adapted strains of virus, but after passage in TC, all the viruses showed some loss of pathogenicity for mice.

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Received November 21, 1960. P.S.E.B.M., 1961, v106.

## A Biochemical Comparison of 6-Aminopenicillanic Acid, Benzylpenicillin, and 2,6-Dimethoxyphenylpenicillin. (26295)

HARRY G. STEINMAN
*Laboratory of Clinical Investigation, National Institute of Allergy and Infectious Diseases, Bethesda, Md.*

With the advent of many new semi-synthetic penicillins as the result of the availability of 6-aminopenicillanic acid(1) as precursor material, it will be possible to examine in greater detail the problem of correlation of chemical structure of the penicillins with their biological and biochemical activities. In this paper one of the newer penicillin derivatives, 2,6-dimethoxyphenylpenicillin, which has shown promise in treatment of penicillin-resistant staphylococcal infections(2,3), benzylpenicillin, and 6-aminopenicillanic acid are compared as substrates for penicillinase, as growth-inhibitory agents, as inducers of penicillinase, and as selectors of penicillin-resistant mutants.

*Materials and methods.* 2,6-Dimethoxyphenylpenicillin (DMP)* was supplied by Bristol Laboratories, benzylpenicillin (penicillin G) by Squibb Research Inst., and 6-aminopenicillanic acid (6-APA) by Pfizer Research Laboratories. *Hydrolysis* of penicillin by penicillinase was measured manometrically in the Warburg apparatus through liberation of $CO_2$ from a $NaHCO_3$ buffer system by formation of penicilloic acid(4). The enzymes used were cell-free preparations of penicillinase obtained from *Bacillus cereus* and from *Staphylococcus aureus*, which were partially purified and concentrated by isoelectric precipitation(5). *Minimum* growth-inhibitory concentration of penicillin was determined by turbidimetric observations, over a 5-hour period, of the rate of growth of cultures in trypticase soy broth(6). The cultures were shaken in an air-bath (New Brunswick Gyrotory) at 37°C in 300 ml Erlenmeyer flasks equipped with side-attached test tubes for optical density measurements (Bellco Glass, Inc.). The test organisms were penicillinase-producing strains of *B. cereus* (ATCC 10876; NRRL B-569) and *S. aureus*(7). Induced formation of penicillinase by *B. cereus* and by *S. aureus* was carried out as described previously(7). *Selection* of strains resistant to penicillin was accomplished by serial passage of the bacteria through media containing increasingly higher concentrations of the antibiotic(8). Both penicillin-sensitive and penicillin-resis-

* 6-(2,6-dimethoxybenzamido)penicillanic acid (Staphcillin, Celbenin).

TABLE I. Relative Rates of Hydrolysis of Several Penicillins by 2 Different Penicillinases.

| Compound | Source of penicillinase | |
|---|---|---|
| | *B. cereus* | *S. aureus* |
| Penicillin G | 100 | 100 |
| DMP | 1.6 | .35 |
| 6-APA | .8 | .1 |

Rates of enzymatic hydrolysis of the various penicillins, at 0.01 M concentrations, were measured by rate of release of $CO_2$ from a $NaHCO_3/CO_2$ buffer system (pH 7.4) at 37°C in presence of $10^{-3}$M p-chloromercuribenzoate and compared relative to the rate for penicillin G. Ratios were calculated from the initial linear portions of the reaction curves. The penicillinase solutions were cell-free preparations, assaying 10 to 100 enzyme units (μmoles penicillin G hydrolyzed/hr) per ml. The extracellular enzyme obtained directly from *B. cereus* was concentrated by isoelectric precipitation. The intracellular enzyme from *S. aureus* was released into solution by means of the Nossal bacterial cell disintegrator and concentrated also by isoelectric precipitation.

6-APA = 6-aminopenicillanic acid; DMP = 2, 6-dimethoxyphenylpenicillin.

tant strains of *S. aureus* were employed.

*Results. Relative susceptibility of penicillins to destruction by penicillinase.* The intracellular penicillinase of *S. aureus* and the extracellular penicillinase of *B. cereus,* although different in some of their physicochemical properties(5), attack penicillin in the same manner. DMP was hydrolyzed by staphylococcal penicillinase at a very low rate compared to the rate for benzylpenicillin, the ratio being about 1 to 300 (Table I). The rate of hydrolysis of 6-APA was even less, the ratio to benzylpenicillin being about 1 to 1000. Batchelor *et al.*(1) had found 6-APA to be destroyed by the penicillinase from *B. cereus* similarly at a much slower rate than benzylpenicillin. On comparing the 2 enzymes, it was observed that rates of hydrolysis of DMP and of 6-APA by the penicillinase from *B. cereus,* although still quite low, were 4 to 8-fold greater, relative to penicillin G, than by the enzyme from *S. aureus.*

*Inhibition of growth of B. cereus and S. aureus by penicillins.* Inhibitory levels of the penicillins against *B. cereus* and *S. aureus* were determined by their effect on rate of growth of the organism. The minimal concentrations of the 3 compounds required for inhibition of *S. aureus* covered a wide range (Table II). For the large number of organisms used in the experiment, DMP was effective at a concentration less than 1 to 1000 that of penicillin G. Surprisingly, 6-APA was significantly more effective than penicillin G. Against *B. cereus,* however, DMP and benzyl-penicillin were about equally effective, whereas 6-APA was rather ineffective.

