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Stabilization and Breakdown of *Escherichia coli* Messenger Ribonucleic Acid in the Presence of Chloramphenicol†

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ABSTRACT: In *Escherichia coli* chloramphenicol stabilizes the fraction of preexisting mRNA which is associated with polyribosomes. mRNA not bound to ribosomes is not protected against breakdown. mRNA synthesized in the presence of chloramphenicol enters into polyribosomes and is turning over as rapidly as in exponentially growing cells. Consequently there is no significant accumulation of mRNA in chloramphenicol-treated cells.

Chloramphenicol at appropriate concentrations stops protein synthesis abruptly but allows RNA synthesis to continue for a considerable period. RNA synthesized in the presence of chloramphenicol contains all the RNA species encountered in normal, exponentially growing cells. Ribosomal RNA accumulates and is mainly localized in the so-called chloramphenicol particles, the nature of which is still unclear (Osawa, 1968; Schleif, 1968). The preexisting total mRNA (Levinthal *et al.*, 1963) as well as the specific lactose mRNA (Varmus *et al.*, 1971) and tryptophan mRNA (Morse *et al.*, 1969) seem to be stabilized by chloramphenicol. The data concerning the fate of mRNA synthesized in the presence of chloramphenicol are controversial. Several reports indicate that under these conditions lactose mRNA is either not synthesized at all (Artman and Ennis, 1972) or is rapidly degraded (Varmus *et al.*, 1971). On the other hand, Gurgo *et al.* (1969) reported that there was an accumulation of mRNA in polyribosomes of cells exposed to chloramphenicol. This conclusion was inferred

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from the finding that pulse-labeled RNA synthesized in the presence of chloramphenicol entered into and accumulated in polyribosomes, the assumption being that all pulse-labeled RNA found in polyribosomes was mRNA. That this assumption may be incorrect is borne out by two different observations: (1) pulse-labeled RNA associated with polyribosomes in exponentially growing cells consists of both the newly synthesized rRNA and mRNA (Fry and Artman, 1969); and (2) part of chloramphenicol particles has been found combined with ribosomes forming polyribosome-like material (Muto *et al.*, 1966).

We now report the effect of chloramphenicol on the metabolic stability and turnover of mRNA synthesized before and after the exposure of cells to this antibiotic. In these studies the mRNA component of pulse-labeled RNA was identified by means of hybridization competition assay.

Materials and Methods

Cells. Escherichia coli strain B was used throughout the work. The cells were grown with agitation at 37° in the glucose–salts medium of Davis and Mingioli (1950), which contained per liter: 7 g of K_2HPO_4, 3 g of KH_2PO_4, 1 g of $(NH_4)_2SO_4$, 0.1 g of $MgSO_4 \cdot 7H_2O$, 0.5 g of trisodium citrate dihydrate, and 0.2 g of D-glucose.

Incorporation of Radioactive Precursors into Protein and RNA. Exponentially growing cells or chloramphenicol-treated cultures at a density of 2×10^8 cells/ml of medium were exposed to [^{3}H]leucine, 0.2 μCi/ml (sp act. 3 mCi/mmole) or [2-^{14}C]uracil, 0.2 μCi/ml (sp act. 20 mCi/mmole). At zero time and after various time intervals, 1-ml samples were added to an equal volume of ice-cold trichloroacetic acid, 10% (w/v). Cells with incorporated radioactive leucine were heated at 90° for 20 min, cooled, left at 4° for 30 min, filtered through Millipore cellulose nitrate filters, dried, and counted. For samples with incorporated radioactive uracil, the heating step was omitted.

Preparation of Spheroplasts, Polyribosomes, and Nonfragmented Pulse-Labeled RNA. Spheroplasts were made by a modification of the method of Kohn (1960). Cells (100 ml) were grown in glucose–salts medium with glucose as a carbon source to a density of 2×10^8 cells/ml, pulse labeled under the various experimental conditions, poured onto crushed frozen 10 mM Tris-HCl buffer (pH 7.75), containing 30% (w/v) sucrose (ribonuclease-free; Mann Research Laboratories Inc., New York, N. Y.) and 15 mM magnesium acetate, centrifuged in the cold, washed once with 10 ml of the same buffer, and resuspended in 0.5 ml of the above buffer to which 0.6 mg of egg-white lysozyme (Sigma Chemical Co., St. Louis, Mo.) was added. The cells were twice frozen in an acetone–solid CO_2 bath and thawed in an ice bath. For the preparation of polyribosomes, the spheroplasts were lysed by incubation with 0.015 ml of 10% (w/v) sodium deoxycholate for 3 min at 0° (Ron *et al.*, 1966). The released DNA and cell debris were sedimented by centrifugation at 15000*g* for 15 min in the cold, and the supernatant fraction containing polyribosomes was analyzed by sucrose density gradient centrifugation.

