The Control of Ribonucleic Acid Synthesis in Bacteria

THE SYNTHESIS AND STABILITY OF RIBONUCLEIC ACID IN CHLORAMPHENICOL-INHIBITED CULTURES OF *ESCHERICHIA COLI*

By J. E. M. MIDGLEY AND W. J. H. GRAY

*Department of Biochemistry, University of Newcastle upon Tyne,
Newcastle upon Tyne NE1 7RU, U.K.*

(Received 7 September 1970)

The rate of polymerization of ribosomal ribonucleic acid chains was estimated for steadily growing cultures of *Escherichia coli* M.R.E.600, from the kinetics of incorporation of exogenous [5-³H]uracil into completed 23S rRNA molecules. The analytical method of Avery & Midgley (1971) was used. Measurements were made at 37°C, in the presence or the absence of chloramphenicol, in each of three media; enriched broth, glucose-salts or sodium lactate-salts. The rate of chain elongation of 23S rRNA was virtually constant in all media at 37°C, as 24 ± 4 nucleotides added/s. Accelerations in the rate of biosynthesis of rRNA by chloramphenicol in growth-limiting media are due primarily to an increase in the rate of initiation of new RNA chains, up to the rates existing in cultures growing rapidly in broth. Thus, in poorer media, only a small fraction of the available DNA-dependent RNA polymerase molecules are active at any given instant, since the chain-initiation rate is limiting in these conditions. In cultures growing rapidly in enriched broth, antibiotic inhibition caused a rise of some 12% in the rate of incorporation of exogenous uracil into total RNA. This small acceleration was due entirely to the partial stabilization of the mRNA fraction, which accumulated as 14% of the RNA formed after the addition of chloramphenicol. In cultures growing more slowly in glucose-salts or lactate-salts media, chloramphenicol caused an immediate acceleration of two- to three-fold in the overall rate of RNA synthesis. Studies by DNA-RNA hybridization showed that the synthesis of mRNA was accelerated in harmony with the other affected species. However, just over half the mRNA formed after the addition of chloramphenicol quickly decayed to acid-soluble products, whereas the remainder was more stable and accumulated in the cells. The mRNA fraction constituted about 6% of the total cellular RNA after 3h inhibition. A model was suggested to explain the partial stabilization and accumulation of the mRNA fraction and the acceleration in the rate of synthesis of mRNA when chloramphenicol was added to cultures in growth-limiting media.

When bacterial cultures are inhibited by chloramphenicol, the cells accumulate considerable quantities of RNA (Pardee, Paigen & Prestidge, 1957; Nomura & Watson, 1959; Kurland & Maaloe, 1962). Examination of the detailed kinetics of RNA synthesis in inhibited cells indicates significant changes in the gross rates of formation of nucleic acids. In particular, the rate of RNA synthesis is almost instantaneously increased when the antibiotic is added to cultures growing slowly in simple defined media such as glucose-salts (Fraenkel & Neidhardt, 1961; Kurland & Maaloe, 1962; Maaloe & Kjeldgaard, 1966). However, in cultures growing at optimum rates in enriched broth, or in amino acid-enriched media, such accelerations are barely detectable (Fraenkel & Neidhardt, 1961;

Kurland & Maaloe, 1962). Although there has been no detailed examination of the possibilities, it has been assumed that accelerations in RNA synthesis primarily concern stable RNA (tRNA+rRNA) (Kurland & Maaloe, 1962). The position is complicated by the fact that the mRNA fraction is at least partly stabilized by chloramphenicol and accumulates in the inhibited cells (Midgley & McCarthy, 1962; Midgley, 1963; Willson & Gros, 1964; Nakada, 1965; Friesen, 1966; Gurgo, Apirion & Schlessinger, 1969).

It is thus of interest to consider whether the rate of synthesis of the mRNA fraction is affected by chloramphenicol in the same way as that of stable RNA. Opinion seems as yet to be divided between those who have suggested that the synthesis of

these RNA species is under separate control (e.g. Morris & DeMoss, 1966; Edlin, Stent, Baker & Yanofsky, 1968; Lavallo & de Hauwer, 1968; Morris & Kjeldgaard, 1968; Venetianer, 1969) and those who consider that mRNA and rRNA biosynthesis is in some way co-ordinated (e.g. Friesen, 1966).

Gurgo *et al.* (1969) have shown that, in cultures of *Escherichia coli* inhibited by high concentrations of chloramphenicol, newly formed mRNA continued to enter the polyribosomal fractions of the cells at a rate comparable with that found in normally growing bacteria. To account for their observations, Gurgo *et al.* (1969) suggested that the ribosomes continued to bind with and translocate the mRNA strands during inhibition by chloramphenicol, even though translation of the mRNA and protein biosynthesis was prevented. This concept was in contrast with the suggestions of Das, Goldstein & Kanner (1966) that chloramphenicol inhibited the movement of ribosomes in the polyribosomal complexes. The drug also appears to stabilize the polyribosomal complexes *in vitro*, as Kaempfer (1968) showed that it caused a greatly diminished release and rate of recycling of ribosomal subunits. However, Midgley & McCarthy (1962) showed that, when the drug was injected into growing cultures and [^{32}P]phosphate was added, the apparent nucleotide base composition of the newly formed RNA altered with time from that typical of a mixture of mRNA and rRNA precursor (1:2 weight ratio) to a final composition more like that of the total cellular RNA. This showed that at least some part of the mRNA was still unstable in chloramphenicol-inhibited cells.

