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Action of Chloramphenicol and Its Isomers on Secondary Biosynthetic Processes of *Bacillus*

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ABSTRACT

WEINBERG, EUGENE D. (Indiana University, Bloomington), AND SUE M. TONNIS. Action of chloramphenicol and its isomers on secondary biosynthetic processes of *Bacillus*. Appl. Microbiol. **14**:850–856. 1966.—Chloramphenicol or its isomers, if supplied within 4 hr after the addition of Mn^{+2}, prevented sporulation of *Bacillus megaterium* at concentrations that partially inhibited protein synthesis but which were neither bacteriostatic nor bactericidal. Likewise, sub-bactericidal quantities of the compounds, if supplied within 2 hr after the addition of Mn^{+2}, suppressed formation of bacitracin by *B. licheniformis*. In contrast to previous reports that chloramphenicol is less active than its isomers against sporulation and peptide formation, the results of the present study indicated that the order and extent of these activities of the compounds is similar to that of their ability to prevent growth and to suppress protein synthesis; i.e., D(−)-threo > L(+)-erythro > D(−)-erythro.

Studies of the action of chloramphenicol and its isomers against species of *Bacillus* have yielded a collection of rather unexpected and somewhat conflicting observations. For example, although the antibiotic is bacteriostatic for most genera, for the genus *Bacillus* it is bactericidal (4). The D(−)-threo isomer, which inhibits protein synthesis and has clinical usefulness, predictably suppresses sporulation at growth inhibitory concentrations (5, 17); but, surprisingly, a mixture of the L(+)- and D(−)-erythro isomers was reported to be more active than the D(−)-threo form in preventing spore formation (3). The L(+)-erythro isomer, which has only 1 to 2% of the bacteriostatic potency of the D(−)-threo compound for *Escherichia coli* (12), suppresses D-glutamyl peptide formation of *B. subtilis* at concentrations that affect neither growth nor protein synthesis, whereas the D(−)-threo form is inactive (7). On the other hand, a mixture of the DL-erythro isomers prevents accumulation of *both* D- and L-glutamyl peptide, but only if growth is slowed and the rate of death increased by limiting concentrations of Mn^{-2} (9). Formation of bacitracin, a peptide that contains both D- and L-amino acids, is suppressed by neither the D(−)-threo nor the DL-erythro isomers (3).

At the end of the exponential phase of growth, a shift to secondary metabolism occurs in cultures of *Bacillus* if the quantity of available Mn^{+2} is at least 1 to 2 log units higher than the concentration of 5×10^{-8} M required for vegetative growth (18). The onset of secondary metabolism can be delayed by withholding Mn^{+2}, but, if the period of withholding is prolonged, death intervenes (10, 18). In the normal sequence of events in secondary metabolism of *Bacillus*, production of peptides that contain both D- and L-amino acids occurs first and is followed by sporulation (2, 11); it is not known whether the presence of the peptides is a prerequisite for spore formation. Five possible functions of the peptides have been suggested, but little evidence is available in support of any of these (Weinberg, *in press*).

The experiments described in the present study were performed to answer the following questions: (i) is the ability of the D(−)-threo, L(+)-erythro, and D(−)-erythro isomers of chloramphenicol to suppress growth and protein synthesis parallel or antipodal to their ability to prevent sporulation; (ii) is the antisporogenic effect of the compounds simply a consequence of their bactericidal activity; and (iii) can any of the isomers influence the quantity of a peptide (bacitracin) that accumulates in the cultures?

MATERIALS AND METHODS

Strains and culture media. B. megaterium strain WS (Indiana University culture collection) was used for the studies on viability, sporulation, and protein synthesis; strain ATCC 10716 of *B. licheniformis* was used for the experiments on production of bacitracin. Cultures of *B. megaterium* were grown and tested in a

