Antibiotics and Polyribosomes. II. Some Effects of Lincomycin, Spiramycin, and Streptogramin A *in Vivo**

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ABSTRACT: In previous work, polyribosomes were shown to be completely preserved *in vivo* in the presence of high concentrations of chlortetracycline but were partially degraded when lower drug concentrations were used. Similar observations have since been made using chloramphenicol, sparsomycin, pactamycin, and erythromycin. It is apparent that these drugs, and chlortetracycline, all inhibited protein synthesis in such a way that ribosomes were immobilized on messenger ribonucleic acid. In the present work lincomycin, spiramycin, and streptogramin A caused extensive breakdown of polyribosomes at all drug concentrations, in marked contrast to those mentioned above. This breakdown was much more rapid than that induced by actinomycin D, but in all cases the polyribosomes were degraded mainly to 50S and 30S ribosomal subunits rather than 70S ribosomes. Possible explanations of these results include the following: the drugs may cause premature detachment of ribosomes from messenger ribonucleic acid or alternatively they may selectively inhibit the initiation of polypeptide synthesis.

In a previous communication (Cundliffe, 1967) the effects of chlortetracycline *in vivo* on the size and content of polyribosomes were discussed. At low drug concentrations polyribosomes were partially degraded whereas with higher concentrations of chlortetracycline (around 100 μg/ml) they were preserved. Qualitatively similar results have since been obtained using chloramphenicol, sparsomycin, erythromycin, and pactamycin (E. Cundliffe, unpublished data). Each of these drugs preserved polyribosomes essentially completely when used at a high enough concentration (usually 100–200 μg/ml). It is clear that these antibiotics all stop protein synthesis without causing the detachment of ribosomes from mRNA. The object of this report is to discuss the mode(s) of action of three drugs which do not act in this manner.

Lincomycin, streptogramin A, and spiramycin inhibit protein synthesis *in vivo* and *in vitro* in 70S ribosomal systems (see reviews by Chang and Weisblum, 1967a; Vazquez, 1967a,b). Each drug inhibits the uptake of [^{14}C]chloramphenicol by bacteria (Vazquez, 1966) and also competes with chloramphenicol for binding sites on 50S ribosomes (Vazquez, 1966; see also Chang and Weisblum, 1967b). The binding of lincomycin to the 50S ribosome is reversible but streptogramin A and spiramycin bind irreversibly (Vazquez, 1967c). It is known that the three drugs do not prevent the formation of aminoacyl-tRNA and it has been suggested that none of them acts primarily by preventing the binding of aminoacyl-tRNA to the ribosome–mRNA complex (Vazquez and Monro, 1967; see, however, Chang *et al.*, 1966).

Protoplasts of *Bacillus megaterium* were used throughout this work since they could be lysed promptly and gently to yield polyribosomes which, hopefully, were not degraded during the preparation of lysates. These protoplasts were very active metabolically and techniques for obtaining them in a reproducible state have been described (Cundliffe, 1967, 1968a).

Methods

All the methods used in this work have been described fully elsewhere (cited above) and only a brief outline is given here.

Growth of Bacteria and Preparation of Protoplasts. *Bacillus megaterium* KM was steady-state labeled with [^{32}P]orthophosphate by growth in the protoplast medium supplemented with peptone and was converted into protoplasts using lysozyme. Suspensions of protoplasts were routinely incubated for about 20–25 min before experiments were commenced, by which time the optical density (600 mμ) was increasing rapidly. In all the experiments described here protoplasts in this reproducible, active state were used. "Control" samples were taken and incubation of the remaining protoplasts was continued in the presence of drugs as indicated in the text. Samples were taken as below.

Sampling Procedure. Samples of suspensions of protoplasts (usually 0.3 ml) were pipetted into thin-glass vials standing in a metal block in ice. The vials contained deoxyribonuclease, Triton X100, and, sometimes, pancreatic ribonuclease as previously described. In this way chilled lysates were prepared instantly.

Sucrose Density Gradient Analysis. Lysates (0.2 ml) were layered on linear sucrose density gradients (15–40% w/v, volume 4.8 ml) which were centrifuged at 0° in a Spinco L2 ultracentrifuge as indicated in the

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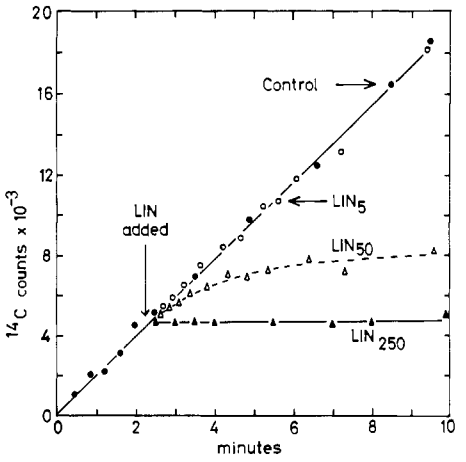

FIGURE 1: Inhibition of protein synthesis by lincomycin. Protoplasts were preincubated (see Methods) then the uptake of [^{14}C]leucine into hot trichloroacetic acid insoluble material was followed in the presence and absence of drug. In this and subsequent figures, subscripts to abbreviations (*e.g.*, lincomycin$_{250}$) indicate drug concentrations expressed as micrograms per milliliter.