RNA Preparations. Stable RNA free of mRNA was extracted from glucose-starved cells (Fry and Artman, 1969). Unlabeled RNA containing mRNA was prepared from exponentially growing cells. For pulse labeling, exponentially growing cells or chloramphenicol-treated cultures were exposed to [5-^{3}H]uracil, 2.5 μCi/ml of medium (sp act. 23 Ci/mmole), for 45 sec (0.01 of the generation time) and quickly chilled by pouring the cells onto crushed frozen 10 mM Tris-HCl buffer (pH 7.4) of the desired composition. In pulse-chase experiments, the pulse-labeled cells were incubated for an additional 10 min with a 200-fold excess of nonradioactive uridine. For the preparation of RNA from pulse-labeled and chased cultures the cells were converted into spheroplasts. The spheroplasts were gently suspended, with a wide bore pipet, in a small volume of ice-cold 10 mM Tris-HCl buffer (pH 7.4), containing 10 mM magnesium acetate, and lysed by adding 0.1 volume of 15% (w/v) sodium dodecyl and incubating the mixture at 0° for 1–3 min (Fry and Artman, 1968). RNA from glucose-starved cultures was extracted after the cells were disrupted for 2 min in the 20-KHz MSE ultrasonic disintegrator.

RNA extraction from spheroplast lysates and cell sonicates was described in detail (Fry and Artman, 1969).

Hybridization Competition Experiments. The hybridization technique of Gillespie and Spiegelman (1965) was used.

Scintillation Counting. All samples were counted in a Packard TriCarb scintillation counter. The scintillation fluid contained 0.4% of 2,5-diphenyloxazole and 0.01% of 1,4-bis(5-phenyloxazol-2-yl)benzene in toluene.

[^{3}H]Leucine (sp act. 2 Ci/mmole) and [5-^{3}H]uracil (sp act. 23 Ci/mmole) were purchased from Schwarz BioResearch, Orangeburg, N. Y. [2-^{14}C]Uracil (sp act. 50 mCi/mmole) was obtained from the Radiochemical Centre, Amersham, Bucks, England.

Chloramphenicol was purchased from Sigma Chemical Co., St. Louis, Mo. All chemicals used were of analytical grade.

Results

Protein and RNA Synthesis in the Presence of Chloramphenicol. In the presence of chloramphenicol, 100 μg/ml of medium, protein synthesis and bacterial growth stop abruptly. Incorporation of radioactive uracil into RNA continues for 30 min at a rate comparable to that observed in exponentially growing cells. Incubation of *E. coli* cells with chloramphenicol for longer periods leads to a decline and cessation of RNA synthesis (Figure 1). These results are in accord with those reported by other authors (Gale and Folkes, 1953; Nomura and Watson, 1959; Gurgo *et al.*, 1969).

Relative Amounts of mRNA Synthesized by Exponentially Growing and Chloramphenicol-Treated Cells. E. coli were grown in glucose–salts medium with agitation at 37° to a density of 2×10^8 cells/ml and divided into two portions. One portion was pulse labeled with tritiated uracil and the other was exposed to chloramphenicol for 5, 10, 20, and 30 min and then pulse labeled. All pulse-labeled cells were converted into spheroplasts and their RNA was extracted. In order to distinguish between the newly synthesized rRNA and mRNA, the two components of pulse-labeled RNA, hybridization competition experiments were performed. In these experiments, a constant amount of pulse-labeled RNA from exponentially growing and chloramphenicol-treated cells was mixed with increasing amounts of unlabeled stable RNA, assumed to be free of mRNA, and annealed to heat-denatured *E. coli* DNA bound to Millipore cellulose nitrate membrane filters at a DNA:pulse-labeled RNA ratio of 100:1. The efficiency of annealing without competitor RNA was about 45%. The amount of radioactivity hybridized without competitor RNA was regarded as 100% hybridization. Figure 2 shows the results of such hybridization competition experiments. It can be seen that with all the pulse-