basal medium of the following composition: glucose, 1.0 g; KH_2PO_4, 5.0 g; $(NH_4)_2HPO_4$, 1.0 g; NaCl, 1.0 g; $MgSO_4 \cdot 7H_2O$, 0.41 g; $FeSO_4 \cdot 7H_2O$, 18 mg; $ZnSO_4 \cdot 7H_2O$, 18 mg; $CaCl_2 \cdot 2H_2O$, 6.6 mg; deionized water, 1.0 liter. Solutions of each ingredient were sterilized separately, and appropriate volumes were combined for the complete medium; the latter was then adjusted to *p*H 7.3 with sterile 1 N NaOH. The composition of the medium was similar to that used by Slepecky and Foster (15), except that glucose was substituted for sucrose and $MnSO_4$ was not included. The small quantity of Mn^{+2} required for vegetative growth is supplied as a contaminant of glucose and the inorganic salts. Cultures of *B. licheniformis* were grown and tested in nutrient broth which consisted of 0.3% Beef Extract (Difco), 0.5% Polypeptone (BBL), and deionized water, *p*H 7.0. This complex medium, provided that deionized water is used, contains a biologically available concentration of Mn^{+2} of $<10^{-6}$ M (17; *unpublished data*); to obtain formation of spores and detectable quantities of bacitracin, additional Mn^{+2} must be supplied.

Inocula for the experiments were obtained by subculturing three times, in the appropriate test medium, organisms originally obtained from nutrient agar slants. Cultures used in the serial passages, as well as in the experiments, were grown in 10-ml portions of the respective media contained in 50-ml Erlenmeyer flasks and incubated at 37 C on a reciprocating shaker. Inocula for the actual tests consisted of 0.1-ml quantities of suspensions of the third subcultures obtained at the end of the period of logarithmic growth.

Assays for viable organisms, spores, protein synthesis, and bacitracin. For counts of total viable cells and of viable spores of either *B. megaterium* or *B. licheniformis*, 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. To rule out the possibility that, under some conditions, heat-sensitive spores were being formed, wet mounts of all cultures were examined for spores under the phase microscope. In no case were samples observed to contain spores that were visible under the microscope but which could not survive the heating process.

For studies on protein synthesis, 14-hr basal medium cultures of *B. megaterium* were adjusted to *p*H 7.3 and supplemented with 5×10^{-6} M Mn^{+2}. Appropriate concentrations of either the D(−)-threo or L(+)-erythro isomer of chloramphenicol were added, and 1-ml samples were then incubated in a water-bath shaker at 37 C for 5 min. A 0.02-ml amount of a solution of uniformly labeled L-leucine-C^{14} was added to each tube; the quantity of leucine per sample was 0.012 mg, and the activity was 0.1 μc. A preliminary experiment demonstrated that uptake of radioactivity in control cells would be linear with time for at least 120 min; in the actual tests, a reaction time of 30 min was selected. The reaction was terminated by addition of 1 ml of 10% trichloroacetic acid, and the tubes were steamed for 20 min. The samples were filtered through membrane filters (type HA, 25 mm; Millipore Filter Corp., Bedford, Mass.); the filters were washed with 3 ml of 5% trichloroacetic acid, glued to planchets, and dried. Radioactivity was counted in a Nuclear-Chicago gas-flow counter with thin window for at least 1,000 counts.

Bacitracin produced by *B. licheniformis* was measured by assay with a stock laboratory strain of *Staphylococcus aureus*. Portions of 18-hr nutrient broth cultures of *S. aureus* were diluted 1:100 in nutrient agar at 48 C. Portions (3-ml) of the seeded agar were added to petri dishes containing a base layer of 15 ml of nutrient agar. To obtain maximal expression of bacitracin activity, the nutrient agar was enriched with 5×10^{-5} M Zn^{+2} (19). The seeded plates were stored at 2 C until needed. Holes were then made in the agar by use of a no. 5 cork borer. Portions (0.1-ml) of solutions of authentic bacitracin or of culture supernatant fluids to be assayed were added to the holes; each sample was tested in duplicate. The petri plates were then incubated at 37 C for 24 hr. Zones of inhibition were measured with a Fisher Lilly Antibiotic Zone Reader. Under these conditions, a straight-line relationship was obtained when zone diameters between 12 and 24 mm were plotted against the logarithms of doses of authentic bacitracin between 10 and 100 μg (0.54 and 5.4 units) per ml. Cultures to which chloramphenicol or its isomers were added were included as controls, to ascertain that these agents would not be present in the supernatant fluids in sufficient quantity to interfere with the biological assay for bacitracin. Under conditions in which bacitracin could not be formed (i.e., absence of Mn^{+2} or presence of large amounts of chloramphenicol or its isomers), no zones of inhibition were obtained.

