

out on the blood samples of 37 post-age 50 healthy control subjects (17 males and 20 females), 11 patients with advanced breast cancer and not on hormone therapy, and on 12 patients with advanced breast cancer receiving estrogen therapy. It was found that the control male subjects had both a higher serum glucuronidase and serum esterase than did the control female subjects. An inverse

relationship was found for the changes in sera activity levels of glucuronidase and esterase in the patients with breast cancer subjected to variations in estrogen therapy.

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### Synnematin, an Antibiotic Produced by *Tilachlidium*. (18473)

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(Introduced by C. W. Muehlberger)

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In a search for microorganisms capable of producing antibiotic substances, a mold belonging to the genus *Tilachlidium* Preuss which inhibits bacterial growth was isolated. In liquid medium the mold forms a substance which is more active against certain species of *Salmonella* than against *Escherichia coli* and which has low toxicity for mice. On the basis of the characteristic hyphal fascicles (Synnemata) of *Tilachlidium*, the name "synnematin" is proposed for the antibiotic.

**Materials and methods.** (a) *Method of assay.* One synnematin unit is defined as the minimum quantity/ml which completely inhibits the growth of *S. typhimurium* for 24 hours. Activity is assayed by the broth dilution test with *S. typhimurium* (Dr. P. R. Edwards, strain 9) as the test organism. Samples for assay are sterilized by Seitz filtration. F. D. A. broth\* at pH 7.0 is inoculated with 1% of an 18 to 24 hour culture and 2-fold dilutions of the antibiotic are made with 2.0 ml volumes of the seeded broth. (b) *Production.* The synnematin used in these studies was produced by growing *Tilachlidium* sp. strain MDH 3590A, on a medium composed of Bacto Casamino Acids, 10.0 g; glucose, 1.0 g;  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ , 0.25

g; and  $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ , 0.1 g/liter of tap water. The medium was adjusted to pH 6.7-6.9, dispensed in 2-quart milk bottles in 300 ml volumes and autoclaved at 121°C for 30 minutes. Each bottle was seeded with 6 ml of a 4-day-old culture in Czapek-Dox medium and incubated on the flat side at 24°C. Peak activity, 8 to 32 units, was obtained in 6 to 9 days. At this time the pH was 7.9 to 8.3. (c) *Purification.* The culture liquid was adjusted to pH 6 with  $\text{H}_3\text{PO}_4$ , mixed with 0.25% Nuchar C-190-N and filtered with the aid of 0.5% Standard Super-Cel. The antibiotic was then adsorbed from the filtrate on 1% Darco G-60. Synnematin was eluted from the Darco with 0.1 volume of 75% acetone, concentrated in vacuum at a temperature under 30°C and the residue was clarified with 2% Nuchar. The resulting filtrate was shell-frozen and dried. Crystalline synnematin has not been isolated; the most pure material thus far obtained contains 32 units/mg.

**Characteristics.** (a) *Stability.* Synnematin

TABLE I. Effect of pH on Activity of Synnematin.

| Broth, pH | Units/ml for inhibition |                    |
|-----------|-------------------------|--------------------|
|           | <i>S. typhimurium</i>   | <i>M. pyogenes</i> |
| 5.8       | 1                       | 2                  |
| 7.0       | 1                       | 4                  |
| 7.9       | 2                       | 8                  |

\* Unless otherwise stated, "broth" or F. D. A. broth refers to the nutrient broth portion of penicillin assay agar(1).

1. Federal Register, 1945, 10F.R., 11478.

TABLE II. Activity of Synnematin Against Bacteria and Molds.