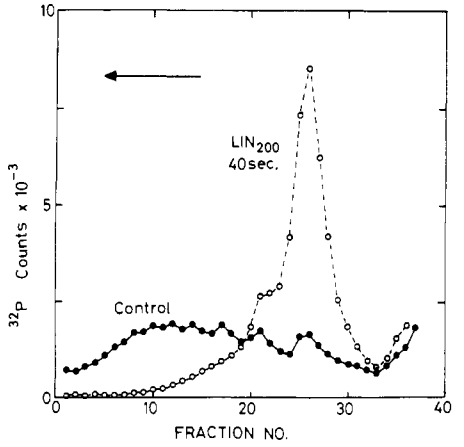

FIGURE 2: Breakdown of polyribosomes in the presence of lincomycin. ^{32}P-Labeled protoplasts were preincubated as usual then incubation was continued in the presence of lincomycin. Samples were taken at the times indicated and lysates were analyzed on sucrose density gradients. Gradients were centrifuged at 38,000 rpm for 75 min using the Spinco SW39 rotor. In Figures 2–4 sedimentation is right to left. (●) Protoplasts sampled immediately before addition of drug. (○) Protoplasts sampled 40 sec after addition of lincomycin (200 μg/ml).

legends to the figures. In this work the gradients contained 5 mM Mg^{2+} instead of 10 mM Mg^{2+} as used in earlier studies (Cundliffe, 1967). Changing the magnesium ion concentration in this way does not affect polyribosomes but, under the conditions employed here, allows ribosomal subunits to be clearly separated (Cundliffe, 1968b). After spinning, 40 fractions were collected from each gradient, precipitated with cold trichloroacetic acid, filtered on glass fiber disks, rinsed, dried, and counted in a liquid-scintillation counter as previously described.

Inhibition of Protein Synthesis. Protoplasts (not labeled with ^{32}P) were prepared and incubated as usual. Then the uptake of [^{14}C]leucine into material precipitable by hot trichloroacetic acid was followed before and after the addition of drugs. L-[1-^{14}C]Leucine (8.5 mCi/mmole) was used at a final activity of 0.25 μCi/ml. Samples (0.5 ml) were taken into trichloroacetic acid, held at 90° for 10 min, then filtered, etc., as above.

Materials

The antibiotics used in this work were most generously provided by the following organizations: Merck and Co., Inc., Rahway, N. J. (actinomycin D and streptogramin A); The Upjohn Co., Kalamazoo, Mich. (lincomycin); Rhône-Poulenc, France, *via* May and Baker, Ltd. Dagenham, England (spiramycin III).

Results

Inhibition of Protein Synthesis. Lincomycin, streptogramin A, and spiramycin III, all inhibited the incorporation of [^{14}C]leucine into protein of protoplasts. Qualitatively similar results were obtained with all three drugs; those for lincomycin are given in Figure 1. In this and subsequent figures, subscripts to abbreviations (*e.g.*, lincomycin$_{200}$) indicate drug concentrations expressed as micrograms per milliliter. It was concluded that each drug inhibited protein synthesis rapidly and essentially completely at concentrations of 5×10^{-4} M (*i.e.*, lincomycin$_{200}$, streptogramin$_{250}$, and spiramycin III$_{500}$).

Breakdown of Polyribosomes. When protoplasts were incubated in the presence of lincomycin, streptogramin A, or spiramycin, polyribosomes were degraded (Figure 2). With each drug the amount and rate of breakdown increased until a maximum was reached around $3–5 \times 10^{-4}$ M drug. Increasing the drug concentration beyond this value did not protect polyribosomes, which were degraded to the maximal extent in the presence of lincomycin at 2 mg/ml (5×10^{-3} M), streptogramin A at 750 μg/ml (1.5×10^{-3} M), and spiramycin at 1 mg/ml (10^{-3} M). It is therefore clear that breakdown under these conditions was not due to incomplete inhibition of protein synthesis. Figure 2 shows the maximal extent of breakdown in the presence of lincomycin; closely similar results were obtained with streptogramin A and spiramycin.

