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Manganese Requirement for Sporulation and Other Secondary Biosynthetic Processes of *Bacillus*[1]

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ABSTRACT

WEINBERG, EUGENE D. (Indiana University, Bloomington). Manganese requirement for sporulation and other secondary biosynthetic processes of *Bacillus*. Appl. Microbiol. **12**:436-441. 1964.—Concentrations of manganese, considerably in excess of the quantity required for normal vegetative growth, are needed by *Bacillus* for (i) synthesis of such secondary metabolites and structures as antibiotics, D-glutamyl peptide, endospores, bacteriophage, and protective antigen; and (ii) longevity of vegetative cell cultures. No other biologically active element can substitute for manganese, and no secondary biosynthetic process of *Bacillus* has been found in which the requirement for manganese is absent. In the present study, manganese could induce sporulation of *B. megaterium* even when added to synthetic broth cultures as late as 100 hr after inoculation. When sub-bactericidal concentrations of various biologically active trace elements were supplied within 2 hr of manganese addition, suppression of spore formation occurred in cultures exposed to elements of group VI (chromium, molybdenum, tungsten, selenium, tellurium) and one of group VIII (nickel); of the six interfering elements, selenium and nickel were most potent.

The synthesis of a wide variety of metabolites as well as the execution of many physiological processes by microbial, plant, or animal cells are often influenced markedly by the presence of one or a few transition metals in concentrations that differ from those required for vegetative growth (Weinberg, 1962). Notable examples are the requirement of various quantities of zinc for a wide diversity of metabolic reactions in fungi (Cochrane, 1958) and of high concentrations of manganese for secondary biosynthetic processes in *Bacillus*. In a stimulating essay, Bu'Lock (1961) defined secondary metabolites as those which (i) have a restricted distribution that is almost species-specific, (ii) possess no obvious function in general metabolism, and (iii) are synthesized most rapidly or entirely when the cells in the culture are no longer increasing in number. In the present paper, the phrase "secondary biosynthetic processes" is used to denote the formation by *Bacillus* not only of such secondary metabolites as antibiotics and D-glutamyl peptide but also of such specialized entities as endospores and bacteriophage.

[1] A portion of the material was presented at the Annual Meeting of the American Society for Microbiology in Washington, D.C., 3-7 May 1964.

Attempts were made to interfere with or even to eliminate the need for manganese in one secondary biosynthetic process, namely, sporulation. The attempts involved (i) withholding from vegetative cell cultures the required quantity of the metal for various periods of time, (ii) supplementing or replacing manganese by other trace elements at different growth phases, and (iii) reducing the cell density of vegetative cell cultures at the time at which spore formation normally would begin if the metal were present.

MATERIALS AND METHODS

Culture media and sporulation assay. Cultures of a stock laboratory strain of *B. megaterium* were maintained and tested in a liquid medium of the following composition: glucose, 1.0 g; KH_2PO_4, 5.0 g; $(NH_4)_2PO_4$, 1.0 g; NaCl, 1.0 g; $MgSO_4$, 0.2 g; $FeSO_4$, 10 mg; $ZnSO_4$, 10 mg; $CaCl_2$, 5.0 mg; deionized water, 1.0 liter. The solutions of glucose and phosphates were sterilized separately prior to addition to the medium; the pH of the complete broth was 7.0. The composition of the medium was similar to that used by Slepecky and Foster (1959) except that glucose was substituted for sucrose and $MnSO_4$ was not included. The quantity of manganese supplied to the medium as a contaminating ingredient of the particular sample of $FeSO_4$ employed (Baker Analyzed reagent grade) was 5×10^{-8} M, which is sufficient for vegetative growth but not for sporulation.

Portions (10 ml) of medium in 50-ml Erlenmeyer flasks were inoculated with 0.1 ml of a 20-hr culture and incubated at 37 C on a reciprocating shaker. In a few tests to be described in the results, nutrient broth [0.5% Polypeptone (BBL), 0.3% Beef Extract (Difco)] was used in addition to the synthetic medium; the inocula of vegetative cells employed for each medium always were obtained from 20-hr nonsporulated cultures in synthetic broth.

