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

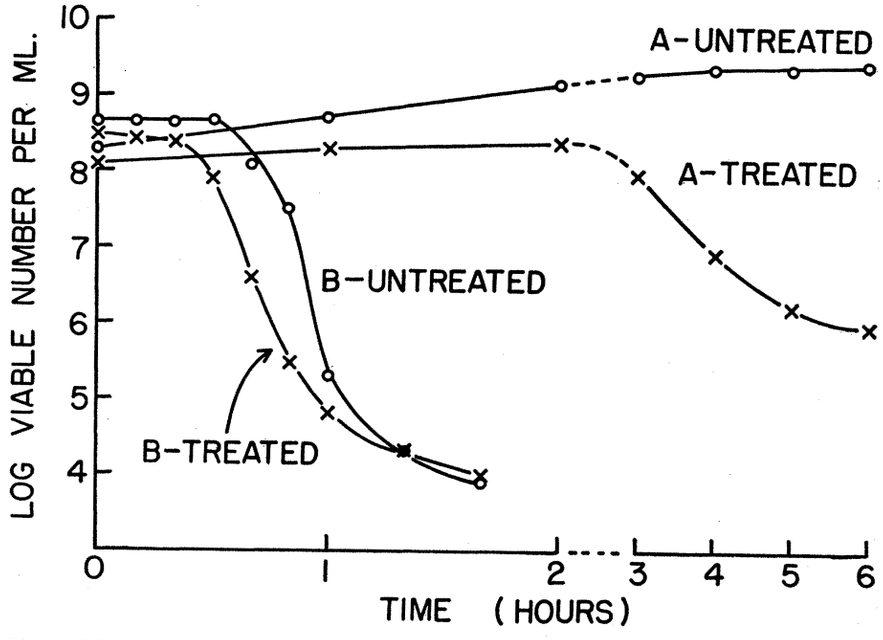

Fig. 1. Effect of penicillin treatment on subsequent killing by streptomycin. *E. coli* ML-35 was grown in minimal medium A (*15*), containing 0.5-percent glucose, on a Brunswick rotary shaker at 37°C. Penicillin G (60 units/ml) was added to a portion of the exponentially growing culture when it reached 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.

cells, however, could be readily demonstrated: the penicillin-treated cells showed increased ability (in fresh medium) to use lactose as a sole carbon source for growth and division, indicating a decrease in crypticity (*10*).

As Fig. 1 shows, this treatment with penicillin hastened the onset of killing by streptomycin (40 μg/ml) in fresh medium. In addition, it lowered the threshold concentration of streptomycin: 10 μg/ml was sufficient to kill penicillin-treated cells, whereas this low concentration did not affect untreated cells during several generations of growth. Chloramphenicol, which protects normal cells from the lethal action of streptomycin (*6*), also protected penicillin-damaged cells.

In the reverse sequence, brief treatment with streptomycin failed to hasten the subsequent killing by penicillin. Indeed, longer treatment, into the phase of exponential decline of viability, even slowed the killing of survivors by penicillin.

Even after penicillin-treated cells were allowed to grow for one generation without the drug, they were killed faster than untreated cells on exposure to streptomycin. It therefore appears that the damage caused by penicillin is not repaired immediately but persists through further growth and division.

