AN EFFECT OF PACTAMYCIN ON THE INITIATION OF PROTEIN SYNTHESIS IN RETICULOCYTES*

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Summary: Low levels of pactamycin (about 10^{-6} M) stop polypeptide synthesis by intact reticulocytes and their lysates after a lag of about two minutes. This effect which is similar to that of poly A, an inhibitor of polypeptide chain initiation, is not additive to that of poly A. Cells which have been pre-treated with NaF and must form new polypeptide chains are more sensitive to pactamycin than cells which are able to complete pre-existing chains. High levels of pactamycin ($\geq 10^{-5}$ M) inhibit elongation in addition to initiation. ^{3}H-pactamycin binds to 80S ribosomes and the 40S subunits at 0°. Monosomes produced by NaF treatment of reticulocytes bind ^{3}H-pactamycin very effectively, whereas those produced by RNase treatment of polyribosomes do not.

Pactamycin (PM) is a potent antibiotic inhibitor of protein synthesis in whole cells and extracts of procaryotic and eucaryotic cells.[1-5] It has been shown that PM binds to the 30S ribosomal subunit of E. coli and affects the structure of the initiator complex (with N-formylmethionyl-tRNA, N-acetyl-phenylalanyl-tRNA, or polylysyl-tRNA as "initiator"-tRNA), resulting in its disassociation at low Mg^{++} concentrations.[5-7] Furthermore, PM inhibition of polypeptide synthesis initiated by N-acetylphenylalanyl-tRNA is greater when the antibiotic is present before polypeptide synthesis has started; the extent of inhibition is also greater at low Mg^{++} concentrations.[5] PM, however, does not interfer with peptide bond formation itself, as judged by its lack of effect on the puromycin reaction.[2,4,5,7]

These data, coupled with the finding of Colombo, et al.,[1] that PM induces the conversion of rabbit reticulocyte polyribosomes to 80S ribosomes with the release of soluble, newly synthesized protein, suggest that this antibiotic may

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affect the initiation of polypeptide synthesis. This view finds support in experiments (M. Stewart, I. Yanowitz and I.H. Goldberg, in preparation), where low levels of PM (about 10^{-6} M, dependent on the number of ribosomes) rapidly and almost quantitatively produce single ribosomes from polyribosomes in lysates from rabbit reticulocytes with the release of completed hemoglobin chains.

We now show that low concentrations of PM mainly affect polypeptide chain initiation in reticulocytes and their lysates, but that higher concentrations also inhibit elongation. Furthermore, the ribosomal binding site for PM (at 0^{o} and 10^{-7} M PM) is absent in ribosomes bearing a fragment of mRNA, such as are produced by RNase treatment of polyribosomes.

Materials and Methods: The preparation and incubation of rabbit reticulocytes and cell-free lysates were as described by Colombo, et al.,[1] except that in the lysate system the incorporation of (^{14}C) valine into hot trichloroacetic acid-precipitable protein was assayed by the filter paper disc method described by Bollum[8]. The binding of (^{3}H) PM, prepared as previously reported,[6] to reticulocyte ribosomes was measured on 15-30% sucrose density gradients containing 0.01 M Tris, pH 7.4, 0.01 M KCl, and 0.0015 M $MgCl_2$. After centrifugation in the Spinco rotor SW 41 at 41,000 RPM for 90 min. at 4^{o}, fractions were collected from the bottom of the tube through a Gilford absorbance recorder for radioactivity analysis as previously described.[6]

Results and Discussion: Hardesty, et al.,[9,10] have shown that poly A inhibits polypeptide synthesis by reticulocyte lysates by interfering with the initiation of new hemoglobin chains. The extent of inhibition varies with the degree to which the particular lysate system depends on the formation of new chains for the incorporation of labelled amino acids. Figure 1 shows that in the presence of poly A the synthesis of polypeptide proceeds at control rates for almost two minutes, at which time there is an abrupt cessation of further synthesis. This figure also shows that 10^{-6} PM gives essentially the same level and kinetics of inhibition as does poly A. Furthermore, when poly A and 10^{-6} PM are both present,