For counts of total viable cells and of viable spores, diluted samples were plated in nutrient agar; for the spore counts, vegetative cells were inactivated prior to plating by exposure of the cultures to 72 C for 30 min. Microscopic examination of all cultures was performed immediately before heat inactivation to ascertain whether spores might have formed that would not be viable after exposure to heat. Microscopic studies and plate counts

were performed prior to and after the addition of the various inorganic salts at appropriate time intervals.

Inorganic salts. Various concentrations of $MnCl_2$ were added to the cultures at times to be described. The particular sample (Baker Analyzed reagent grade) contained 0.02% zinc and 0.0002% iron, but these metals were present in the basal medium at concentrations far in excess of the amounts contributed by the manganese salt. The following reagent-grade salts were tested to learn whether they could either replace or antagonize the manganese requirement for sporulation: $AlCl_3$, $VOCl_2$, $CrCl_3$, $CoCl_2$, $NiCl_2$, $CuCl_2$, Na_2Se, Na_2SeO_3, Na_2SeO_4, $ZrOCl_2$, Na_2MoO_4, $SnCl_2$, K_2TeO_3, and Na_2WO_4. The concentrations tested ranged between 1.0 and 20×10^{-6} M plus additionally higher quantities for selenium and nickel (described below). At the concentrations used, manganese contamination of each of the 14 salts was negligible; for example, 5×10^{-6} M $CrCl_3$ supplied less than 5×10^{-11} manganese (R. W. Kreinest, *personal communication*); therefore, if replacement of manganese activity were to occur, such action would not be the result of contamination of the test salt by the metal.

Reduction of cell density of postlogarithmic-phase cultures. Cells were removed from their staled medium and resuspended in homologous-staled broth at concentrations of 1.0, 0.1, and 0.01 times the original density. For each of the three densities, a range of concentrations of $MnCl_2$ was added at the time the cells were replaced in the staled medium. The replacement cultures were then shaken at 37 C for 24 hr; during this period, the usual assays for total viable organisms and for viable spores were performed.

RESULTS

Vegetative growth, persistence of viability of vegetative cells, and sporulation in basal medium and in medium supplemented with manganese. The increase in number of total viable cells and of spores in the 24-hr period after addition of 5×10^{-6} M manganese to cultures of various ages is shown in Fig. 1. In the basal medium with or without added manganese, a 100-fold increase in number of vegetative cells occurred within the first 14 hr of incubation; in manganese-enriched medium, sporulation occurred within the subsequent 10 hr. When manganese was withheld, the number of total viable cells in maximal stationary-phase cultures gradually decreased (to about 0.1% at 100 hr of the number present at 20 hr), and the number of spores gradually increased (from 10^2 at 20 hr to 1.2×10^4 at 80 hr). This slight amount of sporulation may have occurred because of the release of manganese from dying vegetative cells that had sequestered the metal during growth; the decrease in number of spores between 80 and 100 hr presumably indicates spore germination rather than a sporocidal action in the aged culture broth.

The significant increases in number of spores, however, occurred within the respective 24-hr periods after addition of manganese at either 0, 20, 40, or 60 hr, and even to some extent at 80 or 100 hr. When nutrient broth was employed instead of the synthetic medium, sporulation occurred only if manganese was added within the first 10 hr after inoculation. The pH of nutrient broth cultures rose from 7.0 to 8.0 as vegetative growth occurred, whereas in the synthetic medium it remained at 7.0. Possibly, the rise in pH in the complex medium permitted the formation or persistence of antisporulation factors, or both.

Various concentrations of manganese were added to 20-hr synthetic medium cultures, and the numbers of total viable cells and of viable spores present after an additional 24 hr of incubation are shown in Fig. 2. As the concentration of added manganese was increased from 0.1 to 10×10^{-6} M, there was a slight increase in total viable cells and a very large increase in number of viable spores. Microscopic examination of specimens from the cultures confirmed these results inasmuch as, in the presence of less than 10^{-6} M manganese, there was less than 0.1% spores in the preparations.

