CHLORAMPHENICOL PROTECTS POLYRIBOSOMES

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Chloramphenicol (CAM) inhibits protein synthesis in bacteria by preventing the growth of nascent protein chains on polyribosomes (Das et al. 1966, Weber and DeMoss 1966, Monro and Vasquez 1967, Cundliffe and McQuillen 1967). It apparently does this by blocking the transfer of amino acids from transfer RNA (tRNA) to the growing polypeptide chains. The drug has no effect upon the interaction of messenger RNA (mRNA) and ribosomes, nor is there preferential inhibition of chain initiation or termination. These properties have led a number of workers (Mangiarotti and Schlessinger 1966, Hotham-Iglewski and Franklin 1966, Flessel et al. 1966) to employ CAM to "freeze" protein synthesis during extraction of bacterialpolyribosomes. Evidence is presented here that CAM protects polysomes from breakdown. The in vivo breakdown of polysomes observed in the presence of puromycin or actinomycin can be largely inhibited by the concomitant addition of CAM. This effect at the level of the polysome is consistent with earlier studies of these inhibitors on total protein and RNA synthesis.

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MATERIALS AND METHODS

Biochemicals: Tryptone and yeast extract were obtained from Difco Laboratories. Sucrose was a product of Merck Company. Chloramphenicol was donated by Parke, Davis and Company and actinomycin D by Merck, Sharp and Dohme. Puromycin was purchased from Nutritional Biochemicals. Uracil-2-C^{14} and C^{14}-reconstituted protein hydrolysate were obtained from the New England Nuclear Corporation. Deoxyribonuclease (DNase) electrophoretically pure was purchased from Worthington. All other chemicals were of reagent grade.

Bacterial culture conditions: Micrococcus lysodeikticus, obtained from Dr. Gene M. Brown, Department of Biology, Massachusetts Institute of Technology, was grown at 32° C in yeast extract (0.5%)-tryptone (1%) broth containing 1% glucose and 10^{-2} M $MgSO_4$ in a gyrotary shaker bath. Experiments were carried out on exponential cultures at cell densities of 4-8 x 10^8 colony forming units per ml. Under these conditions the optical density doubling time was about 100 minutes.

Preparation and Analysis of extracts: Following drug treatment and/or radioactive labeling, the culture was poured over an equal volume of ice and harvested by centrifugation in the cold. Protoplasts were formed using lysozyme at 0°C and the polysomes extracted by detergent lysis in the presence of DNase, as previously described (Flessel et al. 1967), except that no CAM was added during the extraction. Polysome extracts were analyzed by sucrose gradient centrifugation in NMT buffer (0.05 M NH_4Cl, 0.01 M $MgSO_4$, 0.01 M Tris-HCl pH 7.4). Centrifugation times and gradients used are indicated in figure legends. After centrifugation, the contents of each tube were pumped through a Gilford Model 2000 recording spectrophotometer and the absorbance at 260 mμ monitored continuously.

Radioactivity measurements: In RNA labeling experiments samples were precipitated in cold trichloroacetic acid (TCA) at a final concentration of 10%. The precipitate was collected on Millipore filters, washed and counted. In protein labeling experiments, samples precipitated in 5% TCA were heated to 90° C for 15 minutes prior to filtration. Counting was done in a Nuclear Chicago gas flow counter with an efficiency of approximately 20%.

RESULTS

Effect of chloramphenicol: Chloramphenicol and puromycin each inhibit incorporation of radioactive amino acids into hot TCA insoluble material by 80-85% at a concentration of 500 μg/ml. As shown in figure 1, addition of chloramphenicol to cultures for short times prior to harvest has little effect on the polysome profiles obtained. However, if cells are treated with CAM for prolonged periods (greater than about 10 minutes at 32° C), polysome breakdown does occur.

