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Regulation of Penicillinase Synthesis: Evidence for a Unified Model

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The kinetics of penicillinase induction in *Bacillus cereus* 569 was investigated. An increase in the rate of penicillinase synthesis was demonstrated within 30 sec of the addition of inducer (benzylpenicillin); however, the maximum induced rate of penicillinase synthesis was not attained until at least 30 min after the addition of inducer. In contrast to earlier claims, a quantitative estimate showed that the penicillinase messenger ribonucleic acid (mRNA) half-life is approximately 2 min. These findings strongly suggest that the rate of synthesis of penicillinase mRNA increases continuously during most of the 30-min latent period. A model for the regulation of penicillinase synthesis in three gram-positive organisms is presented which is consistent with a nondiffusible inducer, a short-lived mRNA, a relatively long latent period (i.e., an apparently slow inactivation of penicillinase repressor), and the existence of at least two regulatory genes.

Although the regulation of penicillinase synthesis has been studied extensively (2, 22, 26) a unified concept, or model, consistent with the published data has not been provided. The lack of a realistic working model is due in part to inconsistencies in the various biochemical data which have been reported and in part to a dearth of genetic data. Recently, a reliable procedure for transformation of *Bacillus licheniformis* was established, and as a result Sherratt and Collins (28; *private communication*) have been conducting an analysis of the genetic determinants which are responsible for the synthesis and regulation of penicillinase in *B. licheniformis*. These data are consistent with an earlier description of the genetics of penicillinase regulation in *Staphylococcus aureus* (3, 24, 25). Therefore, it appeared that a reexamination of the biochemical aspects of penicillinase regulation might permit the construction of a realistic model for the regulation of penicillinase synthesis in these gram-positive organisms.

This report provides basic biochemical data which describe the induction of penicillinase synthesis in *B. cereus* 569. These data, combined with recent genetic evidence and other well-established observations, permit the construction of a general model for the regulation of penicillinase synthesis in *B. cereus* 569, *B. licheniformis* 749, and *S. aureus*. A description of this model is presented.

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MATERIALS AND METHODS

Cell growth. *B. cereus* 569 (inducible for penicillinase) was used in these studies. Exponentially growing cultures were prepared by incubating approximately 2×10^4 spores for 10 hr. Unless stated otherwise, all cultures were grown with vigorous shaking in a Mickle shaker at 37 C on the standard Casamino Acids-glucose-salts-gelatin medium (13). Prior to the start of an experiment, the cells were collected by centrifugation (4,000 × *g* for 4 min at room temperature) and resuspended in prewarmed (37 C) medium. They were then recentrifuged, and the cell pellet was suspended in the required volume of medium. Cell growth was measured turbidimetrically, at 620 nm, by use of a Unicam SP 500 spectrophotometer. The doubling time of both cells and protoplasts was approximately 35 min.

Enzyme assay. Culture samples to be assayed for penicillinase activity (usually 5 ml) were transferred to tubes which contained 0.1 volume of either chilled 1.6×10^{-3} M 8-hydroxyquinoline (19) or crushed ice composed of 2×10^{-2} M tris(hydroxymethyl)aminomethane, *p*H 7.3, 400 μg of chloramphenicol per ml, and 0.02 M NaN_3 (16). The samples were mixed vigorously and maintained in ice until assayed. When only cell-bound penicillinase was to be assayed, 20-ml culture samples were taken. The mixtures were centrifuged as described above, and the cell pellet was suspended in 10 ml of cold 0.3 M potassium phosphate buffer, *p*H 7.0. The centrifugation was repeated, and the resulting pellet was suspended in 2.2 ml of

cold 0.3 M phosphate buffer. Penicillinase activity was assayed by the standard Perret procedure (18). A unit of penicillinase is defined as the amount of enzyme activity required to hydrolyze 1 μmole of benzylpenicillin per hr at *p*H 7.0 and 30 C. Specific activity is defined as units of penicillinase per milligram (dry weight) of cells.