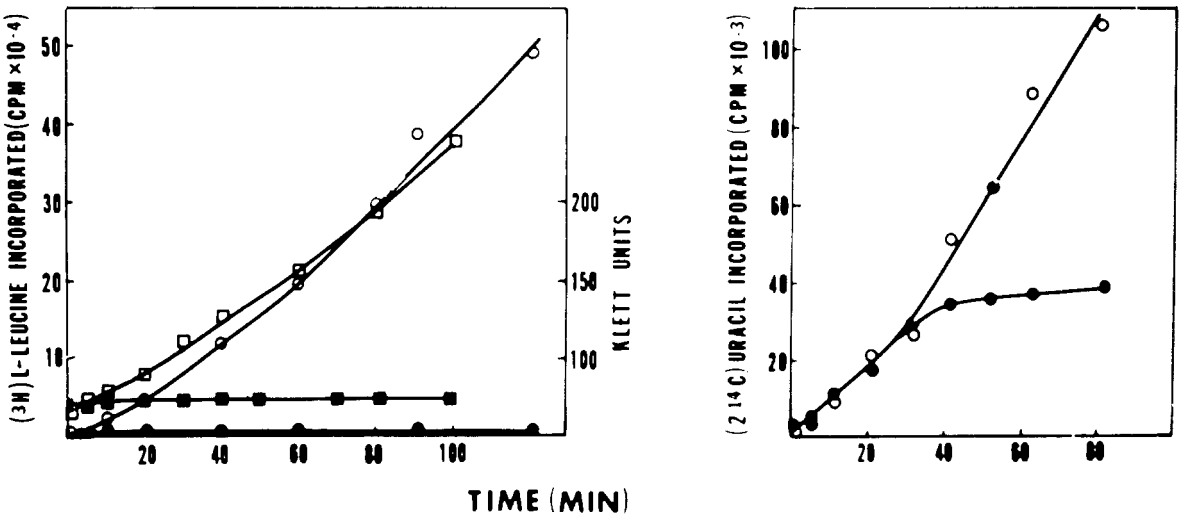

FIGURE 1: Growth, protein, and RNA synthesis in the presence of chloramphenicol. Exponentially growing *E. coli* at a density of 2×10^8 cells/ml of medium was divided into two portions. One portion was exposed to chloramphenicol (100 μg/ml), the other portion served as control. To both portions either [^{3}H]leucine, 1 μCi/ml (sp act. 2 μCi/mmole), or [2-^{14}C]uracil, 0.2 μCi/ml (sp act. 20 mCi/mmole), was added. At zero time and after various time intervals the incorporation of precursors into protein and RNA was determined as described in the text. Left: Growth and incorporation of [^{3}H]leucine into proteins: control culture, E_{450} (□---□); chloramphenicol-treated culture, E_{450} (■---■); incorporation of [^{3}H]leucine by control culture (○---○); incorporation of [^{3}H]leucine by chloramphenicol-treated cells (●---●). Right: Incorporation of [2-^{14}C]uracil by: control culture (○---○); chloramphenicol-treated cells (●---●).

labeled preparations tested, the addition of increasing quantities of unlabeled RNA devoid of mRNA resulted in a steady lowering of radioactivity bound to DNA. At a ratio of unlabeled RNA:pulse-labeled RNA of 200:1 a value of 35–40% hybridization was attained. Further addition of unlabeled stable RNA did not decrease this value. It is evident, therefore, that exponentially growing cells and cultures exposed to chloramphenicol for periods up to 30 min synthesize approximately the same amounts of mRNA, which comprises 35–40% of the total pulse-labeled RNA.

Metabolic Turnover of E. coli mRNA. Messenger RNA is metabolically unstable in exponentially growing bacterial cells (Levinthal *et al.*, 1962; Artman and Engelberg, 1964; Salser *et al.*, 1968; Midgley, 1969; Fry, 1970), but is protected against degradation by chloramphenicol (Levinthal *et al.*, 1963). These conclusions were based on the results of experiments in which the loss of acid-insoluble radioactivity of pulse-labeled RNA was determined in cells exposed to actinomycin D. We examined the metabolic turnover of *E. coli* mRNA directly using the competition hybridization assay. To this end we determined the fraction of pulse-labeled RNA that does not compete with stable RNA immediately after pulse labeling and following chase with a 200-fold excess of unlabeled uridine. In these experiments, the metabolic fate of mRNA synthesized in the absence and presence of chloramphenicol was studied.