Test reagents. Various concentrations of $MnCl_2 \cdot 4H_2O$ or one of three chloramphenicol isomers, or both, were added to the cultures of *B. megaterium* or *B. licheniformis* at times to be described in Results.

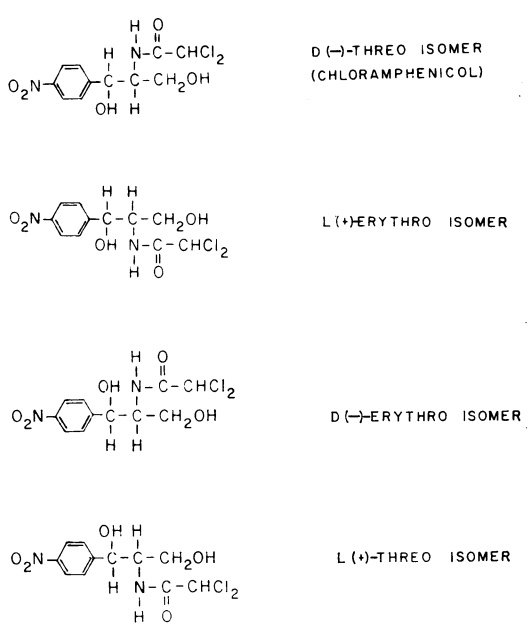

FIG. 1. *Chloramphenicol and its stereoisomers.*

The particular manganous salt ("Baker Analyzed" reagent grade, lot 23422; J. T. Baker Chemical Co., North Phillipsburg, N.J.) was reported by the processor to contain 0.02% zinc and 0.0002% iron, but these metals were present in both the basal and complex media at concentrations far in excess of the amounts contributed by the salt. The chloramphenicol isomers (obtained from Parke, Davis & Co., Detroit, Mich., through the courtesy of H. M. Crooks, Jr.) consisted of the first three of the four compounds whose structural formulas are shown in Fig. 1. The sample of bacitracin (lot 80163-3) was obtained from Commercial Solvents Corp., Terre Haute, Ind., through the courtesy of M. C. Bachman. Radioactive leucine was purchased from Schwarz Bio Research, Inc., Orangeburg, N.Y.

RESULTS

Induction by Mn^{+2} of sporulation in B. megaterium, and of bacitracin and spore formation in B. licheniformis. The dose-response curves of spore formation and bacitracin production to addition of Mn^{+2} to cultures in the maximal stationary phase are shown in Fig. 2 and 3. For *B. megaterium*, the time of addition of the metallic ion was 14 hr; for *B. licheniformis*, 8 hr. To obtain maximal sporulation and efficient production of bacitracin, the *p*H of 14-hr *B. megaterium* cultures was changed from 6.6 to 7.3, and of 8-hr *B. licheniformis* cultures from 7.8 to 7.3.

Also presented in Fig. 3 are the quantities of bacitracin formed when Mn^{+2} was added to cultures of *B. licheniformis* at the time of inoculation. The reason for the decrease in ability to form the peptide by cells of cultures deprived of Mn^{+2} during the logarithmic phase of growth is not known. The decrease was not a function of a change in cell numbers, inasmuch as neither the generation time of 40 min nor the total cell yield was altered by the delay in addition of the metallic ion. When Mn^{+2} was supplied at the time of inoculation, bacitracin was first detected in 8-hr cultures (i.e., 2 hr after exponential growth had ceased); when the metallic ion was added to maximal stationary-phase cultures, the peptide was first detected within 4 hr.

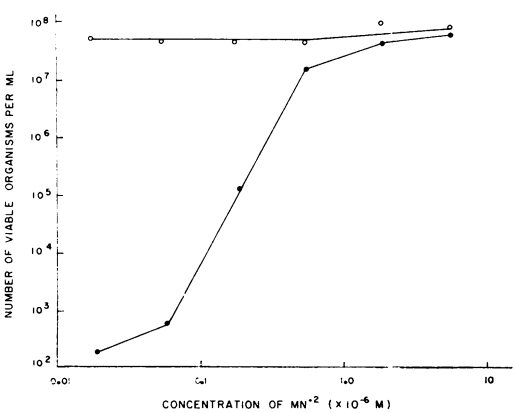

FIG. 2. *Number of total viable organisms* (○) *and of viable spores* (●) *of Bacillus megaterium 24 hr after the addition to 14-hr cultures of various concentrations of Mn^{+2}. The 14-hr cultures, which contained 4.2 × 10^7 total viable cells and 10^2 viable spores per milliliter, were adjusted to pH 7.3 prior to addition of Mn^{+2}.*