^{14}C-penicillin binding. The amount of radioactive benzylpenicillin bound to *B. cereus* 569 was measured by a modification of the procedure by Pollock and Perret (23). A 2-liter culture of log-phase cells was collected rapidly with a Sorvall RC2 centrifuge equipped with a Szent-Gyorgyi and Blum continuous-flow system (15,000 × *g* for 5 min at 2 C). The cell pellets were combined in 120 ml of cold 0.01 M potassium phosphate buffer, *p*H 7.0, and centrifuged. The washed pellets were suspended in 210 ml of cold 0.01 M phosphate, the suspension was divided equally into seven samples, and various amounts of ^{14}C-penicillin added. After stirring, the samples were allowed to stand in ice for 1 hr. The cells were collected by centrifugation (10,000 × *g* for 5 min at 2 C), and each pellet was resuspended in 30 ml of cold buffer, shaken vigorously, and centrifuged. The washing procedure was repeated three times. The final pellet from each sample was suspended in 10 ml of cold 0.01 M phosphate buffer and 0.1 ml of a 5% suspension of Primafloc was added. The flocculated cells from each sample were collected on several glass-fiber discs which were then placed in a scintillation vial and allowed to dry overnight at 60 C. After cooling, 15 ml of scintillation fluid (toluene plus phosphors) was added to each vial, and the radioactivity was measured with a Beckman LS 200 liquid scintillation spectrometer (6).

Protoplasts. Protoplasts were prepared essentially by the procedure of Duerksen (7, 8). Early log-phase cells were collected by centrifugation (4,000 × *g* for 4 min at room temperature), washed in an equal volume of 0.01 M phosphate buffer, *p*H 7.0, centrifuged, and resuspended in 0.2 volumes of buffered sucrose solution (8) which contained 100 μg of lysozyme per ml. The suspension was allowed to stand at 37 C for 1 hr. Microscopic examination revealed that at least 95% of the cells had become spherical during the first 30 min of the treatment, and a standard staining procedure (5) failed to detect any cell wall material associated with the protoplasts. The protoplasts were collected by centrifugation (*see above*). They were then spread on glass-fiber discs and assayed for radioactivity, or they were resuspended gently in prewarmed (37 C) medium supplemented with 0.05 M $MgSO_4$, 0.3 M sucrose, and 1% glucose (7), and allowed to grow with gentle shaking. Penicillinase activity was assayed as described above.

Materials. Labeled benzylpenicillin [potassium 6-phenyl (acet-1-^{14}C) amidopenicillinate], specific activity of 20.7 μc/μmole, was obtained from Amersham-Searle, Des Plaines, Ill.; rifampicin, from Ciba Ltd., Basel, Switzerland; and Primafloc, from the British Drug Houses, Poole, England.

RESULTS

Kinetics of induction. It has been reported (e.g., 10, 26) that there is a lag of about 10 min between the time of addition of the inducer and the initial increase in penicillinase activity. That this is not the case is shown in Fig. 1. These data show that the specific activity of cell-bound penicillinase increases within 30 sec of the addition of inducer. Furthermore, the data presented in Fig. 2 show that the rate of penicillinase accumulation follows a smooth, continuous time path.

It has also been reported that, following the addition of inducer, the rate of penicillinase synthesis continues to increase for approximately 15 min, after which time synthesis proceeds linearly (20, 22). The data provided in Fig. 2 show that the rate of penicillinase synthesis increases for at least 30 min after the addition of inducer. Indeed, a differential plot constructed from these data (i.e., units penicillinase synthesized per milliliter per minute plotted against time) produces an approximately symmetrical sigmoid curve with an inflection point at 16 min. It should be stressed that the increase in the rate of penicillinase synthesis which occurs more than 5 min after the addition of inducer results from expression of the previously "bound" inducer and not from a greater uptake of inducer. This statement is based on the fact that approximately 90% of the penicillinase activity synthesized under these conditions is secreted by the cells into the culture medium, and that the extracellular penicillinase which has accumulated

