COMPARATIVE STUDIES OF PENICILLINASE INDUCTION IN MICROORGANISMS BY NATURAL AND SEMI-SYNTHETIC PENICILLINS*

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The isolation of 6-aminopenicillanic acid from penicillin fermentations (Batchelor et. al., 1959) occasioned the synthesis of a large series of pencillins with different side chains. A major objective was to prepare new drugs active against penicillin-resistant organisms, since such organisms, as encountered clinically, often owe their resistance to their inducible penicillinase. Abraham and Chain (1940) showed clearly that a specific enzyme, penicillinase, was involved in the inactivation of penicillin. Harper (1943) was able to demonstrate that both Gram-positive and Gram-negative organisms produced the enzyme, penicillinase. The inactivation of penicillin is due to the hydrolysis of the β-lactam ring by penicillinase. If the β-lactam ring is the "functional group" (Cooper, 1956), then the rest of the molecule might not be necessary. But a reactive molecule such as penicillin would probably afford some means of specifically limiting its reactivity which may well be the role of the side chain. Spink and Ferris (1945), Bondi and Dietz (1948), and Pollock (1957a), have shown that the only clear-cut

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case of drug resistance being due to a specific enzyme is that of penicillinase. This relationship is clearly illustrated by Pollock (1950, 1951, 1953), on the induction of penicillinase formation in *Bacillus cereus* by treatment of cells with penicillin. The effect of penicillin is very rapid and even small doses of the antibiotic will stimulate cells to increase their rate of enzyme formation up to 800 times, with concomitant increase in their penicillin resistance. Geronimus and Cohen (1957) have shown that many strains of staphylococci respond in a similar manner. Other workers studied the comparative activities of various synthetic and semi-synthetic penicillins against penicillinase producing and non-penicillinase producing organisms. Wallmark (1954) and Wallmark and Finland (1961) examined the comparative activity of various penicillins against penicillinase producing and non-penicillinase producing staphylococci with particular reference to the effect of the size of the inoculum. Steinman (1961) made comparisons of the action of various penicillins on different strains of *B. cereus* and *Staphylococcus aureus*. The work reported in this paper is the study of the differences in levels of penicillinase produced by various bacilli in response to treatment with penicillins of widely different side chains.

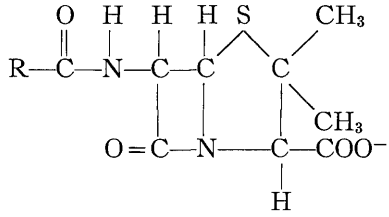
METHODS AND MATERIALS

Organisms

Strains of *B. cereus* N.R.R.L. 569, *B. cereus* NCTC 9945—569/H, and *B. cereus* NCTC 9946—5/B, were kindly sent by Dr. M. R. Pollock, Medical Research Council, London, U. K., and a strain of *B. subtilis*—ATCC 6633, were used in the studies.

Penicillins

Penicillins used in these investigations are described in table 1. The generalized structure of the penicillins can be given as



where R = side chain.

Medium

The medium used was prepared according to Pollock's (1957b) formula for casein hydrolyzate, designated CH/C.

Production of Cultures

An inoculum of the appropriate organism from a nutrient agar slant transferred by wire loop was used to inoculate 125 ml of the culture medium. The culture was incubated under stationary conditions at 37 C until the growth became visible, whereupon it was shaken in an Erlenmeyer flask on a Burrell, Size—T, wrist action shaker, at a setting of 2, aerobically at 37 C for about 16 hr. Efficient aeration was necessary for maximum enzyme formation.

INDUCTION

Continued Growth Method

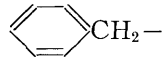
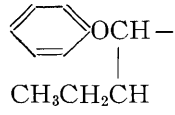
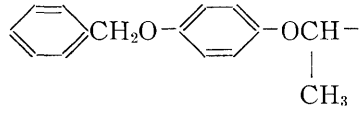
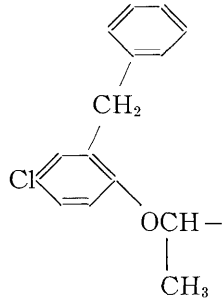
After 16 hr the cultures were treated with penicillin solutions having the concentrations of $\sim 10^{-7}$ M. The treated organisms were allowed to grow on a shaker at 37 C for 2½ hr. After 2½ hr the cultures were centrifuged in an International Refrigerated Centrifuge, Model PR–1 set at <0 C, and the supernatant fluid containing the exopenicillinase were stored in polyethylene bottles at 0 C.

Cold Pretreatment Method

This method is a variation of the procedure described by Pollock (1950). After 16-hr growth periods the cultures were centrifuged and suspended in equal volumes of CH/C, subdivided into equal portions, cooled at 0 C then treated either with penicillin or left untreated as a control. After 1 hr at 0 C the cells were centrifuged and resuspended in equal volumes of CH/C and allowed to grow at 37 C. After 2½ hr the cultures were centrifuged and the supernatant fluids containing the exopenicillinase were collected in polyethylene bottles.