Effect of other trace elements on cell viability and sporulation when added with and without manganese. When added to 20-hr synthetic broth cultures, none of the 14 inorganic salts listed in the methods section could replace the manganese requirement for sporulation; nor did they have any significant effect, at the low concentrations tested, on vegetative cell viability. When added with manganese, the elements of group VI (chromium, molybdenum, tungsten, selenium, and tellurium) and one of the elements of group VIII (nickel) were able to interfere with spore formation. Of the six active elements, only partial interference with manganese was obtained with chromium, molybdenum, tungsten, and tellurium, and their action could be suppressed by moderately increasing the concentration of

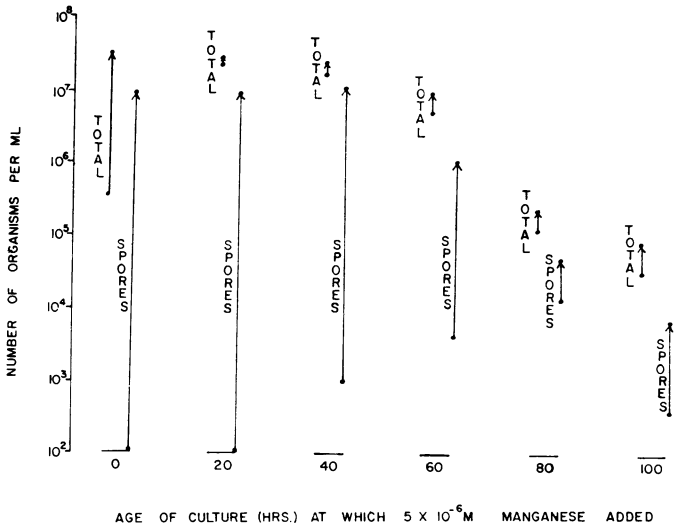

FIG. 1. *Height of lines indicates increase in number of total viable organisms and of viable spores in the 24-hr period after the addition of manganese to basal medium cultures at 0, 20, 40, 60, 80, and 100, hr, respectively.*

manganese. With each of the three selenium salts and with nickel, however, marked interference with manganese action was obtained and, to counteract the potency of these elements, the concentration of manganese had to be increased considerably. To obtain maximal sporulation in the presence of 13×10^{-6} M selenium, a tenfold increase in manganese was required (Fig. 3). At higher concentrations of selenium, the bactericidal action of the element was increased, whereas its sporulation-suppressing power remained constant; and, unexpectedly, high quantities of manganese aided the bactericidal action of large amounts of selenium (Fig. 3).

Each of the six elements that interfered with spore formation consistently was more active when added between 1 and 2 hr after, rather than at the same time as, manganese. For example (Table 1), when added simultaneously with manganese, nickel permitted 1.2% sporulation, but, when added 2 hr later than manganese, allowed only 0.2% of the viable cells to form spores. Interference by each of the six elements was diminished if they were withheld for more than 3 hr after the addition of manganese; at 6 hr, they were inactive, except for nickel which still retained some of its potency. At 8 hr, mature spores had begun to appear in the population, and nickel was no longer active if added at that time. None of the elements, at the low concentrations tested, interfered with spore germination in nutrient agar, as demonstrated by their lack of germicidal activity when added to fully sporulated cultures.