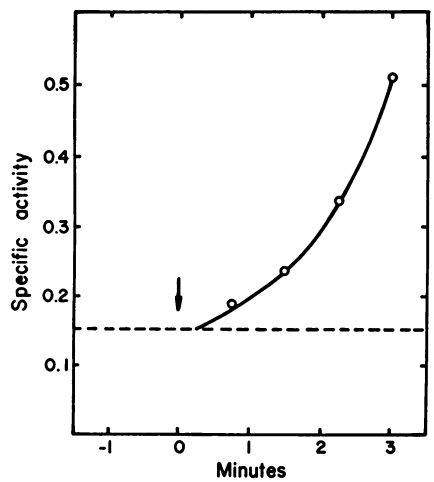

FIG. 1. *Initial kinetics of penicillinase induction. Inducer (2 units of benzylpenicillin/ml) was added at zero time. Only cell-bound penicillinase was assayed.*

in the culture medium during the first 5 min of induction is sufficient to destroy essentially 100% of the exogenous inducer.

Penicillin binding. *B. cereus* 569 is known to bind penicillin essentially irreversibly (7, 23). Although it has been estimated that 80 to 200 molecules of penicillin are bound per cell (22, 23), the data presented in Fig. 3 show that there are approximately 1,000 molecules of ^{14}C-penicillin bound per cell. These data also show that approximately 50% of the maximum specific binding is achieved in the presence of 0.3 unit of benzylpenicillin per ml. The earlier, and smaller, numbers were obtained by plating to infinite thickness and using a gas-flow detector (23), whereas the data represented in Fig. 3 were obtained by use of liquid scintillation spectrometry, which permits radioactivity to be expressed directly as disintegrations per minute.

Protoplasts. Several attempts have been made to determine the nature and location of the factor to which penicillin is bound in *S. aureus* (4, 27); however, only one such study has been reported to date for *B. cereus* 569 (7). In that report (7), it was concluded that the bound

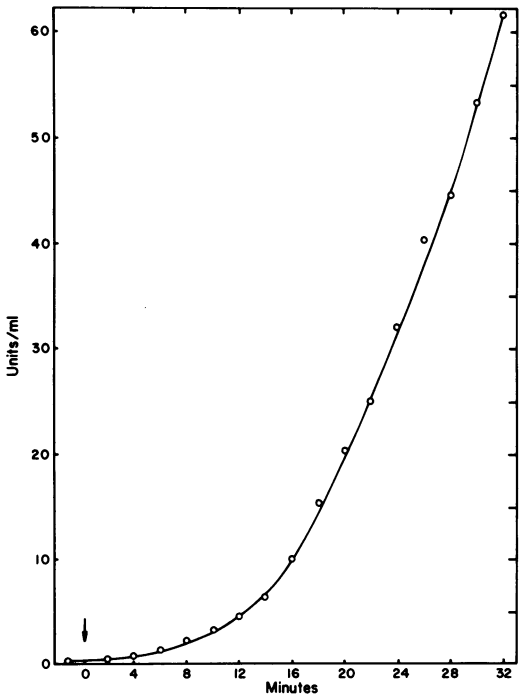

FIG. 2. *Kinetics of penicillinase induction. Inducer (2 units of benzylpenicillin/ml) was added at zero time. Total penicillinase activity (i.e., extracellular and cell-bound enzyme) was assayed.*