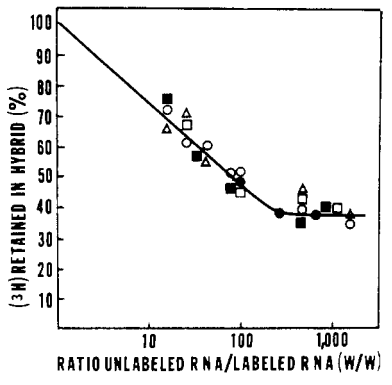

FIGURE 2: Competition experiments. Increasing amounts of unlabeled stable RNA were annealed to 60 μg of homologous DNA immobilized on filters in the presence of 0.5 μg of pulse-labeled RNA extracted from: exponentially growing cells (○---○); cells exposed to chloramphenicol for 5 min (■---■); cells exposed to chloramphenicol for 10 min (□---□); cells exposed to chloramphenicol for 20 min (●---●); cells exposed to chloramphenicol for 30 min (Δ---Δ). The amount of radioactivity hybridized without competitor RNA represents 100% hybridization. Background corrections were made for each point of the curve from control samples containing pulse-labeled RNA, the appropriate amount of unlabeled RNA, and filters without DNA.

Figure 3a shows that mRNA in exponentially growing cultures is turning over rapidly. After 10 min of chase with excess of unlabeled uridine about 90% of the RNA fraction which immediately after pulse labeling was identified as mRNA became converted into stable RNA. The addition of chloramphenicol to exponentially growing pulse-labeled cells resulted in a marked decrease in the rate of mRNA turnover. Figure 3b shows that after 10 min of chase with uridine the preexisting mRNA remained undegraded. When the cells were pulse labeled 5 min after the addition of chloramphenicol, the 10-min chase with unlabeled uridine led to the conversion of the bulk of mRNA into stable RNA species, *i.e.*, the results were analogous in all respects to those obtained with exponentially growing cells (Figure 3c). The results of these experiments indicate that in *E. coli* the preexisting mRNA is stabilized by chloramphenicol, but mRNA synthesized in the presence of this antibiotic is turning over as rapidly as in exponentially growing cells. These results also suggest that there should not be any significant accumulation of mRNA in cells exposed to chloramphenicol.

Lack of Accumulation of mRNA in Cells Exposed to Chloramphenicol. Evidence for the lack of accumulation of mRNA in cells incubated with chloramphenicol was provided by experiments in which the amount of mRNA synthesized by

