KINETICS OF PENICILLINASE INDUCTION AND VARIATION OF PENICILLINASE TRANSLATION IN *STAPHYLOCOCCUS AUREUS*

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ABSTRACT At neutral pH, the rate of penicillinase synthesis by staphylococci declines gradually after removal of free inducer, while at pH 5.4 enzyme formation is generally linear for an extended period. Linear synthesis of penicillinase was observed at neutral pH in nonsaturating concentrations (1 μg/ml) of actinomycin D. The rate of enzyme synthesis, corrected for inhibition of growth caused by the antibiotic, was relatively independent of the time of actinomycin addition. The lag preceding linear enzyme formation increased with the interval between induction and the addition of actinomycin. The findings are consistent with the concept that, at neutral pH, "operons" activated by induction are rapidly repressed, while at pH 5.4, this process is delayed.

At a concentration of 4 μg/ml, actinomycin D blocked penicillinase messenger synthesis and also elicited a short-lived acceleration of the increase of penicillinase activity in uninduced and, late after induction, in induced cultures. This effect did not require a functional genomic repressor mechanism since it occurred also in a penicillinase-constitutive strain. It required protein synthesis and could not be attributed to a greater enzyme stability in the presence of actinomycin. The results suggest enhanced penicillinase translation after addition of actinomycin D.

INTRODUCTION

The kinetics of induction of staphylococcal penicillinase are affected by the ionic composition of the medium (1, 9). At pH 5.4, with appropriate concentrations of inorganic ions, penicillinase is formed at a constant rate for an extended period after removal of all external inducer, in a fashion similar to that found with *Bacillus cereus* at neutral pH (19). The rate of staphylococcal penicillinase accumulation by induced organisms at neutral pH declines progressively after removal of inducer and returns to the basal level within two generations. This decline is not due to accelerated inactivation of enzyme (9). In an attempt to elucidate the underlying mechanism, we tested two hypotheses. One was inspired by Pollock's suggestion that the prolonged synthesis of penicillinase by induced cells of *B. cereus* after removal of external inducer was caused by a mRNA of more than usual functional stability for

an inducible bacterial enzyme (20). Thus, an unusually stable messenger for staphylococcal penicillinase whose functional life varied nonetheless with external conditions, notably H^+ and Fe^{++} concentrations, could account for the differences in kinetic patterns with growth conditions. The other hypothesis was that gradual restoration of repression caused the decay of penicillinase synthesis at neutral pH.

Actinomycin D was employed in the experiments designed to test these hypotheses. In our work with this agent we observed a short-lived acceleration of the increase of penicillinase activity that occurred even at antibiotic concentrations that blocked penicillinase messenger formation. We investigated this phenomenon further and include the results in this paper.

MATERIALS AND METHODS

Organisms. *Staphylococcus aureus* 55Cl, a penicillinase-inducible strain, was the routine test organism (4). *S. aureus* 8325αi_1^-, constitutive for penicillinase, was obtained from Novick (14).

Media. The chemically defined medium WMa (8), a modification of that described by Wright and Mundy (24), was supplemented with 10^{-7} M $CaCl_2$ and deferrated as described elsewhere (1). In a number of experiments, cells were shifted from tryptic digest broth (TD) (Baltimore Biological Laboratory, Inc., Baltimore, Md.) to WMa. Occasionally, as indicated in the text, a deferrated broth was used. This was obtained as follows: double strength TD pH 5.4 was passed in 1 liter amounts through a 2.5 × 15 cm column of Dowex 50 Na^+ form, 100–200 mesh ('Baker Analyzed' Reagent, J. T. Baker Chemical Co., Phillipsburg, N. J.); the eluate was supplemented with 0.8 g L-arginine·HCl, 1.7 g L-lysine·HCl, and 0.1 g $MgSO_4$; the pH was adjusted with NaOH to 7.2 and the volume brought to 2 liters with deionized water.

