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Accumulation of Ribonucleoprotein Particles in a Relaxed Mutant of *Escherichia coli*

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ABSTRACT

SYPHERD, PAUL S. (University of California, San Diego). Accumulation of ribonucleoprotein particles in a relaxed mutant of *Escherichia coli*. J. Bacteriol. **90**:403–410. 1965.—The synthesis of ribonucleic acid during amino acid deprivation of a "relaxed" mutant was investigated. Aspects of the stability of the macromolecular ribonucleic acid (RNA) were studied, and standard conditions were established to allow maximal recovery of the larger RNA's (i.e., 16*S* and 23*S*). These RNA's, representing 75% of the total RNA produced during starvation, were present in particles with nominal *S*-values of 20, 30, and 43. The particles are extremely sensitive to nuclease action, being completely destroyed in the presence of 2 μg/ml of pancreatic ribonuclease at 15 C for 30 min. The particles containing the bulk of the RNA were shown to be ribonucleoprotein, consisting of 26 to 28% protein by weight. It was shown that no mature 70*S* ribosomes were formed during the accumulation of the lighter, protein-deficient particles.

Numerous investigations in mammalian, plant, and microbial systems have shown that ribosomes are the structural cellular elements of the protein biosynthetic process. Various properties of ribosomes have been investigated, with the aim of understanding more precisely the biochemical role which ribosomes play in protein formation.

Aside from the participation of ribosomes in protein synthesis, there is an intrinsic interest in their complex structure and the sequences involved in their biosynthesis. With the aim of studying the coordinated biosynthesis of ribosomal protein and ribonucleic acid (RNA), aspects of ribosome formation in a "relaxed" amino acid auxotroph (Borek, Ryan, and Rockenback, 1955) of *Escherichia coli* have been under investigation. This particular mutant was shown to possess a genetic lesion (in the RC gene) which makes RNA synthesis no longer dependent on amino acids (Stent and Brenner, 1961). As a result, when a required amino acid is withheld from this mutant, RNA synthesis will proceed. Such relaxed (RC^{rel}) mutants may accumulate two to three times their normal quantity of RNA in the absence of net protein synthesis (Neidhardt, 1963).

The RNA which is accumulated during amino acid depletion of the RC^{rel} organism was reported to be "normal," in the sense that RNA molecules are found which are present in normally grown cells (Mandel and Borek, 1962; Neidhardt and Eidlic, 1963). However, these previous investigations showed that the distribution of these RNA molecules is abnormal, so that when a starved RC^{rel} culture overproduces RNA, two to three times the normal amount of 4*S* RNA is synthesized, and proportionately less 16 and 23*S* ribosomal RNA is formed. Dagley, Turnock, and Wild (1963) concluded that the RNA which is overproduced during starvation is present in ribonucleoprotein particles with sedimentation values somewhat less than those for normal mature ribosomes. Their studies also showed that the RNA-protein ratio of these "relaxed particles" was about the same as that reported for mature ribosomes (Tissieres et al., 1959).

In the course of our studies, some observations were made which are at variance with previous ones concerning the distribution of the RNA's (Mandel and Borek, 1962; Neidhardt and Eidlic, 1963), and the properties of the ribonucleoprotein formed during relaxed starvation.

MATERIALS AND METHODS

Bacterial cultures. The experiments reported here were performed with a histidine-arginine double auxotroph carrying the RC^{rel} allele of the RC gene. This organism was obtained from G. S. Stent, and was produced by crossing Hfr·H:RC^{rel}, *met*$^-$ by F$^-$:RC^{st}, B_1^-, *thr*$^-$, *leu*$^-$, *try*$^-$, *his*$^-$, and *arg*$^-$. This recombinant is referred to here as

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HAR. For certain control experiments a "stringent" (RC^{st}) organism was employed. This strain, *Escherichia coli* W2961, which required *thr*, *leu*, *pro*, and B_1, was obtained from E. A. Adelberg and designated AB266 in his collection. The bacteria were maintained on nutrient agar. For experimental purposes, bacteria were grown in a mineral salts medium (Sypherd and Strauss, 1963) with 0.5% glucose, and supplemented with the necessary amino acids at 50 μg/ml.