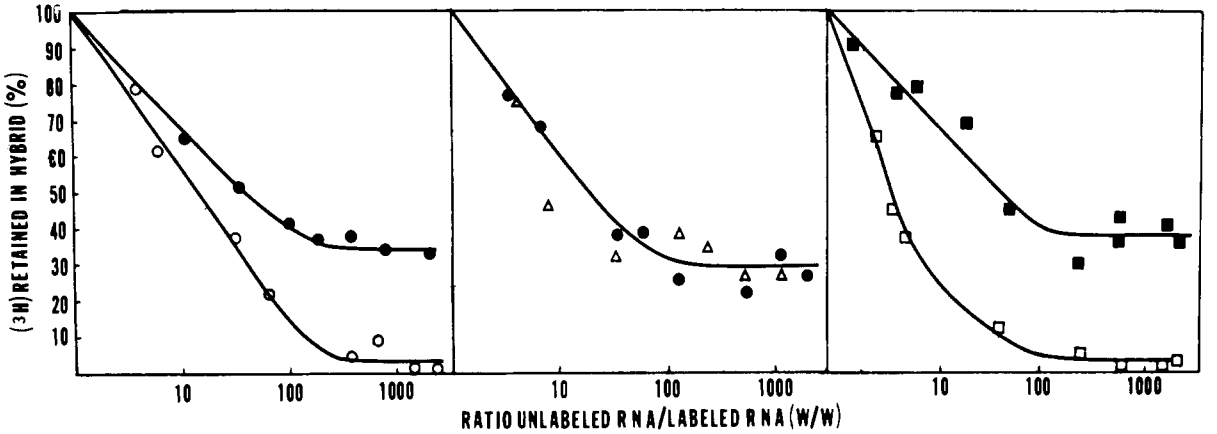

FIGURE 3: Competition experiments. Increasing amounts of unlabeled stable RNA free of mRNA were annealed to 60 μg of homologous DNA immobilized on filters in the presence of 0.3 μg of pulse-labeled RNA: synthesized by exponentially growing cells (●---●); synthesized by exponentially growing cells after 10-min chase with uridine (○---○); synthesized by exponentially growing cells, chase in the presence of chloramphenicol (Δ---Δ); synthesized in the presence of chloramphenicol (■---■); synthesized in the presence of chloramphenicol after chase in the presence of chloramphenicol (□---□).

cells exposed to chloramphenicol was measured and compared with that found in exponentially growing cells. In these experiments, exponentially growing cells were pulse labeled with radioactive uracil and their RNA was extracted and used in hybridization competition experiments with the following unlabeled RNA preparations: (1) RNA extracted from glucose-starved cells and actinomycin D treated cultures and assumed to be devoid of mRNA; (2) RNA extracted from exponentially growing cells and containing the normal amount of mRNA; and (3 and 4) RNA extracted from cells exposed to chloramphenicol for 5 and 30 min and containing an unknown amount of mRNA.

In these experiments, a constant amount of radioactive mRNA from exponentially growing cells was annealed to homologous DNA with increasing amounts of one of the above different unlabeled RNA preparations. From the efficiency with which the unlabeled RNA preparations competed with labeled mRNA for homologous sites on DNA, their relative mRNA content was determined. The rationale for this kind of experiment was provided by Bolle *et al.* (1968) who showed that the sensitivity of hybridization competition assays is sufficiently high to allow determination of mRNA concentrations even if they vary over a wide range in different RNA preparations. The results of these experiments presented in Figure 4 are as follows: (1) mRNA in exponentially growing cells comprises about 40% of pulse-labeled RNA; (2) the addition of similar amounts of unlabeled RNA from exponentially growing cells and from cultures exposed to chloramphenicol for different periods up to 30 min brought about a similar exclusion of radioactive mRNA from the hybrids. The essentially similar efficiency of competition displayed by RNA preparations obtained from exponentially growing and chloramphenicol-treated cells strongly suggests that the mRNA content of cells incubated with chloramphenicol for as long as 30 min is not significantly different from that found in exponentially growing cells.

Distribution of mRNA Synthesized in the Presence of Chloramphenicol. Eighty-five to 90% of mRNA in exponentially growing cells is associated with ribosomes, usually aggregated in the form of polyribosomes. Chloramphenicol when added to actively growing cells stops protein synthesis by specifically inhibiting peptidyl tRNA transferase (Monro and Vasquez, 1967), thus arresting the movement of ribosomes along the mRNA tape. Under these conditions mRNA becomes protected against enzymatic degradation. Messenger RNA synthesized in the presence of chloramphenicol, however, is rapidly degraded. Were the localization of mRNA synthesized in the presence of chloramphenicol different from that in growing cells its lability could be explained. At present there are no data concerning the intracellular distribution of mRNA synthesized by cells exposed to chloramphenicol. All we know is that pulse-labeled RNA synthesized in the presence of chloramphenicol enters into polyribosomes (Gurgo *et al.*, 1969; Cameron and Julian, 1968; Weber and DeMoss, 1966). Since identification of the mRNA fraction of pulse-labeled RNA can only be achieved by means of competition hybridization assay we examined the distribution

