Mechanism of action of chloramphenicol : effect on T4D head coat protein synthesis
by Charles Edward Green

A thesis submitted to the Graduate Faculty in partial fulfillment of the requirements for the degree of DOCTOR OF PHILOSOPHY in Genetics
Montana State University

Abstract:
Inhibition of protein synthesis by chloramphenicol (CAP) was completely reversible on bacteriophage T4 mRNA, nascent peptides, and host E. coli ribosomes after removal of CAP. This was established by using T4D w.t. and T4 amber mutants amB17 and amN54 with density gradient polysome analysis and polyacrylamide disc gel electrophoresis.

The mRNA continues to code for protein synthesis after transient treatment with CAP. This finding was demonstrated by first treating T4 infected cells with rifampicin to prevent further mRNA synthesis, which was followed by treatment with CAP to inhibit protein synthesis. The CAP was subsequently removed and the utilization of mRNA which had been formed prior to rifampicin treatment was then tested by observing the rate of C^14-arginine incorporation. This incorporation increased steadily during the first 10 minutes after CAP removal, but 20 minutes later its rate had declined only slightly. This result indicated that CAP treated mRNA remained functional and continued to code for protein synthesis after removal of the antibiotic.

Messenger RNA-ribosome complexes which are subjected to CAP treatment do resume protein synthesis after removal of the antibiotic. Nascent peptides labeled with C^14-leucine were trapped in the polysome region of a sucrose density gradient if CAP was added to T4 infected cultures. With these cells C^14-leucine could be chased out of the polysome region when C^l2-leucine was added after CAP was removed. Eighty-five percent of this label was removed from this region in a 10 minute chase indicating that ribosomal movement and function have been restored.

Nascent peptides are completed and form normal head coat protein after transient treatment with CAP. This was demonstrated on polyacrylamide gels by the appearance of the head coat protein band when labeled nascent peptides in CAP treated cells were completed after the removal of CAP to form normal head coat protein.

This was demonstrated by a second method: incomplete head coat peptides of cells transiently treated with CAP were chased into the protein aggregate region of polyacrylamide gels . Therefore, nascent peptides in CAP treated cultures are completed and released normally after removal of the antibiotic.

MECHANISM OF ACTION OF CHLORAMPHENICOL: EFFECT ON T4D HEAD COAT PROTEIN SYNTHESIS

by

CHARLES EDWARD GREEN, JR.

A thesis submitted to the Graduate Faculty in partial fulfillment of the requirements for the degree

of

DOCTOR OF PHILOSOPHY

in

Genetics

Approved:

P D Skaar
Head, Major Department

Chairman, Examining Committee

Graduate Dean

MONTANA STATE UNIVERSITY
Bozeman, Montana

June, 1970

ACKNOWLEDGEMENTS

The author thanks:

DR. GORDON R. JULIAN for his patient guidance, understanding, and constructive criticism throughout this study.

DR. SAMUEL J. ROGERS for his intellectual encouragement and his assistance in manuscript preparation.

DR. PALMER D. SKAAR for his advice and assistance in manuscript preparation.

DR. HELEN J. CAMERON for her continued interest and helpful discussion.

DOROTHY SPAIN GREEN for her unwavering support and encouragement during this study.

The National Institutes of Health for Predoctoral Fellowship 5 FOI GM38314.

TABLE OF CONTENTS

TABLE OF FIGURES

LIST OF TABLES

ABSTRACT

Inhibition of protein synthesis by chloramphenicol (CAP) was completely reversible on bacteriophage T4 mRNA, nascent peptides, and host E. coli ribosomes after removal of CAP. This was established by using T4D w.t. and T4 amber mutants amB17 and amN54 with density gradient polysome analysis and polyacrylamide disc gel electrophoresis.

The mRNA continues to code for protein synthesis after transient treatment with CAP. This finding was demonstrated by first treating T4 infected cells with rifampicin to prevent further mRNA synthesis, which was followed by treatment with CAP to inhibit protein synthesis. The CAP was subsequently removed and the utilization of mRNA which had been formed prior to rifampicin treatment was then tested by observing the rate of C^{14}-arginine incorporation. This incorporation increased steadily during the first 10 minutes after CAP removal, but 20 minutes later its rate had declined only slightly. This result indicated that CAP treated mRNA remained functional and continued to code for protein synthesis after removal of the antibiotic.

Messenger RNA-ribosome complexes which are subjected to CAP treatment do resume protein synthesis after removal of the antibiotic. Nascent peptides labeled with C^{14}-leucine were trapped in the polysome region of a sucrose density gradient if CAP was added to T4 infected cultures. With these cells C^{14}-leucine could be chased out of the polysome region when C^{12}-leucine was added after CAP was removed. Eighty-five percent of this label was removed from this region in a 10 minute chase indicating that ribosomal movement and function have been restored.

Nascent peptides are completed and form normal head coat protein after transient treatment with CAP. This was demonstrated on polyacrylamide gels by the appearance of the head coat protein band when labeled nascent peptides in CAP treated cells were completed after the removal of CAP to form normal head coat protein.

This was demonstrated by a second method: incomplete head coat peptides of cells transiently treated with CAP were chased into the protein aggregate region of polyacrylamide gels. Therefore, nascent peptides in CAP treated cultures are completed and released normally after removal of the antibiotic.

INTRODUCTION

Chloramphenicol

Ever since its discovery in 1947, chloramphenicol (CAP) has eluded the biochemist in his attempt to understand its mechanism of action. Since chloramphenicol was found to inhibit effectively protein synthesis in bacteria (1,2,3), its common use in molecular biology as a biochemical tool made it desirable that its action be understood in precise molecular terms. The problem has been studied imaginatively by many workers using various bacteria, principally Escherichia coli. Despite these efforts its mechanism is rather poorly understood, perhaps because it acts on the complex 50S ribosomal subunit of bacteria (4,5,6,7,8).

Chloramphenicol does not interfere with amino acid activation (9) nor with the esterification of transfer ribonucleic acid with these activated amino acids (10). It does not inhibit binding of amino acyl-tRNA to ribosomes (11,12,13,14) nor the binding of messenger ribonucleic acid (mRNA) to ribosomes (7,15,16,17,18).

In vitro studies with C^{14}-CAP have demonstrated a weak but stereospecific binding to 70S ribosomes (19,13). This binding was on the 50S ribosomal particle and was readily reversible (4,20,13). A 1:1 equivalence between the number of ribosomes and the number of bound CAP molecules suggested the presence of only one binding site for

CAP (13). Other workers find the 1:1 equivalence at concentrations below 50 μg/ml CAP but show binding of approximately two molecules of CAP/ribosome at 200 μg/ml (5).

Analysis of lysine peptides from a CAP inhibited polyadenylic acid *in vitro* protein synthesis system indicated the formation of larger peptides (4-11 lysine residues) was inhibited (21). This was extended when it was shown that nascent peptides did not form on *E. coli* polysomes in the presence of CAP (5,17). These results have been interpreted to mean that CAP directly blocks some step in peptide bond formation.

The antibiotic puromycin, because of its structural resemblance to aminoacyl-tRNA, enters the aminoacyl site of ribosomes and becomes incorporated into the growing peptide chain. This causes the release of the puromycin-polypeptide chain from the ribosome. Many workers have found CAP to inhibit this reaction (22,23,24,25,26) and have argued that CAP inhibited the ribosomal peptidyl transferase reaction. Careful analysis of the effect of guanosine triphosphate (GTP) in a similar system caused Weber and DeMoss (27) to reject the peptidyl transferase reaction as measured by the puromycin induced release of peptides from tRNA, but rather inhibited some step in the peptide synthesis cycle prior to this reaction.

When amino acid auxotrophs of *E. coli* Rel^{+}(RC^{st}) were starved for required amino acids RNA synthesis stops and the polysome region

in sucrose density gradients was found to be reduced (18) or entirely depleted (16). The addition of CAP after starvation in $\underline{Rel}^+$ cells regenerated normal sedimenting polysomes in one minute, whereas 8-10 minutes were required for an equivalent regeneration when the required amino acid was added to starved cells in the absence of CAP (16).

Gurgo, Apirion, and Schlessinger (28) suggest that CAP uncouples ribosomal movement from peptide bond formation because it allows the continued formation of polysomes in the absence of protein synthesis. Other workers have demonstrated that C^{14}-labeled nascent peptides initiated in the absence of CAP were not chased off these polysomes during a 90 second chase with C^{12}-amino acids in the presence of CAP (17). Furthermore, CAP generated polysomes in $\underline{Rel}^+$ amino acid starved cells did not contain nascent peptides (16). The results of Weber and DeMoss and of Morris and DeMoss were confirmed and extended to show that CAP promoted 50S and 30S ribosomal subunits into polysomes, and that CAP generated polysomes did not possess nascent peptides 5 minutes after the removal of the antibiotic (18). However, after 25 minutes, large amounts of nascent peptides were present in this polysome region.

