A Penicillinase-Specific Ribonucleic Acid Component from *Bacillus cereus*. I. Ribonucleic Acid Extraction and Definition of the *in Vivo* Test System*

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ABSTRACT: Ribonucleic acid (RNA) extracts obtained from *Bacillus cereus* 569/H (penicillinase constitutive) membranes caused an increased differential rate of penicillinase production in inducible, microconstitutive, and absolute negative recipient cultures (*B. cereus*, *Bacillus subtilis*, and *Staphylococcus aureus*). The generally RNase-sensitive and deoxyribonuclease- (DNase) resistant active component of the 569/H RNA rapidly became refractory to RNase action upon addition to recipient cells. The response of recipient cells was found to be RNA concentration dependent and was highly competitively inhibited by 4S RNA and to a lesser degree by ribosomal ribonucleic acid (rRNA). *B. cereus* 569 (inducible) RNA contains small but measurable amounts of the active component as well as competitive and noncompetitive species of RNA. Irrespective of the recipient cell species tested, the enzyme activity produced was shown to be serologically indistinguishable from constitutive *B. cereus* penicillinase. Synthesis of the 569/H penicillinase, *de novo*, by the recipient cultures was suggested by the resistance to or enhancement by actinomycin D of the 569/H RNA-stimulated production as opposed to sensitivity of penicillin-induced synthesis. Chloramphenicol, streptomycin, and puromycin inhibition of penicillinase synthesis in 569/H RNA-stimulated and penicillin-induced cells was the same. The candidacy for the active 569/H RNA component of DNA, internal inducer, a repressor-destroying enzyme, or inactive penicillinase protein has been tentatively eliminated. A penicillinase-specific RNA species has been suggested as the active fraction.

A class of RNA of probable significance in transfer of genetic messages has been extensively characterized (Singer and Leder, 1966). The properties largely fulfill the qualities predicted for mRNA (Jacob and Monod, 1961). The message-bearing functional role of this class of RNA, however, needs to be demonstrated by its activity in an *in vivo* system. Several systems recently have been utilized in attempting such *in vivo* demonstration.

Firstly, transformation of *Pneumococcus* from sulfanilamide sensitivity to resistance was observed by the addition of RNA-containing material extracted from resistant organisms (Evans, 1964). Partial sensitivity to both DNase[1] and RNase suggested the nucleic acid nature of the active component. Secondly, the acquisition of new immunological properties by normal lymphoid cells after treatment with RNA extracted from immunized animals affords a number of further examples of attempts at a demonstration of RNA transformation (Friedman, 1964; Wilson and Wecker, 1966; Adler *et al.*, 1966). All these active RNA preparations were sensitive to RNase action; however, the presence of carry-over antigen in RNA extracts may have been responsible for at least some of the antibody synthesized. A recent report (Gottlieb *et al.*, 1967) indicated that an immunogenic RNA–protein complex may be the essential means by which the information eliciting specific antibody production is processed, even though the RNA itself seems not to be specific. Thirdly, an *in vivo* demonstration of a specific template RNA was preliminarily indicated, though not confirmed by others, using the penicillinase system of *Bacillus cereus* (Csanyi *et al.*, 1960). Stimulation of the rate of enzyme synthesis in inducible *B. cereus* in the absence of inducer, was obtained by addition of RNA extracted from the constitutive mutant but not from the inducible strain; pretreatment of recipient cells with RNase apparently being necessary for the demonstration of the phenomenon.

The penicillinase system of *B. cereus* seems unusually suitable to study the biological activity of RNA fractions introduced into recipient cultures. Inducible and constitutive variants are easily obtained (Sneath, 1955) and synthesize the same enzyme (Pollock, 1956) about 85% of which is exoenzyme and which is easily purified

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[1] The trivial name, penicillinase, is used for this enzyme. The name suggested by the Commission on Enzymes of the International Union of Biochemistry is pencillin amidohydrolase (EC 3.5.2.6). Other abbreviations used are: DNase, deoxyribonuclease (deoxyribonucleate oligonucleotido-hydrolase, EC 3.1.4.5); RNase, ribonuclease (polyribonucleotide 2-oligonucleotidotransferase, EC 2.7.7.16); lysozyme or muramidase (*N*-acetylmuramide glycanohydrolase, EC 3.2.1.17); SLS, sodium lauryl sulfate; MAK, methylated serum albumin Kieselguhr; SSC, standard saline–citrate buffer.

(Kogut *et al.*, 1956; Citri *et al.*, 1960). Furthermore, the corresponding mRNA seems to be relatively stable metabolically (Pollock, 1963; Duerksen, 1964; Yudkin, 1966), so that most of the relevant mRNA molecules extracted from a culture should be complete. Here we present evidence for the biological activity ("messenger function") of RNA extracts from a penicillinase constitutive *B. cereus* strain.

Materials and Methods

Strains and Growth. *B. cereus* 569 (inducible for penicillinase), its constitutive mutant 569/H, *B. cereus* 5 (microconstitutive), and *Bacillus subtilis* ICI strain were grown in casein–hydrolysate (CH) medium according to the method of Pollock (1957) from an inoculum of germinated spores. *Staphylococcus aureus* 187 (NCTC 9874, inducible for penicillinase) and *S. aureus* 8325 (N) (absolute negative for penicillinase; Novick, 1963) were grown in 0.5 CY medium of Novick (1963). *E. coli* (penicillinase negative) was grown in CH medium.

Extraction of RNA from Protoplasts. Protoplasts were prepared from log-phase cultures of *B. cereus* 569/H by the modified lysozyme method (Duerksen and O'Conner, 1963). DNase at a concentration of 1 μg/ml was included during protoplast formation to decrease the viscosity in subsequent steps. Centrifugation at 10,000*g* for 15 min sedimented the protoplasts. Preliminary investigations indicated that treatment of protoplasts with 0.5% sodium lauryl sulfate (Gros *et al.*, 1961) alone resulted in preparations which were either biologically inactive or contained measurable residual penicillinase activity. If the SLS treatment was followed by the phenol–ether method of Nomura *et al.* (1960) biologically active RNA preparations free of measurable protein or penicillinase activity were obtained.

Extraction of RNA from Membranes. *B. cereus* 569/H protoplasts, after sedimentation, were immediately lysed with cold buffer (Tris-KCl-Mg^{2+}, pH 7.3, and Tris 0.01 M, pH 7.3, containing 0.05 M each of potassium chloride and magnesium acetate). The membrane fraction was sedimented at 10,000*g* for 15 min, washed two to three times with small volume of the same buffer, and resuspended in Tris-KCl-Mg^{2+} buffer. The membrane suspension was incubated at room temperature for 15 min with 0.5% SLS added as a 5% solution in 45% ethanol. RNA was extracted by the method of Nomura *et al.* (1960). After removal of ether, RNA usually was treated with washed bentonite (Fraenkel-Conrat *et al.*, 1960) at 100 μg/ml for 30 min in cold. Centrifugation at 10,000*g* for 10 min removed most of the bentonite yielding a clear supernatant apparently free of nucleases. In some of the extractions, washed bentonite was added just prior to the phenol step. RNA thus extracted contained only traces of DNA and was free of measurable protein. It was dialyzed overnight against three changes of cold buffer (100 volumes).

