Inhibition of the Growth of *Escherichia coli* by Chlortetracycline

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1. This study extends previous work concerned with the ribonucleic acid made by *Escherichia coli* during inhibition of protein synthesis by chlortetracycline. 2. The antibiotic caused an initial stimulation in the rate of RNA synthesis. 3. RNA made during inhibition was stable during continued incubation in the presence of the antibiotic although it was extensively degraded in resting cell suspensions. 4. Most of the RNA accumulated during chlortetracycline action was in particles that sedimented more slowly than ribosomes. During the recovery of cells from the effects of the antibiotic, accumulated RNA was apparently not degraded and ribosomes were synthesized from the RNA in the particles.

In certain circumstances, the synthesis of RNA by bacteria can be dissociated from protein synthesis and cells are produced that contain more than their normal complement of RNA. In *Escherichia coli* this occurs, for example, during inhibition by chloramphenicol (Gale & Folkes, 1953), puromycin (Takeda, Hayashi, Nakagawa & Suzuki, 1960) or streptomycin (Anand & Davis, 1960). It is also found when a transition to a faster growth rate takes place (Kjelgaard, Maaløe & Schaechter, 1958), when protein synthesis is abruptly halted in 'relaxed' mutants (Borek, Ryan & Rockenbach, 1955) and also during K⁺ ion starvation of a mutant with a defective mechanism for the transport of these ions (Lubin & Ennis, 1961).

In such cases most of the RNA that accumulates is similar to ribosomal RNA in its sedimentation properties but is present in particles that contain only a small amount of protein and sediment more slowly than 30s ribosomes. These particles also differ from ribosomes in their sensitivity to ribonuclease and sonic vibrations and in their behaviour during chromatography on DEAE-cellulose (Nomura & Watson, 1959; Dagley, Turnock & Wild, 1963; Sells, 1964; Dubin, 1964; Ennis & Lubin, 1965a).

Previously we studied some events that occur during the inhibition of protein synthesis in *E. coli* by antibiotics of the tetracycline group. The five that have been examined (tetracycline, chlortetracycline, oxytetracycline, 5-hydroxy-6-methylene-tetracycline and demethylchlortetracycline) permit the continued synthesis of RNA by inhibited cells, whose RNA content may nearly double in the virtual absence of protein synthesis; after inhibition, particles sedimenting more slowly than

30s ribosomes are visible in schlieren diagrams of cell-free extracts (Holmes & Wild, 1966). A closer examination of events during inhibition by chlortetracycline (Holmes & Wild, 1965) has shown that although about 25% of the RNA made during inhibition has the sedimentation characteristics of transfer RNA, the remainder is in the particles ('chlortetracycline particles') that are visible in the ultracentrifuge diagrams and whose properties are similar to those of the particles that arise in the other cases mentioned above.

In this paper are presented some further properties of the RNA accumulated during inhibition by chlortetracycline, together with studies on the fate of the chlortetracycline particles in cells that are recovering from the effects of the antibiotic. The results confirm previous suggestions (Holmes & Wild, 1965) of the essential similarity of events that take place during inhibition by chlortetracycline to those found in the other situations in which bacteria are produced that contain a considerable excess of RNA.

EXPERIMENTAL

Organism and growth conditions. Exponentially growing cells of *E. coli* strain MRE 600 were used. This organism, which lacks ribonuclease (Cammack & Wade, 1965), had a mean generation time of about 60 min. during growth at 30° with aeration in a medium adjusted to pH 7.0 with NaOH and containing, per l.: KH₂PO₄, 9g.; (NH₄)₂SO₄, 1g.; MgSO₄·7H₂O, 1.6g.; glucose, 10g. Extinctions of cultures were measured at 540mμ with cuvettes of 1cm. light-path.