Products of Breakdown of Polyribosomes. When lysates similar to that used for Figure 2 were subjected to prolonged centrifugation it became clear that the products of breakdown under these conditions accumulated as ribosomal subunits (assumed to be 50 and 30 S) and not as 70S ribosomes. Typical results obtained with streptogramin A are shown in Figure 3 and with spiramycin in Figure 4. The results obtained using lincomycin were closely similar to those with spiramycin and are not shown here.

These figures also show "control" lysates which were prepared immediately before the addition of a given drug to the remaining protoplasts. The control lysates, which originally contained undegraded polyribosomes,

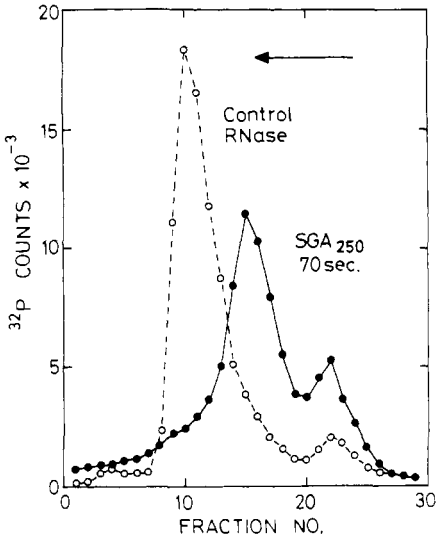

FIGURE 3: Products of breakdown of polyribosomes in the presence of streptogramin A. ^{32}P-Labeled protoplasts were preincubated then a control sample was taken, and incubation of the remaining protoplasts was continued in the presence of streptogramin A (250 μg/ml). Other samples were taken at various times after the addition of streptogramin A. The control lysate was treated with pancreatic ribonuclease (5 μg/ml) at 0° for 5 min before gradient analysis. The samples containing streptogramin A were not treated with ribonuclease. Gradients were centrifuged at 45,000 rpm for 150 min using the Spinco SW50 rotor. (○) Control lysate treated with ribonuclease. (●) Protoplasts sampled after 70-sec incubation with streptogramin A.

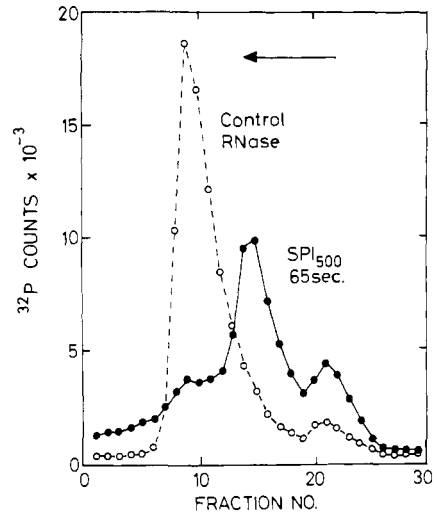

FIGURE 4: Products of breakdown of polyribosomes in the presence of spiramycin. Experiment carried out as in Figure 3 except that spiramycin (500 μg/ml) was used instead of streptogramin A. (○) Control lysate treated with ribonuclease. (●) Protoplasts sampled after 65-sec incubation with spiramycin.

were treated *in vitro* with pancreatic ribonuclease under mild conditions (5 μg of RNase/ml at 0° for 5 min) before sucrose gradient analysis. It was previously shown that such ribonuclease treatment converted polyribosomes almost quantitatively into 70S material and only slightly increased the proportion of ribosomal subunits (Cundliffe, 1968b; see also Mangiarotti and Schlessinger, 1966). Such lysates can be used to calibrate sucrose density gradients by defining the "70S" region.

Rates of Breakdown of Polyribosomes. The results of several experiments are collected in Figure 5. Essentially two rates of breakdown of polyribosomes were observed: rapid, incomplete breakdown with lincomycin, spiramycin, and streptogramin A and slower breakdown with actinomycin D. Although it is not shown in Figure 5, more than 90% of the polyribosomes decayed, eventually, in the presence of actinomycin. In each case the products of decay were predominantly ribosomal subunits (see also Cundliffe, 1968b). It should be emphasized here that in performing the calculations relating to Figure 5 material sedimenting at 70 S was included with the polyribosomes and not with the breakdown products.

Other Data. Lincomycin, spiramycin, and streptogramin A had no effect upon polyribosomes when added to "control" lysates *in vitro*. This shows that the drugs caused polyribosome breakdown *in vivo* and not subsequent to the formation of lysates. It was also established that, at the concentrations used here, none of these three drugs inhibited RNA synthesis during a 10-min time course. Therefore breakdown of polyribosomes in the presence of these antibiotics was not due to the depletion of messenger RNA, as with, *e.g.*, actinomycin D.