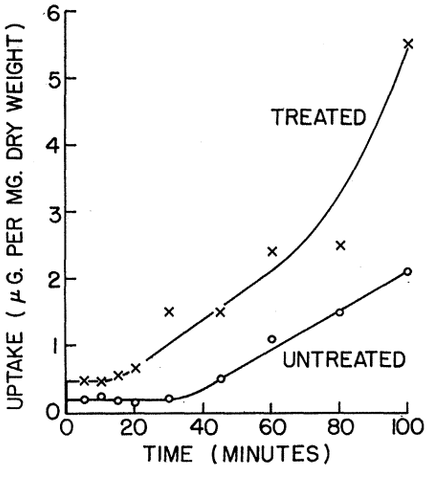

Fig. 2. Effect of penicillin treatment on uptake of streptomycin-C^{14}. The results are from expt. *B* of Fig. 1. Streptomycin-C^{14} (40 μg/ml; 240 counts per minute per microgram) was added. At appropriate intervals 1-ml samples were pipetted onto a membrane filter (Millipore HA), washed twice with 5 ml of water (*12*), and dried. Radioactivity was determined on a thin-window gas-flow counter (Nuclear-Chicago Corp.). The 5- to 20-minute values are based on duplicate samples. All samples had at least 35 counts per minute. The data are corrected for the blank values (about 20 counts per minute) obtained by filtering medium containing streptomycin-C^{14} but no cells. The primary uptake with *E. coli* strain ML-35 is considerably lower, and the secondary uptake is somewhat later, than the values previously found (*12*) with strain W.

To relate the synergism to possible changes in access to binding sites, we observed the effects of penicillin treatment on the uptake of streptomycin-C^{14} (*11*). As noted above, the kinetics of streptomycin uptake follow a biphasic curve: an immediate uptake occurs with both sensitive and resistant cells; and after a lag it is followed, with sensitive cells only, by a gradually increasing secondary uptake (*8*). As Fig. 2 shows, penicillin treatment hastened the onset of the secondary uptake of streptomycin, which represents (*8, 12*) an increase in the number of freely accessible binding sites.

Figure 2 shows that the level of the primary uptake also was increased by penicillin treatment. This effect, however, may involve sites irrelevant to streptomycin action since it occurred to an equal extent with resistant cells, which exhibited (see below) no response to streptomycin.

Our results suggested that penicillin treatment might also increase the sensitivity of streptomycin-resistant cells, if the resistance were associated with a change in the membrane rather than in an intracellular site. Indeed, Lederberg and St. Clair (*13*) reported that with protoplasts of streptomycin-resistant *Escherichia coli* K-12, prepared by penicillin treatment or by diaminopimelic-acid starvation in hypertonic medium, growth on solid medium was inhibited by low levels of streptomycin. They considered this response to be due to "negation of the S^r effect when the wall is stripped." We found, however, that a streptomycin-resistant mutant of *E. coli* ML-35, treated with penicillin as described above, immediately resumed exponential growth in fresh medium A whether or not streptomycin (1000 μg/ml) was present. Furthermore, the presence of streptomycin (1000 μg/ml) did not accelerate the killing during longer exposure to 60 units of penicillin per milliliter. The site of streptomycin resistance thus appears uncertain, and the effect of streptomycin on the growth of resistant protoplasts requires further investigation.

Our findings provide evidence that the damaging effect of penicillin on the growing membrane is responsible for the synergism of this drug with streptomycin. While these findings suggest that membrane damage by either drug may be only a precursor to lethal action of streptomycin at an intracellular site, the possibility has not been excluded that the membrane is itself the site of streptomycin's lethal action (*14*).

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9. During this exposure there was no change in viable number in the pencillin-treated culture, while the untreated culture continued to grow exponentially.
10. Membrane damage was also shown by a decrease in crypticity to *o*-nitrophenyl-β-D-galactopyranoside. This index, however, is not suitable for present purposes since most of the damage could be shown to reside in cells that had been rendered nonviable (to be published).
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Iodine-125 as a Protein Label in Immunology

Abstract. Iodine-125 has the relatively long half-life of 60 days, and emits 27.3- to 35.5-kev photon radiation, permitting counting in an ordinary well crystal scintillation counter with 30 to 40 percent efficiency. The emission of low-energy electrons having the same general energy range as tritium beta radiation electrons allows high-resolution autoradiography. These properties, together with the other advantages of radioactive iodine, make this nuclide a particularly satisfactory label for immunologically active proteins.

There is need for more adequate methods to study tissue and cellular localization and metabolic fate of antigens. Various radionuclides have been used for this purpose. Carbon-14, tritium, and sulfur-35 have the disad-