TABLE 1

Chemical names, side chains, and designations of the penicillins used in this investigation.

Chemical Name	R—	Designation
Benzyl penicillin		G[1]
6-*N*-(α-phenoxy)pentanoyl-aminopenicillanic acid		PPA[2]
6-*N*-(α-(*p*-benzyloxyphenoxy)-propionylaminopenicillanic acid		BPP[2]
6-*N*-(*o*-benzyl-*p*-chlorophenoxy)-propionylaminopenicillanic acid		BCP[2]
6-Aminopenicillanic acid		6-APA[3]

[1]Purchased from Nutritional Biochemical Corporation.

[2]Kindly supplied by Bristol Laboratories. The names are given by the authors which correspond to Bristol Laboratories code Nos. BL-P 299 for PPA, BL-P 383 for BPP and BL-P 302 for BCP respectively.

[3]6-aminopenicillanic acid was kindly sent by Eli Lilly and Company.

Growth Measurements

Growth measurements were carried out after the treatment of organisms with different penicillins. Samples were taken at half-hour intervals and the optical densities were measured on a Bausch and Lomb, "Spectronic 20" colorimeter at 540 mμ. Growth measurements were carried out in similar fashion for the cold pretreatment method also.

Penicillinase Assay

Each supernatant fluid containing exopenicillinase was assayed by an iodometric method very similar to that described by C. J. Perret (1954), modified by D. A. Wolf (1960). The supernates in proper dilution (usually 100x) were warmed to 30 C and mixed with penicillin G sodium solution having a concentration of 8000 μg/ml also at 30 C, in a volume ratio of 1:6. After 1 hr, aliquots of

1 ml were withdrawn and mixed with 1 ml of 0.2 M KH_2PO_4 and 10 ml of 0.01 N KI_3. After exactly 5 min the excess iodine was titrated with 0.01 N $Na_2S_2O_3 \cdot 5H_2O$ using a drop of 2 per cent starch solution as indicator. A blank was run along with enzyme-substrate reaction.

Enzyme activities are expressed in units defined by Pollock (1957b) as "that amount which will hydrolyze one micromole of benzyl penicillin per hour at 30°C. and pH 7.0 under conditions where enzyme is saturated with substrate."

RESULTS AND DISCUSSION

The results presented in table 2 show significant differences in the effects produced by various penicillins used as inducers. These effects are seen consistently. The quantity of enzyme obtained by induction using different penicillins does vary depending upon the inducer penicillin. It can be seen from the data that penicillins BPP, BCP and PPA are better inducers than penicillin G and 6–APA. In *B. cereus* 569 and *B. subtilis*, 6–APA shows inducer activity equivalent to penicillin G, but in constitutive strains of *B. cereus* 569/H and 5/B, 6–APA behaves as a repressor. The molarity of penicillin solutions were not identical, but the variations do not significantly alter the effects observed nor vitiate the interpretation. The effects produced by larger differences (orders of magnitude) in pencillin concentrations will be discussed elsewhere.

TABLE 2

Comparative activity of penicillinase obtained by using different penicillin solutions of $\sim 10^{-7}$ M concentration as inducers, continued growth method.[1]

Penicillin	Concentration	*B. subtilis*	*B. cereus* 569	*B. cereus* 569/H	*B. cereus* 5/B
G	$2.8 \times 10^{-}$ M	7120	1740	3640	6850
PPA	2.4×10^{-7} M	7500	1950	4000	7710
BPP	2.0×10^{-7} M	9570	2470	4470	9070
BCP	1.9×10^{-7} M	8140	2380	4220	8220
6–APA	4.6×10^{-7} M	6850	1740	2090	6610
Basal	————	77	24	2770	6800

[1]The activity is expressed as units of enzyme activity per ml.

Along with the enzyme activity measurements, observations were also made of the growth of the organisms, since the repressive effect might be part of a general growth inhibition. The studies were made in the penicillin containing medium, i.e., cultures treated with $\sim 10^{7}$ M concentration solutions of penicillins under conditions of the "continued growth method." Growth was also observed in a medium from which penicillin is removed, i.e., under conditions of "cold pretreatment method." It was observed that with the former, the organisms grew steadily for about 2½ hr, but then the optical density declined, due in part to sporulation. Figure 1 shows the results for *B. cereus* 569, *B. cereus* 569/H, *B. cereus* 5/B and *B. subtilis*. It should be noted that under the conditions used the cultures are several hours from the end of the log phase at the time of the penicillin treatment. In figure 1 it can be seen that the organisms which are not treated with penicillins show the greatest growth.

