Inhibition of Synthesis of New Globin Chains in Reticulocyte Lysates by Pactamycin*

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ABSTRACT: Low concentrations of pactamycin inhibit the formation of *new* polypeptide chains in cell-free systems from rabbit reticulocytes. The following data are presented in support of this view. (1) Pactamycin is most effective in inhibiting polypeptide synthesis where there is active formation of new chains and is least effective where the system acts primarily to complete existing nascent peptide. (2) NH_2-terminal valine analysis shows that few, if any, new hemoglobin chains are made in the presence of the antibiotic. (3) Pactamycin induces the rapid and almost complete degradation of polyribosomes to 80S ribosomes through readoff with the release of labeled soluble protein. (4) Antibiotics such as sparsomycin, fusidic acid, or cycloheximide, which interfere with peptide elongation, block the pactamycin-induced polyribosomal decay and polypeptide release. (5) Analysis with polyacrylamide gel electrophoresis shows that all the radioactive polypeptide released into the soluble fraction in the presence of pactamycin is completed globin chains, excluding the premature release of incomplete peptide or peptidyl-tRNA. (6) When the incorporation of radioactive amino acids by prelabeled polyribosomes is prevented by the addition of an excess of unlabeled amino acids, the amount of radioactive hemoglobin chains formed and released is the same whether pactamycin is present or not, indicating that chain elongation and release are unaffected by the antibiotic. (7) The sequential conversion of polyribosomes to 80S ribosomes by pactamycin takes less than 2 min and is consistent with the continued normal readoff associated with a block in the formation of new polyribosomes. At high levels of pactamycin ($\geq 10^{-5}$ M) polypeptide elongation is also affected. This results in a much slower decay of the polyribosomes and release of globin chains.

Pactamycin, an inhibitor of protein synthesis in cells and extracts of procaryotic and eucaryotic cells (Colombo *et al.*, 1966; Felicetti *et al.*, 1966; Bhuyan, 1967; Cundliffe and McQuillan, 1967; Cohen and Coldberg, 1967; Cohen *et al.*, 1969b), has been shown to alter the structure and function of the initiation complex by binding to the smaller ribosomal subunit (Cohen *et al.*, 1969b,a; Macdonald and Goldberg, 1970). In lysates prepared from rabbit reticulocytes, indirect evidence has been presented to show that pactamycin at low concentrations (10^{-6} M) blocks *de novo* protein synthesis, while allowing completion of nascent peptide on ribosomes; at higher levels ($\geq 10^{-5}$ M) of the antibiotic, elongation of peptide appears to be inhibited as well (Macdonald and Goldberg, 1970).

In this paper we present evidence of a more direct nature in support of a primary action of low levels of pactamycin on the formation of new polypeptide chains. We show that expression of the effect of low levels of pactamycin requires polypeptide-chain initiation by reticulocyte ribosomes. Furthermore, the nascent peptide on polyribosomes can be converted into completed globin chains in the presence of a concentration of pactamycin which completely blocks the formation of new chains and of new polyribosomes, while permitting the breakdown of existing polyribosomes through mRNA readoff.

Experimental Section

Preparation of Reticulocytes. New Zealand, white male rabbits (2–3 kg) were injected on days 1, 2, 4, and 5 with fresh solutions of 1.5% (v/v) phenylhydrazine, 0.5 ml/kg per day. Blood was collected into heparinized syringes by cardiac puncture on day 7 and quickly chilled. After centrifugation, the cells were washed twice in special saline (Colombo *et al.*, 1965) for lysate preparation or in the saline solution described by Lingrel and Borsook (1963) for ribosome and enzyme fractionation.

Preparation of Reticulocyte Lysate. Washed, packed cells were lysed with an equal volume of 1.5×10^{-3} M $MgCl_2$ and the lysate clarified at 15,000*g* for 15 min. Ribosome concentrations in the lysates were calculated from automatically integrated areas on sucrose gradients and compared to known standards sedimented under equivalent conditions. The extinction coefficient used was 11 $A_{260\,m\mu}$/mg per ml (Felicetti and Lipmann, 1968). Lysates were immediately stored in liquid nitrogen and thawed only once prior to use.

Assay of Protein Synthesis in Lysates. Protein synthesis by lysates was measured as described by Adamson *et al.* (1968), with some modifications. Reactions incubated at 37° contained in a final volume of 1 ml the following components: 2 μmoles of $MgCl_2$, 100 μmoles of ammonium acetate, 10 μmoles of Tris-HCl (pH 7.6), 5.35 μmoles of 2-mercaptoethanol, 1.2 μmoles of ATP, 0.36 μmole of GTP, 5 mg of creatine phosphate, 0.25 mg of creatine phosphokinase, 0.04 μmole each of 20 amino acids plus 4 μCi of a [^{14}C]amino acid mixture or 0.055 μmole each of 18 amino acids plus 0.11 μmole each of L-[^{14}C]valine (230 μCi/μmole) and L-[^{14}C]leucine (250 μCi/μmole), 0.04 μmole of hemin, and 600 μl of 1:1 lysate. The order of addition and preparation of the hemin solution was as described by Maxwell and Rabinovitz (1969) except that the hemin was diluted in 10^{-3} M KCN just prior to use (T. Hunt, personal communication). Antibiotics were added

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in volumes of solution not exceeding 10% of the reaction mixture; parallel additions of water were added to control reactions.

For determination of hot trichloroacetic acid insoluble radioactivity 10-μl aliquots were removed as indicated from reaction mixtures containing [^{14}C]valine and [^{14}C]leucine and quickly chilled. Incorporation into hot trichloroacetic acid precipitable material was as previously described (Macdonald and Goldberg, 1970) except that precipitates were collected on Millipore filters, dried, and placed in scintillation vials; 0.6 ml of concentrated NH_4OH was then added to the vials and after 15 min at room temperature, the filters were dissolved in Bray's counting solution. Samples were counted in a Packard scintillation spectrometer with an efficiency for ^{14}C of 65%.

Ribosomes and Enzyme Fraction. Lysis of washed, packed cells was as described by Allen and Schweet (1962). After clarification at 15,000*g* for 15 min, ribosomes were collected by centrifugation in the Spinco 30 rotor at 30,000 rpm for 2.5 hr and resuspended in 0.25 M sucrose, 0.05 M Tris-HCl (pH 7.3), and 2×10^{-3} M $MgCl_2$ (Felicetti and Lipmann, 1968). Preparations (approximately 450 $A_{260\,m\mu}$/ml) were stored in liquid nitrogen and thawed only once prior to use.

A crude enzyme fraction was prepared by the method of Felicetti and Lipmann (1968) from the supernatant obtained above. After precipitation of the pH 5.0 supernatant fraction with 70% $(NH_4)_2SO_4$, the pellet was dissolved in buffer (0.25 M sucrose, 0.05 M Tris-HCl (pH 7.4), 10^{-4} M EDTA, and 10^{-2} M 2-mercaptoethanol), dialyzed against the same buffer, clarified at 10,000*g* for 10 min, and stored in liquid nitrogen.