*Induction of penicillinase in B. cereus and S. aureus by penicillins.* Penicillinase-producing cultures of *S. aureus,* as well as *B. cereus,* are stimulated by the presence of penicillin G to elaborate penicillinase many times the basal level(7). In Fig. 1 is shown the rate of enzyme formation in *S. aureus* resulting from various inducing concentrations of penicillin G. It can be seen that the rate of production of enzyme was dependent on the initial concentration of penicillin. From Fig. 2 it is apparent that there was a similar pattern when the formation of penicillinase in *S. aureus* was induced by DMP.

The concentration required to obtain half the maximum rate of enzyme formation which can be achieved is considered as the "induction constant"(9). In Table III are summarized the "induction constants" for the 3 penicillins, with both *B. cereus* and *S. aureus* obtained from graphs in which relative amounts of enzyme formed were plotted against inducing concentrations of the penicillin (cf. 7). For *B. cereus,* benzylpenicillin

TABLE II. Growth-Inhibitory Concentrations of Several Penicillins for Two Organisms.

| Antibiotic | Organism | |
|---|---|---|
| | *B. cereus,* μg/ml | *S. aureus,* μg/ml |
| Penicillin G | 20 | 4,000 |
| DMP | 20 | 2 |
| 6-APA | 2,000 | 200 |

Bacteriostatic concentrations of the 3 penicillins were determined by turbidimetric measurements of rate of growth at 37°C of shaking cultures of *B. cereus* and *S. aureus.* The test systems containing a series of penicillin concentrations were seeded with 1-100 dilution of over-night culture to yield an initial concentration of about $10^7$ organisms/ml. Under these conditions growth was exponential. The generation time for *B. cereus* was about 30 min. and for *S. aureus* about 50 min. The bacteriostatic level is defined as the minimal concentration of antibiotic in which there was no significant increase in turbidity of the culture in 5 hr.

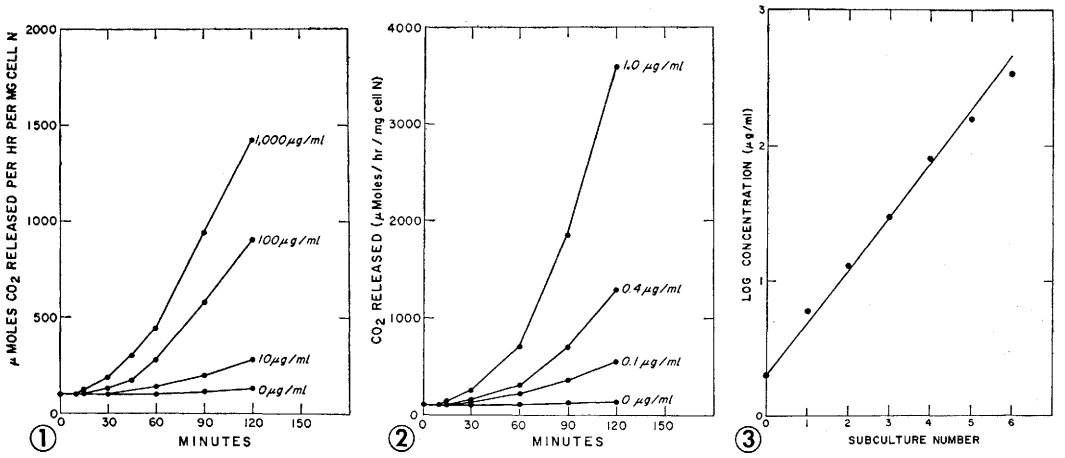


FIG. 1. Induced formation of penicillinase by *S. aureus* in response to various concentrations of benzylpenicillin. Test organisms were washed cells from an overnight culture in trypticase soy broth and were added at an initial concentration of 50 μg (dry wt)/ml. Induction was carried out in trypticase soy broth containing from 10 μg to 1,000 μg penicillin G/ml in an incubated shaker at 37°C. Samples were removed from a 20 ml reaction mixture at periodic intervals and the induction reaction was halted by inactivation of the organisms with $10^{-3}$M p-chloromercuribenzoate. The penicillinase content thus stabilized was determined manometrically. The resultant penicillinase activities, corrected for growth during the course of the reaction, were plotted against time of withdrawal of the sample.