RNA and protein synthesis. Chemical determinations of the RNA and protein contents of whole cells were carried out on culture samples (5ml.) as described by Fraenkel &

Neidhardt (1961), by using orcinol and Folin methods for RNA and protein respectively. RNA and protein synthesis was also followed by the inclusion of $[2-^{14}\text{C}]\text{uracil}$ or $\text{DL-}[^{14}\text{C}]\text{leucine}$ in the medium at concentrations and specific activities given in association with the experiments in question. At intervals, samples (1 ml.) were pipetted into 1 ml. of 10% (w/v) trichloroacetic acid at 0° for the assay of radioactivity as described below.

Other methods. Extracts of bacteria were prepared by disrupting the cells in a Hughes (1951) press cooled to about -15° and centrifuging the extracts at $15000g$ for 60 min. at 5° . For examination in the analytical ultracentrifuge (at room temperature), or for sucrose-density-gradient analysis, extracts were made in 15 mM- KH_2PO_4 , pH 7.0, containing 0.1 mM-magnesium acetate. For chromatography on DEAE-cellulose, the buffer used to extract the cell contents was 10 mM-tris chloride, pH 7.2, containing 20 mM- MgCl_2 . Deoxyribonuclease (5 $\mu\text{g./ml.}$) was included in both buffers.

Methods of sucrose-density-gradient analysis and chromatography on DEAE-cellulose were as previously described (Holmes & Wild, 1965). Radioactivity measurements were made on material insoluble in 5% (w/v) trichloroacetic acid at 0° . Such precipitates (derived either from whole cells or from fractions after chromatography and sedimentation analysis), were collected on Oxoid (Oxo Ltd., London, E.C.4) membrane filters that were gummed to aluminium planchets and counted for a minimum of 1000 counts with a thin-window gas-flow counter (Nuclear-Chicago Corp., Des Plaines, Ill., U.S.A.).

RESULTS

Effect of chlortetracycline on RNA and protein synthesis. RNA continues to be made in the virtual absence of protein synthesis by cells of *E. coli* K 12 that are inhibited in a peptone medium by 15 $\mu\text{g.}$ of chlortetracycline/ml. (Holmes & Wild, 1965). Strain MRE 600 (the organism used in the present work) was more sensitive than K 12 to the effects of chlortetracycline but, as shown in Fig. 1, the antibiotic caused preferential synthesis of RNA over the concentration range tested (0.05–10 $\mu\text{g./ml.}$) during inhibition for 90 min. At concentrations of 0.3 $\mu\text{g./ml.}$ and above, the synthesis of RNA was appreciably inhibited but much less than that of protein; at low concentrations of chlortetracycline (0.05 and 0.1 $\mu\text{g./ml.}$), although protein synthesis was decreased, the amount of RNA made was rather greater than in control cultures. Such a stimulation of RNA synthesis was also observed at the higher concentrations when samples were taken 10 min. after the addition of the antibiotic; the incorporation of $[^{14}\text{C}]\text{uracil}$ was then 30–40% greater than in the corresponding control culture. However, this increased rate of synthesis did not persist and a net inhibition was recorded in samples taken at 30 min.

Fig. 2 shows that the RNA that is made by cells inhibited by 1 $\mu\text{g.}$ of chlortetracycline/ml. is apparently largely stable throughout continued

Fig. 1. Inhibition of RNA and protein synthesis by chlortetracycline. Uracil (15 $\mu\text{g./ml.}$) and DL-leucine (100 $\mu\text{g./ml.}$) were added to portions (50 ml.) of exponentially growing cultures ($E_{540\text{ m}\mu}$ 0.1–0.15) and synthesis of RNA and protein followed separately by the addition of 20 $\mu\text{m}\mu\text{C}$ of $[^{14}\text{C}]\text{uracil/ml.}$ or 40 $\mu\text{m}\mu\text{C}$ of $[^{14}\text{C}]\text{leucine/ml.}$ in the presence and absence of the antibiotic. Inhibition was for 90 min. The incorporation of radioactivity (counts/min./ml.) by inhibited cultures into material insoluble at 0° in 5% trichloroacetic acid is expressed as a percentage of the incorporation in the corresponding control culture. Shaded areas: protein synthesis; unshaded areas: RNA synthesis.