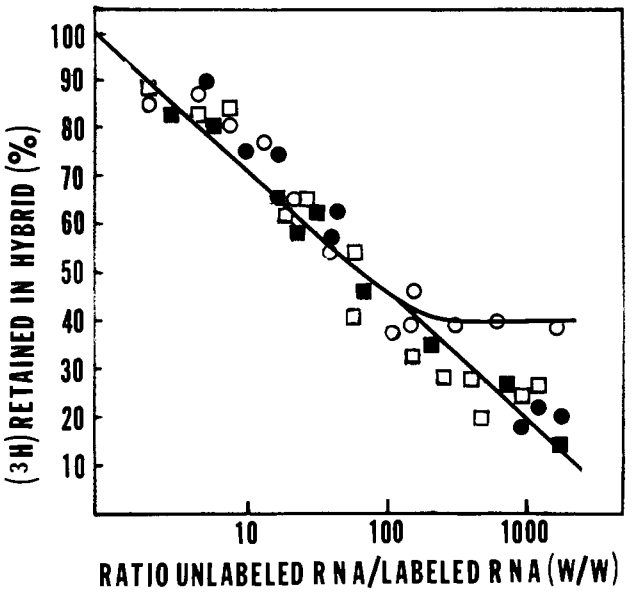

FIGURE 4: Competition experiments. Pulse-labeled RNA (0.5 μg) from exponentially growing cells was annealed to 60 μg of DNA immobilized on filters in the presence of increasing amounts of unlabeled RNA extracted from: glucose-starved cells (○---○); exponentially growing cells (●—●); cells exposed to chloramphenicol for 5 min (□—□); cells exposed to chloramphenicol for 30 min (■—■).

TABLE I: Distribution of Pulse-Labeled mRNA between the Various Ribosomal Classes before and after Chase with Excess of Unlabeled Uridine.

	Intracellular Distribution of mRNA Synthesized by									
	Exponentially Growing Cells						Chloromphenicol-Treated Cells			
		mRNA, % of Total Pulse-Labeled RNA						mRNA (% of Total Pulse-Labeled RNA)		
RNA Extracted from	Localization of mRNA (% of Total mRNA)	Before Chase	After Chase	Degraded (%)	After Chase in Chloramphenicol	Degraded (%)	Localization of mRNA (% of Total mRNA)	Before Chase	After Chase	Degraded (%)
Whole lysates	100.0	35.0	1.4	96.0	25.0	28.5	100.0	34.0	5.6	84.0
Polyribosomes and monosomes	85–90	42.0	3.2	92.4	29.0	31.0	85–90	42.0	9.1	78.5
Ribosomal subunits										
Top fractions of the gradient	10–15	26.5	8.4	68.0	10.9	59.0	10–15	25.0	7.6	70.0

of mRNA synthesized by chloramphenicol-treated cells by means of this assay. The following experimental conditions were employed. (1) Exponentially growing cells were pulse labeled with radioactive uracil for 0.01 of the generation time and divided into three portions. One portion was used immediately. The remaining two portions were chased for 10 min with a 200-fold excess of unlabeled uridine, one in the presence and the other in the absence of chloramphenicol. (2) Exponentially growing cells were exposed to chloramphenicol for 5 min, pulse labeled with radioactive uracil, and divided into two portions. One portion was used immediately and the other was chased for 10 min with unlabeled uridine. All cells were converted into spheroplasts and lysed with deoxycholate, and the lysates fractionated by sucrose gradient centrifugation. RNA was extracted from the different sucrose gradient fractions, as shown in Figure 5, and used for mRNA identification by means of the competition hybridization assay.

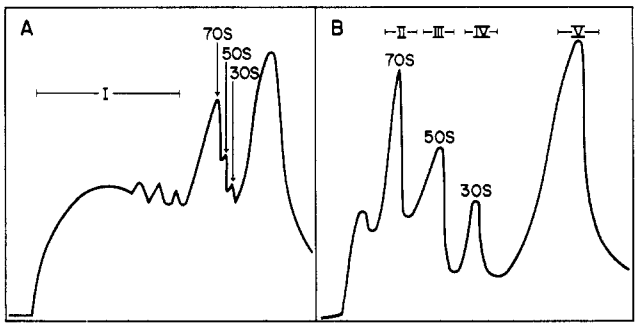

FIGURE 5: Sedimentation pattern of a lysate of *E. coli*. The total cell lysate from 40 ml of a pulse-labeled culture was layered on a 4.4-ml sucrose density gradient (15–30%, w/v) in 10 mM Tris-HCl buffer (pH 7.4), containing 10 mM magnesium acetate and 60 mM KCl, and centrifuged in the SW 39 rotor at 37000 rpm at 4° for (a) 70 min and (b) 3.5 hr. The profile was divided into five regions: I, polyribosomes; II, 70S ribosomes; III, 50S ribosomal subunits; IV, 30S ribosomal subunits; and V, the upper part of the gradient. The RNA extracted from the different regions was used in hybridization competition experiments. These are the original recordings from the continuous flow recording system.

