METABOLIC CONTROL OF PENICILLINASE BIOSYNTHESIS IN *BACILLUS CEREUS*¹

LILY C. YIP, RAMESH SHAH, AND RICHARD A. DAY

Department of Chemistry, University of Cincinnati, Cincinnati, Ohio

Received for publication 2 March 1964

ABSTRACT

YIP, LILY C. (University of Cincinnati, Cincinnati, Ohio), RAMESH SHAH, AND RICHARD A. DAY. Metabolic control of penicillinase biosynthesis in *Bacillus cereus*. *J. Bacteriol.* **88**:297-308, 1964.—Penicillinase production in strains 5 and 5/B of *Bacillus cereus* in response to treatment by 6-aminopenicillanic acid (APA), penicillin G, (6-*N*- α -(*p*-benzyloxyphenoxy)-propionylamino-penicillanic acid, and cephalosporin C (CC) was found to be analogous to that seen in constitutive strains. Strain 5 did not release penicillinase into the medium to any great extent. Penicillinase production and the effect of the above penicillins on it were found to decline with increasing density of the culture. The penicillins were shown to accelerate or retard the production of penicillinase activity in strain 5 cells during pretreatment at 0 C and during incubation at 37 C. Strains 5 and 5/B gave qualitatively similar responses to penicillin treatment. At 0 C, the specific activity of penicillinase in strain 5 passes through a period of rapid increase at 0 hr and a period of little change at approximately 1 hr, followed by an increased rate of change towards 2 hr. The effect of APA or CC on specific activity of strain 5 cells during treatment at 0 C could not be reversed by one another, but Hg could reverse the increase caused by CC to some extent and the repression caused by APA. The production of penicillinase in the microconstitutive strain 5 of *Bacillus cereus* in response to treatment with CC was influenced by various inhibitors. 8-Azaguanine inhibited the production of the enzyme both during a pretreatment of the cells with CC at 0 C and during the subsequent incubation at 37 C. Actinomycin D, 6-azauracil, 6-thioguanine, and 2-thiocytosine inhibit the increase in penicillinase arising after the pretreatment at 0 C. 6-Azathymine has very little effect on the change of penicillinase activity. The CC-induced change occurring during the 0 C

period was postulated to be a process at the level of protein biosynthesis itself; change at 37 C, constituting a delayed response, was considered a process at the level of messenger ribonucleic acid synthesis.

As was previously noted, the production of penicillinase by two so-called constitutive strains of *Bacillus cereus*, designated 569/H and 5/B, is not insensitive to penicillin treatment (Day and Shah, 1962; Shah and Day, 1963a). Prior to our work, it had been reported that these mutants do not give different levels of penicillinase when treated with penicillin (Pollock, 1957b). The increase in the levels of penicillinase produced in these constitutive strains when treated with certain penicillins was much smaller than the change in the level of penicillinase seen in inducible strains after penicillin treatment; also, the repression of penicillinase production by one penicillin was much less than the repression seen in the case of most repressible enzyme systems. The smaller response in these two strains may be taken as being indicative of a qualitatively different response than that seen in the many cases of inducible and repressible enzyme systems.

Further observations made with one of the above strains, 5/B, and a related strain, *B. cereus* 5, termed a "microconstitutive" strain (Pollock, 1957b) are detailed below. Although overall penicillinase production is much retarded by low temperatures in *B. cereus*, cells grown at 37 C when exposed to penicillin at 0 C undergo a rapid increase in penicillinase level; the magnitude of the increase is a function of the penicillin. These data were interpreted as supporting the concept that an inducer, or a repressor, may exert its metabolic control of de novo protein synthesis at both the level of the gene and at the level of protein synthesis.

To differentiate more clearly between the changes of levels of penicillinase occurring at 0 C and those occurring during the incubation at 37 C,

¹ Preliminary reports of this and related work were presented in part at the 143rd Meeting of the American Chemical Society, 13 to 17 January 1963 and at the Sixth International Congress of Biochemistry, 26 July to 1 August 1964.

various analogues of the purines and pyrimidines were added in various combinations with cephalosporin C to cultures of strain 5. These bases interact with the process of protein biosynthesis at various levels. Both adenine and guanine are required as their nucleoside triphosphates, although in entirely different roles. The usual bases found in ribonucleic acid (RNA) are essential for the three types of RNA shown to be essential for protein biosynthesis; of these three, messenger RNA has the highest turnover rate, and hence would be expected to be affected by the presence of analogues more quickly than the other two types. The incorporation of an analogue into the RNA of *B. cereus* has been established (Matthews and Smith, 1956).

MATERIALS AND METHODS

Organisms. *B. cereus* strain 5/B (NCTC 9946) and strain 5 (ATCC 10702) were used. The term "macroconstitutive" will be used when appropriate to describe both strains 569/H and 5/B, since there was no great difference between the two with respect to the changes in penicillinase activity discussed here. [Pollock (1957b) used the terms "pleno-" and "magnconstitutive" for 569/H and 5/B, respectively.]

