Inhibition by Chloramphenicol of the Growth of Nascent Protein Chains in *Escherichia coli*

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SUMMARY

Inhibition of protein synthesis by chloramphenicol has been studied in intact cells of *Escherichia coli* at 37° and at 0°. The drug has no effect upon the association of ribosomes and messenger RNA, and no preferential effect upon the initiation of new protein chains or upon chain termination and detachment from ribosomes. The primary action of the drug is to block the addition of new residues to the growing nascent protein chains, which remain attached to the ribosomes. There appears to be no selective blockade of the addition of any particular amino acid, nor is there preferential inhibition of the synthesis of any particular kind of protein. Chloramphenicol is bound to ribosomes at 0° in the same concentration range in which it produces a graded inhibition of protein synthesis. A possible mechanism is proposed, whereby chloramphenicol might act by competing with the carboxy-terminal amino acid of the growing chain of nascent protein (attached to transfer-RNA) for a stereospecific locus associated with one of the transfer-RNA binding sites on the ribosome.

INTRODUCTION

CAM[1] (Fig. 1) causes a very rapid cessation of protein synthesis in sensitive bacteria under normal growth conditions, but the exact step at which it acts is still unclear. Studies with cell-free systems have indicated that the drug does not affect the esterification of amino acids to transfer RNA but inhibits a subsequent step (1, 2). It is ordinarily inactive in mammalian protein-synthesizing systems, but will inhibit protein synthesis if syn-

[1] Abbreviations used: CAM, chloramphenicol; mRNA, messenger ribonucleic acid; tRNA, transfer RNA; poly-UC, mixed polymer of uridylic acid and cytidylic acid; poly-U, polymer of uridylic acid; poly-A, polymer of adenylic acid; O.D., optical density; Tris, tris(hydroxymethyl)-aminomethane acetic acid buffer, pH 7.4; SDS, sodium dodecyl sulfate; TCA, trichloroacetic acid; DEAE, diethylaminoethyl.

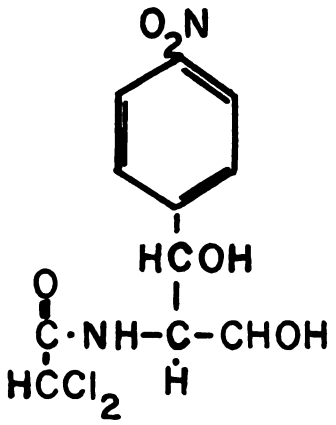

Fig. 1. *Chloramphenicol*

In the biologically active D(—)-*threo* isomer the configuration about the C atom adjacent to the primary alcohol group is identical to that of the α carbon in the L-amino acids. The secondary alcohol group must be on the same side as the primary alcohol, as shown.

thetic template is employed, suggesting that it impairs the attachment of mRNA to the ribosomes (3). This conclusion, however, was rendered doubtful when other investigators (4), although able to obtain similar results with viral RNA as externally added template, could not demonstrate any inhibition by CAM of the attachment of poly-UC to ribosomes; yet the polymerization of amino acids promoted by poly-UC was the most sensitive to CAM of all those tested. Conflicting reports show failure to find any inhibition by CAM of poly-A binding to ribosomes (5) but partial inhibition of poly-U binding (6, 7). Formation of the ternary complex of ribosomes, poly-U, and phenylalanyl-tRNA was not inhibited by CAM (8). Recent findings suggest that in a cell-free system stimulated by poly-A, CAM inhibits the peptide-linking step (9).

Most of the experiments reported so far to determine the site of action of CAM have employed cell-free systems. The ease of control and of manipulation *in vitro* are attractive, but it is by no means certain that the results reflect the true state of affairs inside a living cell. Many differences have been brought to light between synthetic polynucleotides and natural mRNA with regard to interaction with ribosomes, promotion of protein synthesis, and sensitivity toward CAM (10). Moreover, the concentration of CAM used to inhibit protein synthesis in cell-free systems is usually much higher than required for bacteriostasis.

An obvious reason why more use has not been made of whole bacterial cells is that reaction rates are too fast, making recognition of transient metabolic steps extremely difficult. For example, in *Escherichia coli* the estimated average assembly time of a protein of molecular weight 5×10^4 is around 5 sec (11, 12). Thus, a drug that interfered with any one of the many steps between transcription of the genetic message and detachment of completed protein from the ribosome would appear to act almost instantaneously. A pool of free and sRNA-bound amino acids on the order of 10^7 molecules/cell (13), given 5000 nascent protein chains per cell (11), would suffice for only about 6 assembly cycles; so even a drug that depleted an animo acid or blocked its activation would take effect in well under a minute. Temperature reduction to 0° provides an opportunity to study events within a slowed-down assembly cycle of protein synthesis (11). In many respects it is an ideal method for examining, in intact cells, the inhibition of biosynthetic processes by drugs. We describe here its use in analyzing the mode of action of CAM.

MATERIALS AND METHODS

Materials. Chloramphenicol (D(—)-*threo* isomer) was a gift from Parke, Davis and Company; ^{14}C-L-leucine (uniformly labeled), ^{14}C-L-tyrosine (uniformly labeled) hydrochloride, ^{14}C-L-lysine (uniformly labeled), and ^{14}C-chloramphenicol (methylene labeled) were purchased from Nuclear Chicago Corporation; ^{3}H-L-leucine (4,5-H labeled) and ^{14}C-uridine (2-C labeled) were from New England Nuclear Corporation; and ^{14}C-L-phenylalanine (carboxyl labeled), ^{14}C-DL-tryptophan (1′-C labeled) and ^{14}C-carboxylinulin (carboxyl labeled) were from California Corporation for Biochemical Research.

Cells. For most of the experiments a leucine-proline-uracil auxotroph of *Escherichia coli* K12 was grown at 37° in a phosphate-buffered glucose salts medium containing leucine, proline, and uridine; harvested during exponential growth (about 0.15 mg dry weight/ml) on Millipore filter, and resuspended at 0° in about 1/50 the original volume of medium to a bacterial density of 6–8 mg dry weight per ml, as described fully elsewhere (11). When radioactive leucine was to be incorporated at 0°, the culture at 37° was starved for leucine for 30 min before harvesting; when radioactive uridine was to be incorporated no starvation was necessary.