In this paper, we have examined the kinetics of synthesis and breakdown of mRNA in chloramphenicol-inhibited cultures of *E. coli* by using the technique of DNA-RNA hybridization. In addition, we have further considered the basic regulatory mechanisms for rRNA and mRNA synthesis by measuring the rate of RNA chain polymerization in various media in the presence or absence of chloramphenicol. The overall rate of RNA biosynthesis depends on the product of two sequential processes, the rate of formation of the initiation complex between DNA-dependent RNA polymerase molecules and the initiator region of the cistrons, and the subsequent rate of polymerization of the growing RNA chains (Maaloe & Kjeldgaard, 1966). Several estimations of the rate of RNA-chain polymerization have been made (Zimmermann & Levinthal, 1967; Bremer & Yuan, 1968*a,b*; Mangiarotti, Apirion, Schlessinger & Silengo, 1968; Manor, Goodman & Stent, 1969; Winslow & Lazzarini, 1969*a,b*). Our chosen method of analysis was that originally devised by Mangiarotti *et al.* (1968), as modified by Avery & Midgley (1971).

Preliminary accounts of this work have been given (Gray, 1970; Midgley, 1970).

MATERIALS AND METHODS

Organism. *Escherichia coli* M.R.E.600 was used.

Bacterial growth media. The three media used were as described by Gray & Midgley (1970). Growing cultures were inhibited by 0.2g of chloramphenicol/litre, the antibiotic being injected in ethanolic solution.

Preparation of RNA and DNA-RNA hybridization. The labelling of cultures by exogenous additives, the harvesting of cultures, disruption of cell pellets and the purification of RNA was as described by Pigott & Midgley (1968). The technique of DNA-RNA hybridization was essentially that of Gillespie & Spiegelman (1965). tRNA and rRNA were fractionated by the method of Gray & Midgley (1970).

Sucrose-density-gradient sedimentation. The method was that of Avery & Midgley (1969).

Radiochemicals. [$2\text{-}^{14}\text{C}$]Uracil (specific radioactivity 54.5 mCi/mmol), [$5\text{-}^3\text{H}$]uracil (specific radioactivity 1 Ci/mmol) and L-[$1\text{-}^{14}\text{C}$]leucine (specific radioactivity 62 mCi/mmol) were obtained from The Radiochemical Centre, Amersham, Bucks., U.K.

Antibiotics. Chloramphenicol was obtained from Sigma (London) Chemical Co. Ltd., London S.W.6, U.K. Rifampicin was a kind gift from Lepetit Pharmaceuticals Ltd., Maidenhead, Berks., U.K.

RESULTS

Inhibition of protein synthesis by chloramphenicol. In our studies on RNA synthesis in *E. coli* cultures inhibited by chloramphenicol, we used a concentration of the antibiotic that was sufficient to prevent protein synthesis completely *in vivo*. This was

Fig. 1. Incorporation of L-[^{14}C]leucine into protein in cultures of *E. coli* M.R.E.600 inhibited by chloramphenicol. For details see the text. Chloramphenicol added/ml of culture: ○, None; ▲, 1 $\mu\text{g/ml}$; △, 5 $\mu\text{g/ml}$; ■, 10 $\mu\text{g/ml}$; ●, 50 $\mu\text{g/ml}$.

necessary to avoid the argument that changes in the rates of RNA synthesis brought about by the drug were due to a residual synthesis of proteins that might be important in controlling this process. Growing cultures of *E. coli* M.R.E.600 were incubated in the presence of various concentrations of the drug, in each of the three growth media used. Fig. 1 shows the kinetics of incorporation of L-[1-¹⁴C]leucine (specific radioactivity 2mCi/mmol) into cultures inhibited by concentrations of chloramphenicol over the range 0–0.05g/l. Cultures grown in glucose-salts media were typical of the others in their response to the drug. The formation of labelled protein was monitored by precipitating samples (3ml) at various times with an equal volume of ice-cold 10% (w/v) trichloroacetic acid and by measuring the precipitable radioactive material (c.p.m.) that was resistant to incubation at 100°C for 30min. In this strain of *E. coli*, leucine incorporation into protein is completely inhibited at drug concentrations greater than 0.05g/l. In all following experiments, where chloramphenicol was added, concentrations of 0.2g/l were used.

Kinetics of RNA labelling in inhibited cultures. To cultures growing steadily in each of the three media, unlabelled uracil was added (0.5mg/l) at a cell density of E_{650} 0.5. After 5min, when the pathways of incorporation of exogenous uracil were stabilized, [5-³H]uracil (1μCi/ml) was injected. Samples (1ml) were pipetted into 4ml of ice-cold 10% trichloroacetic acid at intervals of 15s for a period of 3min. Then chloramphenicol (0.2g/l) was added, and further samples were taken for another 3min. The acid-precipitable radioactivity in the nucleic acids was measured. As only about 5–10% of the label in the total cell nucleic acids was in the DNA fraction (W. J. H. Gray, unpublished work) this could be taken as a measure of the labelled RNA in the cells. The results for cultures growing in broth, glucose-salts or lactate-salts media are shown in Fig. 2. Two observations were made. First, in glucose-salts or lactate-salts media, where the cells are dividing more slowly, chloramphenicol caused a two- to three-fold acceleration in the rate of labelling of the RNA. In enriched broth, where amino acids are in plentiful supply, the acceleration was only about 12%. This finding confirmed the studies of Fraenkel & Neidhardt (1961) and Kurland & Maaloe (1962). Secondly, the attainment of the new rate of RNA synthesis when the drug was added was not truly instantaneous, but required about 30s to 1min to establish itself. Although a small part of the delay may be the time required for the drug to act inside the cell, it seems to be too great to be explained by this factor alone.