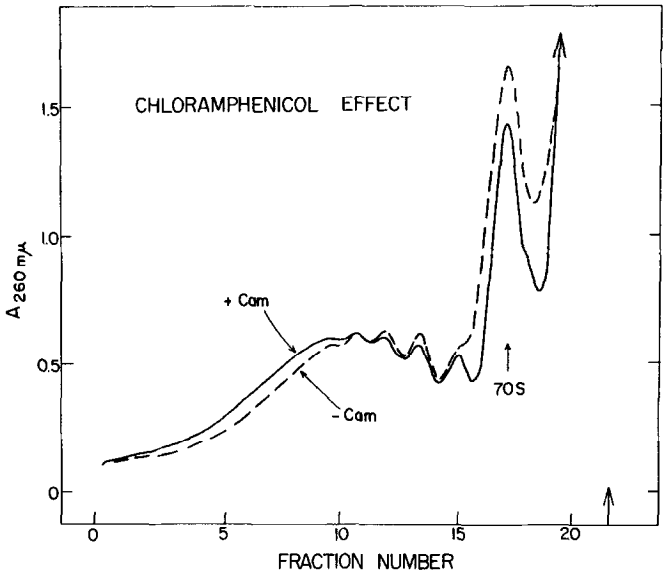

Figure 1. Efffect of Chloramphenicol on Polysomes. Chloramphenicol (final concentration 500 μg/ml) was added to a 20 ml portion of the culture and thirty seconds later the culture poured over crushed ice. The control cells received no chloramphenicol. Cells were harvested and the polysomes extracted as described. One ml of extract was layered on a 28 ml 5-20% sucrose density gradient in the standard buffer and centrifuged for 120 minutes at 25,000 r.p.m. in an SW25.1 Spinco rotor with the temperature maintained near 0° C. The arrow at the lower right indicates the top of the gradient.

Puromycin induced polysome decay and protection by Chloramphenicol:

Puromycin functions as an analogue of amino acyl-tRNA and releases the growing peptide chain from the messenger-ribosome complex with the formation of peptidyl puromycin (Allen and Schweet 1962). The addition of puromycin to cells prelabeled with radioactive amino acids results in

the rapid release of nascent chains from ribosomes (figure 2). However, the simultaneous addition of CAM and puromycin results in a much slower rate of release of nascent chains than with puromycin alone (figure 2). This confirms the earlier findings of Goldberg and Mitsugi (1966) and Cundliffe and McQuillen (1967) who also observed that CAM prevents puromycin-induced release of nascent polypeptides in E. coli.

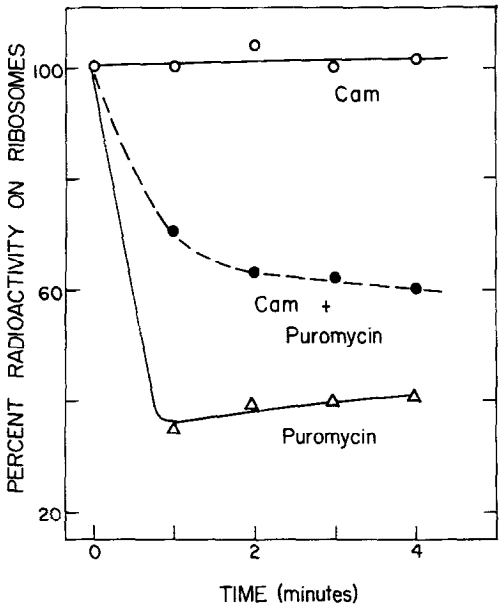

Figure 2. Puromycin Release and Chloramphenicol Protection of Nascent Chains. Three portions of culture were simultaneously prelabeled for three minutes with C^{14} amino acids (final concentration was 0.3 μC/ml). Then 10 ml portions of each culture were withdrawn and pipetted over ice (t=0). Immediately thereafter chloramphenicol (500 μg/ml), puromycin (500 μg/ml) or chloramphenicol plus puromycin was added to the appropriate culture. At one minute intervals, 10 ml portions were withdrawn and chilled. Lysates were prepared and 1 ml layered over 8 mls of 15% sucrose in NMT. Ribosomes were pelleted by centrifugation for 8 hours at 50,000 r.p.m. in a No. 50 Spinco rotor. Following centrifugation the radioactivity in the hot TCA insoluble material of the pellet was determined.

The effect of puromycin on polysomes is shown in figure 3a. Addition of puromycin is followed by the loss of polysomes and the formation of 70S monomers. Since the release of nascent chains preceeds or accompanies polysome breakdown and CAM can prevent this release, then CAM should also block polysome decay by puromycin. This result is shown in figure 3b.