Starvation conditions. In experiments with strain HAR, the cells were grown into exponential phase (about 5×10^8 cells per milliliter), then removed from the culture medium by use of filtration onto Millipore filters at room temperature. The cells were washed on the filters with 10 ml of distilled water and were then resuspended in the same volume of mineral salts medium lacking the specific amino acid in question. Washing the filtered cells with water proved effective in removing pools of amino acids and uracil, so that starvation was effected at the time of resuspension. The removal of uracil pools by use of this procedure was demonstrated by the fact that radioactive uracil incorporation could be halted immediately upon resuspension in medium containing the nonradioactive isotope. This is in contrast to the difficulty in "chasing" uracil-C^{14} pools with excess uracil-C^{12}, as shown by McCarthy and Aronson (1961). This procedure made it possible to establish large intracellular concentrations of "cold" uracil to act as trapping pools for any radioactive material released by degradation of previously labeled RNA.

In some experiments, the culture of HAR was depleted of histidine, and the incorporation of arginine-C^{14} was measured. This was done by first establishing the starvation with respect to histidine in the presence of arginine-C^{12} (1 μg/ml). The cells were filtered again, washed with distilled water, and suspended in the absence of histidine and arginine. Arginine-C^{14} was then added at the desired specific activity.

Biochemical and radioactivity determinations. Protein determinations were performed on trichloroacetic acid-insoluble material by the method of Lowry et al. (1951). A standard curve was constructed with *E. coli* protein. The latter was prepared by centrifuging a crude extract at $150{,}000 \times g$ for 120 min to remove ribosomes. The supernatant solution was extracted five times with hot trichloroacetic acid. The insoluble material was then extracted several times with ethyl alcohol-ether (5:3, v/v). The remaining material was devoid of orcinol-reacting material, and had a 280–260 mμ ratio of 1.8.

Nucleic acid was determined by use of the orcinol procedure (Dische, 1955), with a standard of D-ribose. A calculated conversion factor (5.25) was applied to express the data in micrograms of RNA.

Bacterial extracts were prepared by passing a 10 to 20% suspension of cells through a French pressure cell at 10,000 psi. The effluent was centrifuged at $5{,}000 \times g$ for 15 min, and the supernatant fraction was centrifuged at $15{,}000 \times g$ for 20 min. The resulting supernatant liquid was referred to as the clarified crude extract. Except where noted, extracts were prepared and analyzed in 10^{-2} M tris(hydroxymethyl)aminomethane (Tris) buffer containing 10^{-2} M magnesium acetate. All extracts were prepared in the presence of 5 μg/ml of deoxyribonuclease.

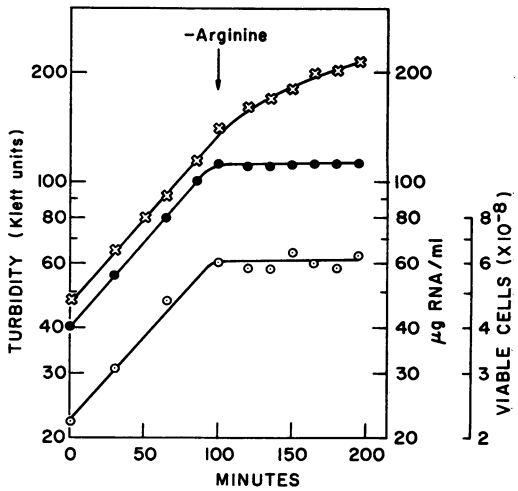

FIG. 1. *Arginine deprivation of the relaxed mutant HAR. A culture in glucose-salts medium was grown into early exponential phase, filtered from the medium, and resuspended (at the arrow) in the absence of arginine. Samples were removed at intervals for determinations of protein* (●) *and RNA* (×) *content, and for viable-cell counts* (⊙).