Chemicals. Amino acids were from Nutritional Biochemicals Corp., Cleveland, Ohio or Calbiochem, Los Angeles, Calif. (A grade), uracil, cytidine and purine ribonucleosides from Pabst Research Laboratories, Milwaukee, Wisc.; thymidine (A grade) was purchased from Calbiochem. Uracil-^{14}C was obtained from New England Nuclear Corp., Boston, Mass.; Actinomycin D and chloramphenicol were gifts from Merck Sharpe and Dohme, West Point, Pa., and Parke, Davis and Co., Detroit, Mich. respectively. Glucose and salts were reagent grade.

Experimental Conditions. Cultural conditions were essentially those of previous work (8, 9). However, except for early experiments conducted at 37°C, cells were grown at 30°C, since determinations of penicillinase messenger stability were facilitated at this lower temperature. Benzylpenicillin was the inducer. Details of induction, measurement of cell mass and sampling have been reported (9). Actinomycin was dissolved in 70% ethanol at a concentration of 1 mg/ml and stored in the dark at 4°C. Penicillinase was assayed by the iodometric method of Perret (17) as modified by Novick (13). To transfer cells from TD to WMa, the organisms were removed by filtration (Millipore DA, 47 mm), washed with WMa at 30°C, suspended in 2 ml WMa, and inoculated into the prewarmed medium. Care was taken to prevent drying of cells during filtration.

Enzyme stability in shifted cultures was tested as follows: Cells growing in TD at 30°C were induced with benzylpenicillin 1 μg/ml. 15 min later, the cells were transferred to WMa and growth was resumed at 30°C. 40 min after induction, portions of the culture received

chloramphenicol 100 μg/ml. Penicillinase activity was followed for 48 min. In six out of seven independent determinations, the decline of enzyme activity was 2–11%/hour. In one determination, a discordant 37%/hour loss was measured. Purine ribonucleosides and actinomycin had no significant effect on enzyme stability. In the same medium, but under somewhat different conditions, a substantially greater loss of enzyme activity (up to 30% in 30 min) was reported (1). The greater stability of the enzyme in shifted cultures may be due to the removal of proteolytic enzymes by filtration and possibly to the lower temperature used in these experiments.

Inhibition by actinomycin of uracil incorporation into the cold trichloracetic acid–insoluble fraction was determined in an induced culture of strain 55Cl growing in WMa supplemented with uracil, cytidine and thymidine 20 μg/ml each. 22 min after induction, fractions of this culture received actinomycin, followed 1 min later by uracil-^{14}C 0.2 μc/ml. A control culture received uracil-^{14}C only. At various intervals, 2- or 4-ml samples were collected in an equal volume of ice-cold 10% trichloracetic acid. The suspensions were filtered (Millipore DA, 25 mm), the cells washed with cold 5% trichloracetic acid, the pads pasted on to 2-inch planchettes and dried under infrared light. Radioactivity was measured in a Beckman Low-beta II counter (Beckman Instruments, Inc., Fullerton, Calif.)

RESULTS

Functional Stability of Penicillinase mRNA. We determined the functional half-life of staphylococcal penicillinase messenger from the decay of enzyme-forming capacity in an induced culture after suppression of messenger synthesis with actinomycin D, 4 μg/ml (5). At this concentration, actinomycin reduced uracil-^{14}C incorporation into the cold trichloracetic acid–insoluble fraction by over 85% and prevented induction when added immediately after the inducer. In the presence of actinomycin, the enzyme-forming capacity of an induced culture decayed exponentially, with a half-life of 2.2 min at 30°C (Fig. 1). This decay was not affected by the pH or Fe^{++} concentration of the medium (Table I). Chloramphenicol (100 μg/ml), added to an actinomycin-containing culture before penicillinase synthesis ceased, blocked further increase of enzyme activity. The exponential decay of enzyme-forming capacity and the effect of chloramphenicol suggest that we were indeed measuring *de novo* penicillinase protein synthesis.