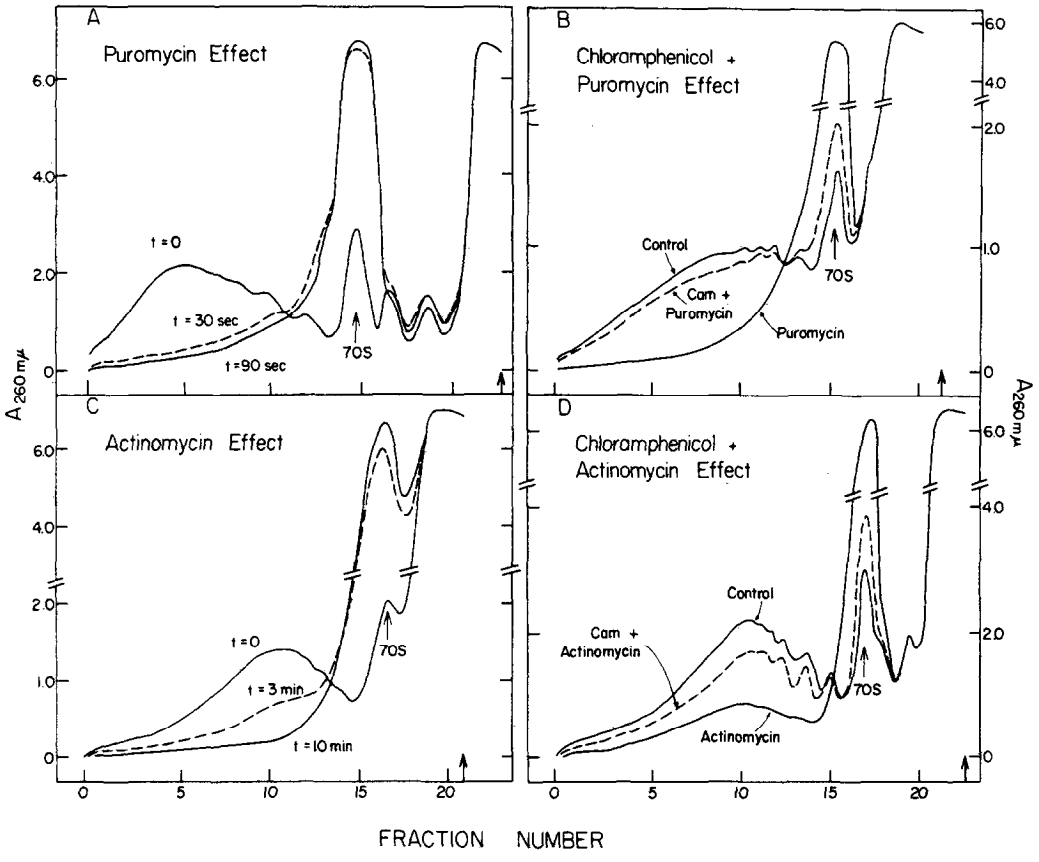

Figure 3. Puromycin and Actinomycin Decay of Polysomes and Protection by Chloramphenicol.

A. Puromycin effect. Twenty milliliter portions of culture were treated with puromycin at a final concentration of 500 μg/ml for 30 seconds or 90 seconds. No puromycin was added to the control (t=0). Extracts were prepared and layered on 28 ml 15-45% sucrose gradients and centrifuged for 5 hours at 25,000 r.p.m. in an SW25.1 rotor.

B. Puromycin plus chloramphenicol effect. Chloramphenicol (500 μg/ml) was added to 20 mls of culture for 30 seconds, then puromycin (500 μg/ml) was added for an additional 30 seconds prior to pouring the cells onto ice. A second portion of culture received puromycin alone for 30 seconds. No additions were made to the control. Polysome extracts were layered on 26 ml 15-30% sucrose gradients and centrifuged for 150 minutes at 25,000 r.p.m. in an SW 25.1 rotor.

C. Actinomycin effect. Two forty milliliter portions were treated for 3 and 10 minutes, respectively, with 20 μg/ml actinomycin. No actinomycin was added to the control (t=0). Extracts were analyzed as in 3B.

D. Actinomycin plus chloramphenicol effect. To one forty milliliter portion of culture, actinomycin (20 μg/ml) was added. To a second portion, both actinomycin (20 μg/ml) and chloramphenicol (100 μg/ml) were added. After 90 seconds, the two cultures, along with an untreated control were stopped. Extracts were layered on 16 ml 15-45% sucrose gradients and centrifuged for 13 hours at 17,000 r.p.m. in a SW 25.3 rotor.

Polysomes isolated from a culture treated briefly with puromycin in the presence of CAM are only slightly degraded relative to the untreated control. Few polysomes remain in the puromycin treated culture. Therefore CAM effectively blocks the puromycin-induced breakdown of polysomes.

Polysome decay in actinomycin and its inhibition by chloramphenicol: Actinomycin inhibits DNA-dependent RNA synthesis. This property has been fully exploited by numerous workers (see for examples Levinthal et al. 1961, Hartwell and Magasanik 1963, Kepes 1963, Leive 1965) to measure the in vivo decay of messenger RNA in bacteria. From such studies the half life of messenger RNA in bacteria has been estimated to be of the order of several minutes.