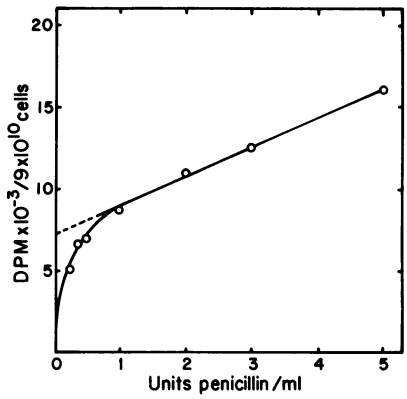

FIG. 3. *Binding of ^{14}C-benzylpenicillin by Bacillus cereus 569. The extrapolated line indicates the amount of nonspecific binding.*

penicillin is associated with a particulate material which resides in the space between the cell wall and membrane and which is liberated from the cell during protoplast formation. The data listed in Table 1 show that approximately 75% of the ^{14}C-penicillin "bound" to the cell after 1 min, or after 20 min of normal cell growth, is retained by the cell after protoplast formation. This finding is supported by the data presented in Fig. 4, which show: (i) that protoplasts prepared from uninduced cells produce penicillinase at a rate which is only slightly higher than that of uninduced cells, regardless of whether or not penicillin is added to exponentially "growing" protoplasts; (ii) that protoplasts prepared from cells which were exposed to penicillin 60 min prior to their conversion to protoplasts synthesize penicillinase at approximately the same rate as induced control cells; and (iii) that the rate of penicillinase synthesis by protoplasts prepared from cells which were exposed to penicillin 15 min prior to their conversion to protoplasts increases with time as the protoplasts are increasing in mass. These results suggest that the "bound" penicillin is retained by the protoplasts but that protoplasts themselves, as Duerksen has reported (7), are apparently incapable of binding penicillin.

Messenger ribonucleic acid (mRNA) half-life. Previous reports suggested that the penicillinase produced by *B. cereus* 569 is translated from an mRNA whose life time was estimated to be 20 min (10) to 40 min (21). Indeed, continued synthesis of penicillinase by protoplasts was assumed to result from a long-lived mRNA (7). Since the results presented in Table 1 and Fig. 4 strongly suggest that the inducer, penicillin, is

TABLE 1. *Retention of ^{14}C-penicillin by protoplasts*[a]

Sample	Net disintegrations/min	Disintegrations per min per 40 ml	Percentage of ^{14}C retained by protoplasts
1 min			
Cells	4,100	8,200	
Protoplasts	6,100	6,100	74
21 min			
Cells	4,340	8,680	
Protoplasts	7,110	7,110	82

[a] Early log-phase cells (154 mg, dry weight) were collected from 640 ml of culture medium by centrifugation. The washed cell pellets were suspended in 100 ml of fresh, prewarmed medium and, after vigorous shaking, 0.5 unit of ^{14}C-benzylpenicillin per ml was added. Shaking at 37 C was continued for 60 sec, the culture was evenly divided, and 5 ml of 8-hydroxyquinoline was added to one fraction (A) to prevent further cell metabolism. Both fractions (A and B) were centrifuged, and the cell pellet from B was suspended in 320 ml of warm medium and allowed to grow (and "transport" the bound penicillin if necessary) for 20 minutes at 37 C. Cells from fraction A were suspended in 60 ml of 0.01 M PO_4, *p*H 7.0, at 37 C, divided into two fractions (A-20 ml and A-40 ml), and both fractions were centrifuged. The pellet from A-20 was suspended in 10 ml of 0.01 M PO_4; the cells were precipitated with Primafloc, collected on glass-fiber discs, and assayed for radioactivity. The cell pellet from A-40 was suspended in 50 ml of buffered sucrose plus lysozyme, and the suspension was allowed to stand at 37 C for 60 min. Cells from fraction B were harvested by centrifugation, washed, recentrifuged, suspended in 60 ml of 0.01 M PO_4, divided into B-20 ml and B-40 ml, and then processed like the cells from fraction A.

retained by the protoplasts, and since it has been reported that the penicillinases produced by *S. aureus* (15) and *B. licheniformis* 749 (J. W. Davies, J. Gen. Microbiol., *in press*) are coded by a "short-lived" mRNA, an attempt was made to estimate quantitatively the functional half-life of the penicillinase mRNA in *B. cereus* 569. As shown in Fig. 5, rifampicin, which is known to inhibit bacterial RNA polymerase activities (29), inhibits both cell growth and penicillinase synthesis. Since the concentration of rifampicin used (2.5 μg/ml) is sufficient to inhibit RNA synthesis at least 95% (Davies, *in press*), one can estimate quantitatively the functional half-life of penicillinase mRNA (Fig. 6). These results suggest that the half-life is approximately 2 min. A similar value was obtained when 2.0 μg of actinomycin D per ml was used in place of rifampicin (Imsande and Davies, *unpublished results*).