A unique action of chromium was observed when large quantities were added to vegetative cell cultures 3 hr after the addition of suboptimal concentrations of manganese

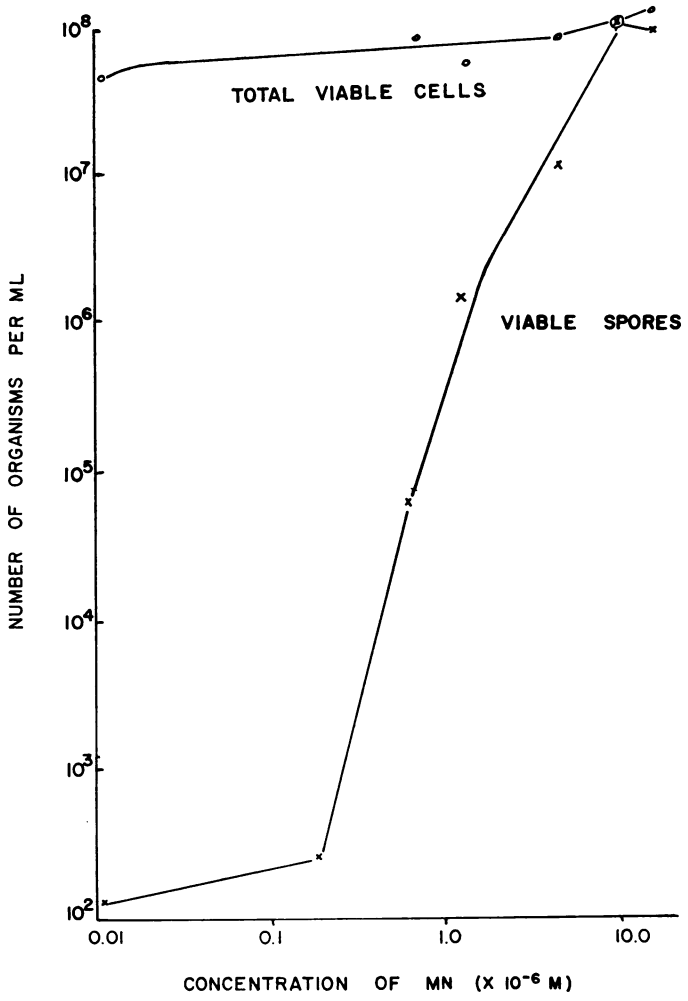

FIG. 2. *Number of total viable organisms and of viable spores 24 hr after the addition (to 20-hr basal medium cultures) of various concentrations of manganese. At 20 hr, prior to manganese addition, the cultures contained 6 × 10⁷ total viable organisms and 10² viable spores per ml.*

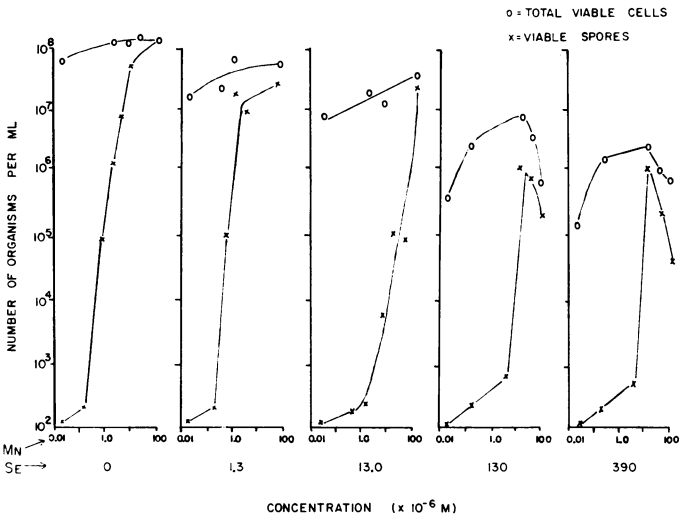

FIG. 3. *Number of total viable organisms and of viable spores 24 hr after the addition (to 20-hr basal medium cultures) of various concentrations of manganese and selenium. At 20 hr, prior to manganese and selenium addition, the cultures contained 6 × 10⁷ total viable organisms and 10² viable spores per ml.*

TABLE 1. *Effect of selenium, nickel, or chronium additions on Bacillus sporulation at various time intervals after manganese addition to 20-hr basal medium culture*

