7H_2O$	Coleman-Bell	cp	Not listed	0.025	Occasional	Maximum
$Fe(NH_4)_2(SO_4)_2 \cdot 6H_2O$	Coleman-Bell	cp	0.015%	0.030	Occasional	Maximum
$Fe(NH_4)_2(SO_4)_2 \cdot 6H_2O$	Mallinckrodt	Analytical	0.01%	<0.010	Occasional	Maximum
$Fe(NH_4)_2(SO_4)_2 \cdot 6H_2O$	Baker	cp special	0.000%	<0.010	Occasional	Maximum
$FeCl_2 \cdot 4H_2O$	Baker	cp	0.04%	0.025	Occasional	Maximum
$FeCl_2 \cdot 4H_2O$	Mallinckrodt	Analytical	Not listed	0.040	Occasional	Maximum
$FeC_2O_4 \cdot 2H_2O$	Fisher	Pure	Not listed	0.050	Occasional	Maximum

TABLE 4

Effect on pH and sporulation of initial addition of glucose to nutrient broth containing 10^{-6} M Mn^{++}

Final Concentration of Added Glucose	0 hr		12 hr		24 hr		48 hr	
	pH	Sporulation	pH	Sporulation	pH	Sporulation	pH	Sporulation
0	7.0	None	7.9	None	8.6	Maximum	8.9	Maximum
5×10^{-3} M	7.0	None	7.2	None	8.6	Maximum	8.7	Maximum
5×10^{-2} M	7.0	None	5.7	None	7.6	None	8.3	Occasional

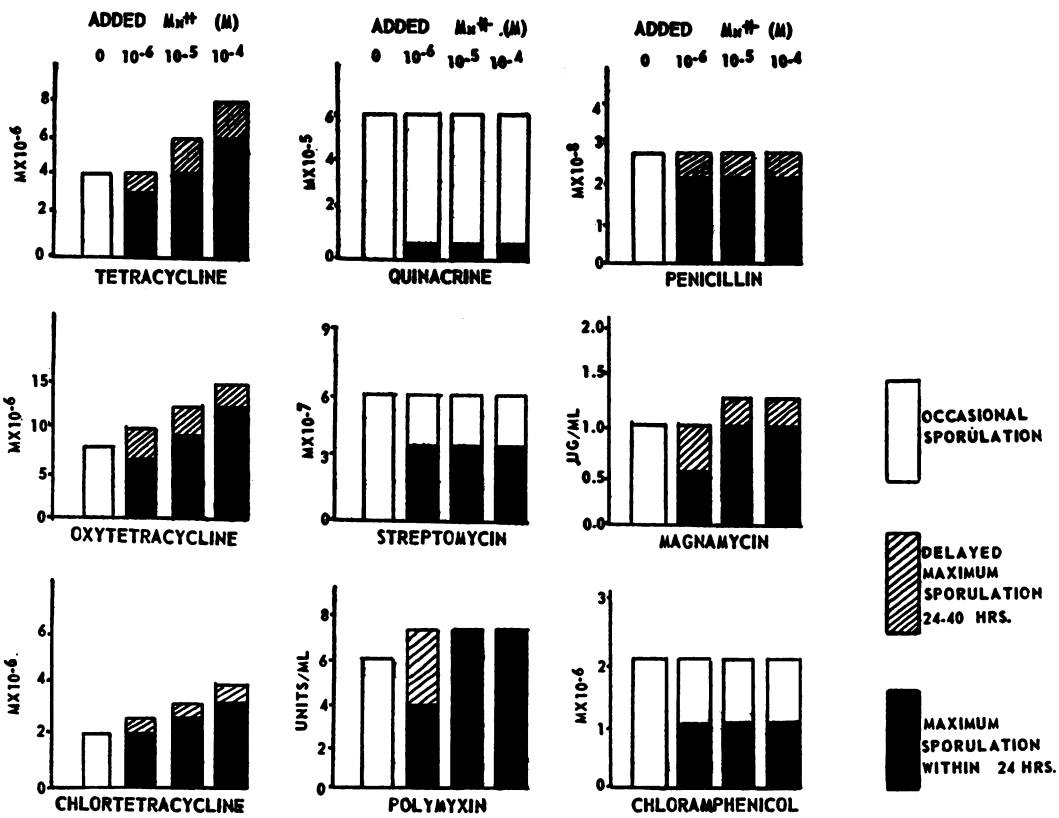

Figure 2. Effect of initial addition to nutrient broth of 9 antimicrobial drugs and Mn^{++} on growth and sporulation. The height of the bars indicates the concentrations of drugs that permitted maximum vegetative growth within 40 hr.