Preparation of Rabbit Hemoglobin. Normal rabbit hemoglobin used in the fractionated system, as marker in polyacrylamide gels, and as carrier where indicated was prepared by chromatography on CM-Sephadex C-50 according to the method described by Winterhalter and Huehns (1964).

Sucrose Density Gradient Centrifugation. Linear, 15–30% sucrose density gradients in a final volume of 11 ml were prepared in standard buffer (10^{-2} M Tris-HCl (pH 7.4), 1.5×10^{-3} M $MgCl_2$, and 10^{-2} M KCl). For analysis, 200-μl reaction mixtures were diluted in 350 μl of cold standard buffer and 500-μl portions of the diluted sample were layered onto gradients. Centrifugation was at 41,000 rpm in the SW 41 rotor at 4° for 85 min unless otherwise indicated. For fractionation, tubes were pierced through the bottom and the sample displaced upward by 2 M sucrose through a Beckman fraction recovery system. Gradients were continuously monitored beginning with the supernatant fraction by a Gilford spectrophotometer equipped with an International Equipment 2-mm path flow cell and a Disc integrator.

Unless otherwise indicated, distribution and quantitation of ribosome-bound peptide were determined by collecting fractions directly into scintillation vials containing Bray's solution. Samples were then counted in a Packard scintillation spectrometer at an efficiency of 75%.

In some experiments, the uppermost 1.5 ml of each gradient was carefully removed prior to fractionation. Aliquots (20 μl) of this pooled supernatant fraction (protein concentration 10 mg/ml) were analyzed as described above for hot trichloroacetic acid precipitable radioactivity, and where indicated, samples were prepared for analysis by neutral sodium dodecyl sulfate polyacrylamide gel electrophoresis. The remainder of the gradient was fractionated as described above.

Distribution of Radioactivity between Supernatant and Ribosomal Fractions. Recovery of Nascent Chains. The distribution of [^{14}C]leucine- and [^{14}C]valine-labeled peptide synthesized in reaction mixtures was also analyzed by separating supernatant protein from ribosome-bound nascent peptide in discontinuous gradients composed of 4.5 ml of 20% sucrose and a layer on the bottom of 200 μl of 75% sucrose prepared in standard buffer plus 2-mercaptoethanol (0.01 M). Centrifugation was for 2.5 hr at 4° in the Spinco SW 50.1 rotor at 50,000 rpm.

For quantitation of supernatant protein, the uppermost 1.0 ml and the next 3.0 ml were removed separately. Because protein concentrations in each pool differed four- to fivefold, duplicate aliquots of 25 and 100 μl, respectively, were removed from each pool and analyzed for hot trichloroacetic acid precipitable material as described above. Radioactivity measured in these fractions was taken together for calculations of total peptide released in synthesis.

Polysome-bound nascent chains, largely freed of supernatant protein by the above procedure, were recovered by the following method (Hunt *et al.*, 1968). Ribosomal pellets were resuspended by homogenization in approximately 100 μl of the 75% sucrose immediately above them. Ribonuclease and 2-mercaptoethanol were added to a final concentration of 100 μg/ml and 1%, respectively. After incubation at 37° for 5 min, volumes were made up to 250 μl with 0.01 M phosphate buffer (pH 7.0), 25 μl of a 10% sodium dodecyl sulfate solution was added, and samples were transferred to a 100° bath for 2 min. After cooling to room temperature, 20-μl aliquots of the clear solutions were removed, 50 μg of rabbit hemoglobin carrier was added, and hot trichloroacetic acid precipitable radioactivity was determined as described above. Samples to be analyzed by polyacrylamide gel electrophoresis were then dialyzed overnight in small cells against buffer A (10^{-2} M phosphate buffer (pH 7.0) containing 0.1% 2-mercaptoethanol and 0.1% sodium dodecyl sulfate).

Preparation of Supernatant Fractions for Electrophoretic Analysis. Supernatants (1.5 ml) from linear 15–30% sucrose gradients were prepared for polyacrylamide gel electrophoresis by the following procedure. 2-Mercaptoethanol was added to a final concentration of 1% and dry sodium dodecyl sulfate equivalent to five times the protein concentration in each fraction was added (Maizel, 1969). Samples were then heated at 100° for 2 min, cooled, and dialyzed at room temperature overnight against two changes of 1000 volumes of buffer A. Omitting the detergent, 100° treatment resulted in a significant loss of hot trichloroacetic acid precipitable material upon subsequent dialysis of supernatant fractions at room temperature. On the other hand, while essentially no hot trichloroacetic acid precipitable material appeared to be lost during incubation of the ribosome fractions with ribonuclease for 5 min at 37°, aliquots taken before and after dialysis indicated that approximately 15% of the radioactive peptide was dialyzable. Given the population of completed chains remaining (approximately 50%, Figure 9), it is possible that as much as 30% incompleted chains were, in fact, dialyzable.

Polyacrylamide Gel Electrophoresis. Dialyzed [^{14}C]valine- and [^{14}C]leucine-labeled peptides released from polyribosomes in synthesis reactions and isolated from gradient supernatants were analyzed as described by Weber and Osborn (1969) and Maizel (1969) with some modifications. Supernatant protein (250 μg) from each sample was diluted into a final volume of 250 μl in 0.01 M phosphate buffer (pH 7.0), 1.0% 2-mercaptoethanol, 1.0% sodium dodecyl sulfate, and 10% glycerol. Samples were heated at 100° for 1 min and cooled to room temperature.

Bromophenol blue (6 μl of 0.05% solution) was then added

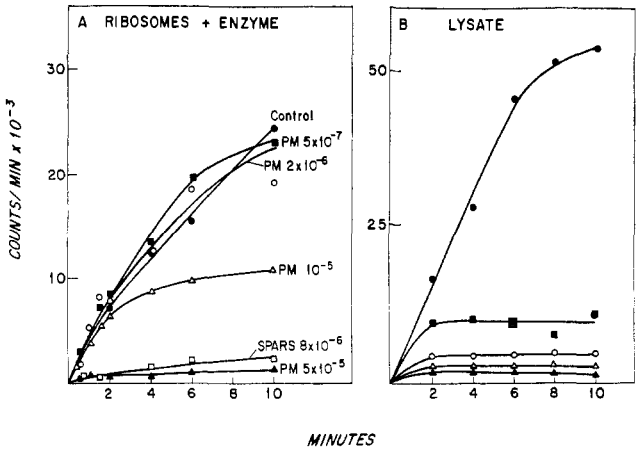

FIGURE 1: Effect of pactamycin on protein synthesis in fractionated and whole lysates. Reaction mixtures containing [^{14}C]valine and [^{14}C]leucine were prepared as described in the Experimental Section. Reactions were identical except that in part A, ribosomes were isolated from the lysate and a crude enzyme fraction was prepared from the supernatant by pH 5 treatment and $(NH_4)_2SO_4$ precipitation, as described in the Experimental Section. The ribosome concentration in each case was 2.5-mg/ml reaction mixture. In addition to ribosomes, reactions in A also contained in a final volume of 1.0 ml: 4.0 mg of crude enzyme protein, 113 μg of stripped rabbit liver tRNA, and 500 μg of hemoglobin, essentially as described by Schaeffer *et al.* (1969). Antibiotics (PM, pactamycin; SPARS, sparsomycin) were added to reactions prior to incubation at 37°. At times indicated, 10-μl aliquots were removed and analyzed for hot trichloroacetic acid precipitable radioactivity as described in the Experimental Section.