The metabolic stability of staphylococcal penicillinase messenger is thus no greater than that of other inducible bacterial enzyme messengers [e.g. *Escherichia coli* β-galactosidase (12, 18); *Bacillus subtilis* histidase (5)] and does not explain the variably protracted enzyme synthesis in the absence of external inducer.

The Role of Repression in the Decay of Penicillinase Synthesis. The declining rate of penicillinase synthesis in induced organisms after removal of free inducer may reflect a gradual restoration of repression. To test this hypothesis we attempted to prevent the decline of enzyme synthesis by interfering with its regulation. Pollock (20) observed a doubling of penicillinase formation by inducible strains of *Bacillus licheniformis* at very low concentrations of actinomycin which he attributed to more effective inhibition of the penicillinase regulatory gene than of the corresponding

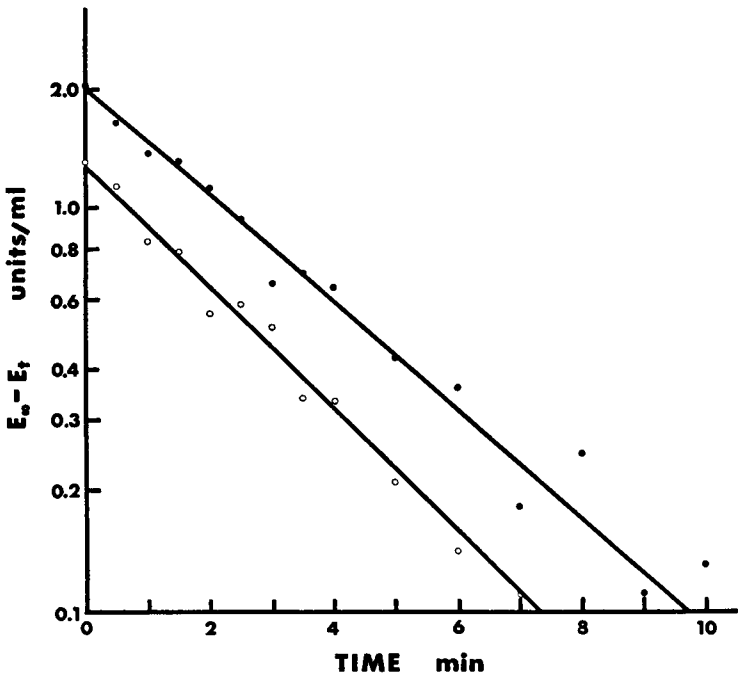

FIGURE 1 Decay of penicillinase-forming capacity at 30°C in the presence of actinomycin (4 μg/ml) in two induced cultures. Cells grown in TD were transferred to WMa and induced with benzylpenicillin (1 μg/ml) after one generation of growth in the new medium. Penicillinase-forming capacity, i.e. maximal enzyme content (E_∞) less content at time indicated (E_t), is plotted against time after actinomycin addition. ○ = Fe^{++} content of the medium < 10^{-7} M, actinomycin added 30 min after induction; ● = medium supplemented with 10^{-5} M Fe^{++}, actinomycin added 15 min after induction.

TABLE I

HALF-LIFE OF PENICILLINASE MESSENGER AT 30°C

pH	Fe^{++} conc	Half-life
	M	*min*
7.4	$<10^{-7}$	2.2
7.4	5.10^{-6}	2.0
7.4	10^{-5}	2.2
5.4	2.10^{-5}	2.4

structural gene. Although in staphylococci the genetic system of penicillinase is extrachromosomal, it consists of structural and regulatory loci (15) like the genome of chromosomally inherited inducible bacterial enzymes. Furthermore, actinomycin affected penicillinase synthesis as it does the formation of enzymes having chromosomal determinants (cf. previous section). Hence, it was conceivable that the regulatory gene of penicillinase in staphylococci, like its homologue in *B. licheniformis*,

would be more sensitive to actinomycin than the corresponding structural gene. If this were so, nonsaturating concentrations of the antibiotic should prevent the progressive decline of penicillinase synthesis at neutral pH, after removal of external inducer. In fact, after a variable lag, enzyme synthesis was linear for at least 60 min in the presence of 1 μg actinomycin/ml (Fig. 2). Growth in these cultures was exponential but at a considerably slower rate; growth inhibition was 61 % (*a*) and 34 % (*b*) respectively. The rate of linear enzyme synthesis, corrected for growth inhibition, was relatively independent of the time of actinomycin addition: 1.33 units/generation time (*a*) and 1.56 units/generation time (*b*). This is consistent with a differential