| Organism                                              | Strain No. | Test medium                | Units/ml required for inhibition |
|-------------------------------------------------------|------------|----------------------------|----------------------------------|
| <i>Aerobacter aerogenes</i>                           | 527        | FDA broth                  | >64                              |
| " "                                                   | 600        | " "                        | >64                              |
| " "                                                   | 8308       | " "                        | 64                               |
| " "                                                   | 8329       | " "                        | >64                              |
| " "                                                   | 618        | " "                        | >64                              |
| <i>Bacillus subtilis</i>                              | 231        | " "                        | 8                                |
| <i>Brucella abortus</i>                               | 819a       | Tryptose broth             | 2                                |
| " <i>melitensis</i>                                   | 812        | " "                        | 1                                |
| " <i>suis</i>                                         | 811        | " "                        | 1                                |
| <i>Clostridium perfringens</i>                        | BP6K       | Mueller's seed medium      | 16                               |
| <i>Corynebacterium diphtheriae</i>                    | PWS        | Veal infusion broth        | 0.25                             |
| <i>Diplococcus pneumoniae</i> Type III                |            | Felton's medium            | 1                                |
| <i>Escherichia coli</i>                               | 8090       | FDA broth                  | >64                              |
| " " var. <i>communior</i>                             | 603        | " "                        | 32                               |
| " " var. "                                            | 621        | " "                        | 32                               |
| " " var. <i>communis</i>                              | 605        | " "                        | 64                               |
| <i>Hemophilus pertussis</i>                           |            | B-G medium                 | 16                               |
| <i>Klebsiella pneumoniae</i>                          |            | FDA broth                  | >32                              |
| <i>Micrococcus pyogenes</i> var. <i>aureus</i>        | SM         | " "                        | 1                                |
| " " var. "                                            | 209P       | " "                        | 2                                |
| " " var. "                                            | B314       | " "                        | 2                                |
| <i>Mycobacterium tuberculosis</i> var. <i>hominis</i> | H37        | Glycerol beef extract agar | >12                              |
| " <i>phlei</i>                                        | 1201       | " " " "                    | >12                              |
| <i>Proteus</i> sp.                                    | 1414       | FDA broth                  | 1                                |
| " <i>morganii</i>                                     | 548        | " "                        | >64                              |
| <i>Pseudomonas aeruginosa</i>                         | R-P-CC     | " "                        | >32                              |
| <i>Salmonella choleraesuis</i>                        | 550        | " "                        | 1                                |
| " <i>enteritidis</i>                                  | 575        | " "                        | 1                                |
| " <i>paratyphi</i>                                    | 577        | " "                        | 1                                |
| " <i>pullorum</i>                                     | 75         | " "                        | 0.5                              |
| " <i>schottmüller</i>                                 | 576        | " "                        | 1                                |
| " <i>typhimurium</i>                                  | 9          | " "                        | 1                                |
| " "                                                   | 569        | " "                        | 1                                |
| " "                                                   | SB682      | " "                        | 2                                |
| " "                                                   | SB683      | " "                        | 2                                |
| " <i>typhosa</i>                                      | 710        | " "                        | 0.5                              |
| " "                                                   | 17,001     | " "                        | 0.5                              |
| <i>Shigella alkalescens</i>                           | 801        | " "                        | 16                               |
| " "                                                   | 802        | " "                        | 4                                |
| " "                                                   | 1044       | " "                        | 8                                |
| " <i>dysenteriae</i> Flexner X                        | 3001       | " "                        | 32                               |
| " <i>Luxington</i>                                    |            | " "                        | 1                                |
| " <i>madampensis</i> Dispar I                         |            | " "                        | 64                               |
| " <i>paradyenteriae</i>                               | 1006V      | " "                        | 64                               |
| " "                                                   | 2003W      | " "                        | 64                               |
| " "                                                   | 8031       | " "                        | 64                               |
| " "                                                   | 9001       | " "                        | 64                               |
| " "                                                   | 10,001     | " "                        | 64                               |
| " "                                                   | 11,001     | " "                        | 64                               |
| " "                                                   | 12,001     | " "                        | 64                               |
| " "                                                   | 15,001     | " "                        | 4                                |
| " "                                                   | 15,401     | " "                        | 32                               |
| " "                                                   | 15,802     | " "                        | 8                                |
| " "                                                   | 15,601     | " "                        | 64                               |
| " <i>sabaulensis</i>                                  | 16,002     | " "                        | 16                               |
| " <i>schmitzii</i>                                    | 202        | " "                        | 4                                |
| " <i>shiga</i> Shiga I                                |            | " "                        | 32                               |
| " <i>sonnei</i>                                       | 401        | " "                        | 64                               |
| <i>Streptococcus hemolyticus</i>                      | 98A        | Felton's                   | 1                                |
| " "                                                   | 286        | " "                        | 32                               |
| " "                                                   | 314B       | " "                        | 4                                |
| " "                                                   | 316A       | " "                        | 32                               |
| " "                                                   | 594        | " "                        | 2                                |
| " "                                                   | M1-1A      | " "                        | 2                                |

