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Induction of Penicillinase with Inorganic Phosphate

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Phosphate stimulates penicillinase formation in *Bacillus cereus* 569. The rate of penicillinase synthesis in the presence of 0.3 M phosphate, *p*H 7.0, is approximately 10-fold greater than that for uninduced cells, while the rate of synthesis in the presence of 0.3 M phosphate and 1 unit/ml of penicillin is approximately fourfold greater than in the presence of penicillin alone. When phosphate-induced cells are transferred to low phosphate medium, the rate of penicillinase synthesis rapidly reverts to that of uninduced cells. Furthermore, the phosphate-induced synthesis of the enzyme is inhibited by either chloramphenicol or actinomycin D. These antibiotics are known to inhibit protein synthesis and deoxyribonucleic acid-dependent ribonucleic acid (RNA) synthesis, respectively. Thus, phosphate appears to induce the synthesis of a species of RNA that is required for the synthesis of penicillinase in *B. cereus* 569. The penicillin-dependent induction lag for penicillinase was compared in high and low phosphate media. It was found that, at 37 C, the penicillin-dependent lag is approximately 3 min in the presence of 0.3 M phosphate and approximately 6 min in low phosphate medium.

The mechanism of penicillinase induction in *Bacillus cereus* 569 is reported (5, 12) to differ in three ways from general enzyme induction in *Escherichia coli*; (i) length of induction lag, (ii) effect of the inducer and removal of the inducer on enzyme induction and de-induction, respectively, and (iii), half-life of the messenger ribonucleic acids (RNA). Recently, Csányi and collaborators described a fourth difference, namely, the induction of penicillinase by electrolytes (Csányi, *in press*). Enzyme induction in *B. cereus* by electrolytes is not unique to penicillinase (4), yet this phenomenon has not been reported for *E. coli*. Although there is no reason *a priori* why all inducible systems should be exactly alike, neither is there a clear reason why they should differ so widely as the reports on penicillinase and β-galactosidase suggest. Therefore, we undertook a study on the induction of penicillinase in *B. cereus* 569 in an attempt to determine some of the features common to both the penicillinase and β-galactosidase systems, and thus to provide a better understanding of the phenomenon of enzyme induction.

The data presented in this communication show that 0.3 M phosphate, *p*H 7.0, induces penicillinase synthesis in *B. cereus* 569 in the absence of penicillin, and that the amount of penicillinase produced in the presence of penicillin is greatly increased when phosphate is added to the culture medium. The induction lag for enhanced penicillinase production following the addition of penicillin to a phosphate-induced culture of *B. cereus* is approximately 3 min (a value equal to that established for β-galactosidase induction in *E. coli*) (11). Furthermore, when phosphate-induced cells are transferred from a high phosphate medium to a low phosphate medium, penicillinase synthesis promptly returns to the uninduced level. These and other data presented suggest that, under certain defined conditions, the mechanism of penicillinase induction may not differ as widely from β-galactosidase induction in *E. coli* as had been previously reported.

MATERIALS AND METHODS

B. cereus 569 (inducible for penicillinase) was obtained from the American Type Culture Collection. Exponentially growing cultures were prepared by incubating spores for 3 to 4 hr in either Csányi's or Pollock's low phosphate medium. All cultures were grown in a New Brunswick gyratory shaker at 37 C unless indicated otherwise.

Media. Csányi's medium (1) consisted of (per liter): trisodium citrate·$2H_2O$, 1.25 g; $MgSO_4 \cdot 7H_2O$, 0.719 g; KH_2PO_4, 2.72 g; casein hydrolysate (Difco), 10 g; glucose, 4 g; Tween 80, 1 ml; and trace minerals at approximately the following concentrations; 2.5 mg of ferric citrate·XH_2O, 10^{-2} mg of $MnCl_2 \cdot 4H_2O$, 10^{-3} mg of $ZnSO_4 \cdot 7H_2O$, 10^{-3} mg of $CoCl_2 \cdot 6H_2O$, 10^{-3} mg of $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$, and 10^{-4} mg of $CuSO_4 \cdot 5H_2O$. Pollock's growth medium (14) consisted of (per liter): 5.9 g of trisodium citrate·$2H_2O$; 0.52 g of $MgSO_4 \cdot 7H_2O$; 2.72 g of KH_2PO_4; 2.0 g of $(NH_4)_2SO_4$; 10 g of casein hydrolysate; and 2 g of glucose. Gelatin

(0.1%) was present in both media, and the *p*H was adjusted to 7.0.