Extraction of DNA. The extraction procedure of Saito and Miura (1963) for obtaining DNA of high-transforming activity free of measurable RNA was followed.

Treatment of Recipient Cells. The recipient cells were grown as previously described to a density of 0.15–0.3 mg dry wt/ml. The ratio of culture to RNA volume was 5 to 1 unless otherwise stated. Use of bentonite in the extraction procedure enabled equilibration of the RNA solution at 37° prior to the addition of recipient cells. With some extractions (bentonite omitted) incubation-sensitive active RNA extracts prevented temperature equilibration. The treated cultures were shaken at 37° and 1-ml samples were removed at indicated intervals for 1–2 hr to tubes containing an equal volume of oxine–gelatin solution (Pollock and Perret, 1951). Control cultures with buffer and nonspecific RNA for the basal rate of penicillinase synthesis and penicillin at 0.3–10 units/ml for the induced rate of enzyme synthesis were routinely run. The combination of RNA and recipient cells is referred to as donor strain RNA-strain name of the recipient cells. Enzyme synthesized in response to RNA in these cells is referred to similarly (*e.g.*, 569/H RNA-569 penicillinase).

Enzyme Assay. A modified iodometric assay procedure (Novick, 1962; Duerksen and O'Conner, 1963) was used for estimating penicillinase activity in the samples. Enzyme units are expressed as micromoles of penicillin hydrolyzed per minute per milliliter of culture at 25°. The differential rate of enzyme synthesis (K) was calculated as units of enzyme synthesized per milligram dry weight of cells.

Purification of Penicillinase. The glass adsorption–elution procedure of Kogut *et al.* (1956) and the Celite (535) column adsorption–elution method of Citri *et al.* (1960) were followed. Ballotini beads (0.17–0.18 mm in diameter) were found suitable for the glass adsorption procedure. For complete adsorption on these glass beads stirring in cold for at least 18 hr was necessary. The enzyme, either from constitutive *B. cereus* 569/H supernatant or from RNA-stimulated *S. aureus* 8325 (N) supernatant, could be purified by either of the two methods. Generally, the procedures were employed sequentially.

Anti-α-penicillinase Serum. Antiserum against 569/H penicillinase was obtained from rabbits given two immunization series. The enzyme used for inoculation was purified prior to each inoculation series by two Celite column passages and dialysis. Non-α-penicillinase antibodies possibly present in the heat-inactivated (56° for 30 min) anti-569/H penicillinase serum were removed by adsorption with 569 culture supernatant, containing comparatively small amounts of penicillinase, purified in the same manner. The serum supernatant obtained after centrifugation was free of penicillinase activity but still retained the majority of its neutralizing capacity. A constant antigen titration procedure (Pollock, 1956) was used to determine the antibody titer. Known units of penicillinase activity, varied dilutions of nonabsorbed or absorbed anti-569/H penicillinase serum, and oxine–gelatin solution were mixed in 1 : 1 : 2 proportions. After 35-min incubation at room temperature, the samples were assayed for penicillinase activity. The neutralizing unit (nu) of antiserum is expressed as milliliters of antiserum capable of neutralizing one unit of penicillinase at room tempera-

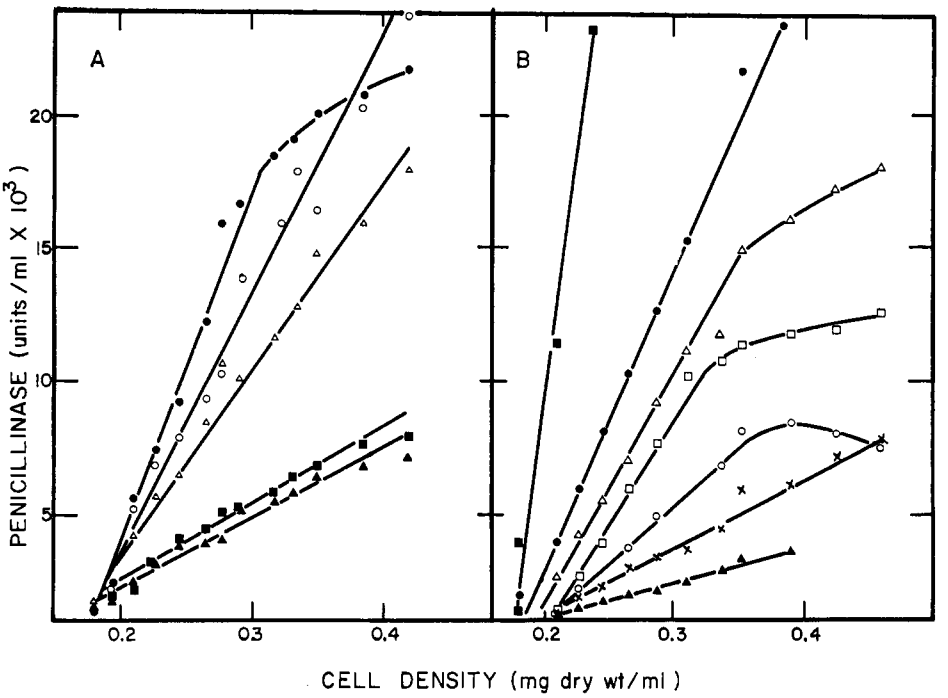

FIGURE 1: Biological activity of *B. cereus* 569/H membrane RNA. Extraction of RNA, and treatment of the recipient cells, *B. cereus* 569, were as described in Materials and Methods. Penicillinase synthesis was followed in each recipient culture and the differential rate (K) was calculated. (A) Response in the differential rate of synthesis upon RNA addition and the effect of RNase treatment of the added RNA on the response. Bentonite-treated RNA was used. (▲—▲) Uninduced (K = 0.026); (○—○) RNA at 9.0 μg/ml (K = 0.093); (■—■) RNase-treated RNA at 8.3 μg/ml (K = 0.028); (△—△) preincubated RNA at 8.3 μg/ml (K = 0.068); (●—●) RNA (bentonite treatment omitted) at 9.8 μg/ml (K = 0.12). (B) Response in the rate of synthesis as a function of added RNA concentration. Bentonite treatment of RNA was omitted. (▲—▲) Uninduced (K = 0.013); (■—■) penicillin at 10 units/ml (K = 0.45); (●—●) penicillin at 0.3 unit/ml (K = 0.106); (×—×) RNA at 1.7 μg/ml (K = 0.026); (○—○) RNA at 3.4 μg/ml (K = 0.45); (□—□) RNA at 8.5 μg/ml (K = 0.080); (△—△) RNA at 17 μg/ml (K = 0.089).

ture, and the neutralizing titer is the slope of the linear portion of the neutralizing curve. The antiserum (1 ml) (nonabsorbed) obtained after the first series of inoculations contained 3.1 nu and the (absorbed) after the second series contained 76 nu.

Nuclease Treatment. Aliquots of 569/H RNA extracts were usually treated with RNase, DNase, or a combination of both nucleases each at 100 μg/ml of RNA solution at 37° for 30 min and tested for biological activity. The RNase sensitivity of biologically active 569/H RNA subsequent to addition to recipient cells was tested by adding RNase (100 μg/ml of culture) to recipient culture aliquots removed at various time intervals after RNA addition. Stock RNase solutions at 2-mg/ml SSC buffer (0.15 M NaCl–0.015 M sodium citrate, pH adjusted to 5 with 1 M citric acid) were heated at 100° for 10 min. Stock DNase solutions (1 mg/ml) were made up in TS2M buffer (pH 7.2) (10 mM Tris-(2-amino-2-(hydroxymethyl)-1,3-propanediol), 4 mM succinic acid, and 10 mM magnesium acetate).

