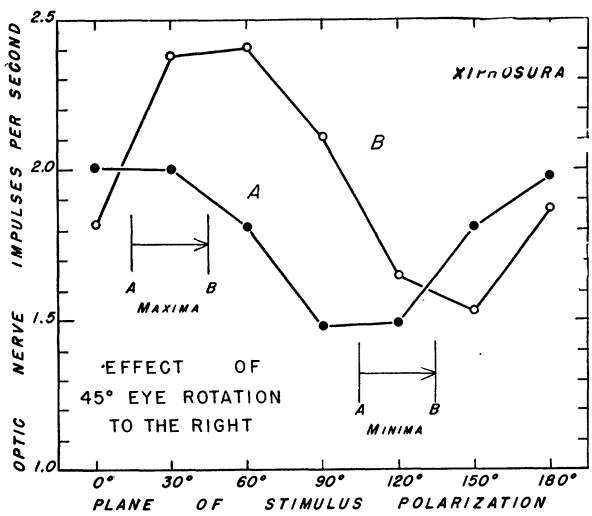

FIG. 4. Control data demonstrating that the polarization effect observed is a function of the biological system. After measurements were made as in previous experiments (A), the limulus eye itself was rotated clockwise through 45° and a second series of measurements taken. Corresponding displacements of both maxima and minima of the response curve occurred.

First, a quantitative study should be made to determine the extent of this effect through the full ranges of intensity and wavelength to which this visual system is sensitive. Then an analytical search should be instituted to discover the structures constituting the polarization analyzer involved. Some clues for such research should be sought in the studies of polarization optics of biological materials in general (*9*) and of eyes in particular (*2*). The dioptric properties of the limulus eye and possible birefringence of various of its elements, including the retinula cells, clearly should be investigated in this connection. Third, the behavior of *Xiphosura* should be examined to see whether polarized light has any normal functional significance for this arthropod. Finally the possible relationship of the present observations to von Frisch's work on bees, mentioned in the introduction, should be worked out. Particularly pertinent here would be the determination of the over-all pattern of sensitivity and orientation of the individual polarization analyzers in the whole retina. In the solution of the various structural and functional problems involved here, one may expect to find answers to certain of the specific questions asked, but one may also confidently expect in the process to learn much that will contribute toward our broad understanding of the compound eye.

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The Combined Action of Penicillin with Streptomycin or Chloromycetin on Enterococci *in Vitro*[1]

E. Jawetz, J. B. Gunnison, and V. R. Coleman

Division of Bacteriology,

University of California Medical School, San Francisco

Synergistic effects have been demonstrated with a number of chemotherapeutic agents (*1, 2, 6–10*). A synergistic effect of penicillin-streptomycin mixtures *in vitro* against certain staphylococci and a hemolytic streptococcus has been reported by Nichols (*8*). The combined action was greater than a simple additive effect of the drugs but the synergism was of low order. Clinical reports (*4, 11, 13*) indicate that combined therapy with penicillin and streptomycin is often successful in curing bacterial endocarditis due to enterococci, which ordinarily fails to respond to either drug alone, even when administered in high dose over a long period. With enterococci it has been shown (*5*) that mixtures of penicillin and streptomycin *in vitro* rapidly brought about death of the entire bacterial population, whereas streptomycin alone had no effect, and penicillin alone had mainly bacteriostatic properties. The experiments presented here may add to the understanding of this antibiotic synergism. In the course of our studies it was also noted that Chloromycetin[2] interfered with the action of penicillin on many strains of enterococci *in vitro*. This drug was therefore included in these experiments to compare streptomycin-penicillin synergism with apparent Chloromycetin-penicillin antagonism.

The bacteriological culture media used were Proteose-Peptone #3 agar (Difco) and a broth having the same base. Crystalline Sodium Penicillin G, Streptomycin sulfate, and Chloromycetin (Rx 117344) were dissolved in sterile saline. The Chloromycetin solution was sterilized by Seitz filtration. Final dilutions of the drugs were made in broth in a total volume of 20 ml. The bacterial inoculum consisted of 1 ml of an 18-hr broth culture containing 10^8-10^9 organisms/ml. All cultures were incubated at 37° C. At intervals aliquots were removed from the test mixtures, and the number of viable organisms determined by serial dilution and plate count. In other aliquots the penicillin was inactivated with penicillinase (Bacto-Penase), in order to permit penicillin-inhibited bacteria to grow. Absence of growth after the addition of penicillinase was interpreted as absence of

[1] Supported in part by a grant from the Research Committee of the University of California Medical School.

[2] Commercial brand of chloramphenicol. Generous supplies of this drug were kindly made available by Dr. G. Rieveschl, Parke Davis and Company, Detroit, Michigan.

viable bacteria. Nine strains of enterococci were studied. The organisms were isolated from the blood stream or urinary tract of patients and corresponded to the description given by Sherman (*12*). The strain[3] most extensively studied (#16), shown in Fig. 1, originated in a patient with subacute bacterial endocarditis subsequently cured by combined penicillin-streptomycin therapy.