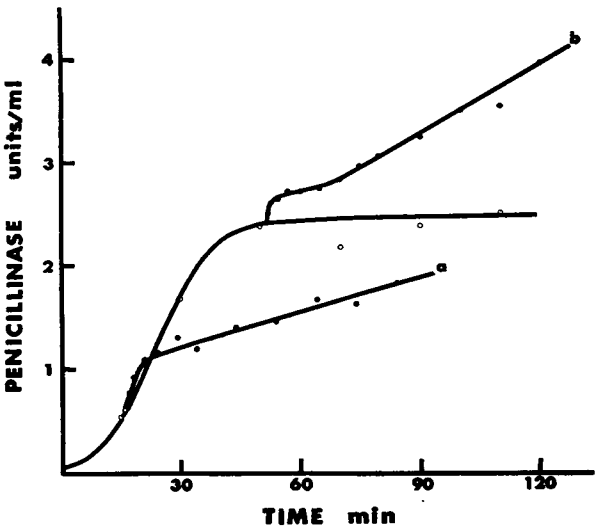

FIGURE 2 Effect of nonsaturating concentrations (1 μg/ml) of actinomycin on an induced culture at pH 7.4. Cells growing in WMa at 37°C were induced with benzylpenicillin (2 μg/ml) at a cell concentration of 14 μg of protein/ml. The generation time of the culture was 46 min. Portions of the culture received actinomycin 16 min (*a*) and 52 min (*b*) after induction. ○ = control culture; ● = cultures containing actinomycin.

sensitivity to actinomycin of the regulatory and structural genes, for the steady-state rate of penicillinase synthesis should be determined by the opposing influences of actinomycin on repression and the readout of the structural gene and therefore should not depend on the time of addition of the antibiotic. Implicit in this argument is the assumption that penicillinase translation was equally affected by actinomycin in both instances (see text following).

Besides preferential inhibition of repressor formation, the effect of actinomycin on repression could also be interpreted as an interference with repressor function. Thus, binding of actinomycin to the penicillinase operator or to adjacent sites may hinder the attachment of repressor without fully preventing messenger transcription.

The lag (i.e. the time between addition of actinomycin and onset of linear enzyme

synthesis) increased with the interval between induction and the addition of actinomycin (Fig. 2). This lag was 5–6 min if actinomycin was added early after induction, when enzyme synthesis was rapid (*a*), and about 15 min if actinomycin was added later, when enzyme formation had nearly returned to its basal level (*b*). Since onset of linear enzyme synthesis is determined by the time required to reach a steady-state messenger concentration, inhibition of the functioning of the structural gene should determine the lag in the largely derepressed state (*a*), while blocking of regulatory function would be the determining factor in nearly fully repressed cells (*b*). The latter requires either a decay of accumulated repressor or displacement of repressor from operator sites by actinomycin before transcription can reach a steady-state level, hence the longer lag.