M. lysodeikticus, a Gram-positive organism, is very sensitive to actinomycin. Concentrations of 5 μg/ml completely inhibit RNA synthesis for at least 30 minutes. The expected rapid decay of pulse-labeled RNA and of the rate of protein synthesis is also observed. The half-life of RNA decay is about 2 minutes under these conditions. As a consequence of normal messenger RNA decay, polysomes disappear in the presence of actinomycin (figure 3c). After 3 minutes about two-thirds of the polysomes have decayed and after 10 minutes their conversion to 70S ribosomes is complete. Polysome decay therefore parallels the decay of messenger RNA and protein synthetic rate.

Levinthal et al. (1963) have observed that the decay of messenger RNA in B. subtilis could be arrested if peptide bond formation was stopped either by using chloramphenicol or by subjecting the cells, growing on succinate, to anaerobic conditions. I have confirmed this effect of chloramphenicol on RNA stability in M. lysodeikticus. There is less than 10% decay of three minute pulse-labeled RNA after simultaneous addition of actinomycin and CAM, while almost half of the RNA decays following addition of actinomycin alone. CAM alone does not measurably affect RNA synthesis over the duration of the experiment.

Most of the cell's messenger RNA is found on polysomes in E. coli. (Mangierotti and Schlessinger 1967, Friesen 1968). I have found this to be true in M. lysodeikticus too. About 75% of the newly made RNA is found on polysomes after a 30 second uracil pulse label. CAM protects messenger RNA from decay in actinomycin. Thus we would predict that it should

also protect polysomes from decay. As shown in figure 3D, this is indeed the case. In cells treated with actinomycin for 90 seconds, the amount of polysome material has already been reduced by more than one half, while in the culture treated with actinomycin and CAM for the same period, the decay of polysomes is markedly reduced. Therefore, chloramphenicol can prevent not only the puromycin-induced decay of polysomes, but also the breakdown of polysomes which occurs in the presence of actinomycin.

Discussion and Summary: The use of inhibitors to isolate specific steps involved in protein synthesis has proved extremely valuable in understanding the details of peptide bond formation. Conversely, these studies have also shed further light on the mechanisms of action of the inhibitors themselves. Of interest is the present observation that when polysomes break down in the presence of puromycin, 70S ribosomes, and not 50S and 30S ribosomal subunits, are observed. This is in contrast to the in vitro experiments of Schlessinger et al. (1967) which showed that when nascent chains are released from 70S ribosomes by puromycin, ribosomal subunits are formed. Their results imply that the peptidyl-tRNA in some way stabilizes the 50S and 30S couple. In the present study, polysome breakdown by puromycin in vivo may have resulted in the formation of subunits. However, if any subunits are formed they appear to have reassociated to 70S ribosomes. This may reflect the fact that protein synthesis was inhibited by only 80-85% by puromycin which could allow for subunit reassociation.

I also observe that in the presence of actinomycin polysomes are degraded to 70S ribosomes and not to subunits. In similar experiments Zimmerman and Levinthal (1967) have shown that either result can be obtained depending on the growth medium used. On Tris-glucose medium subunits are formed, while on phosphate medium, polysomes were degraded to 70S ribosomes. They suggest that high concentrations of phosphate prevent "complete digestion of mRNA, leaving oligonucleotides attached to the ribosomes which thus stabilize them". Similar stabilization may also have occurred in the present study in which a complex broth medium was used.

It is now believed that chloramphenicol acts by inhibiting the peptidyl transferase reaction in which an activated amino acid is joined to the grow-

ing nascent chain (Munro and Vasquez 1967). By virtue of its ability to inhibit peptide bond formation, CAM can also prevent the puromycin-induced release of nascent protein chains (Goldberg 1966, Cundliffe and McQuillen 1967) and can also block the decay of messenger RNA in the presence of actinomycin (Levinthal et al. 1963, Gros et al. 1963). This suggests a mode of translational control discussed by Gros et al. (1963) in which the lifetime of a messenger RNA molecule can be influence by the rate at which it is translated. The ability of the cell to mimic CAM inhibition of peptidyl transferase activity could conceivably prove useful in conserving messenger RNA under certain growth conditions, thus improving the overall economy of the organism. The present data confirm these earlier results on total RNA and protein synthesis while extending them to the level of the polysome. The experiments described here show that chloramphenicol protects polyribosomes from degradation in the presence of puromycin and actinomycin.

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