Penicillins and antimetabolites. The penicillins, their designations, and sources of supply were as follows: benzyl penicillin, Nutritional Biochemicals Corp., Cleveland, Ohio; 6-aminopenicillanic acid (APA), Eli Lilly & Co., Indianapolis, Ind.; 6-*N*- α -(*p*-benzyloxyphenoxy)-propionyl-aminopenicillanic acid (BPP), Bristol Laboratories, Inc., Syracuse, N.Y.; and cephalosporin C (CC) Eli Lilly & Co. 8-Azaguanine, 6-azathymine 6-azauracil, 6-thioguanine, and 2-thiocytosine were obtained from Nutritional Biochemicals Corp. Actinomycin D was obtained from the Department of Biological Sciences, University of Cincinnati.

Culture medium. The casein hydrolysate medium (CH/C) described by Pollock (1957c) was employed throughout. Casein hydrolysate was obtained from Nutritional Biochemicals Corp.; the other constituents were reagent-grade chemicals.

Preparation of cultures. Suitable quantities of CH/C were inoculated with *B. cereus* 5 by wire-loop transfer from a slant. The cultures were shaken on a Burrell wrist-action shaker with sufficient arc to maintain splashing for aeration.

Temperatures were controlled at 37 ± 0.2 C in a "reach-in" incubator, or at 37 ± 0.1 C in a water bath. The latter was used principally for the induction. All of the data in any one figure were obtained from a single culture subdivided into the appropriate number of samples.

Penicillin treatment. Three methods of penicillin treatment were used. (i) For the cold-pretreatment method, an appropriate volume of log-phase cells was centrifuged in the cold, washed with 0.02 M sodium phosphate buffer (pH 6.5), resuspended in fresh cold phosphate buffer containing the indicated concentration of penicillin, and stored at 0 C for 1 hr. The cells were centrifuged in the cold, washed with 0.02 M phosphate buffer (pH 6.5) precooled to 0 C, centrifuged in the cold, and then resuspended in cold CH/C and immediately placed in a water bath at 37 C. The total time elapsing between the end of the cold pretreatment and the beginning of the incubation at 37 C was approximately 1 hr. Portions removed from each sample were added to test tubes containing a few crystals of 8-quinolinol to stop further production of penicillinase, and were stored at ca. 0 C until assayed. In general, the tubes were assayed within 24 hr of collection. Addition of gelatin to preserve penicillinase activity was not found necessary for strain 5 cultures. No growth inhibition in cultures treated with penicillin by this method was ever detected. (ii) As the standard method, essentially the method of Pollock (1957a) was used. (iii) The third method was the washed-cell method. The cells were washed at 0 C with 0.02 M sodium phosphate buffer (pH 6.5), centrifuged with the centrifuge maintained at <0 C, and resuspended in CH/C containing the additions indicated. Incubation at 37 C was commenced immediately.

Measurement of growth. Growth was determined by comparison of optical densities at 650 m μ observed in a Bausch & Lomb Spectronic-20 colorimeter with a standard curve relating milligrams of dry weight per milliliter to optical densities.

Terms. Plenoconstitutive designates a strain producing penicillinase at a high level in the absence of penicillin. The level is relatively insensitive to penicillin treatment. The organism is a mutant of an inducible organism (Pollock, 1957b). Magnconstitutive is the same as plenoconstitutive, except that it describes a mutant derived from a microconstitutive strain (Pollock,

1957b). Microconstitutive designates a strain which produces penicillinase at a very low level and is not inducible (Pollock, 1957b), but will give altered levels of penicillinase upon penicillin treatment. Transcription is used as defined by Spiegelman (1963); i.e., it is the process of DNA-dependent synthesis of messenger RNA. Translation is the messenger RNA-directed synthesis of the protein polypeptide chain (Spiegelman, 1963). Modulation means control of rate of translation.

Assay of penicillinase. Penicillinase was assayed by essentially the method of Perret (1954). Whole-cell suspensions of strain 5 were utilized, since almost all of the penicillinase is cell-bound. The penicillinase levels are represented as units per milligram of dry cells in the cultures treated by the cold pretreatment method.

Antimetabolite treatment. Cultures of late log-phase cells of strain 5 were subdivided in each case into the requisite number of samples. Except when noted otherwise, the subdivisions made were treated with various combinations of CC (10^{-4} M) and antimetabolite. The concentrations and combinations are indicated in the legends during the cold pretreatment and during the subsequent inoculation in fresh CH/C at 37 C.

Several controls were employed for most, but not all, of the antimetabolites used. One subdivision represents the control on basal culture. A second subdivision gives the induction by CC; a third affords a measure of the effect of the antimetabolite on the delayed response after CC treatment. A fourth provides a measure of the action of antimetabolite on the basal level, and a fifth provides an index for the action of the antimetabolite when the cells are exposed to it only in the cold pretreatment concurrently with CC. A sixth subdivision provides an index of the action of the antimetabolite at 37 C on cultures subjected to the cold pretreatment with CC.

No addition of CC during the incubation at 37 C was made because of the growth inhibition; cold pretreatment with 10^{-4} M CC causes no measurable growth inhibition. The fifth subdivision was used to determine whether the antimetabolite permeated the cell at 0 C and had any effect on subsequent penicillinase specific activity values.