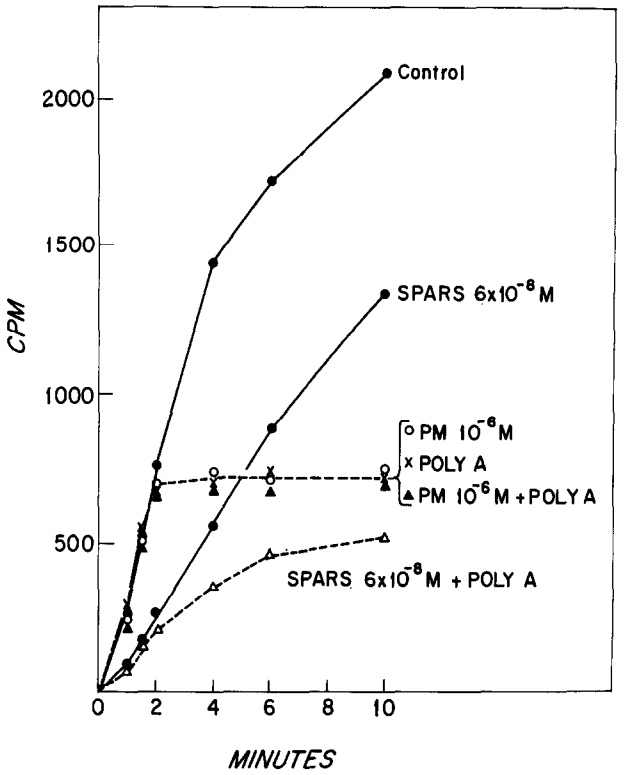

Figure 1. Effect of antibiotics and poly A on cell-free protein synthesis. The incorporation of (^{14}C) valine (35 µc/µmole) into protein by reticulocyte lysates was followed as previously described.[1] The volume of the reaction mixture was 500 µl; 75 µl was removed for assay at each of the indicated times. Poly A (100 µg/ml), PM and sparsomycin were added from the start of the incubation, as indicated

the pattern and extent of inhibition is that of either one alone; there is no additive effect, suggesting that both agents are exerting maximal actions at the same overall step in protein synthesis.

During the two minute lag in onset of the inhibitory action of PM (and poly A) nascent peptide chains are presumably being completed and released from the ribosome. In reticulocytes or lysates where new chains are being formed continuously, the lag in onset of the PM (10^{-6} M) inhibition is always found, although the final extent of inhibition increases as the number of rounds of synthesis of chains increases in the control. Since PM binds to the ribosome, the inhibitory effect depends on both the PM and ribosome concentrations.

Increasing the number of ribosomes but not the amount of soluble protein decreases the PM effect (Cohen and Goldberg, unpublished results).

A very different result is found with sparsomycin, which is an inhibitor of peptide bond formation.[11] At the low level of sparsomycin (6 x 10^{-8} M) used in Figure 1, the rate of protein synthesis is slowed down from the very start and this effect is additive with that due to poly A. It should be noted (Figure 2A) that at higher concentrations of PM (10^{-5} M), the initial rate of protein synthesis is also inhibited, suggesting that at high levels of antibiotic polypeptide chain elongation is also affected. As expected, poly A does not contribute to this inhibition. The inhibition of chain elongation found at high PM concentrations probably results from an effect on translocation since peptide bond formation (the puromycin reaction) remains intact (I. Yanowitz and I.H. Goldberg, unpublished data).