Analysis of nascent peptides on $\underline{E.\ coli}$ polysomes to resolve the above controversies have encountered serious difficulties. Treatment of $\underline{E.\ coli}$ with CAP allowed continued RNA synthesis and an accumulation of CAP particles (29). These particles were completed and became

functional after the removal of the antibiotic. Therefore, any labeled amino acids added after CAP removal can become incorporated into ribosomal proteins used to complete the CAP particles. These labeled ribosomal proteins would obscure nascent peptide protein synthesis. Although well defined, the polysome region of E. coli lacks a predominant species of mRNA during growth which would code for a single predominant protein. In general, E. coli polysomes have not been useful tools in the study of the mechanism of action of CAP.

The use of bacteriophage T4 circumvents some of these difficulties and was used in these studies to contribute to knowledge about the mechanism of action of CAP. Specifically examined were the fate of ribosomes, mRNA, and nascent peptides after transient treatment with CAP.

Rationale

When the phage T4 infects a host bacterium several characteristic events occur. After injection of the viral DNA, no further host DNA or RNA synthesis occurs (30,31). Synthesis of host protein and ribosomes is also stopped shortly after phage infection (32). Thus, one is allowed to treat T4 infected E. coli with CAP and not induce the formation of CAP particles. The fate of ribosomes, mRNA and nascent peptides can now be studied by polysome analysis on sucrose density gradients.

Cells infected with bacteriophage T4D produced polysomes near the end of the eclipse period in the phage intracellular life cycle. These polysomes were observed as a peak migrating in density gradients at 280S (33). Chymotryptic cleavage of the isolated nascent peptides found in this peak revealed that the major product was from phage gene 23 (34). The gene 23 product (P23) (35) was shown to be the major head coat protein of mature T4 (36).

It was found that the head coat protein of T4 made up 85% of the total phage protein (37), and that 60-70% of protein synthesis occurring late in the phage life cycle was devoted to production of this product (38). Consistent with this was the finding that the magnitude of the 280S polysome peak increased with increasing time after infection until a maximum was reached late in infection (34,39).

It was therefore possible to study a polysome region which possessed a well defined peak responsible for the synthesis of the major phage protein which itself was well characterized.

Phage proteins, specifically P23, were assayed by disc gel electrophoresis (40,41, 42,43) and autoradiography (40,41). This allowed rapid high resolution analysis of phage proteins by staining or by their incorporated radioactivity (44). Amber mutants of T4 (45,46, 47) were used to establish gene function with discrete proteins on polyacrylamide gels (40,41). The protein P23 was found to be the major

radioactive band in these gels during late infection and was monitored with relative ease.

In conclusion, the use of bacteriophage T4 to study the mechanism of action of CAP eliminated many problems which developed in *in vitro* *E. coli* studies. The advantages included: no new host ribosome synthesis after infection and therefore no CAP particles, a well defined polysome region which synthesized principally the phage head coat protein, and polyacrylamide analysis of this coat protein. Therefore, it was possible to determine the fate of mRNA, ribosomes and nascent peptides after treatment and removal of CAP.

MATERIALS AND METHODS

Bacterial and Bacteriophage Strains

The bacterial strains used were Escherichia coli B wild type (w.t.), which is a restrictive host for T4 amber mutants, obtained from Dr. P. D. Skaar, Montana State University, Bozeman, Montana and E. coli CR63, which is a suppressor host for T4 amber mutants, obtained from Dr. G. Streisinger, University of Oregon, Eugene, Oregon.

The phage strains used were T4D w.t. and amber mutants T4amB17 and T4amN54. All were obtained from Dr. G. Streisinger, University of Oregon, Eugene, Oregon.

Media and Supplements

All media and supplements were sterilized in an autoclave for 20 minutes at 15 pounds pressure unless otherwise indicated. Final concentrations are given in grams (g) or milligrams (mg)/liter (l) of distilled water unless otherwise indicated.

M9 minimal (M9): Na_2HPO_4, 5.9 g; KH_2PO_4, 3.0 g; NH_4Cl, 1.0 g; $MgSO_4$-7 H_2O, 0.25 g; glucose, 4.0 g; L-tryptophan, 20 mg; $FeCl_3$, 2.7 mg. All experiments employing C^{14}-isotopes with T4 were conducted in M9 minimal media.

M9S: Same as M9 media, except that the organic compounds glucose and L-tryptophan were omitted. M9S was used in serial dilutions of T4 for determining phage titers.

Nutrient broth (NB): Bacto Nutrient Broth, 10 g; NaCl, 5 g.

Nutrient agar (NA): Bacto Nutrient Broth, 10 g; Bacto-Agar, 12 g; NaCl, 5 g.

Top layer agar (TL): Bacto Nutrient Broth, 10 g; Bacto-Agar, 7 g; NaCl, 5 g.

Chloramphenicol (CAP): This was a gift from Parke Davis Co. and was used in 2 mg/ml stock solutions. This unautoclaved solution was stored at 4°C and replaced at least once per month.

Rifampicin (RIF): This is a macrocyclic antibiotic used to inhibit DNA dependent RNA polymerase (48,49). Unautoclaved 2 mg/ml stock solutions were stored at 4°C in the dark.

L-Arginine (C^{12}-arg): 2 mg/ml stock solutions used in C^{14}-arginine experiments.

L-Leucine (C^{12}-leu): 2 mg/ml stock solutions used in C^{14}-leucine experiments.

Uracil (C^{12}-uracil): 2 mg/ml stock solutions used in C^{14}-uracil experiments.

Bacterial and Bacteriophage Stock Preparation

Bacterial strains: E. coli B and CR63 were grown and stored at $4^{o}C$ on NA slants. Cultures were transferred to new slants once per month to ensure rapid growth of the overnight M9 minimal cultures.

Bacteriophage strains: Phage stock solutions were obtained by growing E. coli B to $5 X 10^{8}$ cells/ml at $37^{o}C$ and infection with T4D w.t. at a multiplicity of infection (MOI) of 5. Five minutes after infection these cells were superinfected with a multiplicity of superinfection (MOSI) of 5. Aeration at $37^{o}C$ in a New Brunswick gyratory incubator shaker was continued for 4 hours when 1 ml/l of chloroform was added. The suspension was then thoroughly mixed, and allowed to stand for 15 minutes at $4^{o}C$. The bacterial debris from this lysate was removed by spinning in sterile centrifuge tubes for 15 minutes at 5000 rpm (4080 Xg) in the Sorvall centrifuge GSA rotor at $4^{o}C$. This supernatant was placed into new sterile centrifuge tubes and spun for 20 minutes at 5000 rpm in the same rotor. This supernatant was carefully decanted and stored at $4^{o}C$ over chloroform and had an initial titer of $2-8 X 10^{10}$ T4D/ml.

These phage stock solutions were concentrated further when necessary, by pelleting at 12,000 rpm (21,500 Xg) for 60 minutes in the Sorvall GSA rotor at $4^{o}C$. The supernatant was decanted and the pellet covered with 5 ml of cold LB buffer and allowed to dissolve for 12 hours in the cold. The insoluble debris was removed by a final low speed

spin. The opalescent supernatant was titered and diluted to a working concentration of 2-4X10^{11} T4D/ml. This stock preparation maintained a stable titer for up to 12 months.

The preparation of T4 amber mutant stock solutions was identical to the preceding method except that E. coli CR63 was used in place of E. coli B and these cultures were allowed to grow for 8 hours with aeration at 37°C.

Bacteriophage Assay

Phage titers were determined by the agar overlay method (50). Sterile, 10 cm petri dishes were filled with 20 ml of NA. When these solidified they were dried at 37°C for at least 12 hours. Two and one-half ml of soft 45°C TL agar was inoculated with 0.20 ml of a 5X10^{8} cells/ml culture of E. coli B for titering T4D w.t. or E. coli CR63 for titering T4 amber mutants. Phage serial dilutions were made with 0.1 ml of the appropriate dilution added to the TL agar containing the host bacteria. This TL agar was mixed and poured onto the NA plates. When it had hardened, the plate was developed at 37°C for 12 hours.

Growth Temperatures

The preparation of phage stock solutions, growth of overnight cultures, and phage titrations was carried out at 37°C. All other studies were conducted at 30°C in order to slow phage intracellular development, thus allowing more time to perform experimental procedures. At

30°C, spontaneous host lysis did not begin until 40 min after infection in M9 minimal media.

One Step Growth Curves

These were carried out according to the method of Ellis and Delbrück (51), except that the culture samples were treated with chloroform to lyse the bacteria in which phage endolysin was being formed (52). Each sample was serially diluted in M9S and titered by the agar overlay method.

Biochemicals, Reagents, and Supplies

Hydrochloric acid: Reagent grade; E. I. Dupont Company.