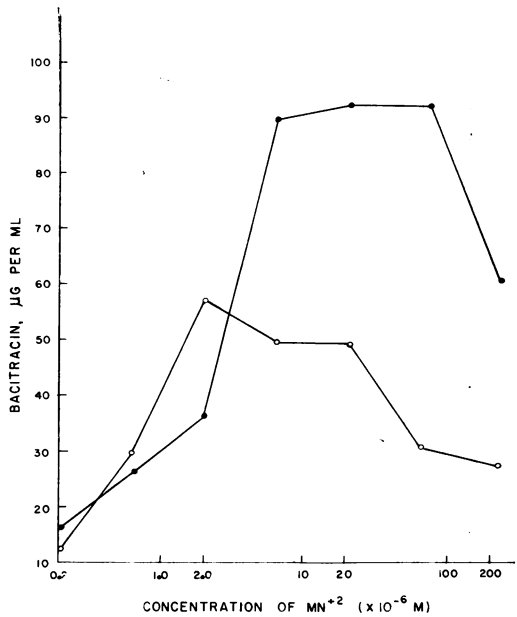

FIG. 3. *Quantity of bacitracin in supernatant fluids of cultures of Bacillus licheniformis to which various concentrations of Mn^{+2} had been added at the time of inoculation* (●) *and at 8 hr after inoculation* (○). *Data were obtained 24 hr after Mn^{+2} was added. At a concentration of 0.06 × 10^{-6} M Mn^{+2}, a slight amount of bacitracin was detected; at 0.02 × 10^{-6} M Mn^{+2}, no bacitracin was found.*

The minimal quantity of Mn^{+2} needed for sporulation of *B. licheniformis* was identical to that required for bacitracin production. As with the latter, the amount of sporulation was less when the metallic ion was added after exponential growth had occurred than if added at the time of inoculation. To learn whether the cell density has an effect on the quantity of Mn^{+2} required for secondary biosynthetic processes, the normal postlogarithmic concentration of 10^9 viable cells per milliliter was increased 10-fold at the same time as the addition of the metallic ion. The amount of Mn^{+2} needed for maximal sporulation and bacitracin production was identical to that required in systems in which a cell density of 10^9 per milliliter was used.

Suppression of sporulation by chloramphenicol isomers. Each of the three isomers tested was able to prevent sporulation of *B. megaterium* in concentrations less than those required to prevent growth or to kill vegetative cells. Data for the D(−)-threo and L(+)-erythro isomers are summarized in Fig. 4; the D(−)-erythro form possessed about half of the antisporogenic, bacteriostatic, and bactericidal activity of the L(+)-erythro compound. Amounts of added Mn^{+2} as high as 5×10^{-4} M were unable to reverse the effects of any of the three isomers. Figure 5 shows that the D(−)-threo isomer could prevent sporulation only if added within a period of 4 to 5 hr after the introduction of 5×10^{-6} M Mn^{+2}; this was true also for each of the other two isomers.

Similarly, with *B. licheniformis*, the D(−)-threo and L(+)-erythro isomers prevented sporulation at concentrations that were without effect on viability (Table 1). However, the effective concentration of the former compound was 10-fold higher for this organism than for *B. megaterium*, whereas that of the latter isomer was essentially identical for both species. The difference in activity of the D(−)-threo compound did not result from the difference in media used for the two test organisms; when *B. megaterium* was grown in nutrient broth, spore formation was prevented by the same low concentration effective in the synthetic medium.