The results observed with 9 strains of enterococci were fairly uniform and are represented by the experiment shown in Fig. 1. The numbers of viable enterococci in populations exposed to penicillin (6 μg/ml), streptomycin (25 μg/ml), Chloromycetin (10 μg/ml), or combinations of these drugs, are plotted against time of incubation.

The concentration of penicillin used (6 μg/ml) falls into the "optimal zone" (*3*, *5*) for this enterococcus

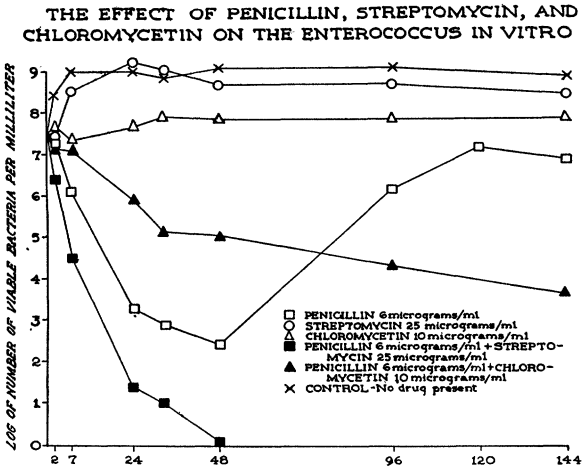

FIG. 1.

strain and results in a maximal bactericidal rate. Lower concentrations produce only slight bacteriostasis, whereas concentrations greater than 15 μg/ml have a much reduced rate of bactericidal action. It is seen in Fig. 1 that with 6 μg/ml of penicillin the count of viable bacteria rapidly decreased to low levels. However, the number of bacteria did not reach zero but after an additional 24 to 48 hr the population again increased and remained for at least 10 days at levels somewhat below those of the controls without drug. With higher concentrations of penicillin, (e.g., 300 μg/ml) the decrease in viable bacteria occurred more slowly and again not all bacteria were killed in 7 to 10 days.

Streptomycin in concentrations of 25 to 50 μg/ml completely failed to inhibit the large bacterial inocula used in these experiments. When these amounts of streptomycin were added to penicillin, two striking effects were noted: a) The rate of bactericidal action was greater than with penicillin alone. This increase in rate was observed with concentrations of penicillin from 6 to 300 μg/ml and concentrations of streptomycin of 25 to 100 μg/ml. b) The mixture of streptomycin and penicillin usually produced complete sterilization of the medium; within 3 to 5 days all enterococci were nonviable, failing to grow in subculture even after adequate amounts of penicillinase had been added.

The combined effect of streptomycin and penicillin on enterococci is evidently more than a summation of the individual drug effects. In the concentrations used, streptomycin alone had no demonstrable action whatever on these bacteria. The concentration of penicillin was in the "optimal zone" of the drug for the particular organism, i.e., the bactericidal rate was greater at 6 μg/ml than at 3, 15, 30, or 300 μg/ml. Thus in terms of bactericidal rate, penicillin action alone was already at an optimum which could not be increased by raising the concentrations of that drug. Hence, the increased effect of penicillin-streptomycin mixtures must be a true synergism of the two drugs. Likewise, in terms of the rapid sterilization of the medium by streptomycin-penicillin combinations, the effect is not merely a summation of individual drug actions. Streptomycin alone, in concentrations of 25 to 500 μg/ml, even after long incubation at 37° C, does not kill the entire bacterial population, penicillin alone in concentrations 5 to 50 times greater than that used here sometimes killed all exposed organisms, but only after incubation periods exceeding 5 to 7 days at 37° C. The rapid death of the entire enterococcal population exposed to streptomycin-penicillin mixtures indicates a considerable (at least tenfold) potentiation of penicillin action. This rapid sterilization might be a function of the greatly accelerated rate of bactericidal action.

The questions naturally arise whether the few enterococci remaining viable after exposure to 6 μg/ml of penicillin alone for 24 hr represent resistant mutants, and whether their subsequent multiplication at a normal rate is due to such resistance. If that were the case, the effect of streptomycin-penicillin mixtures might be explained on the basis of inhibition of penicillin-resistant forms by another antibiotic. To test this hypothesis, organisms were removed from penicillin action by adding penicillinase to the medium after most of the bacterial population had become nonviable. Sensitivity tests performed with such organisms indicated no increase in their penicillin resistance over that of the original population. Furthermore, there is to date no indication that these organisms are more susceptible to the action of streptomycin than the original population. Thus the main demonstrable effect of streptomycin-penicillin synergism on enterococci appears to be the increase in rate of bactericidal action beyond the optimum obtainable with penicillin alone.