TABLE I: NH_2-Terminal [^{14}C]Valine in Hemoglobin Synthesized in the Cell-Free System.[a]

Incubation Conditions	Total [^{14}C]Valine Incorp (cpm)	NH_2-Terminal [^{14}C]Valine (%)
Lysate	2.1×10^5	5.90
Lysate + pactamycin	4.1×10^4	0.76
Lysate + poly(A)	4.7×10^4	0.90
Fractionated system	1.3×10^5	0.75
Fractionated system + pactamycin	1.2×10^5	0.65
Fractionated system + poly(A)	1.2×10^5	0.70

[a] Incubation conditions were as described in Figure 1 except that incubation was for 1 hr. When present, pactamycin was at 2×10^{-6} M and polyadenylic acid at 150 μg/ml. Total hot trichloroacetic acid precipitable radioactivity in each reaction was determined by removal of a 10-μl aliquot prior to separation of ribosomes and supernatant as described by Hardesty *et al.* (1963). The amount of [^{14}C]valine in NH_2-terminal position in the supernatant protein was determined (Hardesty *et al.*, 1963) by Dr. K.-K. Chan. The theoretical value for uniformly labeled rabbit hemoglobin is 8.3% NH_2-terminal valine.

to each. Samples containing purified hemoglobin marker and other proteins (*e.g.*, porcine ACTH and trypsin) used for gel calibration were treated similarly. Samples containing dialyzed nascent chains (corresponding by volume to the related supernatant fraction used for gels) were prepared as described above except that 20 μg of hemoglobin carrier was added to each.

Electrophoresis buffer was 0.1 M phosphate (pH 7.0) containing 0.1% sodium dodecyl sulfate. Samples were layered onto 20-cm, 15% acrylamide gels (0.6 cm in diameter). Electrophoresis was at room temperature for 17–18 hr at 90 V in a modified Canalco gel electrophoresis unit. When the marker was within 2 cm of the bottom, gels were removed, the region below the marker was discarded, and samples were fractionated from front to origin in a Savant automatic gel fractionator equipped with a syringe mechanism providing a 13:1 water-to-gel ratio. Fractions corresponding to 1.3 mm were automatically collected into scintillation vials and counted at an efficiency of 85% in 10 ml of a solution prepared by mixing 1 l. of Triton X-100 and 2 l. of toluene plus 330 ml of Liquifluor (New England Nuclear).

As described by Weber and Osborn (1969), peptide samples to be stained were treated with coomassie brilliant blue at room temperature for 1.5 hr, washed and destained in a Canalco electrophoretic destainer for approximately 1 hr.

Miscellaneous. Sucrose density gradient "ribonuclease-free" grade sucrose (Mann Research Biochemicals) was used for preparation of all sucrose solutions. Equine hemin (twice crystallized) was purchased from Mann Research Biochemicals. Dialysis tubing was boiled for 10 min, rinsed in distilled water, and finally in dialysis buffer before use. All magnesium stock solutions were measured in a Perkin-Elmer Model 203 fluorescence spectrometer according to the method of Schachter (1961). Protein concentrations were determined by the method of Warburg and Christian (1942). The incorporation of [^{14}C]valine into the NH_2-terminal position of hemoglobin was measured by the dinitrofluorobenzene method as described by Bishop *et al.* (1960).

Results

Dependence of Sensitivity to Pactamycin on Peptide-Chain Initiation. Two cell-free hemoglobin-synthesizing systems which differ in their ability to start new peptide chains were compared for their sensitivity to different concentrations of pactamycin. Figure 1A shows results with the fractionated system of Bishop *et al.* (1960), which has been found to initiate very poorly but to complete existing nascent peptide; in Figure 1B use was made of the reticulocyte lysate system which initiates new peptide chains efficiently in the presence of added hemin (Zucker and Schulman, 1969). Comparison of A and B reveals that polypeptide synthesis in the lysate system is considerably more sensitive to inhibition by pactamycin than is that in the fractionated system. The latter, however, remains sensitive to an inhibitor of peptide-chain elongation, such as sparsomycin (Goldberg and Mitsugi, 1967; Jayaraman and Goldberg, 1968; Herner *et al.*, 1969; Monro *et al.*, 1969). At levels of pactamycin of 10^{-5} and above, the fractionated system is also inhibited, suggesting that high concentrations of pactamycin affect peptide-chain elongation as well. As expected from the foregoing, we have found that at this ribosome concentration 5×10^{-5} M aurintricarboxylic acid, which has been found to block initiation in bacterial and mammalian cell-free systems (Grollman and Stewart, 1968; Stewart *et al.*, 1971), is ineffective in the fractionated system used in A but highly effective in the lysate system.

NH_2-terminal [^{14}C]valine analysis of the labeled hemoglobin synthesized by the lysate and fractionated systems is shown in

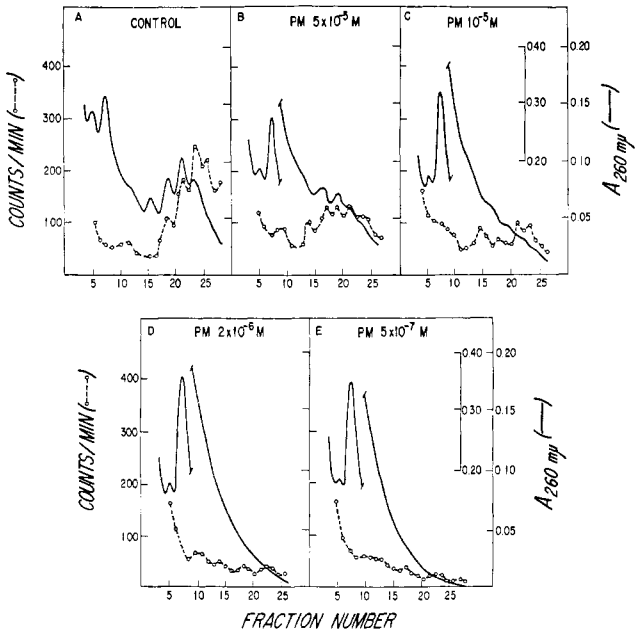

FIGURE 2: Effect of pactamycin concentration of the breakdown of polyribosomes and release of peptide in lysates. Five, 200-μl reaction mixtures containing [^{14}C]amino acids and 2.7 mg/ml of lysate ribosomes were incubated for 3 min. Pactamycin was then added to four reactions at the indicated final concentrations. After further incubation for 2.25 min, samples were diluted in 350 μl of cold standard buffer and subjected to sucrose gradient centrifugation. Fractions (0.4 ml) were collected directly into 2.0 ml of ice-cold 5% trichloroacetic acid. Carrier bovine serum albumin (100 μg) was added and each fraction analyzed for hot trichloroacetic acid precipitable material as described in the Experimental Section except that dried Millipore filters were counted in toluene–Liquifluor counting solution. Sedimentation is from left to right. The inserted scale, 0.2–0.4 A_{260}mμ, refers to the left side of the optical density profile where applicable in this and other figures. Not shown are the optical density and labeling pattern after the initial 3-min incubation. These were essentially the same as that shown in part A.