Glacial acetic acid: Reagent grade; E. I. Dupont Company.

Trichloroacetic acid: Analytical reagent; Mallinckrodt Chemical Works.

Sucrose: Mann Ultra Pure; Mann Research Laboratories, Inc.

Yeast RNA: Yeast nucleic acid; Mann Research Laboratories, Inc.

PPO: 2,5-Diphenylaxozale; Packard Instrument Co., Inc.

Hyamine: Hydroxide of hyamine 10X; Packard Instrument Co., Inc.

Lysozyme: Egg white, 3X crystallized; Sigma Chemical Company.

Trizma: Trizma Base, reagent grade; Sigma Chemical Company.

DNase I: Deoxyribonuclease, RNase free, 2150 μ/ml; Worthington Biochemical Corporation.

RNase B: Ribonuclease, chromatographically pure; Worthington Biochemical Corporation.

X-ray film: 8" X10", NS54T; Eastman Kodak Co.

Kodak liquid developer: Eastman Kodak Co.

Kodak liquid fixer: Eastman Kodak Co.

Millipore filters: HAWP, nitrocellulose; Millipore Corporation.

Radioisotopes

The following C^{14}-isotopes were obtained in 50 μCi lots from Amersham-Searle Corporation. They were diluted to a working concentration of 1 μCi/100 μl with glass distilled water and stored frozen when not in use.

L-Arginine-C14(U) (C^{14}-arg): 312 mCi/mM.

L-Leucine-C14(U) (C^{14}-leu): 312 mCi/mM.

Uracil-2-14 (C^{14}-uracil): 52.5 mCi/mM.

Buffers

The following buffers were made with glass distilled water which was house distilled water redistilled in a water distillation apparatus.

The pH of these buffers was adjusted using glass distilled hydrochloric acid which was the middle third distillate of a 1:1 dilution with glass distilled water of reagent grade hydrochloric acid.

These buffers were treated with 2 drops/l of a bentonite suspension for 2 hours. This colloid was known to bind RNase and was therefore used to remove this enzyme from the buffers.

B: Trizma base, 12.11 g; KCl, 3.72 g; $Mg(OAc)_2$, 2.359 g. These crystalline compounds were dissolved in 1 liter water and the pH adjusted to 7.8 at 4°C. After treatment with bentonite, this buffer was filtered with HAWP Millipore filters into heat treated reagent bottles and stored at 4°C.

LB: Trizma base, 12.11 g; NaCl, 5.84 g; $Mg(OAc)_2$, 2.16 g. This buffer was treated and stored the same as Buffer B above.

Ribonuclease Removal

Ribonuclease (RNase) was removed from experimental hardware by two methods: heating for 2 hours at 210°C in a hot air oven or autoclaving for 15 minutes at 15 pounds pressure. RNase was removed from buffers by adding 2 drops/l of a bentonite suspension which was removed by filtration through Millipore filters.

This procedure was particularly important in polysome analysis because any residual RNase on glassware or in buffers contributed to the 70S ribosome regions by degradation of mRNA-ribosome complexes.

General Experimental Procedure

An overnight culture of E. coli B was diluted 1:10 with prewarmed M9 and aerated gently at 30°C in a New Brunswick metabolyte water bath shaker. The optical density (O.D.) of this culture was measured periodically with a Bausch and Lomb Spectronic 20 spectrophotometer at 525 mμ until 0.20 O.D. was reached (approximately 2×10^8 cells/ml).

These cells were infected at O.D. = 0.20 with T4D w.t. or T4 amber mutants at an MOI = 5 and after 5 minutes superinfected with an MOSI = 5. This culture was divided into 10 or 20 ml aliquots and aerated at 30°C until 15 or 20 minutes after infection. Carbon^{14}-isotopes were added at these times since the synthesis of head coat protein (P23) was occurring at a near maximal rate and 20 minutes still remained in the phage intracellular life cycle before spontaneous lysis began. One-half or one μCi aliquots of C^{14}-arginine or C^{14}-leucine labeled these infected cells adequately during a one minute pulse. This produced a well defined polysome region.

The antibiotics RIF and CAP were used at varying times after infection but always at concentrations where mRNA or protein synthesis were completely inhibited. Whenever C^{14}-isotopes or CAP were removed from these cells one of two methods was used: a 1:100 dilution of cells with prewarmed M9 or by pelleting the cells at 8000 rpm for 5 minutes in the Sorvall SS-1 rotor. These cells were chilled before

centrifugation to 0°C with one-half volume of ice and were resuspended in prewarmed M9 to continue the experiment.

Carbon^{14}-arginine, C^{14}-leucine, and C^{14}-uracil were effectively chased with 50 µg/ml of the unlabeled corresponding compound. Cell growth was stopped by pouring the cells over one-half volume of ice and pelleting them in the Sorvall centrifuge.

Cell Lysis Procedure

All steps in this lysis procedure were carried out at 0-4°C unless otherwise indicated.

The supernatant from pelleted cells was poured off and any remaining droplets wiped from the upper half of the centrifuge tubes. The remaining cell pellet was resuspended with 0.7 ml cold LB buffer and then quantitatively transferred to heat treated 12 ml conical centrifuge tubes.

Egg white lysozyme (1.2 mg) was added in 0.3 ml of LB buffer along with 4 µg of deoxyribonuclease (DNase I). These cells were frozen in a -90°C alcohol bath, thawed for 30 minutes in an ice-water bath, heated to 30°C for 2 minutes and then chilled to 0°C. This freeze-thaw treatment was repeated. These lysed cells were immediately layered on sucrose density gradients with heat treated 1 ml serological pipettes, or made 8M with respect to urea by adding 0.48 g of crystalline urea to the cell lysate as the experiment required.

This lysis procedure was used for all T4D studies except for amber mutant T4amN54 which was lysed by three cycles of freeze-thawing without heating to 30°C for 2 minutes. These lysed cells were spun at 4°C for 30 minutes at high speed in the #221 swinging bucket head of the International clinical centrifuge. This supernatant was then carefully layered on 15-30% sucrose gradients.

Ribonuclease Treatment

A classical method in characterizing polysome material found sedimenting faster than 70S ribosome monomers in a sucrose density gradient, is to demonstrate their sensitivity to low levels of RNase (53). If this fast sedimenting material represents polysomes it is degraded and found as a large peak in the 70S region of a density gradient. The cell lysate in these studies was treated with 2 µg/ml RNase B for 10 minutes at 30°C. This lysate was immediately chilled to 0°C and layered on sucrose gradients.

Sucrose Density Gradient Procedure

Preparation: Sucrose solutions of 15, 30 and 50% (w/w) were made by adding 150, 300 and 500 g respectively of sucrose to make one kilogram solutions. These were stored in sterile reagent bottles in the 4°C cold room where they were formed into linear gradients. The gradients were constructed by adding 0.5 ml 50% sucrose to the bottom of Spinco sw 25.3 rotor cellulose nitrate centrifuge tubes, then layering a

linear 13 ml, 15 to 30% (w/w) sucrose gradient into these tubes. The gradients were made up at least six hours prior to centrifugation so that slight density irregularities could equilibrate.

Centrifugation: One ml of cell lysate was carefully layered onto these gradients with heat-treated 1 ml serological pipettes. They were spun 2.25 hours at 25,000 rpm in the Spinco L-2 ultracentrifuge sw 25.3 rotor. At the conclusion of the run the rotor was allowed to stop with the centrifuge brake in the OFF position. The centrifuge buckets were removed from the rotor and stored in a refrigerator until fractionation.

Fractionation: Sixteen drop fractions of the sucrose density gradient were collected in heat-treated 12 X 150 mm tubes with a drop-counting fraction collector by puncturing the bottom of the gradient tube. During fractionation the gradient was held in a 4°C cooling jacket.

Precipitation and Plating Procedure for Density Gradient Fractions

Carrier yeast RNA (1 mg/gradient fraction) was added to each fraction, thoroughly mixed, and chilled to 0°C. Three-fourths ml of cold 10% trichloroacetic acid (TCA) was added to each fraction, mixed, and the resulting precipitate allowed to floculate at 4°C for at least 30 minutes.

The precipitate from each fraction was collected on Millipore filters using a 30 sample Millipore manifold. These filters were

washed twice with 2-3 ml of 5% TCA in order to wash out all unprecipitated radioactivity.

These filters were mounted on copper discs which were placed in disc holders, dried for 10 minutes at 160°C, allowed to cool to room temperature and counted in a Nuclear Chicago low background planchette counter.

Acidic Polyacrylamide Urea Disc Gel Electrophoresis

These procedures were carried out according to the methods of Davis (42) with the following modifications. Acidic polyacrylamide gel procedures and solutions were adopted from Reisfield (54) and the incorporation of 8M urea into these gels from Hosoda and Levinthal (40). After separation of the T4 proteins, followed by staining and destaining, these gels were sliced longitudinally and dried by the methods of Fairbanks (44).

