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.

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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

stability when dried from a methyl alcohol solution. Subtenolin has very interesting chemical properties. It is extremely sensitive to drying in the presence of water. However, in aqueous solution, it is very stable to heat. This antibiotic has strong enolic properties. The bacteriological study of the antibiotic is described in the preceding paper.[1]

Experimental. Isolation of the Antibiotic. Ten liter portions of the medium, the composition of which has been given in the preceding article,[1] were processed each time. The pH of the broth was usually between 6.7 and 6.8. The pellicles were removed by screening through several layers of cheesecloth, then by centrifuging in a Sharples supercentrifuge. Forty grams of charcoal (Darco G-60) were added per liter of culture fluid. The mixture was shaken during a period of 20 minutes and filtered on Buchner funnels. The carbon cake was washed with 2 liters of water and transferred to a 5-liter bottle. The antibiotic was eluted with 2 liters of methyl alcohol by shaking for 30 minutes. After filtration the eluate was concentrated *in vacuo* at 40°C to 30 ml.

The concentrated eluate was poured into 1500 ml of butanone (contained in a beaker) and stirred with a glass rod until solidification of the antibiotic took place. The precipitate was washed twice with 100 ml portions of butanone and twice with 100 ml portions of ether. The antibiotic was extracted from the precipitate with one 80 ml and two 30 ml portions of methyl alcohol. The mixture was filtered to remove undissolved material. The white residue on the filter paper was washed with two 30 ml portions of methyl alcohol. Upon solution in water this material showed insignificant activity. The combined methyl alcohol extracts were evaporated to 8 ml *in vacuo* at 45°C. A small amount of inactive material which separated at this point was removed by centrifuging and discarded. The methyl alcohol solution was evaporated to dryness *in vacuo* at 45°C. The dried preparation was stored *in vacuo* over $CaCl_2$.

The yield of subtenolin was about 1.0 g of light yellow powder per 10 liters of medium. Preparations with 1000 to 1600 dilution units per milligram, representing 25 to 50% recovery, were obtained. It is important to note that when solutions of the antibiotic containing more than traces of water are evaporated to dryness, the antibiotic is largely destroyed. Apparently most of the water present in the concentrated eluate was removed by the butanone treatment without inactivating the antibiotic.

Chemical Properties of Subtenolin. The antibiotic is very soluble in water, in ethylene glycol, in 95% methyl alcohol, and in many organic solvents containing a small amount of water. It is insoluble in acetone, butanone, ether, and 95% ethyl alcohol. It is not precipitated by acids or alkalis. Subtenolin is very heat stable. No loss of activity occurs when a solution in water is autoclaved for 15 minutes at 15 lb pressure. No destruction takes place at pH 2.0 during 18 hours at room temperature. The dried preparations may be kept for months in a vacuum without loss of potency. Subtenolin dialyzes readily through cellophane membranes and is somewhat hygroscopic in the dry state. The antibiotic in neutral solution gives a strong "peroxidase" test with p-phenylenediamine and hydrogen peroxide (purple color).[2] It gives a strong enol test,[2] a typical Molisch test, a blue color with ninhydrin, and a dark brown color with ferric chloride. It forms a crystalline hydrazone with 2,4-dinitro-phenylhydrazone in alcoholic solution. When a saturated solution of picric acid in ethyl alcohol is added to a concentrated aqueous solution of subtenolin, the solution turns brown and a crystalline substance is obtained in the form of rosettes. This crystalline material is inactive. Subtenolin reduces permanganate, iodine, and bromine in the cold, and Benedict's qualitative sugar reagent and ammoniacal silver nitrate solution on boiling. It reduces Shaffer-Somogyi's sugar reagent one-half as much as penicillin G and about one-sixth as much as glucose when two milligrams of each of the substances were used

[1] Hirschhorn, H. N., Bucca, M. A., and Thayer, J. D., PROC. SOC. EXP. BIOL. AND MED., 1948, **67**, 429.

[2] Feigl, F., *Qualitative Analysis by Spot Tests*, Nordemann Publishing Company, Inc., New York, 1939.

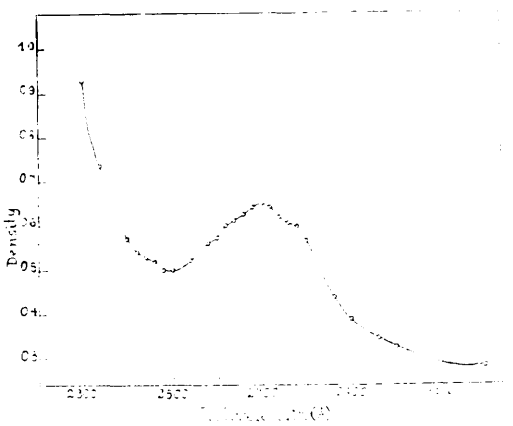

FIG. 1.
Adsorption spectrum of subtenolin in the ultraviolet region. One cc contained 0.4 mg of the antibiotic in distilled water. The pH was 6.52.

per test solution. The purified product contains 51.4% carbon, 7.00% hydrogen, 7.88% nitrogen, and 0.81% sulfur.

A solution of subtenolin in normal hydrochloric acid becomes rose colored upon boiling. Upon the addition of an excess of alkali the solution turns yellow but returns to rose color when reacidified. Subtenolin is destroyed by hydrogen sulfide but not by cysteine. Pepsin (Parke, Davis & Company, 1:3000), trypsin (Difco Laboratories, 1:250), polidase-S (Schwarz Laboratories), and tyrosinase (1830 units per ml)[3] have no effect on the antibiotic.