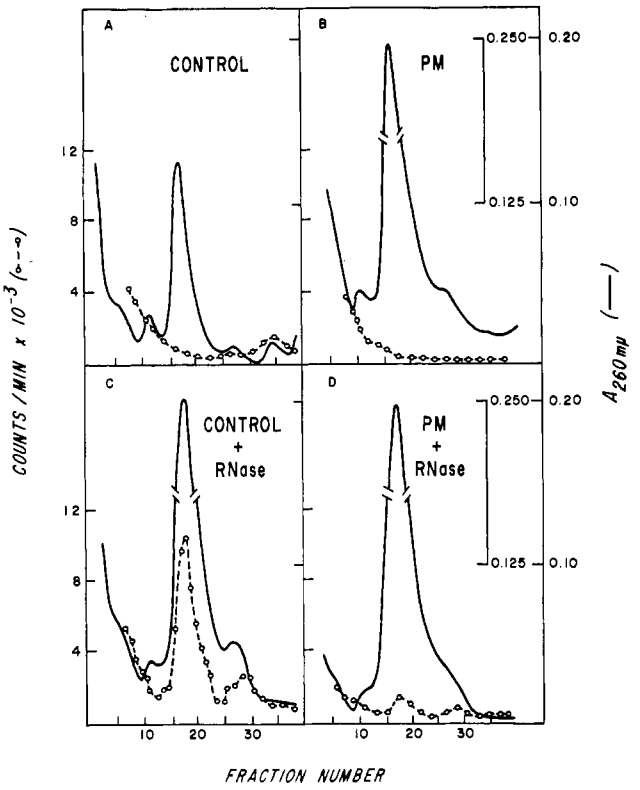

FIGURE 3: Effect of pactamycin and ribonuclease on monomer, dimer, and trimer ribosomal regions. Two identical 500-μl reaction mixtures containing [^{14}C]valine plus [^{14}C]leucine and 2.1 mg of lysate ribosomes were incubated as described in the Experimental Section. At 1.25 min, pactamycin (final concentration 3.5×10^{-6} M) was added to one reaction and incubation was continued for 3 min. After chilling to 0° each reaction was divided in two and ribonuclease (1 μg/ml) was added to parts C and D. After standing at 0° for 15 min, each reaction was analyzed by centrifugation at 41,000 rpm for 175 min in 15–30% sucrose gradients. Not shown are equivalent reactions which were centrifuged for 85 min at 41,000 rpm to ascertain completion of RNase and pactamycin effects.

Table I, confirming the marked differences in the extent of new chain formation in the two systems. Over 70% of the radioactivity in the lysate system is in chains synthesized *de novo*, whereas in the fractionated system less than 10% of the radioactivity can be accounted for by newly made chains. In the presence of pactamycin (2×10^{-6} M) the NH_2-terminal [^{14}C]valine content is markedly decreased, indicating that few, if any, new chains are made in the presence of the antibiotic. Polyadenylic acid, which has been shown to have a predominant effect on peptide-chain initiation in the reticulocyte cell-free system resembles pactamycin in its effect on NH_2-terminal [^{14}C]valine incorporation (Hardesty *et al.*, 1963). These data and those of Figure 1 have been taken to indicate that pactamycin (2×10^{-6} M) has its major action on polypeptide-chain initiation.

Pactamycin-Induced Degradation of Polyribosomes in Reticulocyte Lysate. If pactamycin acts predominantly by blocking new polypeptide chain synthesis, the antibiotic should prevent the formation of polyribosomes while allowing preexisting ones to decay upon completion and release of their peptide chains. An effect of this sort has been described by Colombo *et al.* (1966) in intact reticulocytes treated with 10^{-4} M pactamycin. The observed polyribosome degradation, however, was exceedingly slow, requiring up to 30 min for completion. Such slow kinetics of polyribosome degradation would not be expected if initiation were the sole site of action of pactamycin. We, therefore, undertook to study this in greater detail in reticulocyte lysates. In these experiments, conditions were used (*e.g.*, hemin addition) such that at the time of addition of the antibiotic the system was actively initiating new chains as evidenced either by the maintenance of the polyribosomal pattern or the continual increase in polyribosomes and a reciprocal decrease in 80S monomers to approximately 20% of this total optical density over the first 5- to 7-min incubation. Any breakdown of polyribosomes would then be due to the added pactamycin.

At all of the concentrations of pactamycin used in the experiments of Figure 2 there is decay of the polyribosomes in the sequence from larger to smaller ones with the formation of 80S monosomes concomitant with the release of soluble radioactive polypeptide (quantitated in Figure 5) within 2.25-min incubation. This effect is most pronounced at the lower concentrations of pactamycin used in D and E. At this ribosome concentration (2.5 mg/ml), the polyribosome pattern is better preserved as the level of pactamycin is increased above 2×10^{-6} M (B and C) or decreased below 5×10^{-7} M (not shown). The higher levels of antibiotic which inhibit polyribosome breakdown are those which inhibited polypeptide synthesis in the noninitiating systems (Figure 1A), presumably by affecting chain elongation. As the concentration is decreased below 5×10^{-7} M, the pattern approaches that in the

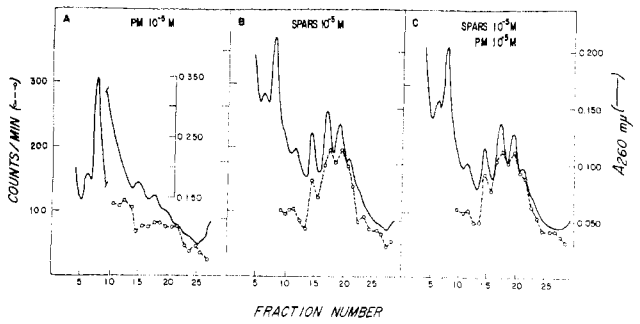

FIGURE 4: Effect of sparsomycin on pactamycin-induced polyribosome breakdown. Three 200-μl mixtures containing [^{14}C]amino acids and 2.7 mg/ml of ribosomes were prepared and incubated as described in Figure 2. At 3 min, pactamycin and sparsomycin were added to reactions at the concentrations indicated and incubation was continued for 3 min. Sparsomycin was added to part C 15 sec before addition of pactamycin. Reactions were quickly chilled in cold standard buffer and analyzed by sucrose gradient centrifugation.