RESULTS

To determine whether penicillinase production in the microconstitutive strain 5 was sensitive to

FIG. 1. Production of penicillinase by *Bacillus cereus* 5 with the washed-cell method with 10^{-4} M penicillins. The cells were treated as described in the text. The cells were incubated in fresh CH/C at 37 C with the following additions: ○, basal; △, penicillin G; □, cephalosporin C; and ●, 6-amino-penicillanic acid.

exposure to penicillin, the organism was exposed to penicillin under two sets of conditions. Figure 1 shows that *B. cereus* 5, when treated with penicillins at a concentration of 10^{-4} M by the washed-cell method, behaves qualitatively in the same manner as the macroconstitutive strains 5/B and 569/H (Day and Shah, 1962; Shah and Day, 1963a, b). That is, strain 5 exhibits an enhancement effect in response to the incubation with CC and repression in response to incubation with APA. As can be seen here, there is a lag period also typical of penicillinase production in *B. cereus* 569, an inducible strain (Pollock, 1950). Under this condition, penicillinase production terminated about 2 hr after the penicillin had been added; this also marks the time of transition of the strain 5 cells from late log phase to a stationary phase prior to sporulation.

The possibility that part of the change in levels of penicillinase could have been caused by contamination of the cultures with inducible cells, or that during the culturing enough spontaneous mutations occurred to give part or all of the changes seen, has not been ruled out entirely. The rate of mutation of strain 5 to 5/B is small enough (Sneath, 1955) to make it unlikely that the changes observed could be accounted for quantitatively by such mutations. The magnitude of the response in cultures of strain 5/B and 569/H (Shah and Day, 1963b) is large enough to require the presence of a relatively large fraction

FIG. 2. Comparison of the production of penicillinase by *Bacillus cereus* 5 and 5/B with the washed-cell method. The penicillins were added to give 10^{-7} M. The symbols, the bacterial strain, and the penicillin are, respectively: ○, 5/B, none; △, 5/B, benzylpenicillin; □, 5/B, cephalosporin C; ●, 5, none; ▲, 5, benzyl penicillin; ■, 5, cephalosporin C; and ○, 5/B, basal; △, 5/B, penicillin G; □, 5/B, cephalosporin C; ●, 5, basal; ▲, 5, penicillin G; ■, 5, cephalosporin C.

of inducible cells; cultures from single-cell isolates have never been found to be inducible. The fact that benzyl penicillin has a smaller effect on the inducible strain 569 or constitutive strain 5/B at concentrations where it gives a maximal response with strain 5 tends to rule out the possibility that the penicillinase measured is being made in any great part by inducible cells or 5/B mutants.

The qualitative similarity of the responses of strain 5 and the affiliated strain 5/B treated by the washed-cell procedure is illustrated in Fig. 2. Strain 5 exhibits some differences in the levels of penicillinase activity at 0 hr, which represents a change that has occurred during the brief interval between addition of the penicillin and the assay of a sample. This change is described in more detail below. Samples of strain 5/B showed no measurable differences among themselves at 0 hr, in contrast to strain 5. Also, the penicillinase

activity after CC treatment was ca. twofold higher than basal at 1.5 hr for both strains 5 and 5/B. The relative difference in activity became less for strain 5/B, but more for strain 5. This is related to the growth inhibition exhibited by the latter.

Figure 3 shows the concentration dependence of the enhancement of penicillinase activity under the "standard conditions" with strain 5/B. The pattern of response to different levels of benzyl penicillin is similar to 569/H and to that reported for strain 569 (Pollock, 1950), but differed from the response of strain 5, which showed a maximal enhancement at higher concentrations of benzyl penicillin. On the other hand, strains 5 and 5/B gave maximal relative activities with CC at a concentration of 10^{-4} M.

It is important to note that strain 5 differs qualitatively from strain 5/B in an important aspect of penicillinase production; i.e., we have found that strain 5 does not release a significant

FIG. 3. Concentration dependence of the response of *Bacillus cereus* 5/B to treatment by various penicillins by the standard method. The values plotted represent the increments in penicillinase activity produced by the standard method. The values were taken 2.5 hr after addition of the penicillins to give the desired concentrations. The symbols and penicillins tested are: ○, 6-N- α -(p-benzoyloxyphenoxy)-propionylaminopenicillanic acid; △, 6-aminopenicillanic acid; □, cephalosporin C; ●, penicillin G.

amount of its penicillinase into the culture medium. By contrast, both inducible strain 569 and the constitutive strains 569/H and 5/B release approximately 85% of the penicillinase into the medium (Pollock, 1956).

In Fig. 4 is shown the variation of specific activity vs. time at 0 C. It can be seen that after 1 hr the specific activity is not changing appreciably, but that it increases again toward 3 hr. That the second increase in specific activity may be related to the delayed response is indicated by the observation (Fig. 5) that the specific activity rises rapidly after two 1-hr treatments at 0 C with CC, where the cells are suspended in CH/C at 37 C. Because of these observations and because of Pollock and Perret's (1951) finding that *B. cereus* had acquired 85% of its specifically bound S³⁵ penicillin after 1 hr at 0 C, a 1-hr pretreatment period was considered the most suitable.

Figure 6 represents data obtained with strain 5 under conditions of the cold pretreatment

FIG. 4. Increase of penicillinase activity during the pretreatment at 0 C. The appropriate number of samples were withdrawn from a culture of *Bacillus cereus* 5 grown on casein hydrolysate medium (CH/C) and treated with 10^{-4} M cephalosporin C at 0 C for the intervals indicated.