Liquid Scintillation Counting of Polyacrylamide Gels

The separation gel portion of polyacrylamide gels containing C^{14}-isotope to be assayed by liquid scintillation counting were sliced into 0.5 cm lengths immediately at the conclusion of electrophoresis without fixing in 7% acetic acid or staining the protein. Each fraction was placed in a 12 X 150 mm Pyrex tube and finely ground with a homogenizer. One milliliter of hyamine protein solubilizer was added to each fraction and allowed to stand for 12 hours to extract all possible

protein. These hyamine solutions were placed in vials with 10 ml of scintillation fluid containing 507 ml toluene, 415 ml absolute ethanol, 77 ml formamide, and 2.9 g PPO/liter. These samples were counted in a Nuclear Chicago liquid scintillation spectrometer.

Autoradiogram Technique

Autoradiograms of C^{14}-T4 proteins were made by drying the polyacrylamide gels according to the methods of Fairbanks (44) and placing these flat, dried gel slices in X-ray film containers. The X-ray film was exposed to the C^{14}-isotope in the dried gels for varying lengths of time, but commonly for 24 hours when 1 μCi C^{14}-leucine was pulsed for 1 minute into 20 ml of T4 infected E. coli B at 30°C.

These films were developed with Kodak liquid developer for 4 minutes and development stopped with dilute acetic acid treatment for 1 minute. The films were fixed with Kodak liquid fixer for 10 minutes, soaked in distilled water for 30 minutes, and air dried.

Terminology

Polysome: In this manuscript the term polysome is applied to material sedimenting faster than 70S ribosomal monomers in sucrose density gradients. It was considered to contain ribosomes held together by mRNA (55, 56, 57) which can be degraded with pancreatic ribonuclease (RNase) forming free 70S ribosomes but not degraded with proteinases or DNase (53). The site of cellular protein synthesis was

considered to be the polysome because C^{14}-amino acids could be pulsed into it and then chased out with C^{12}-amino acids during protein synthesis.

Amber mutants: These are mutations in T4 which cause polypeptide chain termination prematurely. They are the result of a base mutation which converts an amino acid specifying codon into the base sequence UAG, which signals chain termination.

EXPERIMENTAL RESULTS

Effect of CAP and RIF on Mature Phage Formation

The continued growth of bacteria after a brief treatment with CAP has been extensively studied (58,59,60) but little is known about the reversibility of processes in phage infected bacteria. The first step in the present studies examines the sensitivitity of the phage growth to CAP and RIF and the kinetics of phage formation after transient antibiotic treatment.

The data indicated in Figs. 1 and 2 show that formation of mature phage was completely inhibited by 8 μg/ml CAP or 40 μg/ml RIF. These inhibition curves were used to select antibiotic concentrations where the bulk of protein synthesis and virtually all the mature phage formation were inhibited.

The effect of 50 μg/ml CAP on phage production was completely reversible (Fig. 3). By contrast, the effect of 50 μg/ml RIF on phage production was irreversible (Fig. 4). No recovery in phage production was found when RIF treated cells were diluted 1:100 into fresh prewarmed M9 media, or when RIF was retained in the cultures throughout the experiment.

Effect of CAP on T4 Polysomes

Inferences regarding the effect of CAP on polysomes and their constituents (ribosomes, mRNA, and nascent peptides) could not be

made from the preceding experiments. By using the T4 system, the following questions could be posed: is mRNA allowed to code for further protein after CAP inhibition; do transiently inhibited ribosomes continue to synthesize protein; do nascent peptides complete normally after removal of CAP?

Rapidly sedimenting polysomes were found in 15-30% sucrose gradients when T4D infected E. coli B were lysed near the end of the eclipse period (33,34). This polysome region contained a relatively homogenous, well defined peak at gradient fractions 10-15 as detected by C^{14}-labeled nascent peptides (Figs. 5, 13) or C^{14}-labeled mRNA (Fig. 9). This polysome region was extremely labile to low levels of RNase B and was moved from gradient fractions 10-14 to fractions 21-22 which represented free 70S ribosomes (Figs. 5, 7, 9, 14). The presence of 70S ribosomes in fractions 21-22 was verified by sedimenting purified 70S preparations into 15-30% sucrose density gradients.

Quantitative differences in reference polysome peaks resulted from varied lysis procedures with egg white lysozyme (Fig. 6). Two cycles of freeze-thaw lysis resulted in a small polysome peak, while two cycles of freeze-thaw lysis with two minutes of heat at 30°C dramatically increased this peak. However, when this heating was increased to four minutes, degradation of polysomes and the appearance of a 70S peak occurred.

When C^{14}-arginine was pulsed into T4D w.t. polysome peaks (Fig. 7), then chased for 10 minutes with 10 μg/ml C^{12}-arginine, an un-unexpected result was found. Instead of being readily removed, the C^{14}-level in the peak was increased dramatically, as compared to the 1 minute C^{14}-arginine pulse. The unchased polysome peak was completely sensitive to RNase (Fig. 7), while the chased peak was entirely RNase resistant (Fig. 8). Thus, T4D polysome C^{14}-nascent peptides were chased into an RNase resistant peak which was superimposed on the RNase sensitive polysome peak in the density gradients (Fig. 8).

Only a small amount of C^{14}-uracil was incorporated into mRNA in the presence of RIF. This was demonstrated with a culture containing 100 μg/ml RIF with a 5 minute C^{14}-uracil pulse (Fig. 9). The C^{14}-uracil incorporated into this polysome region both in the absence and presence of RIF was entirely RNase sensitive. Since RIF effectively stopped synthesis of mRNA, it was used to examine protein synthesis directed by mRNA made previous to the transient treatment with CAP in order to answer the following question. Is mRNA allowed to code for further protein synthesis after removal of CAP?

When C^{14}-arginine was added at 1, 10, and 20 minutes after the removal of RIF from cultures, a steady loss in C^{14}-incorporation with increasing time was observed (Figs. 10, 11, 12). After 20 minutes, no C^{14}-arginine was found in the polysome region. When cultures were

treated with CAP and RIF the labeled polysome pattern was very different (Figs. 10, 11, 12). Incorporation of isotope in these polysome regions (Figs. 10, 11, 12) increased for 10 minutes after removal of the drugs. While a slight drop was noted at 20 minutes (from 63% to 54%), a large amount of activity remained. A summary of data from this experiment is found in Table I of the discussion.

The infection of E. coli B with T4amN54 produced a polysome region with distinct differences from T4D w.t. (Fig. 13). A separate species of polysome was seen at gradient fraction 4 along with the major polysome peak at fractions 10-14. When this C^{14}-lecuine labeled polysome region was chased for 10 minutes at 30°C with 50 µg/ml C^{12}-leucine, virtually all of the radioactivity was moved into the supernatant region of the gradient (Fig. 14). This polysome region was also completely sensitive to RNase.

Polysome analysis of T4amN54 infected cultures allowed examination of the following question: do CAP inhibited ribosomes in mRNA-ribosome complexes continue to synthesize protein after removal of CAP?

These infected cultures were transiently treated with 50 µg/ml CAP and then chased with 50 µg/ml C^{12}-leucine with the following results. After a 10 minute chase 85% of the C^{14}-leucine was chased out of the CAP treated polysome region (Fig. 13). This was increased to 97% after a 20 minute chase.

The results from the RIF treated cultures indicated that mRNA made prior to CAP treatment can be used in coding for protein synthesis after removal of CAP. The finding that nascent peptides are chased out of the T4amN54 polysome region when CAP was removed indicates that ribosomal movement continues, and implies, but does not prove, that the nascent peptides are released normally.

Effect of CAP on T4 Head Coat Protein

The preceding experiments did not provide convincing evidence that transiently inhibited nascent peptides formed completed phage protein after removal of CAP. The analysis of T4 proteins on polyacrylamide gel electrophoresis was undertaken to answer this question.

The proteins of T4 have been separated and many have been identified as to gene function by subjecting T4 amber and temperature sensitive mutant lysates to polyacrylamide gel electrophoresis (40,41, 35). Separation of T4 phage proteins by acidic urea polyacrylamide gels was shown (Fig. 15). This diagram displays duplicate gels, stained with amido schwarz, the dried sections of these gels, and autoradiograms of the same dried gels. Gel autoradiogram "c" shows the intense radioactive band marked P23 which was chased out of gel autoradiogram "f" with C^{12}-leucine.

The head coat protein P23 was identified by introducing C^{14}-leucine at increasing times after infection and observing the

appearance of an intense radioactive band (Fig. 16). The use of T4amB17, an amber mutant in gene 23 which lacks the complete P23 protein, was also utilized to establish the position of this band in the polyacrylamide gels.