FIG. 2. Formation of induced penicillinase by *S. aureus* in response to various concentrations of 2,6-dimethoxyphenylpenicillin. The procedure was that described in the legend of Fig. 1. Data are shown for inducing concentrations ranging from 0.1 μg to 1 μg/ml. Above 1 μg/ml the staphylocidal action of the drug inhibited the induction reaction.

FIG. 3. Development of resistance to 2,6-dimethoxyphenylpenicillin by *S. aureus*. An overnight culture of a penicillinase-producing strain of *S. aureus* was inoculated, at a 1-100 dilution, into fresh trypticase soy broth medium. After a 2 hr incubation period, in which the concentration of organisms increased to about $6 \times 10^7$ cells/ml, the staphylococci were inoculated, at a 1-10 dilution, into tubes of trypticase soy broth containing a series of concentrations of 2,6-dimethoxyphenylpenicillin ranging from 1 μg to 10 μg/ml. Cultures were incubated at 37°C for 24 hr without shaking. Subcultures, at a 1-100 dilution, were made of the tubes containing the highest concentrations of penicillin which showed growth into fresh medium without antibiotic. After a 2 hr growth period in the "starting" medium, the cells were transferred at a 1-10 dilution to yield a concentration equivalent to about a 1-300 dilution of original subculture, into tubes of broth medium now containing a slightly higher series of concentrations of the penicillin. As the organisms became more resistant to 2,6-dimethoxyphenylpenicillin, growth rate decreased. The figure shows the lowest concentration of the drug which prevented growth of each successive subculture.

and DMP were equally effective. For *S. aureus*, however, a hundred-fold higher concentration of benzylpenicillin was required for half-maximum induction than of 2,6-dimethoxyphenylpenicillin.

Table IV summarizes the results on the increase in amount of penicillinase obtained with optimal concentrations of each inducer after 120 minutes induction. It is evident that DMP was a very good inducer for both *B. cereus* and *S. aureus*. However, 6-APA and benzylpenicillin were less effective inducers for *S. aureus* than they were for *B. cereus*. Weak induction systems (penicillin G with *S. aureus;* 6-APA with both *B. cereus* and *S. aureus*) were characterized not only by lesser total amounts of enzyme produced but also by higher inducing concentrations required for induction (Table III).

TABLE III. Concentration of Inducer Producing One-Half the Maximum Amount of Induction.

| Inducer | Organism | |
|---|---|---|
| | *B. cereus*, μg/ml | *S. aureus*, μg/ml |
| Penicillin G | .05 | 40 |
| DMP | .05 | .5 |
| 6-APA | 20 | 2 |

Amounts of enzyme formed after 120 min. induction with various concentrations of inducer were plotted against concentrations. From each such plot was derived the concentration of penicillin inducer required to obtain one-half the maximum amount of enzyme.

TABLE IV. Ratio of Induced Penicillinase Activity to Basal Activity.

| Inducer | Organism | |
|---|---|---|
| | *B. cereus* | *S. aureus* |
| Penicillin G | 500 | 40 |
| DMP | 800 | 300 |
| 6-APA | 100 | 20 |

Total penicillinase activity (per mg cell N) resulting from 120 min. induction with optimal concentrations of each inducer was compared relative to basal activity (per mg cell N) in the absence of inducer. The test organisms were logarithmically growing broth cultures. The penicillins were added when optical density reached a standard value corresponding to $10^8$ cells/ml.

6-APA was a poor inducer of penicillinase in *B. cereus,* as measured by the "induction constant." However, because of its relatively low toxicity for *B. cereus* (Table II), it could be used at much higher concentrations than could the other penicillins. For *S. aureus,* 6-APA was intermediate in the concentration required to obtain half the maximum amount of enzyme, but induced at a very low rate (Table IV).

*Production of mutants of S. aureus resistant to the penicillins.* 6-APA, benzylpenicillin, and 2,6-dimethoxyphenylpenicillin were compared with respect to the ease with which resistant mutants could be selected by means of subculture in increasing concentrations of the antibiotics. Both a penicillinase- and a nonpenicillinase-producing strain of *S. aureus* were studied. Large inocula of organisms were used in each subculture since the mutation rate for penicillin resistance in *S. aureus* had been shown to be of the order $10^8$(10). Moderate increases in resistance to each of the penicillins was readily achieved with the non-penicillinase producing strain of *S. aureus.* Only a slight increase in resistance of the penicillinase-producing strain of *S. aureus* to 6-APA was obtained. Similarly, only a 3-fold increase in resistance to penicillin G was observed after several subcultures. However, large inocula of penicillinase-producing strain of *S. aureus* were already resistant to 10,000 μg penicillin G per ml, a rather massive dose. In contrast, a 170-fold increase in resistance to DMP (from 2 μg per ml to 350 μg per ml) resulted after 6 successive subcultures (Fig. 3). The initial subculture of the organism was fully induced with respect to penicillinase. Resistance does not appear, however, to be due to the increased amount of internal penicillinase since there was no further elevation in enzyme content as resistance increased in subsequent subcultures.