The antibiotic exhibits a striking absorption of light in the ultraviolet region of the spectrum (see Fig. 1). The curve has a high sharp peak at 2700 A and shows a minimum absorption at 2500 A. The Beckman spectrophotometer was used.

Discussion and Summary. It may be seen from Table I that subtenolin is quite different chemically from other antibiotics such as streptomycin, penicillin G, gramicidin, and bacitracin. Subtenolin, bacitracin, bacillin, subtilin, and eumycin are products of various strains of *B. subtilis*. Gramicidin[4] and bacitracin[5] appear to be peptides. Subtilin, which

TABLE I. Some Chemical Properties of Subtenolin and Other Antibiotics.

	Subtenolin, 1000 u/mg	Penicillin G Sodium (Com'l Solvents Corp. 1622 u/mg)	Streptomycin (Merck & Co. CaCl2 Complex Crystalline)	Gramicidin (Wallerstein Co.)	Bacitracin (Com'l Solvents Corp., 34 u/mg)
Molisch's Test	++	—	—	—	—
Benedict's Qualitative Sugar Test	++	+	—	—	—
Millon's Test	—	—	—	—	+
Hopkins-Cole Test	—	—	Light blue	Intense purple	Purple
Biuret Test	Yellow on boiling	—	" "	Intense violet	"
Ninhydrin Test	Blue	—	—	Blue	"
Trichloroacetic acid (10%)	—	—	—	Heavy precipitate	Turbid
Ferric Chloride	Dark brown	White precipitate	Yellow	—	Yellow
Enol Test	+	—	—	—	—
Indicator Test	Rose (turns yellow in alkaline sol.)	—	—	—	—
p-Phenylenediamine Test, In neutral sol.	Dark purple	Same as control	Same as control	Same as control	Same as control
In acid sol.	Yellow	" " "	" " "	" " "	" " "

In all tests 5 mg of the respective antibiotics were used except in the *p*-phenylenediamine test in which 0.5 mg of each of the antibiotics was used. The *p*-phenylenediamine test was carried out by adding 0.5 ml M phosphate buffer pH 7.0, 0.5 ml of 1% *p*-phenylenediamine in water, and 0.5 ml 3% hydrogen peroxide to 0.1 ml of the respective antibiotic solutions. When th is test was carried out in acid medium 2 M acetic acid was substituted for the phosphate solution.

[3] Miller, W. H., Mallette, M. F., Roth, L. J., and Dawson, C. R., *J. Am. Chem. Soc.*, 1944, **66**, 514.

[4] Hotchkiss, R. D., *Adv. Enzymol.*, 1944, **4**, 153.

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

is extracted from the pellicles of a particular strain of *B. subtilis,* is of unknown chemical composition.[6] Eumycin is prepared by acid precipitation of the medium.[7] The chemical relationship between bacillin and subtenolin has not been ascertained since data pertaining to the chemical nature of bacillin is not available for comparison.[8]

Subtenolin has a low molecular weight. Its chemical and physical properties indicate that it contains a resonating double bond, phenolic groups, a very active enolic group, and an aromatic aldehyde radical. This antibiotic gives typical color reactions such as the Molisch and enol reaction. These tests, and the indicator test shown in Table I, may be used in its identification. Although the antibiotic has not been obtained in a pure state, its chemical and antibiotic properties, particularly its stability in aqueous solution or when dry, make it an interesting object for further studies.

[6] Dimick, K. P., Alderton, G., Lewis, J. C., Lightbody, H. D., and Fevold, H. L., *Arch. Biochem.,* 1947, **15**, 1.

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

[8] Woodruff, H. B., and Foster, J. W., *J. Bact.,* 1946, **51**, 371.

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Respiratory Arrest in Rabbits Exposed to Hypoxia after Dibenamine.*

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The observations here reported were made as part of a systematic elucidation of the effect of alteration of function of the autonomic nervous system on the response of the intact animal to hypoxia.

Rabbits underwent early and sudden respiratory arrest on exposure to hypoxia after receiving Dibenamine (N, N-dibenzyl-β-chloroethylamine) in ethyl alcohol and propylene glycol aa by vein. Control animals underwent such arrest very rarely even under more prolonged similar exposure to hypoxia.

Procedure. Normal rabbits (weight 1.5 to 4 kg) under intravenous pentothal anesthesia were subjected to tracheotomy. Procaine hydrochloride 2% was infiltrated through the margins of the incision and skin closure around the cannula was made with skin clips.

The rabbits were restrained in the supine, head low position on a table offering a slope of 15° from the horizontal in order to aid the venous return. After recovery (minimum 30 min.) from the general anesthesia the inspiratory ventilation volume on room air was measured by spirometer. This was made possible by an hydraulic flutter valve‡ interposed between the tracheal cannula and the spirometer. The dead space of the system was 3 to 5 cc. This compares favorably with the dead space of the eliminated respiratory tract above the cannula in the rabbit.

Measurements were then continued as the animal was abruptly shifted to an atmosphere of 5% oxygen in helium for a period of 5 minutes. After one or a series of such control

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† Work done in partial fulfillment of the requirements for the degree of Master of Arts, Boston University Graduate School.

‡ A modification of the type pictured on page 206, Jackson, D. E., *Experimental Pharmacology and Materia Medica,* 2nd Edition, 1939. C. V. Mosby Co., St. Louis, Mo.