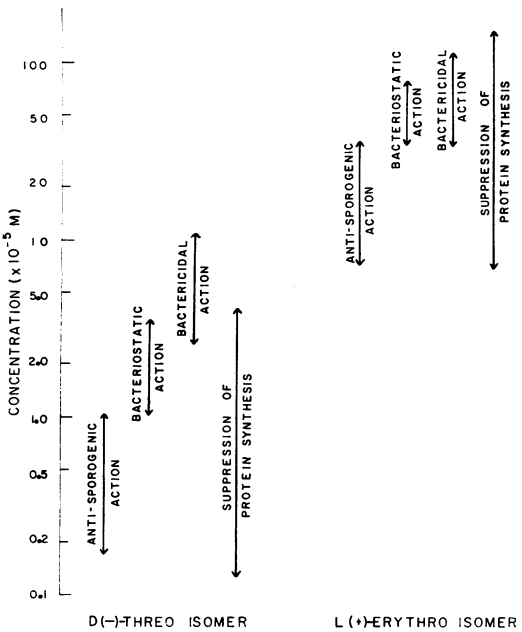

FIG. 4. *Concentrations of D(−)-threo and L(+)-erythro isomers of chloramphenicol required to suppress sporulation, prevent growth, destroy viability, and suppress protein synthesis of Bacillus megaterium. Doses below arrows had no effect; those above arrows had complete effect.*

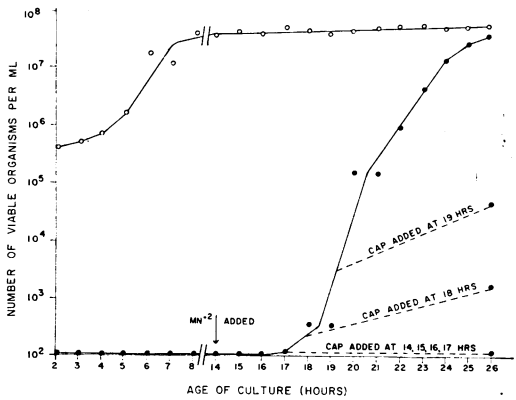

FIG. 5. *Number of total viable organisms (○) and of viable spores (●) of Bacillus megaterium at various times after inoculation of 4 × 10⁵ vegetative cells per milliliter. At 14 hr, 5 × 10⁻⁶ M Mn^{+2} was added, and the pH was adjusted to 7.3. Addition of 10⁻⁵ M D(−)-threo isomer of chloramphenicol (CAP) at 14, 15, 16, or 17 hr completely suppressed spore formation; addition at 20 hr or later had no action on sporulation; effect of addition at 14 through 19 hr is indicated on the figure. Cell viability was not altered in any of the samples.*

Effect of chloramphenicol isomers on cultural longevity and on protein synthesis of B. megaterium. The decline in number of living organisms in control cultures and in cultures supplemented with Mn^{+2} plus sub-bactericidal concentrations of either the D(−)-threo or the L(+)-erythro isomers is shown in Fig. 6. It may be noted that cultures retained viability only when provided with Mn^{+2} in the absence of the antibiotic isomers. Longevity of the cultures appeared to be associated, in part, with sporulation; however, not all of the viable organisms in the Mn^{+2}-supplemented cultures formed spores. The rate of killing of vegetative cells by a bactericidal concentration of the D(−)-threo isomer (10-fold higher than the quantity used for the experiments summarized in Fig. 6) is shown in Fig. 7. The dose-response curves to the D(−)-threo and L(+)-erythro isomers with respect to suppression of protein synthesis are given in Fig. 8. The activity of the latter isomer was approximately 3% of that of the D(−)-threo compound. In a cell-free system of *E. coli*, the isomer was found to have about 12% of the activity of chloramphenicol (13).

Effect of chloramphenicol isomers on formation of bacitracin by B. licheniformis. Each of the three isomers tested was able to prevent formation of bacitracin at concentrations less than those required to kill vegetative cells (Table 1). Complete

TABLE 1. *Concentrations of chloramphenicol and its isomers[a] required to prevent growth, bacitracin formation, and sporulation, and to kill vegetative cells of Bacillus licheniformis[b]*

Concn of I, II, or III ($\times 10^{-5}$ M)	Bacteriostatic activity[c]			Suppression of bacitracin formation[d]			Suppression of sporulation[d]			Bactericidal activity[d]		
	I	II	III	I	II	III	I	II	III	I	II	III
zero	0	0	0	0	0	0	0	0	0	0	0	0
3	0	0	0	0	0	0	0	0	0	0	0	0
6	+	0	0	+	0	0	0	0	0	0	0	0
10	++	0	0	++	0	0	++	0	0	0	0	0
20	++	0	0	++	+	0	++	0	0	0	0	0
30	++	+	0	++	++	+	++	0	0	0	0	0
60	++	++	0	++	++	++	++	++	0	0	0	0
100	++	++	+	++	++	++	++	++	0	+	0	0

[a] Symbols used for isomers: I, D(−)-threo; II, L(+)-erythro; III, D(−)-erythro.
[b] Results expressed as: 0, no effect; +, partial effect; ++, complete effect.
[c] Isomer added with 2×10^{-6} M Mn^{+2} at time of inoculation.
[d] Isomer added with 2×10^{-6} M Mn^{+2} to 8-hr cultures.