The high phosphate medium was prepared by adding KH_2PO_4 to the low phosphate medium to give a final molarity of 0.32 for PO_4, and by adjusting the *p*H to 7.0 with KOH pellets. Growth was measured turbidimetrically on a Zeiss spectrophotometer at 620 mμ.

Enzyme assay. Penicillinase production was followed by transferring a 2-ml sample from the culture at the desired time intervals to a tube that contained 0.4 ml of 8×10^{-4} M 8-hydroxyquinoline (which inhibits further enzyme synthesis). The sample was mixed, centrifuged, and the supernatant fluid was assayed for penicillinase activity by the Pardee iodine method which is as follows: 2.8 ml of a solution composed of 60 ml of water, 15 ml of 0.5 M phosphate, *p*H 7; and 3.0 ml of 3.5×10^{-3} M I_2 plus 2 M KI; and 0.1 ml of culture supernatant fluid (or a sample of supernatant fluid diluted to 0.1 ml). After the mixture had equilibrated at room temperature, 0.1 ml of penicillin substrate (2.8 mg/ml) was added, and the rate of change in optical density of the iodine solution at 350 mμ was observed for 5 min on a Zeiss spectrophotometer. The micromoles of penicillin hydrolyzed were calculated from the rate of change of optical density (OD). A ΔOD_{350} of 3.7 = 0.1 μmole of penicillin hydrolyzed.

Chemicals. Bacitracin and D-cycloserine were obtained from the Sigma Chemical Co., St. Louis, Mo., and penicillin from Calbiochem, Los Angeles, Calif. Actinomycin D and chloramphenicol were gifts from Merck Chemical Co. and Parke-Davis and Co., respectively.

RESULTS

Induction with phosphate. Penicillinase, like all inducible enzymes, is produced in low amounts in the absence of an inducer. Upon the addition of the specific inducer, penicillin, the rate of penicillinase synthesis increases sharply. Recently, Csányi and collaborators reported that electrolytes also induce penicillinase synthesis (Csányi, *in press*). Data represented in Fig. 1 (curves A and B) show that the amount of penicillinase produced by *B. cereus* 569 is approximately 12-fold above the noninduced level when 0.3 M phosphate, *p*H 7.0, is added to the culture medium. Similarly, curves C and D of Fig. 1 show that the amount of penicillinase produced in the presence of penicillin is also increased (approximately fivefold) upon the addition of 0.3 M phosphate.

Penicillinase is known to adhere to glass surfaces, while high salt concentrations or gelatin (10) have been shown to prevent this adsorption. Therefore, gelatin was added routinely to Csányi's medium to ensure that the increased level of penicillinase in the presence of 0.3 M phosphate was not due simply to a decrease in adsorption onto the surface of the glass culture flask. Since