Tests for RNA Purity. RNA preparations were routinely tested for the presence of penicillinase activity, RNA, DNA, and protein. RNA solutions were also incubated with penicillin (0.1 μmole/ml) for 10–20 min at room temperature and assayed for enzyme activity to rule out the possible presence of reversibly inactivated enzyme (Citri *et al.*, 1960) in the RNA preparation. Treatment of each RNA preparation with RNase was followed by a check for penicillinase activity.

Effects of Chloramphenicol, Streptomycin, Puromycin, and Actinomycin D. Inducible *B. cereus* 569 was used in all antibiotic experiments. Recipient cells grown to desired density were distributed in three flasks. Penicillin (10 units/ml) was added to flask 1, 569/H membrane RNA to flask 2, and Tris-KCl-Mg^{2+} buffer to flask 3. All the flasks were incubated at 37° with shaking. At indicated time intervals aliquots were removed from these flasks and added to chloramphenicol-containing flasks (30 μg/ml of culture). The synthesis of penicillinase was followed in these flasks for 1 hr at 37° in the usual manner. Similar experiments were repeated with streptomycin (50 μg/ml of culture) and puromycin (20 μg/ml of culture). The former antibiotic was at a partially growth-inhibiting concentration whereas the latter two were at bacteriostatic concentrations.

Experiments with actinomycin D were carried out in a more detailed manner. Additional controls, such as pretreatment of recipient cells with the antibiotic at 37° for 20 min prior to 569/H RNA addition and incubation of RNA with the antibiotic at 37° for 20 min prior to the addition of recipient cells, were included in the protocol described for the other antibiotics. Actinomycin D was tested at 0.055 and 0.1 μg/ml representing partially growth-inhibiting and bacteriostatic concentrations, respectively.

Analytical Procedures. RNA concentrations were estimated by the Orcinol method (Schneider, 1957). For determination of DNA the diphenylamine reaction

(Dische, 1955) was used. The Folin–Ciocalteu method (Lowry *et al.*, 1951) was followed for estimation of protein.

Enzymes and Antibiotics. DNase (pancreas) B grade and lysozyme (three-times crystallized) were obtained from California Corp. for Biochemical Research, Los Angeles. Pancreatic crystalline ribonuclease (without protease activity) was obtained from Mann Research Laboratories, Inc., New York, N. Y., and California Corp. for Biochemical Research, Los Angeles, Calif. Actinomycin D (Merck Sharp and Dohme Research Laboratories, West Point, Pa.), 100 mg/ml, aqueous solution stored at −20° until used; chloramphenicol (chloromycetin–sodium succinate, Parke Davis and Co., Detroit, Mich.), 1.0 mg/ml, solution in phosphate buffer (pH 7.0); streptomycin sulfate (USP, E. R. Squibb and Sons, New York), 250 mg/ml, solution in phosphate buffer (pH 7.0); and puromycin (aminonucleoside from Nutritional Biochemicals Co., Cleveland 28, Ohio), 200 mg/ml, were the stock antibiotic solutions used.

Results

Biological Activity of 569/H RNA Extracts. RNA, free of measurable protein and penicillinase activity, extracted from 569/H protoplasts by the SLS–phenol method significantly increased the differential rate of penicillinase synthesis in recipient uninduced 569 cultures (K = 0.04–0.1). Extraction of washed protoplast membranes often yielded RNA preparations causing an increased differential rate of synthesis (K = 0.1–0.3) approaching 20% maximal induction with penicillin. These nonbentonite-treated RNA extracts were relatively unstable since incubation for 30 min at 30° destroyed the stimulatory activity. After inclusion of bentonite (Fraenkel-Conrat *et al.*, 1960) in the extraction procedure, incubation alone had no effect on the active component of the RNA preparation, whereas incubation with RNase destroyed the activity completely (Figure 1A). A decreasing differential rate of penicillinase synthesis after approximately 30 min is observed in the recipient cells treated with nonbentonite RNA preparations whereas the cells receiving bentonite-treated RNA extracts continued a linear differential rate of enzyme synthesis for at least 120 min. Generally the K values of basal:membrane RNA induced:penicillin-induced penicillinase synthesis exhibited a ratio of approximately 1:10:100. Addition of purified 569/H penicillinase to membrane suspensions prior to extraction of RNA did not increase the stimulative activity of RNA extracts when added to recipient cells. In all of the experiments reported here the active component in the RNA extracts as well as in the purified fractions was RNase sensitive. RNase added to recipient cultures at various intervals subsequent to 569/H RNA addition (Figure 2) showed that approximately 3 min is required for the active component to become completely resistant to RNase. At 20–30 min after addition to the recipient cells, the active component of the RNA extracts showed partial sensitivity to RNase and the subsequent differential rate of penicillinase synthesis decreased almost 50%.

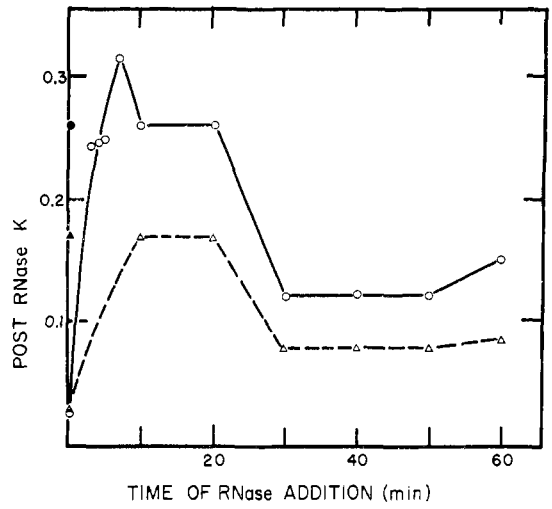

FIGURE 2: RNase sensitivity of the active component of *B. cereus* 569/H membrane RNA subsequent to addition to recipient cells. To *B. cereus* 596 recipient cells (0.19 mg dry wt/ml) and RNA extract was added at a volume ratio of 1 to 20. At the indicated time interval aliquots of culture were removed to RNase- (100 μg/ml) containing flasks and the subsequent rate of penicillinase synthesis was followed. The differential rate was determined. Parallel experiments using two different RNA preparations were carried out. (○—○) RNA at 3.2 μg/ml; (△—△) RNA at 18 μg/ml; (● and ▲) depict, respectively, the K values obtained in control RNA-treated cultures.