In some experiments the incorporation of various amino acids into wild-type cells of *E. coli* K12 was followed. The cultures were grown at 37° in the presence of an

amino acid (20 μg/ml). Then at 0°, after harvesting and resuspending, the same amino acid was furnished (1 μg/ml) in radioactive (^{14}C) form.

Determination of radioactivity. For leucine incorporation into total cell protein the cells were sampled directly into chilled 5% TCA, then extracted for 30 min at 80–90°, again chilled and washed 5 times with cold 5% TCA on a Millipore filter. The filter was placed in a scintillation vial, 5 ml scintillation mixture (14) was added, and radioactivity was determined in a Packard Tri-Carb scintillation spectrometer. For tritium assay the precipitate on the filter was dissolved in 0.05 N NaOH and 1 ml was placed in the vial with 1 ml hyamine hydroxide and 10 ml scintillation mixture. For uridine incorporation into nucleic acids the whole procedure was the same except that the hot extraction step was omitted.

CAM particles. CAM (10 μg/ml) was added to *E. coli* cells at 37° in the exponential growth phase. Radioactive uridine was added 1 min later. Samples from the cell suspension (50 ml) were withdrawn periodically, collected on Millipore filters, resuspended in 1 ml 10^{-2} M Tris, 10^{-2} M magnesium acetate and were treated for 1 min at 0° in the MSE-Mullard 20 kc/sec Ultrasonic Disintegrator. The lysate was centrifuged at 8000 *g* for 10 min, and the sediment was discarded. The supernatant solution was made up to about 10 ml with 10^{-2} M Tris, 10^{-2} M Mg^{++} and centrifuged for 3 hours at 50,000 rpm in the No. 50 rotor of the Beckman Model L Ultracentrifuge. The pellet was washed once by resuspension in the same Tris-Mg^{++} buffer, and recentrifuged for 3 hours at 50,000 rpm. The final pellet was suspended in 10^{-2} M Tris, 10^{-4} M Mg^{++} (0.8 ml) and 0.2 ml was layered on a linear sucrose gradient (5 ml, 5–20%), containing 10^{-2} M Tris, 10^{-4} M Mg^{++} and centrifuged for 3 hours at 39,000 rpm in the Beckman SW39 swinging-bucket rotor. The gradient was run through a flow cell in the Gilford Model 2000 absorbance recorder into scintillation vials. Absorbance was measured at 260 mμ. Radioactivity was determined in the scintillation spectrometer after addition of equal volume of hyamine hydroxide and 5 ml of the scintillation mixture. The dead space between the flow cell and the delivery tip was determined, so that the radioactivity data could be superimposed on the absorbance record.

Entry of CAM into cells at 0°. E. coli cells were grown, harvested, and resuspended as in experiments for leucine incorporation. The suspension was then incubated at 0° with leucine (1 μg/ml) and radioactive CAM (10 μg/ml). Samples (1 ml, about 8 mg dry weight of cells) were collected at 2° on Millipore filters (47 mm, 45 μ). Some cells were scraped off gently and resuspended in water. Turbidity of the suspension was determined, and a sample was counted with hyamine hydroxide and scintillation mixture. A similar experiment was done with radioactive carboxylinulin to determine the volume of trapped fluid between the cells.

Binding of CAM to ribosomes. Ribosomes were prepared by lysing cells in the sonic disintegrator in medium containing 0.02 M magnesium acetate, 0.18 M KCl. After removal of "debris" at 8000 *g* for 10 min the supernatant ribosomes were sedimented at 50,000 rpm for 1 hr in the No. 50 rotor, then resuspended in 0.016 M magnesium acetate, 0.06 M KCl, 0.01 M Tris acetate, pH 7.6. The ribosomes were sedimented again in the same way and resuspended in fresh buffer. All procedures were carried out in the cold.

For binding studies aliquot portions of the ribosome suspension were added to buffer containing CAM at 0°. Each sample contained ribosomes from 12–14 mg dry weight of cells ($5–6 \times 10^{14}$ ribosomes) in 2.2 ml reaction volume. The samples all contained the same amount of ^{14}C-CAM (15 μg, specific radioactivity 7.75 μC/μmole) and different amounts of nonradioactive CAM. The drug concentrations varied from 6.8 to 1050 μg/ml, and the specific radioactivities from 7.75 to 0.05. The ribosomes were separated from the reaction mixtures by sedimentation at 50,000 rpm for 2 hr. The supernatant fluids were discarded, the tube walls were

carefully rinsed, and the pellets were taken up in 0.1 N NaOH and transferred to vials for counting. Internal standards were used to obtain counting efficiencies.

As described by Wolfe and Hahn (15), since the concentration of ^{14}C-CAM was the same in all the reaction mixtures, regardless of the total CAM concentration, the radioactivity due to water entrapped in the pellets should be constant. However, the radioactivity due to binding of any given amount of CAM should vary inversely with the total concentration. Thus a graph of the sedimented radioactivity as a function of specific radioactivity will be a horizontal line, representing entrapped counts, if there is no binding. If the amount bound is constant, a straight line with positive slope will be found. If the amount bound varies with concentration, a curvilinear relationship results. In all three cases the extrapolated value at zero specific radioactivity gives the entrapped radioactivity. By subtraction, then, and the known specific radioactivities of the several samples, it is possible to find the molecules of CAM bound per ribosome at each drug concentration.

Isolation of RNA. RNA was isolated from sonic lysates as described elsewhere (11) by a modification of the phenol-SDS method of Kjeldgaard and Kurland (16). tRNA was isolated by the method of Zubay (17).

