having a generally spherical form, median cell diameter was 15.6 μ, and the range was 5.2 to 23.4 μ.

Chromosomes were prepared for enumeration by procedures adopted from the methods of Tjio and Puck (*9*) and Hsu and Kellogg (*10*). Counts were made on 50 cells and the modal chromosome number was 59 with the range being 49 to 71 and 56 percent of the cells having 59 ± 2. The diploid number for the species is 60.

The line is highly susceptible to the virus of infectious pancreatic necrosis of trouts and supports a rise of virus titer of about $10^{8.5}$ per milliliter.

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Inhibitors of Deuterophoma tracheiphila in Citrus Varieties Resistant to "Mal Secco"

Abstract. Growth of colonies of the fungus *Deuterophoma tracheiphila*, the causal agent of the "mal secco" disease of lemons, is inhibited by two substances, C_1 and CS_2, which are found in resistant mandarin varieties but not in susceptible lemons and related species. Some properties of these substances are reported. CS_2 may be naringenin, but the stronger inhibitor, C_1, has not yet been identified.

The "mal secco" disease caused by *Deuterophoma tracheiphila* is the main limiting factor of lemon growing in many areas of the Mediterranean basin (*1*). The fungus may attack either through the roots or through the branches. Diseased trees die progressively by loss of leaves and subsequent drying of branches till their eventual death. Research for resistant rootstocks and lemon varieties is very active in countries where citrus trees are affected. Other citrus species such as mandarins, oranges, and grapefruit are generally not affected.

In the course of studies of physiological reactions of the lemon scion to fungal attack and on the reasons for the difference in susceptibility of different citrus species (*2*) natural inhibitors of fungal growth in resistant species have been detected. This is the first instance known to us of detection of such inhibitors of *D. tracheiphila*.

We first investigated the influence of water extracts of bark of different citrus species on the growth of fungus on carrot-agar dishes. The water extract was prepared by blending 50 g of bark, previously cut to small pieces, in 250 ml of distilled water. The extract was boiled for 30 min, brought to volume, and filtered through a Buchner funnel; the filtrate was further sterilized for 30 min at 15 lb pressure. The extract was mixed with agar; dishes were poured, inoculated, and incubated at 21°C for several days. Growth experiments are summarized in Table 1. Only measurements at 8 (or 9) and 12 days are reported.

Table 1 shows that there is a very clear inhibition of growth by Cleopatra extract, a lesser inhibition due to sour orange extract, and a slight initial promotion of growth by lemon extracts, especially by extract from the highly susceptible Eureka lemon. Cleopatra extract inhibited the normal spread of the colonies, causing an initial increase in height.

In order to separate and identify the factors responsible for growth inhibition, the water extracts (after a fivefold concentration) were chromatographed by descending procedure on Whatman paper No. 1 with butanol, acetic acid, and water (5 : 1 : 4) for 20 hours.

Table 1. Growth in diameter of fungal colonies of *Deuterophoma tracheiphila* as influenced by water extracts of bark. Growth is expressed in percentage of growth in water controls.

Fruit	Growth (%)					
	Expt. 1: 400 mg of bark extracted		Expt. 2: 1000 mg of bark extracted		Expt. 3: 1000 mg of bark extracted	
	9 days	12 days	8 days	12 days	8 days	12 days
Water control	100.0	100.0	100.0	100.0	100.0	100.0
Eureka lemon	107.6	108.2	112.0	100.0	101.4	90.7
Interdonato lemon					103.6	93.8
Monachello lemon					99.3	87.6
Sour orange			99.1	87.1	95.6	83.0
Cleopatra mandarin	58.5	64.1	61.2	54.1	54.3	46.1
Standard errors	0.5	4.5	1.9	1.5	1.7	1.1

Each spot consisted of the extract from 50 mg of bark.

The examination of chromatograms in ultraviolet light (320 mμ) showed a yellow fluorescent spot at R_F 0.87 in Cleopatra and sour orange extracts, and a yellow fluorescent spot at R_F 0.92 in Cleopatra extracts only. Both spots were absent from Eureka and other lemon extracts. Other differences between chromatograms were found to be irrelevant.