Age of culture at which metal(s) added			Per cent sporulation at 44 hr	Age of culture at which metal(s) added		Per cent sporulation at 44 hr
Manganese (5×10^{-6})	Selenium (50×10^{-6} M)	Nickel 50×10^{-6} M)		Manganese (0.5×10^{-6} M)	Chromium (60×10^{-6} M)	
hr	*hr*	*hr*		*hr*	*hr*	
—	—	—	0.01	—	—	0.01
—	20	—	0.00	—	20	0.01
—	—	20	0.00	20	—	0.30
20	—	—	18.00	20	20	0.10
20	20	—	0.60	20	21	0.00
20	21	—	0.00	20	22	0.10
20	22	—	0.20	20	23	0.90
20	23	—	1.50	20	24	0.30
20	24	—	7.00			
20	—	20	1.20			
20	—	21	0.50			
20	—	22	0.20			
20	—	23	2.10			
20	—	24	3.00			

(Table 1). Chromium actually stimulated the production of more spores than would have been formed with manganese alone. This action was not due to manganese contamination, since the added chromium contained less than 0.0006×10^{-6} M manganese, which was lower than the amount of the latter metal present in the basal medium; moreover, 0.02×10^{-6} M manganese added in place of chromium at 3 hr could not replace the activity of the latter. Chromium was unable to stimulate spore formation when added to cultures which, 3 hr previously, had received concentrations of manganese either greater than 0.5×10^{-6} M or none at all.

Concentrations of manganese required to induce sporulation in cultures of reduced cell density. A possible reason for the need for increased quantities of manganese for sporulation over vegetative cell growth is that production of spores occurs after exponential growth takes place, and thus many more cells (100-fold in the present tests) are competing for the small amount of the metal provided in the basal medium. However, when basal medium cultures at 20 hr were centrifuged and the cells were resuspended in homologous-staled medium at densities of 1.0, 0.1, and 0.01 the original density, sporulation did not occur in the absence of added manganese in any of the replaced cultures. Rather, regrowth of the diluted cultures occurred and then spore formation took place; the dose-response curve for manganese was identical to that shown in Fig. 2. Unfortunately, vegetative cells of the test organism used in this study were unable either to survive or to form spores when resuspended (with or without manganese) in a replacement menstruum such as phosphate buffer. It would be of interest to determine the amount of added manganese that would be needed for endotrophic sporulation at various cells densities; indeed, such cells might not be able to utilize the metal unless it had been supplied prior to their replacement in the endotrophic system.

DISCUSSION

Various metabolites or structures produced by strains of *Bacillus* are designated in Table 2 as either primary or secondary. Proteinase and α-amylase have obvious metabolic functions and are not restricted in distribution to *Bacillus*; depending on cultural conditions, they may be produced either during or after rapid cellular multiplication, or at both times, and are, therefore, considered to be primary materials. Likewise, it is clear that flagella are primary structures since they are not restricted to *Bacillus*, have an obvious function, and are synthesized during the

TABLE 2. *Transition metals required by Bacillus for synthesis of specific metabolites or structures and for cultural longevity*

Metabolite or structure	Transition metals[a]							Reference(s)
	Cr	Mn	Fe	Co	Ni	Cu	Zn	
Primary								
Proteinase		+, 0					0[b]	Stockton and Wyss, 1946 Fukumoto, Yamamoto, and Tsuru, 1957
α-Amylase		+, 0[c]	+, 0[d]					Fukumoto et al., 1957 Coleman and Elliott, 1962
Flagella		0	0	0	0	0	0	Weinberg and Brooks, 1963
Secondary								
Bacillin		+	0				0	Foster and Woodruff, 1946
Bacitracin	0	+	0[b]		0	0	0	Hendlin, 1949
Mycobacillin		+	+	0	0	0	0	Majumdar and Bose, 1960
Subtilin		+	0			0		Jansen and Hirschmann, 1944
D-Glutamine		+		0			0	Leonard, Housewright, and Thorne, 1962
D-Glutamyl peptide		+	0	+, 0[e]			+, 0[e]	Leonard et al., 1958 Leonard and Housewright, 1963
Protective antigen		+						Wright, Hedberg, and Slein, 1954
Endospores		+	0	0	0	+, 0[f]	0[b]	Halvorson, 1963 Slepecky and Foster, 1959 Kolodziej and Slepecky, 1962
Bacteriophage		+	+, 0[g]	0		0	0	Huybers, 1953
Cultural longevity[h]		+	0	0			0	Leonard et al., 1958