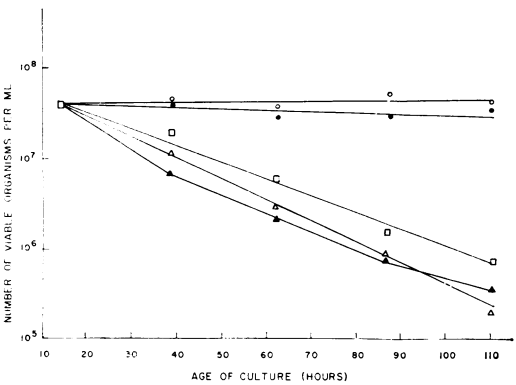

FIG. 6. *Number of total viable organisms of Bacillus megaterium at various times after addition to 14-hr cultures (adjusted to pH 7.3) of 5×10^{-6} M Mn^{+2} (○), 5×10^{-6} M Mn^{+2} plus 10^{-5} M D(−)-threo isomer (△), or 5×10^{-6} M Mn^{+2} plus 20×10^{-5} M L(+)-erythro isomer (▲). Control (neither Mn^{+2} nor isomers), □. Sporulation (●) occurred only in cultures in which Mn^{+2} was the sole addition; the number of spores in all other cultures remained at $<10^2$.*

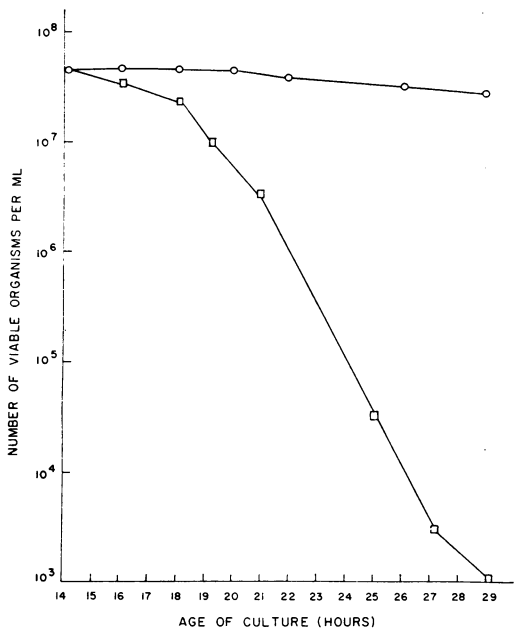

FIG. 7. *Number of viable organisms of Bacillus megaterium at various times after addition of 10^{-4} M D(−)-threo isomer of chloramphenicol to 14-hr cultures. Control, ○; chloramphenicol, □.*

suppression of bacitracin formation without interference with sporulation was obtained by the addition of a concentration of either 30×10^{-5} M of the L(+)-erythro isomer or 60×10^{-5} M of the D(−)-erythro isomer. Quantities of added Mn^{+2} as high as 50×10^{-5} M were unable to reverse the action of either chloramphenicol or its isomers.

The D(−)-threo isomer could prevent bacitracin formation only when added within a period of 2 hr after the introduction of 5×10^{-6} M Mn^{+2} (Table 2); this was true also for each of the other isomers. Chloramphenicol and its isomers had no effect on bacitracin production when added to 8-hr cultures that had been supplied with Mn^{+2} either at the time of inoculation or during exponential growth. In a previous study (3), exposure of cultures of strain A-5 of *B. licheniformis* to high concentrations of chloramphenicol or its isomers resulted in apparent reduction of the compounds to the corresponding aryl amines with subsequent lysis of the cells. In the present study with strain 10716, neither the