Zone centrifugation of crude extracts was performed in 20 to 5% linear gradients of sucrose. The gradients were centrifuged in the 25sw rotor in a Spinco model L centrifuge for 5.5 hr at 5 to 7 C. After centrifugation, fractions of equal volume were collected from the bottom of the tube. The fractions were read at 260 mμ, then precipitated with trichloroacetic acid in the presence of sufficient carrier albumin to cause maximal self-absorption during counting. The precipitates were collected on Millipore filters, and the filters were dried and placed in scintillation counting vials.

Isolation of relaxed particles. Relaxed particles were isolated from extracts prepared in 10^{-2} M Mg^{++}. The clarified crude extract (see above) was centrifuged at $30{,}000 \times g$ for 30 min. The supernatant fluid (S-30 fraction) was then centrifuged at $100{,}000 \times g$ in a Spinco ultracentrifuge for 60 min to remove ribosomes of 70*S* and larger. This supernatant fluid (S-100 fraction) contained the relaxed particles and native 50*S* and 30*S* ribosomes (*see* Fig. 3*B*). From the total adsorbancy at 260 mμ of the S-100 fraction from nonstarved and starved cells, it was estimated that 10 to 15% of the nucleic acid in this fraction of starved cells is contributed by native 50*S* and 30*S* particles.

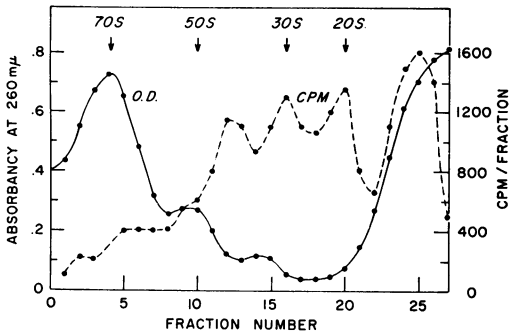

FIG. 2. *Distribution of uracil-C^{14} (broken line) incorporated in RNA during arginine deprivation of the mutant HAR. The cells were grown as in Fig. 1. After 30 min of incubation in the absence of arginine, uracil-C^{14} was introduced into the medium and incorporated for an additional 60 min. An extract was prepared and analyzed on a sucrose gradient in the Spinco 25sw rotor. The absorbancy profile (solid line) is contributed by the 25-fold excess of carrier nonstarved cells.*

The relaxed particles could be quantitatively recovered as a pellet by centrifuging the S-100 fraction for 3 hr at 150,000 × *g*.

RESULTS

The general characteristics of RNA synthesis during amino acid deprivation of an RC^{rel} auxotroph have been reported by others (*see* Neidhardt, 1963). Figure 1 shows the effect of arginine deprivation on viability and protein and RNA synthesis in the RC^{rel} mutant used in the present studies. Within the limits of the viable-count assay, there was no apparent loss of viability during the period of starvation, and concomitant over-synthesis of RNA.

Some of the macromolecular events which occurred during the starvation were determined by isotopically labeling the nucleic acids with uracil-C^{14}. After 90 min of starvation, the distribution of labeled molecules was examined by use of zone centrifugation of crude extracts. Approximately 30% of the total radioactivity incorporated was located in the 4 to 5*S* region of the gradient (Fig. 2). The remaining 70% was present in three major peaks, with nominal *S*-values of 20, 30, and 45 in 10^{-2} M Mg^{++}. These "relaxed" particles were isolated by differential centrifugation (Fig. 3*A* and *B*), and the RNA was extracted by the phenol-dupanol method (Kirby, 1956). The RNA sedimented in a sucrose gradient as two components, 16*S* and 23*S* (Fig. 3*C*). The distribution of relaxed RNA (Fig. 2) was variable, and the demonstration of the larger *S*-value material depended on rigorously maintaining the labeled cells and extracts below 5 C. In addition, the larger material was slowly lost when extracts were allowed to stand even at refrigerator temperature (i.e., as in dialysis). In the course of these experiments, as much as 60% of the total RNA made during relaxed starvation was in the 4*S* zone in extracts that were either dialyzed overnight against buffer with 10^{-4} M Mg^{++}, or prepared in potassium phosphate buffer with 10^{-4} M Mg^{++}. The lability of the relaxed particles