Synthesis of tRNA and rRNA after inhibition with chloramphenicol. The RNA labelled after chloramphenicol was added to each medium was assayed

Fig. 2. Kinetics of incorporation of [³H]uracil into bacterial RNA after the addition of 0.2mg of chloramphenicol/ml of culture. The arrow represents time of chloramphenicol addition. (a) Broth culture; (b) glucose-salts culture; (c) lactate-salts culture. Other details are given in the text.

for its content of tRNA and rRNA+mRNA, by the technique of Gray & Midgley (1970). It was considered that, in drug-inhibited cells, the instability of the large rRNA molecules formed (see below) would render measurements based solely on the molecular size of isolated RNA inaccurate. In this case, the most satisfactory method of separating labelled tRNA and larger RNA species was by ion-exchange column chromatography on DEAE-cellulose. This method probably fractionates RNA

according to its secondary structure (Stanley & Bock, 1965), as even when rRNA is degraded to a size comparable with that of native tRNA, it still displays the chromatographic characteristics of undegraded material (Midgley, 1962). The 5S rRNA probably contaminates the tRNA fraction eluted from DEAE-cellulose columns, but this can be considered as a negligible component.

Cultures of *E. coli* were inhibited with 0.2 g of chloramphenicol/l and 5 min later, [$2\text{-}^{14}\text{C}$]uracil was injected and the precursor was incorporated into the cells for 10 min. The RNA was isolated and then fractionated on DEAE-cellulose columns (Gray & Midgley, 1970). The results are shown in Table 1. Our findings suggest that when, on addition of chloramphenicol, accelerations occurred in the rate of synthesis of stable RNA, the rRNA fraction was chiefly affected. The tRNA (defined as that RNA fraction eluted from DEAE-cellulose with the same chromatographic characteristics as a pure specimen) seemed to be formed at virtually the same rate as before. However, in broth, where both tRNA and rRNA are produced at maximum rates, there was little, if any, change in the relative rate of formation of these fractions when the drug was added. The small acceleration of 12%, noted after inhibition of broth cultures by chloramphenicol, has been shown to be due entirely to the partial stabilization and accumulation of the mRNA fraction (see below).

Kinetic analysis of the labelling of 23S rRNA. The rate of polymerization of 23S rRNA chains was measured by the kinetic analysis of labelling completed 23S molecules (Mangiarotti *et al.* 1968; Avery & Midgley, 1971). To cultures of *E. coli* M.R.E.600, growing steadily in each of the three media at 37°C,

Table 1. *Rate of incorporation of exogenous labelled uracil into various RNA fractions in normally growing and chloramphenicol-inhibited cultures of E. coli M.R.E.600*

The rate of synthesis of total RNA in normally growing cultures was taken to be 100 units/unit time. Values for chloramphenicol-inhibited cultures = (acceleration in RNA labelling after the addition of drug) $\times$ (fraction of the radioactivity entering the particular RNA species in unit time).

Growth medium	Chloramphenicol	Relative rate of synthesis of RNA fractions (arbitrary units)	
		tRNA + 5S rRNA	16S + 23S rRNA + mRNA
Broth	—	21.2	78.8
	+	25.0	88.2
Glucose-salts	—	34.7	65.3
	+	38.2	181.4
Lactate-salts	—	51.7	48.3
	+	46.1	301.0

0.5 mg of unlabelled uracil/l was added, at a cell density E_{650} 0.5. After 5 min, [$5\text{-}^3\text{H}$]uracil was injected, and at intervals samples of the culture were poured on crushed ice. The RNA was then extracted and the various species were fractionated on sucrose density gradients (Avery & Midgley, 1969). The specific radioactivity of the 23S rRNA peak was then measured. Corrections were made, by DNA—

Fig. 3. Kinetics of labelling of completed chains of 23S rRNA in exponentially growing cultures of *E. coli* at 37°C. (a) Broth culture; (b) glucose-salts culture; (c) lactate-salts culture. Other details are given in the text. ●, Completed 23S rRNA chains; ----, uncompleted + completed 23S rRNA chains. The extrapolation of the line for completed 23S chains to the abscissa cuts it at half the time required to complete a 23S rRNA molecule (Mangiarotti *et al.* 1968; Avery & Midgley, 1971). Half-times so obtained are: (a) broth cultures, 1.1 min; (b) glucose-salts culture, 1.2 min; (c) lactate-salts culture, 1.3 min.

Fig. 4. Kinetics of labelling of completed chains of 23S rRNA in chloramphenicol-inhibited cultures of *E. coli* at 37°C. (a) Broth culture; (b) glucose-salts culture; (c) lactate-salts culture. Other details are given in the text. ●, Completed 23S rRNA chains; ----, uncompleted + completed 23S rRNA chains. Half-times for completion of 23S rRNA by extrapolation (see Fig. 3 legend) are: (a) broth culture, 1.0 min; (b) glucose-salts culture, 1.0 min; (c) lactate-salts culture, 1.0 min.

RNA hybridization of fractions under the 23S peak, for its contamination by large fragments of mRNA (Avery & Midgley, 1971). As found by these authors, the contribution of labelled mRNA to the labelled 23S peak was small, and did not exceed 20% of the radioactivity in this region even in the

Table 2. Rate of chain polymerization of 23S rRNA in growing and chloramphenicol-inhibited cultures

Details are given in the text.

