If this one determination represented the average serum concentration of chloromycetin during the course of treatment one would expect the slight borderline therapeutic effect which was noted since this concentration of chloromycetin, while it does not completely inhibit the growth of H37Rv strain *in vitro,* will partially retard the multiplication of the bacilli.

Summary and Conclusions. The antibiotic chloromycetin has been found to be only moderately bacteriostatic for virulent human type tubercle bacilli *in vitro* as compared with streptomycin or para-aminosalicylic acid. The majority of 19 human type strains studied were completely inhibited in their growth by between 6.25 and 12.5 micrograms or more of the drug in the presence of serum. One bovine strain was equally sensitive. This degree of bacteriostatic activity was not markedly affected by the number of tubercle bacilli present nor was the bacteriostatic activity of para-aminosalicylic acid or streptomycin enhanced by the addition of chloromycetin.

When administered subcutaneously, chloromycetin has been shown to be ineffective, whereas, when admixed with the diet in concentrations of 0.5 and 0.25%, it was slightly effective for the suppression of experimental murine tuberculosis.

16330

Subtenolin. An Antibiotic from *Bacillus subtilis.* I. Bacteriologic Properties.

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(Introduced by Henry Tauber.)

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In the course of a search for antibiotic agents in this laboratory, an organism was isolated in February, 1945, that produced a substance showing antibiotic activity for certain gram-positive and gram-negative bacteria. The organism was isolated from nutrient agar plates that had been seeded with *Escherichia coli* and *Staphylococcus aureus,* respectively, and inoculated with material from dusty surfaces in the laboratory. It was identified as *Bacillus subtilis.** Because of its microbic source and strong enolic properties[1] the name, subtenolin, has been given to the antagonist. The bacteriologic properties of the antibiotic are the subject of this report; its isolation from the harvest and its chemical properties are described in the succeeding report.[1]

Production of Subtenolin. Mediums containing peptones and other complex organic enrichments were found to be very poor for the production of subtenolin. A medium containing *dl*-alanine produced the highest yields and least destruction. With subtenolin, as with subtilin,[2] strong stimulatory action by manganese was observed. Production was slightly stimulated by copper.

The following medium was adopted for use in the production of subtenolin:

* Acknowledgment is made to N. R. Smith of the U. S. Department of Agriculture, Beltsville, Maryland, for the final identification of the organism.

1 Howell, S. F., and Tauber, H., PROC. SOC. EXP. BIOL. AND MED., 1948, **67**, 432.

2 Jansen, E. F., and Hirschmann, D. J., *Arch. Biochem.*, 1944, **4**, 297.

	%
dl-alanine	0.3
Potassium dihydrogen phosphate	0.5
Magnesium citrate • $14H_2O$	0.25
Magnesium sulfate • $7H_2O$	0.05
Glycerol	2.0
Manganous sulfate • $5H_2O$	0.0004
Cuprie sulfate • $5H_2O$	0.00005
Water, distilled, to volume	

The medium was adjusted to pH 6.8-7.1, dispensed 300 ml per one-gallon bottle, and autoclaved at 15 lb for 15 minutes. The bottles were inoculated with 0.5 ml of a standardized spore suspension (tube No. 0.5 McFarland barium sulfate standard) that had been prepared from discrete colonies. To obtain maximum surface exposure, the bottles were incubated in a horizontal position at 36°C for 3 days. Subtenolin activity reached its peak when growth was greatest (3 to 4 days) and remained constant until one or 2 days later when antibiotic activity steadily decreased with increased alkalinity of the culture medium.

Lyophilization of the spores in 25% gelatin proved to be the best method for maintaining the strain of *Bacillus subtilis* for consistent maximum production of subtenolin. By preparing a new batch of lyophilized spores at least every 3 months, the level of subtenolin production was held fairly constant throughout the period of this work.

In practice, the inoculum was prepared by reconstituting the contents of an ampoule of lyophilized spores and preparing tenfold dilutions in saline for plating on FDA agar.[3] After incubation for 4 to 7 days at 30°C, individual rough colonies were selected and suspended in 0.85% NaCl solution. This suspension was standardized and inoculated in the medium described above. Harvesting of the culture was done by screening through several layers of gauze and cotton to remove most of the bacterial pellicle.

General Properties of Subtenolin. Subtenolin diffuses very readily through agar, producing large and clearly defined zones with *Staphylococcus aureus* as the test organism. Many preparations showed the presence of isolated, resistant colonies within the clear area of inhibition.