FIG. 5. Effect of successive cold pretreatments with cephalosporin C (CC) and 6-aminopenicillanic acid (APA) on penicillinase production in *Bacillus cereus* 5. The cells were exposed for two 1-hr periods at 0 C to CC (10^{-4} M) and APA (10^{-4} M). The symbols and the penicillin added during the first and during the second pretreatment are, respectively: ○, none, none; △, CC, CC; □, CC, APA; ●, APA, CC; ▲, APA, APA. The end of the first treatment is "time 0" and of the second, "time 0'". The cells were washed twice at the end of each pretreatment and then incubated in fresh CH/C at 37 C.

method. The age of the culture is critical, the penicillinase production and the response declining with age. In Fig. 7 is shown the variation in response to penicillin treatment with the "age" (absorbancy at 650 mμ) of the culture. The younger the culture, the greater is the difference in response to penicillin treatment. The measurements cannot be carried out on very young cultures of strain 5 because of the low absolute level of penicillinase in them.

Some evidence for two types of changes may be adduced from Fig. 4 and 6. The first is the increase in activity that occurs during the cold pretreatment. In Fig. 6 is shown the change in penicillinase activity which occurred during the cold pretreatment period, during the period required for washing, centrifuging, and resuspen-

FIG. 6. Production of penicillinase by *Bacillus cereus* 5 during and after the cold pretreatment. The dashed lines from 1 to 0 connect the points representing activity measured at the end of the 1-hr period at 0 C to that measured after washing and resuspension in fresh CH/C; this marks the beginning of the incubation at 37 C. The additions were: ○, none; △, benzylpenicillin; □, cephalosporin C; and ●, 6-aminopenicillanic acid.

sion, and during the subsequent incubation at 37 C. It is apparent that APA-treated cells do not undergo an appreciable change in penicillinase activity during the 1-hr interval at 0 C. The basal level increases slightly during this time, and the level of benzyl penicillin and CC increases still more. The second type of event is the rise in relative rate of synthesis after a lag period. We see a more rapid response to CC and benzyl penicillin treatment than to APA. In fact, it is questionable whether there actually is a lag period with APA of the same type as is seen with benzyl penicillin and CC, which may reflect a qualitative difference in its action. However, the level of penicillinase activity does rise with all three penicillin-treated samples, after a time, by both the washed-cell and the cold-pretreatment methods. The results in Fig. 1 are not directly comparable with the results in Fig. 6 because of

the different units used for ordinates. Since the penicillins produce morphological changes in the cells by the washed-cell method, which in turn alters the relationship of optical density to cell mass, we did not attempt to represent activity as units per milligram of cells in these cases.

Since it has been reported that no appreciable amount of penicillinase is synthesized by *B. cereus* at 18 C (Pollock, 1950), although the organism grows well at that temperature, it seemed likely that penicillinase activity arising at 0 C represents a different rate-limiting step than is operable at 37 C. The possibility that this increase in specific activity at 0 C may represent conversion of a prepenicillinase polypeptide chain into penicillinase by completion of folding of the chain was

FIG. 7. Effect of the age of *Bacillus cereus* 5 cultures on production of penicillinase and the effect of various penicillins on it. The cold-pretreatment method was used here, the assays being taken 0.5 hr after the beginning of the incubation at 37 C. The optical density of the culture was measured at the same time. Because of changes in volumes during the treatment, the optical density of each sample taken at intervals to be subdivided for treatment with each of the penicillins was approximately two-fold greater than optical density after 0.5 hr at 37 C. The additions were: ○, none; △, benzylpenicillin; □, cephalosporin C; and ●, 6-aminopenicillanic acid.

tested with Hg^{++} ion; Hg^{++} has been shown to stabilize protein conformation (Martin, 1963). Treatment of strain 5 cells with various combinations of mercuric ion, CC, and APA shows that the Hg^{++} abolishes both the increase in level of penicillinase activity occasioned by CC and the static effect of APA on the cells during the 0 C pretreatment (Table 1).

Table 2 shows that the 1-hr cold pretreatment of relatively young strain 5 cells with APA or CC is irreversible in the same sense that the binding of benzyl penicillin and other penicillins had been found to be irreversible for strain 569 cells (Pollock and Perret, 1951; Pollock, 1957a). Cold pretreatment of the cells with APA for the usual 1-hr period at 0 C, followed by washing and treatment with CC at 0 C for another 1-hr period, shows that the repression by APA was not reversed by the subsequent CC treatment. The converse was shown by first treating with CC at 0 C and then with APA.

Figure 5 shows the results obtained with strain 5 cells for two successive cold pretreatments with CC and APA followed by incubation at 37 C. The age of the culture is important; the data in Fig. 5 were obtained from an older culture. A younger culture does not give an identical pattern. However, the response was similar for both the older and the younger cultures, except for one important difference with respect to the cells that were cold-pretreated with CC followed by the cold pretreatment with APA. For the older culture, the delayed response of the typical CC cold-pretreated cells was strongly diminished; in the younger culture, the delayed response was diminished less, although it is still not so prominent as in the one that was treated with CC in both pretreatments. The sample treated with APA and then CC (Fig. 5) bears a relationship to the basal similar to that given cold pretreatment with APA alone (Fig. 6); CC seems to have no effect on the APA-treated cells. On the other hand, APA treatment of the CC-treated cells considerably diminishes the "delayed response."