Chloromycetin (10 μg/ml) alone had no significant effect on the bacterial population (Fig. 1). However, when mixed with 6 μg/ml of penicillin this concentration of Chloromycetin had a notable effect. The rate of bactericidal action was less than with penicillin alone. The low number of viable bacteria attained with penicillin alone in 24 hr was not reached with the Chloromycetin-penicillin mixture until the 6th to 12th day. Thus it appeared that Chloromycetin somehow interfered with the

[3] Dr. L. A. Rantz kindly supplied this organism.

early bactericidal effects of penicillin on enterococci, i.e., had some antagonistic action to that of penicillin. This lowering of the rate of bactericidal action occurred in combinations of Chloromycetin (10 μg/ml) with a wide range of concentrations of penicillin (6 to 60 μg/ml). This interference phenomenon was observed with all nine strains of enterococci but not always to the same degree.

Another observation should be noted: Whereas penicillin (6 μg/ml) alone permitted a great increase in the numbers of viable bacteria after the original sharp decline (Fig. 1), Chloromycetin-penicillin mixtures resulted in a slow but steady decrease of the population. Thus the late effects of this antibiotic mixture resembled those of high concentrations of penicillin (e.g., 300 μg/ml) alone, far outside the optimal zone. In both instances the number of viable bacteria diminished more slowly than at the optimal concentration of penicillin alone (6 μg/ml) but after long periods of incubation occasionally all enterococci succumbed.

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Cytologic Demonstration of Nucleic Acids in Tissue Culture[1]

Robert J. Stein and Horace W. Gerarde[2]

Department of Obstetrics and Gynecology,
University Hospitals,
State University of Iowa, Iowa City

The tissue culture technique offers a means of investigating a great variety of biochemical and physiologic processes such as the nutritional requirements of the various cell types (*6, 15*), the action of drugs and other chemicals on growing tissue (*12*), the nature of various metabolic reactions and other biochemical changes in the medium (*7, 8*), and many other vital manifestations of growth phenomena (*9*).

The authors' interest in nucleic acid metabolism and the effect of nucleic acid inhibitors on growth and their relation to protein synthesis suggested the possibility of using tissue culture techniques in these investigations. It has been shown recently (*5*) that cells grown *in vitro* may be analyzed biochemically for desoxyribonucleic (DNA) and ribonucleic (RNA) acids, and that cytochemical methods are available for the demonstration of the presence of these nucleic acids in fixed sections of tissue culture. The following procedures have proved successful in demonstrating nucleic acids in cells grown *in vitro*.

The classical double cover slip technique of Maximow was used. Tissue (chick embryo muscle) was cut into small fragments (1 mm^3), embedded in a medium composed of equal parts of 50% chick embryo extract and rooster plasma,[3] incubated at 37° C for 72 hr, and fixed.

The following three fixatives were used: *Cowdry's*—equal parts saturated aqueous solution of mercuric chloride and 95% ethyl alcohol for 3 hr. *Carnoy's*—acetic acid-alcohol (1: 3) for 1 hr. *Serra's*—alcohol-formol-acetic acid (6: 3: 0.5) for 3 hr. Although Carnoy's and Serra's are not ideal fixatives, nevertheless they preserve nucleic acids well and permit excellent staining (*2*).

The cover slip preparations were washed thoroughly in running water after fixation in Cowdry's and Serra's fluids, and washed in descending strengths of alcohol after Carnoy's. They were rinsed in distilled water and dried slowly in air (*4*). This drying technique has proved very successful in eliminating the opacity produced in the plasma coagulum by fixation.

The basic dyes, methyl green and pyronin, have been shown to be specific for the two nucleic acids DNA and RNA (*1, 10*). By employing these stains in conjunction with specific enzyme digestion (desoxyribonuclease and ribonuclease), it is possible further to identify these acids. The Feulgen reaction was also used to demonstrate the presence of DNA.

Staining solutions used were:

Methyl green[4] (Grübler or National Aniline)	0.15 g
Pyronin (Grübler or Eastman)	0.25 g
Ethyl alcohol, 95%	2.5 ml
Glycerine, C. P.	20.0 ml
Carbolic acid-water (0.5%)	77.5 ml

The method of staining used was as follows: The cover slip preparations were stained in dye solutions for 20–30 min. They were rinsed in distilled water, blotted with filter paper, differentiated and dehydrated in tertiary butyl alcohol for 1–3 hr, then cleared in xylol and mounted in clarite. By this method, chromatin is stained green, nucleoli and cytoplasm red.

In studying enzyme digestion of desoxyribonuclease,[5]

[1] Supported in part by a grant from the Iowa Division, American Cancer Society.

[2] Research Fellow in Medicine, American College of Physicians, 1949–1950.

[3] The lyophilized embryo extract and plasma were kindly furnished by Dr. C. W. Christenson of Difco Laboratories, Inc., Detroit, Michigan.

[4] This dye contains a small amount of a violet compound which stains nonspecifically and must be removed. To achieve this, the chloroform purification method described by Pollister (*11*) was used.

[5] The desoxyribonuclease and ribonuclease were obtained commercially from Worthington Biochemical Laboratory, Freehold, New Jersey.