TABLE II (continued).

|                                                           |       |          |     |
|-----------------------------------------------------------|-------|----------|-----|
| <i>Streptococcus hemolyticus</i>                          | SF IV | Felton's | 1   |
| " "                                                       | K64C  | " "      | 4   |
| <i>Aspergillus flavus</i>                                 | 1035  | FDA agar | >12 |
| " <i>niger</i>                                            | 7416  | " "      | >12 |
| <i>Histoplasma capsulatum</i>                             |       | " "      | >12 |
| <i>Mucor</i> sp.                                          | 8445  | " "      | >12 |
| <i>Penicillium notatum</i>                                | 832   | " "      | >12 |
| <i>Streptomyces griseus</i>                               | 4     | " "      | >12 |
| <i>Trichophyton mentagrophytes</i> var.<br><i>gypseum</i> |       | " "      | >12 |

is labile to acid and to heat. At pH 2, the activity disappears after 20 minutes at 70°C or 3 hours at room temperature but remains unchanged at 5°C for 45 minutes. Heating at 117°C for 10 minutes reduces the activity at pH 5 or 7.5 and destroys it at pH 9. Dried synnematin is stable for 11 months but shows a decrease in potency after 17 months at 5°C. (b) *Extractability*. Synnematin is not extracted from culture filtrates by amyl acetate, n-butanol, chloroform or diethyl ether at pH 2, 4, 6 or 8. (c) *Solubility*. It is readily soluble in water, methanol, 75% acetone or 50% ethanol and insoluble in ethanol, acetone and diethyl ether.

*Effect of pH, addition of serum, and size of inoculum on activity.* The data in Table I show that the antibiotic is more active in a slightly acid medium than in an alkaline one. In broth containing 20% horse serum, approximately twice as much synnematin is required to inhibit *S. typhimurium* and *M. pyogenes* as is needed in plain broth. The size of inoculum has little effect on the concentrations necessary to prevent growth. One unit inhibited concentrations of *S. typhimurium* whether the number of organisms used for inoculum was 7,000 or 70,000,000/ml.

*Biological properties.* (a) *Activity of Synnematin against bacteria and molds.* The antimicrobial spectrum of synnematin against various bacteria and molds is given in Table II. All of the tests were read in 24 hours except those for *Corynebacterium diphtheriae* which was read after 3 days incubation; *M. tuberculosis*, 42 days; *M. phlei*, 7 days; and the molds, 8 to 14 days. The inoculum was 1% of a 24-hour culture of the organisms in all the tests except for *C. diphtheriae* in which a loopful of pellicle from a 3-day

culture was floated on the surface of the tubes of serially diluted synnematin and for *H. pertussis*, *M. tuberculosis*, *M. phlei* and the molds which were inoculated by streaking the surface of the agar slants containing dilutions of synnematin. (b) *Bactericidal activity of synnematin.* Table III shows that synnematin reduced the number of organisms but even 16 to 32 units does not sterilize the cultures. A series of tubes containing 2-fold dilutions of synnematin in broth was inoculated with an equal quantity of broth seeded with 1% of an 18 to 24 hour culture *M. pyogenes*. Another series of tubes was similarly inoculated with *S. typhimurium*. The number of organisms present before and after 24 hours incubation at 37°C was determined by plating on nutrient agar. In the test made with *M. pyogenes* there were more organisms present in the tube containing 16 or 32 units than in the tube containing only one unit of synnematin. Similar results were obtained in several other experiments conducted in the same manner. It is possible that this phenomenon is the same as the one described for

TABLE III. Reduction of No. of Organisms in Presence of Synnematin.