Since hydrolysis of the penicillins by the penicillinase already present is undoubtedly a factor in determining the effectiveness of a penicillin in producing the effects described, the relative rates of hydrolysis by the exo-penicillinase of strain 5/B were evaluated. To do this the enzyme was exposed to concentrations of penicillins far in excess of the K_m value of 28.5 units per ml of

TABLE 1. *Effect of mercuric ion on the level of penicillinase activity in penicillin-treated and untreated cells*

Addition during first treatment*	Penicillinase activity	Addition during second treatment†	Penicillinase activity
	units/mg		units/mg
—	32	Hg^{++} (10^{-7} M)	38
CC	42	Hg^{++} (10^{-7} M)	40
CC	42	Hg^{++} (10^{-4} M)	33
APA	20	Hg^{++} (10^{-7} M)	30
APA	21	Hg^{++} (10^{-4} M)	36

* The first treatment consisted of the usual pretreatment at 0 C with the penicillins at 10^{-4} M.

† The cells were washed, resuspended in water containing Hg^{++} at the concentrations indicated, and stored at 0 C for 1 hr. The cells were washed with 0.1 M cysteine and then assayed.

TABLE 2. *Penicillinase activity in Bacillus cereus 5 after two successive 1-hr treatments at 0 C*

Addition during first period	Addition during second period	Penicillinase activity
		units/mg
—	—	72
CC	—	127
CC	APA	113
APA	—	46
APA	CC	50

benzyl penicillin reported for the enzyme of strain 569 (Pollock, 1956). The values normalized to 1.00 for benzyl penicillin are 0.65, 0.28, and 0.31 for BPP, CC, and APA, respectively. This indicates that hydrolysis of the penicillins will be an important factor for evaluating the effects of all of the penicillins used here. There will be less uncertainty from this factor in the cold-pretreatment method than in the standard or washed-cell methods, even for strain 5 with its low level of penicillinase activity.

To determine levels of the antimetabolites causing a pronounced repression of the increase in specific activity in the delayed response, the degree of repression was compared with growth inhibition over the same concentration range. Significant repression of the delayed response could not be achieved without some degree of growth inhibition in any case.

Treatment of *B. cereus* 5 cells with all of the antimetabolites listed, except 6-azathymine, has one thing in common; i.e., cold pretreatment with

FIG. 8. Effect of 8-azaguanine (AG) on the response of *Bacillus cereus* 5 to treatment with cephalosporin C (CC). CC was 10^{-4} M and AG was 3×10^{-4} M. The symbols designating each combination of treatments, and the additions during the cold pretreatment period, during the incubation at 37 C are, respectively: ○, none, none; △, CC, none; □, CC, AG; ●, CC and AG, none; ▲, CC and AG, AG.

the antimetabolite and CC concurrently led to a partial repression of the increase in specific activity (the delayed response) occasioned by CC pretreatment alone. Both the RNA-pyrimidine analogues tested, 6-azauracil and 2-thiocytosine, and the RNA-purine analogues, 6-thioguanine and 8-azaguanine, seemed to be of comparable effectiveness in permeating the cell at 0 C and diminishing the delayed response. Not all of the antimetabolites exerted a repressive effect on the change in specific activity during the cold pretreatment period. 8-Azaguanine (Fig. 8) showed the most complete repression of this change, and actinomycin D (Fig. 9) showed the least. The other RNA-base analogues exerted intermediate repressive effects.

Cold pretreatment with these analogues concurrently with CC blocked the delayed increase in specific activity, even though the excess antimetabolite was removed prior to the incubation

in fresh CH/C at 37 C. This clearly shows that the antimetabolites interfere with a process stimulated by CC. Similarly, the delayed rise in specific activity initiated by the pretreatment with CC alone can be partially abolished by incorporating the antimetabolites into the fresh CH/C used in the incubation at 37 C.

In each case, the antimetabolites kept the specific activity at a value below that of the basal. This difference between the basal and basal plus metabolite is small. 6-Azathymine exerts very little effect on the change of specific activity of the control on CC-treated cultures. Assuming

FIG. 9. Effect of actinomycin D (AD) on the response of *Bacillus cereus* 5 to the treatment with cephalosporin C (CC) by the cold-pretreatment method. The concentration of CC was 10^{-4} M, and the concentration of AD was 1.19 μ g/ml. The AD solutions were made up by two serial dilutions from an ethanolic stock solution. The final concentration of ethanol in the cultures was 0.095%. The designations and the additions during the cold pretreatment period and during the incubation at 37 C for each combination of treatments are, respectively: ○, none, none; △, CC, none; □, CC and AD, AD; ●, AD, AD; ▲, 0.095% ethanol, 0.095% ethanol. The dashed lines connect the points representing the specific activity of the cultures before subdivision to the activities found after the cold pretreatment.

that it permeates the *B. cereus* cells, one is led to the conclusion that it does not interfere with the processes that are retarded by the RNA-base analogues and actinomycin D.