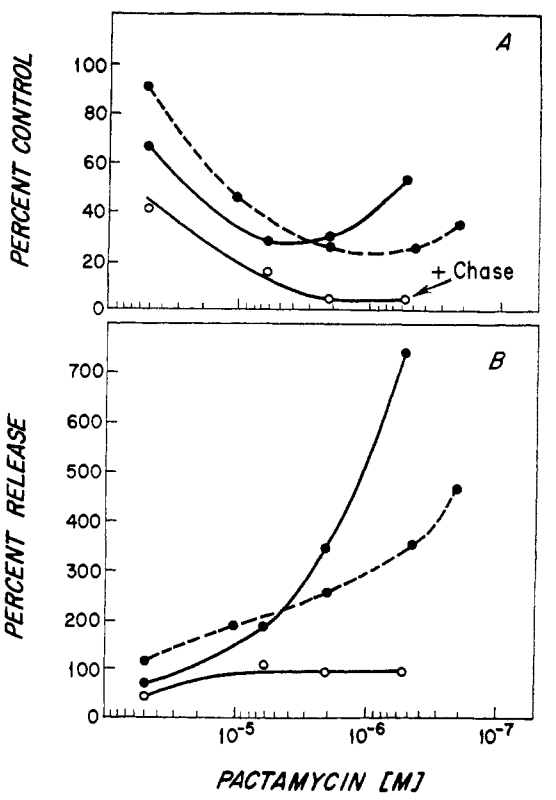

FIGURE 5: Distribution of peptide between supernatant and ribosomal fractions isolated from reactions incubated with varying concentrations of pactamycin. Reaction mixtures containing [^{14}C]-valine plus [^{14}C]leucine in 500 μl were incubated as described in the Experimental Section. Solid and dashed lines represent reactions containing 4.3 and 1.7 mg per ml of ribosomes, respectively. At 1.25 min (zero time), a control reaction for each ribosome concentration was rapidly chilled and pactamycin was added at the indicated final concentrations to the other reactions. To one series of mixtures (+ chase, ribosome concentration 4.3 mg/ml), 15 μmoles each of unlabeled valine and leucine were added together with pactamycin. An equivalent addition to reactions containing 1.7 mg/ml of ribosomes is not shown. After further incubation for 3 min, reactions were chilled and 400-μl aliquots layered onto discontinuous gradients. Distribution of hot trichloroacetic acid precipitable radioactivity in ribosomal and supernatant fractions was determined as described in the Experimental Section. (A) Per cent nascent peptide remaining on ribosomes after incubation with pactamycin. Numbers are expressed as per cent peptide present at 1.25 min (for 1.7 mg/ml of ribosomes, 9.5×10^4 cpm; for 4.3 mg/ml of ribosomes, 1.5×10^5 cpm. (B) Per cent nascent peptide released. Numbers are expressed as per cent nascent peptide released to supernatant fractions. Zero time backgrounds (for 1.7 mg/ml of ribosomes, 9.3×10^4 cpm; for 4.3 mg/ml of ribosomes, 2.0×10^5 cpm) have been subtracted from each. Protein synthesis was linear for the time interval assayed. After incubation for 4.25 min, control supernatants contained 8.2×10^5 and 1.1×10^6 cpm (representing an increase of 880 and 800% of the nascent chains) for the low and high ribosome concentrations, respectively. In each case, corresponding polysome-bound peptide increased by 15%. The same amount of labeled peptide was found on the ribosome or released into the supernatant in "chase" reactions from which pactamycin was omitted as in those in which it was present at its lowest shown concentration.

control reaction; both polyribosome degradation and inhibition of protein synthesis disappear simultaneously, so that by 2×10^{-8} M both polyribosome formation and protein synthesis are normal. We have obtained similar results with intact reticulocytes.

In some experiments the sucrose density gradient centrifugation was for a longer time so as to reveal more clearly the region of the smaller polyribosomes (dimers and trimers) and the ribosomal subunits. Pactamycin at concentrations which produced maximal polyribosome decay did not lead to the accumulation of any radioactive polypeptide on the small polyribosomes (Figure 3). The small amount of radioactivity which remained associated with the larger polyribosomes after pactamycin addition could be converted into labeled 80S ribosomes by mild RNase treatment (Figure 3D). Furthermore, the decrease in $A_{260\,m\mu}$ associated with the polyribosomes could be entirely accounted for by the increase in 80S ribosomes; there was no increase in ribosomal subunits (unlike the effect produced by aurintricarboxylic acid, unpublished data). The lack of accumulation of subunits with pactamycin may indicate that in this system the antibiotic allows for the formation of an 80S initiation complex which is defective in the subsequent steps of protein synthesis.

Evidence that the pactamycin-induced breakdown of polyribosomes and release of labeled protein required peptide-chain elongation was provided by experiments using antibiotic inhibitors of this step, such as sparsomycin (Figure 4), fusidic acid, or cycloheximide. At concentrations of these agents which block protein synthesis nearly completely, the polyribosome pattern remains the same as when the agent was first added, whether pactamycin is present or not. Furthermore, the pactamycin-induced decay of polyribosomes and release of peptide was not found if incubation of nascent peptide-containing polyribosomes at 37° was carried out in buffer in the absence of the other components necessary for polypeptide synthesis.

Completion and Release of Polypeptide at Different Pactamycin Concentrations. Since pactamycin acts by binding to and altering the function of the ribosome, it is to be expected that the overall effect will depend on the ratio of pactamycin to ribosomes. The effect of this relationship on the release of radioactive polypeptide into the soluble fraction is shown in Figure 5. In this experiment, both the decrease in radioactive polypeptide associated with the ribosomes (A) and the appearance of labeled polypeptide in the soluble fraction (B) were followed in lysates containing two concentrations of ribosomes and several different concentrations of pactamycin. At the lower level of ribosomes, the U-shaped inhibition curve is shifted to the right since less pactamycin is required for maximal release of labeled polypeptide from the ribosomes