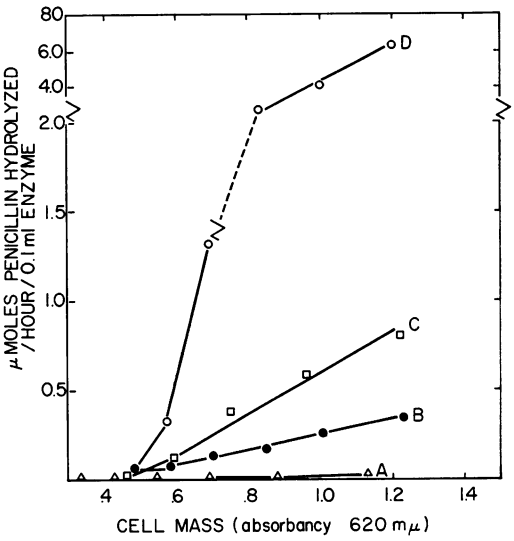

FIG. 1. *Effect of orthophosphate on penicillinase induction (Csányi medium). A culture of Bacillus cereus 569 growing exponentially in low PO_4 Csányi medium was collected on a membrane filter (Millipore Corp., Bedford, Mass.) and resuspended in fresh Csányi medium that was supplemented as follows: △, no supplement; ●, 0.3 M PO_4; □, 1 unit/ml of penicillin; ○, 0.3 M PO_4 plus 1 unit/ml of penicillin. The amount of enzyme activity in each culture was determined as the cell mass increased.*

Csányi's medium contains a nonionic detergent, and thereby differs from the simple Casamino Acids-glucose-salts-gelatin medium (Pollock's medium) generally used for the studies on penicillinase induction in *B. cereus* 569, the phenomenon of phosphate induction of penicillinase was examined using the standard medium. These results, depicted in Fig. 2, show that 0.3 M phosphate also induced penicillinase formation in this medium. Furthermore, upon comparing the data presented in Fig. 1 and 2, it is apparent that more penicillinase accumulates in Pollock's medium than in Csányi's medium.

The lower activity in Csányi's medium is not due to inhibition of the enzyme, because enzyme formed in Pollock's medium is not inhibited when placed in Csányi's medium.

De-induction studies. When *B. cereus* 569 is exposed to penicillin, about 200 molecules of the inducer are bound per cell (14). Furthermore, penicillin-induced cells continue to form penicillinase for several generations in the absence of free penicillin. It is not clear whether the continued synthesis of penicillinase in the absence of free inducer is due to the continued synthesis of penicillinase messenger ribonucleic acid (RNA) induced by the membrane-bound penicillin, or

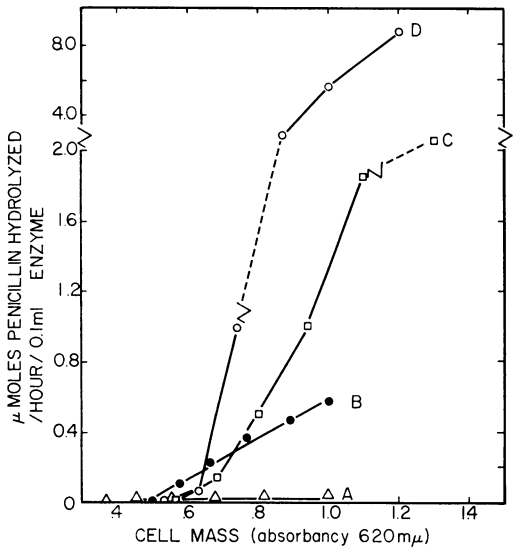

FIG. 2. *Effect of orthophosphate on penicillinase induction (Pollock medium). A culture of Bacillus cereus 569 growing exponentially in low PO_4 Pollock medium was collected on a membrane filter and resuspended in Pollock medium that was supplemented as follows: Δ no supplement; ●, 0.3 M PO_4; □, penicillin, 1 unit/ml; ○, 0.3 M PO_4 plus penicillin, 1 unit/ml. The amount of enzyme activity in each culture was determined as the cell mass increased.*

to the existence of a long-lived messenger RNA, or both. Although actinomycin D has been used in the examination of this question, the answer is not firmly established. Therefore, cells induced by phosphate were examined for penicillinase synthesis following deinduction. The cells were phosphate-induced, collected, washed, and resuspended in low phosphate medium. Samples were taken from de-induced and control cultures at various times and assayed for penicillinase activity. The results of these studies, presented in Fig. 3, show that the phosphate-induced cells rapidly lose their ability to make high (induced) levels of penicillinase when phosphate is removed from the culture medium. These results could be explained by any of the three following possibilities: (i) phosphate induces the synthesis of a short-lived penicillinase messenger in *B. cereus*; (ii) phosphate induces the synthesis of a penicillinase messenger whose translation requires the presence of a high concentration of phosphate; or (iii) the mechanism whereby phosphate produces penicillinase accumulation does not include messenger synthesis.