DISCUSSION

That a two-point control system may be significant in the penicillinase-synthesizing system was supported by the observation that the levels of penicillinase bound to ribosomes of non-induced, induced, and constitutive strains of *B. cereus* (Duerksen and O'Connor, 1963) are not of the same ratio as the change from noninduced to induced (Steinman, 1961) or from noninduced to constitutive cells of *B. cereus* (Pollock, 1957*b*). This is indicative of a change in the rate of turnover of penicillinase per ribosomal site. A similar situation seems to hold for a β -galactosidase system in which the increase of a specific messenger RNA is 30-fold (Hayashi et al., 1963), whereas maximal induction brings about at least a 1,000-fold increase in the level of the enzyme (Hogness, 1959). Evidence supporting a two-point metabolic control of de novo protein synthesis in various pathways has been advanced (Hauge et al., 1961; MacQuillan and Halvorson, 1962; McFall and Mandelstam, 1963). Strong evidence for a regulatory mechanism in protein synthesis that operates at the level of polypeptide assembly—the translational level—has been found (Ohtaka and Spiegelman, 1963). Arginine biosynthesis may be controlled at the metabolite and genetic levels (Gorini and Gundersen, 1961; Gorini, 1963), that is, at the levels of translation and transcription.

There is a response in the cold-pretreatment method which we interpret as indicating the existence of two distinct steps in the biosynthesis. These are (i) a step with a relatively low temperature coefficient since it occurs at 0°C, and (ii) a step that manifests itself after a lag period during the incubation at 37°C. The first, we feel, is a change occurring at the level of protein biosynthesis of a critical RNA. Since it has been shown that reversible conformational changes in the penicillinase of *B. cereus* occur readily at low temperature (Garber and Citri, 1962) and that overall penicillinase production is strongly retarded at low temperatures (Pollock, 1950), we conclude that the change in penicillinase activity is related to a change in protein conformation,

possibly completion of the folding at the behest of the penicillin.

The evidence, in addition to the inference of the data of Duerksen and O'Connor (1963), that biosynthesis of penicillinase is controlled at the level of protein formation is, first of all, that the relative order of penicillinase-inducing power among various penicillins is the same for an inducible strain of *B. cereus* and of *B. subtilis* (Steinman, 1961; Day and Shah, 1962; Shah and Day, 1963*a, b*), two macroconstitutive strains of *B. cereus* (Day and Shah, 1962; Shah and Day, 1963*a, b*), and for the microconstitutive strain 5 of *B. cereus*. If we assume that Szilard's (1960) model of enzyme induction represents a mode of control that is, in fact, operating, then it is easy to see that the nature of the penicillin bound to the protein-synthesizing site will have a characteristic influence on the rate of appearance of penicillinase activity if it effectively modulates a rate-limiting step.

Secondly, the effect of mercuric ion (Table 2) may be interpreted as being linked to conformational changes; precedent for this has been found (Martin, 1963). The reversal of the CC-catalyzed increase and the APA-linked blockage of increase of penicillinase activity during a cold pretreatment may represent conversion of two less stable conformations to a more stable one(s) with activities closer to the basal level. Perhaps the most important point to be seen in Table 2 is the increase occasioned by mercuric ion after APA treatment; this may be a direct effect on conformation. A secondary effect could arise through the release of the bound penicillin, which may be held by a covalent bond (Schepartz and Johnson, 1956) sensitive to mercuric ion.

The delayed response in the production of penicillinase in *B. cereus* is interpreted as stemming from the time required to synthesize a critical RNA (probably messenger RNA) and the subsequent time required for synthesis of the polypeptide chain. This is consistent with observations on the effect of 8-azaguanine on levels of penicillinase in a constitutive and an inducible mutant of *B. cereus* (Chantrenne, 1958; Chantrenne and Leclercq-Calingaert, 1963) and our observations on the effect of 8-azaguanine and other analogues on the penicillinase levels in strain 5. Experiments with actinomycin D confirm the duality of the control system; that actinomycin D specifically blocks the synthesis

of messenger RNA for penicillinase in *B. cereus* has found considerable support (Pollock, 1963).

The results of measuring the response of strain 5 to CC in the presence of various pyrimidine and purine analogues and actinomycin D lend support to the idea that the delayed response seen after a 1-hr cold pretreatment is a function of RNA synthesis and penicillin treatment. The guanine analogue, 8-azaguanine, has been found to be incorporated into RNA of various organisms (Matthews and Smith, 1956). The analogue of uracil, 6-azauracil, was found to be an inhibitor of nucleic acid synthesis; it apparently functions by blocking the pathways of uridylic acid synthesis (Skoda and Sorm, 1958, 1959). Since 2-thio-uracil was found to be incorporated into the RNA of *B. megaterium* (Hamers, 1956; Amos, Vollmayer, and Korn, 1958), it would not be unreasonable to believe that the thio analogues used here may be incorporated into the RNA of *B. cereus* 5. Thus, if the delayed response of the cells given the cold pretreatment with CC is caused by the increased synthesis of the messenger RNA coded for penicillinase, it would be reasonable to predict that these analogues of RNA bases used in this study would have some effect on the delayed response to penicillin treatment. This was, in fact, observed; the abolition or reduction of the delayed response of the strain 5 cells was effected. Optical density measurements of the cultures showed that there was also some degree of growth inhibition caused by these analogues. The inhibition was slight at concentrations producing strong effects on levels of penicillinase. Since 6-azathymine is an analogue of thymine and could be expected to appear in deoxyribonucleic acid instead of RNA, it is not unexpected that there was very little response in the relatively short time the cells were studied after exposure to this analogue.