incubation in growth medium containing the antibiotic. For this experiment, $[^{14}\text{C}]\text{uracil}$ was included in the medium and at intervals during the inhibition a 20-fold excess of non-radioactive uracil was added to portions of the culture to stop the further formation of radioactive RNA. Samples were taken subsequently for the measurement of trichloroacetic acid-insoluble radioactivity. The results show that the RNA accumulated for 30 or 60 min. in the presence of chlortetracycline is almost completely stable during the further incubation period; RNA made during exposure for 90 min. is somewhat degraded but the loss of radioactivity then amounts to only about 5%/hr.

However, although this experiment gives little evidence for turnover of the RNA made during chlortetracycline action, such RNA is broken down extensively in resting cell suspensions. For the experiment of Fig. 3, cells were incubated with chlortetracycline and $[^{14}\text{C}]\text{uracil}$, then harvested, washed once and resuspended in a complete medium without antibiotic but containing excess of uracil. Portions of this culture were removed at intervals and the cells incubated in medium containing excess of uracil but lacking glucose.

The results (Fig. 3) show the trichloroacetic acid-insoluble radioactivities of samples taken from the resting cell suspensions. The RNA made during

Fig. 2. Stability of the RNA synthesized during inhibition by chlortetracycline. The incorporation of radioactivity into material insoluble in 5% trichloroacetic acid was followed in portions (50 ml.) of a culture in the presence of [14 C]uracil (15 m μ C/ml. and 5 μ g./ml.). After incubation for 30 min. (■), 60 min. (○) and 90 min. (●), excess of uracil (100 μ g./ml.) was added to a portion and measurements of radioactivity were continued.

inhibition was appreciably unstable and the portion of the culture harvested at zero time lost more than 50% of its radioactivity in 150 min. However, during suspension in the complete drug-free medium, the extinction of the culture increased slightly and this recovery was associated with an increasing stability of the RNA made during inhibition; after recovery for 60 and 120 min., 40 and 15% respectively of the radioactivity was lost during the subsequent incubation in glucose-free medium. Thus as cells recover from inhibition by chlortetracycline the accumulated RNA is converted into a more stable form. The experiments below indicate what is involved in this conversion.

Events during recovery from inhibition by chlortetracycline. For a more detailed examination of events during recovery from inhibition, cells were incubated for 90 min. with 0.5 μ g. of chlortetracycline/ml. This concentration of antibiotic was chosen because after exposure to higher concentrations, cells recovered with difficulty, if at all, from the effects of the drug; at concentrations less than 0.5 μ g./ml. the increasing extent of protein synthesis during inhibition (Fig. 2) resulted in appreciable incorporation of accumulated RNA into completed ribosomes. The rate of recovery was rather variable after incubation with 0.5 μ g. of antibiotic/ml. and

Fig. 3. Stability in medium lacking glucose of the RNA made during inhibition by chlortetracycline. Cells were incubated for 90 min. with chlortetracycline (1 μ g./ml.) and [14 C]uracil (20 m μ C/ml. and 7.5 μ g./ml.), harvested, washed once and resuspended in the same volume of drug-free medium containing excess of uracil (100 μ g./ml.). Immediately after resuspension, a portion (50 ml.) of the culture was removed, and the cells were washed and resuspended in the mineral salts medium containing excess of uracil (100 μ g./ml.) but lacking glucose (■). Further portions were removed after recovery for 60 min. (○) and 120 min. (●) and treated similarly. Trichloroacetic acid (5%) -insoluble radioactive material was assayed on samples of the cultures in the media without glucose; results are expressed as percentages of the values found at the beginning of the suspension in this solution.

the time-scale of events varied somewhat in different experiments; however, the nature and extent of the accumulation of RNA during inhibition were comparable with those recorded previously (Holmes & Wild, 1965) when higher concentrations of chlortetracycline were used.