Latent period. If one assumes that mRNA is

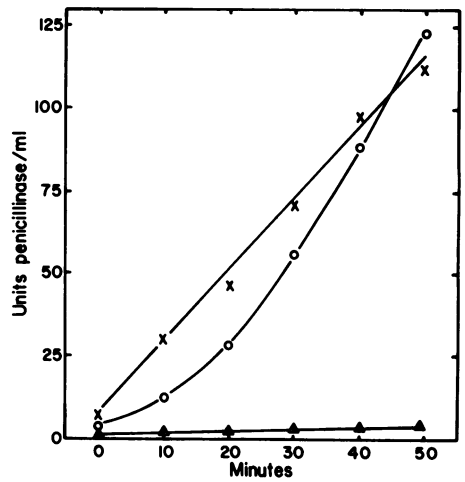

FIG. 4. *Synthesis of penicillinase by protoplasts prepared from preinduced cells. Cells were exposed to 1 unit of benzylpenicillin per ml 15 (○) or 60 (×) min prior to protoplast formation. Protoplasts prepared from uninduced cells were grown in the absence of penicillin (●) or in the presence of 1, 10, or 100 units of benzylpenicillin per ml (△).*

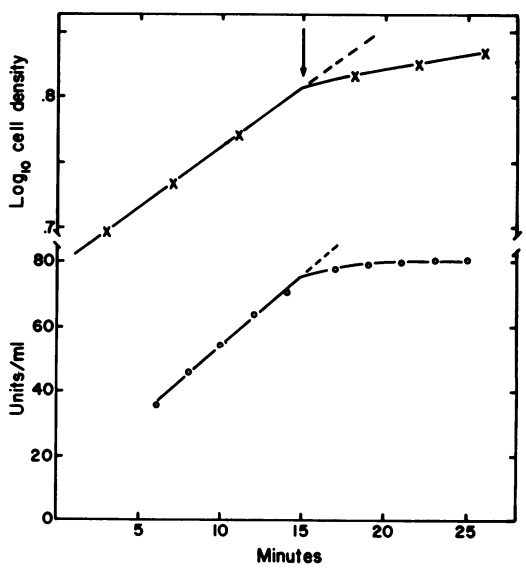

FIG. 5. *Inhibition of cell growth and penicillinase synthesis by rifampicin. Cells were exposed to 1 unit of benzylpenicillin per ml 60 min prior to zero time. Rifampicin (2.5 μg/ml) was added at 15 min. (Increases in absorbancy at 620 nm after the addition of rifampicin are due to cell wall synthesis and cell elongation and not to net protein synthesis.)*

rate-limiting in enzyme synthesis during exponential cell growth, then a shift from the basal level of enzyme formation (steady state 1) to the induced level of enzyme synthesis (steady state 2) must be accompanied by a corresponding increase in the amount of that specific mRNA per cell. Furthermore, if the rate of mRNA synthesis is shifted abruptly from the basal rate to the induced rate, as appears to be the case for most inducible bacterial enzyme systems, then the time path by which mRNA accumulates within the newly induced cell is determined by the functional half-life of that mRNA. This conclusion can be represented by the following mathematical formulations.

Let X equal the new constant rate of mRNA synthesis. When the cell reaches the new steady state, it will have accumulated N molecules of the specific mRNA. The rate of decay of this mRNA is $-dN/dt$, where $-dN/dt = \lambda N$ and λ is the decay constant. Now, at the new steady state the rate of mRNA synthesis (X) must equal the rate of mRNA decay ($-dN/dt$). Therefore, $X = \lambda N$. Since the half-life of the penicillinase mRNA is approximately 2 min (Fig. 6), and since $\lambda = \ln 2/t_{1/2}$, then $N = (2)(X)/0.693 = 2.89\ X$.