Growth medium	No. of cell divisions/h at 37°C	RNA chain-elongation rate (nucleotides added/s)
Broth	1.9	24
Broth + chloramphenicol	—	27
Glucose-salts	1.0	22
Glucose-salts + chloramphenicol	—	26
Lactate-salts	0.4	20
Lactate-salts + chloramphenicol	—	26

earliest samples. Similar experiments were carried out on the corresponding cultures inhibited 10 min previously by 0.2 g of chloramphenicol/l. As the rate of turnover of the RNA labelled in the presence of the drug was fairly low compared with its rate of synthesis, RNA instability was considered to have a negligible effect on the kinetics of RNA labelling over the short time-interval used. Typical labelling curves for completed 23S chains are shown for normally growing and drug-inhibited cultures in Figs. 3 and 4.

A summary of the times required for synthesis of 23S rRNA chains in the various conditions is given in Table 2. Chain-polymerization rates, expressed as nucleotide residues added/s to the growing RNA molecules, were calculated from the 23S rRNA labelling curves as described by Mangiarotti *et al.* (1968) and Avery & Midgley (1971). It was assumed that 23S rRNA is a single chain of 3100 nucleotide residues (Kurland, 1960). The evidence so far available suggests that newly formed rRNA, destined to become the 23S rRNA in mature 50S ribosomes, has a molecular weight virtually indistinguishable from that isolated from the ribosomes themselves (Adesnik & Levinthal, 1969; Pace, Peterson & Pace, 1970). We can thus rule out the possibility that chain-elongation rates of this fraction are greatly underestimated by the later processing of significantly larger precursors of 23S rRNA to a smaller end-product.

The results show that, except possibly for cultures growing slowly in lactate-salts media, the rate of chain polymerization of *E. coli* 23S rRNA is virtually independent of the nature of the growth medium or the presence of inhibitory concentrations of chloramphenicol. The maximum variation in chain-elongation rates that can be permitted in the three conditions is not more than 25%, even though the growth rates of the culture may vary by a factor of more than three. Thus, it seems that if the results

for 23S rRNA can be extended to other species of RNA, chain polymerization rates in general are constant over a wide range of growth conditions.

Synthesis of mRNA in chloramphenicol-inhibited cultures. The kinetics of labelling of mRNA was studied in various cultures during a period of drug inhibition. The technique of DNA-RNA hybridization was used (Gillespie & Spiegelman, 1965). Hybridization of mRNA was maximized and the binding of other RNA fractions was minimized by challenging 160 μ g of denatured DNA/nitrocellulose membrane filter with 40 μ g of RNA (Pigott & Midgley, 1968). In all experiments, the efficiency of hybridization was measured by including filters bearing DNA that were challenged with only 0.5 μ g of RNA, when in theory all the mRNA and rRNA could be bound (Pigott & Midgley, 1968). tRNA was very poorly hybridized in these conditions (Kennell, 1968), and a small correction was made to take account of its contribution to the labelling patterns, especially important over longer periods of labelling in chloramphenicol-inhibited cultures (Maaloe & Kjeldgaard, 1966). This correction was made by comparing the percentage of labelled RNA hybridized at a DNA/RNA ratio 200:1 with the percentage hybridized in a rapidly labelled RNA specimen known to contain no labelled tRNA, at the same DNA/RNA ratio. As tRNA is not hybridized efficiently at such ratios (Kennell, 1968) any extra labelled RNA that was not hybridized in the first case was taken to be tRNA.

To study the stability of the mRNA fraction in *E. coli* immediately after the addition of a high concentration of chloramphenicol, steadily growing

cultures were first given 0.5 μ g of unlabelled uracil/ml at a cell density of E_{650} 0.5. After 5 min, [3 H]-uracil was injected, and 50 ml samples of the cultures were removed at intervals and poured on crushed ice. After a further 3 min, chloramphenicol (0.2 g/l) was added and samples were taken over a further 30 min. The total RNA was extracted and hybridized with DNA for 16–18 h at 66°C. Throughout the period of labelling, samples (1 ml) were precipitated by ice-cold 10% (w/v) trichloroacetic acid. This gave a measure of the labelling of the total cellular RNA. The results for cultures grown in broth, glucose-salts or lactate-salts media (Gray & Midgley, 1970) are shown in Fig. 5. Several points emerge from these figures. First, the addition of chloramphenicol caused a rapid acceleration in the rate of RNA labelling in glucose-salts or lactate-salts media, but in broth, the acceleration was much smaller, giving at maximum a 15% rise in the overall rate of labelling. Secondly, in all media, the initial proportions of labelled mRNA in the short period before the addition of chloramphenicol were constant, as about 30% of the rapidly labelled fraction (Gray & Midgley, 1970). Thirdly, whether or not an overall acceleration occurred in the rate of RNA synthesis with chloramphenicol, the mRNA fraction accumulated as a constant 14–16% of the RNA formed in the presence of the antibiotic. However, especially in experiments with broth cultures, the kinetics of mRNA labelling indicated a discontinuity in the curve close to the time at which the drug was added to the cultures. An explanation of this phenomenon was next sought.

Experiments were carried out over a longer period

Fig. 5. Kinetics of labelling of mRNA and total RNA in *E. coli* cultures before and after the addition of chloramphenicol. (a) Broth-grown culture; (b) glucose-salts culture; (c) lactate-salts culture. Other details are given in the text. O, Radioactivity (c.p.m./ml of culture) in total RNA; ●, radioactivity (c.p.m./ml of culture) in mRNA. In (a) the broken line is a projection of the labelling curve for mRNA at early times in steadily growing cultures. In (b) and (c), the upper broken line is a projection of the kinetic curve for labelling of total RNA before the addition of chloramphenicol. Similarly, the lower broken line is a projection of the labelling curve for mRNA before chloramphenicol was added (indicated by the arrow).