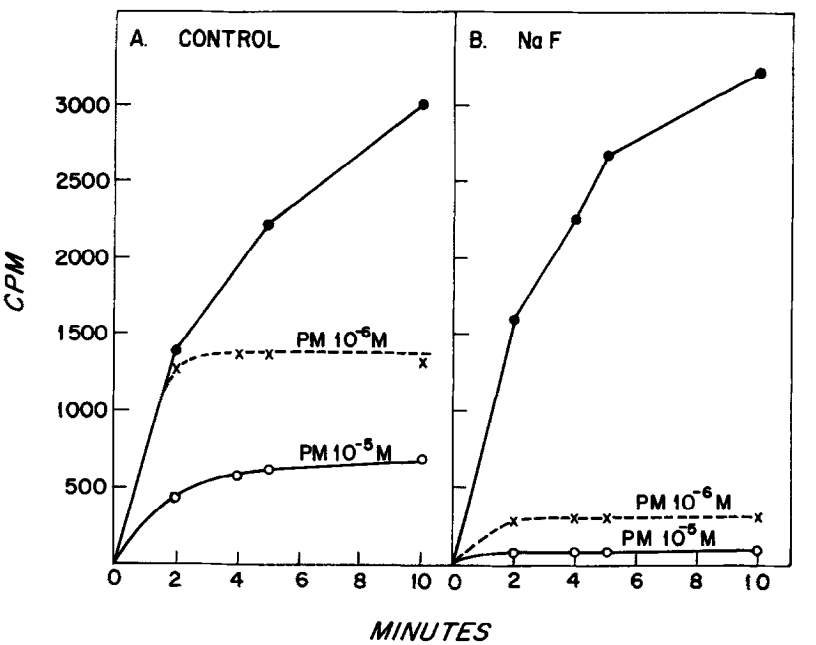

Figure 2. Sensitivity of NaF-treated and untreated reticulocytes to PM. Reticulocytes were incubated as previously described[1] without (A) or with (B) 0.01 M NaF for 30 min. at 37°. After addition of equal volumes of cold saline, the cells were washed four times with the same buffer prior to reincubation under standard conditions (0.8 µc of ^{14}C-amino acid mix (1.5 mc/mg) per reaction)[1] in the presence or absence of PM. At the indicated times 100 µl aliquots were removed for the analysis of ^{14}C incorporation into protein.

Figure 2 also shows that the pretreatment of reticulocytes with NaF, which converts most of the polyribosomes to single ribosomes,[12] increases the sensitivity of polypeptide synthesis to PM. In this system, polypeptide synthesis is almost entirely dependent on the initiation of new hemoglobin chains. Similar results have been found with protein synthesis in lysates prepared from NaF treated cells (M. Stewart, I. Yanowitz and I.H. Goldberg, unpublished results).

In a system (polyphenylalanine synthesis) that is not dependent on the physiological mechanism for the initiation of protein synthesis, PM (10^{-5} M) affects the overall synthesis only to a small degree and, as with sparsomycin, acts mainly by decreasing the initial rate of synthesis (Figure 3).

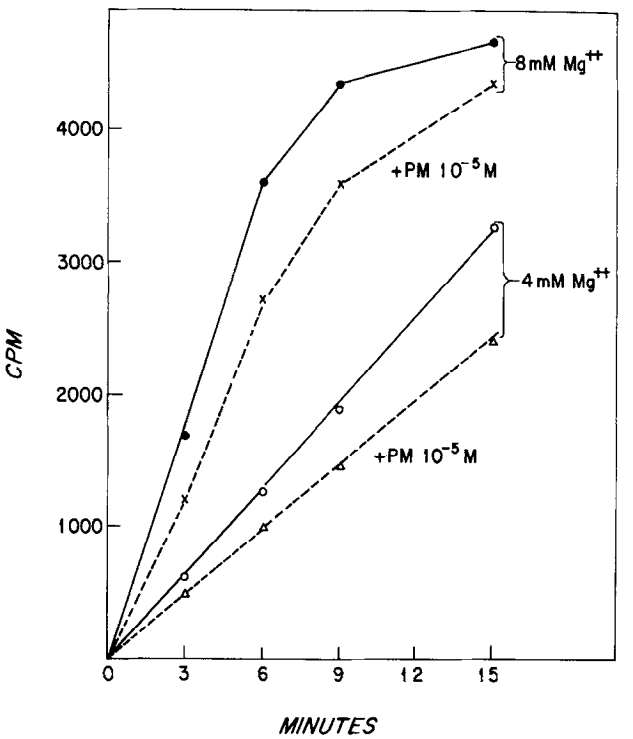

Figure 3. Effect of PM on poly U-dependent polyphenylalanine synthesis. S-100 and ribosomal fractions were obtained by centrifugation of a 1:1 reticulocyte lysate at 105,000xg for 2 hours. NH_4Cl washed ribosomes were prepared as described by Felicetti and Lipmann.[13,4] Polyphenylalanine synthesis was followed by incubating the following in a volume of 200 µl: 40 µg E. coli (^{14}C) phe-tRNA (330 cpm/µg), 10 µmoles Tris, pH 7.4, 16 µmoles KCl, 0.8 or 1.6 µmoles $MgCl_2$, 3.2 µmoles dithiothreitol, 0.8 µmoles GTP, 2 units ($A_{260m\mu}$) ribosomes, 40 µg poly U, and 30 µl S-100. At the indicated times 30 µl aliquots were removed for assay on filter paper discs.[8] PM (10^{-5} M) was added as indicated. In the absence of poly U, 200 cpm were incorporated at 15 min.