This formulation provides neither the rate of mRNA synthesis nor the number of molecules of mRNA that have accumulated; however, the time path by which the rate of enzyme synthesis changes during an abrupt shift from one steady state to another can be calculated (Fig. 7). It can be seen that 95% of the asymptotic value is reached approximately 6 min after the addition of inducer. Although the theoretical curve (Fig. 7, curve D) presented is in excellent agreement with the available experimental data for the accumulation of β-galactosidase following its induction in *Escherichia coli* (17), the experimental curve (Fig. 7, curve E) for the accumulation of penicillinase following its induction in *B. cereus* 569 differs markedly from the theoretical curve. This observation is offered as evidence that the rate of synthesis of penicillinase mRNA does not change abruptly from one steady state to another; rather, the rate of synthesis of penicillinase mRNA increases gradually during the latent period. As stated above (*see* Kinetics of induction), the differential rate of penicillinase synthesis after the addition of inducer proceeds by an approximately symmetrical sigmoid curve with an inflection point at 16 min. By curve-fitting, an idealized induction response curve (i.e., the percentage of the maximum response acquired per minute) can be obtained (Fig. 8, curve A). The integration of the idealized induction response curve generates a theoretical maximum response curve (or mRNA accumulation curve; Fig. 8, curve B), which in turn gives rise to a theoretical induction curve (Fig. 8, curve C). Since the theoretical induction curve (curve C) is essentially identical to the experimentally determined induction curve (Fig. 2)

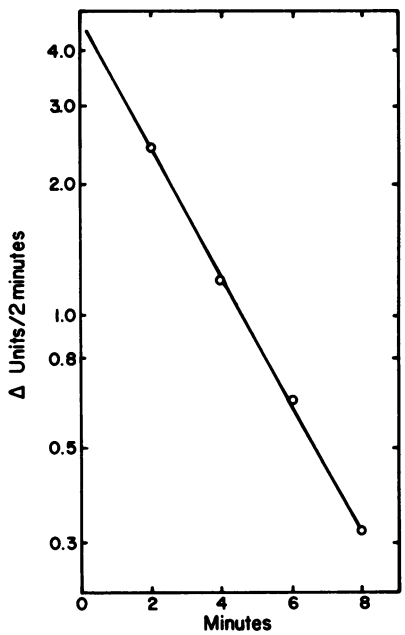

FIG. 6. *Differential rate of penicillinase synthesis after inhibition by rifampicin. See Fig. 5.*

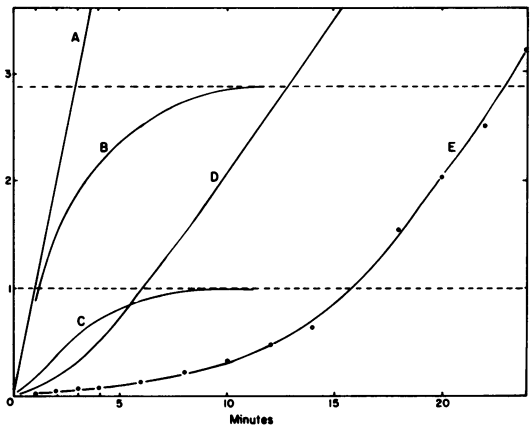

FIG. 7. *Theoretical curves for the induction of penicillinase. Curve A, rate of synthesis of penicillinase mRNA. Curve B, rate of accumulation of penicillinase mRNA, assuming* $t_{1/2} = 2$ *min. Curve C, rate of degradation of penicillinase mRNA, assuming* $t_{1/2} = 2$ *min. Curve D, theoretical penicillinase induction curve. Curve E, experimentally determined penicillinase induction curve.*