Effect of Varying Concentrations of B. cereus 569/H RNA and Competition of RNA-Induced Penicillinase Synthesis by Various RNA Species. Variations observed in stimulatory activity from one RNA preparation to another could be due to either concentration variation of the RNA extracts, the presence of competing RNA species in the preparations, or both. Results of 569/H RNA addition at varying concentrations to recipient cells are represented by the differential plot of Figure 1B. These results along with two other examples (bentonite and nonbentonite 569/H RNA) as well as the response of recipient cultures to *B. cereus* 569 RNA are plotted as K *vs.* RNA concentration in Figure 3A; representation of the data on a double-reciprocal plot is seen in Figure 3B. The saturation kinetics observed are reminiscent of the dependence of the number of transformants on DNA concentration (Spizizen *et al.*, 1966). The saturation level is dependent on the RNA preparation and suggests the presence of varying amounts of "biologically inert" RNAs which compete with the stimulating capacity of the active RNA component. The effect of the ratio of the active component to competing RNA species on the increased differential rate of penicillinase synthesis in the recipient cells is quite pronounced with the RNA extracted from uninduced 569 membranes. Bentonite, aside from removing trace amounts of nucleases, apparently also eliminated to a greater or lesser extent some of the competing species of RNA since higher saturating concentrations are demonstrated with the bentonite-treated 569/H RNA.

A definite competitive inhibition of 569/H RNA induction of penicillinase synthesis was observed with total *E. coli* RNA (data not presented). The response of

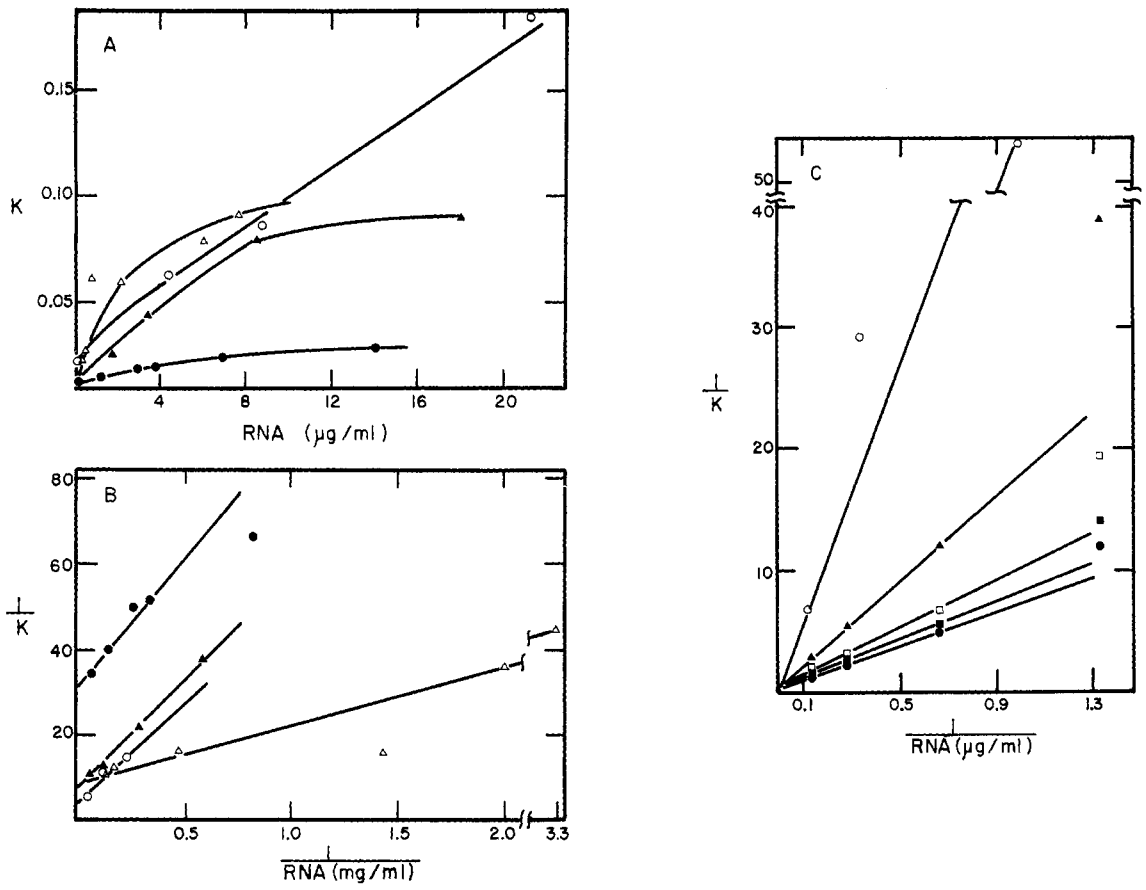

FIGURE 3: Effect of RNA concentration on recipient cell response. (A) Comparison of the stimulatory activity of different RNA preparations as a function of their RNA concentration. Conditions for RNA addition and sampling for biological activity are described in Materials and Methods. The differential rate of enzyme synthesis (K) was determined for each culture at various concentrations of RNA. (●—●) Bentonite-treated 569 (uninduced) RNA; (○—○) bentonite-treated 569/H RNA; (△—△ and ▲—▲) 569/H RNAs (bentonite treatment omitted) from different extractions. (B) Kinetics of the recipient cell response to varying concentrations of the different RNA preparations. Data from A are represented on an inverse plot of K *vs.* RNA concentration. (C) Inhibition by 4S and 23S RNA species, isolated from a *B. cereus* 569/H membrane RNA extract, of the partially purified biologically active component as a function of RNA concentration. The active component (represented by MAK column fractions eluting between 0.6 and 0.7 M NaCl) at various concentrations was mixed with one concentration of 4S RNA (0.45 M NaCl fractions) and three different concentrations of 23S RNA (0.8 M NaCl fractions) and added to recipient *B. cereus* 569 cultures. Penicillinase synthesis was followed in each culture for 1 hr after addition of each mixture. K values for the cultures were determined and are represented as in B. (●—●) Active component alone; (○—○) active component plus 4S RNA (38-μg/ml final concentration); (■—■) active component plus 23S RNA (15 μg/ml); (□—□) active component plus 23S RNA (30 μg/ml); (▲—▲) active component plus 23S RNA (65 μg/ml).

recipient cells to 569/H RNA was inversely proportional to the *E. coli* RNA concentration. More definitive competition experiments were possible using the various isolated species of RNA from MAK column fractionations (Kirtikar and Duerksen, 1968). Sufficient quantities of 4S, 16S rRNA, 23S rRNA and the component specific for penicillinase synthesis were obtained from several fractionations of 569/H RNA on MAK columns by ethanol precipitation of the relatively well-resolved peak areas. The rate of penicillinase synthesis in the recipient cells, observed to be directly proportional to the active component, with the highest concentration used approached one-third the fully induced rate (penicillin). The competitive nature of the inhibition by 23S rRNA and 4 S is obvious (Figure 3C). The differential rate of penicillinase synthesis is inversely proportional to the ratio of inhibitor RNA:active component. A fairly high concentration of the rRNA is necessary to bring about a marked inhibition whereas considerably less 4S RNA is needed.

When the RNA donor cells (*e.g.*, uninduced *B. cereus* 569) are removed from the relatively rich CH medium into TM (Tris-Mg^{2+}) buffer plus a carbon source and shaken for 30 min at 37° prior to protoplast formation, the RNA extracted from the membranes apparently contained higher proportions of the active component. Approximately a 15-fold increase in the ratio $K_{RNA}:K_{basal}$ of the "transition" 569-RNA-treated recipient cells over the control cells is observed.