(a) RNA and protein synthesis. Fig. 4 shows the synthesis of RNA and protein and changes in extinction during the recovery of cells from inhibition by 0.5 μ g. of chlortetracycline/ml. Inhibited cells were washed once and then resuspended in a volume of drug-free medium equal to that used for the inhibition. After 2 hr., protein and RNA had increased by 69% and 40% respectively, although during the first hour the increases in RNA were rather the greater. The analyses for RNA suggest that there was a slight immediate synthesis and then a lag before more RNA was made; similar

Fig. 4. Growth and synthesis of RNA and protein during recovery from chlortetracycline action. Cells were inhibited for 90 min. with $0.5 \mu\text{g.}$ of chlortetracycline/ml., harvested, washed once and resuspended in the same volume of drug-free medium. (a) Extinction at $540 \text{ m}\mu$ (■) of the culture during recovery. (b) Samples (5 ml.) were taken for chemical analyses of RNA (○) and protein (●). Results are expressed as percentage increases of the values at the beginning of the resuspension.

kinetics were detected in measurements of $[^{14}\text{C}]$ uracil incorporation during recovery.

These rather similar increases in RNA and protein are surprising in that a much more pronounced preferential synthesis of protein accompanies the recovery of cells that have, in other circumstances, accumulated an excess of RNA

(Sells, 1964; Turnock & Wild, 1965; Ennis & Lubin, 1965b); we have also found a considerable over-synthesis of protein to occur during the recovery of strain MRE 600 from inhibition by chloramphenicol. The response observed during recovery from chlortetracycline action could be due to retention of the antibiotic by the washed cells, although synthesis of RNA and protein and the rate of increase in extinction of a culture during incubation in chlortetracycline-free medium were found not to be altered by more extensive washing procedures.

(b) Synthesis of ribosomes from RNA made during inhibition. For the experiments below, the RNA made by cells incubated for 90 min. with $0.5 \mu\text{g.}$ of chlortetracycline/ml. was labelled by the inclusion of $[^{14}\text{C}]$ uracil in the medium. The cells were harvested, washed once and allowed to recover in drug-free medium containing excess of non-radioactive uracil. Samples were taken at intervals for the determination of trichloroacetic acid-insoluble radioactivity and showed that, in the different experiments, RNA made during inhibition was almost completely stable as growth resumed. Losses of radioactivity up to a maximum of only 5% were recorded in the first 2 hr. of recovery.

Fig. 5 shows schlieren diagrams for extracts (in phosphate buffer containing 0.1 mM -magnesium acetate) obtained from cells at intervals during recovery. At zero time (Fig. 5a) the diagram is typical of that for cells that have accumulated RNA during inhibition by tetracyclines and shows two boundaries additional to those caused by the 50s and 30s ribosomes and 'soluble' cell contents. These new boundaries are caused by the chlortetracycline particles that accumulate during inhibition. After recovery for 25 min. there is little change in the pattern (Fig. 5b), although the particles are rather less well resolved from the 30s ribosomes. In

Fig. 5. Schlieren diagrams after centrifuging cell-free extracts of strain MRE 600 for 16 min. at $187000g$. Cells were inhibited for 90 min. in medium containing $20 \mu\text{g.}$ of uracil/ml. and $0.5 \mu\text{g.}$ of chlortetracycline/ml., harvested, washed and resuspended in the same volume of drug-free medium containing excess of uracil ($100 \mu\text{g./ml.}$). Extracts were prepared from cells after recovery for (a) 0 min., (b) 30 min., (c) 75 min., (d) 120 min. Sedimentation is to the left. The bar angle was 40° . The protein concentrations of the extracts were adjusted to 1% before centrifuging.

samples taken after recovery for 75 min. (Fig. 5c) and 120 min. (Fig. 5d), the number of particles is progressively less and there is an increase in the area of the ribosome boundaries. Thus the chlortetracycline particles largely disappear during recovery and the schlieren diagrams are consistent with their conversion into ribosomes or ribosome-like particles.