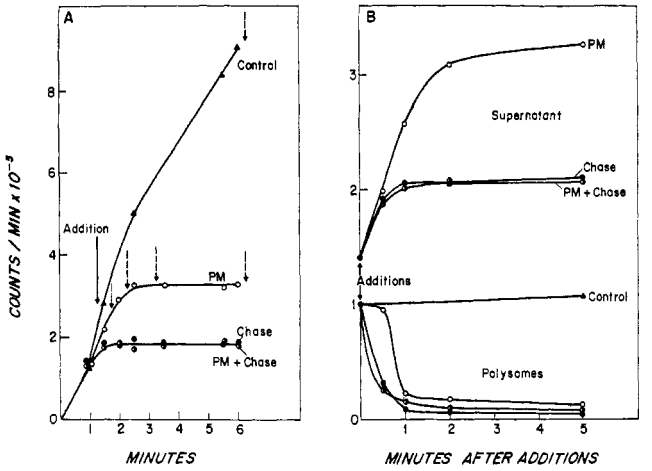

FIGURE 6: Effect of pactamycin (3.5×10^{-6} M) on polypeptide synthesis and distribution as a function of time. Five, 1.0-ml reaction mixtures each containing 4.2 mg of lysate ribosomes plus [^{14}C]leucine and [^{14}C]valine were prepared as described in the Experimental Section. At 1.25 min (additions, A and B), solutions containing pactamycin, pactamycin + chase (0.01 M final concentration each unlabeled valine and leucine), and a chase solution without pactamycin were added as indicated, to three of the reactions. At the same time, a control reaction was chilled, a 200-μl aliquot was used for the zero time point in part B and Figure 7A, and a 400-μl aliquot was layered onto a discontinuous gradient for isolation of nascent chains and analysis by polyacrylamide gel electrophoresis (Figure 9). At times indicated by points in part A, 10-μl aliquots were removed from each incubation and analyzed for hot trichloroacetic acid precipitable radioactivity. At times indicated by dashed arrows, aliquots (200 μl) were removed for analysis in sucrose gradients to obtain the data shown in part B. The ordinate in part A represents the total acid-precipitable radioactivity in the fraction applied to gradients. After centrifugation, the upper 1.5 ml was removed; acid-insoluble radioactivity in 40-μl aliquots determined (part B, supernatant) and expressed as the total acid-precipitable radioactivity recovered in the supernatant fraction. The rest of each sample was prepared for analysis in polyacrylamide gel (Figure 9). The polyribosome region of each gradient was fractionated and counted directly. Polyribosome-bound radioactivity was calculated from timeric and larger polyribosomes.

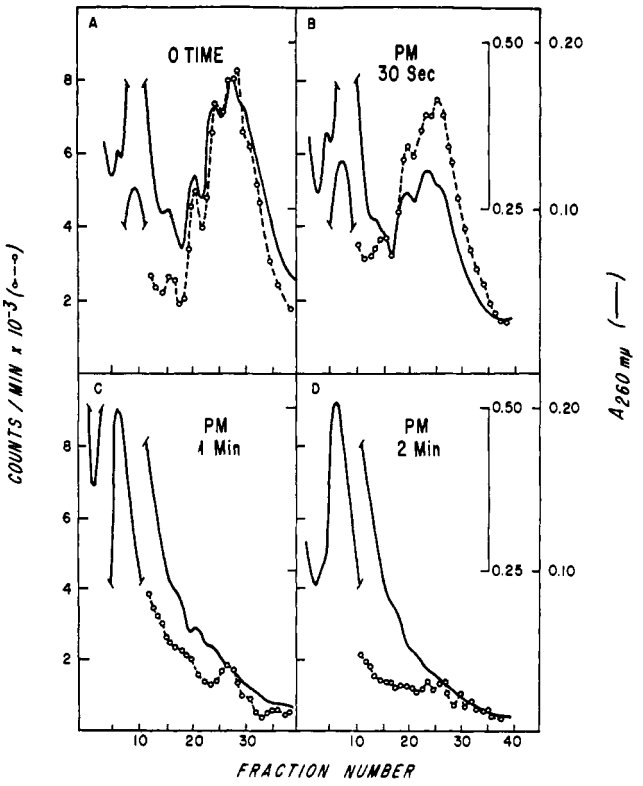

FIGURE 7: Sucrose gradient analysis of polyribosome-peptide complexes as a function of time incubated with 3.5×10^{-6} M pactamycin. Samples were removed and analyzed by sucrose density centrifugation at times indicated by dashed arrows in Figure 6A. (A) The polysome profile at a time of addition of pactamycin (after 1.25-min preincubation). Subsequent sampling times are as indicated. "Chase" reactions were performed but not shown.

(and for maximal polyribosome decay), while permitting extensive inhibition of overall protein synthesis, as measured by the amount of radioactive polypeptide accumulating in the soluble fraction. The curve for the pactamycin-induced release of radioactivity from the ribosomes exhibits a trough in the region of the concentrations of pactamycin where release (and polyribosome decay) is maximal (A). The U shape of these curves can be understood if the trough represents concentrations of pactamycin which maximally inhibit the initiation of new polypeptide chains, while minimally affecting chain elongation, resulting in polyribosome decay and decrease in label on the ribosomes. At the higher pactamycin concentrations, elongation is increasingly inhibited and peptide release from the ribosomes is decreased; at the lower pactamycin concentrations, the block in initiation (and elongation) is less and the ribosomes maintain a more normal labeling pattern. At the lower ribosome concentration (1.7 mg/ml) aurintricarboxylic acid (5×10^{-5} M) gave results almost identical with those with 10^{-6} M pactamycin. At the higher ribosome concentration, this concentration of aurintricarboxylic acid was almost ineffectual over the time interval assayed.

Why is there still residual radioactivity, equivalent to about 25% of that present at the time of pactamycin addition, remaining on the ribosomes at the pactamycin concentration most favorable for release? The residual label is in large part due to the continued incorporation of radioactive amino acids associated with peptide-chain elongation and completion, since the addition of excess unlabeled amino acids under these conditions results in a significant decrease in this fraction to 7%. Furthermore, elongation is probably also slowed down to some extent even at low pactamycin concentrations (see Figure 6). Whether this is a direct effect of pactamycin or secondary to that on the initiation of new polypeptide chains is not known. The continued incorporation of radioactive acids associated with elongation in the absence of initiation also accounts for the slightly more than twofold increase in labeled protein released into the soluble fraction (B). This value is what would be expected if at the time of addition of pactamycin the existing peptide was on the average somewhat less than about half-finished. The experiments in which excess unlabeled amino acids were added support this interpretation. It should be noted that even though the highest concentration of pactamycin (5×10^{-5} M) permits the release of only approximately 30% of the nascent peptide on ribosomes (with 4.3 mg/ml of ribosomes), 50% of that remaining is "chaseable" with unlabeled amino acids. Elongation and release appear, therefore, to continue in the presence of high concentrations of pactamycin, although at a slower rate. Gradient analysis of aliquots from the experiments in Figure 5 are compatible with this concept, since the addition of 5×10^{-5} M pactamycin together with excess unlabeled amino acids to the incubation results in a uniform decrease in labeled peptide across the gradient, as is observed in the control reaction containing no inhibitor; while the addition of pactamycin