Chloramphenicol or RIF treatment of cultures did not alter the chasing of C^{14}-leucine from the P23 band by C^{12}-leucine (Fig. 18). Cultures transiently treated with both CAP and RIF also allowed C^{14}-leucine to be chased from the P23 band (Figs. 17 and 18). The radioactivity in P23 was completely chased with C^{12}-leucine in untreated cells in 10 minutes and essentially completed in CAP and RIF treated cells in 20 minutes.

Incomplete P23 peptides from amber mutants in gene 23 have been shown to possess characteristic mobilities in polyacrylamide gels. This mobility is in general inversely proportional to their length (35,40). The uncompleted P23 peptides from CAP treated polysomes are shown in Fig. 19. When the CAP was removed from these cells the incomplete peptides were chased out of polyacrylamide gel fractions 4-8 in 10 minutes and appeared in fraction one. After a 20 minute C^{12}-leucine chase the C^{14}-leucine did not enter the gels at all.

A 15 second pulse of C^{14}-leucine in T4amN54 infected E. coli B produced a diffuse radioactive region near P23 but after a 10 minute chase with C^{12}-leucine a sharp well defined band appeared at the P23 position (Fig. 20). Cells treated with 100 μg/ml CAP at the end of the

15 second C^{14}-leucine pulse also showed the absence of the P23 band. After removal of the CAP and a 20 minute chase with C^{12}-leucine the P23 band appeared.

The data from these experiments suggest that the nascent peptides whose elongation has been inhibited by CAP are completed and released normally after removal of the antibiotic.

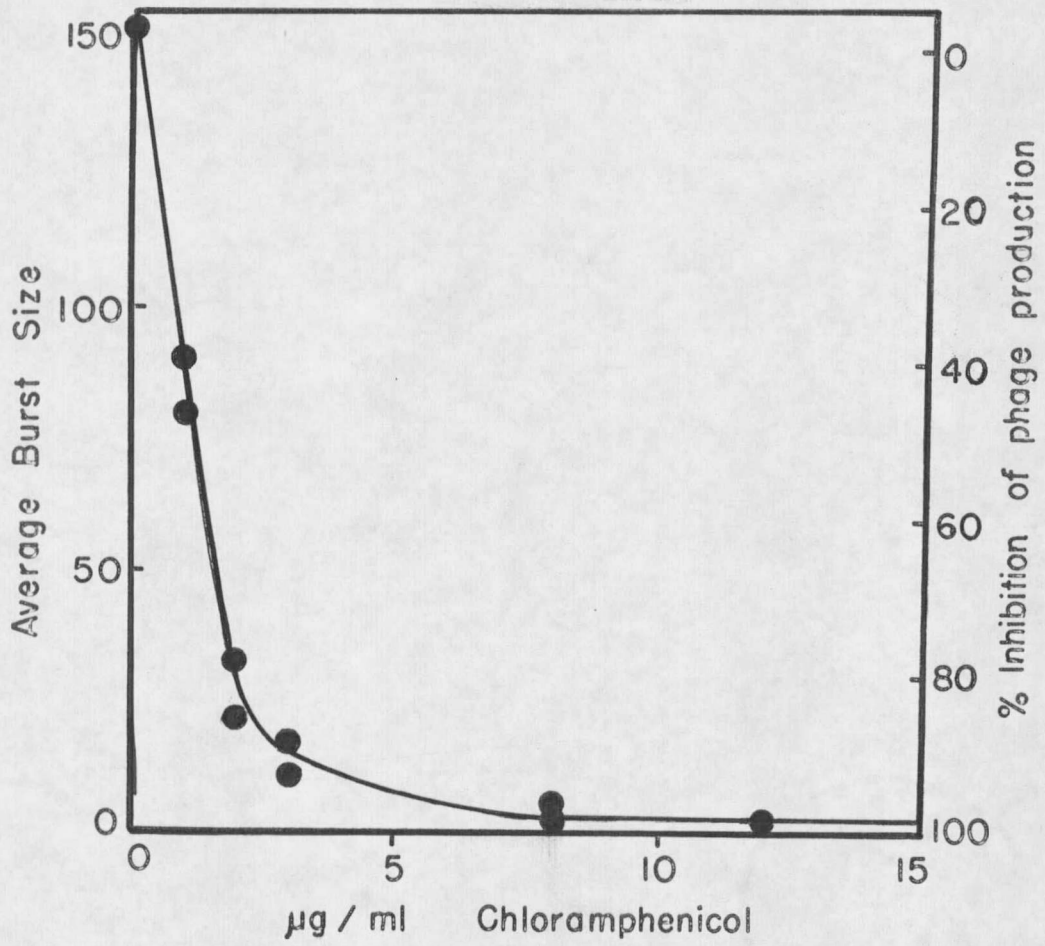

Figure 1. Chloramphenicol Inhibition Curve. E. coli B was infected at 30°C in M9 media with T4D w.t. at an MOI = 5. Chloramphenicol was added at varying concentrations at 2 min and growth continued to 90 min after infection when 0.5 ml samples were chilled and treated with chloroform for 5 min. Phage titers were determined by the agar layer method (50).

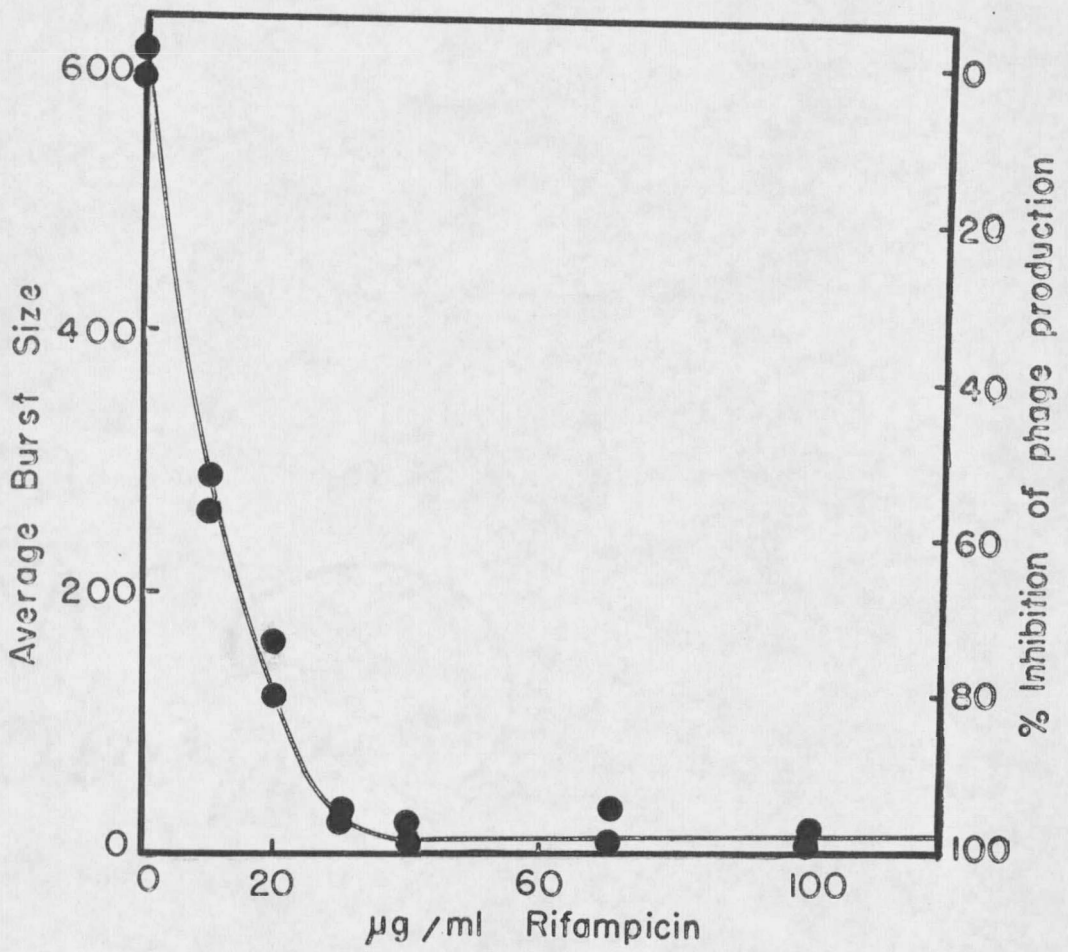

Figure 2. Rifampicin Inhibition Curve. Method was the same as Fig. 1, except that RIF was added at varying concentrations at 12 min after infection.

Figure 3. Chloramphenicol One Step Growth Curve. E. coli was infected at 30°C in M9 with an MOI = 5 and after 5 min superinfected with an MOSI = 5. Fifty μg/ml CAP was added at 2 min and diluted 1:100 with prewarmed M9 at 10 min after infection. Samples of 0.5 ml were removed at the indicated times, chilled, treated with chloroform for 5 min, and assayed by the agar layer method. Control single burst curve (O—O); 50 μg/ml CAP added at 2 min, diluted 1:100 at 10 min (●—●); and 50 μg/ml CAP added at 2 min (□—□).