DEAE cellulose fractionation. Cells were grown in ^{3}H-leucine at 37°, starved for leucine for 30 min in the exponential growth phase (0.15 mg dry weight/ml), harvested, and resuspended at 0° in 1/50 the original volume of growth medium containing no leucine. ^{14}C-leucine (1 μg/ml) and CAM (10 μg/ml) were added simultaneously and the mixture was incubated at 0° for 1 hr. The cells were lysed and centrifuged with 10^{-2} M Tris, 10^{-2} M Mg^{++} for 1 hr at 50,000 rpm as described above. The supernatant solution was dialyzed against 5×10^{-3} M Tris overnight at 2° with four changes of buffer. The dialyzed sample (9 ml) was run onto a 1.1 cm × 10 cm column of DEAE-cellulose (Calbiochem, washed with 0.02 N NaOH in 2 M NaCl, 2 M NaCl in 5×10^{-3} M neutral Tris, 5×10^{-3} M Tris). Elution was done with NaCl solution in 5×10^{-3} M Tris changing the concentration of NaCl in discontinuous steps, from 0.02 M to 2.0 M. Each fraction was 5.0 ml and collected at a flow rate of 1.3 ml/min. To eliminate acid-soluble radioactive material, the first 15 fractions were precipitated and extracted with hot 5% TCA. Equal portions (1 ml) of all fractions were counted with hyamine and scintillation mixture.

Incorporation of leucine into nascent and finished proteins. To 1 ml of incubation mixture at 0° was added 0.1 ml of 1.5 M magnesium acetate and the cells were lysed as described earlier. Low speed and high speed centrifugations were carried out as described for chloramphenicol particles except that the one at 50,000 rpm lasted only 1 hr. A sample from the supernatant solution, which contained the finished proteins, was extracted with hot 5% TCA, then chilled, and collected on Millipore filters for determination of radioactivity. The ribosomal pellet was treated in the same way as the supernatant solution.

We consider that in these experiments whatever radioactive leucine was associated with the ribosomal pellet in 10^{-2} M Mg^{++} represented nascent protein chains, for the following reasons:

1. Almost none of it could be extracted by hot TCA.

2. Most of it could be detached and obtained in the supernatant solution if the pellet was resuspended in the absence of Mg^{++} and centrifuged again at 50,000 rpm for 3 hr.

3. If the cells were incubated for 5 min at 37° with nonradioactive leucine, then lysed and centrifuged at 50,000 rpm, most of the TCA-insoluble radioactivity that had been in the ribosomal pellet was now found in the supernatant protein.

4. Addition of radioactive finished proteins to an unlabeled cell lysate did not result in any binding of radioactivity to the ribosomal pellet.

Incorporation of leucine into NH_2-termi-

nal positions of proteins. Determination of radioactivity in NH_2-terminal leucine was done by the fluorodinitrobenzene method as described earlier (11).

Attachment of mRNA to ribosomes. Radioactivity in mRNA bound to ribosomes was determined in the same way as leucine incorporation into nascent proteins except that here the incubation was done with radioactive uridine instead of leucine, and the TCA precipitates were not subjected to hot extraction. The identification of ^{14}C-uridine in the ribosomal pellet as ribosome-bound mRNA rests upon the following findings, to be published in detail elsewhere:

1. Even in several hours at 0° no 23 S or 16 S ribosomal RNA was synthesized, as determined by ^{14}C-uridine incorporation and sucrose density gradient centrifugation of the extracted RNA. Practically all the label appeared in a single peak at about 12 S, indistinguishable from that seen after 20 sec of incorporation at 37°.

2. The association of newly synthesized mRNA with the ribosome pellet was evidently not an artificial aggregation occurring after cell lysis; when protein synthesis in these cells fails after several hours at 0° this binding no longer occurs.

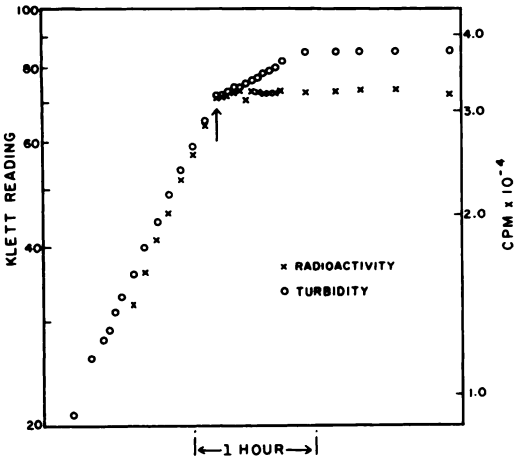

FIG. 2. *Effect of CAM (10 μg/ml) on growth and protein synthesis in E. coli at 37°*

The cell culture was allowed to incorporate ^{14}C-leucine. Samples were taken periodically for turbidimetry in the Klett-Summerson colorimeter with No. 42 filter, and for TCA precipitation and determination of radioactive protein as described in Methods. CAM (10 μg/ml) was added at the arrow.

RESULTS

Figure 2 shows that CAM at 10 μg/ml, added to a culture at 37°, stopped protein synthesis immediately. The turbidity then increased slowly for about one normal doubling time, presumably because of continued nucleic acid synthesis in the presence of the drug (18, 19). If cells inhibited by CAM (35 min exposure) were harvested on Millipore filters and resuspended in the absence of CAM, growth and protein synthesis resumed promptly at the usual rate.

The O.D. pattern in Fig. 3 shows that

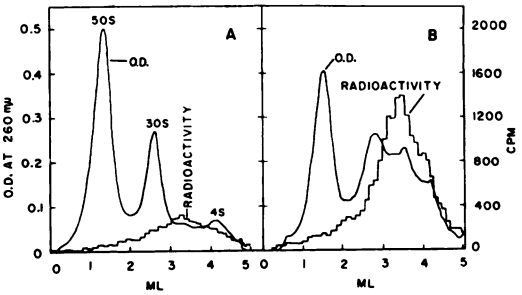

FIG. 3. *Sucrose density gradient centrifugation of CAM particles*

CAM (10 μg/ml) was added to an exponentially growing culture at 37°. One minute later ^{14}C-uridine was added. After another 4 min, half of the culture was harvested (A). At 45 min after addition of CAM the remainder of the culture was harvested (B). Both samples were lysed and worked up as described in Materials and Methods. The washed pellets were resuspended and layered on linear (5 ml, 5–20%) sucrose gradients containing 10^{-2} M Tris and 10^{-4} M Mg^{++}. The gradients were centrifuged at 39,000 rpm for 3 hr and collected through a flow cell in the Gilford absorbance recorder. The bottom of the tube is at the left.