PM binds at 0° to the 80S ribosome and the 40S ribosomal subunit of the reticulocyte, but not the polyribosome (Figure 4). The specific activity of PM bound to 40S subunits is much higher than that bound to 80S ribosomes,

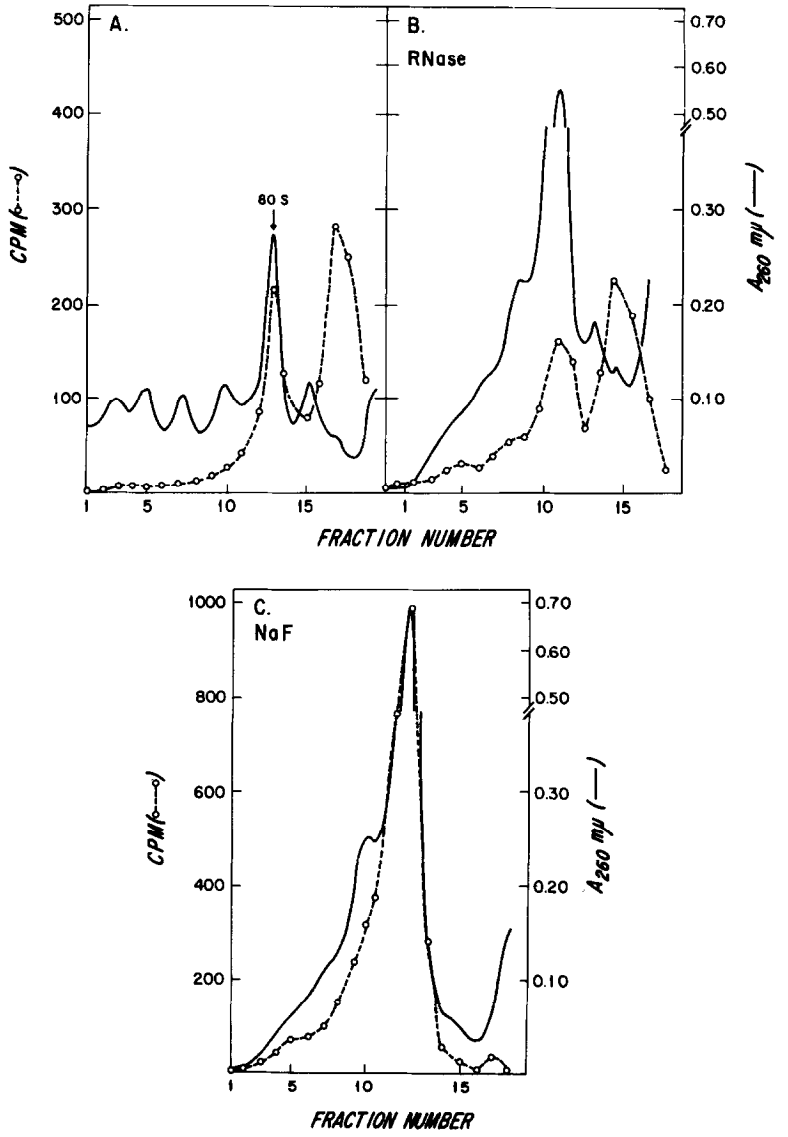

Figure 4. Binding of (^{3}H) PM to reticulocyte ribosomes. (^{3}H) PM (10^{-7} M, 6 x 10^4 cpm/reaction) was added at 0° to 3 units ($A_{260m\mu}$) of ribosomes in 1 ml of the same buffer as in the sucrose gradient. This solution was layered on the sucrose gradient and analyzed as described in Materials and Methods. A) control ribosomes, B) prior to addition of PM, control ribosomes were treated with 0.15 μg crystallin pancreatic RNase for 10 min at 0°, C) ribosomes from NaF-treated cells (prepared as in Figure 2).

suggesting that only a fraction of the 80S ribosomes bind PM. At 37° binding to the polyribosomes can also be found. Increasing the amount of 80S ribosomes by treatment of the polyribosomes with RNase does not increase the amount of PM bound at 0°. On the other hand, NaF treatment of reticulocytes which produces an equivalent amount of 80S ribosome in the lysate by blocking initiation of protein synthesis,[14,15] greatly increases the amount of PM bound at 0°. As found by Colombo, et al.,[16] NaF treatment decreased ribosome subunits; PM binding to the 40S subunit is similarly markedly decreased.

The single ribosomes formed by treatment of reticulocytes with NaF are produced by the run-off of ribosomes from polyribosomes during the course of peptide chain completion and release, and presumably lack mRNA, peptide and possibly protein factors. On the other hand, RNase-produced single ribosomes carry a fragment of mRNA, peptide and possibly protein factors. It is likely that the marked increase in binding of ^{3}H-PM found with the NaF-produced ribosomes is due to the availability of a binding site for PM on such ribosomes (present on the 40S subunit) which is lacking, possibly because of steric hindrance or allosteric change, on the RNase-produced ribosomes. It is possible that once the initiation complex is formed and peptide-bond formation has taken place, the particular PM binding site responsible for the inhibition of initiation is no longer accessible to the antibiotic.

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