| Test organism         | Exp. No. | Units of antibiotic | No. of organisms before incubation, $\times 1000$ | No. of organisms after incubation. |
|-----------------------|----------|---------------------|---------------------------------------------------|------------------------------------|
| <i>M. pyogenes</i>    | 1        | 1                   | 540                                               | 90                                 |
|                       |          | 32                  | 540                                               | 260000                             |
|                       |          | 1                   | 500                                               | 300                                |
|                       |          | 16                  | 500                                               | 100000                             |
| <i>S. typhimurium</i> | 1        | 1                   | 1300                                              | 80                                 |
|                       |          | 32                  | 1300                                              | 270                                |
|                       | 2        | 1                   | 920                                               | 36                                 |
|                       |          | 32                  | 920                                               | 20                                 |

TABLE IV. Resistance to Synnematin Induced in Three Organisms by Daily Transfer in Broth Containing the Antibiotic.

| Transfer No. | Units of synnematin required for complete inhibition |                    |                               |
|--------------|------------------------------------------------------|--------------------|-------------------------------|
|              | <i>S. typhimurium</i>                                | <i>M. pyogenes</i> | <i>D. pneumoniae</i> Type III |
| 1            | 1                                                    | 4                  | 1                             |
| 2            | 4                                                    | 4                  | 2                             |
| 3            | 8                                                    | 8                  | 2                             |
| 4            | 8                                                    | 8                  | 2                             |
| 5            | 8                                                    | 8                  | 4                             |
| 6            | 8                                                    | 8                  | 4                             |
| 7            | 16                                                   | 16                 | 4                             |
| 8            | 16                                                   | 16                 | 4                             |
| 9            | 16                                                   | 16                 | 8                             |
| 10           | 32                                                   | 16                 | 8                             |
| 11           | 32                                                   | 32                 | 8                             |
| 12           | 32                                                   | 32                 | 8                             |

TABLE V. Effect of Synnematin on Mice Infected with *D. pneumoniae* Type III. (6 mice in each series).

| Dilution of culture | Dose of synnematin                | No. survived |
|---------------------|-----------------------------------|--------------|
| 1:10000000          | None, control                     | 4            |
| 1:1000000           | None, control                     | 1            |
| 1:10000             | 800 units in 1 dose               | 6            |
| 1:100               | 800 units in 1 dose               | 3            |
| 1:100               | 1600 units in 2 doses, 8 hr apart | 6            |

penicillin by Eagle(2). (c) *Induced resistance developed toward synnematin.* Serial 2-fold dilutions of synnematin containing 128 units/ml were made in broth seeded with *M. pyogenes* or *S. typhimurium* or, in Felton's broth seeded with *D. pneumoniae*. Twenty-four hours later a similar series of tubes was made in which the 1% inoculum was taken from the tube containing the highest concentrations of antibiotic permitting growth. These daily transfers were repeated 12 times. The results given in Table IV show that the resistance of *S. typhimurium* toward synnematin increased 32 fold, but in the case of *M. pyogenes* and *D. pneumoniae* the resistance increased only 8-fold. (d) *Toxicity.* When administered intraperitoneally, 100 mg of partially purified synnematin was tolerated by 20 g mice. Four hundred mg was not lethal when 100 mg of the material was injected in 2 equally divided doses daily over a period of 4 days. One hundred mg con-

tained 3200 synnematin units. Injection of larger doses awaits further purification of the antibiotic. (e) *In vivo activity.* Preliminary experiments were made to determine whether synnematin protected mice after injection with *D. pneumoniae* Type III and chick embryos after injection with *S. pullorum*.

Table V shows the effect of the treatment of mice with synnematin after intraabdominal injection of 0.1 ml of dilutions of a 24-hour culture of *D. pneumoniae* Type III. The single dose, or the first dose when 2 doses were given, was administered subcutaneously immediately after the injection of the organisms. The animals were observed for 14 days.

Table VI shows the effect of the treatment of chick embryos after infection with 0.1 ml of dilutions of a 24-hour culture of *S. pullorum*. Eleven-day-old chick embryos were used and all injections were made into the allantoic cavity. The synnematin was given immediately after the injection of organisms. After further incubation for 8 days the eggs were cultured in both nutrient and tetrathionate broths. Cultures were plated on SS agar and on bismuth sulfite agar.