hr but the ability of the cells to sporulate was lost. If Mn^{++} was not present, sporulation did not occur with either concentration of glucose. In a single experiment, an attempt was made to imitate in nutrient broth plus 10^{-6} M Mn^{++} the temporary acid pH shift obtained with 5×10^{-2} M glucose. This was done by adding appropriate amounts of HCl at hourly intervals throughout the first 24 hr of growth. Under these conditions, no spores were formed.

The effect of the initial addition of the antimicrobial drugs to nutrient broth on growth and sporulation in the presence of varying concentrations of initially added Mn^{++} is summarized in figure 2. The ability of oxy-, chlor-, and tetracycline to inhibit growth was slightly reversed by Mn^{++}. Subbacteriostatic concentrations of these antibiotics and polymyxin, penicillin, and magnamycin did not inhibit but merely delayed the appearance of spores by extending the time required for the occurrence of logarithmic growth. Streptomycin and chloramphenicol inhibited sporulation at one-half the concentration required to inhibit growth. Quinacrine prevented

TABLE 5

Sporulation of cells removed from nutrient broth and resuspended in other nutrient broths or in phosphate buffer

Origin of Cells	Cells Resuspended in:					
	10 hr nutrient broth		24 hr nutrient broth		pH 8.0 phosphate buffer plus 7.5×10^{-3} M glutamic acid	
	No Mn^{++}	10^{-6} M Mn^{++}	No Mn^{++}	10^{-6} M Mn^{++}	No Mn^{++}	10^{-6} M Mn^{++}
10 hr cells grown in nutrient broth + 10^{-6} M Mn^{++}	Maximum within 12 hr		Partial within 12 hr*		Maximum within 12 hr	
10 hr cells grown in plain nutrient broth	Occasional	Maximum within 12 hr	Partial within 24–36 hr		Partial within 12 hr*	Maximum within 12 hr
24 hr cells grown in plain nutrient broth	Occasional	Partial within 24–36 hr	Occasional		Occasional	Partial within 24–36 hr

* No increase in sporulation during subsequent 24 hr.

spore formation at one-tenth of the bacteriostatic concentration. Spore inhibiting amounts of the latter drug also caused vegetative cell autolysis to occur between 24 and 48 hr. Autolysis was not observed under any other conditions in this study. None of the 9 drugs affected the alkaline pH shift that accompanied vegetative growth.

In one series of experiments, tetracycline was added in larger quantities at the completion of logarithmic growth than could have been added initially. Viable cell counts at the end of 24 hr incubation indicated that a drug concentration of 16 (but not 24) $\times 10^{-6}$ M was subbacteriostatic and could be safely added at 10 hr. If 10^{-4} M Mn^{++} had been included initially, the 10 hr addition of 16×10^{-6} M tetracycline delayed the appearance of spores for 8 hr. If the cation and drug were added simultaneously at 10 hr the same delay occurred and, in addition, only partial sporulation was obtained.

Sporulation of cells removed from nutrient broth and suspended in either older or fresher broth or in phosphate buffer. In some experiments, cells were taken by centrifugation from nutrient broth at either 10 hr or 24 hr and were resuspended in 24 or 10 hr broth whose native cells had been removed and discarded. In other experiments, 10 or 24 hr cells were removed from their homologous broths, washed once in M/30 phosphate buffer, and resuspended in a volume of buffer equal to the volume of the discarded nutrient broth. Buffer solutions of pH 7.0 and 8.0 were employed; in half of the solutions, 7.5×10^{-3} M glutamic acid was included. All resuspended cells were then shaken at 37 C and observed for sporulation at 12, 24, and 36 hr.