CAM particles began to appear when protein synthesis had ceased, and accumulated progressively thereafter. The sample taken 5 min after addition of CAM (i.e., almost 5 min after protein synthesis had stopped) contained normal 50 S and 30 S ribosomal subunits in their usual 2:1 O.D. ratio (Fig. 3A), and a small 4 S peak. There

was also the suggestion of a peak at about 19S characteristics of CAM particles and containing ^{14}C-uridine radioactivity. After 45 min in CAM (Fig. 3B) the 19S O.D. peak had become very prominent and a large amount of new RNA (^{14}C-uridine) was associated with it. There was little degradation of existing ribosomes, and no new RNA appeared in the ribosome peaks.

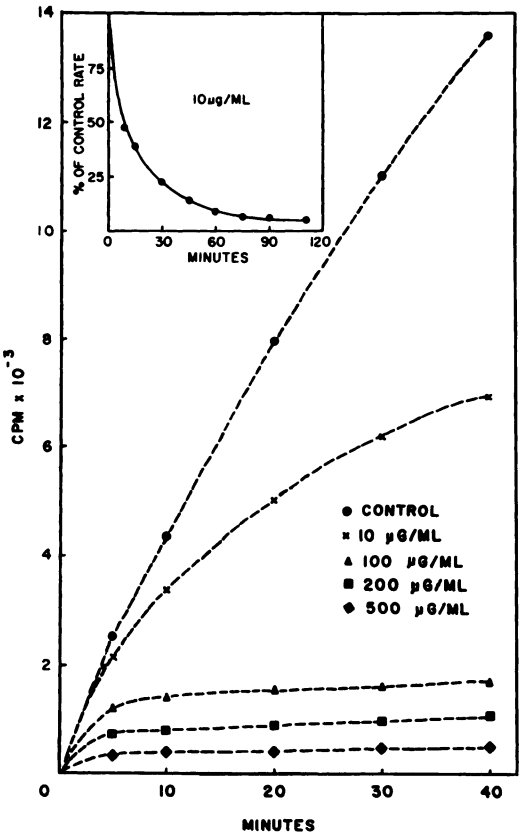

FIG. 4. *Effect of CAM on the incorporation of leucine into E. coli protein at 0°*

CAM at several concentrations was added together with ^{14}C-leucine at 0°. "Dead-cell" radioactivity (a control in which ^{14}C-uridine is added to cells in TCA) (here 32 cpm) has been subtracted. The inset is a plot of incorporation rate relative to the control for a longer period of time. The comparisons with control rates were made at the same cumulative incorporations rather than at the same elapsed time. This procedure is justified by our finding (unpublished) that at 0° the control rate at any time is determined by the total cumulative incorporation up to that time.

Figure 4 shows the effects of several concentrations of CAM on the incorporation of ^{14}C-leucine into whole-cell protein at 0°. With 10 µg/ml the onset of action was slow and the inhibition was incomplete, in contrast to the effect at 37°. The inset diagram on Fig. 4 shows that the maximum inhibition of incorporation rate took about an hour to develop. At 100 µg/ml and above, however, the onset was immediate and the inhibition was almost complete within a few minutes. Reversibility at 0° was as prompt as at 37°; however, for reasons we do not understand, the rate of incorporation after dilution of cells into fresh radioactive medium was only one-third its previous value, in controls as well as in CAM-treated cells.

Figure 5 shows that at 0° CAM (10 µg/ml) took only 10 min to reach the maximum intracellular concentration. Moreover, the initial entry was very fast, 75% of the ultimate level being attained in the first minute. The curve seems to

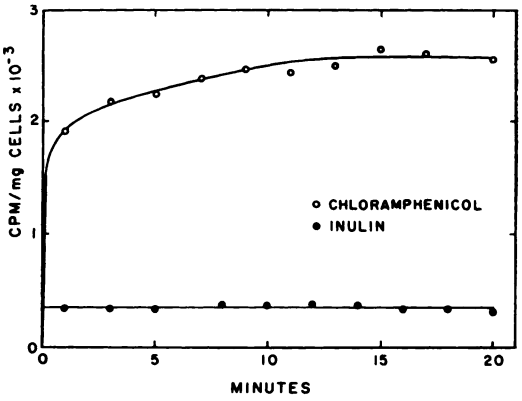

FIG. 5. *Kinetics of entry of CAM into E. coli cells at 0°*

E. coli cells (7.5 mg/ml) were incubated at 0° with ^{14}C-CAM (10 µg/ml, 6.81 µC/µmole) added at time zero. The upper curve represents ^{14}C-CAM associated with the mat of cells scraped from the Millipore filter. A similar experiment was done with ^{14}C-carboxylinulin (1.21 µC/mg). The data obtained with carboxylinulin, adjusted for the difference in specific radioactivity, are shown in the lower curve, which therefore represents the amount of trapped ^{14}C-CAM in the intercellular water.

consist of a fast and a slow component. The possibility was considered that the bulk of the counts measured were due to binding of CAM to cell wall or other surface. If that were the case, then increasing the concentration of nonradioactive CAM (without altering the concentration of radioactive CAM) should reduce the counts associated with the cells. The same experiment was therefore repeated in the presence of a tenfold excess of nonradioactive CAM. The fast component of the curve remained unchanged; the slow component was substantially reduced. The former can therefore be accounted for as the equilibration of cell water, the latter as a binding phenomenon corresponding to a little more than one CAM molecule per ribosome.[2]

Essentially the same method was used to estimate CAM trapped in the water phase of a ribosomal pellet (15), and thus to determine, by difference, the ribosome-bound CAM. Figure 6 shows that within approximately the same concentration range in which CAM caused a graded inhibition of protein synthesis at 0°, the binding increased from nil to about 1 molecule per ribosome. At higher concentrations additional binding sites seems to become available.