The repressive activity of 6–APA led us to study the effect under the conditions of the "cold pretreatment method" in order to rule out the possibility of general growth inhibition. The data of table 3 show that the behavior of 6–APA was similar to that of the "continued growth method." It can also be seen that other penicillins show similar effects as inducers under the "cold pretreatment method."

Pollock (1950) has reported that certain strains of *B. cereus* irreversibly bind

penicillin G, and the "bound" penicillin is not removed by washing. After treating the organisms at 0 C for 1 hr, the penicillins were removed from the medium by centrifuging the culture, the temperature of the centrifuge chamber remaining at <0 C. The organisms resuspended in fresh medium were grown at 37 C. Table 4 shows that there is no inhibition in the growth and it is the same as the basal level after 5 hr. This contrasts with the results shown in figures 1–4 which shows that the penicillins act as inhibitors of the growing organisms under conditions of the "continued growth method."

TABLE 3

Activity of penicillinase produced by B. cereus *569*, B. cereus *569/H, and* B. cereus *5/B, by the treatment with different penicillin solutions having concentrations of* $\sim 10^{-7}$M *at 0 C, cold pretreatment method.*[1]

Penicillins	Concentration	*B. cereus* 569	*B. cereus* 569/H	*B. cereus* 5/B
G	2.8×10^{-7}M	4640	5120	7790
PPA	2.4×10^{-7}M	4780	5170	8200
BPP	2.0×10^{-7}M	5010	5210	8790
BCP	1.9×10^{-7}M	4860	5180	8670
6-APA	4.6×10^{-7}M	4750	4810	5560
Basal	————	45	5120	7050

[1]The activity is expressed as units of enzyme activity per ml.

TABLE 4

Measurement of growth of B. cereus *569*, B. cereus *569/H, and* B. cereus *5/B in a medium which does not contain penicillin—cold pretreatment method—at 37 C.*[1]

Penicillins	*B. cereus* 569			*B. cereus* 569/H			*B. Cereus* 5/B		
	0	2½	5 hr	0	2½	5 hr	0	2½	5 hr
G	0.80	0.97	1.70	0.32	0.45	0.62	0.60	0.70	0.95
PPA	0.80	0.99	1.70	0.32	0.47	0.61	0.60	0.70	0.95
BPP	0.80	0.99	1.70	0.32	0.47	0.62	0.60	0.70	0.95
BCP	0.80	0.99	1.70	0.32	0.48	0.62	0.60	0.70	0.95
6-APA	0.80	1.10	1.68	0.32	0.47	0.62	0.60	0.68	0.95
Basal	0.80	0.95	1.70	0.32	0.45	0.62	0.60	0.71	0.92

[1]The results are in terms of optical density measured on a Bausch and Lomb, "Spectronic 20", colorimeter, at 540 mμ.

From the information obtained it can be seen that penicillins do act as inducers. The five penicillins showed differences in their activities as inducers. This can be represented in decreasing order as:

$$\text{BPP} > \text{BCP} > \text{PPA} > \text{G} \sim \text{6-APA}$$

Apparently the larger the side chain and bulkier the group in penicillin molecules, the greater is the induction effect. Also, it can be concluded that inhibition in growth follows the same pattern.

Lastly, it is observed that the constitutive strains of *B. cereus* show increases in enzyme production upon penicillin treatment. The results reported here have shown marked differences in the enzyme production in these strains. Penicillin G

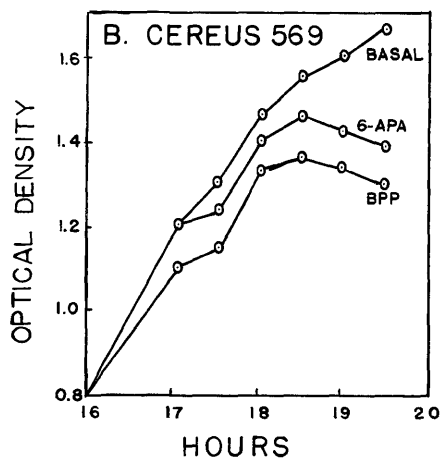


Figure 1

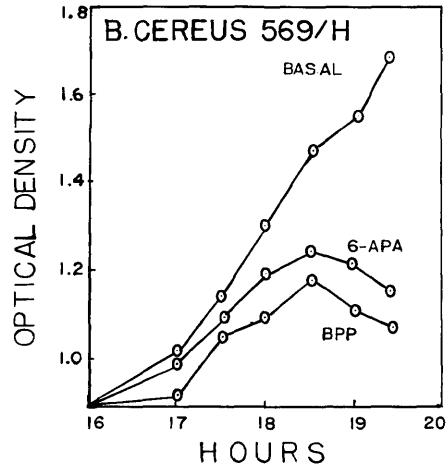


Figure 2

FIGURE 1. Inhibition of the growth of *B. cereus* 569 by the penicillins BPP and 6-APA at concentrations of *ca.* 10^{-7}M. The penicillins were added to the cultures after 16 hours of growth. The growth was measured in terms of optical density using a Bausch and Lomb "Spectronic 20" colorimeter at 540 mμ. The effects of penicillin G, PPA, and BCP were similar to BPP, the results falling between the values found with BPP and 6-APA, and hence are not represented in this figure.