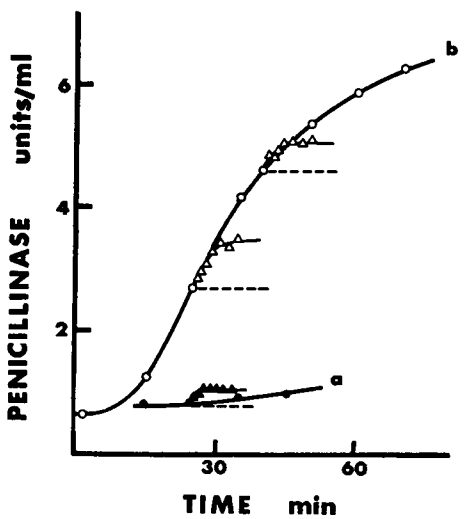

FIGURE 3 Residual penicillinase formation in the presence of actinomycin. Portions of two cultures, growing at 30°C in WMa, one uninduced (*a*) the other induced with benzylpenicillin (1 μg/ml) at time 0 (*b*), received actinomycin 4 μg/ml at times indicated. Circles indicate cultures without actinomycin; triangles, portions containing actinomycin. The interrupted lines mark the level of penicillinase when the actinomycin was added.

Early Effect of Actinomycin on Penicillinase Formation. A rapid increase of penicillinase activity above control levels occurred immediately after the addition of actinomycin in portion *b* (Fig. 2). A similar increase was also observed when actinomycin was added at fully inhibitory concentrations (4 μg/ml) to an uninduced culture (Fig. 3,*a*) or, late after induction, to an induced culture (Fig. 3,*b*; at 40 min). This increase of activity was dependent on concomitant protein synthesis since it did not occur in the presence of chloramphenicol (100 μg/ml). It was not the result of greater stability of penicillinase in the presence of actinomycin, for, when protein synthesis was blocked by chloramphenicol, penicillinase activity declined at the same slow rate whether actinomycin was present or not. It did not require a functional genomic repressor mechanism since it occurred also in the penicillinase constitutive strain $8325\alpha_i^-$. Hence, it would appear that the penicillinase messenger is expressed with greater efficiency in the presence of actinomycin than in its absence. This phe-

nomenon could be a trivial consequence of special physico-chemical properties of the penicillinase messenger, for example greater stability than other messengers, or a slower rate of association with ribosomes. Either of these properties could lead to an enhanced efficiency of translation during the transition from a state of intense competition for ribosomes, as in balanced growth, to a situation when ribosomes are more accessible, as after addition of actinomycin. However, the penicillinase messenger, with a functional half-life of 2.2 min at 30°C, is not unusually stable, and

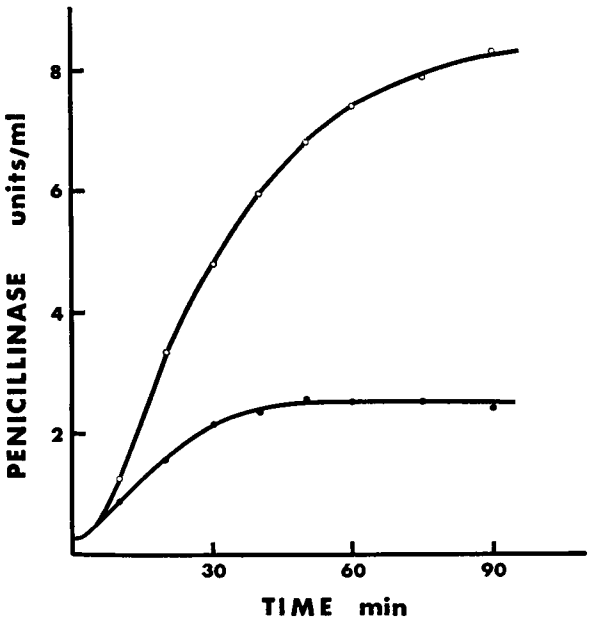

FIGURE 4 Effect of a shift of medium on penicillinase formation. ○ = cells transferred to WMa; ● = cells returned to TD.

therefore unlikely to be significantly more stable than the majority of staphylococcal messengers. As for the association between messenger and ribosomes, its rate limiting effect has been established in vitro only (6); whether this effect is duplicated in vivo is unknown. At any rate, any difference in this respect between penicillinase messenger and most other messengers would have to be very large to account for the result of Fig. 3,*a*.