[a] Plus sign indicates that metal is required (zero sign = not required; blank space = not tested) in concentration higher than the minimal quantity present in the basal medium in which normal vegetative growth occurs.

[b] Supplements activity of, but cannot replace requirement for, manganese.

[c] Required by resting but not by multiplying cells.

[d] Required by multiplying but not by resting cells.

[e] Can replace manganese in whole cells but not in cell-free system.

[f] Required in some systems (in addition to manganese) with or without molybdenum.

[g] Can replace manganese if present during period from 20 min prior to ultraviolet irradiation through maturation phase; cannot replace manganese if present only during maturation phase.

[h] Under conditions in which sporulation cannot occur.

logarithmic phase of growth. Placement of the antibiotics, D-glutamyl peptide, endospores, anthrax protective antigen, and bacteriophage in the secondary category is justified on the basis that these materials have no obvious function in general metabolism and are synthesized in cultures whose cells have stopped increasing at an exponential rate.

For synthesis of materials in the secondary category, as well as for longevity of nonsporulating vegetative cells in the maximal stationary phase, manganese consistently is required (Table 2). In only one case (that of mycobacillin and iron) can another transition metal be substituted for manganese; and it is predicted that manganese will be found to be essential for other secondary biosynthetic processes of *Bacillus* for which transition metals have not yet been tested. In no case can metals outside the transition series (e.g., potassium, magnesium, or calcium) replace manganese. In contrast, the synthesis of primary metabolites or structures may or may not require a quantity of manganese in excess of that needed for normal vegetative growth.

Comparisons of the concentration of manganese required for vegetative growth with the quantities needed for specific biosynthetic processes or for cultural longevity are presented in Table 3. It might be predicted that the concentration needed for a particular function would vary as the organic constituents of the medium are changed, since some of these would be expected to (i) suppress or enhance cellular assimilation of the metal, (ii) reduce the requirement for the metal (e.g., if manganese is needed for synthesis of small organic molecules that perhaps would be supplied preformed in a compex medium), or (iii) raise the requirement (e.g., by shifting metabolic systems away from the function), or to perform all three functions. Surprisingly, however, there is comparatively little variation in the quantity of manganese needed for a specific function in different nutritional environments. For example, sporulation requires that 1.0 to 10×10^{-6} M manganese be added to different chemically defined media (Charney et al., 1951; Slepecky and Foster, 1959) as well as to such complex media as nutrient broth (Weinberg, 1955) and Trypticase Soy Broth (Charney, Fisher, and Hegarty, 1951). Likewise, similar concentrations of manganese are needed for the synthesis of bacitracin in chemically defined (Hendlin, 1949) and in complex (*unpublished data*, this laboratory) media. Fortunately, contamination of media with sufficient quantities of selenium and nickel for suppression of sporulation would rarely occur.

TABLE 3. *Minimal concentrations of manganese required for various functions of Bacillus*

Function	Concn
	M
Normal vegetative growth*	5×10^{-8} to 5×10^{-7}
Synthesis of	
Bacitracin	10^{-6} to 10^{-5}
D-Glutamine	10^{-6} to 10^{-5}
Mycobacillin	10^{-6} to 10^{-5}
Endospores	10^{-6} to 10^{-5}
Protective antigen†	10^{-6} to 10^{-5}
Proteinase	10^{-5} to 10^{-4}
D-Glutamyl peptide	10^{-5} to 10^{-4}
Subtilin	10^{-5} to 10^{-4}
α-Amylase	10^{-4} to 10^{-3}
Bacillin	10^{-4} to 10^{-3}
Bacteriophage	10^{-4} to 10^{-3}
Cultural longevity	10^{-4} to 10^{-3}

* Hendlin, 1949; Leonard et al., 1958; Lankford et al., 1957.
† Synthesis suppressed by concentrations above 2×10^{-5} M.