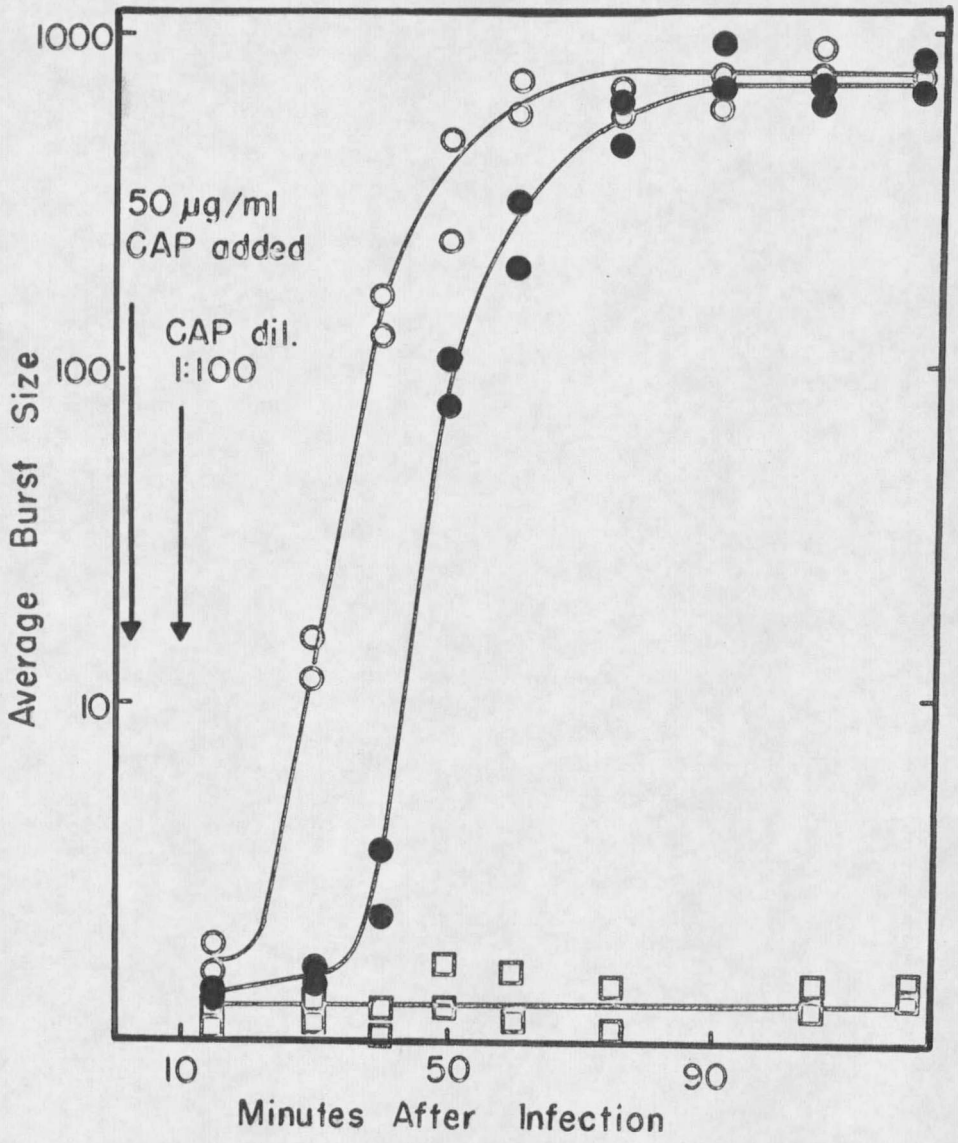

Figure 3. Chloramphenicol One Step Growth Curve.

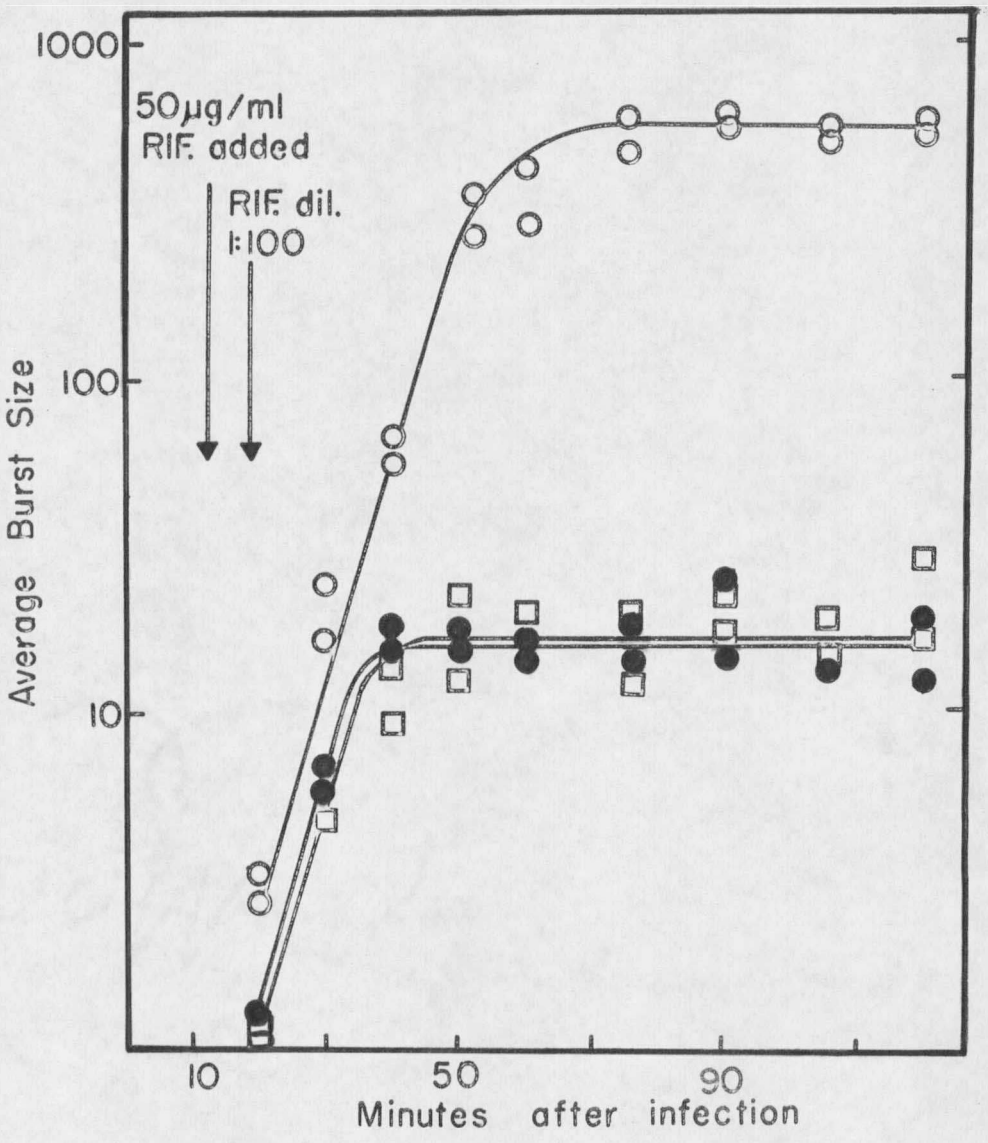

Figure 4. Rifampicin One Step Growth Curve. Method was the same as Fig. 3, except that 50 μg/ml RIF was added at 12 min and diluted with prewarmed M9 at 18 min after infection. Control single burst curve (O—O); 50 μg/ml RIF added at 12 min, diluted 1:100 at 18 min (●—●); 50 μg/ml RIF added at 12 min (□—□).

Figure 5. T4D w.t. Polysomes. Ten ml of E. coli B at 30°C were infected with T4D w.t. at an MOI = 5 and superinfected at 5 min with an MOSI = 5. At 15 min after infection 0.5 μCi C^{14}-Arg was added to a 1 min pulse, cells chilled, pelleted, and lysed by 2 cycles of freeze-thaw-2 min heating with lysozyme as in "Materials and Methods". The lysate was spun for 2.25 hours at 25,000 rpm on 15-30% sucrose gradients, fractions collected, precipitated by cold 5% TCA, and the radioactivity counted in a Nuclear Chicago planette counter. One min C^{14}-arg pulse (O—O); 1 min C^{14}-arg pulse followed by 2 μg/ml RNase B for 10 min at 30°C (●—●).