In order to discover whether CAM blocked the incorporation of any particular amino acid preferentially, the experiments shown in Fig. 7 were carried out. The incorporation of a radioactive amino acid at 0° in these nonstarved cells showed a lag of a few minutes and took even longer to become linear; presumably this was the time required to replace the nonradioactive

[2] Water content of *E. coli* cells is 78% (20) so each milligram dry weight is associated with about 0.004 ml water. After subtraction of counts trapped in the intercellular water (from the carboxylinulin data), the CAM radioactivity was found to be 560×10^3 cpm/ml inside cells and 330×10^3 cpm/ml outside. The CAM specific radioactivity corresponded to 1.7×10^{-11} cpm/molecule. Since a cell weighs 4.2×10^{-7} μg and contains 1.8×10^4 ribosomes (11), it follows that the excess of internal over external CAM could be accounted for by the binding of 1.3 CAM molecules per ribosome.

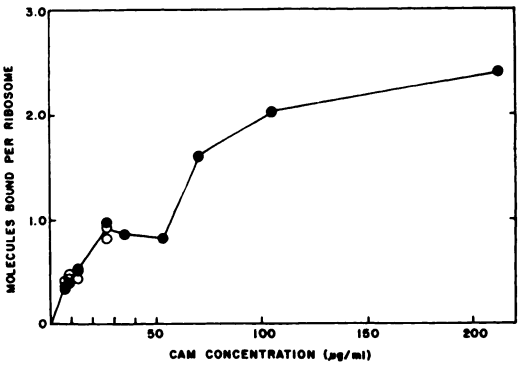

FIG. 6. *Binding of CAM to ribosomes at 0°*

Each point represents a sample of about 5–6 × 10^{14} ribosomes, washed with buffer, then exposed to radioactive CAM at 0° and sedimented for 2 hr at 50,000 rpm in the No. 50 rotor. Corrections for entrapped fluid containing radioactive CAM were made as described under Materials and Methods; they were 6.1 and 6.4 × 10^3 dpm in the two experiments shown. The totals in the pellets varied from 7.4 × 10^3 dpm at 212 μg/ml, to 12.5 × 10^3 dpm at 6.8 μg/ml, so that the points of this figure corresponding to high concentrations of CAM (low specific radioactivities) are less reliable than those for low concentrations.

amino acid attached to tRNA. The effects of CAM (10 μg/ml) were not exactly the same with all five amino acids, but no remarkable sensitivity or insensitivity to the CAM inhibition was apparent.

The DEAE-cellulose fractionation demonstrates the qualitative identity of the proteins formed in the absence and presence of CAM (10 μg/ml) (Fig. 8). There was preferential synthesis of the early peaks (more basic proteins), as evidenced by the higher ^{14}C:^{3}H ratios, but this is typical of protein synthesis at 0° (11); CAM did not inhibit the synthesis of any particular type of protein preferentially.

Figure 9 shows that the earliest inhibitory action of CAM (at 2 min) affected both nascent and finished proteins simultaneously. The radioactivity in the nascent proteins ultimately reached a plateau in the control experiment (Fig. 10A), in about one assembly time at 0°. CAM (10 μg/ml) only slowed the building of the nascent protein chains, but the eventual total radioactivity was almost the same as in the

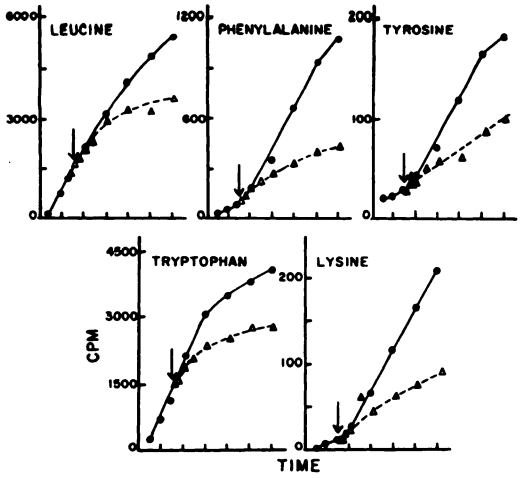

FIG. 7. *Effect of CAM (10 μg/ml) on the incorporation of leucine, phenylalanine, tyrosine, tryptophan, and lysine into proteins of wild-type E. coli K12 at 0°*

CAM (10 μg/ml) was added 15 min after the radioactive amino acid at the points indicated by the arrows. The solid lines represent the control and the broken lines represent incorporation in the presence of CAM. The ordinates represent cpm (note different scales), and each division on the abscissa is 10 min of incubation at 0°.

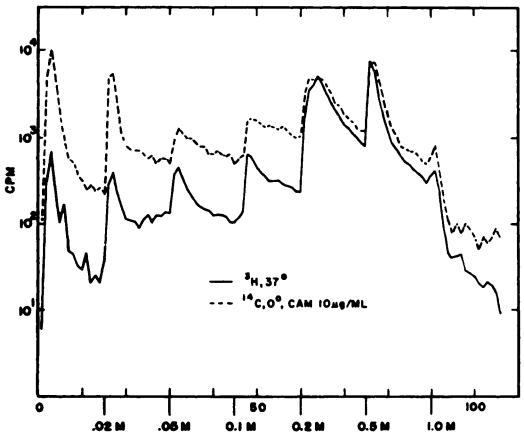

FIG. 8. *DEAE-cellulose column fractionation of proteins*

Abscissa shows fraction numbers and NaCl concentrations. Cells were prelabeled with ^{3}H-leucine at 37°, then incorporated ^{14}C-leucine at 0° for 1 hr in the presence of CAM (10 μg/ml). Each fraction was 5 ml. The first 15 fractions were precipitated and extracted with hot 5% TCA. One-milliliter aliquots of the other fractions were counted directly (^{14}C and ^{3}H) in the Packard liquid-scintillation spectrometer.