Further insight into this problem was gained from an independent investigation on the effect of a shift of medium on induced penicillinase formation. Fig. 4 illustrates a typical experiment. Cells of strain 55Cl, grown in deferrated tryptic digest broth (TD) at 37°C, were suspended in 0.01 M phosphate buffer, pH 7.35, at 0°C and exposed to benzylpenicillin 2 μg/ml for 30 min. Any residual free inducer was removed by filtration. The washed cells were divided into two portions: one was

returned to deferrated TD, the other inoculated into WMa, consisting of amino acids, vitamins, glucose and salts. While the two resulting cultures grew at the same rate (as determined by optical density and cell-protein measurements), the cells transferred to WMa formed three times as much enzyme as those returned to TD. Results were similar when WMa supplemented with purine bases or nucleosides was substituted for TD after induction. Lowered penicillinase synthesis in the presence of purine bases had been observed previously (7).

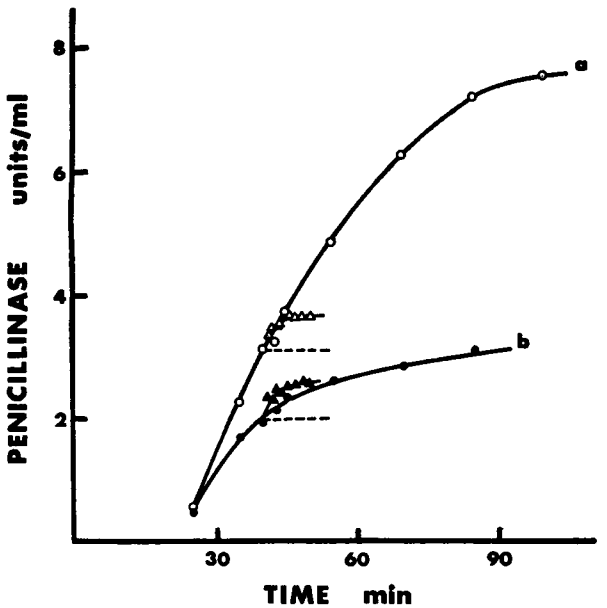

FIGURE 5 Residual penicillinase synthesis in the presence of actinomycin (4 μg/ml) in a pair of shifted cultures. *a* = no supplements; *b* = adenosine and guanosine (95 μg/ml). (Similar results were obtained with nucleoside concentrations of 2 μg/ml.) Both cultures had an initial cell concentration of 48 μg of protein/ml and a generation time of 57 min. Circles indicate cultures without actinomycin; triangles, portions containing actinomycin. The interrupted lines mark the level of penicillinase when the actinomycin was added.

The effect of actinomycin on a pair of shifted cultures is shown in Fig. 5. Here, benzylpenicillin 1 μg/ml was added to a culture growing in TD at 30°C. 15 min later, the cells were transferred to WMa. One portion of the resulting suspension (*b*) received a supplement of adenosine and guanosine. Growth was resumed at 30°C. 40 min after induction, a portion of each culture received actinomycin 4 μg/ml. Although twice as much enzyme was accumulated in *a* as in *b*, the initial rate of penicillinase synthesis in the presence of actinomycin, as estimated from the residual increase of enzyme activity, was about the same in both portions. Actinomycin accelerated enzyme synthesis in *b* but not in *a*. The experiment furnishes additional evidence that this effect could not be due to a stabilization of enzyme by the anti-

biotic since enhanced increase of enzyme activity occurred only in one of the two portions receiving actinomycin.

Two alternate interpretations of these results seem plausible:

1. The same amount of penicillinase messenger is present in both cultures, but a regulatory mechanism affecting translation modulates the expression of messenger under the influence of environmental factors. Actinomycin relieves messenger function from regulation, equalizing its expression in the two cultures.