Strain and Species Specificity of the Response to 569/H RNA. When strain *B. cereus* 5, a microconstitutive (Pollock, 1957) was used as recipient cells for 569/H RNA, a sevenfold increase over basal in the differential rate of penicillinase synthesis was observed (Figure 4A). With *B. subtilis* ICI strain (basal penicillinase synthesis negligible) and *S. aureus* 187 (peni-

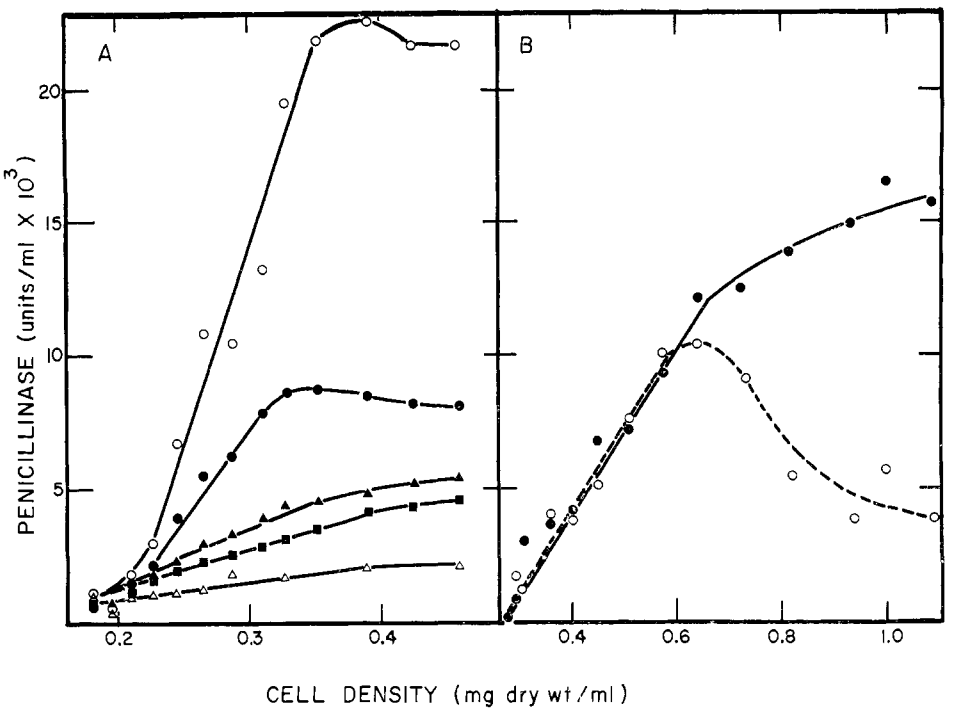

FIGURE 4: Strain and species specificity of recipient cell response to *B. cereus* 569/H membrane RNA. (A) Test of strain specificity of recipient cell response. Conditions for testing biological activity of 569/H RNA in recipient cells were described in Materials and Methods. (▲—▲) Uninduced *B. cereus* 569 (K = 0.012); (○—○) *B. cereus* 569 with RNA at 22 μg/ml (K = 0.16); (■—■) uninduced *B. cereus* 5 (K = 0.010); (●—●) *B. cereus* 5 with RNA at 22 μg/ml (K = 0.072); (△—△) penicillin-(0.3 unit/ml) treated *B. cereus* 5 (K = 0.003). (B) Test of species specificity of recipient cell response. Except for variations noted below, biological activity of RNA was tested as outlined in Materials and Methods. To a 175-ml culture of *S. aureus* 8325 (N) (at 0.275 mg dry wt/ml), 10 ml of bentonite-treated 569/H RNA was added to give a final concentration of 4.5 μg/ml. Duplicate samples were removed at intervals. One set of samples was placed in oxine-gelatin for assay as described in methods while the other set was subjected to quick-freeze, quick-thaw treatment three times. The cells were removed by centrifugation at 10,000g for 10 min and an aliquot of the supernatants was placed in oxine-gelatin for assay. (○—○) Normal samples; (●—●) freeze-thawed samples. Initial K values for both the samples are the same (K = 0.32).

cillinase microinducible strain) the response to 569/H RNA, although significant, was not as great when compared with homologous donor RNA-recipient species. Only rates comparable to basal *B. cereus* 569 synthesis were obtained. The 569/H RNA extracts usually evoked a marked response in penicillinase production in the penicillinase-negative *S. aureus* 8325 (N) (Figure 4B). As can be expected from a strain that has lost the genetic capacity for its own penicillinase synthesis (Novick, 1963), no penicillinase activity is measurable in the control culture. A considerable drop in the culture enzyme activity, as also seen with penicillin-induced *Staphylococci* (Swallow and Sneath, 1962), was observed after approximately 30 min. This drop can be explained by a readsorption phenomenon causing some of the enzyme activity to become latent. This latent enzyme activity can be measured merely by a freeze-thaw technique as has been done in the parallel samples of Figure 4B. The kinetics of pencillinase synthesis in this recipient *S. aureus* then becomes similar to that in recipient *B. cereus*.

Neutralization of the Penicillinases Produced by the Various Recipient Cells by Anti-569/H Penicillinase Serum. Using a nonabsorbed anti-569/H penicillinase serum, serological identity of the penicillinase produced by recipient cells with that of constitutive *B. cereus* enzyme was indicated by similar neutralization slopes (3.05–3.60) for purified 569/H penicillinase, crude 569/H RNA *B. cereus* 5 penicillinase, and crude 569/H RNA *S. aureus* 8325 (N) penicillinase. These observations were substantiated using the absorbed anti-569/H penicillinase serum which gave neutralization slopes of 76, 58, and 40 for purified 569/H penicillinase, crude 569/H RNA *B. cereus* 5 penicillinase, and purified 569/H RNA *S. aureus* 8325 (N) penicillinase, respectively (Figure 5). The lower value for the neutralization slope in 569/H RNA 8325 (N) penicillinase and presence of some residual activity could be due to an insufficient number of determinations in the linear portion of the titration curve and incomplete reconversion of γ- into α-penicillinase upon elution from glass beads (Citri and Garber, 1958).

Effect of Actinomycin D. The results presented in Figure 6A are in agreement with the earlier observations (Pollock, 1963) on the temporary resistance of pre-induced cells to low concentrations (0.055 μg/ml) of actinomycin. This concentration of the antibiotic which inhibits induced penicillinase synthesis after approximately 30 min has no inhibitory effect on the 569/H RNA-stimulated enzyme synthesis irrespective of the time interval between RNA and antibiotic addition (Figure 6B). The increase in the rate of penicillinase synthesis in the RNA-induced culture after approximately 60 min (Figure 6B) is as yet unexplained. When