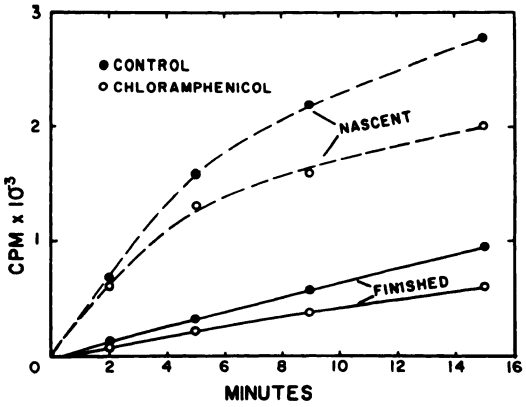

FIG. 9. *Early effect of CAM (10 μg/ml) on the incorporation of leucine into nascent and finished proteins of E. coli at 0°*

CAM was added together with ^{14}C-leucine at time zero. Nascent protein represents counts in the ribosome pellet at 10^{-2} M Mg^{++}, finished protein represents supernatant counts, as described under Materials and Methods.

control. At 100 μg/ml, however, the incorporation of leucine into the nascent proteins was inhibited quickly and completely (Fig. 10B).

Incorporation of leucine into NH_2-terminal positions was inhibited by CAM (Fig. 11A) but the ratios of NH_2-terminal to total incorporation remained unchanged (Fig. 11B), i.e., chain initiation was not preferentially blocked.

Preincubation of cells at 0° for 20 min with CAM (10 μg/ml) had no effect on the attachment of newly synthesized labeled mRNA strands to ribosomes. Increasing the concentration of CAM 10-fold or prolonging the preincubation to 90 min was equally ineffective. Nor could the drug displace mRNA already attached to ribosomes (Table 1). Experiment C of Table 1 was repeated, but the cell lysate was placed directly on a sucrose density gradient (5–20%) at 10^{-2} M Mg^{++} and centrifuged 2 hr at 39,000 rpm in the SW39 rotor. Under these conditions practically all ribosomes appear in 70S (monomer) and 100S (dimer) peaks; polysomes are evidently degraded. CAM (100 μg/ml) had no effect upon the association of the radioactive mRNA strands with the ribosomal peaks

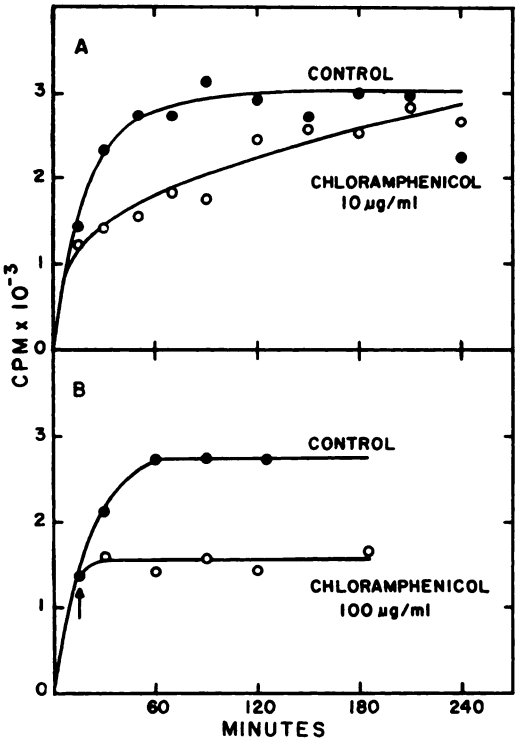

FIG. 10. *Long-term effect of CAM on the incorporation of leucine into nascent proteins of E. coli at 0°*

For the operational definition of nascent proteins, see Materials and Methods. (A) CAM (10 µg/ml) was added together with ^{14}C-leucine at time zero. (B) CAM (100 µg/ml) was added 15 min after ^{14}C-leucine at the point indicated by the arrow.

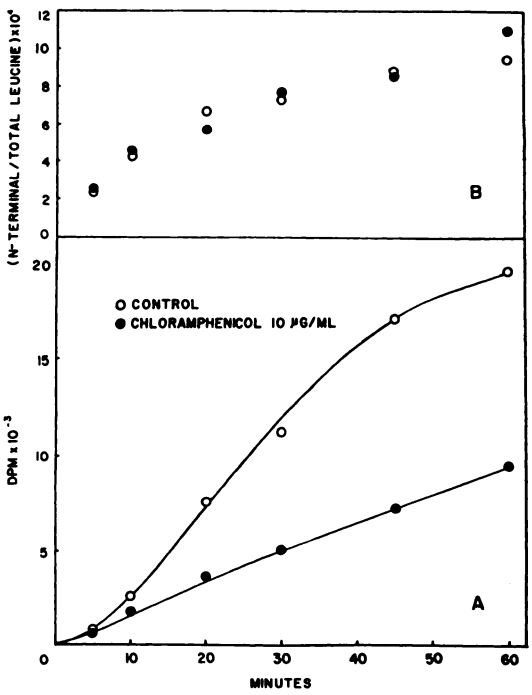

FIG. 11. *Effect of CAM (10 µg/ml) on the incorporation of leucine into NH_2-terminal positions of E. coli proteins at 0°*

CAM was added together with ^{14}C-leucine at time zero. Samples were subjected to treatment with fluorodinitrobenzene as described under Materials and Methods. (A) Kinetics of the incorporation of leucine into NH_2-terminal positions in the presence and absence of CAM (10 µg/ml). (B) Ratios of NH_2-terminal to total incorporation.

as compared with a control, nor was there any abnormality of the O.D. pattern.

CAM (Fig. 1) has a close structural similarity to an aromatic amino acid. Structure-activity studies (21) have shown that the configuration about the carbon atom adjacent to the primary alcohol group must be the same as in the L-amino acids, and that a blocked amino group is absolutely essential. We hypothesized that CAM itself might be inactive until sensitive cells oxidized its primary alcohol group to —COOH, thereby converting it to an amino acid derivative. This would become attached to one of the kinds of tRNA and thus enter the ribosomes. Blockade of chain growth of proteins might then result from inability of the blocked amino group to form a peptide bond with the previously added amino acid. Alternatively, if the blocked amino group were deacylated within the cells, CAM or a metabolic derivative might somehow disturb the further growth of protein chains by being incorporated into them. The outcome of experiments designed to test key aspects of this hypothesis permitted its decisive rejection.