The ability of resuspended cells to form spores in heterologous nutrient broths and in pH 8.0 phosphate buffer containing glutamic acid is summarized in table 5. Of particular interest are the observations that (a) 10 hr cells grown in the presence of Mn^{++} partially sporulated when suspended in 24 hr nutrient broth; (b) 10 hr cells grown in the absence of Mn^{++} slowly and partially sporulated in 24 hr broth as well as in phosphate buffer that contained no Mn^{++}; and (c) 24 hr cells from nutrient broth without Mn^{++} could not form spores unless the cation were present in the new environment and then only slowly and partially in 10 hr broth or in phosphate buffer. In buffer solutions to which glutamic acid had not been added, only slight sporulation was obtained even in the presence of 10 hr cells and Mn^{++}. When buffer adjusted to pH 7.0 was employed, spore formation was slower under all conditions than in pH 8.0 buffer. In the neutral buffer, the alkaline pH shift (similar to that in nutrient broth) occurred, provided that glutamic acid had been included.

DISCUSSION

At least 2 important facts are apparent from the previous and current studies: (a) a larger concentration of Mn^{++} is required for sporulation

than for growth of *Bacillus subtilis*; and (b) Mn^{++} can stimulate spore formation at only 1 stage during the growth cycle of a culture; if the cation is withheld until this stage has passed and is then added, maximum sporulation does not occur.

With respect to (a), it may be noted that in recent studies on sporulation in synthetic media, excess concentrations of Mn^{++} have been usually present. For example, Hardwick and Foster (1952) and Krask (1953) included in their media approximately 5×10^{-5} M Mn^{++}. In an older study in which synthetic media were used (Williams, 1929), Mn^{++} was not included; the author obtained 10 per cent sporulation in the presence of 6×10^{-4} M Fe^{++} and meager to scanty sporulation in the absence of this ion. The activity of iron salts in discussed below. The majority of studies on sporulation have been conducted in complex media to which, prior to 1951, Mn^{++} had not been added. The results obtained in such studies, therefore, must have depended in part upon the trace amounts of the cation in the various constituents of the many different media employed. An obvious source of Mn^{++} might have been agar, and it is generally recognized that sporulation occurs more readily in a complex medium to which agar has been added than in the complex broth itself. In this laboratory the persulfate assay method for Mn^{++} revealed a slight trace of the ion in agar (Difco) and 3×10^{-6} M in BBL flake agar; however, no detectable traces were found in samples of Difco Noble, Aico, Aloe, Penick, or Nutritional Biochemical agars. Possible functions of Mn^{++} in spore formation are suggested by the observations of Stockton and Wyss (1946) that the ion is essential in synthesis of proteinases and of Gale (1949) that the ion is required for transport of glutamic acid into growing cells.

The only salts that have been found to substitute for the Mn^{++} requirement are those containing $Fe^{++(+)}$. Curran and Evans (1954) reported that approximately 4×10^{-3} or 1.2×10^{-2} M $Fe^{++(+)}$ was required to obtain maximum sporulation in nutrient broth or in skim milk, respectively. However, if the samples of iron salts used by these investigators were contaminated with as little as 0.010 to 0.003 per cent Mn^{++} (approximately 10^{-6} M), the activity of the salts could have been caused by the trace amount of Mn^{++}. In the present study, 7 ferrous salts were selected at random and were assayed by the ammonium persulfate method for Mn^{++} contamination. Five salts contained between 0.025 and 0.050 per cent Mn^{++}, and two contained less than 0.010 per cent. Each of the 7 salts stimulated sporulation at a concentration of Fe^{++} of 10^{-3} but not 10^{-4} M. It is, therefore, suggested that the sporulation stimulating activity of high concentrations of iron salts observed by Williams (1929), Curran and Evans (1954), and the present author can be attributed to a considerable extent to the slight amount of Mn^{++} that is usually associated with such salts.