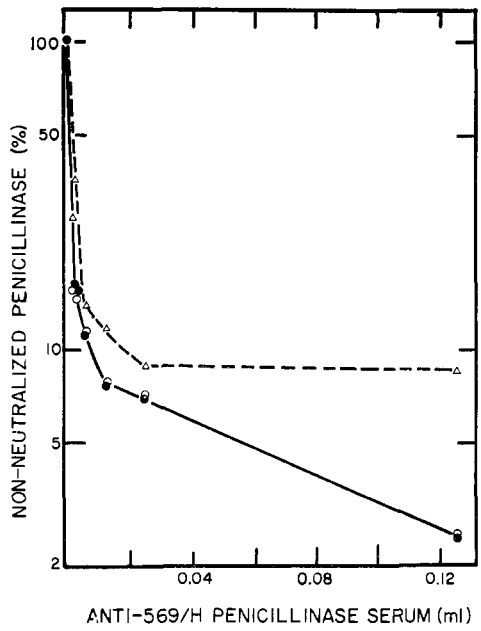

FIGURE 5: Neutralization of various recipient cell penicillinases using a partially absorbed anti-569/H penicillinase serum. The serum was prepared as outlined in Material and Methods. Constant antigen titrations were carried out. (●—●) Glass absorption-Celite column-purified *B. cereus* 569/H penicillinase at a reaction mixture concentration of 0.106 unit/ml; (○—○) crude 569/H RNA *B. cereus* 5 penicillinase at a reaction mixture concentration of 0.0431 unit/ml; (△—△) glass absorption-elution-purified 569/H RNA *S. aureus* 8325 (N) penicillinase at a reaction mixture concentration of 0.0428 unit/ml.

the concentration of actinomycin was increased to a growth inhibitory level (0.1 μg/ml), the penicillin-induced synthesis was halted essentially within 5 min (Figure 6C). This result agrees well with Yudkin's finding (1966) demonstrating that actinomycin at 1 μg/ml of *Bacillus licheniformis* culture, completely stopped penicillin-induced penicillinase synthesis within 8 min. Corroborating the results at 0.055 μg/ml, 0.1 μg/ml has no inhibitory effect on the RNA-induced synthesis (Figure 6D). Treatment with actinomycin shortly after 569/H RNA addition caused a noticeable increase in the rate of penicillinase synthesis over the 569/H RNA-recipient cell control. Verification of this preferential synthesis of penicillinase by recipient cells in the presence of 569/H RNA and actinomycin (0.055 μg/ml) is evident in Figure 6E. Preincubation of 569/H RNA with actinomycin caused an increased rate of enzyme synthesis over the RNA-induced control. Preaddition (20 min) of actinomycin to the recipient cells caused a 45% inhibition of penicillin-induced penicillinase synthesis, a finding previously reported by Pollock (1963). However, considerable enhancement of penicillinase synthesis (threefold over RNA-induced control) was observed when 569/H RNA was added to such pretreated cells. If the recipient cells are pretreated for 60 and 120 min with actinomycin at 0.055 μg/ml, washed free of the antibiotic, and resuspended in growth medium at a density approaching resting cell conditions, similar results are obtained; *viz.*, pretreatment with actinomycin decreases the penicillin-induced penicillinase synthesis whereas the RNA-stimulated rate of enzyme synthesis is enhanced in such pretreated recipient cells.

Effect of Chloramphenicol, Streptomycin, and Puromycin. With streptomycin and puromycin, cessation of enzyme synthesis in both penicillin-induced and RNA-stimulated cells was immediate (Figure 7A–D). With chloramphenicol (Figure 7E,F), even though complete cessation of penicillinase synthesis is not achieved, significant inhibition of both penicillin-induced and RNA-stimulated enzyme synthesis is evident. In fact, since this concentration (30 μg/ml) of chloramphenicol was not completely bacteriostatic, "apparent" differential rate of synthesis could be compared. It became evident that, under these conditions, the RNA-induced penicillinase synthesis was considerably more sensitive than the penicillin-induced synthesis, especially when chloramphenicol is added at a later time (82% inhibition *vs.* 71 and 68 *vs.* 23% at 0- and 60-min addition of chloramphenicol, respectively).

Discussion

Extraction of RNA and Concentration Effects of the Active Component and Inhibiting RNA Species. The results presented in this article suggest that RNA extracted from washed protoplast membranes of a penicillinase constitutive *B. cereus* contains a factor specific for penicillinase synthesis. The biological activity of this component is manifested in the non-induced recipient *B. cereus* cells by an increase in the differential rate of penicillinase synthesis. Increase in the relative amounts of the active component by RNA extraction from the protoplast membrane (as was usually done) rather than the whole protoplast would be expected considering the cellular components demonstrated to be associated with the membrane (Schlessinger, 1963; Suit, 1963). "Stepdown transition" (Hayashi and Spiegelman, 1960; Gros *et al.*, 1961) of the 569 RNA donor cell appears to increase the amount of the active component in the membrane RNA extract either by preferential synthesis, extraction, or stabilization of the active component. The presence of chloramphenicol during protoplast formation did not increase the proportion of the active component.

The recipient cell response to the concentration of RNA showed saturation kinetics, but these kinetics varied with the RNA preparation. This variation suggested differences in the extracts in the ratio of the active fraction to both competitive and noncompetitive inhibiting RNA species. The competitive nature of some of the various RNA species, especially tRNA, was definitively demonstrated. These results do not indicate, however, what component(s) in the 569 RNA extract cause the noncompetitive type of inhibition nor do they allow the determination of the actual site(s) at which the inhibition takes place. In addition, however, the greater activity of membrane RNA would be expected, simply because of the elimination of a large amount of competing RNA species present in the cytoplasm. The removal of these competing species by MAK column fractionation demonstrated conclusively