(1) *Cells do not oxidize CAM.* In the Warburg manometer with 18 mg dry weight of cells per vessel, KOH in center well, air atmosphere, 37°, the endogenous O_2 consumption was 0.078 µmole/mg·hr. The addition of CAM at 450 µg/ml led to no dis-

TABLE 1

Effect of CAM on attachment of ribosomes to newly synthesized regions of mRNA

Cells were grown at 37° and harvested in exponential growth (0.15 mg/ml). In *experiment A* the cells were resuspended in a complete medium and preincubated at 0° for 20 min with and without CAM (10 μg/ml). ^{14}C-Urudine was then added and the incubation was continued for another 10 min at 0°. Cells were lysed by sonic oscillation in presence of 10^{-2} M Mg^{++}, ribosomes were isolated by differential centrifugation, and radioactivity was determined. In *experiment B* the preincubation was done for 90 min instead of 20 min with and without CAM (10 μg/ml). The concentration of CAM in *experiment C* was 100 μg/ml and the preincubation was for 5 min. In *experiment D* the cells were first incubated for 10 min at 0° with ^{14}C-uridine, filtered, washed and resuspended in a complete medium containing 1000-fold excess of uridine, then reincubated at 0° with and without CAM (10 μg/ml) for 25 min.

Experiment	Treatment besides incubation with ^{14}C-uridine	Radioactivity associated with ribosomes/mg cells (cpm)[a]
A	Preincubated for 20 min	
	—without CAM	1191
	—with CAM (10 μg/ml)	1126
B	Preincubated for 90 min	
	—without CAM	1881
	—with CAM (10 μg/ml)	1704
C	Preincubated for 5 min	
	—without CAM	1461
	—with CAM (100 μg/ml)	1467
D	Postincubated for 25 min	
	—without CAM	1881
	—with CAM (10 μg/ml)	1828

[a] The different experiments were not done with ^{14}C-uridine of the same specific activity, so the cpm between experiments cannot be compared.

cernible increase in O_2 consumption over a period of 2 hr, yet an uptake of less than 0.5 mole O_2 per mole CAM could have been measured easily. Addition of glucose to the same vessels at the end of the experiment produced an 18-fold increase over the endogenous rate.

(2) *CAM does not become attached to tRNA.* ^{14}C-CAM (11 μg/ml, 7 μC/μmole) was incubated in complete medium with a cell suspension at 0° for 30 min. The cells were lysed by sonic oscillation, and tRNA was prepared by the method of Zubay (see Materials and Methods), then dissolved in NaOH and counted with hyamine and scintillation mixture. The 5 cpm above background that were observed signify fewer than 2 molecules of CAM per cell, or a ratio of less than one CAM-tRNA to 1.4×10^5 total tRNA molecules. Neither was any CAM found in the ribosomal (NaCl-insoluble) RNA.

(3) *CAM is not incorporated into nascent protein.* ^{14}C-CAM (100 μg/ml, 3 μC/μmole) was incubated with a cell suspension in complete medium at 0° for 30 min. Mg-acetate and KCl were added, to 0.03 M and 0.17 M, respectively, then the cells were lysed by sonic oscillation. "Debris" was removed at 8000 *g* for 10 min, and a ribosome pellet was prepared as described in Materials and Methods. The ribosomes were resuspended, heated for 30 min at 80–90° in 5% TCA, chilled, collected on a Millipore filter, washed 5 times with TCA, and placed in scintillation fluid for counting. In a "dead-cell" blank worked up in parallel, the radioactive CAM had been added after sonic lysis. We found no detectable incorporation of ^{14}C-CAM into the ribosomes, which carry nascent protein under these conditions (see Materials and Methods). There were 69 cpm in the "dead-cell" blank, 38 cpm in the experimental sample, background was 13 cpm (already subtracted). If one molecule of CAM had been incorporated into each of the estimated 5000 chains of nascent protein, we would have observed 680 cpm in the experimental sample. CAM bound reversibly to the ribosomes would have been removed in the hot acid extraction.

DISCUSSION

Our present understanding of the whole process of protein synthesis is summarized diagrammatically in Fig. 12. Where in this scheme does CAM act?

CAM particles have long been recognized as defective partially formed ribosomes. The drug could conceivably interfere with protein synthesis by causing disintegration

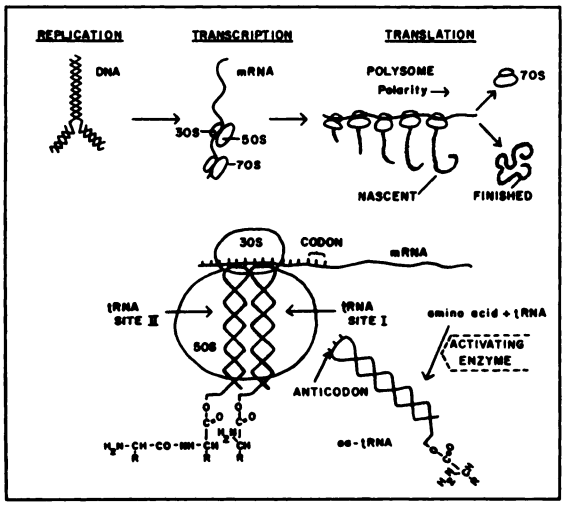

FIG. 12. *The mechanism of protein synthesis, 1965*

The 30 S ribosomal subunit may attach to mRNA initially, followed by the 50 S subunit. The configurations of the tRNA molecules are probably more complex than shown.

of existing ribosomes and defective synthesis of new ones. Such a mechanism has not been thought likely, and our experiments show clearly that the rates of appearance of CAM-particles and of the cessation of protein synthesis are completely unrelated. At 37°, with CAM at 10 μg/ml, the particles make their appearance relatively slowly, while the old ribosomes persist; whereas the blockade of protein synthesis develops within less than a minute. At 0° the inhibition of protein synthesis by CAM at 100 μg/ml is practically complete in a few minutes but no CAM particles can be seen up to at least 15 minutes.