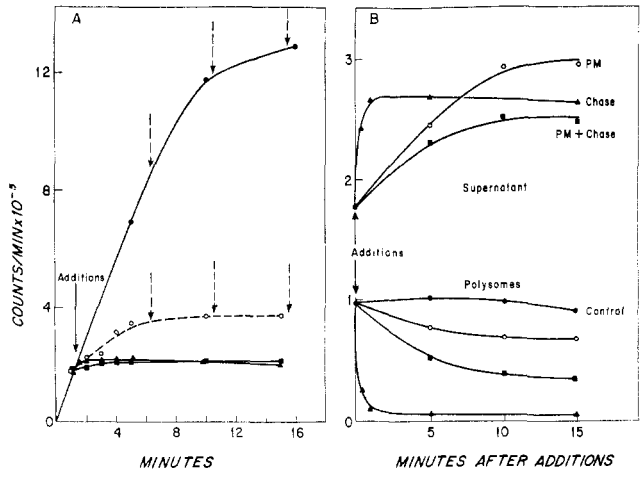

FIGURE 8: Effect of 5×10^{-5} M pactamycin on peptide distribution as a function of time. Reaction mixtures and conditions were as described in Figure 6. Pactamycin (5×10^{-5} M) was added at 1.25 min. Incubation was continued and aliquots removed at times indicated for analysis as described in Figure 6. Times apply to pactamycin + chase and chase reactions also. Hot acid-precipitable radioactivity present in the uninhibited control supernatant at 6.25 min was 8.3×10^6 cpm or 870% of the nascent chains present at zero time.

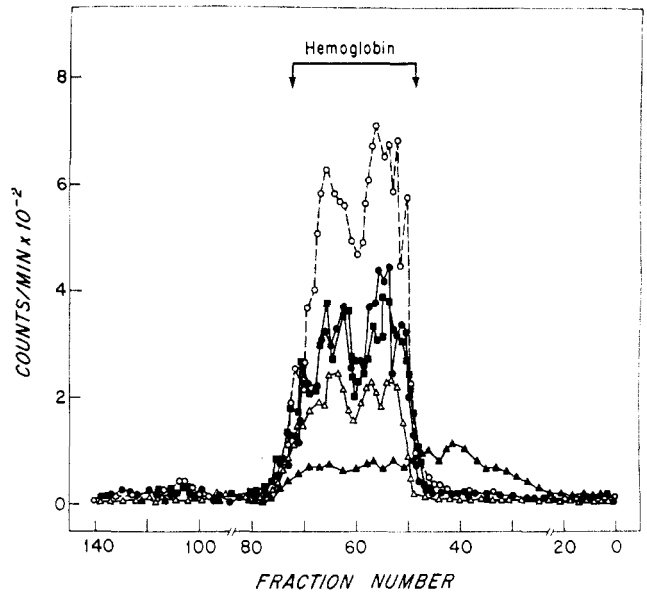

FIGURE 9: Polyacrylamide gel analysis of reaction products. Gradient supernatants from reactions described in Figure 6 were analyzed by polyacrylamide gel electrophoresis as described in the Experimental Section. Samples were as follows: (▲) control nascent chains (zero time), 3562 cpm; (Δ) control supernatant (zero time), 5636 cpm; (■) chase (1 min), 7584 cpm; (●) pactamycin + chase (1 min), 7311 cpm; (○) pactamycin (1 min), 11,043 cpm. Mobility of hemoglobin marker (250 μg) is bracketed by arrows. Two other markers (ACTH (porcine) molecular weight of approximately 3500, and trypsin, molecular weight of 23,000–25,000) were located in gels corresponding to fractions 20–22 and 106–108, respectively. The sample containing nascent chains was run primarily as a marker to indicate mobility of dialyzed incomplete polypeptide in these gels.

alone shows a shift of labeled material to the lighter region of the gradient.

Time Course of Pactamycin Action. The pactamycin effect was nearly completely expressed within 2 min of the addition of 3.5×10^{-6} M pactamycin (the optimal concentration determined for this lysate as described in Figure 5) to the protein-synthesizing system (Figures 6 and 7). At the 30-sec point, however, there appeared to be a dissociation between polyribosome decay and radioactive peptide released (Figures 6B and 7B). The radioactivity which persists on the polyribosomes for the first 30 sec results from the continued addition of radioactive amino acids onto elongating peptide, since the addition of excess unlabeled amino acids obliterates the lag in the release of radioactivity from the polyribosomes. There appears to be a slow release of the small amount of radioactivity still remaining on the polyribosomes after 1 min. As expected of an agent which predominantly blocks polypeptide-chain initiation, the patterns of release of radioactivity from polyribosomes and of accumulation in the supernatant fraction are practically identical with the control when further incorporation of radioactivity is prevented by excess unlabeled amino acids. It should also be noted that the fact that labeled nascent peptide is better maintained on the polyribosomes at 30 sec than the polyribosomes themselves is further evidence against the premature release of incomplete polypeptide chains due to pactamycin.

The previously mentioned results obtained by Colombo *et al.* (1966) in whole reticulocytes at a pactamycin concentration of 10^{-4} M can be understood by reference to the results in Figures 2 and 8. In the latter experiment, the time course of release of peptide from polyribosomes into the soluble fraction of linear gradients was followed at 5×10^{-5} M pactamycin. It can be seen that release is very slow, as suggested by experiments with discontinuous gradients at high concentrations of pactamycin (Figure 5), and continues for a prolonged time, approaching the control in the "chase" experiment. Such a result would be expected if the high level of pactamycin slows down the rate of elongation and/or release in addition to blocking initiation. It is important to note, however, that the system is not frozen. Analysis of polyribosome patterns reveals similar kinetics for their decay as is found for polypeptide release.

Completion and Release of Globin Chains in the Presence of Pactamycin. To determine whether the radioactive protein released into the soluble fraction in the presence of pactamycin (3.5×10^{-6} M) was globin chains or prematurely released incomplete polypeptide chains, gradient supernatants from the experiment shown in Figure 7 were removed before fractionation of the polyribosome region (Figure 7) and the radioactive product of the incubation analyzed by polyacrylamide gel electrophoresis (Figure 9). Radioactivity was found to be associated only with the two globin chains and not with smaller peptides, whether or not pactamycin was included in the incubation. On the other hand, the labeled protein extracted from the ribosomes consisted of smaller incomplete peptides which migrated on electrophoresis more rapidly than globin, in addition to a small amount of probably contaminating globin from the supernatant fraction. The quantity of labeled globin chains released in the incubation in which excess unlabeled amino acids were added after a preincubation with radioactive amino acids was the same with or without pactamycin. As expected, in the incubation where the "chase" with unlabeled amino acids was omitted, pactamycin permitted more than double the amount of completed radioactive globin chains to be released, but again no incomplete chains.

Polyacrylamide gel analysis of the radioactive soluble products from incubations containing high levels of pactamy-

cin also revealed that label was limited to the two globin chains. Similarly, the product synthesized and released in the fractionated lysate (Figure 1A) was also globin. In the presence of 10^{-5} M pactamycin, the amount of soluble labeled globin was reduced proportionately to the inhibition of overall synthesis.