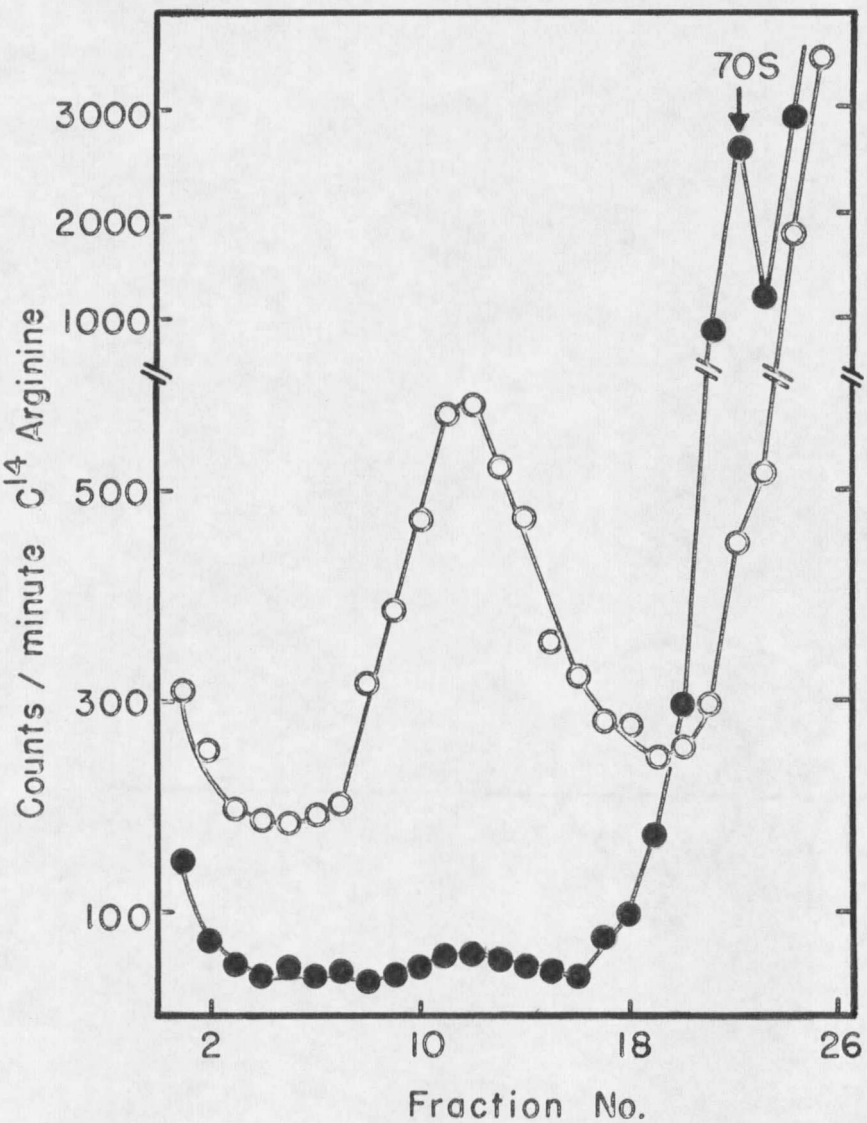

Figure 5. T4D w.t. Polysomes.

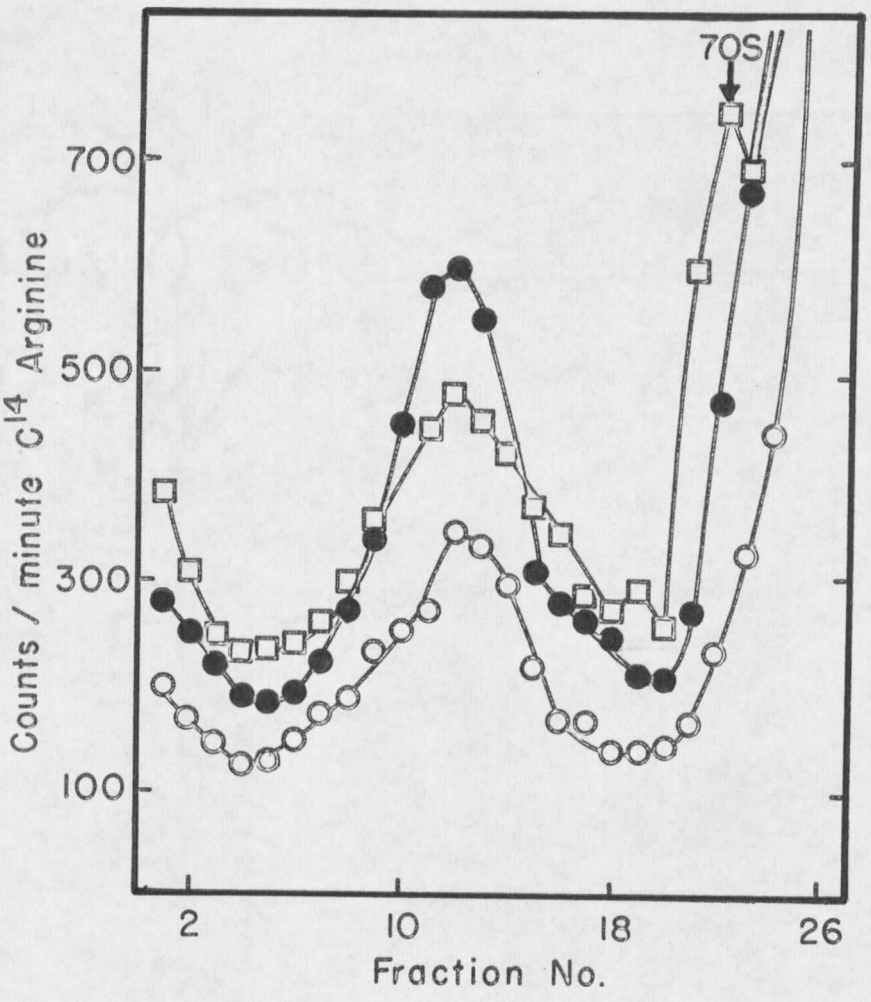

Figure 6. T4D w.t. Polysome Lysis Controls. Method was the same as Fig. 5, except for the lysis procedure. The cells were frozen in a -90°C ethanol bath and thawed in a 0°C ice-water bath for 2 cycles. Two cycles freeze-thawing only (O—O); freeze-thaw-heating for 2 min at 30°C, 2 cycles (●—●); freeze-thaw-heating for 4 min at 30°C, 2 cycles (□—□).

Figure 7. T4D w.t. Chased Polysomes. Method was the same as Fig. 5 except that 1 μCi C^{14}-arg was added for a 1 min pulse 20 min after infection. One min C^{14}-arg pulse (O—O); 1 min C^{14}-arg pulse followed by 2 μg/ml RNase B for 10 min at 30°C (●—●); 1 min C^{14}-arg pulse followed by 10 min chase with 10 μg/ml C^{12}-arg (□—□).