Fig. 6. Kinetics of labelling of mRNA and total RNA in antibiotic-inhibited cultures of *E. coli*. (a) Broth-grown culture; (b) glucose-salts culture; (c) lactate-salts culture. Other details are given in the text. O, Radioactivity (c.p.m./ml of culture) in total RNA; ●, radioactivity (c.p.m./ml of culture) in mRNA. In (a) the fall in the absolute amount of radioactive RNA after 60 min inhibition by chloramphenicol is due to a slow partial lysis of the bacteria in broth media. This phenomenon was not noted in inhibited cultures in glucose-salts or lactate-salts media (b and c).

of inhibition with chloramphenicol, in which the cultures were inhibited 5 min before the addition of [5-³H]uracil. Samples were again taken at intervals and the mRNA content of the labelled RNA was estimated by hybridization. Fig. 6 shows the results obtained.

Table 3. *Percentage of labelled mRNA in the rapidly ³H-labelled RNA of chloramphenicol-inhibited cultures*

mRNA was identified with that fraction of labelled RNA that hybridized with DNA at low DNA/RNA ratios (see the text). The results have been corrected for a small degree of rRNA hybridization and for the incompleteness of the hybridization process.

Time of labelling with [5- ³ H]uracil (min)	Percentage of labelled RNA as mRNA		
	Broth	Glucose-salts	Lactate-salts
2	30.5	33.1	32.8
5	21.4	23.8	19.6
10	14.7	16.0	13.2
15	12.4	15.1	14.7
20	14.3	16.1	15.0

Table 4. *Percentage of mRNA in rapidly ³H-labelled RNA obtained by the incorporation of [5-³H]uracil for 20 s into chloramphenicol-inhibited cultures, at various times after the commencement of inhibition*

All samples of rapidly labelled RNA were obtained from cultures at 37°C in glucose-salts media, with the addition at zero time of 0.2 g of chloramphenicol/l. Further details are given in the text.

Time of incubation of cultures with chloramphenicol (min)	Percentage of labelled RNA as mRNA
5	34.8
10	32.1
30	33.7
60	31.1
90	31.8

All of the earliest samples taken after addition of labelled uracil contained a detectably higher proportion of hybridizable mRNA than samples taken at later times (Table 3). This indicated that some of the mRNA fraction produced in chloramphenicol-inhibited cells was still more unstable than the rest of the RNA in the early period of inhibition. Thus, the addition of the drug to growing bacterial cultures had no immediate effect on the proportions of labelled mRNA and other RNA species formed, even when there were quite considerable overall changes in the rate of RNA synthesis.

To check whether this partial instability of mRNA persisted throughout the 2 h inhibition with chloramphenicol, samples of the culture were labelled for 20 s at various times, and the proportion of mRNA in the rapidly labelled material was estimated. The results are shown in Table 4 for glucose-salts

Fig. 7. Stability of mRNA in normally growing and chloramphenicol-inhibited cultures of *E. coli* in glucose-salts media. Details are given in the text. ○, Drug-inhibited cells; ●, growing cells.

cultures inhibited by chloramphenicol, as an example typical for all three media. They demonstrate that the proportion of mRNA in the rapidly labelled RNA was at all times identical with that found in normally growing cultures, as some 30% of the labelled material (Gray & Midgley, 1970). However, the results from Figs. 5 and 6 and from Table 3 showed equally clearly that, over long periods of labelling, the accumulated mRNA accounted for only half of that originally entering the rapidly labelled fraction. Thus, half of the mRNA produced in drug-inhibited cultures was unstable and decayed quickly to non-hybridizable material, and half had the same stability as the whole of the RNA formed with chloramphenicol. A more detailed examination of the early kinetics of labelling of the mRNA is shown in Fig. 7, in comparison with those typical of normally growing cultures. The unstable portion of the mRNA decayed at a rate that was very similar to that found in uninhibited cells.

A calculation was made of the amount of mRNA present in chloramphenicol-inhibited cultures. As the cellular RNA content increased by about 60% over the whole period of drug inhibition, and the mRNA was about 15% of the RNA formed in the presence of the drug, then it would constitute $60/160 \times 15 = 6\%$ of the total. The results indicate that the amount of mRNA in chloramphenicol-inhibited cultures was at maximum almost three times that found in normal cells (Friesen, 1966).

We wished to check that none of our measurements were made grossly inaccurate during inhibition with chloramphenicol by problems arising from the labelling of the intracellular-nucleotide pools or by a rapid hidden turnover of RNA that might dilute the incoming labelled precursors. Experiments were carried out on cultures randomly labelled with $[5\text{-}^3\text{H}]\text{uracil}$. Steadily growing cultures at a cell density E_{650} 0.05 were given $[5\text{-}^3\text{H}]\text{uracil}$ and the

Table 5. *Estimation of the mRNA content of E. coli cultures after various periods of inhibition by 0.2 g of chloramphenicol/l, by the technique of 'coincidence hybridization'*

DNA-RNA hybridization was carried out at DNA/RNA ratio 4:1. Small corrections were made for the hybridization of rRNA and for spurious RNA binding to the filter (see the text).