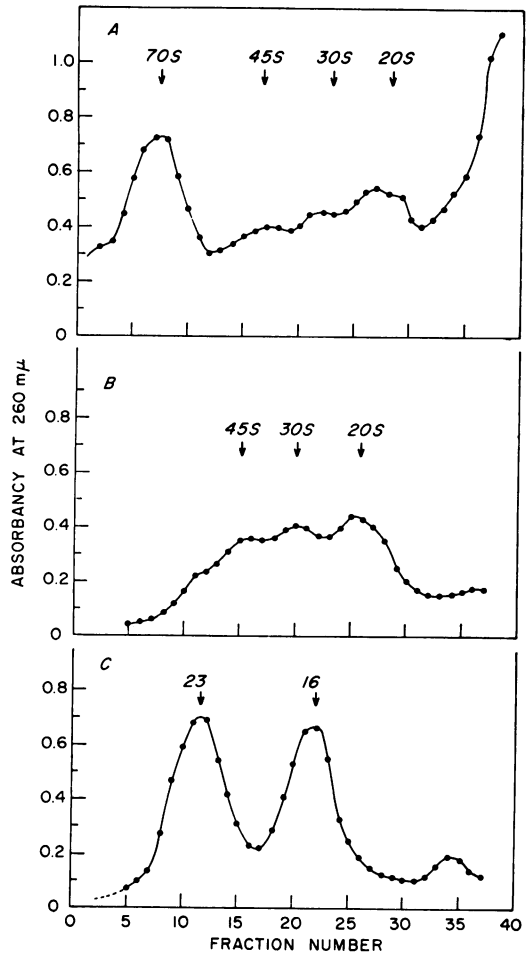

FIG. 3. *Isolation of relaxed particles by differential centrifugation. (A) Absorbancy profile of a crude extract prepared from HAR cells starved for arginine for 120 min. A portion of the crude extract was centrifuged to remove ribosomes that sediment greater than 70S (see Materials and Methods). (B) Relaxed particles recovered from the supernatant solution and analyzed on a second sucrose gradient. (C) Sedimentation of RNA extracted from the relaxed particles shown in (B). The RNA was extracted with phenol-dupanol and analyzed on a 20 to 5% linear gradient of sucrose in Tris buffer (10^{-2} M).*

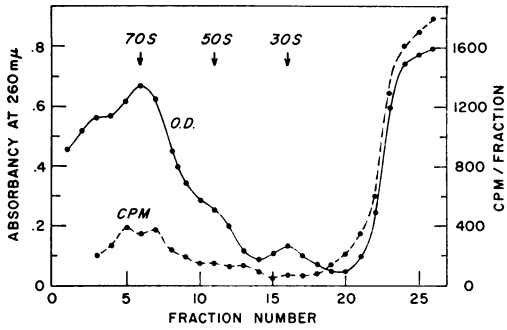

FIG. 4. *Distribution of uracil-C^{14} incorporated during starvation of HAR. The extract from these cells was incubated with pancreatic ribonuclease (2 μg/ml) at 15 C for 30 min. The extract was then analyzed on a sucrose gradient.*

pointed out another important aspect in the analysis of crude extracts on sucrose gradients. The distribution of relaxed RNA between the soluble and particulate fractions was affected by the pressure employed in making the extracts in the French cell. Above 10,000 psi, the percentage of relaxed RNA in the 4 to 5*S* region increased in proportion to the pressure. In all the experiments reported here, extracts were prepared at 8,000 to 10,000 psi. In view of the reports (Mandel and Borek, 1962; Neidhardt and Eidlic, 1963) that abnormally high ratios of soluble-to-ribosomal RNA's are produced during relaxed starvation, the results shown here suggest that much of the 4*S* RNA is degraded ribosomal RNA. Neidhardt and Eidlic (1963) showed that, although the 4*S* RNA increases disproportionately during relaxed starvation, the amino acid acceptor activity increases proportionately to the total RNA formed. Their finding suggests that not all the 4*S* material is transfer RNA.