FIGURE 2. Inhibition of growth of *B. cereus* 569/H. The other details are the same as for fig. 1.

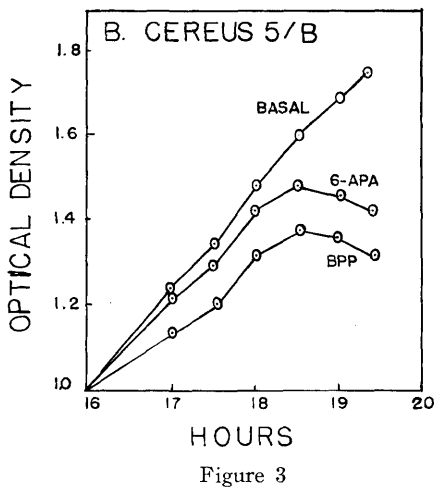


Figure 3

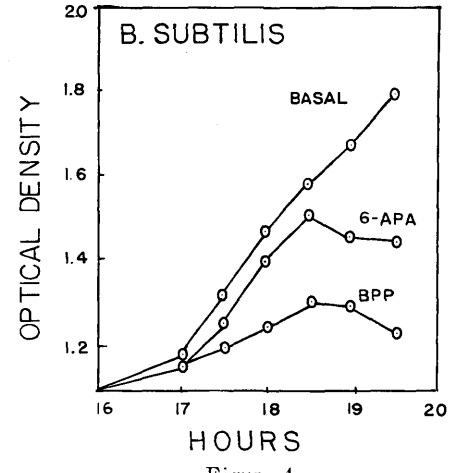


Figure 4

FIGURE 3. Inhibition of growth of *B. cereus* 5/B. The other details are the same as for fig. 1.
FIGURE 4. Inhibition of growth of *B. subtilis*. The other details are the same as for fig. 1.

does not show much of the difference but synthetic penicillins, BPP, BCP and PPA do show differences. Thus, the production of enzyme in constitutive strains of *B. cereus* is enhanced by inducer penicillins. On the other hand, in these strains 6-APA behaves as a repressor.

Of the various enzyme induction models proposed, we will limit the comparision of our data to only one, namely, that proposed by Szilard (1960). His model contains the concept that the inducer competes with a repressor for a "controlling

site." If the "controlling site" is occupied by the "repressor substance" then release of the enzyme protein (here the completed penicillinase structure) is blocked. If an inducer substance, which may or may not be identical with the inducer added to the medium, is bound then the enzyme may be released by the agency of a hypothetical "universal enzyme." If no repressor is present, then the situation that prevails is that found in constitutive mutants, e.g., *B. cereus* 569/H and 5/B.

Szilard does not postulate the nature of the action of the repressor and inducer in a specific manner. We propose that it is partially steric. The data presented here is consistent with a steric factor being involved in the complex interaction of the ribosomal template, the inducer, the hypothetical repressor, and preformed (but bound) penicillinase. In these constitutive strains the penicillins with large and bulky groups in the side chain may introduce steric factors which weaken the bonds of the enzyme with the template. The greater the side chain, the greater will be the effect on bonds connecting the pre-enzyme protein to the template. The 6–APA on the other hand, does not have an acyl side chain, and thus, has a different effect on enzyme release. But, possibly due to the small molecular structure and its ionic character, it may behave as a repressor, i.e., it binds the pre-enzyme to the template to some degree and hence represses the enzyme formation. Thus, from the data it can be seen that Szilard's model may offer a suitable explanation for the association of inducers with RNA-template only with some modification, as it does not account for the permanent effect (Pollock, 1950) of penicillin on *B. cereus* or the enhancement phenomenon we have reported here.

SUMMARY

(1) An investigation was made of the effects of several semi-synthetic penicillins and penicillin G on the induction of exopenicillinase in *B. cereus* and *B. subtilis*.

(2) Penicillins BPP, BCP, and PPA were better inducers than penicillin G and 6–APA. They also enhanced penicillinase production in the constitutive strains of *B. cereus*.

(3) All of the penicillins acted as growth inhibitors. Removal of penicillins from the medium allowed the organisms to continue normal growth.

(4) BPP, BCP, and PPA enhanced penicillinase production in the constitutive strains of *B. cereus* 569/H and 5/B.

(5) The 6–APA appears as a repressor in the constitutive strains of *B. cereus* 569/H and 5/B.

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