Time of inhibition (min)	Radioactivity in hybridized RNA (c.p.m.)		$^{14}\text{C}/^3\text{H}$ ratio	Percentage of mRNA
	^{14}C	^3H		
0	3189	1215	2.62	2.2
	3356	1288	2.61	
	2989	1140	2.60	
15	3175	1453	2.19	2.63
	3003	1481	2.03	2.81
	3498	1570	2.10	2.74
30	3111	2198	1.41	4.11
	3418	2483	1.38	4.17
	2248	1585	1.42	4.09
60	3346	3268	1.03	5.60
	3619	3501	1.04	5.52
	3039	2953	1.03	5.60
90	3407	3848	0.87	6.53
	3188	3595	0.88	6.46
	2657	3053	0.87	6.53
150	3091	3635	0.85	6.78
	3316	3865	0.86	6.70
	3353	3851	0.87	6.53

cultures were grown with a steady incorporation of isotope to a density E_{650} 0.6. Chloramphenicol was then added, and samples of randomly ^3H -labelled RNA were obtained. RNA randomly labelled with $[2\text{-}^{14}\text{C}]\text{uracil}$ was similarly prepared from a steadily growing culture in glucose-salts medium at 37°C . It was known that the amount of mRNA in this culture was 2.2% of the total (Gray & Midgley, 1970). Mixtures were then made, consisting of equal weights of ^{14}C -labelled and ^3H -labelled RNA. As both cultures were randomly labelled, the $^{14}\text{C}/^3\text{H}$ c.p.m. ratio was constant in all specimens. The mixtures were then hybridized with DNA at a DNA/RNA ratio 4:1 and the $^{14}\text{C}/^3\text{H}$ c.p.m. ratio was measured in the hybrids. Small corrections were made for the binding of rRNA and for spurious hybrids, knowing that the $^{14}\text{C}/^3\text{H}$ c.p.m. ratio for these fractions was the same as in the input material. From the corrected c.p.m. ratio in the hybrids, the percentage of mRNA in the ^3H -labelled fractions was calculated. This technique of 'coincidence hybridization' had the considerable advantage that an internal standard was present in each hybridization experiment and that the interference of

spurious hybrids and rRNA binding, giving small uncertainties that might be important in direct estimations, was very greatly minimized. The results are shown in Table 5. These results confirmed those obtained by examining the kinetics of mRNA labelling during inhibition with chloramphenicol, in that a maximum of 6.5% of the total RNA was mRNA in cultures inhibited over a long period by the drug. Thus, no significant difficulties in interpretation were caused in the kinetic studies by hidden RNA turnover and by pool dilution.

It was nevertheless important to estimate the degree of stability of the total RNA formed in the presence of chloramphenicol. This would allow us to compare the rapid turnover of half the mRNA fraction with any general turnover that might

affect the whole of the material formed in the presence of the drug. Accordingly, to cultures growing steadily in glucose-salts media, 0.2g of chloramphenicol/l was added, and 5min later, 3mg of [2-¹⁴C]uracil was added. To one culture, 0.1g of rifampicin/l was added after a further 5min, and to another the antibiotic was added 30min later. It is known that rifampicin inhibits RNA synthesis by preventing the initiation of new RNA chains, but does not prevent the completion of chains that were already in the process of polymerization on the DNA cistrons (Hartmann, Honikel, Knüsel & Nüesch, 1967; Lancini & Sartori, 1968; Sippel & Hartmann, 1968; Wehrli, Nüesch, Knüsel & Staehelin, 1968). Thus the stability of the RNA, formed in the presence of chloramphenicol before the addition of rifampicin, could be estimated. Fig. 8 shows the results obtained.

In both cases, the RNA formed in the presence of chloramphenicol was not completely stable. However, if we take into account the mRNA that turned over very rapidly, it appeared that the slow rate of degradation of the total labelled RNA was very similar in both experiments. An experiment in which the inhibited cultures were given [2-¹⁴C]uracil for 90min before the addition of rifampicin showed virtually the same results, except that, by the time the second antibiotic was added, turnover of RNA had reached such proportions that the net rate of incorporation of labelled uracil into RNA was very small (Fig. 8). If the assumption can be made that the rifampicin does not trigger off the instability of RNA formed in the presence of chloramphenicol, then, over the whole period of drug inhibition, a slow turnover of the RNA is taking place.

DISCUSSION

The results presented in this paper have confirmed that the overall rate of rRNA synthesis in growing bacteria is governed primarily by the rate of initiation of new RNA chains on the DNA cistrons and not by the subsequent rate of polymerization of already initiated chains (Bremer & Yuan, 1968*a,b*; Manor *et al.* 1969). The failure of chloramphenicol to accelerate significantly the rate of RNA synthesis in cultures growing rapidly in broth media suggests that, in these conditions, the available DNA-dependent RNA polymerase molecules transcribe the cistrons specific for rRNA with optimum efficiency. On the other hand, in growth-limiting media such as glucose-salts or lactate-salts, there is a pool of polymerases in excess of the steady-state requirements for RNA biosynthesis. This pool can be brought into play by the action of chloramphenicol, by over-riding the normal control mechanisms for limiting the rate of RNA synthesis and

Fig. 8. Stability of RNA synthesized in chloramphenicol-inhibited cultures of *E. coli* originally grown in glucose-salts media. (a) RNA labelled with [5-³H]uracil for 5 min during inhibition with chloramphenicol, and (b) for 30 min before the addition of 0.1 g of rifampicin/l. The time of rifampicin addition is indicated by the arrows. ○, RNA labelling; ●, DNA labelling.

chain initiation. A rapid acceleration then occurs in the rate of RNA synthesis (Kurland & Maaloe, 1962; Maaloe & Kjeldgaard, 1966), which requires a small, though finite, time to establish itself. An explanation of this delay could be that, in growth-limiting conditions, the enzymes engaged in rRNA transcriptions are, on average, more widely spaced along the cistrons than in conditions where the enzymes are fully active, as in enriched broth. On addition of chloramphenicol, the rate of initiation of the available polymerases may rise almost instantaneously, causing a 'wavefront' of enzymes, more closely spaced along the cistrons, to traverse their length. It should take about 2 min for this 'wavefront' to complete the transcription of the cistrons for 23S rRNA. The kinetic delay observed in the acceleration of RNA labelling overall was smaller than this. However, the accelerated labelling of mRNA, together with its partial stabilization by chloramphenicol, would be expected to obscure considerably the labelling kinetics of normally stable RNA molecules.