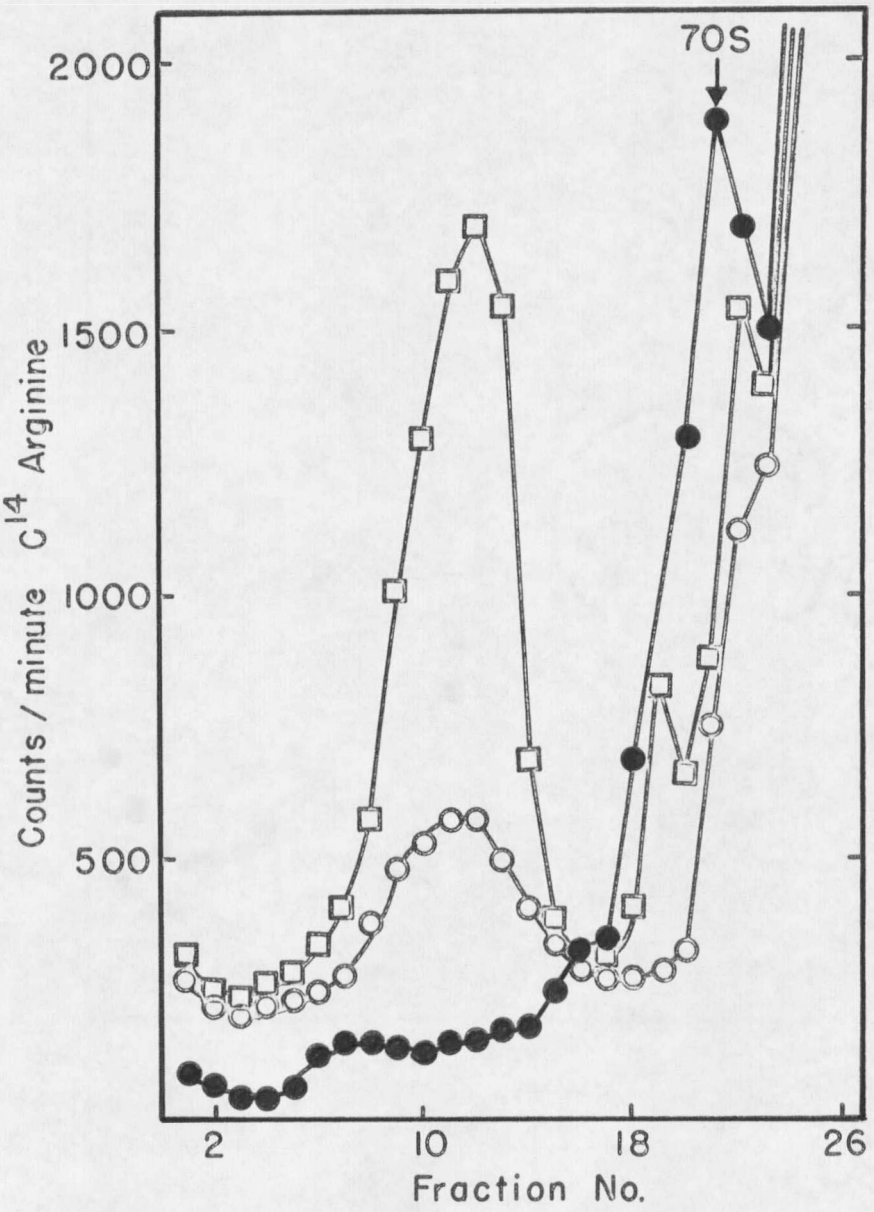

Figure 7. T4D w.t. Chased Polysomes.

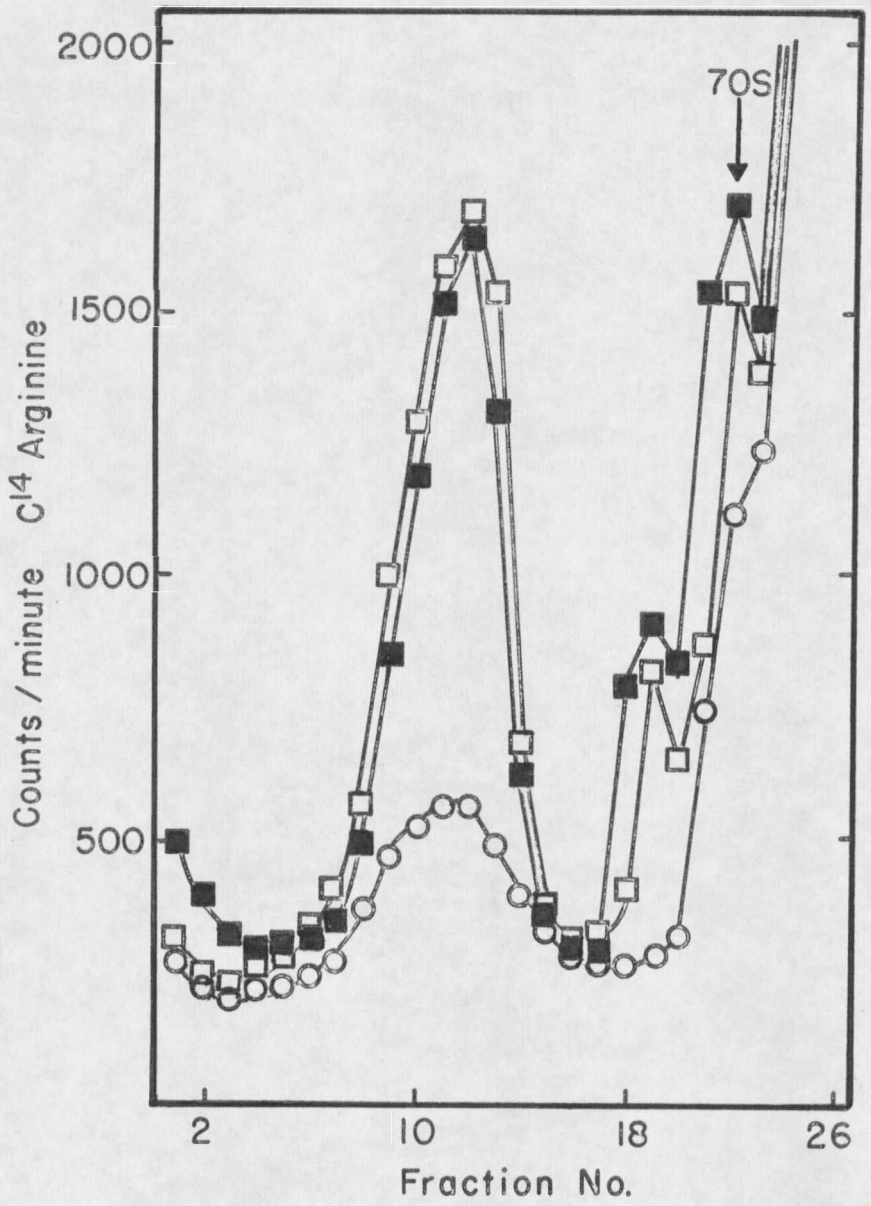

Figure 8 T4D w.t. Chased Polysomes. Method was the same as Fig. 7. One min C^{14}-arg pulse followed by a 10 min chase with 10 μg/ml C^{12}-arg (□—□); one min C^{14}-arg pulse (○—○); 1 min C^{14}-arg pulse followed by 2 μg/ml RNase B for 10 min at 30°C (■—■).

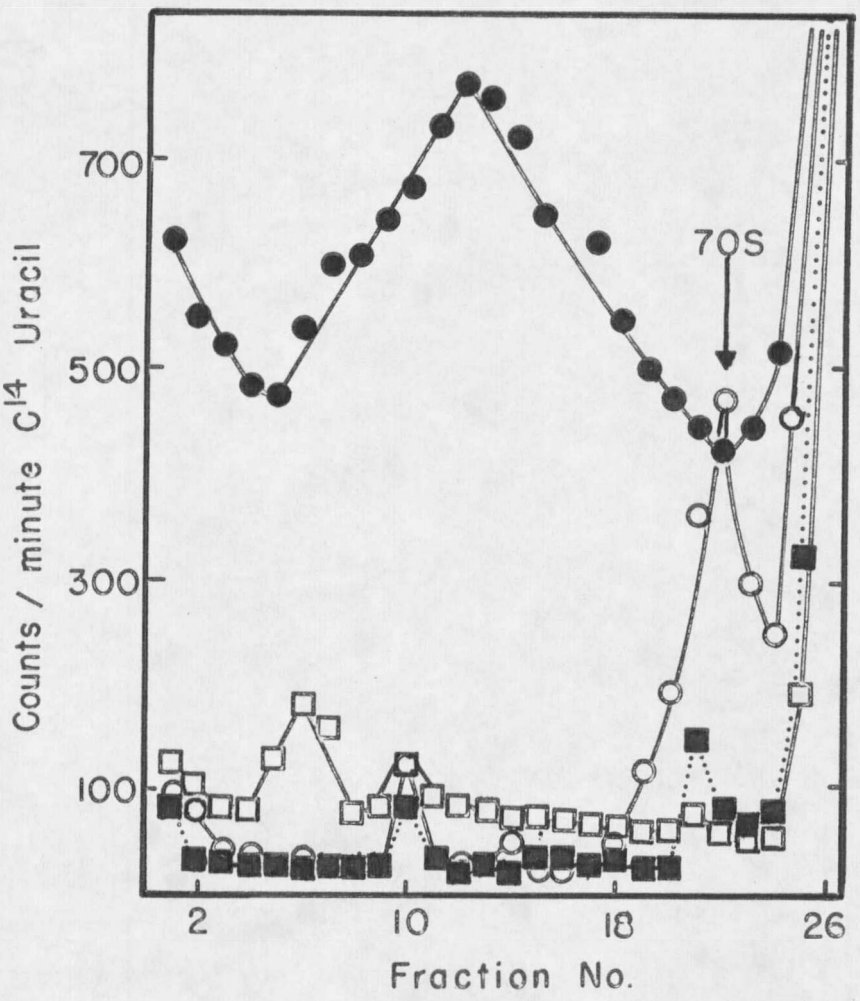

Figure 9. Rifampicin Treated T4D w.t. Polysomes. Method was the same as Fig. 5 except that 2 μCi C^{14}-uracil was added at 20 min after infection for 1 min in control cells and 5 min in RIF treated cells. One min C^{14}-uracil pulse (●—●); 1 min pulse followed by a 2 μg/ml RNase B treatment for 10 min at 30°C (○—○); 100 μg/ml RIF added at 17 min followed by a 5 min C^{14}-uracil pulse at 20 min (□—□); 100 μg/ml RIF at 17 min, 5 min C^{14}-uracil pulse at 20 min and a 2 μg/ml RNase B treatment at 30°C for 10 min (■—■).