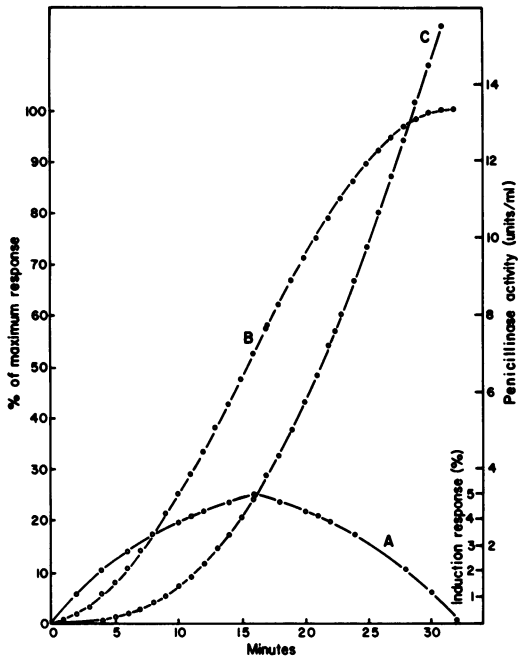

FIG. 8. *Idealized inducer-response curves.*

one can assume that the induction response is accurately described by curve A, Fig. 8. Thus, the kinetics of induction, when combined with genetic evidence which shows that penicillinase synthesis is regulated at least in part by negative control (24), suggests that the penicillinase repressor molecules are inactivated very slowly.

DISCUSSION

In this investigation, some of the biochemical aspects of penicillinase induction in *B. cereus* 569 were examined. It was shown that a minimum of 1,000 molecules of penicillin are bound per cell and that approximately 80% of the essentially irreversibly bound inducer is retained by the protoplast after the removal of the cell wall. The half-life of penicillinase mRNA was estimated quantitatively. In contrast to previous reports (10, 21), the functional half-life of penicillinase mRNA was shown to be approximately 2 min. Also, the latent period (i.e., the time required, following the addition of inducer, for the attainment of the maximal response to the inducer) was examined. Although the exact cause of the relatively long latent period in penicillinase induction is unknown, it can be analyzed theoretically. If one assumes that mRNA is rate-limiting in penicillinase synthesis (and this appears to be the case), and that its half-life is approximately 2 min, then one must conclude, because of the kinetics of penicillinase induction, that the rate of penicillinase mRNA accumulation is inconsistent with the theory that the rate of penicillinase mRNA synthesis shifts immediately and abruptly from the basal rate to the final induced rate upon the addition of inducer. Rather, these data suggest that the rate of mRNA synthesis increases gradually from the basal rate to the final induced rate, and that this gradual increase in the rate of mRNA synthesis results in mRNA accumulation at a rate which is slower than would be observed if there were an immediate and abrupt shift in mRNA synthesis from one steady state to another.

The gradual increase in the rate of penicillinase mRNA synthesis following the addition of inducer is in sharp contrast to that observed after the induction of the lactose (17), tryptophan (11), or histidine (1) operons of *E. coli*. Yet, similar to the inducible *E. coli* enzymes cited, penicillinase synthesis is reported to be regulated, at least in part, by a soluble repressor (24). However, there is one major difference between the penicillinase system and the *E. coli* systems cited which can readily account for this apparent paradox; namely, for the *E. coli* systems the inducer, or effector, is transported into the cell where it is free in solution at a relatively high concentration, whereas the few molecules of penicillin, the apparent inducer of penicillinase, are reported to be irreversibly bound to a particulate component of the cell which is thought to be associated with the cell membrane (4, 7, 27).

The concept of a "bound" inducer, to date, is apparently unique to the penicillinase system; however, it is a feature common to all three of the gram-positive organisms in which penicillinase induction has been studied extensively. Indeed, the regulation of penicillinase synthesis in *B. cereus* 569, *B. licheniformis* 749, and *S. aureus* has at least three important features in common: (i) all of the strains of these three species which have been examined and are inducible for penicillinase (also some strains which are not inducible for penicillinase) bind penicillin specifically and essentially irreversibly (Fig. 3; Davies, *unpublished results*; 28); (ii) they all display a relatively long latent period during penicillinase induction (Fig. 2; Davies, *in press*; 14); and (iii) the two organisms, *S. aureus* (3, 25) and *B. licheniformis* (Sherratt and Collins, *private communication*), in which the regulation of penicillinase synthesis has been examined genetically each contain at least two penicillinase regulatory genes.