The lability of the larger RNA's may be accounted for by the ribonuclease sensitivity of the relaxed particles. Figure 4 shows that treatment at 15 C for 30 min with 2 μg/ml of pancreatic ribonuclease destroys the 20 to 45*S* components. This treatment does not appear to destroy significant numbers of native 70*S*, 50*S*, and 30*S* ribosomes, as can be seen from the absorbancy profiles of ribosomes of the carrier extract before and after treatment with the enzyme.

In addition to the in vitro instability of the larger RNA's formed during relaxed starvation, there was an apparent instability in vivo during starvation. This was demonstrated by labeling with uracil-C^{14} the RNA formed during the first 20 min of starvation, and allowing the cells to continue synthesizing RNA for the remaining 70 min in the presence of excess nonradioactive uracil. The cells were examined immediately after exhausting a 20-min supply of uracil-C^{14} and at intervals after exhaustion. The zone-centrifugation analyses of these cells are shown in Fig. 5*A*. It can be seen that the distribution of radioactivity at 20 min is similar to that shown in Fig. 2. However, at subsequent times after exhaustion of the isotope, the radioactivity in the 20 to 45*S* region decreases, while that in the 4 to 5*S* region increases. In this experiment, approximately 22% of the total RNA was present in the 4 to 5*S* zone at the 20-min point. This percentage increased to 40% when RNA synthesis continued for an additional 70 min in the presence of excess nonradioactive uracil (Fig. 5*B*). The percentage changes were accounted for by net losses of radioactivity in the 20 to 45*S* region, and net gain in the 4 to 5*S* region of the gradient. These results suggest that larger RNA's are degraded during RNA synthesis in vivo. The degradation apparently stops at the level of 4 to 5*S* since there was little net loss of total radioactivity from the cells during the entire starvation period. The data do not indicate the cause of this apparent in vivo degradation of the larger RNA's.

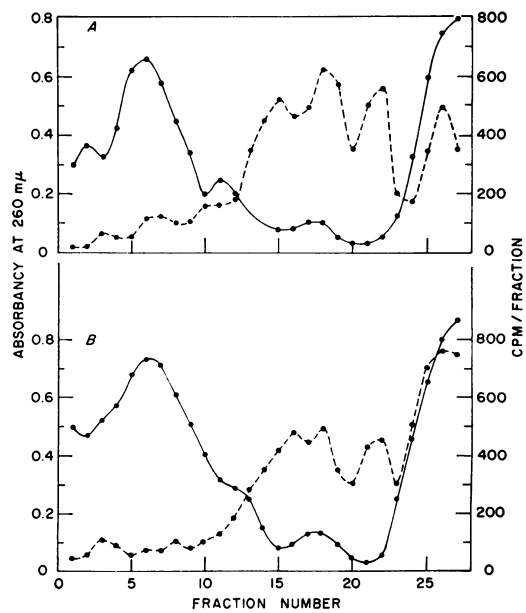

FIG. 5. *Change in distribution of relaxed RNA, labeled with uracil-C^{14} (broken line), during the course of starvation. (A) Distribution of radioactivity after a 20-min exposure to uracil-C^{14} during the starvation period. (B) Distribution of radioactivity after the culture in (A) synthesized relaxed RNA for an additional 70 min in the presence of an excess of uracil-C^{12}. Sucrose gradient analysis was performed as in Fig. 2.*