When chloramphenicol-induced accelerations in RNA synthesis occurred, the hybridizable mRNA fraction was affected to the same extent as the rRNA fraction. The mRNA constitutes 30–33% of the rapidly labelled RNA in normally growing and in chloramphenicol-inhibited cultures (cf. Gray & Midgley, 1970). This confirmed the original findings of Midgley & McCarthy (1962), who showed that the apparent nucleotide base compositions of ^{32}P -labelled RNA in normal and chloramphenicol-inhibited cultures of *E. coli* were identical in the early stages of labelling. The results are also in general agreement with those of Friesen (1966), if allowance is made for the efficiency of DNA–RNA hybridization processes, which was not considered by this author. The results favour the concept of a co-ordinate control of mRNA and rRNA synthesis in bacteria, directed towards chain initiation of RNA molecules (Stent & Brenner, 1961; Friesen, 1966; Stent, 1966; Gallant & Cashel, 1967). Chloramphenicol appears to act by freeing the rate-limiting regulatory processes involved in initiation of both mRNA and rRNA, giving a co-ordinate acceleration in their rates of synthesis.

A further finding is that part of the newly formed mRNA is, throughout the period of drug inhibition, as unstable as in cells undergoing normal growth. Thus, about half of the mRNA produced in unit time in chloramphenicol-inhibited cells rapidly decays, and half is stabilized and accumulates as some 6% of the total cellular RNA after 2 h inhibition. This again is basically in agreement with Friesen's (1966) results, except that his studies indicated a much slower rate of initial decay of the unstable fraction of mRNA in chloramphenicol-inhibited cultures. However, in agreement with

Friesen's (1966) results, the mRNA content of drug-inhibited cells is finally two to three times that found in normally growing organisms (Gray & Midgley, 1970). The differential decay of the two fractions of mRNA confirms the findings of Midgley & McCarthy (1962), who showed that the apparent nucleotide base composition of rapidly labelled RNA in chloramphenicol-inhibited cultures changed quickly with time from one that was intermediate between mRNA and rRNA (1:2 w/w ratio) to one very like that of the total RNA. Their estimation of the amount of mRNA accumulating in the inhibited cells has also been confirmed.

The question immediately arises as to how the two fractions of mRNA develop such different stabilities in the inhibited cultures. One explanation could be that two different classes of mRNA molecules exist, having different susceptibilities to degrading nucleases (Kuвано, Schlessinger & Apirion, 1970*a,b*). However, as there is no convincing evidence for the existence of two such classes in normally growing cultures, it must be that chloramphenicol is in some way responsible for stabilizing some fractions of mRNA and not others. A more attractive explanation is that some part of all mRNA molecules produced in the presence of chloramphenicol is unstable, and is thus readily degraded by the polar exonuclease described by Kuвано *et al.* (1970*a*). Chloramphenicol is known to inhibit the release and rate of recycling of ribosomal subunits *in vitro* (Kaempfer, 1968). Thus, we may propose that, *in vivo*, the ribosomes attach normally at the 5'-ends of the mRNA chains and translocate towards the distal 3'-termini without translation (Gurgo *et al.* 1969). If the rate of mRNA breakdown is related to the rate of ribosomal translocation (Kuвано *et al.* 1970*a*) then it would seem that the translocation of the mRNA chains in chloramphenicol-inhibited cells proceeds at the normal rate. Ribosomes may then proceed to the distal 3'-terminus of the mRNA chains, there to be held and released at a slow rate (Kaempfer, 1968). Further ribosomes will translocate the mRNA and accumulate behind the first. Thus, stable, immobilized polyribosomes will be produced (Das *et al.* 1966; Weber & DeMoss, 1966). For every ribosome that attaches to the mRNA chains, we propose that there is a constant chance that, instead, a nuclease is bound, and degrades the mRNA strand as far as the accumulated immobilized ribosomes. These will then protect the remainder of the mRNA molecules from further degradation at the initial rapid rate, the subsequent rate of turnover and degradation depending on the low rate of release of the ribosomes from the 3'-terminus of the mRNA. Our model for the action of chloramphenicol proposes that the mRNA fragments that accumulate in inhibited cultures are

richer in sequences from the distal regions of the mRNA chains (i.e. those near the 3'-terminus). Further, this explains why smaller polyribosomes accumulate in chloramphenicol-inhibited cultures, since half an mRNA molecule, on average, survives the initial rapid degradation by the nuclease (Friesen, 1966; Gurgo *et al.* 1969; Weber & DeMoss, 1966).

In summary, our model explains satisfactorily the main features of bacterial response to inhibition by chloramphenicol. The basic parts of the transcription and translation processes disrupted by this drug are those that control the rate of RNA chain initiation and the rate of ribosome release from the mRNA molecules. The latter process may be a result of the inhibition of translation by the drug. Further work (W. J. H. Gray & J. E. M. Midgley, unpublished work) has indicated that RC^{ret} mutants of *E. coli* that are also amino acid auxotrophs behave in an identical way to chloramphenicol-inhibited cultures when the amino acid is withdrawn from the growth medium. Thus, the patterns of RNA synthesis and stability in drug-inhibited or RC^{ret} strains of *E. coli* appear to have a common origin, due to a release of the control of RNA-chain initiation.