Although a realistic model for the regulation of penicillinase synthesis in these three gram-positive organisms has not been reported to date, a useful working model can be constructed which

is consistent with an mRNA of a short half-life and the three general properties noted above.

If one assumes that penicillin is the primary inducer and that it is irreversibly bound to a lipoprotein complex associated with the membrane (i.e., that the inducer is not freely diffusible), and if one accepts the genetic evidence for the existence of a diffusible penicillinase repressor molecule which has the ability to be inactivated by the bound inducer, or inducer complex, then it is immediately clear that in order for the repressor molecule to be inactivated it must diffuse over to and interact with the membrane-associated inducer complex. Furthermore, since approximately 1,000 molecules of penicillin are associated with a membrane which is relatively much larger than the penicillin molecules, the probability that the diffusible repressor molecule will strike and interact with an inducer complex on any given collision with the membrane is very small. Indeed, it can be estimated that a diffusible repressor molecule would collide with the cell membrane perhaps 10^4 times before it became inactivated by the inducer complex. Thus, it is not surprising that penicillinase would become induced more slowly than β-galactosidase and similar systems where the freely diffusible inducer is actually concentrated within the cell. This model also predicts that the repressor must dissociate from the hypothetical penicillinase operator spontaneously, because the inducer is not free to interact with the repressor-deoxyribonucleic acid complex as has been shown to be necessary to account for the rapid rate of β-galactosidase induction (9). This is also likely to be a relatively slow process. It should be noted, however, that the induction response curve (Fig. 8, curve A) suggests that repressor inactivation is not coupled directly to the rate of chromosome replication. This statement is based on the fact that such an association would produce a linear induction response curve with a slope of zero.

A model for the regulation of penicillinase synthesis which is consistent with the facts and assumption presented above is summarized in Fig. 9. According to this model, the product of one of the penicillinase regulatory genes is an integral part of the inducer-binding site complex, and the product of the other is analogous to the cytoplasmic repressor in the classical Jacob-Monod scheme (12). Also, according to this model only two regulatory genes are involved in the control of penicillinase synthesis, even though three penicillinase regulatory genes have been described in *S. aureus* (3, 25). However, Richmond has suggested that the cytoplasmic repressor is composed of two different subunits and that the two regulatory genes which are tightly linked to the penicillinase structural gene code for the synthesis of the repressor subunits (25). Therefore, a necessary function exists for all three of the regulatory genes.

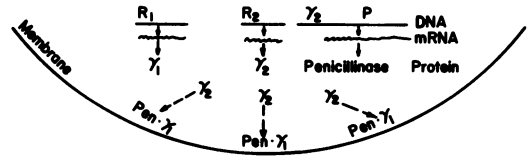

FIG. 9. *Model for the regulation of penicillinase synthesis. R_1 = regulator gene 1, the product of which (r_1) is a part of the inducer-binding site. R_2 = regulator gene 2, the product of which (r_2) is the cytoplasmic repressor. (R_1 and R_2 function constitutively; however, a mutation in either of these genes could render the cell constitutive, noninducible, meso-constitutive, or meso-inducible for penicillinase synthesis.) P = penicillinase structural gene. Pen = penicillin, the inducer. Pen-r_1 = inducer-repressor binding site complex.*

This model is also consistent with the gradual de-induction of penicillinase after the removal of exogenous inducer. However, since both the distribution of the bound penicillin molecules among the daughter cells and the mechanism of repressor inactivation are unknown, an understanding of the de-induction of penicillinase will require further investigation. It seems very likely that this general model of control is widely employed by multicellular systems.

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