Effect of chloramphenicol or actinomycin D on phosphate induction of penicillinase. Chloramphenicol is known to inhibit protein synthesis in *B. cereus* 569 (8). Therefore, chloramphenicol was added to cultures of phosphate-induced *B. cereus* in an effort to establish whether phosphate induces penicillinase synthesis de novo, or whether it, in some way, stabilizes or activates the enzyme. Data presented in Fig. 4 show that the induction of penicillinase by phosphate is inhibited by chloramphenicol. Furthermore, the activity of penicillin-induced penicillinase is not increased when exposed to 0.3 M phosphate. Thus, it appears that the phosphate-dependent accumulation of penicillinase results from an increased rate of synthesis of the enzyme.

Actinomycin D, an inhibitor of deoxyribonucleic acid (DNA)-dependent RNA synthesis (15), was employed in an attempt to determine whether or not the phosphate-induced synthesis of penicillinase requires RNA synthesis. Since induction is inhibited by actinomycin D, these studies (Fig. 4) suggest that phosphate may, in some way, induce the synthesis of a penicillinase-specific messenger RNA.

Effect of inhibitors of cell-wall synthesis on penicillinase induction. Penicillin inhibits cell wall syn-

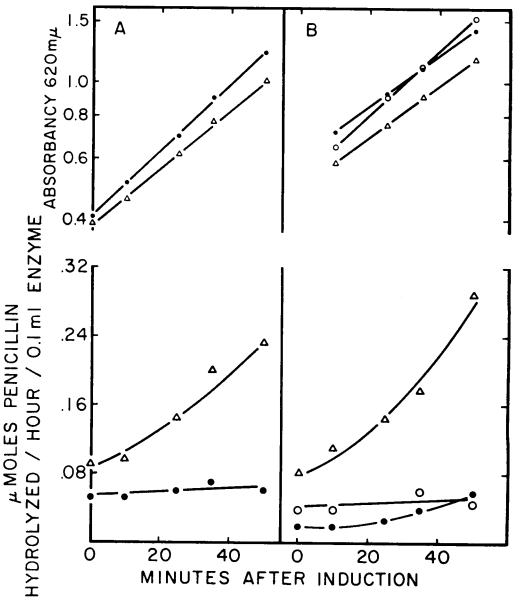

FIG. 3. *Effect of phosphate removal on penicillinase production. A culture of Bacillus cereus 569 growing exponentially in low PO_4 Csányi medium was collected on a membrane filter and resuspended in: (●) low PO_4 Pollock medium or (△) 0.3 M PO_4 Csányi medium (A). After 50 min of growth, the culture incubated in the presence of high PO_4 was collected on a membrane filter, washed with low PO_4 medium, and transferred to: ●, low PO_4 Pollock; △, 0.3 M PO_4 Csányi; or ○, low PO_4 Csányi media (B).*