The time of sporulation in the present study in Mn^{++} enriched nutrient broth was at least 12 hr earlier than that recorded by Charney *et al.* (1951) and by Curran and Evans (1954). However, the former investigators employed different strains and incubated at 30 C rather than 37 C, and the latter workers employed stationary rather than shaken cultures. In the experiments reported here, the per cent of vegetative cells that sporulated in the presence of optimum concentrations of Mn^{++} fluctuated between 40 and 60. Fifty per cent sporulation in nutrient broth plus the cation was also obtained by Charney *et al.* (1951) whereas in synthetic media containing Mn^{++}, 80 to 100 per cent sporulation has often been observed (Charney *et al.*, 1951; Krask, 1953). Nutrient broth undoubtedly contains antisporulation factors such as those described by Hardwick *et al.* (1951) and which are not originally present in synthetic media although they may subsequently be formed during vegetative growth.

With respect to fact (b), the generalization is often made that sporulation commences after the logarithmic period of vegetative growth, but the possibility that cultures may lose the ability to sporulate after a period of maximum stationary growth is rarely mentioned. However, in the case of initial Mn^{++} deprivation and belated addition, the latter phenomenon is readily observed. If the cation were to be supplied at the time it is required for sporulation, but some other essential nutrilite (glutamate?) withheld for 6 to 12 hr, would the phenomenon also occur?

Do the cells lose the ability to sporulate after 6 hr in the maximum stationary phase because they have metabolized substances that might have been utilized in spore formation at the beginning of the phase? Or are the antisporulation factors that the cells synthesize during the phase (demonstrated by Hardwick *et al.*, 1951) suffi-

cient to suppress the cells' ability to sporulate? In the present study, 24 hr cells could slowly and partially sporulate if resuspended in 10 hr broth or phosphate buffer plus Mn^{++}; perhaps maximum spore formation was prevented by antisporulation factors contained in the aged cells. On the other hand, 10 hr cells could slowly and partially form spores in 24 hr broth; apparently the young cells can overcome, to some extent, an antisporulating environment. Oddly enough, under the latter conditions, Mn^{++} was not required, nor did its presence enhance sporulation. In future work, these phenomena might be more efficiently investigated in synthetic media.

The inhibition of sporulation by glucose with the accompanying development of an acid pH has been discussed by Knaysi (1948). In the present study it was observed that although the pH of glucose containing cultures subsequently became alkaline (in 24 to 48 hr), spore formation did not subsequently occur. This observation indicates also that the opportunity for sporulation had expired.

The other compounds in this study that inhibited sporulation (streptomycin, chloramphenicol, and quinacrine) did not affect pH, nor did they apparently act by combining with Mn^{++}. The 6 inactive drugs evidently do not attack the mechanisms of spore formation in concentrations lower than those required to antagonize vegetative growth.

The inability of the tetracycline drugs to interfere with sporulation by combining with Mn^{++} may be explained by postulating either that the enzymatic sites in the cell that require the cation have a stronger affinity for Mn^{++} than do the drugs or that the tetracyclines cannot penetrate to these sites. Although the cation combines chemically with the drugs and reverses their inhibition of a cell-free nitro reductase, Mn^{++} is not an efficient reversing agent for inhibition of growth by the drugs (Weinberg, 1954). In some systems, and at some concentrations, in fact, the ion enhances the activity of the tetracyclines (Little *et al.*, 1953; Weinberg, 1954).

When a large quantity of tetracycline and Mn^{++} were added simultaneously at 10 hr, the drug partially suppressed sporulation whereas it could only delay the appearance of spores if Mn^{++} had been added at 0 hr. This experiment indicates that the drug can compete to some extent with the cells for the cation in the environment but cannot as readily combine with Mn^{++} that had been presumably assimilated by the cells during logarithmic growth.

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SUMMARY

The observation that cells of *Bacillus subtilis* require Mn^{++} for sporulation in nutrient broth is confirmed. The addition of as little as 10^{-6} M Mn^{++} (which has no effect on vegetative growth) to the medium permits maximum sporulation within 24 hr. However, if the ion is withheld for more than 6 hr after logarithmic growth has ceased, it is subsequently unable to stimulate spore formation.