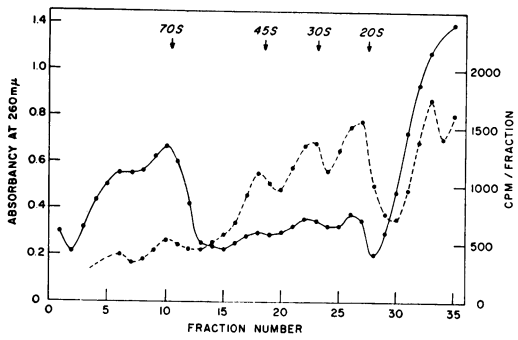

FIG. 6. *Incorporation of arginine-C^{14} into the regions occupied by relaxed RNA. Absorbancy (solid line) and radioactivity (broken line) determinations were performed on the same fractions taken from the centrifuge tube.*

From the preceding experiments, it is clear that two factors affect the estimations of the relative amounts of soluble and particulate RNA's formed during relaxed starvation. One factor, which apparently cannot be eliminated, is the continual loss of larger RNA's during the starvation period. The second factor, which can be largely eliminated, is the lability of the larger RNA's in vitro. The data indicate that the degradation of 20 to 45*S* material in both cases results in the formation of 4 to 5*S* RNA.

Using conditions which gave maximal recovery of 20 to 45*S* material, the complex nature of the accumulated particles was investigated. That the particles are partly composed of protein was shown by the incorporation of C^{14} amino acids into the particle. Figure 6 shows that arginine-C^{14} incorporated into cells starved for histidine appeared prominently in the relaxed-particle regions of the gradient. Since there was no net protein synthesis in these cultures, protein which was incorporated in the relaxed particles, and labeled with exogenously provided C^{14} amino acid, was derived from protein turnover. Amino acid-incorporation data make it appear as if the resynthesis of protein during this turnover period favors the formation of ribosomal protein. This deduction was made by calculating the expected distribution of two amino acids—arginine-C^{14} and leucine-C^{14}—between particulate and soluble protein, and comparing the expected values with those obtained by incorporation studies. The calculations were made by using the data of Sphar (1962) for the amino acid composition of ribosomal and soluble proteins, and the fact that 30% of the total cellular protein is particulate (i.e., sediments greater than 10*S* in sucrose gradients) when the cells are grown under stringent conditions. Table 1 shows that the particulate-soluble protein ratio determined for each amino acid is about twice that calculated by assuming equal resynthesis of all classes of proteins during the turnover period. It is apparent, then, that ribosomal protein was preferentially formed during the starvation and concomitant oversynthesis of RNA by the relaxed mutant. It might be added that labeling the protein of a starved stringent mutant yields particulate-soluble protein ratios very near those calculated for stringent growth in Table 1. Thus, the shift in distribution of proteins synthesized during amino acid deprivation is peculiar to the relaxed mutant, and is probably related to the oversynthesis of RNA by this organism.

Simultaneous incorporation of uracil-H^3 and arginine-C^{14} show that both protein and nucleic acid appear in the region of the sucrose gradient which is occupied by relaxed particles (Fig. 7*A*). Two lines of evidence show that both these labeled precursors are incorporated into the same particle structures. Figure 7*B* shows the effect on the 20 to 45*S* material of preparing and analyzing the extract in 10^{-4} M Mg^{++}. In addition to the usual breakdown of 70*S* ribosomes to 50*S* and 30*S* particles, there is a simultaneous shift in the distribution of uracil-H^3 and arginine-C^{14} from peaks of 20, 30, and 45*S* in 10^{-2} M Mg^{++} to a predominant peak of 16*S* in 10^{-4} M Mg^{++}. Figure 6 shows that not all the radioactivity from arginine-C^{14} is present in the 16 and 20*S* regions in low Mg^{++}, but that the 30 and 50*S* particles are also labeled. Further experiments showed that this radioactivity was derived from 70*S* ribosomes and native 50 and 30*S* particles in about

TABLE 1. *Distribution of C^{14} amino acids between soluble and particulate fractions of histidine-starved RC^{rel} bacteria*

Amino acid	Fraction of total radioactivity		Ratio of particulate to soluble fraction
	Particulate*	Soluble	
Leucine-C^{14}			
Calculated†	0.30	0.70	0.43
Experimental	0.46	0.54	0.85
Arginine-C^{14}			
Calculated	0.48	0.52	0.92
Experimental	0.69	0.31	2.2

* The total radioactivity in relaxed and ribosomal particles.