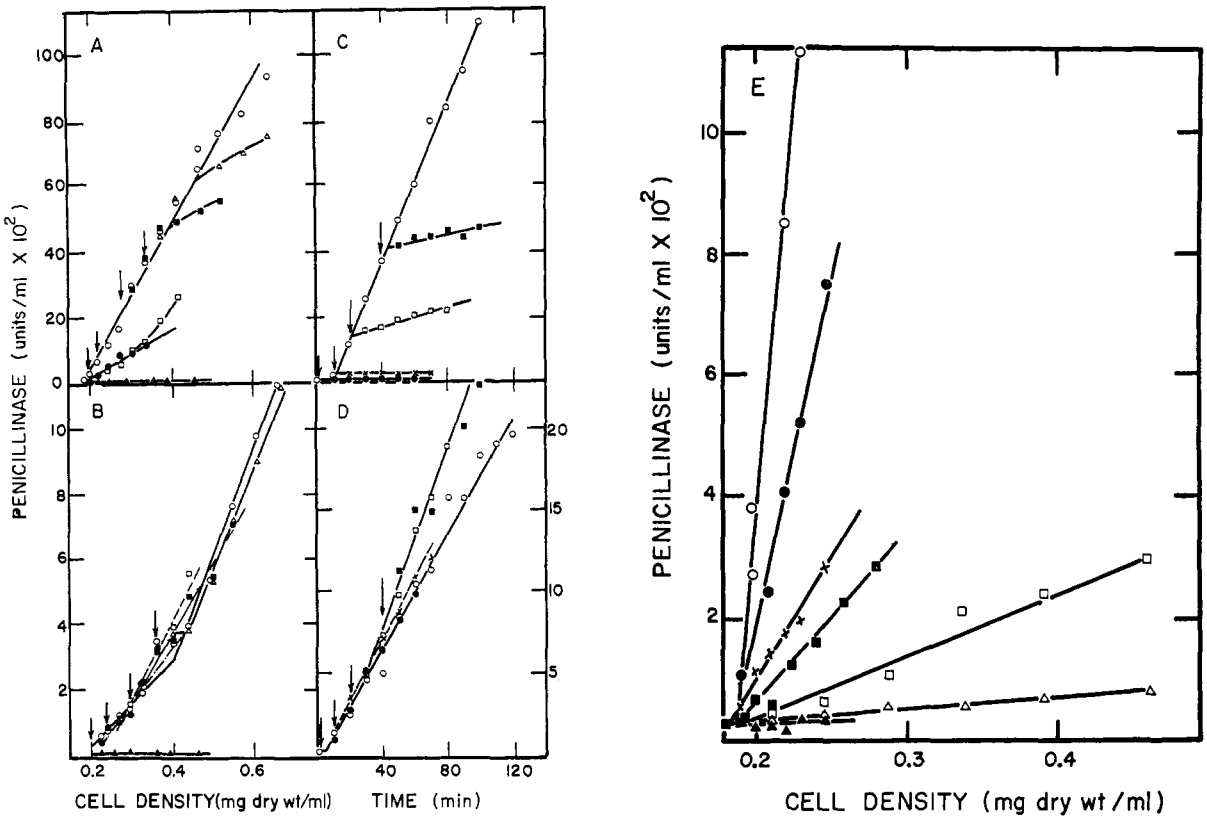

FIGURE 6: Effect of actinomycin D on penicillinase synthesis. (A) Penicillin-induced system. *B. cereus* 569 recipient cells at 0.18 mg dry wt/ml were induced with penicillin (10 units/ml) and aliquots of the culture were treated with actinomycin at 0.055 μg/ml of culture at the time intervals indicated by the arrows. The rate of penicillinase synthesis was followed for 1 hr in all the cultures as outlined in Materials and Methods. (●—●) Actinomycin at 0 min; (□—□) at 20 min; (■—■) at 40 min; (△—△) at 60 min; (○—○) induced control without actinomycin (K = 2.19); (▲—▲) uninduced control without penicillin or actinomycin (K = 0.02). (B) RNA-stimulated system. Experimental conditions were similar to A with the exception that 569/H RNA (2.5 μg/ml) was added instead of penicillin. K value for control culture (without actinomycin) was 0.25 for the first 60 min increasing to 0.34 during the latter period. (C) Penicillin-induced system. Actinomycin at 0.1 μg/ml of culture was added to culture aliquots at the time intervals indicated by the arrows. Experimental conditions were similar to A with the exception of the actinomycin D concentration. (○—○) Absence of actinomycin; (●—●) actinomycin at 0 min; (×—×) at 10 min; (□—□) at 20 min; (■—■) at 40 min. (D) RNA-stimulated system. Experimental conditions are similar as in C with 569/H RNA (15.4 μg/ml) added instead of penicillin. (E) Effect of pretreatment of recipient cells with actinomycin D on the subsequent rate of penicillinase synthesis. A. *B. cereus* 569 culture grown to a cell density of 0.18 mg dry wt/ml was divided into two series of 10-ml cultures. The cultures of the second series were incubated with actinomycin at 0.055 μg/ml for 20 min. Various indicated additions were made at time zero (20 min after addition of actinomycin to the second series) to the cultures of both the series and the rate of penicillinase synthesis was followed for 1 hr in all the cultures. Untreated series: (△—△) uninduced (K = 0.018); (○—○) penicillin (10 units/ml) induced (K = 2.2); (□—□) RNA (1.5 μg/ml) stimulated (K = 0.113); (■—■) RNA (preincubated with actinomycin for 20 min) stimulated (K = 0.28). Actinomycin-pretreated series: (▲—▲) uninduced (K = 0.016); (●—●) penicillin (10 units/ml) induced (K = 1.15); (×—×) RNA (1.5 μg/ml) stimulated (K = 0.38).

the direct proportionality of recipient cell response to RNA concentration. The increased response of the recipient cells began to approach a level of full penicillin induction, a situation touching upon the question of recipient cell competency and distribution of the active RNA component. The isolation of sufficient purified labeled specific RNA should permit the resolution of this question as well as those of RNA penetration and binding.

Fate of the Active Component and Tentative Elimination of Some Cell Fraction Candidates. Discussion of the nuclease sensitivity and nature of the active RNA component shall be left largely to the paper on characterization of the active component (Kirtikar and Duerksen, 1968). It will be sufficient for our present purpose to say that the loss of the stimulatory activity of the RNA extracts examined here by RNase treatment supports the conclusion that a specific RNA species is responsible for the biological activity. Development of resistance of the specific RNA component to RNase attack within 3 min after addition to the recipient cells implies penetration of the active component into at least some part of the cell. RNase penetration and/or an increase in accessibility of the active component to its action as well as localization of the binding site near the cell surface, is indicated by the decrease in the RNA-elicited response observed upon the addition of RNase 30 min after the addition of RNA to the recipient cells. Existence of the active RNA component in an autonomous or cytoplasmic state is also suggested. Relative persistence of this acquired trait (an increased linear differential rate of penicillinase synthesis) through at least three generations of recipient cultures implies replication of the introduced

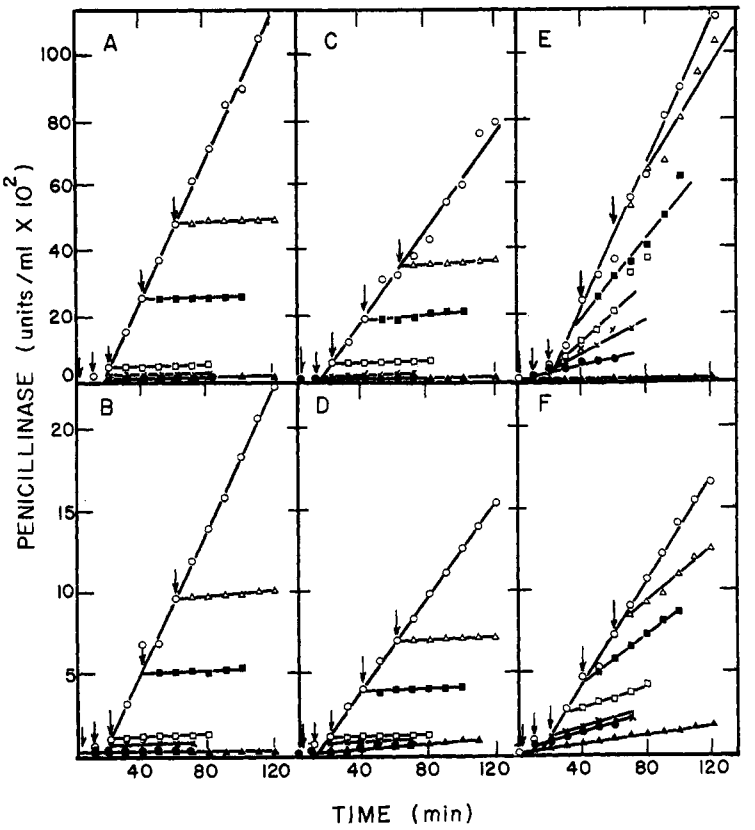

FIGURE 7: Comparison of streptomycin, puromycin, and chloramphenicol inhibition of penicillin-induced and RNA-stimulated penicillinase synthesis. Experimental conditions and sampling were similar to that described in Figure 6. Streptomycin (50 μg/ml) inhibition of (A) penicillin (10 units/ml) induction and (B) RNA (14.9 μg/ml) stimulation. Puromycin (20 μg/ml) inhibition of (C) penicillin (10 units/ml) induction and (D) RNA (15.4 μg/ml) stimulation. Chloramphenicol (30 μg/ml) inhibition of (E) penicillin (10 units/ml) induction and (F) RNA (16.8 μg/ml) stimulation. (▲—▲) Uninduced control (no antibiotic or inducer); (○—○) induced control (no antibiotic); (●—●) antibiotic at 0 min; (×—×) at 10 min; (□—□) at 20 min; (■—■) at 40 min; (△—△) at 60 min.

fraction by cellular polymerases to a limited extent in some unknown manner; a suggestion already made for the sulfanilamide resistance RNA fraction of *Pneumococcus* (Evans, 1964; Spizizen *et al.*, 1966). This suggestion requires experimental confirmation.