Discussion

When lincomycin, spiramycin, or streptogramin A was added to protoplasts *in vivo*, 60–70% of the polyribosomes was rapidly degraded to ribosomal subunits, the remainder being present as small polyribosomes (see Figure 2) after decay was completed. However in the presence of actinomycin D polyribosomes were completely degraded. Incomplete breakdown of polyribosomes has been observed previously in this laboratory (Cundliffe, 1967) when protoplasts were incubated with low concentrations of drugs active against protein synthesis, but never when protein synthesis was fully inhibited.

An obvious *ad hoc* explanation of these findings is that a special class of small polyribosomes exists, comprising 30–40% of the total, but there is no independent evidence to suggest this. Therefore alternative hypotheses are preferred, according to which all polyribosomes are assumed to behave similarly in these experiments. Two such alternatives are suggested.

Hypothesis 1: lincomycin, spiramycin, and streptogramin A caused displacement of 70% of the ribosomes from polyribosomes and left the remainder unaffected. This might possibly have involved premature detachment of nascent peptides thereby causing ribosome–mRNA complexes to become unstable. Or possibly the drugs interfered with the coupling of 50S and 30S subunits in some other fashion. However, if so, it is not clear why all the ribosomes were not affected and for this reason the following hypothesis is preferred.

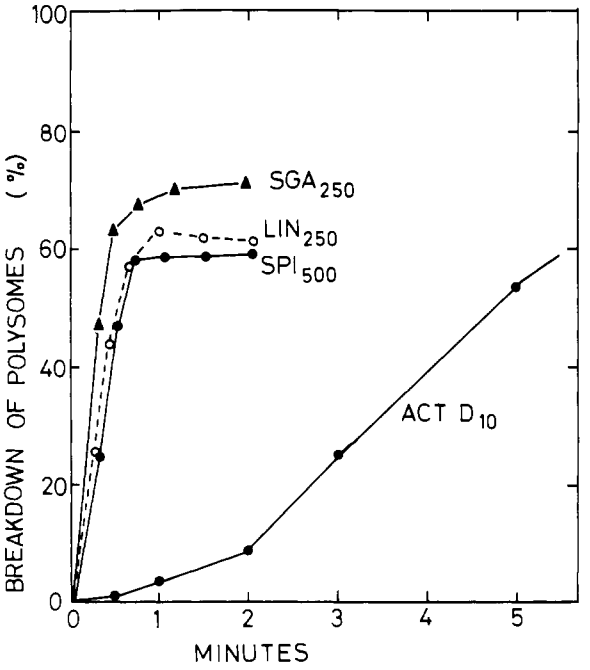

FIGURE 5. Rates of breakdown of polyribosomes to ribosomal subunits in the presence of various drugs. A series of experiments similar to those described in Figures 3 and 4 were carried out together with identical ones using actinomycin D (10 μg/ml). In each case a control lysate was treated with ribonuclease to define the 70S region of the gradients and to reveal the total amount of polyribosomal material (present at 70 S after RNase). Breakdown of polyribosomes was computed as [(50 + 30 S after breakdown) − (50 + 30 S in controls)]/70 S in control after RNase.

Hypothesis 2: the drugs affected some of the ribosomes in polyribosomes and immobilized them on mRNA in such a way that about 70% of the ribosomes was still able to proceed to the normal detachment position(s). This raises the possibility that these drugs specifically act at the 5′ end of mRNA (or cistrons thereof) by inhibiting some aspect of polypeptide chain initiation. According to this scheme the residual "polyribosomal" material present after breakdown would consist of inhibited initiation complexes (Nomura and Lowry, 1967; see also Luzzatto *et al.*, 1968) plus polyinitiation complexes in those cases where the drugs were acting at the 5′ ends of multiple cistrons of polycistronic messengers.

The other curious aspect of the decay of polyribosomes induced by these three drugs was the rapidity of the process compared with that induced by actinomycin D. A proper evaluation of this phenomenon requires a fuller knowledge of the metabolism of polyribosomes and of their fate *in vivo* after addition of actinomycin D.

During this discussion it has been assumed, for convenience of presentation, that lincomycin, spiramycin, and streptogramin A acted similarly. However the three drugs are chemically very different and it remains to be seen whether their modes of action are identical. The data presented in Figures 3–5 might indicate certain differences since streptogramin A caused more breakdown of polyribosomes than did the other two drugs and there was less 70S material present after treatment with streptogramin A. These differences, although reproducible, were not very great however and it is not possible at this time to evaluate their significance.

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