W. J. H. G. thanks the Medical Research Council for a postgraduate studentship. J. E. M. M. is a Senior Research Associate in the Medical Research Council Research Group in the Structure and Biosynthesis of Macromolecules. We thank Mrs P. Hislop for her excellent technical assistance.

REFERENCES

- Adesnik, M. & Levinthal, C. (1969). *J. molec. Biol.* **46**, 281.
- Avery, R. J. & Midgley, J. E. M. (1969). *Biochem. J.* **115**, 383.
- Avery, R. J. & Midgley, J. E. M. (1971). *Biochem. J.* **122**, 139.
- Bremer, H. & Yuan, D. (1968a). *J. molec. Biol.* **34**, 527.
- Bremer, H. & Yuan, D. (1968b). *J. molec. Biol.* **38**, 163.
- Das, H. K., Goldstein, A. & Kanner, L. C. (1966). *Molec. Pharmac.* **2**, 158.
- Edlin, G., Stent, G. S., Baker, R. F. & Yanofsky, C. (1968). *J. molec. Biol.* **37**, 257.
- Fraenkel, D. G. & Neidhardt, F. C. (1961). *Biochim. biophys. Acta*, **53**, 96.
- Friesen, J. D. (1966). *J. molec. Biol.* **20**, 559.
- Gallant, J. & Cashel, M. (1967). *J. molec. Biol.* **25**, 545.
- Gillespie, D. & Spiegelman, S. (1965). *J. molec. Biol.* **12**, 829.
- Gray, W. J. H. (1970). *Biochem. J.* **117**, 60P.
- Gray, W. J. H. & Midgley, J. E. M. (1970). *Biochem. J.* **120**, 279.
- Gurgo, C., Apirion, D. & Schlessinger, D. (1969). *J. molec. Biol.* **45**, 205.
- Hartmann, G., Honikel, K. O., Knüsel, F. & Nüesch, J. (1967). *Biochim. biophys. Acta*, **145**, 843.
- Kaempfer, R. (1968). *Proc. natn. Acad. Sci. U.S.A.* **61**, 106.
- Kennell, D. (1968). *J. molec. Biol.* **34**, 85.
- Kurland, C. G. (1960). *J. molec. Biol.* **2**, 83.
- Kurland, C. G. & Maaloe, O. (1962). *J. molec. Biol.* **4**, 193.
- Kuwano, M., Schlessinger, D. & Apirion, D. (1970a). *Nature, Lond.*, **226**, 514.
- Kuwano, M., Schlessinger, D. & Apirion, D. (1970b). *J. molec. Biol.* **51**, 75.
- Lancini, G. C. & Sartori, G. (1968). *Experientia*, **24**, 1105.
- Lavalle, R. & de Hauwer, G. (1968). *J. molec. Biol.* **37**, 269.
- Maaloe, O. & Kjeldgaard, N. O. (1966). *Control of Macromolecular Biosynthesis*. New York: W. A. Benjamin Inc.
- Mangiarotti, G., Apirion, D., Schlessinger, D. & Silengo, L. (1968). *Biochemistry, Easton*, **7**, 456.
- Manor, H., Goodman, D. & Stent, G. S. (1969). *J. molec. Biol.* **39**, 1.
- Midgley, J. E. M. (1962). *Biochim. biophys. Acta*, **61**, 513.
- Midgley, J. E. M. (1963). *Biochim. biophys. Acta*, **68**, 354.
- Midgley, J. E. M. (1970). *Biochem. J.* **117**, 60P.
- Midgley, J. E. M. & McCarthy, B. J. (1962). *Biochim. biophys. Acta*, **61**, 696.
- Morris, D. W. & DeMoss, J. A. (1966). *Proc. natn. Acad. Sci. U.S.A.* **56**, 262.
- Morris, D. W. & Kjeldgaard, N. O. (1968). *J. molec. Biol.* **31**, 145.
- Nakada, D. (1965). *J. molec. Biol.* **12**, 695.
- Nomura, M. & Watson, J. D. (1959). *J. molec. Biol.* **1**, 204.
- Pace, B., Peterson, R. L. & Pace, N. R. (1970). *Proc. natn. Acad. Sci. U.S.A.* **65**, 1097.
- Pardee, A. B., Paigen, K. & Prestidge, L. S. (1957). *Biochim. biophys. Acta*, **24**, 215.
- Pigott, G. H. & Midgley, J. E. M. (1968). *Biochem. J.* **110**, 251.
- Sippel, A. & Hartmann, G. (1968). *Biochim. biophys. Acta*, **157**, 218.
- Stanley, W. J., jun. & Bock, R. M. (1965). *Biochemistry, Easton*, **4**, 1302.
- Stent, G. S. (1966). *Proc. R. Soc. B*, **164**, 181.
- Stent, G. S. & Brenner, S. (1961). *Proc. natn. Acad. Sci. U.S.A.* **47**, 2005.
- Venetianer, P. (1969). *J. molec. Biol.* **45**, 375.
- Weber, M. J. & DeMoss, J. A. (1966). *Proc. natn. Acad. Sci. U.S.A.* **55**, 1224.
- Wehrli, W., Nüesch, J., Knüsel, F. & Staehelin, M. (1968). *Biochim. biophys. Acta*, **157**, 215.
- Willson, G. & Gros, F. (1964). *Biochim. biophys. Acta*, **80**, 478.
- Winslow, R. M. & Lazzarini, R. A. (1969a). *J. biol. Chem.* **244**, 1128.
- Winslow, R. M. & Lazzarini, R. A. (1969b). *J. biol. Chem.* **244**, 3387.
- Zimmermann, R. A. & Levinthal, C. (1967). *J. molec. Biol.* **30**, 349.