† Calculated from the amino acid composition of ribosomal and soluble protein (Spahr, 1963) and the distribution of cellular protein between particulate and nonparticulate fractions, assuming equal resynthesis of all classes of protein during protein turnover.

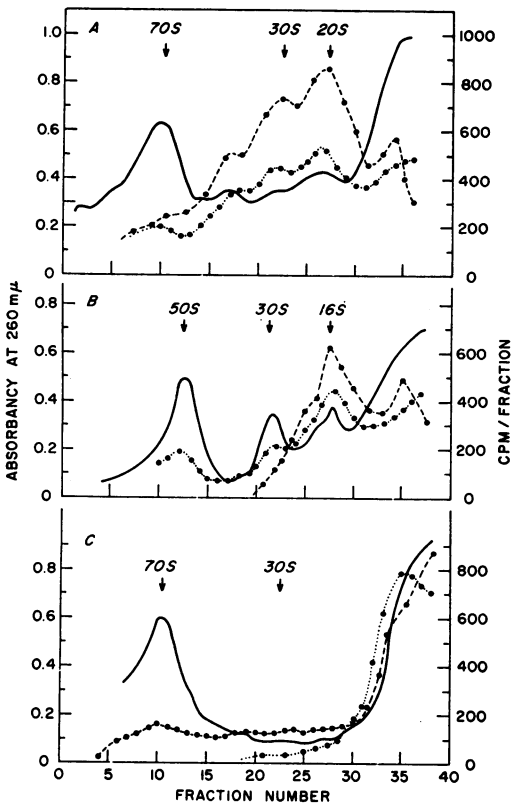

FIG. 7. *(A) Incorporation of arginine-C^{14} and uracil-H^3 into the relaxed particles of a culture of HAR deprived of histidine. (B) Effect on the distribution of both isotopes of lowering the Mg^{++} concentrate to 10^{-4} M. (C) Effect of pancreatic ribonuclease (2 μg/ml; 30 min at 30 C) on the distribution of both isotopes. Absorbancy (solid line), arginine-C^{14} (dotted line), and uracil-H^3 (dashed line) determinations were all performed on the same fraction sample.*

equal proportions. Apparently the protein moiety of these ribosomal particles was labeled (by turnover), and the nucleic acid was not.

The simultaneous conversion of RNA and protein, labeled during starvation, to a lighter particle size in low Mg^{++} indicated that these molecules reside together on the 20 to 45*S* particles seen in the high Mg^{++}. Further evidence on this point may be obtained by taking advantage of the extreme ribonuclease sensitivity (Fig. 2) of the relaxed particles. When relaxed particles doubly labeled with arginine-C^{14} and uracil-H^3 were treated with ribonuclease, the relaxed particles disappeared from the heavier regions of the sucrose gradient (Fig. 7*C*). There was a subsequent increase in C^{14} radioactivity in the soluble region of the gradient, and a loss of much of the total trichloroacetic acid-insoluble H^3 radioactivity.