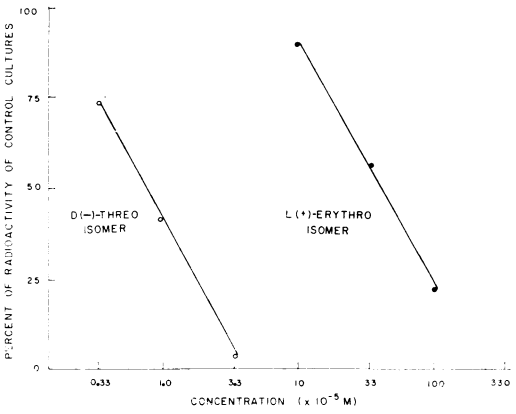

FIG. 8. *Suppression of protein synthesis of Bacillus megaterium by various concentrations of the* D(−)-*threo and* L(+)-*erythro isomers of chloramphenicol. Values were obtained after 30 min of exposure of cells to* C^{14}*-leucine; control samples averaged 1,786 counts per min.*

TABLE 2. *Quantity of bacitracin produced by Bacillus licheniformis after addition of* Mn^{+2} *or chloramphenicol, or both, to maximal stationary-phase cultures*

Age of culture when test substance added (hr)		Quantity of bacitracin (μg/ml) in culture supernatant fluid at				
Mn^{+2} (2×10^{-6} M)	Chloramphenicol (10×10^{-5} M)	8 to 11 hr	12 hr	13 hr	14 hr	15 hr
—	—	0	0	0	0	0
—	8	0	0	0	0	0
8	—	0	8	25	50	58
8	8–10	0	0	0	0	0
8	11	0	0	12	31	42

change in color indicative of aryl amine formation nor cell lysis was observed.

DISCUSSION

The results obtained in this study provide evidence that the ability of chloramphenicol and its isomers to prevent sporulation parallels the extent of their ability to inhibit protein synthesis. Furthermore, effective antisporogenic concentrations for each of the three isomers are less than the quantities needed to prevent growth and to kill vegetative cells. In contrast to a previous observation (3) that the DL-erythro isomers have strong antisporogenic action, we found that the order and relative proportion of activity of chloramphenicol and its isomers are similar to the antibiotic potency of the compounds for species of *Bacillus* and, as reported by other workers (12), for *E. coli*.

In addition to suppression of sporulation, subbacteriostatic concentrations of chloramphenicol and its isomers interfere with maintenance of Mn^{+2}-dependent cultural longevity. Apparently, the antibiotics suppress the synthesis of a specific protein(s) that is required for viability of cells in maximal stationary-phase cultures. Excess quantities of Mn^{+2} are unable to neutralize any of the activities of chloramphenicol or its isomers.

In the present study, a concentration of 10^{-5} M chloramphenicol completely inhibited sporulation of *B. megaterium*, but suppressed total protein synthesis by only 58%. The formation of an enzyme that catalyzes the synthesis of L(+)-*N*-succinyl glutamic acid by presporulating cells of *B. megaterium* is suppressed by 12×10^{-5} M chloramphenicol (1). Presporulating cells of *B. cereus* are unable to synthesize acetoacetyl coenzyme A reductase (8) and total protein (16) in the presence of 3×10^{-5} M and 33×10^{-5} M chloramphenicol, respectively. In cells of *B. subtilis*, 15×10^{-5} M chloramphenicol suppresses protein synthesis and spore coat formation (14). As was true in the present study, the antibiotic begins to lose effectiveness when added to cultures in which heat-resistant spores have begun to appear (16).

When sufficient Mn^{+2} is available in cultures of growing cells, so that enzymes required for the assembly of bacitracin can be preformed, chloramphenicol added to postlogarithmic-phase cultures cannot interfere with synthesis of the peptide. The present finding is in agreement with previous reports (3, 6). Such enzymes might include racemases needed for synthesis of the four amino acids present in bacitracin in the D configuration. However, when Mn^{+2} is withheld from the cells, one or more enzymes are presumably absent, since no detectable bacitracin is synthesized. Addition of Mn^{+2} to maximal stationary-phase cells results in appearance of the peptide within 4 hr. When either chloramphenicol or its isomers are supplied within 2 hr after addition of the metallic ion, bacitracin is not produced; apparently, synthesis of either the racemases or the peptide assembly enzymes is suppressed. Predictably, the order of activity of the compounds is D(−)-threo > L(+)-erythro > D(−)-erythro.

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