The antibacterial activity of subtenolin is variously reduced by complex organic substances. In whole, defibrinated rabbit blood there is almost complete loss of activity in 2 hours. Several peptones also reduced the antibacterial effect of subtenolin. However, rabbit or horse serum, FDA broth,[3] and heart infusion broth had no effect when tested by the agar streak method.

Toxicity studies by intraperitoneal injection of mice weighing 17 to 23 g showed the LD_{50} to be 30 to 60 mg (1000 units mg). Considerable subtenolin activity was found in the urine 15 minutes after injection and, depending upon the dosage, continued 4 to 10 hours. With a dose of 50 to 60 mg (1000 units mg), subtenolin continued to be excreted for 10 hours after intraperitoneal injection. On the average, 30 to 50% of the administered dose was found in the urine.

Assay Methods and Antibacterial Spectrum. The serial dilution method using FDA broth[3] was the procedure adopted for subtenolin assay. Since there was reduction of subtenolin activity after overnight incubation but none after 5 hours, the tests were read at 5 hours and again at 22 hours. The endpoint selected was the highest dilution of a standard subtenolin solution showing complete inhibition of the test organism. For the more fastidious organisms, heart infusion broth containing 10% rabbit serum and 1% dextrose was used. This medium reduced subtenolin activity 50 to 80% after overnight incubation although no reduction in activity was observed after 5 hours incubation.

The antibacterial spectrum of subtenolin is summarized in Table I. The most susceptible organisms were: *Staphylococcus aureus, Staphylococcus albus, Eberthella typhosa, Escherichia coli, Salmonella enteritidis, Micrococcus conglomeratus,* and *Salmonella schottmuelleri.* Two strains of *Pasteurella* showed marked susceptibility to the antibiotic upon overnight incubation. The growth of *Mycobacterium tuberculosis,* H37Rv in Dubos medium[4] was partially inhibited by a concentration of 2000 units/ml of subtenolin.

Differentiation of Subtenolin from Other Antagonists. Antibiotic substances produced

[3] *Federal Register*, Oct. 17, 1946, **11**, 12128.

TABLE I.
The Antibacterial Spectrum of Subtenolin.

	Minimal inhibiting quantity (mg)	
	5 hr	22 hr
Preparation No. 165, 300 units/mg*		
1. *Staphylococcus aureus*, FDA 209P	.004	0.55
2. " " MB 111	.004	n.i.†
3. " " 30393	.004	n.i.
4. " " H	.0065	n.i.
5. " *albus*, 30389	.0023	0.47
6. *Streptococcus*, sp. (enterococcus group VD)	.023	1.25
7. *Bacillus anthracis*, ATCC 8705	2.0	n.i.
8. *Micrococcus conglomeratus*, MB 78	.024	n.i.
9. *Escherichia coli*, ATCC 7011	.014	1.67
10. *Salmonella enteritidis*	.015	n.i.
11. *Eberthella typhosa*, MB 59	.019	1.0
12. *Salmonella schottmuelleri*, M	.047	2.0
13. " *typhimurium*, ATCC 9148	.078	n.i.
14. *Serratia marcescens*, D	.16	n.i.
15. *Aerobacter aerogenes*, ATCC 8308	.16	n.i.
16. *Klebsiella pneumoniae*, ATCC 9997	.71	n.i.
17. *Pasteurella pestis*, A 1122	i.g.‡	0.055
Preparation No. 176, 550 units/mg*		
1. *Staphylococcus aureus*, FDA 209P	.0021	0.28
2. *Streptococcus pyogenes*, C203	.54	2.7
3. *Diplococcus pneumoniae*, type III	2.72	n.i.
4. *Clostridium novyi*, AMS	i.g.	0.55
5. " *perfringens*, AMS	i.g.	0.55
6. " *fallax*, AMS	i.g.	0.91
7. " *tertium*, AMS	i.g.	0.91
8. " *histolyticum*, AMS	i.g.	1.1
9. " *oedematis maligni*, AMS	i.g.	1.4
10. *Bacillus subtilis* (subtenolin producing strain)	n.i.	n.i.
11. " " R	n.i.	n.i.
12. *Micrococcus lysodeikticus*, MB 79	.17	n.i.
13. *Pasteurella*, sp., MB 90	i.g.	0.041
14. *Eberthella typhosa*, PCI 412	.0065	0.303
15. *Escherichia coli*, P6	.018	0.68
16. " " MB 60	.064	2.7
17. " " VD	.026	n.i.
18. *Pseudomonas aeruginosa*	2.7	n.i.
19. *Neisseria gonorrhoeae*, strain 1	i.g.	1.1
20. " " strain 2	i.g.	1.1
Preparation No. 189, 850 units/ mg*		
1. *Staphylococcus aureus*, FDA 209P	.0014	0.17
2. *Brucella abortus*, USDA 2610	i.g.	n.i.
3. " *melitensis*, USDA K1957	i.g.	n.i.
4. " *suis*, USDA, 8452	i.g.	n.i.
5. *Salmonella paratyphi*, ATCC 9150	n.i.	n.i