*Discussion.* Rolinson *et al.*(11), using whole cell preparations of *S. aureus,* have reported DMP to be stable to staphylococcal penicillinase. By employing concentrated cell-free preparations, DMP was found, in the present studies, to be hydrolyzed at measurable rates by *S. aureus* penicillinase as well as *B. cereus* penicillinase. However, DMP approaches Cephalosporin C(12) in its relative resistance to the destructive action of penicillinase.

The insensitivity of DMP to penicillinase presumably permits the intrinsic staphylocidal activity of the drug to exert itself effectively against both small and large inocula of penicillinase-producing staphylococci(13). This is in marked contrast to the failure of benzylpenicillin, at concentrations which are effective against small numbers of penicillinase-producing organisms, to inactivate larger numbers(14).

Induced formation of penicillinase in both *B. cereus* and *S. aureus* occurred with equally low concentrations of DMP. A much higher concentration of benzylpenicillin, however, was required to induce *S. aureus* than *B. cereus.* In this instance, also, the persistence of DMP against the destructive action of penicillinase, which occurs in higher concentrations in *S. aureus* than in *B. cereus,* may have contributed to its greater inducing activity.

Development of penicillin-resistant mutants of *S. aureus* appeared to proceed more readily with DMP as a selecting agent than with benzylpenicillin. Presumably the increased resistance of a penicillinase-producing strain of *S. aureus* to DMP is akin to the well-known increased resistance of nonpenicillinase-producing strains of *S. aureus* to penicillin G *in vitro,* which has yet to be adequately explained. However, the role of the penicillinase-insensitivity of DMP is dif-

ficult to assess in this situation until further study.

*Summary.* Benzylpenicillin, 2,6-dimethoxyphenylpenicillin, and 6-aminopenicillanic acid were compared with respect to several biochemical activities. 2,6-Dimethoxyphenylpenicillin and 6-aminopenicillanic acid were hydrolyzed at very low rates, compared to the rate for benzylpenicillin, by partially purified concentrated cell-free preparations of penicillinase derived from *B. cereus,* and at still lower rates by analogous preparations from *S. aureus.* For *S. aureus,* but not *B. cereus,* the growth-inhibitory concentrations of the two penicillinase-resistant compounds, particularly 2,6-dimethoxyphenylpenicillin, were much lower than that of benzylpenicillin. 2,6-Dimethoxyphenylpenicillin was a very effective inducer of penicillinase formation in *B. cereus* and in *S. aureus.* 6-Aminopenicillanic acid and benzylpenicillin were considerably less active as inducers of penicillinase in *S. aureus* than in *B. cereus,* as measured by effective inducing concentrations and by the amounts of enzyme formed. A mutant of a penicillinase-producing strain of *S. aureus* of increased resistance to 2,6-dimethoxyphenylpenicillin was obtained by successive subculture in increasing concentrations of the antibiotic. Its resistance did not appear to be due to the increased amount of its penicillinase content.

It is a pleasure to acknowledge the skillful assistance of Miss Emmy Lou Denny.

ADDENDUM: Examination of the manometric assay of the rate of hydrolysis of 6-APA has revealed that this method yields artifactually low results with this particular compound because the product of enzymatic hydrolysis combines with some of the $CO_2$ liberated from the $NaHCO_3$ buffer system. Iodometric assay of this reaction shows the real rates of hydrolysis to be several-fold higher than given in Table I.

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Received November 21, 1960. P.S.E.B.M., 1961, v106.

## Biological Activities of 16α, 17α Dihydroxyprogesterone Derivatives. (26296)

LEONARD J. LERNER, DAVID M. BRENNAN* AND ALECK BORMAN
(Introduced by O. Wintersteiner)
*Squibb Institute for Medical Research, New Brunswick, N. J.*

Since the demonstration by Inhoffen and Holweg that ethisterone (17α-ethinyl testosterone) is an orally active progestational agent(1), a wide variety of steroidal compounds possessing progestational activity have been reported(2-12). Fried *et al.* have recently reported on the synthesis and progestational activity of 16α, 17α ketal derivatives of 16α, 17α-dihydroxyprogesterone (DHP), including those with acetophenone (P-DHP) and acetone (A-DHP), as well as their 6-methyl, and 6-fluoro analogues(13,

* Present address: Eli Lilly and Co., Indianapolis, Ind.