The ability of 9 antimicrobial drugs to prevent sporulation in the presence of varying concentrations of Mn^{++} was examined. The ability of oxy-, chlor-, and tetracycline, but not of the other 6 drugs, to inhibit growth was slightly reversed by Mn^{++}. Subbacteriostatic concentrations of the 3 tetracyclines and polymyxin, penicillin, and magnamycin did not inhibit sporulation. Streptomycin, quinacrine, and chloramphenicol inhibited spore formation at lower concentrations than were required to inhibit growth. Mn^{++} could not reverse this inhibition.

REFERENCES

ALBERT, A. 1953 Avidity of terramycin and aureomycin for metallic cations. Nature, **172**, 201.

BARTHOLOMEW, J., AND MITTWER, T. 1950 Simplified bacterial spore stain. Stain Technol., **25**, 153–156.

CHARNEY, J., FISHER, W. P., AND HEGARTY, C. P. 1951 Manganese as an essential element for sporulation in the genus *Bacillus*. J. Bacteriol., **62**, 145–148.

CURRAN, H. R., AND EVANS, F. R. 1954 The influence of iron or manganese upon the

formation of spores by mesophilic aerobes in fluid organic media. J. Bacteriol., **67**, 489–497.

GALE, E. F. 1949 The assimilation of amino acids by bacteria. J. Gen. Microbiol., **3**, 369–386.

HARDWICK, W. A., AND FOSTER, J. W. 1952 On the nature of sporogenesis in some aerobic bacteria. J. Gen. Physiol., **35**, 907–927.

HARDWICK, W. A., GUIRARD, B., AND FOSTER, J. W. 1951 Antisporulation factors in complex organic media. II. Saturated fatty acids as anti-sporulation factors. J. Bacteriol., **61**, 145–151.

KRASK, B. J. 1953 Methionine sulfoxide and specific inhibition of sporulation in *Bacillus subtilis*. J. Bacteriol., **66**, 374.

KNAYSI, G. 1948 The endospore of bacteria. Formation. Bacteriol. Revs., **12**, 50–63.

LITTLE, P. A., OLESON, J. J., AND WILLIAMS, J. H. 1953 Factors influencing the sensitivity of protozoa to antibiotics. Antibiotics and Chemotherapy, **3**, 29–34.

NEWTON, B. A. 1953 Reversal of the antibacterial activity of polymyxin by bivalent cations. Nature, **172**, 160–161.

PEPPER, W. F., SLINGER, S. J., AND MOTZOK, I. 1953 Effect of aureomycin on the niacin and manganese requirements of chicks. Poultry Sci., **32**, 656–660.

ROSEN, W. G. 1954 Plant growth inhibition by streptomycin and its prevention by manganese. Proc. Soc. Exptl. Biol. Med., **85**, 385–388.

SAZ, A. K., AND SLIE, R. B. 1954 Reversal of aureomycin inhibition of bacterial cell-free nitro reductase by manganese. J. Biol. Chem., **210**, 407–412.

SILVERMAN, M. 1948 Metal antagonism of the antibacterial action of atabrine and other drugs. Arch. Biochem., **19**, 193–198.

STOCKTON, J. R., AND WYSS, O. 1946 Proteinase production by *Bacillus subtilis*. J. Bacteriol., **52**, 227–228.

THEROUX, F. R., ELDRIDGE, E. F., AND MALLMAN, W. L. 1943 Manganese, colorimetric method, pp. 29–30. In *Laboratory manual for chemical and bacterial analysis of water and sewage*. McGraw-Hill, New York.

WEINBERG, E. D. 1954 The reversal of the toxicity of oxytetracycline by multivalent cations. J. Infectious Diseases, **95**, 291–301.

WILLARD, H. H., AND GREATHOUSE, L. H. 1917 The colorimetric determination of manganese by oxidation with periodate. J. Am. Chem. Soc., **39**, 2366–2377.

WILLIAMS, O. B. 1929 The heat resistance of bacterial spores. J. Infectious Diseases, **44**, 421–465.

WYNNE, E. S. 1952 Symposium on the biology of bacterial spores. Spore formation. Bacteriol. Revs., **16**, 101–104.