* One unit is defined as the minimal concentration of subtenolin completely inhibiting the growth of *Staphylococcus aureus*, FDA 209P in total volume of one ml after five hours incubation period. In the determination of the antibacterial spectrum, a reference solution was prepared by dissolving subtenolin in distilled water to give a concentration of 15,000 units/ml.

† n.i. = no inhibition.

‡ i.g. = insufficient growth.

by strains of *Bacillus subtilis* include subtilin,[2] bacitracin,[5] bacillin,[6] eumycin,[7] and subtilysin, a lytic principle obtained by Vallée[8] from culture filtrates. In addition, coli-

[4] Dubos, R. J., and Davis, B. D., *J. Exp. Med.*, 1946, **83**, 409.

[5] Johnson, B. A., Anker, H., and Meleney, F. L., *Science*, 1945, **102**, 376.

[6] Foster, J. W., and Woodruff, H. B., *J. Bact.*, 1946, **51**, 363.

[7] Johnson, E. A., and Burdon, K. L., *J. Bact.*, 1946, **51**, 591.

[8] Vallée, M., *C. R. Soc. biol.*, 1945, **139**, 148.

statin, apparently produced by an unidentified member of the genus *Bacillus*, has been reported.[9] Subtenolin differs from these antagonists of similar origin as described below.

Subtilin. Subtilin is extracted from the cellular material of the pellicle and is light-sensitive. Subtenolin is extracted from the culture medium and is not light-sensitive.

Bacitracin. Bacitracin does not inhibit *Escherichia coli*, *Aerobacter aerogenes* nor *Eberthella typhosa*, but these organisms are susceptible to the action of subtenolin. Unlike bacitracin, subtenolin is relatively inactive against *Streptococcus pyogenes*, pneumococcus and clostridia.

Bacillin. Subtenolin and bacillin display a loss of activity in the presence of certain complex organic substances (although not to the same degree), and their production is stimulated by manganese. On the other hand, carbohydrates promote the production of bacillin but interfere with the production of subtenolin. Also, carbohydrates are required for demonstrating the anticoli activity of bacillin but not of subtenolin. Bacillin is not excreted in the urine of mice† but subtenolin activity has been found in the urine of mice after intraperitoneal injections. *Streptococcus pyogenes* and *Diplococcus pneumoniae* Type III are relatively susceptible to bacillin but resistant to the action of subtenolin. In comparative studies with bacillin, subtenolin was found to be 5 times as active against *Staphylococcus aureus* 209 P on FDA agar.

Eumycin. Eumycin was reported to have no activity against *Eberthella typhosa* and colon bacilli and only slight activity against staphylococci. Subtenolin definitely inhibited these organisms, especially staphylococci.

Subtilysin. Subtilysin shows activity for various clostridia and none for staphylococci. Subtenolin shows the opposites of these activities.

Colistatin. The presence of glucose in the medium is required for colistatin production, but this sugar is not favorable for subtenolin production. Contrary to subtenolin, colistatin inhibits the pneumococcus.

Summary and Conclusions. 1. An antibiotic, tentatively designated subtenolin, was isolated from a strain of *Bacillus subtilis*.

2. Although the antagonist displays its greatest activity against staphylococci, the growth of certain gram-positive as well as gram-negative organisms is inhibited.

3. The growth of *Mycobacterium* tuberculosis is partially inhibited by subtenolin.

4. Subtenolin activity has been detected in urines recovered from intraperitoneally inoculated mice.

5. Acute toxicity studies show that the LD_{50} of subtenolin for mice inoculated intraperitoneally is 30 to 60 mg (30,000-60,000 units).

[9] Gause, G. F., *Science*, 1946, **104**, 289.

† Personal communication from H. B. Woodruff of Merck and Company.

16331

Subtenolin. An Antibiotic from *Bacillus subtilis*. II. Isolation and Chemical Properties.

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The method of isolation and the chemical properties of subtenolin, an antibiotic produced by a strain of *Bacillus subtilis*, are described in this paper. The method used in the isolation of the antibiotic is based on its ability to be concentrated by adsorption on activated carbon, its solubility in methyl alcohol, its insolubility in butanone, and its