A concentration of CAM (10 μg/ml) well above the bacteriostatic threshold, which blocked protein synthesis very quickly at 37°, acted slowly at 0°. We showed that this slow onset of action was not due to slow penetration of CAM into the cells; on the contrary, the equilibration of cell water was a rapid process, but the increment in internal concentration that is believed to represent binding to the ribosomes developed slowly. The inhibition caused by this same concentration of CAM was total at 37° but only partial at 0°. That some concentration should cause incomplete inhibition is consistent with the demonstrated reversibility of CAM action, but it is not clear why the efficacy should be lower at 0° than at 37°. We have shown, in agreement with others (15, 22, 23), that CAM is bound to ribosomes. It is noteworthy that the binding increased from zero to one molecule of CAM per ribosome in just the concentration range through which the graded inhibition of protein synthesis is demonstrable at 0°: no binding at 1 μg/ml, 0.5 CAM per ribosome at 10 μg/ml, about 1.0 CAM per ribosome at 25–50 μg/ml.

The range of partial inhibition at 0° was exploited to carry out certain experiments not otherwise possible. With five different amino acids (including those closest in structure to CAM) we found that no one of them had its incorporation blocked preferentially.[3] We also observed, by means of DEAE-cellulose fractionation of finished proteins, that the incomplete inhibition reflected a partial effect on the synthesis of most cell proteins, not a preferential blockade of a restricted class of proteins.

We sought evidence that CAM might be oxidized to an amino acid derivative, which would be the active agent in blocking protein synthesis, but we could find none. And we also decisively ruled out the hypothesis that CAM (or a derivative) is incorporated into a tRNA complex (analogous to amino acyl-tRNA) or into nascent protein.

CAM did not preferentially inhibit the initiation of new protein chains, as measured by the entry of amino acids into NH_2-terminal positions. Initiation of new chains, addition of new residues to nascent chains, and release of completed chains from the

[3] The hypothesis under test was that CAM blocked the entry of one kind of amino acid into growing nascent chains. Under conditions of rapid assembly (37°) every nascent chain would be blocked so quickly that a preferential block of this kind could not be distinguished. At 0°, however, we should expect to see continued growth of every chain until the blocked amino acid was required. Thus most amino acids should continue to be incorporated, at a slowly diminishing rate, but the blocked amino acid should not be incorporated at all.

ribosomes were all blocked by CAM in a coordinated fashion. In contrast to the "puromycin effect," in which nascent protein is released from the ribosomes (24, 25), nascent protein chains whose extension had been blocked by CAM remained attached to the ribosomes.

The effect of CAM upon protein synthesis in *E. coli* has nothing to do with the process of attachment of ribosomes to mRNA. If the drug acted to prevent such attachment, then ribosomes already functioning (as polysomes) should complete their nascent protein chains but be prevented from starting new ones. Thus even with high enough CAM concentration to block all attachment immediately, protein synthesis should not stop for about one assembly time, 30 min at 0°. On the contrary, we have shown that at 0° CAM (100 μg/ml) causes complete blockade of protein synthesis in a few minutes. Direct measurement of the association between mRNA and ribosomes in inhibited cells also failed to reveal any effect of CAM under a variety of different conditions.

We shall present evidence elsewhere (manuscript in preparation) that ribosomes attach to nascent mRNA while transcription is still in progress. The addition of new residues at the growing 3′ end (26), fixed to the DNA template and RNA polymerase, results in an outward propagation of the mRNA strand and its free 5′ end. This movement of mRNA consequent to its synthesis could be coordinated with the movement of ribosomes in the direction 5′→3′ relative to the messenger strand during the translation process; certainly the rate of translation of the nascent message must be limited by the rate of mRNA synthesis. The binding of newly incorporated ^{14}C-uridine residues to ribosomes (as measured, for example, in the experiments shown in Table 1) could reflect the movement of new nucleotide residues onto the proximal ribosomes in polysomes already attached to the nascent mRNA, in the manner of a magnetic tape moving through the first of a series of reading heads. According to this interpretation the failure of CAM in our experiments to affect the ribosome-^{14}C-uridine interaction (Table 1) would indicate that although protein synthesis is inhibited, the movement of ribosomes along mRNA strands is not. This may be related to the well-known "uncoupling" by CAM of the dependence of RNA synthesis upon protein synthesis in stringent strains.[4]

Our findings are more decisive in showing how CAM does not act than in elucidating any concrete mechanism of its action. The data are consistent with a reversible binding at a ribosomal site, preventing further growth of nascent protein chains, which remain attached to the ribosomes. Others have shown (6, 8, 15, 27) that CAM does not block the binding of aminoacyl-tRNA to ribosomes. The configurational relationship of the drug to the L-amino acids suggests a competition for some stereospecific locus. Suppose site I (Fig. 12) contained such a locus, in which the incoming amino acid (attached to tRNA) could be held in position for peptide bond formation. If CAM could compete there, then we should expect free amino acids to act similarly. We have found, however, that very high concentrations of free amino acids do not inhibit protein synthesis at 0°; neither do they affect the binding of CAM to ribosomes (unpublished observations). Alternatively, CAM may be regarded as a structural analog of the terminal amino acid of a nascent protein chain, inasmuch as its amino group is combined in amide linkage. Site II might well contain a locus to accommodate the carboxy-terminal amino acid of the nascent protein, especially since the nascent protein-tRNA (in contrast to the aminoacyl-tRNA in site I) is bound especially tightly to the ribosome (27). The essential step in peptide bond formation is evidently a shift of the terminal carbonyl C of the nascent protein chain from tRNA-adenosine in site II to amino N of aminoacyl-tRNA in site I, i.e., a momentary transfer of the nascent chain from site II to site I. The subsequent step (whatever

[4] We are indebted to Dr. Gunther S. Stent for calling attention to these implications of our findings.

its detailed mechanism may be) can be thought of as a "hinge-flexion" of the ribosome, so that the nascent chain with its newly attached tRNA is recaptured by site II. This step entails the translocation of the ribosome by one reading-frame (approximately 10 A) relative to the mRNA. If the translocation process continues in the presence of CAM, while the nascent protein chains remain attached to the ribosomes, it would seem possible that the primary effect of the drug is to prevent site II from accepting the nascent protein-tRNA from site I. It seems fruitless to speculate further until more is known about the mechanism of peptide bond formation and of ribosome movement along the mRNA.

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