With the conclusion that the RNA overproduced by the amino acid-starved relaxed mutant is in the form of ribonucleoprotein, attempts were made to determine the ratio of nucleic acid to protein in these particles. Because the relaxed particles sediment with native 30*S* and 50*S* ribosomes in high Mg^{++}, it was necessary to effect some discrimination between relaxed and native particles. Dagley et al. (1963) relied on differences in sedimentation between these particle types in low Mg^{++} to recover the relaxed material by use of differential centrifugation. Two considerations made this approach unacceptable. (i) As already shown, preparation of extracts in low (10^{-4} M) Mg^{++} resulted in the loss of some RNA from the particulate regions of the gradient. (ii) It was found that considerable "soluble" protein was sedimented by the gravitational forces necessary to pellet the relaxed material from extracts in low Mg^{++}. Therefore, the method chosen for discriminating between the ribonucleoprotein of relaxed particles and of native 30*S* and 50*S* ribosomes was the ribonuclease sensitivity of the former (*see* Fig. 4). Determinations of particulate RNA and protein before and after ribonuclease treatment (2 μg/ml, 30 min at 15 C) gave the composition of the relaxed particles by difference. From several independent determinations, the ratio of RNA to protein in the relaxed (ribonuclease-sensitive) particles varied from 2.5 to 2.9. This would put the protein composition at 26 to 28% of the total mass. No further attempts were made to determine these ratios for each of the particle sizes formed during starvation.

DISCUSSION

Beginning with the initial studies of Borek et al. (1955), various aspects of the metabolism of "relaxed" mutants have been investigated. Since the bulk of cellular RNA (80 to 85% in *E. coli*) is associated with protein as a ribonucleoprotein complex, my interest in the RC^{rel} organism was to study a possible coordination of synthesis of ribosomal protein and RNA. In pursuing this line of investigation, several questions arose concerning the nature of the RNA synthesized during "relaxed" starvation. The work of Neidhardt and Eidlic (1963) and of Mandel and Borek (1962) showed an abnormal distribution in the RNA's formed during amino acid depletion. Their data showed that from 40 to 60% of this RNA was present as 4 to 5*S* material on sucrose gradients.

In exponentially growing cells, 15 to 20% of the total RNA is present as 4 to 5*S* material. These results seemed to indicate that the RC gene, if it is a regulatory gene, has a differential effect on the various RNA's which a bacterial cell may synthesize. Under the conditions employed in the present study, which seem to minimize degradation of the larger RNA's, nearly normal distribution of particulate and soluble RNA's was found. This was in spite of an apparently uncontrollable loss of the larger RNA's during the course of the starvation. These findings lead to the conclusion that the RC gene controls equally the formation of soluble and ribosomal RNA's. The data strongly suggest that large shifts in RNA distribution during the starvation period are the result of continuous degradation of RNA's with high molecular weight to the 4 to 5*S* size.

A second problem which was investigated was that of the state of the RNA formed without concomitant protein synthesis. The RC^{rel} mutant was allowed to accumulate RNA during a standard starvation period, and crude extracts of the bacteria were analyzed by use of zone centrifugation in 10^{-2} M Mg^{++}. These analyses showed that the bulk of the RNA formed under relaxed conditions occurred as three primary peaks with nominal *S*-values of 20, 30, and 45. The amino acid-incorporation studies demonstrated that these peaks were occupied by ribonucleoprotein. Chemical determinations indicated that the relaxed particles were RNA-rich, with 26 to 28% of the particle mass comprised of protein moiety. These numbers are close to the values reported for the protein composition of chloramphenicol particles (Nomura and Watson, 1959). Because there is no net protein synthesis during the amino acid depletion, protein in the relaxed ribonucleoprotein particles could come from only two sources; protein turnover or a previously existing pool of ribosomal protein. Our data show that the protein moiety of relaxed particles is formed from turnover of existing protein. However, there is no evidence against the possibility that a soluble pool of ribosomal protein exists. The results also demonstrate a diversion of the protein synthetic capacity to favor the formation of ribosomal protein during the starvation period.

The relaxed particles have nominal sedimentation values approximating those of ribosome precursors formed under stringent conditions (Boezi et al., 1961). One might easily infer, then, that relaxed particles are normal precursors halted in the process of ribosome formation. The question of whether the relaxed particles are equivalent to normal precursors has been examined and is the subject of the accompanying report (Sypherd, 1965).

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