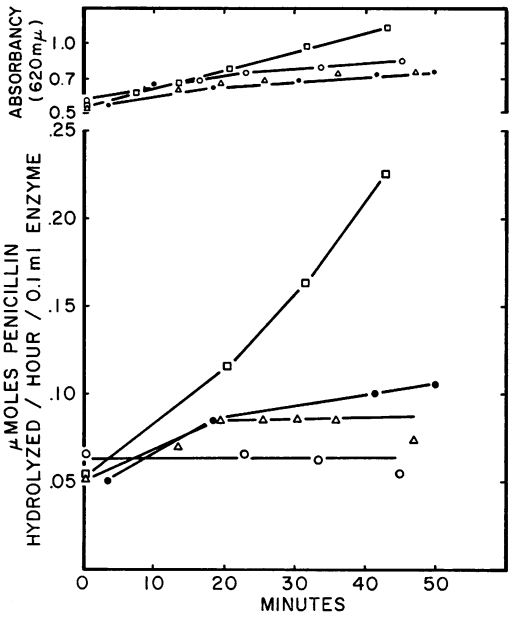

FIG. 4. *Effect of actinomycin or chloramphenicol on the phosphate-dependent induction of penicillinase. An exponentially growing culture of Bacillus cereus 569 was collected on a membrane filter and resuspended in Csányi 0.3 M phosphate medium which was supplemented as follows: □, control; ○, chloramphenicol (20 μg/ml); △, actinomycin D (0.24 μg/ml); ●, 0.24 μg/ml of actinomycin D and penicillin (1 unit/ml) added 3 min after actinomycin.*

thesis. In addition, it binds to the cell membrane of *B. cereus* 569 and apparently exerts its influence as an inducer of penicillinase while bound to the cell membrane (2). High concentrations of phosphate also induce penicillinase synthesis, and it is possible that media of high ionic strength may alter the properties of the cell membrane because of its hypertonicity. Therefore, antibiotics known to interfere with cell wall synthesis were examined to see if general disruption of the cell wall, and perhaps the cell membrane, is sufficient to induce penicillinase synthesis.

These data, presented in Fig. 5, show that bacitracin and cycloserine inhibit cell growth but apparently do not enhance the synthesis of penicillinase. Thus, simple disruption of the cell wall, and perhaps of the cell membrane, does not appear to account for the phosphate-induced synthesis of penicillinase in *B. cereus* 569.

Induction lag. Data presented in Fig. 1 and 2 suggest the possibility that phosphate may alter the length of the induction lag observed when penicillinase is induced by penicillin. Therefore, a careful investigation of the effect of phosphate on the lag was undertaken. For the experiments shown in Fig. 6, cells were grown on Csányi's low phosphate medium for several generations, then collected, and resuspended in fresh medium supplemented with 0.3 M phosphate. Samples were removed from the culture at the times indicated and assayed for enzyme activity. Experiments were performed at 30, 37, and 39 C to determine the effect of temperature on the induction lag. These data indicate that the length of the initial lag (i.e., the induction of penicillinase with phosphate) is approximately 10 min and comparable to the lag observed when penicillinase is induced with penicillin in low phosphate media (Fig. 1, 2, and 7). On the other hand, the second induction lag, i.e., the time lag between the addition of penicillin to the phosphate-induced culture and the greatly enhanced synthesis of the enzyme, is approximately 3 min at 37 and 39 C, while at 30 C it is approximately 6 min. The important observation in these studies, however, is the fact that the lag following penicillin induction in high phos-

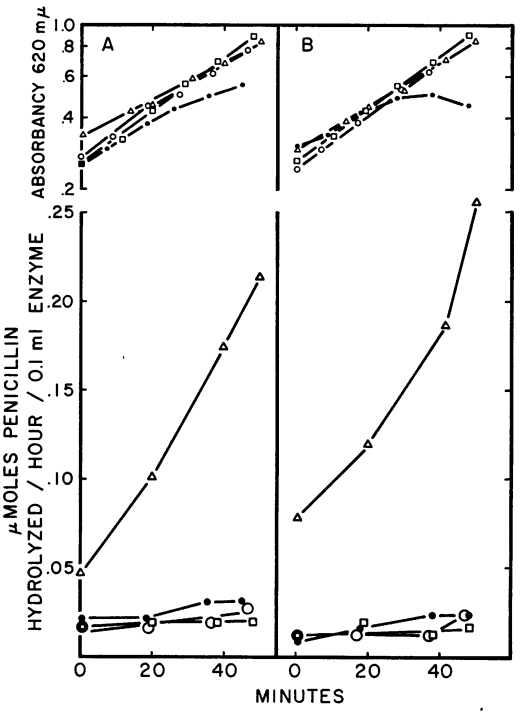

FIG. 5. *Effect of inhibitors of cell wall synthesis on penicillinase induction. Bacillus cereus 569 growing exponentially in low PO_4 Csányi medium was collected on a membrane filter and resuspended in Csányi medium containing: □, no supplement; △, 0.3 M PO_4; ○, 0.7 mg/ml of bacitracin; or ●, 3.0 mg/ml of bacitracin (A): or □, no supplement; △, 0.3 M PO_4; ○, 0.010 mg/ml of D-cycloserine; or ●, 0.025 mg/ml of D-cycloserine (B).*