Undoubtedly, one reason for the quantitative consistency of the manganese requirement is that the amount of the metal that normally contaminates various components of media is rather low, and thus does not alter appreciably the known quantity that must be added to achieve the specific desired synthesis. For example, the approximate concentration of manganese that might be contributed to a medium by a representative sample of 1.0% reagent-grade glucose is 10^{-8} M (Noguchi and Johnson, 1961), by 0.5% peptone (Difco) is 6.5×10^{-8} M (Sykes, 1956), by 0.1% yeast extract is 4.0×10^{-8} M (Grant and Pramer, 1962), and by 0.001% "chemically pure" iron salts is 4.0×10^{-8} M (Weinberg, 1955). Occasionally, however, a sample of iron salt is encountered that has an unusually large amount of manganese contamination (Leonard, Housewright, and Thorne, 1958); the other potential source of excessive contamination by the element is improperly deionized water or tap water, and some supplies of the latter, although considered by U.S. Public Health Service standards to be free from objectionable quantities of manganese, may contribute as much as 4.0×10^{-6} M (Anonymous, 1959) to the medium. Mammalian blood has a normal content of 5×10^{-7} M manganese (Cotzias, 1958); this concentration is sufficient to permit *B. anthracis* to multiply but not to sporulate in the circulatory system of the host.

In some cases, of course, metal contamination in the order of 10^{-7} M can be critical. For example, a particular mixture of chemically pure salts of iron, zinc, manganese, and calcium at a concentration of 0.0032% contributed approximately 2.0×10^{-7} M copper to the medium; this quantity of copper (in addition to manganese) was required for sporulation and had to be added when "spectrographically pure" salts were employed; moreover, copper was active only if added not later than 6 hr after spore germination of the inoculum had occurred (Kolodziej and Slepecky, 1962). It will be of considerable interest to learn whether copper, in addition to manganese, is essential not only for sporulation of various species of *Bacillus* but also for the synthesis of other secondary metabolites and structures.

Another area in which studies are needed concerns the precise period in which manganese is required during the secondary biosynthetic process. In sporulation, for example, it is not known whether the metal need be present only for a brief initial period or for the entire 8 hr. Moreover, it is not yet apparent whether the metal must be available to (i) participate in derepression of the segment of the genome associated with sporulation, (ii) activate enzymes engaged in the degradation of vegetative materials or synthesis of spore materials, or both, or (iii) suppress enzymes that favor continuation of primary biosynthetic processes. Manganese is the predominant metal involved in general enzymatic decarboxylation and hydrolysis (Nason, 1958), and extensive degradation of vegetative-cell proteins and of poly-β-hydroxybutyric acid occurs prior to sporogenesis (Halvorson, 1963). The element might also have a critical role in activation of enzymes necessary for biosynthesis of metabolites and components of secondary structures in addition to spores; for example, polyglutamic acid synthetase has an absolute manganese requirement (Leonard and Housewright, 1963). A similarity in the synthesis by *Bacillus* of simple peptides, antibiotics, and spore coats has been suggested (Bernlohr and Sievert, 1962), but it is not clear how the synthesis of bacteriophage might fit into this group of substances. The mechanism whereby manganese prolongs longevity of vegetative cell cultures of nonsporulating strains of *Bacillus* (Leonard, Housewright, and Thorne, 1958) is likewise obscure. Does death occur in the absence of high concentrations of the metal because of (i) inability of the cells to produce a specific and as yet unrecognized secondary metabolite or (ii) a general need for high levels of manganese-catalyzed protease or synthetase activity, or both, during the maximal stationary phase?

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