Particularly significant is the fact that, during the 1-hr cold pretreatment of the cells with CC, 8-azaguanine showed the strongest inhibitory effect on the increase of penicillinase activity occurring during this period among the anti-metabolites examined. If the increase in penicillinase at this point is caused by agency of the permanently bound penicillin with a mechanism other than that involved in the overall synthesis (that is, one not involved in controlling the formation of covalent bonds), one would be unable to predict the effect of any particular

nucleic acid base analogue. On the other hand, it has been noted that 8-azaguanine has a "secondary effect" on penicillinase production in *B. cereus* (Chantrenne and Leclercq-Calingaert, 1963), although this does not appear to be related to the phenomenon arising during the cold pretreatment. A specific role has been assigned to guanosine triphosphate (GTP) in protein biosynthesis in *Escherichia coli* (Lipmann, 1963); we propose that the 8-azaguanine is converted to the corresponding analogue of GTP and interferes with protein synthesis at this level, as well as being incorporated into messenger RNA and causing the reduction in the delayed response. If the analogue triphosphate is interfering with the formation of the polypeptide chain, then it follows that the total time for synthesis of the polypeptide chain is very short. However, at this point we are unable to decide whether the effect arising at 0 C is linked to conformational changes, to synthesis of polypeptide bonds, to release of the enzyme from its site of synthesis, or to other factors not enumerated here.

The influence of actinomycin D on penicillinase production by strain 5 is in accord with Pollock's (1963) observation. The rise in the level of penicillinase during the 0 C period was little affected, but the delayed response in the second phase of the response to treatment with CC was strongly reduced. This is in good agreement with the

TABLE 3. Comparison of differences in penicillinase activities during two phases of the response in the cold-pretreatment method

Penicillin	Treated minus basal*	Increments from 0 min to maximal response†	Time of maximal delayed response‡
	units/mg	units/mg	min
CC	80	66	30
G	12	40	52
—	0	—15	75
APA	—80	23	75

* The values listed are those measured at the end of the 0 C pretreatment less the basal value.

† The increments of penicillinase activity from the beginning of the 37 C treatment to the peak of the delayed response.

‡ The time of maximal delayed response is also recorded; the delayed response for the basal level is taken as the point of maximal deviation from a smooth, monophasic curve, interpolated from 45 to 90 min.

proposal that the change occurring at 0 C is at the level of completion or release, whereas the delayed response is largely an expression of increased messenger RNA synthesis.

Another argument we will raise here is that the influence of penicillin on the synthesis of penicillinase as seen in strain 5 is seen in at least two levels of the overall process in the quantitative differences among the responses to various penicillins seen during the 0 C pretreatment and the subsequent delayed response. In Table 3 are tabulated values obtained from a younger culture of a higher specific activity and at a greater susceptibility to influence by the penicillins. If the only process influenced by the inducer were an increase in the rate of synthesis of messenger RNA, it would be expected that the ratios of the values for the two stages, as represented in columns 2 and 3, would be in closer agreement among the various penicillins.

ACKNOWLEDGMENTS

This work was supported in part by Public Health Service grant AI-04326 from the National Institutes of Health. We thank Eli Lilly & Co. and Bristol Laboratories, Inc., for the penicillins provided and A. H. Romano for the actinomycin D.