2. Under physiologic conditions, the degree of messenger expression is constant, i.e., the rate of enzyme synthesis is directly proportional to messenger concentration. Actinomycin, however, enhances translation under selective environmental conditions, e.g., in the presence of purine ribonucleosides. In this model at least half the translative potential of penicillinase messenger would normally be wasted.

There is not sufficient evidence to sustain either hypothesis. The mRNA-DNA hybridization technique (16) could possibly furnish the lacking evidence to determine which hypothesis applies. Should the first interpretation be correct, declining messenger translation, generally detectable in induced cultures, would accelerate the dacay of penicillinase synthesis due to decreasing transcription at neutral pH (Fig. 3,*b*).

DISCUSSION

The accelerated increase of penicillinase activity in the presence of actinomycin had the following properties: it was elicited by the antibiotic at nonsaturating concentrations (e.g. 1 μg/ml) as well as at concentrations that inhibited penicillinase messenger synthesis (4 μg/ml); it occurred in induced and uninduced cultures of strain 55Cl and also in the penicillinase constitutive strain 8325$\alpha_{i_1}^-$; the effect occurred without detectable delay; it required protein synthesis and was not due to a stabilization of enzyme by the antibiotic. These results are best explained by enhanced penicillinase translation. The faster rate of enzyme synthesis could be due either to an enhanced average rate of translation of functionally active messengers or to a decoding of previously idle messengers. The rapid response of enzyme synthesis to actinomycin would favor the first interpretation.

Enzyme synthesis was not increased by actinomycin in a culture shifted from TD to WMa medium unless the latter was supplemented with purines (Fig. 5). However, this fact appeared attributable to the faster enzyme synthesis before actinomycin addition in the medium lacking purines (*a*). Indeed, the initial rate of enzyme formation after actinomycin addition was essentially the same in both cultures. The role of purines is not understood.

Regulation of protein synthesis at the level of translation has been reported in several instances, primarily in Metazoa. Hence, while control of DNA transcription may be the most common means of specific regulation, it is not the only one. Protein synthesis is initiated through regulation of mRNA translation, for example, in the

fertilized sea urchin egg during the period of cleavage (23); in the heme-stimulated globin synthesis in erythroid cells (10); in the ACTH-induced formation by the rat adrenal of an unidentified enzyme along the biosynthetic pathway of corticosterone (3). Of particular interest are two enzymes in rat liver, tryptophan pyrrolase and tyrosine transaminase, whose synthesis is induced by hydrocortisone. Initiation and programmed termination of their synthesis are both actinomycin-sensitive steps suggesting a "mixed" regulatory system: induction at the level of DNA transcription and repression at the level of mRNA translation by a repressor synthesized under genomic control. Repression is reversible by actinomycin D indicating a stable messenger and probably a short-lived repressor (2). A similar control system appears to regulate the synthesis of thymidine kinase in poxvirus-infected HeLa cells. However, here repression is not reversible by actinomycin and may therefore entail the destruction rather than the inhibition of messenger (11). In the unicellular alga *Acetabularia crenulata*, the synthesis of an alkaline phosphatase is correlated with the formation of a cap. Although regeneration of the cap, after its removal, requires several weeks, cell enucleation does not interfere with this mechanism (22). From a toxinogenic strain of *Corynebacterium diphtheriae*, grown at toxin-inhibiting concentrations of iron, Sato and Kato (21) isolated a protein fraction which inhibits selectively toxin formation in a cell-free system of the same strain grown at low iron concentrations. The kinetics of this inhibition indicate interference with messenger function.

Short-lived mRNA is prerequisite for a rapid response of protein synthesis to direct genomic regulation. Hence, variation of messenger function provides the only effective means of regulation when a protein is coded by a relatively stable messenger. Staphylococcal penicillinase messenger, however, is short-lived and available evidence indicates that induction of penicillinase affects transcription. If the variation of translation, observed by us, were part of the physiologic regulation of this enzyme, it would confer additional flexibility to the control of penicillinase synthesis. The advantage to the cell of such combined regulation is, however, not immediately apparent. This question merits further investigation.

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