The chromatograms were marked under ultraviolet light so that the spots could be identified, and five main zones were eluted separately. Paper portions, corresponding to extract from 150 mg of bark spotted on the origin, were extracted with 5 ml of 80-percent ethyl alcohol. After 1 hour the alcohol was collected and the paper strips were washed with 2 ml of water. The combined elutions were evaporated on a water bath to a volume of 1 ml, added to carrot agar, and sterilized for 30 min. Four dishes were poured for each zone and extract, and inoculated with fungus. Only the elution from zone 1 (R_F 0.8 to 0.95) caused inhibition of growth. When measured 11 days after inoculation, growth over extract from Eureka lemon was 104 percent of control, but over extract from sour orange growth was only 87.5 percent of control, and over extract from Cleopatra mandarin growth was only 58.7 percent of control.

In another experiment, we tested the effect on growth of higher concentrations of the inhibitors from Cleopatra. Growth was increasingly inhibited at higher concentrations of the extract: it decreased from 82 percent of control with extract from 100 mg of bark to about 65 percent with extract from 500 mg and 59 percent with extract from 1000 mg. Colonies were measured 18 days after inoculation.

The area from R_F 0.80 to 0.95 was

further separated into two areas of inhibitions by two-dimensional chromatography; water-saturated ethyl acetate was used in one direction and isopropanol and water in the other. The first inhibitor, C_1, has an R_F of 0.92 in butanol–acetic acid–water and 0.95 in isopropanol–water, and does not react with $FeCl_3$, Folin-Denis phenolic reagent (*3*), or Diazo reagent (*4*). The second inhibitor, CS_2, found in Cleopatra and sour orange, has an R_F of 0.87 in butanol–acetic acid–water and 0.91 in isopropanol–water. The spot takes on a gray-orange tinge when sprayed with $FeCl_3$, gives a strong blue-grey reaction when treated with Folin-Denis reagent, and becomes light red and then a deeper red when treated with Diazo reagent followed by ammonia vapor. These reactions suggest that CS_2 might be naringenin.

From chromatograms of 300 mg of Cleopatra bark (solvent, isopropanol and water), C_1 and CS_2 were eluted with 80-percent ethyl alcohol, evaporated to 1 ml, and transferred to agar. Colonies of *D. tracheiphila* were measured after 14 days. Growth was inhibited most by C_1 extract (70.2 percent of control, $p<0.05$). The CS_2 did not inhibit growth significantly (89.5 percent of control).

Mandarin varieties (*Citrus reticulata* Bl.) that are resistant to "mal secco" (*1*, *2*) such as Cleopatra, Dancy, and Clementine, contain both C_1 and CS_2, inhibitors of *D. tracheiphila*. The Marsh seedless grapefruit, also resistant, contains only CS_2. Shamouti sweet orange, although resistant, contains neither inhibitor. In striking contrast, susceptible citrus trees such as sour orange, Eureka, Interdonato and Monachello lemons, and the rough lemon and sweet lime do not contain the effective C_1 inhibitor. Sour orange contains the weak CS_2, but it does not prevent the tree from being susceptible (*5*).

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Synergism between Streptomycin and Penicillin: A Proposed Mechanism

Abstract. Brief treatment of growing *Escherichia coli* with penicillin hastened subsequent killing of these cells by streptomycin. It also hastened the secondary uptake of streptomycin, which represents an increase in the number of freely accessible binding sites. In contrast, brief treatment with streptomycin failed to affect subsequent killing by penicillin. These findings suggest that the synergism of penicillin with streptomycin depends on the damaging effect of penicillin on the cell membrane, which promotes further damage by streptomycin and increases its subsequent access to intracellular sites. Observations on a streptomycin-resistant mutant are also reported.

While synergism between streptomycin and penicillin is well established in vitro (*1*) and in some clinical infections (*2*), the mechanism is unknown. It is difficult to visualize a reasonable explanation in terms of inhibition of intracellular enzymes. A possibility is suggested, however, by findings that have implicated the cell membrane in the action of both drugs.