LITERATURE CITED

- AMOS, H., E. VOLLMAYER, AND M. KORN. 1958. Thiouridylic acid formation and bacterial growth. *Arch. Biochem. Biophys.* **77**:236-238.
- CHANTRENNE, H. 1958. Nucleic acid metabolism and induced enzyme formation. *Rec. Trav. Chem.* **77**:586-601.
- CHANTRENNE, H., AND M. LECLERCQ-CALINGAERT. 1963. Secondary effect of 8-azaguanine on the induced or constitutive synthesis of penicillinase in *Bacillus cereus*. *Biochim. Biophys. Acta* **72**:87-91.
- COOPER, P. D. 1956. Site of action of radiopenicillin. *Bacteriol. Rev.* **20**:28-48.
- DAY, R. A., AND R. SHAH. 1962. Penicillinase production in some bacilli. *Science* **138**:1108-1109.
- DUERCKSEN, J. D., AND M. L. O'CONNOR. 1963. The demonstration of ribosome bound penicillinase in *Bacillus cereus*. *Biochem. Biophys. Res. Commun.* **10**:34-39.
- GARBER, N., AND N. CITRI. 1962. The interaction of penicillinase with penicillins. I. Effect of substrates and of a competitive inhibitor on native and urea-treated enzyme. *Biochim. Biophys. Acta* **62**:385-396.
- GORINI, L. 1963. Symposium on multiple forms of enzymes and control mechanisms. III. Control by repression of a biochemical pathway. *Bacteriol. Rev.* **27**:182-190.
- GORINI, L., AND W. GUNDERSEN. 1961. Induction by arginine of enzymes of arginine biosynthesis in *Escherichia coli* B. *Proc. Natl. Acad. Sci. U.S.* **47**:961-971.
- HAMERS, R. 1956. Incorporation at ³⁵S thiouracil in *Bacillus megaterium*. *Biochim. Biophys. Acta* **21**:170-171.
- HAUGE, J. G., A. M. MACQUILLAN, A. L. CLINE, AND H. O. HALVORSON. 1961. The effect of glucose repression on the level of ribosomal-bound β -glucosidase. *Biochem. Biophys. Res. Commun.* **5**:267-269.
- HAYASHI, M., S. SPEIGELMAN, N. C. FRANKLIN, AND S. E. LURIA. 1963. Separation of the RNA message transcribed in response to a specific inducer. *Proc. Natl. Acad. Sci. U.S.* **49**:729-736.
- HOGNESS, D. S. 1959. Induced enzyme synthesis, p. 256-268. In J. L. Oncley et al. [ed.], *Biophysical science, a study program*. John Wiley & Sons, Inc., New York.
- LIPMANN, F. 1963. Biotechnology of protein synthesis. Abstr. 143rd Meeting. Am. Chem. Soc., p. 39A.
- McFALL, E., AND J. MANDELSTAM. 1963. Specific metabolic repression of three induced enzymes in *Escherichia coli*. *Biochem. J.* **89**:391-398.
- MACQUILLAN, A. M., AND H. O. HALVORSON. 1962. Metabolic control of β -glucosidase synthesis in yeast. *J. Bacteriol.* **84**:23-30.
- MARTIN, R. G. 1963. The first enzyme in histidine biosynthesis: the nature of the feedback inhibition by histidine. *J. Biol. Chem.* **239**:257-268.
- MATTHEWS, R. E., AND J. D. SMITH. 1956. Distribution of 8-azaguanine in the nucleic acids of *B. cereus*. *Nature* **177**:271-272.
- OHTAKA, Y., AND S. SPIEGELMAN. 1963. Translational control of protein biosynthesis in a cell-free system directed by a polycistronic viral RNA. *Science* **142**:493-497.
- PERRET, C. J. 1954. Iodometric assay of penicillinase. *Nature* **174**:1012-1013.
- POLLOCK, M. R. 1950. Penicillinase adaptation in *B. cereus*: adaptive enzyme formation in the absence of free substrate. *Brit. J. Exptl. Pathol.* **31**:739-753.
- POLLOCK, M. R. 1956. The cell-bound penicillinase of *B. cereus*. *J. Gen. Microbiol.* **15**:154-169.
- POLLOCK, M. R. 1957a. The activity and specificity of inducers of penicillinase production in *B. cereus*, strain NRRL 569. *Biochem. J.* **66**:419-428.

- POLLOCK, M. R. 1957b. Penicillin-induced resistance to penicillin in cultures of *Bacillus cereus*. Ciba Found. Symp. Drug Resistance Micro-organisms, p. 78-101.
- POLLOCK, M. R. 1957c. A simple method for the production of high titre penicillinase. J. Pharm. Pharmacol. **9**:609-611.
- POLLOCK, M. R. 1958. Precursors of bacterial penicillinase. Proc. Intern. Symp. Enzyme Chem. Tokyo Kyoto 1957, p. 369-374.
- POLLOCK, M. R. 1963. The differential effect of actinomycin D on the biosynthesis of enzymes in *Bacillus subtilis* and *Bacillus cereus*. Biochim. Biophys. Acta **76**:80-93.
- POLLOCK, M. R., AND C. J. PERRET. 1951. The relation between fixation of penicillin sulphur and penicillinase adaptation in *B. cereus*. Brit. J. Exptl. Pathol. **32**:387-396.
- SCHEPARTZ, S. A., AND M. J. JOHNSON. 1956. The nature of the binding of penicillin by bacterial cells. J. Bacteriol. **71**:84-90.
- SHAH, R., AND R. A. DAY. 1963a. Comparative studies of penicillinase induction in microorganisms by natural and semi-synthetic penicillins. Ohio J. Sci. **63**:217-224.
- SHAH, R., AND R. A. DAY. 1963b. Effect of certain penicillins on penicillinase production by some constitutive strains of bacilli. Abstr. 143rd Meeting Am. Chem. Soc., p. 8A.
- SKODA, J., AND F. SORM. 1958. Accumulation of nucleic acid metabolites in *Escherichia coli* exposed to the action of 6-azuracil. Biochim. Biophys. Acta **28**:659-660.
- SKODA, J., AND F. SORM. 1959. The accumulation of orotic, uracil and hypoxanthine by coli in the presence of 6-azauracil and the biosynthesis of 6-azauridylic acid. Collection Czech. Chem. Commun. **24**:1331-1337.
- SNEATH, P. H. A. 1955. Proof of the spontaneity of a mutation to penicillinase production in *B. cereus*. J. Gen. Microbiol. **13**:561-568.
- SPIEGELMAN, S. 1963. Information transfer from the genome. Federation Proc. **22**:36-54.
- STEINMAN, H. G. 1961. A biochemical comparison of 6-aminopenicillanic acid, benzyl penicillin, and 2,6-dimethoxyphenylpenicillin. Proc. Soc. Exptl. Biol. Med. **106**:227-231.
- SZILARD, L. 1960. The control of the formation of specific proteins in bacteria and in animal cells. Proc. Natl. Acad. Sci. U.S. **46**:277-292.