Discussion

Pactamycin induces the breakdown of prelabeled reticulocyte polyribosomes and the release of radioactive peptide by inhibiting the formation of new polypeptide chains. These effects are not due to the premature dissociation of the polyribosomes with the release of peptidyl-tRNA or to the continued readoff of mRNA by the ribosomes, without concomitant peptide-bond formation, and the release of incomplete peptides. The only labeled products of this reaction are completed globin chains. Furthermore, if the protein-synthesizing complex were made to disintegrate by pactamycin, one would expect that higher levels of the antibiotic would be even more effective in this respect, contrary to the observations. Similarly, it was found that at very early times peptide-chain elongation continued in the presence of pactamycin while the polyribosomes were being degraded. Finally, it was shown that continued polypeptide synthesis was essential for expression of the pactamycin-induced decay of polyribosomes.

While at low levels of pactamycin elongation and release of peptide as completed globin chains proceed nearly normally, at higher concentrations the rate of readoff is also slowed down. A possible explanation for some of these findings might be a mechanism of pactamycin action similar to that proposed by Cundliffe (1967) for the tetracycline-induced degradation of *Bacillus megaterium* protoplast polyribosomes. In this scheme, at an optimal concentration of tetracycline which allows for about one antibiotic molecule to be bound per polyribosome, the ribosomes on the mRNA distal to the inactivated ribosome (which is randomly selected by the antibiotic from among the other ribosomes of the polyribosomal structure) continue to readoff and complete their peptide, while those proximal to the obstruction cannot proceed further along the messenger. Such a mechanism permits, on the average, the decay of half the polyribosomes. It seems unlikely, however, that this mechanism fits the situation with pactamycin for the following reasons: (1) polyribosome decay and peptide release considerably exceed 50%; (2) even at high pactamycin levels elongation proceeds, although slowly, so that the affected ribosome cannot be considered to be "frozen" in its path; (3) control amounts of radioactive, complete globin chains are released in the presence of low levels of pactamycin which cause maximal polyribosome breakdown and marked inhibition of overall protein synthesis in the "chase" experiments; and (4) the fractionated system in which peptide-chain completion is the major event is insensitive to pactamycin concentrations which induce maximal polyribosome decay in lysates.

The precise mechanism whereby pactamycin interferes with new polypeptide-chain formation by reticulocyte ribosomes is not yet known. Such as overall effect could result from any of three possible actions: (1) prevention of one of the discrete steps in initiation complex formation from ribosomal subunits, mRNA, and initiator tRNA; (2) inactivation of the formed initiation complex, or (3) block in the production of active ribosomal subunits from "runoff" ribosomes, such as has been reported to occur with NaF (Colombo *et al.*, 1968). In the bacterial system evidence has been provided by the study of partial reactions for an effect of pactamycin on the structure and stability of the initiation complex, suggesting that an inactive complex has been found. Whether this also holds for the complete protein-synthesizing system, remains to be shown. The finding that pactamycin binds selectively at 0° to the reticulocyte 40S ribosomal subunit and to the 80S ribosome lacking mRNA (Macdonald and Goldberg, 1970; M. L. Stewart-Blair and I. H. Goldberg, unpublished data) is compatible with all three mechanism. The lack of accumulation of ribosomal subunits, in contrast to the finding with aurintricarboxylic acid, favors the last two mechanisms, although such an effect might have a more complicated explanation. It is of interest that several of the effects of pactamycin on the reticulocyte protein-synthesizing system resemble those of NaF, which has been shown to produce inactive initiation complexes (Hoerz and McCarthy, 1969; Culp *et al.*, 1970; Pawelek *et al.*, 1971). At higher pactamycin concentrations, the function of ribosomes at steps beyond initiation is also blocked. This effect may be a less specific one and due to antibiotic bound to sites on the ribosome other than those occupied at the lower pactamycin concentrations.

It seems likely that pactamycin alters the various functions of the smaller ribosomal subunit by binding to it. Thus pactamycin may alter the structure of the A (acceptor aminoacyl-tRNA) site and possibly also the P (donor initiator tRNA or peptidyl-tRNA binding) site on the whole ribosome to which the smaller subunit contributes. Such actions would account for the decreased stability of ribosome-bound peptidyl-tRNA, initiator tRNA, and aminoacyl-tRNA at low Mg^{2+} concentrations (Cohen and Goldberg, 1967; Cohen *et al.*, 1969a,b). Since peptide-bond formation, as judged by the puromycin reaction, is unaffected by even high levels of pactamycin (Felicetti *et al.*, 1066; Cohen and Goldberg, 1967; Cundliffe and McQuillan, 1967; Cohen *et al.*, 1969b), the peptidyl transferase which resides only on the larger ribosomal subunit is not affected. While it is possible that the translocation process is in some way inhibited by these levels of antibiotic, evidence from a bacterial system (Cundliffe and McQuillan, 1967) suggests that this is not so.

Some of these features of pactamycin action are similar to those described for streptomycin (Modolell and Davis, 1969, 1970; Schlessinger *et al.*, 1969; Lennette and Apirion, 1970). On the other hand, in *Escherichia coli* extracts programmed with f2 RNA, inhibitory concentrations of pactamycin do not induce polyribosome breakdown (sequential or otherwise) and very little peptide is released to gradient supernatants in the presence of the antibiotic (M. L. Stewart-Blair and I. H. Goldberg, unpublished data). In addition, pactamycin does not induce codon misreading in the *E. coli* system (L. B. Cohen and I. H. Goldberg, unpublished results) and is active against both procaryotes and eucaryotes. Furthermore, streptomycin has little effect on pactamycin binding to *E. coli* ribosomes (M. L. Stewart-Blair and I. H. Goldberg, in preparation).

Cycloheximide has also been found to block peptide-chain initiation and elongation (translocation) in intact mammalian cells and extracts (Lin *et al.*, 1966; Godchaux *et al.*, 1967; Baliga *et al.*, 1969; McKeehan and Hardesty, 1969). In cell-free systems chain initiation is more sensitive to cycloheximide than is chain elongation (Baliga *et al.*, 1969), whereas the opposite is true for intact cells (Stanners, 1966; Godchaux *et al.*, 1967). In this respect pactamycin differs from cycloheximide, since in both reticulocytes and their lysates peptide chain initiation is the more sensitive step, as has also been bound for NaF (Lin *et al.*, 1966). Furthermore, while cycloheximide inactivates transferase II (Baliga *et al.*, 1969; Mc-

Keehan and Hardesty, 1969) and involves the 60S ribosomal subunit (Rao and Grollman, 1967), pactamycin inactivates the ribosome (Felicetti *et al.*, 1966) and binds to its 40S subunit (Macdonald and Goldberg, 1970). Finally, cycloheximide activity is limited to eucaryotes, whereas pactamycin works on procaryotes as well.

Added in Proof

Since submission of this paper additional evidence that pactamycin inhibits the initiation of globin synthesis has appeared (Lodish *et al.*, 1971).

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