The results of these experiments can be summarized as follows (Table I). mRNA whether synthesized in the presence or absence of chloramphenicol comprises about 35–40% of the total pulse-labeled RNA and is mainly associated with polyribosomes and monosomes (85–90% of the total mRNA). There is no mRNA in the region of native ribosomal subunits. A small fraction of mRNA (10–15% of the total) is not bound to ribosomes and can be detected in the upper third of the gradient. Polyribosome-bound mRNA synthesized by exponentially growing cells was degraded to the extent of 93% during uridine chase in the absence of chloramphenicol. Only 29% of the mRNA was degraded when the chase took place in the presence of chloramphenicol. Polyribosome-bound mRNA synthesized in the presence of chloramphenicol was degraded to the extent of 84% during uridine chase. The mRNA fraction not associated with any of the known ribosomal classes was broken down during uridine chase to the extent of 70% regardless of whether it was synthesized in the presence or absence of chloramphenicol.

Discussion

The different metabolic stability of various RNA species in bacterial cells is a well-known phenomenon. In actively growing cells and in cultures exposed to chloramphenicol for short periods, rRNA and transfer RNA are stable. On the other hand, mRNA in growing cells is metabolically unstable. Chloramphenicol stabilizes preexisting mRNA but allows rapid turnover of mRNA synthesized in its presence. It is difficult to explain the different metabolic fate of mRNA observed under these conditions for the reason that our knowledge of the mechanism underlying mRNA lability is poorly understood. Recently a new *E. coli* exonuclease, RNase V, has been implicated in mRNA degradation. RNase V was reported to be specific for mRNA and inhibited by chloramphenicol (Kuwano *et al.*, 1969). This finding could explain the stability of preexisting mRNA by chloramphenicol. The degradation of mRNA synthesized in the presence of chlor-

amphenicol, however, indicates that other enzyme(s) than RNase V may be involved in mRNA degradation.

Messenger RNA synthesized by exponentially growing and chloramphenicol-treated cells is mainly found in polyribosomes, but there is a small fraction of mRNA not bound to ribosomes. Chloramphenicol protects only the preexisting polyribosome-bound mRNA. The reason for the stability of polyribosome-associated mRNA in the presence of chloramphenicol shoud be perhaps sought in the different manner in which the preexisting mRNA and that synthesized in the presence of chloramphenicol are associated with polyribosomes. In growing cells the formation of polyribosomes results from the sequential addition of ribosomes at the 5′ end of the messenger during protein synthesis. Movement of ribosomes along messenger tape during protein synthesis is called "productive translocation." When chloramphenicol is added to growing cultures "productive translocation" is arrested and protein synthesis stops. At the same time mRNA continues to be synthesized and to enter polyribosomes. According to Cameron and Julian (1968) the formation of chloramphenicol-promoted polyribosomes results from addition of ribosomal material to mRNA in a mode other than the sequential addition of ribosomes to the 5′ end of mRNA. Polyribosomes formed in this way are nonfunctional and were called "abortive complexes." The movement of ribosomes in these complexes may accordingly be called "abortive translocation." Thus the stability and lability of mRNA in polyribosomes can be tentatively explained in the following way. For the breakdown of mRNA to occur, translocation of ribosomes, either productive or abortive, is required, hence, the breakdown of mRNA during protein synthesis and of mRNA synthesized in the presence of chloramphenicol. On the other hand the arrest by chloramphenicol of productive translocation of ribosomes along preexisting mRNA results in the protection of the latter against degradation.

A somewhat similar explanation was offered by Morse (1970, 1971) for the "artificial polar effect" on *E. coli* tryptophan operon mRNA produced by chloramphenicol (this effect can be releaved by the suA allele). In this case, however, there is no direct evidence for a rapid degradation of the distal tryptophan mRNA synthesized in the presence of chloramphenicol, and the possibility that coupling of translation to transcription prevents the synthesis of distal tryptophan mRNA by chloramphenicol and that suA may "relax" such coupling was not ruled out. Experiments on the metabolic fate of mRNA in suA mutants of *E. coli* are now in progress in our laboratory.

Finally, the degradation of mRNA not bound to ribosomes indicates that for the enzyme(s) responsible for mRNA breakdown to function its association with ribosomes is not obligatory.

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