*Discussion.* The form-genus *Tilachlidium* includes those imperfect fungi having *Cephalosporium*-like conidial heads and conidiophores aggregated into synnemata. Waksman and Horning(3) included the *Cephalosporium-Fusarium* group among the fungi known to be capable of producing antibiotics, and there may be a phylogenetic relationship between the strains reported here and other antibiotic-forming molds. In fact, broth cultures of *Cephalosporium charticola* Lindau have

TABLE VI. Effect of Synnematin on Chick Embryos Infected with *S. pullorum*. (6 embryos in each series).

| Dilution of culture | Dose of synnematin                | No. of uninfected embryos |
|---------------------|-----------------------------------|---------------------------|
| 1:1000000000        | None, control                     | 5                         |
| 1:100000000         | None, control                     | 1                         |
| 1:10000000          | None, control                     | 0                         |
| 1:100000            | 320 units in 2 doses, 24 hr apart | 6                         |

3. Waksman, S. A., and Horning, E. S., *Mycologia*, 1943, v35, 47.

been found to contain synnematin(4). All the tests were made with partially purified synnematin and further purification may establish the presence of more than one chemical entity. The material investigated has certain attributes of a clinically desirable antibiotic such as: (1) It is soluble in water. (2) It is stable and active near neutrality. (3) It is active against a large number of species of bacteria. (4) The size of inoculum has little effect on the concentrations necessary to prevent growth. (5) Organisms do not appear to develop resistance rapidly. (6) It has a low toxicity for mice.

**Summary.** (a) A mold, a species of *Til-*

4. Gottshall, R. Y., Roberts, J. M., Portwood, Lucile M., and Jennings, J. C., unpublished observations.

*achlidium*, was found to produce a water soluble antibiotic, synnematin, which was partially purified. (b) Crude preparations inhibit *in vitro* certain species of *Brucella*, *Corynebacterium*, *Micrococcus*, *Streptococcus*, all species of *Salmonella* tested and some species of *Shigella*. Synnematin is inactive against the tested strains of *Aerobacter*, *Escherichia*, *Mycobacterium*, filamentous molds and most species of *Shigella*. (c) *In vivo* activity is demonstrated by the ability of synnematin to protect mice against infection with *D. pneumoniae* and to protect egg embryos against infection with *S. pullorum*. (d) Its low toxicity and other properties are reported.

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## Influence of Storage on Viability of BCG Vaccine. (18474)

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The use of BCG vaccine has grown at a tremendous rate since the war despite the unsatisfactory nature of any prophylactic that consists of living and unstable microorganisms. The lack of a wholly acceptable preparation should not prevent such improvements as are possible within the formula originally established by Calmette(1) and, with this in mind, efforts to standardize BCG vaccine have been underway in the Division during the past five years. The present report of the uniformity of 204 consecutive weekly lots of vaccine and of their viability following storage is part of those studies.

**Materials and methods.** The methods have been described elsewhere(2). The last 144 lots of vaccine were processed from larger amounts of semidry BCG culture and were dispersed in a modified homogenizer making 60 r.p.m. In addition, the diluent has been

replaced by a glycerine-free solution consisting of one part Sauton and three parts of phosphate buffer solution, pH 7.2. It is recognized that the homogenization of semidry BCG culture is incomplete. Direct examination shows small clumps of bacilli mixed with the more uniformly dispersed ones. The number of living BCG aggregates in each weekly lot of vaccine was determined by serial tenfold dilutions each seeded on five tubes of Löwenstein's egg medium. The colony counts were made after four and eight weeks' incubation at 37.5°C. The effect of storage was determined by vigorously shaking by hand various samples of vaccine that had been stored for as long as three and one-half years at 2-4°C. The viability tests were made of serial tenfold dilutions in Dubos' medium and on Löwenstein's egg medium.

**Results.** For purposes of simplicity, the results of the colony counts are shown in Table I as the mean of twelve consecutive weekly tests. As may be seen from the table,

1. Calmette, A., *L'Infection bacillaire et la Tuberculose*. Masson et Cie, Paris, 1936, p. 912.

2. Birkhaug, K., *Am. Rev. Tuberc.*, 1949, v59, 567.