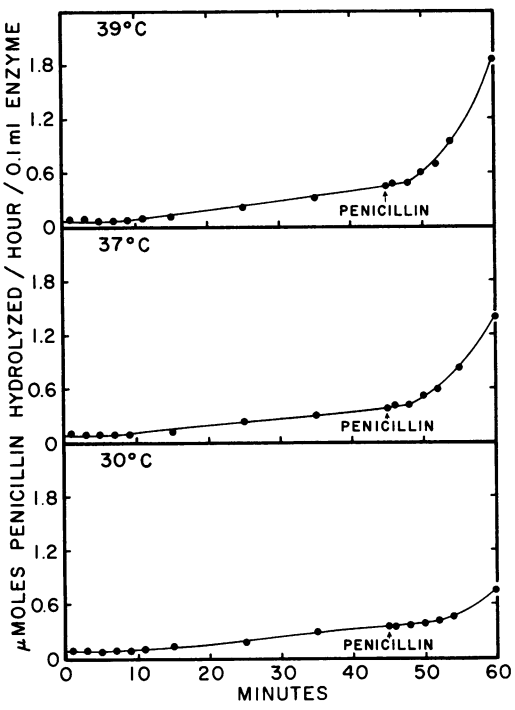

FIG. 6. *Lag period following penicillinase induction by penicillin in phosphate-induced cells. Bacillus cereus 569 growing exponentially in low PO_4 Csányi medium at 37 C at 30, 37, or 39 C. After 45 min of growth, penicillin (1 unit/ml) was added to the PO_4-induced cells.*

phate medium is approximately 3 min, while the comparative lag in low phosphate medium is reported routinely to be more than 6 min (12).

DISCUSSION

Although the mechanism of penicillinase induction has been studied extensively (5, 7, 12, 14), it is not well understood. The doubling times for *E. coli* and *B. cereus* 569 grown on rich medium are similar; yet the induction lag for β-galactosidase at 37 C is 3 min (11) and that reported for penicillinase is 6 to 12 min (12). The time required for the synthesis of a molecule of messenger RNA in *E. coli* is approximately 2 min (6), whereas a protein molecule is synthesized in a few seconds (9). A 3-min lag period is not inconsistent with these rates of synthesis. On the other hand, a lag period of 6 to 12 min cannot be explained readily in these terms. Similarly, the half-life of messenger RNA in exponentially growing *E. coli* is 1 to 4 min (3, 9), while the penicillinase messenger of *B. cereus* is estimated to be 20 to 60 min (5, 13). The half-life of *E. coli* messenger has been substantiated with a number of experimental techniques. The longevity of the penicillinase messenger, however, is based solely on experiments with actinomycin D.

The observation by Csányi et al. (*in press*) that phosphate induces penicillinase in *B. cereus* 569 provides an opportunity for the reexamination of some of the parameters of the inducible penicillinase systems. The data presented in Fig. 1 and 2, coupled with the controls described in Results, strongly suggest that penicillinase synthesis in *B. cereus* is induced by 0.3 M phosphate, *p*H 7.0. The data provided in Fig. 3 show that when cells induced by 0.3 M phosphate are transferred to a low phosphate medium, they immediately lose their ability to form penicillinase at the induced level. These findings are not inconsistent with the notion that 0.3 M phosphate induces the formation of short-lived penicillinase messenger.