The results of sucrose-density-gradient analyses and chromatography on DEAE-cellulose confirm this conclusion. Fig. 6(a) shows an analysis on a sucrose gradient of an extract prepared from inhibited cells immediately after their resuspension in fresh medium. Most of the radioactivity is associated with material that has sedimented rather less than the 30s ribosomes and corresponds to the particles observed in the schlieren diagrams; a second radioactive component that has sedimented little during centrifugation is probably transfer RNA, and the radioactivity profile in fractions containing 50s ribosomes suggests that some completed ribosomes were made during inhibition by the low concentration of antibiotic.

After recovery for 2 hr., a similar analysis (Fig. 6b) shows a nearly complete correspondence between the extinction and radioactivity profiles in the regions of both 50s and 30s ribosomes. This corresponds to the synthesis of ribosomes from particles. Analytical ultracentrifuging of this extract showed an almost complete absence of particles from the schlieren diagrams.

The synthesis, during recovery, of ribosomes from particles is also suggested by the results of DEAE-cellulose chromatography given in Fig. 7. For this experiment, inhibited cells were washed and resuspended in drug-free medium; extracts were made in tris buffer containing 20 mM-magnesium chloride and a ribosome plus particle fraction was collected by centrifugation. Fig. 7(a) shows the results of chromatography of a sample prepared from cells immediately after their resuspension in the new medium; as has previously been noted (Holmes & Wild, 1965), the chlortetracycline particles are eluted at a higher molarity of sodium chloride than the ribosomes; these components are thus well resolved. However, fractions containing the ribosomes are appreciably radioactive, so that, as in the previous experiment, there was some synthesis of completed ribosomes during the initial inhibition with chlortetracycline. After recovery for 90 min. (Fig. 7b), the particles accumulated during inhibition have been lost and the radioactivity and extinction profiles are very similar.

DISCUSSION

The results presented in this paper, and previously (Holmes & Wild, 1965), stress the marked similarity

Fig. 6. Sucrose-density-gradient analyses of extracts made from cells during recovery from inhibition by chlortetracycline. Cells were inhibited for 90 min. in medium containing [^{14}C]uracil (13 mμC/ml. and 20 μg./ml.) and chlortetracycline (0.5 μg./ml.), harvested, washed and resuspended in the same volume of drug-free medium containing excess of uracil (100 μg./ml.). Extracts prepared from cells after recovery for (a) 0 min. and (b) 120 min. were centrifuged in the SW 25.1 rotor of the model L ultracentrifuge for 16.5 hr. at 33000g. After centrifugation, 10-drop fractions were collected into 2.5 ml. of buffer (15 mM- KH_2PO_4 , pH 7.0, containing 0.1 mM-magnesium acetate) for measurement of extinction at 260 mμ (○) and radioactive material insoluble in 5% trichloroacetic acid (●).

of events taking place in cells inhibited by chlortetracycline to those occurring in other circumstances that involve the preferential synthesis of

Fig. 7. Chromatography on DEAE-cellulose of a fraction containing ribosomes and particles prepared from cells during recovery from chlortetracycline action. Cells were inhibited for 90 min. in medium containing [^{14}C]uracil (13 $\mu\text{C}/\text{ml}$. and 20 $\mu\text{g}/\text{ml}$.) and chlortetracycline (0.5 $\mu\text{g}/\text{ml}$.), harvested, washed and resuspended in the same volume of drug-free medium containing excess of uracil (100 $\mu\text{g}/\text{ml}$.). Extracts were prepared from cells after recovery for (a) 0 min. and (b) 90 min. and a ribosome plus particle fraction obtained by centrifugation of the extracts at 102000g for 4 hr. A portion of the preparation was loaded on the column and eluted with a concentration gradient of NaCl (0–1.0M) in 200 ml. of tris chloride buffer (pH 7.2) containing 20 mM-MgCl₂. Measurements of extinction at 260 m μ (○) and 5%-trichloroacetic acid-insoluble radioactive material (●) were made on fractions of volume about 2.6 ml.