The possibility of transformation by the trace amounts of 569/H (constitutive) DNA present in the RNA extracts is not completely excluded by sensitivity to RNase, resistance to DNase, and lack of stimulating activity in isolated 569/H DNA. From our results such a possibility requires that constitutivity be dominant over inducibility and that this dominance be expressed without a noticeable integration or phenotypic lag. Dominance of inducibility, over constitutivity, however, seems to be the rule (Jacob and Monod, 1961; Richmond, 1965). The inability to demonstrate any transformation to increased penicillinase synthesis with constitutive mutant DNA and failure of RNA-treated cells to form constitutive colonies diminishes the possibility of DNA transformation. In addition, transfer of streptomycin resistance and dependence markers with corresponding *B. cereus* 569 DNA to streptomycin-sensitive *B. cereus* 569/H was unsuccessful (Sr. M. S. P. Lorenz and J. D. Duerksen, unpublished results), and the presence of extrachromosomal genetic elements for penicillinase synthesis in *B. cereus* are tentatively eliminated by use of the elevated growth-temperature technique. It seems unlikely that the introduction into recipient *B. cereus* of DNA fragments carrying the penicillinase operator region with or without the structural gene (P) in numbers sufficient to reduce the repressor concentration would be the cause of the increased rate of enzyme synthesis; but the possibility cannot be eliminated on the basis of our results. Of course, only the introduction of the structural gene fragments into recipient *S. aureus* 8325 (N) along with limited transcription would be necessary. Our results, however, would dictate lack of integration and at most limited replication of these fragments.

Two other possible cell fraction candidates must be considered. A repressor-destroying enzyme as well as an internal inducer (Pollock, 1953) present in the 569/H RNA preparations would be expected to cause an increased differential rate of penicillinase synthesis in *B. cereus* recipient cells but certainly not in *S. aureus* 8325 (N) which lacks a structural gene for any penicillinase synthesis. Though these two candidates would be expected to be resistant to RNase action, the results of Gottlieb *et al.* (1967) suggest that they could exist as an RNase-sensitive RNA–protein complex. Also, on the basis of these worker's results, a penicillinase protein–RNA complex (inactive or incomplete penicil-

linase protein) present in the biologically active 569/H RNA extracts could be RNase sensitive. Carry-over penicillinase protein in the 569/H RNA extracts as a possible RNA complex is a serious candidate for the active fraction of 569/H RNA preparations; a candidacy eliminated only by a demonstration of *de novo* penicillinase synthesis in 569/H RNA recipient cultures.

Effect of Antibiotics as Presumptive Evidence for de Novo Penicillinase Synthesis. The failure of actinomycin D to inhibit the 569/H RNA-stimulated increased rate of penicillinase synthesis in recipient *B. cereus* 569 cells coupled with the expected actinomycin D inhibition of penicillin-induced synthesis of penicillinase (Pollock, 1963; Yudkin 1966) is consistent with the proposed mode of action of this drug as a potent inhibitor of transcription (Reich, 1966; review by Singer and Leder, 1966). Actinomycin has a negligible effect on protein synthesis by a ribosome–mRNA complex. Assuming that inhibition of genetic transcription makes more ribosomes available to the added RNA, the enhancement of the 569/H RNA-stimulated rate of penicillinase synthesis in recipient cells pretreated with actinomycin was expected.

While actinomycin D causes inhibition of protein synthesis at the transcription level, streptomycin, chloramphenicol, and puromycin inhibit at the translation level; the first two by binding to the ribosome subunits (for review, see Brock, 1966; Moldave, 1965; Schweet and Heintz, 1966; Vazquez, 1966), while the latter acts as an analog for aminoacyl-tRNA causing termination of peptide-chain formation (Smith *et al.*, 1965; for review, see Schweet and Heintz, 1966). Consequently our results showing immediate cessation or marked inhibition of penicillinase synthesis in *B. cereus*, whether RNA induced or penicillin induced, after addition of either of these three antibiotics to the culture, suggest a marked similarity in both systems subsequent to the transcription step in the synthesis of penicillinase. These findings coupled with the actinomycin results implicate *de novo* penicillinase synthesis in recipient cells directed by a component found in the added 569/H (constitutive) membrane RNA preparations.

Some [^{14}C]amino acid incorporation experiments not reported here, though not conclusive, lend support to this suggested *de novo* penicillinase synthesis. The specific activity of the labeled, partially purified penicillinase produced by *S. aureus* 8325 (N) in the presence of 569/H RNA was several 1000-fold that of the negligibly labeled "penicillinase-like" protein synthesized in its absence. The amount of possible labeled penicillinase protein found in 569/H RNA extracts was usually considerably less than the penicillinase activity found in recipient cultures after 30-min incubation in the presence of the 569/H RNA. When the amount of incorporated [^{14}C]amino acids found in the donor RNA extract indicated a higher level of possible penicillinase protein, little of this radioactivity found its way into the partially purified recipient culture (8325 (N)) penicillinase. However, the identity of a two-dimensional fingerprint analysis of the purified ^{14}C-labeled recipient culture penicillinase with that of *B. cereus* 569/H penicillinase is minimally necessary for an irrefutable conclusion of *de novo* penicillinase synthesis.

Recipient Cell Specificity and Serological Identity of Recipient Cell Penicillinases. B. cereus 5, for several possible reasons (Jacob and Monod, 1961), exhibits noninducibility and a markedly decreased penicillinase synthesis. Assuming a block at the transcription level, the addition of 569/H membrane RNA bypasses this block (or any other) and causes an increased differential rate of penicillinase synthesis, albeit apparently a *B. cereus* 569/H penicillinase. *B. cereus* 5/B penicillinase can be distinguished from *B. cereus* 569 enzyme by partially absorbed antiserum to either enzyme (Pollock, 1956). On the other hand, complete antigenic and physical–chemical dissimilarity of *B. cereus* and *S. aureus* penicillinase has been demonstrated thoroughly (Pollock, 1956; Kushner, 1960). Consequently, the serologically identified *B. cereus* 569/H penicillinase produced by the absolute negative *S. aureus* 8325 (N) cultures receiving 569/H RNA becomes significant. Whether or not there are any differences between the penicillinase produced by 569/H RNA recipient *S. aureus* 8325 (N) and *B. cereus* 569/H remains the question of further experimentation.

In conclusion, it seems clear that RNA extracts obtained from *B. cereus* 569/H membranes contains a specific fraction causing recipient cells, *B. cereus or S. aureus*, to produce a *B. cereus* 569/H penicillinase apparently by *de novo* enzyme synthesis. It shall be the purpose of the next paper (Kirtikar and Duerksen, 1968) to partially characterize the active RNA component.

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