In an attempt to determine the mechanism whereby phosphate produces penicillinase accumulation, phosphate-induced cultures were treated with chloramphenicol or actinomycin D. As shown in Fig. 4, the phosphate-dependent accumulation of penicillinase is chloramphenicol-sensitive. This observation suggests that the phos-

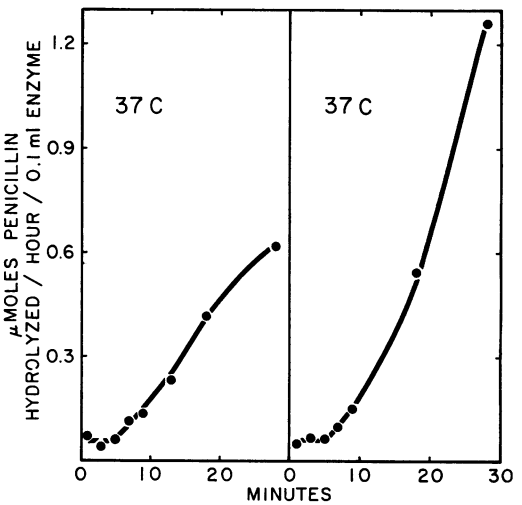

FIG. 7. *Lag period following penicillinase induction by penicillin in low PO_4 Csányi or Pollock medium at 37 C. Bacillus cereus 569 growing exponentially in low PO_4 Csányi medium was collected on a membrane filter and resuspended in either low PO_4 Csányi medium (A) or low PO_4 Pollock medium (B). Penicillin (1 unit/ml) was added to each flask at zero time.*

phate-induced accumulation of penicillinase activity results from de novo synthesis of penicillinase or some other protein which is required to activate a preexisting penicillinase precursor. When actinomycin D and phosphate are added to the culture medium simultaneously, penicillinase accumulation does not occur (see Fig. 4). This observation suggests that the phosphate-dependent accumulation of penicillinase requires RNA synthesis. These data, taken collectively, suggest that phosphate induces the formation of a short-lived messenger that is required for penicillinase synthesis or that phosphate induces the formation of a long-lived messenger RNA for penicillinase that requires the presence of 0.3 M phosphate for its translation. This conclusion leads to the prediction, which is contradictory to previous reports (5, 13), that the membrane-bound penicillin stimulates the synthesis of penicillinase messenger in the presence of low levels of actinomycin D or that the membrane-bound penicillin influences the translation of the penicillinase messenger, perhaps through messenger stabilization (7).

The data presented in Fig. 1 and 2 suggest the possibility that phosphate may shorten the penicillin-dependent induction lag. Therefore, the penicillin-dependent induction lag was carefully compared in high and low phosphate media. It was found (Fig. 6 and 7) that the penicillin-dependent lag in the presence of 0.3 M phosphate is approximately 3 min at both 37 and 39 C, while in low phosphate the lag is about 6 min. These data also show that the phosphate-dependent induction lag in the absence of penicillin is approximately 6 min at 37 C.

It appears, therefore, that at 37 C, the phosphate-dependent lag is similar in duration to the penicillin-dependent lag in low phosphate medium, and that phosphate induction shortens the penicillin-dependent lag from 6 min to approximately 3 min.

The results reported in this communication suggest that under certain defined conditions the mechanism of penicillinase induction may not differ as drastically from that of β-galactosidase induction in *E. coli* as has been reported. This statement is based on the following observations: (i) the induction lag for β-galactosidase is approximately 3 min (11) and the penicillin-dependent induction lag for penicillinase in the presence of 0.3 M phosphate is of the same duration; (ii) removal of the β-galactosidase inducer from the medium causes β-galactosidase synthesis to revert rapidly to the uninduced level (9, 11), and the phosphate-dependent synthesis of penicillinase likewise reverts to the uninduced level when cells are transferred from high phosphate to low phosphate medium; (iii) the half-life of the β-galactosidase messenger has been estimated to be approximately 1 min (9), while the actinomycin-sensitivity of phosphate-dependent synthesis of penicillinase, coupled with the phosphate de-induction studies reported here, are not inconsistent with a short half-life for the penicillinase messenger.

ACKNOWLEDGMENT

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