containing the antibiotic but is more rapidly lost from resting cell suspensions (Horowitz, Lombard & Chargaff, 1958); a similar instability in buffer solutions has been found for the RNA accumulated during starvation of a relaxed mutant (Nakada, Anderson & Magasanik, 1964) and also for that made by a transport-defective mutant in a medium lacking K⁺ ions (Ennis & Lubin, 1965a). Similarly, a stimulation of RNA synthesis in the early phases of RNA accumulation occurs during inhibition by chloramphenicol (Kurland & Maaløe, 1962), or streptomycin (Dubin, 1964). Such an increased rate of synthesis indicates that in exponentially growing cells, RNA is made at less than the maximum rate of which the cells are capable; possible reasons for the temporary capacity for faster synthesis have been advanced (Kurland & Maaløe, 1962; Dubin, 1964).

Most of the RNA made during inhibition of *E. coli* by chlortetracycline is similar to ribosomal RNA and is contained in particles that resemble those that accumulate when preferential synthesis of RNA occurs in other situations (Holmes & Wild, 1965). The fate of these particles during a subsequent return to more normal conditions has been investigated in some detail. During recovery from inhibition by chloramphenicol or puromycin, or when protein synthesis resumes in starved cells of a relaxed mutant, cells preferentially make ribosomal protein and ribosomes are formed from the RNA in the particles, which disappear from schlieren diagrams (Aronson & Spiegelman, 1961; Dagley, White, Wild & Sykes, 1962; Sells, 1964; Turnock & Wild, 1965). Our experiments show that chlortetracycline particles are in this same category but do not prove the direct synthesis of ribosomes from particles. Although the stability of chloramphenicol particles during the accumulation of RNA may be more apparent than real (Dubin & Elkort, 1965), there is evidence for the conversion of chloramphenicol particles, puromycin particles and relaxed particles into mature ribosomes without degradation (Nakada *et al.* 1964; Nomura & Hosokawa, 1965; Horowitz & Hills, 1966).

The RNA content of a growing bacterial cell is a direct function of its growth rate and thus of the overall rate of protein synthesis. Although amino acid activation is a requisite for the synthesis of RNA (Fangman & Neidhardt, 1964; Eidlic & Neidhardt, 1965), it is not yet clear how this synthesis is regulated nor are reasons apparent for the breakdown in the regulation represented by the continued synthesis of RNA during the inhibition of protein synthesis by antibiotics such as chlortetracycline, chloramphenicol, puromycin and streptomycin. However, these drugs are thought to interfere with the assembling of components for protein synthesis on ribosomes (Yarmolinsky &

RNA by bacteria. Thus RNA made in the absence of protein synthesis during inhibition by chloramphenicol is only slowly degraded in growth medium

de la Haba, 1959; Nathans & Lipmann, 1961; Speyer, Lengyel & Basilio, 1962; Hierowski, 1965); they therefore block the manufacture of proteins at stages after the formation of aminoacyl-transfer RNA complexes. One hypothesis is that RNA synthesis is repressed and thus controlled by transfer RNA uncombined with amino acids (Kurland & Maaløe, 1962); the concentration of this species might not alter immediately in response to inhibition by the antibiotics. However, this suggestion may need modification in view of an apparent lack of correlation between the rate of RNA synthesis and the concentration of free transfer RNA (Morris & DeMoss, 1965). An alternative view (Morris & DeMoss, 1965) is that RNA synthesis is de-repressed in the presence of an 'active' protein-synthesizing unit (composed of ribosomes, messenger RNA, aminoacyl-transfer RNA, Mg^{2+} ions and polymerizing enzymes); this unit is maintained in the correct configuration for RNA synthesis when protein synthesis is inhibited by the antibiotics.

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