With penicillin, which interferes with synthesis of the cell wall (*3*), gross lysis of growing bacteria is preceded by membrane damage. The evidence includes reduced ability to concentrate some amino acids (*4*), outward leakage of compounds absorbing light at 260 mμ (*5*), and inward leakage of β-galactosides (*5*). With streptomycin, recent work in this laboratory has revealed similar evidence of membrane damage (without lysis) in growing bacteria (*6*, *7*). Furthermore, streptomycin exhibits a biphasic uptake and a delayed lethal action (*8*), suggesting a two-stage mechanism in which membrane damage precedes binding of the drug to intracellular sites. It seemed possible, then, that synergism would result if penicillin contributed to the membrane damage required for the lethal action of streptomycin. Such damage should lead to synergism not only on simultaneous exposure of cells to the two drugs, as in the usual test, but also in sequential exposure, with penicillin first.

We tested this hypothesis with *Escherichia coli* strain ML-35 (kindly furnished by Dr. J. Monod), which is cryptic and constitutive for β-galactosidase. Cells were briefly exposed to penicillin in an osmotically nonprotective medium (*9*), after which they still appeared normal under a light microscope and, on transfer to drug-free medium, resumed exponential growth with little or no lag. Membrane damage in still viable

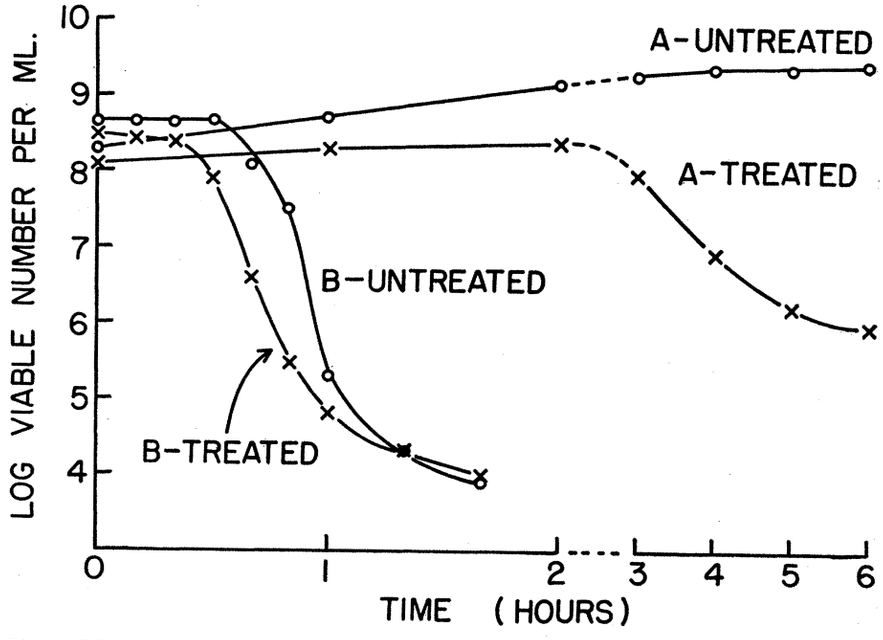

Fig. 1. Effect of penicillin treatment on subsequent killing by streptomycin. *E. coli* ML-35 was grown in minimal medium A (*15*), containing 0.5-percent glucose, on a Brunswick rotary shaker at 37°C. Penicillin G (60 units/ml) was added to a portion of the exponentially growing culture when it reached about 0.15 mg (dry weight) per milliliter. After 10 minutes (expt. *A*) or 15 minutes (expt. *B*), treated and untreated cells were recovered by centrifugation in the cold, were resuspended in fresh medium containing streptomycin (expt. *A*, 10 μg/ml; expt. *B*, 40 μg/ml), and were reincubated. For viability counts, samples taken at appropriate intervals were diluted with medium without glucose and rapidly poured in plates of tryptic digest 1-percent agar.